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Neutrophil apoptosis & chemotaxis and the complex systemic host response

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Abstract - English

Multiple Organ Dysfunction Syndrome (MODS) is the leading cause of mortality in Intensive Care Unit (ICU) patients. Although essential for host defense, the polymorphonuclear neutrophil (PMN) contributes to endothelial cell and end organ injury in disease states such as MODS. PMN chemotaxis and apoptosis are principally involved in neutrophil delivery and clearance, and their evaluation is performed with the goal of developing effective therapeutic intervention that would attenuate neutrophil mediated host injury.

To investigate the role of neutrophil membrane receptor expression in the regulation of neutrophil apoptosis and chemotaxis, human circulating PMN (venipuncture, healthy controls), exudate PMN (skin window skin blister) and septic PMN (venipuncture, patients with sepsis) were evaluated for apoptosis rates (flow cytometry), chemotactic function (Boyden chambers), and receptor expression (flow cytometry - Fas, FasL, TNFRI, TNFRII, IL-8RA, IL-8RB, C5aR). Experiments were coupled with a theoretical evaluation of the assumptions and clinical implications inherent to analytical research of the host response.

Following transmigration, exudate neutrophils demonstrate delayed constitutive and induced (TNF-α & Fas Ab) apoptosis. Decreased binding to TNF-α (not receptor expression) was found in association with decreased TNF-α induced apoptosis in exudate PMN. In contrast to circulating PMN, inhibition of protein synthesis in exudate PMN does not augment apoptosis; NF-κB does not mediate this effect as inhibition of NF-κB augments apoptosis in circulating and exudate PMN. Evaluation of chemoattractant receptors and chemotactic function revealed the following: exudate PMN displayed increased C5a receptors & C5a chemotaxis and reduced Interleukin-8 receptors (both IL-8RA & IL-8RB) & IL-8 chemotaxis. Septic PMN displayed reduced C5a & IL-8 receptors, and decreased C5a chemotaxis. These results suggest that alteration in chemoattractant receptor expression serves to regulate PMN chemotaxis *in vivo*, exudate PMN chemotaxis depends more on C5a than IL-8, and diminished chemoattractant receptors and chemotaxis in septic PMN may explain decreased PMN delivery in these patients.

Therapeutic interventions for patients with complex inflammatory disease states such as MODS remains elusive despite immense growth in understanding the mechanisms involved in the host response. To complement analytical research, the systemic host response to trauma, shock or sepsis may be evaluated as a complex system. This offers a novel explanation for the failure of anti-mediator trials in the treatment of patients with sepsis and MODS, and suggests innovative means of monitoring critical care patients. The following hypothesis is presented with the goal of improving outcome in patients with MODS, that analysis of variability and connectivity of the variables of a complex system offer a means of evaluating, differentiating and altering the systemic properties of that system. Present and future applications of evaluating variability and connectivity are discussed, and specific hypotheses regarding future research are presented to help bridge the gap between laboratory bench and ICU bedside.

Résumé – Français

Le syndrome d'atteinte multisystémique (SAM) est la principale cause de décès chez les patients traités aux soins intensifs. Bien qu'il soit essentiel à l'organisme pour se défendre contre les infections, le neutrophile polynucléaire (*PMN*) contribue aux lésions des cellules endothéliales et des organes cibles dans les maladies comme le SAM. La chimiotaxie et l'apoptose du PMN sont responsables du recrutement et de l'élimination des neutrophiles; l'étude de ces deux phénomènes vise à élaborer une intervention thérapeutique qui permettrait d'atténuer les dommages causés à l'hôte par les neutrophiles.

Pour étudier le rôle de l'expression des récepteurs de la membrane dans la régulation de la chimiotaxie et de l'apoptose des neutrophiles, on a évalué chez l'humain les PMN circulants (ponction veineuse, témoins en santé), les PMN de l'exsudat (technique des fenêtres cutanées) et les PMN de patients septiques (ponction veineuse, patients infectés) afin de déterminer les taux d'apoptose (cytométrie de flux), la fonction chimiotactique (épreuve de Boyden), et l'expression des récepteurs (cytométrie de flux - Fas, FasL, TNFRI, TNFRII, IL-8RA, IL-8RB, C5aR). On a jumelé le volet expérimental à une évaluation théorique des hypothèses et des conséquences cliniques inhérentes à la recherche analytique de la réponse de l'hôte.

Suite à la diapédèse des PMN obtenus par exsudat, on a observé une apoptose retardée, soit constitutive, soit induite (TNF-α et Fas Ab). Une réduction de la capacité de liaison au TNF-α (non reliée à l'expression des récepteurs) a été observée en association avec la diminution de l'apoptose induite par TNF-α des PMN de l'exsudat. Contrairement à l'inhibition de la synthèse des protéines des PMN circulants, celle des PMN de l'exsudat n'augmente pas l'apoptose; le NF-κB n'est pas le médiateur de cet effet puisque l'inhibition de NF-κB augmente l'apoptose dans les NPN de l'exsudat et les NPN circulants. L'évaluation des récepteurs chimiotactiques et de la fonction chimiotactique a révélé les résultats suivants : augmentation des récepteurs de la C5a et de la chimiotaxie reliée à la C5a et réduction des récepteurs de l'interleukine-8 (tant IL-8RA que IL-8RB) et de la chimiotaxie reliée à l'IL-8 dans les PMN de l'exsudat; réduction des récepteurs de la C5a et de l'IL-8 de même que diminution de la chimiotaxie reliée à la C5a dans les PMN de patients septiques. Ces résultats suggèrent que la modification de l'expression des récepteurs chimiotactiques module la chimiotaxie des PMN *in vivo* et que la chimiotaxie des PMN de l'exsudat dépend plus de la C5a que de l'IL-8. De plus, une baisse des récepteurs chimiotactiques et de la chimiotaxie dans les PMN de patients septiques pourrait expliquer la mobilisation réduite des PMN chez ces patients.

Malgré une meilleure compréhension des mécanismes en jeu dans la réponse de l'hôte, il est encore difficile de traiter les patients souffrant de maladies inflammatoires complexes comme le SAM. Pour compléter la recherche analytique, on peut évaluer la réponse systémique de l'hôte au traumatisme, au choc ou à la septicémie en tant que système complexe. Ceci permet de fournir une nouvelle explication à l'échec des essais anti-médiateur dans le traitement de patients atteints de septicémie et d'atteinte multisystémique et de suggérer des moyens novateurs de surveillance des patients aux soins intensifs. Dans cette thèse, afin d'améliorer le pronostic en cas d'atteinte multisystémique, on présente l'hypothèse selon laquelle l'analyse de la variabilité et des liens entre les variables d'un système complexe permet d'évaluer, de différencier

et de modifier les propriétés globales de ce système. On y examine les applications présentes et futures relatives à l'évaluation de la variabilité et des liens, et on y présente des hypothèses précises concernant la recherche future pour aider à combler le fossé entre la situation en laboratoire et le chevet du patient aux soins intensifs.

Thesis Overview

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- The neutrophil and Multiple Organ Dysfunction Syndrome (MODS)

II Experimental research evaluating neutrophil membrane expression and the regulation of neutrophil apoptosis and chemotaxis

- Neutrophil apoptosis and chemotaxis
- Manuscript 1: Neutrophils display a reduction in cell surface expression of TNF receptors but not Fas following transmigration: Implications for the regulation of neutrophil apoptosis
- Manuscript 2: Delayed constitutive and induced apoptosis in human exudate neutrophils is not mediated by NF-κB
- Manuscript 3: Alteration of chemoattractant receptor expression regulates human neutrophil chemotaxis *in vivo*
- Manuscript 4 (discussion): Neutrophil membrane expression regulates neutrophil delivery, function and clearance

III Theoretical research evaluating the clinical application of analytical research to critically ill patients with organ failure

- Clinical significance of analytical experimental research
- Manuscript 5: Multiple Organ Dysfunction Syndrome: Exploring the paradigm of complex non-linear systems
- Treatment of sepsis and MODS: hypotheses for investigation

IV Conclusion

- Conclusions of bench-to-bedside, experimental & theoretical research

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Contribution of Authors

Manuscript 1:

Andrew J.E. Seely wrote and edited the manuscript. Daniel E. Swartz, co-worker in the laboratory, assisted with interpretation and discussion of the data and editing. Betty Giannias, senior laboratory technical assistant, assisted with FACS analysis and ELISA assays. Nicolas V. Christou was research supervisor, providing funding and educational opportunities, providing direction, interpretation, discussion and editing.

Manuscript 2:

Andrew J.E. Seely wrote and edited the manuscript. Betty Giannias, senior laboratory technical assistant, assisted with FACS analysis and laboratory studies. Lorenzo E. Ferri and Jose L. Pascual, co-workers in the laboratory, assisted with interpretation and discussion of the data and editing. Nicolas V. Christou was research supervisor, providing direction, interpretation & discussion of the data, and editing.

Manuscript 3:

Andrew J.E. Seely wrote and edited the manuscript. Jean-Francois Naud performed the chemotaxis assays. Giuseppina Campisi assisted with collection of exudate neutrophils. Betty Giannias senior laboratory technical assistant, assisted with FACS analysis and ELISA assays. Shuqing Liu and Antonio DiCarlo performed the gene expression analysis, supervised by Jean Tchervenkov. Lorenzo E. Ferri and Jose L. Pascual, co-workers in the laboratory, assisted with interpretation, discussion and editing. Nicolas V. Christou was research supervisor, providing direction, interpretation, discussion and editing.

Manuscript 4:

Andrew J.E. Seely wrote and edited the manuscript. Jose L. Pascual created the tables & figures. Nicolas V. Christou was research supervisor, providing interpretation and discussion.

Manuscript 5:

Andrew J.E. Seely wrote and edited the manuscript. Nicolas V. Christou was research supervisor, providing funding and educational opportunities.

Other sections were written and edited by Andrew J.E. Seely.

Introduction to the Neutrophil and Multiple Organ Dysfunction Syndrome

The human polymorphonuclear neutrophil (PMN) has long been the focus of scientific inquiry by surgical investigators. The interest in neutrophil biology has been driven by the neutrophil's paradoxical roles within the human host response, namely its physiologic role in human host defense against bacterial or fungal infection, and its pathologic role in causing host injury in disease states characterized by persistent inflammation. Clinician scientists have thus focused on the neutrophil with one of the following two objectives: (1) understand and minimize impairment in neutrophil mediated host defense in order to decrease rates of infection, or conversely, (2) minimize neutrophil mediated host injury and improve outcome in inflammatory disease. As surgical patients' continue to "live to survive our (neutrophils') paradoxes", surgical scientists investigate neutrophil biology, including PMN delivery, function and clearance, in order to improve the outcome of their patients.

As neutrophils comprise greater than 90% of circulating phagocytes, the first and most abundant cell to be delivered to a site of inflammation, the circulating neutrophil is a major participant and contributor to the inflammatory host response. Other polymorphonuclear phagocytic leukocytes, namely eosinophils and basophils, have more specific functions, and are less involved in the local inflammatory response. Neutrophils contribute to the non-specific immune response, providing defense against bacterial and fungal infection. Mild to moderate abnormalities in neutrophil function are well defined, result in serious impairment of host defense, and manifest clinically as recurrent life-threatening infection; these abnormalities have provided insights to the mechanisms involved in normal neutrophil function.[1, 2] In order to pursue its defense-oriented function, the neutrophil must be delivered to the site of inflammation, migrate from the circulation to the exudate inflammatory environment, and survive to function appropriately and destroy the offending pathogen. Armed with potent means to destroy pathogens, the neutrophil must accomplish its defense-oriented task while minimizing damage to the host.

There are numerous disease states where persistent activation of the immuno-inflammatory host response is responsible for damage to the host. Illness such as rheumatoid arthritis, inflammatory bowel disease, Adult Respiratory Distress Syndrome (ARDS), and Multiple Organ Dysfunction Syndrome (MODS) are characterized by persistent, unremitting, over stimulation of the inflammatory components of the host response. As the effector cell of the inflammatory response, the neutrophil is implicated in host injury. Recent reviews highlight the neutrophils' role in the pathophysiology of rheumatoid arthritis [3-5], ARDS [6], inflammatory bowel disease [7, 8], ischemia-reperfusion [9], and organ damage in MODS[10, 11]. Thus, parallel investigation has been performed investigating the pathophysiologic roles of the neutrophil in the multiple disease states.

Since Tilney first described the sequential failure of organ systems after abdominal aortic aneurysm rupture in 1973, MODS remains "an unsolved problem in postoperative care".[12] MODS is a relatively new clinical entity, thought to be secondary to advances in the acute care of post-operative and post-injury patients, as well as improvements in intensive care medicine.[13-15] The mortality of MODS is between 30-100%, depending on the number of failing organ systems,[16] and it remains the leading cause of mortality in ICU patients. [17-19] Given its clinical importance, our inability to impact its clinical progression and its challenging nature, Multiple Organ Dysfunction Syndrome remains the focus for investigation and discussion within the experimental and theoretical research presented in this thesis.

Several lines of evidence implicate neutrophils in the pathophysiology of MODS. Armed with potent means to protect the host from infection, neutrophils possess a variety of means of inducing tissue injury, including oxygen dependent mechanisms and oxygen independent mechanisms.[20] The PMN plasma membrane contains the enzyme NADPM oxidase that allows the PMN to generate reactive oxygen metabolytes (ROM's). Also, intracellular granules contain microbicidal peptides, proteins and enzymes, including elastase, proteinases and

myeloperoxidase. When a PMN is triggered by any one of a wide variety of inflammatory mediators, there is an almost simultaneous activation of NADPH oxidase (called the respiratory burst) and fusion of the intracellular granules with the plasma membrane releasing proteolytic enzymes into the extracellular environment. The combination of neutrophil derived oxygen radicals (O_2.-), hydrogen peroxidase (H_2O_2), myeloperoxidase, hypochlorous acid (HOCl)[21] and the hydroxyl radical (OH.-)[22] overwhelm protective anti-oxidants and protease inhibitors within the extracellular matrix (such as α_1- proteinase inhibitor, α_2- macro globulin and secretory leukoproteinase inhibitor[23]) leading to endothelial cell and tissue injury.

In addition to possessing the means to cause injury, neutrophil number and neutrophil products are correlated with inflammatory disease and its severity. In animal models, intravascular administration of lipopolysaccharide will enhance lung neutrophil sequestration and increase lung vascular permeability and endothelial injury.[24] Significant leukosequestration (neutrophil deposition) was demonstrated in a burn model in rats in lung, gut, kidney, skin and brain 5 hr. post burn.[25] Injury to the intestinal tract is hypothesised to "prime" neutrophils, and a subsequent secondary event will "activate" the neutrophils to produce distant organ injury.[26, 27] In the lung, the potential for neutrophil mediated damage is illustrated in patients who lack α_1-antitrypsin (hereditary emphysema), a protease inhibitor; these patients are unable to prevent injury secondary to neutrophil elastase.[28] In patients with ARDS, commonly associated with MODS, the degree of lung inflammation correlated with the level of activation of bronchioalveolar lavage (BAL) neutrophils, as well as local levels of pro-inflammatory cytokines.[29] In a separate study, pulmonary vascular permeability was correlated with the number of neutrophils found in the BAL fluid in intensive care patients with ARDS.[30] Granulocyte elastase was found to be significantly elevated in bronchioalveolar fluid of patients with septic shock, compared to hemorrhagic shock.[31] Thus both animal and human studies have demonstrated neutrophils to be capable of, primed for, and correlated with tissue injury.

Prevention of neutrophil sequestration or delivery leading to reduced tissue injury has been demonstrated in multiple animal models. Following rat mesenteric ischemia-reperfusion, both liver and lung injury were eliminated in neutrophil depleted animals.[32] Similarly, blocking CD 11b, a principal component of the integrin receptor necessary for PMN delivery, decreased pulmonary vascular permeability in the same model.[33] Blocking E and L-selectin (also necessary for PMN delivery) significantly reduced neutrophil accumulation in lung and attenuated sepsis-induced lung injury in a porcine sepsis model.[34, 35] In a guinea pig pneumonia model, neutrophils protect against lung injury during low-level bacterial challenge, but enhance lung injury and contribute to mortality during high-level bacterial challenge.[36] Lastly, resolution of inflammation is associated with neutrophil clearance. Apoptosis (physiologic cell death) and subsequent macrophage phagocytosis is the principal mode by which aging neutrophils are cleared from circulation. The resolution of inflammation has been linked to increased macrophage engulfment of apoptotic neutrophils.[37]

Given that neutrophils are capable of and correlated with inflammatory host injury, and blocking neutrophil delivery will attenuate host damage in animal models, the neutrophil is widely believed to play a role in the pathogenesis of persistent inflammatory disease and MODS. In addition, acquired or congenital deficiency of neutrophil function is associated with increased incidence and severity of bacterial and fungal infection. As a principal mediator of physiologic and pathologic function within the host response, investigators have focussed on the neutrophil in an effort to eventually be capable of modulating neutrophil function within the host response such that clinical outcome is improved.[38]

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Commentary

Given the fundamental objective of improving clinical outcome in critically ill patients with illness characterised by overwhelming and persistent inflammation, the thesis is divided into two distinct components. The first part of the thesis represents experimental investigation, including a discussion and review of the literature regarding the role of the neutrophil cell membrane in the regulation of neutrophil delivery and clearance, both in health and in disease. Given that surgeons are engaged in the care of patients, all basic science experimental "bench" surgical research is performed with the goal of bringing it to the "bedside", and contributing to the development of effective therapeutic intervention.

While performing this experimental research investigating the role of the neutrophil in the human host response, it became evident that there is a vast literature of parallel streams of investigation regarding the cellular and molecular mechanisms inherent to host defense and inflammation. Thus, the second component of the thesis represents a theoretical evaluation investigating the role and significance of analytical experimental research within the systemic host response. No different from experimental work, theoretical research endeavors to improve clinical outcome by providing novel and testable hypotheses, in this discussion, applied to the clinical problem of organ dysfunction. A hallmark of surgical scientist research, the bench-to-bedside theme demands critical appraisal of the clinical significance of laboratory investigation.

Introduction to Neutrophil Apoptosis and Chemotaxis

As the principal circulating phagocyte, the neutrophil is the first inflammatory cell delivered to a site of inflammation, then stimulating further inflammatory events such as the emigration of monocytes[1] and the generation of tissue edema.[2] Neutrophils possess the means to cause tissue injury,[3] and in large enough numbers, induce host injury to both endothelial cells[4] and parenchymal cells.[5] Neutrophils amplify the inflammatory process by degrading matrix proteins into chemotactic fragments[6] and releasing chemoattractants and pro-inflammatory mediators.[7] The subsequent tissue injury and clinical sequelae of neutrophil accumulation in the exudate environment are strongly related to the tissue load or number of cells present. As the neutrophil is a terminally differentiated cell, incapable of self-renewal, the regulation of neutrophil number at inflammatory sites or otherwise is a function of both their rate of delivery, as well as their rate of clearance from that environment.

Neutrophil delivery to, function in, and clearance from the inflammatory microenvironment have been extensively studied, and are reviewed in greater detail within the discussion paper regarding the experimental research (Manuscript #3). Response to chemoattractants and neutrophil chemotaxis is the principal determinant of neutrophil delivery,[8] and neutrophil cell death by apoptosis is the principal means by which neutrophils are cleared from the exudate environment.[9] Thus, neutrophil chemotaxis and apoptosis and their regulation are evaluated to understand neutrophil delivery and clearance, and the regulation of the physiologic and pathologic impact of neutrophil sequestration.

Neutrophil Delivery and Chemotaxis

Leukocytes have long been known to follow chemical signals both *in vitro* and *in vivo*. For over two decades, N-formylated peptides produced by bacteria (e.g. FMLP), polypeptides (e.g. C5a) and lipids (e.g. leukotriene B4) have been known to act as chemoattractants for various

leukocyte populations[10-12]. C5a is formed during complement activation, and serves as a general leukocyte chemoattractant, acting upon monocytes, neutrophils, eosinophils and basophils and leading to aggregation and degranulation of neutrophils.[13] The C5a receptor (C5aR) has been identified on neutrophils and mediates the C5a effect on PMN.[14, 15] Other leukocyte chemoattractants serve to attract specific sub-populations of leukocytes. Interleukin-8 (IL-8) is the prototype of a family of cytokines that possess specific chemotactic activity for neutrophils. IL-8 is produced by virtually all nucleated cells in response to inflammatory stimuli such as endotoxin, TNF-α and interleukin-1 (IL-1).[16] There are two receptors for IL-8, type A receptor, IL-8 RA (C-X-C R1) and type B receptor, IL-8 RB (C-X-C R2). Both receptors coupled to G proteins, are expressed on neutrophils, differ in their ligand selectivity, and have uncertain biologic differences.[17]

Chemokines that are chemotactic to neutrophils include IL-8, epithelial cell derived neutrophil activating peptide (ENA-78), neutrophil activating peptide-2 (NAP-2), growth related oncogene (GRO-alpha, beta, gamma), macrophage inflammatory protein-2 (MIP-2) alpha and beta. These chemokines are structurally similar, consisting of the first two cysteines seperated with an amino acid; thus are referred to as C-X-C chemokines. A separate family of chemokines are known as the C-C chemokines, as the first two cysteine amino acid residues are in juxtaposition. Monocyte chemoattractant protein-1,2 and 3 (MCP-1, MCP-2, MCP-3), macrophage inflammatory protein-1 (MIP-1) alpha and beta, and RANTES are members of the C-C family. The activity of the C-C supergene family of chemokines is predominantly oriented towards monocytes. [18]

Recruitment of specific subpopulations of leukocytes to inflamed tissue is a property of the immune host response. For example, neutrophil accumulation occurs in response to acute bacterial infection, eosinophils will be recruited to sites of chronic allergic inflammation or parasitic infection, and monocytes are found in abundance in chronic inflammatory diseases. The discovery of chemotactic cytokines, or chemokines, with selective activity for specific

leukocyte subsets has assisted in the understanding of mechanisms leading to specific leukocyte delivery.

Measurement of Chemotaxis

The measurement of chemotaxis involves a measure of the ability of a cell to undergo directional migration in response to a stimulus. Chemotaxis represents a cell function, and must be measured over a specific time period. Although variations exist, chemotaxis assays evaluate the movement of cells incubated on one side of an inert substance such as a filter or membrane, in response to a chemoattractant gradient provided by a solution on the other side: a technique developed by Boyden in 1962. By varying chemoattractants and their concentration, the chemotactic function of a population of cells may be determined by counting the number of cells within the filter or membrane. In addition, analogous transmigration assays evaluate the number of cells that migrate across an intact endothelial cell monolayer in response to a chemoattractant gradient. This technique evaluates the response of the leukocyte to a chemoattractant along with the presence of endothelial cells. Both techniques determine the impact of individual chemoattractants independent of the presence of other chemoattractants.

Neutrophil Clearance and Apoptosis

The neutrophil has long been known to possess powerful histotoxic properties; however, the fate of the neutrophil at an inflammatory site, and the means by which it is cleared has received little scientific inquiry until recently. Although it was assumed that neutrophils would undergo necrotic cell death followed by macrophage phagocytosis, the lack of inflammation associated with normal neutrophil delivery to healthy tissues and the inability for macrophages to clear large numbers of neutrophils do not support this means of neutrophil clearance. The further elucidation of neutrophil clearance awaited the discovery of a process leading to cell death that is entirely distinct from necrosis.

Macrophages have been noted to be phagocytose neutrophils since Metchnikoff studied the resolution of inflammation in the latter 19'th century. In 1982, macrophages were confirmed to clear neutrophils from the peritoneal cavity following peritonitis.[19] In the same year, macrophages were noted to ingest neutrophils only following an aging process; freshly isolated neutrophils from humans were not ingested, but increasing numbers of neutrophils were phagocytosed over time.[20] John Savill and Chris Haslett demonstrated that senescent neutrophils are cleared through the process of apoptosis, and this process leads to their recognition and engulfment by macrophages.[21] Macrophage ingestion of neutrophils has subsequently been reported in a variety of tissues including joints,[21] lungs,[22] and kineys[23].

Apoptosis, a term introduced by Kerr in 1972 from the Greek word for "falling off",[24] denotes a form of cell suicide. A cell undergoing apoptosis shrinks, loses intercellular contacts, the nucleus undergoes karyorrhexis (fragmentation) and karyolysis (dissolution), chromatin undergoes condensation, DNA undergoes internucleosomal cleavage (leaving DNA in finely chopped pieces of ~180-200 base pairs), and the cell ultimately breaks up into apoptotic bodies containing pyknotic nuclear debris.[25] Surrounding cells, notably macrophages, as well as cells that are not "professional phagocytes" such as epithelial cells, phagocytose the apoptotic bodies. Considered the counterpart of mitosis, apoptosis is a remarkable means by which cells undergo cell suicide with no resultant inflammation, as no cytosolic contents are released into the extracellular environment. This is distinct from cell death secondary to oncosis and necrosis, where spillage of cellular contents and cell destruction lead to an inflammatory response.[25]

Once neutrophils undergo delivery from the intravascular to the extravascular milieu, it is generally accepted that they do not re-enter the circulation, and instead meet their fate at the site of inflammation to which they have been delivered.[9] Because of the rapidity of the process of apoptosis, and the lack of inflammation associated with it, the process of apoptosis is a

highly efficient means of clearing neutrophils delivered to various organs. Although neutrophils may be cleared by other means, such as direct expulsion in sputum or feces, or may re-enter the circulation if only sequestered in capillaries, the majority of exudate neutrophils trapped at inflammatory sites are cleared through the process of apoptosis.[9, 26]

Neutrophils undergo both constitutive and induced apoptosis. Constitutive apoptosis is pre-programmed, whereas induced apoptosis is stimulated by surface receptors bearing the cytoplasmic death domain. These include the TNF-R1 receptor and Fas, both constitutively expressed on neutrophils. Although TNF-α appears to have differential effects on neutrophil apoptosis depending on the activation state of the neutrophil,[32] if circulating resting neutrophils are taken from human controls, TNF-α will dramatically increase apoptosis rates.[33] In addition to TNF-α mediated apoptosis, Fas Ligand (FasL) induced apoptosis of phagocytes has received considerable attention as it plays a critical role in the regulation T cell development and apoptosis,[34-36] anti-Fas antibodies accelerate neutrophil apoptosis to a greater degree than lymphocytes and monocytes,[37] and activated T-cells secrete soluble FasL.[38] Constitutive expression of both Fas and FasL on the neutrophil cell surface (a unique feature amongst the phagocytes) suggests that the neutrophil may be irrevocably committed towards an apoptotic cell death.[39] The importance of soluble FasL, soluble TNF-α, and membrane bound FasL in the regulation of neutrophil apoptosis in the exudate environment remains under investigation.

Both constitutive and induced neutrophil apoptosis are subject to modulation by surrounding mediators. Although neutrophils are highly susceptible to rapid apoptosis following incubation with anti-Fas IgM antibody, this may be suppressed with a variety of inflammatory mediators, including G-CSF, GM-CSF, IFN-γ, and TNF-α. [39, 40] Thus, elevated levels of TNF-α in the exudate inflammatory environment stimulating the release of these mediators may prolong neutrophil survival by inhibiting the Fas-FasL apoptotic pathway. Other inflammatory mediators that delay constitutive neutrophil apoptosis include Interleukin-6[41], Interleukin-2[42], G-CSF[43], GM-CSF, C5a and lipopolysaccharide (LPS)[33, 44]. Neutrophils also undergo apoptosis following

bacterial ingestion providing a mechanism for neutrophil clearance in the inflammatory milieu.[45] Immune complexes modulate the rate of neutrophil apoptosis.[46] Engagement of adhesion molecules will impact upon neutrophil apoptosis; Watson demonstrated a delay in apoptosis after cross-linking CD11a and CD11b, however, cross-linking L-selectin accelerated neutrophil apoptosis.[47] Thus, neutrophil apoptosis demonstrates remarkable ability for modulation, providing a glimpse of the complexity of the host response.

Measurement of Apoptosis

Neutrophil apoptosis has only recently come under scrutiny with the advent of accurate techniques in evaluating apoptosis rates. Techniques that have been developed to measure apoptosis involve counting of cells displaying apoptotic morphology under light microscopy. Limitations of this technique include the time consuming nature and interpretation bias inherent to manual counting. Other techniques make use of specific cellular alterations during apoptosis. Given the internucleosomal cleavage of DNA during apoptosis, the demonstration of DNA laddering on gel electrophoresis (first introduced by Wyllie in 1984) provides a reliable means of determining the presence or absence of apoptosis in a population of cells. For example, this technique may be useful to detect the apoptosis under separate culture conditions.

Flow cytometric methods include means of quantifying apoptosis rates. These include staining cellular DNA with Propidium Iodide (PI)[27]; following permeabilization of the neutrophil membrane, cells undergoing apoptosis and DNA cleavage stain with a hypodiploid DNA peak, non-apoptotic cells maintain a diploid peak, and both can be seen and quantified with flow cytometry. Also utilising flow cytometry, the use of ANNEXIN V and PI can differentiate apoptotic and necrotic cells. Phosphatidylserine (PS), which normally resides on the inner membrane leaflet, is expressed on the outer membrane as an early feature of apoptosis, and is implicated in macrophage recognition of apoptotic cells.[28, 29] Using ANNEXIN V to specifically bind PS positive cells, early apoptosis is detected by staining positively with ANNEXIN conjugated with FITC; cells staining PI are recognised as necrotic as their cell membrane is not

intact (no membrane permeabilization step performed).[30, 31] Advantages of the PS binding technique include a clear differentiation between necrotic and apoptotic cells, the detection of early apoptosis, and reproducible results with small numbers of cells. With this technology, it is also possible to perform immunohistochemistry to stain apoptotic cells in tissue sections not amenable to flow cytometry; numerous staining kits are commercially available.

Regarding the evaluation of apoptosis, it is essential to recognise that certain techniques of measurement reveal the presence or absence of apoptosis (e.g. DNA laddering), or reveal a rate of apoptosis, subject to change over time. Given that different techniques evaluate apoptotic cells at varying stages undergoing the process of apoptosis, different techniques will yield differing results regarding the absolute proportion of cells undergoing apoptosis at any given time. For example, the expression of PS on the outer surface of the neutrophil membrane is an early feature of apoptosis, compared to endonucleosomal DNA cleavage, a late feature of apoptosis. Thus, evaluating apoptosis rate in human neutrophils isolated and cultured for 6 hours will reveal a higher rate of apoptosis if binding PS with ANNEXIN, compared with PI hypodiploid peak staining cells with nuclear changes. Nonetheless, apoptosis measurement techniques can reliably and reproducibly demonstrate changes in apoptotic rates.

In discussing neutrophil apoptosis rates, and their means of measurement, it is useful to observe that neutrophil apoptosis rates are sigmoid shaped curves akin to the hemoglobin dissociation curve, and are expressed as percent apoptosis vs. time. Changes in apoptosis rates reflect a shift in this curve to the right (delayed apoptosis) or the left (increased apoptosis). Different techniques capture a separate time curve with separate coordinates on the (time) x-axis, and measurement of apoptosis rates at distinct time points yield different values. If cultured long enough, all techniques demonstrate 100% cell death, for example, in ~12 hours as detected by ANNEXIN staining, and ~ 48 hrs using Propidium Iodide staining. Thus, comparison of apoptosis rates can be performed using standardised culture conditions, apoptosis measurement techniques, and incubation times. The apoptosis curve, or rate of cell

death, must also be considered when discussing the clinical implications of neutrophil apoptosis.

Therapeutic Implications

As critical components to delivery and clearance, the study of neutrophil chemotaxis and apoptosis, is performed with the goal of altering outcome in disease. Authors seek to identify how their research may impact upon disease incidence or progression. Experimental papers often refer to this issue in the final paragraph of the discussion, As an example, the elucidation of adhesion molecules and the step-wise events involved in leukocyte delivery have led to the suggestion of targeted therapy to help minimise inflammation.[48] Evaluation of the differences between TNF RI and TNF RII has led to the suggestion of focused anti-inflammatory therapies directed at one of the receptors.[49] In analogous fashion, understanding the regulation of neutrophil apoptosis is performed with the goal of developing strategies that minimise inflammation by altering apoptosis rates.[50] The fundamental goal of all research regarding neutrophil delivery and clearance is thus appropriately oriented towards the improvement of clinical outcome.

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Commentary

The experimental component of this thesis began with an interest in the human neutrophil apoptosis; literature review along with preliminary investigations in the laboratory led to the investigation of neutrophil apoptosis in circulating and exudate neutrophils in human volunteers. Given that TNF-α stimulates neutrophil delivery to inflammatory sites, and is also reported as causing rapid neutrophil apoptosis, neutrophils were hypothesised to shed their TNF receptors following transmigration so that they would not undergo TNF induced apoptosis. The first manuscript represents the evaluation of that hypothesis.

Manuscript #1

Neutrophils display a reduction in cell surface expression of TNF receptors but not Fas following transmigration: Implications for the regulation of neutrophil apoptosis

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Manuscript 1: Neutrophils display a reduction in cell surface expression of TNF receptors but not Fas following transmigration: Implications for the regulation of neutrophil apoptosis

Abstract

Objectives: To test the hypothesis that loss of polymorphonuclear neutrophil (PMN) TNF-α receptors (TNFR) during transmigration renders the exudate PMN refractory to TNF-α mediated stimulation of apoptosis; and to investigate the surface expression of Fas on both circulating and exudate neutrophils.

Design: A prospective cohort study.

Setting: A tertiary care hospital surgical laboratory.

Participants: 21 healthy human volunteers.

Interventions: All subjects had circulating PMN (venipuncture) and exudate PMN collected (skin window method).

Main Outcome Measures: Circulating and exudate PMN were incubated in culture medium (1.0×10^6 PMN/ml) alone or with TNF-α [100 ng/ml]. Apoptosis was evaluated using flow cytometry (ANNEXIN V-FITC and Propidium Iodide). TNF-α-PE and anti-human Fas-FITC were used to evaluate PMN TNF receptors (TNFR) and surface expression of Fas.

Results: Exudate PMN have a significant delay in apoptosis rates when compared to circulating PMN. The % PMNs expressing TNFR was significantly diminished after exudation (80 ± 15 % vs. 33 ± 9%; p=0.0001), as was the median channel number of TNF-α PE fluorescence (8.1 ± 1.6 vs. 5.2 ± 0.5; p=0.001). However, the expression of Fas was unchanged following transmigration (% positive for Fas: 98.7 ± 0.7 % vs. 92.8 ± 3.4%, NS; Fas Ab-FITC median channel fluorescence: 12.2 ± 1.1 vs. 13.1 ± 1.2, NS). Exposure of exudate PMNs to TNF-α failed to increase their rate of apoptosis.

Conclusions: Exudate PMN are confirmed to have delayed apoptosis. Loss of TNF-α receptors during transmigration leads to increased PMN survival in the extravascular inflammatory milieu.

Neutrophils display a reduction in cell surface expression of TNF receptors but not Fas following transmigration: Implications for the regulation of neutrophil apoptosis

Introduction

The study of neutrophil apoptosis has been stimulated by the belief that neutrophils are implicated in the pathogenesis of persistent inflammatory states, including Multiple Organ Dysfunction Syndrome (MODS) and Adult Respiratory Distress Syndrome (ARDS). Neutrophils possess a variety of means of inducing tissue injury[1], including the secretion of connective tissue proteases capable of tissue destruction in septic shock[2]. There is an association between neutrophil number and level of activation and the degree of inflammation[3], as well as pulmonary vascular permeability[4], in patients with ARDS. Prevention of neutrophil sequestration will lead to reduced tissue injury in multiple animal models.[5,6,7] In addition, clearance of neutrophils is associated with resolution of inflammation.[8] Neutrophil apoptosis, a genetically programmed form of cell death fundamentally distinct from necrosis, is the principal mode by which senescent neutrophils are cleared from circulation.[9] It has been suggested that an imbalance of granulocyte apoptosis and necrosis may be important in the pathogenesis of inflammatory disease.[10] Thus, the study of neutrophil apoptosis is vital to better understand the persistent inflammation common to patients with MODS and ARDS.

Materials and Methods

Subjects: 21 human healthy volunteers were recruited for the study. Exclusion criteria included history of infection within the previous 48 hours, severe chronic illness, immunosuppressive medication, and known malignancy. The study was approved by the Committee of Human Experimentation, Royal Victoria Research Institute.

Materials and reagents: All preparations of neutrophils were kept in polypropylene tubes to prevent adherence. Neutrophil incubation was in dMEM (Gibco, Ontario, Canada), supplemented with 10% FBS, 1% streptomycin/penicillin and 1% L-glutamine (Gibco, Ontario, Canada). Recombinant human (rh) TNF-α (Sigma Chemical Co.) was stored at -70°C in aliquots of 60 μl at a concentration of 2 ng/μl.

Isolation of circulating neutrophils : 7 cc of whole blood was obtained from the subjects using heparinized vacuum-sealed tubes (Becton Dickenson, Franklin Lakes, NJ) and circulating neutrophils were isolated using Macrodex/Dextran-70 (Pharmacia Laboratories, Piscataway, NJ), gravity sedimentation (60 minutes) followed by Ficoll-Hypaque (Pharmacia Laboratories) centrifugation. Cells were counted with hemocytometer following staining with Turk's solution, and suspended in media at a concentration of $1x10^6$cells/ml.

Isolation of exudate neutrophils: Skin window chambers were manufactured at the McGill University Workshop and used as previously described.[11] The techniques follows the one described by Zimmerli and Galin.[12] Briefly, exudate neutrophils were collected from skin windows placed on the volar aspect of the forearm. The forearm was sterilized with 10% proviodine-iodine topical antiseptic followed by 70% isopropyl alcohol. 360 mm Hg vacuum suction was applied through a plastic template for 60-90 minutes until 4 even 1.0 X 1.0 cm blisters formed. These blisters were unroofed using sterile scissors and a template consisting of four 1.0 X 1.0 cm open-bottomed chambers was tightly applied using wide adhesive tape. The chambers were filled with 10% autologous serum and the superior apertures sealed with a sterile covering. After 16-18 hours, the exudate fluid, consisting of an almost pure (>98%) suspension of neutrophils, was aspirated. The chambers were rinsed three times with normal saline and the fluid was transported immediately on ice to the laboratory. The neutrophils were sedimented at 400xg at 4°C for 5 minutes. Neutrophil viability was confirmed to be > 95% using Propidium iodide exclusion. Neutrophils collected from skin windows were counted using

a hemocytometer after staining with Turk's solution, and suspended in media at a concentration of $1x10^6$ cells/ml.

Neutrophil Incubation: Both circulating and exudate neutrophils were incubated in culture medium @ 37°C with 5% CO2 for variable periods of time. Gentle shaking of the cells was accomplished with a Thermolyne Rotomix (Diamed, Mississauga, Ontario). $1x10^6$ cells were incubated in 1 ml of media in polypropylene tubes. Varying concentrations of TNF-α (see results) were added to the cell suspension immediately prior to cell incubation.

Quantification of neutrophil apoptosis and necrosis: Following incubation, cells were removed from the incubator, sedimented (400xg @ 4°C for 5 minutes), washed with PBS, sedimented and suspended in 1 ml binding buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 100 µl of the cell suspension in binding buffer was added to 5 µl Annexin V-FITC (Pharmingen Canada, Mississauga, Ontario) and 10 µl Propidium Iodide (PI - Sigma, Oakville, Ontario) and incubated in the dark for 30 minutes @ room temperature. Following incubation, 400 µl of binding buffer was added to cell suspension, and flow cytometric analysis followed immediately.

Cells were analysed with a Becton Dickenson FACScan® cytofluorometer. This technique takes advantage of a redistribution of plasma membrane phospholipids in the early stages of apoptosis. Phosphatidylserine (PS), which normally resides on the inner membrane leaflet, has been shown to be expressed on the outer membrane as an early feature of apoptosis regardless of initiating stimulus.[13] Annexin V conjugated to FITC will bind specifically to PS and thus can be used to quantify the number of cells expressing PS and thus undergoing apoptosis.[14,15] PI was used to stain cellular DNA. For PI to stain DNA, the cellular membrane must not be intact. These cells are thus determined to be necrotic. PI fluorescence (FL2) was plotted versus Annexin V FITC fluorescence (FL1); the data were registered on a logarithmic scale. At least 5000 events were recorded for each sample.

Cell surface analysis: Whole blood, collected in 7 cc sterile vacutainer® tubes using EDTA as anti-coagulant (Becton Dickinson, Franklin Lakes, NJ) was kept on ice until analysis (begun immediately). Exudate neutrophils were analysed as soon as they were suspended in media at a concentration of $1x10^6$ cells/ml. TNF receptor density was analysed using TNF-PE (R&D Systems, Minneapolis, Minn.) in the following manner. In 5 ml tubes, 10 µl of TNF-PE was incubated along with 100µl of whole blood for 30 minutes at room temperature in the dark. Subsequently, 2 ml monoclonal lysing solution (1L dH_2O, 8.26 g NH_4Cl, 1 g. $KHCO_3$, 0.037 G Na_2EDTA) was added to the mixture, and again stored in the dark for 10 minutes. After sedimenting the neutrophils (400xg @ 4°C for 5 minutes), the cells were washed twice with PBS with 1% FBS and 0.1% Azide (Sigma, Oakville, Ontario). Cells were finally suspended in 2% paraformaldehyde (Fisher, Ontario) and promptly analysed by Flow Cytometry. Specificity of the TNF-PE reaction was ascertained using a TNF blocking antibody (R&D Systems, Minneapolis, Minn.) prior to incubation. Exudate neutrophils were prepared in exactly the same manner, except the lysing step was omitted. An antibody (mouse anti-human IgG1) to Fas conjugated with FITC (Pharmingen Canada, Miss., Ontario) was used to assess the density of expression of Fas on the outer membrane of the neutrophil in the same manner (10 µl Fas Ab-FITC and 100 µl of whole blood or exudate neutrophils in media). Median intensity of fluorescence was chosen over mean as the fluorescence distributions were often skewed. The threshold to determine positivity was fixed so that the negative control was at least 95% negative. The negative control used for TNF-PE was Streptavidin-PE (R&D Systems, Minneapolis, Minn.), and the negative control for the Fas-FITC was IgG1-FITC (Pharmingen Canada, Miss., Ontario).

Flow Cytometer calibration: The FACScan was calibrated weekly to ensure no overlap of FL1 and FL2 spectra using Calibrite® beads (Becton Dickinson, Mississauga, Canada . In addition, QC3 beads (Becton Dickinson, Mississauga, Canada) were used to calibrate the intensity of fluorescence for every use of the FACScan. The QC3 beads allowed for standardizing of the

intensity of fluorescence, which is proportional to the density of surface receptors. The settings both for apoptosis and for cell surface analysis were altered when required to ensure the intensity of fluorescence of the beads remained the same throughout the study. Cells were plotted on forward scatter vs. side scatter; debris was excluded using a neutrophil gate.

Data analysis: All results are expressed as mean ± standard deviation. Statistical significance ($p<0.05$) was determined using the paired student's T-test, with the Bonferroni correction when multiple tests were performed simultaneously. Statistical significance is indicated on the figures to follow. The number of subjects used for each test may vary as the yield of the exudate cells would also vary.

Results

Exudate neutrophil apoptosis was delayed when compared to circulating neutrophil apoptosis (Figure 1). PMN apoptosis of both all neutrophils equalized and reached a plateau after 24 hours.

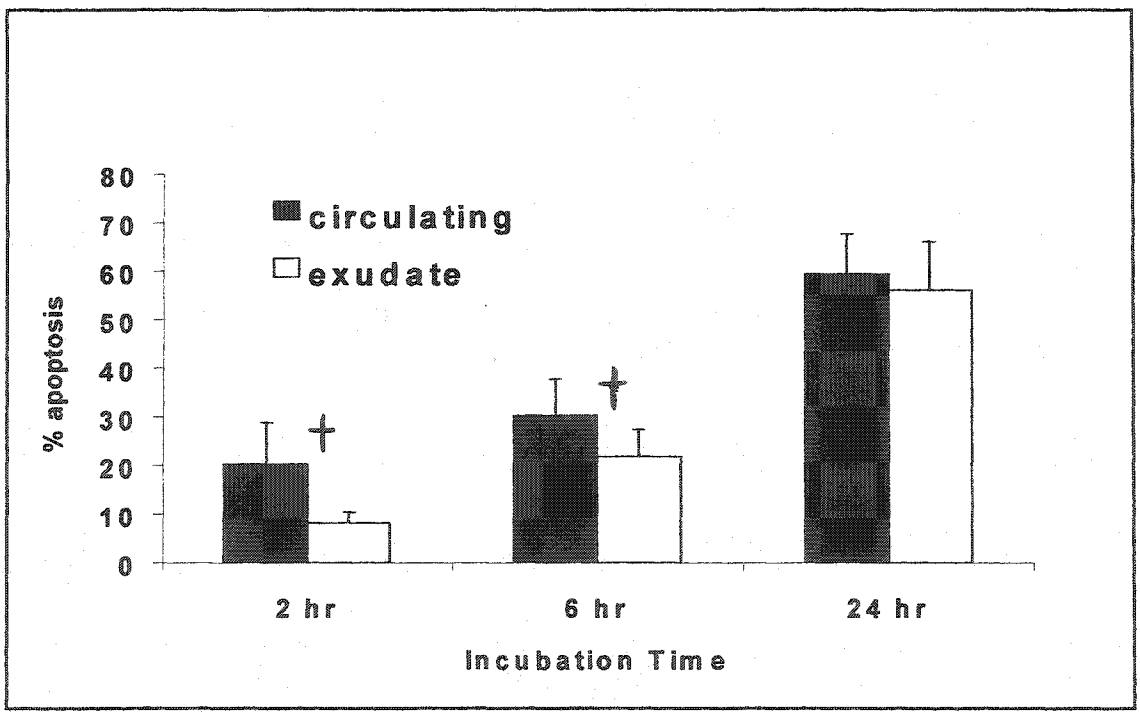

FIGURE 1: Baseline apoptosis is significantly decreased in exudate PMN following 2 & 6hr, but not 2(hr incubation in media, compared to circulating PMN († $p \leq 0.001$).

A dose response curve for TNF-α with circulating PMN from seven healthy controls was performed (Figure 2). PMN apoptosis increased from a 15% baseline to 28% at 100 ng/ml TNF-α ($P<0.05$).

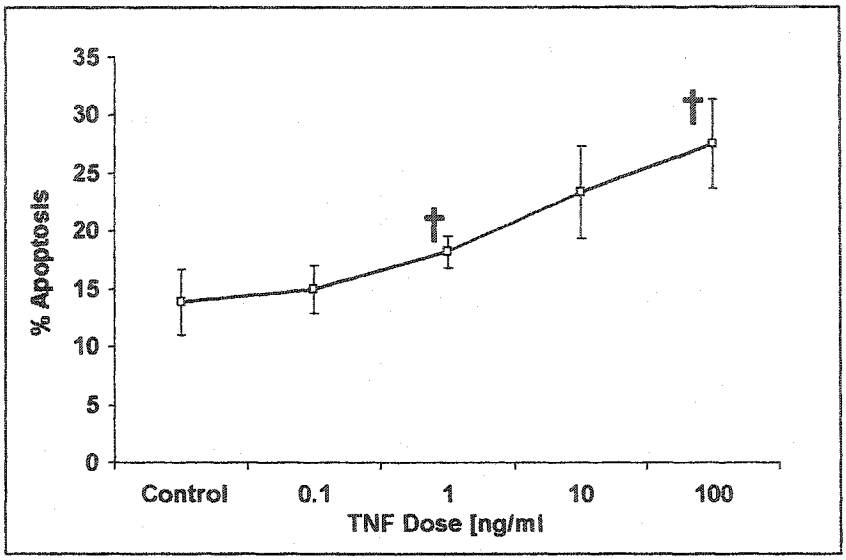

Figure 2: TNF dose response curve in circulating PMN (2 hr incubation, n=7; mean ± SD, † $p < 0.05$).

In contrast to circulating PMN, incubation with low dose or maximal dose TNF-α (Figure 3) failed to accelerate apoptosis in exudate neutrophils following 2, 6, or 24 hours of incubation.

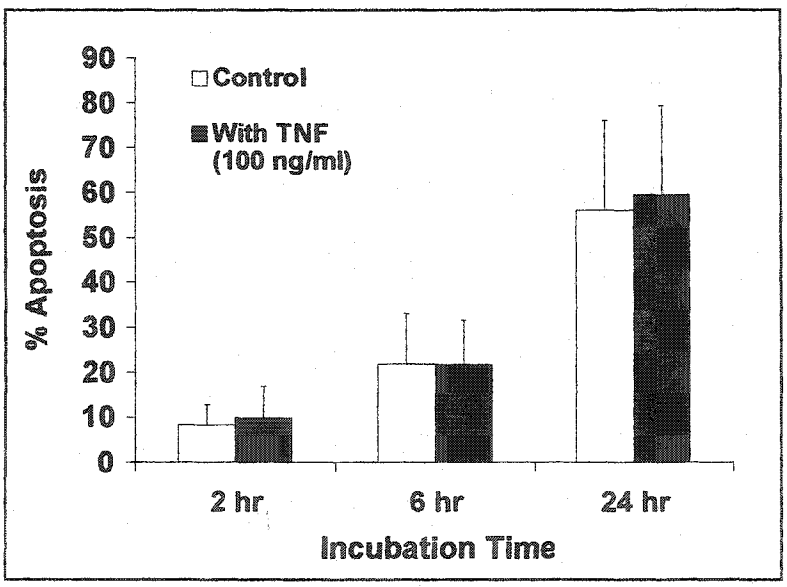

Figure 3: Exudate PMN apoptosis incubated with TNF-α (TNF 100 ng/ml, mean±SD, 2hr (n=2), 6hr (n=20), & 24hr (n=8), all NS).

Exudate and circulating neutrophils were analysed by FACScan to determine their binding of TNF, conjugated with PE for FACS analysis. Median intensity of fluorescence (MIF) of TNF-PE was significantly decreased in exudate neutrophils when compared to circulating cells (Figure 4); and the number of neutrophils positively staining with TNF-PE was significantly reduced in exudate

neutrophils compared to circulating neutrophils (Figure 5). The median intensity of fluorescence as well as the number of cells that were positively expressing Fas were not significantly different between circulating and exudate neutrophils (Figure 4 and 5 respectively).

Discussion

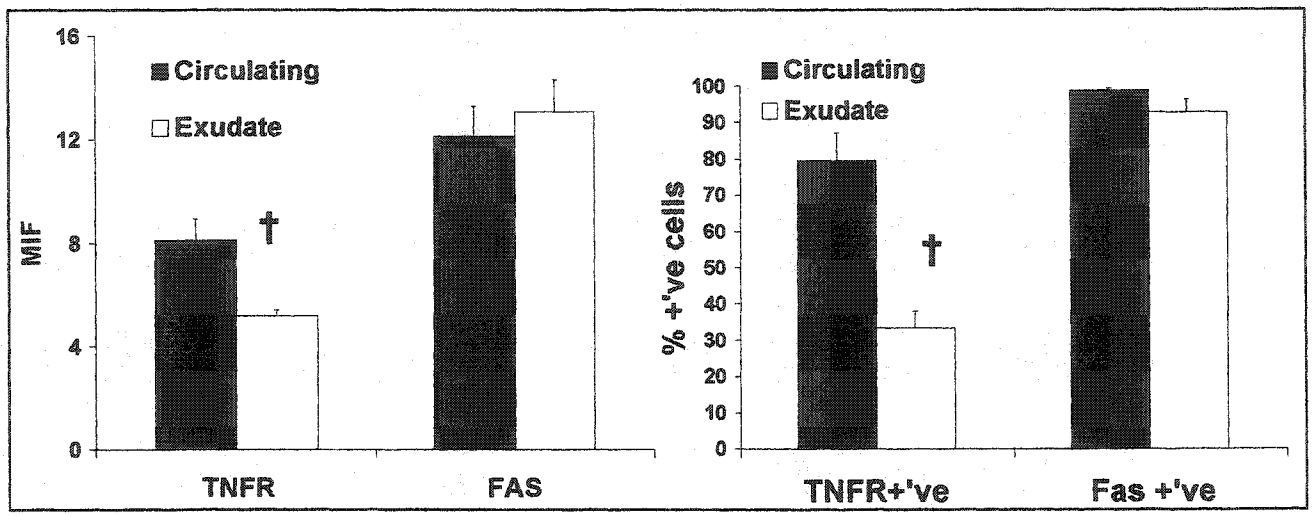

Figure 4: Median intensity fluorescence (MIF) for circulating & exudate PMN (TNFR & FAS expression, mean±S.D, † p=0.001)

Figure 5: Percentage of circulating & exudate PMN expressing TNFR & Fas (mean ± S.D, † p<0.0001).

There are two potential mechanisms suggested by previous studies to explain the observed delay in apoptosis following transmigration. First, neutrophil adhesion to the endothelial cell during transmigration may cause a delay in apoptosis. Watson has demonstrated a delay in apoptosis after cross-linking CD11a and CD11b, however, cross-linking L-selectin accelerated neutrophil apoptosis.[16] The authors conclude that adhesion molecules may serve as modulators of neutrophil apoptosis. The second mechanism capable of causing delayed neutrophil apoptosis in exudate cells involves the products of the inflammatory process. These include lipopolysaccharide (LPS), and a host of inflammatory cytokines including IL-1, IFN-γ, G-CSF & GM-CSF, and IL-2.[17-20] As neutrophil survival in the extravascular environment is necessary for eradication of the inflammatory stimulus, it is likely that multiple pathways may lead to a delay in apoptosis in the exudate inflammatory environment.

As with the delay in apoptosis during transmigration, the loss of TNF-α receptors may be secondary to engagement of adhesion molecules. One report suggests that neutrophil adherence, involving L-Selectin and the CD11/CD18 integrins, mediates downregulation of the TNF receptor.[17] Thus, the neutrophil may shed its TNF receptor prior to even entering the exudate environment. This would suggest that TNF-α will influence the neutrophil in an endocrine fashion only (i.e. in circulation), and not through a paracrine manner (i.e. in the exudate environment). This may help explain the uncontrolled neutrophil activation seen in severe sepsis when systemic levels of TNF-α are elevated.

Neutrophil activation in the inflammatory milieu may provide the second mechanism for loss of neutrophil expression of TNF-α receptors. TNF-α receptors are shed when neutrophils are activated *in vitro* with FMLP.[18] The authors also noted that neutrophils shed their TNF-α receptors rather than internalize them and/or decrease their synthesis of the TNFR. TNF-α itself has also been demonstrated to cause a loss of neutrophil TNF receptors (due to receptor endocytosis), in addition to other inflammatory mediators, including C5a, Platelet activating Factor, leukotriene B4, endotoxin, and FMLP.[19] The same authors found that TNF-α mediated activation *in vitro* is more potent when neutrophils are adherent, rather than in suspension. As TNF-PE was used to detect both TNF receptors on the neutrophil in this experiment, the observed reduction in TNF binding may be secondary to downregulation of either the 55 kD (TNFR1), the 75 kD receptor (TNFR2), or both. Nonetheless, the loss of TNF receptors on neutrophils appears to be fundamentally important to neutrophil survival in the inflammatory exudate environment.

Although the loss of TNF-α receptors helps explain neutrophil survival in the extravascular inflammatory environment, the mechanism leading to neutrophil apoptosis and clearance and the resolution of inflammation remains unsolved. The Fas-Fas Ligand (FasL) apoptotic pathway has received considerable attention in the regulation of phagocyte apoptosis as it

plays a critical role in the regulation T cell development and apoptosis.[20,21,22] Although neutrophils are highly susceptible to rapid apoptosis following incubation with anti-Fas IgM antibody, this may be suppressed with a variety of inflammatory mediators, including G-CSF, GM-CSF, IFN-γ, and TNF-α.[23] Thus, elevated levels of TNF-α in the exudate inflammatory environment may prolong neutrophil survival by inhibiting the Fas-FasL apoptotic pathway. The importance of soluble FasL versus membrane bound FasL in the exudate environment remains under investigation. Activated T-cells has been shown to secrete soluble FasL.[24] Constitutive expression of both Fas and FasL on the neutrophil cell surface (a unique feature amongst the phagocytes) suggests that the neutrophil may be irrevocably committed towards an apoptotic cell death.[25] Regardless of the exact mechanism, our demonstration that expression of Fas is maintained following transmigration lends further support to the Fas-FasL pathway in PMN clearance in the exudate environment. Neutrophils also undergo apoptosis following bacterial ingestion[26] providing a second possible mechanism for neutrophil clearance in the inflammatory milieu. The full understanding of neutrophil transmigration and subsequent clearance from the exudate inflammatory environment may provide important clues to prevent or treat persistent inflammatory states responsible for significant morbidity and mortality in the Surgical Intensive Care Unit.

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Commentary

Completion of the first experiment and manuscript further developed an interest in evaluating changes in neutrophil membrane receptor expression in humans *in vivo*. Receptor alteration appeared to be an interesting means by which cell function was altered. However, although a decrease in TNF receptors in exudate PMN the first manuscript was reported, the assay actually evaluated TNF binding to neutrophils (intensity of fluorescence of binding to TNF conjugated with PE), not receptor expression explicitly. Further preliminary experiments in the laboratory suggested that Fas Ligand (FasL) was downregulated in exudate neutrophils. Because neutrophils express both Fas and FasL, and because exudate PMN exist in close proximity at a site of inflammation, it was hypothesised that circulating neutrophils in close proximity would undergo *contact apoptosis* (apoptosis on one neutrophil expressing FasL causing apoptosis of an adjacent neutrophil expressing Fas); and the loss of FasL would render exudate neutrophils refractory to this novel apoptotic mechanism, allowing for large numbers of neutrophils to survive en masse at sites of inflammation. The hypothesis of membrane alteration leading to altered cellular function also suggests that interventions designed to alter membrane expression may be possible and potentially therapeutic. Thus, a second observational experiment was designed to evaluate constitutive, induced and contact apoptosis as well as receptor expression in human circulating and exudate neutrophils.

Despite initially suggestive data, this hypothesis was not substantiated with repeated experiments. Neutrophils undergoing cell-cell contact, incubated in close proximity (high density), did not demonstrate altered apoptosis. Thus, cell contact alone does not appear to be induce FasL-Fas induced apoptosis. In addition, decrease in FasL expression and TNF receptor downregulation in exudate neutrophils were not confirmed. These observations stimulated the investigation of processes inside the neutrophil membrane that regulate apoptosis. These are reported in the following manuscript.

Manuscript #2

Delayed constitutive and induced apoptosis in human exudate neutrophils is not mediated by NF-κB

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To be submitted for publication in *Critical Care Medicine*.

Keywords: Neutrophil, PMN, apoptosis, exudate, transmigration, Fas, FasL, TNF RI, TNF RII.

Manuscript 2: Delayed constitutive and induced apoptosis in human exudate neutrophils is not mediated by NF-κB

Abstract

The regulation of constitutive and induce neutrophil apoptosis in circulating and exudate neutrophils is important to understanding physiologic host defense, and pathologic over stimulation of the inflammatory response leading to host injury. The transcription factor NF-κB has been identified as a regulator of apoptosis in circulating neutrophils; its role in exudate neutrophils is not known. Circulating (venipuncture) and exudate neutrophils (skin blister skin windows) were harvested from 22 healthy human volunteers, and evaluated for constitutive apoptosis, and TNF-α and Fas induced apoptosis, both with and without protein synthesis inhibition (cycloheximide). The effect of NF-κB inhibition on constitutive circulating and exudate apoptosis was also evaluated (PDTC). Flow cytometry was used to evaluate expression of Fas, Fas Ligand, TNF RI and TNF RII, as well as the evaluation of apoptosis using two techniques (ANNEXIN V staining of apoptotic cells and Propidium Iodine identification of hypodiploid DNA) Compared to circulating PMN, exudate PMN displayed delayed constitutive apoptosis, and this delay was not altered by protein synthesis inhibition. In contrast to circulating PMN, exudate PMN were refractory to both TNF and Fas induced apoptosis. Although inhibition of all generalised protein synthesis did not augment apoptosis in exudate PMN, specific inhibition of NF-κB regulated protein synthesis did augment apoptosis equally in both circulating and exudate PMN. These results demonstrate delayed constitutive and induced apoptosis in exudate PMN, but do not implicate NF-κB as the mediator of increased exudate PMN survival.

Introduction

The polymorphonuclear neutrophil (PMN) is the most abundant circulating phagocyte in humans. The interest in neutrophil biology and function has been driven by its paradoxical roles within the host response, including the physiologic role of the neutrophil in host defense against bacterial or fungal infection, and the pathologic role of the neutrophil in causing tissue injury in diseases characterized by persistent inflammation.[1] Abnormalities in neutrophil function result in serious impairment of host defense, manifesting clinically as recurrent life-threatening infection.[2, 3] Neutrophils are also implicated in the pathogenesis of illness characterised by persistent inflammation such as rheumatoid arthritis [4-6], ARDS [7], inflammatory bowel disease [8, 9], ischemia-reperfusion [10], and organ damage in Multiple Organ Dysfunction Syndrome (MODS)[11, 12].

The data to support the role of neutrophils in host injury during persistent inflammation is compelling. Neutrophils possess the means of inducing tissue injury including oxygen-independent and oxygen-dependent mechanisms,[13] neutrophils and neutrophil products are found at sites of severe inflammation and correlate with degree of inflammation,[14-16] prevention of neutrophil sequestration will lead to reduced tissue injury in multiple animal models,[17-21] and clearance of neutrophils is associated with resolution of inflammation.[22] Neutrophil apoptosis, or genetically programmed cell death, is the principal mode by which senescent neutrophils are cleared from circulation.[23] Apoptosis, cell death without inflammation, is fundamentally distinct from necrosis, and an imbalance of granulocyte apoptosis and necrosis may contribute to the pathogenesis of inflammatory disease.[24, 25]

Committed to cell death at a pre-programmed rate over time, neutrophils display constitutive apoptosis. In addition, neutrophils may undergo induced apoptosis, following ligand binding of death receptors such as TNFRI and Fas by various soluble extracellular factors, which engage the intracellular pathways leading to apoptosis.[26] *In vitro*, inflammatory soluble

mediators delay constitutive neutrophil apoptosis, as well as TNF induced apoptosis.[27-31] Anti-inflammatory cytokines counterbalance this process by accelerating apoptosis,[28, 32] thus the potential for variability in neutrophil apoptosis evaluated *in vivo*.

Distinct populations of neutrophils collected *in* vivo display different rates of constitutive and induced apoptosis. Exudate neutrophils are human neutrophils that have undergone transmigration from the intravascular to the extravascular exudate inflammatory microenvironment. Collected from human skin, neutrophils display reduced constitutive apoptosis and TNF induced apoptosis associated with decreased binding to TNF-α.[33] The delay in apoptosis may be explained by engagement of adhesion molecules during transmigration,[34] inflammatory mediators[27-31] or decreased neutrophil TNF receptors and/or binding to TNF-α in the exudate milieu[33, 35]. Inside the neutrophil, intracellular protein synthesis leads to a tonic inhibition of constitutive apoptosis;[36] arresting ongoing protein synthesis will prevent dexamethasone delay in neutrophil apoptosis,[37] and arresting protein synthesis will accelerate TNF-α induced apoptosis in circulating and salivary neutrophils.[38]

NF-κB (Nuclear Factor - κB) is a transcriptional regulatory protein consisting of two subunits (one of five potential proteins: c-Rel, p65 (RelA), RelB, p50, p52) normally sequestered in the cytoplasm of a cell, and has been implicated as a cell survival mechanism, causing delay of the cell death program. Following exposure to inflammatory stimuli such as LPS, TNF-α, and FMLP,[39] NF-κB is released from its cytoplasmic inhibitory partner (I-κB - one of several inhibitory proteins), migrates to the nucleus, and binds to a specific promoter/enhancer region of multiple genes, including those that produce cell adhesion molecules, cytokines, growth factors, immunoreceptors, chemokines, acute phase proteins, other transcription factors, and more.[40] Investigating the role of NF-κB in constitutive apoptosis, using Gliotoxin to inhibit the activity of NF-κB (by preventing the degradation of I-κB, the inhibitory regulator of NF-κB),[41]

NF-κB inhibition upregulated constitutive neutrophil and eosinophil apoptosis *in vitro*, suggesting that NF-κB activation is an important survival mechanism in granulocytes.[42]

In terms of inducible apoptosis, inactivation of NF-κB will augment the potential for TNF-α to cause apoptosis, a finding reported simultaneously by three separate labs.[43-45] NF-κB activation has also been demonstrated in critically ill patients, including patients with ARDS.[46, 47]

Taken together, these results suggest that NF-κB mediates prolongation of neutrophil survival and the secretion of pro-inflammatory cytokines, and may be associated with neutrophil mediated host injury. It is not known if NF-κB mediates increased cell survival in the exudate environment. In addition, although we had previously demonstrated exudate neutrophils were refractory to TNF-α induced apoptosis, we wished to investigate Fas induced apoptosis in exudate neutrophils. Given that exudate neutrophils already displayed delayed constitutive apoptosis, we hypothesised that ongoing protein synthesis and NF-κB activation were not necessary for survival in exudate neutrophils. In order to do so, in this experiment, constitutive and induced (with Fas Ab and TNF-α) apoptosis rates with or without protein synthesis arrested, and constitutive apoptosis with inhibition of NF-κB were evaluated in both human circulating and exudate neutrophils. In order to evaluate the importance of receptor expression as a follow up of past experiments,[33] monoclonal antibody analysis of TNF receptors, Fas and Fas Ligand were performed on circulating and exudate neutrophils.

Materials and Methods

Subjects

Subjects under evaluation in this study included 22 human healthy controls (age 18-50, mean 30±8). Exclusion criteria for the control cohort included history of infection within the previous 48 hours, severe chronic illness, immunosuppressive medication, and known malignancy. Informed consent was obtained from all controls. Informed consent was obtained in all cases. The Committee of Human Experimentation, Royal Victoria Research Institute, approved the study prior to experiments.

Reagents

Neutrophil incubation was in Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium (Flow Laboratories). All preparations of neutrophils were kept in polypropylene tubes to prevent adherence. Recombinant human TNF-α (Sigma Chemical Co., St. Louis, MO) was stored at -70°C in 0.1mL aliquots at a concentration of 100 ng/mL, and Fas Ab (Immunotec, Catalog #1504) was diluted with DMEM + 10% fetal calf serum and stored at -70°C in 0.1mL aliquots at concentrations of 5 - 50 ng/μL). All glassware was baked at 250°C for at least 6 hours. Disposable sterile plastic pipettes and polypropylene tubes were used whenever possible.

Isolation of circulating neutrophils

7 cc of whole blood was obtained from the subjects using heparinized vacuum-sealed tubes (Becton Dickenson, Franklin Lakes, NJ). The blood was immediately added to 1.5 ml Macrodex/Dextran-70 (Pharmacia Laboratories, Piscataway, NJ), and gently mixed. The erythrocytes were gravity sedimented for 60 minutes at room temperature. The leukocyte-rich supernatant was removed and centrifuged at 400xg for 5 minutes at 4°C. The pellet was gently resuspended in Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium (Flow Laboratories) and layered on 3.0 ml of Ficoll-Hypaque (Pharmacia Laboratories). Centrifugation continued at 400xg for 25 minutes at 4°C. The plasma,

lymphocyte interface and Ficoll were removed, and the pellet resuspended in Elyse (Cardinal Associates, Santa Fe, New Mexico) in order to lyse the erythrocytes. After re-pelleting the neutrophils (400xg for 25 minutes at 4°C), they were washed with and resuspended in iced PBS. Cells were counted with hemocytometer following staining with Turk's solution, and suspended in media at a concentration of $1x10^6$cells/ml. Cell viability, as measured by Propidium iodide (Sigma Chemical Co., St. Louis, MO) or trypan blue exclusion, and purity, assessed by flow cytometry or microscopic field examination, were in excess of 90% in all experiments.

Isolation of exudate neutrophils

Skin window chambers were manufactured at the McGill University Workshop and used as previously described.[48] The technique follows the one described by Zimmerli and Galin.[49] Briefly, exudate neutrophils were collected from skin windows placed on the volar aspect of the forearm. The forearm was sterilized with 10% proviodine-iodine topical antiseptic followed by 70% isopropyl alcohol. 360 mm Hg vacuum suction was applied through a plastic template for 45-90 minutes until 4 even 1.0 X 1.0 cm blisters formed. These blisters were unroofed using sterile scissors and a template consisting of four 1.0 X 1.0 cm open-bottomed chambers was tightly applied using wide adhesive tape. The chambers were filled with 10% autologous serum through two mm superior apertures. The superior apertures were subsequently sealed with a sterile covering. After 18-22 hours, the exudate fluid, consisting of an almost pure suspension of neutrophils, was aspirated. The chambers were rinsed three times with normal saline and the fluid was transported immediately on ice to the laboratory. The neutrophils were sedimented at 400xg at 4°C for 5 minutes. Neutrophil viability was confirmed to be > 95% using propidium iodide exclusion. Neutrophil purity was > 98%, assessed by flow cytometry or microscopic field examination. Neutrophils collected from skin windows were counted using a hemocytometer after staining with Turk's solution, and resuspended in iced PBS for immediate staining with monoclonal antibodies to

chemoattractant receptors. Using this technique, a population of approximately 2-10 x 10^6 pure and viable exudate PMN are collected for each healthy control.

Quantification of surface receptors

Circulating neutrophils were collected in 7 cc sterile vacutainer® tubes using EDTA as anti-coagulant (Becton Dickinson, Franklin Lakes, NJ). The whole blood was kept on ice until analysis (begun immediately). Exudate neutrophils were analysed as soon as they were suspended in iced PBS at a concentration of $1x10^6$ cells/ml. Neutrophil membrane expression of TNF receptors, Fas and Fas Ligand were analysed using specific immunofluorescent-labeled monoclonal antibodies. Mouse Phycoerythrin(PE)-labeled anti-human (IgG_1) TNF-RI (Cedarlane Laboratories, Hornby, Ontario), mouse anti-human (IgG_{2A}) TNF-RII-PE (R&D Systems Inc.), mouse anti-human (IgG_1) FITC-labelled anti-Fas Ligand (Cedarlane Laboratories, Hornby, Ontario), and mouse anti-human (IgG1) anti-Fas (CD95) conjugated with FITC (Pharmingen, Missassauga, Ontario), were used to detect the chemoattractant receptors in the following manner. In 5 ml tubes, 10 µl (1-2 µg) of the immunofluorescent monoclonal antibody was incubated along with 100 µl of whole blood for 30 minutes at room temperature in the dark. Subsequently, 2 ml monoclonal lysing solution (1L dH_2O, 8.26 g NH_4Cl, 1 g. $KHCO_3$, 0.037 G Na_2EDTA) was added to the mixture, and again stored in the dark for 10 minutes. After sedimenting the neutrophils (400xg at 4°C for 5 minutes), the cells were washed twice with PBS with 1% FBS and 0.1% Azide (Sigma, Oakville, Ontario). Cells were finally suspended in 2% paraformaldehyde (Fisher, Ontario) and promptly analysed by Flow Cytometry. Exudate neutrophils were also washed with PBS with 1% FBS and 0.1% Azide, and suspended in 2% paraformaldehyde in the same manner. The median intensity of fluorescence for each receptor, which is directly proportional to the density of surface receptors per cell, was recorded for each PMN population, following at least 5000 counts. Median intensity of fluorescence was chosen over mean, as the fluorescence distributions were often skewed. Cells were plotted on forward scatter vs. side

scatter; and a gate was used to isolate the neutrophils in whole blood. Monoclonal isotype controls included FITC conjugated mouse IgG_{2a}, PE conjugated IgG_{2b} (IL-8 RA) and PE conjugated IgG_1 (IL-8 RB).

Flow Cytometer Calibration

The FACScan was calibrated bi-weekly to ensure no overlap of FL1 and FL2 spectra using Calibrite™ beads (Becton Dickinson, Mississauga, Canada). In addition, QC3™ microbeads (Becton Dickinson, Mississauga, Canada) were used to standardize the intensity of fluorescence on a bi-weekly basis or sooner if instrument maintenance was performed, thus correcting for any variations in flow cytometer performance. By making small alterations in the cytometer settings, the cytometer was calibrated such that the FACScan read the same FITC and PE target channels for the QC3™ beads from week to week.

Quantification of neutrophil apoptosis

Following incubation, cells were removed from the incubator, sedimented (400xg @ 4°C for 5 minutes), washed with PBS, sedimented and suspended in 1 ml binding buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 100 µl of the cell suspension in binding buffer was added to 5 µl Annexin V-FITC (Pharmingen Canada, Mississauga, Ontario) and 10 µl Propidium Iodide (PI - Sigma, Oakville, Ontario) and incubated in the dark for 30 minutes @ room temperature. Following incubation, 400 µl of binding buffer was added to cell suspension, and flow cytometric analysis followed immediately.

Cells were analysed with a Becton Dickenson FACScan® cytofluorometer. This technique takes advantage of a redistribution of plasma membrane phospholipids in the early stages of apoptosis. Phosphatidylserine (PS), which normally resides on the inner membrane leaflet, has been shown to be expressed on the outer membrane as an early feature of apoptosis

regardless of initiating stimulus.[50] Annexin V conjugated to FITC will bind specifically to PS and thus can be used to quantify the number of cells expressing PS and thus undergoing apoptosis.[51, 52] PI was used to stain cellular DNA. For PI to stain DNA, the cellular membrane must not be intact. These cells are thus determined to be necrotic. PI fluorescence (FL2) was plotted versus Annexin V FITC fluorescence (FL1); the data were registered on a logarithmic scale (see **Figure 1**). At least 5000 events were recorded for each sample.

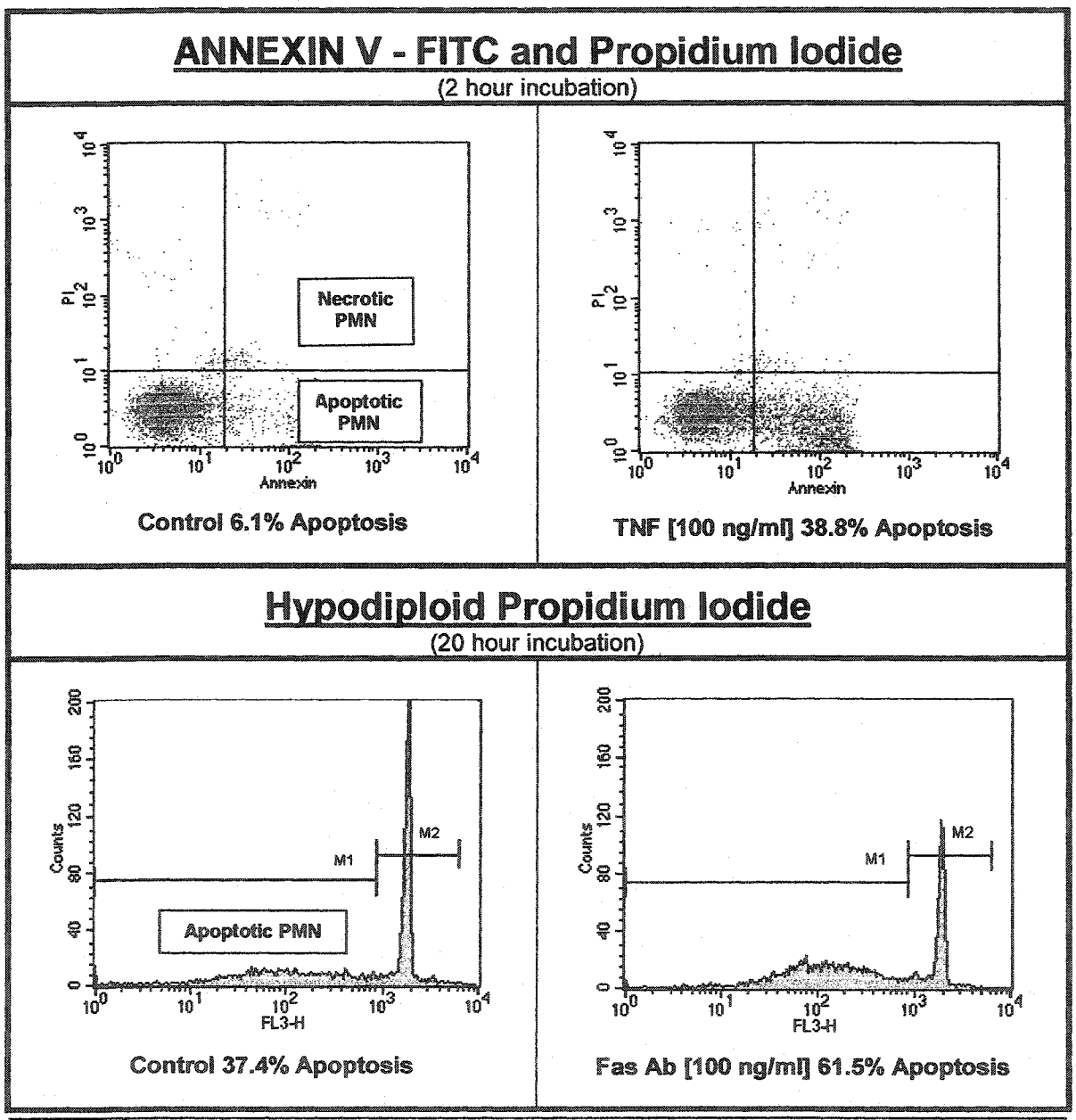

FIGURE 1: Comparison of simultaneous apoptosis measurements with ANNEXIN V cell surface binding and Propidium Iodide hypodiploid (HYPO PI) DNA staining (n=5).

The second technique involved the use of Propidium Iodide to identify cells with a hypodiploid DNA peak.[53] As others have performed for neutrophils,[54] cell suspensions were sedimented (400xg @ 4°C for 5 minutes) and placed in hypotonic solution containing 50 ug/ml propidium iodide, 3.4 mmol/L sodium citrate, I mmol/L TrIS buffer, 0.1 mmol/L edetic acid and 0.1% Triton X-100), stored in the dark for 10 minutes at 4°C. Cells were analysed with a Becton Dickenson FACScan® cytofluorometer. PI fluorescence (FL3) was plotted as a frequency histogram, the hypodiploid peak appearing to the left of the diploid peak); the data were registered on a logarithmic scale (see **Figure 1**). At least 5000 events were recorded for each sample.

Statistics

All results are expressed as mean ± standard deviation. SYSTAT® 6.0 for Windows was used for all statistical analysis. Statistical significance ($p<0.05$) was determined using Analysis of Variance between groups, with the Bonferroni correction when multiple tests were performed simultaneously, followed by student's T-test using paired analysis. Statistical significance is indicated on the figures to follow. If the number of subjects used for a figure differs from the number listed above, it is stated in the legend for the figure.

Results

Evaluation of apoptosis rates in two distinct populations of human neutrophils revealed different

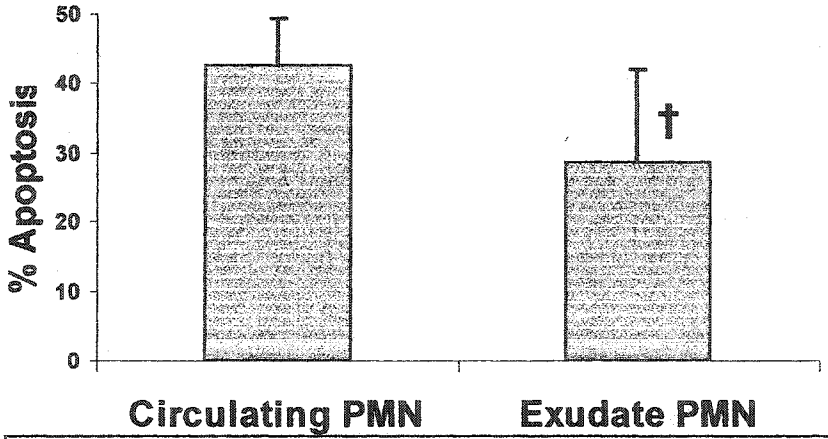

FIGURE 2: Baseline apoptosis is significantly decreased in exudate PMN following 20 hr incubation in media, compared to circulating PMN († $p \leq 0.001$).

apoptosis rates (see **Fig 2**). Compared to circulating PMN from control subjects (mean ± SD: 42.6 ± 6.7%), exudate PMN (28.6 ± 13.2% apoptosis following 20 hr incubation; p = 0.001) displayed a reduction in apoptosis rates. Thus, following transmigration, PMN constitutive apoptosis was decreased in healthy human controls.

The relative importance of *de novo* protein synthesis was evaluated with cycloheximide, an inhibitor of protein synthesis, preventing initiation and elongation on 80S ribosomes. A dose response curve was performed for cycloheximide in circulating cells, revealing the following apoptosis rates following 20 hrs incubation for n=8 individuals: control (media alone): 21.7 ± 9.3%; cyclo. 0.1 μg/ml: 21.0 ± 10.9% (NS vs. control); cyclo. 1 μg/ml: 28.5 ± 6.3% (NS vs. control); cyclo. 10 μg/ml: 36.4 ± 11.8% (p=0.09 vs. control); cyclo. 100 μg/ml: 41.3 ± 12.0% (p=0.01 vs. control). 100 μg/ml was subsequently used to evaluate the impact of cycloheximide on the the study population of circulating and exudate PMN.

PMN incubation with cycloheximide revealed a significant increase in apoptosis rates in circulating PMN (see **Fig 3**) in control PMN (42.6 ± 6.7% increased to 54.0 ± 8.5%, p<0.001), but not exudate PMN (28.6 ± 13.2% vs. 27.0 ± 8.8%). Thus, in contrast to circulating PMN, inhibition of protein synthesis yielded no increase in constitutive apoptosis rates in exudate PMN.

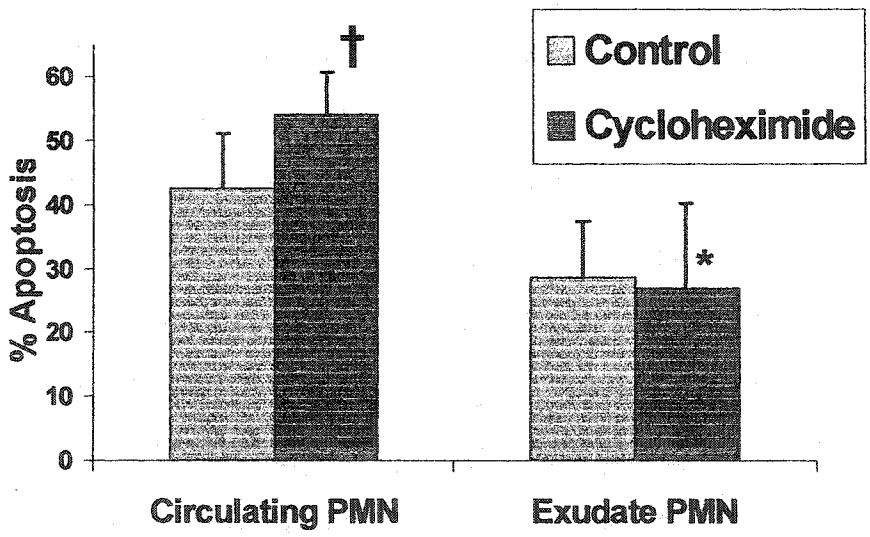

FIGURE 3: Protein synthesis inhibition with 100 μg/ml cycloheximide augments PMN apoptosis in circulating PMN, but not exudate PMN († p ≤ 0.001, * NS).

Induced apoptosis was evaluated with TNF-α (see **Fig 4**). Incubation of control circulating PMN with TNF-α alone for 20 hrs produced a significant increase in PMN apoptosis (56.2 ± 15.6% with TNF-α, p=0.008 vs. controls). The addition of cycloheximide to TNF-α further increased TNF induced apoptosis rates (69.5 ± 8.1%, p = 0.05 vs. TNF-α alone, p=0.002 vs. cycloheximide alone, and p<0.001 vs. control). In exudate PMN, TNF-α was not capable of augmenting PMN apoptosis (22.9 ± 9.5% with TNF-α vs. 28.6 ± 13.2% in control PMN, p=0.7); furthermore, the addition of cycloheximide did not increase the capacity of TNF-α to induce apoptosis in the exudate PMN (TNF-α & cycloheximide: 49.8 ± 18.9%, p=0.12 vs. TNF-α alone, p=0.3 vs. cycloheximide alone, p=0.65 vs. control).

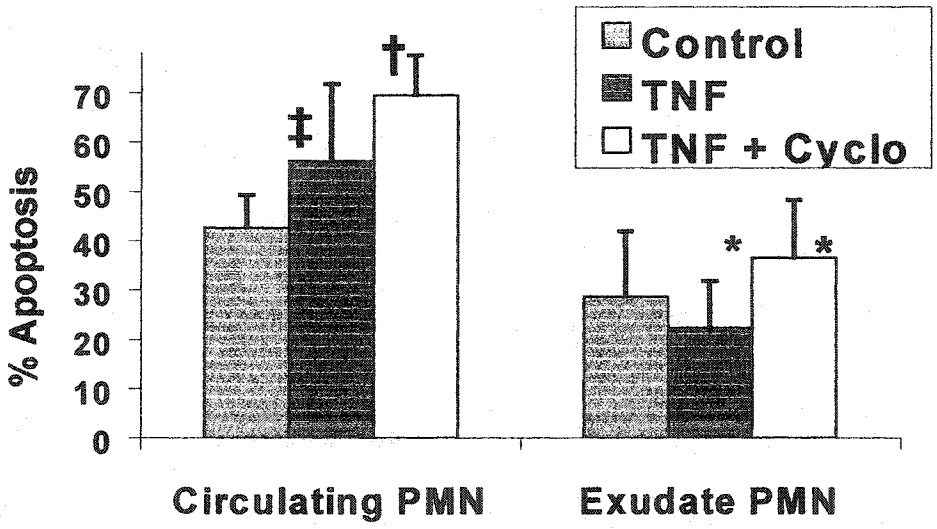

FIGURE 4: Response to TNF-α [100 ng/ml] & TNF-α with cycloheximide [100 μg/ml] in circ & exudate PMN (n=7): † $p \leq 0.001$; ‡ $p < 0.01$; * NS vs. control.

Fas induced apoptosis was also evaluated with and without protein synthesis inhibition (see **Fig 5**). In circulating PMN, incubation with anti-Fas antibody (Fas Ab) yielded a significant increase in apoptosis (54.7 ± 8.0%, p<0.001 vs. controls). Adding cycloheximide to Fas Ab did not significantly further apoptosis rates (60.2 ± 10.0, p=0.42 vs. Fas Ab alone, p=0.2 vs. cycloheximide alone, p<0.001 vs. controls). In exudate PMN, Fas alone did not increase apoptosis rates (33.4 ± 7.5, p=0.88 vs. control), however, the addition of cycloheximide did

lead to an increase in apoptosis compared to control conditions (38.6 ±8.6%, p=0.005 vs. cycloheximide alone p=0.42 vs. Fas Ab alone, p=0.02 vs. control).

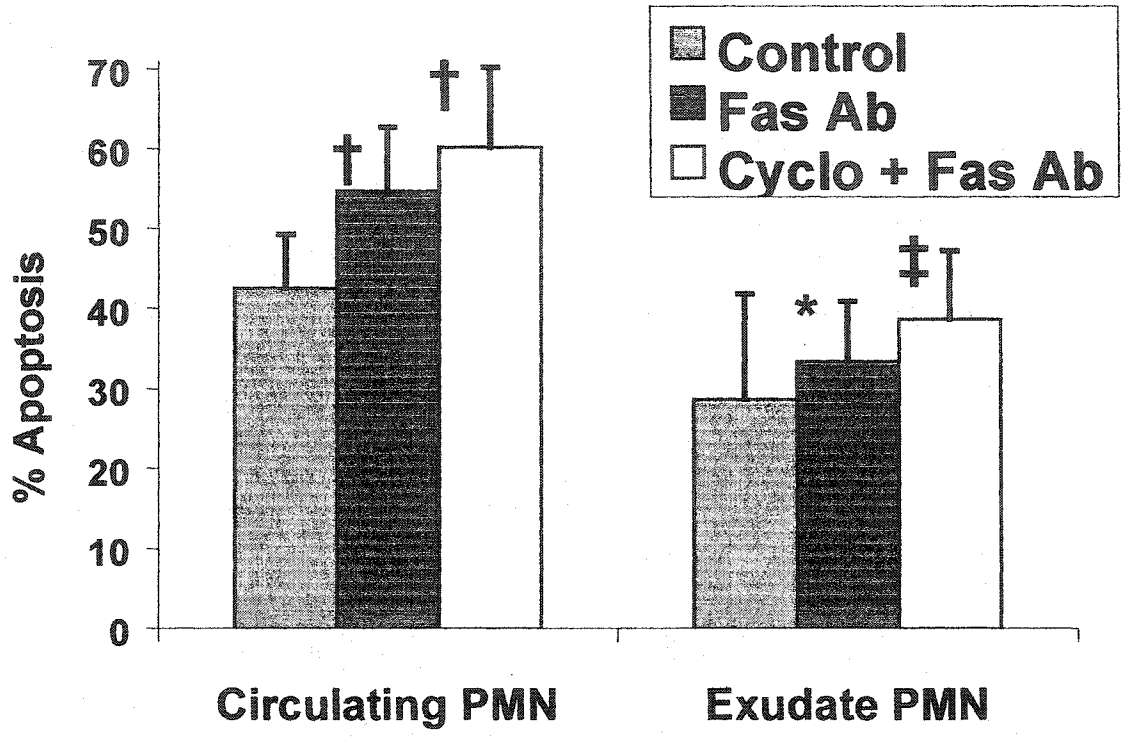

FIGURE 5: Response to Fas Ab [100 ng/ml] and Fas Ab with cycloheximide [100 μg/ml] in circulating and exudate PMN (n=15 circulating and exudate PMN): † $p \leq 0.001$: ‡ $p < 0.01$: * NS vs. control PMN.

To assess the role of NF-κβ, we evaluated the effect of pyrrolidinedithiocarbamate (PDTC), an NF-κβ inhibitor, on circulating and exudate PMN apoptosis (see **Fig 6**). A stock solution of 15 mM PDTC was used to generate the dose response characteristics; 1, 10 and 100 μl of 15 mM PDTC were added to a 0.5 ml suspension of 5 x 10^5 PMN to generate final concentrations of 30 μM,

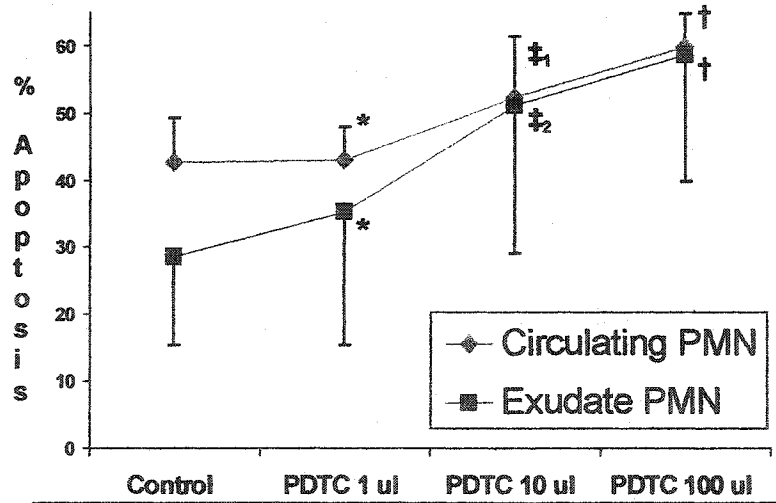

FIGURE 6: PDTC dose response curve: † $p \leq 0.001$; ‡1 $p = 0.08$; ‡2 $p = 0.03$; * NS; all compared to control (incubation with media alone) in circulating (n=4) and exudate (n=6) PMN.

300 μM and 2.5 mM PDTC. Both circulating and exudate PMN demonstrated augmented apoptosis secondary to incubation with PDTC. Incubation with 300 μM PDTC led to increased apoptosis in circulating PMN (52.3 ± 9.2% vs. 42.6 ± 6.7% in control PMN, p=0.08) and exudate PMN (51.1 ± 22.1% vs. 28.7 ± 13.2% in controls, p=0.033). Incubation with 2.5 mM PDTC further increased apoptosis in both circulating PMN (59.9 ± 5.0%, p<0.001 vs. control) and in exudate PMN (58.6 ± 18.9%, p=0.002).

Receptor expression for circulating and exudate PMN is included as Table 1. There were no significant differences in membrane expression between circulating and exudate PMN.

PMN Type	Fas	FasL	TNF R1	TNF R2
Circulating PMN	8.5 ± 1.2	9.7 ± 5.6	9.1 ± 1.0	20.8 ± 3.2
Exudate PMN	10.5 ± 1.6	8.4 ± 5.6	11.2 ± 3.6	27.4 ± 10.9

TABLE 1: Receptor expression (median intensity of expression) for apoptosis receptors in circulating and exudate (both n=20); no significant differences.

Discussion

Given the role of the human neutrophil in host defense and its participation in host injury during persistent inflammation, the regulation of neutrophil delivery and clearance has undergone extensive investigation. Apoptosis represents the means by which senescent neutrophils are cleared from circulation, and from the exudate environment. In this experiment using a human transmigration model, we have confirmed that when neutrophils undergo transmigration from the circulating to the exudate environment, they demonstrate delayed constitutive apoptosis, and remain refractory to stimulation with protein synthesis inhibition. In addition, in contrast to circulating PMN, exudate PMN are refractory to both TNF-

α and Fas Ab induced apoptosis. When protein synthesis in inhibited, exudate PMN only respond to Fas induced apoptosis, and are refractory to TNF-α induced apoptosis. Alterations in apoptosis rates in circulating and exudate PMN was not explained by alteration in receptor expression. Last, specific inhibition of NF-κB revealed similar increase in apoptosis in both circulating and exudate apoptosis.

These results demonstrate that following transmigration to the extravascular inflammatory environment, exudate neutrophils display both delayed constitutive and induced apoptosis. Exudate neutrophils remain refractory to TNF-α, even with protein synthesis arrested. Ongoing synthesis of intracellular proteins in exudate neutrophils does not further inhibit apoptosis as it does in circulating neutrophils. Nonetheless, we have demonstrated that protein synthesis directed by the transcription factor NF-κB does inhibit apoptosis and prolong cell survival in both circulating and exudate neutrophils.

The results of the experiment resulted from a direct comparison between circulating PMN, collected by venipuncture and exudate PMN, harvested using the skin blister skin window technique, collected in the same healthy controls. The skin blister skin window technique provides an accessible means of collection of neutrophils that have necessarily undergone transmigration to the extravascular environment. The strengths of the techniques include the ability to harvest a pure and viable population of exudate neutrophils from humans *in vivo* without manipulation or stimulation. Drawbacks include the limited yield of neutrophils, mild discomfort and occasional scarring of the procedure, and thus the difficulty of studying patients.

Although results for apoptosis were provided as histograms, apoptosis is better considered as a rate of cell death for a population of cells, a sigmoid shaped curve with apoptosis on the ordinate (y-axis) and time on the abscissa (x-axis). The curve may be shifted to the right or left depending upon if the rate of apoptosis is delayed or accelerated, respectively. The curve

may also differ based upon technique of evaluating apoptosis given that different techniques evaluate cells at different stages of apoptosis. In general, apoptosis rate are measured on the steep part of the sigmoid shaped curve, in order to demonstrate differences in apoptosis rates (shift of the curve) with varying conditions.

Not all authors have found delayed apoptosis in exudate neutrophils. In vitro endothelial transmigration stimulated by FMLP led to increased neutrophil apoptosis,[55] and inflammatory neutrophils isolated from the joints of patients with rheumatoid arthritis display augmented constitutive apoptosis, and no change in Fas induced apoptosis.[56] However, these results substantiate our previous experiments documenting delayed apoptosis in exudate human neutrophils from skin.[33] Exudate salivary neutrophils also demonstrate delayed apoptosis.[38] In animal studies, exudate pulmonary neutrophils[35] and peritoneal neutrophils[57] display refractory constitutive apoptosis. In addition, *in vitro* endothelial transmigration and binding adhesion molecules will lead to delayed neutrophil apoptosis.[34] It is likely that the process of transmigration affects neutrophil apoptosis differently in separate organs, modulated by the degree of inflammation at those sites.

In contrast to circulating neutrophils, the delayed apoptosis in exudate neutrophils was found to be unaffected by inhibition of protein synthesis. These findings confirm other reports in exudate neutrophils in salivary neutrophil,[38] in peritonea neutrophils in an animal model.[57] The results imply that tonic inhibition of apoptosis secondary to protein synthesis is not present in exudate neutrophils, as they are committed to a delayed rate of apoptosis.

Given that NF-κB plays an important role incell survival by tonic inhibition of granulocyte apoptosis (inhibition of NF-κB is associated with the onset of apoptosis),[42] and given that global protein synthesis inhibition did not impact upon exudate neutrophil apoptosis, we hypothesised that NF-κB would not act to promote cell survival in exudate neutrophils. However, inhibition of NF-κB produced identical augmentation of apoptosis in both circulating

and exudate neutrophils. Thus, the delay in apoptosis in exudate neutrophils does not appear to be mediated by alteration of the regulatory transcription factor, NF-κB.

Given previous experiments documenting a decreased binding of TNF-α to exudate PMN, we reported decreased TNF receptors in exudate neutrophils,[33] however, we have not substantiated this conclusion. Apoptosis is mediated through the TNF RI or 55 kDa receptor[58] and facilitated by the TNF RII or 75 kDa receptor.[59] In this experiment, using specific monoclonal antibodies to TNF RI and TNF RII, we did not observe any alteration in TNF receptor expression between circulating and exudate neutrophils. Although other reports also found no alteration in TNF receptors in exudate neutrophils isolated from arthritic joints,[60] others have found activation and/or adherence of neutrophils will stimulate release of TNF receptors,[61, 62] and observed decreased TNF RI expression in rat pulmonary exudate neutrophils.[35] Multiple authors have observed that exudate neutrophils are refractory to TNF-α; whether this is mediated by decreased TNF-α binding or a reduction in receptor expression requires further investigation.

The process of apoptosis plays a central role in neutrophil clearance, within the circulating and the exudate inflammatory environments. Delayed apoptosis in exudate neutrophils allows for the neutrophil to perform its defense oriented function, however, this delay contributes to host injury in the presence of persistent and overwhelming infection. Interventions to modulate apoptosis will be complicated by alterations in apoptosis over time and space. A delay in exudate neutrophil apoptosis may be physiologic, necessary for host defense in the urinary bladder, while simultaneously, delayed neutrophil apoptosis leads to host injury in the lungs. Thus, organ specific targeting, and interventions at certain specific times based upon individualised patient response may be necessary to impact upon patient outcome.

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Commentary

While performing the experiments investigating neutrophil apoptosis, related experiments were done evaluating neutrophil chemoattractant receptors and chemotaxis. This was in keeping with the principal hypothesis that change in receptor expression occurs to alter cell function *in vivo*. Thus, an investigation was performed evaluating the hypothesis that chemoattractant receptor alteration led to altered chemotactic function in neutrophils *in vivo*. In addition to evaluating exudate neutrophils, septic neutrophils (PMN collected from critically ill patients with the sepsis syndrome) were also harvested and compared to control circulating PMN. Thus the evaluation of the changes in receptor expression and cell function were performed in two unique and altered neutrophil populations, namely exudate and septic PMN, both compared separately to control circulating PMN. The following manuscript represent the results from the evaluation of this hypothesis regarding neutrophil chemotaxis.

Manuscript #3

Alteration of chemoattractant receptor expression regulates human neutrophil chemotaxis *in vivo*

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Manuscript 3: Alteration of chemoattractant receptor expression regulates human neutrophil chemotaxis *in vivo*

Mini abstract

This study demonstrates both increased and decreased expression of human neutrophil chemoattractant receptor expression *in vivo*, with parallel alteration in neutrophil chemotaxis. Following transmigration, exudate neutrophils display increased C5aR and C5a chemotaxis, but decreased IL-8R and IL-8 chemotaxis. Neutrophils from septic patients have diminished chemoattractant receptors, and decreased C5a chemotaxis.

Abstract

Alterations in human polymorphonuclear neutrophil (PMN) chemoattractant receptor expression and chemotactic function *in vivo* were evaluated in two distinct experiments: exudate PMN (PMN that have undergone transmigration to skin window blisters in controls) and septic PMN (circulating PMN from septic ICU patients, APACHE II 23.6±7.8) were both separately compared with control circulating PMN. Exudate PMN displayed increased C5a receptors & C5a chemotaxis, and reduced Interleukin-8 receptors (both IL-8RA & IL-8RB) & IL-8 chemotaxis. Septic PMN displayed reduced C5a & IL-8 receptors, decreased C5a chemotaxis, but no change in IL-8 chemotaxis. IL-8, but not C5a receptor gene expression decreased in parallel to receptor alteration. These results suggest: (1) change in PMN chemoattractant receptor expression serves to regulate PMN chemotaxis *in vivo*; (2) exudate PMN chemotaxis depends more on C5a than IL-8, and (3) diminished chemoattractant receptors and chemotaxis in septic PMN may explain decreased PMN delivery in these patients.

Introduction

Appropriate and effective host response to infection is of fundamental importance to host survival. Thus, a thorough understanding of the alterations in host response in critically ill patients has long been of particular interest.[1,2] Appropriate recruitment of polymorphonuclear neutrophil (PMN) to a site of inflammation is a principal component to effective host defense against bacterial and fungal infection.[3] We have previously demonstrated that septic patients have reduced delivery of neutrophils to skin blisters,[4,5] and believe that diminished PMN delivery to remote sites may contribute to sepsis-related immunosuppression, leading to "second front" infections, subsequent organ dysfunction and mortality. Given the essential role of the PMN in both health and disease, our investigations have focused on the regulation of human PMN delivery *in vivo*.

Directional migration or chemotaxis along the concentration gradient of a leukocyte chemoattractant is essential for effective leukocyte delivery to a site of infection. PMN will undergo directional migration or chemotaxis towards an increasing concentration of a variety of chemoattractant substances. C5a, the most potent pro-inflammatory and chemotactic anaphylatoxin, is formed during complement activation, and serves as a non-specific chemoattractant for monocytes, neutrophils, eosinophils and basophils.[6] The effect of C5a on PMN is mediated by the C5a receptor (C5aR), identified on neutrophils and other peripheral blood leukocytes.[7] It is a member of the superfamily of rhodopsin-type receptors, all containing seven transmembrane loops.[8] In addition to chemotaxis, C5aR also mediates a pro-inflammatory response in PMN, including the production of superoxide anions[9], and the release of proteolytic enzymes[10]. The capacity of mediators to alter C5aR expression *in vitro* is well known.[11,12] When C5a binds to C5aR, both ligand and receptor are internalized, with the potential for cell surface re-expression of the receptor, approximately 100 minutes later.[13,14] Although C5aR have been shown to be decreased in anergic patients,[5] the functional significance of increased or decreased C5aR expression *in vivo* is unknown.

Interleukin-8 (IL-8) is the prototype of a supergene family of chemokines (chemotactic cytokines) that possess specific chemotactic activity for neutrophils, and is implicated in the pathogenesis of inflammatory disease.[15] IL-8 is produced by virtually all nucleated cells in response to inflammatory stimuli such as endotoxin, TNF-α and interleukin-1 (IL-1).[16] There are two receptors for IL-8: type A receptor or IL-8 RA (also named C-X-C R1) and type B receptor or IL-8 RB (C-X-C R2). Both IL-8 receptors have seven transmembrane regions, are coupled to G proteins, and share 29% amino acid sequence homology with C5aR.[17] IL-8 receptors are expressed on monocytes, a subset of NK cells and T cells, but are most strongly expressed on neutrophils.[18] IL-8RA and IL-8RB differ in their selectivity for other ligands, and their biologic differences remain to be clarified.[19] Though numerous mediators modulate IL-8R expression, including G-CSF, lipopolysaccharide (LPS) and IL-8,[20,21] *in vivo* regulation of IL-8 RA, IL-8 RB and their functional significance remain to be determined.

Although chemoattractant receptors are known to be responsible for mediating PMN response to various chemotactic factors including both C5a and IL-8, *in vivo* alteration of chemoattractant receptors, the mechanism for their alteration, and the functional significance of receptor alteration are unclear. We hypothesised that PMN chemoattractant receptor expression display significant alteration in humans *in vivo*, that receptor alteration may be increased or decreased, and that PMN receptor alteration is associated with a parallel functional change in PMN chemotaxis. To investigate these hypotheses, we evaluated the simultaneous alterations in PMN chemoattractant receptors and PMN chemotaxis in two human PMN populations that are functionally distinct from control circulating PMN. Thus, in two separate but related experiments, we measured the change from control circulating PMN in chemoattractant receptor expression and PMN chemotaxis in (1) exudate PMN (PMN which have necessarily undergone transmigration to skin blisters), and (2) septic PMN (circulating PMN exposed to increased levels of circulating pro-inflammatory mediators); changes in C5a and IL-8 receptor expression were compared with changes in PMN function, or chemotaxis to C5a and IL-8. To evaluate the mechanism for receptor alteration, receptor

gene expression was evaluated in all three PMN populations. The purpose of these investigations includes the further elucidation of the mechanisms that regulate human neutrophil delivery *in vivo*, as well as the mechanisms that lead to observed reduction in PMN delivery to remote sites in septic patients.

Materials and methods

Subjects

The subjects under evaluation in this study were healthy human controls and septic patients. Circulating PMN were isolated from septic patients (septic PMN), whereas, both circulating PMN and exudate PMN were collected simultaneously from healthy controls (see below). Sepsis was defined by the presence of active infection requiring antibiotic treatment along with the Systemic Inflammatory Response Syndrome (SIRS - identified by having two or more of the following criteria: body temperature > 38°C or < 36°C; heart rate > 90; tachypnea (respiratory rate > 20 or $PaCO_2$ < 32 mm Hg); WBC count > $12.0X10^9$/L or < $4.0X10^9$/L).[22] All patients were being treated for active infection. Exclusion criteria for septic patients included the following: having received a transfusion of greater than 5 units blood or blood products within 48 hours, chemotherapy or radiotherapy in the previous three months, steroid administration, or hemodialysis; liver failure (Child's B or C), known HIV positivity, hypovolemic shock, or hypotension requiring vasoactive drugs (e.g. dopamine at > 8 μg/kg/min or norepinephrine > 8 μg/min). Exclusion criteria for the control cohort included a history of infection within the previous 48 hours, severe chronic illness, immunosuppressive medication, and known malignancy. Informed consent was obtained from all patients and controls. The study was approved by the Committee on Human Experimentation, McGill University Health Center Research Institute.

Reagents

All preparations of neutrophils were kept in polypropylene tubes to prevent adherence. Neutrophil incubation was in Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium (Flow Laboratories). Recombinant human C5a (Sigma Chemical Co., St. Louis, MO) and IL-8 (Sigma Chemical Co.) were stored at -70°C in 0.1mL aliquots at a concentration of 100 ng/mL and 10 ng/mL, respectively. All glassware was baked at 250°C for at least 6 hours. Disposable sterile plastic pipettes and polypropylene tubes were used whenever possible.

Isolation of circulating neutrophils

7 ml of whole blood was obtained from the subjects using heparinized vacuum-sealed tubes (Becton Dickenson, Franklin Lakes, NJ). The blood was immediately added to 1.5 ml Macrodex/Dextran-70 (Pharmacia Laboratories, Piscataway, NJ), and gently mixed. The erythrocytes were gravity sedimented for 60 minutes at room temperature. The leukocyte-rich supernatant was removed and centrifuged at 400xg for 5 minutes at 4°C. The pellet was gently resuspended in Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium (Flow Laboratories) and layered on 3.0 ml of Ficoll-Hypaque (Pharmacia Laboratories). Centrifugation continued at 400xg for 25 minutes at 4°C. The plasma, lymphocyte interface and Ficoll were removed, and the pellet resuspended in Elyse (Cardinal Associates, Santa Fe, New Mexico) in order to lyse erythrocytes. After re-pelleting the neutrophils (400xg for 25 minutes at 4°C), they were washed with, then resuspended in iced PBS. Cells were counted with a hemocytometer following staining with Turk's solution, and suspended in media at a concentration of $1x10^6$cells/ml. Cell viability (measured by Propidium iodide (Sigma Chemical Co., St. Louis, MO) or trypan blue exclusion) and purity (assessed by flow cytometry or microscopic field examination) were in excess of 90% in all experiments.

Isolation of exudate neutrophils

Skin window chambers were manufactured at the McGill University Workshop and used as previously described.[23] The technique follows the one described by Zimmerli and Galin.[24] Briefly, exudate neutrophils were collected from skin windows placed on the volar aspect of the subject's forearm, previously sterilized with 10% proviodine-iodine topical antiseptic and 70% isopropyl alcohol. 360 mm Hg vacuum suction was applied using a plexiglass template (contains four separate 1.0 cm diameter chambers attached to two side ports that connect to suction) for 45-90 minutes until 4 1.0 X 1.0 cm blisters formed. These blisters were unroofed using sterile scissors, and a second template consisting of four open-bottomed chambers (1.0 cm diameter inferior opening next to skin) was tightly applied using wide adhesive tape. The chambers were individually filled with 10% autologous serum through superior portholes (0.2 cm diameter). The portholes were subsequently sealed with a sterile covering (OpSite™, Smith & Nephew, Hull, England). After 18-22 hours, sterile covering was removed, and the exudate fluid was aspirated through the portholes. Neutrophil purity within the exudate fluid was > 98% assessed by microscopic field examination; no red blood cells were present. To maximize cell yield, chambers were rinsed three times with normal saline. Neutrophils were sedimented at 400xg at 4°C for 5 minutes. Neutrophil viability was confirmed to be > 95% using propidium iodide exclusion. Neutrophils were counted using a hemocytometer after staining with Turk's solution, and resuspended in iced PBS at a concentration of $1x10^6$ cells/ml for immediate quantification of surface receptors. Using this technique, a population of approximately 2-10 x 10^6 pure and viable exudate PMN was collected from each healthy control.

Quantification of surface receptors

Circulating neutrophils were collected in 7 ml sterile Vacutainer® tubes containing EDTA as anti-coagulant (Becton Dickinson, Franklin Lakes, NJ). The whole blood was kept on ice until analysis (begun immediately). Exudate neutrophils were analysed as soon as they were suspended in iced PBS ($1x10^6$ cells/ml). Chemoattractant receptor density was analysed using specific immunofluorescent-labeled monoclonal antibodies to the C5aR (CD88), IL-8

RA (CXCR1) and IL-8 RB (CXCR2). Mouse anti-human (IgG_1) CD88-FITC (Serotec Ltd., Oxford, England), mouse anti-human (IgG_{2b}) IL-8 RA-PE (Pharmingen Canada, Miss. Ontario) and mouse anti-human (IgG_1) IL-8 RB-PE (Pharmingen Canada, Miss. Ontario) were used to detect the chemoattractant receptors in the following manner. In 5 ml tubes, 10 µl (1-2 µg) of immunofluorescent monoclonal antibody was incubated along with 100µl of whole blood for 30 minutes at room temperature in the dark. Subsequently, 2 ml of monoclonal lysing solution (1L dH_2O, 8.26 g NH_4Cl, 1 g $KHCO_3$, 0.037 g Na_2EDTA) was added to the mixture, and again stored in the dark for 10 minutes. After neutrophil sedimentation (400xg at 4°C for 5 minutes), cells were washed twice with PBS with 1% FBS and 0.1% Azide (Sigma, Oakville, Ontario), and were finally suspended in 2% paraformaldehyde (Fisher, Ontario) and promptly analyzed by Flow Cytometry. Exudate neutrophils were also washed with PBS with 1% FBS and 0.1% Azide, and suspended in 2% paraformaldehyde in the same manner. The median intensity of fluorescence for each receptor, which is directly proportional to the density of surface receptors per cell, was recorded for each PMN population, following at least 5000 counts (see **Fig 1**). Median intensity of fluorescence was chosen over mean, as the fluorescence distributions were often skewed. Cells were plotted on forward scatter vs. side scatter; and a gate was used to isolate the neutrophils from other cellular elements in whole blood. Monoclonal isotype controls included FITC conjugated mouse IgG_1 (C5aR), PE conjugated IgG_{2b} (IL-8 RA) and PE conjugated IgG_1 (IL-8 RB).

Flow Cytometer Calibration

The FACScan was calibrated bi-weekly to ensure no overlap of FL1 and FL2 spectra using Calibrite™ beads (Becton Dickinson, Mississauga, Canada). In addition, QC3™ microbeads (Becton Dickinson, Mississauga, Canada) were used to standardize the intensity of fluorescence on a bi-weekly basis or sooner if instrument maintenance was performed, thus correcting for any variations in flow cytometer performance. By making small alterations in the cytometer settings, the cytometer was calibrated such that the FACScan read the same FITC and PE target channels for the QC3™ beads from week to week.

In addition to FACScan calibration, standardized phycoerythrin (PE)-conjugated beads were used to convert from intensity of fluorescence to number of receptors per cell. Quantibrite™ beads (Becton Dickinson, Mississauga, Canada) with known numbers of PE molecules per bead have been previously described and validated.[25,26] A mixture of four pre-calibrated beads was used to create the calibration curve. Antibodies to the IL-8 receptor used in this experiment had one PE molecule per antibody. By verifying that the receptors on both circulating and exudate PMN were saturated with antibody (doubling the concentration of antibody did not alter fluorescence), the number of antibodies bound to the cells were assumed to be identical to the number of receptors per cell. Thus, a standardized curve relating intensity of fluorescence to number of receptors per cell was used to calculate IL-8 receptors on neutrophils; the correlation coefficient for the linear regression performed on the calibration curve was 0.99993 ($p=0.00015$). The C5a receptor was marked with a FITC labeled antibody, thus it was not possible to convert from intensity of fluorescence to number of C5a receptors per PMN.

Chemotaxis assay

The chemotaxis assay was carried out using a modified Boyden's chemotaxis assay, originally described by Boyden[27]. Briefly, neutrophils were placed in the top wells of a Boyden chamber (Neuro Probe, Gaithersburg, MD). The chemoattractants, C5a, IL-8, Zymosan–activated human serum (ZAS - positive control), or Phosphate Buffered Saline (PBS - negative control) were placed in the bottom wells. The two wells were separated by a 25 x 80 mm 3 micrometer-pore-size polyvinylpyrolidone-free polycarbonate membrane (Neuro Probe, Gaithersburg, MD). Following a 55-minute incubation period, the membrane was removed, stained using Hema 3 staining solutions for leukocytes (Biochemical Sciences, Swedesboro, NJ), and examined under a microscope at 400x magnification. Three microscopic fields were observed for each well, and average cell counts per field were calculated in order to yield chemotaxis values. Samples were always evaluated in triplicate.

A dose response curve was performed for IL-8 and C5a to establish the appropriate chemoattractant concentrations for use in the study.

Assay of Gene Expression

RNA isolation of was performed on PMN suspensions; 1-3 million PMN were collected and suspended in 1 ml of Phosphate Buffered Saline (PBS). Following sedimentation (400xg at 4°C for 5 minutes), cells were lysed in 1 ml of TRIzol (Life Technologies, Burlington, Ontario) and total RNA was collected as described by the manufacturer's protocol. The RNA pellet was then suspended in 20 μl of DEPC treated double distilled water.

Reverse Transcription (RT) was performed on 2 μg of total RNA using the ThermoScript RT-PCR System (Life Technologies, Burlington, Ontario) and the manufacturer's supplied oligo dT primer. The manufacturer's suggested conditions were adhered to. A total reaction volume of 20 μl was used.

Selective gene amplification from the cDNA was achieved by performing Polymerase Chain Reaction (PCR) using Platinum Taq DNA Polymerase (Life Technologies, Burlington, Ontario). PCR conditions employed were as follows: GAPDH required 0.8 μl of rt product, annealing temperature 60 °C and 30 cycles, CXC R1 required 2.4 μl rt product, annealing temperature 55 °C and 35 cycles, CXC R2 required 1.6 μl of rt product, annealing temperature 53 °C and 30 cycles, C5aR required 0.5 μl of rt product, annealing temperature 55 °C and 33 cycles. The specific oligonucleotide primers used and amplified fragment lengths were: GAPDH 5' ACC ACC ATG GAG AAG GCT GG 3' and 5'CTC AGT GTA GCC CAG GAT GC 3' (527 bp) (modified from [28]), CXCR1 5' CAG ATC CAC AGA TGT GGG AT 3' and 5' TCC AGC CAT TCA CCT TGG AG 3' (296 bp),[29] CXCR2 5' CTT TTC TAC TAG ATG CCG C 3' and 5'GAA GAA GAG CCA ACA AAG G 3' (966 bp),[29] C5aR 5'ATG AAC TCC TTC AAT TAT ACC 3' and 5'TGG TGG AAA GTA CTC CTC CCG 3' (551 bp)[30].

After amplification, 10 ul of PCR product were electrophoresed on an ethidium-bromide stained Agarose gel and imaged with the Alpha Imager 2000 (Alpha Innotech Corporation, San Leandro, CA). Band density was determined with the ChemiImager Software (Alpha Innotech Corporation). Analysis was performed by comparing the proband genes as ratios to the housekeeping gene GAPDH.

Statistics

All results are expressed as mean ± standard deviation (SD). SYSTAT® 8.0 for Windows was used for all statistical analysis. Statistical significance ($p<0.05$) was evaluated with student's T-test or Analysis of Variance between groups, with Bonferroni correction when multiple tests were performed simultaneously. Statistical significance is indicated on the figures to follow. If the number of subjects used for a figure differs from the number listed below, it is stated in the legend for the figure.

Results

All subjects were prospectively enrolled from 01/2000 to 05/2000. 12 ICU patients were identified and consented (APACHE II range 11-37, mean and standard deviation of 23.6 ± 7.8). Patient infections were varied, including pneumonia, intra-abdominal abscess with peritonitis, infected acute pancreatitis, pyelonephritis, endocarditis, and infected aortic prosthesis. 19 human healthy volunteers were studied during the same period of time.

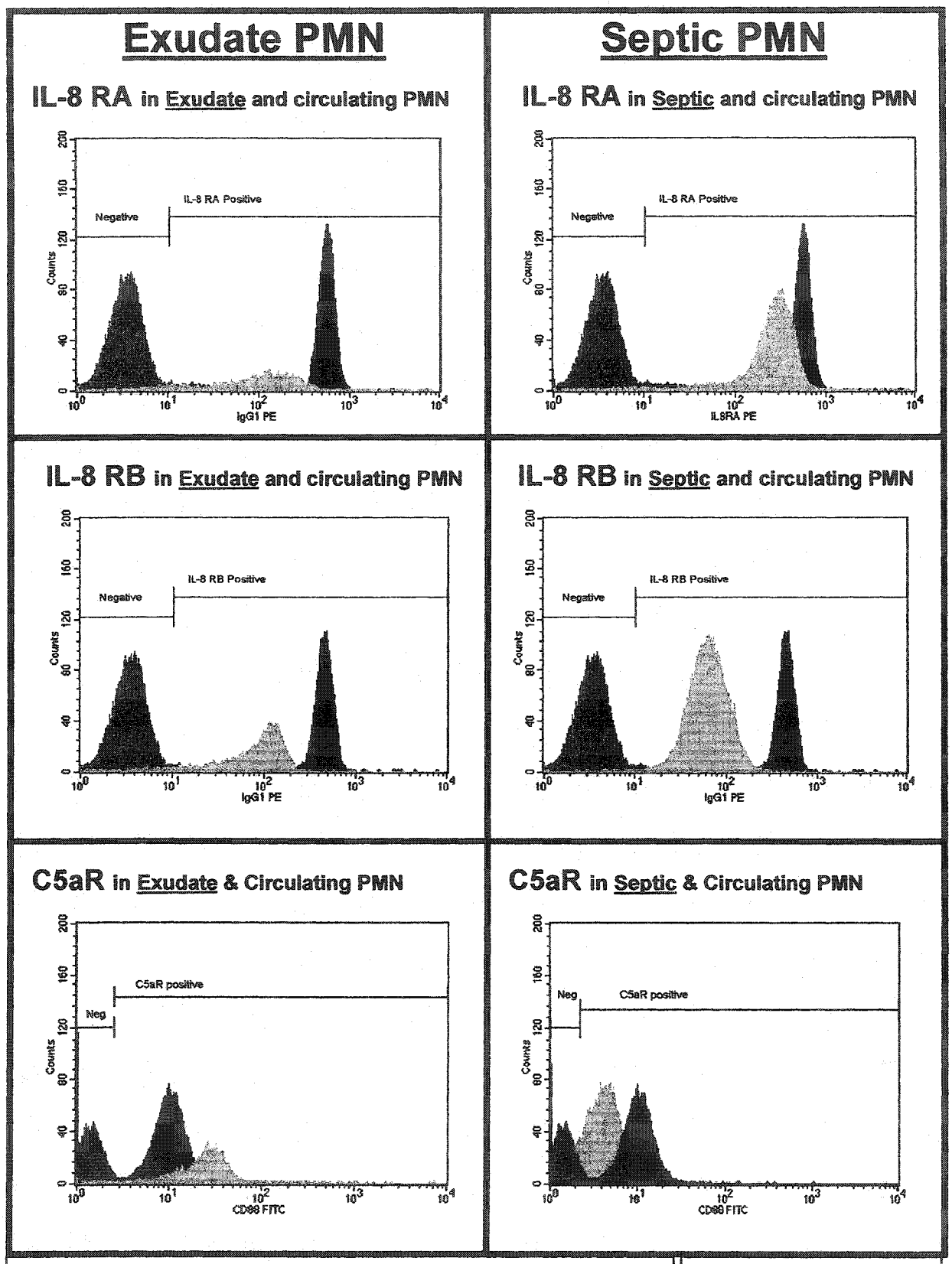

FIGURE 1: Flow Cytometer analysis. Histograms with intensity of fluorescence (log scale, x-axis); left peak (dark gray) - isotype negative control; right (black) peak - control circulating PMN; gray peak - exudate PMN. Histograms for IL-8 RA, IL-8 RB, & C5aR.

- Negative Control
- Exudate PMN
- Circulating PMN

Exudate PMN

Analysis of chemoattractant receptor expression in healthy controls demonstrated that exudate neutrophils (see **Fig 1 and 2**) display significantly greater expression of C5a receptors as compared to fluorescence (MIF): 31.5 ± 15.9 in exudate PMN vs. 16.8 ± 7.0 in circulating PMN; p<0.001). In contrast to increased C5aR, exudate PMN have decreased expression in both IL-8 RA and IL-8 RB when compared to circulating PMN (IL-8RA: 12 200 ± 7400 receptors per cell in exudate PMN vs. 52 800 ± 6300 in circulating PMN; IL-8RB: 7200 ± 3900 in exudate PMN vs. 44 800 ± 3100 in circulating PMN; both p<0.001). Evaluation of chemotaxis in exudate PMN (see **Fig 2**) from healthy controls demonstrated increased migration to C5a and ZAS, both significantly higher than that of circulating PMN. Exudate PMN migration to C5a was 40.86 ± 12.82 PMN per microscopic field (mf), as compared to 32.17 ± 8.86 PMN/mf for circulating neutrophils (p=0.028). Similarly, ZAS, a source of C5a, induced an exudate PMN chemotaxis of 50.84 ± 19.43 PMN/mf, which was significantly higher than with circulating PMN, 34.90 ± 8.53 PMN/mf (p=0.004). Exudate PMN displayed

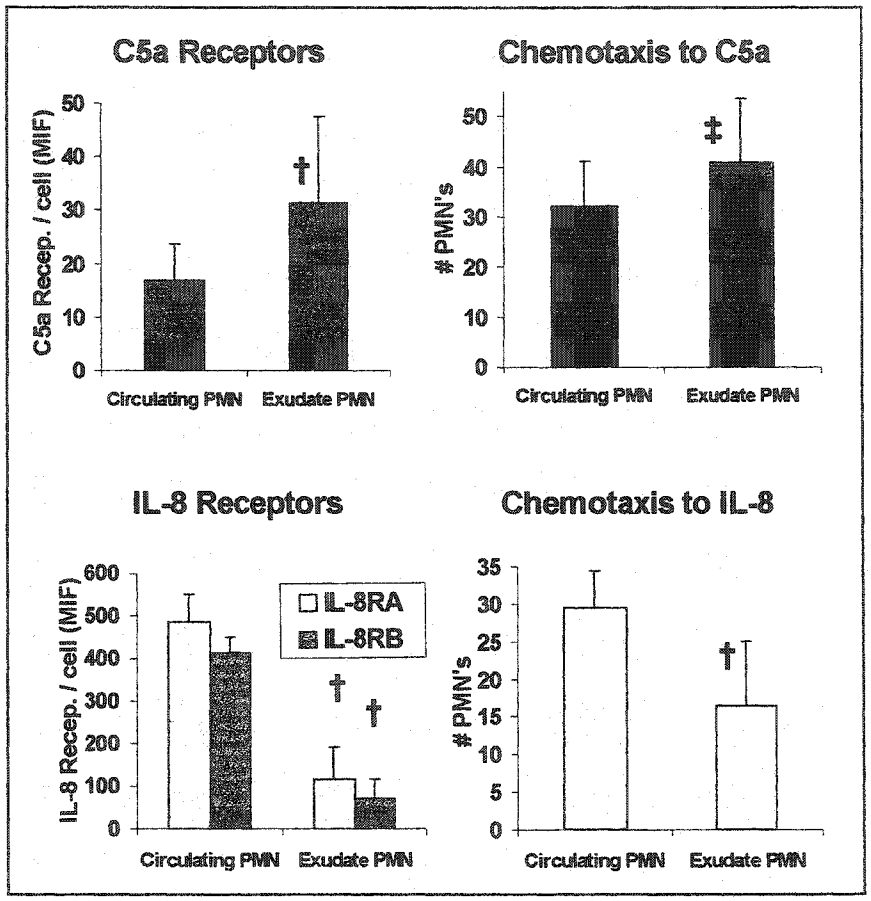

FIGURE 2: Circulating vs. Exudate PMN: Receptor expression (left) and chemotactic function (right) for C5a (top) and IL-8 receptors (bottom) Results: Mean ± SD; MIF = Median Intensity Fluorescence; † ⇒ p<0.001, ‡ ⇒ p<0.05 vs. Circulating PMN.

reduced chemotactic migration to IL-8 when compared to circulating PMN in the same individuals (29.5 ± 4.9 PMN/mf in circulating PMN vs. 16.48 ± 8.42 PMN/mf in exudate PMN; $p<0.001$). Chemotaxis to the negative control (PBS) was similar in exudate and circulating neutrophils (circulating PMN 5.28 ± 2.41 PMN/mf vs. exudate PMN: 6.3 ± 3.2; $p = 0.3$). Semi-quantitative gene expression analysis revealed significant differences between IL-8 mRNA expression, but similar C5aR mRNA expression in circulating vs. exudate PMN (see **Table 1**).

	Control Circulating PMN	Exudate PMN	Septic PMN
C5aR	0.55 ± 0.40	0.48 ± 0.83 *	0.52 ± 0.19 *
IL8-RA	1.66 ± 0.87	0.95 ± 0.30 ‡$_1$	0.91 ± 0.39 ‡$_2$
IL8-RB	1.83 ± 0.80	0.72 ± 0.23 †	1.48 ± 0.43 *

TABLE 1: C5a & IL-8 Receptor mRNA in Control Circulating, Exudate and Septic PMN. Analysis: † ⇒ $p<0.001$, ‡$_1$ ⇒ $p=0.07$, ‡$_2$ ⇒ $p=0.06$, * ⇒ NS, vs. control circulating PMN.

Septic PMN

Septic PMN from 12 septic patients demonstrated a significantly decreased expression of all three chemoattractant receptors evaluated (see **Fig 1 and 3**), including C5aR (MIF: 7.2 ± 3.6 in septic PMN vs. 16.8 ± 7.0 in circulating PMN; $p<0.001$), IL-8 RA (# receptors: 36 200 ± 13 800 in septic PMN vs. 52 800 ±

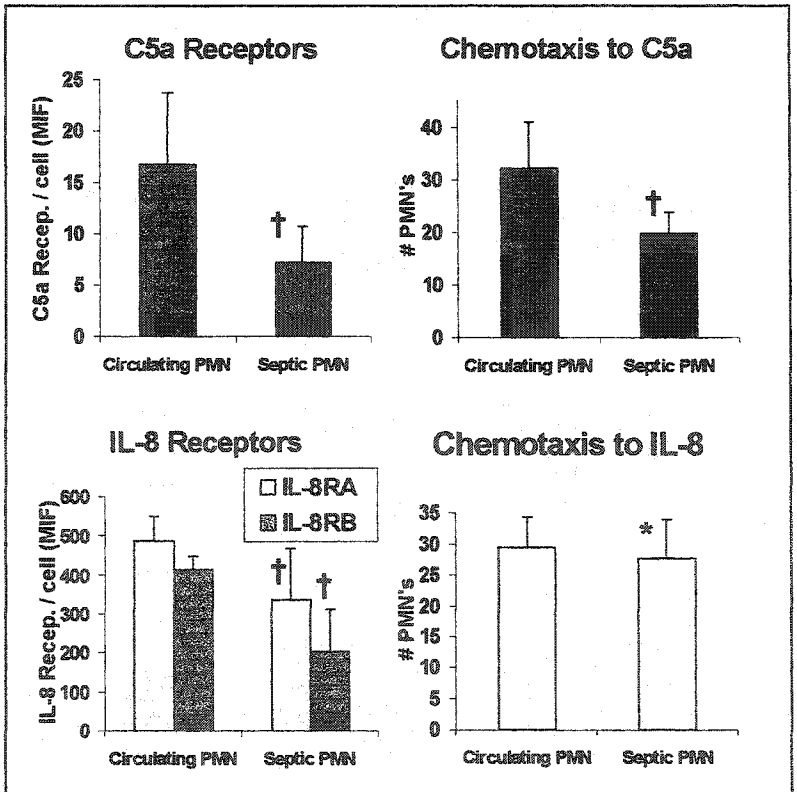

FIGURE 3: Circulating vs. Septic PMN: Receptor expression (left) and chemotactic function (right) for C5a (top) and IL-8 receptors (bottom) Results: Mean ± SD; MIF = Median Intensity Fluorescence; † ⇒ $p<0.001$; * ⇒ NS vs. Circ PMN.

6300 receptors in circulating PMN; p<0.001) and IL-8 RB (21 500 ± 11 100 in septic PMN vs. 44 800 ± 3100 in circulating PMN; p<0.001). Evaluation of chemotactic function in circulating PMN in 12 septic ICU patients revealed that chemotaxis to C5a was significantly reduced in septic PMN (see **Fig 3**) when compared to the control cohort (19.7 ± 4.0 in septic PMN vs. 32.2 ± 8.9 PMN/mf in circulating PMN; p < 0.001). The observed response to the positive control, ZAS paralleled the alterations in C5a mediated chemotaxis (23.87 ± 6.58 in septic PMN vs. 34.89 ± 8.53 PMN/mf in circulating PMN; p=0.001). There was no alteration in chemotaxis to IL-8 observed in septic PMN (27.8 ± 6.1 in septic PMN vs. 29.5 ± 4.9 PMN/mf in circulating PMN; p=0.42). Chemotaxis to the negative control (PBS) in septic PMN was similar to circulating neutrophils (septic PMN: 8.23 ± 5.99 PMN/mf vs. control circulating PMN 5.28 ± 2.41 PMN/mf; p=0.08). Semi-quantitative gene expression analysis revealed significant differences between IL-8 RA mRNA expression, slightly reduced Il-8 RB gene expression, and similar C5aR mRNA expression in circulating vs. septic PMN (see **Table 1**).

Discussion

Polymorphonuclear neutrophil delivery to the inflammatory exudate microenvironment is essential for effective host response to infection. In order to investigate the importance of *in vivo* alteration of chemoattractant receptors in determining PMN chemotactic function, as well as evaluate potential mechanisms underlying the observed reduction in PMN delivery to skin blisters in septic patients, we studied the alteration from control circulating PMN in healthy subjects in chemoattractant receptor expression and chemotactic function in two distinct PMN populations: exudate neutrophils in healthy controls, and circulating PMN in septic patients. Both (1) exudate PMN, which have undergone transmigration from the intravascular to the interstitial environment and have been exposed to inflammatory mediators in the exudate milieu, and (2) septic PMN, which have been exposed to increased levels of various circulating mediator mediators, cells and cytokines within the serum of septic patients, represent functionally separate populations of PMN. They have undergone physiologic

alteration from "resting" circulating PMN in the bloodstream of healthy individuals. Comparison of both healthy exudate PMN and septic circulating PMN to control healthy circulating PMN reveals that receptor alteration may be a physiologic mechanism occurring *in vivo* that leads to change in chemotactic function. In addition, the results have specific implications regarding PMN delivery in patients with sepsis, and PMN chemotaxis in the exudate environment.

In this experiment, exudate PMN of septic patients were not investigated. First, it is important to note that the skin blister method is associated with some discomfort, and critically ill patients' families are often reluctant to grant consent. Second, our previous data had demonstrated similar receptor alterations in exudate PMN, regardless of whether the subject was septic or healthy. For example, in septic patients, we observed a similar loss of expression of both IL-8 RA (69% reduction; $p<0.00001$) and IL-8 RB (72% reduction; $p=0.00007$) in the exudate PMN; however, as opposed to the current study, chemotactic function was not evaluated in this previous experiment.[39] Given that alterations found in exudate PMN appear to be consistent in septic patients and controls, and wishing to minimise patient discomfort, we elected not to collect exudate PMN in septic patients.

PMN Receptor	**Exudate PMN**	
	Receptors	**Chemotaxis**
C5a R	⇑ **88%**	⇑ **27%** ‡
IL-8 RA	⇓ **76%**	⇓ **46%**
IL-8 RB	⇓ **82%**	

TABLE 2: Percent change in C5a and IL-8 receptors and in chemotaxis in <u>exudate PMN</u> when compared to circulating PMN in healthy controls. All percent changes are significant ($p<0.001$, except ‡ ⇒ $p<0.05$).

The first component of our study involved the evaluation of alterations in chemoattractant receptor expression and chemotaxis following the process of transmigration. Using a human *in vivo* model of PMN transmigration, we have demonstrated that exudate PMN display an upregulation of both C5a receptors and chemotaxis to C5a compared to circulating PMN; conversely, marked downregulation of both interleukin-8 receptor type A & B and a parallel decrease in chemotaxis to IL-8 was observed in exudate PMN (see **Table 2**). Our observed augmentation of both C5aR and chemotaxis to C5a in exudate PMN has not been previously reported. The increase in C5a receptors and chemotaxis and decrease in IL-8 receptors and chemotaxis in exudate PMN suggests that C5a, a potent PMN activator,[9,10] may be more important for neutrophil delivery and activation in the exudate environment once transmigration has occurred.

Our observation of decreased IL-8 receptors and IL-8 chemotaxis in exudate PMN confirms previous reports demonstrating diminished IL-8 receptors in exudate PMN harvested from bronchioalveolar lavage fluid in patients with chronic respiratory tract infections (chemotactic function was not evaluated), [31] and reduced chemotaxis to IL-8, FMLP, leukotriene B4 and C5a in pustule (exudate) PMN in a single patient with relapsing bullous staphyloderma[32]. The importance of IL-8 to the proximal component to neutrophil delivery, including upregulation of PMN integrins, leading to neutrophil trans-endothelial cell migration, is well established.[33,34] The importance of IL-8 for circulating PMN chemotaxis in contrast to the downregulation of IL-8 receptors and chemotaxis in the exudate environment suggest that IL-8 may be representative of a class of PMN-specific *regional* chemoattractants, recruiting PMN from the circulation to a particular region or specific area of infection or inflammation based on production of inflammatory mediators and endothelial cell activation as the first step in PMN delivery. C5a may be representative of a class of *local* chemoattractants, serving to attract and activate the PMN within the inflammatory exudate microenvironment. Analogous to PMN endothelial cell interactions guided by a stepwise progression of adhesive interactions, neutrophil delivery may also involve a stepwise progression of chemoattractant exposure.

The second component to the study included the evaluation of septic PMN, namely circulating PMN in patients with the presence of infection and altered host response. As we have previously demonstrated that neutrophil delivery to skin blister sites is reduced by 72% in septic patients with active infection and SIRS,[4] we hypothesised that neutrophil chemoattractant receptors and chemotaxis in the circulating PMN of septic patients would be diminished. In this experiment, we found a significant reduction in C5a receptors on circulating PMN from septic patients and a corresponding decrease in chemotaxis to C5a. There were significant reductions in both IL-8 RB (51% reduction) and IL-8 RA (31% reduction) in septic PMN, but no alteration in IL-8 stimulated chemotaxis (see **Table 3**). In addition, we have previously demonstrated that both IL-8 and C5a are present in skin window fluid of healthy subjects at concentrations much greater than serum, thus creating a chemotactic gradient for neutrophils undergoing transmigration. However, the C5a gradient is absent in septic patients secondary to elevation in serum C5a [4]. Thus, our data to date suggests a defect in C5a mediated chemotaxis in sepsis, due to both a lack of a C5a gradient as well as a significant loss of C5a receptors and C5a chemotactic response in circulating PMN. These results provide a mechanism to explain the observed decrease in PMN delivery to skin window blisters in septic patients.

PMN Receptor	**Septic PMN**	
	Receptors	**Chemotaxis**
C5aR	**⇓ 57%**	**⇓ 76%**
IL-8 RA	**⇓ 31%**	**No significant change**
IL-8 RB	**⇓ 51%**	

TABLE 3: Percent change in C5a and IL-8 receptors and in chemotaxis in septic PMN when compared to circulating PMN in healthy controls. All percent changes are significant ($p<0.001$).

In this experiment, IL-8 and C5a receptor expression and chemotaxis to IL-8 and C5a demonstrated parallel alterations in exudate PMN (see **Table 2**) and in septic PMN (see **Table 3**). These data support receptor alteration as a mechanism for change in cell function *in vivo*, a conclusion suggested but not confirmed from previous *in vitro* investigations. Although receptor downregulation occurs in conjunction with concurrent decrease in chemotactic function *in vitro*,[20] the effects of C5a receptor antagonists, which block C5aR both *in vitro* and *in vivo*,[35] and inflammatory mediators, including TNF-α and GM-CSF, may attenuate C5a mediated chemotaxis in PMN *in vivo* despite the presence of the receptor.[11] In this study, both upregulation and downregulation of C5a receptors were associated with parallel alterations in C5a chemotaxis. The reduction in IL-8 receptors with no alteration in IL-8 chemotaxis observed in circulating PMN in septic patients demonstrates that receptor alteration alone may not be necessarily sufficient to alter PMN chemotaxis; however, chemotaxis to other IL-8 receptor (IL-8 RB) ligands, which may be decreased, was not evaluated. Taken together, these observations support the hypothesis that chemoattractant receptor alteration is a physiologic mechanism by which chemotactic function is altered *in vivo*.

The biologic mechanisms leading to receptor alteration in human neutrophils have undergone extensive investigation. In this experiment, semi-quantitative analysis of gene expression demonstrated moderate downregulation of IL-8 receptor, but not C5a receptor gene expression was noted in parallel with receptor alteration. Despite the inherent limitations with semi-quantitative mRNA isolation and densitometry using ratios of proband to housekeeping genes, these data support previous observations suggesting decreased receptor gene expression mediates alterations in IL-8 receptors.[20] In addition, other mechanisms result in IL-8 receptor loss *in vitro*, including incubation with IL-8 itself, [21, 36] TNF-α and LPS[37]; whereas G-CSF will stimulate IL-8 R expression of human PMN.[20] Other factors, including hypoxia and/or hypoxia with reoxygenation will affect matrix protein regulation of PMN IL-8 receptors.[38] C5a is known to mediate a decrease in C5aR. When compared to controls, C5a

concentration is elevated in the serum of septic patients; exudate fluid C5a concentration is also elevated in the exudate fluid, compared to serum levels in healthy controls.[4] Thus, multiple factors are likely involved in the *in vivo* regulation of cell surface chemoattractant receptor expression.

By evaluating the change in receptor expression and chemotactic function in exudate PMN (skin window skin blister method in healthy controls) and septic PMN (circulating PMN in septic patients), and comparing both with control circulating PMN (from healthy controls), we have demonstrated the following: (1) chemoattractant receptor alteration is variable, dependent upon the receptor in question, capable of both significant increase or decrease; (2) receptor alteration is associated with a concurrent parallel change in chemotaxis, while the receptor may not be necessarily sufficient to alter PMN chemotaxis; (3) alteration in gene expression may explain alteration in IL-8, but not C5a receptors; (4) exudate PMN chemotaxis relies more on C5a than IL-8; and (5) diminished chemoattractant receptors and chemotaxis in septic PMN may explain decreased PMN delivery in these patients. These data support the conclusion that receptor alteration is a principal means by which PMN chemotaxis is regulated in humans *in vivo*.

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Commentary

During the performance of these experiments, the implications for neutrophil biology and the clinical significance of the results were always considered. The literature regarding neutrophil delivery to, function in, and clearance from a site of inflammation was reviewed. Not previously highlighted in the literature, the membrane of the neutrophil provides the means for the neutrophil to interact with surrounding cells and mediators. It therefore was appropriate to perform a review and analysis of available data regarding the role of the neutrophil membrane in the regulation of the neutrophil life cycle. Thus, the following manuscript represents a generalisation of the results already presented, and a discussion regarding how the neutrophil membrane participates in and mediates neutrophil delivery, function and clearance.

Manuscript #4

Neutrophil membrane expression regulates neutrophil delivery, function and clearance

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Manuscript 4: Neutrophil membrane expression regulates neutrophil delivery, function and clearance

Abstract

As the principal cellular component of inflammatory host defense, and a major contributor to host injury following severe physiologic insult, the neutrophil is inherently coupled to patient outcome in health and disease. Extensive research has thus focused on the mechanisms and processes of neutrophil delivery to, function in, and clearance from the inflammatory microenvironment. The neutrophil cell membrane expresses a complex array of adhesion molecules and receptors for various ligands, including soluble mediators, cytokines, immunoglobulin, and membrane molecules on other cells; the membrane is the principal means a cell interacts with its environment. This article reviews the evidence that receptors play a central role in the regulation of neutrophil delivery (including neutrophil production, rolling, adhesion, diapedesis, and chemotaxis), neutrophil function (including priming and activation, microbicidal activity and neutrophil mediated host injury), and neutrophil clearance (apoptosis and necrosis). In addition to mediating these processes within the neutrophil life cycle, change in neutrophil receptor expression is a means by which neutrophils alter their function *in vivo*. In summary, neutrophil cell surface expression, representative of the connectivity of the neutrophil, mediates the processes inherent to the neutrophils' life cycle; and alteration of neutrophil surface expression contributes to and is synonymous with change in neutrophil function.

Introduction

Tissue inflammation, manifesting clinically as *rubor*, *calor*, *tumor* and *dolor,* has been a source of investigation since the beginning of medical science. Inflammation may be defined as a condition or state, which tissues enter as a response to injury or insult. The neutrophil is the most important and the most extensively studied cell involved in the inflammatory response. As the principal circulating phagocyte, the neutrophil is the first and most abundant leukocyte to be delivered to a site of infection or inflammation, an integral component of non-specific host defense. In addition to its role in host defense, the neutrophil is implicated in the pathogenesis of tissue injury and of persistent inflammatory diseases. The paradoxical roles of the neutrophil in host defense and host injury have fueled intense scientific inquiry regarding the processes of neutrophil delivery to a site of inflammation, neutrophil function within the inflammatory environment, and neutrophil clearance from that milieu. The aim of this review is to highlight the importance of neutrophil cell membrane expression in the participation and regulation of neutrophil delivery, function, and clearance from its environment. The relationship between altered receptor expression and altered neutrophil function in humans and *in* vivo will be emphasized. The review will conclude with a brief discussion and interpretation regarding the importance of membrane receptor expression as a measure of cellular "connectivity", and suggestions for future research regarding the role of neutrophils in the inflammatory response.

Neutrophil Delivery to the Inflammatory Microenvironment

1) Neutrophil production and storage

The neutrophil life cycle begins with a bone marrow phase, is followed by a circulating phase, and ends with a tissue phase. Within the bone marrow, neutrophils originate from self-renewing myeloid stem cells: the myeloblast differentiates into the promyloblast, and then into

the myelocyte. These cells differentiate into metamyelocytes, as well as segmented band neutrophils, which are occasionally seen in circulation during a stress response. The metamyeolcyte is the precursor to polymorphonuclear leukocytes, commonly referred to as granulocytes, including eosinohphils, basophils and neutrophils. The process of neutrophil maturation and differentiation within the marrow takes approximately 14 days, and has undergone considerable investigation.[1] Neutrophil production is estimated to vary from 10^8 to 10^{11} cells/day depending on measurement technique.[1, 2] This is mediated by a variety of hematopoietic growth factors, most notably granulocyte colony stimulating factor (G-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF).[3]

Growth factors exert their effect through interaction with membrane receptors, with subsequent induction of intracellular tyrosine phosphorylation and activation of multiple signaling cascades.[4] Variation in receptor expression and modulation by soluble mediators occurs during cell maturation.[5] In addition to others, GM-CSF and G-CSF mediate proliferation and differentiation of neutrophil bone marrow stem cells allowing for substantial variation in neutrophil production, increasing as much as 10-fold during a stress response.[2] Pathologic function of growth factor receptors leads to hematologic illness;[6, 7] and a reduction in marrow G-CSF receptor expression is associated with myeloid maturation arrest and neutropenia following severe burn injury[8]. Thus, neutrophil production, differentiation and maturation depend upon physiologic interaction of growth factors with receptors on neutrophil myeloid precursors.

After release from the bone marrow, neutrophils enter the circulating compartment, the second phase of their life cycle. In circulation, neutrophils have a half-life of 6-9 hours. Neutrophils comprise >50% of circulating leukocytes, and >90% of circulating phagocytes, and reversibly move from circulating to marginating pools. Marginated neutrophils are those "stored" in the capillaries of certain tissues, most notably in the lung, and form a much greater number than those free in circulation at any given time.[9] The lung harbours large numbers of

marginating neutrophils due to the tremendous number of small capillaries (diameter < neutrophil), forcing neutrophils to deform in order to pass through these capillaries.[10] The marginating pool of neutrophils allows for rapid mobilization in response to infection or other stresses. Despite the rapid turnover, human neutrophil counts are relatively stable, averaging 3000 to 4000 neutrophils/mm^3. Neutrophil delivery occurs in the post-capillary venule as a sequential series of well-studied processes (see **Figure 1**)

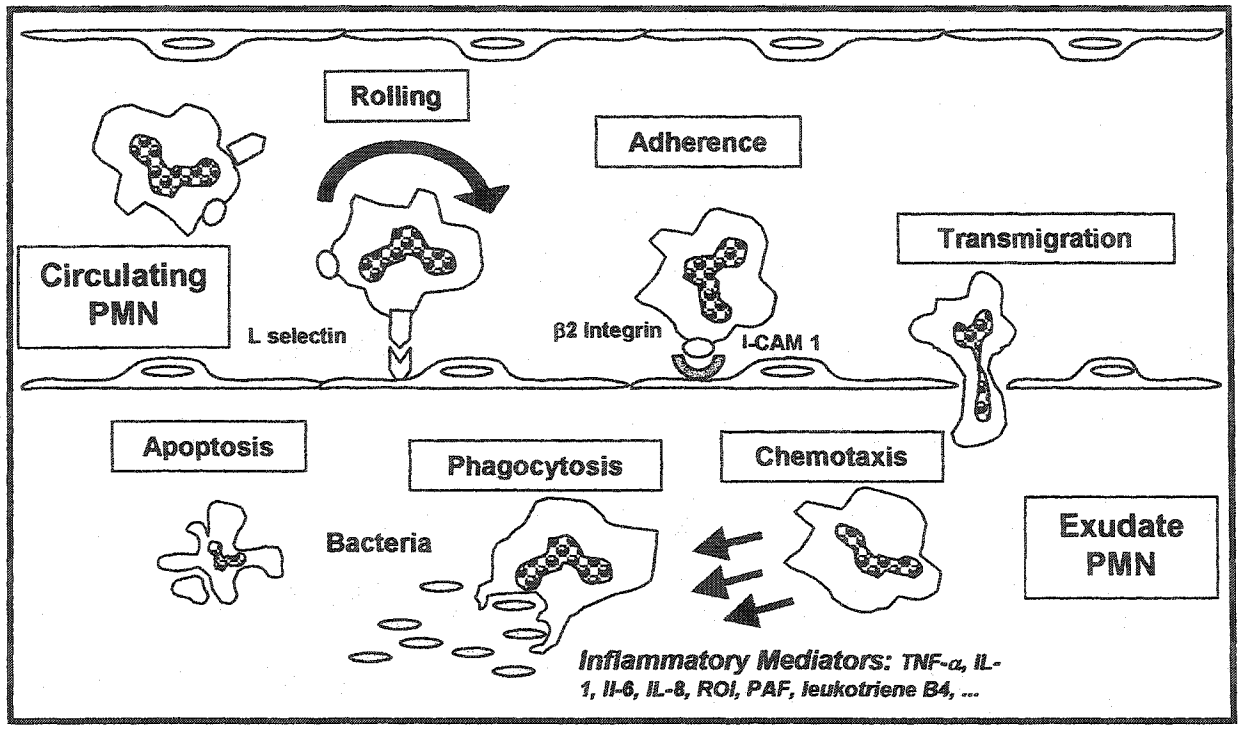

Figure1: Neutrophil Delivery within the Post-Capillary Venule

2) Margination

Neutrophil transmigration from the intravascular to the extravascular (exudate) milieu predominantly occurs in the post-capillary venule, facilitated by a combination of mechanical, chemical and molecular factors. The first step is *margination*, or movement of the neutrophil from the central stream to the periphery of a vessel. In post-capillary venules, when the vessel diameter is 50% larger than the diameter of the leukocyte, erythrocytes move faster than the larger leukocytes, especially in the center of the vessel, pushing leukocytes to the

vessel periphery.[11] Physical forces involved in the erythrocyte-leukocyte interactions govern this radial movement of leukocytes. The importance of erythrocytes has been demonstrated in a rat mesenteric perfusion model, where no leukocyte margination was observed in the absence of red cells.[12] Neutrophil margination allows for a molecular interaction between the cell surface of the neutrophil and endothelial cell to occur, resulting in neutrophil rolling on the vessel wall.

3) Rolling

A state of weak adhesive interaction between the neutrophil and endothelial cell allows the neutrophil to roll along the surface of the post-capillary venule. *Rolling* is dependent upon both physical and molecular forces. The neutrophil's ability to roll and adhere to endothelial cells is inversely proportional to the vessel shear rate (i.e. faster moving blood decreases the ability of leukocytes to adhere).[13] Neutrophil rolling velocity is also directly proportional to luminal red blood cell velocity.[14] Once in proximity to the endothelial cell, a low affinity adherence occurs, and in conjunction with the shear stress of passing erythrocytes, the neutrophil begins to roll along the endothelial lining of the vessel.

Selectins: Interactions between the surface of the neutrophil and the endothelial cell allow for rolling, and subsequently, adherence and diapedesis. The low affinity interaction involved in rolling is largely governed by *selectins* and their ligands (see **Table 1**). Selectins are a family of glycoprotein surface adhesion molecules that include L-selectin (expressed exclusively of leukocytes), E-selectin (expressed exclusively of endothelial cells) and P-selectin (expressed on platelets and endothelial cells). Constitutive expression of L-selectin is maintained on all circulating quiescent leukocytes (except for certain subpopulations of memory T-cells).[15] Animal intravital microscopy has demonstrated that blocking L-selectin and/or P-selectin with high dose selectin-binding carbohydrate (fucoidin) decreased both neutrophil rolling and adherence following ischemia-reperfusion.[16] L- and P-selectin gene-deficient mice

demonstrate diminished rolling.[17] The ligands for neutrophil L-selectin are multiple sialylated carbohydrate determinants, which are linked to mucin-like molecules.[15, 18] These selectin ligands on endothelial cells are inducible with LPS or a variety of inflammatory cytokines.[19] In addition to L-selectin mediated rolling, endothelial cell expression of E-selectin is necessary for normal leukocyte recruitment and may initiate leukocyte rolling in certain models.[20, 21]

Table 1: Neutrophil & Endothelial Cell Adhesion Receptors

Receptor	Cell	Ligand	Cell type	Purpose
L-Selectin	Neutrophil	sLe^a , sLe^x	Endothelium	Rolling and weak adhesion of PMN on EC
CD11a/CD18	Neutrophil	ICAM-1, ICAM-2, ICAM-3	Endothelium	Adhesion of PMN on EC
CD11b/CD18	Neutrophil	ICAM-1,	Endothelium	Adhesion of PMN on EC
		iC3b	Complement	Phagocytosis?
		Fibrinogen	-	-
		Factor X	-	-
CD11c/CD18	Neutrophil	iC3b	Complement	Phagocytosis?
		Fibrinogen	-	-
E-Selectin	Endothelium	sLe^x	Neutrophil	Firm PMN/EC adhesion
P-Selectin	Endothelium Platelets	sLe^x	Endothelium	Firm PMN/EC adhesion
		PSGL-1	Neutrophil	Firm PMN/EC adhesion
PECAM-1	Endothelium	CD31/alpha$_v$	Leukocytes	Diapedesis of PMN through EC
	Neutrophil		-	-
ICAM-3	Neutrophil	CD11a/CD18	Leucocytes	Antigen presentation

The rolling governed by a weak molecular interaction is a prerequisite for a stronger molecular interaction, namely adherence. This has been demonstrated using intra-vital microscopy in the rat mesenteric microcirculation,[22] in human neutrophils in rabbit mesenteric venules,[23] and in a cat mesenteric perfusion model.[14] However, others have demonstrated that antibodies to P-selectin will attenuate rolling, but not impact adherence.[24] Blocking L-selectin in animal models has reduced neutrophil-mediated tissue injury, thought to be dependent on neutrophil adherence.[25] Thus, these studies suggest that selectins not only mediate rolling, but are required for ensuing leukocyte adherence.

4) Adherence

As with rolling, the cell surface of the neutrophil determines its ability to undergo *adherence*. In contrast to rolling, a dynamic low-affinity adhesive interaction, adherence is a stationary high-affinity (strong) adhesive interaction between the neutrophil and endothelial cell. This interaction is largely mediated by a separate set of adhesion molecules, the integrins and their ligands. The importance of integrin mediated adhesion to neutrophil delivery and host defense was first demonstrated in patients with Leukocyte Adhesion Deficiency-1 (LAD-1).[26] These patients develop life-threatening bacterial infections, as neutrophils are unable to undergo transmigration to sites of inflammation due to a genetic mutation in CD18, the β subunit of the integrin family of adhesion molecules. Neutrophils from healthy controls incubated with monoclonal antibodies to integrins, or neutrophils from patients with LAD-1 both demonstrate deficient adhesion and transmigration through activated endothelial monolayers.[27]

Integrins and ICAMs: Integrins are a family of heterodimeric proteins (made up of two different subunits - α and β subunits) expressed on the cell surface, and are integral to the process of cell adhesion. The β_2-integrins are restricted to leukocytes, and are essential to

normal leukocyte trafficking. They consist of three distinct α subunit (CD11a, CD11b, CD11c) bound to a common β subunit (CD18). Although the distribution of β_2-integrins subclasses differs amongst leukocyte populations, neutrophils express all three classes. The relative contribution of each α subunit to leukocyte adherence may vary and depend upon the stimulus leading to adherence and transmigration.[28] Neutrophil integrins interact with complementary surface molecule ligands on endothelial cells in order to generate the high affinity bond that characterizes adherence (see **Table 1**). Particularly important to neutrophils, intercellular adhesion molecule 1 (ICAM-1) on endothelial cells serves as the ligand for both CD11a/CD18 and CD11b/CD18, whereas ICAM-2 is capable of binding CD11a only.[29] Animal intravital microscopy has demonstrated the importance of integrin β subunit CD18 to adhesion, but not to rolling.[30, 31] Multiple studies have demonstrated that anti-CD11/CD18 antibodies have reduced inflammation and injury in model of allograft rejection, endotoxin challenge, hemorrhagic shock, aspiration pneumonia, pneumonia, ischemia reperfusion and more.[32] While CD18-dependent neutrophil transmigration is essential for physiologic neutrophil delivery, CD18-independent neutrophil transmigration has been demonstrated in rabbit models of respiratory and peritoneal infection, respiratory and hepatic ischemia-reperfusion;[33-36] and may depend on the type of bacteria at a site of infection.[37] Despite the complexity of adhesion molecules, the membrane of the neutrophil and of the endothelial cell must interact and undergo firm adhesion in order for the process of neutrophil transmigration to progress.

Both integrins on neutrophils, as well as ICAMs on endothelial cells demonstrate marked variability of expression and adhesiveness. Augmented neutrophil expression of CD11b/CD18 is induced from intracellular pools by various cytokines, including FMLP, GM-CSF, C5a, TNF-α and others; however, increased neutrophil adhesiveness may be more significantly related to conformational changes in the CD11b/CD18 protein complex. [38] Chemoattractants such as the chemokine IL-8 will activate integrin adhesiveness as well as help to direct leukocyte migration.[39, 40] In addition to constitutive expression of ICAM-1 and

ICAM-2 on endothelial cells, ICAM-1 expression may be augmented by numerous inflammatory mediators.[41-43] Thus, under the influence of inflammatory mediators, changes in number and conformation of neutrophil integrins and upregulation of endothelial cell ICAM expression will induce a transition from selectin-dependent rolling to integrin/ICAM-dependent adherence,[44] subsequently leading to diapedesis, the next step in neutrophil delivery.

5) Diapedesis

Following adherence, the neutrophil must pass through the endothelial monolayer and basement membrane to enter the extravascular inflammatory (exudate) environment. *In vitro* adherence of neutrophils on activated endothelial cells will cause a disruption of endothelial cell-cell interaction and augment endothelial cell permeability, an effect blocked with anti-integrin monoclonal antibodies.[45] Transmission electron microscopy in a human umbilical vein neutrophil transmigration model suggests that diapedesis of neutrophils occurs at endothelial cell tri-cellular corners (the intersection of three endothelial cells).[46] Endothelial adhesion molecules are necessary for diapedesis and transmigration. Leukocyte adherence and emigration observed post ischemia/reperfusion and in response to leukotriene B4 or platelet activating factor, is decreased with monoclonal antibodies to various adhesion glycoproteins, including CD18, CD11b, ICAM-1 and L-selectin.[47, 48] Thus, membrane mediated adherence is a prerequisite for diapedesis, a process also mediated by neutrophil-endothelial cell membrane interaction.

PECAM-1: Other adhesion molecules, such as platelet-endothelial cell adhesion molecule-1 (PECAM-1) are specifically involved with the process of diapedesis. PECAM-1 is constitutively expressed and concentrated on the lateral borders of endothelial cells where diapedesis is observed to take place, as well as on the surface of neutrophils, some T cells, monocytes, and platelets. Blocking PECAM-1 with monoclonal antibodies will increase neutrophil adhesion to endothelial cells mediated by CD11b/CD18,[49, 50] thus inhibiting the

ability of the neutrophil to undergo diapedesis. Monoclonal antibodies to PECAM-1 will arrest leukocytes transmigration by 70-90% without interfering with normal leukocyte adhesion to endothelial monolayers; leukocytes remain tightly bound to the apical surface of the endothelial cell, precisely over the intercellular junction.[51] The importance of endothelial and neutrophil expression of PECAM-1 was confirmed using in vivo murine intravital microscopy.[52] Thus, PECAM-1 appears to allow the neutrophil to evade adhesion at intercellular junctions so that diapedesis leading to neutrophil transmigration may take place.

In summary, the process of neutrophil transmigration is regulated by a multi-step process that involves sequential events, each necessary for progression to the next. Theses cellular processes are governed by molecular interactions between receptors and their ligands expressed on neutrophils and endothelial cells. The cell membrane of the neutrophil is what allows it to interact with endothelial cells. By altering the expression and efficacy of the various adhesion receptors dynamically *in vivo,* leukocyte delivery is regulated leading to site-specific leukocyte accumulation. In addition to adhesion receptors and ligands mediating neutrophil-endothelial cell interactions, leukocyte delivery requires further neutrophil cell membrane participation, specifically responding to soluble mediators in the extracellular inflammatory environment.

6) Chemotaxis

In addition to intercellular adhesion, leukocytes require a chemoattractant gradient in order to complete the process of transmigration. Chemoattractants are soluble molecules that cause directionality to cell movement; cells migrate in the direction of increasing concentration of a chemoattractant in a process called chemotaxis. For over three decades, neutrophils have been known to undergo chemotaxis towards damaged or inflamed tissue.[53] The production of chemoattractants in the inflammatory environment is due to a combination of sources, including bacterial breakdown products, complement factors, and chemokines produced by

inflammatory and non-inflammatory cells. For example, in addition to neutrophils themselves,[54] monocytes, smooth muscle cells, epithelial cells, endothelial cells and fibroblasts are capable of generating Interleukin-8 (IL-8), a potent neutrophil chemoattractant, when stimulated with an pro-inflammatory agonist, such as IL-1 or TNF-α.[55] Chemoattractants serve not only to direct leukocytes to specific areas of inflammation, but also recruit specific subpopulations of leukocytes to inflamed tissue, such as neutrophils in response to acute bacterial infection, eosinophils at sites of chronic allergic inflammation or parasitic infection, or monocytes in chronic inflammatory diseases. Chemoattractant mediators may thus be classified depending upon their spectrum of leukocyte activity (see **Table 2**). Classical chemoattractants include N-formylated peptides produced by bacteria such as f-Met-Leu-Phe (FMLP), polypeptides (e.g. C5a) and lipids (e.g. leukotriene B4), which act as chemoattractants for various non-specific leukocyte populations.[56-58] Chemoattractant cytokines, or chemokines are a novel family of chemoattractants that confer specificity to leukocyte subset responsiveness, and are well reviewed elsewhere.[59, 60] Extensive *in vitro* and *in vivo* investigation has identified IL-8 as a principal factor in neutrophil delivery.[61,62,63,64] Other chemokines specific for neutrophils include epithelial cell derived neutrophil activating peptide (ENA-78), neutrophil activating peptide-2 (NAP-2), growth related oncogene (GRO-α, GRO-β, GRO-δ), macrophage inflammatory protein-2 (MIP-2) alpha and beta. These chemokines are structurally similar, consisting of the first two cysteine (C) amino acid residues separated with a separate amino acid (X), and are referred to as CXC chemokines or α chemokines. A separate family of chemokines are known as the CC chemokines, as the first two cysteine residues are in juxtaposition. Monocyte chemoattractant protein-1,2 and 3 (MCP-1, MCP-2, MCP-3), macrophage inflammatory protein-1 (MIP-1) alpha and beta, and RANTES are members of the CC family, or are β chemokines. The activity of the CC supergene family of chemokines is predominantly oriented towards monocytes.[65] Thus, chemoattractants help explain how leukocytes localize to specific inflammatory sites, and how specific leukocyte populations are recruited to these sites.

Table 2: Neutrophil Chemoattractants

Neutrophil specific	Leukocyte Non-specific
IL-8	C5a
Granulocyte chemotactic protein 2 (GCP- 2)	TNF
Epithelial cell-derived neutrophil attractant 78 (ENA-78)	Monocyte chemoattractant protein-1,2,3,4 (MCP-1,2,3,4)
Neutrophil Activating Pepetide-2 (NAP-2)	FMLP
Growth Related Oncogene (GRO-alpha, beta, gamma)	Macrophage chemotactic and activating factor (MCAF)
Macrophage inflammatory protein-1,2 (MIP-1,2)	PAF
Platelet factor 4 (PF4)	*RANTES*
Mast cell-derived chemotactic factor	I-309
5-Hydroxyeicosatetraenoic acid	Casein
	Leukotriene B4 (LTB4)

Chemoattractant receptors: Leukocyte delivery is further regulated by chemoattractant receptors that display specificity for both the type of leukocyte on which they are expressed, and the ligand to which it will bind. The specificity of chemoattractant induced leukocyte chemotaxis is related to differential expression of chemokine receptors, a superfamily of G-protein coupled receptors with seven transmembrane regions.[66, 67] Although chemokine receptors share similar structure, they differ in their ligand specificity (see **Table 3**). For example, IL-8 receptor A (IL-8RA or CXC R1) and IL8-RB (CXC R2) have a 78% identical amino acid sequence, and will both bind IL-8; however, while IL8RA is specific for IL-8, IL8RB has multiple agonists, including other CXC chemokines such GRO-α, GRO-β, GRO-δ, neutrophil-activating peptide-2 and epithelial cell-derived neutrophil activating peptide-78.[68] Neutrophil transmigration appears to depend to a greater degree on IL-8RA than IL-8RB, as antibodies directed against IL-8 RA inhibited the majority (78%) of IL-8 induced chemotaxis.[69] In contrast, IL-8RB has been implicated in the transendothelial migration of T-cells.[70] In addition, chemoattractant receptors are expressed on specific leukocyte subsets (see **Table 3**): whereas receptors to the classical chemoattractants are expressed on monocytes, neutrophils, eosinophils and basophils, CXC chemokine receptors are primarily restricted to neutrophils.[15] Thus, chemokine receptors display both ligand and leukocyte specificity. These

complex rules defining the interactions between specific chemoattractants and leukocytes are believed to allow the host response to deliver specific subsets of leukocytes to localized areas of infection or inflammation. Chemoattractant receptors not only mediate the process of chemotaxis, but changes in receptor expression within the inflammatory environment confer changes to cell function. Prior to discussing changes in neutrophil receptor expression, neutrophil function & clearance from the inflammatory microenvironment are discussed.

Table 3: Neutrophil Chemoattractant Receptors & Ligands

Class	Receptors	Ligands
C-X-C Receptors	CXCR-1 (IL-8 RA)	IL-8
	CXCR-2 (IL-8 RB)	IL-8,GRO, NAP-2, ENA-78, GCP-2
	CXCR-3	Mig, IP-10
	CXCR-4	SDF-1
C-C Receptors	CCR-1	MIP-1α, MIP-1β, MCP-3
	CCR-2A,2B	MCP-1, MCP-3
	CCR-3	Eotaxin, RANTES, MCP-3
	CCR-4	MIP-1α, RANTES, MCP-1
	CCR-5	MIP-1α, MIP-1β, RANTES
	CCR-6	MIP-3α
	CCR-7	ELC
	CCR-8	I-309
Non-C-X-C	C5aR	C5a
	FMLPr	FMLP

Neutrophil function in the Inflammatory Microenvironment

7) Neutrophil priming and activation

Neutrophils are capable of existing in different stable functional states. Different neutrophil states are associated with distinct patterns of altered membrane expression and altered function (see **Table 4**). For example, quiescent neutrophils can be "activated" by various inflammatory mediators in order to produce reactive oxygen metabolites (the respiratory burst) and destructive proteolytic enzymes (see below). In addition to being activated, the neutrophil can be "primed" to produce an augmented or exaggerated response to an activating stimulus. Priming is defined as an enhancement or amplification of the neutrophil

respiratory burst in response to a given activating stimulus following exposure to the priming agent.[71] Altering the neutrophil from a "resting" state to a "primed" state does not activate the respiratory burst directly but will potentate the neutrophil response to a subsequent stimulus.[72] Various mediators have been known to cause neutrophil priming, including ATP[73], platelet activating factor (PAF)[74], IL-8[75], IL-6[76], lipopolysaccharide[77] and leukotriene B4[78]. An alteration in cell surface receptor expression has been proposed to mediate the priming phenomenon; for example, GM-CSF and TNF will cause an increase in neutrophil FMLP receptor expression when primed.[79, 80] Yet, others have demonstrated diminished or unchanged numbers of receptors with other priming agents, or that the priming effect was temporally unrelated to increase in receptor numbers.[81-83] Separate investigators found that the priming effect altered the signal transduction cascade distal to the FMLP receptor, involving a direct activation of G-proteins.[84] An immediate and rapid rise in intracellular [Ca^{2+}] is implicated in the ability of a group of agents to cause priming, including IL-8, ATP, leukotriene B4 and PAF.[85,73] However, under certain conditions priming secondary to FMLP occurs without any rise in [Ca^{2+}].[86] Other priming agents, such as TNF-α, GM-CSF or LPS are associated with less rapid rises in intracellular [Ca^{2+}], and require longer incubation periods to achieve the priming effect.[87] Priming effects are further complicated by the fact that priming agents display synergy.[88, 89] Neutrophil priming and subsequent activation has been hypothesized to play an important role in endothelial cell and end organ injury and the pathogenesis of multiple organ dysfunction,[90] supported by data from an animal ischemia-reperfusion model,[91] and observations of human neutrophils following trauma.[92]

Table 4: Human neutrophil states

PMN States	PMN Receptors	PMN Function
Circulating PMN **Resting bloodstream PMN, collected by venipuncture**	**Adhesion receptors:** Constitutive expression L-selectin, PECAM-1, ... **Chemoattractant receptors:** Constitutive expression of IL-8RA, IL-8RB, C5aR, ... **Apoptosis receptors:** Constitutive expression of TNFαR1, Fas and FasL. **Other:** CD14	**PMN-EC Interactions:** Baseline PMN rolling, adhesion on activated endothelium and transmigration. **Chemotaxis:** Will undergo chemotaxis to PMN-specific and leukocyte non-specific chemoattractants **Function:** Minimal PMN respiratory burst (ROI·) and microbicidal activity (proteolytic enzymes) **Apoptosis:** Constitutive apoptosis (PMN half-life ~ 6hrs.)
Primed PMN **PMN stimulated with priming agent *in vitro***	**Adhesion receptors:** Increased expression CD11b, L-selectin, PECAM-1, ⇔FMLPr **Chemoattractant receptors:** ?IL-8RA, ?IL-8RB ⇔C5aR, **Apoptosis receptors:** ?TNFαR1, ?Fas, ?FasL **Other:** CD14, ⇑ LTB4r, ⇑ PAFr	**PMN-EC Interactions:** Unclear impact on rolling, adhesion, diapedesis. **Chemotaxis:** No change chemotaxis **Function:** Increased respiratory burst & microbicidal activity after activation **Apoptosis:** Delayed constitutive apoptosis, ? induced apoptosis.
Activated PMN **PMN stimulated with activating agent *in vitro***	**Adhesion receptors:** ⇑ CD11b, ⇑ FMLPr, ? L-selectin, ? PECAM-1, **Chemoattractant receptors:** ⇓ IL-8RA, ⇓ IL-8RB ⇔ C5aR **Apoptosis receptors:** Unknown **Other:** ⇓ C3br, ⇓1C3b	**PMN-EC Interactions:** ⇑ PMN rolling & adhesion; ?transmigration. **Chemotaxis:** ⇔ Chemotaxis to C5a ⇑? Chemotaxis to FMLP Chemotaxis to LTB4/ZAS **Function:** ⇑ Respiratory burst (ROI·) and microbicidal activity (proteolytic enzymes), ⇔ Phagocytosis **Apoptosis:** Apoptosis delayed
Exudate PMN **PMN collected from dermal exudate milieu *in vivo***	**Adhesion receptors:** ⇑ CD11b, ⇑ Mac-1, ⇓ L-selectin, ⇓ PECAM-1 **Chemoattractant receptors:** ⇓ IL-8RA, ⇓ IL-8Rb, ⇑ C5ar **Function:** ⇑ FMLPr **Apoptosis receptors:** ⇓ binding to TNF-α, ?⇓ TNF RI ⇔ Fas, FasL	**PMN-EC Interactions:** Unknown. **Chemotaxis:** ⇑ baseline CTX, ⇓ chemotaxis to IL-8 ⇑ chemotaxis to C5a **Function:** ⇑ Respiratory burst (ROI·) ⇑ Microbicidal activity and Phagocytosis **Apoptosis:** ⇓ constitutive Apoptosis ⇓ TNF-α induced, not Fas induced apoptosis
Septic PMN **PMN collected from circulation in septic patients *in vivo***	**Adhesion receptors:** ⇓ L-selectin, ?CD11b, ?FMLPr, ? PECAM-1, **Chemoattractant receptors:** ⇓ IL-8RA, ⇓ IL-8RB ⇓ C5aR **Apoptosis receptors:** ⇓ TNFα R1, ?Fas, ?FasL	**PMN-EC Interactions:** Unknown **Chemotaxis:** ⇓ Chemotaxis to IL-8 and C5a **Function:** ?⇑ Respiratory burst (ROI·) ?⇑ Microbicidal activity and Phagocytosis **Apoptosis:** ⇓ constitutive Apoptosis ⇓ TNF-α induced, not Fas induced apoptosis
Unresponsive or apoptotic PMN	**Adhesion receptors:** ⇓ L-selectin, ?CD11b, ?PECAM-1, **Chemoattractant receptors:** Unknown **Apoptosis receptors:** ?⇓ TNFRI, ?Fas, ?FasL **Other:** ⇓ PAFr	**PMN-EC Interactions:** No interaction. **Chemotaxis:** ⇓ Chemotaxis **Function:** ⇓ Respiratory burst (ROI·) ⇓ Microbicidal activity or phagocytosis **Apoptosis:** Unresponsive PMN are undergoing apoptosis.

In summary, neutrophil priming occurs through differing, interconnected pathways marked by redundancy and synergy, is mediated by alteration in surface receptor expression and intra-cellular pathways, and has been associated with the development of organ injury following severe physiologic insult.

Strongly related to priming, neutrophil activation is an integral component to the systemic host response. As the most abundant inflammatory cell, neutrophil activation is essential for host defense against bacterial or fungal infection, as well as principally involved in host injury in states of persistent inflammation. Our patients live to survive the balance between the paradoxical roles of the neutrophil. Although this subject has undergone comprehensive review,[93, 94] the physiologic and pathologic roles of the neutrophil are presented, highlighting the role of the neutrophil cell membrane. Both neutrophil microbicidal activity and neutrophil induced tissue injury are representative of the function of the activated neutrophil within the exudate inflammatory microenvironment.

8) Neutrophil microbicidal activity and neutrophil-induced tissue injury

As the principal phagocyte delivered to inflammatory sites, the role of the neutrophil is to destroy and ingest pathogens in the circulating and exudate milieu, an important component to non-specific immunity. Deficiencies in neutrophil function are well studied and are clearly linked to increased frequency and severity of bacterial and fungal infections.[95] Simultaneously, the neutrophil's destructive capacity leads to host injury in numerous disease states.[96] This paradox is at the heart of the difficulty in creating effective immunomodulation for critically ill patients.

Cell surface receptors on the neutrophil are essential to the process of phagocytosis and simultaneous activation of microbicidal mechanisms. Using mechanisms similar to those used in chemotactic movement, the membrane of the neutrophil is capable of extending

pseudopodia and engulfing microorganisms. Opsonins will bind to neutrophil receptors and trigger phagocytosis. Opsonins principally include complement fragments and antibodies. For example, neutrophil receptors for the Fc domain of IgG antibodies will recognize particles coated with IgG. Change in receptors may alter the ability of neutrophils to respond to opsonins. For example, Fcγ RIII (CD16) and Fcγ RII (CD 32) are low affinity receptors for IgG and are expressed constitutively on circulating neutrophils in healthy controls, whereas Fcγ RI (CD64), a high affinity IgG receptor is induced by inflammatory cytokines[97], and expressed in circulating neutrophils in patients with bacterial infections.[98] Following binding to neutrophil receptors, phagocytosis takes place; cytosolic granules subsequently fuse with phagolysosomes into which they release their toxic contents.

Neutrophil toxins are divided into two groups based upon their localization within the cell: intracellular granules and plasma membrane.[96] Intracellular granules contain microbicidal peptides, proteins and enzymes, including elastase, proteinases and myeloperoxidase. These enzymes are also released into phagocytic vacuoles, destroying targets ingested by the neutrophil. When a neutrophil is activated, there is fusion of intracellular granules with its plasma membrane releasing proteolytic enzymes into the extracellular environment. Concurrently, neutrophil membrane NADPH oxidase is activated. The activated NADPH oxidase converts oxygen (O_2) to the superoxide anion ($0_2^{\bullet}$-), a process known as the respiratory burst. The majority of $O_2^{\bullet}$- then dismutates to hydrogen peroxide H_2O_2. Hypochlorous acid (HOCl) is formed when myeloperoxidase oxidases Cl- in the presence of H_2O_2. In addition to the direct toxic effects of superoxide ion, proteolytic enzymes and hypochlorous acid, neutrophil endothelial cell injury may also occur through combination of H_2O_2 with reduced iron within the endothelial cell, forming the highly reactive and toxic hydroxyl ($OH^{\bullet}$-) radical.[99] Reactive nitrogen species, including nitric oxide ($NO^{\bullet}$), act independently and synergistically with reactive oxygen species to augment neutrophil delivery, and form secondary cytotoxic species.[93] Thus, neutrophil microbicidal activity is

mediated by a synergistic combination of membrane respiratory burst and intracellular granules.

Neutrophil-mediated tissue injury is dependent upon a balance of competing protective and destructive pathways. To protect the host against the damaging products generated by neutrophils, there exist anti-oxidants and powerful protease inhibitors within the extracellular matrix, such as x_1- proiteinase inhibitor, x_2- macroglobulin and secretory leukoproteinase inhibitor.[100] To counteract the neutralizing effect of the protease inhibitors, hypochlorous acid will inactivate the anti-proteases in the immediate vicinity of the neutrophil.[96] Neutrophils also contain an endogenous supply of anti-oxidants, protecting themselves and surrounding tissue. Also contributing to the balance of inflammation, the rate of clearance of neutrophils through apoptosis is correlated with degree and resolution of inflammation, and is subsequently discussed in greater depth. The balance of inflammatory and anti-inflammatory mediators is coupled with the neutrophil's paradoxical roles. An inflammatory response associated with severe sepsis may be harmful, whereas the inflammatory response is necessary to clear infection, as demonstrated in an elegant murine cecal ligation and puncture model utilising variable caliber of puncture. Inflammatory response may be localized or systemic, and interventions that yield a reduction in neutrophil-mediated inflammatory injury in one organ, may predispose to infection at other sites. Genetic factors are clearly involved in determining host response to physiologic insult, and are only recently under active investigation. Improved understanding of these factors will be essential to better understand how to intervene effectively in patients with overwhelming persistent inflammation.

Neutrophil clearance from the Inflammatory Microenvironment

9) Apoptosis and necrosis

Apoptosis is the means by which physiologic cell death occurs (see **Figure 2**). It is a highly orchestrated, much studied, form of cell death whereby cells commit suicide, by cleaving their

DNA into relatively uniform short segments, and dividing the cell into membrane-packaged parcels of intracellular contents (including intact organelles), which are then phagocytosed by surrounding cells. Physiologic cell death is crucial to the varied functions of multicellular organisms, including normal tissue development, homeostasis, and neural and immune system development.[101] As illness may reflect an altered balance between cell proliferation and cell death, too little or too much apoptosis has been implicated in human diseases such as Alzheimer's and cancer.[102] Apoptosis, a term introduced by Kerr in 1972[103], denotes a form of cell death under genetic control that results in removal of a cell with no inflammatory reaction. A cell undergoing apoptosis will shrink. Its nucleus will undergo karyorrhexis (fragmentation) and karyolysis (dissolution), its DNA undergoes specific inter-nucleosomal cleavage (resulting in DNA segments of approximately 185 base pairs in length), and the cell will ultimately break up into apoptotic bodies containing pyknotic nuclear debris.[104] Surrounding cells, even those that are not "professional phagocytes" such as epithelial cells, will phagocytose the apoptotic bodies. The phagocytosis of apoptotic bodies containing intact cellular organelles allows for efficient recycling of valuable intracellular contents, without causing an inflammatory response. The lack of inflammation associated with apoptosis is crucial to the distinction between apoptosis and other forms of cell death. For example, ischemic cell death (termed oncosis) is characterized by cellular swelling, organelle swelling, blebbing and increased membrane permeability, non-specific DNA breakup, which will evolve to cell membrane dissolution, or necrosis.[104] Particularly important to the neutrophil, oncosis and necrosis involve the spillage of intracellular contents into the extracellular environment with resultant inflammation. The lack of inflammation associated with neutrophil clearance through apoptosis has led to intensive investigation regarding the regulation of neutrophil apoptosis. This review will focus on the role of neutrophil membrane expression to the process of apoptosis. First, alteration in receptor expression occurs during the process of apoptosis providing a means to detect apoptosis, and second, the neutrophil membrane mediates the activation of apoptosis through death receptors.

Alterations of cell membrane expression in apoptotic cells may be used to detect apoptosis in the laboratory. It was noted that phagocytosis is inhibited by phosphatidylserine (PS), regardless of species (human or murine) or type of apoptotic cell (lymphocyte or neutrophil).[105] PS normally resides on the inner membrane leaflet, but is expressed on the outer membrane as an early feature of apoptosis,[106] and is implicated in macrophage recognition of apoptotic cells.[107] Flow Cytometry analysis using a fluorescent-labeled molecule (Annexin V) that specifically binds to PS facilitates the quantification of cells expressing PS and undergoing apoptosis.[108, 109] The PS binding technique detects early apoptosis, and provides a clear differentiation between necrotic and apoptotic cells.

Death receptors: In addition to genetically controlled, pre-programmed apoptosis, cells may be instructed to undergo apoptosis by the binding of neutrophil membrane death receptors, which transmit signals initiated by the binding of a death ligand.[110] Death receptors are part of the TNF receptor gene superfamily, and contain a cytoplasmic sequence that has been named the "death domain" – a sequence of approximately 80 base pairs near the C-terminus, which is located within the intracellular region of the receptor and mediates its cytotoxicity.[111, 112] The best characterized and presumably most important death receptors are Fas (CD95) and TNF RI (the p55 or 55 kilo dalton TNF receptor).[112, 113] Neutrophils express both of these receptors, which may be activated by their ligands to induce rapid cell death. Other more recently discovered death receptors include death receptor 3 (DR3), DR4, and DR5; these receptors are either not expressed on neutrophils, have not yet been investigated with respect to neutrophil apoptosis, or are not recognized as significant to neutrophil homeostasis.[110] Following activation of a death receptor, a receptor-specific complex cascade of intracellular events results in apoptosis.

Fas: When Fas ligand (FasL) interacts with Fas, a death receptor, the cell expressing the Fas will undergo rapid apoptosis.[114, 115] The Fas-Fas Ligand (FasL) apoptotic pathway has been demonstrated to play important roles involved in immune system development and function,

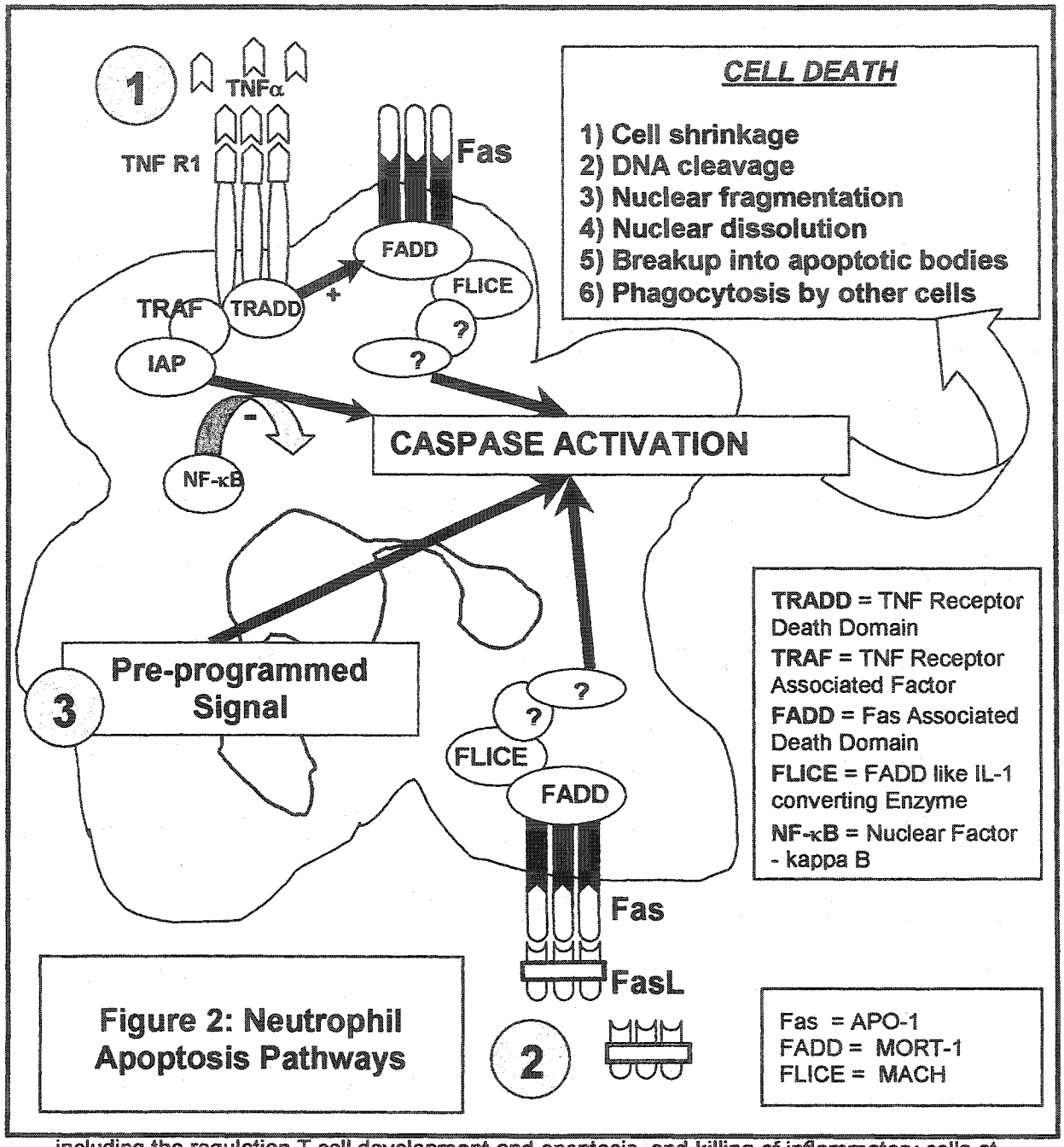

Figure 2: Neutrophil Apoptosis Pathways

including the regulation T cell development and apoptosis, and killing of inflammatory cells at "immune-privileged" sites.[116-119] Fas and FasL are of crucial importance to initiation of apoptosis in human neutrophils. Anti-Fas antibodies will accelerate neutrophil apoptosis to a greater degree than lymphocytes and monocytes.[120] FasL exists either in soluble form or as a cell surface molecule, forming part of the tumour necrosis factor family. FasL will bind three Fas molecules simultaneously, cause clustering of the death domains, which leads to the binding of specific intracellular proteins. These include FADD (Fas-associated death domain)

and FLICE (FADD like IL-1β converting enzyme), when bound to the death domain, activate a family of specific cysteine proteases, called *caspases*.[110] Caspases represent the machinery of cell death: they inactivate proteins that protect against apoptosis, disable and deregulate proteins in general, participate in direct disassembly of cell structures, reorganization of the cytoskeleton, and disruption of the nucleus and its contents.[121]

TNF RI: TNF-α will dramatically increase apoptosis rates in circulating neutrophils of healthy human controls.[122, 123] Similar to FasL, three TNF-α will trimerize TNF RI leading to clustering of death domains, and leading to binding by TRADD (TNFR-associated death domain). Two distinct and independent signaling pathways then proceed: activation of NF-κB, and activation of the caspase pathway leading to apoptosis (mediated through FADD, similar to the Fas pathway).[124] NF-κB regulates a wide variety of genes involved in the synthesis of hematopoeietic growth factors, chemokines, and leukocyte adhesion molecules.[125-127] Recent evidence also implicates NF-κB activation as an important survival mechanism in granulocytes. It has been shown to downregulate TNF-mediated apoptosis in a negative feedback mechanism.[126-130] The survival mechanism mediated by NF-κB explains why TNF-α may not trigger apoptosis unless protein synthesis is blocked. Given that activation of the death receptor TNF R1 will lead to competing pathways, TNF-α will have differential effects on neutrophil apoptosis depending on the activation state of the neutrophil.[131]

The signaling pathways initiated by both TNF and FasL may be "modulated" by a variety of mediators in the inflammatory environment. Specifically, delayed apoptosis in states of persistent inflammation has been extensively investigated. Many inflammatory mediators cause a delay in constitutive neutrophil apoptosis, and include Interleukin-2,[132] Interleukin-6,[133] Interleukin-8,[134] G-CSF,[135] GM-CSF[136], C5a and lipopolysaccharide (LPS)[123, 137]. In addition to constitutive apoptosis, inducible apoptosis mediated by the Fas pathway is suppressed with a variety of inflammatory mediators, including IL-8,[138] G-CSF, GM-CSF, IFN-γ, and TNF-α.[139] This delay in Fas mediated apoptosis secondary to inflammatory cytokines

may be diminished in elderly subjects[140]. In addition, inflammatory mediators may alter intracellular factors within neutrophils in order to delay apoptosis; these factors include mitochondrial stability and caspases activity[141], in addition to NF-κB activation. Other agents in the inflammatory microenvironment that have been demonstrated to modulate neutrophil apoptosis include immune complexes[142], reactive oxygen intermediates,[143] and red blood cells (possibly secondary to scavenging oxidants)[144]. In addition, engagement of neutrophil adhesion receptors will delay apoptosis.[145] Thus, through alterations occurring during and following neutrophil delivery to the exudate environment, numerous agents modulate the rate of constitutive and inducible neutrophil apoptosis.

Neutrophil cell surface expression in the exudate environment

Neutrophils display altered membrane expression and cell function following transmigration. Using monoclonal antibodies directed towards surface molecules, characterization of the neutrophil cell surface reveals significant and consistent alteration in exudate neutrophil membrane expression. Our laboratory has previously demonstrated that exudate polymorphonuclear neutrophils have enhanced microbicidal activity, superoxide production, and augmented expression of CD16 and the FMLP receptor, and are refractory to further stimulation with TNF.[146] Multiple studies have confirmed that human exudate neutrophils collected in skin windows are primed for enhanced metabolic activation and phagocytic activity.[147-150] In addition to altered function within the inflammatory environment, exudate neutrophils demonstrate altered membrane expression, including receptors that mediate adhesion, chemotaxis, and function.

Adhesion receptors are altered following transmigration. Our laboratory and others have found increased expression of CD11b, decreased L-selectin, and decreased PECAM-1 expression in exudate neutrophils following transmigration.[146, 151, 152, 52] The loss of PECAM-1

is particularly interesting as it mediates adhesion to endothelial cell corners and is necessary for diapedesis (see discussion above).[51, 153] The alteration of adhesion molecule expression may allow the neutrophil to complete the process of diapedesis, and undergoes chemotaxis to a site of inflammation or infection.

Evidence suggests that change in the membrane expression in exudate neutrophils is closely tied to the mobilization of secretory vesicles. Exudate neutrophils collected in skin windows displayed increased surface expression of alkaline phosphatase, complement receptor 1, and CD11b/CD18, but a complete loss of L- selectin following transmigration; and the increase in the content of surface molecules in the plasma membrane was correlated with complete mobilization of secretory vesicles.[154] Loss of specific granules was also correlated with increased number of FMLP receptors in exudate neutrophils.[150] Thus, the changes to membrane expression are intrinsic to the change in neutrophil function.

Exudate neutrophils display a reduced number of chemoattractant receptors along with reduced chemotaxis. In animal models, exudate neutrophils demonstrate reduced chemotactic response.[155] In humans, neutrophils isolated from skin windows have diminished chemotactic ability when compared to circulating neutrophils.[148] Exudate neutrophils from pustules in a single patient revealed markedly reduced chemotaxis to C5a, FMLP, leukotriene B4 and IL-8 when compared to circulating neutrophils.[156] Bronchioalveolar lavage neutrophils in patients with chronic respiratory tract infections have reduced IL-8 RA and IL-8 RB when compared to circulating neutrophils.[157] Circulating neutrophils taken from humans *in vivo* demonstrate dynamic and variable alterations in surface expression of chemoattractant receptors that correlate with changes in cell function (chemotaxis). We have demonstrated that exudate neutrophils displayed increased C5a receptors & C5a chemotaxis, and reduced Interleukin-8 receptors (both IL-8RA & IL-8RB) & IL-8 chemotaxis. Septic neutrophils displayed reduced C5a & IL-8 receptors, decreased C5a chemotaxis, but no change in IL-8 chemotaxis. These results suggest: (1) change in neutrophil chemoattractant receptor

expression serves to regulate neutrophil chemotaxis *in vivo*; (2) exudate neutrophil chemotaxis depends more on C5a than IL-8, and (3) diminished chemoattractant receptors and chemotaxis in septic neutrophils may explain decreased neutrophil delivery in these patients (Seely AJE et al, manuscript submitted).

Exudate neutrophils have delayed rates of physiologic cell death, or apoptosis. In humans, exudate neutrophils have decreased surface expression of FcγRIII [146]; and FcγRIII is known to be decreased in apoptosis[158, 159] In an animal model, pulmonary exudate neutrophils exhibited delayed constitutive and induced apoptosis.[160] In humans, using a sterile skin blister skin window technique, we found that exudate neutrophils had delayed apoptosis, a reduction in TNF-α membrane binding, and a decreased susceptibility to TNF-α induced apoptosis.[161] Human salivary neutrophils do not respond to a combination of TNF-α and cycloheximide, as circulating neutrophils do.[162] Thus, exudate neutrophils have delayed apoptosis, and will be less responsive to certain apoptotic stimuli such as TNF.

In summary, following delivery to the inflammatory environment, neutrophils are primed for enhanced bacteriocidal activity, have altered expression of chemoattractant receptors and chemotaxis, and are refractory to cell death. These mechanisms have presumably developed in order to facilitate neutrophil effector function in host defense. Participating in and being altered by the multiple sequential steps involved in the neutrophil's path from the circulation to the inflammatory environment, the neutrophil membrane is changed into a new configuration, reflecting the fact that the function of the neutrophil, its overall properties, have changed too.

Conclusions

As the principal means by which the neutrophil interacts and communicates with its environment, it is not surprising that it mediates the processes inherent to the neutrophil life

cycle. As a complementary interpretation, the neutrophil membrane offers a measure of neutrophil *connectivity*, that is, the degree of interconnectedness of the neutrophil with other elements within the host response. Applying a systems paradigm to the host response,[163] the neutrophil itself may be regarded as a complex system with its own emergent properties. It is hypothesised that changes in variability and connectivity may be utilised to monitor changes to the emergent properties of a complex system.[163] By demonstrating that altered neutrophil membrane expression is associated with altered neutrophil function, it is an exhibition of how connectivity, investigated as membrane expression, can identify changes in the emergent properties (function) of the cell. Future investigations must focus on identifying which patients might benefit from attempted modulation of the host response, when intervention should occur, and if specific organ system targeting is warranted.

As the principal circulating phagocyte essential to normal effective host defense, and responsible for host tissue injury in states of persistent inflammation, the neutrophil has undergone extensive investigation. This evaluation has revealed two principal conclusions: (1) the neutrophil membrane mediates the processes integral to neutrophil delivery, function and clearance, and (2) alterations in membrane expression occur with change in cell function. Neutrophil membrane mediates neutrophil delivery, including neutrophil-endothelial cell interactions, rolling, adhesion, and diapedesis. During this process, and in the interstitial inflammatory environment, the neutrophil will respond to various chemoattractants based upon the presence and binding capacity of the appropriate receptors. In the inflammatory environment, neutrophil membrane receptors participate in phagocytosis, priming and activation, leading to release of a toxic arsenal of granules and activation of the membrane bound respiratory burst. Following completing its function, the neutrophil is cleared through the physiologic cell death or apoptosis, a process activated by membrane bound death receptors. In summary, as the neutrophil membrane is the principal means by which the cell interacts with its surrounding, it is the principle mediator of neutrophil development during the neutrophil life cycle. The second principal conclusion from the evaluation of neutrophil

membrane expression and function is that alterations of the neutrophil membrane are synonymous with alterations in cell function. Thus, in addition to the neutrophil membrane mediating cell processes during the neutrophil life cycle, change in membrane expression allows for *in vivo* regulation of cellular function.

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Commentary

Performing experimental research and reviewing the literature regarding neutrophil delivery and clearance, the question of how this might contribute to patient care continued to dominate the interpretation of the results. Cell membrane receptors are particularly amenable to intervention with widely available monoclonal antibodies that block, or bind various membrane bound molecules. As the neutrophil membrane is principally involved in neutrophil biology, interventions directed at the cell surface appear promising. However, questions remain. The neutrophil is simply one component of a myriad of interconnected variables involved in the human host response to sepsis, shock or trauma. How might modulation of neutrophil function alone be beneficial? In which patient and when might this be attempted? Would it not cause harm in certain patients given the simultaneous roles of the neutrophil in host defense and injury? New layers of understanding of the host response and its importance to patient outcome have unfolded, yet no ability to effectively modulate the host response has been developed. As a surgical resident, one is intimately aware of the altered host response in patients with Multiple Organ Dysfunction Syndrome (MODS) and our inability to actively treat those patients; it seemed that there was a gap between analytical science and patient care. An interest in mathematics, chaos theory and complex systems stimulated the theoretical re-evaluation of the clinical implications of analytical research such as the evaluation of receptor expression on neutrophils. This shift in theoretical viewpoint demanded that the assumptions and conclusions of basic science be reexamined, and complimented with something new.

The clinical significance of analytical experimental research

The implicit or explicit objective of medical and surgical research is to contribute to the improvement of patient care. In pursuit of this goal, experimental basic science papers address the means by which the findings may lead to the development of successful therapeutic interventions, often in the final paragraph(s) of the paper. It is essential that this question be addressed closely, for there has been much difficulty with the direct application of bench research to the bedside, particularly in the treatment of patients with sepsis and/or MODS. It is imperative to evaluate the underlying assumptions that guide the investigation of the host response with the goal of improving patient care. Thus, performing the experimental research within this thesis investigating the neutrophil in health and in sepsis begs the question of how might this research might benefit a critically ill patient.

Why perform research investigating the role of neutrophil membrane expression in the regulation of chemotaxis and apoptosis? As mentioned, it is clear that the neutrophil, which comprises >90% of circulating phagocytes is principally involved both in host defense against bacterial and fungal infection, and is also implicated in the pathogenesis of disease states characterised by unremitting and/or overwhelming inflammation. Thus, it is believed that a greater understanding of the mechanisms that regulate neutrophil delivery in health and disease will help design an intervention that will impact upon neutrophil delivery and/or function in a clinically beneficial way. As the cell membrane of neutrophils is readily altered by monoclonal antibodies to specific membrane molecules, one might imagine designing an intervention that would block one or more specific receptors may lead to a successful intervention. For example, authors have suggested that decreasing neutrophil delivery by blocking adhesion molecules, or enhancing neutrophil apoptosis are potential strategies that will deliver effective therapeutic intervention.

Basic science research is also interested in pushing back the frontiers of knowledge, and is considerably removed from bedside intervention. At times, it is assumed that the clinical application of basic science is yet to be discovered, or may be considered mundane, unworthy of concern by scientists, because one can not predict when there shall be a breakthrough in clinical application of basic investigation. Numerous anecdotes of past scientific serendipity support this impression. However, despite the clear importance of improving our understanding of the mechanisms and processes of the human physiology, it is imperative to directly address how this improved understanding will assist in the care of patients. This sentiment is bolstered by a sense of frustration amongst clinician investigators, that despite the enormous gains of our understanding of the multiple, interconnected processes, pathways, and players of the systemic host response to sepsis, shock or trauma, there has been a disproportionately inadequate improvement in our ability to treat patients and improve outcome.

The discussion regarding neutrophils may be expanded to include analogous research investigating the systemic host response. It is widely observed that, in controlled animal sepsis models, and in certain patients following significant physiologic insult, the systemic host response is altered, becoming dysregulated and pathologic, and this leads to progressive organ dysfunction and death. The altered host response is often portrayed as a sequence of events, with activation of pro-inflammatory mediators following the insult, followed by a dysfunctional host defense leading to potential secondary infection, resulting in the clinical sequelae characteristic of patients with organ dysfunction, including capillary leak, non-cardiogenic pulmonary edema or ARDS, renal failure, coagulopathy, hepatic dysfunction, coma, circulatory failure and death. In analogous but divergent fields of basic science investigation regarding the mechanisms inherent to the pathologic host response in critically ill patients, multiple investigators suggest specific agents to improve outcome, usually to decrease inflammation. We identify factors associated with illness and mortality in humans using epidemiological techniques or in animal models, and then seek to block or reduce those

factors in the laboratory *in vitro*, and subsequently in interventional studies using animal models; only if repeatedly successful in animal models, clinical testing is attempted. Factors may also be found to be statistically associated with health rather than disease, and re-establishment or augmentation of those factors may be attempted in those that are ill. All investigations are performed in an attempt to bring the bench to the bedside, that is, develop interventions in a controlled laboratory environment, and apply it to the patient at the bedside.

What are the strategies and underlying assumptions inherent to this process of understanding and attempting to intervene in this altered and dysregulated host response? (1) Factors statistically associated with illness or illness severity are assumed to be causative, and blocking those factors assumed to be therapeutic; (2) successful therapeutic intervention in a controlled animal model is assumed to apply to uncontrolled human patients; (3) interventions statistically beneficial for a cohort of patients should then be given to all patients; and (4) the properties of the host response that lead to disease can be understood by understanding the properties of the components to that system.

An example of this research strategy is provided by the investigation of TNF-α. Following the realisation that infection alone was not the cause of organ failure, but rather the patients host response was strongly correlated with outcome, discovery of TNF-α lead to a great excitement regarding the potential for effective immunomodulation. TNF-α was strongly associated with sepsis in animal models and human patients; it appeared to initiate a cascade of inflammatory events initiated by endotoxin, a component to the gram negative bacterial wall. In multiple randomised controlled animal models, including primate studies, blocking TNF-α either decreased or completely eliminated mortality from sepsis or endotoxemia. However, multiple randomised clinical trials (RCT's) could not reproduce a clinical benefit from various anti-TNF-α strategies. The experience with anti-TNF-α is mirrored by numerous other agents with similar background investigation. In addition to anti-TNF-α therapies, over 30 RCT's of numerous anti-inflammatory therapies have found no

benefit. This has not been due to inadequate laboratory investigation; all trials of attempted immunomodulation have had extremely supportive basic science, including *in vitro* and *in vivo* data as described for TNF-α. This experience forces the re-evaluation of the assumptions and beliefs that underlie research regarding the systemic host response.

The fundamental paradigm of the vast majority of medical and surgical research is that of analytical science. By breaking up a whole into its component parts, and studying the properties and interactions of the parts, it is assumed that the properties of the whole will be understood. This methodology has been immensely successful, allowing us to understand and treat a great deal of human illness. In addition, the physical technology that Newtonian science has created using analytical science is astounding, from the infrastructure of our cities to the space shuttle. Treating multiple diseases such as infection, diabetes, heart disease, organ specific care and much more have benefited tremendously from analytical investigation. It has well served scientists and humankind for over 300 years.

Nonetheless, disease states such as Multiple Organ Dysfunction Syndrome (MODS) remain elusive. In patients with MODS, altering disease progression has not been successful, rather, we support organ function and fight infection, maximizing the chance a patient may survive. For clinical problems like MODS, where disease is the result of an alteration in an complex web of interactions, involving a myriad of inter-dependent and changing variables, a complimentary outlook may be necessary to deliver successful active immunomodulation in the intensive care unit. There is need for technology to evaluate the properties of the whole system, not just the components of the system. Our knowledge of the mechanisms of the host response has grown enormously over the past three decades; however, theoretical research may help bridge the gap between bench and bedside, to realise the clinical potential of understanding the systemic properties of the human host response.

Manuscript #5

Multiple Organ Dysfunction Syndrome: Exploring the paradigm of complex non-linear systems

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Manuscript 5: Multiple Organ Dysfunction Syndrome: Exploring the paradigm of complex non-linear systems

Abstract

Objectives: The objectives of this article are to introduce and explore a novel paradigm based on complex non-linear systems, and evaluate its application to critical care research regarding the systemic host response and Multiple Organ Dysfunction Syndrome (MODS).

Data Sources: Published original work, review articles, scientific abstracts and books, as well as the authors' personal files.

Study Selection: Studies were selected for their relevance to the applications of non-linear complex systems, to critical care medicine, and for their relevance to the concepts presented.

Data Extraction: The authors extracted all applicable data.

Data Synthesis: Following a brief review of MODS, an introduction to complex non-linear systems is presented, including clear concepts, definitions and properties. By examining (1) the multiple, non-linear, interrelated and variable interactions between the metabolic, neural, endocrine, immune and inflammatory systems, (2) data regarding interconnected antibody networks, and (3) the redundant, non-linear, interdependent nature of the inflammatory response, we present the hypothesis that the systemic host response to trauma, shock or sepsis must be evaluated as a complex non-linear system. This model provides a new explanation for the failure of trials utilizing various anti-mediator therapies in the treatment of patients with sepsis and MODS. Understanding the host response as a complex non-linear system offers innovative means of studying critical care patients, specifically by suggesting a greater focus on systemic properties. We hypothesize that analysis of variability and connectivity of individual variables offer a novel means of evaluating and differentiating the systemic properties of a complex non-linear system. Current applications of evaluating variability and connectivity are discussed, and insights regarding future research are offered.

Conclusion: The paradigm offered by the study of complex non-linear systems suggests new insights to pursue research to evaluate, monitor and treat patients with MODS.

Multiple Organ Dysfunction Syndrome: Exploring the paradigm of complex non-linear systems

Introduction:

Multiple Organ Dysfunction Syndrome (MODS) represents the most common cause of death in the intensive care unit in the 1990's.[1] MODS is characterized by a complex and overwhelming host response involving immune, metabolic, neuroendocrine and inflammatory mechanisms that usually occurs following massive injury or infection. Treatment for patients with MODS is largely supportive. Despite an immense literature describing the immuno-inflammatory response to trauma, shock and sepsis, over two dozen clinical studies of mediator-targeted therapy for these patients have been universally disappointing. The principal objective of this paper is to propose a new paradigm based on complex non-linear systems, and evaluate its potential application to patients with MODS. First, a review of MODS and the attempts at therapeutic intervention in these patients will be presented. As will be shown, the vast majority of research in this field has been analytical or reductionist, focusing on individual immunologic mechanisms, and assuming linear relationships. Second, an introduction to the history, definitions and concepts of complex non-linear systems will be followed by a description of the properties of such systems particularly important to clinicians. Third, it will be demonstrated that the host response to trauma, shock or sepsis is indeed a complex non-linear system. Understanding the host response in this manner allows for a reevaluation of existing evidence and treatment strategies. Current applications of non-linear dynamics to critical care medicine will be reviewed. Lastly, future prospects to evaluate the systemic host response as a complex non-linear system will be presented.

Multiple Organ Dysfunction Syndrome

Remarkable advances have been made in this century in the care of the post-operative and post-injury patient. Because of improvements in the care of acutely injured persons as well as advancements in providing organ-specific supportive care, a new class of patients has been created; these patients represent the *chronically critically ill*. Multiple Organ Dysfunction Syndrome (MODS) is the clinical syndrome characteristic of these patients.

In 1969, Skillman first noted the lethal syndrome of respiratory failure, hypotension, sepsis and jaundice[2], and then Tilney described the sequential failure of organ systems after abdominal aortic aneurysm rupture in 1973, and appropriately referred to it as "an unsolved problem in postoperative care".[3] Sequential organ failure usually occurs after a lag period of days to weeks following various physiologic insults, which may include pancreatitis[4], trauma[5], burns[6], shock[7], severe infection, aspiration, multiple blood transfusions and pulmonary contusions[8]. Organ failure often begins with respiratory failure, followed by intestinal, hepatic, renal failure, hematologic and cardiac failure; the exact order may vary due to pre-existing disease or the nature of the precipitating insult.[1] Mortality is strongly correlated with the number of organ systems failing, as well as age and duration of organ failure.[9] The mortality of MODS is between 30-100% depending on these factors. MODS is associated with costs exceeding $150,000 per patient, and is the leading cause of death in intensive care units responsible for 50-80% of total ICU mortality.[1]

For two decades, investigators have focused on finding a specific agent as the "cause" of MODS. Initially, uncontrolled infection was thought to precipitate organ failure,[10] however it became clear that infection is the initiating stimulus in only half of the patients with MODS; in the remaining half, the syndrome occurs without a clinically identifiable focus of infection.[11]

As host related factors were shown to be associated with patient outcome,[12,13] the focus has shifted to the study of host response to trauma, shock and sepsis. The discovery of multiple pro-inflammatory mediators, including endotoxin, tumour necrosis factor (TNF-α) and interleukin-1 (IL-1), brought about new theories regarding the pathophysiology of MODS and great optimism in the potential to treat the syndrome. The pro-inflammatory mediators were seen as causing a generalized, persistent, and overwhelming inflammatory host response. In reference to Ehrlich, this excessive immuno-inflammatory autoimmune response was coined a modern "horror autotoxicus".[14]

The search for the various mediators that cause sepsis and subsequently MODS has led to attempts to treat with anti-mediator therapies. Despite compelling data from basic science and animal experiments supporting the biologic basis for these trials, clinical trials examining the attempts to attenuate the host response to injury have been uniformly disappointing. Over two dozen studies including antibodies to *E. Coli.* core glycolipid J5, murine anti-endotoxin monoclonal IgM antibodies, HA-1A human monoclonal antibody to endotoxin, antibradykinins, corticosteroids, anti-TNF monoclonal antibodies, soluble TNF receptors, interleukin-1 receptor antagonist, antiprostaglandins and platelet activating factor antagonist have demonstrated no consistent benefit, and have even been associated with deleterious effects. A review of anti-inflammatory clinical trials from 1986 to the present has been recently published in this journal.[15] Various explanations have been put forth to explain the failure of these trials, including the heterogeneity of the enrolled patients, the timing of the intervention, the dose of the intervention, or the focus on the pro-inflammatory mediators.[16] In this paper, we present the hypothesis that the failure of critical care clinical trials does not represent study design problems, but rather, the current assumptions and set of beliefs underlying the study of the host response may be inaccurate. Following an introduction to complex non-linear systems, the failed attempts at therapeutic intervention will be reexamined.

Introduction to Complex Non-Linear Systems

In order to appreciate the novel outlook offered by the study of complex non-linear systems, we must first outline the current research paradigm. Science has employed the analytical, mechanistic or reductionist approach with great success in order to address problems large and small. An *analytical approach* consists of breaking up the whole into its component parts, in order to understand the whole through the properties of its parts. This analytical, mechanistic or reductionist philosophy began with René Descartes (1596-1650) and Galileo Galilei (1564-1642), who introduced the method of analytical thinking, and was triumphantly taken to completion by Sir Isaac Newton (1642-1727). With four simple equations, Newton could predict the trajectory of the celestial objects that had perplexed scientists for hundreds of years. Newton's awesome achievement enshrined both mathematics and mechanistic thinking as the means to search for truth in science, and has influenced human scientific pursuit ever since.

In the nineteenth century, it became apparent that there were limits to the applications of Newtonian Mechanics to real life problems. For example, problems such as friction or turbulence could not be solved using Newton's equations; in fact, the linearity of Newton's equations are approximations for the ubiquitous non-linearity that exists in the physical and biologic world. Specifically, biologic systems cannot be modeled using linear equations. Henri Poincaré (1854-1912) realized that certain systems could only be described by non-linear differential equations. He began the development of mathematical tools necessary to solve non-linear problems. Further developments awaited the development of the computer, pioneered by Alan Turing (1912-1954) and John von Neumann (1904-1958). At present, a new understanding of complex systems is being pursued by a variety of scientists in physical, geological, biological, psychological and behavioral disciplines.[17]

Non-linear systems theory has also been referred to as complexity theory, complex systems theory, dynamical systems theory, or chaos theory; non-linear dynamics represents the corresponding field of mathematics. It is necessary to define certain terms that underlie the concepts of this discussion. *Linear systems* are those where the magnitude of a response may be expressed as a sum of multiple, mutually independent variables, each with its own independent linear coefficient. A simple example involves the equations of motion for a ball thrown into the air; the forces acting on the ball and resultant velocity may all be independently separated into x, y, and z variables, in order to calculate its exact trajectory (ignoring friction). A *non-linear system* involves variables that are not mutually independent (i.e. alteration in certain variables will alter relationships between other variables), and the magnitude of effect may be expressed in products or powers of variables. For this discussion, we define a *complex non-linear system* as follows: **a system or whole consisting of an extremely large and variable number of component parts, where the individual components display marked variability over time, and are characterized by a high degree of connectivity or interdependence between variables.** Complex non-linear systems have unique properties; most importantly, they are capable of creating the remarkable stability and order we observe everyday in the world around us. Complex non-linear systems are ubiquitous in nature; they include weather patterns, the biosphere of our planet, the stock market, the ecosystem of a tropical rain forest, the central nervous system, and as will be subsequently discussed, the host response to trauma, sepsis or shock.

Properties of Complex Non-Linear Systems

What exactly is a complex non-linear system? Imagine a system (the whole) composed of virtually infinite numbers of interconnected variables (the parts), the exact quantity of which is constantly changing and unimportant. Variables can be anything: molecules, cells, people, or stock prices. The interactions between variables are constantly being altered, and are

dependent on other variables, but not always the same variables. It is a fluid, dynamic and complex web of interactions. In such a system, *(1) the concept of linear proportionality does not hold (i.e. relationships are non-linear); (2) variables are not independent; there is marked connectivity between variables; (3) individual components display systematic variability, (4) small alterations in variables can result in completely different outcomes, thus there is a sensitive dependence on initial conditions; (5) the system is deterministic (specific rules govern interactions between variables); (6) the system displays apparent negative entropy, capable of producing emergent order.* Given these properties, it is evident that alteration in one variable will likely impact other variables as well as interactions between other variables. **The important components of a non-linear complex system are the relationships between variables, the interconnections, rather than the variables themselves.** The *connectivity* of a non-linear system represents the nature and degree of interdependence between the variables of the system. The sensitive dependence on initial conditions can be demonstrated with extremely simple non-linear equations.[18] A small perturbation may be magnified or dampened depending on the state of the system. The ability of a small perturbation to unpredictably cause a large change in outcome has been termed the *Butterfly effect.*[19]

The most important and exciting property of complex non-linear systems is their ability to generate negative entropy (i.e. less disorder). This property, called *emergent order*, explains why complex non-linear systems are ubiquitous in nature. These properties are termed "emergent" because they "emerge" at increasing and specific levels of complexity and connectivity of the system. They arise naturally from the organizing relations of the parts. Emergent properties are the important systemic properties (i.e. they define the system), properties which none of the parts have individually. Emergent order arises precisely because *the whole is greater than the sum of its parts.* When a system is dissected into isolated

elements, these emergent properties are destroyed.[17] Emergent properties cannot be studied or predicted by studying the parts of the system in isolation.

The emergent defining "systemic" properties of a complex non-linear system arise from the relationships between the components of the system. Because a complex non-linear system is a dynamic fluid network of interactions, it may exist in a virtually infinite number of states. Through a process called "self-organization", the system will "naturally" settle into a reduced number of "stable" configurations, called emergent order. The emergent order is dependent on the number, bias and type of interconnections among the system's constituents.[20] Feedback loops are the building blocks of emergent order. Feedback loops, either positive (self-reinforcing) or negative (self-balancing), are ubiquitous in the biologic world, and form a key component of the emergent order of complex non-linear systems.[17]

An excellent illustration of emergent order is provided by the NK Model developed by Stuart Kauffman[19], recently well explained in this journal[21], to investigate the process by which a cell "turns on" the appropriate genes during ontogeny. The NK model can be thought of as an interconnected array of lightbulbs, where N represents the *size* of the system or number of lightbulbs, and K represents the *connectivity* or number of interconnections (wires) between lightbulbs. Interconnections are governed by Boolean operators (eg. AND, NOT, OR); thus, the system is *deterministic*, that is, specific rules govern the interactions between the variables. Theoretically, as lightbulbs can be either on or off, there are 2^N possible states; however, Kauffman has demonstrated that the system will be "attracted" to a markedly reduced number of states. For example, if N is set to 100,000 and K to 2, when the array of lightbulbs is turned on, they blink on/off, and then settle into stable patterns. These stable patterns may have large areas where the lights are always on, others always off; there would be lights that cycle regularly (i.e. feedback loops), and lights that appear to twinkle randomly. Despite a possible $2^{100,000}$ states for the network of lightbulbs, there are only 317 stable

configurations! When compared to ontogeny, this result is that much more impressive. There are approximately 100,000 genes in the human genome. If one assumes that each gene could be either activated or not activated during embryogenesis, there are $2^{100,000}$ different states. However, during embryogenesis, 256 different human cell types are formed, a number remarkable close to the number of states found with the NK model. A similar relationship between number of genes and number of discrete cell types has been obtained with other species, suggesting interesting hypotheses regarding ontogeny. However, the reason for presenting the NK model is that it provides an excellent demonstration of emergent order in a complex non-linear system.

In summary, complex non-linear systems are attracted to specific states, which are robust, capable of maintaining the same essential structure despite small perturbations. While the system may exist in a stable configuration, certain elements of the system may display a large degree of variability, whereas others are relatively constant. In fact, when certain physiologic variables are measured precisely and continuously, health is marked by high degrees of variability, and disease occurs with a reduction in variability.[22,23] Heart rate variability provides a ready example of this type of phenomenon, and will be discussed further below. As the emergent order of a complex system is created by the dynamic interactions between the elements of the system, it is thus impossible to study the emergent properties of a system using a reductionist or mechanistic approach. If one is interested in the study of the emergent properties of a system, and given that the emergent properties of the whole system are lost when you break the system into its component parts, studying the individual interactions between components of a system under controlled conditions will be of questionable merit in interpreting the properties of the system. To use a biologic analogy, the emergent properties of a beehive cannot be understood by the study of bees. Lewis Thomas observed "you'll never understand how bees make honey no matter how carefully you dissect a single bee".[24] Although analytical methods continue to be the cornerstone of scientific

enquiry, there is a need to find means to evaluate the entire system as a whole. The study of the components of a system is vital to the further understanding of the mechanisms of various specific interrelationships, however that understanding is of dubious merit in determining the emergent properties of the system. We propose that the paradigm of complex non-linear systems offers new insights into the evaluation of the system as a whole. Following a demonstration that the neuroendocrine and immune response to sepsis, shock or trauma behaves as a non-linear complex system, we will re-examine how we may evaluate the emergent properties of that system.

Host response as a Complex Non-Linear System

The host response to trauma, shock or sepsis behaves as a complex non-linear system involving great numbers of variables and systems of variables. Three lines of evidence support this statement. First, the evaluation of the "immune response" as separate from the metabolic, endocrine and neural responses ignores the increasing data suggesting a close interaction between all these systems. Second, data will be presented to suggest that the immune system behaves as a non-linear network, rather than the defense oriented function traditionally associated with immune responses. Lastly, the non-linear nature of the inflammatory response will be demonstrated and the pathophysiology of MODS will be reviewed.

Numerous developments over the past two decades have demonstrated relationships between the immune system and the nervous system, endocrine system, and behavioural and emotional states. The term "psychoneuroimmunology" has been coined by Dr. Robert Ader to represent the study of these interactions.[25] This is particularly important to the field of surgical trauma as the autonomic, metabolic and endocrine response to injury and/or

surgery is often activated concurrently or immediately prior to an infectious stimulus. There are numerous neural-immune, endocrine-immune and behavioural-immune interactions demonstrated in humans and animal models.[26,27] For example, pro-inflammatory cytokines cause activation of the hypothalamic-pituitary-adrenal axis.[28] Sympathetic enervation of lymphoid tissue has variable effects, including potentiating B Cell IgM antibody response.[29] Lymphocytes bear receptors for substance P, somatostatin, vasoactive intestinal peptide,[30] corticotropin-releasing factor, ACTH and endogenous opioids[31], and all mediate significant immune alteration. Cytokines are known to possess variable effects in the CNS, and on endocrine and immune function. Given the wealth of evidence supporting these interactions, the "host response" to sepsis, shock or trauma must be considered as a "systemic response", involving multiple interactions between metabolic, neural, endocrine, behavioural and immune processes.

In addition to these systemic interactions, if the immune system is considered separately, there is mounting evidence that the immune response can be thought of as a highly interconnected self-reactive non-linear network. Contrary to the ideas of a defense oriented immune system, with a learned recognition between self and non-self (introduced by Jerne[32] and modified by Burnet[33]), the immune system can and routinely does recognize self-antigen. Contradicting the theories of clonal selection, animals bred in antigen-free environment develop relatively normal immune parameters.[34,35] Antibodies that are reactive to both intracellular and cell surface self-antigens have been demonstrated.[36,37,38] Thus, there are two repertoires of antibody variable regions (V-regions: site of antigen binding): one reactive to self, and the other non-reactive to self. Self-reactive immunoglobulin G (IgG) levels are stable throughout life, whereas there is an age-dependent diversification of IgG repertoires that are directed towards external antigen.[39] There is a high degree of autoreactivity or connectivity between elements in the immune system, specifically between variable regions (V-regions) on antigen binding sites of immunoglobulin. Anti-idiotypic antibodies (antibodies

that recognize self-antibodies) have been identified, and their degree of autoreactivity is dependent on T cells in mice.[40] Given this data, it has been postulated that there co-exists two fundamentally distinct components in mammalian immune systems, the first is devoid of both autoreactivity and V-region connectivity and serves the traditional defense function of the immune response; the second involves a network of V-regions on antibodies and T cell receptors that are autoreactive, and highly interconnected.[41] The latter set is thought to represent the continuous assertion of the antigenic composition of an individual, or the "immunological homunculus".

The connectivity of the elements of the immune system is related to disease states, which we hypothesize may be secondary to emergent properties of the immune system. The immunologic homunculus appears to be well conserved throughout life from individual to individual[42] and regardless of the degree of external antigenic simulation.[43] However, deviations in the interconnected autoreactive set of V-regions have been suggested to be associated with autoimmune pathology. Recently, in an experiment involving controls and patients with systemic lupus erythematosus (SLE), distinguishable patterns of IgG connectivity were found in the patients with SLE when compared to controls.[44] It has been suggested that if autoimmune disease is accompanied by or even caused by defects in immunoglobulin connectivity, then interventions to stimulate connectivity (eg. intravenous immunoglobulin) may prove therapeutic.[41] This network of dynamic tightly meshed interconnecting elements is thought to display the properties of a complex non-linear system.[45] Altered immunoglobulin connectivity has not been investigated in patients with multiple organ dysfunction. In summary, normal immune function includes a complex non-linear network of self-reactive immunoglobulin; the study of the connectivity of this network may provide valuable information regarding altered emergent properties of the immune system, which lead to autoimmune disease.

The third argument for analyzing the host response as a complex non-linear system is the complex non-linear nature of the inflammatory response. As stated previously, investigators have pursued the study of the inflammatory response for over two decades, in search of one or more causative agents in the development of systemic inflammation and organ dysfunction. The cytokine network has failed to yield such a singular causative agent. Recently, much focus has turned to the anti-inflammatory cytokine network. Bone proposed that these anti-inflammatory mediators, if sufficiently elevated, manifest clinically as the "compensatory anti-inflammatory response syndrome" (CARS).[16] The concept that the pro- and anti-inflammatory mediators simply counter each other may be a simplification of a far more complex interaction. In patients who go on to develop MODS, there is activation of both the pro-inflammatory and the anti-inflammatory response. For example, in patients with septic shock, IL-10 plasma levels were positively correlated with TNF levels and parameters of shock severity.[46]

It has been repeatedly demonstrated that cytokines exhibit marked interdependence, pleiotropy (cytokines have multiple effects) and redundancy (multiple cytokines with the same effect). The effects of cytokines may dampen, or amplify the effects of other cytokines. For example, TNF-α and IL-1 act synergistically, whereas IL-10 decreases serum TNF-α and IL-1.[47] The effect of a cytokine may vary with its concentration in a non-linear fashion. For example, TNF-α will cause neutrophilia at low doses, and neutropenia at high doses in healthy humans.[48] Plasma cytokine concentrations fluctuate from day to day, and correlate poorly with physiologic parameters in septic patients.[49] Cytokine receptors are highly variable on a variety of cells. Endothelial cells, neutrophils, TH1 cells, TH2 cells, cytotoxic T cells, monocytes, macrophages, NK cells and more are all players in a fluid network of cells and cellular mediators. Feedback loops, either positive or negative, are ubiquitous within the cytokine-cellular network. The numbers of mediators are extremely large, and continue to be

identified. The inflammatory cellular and cytokine network truly consists of a complex non-linear system.

In summary, *the systemic host response to trauma, shock or sepsis is a complex, non-linear, highly variable web of interconnections between metabolic, neural, endocrine, inflammatory and immune variables.* Despite the dynamic nature of the variables of this complex non-linear system, the properties of the whole system remain remarkably stable in the vast majority of individuals. Understanding the host response as a complex non-linear system may explain the lack of success in anti-mediator therapies in patients with sepsis. In such a system, blocking one agent with an antibody will have unpredictable results that will depend on the state of the network at any particular time. For example, anti-TNF strategies in animals have variable effects depending on the model used. A controlled animal sepsis laboratory experiment will produce similar alterations in host response in different animals, thus the majority of animals may benefit from the same intervention. However, demonstrating a relationship with a controlled experiment in the animal laboratory has little application to a highly heterogeneous clinical cohort of human patients, all with a markedly different host response. It is noted that successful anti-mediator therapy has been successful in clinical syndromes with more homogeneous alterations in the immune response, such as Rheumatoid Arthritis. Sepsis and MODS has not proven to be amenable to single agent anti-mediator therapy. The analytical or reductionist approach has proven to be successful in the elucidation of immunologic mechanisms, however it has not been successful in the synthesis of these mechanisms, in the evaluation of the whole system, and has not provided for effective clinical intervention in patients with sepsis or MODS.

If an analytical approach is not effective in evaluating the emergent properties of the system, how can one study and understand non-linear complex systems? Is the system too complex to possibly comprehend? How can complex non-linear systems be analyzed and monitored?

Can the system be modulated or controlled in some way? Following a review of applications of non-linear dynamics to critical care medicine, we will attempt to address these questions with respect to the human host response.

Application of Non-Linear Dynamics to Critical Care Medicine

There have been a growing number of scientists and clinicians who have been interested in the applications of non-linear dynamics to biology and medicine. Alan Perelson has pioneered mathematical models of diverse biologic phenomena.[50] Direct study of human biologic non-linear systems has only recently begun. **We propose that in order to study the emergent properties of a complex non-linear system, one can evaluate the connectivity and variability of the components of the system. We believe the connectivity and variability contain more information regarding systemic emergent properties than the absolute value of individual variables at precise moments in time. Connectivity and variability might be thought of as a means of assessing the nature of the constantly changing web of interrelationships and feedback loops that form the building blocks of the system's emergent order.** *Connectivity* represents a measure of the interrelationships of the system, and *variability* is the measure of change over time of individual components of the system. As mentioned previously, the evaluation of immunoglobulin variable region connectivity is under evaluation in health and autoimmune disease, led by Antonio Coutinho.[41] In addition to studying connectivity, the variability of individual physiologic variables has undergone considerable investigation. Variability is a principal component of the *dynamics* of a variable, which refers to its *pattern of change over time*. Glass and Mackey have labeled pathologic states with abnormal temporal organization as *Dynamical Diseases*.[51] By analyzing the dynamics of physiologic variables, the degree of variability can

be measured. For the rest of the discussion, we shall focus on alteration the variability of physiologic variables, as it is particularly applicable to the Intensive Care Unit.

Heart Rate Variability

Heart rate variability has been extensively studied and is of particular importance in critical care medicine. Heart rate variability (HRV) is reliably and accurately measured as the change in the time interval between QRS complexes on a standard EKG (measured as RR' interval). Heart rate variability is quantified in different ways, based on time domain (statistical and geometric) or frequency domain measurements (power spectral density), and is well reviewed elsewhere.[52,53,54] Whereas a normal heart rate displays considerable beat to beat variability, a decrease in HRV is associated with several pathologic states. Several studies have demonstrated that a reduction in HRV is associated with increased mortality after acute myocardial infarction. In one study, patients with a decrease in HRV had a fourfold increase in 3-yr. mortality rates post MI (9.0% to 34.4%).[55] A reduction in HRV is superior to left ventricular ejection fraction (EF) in predicting post-MI arrhythmias and equal to left ventricular EF in predicting mortality.[56] In addition, decreased HRV has been shown to be associated with cardiac sudden death,[57,58] post-operative congestive heart failure,[59] and death secondary to CHF[60]. Heart rate variability has been evaluated in animal models of sepsis and septic patients. Endotoxin in rabbits has been shown to cause a loss in HRV.[61] A reduction in HRV was found in septic patients, and the observed loss was correlated with disease severity.[62] This was recently extended to include critically ill and injured pediatric patients.[63] In a separate study of non-cardiac intensive care unit patients, low HRV was associated with an increased risk for mortality.[64]

Why does a reduction in HRV signify greater risk for mortality? The reduction in HRV was suggested to represent a sympatho-vagal imbalance.[65] It has also been suggested that reduction in HRV may also represent increased isolation of the heart from its interaction with other organs. This hypothesis, presented by Godin and Buchman,[20] suggests organ systems act as biologic oscillators and are coupled to one another. The hypothesis stems from work by Pincus, who demonstrated that the uncoupling of stochastic (random) oscillators causes a loss in variability in each oscillator.[66] Variability in each oscillator reflects the "health" of its interconnections with other oscillators. Godin and Buchman suggest that reduction in heart rate variability is caused by the exaggerated immunoinflammatory response in the septic inflammatory response syndrome (SIRS), and subsequently the uncoupling of organ systems is the cause of MODS. The uncoupling of organs from each other is said to be "a consequence of SIRS, and the cause of MODS".[20] In support of this hypothesis, the authors demonstrate a loss in HRV following intravenous endotoxin infusion in healthy humans;[67] however they do not remark on the fact that although endotoxin leads to decreased heart rate variability, it is not sufficient to induce MODS in these healthy human volunteers. Thus, diminished HRV can not alone be considered a cause of MODS. Further evaluation will be required to validate their hypothesis, especially the concept of organs as biologic oscillators, yet it remains the first application of non-linear dynamics to the pathogenesis of MODS.

In summary, a reduction in heart rate variability has proven to predict arrhythmias and mortality in cardiac patients, is correlated with severity of sepsis, and is seen following endotoxin administration in animal models and humans. **Thus, the continuous and precise evaluation of both heart rate variability and the absolute value of heart rate provide for a superior evaluation of the patient compared to the absolute value of heart rate alone.** This data demonstrates the potential value of analysis of the dynamics of a particular physiologic variable in addition to its absolute value. Expanding upon this concept, the future prospects of the applications of non-linear dynamics to the patient with MODS are presented.

Future prospects

What is the use of the evaluation of the host response to trauma, shock or sepsis as a non-linear complex system? What advantage does it have in providing effective therapeutic intervention for patients with organ dysfunction? These questions are challenges for the future. Dynamical systems' thinking has been criticized in the past for not producing solutions to substantive problems.[68] However, utilizing the concepts and set of beliefs underlying the study of complex non-linear systems, novel theories and hypotheses can be tested. First, the systemic host response is must be thought of and evaluated as a complex non-linear system, whose emergent properties are not amenable to analytical or reductionist study. A greater focus on feedback loops, the interconnections between and within systems, and on systemic emergent properties is suggested by the study of complex non-linear systems. If one accepts the designation of the systemic host response as a complex non-linear system with emergent properties, then it is a challenge to direct research at characterizing, differentiating, analyzing and modulating the emergent properties of such a system. This will require original approaches, hypotheses and technology, as systemic thinking truly reflects a paradigm shift from the present. For example, attention might be directed towards defining and studying the physiologic and pathologic emergent properties of the host response. We have hypothesized in this article that altered variability and connectivity of individual physiologic variables may better reflect altered emergent properties of the host response, offering a means of monitoring the instantaneous state of the individual patient. The technology of rapidly evaluating variability and connectivity of various systemic, cellular, and local variables must be developed. If alterations in variability and connectivity can reliably differentiate between pathologic and physiologic emergent properties of the host response, then continuous, instantaneous individualized patient monitoring may provide the means of determining benefit for a given intervention in a particular patient in the future. These concepts need further

development and investigation, and will ultimately be judged depending on their ability to lead to improved outcome in critically ill patients with Multiple Organ Dysfunction Syndrome.

Conclusion

Non-linear dynamics is emerging in a variety of scientific disciplines, offering a different paradigm in which we ask questions and seek solutions. Reductionism and linear approximations of physical and biologic phenomena have been tremendously successful and have led to great advances in our understanding of the host response. These processes will remain unintelligible if not complemented by the evaluation of the emergent properties of the system as a whole.[45] By evaluating the connectivity and variability of the components of a complex non-linear system, the emergent properties of the system can be studied. Effective immunomodulation of a patient with MODS represents the most difficult challenge facing critical care medicine. It is hoped that the history of analytical thinking, the introduction to complex non-linear systems and its applications to critical care medicine, and the future prospects presented will stimulate both thought and experiment in the future.

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Treatment of patients with sepsis and MODS: hypotheses for investigation

What is the use of the evaluation of the host response to trauma, shock or sepsis as a complex system? What advantage does it have in providing effective therapeutic intervention for patients with sepsis and/or organ dysfunction? Dynamical systems' thinking has been criticized in the past for not producing solutions to substantive problems, thus concrete testable hypotheses are required. Utilizing a complex systems paradigm, the following research proposal and hypotheses regarding the definition, characterization, monitoring and treatment of Multiple Organ Dysfunction Syndrome (MODS) are presented. An explanation of each point along with specific testable research questions will be included following the proposal.

(1) The systemic human host response is a complex system. (2) The emergent properties of the host response are stable and physiologic. (3) Unpredictably, in certain patients following a significant physiologic insult, the emergent properties of the host response are altered, resulting in a pathologic, maladaptive, yet stable state, that manifests clinically as MODS. (4) The alteration in emergent properties of the host response from physiologic to pathologic can be detected by alterations in connectivity and variability. (5) By using variability and connectivity to continuously differentiate between physiologic and pathologic emergent properties of the host response in individual patients, interventions may be selected for therapeutic benefit, such that outcome is improved in critically ill patients with organ dysfunction.

(1) The systemic human host response is a complex system. As such, the system has emergent order, or emergent properties that are dependent on the whole system. The normal function of the neuroendocrine, immunoinflammatory response represents the emergent properties of the system itself. This includes an appropriate hemodynamic and neuroendocrine response to shock preserving perfusion to vital organs, delivery and subsequent clearance of neutrophils to a site of infection, wound healing, cohesive parallel and interconnected functioning of complement, immunoglobulin, and cellular defense, and innumerable properties of normal immune, inflammatory, endocrine, neural and psychological function. As suggested in the second statement, **the emergent properties of the host response are stable and physiologic.** In the majority of patients, responses to inflammation, infection, shock or trauma are physiologic, with appropriate resolution of the insult. Most patients resolve inflammation and infection, heal wounds, regain hemodynamic stability following resuscitation and do not deviate from a stable, physiologic host response. There have been tremendous advances in our understanding and the definition of these physiologic properties of the host response, from molecular to the cellular, from inflammatory mediators to organ function. As the complexity of the host response becomes more unraveled and apparent, it is readily identified as a complex system.

(3) Unpredictably, in certain patients following a significant physiologic insult, the emergent properties of the host response are altered, resulting in a pathologic, maladaptive, yet stable state, which manifests clinically as MODS. This alteration in the systemic host response from physiologic to pathologic is postulated to precede organ dysfunction and be the *cause of MODS*. Thus, no single agent or agents causes MODS; more accurately, the new configuration of the dynamic web of interactions that make up the host response will manifest clinically as MODS. The persistent maladaptive state and subsequent organ dysfunction will arise "naturally" as an emergent property of the altered host response following a physiologic insult. Examples of pathologic emergent properties of the host response include inability to maintain vascular tone, widespread capillary leak,

coagulopathy, endothelial cell injury, activation of both pro-inflammatory and anti-inflammatory cascades, cytokines and pathways, a failure of normal cellular oxygen extraction, and more. Hypothesis (3) stipulates that the pathologic emergent state of the host response is stable. This is supported by clinical observation in ICU patients who survive for days to weeks with persistently failing, yet stable organ dysfunction, and then most commonly, do not survive.

The concept of emergent discrete states of the complex systemic host response is reminiscent of discrete energy levels in quantum physics. Complex systems appear to exist is separate stable configurations, discrete energy levels, and function. In terms of the human function of the circulatory, neuroendocrine, immunoinflammatory systemic host response, the emergent properties of that system demonstrate marked stability, altered over long periods in time by lifestyle and environment, or altered fully completely and dramatically by a massive traumatic injury. As suggested by Per Bak and others discuss, complex systems are self-organizing attracted to critical and stable states. Complex systems exist far from thermodynamic equilibrium, requiring energy input and energy dissipation, or a flow of energy through the complex system, analogous to dissipative structures presented by Ilya Prigogine. Complex systems create order which reduces the amount of entropy (randomness) within the system. In a very recent and provocative hypothesis, assuming health is governed by a complex system just the right distance from thermodynamic equilibrium, Peter Macklem has suggested that variability may reflect the distance from thermodynamic equilibrium of a system: too close means too little entropy, high energy dissipation and high variability vs. too far from thermodynamic equilibrium, too little energy dissipation and low variability. Returning to the human systemic host response, it is hypothesised to exist in stable states, and they may be defined as physiologic or pathologic, depending on the function of the various organ systems.

The pathologic alterations to the host response leading to organ failure occur unpredictably, although there are epidemiological risk factors that relate to the patient and the insult. Patient factors include age, past medical history, physiologic reserve, genetic predisposition and more. Factors relating to the physiologic insult relate to the severity and timing of the insult, or insults. Although these factors may determine the risk or probability of developing MODS, they are not predictive. Despite this, hypothesis (3) above suggests that the alteration in the host response *precedes* organ dysfunction, thus potentially allowing a window of opportunity for therapeutic intervention prior to the clinical manifestations of MODS. In order to intervene and modulate the host response, there must be a improved means of detecting the altered emergent properties of the host response, as well as detecting if an intervention is therapeutic.

(4) The alteration in emergent properties of the host response from physiologic to pathologic can be detected by alterations in variability and connectivity. If the normal functioning of the immune, endocrine and inflammatory systems represents a healthy and stable state, and MODS represents a maladaptive and stable state, hypothesis (4) suggests that both will have identifiable and distinct patterns connectivity and variability, due to the alteration in the dynamic web of interactions of the system.

Using continuous, individualized analysis of the variability of a panel of variables, it is hypothesised that it is possible to identify whether an individual patient is in a physiologic or pathologic emergent state at any given moment. Thus, this panel of multiple parameter variability would form a type of dynamic fingerprint, or recognizable pattern, which would allow identification of the current state of the patient. Analogous to the NK model, the overall emergent states of the system is stable, yet with individual variables demonstrating high levels of variability. Variability evaluates parameters over the added dimension of time, and hypothesis (4) suggests altered patterns of variability will represent altered systemic

properties. Although this has never been attempted, there is increasing evidence to suggest that variability of individual variables contains clinically significant information. For example, decreased heart rate variability is associated with poor outcome in patients post myocardial infarction, in heart failure and with sepsis. Respiratory variability is increased in patients with asthma, or reduced with severe neurologic injury. In these and other parameters, altered variability is associated with illness.

Measuring patient parameters like heart rate, respiratory resistance, temperature, white cell count, glucose level, etc provides a great deal of useful information to the clinician, and may be considered "first-order" patient monitoring (i.e. measuring the absolute value of parameters). Measuring variability, documenting patterns of change over time, may then be considered second-order patient monitoring (analogous to measuring velocity). The hypotheses outlined here seek to evaluate patterns of variability, or "third-order" monitoring (akin to evaluating acceleration) in order to identify alterations to the emergent properties of the systemic host response.

Hypothesis 4 also stipulates that connectivity may assist in the distinction between physiologic and pathologic emergent states. However, connectivity is more difficult to evaluate, and is not easy to measure. As with variability, connectivity represents a parameter reflective of the entire system, providing a measure of the interconnectedness of the elements of that system. Given that a complex system is made up of a near infinite number of parts, variability represents the analysis of the parts over time, and connectivity represents an analysis of the parts over space, both adding an additional dimension of analysis.

There are examples of where altered connectivity is associated with altered systemic properties of a complex system. Gathering increasing interest, the connectivity of immunoglobulin variable regions (discussed above) has been investigated and is altered in

autoimmune disease; autoimmune disease may be considered caused by a stable pathologic altered state of the complex immune system.

A second example of connectivity providing a representation of emergent order relates to the earlier discussion regarding the membrane expression of neutrophils. As neutrophils require the appropriate and functioning receptors to "see" extracellular ligands, the cell's membrane expression represents a measure of its connectivity. Considering a neutrophil as a complex system, its membrane expression (connectivity) is altered in conjunction with its cellular function (emergent property). This statement merits further investigation with the analysis of larger numbers of membrane receptors.

Novel ideas regarding the measurement of connectivity must be developed. Greater scientific evaluation of connectivity requires the technology to measure it. As with variability, the analysis of connectivity over time may offer an improved evaluation of the immediate state of the system and its evolution. In summary, both connectivity and variability are hypothesised to allow for better distinction between the physiologic and pathologic emergent states of a complex system.

(5) By using variability and connectivity to continuously differentiate between physiologic and pathologic emergent properties of the host response in individual patients, interventions may be selected for therapeutic benefit, such that outcome is improved in critically ill patients with organ dysfunction. Fundamentally important to this statement are the concepts of continuous and individualized patient monitoring of variability and connectivity, thus allowing the response to an intervention (perturbation) to guide further intervention. Applying a complex systems approach suggests that it is expected that patients may respond completely differently and unpredictably to the same intervention, and thus *only the response to an intervention may determine if it is effective for that individual patient at that*

time. Interventions (perturbations) might include an anti-inflammatory treatment (eg. TNF-α, IL-1, IL-6, etc), or conversely a pro-inflammatory agent (eg. anti-TNF-α, IL-10, IL-4), intravenous immunoglobulin, or any of the therapies used in failed clinical trials for sepsis. With the ability to evaluate the response of each individual patient to each perturbation, in addition to the knowledge of what a healthy pattern represents, hypothesis (5) states that the systemic host response could be purposefully directed with repeated interventions towards the healthy configuration (i.e. active therapeutic immunomodulation). It is realized that different patients may require completely different interventions in an unpredictable fashion, thus an individualized approach based upon protocols that maximize physiologic variability and connectivity would be necessary.

Applying the same reasoning to patients with Lupus suggests the following hypothesis. As autoimmune disease have associated altered patterns of immunoglobulin connectivity, then interventions directed at altering immunoglobulin connectivity (e.g. with intravenous immunoglobulin) towards a more "normal" or physiologic patterns of connectivity should improve outcome in those patients. The possibility for wide variations in response to interventions would mandate small interventions followed over time to prevent harmful effects.

This concept of continuous individualized monitoring of the variability of physiologic parameters or their connectivity in order to determine the effectiveness of a therapeutic intervention to guide future intervention is in contradistinction to the current practice of clinical trials. In randomized controlled trials (RCT's), all patients receive the same intervention (vs. placebo), and an intervention is deemed effective if the impact on the intervention cohort is both statistically and clinically significant. RCT's demonstrate an impact on cohorts or groups of patients, but are unable to detect impact on individuals within the cohort. Despite a negative statistical result in a trial, certain patients may have benefited (or were harmed) from

an intervention. Individualized continuous monitoring allows the selection of appropriate therapeutic intervention based upon observed therapeutic impact. This approach can still be subjected to a controlled trial; however, it must be a trial of a protocol, rather than a trial of a specific intervention.

If the host response truly represents a complex system comprised of continuous dynamic alteration of physiologic variables, then in order to impact that system, we must develop the technology to monitor the variability and connectivity of those variables in health and in disease. It is hypothesised that patients with MODS will display stable patterns of variability distinct from healthy controls, and interventions performed to reduce pathologic variability and improve physiologic variability on an individualized patient basis based upon continuous monitoring, mortality in patients with multiple organ dysfunction will be improved.

These hypotheses raise many questions that depend upon the observed patterns of physiologic and pathologic variability. Are there similarly altered patterns of variability in different patients with MODS? How many variables must one follow in order to accurately differentiate a pathologic from physiologic host response? Does the alteration in the dynamics of physiologic variables in MODS precede the clinical manifestations, and if so, by how long? Is variability always reduced, or do certain variables display increased variability in patients with MODS? Does MODS represent the clinical sequelae of multiple stable emergent states with separate patterns of variability, or a common one? It is unlikely that the host response exists in simply two states, either physiologic or pathologic; are there identifiable emergent states that bridge the two states? Are the dynamics of immuno-inflammatory variables altered in MODS (e.g. circulating neutrophil numbers, serum TNF-α concentration)? How can assays be developed to facilitate precise and frequent determination of these immunoinflammatory mediators? Models of complex non-linear phenomena (such as the NK model) could be used to theoretically determine the number of variables that would be required in order to classify the entire state of the system, as well as

how detailed the knowledge of their dynamics must be. When would perturbations to the host response be most effective? Will some patients be refractory to any and all attempts to modulate the host response? How can those patients be identified? Is there a specific window of opportunity for therapeutic intervention? Are there demonstrable patterns variability that might predict if an intervention will be successful? Can one predict which specific therapeutic intervention might be effective based on patterns of variability?

The hypotheses presented merit further investigation. The extensive analytical research regarding the host response requires complimentary technology to evaluate the systemic properties of the host response. Variability and connectivity may represent that technology. As is often the case with novel ideas, they raise more questions than they answer. The investigation of the hypotheses, seeking the answers to the above questions, will no doubt be exciting, educational, fun and will ultimately determine the clinical use of variability analysis.

Conclusion

The polymorphonuclear neutrophil is a crucial component to the human systemic host response. It participates in physiologic processes essential for non-specific host defense, and contributes to host injury in pathologic states characterised by persistent inflammation. As such, it is imperative that the neutrophil receive close analytical scrutiny, to improve our understanding of the processes involved in neutrophil delivery, function and clearance within the inflammatory microenvironment.

Neutrophil research must also be performed with the full understanding that it the neutrophil is but one small component of an exceedingly complex, dynamic web of interactions, known as the systemic host response. As a complex system, it is the defining, emergent properties of the whole system that manifest themselves as illness. Complimenting the analytical analysis of the elements of a complex system, we must develop the technology to evaluate and characterise the properties of the system itself. Variability and connectivity are hypothesised to provide that technology, such that individualised patient monitoring may help identify patients that are candidates for therapeutic modulation of the host response, and to determine if the intervention was successful or not.

Ironically, using analytical science, there developed tremendous advances in our understanding of the host response, which led to the realization of its complexity and the limitations of solely utilising the analytical approach. By studying the components of a complex system, analytical science can identify potential interventions that may alter the systemic host response. This means of investigation may be complimented with continuous individualised analysis of variability and connectivity, the evaluation of the components of the system over time and space in order to bridge the gap between the parts and the whole. This provides an evaluation of the properties of the whole system (health vs. illness), thus the means to determine if an intervention is therapeutic. Using this partnership, it is hoped that therapeutic modulation of the host response in critically ill patients may become a reality.

Original Scholarship

The thesis presented represents a combination of experimental and theoretical research, both of which represent original scholarship.

In terms of neutrophil apoptosis, the articles presented contribute to the knowledge base concerning the alterations in apoptosis following transmigration. Contributions include the confirmation that both constitutive and induced exudate neutrophil apoptosis are delayed in humans (using an *in vivo* means of collecting human exudate neutrophils), elucidation of the decrease in TNF induced apoptosis in exudate neutrophils, and the novel investigation of the role of NF-κB in exudate neutrophil apoptosis.

In the realm of neutrophil chemotaxis, observed simultaneous concomitant alterations (both increased and decreased) in chemoattractant receptor expression and chemotactic function in distinct human neutrophil populations collected *in vivo* contribute to existing data supporting the role of receptor alteration regulating chemotactic function in humans *in vivo*. The observation of increased C5aR and chemotaxis to C5a in exudate neutrophils is novel, and suggests that while IL-8 is important during the initial stages of transmigration, C5a is more important within the exudate inflammatory milieu.

The experimental data presented were accepted for presentation at internationally recognised conferences following peer review of abstracts. This included the Surgical Infection Society (awarded first prize, resident presentation award, 1998) and the American College of Surgeons *Surgical Forum* (1998 and 2000).

The theoretical research included in the theses is novel, and possesses greater potential for sustainable contribution to the literature regarding the host response. The manuscript reviewing the host response as a complex system represents a synthesis of ideas and a

review of divergent fields of investigation, and presents an original evaluation of the host response as a complex system. The hypothesis suggesting that both variability and connectivity may be utilised as technology to identify alterations in the emergent order of complex systems and its application to the human host response is, to the best of my knowledge, an original contribution to the literature. This hypothesis has significant and exciting potential for the understanding and modulation of complex systems, and the treatment of illness in critically ill patients. The article (manuscript 5) was published in *Critical Care Medicine* as a lead article, along with a supportive editorial.

The ideas regarding the continuous individualised variability assessment (CIVA) in ICU patients were patented in July 2000. This was performed with the fundamental objective of determining if continuous variability assessment is clinically useful, first to evaluate the prognostic potential of CIVA in ICU patients and most importantly, determine if it enables effective therapeutic intervention, furthering the concept of therapeutic monitoring.

Clinician scientists are uniquely poised to evaluate the application of basic science research to clinical surgery and medicine. Although intellectually stimulating, theoretical research remains exciting but unfulfilled potential, until validated by experimental science. In contrast, experimental basic science is often considerably removed from the care of ill patients, with speculative clinical benefit. It is the combination of both theoretical and experimental research that is necessary to apply laboratory research to the treatment of patients. It is hoped that this thesis and the future research suggested will assist in bridging the gap between laboratory bench and ICU bedside.

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