Functional and clinical translation of asthma and allergy associated genetic variants in IL33 and IL1RL1

Ketelaar, Marlies

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Chapter 7

The challenge of measuring IL-33 in serum using commercial ELISA: lessons from asthma

M. E. Ketelaar[1,2,3], M. C. Nawijn[2], D. E. Shaw[1], G. H. Koppelman[3,a] and I. Sayers[1,a]

1.Division of Respiratory Medicine, University of Nottingham, Nottingham, UK,
2.University Medical Center Groningen, Department of Pathology and
Medical Biology, Laboratory of Allergology and Pulmonary Diseases, University of Groningen, Groningen, The Netherlands and
3.University Medical Center Groningen, Pediatric Pulmonology and Pediatric Allergology, Beatrix Children's Hospital, Groningen Research Institute for Asthma and COPD, University of Groningen, Groningen, The Netherlands

Abstract

Background

Interleukin-33 (IL-33) has been subject of extensive study in the context of inflammatory disorders, particularly in asthma. Many human biological samples, including serum, have been used to determine the protein levels of IL-33, aiming to investigate its involvement in asthma. Reliable methods are required to study the association of IL-33 with disease, especially considering the complex nature of serum samples.

Objective

We evaluated four IL-33 ELISA kits, aiming to determine a robust and reproducible approach to quantifying IL-33 in human serum from asthma patients.

Methods

IL-33 levels were investigated in serum of well-defined asthma patients by the Quantikine, DuoSet (both R&D systems), ADI-900-201 (Enzo Life Sciences), and SKR038 (GenWay Biotech Inc San Diego USA) immunoassays, as well as spiking experiments were performed using recombinant IL-33 and its soluble receptor IL-1RL1-a.

Results

We show that 1) IL-33 is difficult to detect by ELISA in human serum, due to lack of sensitivity and specificity of currently available assays; 2) human serum interferes with IL-33 quantification, in part through IL-1RL1-a; and 3) using non-serum certified kits may lead to spurious findings.

Conclusion and Clinical Relevance

If IL-33 is to be studied in the serum of asthma patients and other diseases, a more sensitive and specific assay method is required, which will be vital for further understanding and targeting of the IL-33/IL-1RL1 axis in human disease.

Keywords

Asthma, IL-1RL1, IL-33, immunoassay, interference, sensitivity, serum, specificity

7

To the editor

Accumulating evidence suggests that interleukin-33 (IL-33) and its receptor interleukin-1-receptor-like-1 (IL-1RL1, also called ST2) play a critical role in the pathogenesis of asthma. Multiple genetic association studies and meta-analyses have identified several independent polymorphisms in the IL33 and IL1RL1 regions that are associated with human asthma (reviewed in (26,198)). IL-33 is an alarmin released from damaged cells, including the lung epithelium, following allergic, viral or bacterial stimuli (26,251). Extracellular IL-33 induces IL-1RL1 signalling, activating innate and adaptive immune cells, including innate type 2 helper cells (ILC2) and Th2 cells, resulting in increased production of type 2 cytokines IL-4, IL-5, and IL-13 3.

IL-33 is produced as a full length (IL-33FL) 32 kDa protein, which is the active form of IL-33, released upon necrosis of structural cells (26,251). IL-33FL can be cleaved by caspases-1, caspases-3, and caspases-7 at D178, resulting in IL-33$_{179-270}$, which is believed to result in inactivation of IL-33 during apoptosis, a non-immunogenic programmed cell death (252). IL-33FL can also be cleaved by the (neutrophil) enzymes elastase (IL-33$_{95-270}$,) cathepsin G (IL-33$_{99-270}$), and proteinase 3 (IL-33$_{109-270}$), while retaining its activity 4.

When not appropriately regulated, extracellular IL-33 could contribute to chronic or exaggerated type 2 inflammation. A well-described regulatory mechanism of (extracellular) IL-33 is its capture by the soluble form of its receptor (IL-1RL1-a or sST2), thereby preventing the immunogenic activity of extracellular IL-33 (26,252). In asthma, the IL-33/IL-1RL1 pathway seems to be dysregulated. Indeed, IL-33 levels have been reported to be increased in induced sputum, bronchial lavage fluid (BALF), and lung epithelial cells of patients with asthma compared to non-asthmatic controls (51,124). Despite these studies, it is unknown whether serum levels of IL-33 represent a suitable biomarker for asthma or subgroups of asthma.

In this study, we set out to investigate the level of IL-33 in serum of well-characterized patients with asthma, aiming to evaluate its suitability as a biomarker for asthma phenotypes. As this requires a robust method to quantify IL-33 in human serum, we evaluated four human IL-33 ELISA kits including three assays specifically designed for serum samples (Quantikine® by R&D systems Abingdon UK, ADI-900-201® by Enzo LifeScience Exeter UK, SKR038® by GenWay Biotech San Diego USA). We also included an assay for quantification of IL-33 in general biological samples (DuoSet® by R&D systems Abingdon UK). For IL-33 quantification, we obtained serum samples from 45 asthma patients recruited from both Groningen and Nottingham (mild asthma n = 25, moderate asthma n = 10, severe asthma n = 10, based on the GINA 2012 criteria, http://www.ginasthma.org/), of whom n = 17 (mild asthma n = 7, moderate asthma n = 5, severe asthma n = 5) were used across all four methods. Serum was separated from whole blood samples in serum separating tubes using centrifugation after a 20–40 min clotting time at room temperature. Serum was aliquoted and immediately frozen at −80°C. Transportation from the hospitals to the research facility was carried out on dry ice. Stocks were kept at −80°C, while working tubes were stored at

7

−20°C. Care was taken to keep identical freeze/thaw cycles among the comparisons.

Both studies had approval of local medical ethical committees, and patients had given written informed consent. Recombinant IL-33 (from each of the kits) and IL-1RL1-a (sST2; from R&D) were used to determine specificity, sensitivity, and interference of IL-1RL1 in each of the assays.

Using the three kits specifically designed for serum, the level of IL-33 was at or below the lower limit of detection (LLD) in serum from asthma patients (Table 1 and Fig. 1, LLD being the detection limit as defined by each manufacturer). In contrast, the DuoSet showed a clear signal above the LLD in 76% of the samples, and a higher signal in ~17.5% of the serum samples. Indeed, when directly compared to the other assays, the DuoSet assay showed multiple deviating values, some of which were in a tenfold higher range (Fig. 1b). This discrepancy is striking and could not easily be explained by possible confounding factors, such as asthma severity.

To evaluate specificity and sensitivity of the assays, we tested whether these were able to accurately quantify a known concentration of IL-33 when serum was present or in the presence of its soluble receptor IL-1RL1-a. This is important as IL-33 may exist in both free and IL-1RL1-a complexed forms in serum (26,252). Both serum and recombinant IL-1RL1-a interfered significantly with the quantification of IL-33 in two of these ELISA kits (Fig. 1c). The Quantikine kit showed accurate detection of recombinant IL-33 irrespective of serum dilution and the presence of IL-1RL1-a, indicating its technical feasibility of measuring IL-33 in serum, at least up to its LLD. These data strongly suggest that the high IL-33 measurements using the DuoSet kit in our asthma patients are unreliable, as the more sensitive and specific Quantikine kit did not measure IL-33 in any of these serum samples.

The overall lack of IL-33 detection in our serum samples suggests that IL-33 is present at very low levels in serum of these patients limiting our ability to study serum IL-33. Therefore, the activity of the IL-33/IL-1RL1 pathway might specifically be increased in severe asthma or during exacerbations, as suggested by studies correlating IL-33/IL-1RL1 levels with asthma severity (51,124,253), or it may indicate that serum is not the best compartment to study IL-33 levels, as IL-33 protein has previously been detected in BALF of patients with mild and moderate asthma severity (124).

Another explanation for the lack of detection of IL-33 in serum samples would be that the IL-33 present in serum has undergone posttranslational modifications that preclude its detection by the antibodies used in these kits, such as cleavage by cathepsins or elastase. Unfortunately, detailed information on the exact IL-33 epitope detected by the antibodies used in these ELISA kits is lacking to experimentally test this. As far as we are aware, no (proteomics) study has investigated the exact protein isoforms of IL-33 present in human serum, which we think are useful to enable the development of methods to detect specific IL-33 isoforms and to improve the availability of such information.
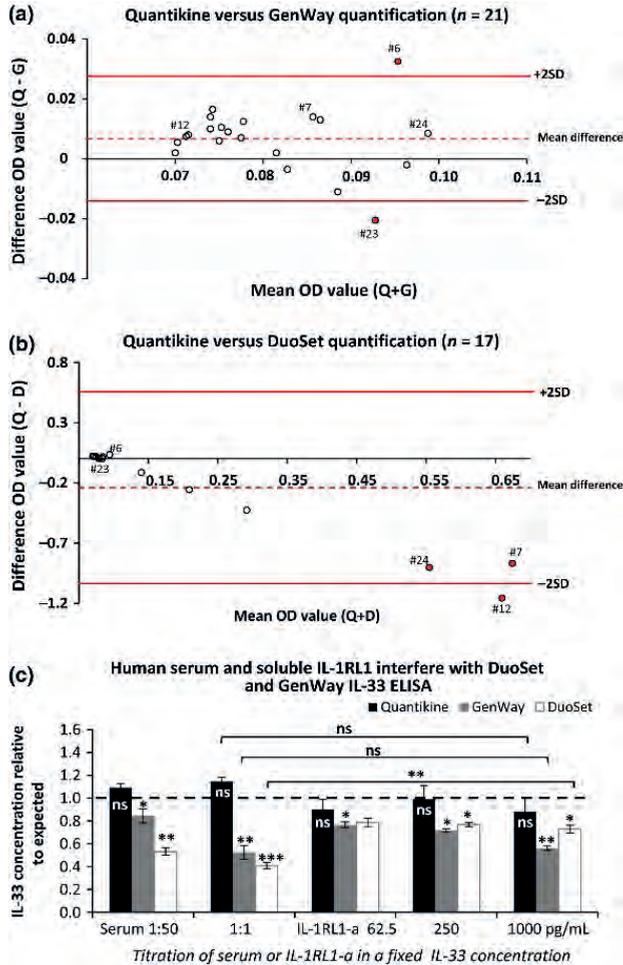
Figure 1.

Comparison of IL-33 immunoassays and identification of potential confounders. The upper Bland–Altman plot [Fig. a] compares optical density (OD) values of the IL-33 immunoassays Quantikine (Q), and GenWay (G), (n = 21); similar data were observed when comparing Quantikine or GenWay to the EnzoLS kit (data not shown, n = 17). The lower Bland–Altman plot [Fig. b] compares the OD values of the Quantikine kit with the DuoSet (D) kit (n = 17), which is representative for the comparison of the EnzoLS and GenWay assay to DuoSet (not shown, n = 17). In the plots, the difference in OD value of each sample as measured by Quantikine versus GenWay (Q-G = Quantikine minus GenWay), respectively, Quantikine versus DuoSet (Q-D = Quantikine minus DuoSet) is depicted on the y-axis. This is set against the average of the OD value for each sample as measured by the two compared kits (x-axis: Q+G= mean OD value of Quantikine and GenWay for each sample, Q+D = mean OD value of Quantikine and DuoSet). Red dots indicate deviating samples (> 1SD from the mean difference). Unique identifiers (#) labelling these deviating samples enable comparison between panels (a) and (b). In Fig. c, the influence of serum or IL-1RL1-a on IL-33 detection is shown. X-axis: Serum (1:50 or 1:1) or IL-1RL1-a (62.5, 250, 1000 pg/mL final concentration) was spiked into a fixed (end) concentration of recombinant IL-33, being the middle of each kits' standard curve (IL-33 concentration of 50 pg/mL, 250 pg/mL, 187.5 pg/mL for Quantikine, GenWay, and DuoSet, respectively). Y-axis: The measured IL-33 concentration is expressed as proportion of the expected IL-33 concentration. Groups are compared by anova, using Games–Howell post hoc testing. Results of the statistical comparison between measured IL-33 concentration and expected IL-33 concentration are indicated, as well as differences in measured IL-33 concentrations after addition of 1000 pg/mL IL-1RL-1a versus addition of 1:1 serum. Note: 1000 pg/mL represents the maximum interfering effect of IL-1RL-1a, as no differences with 2000 pg/mL and 4000 pg/mL were found (P > 0.3 compared to 1000 pg/ml IL-1RL-1a, not shown.) Values are based on duplicate readings of an N = 2–3 experimental replicates. *P < 0.05, **P < 0.01, ***P < 0.001, ns= not statistically significant, dotted line (----) indicates the expected IL-33 concentration set as '1'.

Finally, the low levels of IL-33 detected in serum may be explained by the presence of interfering factors in serum such as IL-1RL1-a, as we find that both serum and recombinant IL-1RL1-a (sST2) interfere with accurate quantification of IL-33 in a dose-dependent manner in the GenWay and DuoSet ELISA kits. Hence, the level of IL-1RL1-a in serum should perhaps be treated as a covariate when using these kits. Nevertheless, as the maximum interfering effect of IL-1RL1-a is less than the effect of serum, at least in the DuoSet (see Fig. 1c), other factors may be present in serum that interfere with accurate detection of IL-33, such as serum globulins and albumins, potentially further complicating the use of these assays.

Other studies in allergic disorders have reported difficulties in detecting IL-33 in serum using ELISA, ranging from zero positive samples (n = 24 allergic rhinitis patients using the Quantikine kit (254)) to detectable values in every sample, but at the lower range of the standard curve (n = 37 wheezing children using the GenWay kit (51)). Furthermore, also in a non-allergic inflammatory disorder (Sjögren), measurement of IL-33 by the currently used immunoassays was shown to be problematic (255). Digital or multiplex immunoassays could be promising novel methods in the measurement of IL-33 (256).

In summary, we show that 1) IL-33 is difficult to detect by ELISA in human serum, due to lack of sensitivity and specificity of currently available assays, 2) human serum interferes with IL-33 quantification in part through IL-1RL1-a, and 3) using non-serum certified kits may lead to spurious findings. We suggest that the data generated in the current study should be taken into account for prospective and retrospective studies of human serum IL-33 levels using these existing kits.

To take home

∞ Currently available immune-assays to detect IL-33 protein in human serum lack sufficient sensitivity and specificity, future studies would benefit from assays that take into account inhibiting factors including serum carrier proteins and soluble IL-1RL1 to accurately quantify IL-33.

7