Delineating the deranged immune system

in systemic lupus erythematosus and antiphospholipid syndrome

PhD Thesis

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Het immuunsysteem ontrafeld
in systemische lupus erythematodes en het antifosfolipiden syndroom
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

CHAPTER 1

General Introduction

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic and systemic autoimmune disease that typically affects women of child-bearing age. In SLE, immune complexes of autoantibodies directed against nuclear proteins form immune complexes and are deposited in tissues. This initiates tissue inflammation and subsequent tissue damage[1]. Several organs can be affected, including the skin, joints and kidneys. In depth analyses on the pathophysiology of SLE, ranging from genetic studies to in vitro studies and mouse models implicate a broad derailment of both the innate and adaptive immune system in the pathogenesis of SLE[2]. Such studies have led to the development of drugs targeting specific pathways to treat SLE as well as the identification of biomarkers which aid clinical decision making. In particular, (1) high levels of interferons, (2) abnormally activated neutrophils and (3) hyperactivity of B-cells form the cornerstones of the pathophysiology of SLE[3].

1. Interferon alpha: the signature cytokine of SLE

Interferons are antiviral proteins which are produced by immune cells upon viral infections[4]. In the 1980s high levels of type I interferons (IFNα or IFNβ) were found in the circulation of patients with SLE[5,6]. When in the early 2000s the first transcriptomic studies in SLE were performed, a striking upregulation of IFNα-inducible genes was found in these patients, termed the IFN signature[7,8]. Measuring this IFN signature is widely used to reflect increased levels of IFNα in autoimmune diseases, as for technical reasons it is difficult to detect IFNα in the circulation. In SLE, the IFN signature correlates with disease activity, vascular disease and is associated with future flares[9–11]. The importance of type I IFN in the pathophysiology of SLE is reflected by the clinical efficacy of anifrolumab[12], a monoclonal antibody against IFNAR, the receptor for type I IFN.

In SLE, IFNα is produced when plasmacytoid dendritic cells (pDCs) are activated by DNA- or RNA- containing immune complexes[13]. pDCs are reduced in peripheral blood of patients with SLE[14] and have been found in affected skin[15] suggesting active migration of pDCs towards inflamed areas. In line with this, tissue samples of SLE patients display an IFN signature[16]. pDC-derived IFNα may in turn activate key pathways central to the pathogenesis of SLE, including the release of neutrophil extracellular traps (NETs, see below)[17], the production of the B-cell growth factor BAFF[18] (B-cell activating factor, see below). As such, IFNs amplify the immunopathology of SLE.

2. The NET-effect of neutrophils and low-density granulocytes in SLE

Neutrophils are the most abundant leukocyte in peripheral blood. They form a first line of defence against invading pathogens which they kill either intracellularly after phagocytosis or extracellularly after degranulation of antimicrobial peptides. In 2003 a novel defence mechanism of neutrophils was described. Upon encountering a pathogen, neutrophils can release their chromatin content decorated with antimicrobial peptides in web-like lattices in a literal attempt to trap and kill the encountered pathogen. These structures are called Neutrophil Extracellular Traps (NETs)[19]. Soon it was discovered that NET-release is augmented in patients with SLE, and autoantibodies present in the serum of patients with SLE induce the release of NETs[17,20,21]. Uncontrolled NET release induces tissue damage in for instance lupus nephritis[22]. NET-release is potentiated by IFNα[17] and NETs may activate pDCs to produce IFNα through stimulation of Toll-like receptors (TLRs) [17,20,22], creating a pathogenic loop of NET-release and IFNα production. In SLE, NETs are thought to be primarily released by a specific subset of neutrophils termed low-density granulocytes (LDG), which are present in patients with SLE[23]. These LDGs co-purify with peripheral blood mononuclear cells (PBMCs) upon ficoll density gradient centrifugation and are the source of the "granulopoiesis" signature found in PBMCs of SLE patients[3,7]. LDGs induce damage to endothelial cells and may synthesize IFNs and spontaneously release NETs[22,23].

3. Baffled B-cells in SLE

The hallmark of SLE is the finding of autoantibodies against nuclear components, most notably antibodies against double stranded DNA. These autoantibodies are produced by self-reactive B-cells and induce tissue damage and activate key players in the pathophysiology of SLE including pDCs and neutrophils as described above. As a result, B-cells are strongly implicated in the pathophysiology of SLE and B-cells are therapeutic targets in SLE[24].

From their origin in the bone marrow, B-cells undergo several selection and maturation steps, ultimately leading to the formation of memory B-cells and plasma cells which secrete high amounts of antibodies. The importance of B-cells in the pathophysiology of SLE is supported by flow cytometric analysis of the B-cell subsets [25,26], as well as transcriptomic studies of circulating leukocytes [3,7] in patients with SLE, showing increased levels of circulating memory B-cells and plasmablasts in association with disease activity in SLE.

However, in contrast to their alleged central role in the pathophysiology of SLE, B-cell depleting therapy with rituximab has yielded negative results in SLE clinical

trials [24]. The lack of success of rituximab is partly blamed on trial design and as a result, rituximab is sometimes used as a last resort off-label treatment for patients with SLE[27].

In contrast to rituximab, targeting BAFF (B-cell activating factor), a key B-cell growth factor, has yielded positive results in SLE clinical trials [28,29]. BAFF is produced by myeloid cells upon stimulation by IFNs[18] and interacts with one of its three receptors: the BAFF-receptor BAFF-R, transmembrane activator and CAML interactor (TACI) and B-cell maturation antigen (BCMA) which are highly expressed by B-cells[30]. Signaling through these receptors induces canonical and non-canonical NFκB signalling promoting in cell survival[30]. BAFF is implicated in the pathophysiology of SLE as mice transgenic for BAFF spontaneously develop SLE[31] and patients with SLE have elevated levels of BAFF, which correlate with disease activity and auto-antibody levels [32]. In 2011, belimumab, a monoclonal antibody against BAFF, was the first drug in several decades to be approved for the treatment of SLE and other drugs targeting BAFF are currently under development as therapeutics in SLE[33].

Antiphospholipid syndrome

In 1952 Conley and Hartmann reported two patients with SLE that had a "peculiar hemorrhagic disorder attributable to a pronounced coagulation defect". The patients' lab results showed prolonged clotting times which were due to the presence of an anticoagulant, as the addition of "one part of the patient's plasma to 200 parts of normal blood significantly prolonged clotting times of the latter". Although the authors were unable to identify the cause of the clotting defect, they did notice that both patients had a false positive serologic test for syphilis[34]. In subsequent years such coagulation inhibitor(s) were reported in other patients and became known as "lupus anticoagulants" (LA) [35,36]. In the following decades it became apparent that lupus anticoagulant, in contrast to its in vitro property, was not associated with serious bleeding events[35] but rather with a "striking" tendency for thrombosis, as well as pregnancy morbidity[37–40].

Antibodies with reactivity to phospholipids, a constituent of cellmembranes, were identified as a potential cause of lupus anticoagulant[41] and such antibodies are collectively termed antiphospholipid antibodies. As LA was often found in patients with a false positive syphilis test, which used cardiolipin derived from bovine heart cells in its reaction, anticardiolipin antibodies were proposed as the antibody causative of LA. Indeed, after the development of a sensitive assay to detect anticardiolipin

(aCL) antibodies, a strong correlation with LA was found in patients with SLE[42]. Subsequently, β2 glycoprotein I (β2GPI), an abundant plasma protein, was found to be an important co-factor for the binding of aCL antibodies to cardiolipin[43].

The triad of the detection of antiphospholipid antibodies in patients suffering from thrombosis and/or pregnancy morbidity became known as the antiphospholipid syndrome[39,44]. Currently, classification criteria for APS exist, in which APS is defined as the detection of either LA, significant titres of IgG or IgM of anticardiolipin (aCL) or anti-β2 glycoprotein I (β2GPI) antibodies in patients who have suffered from at least one thrombotic event or pregnancy complication[45]. However, thrombosis and pregnancy morbidity are not the only clinical symptoms seen in APS. Other, so-called "non-criteria manifestations" include thrombocytopenia, livedo reticularis, skin ulcers, haemolytic anaemia and heart valve lesions[46,47]. Up to 30% of SLE patients have APS. On the other hand, of all APS patients, 50% do not have SLE or another underlying autoimmune disease and these patients are referred to as *primary* APS (PAPS)[44,46].

Research in the past decades has clearly shown a pathological role for aPL in the clinical manifestations of APS. For instance, aPL activate endothelial cells and thrombocytes in vitro, resulting in a procoagulant phenotype of endothelial cells and thrombocytes[48]. Furthermore aPL decrease trophoblast viability[49]. *In vivo*, the infusion of aPL promotes thrombus formation[50] and induces fetal resorption in pregnant mice[51]. However, in contrast to other autoimmune diseases, little attention has been given to the immunological aberrancies seen in patients with APS and little attention has been given to the presence of an underlying disease (i.e. SLE) when studying the pathophysiology of APS.

Thesis outline

Primary antiphospholipid syndrome: a role for the immune system?

In this thesis we set out to investigate the immunological abberancies in APS patients as compared to SLE and secondary APS patients. Particular emphasis was given to the pillars of the pathophysiology of SLE (activation of pDCs, NET-release and B-cell hyperactivity) studied in patients with PAPS. As such, we investigated to what extent PAPS, on a pathophysiological level, is part of the SLE spectrum. In **chapter 2** we review the literature on APS and describe for each immune cell the current knowledge on their role in APS and indicate gaps in the current literature.

In the following chapters, we studied immune cell aberrancies in patients with PAPS and compared them with patients with APS secondary to SLE (SLE+APS) and SLE patients without APS (typically referred to as just "SLE" throughout this thesis). In **chapter 3** we evaluated the presence of a type I IFN signature in patients with PAPS and report its prevalence in comparison to patients with SLE and SLE+APS. In **Chapter 4** we studied the potential of serum biomarkers, in particular galectin-9, as easy to measure biomarkers for the detection of the IFN signature in both SLE and APS, overcoming the hurdle of laborious gene-expression testing. In **Chapter 5** and **Chapter 6** we studied neutrophil extracellular trap release and low-density granulocytes in PAPS, in comparison with patients with SLE and SLE+APS and in relation to the IFN signature in these conditions. In **Chapter 7** we investigated whether, similar to SLE, BAFF levels are elevated in PAPS.

Dendritic cells and the IFN signature: drivers of the pathophysiology of systemic autoimmune diseases

In the next part, we studied dendritic cells (DCs) isolated from patients with SLE and APS. DCs are the sentinel cells of the immune system which capture, process and present antigens to T-cells. DCs present antigen loaded into major histocompatibility complex (MHC) molecules and can provide co-stimulatory signals to T-cells. As such, DCs are the initiators of the immune response and are implicated in the pathophysiology of autoimmune diseases. DCs come in two principal subsets: classical DCs (cDCs) and pDCs, the latter of which were introduced earlier in this chapter as major producers of IFNα. Both subsets are present in low numbers in peripheral blood (<0.5% of leukocytes) which has hampered their study. In chapter 8 and 9 we studied isolated cDCs and pDCs of patients with SLE, SLE+APS and PAPS, and we investigated the transcriptomic dysregulation of these cells by RNA sequencing and microRNA profiling.

MicroRNAs are short non-coding RNAs that regulate gene-expression at a post-transcriptional level. Alterations in microRNA expression are suggested to play an important role in immune cell activation in autoimmune diseases[52]. However, most studies on microRNAs in autoimmune diseases have used bulk cells obtained from the circulation and therefore the changes observed in microRNA expression may reflect alterations in numbers of circulating cells. In **Chapter 8** we integrated microRNA expression profiling data with mRNA sequencing data obtained from pDCs of patients with SLE and APS in order to identify key pathways regulated by altered microRNA expression in relation to the IFN signature in pDCs of SLE and APS patients.

In **Chapter 9** we compared the transcriptome of pDCs and mDCs by means of RNA sequencing in patients with SLE and APS and studied how elevated IFNα levels in SLE and APS differentially activate both DC subsets, resulting in a self-perpetuating loop of IFNα production by pDCs in interferon-positive SLE and APS patients.

Previous studies have identified that DCs of SLE patients instruct CD8+ T-cells to produce granzymes[53]. Granzymes are serine proteases that eliminate virally infected or tumour cells by inducing apoptosis. In SLE, granzyme production results in the generation of neo-autoantigens [53]. In **Chapter 10** we studied granzyme levels in peripheral blood and kidney samples of patients with SLE, in relation to the IFN signature and signs of kidney damage.

Innate lymphoid cells (ILCs) are recently discovered immune cells that are implicated in the initiation of inflammation in rheumatic diseases, most notably spondyloarthropaties[54]. As of yet, no studies are available on the frequency and phenotype of ILC in patients with SLE. In **Chapter 11** we studied the frequency and phenotype of ILCs in patients with SLE as well as in patients with Sjögren's syndrome in relation to the IFN signature which affects both diseases.

Immune cell profiling in systemic autoimmune disease by CyTOF

Cytometry by time of flight (CyTOF) is a novel tool to study the expression of markers on cells, similar to flow cytometry. CyTOF differs from flow cytometry as it uses antibodies conjugated to rare metals rather than fluorochrome labelled antibodies, overcoming the hurdle of spectral overlap which limits the maximum detection rate of labels on a single cell. CyTOF holds great promise in immunophenotyping immune cells in patients with autoimmune diseases[55]. In **Chapter 12** we compared the circulating immune cell compartment of patients with SLE with several other autoimmune diseases including Sjögren's syndrome and Systemic sclerosis by means of CyTOF.

References

1. Tsokos G. Systemic Lupus Erythematosus. *N Engl J Med* 2011;**365**:2110–21. doi:10.1007/SpringerReference_61618

2. Tsokos GC, Lo MS, Reis PC, *et al.* New insights into the immunopathogenesis of systemic lupus erythematosus. *Nat Rev Rheumatol* 2016;**12**:716–30. doi:10.1038/nrrheum.2016.186

3. Banchereau R, Hong S, Cantarel B, *et al.* Personalized Immunomonitoring Uncovers Molecular Networks that Stratify Lupus Patients. *Cell* 2016;**165**:551–65. doi:10.1016/j.cell.2016.03.008

4. Isaacs A, Lindenmann J. Pillars Article : Virus Interference . I . The. *Proc R Soc L B Biol Sci* 1957;**147**:258–67.

5. Hooks J, Moutsopoulos H, Geis S, *et al.* Immune interferon in the circulation of patients with autoimmune disease. *N Engl J Med* 1979;**301**:5–8.

6. Preble O, Black R, Friedman R, *et al.* Systemic lupus erythematosus: presence in human serum of an unusual acid-labile leukocyte interferon. *Science (80-)* 1982;**216**:429–31.

7. Bennett L, Palucka AK, Arce E, *et al.* Interferon and Granulopoiesis Signatures in Systemic Lupus. 2003;**197**. doi:10.1084/jem.20021553

8. Baechler EC, Batliwalla FM, Karypis G, *et al.* Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci* 2003;**100**:2610–5. doi:10.1073/pnas.0337679100

9. Somers EC, Zhao W, Lewis EE, *et al.* Type I interferons are associated with subclinical markers of cardiovascular disease in a cohort of systemic lupus erythematosus patients. *PLoS One* 2012;**7**:e37000. doi:10.1371/journal.pone.0037000

10. Kirou KA, Lee C, George S, *et al.* Activation of the interferon-α pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. *Arthritis Rheum* 2005;**52**:1491–503. doi:10.1002/art.21031

11. Hoffman RW, Merrill JT, Alarcón-Riquelme MME, *et al.* Gene Expression and Pharmacodynamic Changes in 1,760 Systemic Lupus Erythematosus Patients From Two Phase III Trials of BAFF Blockade With Tabalumab. *Arthritis Rheumatol* 2017;**69**:643–54. doi:10.1002/art.39950

12. Furie R, Khamashta M, Merrill J, *et al.* Anifrolumab, an Anti-Interferon-Alpha Receptor Monoclonal Antibody, in Moderate to Severe Systemic Lupus Erythematosus. *Arthritis Rheumatol* 2017;**69**:376–86. doi:10.1002/art.39962

13. Eloranta M-L, Alm G V, Rönnblom L. Disease mechanisms in rheumatology--tools and pathways: plasmacytoid dendritic cells and their role in autoimmune rheumatic diseases. *Arthritis Rheum* 2013;**65**:853–63. doi:10.1002/art.37821

14. Blomberg S, Eloranta ML, Magnusson M, *et al.* Expression of the markers BDCA-2 and BDCA-4 and production of interferon-α by plasmacytoid dendritic cells in systemic lupus erythematosus. *Arthritis Rheum* 2003;**48**:2524–32. doi:10.1002/art.11225

15. Farkas L, Beiske K, Lund-Johansen F, *et al.* Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol* 2001;**159**:237–43.

16. Higgs BW, Liu Z, White B, *et al.* Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis* 2011;**70**:2029–36. doi:10.1136/ard.2011.150326

17. Lande R, Ganguly D, Facchinetti V, *et al.* Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* 2011;**3**:73ra19. doi:10.1126/scitranslmed.3001180

18. Sjöstrand M, Johansson A, Aqrawi L, *et al.* The Expression of BAFF Is Controlled by IRF Transcription Factors. *J Immunol* 2016;**196**:91–6. doi:10.4049/jimmunol.1501061

19. Brinkmann V, Reichard U, Goosmann C, *et al.* Neutrophil extracellular traps kill bacteria. *Science* 2004;**303**:1532–5. doi:10.1126/science.1092385

20. Garcia-Romo GS, Caielli S, Vega B, *et al.* Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med* 2011;**3**:73ra20. doi:10.1126/scitranslmed.3001201

21. Van Avondt K, Fritsch-Stork R, Derksen RHWM, *et al.* Ligation of signal inhibitory receptor on leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus. *PLoS One* 2013;**8**:e78459. doi:10.1371/journal.pone.0078459

22. Villanueva E, Yalavarthi S, Berthier CC, *et al.* Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* 2011;**187**:538–52. doi:10.4049/jimmunol.1100450

23. Denny MF, Yalavarthi S, Zhao W, *et al.* A Distinct Subset of Proinflammatory Neutrophils Isolated from Patients with Systemic Lupus Erythematosus Induces Vascular Damage and Synthesizes Type I IFNs. Published Online First: 2010. doi:10.4049/jimmunol.0902199

24. Dörner T, Lipsky PE. Beyond pan-B-cell-directed therapy - new avenues and insights into the pathogenesis of SLE. *Nat Rev Rheumatol* 2016;**12**:645–57. doi:10.1038/nrrheum.2016.158

25. Odendahl M, Jacobi A, Hansen A, *et al.* Disturbed Peripheral B Lymphocyte Homeostasis in Systemic Lupus Erythematosus. *J Immunol* 2000;**165**:5970–9. doi:10.4049/jimmunol.165.10.5970

26. Jacobi AM, Odendahl M, Reiter K, *et al.* Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* 2003;**48**:1332–42. doi:10.1002/art.10949

27. Ryden-Aulin M, Boumpas D, Bultink I, *et al.* Off-label use of rituximab for systemic lupus erythematosus in Europe. *Lupus Sci Med* 2016;**3**:1–9. doi:10.1136/lupus-2016-000163

28. Furie R, Petri M, Zamani O, *et al.* A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum* 2011;**63**:3918–30. doi:10.1002/art.30613

29. Navarra S V., Guzmán RM, Gallacher AE, *et al.* Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: A randomised, placebo-controlled, phase 3 trial. *Lancet* 2011;**377**:721–31. doi:10.1016/S0140-6736(10)61354-2

30. Rickert RC, Jellusova J, Miletic A V. Signaling by the TNFR superfamily in B-cell biology and disease. doi:10.1111/j.1600-065X.2011.01067.x

31. Mackay F, Woodcock S a, Lawton P, *et al.* Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med* 1999;**190**:1697–710. doi:10.1084/jem.190.11.1697

32. Vincent FB, Morand EF, Schneider P, *et al.* The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol* 2014;**10**:365–73. doi:10.1038/nrrheum.2014.33

33. Croft M, Siegel RM. Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases. *Nat Rev Rheumatol* 2017;**13**:217–33. doi:10.1038/nrrheum.2017.22

34. Conley C, Hartman RC. A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosus. *J Clin Invest* 1952;**31**:621–2.

35. Boxer M, Ellman L, Carvalho A. The lupus anticoagulant. *Arthritis Rheum* 1976;**19**:1244–7.

36. Feinstein DI, Rapaport SI. Acquired inhibitors of blood coagulation. *Prog Hemost Thromb* 1972;**1**:75–95.

37. Boey ML, Colaco CB, Gharavi a E, *et al.* Thrombosis in systemic lupus erythematosus: striking association with the presence of circulating lupus anticoagulant. *Br Med J (Clin Res Ed)* 1983;**287**:1021–3.

38. Mueh J, Herbst K, Rapaport S. Thrombosis in Patients With the Lupus Anti-Coagulant. *Ann Intern Med* 1980;**92**:156–9.

39. Hughes GR. Thrombosis, abortion, cerebral disease, and the lupus anticoagulant. *Br Med J (Clin Res Ed)* 1983;**287**:1088–9. doi:10.1136/bmj.287.6399.1088

40. Bowie EJ, Thompson JH, Pascuzzi CA, *et al.* Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. *J Lab Clin Med* 1963;**62**:416–30. doi:10.5555/uri:pii:0022214363900416

41. Laurell AB, Nilsson IM. Hypergammaglobulinemia, circulating anticoagulant, and biologic false positive Wassermann reaction; a study in two cases. *J Lab Clin Med* 1957;**49**:694–707.

42. Harris EN, Boey ML, Mackworth-Young CG, *et al.* Anticardiolipin Antibodies: Detection By Radioimmunoassay and Association With Thrombosis in Systemic Lupus Erythematosus. *Lancet* 1983;**322**:1211–4. doi:10.1016/S0140-6736(83)91267-9

43. McNeil HP, Simpson RJ, Chesterman CN, *et al.* Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci U S A* 1990;**87**:4120–4.

44. Asherson R, Khamashta MA, Ordi-Ros J, *et al.* The 'primary' antiphospholipid syndrome: Major clinical and serological features. *Med (United States)* 1989;**68**:366–74.

45. Miyakis S, Lockshin MD, Atsumi T, *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006;**4**:295–306.

46. Cervera R, Piette JC, Font J, *et al.* Antiphospholipid syndrome: Clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum* 2002;**46**:1019–27.

47. Abreu MM, Danowski A, Wahl DG, *et al.* The relevance of 'non-criteria' clinical manifestations of antiphospholipid syndrome: 14th International Congress on Antiphospholipid Antibodies Technical Task Force Report on Antiphospholipid Syndrome Clinical Features. *Autoimmun Rev* 2015;**14**:401–14. doi:10.1016/j.autrev.2015.01.002

48. Ruiz-irastorza G, Crowther M, Branch W, *et al.* Antiphospholipid syndrome. *Lancet* 2010;**376**:1498–509. doi:10.1016/S0140-6736(10)60709-X

49. Tong M, Viall CA, Chamley LW. Antiphospholipid antibodies and the placenta: a systematic review of their in vitro effects and modulation by treatment. *Hum Reprod Update* 2014;**21**:97–118. doi:10.1093/humupd/dmu049

50. Arad A, Proulle V, Furie RA, *et al.* β2-Glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. *Blood* 2011;**117**:3453–9. doi:10.1182/blood-2010-08-300715

51. Girardi G, Berman J, Redecha P, *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003;**112**:1644–54. doi:10.1172/JCI200318817

52. Chen JQ, Papp G, Szodoray P, *et al.* The role of microRNAs in the pathogenesis of autoimmune diseases. *Autoimmun Rev* 2016;**15**:1171–80. doi:10.1016/j.autrev.2016.09.003

53. Blanco P, Pitard V, Taupin J. Increase in Activated CD8 ϱ T Lymphocytes Expressing Perforin and Granzyme B Correlates With Disease Activity in Patients With Systemic Lupus Erythematosus. *Arthritis Rheum* 2005;**52**:201–11. doi:10.1002/art.20745

54. Wenink MH, Leijten EFA, Cupedo T, *et al.* Review: Innate Lymphoid Cells: Sparking Inflammatory Rheumatic Disease? *Arthritis Rheumatol* 2017;**69**:885–97. doi:10.1002/art.40068

55. Ermann J, Rao D a., Teslovich NC, *et al.* Immune cell profiling to guide therapeutic decisions in rheumatic diseases. *Nat Rev Rheumatol* 2015;**11**:541–51. doi:10.1038/nrrheum.2015.71

CHAPTER 2

Delineating the deranged immune system in the antiphospholid syndrome

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Abstract

The antiphospholipid syndrome (APS) is a systemic autoimmune disease that is characterized serologically by the presence of antiphospholipid antibodies (aPL) and clinically by vascular thrombosis and obstetric complications. The protein β2 glycoprotein I (β2GPI) is identified as the most important autoantigen in this syndrome. Activation of endothelial cells, thrombocytes and placental tissue by anti-β2GPI antibodies relates to the clinical manifestations of APS. This review describes genetic and environmental factors in relation to APS and summarizes current knowledge on abnormalities in components of both the innate and adaptive immune system in APS. The role of dendritic cells, T-cells, B-cells, monocytes, neutrophils and NK-cells as well as the complement system in APS are discussed. Several gaps in our knowledge on the pathophysiology of APS are identified and a plea is made for future extensive immune cell profiling by a systems medicine approach in order to better unravel the pathogenesis of APS, to gain more insight in the role of the immune system in APS as well as having the potential to reveal biomarkers or novel therapeutic targets.

Keywords: Antiphospholipid syndrome, anti-β2 glycoprotein I antibody, dendritic cell, autoreactive T-cell, monocyte, neutrophil.

1.1 Introduction

The antiphospholipid syndrome (APS) is systemic autoimmune disease in which persistent presence of so-called antiphospholipid antibodies (aPL) is the serologic hallmark. In the classification criteria aPL are defined as lupus anticoagulant (LAC) and/or significant titers of IgG and/or IgM class anticardiolipin (aCL) and/or IgG and/or IgM class anti-β2 glycoprotein I antibodies (anti-β2GPI) [1]. The clinical criteria of APS are obstetric complications and thrombosis. The obstetric complications comprise recurrent early abortions, fetal loss and premature birth due to (pre-) eclampsia or recognized features of placental insufficiency. Thrombosis can afflict both arterial and venous blood vessels of any size. Vascular thrombosis and obstetric complications are undisputable manifestations of APS [1]. Other - so called non-criteria - manifestations are seen in APS as well. Among these are thrombocytopenia, livedo reticularis, leg ulcers, cognitive disturbances, nephropathy and heart valve lesions [1–3]. Similarly, other aPL than those included in the classification criteria have been linked to clinical manifestations in APS. These include IgA class antibodies of aCL and anti-β2GPI as well as antibodies directed towards phosphatidylserine/prothrombin (aPS/PT) [4]. The syndrome can occur as a stand-alone disorder (primary APS, PAPS) or in association with another systemic autoimmune disease, most often systemic lupus erythematosus (SLE; secondary APS). A small subset of patients with APS presents with a life-threatening variant, characterized by multi-organ thrombosis, termed catastrophic APS (CAPS) [5,6].

Antibodies against β2GPI are central in the current perception of the pathophysiology of APS [7]. The plasma protein β2GPI consists of five complement control protein (CCP) repeat domains and circulates in a blood in a circular form. After binding of the fifth domain to negatively charged surfaces, the β2GPI molecule opens up and obtains a characteristic fishhook appearance that exposes a hidden epitope in the first CCP domain [8]. Pathogenic anti-β2GPI are mostly directed towards this hidden epitope [9]. Nonetheless, clinical studies clearly show that single positivity for IgG- or IgM-anti-β2GPI or IgG- or IgM- aCL antibodies are not or only slightly associated with clinical APS manifestations. In contrast, presence of LAC and/or triple positivity (defined as presence of LAC and significant levels of aCL and anti-β2GPI antibodies) are strongly related to clinical APS manifestations [10–17].

Over the past decades many in vitro and animal studies have been conducted to elucidate the pathophysiological role of aPL in thrombosis and pregnancy morbidity. It has clearly been demonstrated that aPL can activate endothelial cells, platelets and (syncitio-)trophoblast cells in vitro. This holds in particular for anti-β2GPI

antibodies. Several cellular receptors, including Toll-like receptors (TLRs) and apolipoprotein E receptor 2 are involved in the activation of these cells (reviewed in [18]). Animal models strongly suggest that infused aPL, notably anti-β2GPI, are thrombogenic when endothelial cells are compromised by mechanical or chemical injury [19,20] and can induce fetal resorption in rodents [21]. Although much is known about effector mechanisms of aPL on endothelial cells, platelets and throphoblast cells [22–24], less attention has been given to the factors that initiate and sustain immune cell activation in APS. In this review we summarize current knowledge on the origin of aPL and on abnormalities in components of both the adaptive and innate immune system in relation to aPL and APS. Finally aims for future studies are formulated.

2.1 The role of genetics and environmental factors

The identification of families with multiple cases of APS and/or aPL positivity suggests that genetics are of importance for aPL production [25–27]. This is supported by studies that identified genes that are associated with APS and/or aPL positivity (reviewed in [28]). In line with similar findings in other autoimmune diseases, these include several MHC-II haplotypes (such as HLA-DR4 and HLA-DR7) [28–30]. Of interest, PAPS was found to associate with single nucleotide polymorphisms (SNPs) in STAT4-, BLK- and IRF5-genes [31–33]. These genes all are involved in immune cell signaling and have also been associated with SLE [34–36]. Also, polymorphisms in the gene encoding for β2GPI seem relevant for APS. A recent meta-analysis concluded that the Val247Leu SNP is associated with APS, in particular APS with anti-β2GPI antibodies [37]. The position of this polymorphism (247) is near the phospholipid-binding site of β2GPI.

Currently, the most informative way to identify susceptibility loci/genes for diseases is by employing genome-wide association studies (GWAS). Up till now only one GWAS in relation to aPL/APS has been published [38]. In that study, a subset of individuals from a larger GWAS in SLE patients, namely those in which LAC (n=708), aCL (n=670) or anti-β2GPI antibodies (n=496) had been measured was analyzed. Apart from several SNPs that associate with overall aPL production, such as those in the MHC region, the study also detected six SNPs in the gene encoding for β2GPI that associate specifically with presence of anti-β2GPI antibodies, but not with presence of LAC or aCL. The SNP in the β2GPI gene that had the strongest association with anti-β2GPI antibody production was Trp316Ser, which afflicts the fifth domain of β2GPI. In contrast to finding by others [37] no association was found between the Val247Leu SNP and production of anti-β2GPI antibodies [38].

Other genes involved in the immune system that have been linked to APS include TLR4 [39], Fcγ receptor IIa [40,41], CD36 (a scavenger receptor expressed on monocytes) [42] and tumor necrosis factor (TNF) α [43]. With respect to clinical features of APS, polymorphisms in the gene encoding for annexin A5 have been associated with obstetric morbidity in APS [44,45], while several genes involved in coagulation (like polymorphisms in platelet glycoprotein Ia and Ib [46,47] and P-selectin glycoprotein ligand-1 (PSGL-1) [48]) have been linked to thrombosis in APS [49].

Similar to most other autoimmune diseases it is likely that for APS to develop, both environmental factors and the genetic background are important. In a recent study in SLE patients, a history of smoking was found to be associated with the presence of aPL, especially triple positivity [50]. Interestingly, aPL were found even more frequently among former smokers than in current smokers, and aPL were the only autoantibodies that associated with such a history [50]. Several lines of evidence indicate that infectious agents serve as an initial trigger of aPL production, due to molecular mimicry of microbial epitopes with β2GPI. Early studies noted aPL production in mice immunized with microbial derived peptides with structural similarities to parts of β2GPI [51,52]. Recently it was shown that β2GPI binds to peptide H derived from streptococcus pyogenes resulting in a conformational change of β2GPI and the exposure of the cryptic epitope on β2GPI to which aPL can bind [53]. Immunization of mice with peptide H leads to the induction of anti-β2GPI antibodies [53]. Thus, infectious diseases in genetically susceptible individuals are likely involved in the initiation of anti-β2GPI antibody production.

3.1 Dendritic cells in APS

Dendritic cells (DCs) are key sentinel cells of our body and form the classical link between innate and adaptive immunity due to their unique capacity to capture, process and present antigens loaded onto MHC molecules to T-cells. They travel via the lymph to specific T-cell areas in lymphoid tissues where interaction between the antigen loaded MHC complex and an antigen-specific T-cell receptor can take place. DCs originate from the bone marrow and are found in the circulation as well as in lymphoid and non-lymphoid tissue. In humans, circulating dendritic cells can be divided into two types of (classical) myeloid DCs (mDCs), recognized by the expression of either Blood Dendritic Cell Antigen-1 (BDCA-1) or BDCA-3, and a separate group of plasmacytoid DCs (pDCs), identified by their expression of both BDCA-2 and BDCA-4 [54]. Circulating DCs are rare, with percentages reported of less than 0.5% of circulating peripheral blood mononuclear cells (PBMCs) [54]. As

this low frequency hampers their study ex vivo, researchers commonly rely on other sources of DCs. For instance, monocytes can be turned into DCs after culturing them for 5-7 days in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) 4, resulting in monocyte derived DCs (moDCs). Such in vitro derived DCs are frequently used in research as surrogates for naturally occurring myeloid DCs.

The importance of DCs in the development of systemic antibody-mediated autoimmune diseases is illustrated in experiments in mice in which hyperresponsiveness of DCs is induced by DC-specific knock-out of the ubiquitin editing protein A20. Such knock-out leads to the abrogation of the de-ubiquitination of NFκB signaling intermediates, which results in spontaneous maturation and hyperresponsiveness to activation stimuli of DCs. These knock-out mice develop both manifestations of SLE and APS with appearance of aCL, thrombocytopenia and fetal demise [55].

Furthermore, the observation that mice start to produce anti-β2GPI antibodies after injection of (bone marrow derived) DCs that have pinocytosed β2GPI or phagocytosed apoptitic thymocytes, suggests a fundamental role for DCs in aPL formation [56,57]. One of the characteristics of apoptotic cells is a negatively charged cellular surface. The protein β2GPI easily binds these anionic surfaces and thereby functions as an opsonin for apoptotic cells. Experiments with human moDCs showed that binding of β2GPI to an anionic surface strongly enhances both the processing of the protein and the capacity of moDCs to stimulate β2GPI-reactive T-cells [58,59]. Studies in mice showed that presence of anti-β2GPI antibodies in such experiments not only results in facilitation of the uptake of β2GPI-opsonized apoptotic cells by DCs, but also results in a pro-inflammatory state of the DCs. This is expressed by increased cytokine production (IL-1β, IL-10 and TNFα) and presentation of apoptotic cell-derived epitopes to helper T-cells in the context of MHC-II molecules [60,61].

It has well been demonstrated that aPL are among the earliest autoantibodies that can be found in the blood of individuals that develop SLE many years later [62]. One may therefore hypothesize that the facilitated uptake by DCs of apoptotic cells opsonized by β2GPI/anti-β2GPI complexes might be involved in the epitope spread to other self-antigens in SLE. This speculation is supported by findings in mice immunized with β2GPI, in which a similar sequential order of autoantibodies is seen as in SLE patients prior to the onset of their disease [62,63], exemplified by first the appearance of aPL, followed by antinuclear antibodies (ANA), anti-Ro/La and eventually anti-dsDNA, anti-Sm and anti-nRNP antibodies. However, observations

that the development of full blown SLE in patients with longstanding primary APS is rare [64] and that only about 50% of all SLE patients have aPL [65] indicate that this aPL-related hypothesis can at most explain part of the immunopathogenesis of SLE.

In APS patients the proportion of oxidized β2GPI in blood is higher compared to that in (aPL positive or negative) patients with other autoimmune diseases [66]. Of interest, oxidized β2GPI by itself can induce maturation and activation of human moDCs, a process in which NFκB- and Interleukin-1 receptor–associated kinase (IRAK) pathways are involved [67]. This results among others in an increased secretion of pro-inflammatory cytokines and the expression of co-stimulatory molecules. In co-cultures of moDCs activated with oxidized β2GPI and T cells the induction of a T helper-1 response with predominant production of interferon (IFN) γ by T cells was noted [67].

Tolerogenic DCs are a subtype of moDCs that can be derived from monocytes when these are cultured in the presence of TGFβ and IL-10, in addition to the conventional GM-CSF and IL-4. Such tolerogenic DCs can downregulate T-cell responses, as was recently also demonstrated for APS. In autologous co-culture experiments with β2GPI-loaded tolerogenic DCs and β2GPI-reactive T-cells from APS patients, these DCs significantly decreased the proliferative potential and cytokine production of the autoreactive T-cells [68]. All together these observations place DCs on a central stage in the initiation of an immune response towards β2GPI and further show that this response can be downregulated by manipulating DCs to become tolerogenic.

pDCs are capable of presenting antigen to T-cells and activate these cells. However, their main physiological role is sought in antiviral immunity due to their capacity to produce large amounts of the antiviral cytokines IFNα and β (type I interferons), after stimulation of their endosomal TLRs 7 and 9 by respectively virus-derived single-stranded (ss)RNA and unmethylated CpG sequences in microbial DNA [69,70]. Next to antiviral effects, type I IFNs augment T and B-cell responses [69]. Several autoimmune diseases, including SLE have a so-called type I interferon signature [71–73], which stands for the overexpression of a set of genes known as downstream events after type I IFN stimulation. This finding suggests that pDC have an important role in the pathogenesis of systemic autoimmune diseases [74]. Whether this also holds for APS is unclear.

Data on the presence of a type I interferon signature in APS are contradictory. One study using PBMCs from PAPS patients did find an upregulation of type I interferon induced genes in PAPS patients [75], whereas another study that used purified monocytes did not [76]. In both studies gene-expression of P APS patients was compared to SLE patients. In the former study however a different set of type I IFN responsive genes was found to be upregulated in the group of PAPS patients in comparison to SLE patients. For instance, MX1, a classical type I IFN induced gene [71,77], was only found upregulated in patients with SLE [75]. Nonetheless, the authors concluded that based on their data the IFN pathway is prominent in primary APS as well [75].

In vitro, aPL have significant activating effects on pDCs. Both monoclonal aPL, and IgG fractions from patients with either primary or secondary APS prime pDCs by upregulating their TLR7 expression. Such TLR7 upregulation strongly enhances the secretion of proinflammatory cytokines such as IL-1β, TNFα and IFNα [78,79]. aPL mediate the translocation of TLR7 from the endoplasmic reticulum to the endosome, where they induce ROS production and ROS-induced changes in pH. As a consequence pDCs are dramatically sensitized to TLR7 stimulation [78]. Support for a role of pDCs and type I IFN in APS also comes from experiments in mice, where agonists of TLR7 potentiate the induction of APS [80]. In humans, IFNα administration for melanoma is associated with the induction of aPL [81,82]. Also the above mentioned association of SNPs in genes that are important in type I IFN signaling (IRF5, STAT4), suggest a role for type I IFN in PAPS [31–33]. Furthermore, at least one of the mechanisms of action of hydroxychloroquine (HCQ), a drug frequently used in the treatment of SLE, is to downregulate endosomal TLR signaling [83,84], resulting in lower IFNα production by pDCs from SLE patients treated with HCQ [85]. This also might constitute one further mechanism underlying the beneficial clinical effects of HCQ in both obstetric [86,87] and thrombotic [88] APS patients in addition to effects on platelet aggregation and restoration of the anticoagulant properties of Annexin A5 (reviewed in [89,90]).

3.2 T-cells in APS

As MHC class II molecules present peptides to CD4+ T-helper cells, the association of certain MHC class II genes with APS points towards an important role for T-helper cells in this disease. Presence of β2GPI-specific autoreactive T-helper cells has been demonstrated both in blood from patients with APS and, to a lesser extent, in healthy individuals (reviewed in [91,92]). These T-cells almost uniformly react with an epitope located in the fifth domain of β2GPI [93], which covers the

phospholipid-binding site of the molecule [94]. Most studies found that β2GPI-reactive T-cells do not respond to native β2GPI as it circulates in blood, but only to reduced or recombinant β2GPI [59,93–95]. The characterization of T-cells reactive to native β2GPI [96–98] might be attributed to the observed spontaneous oxidative modifications to β2GPI in culture medium [67]. β2GPI reactive T-cell clones are CD4 positive and mainly HLA-DR restricted [94] and have Vβ-chains that are highly conserved between different clones [95]. This strongly suggests that APS is an antigen-driven disorder.

CD4+ T-cells can be subdivided into subclasses based on the profile of cytokines released and the expression of transcription factors and chemokine receptors. The division of these T-helper cell subsets has been studied in many autoimmune diseases. In most autoimmune diseases the frequency and function of Tregs seems to be diminished, whereas the number and activity of Th1 and Th17 cells is increased (reviewed in [99–101]). In PAPS patients several studies on T-helper cell subsets have been performed, mostly focusing on the subdivision of T-cells in naïve and memory phenotype and/or T-cell activation status [75,102–105]. Results are often contradictory so that firm conclusions cannot be made. For instance while in PAPS patients an increase in naïve T-helper cells has been reported [103], a decrease [102] and no significant difference compared to controls were also reported [104].

Several studies reported that stimulation of β2GPI autoreactive T-cells results in predominant secretion of Th1 cytokines like IFNγ [67,94,96,98,102,106]. These autoreactive T-cell clones stimulate B-cells to produce anti-β2GPI antibodies, which is dependent on the production of IL-6 by these T-cells [94]. In serum from PAPS patients the cytokine profiles seem to indicate T-cell skewing towards Th2 [107,108] and Th17 [109] (based on the serum concentrations of major identifying cytokines IL-4 and IL-17 respectively). Culture of PBMCs from healthy individuals with purified IgG from APS patients results in an increase in Th2 and Th17 cells, and a decrease in Th1 and Tregs [110]. In line with this, the number of Tregs was found to be decreased in both primary [111–113] and secondary APS patients [114,115].

3.3 B-cells in APS

Despite the alleged central role B-cells in the pathophysiology of the autoantibody mediated disease APS, relatively few in depth studies on B-cells have been performed in APS.

As is the case in many other autoimmune disorders, several papers describe increased percentages of circulating B-cells that bear CD5 in PAPS patients [102,116–118]. However, these studies were conducted in small patient groups. In one study, involving 36 thrombotic and/or obstetric PAPS patients, no difference in CD5 expression on B-cells between PAPS patients and controls was reported [105]. CD5-positive B-cells are termed B1 cells and secrete natural antibodies of relatively low affinity that can cross-react with selfantigens [119]. Healthy individuals have aCL-bearing memory B-cells, as their presence has been described during acute Epstein Barr virus (EBV) infection [120]. Polyclonal B-cell activation of (aPL producing) B-cells can explain the finding of aPL production early in the course of EBV infection.

In a group of 36 patients with obstetric PAPS, including 10 who also had a history of thrombosis it was found that only the patients with thrombosis had increased percentages and absolute numbers of circulating naïve B-cells and a reduction in memory B-cells [105]. The same study showed an increased proportion of naïve B cells (IgD+CD27-), and reduced unswitched (IgD+CD27+) and switched (IgD-CD27+) memory B cells in trombotic PAPS patients only [105]. Similar results were recently reported in a cohort of thrombotic PAPS patients [118].

B-cell directed therapies targeting CD20 (rituximab) or BAFF (belimumab) are used increasingly in SLE during the last decade. In a thrombotic APS prone mouse model ((NZW × BXSB)F1 mice), BAFF blockade reduced the occurrence of myocardial infarction [121] and in humans with CAPS beneficial clinical effects have been suggested with rituximab treatment [122]. Both BAFF blockade in mice and rituximab treatment in humans did not lead to significant effects on aPL titers [121,123], in a 12 month follow-up period in case of rituximab treatment [123].

The data summarized so far indicate an important role of DCs, T-cells and B-cells in the initiation and perpetuation of immune responses towards β2GPI in APS. In the next section, the role of the innate immune system in APS is discussed.

4.1 Monocytes in APS

Compared to other immune cells, monocytes have been studied most extensively in APS. Monocytes from APS patients, compared to those from healthy controls, are in an activated state, as is expressed among others by upregulation of the NFκB, MEK-1/ERK and p38 MAPkinase pathways [124]. This results both in a procoagulant and a proinflammatory phenotype of APS monocytes. A multitude of cellular receptors have been implicated in the activation of monocytes by β2GPI/

anti-β2GPI antibody complexes. The molecule Annexin A2 is regarded as a likely docking station for β2GP/anti-β2GP antibody complexes. However, as Annexin A2 lacks a transmembrane intracellular signaling tail, this molecule can only result in cellular activation via additional mechanisms such as involvement of Toll-like receptor 4 (TLR4). It has been demonstrated that dimers of β2GPI co-localize on the monocyte surface in membrane micro-domains called lipid rafts together with Annexin A2 and that anti-β2GPI antibodies recruit TLR4 to these lipid rafts [125]. Subsequent signaling via TLR4 leads to phosphorylation of MyD88, TRIF and IRAK and activation of the NF-κB-pathway, which results among others in TNFα secretion and tissue factor expression (TF) [125–128]. In line with this is the finding that blockade of Annexin A2 reduces aPL-induced expression of TF, the main initiator of humoral coagulation, on healthy monocytes [129]. Furthermore, it was shown that monocytes from thrombotic APS patients produce more Annexin A2 than non-thrombotic APS patients or healthy controls [130]. Apart from TLR4 and its co-receptor molecule CD14, a role in monocyte activation has also been suggested for other toll-like receptors, such as TLR1, TLR2, TLR6 and TLR8 [79,131–133]. Gene expression analysis demonstrated that TLR8 and CD14 are up regulated in PBMCs from PAPS patients compared to those from healthy controls or SLE patients [75] and incubation of monocytes with monoclonal aPL increases TLR8 expression and enhances TLR8-mediated TNFα, IL1β and caspase-1 production [79,133].

The most prominent expression of a procoagulant status of monocytes from APS patients is their increased production, surface expression and activity of tissue factor (TF) [124,125,130,131,134–141]. A procoagulant status is also reflected in an increased number of TF-expressing monocyte-derived microparticles in the circulation of APS patients [142–144] and the notion that monocytes from APS patients display increased production and expression of protease-activated receptors (PARs), vascular endothelial growth factor (VEGF) and the VEGF receptor Flt1 [139,140,145]. Incubation of monocytes from healthy controls with aPL showed that blockade of the signaling pathways for p38, ERK-1 MAP-kinase and/or NFκB in these cells lowers their expression of TF and VEGF [124,139,141,146,147]. Of interest, affinity purified IgG from patients with thrombotic APS activate NFκB and p38 MAP-kinase pathways more potently than affinity purified IgG from obstetric APS patients [126]. In line with this are observations that the procoagulant state of monocytes in patients with thrombotic APS is more pronounced than in women with obstetric APS [124,126,130,135,137,139]. Other studies showed that the extent of TF expression on monocytes in APS patients is related to the level of circulating aPL [124,130,139,140]. TF-expression in APS patients is enhanced by

priming of monocytes with an inflammatory stimulus like lipopolysaccharide (LPS) [148], but is independent of serum levels of interleukin (IL)-1β and tumor necrosis factor α (TNFα) [124,135], and the use of aspirin or oral anticoagulants[135,137]. Additionally, TF-expression on monocytes from healthy individuals can be induced by their incubation with serum from APS patients or purified aPL [124,125,127,134,138,145,146,149], and $F(ab)_2$ fragments of aPL [138].

MicroRNAs (miRNA) are short non-coding RNA strands that regulate gene expression through RNA interference. It has been described that the levels of two miRNAs (miR19b and miR20a) are downregulated in monocytes from APS patients compared to monocytes from healthy subjects [150]. In transfection experiments with monocytic-cell lines it was shown that upregulation of these two miRNAs reduces TF expression [150]. This suggests that these miRNAs are important factors in the occurrence of a procoagulant phenotype in APS monocytes.

Monocytes from APS are in a pro-inflammatory status based on enhanced production of cytokines and reactive oxygen species (ROS). After stimulation with LPS, monocytes from APS patients secrete more proinflammatory cytokines than healthy controls [75,151]. This strong cytokine production in APS seems secondary to aPL as incubation of monocytes from healthy donors with aPL stimulates release of TNFα and IL-1β [125,127,131,133,152], probably via aPL-induced activation of the NLRP3-inflammasome [152]. Increased production of ROS by monocytes from APS patients is related to mitochondrial dysfunction that was found in APS patients [145]. Also this phenomenon seems to be secondary to aPL as it can be induced in monocytes from healthy donors through incubation with aPL [145]. Comparison of gene expression profiles of monocytes taken from patients with PAPS, APS with SLE and SLE patients without APS, showed that expression of genes involved in mitochondria biogenesis, function and oxidative stress is a specific feature of APS monocytes [76].

Several drugs interfere with the increased activation state of APS monocytes. For instance, chloroquine reduces TF-expression and TNFα production in aPL activated monocytes through inhibition of aPL mediated endocytosis [132]. Likewise, CoEnzyme Q10 reduces TF, VEGF and Flt1 expression and ROS production [145]. Also a combination of vitamin E and vitamin C [149] or Ubiquinol [153] reduces monocyte TF expression. The same holds for statins [154,155]. The recent finding that losmapimod, an oral drug that inhibits p38 MAP-kinase is beneficial in patients with myocardial infarction [156] in combination with demonstrable up-regulation of the p38 MAPkinase in APS monocytes makes this drug of possible value in APS.

4.2 Neutrophils in APS

Like monocytes, circulating neutrophils from patients with APS are in an activated state as shown by their increased production of reactive oxygen species (ROS) [145], which is associated with altered expression of several miRNAs that regulate ROS production [157]. The increased ROS production apparently is secondary to aPL as in vitro incubation of human neutrophils from healthy donors with murine monoclonal anti-β2GPI antibodies stimulates their ROS production and degranulation, through activation of FcγRII [158]. Of interest, incubation of neutrophils from healthy controls with human monoclonal aPL induce ROS production only after priming of these cells with LPS [159].

In contrast to monocytes from APS patients, the neutrophils from APS patients do not express significantly increased levels of TF, Flt1 and PAR-2 [145]. This may seem remarkable as incubation of both mouse and human neutrophils with aPL results in increased cellular expression of TF and PARs [160,161]. Interestingly, the in vitro induction of TF on human neutrophils by serum from APS patients apparently is mediated by complement, since heat-inactivation or pretreated of serum with a complement inhibitor abolishes this effect [161].This contrasts with findings with monocytes, where it has been shown that $F(ab)_2$ fragments of aPL can induce TF expression on monocytes [138].

In a mouse model for obstetric APS (induction of fetal resorption by high doses of aPL, injected intraperitoneally in pregnant mice) a clear inflammatory response is noted in decidual tissue with abundant presence of neutrophils. As in this model the deleterious effects of the administration of aPL can be abrogated by depleting mice from neutrophils (not by depleting monocytes) [21,162] or by blockade of the complement cascade via inhibition of complement factor C5a, it can be concluded that at least in this model for obstetric APS, there are important pathophysiological roles for both neutrophils and the complement system [21].

An important effector function of neutrophils is their capacity to form extracellular traps (Neutrophil Extracellular Traps, NET), which is a cell death process termed NETosis. The process of NETosis is known to be disturbed in patients with SLE [163–166], and recent studies indicate that NET formation and degradation are equally affected patients with PAPS. It has been found that incubation of neutrophils from healthy controls with aPL stimulates NET formation and also, that neutrophils from PAPS patients are more prone to undergo NETosis than neutrophils from healthy controls [167]. Additional experiments showed that serum from both SLE and primary and secondary APS patients have reduced capacity to degrade NETs,

which implies that these patients are strongly exposed to NETs [168]. As NETs promote platelet activation and thrombosis [169], and are present in placentas from patients with pre-eclampsia [170] strong exposure to NETs is likely relevant for the pathophysiology of APS. When this hypothesis holds true, it may lead to clinical studies in APS patients on clinical effects of inhibition of NETosis by targeting the inhibitory receptor SIRL-1, as was recently proposed for SLE [166].

4.3 Natural Killer cells in APS

Natural killer (NK) cells are well known as effector cells of the innate immune system with cytotoxic responses on virus infected cells and tumor formation. Apart from this, NK cells have a wide variety of other organ specific features [171]. This also holds true for the pregnant uterus, where NK cells are abundant in the decidua (the endometrium of the pregnant uterus that forms the maternal part of the placenta). There, uterine NK cells are thought to control endometrial tissue remodeling, vascular function and the formation of a placenta in the uterus [172]. There are strong indications that an excess of NK cells in the uterus hinders fetal implantation and may underlie recurrent miscarriage in a significant number of patients. In healthy women with recurrent miscarriage the number of circulating NK cells has been found to be increased [173–175].

In women with APS and recurrent miscarriage, the number of circulating NK cells is even higher than in women with recurrent miscarriage but no aPL [175]. The number of circulating NK cells in women with APS also was higher in the subset with recurrent miscarriage compared to those with normal pregnancy outcome [174]. Although this strongly suggest a role for NK cells in aPL-related pregnancy morbidity and that high NK-cell levels in aPL-positive women may indicate a high risk for pregnancy morbidity (as was found in one study [176]), firm conclusions cannot be made yet. There are also studies that report decreased instead of increased numbers for circulating NK cells in PAPS patients with recurrent pregnancy loss [105] or thrombotic PAPS patients [104,105] and in a histopathological study the number of endometrial NK cells in women with recurrent miscarriage was similar in those with and without aPL [177]. However, the activity of these NK-cells has not been studied.

4.4 Complement in APS

The complement system is an integral part of the innate immune system. It consists of a large number of plasma proteins that upon activation react with one another in

a cascade-like fashion. The final results are opsonisation of pathogens and recruitment of immune cells to the site of infection. Besides, the complement system has a major role in opsonisation and subsequent non-inflammatory clearance op apoptotic cells and cellular debris. The complement system can be activated via three separate pathways: the classical, alternative and lectin routes, respectively. These routes differ in their mode of recognition, but converge in the generation of C3 and C5 convertases. As uncontrolled, activation of the complement system is harmful to self, there are many regulatory mechanisms including both membrane-bound (like CR1 and CD59) and soluble regulators (like factor H and factor I) [178].

The structural similarity of β2GPI to factors H and I, with comparable complement control proteins (CCP) repeat domains, formed the basis for research on complement regulatory properties of the β2GPI protein. Gropp et al. [179] showed that the elongated, open structure of β2GPI that occurs when the molecule binds to negatively charged surfaces, as for instance those present on apoptotic cells, acquires the capacity to bind complement components C3 and C3b. After binding of C3 or C3b to membrane bound β2GPI these molecules undergo a conformational change which enables them to bind factor H, which is followed by their degradation by factor I. This complement regulatory function of β2GPI was identified in domain I of the molecule [179]. As this domain also harbors the binding site of most pathogenic anti- β2GPI antibodies [9], one can speculate that these antibodies interfere with the suggested complement regulatory function of β2GPI and that this results in enhanced activation and consumption of complement in APS. In line with this, reduced concentrations of C3 and C4 as well as the CH50 activity have been described in patients with PAPS comparison to healthy controls and patients with non-SLE connective tissue diseases such as systemic sclerosis or Sjögrens syndrome [180]. In contrast, another study reported similar complement levels C3 and C4 between healthy controls and PAPS patients [76]. The simultaneous increased concentrations of their respective breakdown products (C3a and C4a) is compatible with the suggestion that lower complement levels are caused by complement activation [180]. Other studies also described higher plasma concentrations of C3a in patients with LAC [181] and primary APS patients [182]. In the latter study the raised C3a levels were accompanied by increased levels of fragment Bb, another marker for complement activation [182]. Whether these findings are clinically important is not clear as C3a levels do not discriminate between thrombotic and non-thrombotic APS patients in these studies [180–182]. In pregnant women with PAPS it was reported that mean levels of C3 and C4 are lower in all three trimesters compared to levels during pregnancies in healthy women, but that low levels of C3 or C4 levels are not predictive for pregnancy complications [183]. In contrast, another study in

PAPS patients did find an association between low levels of C3 and C4 in serum taken before 20 weeks of gestation and pregnancy outcome [184].

In conclusion, it is conceivable that aPL can induce activation of the complement system, but its relation to clinical manifestations of APS in humans is far from evident. There is no evident deposition of C3 or C3 split products in placental tissue of women with aPL, as concluded in a recent systematic review [23]. This is in sharp contrast with data from experiments in animals where complement activation is an important factor for both aPL-induced fetal resorption, the surrogate equivalent for obstetric APS [21,185] and aPL-induced thrombosis [186].

The generally accepted beneficial effects of (low molecular weight) heparin in human obstetric APS may relate to its complement inhibiting activity [187]. Some suggestions have been made that inhibition of complement activation by the use of an inhibitor of C5 cleavage (eculizumab) may benefit patients with catastrophic APS, but has only been applied sporadically [188].

5.1 Discussion and conclusion

Although the antiphospholipid syndrome manifests as thrombosis and pregnancy morbidity, the presence of anti-β2GPI antibodies underline the underlying autoimmune mechanism. In line with other autoimmune diseases, there are strong indications that genetic and environmental factors form the basis of APS. Research on genetics in APS patients identified polymorphisms that relate to antigen presentation (e.g. MHC class II) and immune cell activation (e.g. IRF5, STAT4) that are shared with other autoimmune diseases, but also identified SNPs in the gene for β2GPI that relate to production of anti-β2GPI antibodies. Of interest, these SNPs in β2GPI result in substitution of a single amino acid in the fifth domain of the β2GPI molecule at sites that harbors not only the phospholipid binding site of the protein, but also the epitope for β2GPI autoreactive T-cells found in APS patients [94]. Further studies that shed light on the interesting link between subtle changes in the fifth domain of β2GPI and autoimmunity should be encouraged.

The fact that APS shares the same genetic risk loci with other autoimmune diseases indicates that additional factors are involved in the induction of APS. With respect to environmental factors that contribute to aPL production, several studies in animals showed that a variety of microbe-derived peptides can, via cross-reactivity with β2GPI, induce the production of aPL. In recent years the role of epigenetics in the pathophysiology of autoimmune diseases is emerging [189,190] but little is

known on epigenetic alterations in APS. The expression of a gene and its translation into protein are regulated by epigenetic factors, such as DNA methylation, histone modifications and miRNAs. Dysregulation of miRNAs in monocyte and neutrophils in relation to their activation state in APS has been reported [150,157]. In coming years, studies on epigenetic alterations in relation to APS will further clarify its contribution to APS.

Characteristics of autoreactive T-cell clones found in APS indicate that the disorder is an antigen-driven autoimmune disease, with β2GPI being the central antigen. In line with this are results of experiments that showed that DCs can play a key role in the immune response to β2GPI. Recent observations have shown that aPL induce the upregulation of TLR7 in pDCs [78], resulting in a hyperactive state of these type I IFN producing cells in APS. Although this points towards an important role for pDCs and IFNα in APS, data on the presence of a type I IFN signature in APS are conflicting and warrant further clarification in coming years. It is of note that, although DCs have been implicated in the pathogenesis of APS, no studies have reported on the frequency, phenotype and function of mDCs and pDCs isolated from APS patients.

There are various publications on the distribution and phenotype of T cell subsets in patients with APS. Unfortunately, it is difficult to draw conclusions, as data from different studies often are conflicting. This might be due to small sample sizes or reflect clinical heterogeneity. Recent findings show skewing of T-helper cell populations towards Th17 subsets in APS, with a decrease in the number of circulating Tregs. Whether Tregs in APS are also functionally impaired is currently unknown, while Th17 cells have not been directly studied in APS. As for the other important cell of adaptive immunity, it is remarkable that few in depth studies on B-cells in APS have been performed and no distinct pattern of B-cell abnormalities has been described.

In patients with APS both monocytes and granulocytes are in an activated state, enabling these cells to contribute to inflammation and to activate coagulation by the expression of TF or release of NETs. This cellular activation can be induced by aPL. Many studies investigated which receptors are involved in monocyte activation by aPL and a role has been suggested for TLR1, TLR2, TLR4 and its co-receptor molecule CD14, TLR6 and TLR8. It is highly unlikely that all these receptors will be equally important, but it cannot be deducted to what extent the results are influenced by methodological differences between individual studies or reflect relevant clinical heterogeneity (reviewed in [191–193]). Additional research on the receptors

and pathways involved in aPL-induced immune cell activation is certainly needed. The finding that neutrophils from patients with APS, like those from patients with SLE, are prone to undergo NETosis and that APS patients show impaired clearance of NETs, is recent expression of overlap between these closely related disorders.

Of interest is the recent discovery of a complement regulatory function of β2GPI. This led to the hypothesis that anti-β2GPI antibodies may interfere with complement homeostasis leading to increased activation of the complement system. Several studies indeed reported increased levels of complement activation products in APS. That such complement activation is important for clinical APS manifestations to develop is not convincingly supported.

An important shortcoming in most studies on the genetic and immunological aspects of APS is that little attention is given to the presence or absence of an underlying disease (i.e. SLE) in the studied APS patients, to their clinical APS phenotype and to specification of the aPL profile. This certainly hampers proper interpretation of results. Many data suggest for instance that there is a lot of concordance in genetic and cellular abnormalities between patients with APS and SLE. However, studies that compare findings in patients with PAPS, SLE with APS and SLE patients without APS are infrequent. Such studies should however be encouraged in the future as this is the only way to make firm conclusions on the role of the immune system in APS.

By not taking into account the clinical phenotype of aPL positive patients (asymptomatic, thrombotic, obstetric) it is impossible to detect that certain immunologic abnormalities specifically relate to clinical subtypes. Over the past few years it has become increasingly clear that not all aPL are equally relevant from a clinical point of view. Presence of LAC and simultaneous presence of LAC, aCL and anti-β2GPI are the aPL-profiles with the highest risk for thrombosis and obstetric complications. Often, immune cells obtained from healthy donors are often incubated with IgG fractions from APS patients or purified aPL, instead of using cells directly isolated from APS patients.

There are indications that at the molecular level, obstetric and thrombotic APS patients are different [126,130], additional studies in this field certainly are relevant. We feel that the time has come to perform in aPL-positive patients extensive immune cell profiling [194] that includes extensive phenotyping of immune cells by mass or flow cytometry as well as the analysis of gene-expression of separate cell subsets. Such a systems medicine approach on well characterized patients (primary APS, secondary APS, asymptomatic aPL carriers, SLE patients and healthy age-,

sex- matched controls) may identify separate clusters of APS patients based on their molecular taxonomy. The ultimate goal of such studies would be a patient tailored treatment, driven by the integrated picture of the clinical phenotype and molecular taxonomy, termed precision medicine [195].

Take-home messages

- The antiphospholipid syndrome (APS) has an antigen driven origin in which genetic and environmental factors shared with other autoimmune diseases form the basis.
- Antiphospholipid antibodies have the potential to activate monocytes, neutrophils and plasmacytoid dendritic cells. Disturbances in T-cell homeostasis reported in APS patients further contribute to the immunopathology of APS.
- An integrated immune cell profiling approach to delineate the immune response in antiphospholipid syndrome is advocated in order to reveal new therapeutic targets, prognostic biomarkers and improve stratification of risks in antiphospholipid syndrome patients.

References

1. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295–306. doi:10.1111/j.1538-7836.2006.01753.x.

2. Abreu MM, Danowski A, Wahl DG, Amigo M-C, Tektonidou M, Pacheco MS, et al. The relevance of "non-criteria" clinical manifestations of antiphospholipid syndrome: 14th International Congress on Antiphospholipid Antibodies Technical Task Force Report on Antiphospholipid Syndrome Clinical Features. Autoimmun Rev 2015;14:401–14. doi:10.1016/j.autrev.2015.01.002.

3. Denas G, Jose SP, Bracco A, Zoppellaro G, Pengo V. Antiphospholipid syndrome and the heart: A case series and literature review. Autoimmun Rev 2015;14:214–22. doi:10.1016/j.autrev.2014.11.003.

4. Bertolaccini ML, Amengual O, Andreoli L, Atsumi T, Chighizola CB, Forastiero R, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. Autoimmun Rev 2014;13:917–30. doi:10.1016/j.autrev.2014.05.001.

5. Berman H, Rodríguez-Pintó I, Cervera R, Gregory S, de Meis E, Rodrigues CEM, et al. Pediatric catastrophic antiphospholipid syndrome: Descriptive analysis of 45 patients from the "CAPS registry." Autoimmun Rev 2014;13:157–62. doi:10.1016/j.autrev.2013.10.004.

6. Cervera R, Rodríguez-Pintó I, Colafrancesco S, Conti F, Valesini G, Rosário C, et al. 14Th International Congress on Antiphospholipid Antibodies Task Force Report on Catastrophic Antiphospholipid Syndrome. Autoimmun Rev 2014;13:699–707. doi:10.1016/j.autrev.2014.03.002.

7. De Laat B, Derksen RHWM, De Groot PG. B2-Glycoprotein I, the playmaker of the antiphospholipid syndrome. Clin Immunol 2004;112:161–8. doi:10.1016/j.clim.2004.02.012.

8. Ağar Ç, Van Os GM, Mörgelin M, Sprenger RR, Marquart JA, Urbanus RT, et al. β2-Glycoprotein I can exist in 2 conformations: Implications for our understanding of the antiphospholipid syndrome. Blood 2010;116:1336–43. doi:10.1182/blood-2009-12-260976.

9. De Laat B, Derksen RHWM, van Lummel M, Pennings MTT, Groot PG De. Pathogenic anti – beta 2 -glycoprotein I antibodies recognize domain I of beta 2 -glycoprotein I only after a conformational change. Hemostasis, Thromb Vasc Biol 2006;107:1916–24.

10. De Laat BH, Derksen RHWM, Urbanus RT, Roest M, De Groot PG. B2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood 2004;104:3598–602. doi:10.1182/blood-2004-03-1107.

11. Ruffatti A, Tonello M, Del Ross T, Cavazzana A, Grava C, Noventa F, et al. Antibody profile and clinical course in primary antiphospholipid syndrome with pregnancy morbidity. Thromb Haemost 2006;96:337–41. doi:10.1160/TH06-05-0287.

12. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. Blood 2003;101:1827–32. doi:10.1182/blood-2002-02-0441.

13. Opatrny L, David M, Kahn SR, Shrier I, Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. J Rheumatol 2006;33:2214–21.

14. Lockshin MD, Kim M, Laskin CA, Guerra M, Branch DW, Merrill J, et al. Prediction of adverse pregnancy outcome by the presence of lupus anticoagulant, but not anticardiolipin antibody, in patients with antiphospholipid antibodies. Arthritis Rheum 2012;64:2311–8. doi:10.1002/art.34402.

15. Pengo V, Ruffatti A, Legnani C, Gresele P, Barcellona D, Erba N, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. J Thromb Haemost 2010;8:237–42. doi:10.1111/j.1538-7836.2009.03674.x.

16. Pengo V, Ruffatti A, Legnani C, Testa S, Fierro T, Marongiu F, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. Blood 2011;118:4714–8. doi:10.1182/blood-2011-03-340232.

17. Alijotas-Reig J, Ferrer-Oliveras R, Ruffatti A, Tincani A, Lefkou E, Bertero MT, et al. The European Registry on Obstetric Antiphospholipid Syndrome (EUROAPS): A survey of 247 consecutive cases. Autoimmun Rev 2014;14:387–95. doi:10.1016/j.autrev.2014.12.010.

18. Meroni PL, Borghi MO, Raschi E, Tedesco F. Pathogenesis of antiphospholipid syndrome: understanding the antibodies. Nat Rev Rheumatol 2011;7:330–9. doi:10.1038/nrrheum.2011.52.

19. Arad A, Proulle V, Furie RA, Furie BC, Furie B. β2-Glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. Blood 2011;117:3453–9. doi:10.1182/blood-2010-08-300715.

20. Pericleous C, Ruiz-Limon P, Romay-Penabad Z, Carrera Marin A, Garza-Garcia A, Murfitt L, et al. Proof-of-concept study demonstrating the pathogenicity of affinity-purified IgG antibodies directed to domain I of 2-glycoprotein I in a mouse model of anti-phospholipid antibody-induced thrombosis. Rheumatology 2014;54:722–7. doi:10.1093/rheumatology/keu360.

21. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. J Clin Invest 2003;112:1644–54. doi:10.1172/JCI200318817.

22. Willis R, Gonzalez EB, Brasier a. R. The Journey of Antiphospholipid Antibodies From Cellular Activation to Antiphospholipid Syndrome. Curr Rheumatol Rep 2015;17:1–11. doi:10.1007/s11926-014-0485-9.

23. Viall CA, Chamley LW. Histopathology in the placentae of women with antiphospholipid antibodies: A systematic review of the literature. Autoimmun Rev 2015;14:1–20. doi:10.1016/j.autrev.2015.01.008.

24. Tong M, Viall CA, Chamley LW. Antiphospholipid antibodies and the placenta: a systematic review of their in vitro effects and modulation by treatment. Hum Reprod Update 2014;21:97–118. doi:10.1093/humupd/dmu049.

25. Exner T, Barber S, Kronenberg H, Rickard KA. Familial association of the lupus anticoagulant. Br J Haematol 1980;45:89–96.

26. Matthey F, Walshe K, Mackie IJ, Machin SJ. Familial occurrence of the antiphospholipid syndrome. J Clin Pathol 1989;42:495–7.

27. Ford PM, Brunet D, Lillicrap DP, Ford SE. Premature stroke in a family with lupus anticoagulant and antiphospholipid antibodies. Stroke 1990;21:66–71.

28. Soriano A, Blank M, Shoenfeld Y. Genetics and Origin of Antiphospholipid Syndrome. In: Meroni PL, editor. Antiphospholipid Antibody Syndrome From Bench to Bedside. 1st ed., Springer; 2015, p. 1–12.

29. Tanimura K, Jin H, Suenaga T, Morikami S, Arase N, Kishida K, et al. b 2-Glycoprotein I / HLA class II complexes are novel autoantigens in antiphospholipid syndrome 2015;125:2835–44. doi:10.1182/blood-2014-08-593624.K.

30. Domenico Sebastiani G, Minisola G, Galeazzi M. HLA class II alleles and genetic predisposition to the antiphospholipid syndrome. Autoimmun Rev 2003;2:387–94.

31. Yin H, Borghi MO, Delgado-Vega AM, Tincani A, Meroni PL, Alarcón-Riquelme ME. Association of STAT4 and BLK, but not BANK1 or IRF5, with primary antiphospholipid syndrome. Arthritis Rheum 2009;60:2468–71. doi:10.1002/art.24701.

32. Fredi M, Tincani A, Yin H, Delgado-Vega A, Borghi MO. IRF5 is associated with primary antiphospholipid syndrome, but is not a major risk factor. Arthritis Rheum 2010;62:1201. doi:10.1002/art.27341.

33. Horita T, Atsumi T, Yoshida N, Nakagawa H, Kataoka H, Yasuda S, et al. STAT4 single nucleotide polymorphism, rs7574865 G/T, as a risk for antiphospholipid syndrome. Ann Rheum Dis 2009;68:1366–7. doi:10.1136/ard.2008.094367.

34. Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman a C, Sturfelt G, et al. Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. Am J Hum Genet 2005;76:528–37. doi:S0002-9297(07)63349-7 [pii]\r10.1086/428480.

35. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 2007;357:977–86. doi:10.1056/NEJMoa073003.

36. Hom G, Ph D, Graham RR, Modrek B, Taylor KE, Ortmann W, et al. Association of Systemic Lupus Erythematosus with C8orf13–BLK and ITGAM–ITGAX. N Engl J Med 2008;358:900–9.

37. Chamorro A-J, Marcos M, Mirón-Canelo J-A, Cervera R, Espinosa G. Val247Leu beta2-glycoprotein-I allelic variant is associated with antiphospholipid syndrome: Systematic review and meta-analysis. Autoimmun Rev 2012;11:705–12. doi:10.1016/j.autrev.2011.12.006.

38. Kamboh MI, Wang X, Kao AH, Barmada MM, Clarke A, Ramsey-goldman R, et al. Genome-Wide Association Study of Antiphospholipid Antibodies. Autoimmune Dis 2013;2013.

39. Pierangeli SS, Vega-Ostertag ME, Raschi E, Liu X, Romay-Penabad Z, De Micheli V, et al. Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies. Ann Rheum Dis 2007;66:1327–33. doi:10.1136/ard.2006.065037.

40. Atsumi T, Caliz R, Amengual O, Khamashta MA, Hughes GR. Fcgamma receptor IIA H/R131 polymorphism in patients with antiphospholipid antibodies. Thromb Haemost 1998;79:924–7.

41. Karassa FB, Bijl M, Davies K a., Kallenberg CGM, Khamashta MA, Manger K, et al. Role of the Fcγ receptor IIA polymorphism in the antiphospholipid syndrome: An international meta-analysis. Arthritis Rheum 2003;48:1930–8. doi:10.1002/art.11059.

42. Kato M, Atsumi T, Oku K, Amengual O, Nakagawa H, Fujieda Y, et al. The involvement of CD36 in monocyte activation by antiphospholipid antibodies. Lupus 2013;22:761–71. doi:10.1177/0961203313490242.

43. Bertolaccini ML, Atsumi T, Lanchbury JS, Caliz AR, Katsumata K, Vaughan RW, et al. Plasma tumor necrosis factor alpha levels and the -238*A promoter polymorphism in patients with antiphospholipid syndrome. Thromb Haemost 2001;85:198–203.

44. Hiddink L, de Laat B, Derksen RHWM, de Groot PG, van Heerde WL. Annexin A5 haplotypes in the antiphospholipid syndrome. Thromb Res 2015;135:417–9. doi:10.1016/j.thromres.2014.12.004.

45. De Laat B, Derksen RHWM, Mackie IJ, Roest M, Schoormans S, Woodhams BJ, et al. Annexin A5 polymorphism (-1C-->T) and the presence of anti-annexin A5 antibodies in the antiphospholipid syndrome. Ann Rheum Dis 2006;65:1468–72. doi:10.1136/ard.2005.045237.

46. Yonal I, Hindilerden F, Hancer VS, Artim-Esen B, Daglar A, Akadam B, et al. The impact of platelet membrane glycoprotein Ib alpha and Ia/IIa polymorphisms on the risk of thrombosis in the antiphospholipid syndrome. Thromb Res 2012;129:486–91. doi:10.1016/j.thromres.2011.10.005.

47. Jiménez S, Tàssies D, Espinosa G, García-Criado a, Plaza J, Monteagudo J, et al. Double heterozygosity polymorphisms for platelet glycoproteins Ia/IIa and IIb/IIIa increases arterial thrombosis and arteriosclerosis in patients with the antiphospholipid syndrome or with systemic lupus erythematosus. Ann Rheum Dis 2008;67:835–40. doi:10.1136/ard.2007.077321.

48. Diz-Kucukkaya R, Inanc M, Afshar-Kharghan V, Zhang QE, López JA, Pekcelen Y. P-selectin glycoprotein ligand-1 VNTR polymorphisms and risk of thrombosis in the antiphospholipid syndrome. Ann Rheum Dis 2007;66:1378–80. doi:10.1136/ard.2007.075945.

49. Castro-Marrero J, Balada E, Vilardell-Tarrés M, Ordi-Ros J. Genetic risk factors of thrombosis in the antiphospholipid syndrome. Br J Haematol 2009;147:289–96. doi:10.1111/j.1365-2141.2009.07831.x.

50. Gustafsson JT, Gunnarsson I, Källberg H, Pettersson S, Zickert A, Vikerfors A, et al. Cigarette smoking, antiphospholipid antibodies and vascular events in Systemic Lupus Erythematosus. Ann Rheum Dis 2014:1537–43. doi:10.1136/annrheumdis-2013-205159.

51. Gharavi AE, Pierangeli SS, Espinola RG, Liu X, Colden-Stanfield M, Harris EN. Antiphospholipid antibodies induced in mice by immunization with a cytomegalovirus-derived peptide cause thrombosis and activation of endothelial cells in vivo. Arthritis Rheum 2002;46:545–52. doi:10.1002/art.10130.

52. Blank M, Krause I, Fridkin M, Keller N, Kopolovic J, Goldberg I, et al. Bacterial induction of autoantibodies to B2-glycoprotein-I accounts for the infectious etiology of antiphospholipid syndrome. J Clin Invest 2002;109:797–804. doi:10.1172/JCI200212337.

53. Van Os GMA, Meijers JCM, Agar Ç, Seron M V., Marquart JA, Åkesson P, et al. Induction of anti-β 2-glycoprotein I autoantibodies in mice by protein H of Streptococcus pyogenes. J Thromb Haemost 2011;9:2447–56. doi:10.1111/j.1538-7836.2011.04532.x.

54. Dzionek a, Fuchs a, Schmidt P, Cremer S, Zysk M, Miltenyi S, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165:6037–46. doi:10.4049/jimmunol.165.11.6037.

55. Kool M, van Loo G, Waelput W, De Prijck S, Muskens F, Sze M, et al. The ubiquitin-editing protein A20 prevents dendritic cell activation, recognition of apoptotic cells, and systemic autoimmunity. Immunity 2011;35:82–96. doi:10.1016/j.immuni.2011.05.013.

56. Bondanza A, Rovere-Querini P, Zimmermann VS, Balestrieri G, Tincani A, Sabbadini MG, et al. Requirement for dendritic cells in the establishment of anti-phospholipid antibodies. Autoimmunity 2007;40:302–6. doi:10.1080/08916930701356572.

57. Bondanza A, Zimmermann VS, Dell'Antonio G, Dal Cin E, Balestrieri G, Tincani A, et al. Requirement of Dying Cells and Environmental Adjuvants for the Induction of Autoimmunity. Arthritis Rheum 2004;50:1549–60. doi:10.1002/art.20187.

58. Kuwana M, Matsuura E, Kobayashi K, Okazaki Y, Kaburaki J, Ikeda Y, et al. Binding of beta 2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells. Blood 2005;105:1552–7. doi:10.1182/blood-2004-08-3145.

59. Yamaguchi Y, Seta N, Kaburaki J, Kobayashi K, Matsuura E, Kuwana M. Excessive exposure to anionic surfaces maintains autoantibody response to beta(2)-glycoprotein I in patients with antiphospholipid syndrome. Blood 2007;110:4312–8. doi:10.1182/blood-2007-07-100008.

60. Rovere P, Sabbadini MG, Vallinoto C, Fascio U, Rescigno M, Crosti M, et al. Dendritic Cell presentation of antigens from apoptotic cells in a proinflammatory context Role of opsonizing anti B2 -Glycoprotein I Antibodies. Arthritis Rheum 1999;42:1412–20.

61. Rovere P, Manfredi AA, Vallinoto C, Zimmermann VS, Fascio U, Balestrieri G, et al. Dendritic Cells Preferentially Internalize Apoptotic Cells Opsonized by Anti- 2-glycoprotein I Antibodies. J Autoimmun 1998;11:403–11.

62. Arbuckle M, McClain M, Rubertone M, Scofield R, Dennis G, James J, et al. Development of Autoantibodies before the Clinical Onset of Systemic Lupus Erythematosus. N Engl J Med 2003;349:1526–33. doi:10.1056/NEJM200401153500320.

63. Levine JS, Subang R, Nasr SH, Fournier S, Lajoie G, Wither J, et al. Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. J Immunol 2006;177:6504–16. doi:10.4049/jimmunol.177.9.6504.

64. Ara FD, Reggia R, Taraborelli M, Andreoli L, Taglietti M, Frassi M, et al. Patients with longstanding primary antiphospholipid syndrome : retrospective analysis of organ damage and mortality. Lupus 2014;23:1255–8.

65. Petri M. Update on anti-phospholipid antibodies in SLE: the Hopkins' Lupus Cohort. Lupus 2010;19:419–23. doi:10.1177/0961203309360541.

66. Ioannou Y, Zhang J-Y, Qi M, Gao L, Qi JC, Yu D-M, et al. Novel assays of thrombogenic pathogenicity in the antiphospholipid syndrome based on the detection of molecular oxidative modification of the major autoantigen β2-glycoprotein I. Arthritis Rheum 2011;63:2774–82. doi:10.1002/art.30383.

67. Buttari B, Profumo E, Mattei V, Siracusano A, Ortona E, Margutti P, et al. Oxidized beta2-glycoprotein I induces human dendritic cell maturation and promotes a T helper type 1 response. Blood 2005;106:3880–7. doi:10.1182/blood-2005-03-1201.

68. Torres-Aguilar H, Blank M, Kivity S, Misgav M, Luboshitz J, Pierangeli SS, et al. Tolerogenic dendritic cells inhibit antiphospholipid syndrome derived effector/memory $CD4^+$ T cell response to β2GPI. Ann Rheum Dis 2012;71:120–8. doi:10.1136/annrheumdis-2011-200063.

69. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. Nat Rev Immunol 2015;15:471–85. doi:10.1038/nri3865.

70. Gilliet M, Cao W, Liu Y-J. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 2008;8:594–606. doi:10.1038/nri2358.

71. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, et al. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med 2003;197:711–23. doi:10.1084/jem.20021553.

72. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A 2003;100:2610–5. doi:10.1073/pnas.0337679100.

73. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, et al. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. Ann Rheum Dis 2011;70:2029–36. doi:10.1136/ard.2011.150326.

74. Eloranta M-L, Alm G V, Rönnblom L. Disease mechanisms in rheumatology--tools and pathways: plasmacytoid dendritic cells and their role in autoimmune rheumatic diseases. Arthritis Rheum 2013;65:853–63. doi:10.1002/art.37821.

75. Bernales I, Fullaondo a, Marín-Vidalled MJ, Ucar E, Martínez-Taboada V, López-Hoyos M, et al. Innate immune response gene expression profiles characterize primary antiphospholipid syndrome. Genes Immun 2008;9:38–46. doi:10.1038/sj.gene.6364443.

76. Perez-Sanchez C, Barbarroja N, Messineo S, Ruiz-Limon P, Rodriguez-Ariza A, Jimenez-Gomez Y, et al. Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in antiphospholipid syndrome, systemic lupus erythematosus and antiphospholipid syndrome with lupus. Ann Rheum Dis 2014;74:1441–9. doi:10.1136/annrheumdis-2013-204600.

77. Crow MK. Type I interferon in the pathogenesis of lupus. J Immunol 2014;192:5459–68. doi:10.4049/jimmunol.1002795.

78. Prinz N, Clemens N, Strand D, Pütz I, Lorenz M, Daiber A, et al. Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. Blood 2011;118:2322–32. doi:10.1182/blood-2011-01-330639.

79. Hurst J, Prinz N, Lorenz M, Bauer S, Chapman J, Lackner KJ, et al. TLR7 and TLR8 ligands and antiphospholipid antibodies show synergistic effects on the induction of IL-1beta and caspase-1 in monocytes and dendritic cells. Immunobiology 2009;214:683–91. doi:10.1016/j.imbio.2008.12.003.

80. Aguilar-Valenzuela R., Nickerson K., Romay-Penabad Z., Shlomchik M.J., Vargas G., Shilagard T. PSS. Involvement of TLR7 and TLR9 in the production of antiphospholipid antibodies. Arthritis Rheum 2011;63:SUPPL 1.

81. Becker JC, Winkler B, Klingert S, Bröcker EB. Antiphospholipid syndrome associated with immunotherapy for patients with melanoma. Cancer 1994;73:1621–4.

82. Stavropoulou-giokas C, Frangia K, Markopoulos C, Bafaloukos D, Pectasides D, Fountzilas G, et al. Prognostic Significance of Autoimmunity during Treatment of Melanoma with Interferon. N Engl J Med 2006;354:709–18.

83. Kuznik A, Bencina M, Svajger U, Jeras M, Rozman B, Jerala R. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. J Immunol 2011;186:4794–804. doi:10.4049/jimmunol.1000702.

84. Wallace DJ, Gudsoorkar VS, Weisman MH, Venuturupalli SR. New insights into mechanisms of therapeutic effects of antimalarial agents in SLE. Nat Rev Rheumatol 2012;8:522–33. doi:10.1038/nrrheum.2012.106.

85. Sacre K, Criswell L a, McCune JM. Hydroxychloroquine is associated with impaired interferon-alpha and tumor necrosis factor-alpha production by plasmacytoid dendritic cells in systemic lupus erythematosus. Arthritis Res Ther 2012;14:R155. doi:10.1186/ar3895.

86. Mekinian A, Lazzaroni MG, Kuzenko A, Alijotas-Reig J, Ruffatti A, Levy P, et al. The efficacy of hydroxychloroquine for obstetrical outcome in anti-phospholipid syndrome: Data from a European multicenter retrospective study. Autoimmun Rev 2015:6–10. doi:10.1016/j.autrev.2015.01.012.

87. De Carolis S, Botta a., Salvi S, di Pasquo E, Del Sordo G, Garufi C, et al. Is there any role for the hydroxychloroquine (HCQ) in refractory obstetrical antiphospholipid syndrome (APS) treatment? Autoimmun Rev 2015;14:760–2. doi:10.1016/j.autrev.2015.04.010.

88. Schmidt-Tanguy a, Voswinkel J, Henrion D, Subra J, Loufrani L, Rohmer V, et al. Anti-thrombotic effects of hydroxychloroquine in primary antiphospholipid syndrome patients. J Thromb Haemost 2013;11:1927–9. doi:10.1111/jth.12363.

89. Belizna C. Hydroxychloroquine as an anti-thrombotic in antiphospholipid syndrome. Autoimmun Rev 2015;14:358–62. doi:10.1016/j.autrev.2014.12.006.

90. Comarmond C, Cacoub P. Antiphospholipid syndrome: From pathogenesis to novel immunomodulatory therapies. Autoimmun Rev 2013;12:752–7. doi:10.1016/j.autrev.2012.12.006.

91. Kuwana M. Autoreactive CD4 T cells to b 2 -glycoprotein I in patients with antiphospholipid syndrome. Autoimmun Rev 2003;2:192–8. doi:10.1016/S1568-9972.

92. Kuwana M. Beta2-glycoprotein I: antiphospholipid syndrome and T-cell reactivity. Thromb Res 2004;114:347–55. doi:10.1016/j.thromres.2004.06.029.

93. Hattori N, Kuwana M, Kaburaki J, Mimori T, Ikeda Y, Kawakami Y. T cells that are autoreactive to beta2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. Arthritis Rheum 2000;43:65–75. doi:10.1002/1529-0131(200001)43:1<65::AID-ANR9>3.0.CO;2-I.

94. Arai T, Yoshida K, Kaburaki J, Inoko H, Ikeda Y, Kawakami Y, et al. Autoreactive CD4+ T-cell clones to beta2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site. Blood 2001;98:1889–96. doi:10.1182/blood.V98.6.1889.

95. Yoshida K, Arai T, Kaburaki J, Ikeda Y, Kawakami Y, Kuwana M. Restricted T-cell receptor beta -chain usage by T cells autoreactive to beta 2-glycoprotein I in patients with antiphospholipid syndrome. Blood 2002;99:2499–504. doi:10.1182/blood.V99.7.2499.

96. Visvanathan S, McNeil HP. Cellular immunity to beta 2-glycoprotein-1 in patients with the antiphospholipid syndrome. J Immunol 1999;162:6919–25.

97. Davies ML, Young SP, Welsh K, Bunce M, Wordsworth BP, Davies K a, et al. Immune responses to native beta(2)-glycoprotein I in patients with systemic lupus erythematosus and the antiphospholipid syndrome. Rheumatology (Oxford) 2002;41:395–400.

98. Conti F, Spinelli FR, Alessandri C, Pacelli M, Ceccarelli F, Marocchi E, et al. Subclinical Atherosclerosis in Systemic Lupus Erythematosus and Antiphospholipid Syndrome: Focus on β2GPI-Specific T Cell Response. Arterioscler Thromb Vasc Biol 2014;34:661–8. doi:10.1161/ATVBAHA.113.302680.

99. Grant CR, Liberal R, Mieli-Vergani G, Vergani D, Longhi MS. Regulatory T-cells in autoimmune diseases: Challenges, controversies and—yet—unanswered questions. Autoimmun Rev 2015;14:105–16. doi:10.1016/j.autrev.2014.10.012.

100. Singh RP, Hasan S, Sharma S, Nagra S, Yamaguchi DT, Wong DT., et al. Th17 cells in inflammation and autoimmunity. Autoimmun Rev 2014;13:1174–81. doi:10.1016/j.autrev.2014.08.019.

101. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmun Rev 2014;13:668–77. doi:10.1016/j.autrev.2013.12.004.

102. Karakantza M, Theodorou GL, Meimaris N, Mouzaki A, John E, Andonopoulos AP, et al. Type 1 and type 2 cytokine-producing CD4+ and CD8+ T cells in primary antiphospholipid syndrome. Ann Hematol 2004;83:704–11. doi:10.1007/s00277-004-0910-7.

103. Papo T, Piette JC, Legac E, Frances C, Grenot P, Debre P, et al. T lymphocyte subsets in primary antiphospholipid syndrome. J Rheumatol 1994;21:2242–5.

104. Ames P, Tommasino C, Fossati G, Matsuura E, Margarita a, Saulino a, et al. Lymphocyte subpopulations and intima media thickness in primary antiphospholipd syndrome. Lupus 2005;14:809–13. doi:10.1191/0961203305lu2181oa.

105. Carbone J, Gallego A, Lanio N, Navarro J, Orera M, Aguaron A, et al. Quantitative abnormalities of peripheral blood distinct T, B, and natural killer cell subsets and clinical findings in obstetric antiphospholipid syndrome. J Rheumatol 2009;36:1217–25. doi:10.3899/jrheum.081079.

106. Ito H, Matsushita S, Tokano Y, Nishimura H, Tanaka Y, Fujisao S, et al. Analysis of T cell responses to the beta 2-glycoprotein I-derived peptide library in patients with anti-beta 2-glycoprotein I antibody-associated autoimmunity. Hum Immunol 2000;61:366–77.

107. Forestiero R. Imbalance towards Th2-Type Response in Patients with Definite Primary Antiphospholipid Syndrome. Thromb Haemost 2001;453:934–5.

108. Soltesz P, Der H, Veres K, Laczik R, Sipka S, Szegedi G, et al. Immunological features of primary anti-phospholipid syndrome in connection with endothelial dysfunction. Rheumatology (Oxford) 2008;47:1628–34. doi:10.1093/rheumatology/ken349.

109. Popovic-Kuzmanovic D, Novakovic I, Stojanovich L, Aksentijevich I, Zogovic N, Tovilovic G, et al. Increased activity of interleukin-23/interleukin-17 cytokine axis in primary antiphospholipid syndrome. Immunobiology 2013;218:186–91. doi:10.1016/j.imbio.2012.03.002.

110. Xiao J, Zhu F, Liu X, Xiong J. Th1/Th2/Th17/Treg expression in cultured PBMCs with antiphospholipid antibodies. Mol Med Rep 2012;6:1035–9. doi:10.3892/mmr.2012.1055.

111. Dal Ben ERR, do Prado CH, Baptista TSA, Bauer ME, Staub HL. Decreased levels of circulating CD4+CD25+Foxp3+ regulatory T cells in patients with primary antiphospholipid syndrome. J Clin Immunol 2013;33:876–9. doi:10.1007/s10875-012-9857-y.

112. Soroka N, Talako T, Smolnikova V. THU0504 Regulatory T Cells in Peripheral Blood of Patients with Antiphospholipid Syndrome. Ann Rheum Dis 2014;73:357–8. doi:10.1136/annrheumdis-2014-eular.4485.

113. Staub HL, Dal Ben ERR, Bauer ME. The antiphospholipid syndrome and Tregs. Autoimmun Rev 2014;13:697–8. doi:10.1016/j.autrev.2013.08.004.

114. Dal Ben ERRD, Do Prado CH Do, Baptista TSA, Bauer ME, Staub HL. Patients with systemic lupus erythematosus and secondary antiphospholipid syndrome have decreased numbers of circulating CD4+CD25+Foxp3+ Treg and CD3– CD19+ B cells. Rev Bras Reumatol (English Ed 2014;54:241–6. doi:10.1016/j.rbre.2013.09.001.

115. Lai Z, Marchena-Mendez I, Perl A. Oxidative stress and Treg depletion in lupus patients with antiphospholipid syndrome. Clin Immunol 2015;158:148–52. doi:10.1016/j.clim.2015.03.024.

116. Mahmoud F, Diejomaoh M, Omu a E, Abul H, Haines D. Lymphocyte subpopulation frequency and presence of anti-cardiolipin and anti-nuclear antibodies in peripheral blood of Kuwaiti women experiencing recurrent pregnancy loss. J Obstet Gynaecol 2001;21:587–90. doi:10.1080/01443610120087805.

117. Velasquillo MC, Alcocer-Varela J, Alarcón-Segovia D, Cabiedes J, Sánchez-Guerrero J. Some patients with primary antiphospholipid syndrome have increased circulating CD5+ B cells that correlate with levels of IgM antiphospholipid antibodies. Clin Exp Rheumatol 1991;9:501–5.

118. Simonin L, Pasquier E, Leroyer E, Cornec D, Bendaoud B, Hillion S, et al. Among patients with venous thrombo-embolism, B cell subset disturbances characterise those with primary antiphospholipid syndrome. Ann Rheum Dis 2015;74:SUPLL 2: 572.

119. Sigal LH. Basic Science for the Clinician 49. JCR J Clin Rheumatol 2009;15:90–4. doi:10.1097/RHU.0b013e31819c3a49.

120. Lieby P, Soley A, Knapp A-M, Cerutti M, Freyssinet J-M, Pasquali J-L, et al. Memory B cells producing somatically mutated antiphospholipid antibodies are present in healthy individuals. Blood 2003;102:2459–65. doi:10.1182/blood-2003-01-0180.

121. Kahn P, Ramanujam M, Bethunaickan R, Huang W, Madaio MP, Factor SM, et al. Prevention of Murine Antiphospholipid Syndrome by BAFF Blockade. Arthritis Rheum 2008;58:2824–34. doi:10.1002/art.23764.Prevention.

122. Berman H, Rodríguez-Pintó I, Cervera R, Morel N, Costedoat-Chalumeau N, Erkan D, et al. Rituximab use in the catastrophic antiphospholipid syndrome: Descriptive analysis of the CAPS registry patients receiving rituximab. Autoimmun Rev 2013;12:1085–90. doi:10.1016/j.autrev.2013.05.004.

123. Erkan D, Vega J, Ramón G, Kozora E, Lockshin MD. A pilot open-label phase II trial of rituximab for non-criteria manifestations of antiphospholipid syndrome. Arthritis Rheum 2013;65:464–71. doi:10.1002/art.37759.

124. López-Pedrera C, Buendía P, Cuadrado MJ, Siendones E, Aguirre MA, Barbarroja N, et al. Antiphospholipid antibodies from patients with the antiphospholipid syndrome induce monocyte tissue factor expression through the simultaneous activation of NF-kappaB/Rel proteins via the p38 mitogen-activated protein kinase pathway, and of the MEK-1/ERK . Arthritis Rheum 2006;54:301–11. doi:10.1002/art.21549.

125. Sorice M, Longo A, Capozzi A, Garofalo T, Misasi R, Alessandri C, et al. Anti-beta2-glycoprotein I antibodies induce monocyte release of tumor necrosis factor alpha and tissue factor by signal transduction pathways involving lipid rafts. Arthritis Rheum 2007;56:2687–97. doi:10.1002/art.22802.

126. Lambrianides A, Carroll CJ, Pierangeli SS, Pericleous C, Branch W, Rice J, et al. Effects of polyclonal IgG derived from patients with different clinical types of the antiphospholipid syndrome on monocyte signaling pathways. J Immunol 2010;184:6622–8. doi:10.4049/jimmunol.0902765.

127. Xie H, Zhou H, Wang H, Chen D, Xia L, Wang T, et al. Anti-β(2)GPI/β(2)GPI induced TF and TNF-α expression in monocytes involving both TLR4/MyD88 and TLR4/TRIF signaling pathways. Mol Immunol 2013;53:246–54. doi:10.1016/j.molimm.2012.08.012.

128. Colasanti T, Alessandri C, Capozzi A, Sorice M, Delunardo F, Longo A, et al. Autoantibodies specific to a peptide of β2-glycoprotein I cross-react with TLR4, inducing a proinflammatory phenotype in endothelial cells and monocytes. Blood 2012;120:3360–70. doi:10.1182/blood-2011-09-378851.

129. Zhou H, Ling S, Yu Y, Wang T, Hu H. Involvement of annexin A2 in anti-beta2GPI/beta2GPI-induced tissue factor expression on monocytes. Cell Res 2007;17:737–9. doi:10.1038/cr.2007.33.

130. López-Pedrera C, Cuadrado MJ, Herández V, Buendïa P, Aguirre MA, Barbarroja N, et al. Proteomic analysis in monocytes of antiphospholipid syndrome patients: deregulation of proteins related to the development of thrombosis. Arthritis Rheum 2008;58:2835–44. doi:10.1002/art.23756.

131. Satta N, Kruithof EKO, Fickentscher C, Dunoyer-Geindre S, Boehlen F, Reber G, et al. Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies. Blood 2011;117:5523–31. doi:10.1182/blood-2010-11-316158.

132. Brandt KJ, Fickentscher C, Boehlen F, Kruithof EKO, de Moerloose P. NF-κB is activated from endosomal compartments in antiphospholipid antibodies-treated human monocytes. J Thromb Haemost 2014;12:779–91. doi:10.1111/jth.12536.

133. Döring Y, Hurst J, Lorenz M, Prinz N, Clemens N, Drechsler MD, et al. Human antiphospholipid antibodies induce TNFalpha in monocytes via Toll-like receptor 8. Immunobiology 2010;215:230–41. doi:10.1016/j.imbio.2009.03.002.

134. Kornberg A, Blank M, Susana K, Yehuda S. Induction of Tissue Factor-like Activity in Monocytes by anti-cardiolipin antibodies. J Immunol 1994;153:1328–32.

135. Cuadrado MJ, López-Pedrera C, Khamashta M a, Camps MT, Tinahones F, Torres a, et al. Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. Arthritis Rheum 1997;40:834–41. doi:10.1002/1529-0131(199705)40:5<834::AID-ART8>3.0.CO;2-#.

136. Reverter J, Tassies D, Font J, Khamashta MA, Ichikawa J, Cervera R, et al. Effects of human monoclonal anticardiolipin antibodies on platelet function and on tissue factor expression on monocytes. Arthritis Rheum 1998;41:1420–7.

137. Dobado-Berrios PM, López-Pedrera C, Velasco F, Aguirre M a, Torres a, Cuadrado MJ. Increased levels of tissue factor mRNA in mononuclear blood cells of patients with primary antiphospholipid syndrome. Thromb Haemost 1999;82:1578–82.

138. Zhou H, Wolberg AS, Roubey R a S. Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. Blood 2004;104:2353–8. doi:10.1182/blood-2004-01-0145.

139. Cuadrado MJ, Buendía P, Velasco F, Aguirre M a, Barbarroja N, Torres L a, et al. Vascular endothelial growth factor expression in monocytes from patients with primary antiphospholipid syndrome. J Thromb Haemost 2006;4:2461–9. doi:10.1111/j.1538-7836.2006.02193.x.

140. López-Pedrera C, Aguirre MA, Buendía P, Barbarroja N, Ruiz-Limón P, Collantes-Estevez E, et al. Differential expression of protease-activated receptors in monocytes from patients with primary antiphospholipid syndrome. Arthritis Rheum 2010;62:869–77. doi:10.1002/art.27299.

141. Xia L, Zhou H, Hu L, Xie H, Wang T, Xu Y, et al. Both NF-κB and c-Jun/AP-1 involved in anti-β2GPI/β2GPI-induced tissue factor expression in monocytes. Thromb Haemost 2013;109:643–51. doi:10.1160/TH12-09-0655.

142. Nagahama M, Nomura S, Kanazawa S, Ozaki Y, Kagawa H, Fukuhara S. Significance of Anti-oxidized LDL Antibody and Monocyte-derived Microparticles in Anti-phospholipid Antibody Syndrome. Autoimmunity 2003;36:125–31. doi:10.1080/0891693031000079257.

143. Vikerfors a, Mobarrez F, Bremme K, Holmström M, Ågren a, Eelde a, et al. Studies of microparticles in patients with the antiphospholipid syndrome (APS). Lupus 2012;21:802–5. doi:10.1177/0961203312437809.

144. Chaturvedi S, Cockrell E, Espinola R, Hsi L, Fulton S, Khan M, et al. Circulating microparticles in patients with antiphospholipid antibodies: Characterization and associations. Thromb Res 2015;135:102–8. doi:10.1016/j.thromres.2014.11.011.

145. Perez-Sanchez C, Ruiz-Limon P, Aguirre MA, Bertolaccini ML, Khamashta M a, Rodriguez-Ariza A, et al. Mitochondrial dysfunction in antiphospholipid syndrome: implications in the pathogenesis of the disease and effects of coenzyme Q(10) treatment. Blood 2012;119:5859–70. doi:10.1182/blood-2011-12-400986.

146. Bohgaki M, Atsumi T, Yamashita Y, Yasuda S, Sakai Y, Furusaki A, et al. The p38 mitogen-activated protein kinase (MAPK) pathway mediates induction of the tissue factor gene in monocytes stimulated with human monoclonal anti-beta2Glycoprotein I antibodies. Int Immunol 2004;16:1633–41. doi:10.1093/intimm/dxh166.

147. Kubota T, Fukuya Y, Hashimoto R, Kanda T, Suzuki H, Okamura Y, et al. Possible involvement of chemokine-induced platelet activation in thrombophilic diathesis of antiphospholipid syndrome. Ann N Y Acad Sci 2009;1173:137–45. doi:10.1111/j.1749-6632.2009.04648.x.

148. Williams FM, Jurd K, Hughes GR HB. Antiphospholipid Syndrome Patients ' Monocytes Are " Primed " to Express Tissue Factor. Thromb Haemost 1998;80:864–5.

149. Ferro D, Saliola M, Meroni PL, Valesini G, Caroselli C, Praticò D, et al. Enhanced monocyte expression of tissue factor by oxidative stress in patients with antiphospholipid antibodies: effect of antioxidant treatment. J Thromb Haemost 2003;1:523–31.

150. Teruel R, Pérez-Sánchez C, Corral J, Herranz MT, Pérez-Andreu V, Saiz E, et al. Identification of miRNAs as potential modulators of tissue factor expression in patients with systemic lupus erythematosus and antiphospholipid syndrome. J Thromb Haemost 2011;9:1985–92. doi:10.1111/j.1538-7836.2011.04451.x.

151. Martirosyan A, Petrek M, Navratilova Z, Blbulyan A, Boyajyan A, Manukyan G. Differential regulation of proinflammatory mediators following LPS- and ATP-induced activation of monocytes from patients with antiphospholipid syndrome. Biomed Res Int 2015;2015:292851. doi:10.1155/2015/292851.

152. Müller-calleja N, Köhler A, Siebald B, Canisius A, Orning C, Radsak M, et al. Cofactor-independent antiphospholipid antibodies activate the NLRP3-inflammasome via endosomal NADPH-oxidase : implications for the antiphospholipid syndrome 2015:1–13.

153. Lopez-Pedrera C, Perez-Sanchez C, Zamorano A, Barbarroja N, Ruiz-Limon P, Jimenez Gomez Y, et al. Beneficial Effects of in Vivo Ubiquinol Supplementation on Athero- Thrombosis Prevention in Antiphospholipid Syndrome Patients. Arthritis Rheumatol (Hoboken, NJ) 2014.

154. Erkan D, Willis R, Murthy VL, Basra G, Vega J, Ruiz-Limón P, et al. A prospective open-label pilot study of fluvastatin on proinflammatory and prothrombotic biomarkers in antiphospholipid antibody positive patients. Ann Rheum Dis 2014;73:1176–80. doi:10.1136/annrheumdis-2013-203622.

155. López-Pedrera C, Ruiz-Limón P, Aguirre MÁ, Barbarroja N, Pérez-Sánchez C, Buendía P, et al. Global effects of fluvastatin on the prothrombotic status of patients with antiphospholipid syndrome. Ann Rheum Dis 2011;70:675–82. doi:10.1136/ard.2010.135525.

156. Newby LK, Marber MS, Melloni C, Sarov-Blat L, Aberle LH, Aylward PE, et al. Losmapimod, a novel p38 mitogen-activated protein kinase inhibitor, in non-ST-segment elevation myocardial infarction: a randomised phase 2 trial. Lancet 2014;384:1187–95. doi:10.1016/S0140-6736(14)60417-7.

157. Perez-Sanchez C., Ruiz-Limon P., Aguirre M.A., Carretero R.M., Barbarroja N., Collantes E., Gonzalez-Conejero R., Martinez C, Cuadrado MJ. Characterization of micrornas involved in the regulation of atherotrhombosis in antiphospholipid syndrome and systemic lupus erythematosus. Ann Rheum Dis 2014;73:SUPPL 2.

158. Arvieux J, Jacob M-C, Roussel B, Bensa J-C, Colomb M. Neutrophil antibodies activation by anti-B2 glycoprotein I monoclonal antibodies via Fcg receptor II. J Leukoc Biol 1995;57:387–94.

159. Gladigau G, Haselmayer P, Scharrer I, Munder M, Prinz N, Lackner K, et al. A role for Toll-like receptor mediated signals in neutrophils in the pathogenesis of the anti-phospholipid syndrome. PLoS One 2012;7:e42176. doi:10.1371/journal.pone.0042176.

160. Redecha P, Franzke C, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor / Factor VIIa / PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. J Clin Invest 2008;118:3453–61. doi:10.1172/JCI36089.loss.

161. Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, et al. A Novel C5a Receptor-Tissue Factor Cross-Talk in Neutrophils Links Innate Immunity to Coagulation Pathways. J Immunol 2006;177:4794–802.

162. Redecha P, Tilley R, Tencati M, Salmon JE, Kirchhofer D, Mackman N, et al. Tissue factor : a link between C5a and neutrophil activation in antiphospholipid antibody – induced fetal injury Tissue factor : a link between C5a and neutrophil activation in antiphospholipid antibody – induced fetal injury. Blood 2007;110:2423–31. doi:10.1182/blood-2007-01-070631.

163. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med 2011;3:73ra19. doi:10.1126/scitranslmed.3001180.

164. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med 2011;3:73ra20. doi:10.1126/scitranslmed.3001201.

165. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol 2011;187:538–52. doi:10.4049/jimmunol.1100450.

166. Van Avondt K, Fritsch-Stork R, Derksen RHWM, Meyaard L. Ligation of signal inhibitory receptor on leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus. PLoS One 2013;8:e78459. doi:10.1371/journal.pone.0078459.

167. Yalavarthi S, Gould TJ, Rao AN, Mazza LF, Morris AE, Núñez-Álvarez C, et al. Antiphospholipid antibodies promote the release of neutrophil extracellular traps: A new mechanism of thrombosis in the antiphospholipid syndrome. Arthritis Rheumatol (Hoboken, NJ) 2015. doi:10.1002/art.39247.

168. Leffler J, Stojanovich L, Shoenfeld Y, Bogdanovic G, Hesselstrand R, Blom AM. Degradation of neutrophil extracellular traps is decreased in patients with antiphospholipid syndrome. Clin Exp Rheumatol 2014;32:66–70.

169. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A 2010;107:15880–5. doi:10.1073/pnas.1005743107.

170. Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum Immunol 2005;66:1146–54. doi:10.1016/j.humimm.2005.11.003.

171. Shi F-D, Ljunggren H-G, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. Nat Rev Immunol 2011;11:658–71. doi:10.1038/nri3065.

172. Moffett A, Loke C. Immunology of placentation in eutherian mammals. Nat Rev Immunol 2006;6:584–94. doi:10.1038/nri1897.

173. King K, Smith S, Chapman M, Sacks G. Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage. Hum Reprod 2010;25:52–8. doi:10.1093/humrep/dep349.

174. Perricone C, De Carolis C, Giacomelli R, Zaccari G, Cipriani P, Bizzi E, et al. High levels of NK cells in the peripheral blood of patients affected with anti-phospholipid syndrome and recurrent spontaneous abortion: a potential new hypothesis. Rheumatology (Oxford) 2007;46:1574–8. doi:10.1093/rheumatology/kem197.

175. Kwak JY, Beaman KD, Gilman-Sachs A, Ruiz JE, Schewitz D, Beer AE. Up-regulated expression of CD56+, CD56+/CD16+, and CD19+ cells in peripheral blood lymphocytes in pregnant women with recurrent pregnancy losses. Am J Reprod Immunol 1995;34:93–9.

176. Heilmann L, Ph D, Schorch M, Hahn T, Adasz G, Schilberz K, et al. Pregnancy Outcome in Women with Antiphospholipid Antibodies : Report on a Retrospective Study. Semin Thromb Hemost 2008;34:794–802. doi:10.1055/s-0029-1145261.

177. Mariee NG, Tuckerman E, Laird S, Li TC. The correlation of autoantibodies and uNK cells in women with reproductive failure. J Reprod Immunol 2012;95:59–66. doi:10.1016/j.jri.2012.04.003.

178. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nat Rev Immunol 2009;9:729–40. doi:10.1038/nri2620.

179. Gropp K, Weber N, Reuter M, Micklisch S, Kopka I, Hallström T, et al. β2-glycoprotein I, the major target in antiphospholipid syndrome, is a special human complement regulator. Blood 2011;118:2774–83. doi:10.1182/blood-2011-02-339564.

180. Oku K, Atsumi T, Bohgaki M, Amengual O, Kataoka H, Horita T, et al. Complement activation in patients with primary antiphospholipid syndrome. Ann Rheum Dis 2009;68:1030–5. doi:10.1136/ard.2008.090670.

181. Devreese KMJ, Hoylaerts MF. Is there an association between complement activation and antiphospholipid antibody-related thrombosis? Thromb Haemost 2010;104:1279–81. doi:10.1160/TH10-06-0410.

182. Breen KA, Seed P, Parmar K, Moore GW, Stuart-smith SE, Hunt BJ. Complement activation in patients with isolated antiphospholipid antibodies or primary antiphospholipid syndrome. Thromb Haemost 2012;107:423–9. doi:10.1160/TH11-08-0554.

183. Reggia R, Ziglioli T, Andreoli L, Bellisai F, Iuliano A, Gerosa M, et al. Primary anti-phospholipid syndrome: any role for serum complement levels in predicting pregnancy complications? Rheumatology (Oxford) 2012;51:2186–90. doi:10.1093/rheumatology/kes225.

184. De Carolis S, Botta A, Santucci S, Salvi S, Moresi S, Di Pasquo E, et al. Complementemia and obstetric outcome in pregnancy with antiphospholipid syndrome. Lupus 2012;21:776–8. doi:10.1177/0961203312444172.

185. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS, et al. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. J Exp Med 2002;195:211–20. doi:10.1084/jem.200116116.

186. Fischetti F, Durigutto P, Pellis V, Debeus A, Macor P, Bulla R, et al. Thrombus formation induced by antibodies to β2-glycoprotein I is complement dependent and requires a priming factor. Blood 2005;106:2340–6. doi:10.1182/blood-2005-03-1319.

187. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody – induced fetal loss by inhibiting complement activation 2004;10:1222–6. doi:10.1038/1121.

188. Erkan D, Aguiar CL, Andrade D, Cohen H, Cuadrado MJ, Danowski A, et al. 14th International Congress on Antiphospholipid Antibodies: task force report on antiphospholipid syndrome treatment trends. Autoimmun Rev 2014;13:685–96. doi:10.1016/j.autrev.2014.01.053.

189. Zhang Z, Zhang R. Epigenetics in autoimmune diseases: Pathogenesis and prospects for therapy. Autoimmun Rev 2015. doi:10.1016/j.autrev.2015.05.008.

190. Broen JCA, Radstake TRDJ, Rossato M. The role of genetics and epigenetics in the pathogenesis of systemic sclerosis. Nat Rev Rheumatol 2014;10:671–81. doi:10.1038/nrrheum.2014.128.

191. Poulton K, Rahman A, Giles I. Examining how antiphospholipid antibodies activate intracellular signaling pathways: a systematic review. Semin Arthritis Rheum 2012;41:720–36. doi:10.1016/j.semarthrit.2011.09.004.

192. Du VX, Kelchtermans H, De Groot PG, De Laat B. From antibody to clinical phenotype, the black box of the antiphospholipid syndrome: Pathogenic mechanisms of the antiphospholipid syndrome. Thromb Res 2013;132:319–26. doi:10.1016/j.thromres.2013.07.023.

193. Brandt KJ, Kruithof EKO, de Moerloose P. Receptors involved in cell activation by antiphospholipid antibodies. Thromb Res 2013;132:408–13. doi:10.1016/j.thromres.2013.08.015.

194. Ermann J, Rao D a., Teslovich NC, Brenner MB, Raychaudhuri S. Immune cell profiling to guide therapeutic decisions in rheumatic diseases. Nat Rev Rheumatol 2015. doi:10.1038/nrrheum.2015.71.

195. Jameson J, Longo D. Precision Medicine — Personalized, Problematic, and Promising. N Engl J Med 2015;372:2229–34.

CHAPTER 3

Monocyte type I interferon signature in antiphospholipid syndrome is related to pro-inflammatory monocyte subsets, hydroxychloroquine and statin use

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Several autoimmune diseases, most notably systemic lupus erythematosus (SLE), show an overexpression of type I interferon (IFN) inducible genes, termed the IFN signature[1]. The IFN signature is associated with endothelial progenitor cells (EPC) dysfunction in SLE[2] and the recent report by Grenn et al.[3] provides evidence for this link in antiphospholipid syndrome (APS) as well.

The authors show that primary APS (PAPS) patients have a reduced number of circulating EPC that differentiate into endothelial cell-like cells. This is supported by a similar reduction in EPC differentiation when EPC from healthy controls (HC) are cultured with serum from PAPS patients. Whereas depletion of antiphospholipid antibodies (aPL) has no effect, blockade of the receptor for type I IFN restores EPC differentiation. Hence, targeting type I IFN might mitigate vascular disease in APS patients by restoring EPC differentiation. In support of this assumption, the authors demonstrate the presence of an IFN signature in peripheral blood mononuclear cells (PBMC) of PAPS patients.

Previous hints in the literature further confirm the presence of an IFN signature in PBMC of PAPS patients[4,5]. Interestingly, a micro-array study on isolated monocytes of SLE, SLE+APS and PAPS patients reported the presence of an IFN signature only in monocytes of SLE and SLE+APS patients, not in PAPS[6]. Monocytes are considered key players in the pathogenesis of APS[4], amongst others due to an overexpression of tissue factor (TF), the main initiator of humoral coagulation, while shifts in monocyte subsets are linked to vascular disease in rheumatic diseases[7,8].

Here we report the prevalence of the IFN signature in monocytes from PAPS, SLE+APS and SLE patients in relation to TF expression and increases in pro-inflammatory monocyte subsets and show that PAPS patients treated with hydroxychloroquine and statins have reduced IFN signatures.

Prevalence of a type I IFN signature in monocytes of APS patients

Significantly increased IFN scores were observed in SLE, SLE+APS and PAPS patients as compared with HC. Interestingly, SLE patients had higher IFN scores than PAPS patients (**Fig 1A**). Using a cut-off set at the 95th percentile of HC, an IFN signature (IFN+) was observed in 77%, 71% and 46% of SLE, SLE+APS and PAPS patients respectively (**Fig 1B**). The previously mentioned micro-array study in monocytes of SLE, SLE+APS and PAPS patients[6], used only genes differentially expressed by at least twofold for further pathway analysis. This threshold explains why the IFN signature was not detected in PAPS. A reanalysis of the publically

available dataset (GEO dataset ID 200050395) is in line with our data, showing increased expression of type I IFN inducible genes in monocytes of PAPS patients as compared with HC, with higher expressions in SLE and SLE+APS patients.

In our cohort, the IFN score was neither associated with the presence or absence of lupus anticoagulant, anti-cardiolipin, anti-β2 glycoprotein I antibodies or triple positivity nor with the type of thrombosis (arterial or venous), both in PAPS and in SLE+APS patients (data not shown). Monocytes of SLE+APS and PAPS patients had increased expression of TF, with a similar trend in SLE patients. Although TF expression was on average higher in patients with a type I IFN signature (IFN+) as compared to patients without (IFN-), this did not reach statistical significance in any of the patient groups (IFN+ vs IFN- in SLE, SLE+APS and PAPS patients p=0.10, p=0.055 and p=0.67, respectively) (**Fig 1C**).

Type I IFN signature associates with increases in pro-inflammatory monocyte subsets in APS

Monocytes can be subdivided into three different subsets based on the expression of CD14 and CD16. The majority of monocytes are CD14++CD16- and termed classical monocytes, whereas CD14++CD16+ and CD14+CD16+ are termed intermediate and non-classical monocytes respectively. No differences were observed in the proportion of monocyte subsets between SLE+APS and PAPS patients (data not shown). However, the IFN scores correlated positively with the proportions of intermediate and non-classical monocytes ($r=0.47$, $p<0.001$ and $r=0.39$, $p=0.006$ respectively) and negatively to the proportion of classical monocytes ($r=-0.47$, $p<0.001$) in APS (**Fig 1D-F**). As increases in intermediate and non-classical monocytes are associated with preclinical cardiovascular disease (CVD) in SLE and rheumatoid arthritis[7,8], this observation further substantiates the link between type I IFN and vascular disease in APS, as was proposed by Grenn et al.

Type I IFN signature in relation to lupus-like serology, hydroxychloroquine and statin use in PAPS

As IFN scores are higher in SLE patients, it might be speculated that the presence of an IFN signature predisposes PAPS patients to develop SLE. None of the PAPS patients studied had clinical evidence of SLE, fulfilled the ACR criteria for SLE or was treated with immunosuppressants (**Table 1**). The presence of lupus-like serology such as antinuclear (ANA), anti-double stranded DNA (anti-dsDNA) or anti-nucleosome antibodies is associated with the progression of PAPS towards

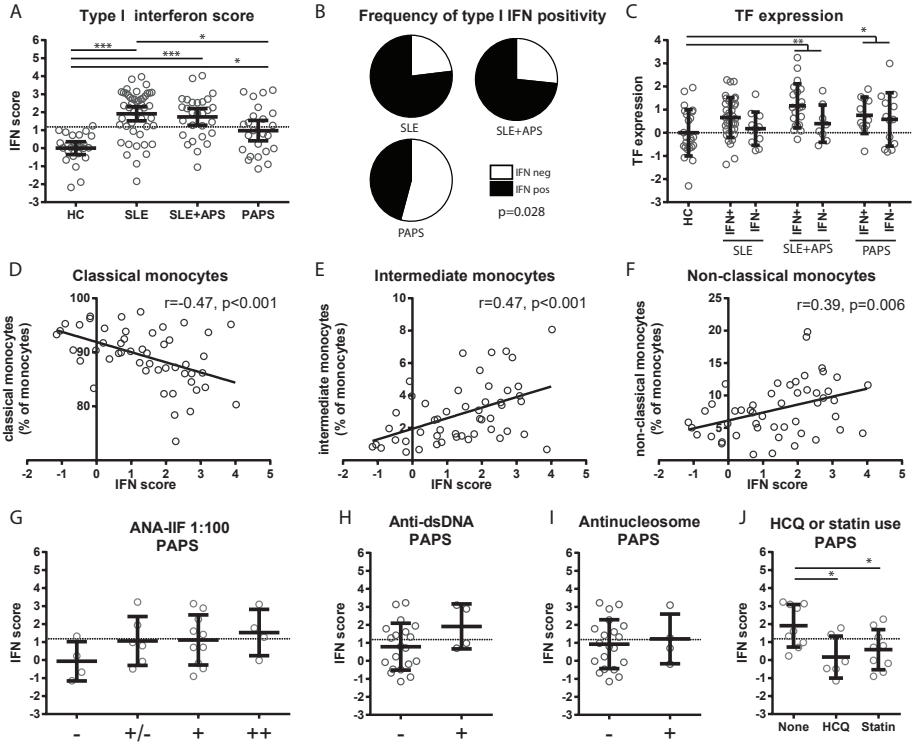


Figure 1: Prevalence of a type I interferon signature in monocytes of SLE, SLE+APS and PAPS patients in relation to tissue factor expression, monocyte subsets, lupus-like serology, hydroxychloroquine and statin use

(A) Distribution of type I IFN scores among SLE, SLE+APS and PAPS patients (means±95%CI). IFN scores were determined as previously described[12]. Statistics: ANOVA with post-hoc Tukey's HSD. **(B)** Proportion of patients with or without a type I IFN signature by setting a threshold at the 95th percentile of HC. Statistics: chi-square. **(C)** Tissue Factor expression z-scores in patients when stratified by the presence (IFN+) or absence (IFN-) of a type I IFN signature (means±SD). Statistics: ANOVA with post-hoc Dunnett's and Student's T-test (comparison of IFN+ with IFN- subgroups) **(D-F)** Correlations of the type I IFN score with the proportion of classical, intermediate and non-classical monocytes in APS patients (SLE+APS and PAPS) as determined by flowcytometry on isolated monocytes. Statistics: Pearson correlation coefficients. **(G)** Type I IFN scores of PAPS patients stratified by result of ANA-IIF test (at 1:100 serum dilution). Only ++ samples also tested positive at 1:1000 serum dilution, **(H)** the presence or absence of anti-dsDNA antibodies (ELiA) or **(I)** anti-nucleosome antibodies (line-blot) (means±SD). Statistics polynominal ANOVA (G) or Student's t-test (H-I). **(J)** PAPS patients stratified by their use of hydroxychloroquine (HCQ) or statins (means±SD). Statistics: ANOVA with post-hoc Dunnett's. Each dot represents one subject. * = p<0.05, ** = P<0.01, *** = p<0.001.
The dotted horizontal line in G-J indicates the 95th percentile of IFN scores in HC as in A.

SLE[9,10]. Insignificant trends towards higher IFN scores were observed in PAPS patients with higher level of ANA (**Fig 1G,** p=0.12) and with elevated anti-dsDNA

Table 1

	HC (n=24)	SLE (n=47)	SLE+APS (n=28)	PAPS (n=24)
Age (y)	43 (35 - 50)	43 (28 - 52)	45 (39 - 54)	39 (31 - 50)
Female	91%	96%	100%	96%
SLE duration (y)		12 (4 - 23)	16 (12 - 26)	N.A.
APS duration (y)		N.A.	15 (8 - 21)	6 (3 - 19)
Body Mass Index (kg/m2)		24 (22 - 27)	26 (22 - 32)	23 (21 - 31)
aPL profile				
Lupus Anticoagulant		7%	71%	83%
anti-cardiolipin IgG		17%	71%	88%
anti-cardiolipin IgM		10%	11%	38%
anti-β2 Glycoprotein I IgG		8%	30%	38%
anti-β2 Glycoprotein I IgM		6%	0%	8%
Triple positive (IgG or IgM)		2%	21%	42%
APS manifestations				
Arterial thrombosis		13%	54%	58%
Venous thrombosis		4%	54%	42%
Obstetric complications		6%	25%	29%
ACR criteria				
Lupus Nephritis		51%	39%	0%
Arthritis		70%	71%	0%
Discoid rash		21%	18%	0%
Malar rash		64%	54%	0%
Photosensitivity		47%	54%	0%
Oral Ulcers		32%	36%	0%
Serositis		28%	14%	0%
Neurologic disorder		4%	21%	13%*
Hematologic disorder		66%	86%	33%**
Actual Drug use				
Oral anticoagulant		2%	68%	67%
Aspirin		21%	36%	50%
Hydroxychloroquine		75%	50%	25%
Statin		19%	32%	38%
Prednisone		53%	39%	0%
Prednisone dose (mg/day)		7.5 (5-10)	7.5 (5-10)	-
Azathioprine		38%	32%	0%
MMF		11%	7%	0%

Medians with interquartile range or percentage of total
* seizures, ** thombocytopenia

antibodies (**Fig 1H,** p=0.12). No relation between anti-nucleosome antibodies and the IFN signature was observed in PAPS (**Fig 1I** p=0.70). Importantly, 45% of the PAPS patients without anti-dsDNA antibodies had an IFN signature defined as above the 95th percentile of HC (**Fig 1H**). There is a growing interest in the use of hydroxychloroquine and statins as immunomodulators in PAPS[11]. Interestingly, PAPS patients treated with hydroxychloroquine and statins had lower IFN scores as compared with patients not using these drugs (**Fig 1J**).

In support of the data by Grenn et al. we conclude that monocytes of PAPS patients display an IFN signature. However, based upon our data we conclude that the IFN signature is more outspoken in SLE patients. Besides its association with EPC dysfunction, we report that the IFN signature is associated with increases in CVD-associated monocyte subsets and is not restricted to PAPS patients with lupus-like serology. Finally, hydroxychloroquine and statin treatment might serve to control type I IFN related immune activation in PAPS.

References

1. Higgs BW, Liu Z, White B, et al. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis*. 2011;70(11):2029–36. doi:10.1136/ard.2011.150326.

2. Lee PY, Li Y, Richards HB, et al. Type I interferon as a novel risk factor for endothelial progenitor cell depletion and endothelial dysfunction in systemic lupus erythematosus. *Arthritis Rheum*. 2007;56(11):3759–3769. doi:10.1002/art.23035.

3. Grenn RC, Yalavarthi S, Gandhi AA, et al. Endothelial progenitor dysfunction associates with a type I interferon signature in primary antiphospholipid syndrome. *Ann Rheum Dis*. 2016:Online First 2016 jul 18. doi:10.1136/annrheumdis-2016-209442.

4. Van den Hoogen LL, van Roon JAG, Radstake TRDJ, Fritsch-Stork RDE, Derksen RHWM. Delineating the deranged immune system in the antiphospholipid syndrome. *Autoimmun Rev*. 2016;15(1):50–60. doi:10.1016/j.autrev.2015.08.011.

5. Bernales I, Fullaondo a, Marín-Vidalled MJ, et al. Innate immune response gene expression profiles characterize primary antiphospholipid syndrome. *Genes Immun*. 2008;9(1):38–46. doi:10.1038/sj.gene.6364443.

6. Perez-Sanchez C, Barbarroja N, Messineo S, et al. Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in antiphospholipid syndrome, systemic lupus erythematosus and antiphospholipid syndrome with lupus. *Ann Rheum Dis*. 2015;74(7):1441–9. doi:10.1136/annrheumdis-2013-204600.

7. Mikołajczyk TP, Osmenda G, Batko B, et al. Heterogeneity of peripheral blood monocytes, endothelial dysfunction and subclinical atherosclerosis in patients with systemic lupus erythematosus. *Lupus*. 2015;25(1):18–27. doi:10.1177/0961203315598014.

8. Winchester R, Giles JT, Nativ S, et al. Association of Elevations of Specific T Cell and Monocyte Subpopulations in Rheumatoid Arthritis with Subclinical Coronary Artery Atherosclerosis. *Arthritis Rheumatol*. 2016;68(1):92–102. doi:10.1002/art.39419.

9. Derksen R, Gmelig-Meijling F, de Groot P. Primary antiphospholipid syndrome evolving into systemic lupus erythematosus. *Lupus*. 1996;5(1):77–80.

10. Abraham Simón J, Rojas-Serrano J, Cabiedes J, Alcocer-Varela J. Antinucleosome antibodies may help predict development of systemic lupus erythematosus in patients with primary antiphospholipid syndrome. *Lupus*. 2004;13(3):177–81.

11. Erkan D, Aguiar CL, Andrade D, et al. 14th International Congress on Antiphospholipid Antibodies: task force report on antiphospholipid syndrome treatment trends. *Autoimmun Rev*. 2014;13(6):685–96. doi:10.1016/j.autrev.2014.01.053.

12. Brkic Z, Corneth OB, van Helden-Meeuwsen CG, et al. T helper 17 cell cytokines and interferon type I: partners in crime in systemic lupus erythematosus? *Arthritis Res Ther*. 2014;16(2):R62. doi:10.1186/ar4499.

Supplementary Methods

Patients and healthy controls

Consecutive patients with SLE and/or APS were recruited from the department of rheumatology and clinical immunology at the university medical center Utrecht. Healthy controls (HC) were recruited from the in-house healthy donor service. Controls were anonymized except for age and gender. All patients and controls signed informed consent prior to donating blood. Patients were classified as SLE when fulfilling the 1997 American College of Rheumatology (ACR) criteria for SLE. Patients were classified as APS when fulfilling the revised Sapporo classification criteria for APS. Patients that fulfilled both SLE and APS criteria were classified as SLE+APS. Patients were classified as PAPS when they did not fulfill ACR-endorsed classification criteria for rheumatic diseases, notably SLE.

None of the PAPS patients had a clinical diagnosis of SLE or (histories of) lupus skin rash, arthritis, serositis, lupus nephritis and oral ulcers. Tests for antinuclear antibodies (ANA), antibodies against double stranded DNA (anti-dsDNA) or anti-nucleosome (anti-NCS) antibodies were obtained from the patients' charts. ANA were tested by indirect immunofluorescence on Hep-20-10 cells (EuroImmun) at a serum dilution of 1:100. Based on intensity of fluorescence results were scored as negative (-), weakly positive (+/-), intermediate (+) and strongly (++) positive according to the manufacturer's instructions. Antibodies against double stranded DNA (anti-dsDNA) were tested by ELiA (Thermofisher). Anti-dsDNA titers above 30U/mL were considered positive. Anti-NCS antibodies were tested by profile 3 lineblot (EuroImmun) and reported as either positive or negative.

Monocyte isolation

PBMCs were isolated from peripheral blood by density gradient isolation using ficoll-hypaque (GE healthcare). Monocytes were isolated by CD14 magnetic bead (Miltenyi) isolation using an autoMACSpro according to the manufacturer's instructions.

Assessment of the IFN signature

RNA was isolated from purified monocytes using the All prep Universal Kit (Qiagen) by the Qiacube (Qiagen) according to the manufacturer's instructions. cDNA was generated using the iScript cDNA synthesis kit (BioRad). Four type I IFN inducible genes that were previously shown to explain 95% of the variance of a total of 11 type I IFN inducible genes in monocytes of SLE patients (Brkic et al. Arthritis Research and Therapy 2014) were quantified by RT-qPCR using Taqman predesigned primer/probe sets (Thermofisher). RT-qPCR was conducted in duplicate on

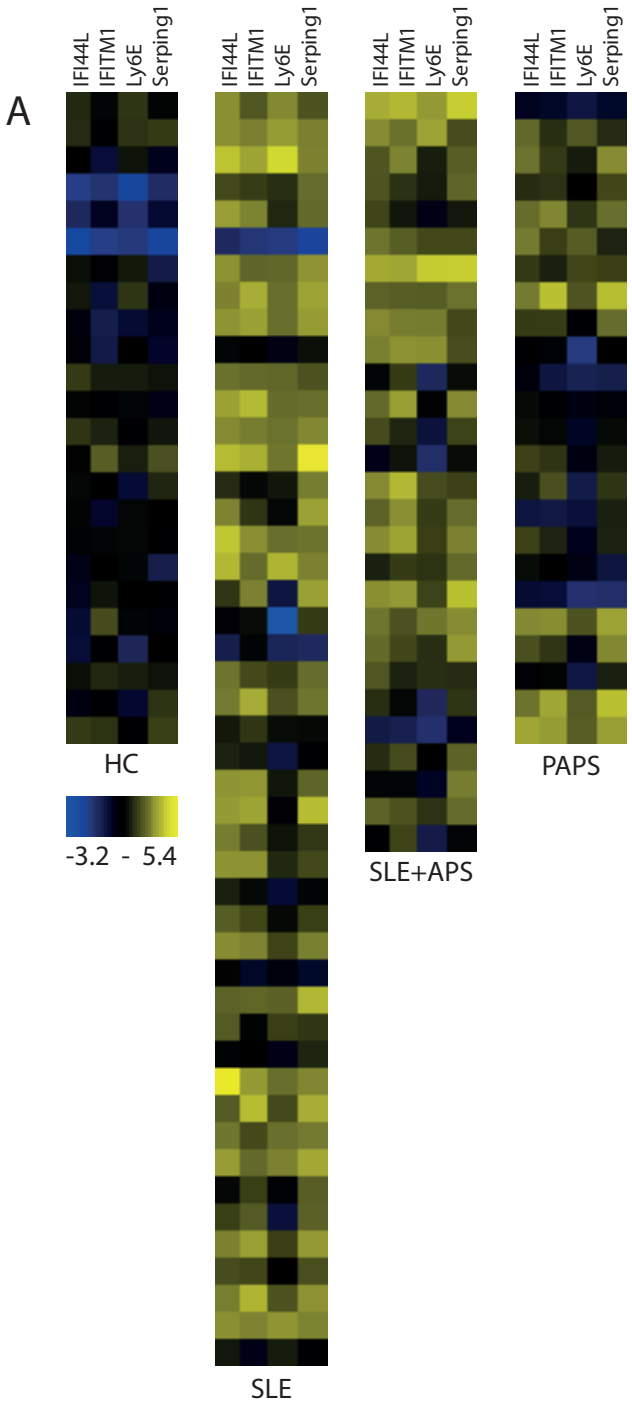
the following four type I IFN inducible genes Ly6E, IFITM1, Serping1 and IFI44L and F3 (TF). Gene-expression was normalized to the housekeeping gene GUSB (**Supplementary Figure 1**).

Flow cytometry

10^4 purified monocytes were stained for 20 minutes at 4°C with CD45-PerCP (Biolegend), CD16-PE (Dako) and CD14-FITC (Miltenyi) to assess the purity of the isolated monocytes. All samples had a purity of >90%. Classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{+}$) monocyte subsets were identified on the basis of CD14 and CD16 expression and reported as the proportion of total monocytes.

Statistical analysis

RT-qPCR data were analyzed using the ΔΔCt method. Z-scores were calculated for each gene as previously described by Petri et al. (Lupus 2009). The z-scores of the 4 type I IFN inducible genes were averaged to define the IFN-score per subject. To define the percentage of patients with a type I IFN signature, a threshold was set at the 95^{th} percentile of the type I IFN scores of HC. Patients above or below this threshold were considered as with (IFN+) or without (IFN-) a type I IFN signature. Flowcytometry data were analyzed by FlowJo. Statistical analysis was performed using SPSS (version 21) and Graphpad Prism (version 6). Statistical tests were conducted as described in the figure legends. All tests were conducted two-sided with an alpha level of 0.05.



Supplementary figure 1: heatmap of gene-expression values to calculate the type I IFN scores

CHAPTER 4

Galectin-9 is an easy to measure biomarker for the interferon signature in systemic lupus erythematosus and antiphospholipid syndrome

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submitted for publication

Abstract

Objective

The interferon (IFN) signature is related to disease activity and vascular disease in systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) and represents a promising therapeutic target. Quantification of the IFN signature is currently performed by gene-expression analysis, limiting its current applicability in clinical practice. Therefore, the objective of this study was to establish an easy to measure biomarker for the interferon signature.

Methods

Serum levels of galectin-9, CXCL-10 (IP-10) and TNF-RII were measured in patients with SLE, SLE+APS and primary APS (PAPS) and healthy controls (n=148) after an initial screening of serum analytes in a smaller cohort (n=43). Analytes were correlated to measures of disease activity and the IFN signature. The performance of galectin-9, CXCL-10 and TNF-RII as biomarkers to detect the IFN signature was assessed by receiver operating characteristics (ROC) curves.

Results

Galectin-9, CXCL-10 and TNF-RII were elevated in patients with SLE, SLE+APS and PAPS ($p<0.05$) and correlated with disease activity and tissue factor expression. Galectin-9 correlated stronger than CXCL-10 or TNF-RII with the IFN score ($r=0.70$, $p<0.001$) and was superior to CXCL-10 or TNF-RII in detecting the IFN signature (AUC 0.86). Importantly, in patients with SLE(±APS), galectin-9 was also superior to anti-dsDNA antibody (AUC 0.70), or complement C3 (AUC 0.70) and C4 (AUC 0.78) levels in detecting the IFN signature.

Conclusion

Galectin-9 is a novel, easy to measure hence clinically applicable biomarker to detect the IFN signature in patients with systemic autoimmune diseases such as SLE and APS.

Keywords

Systemic lupus erythematosus, antiphospholipid syndrome, interferon signature, galectin-9, biomarker

Introduction

Systemic lupus erythematosus (SLE) is a chronic relapsing autoimmune disease in which immune complexes of autoantibodies are deposited in tissues inducing tissue damage. Approximately 20% of SLE patients have antiphospholipid syndrome (APS), defined as the persistent presence of antiphospholipid antibodies (aPL) in patients who experienced at least one thrombotic or predefined obstetric complication. APS also affects patients without an underlying disease and is then termed primary APS (PAPS). The pathogenesis of SLE and APS is partly overlapping including shared genetic risk loci and perturbations in both the innate and adaptive immune system, although both conditions are only rarely studied together[1].

Transcriptomic studies in SLE and APS have revealed a markedly increased expression of IFN inducible genes, known as the IFN signature which is present in ~75% of SLE(±APS) patients and ~50% of PAPS patients[2–5]. In SLE, the IFN signature is associated with elevated autoantibody levels, disease activity, future flares and congenital heart block [3,4,6,7]. In (P)APS, the IFN signature has only recently been reported and has been linked to endothelial progenitor cell dysfunction and increases in pro-inflammatory monocytes[2,5].

The IFN signature is a promising therapeutic target. Anifrolumab, a monoclonal antibody against IFNAR, the receptor for type I IFN, showed clinical efficacy in a phase IIb clinical trial in SLE, particularly in those patients with an IFN signature[8]. The detection of the IFN signature may therefore soon be used to guide treatment decisions in SLE and possibly other autoimmune diseases.

The IFN signature is measured by gene-expression analysis. Therefore, easier to measure biomarkers for IFN signature detection are urgently needed. Measuring IFNα in peripheral blood seems the most straight-forward method. However, IFNα is difficult to detect by standard techniques[9] and the 12 different IFNα proteins cannot be measured by a single assay. In addition, other type I IFNs (including IFNβ) as well as type II and III IFN (IFNγ and IFNλ) contribute to the IFN signature[10]. Therefore surrogate markers for the IFN signature were searched for: Siglec-1 expression on monocytes and serum levels of CXCL-10 correlate with the IFN signature in SLE and serve as alternatives to detect the IFN signature in SLE[6,11].

Here, using an identification and replication approach we identified that galectin-9, CXCL-10 and TNF-RII correlate with the IFN signature and disease activity in patients with SLE and APS. Importantly, galectin-9 outperformed CXCL-10 or

traditional markers of disease activity as an easy measurable biomarker to detect the IFN signature both in SLE and APS.

Methods

Blood was drawn from patients with SLE, SLE+APS, PAPS and healthy controls (HC, **Table 1**). PAPS patients did not meet ACR criteria for SLE[12]. The medical ethical committee of the UMC Utrecht approved the study and written informed consent was obtained.

Clinical and laboratory assessments

Serum analytes were measured as described[13] in an initial identification cohort (n=43), followed by a replication step (n=148). For serum analytes, elevated levels were defined as 2 standard deviations above the mean of HC. Anti-dsDNA antibodies and complement levels were determined by ELiA or nephelometry. The IFN signature and tissue factor expression was quantified by qPCR in CD14+ monocytes as described[5]. mRNA expression of galectin-9 was assessed in circulating dendritic cells as described in the supplementary methods.

Statistics

All tests were conducted two-sided at an alpha level of 0.05. Statistical tests used are provided in the figure legends.

Results

Galectin-9, CXCL-10 and TNF-RII are biomarkers of disease activity in SLE and APS

In an initial screening of 22 serum analytes, galectin-9 ($r=0.81$, $p<0.001$), CXCL-10 ($r=0.72$ $p<0.001$) and TNF-RII ($r=0.42$, $p=0.007$) correlated significantly with the IFN score in patients with SLE, SLE+APS and PAPS (n=43, **supplemental table and figure 1**). We next assessed these analytes in a larger replication step (n=148). Galectin-9, CXCL-10 and TNF-RII levels were significantly elevated in patients with SLE, SLE+APS and PAPS as compared with HC (**Fig 1A**). Elevated levels of galectin-9 were present in 74%, 68% and 41% of SLE, SLE+APS and PAPS patients, respectively, compared with 46%, 55% and 45% for CXCL-10 and 46%, 50% and 41% for TNF-RII. All three analytes correlated with anti-dsDNA antibodies and complement levels in patients with SLE (**Supplementary Figure 2**). Furthermore, galectin-9 and TNF-RII, not CXCL-10, correlated with disease activity as assessed by SLEDAI (**Fig 1B**). In patients with APS the increased expression of tissue factor (TF) by monocytes is induced by aPL and reflects a prothrombotic phenotype[1]

Table 1: Clinical Characteristics

	HC (n=27)	SLE (n=50)	SLE+APS (n=40)	PAPS (n=29)
Female	93%	96%	95%	97%
Age	43 (34-50)	40 (28-48)	45 (37-53)	40 (33-50)
Disease manifestations				
SLEDAI		4 (2-6	4 (1-5)	-
Malar Rash		66%	55%	0%
Discoid Rash		22%	18%	0%
Photosensitivity		42%	53%	0%
Oral Ulcers		36%	35%	0%
Arthritis		70%	65%	0%
Serositis		28%	20%	0%
Lupus Nephritis		60%	45%	0%
Neurologic disorder		4%	18%	10%
Hematologic disorder		60%	85%	35%
Arterial Thrombosis		10%	43%	59%
Venous Thrombosis		4%	60%	38%
Obstetrical morbidity		6%	23%	31%
Current drug use				
Hydroxychloroquine		80%	55%	21%
Prednisone		64%	45%	0%
Azathioprine		34%	33%	0%
MMF		16%	10%	0%
Oral anticoagulant		2%	75%	62%
Aspirin		22%	28%	48%
Serology				
C3 (g/L)		0.88 (0.69-1.03)	0.79 (0.68-0.92)	-
C4 (g/L)		0.16 (0.13-0.20)	0.15 (0.11-0.22)	-
a-dsDNA (IU/mL)		27 (6-114)	14 (5-58)	-
Lupus anticoagulant		13%	65%	82%
IgG aCL		15%	78%	86%
IgM aCL		11%	15%	38%
IgG aβ2GPI		7%	26%	35%
IgM aβ2GPI		7%	5%	10%

Medians with interquartile range or percentage of total.

Abbreviations: MMF: mycophenolate mofetil, a-dsDNA: anti-double stranded DNA antibodies, aCL: anticardiolipin antibodies, aβ2GPI: anti-β2 Glycoprotein I antibodies

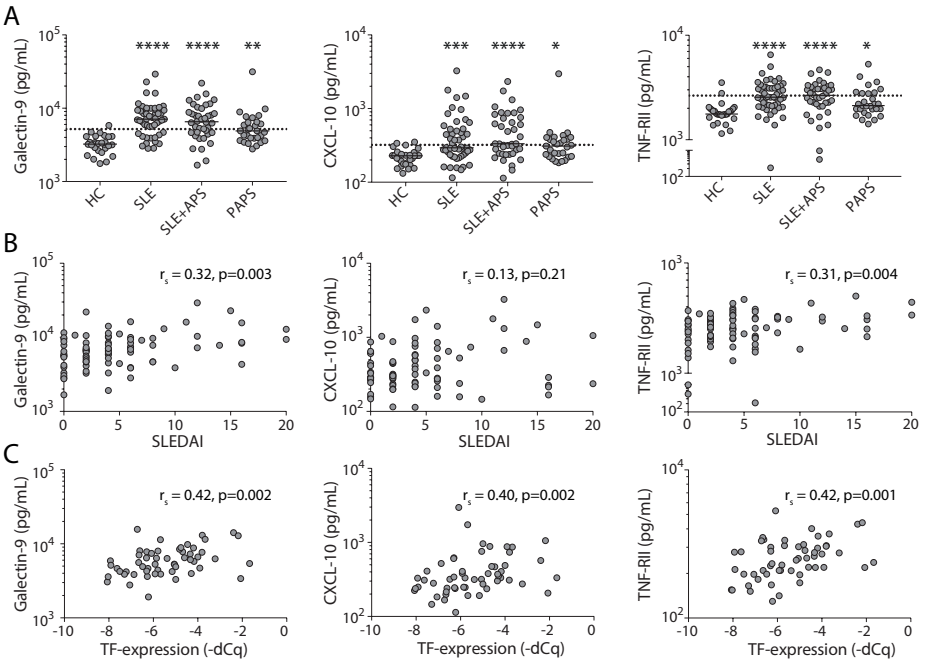


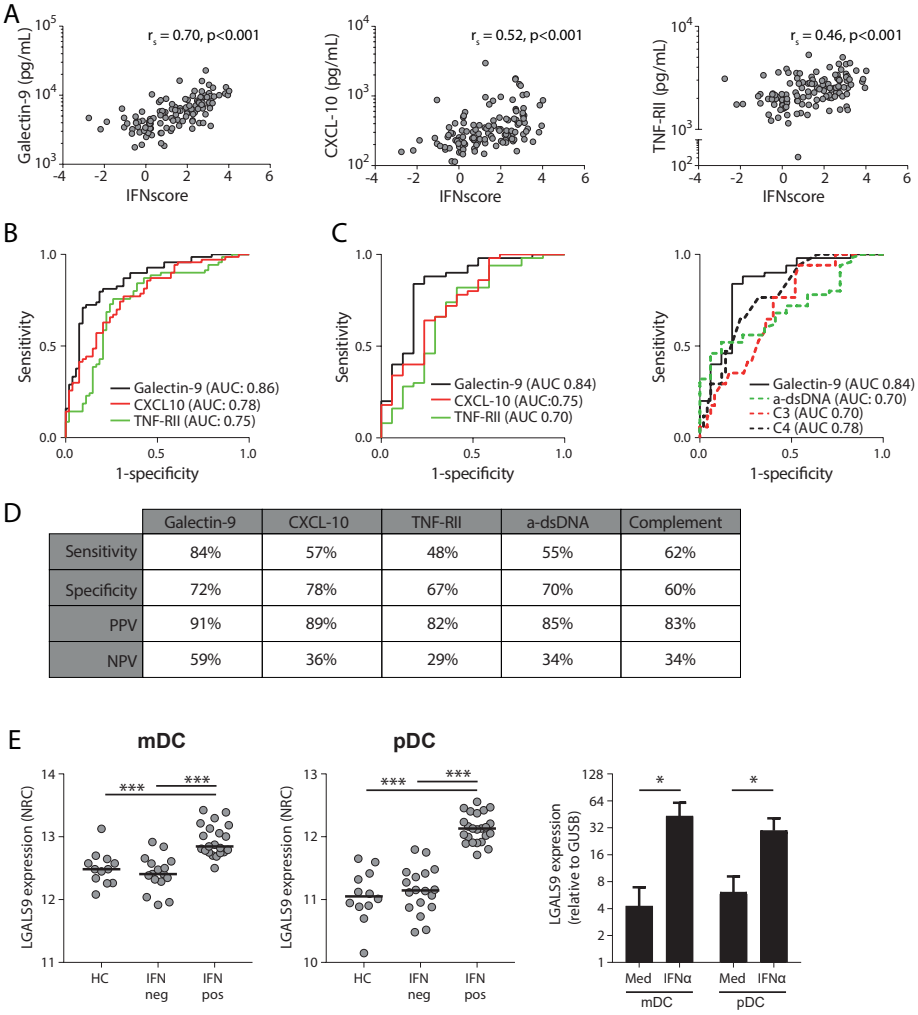
Figure 1: Galectin-9 is a biomarker for disease activity and Tissue Factor expression in SLE and APS

(A) Galectin-9, CXCL-10 and TNF-RII were measured by Luminex in sera of SLE, SLE+APS and PAPS patients (Statistics: Kruskall Wallis with post-hoc Dunn's). Dotted line represents the mean of HC+2SD (B) Correlation of galectin-9, CXCL-10 and TNF-RII with disease activity in patients with SLE and SLE+APS). (C) Correlation of galectin-9, CXCL-10 and TNF-RII tissue factor (TF) expression in monocytes in SLE+APS and PAPS patients as assessed by qPCR (-dCq: delta quantification cycle). Statistics: A) Kruskall-wallis followed by post-hoc Dunn's. B+C) Spearman Correlation coefficients

and we found significant correlations between galectin-9, CXCL-10 and TNF-RII and the expression of TF by monocytes in APS patients (**Fig 1C**).

Galectin-9 is a biomarker to detect the IFN signature in SLE and APS

A superior correlation of galectin-9 with the IFN score (r=0.70, p<0.001) as compared to CXCL-10 (r=0.52, p<0.001) and TNF-RII (r=0.46, p<0.001) was found (**Fig 2A**). ROC-curve analysis revealed the highest area under the curve (AUC, 0.86) for galectin-9, followed by CXCL-10 (0.78) and TNF-RII (0.75) for the detection of the IFN signature (**Fig 2B**) among patients with SLE or APS. When focusing on SLE(±APS) patients only, galectin-9 (AUC 0.84) remained a superior biomarker to detect the IFN signature compared with CXCL-10 (AUC 0.75) or TNF-RII (AUC 0.70) or traditional markers of SLE disease activity such as anti-dsDNA antibodies (AUC 0.70), or complement levels (C3: AUC 0.70, C4: AUC 0.78) (**Fig 2C**). El-



D

	Galectin-9	CXCL-10	TNF-RII	a-dsDNA	Complement
Sensitivity	84%	57%	48%	55%	62%
Specificity	72%	78%	67%	70%	60%
PPV	91%	89%	82%	85%	83%
NPV	59%	36%	29%	34%	34%

Figure 2: In SLE and APS Galectin-9 outperforms CXCL-10 and traditional markers for disease activity as biomarker for the IFN signature and is upregulated in dendritic cells

(A) Correlation of galectin-9, CXCL-10 and TNF-RII with the IFN score. (B) ROC-curve analysis of galectin-9, CXCL-10 and TNF-RII in the detection of the IFN signature in patients with SLE, SLE+APS and PAPS. (C) ROC-curve analysis of galactin-9, CXCL-10 and TNF-RII as compared with traditional markers of SLE disease activity in detecting the IFN signature among patients with SLE±APS. (D) Test characteristics of elevated galectin-9, CXCL-10, TNF-RII, anti-dsDNA antibodies or reduced complement levels to detect the IFN signature in patients with SLE(±APS) (E) Expression of galectin-9 coding gene LGALS9 in myeloid and plasmacytoid DCs as assessed by RNAseq in SLE and APS patients stratified by IFN status. (F) Expression of LGALS9 assessed by qPCR after in-vitro stimulation of DCs with IFNα for 3 hours (n=3/group).

Statistics: A) Spearman correlation coefficients. E) ANOVA with post-hoc Tukey's or Student-Test.

evated levels of galectin-9 had a sensitivity of 84% and a positive predictive value of 91% of detecting the IFN signature among patients with SLE(±APS) (**Fig 2D**). In multivariate linear regression analysis galectin-9 was an independent predictor for the IFN score, and remained significant after correction for anti-dsDNA antibodies, complement levels and disease activity.

Increased expression of galectin-9 in dendritic cells of IFN-positive SLE and APS patients

Galectin-9 is a β-galactoside-binding lectin, encoded by the gene LGALS9 which is highly expressed in dendritic cells[14]. Therefore, we explored the expression of LGALS9 in myeloid and plasmacytoid DCs (mDCs/pDCs) in SLE and APS. LGALS9 expression was markedly higher in circulating DCs of SLE and APS patients with an IFN signature and IFNα increased LGALS9 expression *in-vitro* in mDCs and pDCs (**Fig 2E**).

Discussion

Here we report that galectin-9, CXCL-10 and TNF-RII are elevated and associated with disease activity in SLE and APS. Notably, we identified galectin-9 as a robust and easy to measure biomarker to detect the IFN signature, outperforming traditional biomarkers for the IFN signature such as CXCL-10.

Measuring the IFN signature in patients with SLE might in the future be used in clinical decision making[8]. The necessity to quantify gene expression and the lack of a uniformly accepted gene set or scoring system hampers its implementation in clinical practice. In this respect galectin-9 is a promising stable biomarker, which is superior to CXCL-10 in the detection of the IFN signature, both in SLE and APS, and can furthermore be reliably detected in serum samples stored for long periods.

Besides serving as a biomarker, galectin-9 may play a role in the pathogenesis of SLE and APS. Galectin-9 induces the maturation of DCs by increasing the expression of HLA-DR and co-stimulatory molecules and galectin-9 produced by DCs activates T-cells[14]. Galectin-9 expression was markedly increased in DCs of IFN-positive SLE and APS patients and may therefore drive the unabated activation of DCs seen in these patients. Furthermore, in the pristane induced SLE mouse model, knock-out of LGALS9 results in reduced nephritis and arthritis[15], supporting a pathogenic role for galectin-9 in the pathogenesis of SLE.

The treatment of SLE and APS has remained largely unchanged for decades. The revolution in the treatment of other rheumatic conditions, most notably rheumatoid arthritis, in the last decades has not been paralleled in SLE and APS in which few clinical trials have met their primary outcome. A major reason for the failure of these trials is the molecular heterogeneity of these diseases. Consequently, for SLE and APS there is an urgent need for a more personalized treatment approach, guided by the molecular phenotype rather than clinical diagnosis. Treatment of patients on the basis of the presence of an IFN signature, could represent such a molecular phenotype, supported by the results from the anifrolumab trial[8]. Galectin-9 may serve as a robust and easy to measure biomarker to detect the IFN signature in patients with SLE and APS, as well as other (systemic) autoimmune diseases.

Acknowledgements

We would like to thank the Multiplex Core Facility of the Laboratory of Translational Immunology (UMC Utrecht) for performing the in-house developed and validated multiplex immunoassays.

References

1. van den Hoogen LL, van Roon JAG, Radstake TRDJ, *et al.* Delineating the deranged immune system in the antiphospholipid syndrome. *Autoimmun Rev* 2016;**15**:50–60. doi:10.1016/j.autrev.2015.08.011

2. Grenn RC, Yalavarthi S, Gandhi AA, *et al.* Endothelial progenitor dysfunction associates with a type i interferon signature in primary antiphospholipid syndrome. *Ann Rheum Dis* 2017;**76**:450–7. doi:10.1136/annrheumdis-2016-209442

3. Kirou KA, Lee C, George S, *et al.* Activation of the interferon-α pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. *Arthritis Rheum* 2005;**52**:1491–503. doi:10.1002/art.21031

4. Hoffman RW, Merrill JT, Alarcón-Riquelme MME, *et al.* Gene Expression and Pharmacodynamic Changes in 1,760 Systemic Lupus Erythematosus Patients From Two Phase III Trials of BAFF Blockade With Tabalumab. *Arthritis Rheumatol* 2017;**69**:643–54. doi:10.1002/art.39950

5. van den Hoogen LL, Fritsch-Stork RD, Versnel MA, *et al.* Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. *Ann Rheum Dis* 2016;**75**:e81. doi:10.1136/annrheumdis-2016-210485

6. Rose T, Grützkau A, Klotsche J, *et al.* Are interferon-related biomarkers advantageous for monitoring disease activity in systemic lupus erythematosus? A longitudinal benchmark study. *Rheumatology* 2017;**56**:1618–26. doi:10.1093/rheumatology/kex220

7. Lisney AR, Szelinski F, Reiter K, *et al.* High maternal expression of SIGLEC1 on monocytes as a surrogate marker of a type i interferon signature is a risk factor for the development of autoimmune congenital heart block. *Ann Rheum Dis* 2017;**76**:1476–80. doi:10.1136/annrheumdis-2016-210927

8. Furie R, Khamashta M, Merrill J, *et al.* Anifrolumab, an Anti-Interferon-Alpha Receptor Monoclonal Antibody, in Moderate to Severe Systemic Lupus Erythematosus. *Arthritis Rheumatol* 2017;**69**:376–86. doi:10.1002/art.39962

9. Rodero MP, Decalf J, Bondet V, *et al.* Detection of interferon alpha protein reveals differential levels and cellular sources in disease. *J Exp Med* 2017;**214**:1547–55. doi:10.1084/jem.20161451

10. Chiche L, Jourde-Chiche N, Whalen E, *et al.* Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type i and type ii interferon signatures. *Arthritis Rheumatol* 2014;**66**:1583–95. doi:10.1002/art.38628

11. Rose T, Grützkau A, Hirseland H, *et al.* IFNα and its response proteins, IP-10 and SIGLEC-1, are biomarkers of disease activity in systemic lupus erythematosus. *Ann Rheum Dis* 2013;**72**:1639–45. doi:10.1136/annrheumdis-2012-201586

12. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;**40**:1725. doi:10.1002/1529-0131(199709)40:9<1725::AID-ART29>3.0.CO;2-Y

13. Bellutti Enders F, Van Wijk F, Scholman R, *et al.* Correlation of CXCL10, tumor necrosis factor receptor type II, and galectin 9 with disease activity in juvenile dermatomyositis. *Arthritis Rheumatol* 2014;**66**:2281–9. doi:10.1002/art.38676

14. John S, Mishra R. Galectin-9: From cell biology to complex disease dynamics. *J Biosci* 2016;**41**:507–34. doi:10.1007/s12038-016-9616-y

15. Zeggar S, Watanabe KS, Teshigawara S, *et al*. Lgals9 deficiency attenuates nephritis and arthritis in pristane-induced lupus model of BALB/c mice. *Arthritis Rheumatol* Published Online First: 2018. doi:10.1002/art.40467

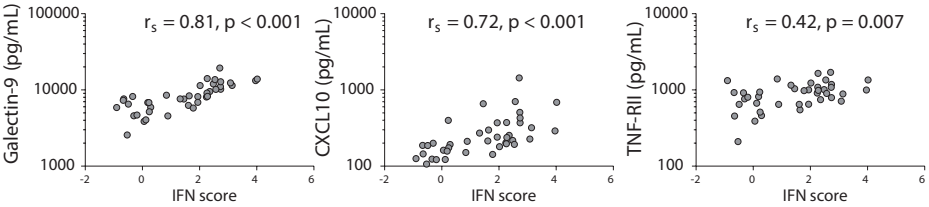
Supplementary data

Supplementary Table 1: correlation of serum cytokines with IFN score in patients with SLE and APS

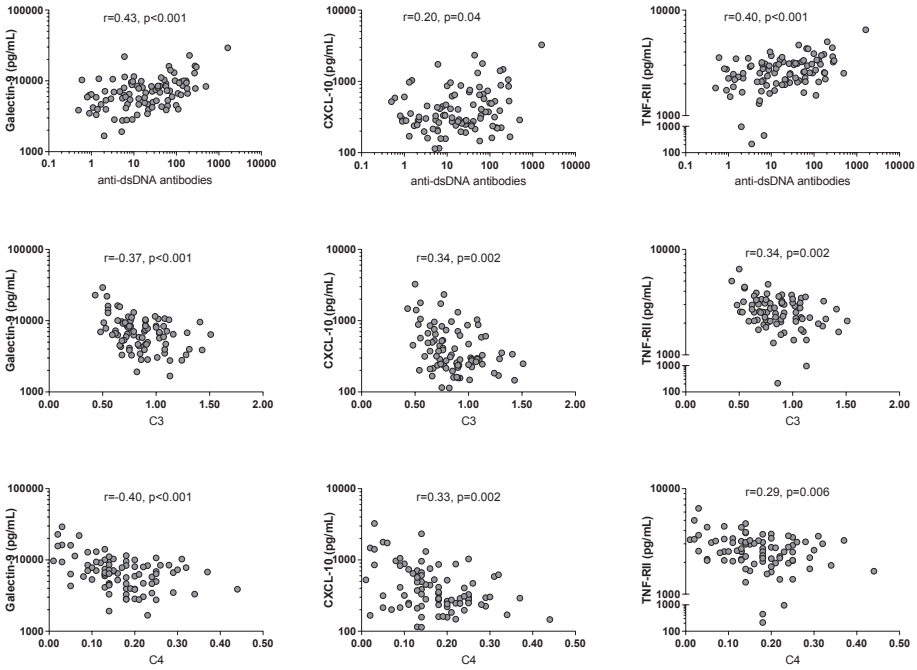
Analyte	Rho	p-value	FDR-correction
Galectin-9	0.8126	0.0001	0.001
CXCL-10	0.7182	0.0001	0.001
TNF-RII	0.4211	0.0068	0.050
CCL27	0.365	0.0206	ns
C5a	-0.3442	0.0319	ns
IL-18	0.3161	0.0469	ns
CCL2	0.3117	0.0503	ns
VCAM	0.1883	0.2446	ns
Galectin-3	0.1662	0.3054	ns
Endoglin	-0.1284	0.4296	ns
sVEGF-R1	-0.1254	0.4406	ns
Angiopoietin-1	-0.1056	0.5165	ns
CXCL-12	-0.0895	0.5829	ns
P-selectin	0.08828	0.588	ns
CCL22	0.0775	0.6346	ns
VEGF	-0.07102	0.6632	ns
Galectin-1	0.06914	0.6716	ns
CCL17	0.06295	0.6996	ns
ICAM	0.05291	0.7457	ns
CXCL-9	-0.05135	0.7562	ns
E-selectin	0.04808	0.7683	ns
CCL18	-0.03321	0.8388	ns

FDR: false discovery rate

Serum analytes were correlated with the IFN score as assessed by qPCR in an initial identification cohort (n=43) consisting of 7 HC, 16 patients with SLE, 11 patients with SLE+APS and 9 patients with primary APS. After correcting for multiple testing by FDR three analytes (galectin-9, CXCL-10 and TNF-RII) were found significantly correlated with the IFN score.



Supplementary figure 1: correlation of galectin-9, CXCL-10 and TNF-RII with the IFN score in the identification cohort.



Supplementary figure 2: Galectin-9, CXCL-10 and TNF-RII correlate with anti-dsDNA antibodies and complement levels in SLE

Supplementary Methods

Galectin-9 expression in dendritic cells

Plasmacytoid dendritic cells (pDC) and CD1c+ myeloid dendritic cells (mDC) were isolated from peripheral blood mononuclear cells (PBMCs) by positive selection using the BDCA4 or BDCA1 dendritic cell isolation kit respectively according to the manufacturer's instruction (Miltenyi). The purity of the isolated cells was assessed by flow cytometry using CD123 and BDCA2 or CD19 and BDCA1 fluorochrome labeled antibodies for pDC and mDC respectively showing a purity >85% for all samples. RNA was extracted using the Allprep Universal Kit (Qiagen) and used for RNAsequencing performed at BGI Tech solutions. RNA-seq libraries were generated with the TruSeq RNAseq RNA Library Prep Kit (Illumina) and sequenced on an Illumina HiSeq 4000 generating approximately 20 million 100bp paired-ended reads for each sample. The sample qualities were assessed by FastQC and the sequencing reads were aligned to human genome (GRCh38 build 79) using STAR aligner. Gene expression data for the annotated genes was generated using HTSeq-count. The variance-stabilizing transformation normalized expression values (normalized readcounts, NRC) for the genes were calculated using R package DESeq2.

For in vitro studies, 100.000 pDCs or mDCs were cultured for 3 hours in RPMI supplemented with 10% fetal calf serum in the presence or absence of interferon-alpha-2a (Cell Sciences) at a concentration of 1000U/μL. The expression of galectin-9 was assessed by real-time qPCR and normalized to the expression of GUSB. LGALS9 primers: CCGAGGAGAGGAAGACACAC, CCCGTTCAC-CATCACCTTGA and GUSB primers: CACCAGGGACCATCCAATACC, GCAGTCCAGCGTAGTTGAAAAA.

CHAPTER 5

Low density granulocytes are increased in the antiphospholipid syndrome and are associated with anti-β2GPI antibodies

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Dear Editor,

With great interest we read the article by Yalavarthi et al. providing evidence for aPL-induced NETosis as a novel prothrombotic mechanism in the antiphospholipid syndrome (APS) (1). In follow up of the impaired NET-degradation in APS patients that has been reported previously, the authors show that primary APS (PAPS) patients have more neutrophil extracellular traps (NETs) in their circulation, that neutrophils isolated from PAPS patients have an enhanced tendency to release NETs spontaneously and that antiphospholipid antibodies (aPL), more precisely anti-β2 glycoprotein I (aβ2GPI) antibodies, activate granulocytes and induce the release of NETs from healthy control neutrophils through stimulation of Toll-like receptor 4. As similar findings with respect to NETosis were previously observed in SLE (2), it seems that NETosis is affected in both APS and SLE patients, two diseases that share many features and are often both observed simultaneously in patients.

In patients with SLE and other inflammatory diseases a subset of neutrophils, termed low density granulocytes (LDGs) has been identified (2-4). LDGs are proinflammatory as shown by increased production of cytokines and are toxic to endothelial cells (3). Most notably, LDGs are extremely potent NET secretors, even in the absence of any triggering stimulus (5). In SLE patients the frequency of LDGs among peripheral blood mononuclear cells (PBMCs) correlates with the presence of circulating NETs (6). Yalavarthi et al. derive their findings from experiments in normal density neutrophils; in their supplementary data they report an unaltered frequency of LDGs in patients with PAPS, based on the assessment of a limited number of patients (PAPS: n=9, HC: n=7, SLE: n=1), possibly hampering the informative value due to patient heterogeneity and low statistical power.

We analyzed a large group of patients with SLE with and without aPL, SLE with APS (SLE+APS) and PAPS and observed a significantly increased frequency of LDGs in patients with SLE with aPL, SLE+APS and PAPS as compared to healthy controls (HC). Interestingly the frequency of LDGs in patients with SLE+APS and PAPS as compared to SLE patients without aPL was also significantly increased. SLE patients with aPL showed the same trend towards more LDGs as compared to SLE patients without aPL (Fig 1A).

APS patients with anti-β2GPI IgG antibodies had an increased LDG frequency as compared to APS patients without (Fig 1B), corroborating the correlation of anti-β2GPI IgG antibodies with the presence of circulating NETs in PAPS patients in the article by Yalavarthi et al. (suppl fig 3A). LDG percentages did not differenti-

ate between APS patients with arterial and/or venous thrombosis (Fig 1C). APS patients treated with vitamin K antagonists (VKA) had higher percentages of LDGs than APS patients not treated with VKA, although this might be related to more severe disease such as recurrent arterial events. No differences with respect to other treatments for APS including low dose aspirin (LDA), statins and hydroxychloroquine (HCQ) were observed (Fig 1D).

Therefore, we conclude that LDGs are increased in patients with APS and are associated with anti-β2GPI IgG antibodies and VKA use. LDGs thus are likely to form a novel source of NETs, contributing to the immunopathology of APS which warrants further investigation (7).

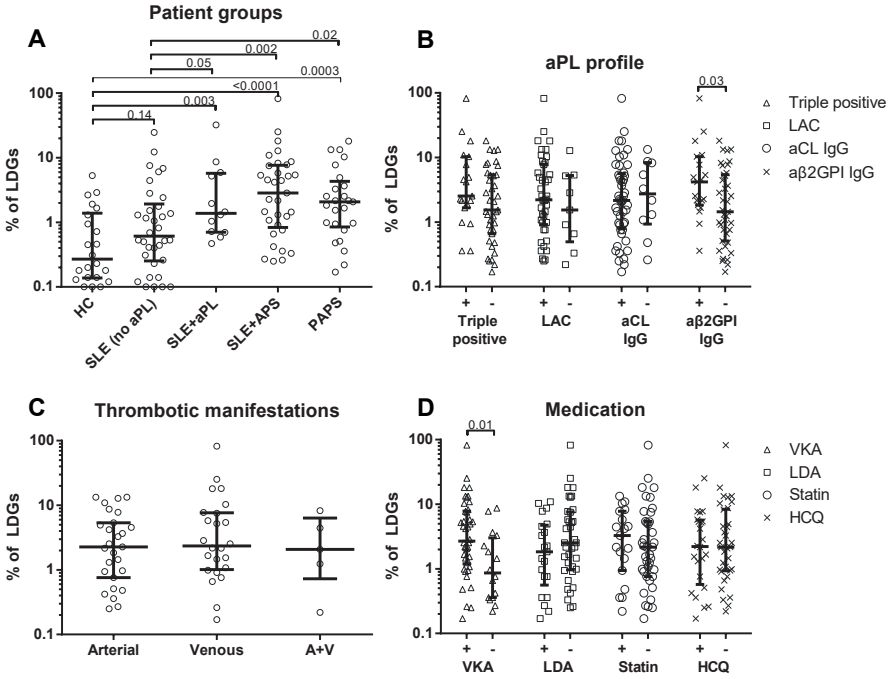


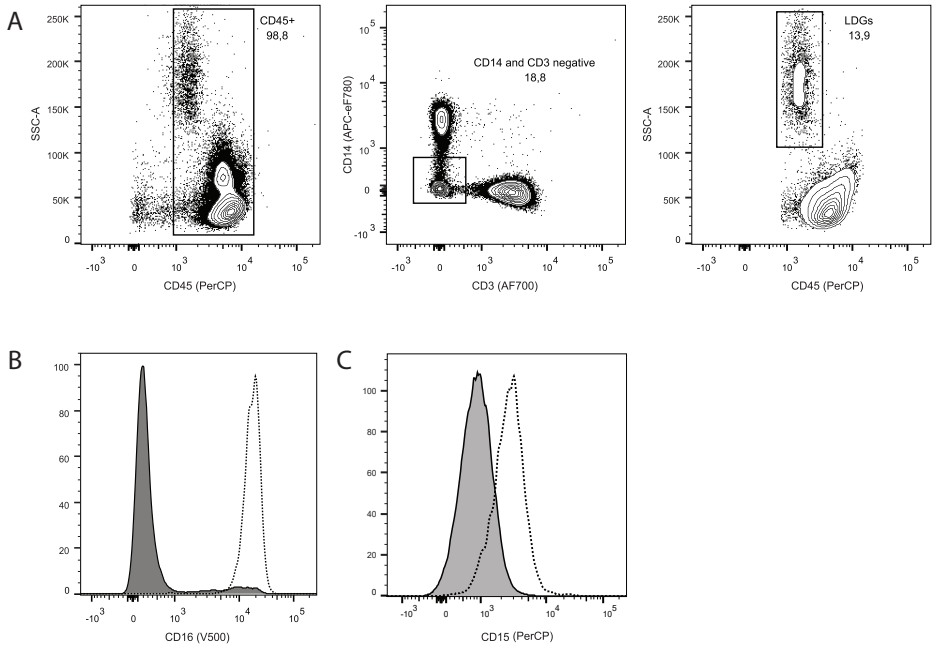
Figure 1. The frequency of LDGs is increased in APS patients, in particular in anti-β2GPI antibody positive patients and patients treated with vitamin K antagonists.

The frequency of LDGs was determined by flowcytometry in freshly isolated ficoll-hypaque separated PBMCs in 126 subjects, of whom 22 healthy controls (HC), 34 SLE patients without aPL (SLE no aPL), 12 SLE patients with aPL without APS (SLE+aPL), 33 patients with APS secondary to SLE (SLE+APS) and 25 patients with primary APS (PAPS) (A). All APS patients (n=58) were stratified according to their serological aPL profile (B), the history of arterial and/or venous thrombotic manifestations (C) and the use of medication (D). Median ± interquartile range, Mann Whitney test Abbreviations: Triple positive: positive for LAC and aCL (IgM or IgG) and aβ2GPI (IgM or IgG), LAC: lupus anticoagulant, aCL: anticardiolipin antibody, A+V: arterial and venous thrombosis.

References

1. Yalavarthi S, Gould TJ, Rao AN, Mazza LF, Morris AE, Núñez-Álvarez C, et al. Antiphospholipid antibodies promote the release of neutrophil extracellular traps: A new mechanism of thrombosis in the antiphospholipid syndrome. Arthritis Rheumatol 2015;67:2290-3003.
2. Grayson PC, Kaplan MJ. At the Bench: Neutrophil extracellular traps (NETs) highlight novel aspects of innate immune system involvement in autoimmune diseases (review). J Leukoc Biol 2015;99.
3. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. J Immunol 2010;184:3284–97.
4. Hacbarth E, Kajdacsy-Balla A. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. Arthritis Rheum 1986;29:1334–42.
5. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol 2011;187:538–52.
6. Zhang S, Lu X, Shu X, Tian X, Yang H, Yang W, et al. Elevated Plasma cfDNA May be Associated with Active Lupus Nephritis and Partially Attributed to Abnormal Regulation of Neutrophil Extracellular Traps (NETs) in Patients with Systemic Lupus Erythematosus. Intern Med 2014;53:2763–71.
7. van den Hoogen LL, van Roon JAG, Radstake TRDJ, Fritsch-Stork RDE, Derksen RHWM. Delineating the deranged immune system in the antiphospholipid syndrome (review). Autoimmun Rev 2016;15:50-60.

Supplementary data



Supplementary Figure 1: identification of LDGs among PBMCs in patients with APS

LDGs were identified from the single cell population by high side scatter signal and were negative for CD14. The percentage of LDGs was determined as the percentage of LDGs from the total of $CD45^+$ PBMCs (A). LDGs are positive for CD16 (dashed line) as compared to the total of $CD45^+$ PBMCs (grey filled area) (B). Although no surface marker distinguishes LDGs from normal density neutrophils, LDGs (dashed line) have a higher expression of CD15 as compared to autologous conventional density granulocytes (grey filled area) as described elsewhere [19,27] suggesting the correct identification of LDGs (C)

CHAPTER 6

Neutrophil extracellular trap release is associated with antinuclear antibodies in systemic lupus erythematosus and antiphospholipid syndrome

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[#]Timothy RDJ Radstake and Linde Meyaard contributed equally to this study.

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Abstract

Objectives

Increased release of neutrophil extracellular traps (NETs) is implicated in the activation of plasmacytoid dendritic cells, vascular disease and thrombosis in systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). However, studies comparing NET release between patients with SLE and APS are lacking. Here we evaluated plasma-induced NET release in a large cohort of patients with SLE, SLE+APS and primary APS in relation to clinical and serological parameters.

Methods

Neutrophils from healthy controls (HC) were exposed to plasma of heterologous HC (n=27), SLE (n=55), SLE+APS (n=38), or PAPS (n=28) patients and NET release was quantified by immunofluorescence. In a subset of SLE patients, NET release was assessed in longitudinal samples before and after a change in treatment.

Results

Plasma-induced NET release was increased in SLE and APS patients, with the highest NET release found in patients with SLE(±APS). Plasma of 60% of SLE, 61% of SLE+APS and 45% of PAPS patients induced NET release. NET release did not correlate with disease activity in SLE or APS. However, increased levels of antinuclear and anti-dsDNA autoantibodies were associated with increased NET release in SLE and APS. Only in SLE patients, elevated NET release and an increased number of low-density granulocytes were associated with a high interferon signature.

Conclusion

Increased NET release is associated with autoimmunity and inflammation in SLE and APS. Inhibition of NET release thus could be of potential benefit in a subset of patients with SLE and APS.

Keywords: Systemic Lupus Erythematosus, Antiphospholipid syndrome, Neutrophil extracellular traps, Autoantibodies, Interferon signature

Key messages:

- Plasma of ~60% of systemic lupus erythematosus (± antiphospholipid syndrome) and ~45% of primary antiphospholipid syndrome patients induce elevated neutrophil extracellular trap release compared to 7% of healthy controls
- Plasma-induced neutrophil extracellular trap release is associated with increased antinuclear and anti-dsDNA antibodies in systemic lupus erythematosus (± antiphospholipid syndrome) and primary antiphospholipid syndrome
- In systemic lupus erythematosus, but not antiphospholipid syndrome, plasma-induced neutrophil extracellular trap release is associated with the interferon signature

Introduction

Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) are overlapping autoimmune diseases that can occur separately or in the same patient. In SLE, immune complexes (IC) of autoantibodies are deposited into tissues leading to inflammation in several organs, including kidney, skin and joints. In APS, antiphospholipid antibodies (aPL) activate endothelial cells and trophoblasts resulting in thrombosis and pregnancy morbidity. APS is termed primary APS (PAPS) when no underlying disease such as SLE is present. In APS, most research has focussed on the pro-thrombotic role of aPL. However, research over the last years indicates an important role for immune cells in the pathogenesis of (P)APS, often in a similar fashion as in SLE, although studies that compare immunopathology between SLE, SLE+APS and PAPS patients are scarce [1].

There is a growing interest in the role of neutrophils in rheumatic diseases [2]. Neutrophils act as a first line of defence against infectious invaders by, amongst other strategies, the release of neutrophil extracellular traps (NETs). NETs consist of decondensed chromatin decorated with neutrophil derived proteases and antimicrobial peptides which trap and kill pathogens [3]. Neutrophils from SLE and PAPS patients are prone to release NETs spontaneously [4,5]. In addition, healthy neutrophils release NETs when stimulated *in vitro* with autoantibodies present in sera of SLE or APS patients [5,6]. Furthermore, DNase activity is decreased in SLE and APS resulting in increased NET exposure [2,7] and SLE and APS patients have increased numbers of circulating low-density granulocytes (LDG) [8,9], a subset of neutrophils prone to undergo NET formation. As a result, SLE and APS patients have elevated levels of NET remnants in the circulation [2,7] and NETs are present in affected tissues such as the skin or kidney in SLE or in aPL-induced thrombi [9,10].

Uncontrolled NET release triggers a pathological cascade of events relevant for the pathophysiology of SLE and APS. NETs induce tissue damage [2], activate the clotting system to promote thrombus formation [5], induce endothelial dysfunction [11] and represent a source of autoantigens [4]. Moreover, *in vitro,* NETs activate plasmacytoid dendritic cells (pDC) to produce interferon alpha (IFNα) [4,9,12] which might explain the interferon signature in SLE and APS patients [13].

Until now, NET release has only been studied in SLE and APS separately in small-scale studies. The different methodologies to induce and quantify NET release hamper the comparison across studies. Recently we developed a novel NET assay which specifically measures NET release as it distinguishes NET release from other forms

of neutrophil death while the automatic quantification avoids subjectivity [14]. Here, we employed our assay to investigate plasma-induced NET release in a large cohort of SLE, SLE+APS and PAPS patients in relation to clinical and serological parameters including the interferon signature.

Methods

A detailed description of the methods is described in the supplementary methods.

Study population

SLE, SLE+APS and PAPS patients and age/sex matched healthy controls (HC) were recruited from our outpatient clinic or in-house healthy donor service. PAPS patients did not meet classification criteria for SLE, nor had clinical evidence of SLE. None of the patients had evidence of an on-going infection. Patients were stratified by a high or low interferon signature as previously described [13]. The UMCU medical ethical committee approved this study and all study participants signed informed consent.

Quantification of NETs

HC neutrophils were cultured with 10% (heterologous) plasma of patients or controls for 4 hours in a 96-wells plate. NET release was quantified as previously described, with or without fixation after four hours. Sytox Green images were used to quantify NET area [14] (Fig.1A).

Statistical analysis

The NET area of 20 different microscopic fields per well was averaged. The mean of log-transformed NET areas per plasma donor of four independent experiments was reported as the mean NET area. The J-statistic of the Youden index of the Receiver Operating Characteristic (ROC) curve of NET release in patients (SLE, SLE+APS and PAPS) as compared with HC was used to define a cut-off to stratify patients into high or low NET inducers. Differences between groups were tested two-sided by ANOVA and Tukey's post-test or t-test as appropriate (α=0.05) using SPSS (v22).

Results

Validation of a high-throughput assay to measure plasma-induced NET release

Our live-imaging assay to monitor NET release over time revealed NET release within 30 min after exposure to SLE plasma (**Fig.1B**, videoS1). The presence of

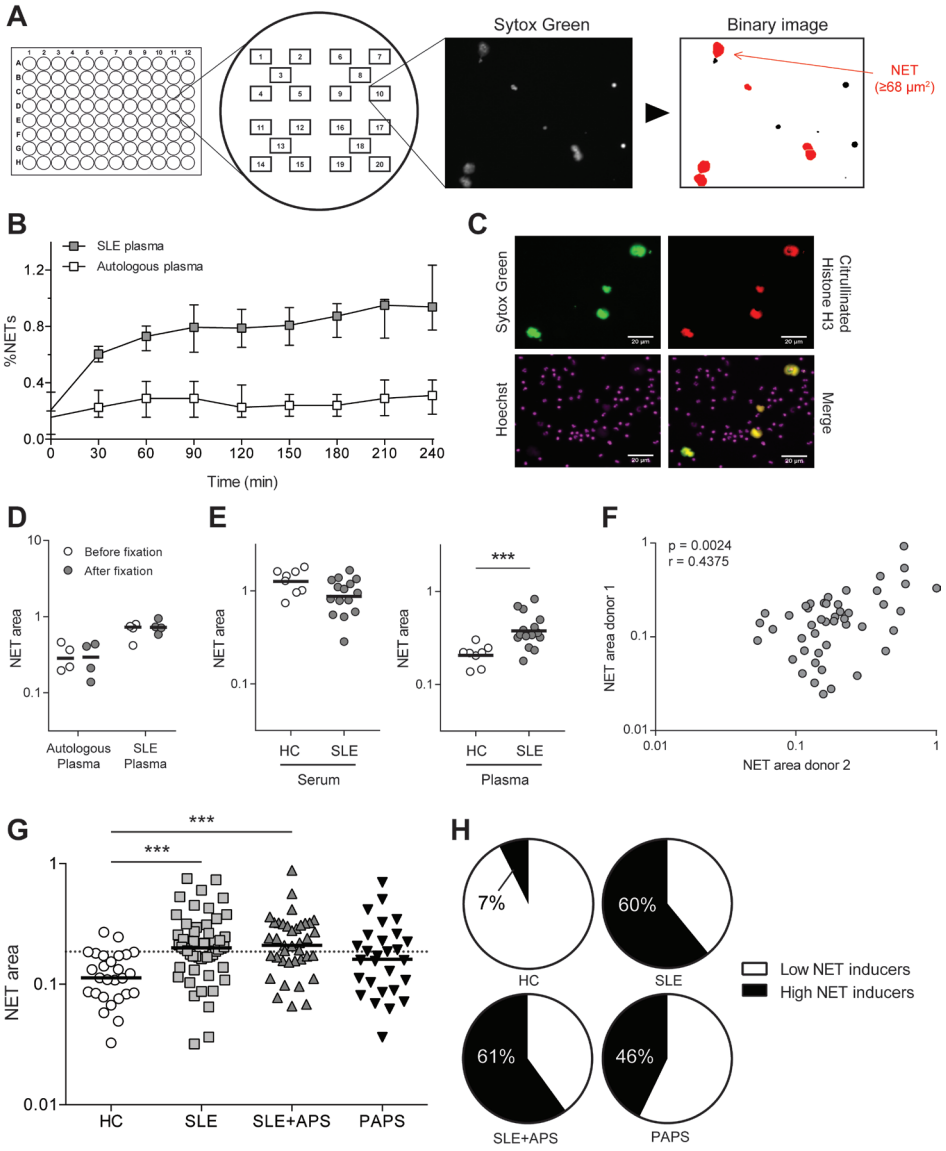

Figure 1: NET release in response to plasma of SLE, SLE+APS and PAPS patients.

(**A**) Experimental approach to measure NET release in a fixed time point. Twenty fields of view were captured per condition and Sytox Green images were used to analyse NET area. (**B**) Live imaging showed enhanced NET release of HC neutrophils when exposed to SLE plasma compared to autologous plasma. Data points represent median ± interquartile range of four independent experiments with four plasma samples each. (**C**) Citrullinated histone H3 staining confirmed the presence of actual NETs and (**D**) fixation after 4 hours did not affect NET quantification. In a pilot experiment, serum and plasma from HC (n=8) and SLE (n=15) patients was used to induce NET release in HC neutrophils. NET area of independent experiments with neutrophils from 3 different HC donors is pre-

sented. (**E**) Elevated NET release was shown in neutrophils exposed to plasma, not serum, from SLE patients compared with those exposed to plasma or serum from heterologous HC. (**F**) Plasma induced NET release correlated between independent experiments. (**G**) HC neutrophils exposed to heterologous plasma from SLE (n=55), SLE+APS (n=38) and PAPS (n=28) patients displayed increased NET release compared with those exposed to plasma from HC (n=27). NET area of independent experiments with neutrophils from 4 different HC donors is presented. (**H**) Prevalence of high NET release in patients with SLE, SLE+APS or PAPS. The data in **D**, **E** and **G** are presented as mean, $^{***}P<0.001$. The images in **C** are representative of at least three experiments with neutrophils from different donors.

citrullinated histone H3 in the extracellular DNA confirmed the formation of NETs (**Fig.1C**). To allow the measurement of >100 samples without a time difference between the first and last sample, we introduced a fixation step after 240 minutes, which did not affect the quantification of NETs (**Fig.1D**). Pilot experiments with plasma and serum samples from SLE (n=15) and HC (n=8), showed increased NET induction by plasma from SLE patients as compared with HC. Although serum of both patients and controls had higher NET-induction capacity than plasma, no difference was observed between SLE and HC (**Fig.1E**). In these pilot experiments, we observed a moderate (r=0.4375, p=0.002) correlation of NET release between independent experiments using different neutrophil donors (**Fig.1F**).

Increased plasma-induced NET release in SLE, SLE+APS and PAPS

We next used plasma samples of HC (n=27), SLE (n=55), SLE+APS (n=38) and PAPS (n=28, **Supplementary Table 1**) patients to induce NET release in neutrophils of four HC donors in four independent experiments. Confirming our pilot experiments, the mean NET release of four independent experiments was higher using plasma from SLE and SLE+APS patients as compared with HC plasma (p<0.001), with a similar trend when using PAPS plasma ((p=0.14, Fisher's LSD: p=0.03) **Fig.1G, Fig.S1**). NET release did not differ among SLE, SLE+APS or PAPS patients (ANOVA, p=0.19). Setting a threshold by ROC-curve analysis (**Fig. S2**), plasma samples from 33/55 (60%) of SLE, 23/38 (61%) of SLE+APS and 13/28 (46%) of PAPS patients had high NET release (**Fig.1H**), as compared with 2/27 (7%) of HC plasmas. Thus, our data show that plasma from the majority of SLE and APS patients induces NET release.

NET release did not correlate with clinical measures of disease activity, including SELENA-SLEDAI for SLE(±APS) patients (p=0.57, **Fig.S2A**) and the adjusted global anti-phospholipid syndrome score (aGAPSS) for (P)APS patients (p=0.88, **Fig.S2B**). Furthermore, there were no significant differences among clinical phenotypes including (active) lupus nephritis in SLE patients or APS patients with or without arterial or venous thrombosis or pregnancy morbidity. Also, NET release

did not differ between patients treated with or without prednisolone, azathioprine, aspirin or other immunosuppressants (*P*>0.05, data not shown).

Plasma-induced NET release is associated with antinuclear and anti-dsDNA antibodies

In vitro studies implicate NET release as a source of autoantigens eliciting the production of autoantibodies against nuclear components in SLE [5]. In line with these observations, SLE patients with high NET release had increased levels of anti-dsDNA antibodies compared to patients whose plasma induced low NET release (p=0.008, **Fig.2A**). Likewise, PAPS patients with high NET release had elevated ANA staining intensities (p<0.05, **Fig.2B**). In longitudinal samples, collected from SLE patients before and after a change in immunosuppressive therapy, a decline in anti-dsDNA antibodies (p=0.02, **Fig.2C**) was paralleled by a decline in NET release (p=0.03, **Fig.2D**), whereas patients with stable or increasing anti-dsDNA antibodies (**Fig.2E**) between two time points did not have a decrease in plasma-induced NET release (p=0.48, **Fig.2F**). Among SLE or APS patients, no specific association between the presence or absence of anti-β2 Glycoprotein I and anti-ribonucleoprotein (RNP) antibodies was observed.

NET kinetics is similar in SLE and PAPS

We previously showed that the kinetics of NET release differs between stimuli [14]. We observed no difference in the kinetics of plasma-induced NET release among high-inducing plasma samples of SLE or PAPS patients (**Fig.2G**). In comparison to patient plasma, exposure of neutrophils to immune complexes induced abundant NETs, approximately 30% compared to 3% in patient plasma samples. DPI inhibits, amongst others, NADPH oxidase and NET release in response to immune complexes was inhibited by 60-70% in the presence of DPI while PAPS and SLE plasma-induced NET release was not inhibited in the presence of DPI (**Fig.2H**).

NET release and low-density granulocytes are associated with the IFN signature in SLE

In vitro experiments implicate NETs and LDGs as a trigger for IFNα production by pDC [4,9], although no studies have explored NET release in relation to the presence or absence of the interferon signature. SLE patients with a high interferon signature (IFN-high) had higher NET release than patients with a low interferon signature (IFN-low) (p<0.01, **Fig.2I**). Corroborating this finding we observed that IFN-high SLE patients had increased numbers of circulating LDGs (p<0.01, **Fig.2J**). Interestingly, these associations were not seen in APS patients, neither in SLE+APS nor in PAPS (p>0.05).

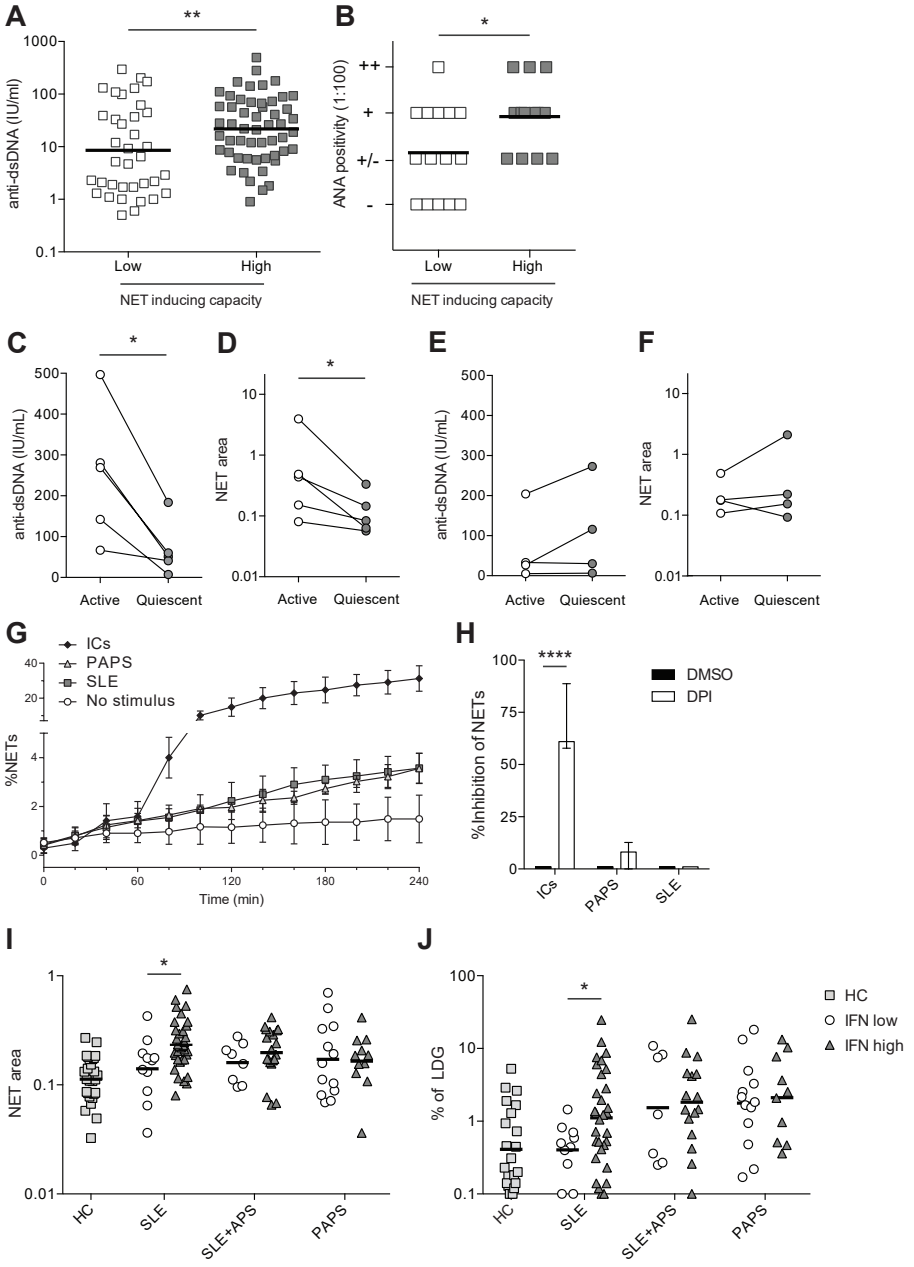

Figure 2: Plasma-induced NET release is associated with antinuclear antibody levels in plasma SLE and PAPS

(A) SLE patients classified as high NET inducers contained elevated levels of anti-dsDNA antibodies, compared with those that are classified as low NET inducers. (**B**) PAPS patients classified as high NET inducers contained increased ANA staining intensities, compared with those that are classified

as low NET inducers (- negative, +/- weak, + positive, ++ strongly positive). Longitudinal samples were collected from SLE patients at time of active disease as well as subsequent quiescent disease. (**C**) Anti-dsDNA antibody level and (**D**) NET area was increased in SLE patients (n=5) with an active disease compared with those with a quiescent disease state. Patients (n=4) with (**E**) stable or increasing anti-dsDNA antibodies between two time points (**F**) did not have a decrease in plasma-induced NET release. (**G**) NET release in response to IC was high compared to SLE and APS plasma-induced NET release. Data points represent mean ± SD of independent experiments with neutrophils of three HC donors and four plasma samples per group. (**H**) DPI, NADPH oxidase inhibitor, supressed IC-induced NET release while SLE and PAPS plasma-induced NET release was independent of NADPH oxidase. The percentage of inhibition of NET release was calculated based on the area under the curve relative to neutrophils exposed to IC, PAPS or SLE plasma in the presence of DMSO. (**I**) Plasma from SLE patients classified as high IFN signature displayed elevated levels of NET release compared to those that were classified as low IFN signature. (**J**) Increased amount of LDGs were present in SLE IFN high patients compared with SLE IFN low patients. Differences of LDG amount between high and low IFN patients were not shown in SLE+APS and PAPS patients. The data in **A**, **B**, **H**, **I** and **J** are presented as mean, $*P<0.05$ and $**P<0.005$.

Discussion

Using a novel high throughput assay we show that plasma of SLE, SLE+APS and PAPS patients induces NET release, which is associated with antinuclear antibodies in PAPS and anti-dsDNA autoantibodies and the IFN signature in SLE patients. This study highlights the potential role of NET release in relation to autoimmunity and inflammation in SLE and APS and compares NET release in a large cohort of SLE and APS patients.

Previous studies have shown induction of NET release using serum of SLE or APS patients [6,15]. In small pilot studies, when comparing serum with plasma, serum induced a higher release of NETs than plasma, both in patients and HC, and no difference between HC and patients was observed. The generation of serum leads to platelet activation, which is a strong NET inducer [16] and is a likely cause for the higher NET release induced by serum samples. As a result, to avoid potential effects of platelet activation, we used patient plasma to trigger NET release in further experiments. Importantly, although similar trends were observed when using different neutrophil donors, the amount of NETs formed differed between neutrophil donors and therefore our results stress the need to use different neutrophil donors when studying NET release [16].

Besides the amount of NETs, the kinetics of NET release differ between stimuli [14]. We observed a rapid release of NETs (within 30 minutes) upon exposure to patient plasma in both SLE and PAPS patients. As the composition of NETs differs between stimuli [17], we speculate that the content of NETs could differ between

SLE and APS, since NET release was differentially associated with the IFN signature in SLE and APS.

NET release in the context of SLE and APS has been mainly studied using purified antibodies or cytokines to trigger NET release, including anti-β2 Glycoprotein I, anti-RNP, anti-human neutrophil protein (HNP), anti-LL37, anti-matrix metalloproteinase 9 antibodies and interleukin-18 and hyperacetylated microparticles [4–6,11,12,18–20]. As multiple factors present in patient plasma may induce NET release, it is unknown which stimulus is responsible for NET release in our assay, however the association of autoantibodies and the IFN signature with NET release suggest their involvement and this is clearly different in plasma from healthy individuals. Nevertheless NET release by purified factors should be interpreted with caution since the concentration and composition of these factors in patients' plasma might be different. Indeed, in our study NET release in response to immune complexes was much higher than in response to patient plasma. Moreover, immune complex-induced NET release is dependent on NAPDH oxidase, in contrast to plasma-induced NET release, although it is unknown whether the magnitude of NET release *in vitro* can be directly translated to *in vivo* situations.

Enhanced NET release is considered a major pathogenic factor linked to tissue damage, the interferon signature and other disease manifestations in both SLE and APS [4,5,10]. Consistent with this, we report increased NET release in patients with elevated autoantibodies or the interferon signature in SLE and APS. Treatment options that mitigate NET release could therefore be of added clinical value. Inhibition of NET release ameliorates mouse models of SLE and APS [10,21]. Several small inhibitory molecules reduce NET release *in vivo* [22] while hydroxychloroquine, a treatment for SLE, inhibits NET release *in vitro* [2]. We previously reported that triggering the inhibitory receptor SIRL-1 attenuates SLE plasma and autoantibody-induced NET release [6]. Our current results indicate that only a subset of patients, i.e. ~60% of SLE(±APS) and ~45% of PAPS patients would benefit from inhibiting NET release.

References

1. L.L. van den Hoogen, J.A.G. van Roon, T.R.D.J. Radstake, R.D.E. Fritsch-Stork, R.H.W.M. Derksen, Delineating the deranged immune system in the antiphospholipid syndrome., Autoimmun. Rev. 15 (2016) 50–60. doi:10.1016/j.autrev.2015.08.011.

2. P.C. Grayson, C. Schauer, M. Herrmann, M.J. Kaplan, Neutrophils as Invigorated Targets in Rheumatic Diseases, Arthritis Rheumatol. 68 (2016) 2071–2082. doi:10.1002/art.39745.

3. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria., Science. 303 (2004) 1532–5. doi:10.1126/science.1092385.

4. R. Lande, D. Ganguly, V. Facchinetti, L. Frasca, C. Conrad, J. Gregorio, S. Meller, G. Chamilos, R. Sebasigari, V. Riccieri, R. Bassett, H. Amuro, S. Fukuhara, T. Ito, Y.-J. Liu, M. Gilliet, Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus., Sci. Transl. Med. 3 (2011) 73ra19. doi:10.1126/scitranslmed.3001180.

5. S. Yalavarthi, T.J. Gould, A.N. Rao, L.F. Mazza, A.E. Morris, C. Núñez-Álvarez, D. Hernández-Ramírez, P.L. Bockenstedt, P.C. Liaw, A.R. Cabral, J.S. Knight, Antiphospholipid antibodies promote the release of neutrophil extracellular traps: A new mechanism of thrombosis in the antiphospholipid syndrome, Arthritis Rheumatol. 67 (2015) 2290–3003. doi:10.1002/art.39247.

6. K. Van Avondt, R. Fritsch-Stork, R.H.W.M. Derksen, L. Meyaard, Ligation of signal inhibitory receptor on leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus., PLoS One. 8 (2013) e78459. doi:10.1371/journal.pone.0078459.

7. A. Hakkim, B.G. Fürnrohr, K. Amann, B. Laube, U.A. Abed, V. Brinkmann, M. Herrmann, R.E. Voll, A. Zychlinsky, Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis., Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 9813–8. doi:10.1073/pnas.0909927107.

8. L. van den Hoogen, R.D.E. Fritsch-Stork, J.A.G. van Roon, T.R.D.J. Radstake, Low density granulocytes are increased in the antiphospholipid syndrome and are associated with anti-β2GPI antibodies, Arthritis Rheumatol. 68 (2016) 1320–1321. doi:doi: 10.1002/art.39576.

9. E. Villanueva, S. Yalavarthi, C.C. Berthier, J.B. Hodgin, R. Khandpur, A.M. Lin, C.J. Rubin, W. Zhao, S.H. Olsen, M. Klinker, D. Shealy, M.F. Denny, J. Plumas, L. Chaperot, M. Kretzler, A.T. Bruce, M.J. Kaplan, Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus., J. Immunol. 187 (2011) 538–52. doi:10.4049/jimmunol.1100450.

10. H. Meng, S. Yalavarthi, Y. Kanthi, L.F. Mazza, M. a. Elfline, C.E. Luke, D.J. Pinsky, P.K. Henke, J.S. Knight, In Vivo Role of Neutrophil Extracellular Traps in Antiphospholipid Antibody–Mediated Venous Thrombosis, Arthritis Rheumatol. 69 (2017) 655–667. doi:10.1002/art.39938.

11. C. Carmona-Rivera, W. Zhao, S. Yalavarthi, M.J. Kaplan, Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2., Ann. Rheum. Dis. (2014) 1–8. doi:10.1136/annrheumdis-2013-204837.

12. G.S. Garcia-Romo, S. Caielli, B. Vega, J. Connolly, F. Allantaz, Z. Xu, M. Punaro, J. Baisch, C. Guiducci, R.L. Coffman, F.J. Barrat, J. Banchereau, V. Pascual, Netting neutrophils are major inducers of type I IFN

production in pediatric systemic lupus erythematosus., Sci. Transl. Med. 3 (2011) 73ra20. doi:10.1126/scitranslmed.3001201.

13. L.L. van den Hoogen, R.D. Fritsch-Stork, M.A. Versnel, R.H. Derksen, J.A. van Roon, T.R. Radstake, Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use, Ann. Rheum. Dis. 75 (2016) e81. doi:10.1136/annrheumdis-2016-210485.

14. M. van der Linden, G.H. a. Westerlaken, M. van der Vlist, J. van Montfrans, L. Meyaard, Differential Signalling and Kinetics of Neutrophil Extracellular Trap Release Revealed by Quantitative Live Imaging, Sci. Rep. 7 (2017) 6529. doi:10.1038/s41598-017-06901-w.

15. T. Kraaij, F.C. Tengström, S.W.A. Kamerling, C.D. Pusey, H.U. Scherer, R.E.M. Toes, T.J. Rabelink, C. Van Kooten, Y.K.O. Teng, Autoimmunity Reviews A novel method for high-throughput detection and quanti fi cation of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes, Autoimmun. Rev. 15 (2016) 577–584. doi:10.1016/j.autrev.2016.02.018.

16. T. Hoppenbrouwers, A.S. a. Autar, A.R. Sultan, T.E. Abraham, W. a. Van Cappellen, A.B. Houtsmuller, W.J.B. Van Wamel, H.M.M. Van Beusekom, J.W. Van Neck, M.P.M. De Maat, In vitro induction of NETosis: Comprehensive live imaging comparison and systematic review, PLoS One. 12 (2017) 1–29. doi:10.1371/journal.pone.0176472.

17. R. Khandpur, C. Carmona-Rivera, A. Vivekanandan-Giri, A. Gizinski, S. Yalavarthi, J.S. Knight, S. Friday, S. Li, R.M. Patel, V. Subramanian, P. Thompson, P. Chen, D. a Fox, S. Pennathur, M.J. Kaplan, NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis., Sci. Transl. Med. 5 (2013) 178ra40. doi:10.1126/scitranslmed.3005580.

18. J.M. Kahlenberg, C. Carmona-Rivera, C.K. Smith, M.J. Kaplan, Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages., J. Immunol. 190 (2013) 1217–26. doi:10.4049/jimmunol.1202388.

19. Rother N, Pieterse E, Lubbers J, Hilbrands L, van der Vlag J. Acetylated Histones in Apoptotic Microparticles Drive the Formation of Neutrophil Extracellular Traps in Active Lupus Nephritis. Front Immunol. 2017;8:1136. doi:10.3389/fimmu.2017.01136

20. Dieker J, Tel J, Pieterse E, Thielen A, Rother N, Bakker M, et al. Circulating Apoptotic Microparticles in Systemic Lupus Erythematosus Patients Drive the Activation of Dendritic Cell Subsets and Prime Neutrophils for NETosis. Arthritis Rheumatol. 2016;68(2):462-72. doi:10.1002/art.39417

21. C. Lood, L.P. Blanco, M.M. Purmalek, C. Carmona-Rivera, S.S. De Ravin, C.K. Smith, H.L. Malech, J. a Ledbetter, K.B. Elkon, M.J. Kaplan, Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease., Nat. Med. 22 (2016) 146–53. doi:10.1038/nm.4027.

22. M. van der Linden, L. Meyaard, Fine-tuning neutrophil activation: Strategies and consequences, Immunol. Lett. 178 (2016) 3–9. doi:10.1016/j.imlet.2016.05.015.

Supplementary Methods

Study population

Patients were classified as either SLE or APS when fulfilling their respective classification criteria [1,2]. Patients that fulfilled both sets of criteria were classified as SLE+APS whereas APS patients that did not fulfil the classification criteria for SLE or another rheumatic disease were classified as PAPS. Within 2 hours of collection whole blood was centrifuged for 10 minutes at room temperature at 1500xg and plasma (from lithium-heparin vacutainers) or serum (from serum tubes) were stored at -80°C until further use. Disease activity was scored by SELENA-SLEDAI in patients with SLE and by the adjusted global antiphospholipid syndrome score (aGAPSS) [3] in patients with APS. Anti-dsDNA antibodies were measured by ELiA (Euroimmun), antinuclear antibodies were tested by indirect immunofluorescence on HEP-2000 cells at a serum dilution of 1:100. Results were reported as negative, weakly positive, positive or strongly positive. All study participants signed informed consent approved by the Medical Research Ethics Committee University Medical Centre (UMC) Utrecht review broad prior to the donation of blood in accordance with the declaration of Helsinki.

Preparation of Immune Complexes

Insoluble immune complexes were formed by using human serum albumin (HSA; Sanquin) and rabbit polyclonal anti-HSA IgG (Sigma Aldrich) as described previously [4]. Briefly, a mix of 5 μg HSA and 45 μg rabbit-anti-HSA antibody was made in a final volume of 50 μl PBS. After 1 h incubation at 37 °C, insoluble ICs were formed and used for stimulation of neutrophils in a concentration of 10 μg/ml.

Quantification of the IFN signature

The IFN signature was quantified on isolated monocytes as previously described [5]. In brief, PBMC were isolated by ficol density gradient separation. Subsequently, monocytes were isolated by CD14+ selection using the autoMACS pro (Miltenyi). RNA was extracted using the all-prep universal kit (Qiagen) and complementary DNA was generated using iScript (Biorad). qPCR using predesigned primer/probe sets (Thermofisher) on the following type I IFN inducible genes: IFI44L, Serping1, IFITM1 and Ly6E and normalized to the expression of GUSB. Normalized gene-expression values were used to calculate a type I IFN score as previously described [6].

Isolation of human neutrophils

Neutrophils from healthy controls were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation, after which erythrocytes were lysed in ammonium

chloride buffer (155 mM NH4Cl; 10 mM KHCO3; 0.1 mM EDTA in double-distilled H2O; pH = 7.2). Cells were resuspended in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) heat-inactivated (HI) foetal bovine serum (FBS; Biowest) and 50 U/ml Penicillin-Streptomycin (Life Technologies).

Microscopic imaging of NET release

NET release by human neutrophils was analysed by a quantitative live imaging NET assay as described before [4]. Briefly, nuclear DNA of neutrophils was stained with Hoechst 33342 (AnaSpec Inc.) and neutrophils were challenged with 10 μg/ml immune complexes or 10% (v/v) plasma from HC and SLE patients in medium containing Sytox Green (Life Technologies). Autologous plasma was used as a negative control. Where indicated, neutrophils were pre-treated with 1 μM DPI for 30 min at 37°C. NETs were confirmed to be positive for citrullinated histone H3. NET release was recorded on the Pathway 855 bioimaging system (BD Biosciences) with a 10x objective. A set of two images (Hoechst and Sytox Green) was taken with an Orca high-resolution CCD camera and four fields of view per condition were captured. The system was controlled by the AttoVision software (version 1.7/855).

For fixed time point NET release, neutrophils were incubated in RPMI 1640 (without phenol red) supplemented with 2% FBS, 50 U/ml Pen-Strep and 10 mM HEPES (Life Technologies) containing 20 μM Hoechst 33342 for 30 min at 37°C. A total of 1 x 105 neutrophils seeded in 0.001% poly-L-lysine (Sigma-Aldrich) pre-coated wells of a clear bottom 96-wells plate (Ibidi) and incubated with plasma from (heterologous, age-sex matched) HC, SLE, SLE+APS and PAPS patients for 3 hours and 45 min at 37°C and 5% CO2. PMA was used as a positive control for NET release. Extracellular DNA was stained with 0.2 μM Sytox Green for 15 min at 37°C and fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 min at room temperature. Fixed NETs were imaged with a 10x objective. A set of two images (Hoechst and Sytox Green) was taken and twenty fields of view per condition were captured.

Semi-automatic NET quantification

Quantification of live imaging NET release was performed as described previously [4]. For automatic quantification of fixed time point NETs, a macro was created that contains the following steps: 1) Convert Sytox Green images in 8-bit grey scale images, 2) Thresholding with "Li" logarithm, 3) Watershed segmentation and 4) Analysis of NETs (>68 μm2). A schematic overview of the procedure of the macro is presented in figure 1C. The quantification approach was controlled by Fiji software (version 2.0.0-rc-43/1.51d). NET area, the percentage of pixels per microscopic

field of view that have been highlighted after thresholding, was log transformed and presented on the y-axes.

Identification of low-density granulocytes

Freshly isolated PBMCs were stained with CD14-APC-eF780 (eBioscience), CD45-PerCP (Biolegend) and CD16-V500 (BD). Samples were acquired on a LSR fortessa (BD). From the single cell population LDGs were defined as CD45dim cells with a high side scatter signal and negative for CD14 and reported as percentage of PBMC.

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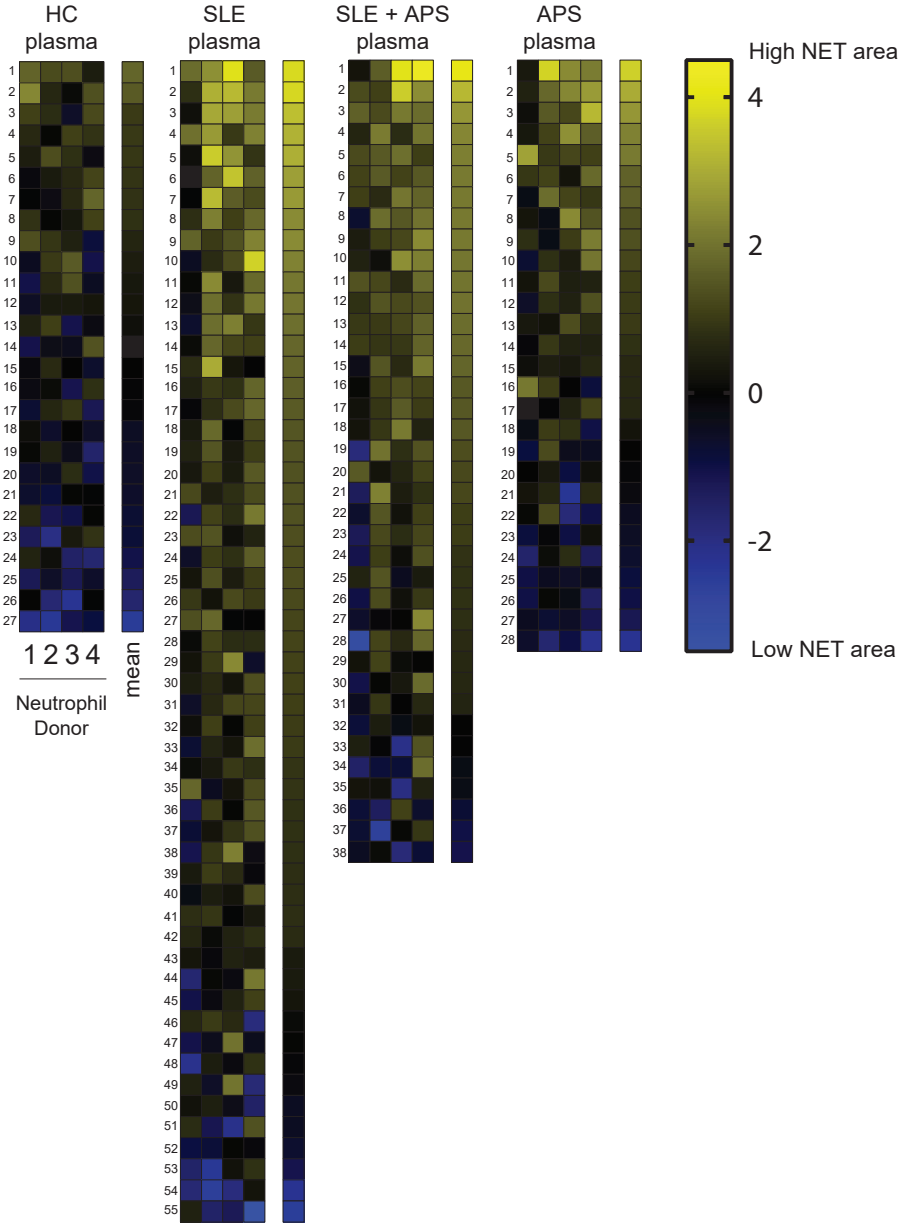
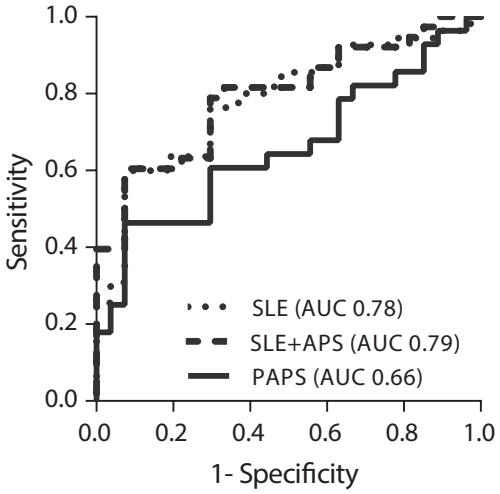
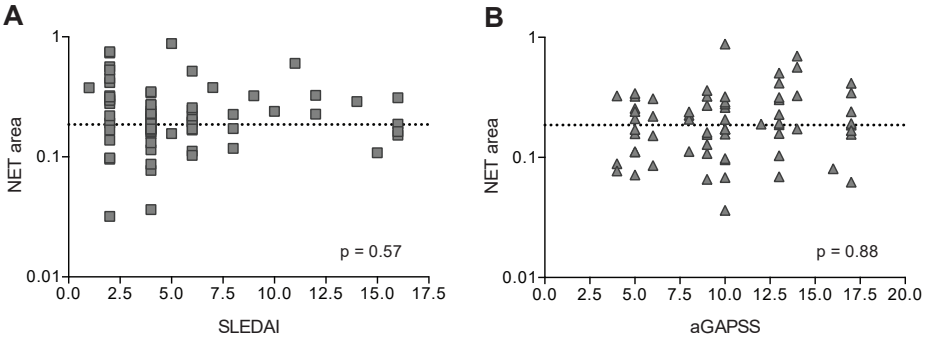


Supplementary figure 1: heatmap of NET-release in 4 independent HC neutrophil donors when stimulated with plasma from patients with SLE, SLE+APS and PAPS or heterologous HC.



Supplementary figure 2: ROC-curve analysis of NET-release in patients with SLE, SLE+APS and PAPS as compared with HC.



Supplementary Figure 3: Correlation of NET release with SLEDAI or aGAPSS in patients with SLE or APS respectively

Supplementary references:

1. M.C. Hochberg, Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus, Arthritis Rheum (1997) Sep;40(9):1725.
2. S. Miyakis, M.D. Lockshin, T. Atsumi, D.W. Branch, R.L. Brey, R. Cervera, R.H. Derksen, P.G. de Groot, T. Koike, P.L. Meroni, G. Reber, Y. Shoenfeld, A. Tincani, P.G. Vlachoyiannopoulos, S.A. Krilis, International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS), J Thromb Haemost (2006) Feb;4(2):295-306.
3. S. Sciascia, G. Sanna, V. Murru, D. Roccatello, M.A. Khamashta, M.L. Bertolaccini, The global anti-phospholipid syndrome score in primary APS, Rheumatology (Oxford) (2015) Jan;54(1):134-8.
4. M. van der Linden, G.H. a. Westerlaken, M. van der Vlist, J. van Montfrans, L. Meyaard, Differential Signalling and Kinetics of Neutrophil Extracellular Trap Release Revealed by Quantitative Live Imaging, Sci. Rep. 7 (2017) 6529.
5. L.L. van den Hoogen, R.D. Fritsch-Stork, M.A. Versnel, R.H. Derksen, J.A. van Roon, T.R. Radstake, Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use, Ann. Rheum. Dis. 75 (2016) e81.
6. M. Petri, S. Singh, H. Tesfasyone, R. Dedrick, K. Fry, P. Lal, G. Williams, J. Bauer, P. Gregersen, T. Behrens, E. Baechler, Longitudinal expression of type I interferon responsive genes in systemic lupus erythematosus, Lupus (2009) Oct;18(11):980-9.

CHAPTER 7

Increased B-cell activating factor BAFF / B-lymphocyte stimulator BLyS in primary antiphospholipid syndrome is associated with higher adjusted global antiphospholipid syndrome scores

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submitted for publication

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by antiphospholipid antibodies (aPL), thrombosis and obstetric complications. Other manifestations seen in APS include thrombocytopenia, heart valve disease and leg ulcers. APS was first described in patients suffering from systemic lupus erythematosus (SLE). However, APS may also affect patients not suffering from SLE or another underlying disease, termed primary APS (PAPS). Current treatment strategies for APS relying on anticoagulants are suboptimal, particularly for non-thrombotic manifestations and immunomodulatory drugs have been suggested as potential novel drug candidates in APS[1,2].

B-cell activating factor (BAFF), also known as B-lymphocyte stimulator (BLyS) is an important growth factor for B-cells. The importance of BAFF in SLE is supported by animal models as mice transgenic for BAFF spontaneously develop SLE-like autoimmunity including the production of antibodies against double stranded DNA (anti-dsDNA)[3]. In humans, patients with SLE have elevated levels of BAFF which correlate with disease activity[4,5]. Moreover, belimumab, a monoclonal antibody against BAFF is approved for the treatment of SLE and several other BAFF targeting therapies are under development for the treatment of SLE[4].

In contrast to SLE, little is known on the role of BAFF in APS. Case reports suggest a beneficial effect of belimumab in the treatment of non-thrombotic manifestations of PAPS patients [6] and blockade of the BAFF-receptor BAFF-R prevents the development of APS in a murine model for APS [7]. Post-hoc analyses of the belimumab trials reveal that belimumab reduces anticardiolipin antibodies in patients with SLE[8]. As a result, belimumab is a promising drug candidate for APS. However, no studies are available that compare the expression of BAFF and its receptors in patients with SLE and APS.

We measured BAFF levels by ELISA in sera of patient with SLE, SLE+APS and PAPS (**Table 1**) and found increased serum levels of BAFF in patients with PAPS compared to healthy controls (HC), similar as in patients with SLE and SLE+APS (all $p<0.05$, **Fig 1A**). Setting a threshold using the mean plus two standard deviations of HC, the prevalence of increased BAFF levels in PAPS was 7/29 (24%) as compared with 21/54 (39%) and 17/40 (43%) in SLE and SLE+APS patients respectively.

The adjustged global antiphospholipid syndrome score (aGAPSS) is a validated tool for risk stratification in patients with PAPS[9] and serum levels of BAFF correlated with higher aGAPSS scores in patients with PAPS ($r=0.40$, $p=0.03$) (**Fig 1B**). In patients with SLE, serum levels of BAFF significantly correlated with higher levels

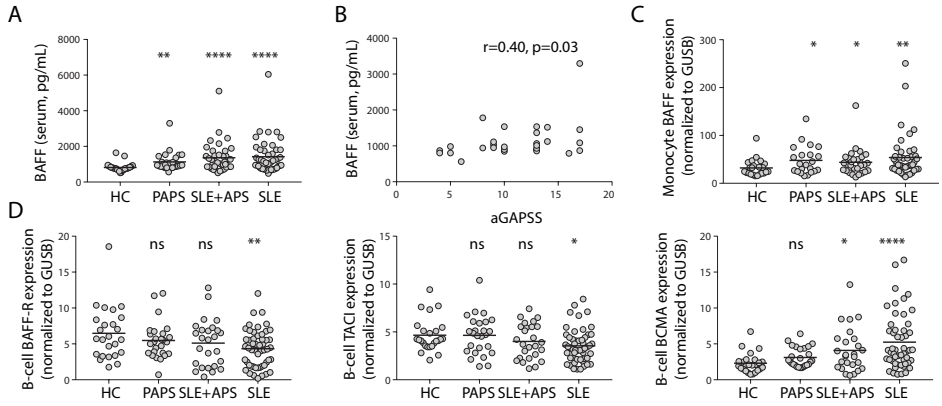

Figure 1: BAFF is increased in primary antiphospholipid syndrome and is associated with higher adjusted global antiphospholipid syndrome scores

A) BAFF was measured by ELISA in the sera of patients with PAPS, SLE+APS and SLE and HC. B) Correlation of adjusted global antiphospholipid syndrome scores (aGAPSS) with BAFF serum levels in patients with PAPS. C) mRNA expression of BAFF (TNFSF13B) was assessed by RT-qPCR in purified monocytes of patients with PAPS, SLE+APS and SLE and HC. D) mRNA expression of BAFF-R (TNFRSF13C), TACI (TNRSF13B) and BCMA (TNFRSF17) was assessed in purified total $CD19^+$ B-cells in patients with PAPS, SLE+APS and SLE and HC. *: p<0.05, **: p<0.01, ****: p<0.0001, ns: non-significant. Statistics: Kruskall-Wallis followed by post-hoc Dunn's (A, C and D) or spearman's correlation coefficient (B).

of anti-dsDNA antibodies (r=0.39, p<0.001) and lower levels of complement component C3 (r=-0.26, p=0.02, data not shown). Monocytes are major producers of BAFF, therefore we evaluated mRNA expression of BAFF in purified monocytes obtained from patients with SLE, SLE+APS and PAPS and found increased mRNA expression in all three patient groups as compared with HC (all p<0.05 **Fig 1C**).

BAFF is recognized by one of its three receptors which are highly expressed by B-cells: BAFF-R, transmembrane activator and CAML interactor (TACI) and B-cell maturation antigen (BCMA). Binding of BAFF to these receptors promotes B-cell survival and maturation through activation of NFκB[4]. In purified B-cells of patients with SLE we found a lower expression of BAFF-R and TACI, in line with previous studies[5]. However, in patients with APS, both in SLE+APS and PAPS we found no changes in the mRNA expression of BAFF-R and TACI as compared with HC (all p>0.05, **Fig 1D**). In contrast, patients with SLE and SLE+APS but not patients with PAPS had an increased expression of BCMA compared with HC (all p<0.05 **Fig 1D**). BCMA is predominantly expressed by plasmablasts and plasmacells [4] which are increased in patients with SLE, potentially explaining the difference in BCMA expression between PAPS and SLE.

	HC (n=29)	PAPS (n=29)	SLE+APS (n=40)	SLE (n=54)
Clinical manifestations				
Age	43 (34 - 50)	40 (33 - 50)	45 (37 - 53)	36 (28 - 48)
Female	93%	97%	95%	96%
SELENA-SLEDAI		-	4 (1 - 5)	4 (2 - 6)
Malar rash		0%	55%	65%
Discoid rash		0%	18%	19%
Photosensitivity		0%	53%	43%
Oral Ulcers		0%	35%	32%
Arthritis		0%	65%	72%
Serositis		0%	20%	32%
Lupus Nephritis		0%	45%	69%
Neurologic disorder		10%*	18%	4%
Hematologic disorder		35%**	85%	67%
Arterial Thrombosis		59%	43%	9%
Venous Thrombosis		38%	60%	2%
Obstetric morbidity		31%	23%	6%
Current Drug use				
Hydroxychloroquine		21%	55%	76%
Prednisone		0%	45%	67%
Azathioprine		0%	41%	33%
Mycophenolate mofetil		0%	10%	19%
Oral anticoagulant		62%	75%	0%
Aspirin		48%	28%	20%
Serology				
anti-dsDNA (IU/mL)		-	14 (5 - 58)	27 (6 -99)
C3		-	0.79 (0.68 - 0.92)	0.86 (0.70 - 1.03)
C4		-	0.14 (0.10 - 0.22)	0.15 (0.12 - 0.20)
Lupus anticoagulant		82%	65%	14%
anti-Cardiolipin IgG		86%	78%	20%
anti-Cardiolipin IgM		38%	15%	10%
anti-β2 Glycoprotein I IgG		35%	26%	4%
anti-β2 Glycoprotein I IgM		10%	5%	7%

Medians with interquartile range or percentages of total, * seizures, ** thrombocytopenia

In conclusion, similar to SLE, both mRNA and serum levels of BAFF are elevated in PAPS, in particular in PAPS patients with higher aGAPSS which are at higher risk for thrombotic events[9]. As the treatment response to belimumab in SLE patients is associated with higher serum levels of BAFF[10], belimumab might be a therapeutic option in a subset of PAPS patients with increased BAFF levels.

References

1. Erkan D, Aguiar CL, Andrade D, *et al.* 14th International Congress on Antiphospholipid Antibodies: task force report on antiphospholipid syndrome treatment trends. *Autoimmun Rev* 2014;**13**:685–96. doi:10.1016/j.autrev.2014.01.053

2. van den Hoogen LL, Fritsch-Stork RD, Versnel MA, *et al.* Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. *Ann Rheum Dis* 2016;**75**:e81. doi:10.1136/annrheumdis-2016-210485

3. Mackay F, Woodcock S a, Lawton P, *et al.* Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med* 1999;**190**:1697–710. doi:10.1084/jem.190.11.1697

4. Vincent FB, Morand EF, Schneider P, *et al.* The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol* 2014;**10**:365–73. doi:10.1038/nrrheum.2014.33

5. Salazar-Camarena DC, Ortiz-Lazareno PC, Cruz A, *et al.* Association of BAFF, APRIL serum levels, BAFF-R, TACI and BCMA expression on peripheral B-cell subsets with clinical manifestations in systemic lupus erythematosus. *Lupus* 2016;**25**:582–92. doi:10.1177/0961203315608254

6. Yazici A, Yazirli B, Erkan D. Belimumab in primary antiphospholipid syndrome. *Lupus* 2016;:1123–4. doi:10.1177/0961203316682102

7. Kahn P, Ramanujam M, Bethunaickan R, *et al.* Prevention of Murine Antiphospholipid Syndrome by BAFF Blockade. *Arthritis Rheum* 2008;**58**:2824–34. doi:10.1002/art.23764.

8. Vilas-Boas A, Morais SA, Isenberg D. Belimumab in Systemic Lupus Erythematosus. *RMD open* 2015;**1**:e000011. doi:10.4103/0019-5154.190107

9. Sciascia S, Sanna G, Murru V, *et al.* The global anti-phospholipid syndrome score in primary APS. *Rheumatol (United Kingdom)* 2014;**54**:134–8. doi:10.1093/rheumatology/keu307

10. Roth DA, Thompson A, Tang Y, *et al.* Elevated BLyS levels in patients with systemic lupus erythematosus: Associated factors and responses to belimumab. *Lupus* 2016;**25**:346–54. doi:10.1177/0961203315604909

CHAPTER 8

microRNA downregulation in plasmacytoid dendritic cells in interferon positive systemic lupus erythematosus and antiphospholipid syndrome

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Abstract

Objective

To investigate microRNA expression in relation to transcriptomic changes in plasmacytoid dendritic cells (pDCs) in systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). pDCs are major producers of interferon alpha (IFNα) in SLE and APS and microRNAs are emerging as regulators of pDC activation.

Methods

microRNA and mRNA expression were measured by OpenArray and RNA-sequencing in pDCs of SLE, SLE+APS and primary APS (PAPS) patients. The microRNA profile of patients was compared to the microRNA pattern of TLR7-activated pDCs.

Results

Among 131 microRNAs detected in pDCs, 35, 17 and 21 were significantly lower expressed in SLE, SLE+APS and PAPS patients as compared to HC. Notably, the microRNA profile did not significantly differ between SLE and APS, but was driven by the presence or absence of an IFN signature. TLR7 stimulation induced a general downregulation of microRNAs, similar to the pattern observed in patients. miR-361-5p, miR-128-3p and miR-181a-2-3p expression was lower in patients with a high IFN signature (FDR<0.05) as compared to patients with a low IFN signature and HC. Pathway enrichment on the overlap of microRNA targets and upregulated genes from the RNAseq indicated that these miRNAs are involved in pDC activation and apoptosis.

Conclusion

Lower miRNA expression in pDCs is shared between SLE, SLE+APS and PAPS and is related to the IFN signature. As pDCs are the alleged source of the IFN signature in these patients, a better understanding of the molecular mechanisms/pathways leading to pDC dysregulation in SLE and APS might open novel pathways for therapeutic intervention.

Key words: microRNA, plasmacytoid dendritic cell, systemic lupus erythematosus, antiphospholipid syndrome, Toll-like receptor, interferon signature, epigenetics

Key messages

- plasmacytoid dendritic cell microRNAs are downregulated in SLE and APS
- Downregulation of pDC microRNAs reflects pDC activation in SLE and APS
- Reduced expression of miR-361-5p, miR-128-3p and miR-181a-2-3p affects pathways involved in pDC activation

Introduction

Systemic lupus erythematosus (SLE) is a chronic relapsing autoimmune disease that affects women of child-bearing age. In SLE, immune complexes are deposited into tissues, including skin, joint and kidneys, leading to tissue inflammation. The pathogenesis of SLE is complex and includes derailments of both the innate and adaptive immune system[1]. Antiphospholipid antibodies (aPL) are strongly associated with thrombotic events and pregnancy morbidity among patients with SLE. Approximately 20% of SLE patients have antiphospholipid syndrome (APS), defined as the persistent presence of aPL in patients who experienced at least one thrombotic or obstetric complication[2]. APS also affects patients without an underlying disease and is then termed primary APS (PAPS). SLE and PAPS share many pathologic features, from genetics to perturbations in immune cells[3], although both conditions are rarely studied together.

Both SLE and PAPS are characterized by an interferon (IFN) signature[4–6], which stands for an increased expression of IFNα inducible genes. The IFN signature is detected in ~75% of SLE(±APS) patients and ~50% of PAPS patients[5] and is linked to disease activity in SLE[6,7] and vascular disease in both SLE and PAPS[4]. Plasmacytoid dendritic cells (pDCs) are considered the source of elevated IFNα levels in SLE and APS as immune complexes and aPL induce IFNα production by triggering Toll-like receptor (TLR)7 in pDCs[8,9]. However, due to their scarcity in the circulation (<0.5% of leukocytes), little is known on the molecular alterations of pDCs in patients with SLE and APS.

MicroRNAs (miRNAs) are short (18-25 nucleotides) non-coding RNAs that regulate gene-expression at a post-transcriptional level by binding to complementary sequences in target genes, resulting in the reduced translation of these target genes. miRNAs fine-tune cellular activation and differentiation and their dysregulation is emerging as an underlying cause of autoimmunity[10]. The expression of several miRNAs is altered in patients with autoimmune diseases[10,11], however most studies used bulk peripheral blood cells or plasma and are therefore of limited value in identifying the pathways regulated by altered miRNA expression in a cell-specific manner.

Recent evidence suggests an important role for miRNAs in regulating pDC activation[12,13] and type I IFN responses[10]. Here we performed miRNA-profiling and RNA sequencing (RNAseq) on purified pDCs from patients with SLE, SLE+APS and PAPS in order to identify changes in miRNA expression and their target genes. Our results support an increased activation state of pDCs in SLE and APS as reflected by a reduced expression of miRNAs in patients with high IFN signatures.

Methods

pDC isolation

pDCs were isolated from patients with SLE (n=20), SLE+APS (n=10) and PAPS (n=10) and healthy controls (HC, n=12, **Supplementary table**) from peripheral blood mononuclear cells by positive selection using the BDCA4 isolation kit following the manufacturer's instruction (Miltenyi). Classification criteria were used to classify patients as SLE, SLE+APS or PAPS[2,14]. All participants signed informed consent prior to the donation of blood and approval by the ethical committee from the UMC Utrecht was obtained.

The purity of isolated pDCs was assessed by flow cytometry using CD123 and BDCA2 fluorochrome-labeled antibodies. The average purity was 92% (±3) and did not differ between patients and controls.

miRNA profiling and RNAseq

RNA was extracted from isolated pDCs using the Allprep universal kit (Qiagen) according to the manufacturer's instructions. 10ng of extracted RNA was reverse transcribed by using the miRNA multiplex RT primer pools, either v2.1 for pool A or v3.0 for pool B, and the TaqMan miRNA reverse transcription kit (Life Technologies), allowing the detection of 758 miRNA including housekeeping snRNA. RT products were pre-amplified using the Megaplex PreAmp Primer pools A and B in the presence of the TaqMan PreAmp Master Mix (Life Technologies), by using the following thermal cycler conditions: 10 min, 95 °C; 2 min, 55 °C; 2 min, 72 °C and 16 cycles of 15 sec, 95 °C and 4 min, 60 °C and one single cycle of 10 min, 96 °C. The miRNA Open Array profiling was performed on the amplified cDNA, diluted 1:40, with 0.1 × TE buffer (pH 8.0) and subsequently 1:2 with TaqMan Open Array MasterMix on the QuantStudio 12K Flex Real-Time PCR System (Life Technologies).

miRNA profiling data was analyzed using the Thermofisher Cloud (www.thermofisher.com). miRNA expression was normalized to the mean expression of RNU44 and RNU48. Normalized expression values (ΔΔCrt) were used for statistical analysis. miRNAs with Crt values >27 were set at 27. Only miRNAs with <10% of missing values were included for further analysis. A publicly available miRNA profile (GSE21160) generated using the same experimental approach in pDCs activated by TLR7-agonist R837 (imiquimod) was reanalyzed using the same criteria.

RNAseq was performed on the same pDCs used for miRNA profiling. RNAseq libraries were generated with the TruSeq RNA Library Prep Kit (Illumina) and

sequenced on an Illumina HiSeq 4000 generating approximately 20 million 100bp paired-ended reads. The sample qualities were assessed by FastQC and the sequencing reads were aligned to human genome (GRCh38 build 79) using STAR aligner. Gene expression data for the annotated genes was generated using HTSeq-count. The variance-stabilizing transformation normalized expression values (normalized readcounts, NRC) were calculated using R-package DESeq2. Type I IFN scores were calculated from NRC as previously described[5]. Patients were stratified by the presence (IFN-high) or absence (IFN-low) of a type I IFN signature by setting a threshold at the highest HC.

Data handling and statistics

miRWALKm2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) was used to search Targetscan and miRANDA for potential target genes of selected miRNAs. Pathway enrichment based on the KEGG database was performed on the intersect of the miRNA predicted targets and upregulated genes from the RNAseq, using TopFunn (https://toppgene.cchmc.org/enrichment.jsp). Pathways related to specific infections were excluded.

Statistical tests were conducted two sided with an alpha level of 0.05. Differences between two groups were tested by Welch T-test. Correction for multiple testing was performed by FDR at a q-value of 0.05.

Results

Global downregulation of pDC microRNAs in SLE and APS patients

Out of 755 analyzed, 131 miRNAs were expressed in pDCs. Principal component analysis (PCA, **Fig 1A**) and hierarchical clustering (**Fig 1D**) of all expressed miRNAs did not cluster SLE patients from APS patients, suggesting that these groups do not have a strong differential miRNA pattern. When comparing miRNA expression in SLE, SLE+APS and PAPS patients with HC, respectively 36, 17 and 21 miRNA were found differentially expressed (**Fig 1B, Supplementary Table 1**). Remarkably, with the exception of 1 miRNA (miR-222), all differentially expressed miRNAs had a lower expression in patients as compared with HC, as evident from the left-skewed volcano plots of these comparisons (**Fig 1B**); whereas the miRNA profile of patients with SLE, SLE+APS and PAPS did not show strong differences (**Fig 1C**). miRNA expression in pDCs is therefore similarly affected in SLE and APS.

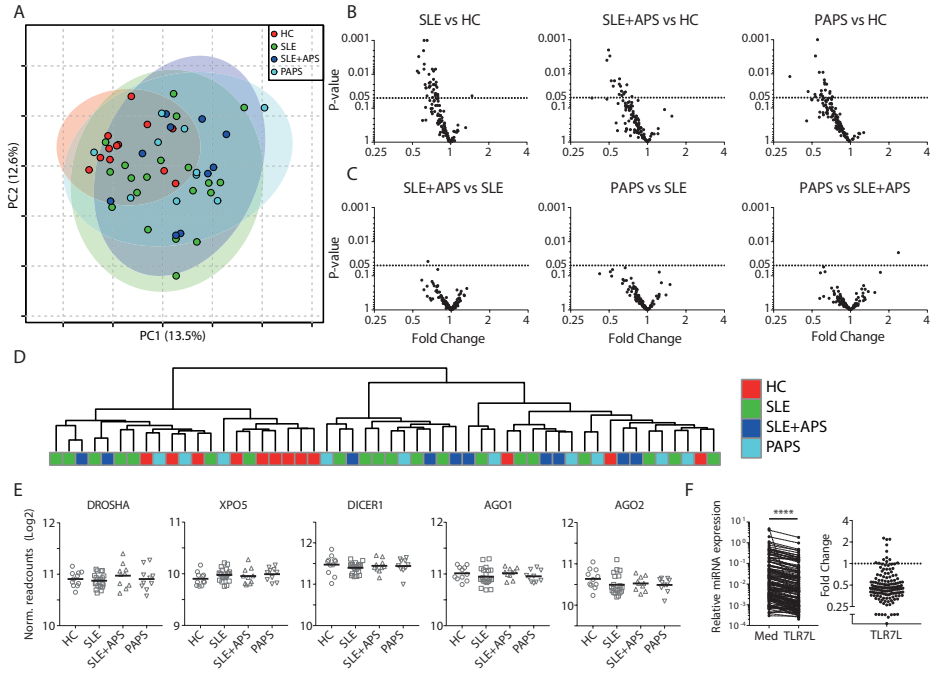

Figure 1: Downregulation of pDC miRNAs in SLE, SLE+APS and PAPS

A) Principal Component Analysis of all miRNAs expressed in pDCs of SLE, SLE+APS and PAPS patients and HC. B) Volcanoplots of miRNA expression in SLE, SLE+APS and PAPS patients as compared to HC C) or among patients D) Hierarchical clustering of pDC miRNAs. E) Expression of genes related to the biogenesis and transport of miRNA in pDCs as assessed by RNAseq. F) Changes in the expression in purified pDCs upon stimulation by TLR7 agonist R837. Relative miRNA expression was calculated as 2^{-dCq}, Fold Change was compared to medium control. ****: p<0.0001

TLR7 signaling induces a downregulation of pDC microRNA expression

Several proteins are involved in the formation, transportation and stabilization of miRNAs, including the nucleases DICER and DROSHA, which cut pre-miRNA transcripts into mature miRNAs, exportin-5 (XPO5) which exports the miRNA out of the nucleus and argonaute proteins (AGO1/AGO2) which form the RNA-induced silencing complex (RISC). Reduced expression of these genes in neutrophils of SLE and APS patients results in globally reduced miRNA expression[15]. RNAseq on the same pDC samples that were used for miRNA profiling however did not show significant differences in the expression of these genes (**Fig 1E**). The global miRNA downregulation in pDCs of SLE and APS seemed therefore not due to alterations in the miRNA-machinery. In contrast, activation of pDCs by TLR7 agonists induced a global downregulation of miRNA, to a similar extent as seen in the patient samples

(mean fold-change 0.45, **Fig 1F**), suggesting that the downregulation of miRNA in pDCs in SLE and APS reflects pDC activation.

pDC microRNA expression is related to the type I IFN signature in pDCs

RNAseq revealed increased type I IFN scores in pDCs of SLE, SLE+APS and PAPS, which was strongest in the patients with SLE and SLE+APS (**Fig 2A**). 58% of the patient samples (n=23) classified as IFN-high (**Fig 2B**). The IFN scores in pDCs strongly correlated with the IFN scores we previously reported in monocytes of the same patients[5] (**Fig 2C**).

Upon stratification of the patients into those with or without an IFN signature, miRNA expression was more strongly reduced in IFN-high patients as compared to the IFN-low patients (**Fig 2D**). Consequently, principal component analysis and hierarchical clustering on all miRNA showed a better separation of patients on the basis of their IFN status as compared to their clinical disease (**Fig 2E, 2F)**. Three miRNAs (miR-361-5p, miR-128-3p and miR-181-2-3p) were expressed at lower levels ($p<0.001$, FDR<0.05) in IFN-high patients compared to those without an IFN signature and HCs (**Fig 2G**). These miRNA were also downregulated upon TLR7 ligation, whereas in SLE only the expression of mir-361-5p was significantly lower in patients with high disease activity (SLEDAI ≥4 (n=12) as compared to patients with low disease activity (SLEDAI <4, n=18, $p=0.03$, data not shown).

Altered miRNA expression affects key signaling pathways involved in pDC activation

One miRNA may regulate the expression of several hundreds of genes. By integrating miRNA and mRNA expression data we investigated the potential pathways affected by altered miRNA expression in pDCs. An intersection of the predicted target genes of miR-361-5p, miR-128-3p and miR-181-2-3p and the upregulated genes from IFN-high patients revealed 680 genes in common. Pathway enrichment on these genes revealed that these miRNAs are regulating the expression of genes involved in pDC activation (RIG-I receptor and NOD-like receptor and TNF signaling) and apoptosis (**Fig 1H**).

Discussion

Here we studied pDCs directly isolated from peripheral blood of patients with SLE and APS and for the first time analyzed both their miRNome and transcriptome. Strikingly, the overall miRNA expression was lower in both SLE and APS patients versus controls, which was most pronounced in patients with a high type I IFN signature. TLR7 activation induced a similar profile of downregulated miRNA

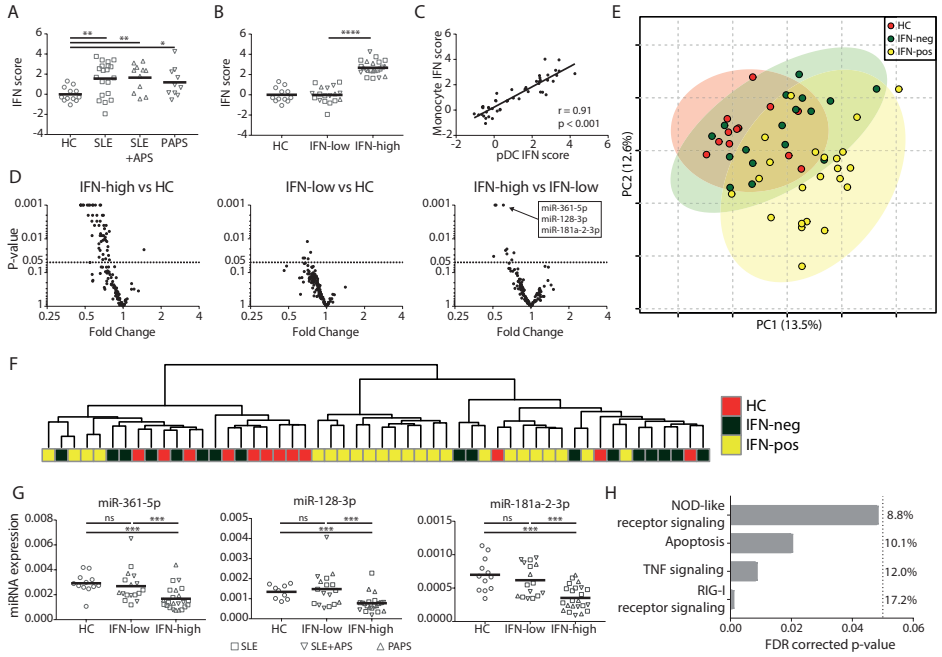

Figure 2: Downregulation of pDC miRNAs is associated with a pDC IFN signature

A) IFN scores of purified pDCs in patients stratified by clinical diagnosis or B) IFN status. C) Correlation of IFN scores between monocytes and pDCs. D) Volcanoplots of pDC miRNA expression in patients stratified by IFN status. E-F) Principal Component Analysis and hierarchical clustering of patients based on all miRNA stratified by IFN status. G) Expression (2^{-dCq}) of the three most significant miRNA between patients with or without an IFN signature. H) Pathway enrichment of predicted target genes of miR-361-5p, miR-128-3p and miR-181a-2-3p and upregulated genes in pDC of patients with high IFN signatures. Percentages indicate the number of affected genes over the total genes annotated in the pathway. *: p<0.05, **: p<0.01 ***: p<0.001, ns: non-significant

expression in pDCs. Therefore, this indicates that the profile of miRNA expression in pDCs of SLE and APS patients is a reflection of pDCs activation *in vivo* in these patients.

A reduced expression of miRNAs has previously been reported in neutrophils of SLE and APS patients[15]. This reduced miRNA expression in neutrophils was shown to be due to the decreased expression of the miRNA machinery in these cells[15]. In pDCs we did not see differential expression of these genes. As TLR7 signaling is driving the production of IFNα by pDCs in SLE and APS[8,9] we assessed changes in miRNA expression after TLR7 activation and observed that miRNA expression in pDCs is highly sensitive to TLR7 stimulation, resulting in the downregulation of

the majority (92%) of miRNAs. Thus, the mechanism behind miRNA downregulation in neutrophils and pDCs of SLE and APS patients is cell-specific.

Two previous studies on miRNA expression in monocytes and neutrophils of SLE and PAPS patients reported no differences in miRNA expression between patients with either SLE or PAPS[15,16]. Similarly, we observed no major differences in miRNA expression between SLE, SLE+APS and PAPS in pDCs. The dysregulation of pDCs in terms of miRNA expression is therefore another shared immunologic abnormality between patients with SLE and APS[3] while the presence or absence of an IFN signature, rather than having SLE or APS, is defining miRNA expression in pDCs.

miR-361-5p, miR 128-3p and miR-181a-2-3p have not been previously linked to the pathogenesis of SLE or APS[10]. These miRNAs are specifically reduced in pDCs of patients with high IFN signatures. The expression of the targets of these miRNA in pDC of the same patients suggests that these miRNA are regulating the expression of genes involved in key signaling pathways participating in pDC activation such as RIG-I signaling as well as pDC apoptosis.

As pDCs are the alleged source of the type I IFN signature, we believe that a better understanding of the factors (including miRNAs) underlying the dysregulation of pDCs in SLE and APS can yield novel insights into the pathogenesis of SLE and APS. Our data suggest that aberrances in miRNA expression may be key mediators in regulating pDC activity and immunopathology of SLE and APS.

References

1. Tsokos GC, Lo MS, Reis PC, Sullivan KE. New insights into the immunopathogenesis of systemic lupus erythematosus. Nat Rev Rheumatol. 2016;12(12):716–30.
2. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4(Feb):295–306.
3. van den Hoogen LL, van Roon JAG, Radstake TRDJ, Fritsch-Stork RDE, Derksen RHWM. Delineating the deranged immune system in the antiphospholipid syndrome. Autoimmun Rev. 2016;15(1):50–60.
4. Grenn RC, Yalavarthi S, Gandhi AA, Kazzaz NM, Núñez-Álvarez C, Hernández-Ramírez D, et al. Endothelial progenitor dysfunction associates with a type i interferon signature in primary antiphospholipid syndrome. Ann Rheum Dis. 2017;76(2):450–7.
5. van den Hoogen LL, Fritsch-Stork RD, Versnel MA, Derksen RH, van Roon JA, Radstake TR. Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. Ann Rheum Dis. 2016;75(12):e81.
6. Kirou KA, Lee C, George S, Louca K, Peterson MGE, Crow MK. Activation of the interferon-α pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. Arthritis Rheum. 2005;52(5):1491–503.
7. Rose T, Grützkau A, Klotsche J, Enghard P, Flechsig A, Keller J, et al. Are interferon-related biomarkers advantageous for monitoring disease activity in systemic lupus erythematosus? A longitudinal benchmark study. Rheumatology. 2017;56:1618–26.
8. Prinz N, Clemens N, Strand D, Pütz I, Lorenz M, Daiber A, et al. Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. Blood. 2011;118(8):2322-32.
9. Eloranta M-L, Alm G V, Rönnblom L. Disease mechanisms in rheumatology--tools and pathways: plasmacytoid dendritic cells and their role in autoimmune rheumatic diseases. Arthritis Rheum. 2013;65(4):853-63.
10. Chen JQ, Papp G, Szodoray P, Zeher M. The role of microRNAs in the pathogenesis of autoimmune diseases. Autoimmun Rev. 2016;15(12):1171–80.
11. Carlsen AL, Schetter AJ, Nielsen CT, Lood C, Knudsen S, Voss A, et al. Circulating microRNA expression profiles associated with systemic lupus erythematosus. Arthritis Rheum. 2013 May;65(5):1324–34.
12. Zhou H, Huang X, Cui H, Luo X, Tang Y, Chen S, et al. interferon production by human plasmacytoid dendritic cells miR-155 and its star-form partner miR-155 * cooperatively regulate type I interferon production by human plasmacytoid dendritic cells. Blood. 2012;116(26):5885–94.
13. Rossato M, Affandi AJ, Thordardottir S, Wichers CGK, Cossu M, Broen JCA, et al. Association of MicroRNA-618 Expression With Altered Frequency and Activation of Plasmacytoid Dendritic Cells in Patients With Systemic Sclerosis. Arthritis Rheumatol. 2017;69(9):1891–902.
14. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40(9):1725.

15. Pérez-Sánchez C, Aguirre MA, Ruiz-Limón P, Barbarroja N, Jiménez-Gómez Y, de la Rosa IA, et al. 'Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients.' Sci Rep. 2016;6:31375.

16. Teruel R, Pérez-Sánchez C, Corral J, Herranz MT, Pérez-Andreu V, Saiz E, et al. Identification of miRNAs as potential modulators of tissue factor expression in patients with systemic lupus erythematosus and antiphospholipid syndrome. J Thromb Haemost. 2011;9(10):1985–92.

Supplementary Table 1: Clinical characteristics

	HC (n=12)	SLE (n=20)	SLE+APS (n=10)	PAPS (n=10)
Demographics				
Female	100%	100%	100%	90%
Age	42 (35 – 49)	42 (32 – 52)	48 (42 – 53)	38 (30 – 47)
SLE duration (years)		15 (9 - 24)	16 (14 - 32)	-
APS duration (years)		-	13 (8 - 15)	6 (3 - 12)
SELENA-SLEDAI		2 (1 – 4)	2 (0 – 4)	-
Disease manifestations				
Malar Rash		80%	50%	0%
Discoid Rash		20%	30%	0%
Photosensitivity		60%	30%	0%
Oral ulcers		35%	30%	0%
Arthritis		50%	70%	0%
Serositis		25%	30%	0%
Renal disorder		50%	40%	0%
Neurologic disorder		5%	20%	20%
Hematologic disorder		55%	80%	30%
Arterial Thrombosis		5%	60%	60%
Venous Thrombosis		0%	30%	40%
Obstetric morbidity		10%	40%	20%
Actual Drug use				
Hydroxychloroquine		75%	70%	40%
Prednisone		45%	30%	0%
Prednisone dose (mg)		7.5 (7.5 - 10)	7.5 (6.25 - 8.75)	-
Azathioprine		25%	0%	0%
Mycophenolate mofetil		25%	0%	0%
Oral anticoagulant		0%	70%	70%
Aspirin		10%	60%	50%
Serology				
ANA-IIF (≥1:100)		100%	100%	70%
anti-dsDNA antibodies (ever)		85%	100%	10%
Anti-Ro		65%	30%	10%
Anti-Sm		55%	20%	0%
aPL Triple positivity		5%	30%	30%
Lupus anticoagulant		10%	100%	90%
Anti-cardiolipin (IgG)		25%	60%	80%
Anti-cardiolipin (IgM)		10%	10%	50%
Anti-β2 Glycoprotein I (IgG)		10%	30%	30%
Anti-β2 Glycoprotein I (IgM)		0%	0%	10%

Values are reported as percentages of total or median with interquartile range

CHAPTER 9

Differential activation of dendritic cell subsets by interferon α amplifies the type I interferon signature in systemic lupus erythematosus and antiphospholipid syndrome

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Equal contribution*

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Abstract

Background/Purpose

Dendritic cells (DC) are key cells in the pathogenesis of autoimmune diseases by potently activating T-cells. Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) are characterized by an IFN signature, caused by elevated levels of IFNα. Plasmacytoid DC (pDC) are held responsible for the increased levels of IFNα in SLE and APS. The molecular mechanisms underlying the increased activation of pDC in SLE and APS are unknown. Using RNA sequencing (RNAseq) and further in vitro validation experiments on plasmacytoid and myeloid DC (mDC) obtained from patients with SLE and APS we assessed the causes and consequences of increased type I IFN signaling on the dysregulation of both pDC and mDC in patients with SLE and APS.

Methods

RNAseq was performed on pDC and mDC isolated from peripheral blood of patients with SLE, SLE+APS and primary APS (PAPS) and healthy controls (n=54). Weighted gene correlation network analysis (WGCNA) was used to identify pDC- and mDC-specific gene modules and to stratify patients into those with (IFN-high) or without (IFN-low) an IFN signature. The response of pDC and mDC to IFNα and TLR agonists were analyzed by RT-qPCR and flow cytometry to functionally validate RNAseq data.

Results

WGCNA identified IFN modules in pDC and mDC that perfectly stratified patients from HC. Comparing the IFN modules of pDC and mDC revealed cell specific alterations related to the IFN signature. Increased expression of TLR7 and its downstream intermediates was confined to IFN-high patients in pDC. In contrast, genes involved in the activation of T-cells in mDC were related to the IFN module in mDC. Both pDC and mDC showed increased expression of BAFF. In vitro, IFNα upregulated TLR7 in pDC and augmented TLR7-mediated IFNα production. In contrast to pDC, IFNα primed mDC for enhanced T-cell activation via the upregulation of co-stimulatory molecules.

Conclusion

pDC and mDC are differentially affected by IFNα in SLE and APS. IFNα primes pDC for enhanced IFNα production and potentiates mDC to activate T-cells thereby sustaining the IFN signature in SLE and APS. Intervening in this loop potentially attenuates the dysregulation of DC in SLE and APS.

Introduction

Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) are autoimmune diseases which frequently occur in the same patient. SLE is characterized by the production of autoantibodies against nuclear components to form immune complexes which are deposited in tissues where they locally induce inflammation. Frequently affected tissues in SLE are the skin, joints and kidneys although virtually any organ can be affected[1]. APS on the other hand is characterized by antibodies against phospholipid binding proteins, collectively termed antiphospholipid antibodies (aPL), which activate endothelial cells, trophoblasts and immune cells resulting in thrombosis and pregnancy morbidity[2]. Roughly 20% of SLE patients also have APS (SLE+APS), although APS may also occur as a stand-alone disorder, termed primary APS (PAPS).

Dendritic cells (DC) are the sentinel cells of the innate immune system specialized in the presentation of antigen to T-cells. DCs express high levels of class II HLA and upon activation provide co-stimulatory and differentiation signals to T-cells. DCs are present in low numbers in peripheral blood, from which they may migrate into tissues or lymph nodes. In humans, circulating DCs are divided in two principal subsets: myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC). mDC, also known as classical DCs, are potent activators of CD4 (BDCA1+ mDC) and CD8 (BDCA3+ mDC) T-cells. pDC can activate T-cells but their main physiological role is to induce antiviral immunity due to their unique capacity to produce large amounts of the antiviral type I interferons, such as interferon alpha (IFNα).

Compelling evidence over the past decades places the cytokine interferon alpha (IFNα) in the center of the pathogenesis of SLE. Patients with SLE elevated levels of IFNα, correlating with disease activity [3,4] and a striking upregulation of IFN inducible genes termed the IFN signature is found in peripheral blood and tissues of patients with SLE[5–7]. Notably, anifrolumab, a monoclonal antibody against IFNAR, the receptor for IFNα, decreases disease activity in patients with SLE[8]. The IFN signature is shared between SLE and several other autoimmune diseases and recently, we [9,10] and others [11] reported the presence of an IFN signature in patients with PAPS.

In vitro, IFNα activates key pathways involved in the pathogenesis of SLE. For instance, IFNα induces the maturation monocytes into monocyte-derived dendritic cells[12], the differentiation of autoantibody producing plasma cells[13], activates the endothelium[14] and potentiates the release of neutrophil extracellular traps[15]. Toll-like receptor (TLR) 7 and 9 are located in the endosome of pDCs and sense

either RNA or DNA which leads to IFNα production. pDCs are considered the source of elevated IFNα levels in SLE and APS as immune complexes[16] and aPL[17] activate pDCs resulting in the production of IFNα. However, due to their scarcity in peripheral blood, little is known on the molecular alterations of pDCs in patients with SLE or APS. Here we investigated the role of both DC subsets in relation to the type I IFN signature in SLE and APS.

Methods

Patients and controls

Blood was drawn from patients with SLE, SLE+APS and PAPS and healthy controls (HC). Patients were classified as SLE or APS using their respective classification criteria [18,19]. This study was performed according the guidelines of our institution's medical ethical committee and all patients and controls signed informed consent prior to the donation of blood.

Cell isolation

Blood was collected in Lithium Heparin vacutainers (BD) and peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation using Ficoll Paque Plus (GE Healthcare) within one hour after collection. Specific cell populations were positively isolated by positive selection using magnetic beads with an autoMACSpro (all Miltenyi), following manufacturer's protocols. BDCA4 microbeads, the CD1c dendritic cell isolation kit and CD3 microbeads were used to isolate pDCs, mDCs and T-cells, respectively. Purity was assessed by flow cytometry and all samples exceeded 85% purity.

RNA sequencing (RNAseq)

RNA was extracted using the Allprep Universal Kit (Qiagen) and used for RNA sequencing performed at BGI Tech solutions. RNA libraries were generated with the TruSeq RNAseq RNA Library Prep Kit (Illumina) and sequenced on an Illumina HiSeq 4000 generating approximately 20 million 100bp paired-ended reads for each sample. The sample qualities were assessed by FastQC and the sequencing reads were aligned to human genome (GRCh38 build 79) using STAR aligner. Gene expression data for the annotated genes was generated using HTSeq-count. The variance-stabilizing transformation normalized expression values (normalized read counts, NRC) for the genes were calculated using R package DESeq2. Because of low RNA yields RNA sequencing could not be performed on both pDCs and mDCs for all patients, 46 samples had both pDCs and mDCs sequenced. For the

pDCs 12 HC were compared with 41 patient samples (SLE n=21, SLE+APS n=10, PAPS n=10) and for the mDCs, the same 12 HC were used and 37 patients samples (SLE n=16, SLE+APS n=11, PAPS n=10).

RT-qPCR

Isolated pDCs and mDCs were cultured in RPMI 1640 (Life technologies) at a density of 1x10^6 cells/ml in round bottom plates in a final volume of 200 ul. Cells were stimulated for three hours with IFNα-2a (Cell sciences) at a concentration of 1000U/mL or Loxoribine (1μM) or left untreated. RNA was extracted using the Allprep universal kit (Qiagen) following the manufacturer's instructions. The expression of TLR7 and BAFF were normalized to the expression of GUSB.

Cytokine production of pDCs and mDCs

PBMCs were cultured at a density of $2x10^6$ cells/ml in flat-bottom plates in RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum (heat inactivated, Biowest), 5% Penicillin and Streptomycin (both from Life Technologies), in a final volume of 1 ml, at 37°C, 5% CO2. PBMCs were primed overnight with IFNα or left untreated. The following day samples were stimulated for four hours with loxoribine (Invivogen 1 uM) or left untreated. For intracellular cytokine production, after one hour, brefeldin A (eBiosciences, 10ug/mL) was added to the cultures. Samples were assessed by flowcytometry as described below.

T-cell proliferation

pDCs and mDCs were cultured overnight with or without IFN-α-2a. Peripheral blood lymphocytes (PBLs) were collected the same day from the same donor, cryopreserved and the subsequent day, CD3+ T cells were isolated as described above. T-cells were stained with Cell Trace Violet (CTV, fluorescent dye, 1.5 uM; Invitrogen). CD3+ T cells were plated together with either mDCs or pDCs, in a final volume of 150 ul. DCs and CD3+ T cells were seeded in a ratio 1:5. In some experiments, Abatacept was added to the cells (Orencia®, Bristol-Myers Squibb, working concentration 10 ug/ml). After 4 days, cells were harvested and proliferation was assessed by flow cytometry.

Flow cytometry for co-stimulatory markers and mBAFF

PBMCs were cultured as described above for 24 hours with or without IFNα. Cells were incubated with fixable viability dye eFluor780 to allow exclusion of dead cells. Cells were then washed and further incubated with human FcR blocker (Miltenyi) in a 1:10 dilution (10 ul final volume), to prevent nonspecific Ag binding. Cells were

stained for 20 min at 4°C with fluorochrome-conjugated antibodies. Fluorescence Minus One (FMO) conditions were used to discriminate positive populations.

The "Fix&Perm" fixation and permeabilization kit (cat: GAS004, Life Technologies) was used following the manufacturer's instructions to detect TLR7 expression (Novus Biologicals). Cells were then washed once with FACS buffer before measuring and acquired on a LSR Fortessa Flow Cytometer (BD). pDCs and mDCs were gated on using CD123 and BDCA2 and CD1c and CD19 respectively. Data were analyzed with FlowJo software (Tree Star). Data were represented as ΔMFI (MFI marker of interest minus FMO) or the percentage of positive cells for a specific cell marker.

BAFF production

BAFF was measured by ELISA using the Human BAFF/BLyS/TNFSF13B Quantikine ELISA Kit in the supernatant of 100.000 purified pDCs and mDCs stimulated with IFNα for 24 hours. Untreated samples were used as control.

Statistical Analysis

Graphpad Prism software was used to perform statistical analysis. Differences between more than two groups were tested by ANOVA followed by Tukey's post-test. Paired t-tests to compare the effects of in vitro stimulation with IFNα. All tests were conducted two sided with an alpha level set at 0.05.

Results

RNAsequencing on pDCs and mDCs reveals shared affected pathways in SLE and APS

Multidimensional scaling (MDS) plots of differentially expressed genes (DEG) in SLE, SLE+APS and PAPS patients in pDCs (**Fig 1A**) and mDCs (**Fig 1B**) separated patients from healthy controls. Little separation was seen between SLE and APS. Pathway enrichment on the DEG in SLE, SLE+APS and PAPS patients further confirmed homogeneity of affected pathways, both in pDCs (**Fig 1C**) and mDCs among the diseases (**Fig 1D**). Notably, several pathways related to gene-translation, inflammation and interferon signalling were enriched among the DEG of patients with SLE and APS, both in pDCs and mDCs, suggesting that the gene-expression pattern is shared between SLE and APS as well as between pDCs and mDCs.

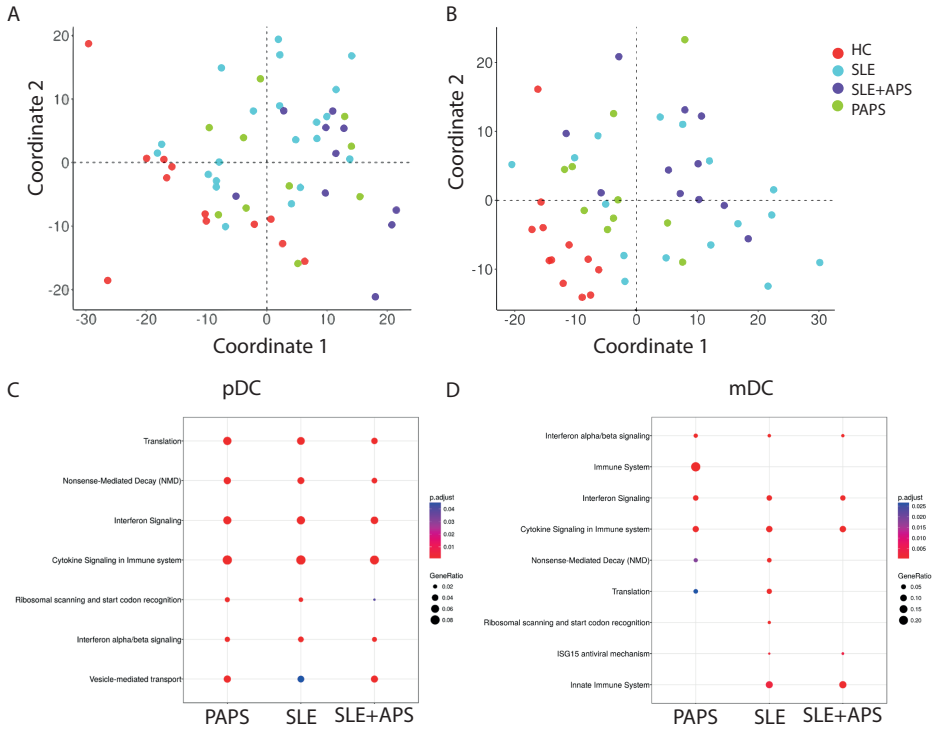

Figure 1. RNA sequencing of pDCs and mDCs in SLE, SLE+APS and PAPS reveals common affected gene expression profiles and pathways.

MDS plots of DEGs in pDCs (A) and mDCs (B) in patients with SLE, SLE+APS and PAPS. DEGs were subsequently used for pathway enrichment analyses in pDCs (C) and mDCs (D). GeneRatio represents the proportion of genes of the pathway.

The IFN signature stratifies SLE and APS patients from HC

Weighted gene co-expression network analysis (WGCNA) on DEG identified a gene module highly enriched for IFN-related pathways in both pDCs and mDCs, further referred to as the IFN module (**Fig 2A**). Stratification of patients by their eigengene value in this IFN module in the same (negative) or opposite (positive) direction of HC, identified 25 and 22 patients as positive for this IFN module (IFNpos) in pDCs and mDCs respectively and 16 and 15 patients, plus all HC, as negative for this IFN module (IFNneg). Using the IFN module to stratify patients revealed a complete separation of IFNpos patients from HC in MDS plots (**Fig 2B**), suggesting that this IFN module is a dominant gene module in DCs of SLE and APS patients. Consistently, the number of DEG between IFN-negative patients and HC was relatively low (pDCs: n=484, mDCs: n=90) in comparison to IFN positive patients versus HC (pDCs: n=3001, mDCs: n=2278).

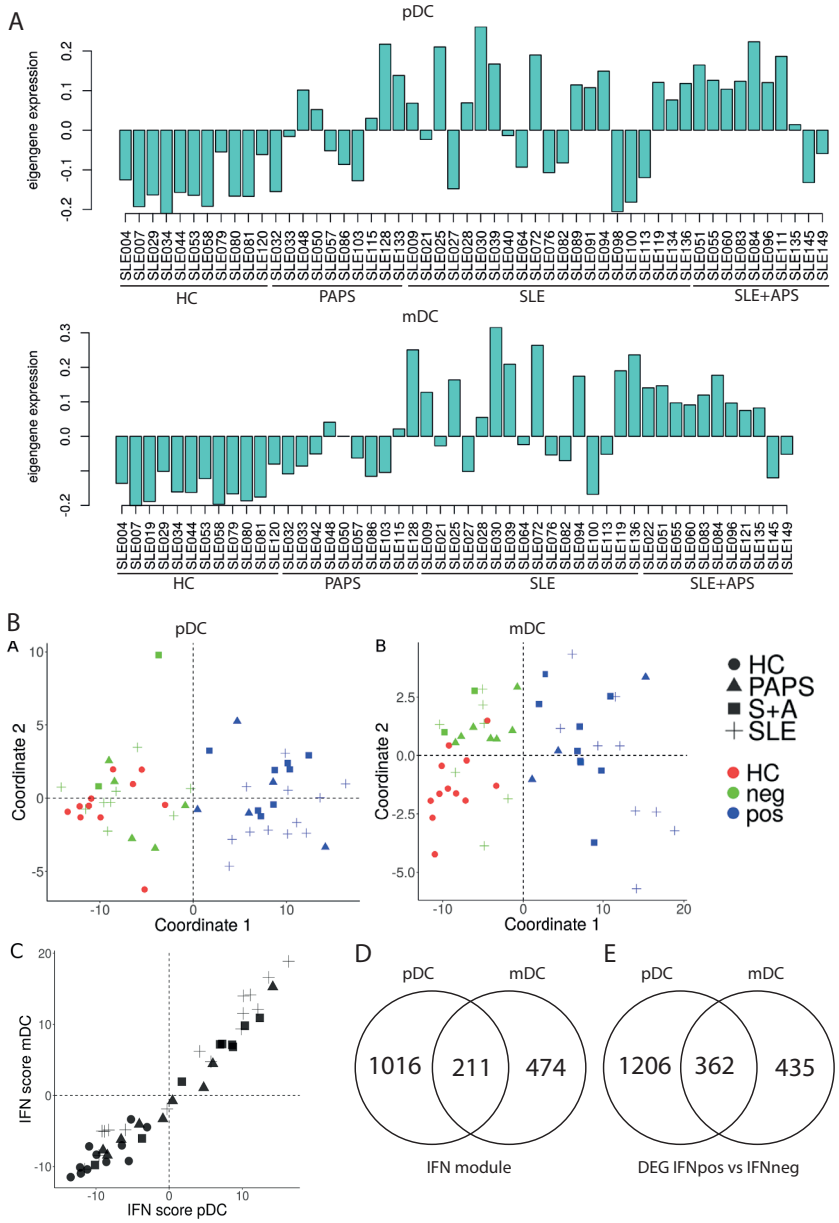

Figure 2: The IFN module segregates pDCs and mDCs from SLE, SLE+APS and PAPS and is associated with largely different gene expression profiles in mDCs and pDCs.

Eigen-gene values of the IFN module in all samples of pDCs and mDC (A). MDS plot on DEG when stratified by the IFN module in pDCs and mDC (B). Correlation of IFN scores derived from the first coordinates of the MDS in pDC versus mDC (C). Venn diagram of the genes allocated to the IFN module in pDC versus mDC (D). Venn diagram of DEG between IFNpos and IFNneg patients in pDC and mDC (E).

There was a strong correlation of IFN-scores between pDCs and mDCs (**Fig 2C**), and the same patients were classified as IFN-positive or negative by using the IFN module from either pDCs or mDCs (**Fig 2A**). However, the genes that made up the IFN module partly overlapped but largely differed between pDCs and mDCs (**Fig 2D**). The genes shared between the IFN module of pDCs and mDCs included several classical IFN-inducible genes such as IFITM1, IFI44, IFI44L, MX1, Ly6E, OAS2. Furthermore, the genes that were differentially expressed between IFNpos and IFNneg patients largely differed between pDCs and mDCs (**Fig 2E**). Thus, although the IFN signature is shared between pDCs and mDCs in SLE and APS, the genes that are affected by the IFN signature are largely distinct between DC subsets.

Increased TLR7 expression in pDCs amplifies type I IFN production in SLE and APS

Since pDCs are the major cellular source of type I IFN we next analysed whether increased mRNA of any of the type I IFNs could be detected in pDCs. Surprisingly no gene-transcripts of any of the type I IFNs were found in pDCs at all (data not shown). However, when we evaluated the expression of Toll-like receptors (TLR) in pDCs of patients with or without an IFN signature we observed that TLR7 was significantly higher expressed in IFNpos patients as compared with IFNneg patients (**Fig 3A and supplementary table 1**). Besides, the TLR7-adaptor molecule MyD88 and IRF7, the transcription factor that induces IFNα transcription, were similarly upregulated in pDCs of IFN positive patients (**Fig 3A**), suggesting that pDCs of IFN-positive SLE and APS patients are primed for IFNα production through increased expression of TLR7. Therefore we next evaluated the effects of IFNα stimulation on TLR7 expression in cultured pDCs.

In vitro, IFNα upregulated the expression of TLR7 in pDCs of HC, both at mRNA and protein level (**Fig 3B**). As a result, priming of pDCs with IFNα greatly enhanced TLR7-mediated IFNα production (**Fig 3C**). However, no increase in TNFα production was seen, and the amount of TNF produced per cell as measured by median fluorescence intensity even decreased (**Fig 3D**). As expected, mDCs did not produce IFNα upon TLR7 triggering, nor after IFNα priming (data not shown).*Interferon alpha enhances antigen presentation in mDCs*

mDCs of IFN-positive SLE and APS patients had an increased expression of CD80 and CD86 (**Fig 4A**). In contrast to pDCs, mDCs upregulated protein expression of CD80, CD86 and HLA-DR upon activation by IFNα (**Fig 4B**). Of these proteins, the gene encoding for CD80 was allocated to the IFN module in mDCs, not in pDCs. We therefore hypothesized that IFNα priming enhances promotes T-cell stimulation in mDCs, not pDCs.

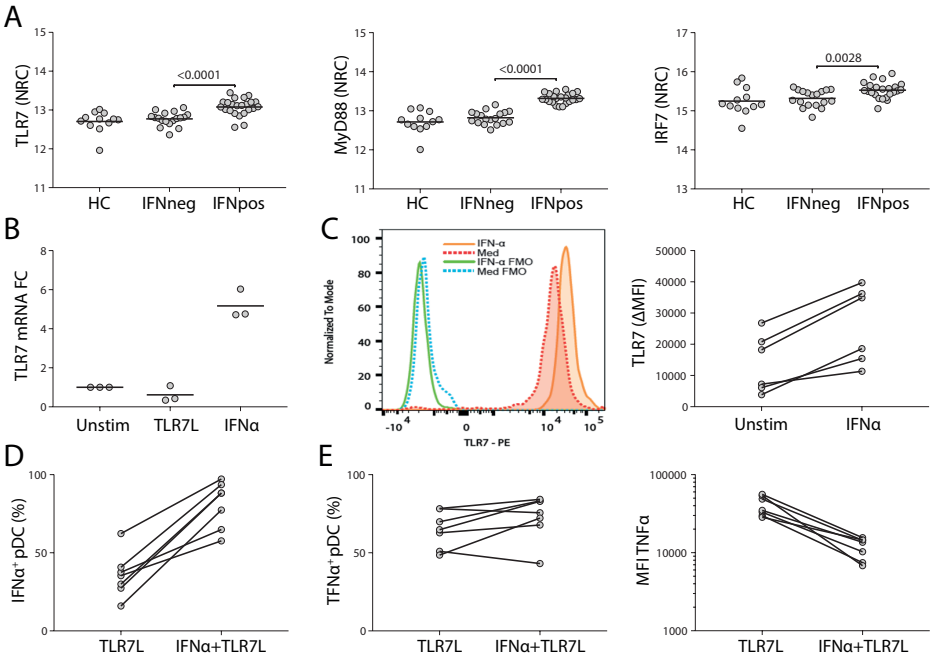

Figure 3. Increased expression of TLR7 amplifies type I IFN production by pDCs in SLE, SLE+APS and PAPS.

Expression (normalized read counts, NRC) of TLR7, MyD88 and IRF7 in pDCs of patients with SLE, SLE+APS and PAPS according to IFN status (A). mRNA expression of TLR7 in purified pDCs cultured for three hours with IFNα or TLR7 agonist loxoribine (B). Intracellular protein expression as assessed by flowcytometry in pDCs stimulated overnight with IFNα (C). IFNα production by pDCs as assessed by intracellular flow cytometry after stimulation with TLR7 agonist loxoribine with or without overnight priming of cells with IFNα (D). TNFα production by pDCs as assessed by intracellular flow cytometry after stimulation with TLR7 agonist loxoribine with or without overnight priming of cells with IFNα (E).

Upon co-culture with T-cells, IFNα primed mDCs induced higher T-cell proliferation than non-primed mDCs. The CTLA4-Ig Abatacept, which blocks the stimulatory signal provided by CD80/86 on DCs completely abolished T-cell proliferation (**Fig 4C**). In contrast to mDCs, pDCs induced no T-cell proliferation and IFNα priming of pDCs had no effect on T-cell proliferation (**Fig 4D**).

IFNα induces the production of BAFF by pDCs and mDCs

Activation of B-cells and their production of autoantibodies forms the pathologic cornerstone of autoimmune diseases. Autoantibodies, including those found in patients with SLE and APS activate pDCs to produce IFNα[16,17]. To investigate

whether DCs in SLE and APS contribute to B-cell pathology we studied the expression of the key B-cell growth factor B-cell activating factor (BAFF).

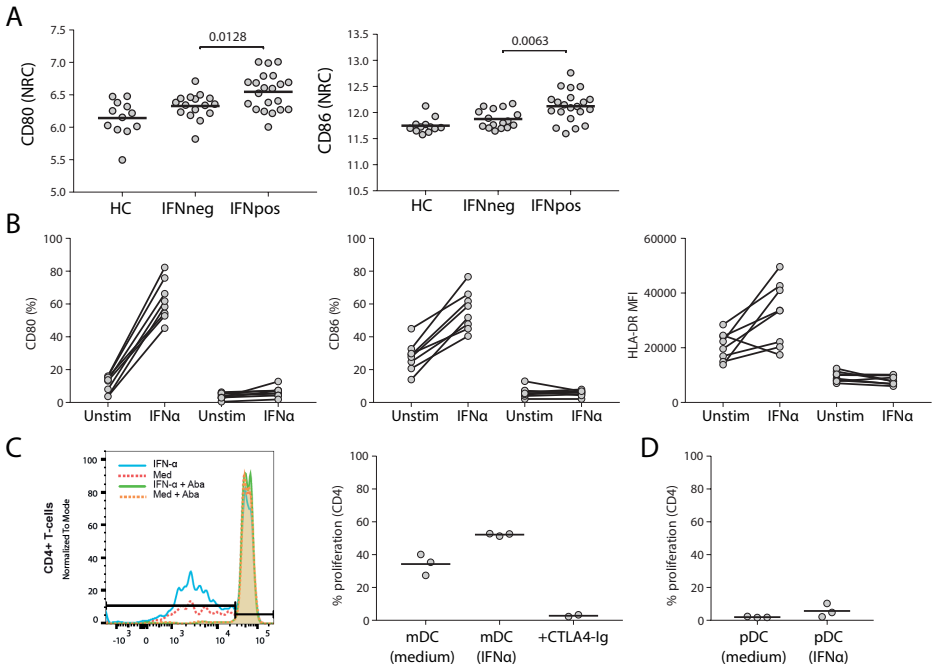

Figure 4: Increased expression of co-stimulatory molecules on mDCs of IFN-positive SLE, SLE+APS and PAPS patients induces enhanced T-cell proliferation.

Expression (normalized read counts NRC) of CD80 and CD86 in mDCs of patients with SLE, SLE+APS and PAPS according to IFN status (A). Expression of CD80, CD86 and HLA-DR in mDCs and pDCs stimulated overnight with IFNα (B). Proliferation of T-cells after co-culture with mDCs primed or not for 24 hours with IFNα (C). Proliferation of T-cells after co-culture with pDCs primed or not for 24 hours with IFNα (D).

B-cell activating factor (BAFF) plays a crucial role in the activation of B-cells in autoimmune diseases [20]. BAFF is produced by several myeloid cells including DCs when stimulated with IFNα[21]. Consistently, both in pDCs and mDCs of patients with SLE and APS we observed increased BAFF mRNA expression in IFN-positive patients (**Fig 5A**). Furthermore, in vitro, IFNα upregulated BAFF mRNA expression in both pDCs and mDCs of HC (**Fig 5B**). BAFF may be either expressed on the cell membrane or cleaved and released. Although membrane bound BAFF (mBAFF) was higher in unstimulated mDCs as compared with pDCs (**Fig 5C**), IFNα decreased mBAFF in mDCs, not pDCs, suggesting that in mDCs mBAFF is cleaved and secreted. However, both mDCs and pDCs secreted BAFF in the supernatant to a similar extent (**Fig 5D**).

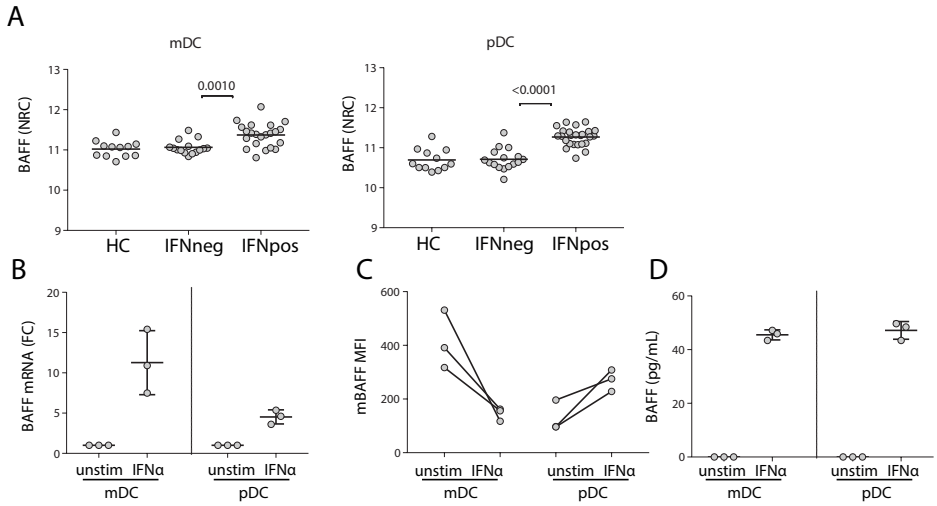

Figure 5: BAFF expression is increased in pDCs and mDCs of type I IFN+ SLE, SLE+APS and PAPS patients and is induced by IFNa in mDCs and pDCs.

Expression (normalized readcounts NRC) of BAFF (TNFSF13B) in pDCs and mDCs of patients with SLE, SLE+APS and PAPS according to IFN status (A). mRNA expression of BAFF in purified pDCs and mDCs stimulated for 3 hours with IFNα (B). membrane-bound BAFF (mBAFF) expression in mDCs and pDCs stimulated overnight with IFNα (C). Secretion of BAFF in the supernatant of pDCs and mDCs stimulated for 24 hours with IFNα (D).

Discussion

Here we studied the transcriptome of both pDCs and mDCs isolated from patients with SLE, SLE+APS and PAPS. There was considerable homogeneity between SLE and APS patients in altered pathways in both pDCs and mDCs. The IFN signature as assessed by gene module analysis strongly effected the transcriptome of both pDCs and mDCs in SLE and APS. Nonetheless, there was a remarkable difference in genes affected by the IFN signature in IFN-positive patients between pDCs and mDCs, which related to functional differences among these DC subsets.

According to our observations, IFNα primes pDCs for more IFNα production through upregulation of TLR7, whereas mDCs upregulate co-stimulatory molecules resulting in an increased T-cell stimulatory capacity. Both pDCs and mDCs upregulated and produced BAFF upon exposure to IFNα. As a result, the IFNα produced by pDCs not only amplifies its own production but through its effects on mDCs it induces T and B-cell activation through upregulation of co-stimulatory molecules and BAFF. This in turn may lead to more autoantibody production, which may results in more tissue damage and IFNα production[16,22].

The increased expression of TLR7 induced by IFNα resulted in increased IFNα production by pDCs when activated by TLR7 agonists. This was not true for TNFα secretion, which was not potentiated by IFNα priming of pDCs and the amount of TNF produced per pDC even decreased in IFNα primed pDCs. Although the mechanisms underlying this difference are unclear, we speculate that IFNα exerts different effects on the different downstream intermediates of TLR7 which could result in the activation of IRF7 or NFκB pathways, the former resulting in the production of type I IFN, the resulting in the secretion of other cytokines including TNF [23].

Abatacept inhibited the T-cell proliferation of IFNα primed mDCs. In clinical trials abatacept did not reach the primary endpoint in patients with SLE[24–26]. However, a recent post-hoc analysis, which grouped patients based on a transcriptomic based full blood deconvolution identified that a subgroup of patients, characterized by increased B-cell and DC activity showed a favourable response to abatacept[27]. This cluster of patients was the only subgroup with elevated anti-dsDNA antibodies. As anti-dsDNA antibodies correlate with the IFN signature[28], we speculate that this cluster represents patients with a high IFN signature and hence, the IFN signature may predict treatment response to abatacept. The increased expression of CD80/86 on mDCs of IFN-positive patients may serve as an explanation for the favourable response of IFN-positive patients to abatacept. However, future research should determine indeed if abatacept is clinically effective in IFN positive SLE patients.

Patients with SLE have elevated serum levels of BAFF which correlate with disease activity and autoantibody levels[20]. Belimumab, a monoclonal antibody that neutralizes BAFF is clinically effective in patients with SLE[29,30]. Expression of BAFF was increased in both pDCs and mDCs of IFN positive patients, suggesting that pDCs and mDCs directly contribute to B-cell hyperactivity in SLE and APS. BAFF mRNA expression was induced in both pDCs and mDCs upon activation by IFNα. Although in both cell types this led to secretion of BAFF in the supernatant, the expression of membrane bound BAFF decreased in mDCs upon activation by IFNα. Nonetheless, the soluble form of BAFF is superior in B-cell activation than mBAFF[31]. To our knowledge, BAFF production by pDCs and mDCs has not been previously studied in autoimmune diseases. Other cells besides pDCs and mDCs may produce BAFF as well, however DCs migrate towards lymph nodes upon activation where they come in close proximity of B-cells and thus DC derived BAFF may be crucial for the B-cell hyperactivity seen in autoimmune diseases.

Although the IFN signature is equally strong between pDCs and mDCs, the genes affected by the IFN signature are largely distinct between the cell subsets, resulting in a differential activation of pDCs and mDCs by IFNα in vitro. IFNα therefore activates different pathways in cell subsets downstream of IFNAR, the receptor for type I IFN. Type I IFN is crucial for our defence against viruses and as a result, an increased risk for viral infections, most notably herpes zoster, is seen in SLE patients treated with anifrolumab[8]. Hence a better understanding of how IFNα differentially activates cell subsets may help to identify novel therapeutics that prevent the damaging effects of IFNα in autoimmune diseases while retaining its antiviral effects.

References

1. Tsokos G. Systemic Lupus Erythematosus. *N Engl J Med* 2011;**365**:2110–21. doi:10.1007/SpringerReference_61618
2. Ruiz-irastorza G, Crowther M, Branch W, *et al.* Antiphospholipid syndrome. *Lancet* 2010;**376**:1498–509. doi:10.1016/S0140-6736(10)60709-X
3. Hooks J, Moutsopoulos H, Geis S, *et al.* Immune interferon in the circulation of patients with autoimmune disease. *N Engl J Med* 1979;**301**:5–8.
4. Preble O, Black R, Friedman R, *et al.* Systemic lupus erythematosus: presence in human serum of an unusual acid-labile leukocyte interferon. *Science (80-)* 1982;**216**:429–31.
5. Higgs BW, Liu Z, White B, *et al.* Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis* 2011;**70**:2029–36. doi:10.1136/ard.2011.150326
6. Bennett L, Palucka AK, Arce E, *et al.* Interferon and Granulopoiesis Signatures in Systemic Lupus. 2003;**197**. doi:10.1084/jem.20021553
7. Baechler EC, Batliwalla FM, Karypis G, *et al.* Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci U S A* 2003;**100**:2610–5. doi:10.1073/pnas.0337679100
8. Furie R, Khamashta M, Merrill J, *et al.* Anifrolumab, an Anti-Interferon-Alpha Receptor Monoclonal Antibody, in Moderate to Severe Systemic Lupus Erythematosus. *Arthritis Rheumatol* 2017;**69**:376–86. doi:10.1002/art.39962
9. Van Den Hoogen LL, Fritsch-Stork RDE, Versnel MA, *et al.* Monocyte type i interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. *Ann Rheum Dis* 2016;**75**. doi:10.1136/annrheumdis-2016-210485
10. van den Hoogen LL, van Roon JAG, Radstake TRDJ, *et al.* Delineating the deranged immune system in the antiphospholipid syndrome. *Autoimmun Rev* 2016;**15**:50–60. doi:10.1016/j.autrev.2015.08.011
11. Grenn RC, Yalavarthi S, Gandhi AA, *et al.* Endothelial progenitor dysfunction associates with a type i interferon signature in primary antiphospholipid syndrome. *Ann Rheum Dis* 2017;**76**:450–7. doi:10.1136/annrheumdis-2016-209442
12. Blanco P, Palucka a K, Gill M. Induction of Dendritic Cell Differentiation by IFN-α in Systemic Lupus Erythematosus. *Science (80-)* 2001;**294**:1540–4.
13. Jego G, Palucka AK, Blanck J-PJ, *et al.* Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* 2003;**19**:225–34. doi:S1074761303002085 [pii]
14. Kaplan MJ, Salmon JE. How Does Interferon-a Insult the Vasculature ? Let Me Count the Ways. *Arthritis Rheumatol* 2011;**63**:334–6. doi:10.1002/art.30161
15. Lande R, Ganguly D, Facchinetti V, *et al.* Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* 2011;**3**:73ra19. doi:10.1126/scitranslmed.3001180

16. Lövgren T, Eloranta ML, Båve U, *et al.* Induction of interferon-α production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum* 2004;**50**:1861–72. doi:10.1002/art.20254

17. Prinz N, Clemens N, Strand D, *et al.* Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. *Blood* 2011;**118**:2322–32. doi:10.1182/blood-2011-01-330639

18. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;**40**:1725. doi:10.1002/1529-0131(199709)40:9<1725::AID-ART29>3.0.CO;2-Y

19. Miyakis S, Lockshin MD, Atsumi T, *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006;**4**:295–306.

20. Vincent FB, Morand EF, Schneider P, *et al.* The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol* 2014;**10**:365–73. doi:10.1038/nrrheum.2014.33

21. Sjöstrand M, Johansson A, Aqrawi L, *et al.* The Expression of BAFF Is Controlled by IRF Transcription Factors. *J Immunol* 2016;**196**:91–6. doi:10.4049/jimmunol.1501061

22. Prinz N, Clemens N, Strand D, *et al.* Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. *Blood* 2011;**118**:2322–32. doi:10.1182/blood-2011-01-330639

23. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol* 2015;**15**:471–85. doi:10.1038/nri3865

24. Wofsy D. Treatment of lupus nephritis with abatacept: the Abatacept and Cyclophosphamide Combination Efficacy and Safety Study. *Arthritis Rheumatol (Hoboken, NJ)* 2014;**66**:3096–104. doi:10.1002/art.38790

25. Furie R, Nicholls K, Cheng TT, *et al.* Efficacy and safety of abatacept in lupus nephritis: A twelve-month, randomized, double-blind study. *Arthritis Rheumatol* 2014;**66**:379–89. doi:10.1002/art.38260

26. Merrill JT, Burgos-Vargas R, Westhovens R, *et al.* The efficacy and safety of abatacept in patients with non-life-threatening manifestations of systemic lupus erythematosus: Results of a twelve-month, multicenter, exploratory, phase IIb, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 2010;**62**:3077–87. doi:10.1002/art.27601

27. Bandyopadhyay S, Connolly SE, Jabado O, *et al.* Identification of biomarkers of response to abatacept in patients with SLE using deconvolution of whole blood transcriptomic data from a phase IIb clinical trial. *Lupus Sci Med* 2017;**4**. doi:10.1136/lupus-2017-000206

28. Kennedy WP, Maciuca R, Wolslegel K, *et al.* Association of the interferon signature metric with serological disease manifestations but not global activity scores in multiple cohorts of patients with SLE. *Lupus Sci Med* Published Online First: 2015. doi:10.1136/lupus-2014-000080

29. Furie R, Petri M, Zamani O, *et al.* A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum* 2011;**63**:3918–30. doi:10.1002/art.30613

30. Navarra S V., Guzmán RM, Gallacher AE, *et al*. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: A randomised, placebo-controlled, phase 3 trial. *Lancet* 2011;**377**:721–31. doi:10.1016/S0140-6736(10)61354-2

31. Bossen C, Tardivel A, Willen L, *et al*. Mutation of the BAFF furin cleavage site impairs B-cell homeostasis and antibody responses. *Eur J Immunol* 2011;**41**:787–97. doi:10.1002/eji.201040591

Supplementary table 1:
Expression of Toll-like receptors in pDCs in IFN-pos versus IFN-neg patients in order of statistical significance.

hgnc_symbol	baseMean	Fold change (Log2)	P-value	Adjusted p-value (FDR)
TLR7	7579.33	0.31	1.95E-09	1.81E-07
TLR2	756.45	0.22	6.53E-02	2.66E-01
TLR1	851.64	0.11	8.75E-02	3.14E-01
TLR6	763.84	-0.11	9.40E-02	3.28E-01
TLR4	869.77	0.18	1.39E-01	4.05E-01
TLR8	460.48	0.18	1.48E-01	4.20E-01
TLR5	116.28	0.11	3.99E-01	6.85E-01
TLR10	1357.29	-0.05	7.02E-01	8.76E-01
TLR3	2.25	0.01	8.89E-01	NA
TLR9	23.52	-0.28	2.64E-02	NA

Supplementary table 2:
Primer lists

TLR7_Fw	CAAGAAAGTTGATGCTATTGGGC
TLR7_Rv	TGGTTGAAGAGAGCAGAGCA
TNFSF13B_Fw (BAFF)	TGGGGATGAATTGAGTCTGGTG
TNFSF13B_Rv (BAFF)	GCAATGCCAGCTGAATAGCA
GUSB_Fw	CACCAGGGACCATCCAATACC
GUSB_Rv	GCAGTCCAGCGTAGTTGAAAAA

CHAPTER 10

Systemic and local Granzyme B levels are associated with disease activity, kidney damage, and IFN signature in systemic lupus erythematosus.

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Abstract

Objectives: Granzymes (Gr) are serine proteases that eliminate virally infected or tumor cells by inducing apoptosis. Granzyme B has been associated to the pathophysiology of systemic lupus erythematosus (SLE), whereas the role of the other granzymes in SLE remains unknown.

Methods: Granzyme levels were determined in serum of SLE patients and controls, and linked to SLE activity parameters, including the interferon signature. In addition, GrB expression was investigated in lupus nephritis (LN) biopsies and correlated to kidney function parameters and disease severity.

Results: Serum GrK and GrM levels were not elevated in SLE and did not correlate with disease activity. In contrast, GrB was increased in SLE serum, which correlated to both SLEDAI and interferon signature. GrB expression was detected in LN tissue biopsies. The amount of GrB positive cells in tissue correlated to several kidney function parameters (e.g., serum creatinine, proteinuria) and to the LN chronicity index.

Conclusions: GrB, but not GrK and GrM, is increased in serum and kidney of patients with SLE and correlates with measures of poor prognosis in LN. These data suggest that GrB may contribute to the pathogenesis of SLE/LN, which opens the possibilities for GrB as a biomarker and/or a therapeutic target.

Key messages:

- Circulating GrB but not GrK and GrM levels are elevated in SLE patients
- Circulating GrB concentration correlates with SLEDAI and the interferon signature
- GrB expression in lupus nephritis biopsies correlates with signs of kidney damage

Introduction

Systemic lupus erythematosus (SLE) is a chronic and systemic autoimmune disease characterized by the presence of autoantibodies directed against nuclear components. The pathophysiology of SLE is complex and includes genetic and environmental factors, defects in the clearance of apoptotic cells and the activation of immune cells. Overexpression of type I interferon (IFN) inducible genes due to the activation of plasmacytoid dendritic cells (pDC) and alterations in B-cells as well as helper and cytotoxic T-cell subsets drive inflammation in SLE (1). Several organs can be affected in SLE and up to 50% of patients develop kidney disease, termed lupus nephritis (LN). In LN immune complexes are deposited in the glomeruli, initiate inflammation, and induce tissue damage. LN is diagnosed by kidney biopsy and classified into different classes based on histologic findings. Despite improvements in its treatment, LN is still associated with substantial morbidity and mortality in patients with SLE (2).

Granzymes are serine proteases mainly produced by cytotoxic lymphocytes such as CD8+ T cells, γδ T cells, Natural Killer (NK) cells, and NKT cells as well as by pDCs (3). In humans, five different granzymes have been described: granzyme A (GrA), B, H, K, and M. All five are cytotoxic proteases that induce apoptosis in abnormal cells such as virally infected or cancerous cells (4). Next to this, granzymes fulfill extracellular functions in inflammation or autoimmune diseases where they trigger cytokine production and induce tissue damage by cleavage of extracellular matrix proteins (5). Extracellular GrB is thought be involved in the pathogenesis of SLE, by cleaving autoantigens resulting in the formation of immunogenic neo-epitopes leading to the formation of pathogenic autoantibodies (6). Increased levels of GrB expressing CD8+ T-cells and NK cells associate with disease activity in SLE (7). Similarly GrB positive CD8+ T-cells infiltrate affected skin of SLE patients (8). GrB expression in patients with SLE in frequently affected tissues such as the kidney has not been analyzed. Likewise, the role of granzymes other than GrB has not been studied in SLE and their implication in SLE and/or LN pathogenesis remains unknown. In this study we investigated serum levels of GrB, GrK and GrM according to disease activity and interferon signature in SLE. In addition, renal GrB expression was locally examined in relation to LN class and other signs of kidney damage.

Methods

Serum Granzyme levels

Serum from 30 patients with SLE fulfilling the 1997 ACR criteria for SLE and 20 age and sex matched healthy controls (HC) was collected and stored at -80°C until further use. Clinical characteristics were obtained from the patients' charts, disease activity was assessed by SELENA-SLEDAI. None of the patients had evidence of an active viral infection at inclusion. Serum levels of GrB, GrK, and GrM were determined using ELISA (Uscn Life Science Inc) according to the manufacturer's instructions.

Interferon signature

From 20 patients with SLE and 20 HC peripheral blood mononuclear cells were isolated from peripheral blood by density gradient isolation using ficoll-hypaque (GE healthcare). Monocytes were isolated by CD14 magnetic bead (Miltenyi) isolation using an autoMACSpro according to the manufacturer's instructions. Purified monocytes (10^4) were stained for 20 minutes at 4°C with CD45-PerCP (Biolegend), CD16-PE (Dako) and CD14-FITC (Miltenyi) to assess the purity of the isolated monocytes. All samples had a purity of >90% (Data not shown). RNA was isolated from purified monocytes using the All prep Universal Kit (Qiagen) by the Qiacube (Qiagen) according to the manufacturer's instructions. cDNA was generated using the iScript cDNA synthesis kit (BioRad). RT-qPCR was conducted in duplicate on the following four type I IFN inducible genes (Ly6E, IFITM1, Serping1 and IFI44L). Gene-expression was normalized to the housekeeping gene GUSB. Normalized gene-expression values were used to calculate type I IFN scores as previously described (9).

Granzyme B evaluation in lupus nephritis kidney

Formalin fixed paraffin embedded renal biopsies of (a historic cohort of) 34 histologically proven proliferative LN patients (including 3 patients with re-biopsies) were cut into 3 μm slides and used for immunohistochemistry (IHC). LN was diagnosed by light microscopy on hematoxylin and eosin, Periodic acid-Schiff, or silver stained sections in combination with immunofluorescence for IgG, IgM, IgA, C1q, C3, kappa, and lambda light chains using the revised classification criteria for glomerulonephritis in SLE (10) by two experienced renal pathologists (RG and TQN). Activity index and chronicity index were scored based on histology slides as described by Austin et al (11). GrB expression was determined by IHC using the mouse IgG -7 clone (Thermo Fisher) as described before (12). The number of GrB positive cells was corrected for biopsy size and recorded using Aperio Image scope. In healthy renal tissue sections, GrB cannot be detected as described previously (12).

Serum creatinine levels (μmol/L), 24 hour urinary protein excretion (g/L) and urine sediment prior to biopsy were obtained from the patients' charts.

Ethics

For the serum samples, all patients and healthy donors signed an informed written consent form approved by the local institutional review boards prior to participation in the study.

For the renal biopsies, we are using archival pathology material which does not interfere with patient care and does not involve physical involvement of the patient. Therefore, no ethical approval is required according to Dutch legislation (13). Use and storage of pseudo-anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients and therefore informed consent procedure was not required for the tissue sections according to our institutional medical ethical review board (14).

Statistics

Comparisons between groups were made using Mann-Whitney U test when comparing 2 groups or Kruskall-Wallis with post-hoc Dunn's when comparing more than 2 groups. Correlations between parameters were tested using the spearman rank correlation. All tests were conducted two-sided with an alpha level set at 0.05. Statistical analysis were performed using graphpad prism (version 6).

Results

Serum GrB but no1t GrK and GrM levels are elevated in SLE patients.

Elevated levels of GrB ($p<0.001$) but not GrK ($p=0.12$) and GrM ($p=0.40$) were found in the sera of the SLE patients (Fig. 1A-C). Only serum GrB levels correlated to disease activity as measured by SELENA-SLEDAI ($r=0.38$, $p=0.04$, Fig. 1D). Increased levels of GrB were not confined to active SLE patients, as patients with low disease activity (defined as SELENA-SLEDAI ≤4, n=20), also had elevated serum levels of GrB compared to HC ($p=0.005$, data not shown). Furthermore, GrB levels were strongly associated with the type I IFN score ($r=0.754$, $p<0.0001$, Fig. 1E). There were no significant differences in serum GrB levels in patients to levels of anti-dsDNA antibodies, complement, or leukocyte count, nor between patients with or without medication or immunosuppressant use (Data not shown). No correlations between disease activity nor the type I IFN score and GrK or GrM levels were observed (data not shown).

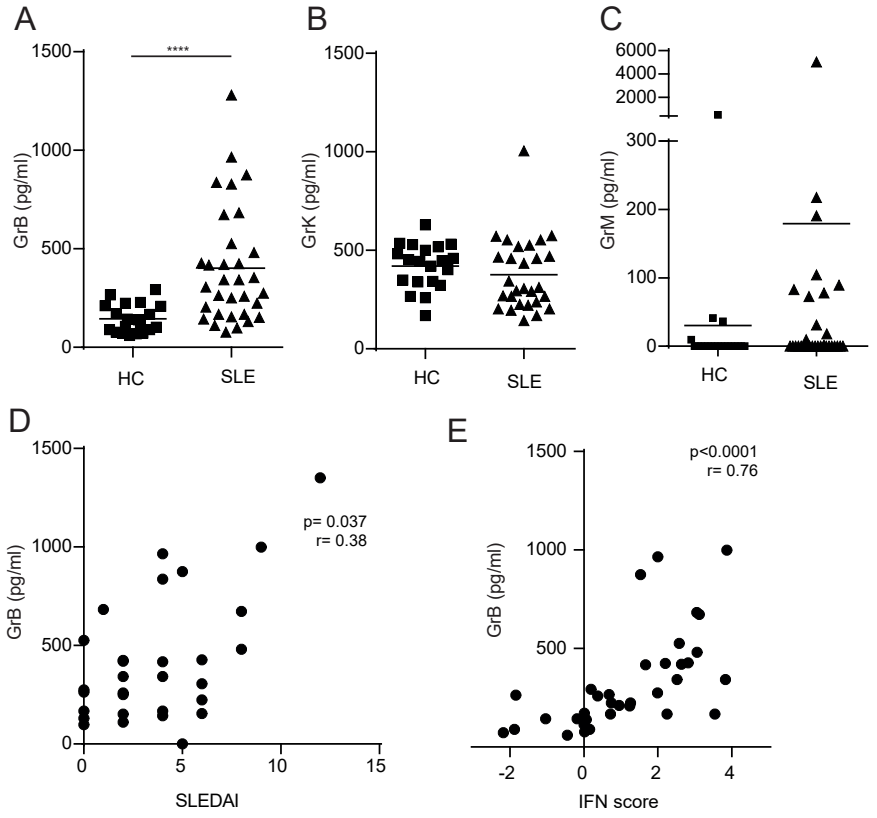

Figure 1. Serum GrB, GrK, and GrM levels correlate to SLE disease parameters.
Serum levels of GrB (A), GrK (B) and GrM (C) in SLE patients and healthy controls were measured using ELISA. Serum GrB levels were correlated to SLEDAI index (D). GrB of all available samples (SLE and HC, n=35) were correlated with the type I IFN score (E). Mean granzyme levels are depicted and statistical analysis was performed using the Mann-Whitney U-test. **** $p < 0.0001$ compared to healthy control.

Granzyme B is expressed in the kidney in lupus nephritis and associates with signs of kidney damage.

To assess relevant tissue granzyme expression beyond the already described GrB presence in SLE skin, we analyzed GrB expression in the kidney cortex of proliferative LN from patients with active urinary sediment based on SELENA-SLEDAI. GrB positive cells were seen in the majority of biopsies predominantly located in the insterstitium between tubules rather than in glomeruli or around blood vessels (Fig. 2A). Staining with an isotype control antibody was negative (data not shown). Whereas no correlation was found between local GrB levels and the LN activity index (r=0.28 p=0.13) (Fig. 2B), a significant correlation was seen with the LN chronicity index (r=0.51 p=0.003) (Fig. 2C). Furthermore, GrB expression cor-

related with signs of kidney damage, such as proteinuria (r=0.53, p=0.003) (Fig. 2D) and serum creatinine levels (r=0.41, p=0.03) (Fig. 2E). GrB expression differed between proliferative LN class III and class IV (p=0.035) (Fig. 2F). Finally, there was no significant difference in GrB expression between proliferative classes (III and IV) compared to non-proliferative classes (II and V) (Fig. 2G). Class IV GrB levels were significantly higher than classes II, III and V (p=0.029) (data not shown).

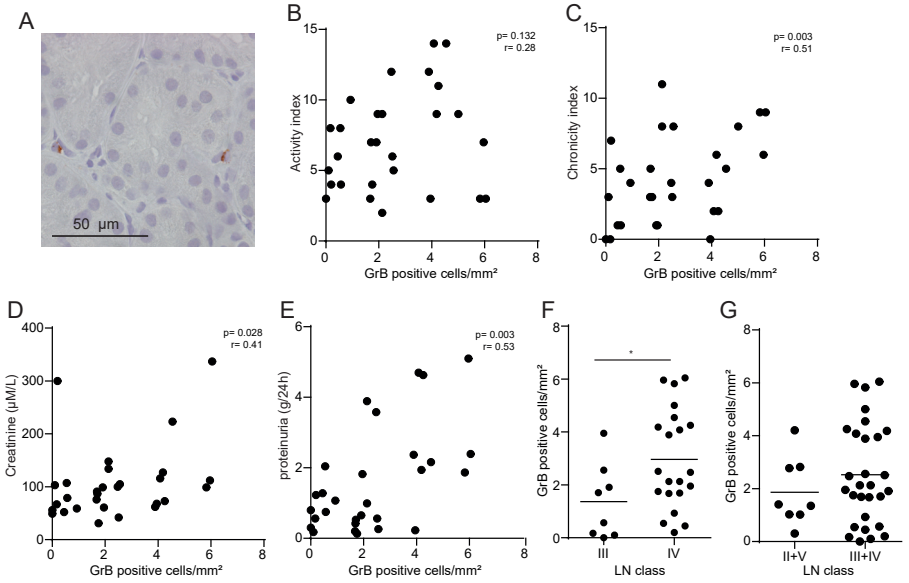

Figure 2: Local GrB in lupus nephritis biopsies correlates with kidney damage.
A representative picture of a GrB positive cell in a LN biopsy is shown in (A). GrB positive cells/mm^2 of LN biopsies were determined and correlated with the lupus nephritis activity index (B), chronicity index (C), creatinine (D) and 24h proteinuria (E). GrB positive cells in LN biopsies were observed in different LN classes (F) and proliferative classes (II+V) versus proliferative classes were shown (G). * p < 0.05 compared to other LN class

Discussion

In the present study, the expression levels of several granzymes were assessed in serum and kidney samples of patients with SLE. Serum levels of GrK and GrM were not elevated in SLE and did not correlate with disease activity. In contrast, serum GrB levels were increased and correlated with disease activity and the IFN signature. Additionally, GrB levels were associated with kidney damage in LN patients.

Similar to the results of our cohort of patients with SLE, GrK levels were shown not to be altered in the sera of patients with rheumatoid arthritis (15). In contrast,

GrK has previously been found elevated in the circulation of patients with sepsis (16) and during viral infections (15). This discrepancy suggests that serum levels of specific granzymes are differentially regulated in infection driven as compared to auto-immune induced inflammation.

Serum GrB levels correlated with the type I IFN signature in SLE patients. The type I IFN signature, a typical feature of SLE immune pathology, is thought to be caused by the activation of pDC endosomal Toll-like receptor (TLR) ligands (1). pDC are able to express and release GrB (3). However, stimulation of pDC with endosomal TLR agonists inhibits GrB release (17). Hence, it is unlikely that TLR triggering leads to the release of both type I IFN and GrB by pDC in patients with SLE. However, elevated levels of type I IFN in the sera of patients with SLE drive DC to induce GrB expression in CD8+ cytotoxic T-cells in patients with SLE (7). Therefore, pDC, by producing type I IFN, might have a more indirect effect on GrB release via DC and CD8+ T-cells.

In this study, we find positive correlations between SLE disease severity and serum GrB or GrB-positive cell influx in the kidney. Consistent with this, increased levels of GrB+ CD8+ T-cells in peripheral blood of patients with SLE have been shown to be associated with disease activity (7). Contradictory to our findings, a decrease of Granzyme B-positive SLAMF4+ CD8+ T cells and GrB positive NK cells in the circulation of patients with SLE has also been demonstrated (18, 19). This discrepancy could be explained by multiple reasons. First, GrB can be expressed by multiple cell types other than SLAMF4+ CD8+ T cells or NK cells, including CD4+ T cells, Treg cells, γδ T cells, NKT cells, neutrophils, basophils, dendritic cells, macrophages and mast cells (20). Second, GrB might be released from these specific cell types leading to a selective loss of these T cells and NK cells in SLE (18). Alternatively, we cannot rule out the possibility that NK cells or GrB+ SLAMF4+ CD8+ T cells migrate from the circulation into the kidney. A correlation between circulating GrB levels and SLEDAI in patients with active SLE (mean SLEDAI 31, range 12-48) was reported (21). In our cohort, the median SLEDAI was 4 (range 0-12) indicating a more quiescent and representative SLE cohort, which nevertheless expressed an elevated GrB compared to HC.

Besides elevated systemic levels, also an increased expression of GrB in the kidney was detected. In contrast to immune complexes which are deposited in the glomeruli, we observed GrB expressing cells mostly in tubolointerstitial areas. This pattern of GrB expression is in line with a previous report on GrB expression in renal allografts during acute rejection where the recipient's T-cells are thought to primarily

target tubular epithelial cells (12). Although GrB expression was not observed in the glomeruli, its expression correlated with proteinuria and serum creatinine levels.

Non-proliferative classes II and V showed comparable GrB expression as compared to proliferative classes III and IV. In this context it should be mentioned that 30% of class II biopsies are usually sample errors from class III (22). Therefore we cannot fully exclude the possibility that we overestimate Granzyme B expression in non-proliferative classes. This is compatible with the notion that the 3 non-proliferative cases with highest GrB expression are class II cases. Furthermore, GrB expression correlated to the chronicity index. Since the degree of proteinuria, higher serum creatinine levels, and higher chronicity index are associated with poor clinical outcome of LN (23, 24), local GrB expression might be of influence to the prognosis of LN.

Whether or not GrB exerts more impact on lupus related damage or on SLE disease activity remains an open question. Besides the role of GrB in immune cell-mediated cytotoxicity, GrB may also contribute to autoantigen processing, cytokine responses or matrix remodeling in (renal) fibrosis by cleaving extracellular matrix proteins (6, 7). This is compatible with potential roles of GrB in the interstitial facet of LN as well as systemically in SLE, influencing the overall prognosis (25). Further research is required to elucidate the importance of GrB in SLE and LN pathogenesis, to determine how the biomarker value of GrB might be further increased, and whether inhibition of GrB might be a valid target for novel therapies.

References

1. van den Hoogen LL, Sims GP, van Roon JA, Fritsch-Stork RD. Aging and Systemic Lupus Erythematosus - Immunosenescence and Beyond. Curr Aging Sci. 2015;8(2):158-77.
2. Hanly JG, Su L, Urowitz MB, Romero-Diaz J, Gordon C, Bae SC, et al. A Longitudinal Analysis of Outcomes of Lupus Nephritis in an International Inception Cohort Using a Multistate Model Approach. Arthritis Rheumatol. 2016 Aug;68(8):1932-44.
3. Jahrsdorfer B, Vollmer A, Blackwell SE, Maier J, Sontheimer K, Beyer T, et al. Granzyme B produced by human plasmacytoid dendritic cells suppresses T-cell expansion. Blood. 2010 Feb 11;115(6):1156-65.
4. Bovenschen N, Kummer JA. Orphan granzymes find a home. Immunol Rev. 2010 May;235(1):117-27. Epub 2010/06/12. eng.
5. Wensink AC, Hack CE, Bovenschen N. Granzymes regulate proinflammatory cytokine responses. J Immunol. 2015 Jan 15;194(2):491-7. Epub 2015/01/04. eng.
6. Graham KL, Utz PJ. Sources of autoantigens in systemic lupus erythematosus. Curr Opin Rheumatol. 2005 Sep;17(5):513-7.
7. Blanco P, Pitard V, Viallard JF, Taupin JL, Pellegrin JL, Moreau JF. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. Arthritis Rheum. 2005 Jan;52(1):201-11. Epub 2005/01/11. eng.
8. Grassi M, Capello F, Bertolino L, Seia Z, Pippione M. Identification of granzyme B-expressing CD-8-positive T cells in lymphocytic inflammatory infiltrate in cutaneous lupus erythematosus and in dermatomyositis. Clin Exp Dermatol. 2009 Dec;34(8):910-4. Epub 2009/05/22. eng.
9. van den Hoogen LL, Fritsch-Stork RD, Versnel MA, Derksen RH, van Roon JA, Radstake TR. Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. Ann Rheum Dis. 2016 Dec;75(12):e81.
10. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. J Am Soc Nephrol. 2004 Feb;15(2):241-50.
11. Austin HA, 3rd, Muenz LR, Joyce KM, Antonovych TT, Balow JE. Diffuse proliferative lupus nephritis: identification of specific pathologic features affecting renal outcome. Kidney Int. 1984 Apr;25(4):689-95.
12. Kummer JA, Wever PC, Kamp AM, ten Berge IJ, Hack CE, Weening JJ. Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. Kidney Int. 1995 Jan;47(1):70-7.
13. CCMO website:. Central Committee on Research involving Human Subjects (Centrale Commissie Mensgebonden Onderzoek) (text in Dutch).
14. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. For. BMJ. 2002 Sep 21;325(7365):648-51.
15. Bade B, Lohrmann J, ten Brinke A, Wolbink AM, Wolbink GJ, ten Berge IJ, et al. Detection of soluble human granzyme K in vitro and in vivo. Eur J Immunol. 2005 Oct;35(10):2940-8.
16. Rucevic M, Fast LD, Jay GD, Trespalcios FM, Sucov A, Siryaporn E, et al. Altered levels and molecular forms of granzyme k in plasma from septic patients. Shock. 2007 May;27(5):488-93.

17. Karrich JJ, Jachimowski LC, Nagasawa M, Kamp A, Balzarolo M, Wolkers MC, et al. IL-21-stimulated human plasmacytoid dendritic cells secrete granzyme B, which impairs their capacity to induce T-cell proliferation. Blood. 2013 Apr 18;121(16):3103-11.

18. Kis-Toth K, Comte D, Karampetsou MP, Kyttaris VC, Kannan L, Terhorst C, et al. Selective Loss of Signaling Lymphocytic Activation Molecule Family Member 4-Positive CD8+ T Cells Contributes to the Decreased Cytotoxic Cell Activity in Systemic Lupus Erythematosus. Arthritis Rheumatol. 2016 Jan;68(1):164-73.

19. Henriques A, Teixeira L, Ines L, Carvalheiro T, Goncalves A, Martinho A, et al. NK cells dysfunction in systemic lupus erythematosus: relation to disease activity. Clin Rheumatol. 2013 Jun;32(6):805-13. Epub 2013/02/05. eng.

20. Boivin WA, Cooper DM, Hiebert PR, Granville DJ. Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. Lab Invest. 2009 Nov;89(11):1195-220. Epub 2009/09/23. eng.

21. Shah D, Kiran R, Wanchu A, Bhatnagar A. Soluble granzyme B and cytotoxic T lymphocyte activity in the pathogenesis of systemic lupus erythematosus. Cell Immunol. 2011;269(1):16-21. Epub 2011/04/05. eng.

22. Lee SG, Cho YM, So MW, Kim SS, Kim YG, Lee CK, et al. ISN/RPS 2003 class II mesangial proliferative lupus nephritis: a comparison between cases that progressed to class III or IV and cases that did not. Rheumatol Int. 2012 Aug;32(8):2459-64.

23. Contreras G, Pardo V, Cely C, Borja E, Hurtado A, De La Cuesta C, et al. Factors associated with poor outcomes in patients with lupus nephritis. Lupus. 2005;14(11):890-5.

24. Soliman S, Mohan C. Lupus nephritis biomarkers. Clin Immunol. 2016 Aug 3.

25. Buzza MS, Zamurs L, Sun J, Bird CH, Smith AI, Trapani JA, et al. Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. J Biol Chem. 2005 Jun 24;280(25):23549-58.

Supplementary table 1: clinical and demographic patient characteristics from SLE patients – serum cohort

	HC (n=20)	SLE (n=30)
Age	44 (35 – 50)	43 (35 – 53)
Female	90%	93%
SLENA-SLEDAI		4 (2-6)
anti-dsDNA (EliA, IU/mL)		20 (3 – 66)
C3 (g/L)		0.84 (0.75 – 0.99)
C4 (g/L)		0.15 (0.12 – 0.21)
SLE duration (y)		12 (8 – 21)
Lupus Nephritis (history of)		50%
eGFR <90 ml/min		30%
Current drug use		
Prednisone		57%
Prednisone dose (mg/day)		7.5 (5 – 10)
Hydroxychloroquine		53%
Azathioprine		40%
Mycophenolate mofetil		10%

Table 1: patient characteristics. Table shows medians ± IQR or percentage of total.

Supplementary table 2: Patient characteristics from SLE patients – biopsy cohort

Kolom1	SLE n=38
Biopsies	38
Number of patients	34
Female	78%
Age	33 (23 – 51)
Creatinine (uMol/L prior to biopsy)	87 (60 – 107)
Proteinuria (g/L, prior to biopsy)	1.15 (0.52 – 2.8)
Renal SLEDAI (prior to biopsy)	12 (8 – 16)

CHAPTER 11

Increased expression of Fas on group 2 and 3 innate lymphoid cells is associated with a type I interferon signature in systemic lupus erythematosus and primary Sjögren's syndrome patients

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Abstract

Objective

The role of innate lymphoid cells (ILCs) in the pathophysiology of rheumatic diseases is emerging. Evidence from animal studies implicate type I interferon (IFN), produced by plasmacytoid dendritic cells (pDCs), to be involved in regulating the survival of group 2 and group 3 ILCs (ILC2s and ILC3s) via the upregulation of Fas (CD95) expression. For the first time, we explored the frequency and phenotype of circulating ILCs in systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS) in relationship to the IFN signature.

Methods

Frequencies and phenotypes of ILC subsets and pDC were assessed by flow cytometry in peripheral blood of patients with SLE (n=20), pSS (n=20) and healthy controls (n=17). Patients were stratified by the presence or absence of an IFN signature as assessed by RT-qPCR on circulating mononuclear cells.

Results

ILC1 frequencies were increased in peripheral blood of patients with SLE and in pSS patients with high disease activity as compared to healthy controls. Overall, the frequencies of ILC1s, ILC2s or ILC3s did not differ between patients with SLE, pSS and healthy controls. However, when stratifying patients based on the IFN signature, patients with a high type I IFN signature expressed elevated levels of Fas on ILC2s and ILC3s, which coincided with decreased frequencies of these cells.

Conclusion

The presence of a type I IFN signature is related to Fas expression and frequencies of circulating ILC2s and ILC3s in patients with SLE and pSS, potentially altering the homeostatic balance of ILCs.

Introduction

Innate lymphoid cells (ILCs) are immune cells of lymphoid morphology that play an important role in epithelial homeostasis. ILCs lack the expression of T cell receptors and other "lineage" markers characteristic for other hematopoietic cells. Besides their location at barrier sites, ILCs can be found in low numbers in blood. In response to environmental triggers ILCs rapidly release cytokines. The expression of transcription factors and the production of cytokines distinguishes three subsets of ILCs, that mirror the well-known subsets of helper T cells (Th). ILC1s express T-bet and produce interferon (IFN) γ and tumor necrosis factor (TNF) α, ILC2s express GATA-3 and produce interleukin (IL) 4, 5 and 13 and ILC3s express RORγt and produce IL-17 and 22, analogous to Th1, Th2 and Th17 cells, respectively.[1,2] Due to their high expression of the IL-7 receptor alpha chain (CD127), survival of ILCs, in particular ILC2s and ILC3s [2], is critically dependent on IL-7.

Recent studies have linked alterations in the frequency and function of ILCs to the pathophysiology of rheumatic diseases, in particular rheumatoid arthritis (RA) and spondyloarthropathies.[1,2] SLE and pSS are systemic autoimmune diseases with involvement of epithelial tissue including the salivary and lacrimal glands, skin and oral mucosa. Central to the pathogenesis of SLE and pSS is the increased activity of T and B cells leading to production of pathogenic autoantibodies. Increased activity of the IL-7 axis drives T and B cell hyperactivity in SLE and pSS and is related to the major pathologic features such as dryness and lupus nephritis.[3,4]

Type I IFNs (eg. IFNα) play an important role in SLE and pSS, as increased levels are associated with disease activity and IFNs regulate multiple key immunological processes, including B cell hyperactivity. Mechanistically, In SLE and pSS, immune complexes of RNA binding autoantibodies have been shown to activate plasmacytoid dendritic cells (pDC) to produce type I IFN (IFNα), resulting in an overexpression of type I IFN-inducible genes, known as the IFN signature.[5]

Evidence from animal studies that model HIV and asthma indicate a close relationship between IFNα-producing pDCs and the fate of group 2 and group 3 ILCs. pDC-derived IFNα induces the expression of Fas (First apoptosis signal receptor, CD95) and other proapoptotic molecules on ILC2s and ILC3s rendering them susceptible to apoptosis.[6–8] In patients with HIV, ILC2s and ILC3s are decreased in frequency and characterized by a high expression of Fas.[9] However, no previous studies have investigated ILCs in patients with SLE and pSS. Here we explored the frequency and phenotype of circulating ILCs in SLE and pSS in relation to pDC activation and the subsequent IFN signature.

Methods

Patients

Peripheral blood mononuclear cells (PBMCs) from patients and healthy controls were isolated by ficoll density gradient isolation and cryopreserved in liquid nitrogen until further use. A total of 20 patients with SLE, 20 patients with pSS and 17 age- and sex-matched healthy controls were included in this study. SLE patients met the American college of rheumatology (ACR) classification criteria and patients with pSS met the American-European Consensus Group criteria (AECG). None of the included patients had clinical evidence of an ongoing infection. This study was approved by the ethical committee of the UMC Utrecht, in accordance with the Helsinki declaration. All patients and healthy controls signed informed consent prior to the donation of blood. Patient characteristics can be found in **supplementary table 1**.

Flow cytometry

ILCs were identified as previously described by our group.[10] A list of used antibodies can be found in **supplementary table 2**. 5-10x10e6 PBMC were stained and subsequently acquired on an LSR Fortessa (BD). Dead cells were excluded using the fixable viability dye eF506 (eBioscience). From the lymphocyte gate, ILCs were identified as Lin-CD127+. The expression of CD117 (c-Kit) and CD294 (CRTH2) were used to identify ILC1s (CD117-CD294-), ILC2s (CD294+) and ILC3s (CD117+CD294-) subsets.

A second flow cytometry panel was used to identify the frequency of pDCs among CD45+ cells (defined as CD123+BDCA2+BDCA4+ cells) and the expression of Siglec-1 (Sialic acid-binding immunoglobulin-type lectin-1, CD169, an IFN inducible protein [11]) on CD14+ monocytes.

qPCR

RNA was extracted from PBMCs using the Allprep universal kit (Qiagen) according to the manufacturer's instructions. Complementary DNA was synthesized from 200ng RNA using iScript (BioRad). RT-qPCR was performed on a Quantstudio 12k Flex (Thermofisher). The expression of IFI44, IFI44L, MX1, Ly6E and IFIT3 normalized to the housekeeping gene GUSB were used to calculate an IFN scores. A list of primer sequences can be found in **supplementary table 3**.

Statistics

The type I IFN scores were calculated as previously described.[12,13] An optimal cut-off to discern IFN-high and IFN-low patients subgroups was identified at the

maximum J-statistic of the Youden's index of the receiver operating characteristics curve as described[13]. Mann Whitney U tests and spearman rank correlations were used to test statistical significance. All tests were conducted two sided at an alpha level of 0.05.

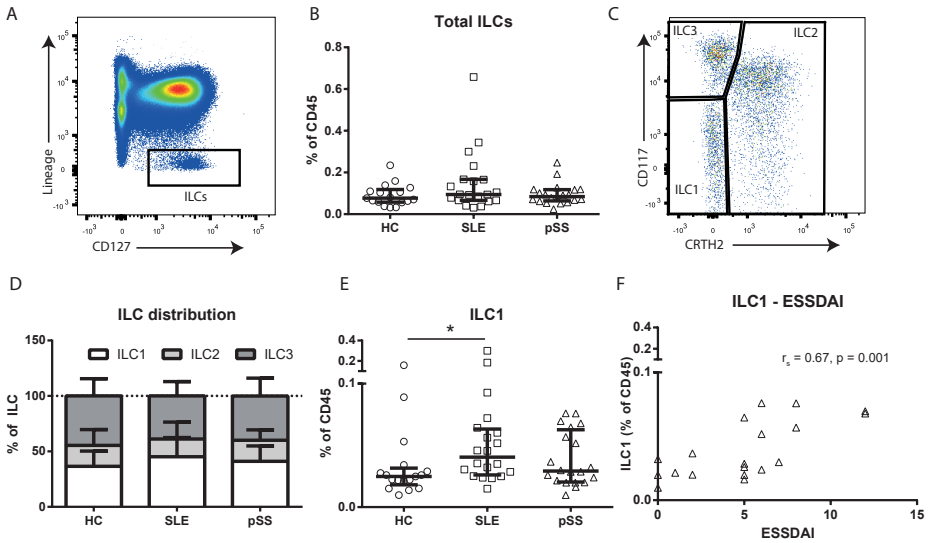


Figure 1. Increased circulating ILC1 frequency in patients with SLE and pSS with high disease activity. (**A**) Identification of ILCs in the peripheral blood of SLE and pSS patients based on lack of expression of lineage markers and expression of IL-7Rα (CD127). (**B**) Frequency of total ILCs in patients with SLE and pSS does not differ from HCs. (**C**) Identification of the three ILC subsets on the basis of the expression of CD117 and CD294. (**D**) ILC1s are increased in patients with SLE. (**E**) Increased frequencies of ILC1s are correlated to higher disease activity in patients with pSS.

Results

ILC1s are increased in SLE and are associated with disease activity in pSS

We readily identified circulating ILCs among PBMCs by their high expression of CD127 and the absence of lineage markers (**Figure 1A**). The total frequencies of ILCs did not differ among SLE and pSS patients as compared with HCs (**Figure 1B**). Using the expression of CD117 and CD294 we discerned three populations of ILCs: CD117-CD294- ILC1s, CD294+ ILC2s and CD117+CD294- ILC3s (**Figure 1C**). There were no changes in the proportions of ILC subsets among total ILCs among patients with SLE, pSS and HCs (**Figure 1D**). With the exception of ILC1s being increased in patients with SLE as compared with HCs (**Figure 1E**), no differences were found in the frequencies of ILCs subsets among patients with SLE, pSS and HCs (**Supplementary figure 1**). Within the pSS group, higher ILC1

frequencies were associated with higher disease activity as measured by ESSDAI (r=0.68, p=0.001, **Figure 1F**) as well as serum IgG levels (r=0.53, p=0.015, data not shown) and with presence of anti-La/SSB autoantibodies (p=0.01, data not shown). No correlation with SLE clinical features were found.

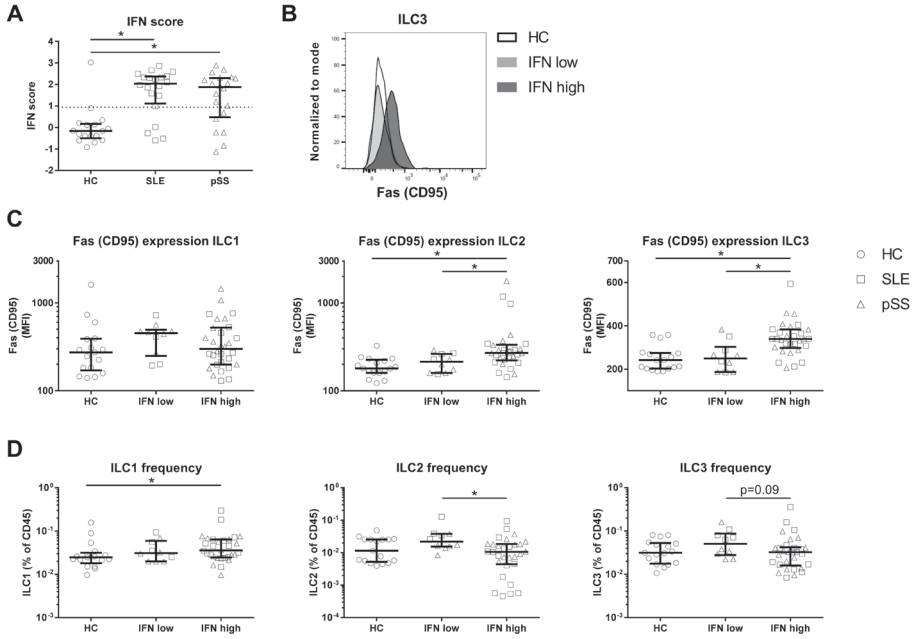


Figure 2. Fas expression is elevated on ILC2s and ILC3s from patients with a high type 1 IFN signature in association with decreased frequencies of these cells. (**A**) Elevated IFN scores are present in a subgroup of patients with SLE and pSS. (**B** representative FACS plot, **C**) Increased expression of Fas (CD95) on ILC2s and ILC3s in patients with a high type I IFN signature. (**D**) Decreased frequencies of ILC2s and ILC3s in patients with a high type I IFN signature.

The presence of a type I IFN signature is associated with elevated Fas expression on ILC2 and ILC3 subsets and decreased cell frequency

Elevated IFN scores were observed in patients with SLE and pSS (p<0.001, **Figure 2A**), which strongly correlated to monocyte Siglec-1 (CD169) expression (r=0.82, p<0.001, **supplementary figure 2**), confirming the robust identification of the IFN signature. Setting a threshold using the HCs, 16/20 (80%) and 14/20 (70%) patients with SLE and pSS respectively were characterized by a high type I IFN signature (IFN-high, **Figure 2A**). In patients with a high IFN signature, Fas expression was upregulated on both ILC2 and ILC3 subsets (**Figure 2B, C**). In contrast, Fas expression on ILC1s was not related to the IFN signature (**Figure 2C/data not shown**). The increased expression of Fas on the ILC2 and ILC3 subsets in IFN-high

patients was associated with a decrease in ILC2s (p=0.01, **Figure 2D**) and a similar trend in ILC3s (p=0.09, **Figure 2D**) as compared with IFN-low patients. ILC1 frequencies did not differ between IFN-high and IFN-low patients (**Figure 2D/data not shown**). Circulating pDCs were decreased in SLE and pSS and correlated with Fas expression on ILC2 and ILC3 subsets (r=-0.39, p=0.01, and r=-0.38, p=0.02 for ILC2 and ILC3 respectively, **Supplementary figure 3**).

Discussion

Here we evaluated for the first time the frequency and phenotype of circulating ILC subsets in patients with SLE and pSS. ILC1s were found elevated in blood of patients with SLE and were associated with disease activity in pSS. When comparing SLE and pSS, no differences were observed in the frequencies of ILC subsets. However, high expression of Fas (CD95) on circulating ILC2 and ILC3 subsets was associated with an increased IFN signature, possibly rendering these cells more susceptible to apoptosis. These observations corroborate previous studies in mice that reported a link between type I IFN, pDC activation and apoptosis of circulating ILC2s and ILC3s [6–8]. We here report unprecedented data on a potential similar regulation in the blood of patients with systemic autoimmune diseases.

ILCs were only recently identified. In the pre-ILC era, a subset of ILC1-like natural killer (NK) cells were reported increased in patients with SLE as compared to HC.[2] Here we report that circulating ILC1s, classified according to current phenotypical definitions, are increased in patients with SLE and in patients with pSS with higher disease activity.

In mice models of rheumatoid arthritis (RA), the ILC2 subset is crucial for the resolution of autoimmune inflammation by producing IL-9 which promotes regulatory T cell function and restores immune homeostasis. In humans, circulating ILC2 are reduced in active RA and normalize to normal levels in remission.[14] Whether such differences represent systemic cell death or (re-)circulation events remains to be established. In our cohort, the frequency of circulating ILC2s is reduced in patients with a high IFN signature, which is associated with disease activity in these patients. Hence, possibly the decrease in ILC2s in IFN-high SLE and pSS patients may contribute to ongoing inflammation via similar mechanisms as in RA.

In systemic sclerosis, another disease characterized by a type I IFN signature, ILC2s are elevated in the skin.[15] Migration of ILCs into target tissues might therefore represent another explanation for the reduced levels of circulating ILC2s and ILC3s

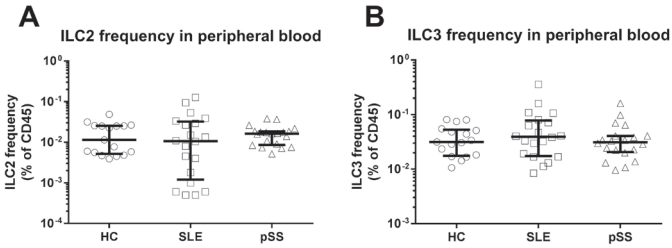
in IFN-high patients. No studies so far have assessed ILCs in affected tissues of patients with SLE. In pSS, IL-22 producing NKp44^{+} cells, which might represent ILC3, are found in the salivary glands of patients with pSS and correlate with the amount of lymphocytic infiltration.[16]

Survival of ILCs, including lymphoid tissue inducer cells (LTi) is dependent on IL-7, which is elevated in salivary glands of pSS patients[17]. The IL-7/IL-7R axis plays an important role in formation of ectopic lymphoid structures in non-lymphoid tissues, including the salivary gland, and this has been shown to require presence of LTi cells[18]. Altogether, this suggests a role for ILCs in development of lymphocytic infiltrates, including germinal center-like structures, in the glands of pSS patients.

Perturbations in the immune system are often shared between different autoimmune diseases, with the IFN signature as a clear example for SLE and pSS. It is becoming increasingly clear, that both from an immunological as well as a clinical perspective, stratifying patients with SLE and pSS on a molecular level, such as the IFN signature, aids in the identification of subgroups with different immunological alterations and even response to therapy.[12,13,19] Here we report that alterations in the frequency and phenotype of circulating ILCs is related to the IFN signature in SLE and pSS. On the basis of our observations, we propose that future studies on ILCs in patients with autoimmune diseases, whether in blood or tissue, should take into account the presence or absence of the (local) IFN signature, since this may significantly impact their function and role in autoimmune diseases.

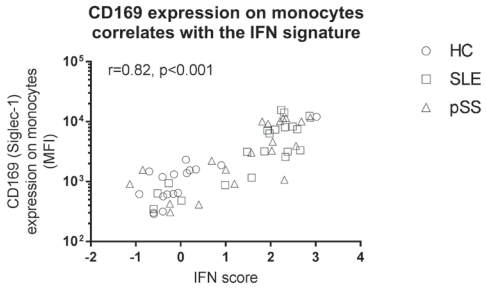
Supplementary Figures

1



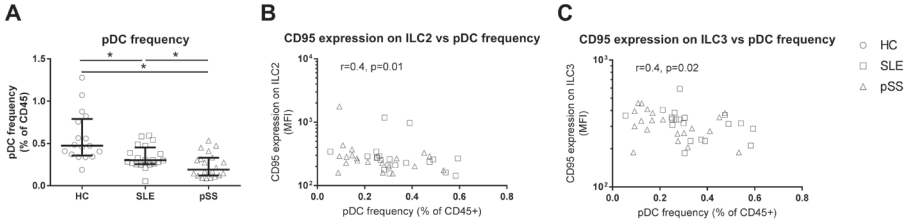
Supplementary figure 1. Peripheral blood frequencies of ILC2s and ILC3s do not differ between HC, SLE and pSS. Frequencies of (**A**) ILC2 and (**B**) ILC3 in peripheral blood of HC and patients with SLE and pSS.

2



Supplementary figure 2. CD169 expression by monocytes correlates with the IFN signature in SLE and pSS patients. Correlation between CD169 (Siglec-1) expression by monocytes and the IFN signature in HC, SLE and pSS.

3



Supplementary figure 3. Fas expression by ILC2s and ILC3s correlates with pDC frequency in SLE and pSS patients. (**A**) Plasmacytoid DC frequencies in peripheral blood of HC, SLE and pSS. Correlation between pDC frequency and Fas expression by (**B**) ILC2 and (**C**) ILC3 in SLE and pSS patients.

References

1. Wenink MH, Leijten EF, Cupedo T, RDJ Radstake T, Radstake T. Innate lymphoid cells: sparking inflammatory rheumatic disease? n.d. doi:10.1002/art.40068.
2. Shikhagaie MM, Germar K, Bal SM, Ros XR, Spits H. Innate lymphoid cells in autoimmunity: emerging regulators in rheumatic diseases. Nat Rev Rheumatol 2017;13:164–73. doi:10.1038/nrrheum.2016.218.
3. Hillen MR, Blokland SLM, Risselada a P, Bikker a, Lauwerys BR, Kruize a a, et al. High soluble IL-7 receptor expression in Sjögren's syndrome identifies patients with increased immunopathology and dryness. Ann Rheum Dis 2016;0:annrheumdis – 2016–209236. doi:10.1136/annrheumdis-2016-209236.
4. Badot V, Luijten RKM a C, van Roon J a, Depresseux G, Aydin S, Van den Eynde BJ, et al. Serum soluble interleukin 7 receptor is strongly associated with lupus nephritis in patients with systemic lupus erythematosus. Ann Rheum Dis 2013;72:453–6. doi:10.1136/annrheumdis-2012-202364.
5. Rönnblom L, Eloranta M-L. The interferon signature in autoimmune diseases. Curr Opin Rheumatol 2013;25:248–53. doi:10.1097/BOR.0b013e32835c7e32.
6. Maazi H, Banie H, Aleman G, Patel N, Wang B, Sankaranarayanan I, et al. Activated Plasmacytoid Dendritic Cells regulate Type 2 Innate Lymphoid Cells-mediated Airway Hyperreactivity. J Allergy Clin Immunol 2017. doi:10.1016/j.jaci.2017.04.043.
7. Zhang Z, Cheng L, Zhao J, Li G, Zhang L, Chen W, et al. Plasmacytoid dendritic cells promote HIV-1 – induced group 3 innate lymphoid cell depletion. J Clin Invest 2015;125:3692–703. doi:10.1172/JCI82124DS1.
8. Duerr CU, McCarthy CDA, Mindt BC, Rubio M, Meli AP, Pothlichet J, et al. Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. Nat Immunol 2015;17:65–75. doi:10.1038/ni.3308.
9. Klöverpris HN, Kazer SW, Mj??sberg J, Mabuka JM, Wellmann A, Ndhlovu Z, et al. Innate Lymphoid Cells Are Depleted Irreversibly during Acute HIV-Infection in the Absence of Viral Suppression. Immunity 2016;44:391–405. doi:10.1016/j.immuni.2016.01.006.
10. Leijten EFA, van Kempen TS, Boes M, Michels-van Amelsfort JMR, Hijnen D, Hartgring SAY, et al. Brief report: enrichment of activated group 3 innate lymphoid cells in psoriatic arthritis synovial fluid. Arthritis Rheumatol (Hoboken, NJ) 2015;67:2673–8. doi:10.1002/art.39261.
11. Rose T, Grutzkau a., Hirseland H, Huscher D, Dahnrich C, Dzionek a., et al. IFN and its response proteins, IP-10 and SIGLEC-1, are biomarkers of disease activity in systemic lupus erythematosus. Ann Rheum Dis 2012:1639–45. doi:10.1136/annrheumdis-2012-201586.
12. Van den Hoogen LL, Fritsch-Stork RD, Versnel MA, Derksen RH, van Roon JA, Radstake TR. Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. Ann Rheum Dis 2016;75:e81. doi:10.1136/annrheumdis-2016-210485.
13. Brkic Z, Maria NI, Helden-meeuwsen CG Van, De JP Van, Daele PL Van, Dalm VA, et al. Prevalence of interferon type I signature in CD14 monocytes of patients with Sjögren ' s syndrome and association with disease activity and BAFF gene expression 2013:728–35. doi:10.1136/annrheumdis-2012-201381.

14. Rauber S, Luber M, Weber S, Maul L, Soare A, Wohlfahrt T, et al. Resolution of inflammation by interleukin-9-producing type 2 innate lymphoid cells. Nat Publ Gr 2017. doi:10.1038/nm.4373.

15. Wohlfahrt T, Usherenko S, Englbrecht M, Dees C, Weber S, Beyer C, et al. Type 2 innate lymphoid cell counts are increased in patients with systemic sclerosis and correlate with the extent of fibrosis. Ann Rheum Dis 2016;75:623–6. doi:10.1136/annrheumdis-2015-207388.

16. Ciccia F, Guggino G, Rizzo A, Ferrante A, Raimondo S, Giardina A, et al. Potential involvement of IL-22 and IL-22-producing cells in the inflamed salivary glands of patients with Sjogren's syndrome. Ann Rheum Dis 2012;71:295–301. doi:10.1136/ard.2011.154013.

17. Van Roon J a G, Kruize A a., Radstake TRDJ. Editorial: Interleukin-7 and its receptor: The axis of evil to target in Sj??gren's syndrome? Arthritis Rheum 2013;65:1980–4. doi:10.1002/art.38002.

18. Pitzalis C, Jones GW, Bombardieri M, Jones S a. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. Nat Rev Immunol 2014;14:447–62. doi:10.1038/nri3700.

19. Furie R, Khamashta M, Merrill J, Werth V, Kalunian K, Brohawn P, et al. Anifrolumab, an Anti-Interferon-Alpha Receptor Monoclonal Antibody, in Moderate to Severe Systemic Lupus Erythematosus. Arthritis Rheumatol 2016;Accepted A. doi:doi:10.1002/art.39962.

Supplementary table 1. Patient characteristics

	HC (n=17)	SLE (n=20)	pSS (n=20)
Age	50 (40 - 56)	43 (27 - 55)	58 (46 - 67)
Female	100%	100%	100%
Current drug use			
Hydroxychloroquine		65%	10%
Prednisone		45%	10%
Azathioprine		25%	5%
Mycophenolate mofetil		15%	0%
Serology			
Antinuclear antibodies		100%	85%
anti-dsDNA antibodies (ELiA, IU/mL)		7 (2 - 14)	-
C3 (g/L)		0.94 (0.86 - 1.04)	1.09 (0.95 - 1.16)
C4 (g/L)		0.18 (0.14 - 0.21)	0.20 (0.16 - 0.27)
Anti-Ro/SSA		45%	80%
Anti-La/SSB		20%	50%
ESR (mm/hr)		7 (3 - 11)	16 (11 - 22)
Serum IgG (g/L)		-	14 (12 - 17)
Clincial manifestations			
ESSDAI		-	5 (2 - 7)
ESSPRI		-	6 (5 - 7)
Lymphocytic focus score (foci/4mm^2)		-	2 (1.3 - 2.4)
Schirmer (mm/5min)		-	10 (5 - 20)
SLEDAI		2 (0 - 4)	-
History of Lupus Nephritis		50%	-

Percentages of total or medians with interquartile range. Abbreviations: ESR: erythrocyte sedimentation rate. ESSDAI: EULAR Sjögren's syndrome disease activity index. ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index. SLEDAI: SLE disease activity index.

Supplementary table 2. Flow cytometry antibodies

ILC panel

Laser	Marker	Label	Company	Clone
Blue1	CD3	FITC	BD	SK7
	CD19	FITC	BD	HIB19
	CD14	FITC	BD	M5E2
	CD16	FITC	BD	3G8
	CD94	FITC	eBioscience	DX22
	CD11c	FITC	Sony Biotechnology	Bu15
	CD141	FITC	Miltenyi	14H12
	CD34	FITC	Sony Biotechnology	561
	BDCA2	FITC	Miltenyi	AC144
	CD123	FITC	eBioscience	6H6
	FCeR1	FITC	eBioscience	AER-37
Red1	CD127 (IL7Ra)	AF647	Sony Biotechnology	HCD127
Red1/1	CD45	AF700	Sony Biotechnology	F10-89-4
YeGr2	CD294 (CRTH2)	PE-CF594	BD Horizon	BM16
Violet1	CD95	ef450	eBioscience	DX2
Violet3	CD117 (cKit)	BV605	Biolegend	104D2
Violet2	Fixable viability dye	ef506	eBioscience	

pDC panel

Laser	Marker	Label	Company	Clone
red2	CD14	APC-ef780	eBioscience	61D3
violet1	CD16	PB	BD	3G8
blue3	BDCA2 (CD303)	PerCP-Cy5.5	Sony Biotechnology	201A
red1	BDCA4 (CD304)	APC	Miltenyi	AD5-17F6
red1/1	CD45	AF700	Sony Biotechnology	F10-89-4
blue1	CD123	FITC	eBioscience	6H6
Yegr1	CD169	PE	eBioscience	7-239

Supplementary table 3: Primers used for assessment of the IFN signature

Gene symbol	Forward primer	Reverse primer
Ly6E	ATCTGTACTGCCTGAAGCCG	GTCACGAGATTCCCAATGCC
IFIT3	ACTGTTTCAACGGGTGTTGG	CCTTGTAGCAGCACCCAATC
IFI44L	CCACCGTCAGTATTTGGAATGT	ATTTCTGTGCTCTCTGGCTT
GUSB	CACCAGGGACCATCCAATACC	GCAGTCCAGCGTAGTTGAAAAA
MX1	GCATCCCACCCTCTATTACTG	CGCACCTTCTCCTCATACTG
IFI44	TTTGCTCTTTCTGACATCTCGGT	TCCTCCCTTAGATTCCCTATTTGC

CHAPTER 12

Immune cell profiling by Cytometry by Time of Flight identifies shared and unique signatures across patients with various systemic autoimmune diseases

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in preparation

Introduction

Systemic sclerosis (SSc), systemic lupus erythematosus (SLE) and primary Sjogren's syndrome (pSS) are clinically distinct systemic autoimmune diseases (SAD), which are characterized by signs of autoimmunity that include the production of antinuclear antibodies and disease specific organs involvement. Where cardinal features of SSc include fibrosis of skin and internal organs, SLE is typified by immune complex deposition culminating in tissue damage in virtually any organ system including the skin, kidneys and joints. pSS patients mainly but not exclusively suffer from dryness of the eyes and mouth caused by inflammation in salivary and lacrimal glands.

In contrast, many features of immunopathology are shared among SSc, SLE and pSS. For instance, genome-wide association studies have revealed an overlapping genetic background among SSc, SLE and pSS and transcriptomic studies indicate an important role for type I interferons in all three conditions [1–4]. Although these observations suggest a common background of SSc, SLE and PSS, few studies are available that directly compare immunopathology among large groups of patients with SSc, SLE and PSS.

Phenotypic analysis of circulating immune cells by flow cytometry, using fluorochrome labeled antibodies, has yielded insights in our understanding of rheumatic diseases [5]. For instance, shifts increases in CD27hi plasmablasts are associated with disease activity in SLE [6], while increases in CD16+ monocytes are associated with more extensive skin fibrosis in SSc [7]. However, flow cytometry is limited by its maximum amount of analyzable parameters due to spectral overlap of fluorescent signals. Cytometry by Time of Flight (CyTOF) is a new technique that overcomes this problem by using antibodies conjugated to isotopically purified heavy metal atoms with unique masses allowing the simultaneous detection of a large amount antigens on a single cell using a spectrometer.

CyTOF holds promise as a novel tool to dissect the pathogenesis of complex diseases but has not been employed in patients with SLE and SSc [5]. A recent study using CyTOF analysis of circulating immune cells in patients with PSS identified distinct subtypes of PSS patients related to changes in the abundance of specific immune cells [8] although it is unknown how these changes relate to patients with other SAD and whether such clusters can also be identified in those patients. Here, we used CyTOF to profile the peripheral immune compartment of patients with SSc, SLE or pSS using identification and replication cohorts. Our results show replicated cellular profiling of both overlapping and distinct alterations among patients with

SSc, SLE and pSS. Importantly, by including patients with preclinical SSc (early SSc) we observed that these changes already occur in preclinical phases of disease.

Methods and materials

Study participants

Independent identification and replication cohorts consisting of patients with SSc, SLE and PSS and healthy controls (HC) were studied. Samples of patients were obtained in the clinics of the University medical center Utrecht, the Erasmus medical center Rotterdam, the Radboud university medical center Nijmegen and the Scleroderma Unit of the Fondazione IRCCS Ca' Granda Policlinico di Milano. Patients with SSc, SLE and pSS fulfilled their respective classification criteria [9–11]. SSc patients were stratified as having limited or diffuse cutaneous SSc (LcSSc or DcSSc respectively) based on the extent of skin fibrosis. SSc patients that met the classification criteria but did not have skin fibrosis (modified rodnan skin score = 0) were classified as non-cutaneous SSc [12]. Furthermore patients with early SSc (eaSSc) according to the Leroy and Medsger criteria were included [13]. EaSSc patients did not meet the classification criteria for SSc. Additionally, patients presenting with a typical phenotype of localized scleroderma (LoS) were included. The study was approved by the local research ethics committees, and informed written consent was obtained from all patients and control subjects.

Disease activity was scored by measuring the modified Rodnan skin score (mRSS) the SELENA modification of the SLE disease activity index (SLEDAI) in SLE, the European Sjogren's Syndrome Disease activity score (ESSDAI) in PSS and the modified Localized Scleroderma Skin Severity Index (mLOSSI) in LOS. The presence of interstitial lung disease (ILD) in SSc patients was identified as typical involvement of the lung parenchyma >5% on high resolution CT accompanied by a reduced forced vital capacity (FVC) or a diffusing capacity for carbon monoxide (DLco) < 80% of predicted in patients with SSc.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient from whole blood within 2 hours after drawing of the blood into lithium-heparin tubes. PBMCs were washed with low-barium PBS and resuspended at 6 million per ml in low barium PBS and fixed in 2% methanol-free formaldehyde (Thermofisher scientific). The PBMCs were washed twice with PBS–0.5% BSA–0.02% sodium azide after which cells were resuspended in freshly made PBS–0.5% BSA–0.02% sodium azide. Aliquots containing 2 million cells each were quickly frozen on dry

ice and stored at –80°C until shipment to Biogen (Cambridge, Mass) for mass cytometry analysis.

Mass cytometry

Paraformaldehyde-fixed and frozen cell suspensions were thawed on ice. Samples were stained and prepared for CyTOF analysis, as previously described [8] using an optimized cocktail of metal-conjugated antibodies designed to identify human blood cell subsets (see supplementary Table). The identification cohort was acquired on a CyTOF I device and the replication cohort was acquired on the CyTOF II (Fluidigm, South San Francisco, Calif). The data was normalized to internal bead standards. viSNE plots were made using CellAccense Software [14] to identify populations of cells. Cell subsets were subsequently identified by means of manual gating using FlowJo software (TreeStar) and the resulting cellular proportions were exported into SPSS 21 (IBM) for statistical analysis and Prism (GraphPad Software) for visualization of the data. Frequencies of cells were reported as percentage of CD45+ cells after exclusion of death cells and doublets. For subsets of specific lineages (T-cells, B-cells, monocytes and NK-cells), percentages were also reported as proportion of the subset.

Statistics

T-tests were used to compare the cell frequencies between patient groups and healthy donors after log-transformation. Differences between patients with SSc, SLE and pSS were compared with HC in the identification cohort at an explorative p-value of 0.1. Subsets with a p-value <0.05 in the replication cohort were considered as validated populations. Correlation heat maps and dot plots show the nonparametric Spearman rank correlation coefficient. Using R base cell frequencies were quantile normalized and clustering was performed based on Euclidean distance and wards minimal variance method.

Results

Clinical characteristics

In total 213 subjects were included. The identification cohort consisted of 19 SSc patients, 13 SLE patients, 8 pSS patients and 22 healthy controls (HC). The replication cohort consisted of 69 SSc patients, 18 SLE patients, 15 pSS patients and 22 HC. In addition, 12 patients with localized scleroderma (LOS) and 15 patients with early SSc were studied (**Table 1**).

Table 1: Clinical Characteristics

	SSc (n=88)	SLE (n=31)	pSS (n=23)	eaSSc (n=15)	LoS (n=12)
Age	54 (48 – 66)	43 (34 - 54)	56 (51 - 64)	66 (46 - 77)	52 (33 - 65)
Female	83%	100%	100%	93%	67%
Antinuclear antibodies	93%	100%	87%	93%	NA
Anticentromere antibodies	40%	NA	NA	73%	NA
Anti-Scl70 antibodies	27%	NA	NA	13%	NA
Interstitial Lung Disease	28%	NA	NA	0%	NA
Disease activity*	5 (3 - 10)	4 (1 - 6)	5 (2 - 6)	NA	17 (8 - 23)
Leukocyte count	7.5 (6.0 - 9.4)	6.1 (4.7 - 8)	4.8 (3.6 - 6.3)	6.6 (4.6 - 7.6)	NA
Prednisone	28%	48%	9%	13%	8%
Immunosuppressants (other than prednisone)	25%	84%	17%	7%	NA
anti-dsDNA antibodies (IU/mL)	NA	12 (2 - 93)	NA	NA	NA
C3 (g/L)	NA	0.89 (0.70 - 1.08)	NA	NA	NA
C4 (g/L)	NA	0.18 (0.13 - 0.23)	NA	NA	NA
Anti-Ro antibodies	NA	39%	83%	NA	NA
Anti-La antibodies	NA	16%	65%	NA	NA
Lymphocytic Focusscore	NA	NA	2 (2 - 2)	NA	NA
Serum IgG (g/L)	NA	NA	14 (12 - 18)	NA	NA
Subtype					
	ncSSc 32%	Nephritis 45%			Generalized 25%
	LcSSc 42%				Linear 42%
	DcSSc 26%				Linear + deep 8%
					MEP 16%
					Linear+MEP+ECDS 8%

*SSc: mRSS, SLE: SLEDAI, pSS: ESSDAI, LOS: mLOSSI

NA: not assessed, MEP: morphea en plaque, ECDS: en coup de sabre

Medians with interquartile range or percentage of total

Of the 88 SSc patients, 28 were classified as ncSSc, 37 as lcSSc and 23 patients as dcSSc. 83%, 100%, 100%, 93%, 67% and 93% of patients with SSc, SLE, pSS, eaSSc, LOS and HC were female respectively with a median (IQR) age of 54 (48 – 66) for SSc patients, 43 (34 – 54) for SLE patients, 56 (51 – 64) for pSS patients, 66 (46 – 77) for eaSSc patients, 52 (33 – 65) for LOS patients and 50 (40 – 55) for HC. For SSc patients the median modified Rodnan skin score was 5 (interquartile range (IQR) 3 – 10), for SLE patients the median SLEDAI was 4 (IQR 1 – 6), for pSS patients the median ESSDAI was 5 (IQR 2 – 6) and for LOS patients the median mLOSSI was 17 (IQR 8 – 23).

Identification and validation of distinct and shared altered cellular frequencies in SSc, SLE and pSS

t-distributed stochastic neighbour embedding (t-SNE) was used to identify cell populations which were then gated manually (**Supplemental Fig 2**). A total of 44 unique populations were identified, including rare cell types such as BDCA3+ dendritic cells. We first compared the relative frequency of the cell populations of three autoimmune conditions (SSc, SLE and pSS) with HC in the identification cohort. In SSc, SLE and pSS, 17, 21 and 12 subsets were respectively decreased and 7, 7 and 10 subsets were respectively increased as compared with HC. In the replication cohort 17, 22 and 14 subsets were decreased and 11, 9 and 21 subsets were increased in SSc, SLE and pSS, respectively (**Fig 1A**). Overall, in patients with SSc, 12 populations were consistently altered as compared to HC in both identification and replication cohorts, of which 7 were decreased as compared with HC and 5 were increased. In SLE, 9 populations were decreased and 1 was increased consistently. In pSS 3 populations were decreased and 4 were increased consistently as compared with HC (**Fig 1A**).

Heatmaps of both identification cohort (dotted line) and replication cohort (continued line) showed that these altered frequencies in patients with SSc, SLE and pSS overall showed similar trends among the three diseases (**Fig 1B and supplemental fig 1**), albeit not reaching statistical significance in all diseases. For instance, HLA-DR positive CD8 T-cells (DR^+ CD8) were only significantly increased in both identification and replication cohort in pSS patients, although similar trends were seen in SSc and SLE (**Fig 1D and Supplemental figure 1**). On the other hand, as an example the decrease in $CD56^{hi}$ NK-cells seemed to be relative specific for SSc (**Fig 1D and supplemental fig 1** median (±IQR) 0.21% (0.08-0.36) in SSc as compared with 0.37% (0.22-0.57), 0.28% (0.17-0.50) and 0.38% (0.27-0.65) in HC, SLE and pSS respectively). Taking into account all data, the only cellular phenomenon that was shared across all three diseases both in the identification as well as in the

replication cohort was the significantly reduced frequency of plasmacytoid dendritic cells (pDCs) (**Fig 1B, C & D**).

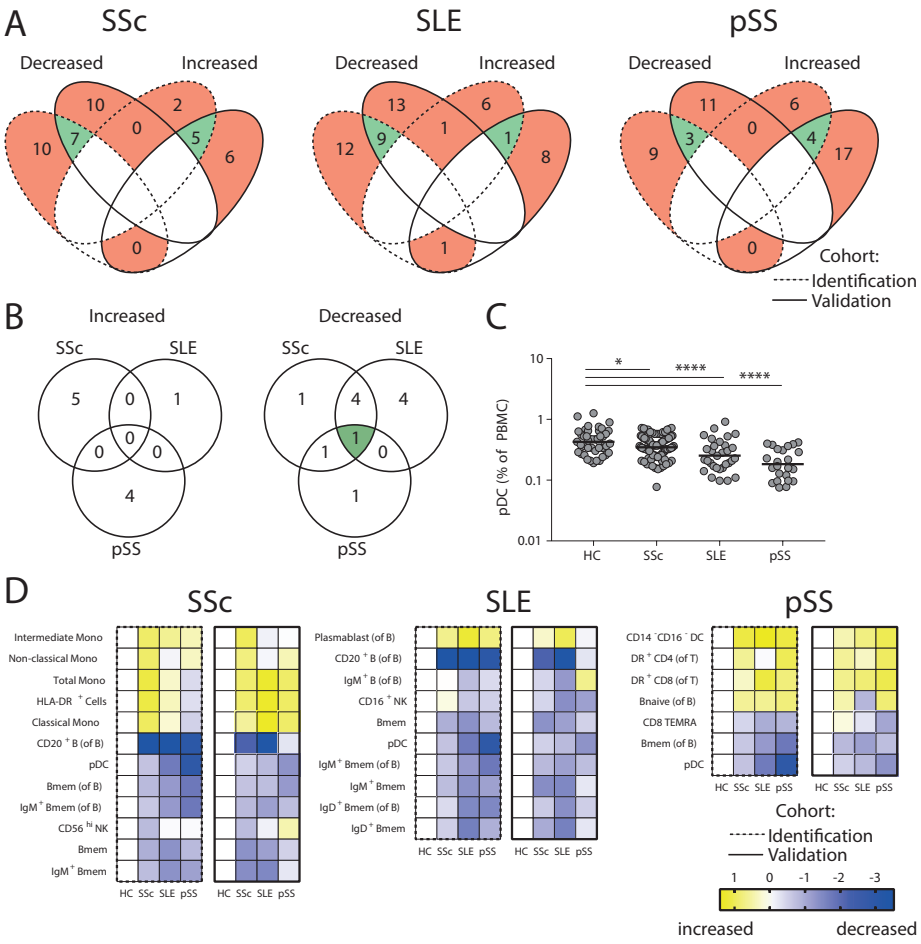


Figure 1. Cytof analysis identifies differentially altered and shared cell frequency differences in SSc, SLE and pSS patients A) Venn diagrams of cell populations with increased or decreased frequencies in patients with SSc, SLE or pSS in identification (dashed line) and replication (continuous line). Green indicates validated populations. B) Venn diagram of validated populations with increased or decreased frequencies. C) decrease in plasmacytoid dendritic cells is the only shared alteration between SSc, SLE and pSS. D) Heatmaps of validated populations in SSc, SLE and pSS in both identification and replication cohorts. *: $p<0.05$, **** $p<0.0001$. Colours in heatmaps represent the mean z-score as compared with HC (yellow represents increased and blue represents decreased as compared with HC).

In patients with pSS, out of the six populations that were previously identified as a "Sjogren's signature" by Mingueneau et al[8] by CyTOF, we were able to replicate the increase in DR^+ CD4 and DR^+ CD8 T-cells and the decrease in memory B-cells

(Bmem) and pDCs in patients with pSS (**Fig 1C**). In our cohort, the increase in plasmablasts and decrease in $CD4^+$ T-cells was not seen in patients with pSS (**Fig 1D** and data not shown). In patients with SLE, only the increase in plasmablasts ($p=0.01$) and decrease in IgM^+ B-cells ($p=0.03$), both as a proportion of total B-cells, were found significantly altered in patients with high disease activity (SLEDAI score ≥6 (n=9)) as compared to patients with low disease activity (SLEDAI < 6 (n=22)). These populations also significantly correlated with the levels of anti-dsDNA antibodies ($\rho=0.515$, $p=0.003$ and $\rho=-0.479$, $p=0.006$ respectively).

Changes in monocyte and other immune cell frequencies correlate with disease severity in SSc patients

We next focused on the subgroup of patients with SSc by performing linear correlations of the validated altered cell frequencies with various clinical parameters of SSc patients in the entire cohort **(Fig 2A)**. The increased monocyte frequencies observed in the circulation of SSc patients correlates significantly with the extent of skin fibrosis as measured by the modified Rodnan skin score ($r=0.48$, $p=0.0001$) **(Fig 2A-B)**. Additionally, we found that SSc patients with ILD (N=25) have significantly more classical monocytes in circulation than patients without ILD (N=63) ($P=0.01$, data not shown). In line with this finding, we found an inverse correlation between the number of circulating total monocytes and the DLco **(Fig 2A)**.

Hierarchical clustering of SSc patients based on the frequencies of the altered cell populations in SSc identified 4 clusters of SSc patients **(Fig 2C)**. Cluster 1 (N=16) was overall characterized by high CD16+ monocytes and low memory B-cell subsets, cluster 2 (N=25) was mainly marked by increased classical monocytes, cluster 3 (N=8) had larger amounts of memory B-cells while patients in cluster 4 (N=37) typically had lower numbers of classical monocytes in their circulation. In line with our univariate analysis we found an enrichment of patients with diffuse cutaneous disease in cluster 2 and present with markedly increased circulating monocytes and high mRSS. On the contrary, patients in cluster 4 are often patients with limited or no skin involvement and were commonly positive for anti-centromere antibodies (ACA) and present with monocyte frequencies similar HC and had lower mRSS **(fig 2D-G)**. These findings indicate that the monocyte alterations identified when comparing healthy donors with SSc patients also segregate patients with clinically more severe disease from those with less severe disease.

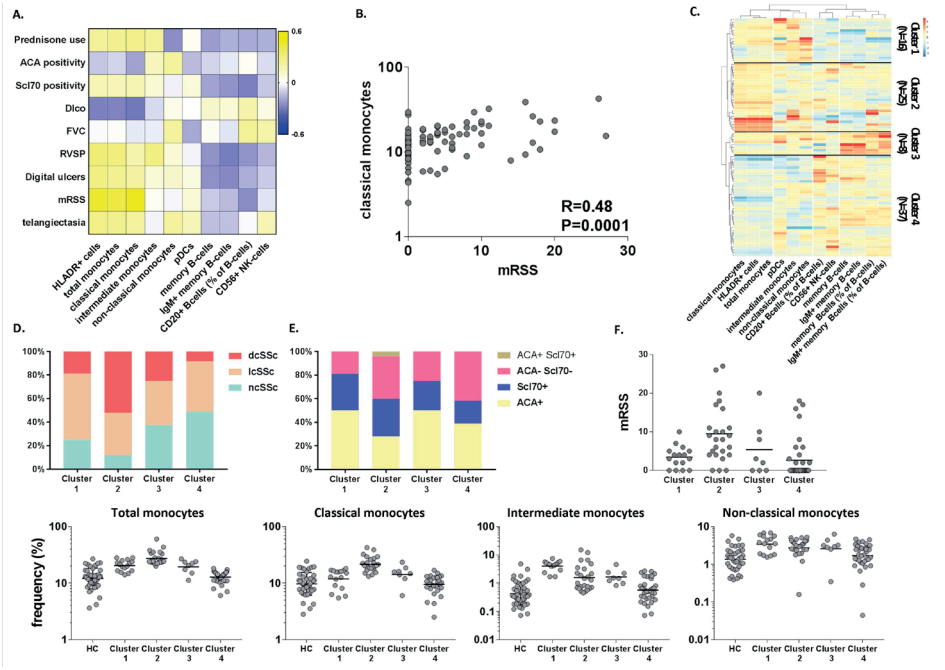


Figure 2 Clinical associations of altered cell frequencies in SSc

A) Clinical correlations of altered cell frequencies in SSc patients. B) Correlation of increases in classical monocyte subsets with modified Rodnan skin score. C) Hierarchical clustering analysis of altered cell frequencies identifies 4 clusters of SSc patients. D&E) Prevalence of clinical and serological SSc subsets among the identified clusters. F) mRSS of patients among the four clusters. G: frequency of total monocytes and monocyte subsets among the four clusters.

Changes in immune cell frequencies typical for SSc patients are already detectable in the early stage of the disease and to lesser extent in patients with localized scleroderma

Before the onset of overt skin fibrosis and other organ involvement, patient with Raynaud's phenomenon and positivity for SSc-specific autoantibodies and/or typical nailfold capillaroscopy abnormalities are considered "earlySSc" patients [13]. Considering the irreversible nature of skin and organ fibrosis and the negative impact thereof on the quality of life and the prognosis of SSc patients, this early phase is considered a window of opportunity for disease intervention [15]. Comparing the earlySSc patients with healthy donors revealed that 9 out of 12 cell subsets whose frequency was significantly changed in SSc patients are already significantly altered in patients with the early phases of the disease, including increases in non-classical monocytes **(Fig 3A).**

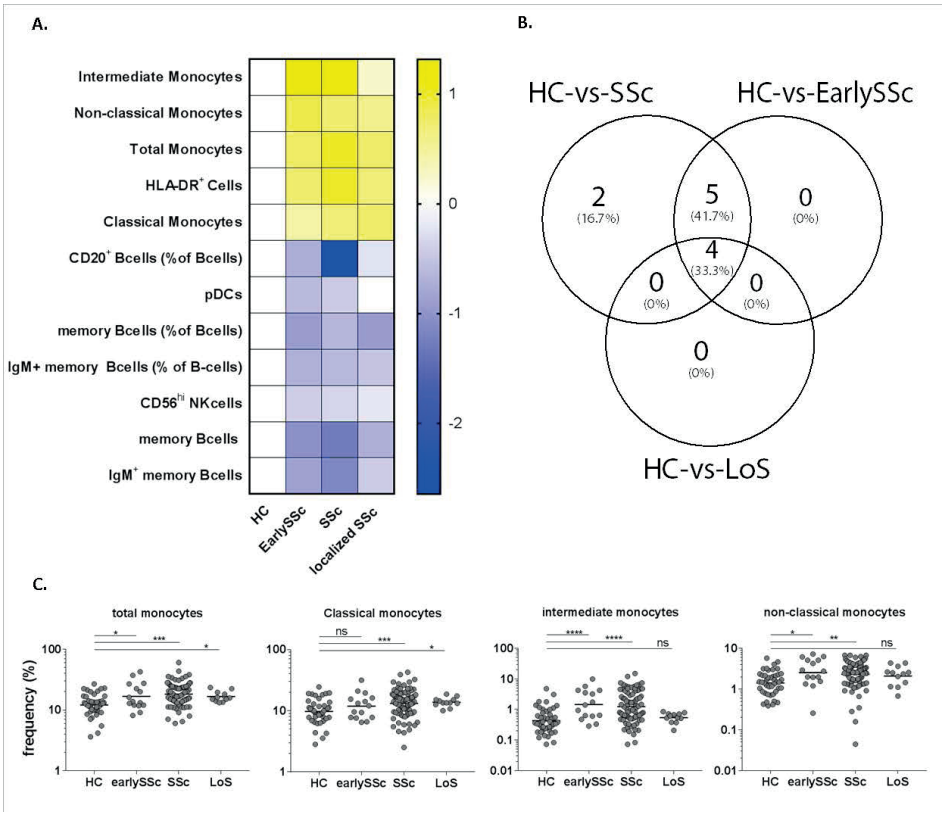


Figure 3. Comparison of altered cell frequencies between SSc, early SSc and LOS

A) Venn diagrams of cell populations with altered frequencies in pre-clinical SSc (early SSc) and localized scleroderma. B) Heatmap of altered cell frequencies of SSc patients in patients with pre-clinical SSc an localized scleroderma. C) frequency of monocyte subsets in SSc, early SSc and localized SSc as compared with HC. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001. Colours in heatmaps represent the mean z-score as compared with HC (yellow represents increased and blue represents decreased as compared with HC).

Localized scleroderma (LoS) patients classically present with fibrosis confined to the skin and underlying tissues. Although the changes in the frequencies of circulating immune cells were mild compared to patients with SSc in these patients we found that 5 out of the aforementioned 12 cell subsets were also significantly altered in LoS patients **(Fig 3B)**, including classical monocytes and memory B-cells. Although statistical significance was not reached, other cell frequencies showed a similar trend in LoS as compared with SSc **(fig 3B)**, nonetheless, the increase in classical as well as intermediate and non-classical monocytes seems to be more pronounced in patients with systemic disease **(Fig 3C)**.

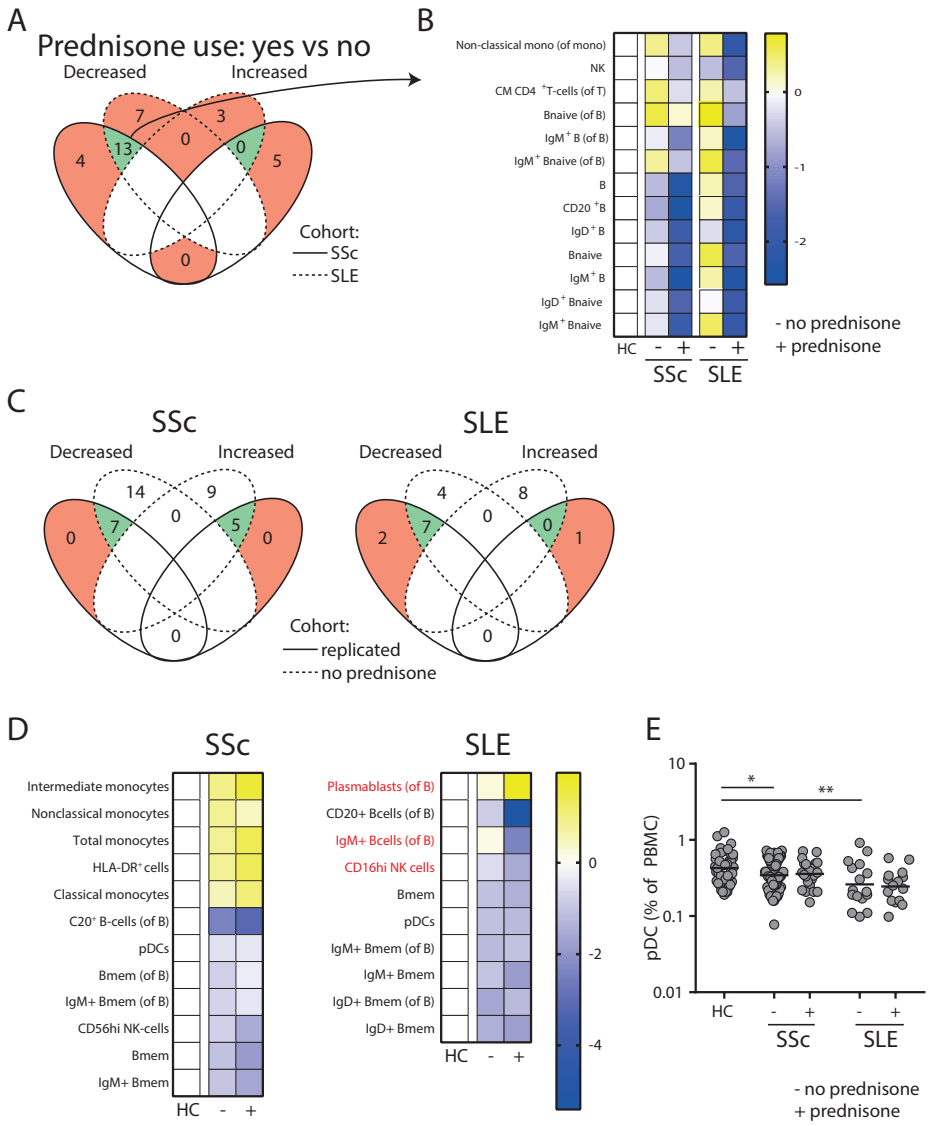


Figure 4. Effects of glucocorticoids on circulating cell frequencies in SSc and SLE. A): Altered cell frequencies between patients treated with or without glucocorticoids in SSc (continuous line) or SLE (dashed line). B) Heatmap of decreased cell populations in patients treated with GC in both SSc and SLE. C) Significantly differentially expressed cell populations in SSc or SLE patients not treated with GC as compared with HC in comparison to "validated" altered cell frequencies. D) Heatmap of validated altered cell frequencies in patients treated with or without GC. Populations in red are not significantly altered in patients not treated with prednisone as compared with HC E) Decrease in plasmacytoid dendritic cells is also present in patients not treated with GC. * $p<0.05$, ** $p<0.01$. Colours in heatmaps represent the mean z-score as compared with HC (yellow represents increased and blue represents decreased as compared with HC).

Effects of prednisone use on immune cell composition

Immunosuppressants may alter the frequency of immune cell populations and a substantial proportion of patients with SSc (28%) and SLE (43%) were treated with prednisone. When comparing between patients treated with or without prednisone both in SLE and SSc, revealed 13 populations significantly reduced in patients treated with prednisone and no population being increased in patients treated with prednisone as compared with patients not treated with prednisone both in SSc and SLE (**Fig 4A**). These populations mainly consisted of B-cells and B-cell subsets, suggesting a strong effect of prednisone on reduced B-cell numbers, both in SLE and SSc (**Fig 4B**).

To evaluate to what extent the use of prednisone affected our results, we assessed the frequency of the altered cell populations as reported by only studying SSc or SLE patients not treated with prednisone. In SSc, all populations that were validated as decreased or increased (7/7 decreased and 5/5 increased) significantly altered in patients not treated with prednisone as compared with HC. Therefore, the use of prednisone was not strongly influencing our results in SSc patients (**Fig 4C**). In patients with SLE 7/9 and 0/1 populations that were validated as decreased or increased respectively were also significantly decreased or increased in patients not treated with prednisone. Of these, the increases in plasmablasts and decreases in CD16-hi NK cells and IgM+ B-cells were not found in SLE patients not treated with prednisone (**Fig 4D**). The reduction of pDCs was found to be not affected by prednisone, as both SSc and SLE patients not treated with prednisone had reduced circulating levels of pDC as compared with HC (**Fig 4D**).

Discussion

SADs are heterogeneous multifactorial diseases marked by a dysregulation of immune cells. Previous research on the pathophysiology of SADs implies that it is unlikely that a single cell is the root of the problem. Rather a complex interplay of various immune cells could lead to the specific phenotype in each of these diseases. Here we report results from Cytometry by Time of Flight (CyTOF) analysis to phenotype the peripheral immune compartment in large identification and replication cohorts of patients with SSc, SLE and pSS. We demonstrate decreased numbers of pDCs in all three conditions whereas increases in monocytes typify patients with SSc, even before overt fibrosis occurs.

Immune cell profiling by CyTOF may bring insights into which compartments of the immune system are involved in the pathogenesis of SADs. Simultaneous

quantification of a large number immune cell subsets in one sample and their correlation with clinical and biological markers of disease may be helpful to gain insights in the pathophysiology of these SADs and how they may relate to one another. Further research is warranted to investigate the role of the cell-types identified by new molecular markers found to be dysregulated in the various SADs and to investigate biological differences in these cells that could be exploited for biomarker and therapeutic purposes.

pDCs are major producers of IFNα and pDCs are therefore considered the source of the type I IFN signature which is shared among SSc, SLE and pSS [16]. The reduction in pDC may reflect migration of pDCs into tissues where they may locally produce type I IFNs and indeed pDCs have been found in affected skin of SSc and SLE patients and in salivary glands of pSS patients [17–19]. An alternative explanation for the decrease in pDCs would be the decreased survival of these cells. In line with this, we recently reported that the increased expression of microRNA-618 in pDCs of patients with SSc inhibits their development by reducing the expression of interferon regulatory factor 8 (IRF8)[20]. Similarly, in SLE we found that the reduced expression of miRNAs in pDCs was affecting pathways related to apoptosis (L.L. van den Hoogen submitted). Decreased survival of pDCs may therefore also be a result of increased apoptosis and dysregulated miRNA expression is emerging as an underlying cause.

Notably, where pDCs were found reduced in all diseases, increases in monocyte subsets were found to be more specific for SSc. The extent of the increase in circulating monocytes was related to disease severity, but the increase is already observed in patients with preclinical evidence of SSc. Using hierarchical clustering on the frequency of cell subsets, we identified a cluster of SSc patients that are characterized by increases in monocyte subsets suffering from more extensive skin fibrosis. These observations corroborate previous findings of increased numbers of monocytes in circulation and skin of SSc patients [21] and an increase of CD16+ non-classical monocytes is observed in diffuse SSc patients [7].

Corroborating the fact that monocytes might be central mediators in the pathophysiology of SSc it was recently shown that the interferon signature is present in SSc monocytes even before the onset of overt fibrosis [3]. Furthermore, a pronounced profibrotic phenotype [22] as well as increases in the amount of profibrotic mediators such as CCL18 and IL-10 released by SSc monocytes upon stimulation have been described [23]. Furthermore, monocytes in the circulation of SSc patients present an M2-type phenotype and differentiation after subsequent monocyte mi-

gration into the affected tissues may play a pivotal role in the over production of extracellular matrix [24].We found that the majority of changes identified in fibrotic SSc patients are already apparent in the pre-fibrotic stage of this disease, suggesting that perhaps early intervention could prevent exacerbation of the disease.

Treatment options for patients with SSc are limited and substantial morbidity and mortality exists[25]. By clustering SSc patients based on their blood-immune cell phenotype we were able to identify signatures linked to SSc disease severity, analogous to the approach by Mingueneau et al in pSS patients[8]. The identification of such cellular signatures may ultimately lead to the identification of distinct molecular phenotypes among SSc patients and improve risk stratification.

References

1. Higgs BW, Liu Z, White B, *et al.* Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis* 2011;**70**:2029–36. doi:10.1136/ard.2011.150326

2. Brkic Z, Maria NI, Helden-meeuwsen CG Van, *et al.* Prevalence of interferon type I signature in CD14 monocytes of patients with Sjögren ' s syndrome and association with disease activity and BAFF gene expression. *Ann Rheum Dis* 2013;**72**:728–35. doi:10.1136/annrheumdis-2012-201381

3. Brkic Z, van Bon L, Cossu M, *et al.* The interferon type I signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high BAFF gene expression and high collagen synthesis. *Ann Rheum Dis* 2015;**0**:1–7. doi:10.1136/annrheumdis-2015-207392

4. van den Hoogen LL, Fritsch-Stork RD, Versnel MA, *et al.* Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. *Ann Rheum Dis* 2016;**75**:e81. doi:10.1136/annrheumdis-2016-210485

5. Ermann J, Rao D a., Teslovich NC, *et al.* Immune cell profiling to guide therapeutic decisions in rheumatic diseases. *Nat Rev Rheumatol* 2015;**11**:541–51. doi:10.1038/nrrheum.2015.71

6. Jacobi AM, Odendahl M, Reiter K, *et al.* Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* 2003;**48**:1332–42. doi:10.1002/art.10949

7. Lescoat A, Lecureur V, Roussel M, *et al.* CD16-positive circulating monocytes and fibrotic manifestations of systemic sclerosis. *Clin Rheumatol* 2017;**36**:1649–54. doi:10.1007/s10067-017-3597-6

8. Mingueneau M, Boudaoud S, Haskett S, *et al.* Cytometry by time-of-flight immunophenotyping identifies a blood Sjogren's signature correlating with disease activity and glandular inflammation. *J Allergy Clin Immunol* 2016;**137**:1809–1821.e12. doi:10.1016/j.jaci.2016.01.024

9. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;**40**:1725. doi:10.1002/1529-0131(199709)40:9<1725::AID-ART29>3.0.CO;2-Y

10. Vitali C, Bombardieri S, Jonsson R, *et al.* Classification criteria for Sjögren´s syndrome: a revised version of the European criteria prposed by the American-European Consensus Group. *Ann Rheum Dis* 2002;**61**:554–8.

11. Van Den Hoogen F, Khanna D, Fransen J, *et al.* 2013 classification criteria for systemic sclerosis: An american college of rheumatology/European league against rheumatism collaborative initiative. *Arthritis Rheum* 2013;**65**:2737–47. doi:10.1002/art.38098

12. Chouri E, Servaas NH, Bekker CPJ, *et al.* Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis. *J Autoimmun* Published Online First: 2018. doi:10.1016/j.jaut.2017.12.015

13. Leroy, E. C.; Medsger TA. Criteria for the Classification of Systemic Sclerosis (Scleroderma). *J Rheumatol* 2001;**23**:1573–6.

14. Shekhar K, Brodin P, Davis MM, *et al.* Automatic Classification of Cellular Expression by Nonlinear Stochastic Embedding (ACCENSE). *Proc Natl Acad Sci* 2014;**111**:202–7. doi:10.1073/pnas.1321405111

15. Matucci-Cerinic M, Bellando-Randone S, Lepri G, *et al.* Very early versus early disease: The evolving definition of the 'many faces' of systemic sclerosis. *Ann Rheum Dis* 2013;**72**:319–21. doi:10.1136/annrheumdis-2012-202295

16. Eloranta M-L, Alm G V, Rönnblom L. Disease mechanisms in rheumatology--tools and pathways: plasmacytoid dendritic cells and their role in autoimmune rheumatic diseases. *Arthritis Rheum* 2013;**65**:853–63. doi:10.1002/art.37821

17. van Bon L, Affandi AJ, Broen J, *et al.* Proteome-wide analysis and CXCL4 as a biomarker in systemic sclerosis. *N Engl J Med* 2014;**370**:433–43. doi:10.1056/NEJMoa1114576

18. Gottenberg J-E, Cagnard N, Lucchesi C, *et al.* Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome. *Proc Natl Acad Sci U S A* 2006;**103**:2770–5. doi:10.1073/pnas.0510837103

19. Farkas L, Beiske K, Lund-Johansen F, *et al.* Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol* 2001;**159**:237–43.

20. Rossato M, Affandi AJ, Thordardottir S, *et al.* Association of MicroRNA-618 Expression With Altered Frequency and Activation of Plasmacytoid Dendritic Cells in Patients With Systemic Sclerosis. *Arthritis Rheumatol* 2017;**69**:1891–902. doi:10.1002/art.40163

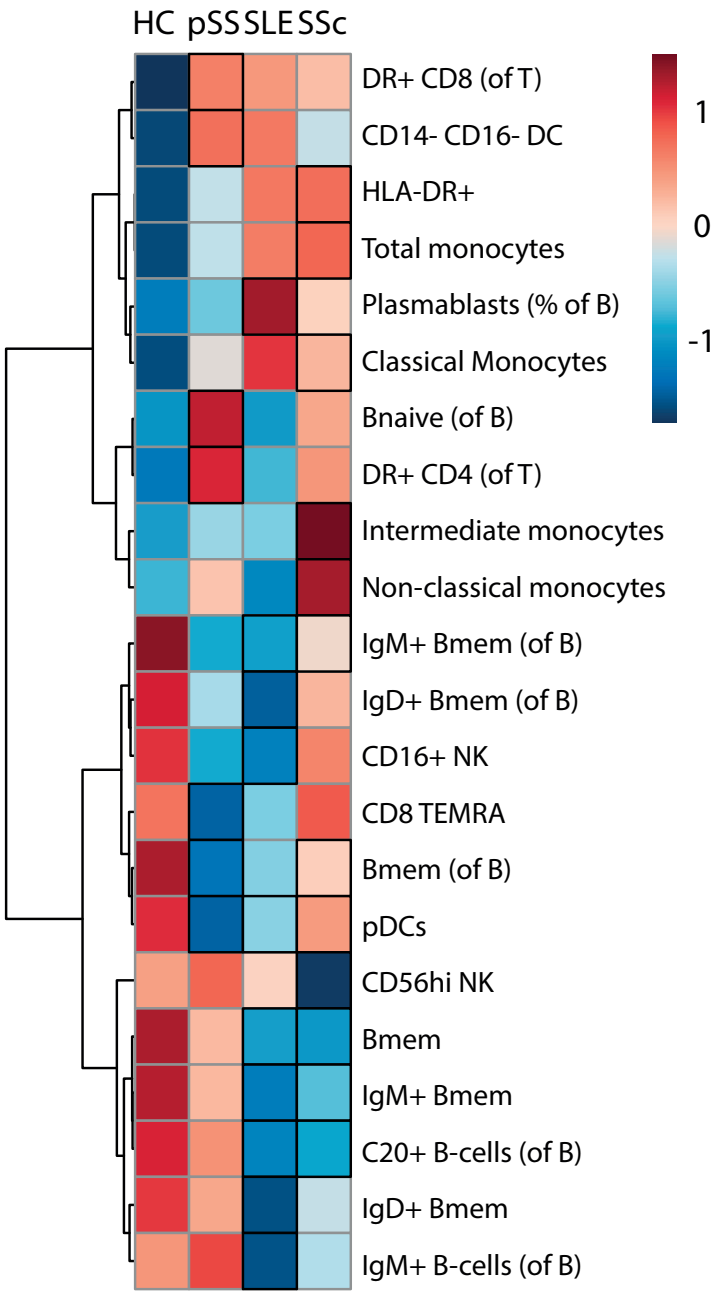
21. Higashi-Kuwata N, Jinnin M, Makino T, *et al.* Characterization of monocyte/macrophage subsets in the skin and peripheral blood derived from patients with systemic sclerosis. *Arthritis Res Ther* 2010;**12**:R128. doi:10.1001/archderm.1961.01580150005001

22. Mathai SK, Gulati M, Peng X, *et al.* Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. *Lab Invest* 2010;**90**:812–23. doi:10.1038/labinvest.2010.73

23. van Bon L, Popa C, Huijbens R, *et al.* Distinct evolution of TLR-mediated dendritic cell cytokine secretion in patients with limited and diffuse cutaneous systemic sclerosis. *Ann Rheum Dis* 2010;**69**:1539–47. doi:10.1136/ard.2009.128207

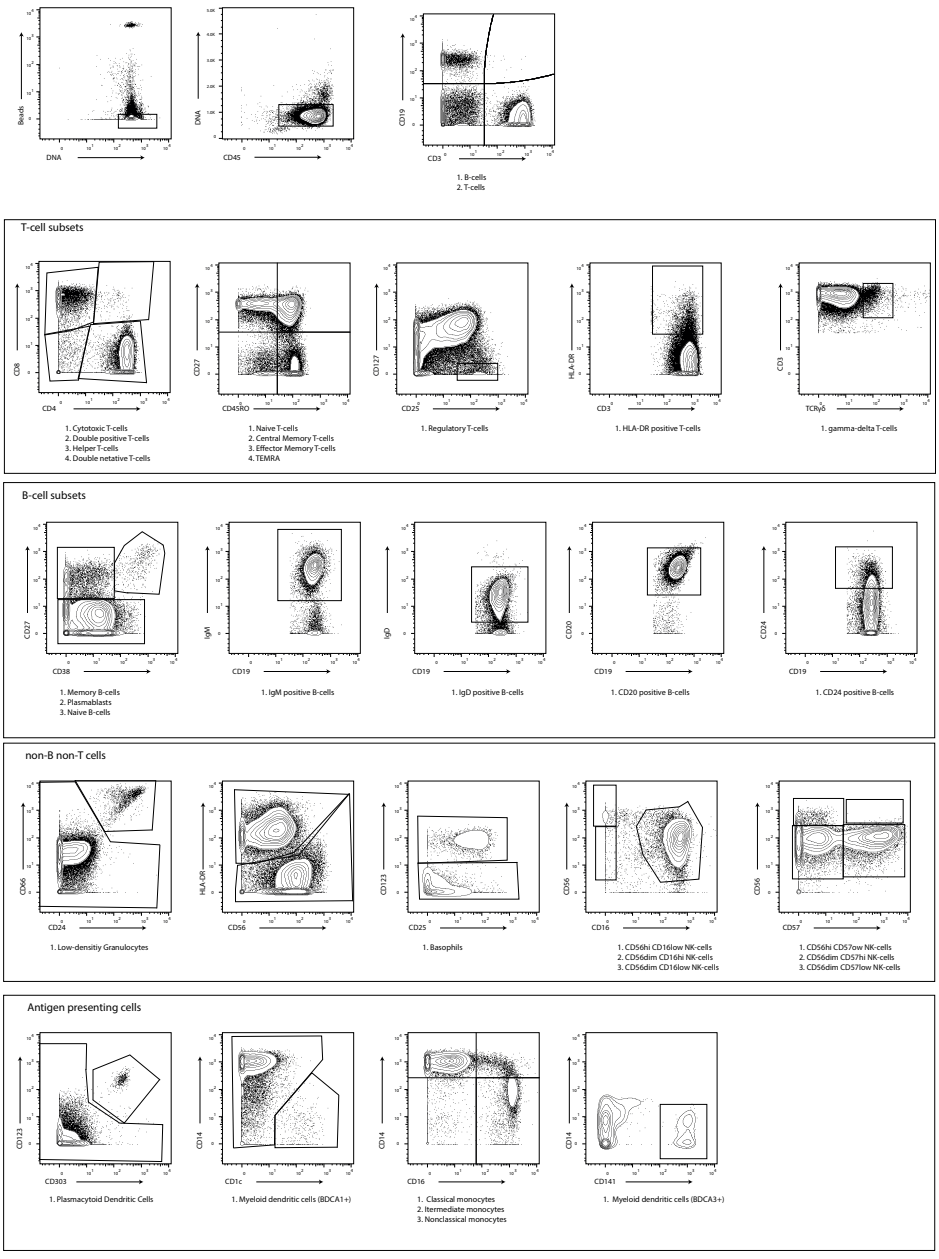
24. Christmann RB, Lafyatis R. The cytokine language of monocytes and macrophages in systemic sclerosis. *Arthritis Res Ther* 2010;**12**:146. doi:10.1186/ar3167

25. Elhai M, Meune C, Boubaya M, *et al.* Mapping and predicting mortality from systemic sclerosis. *Ann Rheum Dis* 2017;**76**:1897–905. doi:10.1136/annrheumdis-2017-211448



Supplemental Figure 1: Patterns of validated cell frequencies across patients with SSc, SLE and pSS

Heatmap of the validated altered cell frequencies as shown in figure 1 in the identification and replication cohorts combined in patients with SSc, SLE and pSS. Populations in black squares are significantly different in both identification and replication cohort between patients and controls.



Supplementary Figure 2 Gating Strategy of identified populations.

CHAPTER 13

General discussion and future perspectives

Antiphospholipid syndrome – An inflammatory disease?

The first reports on antiphospholipid syndrome as a disease affecting patients with SLE were published in 1983[1]. Soon it was appreciated that APS may also affect patients not suffering from SLE or another underlying disease, a group of patients from then on referred to as "primary" APS [2,3]. Basic and translational research in the subsequent three decades has demonstrated that *in vitro* antiphospholipid antibodies (aPL) activate thrombocytes, endothelial cells and trophoblasts, and *in vivo* aPL induce thrombosis and pregnancy loss when injected into mice, implicating aPL as the causative agent of the clinical symptoms of APS.

Few will dispute that antiphospholipid syndrome is an autoimmune disease, as the detection of autoantibodies form the cornerstone of its diagnosis. Nonetheless, APS is generally not considered to be an inflammatory disease[4]. Exemplary in this regard is a short sentence in the classification criteria for APS, which explicitly states that thrombosis in APS should be without evidence of histological signs of vascular inflammation[5]. In addition, clinical manifestations of APS are managed with anticoagulants rather than immunosuppressive therapy. Therefore, it seems obvious why, compared to other autoimmune conditions, relatively little attention has been given to the immunopathogenesis of APS.

In this thesis we set out to delineate the deranged immune system in PAPS as compared to SLE and secondary APS. First in **chapter 2** we reviewed the current literature on the role of the immune system in APS. We report that almost every component of the immune system, from the complement system to regulatory T-cells (Tregs), has been studied in the context of APS and is suggested to contribute to its pathophysiology. Therefore, inflammation may be an important contributor of the pathophysiology of APS. However, whether it are signs of complement activation or Treg dysfunction, these immunological alterations are not specific for APS, as they are also seen in other autoimmune diseases, most notably SLE. Furthermore, few studies explicitly compare their observations in patients with PAPS to those with SLE, with or without APS.

The immune system in SLE and APS: two sides of the same coin?

In this thesis we explored immunological phenomena in patients with PAPS in comparison with SLE, and SLE+APS patients. We demonstrated that key immunological alterations of SLE such as the interferon (IFN) signature (**chapter 3**), increased release of neutrophil extracellular traps (NETs) (**chapter 6**) and elevated BAFF levels (**chapter 7**) are characteristic of PAPS as well. In **chapter 3** we found that monocytes display an IFN signature in PAPS which is associated with increases

in cardiovascular disease associated monocytes subsets. We specifically focussed on monocytes as these cells have been implicated in the pathophysiology of APS due to an increased expression of tissue factor (TF), which initiates humoral coagulation. Although the IFN signature did not correlate with TF expression, the IFN signature correlated with increases in proinflammatory and cardiovascular disease associated monocyte subsets and was lower in patients treated with statins or hydroxychloroquine. In **chapter 5 and 6** we reported increased low-density granulocytes and plasma induced NET release in patients with PAPS. Uncontrolled NET release may trigger autoimmune responses towards NET components[6] and we observed that NET release was associated with antinuclear antibody levels in PAPS. In **chapter 7** we reported that monocytes of PAPS patients have an increased production of BAFF and BAFF levels are elevated in the serum of PAPS patients, which is associated with higher adjusted global antiphospholipid syndrome scores (aGAPSS). However, the expression of BAFF receptors on B-cells was not altered in PAPS.

Most studies on the immune system in APS often rely on observations made using immune cells from healthy individuals incubated with aPL, as a model for the *in vivo* situation in APS patients. Such research has identified that aPL can activate monocytes, plasmacytoid dendritic cells and neutrophils (**chapter 2**). This approach however suggests that the pathophysiology of APS is fully dependent on aPL, not taking into account the underlying immunological alterations that contribute to the pathophysiology of APS. Therefore, in our studies we specifically focussed on studying material directly obtained from patients with APS.

Although currently there is no place for immunosuppressive drugs in the general management of PAPS, immunomodulating treatments may hold a place in the treatment of (P)APS in the future[7]. We observed that immunological perturbations which are successfully targeted by biologics in SLE such as the IFN signature and BAFF[8–10], also affect patients with PAPS. Besides, in **chapter 3** we observed that patients treated with hydroxychloroquine or statins had lower IFN signatures than patients treated without, suggesting a beneficial effect of these drugs to treat the immunopathology of APS.

Besides targeting type I IFNs by hydroxychloroquine or anifrolumab [11,12], targeting NET-release by DNAse or inhibition of P-selectin glycoprotein ligand-1 (PSGL1)[13–15] and reducing oxidative stress by ubiquinol[16,17] have been tested in (pre)clinical studies as novel treatment options in APS. Whether such treatments are effective in APS would need to be tested in randomized controlled clinical trials. Unfortunately, designing trials in APS is challenging[18] because of

low patient recruitment and a low incidence of (thrombotic) events. Furthermore, validated disease activity scores do not exist for APS. As a result, it is unlikely that randomized clinical trials on novel (immunomodulating) treatments for APS are completed in the near future. Until then, in refractory APS cases, off-label use of immunomodulating drugs, supported by findings from (translational) research, may represent a viable option to improve disease outcome among patients with APS.

In SLE research (and research focussed on other autoimmune diseases), the importance of several perturbations in the immune system is often stressed by finding a correlation of the observed perturbation with disease activity. In this regard there is no uniformly accepted disease activity score available for APS. Furthermore, in contrast to SLE (and many other autoimmune diseases), APS is not a disease characterized by a relapsing and remitting pattern of disease activity. As a result, placing (immunologic) perturbations in PAPS patients into clinical perspective is more difficult. In **chapter 7**, we did observe a correlation between elevated levels of BAFF with the adjusted global antiphospholipid syndrome score (aGAPSS). This score consist of a weighted sum of the detection of the different aPL in combination with hypercholesterolemia and hypertension and predicts future thrombotic events[19]. The development of such scoring systems is welcomed and might accelerate both translational and clinical research in APS.

The inclusion of patients with SLE and SLE+APS in our studies allowed us to place the immunological alterations in perspective. As a result, we observed that the IFN signature (**chapter 3**), NET-release (**chapter 6**) and increased BAFF levels (**chapter 7**) are less prominent in PAPS compared with SLE(±APS). Increased numbers of Low-density Granulocytes (LDGs) forms an exception in this regard as in **chapter 5** we reported that the frequency of LDGs was highest in patients with APS, both in SLE+APS and PAPS. Increases in LDGs have been frequently reported in patients with SLE[20–23], but none of these studies stratified their patients to those with or without APS.

LDGs may form a distinct subset of neutrophils or may represent in vivo activated neutrophils [24]. Anti-β2 Glycoprotein I antibodies activate neutrophils[25], which potentially induces the low buoyancy phenotype of neutrophils in APS. Consistent with this hypothesis, we observed a correlation between the presence of such anti-β2 Glycoprotein I and higher numbers of LDGs among patients with APS. LDGs from patients with SLE are extremely potent NET-inducers, externalize IL-17 and synthesize type I IFNs[20,26]. Importantly, LDGs isolated from RA patients seem to have a different behaviour than those found in SLE patients[27]. Therefore, dif-

ferent LDG populations may exist between RA and SLE and future research should reveal whether LDGs from APS patients are functionally distinct from those found in patients with SLE. Such studies should ideally treat LDGs and normal neutrophil in the same way during the isolation process, and only highly pure neutrophils should be used for transcriptomic studies [28,29].

Primary APS: part of the SLE spectrum?

Given the immunological similarities that we found between SLE and APS, the question arises whether primary APS is a distinct disease or part of the SLE spectrum.

Studies focusing on PAPS may suffer from selection bias of "lupus-like" PAPS patients. For instance, we included PAPS patients referred to the rheumatological outpatient clinic. These patients may have been specifically referred to a rheumatologist because the referring doctor considered SLE as a diagnosis. Many "pure" PAPS patients probably remain undiagnosed, as testing for aPL at the first unprovoked thrombotic event is not routinely performed. As a result, such "pure" primary APS patients remain undiagnosed and are unlikely to be included in clinical trials or translational research which are dominated with more "lupus-like" PAPS patients.

As shown in **chapter 3 and 6** a substantial proportion of the PAPS patients we included had positive tests for antinuclear antibodies (ANA), which correlated with the IFN signature or NET-release. One may argue that these patients should not be included when studying PAPS. The detection of ANA does however not rule out a diagnosis of *primary* APS. Even from the early reports of PAPS, the frequency of ANA was reported to be around ~50% in PAPS[2,3] and in some cohorts, the prevalence of ANA (>1:160) in patients with PAPS is even reported to be as high as 85%[30]. Unfortunately, most studies on PAPS patients do not report such serological abnormalities, which makes it difficult to interpret how "primary" the APS patients were.

The problematic distinction between PAPS and SLE+APS is not new. The early studies on PAPS used the classification criteria for SLE to distinguish between SLE+APS and PAPS[2,3]. However, from 1997 on the detection of aPL (anti-cardiolipin or lupus anticoagulant) have been included in the classification criteria for SLE[31]. The more sensitive but less specific SLICC criteria[32] have been postulated recently and new ACR/EULAR endorsed criteria for SLE are soon expected. Therefore, if SLE classification criteria are used to distinguish SLE+APS from PAPS, it is important to know which set of criteria were used to distinguish PAPS from SLE+APS.

A major conclusion of a large cohort study on the clinical differences between SLE+APS with PAPS reported that the prevalence of arthritis was significantly higher in SLE+APS as compared with PAPS[33]. A series of letters published in the *Journal of Rheumatology* in 1992 between several groups studying PAPS led to the postulation of exclusion criteria to distinguish PAPS from SLE[34]. In these criteria, the detection of ANA was included as an exclusion criterium, although only when exceeding a titre of 1:320. Furthermore, the detection of anti-dsDNA antibodies (detected by radioimmunoassay or Crithidia) were included as an exclusion criteria although nowadays anti-dsDNA antibodies are routinely measured with less specific ELISA(-like) techniques[35,36]. Clinical factors typical of SLE such as malar rashes or "frank" arthritis were also seen as exclusion criteria[34]. However, as the treatment of APS does not differ between SLE+APS and PAPS, one could argue that for clinical practice such criteria are not necessary.

It would be helpful if all studies on PAPS report whether the studied patients had primary and/or secondary APS and how these were defined, to allow the reader to interpret what type of PAPS patients were studied. Based on our findings, in which we used the 1997 ACR criteria for SLE to distinguish SLE+APS from PAPS, we find similar but less prominent immunological perturbations between in PAPS as compared with SLE, suggesting that APS is part of the SLE spectrum.

The IFN signature in SLE and APS: difficult to capture?

The IFN signature is the most prominent immunological alteration seen in patients with SLE[37] and in **chapter 3** we reported that 50% of patients with PAPS also have an IFN signature. Research over the past decade has resulted in the development of drugs that target type I IFN, of which anifrolumab, a monoclonal antibody against IFNAR, showed clinical efficacy in a phase IIb study in patients with active SLE[10]. In this trial, patients with an IFN signature at baseline, measured by the detection of 4 IFN-inducible genes in full blood, was associated with a greater response to anifrolumab as compared to patients without this IFN signature. This suggests that the detection of the IFN signature might in the future be used to guide treatment decisions in SLE.

The detection of the IFN signature, as measured by micro-array on full blood[38] or by measuring serum levels of CXCL10[39], is an independent risk factor for future SLE flares. Also the IFN signature, as measured by the induction of IFN inducible genes by patient sera, is independently associated with subclinical signs of cardiovascular disease[40] and the detection of the IFN signature using the expression of siglec1 on monocytes as assessed by flow cytometry is a risk factor for the develop-

ment of congenital heartblock in woman with anti-Ro/La antibodies[41]. These observations not only stress the importance of the detection of the IFN signature in patients with SLE, they also highlight that the IFN signature can be detected using different techniques.

In **chapter 4** we identified that serum levels of galectin-9 can be used as a reliable and easy to measure biomarker to detect the IFN signature in both SLE and APS. We believe that for the detection of the IFN signature, measuring a serum analyte such as galectin-9 is more clinically applicable than gene-expression analysis which is the current golden standard. Galectin-9 measuring may also be more clinically applicable than the detection of siglec1 expression on monocytes, as this requires the measurement of fluorescence intensities by flow cytometry which is more laborious and more difficult to standardize than measuring a single analyte in serum. Furthermore, we reported that galectin-9 outperforms CXCL-10 as a serum biomarker for the IFN signature.

Galectin-9 has not been previously used as a biomarker for SLE or APS. We tested the performance of this biomarker based on the experience of our colleagues who previously demonstrated that galectin-9 serves as a biomarker for disease activity in juvenile dermatomyositis[42]. As a result, in the UMC Utrecht, measuring galectin-9 levels is now available for clinicians to request when ordering blood tests. As galectin-9 is induced in cells upon stimulation by IFNα, as demonstrated in **chapter 4**, galectin-9 may serve as a biomarker for the IFN signature and/or disease activity in other IFN-mediated autoimmune diseases such as rheumatoid arthritis and Sjögren's syndrome (SjS).

The IFN signature: common denominator of autoimmune diseases?

The IFN signature is not only shared between SLE and APS (**chapter 3**), but is found in several autoimmune diseases[43]. In **chapter 11** we measured the IFN signature in patients with SLE and Sjögren's syndrome, both by qPCR and monocyte expression of Siglec1. Although the IFN signature is well established in SjS[44], to our knowledge no previous comparison on the magnitude of the IFN signature between SLE and SjS exist and we observed that the IFN signature is equally strong in SLE and SjS. In both conditions the IFN signature is associated with disease activity[37,44] but on a closer look the IFN signature is only associated with serological rather than clinical signs of disease activity[45]. Although this may reflect the difficulty in capturing clinical disease activity in these diseases, it also shows the need for further research on the potential clinical implications of measuring the IFN signature in SjS and SLE.

Plasmacytoid dendritic cells: root of the IFN signature in SLE and APS?

Plasmacytoid dendritic cells (pDCs) are held responsible for the IFN signature in systemic autoimmune diseases[46,47]. This is supported by SLE mice models in which the depletion of pDCs ameliorates disease manifestations (reviewed in [47]). In humans, BIIB052, an anti-BDCA2 monoclonal antibody which specifically targets pDCs reduces the IFN signature in patients with SLE (Furie R, EULAR 2017) and anifrolumab, specifically blocking the receptor for type I IFNs reduces disease activity and the IFN signature in patients with SLE[10]. Unfortunately, the scarcity of pDCs in peripheral blood (<0.5% of leukocytes) has hampered in depth analysis of these cells. In **chapter 8** we identified that microRNAs, which are small non-coding RNAs that regulate gene-expression, are downregulated in patient with SLE and APS. This reduced expression of miRNAs was seen most strongly in patients with higher IFN signatures and based on a reanalysis of a publicly available dataset seemed to be a consequence of TLR7 activation.

Several dozen rather than a single miRNA were found to be downregulated in pDCs. As a result, selecting one miRNA to knock-down by several hundred folds to gain insights on its function in pDCs seems not representative. As one miRNA affects the expression of several hundreds of target genes, we attempted to integrate miRNA expression data with mRNA expression data on the same samples. This suggested that the differentially expressed target genes of these miRNA in pDCs of IFN-positive SLE and APS patients are enriched for specific pathways related to pDC activation as well as pDC apoptosis.

In **chapter 9** we further studied the transcriptome of pDCs of patients with SLE and APS as well as the transcriptome of classical (myeloid) CD1c+ DCs. Although pDCs and mDCs are distinct cells, the differentially expressed genes in patients with SLE and APS were enriched for similar pathways in pDCs and mDCs, further supporting the idea that SLE and APS are part of the same spectrum. A dominant role for type I IFN signalling was found among the altered pathways in both pDCs and mDCs. Nonetheless, on a closer look, the genes associated with the IFN signature differed between pDCs and mDCs and resulted in different activation status of pDCs and mDCs by type I IFN.

pDCs are considered a major source of the IFN signature in autoimmune diseases [46]. Therefore, we stratified our patients into those with or without an IFN signature. To our surprise, we did not find mRNA of type I IFNs upregulated in pDCs of IFN positive patients. This may be due to the low resolution of the RNAseq; however a recent study could not detect IFNα protein either in circulating pDCs of

patients with SLE[48]. Therefore, circulating pDCs might not be actively producing type I IFNs in SLE. However, we did observe that pDCs of IFN positive patients are primed for IFNα production through an increased expression of the Toll-like receptor (TLR) 7 pathway in pDCs of IFN positive patients, a situation that we could mimic by culturing pDCs with IFNα. We therefore conclude that circulating pDCs of IFN-positive SLE and APS patients are primed for IFNα production, rather than actively producing IFNα in blood.

Upon recognition of their ligands TLR7 and 9 signal via the adaptor molecule MyD88 resulting in the activation of the transcription factor IRF7, which induces the transcription of type I IFNs[47]. TLR7 and 9 are located in the endosome and recognize RNA or DNA respectively. Previous research has identified that antiphospholipid antibodies prime pDCs for IFNα production through upregulating the expression of TLR7[49]. In that study all APS patients had an increased mRNA expression of TLR7 (in PBMCs) as compared with HC. In our results, the expression of TLR7 in pDCs was strongly related with the IFN signature and IFN-negative APS patients did not have an increased expression of TLR7. Hence, TLR7 mRNA expression in pDCs in SLE/APS patients is rather related to the presence of the IFN signature then to the presence of aPL. The discrepancy between these findings might be explained by studying PBMCs rather than pDCs or perhaps all APS patients included in the former study by chance were IFN-positive.

In the same chapter, we also studied the transcriptome of myeloid CD1c+ DCs. Pathway enrichment on differentially expressed genes in SLE and APS patients showed a remarkable overlap with pDCs and several pathways related to IFN signalling were found. Weighted gene co-expression network analysis (WGCNA) identified several gene modules in both pDCs and mDCs, of which a module enriched for IFN inducible genes was found in both cell types. Stratification of patients using the eigen gene expression of these modules allowed a complete separation of IFN-positive patients from IFN-negative patients and HC.

Although the strength of the IFN signature was very similar between pDCs and mDCs, the genes allocated to the IFN module and hence associated with the IFN signature, differed between cell types. In vitro we found that IFNα upregulated the expression of HLA-DR and co-simulatory molecules on mDCs which led to an increased proliferation of T-cells when cultured with T-cells. Although pDCs can activate T-cells as well[47], IFNα did not enhance their T-cell stimulatory capacity. Thus, IFNα primes pDCs for more IFNα production through upregulation of the TLR7 pathway, at the same time mDCs are primed for T-cell stimulation through

upregulation of (co-)stimulatory molecules. These T-cells may induce further immunopathology and provide help to B-cells. In SLE and APS this may lead to the production autoantibodies, subsequently facilitating the uptake of TLR7 (and/or 9) ligands into the endosome and therefore resulting in IFNα production by pDCs[46,49,50]. Furthermore, we observed that both pDCs and mDCs of IFN-positive SLE and APS patients had an increased expression of BAFF and in vitro IFNα induced BAFF secretion by both cells, further stimulating the B-cells and creating a self-perpetuating loop of IFNα production by pDCs (**Figure 1**).

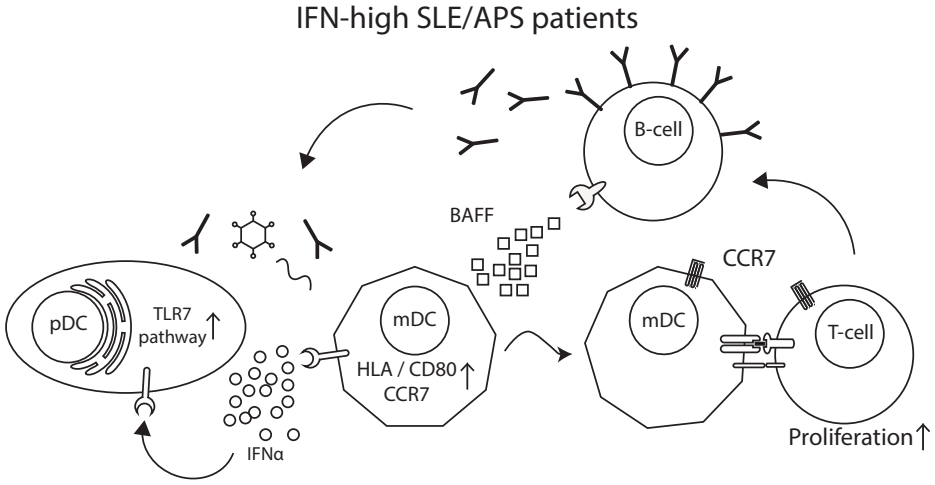


Figure 1 Proposed self-perpetuating loop of IFNα production in pDCs and mDCs of IFN-positive SLE and APS patients. pDCs of IFN-positive SLE and APS patients have an increased expression of toll-like receptor 7 (TLR7) which primes pDCs for IFNα production. IFNα in turn increases the expression of co-stimulatory molecules on mDCs which stimulates T-cells and induces the production of BAFF which may stimulate B-cells to production of (auto)antibodies which could stimulate pDCs to produce IFNα.

IFNα: the signature cytokine of SLE and APS?

Notably, other cells including neutrophils can produce IFNα[51], although not at the same capacity as pDCs, neutrophils far outnumber pDCs and their contribution to the IFN signature cannot be underestimated. Besides IFNα, other IFNs are implicated in the pathophysiology of SLE. IFNβ, which belongs to the type I IFNs, signals through the same receptor as IFNα. IFNβ can be produced by pDCs[52] as well as many other cells including monocytes and fibroblasts. Furthermore, IFNγ which is mainly produced by T-cells and NK-cells has also been implicated in the pathophysiology of SLE as for instance elevated IFNγ levels predate elevated IFNα levels before disease onset [53]. Attempts have been made to distinguish the

contribution of the different IFN subtypes at the gene-signature level [54]. Such studies allocate genes as either IFNα or IFNγ inducible based on experiments in which cells were cultured with either one of the IFNs. However, although some genes might be more strongly induced by one of the IFN subtypes, in general there is a very large overlap between the genes induced by the different IFN subtypes. Of note, IFNγ inducible genes are induced later than IFNα inducible genes [55]. Furthermore, IFNγ may potentiate IFNα responses[53]. Therefore, it remains difficult to pinpoint which exact IFN is the *signature* cytokine of SLE and IFNα produced by pDCs is unlikely to be the sole source of the IFN signature in SLE.

CyTOF: a novel tool to identify immune cell signatures in systemic autoimmune diseases?

In **chapter 12** we made use of a novel tool to interrogate the immune system of patients with systemic autoimmune diseases by using cytometry by time of flight (CyTOF). In CyTOF, cells are stained with antibodies conjugated to rare metals with a specific mass. As such, CyTOF overcomes the hurdle of spectral overlap and the limited amount of fluorochromes used in flow cytometry and has the potential to measure the expression of several dozens of makers on a single cell.

Using an identification and replication phase we identified that the frequency of several immune cells is altered in patients with systemic autoimmune diseases including systemic sclerosis (SSc), SLE and Sjögren's syndrome (SjS). Although at first glance distinct changes for each autoimmune disease were seen, on a closer look a more homogenously altered peripheral immune cell compartment characterized SSc, SLE and SjS. By studying patients with early SSc, i.e. patients with preclinical signs of SSc, we identified that many of the changes seen in patients with SSc were already apparent at an early stage.

Immunosuppressive drugs alter the composition of the peripheral immune system. In our cohort, a substantial proportion of patients with SSc and SLE were treated with prednisone. By comparing patients treated with or without prednisone we identified that several cells, most notably B-cells are reduced in patients treated with prednisone, both in SSc and SLE. The use of immunosuppressive drugs may therefore obscure relevant findings when quantifying immune cells in peripheral blood. Furthermore, it is unknown how the frequencies of circulating cells relate to the presence of such cells in target tissues.

The only change in composition of the peripheral immune compartment that was consistent between SSc, SLE and SjS, both in the training and validation set was

the reduced number of pDCs. This was not due to prednisone use as patients not treated with prednisone also had reduced numbers of pDCs as compared with healthy controls (HC). Although decreases in pDCs have been previously reported in patients with SSc[56], SLE [57] and SjS [58], by including all three diseases in one cohort we found that pDCs were most strongly reduced in SLE and SjS. The general explanation for these reduced levels of pDCs in peripheral blood is their migration into target tissues and indeed, pDCs are found in skin of SSc and SLE patients [59,60] and in salivary glands of SjS patients[61].

An alternative explanation for the decreased levels of pDCs in these diseases is decreased survival of pDCs. In mouse models, viral infections, administration of IFNα and non-viral inflammation decrease pDCs in peripheral blood due to increased apoptosis[62,63]. Our group recently found that the decrease in pDCs in SSc is associated with an increased expression of miR-618 which targets a key transcription factor IRF8 resulting in a decreased pDC development[56]. Besides, in our analysis of pDC microRNA and mRNA expression in **chapter 8** we found apoptosis as one of the most strongly enriched pathways among the microRNA targets. Therefore, decreased survival and/or development of pDCs may be a relevant explanation for the decrease of pDC in these patients.

Conclusion

Several key immunological alterations are often shared among autoimmune diseases, ranging from the IFN signature, present more or less in all auto-antibody positive autoimmune diseases[43] to elevated levels of BAFF which are for instance also found in patients with multiple sclerosis[64]. At first, the lack of specificity of these findings may stress the lack of clinical utility of these observations. On the other hand, we believe that such shared perturbations stress the need for clinical and translational research across the borders of current diagnostic silos. Not all PAPS patients have an IFN signature, neither do all SLE patients. Not all PAPS patients have elevated levels of BAFF, neither do all SLE patients. A better understanding of how such immunological perturbations relate to clinical relevant subsets of patients is needed.

Our observations from studying the immune system in SLE and APS patients suggest that APS is part of the SLE spectrum. Key immunological alterations in SLE such as the IFN signature, high levels of BAFF and increased NET release are found in PAPS as well. In depth molecular analysis of pDCs and mDCs by RNA sequencing and microRNA profiling was unable to distinguish SLE from APS. Immunologically, a dominant role for type I IFNs is present in ~50% of PAPS

patients and ~75% of SLE patients. Such shared immunological perturbations among autoimmune diseases may relate to treatment responses of drugs targeting specific pathways. Hopefully, studying autoimmune diseases based on a molecular taxonomy, rather than classification criteria, improves treatment outcomes in patients with autoimmune diseases[65].

References

1. Boey ML, Colaco CB, Gharavi a E, *et al.* Thrombosis in systemic lupus erythematosus: striking association with the presence of circulating lupus anticoagulant. *Br Med J (Clin Res Ed)* 1983;**287**:1021–3.
2. Asherson R, Khamashta MA, Ordi-Ros J, *et al.* The 'primary' antiphospholipid syndrome: Major clinical and serological features. *Med (United States)* 1989;**68**:366–74.
3. Mackworth-Young CG, Loizou S, Walport MJ. Primary antiphospholipid syndrome: Features of patients with raised anticardiolipin antibodies and no other disorder. *Ann Rheum Dis* 1989;**48**:362–7. doi:10.1136/ard.48.5.362
4. de Groot PG, Urbanus RT. Antiphospholipid Syndrome – Not a Noninflammatory Disease. *Semin Thromb Hemost* 2015;**41**:607–14.
5. Miyakis S, Lockshin MD, Atsumi T, *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006;**4**:295–306.
6. Bosch X, Ph D. clinical implications of basic research Systemic Lupus Erythematosus and the Neutrophil. *N Engl J Med* 2011;**365**:758–60.
7. Erkan D, Aguiar CL, Andrade D, *et al.* 14th International Congress on Antiphospholipid Antibodies: task force report on antiphospholipid syndrome treatment trends. *Autoimmun Rev* 2014;**13**:685–96. doi:10.1016/j.autrev.2014.01.053
8. Furie R, Petri M, Zamani O, *et al.* A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum* 2011;**63**:3918–30. doi:10.1002/art.30613
9. Navarra S V., Guzmán RM, Gallacher AE, *et al.* Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: A randomised, placebo-controlled, phase 3 trial. *Lancet* 2011;**377**:721–31. doi:10.1016/S0140-6736(10)61354-2
10. Furie R, Khamashta M, Merrill J, *et al.* Anifrolumab, an Anti-Interferon-Alpha Receptor Monoclonal Antibody, in Moderate to Severe Systemic Lupus Erythematosus. *Arthritis Rheumatol* 2017;**69**:376–86. doi:10.1002/art.39962
11. Grenn RC, Yalavarthi S, Gandhi AA, *et al.* Endothelial progenitor dysfunction associates with a type i interferon signature in primary antiphospholipid syndrome. *Ann Rheum Dis* 2017;**76**:450–7. doi:10.1136/annrheumdis-2016-209442
12. Van Den Hoogen LL, Fritsch-Stork RDE, Versnel MA, *et al.* Monocyte type i interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. *Ann Rheum Dis* 2016;**75**. doi:10.1136/annrheumdis-2016-210485
13. Yalavarthi S, Gould TJ, Rao AN, *et al.* Antiphospholipid antibodies promote the release of neutrophil extracellular traps: A new mechanism of thrombosis in the antiphospholipid syndrome. *Arthritis Rheumatol* 2015;**67**:2290–3003. doi:10.1002/art.39247
14. Meng H, Yalavarthi S, Kanthi Y, *et al.* In Vivo Role of Neutrophil Extracellular Traps in Antiphospholipid Antibody–Mediated Venous Thrombosis. *Arthritis Rheumatol* 2017;**69**:655–67. doi:10.1002/art.39938

15. Knight JS, Meng H, Coit P, *et al.* Activated signature of antiphospholipid syndrome neutrophils reveals potential therapeutic target. *JCI Insight* 2017;**2**:1–13. doi:10.1172/JCI.INSIGHT.93897

16. Pérez-Sánchez C, Aguirre MÁ, Ruiz-Limón P, *et al.* Ubiquinol Effects on Antiphospholipid Syndrome Prothrombotic Profile: A Randomized, Placebo-Controlled Trial. *Arterioscler Thromb Vasc Biol* 2017;:1923–32. doi:10.1161/ATVBAHA.117.309225

17. Perez-Sanchez C, Ruiz-Limon P, Aguirre MA, *et al.* Mitochondrial dysfunction in antiphospholipid syndrome: implications in the pathogenesis of the disease and effects of coenzyme Q(10) treatment. *Blood* 2012;**119**:5859–70. doi:10.1182/blood-2011-12-400986

18. Erkan D, Unlu O, Sciascia S, *et al.* Hydroxychloroquine in the primary thrombosis prophylaxis of antiphospholipid antibody positive patients without systemic autoimmune disease. *Lupus* 2017;**0**:1–8. doi:10.1177/0961203317724219

19. Sciascia S, Sanna G, Murru V, *et al.* The global anti-phospholipid syndrome score in primary APS. *Rheumatol (United Kingdom)* 2014;**54**:134–8. doi:10.1093/rheumatology/keu307

20. Denny MF, Yalavarthi S, Zhao W, *et al.* A Distinct Subset of Proinflammatory Neutrophils Isolated from Patients with Systemic Lupus Erythematosus Induces Vascular Damage and Synthesizes Type I IFNs. *J Immunol* 2010;**184**:3284–97. doi:10.4049/jimmunol.0902199

21. Hacbarth E, Kajdacsy-Balla a. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. *Arthritis Rheum* 1986;**29**:1334–42.http://www.ncbi.nlm.nih.gov/pubmed/2430586

22. Midgley A, Beresford MW. Increased expression of low density granulocytes in juvenile-onset systemic lupus erythematosus patients correlates with disease activity. *Lupus* 2016;**25**:407–11. doi:10.1177/0961203315608959

23. Pavón EJ, García-Rodríguez S, Zumaquero E, *et al.* Increased expression and phosphorylation of the two S100A9 isoforms in mononuclear cells from patients with systemic lupus erythematosus: a proteomic signature for circulating low-density granulocytes. *J Proteomics* 2012;**75**:1778–91. doi:10.1016/j.jprot.2011.12.020

24. Carmona-Rivera C, Kaplan MJ. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Semin Immunopathol* 2013;**35**:455–63. doi:10.1007/s00281-013-0375-7

25. Arvieux J, Jacob M-C, Roussel B, *et al.* Neutrophil antibodies activation by anti-B2 glycoprotein I monoclonal antibodies via Fcg receptor II. *J Leukoc Biol* 1995;**57**:387–94.

26. Villanueva E, Yalavarthi S, Berthier CC, *et al.* Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* 2011;**187**:538–52. doi:10.4049/jimmunol.1100450

27. Wright HL, Makki FA, Moots RJ, *et al.* Low-density granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. *J Leukoc Biol* 2017;**101**:599–611. doi:10.1189/jlb.5A0116-022R

28. Calzetti F, Tamassia N, Arruda-Silva F, *et al.* The importance of being 'pure' neutrophils. *J Allergy Clin Immunol* 2017;**139**:352–355.e6. doi:10.1016/j.jaci.2016.06.025

29. Zimmermann M, Arruda-Silva F, Bianchetto-Aguilera F, *et al.* IFNα enhances the production of IL-6 by human neutrophils activated via TLR8. *Sci Rep* 2016;**6**:1–13. doi:10.1038/srep19674

30. Nuri E, Taraborelli M, Andreoli L, *et al.* Long-term use of hydroxychloroquine reduces antiphospholipid antibodies levels in patients with primary antiphospholipid syndrome. *Immunol Res* 2017;**65**:17–24. doi:10.1007/s12026-016-8812-z

31. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;**40**:1725. doi:10.1002/1529-0131(199709)40:9<1725::AID-ART29>3.0.CO;2-Y

32. Petri M, Orbai A, Alarco GS, *et al.* Derivation and Validation of the Systemic Lupus International Collaborating Clinics Classification Criteria for Systemic Lupus Erythematosus. 2012;**64**:2677–86. doi:10.1002/art.34473

33. Cervera R, Piette JC, Font J, *et al.* Antiphospholipid syndrome: Clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum* 2002;**46**:1019–27.

34. Piette J, Wechsler B, Frances C, *et al.* Exclusion criteria for primary antiphospholipid syndrome. *J Rheumatol* 1993;**20**:1802–4.

35. Derksen RHWM, Bast EJEG, Strooisma T, *et al.* A comparison between the Farr radioimmunoassay and a new automated fluorescence immunoassay for the detection of antibodies against double stranded DNA in serum. *Ann Rheum Dis* 2002;**61**:1099–102. doi:10.1136/ard.61.12.1099

36. Enocsson H, Sjöwall C, Wirestam L, *et al.* Four anti-dsDNA antibody assays in relation to systemic lupus erythematosus disease specificity and activity. *J Rheumatol* 2015;**42**:817–25. doi:10.3899/jrheum.140677

37. Banchereau R, Hong S, Cantarel B, *et al.* Personalized Immunomonitoring Uncovers Molecular Networks that Stratify Lupus Patients. *Cell* 2016;**165**:551–65. doi:10.1016/j.cell.2016.03.008

38. Hoffman RW, Merrill JT, Alarcón-Riquelme MME, *et al.* Gene Expression and Pharmacodynamic Changes in 1,760 Systemic Lupus Erythematosus Patients From Two Phase III Trials of BAFF Blockade With Tabalumab. *Arthritis Rheumatol* 2017;**69**:643–54. doi:10.1002/art.39950

39. Rose T, Grützkau A, Klotsche J, *et al.* Are interferon-related biomarkers advantageous for monitoring disease activity in systemic lupus erythematosus? A longitudinal benchmark study. *Rheumatology* 2017;**56**:1618–26. doi:10.1093/rheumatology/kex220

40. Somers EC, Zhao W, Lewis EE, *et al.* Type I interferons are associated with subclinical markers of cardiovascular disease in a cohort of systemic lupus erythematosus patients. *PLoS One* 2012;**7**:e37000. doi:10.1371/journal.pone.0037000

41. Lisney AR, Szelinski F, Reiter K, *et al.* High maternal expression of SIGLEC1 on monocytes as a surrogate marker of a type i interferon signature is a risk factor for the development of autoimmune congenital heart block. *Ann Rheum Dis* 2017;**76**:1476–80. doi:10.1136/annrheumdis-2016-210927

42. Bellutti Enders F, Van Wijk F, Scholman R, *et al.* Correlation of CXCL10, tumor necrosis factor receptor type II, and galectin 9 with disease activity in juvenile dermatomyositis. *Arthritis Rheumatol* 2014;**66**:2281–9. doi:10.1002/art.38676

43. Higgs BW, Liu Z, White B, *et al.* Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis* 2011;**70**:2029–36. doi:10.1136/ard.2011.150326

44. Brkic Z, Maria NI, Helden-meeuwsen CG Van, *et al.* Prevalence of interferon type I signature in CD14 monocytes of patients with Sjögren ' s syndrome and association with disease activity and BAFF gene expression. *Ann Rheum Dis* 2013;**72**:728–35. doi:10.1136/annrheumdis-2012-201381

45. Kennedy WP, Maciuca R, Wolslegel K, *et al.* Association of the interferon signature metric with serological disease manifestations but not global activity scores in multiple cohorts of patients with SLE. *Lupus Sci Med* Published Online First: 2015. doi:10.1136/lupus-2014-000080

46. Eloranta M-L, Alm G V, Rönnblom L. Disease mechanisms in rheumatology--tools and pathways: plasmacytoid dendritic cells and their role in autoimmune rheumatic diseases. *Arthritis Rheum* 2013;**65**:853–63. doi:10.1002/art.37821

47. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol* 2015;**15**:471–85. doi:10.1038/nri3865

48. Rodero MP, Decalf J, Bondet V, *et al.* Detection of interferon alpha protein reveals differential levels and cellular sources in disease. *J Exp Med* 2017;**214**:1547–55. doi:10.1084/jem.20161451

49. Prinz N, Clemens N, Strand D, *et al.* Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. *Blood* 2011;**118**:2322–32. doi:10.1182/blood-2011-01-330639

50. Lövgren T, Eloranta ML, Båve U, *et al.* Induction of interferon-α production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum* 2004;**50**:1861–72. doi:10.1002/art.20254

51. Lindau D, Mussard J, Rabsteyn A, *et al.* TLR9 independent interferon α production by neutrophils on NETosis in response to circulating chromatin, a key lupus autoantigen. *Ann Rheum Dis* 2014;**73**:2199–207. doi:10.1136/annrheumdis-2012-203041

52. M. Ziegler S, Beisel C, Sutter K, *et al.* Human pDCs display sex-specific differences in type I interferon subtypes and interferon α/β receptor expression. *Eur J Immunol* 2017;**47**:251–6. doi:10.1002/eji.201646725

53. Munroe ME, Lu R, Zhao YD, *et al.* Altered type II interferon precedes autoantibody accrual and elevated type I interferon activity prior to systemic lupus erythematosus classification. *Ann Rheum Dis* 2016;:annrheumdis-2015-208140. doi:10.1136/annrheumdis-2015-208140

54. Chiche L, Jourde-Chiche N, Whalen E, *et al.* Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type i and type ii interferon signatures. *Arthritis Rheumatol* 2014;**66**:1583–95. doi:10.1002/art.38628

55. Hall JC, Casciola-Rosen L, Berger AE, *et al.* Precise probes of type II interferon activity define the origin of interferon signatures in target tissues in rheumatic diseases. *Proc Natl Acad Sci* 2012;**109**:17609–14. doi:10.1073/pnas.1209724109

56. Rossato M, Affandi AJ, Thordardottir S, *et al.* Association of MicroRNA-618 Expression With Altered Frequency and Activation of Plasmacytoid Dendritic Cells in Patients With Systemic Sclerosis. *Arthritis Rheumatol* 2017;**69**:1891–902. doi:10.1002/art.40163

57. Blomberg S, Eloranta ML, Magnusson M, *et al.* Expression of the markers BDCA-2 and BDCA-4 and production of interferon-α by plasmacytoid dendritic cells in systemic lupus erythematosus. *Arthritis Rheum* 2003;**48**:2524–32. doi:10.1002/art.11225

58. Wildenberg ME, van Helden-Meeuwsen CG, van de Merwe JP, *et al.* Systemic increase in type I interferon activity in Sjögren's syndrome: A putative role for plasmacytoid dendritic cells. *Eur J Immunol* 2008;**38**:2024–33. doi:10.1002/eji.200738008

59. van Bon L, Affandi AJ, Broen J, *et al.* Proteome-wide analysis and CXCL4 as a biomarker in systemic sclerosis. *N Engl J Med* 2014;**370**:433–43. doi:10.1056/NEJMoa1114576

60. Farkas L, Beiske K, Lund-Johansen F, *et al.* Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol* 2001;**159**:237–43.

61. Gottenberg J-E, Cagnard N, Lucchesi C, *et al.* Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome. *Proc Natl Acad Sci U S A* 2006;**103**:2770–5. doi:10.1073/pnas.0510837103

62. Mossu A, Daoui A, Bonnefoy F, *et al.* Plasmacytoid Dendritic Cells Die by the CD8 T Cell–Dependent Perforin Pathway during Acute Nonviral Inflammation. *J Immunol* 2016;**197**:1672–82. doi:10.4049/jimmunol.1501875

63. Swiecki M, Wang Y, Vermi W, *et al.* Type I interferon negatively controls plasmacytoid dendritic cell numbers in vivo. *J Exp Med* 2011;**208**:2367–74. doi:10.1084/jem.20110654

64. Vincent FB, Morand EF, Schneider P, *et al.* The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol* 2014;**10**:365–73. doi:10.1038/nrrheum.2014.33

65. Barturen G, Beretta L, Cervera R, *et al.* Moving towards a molecular taxonomy of autoimmune rheumatic diseases. *Nat Rev Rheumatol* 2018;**14**:75–93. doi:10.1038/nrrheum.2017.220

Nederlandse samenvatting

Het immuunsysteem ontrafeld

in systemische lupus erythematodes en het antifosfolipiden syndroom

Nederlandse samenvatting

Delineating the deranged immune system

in systemic lupus erythematosus and antiphospholipid syndrome

Systemische lupus erythematodes (SLE) is een auto-immuunziekte die wordt gekenmerkt door de vorming van antistoffen tegen verschillende lichaamseigen eiwitten inclusief het eigen DNA. Deze antistoffen activeren het afweersysteem (immuunsysteem) wat kan leiden tot ontstekingen in verschillende orgaansystemen waaronder de huid, gewrichten en nieren. SLE kan op elke leeftijd voorkomen maar wordt het vaakst ontdekt rond het 30e levensjaar en vrouwen worden vaker getroffen dan mannen (de vrouw-man verhouding bedraagt ongeveer 9:1).

Ondanks dat we de precieze oorzaak van SLE niet kennen, weten we dat een ontregeling van het immuunsysteem ten grondslag ligt aan SLE. Dankzij wetenschappelijk onderzoek naar de rol van het immuunsysteem in patiënten met SLE begrijpen we beter welke immuuncellen en ontstekingseiwitten betrokken zijn bij het ontstaan van en in stand houden van SLE. Dergelijk onderzoek heeft geleid tot de ontdekking van aangrijpingspunten voor nieuwe medicijnen alsook de ontdekking van signaalstoffen welke gebruikt kunnen worden om bijvoorbeeld ziekteactiviteit te meten in patiënten met SLE (zogenaamde biomarkers).

Het antifosfolipiden syndroom (Engels: antiphospholipid syndrome, APS) behoort net als SLE tot de auto-immuunziekten. In patiënten met APS worden zogenaamde *antifosfolipiden antistoffen* gevonden (zoals antistoffen tegen β2-glycoproteïne I en cardiolipine of een positieve lupus anticoagulans test). Klassieke uitingen van APS zijn trombose (bijvoorbeeld een trombosebeen of een beroerte) en zwangerschapscomplicaties (bijvoorbeeld herhaalde miskramen). Daarnaast kunnen afwijkingen van de hartkleppen, nieren en een verlaagd aantal bloedplaatjes voorkomen. APS werd voor het eerst beschreven in patiënten met SLE. Ongeveer 20% van de SLE patiënten heeft naast SLE ook APS (*secundair* APS, SLE+APS). Anderzijds kan APS ook voorkomen in patiënten die geen SLE (of andere auto-immuunziekte) hebben en wordt dan *primair* APS genoemd (PAPS).

Wetenschappelijk onderzoek naar APS heeft zich voornamelijk gericht op hoe antifosfolipiden antistoffen de bloedstolling activeren. Trombose (de vorming van bloedstolsels leidend tot een afsluiting van een bloedvat) is immers de belangrijkste uiting van APS. In tegenstelling tot andere auto-immuunziekten weten we echter nog betrekkelijk weinig over de rol van het immuunsysteem in APS. Daarom onderzochten wij in dit proefschrift verschillende typen immuuncellen en ontstekingseiwitten in het bloed van patiënten met APS en vergeleken APS patiënten met SLE patiënten. Zo brachten we de rol van het immuunsysteem in APS en SLE in kaart.

Het immuunsysteem ontrafeld in het antifosfolipiden syndroom

In **hoofdstuk 2** schreven we een overzichtsartikel over wat er tot dan toe onderzocht was met betrekking tot het immuunsysteem in patiënten met APS. Eén van de dingen die ons hierbij opviel is dat er weinig onderzoeken zijn gedaan die APS met SLE vergelijken. In de daarop volgende hoofdstukken beschreven we immunologische fenomenen in patiënten met PAPS in vergelijking tot patiënten met SLE of SLE+APS. Zo beschreven we de aanwezigheid van een *interferon signature* (**hoofdstuk 3**) in patiënten met APS en ontdekten dat deze gemakkelijk te bepalen is door het stofje *galectin-9* te meten (**hoofdstuk 4**). Ook beschreven we dat APS patiënten, net als SLE patiënten, een versterkte neiging tot het produceren van *neutrophil extracellular traps (NETs)* (**hoofdstuk 5 en 6**) en een verhoogde productie van het ontstekingseiwit BAFF (B-cell activating factor, **hoofdstuk 7**) hebben. We beschreven met welke factoren deze immunologische fenomenen samenhangen in patiënten met APS. Aangezien deze immunologische afwijkingen aangrijpingspunten vormen voor nieuwe therapieën in SLE, zouden dergelijke geneesmiddelen in de toekomst misschien ook kunnen worden gebruikt voor de behandeling van APS.

Plasmacytoide dendritische cellen en type I interferon: oorzaak en gevolg in SLE en APS

Plasmacytoide dendritische cellen (pDCs) zijn immuuncellen die grote hoeveelheden *interferon* kunnen produceren. Zij worden daarom gezien als oorzaak van de *interferon signature* in patiënten met SLE en APS. Om te bestuderen waarom en hoe deze cellen *interferon* produceren in SLE en APS isoleerden wij pDCs van deze patiënten en bestudeerden hun genexpressie profiel. We ontdekten dat microRNAs, dit zijn kleine stukjes RNA die de expressie van andere genen reguleren, verlaagd tot expressie komen in pDCs van SLE en APS patiënten, met name in patiënten met een sterkere *interferon signature* (**hoofdstuk 8**). Dit profiel van verlaagde microRNA expressie komt overeen met het microRNA profiel van geactiveerde pDCs en draagt mogelijk bij aan de activatie van pDCs in SLE en APS.

Daarnaast beschreven we gen-expressie profielen in pDCs en vergeleken deze met een ander type dendritische cel, de myeloide dendritische cel (mDC) in patiënten met SLE, SLE+APS en PAPS. Opvallend genoeg waren er meer overeenkomsten dan verschillen in de gen-expressie profielen van deze cellen tussen SLE en APS patiënten waarbij de aanwezigheid van de *interferon signature* een grote invloed had op het gen-expressie profiel. Hoewel in beide cellen *interferon* het gen-expressie profiel sterk beïnvloedt, was het cel specifieke effect van interferon op beide cel typen verschillend met het uiteindelijke effect de *interferon signature* in stand te houden (**hoofdstuk 9**).

Het effect van interferon op immuuncellen in SLE en andere autoimmuunziekten

Granzyme B (GrB) is een stof die wordt uitgescheiden door immuuncellen waaronder pDCs en T-cellen. Een teveel aan GrB kan schade aanrichten aan weefsels. In **hoofdstuk 10** zagen we dat GrB, maar niet andere granzymen, verhoogd aanwezig zijn in het bloed en in de nieren van patiënten met SLE. In het bloed hing dit samen met een hogere *interferon signature* en in het nierbiopt met de chroniciteitsindex zoals bepaald door de patholoog. In **hoofdstuk 11** bestudeerden we de recent ontdekte *innate lymphoid cells* (ILCs) in patiënten met SLE en patiënten met het syndroom van Sjögren (SjS). In andere reumatische ziektebeelden waaronder reumatoïde artritis spelen deze cellen een rol in het in stand houden van ontsteking in het weefsel en wij beschreven dat in SLE en SjS de ILC aantallen samenhangen met de *interferon signature*.

Massacytometry: een nieuwe manier om het immuunsysteem te doorgronden?

Het immuunsysteem bestaat uit verschillende type immuuncellen. Elk type immuuncel brengt specifieke eiwitten tot expressie op zijn celmembraan. Hierdoor kunnen onderzoekers bepalen welke immuuncellen er aanwezig zijn in het bloed of weefsel van patiënten. Cytometry by time of flight (CyTOF) is een nieuwe manier om verschillende typen immuuncellen te meten in bijvoorbeeld bloed van patiënten met auto-immuunziekten. Het voordeel van CyTOF is dat op 1 cel ruim 40 verschillende markers in één keer bestudeerd kunnen worden. In **hoofdstuk 12** gebruikten we CyTOF om de proporties van immuuncellen tussen verschillende auto-immuunziekten in kaart te brengen. Naast SLE bestudeerden we patiënten met verschillende uitingen van systemische sclerose (SSc) en patiënten met het syndroom van Sjögren (SjS). Hoewel er veel overeenkomsten zijn in het voorkomen van subtypen immuuncellen tussen de drie ziektebeelden kent ieder ziektebeeld één of meerdere subtype immuuncellen waarvan de frequentie meer specifiek is aangedaan in dat ziektebeeld.

Conclusie

In dit proefschrift beschreven wij immunologische kenmerken van patiënten met APS in vergelijking tot patiënten met SLE. Daarnaast bestudeerden wij in geselecteerde hoofdstukken ook andere auto-immuunziekten zoals het syndroom van Sjögren en systemische sclerose. Door verschillende immuuncellen en ontstekingseiwitten te bestuderen beschreven we verschillen, maar met name ook overeenkomsten in immunologische fenomenen tussen deze verschillende ziektebeelden. Het herkennen en indelen van patiënten met verschillende auto-immuunziekten op basis van immunologische afwijkingen kan in de toekomst bijdragen aan nieuwe behandelingsmogelijkheden in patiënten met auto-immuunziekten.

Curriculum Vitae

Lucas (Luuk) van den Hoogen was born on the 21st of July 1989 in Nijmegen. He grew up in Malden and attended the Stedelijk Gymnasium Nijmegen from which he graduated in 2007. Afterwards he moved to Utrecht to study medicine at Utrecht University from which he graduated with distinction in 2013. During his studies he completed a clerkship in primary health care in northern Tanzania. He gained an interest in immunology and in his last year performed an internship in the research group of prof. dr. Meyaard studying the role of neutrophil extracellular traps and plasmacytoid dendritic cells in patients with autoimmune diseases. After finishing medicine he started a PhD in the research group of prof. dr. Radstake on the role of the immune system in patients with systemic lupus erythematosus and antiphospholipid syndrome which is presented in this thesis. During his research he was awarded the Abstract Recognition Award (Young Scholars) at the APS meeting in Cyprus 2016, best oral presentation at the Najaarsdagen of the Nederlandse vereniging voor Reumatologie 2016 and the NVLE award 2018. From 2018 he is enrolled in the training programme to become a rheumatologist, starting at the internal medicine department of the St. Antonius hospital in Nieuwegein. Luuk lives in Utrecht with his girlfriend Jocelyn and their son Seb.

List of publications

- Koole D, Zandvoort HJ, Schoneveld A, Vink A, Vos JA, van den Hoogen LL, de Vries JP, Pasterkamp G, Moll FL, van Herwaarden JA. Intraluminal abdominal aortic aneurysm thrombus is associated with disruption of wall integrity. J Vasc Surg. 2013 Jan;57(1):77-83.
- van den Hoogen LL and van den Hoogen FHJ. Medications for skin symptoms, In: Novel Insights into Systemic Sclerosis Management, Unitec House, 2 Albert Place, London N3 1QB, UK (Future Medicine Ltd) 2013 Pages 76-89. Editors: Cutolo M, Smith V
- van den Hoogen LL, Meyaard LM. Inhibitoire receptoren in de klinische praktijk. Ned Tijdschr Allergie en Astma 2014;1:136-42.
- van den Hoogen LL, Sims GP, Fritsch-Stork RDE. Aging and Systemic Lupus Erythematosus Curr Aging Sci. 2015;8(2):158-77.
- van den Hoogen LL, van Roon JA, Radstake TR, Fritsch-Stork RD, Derksen RH. Delineating the deranged immune system in the antiphospholipid syndrome. Autoimmun Rev. 2016 Jan;15(1):50-60.
- van den Hoogen LL, Fritsch-Stork RD, van Roon JA, Radstake TR. Low-Density Granulocytes Are Increased in Antiphospholipid Syndrome and Are Associated With Anti-β2 -Glycoprotein I Antibodies: Comment on the Article by Yalavarthi et al. Arthritis Rheumatol. 2016 May;68(5):1320-1.
- van den Hoogen LL, Fritsch-Stork RD, Versnel MA, Derksen RH, van Roon JA, Radstake TR. Monocyte type I interferon signature in antiphospholipid syndrome is related to proinflammatory monocyte subsets, hydroxychloroquine and statin use. Ann Rheum Dis. 2016 Dec;75(12):e81.
- van den Hoogen LL. Type I-interferon en plasmacytoïde dendritische cellen in het antifosfolipidensyndroom. Ned Tijdschr voor Reumatologie 2016 (4);56-
- Kok HM, van den Hoogen LL, van Roon JAG, Adriaansen EJM, Fritsch-Stork RDE, Nguyen TQ, Goldschmeding R, Radstake TRDJ, Bovenschen N. Systemic and local granzyme B levels are associated with disease activity, kidney damage and interferon signature in systemic lupus erythematosus. Rheumatology (Oxford). 2017 Dec 1;56(12):2129-2134.
- van der Linden M*, van den Hoogen LL*, Westerlaken GHA, Fritsch-Stork RDE, van Roon JAG, Radstake TRDJ, Meyaard L. Neutrophil extracellular trap release is associated with antinuclear antibodies in systemic lupus erythematosus and anti-phospholipid syndrome. Rheumatology (Oxford). 2018 Mar 28. *equal contribution
- Chouri E, Servaas NH, Bekker CPJ, Affandi AJ, Cossu M, Hillen MR, Angiolilli C, Mertens JS, van den Hoogen LL, Silva-Cardoso S, van der Kroef M, Vazirpanah N, Wichers CGK, Carvalheiro T, Blokland SLM, Giovannone B,

Porretti L, Marut W, Vigone B, van Roon JAG, Beretta L, Rossato M, Radstake TRDJ. Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis. J Autoimmun. 2018 Jan 19.

- Rietveld A, van den Hoogen LL, Bizzaro N, Blokland S, Dähnrich C, Gottenberg JE, Houen G, Johannsen N, Mandl T, Meyer A, Nielsen C, Olsson P, van Roon J, Schlumberger W, van Engelen B, Saris C, Pruijn GJM. Autoantibodies to Cytosolic 5'-nucleotidase 1A in primary Sjögren's syndrome and systemic lupus erythematosus. Frontiers in Immunology 2018 (Accepted)
- van den Hoogen LL, Rossato M, Lopes AP, Pandit A, Bekker CJP, Fritsch-Stork RDE, van Roon JAG, Radstake TRDJ. microRNA downregulation in plasmacytoid dendritic cells in interferon positive systemic lupus erythematosus and antiphospholipid syndrome. Rheumatology 2018 (accepted