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Assessing the Immune Status of Critically Ill Trauma Patients by Flow Cytometry

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Abstract

Background—Unintentional injury or trauma remains the leading cause of death among young adults. About one fifth of these trauma patients require care in an intensive care unit (ICU) due to severity of injuries and comorbidities. Patients hospitalized in an ICU are at increased risk for nosocomial infections, such as urinary tract infections, pneumonia, bacteremia, and wound infections. Many of these patients will develop sepsis or septic shock, and some will progress to multiple organ failure (MOF) and death. The balance between the pro-inflammatory and counter-inflammatory immune response appears to be a driving factor in this progression. At present, there is no proposed method for the timely detection of the immune status in trauma patients; making rational decisions to use immune-altering therapies difficult.

Objective—We demonstrate that flow cytometry, with its capabilities to characterize and/or enumerate (a) leukocyte subtypes; (b) leukocyte activation markers; (c) leukocyte-derived cytokines and microvesicles; and (d) leukocyte function is well suited to monitor the immune status of critically ill trauma patients.

Methods—Information for the review was obtained from the extant literature.

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Discussion—We suggest that flow cytometry is a research method that might aid nurse scientists in investigating the immune status of critically ill patients, the recovery status of conditions—such as hemorrhagic shock and tissue injury—and the relationship between cancer disease progression and symptoms. Therefore, flow cytometry has the potential to broaden nursing research priority areas so that a comprehensive approach to understanding the cellular response is attained.

Keywords

cytokines; flow cytometry; inflammation; microvesicles; monocytes; neutrophils; T cells

Unintentional injury (trauma) remains the leading cause of death among young adults age 15–44 in the U.S. (Corso, Finkelstein, Miller, Fiebelkorn, & Zaloshnja, 2006). Of these trauma patients, approximately 20% will require treatment within the intensive care unit (ICU) due to polytrauma, severity of injury, and underlying comorbidities (Duane, Rao, Aboutanos, Wolfe, & Malhotra, 2008). While in the ICU, many patients will develop infection and ensuing sepsis or septic shock. Following sepsis and septic shock, multiple organ failure (MOF) is common and accounts for approximately 50% of all ICU deaths (Brun-Buisson et al., 1995).

Immune system dysregulation appears to be one of the driving factors in the ensuing MOF during sepsis (Wiersinga, Leopold, Cranendonk, & van der Poll, 2014). Immune system dysregulation is also a state in which the white blood cells or leukocytes do not perform host defense functions properly. They are either in an exaggerated condition of activation or suppression (Pinsky, 2004). For example, the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), interferon-gamma (IFN- γ), and interleukin-6 (IL-6)—from stimulated spleen cells—was significantly less in patients who died from sepsis than those who died from non-sepsis causes (Boomer et al., 2011). This demonstrates an example of relative immune suppression in the face of overwhelming infection.

Alternately, the immune response may be so robust in this setting that injury occurs to healthy tissue. Acute Respiratory Distress Syndrome is an example of this tissue injury as a result of exaggerated inflammation (Ware & Matthay, 2000).

Sepsis is a complex process with a temporal course of immune imbalance that is equally complicated. Current research demonstrates that there is an initial hyperinflammatory response in the acute phase, followed by a chronic state of immune suppression (Hotchkiss, Monneret, & Payen, 2013; Stoecklein, Osuka, & Lederer, 2012). Whether an exaggerated response or a relative immune suppression, this immune dysregulation often leads to unintentional tissue injury and subsequent risk for nosocomial infections, respectively (Hotchkiss et al., 2013). The progression to organ failure is multifactorial in these patients. The inciting factors range from the macroscopic sequelae of physiologic responses to overwhelming infection to the microscopic tissue injuries from inflammatory cytokines and activated immune cells (Jean-Baptiste, 2007). These factors, as a result of or combined with nosocomial infection from a relative immunosuppression, contribute to organ failure (Perl, Chung, Garber, Huang, & Ayala, 2006). When the insult becomes severe enough, patients progress to multiple organ failure and ultimately, death.

Currently, immune system dysregulation is not well characterized in ICU patients. A better understanding of the immune imbalance may facilitate better outcomes in immune modulating therapies. Clinical trials are underway, testing immune-modulating agents such as granulocyte macrophage-colony stimulating factor (GM-CSF), showing some success in counteracting this immune system dysregulation (Hall et al., 2011). This example remains in the minority, however, with most immune modulating therapies demonstrating no success. Due to the waxing and waning temporal course and the complex nature of the immune response, present clinical assessment methods are not adequate for monitoring the critically ill trauma patient's immune status. Current methods, like the complete blood count, differential, and CRP, will also be insufficient for monitoring a patient's response to immune-modulating therapies. Therefore, one purpose of this article is to discuss the use of a different method to assess and monitor the immune status of critically ill trauma patients as well as the response to immune modulating therapies. We propose that flow cytometry is this method with its capabilities to characterize and/or enumerate (a) leukocyte subsets; (b) leukocyte activation markers; (c) leukocyte-derived cytokines and microparticles; and (d) leukocyte function. A second purpose of this article is to describe how nursing scientists might use flow cytometry in their research.

Innate and Adaptive Immune Systems

Before discussing flow cytometry capabilities, the innate and adaptive arms of the immune system will be briefly reviewed. The innate and adaptive arms are responsible for a patient's immune status, and both are involved in the inflammatory response in sepsis (Jedynak, Siemi tkowski, & Rygasiewicz, 2012). The innate system is the first arm of the immune system activated upon infection. It is important for the development of an early immune response and for activating the adaptive system. The components of this system are phagocytic cells, soluble proteins that bind the infectious agent, and antimicrobial peptides that all work in tandem to recognize the invading pathogen and phagocytize the microbe (Parham, 2005). These innate cells include monocytes, macrophages, dendritic cells, and neutrophils. Generally, monocytes (which represent immature macrophage) and neutrophils are found in the circulation. Dendritic cells, which can be derived from both myeloid and lymphoid progenitors, and macrophages are found within tissues and at the sites of infection (Shapiro, 2003). Another subtype of innate cells is the innate lymphocytes, namely natural killer (NK) cells and NK T cells. The NK and NK T cells function very similar to myeloid cells during infectious insult. Much like myeloid cells, NK cells are the "first line of defense" against microbial infection or cellular abnormality. These innate immune subtypes can be distinguished based on the presence or expression of certain proteins on the cell surface. These proteins are known as surface markers. Table 1 summarizes these surface markers of relevant immune cells.

The other arm of the immune system—the adaptive arm—also becomes activated during sepsis and consists of both humoral and cell-mediated immunity. The cells that compose the adaptive system are lymphocytes, which are classified as B cells and T cells. These cells rely on activation by the cells of the innate immune arm. Upon activation, B cells mature into plasma cells and mediate humoral immunity through production and secretion of antibodies (Paul, 2013). T cells can be divided into two generalized categories: CD4 and CD8 T cells

(Shapiro, 2003). CD4 T cells are often referred to as helper T cells because of their ability to help promote immune responses through recognition of antigens. Antigens are presented to them in the presence of major histocompatibility complex protein class II (MHCII) by antigen presenting cells of the innate system (e.g., dendritic cells and macrophages). T helper cells can be subdivided into several subclasses and are discussed more in depth in the section on cytokine detection. On the other hand, CD8 T cells are often referred to as cytotoxic T cells due to their ability to eliminate abnormal or infected cells. This cytotoxic ability is predicated on the CD8 cell's recognition of antigens in the presence of major histocompatibility complex protein class I (MHC I). These MHCIs are located on all human cells and function to display antigens, whether normal, from viral infections or altered in the case of cancer cells. T cells can be further divided into subgroups or subtypes based on their activation status, regulatory function, and T cell receptors (Shapiro, 2003). The surface markers used to distinguish various leukocytes are listed in Table 1.

The discovery of these surface markers has led to the characterization of cellular changes that occur during sepsis. For example, an increased NK cell count is associated with mortality on hospital day 1 (Andaluz-Ojeda et al., 2011; Andaluz-Ojeda et al., 2013), suggesting that NK cells are elevated in the acute phase of sepsis. However, in later stages of sepsis, pro-inflammatory T cell subtypes are depleted while the numbers of regulatory T cells remain stable. The net result of a stable number of regulatory T cells coupled with decreased numbers of conventional T cells is a higher proportion or frequency of regulatory T cells. The impact of this would be increased suppression of the immune adaptive arm (MacConmara et al., 2011). Both the innate and the adaptive arms of the immune system are in various states of activation and suppression, depending on the temporal course. The ability to monitor this changing state is one focus of this paper.

Methods to Evaluate Immune Status

Currently, the status of the innate and adaptive systems is assessed by the complete blood count (CBC) with cell differential. In certain cases, a C-reactive protein (CRP) level will be completed along with the CBC with differential.

The CBC quantifies the number of leukocytes and red blood cells, the hemoglobin and hematocrit concentrations, and the platelet count. With the addition of the cell differential, the percentage of neutrophils, monocytes, lymphocytes, eosinophils, basophils, and the presence of any premature neutrophils or "bands" is determined. Of the cells quantified, only the white blood count (WBC), number of neutrophils and number of "bands" is significant to the discussion on immune status. The CBC and differential are typically completed by an automated cell counter which uses flow cytometric principles and requires only minutes to perform. Further identification of anomalies can be performed, but requires a manual examination. This examination can take hours and relies on proportions and percentages of cell per high power microscope field to estimate the actual number. CBC and differential cannot identify surface markers—which limits it to quantification alone, rather than any functional detection of immune differentiation, activation, and suppression states in a trauma patient. CBC lacks the sensitivity necessary to quantify immune status. To increase the sensitivity, additional tests are often ordered, including the CRP.

CRP is an acute phase biomarker commonly used to estimate inflammation within the human body (Pepys & Baltz, 1983; Pepys & Hirschfield, 2003). It is often used to predict early infection prior to clinical appearance (Pepys & Baltz, 1983; Pepys & Hirschfield, 2003; Tillet & Francis, 1930). While CRP is an accurate marker of inflammation, many other conditions, including autoimmune disease and sterile burn injury, will also elevate the CRP (Barati et al., 2008; Szalai, 2004). Therefore, CRP does not have high sensitivity or specificity (Hoffmann, 2009). The limitations of the CBC/differential and CRP, coupled by the complexity of immune system dysregulation, suggest that another approach is needed to assess the immune status of critically ill trauma patients. Flow cytometry significantly enhances the potential for characterization and/or enumeration of leukocyte subtypes, leukocyte activation, leukocyte-derived cytokines and microparticles, and leukocyte function (Roussel et al., 2012). Furthermore, in facilities with access to an instrument, the flow cytometry process will be as efficient and timely as obtaining a CBC and differential.

Flow Cytometry

Description

Flow cytometry is a method for analyzing and detecting particles and cells from tissue or body fluids (Shapiro, 2003). Modern flow cytometers are much smaller than older legacy models and only take up a few linear feet of bench space. After the cells/particles are introduced into the flow cytometer, they are initially separated based on their unique light scatter pattern. The light scatter pattern is produced when the cells/particles suspended in capillary tubing are exposed to an illumination source, typically a laser (Figure 1). The light scatter pattern, consisting of side and forward scatter, is used to separate cells from debris and live cells from dead cells (e.g., live cells are bigger and have less granularity), and then to determine the cells' physical characteristics, such as size and unevenness of the cell surface (granularity). The variation in size and granularity also allows the identification of discrete subtypes, such as lymphocytes, macrophages, and neutrophils (Shapiro, 2003).

Prior to introduction into the flow cytometer, cells can also be labeled with fluorescent probes to further enhance detection and sorting. These fluorescent-labeled antibody probes will attach to specific surface markers on the cells. As each cell passes through the laser, the fluorescent tags on these probes will become excited, and the fluorochrome detectors inside the flow cytometer will detect this excitation state. These probes have different excitation and emission properties that make them distinct, and the number of fluorescent tags are growing rapidly. Common fluorochromes include Pacific Blue, FITC, PE, APC, Per Cy, and Alexa Fluors.

Most flow cytometers are capable of detecting thousands of cells/particles per second. Typically, flow cytometers require approximately 10^6 events, irrespective of the volume, to ensure an adequate sampling (Shapiro, 2003). What is provided in a standard blood collection tube is more than sufficient to run multiple cytometric panels. The volume is minimal such that either pediatric or adult collection tubes will provide adequate volumes for multiple flow cytometric panels. Sample volumes can range from 100–500 μ L, depending on dilution. Sample type ranges from a wide variety cell preparations. Examples of these preparations include cell suspensions prepared from organ or tissue harvest, body

fluids, serum, blood following anticoagulation, and lysis of red blood cells. The data acquired during detection are converted to a data file for analysis. The data are typically projected in a two dimensional histogram, with the X and Y axes representing predetermined cell markers. The population of cells is then further sorted by a technique referred to as “gating” (Figure 2). Gating sets specific limits on the light scatter and fluorescence values to identify specific cell subtypes, such as CD4 T cells from a population of lymphocytes. In addition, flow cytometry generates data in percentages, indicating the proportions of cells positive for the surface markers of interest. To use these data for assessing a clinical outcome, the absolute number of cells will need to be calculated by multiplying the subtype percentage by the total number of cells.

With these capabilities, flow cytometry can characterize and/or enumerate (a) leukocyte subtypes; (b) leukocyte activation; (c) leukocyte-derived cytokines and microparticles; and (d) leukocyte function. In the next four sections, each of these capabilities will be described below. Also, the relationship of these capabilities to sepsis will be discussed.

Capabilities

Leukocyte subtypes—Utilizing light scatter pattern and fluorescent data, flow cytometry can identify and enumerate specific cell types and subtypes in a blood sample that a manual differential cell count cannot. For example, flow cytometry can distinguish CD4+ T cells and CD8+ T cells, whereas a CBC and differential count can only distinguish lymphocytes from other leukocyte types. This delineation of leukocyte subtypes is important because, as described earlier, the number of leukocyte subtypes can change over time during sepsis and based on the patient’s current immune status. For example, initially there will be a marked increase in neutrophils and a subsequent decrease in lymphocytes. The fold difference in these changes can indicate the pathophysiologic severity (Salomao et al., 2009). Therefore, the use of flow cytometry in the acute care setting has the potential to significantly enhance the ability to examine the immune status as it pertains to the temporal nature of the disease process.

Leukocyte activation—Another capability of flow cytometry is the detection of leukocyte activation based on the level of certain surface markers. Table 2 summarizes surface markers that indicate cellular activation and are detectable with flow cytometry. Once the surface markers of activation are detected, the flow cytometry analysis software will generate a percentage of cells that express the activation marker and the amount or level of the activation marker’s expression.

For example, T cell subtypes are easily identified from other lymphocytes or other cells when assessing for the T cell receptor (TCR) or other surface markers, such as CD4 and CD8. To further characterize the activation state of these cells, other cell surface markers can be assessed, including CD25, CD44, CD69, and CD71 (Shipkova & Wieland, 2012). The presence of these four activation markers is consistent with increased T cell functionality. On the other hand, naïve T cells often lack these four markers of activation, but do express CD62L on their surface, so CD62L could be assessed for T cells in an inactive state (De Rosa, Herzenberg, Herzenberg, & Roederer, 2001). Finally, regulatory T

cells and Th17 cells, which are additional CD4⁺ T cell subtypes, express high levels of the transcription factors (FoxP3 or ROR γ T, respectively), so that the presence of these cells can be detected by assessing for these proteins. Th17 cells are responsible for enhanced microbial clearance by a variety of mechanisms, including neutrophil recruitment to sites of infection (Steinman, 2007). Quantifying the levels of these surface markers allows clinicians to better gauge the overall level of immune activation.

For the T cell response during sepsis, flow cytometry may aid in determining the prognosis or progression of sepsis. During the early stages of sepsis, there is robust apoptosis of conventional T cells (Hiramatsu, Hotchkiss, Karl, & Buchman, 1997; Hotchkiss et al., 2001). During the course of sepsis, it is currently hypothesized that if a patient's T cell numbers return to normal levels, the patient is on a path to recovery. In contrast, in the latter stages of sepsis, the observation that the patient has an increased frequency of regulatory T cells and decreased frequency of conventional T cells is consistent with profound immune suppression. Patients with persistent immune suppression are likely to have increased mortality rates. This increased mortality is associated with opportunistic infections that the patient's impaired immune system is unable to overcome (Otto et al., 2011). Thus, flow cytometry assessing T cell numbers/subtypes is a potential means to determine the patient's immune status. In both acute and chronic care settings, accurate assessment of immune status should allow for diagnosis, prognosis, and prediction of long-term sequelae. This will be important in the future when immunomodulatory treatments directed toward T cells might be available (Boomer, Green, & Hotchkiss, 2013).

Similar to T cells, neutrophils also differentially regulate surface markers, depending upon activation status during sepsis. For example, increased CD64 on neutrophils has a sensitivity rate of 95% for detecting sepsis in pediatric sepsis (Bhandari, Wang, Rinder, & Rinder, 2008) and adult patients (Icardi et al., 2009). In fact, in a pediatric cohort, CD64 is more sensitive for sepsis than CRP (Groselj-Grenc et al., 2009). This sensitivity rate is higher than that of CRP, which is estimated to only be around 80% (Simon, Gauvin, Amre, Saint-Louis, & Lacroix, 2004). Therefore, examining expression of CD64 on neutrophils by flow cytometry may be a better diagnostic tool for sepsis than CRP. Additionally, monocytes/macrophages demonstrate altered activation during sepsis (Hotchkiss et al., 2013; Monneret, Venet, Pachot, & Lepape, 2008). When a macrophage takes up antigen, the macrophage becomes activated and upregulates its expression of HLA-DR to enable it to present the antigen to T cells (Paul, 2013). The co-stimulatory surface molecules CD80 and CD86 are also up regulated on macrophages after activation. However, during sepsis, monocytes/macrophages often display decreased levels of HLA-DR, suggesting these macrophages are less capable of presenting antigen to T cells (Monneret et al., 2008). Because of this low HLA-DR expression on monocytes, monocyte expression level of HLA-DR has been used to determine whether to administer septic patients' immune activating treatments, such as IFN- γ or GM-CSF (Boomer et al., 2013). Further, macrophages may also express surface markers, Fas ligand and PD1 ligand, which can interact with T cell co-receptors such that T cell deactivation and even T cell death are increased (Hotchkiss et al., 2013). In this case, treatment with the T cell specific, anti-apoptotic cytokine IL-7 might represent a reasonable course of therapy. Altogether, monitoring expression of surface markers that indicate either

activation or suppression could potentially guide immune-modulating treatment of septic patients in the near future.

In summary, monitoring the activation status of distinct leukocyte subtypes by flow cytometry may enhance diagnosis of infection, and provide more specific information to guide immune modulating treatment of patients with sepsis than the standard CBC with differential and CRP laboratory tests can. This monitoring by flow cytometry will require the same amount of time or sample than these standard laboratory tests so that patients will continue to receive timely assessment and treatment.

Leukocyte-derived cytokines and microparticles—The critically ill patient's response to sepsis and injury involves, in part, a complex immune response that involves both the innate and adaptive immune system. The intercellular cross-talk between these systems is of critical importance in understanding the interplay involved in immune regulation. Changing concentrations of cytokines, chemokines, and microparticles potentiates this systemic regulation. Cytokines are involved in intercellular signaling. These proteins incite a variety of actions to include cellular activation, differentiation, and homeostasis. Chemokines are also involved in cellular signaling and serve to attract immune cells to sites of infection or injury (Paul, 2013). Thus, for monitoring the critically ill patient, it is of interest that these cytokines and chemokines are measured in the blood.

During sepsis, there is an early concurrent production of both inflammatory and counter-inflammatory cytokines. During later stages of sepsis, the immune response becomes paralyzed and there are decreased amounts of cytokines being produced with counter-inflammatory cytokines predominating (Osuchowski, Craciun, Weixelbaumer, Duffy, & Remick, 2012). A goal of future research will be required to standardize the cytokines/chemokines measured in order to determine the immune status during both the early and latter stages of sepsis. Altogether, these assays allow assessment of the amount and types of immune messages being produced in the periphery. One limitation to the measurement of blood soluble mediators is that the analysis of cytokines and chemokines in the periphery may not reflect what is happening at the site of actual infection, and analysis of fluid from the site of infection (such as bronchoalveolar lavage or peritoneal washes) will enable for a more complete analysis of the type of immune response occurring at the site of infection.

Previously, the measurement of soluble mediators was performed by either enzyme-linked immunosorbent assay (ELISA) or Western blot. Both ELISA and Western Blot are limited in that the analysis is time consuming and capable of only one protein at a time. With the development of bead-based arrays, multiple proteins can be examined simultaneously and in rapid fashion. These technologies utilize flow cytometric principles, as well as microspheres, each with a distinct capture antibody specific to the cytokine or chemokine of interest. After the "capture" of the soluble mediator, a second antibody specific to a different epitope to the cytokine or chemokine of interest is utilized to quantify the concentration of the mediator. Not only are there preset kits available with specific cytokines, but the investigator may also develop unique tests using a pan-functional bead along with any capture/detection antibody pair specific for the soluble mediator of interest. Although there are other dedicated multiplex platforms that can be utilized to measure cytokines or

chemokines, if the investigator already has purchased a flow cytometer, then no other large equipment purchase is required.

Flow cytometry can also be used to assess for *in vivo* presence of microparticles, such as microvesicles (MVs). MVs, ranging in diameter from 300 to 1,100 nanometers, are thought to be generated from either activated or apoptotic cells (Théry, Ostrowski, & Segura, 2009) and play a role in the pathophysiology of disease, including inflammation (Anderson, Mulhall, & Garimella, 2010; Prakash, Caldwell, Lentsch, Pritts, & Robinson, 2012) and sepsis (Johnson, Goetzman, Prakash, & Caldwell, 2013; Prakash, Caldwell, Lentsch, Pritts, & Robinson, 2012). These vesicles are absent in uninfected control patients (Prakash et al., 2012). MVs, which circulate in the blood, can exert biological effects to host cells by stimulating extracellular receptors of the target cell or by transferring microvesicle content such as protein, lipids, mRNA, or microRNA to the target cell (Valadi et al., 2007). Therefore, these vesicles can potentially be utilized as biomarkers (Ardoin, Shanahan, & Pisetsky, 2007) for diagnosis and understanding the pathophysiology of sepsis. MVs display surface markers that indicate their cellular origin so that their presence can be detected in the critically ill trauma patient and by flow cytometry (Gelderman & Simak, 2008). In addition, the number of MVs can be quantified by either direct counting by the flow cytometer or indirectly by using counting beads running alongside the MV sample. We also propose that flow cytometry can distinguish whether microvesicles are produced from an apoptotic or nonapoptotic cell on the basis of extracellular phosphatidyl serine (PS) expression on the microvesicle as PS is a marker of apoptosis. Certain MVs exhibit PS on the outer leaflet of the plasma membrane. If the MV expresses PS, this may indicate a suppressed immune system consistent with widespread apoptosis. If the MV lacks PS, this may indicate systemic leukocyte activation or a robust immune response.

Leukocyte function—As discussed previously, immune status can be determined by the expression of surface activation markers. However, activation markers do not provide complete insight into the functional status of individual immune cells. One approach to determine immune cell functional status is to detect posttranslational phosphorylation on intracellular signaling molecules using Phosphorylation Specific Flow Cytometry (PhosphoFlow) (Krutzik & Nolan, 2003; Schulz, Danna, Krutzik, & Nolan, 2012). With PhosphoFlow, the percentage of cells is quantified, or the magnitude of change in phosphorylation events is quantified, by the mean fluorescence intensity (MFI). The MFI is the fluorescence intensity of each event in average and can represent the expression quantity of the parameter chosen, in this case, the phospho-protein of interest. Phosphorylation pathways, Janus kinase signal transducer and activator of transcription (JAK-STAT), nuclear factor kappa B (NF- κ B), and mitogen activated protein kinase (MAPK), are important pathways for immune function in both humans and mice. For example, in a mouse burn model, the MAPK signaling pathway is critical for normal neutrophil function (Adediran et al., 2010). Therefore, this capability of flow cytometry offers a direct approach to detecting the specific intracellular pathways that are involved in immune cell function. By assessing these pathways, a patient's immune status in the acute setting can be determined, a diagnosis can be formulated, and/or immune-modulating therapies can be developed that will target these pathways.

The analysis of cytokines and chemokines in fluids, whether in fluids collected from the site of infection or blood, does not indicate what is happening at a cellular level. In order to determine cytokine production at the cellular level, intracellular cytokine staining can be employed. This method enables investigators to analyze cytokines at the single cell level, and allows a snapshot of a cell at a given moment in time. In intracellular cytokine staining, cells isolated from patients are stimulated *ex vivo* and at the same time protein secretion is blocked. The cells can then be labeled with various cell markers to identify specific cells. The cells are then fixed and permeabilized to allow the antibodies against specific cytokines to enter the cell. The end result is that data are available on what types of cytokines leukocytes are producing.

A caveat to this method is that it reflects the type of cytokines the cells are capable of producing and does not reflect the amount of cytokines being accumulated in blood or fluid as the bead assays do. Secondly, this method can be rather time consuming and may take 6–24 hours to complete. Despite these drawbacks, the PhosphoFlow method coupled with intracellular cytokine staining is useful for the analysis of functional status at the cellular level. These insights gained from these methods can be used to determine the immune status of the patient.

Implications for Nursing Research

Flow cytometry's extensive capabilities such as characterizing and enumerating leukocyte subtypes, leukocyte activation markers, and leukocyte cytokines have the potential to expand nursing research. For example, nurse scientists could be part of a multidisciplinary team to evaluate critically ill trauma patients' responses to GM-CSF therapy. Specifically, using flow cytometry, this team could examine the effect of GM-CSF therapy on immune status, such as monocyte HLA-DR expression, and explore the relationship between immune cell changes (sepsis-induced) and behavioral changes. With the known relationships among stressors, immune function, and behavior and identification of behavioral changes (such as physical dysfunction in sepsis survivors) (reviewed in Leibovici, 2013), a connection between immune status and behavioral changes in sepsis survivors is possible. Additionally, flow cytometry might be a tool to assess the effectiveness of basic nursing care in preventing oral or catheter-related infections. For example, if a sufficient number of cells could be obtained by swabbing the oral mucosa or catheter site, and the cells easily separated into a single cell suspension, then the composition of these cells could be analyzed by flow cytometry to determine whether immune cells indicative of inflammation or infection, such as neutrophils, have been recruited to the site. The accumulation of these cells or the presence of certain cell surface antigens may indicate that the basic nursing care is ineffective in preventing inflammation or infection; whereas, the ongoing absence would indicate this care is effective.

Beyond sepsis, flow cytometry may have applicability for nursing research focused on circulating leukocyte reactive oxygen species (ROS) production. For example, using flow cytometry, Villarroel et al. (2013) examined ROS production by peripheral blood mononuclear cells after rats underwent hemorrhagic shock and resuscitation. The approach included treating these cells with the Mitosox Red dye to detect ROS production and a

second dye, Mitosox Green, to detect cell viability and then exposing the cells to green (ROS) and blue (cell viability) lasers. Although no difference in the percentage of viable, ROS-positive cells was observed among the control, hemorrhagic shock, and resuscitation groups (Villarroel et al., 2013), the same approach could be used to study leukocyte ROS production in ischemia-reperfusion or other injury models of interest to nurse scientists (Dobek, Fulkerson, Nicholas, & Schneider, 2013; Funk et al., 2014).

Finally, flow cytometry may be useful in oncology nursing studies. Currently, flow cytometry can aid in the diagnosis and disease progression of certain leukemias, and its *in vivo* counterpart holds promise in assessing tumor cells circulating in blood or lymph fluid (Woo, Baumann, & Arguello, 2014). Therefore, flow cytometry may strengthen oncology nursing studies by the possible capability to establish a link between a specific cancer cell profile and symptoms or a nursing intervention response.

Conclusion

Overall, flow cytometry offers two major advances for nursing research. First, with its capability to identify immune cell subpopulations, flow cytometry deepens and enriches the focus of nursing research. For instance, the response to a nursing intervention geared toward the phenotype of regulatory T cells can be examined without being confounded by the presence of other T cell subpopulations, such as CD4 or CD8 cells. Second, flow cytometry can offer the capability to also examine function (e.g., detecting cytokines) concurrently with phenotype identification. Therefore, based on these two capabilities, flow cytometry has the potential to broaden nursing research priority areas so that a comprehensive approach to understanding the cellular response is attained.

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FIGURE 1.

Schematic of a flow cytometer. Cells are aligned single file in a capillary tube. The cells are then exposed to a laser beam. The light from the laser is scatter in two directions, forward and to the side at a 90-degree angle. If the cells have been previously labeled with fluorescent antibodies, the light will activate the fluorescence. Multiple sensors detect the forward and side scatter, as well as the fluorescence.

FIGURE 2.

Representative flow cytometry gating scheme. Samples are prepared and labeled with three distinct fluorochromes. Panel A: The cells of interest are selected by size (forward scatter) and granularity (side scatter) in the P1 gate. Panel B: The presence and intensity of the fluorochromes upon the cells selected by the P1 gate are determined. Panel C: The presence and intensity of an activation marker is analyzed in P3 gate.

TABLE 1

Cell Surface Markers

Cell type	Markers	Cell sub-type	Function
T Cells	αβ-TCR and CD4	T Helper Cell	● Regulation of other immune cells
	αβ-TCR and CD8	Cytotoxic T cell	● Elimination of infected or abnormal cells
	αβ-TCR and CD4 or CD8 and T-Bet	CD4 Th(c) 1 Cells	● Stimulation of inflammation and enhanced bactericidal, cytotoxic and anti-viral effects
	αβ-TCR and CD4 and Gata-3	CD4 Th2 Cells	● Mediation of allergic responses
	αβ-TCR and CD4 and FoxP3	CD4 Regulatory T Cells	● Regulation of cytotoxicity
B cells	αβ-TCR and CD4 and RORγT	CD4 Th17 cells	● Recruitment of neutrophils
	CD19, CD20	B Cells	● Antigen recognition
	CD138	Plasma cells	● Production and secretion of antibodies
Innate lymphoid cells	γ-TCRγ and δ-TCR	Gamma-Delta T Cells	● "First line of defense," stimulation of macrophages, enhance cytotoxicity
	CD56 and CD49b	Natural Killer Cells	● Elimination of cells infected with viruses or cells that lack of an MHC1 receptor
	TCR, CD56, and CD49b	Natural Killer T Cells	● Cytokine production following antigen recognition
Innate cells			
	CD66b and CD11b	Neutrophils	● Antigen presentation and antimicrobial elimination
	CD14 and CD11b	Monocytes	● Antigen presentation and antimicrobial elimination
	Clone: 2D7 / Anti-Basophil	Basophils	● Early mast cells, induce allergic reactions
	Siglec 8	Eosinophils	● Anti-parasitic response, function to enhance allergic response

Note. Information summarized from Paul (2013). CD = clusters of differentiation.

TABLE 2**Cell Surface Markers of Activation**

Leukocyte lineage	Markers	Protein/function
Lymphoid	CD62L	L-selectin
	CD44	Receptor for hyaluronic acid
	CD25	IL-2 low affinity receptor
	CD69	Type II membrane protein, C-Lectin family
	CD71	Transferrin receptor protein
Myeloid	CD11b	Integrin
	CD64 ^a	Fc receptor IgG high affinity
	CD80	CD28 and CTLA4 receptor, CD86 tandem
	CD86	CD28 and CTLA4 receptor, CD80 tandem
	HLA-DR	MHC Class II receptor
	PD-1L ^a	B7 homolog, CD80, CD28, CTLA4 receptor

Note. Unlike other markers, CD64 and PD-1L do not indicate a state of activation. CD = clusters of differentiation; CTLA 4 = cytotoxic T-lymphocyte antigen 4; Fc = fragment crystallizable; HLA = human leukocyte antigen; IgG = Immunoglobulin G; IL-2 = interleukin 2; MHC = major histocompatibility complex; PD-1L = programmed cell death ligand 1.