Cytokine biosensors: the future of infectious disease diagnosis?

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Why detect cytokines?

Blood carries evidence of infections, making blood analysis the first and often the best means of diagnosis. The evidence of infections is stored within memory immune cells that arise during cellular immune response and remain 'on file' in case disease reappears at a later time. Hypothetically, all infections eliciting cellular immunity leave behind disease (antigen)-specific T- and B-lymphocytes. These cells may therefore be queried to test for previous exposure to infections. The presence of disease-specific lymphocytes is most frequently determined by monitoring production of cytokines – small signaling proteins released by immune cells in response to antigenic stimulation. The best example of this concept is monitoring IFN-γ for diagnosis of tuberculosis (TB).

The methods for direct detection of TB are either time consuming, as is the case with sputum cultures, or expensive, as is the case with x-rays. Therefore, the field relies on indirect measurement of antigenic responses in skin or blood to quickly diagnose latent TB and identify candidates for further analysis. The blood tests, called IFN-γ release assays, are measuring response of antigen-stimulated T cells and are gradually supplanting the traditional tuberculin skin test [1]. IFN-γ release assays are more quantitative and faster than tuberculin skin test, and importantly, are less likely to yield false positive results in individuals vaccinated with Bacille Calmette–Guerin. Beyond detection of IFN-γ, there is a strong interest in profiling other cytokines to discriminate between active and latent stages of TB [2–4]. For example, recent studies have suggested that TNF-α secretion by CD4+ T cells can be used to discriminate active and latent TB [3]. Given the ease of access, disease diagnosis based on blood biomarkers such as cytokines is appealing for diseases other than TB. For example, granulocyte-colony stimulating factor and IL-8 were shown to be possible biomarkers of bacteremic pneumonia in acute pneumonia diagnosis. Both of these cytokines are responsible for enhancing the antibacterial activity of neutrophils [5].

In the case of viral infection, multiple studies have described cytokines as correlates of HIV progression or suppression [6]. In these studies, presence of Th1 cytokines, IFN-γ and IL-2 was associated with suppression of HIV and was observed in long-term nonprogressors, whereas Th2 cytokines were associated with rapid disease progression. Monitoring the dynamics of proinflammatory and anti-inflammatory cytokines may also be important for cytokine-interference therapy for HIV infection, assisting in lowering the viral setpoint in patients [7]. For parasite infection, cytokines also have an appreciable impact: the polarization to Th2 response in severe malaria infection usually results in higher serum levels of TNF-α in conjunction with lower IL-12 and IFN-γ levels. Therefore, serum TNF-α level can assist in predicting fatal outcomes [8].

What cytokine detection tools are available currently?

There are several powerful methods used for detection of cytokines today, including real-time reverse-transcriptase PCR, intracellular cytokine staining coupled with multiparameter flow cytometry, and cytokine ELISA/enzyme-linked immunospot (ELISPOT) assay [9]. Of these approaches, reverse-transcriptase PCR is most sensitive as it amplifies minute quantities of cytokine mRNA; however, this approach requires skilled labor, extensive laboratory infrastructure and expensive reagents. Another commonly used cytokine detection assay is intracellular cytokine staining coupled with flow cytometry. Cytokine secretion of stimulated cells is firstly blocked by inhibitors. Cells are then fixed and permeated to allow the entering of labeled antibodies to specifically recognize accumulated intracellular cytokines. Although this method provides information that matches cytokine production with specific cell phenotype, the results may not accurately reflect the natural cytokine production while the cells are alive. Moreover, this platform may be limited in scenarios where downstream processing of cells is desired. Yet another cytokine detection approach is single cell level ELISA, called ELISPOT, where cells are seeded and stimulated on plates coated with anticytokine antibodies. Once the cells are removed, the signal is developed with an enzyme-conjugated secondary antibody. This step reveals the presence of 'cell footprints' that reflects the number of cytokine-secreting cells. ELISPOT has been widely used for cytokine analysis in TB infection.

What are the new cytokine biosensors being developed?

Biosensor is a term devised by bioanalytical chemists/bioengineers that describes an approach or device that converts a biological event (e.g., antigen binding) into a physical signal. A biosensor is comprised of a biorecognition element (e.g., antibody), a transducer for signal generation and may also contain a cartridge for sample processing and signal enhancement. The bioengineering community has turned its attention to cytokine detection in the past several years and is making interesting contributions on two fronts: enhancing cytokine signal by miniaturizing volumes and devising new biorecognition and signal transduction schemes. Unlike cytokine levels in serum, where detection is only a function of the sensitivity and specificity of the assay, cellular release of cytokines also depends on the assay design – for example, on the volume into which cytokines are being produced. By keeping the same number of cells but minimizing the volume, one can amplify the signal by orders of magnitude. Small objects and small volumes can be made with high precision and reproducibility using microfabrication approaches – a suite of processes borrowed by bioengineers from semiconductor manufacturing [10,11]. Building cell analysis platforms with micrometer-scale dimensions allows isolating single cells for high-throughput cell-by-cell analysis.

In a recent example of this concept, Heath and coworkers created a culture platform comprised of approximately 1000 compartments, each compartment being 3 nl in volume – large enough to house from one to ten cells [12]. Each compartment also contained micrometer-scale stripes of antibodies for several cytokines. This device was used to load CD8 T cells into the compartments and then detect multiple cytokines from single cells, identifying heterogeneity of tumor-specific antigen responses in this T-cell subset [12].

In another interesting example of microfabrication technology applied to cytokine detection, Love and colleagues have developed a microengraving approach where wells large enough to house single cells are molded into a silicone rubber sheet. After cell seeding, the microwells are covered with a glass slide, creating picolitre volume compartments containing single cells. The surface of the glass slide is precoated with anticytokine antibody and may be immunostained to identify presence of the cytokines [13].

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Our laboratory has used microarraying to print antibodies against cell surface antigens adjacent to anticytokine antibodies. These antibody microarrays were printed onto nonfouling hydrogel, encased inside a miniature flow cell called a microfluidic device and then used to capture pure CD4 or CD8 cells as well as several cytokines associated with each subset. Importantly, the cell capture and cytokine sensing could be performed based on an input of whole blood – that is, limited or no upstream blood processing was required [14,15].

What we have described so far are the efforts to redesign the assay so as to decrease the volume of cytokine release assay and to increase proximity of cytokine-producing cells to sensing elements. These approaches do not alter the bottom line – the need to use antibodies for cytokine detection. While sensitivity and specificity of antibodies is unparalleled, the antibody-based detection suffers from several limitations including the need for multiple washing and labeling steps and lack of insight into real-time dynamics of cellular release.

In light of these drawbacks, our laboratory has been pursuing the use of aptamers for cytokine detection. Aptamers are nucleic acids (either RNA or DNA) that are selected to bind specific target molecules such as cytokines and have a number of advantages over antibodies, including lower cost, chemical stability and potential reusability [16]. Most importantly, aptamers can be engineered to produce optically or electrochemically detectable signals upon binding to targets, allowing aptamer-based biosensors that are suitable for real-time cytokine monitoring. In our laboratory, an IFN-γ aptamer labeled with a primary fluorophore was engineered to form a beacon with a complementary strand conjugated with a quenching fluorophore, using a Förster resonance energy transfer-based mechanism. Upon introducing IFN-γ in solution, the IFN-γ will displace the quencher strand, causing the fluorescence signal to increase [17].

In addition to the Förster resonance energy transfer-based optical method, we have also demonstrated electrochemical biosensors for IFN-γ and TNF-α detection. The aptamer molecules were designed to hybridize into a hairpin structure, had a redox reporter molecule attached on one end and were assembled on an electrode surface. The binding of cytokines caused the hairpin structure to unfold and resulted in decrease of electrical current detected at the electrode. In line with the earlier discussion on miniaturization, devices were designed such that aptamer-containing electrodes were located in the immediate vicinity of small groups of T cells. Release of cytokines, IFN-γ and TNF-α, was monitored continuously in near real time by recording electrical signal from the electrode. This approach could be used to detect cytokines released by as few as 15 cells, 20-min postmitogenic stimulation and could be used to acquire hundreds of cytokine data points over the course of hours [18,19].

Moving forward, we envision that the use of cytokines as biomarkers of disease diagnosis or treatment will expand. Novel cytokine biosensors can help spur this trend by making cytokine assays simpler and thus lowering the relatively high adaption threshold of the current cytokine detection methods. In addition, novel technologies will open new avenues in cytokine analysis, for example, by enabling sensitive, single cell level detection of multiple cytokines or by revealing dynamics of cytokine release over time.

References

1   Lalvani A, Pareek M. Interferon-γ release assays: principles and practice. *Enferm. Infecc. Microbiol. Clin.* 28(4), 245–252 (2010).

2   Casey R, Blumenkrantz D, Millington K *et al.* Enumeration of functional T-cell subsets by fluorescence-immunospot defines signatures of pathogen burden in tuberculosis. *PLoS ONE* 5(12), e15619 (2010).

3   Harari A, Rozot V, Enders FB *et al.* Dominant TNF-α+ Mycobacterium tuberculosis-specific CD4+ T-cell responses discriminate between latent infection and active disease. *Nat. Med.* 17(3), 372–376 (2011).

4   Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. *Nat. Rev. Immunol.* 11(5), 343–354 (2011).

5   Kragsbjerg P, Jones I, Vikerfors T, Holmberg H. Diagnostic value of blood cytokine concentrations in acute pneumonia. *Thorax* 50(12), 1253–1257 (1995).

6   Pantaleo G, Harari A. Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. *Nat. Rev. Immunol.* 6(5), 417–423 (2006).

7   Katsikis PD, Mueller YM, Villinger F. The cytokine network of acute HIV infection: a promising target for vaccines and therapy to reduce viral set-point? *PLoS Pathog.* 7(8), e1002055 (2011).

8   Angulo I, Fresno M. Cytokines in the pathogenesis of and protection against malaria. *Clin. Diagn. Lab. Immunol.* 9(6), 1145–1152 (2002).

9   Turlej E. [Novel methods of cytokine detection: real-time PCR, ELISPOT, and intracellular cytokine staining]. *Postepy. Hig. Med. Dosw.* 63, 213–224 (2009).

10 Toner M, Irimia D. Blood-on-a-chip. *Annu. Rev. Biomed. Eng.* 7, 77–103 (2005).

11 Whitesides GM, Love JC. The art of building small. *Sci. Am.* 9, 39–47 (2001).

12 Ma C, Fan R, Ahmad H *et al.* A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. *Nat. Med.* 17(6), 738–743 (2011).

13 Han Q, Bradshaw EM, Nilsson B, Hafler DA, Love JC. Multidimensional analysis of the frequencies and rates of cytokine secretion from single cells by quantitative microengraving. *Lab Chip* 10(11), 1391–1400 (2010).

14 Stybayeva G, Mudanyali O, Seo S *et al.* Lensfree holographic imaging of antibody microarrays for high-throughput detection of leukocyte numbers and function. *Anal. Chem.* 82(9), 3736–3744 (2010).

15 Zhu H, Stybayeva G, Macal M *et al.* A microdevice for multiplexed detection of T-cell-secreted cytokines. *Lab Chip* 8(12), 2197–2205 (2008).

16 Xiao Y, Lubin AA, Heeger AJ, Plaxco KW. Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor. *Angew. Chem. Int. Ed. Engl.* 44(34), 5456–5459 (2005).

17 Tuleuova N, Revzin A. Micropatterning of aptamer beacons to create cytokine-sensing surfaces. *Cell. Mol. Bioeng.* 3(4), 337–344 (2010).

18 Liu Y, Yan J, Howland MC, Kwa T, Revzin A. Micropatterned aptasensors for continuous monitoring of cytokine release from human leukocytes. *Anal. Chem.* 83(21), 8286–8292 (2011).

19 Liu Y, Kwa T, Revzin A. Simultaneous detection of cell-secreted TNF-α and IFN-γ using micropatterned aptamer-modified electrodes. *Biomaterials* (33)30, 7347–7355 (2012) .