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How clinical Flow Cytometry rebooted sepsis immunology

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Abstract

On May 2017, the World Health Organization (WHO) recognized sepsis as a global health priority by adopting a resolution to improve the prevention, diagnosis and management of this deadly disease. While it has long been known that sepsis deeply perturbs immune homeostasis by inducing a tremendous systemic inflammatory response, pivotal observations based on clinical flow cytometry indicate that sepsis indeed initiates a more complex immune response that varies over time, with the concomitant occurrence of both pro- and anti-inflammatory mechanisms. As a resultant, some septic patients enter a stage of protracted immunosuppression. This paved the way for immunostimulation approaches in sepsis. Clinical flow cytometry permitted this evolution by drawing a new picture of pathophysiology and reshaping immune trajectories in patients. Additional information from cytometry by time of flight mass cytometry and other high-dimensional flow cytometry platform should rapidly enrich our understanding of this complex disease. This review reports on landmarks of clinical flow cytometry in sepsis and how this single-cell analysis technique permitted to breach the wall of decades of unfruitful anti-inflammatory-based clinical trials in sepsis.

Keywords

Sepsis; HLA-DR; monocyte; IL-7; PD-1; flow cytometry; Time of Flight mass spectrometry

1. Sepsis epidemiology and definition

On May 2017, the World Health Organization (WHO) recognized sepsis as a global health priority by adopting a resolution to improve the prevention, diagnosis and management of this deadly disease (1). Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (2). Septic shock is the most severe form of sepsis in which hypotension persists despite adequate volume resuscitation thus requiring the use of

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vasopressors. According to recent definitions, in response to an infectious trigger, the unbalanced host (immune) response is at the center of sepsis pathophysiology (2).

Sepsis represents a major healthcare problem worldwide. As an example, in the USA sepsis is more frequent than myocardial infarction, breast or colon cancers (3–6). Recent modeling of available data determined the number of cases of sepsis in the entire world to over 20–25 million corresponding to > 6 million deaths per annum (7,8). Over years, 28-day mortality has remained high, ranging from 20 % for sepsis to over 40 % for septic shock despite improvement in patients' care (fluid resuscitation, source control, antimicrobial therapy). Whereas few therapeutic options evaluated in phase II clinical trials have demonstrated the potential efficacy of immunostimulation in sepsis, to date, no therapeutic intervention targeting host response has specifically been approved and sepsis remains the leading cause of mortality in intensive care units (ICU)(9,10). In addition, sepsis incidence has dramatically increased over the past decade and is expected to further augment. This rising incidence of documented cases of sepsis has been attributed not only to demographic changes, with a large population of patients with co-morbidities (cancer, diabetes, chronic inflammatory diseases) but also to the expansion of treatments with drugs or invasive medical devices that weaken the immune response. Improved coding of sepsis and its increased awareness has additionally been mentioned as a possible cause (4). More worrisome, the United Nations projects that the global human population over the age of 60 will increase by more than threefold in next decades and will exceed the size of the global population of young individuals by 2050. Thus, the burden of sepsis is expected to increase continuously in the forthcoming years as sepsis is a disease of the elderly (incidence increases exponentially with age). Importantly, according to the Centers for Disease Control and Prevention, over $ 22 billion is spent annually on hospitalizations for sepsis in the USA representing the most expensive hospitalization cause in this country (11). Moreover, during hospitalization, septic survivors are at high risk of developing nosocomial infections (due to induced immunosuppression – see below) that are associated not only with an increased morbidity, mortality and hospital length of stay but also with an important augmentation in patient's hospitalization costs. Even after hospital / ICU discharge, infections remain the leading cause for patients' readmission and death (12–14). Thus, in all respects, the cumulative burden of sepsis on public health is dramatically high and sepsis is likely to become "the quintessential medical disorder of the 21th century" (15).

2. Novel understanding of sepsis immunology

As early as 1975, MacLean, Meakins and colleagues (16–18) reported on the delayed hypersensitivity skin testing in response to recall antigens after injury. This lack of response was associated with enhanced mortality and risk of nosocomial infections which are evocative of immunosuppression. In 1991, the group of Cavaillon observed alterations in inflammatory cytokine response of septic patients' blood upon ex vivo LPS challenge suggesting modifications of innate immunity (19). Meanwhile, the group of Richard Hotchkiss made several contributions to the field by reporting on the massive lymphocyte apoptosis in septic mice and patients supporting impairments of adaptive immunity. Simultaneously, the inability of various anti-inflammatory approaches aimed at blocking exuberant inflammation to show any clinical benefit (20) has led clinicians and researchers

to reconsider their understanding of immune response in sepsis pathophysiology. From early 2000, it was progressively hypothesized that the host response to sepsis associates a pro-inflammatory phase and an anti-inflammatory compensatory response which could become immunosuppressive (21,22). The main mechanisms sustaining this process have been then progressively discovered and are still regularly reinvestigated at the light of recent technological and scientific advances (*e.g.*, bioenergetic, epigenetic, transcriptomic). They have been extensively described in various reviews and they will not be reported here (23–25). In addition, the decades of inconclusive clinical trials with promising host targeted therapies are a reminder that septic patients comprise a heterogeneous group of individuals who are unlikely to respond uniformly to a treatment protocol (26). An important driver of this heterogeneity is patient's individual immune status, which varies with age, sex, comorbidities (including infectious exposure through life), disease progression and underlying pathophysiology. This illustrates the importance of immune monitoring in sepsis to better describe patients' immune status, improve the understanding of pathophysiology and identify novel therapeutic targets and associated biomarkers. In the present review, we will present seminal studies that used flow cytometry to describe sepsis induced immune alterations. We will focus on clinical studies that, by nature, take patients' heterogeneity into account. These studies leveraged flow cytometry as a primary immuno-assay to precisely define immune trajectories in patients suffering from sepsis. Results from these flow cytometry-based clinical studies reshaped our understanding of sepsis immunology by demonstrating the occurrence of immunosuppression in patients. This is particularly important since features of immune failure in septic patients have striking similarities with those seen in cancer for which tailored targeted immunotherapies currently provide spectacular positive results (27,28). This constituted the rationale for translating immunostimulation strategies from cancer to sepsis in recent Randomized Controlled Trial (RCT) using IL-7 or anti-PD-1 antibodies (27). Other candidates that have proven to be efficient as adjunctive anti-cancer agents by acting on immunosuppressive mechanisms could thus be repurposed in sepsis.

3. Landmarks of flow cytometry in sepsis

Decreased monocyte HLA-DR (mHLA-DR) expression

Following several reports describing decreased expression of mHLA-DR after injury, the seminal work by Volk's group in Berlin described the beneficial immunostimulant effect of IFN-γ in a small cohort of septic patients. This highlighted the major interest of this marker in identifying septic patients with altered immune response (29). Indeed, in this small clinical trial, patients' stratification was based on low mHLA-DR expression and positive response to treatment was easily monitored by longitudinal mHLA-DR measurement. Loss of mHLA-DR clinically illustrates the phenomenon of endotoxin tolerance characterized by a reduced responsiveness to a secondary infectious challenge (i.e., refractory state to subsequent endotoxin challenge ex vivo) following a first inflammatory stress. Low mHLA-DR expression also reflects a decreased antigen presentation capacity since HLA-DR molecules belong to histocompatibility complex class II (MHC II) system (23). For now, a consensus exists for considering low monocyte HLA-DR (mHLA-DR) as a surrogate marker of sepsis-induced immunosuppression (30,31). mHLA-DR has been the most studied and

validated biomarker in the field (> 200 publications). In clinics, the magnitude and the persistence overtime of mHLA-DR decrease is demonstrated to be associated with increased mortality and nosocomial infections. It is noteworthy that, after adjustment for usual clinical confounders in multivariate analyses, decreased mHLA-DR remains an independent predictor of mortality / occurrence of nosocomial infections after sepsis (32,33). Thus, immunosuppression illustrated by altered monocyte response contributes to increased risk of adverse events in sepsis and immune recovery is likely a key parameter contributing to patients' favorable outcome. Since these initial studies, there has been a constant increase of citations matching "HLA-DR & sepsis" (figure 1) illustrating the rising interest and use of this biomarker in clinic. mHLA-DR is now used to stratify patients in RCT evaluating immunostimulation in sepsis (e.g., GRID study, NCT02361528) and to monitor drug efficacy (34,35).

It is also important to mention that, at admission, an extremely increased inaugural mHLA-DR value recently helped to unequivocally exclude a diagnosis of septic shock in a patient presenting with organ dysfunctions due to hemophagocytic lymphohistiocytosis (HLH) characterized by tremendous inflammatory responses(36). This preliminary report needs further assessment in various types of HLH. Upon confirmation, as septic shock and HLH would require opposing treatments (i.e., no steroids vs high dose of steroids), mHLA-DR may be of crucial help for clinicians regarding patients' care and management. Thus flow cytometry may be useful clinical tool for the management of sepsis emergency medicine. In addition, it reopens the door for potential use of various immunomodulatory agents that have been initially found ineffective due to lack of stratification whereas they retrospectively demonstrated significant protective effect in the most inflammatory patients (37–39).

The more and more popular use of mHLA-DR is based on a standardized flow cytometry protocol that guaranties results reproducibility between laboratories thus permitting multicentric evaluation of this marker and comparable patients' stratification between sites. In 2005, a group of experts (40) proposed a consensual protocol based on the use of calibrated beads (with known amounts of fluorochrome) to convert means of fluorescence intensities to numbers of antibodies bound per cell (AB/C). As this protocol requires only 2 monoclonal antibodies (anti-HLA-DR and anti-CD14 Abs), it is accessible to most inclusion centers with Flow facilities. Inter-laboratory assessment between centers equipped with cytometers from different manufacturers provided excellent results (41). However strict pre-analytical conditions are mandatory (staining within 1.5 h after blood collection or within 4 h if storage at + 4°C) and limit a wider use of mHLA-DR. To circumvent this drawback, a fully automated table top cytometer with simple operating procedures (injection of patient's sample into a cartridge) was developed for use at the bedside or in emergency labs. Beta site evaluation for mHLA-DR evaluation showed convincing preliminary results (42).

Noteworthy, beyond sepsis, mHLA-DR is progressively becoming a popular immunomonitoring tool in other ICU contexts (traumas, burns), and other clinical conditions (gastroenterology, cancer, hematology, transplantation). Indeed, as monocytes are plastic cells fitted with capacity to both detect danger (and trigger inflammation) and resolve inflammation once danger is eliminated, the rapidly changing HLA-DR level on their surface likely reflects, at a given time point, the resultant of all immune forces applied on

monocytes. Consequently, mHLA-DR regulation is not specific of any disease but rather appears as a relevant global inflammatory indicator of milieu intérieur. As such, it constitutes a useful biomarker in many clinical situations.

Immature neutrophils and myeloid-derived suppressor cells

For long, the markedly increased number of circulating immature neutrophils termed “band cells” is an established immunologic feature found in peripheral blood of septic patients. More recently, two independent groups reported on increased proportion of immature neutrophil with low CD10/CD16 expression and immunosuppressive properties in peripheral blood from septic patients. Increased percentage of these immature neutrophils was associated with increased mortality after sepsis (43,44). These recent results on immature/immunosuppressive neutrophils aggregate with the expanding description of myeloid-derived suppressor cells (MDSC) in infectious contexts (45). MDSC constitute a heterogeneous population of immature myeloid cells including progenitors / precursors of monocytes, neutrophils and dendritic cells (46,47). They are mainly characterized by their suppressive properties (both on innate and adaptive immunity) and are released upon various inflammatory / infectious signals. Recently, Uhel et al., described an association between increased MDSC number in blood and forthcoming occurrence of nosocomial infections after sepsis (48). Unfortunately, to date, Human MDSC definition lacks consensual phenotypic characterization (usable in whole blood for routine basis) and published results were obtained according to various phenotypes, either in whole blood or in Ficoll enriched fraction (49). Thus, further clinical investigations will require better standardization. Upon development of appropriate staining protocol, it is likely that analysis of MDSC populations will provide crucial information in clinical studies.

Lymphocyte alterations

In contrast to the initial innate inflammatory phase, until recently the adaptive immune response has received less attention in the description of sepsis-induced immune alterations. This is striking considering that more than 40 years ago, Meakins et al. described in septic patients the altered delayed–type hypersensitivity (16) which is mostly a reflection of lymphocyte dysfunction. Characterization of lymphocyte response in sepsis has nevertheless improved over the last 10 years.

Absolute lymphocyte count—By using hematological analyzers or flow cytometry, studies have described the major fall in absolute lymphocyte count (ALC) in septic patients. Seminal studies showed that apoptosis was playing a central role in that process and identified an association between the persistence of lymphopenia and increased mortality (50,51). It has been shown that sepsis represents the first cause of lymphopenia among patients in university hospital (52) and that severe lymphopenia is already present on patients’ admission and affects every lymphocyte subpopulations (53). However, the extend of this lymphopenia is variable depending on cell subpopulations, with some subpopulations such as regulatory T cells (Tregs) being less affected (see below). Because ALC is part of the complete blood cell count on hematology analyzers and is thus available 24/7 in every hospital, the association of this cellular alteration and poor outcomes in septic patients is now easily evaluated in large prospective clinical studies and can be retrospectively assessed

in various sepsis cohorts (54–57). Overall, results homogenously show that decreased ALC is associated with initial severity upon ICU admission while persistent lymphopenia is associated with increased mortality and occurrence of nosocomial infections after sepsis. Consequently, lymphopenia depth has recently been selected as a stratification marker in the first clinical trial evaluating IL-7 in septic shock patients. Only patients with ALC < 900 cell/μl were included in the study (58).

Increased regulatory lymphocytes—While the presence of a subpopulation of CD4+ T cells with regulatory properties had been known for long in mice, the exact phenotype usable to identify these cells by flow cytometry in human was only described in 2001 (59,60). Based on this phenotype (i.e., CD4+CD25^{high}) and the subsequent identification of Foxp3 as a specific intracellular marker for these cells (61,62), it was repeatedly observed that the percentage of Tregs is increased in septic shock patients with a maximum at day 3 after shock (63). It was proposed that this relative increase in percentage was due to Treg relative resistance to apoptotic mechanisms induced after sepsis (64). Later on, the role of these cells in sepsis-induced innate and adaptive alterations was showed (65,66). Thanks to improvement in flow cytometry phenotyping such as the addition of CD127 staining and standardization of intracellular Foxp3 staining for its use in clinic (67), large prospective multicenter clinical studies have evaluated this parameter in ICU patients (68). It was showed that increased percentage of Treg in critically ill patients is associated with increased risk of subsequent nosocomial infections and that this parameter could be part of an immunopanel to stratify patients suitable for immuno-intervention in a precision medicine approach (68).

Few data are available regarding B cell response in septic patients. The decrease in circulating absolute B cell number has been shown (53) while their relative percentage among total lymphocytes was increased in patients as observed for Tregs (69). Interestingly, remaining circulating B cells present with an altered phenotype and altered functions (69). While the presence of B cells with regulatory functions has been shown in mice models of infections and in some rare clinical contexts, preliminary data suggest that these cells might be induced after septic shock (70). This deserves to be further explored in sepsis.

Co-inhibitory molecule expressions—Increased co-inhibitory molecule expressions and role of co-inhibitory pathways in T cells exhaustion have been evaluated multiple times in cancer and chronic viral infections (71). In sepsis, a seminal work reported on the increased expressions of PD1 and PDL1 on circulating immune cells on a limited number of septic shock patients and on the role of these molecules in sepsis pathophysiology in mice model of sepsis (72). The associations between increased PD1 and PDL1 expressions and decreased T cell proliferation, increased risk of death and nosocomial infections were subsequently described in a larger cohort of septic shock and trauma patients (73). This observation was confirmed by other groups and was completed by the description of the increased expressions of other co-inhibitory molecules such as BTLA, Tim3, LAG3 (25). Based on these observations and on ex vivo studies showing the efficacy of anti-PD1 and anti-PD-L1 blocking antibodies in improving sepsis-induced T cell alterations (74–76), the first clinical trials evaluating immune checkpoint inhibitors that are currently revolutionizing

cancer treatment have been completed in septic patients (NCT02960854, NCT02576457). Results from these clinical trials are strongly awaited.

Functional testing

Importantly, altered immune cell phenotypes are associated with functional alterations, such as altered cytokine production (23,30). Nevertheless, the potential added value of functional testing in clinical monitoring remains to be demonstrated. This is in particular because, as opposed to immunophenotyping, those tests lack of automatization and standardization: e.g., long incubation time, lengthy cell purification procedures, cell permeabilization, and complex protocols with numerous staining / wash cycles (77,78).

One specific aspect in which functional tests may appear informative is the control of treatments' efficacy (see below). Indeed, functional testing may appear as a gold standard in this context because it directly measures ex vivo the capacity of a cell population to respond to an immune challenge. Such test may indicate whether a given therapy was truly effective in restoring immune functions in patients. Among immune functional assays that have been evaluated in clinic, measurement of intracellular cytokine content by flow cytometry appears as one of the most promising technique. Indeed, novel whole blood flow cytometry protocols provided significant information in septic patients regarding TNF-α production in monocytes (79) or TNF-α, IL-2, IFN-γ production by CD4+ and CD8+ lymphocyte (80). However, technical optimization remains to be done to specifically fit with sepsis monitoring (e.g., nature and concentration of stimulants, incubation time).

Is this immunosuppression?

Immunosuppression lacks specific clinical manifestation. As such, the definition of immunosuppression is based on the combination of immunological alteration (i.e., the quantitative or functional alteration of a given immune cell population) and the documented increased occurrence of infections. This definition describes all patients after sepsis (24,25). Indeed, on the one hand, we described above important immune alterations in patients. On the other hand, increasing numbers of clinical evidences highlight septic patients' diminished capacity to fight secondary infections by weakly virulent germs (including fungi) or reactivation of dormant viruses (TTV, CMV, HSV). In this context, altered immune functions are reported as independent predictors of forthcoming infections in multivariate analyses including classic risk confounders (i.e., prior exposition to antibiotics, exposure to invasive devices such as central venous lines or intubation, comorbidities). In addition, the leading cause of readmission and long-term mortality within first years after surviving sepsis is infection (12–14). In line, an increased risk for cancer has also been recently reported after septic shock (81) that may constitute another clinical event related to chronic immunosuppression.

Taken together, according to definition, these data indicate that septic patients present with immunosuppression that is more or less pronounced and durable depending on individual. Some patients may retain persistent immune defects on a long term basis which may participate in their increased susceptibility to subsequent infections and aggravate occurrence of deleterious outcomes.

Thus, immunostimulation has appeared as a sound therapeutic option (24,25,82). However, appropriate therapeutic strategy relies on our capacity to identify, in a complex situation mixing both pro- and anti-inflammatory responses rapidly varying over time, the most immunosuppressed patients (figure 2). Moreover, it has been shown that variations in the human immune response are largely driven by non-heritable influences (83). Among various components, co-morbidities (including infectious exposure through life) and age are of major importance. As septic patients are older (median around 65 years) and present with various comorbidities, this reinforce the idea that an individual monitoring of immune parameters is of crucial importance as, depending on their own personal history, no patient will immunologically respond like another. International experts agree that, among other reasons, the heterogeneity of septic patients may have participated in the previous failure of clinical trials evaluating host targeted therapies in sepsis (84,85). The use of biomarker for patients' stratification appears thus as a mandatory step in the design of next clinical trials evaluating immunoadjuvant treatments in sepsis (24,30,31). So far, as previously seen in this review, absolute lymphocyte count (including CD4+ and regulatory lymphocytes) and decreased expression of mHLA-DR appear as the most robust markers usable in multicenter studies (figure 3). Both measurements are standardized and their variations are associated with altered functions and deleterious outcomes (either nosocomial infection occurrence or mortality). In addition, monitoring of MDSC and / or immature neutrophils (as markers of bone marrow activity and of chronic low grade inflammation), lymphocyte PD-1 and monocyte PD-L1 expressions (as potential stratification markers for anti-PD-1 / anti-PD-L1 treatments – see below) are good candidates upon further validation in larger cohorts of patients.

4. Current multicenter studies and clinical trials

Early Flow on patients' admission

Most flow cytometric studies of sepsis have focused on delayed immune consequences (i.e., after the first 48 hours). However recent studies have examined the role of flow cytometry in the early management of septic patients. Regarding sepsis diagnosis, increased neutrophil CD64 expression has been shown to be a highly sensitive (>95%) and specific marker for systemic infection and sepsis in adults, neonates and children (86). Low HLA-DR expression associated with PCT determination (i.e., usual biochemical marker of sepsis) tended to reinforce sepsis diagnosis (87) as well as increased CD8/CD19 ratio in another study (88). In contrast, very high values of mHLA-DR may be indicative of HLH (see above and (36)). These studies pave the way for the use of flow cytometry in the diagnosis of sepsis. However, flow cytometry approaches will need to be assessed in comparison with reference markers (i.e., PCT and CRP) since the measurements of these proteins are inexpensive, fully automated (24/7 available) and readily available.

Multicenter studies and improved standardization

Identifying the most severe patients is another opportunity for the clinical application of Flow in sepsis. In a pilot study, percentage of immature granulocytes (CD10low/CD16low) was demonstrated to predict early clinical deterioration at the acute phase of sepsis (44). Those preliminary results were recently confirmed in a multicentric multicolor flow

cytometric study (SEPTIFLUX-2) including 781 patients in 11 centers (56). An increased circulating immature neutrophil percentage at the acute phase of sepsis was linked to clinical worsening, especially when combined with T-cell lymphopenia. The authors concluded that early flow cytometry could help clinicians to target patients at high risk of clinical deterioration. Beyond these interesting results, this study was the first to highlight the feasibility of performing large multicentric multicolor flow cytometry studies in ICU patients. Both Beckman Coulter and BD Biosciences flow cytometers were harmonized as previously established for lymphoma classification (89). In line, the ExPRES-Sepsis study recently reinforced this report. In this study evaluating standardized multi-site flow cytometry in about 400 acutely ill patients with suspected infections from 4 different centers, 47 leukocyte biomarkers were simultaneously assessed (90). Markers of immune suppression (increased neutrophil CD24 and PD-1 and decreased mHLA-DR expressions) showed the strongest associations with clinical outcomes. Moreover, a multicenter, prospective observational cohort study of critically ill patients in four UK ICUs has been published (INFECT study)(68). Three cell surface markers associated with immune cell dysfunctions (CD88 expression on neutrophil, mHLA-DR, percentage of regulatory T cells) were assayed. This study reported that these markers could predict subsequent risk of secondary infections. This study (90) along with SEPTIFLUX-2 (56) and ExPRES-Sepsis (68), all published in 2018, demonstrated that harmonizing Flow protocols is now an achievable objective. Before starting studies, ensuring intercenter reproducibility in setting up thresholds and gates should be performed (e.g., listmodes blindly analyzed). Then, standardization would imply the use of stabilized blood samples as controls (when possible), common standard operating procedures based on antibodies from same batch from a single manufacturer, commercially available standards for results comparison across labs (calibrated beads, e.g., setup and tacking beads) as well as internal (provided by manufacturers) and external controls (e.g., UK NEQAS). In addition, the development of lyophilized pre-formulated antibody panels also represents a major improvement for FCM standardization in RCTs. As an example, FOXP3-lyophilized tubes are currently used for regulatory T cells determination (67) in an RCT evaluating low-dose IL-2 treatment for type 1 diabetes (DIABIL-2 study, NCT02411253). Furthermore, new technical developments (low cost compact portable flow cytometer, bedside flow cytometry or chip-based flow cytometry) are now being proposed that will facilitate the use of Flow at the bedside in ICU (see above in mHLA-DR paragraph (42)). For now, such techniques remain devoted to very simple applications but we may expect major developments in the forthcoming years, including multicolor flow cytometry.

As septic patients form a heterogeneous population, biomarkers characterizing patients' immune trajectories are useful to identify subgroups of patients that may benefit the most from an immune modulatory intervention. These biomarkers should be viewed as markers for patients' enrichment more than tailored stratification *stricto sensu*. So far, patients' stratification was mostly based on Flow which remains the most appropriate tool to individualize immunotherapy (Table 1). Recent phase II trials in the field used either mHLA-DR or lymphocyte count to stratify patients (34,91). In addition, one may expect further developments in PD-1 stratification as anti-PD-1 and anti-PD-L1 are believed to be good candidates in sepsis (92).

Another important application of Flow-based biomarkers is to monitor drug efficacy. The restoration of mHLA-DR expression has been successfully used to control IFN-γ or GM-CSF treatment effects (29,34). Recently, in critically ill patients, a sharp peak of mHLA-DR expression was observed in patients receiving GM-CSF (35). In HLH patient, mHLA-DR was successfully used to increase dosage of corticosteroids to control inflammation process (36). In the recent IRIS study, efficacy of IL-7 was measured by decreased expression of CD127 (i.e., IL-7 receptor) and by a peak of increased Ki67 as surrogate marker of lymphocyte proliferation (58). Of note, in controlling drug efficacy, functional testing might be an appropriate tool as it really reflects cell restoration in response to an immune challenge (i.e, more appropriate than cell counting or phenotyping).

5. The promise of Cytometry by Time of Flight mass spectrometry

The advent of high content immune profiling technologies provides new opportunities for the in-depth characterization of septic patients' immune states. Cytometry by Time of Flight mass spectrometry (CyTOF), or mass cytometry, is a particularly promising single-cell technology, as it allows for the comprehensive monitoring of the distribution and activities of all immune cell subsets present in a blood sample (93,94). Rather than fluorescent reporters used in traditional flow cytometry, mass cytometry utilizes antibodies conjugated to stable isotopes of rare earth metals. By exploiting the resolution, dynamic range and near absence of background noise offered by mass spectrometry, mass cytometry allows for the simultaneous detection of over 50 parameters on a cell-by-cell basis (95,96). The high parametrization afforded by mass cytometry allows for the precise phenotyping and distribution of virtually every immune cell subset, as well as a number of functional attributes which are assessed simultaneously at the single-cell level. Functional attributes routinely measured with a mass cytometry assay include cell proliferation, apoptosis, intracellular signaling responses, and intracellular cytokine production (97). In addition, recent developments in mass cytometry reagents allow the single-cell assessment of epigenetic modifications (98) and mRNA expression (99,100).

The general concept of utilizing mass cytometry to characterize immune states associated with disease progression or response to therapy has been demonstrated in multiple clinical contexts (101), including malignancies (102–104), rheumatological diseases (105), aging (106), traumatic injury (107–109), and pregnancy (110,111). While the use of mass cytometry in sepsis is still in its infancy (112), observational studies in the context of traumatic injury provide the groundwork for the deep immune profiling of complex inflammatory states such as sepsis. Not unlike sepsis, traumatic injury produces a conserved inflammatory response that engages both the innate and adaptive branches of the immune system (113). However, recent mass cytometry analyses of patients undergoing major surgery showed that underlying the canonical immune response to traumatic injury were distinct immune phenotypes associated with surgical recovery outcomes (108,109). In sepsis, the use of mass cytometry in a preliminary study led to the description of novel immune alterations (70). For instance, it was observed that increased checkpoints inhibitor expressions were not uniformly co-expressed on T lymphocyte population (e.g, TIM-3 and PD-1, Figure 3) but that each immune checkpoint was overexpressed on different T lymphocyte subsets. Putative consequences of such disparate expression remain to be

investigated. Another novel result was the identification in septic patients of a very mature subset of B lymphocytes (Figure 3) absent from controls. These cells might be circulating regulatory B cells never reported before in septic patients (70). Thus, the field of sepsis research is primed for similar translational studies that bring mass cytometry “to the bedside”. Exciting applications of this technology will include creating a high-resolution cellular atlas of the human immune response to sepsis, identifying immune signatures predictive of clinical outcomes, and tailoring promising immune therapies such as anti-PD-L1 and IL-7 to individualized immune phenotypes.

The mass cytometry analysis of clinical samples has certain limitations. The technology requires complete ionization of immune cells to their elemental composition, which precludes further cell-based assay once the sample is analyzed. Although the number of antibodies measured per single cell is unparalleled, developing a new mass cytometry assay requires a priori selection of markers of interest. Combining mass cytometry with traditional fluorescence-based cell sorting (114) and untargeted RNA sequencing will become increasingly useful for informing the design of future mass cytometry antibody panels. Most importantly, the increasing dimensionality of mass cytometry datasets – often comprised of thousands of immune features – comes at a price, referred to as the “curse of dimensionality” (115). Extraction of features using traditional analysis by human experts is time consuming a potential source of error (116,117) but a broad range of algorithms have been developed for objective extraction of immunophenotypes from mass cytometry data (118,119). The structure of mass cytometry data is also highly intercorrelated, reflecting the interconnected nature of the human immune system. Novel analytical methods – such as the recently developed cell-signaling Elastic Net algorithm (110) – that account for the dimensionality and correlated nature of large mass cytometry datasets are needed to ensure the statistical validity and generalizability of identified immune predictors of clinical outcomes.

These high dimensional analysis methods will allow not only the integration of multiple classes of single-cell mass cytometry measurements but also the integration of mass cytometry data with other high-throughput biological assays (such as metabolomics, transcriptomics, an proteomics assays) (120). Such multi-omic modeling of inflammation in humans may reveal novel biological connections between functional readouts from discrete cell types and circulating molecular factors, which hold promise for the development of integrative bioassays with high predictive performance (121,122).

Taken together, mass cytometry has now reached the level of technological and computational maturity needed for deep clinical profiling of sepsis to lay the foundation for the design of precision-interventions based on the status of a patient’s immune system.

6. Conclusion

As described in this review, sepsis is entering the era of precision medicine. Clinical Flow has played a critical role in this evolution by drawing a new picture of pathophysiology and by cracking the wall of decades of unfruitful anti-inflammatory-based clinical trials. Considering the current revolution in cancer treatment and the parallels that can be drawn

between the host immune responses to cancer and sepsis, immunotherapeutic interventions hold great promise for the targeted treatment of septic patients. That said, sepsis being a complex syndrome with deleterious consequences on all organs, it is plainly obvious that immunostimulation would not save all septic patients. It will preferentially work in the most immunosuppressed patients and this reasoning is all the more applicable since we can use flow cytometry to stage each patient's immune status and depict immune trajectories. Hopefully, we may soon obtain complementary information from mass cytometry and other high-dimensional flow cytometry platform. So far, mHLA-DR, immature neutrophils and/or MDSC count, lymphocyte count along with PD-1 molecules expression – all measured by flow cytometry - offer relevant information for identifying individual trajectories. Recent IL-7 RCT results and recent observational multicenter studies indicate the feasibility of the approach of flow-based RCT. Taken together, Flow cytometry reshaped sepsis immunology and paved a new way of success for tackling this hitherto deadly disease.

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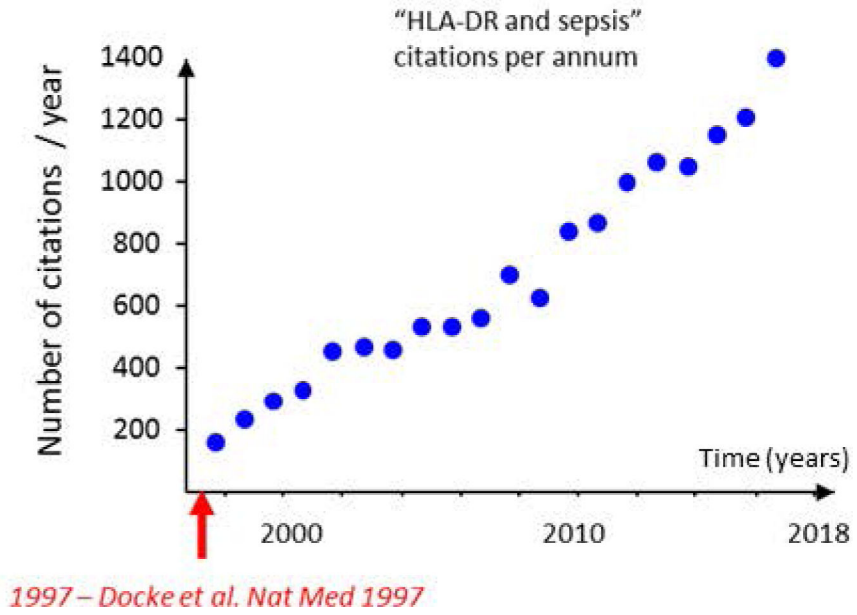

Figure 1. "HLA-DR and sepsis" citations over years.

Yearly number of citations of articles matching terms HLA-DR and sepsis from 1997 to present time (publication year of landmark paper of Docke et al. is marked by a red arrow). Data from Web of Science.

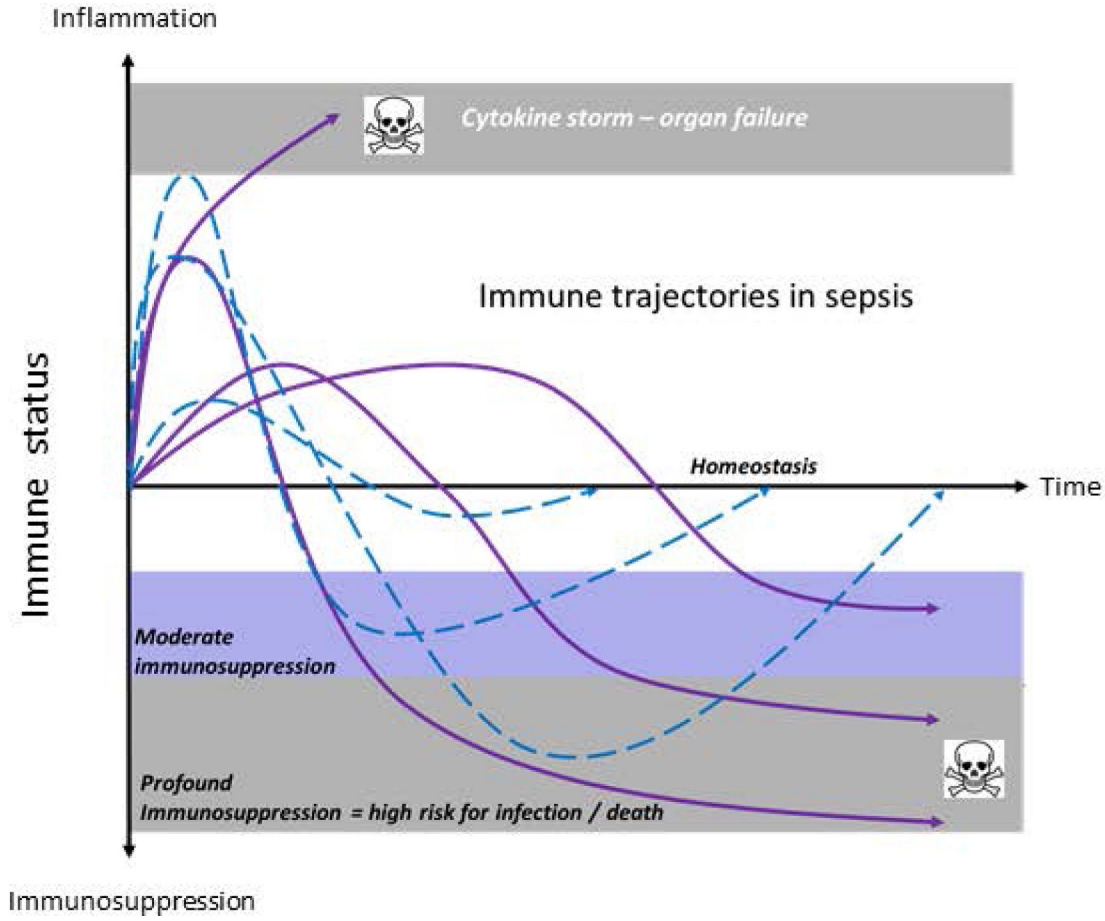

Figure 2. Immune trajectories in sepsis.

Theoretical evolutions over time of immune status of different septic patients are presented. Net inflammatory/suppressive immune responses are very heterogeneous in kinetics, duration and depth. Patients with dashed line return spontaneously to immune homeostasis overtime. Patients with continuous lines present with dysregulated immune responses. These patients should be identified to benefit from host directed immunointerventions.

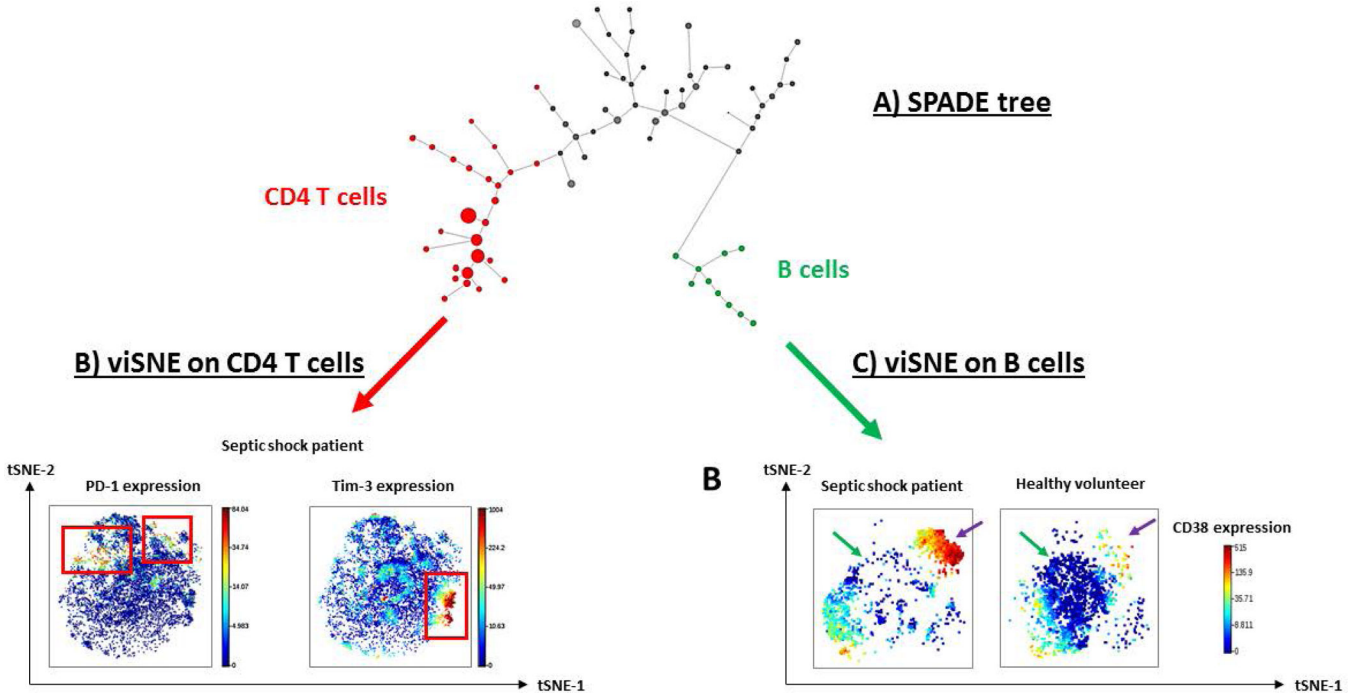

Figure 3. Illustrative example of unsupervised analysis of CyTOF results in healthy volunteers and septic patients.

These results have been obtained from reference (70) **(A)** SPADE clustering analysis was performed in order to group peripheral blood mononuclear cell sub-populations into “nodes” according to phenotype similarities. These nodes and their relationships are represented in a minimum spanning tree format (i.e. SPADE tree). Size of the nodes illustrates the number of cells within the clusters. In this analysis, CD4 expressing cells are highlighted as red nodes while CD19 expressing cells are represented as green nodes. **(B)** viSNE visualization tool was then performed on selected CD4+ cells from SPADE nodes. Based on the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm, viSNE determines the two dimensional representation of single-cell data sets that best matches their respective local and global geometry. Cells get unique x- and y-coordinates (tSNE1 and tSNE2) according to their expression of parameters from CyTOF panel. Their relative positions on the two-dimensional plot indicate similarities in terms of expression pattern. In the current analysis, we used color as a third dimension to visualize specific marker expression levels (PD-1 and Tim-3) in one representative example of a septic patient. Red and blue color intensities respectively represent high and low expression levels of the indicated marker. In this illustrative septic shock patient, PD-1 and Tim-3 are clearly not co-expressed by same CD4 T cell subpopulations (red squares). **(C)** Alternatively, viSNE algorithm was performed on selected CD19+ B cells to compare sepsis vs healthy volunteers. Expression level of CD38, a marker of B cell maturation was visualized on the viSNE plots in one representative septic shock patient and one healthy donor. A depletion of cells from the center of the map (green arrows), but an enrichment within the upper right cell cluster (purple arrows) are noticed in the patient. This latter cluster was constituted of CD38hi cells, suggesting a shift towards differentiated B cells in septic shock patients.

Table 1.

Biomarker-guided clinical cases and trials with immunostimulatory drugs in sepsis.

Interventions	Targeted cells (for stratification)	Biomarkers (at inclusion)	Number of patients	Randomization	Bibliographic Reference or NCT
IFN-γ	Monocytes	HLA-DR	35	No	(29)
IFN-γ	Monocytes	HLA-DR	9	No	(123)
IFN-γ	Monocytes	HLA-DR*	21	yes	(124)
IFN-γ	Monocytes	HLA-DR	1	No	(125)
IFN-γ	Monocytes	HLA-DR	2	No	(126)
IFN-γ	Monocytes	HLA-DR	1	No	(127)
IFN-γ	Monocytes	HLA-DR	Not published yet	yes	NCT03332225
GM-CSF	Monocytes	HLA-DR	38	Yes	(34)
GM-CSF	Monocytes	HLA-DR	Not published yet	Yes	NCT02361528
GM-CSF	Monocytes	HLA-DR	1	No	(128)
GM-CSF	Monocytes	HLA-DR	1	No	(129)
GM-CSF	Monocytes	Whole blood TNF release	14	Yes	(130)
GM-CSF	Monocytes	Whole blood TNF release	Not published yet	Yes	NCT03769844**
GM-CSF	Neutrophils	Phagocytic capacity	38	Yes	(35)
Anti-PD-1	Lymphocytes	PD-1 expression	1	No	(125)
IL-7	Lymphocytes	Absolute count	27	Yes	(58)

*: mHLA-DR was assessed in broncho-alveolar lavages,

**: inclusion criteria is organ failure (including sepsis).