Cytokines

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Cytokines are low molecular weight, soluble proteins produced by immunocompetent cells that communicate with other cells to regulate immune function. Cytokines act via specific receptors and, depending on the particular cytokine and the cell that it binds to, they can up-regulate or down-regulate the activity of other immune cells. Most cytokines have short half-lives and are effective locally, acting on cells in close proximity to their release. Thus, many cytokines are only detectable in peripheral circulation in response to pathology. In contrast, a few cytokines are present at measurable levels in the peripheral blood of most healthy individuals. One example is interleukin(IL)-6 which has a longer half life than many other cytokines and actions that are systemic. IL-6 is involved in the regulation of endocrine hormone production and communicating with the CNS. Cytokines are produced by many cell types in addition to cells of the immune system. There are at least 150 different cytokines and many are involved in the pathogenesis of disease. The table below gives some examples of commonly measured cytokines, their immune cell source, some of their targets and actions.

Cytokine	Cell source	Target	Actions
Proinflammatory Cytokines			
IL-1	Macrophage Dendritic cell	Lymphocytes Endothelial cell CNS Liver	Enhances responses Activates Fever, sickness behavior Synthesis and release of acute-phase proteins
IL-6	Macrophage Dendritic cell Endothelium Th2 cell	Liver B cell	Synthesis and release of acute-phase proteins Proliferation
TNF-alpha	Macrophage Dendritic cell Th1 cell	Endothelial cell Neutrophil Hypothalamus Liver	Activates vascular endothelium – increased permeability and stimulates adhesion molecules Activates Fever Synthesis and release of acute-phase proteins
Anti-inflammatory Cytokines			

IL-10	Macrophage Th2	Macrophage Dendritic cell	Inhibits IL-12 production Inhibits pro-inflammatory cytokine synthesis
Il-12	Macrophage Dendritic cell	CD4+T helper cell NK cell	Th1 differentiation IFN-gamma synthesis
Cytokines Involved in the Acquired Immune Response			
IL-2	T cell	T cell NK Cell B cell	Proliferation Activation and proliferation Proliferation
IL-4	Th2 cell Mast cell	T cell B cell Macrophage	Th2 cell development/proliferation Isotype switch to IgE Inhibit IFN-gamma activation
IFN-gamma	Th1 cell Cytotoxic T cell NK cell	T cell B cell Macrophage	Th1 cell development Isotype switch to IgG Activation

Measurement of Cytokine Levels

Cytokine levels can be measured in a number of bodily fluids, including serum, plasma, urine, supernatants, mucus, and saliva. A range of techniques is available to measure cytokine levels, including enzyme-linked immuno sorbent assay (ELISA), multiplex technology, ELISPOT and flow cytometry. Below are basic descriptions of some of these methods.

Enzyme Linked Immuno Sorbent Assay (ELISA)

What will the assay tell us?

The ELISA is the most commonly used immunoassay to quantify cytokine levels. These assays are available in the form of commercial kits and are widely used because of their specificity, sensitivity, ease, and convenience.

For further details regarding this general method, see **"Common Immune Measures"** on www.pghmbc.org.

How are samples collected and stored?

For plasma samples, whole blood should be collected in sterile tubes with anticoagulants, placed on ice immediately, and spun down as soon as possible, preferable within 30 minutes. Many cytokines have a short half-life and begin to degrade once drawn. Small Aliquots (e.g., .5ml) of plasma are pipetted into freezer tubes and frozen in an ultra-cold freezer. For serum samples, no anti-coagulant is used and samples are allowed to clot at room temperature for 30 minutes before spinning. It is best to avoid repeated freeze-thawing of samples.

What type of ELISA should I use?

Many cytokine ELISA kits come in high and low sensitivity versions. These differ in the range of detectable concentrations, with high sensitivity kits reliably detecting lower

concentrations of cytokines. When available, these kits are better able to detect levels of cytokines in unstimulated samples than the low sensitivity kits.

<u>What are the advantages and disadvantages of an ELISA?</u>
High specificity, reliability, and the relatively low cost and ease of use make the ELISA a good option for assessing cytokine levels. ELISA kits permit the assessment of 38 samples run in duplicate and cost around $400-$600 per kit. One disadvantage of this method is the possibility of capturing biologically inactive cytokines and their fragments. Another issue is the detection range of the ELISA. Often cytokines are present at levels too low for detection in healthy individuals. Thus, it is important to examine whether the cytokine falls within the standard curve for the specific ELISA and thus is present at levels that are reliably detectable. The 'coefficient of variability' (CV) provides information on the reproducibility of an assay and should ideally be < 5% for the intra-assay (between duplicates tested within assay) CV and < 10% for the inter-assay (between assay) CV.

<u>Factors to consider in measuring cytokine levels</u>
A number of factors have been demonstrated to be associated with cytokines levels, including age, sex, and race. Many cytokines also have a clear circadian rhythm, so samples should be drawn at the same time of day for comparisons across individuals. Other heath factors that have been related to cytokine levels include smoking, alcohol use, physical activity, genetics, current acute or chronic illness, and medication use. Findings differ depending on the cytokine of interest, but these factors should be taken into consideration in interpreting findings.

Assays for Cytokine Production

In addition to measuring levels of cytokines in samples, whole blood or isolated cell subtypes (e.g., peripheral blood mononuclear cells (PMBCs)) can be stimulated to produce cytokines in vitro. It has been suggested that measures of cytokine production provide a more biologically meaningful measure of cytokines than plasma levels (Whiteside, 1994). Stimulated cytokine production has been demonstrated to be a stable trait among healthy volunteers (e.g., Friberg et al, 1994), making it a possible risk factor in susceptibility to immune-related disease.

<u>What will the assay tell us?</u>
Spontaneous production of cytokines by cells cultured in the laboratory provides a measure of in vivo activation of these cells. Stimulated production of cytokines by cells exposed to antigens in the laboratory provides a measure of immune potential or competence. More than one cytokine is released in response to stimulation, making it possible to examine cytokine arrays. It is also possible to examine the kinetics of cytokine responses to stimulation, by incubating the culture for varying periods.

<u>How does the assay work?</u>
To examine spontaneous production of cytokines, whole blood or isolated cells are incubated for a period in medium and then the culture supernatant is collected and frozen for future determination of cytokine levels using ELISA. The cells for this assay can be harvested using centrifugation with a density separating agent such as ficoll to isolate peripheral blood mononuclear cells, or using flow cytometry or magnetic beads to isolate a specific type of lymphocyte (e.g., a T cell or B cell).

To assay stimulated cytokine production, whole blood or isolated cells are cultured in medium in the presence of a stimulant. The specific stimulant depends on the immune pathway of interest. For example, lipopolysaccharide (LPS), a component of the cell membrane of gram negative bacteria, is frequently used to stimulate the activation of monocytes/macrophages and thus to stimulate the release of pro-inflammatory cytokines. In contrast phytohemagluttinin (PHA) is predominantly a stimulant of T cells. Following incubation with the stimulant, the culture is spun down and the supernatant harvested and stored in an ultra-low freeze for future assay in batches. Levels of cytokines in culture supernatants can be determined using ELISA or multiplex assay.

Multiplex Bead Assay

<u>What is a multiplex bead assay?</u>
Stimulation of blood or isolated cells results in the release of a cascade of biologically-related cytokines. For example, IL-6, TNF-alpha, and IL-1beta are involved in the inflammatory cascade, IL-2, IFN-gamma, and TNF-alpha characterize Th-1 cell activation, and IL-4, IL-5, and IL-10 are associated with a Th2 type response.
A multiplex bead immunoassay permits the simultaneous measurement of an array of cytokines in a single, small volume sample. Using this method, it is possible to profile cytokine responses. The assay measures levels if a number of different cytokines in a single sample.

<u>How does the assay work?</u>
In this assay, the sample is cultured with distinct fluorescently labeled beads, coated with a cytokine-specific antibody. Beads specific for the different cytokines of interest are placed in a single tube with the sample. The capture antibody binds to the cytokine. Biotin-conjugated detection antibody specific for the cytokine of interest is then added, followed by fluorescein- labeled streptavidin. The level of fluorescence bound to the beads is quantified using the Luminex platform. This is a flow analysis system designed to simultaneously measure multiple analytes.

Kits are commercially available and it is possible to select panels of cytokines (e.g., inflammatory cytokines) or to customize one's own panel

<u>What are the advantages and disadvantages of multiplex bead assays?</u>
This technology makes it possible to detect the levels of many cytokines (>10) in a single 50 ul sample. Thus, this technique is cost effective, saves time and offers flexibility. Although it offers a great system for assessing stimulated levels of cytokines, the detection range is not sensitive to circulating levels of many cytokines in healthy individuals, which often fall below the lower limit of detection. In these instances, it is possible that a high sensitivity ELISA may offer greater sensitivity. As with ELISAs, the investigator must explore whether expected levels will fall within the standard range of the kit. To achieve this, stimulated levels samples will often need to be diluted prior to assay. This may be difficulty when cytokines are present at very different levels within a single sample.

ELISPOT

What will the assay tell us?
ELISPOTs provide a different assay for evaluating cell activation following stimulation with an antigen. This assay is used to determine the frequency of cells in a sample that are capable of releasing cytokines following stimulation.

How does the assay work?
The ELISPOT is an enzyme linked-immune assay that is similar to ELISA and measures immune cell secreted cytokine levels in response to in vitro stimulation with an antigen within the well of a plate coated with antibodies specific for the cytokine of interest.

How are samples prepared?
For this assay, cells are isolated from whole blood. This can be performed using centrifugation with a density separating agent such as ficoll to isolate peripheral blood mononuclear cells, or using flow cytometry or magnetic beads to isolate a specific type of lymphocyte (e.g., a T cell or B cell). The number of isolated cells in the sample has to be determined before cells are added to the ELISPOT plate.

How is the assay conducted?
The isolated cells can be stimulated with a specific antigen or mitogen to produce cytokine either before they are added to the ELISPOT wells, or directly while on the plate. Stimulated cells are added to a plate which is coated with capture antibodies. The plate is then incubated for a specific time at a particular temperature, depending on the cytokine examined. During incubation, the cells react with the capture antibodies on the plate and producing a spot or ring of peptide-antibody complexes that reflects both the kinetics and quality of cytokine production by individual cells during the test period. At the end of incubation, the plate is washed, and an enzyme-linked antibody is added that binds to the cytokine. Chromogenic substrate is then added that depicts the cytokine producing cells as colored points.

How are results interpreted?
The number of colored spots produced in the assay reflects the amount of cytokine produced by the cells. Spots are examined for size, density, and morphology to determine kinetics and type of cells secreting the cytokine. For example, small, dense spots reflect the slow stable release of cytokines. In contrast, larger, more scattered spots reflect faster release. Semi-automated and automated methods exist for reading ELISPOT data, using a plate reader and software programs. The researcher is required to define counting parameters, such as spot size. Data is usually reported as number of spots per a specific number of cells.

What are the advantages and disadvantages of ELISPOT?
ELISPOT offers information about the frequency of specific cells that are activated and producing cytokines in a sample. As with ELISA, a limitation is the possibility of detecting non-biologically active cytokines. They provide a more specific measure of immune function than ELISAs. However, ELISPOT assays are substantially more expensive than ELISAs.

Flow Cytometry Techniques for the Measurement of Extracellular and Intracellular Cytokines

What will the assay tell us?
Flow cytometry can be used to assess cytokines levels in both blood and within single cells.

How does the assay work?
To measure intracellular cytokines, cells are treated so that their cell membranes become permeable, allowing antibody to reach the cytokine inside the cell. This enables the staining of intracellular cytokines. In this technique, cells are treated with a protein transport inhibitor, then fixed and one of a number of methods is used to make the cell more permeable. The cell is then stimulated and exposed to labeled anti-cytokine antibodies that bind to the fixed intracellular cytokines. Flow cytometry is used to quantify cytokines within single cells (See **"Common Immune Measures"** at www.pghmbc.org for further details regarding flow cytometry).

To measure cytokine secretion by single cells, a number of commercial assays are now available. Whole blood or isolated cells are first stimulated by incubation with antigen. Cells are then exposed to a conjugate of anti-CD45 and anti-cytokine antibody. This attaches to the surface of leukocytes and then "catches" the specific cytokine if it is released by the activated cell. Cells are then exposed to a cytokine-specific detection antibody labeled with a fluorochrome which attaches to cytokine-producing cells. The level of fluorochrome is quantified by flow cytometry.

What are the advantages and disadvantages of these flow cytometry methods?
These methods provide a highly sensitive method for determining antigen-specific, cytokine secreting cells. Benefits of this approach include quantifying not only the percentage or number of certain cytokine-producing cells in the sample but also the specific cell type, and the expression of other related molecules.

References

For a general review of cytokine measurement assays, see the following:

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