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Methodological development of cytokine as a biomarker

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Abstract

Biomarker research has rapidly grown in the past few years owing mainly to the advanced development in research tools that provide simultaneous detection of a number of analytes in specific disease states. Cytokines are the polypeptide families released in inflammatory responses that control the immune system and their abnormal levels in the body can lead to multiple diseases. This review provides a detailed and systematic account of the steps in the development of cytokine biomarker. In this regard, serum or blood samples can be obtained from diseased persons and healthy individuals, wherein healthy samples can be regarded as reference standards. After processing according to standard procedures, relative quantitation of analytes can be performed by employing different assays like ELISA or advanced technologies like Luminex multiplex immunoassay and Mesoscale discovery. The statistical analysis can be performed with appropriate software's like Bio-Plex Manager 5.0 and Master Plex QT software's or simply with SPSS. Subsequently, these assays can be validated according to the US Food and Drug Administration guidelines to verify the sensitivity and affinity of biomarker since the development parameters vary in every disease condition. Variations in cytokine levels can serve as a biomarker in certain ailments and careful experimentation paired with advanced technologies can be used for their exploitation.

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Introduction

Cytokines are polypeptides secreted by autocrine or paracrine pathways (Burska *et al.*, 2014) to control immune activity and inflammatory processes (Rang *et al.*, 2011). They have been considered to play major roles in the immune system; however, recent studies have shown that they are produced by and affect the behavior of a range of cells besides the immune cells. The concentration of circulating cytokines is very small (picomolar 10–12 M) however, their amount can increase up to 1,000- fold when essential (Burska *et al.*, 2014). Cytokines are divided into multiple families, including interleukin-1 (IL-1), IL-17, IL-10, IL-6, IL-12, TGF, TNF, PDGF, IFN, beta chain (IL-5, IL-3) and gamma chain (IL-2, IL-7, IL-4, IL-15, IL- 9, TLSP) families, interferons (IFN-α, IFN-β, IFN-γ), chemokines (CC, C, CXC3and CX), tumour necrosis factor, growth factors and colony stimulating factors (Rang *et al.*, 2011; Keustermans *et al.*, 2013). Their levels in the body can be correlated to a number of complications and is thus a candidate biomarker for disease prediction, its prognosis and diagnosis. A biomarker can be defined as an objectively measured and evaluated characteristic that can be used for the assessment of pathogenic processes, normal biologic processes or pharmacological reaction to therapeutics (Dancey *et al.*, 2010).

The purpose of biomarker development is the use of simple, non-invasive tests that can identify any disease state, so that they can be routinely practiced for classification and detection of many diseases (Richens *et al.*, 2010). Cytokine levels can alter in numerous diseases including systemic sclerosis (van Bon *et al.*, 2014), systemic lupus erythematosus (Chun *et al.*, 2007), cancer (Aggarwal *et al.*, 2006), diabetes (Goldberg, 2009), dementia (Chen *et al.*, 2016), multi organ failure (Maier *et al.*, 2007), cardiovascular diseases (Aukrust *et al.*, 2007), rheumatoid arthritis (Khan *et al.*, 2009) and infectious diseases (Garcia-Zepeda *et al.*, 2007). Cytokine biomarker can be used for evaluation of pharmacodynamics such as in the IL-13 targeted therapy of asthma (Ledger *et al.*, 2009), for diagnosis purposes like the differential diagnosis of pancreatic cancer (Shaw *et al.*, 2014), and also for monitoring prognosis of disease like done in the case of lymphomatosis cerebri measuring concentration of IL-10 in CSF (Cerebrospinal fluid) (Hashiguchi *et al.*, 2015). Pro-inflammatory cytokines are also found to be associated with circadian rhythm that alter melatonin and corticosteroids levels and thus can be used to monitor abnormality in cycling (de Jager and Rijkers, 2006). Multiplexing technologies such as Luminex and Mesoscale Discovery are very critical in decoding such disease specific biomolecular patterns and their comparison to ELISA is essential for the purpose of cross-validation (Ashwood *et al.*, 2009; Pokkali and Das, 2009; Richens *et al.*, 2010). Subsequent to successful sample collection, processing, cytokine detection and validation of the assay, cytokines can be used as a successful biomarker for the detection of certain pathological processes and induction into clinical trials. This review examines the available and reported methods of sample collection and processing, cytokine detection and assay validation used to pave the road towards the development of cytokine as a biomarker.

Developmental phases for cytokine biomarker

The major steps involving the development of cytokine biomarkers are candidate selection, sample processing, cytokine detection, validation of assay methods and clinical application as depicted in Fig. 1. The considerations regarding these phases have been discussed further in detail.

Candidate selection

According to the NIH Biomarkers Definitions Working Group, 2001, an immunological biomarker should: (1) have relevance to a disease or pathophysiological process, (2) play a strong role in the biochemical or molecular etiology of the disease, (3) be reliable, (4) be easily used, (5) show practicality, (6) be specific and sensitive to treatment (Metcalf and Orloff, 2004).

Cytokines as candidate biomarkers

Cytokines act as connectors of the innate and adaptive immune systems and alteration in their normal levels can influence auto-inflammatory pathways leading to the progression of certain diseases (Keustermans *et al.*, 2013).

Such a substance that has eminent clinical importance can qualify as a biomarker (Dancey *et al.*, 2010). Cytokines found to be associated with some pathological conditions and their corresponding levels have been given in Table 1; the quantification of cytokine levels is a further step in biomarker development. These altered cytokine levels can serve as diagnostic, prognostic or predictive markers.

Table 1. Pathological conditions, associated cytokines and their reported quantities.

Disease/Condition	Associated cytokines	Cytokine levels (pg/ml)	References
Tuberculosis	G-CSF	1040	(Anbarasu *et al.*, 2013)
	IL-6	7800	
	IL-7	425	
	IL-8	4700	
	IL-9	973	
	PDGF	600	
Dementia	IL-1 beta	0.63	(Chen *et al.*, 2016)
	IL-6	0.42	
	IL-7	0.85	
	IL-8	4.92	
	IL-16	489.37	
	CXCL-10	8.88	
	IL-1 alpha	3.32	
Systemic Sclerosis	CXCL4	25,624	(van Bon *et al.*, 2014)
Systemic lupus erythematosus	IL-6	3.3	(Chun *et al.*, 2007)
	IL-10	8.4	
Chronic Prostatitis	IL-8	15,240	(Penna *et al.*, 2007)
Endometriosis	IL-6	4.41	(Othman *et al.*, 2008)
	CCL-2	37.91	
	INF- γ	19.01	

Sample management and standardization

Collection and handling of samples is pivotal in biomarker development as the mismanagement of samples can considerably change experimental results and generate data that can be contradictory to the biological conditions (Keustermans *et al.*, 2013). Some important aspects regarding sample collection and handling have been discussed as follows.

Sample collection: Plasma or blood cytokines samples can be taken from healthy and diseased persons (Maier *et al.*, 2007; Toedter *et al.*, 2008). Informed consent needs to be filled before sample collection as per the Scientific Committee of Copenhagen and Frederiksberg (Kofoed *et al.*, 2006). The common clotting tubes like those by SST II Advance, BD Bioscience can be used for collecting serum, while sodium heparin (NH), EDTA and sodium citrate tubes can be used for collecting plasma (Shaw *et al.*, 2014). However, on collecting plasma from healthy donors in these tubes, chemokine and cytokines were expressed at higher and lower levels respectively (de Jager *et al.*, 2009). Plasma has been persistently used for the detection of protein biomarker because it contains a variety of disease related proteins (Hu *et al.*, 2006; Farrah *et al.*, 2011).

Whenever possible, non-invasive methods should be preferred over invasive methods, as the collection of non-invasive samples do not require highly professional personnel (Holland *et al.*, 2005). Biopsies of tissues can provide a key demonstration of the tissue related disease processes, however the choice of lysis buffer can affect the level of cytokine concentration (Keustermans *et al.*, 2013; Chen *et al.*, 2016).

Some samples, for example buccal cells can easily be collected by the patient at home and can then be sent to the researcher making studies possible in far away areas (Holland *et al.*, 2005).

Sample storage and management: The collected samples can be processed through numerous methods to obtain serum, red blood cells, buffy coat or blood (whole) can be cryopreserved (Toedter *et al.*, 2008; Zhu *et al.*, 2011). Cytokines' short half-life, release from cell during storage, and the likelihood of degradation during sample management affect the assay measurements and thus makes the standardization of storage time and conditions of proposed specimen types inevitable (Panicker *et al.*, 2007; Dancey *et al.*, 2010). Stability of cytokines is affected by freeze thaw cycles and long term storage. Their stability is nearly two years, but in some cases, cytokines may degrade within a year when they are stored on -80°C (de Jager *et al.*, 2009). No more than one freeze-thaw cycle is allowed in one study (Linkov *et al.*, 2008).

Table 2. Comparison of the validation parameters of different cytokine assay methods; ELISA and Luminex showed high precision while ELISA and Mesoscale Discovery displayed high sensitivity

Assay methods	Validation Parameters		References
	Coefficient of variation (Precision)	Limit of Detection (Sensitivity)	
Luminex	< 25% CV	Up to 3 pg/ml	(Chowdhury *et al.*, 2009)
Mesoscale Discovery	< 25% CV	< 1 pg/ml	(Chowdhury *et al.*, 2009)
ELISA (R&D)	< 10% CV	< 1 pg/ml	(Wang *et al.*, 2005)
LIN Coplex	< 18% CV	3.2 pg/ml	(Wang *et al.*, 2005)
Beadlyte	< 59% CV	Upto 2 pg/ml	(Wang *et al.*, 2005)

The whole sampled blood can be cultured in cases where blood components are not required. For this purpose, true culture syringes can be used. Some results have shown that the cytokine level measured by this system sustain a high degree of stability and these levels were similar when measured in healthy blood donors (Mueller *et al.*, 2012).

Cytokine detection

Cytokine biomarker levels can be checked and quantified by plenty of methods or assays (Keustermans *et al.*, 2013). A relative quantitative assay should be used for such purpose because standard analytes are not available every time in a wholesome characterized form. Samples must be measured at timely period for assays to avoid degradation of cytokines (de Jager *et al.*, 2009). Parameters that should be taken into concern during analysis are range, specificity, affinity, sensitivity, reproducibility and reliability of cytokine because they vary greatly (de Jager and Rijkers, 2006; Bose *et al.*, 2016).

Antibody based arrays

Multiple antibody based arrays are available for cytokine detection and have been successfully used in several biological samples (Srivastava *et al.*, 2006; Shafer *et al.*, 2007; Britschgi and Wyss-Coray, 2009) and biomarker identification (Bafadhel *et al.*, 2009; Miller *et al.*, 2009; Paczesny *et al.*, 2009). Normally, two categories of antibody assays are used; plate-based and bead-based.

Plate based assays

The plate based assays include Sandwich ELISA and Meso Scale Discovery (Chun *et al.*, 2007; Chowdhury *et al.*, 2009; Charbonneau *et al.*, 2012; Keustermans *et al.*, 2013). ELISA makes use of the capture of analytes in the liquid phase by immobilized antibodies in the solid phase.

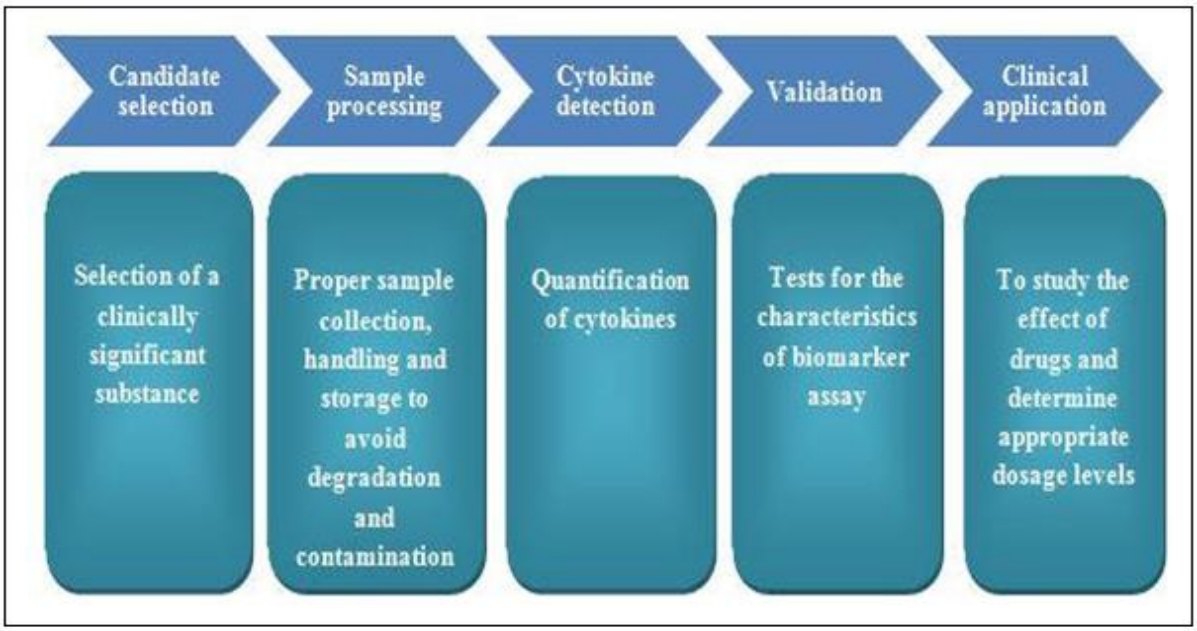

Fig. 1. Phases for the development of cytokine biomarker and their respective purpose.

This binding is measured as a fluorescent or color signal, depending upon the type of substrate employed. The ELISA protocol is highly specific, sensitive, has a wide analytical range and is reproducible (Leng *et al.*, 2008). Mitogen-stimulated whole blood cytokine assays using ELISA can be used for inflammatory cytokine production to test immune cell activation, the detected cytokines are subsequently evaluated by qPCR (Leng *et al.*, 2008; Ryu *et al.*, 2011).

The second plate based assay method is Mesoscale Discovery (MSD) which works on the principle of interaction between electro-chemiluminescent tagged antibody and analyte (Dabitao *et al.*, 2011).

This system employs antibody coated electrode fitted plates. As in sandwich ELISA, the analytes are captured on the electrode and detected by a ruthenium-conjugated secondary antibody. When the electrodes are electrochemically stimulated, ruthenium emits light, allowing the concentration of analyte to be determined for each electrode (Chowdhury *et al.*, 2009). Mesoscale Discovery is highly sensitive and can be used for both qualitative and quantitative analysis, however, it does not differentiate between biologically active and inactive molecules (Keustermans *et al.*, 2013).

Bead based multiplex assays

The underlying principle of bead based assays is same as that of ELISA, the difference lies in the type of surface on which capture antibodies are immobilized. In bead based assays, the antibodies are immobilized on spherical beads rather than a flat surface (Richens *et al.*, 2010). These assay methods encompass multiplex immunoassays (MIA) like Luminex, CBA kit (Richens *et al.*, 2010), Beadlyte by Upstate (Charlottesville, VA), Fluorokine by R&D Systems (Minneapolis, MN), Human Cytokine, Chemokine and Growth Factor Assay Bio-Plex by Bio-Rad (Hercules, CA) and LINCOplex by LINCO (St. Louis, MO) which utilizes interaction of captured antibodies to analytes based on reported markers literature and internal control analytes for validation (Linkov *et al.*, 2008; Khan *et al.*, 2009; Shaw *et al.*, 2014). The bead based methods as a whole have advantages of high sensitivity, high specificity, wide analytical range, rapid detection, reproducibility and use of small sample volume (Keustermans *et al.*, 2013).

Luminex assays based on flow cytometry are the most popular assay method for cytokines and is also the forefront of achieving the parameter goals (Chun *et al.*, 2007; Chowdhury *et al.*, 2009; Charbonneau *et al.*, 2012; Keustermans *et al.*, 2013).

Flow cytometry is a reliable approach since it establishes cytokine production at the single cell level, and has higher specificity (Duramad *et al.*, 2007). Luminex ® xMAP ™ multiplex immunoassay can be performed for both serum assays and EDTA-plasma samples. The Luminex® MAP utilizes differently dyed and antibody coated polystyrene beads. Laser then identifies the bead and analyte's spectral property along with detection of Rphycoerythrin (RPE) labeled secondary antibody (Chowdhury *et al.*, 2009).

Multiple reaction monitoring (MRM)

Another analytical method, a multiple reaction monitoring (MRM) can also be used. Triple-quadrupole mass spectrometer requires a precursor ion (such surrogate peptide used for protein of interest) which is selected by first quadrupole. Another precursor ion (act as protonated integral peptide) is next splitted by the second quadrupole, so that one of the selected fragments is picked by third quadrupole. Then the signal goes to the detector and shows the quantity (Domanski *et al.*, 2012).

Data analysis

For the purpose of quantitation, Bio-Plex Manager 5.0 and Master Plex QT software's can be employed that analyse standard curves obtained from multiplex assays. For EDTA-plasma samples three 1-plex, 5-plex, and 8-plex assays can be used and their quantity can be compared by Pearson correlation coefficients (Kofoed *et al.*, 2006). Analytes that are less than the lower limit of detection and those with no-reading can be detected by using the LLOD [a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator)] for individual samples (Chen *et al.*, 2016). Statistical analysis can also be performed by using the SPSS software (Linkov *et al.*, 2008; de Jager *et al.*, 2009; Anbarasu *et al.*, 2013).

Assay method validation

Validation of any biomarker assay takes into consideration the analytic sensitivity, precision, analytic specificity, inter-along with intra-patient variability in a clinical set-up (Dancey *et al.*, 2010).

Moreover, the validation program prepared by The US Food and Drug Administration guidelines (Food and Administration, 2007) also entails the evaluation of selectivity, LOD (Limit of Detection), linear range, the upper limit of quantification (ULOQ), the lower limit of quantification (LLOQ), precision, stability, complete recovery at room temperature and freezing/thawing stability. It has also been proposed that all combinations of antigens, beads and detection antibodies can be assessed for selectivity. Twofold of the LLOQ concentration can be used for analysis of enriched or non enriched human EDTA-plasma samples. Furthermore, the LOD can be calculated by addition of 3 SD (Standard Deviation) to the mean, median fluorescence intensity (MFI) value of 10 blanks (Kofoed *et al.*, 2006). The recovery of the spiked sample also needs to be within specific ranges that have been predetermined (Toedter *et al.*, 2008). The commercial multiplexing kits used for cytokine detection are highly reliant on the availability of high-quality standard curves (Richens *et al.*, 2010). The validation of such assays and the threshold for acceptance, depends on the stage of development, that needs to be "fit for purpose." Deliberate use of data and the regulatory requirements associated with it, are the considerations of the proposed fit-for-purpose approach towards biomarker method development and validation. Such scheme of development and validation is appropriate for productive biomarker implementation (Lee *et al.*, 2006) wherein, the precision, rather than accuracy can be validated (Mire-Sluis *et al.*, 2004; Lee *et al.*, 2006). Cytokine assays can also be cross-validated by comparing the methodologies available for the quantification of this biomarker (Dancey *et al.*, 2010).

Comparison of validation parameters for different assay methods

ELISA is a well established and validated method of cytokine detection, however the new bead based arrays need to be thoroughly scrutinized for proper validation (Richens *et al.*, 2010). Precision and sensitivity of ELISA, Beadlyte, LINCOplex, Luminex and Mesoscale Discovery have been compared in Table 2.

ELISA was found to have high precision and sensitivity. Mesoscale Discovery showed high sensitivity while LINCOplex was found to have high precision among all techniques (Chowdhury *et al.*, 2009). ELISAs are useful for only few biomarkers verification (Haab *et al.*, 2006) and is expensive and a time taking process (Wang *et al.*, 2009) with limited multiplexing capacities (Krastins *et al.*, 2013), but ELISAs are found to be useful for the final clinical validation assays (Parker *et al.*, 2010; Paulovich *et al.*, 2010). Concentrations obtained in multiplex assays can also be cross validated with ELISA kits (Elshal and McCoy, 2006; Cuellar *et al.*, 2009). Luminex kit results were found to be reliable as opposed to Cytokine bead array when their results were compared with measurements obtained by ELISA (Richens *et al.*, 2010). The antibody array-based biomarker development has the advantage of seamless transition to validation. A certain percentage of biomarkers fail in the validation. However, it is anticipated that the application of new protein chip technologies will accelerate the application of biomarkers into clinical practice (Huang *et al.*, 2012). MRM technique is another detection technique which is identical to ELISA in reproducibility and has low values of coefficients of variation (Addona *et al.*, 2009; Abbatiello *et al.*, 2013; Percy *et al.*, 2014).

Clinical application

Subsequent to successful validation of cytokine assay, the endpoint of biomarker development is the application of these biomarkers in clinics, more simply termed as clinical trials. Biomarkers in clinical trials can be used for the development of therapeutics or the termination of a drug therapy based on the results obtained. The early phase trials involving biomarker need expertise in the required field, funds, infrastructure, considerations on assay properties, scientific rationale and trial design (Dancey *et al.*, 2010).

Conclusion

Cytokines have been related to a number of diseases for instance, cardiovascular diseases, inflammatory diseases and various other infections. Owing to their association with the pathological processes, these inflammatory molecules can be used as a biomarker to monitor diseases.

Standardized sample collection and processing methods are needed to be employed to obtain best results. Decreased freeze thaw cycles, less storage time and non invasive procedures are preferred. A number of quantitative assays exist that can be used according to the disease being studied, the most precise being the Luminex kits. Moreover, validation of these assays is required to assess the validation parameters like precision and accuracy either by cross validation or by making methods, fit to purpose. The cytokine biomarkers can then be implemented in clinical trials to study drug effects. Summing up all, by the employment of standardized sample collection, detection and validation methods, cytokines can be utilized as biomarkers for the detection of certain biological processes in several ailments.

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