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Macrophage characteristics expressed in monocytes in ICU patients

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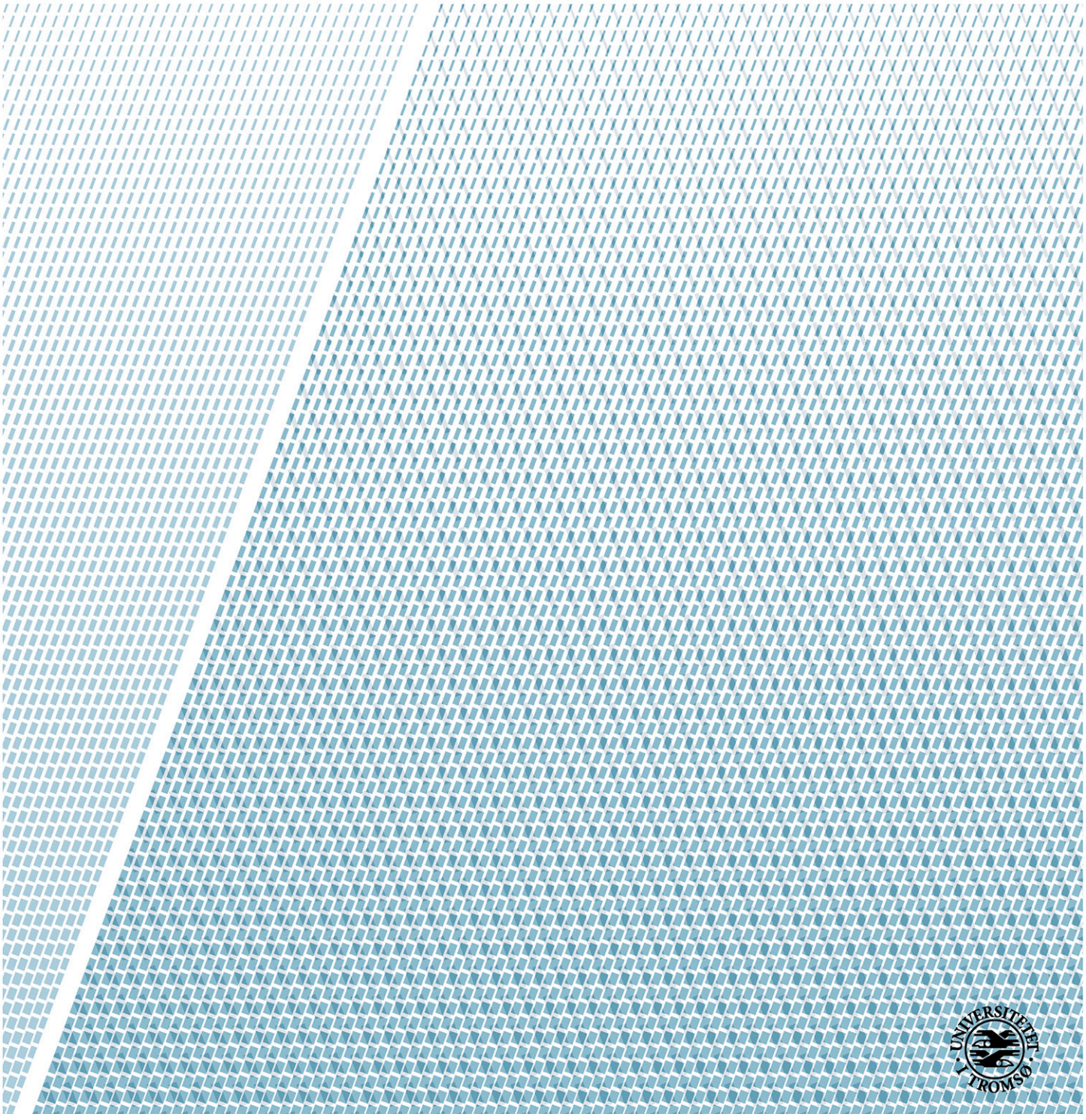


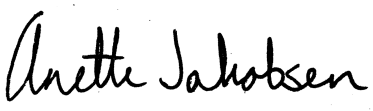
Preface

Ever since I started my medical education, I have had great interest in anaesthesiology. Therefore, choosing a project in this field for my master thesis, was a natural choice for me. When I was presented with this idea, I could not let the opportunity pass me by. The process has been extremely educational, and I have acquired skills in laboratory work, scientific research and writing.

In the early stages of the project, an application to the ethical committee had to be prepared and submitted. To be a part of this process highlighted the importance of ethics in a group of especially vulnerable patients.

The project started in June 2017, and lasted until May 2018. Economical support for the project was provided by internal resources.

I would like to thank my supervisors Lars Marius Ytrebø and Stephen Hodges for the invaluable support they have given me through my laboratory work as well as the writing process. I also want to thank Roy Andre Lysaa for all the help with FACS machine and analysis, Kenneth Larsen for help with the fluorescence microscopy, and all the others from UiT and UNN that helped along the way.



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1 Summary

Introduction: Patients presenting in the intensive care unit with severe injury or infections, typically carry a substantial inflammatory cytokine burden. The pathophysiological changes to leukocytes are poorly defined in these patients. This study aims to look at the macrophage-like characteristics of monocytes in patients entering into the ICU with severe inflammation / sepsis.

Methods: The study has been approved by ”Regional committee for Medical and Health Research Ethics North(REC north). Monocytes were investigated using flow cytometry (FACS analysis) and fluorescence microscopy. For the FACS analysis, 11 healthy adult volunteers and 6 patients were recruited. For the fluorescence microscopy, 1 healthy volunteer and 1 patient were recruited.

Results: The results from the FACS analysis were difficult to interpret, but might suggest that the monocytes from the patient populations have either become highly stressed and are changing morphology, and or, they are rapidly changing morphology and phenotype. The fluorescence microscopy results suggest that this analysis may give a better indication on the changes in monocytes compared to the FACS analyses.

Conclusion: This project has provided an insight into specialist clinical and research areas. It has been an interesting journey to find better ways to understand the patient condition and, hopefully, look at possibilities to eventually improve treatment plans and protocols. Data from an ICU patient revealed marked present changes in their monocytes in a short time frame. These cells might be able to facilitate an understanding of macrophage priming for further differentiation in the future.

2 Abbreviations

APC: Allophycocyanin; a red emitting fluorophore

BV421: Isotype control for the CD68.

BB515: The isotype control for CD206.

CCR2: Chemokine receptor 2

CD14: A lipopolysaccharide-binding protein anchored to the cell surface by linkage to GPI, and functions as an endotoxin receptor. Expressed in high levels on monocytes.

CD16: A low affinity Fc receptor for IgG, responsible for antibody-dependent cellular cytotoxicity.

CD68: Also known as Scavenger receptor class D member 1. Expressed in cytoplasmic granules of monocytes, macrophages, dendritic cells, granulocytes, myeloid progenitor cells and a subset of CD34-positive hemopoietic bone marrow progenitor cells. Binds to a intracellular epitope.

CD163 Acute phase-regulated receptor involved in clearance and endocytosis of haemoglobin/haptoglobin complexes by macrophages and may thereby protect tissues from free haemoglobin-mediated oxidative stress. Expressed in monocytes and mature macrophages. Also expressed in blood.

CD172a α: Also known as signal-regulatory protein alpha(SIRPα). A receptor-type transmembrane glycoprotein expressed on cells of myeoloid orgin, including granulocytes, dendritic cells, macrophages, mast cells and haemtopoietic stem cells.

CD206: Also known as the macrophage mannose receptor or C-type lectin domain family 13 member D. A type I transmembrane glycoprotein expressed on human macrophages, endothelial cells, and cultured dendritic cells. It is not detected on resting monocytes.

DAPI: 4′,6-diamidino-2-phenylindole; a highly specific DNA stain that preferentially binds to AT regions of the DNA molecule.

DCs: Dendritic cells, antigen-presenting cells in the immune system.

MHC:	Major histocompatibility complex, also called HLA (human leukocyte antigens). Group of genes involved in the immunological recognition of self.
PBMCs:	Peripheral blood mononuclear cells
PE:	Phycoerythrin; a yellow emitting fluorophore
PPRs:	Pattern recognition receptors

3 Introduction

Patients presenting in the intensive care unit with severe injury or infections, typically carry a substantial inflammatory cytokine burden. A more comprehensive understanding of the pathophysiology that underpins their condition, may possibly help physicians treat these patients with more targeted interventions.

Finding new and advanced treatment strategies is an important way to develop the field of medicine. However, understanding the background and pathophysiology for the patient's condition is just as important in this process. What happens in a patient admitted to the ICU with inflammation/sepsis is important to understand and facilitate further therapeutic tailoring in order to provide the best possible treatment for each patient. This approach could lead to developing a unique therapeutic individualized regimen.

Research focus has slightly changed during the last few years as it has been more directed towards basic inflammatory processes and how these changed may affect current treatment protocols. This exploratory study is a small portion of the ongoing research project in this specialty, which aim to deliver better care and treatment concepts based on the pathophysiology and clinical features.

3.1 Background

3.1.1 Immune system

The human immune system is a complex and strong defense mechanism against infections, illness and injuries in the body, but it can also contribute directly to the repair process.(1) The system consists of proteins, cells and different immunological organs. It is capable of fighting bacteria, viruses, fungi and parasites to prevent infection through pro- and anti-inflammatory pathways. (1)

The immune system consists of a non-specific and specific part, also called the innate and adaptive immune system. To be effective both parts must be able to identify pathogens that enter the body or respond quickly to inappropriately expressing cell factors. The ability to do so is dependent on recognizing surface molecules on the pathogens/cells, called antigens. The antigens provide a specific label, making it possible for the immune system to identify different pathogens/abnormal cells and differentiate the latter from the body's own cells. (1)

3.1.1.1 Innate immune system

The innate immune system is the immunological system we are born with. The system is capable of inhibiting or killing foreign microorganisms without being exposed to them at an earlier time point, that is, there is not a learning process involved in this immune function. This response is non-specific, which means that their mechanisms work on several different microorganisms and do not react with a stronger effect on repeated infections. (1)

When an antigen is presented, receptors on the surface of the cells, called pattern recognition receptors (PRRs), recognize the antigen and start an immune response in the host, recruiting the phagocytic immune cells; macrophages, neutrophils and dendritic cells, generating pro-inflammatory cytokines through natural killer cells and mast cells. Mast cells also produce histamine and heparin, facilitating blood flow through vasodilation and anticoagulation. Eosinophil granulocytes secrete proteins and free-radicals, while basophil granulocytes release further histamine. Collectively, these systems are responsible for releasing antimicrobial peptides, recruiting leukocytes, and activating the alternative complement pathway form the innate immune system. The system starts functioning immediately after infection and rapidly controls the replication of the infecting pathogen.(1)

3.1.1.2 Adaptive immune system

The adaptive immune system works differently than the innate immune system by being able to differentiate the body's own molecules from foreign ones. This system is specific and directed against antigens. Another difference from the innate system

is that the secondary exposure to an antigen gives a stronger reaction then the primary infection. This “learning” process makes us more resilient to future repeat exposure to infective agents. (1, 2)

The adaptive immune system is organized around two classes of specialized cells called the T and B cells, or the effector cells. These cells need to be produced and differentiated before they become functionally active, which can take up to three or four days. Therefore, the adaptive immune system works slower than the innate, but is better at targeting specific pathogens.(1, 2)

The T cells are responsible for the cellular immunity in our immune system. They are further subdivided into three main types: cytotoxic T-cells(T_c-cells), regulatory T-cells(T_{reg}-cells) and T-helper cells(T_h-cells). Each of these subgroups have their own roles in defeating pathogenic threats, like infections and injuries. (1, 2)

The B cells main task is to make antibodies against specific antigens. These proteins attach themselves to pathogen antigens and mark the cells for destruction. The antibodies are also known as immunoglobulins. (2)

The T and B cells also have the ability to create memory cells. The memory cells are stored in the spleen and lymph nodes and act when a former antigen is presented. In this manner, the immune system can react quickly and vigorously to a repeat infection. (2)

3.1.2 Innate and Adaptive Mediating (Cross-Talk) Immune Functions

There are several ways in which the innate and adaptive immune systems communicate to effectively control infections or recognise aberrant cells. The liver plays a prominent role due to the up-regulation of acute-phase proteins (e.g. haptoglobin) and the concomitant down regulation on non-acute phase proteins (e.g. albumin). (1)

The well recognised complement system is primarily considered to be part of the innate immune system. Activation of a complement can recruit a range of leukocytes and opsonize cells by tagging pathogens and aberrant cells for destruction. (1)

Dendritic cells although phagocytic innate immune cells also interact with the adaptive immune system by presenting pathogen antigens or their surface which are acknowledged by T- and B-cells to produce an adaptive immune response.(1)

3.1.3 Intermediary Leukocytes

There are a group of cells, gamma- delta-T cells, that do not have the classical CD4+ / CD8+ profile but have an alternative T cell receptor. The conditions leading to responses from these cells is not well understood. While these cells are part of the innate immune system they also show characteristics that demonstrate that they have memory of previous insult. (1)

3.1.4 Monocytes

Monocytes are formed from hematopoietic stem cells in the bone marrow. These cells can further differentiate into macrophages and dendritic cells. The monocytes are involved in the immune defence, inflammation process and homeostasis in our bodies. They have the ability to provide a progenitor pool to form the inflammatory dendritic cells. They also play a crucial role in tissue repair. (3)

The monocytes circulate in the blood. They can respond to local signals and become recruited into tissues where they mature into macrophages and take up residence. The monocytes are a cell type with progenitor functions, and their actions are dependent on the context that these cells experience. The monocytes use the PPRs to recognize microorganisms, and have different mechanisms to jumpstart the immune system. They can proliferate in response to infection or injury, secrete chemokines, such as interleukin-8, and phagocytose and presented antigens. (3)

The monocytes have many different functions, and a full understanding of their role in human health and disease is still somehow uncertain. More research could make way for targeted approaches in cases of infection, autoimmunity, cancer and transplantations. (3)

There are three subgroups of monocytes; the classical inflammatory monocytes, the non-classical patrolling monocytes and the intermediate monocytes.

3.1.4.1 Classical monocytes

Classical monocytes are defined as a major population of $CD14^{high}$ $CD16^{low}$ cells, which means they express high levels of CD14 on their cell surface, and low levels of CD16. These monocytes proliferate in the bone marrow in response to infection or injury. They aim for the centre of attention after being released to the circulation in a CCR-2 dependent manner. From the infection/injury site, the monocytes can recruit other immune cells by secreting chemokines and present antigens via MHC2 receptors. This subgroup accounts for 80-90% of peripheral blood monocytes. (3-5)

3.1.4.2 Non-classical monocytes

The non-classical monocytes express low levels of CD14 and high levels of CD16, ($CD14^{low}$ $CD16^{high}$cells). These monocytes are successors to the classical monocytes. The monocytes have circulated in the blood as classical monocytes, and returned to the bone marrow and matured as non-classical monocytes. This process is ruled by a specific leading molecule that facilitates the maturation of the non-classical monocytes. This protein is called Nur77(also known as NGFIB or NR4A1). (4, 6, 7)

3.1.4.3 Intermediate monocytes

There is also a third population in between the classical and non-classical subtypes, called the intermediate monocytes. It is still unclear if these cells truly represent a specific subgroup with their own specific assignments or just variations of the other subgroups. The monocytes express intermediate amounts of CD14 and CD16 and high expression of MHCII. Studies imply that the intermediate monocytes have a closer resemblance to the classical monocytes in comparison to the non-classical monocytes. (5)

3.1.5 Macrophages

As mentioned earlier, monocytes can travel from the blood and under local signals become recruited into tissues where they mature into macrophages and take up

residence. The macrophages are well equipped for phagocytosis in the tissue, and is a very important part of the innate immune system. (8)

It is worth specific mention of sub-sets of macrophages; the liver has a 'resident' population of macrophages called Kupffer cells. There is limited literature on the kidney macrophage population although the cells, intraglomerular mesangial cells, are phagocytotic and derived from monocyte lineage or smooth muscle cell lineage, and have a profound impact on glomerular filtration rate and typically cover 30% of the glomerular capillaries. The gut and lungs have specialist macrophages where the former do not produce cytokines, but retain their phagocytosis capacity and the latter have a significant suppressive function, where alveolar macrophages secrete nitric oxide, prostaglandins, interleukin-4 and -10(IL-4, IL-10), and transforming growth factor-β (TGF-β) to regulate the adaptive immune system. The brain has a particular macrophage with unusual plasticity, adipose tissue contains variable amounts of macrophages, which increases considerably in obesity. (8)

Resident macrophages perform a trophic as well as a homeostatic role in the removal of apoptotic cells. Furthermore, they function as guards that protect against infections or injuries. Terminally, the cells become differentiated in different directions depending on stimuli and the tissue environment. (8)

Macrophages can become functionally polarized depending on which stimulus they receive. They can develop an initial inflammatory or a later anti-inflammatory phenotype. These two subgroups are also called M1 and M2 macrophages. The classical pathway, also termed as the pro-inflammatory pathway, forms M1 macrophages. The alternative pathway, also termed as the anti-inflammatory pathway, leads to M2 macrophages.(9)

3.1.5.1 Classical pathway

The classical activation of macrophages (M1) depends on the activated CD4+ T helper 1 lymphocytes (Th1) or natural killer cells (NK-cells), and the signalling molecules they release. The macrophages can become activated solely from interferon-γ (INF-γ) stimuli. The INF-γ signalling can also be augmented by receiving

other cytokines such as TNFα or Granulocyte-macrophage colony-stimulating factor (GM-CSF), or from xenobiotic materials, such as lipopolysaccharide (LPS) released during microbial infection. The M1 macrophages can directly release a range of inflammatory cytokines, including interleukins (IL)-1β, IL-6, IL-10, IL-12, TNF-α and TGF-β1. These cells are also efficient producers of reactive oxygen and nitrogen free radicle intermediates. (10-13)

3.1.5.2 Alternative pathway

The alternate activation of macrophages (M2) is dependent on the activated CD4+ T helper 2 lymphocytes(Th2), and the cytokines secreted by them. The alternative activated macrophages can become active in response to stimuli from cytokines. The most relevant cytokines in this context are IL-4, IL-10, IL-12 and IL-13 and Transforming-Growth Factor (TGF)-β1. (10, 11, 13, 14)

M2 macrophages can further be divided into subgroups. These are differentiated based on their reaction from other cells. The first group, M2a, is formed in response to the actions of IL-4 or IL-13. The second group, M2b, forms in response to stimuli from immune complexes. This demands presence of a Toll-like receptor ligand. The third group, M2c, becomes activated in response to for example TGF-β1, IL-10 or glucocorticoids. All the stimuli that are capable of activating M2 macrophages have anti-inflammatory effects. (9)

3.1.6 Sepsis

Sepsis is the response to uncontrolled infection, injury or illness that can lead to damage of the tissue, organ failure, or death. The host response which occurs in the human body is overactive and dysregulated. A full understanding of the mechanisms behind the condition has not yet been achieved. Sepsis occurs when our immune system tries to fight of an infection. Instead of fighting the foreign microorganisms, the system starts to fight against itself. This results in the sepsis triad which includes systemic inflammation, hemostatic changes and organ damage. (15)

3.1.6.1 Definition of sepsis

In 2016, the third international consensus definitions for sepsis and septic shock were presented: called Sepsis-3. In the new definition, sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. (16)

In addition to Sepsis-3, the SOFA score was introduced. The SOFA score is a tool used by physicians, and measure the degree of sepsis in the respiratory, circulatory, coagulation status and liver, kidney and central nervous system functions. An acute change in the SOFA score ≥2 points gives associated mortality rate at 10% or more. A simplified clinical instrument was also introduced, qSOFA, which is an indicator for severe illness. The three criterias are: altered mental status(Glasgow Coma Scale (GCS) <15), fast respiratory rate (>22) and low blood pressure (SBP≤100mmHg).(16)

3.1.6.2 Pathophysiology of sepsis

The pathophysiology of sepsis is still poorly understood, and it is still not possible to define the exact cause of the condition. Clinical prognosis is often difficult. A few of the patients die during the initial inflammatory process, in which the hyper activation of the innate immune system seems to be the cause, sometimes referred to as “a cytokine storm”. In this phase, severe illness, a microbial infection or injury leads to a pro-inflammatory cascade with hyper activation of the innate immune response and heavy compliment-activation. This cascade is the initial sign of sepsis. Macrophages and neutrophil granulocytes are produced and act in the direction of stimuli of the cytokines, chemokines and compliments, among others. Secondary mediators, such as reactive oxygen species, increase the inflammation further. Dysfunctional regulatory control of mechanisms in this system can make the inflammation destructive for the host, leading to organ damage/failure. (15)

A minority of patients die from sepsis die during the initial phase, in most cases death occurs during the later phase. Due to a prolonged immunosuppressive stage, patients are exposed to a dysfunction in both the innate and adaptive immune system. Important intracellular signalling pathways are being shut down due to paralysis of the neutrophil granulocytes, and the immune system becomes inefficient.

T_H1-cells, which play an important role early in the process, are transformed to T_H2-cells, and further lead to immunosuppression. (15)

Apart from the immune system, the coagulation and autonomic nervous systems are also profoundly affected in sepsis and it is likely that there are several other mechanisms involved during sepsis, many of which are probably still unknown.

3.1.6.3 Treatment of sepsis

The pathophysiological consequences of sepsis are the main target for treatment. The treatments used in the ICU today are mainly directed against the body's own dysregulated reaction to infection, and on preventing organ failure by bridging the patient through the crisis, often through extra-corporeal support. The approaches used today include plasma glucose control, control of central venous pressure, hourly urine output, ventral venous oxygen saturation and mean artery pressure, as well as antibiotic and corticosteroid treatment. (17)

3.2 Aim of the thesis

As stated earlier the purpose of this thesis is to investigate the macrophage-like character in the monocyte population in leukocytes collected from patients admitted to the intensive care unit. There is little published information on this subject. Therefore, this study aims to develop an initial platform as a proof-of-principle on the nature of the changes in a highly plastic population of leukocytes, the monocytes, in patients with severe metabolic disturbances due to sepsis and escalating inflammation. The long-term intentions are that this study may be extended to examine the potential to monitor patients through their ICU journey looking to distinguish between the individuals that recover and those that do not survive.

4 Material and methods

4.1 Design

The study design used in this thesis was a case-control open “pilot” study. In a case control study a group of patients exposed to an illness is compared to a group of healthy volunteers. The participants in the study were chosen based on their disease on admission to the ICU. This study design can be used to investigate rare diseases with long latency periods and investigate serveral exposures points at the same time. The disadvantages are that it may be difficulties in finding previous information about the patient’s experiences or exposures, and there could be difficulties appraising and interpreting data. (18) This is important in patients with acute illness in the ICU.

4.2 Ethical Committee Approval

The project has been approved by “«Regionale komiteer for medisinsk og helsefaglig forskningsetikk» (2013/1208/REK nord) (Appendix 1), and approved by Personvernombudet UNN, Tromsø.

4.3 Setting and participants

The original intention was to recruit 25 patients and 25 matched controls. Inclusion criteria were age 30-75 years with acute critical illness presenting with severe inflammation or overt sepsis. Patients were recruited in an open-plan study following the patients from entry to discharge. Blood samples were collected (4mL x 2; heparin) within 24 hours after admittance to the intensive care unit. Patients admitted directly from the OR after reoperations were excluded.

For the first study, using fluorescence activated cell sorting (FACS), 6 patients and 11 controls were recruited.

For the second study, using fluorescence microscopy, 1 control and 1 patient were recruited.

Patient sample used in the fluorescence microscopy was obtained from a 40-year-old woman. Admitted to the gastro surgical department in a bad general condition with abdominal pain and nausea. Diagnosed with necrotizing pancreatitis, severe respiratory failure and ARDS. Treated with ECMO (Extracorporeal Life-support) for 3 weeks. Acute renal failure developed and the patient was connected to the CRRT (continues renal replacement therapy) machine.

Blood sample was obtained 6 days after a necrosectomy. This is is suffering from a servere inflammatory process and therefore included as a good candidate for the fluorescence study.

4.4 Methods

The blood samples (4mL) from the patient group were collected within 24 hours after admittance to the intensive care unit. Blood samples from our healthy volunteers were taken by trained phlebotomists or clinical staff at the hospital. Whole blood heparin-anticoagulated samples were immediately taken to the laboratory and stored for a maximum 2 hours. The samples were then mixed with 4 mL phosphate-buffered saline. The samples were carefully layered by pipette onto 6 mL lymfoprep (Stemcell Technologies™) and centrifuged immediately for 30 minutes at 800 g and 20°C to separate the peripheral blood mononuclear cells(PBMCs). The PBMCs were then aspirated using a pipette and mixed with 15 mL calcium/magnesium-free phosphate-buffered saline. The cells were then centrifuged again for 35 minutes at 4°C and 300 g. The cells were resuspended and washed one more time in 15mL with calcium/magnesium-free phosphate buffered saline. After this procedure, the cells were counted using a Millipore Septor™ hand-help instrument(Merck KGaA). The cells were fixed and permeabilized and labelled with fluorescent anti-human antibodies directed to CD14(BD Biosciences), APC Mouse IgG 2a κ isotype (BD Bioisciences), CD68 BV421 (BD Bioisciences) , BV421 Isotype control (BD Bioisciences), CD206 BB515 (BD Bioisciences), BB515 Isotype control (BD Bioisciences) CD163 (Molecular Probes), IgG 1 Isotype Control (Thermo Fisher) CD172a (Thermo Fisher Scientific), CD172a/b (BD Bioisciences), and SIRP (15-414) (Thermo Fisher) surface expressed proteins. The experimental details are described more fully below. The stained cells were cooled in the refrigerator(4°C) for 20 minutes and centrifuged for 10 minutes in 4°C at 300g and then washed. The samples were then run through the flow cytometry with standard settings from the manufacturer.

4.4.1 Lymfoprep

Lymfoprep is an easy and fast way to isolate the PBMCs in whole blood. This product works by exploiting the density of the different cell types. Mononuclear cells have a

lower buoyant density than the polymorphonuclear leucocytes and erythrocytes, and these cells can be isolated by using an isosmotic medium as lymfoprep and then centrifuging the cells. The polymorphonuclear cells and erythrocytes will sediment through the medium while the mononuclear cells will remain in the medium interface. After the centrifuge, the PBMCs are easy to extract from the sample and can be used for further investigation.

4.4.2 Fixating and permeabilization of the cells

The PBMCs were fixed and permabilized before staining them with the antibodies. The permeabilization is done by using a detergent or surfactant. Permeabilization is achived by using a detergent or surfactant which disturbs the packing of lipids in the cell membrane and creates holes. However, before permeabilizing, the cells must undergo a fixation procedure to prevent destruction of the cells.

The cells, were fixed using paraformaldehyde. This is a polymer that depolymerizes in warm water to formaline that can react with enzymes, proteins and other macromolecules and form covalent crossbinding, thereby conserving structural integrity. Many recipes use a 4% paraformaldehyde solution, initial experiments suggested that this may be too extreme for the PBMCs in this study and we reduced the paraformaldehyde concentration to 2%. To permeabillize the cell membrane we used Tween20, a mild polysorbate type nonionic surfactant.

To 98mL phosphate-buffered saline(calcium and magnesium free) at 70°C, 2g paraformaldehyde was added, mixing continuously. After cooling down to room temperature, the pH was adjusted to 7,2 by adding 14 µL 5M potassium hydroxide. To make the permeabilization solution, 200 µL Tween20 was added to 100 ml phosphate-buffered saline (calcium and magnesium free).
Fixing the cells:

1. One million cells were resuspended in 875 µL ice cold phosphate-buffered saline in a BD-test tube and 125 µL ice cold fixating fluid was added and mixed.
2. The cells were incubated in the refrigerator(4°C) for one hour.

3. The cells were then centrifuged in 5 minutes at 300 g and the supernatant was decanted.

Permabilization of the cells:

1. The cell pellet from the fixation proscedure was resuspended gently in 1 mL permabilization fluid at room temperature.
2. The cells were incubated for 15 minutes at 37°C.
3. The cells were then centrifuged for 5 minutes at 300 g at 20^{O}C and the supernatant was decanted.
4. The cells were resuspended in 1 mL staining buffer and the cells were centrifuged for 5 minutes at 300 g and the supernatant was decanted off the product.
5. The cells were then resuspended in 0,5 mL staining buffer containing individual antibodies according to manufacturer´s instructions.

These methodologies reflect the final conditions after optimization, such as titrating the antibody concentration, using control blood samples from healthy controls in order to achieve the best conditions for FACS analyses.

4.4.3 Flow cytometry

Flow cytometry is an effective method to sort, count and profile cells. The instrument measures optical and fluorescence characteristics of single cells. The cells and particles are suspended in a liquid and run through a laser light source. The instrument is able to identify and separate the cells based on the fluorescence wavelength and all other unlabelled cells are ignored. Once tagget with a fluorescent antibody, the instrument can also characterize immune cell subtypes based on morphology and size.

There are two main parameters in the flow cytometry, light scattering and fluorescence. Light scattering occurs when light from the FACS machine hits the particles. The particles will then deflect the light. Forward-scattered light(FSC) measure the diffracted light and is proportional to its size. Side –scattered light(SSC) measures the refracted and reflected light from the particle. The correlation between these two measurements can be used to differentiate the different cells types in the

sample. Fluorescence labelled cells are detected through their targeted proteins. (19)

4.5 Fluorescence microscopy

The use of FACS analysis can tell the operator a great deal about a large number of different cells in a mixed population, but, until very recently. it is not good at investigating single cells for other useful parameters. Single cell analyses is better achieved using fluorescent microscopy.

As a proof-of-principle PBMCs were isolated from a single patient recruited from ICU meeting entrance requirements for the study and a single heathy control. The cells were isolated as described above using lymphoprep through to the wash step in calcium and magnesium-free phosphate buffered saline.

Thereafter, the PBMCs were resuspended in RPMI 1640 culture media containing 10% fetal bovine serum (Merck KGaA) and plated out into 12 well culture plates (Falcon®; Corning Life Sciences) containing 12mm, 1.5 sized circular glass cover slips (Thomas Scientific) previously coated with fibronectin in calcium and magnesium-free phosphate buffered saline (10µg/mL) for 1 hour (Merck KGaA) at a density of 300,000 cells per well. The plates were incubated at 37^{O}C for 1 hour in 5% carbon dioxide/air mixture.

The plates were washed with pre-warmed calcium and magnesium-free phosphate buffered saline before being fixed with the 2% fixation solution described above for 1 hour at 4^{O}C. The cells were washed 3 times with calcium and magnesium-free phosphate buffered saline before being labelled with either mouse anti-human monoclonal anti-CD14 antibody (abcam [1H5D8] 1/150 dilution) or rabbit anti-human monoclonal anti-CD163 antibody (abcam [EPR19518] 1/150 dilution).

The wells were then blocked with 3% goat serum (Thermo Fisher) for 45 minutes at room temperature. The plates were washed with calcium and magnesium-free phosphate buffered saline 3 times before the mouse antibody labelled cells were incubated with goat anti-mouse IgG H&L conjugated with Alexa Fluor® 488 antibody in calcium and magnesium-free phosphate buffered saline containing 1% goat

serum. The rabbit antibody labelled cells were incubated with goat anti-rabbit IgG H&L conjugated with Alexa Fluor® 555 antibody in calcium and magnesium-free phosphate buffered saline containing 1% goat serum. The plates were left at room temperature for 1hr before being washed 5 times with calcium and magnesium-free phosphate buffered saline. On the third wash the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI; 300nM) was added to the calcium and magnesium-free phosphate buffered saline for 10 minutes at room temperature. The cells were washed two more times with calcium and magnesium-free phosphate buffered saline.

The glass cover slips were removed from the wells washed in pure water (resistivity 10-15 MΩ/mL) carefully dried to remove excess water and inverted onto a glass microscope slide with 8µL Abberior Mount Solid Antifade (Abberior GmbH). The slides were catalogued and allowed to stand on a flat surface at room temperature for 30 minutes.

Confocal microscopy was performed on an LSM780 system (Carl Zeiss Microscopy) using a 63X NA1.4 oil immersion objective lens. Identical (non-saturating) acquisition settings were used for all images, and were optimized for the detection of DAPI, AlexaFluor 488 (for CD14) and AlexaFluor 555 (for CD163).

5 Results

5.1 Results from flow cytometry:

For the FACS analysis data from 11 controls and 6 patients was considered. The dot plots from the controls as expect from internal experienced colleagues and from published examples (Fig. 1; all results figures are shown in Appendix 2). The monocyte population being where they were expected to be sited between lymphocytes (Figs. 2). In contrast, in dot plot for the patients, the cells were spread widely throughout the plot, exampled by the positive uptake of CD14, using an example from one patient sample (Fig. 3).

These data would suggest that the monocytes from the patient populations have either become highly stressed and are changing morphology, and or, they are rapidly changing morphology and phenotype. The use of FACS analysis in this context is less rewarding. At this point the FACS study was truncated on ethical grounds and we considered an alternative approach, fluorescence microscopy.

5.2 Results from the fluorescence microscopy:

For the fluorescence microscopy investigation, we used samples from one control and one patient as a proof-of-principle study due to time limitations. The nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) was used as a reference point on the captured images, DAPI positive CD14 positive (green fluorescence) cells being monocytic, while DAPI positive CD14 cells were not of the monocyte lineage. For this study there was a switch to looking at CD163 (red fluorescence) a marker for monocyte lineage cells that scavenge haptoglobin-haemoglobin complex as we considered that CD163 was a likely potential indicator for some of the results that had been found in the FACS analyses. The results showed positive uptake of CD14, CD163 that co-localized with DAPI (Figs. 4 and 5). While single observations cannot be confirmatory the identified $CD14^{+}$, $CD16^{+}$ cell shown for the patient sample (Fig. 4) shows that fluorescence analyses may give a better indication of the changes that the monocytes are experiencing than would be achieved by FACS analyses.

6 Discussion

6.1 Key findings

The literature describing the monocytes in ICU patients is limited. Therefore, to some extent, the findings from this investigation represent novel observations and establish a baseline position for continuing research in this area. In this project, flow cytometry and fluorescence microscopy were used to analyse the blood samples drawn from controls and patients.

In the FACS analysis, it was not able to define monocyte population in the patient samples. Although, for brevity, the results from the CD14 element of the study are shown here, the results were difficult to interpret for the other antibodies that were investigated. The results show a widespread distribution of antibody positive fluorescence. The monocytes, which demonstrate great plasticity, may be exhibiting changes to differing phenotypes that may have morphological impact. Alternatively, the monocytes may also be experiencing morphological changes that are unrelated to phenotypic changes. To complicate the picture further both morphological and phenotypical changes may be occurring at the same time. However, irrespective of the nature of the changes, this study shows that there are profound changes to the monocyte cell population in the ICU patients and that these can be developed very quickly. This is particularly evident when the patient's samples are compared to the observations made on the control subject samples, which followed a standardised pattern with respect to the location of monocyte cells.

Turning to the findings the results from the fluorescence microscopy investigation, it is clear that it was possible to identify $CD14^+$ and $CD16^+$ cells. More work needs to be done but there is a suggestion that the monocytic $CD14^+$ and $CD16^+$ cells in the ICU patient may be larger than witnessed in the control subject used in this snapshot study.

6.2 Comparison with previous studies

Searches of the published literature shows very few publications regarding the potential to express macrophage characteristics in monocytes from patients in the intensive care unit. The need to avoid biopsies in a vulnerable population of patients

often rules out direct tissue sampling and, therefore, tracking the development of M1 and M2 macrophages is difficult. Specifically, there were only two articles presenting results from clinical trials conducted on humans.
The first article studied the adipose tissue in a healthy control group and in non-surviving prolonged critically ill patients. They found an elevated level of gene expression markers such as arginase-1, IL-10 and CD163, which corresponds with M2 macrophages. They also found low levels of markers corresponding with M1 macrophages; TNF-α and inducible nitric-oxide synthase(iNOS). These results suggest that non-surviving patients had an accumulation of M2 macrophages in their adipose tissue and low levels of M1 macrophages. (20)

The second study used bronchial alveolar lavage liquid (BALF), which can provide information about the macrophages in lung microenvironments. They used samples from a healthy control group and samples from patients admitted with pneumonia. This study showed that the M2 subtype dominated in the samples from the healthy control group and that they rapidly were able to polarize to M1 macrophages. (21)

Furthermore, another interesting study, on mouse and zebrafish models, looked at the effect of retinoic acid in treatment of acute kidney injury to reduce injury and fibrosis. This study showed that retinoic acid coordinated a dynamic equilibrium between M1 and M2 macrophages, which reduced the damage in the kidney tissue after acute kidney injury caused by M1 macrophages and enhanced the function of M2 macrophages to remodel the tissue from the injury. (22)

These articles may suggest that the polarization of macrophages have diverse effects in different tissues. More research in this area is necessary to get a deeper understanding of how the polarization of macrophages impact patients admitted to the ICU. This may be particularly relevant in the setting of ICU patients with acute sepsis, infection or suspicion of infection. The potential under these conditions for the monocytes to become ‘primed’ for modification to, initially, become M1 macrophages and later under clinical intervention to become M2 macrophages remains to be investigated. The fact that there have been few of clinical trials on humans regarding this subject, and a longitudinal study of the circulating M1/M2 polarization priming potential in intensive care patients has not been addressed.

6.3 Challenges

In this study, there were many challenges. Research in this area is still unknown territory, and investigating the macrophage-like character in monocytes in patients with severe infections or injuries has not been.

It was necessary to explore different methodologies in the search for monocytes. The original plan, to stain the PBMCs with CD14, CD172, CD68 and CD206 and their isotypes APC, PE, BB515, BV421 would have provided a broad platform to develop an understanding of some of the leukocyte modifications early in the ICU patient's journey. Unfortunately, FACS analyses was not the appropriate tool for this approach. The approach has merits as early in we could not detect from the FACS data any signal from fluorescent labelled CD68 antibody. It was suspected that this might be a problem with the practical procedure and tried several samples to get a better understanding of what might be the problem. However, this antibody binds to an intracellular epitope. It would not be possible for the antibody to bind to the cells unless the cells were first permeabilized.

Furthermore, the leukocytes from patients in the ICU are fragile, and we had to choose a gentle fixation method. We chose to use 2% PFA over a longer exposure time, instead of the standard procedure with 4% PFA.

Additionally, there were specific antibody isotype labelled with the fluorophore PE, where the FACS analysis found that the non-specific binding was the same or even stronger than the specific binding. A new PE labelled isotype was bought in order to overcome this significant limitation.

The most challenging part of the study was to be able to interpret the results. Methodologies were interrogated, in order to ensure that we still had trouble defining where the monocytes were correctly placed from the forward- and side-scatter parameters in the FACS analysis. While this seemed unlikely from multiple control sample analyses, the methodological investigation revealed that the patient results were genuine. In contrast, in the fluorescence microscopy study, we could see that

$CD14^+$ and $CD16^+$ monocytes were present in both the patient and the control samples. However, there is a suggestion that there is an increase in the size of the patient's monocyte.

6.4 Interpretation

Based on the results from the FACS analysis, interpretation is difficult. However, the results indicating that the cells might have altered size and/or morphology. This is in complete contrast to the healthy volunteers, where the monocytes were easy to define based on their size and morphology.

Fluorescence microscopy may have more to offer extended studies, where a return to the study with a broader range of antibodies that are specifically orientated to a fluorescence microscopy study would likely lead to the better understanding of the changes experienced by leukocytes in the ICU patients.

6.5 Strong sides of the thesis

In the study, many different methods were tried to find answer to the thesis. The groundwork done to find the best possible approaches in the methodologies, including choice of antibodies and titrating antibody concentration for optimal results within the instrumentational approaches are strengths, and set the groundwork for future research.

6.6 Limitations

An important factor for the study was the limited time that could be devoted within the structure of a Master's degree. This is a very interesting project that we could easily develop into a longer post-graduate investigation. The study did direct itself towards the ethical requirements of the patient and it was truncated early after sufficient progress could not be engineered with FACS analyses of the blood samples.

7 Conclusion

In its broadest aspects this project has provided the potential to get an insight into specialist clinical and research areas. The former being an understanding of intensive care medicine, while the latter was about gaining an understanding of scientific method in the narrow spectrum of white cell physiology and pathophysiology.

Science begins with defining a question, understanding the problems implicit in the question, developing ways to interrogate the issues at the heart of the problem then applying approaches that can deliver results to be interpreted with respect to the problem. This project has been an interesting journey in medical research and looking at ways to better understand the patient condition and, hopefully, look at possibilities to eventually improve treatment plans and protocols.

Specifically, the obvious differences between the ICU patient's monocytes compared to control subjects became very clear. Ensuring that the approaches were appropriate to give confidence to accept the data was essential. Achieving this required input from a range of people with particular skills.

Why the ICU patient present with such a marked change in their monocytes, often in a short time frame, and if these cells can facilitate an understanding of macrophage priming for later differentiation, remains to be determined.

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9 Appendix

Appendix 1: Approval from the REK-committee.

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK nord			22.03.2017	2017/458/REK nord
			Deres dato:	Deres referanse:
			14.02.2017	

Vår referanse må oppgis ved alle henvendelser

Lars Marius Ytrebø
OPIN klinikken

2017/458 Endringer i immunforsvarets celler ved akutt sykdom
Forskningsansvarlig: Universitetssykehuset Nord-Norge HF
Prosjektleder: Lars Marius Ytrebø

Vi viser til søknad om forhåndsgodkjenning av ovennevnte forskningsprosjekt. Søknaden ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK nord) i møtet 09.03.2017. Vurderingen er gjort med hjemmel i helseforskningsloven (hfl.) § 10, jf. forskningsetikkloven § 4.

Prosjektomtale
Pasienter som innlegges med alvorlig skade eller infeksjoner har typisk en stor betennelsesreaksjon i kroppen (inflammasjon). De hvite blodcellene er en svært viktig del av vårt immunsystem og en undergruppe hvite blodceller kalles monocytter. . Disse sirkulerer i blodbanen i noen timer eller noen få døgn før de vandrer ut fra blodbanen og inn i vevene hvor de blir differensiert til makrofager. Makrofagene kan drepe mikrober, renovere dødt og skadet vev, er med på å sette i gang immunresponser og regulerer diverse prosesser ved hjelp av signalstoffer de skiller ut. Videre kan makrofagene spesialiseres i ulike retninger. De kan bli betennelsesmakrofager(M1) eller reparasjonsmakrofager(M2). I denne studien ønsker vi å karakterisere andelen M1 og M2 makrofager, samt måle nivåene av betennelsesmarkører over tid. Primære mål er å kartlegge profilen til disse celletypene over tid mens pasienten mottar behandling for alvorlig livstruende sykdom.

Vurdering
Data – innsamling og oppbevaring
Data fra pasientjournal. Humant biologisk materiale – fullblod. Biologisk materiale skal destrueres innen to måneder etter prøvetaking. Det skal derfor ikke opprettes biobank.

Data skal oppbevares avidentifisert med koblingsnøkkel og koblingsnøkkel skal oppbevares i låst kontor hos prosjektleder og på UNNs server hvor bare prosjektleder har tilgang.

Metode/design/styrke
Det skal avgis 8 ml fullblod (2x4 ifølge protokoll) under oppholdet i intensivavdelingen, i inntil 7 dager, hver tredje dag mens pasienten er innlagt i sengeavdelingen. Blodprøvene er i tillegg til annen behandling og prøvetaking som gjøres i forbindelse med selve innleggelsen.

Datainnsamling skal gjøres fra minimum 25 kritiske syke pasienter mellom 30-75 år, som innlegges ved intensivavdelingen.

Inklusjon/eksklusjon
Det skal gjøres daglig screening av alle nye innleggelser ved intensivavdelingen for å sjekke om pasienter

oppfyller inklusjons- og eksklusjonskriteriene. Inklusjonskriterier er pasienter som pga. akutt sykdom eller traume antas å ha et aktivert immunsystem. Eksklusjonskriterier er pasienter som innlegges fra operasjonsstuen etter kirurgiske reoperasjoner.

Inkludering av personer uten eller med redusert samtykkekompetanse
Prosjektet er en samtykkebasert studie. Det opplyses at pasientene vil være så syke at de ikke vil være samtykkekompetent og at det ut fra det, forventes at nærmeste pårørende må samtykke til deltakelse i studien. I sin vurdering har komiteen lagt vekt på at det er forsvarlig og nødvending at den omsøkte studien, gjennomføres på denne pasientgruppen, og at studien vurderes å være av vesentlig interesse for samfunnet. Komiteen ser det dog som viktig at det innhentes samtykke fra alle deltakere, noe som også er satt som vilkår for godkjenning av studien.

Det skal tas blodprøver av pasientene i en kritisk situasjon. Før slike prøver tas, skal det innhentes samtykke fra pasienten selv eller pasientens nærmeste pårørende. Ut fra en forståelse av at inklusjon til studien gjøres i det som for både pasient og pårørende er en utfordrende situasjon, settes det som vilkår at der det er pårørende som har samtykket skal det også innhentes et etterfølgende samtykke fra alle pasienter som blir i stand til å samtykke, før man forsker på materialet. Dette innebærer at man kun kan gjøre de analysene som det er helt nødvendig å gjøre før man har avklart et etterfølgende samtykke.

Samtykkeskjema
Under del A – utdypende forklaring om hva studien innebærer, brukes det en del medisinsk terminologi for å forklare prosesser i forhold til hva studien vil se nærmere på. Teksten framstår som tunglest med tanke på at det er alvorlig syke eller deres pårørende som skal lese og forstå innholdet i teksten. Skjema må gjennomgås og forenkles i forhold til dette.

Samarbeid med utlandet
Det er avkrysset i prosjektsøknaden at prosjektet skal samarbeide med utlandet og det opplyses at biveileder er Stephen Hodges, Senior lecturer og jobber ved Royal Veterinary College, U. of London. Det er ingen annen opplysning om annet samarbeid med utlandet og komiteen legger derfor til grunn at ingen personidentifiserende data eller biologisk materiale skal overføres til utlandet.

Vedtak

Med hjemmel i helseforskningsloven §§ 2 og 10 godkjennes prosjektet.

Det stilles som vilkår for godkjenning av prosjektet at det skal innhentes samtykke før det det gjøres blodprøvetaking av pasientene, enten fra pasienten selv eller nærmeste pårørende. Likeledes skal det så langt mulig innhentes samtykke fra pasienten selv før man forsker videre på materialet.

Før prosjektet kan igangsettes må det sendes inn revidert informasjonsskriv i tråd med komiteens merknader. Skrivet sendes som vedlegg i e-post til post@helseforskning.etikkom.no

Sluttmelding og søknad om prosjektendring
Prosjektleder skal sende sluttmelding til REK nord på eget skjema senest 30.06.2019, jf. hfl. § 12. Prosjektleder skal sende søknad om prosjektendring til REK nord dersom det skal gjøres vesentlige endringer i forhold til de opplysninger som er gitt i søknaden, jf. hfl. § 11.

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningsloven § 28 flg. Klagen sendes til REK nord. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK nord, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Med vennlig hilsen

May Britt Rossvoll
Sekretariatsleder

Appendix 2: Results

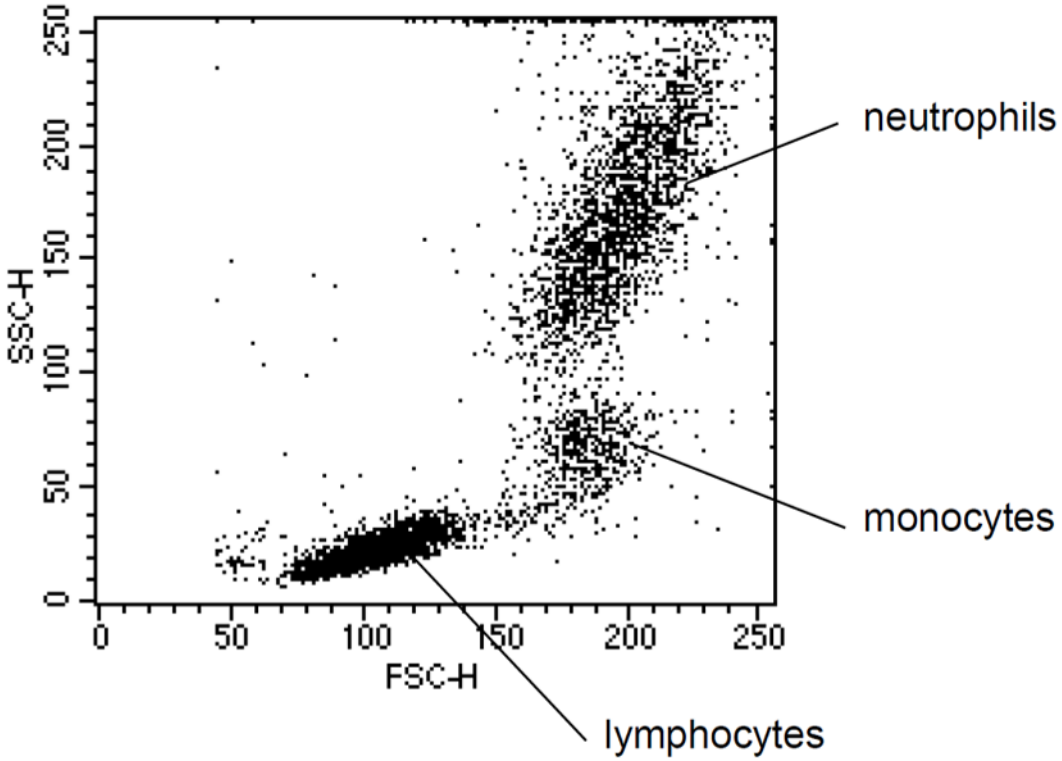

Figure 1: Shows the neutrophil granulocytes, monocytes and the lymphocytes well defined in the dot plot in a healthy adult. (Source: Bongrand, Pierre. Physiologie des cellules monocytaires macrophagiques et dendritiques. 2006)

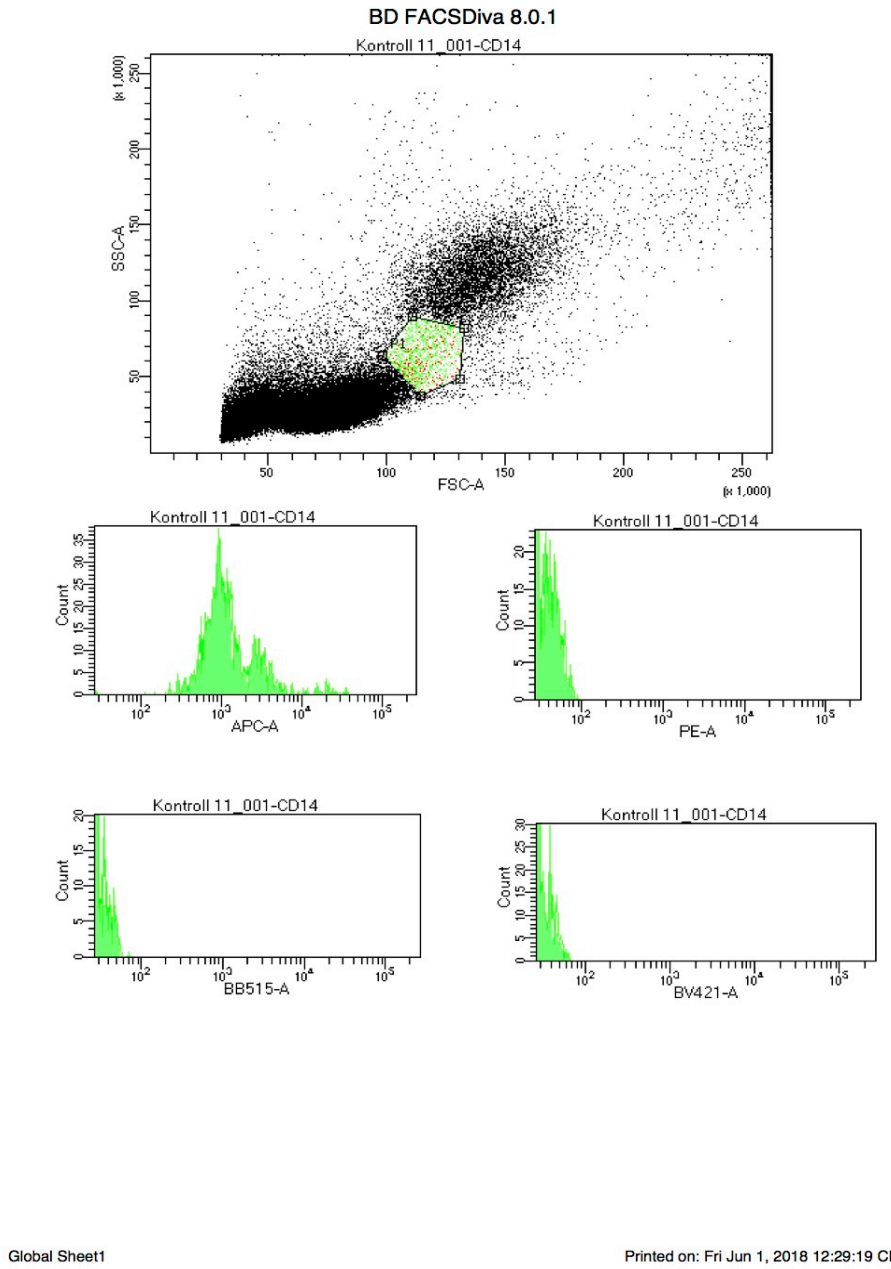

Figure 2: Dot plot is from a control subject showing neutrophils, monocytes and the lymphocytes are well defined. The specific analysis is from cells that have been stained with APC labelled CD14 antibody. The marked area in the dot plot is the area we expect the monocytes to be.

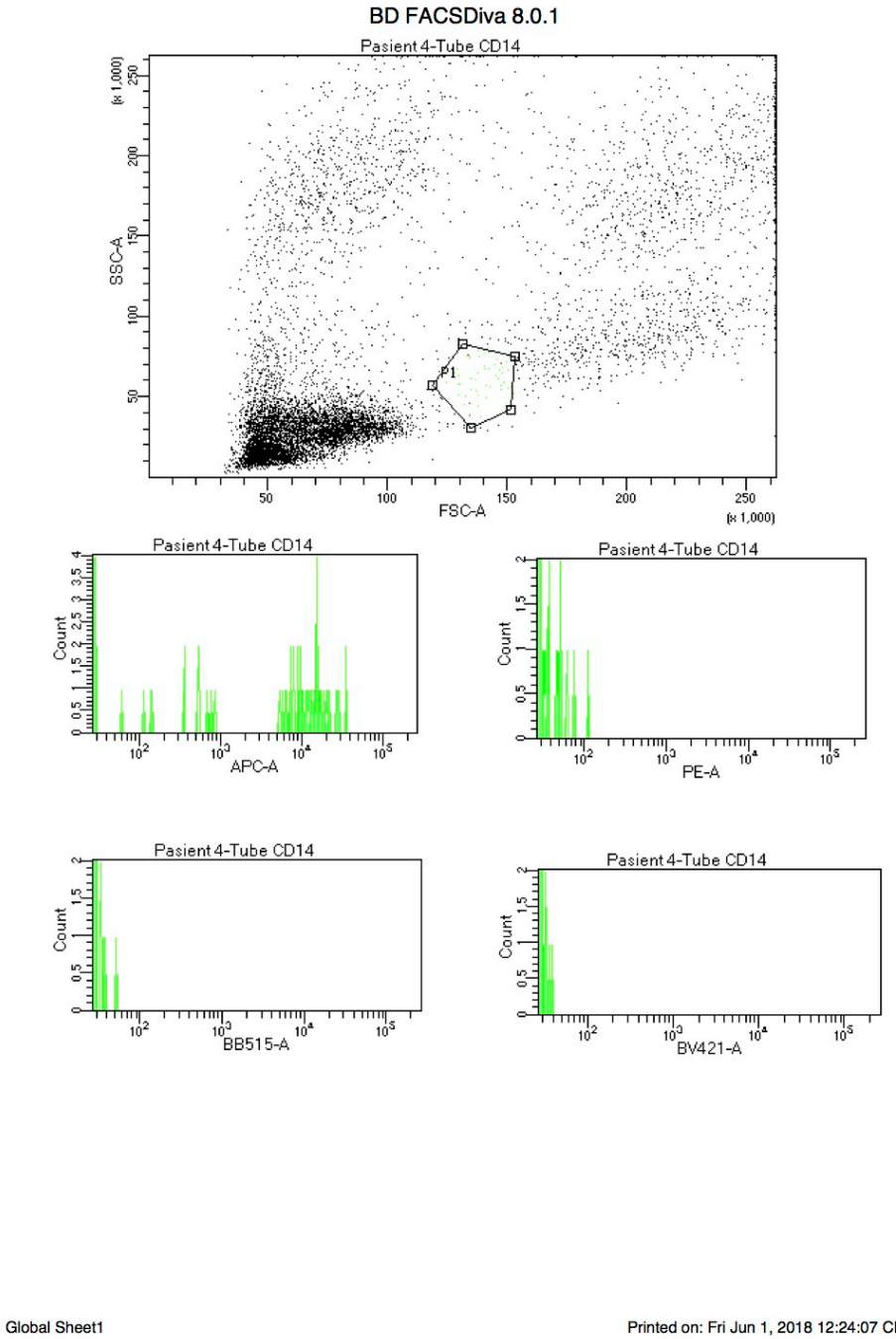

Figure 3: Dot plot from an ICU patient with a substantial inflammatory burden. The cells are spread widely throughout the plot, exampled by the positive uptake of APC labelled CD14 antibody.

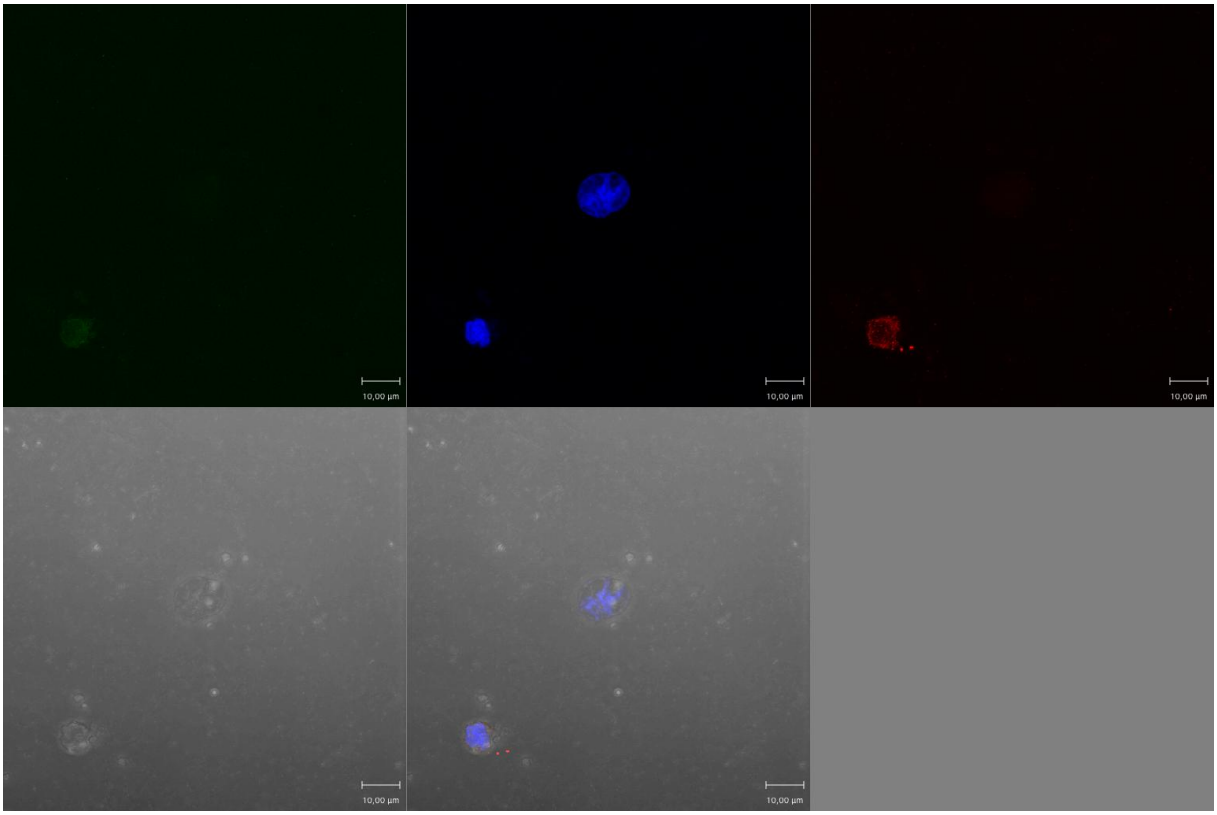

Figure 4: Images from a control sample. The green staining is from CD14 positive cells, the blue from DAPI stained nuclei and the red from CD163 positive cells.

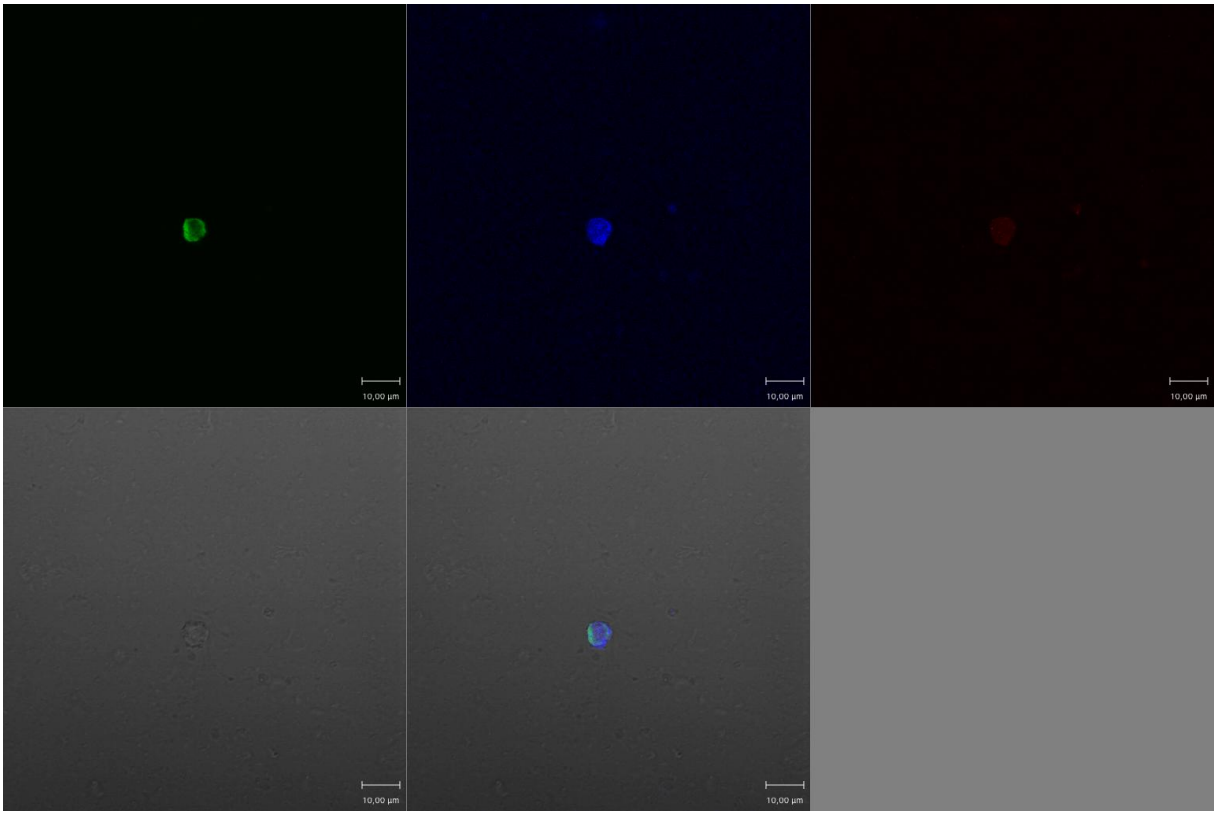

Figure 5: Images from an ICU patient. The green staining is from CD14 positive cells, the blue from DAPI stained nuclei and the red from CD163 positive cells.

Reference: Yoshida Y, Kang K, Chen G, Gilliam AC, Cooper KD. Cellular fibronectin is induced in ultraviolet-exposed human skin and induces IL-10 production by monocytes/macrophages. J Invest Dermatol. 1999;113(1):49-55.			Design: Case-control Documentation level: III Grade: C
Purpose	**Material and method**	**Results**	**Discussion/comments**
We hypothesized that binding of monocyte β1 integrins to ultraviolet-induced extracellular matrix ligands, such as fibronectin, after entry of blood monocytes into the dermis, is involved in the modulation of immunoregulatory monocytic cytokines. Conclusion In conclusion, ultraviolet skin injury results in increased production and deposition of EDA+ cellular fibronectin in the papillary dermis, which may be one of the key signals capable of inducing interleukin-10 but not interleukin-12 in monocytes that infiltrate micromilieu of human skin after ultraviolet exposure. **Country** **USA** **Year** 1999	Immunostaining of human skin and reverse transcriptase–polymerase chain reaction were done to get results in this study. Healthy adult volunteers participated in the study after Institutional Review Board approval of the protocol and informed consent. Keratomes or punch biopsies were performed on the buttocks or hips of volunteers at different time points after four minimal erythemal doses of UV irradiation from a bank of six FS40 bulbs, and nonirradiated sites were used as controls. Inclusion/exclusion criteria not mentioned.	Immunostaining of human skin and reverse transcriptase–polymerase chain reaction studies revealed that the embryonic isoform of cellular fibronectin, in which the extra domain A (EDA) segment is spliced in (EDA+ cellular fibronectin), and confers enhanced binding to β1 integrins, is newly induced and is associated with infiltrating CD11b+ cells post *in vivo* ultraviolet exposure. They tested the effect of fibronectin on resting purified peripheral monocytes *in vitro*. They found that the monocyte interleukin-10, but not interleukin-12, was significantly induced in a concentration-dependent manner by *in vitro*binding to cellular fibronectin (n = 6), but not plasma fibronectin. Tumor necrosis factor-α was also induced in a concentration-dependent manner, but to a lesser extent. Monoclonal antibodies to β1 integrins β-subunit (CD29) also strongly induced tumor necrosis factor-α and interleukin-10 production, but not interleukin-12. Neutralization of tumor necrosis factor-α reduced by 54% the interleukin-10 production that was induced by monocytes binding to cellular fibronectin, indicating that interleukin-10 induction is at least in part dependent upon concomitant autocrine tumor necrosis factor-α release.	This is a case control study with one patient group. A part of their skin was exposed to minimal erythemal doses of UV irradiation, and nonirradiated sites were used as controls. The group consisted of healthy adult volunteers, but no background or characteristics were presented. 6 patients were enrolled in the study. The volunteers were used as both controls and patients, which might leave the results vulnerable to spillover effects. Strong parts of the study: This is an exploratory study that builds a foundation for future research projects. Weak parts of the study: The study enrolled few participants. This might make the results less generalizable. Also, no background story or characteristics about the volunteers were presented. Inclusion/exclusion criteria were not mentioned.

Reference: Langouche L, Marques MB, Ingels C, Gunst J, Derde S, Vander Perre S, et al. Critical illness induces alternative activation of M2 macrophages in adipose tissue. Crit Care. 2011;15(5):R245.	**Design: Case-control**	
	Documentation level	III
	Grade:	C

Purpose	Material and Methods	Results	Discussion/comments
Study the characteristics of adipose tissue macrophage accumulation in critical illness. **Conclusion** Unlike obesity, critical illness evokes adipose tissue accumulation of alternatively activated M2 macrophages, which have local anti-inflammatory and insulin sensitizing features. This M2 macrophage accumulation may contribute to the previously observed protective metabolic activity of adipose tissue during critical illness. **Country** USA **Year** 2006	Macrophage markers with immunostaining and gene expression in visceral and subcutaneous adipose tissue from healthy control subjects (n = 20) and non-surviving prolonged critically ill patients (n = 61) were studied. For comparison, also subcutaneous in vivo adipose tissue biopsies were studied from 15 prolonged critically ill patients. Inclusion/exclusion criteria were not mentioned.	Subcutaneous and visceral adipose tissue biopsies from non-surviving prolonged critically ill patients displayed a large increase in macrophage staining. This staining corresponded with elevated gene expression of "alternatively activated" M2 macrophage markers arginase-1, IL-10 and CD163 and low levels of the "classically activated" M1 macrophage markers tumor necrosis factor (TNF)-α and inducible nitric-oxide synthase (iNOS). Immunostaining for CD163 confirmed positive M2 macrophage staining in both visceral and subcutaneous adipose tissue biopsies from critically ill patients. Surprisingly, circulating levels and tissue gene expression of the alternative M2 activators IL-4 and IL-13 were low and not different from controls. In contrast, adipose tissue protein levels of peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear receptor required for M2 differentiation and acting downstream of IL-4, was markedly elevated in illness. In subcutaneous abdominal adipose tissue biopsies from surviving critically ill patients, we could confirm positive macrophage staining with CD68 and CD163. We also could confirm elevated arginase-1 gene expression and elevated PPARγ protein levels.	This is a case-control study with demographically matched groups. The study has few patients and controls, which makes the results less generalizable. It has included healthy controls, non-surviving and surviving prolonged critically ill patients. The patients characteristics were described thoroughly. This study is exploratory, and little research has been done in this field. Therefore, the researchers have few other studies to support their findings. Strong parts of the study: The authors have described the patient's condition thorough, and have chosen healthy controls to match the patients with. Weak parts of the study: The study has few participants, and therefore it is less generalizable. The study has not considered other factors that might contribute to the results. Inclusion/exclusion criteria were not mentioned.

Reference:	Design: Case-control	
Yang Y, Wu BQ, Wang YH, Shi YF, Luo JM, Ba JH, et al. Regulatory effects of miR-155 and miR-146a on repolarization and inflammatory cytokine secretion in human alveolar macrophages in vitro. Immunopharmacol Immunotoxicol. 2016:1-27.	Documentation level	III
	Grade:	C

Purpose	Material and method	Results	Discussion/comments
Clarify a series of changes at the RNA level in alveolar macrophages under normal and inflammatory conditions **Conclusion** The results indicate that the gene regulation mediated by miR-155 and miR-146a contributes to human alveolar macrophage phenotype repolarization, thus leading to an early switch from pro-inflammatory to anti-inflammatory cytokine production. **Country** China **Year** 2016	Bronchial alveolar lavage liquid (BALF) was collected from healthy volunteers or patients with pneumonia, and compered in density plots. Healthy volunteers were recruited according to the following inclusion criteria: aged 18–65 years, nonsmoker, no respiratory diseases, no hematopathy and immune systems or other underlying diseases. No infectious diseases or use of antibiotics, immunosuppressants or steroid hormones within 1 month before surgery. Routine blood tests, liver and kidney function, blood coagulation tests, electrocardiography (ECG), chest radiography and bacteriological examination from throat swabs were normal within 1 month before surgery. Patients with pneumonia were recruited according to the following inclusion criteria: aged 18–65 years, nonsmoker, no respiratory diseases, no hematopathy and immune systems or other underlying diseases. No use of immunosuppressants or steroid hormones within 1 month before surgery. Blood coagulation tests and ECG examination were normal. Bacterial pneumonia confirmed by bacteriological tests of throat swabs or bronchial alveolar lavage liquid (BALF) and radiological examination.	In this study, the density plots of macrophage subtypes (M1 and M2) in the BALF of healthy volunteers differed from that of the patients with pneumonia. The M2 subtype dominated in healthy volunteers and was rapidly repolarized to M1 in response to miRNA-mediated gene regulation. Differential miRNA expression in the two macrophage subtypes revealed lower expression of miR-155 and MIR-146a in patients with pneumonia compared with healthy volunteers; this may be related to inflammation and the use of anti-inflammatory drugs. We also found increased TNF-α and IL-6 expression at the RNA level, while macrophage galactose-type C-type lectin 1 (MGL-1) expression decreased with downregulation of miR-155 and miR-146a expression.	This is a case-control study with demographically matched groups. One group of healthy controls and one group with pneumonia. The article does not say how many controls and how many patients were enrolled in the study. The background was compered in the case-control groups, and the characteristics were presented. Strong sides of the study: The study is exploratory, and gives information that is valuable for further research. The inclusion and exclusion criteria were defined. Weak sides of the study: How many patients who were enrolled in the study is unclear. The methodology was not described in detail and replicating their experiment can therefore be difficult.

Reference: Baron P, Constantin G, D'Andrea A, Ponzin D, Scarpini E, Scarlato G, et al. Production of tumor necrosis factor and other proinflammatory cytokines by human mononuclear phagocytes stimulated with myelin P2 protein. Proc Natl Acad Sci U S A. 1993;90(10):4414-8.			**Design: Patient series** Documentation level: IV Grade: C
Purpose Examined the effect of myelin P2 protein on some proinflammatory functions exerted by human mononuclear phagocytes. **Conclusion** Since P2 protein is the antigen responsible for the induction of experimental allergic neuritis, these findings identify a potential mechanism involved in the inflammatory reaction and myelin damage during experimental allergic neuritis. **Country** USA **Year collecting data** 1993	**Material and method** The monocytes and monocyte-derived macrophages were perified and cultured. The RNA was isolated and Northern Blot analysis was performed. Antigenic TNF was determined by using a doble-ligand immunoassay, and extracellular antigenic IL-8 was measured by a specific ELISA. H_2O_2 release was assayed by the fluorimetric measurement of the horseradish peroxidase-dependent oxidation of homovanillic acid. Peripheral blood mononuclear cells were obtained from blood buffy coats of healthy donors (8) and were plated. Inclusion/exclusion criteria not mentioned.	Results Northern blot analysis demonstrated that P2 protein selectively induced in monocytes and macrophages mRNA accumulation of tumor necrosis factor (TNF), interleukin 1 beta (IL-1 beta), and interleukin 8 (IL-8) in a time-dependent manner. Natural killer stimulating factor (IL-12) mRNA and protein secretion was strongly induced by lipopolysaccharide but not by P2 protein. Supernatants harvested from P2-stimulated monocytes contained significant amounts of TNF, IL-1 beta, and IL-8, whereas those from macrophages contained only TNF and IL-8. The effect of the P2 protein on TNF and IL-8 mRNA accumulation and secretion was not affected by polymyxin B, which, on the other hand, almost completely abolished the effect of lipopolysaccharide. Finally, P2 protein did not directly trigger hydrogen peroxide release but, through the induced release of TNF, potentiated monocyte respiratory burst capability.	**Discussion/comments** This patient series study was performed with a small selection of people. It included 8 healthy participants. Prior information and characteristics about the patients were not presented. Strong sides of the study: A solid methodology was compiled in this study. It makes a foundation for future research projects in this field. Weak sides of the study: The study has very few participants, and follows the volunteers for a short period of time. Information about health characteristics was not mentioned. The study is exploratory, and therefore it has few articles to support its findings. Inclusion/exclusion criteria were not mentioned.

Reference:	Design: Case-control	
Yum HY, Cho JY, Miller M, Broide DH. Allergen-induced coexpression of bFGF and TGF-β1 by macrophages in a mouse model of airway remodeling: bFGF induces macrophage TGF-β1 expression in vitro. Int Arch Allergy Immunol. 2011;155(1):12-22.	Documentation level	C
	Grade:	IV

Purpose	Material and method	Results	Discussion/comments
Use a mouse model of chronic ovalbumin (OVA) allergen-induced airway remodeling to determine whether bFGF and fibroblast growth factor receptor-1 are expressed and regulated by corticosteroids in the airway, as well as to determine whether bFGF mediates expression of another proremodeling cytokine, transforming growth factor (TGF)-β1. **Conclusion** Overall, this study demonstrates that allergen challenge stimulates peribronchial macrophages to coexpress bFGF and TGF-β1 and that bFGF may potentiate macrophage release of TGF-β1 through autocrine and/or paracrine pathways. **Country** USA **Year** 2011	The methods used in this project were ELISA, immunohistology and image analysis. The airway levels and localization of bFGF, FGF receptor-1 and TGF-β1 were determined by ELISA, immunohistology and image analysis in the remodeled airways of chronic OVA-challenged mice treated with either corticosteroids or diluent. In vitro cultures of bone narrow-derived macrophages were used to determine whether bFGF induced TGF-β1 expression. 16 mice were used as case objects and 16 mice were used as controls and enrolled in the study. The mice were age-and - sex-matched. Inclusion/exclusion criteria, not mentioned.	Mice chronically challenged with OVA developed significant airway remodeling that was associated with significantly increased levels of bFGF and TGF-β1. Immunohistochemistry demonstrated significantly increased bFGF and FGF receptor-1 expression by peri- bronchial F4/80+ cells. Double-label immunofluorescence microscopy studies demonstrated that peribronchial macrophages coexpressed bFGF and TGF-β1. In vitro studies demonstrated that incubation of bone marrow-derived macrophages with bFGF induced expression of TGF-β1. Mice treated with corticosteroids and subjected to chronic OVA challenge had significantly reduced levels of bFGF, FGF receptor-1, peribronchial TGF-β1+ cells and airway remodeling.	This is a case-control study performed one mice. A group of healthy mice were age- and sex-matched with 8-10 weeks old mice who were immunized with ovalbumin for a period of 3 months. The control group was sensitized, but not challenged with ovalbumin. Strong sides of the study: This study is exploratory, and that gives a foundation for future projects in this area. They have enrolled a case and a control group. The methodology was thoroughly explained, and possible to replicate. Weak sides of the study: This is an animal study, and therefore not possible to apply for humans at this stage. Also, there were few models enrolled, and therefore the results might not be generalizable. They have few articles to support their findings. Inclusion/exclusion criteria were not mentioned.