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Measuring autoantibodies against IL-17F and IL-22 in autoimmune polyendocrine syndrome type I by radioligand binding assay using fusion proteins

Bergithe E. V. Oftedal [1,2], Olle Kämpe [3], Anthony Meager [4], Kerstin M. Ahlgren [3], Anna Lobell [3], Eystein S. Husebye [1,2], Anette S. B. Wolff [1]

[1] *Institute of Medicine, University of Bergen, 5021 Bergen, Norway*

[2] *Department of Medicine, Haukeland University hospital, 5021 Bergen, Norway*

[3] *Department of Medical Science, Uppsala University, 75185 Uppsala, Sweden*

[4] *Biotherapeutics, The National Institute for Biological Standards and Control, Blanche Lane, EN6 3QG South Mimms, Herts, United Kingdom*

Correspondence to:

Dr. Anette S. B. Wolff

Section of Endocrinology, Institute of Medicine

Haukeland University Hospital

N-5021 Bergen, Norway

Phone: 55973078, Fax 55972950, e-mail: anette.boe@med.uib.no

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Abstract

Autoantibodies against interleukin (IL) -17A, IL-17F and IL-22 have recently been described in patients with autoimmune polyendocrine syndrome type I (APS I), and their presence is reported to be highly correlated to chronic mucocutaneous candidiasis (CMC). The aim of the present study was to develop a robust high-throughput radioligand binding assays (RLBA) measuring IL-17F and IL-22 antibodies, and to compare them with current enzyme-linked immunosorbent assays (ELISA) of IL-17F and IL-22; moreover to correlate the presence of these antibodies to the presence of CMC. Interleukins are small molecules which make them difficult to express *in vitro*. To overcome this problem, they were fused as dimers, which proved to increase the efficiency of expression. A total of five RLBAs were developed based on IL-17F and IL-22 monomers and homo- or hetero dimers. Analysing the presence of these autoantibodies in 25 Norwegian APS I-patients revealed that the different RLBAs detected anti-IL-17F and anti-IL-22 with high specificity, using both homo- or heterodimers. The RLBAs based on dimer proteins are highly reproducible with low inter- and intra-variation, and have the advantages of high throughput and easily standardisation compared to ELISA, thus proving excellent choices for the screening of IL-17F and IL-22 autoantibodies.

Introduction

The majority of patients with autoimmune polyendocrine syndrome (APS I) develop at least two of the three main components autoimmune adrenocortical failure (Addison's disease), hypoparathyroidism [1] , and chronic mucocutaneous candidiasis (CMC) [2, 3]. The underlying cause is mutations in the autoimmune regulator (*AIRE*) gene, inherited in an autosomal recessive manner [4-6]. AIRE is mainly expressed in the thymic medullary epithelial cells where it orchestrates transcription of tissue-specific proteins for presentation to developing T cells [7-12]. Without a functional AIRE, autoreactive T cells are allowed to escape into the periphery where they later on may be activated and give rise to organ-specific autoimmune diseases.

Until recently, the strong propensity for CMC in APS I has remained an enigma. The recent discovery of high frequencies of IL-17A, IL-17F and IL-22 autoantibodies in sera of APS I patients implies that autoantibody-mediated inhibition of crucial elements in the defence against *C. albicans* may cause these infections [13, 14]. IL-17F is essential in host defence against extracellular bacteria and fungi, by inducing production of cytokines, chemokines and hematopoetic factors [15, 16]. IL-22 belong to a class of cytokines with a predominant effect on epithelial cells and provides protection on all outer barriers by directly stimulate innate immune responses [17]. Both IL-17F and IL-22 are active as homodimers, and IL-17F also bind to its receptor as a heterodimer with IL-17A [15, 18, 19].

Th17-expressed cytokines have previously been reported to be up-regulated in several autoimmune diseases [16], in contrast to patients with isolated hereditary CMC, in whom they are down-regulated [20-21]. Studies using peripheral blood mononuclear cells from APS I patients differs in their results regarding the Th17 response towards *C. albicans*. One study

claims that the IL-17A response is up-regulated while the IL-22 response is down-regulated [22], in contrast to the previous report of unaltered IL-17A and decreased IL-17F and IL-22 responses [13].

Autoantibodies against IL-17F and IL-22 are prevalent in APS I, and may be used as a biomarker of the disease itself and of the presence of CMC. As the frequency of anti-IL-17A previously has been reported to be quite low in APS I patients [13], we aimed to develop reliable assays to measure autoantibodies against IL-17F and IL-22.

Material and Methods

Subjects and serum samples

This study included sera from 25 Norwegian APS I patients (mean age 36.1 years, 9 females and 16 males, 21 of 25 diagnosed with CMC). Clinical phenotype and mutational status have been described previously for most of the patients [6]. In addition, 67 healthy blood donors (anonymous, unknown mean age and gender) recruited from Haukeland University Hospital Blood Bank were used as controls to ensure that the range of the assays were satisfying, and to validate individual results between assays. The study was approved by the local ethical committee and all participating patients signed an informed consent before participation.

Expression and subcloning of IL-17F, IL-22, IL-17F:IL-17F, IL-22:IL-22, and IL-17F:IL-22

IL-17F and IL-22 were expressed directly from the pCMV-vector (OriGene, Rockville, MD, US). To synthesize dimers and chimeric proteins, human IL-17F and IL-22 cDNAs in the pDEST15 vector (Invitrogen, Carlsbad, CA, US) were used as PCR templates with primers introducing new restriction-sites (Table 1). The 5' end of the first part of the fusion-protein cDNA was inserted into the vector, using primers with a mutated stop-codon. The procedure

was then repeated with the second cDNA, using primers with the original stop-codon (Table 1). Amplified PCR-products were extracted using a PCR-cleanup kit (Qiagen, Valencia, CA, US). The PCR-products and the recipient plasmid pcDNA3 (Invitrogen) were then incubated with 10 units (U) of NotI and/or XhoI and BamHI, all in buffer D (Promega, Madison, WI, US) in a total volume of 40 µl at 37 °C for four hours. After electrophoresis using a 1% low melting point agarose gel, the products were excised and melted at 70°C for 10 minutes, and used directly in a in-gel ligation reaction consisting of extracted pcDNA3 (1 µl) and digested and extracted PCR-products (3 µl). The ligation reactions were incubated overnight with 10 U T4 ligase and 10 x ligase buffer (New England Laboratories, Ipswich, MA, US) in a total volume of 30 µl at room temperature. The ligase-solutions were subsequently melted at 70°C for 10 minutes and 3 µl transfected into one-shot TOP10 competent cells (Invitrogen) according to the manufacturer's protocol. The constructs used for protein expression of all products were verified by sequencing to ensure no mismatch in the open reading frame.

Radioligand binding assay (RLBA) of anti-interleukins antibodies

Sera were analysed for antibodies against IL-17F, IL-22, IL-17F:IL-17-F, IL-22:IL-22 and IL-17F:IL-22 using *in vitro* transcribed and translated radiolabelled proteins essentially as described by Husebye et al [23]. Each sample was tested in duplicate (about 30 000 cpm radiolabelled protein per well), and the results expressed as index [(cpm sample – cpm negative control / cpm positive control – cpm negative control) x 1000]. Pooled human sera from healthy donors was used as negative control, and an APS I serum with high antibody titer was used as positive control. Two APS I sera were tested three times in order to calculate inter- and intra- assay variability, expressed as coefficient of variation (CV) (CV (%) = (SD / mean)*100). Intra-variability ranged from 1.3 – 6.4% for all assays, and inter-variability from 5.1 - 8.1%, with the exception of the IL-17F monomer assay (35.1%). Sera from 50 and 67

healthy controls were used to set the normal limit for the monomer and dimer assays, respectively (index mean of blood donors + 3 SD).

To investigate the specificity of the assays, non-radioactive IL-17F, IL-22, and ovalbumin (negative control), were added to the different RLBAs as competition agents. In brief, one APS I serum positive for IL-17F and/or IL-22 was pre-incubated for 1 hr with 0.1 μg, 0.2 μg, 0.4 μg and 1 μg recombinant human IL-17F or IL-22 (1335-IL-025/CF and 782-IL-010/CF, R&D Systems, Minneapolis, MN, US) or ovalbumin (A 5503-1G, Sigma-Aldrich, St. Louis, MO, US) before addition of radiolabelled protein; the rest of the assay was then performed as described above.

For each assay, the sensitivity (true positives (TP) / (TP + false negative)) and specificity (true negative (TN) / (TN + false positive)) of anti-IL-17F and anti-IL-22 in detecting CMC were calculated.

SDS-PAGE

To ensure proper expression of the proteins, approximately 200 000 cpm of the five proteins were separated on a 10 % Nu Page Bis-Tris gel (Invitrogen) and calibrated with SeeBlue Pre-Stained Standard (Invitrogen). The gel was dried, exposed overnight on an imaging plate, and labelled protein bands visualised using Fujifilm BAS-2500 phosphoimager (Fuji Medical Systems, Stamford, CO, USA).

ELISA

Nunc maxi sorb immunosorbent 96-well flat-bottomed plates were coated with 100 μL/well of IL-17F or IL-22 (R&D Systems, Minneapolis, MN, US) with at total of 0.1 μg/ml protein

for each well, diluted in PBS and incubated at 4°C overnight. After removing excess antigens by decanting, non-specific binding sites were blocked for 30 minutes at 37°C with 100 µL PBS with 0.1% Tween-20 (P7949 Sigma-Aldrich) and 3% bovine serum albumin (BSA) (A7030 Sigma-Aldrich). Plates were then washed four times with washing buffer (PBS w/ 0.1% Tween-20) and 100 µL patient or blood donor serum samples diluted 1:50 in blocking buffer were added in duplicates to each well and incubated one hour at room temperature with shaking. The plates were washed four more times and alkaline phosphatise conjugated goat anti-human IgG (diluted 1:2000 in blocking buffer) (A3187-1ML, Sigma-Aldrich) was added, followed by a new one hour incubation at room temperature with shaking. After five final washes, anti-protein binding was visualized by adding SIGMAFAST *p*-nitrophenol phosphate substrate solution according to the manufacturer's protocol (Sigma-Aldrich) and absorbance at 405 nm was read after 20 minutes incubation in the dark using a Tecan spectrophotometer (Männedorf, Switzerland). Intra- and inter-variability was 2.9 % and 7.7 % for IL-17F and 1.6 % and 2.4 % for IL-22, calculated as described for the RLBAs.

Statistical Analysis

Correlations between RLBA and ELISA were calculated using the Spearman r test, confidence interval and p-value (Graphpad version 5.02).

Results

Radioimmunoassays (RLBAs)

SDS-PAGE confirmed good expression levels according to estimated molecular weights of the *in vitro* translated and transcribed proteins (results not shown). The dimers regularly incorporated three times more ^{35}S-methionine than the monomers. The autoantibody indices using monomers and dimers of IL-17F and IL-22 in sera from 25 Norwegian APS I patients, and up to 67 healthy controls are shown in Fig. 1. The results were very similar, but the

homodimer assay of IL-17F identified one additional positive serum compared to the heterodimeric IL-17F/IL-22 assay. Conversely, the monomer IL-22 assay identified one additional positive serum compared to the homodimeric IL-22:IL-22 assay and the chimeric IL-17F/IL-22 assay.

Overall, all RLBAs were reliable in autoantibody detection, illustrated by the trend in antibody-titer for each patient analysed against the different antigens (Fig. 2). The antibody indices for IL-22 were in general higher then those of IL-17F, while the fusion-protein of IL-17F and IL-22 gave intermediate index values, reflecting the dual recognition (Fig. 2).

Specificity of the autoantibody detection

To ensure a highly specific autoantibody-detection in all RLBAs, non-radioactive IL-17F or IL-22 were added at different concentrations, together with a patient serum positive for both anti-IL-17F and anti-IL-22 before addition of the corresponding radioactive protein. Full blocking was observed for all assays, while the negative control using ovalbumin did not interfere with antigen-antibody binding (Fig. 3. A-D). In the chimeric assay, addition of either IL-17F or IL-22 gave approximately 50 % reduction in antibody index, while the combination of the two gave full reduction (Fig. 3E). Using a serum positive exclusively for anti-IL-22, full inhibition was obtained adding non-radioactive IL-22, alone or in combination with IL-17F, while no reduction was observed for the competition with only non-radioactive IL-17F (Fig. 3F).

RLBA correlates to ELISA and to previous studies

Forty-eight percent of the patients were positive for anti-IL-17F as measured by ELISA, compared to 36% when the same patient sera were analysed by RLBA (Fig. 4A). The

opposite was the case for anti-IL-22, with a higher detection-rate in RLBA (80 %) compared to 72 % in ELISA (Fig. 4B). Despite these differences, the correlation between the different RLBAs (index-values) and the ELISAs (A_{405}) were still satisfying, described by Spearman's r, confidence interval and p-values (Table 2).

Autoantibodies against IL-17F, IL-22 and chronic mucocutanous candidiasis

Previously, an almost perfect correlation between anti-IL-17F and anti-IL-22 and CMC has been described [13, 14]. Two patients (siblings) included in this study, both of whom had clinically evident CMC at the time of sampling, lacked both anti-IL-17F and anti-IL-22 antibodies. Moreover, three of the four patients included in this study without CMC had autoantibodies against IL-22. Taken together, the sensitivities of anti-IL-17F to detect previous episodes or ongoing clinically evident CMC was up to 70%, while the sensitivities approached 100% in the different assays (see Table 3 for details). Using anti-IL-22, sensitivities were 70-80 %, but specificities were low; around 25% (Table 3). Some uncertainty remains as candidiasis in the oesophagus may not have been detected in all cases.

Discussion

We have here shown that RLBAs based on IL-17F and IL-22 as monomers or dimers are highly specific in their antigen-detection, and discriminate well between positive and negative samples. Dimeric expression enhances incorporation of radioactivity more than the addition of methionine groups, and makes them less susceptible of degradation by the ubiquitin pathway. The increased size may also give a better refolding of the proteins [24]. The heterodimer introduces the possibility of detecting both antibodies in one assay, although without the possibility of discrimination. It also has the advantages of low analysis time, decreased cost and limited amount of patient-material required.

In general, the performance of ELISA varies widely, especially when used for autoantibody detection. ELISA requires more sample-material, is more costly, and has a lower sensitivity and specificity compared to RLBA [25, 26]. Here, using both methods on the same patient-material, we revealed discrepancies; mainly that ELISA gave a higher number of positive patients than the RLBA when measuring the presence of anti-IL-17F. This might be caused by unspecific binding due incomplete blocking or that the three-dimensional conformation of the protein is affected when binding to the ELISA plates. Yet, some inconsistency in the correlation between the methods should be expected, due to the difference in detection-reagent; as RLBA utilises protein-A to bind IgG, while ELISA may use any commercial antibody binding IgG. The robustness of RLBAs makes them easier to standardise compared to ELISA, as reflected by their low inter- and intra variability. Although ELISA gave similar low inter- and intra variances in these assays, the standardisation of this method is challenging from lab to lab, and the small scale of the raw-data makes it problematic to create comparable titer-values.

The need for a robust method is emphasized when looking to previous studies, where large variations are seen for the reported presence of anti-IL-17F [13, 14, 21] (see overview; table 4). The presence of anti-IL-17F and anti-IL-22 measured by RLBA in patients from Swedish and Finland [22] were in consistency with our findings in the Norwegian population. Using ELISA, a higher frequency of anti-IL-17F positive patients was found in the Norwegian, Finnish and Italian cohort [13], compared our study or to other patient-material investigated [14]. The latter study included analysis by multiplex particle-based flow cytometry, where a very high number of patients with autoantibodies against IL-17F were found, both overall and compared to their results from ELISA [13, 14]. The proposed association between the most common Finnish mutation, p.R357X, and autoantibodies against IL-17F and IL-22 can not

explain this variation, as the Finnish patients were included in two of the studies which gave dissimilar results [13, 22]. The fact that patients of the same origin vary in their autoantibody profile due to choice of method, illustrates the need for robust assays which are easy to standardise. Among the methods investigated in different populations so far, RLBA seems to be much more consistent than ELISA in measuring the presence of autoantibodies against IL-17F and IL-22.

Two patients diagnosed with CMC by clinical experts at the time of sampling were negative for autoantibodies against IL-17F and IL-22. This could reflect variation in autoantibody-titers due to the state of infection, or implies that these autoantibodies are without any functional significance in the pathogenesis. The number of patients included in this study is low, but the specificity of the RLBAs in detecting patients with known CMC, propose the autoantibodies against IL-17F to be the best marker for this phenotype, while autoantibodies against IL-22 might serve as a marker for APS I in suspected cases.

A correlation between autoantibodies against the Th17 derived cytokines and CMC seems like a plausible explanation for this phenotype, as these cytokines are essential in the defence against *C. albicans.* Since both CMC, reported as 97 % in both the Finnish and Norwegian group of patients at the age of 30, and IL-22 autoantibodies occur at high frequency in APS I, the association observed [6, 27] may be coincidental. Iranian Jews with APS I have a unique mutation of *AIRE* and are seldom infected with CMC [28]. Analysing this group of patients for IL-17F and IL-22 autoantibodies could thus provide useful information as to the possible causative relationship between IL-22 autoantibodies and development of CMC.

In conclusion, we here propose RLBA based on dimeric proteins as a highly sensitive and specific method for the detection of autoantibodies against IL-17F and IL-22. RLBA is used routinously in the detection of both organ-specific and the newly discovered cytokine autoantibodies against IFN-ω [29] and IFN-α2 and IFN-α8 (unpublished results). The reliability of this method, being highly reproducible with low inter- and intra-variation, makes it easy to standardise and the method is well-suited for high throughput analysis. These properties make RLBA an excellent choice for detecting IL-17F and IL-22 autoantibodies, and possibly predicting the occurrence of CMC.

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Legends to figures

Figure 1. RLBA-indices of autoantibodies against IL-17F and IL-22 in APS I patients and control samples in five different assays

Titers of autoantibodies against anti-IL-17F and anti-IL-22 are presented as the RLBA indices of APS I patients (APS I) and healthy controls (HC) for the different antigens: IL-17, IL-22, IL-17F:IL-17F, IL-22:IL-22 and IL-17F:IL-22. The limit for positivity for each antigen is marked as horizontal bars in the plot.

Figure 2. Variation in RLBA-indices for the different Th17-antigens among the APS I patients

RLBA indices for the different assays for each APS I patient. The mean of healthy controls (HC) is represented as a black line.

Figure 3. Competition assay for each RLBA

Competition assays were preformed using APS I serum pre-incubated with non-radiolabelled protein of IL-17F, IL-22 or both in combination before addition of the respective radiolabelled protein. As a negative control, the APS I sample was incubated with similar concentrations of Ovalbumin, to ensure that the addition of non-labelled protein did not interfere with the antibody-binding (A-E). For the IL-17F:IL-22 assay, an additional patient-sera with autoantibodies against IL-22 alone was included (F). All results were expressed as indices and the positive control without inhibition was set as 100%.

Figure 4. Corresponding RLBA and ELISA values for each APS I patient

Each patient serum was analysed by RLBA and ELISA, and the corresponding values of index (RLBA) and absorbance at 405 nm (ELISA) are shown for anti-IL-17F (upper panel) and anti-IL-22 (lower panel). Both RLBAs represented are based on the results from the dimer proteins of IL-17F and IL-22.

Table 1. Sequences of the primers and their use in making fusion proteins

Primer name	Primer sequence	Used in fusion constructs
IL-17_Fw_BamHI	5' gaag|gatccaattcaccATGacagtgaagaccctggatg	IL-17F:IL-22 / IL-17F:IL-17F
IL-17F_Rv_NotI (- STOP-codon)	5' ctatgcggcc|gcgcttTTTctgcacatggtggatgac	IL-17F:IL-22 / IL-17F:IL-17F
IL-17F_Fw_NotI	5' atagc|ggccgcaagaaaATGacagtgaagaccctggatg	IL-17F:IL-17F
IL-17F_Rv_XhoI (+ STOP-codon)	5' ctatc|tcgagtttcttTTActgcacatggtggatgac	IL-17F:IL-17F
IL-22_Fw_BamHI	5' gaag|gatccaattcaccATGgccgccctgcagaaatc	IL-22:IL-22
IL-22_Rv_NotI (- STOP-codon)	5' ctatgcggcc|gcgcttTTTaatgcaggcatttctcag	IL-22:IL-22
IL-22_Fw_NotI	5' atagc|ggccgcaagaaaATGgccgccctgcagaaatc	IL-17F:IL-22 / IL-22:IL-22
IL-22_Rv_XhoI (+ STOP_codon)	5' ctatc|tcgagtttcttTCAaatgcaggcatttctcag	IL-17F:IL-22 / IL-22:IL-22

:	Marks the position of the restriction-site
__ :	Mark the enhancer position for the forward-primers, and the inserted bases and truncated stop-codon for the reverse-primers
ATG:	ATG and stop-codon (truncated or not) marked in capital letters

Table 2. Correlation values for ELISA with the corresponding RLBAs

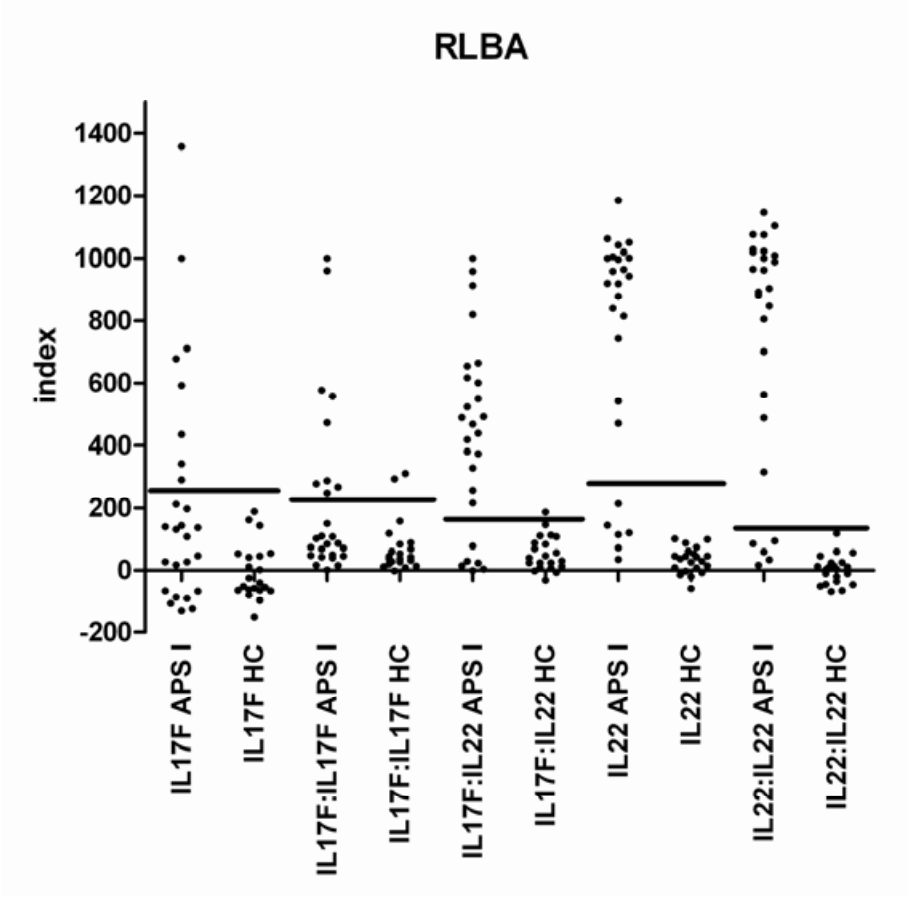
	IL-17F ELISA			IL-22 ELISA		
Parameter	IL-17F RLBA	IL-17F:IL-17F RLBA	IL-17F:IL-22 RLBA	IL-22 RLBA	IL-22:IL-22 RLBA	IL-17F:IL-22 RLBA
Spearman's r	0.7170	0.8049	0.7132	0.7652	0.7252	0.7498
95 % confidence interval	[0.4392, 0.8697]	[0.5928,0.9125]	[0.4329,0.8678]	[0.5214,0.8935]	[0.4527,0.8738]	[0.4946,0.8860]
P-value (two-tailed)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

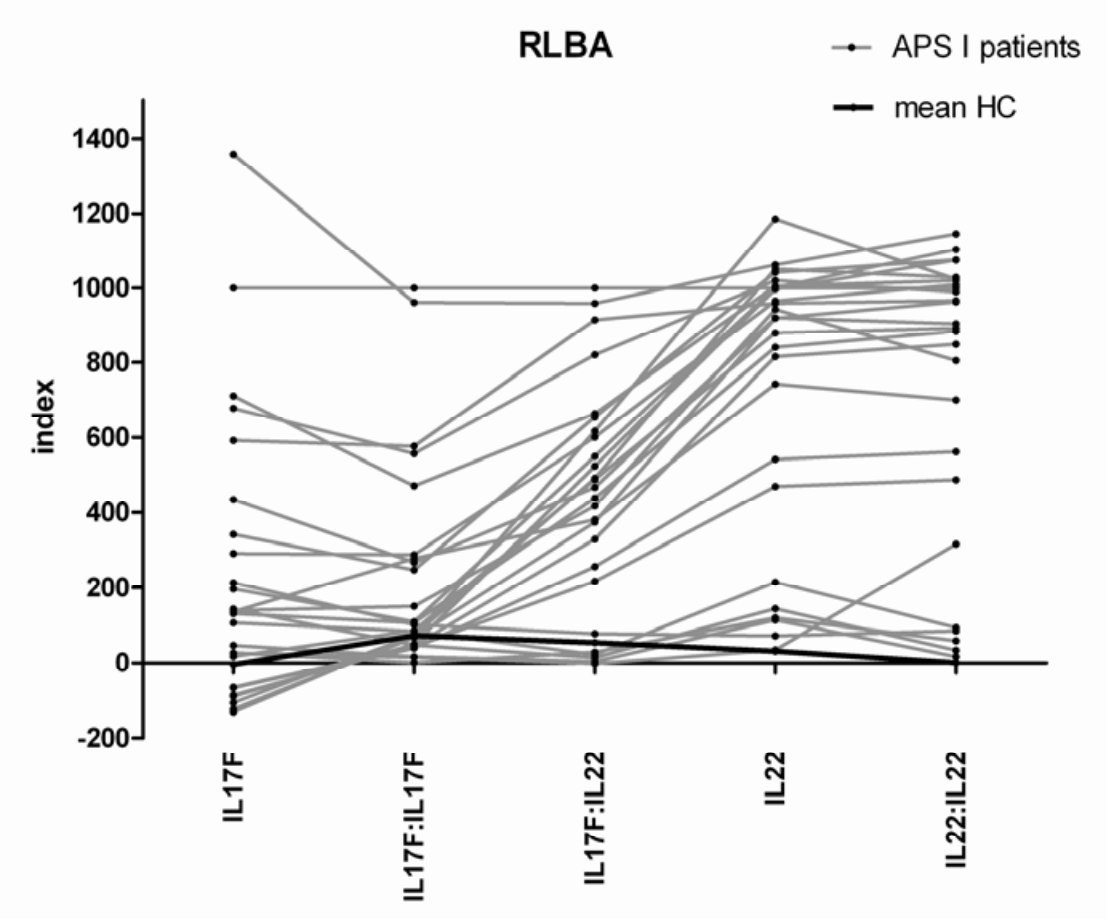
Table 3. Sensitivity and specificity for anti-IL-17F and anti-IL-22 antibodies to detect previous or ongoing clinically evident CMC using different RLBA and ELISA assays

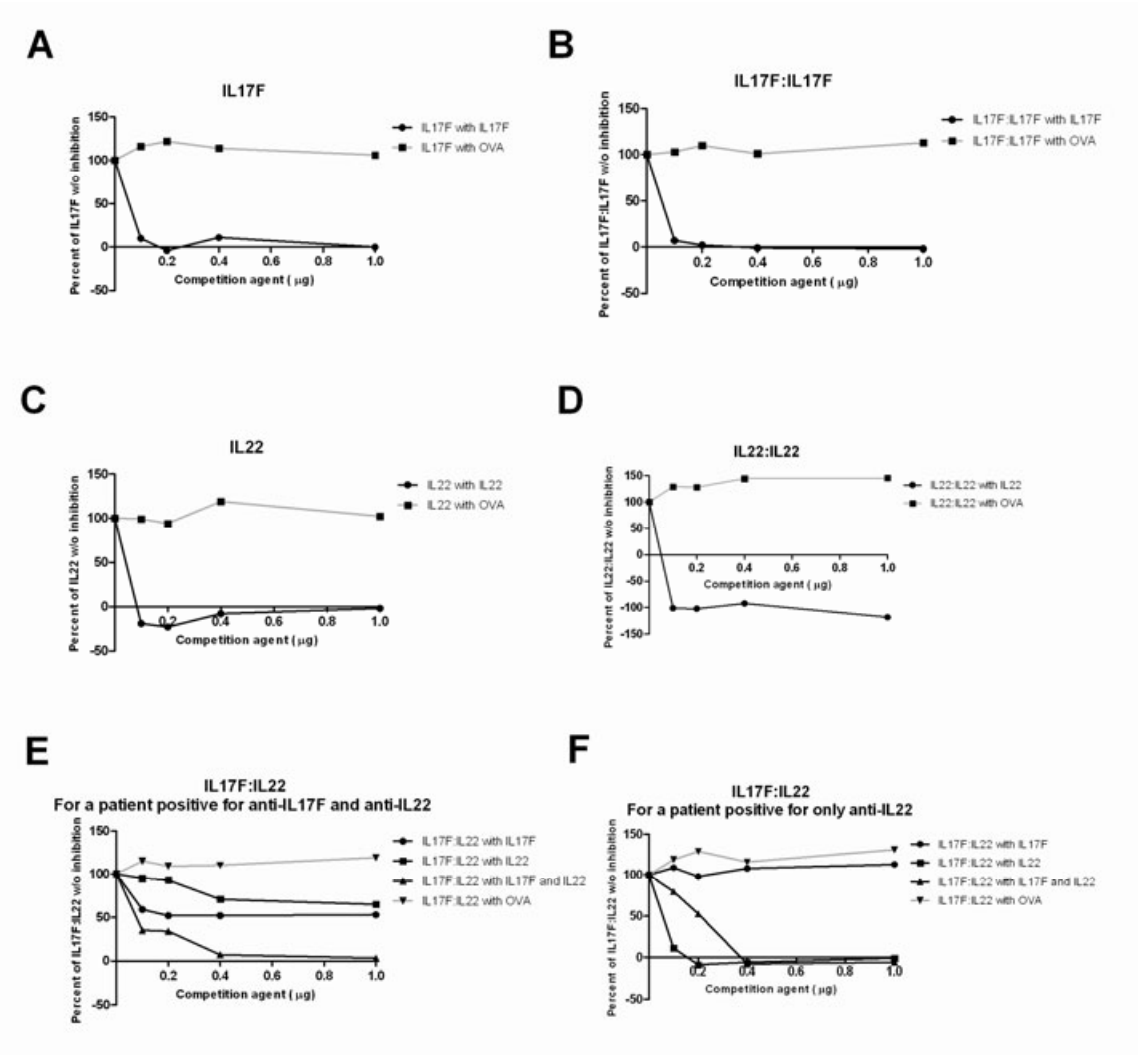
		Sensitivity (%)	Specificity (%)
RLBA	IL-17F	36.4% (8/ 22)	100% (4/ 4)
	IL-22	76.2% (16/ 21)	25% (1/ 4)
	IL-17F:IL-17F	69.2% (9/ 13)	100% (4/ 4)
	IL-22:IL-22	81.0% (17/ 21)	25% (1/ 4))
	IL-17F:IL-22	76.2% (16/ 21)	25% (1/ 4)
ELISA	IL-17F	57.1% (12/ 21)	100% (4/ 4)
	IL-22	71.4% (15/ 21)	25% (1/ 4))

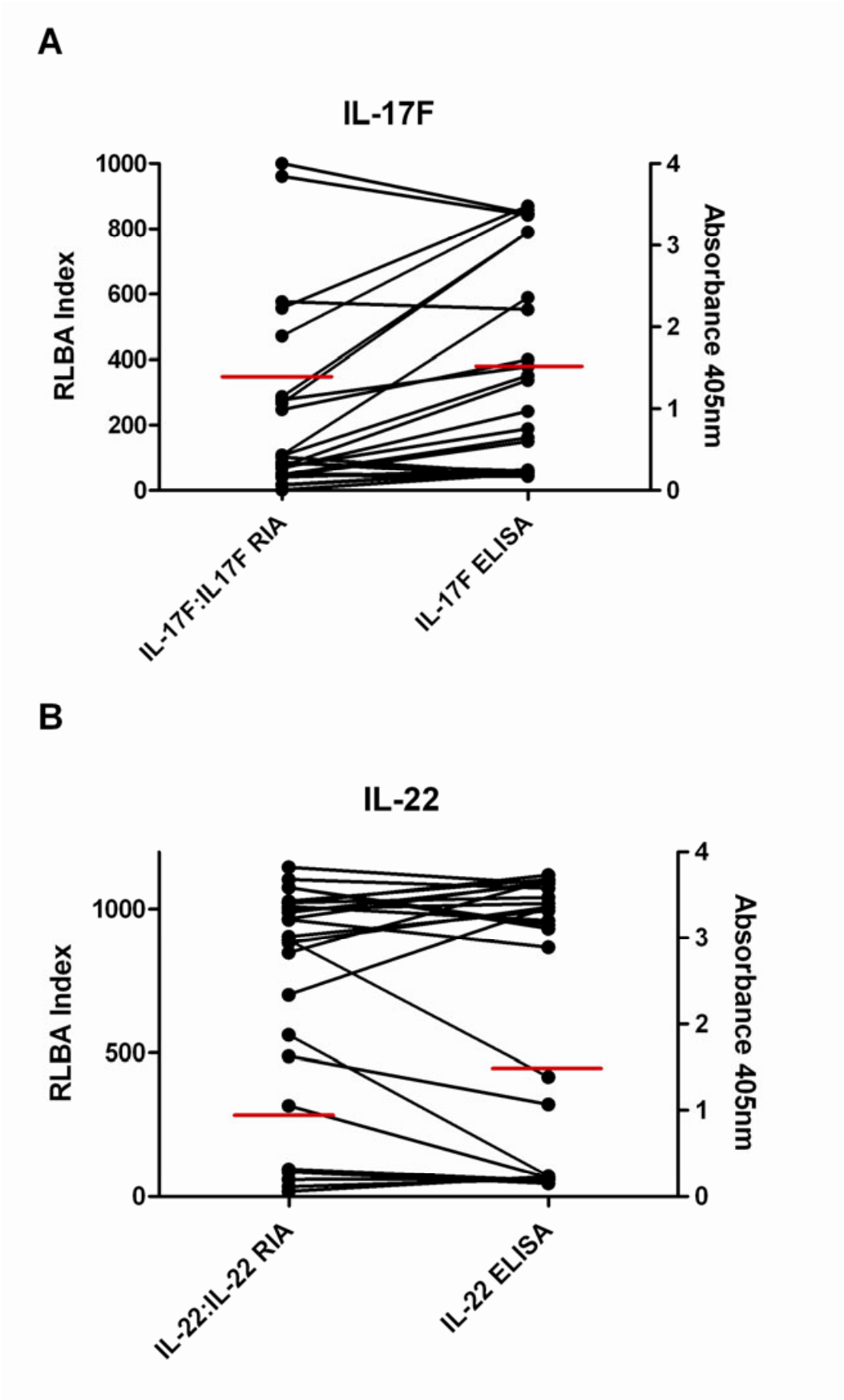
Table 4. Prevalence of autoantibodies against IL-17F and IL-22 using different methods

Method	Auto-Abs against IL-17F, % (n)	Auto-Abs against IL-22, % (n)	Patient origin (Ref)
RLBA (monomer)	32 (25)	76 (25)	Norway (This study)
RLBA (dimer)	36 (25)	80 (25)	Norway (This study)
RLBA	21 (68)	89 (68)	Sweden, Finland (Ahlgren et al 2010)
ELISA	48 (25)	72 (25)	Norway (This study)
ELISA	75 (162)	87 (99)	Italy, Finland, Norway (Kisand et al 2010)
ELISA	33 (30)	40 (30)	Saudi Arabia, Africa, North Africa, France, Ireland, UK, Hungary, Canada (Puel et al 2010)
Multiplex particle-based flow cytometry	94 (33)	91 (33)	Saudi Arabia, Africa, North Africa, France, Ireland, UK, Hungary, Canada (Puel et al 2010)
Neutralization assay	n.d	91 (132)	Italy, Finland, Norway (Kisand et al 2010)


RLBA
index
1400
1200
1000
800
600
400
200
0
-200
IL17F APS I
IL17F HC
IL17F:IL17F APS I
IL17F:IL17F HC
IL17F:IL22 APS I
IL17F:IL22 HC
IL22 APS I
IL22 HC
IL22:IL22 APS I
IL22:IL22 HC


RLBA
APS I patients
mean HC
index
1400
1200
1000
800
600
400
200
0
-200
IL17F
IL17F:IL17F
IL17F:IL22
IL22
IL22:IL22


A
IL17F
Percent of IL17F w/o inhibition
Competition agent (μg)
IL17F with IL17F
IL17F with OVA
B
IL17F:IL17F
Percent of IL17F:IL17F w/o inhibition
Competition agent (μg)
IL17F:IL17F with IL17F
IL17F:IL17F with OVA
C
IL22
Percent of IL22 w/o inhibition
Competition agent (μg)
IL22 with IL22
IL22 with OVA
D
IL22:IL22
Percent of IL22:IL22 w/o inhibition
Competition agent (μg)
IL22:IL22 with IL22
IL22:IL22 with OVA
E
IL17F:IL22
For a patient positive for anti-IL17F and anti-IL22
Percent of IL17F:IL22 w/o inhibition
Competition agent (μg)
IL17F:IL22 with IL17F
IL17F:IL22 with IL22
IL17F:IL22 with IL17F and IL22
IL17F:IL22 with OVA
F
IL17F:IL22
For a patient positive for only anti-IL22
Percent of IL17F:IL22 w/o inhibition
Competition agent (μg)
IL17F:IL22 with IL17F
IL17F:IL22 with IL22
IL17F:IL22 with IL17F and IL22
IL17F:IL22 with OVA


A
IL-17F
RLBA Index
Absorbance 405nm
1000
800
600
400
200
0
4
3
2
1
0
IL-17F:IL17F RIA
IL-17F ELISA
B
IL-22
RLBA Index
Absorbance 405nm
1000
500
0
4
3
2
1
0
IL-22:IL-22 RIA
IL-22 ELISA