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Research paper

Measurement of IL-21 in human serum and plasma using ultrasensitive MSD S-PLEX® and Quanterix SiMoA methodologies

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ABSTRACT

IL-21 is a pleiotropic cytokine that plays a key role in modulating inflammatory responses, including the promotion of autoimmune diseases. Several groups have quantitated circulating levels of IL-21 in plasma and serum samples using various commercial ELISAs. We determined, however, that the most commonly used commercial assays in published literature were not specific or sensitive enough to detect levels of IL-21 in heparin plasma or serum from healthy human individuals. This finding prompted an effort to develop more specific and sensitive methods to quantitate IL-21 in complex biological matrices using proprietary anti-IL-21 antibodies with the Quanterix SiMoA platform and the Meso Scale Discovery (MSD) S-PLEX® format. Assays developed on both technology platforms were characterized in heparin plasma and serum using spike recoveries across a range of concentrations. Each method was able to detect sub-pg/mL levels of IL-21 (predicted Limit of Detection [LOD] of approximately 1.0 fg/mL for both the Quanterix SiMoA and MSD S-PLEX® platforms) which is 200–500 times lower than current commercial assays. Additionally we demonstrated that rheumatoid factor did not interfere with measuring IL-21 in the Quanterix SiMoA assay. Results obtained with the two new ultrasensitive assays showed a strong correlation ($r = 0.9428$; $p < .0001$). Additionally, IL-21 levels were significantly increased in samples from patients with Systemic Lupus Erythematosus (mean +/− SD: $n = 14$, 202.64 +/− 111.47 fg/mL, $p = .0001$ for Quanterix SiMoA and 275.4 +/− 174.66 fg/mL p = .0001 for MSD S-PLEX®) as well as in samples from patients with Sjögren's Syndrome (mean +/− SD: $n = 11$, 122.18 +/− 84.50 fg/mL, $p = .0029$ for Quanterix SiMoA and 183.64 +/− 153.00 fg/mL, $p = .0082$ for MSD S-PLEX®) when compared to healthy donors (mean +/− SD: $n = 11$, 38.1 +/− 27.8 fg/mL for Quanterix SiMoA and 58.1 +/− 30.7 fg/mL for MSD S-PLEX®). These ultrasensitive assays, for the first time, allow for the accurate quantitation of human IL-21 in heparin plasma and serum. In addition, these experiments also provide a direct comparison of the MSD S-PLEX® format and Quanterix SiMoA platform technologies, which may have broader implications to future application of these methods to evaluate low abundance proteins in complex biological matrices.

1. Introduction

Interleukin-21 (IL-21) is a member of the type I cytokine family that includes IL-2, IL-4, IL-7, IL-9 and IL-15 and activates the JAK/STAT pathway through the cognate IL-21 receptor (IL-21R) and the common γ chain (γc) (Ozaki et al., 2000; Parrish-Novak et al., 2000). It is produced by a variety of cell types including natural killer T cells (NKT), T follicular helper (TFH) cells and Th17 cells (Parrish-Novak et al., 2000; Nurieva et al., 2007; Lüthje et al., 2012). IL-21 affects different subtypes of T cells, promoting the activation/function of Th17, TFH and CD8+ T cells and decreasing the expansion of regulatory T cells (Treg) (Korn et al., 2007; Nurieva et al., 2008; Zeng et al., 2005). Furthermore, IL-21 has been shown to be critical in supporting germinal center formation, B cell differentiation and immunoglobulin production (Ettinger et al., 2005; Kuchen et al., 2007; Ozaki et al., 2002; Kotlarz et al., 2014; Zotos et al., 2010).

Because of its broad spectrum of actions on populations of cells with key roles in the pathophysiology of different autoimmune diseases, IL-

Abbreviations: SiMoA, Single Molecule Arrays; LOQ, Limit of Quantitation; LLOQ, Lower Limit of Quantitation; ULOQ, Upper Limit of Quantitation; LOD, Limit of Detection; RF, Rheumatoid Factor; SLE, Systemic Lupus Erythematosus; SS, Sjögren's Syndrome; pSS, Primary Sjögren's Syndrome; MSD, Meso Scale Discovery; IL-21, Interleukin 21; ND, Not Done

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21 has become attractive for therapeutic intervention and biomarker analysis. In order to understand if concentrations in peripheral blood samples can be linked to disease activity and progression, many studies have investigated IL-21 levels in biofluids from autoimmune disorders using commercially-available ELISA kits. Indeed, elevated levels of IL-21 have been observed in patients with Psoriasis (He et al., 2012), primary Sjögren's Syndrome (Kang et al., 2011), Rheumatoid Arthritis (Xing et al., 2018; Gottenberg et al., 2012; Niu et al., 2010), Atopic Dermatitis (Mizutani et al., 2016), and Systemic Lupus Erythematosus (SLE) (Wong et al., 2010; Wang et al., 2014; Nguyen et al., 2009). In these publications, IL-21 was measured in serum or EDTA plasma and was detectable in the pg/mL range in both autoimmune patient and healthy donor samples.

During our efforts to independently verify the results of these experiments, we identified some key limitations with the most widely used commercially-available human IL-21 ELISA kits. We found that these assays lacked the specificity and sensitivity required to accurately quantitate IL-21 in human heparin plasma and serum samples resulting in the need for a new method.

Ultrasensitive ELISA technologies have recently been developed to enable the quantitation of proteins in complex biological matrices in the fg/mL range. Two such systems include the Quanterix SiMoA (Single Molecule Array) and Meso Scale Discovery (MSD) S-PLEX® platforms. The Quanterix SiMoA platform utilizes a fluorescent detection system and antibody-coated microbeads to digitally assess protein binding and concentration. The technology makes use of a digital camera to quantitate reactions occurring in femtoliter-sized reaction chambers each housing a single microbead and has been reported to detect proteins in the single femtomolar range reliably (Rissin et al., 2010). The MSD S-PLEX® electrochemiluminescence detection technology uses SULFO-TAG labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY microplates (Eklund et al., 2017). Both technologies were used to develop orthogonal methods utilizing Lilly proprietary anti-IL-21 antibodies. Each method demonstrated significantly improved specificity and sensitivity, with a Limit of Detection (LOD) of approximately 1.0 fg/mL for both the Quanterix SiMoA and MSD S-PLEX® platforms. Additionally, these independently developed methods demonstrated a high degree of correlation when the same sample cohorts were analyzed on each platform. Furthermore, we found that the concentrations of IL-21 in human plasma and serum are much lower than previously published (i.e., < 100 fg/mL in healthy donors). As others have reported, however, IL-21 levels remain elevated in patients with different autoimmune diseases compared to healthy donors, but not in the concentration ranges formerly identified. These new methods demonstrate the importance of method characterization, even for commercially-validated assays, and now permit accurate and specific quantitation of this important cytokine in heparin plasma and serum samples in two orthogonal ultrasensitive method platforms.

2. Materials and methods

2.1. Samples

Healthy human heparin plasma and serum samples were obtained from the Eli Lilly research blood donation program. Heparin plasma samples from healthy donors, as well as, patients diagnosed with SLE or Sjögren's Syndrome (SS) were obtained from Methodist Hospital in Indianapolis, IN, USA. All samples were collected under Institutional Review Board (IRB)-approved protocols, processed according to standard laboratory methods and placed at −80 °C for long-term storage.

2.2. Spike recovery and ELISA characterizations

Commercially-available human IL-21 ELISA kits from eBiosciences (Cat # 88–8218-22) and PeproTech (Cat # 900-K226) were performed according to the manufacturers' protocols. Three to eight-point calibration curves were freshly prepared from the IL-21 stocks and compared to the concentration of known spikes in pooled heparin plasma or serum samples. All calibration and spike samples had two technical replicates. Spike recoveries were determined as indicated in Section 2.7 Statistical Analysis.

2.3. Antibodies

The anti-IL-21 antibodies used in the ultrasensitive assays were discovered and developed by Eli Lilly. AbM2, Ab2–1, and AbCom3 were transiently expressed in HEK293 or CHO cells and after purification and IL-21 binding was confirmed using a surface plasmon resonance assay on a Biacore 2000 instrument. AbM2 is a modified human IgG4 antibody, while both Ab2–1 and AbCom3 are mouse IgG1 antibodies.

2.4. Immunodepletion of IL-21

All spikes were performed using Lilly recombinant IL-21 and immunoprecipitations with proprietary biotin-conjugated IgG control or anti-IL-21 monoclonal antibodies. Undiluted plasma or serum samples, spiked or unspiked with recombinant human IL-21, were incubated with AbM2-coated streptavidin magnetic beads (Dynabeads, Invitrogen, Cat. # 65001) overnight at 4 °C with rotation and the resulting supernatants assayed for IL-21 on the following day.

2.5. Ultrasensitive immunoassay method development

The Quanterix SiMoA assay was developed by conjugating Lilly proprietary anti-IL-21 antibodies, Ab2–1, AbM2 and AbCom3, to carboxylated paramagnetic beads according to the SiMoA Homebrew Assay Development Kit (Quanterix Corp, Lexington, MA) at 1.0 mg/mL as previously described (Chang et al., 2013; Song et al., 2016; Song et al., 2011). These same antibodies were also biotinylated according to the standard Quanterix protocol (40:1 biotin ratio). For the optimized method, Ab2–1 conjugated beads (approximately 5 million beads/mL) were prepared in Quanterix bead diluent and biotinylated AbM2 antibody (0.5 µg/mL) was diluted in Quanterix sample/detection buffer. Streptavidin-beta-galactosidase (SBG) was prepared in Quanterix SBG diluent at 150 pM. IL-21 recombinant protein or samples (two-fold dilution, final volume of 115uL sample for two technical replicates) were diluted in assay buffer (600 mM NaCl (Sigma), 0.5% Tween 20 (Pierce), 25% FBS (Gibco), 2% BSA (Sigma) and 200 µg/mL heterophilic blocking reagent-1 (Scantibodies) in PBS) at appropriate dilutions. Ab2–1 conjugated beads, biotinylated AbM2 antibody, calibrators, SBG and manufacturer-supplied resorufin-beta-D galactopyranoside reagents were loaded into the instrument and run at room temperature as a two-step Homebrew method according to the SiMoA HD-1 Analyzer User Guide. The assay was characterized for spike recovery and Limit of Detection (LOD).

The MSD S-PLEX® was developed and optimized independently using the Ab2–1, AbM2 and AbCom3 antibodies, following a screen of commercially-available antibodies. MSD's electrochemiluminescence detection technology uses SULFO-TAG labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY microplates. The newly developed S-PLEX® assay platform, a next-generation MULTI-ARRAY technology has significantly higher sensitivity than standard electrochemiluminescence. Samples were diluted two-fold in proprietary MSD buffer prior to analysis (final volume of 50uL for two technical replicates). The assay was characterized for spike recovery and LOD determined in Section 2.7 Statistical Analysis.

2.6. Rheumatoid factor analysis

An elevated Rheumatoid Factor (RF) control plasma, obtained from Meridian Life Science, Inc., was used to evaluate the effect of RF on the

Quanterix SiMoA IL-21 assay only. For this test, heparin plasma samples were prepared with and without a 100 fg/mL spike of recombinant human IL-21 in the absence or presence of a serial dilution of RF (500, 125, 31.25, 7.8125 and 0 IU/mL) in samples containing or lacking the heterophilic blocking reagent HBR-1 (Scantibodies). The spike recovery for each sample was then calculated with results normalized to the 0 IU/mL RF samples, to assess the impact of RF alone on assay performance in the presence or absence of HBR-1.

2.7. Statistical analysis

Spike recoveries were determined by comparing the back-calculated concentrations of the spiked samples including endogenous levels against the known spiked concentration (Sittampalam et al., 2004). LODs were determined by selecting the lowest point on the calibration curve that demonstrated 75–125% recovery against the statistical model (i.e. comparing the calibration samples of the calculated concentration to expected concentration). LOQs were determined by the selection criteria of 75%–125% of the spike recovery samples in biological matrix (i.e. comparing the spike samples of the calculated concentration to expected concentration). Pearson's correlation coefficient and mean difference on log-transformed concentrations were applied to determine the agreement between MSD S-PLEX® and the Quanterix SiMoA methods. One-way ANOVA with Dunnett's multiple comparison test on log-transformed concentrations was used to compare the levels of IL-21 from SLE and SS patients to healthy donors with a significant cutoff at .05.

3. Results

3.1. Assessment of commercially-available IL-21 ELISAs

Sensitivity, specificity and spike recovery are key aspects used to assess the performance of any method prior to sample analysis (Cox et al., 2012; Findlay et al., 2000). The commercial IL-21 ELISA kits from eBiosciences and PeproTech were evaluated for these parameters using IL-21-spiked samples into pooled heparin plasma and serum samples (Table 1). Both ELISAs failed to adequately quantitate IL-21 in heparin plasma. The PeproTech assay for heparin plasma samples demonstrated poor spike recovery, recovering only 33.1–72.8% of the expected recombinant protein, which is below the standard 75–125% acceptable recovery range. Similarly, the eBioscience ELISA only recovered 14.3–77.2% of the spiked recombinant IL-21 in heparin plasma with only a single spike concentration within the acceptable range. The PeproTech assay also failed to demonstrate acceptable recovery in serum, with many spikes failing to calculate a percent recovery, whereas the eBioscience assay did have acceptable spike recovery across the sample range (percent recoveries ranged from 74.3–85.8%) in serum. The ELISA, however, failed to show specificity as anti-IL-21 conjugated beads were unable to immunodeplete endogenous IL-21 in a pooled serum sample compared with an IgG control antibody (Fig. 1). These same beads were able to bind and remove recombinant IL-21 spiked into the serum sample at 100 pg/mL, but not below the endogenous IL-21 levels in the same sample. This indicates that while this ELISA method is capable of detecting recombinant IL-21, it is also detecting an additional analyte in the original sample which is not removed by immunoprecipitation with an IL-21-specific antibody. Endogenous IL-21, in this experiment, should have been reduced by the anti-IL-21 conjugated bead down to the LOD of the method (~7.8 pg/mL). Additionally, the spike recovery of this 100 pg/mL spiked sample above the endogenous level was below specification limits of 75–125% (56%), reflecting further issues with quantitation. These data indicate that the performance of the eBioscience assay in serum was still not acceptable for downstream sample analysis.

Table 1
Assay characterization of PeproTech and eBiosciences IL-21 ELISA methods.

PeproTech IL-21 ELISA			eBioscience IL-21 ELISA		
Spiked Concentration (pg/mL)	Percent Recovery Heparin Plasma	Percent Recovery Serum	Spiked Concentration (pg/mL)	Percent Recovery Heparin Plasma	Percent Recovery Serum
1500	60.9	77.9	750	14.3	74.3
750	40	38.1	375	18.4	77.5
375	33.1	15.2	187.5	24.4	78.1
187.5	32.6	BLQ	93.8	34.1	77.7
93.8	48.4	BLQ	46.9	48.1	80.6
46.9	64.4	BLQ	23.4	61.9	80.3
23.4	72.8	BLQ	11.7	77.2	85.8
LOD	N/A	N/A	LOD	N/A	7.8 pg/mL

Human recombinant IL-21 from each manufacturer was spiked into pooled normal human heparin plasma and serum at the concentrations indicated and percent recoveries determined based on the results of each ELISA (showing representative experiment).

3.2. Development of ultrasensitive IL-21 methods

As a result of the initial characterization work, more sensitive and specific assays were developed using two ultrasensitive formats: the Quanterix SiMoA digital ELISA platform and MSD S-PLEX® platform. IL-21 antibodies, manufactured and developed at Eli Lilly, were used to develop both methods according to manufacturer's protocols. Specifically, for the Quanterix SiMoA digital ELISA platform, the antibodies were bound to the microbeads according to the protocol provided by the manufacturer in order to find optimal binding partners for recombinant IL-21 (Fig. 2). Each antibody was also biotinylated at a 40:1 biotin to antibody ratio for the Quanterix SiMoA platform. Antibodies Ab2–1 and AbM2 proved to be the most sensitive, generating standard curves in the fg/mL range. Other antibody pairs either did not generate an appreciable signal in this assay or were less sensitive. The Ab2–1 and AbM2 antibody pair worked in either orientation of capture and detection, but Ab2–1 as capture and AbM2 as detector were chosen for further assay development and optimization.

Ultrasensitive assays, like conventional immunoassays, often require buffer and method optimization in order to achieve the highest performing method. Indeed, initial characterization work using this pair of antibodies produced reliable spike recovery analysis in standard Quanterix sample buffer, but failed to demonstrate adequate spike recovery when combined with healthy donor heparin plasma or serum (Fig. 3). There was evidence of signal suppression at high spikes of recombinant IL-21 leading to the conclusion that there was an interference present in the sample matrix. Therefore, sample dilution buffers varying in levels of NaCl, BSA, Tween-20 and the addition of newborn calf serum were developed to test for improved spike recovery in heparin plasma. Optimal results were achieved in a buffer containing 600 mM NaCl, 0.5% Tween-20 and 25% newborn calf serum. This buffer combination allowed for better recovery in serum and heparin plasma at a two-fold sample dilution across the entire range of spikes tested (Fig. 3). IL-21 spike recovery in saliva was also characterized with comparable results (data not shown).

It is well known that many patients with rheumatic diseases have elevated rheumatoid factor (RF) which can also cause interference in antibody-based assays (Peen and Mellbye, 2009). Therefore the Quanterix SiMoA assay was further characterized to determine if RF posed any risk for accurate quantitation of IL-21 in patient samples. The positive control (plasma with a known elevated RF level) was diluted into a known low RF concentration plasma sample to create a dose-response of RF-containing samples. These were run in the Quanterix SiMoA IL-21 assay in the presence and absence of heterophilic blocking reagent to quantitate the effect of RF on spike recoveries by the method. As seen in Fig. 4, the spike recoveries were within the 75–125% range in the

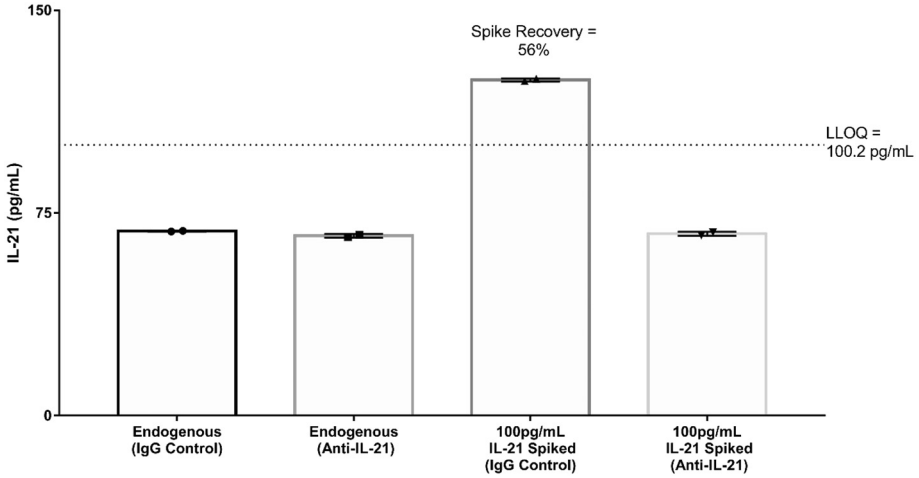

Fig. 1. Immunodepletion of endogenous and recombinant IL-21 in a pooled healthy human serum sample in the eBioscience IL-21 ELISA.
The specificity of the eBioscience IL-21 ELISA was evaluated by attempting to immunodeplete the cytokine using a specific antibody. Recombinant human IL-21 was immunodepleted using this method, but not in an endogenous sample, indicating that the signal detected was not specific for IL-21. Results are mean ± SD of two technical replicates in a pooled healthy serum sample.

presence of the heterophilic blocker. However, in the absence of the blocker, there was a statistically significant reduction in spike recovery at the highest level of RF (500 IU/mL) compared to the no RF control sample (p = .0195, n = 3). We did not perform RF spiking studies on the MSD S-PLEX® assay during method development, but heterophilic blocking reagents were already present within the sample diluents utilized in this method. The results from Fig. 4 indicate that the ultrasensitive IL-21 Quanterix SiMoA assay showed acceptable spike recoveries in the presence of RF up to 500 IU/mL. Therefore, 200μg/mL heterophilic blocking reagent was used for final method characterization and sample analysis for the Quanterix SiMoA method. Spike recoveries of 86–103% in heparin plasma and 75–109.2% for serum were obtained for the Quanterix SiMoA platform (Fig. 5A and Supplemental Table 1).

Independently of the SiMoA assay development, we also explored options to develop an ultrasensitive method on the MSD S-PLEX® platform. Together with the Lilly-proprietary monoclonal IL-21 antibodies, several commercially-sourced antibodies were also evaluated to find the best combination. As with the SiMoA platform, the Lilly antibodies were identified to be optimal for this purpose, selecting Ab2–1 as the capture antibody. Following the independent method development and optimization, we could detect IL-21 across a broad dynamic range of 0.006–35 pg/mL with spike recoveries of 93–109% for the MSD S-PLEX® platform in serum. Heparin plasma was also evaluated for spike recovery, but instead of across the range of the standard curve, only at three levels. Even at this minimal spike recovery, the MSD S-PLEX® assay performed well with recoveries ranging from 88 to 103% at a two-fold sample dilution (Fig. 5B and Supplemental Table 1). At the conclusion of method development, IL-21 freeze/thaw stability was evaluated and found to be stable after four freeze/thaw cycles in spiked and native samples (Supplemental Table 2).

In order to verify the specificity of the assay developed using the Quanterix SiMoA platform, IL-21- immunodepleted heparin plasma samples were prepared. Recombinant human IL-21 (5 pg/mL) was spiked into samples from five healthy donors. IL-21 was immunoprecipitated overnight from both spiked and endogenous samples using anti-IL-21 or control magnetic beads. As shown in Fig. 6, IL-21 was removed to either below the limit of quantitation in the case of the endogenous samples, or to negligible levels for the spiked samples, following immunodepletion. The ability to immunoprecipitate recombinant and endogenous IL-21 distinguishes this method from eBiosciences ELISA, which failed to deplete endogenous. Average spike recovery of the prepared 5 pg/mL samples across five healthy donors was 109% (standard deviation = 25%). The immunodepletion in both untreated and spiked heparin plasma samples supports the specificity of the antibodies and the assay for both recombinant as well as endogenous IL-21. Similar experiments were performed in serum, with comparable results (data not shown).

3.3. IL-21 levels in autoimmune patient samples

Once characterized, the Quanterix SiMoA and MSD S-PLEX® methods were then used to determine the concentrations of IL-21 in heparin plasma samples from SS (n = 11) and SLE patients (n = 14) and age- and sex-matched samples from healthy donors (n = 11) (Fig. 7). As shown above, in both assay formats, all samples were above

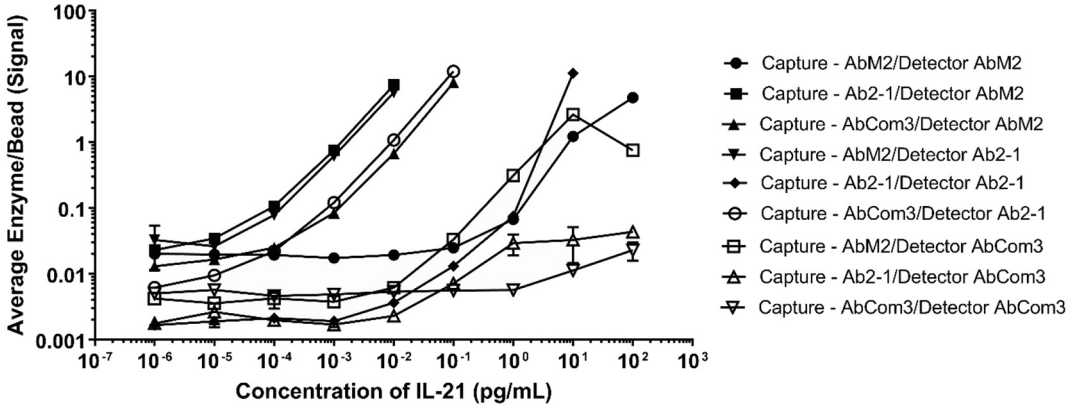

Fig. 2. IL-21 antibody screening in the Quanterix SiMoA assay.
Candidate binding pairs of antibodies tested in the Quanterix SiMoA platform for identification of optimal binding partners to detect recombinant IL-21. Although several antibody pairs detected recombinant IL-21, the AbM2 and Ab2–1 pairing provided the most sensitivity in the assay regardless of orientation.

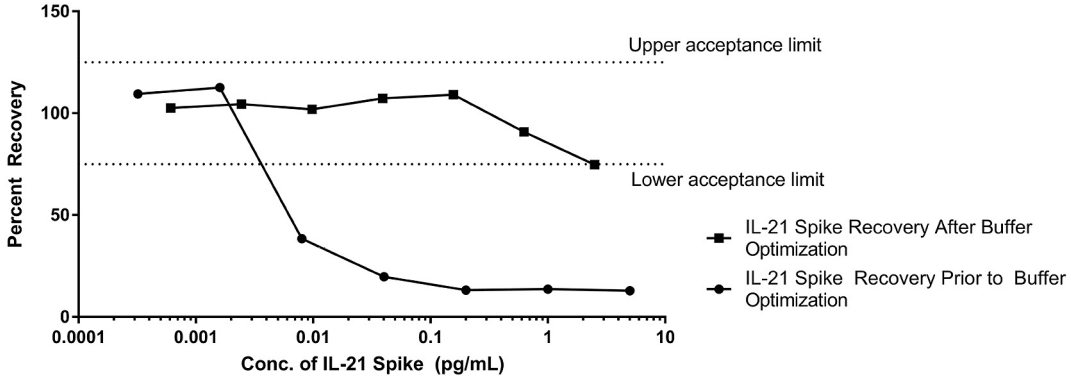

Fig. 3. Comparison of IL-21 spike recovery in pooled healthy donor heparin plasma following optimizing of sample dilution buffer in the Quanterix SiMoA assay. Optimized buffer conditions improved the spike recovery of recombinant IL-21 in heparin plasma and serum.

the LOD for each method and were in the measurable range. A statistically significant difference in IL-21 levels was observed in this small sample set between the plasma samples from healthy donors (mean +/− SD: 38.1 +/− 27.8 fg/mL), SS (mean +/− SD: 122.18 +/− 84.50 fg/mL, $p = .0029$) and SLE patients (mean +/− SD: 202.64 +/− 111.47 fg/mL, $p = .0001$) using the Quanterix SiMoA assay. Likewise, using the MSD S-PLEX® method, a statistically significant difference in IL-21 levels was also observed between the plasma samples from SLE patients (mean +/− SD: 275.4 +/− 174.66 fg/mL, p = .0001) and from SS (mean +/− SD: 183.64 +/− 153.00 fg/mL, $p = .0082$) compared to healthy donors (mean +/− SD: 58.1 +/− 30.7 fg/mL).

3.4. Comparison of ultrasensitive IL-21 methods

In order to compare both methods, fifty heparin plasma samples from healthy donors were added to the results acquired during the analysis of autoimmune cohorts to create a new 86 sample cohort for correlation analysis (Fig. 8). IL-21 levels were readily detected in the additional 50 samples as all samples were above the LOD and fell on the standard curve. A comparison of the CVs between technical replicates for each method is in Supplemental Fig. 1. As shown in Fig. 8, there is a highly significant correlation between the results obtained with both methods (Pearson value of 0.9428 and p-value of $< .0001$). We did notice that the MSD S-PLEX® values generally were two-fold higher than those obtained with Quanterix SiMoA assay (154.9 fg/mL (95% confidence interval of 122.8–186.9 fg/mL) compared to 89.62 fg/mL (95% confidence interval of 70.28–109.0 fg/mL), respectively). These levels were found to be statistically different using a mean difference analysis ($p < .0001$). Thus, while the IL-21 concentrations obtained with both methods highly correlate, the absolute concentrations are different.

4. Discussion

Adequate characterization of any commercially available ELISA is an important step prior to sample analysis. This includes specificity testing through immunodepletion studies and spike recoveries of the desired analyte in the same biological matrix as the test samples. Proper ELISA development and optimization through analysis of different high affinity antibodies and buffer components, including heterophilic blocking reagents, can provide confidence that the resulting signal generated is specific to the analyte and not another source. Additionally, the removal of the analyte from the test sample by immunodepletion should result in a loss of signal towards the LOD of the method. Lastly, however, even when all interferences and background signal have been mitigated, it remains a possibility that the concentration of the analyte is simply not high enough to generate an appreciable signal and an ultrasensitive detection system will be needed. Our analysis of widely used commercially available IL-21 ELISAs demonstrated several shortcomings, including lack of specificity and sensitivity in relevant complex biological matrices. As such, published data on concentrations of IL-21 in health and disease should be considered with caution.

For this reason, quantitating cytokines at lower levels (in the fg/mL range) has recently been reported for several human proteins by combining high-affinity antibodies with next generation ultrasensitive methodologies. It is been reported that interferon alpha can now be measured using the SiMoA digital ELISA technology (Rodero et al., 2017), as well as the difficult-to-measure cytokines IL-17A, IL-13 and IL-5 in samples from different diseases and healthy donors (Gavala and Smith, 2016). Although ultrasensitive assays will clearly play a key role

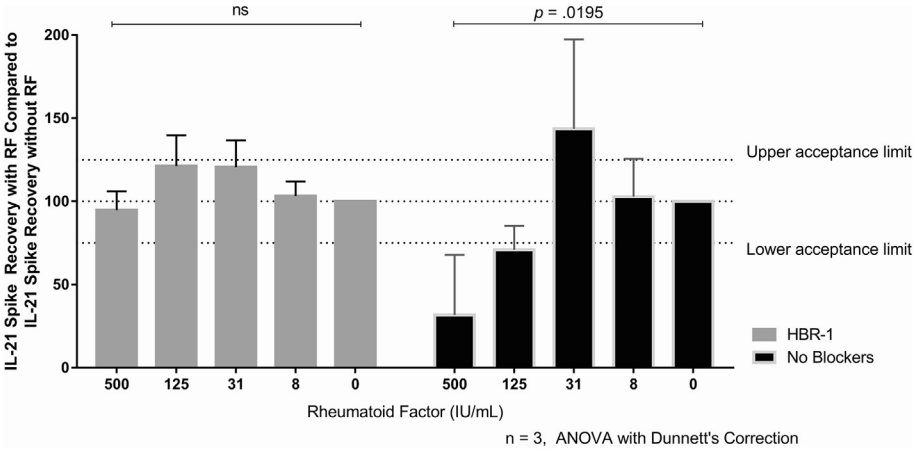

Fig. 4. Percent Recovery of a 100 fg/mL spike is not affected by Rheumatoid Factor using the Quanterix SiMoA Platform. Heterophilic blockers (HBR-1), present in the IL-21 assay buffer, enable acceptable spike recoveries in the presence of up to 500 IU/mL of RF in the Quanterix SiMoA assay. Results are medians from three independent donors.

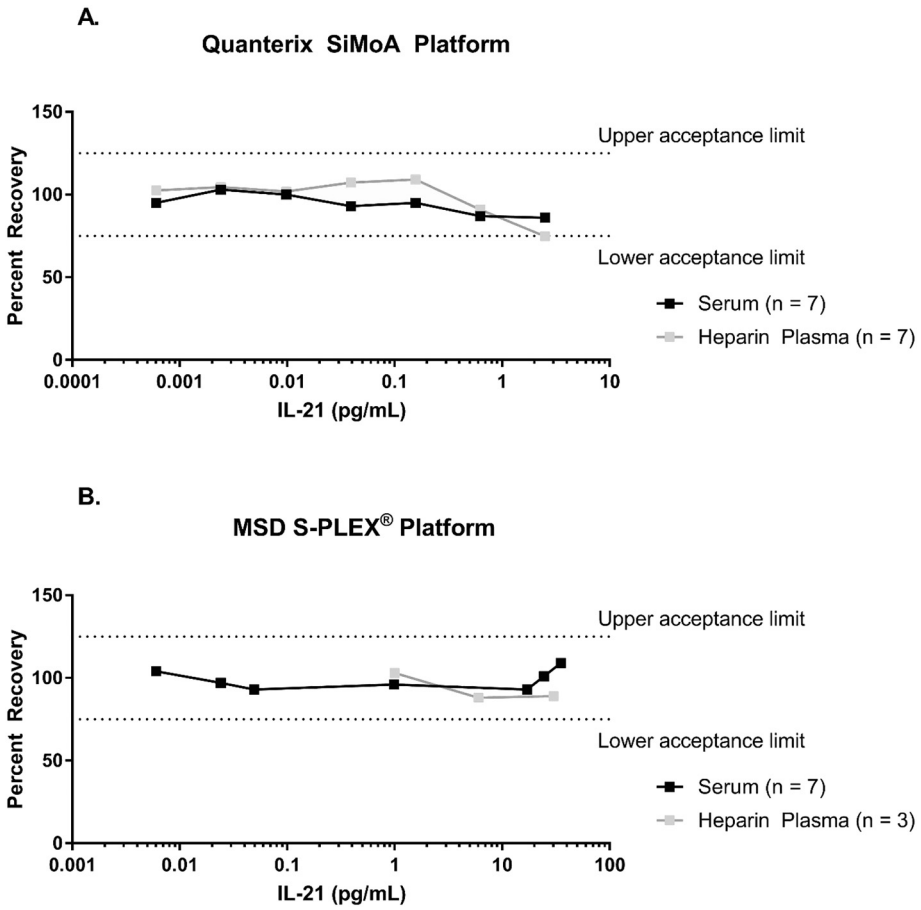

Fig. 5. Dilutional linearity and spike recovery of the IL-21 assays in pooled healthy donor matrices. A. IL-21 spike recovery data for the Quanterix SiMoA IL-21 assay can detect fg/mL levels of IL-21 with a large dynamic range (0.0006–5 pg/mL; LOD of 1.0 fg/mL). B. IL-21 spike recovery data for the MSD IL-21 S-PLEX® method has a LOD of 1.0 fg/mL IL-21 and a range from 0.012–50 pg/mL.

in understanding how cytokines may contribute to disease pathogenesis in a research setting, they also may play a critical role in the future by allowing monitoring of progression of disease or in patient stratification at the onset of treatment. Although there is some conjecture about the relevance of fg/mL levels of cytokines in peripheral blood samples, data from ultrasensitive assays for prostate specific antigen quantitation, a biomarker of cancer recurrence for which fg/mL sensitivity is achievable, may hold positive benefits for patients by allowing earlier detection of disease or disease recurrence and the opportunity to start therapies sooner (Schubert et al., 2015).

For this report, instead of attempting to optimize the commercial assays with novel buffers and reagents, we felt a lack of sensitivity was a significant issue and therefore developed two orthogonal ultrasensitive methods for quantitating IL-21 in human heparin plasma and serum

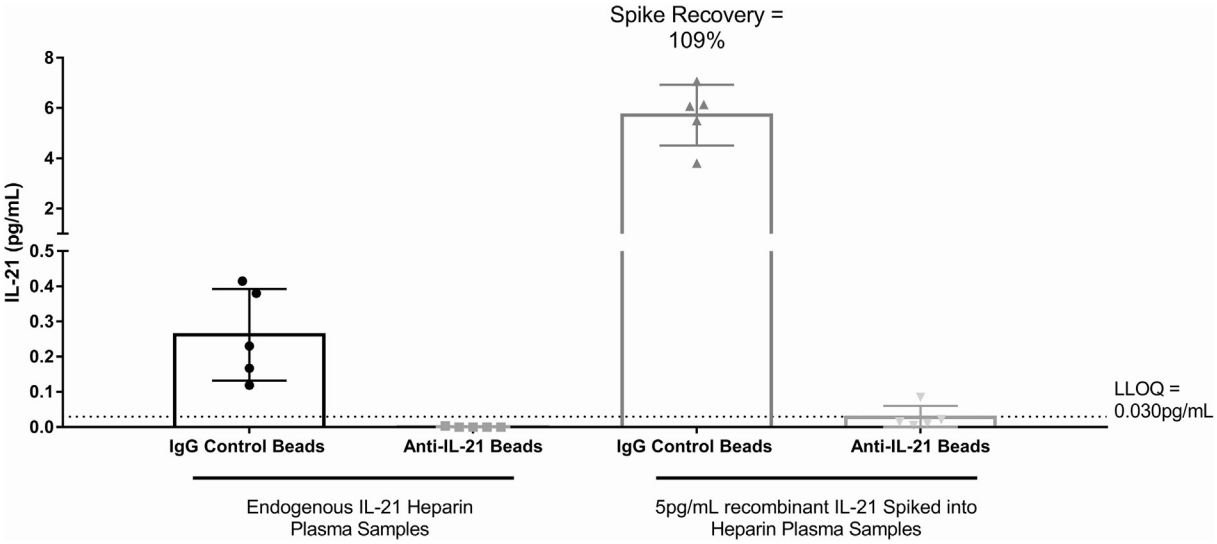

Fig. 6. Specificity testing via immunodepletion of IL-21 from individual healthy donors using the Quanterix SiMoA Platform.
Endogenous IL-21 was removed to below Lower Limit of Quantitation (LLOQ) in five individual healthy donor plasma samples following immunodepletion when using an anti-IL-21 monoclonal antibody conjugated to magnetic beads. Heparin plasma samples spiked with 5 pg/mL recombinant IL-21 were also depleted using the same system, to below the LLOQ of 0.030 pg/mL. Results are mean ± SD of 5 healthy donors.

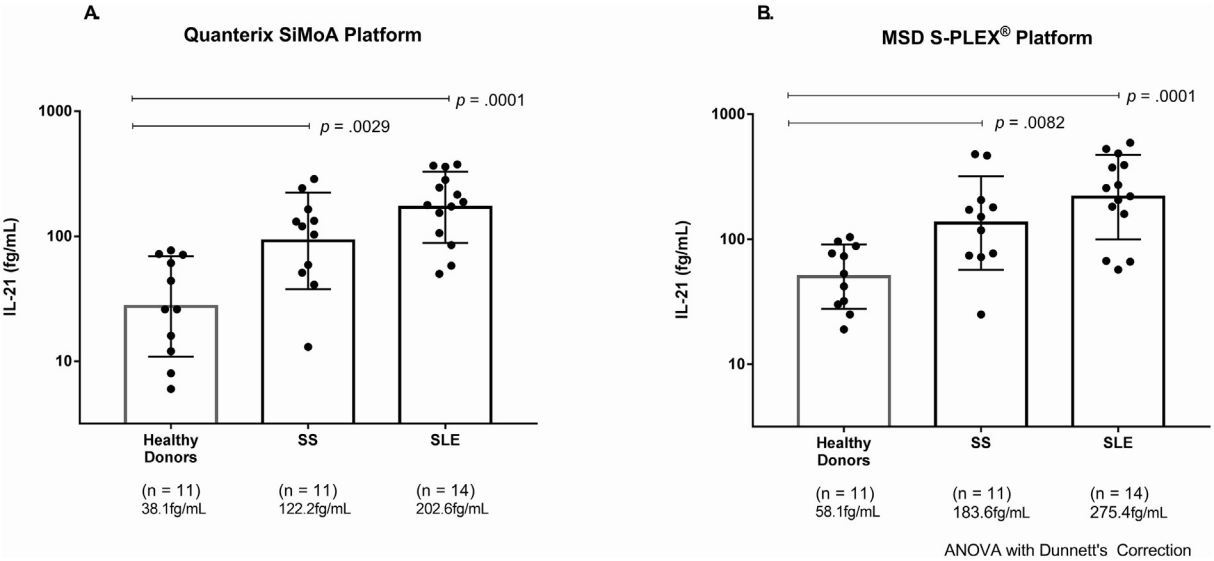

Fig. 7. Determination of IL-21 in Heparin plasma samples from patients with SLE and SS compared to healthy donors.
IL-21 levels were determined using the (A.) Quanterix SiMoA assay and the (B.) MSD S-PLEX® in heparin plasma samples from healthy donors (n = 11), SS (n = 11) and SLE patients (n = 14). SLE and SS patient samples were significantly elevated using either IL-21 ultrasensitive method.

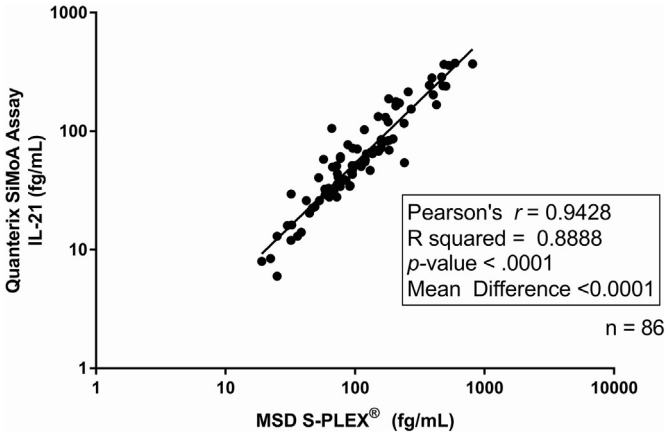

Fig. 8. Correlation of IL-21 concentrations in healthy donor samples using the MSD S-PLEX® Technology and Quanterix SiMoA Platform.
Eighty six heparin plasma samples were analyzed by both the Quanterix SiMoA and MSD S-PLEX® methods for IL-21 levels and added to the values previously obtained in the patient cohorts. There was significant agreement between the two methods (Pearson's r = 0.9428, p < .0001).

samples using proprietary antibodies. While each of these methods are still for research use only and not yet validated to standard Good Laboratory Practice specifications, both methods demonstrated statistically elevated IL-21 levels in samples from SLE and SS patients compared to matched healthy donors. These initial results warrant further analysis is larger, more well-characterized, patient cohorts along with future studies including a more in-depth statistical validation for each method to arrive at a more robust limit of detection and lower limit of quantitation. However, while the SiMoA digital ELISA technology and the MSD S-PLEX® differ in plate set up, sample volume requirements, methodologies, and readout, the two technologies demonstrated highly correlated results in a larger sample that included many healthy donors. While the concentration of IL-21 obtained with the MSD S-PLEX® assay were about two-fold higher overall compared to the Quanterix SiMoA assay with mean difference $p$value of < .0001, both methods were equally able to distinguish patient populations from healthy donors. The observation that both methods are correlated, but fail to have agreement in terms of absolute fg/mL concentrations, indicates that even in well-characterized assays using similar or even the same reagents, technology platform differences could contribute to a different final number. As such, when assays are used to determine a cut-point for clinical decision making, a single method needs to be utilized. For our analysis, either method, therefore, is appropriate for further characterization of IL-21 levels in patient cohorts, making comparisons on absolute values within a given platform.

Since ultrasensitive assays detect minute amounts of analyte, interference by known substances that can alter measurements must be evaluated. RF is one such compound and has a well-documented interference in a variety of ELISA results, in an analyte and assay-specific manner (Tate and Ward, 2004; Ismail et al., 2002). It is present in plasma samples from almost all SS and > 40% of SLE patients (Oxford Handbook of Clinical Medicine, 2014). Subsequently, it was important that the Quanterix SiMoA assay demonstrated no appreciable effect on spike recovery up to 500 IU/mL of RF. This information provides more confidence that the low values measured using this ultrasensitive technology are reliable for further analysis and characterization of patient subsets. In fact, ultrasensitive methodologies as a whole may provide a more robust platform to assess complex biological matrices with interfering analytes due to their enhanced sensitivity and allowance for higher dilutions of samples prior to analysis. RF concentrations can exceed 500 IU/mL but only in a small minority (< 5%) of patients, usually rheumatoid arthritis (Ismail et al., 2002), therefore, future studies could look for interference beyond what was assayed in this study or dilute the sample as appropriate. An effect of RF on the MSD S-PLEX® was not evaluated in this study, but future work could also include this assessment to see if this may account for the small differences observed in our sample sets between the two methods.

It is noteworthy that the IL-21 concentration in peripheral blood samples measured using our ultrasensitive methods was much lower than previously published. Awareness of the actual levels of IL-21 is important given its role in autoimmune disorders. Accurately quantitating IL-21 may provide diagnostic or prognostic advantages that existing assays cannot offer. For example, Song et al. (Song et al., 2016) reported that an ultrasensitive Quanterix SiMoA assay for *C. difficile* toxins A and B in stool samples was used to lower the clinical cut-off to determine patient infections status (Song et al., 2015). In our context, an assay that specifically and accurately detects IL-21 in the fg/mL range could be used to determine novel disease-specific clinical diagnostic cutoffs (e.g. measurement for trial inclusion criteria, and patient

tailoring,) for potential IL-21 antagonist therapies. Additionally, recombinant IL-21 has already been used as a single agent or in combination therapies for cancers, such as metastatic renal cell carcinoma (mRCC) (Bhatia et al., 2014) and metastatic melanoma (Petrella et al., 2012). Since commercial methods of determining levels of IL-21 likely overestimated the concentrations of IL-21, investigators in these studies may have misidentified the pharmacological levels in the patient (Hashmi and Van Veldhuizen, 2010). In addition, some of the adverse toxicological findings observed in these studies may have been obfuscated due to inaccurate measurements of the cytokine using assays with incomplete specificity as exemplified here. Therefore, ultrasensitive assays for IL-21 may provide critical information concerning the endogenous levels of the protein in healthy and patient samples.

In conclusion, two ultrasensitive methods were developed and characterized for the determination of human IL-21 in heparin plasma and serum samples. These assays will be useful to accurately quantitate IL-21 samples in future autoimmunity, cancer and viral infection studies and may open the doors to new insights into clinical cut points and diagnosis.

5. Disclosures

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.12.005.

References

Bhatia, Shailender, et al., 2014. Recombinant interleukin-21 plus sorafenib for metastatic renal cell carcinoma: a phase 1/2 study. Journal for Immunotherapy of Cancer 2.1, 2.

Chang, Lei, et al., 2013. Simple diffusion-constrained immunoassay for p24 protein with the sensitivity of nucleic acid amplification for detecting acute HIV infection. J. Virol. Methods 188 (1), 153–160.

Cox, K.L., Devanarayan, V., Kriauciunas, A., et al., Immunoassay Methods, 2012 May 1. In: Sittampalam, G.S., Coussens, N.P., Brimacombe, K. (Eds.), Assay Guidance Manual [Internet]. Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD) [Updated 2014 Dec 24]. 2004-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK92434/.

Eklund, Emma, et al., 2017. Serum complexed and free prostate specific antigen levels are lower in female elite athletes in comparison to control women. F1000 Research 6.

Ettinger, Rachel, et al., 2005. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. J. Immunol. 175 (12), 7867–7879.

Findlay, J.W.A., et al., 2000. Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. J. Pharm. Biomed. Anal. 21 (6), 1249–1273.

Gavala, Monica L., Smith, Dirk, 2016. Evaluation Of Th2 And Th17 Cytokine Levels In Serum From Asthmatics Using The High Sensitivity Quanterix Simoa Hd-1 Platform. A38. DIAGNOSTIC MARKERS OF ASTHMA AND COPD. American Thoracic Society, pp. A1428.

Gottenberg, Jacques-Eric, et al., 2012. Serum IL-6 and IL-21 are associated with markers of B cell activation and structural progression in early rheumatoid arthritis: results from the ESPOIR cohort. Ann. Rheum. Dis. 71 (7), 1243–1248.

Hashmi, Mehmood H., Van Veldhuizen, Peter J., 2010. Interleukin-21: updated review of phase I and II clinical trials in metastatic renal cell carcinoma, metastatic melanoma and relapsed/refractory indolent non-Hodgkin's lymphoma. Expert. Opin. Biol. Ther. 10 (5), 807–817.

He, Z., et al., 2012. Elevated serum levels of interleukin 21 are associated with disease severity in patients with psoriasis. Br. J. Dermatol. 167 (1), 191–193.

Ismail, Adel A.A., et al., 2002. Interference in immunoassay is an underestimated problem. Ann. Clin. Biochem. 39 (4), 366–373.

Kang, Kwi Young, et al., 2011. Impact of interleukin-21 in the pathogenesis of primary Sjogren's syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. Arthritis Research & Therapy 13.5, R179.

Korn, Thomas, et al., 2007. IL-21 initiates an alternative pathway to induce proinflammatory TH 17 cells. Nature 448 (7152), 484.

Kotlarz, Daniel, et al., 2014. Human IL-21 and IL-21R deficiencies: two novel entities of primary immunodeficiency. Curr. Opin. Pediatr. 26 (6), 704–712.

Kuchen, Stefan, et al., 2007. Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. J. Immunol. 179 (9), 5886–5896.

Lüthje, Katja, et al., 2012. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. Nature Immunology 13.5, 491.

Mizutani, Hiromi, et al., 2016. Serum IL-21 levels are Elevated in Atopic Dermatitis Patients with Acute Skin Lesions. Allergology International.

Nguyen, Vinh, Siaton, Bernadette, Rus, Violeta, 2009. IL-21 is increased in CD4+ T cells and serum from patients with systemic lupus erythematosus (99.36). J. Immunol. 182 (1 Supplement), 36–99.

Niu, Xiaoyin, et al., 2010. IL-21 regulates Th17 cells in rheumatoid arthritis. Hum. Immunol. 71 (4), 334–341.

Nurieva, Roza, et al., 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature 448 (7152), 480.

Nurieva, Roza I., et al., 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 29 (1), 138–149.

Oxford Handbook of Clinical Medicine (9th ed) 2014.

Ozaki, Katsutoshi, et al., 2000. Cloning of a type I cytokine receptor most related to the IL-2 receptor β chain. Proc. Natl. Acad. Sci. 97 (21), 11439–11444.

Ozaki, Katsutoshi, et al., 2002. A critical role for IL-21 in regulating immunoglobulin production. Science 298 (5598), 1630–1634.

Parrish-Novak, Julia, et al., 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 408.6808, 57.

Peen, E., O. J. Mellbye, and H-J. Haga. "IgA rheumatoid factor in primary Sjögren's syndrome." Scand. J. Rheumatol. 38.1 (2009): 46–49.

Petrella, Teresa M., et al., 2012. Interleukin-21 has activity in patients with metastatic melanoma: a phase II study. J. Clin. Oncol. 30 (27), 3396–3401.

Rissin, David M., et al., 2010. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat. Biotechnol. 28 (6), 595–599.

Rodero, Mathieu P., et al., 2017. Detection of interferon alpha protein reveals differential levels and cellular sources in disease. Journal of Experimental Medicine 214 (5), 1547–1555.

Schubert, Stephanie M., et al., 2015. Ultra-sensitive protein detection via Single Molecule Arrays towards early stage cancer monitoring. Scientific Reports 5v.

Sittampalam, G. Sitta, et al., 2004. HPLC-MS/MS for Hit Generation–Assay Guidance Manual.

Song, Linan, et al., 2011. Single molecule measurements of tumor necrosis factor α and interleukin-6 in the plasma of patients with Crohn's disease. Journal of Immunological Methods 372.1, 177–186.

Song, Linan, et al., 2015. Development and validation of digital enzyme-linked immunosorbent assays for ultrasensitive detection and quantification of Clostridium difficile toxins in stool. J. Clin. Microbiol. 53 (10), 3204–3212.

Song, Linan, et al., 2016. A digital enzyme-linked immunosorbent assay for ultrasensitive measurement of amyloid-β 1–42 peptide in human plasma with utility for studies of Alzheimer's disease therapeutics. Alzheimer's Research & Therapy 8.1, 58.

Tate, Jill, Ward, Greg, 2004. Interferences in immunoassay. The clinical biochemist reviews 25 (2), 105.

Wang, Li, et al., 2014. Increased interleukin 21 and follicular helper T-like cells and reduced interleukin 10+ B cells in patients with new-onset systemic lupus erythematosus. J. Rheumatol. 41 (9), 1781–1792.

Wong, C.K., et al., 2010. Elevated production of B cell chemokine CXCL13 is correlated with systemic lupus erythematosus disease activity. J. Clin. Immunol. 30 (1), 45–52.

Xing, Rui, et al., 2018. Autoantibodies against interleukin-21 correlate with disease activity in patients with rheumatoid arthritis. Clin. Rheumatol. 37 (1), 75–80.

Zeng, Rong, et al., 2005. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J. Exp. Med. 201 (1), 139–148.

Zotos, Dimitra, et al., 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism. Journal of Experimental Medicine 207 (2), 365–378.