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Leukocyte Interactions with calcium phosphate biomaterials in vitro

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Dedications:

I dedicate this work to my beloved parents who have the credited to help me achieve the goals. I also want to dedicate the thesis to my girlfriend and all my other family members and friends, who provides me with so much support.

"A journey of a thousand miles, begins at one's feet."
Lao Tzu

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List of Abbreviations:

PMA: Phorbol 12-Myristate 13-Acetate ($C_{36}H_{56}O_8$)

PBC: Peripheral Blood Cells

RBC: Red Blood cells

WBC: White Blood Cells

Hb: Hemoglobin

HSC: Hematopoietic Stem cells

DCs: Dendritic Cells

NK Cells: Natural Killer Cells

MMIF: Macrophage Migration Inhibitory Factor

IL: Interleukin

IgE: Immunoglobin E

T cells: T lymphocytes

B cells: B lymphocytes

CAR-T: Chimeric Antigen Receptor T Cell

CAR-NK: Chimeric Antigen Receptor Natural Killer Cell

CAR-M: Chimeric Antigen Receptor Macrophages

PBMCs: Peripheral Blood Mononuclear Cells

NLRP: Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing

PAMPs: Pathogen-Associated Molecular Patterns

DAMPs: Damage-Associated Molecular Patterns

ILCs: Innate Lymphoid Cells

MCSF: Macrophage Colony Stimulating Factor

RANKL: Receptor Activator of Nuclear Factor kappa-B Ligand

HA: Hydroxyapatite

ECM: Extracellular Matrix

GVHD: Graft Versus Host Disease

HLA: Human Leukocytes Antigens

FFP: Fresh Frozen Plasma

HSCT: Hematopoietic Stem Cell Transplantation

DCPA: Dicalcium Phosphate Anhydrous

MTT Assay: (3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) assay

IL-1 Beta ELISA: Interleukin-1 Beta Enzyme Linked Immunosorbent assay.

HBSS: Hank Balance Salt Solutions

PBS: Phosphate Buffered Saline

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Abstract:

When biomaterials implants or coatings are implanted the initial contact and route for initial potential cell colonization is with the peripheral blood. The objective of this study was to determine whether peripheral blood leukocytes could attach to calcium phosphate materials that are used to repair bone. The initial stage of the work was to develop suitable techniques and parameters for creating reproducible leukocytes cultures on calcium phosphate materials. This enabled the study of leukocyte colonization with biomaterial in vitro. The next phase was to investigate the differences between the human THP-1 lineage cell lines and primary rat leukocyte interactions with calcium phosphate materials. Rat leukocyte separation from the peripheral blood using density centrifugation was challenging, and obtaining reproducible data from their culture was very time consuming. It is decided to switch to human THP-1 cell lines to save time. Rat leukocytes, human THP-1 monocytes and its PMA-derived macrophages were able to attach to the calcium phosphate materials rapidly and proliferate on the materials over the period of days. MTT assay and ELISA assay for IL-1 beta and estimation of cell count and cell size using Image J were used to characterise cultures. Qualitative data was collected using a live-dead assay, H&E staining, immunohistochemistry, MGG and trypan blue staining. Although technically challenging dies to practical difficulties such as observing cells on opaque calcium phosphate granules, autofluorescence of the granules, and inferring cell behaviour on a 3D material, difficulties handling small granules and a limitation on the volume of rat blood it was possible to generate reproducible 3D cell culture with rat leukocytes, human THP-1 monocytes and THP-1 derived macrophages on calcium phosphate granules. Both HA and monetite granules could support rapid leukocyte attachment. It appeared that monetite and HA biomaterials enhanced leukocyte proliferation. MTT measurements indicated higher cell numbers than compared to cells only on tissue culture plastic. Rat leukocytes and human monocytes proliferated most on monetite and human macrophages proliferate most on HA granules.

Resumé:

Lorsque des implants ou des revêtements de biomatériaux sont insérés, le premier contact et la voie potentielle de colonisation cellulaire se produisent avec le sang périphérique. L'objectif de cette étude était de déterminer si les leucocytes du sang périphérique pouvaient se fixer aux matériaux de phosphate de calcium utilisés pour la réparation osseuse. La première étape de ce travail consistait à développer des techniques et des paramètres appropriés pour établir des cultures reproductibles de leucocytes sur les matériaux de phosphate de calcium, ce qui a permis d'étudier la colonisation des leucocytes par les biomatériaux in vitro.La phase suivante consistait à étudier les différences entre les lignées cellulaires humaines THP-1 et les interactions des leucocytes primaires de rat avec les matériaux de phosphate de calcium. La séparation des leucocytes de rat à partir du sang périphérique en utilisant la centrifugation de densité s'est avérée difficile, et obtenir des données reproductibles à partir de leurs cultures a été très chronophage. Par conséquent, la décision a été prise de passer principalement aux lignées cellulaires humaines THP-1 afin de gagner du temps.Les leucocytes de rat, les monocytes humains THP-1 et les macrophages dérivés du PMA ont rapidement adhéré aux matériaux de phosphate de calcium et se sont multipliés sur ces matériaux pendant plusieurs jours. Pour caractériser les cultures, des tests MTT et des tests ELISA pour l'IL-1 bêta ont été réalisés, ainsi que des estimations du nombre de cellules et de la taille des cellules à l'aide de Image J. Des données qualitatives ont également été collectées grâce à des tests de viabilité, des colorations H&E, de l'immunohistochimie, des colorations MGG et du bleu trypan.Malgré les défis techniques, tels que l'observation de cellules sur des granules opaques de phosphate de calcium, l'autofluorescence des granules, l'inférence du comportement cellulaire sur un matériau en 3D, la manipulation de petits granules et la limitation du volume de sang de rat, il a été possible de générer de manière reproductible une culture cellulaire en 3D avec des leucocytes de rat, des monocytes humains THP-1 et des macrophages dérivés de THP-1 sur des granules de phosphate de calcium.

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Introduction:

Peripheral blood cells (PBC) are easily harvested and the wide variety of circulating cells within it make it a very attractive source of cells for therapies and diagnosis.[165] Any blood cell with a round nucleus is referred to as the peripheral blood mononuclear cells (PBMC), which is isolated from the peripheral blood.[164] Recent advances in apheresis and liquid biopsy techniques have further boosted in developing beneficial technologies based around manipulation of PBMC. [166] It is possible that peripheral blood leukocytes are the initial source of cell colonization on biomaterial implants. Despite this, in vitro culture methods remain relatively unchanged for some decades. Many leukocytes are formed in the marrow, an extraordinarily complex intricately structured tissue that renews peripheral blood cells that is protected by being located in the medullary cavities of bone. However, it is become appreciated that quite crude 3D culture systems can provide at least some of microenvironmental cues to create systems capable of undergoing hematopoiesis in vitro. [167]However, there are numerous challenges associated with generating reproducible in vitro 3D microenvironment cell cultures. My hypothesis is to investigate whether peripheral blood white blood cells could attach to, proliferate, and differentiate when co-culture with calcium phosphate granules in 3D microenvironment in vitro. Calcium phosphate biomaterial is the source of instant cells colonization after implantation Furthermore, I am going to compare rat and human leukocytes interactions with calcium phosphate materials to indicate if work with rodents is predictive of the clinical scenario. I will mainly be using two types of calcium phosphate materials and see if there is any difference among the materials interactions with leukocytes. First is monetite (500-1000nm size monetite granules) phenol-hydroxy (500-1000nm size HA granules). The third type of material: pyrite Phosphate (100nm size new granules) is also used in part of the experiment. My research is first to build the ideal reproducible culture parameter for rat leukocytes collected from the peripheral blood using Ficoll density separation protocol. The next step is to investigate if there is a rapid colonization on monetite and HA granules. Characterization and identification of rat leukocytes will be conducted using different stains as well as immunohistochemistry. If rat experiments show promising findings, human THP-1 monocytes cells and THP-1 monocytes derived macrophages will be used to confirm with the findings. Quantification of rat and human leukocytes will be conducted using MTT and ELISA assay, as well as cell count and cell size estimations using Image J. The objective is to see if there is any attachment, proliferation, resorption, and differentiation of human leukocytes on calcium phosphate materials. It is known

that a single hematopoietic stem cell (HSC) can generate the cell types necessary to reproduce ablated marrow.[168] But it is commonly observed yet overlooked that when calcium phosphates are implanted ectopically, marrow genesis can occur with full recapitulation of marrow function.[169] This incidental finding that has largely been overlooked during attempts to generate mechanically functional bone tissue is extremely important because it shows a possible role of calcium phosphates in providing cues for recapitulating boney microenvironments that can allow hematopoiesis. If we observe specificity of cell types that attach and proliferate the potential implication are far reaching, at one level this work can be used to efficiently expand hematopoietic cells, and more impactfully can be used to explore pathological conditions such as osteoporosis caused by inadequate switching from M1 to M2 macrophages, delayed bone and wound as well as pathological inflammatory responses and cancer treatments.

Chapter 1: Peripheral Blood Cells:

1.1 Red Blood Cells

Erythrocytes:

Peripheral blood is the most frequently used system in research and therapy development due to its highly dynamic cell composition and easily accessible collection methods of its WBC population. Peripheral blood cells (PBC) are widely used in all fields of health research in many fields of health research such as basic immunology research, molecular biomarkers, genetic screening, physiological research and many more. Products derived from peripheral blood such as Leukopak, cryopreserved Leukopaks, PBMCs, T cells, antigen-specific T cells, CD56+ NK cells, Monocytes, Dendritic cells and B cells are essential to use in many fields of treatments and medical research.[127] PBC are blood cells that circulate throughout the body which include RBCs, WBCs including peripheral lymphocytes and platelets, suspended in plasma. Commonly, blood is obtained from the peripheral veins and stored in bottles or tubes treated with anticoagulant agent such as heparin for further analysis or to use for whole blood transfer, especially for someone who lost a great portion of their blood because of traumatic events. The blood to anticoagulant ratio should be filled to completion to ensure 9:1 blood-to-anticoagulant ratio and avoid blood clotting, which could impact the quality of the blood test or cell culture.[2]There are three main categories cellular components: erythrocytes known as red blood cells, leukocytes known as white blood cells and thrombocytes known as platelets. Peripheral blood also contains plasma, which is the liquid

component of the peripheral blood that acts as the vehicle that transports PBCs to circulate throughout the body to perform different actions.

PBCs all have a common progenitor, hematopoietic stem cells (HSC), immature cells that possess the ability to give rise to multiples blood cell linages (Figure 1.1). HSC are found in both peripheral blood and the bone marrow. RBC is the most common type of blood cells and continuously produced by erythroblast, a derivative from HSC. Typically, human RBCs have a biconcave shape and the diameter is about 7 to 8 µm and thickness is around 2.5µm, and normal RBCs count range from male 4.7 to 6.1 million cells per microliter, and females range from 4.2 to 5.4 million cells per microliter.[4] In the human, about 1% of RBCs are renewed daily in order to carry normal O_2 and CO_2 exchange functions between lungs and tissues. RBCs turnover result from an average circulatory lifespan of about 120 days.[6] RBCs biconcave shape gives RBCs unique ability for repeated large deformation to squeeze through blood vessels as small as 2-3 µm to its locations and carry its functions without any damage.[5] Its volume compared to the total blood volume refers to the term hematocrit. The normal hematocrit for men should be ranged from 40 to 54% and 36 to 48% for women.[6]

Hemoglobin:

RBCs contain hemoglobin and are covered with a membrane composed of proteins and lipids. Hemoglobin, which carries oxygen from the lung to other parts of the body and takes carbon dioxide back to the lung to be exchanged by inhalation. An adequate number of healthy RBCs is important for maintaining bone health and ensuring proper bone healing.[12] Low production of RBCs, abnormal size and life span of RBCs, various cancers and cancer treatments, low iron levels in blood, and much more can lead to anemia, where patients experience fast heartbeat, fast breathing rate, shortness of breath and chest pain.[9] Anemia patients are associated with reduced cortical bone density.[13] that leads to an increasing chance of bone fractures as well as poor wound and bone healing.[11] Increased risks for hip fracture have been linked to both high and low Hemoglobin levels.[13] Hb is a tetramer protein typically consists of 4 protein chains.[13]Hemoglobin A, which consists of 2 alpha and 2 beta polypeptide chains is the majority types of healthy hemoglobin. Hb A2, and Hb F, accounts for the other two types and a combined of 5% of normal hemoglobin. HbC is an abnormal form of HbC associated with hemolytic anemia and typically caused by a point mutation in the HBB gene.[14] Hb's unique structures give them high affinity for

oxygen and a low affinity for carbon dioxide in arterial circulation. Reversely in venous circulation, hemoglobin has a high affinity for carbon dioxide and other wasteful ions, and a low affinity for oxygen.[13] In order to keep adequate tissue oxygenation, a certain level of hemoglobin must be maintained. The normal Hb level for males is 14 to 18g/dl, and 12 to 16g/dl for females. (Henny H et al). An abnormal Hb level can be caused by serious clinical conditions such as Lymphoma, Leukemia, Anemia, multiple myeloma. [8] Hemoglobin synthesis are a biochemical synthesis of globin production and heme synthesis. Heme synthesis occurs in RBC's cytosol as well as mitochondria. It starts with glycine and succinyl coenzyme A and ends with the production of protoporphyrin IX ring.[12] The last step of heme synthesis is the binding of the protoporphyrin to a $Fe2+$ ion resulting in the heme molecules. Cytosol of RBCs is mainly responsible for the production of globin chain by genetic transcription and translation. A mutation in chromosomes 16 and 11 can lead to abnormal globin chain production that leads to anemia and ultimately results in patient's prolonged wound and bone healing.

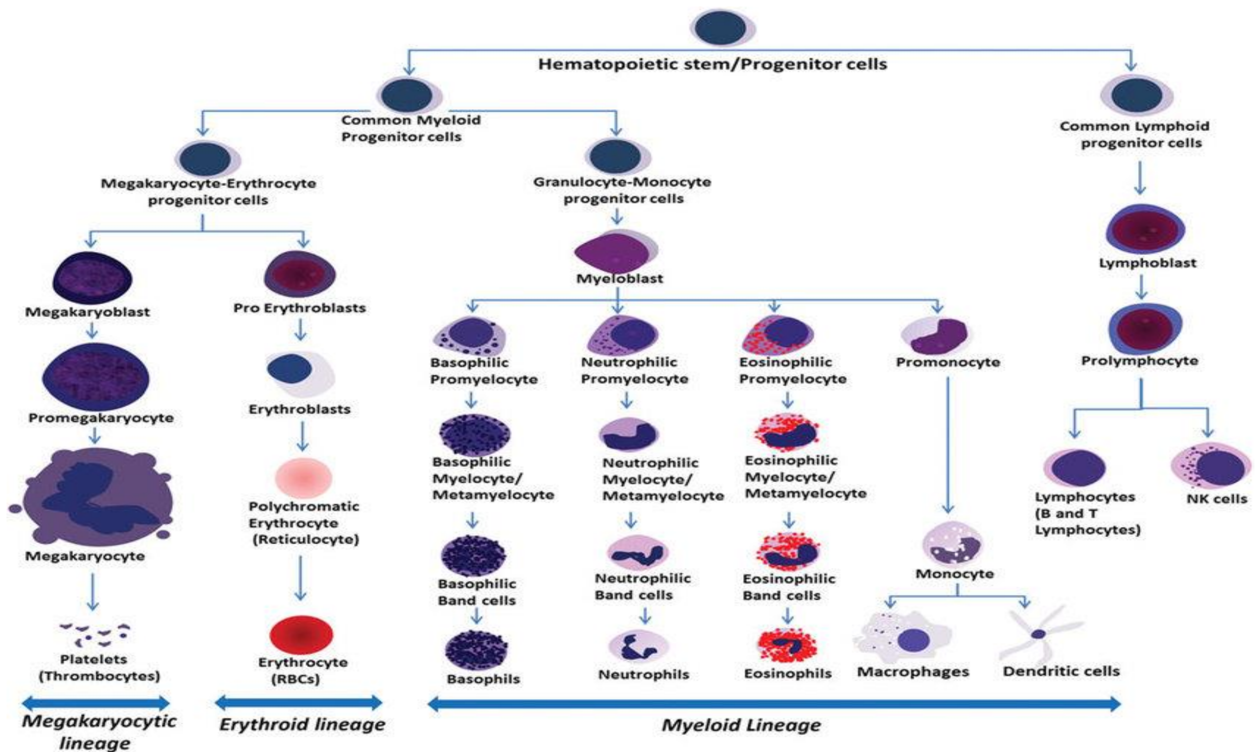

Figure 1.1: Hematopoietic Stem Cells lineages, reproduced from ResearchGate with permission [158]

Macrophage Migration Inhibition Factor

There is mounting evidence that RBCs are much more important for immune function than previously through. The majority of macrophage migration inhibitor factor in the whole blood is stored in RBCs. [10] MIF is a human protein encoded by MIF genes. MIF cytokines is a proinflammatory cytokines that are the major mediators of the innate immune response. In recent times, MIF has been identified as a multipotent cytokine secreted by many cell types in immune response. [15] It works with the cell membrane receptor CD74, which is a type II transmembrane protein. It circulates in the blood stream and upon the stimulation by bacterial antigen, MIF binds to CD74 to trigger immune response. CD74 stimulation in multiple recent studies have shown an improvement in protecting against injury and tissue repairing in multiple parts of the body such as hyperoxic lung injury, sciatic nerve injury and more. [16,17,18] MIF-CD74 signaling pathways activates a pathway to increase survival and proliferation of certain cell types to protect its host during injury. Not only MIF exists in human, but MIF is also expressed ubiquitously in almost all mammalian cells and is vital for numerous biological processes in all species. [19]

RBC Reactions with Biomaterials

Hemocompatibility refers to the blood compatibility with different biomaterials. Blood contacting medical devices and implants are widely used in the medical field to treat different diseases. There are Five test categories associated with the hemocompatibility evaluation in vivo. They are thrombosis, coagulation, platelets, hematology and immunology test on complements and leukocytes. [20]Even until today, thrombogenicity, refers to the tendency of a material to generate blood clotting or thrombus, is still a major problem in cardiovascular implants due to insufficient hemocompatibility. [21]Multiple bioengineering efforts are still needed to change surface properties of the medical implants in order to increase hemocompatibility to reduce the potential side effects of medical implants. Carbon-based coating materials have shown promising results in their augmented ability to suppress thrombo-inflammatory reactions in the fields of biomedical implants.[22] Functional covalent coating also shows an enhanced biocompatibility and hemocompatibility of blood contacting biomaterials.[23] There are numerous biomaterials that can perform and augment the natural functions of the body and biological system. It is still a wide area of research interest to engineer and to modify the new or pre-exist biomaterials to increase the

hemocompatibility and biocompatibility in order to reduce the potential side effects of medical implants.

1.2: Leukocytes

The immune system's function is to protect the host from a wide variety of pathogens, eliminate toxic and oncogenic substances as well as allergenic particles. Another important aspect to immune reactivity is self and non self-discrimination. The immune system is typically divided into innate and adaptive mechanisms to detect and eliminate harmful microbes and to keep a record of the microbes, so the immune system can recognize and react faster when they encounter them again. The immune system also participates in three sequential phases of fracture healing, which the fracture healing process begins with a local inflammatory response, which progresses to the remodeling and repair phases. [132] The bone fracture healing is under the control of immune system. The innate immune response is the first line of defense against invading pathogens and is fast acting with no immunological memory. Adaptive immunity is antigen dependent and antigen-specific and has the memory capacity to ensure host's next immune response to have more rapid and efficient outcomes. [58] Connections and crosstalk between the two types of immune reactions are keys to provide better protections to the host. Defects in either system can provoke diseases like insufficient inflammation, autoimmune disease, immunodeficiency disorders and hyper-sensitivity reactions. [59]

Leukocytes, or white blood cells, circulate in the peripheral blood and participate in both innate and adaptive immune responses. Typically, Leukocytes are classified as granulocytes or agranulocytes depending on the presence of granular structures in their cytoplasm. [24]As indicted in Figure 1.1, leukocytes differentiated from the hemopoietic stem cells presented in bone marrow and peripheral blood following the myeloid cell linage and lymphoid progenitor cells linage. Normal human WBC count should range between 4,000 to 10,000 per microliter[25]. Abnormal WBC count can be an indication of different diseases, inflammations, or infections. Peripheral blood mononuclear cells are cells isolated from peripheral blood and identified as blood cell with round nucleus. PBMCs include lymphocytes: T cells, B cells and NK cells, monocytes, and dendritic cells. Typically, in human, lymphocytes account for 70 to 90%, monocytes account for 10 to 20%, while dendritic cells are rare and only account for 1 to 2% of PBMCs. [60] Neutrophiles, basophils, eosinophils and RBCs are removed from the whole blood by density gradient

centrifugations and will be the main method used to separate PBMCs from the whole blood in the experiments.

Neutrophils

Neutrophils are the most abundant type of WBC, and account for more than half of the WBCs. The neutrophil is a single multilobed nucleus, and range from 12 to 14 µm in cell diameter.[27] Its main function is as a first responder in the immune system to foreign invaders such as bacteria or virus. They are short lived, motile, and phagocytic, typically act in a self-destructive manner to destroy the damaged tissue, infected cells then later removed by macrophages. Upon activations, neutrophils produce substantial amounts of superoxide anion through NADPH oxidase enzyme and reactive oxygen species to kill phagocytized microbes.[61] Abnormal generation of intracellular reactive oxygen species are associated with more susceptibility to infections. While doing so, neutrophiles also send a signal to the body to further modulate the adaptative immune response, critically for B cell maturation and survival. Neutrophils has a complex signaling pathways including G-protein coupled receptors, Fc-receptors, adhesion receptors, cytokine receptors, innate immune receptors. [28]NETosis is a process that results in the development of neutrophil extracellular traps, which are made of chromatin that has been modified and embellished with bactericidal proteins from granules and cytoplasm. In NETosis, the granule components are released into the cytosol, the nuclear envelope is destroyed, histone modifications result in chromatin de-condensation, and plasma membrane pores are formed.[133] Defects in neutrophils lead to cancer-associated neutrophilia and tumor infiltration of neutrophiles are important marker for poor cancer treatment prognosis. [29]

Eosinophils

Eosinophils range from 12 to 17 µm in diameter, are larger than neutrophils, and typically have two lobes in their nucleus. [27]As shown in Figure 1.2, they account for less than 5% of the WBCs, and act in phagocytotic manner. Human eosinophils phagocytose opsonized zymosan, which causes a dose-dependent noncytotoxic release of histaminase, arylsulfatase, and beta-glucuronidase. [134] Eosinophils are involved in combating parasitic, bacterial, viral infections and certain cancers. However, they can also have pathologic roles in diseases including asthma, eosinophilic autoimmune gastrointestinal disorders, autoimmune myocarditis, and responsible for

causing allergic reactions. [30] They can mistakenly recognize something harmless as a foreign invader and incorrectly trigger immune reactions that causes allergy. Eosinophils are equipped with receptors such as heterodimeric receptor for IL-5 and IL-3, chemokine receptor 3, chemokine receptor 1, lipid mediator receptor, pattern recognition receptors, Fc Receptors, Major histocompatibility complex-II, and adhesion receptors that regulates them and allow them to participate in inflammatory reactions. [31]

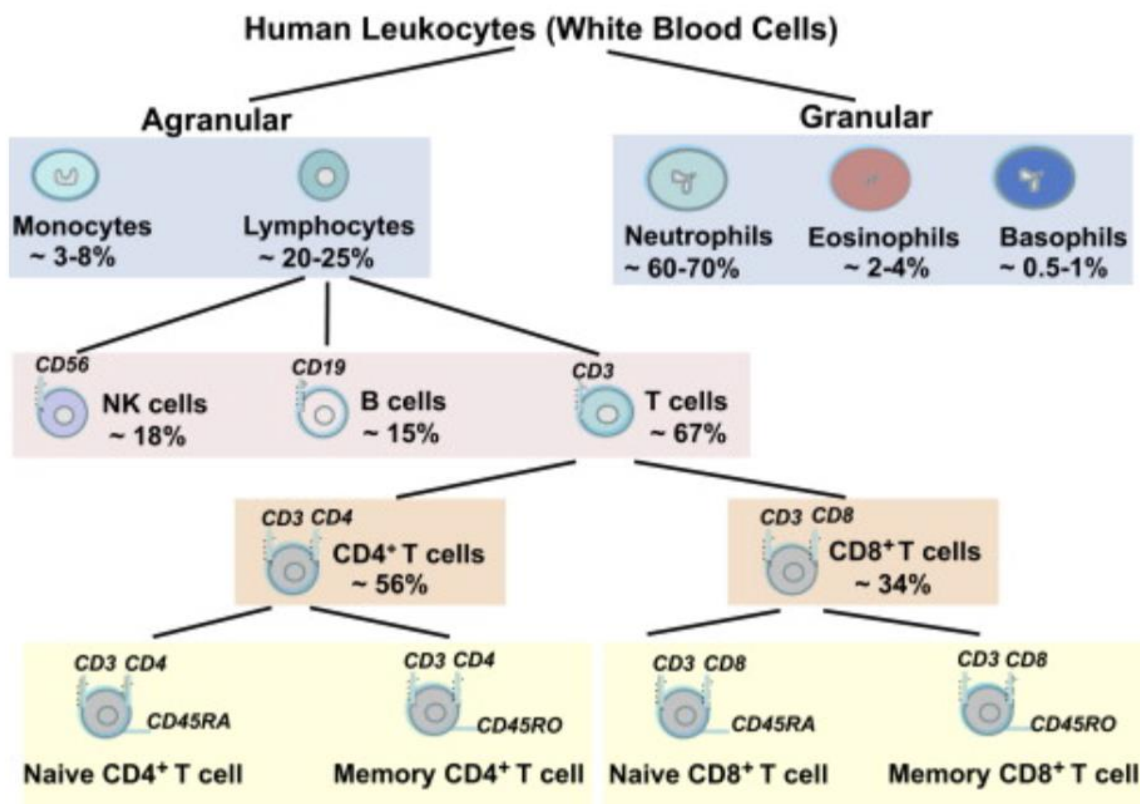

Figure 1.2: Percentage composition of human leukocytes reproduced from with Springer Link with permission[134]

Basophils

Basophils are one of the least common types of WBCs, 14 to 16 μm in diameter.[27] Basophils are mainly involved in immune response to parasites, blood clotting, allergens, and foreign pathogens. They act in phagocytotic fashion, and attack organisms unfamiliar to the body.[32] Basophils acts in allergic reactions by releasing histamine and heparin, where histamine acts to dilate the blood vessels and improve blood flow to allow other immune cells to reach the destination, heparin prevents blood clotting. In addition, basophils have high-affinity IgE receptors on their surface

that binds to IgE antibody to trigger an allergen-specific immune response. IgE antibodies bind to IgE receptor to sensitize basophils to get triggered and promote the release of inflammatory mediators. [33]

Monocytes

Monocytes are the largest and third most common type of WBCs, which can be up to 20µm in diameter with large eccentrically placed kidney bean shaped nucleus and account for up to 10% of WBCs. [27] Monocytes are precursors of macrophages, dendritic cells, and osteoclasts. They are subdivided into classical, intermediate, and non-classical subsets based on their surface expression of CD14 and CD16. Classical monocytes are mainly found to possess the ability to phagocytose, participate in innate immune responses and migrate. Intermediate monocytes express CCR5 and are responsible for antigen presentation, secretion of cytokines, regulation of apoptosis and differentiation into other cell types, non-classical monocytes are involved in complement, Fc gamma mediated phagocytosis and adhesion. [36,37]. A high number of monocytes in PBC can indicate chronic infections, blood disorders, certain cancers, and autoimmune diseases, while a dwindling number of monocytes is a result of bone marrow disorders, chemotherapy and bloodstream infections and disorders. [38]

Macrophages

Macrophages are specialised phagocytic cells derived from bone marrow and monocytes in the peripheral blood. [39] Recent studies have reviewed that despite belonging to the same mononuclear phagocyte system, monocytes, DCs, and macrophages have distinct origins.[44] They typically differentiate and mature in different tissues to exhibit different actions. They are mainly responsible for detection, phagocytosis, and destruction of bacteria and other harmful organisms to ensure the maintenance of tissue homeostasis and tissue regeneration. They can also present antigens to T-cells and initiate inflammatory reaction and active other cells by release various cytokines. [40] Macrophages are divided into M0, M1 and M2, where M0 macrophage are undifferentiated macrophages with the capacity to polarize into distinct macrophages subtypes; M1 macrophages are able to detect and destroy pathogens through phagocytosis and promote inflammation; M2 macrophages are needed for regeneration of connective tissues by producing vascular endothelial growth factor and transforming growth factor, as well as secret digestive enzymes and growth factors to promote wound healing and regenerations. [42,43] The mechanism of

phagocytosis is demonstrated in Figure 1.3, as receptors on the cell surface bind increased ligand molecules, the cell membrane progressively engulfs the target, such as pathogen, dead cells, or bead. Upon full engulfment, a phagosome is formed, which fuses with lysosomes, resulting in the digestion of the target. The abnormal macrophage activation or dysfunctional phagocytic activity in macrophages can lead to several diseases such as acute respiratory distress syndrome, atherosclerosis, HIV related disorders, and chronic inflammations. [41]

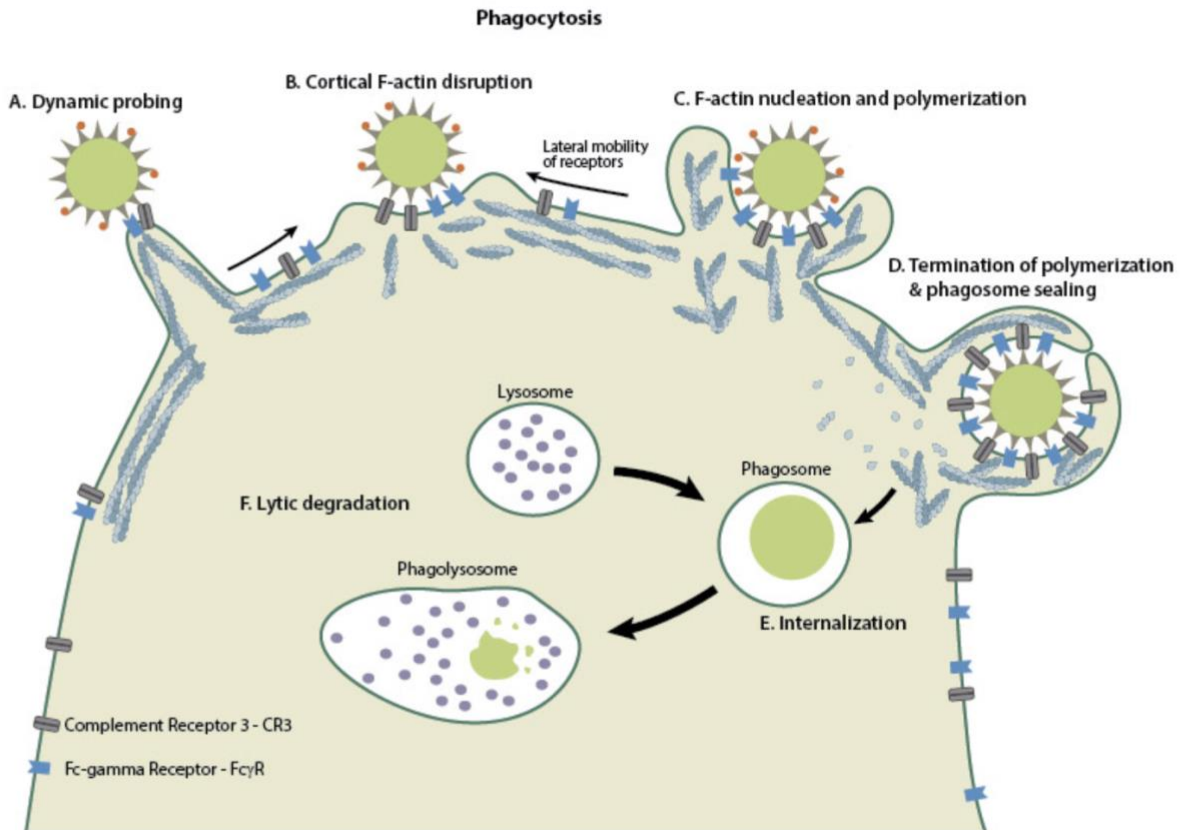

Figure 1.3: The illustration of phagocytosis reproduced from with MBinfo with permission [136]

Dendritic Cells

Dendritic cells connect the innate and adaptive immunity. It is becoming more recognized that DCs originate from progenitors in the bone marrow through hematopoiesis in an independent way distinct from monocytes or macrophages. [44] DCs are traditionally divided into conventional and non-conventional. Conventional DCs are ready to exert their function, normally divide into migratory and lymphoid DCs, whereas non-conventional DCs are only arise in response to certain inflammatory stimuli. DCs are considered antigen presenting cells and essential mediators of the immunity and tolerance. [46]

Osteoclasts

Osteoclasts are large multinucleated cells that possess the ability to degrade and resorb bone in order to start bone remodeling and mediate bone loss in pathologic conditions due to remodeling of extracellular matrix.[47] Bone is consistently being broken down and restructured in response to structural stress or bone injury. Osteoclasts are derived from the circulating monocytes-macrophage linage in the blood, which is in turn derived from the bone marrow. Osteoclasts create these microscopic trenches on the surface of bone trabeculae in the spongy bone seen at the ends of long bones and inside vertebrae by secreting hydrochloric acid and proteases, such as cathepsin K, into an extracellular lysosomal compartment underneath a ruffled part of their basal cell membrane to simultaneously dissolve the mineral and matrix components of bone. It is followed by osteoblasts recruited to the site and mineralize it. [48,137]Osteoclast precursor differentiation is influenced by osteoblasts via RANKL/RANK and ephrinB2/EphB4 signaling pathways. Recent studies have also demonstrated that osteoclasts regulate the differentiation of osteoblast precursors and the movement of hematopoietic stem cells from the bone marrow to the blood stream. [138] Osteoclasts around bone and osteoclast precursors exert other functions as well, such as participating in immune responses and secrete cytokines that can affect their own functions and those of other cells in inflammatory and malignant bone processes. In additionally, they play a role in osteoimmunology and pathologically increased bone resorption. [74,138]

T-Lymphocytes

Lymphocytes are a type of immune WBCs and made in the bone marrow that is mainly found in the peripheral blood and lymphoid tissues. It constitutes of two types of cells, T lymphocytes (T cells) and B lymphocytes (B cells) that works together to help immune system fight against cancer and foreign antigens.[49] T cells are mainly subdivided into natural killer $CD8^+$ T cells, $CD4^+$ T helper cells, and T regulatory cells, and some minor types of T cells. Killer T-cells. [50] Natural Killer T cells are activated by professional antigen presenting cells such as dendritic cells, then migrates into the circulation to execute their targeted function. Helper T cells are activated by professional antigen presenting cells as well such as dendritic cells to produce cytokines to initiate immune reactions from other WBCs. Regulatory T cells produce various inhibitory cytokines and function to end the immune response and inhibit autoimmune processes and possess ability to accelerate wound healing and battle cancer. [51]

B Lymphocytes

B Lymphocytes are part of the immune system exist in various places of the body that works by produce antibodies. They are two types of B cells; plasma cells are short life span effector cells mainly responsible for releasing antibodies in response to certain antigens. Secondly, memory B cells remember particular antigen and start an immune response in a rapid fashion when making contact with these antigens in the future and play a significant in vaccinations. Abnormal B-cells development and functions can result in autoimmune disease such as type 1 diabetes, multiple sclerosis, and rheumatoid arthritis, as well as causing cancers such as acute and chronic lymphocytic leukemia, several types of lymphoma and myeloma. [57] Bed sores known as pressure ulcers, was classically thinking of tissue ischemia induced by prolonged pressure from lying in bed, sitting in a wheelchair, or wearing a cast for prolonged time. Healing process of bedsores can take up to years and can be very costly once they deepen from skins into tissues and bones. [120] In recent studies have shown that compression prevents lymph fluid drainage, which causes increased interstitial fluid and waster build up and contributes to develop of pressure ulcers, in addition to localized ischemia and reperfusion injury to tissues, impaired lymphatic drainage has been shown to contribute to bedsores as well. [119]

Natural Killer Cells

Natural killer Cells are lymphocytes that share a common progenitor with T and B cells and belong to the same family. NK cells respond quickly to the development of tumors as well as intracellular pathogens after infection and on virus-infected cells. In additions, NK cells are best known for destroying virally infected cells and spotting and managing early cancer signs.[139] NK cells can identify stressed or infected cells and kill them in the absence of antibodies and major histocompatibility complex, triggering an immune response much more quickly.

Therapeutic Applications of leukocytes

Chimeric antigen receptor (CAR) is a special engineered receptor created in the laboratory that is meant to bind to certain proteins on certain cells. CAR T-cell immunotherapy has shown impressive result in treating cancers, and six CAR T-cells therapies has been approved by US Food and Drug Administration to treat B-cells lymphomas, different forms of leukemia and multiple myeloma. CAR T-cell treatment works by removing patient’s T-cells and taken to manufactured

in the lab by inserting a gene coded for artificial surface receptor proteins, chimeric immunoreceptors, also knowns as chimeric antigen receptor, to T-cells to induce them express on their surface. Specific proteins, or antigens, on the surface of cancer cells are recognized by the CARs and bound to them.[53] These engineered CAR-T cells are further expanded in cell culture then perfused back to patients. This engineering process aids in the T cells' ability to recognize and destroy cancer cells that express the particular protein that the receptor is made to bind. Although showing promise, CAR-T cells still faces a lot of limitations in currently clinically work such as severe life-threatening toxicities, remarkably-high cost, lack of anti-tumor activity, antigen escape and limited tumor infiltration. [52]

CAR-NK cells are easier to manufactured, have lower toxicity against lung cells and PBMCs[140], lower potentially fatal toxicity, lower cost compared to CAR-T cells treatment, lack of potential for causing graft-versus-host disease, and lower chance of acquiring cytokine release syndromes.[141] CAR-NKs can kill cancer cells through both CAR-dependent and independent pathways such as Fas/FasL pathways and TNF-α cytokine pathways. [54] Create CAR constructs to activate NK cells and control NK receptors to enhance the effectiveness of immunotherapy for cancer. [142]

CAR-M using similar techniques as CAR-T, but instead of T-lymphocytes they engineer CAR receptor proteins on macrophages. It has been an advancement to CAR T cell therapy to use to treat solid tumors. It has begun its first human trials, and work by engineering CAR- M cells to target proteins on cancer cells and penetrate solid tumors, ingest malignant tissue with their phagocytotic ability and stimulate innate and adaptive immunity to treat cancers. [55, 56]

Peripheral Blood Mononuclear Cells:

Peripheral blood mononuclear cells are cells isolated from peripheral blood and identified as blood cell with round nucleus. PBMCs include lymphocytes: T cells, B cells and NK cells, monocytes, and dendritic cells. Typically, in human, lymphocytes account for 70 to 90%, monocytes account for 10 to 20%, while dendritic cells are rare and only account for 1 to 2% of PBMCs. [60] Neutrophiles, basophils, eosinophils and RBCs are removed from the whole blood by density gradient centrifugations and will be the main method used to separate PBMCs from the whole blood in the experiments.

Inter-species variations:

Human, rat, and other mammals have many similarities in functions, identifications, and morphology between WBC. In rats, the normal WBC count range from 3,600 to 14,800 per microliter[26] and the difference between the human WBC and rats WBC are illustrated at Table 1. Mammalian leukocytes have five major categories, neutrophils, eosinophils, basophils, lymphocytes, and monocytes. They are also characterized by granulocytes including the first three and mononuclear cells with the other. [63] Neutrophils are still the dominant type of WBCs; however, many animal species do not have distinct lobe and filament arrangement like humans. Eosinophils granules of iguanas and some birds are pale blue compared to most animals' granules being orange. Horse Basophils are just like human filled with small, round, dark purple granules. Lymphocytes that circulate in healthy animals tend to be small and mature with round nuclei with small rim of light blue cytoplasm. Monocytes are the hardest to identify because their size is not consistent across distinct species. [63] However, they all serve the same function to mediate immune reactions and protect the host from different pathogens.

Table 1. Summary of difference between Human and rat leukocytes.

	Human	Rat
Count(/ml)	4,000 to 10,000 [25]	3,600 to 14,800[26]
Major categories	neutrophils, eosinophils, basophils, lymphocytes, and monocytes.	neutrophils, eosinophils, basophils, lymphocytes, and monocytes.
WBC component:	45 to 65% of neutrophils 1 to 4% of Eosinophils Basophil 1 to 4% Monocyte: 4 to 9% Lymphocytes: 20 to 45% [143]	35 to 71% neutrophil 0 to 4% of Eosinophils Basophil 1 to 4% Monocytes 0 to 4% Lymphocytes: 20 to 50% [143]

Size:	Neutrophil 9-15µm small granules abundant pink color Eosinophil 12 to 17µm granules acidophilic with abundant peroxidase and lysosomal enzymes Basophil 10 to 14 µm, cytoplasmic granules, bulky and disorderly distributed Monocytes 15 to 25 µm granules dense and homogenous cytoplasm Lymphocytes 6 to 15 µm, azurophil granules that are prominent. [144]	Neutrophil 10-12µm granules less dense than specific granules Eosinophil 12 to 15µm granules large round acidophilus Basophil 12 to 14µm small cytoplasmic granules, disorderly distributed Monocytes 12 to 15µm presented as reddest-purple or azurophilic granules Lymphocytes 6 to 10µm Azurophilic granules. [144]

Chapter 2 Leukocytes interactions

2.1 NLRPS activations

Nucleotide-binding Oligomerization Domain (Nod), Leucine rich Repeat and Pyrin domain containing Proteins (NLRP) are members of Nod-like receptor protein family. NLRP are critical in regulating inflammatory reactions and host defense against microbial pathogens mediated by WBCs. NLRP acts as a scaffold protein and aggregates other proteins to form inflammasome or engage transcriptional signaling cascade that results in the production of pro-inflammatory cytokines that regulates several types of WBCs.[65] NLRP proteins have 14 subfamily members and are characterized by the presence of an N-terminal Pyrin domain that allows the recruitment and formation of inflammasome. [64] NLRP3 is activated by PAMPs and DAMPs and subsequently activates caspase-1 that is able to proteolytically cleaves other proteins. This initiates the production and releasing of pro-inflammatory cytokines IL-1 beta and IL18 as shown in Figure 1.4. [66, 145] The most characterized NLRP subfamily type is NLRP3, and is known to be involved in immune response to toxins from the environment and regulate inflammation by mediating pyroptotic cell deaths. The NLRP3 inflammasome is produced by bone marrow derived macrophages, however, abnormalities in NLRP3 inflammasome plays key role in causing osteoarthritis, cancer, atrial fibrillation, Alzheimer's disease, and other diseases. [65] With the better understanding of this signaling pathway, drugs or therapies that target NLPR3 inhibitor to block

chronic inflammation has possess the ability to treat many diseases. For instance, MCC950 is a highly selective and small molecule inhibitor of NLRP3 that has been used as the target to treat Alzheimer's disease, and potentially treat Huntington's disease as well. [66]MNS, CY-09, OLT1177, Glyburide, JC124 and many other NLRP3 inhibitors could have become the therapeutic targets to treat patients with different syndromes. [65]

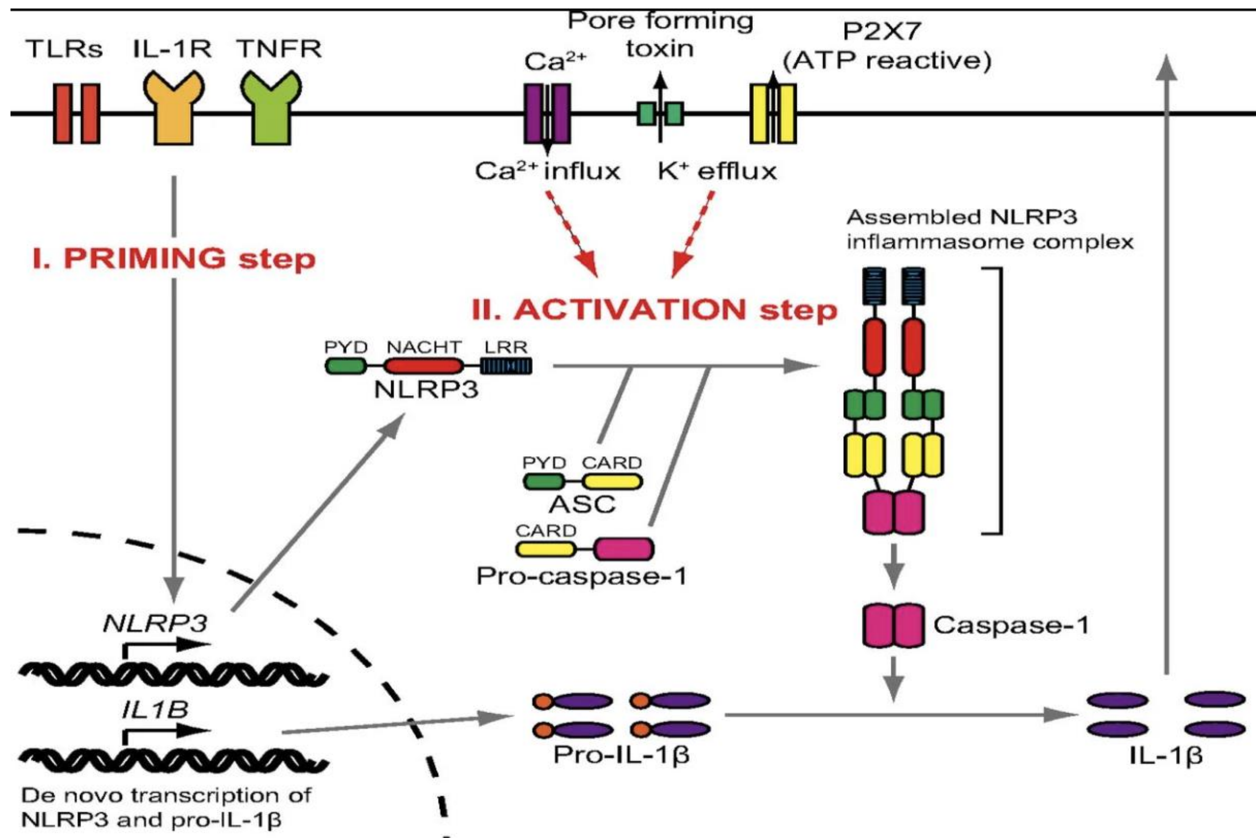

Figure 1.4: The production and activation of NLRP3 via IL-1 beta signaling pathways reproduced from ResearchGate with permission[163]

2.2 NLRPS activation by crystals

Crystalline substances can also induce NLPRs signaling pathways by stimulating caspase-1-activating NLRP3 inflammasome. Uric acid crystals connected to gout, silica crystals and aluminum salts can all activate NLPR3 inflammasome. [145,146] In addition, cholesterol crystals can also induce NLRP3 inflammasome in phagocytes *in vitro,* and when administered intraperitoneally *in vivo* cause acute inflammation, which is suppressed in mice lacking the NLRP3 inflammasome, cathepsin B, cathepsin L, or Il-1 molecules.[147] The potent NLRP is activated cholesterol crystals, which aid in the development of atherosclerotic lesions.

2.3 Pathological Calcification

Calcified plaques in the arteries are clinical markers for atherosclerosis, a chronic inflammatory vascular disease, and the amount of the calcified plaques is correlates to the severity of the cardiovascular disease. The presence of calcium is a sign of how much fatty build up is in the arteries, and plaques in the arteries are the main cause of heart attack and stroke. Calcified plaques can break off from the artery and form blood clot, which results in the blockade of the blood flow and the oxygen supply to your heart and the brain. [68] Inflammation mediated by WBCs play a major part in all phases of atherosclerosis. Stable calcified plaques are characterized by a chronic inflammatory state, whereas vulnerable plaques are characterized by an active inflammation. Evidence suggests that plaques in symptomatic atherosclerosis patients exhibit distinct $CD4^+$ T-cell subset and T cells to be activated and differentiated, whereas in the plaques from asymptomatic patients, there is a raising level of IL-1 beta signaling and activated T cells and macrophages, as well as many other types of WBCs contribute to the progression or immunoregulatory role of calcified plaques. [69] Using monoclonal antibodies against immune cells, proinflammatory cytokines, chemokines, regulatory proteins like PCSK9, a protein that regulate LDLR levels in immune and vascular cells, has shown to be considered to be potential targets for immunotherapy of atherosclerosis. [70]

2.4 Bone, Bioceramics

WBCs have also shown to play a pivot role in bone resorption, remodeling, and healing. Increased production of pro-inflammatory cytokines is associated with osteoclastic bone resorption.[71] In the context of bone injury, immune cells are recruited to the site of injury, and this inflammatory response is required to ensure normal bone healing through angiogenesis, repaired damaged tissue and remodeling. Table 1 has shown the functions of different WBCs in bone healing. Dysfunction and dysregulation of immune cells hinders bone resorption and rejuvenation. [72]Bone is consistently being resorbed and remodeled even under normal conditions to maintain bone health and ion homeostasis by osteoclasts. This process releases minerals and transfers calcium to the blood and is tightly regulated by immune system. [73]Macrophages are present throughout the bone fracture healing process and discharges complex effectors to regulate bone healing. Older adults suffer from weakened bone healing due to abnormal macrophage activations especially in patients with

M1/M2 macrophage ratios. [76] In pathological conditions, the WBCs play a major role in causing chronic inflammations that leads to conditions like rheumatoid arthritis and osteoporosis. [74,75]

Table 2. Summary of cellular constituents of peripheral blood and their main known function in bone formation and resorption.

Cell types	Role in modeling bone
Neutrophils	Neutrophils activates osteoclast bone resorption through RANKL of Toll-like receptor 4[77]
Eosinophils	Typically increased in patients with deficiency of vitamin D, involved in hyper IgE syndromes and pathological connections between allergies and osteoporosis. [78]
Monocytes	Provide signals that promote osteogenic differentiation of mesenchymal stem cells and serve as precursors as well as recruiters to osteoclasts, macrophages, and dendritic cells [79]
Macrophage	M1 macrophages thought to promote bone resorption, M2 promote bone formation, and M1/M2 ratio controlled by estrogen directly relate to bone homeostasis[76]
Dendritic cells	Immature DCs transdifferentiate into osteoclasts[80]
Mast cell	Release pro-inflammatory mediators such as histamine, IL-6 and TNF-alpha to promotes osteoclast productions. [81]
NK cell	Promotes osteoclast formation and osteoclastogenesis by producing RANKL and MCSF [82]
Innate Lymphoid Cells	Produce various cytokines such as RANKL, IL17 that are involved in bone disorders. [83]

Bioceramics are now widely used in dental and orthopedic applications to replace bone and enhance bone healing. [84] Tissue reactions to implant materials broadly classified depending on tissue reaction into Bioactive, bioinert, bioresorbable. Bioactive refers to that the biomaterials are designed to modulate or evoke biological activity; bioinert are the biomaterial that do not initiate a response or interact with other biological tissue; bioresorbable are the biomaterial that upon placement within the human body starts to being resorbed and slowly replaced by advancing tissues. [148] Hydroxyapatite is a bioactive ceramic first used as a coating on implants in 1988 and is the most stable phase of various calcium phosphate. Due to its excellent osteo-conductivity and chemical and structural characteristics similar to the inorganic components of bone and teeth, it is often used as bioactive coating for dental and orthopedic implants.[149] To enhance the mechanical properties and overall effectiveness of implantable biomaterials created for orthopedic applications, HA is combined with other polymers and crosslinkers in form of composites.[177] Calcium phosphate ceramics was first used as implant material in 1980s, and they are used as implant material to be gradually replaced by newly formed bone because they have a biochemical composition, excellent osteo-inductivity, superior biocompatibility, pH-inducible degradability similar to bone and exhibit direct chemical bonding to surrounding bone. [85] The ability of calcium phosphate materials to cause osteoblastic differentiation in progenitor cells has led to their widespread use as biomaterials for the regeneration of bone tissue and creation of calcium phosphate-based nanomaterials.[178] The clinical success of bio-ceramic implants relies on the interaction with the blood leukocytes. Phagocytic cells such as monocytes, macrophages, and neutrophils mediate key events for tissue repair, and the inflammation these cells cause is crucial for accelerating wound healing and re-establishing local homeostasis. [86] Macrophages are observed to have an altered activation when co-culture with different HA materials. [88] The pro-inflammatory activity of these leukocytes can be modulated by bio-ceramics implants by enhancing or suppressing normal immune cell functions.[87] The biomaterial topography can also alter healing *In Vivo*, and Monocytes/Macrophage activation *In Vitro*. Using cDNA microarrays, monocytes and macrophages in-vitro cell culture expressed higher levels of proinflammatory cytokines and chemokines, such as IL-1, IL-6, TNF-α, monocyte chemotactic protein 1, and macrophage inflammatory protein 1-beta.[89] Biomaterials materials and topography play crucial role on monocytes and macrophages' early proinflammatory cytokine production and gene transcription. An in-depth knowledge of the host's inflammatory and wound healing response to implanted

materials is necessary for the design and improve biomaterial-host responses. The search for a viable substitute for current bone regeneration techniques and biomaterials is paramount importance for several significant reasons: enhanced patient outcomes, increased accessibility, reduced surgical complexity, patient customization, biocompatibility, accelerated healing, treatment of complex cases and innovation. This research field holds promise for improving patient outcomes, reducing healthcare costs, and advancing the field of regenerative medicine. It addresses challenges associated with current techniques and materials. It has the potential to make bone regeneration more accessible, efficient and patient-centric.

2.5 Leukocyte reactions with biomaterials

Neutrophils, macrophages, and dendritic cells serve phagocytic and signaling roles, especially in early inflammatory reactions with biomaterials. Other WBCs serve as the immunomodulators. [90] Interactions with biomaterials are typically characterized by hemostatic stage, inflammatory stage, proliferative stage, and remodeling stage. Transition between the inflammatory phase to proliferative phase key for proper healing and tissue regeneration. WBCs possess the ability to alter their own phenotypes and recruiting other cells from the proliferative phase. [91] At the same time, there are regulatory cells such as M2 and T-cells that acts as the inhibitor of chronic inflammation to ensure proper transition between these stages. [90] Specifically, studies have shown that hydroxyapatite phagocytosis is mediated by polymorphonuclear leukocytes, as well as hydroxyapatite biomaterials are not cytotoxic to leukocytes. [92,93] Brushite, Monetite, calcium polyphosphate materials all have shown to have different responses to leukocytes. Coating and scaffolding using these biomaterials have shown enhanced osteo-immunomodulatory functions, thus enhance osteogenesis and dental regeneration. [94,95,96]

Chapter 3: Leukocyte cell culture

3.1 Leukocytes culture parameters

In most current leukocyte culture protocols, the tissue media is supplemented with 10% FBS, and cultured in 95% humidified 5% CO_2 at 37°C. As a first step for isolation and purification, RBC lysis is widely used in many laboratories, which is much quicker than density gradient separation and leaves WBCs relatively undisturbed with a higher yield than density gradient separation. Another widely used protocol is isolation of mononuclear cells by density gradient separation

using 1.077g/ml Ficoll-Hypaque and could additionally followed by using Percoll. Supplemental protocols include enrichment of lymphocytes from mononuclear cells through adherence to plastic or by positive or negative magnetic bead antibody separation. Neutrophils isolation can be accomplished by Percoll gradient centrifugation. The Long-term storage of PBMCs is accomplished by cryopreservation. However, possible alterations of cell function in addition to cell purity and yield may occur using these techniques. [97] More recently studies have shown that PBMC proliferation can be altered with the oxygen concentration, with proliferation rate is higher at 20% oxygen than at 5% and 10% oxygen. [98] In conscious rats, systemic hypoxia increases vascular permeability and leukocytes migration. [100] PBMC can also behave differently under various thermal conditions. Incubation of PBMC at 4°C and 22°C for 1 hour did not affect the apoptotic percentage of cells, and incubation at 4°C for 24 hours resulted in the lower apoptotic percentage compared to 22°C and 37°C.[99]

3.2 Hemopoietic Bioreactors

SA bioreactor refers to a device or system designed to grow cells or tissues. Hemopoietic cultures are complex because they do not reach a steady state and always contain a variety of cell types at various stages of development. These cells interact with their surroundings through the productions of cytokines, growth factors, adhesion molecules and metabolic processes. Despite these challenges, engineers have designed novel bioreactors specifically to grow cell and tissue products.[101] Design of any novel bioreactors require engineers to have profound knowledge in cell's complex signaling pathways, interactions, cytokines release, behavior under stress and metabolic pathways. It is still an active area of research and potential. CAR-T, CAR-M, lymphokine-activated killer cells, tumor infiltrating lymphocytes, cytotoxic T lymphocytes and other adoptive leukocytes cell therapies have all used bioreactors as essential tools to develop high quality cell therapy products. [102]

3.3 Marrow Transplantation and HSC

Marrow transplantation is the procedure to infuse healthy pluripotent blood stem cells in the body replace bone marrow that is malfunctional. Healthy haematopoietic stem cells are collected from the bone marrow, peripheral blood or sometimes umbilical cord blood undergoes apheresis, a protocol to remove parts of the blood that might provoke disease environments. Then they are

cultured, filtered to acquire desired cell types, and re-infused in the patient.[117] Bone marrow transplantation is used to repopulate impaired bone marrow damaged by chemotherapy or radiation, and replace bone marrow that is not functional with the new stem cells. Engraftment is the term used to describe when the implanted marrow cells begin to function and proliferate, and it will take 8 to 14 days from the day of transplantation before blood counts start to recover.[106] For the recovery and long-term survival of patients after allogenic HSCT, the prompt reconstitution and restoration of a donor derived immune system is crucial. In optimal conditions after allogeneic HSCT, innate immunity quickly returns within 20 to 30 days, whereas adaptive immunity takes longer to recover and can take up to a year. [118] Bone marrow transplantation can benefit patients with acute leukemia, aplastic anemia, chronic leukemia, immune deficiencies multiple myeloma, neuroblastoma, and many other diseases. [103] However, if the blood is coming from a healthy donor, there is a risk of developing GVHD, an immunological disorder initiated by mature $CD4^+$ and $CD8^+$ T cells that affect many organs after transplantation.[104,105] The donated immune system recognizes the patients' body as foreign and attacks them leading to different syndromes. Unlike GVHD, transplant rejection occurs when the recipient(patients) immune system recognizes the donating tissues, organs or marrow as foreign pathogen and attack them. Graft refers to the organ and tissue transplanted. Most graft rejections are mediated by recipient immunological mechanism, dominantly by lymphocytes T-cells and the severity of the rejection syndromes is associated with intensity of immune response against the graft.[107]There are three types of rejection, hyperacute, acute and chronic, hyperacute rejection occurs within minutes and hours and caused by the pre-existing anti-donor antibody in receipts WBCs, acute rejection occurs within first weeks to several months after transplantation and affect all organs caused by mismatch in HLA, chronic rejection is associated with long term graft loss and accelerate disease like arteriosclerosis or graft tissue fibrosis. [108] Immunosuppressive drugs and treatments are currently being used to successfully prevent and treat both GVHD and graft rejection, however, chronic inflammation disease caused by both GVHD and graft rejection and is still a major area of research interest. [108,109] Biomaterials are widely used in tissue engineering to repair and replaced damaged tissues and organs, as well as biomaterial scaffolds provide immunomodulatory functions to lower immune response from either the host immune system or the graft. [110,111]

3.4: In vitro blood products, hematopoietic cell colonization on biomaterials

Four of these blood products—RBCs, Fresh frozen plasma (FFP), platelets and cryoprecipitate are more frequently used in general practice than the other blood products that can be prepared and used as replacement therapies in transfusion medicine. FFP, platelets, and cryoprecipitate are utilized for the prevention and treatment of bleeding, while RBC transfusions are primarily given to improve tissue oxygenation in case of anemia or acute blood loss brought on by trauma or surgery. [112] More recently, in a randomised controlled clinical trial, red blood cells were produced in vitro in the laboratory using stem cells from donors and then for the first time in the world administered to volunteers. Laboratory manufactured blood cells could revolutionize treatment for many blood-related disease. [113] Reduction of normal and abnormal WBCs from the allogeneic blood components are needed to minimize the occurrence of adverse immunomodulatory effects such as transfusion rejections, GVDH, alloimmunization to leukocytes or to treat certain blood related diseases. [114] One of the processes is referred as leukoreduction, the removal of WBCs from the blood for blood transfusion. Leukapheresis is a leukoreduction procedure involves the removal of some WBCs from the whole blood using apheresis device, then transferring plasma and WBCs back into the patient in order to treat chronic lymphocytic leukemia. [115,116]

As mentioned earlier, M1 pro-inflammatory macrophages thought to promote bone resorption, M2 pro-reparative state promote bone formation, and M1/M2 ratio controlled by estrogen directly relate to bone homeostasis. Especially in elder generations who suffer from weakened bone healing due to age-related alteration in macrophage activation and M1 to M2 transition. Biomaterials coating are used to design a novel drug delivery system to temporally direct the polarization of macrophages.[42] This potential drug delivery system using biomaterials coating can restore normal macrophage activation and transition and promote proper bone healing in older adults.

Hematopoietic cell colonisation on biomaterials has become an interesting research area with great potentials. One or more biomaterials have been studied on their capacity to promote stem cell differentiate into a particular target.[170] Biomaterials have become a vital frontier for stem cell-based tissue regeneration. Mesenchymal stem cell possesses potentials for multilineage cell differentiation, osteo-modulatory, dental, and immunomodulatory capabilities that can be used for various clinical purposes. A thorough understanding and a better understanding of hematopoietic cell colonization on biomaterials are fundamental for biomaterial based mesenchymal stem cell derivation from pluripotent stem cells. Different biomaterials designs and cellular modifications

have shown to improve mesenchymal stem cell yield, function, and transplantation. [171,172] BMP-9-loaded carbon-based biomaterial scaffolds work together with mesenchymal stem cells had also been chosen as a candidate for osteoporosis therapy and proven to have great clinical potentials. [173]

Chapter 4: Experimental Materials and Method:

4.1: Experiment materials:

Hydroxyapatite (HA) granules (size range from 500nm to 1µm)

The least soluble calcium phosphate at physiological pH is hydroxyapatite, which forms best in neutral or basic environments[129]. Hydroxyapatite is a calcium phosphate mineral with chemical formula [$Ca_{10}(PO_4)_6(OH)_2$], which has attracted great interest from the researchers because they are widely used as biomaterials especially for orthopedic applications such as bone implants. [121] Its excellent biocompatibility, osteoconductive properties, and similarity to the inorganic component of bone led to its use in bone repair, bone regeneration, bone tissue engineering in the forms of granules, blocks, and scaffolds, in a composite form with other ceramics or polymers, or as a coating for dental or orthopedic implants. [122] Hydroxyapatite was made utilizing chemical precursors, particularly calcium and phosphorus, hydroxyapatite can be synthesized using a variety of techniques, including dry, wet, thermal or a combination of these techniques. [174]

Monetite granules (size range from 500nm to 1µm)

Monetite is a unique calcium phosphate [$CaHPO_4$, DCPA] phase known as monetite or dicalcium phosphate anhydrous. Monetite is used as biomaterial for medical implants, bone cements, implant coatings, granules for defect fillers, and scaffolds. Numerous in vitro and in vivo studies demonstrated that monetite products have favorable osteointegration and osteo-conduction properties, as well as a better balance between implant resorption and new bone formation than other calcium phosphate phases.[123]

Both rat and human Leukocytes culture were using RPMI-1640 medium (Gibco™ Catalog number 11875093) with 10% FBS (Gibco™) and 1% Penicilin-Streptomycin (Gibco™ catalog number 15140122). MTT assay (Invitrogen™ V13154), was used to detect cell viability and leukocyte count and manual cell count was conducted with 0.4% Trypan Blue Staining solution (Gibco™ 15250061). Leukocyte attachment and proliferation on biomaterials at different time points and different leukocytes concentrations were observed using Live & Dead Assay (Invitrogen™ LIVE/DEAD™ Viability/Cytotoxicity Kit, catalog number L3224). Another rat and human

Leukocytes viability and quantification was performed using IL-1 beta Human ELISA kit (Invitrogen, ThermoFisher catalog number KHC0011). The amount of the target present in the original specimen relates to how strong this IL-1 beta signal is. Qualitative assay was performed using EdU assay (Abcam ab219801), Hematoxylin and Eosin staining kit (Abcam ab245880), May-Grunwald Giemsa Staining kit (Abcam ab150670) and Immunohistochemistry using first NOX2(MA5-18052), iNOS(ThermoFisher CXNFT), CD4(ThermoFisher GK1.5) and second antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L)).Sterile pH7.4 phosphate buffered saline (Gibco^{TM} 10010023) was used to wash both granules and cells.

4.2 Experiment Methods:

Rat Mononuclear cell isolation, Ficoll protocol

6 to 12 weeks old healthy Wistar rat peripheral blood was collected intravenously (protocol SOP#403) and used in each experiment. Small volume of blood was collected using lateral saphenous vein blood collection and large volume of blood collection was performed using the intracardiac puncture procedure, which is a terminal procedure and should be performed under anesthesia following by CO_2 Euthanasia. The blood was stored in heparinized tubes(10IU/ml) on ice and transferred back to the lab immediately for leukocyte separation.

Peripheral blood collected were then undergone density separation using a Ficoll gradient. Rat peripheral blood was mixed with an equal amount HBSS(Gibco^{tm}) then centrifuged at 400g, 22°C with the brake removed for 40 minutes. Next the yellow mononuclear layer (around 3ml) was extracted using a micropipette and transferred to a new 15ml centrifuge tube. Twice the amount of HBSS(6ml) was added to the tube that was then centrifuged at 400g, 22°C with no brakes for 15 mins. The supernatant was removed, and the cells were further washed with 10ml RPMI-1640 media at 1500rpm, 22°C with brakes for 5 mins. The cells were resuspended in ph7.4 PBS, and centrifuged at 1500rpm, 22°C for 5mins twice to allow rinse them. It is reported that washing can increase the survival rate of rodent leukocytes and lower the cell cytotoxicity. [126] Lastly, the washed cells were resuspended in 1-3ml of media and mixed gently. The cell number was measured using Trypan blue staining.

Rat leukocytes, RBC lysis protocol

10X RBC lysis buffer was also used initially for my experiment to isolate leukocytes. (1ml of peripheral blood + 10ml of RBC lysis buffer) However, the number of live cells and cells that attach to the material were not as good compared to the Ficoll Protocol. In addition, using RBC lysis, the leukocytes will still contain granulocytes that we are not interested in our experiment.

THP-1 cell monocytes and THP derived macrophage cell cultures

Commercially available THP-1 cells were grown in T-75 flask in the 37°C, 5% CO_2 incubator for a period of 10 days. 20ml of RPMI-1640, 10%FPS, 1% anti-fungal media was added to the flask and changed every 2 days. THP-1 cells needed to be centrifuged every time and remove the supernatant media before adding the new media. RPMI-1640 media was pre-warmed to 37°C. THP derived macrophages were prepared by adding Phorbol 12-Myristate 13-Acetatec, and only the adherent macrophages first detached by trypsin 0.25% from the T-75 flask were used for further MTT and Elisa assay. Macrophages did not proliferate as fast as monocytes and so required longer culture times.

Trypan Blue staining:

The initial cells number of leukocytes was estimated using 0.4% Trypan Blue staining solution (Gibco™ 15250061) using a Hemocytometer. 10µl of cells of interested were extracted and mixed with 10µl of Trypan Blue stain, and 10µl of stained cells transferred to the hemocytometer, and covered with the cover slip. Cells were observed under light microscopy to count the total number of leukocytes on 4 counting areas (top left, top right, bottom left and bottom right indicted by the Hemocytometer grid) of the hemocytometer. Record the number of cells observed from each corner 1,2,3,4.

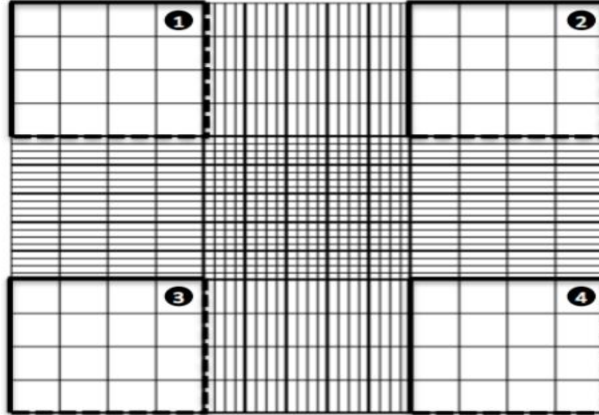

Figure1.5 : Hemocytometer and the grid, cells from area 1,2,3,4 were counted separately, then the average of the 4 corners were calculated

The estimation of number of cells is using the following mathematical formula:

Number of viable cells/ml = $\frac{total\ number\ of\ cells\ observed}{4(number\ of\ quare\ counted)} \times$ Dilution factor (2) × 10,000

Sample calculations:

Rat leukocytes used: (130+150+140+180)/4 × 2 × 10,000 = 3,000,000 cells/ml

Human THP-1 used: (39+35+46+28)/4× 2 × 10,000= 740,000cells/ml

Hematoxylin and Eosin (H&E) Staining:

The rat leukocytes from culture experiments were dehydrated in increasing concentrations of alcohol, then placed in hematoxylin, Mayer's solution (Abcam ab245880), for 5 minutes. It is then washed with distilled H_2O 2 times and stained with blue reagent for 10 to 15 secs followed by washed with distilled H_2O twice and 95% alcohol. Then the sample was treated with Eosin Y solution for 2 minutes then washed with 95% and 100% alcohol twice each. Then the sample was mounted with a cover slip and observed using light microscopy.

EdU(5-ethynyl-2'-deoxyuridine) Staining

The Edu stain/EdU staining proliferation kit (Abcam iFluor488 ab219801) is used to provide a sensitive and robust method to detect and quantify cell proliferation in live mammalian adherent and suspensions cells with fluorescence microscopy. Leukocytes and granules were co-cultured together and treated with Edu solution for overnight then washed with pH7.4 PBS. Three groups were compared: Control cells (+), media alone(-), cells with either monetite or HA granules.

IL-1beta ELISA (Enzyme-Linked Immunosorbent Assay)

Monocytes are main cell type that secrets IL-1, predominantly IL-1 beta. IL-1 is also produced by different forms of activated macrophages and by peripheral neutrophil granulocytes. ELISA is an in-vitro enzyme-linked immunosorbent assay for the quantitative measurement of specific proteins and can be used to estimate protein concentration. Here, media in which rat WBCs, THP-1 monocytes, THP-1 monocytes derived macrophages were collected at 24hrs, 48hrs and 5days and stored in -20°C for 2 weeks before analysis. A standard curve as shown below was made in order to calculate the IL-1 beta protein concentrations from all the samples.

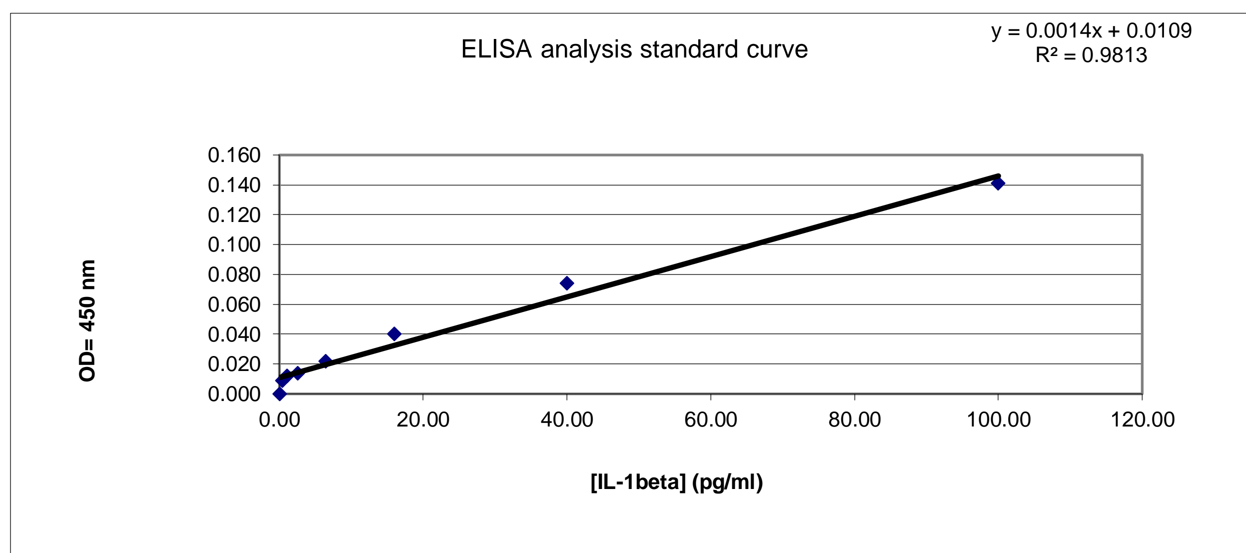

Figure 1.6 IL-1 beta ELISA analysis standard curve created using proteins provided by the ELISA kit.

The standard curve was made using the proteins that are provided with the kit. The plate reader was measured absorbance at 450nm. The ELISA kit chosen for the experiment was RayBio® Human IL-1 beta. The ELISA kit comes with an IL-1 beta 96 wells microplate coated with anti-human IL-1beta, standard proteins, wash buffer, detection antibody, HRP-Streptavidin concentrate, TMB one step substrate reagent, stop solution, assay diluent A and assay diluent B. ELISA kit also provides the Excel-sheet that can be accessed and used for data management and graph generation.

Live-Dead Assay

1µl of 3 mol Calcein, 2µl of EDTH (ethidium homodimer-1) and 0.5µl of Hoechest (Hoechest 34580 Invitrogen™ Catalog H21486) staining were added to a 1.5ml centrifuge tube, then 1ml of

pH7.4 sterile PBS was added to the mixture. The tube was shaken before adding 100µl of the mixture to each sample. Then all samples were further incubated for 10 mins at room temperature then observed using fluorescence microscopy. Leukocytes that attached to the granules and other adherent cells were first fixed with 100-200µl of 4% PFA for 30 minutes then washed with pH7.4 sterile PBS for 5 minutes prior to the live dead assay. The Excitation and emission were set 494/517nm for live green cells, and 517/617nm for red dead cells. The nucleus was stained by Hoechst with blue florescence at 460 to 490nm.

5-diphenyl tetrazodium bromide (MTT) assay:

The MTT assay ((Invitrogen™ V13154) gauges mitochondrial activity by measuring the amount of MTT that living cells convert into formazan crystals. MTT assay is a colorimetric and validated on adherent and suspension cells quantitative assay. The proliferation analysis was conducted using MTT Cell Proliferation assay Kit on a 96 well plate with clear flat bottom, and microplate reader absorbance was set at OD 590nm. Different densities of rat and human leukocytes were placed in duplicate with different materials as well as the background reading with just the media. 50µl of MTT reagent was added into each well. The plate was Incubated at the 37°C, 5% CO_2 for, 24 hrs, 48hrs and 5 days. After incubation period, 150µl of MTT solvent was added into each well and the plate was wrapped with aluminum foil. Then the plate was shake on an orbital shaker for 15 minutes then the absorbance at OD 590nm was measured using the plate reader. The background reading was subtracted, and a calibration curve was produced.

May–Grunwald Giemsa protocol

The MGG stain is frequently used to examine the cytological characteristics of the nucleus in air dried blood smears.[175] The rat leukocytes smear was fixed with methanol for 5 minutes, then stained the May Grunwald solution for 10 minutes, the slide then rinsed with distilled H_2O and was stained with diluted Giemsa for 30 minutes. It was then washed with distilled H_2O then dried. The slide was mounted and examined with light microscopy.

Image-J Image processing

The Leukocytes images taken with the fluorescence microscope were batched processed by using the software Image J (version 1.53). Images were opened and stacked in Image J in multiple files

then processed using batch processing. Image/adjust/brightness contrast were adjusted to create a binary image, then set to scale then analyze particles and eliminate threshold size to 0.02, which you will receive the total cell count as well as the average cell size.

Immunohistochemistry followed by immunofluorescence

The slides were deparaffinized, then NOX2 (Invitrogen™ MA5-18052) antibody was diluted to 1/250, iNOS Monoclonal antibody (ThermoFisher CXNFT) was diluted to 1/100, CD4 monoclonal antibody (ThermoFisher GK1.5) was diluted to 1/400. Cells were incubated with 0.3% triton for 10 mins followed by washing with pH7.4 sterile PBS for 1 min, 3 times. The washed cells were then incubated with serum block for 30 mins and applied each experiment differently with diluted NOXA2, iNOS, and CD4+. Those slides were incubated overnight then the slides were washed with PBS for 1 min, 3 times. The next step was to apply the second antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L)) on all the slides from the previous day for 2 hours, then washed with PBS for 1 min, 3 times again. The last step was to incubate with Hoechst stain for 15 mins and observed the slide under the fluorescence microscopy.

Chapter 5 Result:

5.1: Whole blood Leukocyte culture, troubleshooting:

My research plan was to use both rat and human leukocytes extracted from peripheral blood, and to extract them using density gradient centrifugation following by cell culture in the incubator (37°C, 5% CO_2, 95% humidified). Initially, it seemed that the cells were growing and attaching on the biomaterials. However, when I trypsinized cells from the biomaterials and did the H&E stain and Live-Dead assay, there were mostly small crystals and barely any cells observed. Additionally, the crystals also stained giving a false positive result.

In order to determine if any leukocytes migrated into the granules. They were trypsinized to remove surface located cells and crushed into smaller particles and stained with H&E and MGG to observe if there are cells reside within the granules. However, crushing the granules results in more crystals being produced and as they also stained it was extremely challenging to differentiate them from cells. Leukocytes efficiently navigate microenvironments made of tissue cells and extracellular fibers, which together form complex environments of varying porosity, oxygen concentrations, stiffness, topography, and chemical composition, while consistently moving through multicellular organisms to investigate and respond to pathogens and other harmful insults. [150] Both monetite and HA granules are porous structures that allows cells not only attach to them on the surface but also reside within the granules. A different approach was then followed to investigate the 3-D microenvironment within the granules is fixed these granules first then decalcified these granules using decalcified and put them into Histo-Gel. It was then paraffin embedded sections of granules were cut with a microtome and stained with H&E

However, only a very limited number of leukocytes were visible. It is particularly challenging to identify or quantify them. The next approach to solve this challenge was to increase the cell numbers in the culture. Changing from using DMEM(Gibco™ Catalog number 21331020) media to RPMI-1640 (Gibco™ Catalog number 11875093) media as reported[176] both with 10% FBS serum and 1% Penicillin-Streptomycin However, minor difference was noticed, neither the cell number nor proliferation changed significantly. Attempted to see if changing initial leukocyte separation techniques could increase cell number and survival, but insignificant effect was noted. A deeper literature review suggested that rodent cells in particular were challenging to culture due to a blood component and this could be greatly improved by cell washing with media and neutral sterile PBS.

5.2 The importance of Washing:

In order to provide an optimal and consistent environment that supports ideal cell expansion, the pH of the media is typically kept constant. [151]Changes in pH or a drop in temperature are not tolerated by leukocytes cell culture. It was shown previously that myeloid differentiation is severely inhibited outside of the pH range of 7.2 to 7.4, where if performs the best. [152] Thus, it is very important to keep the cells culturing at the constant pH. Washing cells with PBS during rat leukocytes separation from their peripheral blood was reported to increase both cells' numbers and cell survival rates. [152]

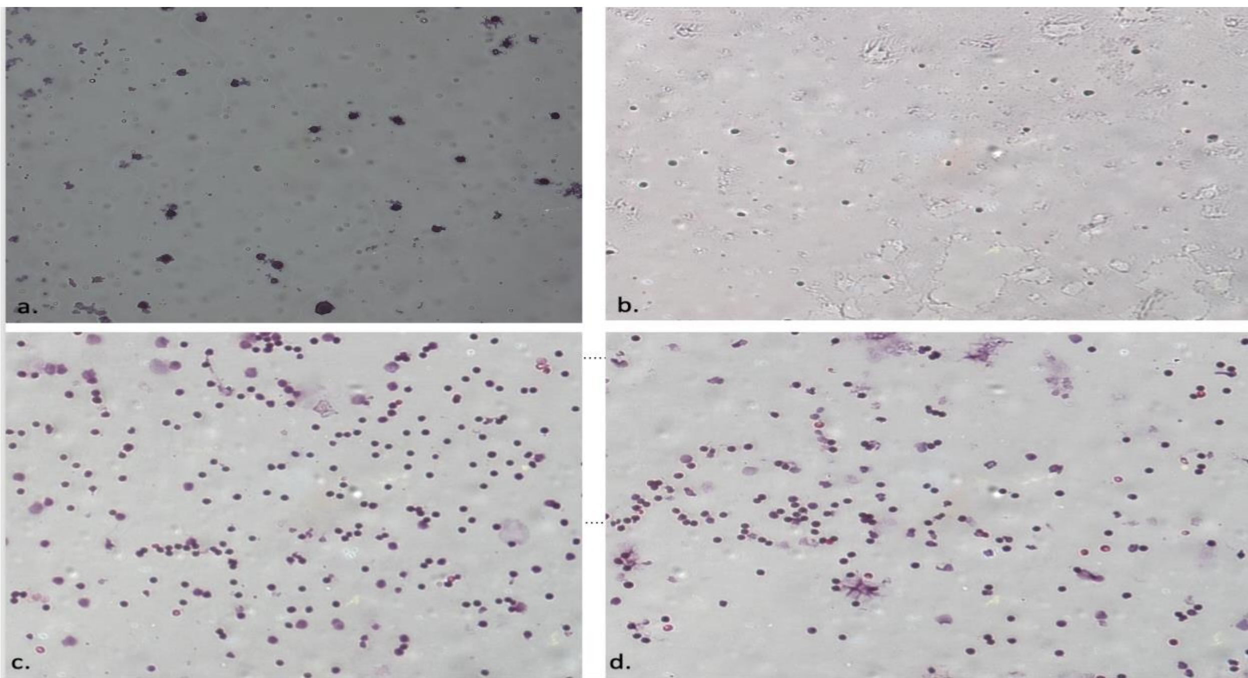

Figure 2.1Leukocytes cell staining with H&E. a) Unwashed rat leukocytes cultured for 24hrs and along with monetite granules then treated with 0.25X Trypsin for 5 mins in the incubator then stained with H&E at the 40X magnifications. b) Unwashed rat leukocytes cultured for 48hrs and along with HA granules then treated with 0.25X Trypsin for 5 mins in the incubator, then stained with H&E at the 40X magnifications c) Washed rat leukocytes with PBS during Ficoll-density gradient separation cultured for 24hrs and with monetite granules then treated with 0.25X Trypsin for 5 mins in the incubator then stained with H&E at the 40X magnifications d) Washed rat leukocytes with PBS during Ficoll-density gradient separation cultured for 48hrs and with HA granules then treated with 0.25X Trypsin for 5 mins in the incubator then stained with H&E at the 40X magnifications.

Figure 2.1 shows the difference between cells washed with PBS during Ficoll-density gradient separation from peripheral blood and cells not washed with PBS during the separation. There was an increase in cell number, from 27 (+/- 5%) to 185 (+/- 5%) in comparison with unwashed rat leukocytes cultured with monetite for 24hrs and the washed rat leukocytes cultured with monetite for 24hrs. Moreover, an increase in cell count from 25(+/- 5%) to 140(+/- 5%) with the washing step for leukocytes culturing with HA granules for 48hrs. More viable cells are trypsinized from both monetite granules at 24hrs and HA granules at 48hrs stained with H&E.

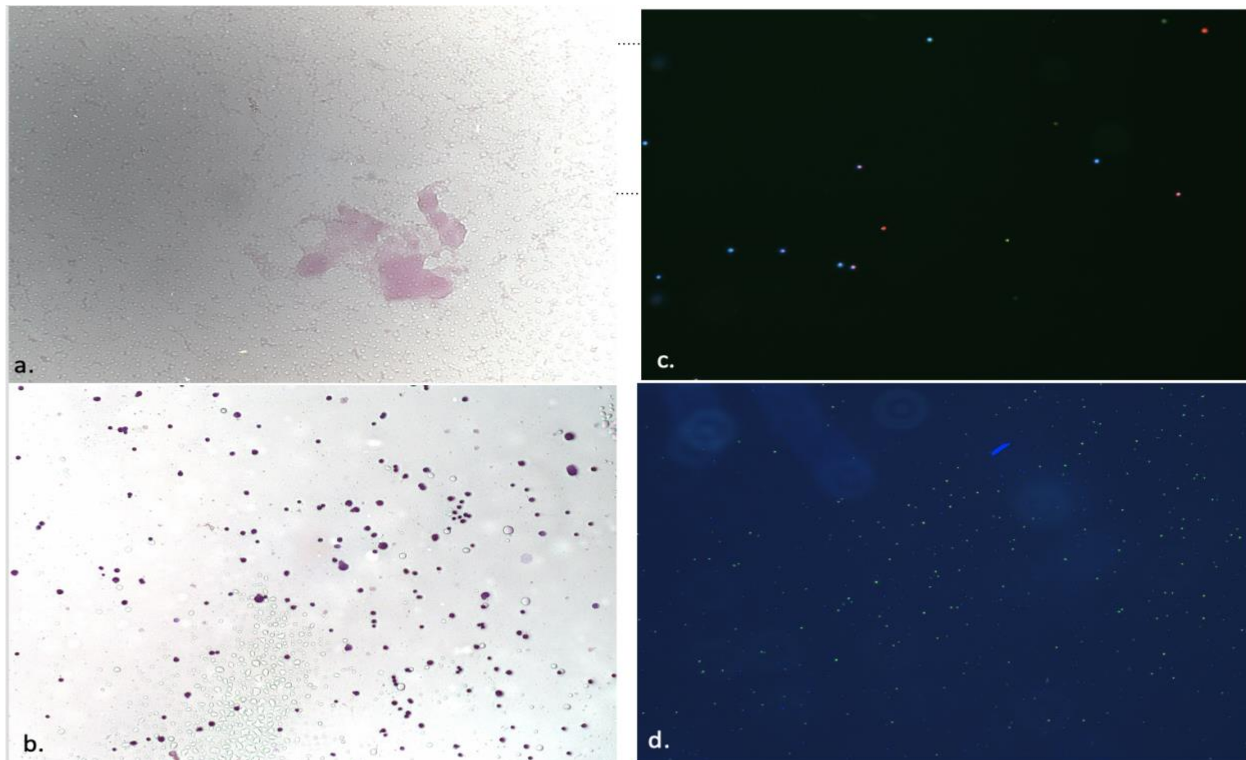

Figure 2.2 Leukocytes cells. a) Unwashed rat leukocytes cultured alone for 15 days then treated with 0.25X Trypsin for 5mins from the plate in the incubator then stained with H&E observed at 40X magnification. b) Washed Rat cultured alone for 15 days then treated with 0.25X Trypsin for 5mins from the plate in the incubator then stained with H&E observed at 40X magnification. c)Live Dead assay for unwashed rat leukocytes culturing alone for 7 days at 5X magnification. Blue stains for the nucleus, green stains for the live cells, red stains for the dead cells. d)Live Dead assay for washed rat leukocytes culturing alone for 7 days at 5X magnification. Blue stains for the nucleus, green stains for the live cells, red stains for the dead cells.

Figure 2.2 shows that after 15 days unwashed leukocytes cultured alone then detached with 0.25% trypsin and H&E no sign of cells could be determined. However, after the washing step with, there were 130 cells observed at day 15 of adherent rat leukocytes culturing alone. Washing cells with PBS twice is thought to remove some blood components that causes cell death and cell cytotoxicity, as well as to increase their ability to attach to granules, thus increase the overall number of viable cells trypsinized from the granules, stained with different stains and observed. In addition, an increase in the overall cell survival rates when they attach to the monetite granules was observed with the washing steps with PBS during the initial density gradient cell separations. Another important observation was that washing the sterilized granules 5 times with PBS prior to co-culture with leukocytes also helped with leukocyte attachment to the biomaterials. Both cells and materials should be under the neutral condition in order to observe the proper cell attachments and cell proliferations.

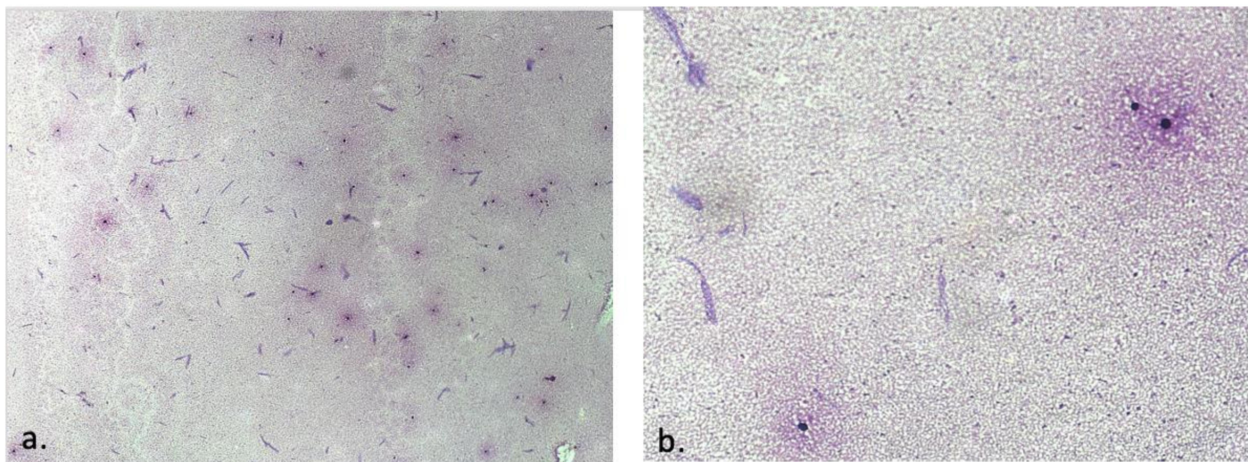

Figure 2.3 MGG stain on rat leukocytes a) MGG stain washed granules and leukocytes co-culture for 7 days, treated with 0.25X trypsin in the incubator for 5mins then stained MGG under 10X magnification. b) MGG stain washed granules and leukocytes co-culture for 7 days, treated with 0.25X trypsin in the incubator for 5mins then stained MGG under 40X magnification.

Figure 2.3 illustrates efforts to utilise another stain. MGG staining stains the cells that attached and trypsinized from the monetite granules. MGG is frequently used to examine the nucleus' cytological characteristics. Previously noted that, H&E-stained crystals released from the biomaterial granules.

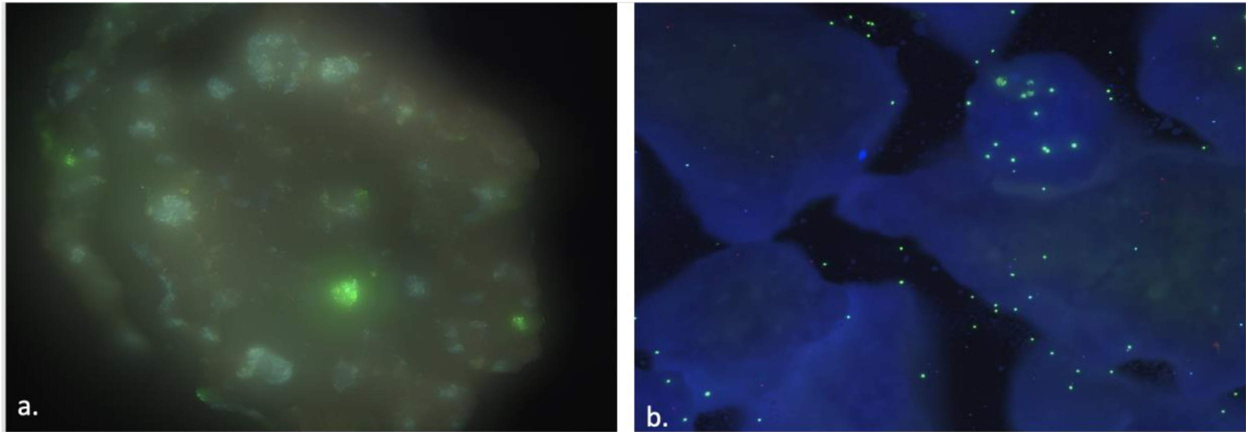

Figure 2.4 Live Dead Assay on rat leukocytes. a) Unwashed leukocytes co-culture with monetite granules for 24hrs then stained with live dead assay, image directly taken from the granules 10X magnification barely any cells observed, but a lot of crystals b)Washed Leukocytes co-culture with monetite granules for 24hrs then stained with live dead assay, image directly taken from the granules 5X magnification, live cells observed and attached to the biomaterials. Two images were taken at different time points and different microscope, emission was set differently.

Figure 2.4 compares the image directly taken with ECO PRO microscope. Washed granules on the right side clearly have more leukocytes attached at 24hrs, whereas on the left side, the size of the live dead does not correspond to the size of cells. They are large crystals from the granules instead of adherent leukocytes. Similar observation is found when washed leukocytes are co-culture with HA granules for 24hrs as shown in figure 2.5. Most of the rat leukocytes are able to attach to the granules, survive and proliferate. Blue edge is the edge of the granules, as observed that most washed cells can attach to the HA granules at 24hrs.

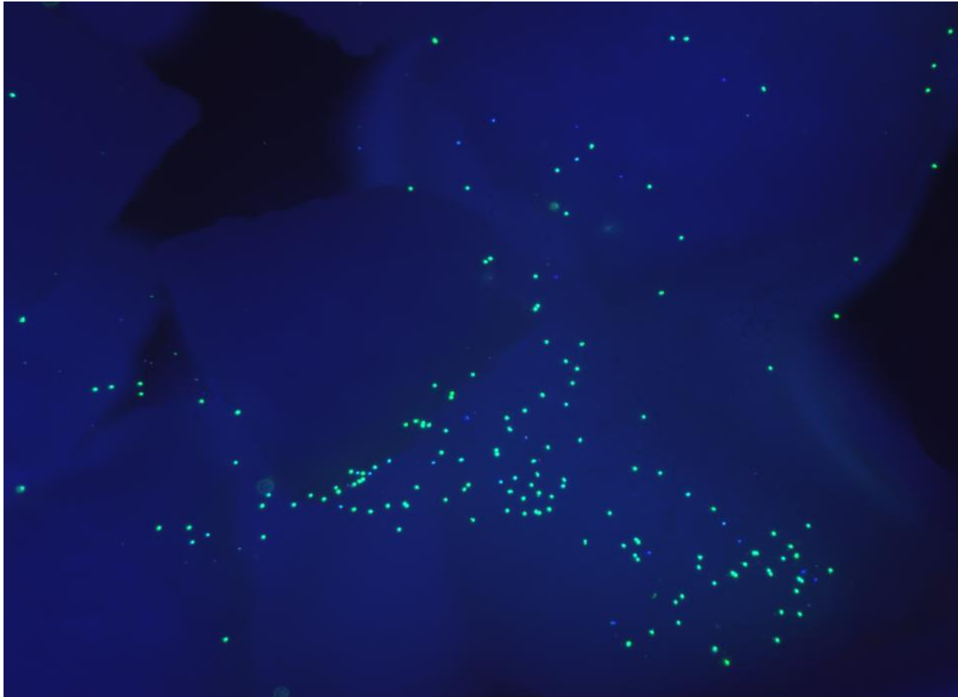

Figure 2.5 HA granules co-culture with washed rat leukocytes for 24hrs 5X magnification stained with Hoechst and Live-Dead stain, blue indicates the nucleus, green indicates the live cells. Granules are self-florescent.

5.3 Identification of the rat leukocytes:

After successfully co-culture rat leukocytes with different biomaterials. The next step was to attempt to identify and quantify these cells. Identification these cells using H&E stain was the original plan to see if certain biomaterials are selective to certain types of rat leukocytes. However, the H&E morphology of the rat leukocytes are not as clear as the human blood smear and makes it exceedingly difficult to identify exactly which type of rat leukocytes. Due to the technical difficulties as not able to use 100X light microscope and the imaging was not available. It makes it challenging to identify exactly types of rat leukocytes using solely light microscope. The next attempt was to use immunohistochemistry to identify exactly types of rat leukocytes that attach to the granules. As shown in figure 2.6, different first antibodies were applied following the immunohistochemistry protocol to the trypsinized rat leukocytes co-cultured with monetite granules for 24hrs. Despite mounting evidence that iNos is involved in the regulation of immune response in addition to its role in pathogen killing, iNos is an important marker of M1 macrophage activation. [153] Phagocytosis is a crucial step in bone shaping and bone remodeling. As shown in

figure 2.6 as well, there are Nox2 + cells were also evident, as Nox2 is an important immunohistochemistry marker for diverse types of phagocytes, Nox2 generates large amount of reactive oxygen species in phagosomes, which function to kill invading microbes. [154]. Another cell type observable was CD4+ T helper cells, which is crucial for the activation of the cells of the innate immune system, cytotoxic T cells, B-lymphocytes as well as regulate nonimmune cells and play a role in immune suppression. [155] Figure 2.6 has shown the presence of rat macrophages, other types of phagocytes as well as CD4+ T cells attach to the monetite granules. However, little quantification can be done as only few markers positive cells were present. Thus, it is difficult to conclude if the monetite granules and other biomaterials are selective to bind certain types of rat leukocytes. The proliferation rate of distinct types of rat leukocytes on the monetite granules seems extremely challenging to measure.

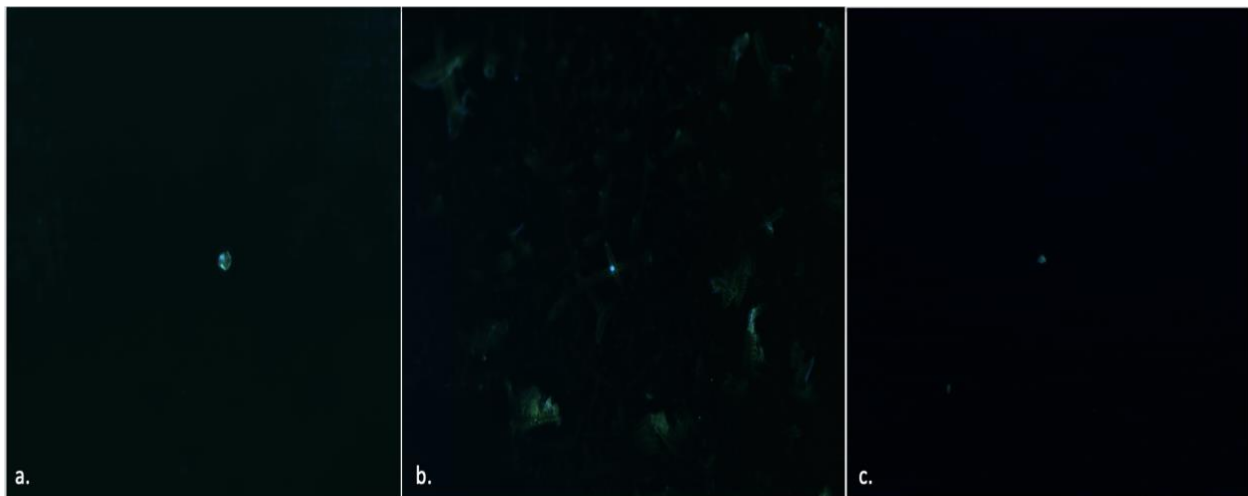

Figure 2.6 Immunohistochemistry identification of rat leukocytes. a)Monetite granules co-culture with rat leukocytes for 24hrs then treated with 0.25X trypsin for 5 mins in the incubator followed by iNOS antibody for ON then 2nd antibody (Alexa Fluor 488 goat anti-rabbit IgG H+L), and Hoechst stain, iNOS+ macrophage.b)Monetite granules co-culture with rat leukocytes for 24hrs then treated with 0.25X trypsin for 5 mins in the incubator followed by Nox2 antibody for ON then 2nd antibody (Alexa Fluor 488 goat anti-rabbit IgG H+L), and Hoechst stain, Nox2+ phagocytes c)Monetite granules co-culture with rat leukocytes for 24hrs then treated with 0.25X trypsin for 5 mins in the incubator followed by CD4 antibody for ON then 2nd antibody (Alexa Fluor 488 goat anti-rabbit IgG H+L), and Hoechst stain, CD4+ T helper cells

Several types of rat leukocytes that attach to the monetite granules and have different morphologies are also seen using live dead assay under the 60X fluorescence microscope. This is another evidence that shows more than one type of rat leukocytes are recruited to the granules, and are able to attach to the granules. It is reasonable to assume that monetite granules are not selective to bind to a certain type of rat leukocytes based on the cells' morphology and the immunohistochemistry stain. However, there could be a difference in the proliferation rate of distinct types of rat leukocytes, but more future work needs to be done, and a better immunohistochemistry quantification technique is needed to develop.

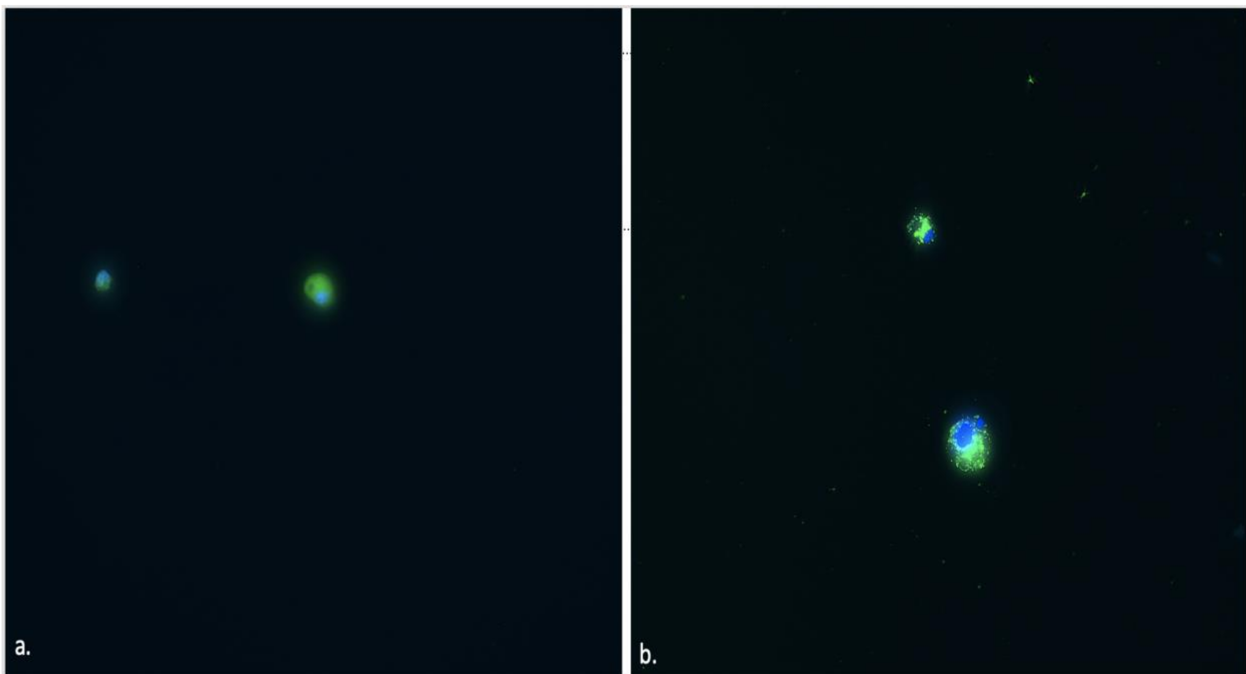

Figure 2.7 Distinct types of trypsinized rat leukocytes. a)0.25X Trypsinized rat leukocytes(phagocytes) from the monetite granules after 24hrs of co-culture, 60X magnification. b) Multi-nucleus rat leukocytes under the 60X magnification.

5.4 Rat leukocyte Quantification:

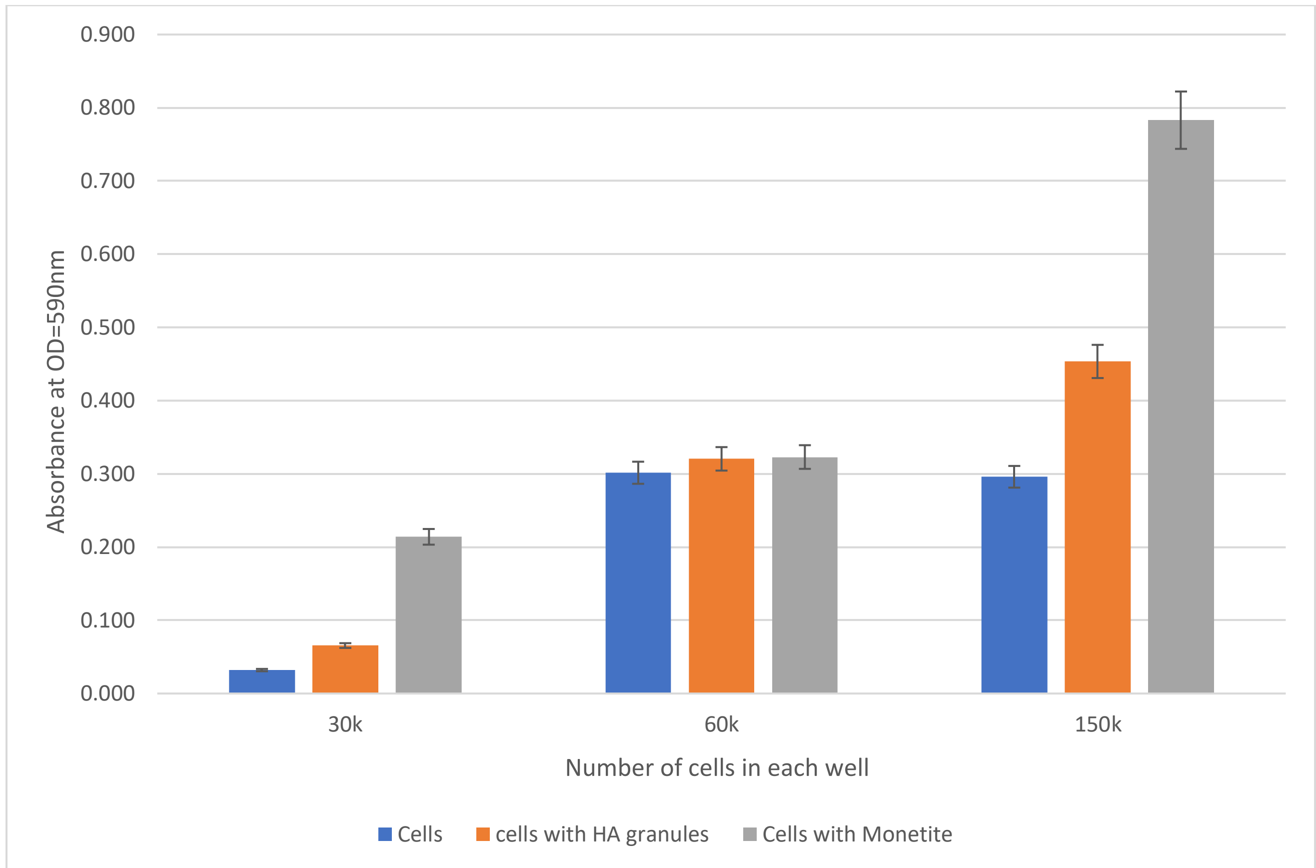

Figure 2.8 The 24h Rat Leukocytes MTT proliferation assay minus the background at different initial cell concentrations 1)The initial amount of rat leukocytes count in each well was estimated using Trypan Blue Stain. 2)The raw data from was processed and subtracted the background reading from the wells with media, MTT reagent and MTT solution only (-control).3) Blue is the reading from wells with material free control only, orange is the wells with cells and HA granules co-culture for 24hrs, grey is the cells with monetite granules co-culture for 24hrs.4) The higher the readings indicate the higher the rat leukocytes proliferations.

Figure 2.8 shows the MTT assay analyses. The three different sections indicate the three different initial number of cells put in each well for the assay. Each well was made with a duplicate, and the average of the two readings was used to create this graph. From all three groups with different concentrations of cells, it is observed that both cells culture with HA granules and cells culture with monetite granules have all higher absorbance readings, thus higher proliferation rate than cells growing alone. Especially at 30,000 and 150,000 cells, cells co-culture with monetite granules

proliferation readings is 6 times and 2.5 times higher than cells culturing alone. Whereas in cells co-culture with HA granules, the cell number was 2 times and 1.5 times higher than the cells growing alone. However, at 60,000 initial cell concentration, the cells co-culture with monetite and HA granules were only slightly higher than the cells growing alone. The overall trend still reflects the finding that rat leukocytes separated from the peripheral blood co-culture with monetite and HA granules have a higher absorbance, thus increased cells proliferation rate compared to material free control where cells were growing alone.

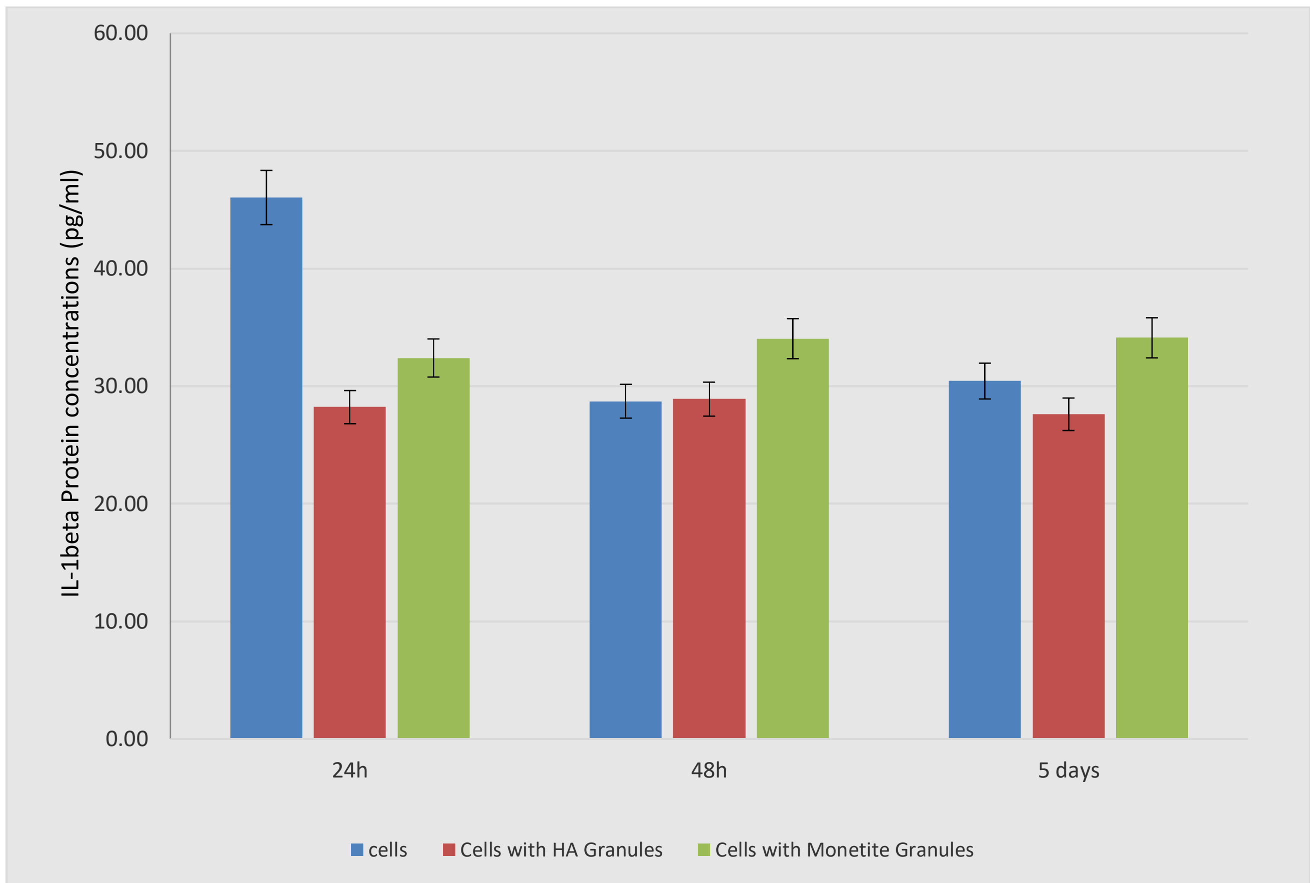

Figure 2.9 ELISA target IL-1beta protein concentrations of rat's Leukocytes alone and with HA granules, Monetite granules at 3 different time points. 1)X-axis indicates the IL-1 beta protein concentrations calculated using the standard protein curve from the method section. 2)The data was processed using the Excel tools provided by the ELISA kit. 3)The data is collected from rat cells growing alone, cells co-culture with HA granules, cells co-culture with monetite granules at 1 day, 2 days, and 5 days. 4)The ELISA assay was conducted using the 96 wells microplate coated with anti-Human IL-1 beta.

Figure 2.9 shows quantification of IL-1 beta released in the media by rat leukocytes either cultured alone or with different materials at 1, 2 and 5 days. At 2 and 5 days, cell cultured with monetite granules release more IL-1 beta protein than other conditions, but the difference was modest. Quantification for the rat leukocytes reveals an interesting trend. While co-culture with HA granules and Monetite granules does not lead to higher concentration of IL-1 beta protein compared to cells growing alone. Cells co-culture with monetite granules exhibit elevated IL-1 beta protein concentrations in the culture media at a later time stage. This could happen due to the fact this ELISA assay was using the human IL-1 beta antibody. It could significantly impact the assay result since we are using the assay for the rat leukocytes. This could be improved with a replication of the assay with the rat leukocytes antibody to validate with the previous finding that co-culture with biomaterials can increase rat leukocytes proliferation.

5.5 THP-1 Monocyte and differentiated Macrophage interaction with HA and Monetite

In order to minimize the potential conflict from culturing human leukocytes separated from the human peripheral blood, we chose to use commercially available monocyte line to save time as well as to obtain a larger number of viable cells. Human monocytic cell line commonly used in research due to its ability to differentiate into macrophage-like cells. THP-1 cells are easy to culture, maintain, and can be manipulated in various experimental settings. Additionally, they provide a consistent and reproducible model system for studying monocyte and macrophage biology and immune responses. Leukocytes adherence to biomaterial is rather rapid[128], and their existence is crucial for evoking inflammatory reactions as well as for modulating injury repairment and bone healing. The rapid attachment to the biomaterials were shown in all the THP-1 human leukocytes interactions with biomaterials.

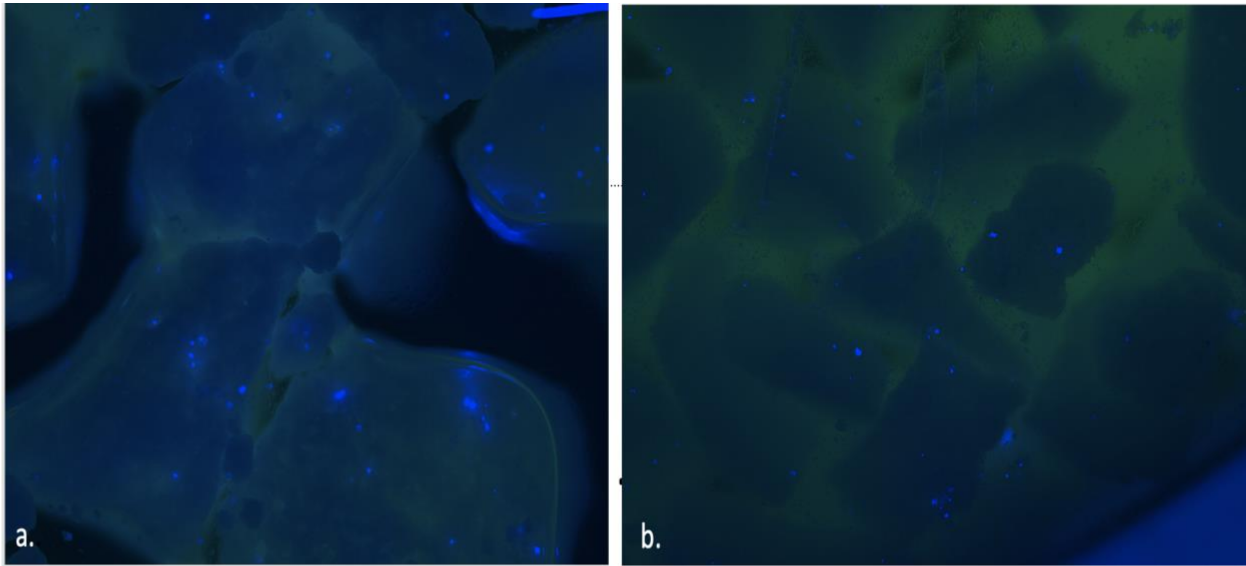

Figure 2.10 Macrophages on biomaterials Live dead assay. a) Monetite granules co-culture with THP-1 Macrophages for 24hrs stained with Edu and Hoechst at 5X magnifications b) HA granules co-culture with THP-1 Macrophages for 24hrs stained with Edu and Hoechst at 5X magnification.

The THP-1 Monocytes were cultured are maintained until confluence with repeatedly adding the media and observation under the microscope to make sure to culture a substantial number of cells. They were then separated into different flasks, and one of the flasks was treated with 5ng/ml PMA from Sigma-aldrich. THP-1 monocytes differentiate into mature macrophages after being exposed the PMA.[156] PMA is frequently used to promote differentiation into cells that resemble human monocytes derived macrophages. The culture media were changed every 48hrs, and cells were checked daily under the microscope to verify appropriate cell morphology.Macrophages are known to be highly adherent cells in culture.[157] Once the cells confluency density reached, they were treated with 0.25X trypsin for 5 minutes in the incubator to be collected. Figure 2.10 shows the rapid attachment of macrophages. Edu staining has been used to indicate cell proliferation. Figure 2.11 indicates that macrophages were able to attach to both materials, more attachment was observed on monetite granules as more cells and larger cells were noticed.

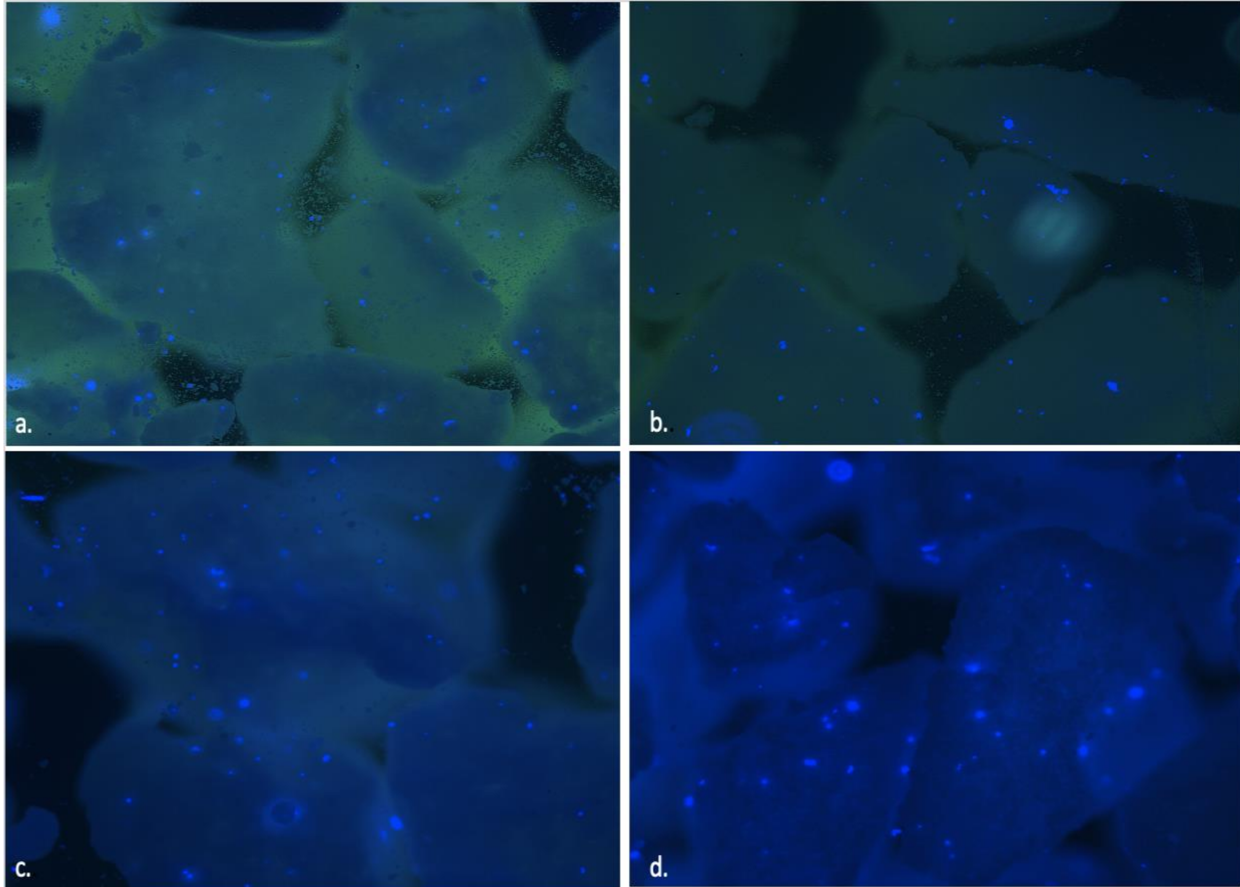

Figure 2.11 Macrphages on biomateirlas Live-Dead Assay. a) Monetite granules co-culture with THP-1 Macrophages for 48hrs stained with Edu and Hoechst at 5X magnification.b) HA granules co-culture with THP-1 Macrophages for 48rs stained with Edu and Hoechst at 5X magnification. c) Monetite granules co-culture with THP-1 Macrophages for 5days stained with Edu and Hoechst at 5X magnification. d) HA granules co-culture with THP-1 Macrophages for 24hrs stained with Edu and Hoechst at 5X magnification.

Comparable results were seen when both biomaterials were co-cultured with macrophages for a longer time period at 2days and 5days. Macrophages still attached to the HA granules and monetite granules very well. There were increasing numbers of cells that attached to both types of granules. Quantification of cells in different biomaterials was performed using Image J batch processing. Five images per timepoint at 5X magnification were quantified. Table 2.12 shows the average cell count and cell size for the 5 different images for cell plate, new granules, HA granules, monetite granules at 24hrs, 48hrs, and 5 days. An additional group of calcium pyrophosphate granules with a particle size around 20μm were included and appeared to have the greatest number of cells.

Although the pyrite phosphate granules appear to have the highest cell count, several factors pose challenges in accurate assessment. Firstly, the average cell size is too small, which aligns with the granules themselves being undersized. Moreover, autofluorescence may contribute to counting inaccuracies. Given the size limitations of the pyrite phosphate granules, conducting further MTT assays is impractical. Therefore, it is necessary to devise an improved method to evaluate cell attachment to the granules and establish a more reliable approach for cell counting and size measurement. These granules themselves autofluorescence and were an equivalent size to cells and so further quantification with this material was abandoned.

Table 2.12 Adherent macrophages c (Cell Count), s (Cell Size), show the average cell count and cell size of cells growing on the plate, cells with new granules, HA granules, and monetite granules using Image J Batch processing.

Adherent Macrophages	1 day (24hrs)	2 days (48hrs)	5 days (120hrs)
Cell plate	c: 54 s: 4.1µm	c: 26 s: 9.7 µm	c: 37 s: 10.3 µm
New Granules (Pyrite Phosphate)	c: 1215 s: 4.0µm	c: 1002 s: 3.4 µm	c: 100 s: 12.0 µm
HA Granules	c: 263 s: 7.7 µm	c: 1206 s: 5.5 µm	c: 778 s: 12.2 µm
Monetite Granules	c: 401 s: 12.1 µm	c: 608 s: 5.5 µm	c: 1083 s: 10.2 µm

The number of cells attach to the plate seems be rather consistent and far less compared to any of the granules. All three biomaterials shown to increase cells number, thus cells proliferations. The data once again demonstrates that monetite granules exhibit a higher number for leukocytes attachment compared to HA granules during the initial 24-hour. An average cell count of 401 cells with a diameter of 12.1µm was observed for monetite granules, whereas HA granules recorded a cell count of 263 cells with a diameter of 7.7µmreflected that at the initial 24h. At 5 days, most of the cells are mature which result in a slightly lower number of cells for HA granules, but bigger cell size. All three types of biomaterials have showed an increase in cell number, thus increased cells proliferation compared to cells culturing on the plate alone.

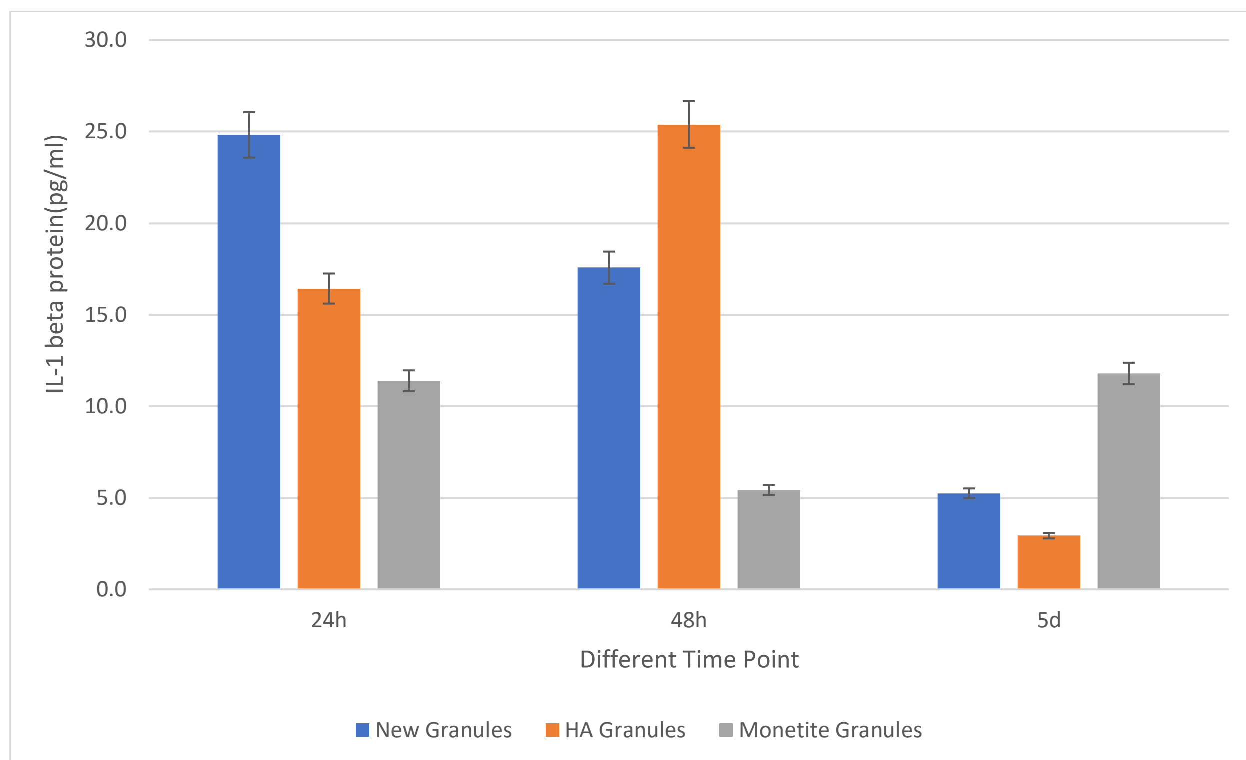

Figure 2.13 ELISA quantification of IL-1 beta in culture medium in which THP-1 macrophages were cultured either alone or with HA, new granules (20μm pyrophosphate granules) and Monetite granules. biomaterials.1) Macrophages culture with different biomaterials over 24h, 48h and 5 days(120hrs), and the media is extracted and stored in the freezer for 2 weeks prior to the ELISA assay. 2) The Y-axis shows the calculated IL-1 beta protein concentrations using the standard curve mentioned earlier, unit is pg/ml. 3) The X-axis is the different calcium phosphate granules used to co-culture with derived human macrophages at 3 different time points. 4) The readings had already subtracted Il-1 beta protein concentrations from macrophages culturing alone, only the exceeded part is shown in the figure.

Figure 2.13 shows the result of ELISA assay. In addition to various activated macrophages and peripheral neutrophil granulocytes, monocytes are the primary source of IL-1 and primary express IL-1 beta. IL-1beta is typically released from macrophages after being processed by caspase-1 after the inflammasome detects infection or danger. [159] The higher the IL-1 beta concentration means that more macrophages are being activated and starts to release this cytokine. Initially, pyrite phosphates granules have the highest IL-1 beta concentrations, this could occur due to the rapid onset of inflammation triggered by the small size of the pyrite phosphate granules. Cells are

immediately recruited and start to release cytokines. Macrophages are recruited to the location and phagocytosis. At 48hrs, HA granules have the highest IL-1 beta protein concentrations, this is the same result as the previous table 2.12, The results revealed that the highest number of macrophages was observed at 48 hours when co-cultured with HA granules. Additionally, at day 5, the highest concentration of IL-1beta was found in monetite granules co-cultured with macrophages. These findings demonstrate that monetite can effectively stimulate macrophages to continuously release IL-1beta, providing direct evidence of macrophage activation by the biomaterials and their ability to release cytokines and engage in phagocytic activity. Such activation is crucial for the healing and remodeling processes of bones and teeth (ref!). Furthermore, macrophages exhibited excellent viability and proliferation at day 5 when co-cultured with monetite granules, as indicated in Table 2.12, where the macrophage count was highest in the monetite granules co-culture. Figure 2.14 presents the MTT reading for the 5-day co-culture of macrophages with biomaterials.

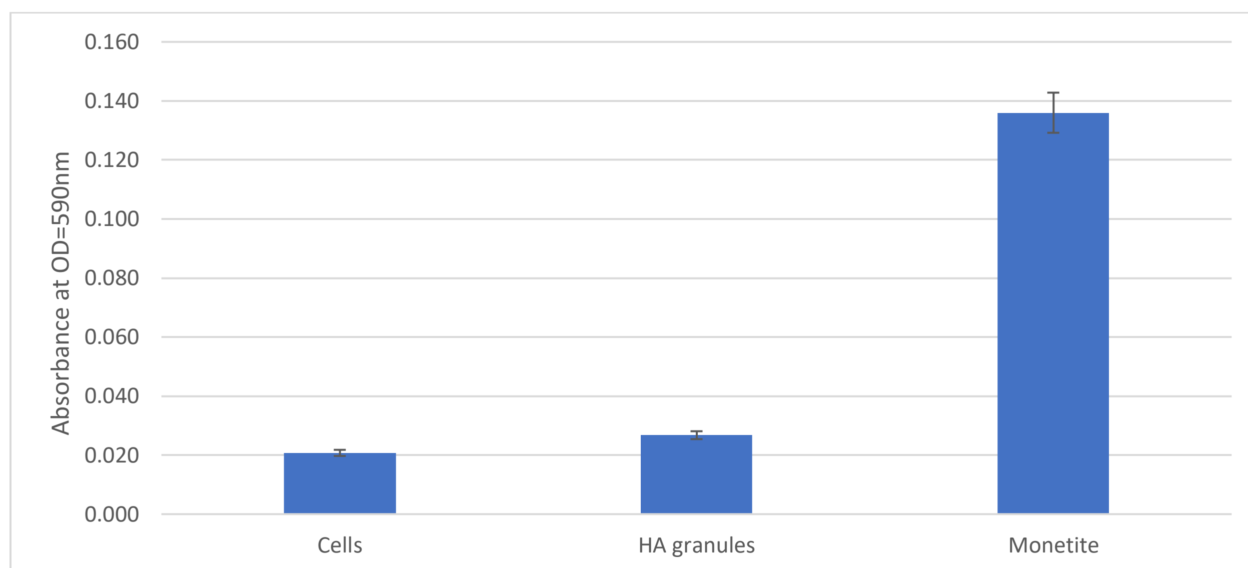

Figure 2.14 5days(120hrs) Human Macrophages culture alone, co-culture with HA and monetite granules MTT average readings subtracted the background readings. 1)The 5 days macrophages are treated with MTT assay and the plate reader is set absorbance at OD=590nm. 2)The higher the reading means the higher the macrophage proliferations. 3.) All three readings, cells culturing alone, cells culture with HA granules, cells culture with monetite granules have already subtract the background (media alone treated with MTT) 4) Each data set came with a duplicate, and the average of the two is taken to generate this graph.

At day 5(120hrs), figure 2.14 has shown MTT assay that measures the macrophages proliferation at 5 days of incubation and cells that are incubated alone and with biomaterials for 5 days prior to the assay. As can be seen, there is no difference between cells alone and cells incubated with HA granules However, there was a significant 6.5-fold increase between the cells alone and those incubated with monetite. Cells culture alone has the reading of 0.020, while cells culture with HA granules has 0.025. There is only a slight increase in proliferation when macrophages are cultured with HA granules. However, an increase from 0.020 in cells growing alone to 0.135 when cells co-cultured with monetite granules is observed. The higher the MTT reading indicates the higher the proliferation rate. Both HA granules and monetite granules improve the number of viable macrophages in-vitro. It can be concluded that both cells culture with HA granules and monetite granules have a higher proliferation rate compared to cells culturing alone. Culturing with biomaterials have positive effect on macrophages proliferations, as well as activations. Cells culture with monetite granules have the highest proliferation, comparing with cells cultured alone and cells with HA granules. Macrophages possess the potential to differentiate into bone resorbing osteoclasts when treated with RANKL[131] that ultimately plays vital role in bone and teeth remodeling and healing.

Quantification of human THP-1 monocytes:

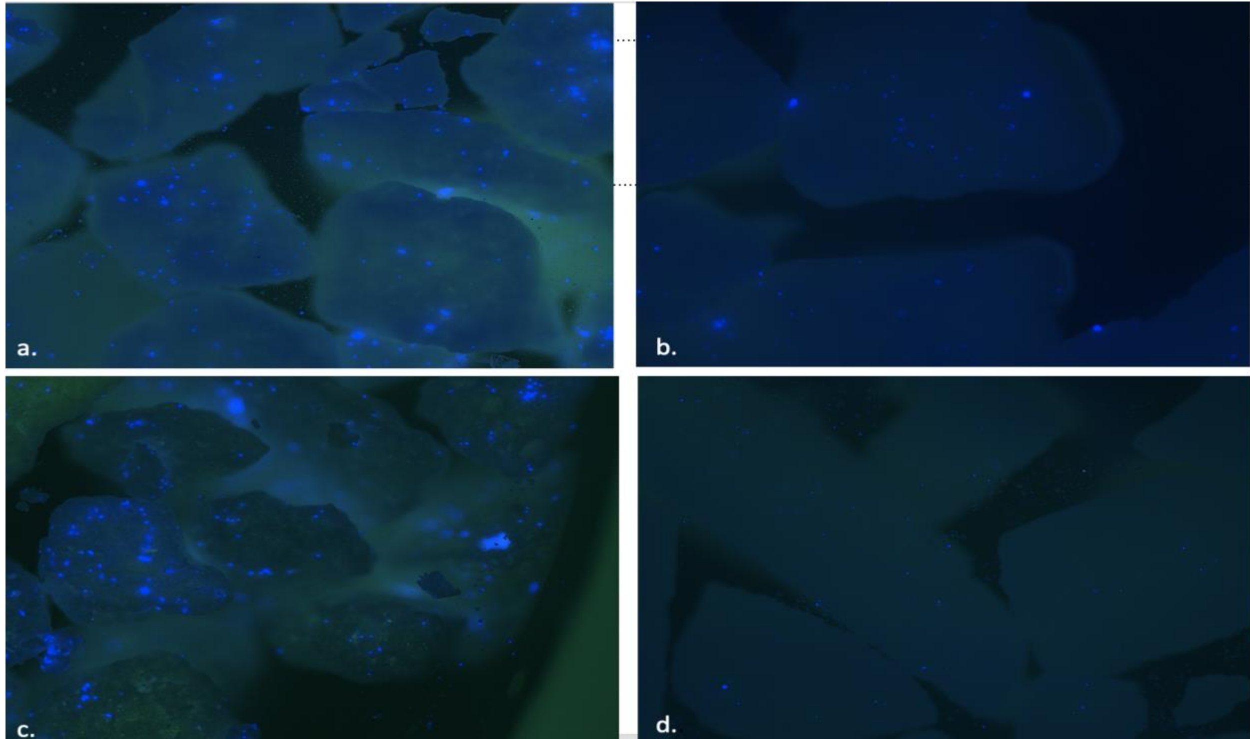

Figure 2.15 Monocytes co-culture with biomaterials. a) Monetite granules co-culture with monocytes for 24hrs, stained with Edu and Hoechst at 5X magnification b) HA granules co-culture with monocytes for 24hrs, stained with Edu and Hoechst at 5X magnification c) Monetite granules co-culture with monocytes for 48hrs, stained with Edu and Hoechst at 5X magnification. d) HA granules co-culture with monocytes for 48hrs, stained with Edu and Hoechst at 5X magnification.

A broken bone can heal because healthy human bone is a material that is constantly changing. Osteoclasts are monocytes derived cells that use ions like calcium and phosphate that are present in blood plasma as well as stored in bones and teeth to degrade or build bone in response to biomechanical stimuli. [130] Recent study has also shown that osteoblastic linage cells have been found exist in the human peripheral blood. [162] Peripheral blood is an easily accessible source of adult stem cells for research and clinical applications. In addition, the transplantation of these PBMC-derived cells can regenerate tissues and restore function after injury. PBMCs have been reported to contain a multitude of distinct multipotent progenitor cell populations and have the capacity to differentiate into blood cells, endothelial cells, hepatocytes, cardiomyogenic cells, smooth muscle cells, osteoclasts, epithelial cells, neural cells or myofibroblasts under appropriate

conditions. [125] Monocyte is an important part of PBMCs and exert important function in immune response as well as injury repair. Figure 2.15 shows the difference between the monocytes culture with monetite and culture with HA granules. At 24hrs, most monocytes are able to attach the monetite granules, while there are not as many cells attach to the HA granules. At 48hrs, similar trend is observed as some large cells or cluster of cells are observed. Monocytes possess the ability to differentiate into macrophages with several factors for phagocytosis as well as osteoclasts that is responsible for bone remodeling. Monetite granules increase the number of the cells and size of the cells attached. There is a clear preference that monocytes attach to the monetite granules better and proliferate more compared to the HA granules. This can potentially be the clinical target for osteoporosis, which is a condition caused by bone resorption exceeds the speed of bone production. If we can positively identify whether monetite promotes certain types of monocytes differentiation, especially with an interest on M1, M2 macrophages, and osteoclasts. Osteogenic substances secreted by M2 macrophages promote the activation and differentiation of pre-osteoblastic cells, such as mesenchymal stem cells, which in turn promotes bone mineralization. [160] If monetite materials can promote a certain type of monocytes differentiation, it can be used as the medical implants to target syndromes like osteoporosis.

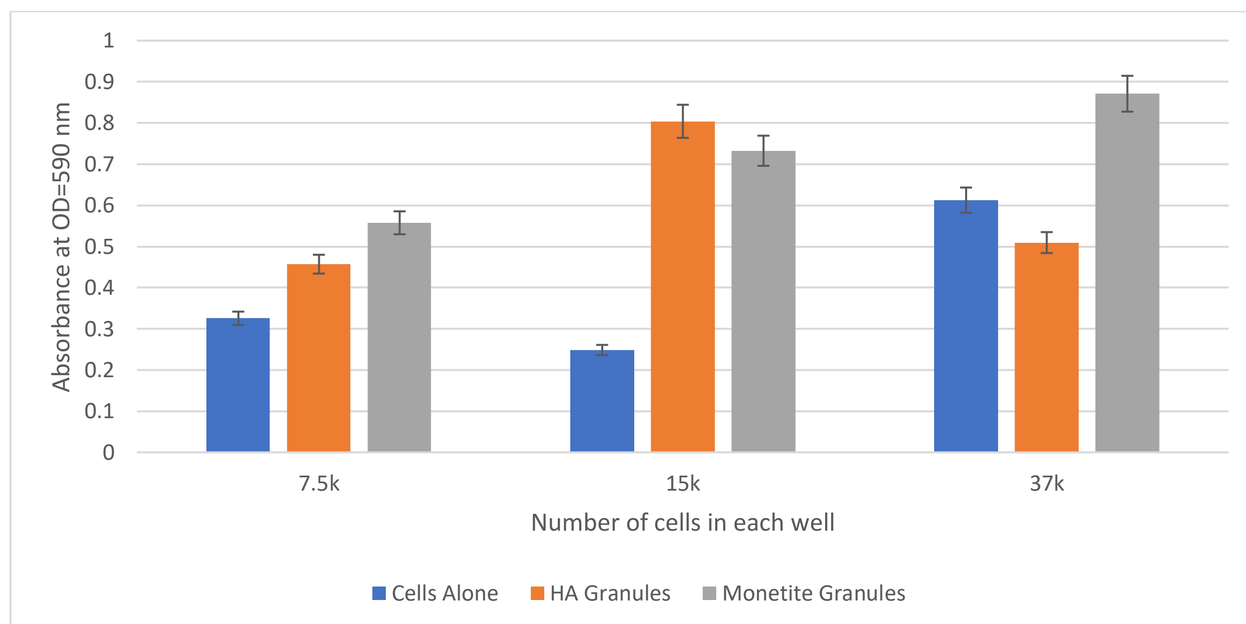

Figure 2.16a 24hrs Human THP-1 monocytes MTT assay for Cells culturing alone, culture with HA granules and Monetite at different Initial cell concentrations subtracted the background reading.

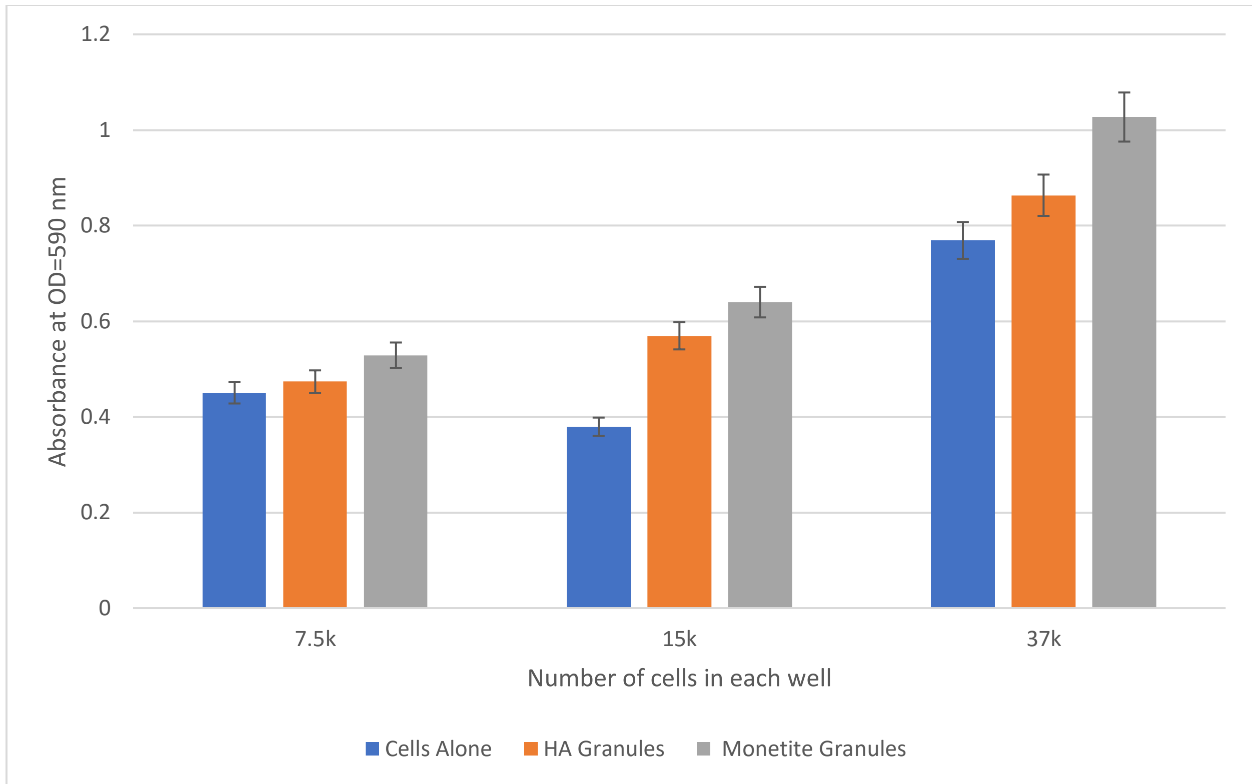

Figure 2.16b 48hrs Human TPH-monocytes MTT assay for Cells culturing alone, culture with HA granules and Monetite at different Initial cell concentrations subtracted the background reading.

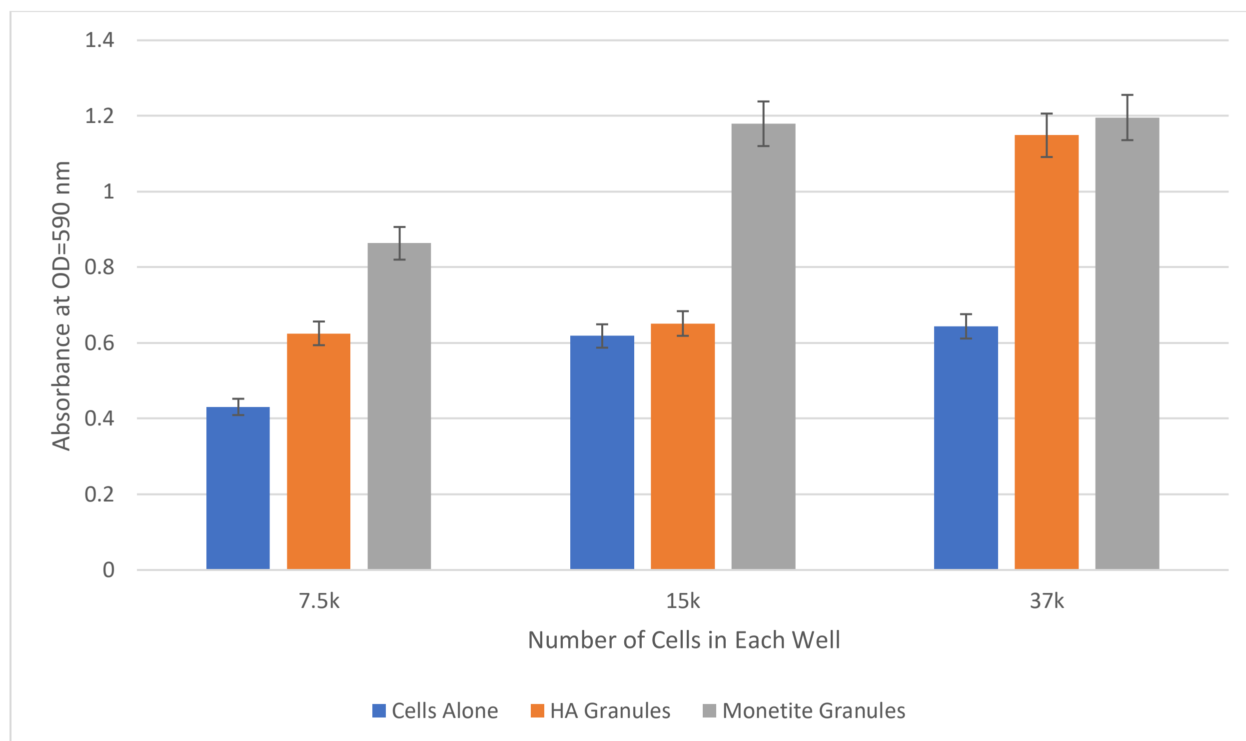

Figure 2.16c 5days(120hrs) Human THP-1 monocytes MTT assay for Cells culturing alone, culture with HA granules and Monetite at different Initial cell concentrations subtracted the background reading. 1) All three graphs use absorbance OD=590nm setting using the plate reader. 2) Three graphs each indicate a different time point, different initial amount of cells numbers is put into the different wells culture alone and co-culture with different biomaterials. 3) All the data readings have subtracted the background readings (RPMI-1640 media alone with MTT), and each well was made with a duplicate. The average of the two is taken to create this graph. 4) Each column represents different culture conditions. Blue is the cells culturing alone, orange column is monocytes culture with HA granules, grey column is monocytes culture with monetite granules. 5) The higher the absorbance reading indicates the higher cells proliferation and better cell viability.

Figure 2.16 a,b,c shows the MTT assay on THP-1 monocytes at three different time points: 24hrs, 48hrs and 5days(120hrs). All the cells culture with HA granules and monetite granules shown a higher absorbance than cells culture alone. With the exception at 24hrs at 37,000 initial cells has a higher absorbance than cells culture with HA granules. This could happen randomly due to the high proliferation of that particular well of cells. However, the rest of the data point at different

time points agrees with the statement that cells co-culture with biomaterial have a higher proliferation and better cells viability compared to cells culturing alone. Especially, monocytes co-cultured monetite granules have showed the most consistent result. In most cases, cells culture with monetite granules have twice the proliferation rate and cell viability compared to the cells culture alone. Human monocytes co-culture with HA granules, on the other hand, also have higher proliferations rate compared to cell culture with no biomaterials, with the exception at 24hrs at 37,000 initial cells. At 24hrs 15,000 initial cells wells, monocytes co-culture with HA granules exceeds the OD=590nm readings than monetite granules. However, in most cases, monocytes co-culture with HA granules has a lower cell proliferations and cell viability compared to monocytes co-culture with monetite granules. Monocytes co-culture with HA granules have a higher MTT absorbance reading than cells growing alone in most cases, hence a higher cells proliferations and cells viability compared to monocytes culturing alone. This finding echoes with the qualitative analysis that more monocytes attach to the monetite than HA granules, while both materials shown to increase THP-1 human monocytes proliferation and viability. Monetite materials have the most positive effect on monocytes proliferations, cell viability. We need to invest to understand the exact differentiation mechanism of cells with the presence of calcium phosphate biomaterials. There is also a need to develop the better characterization and quantification technique of cells differentiated from the monocytes in order to confirm if certain biomaterials promote a certain type of cells differentiation.

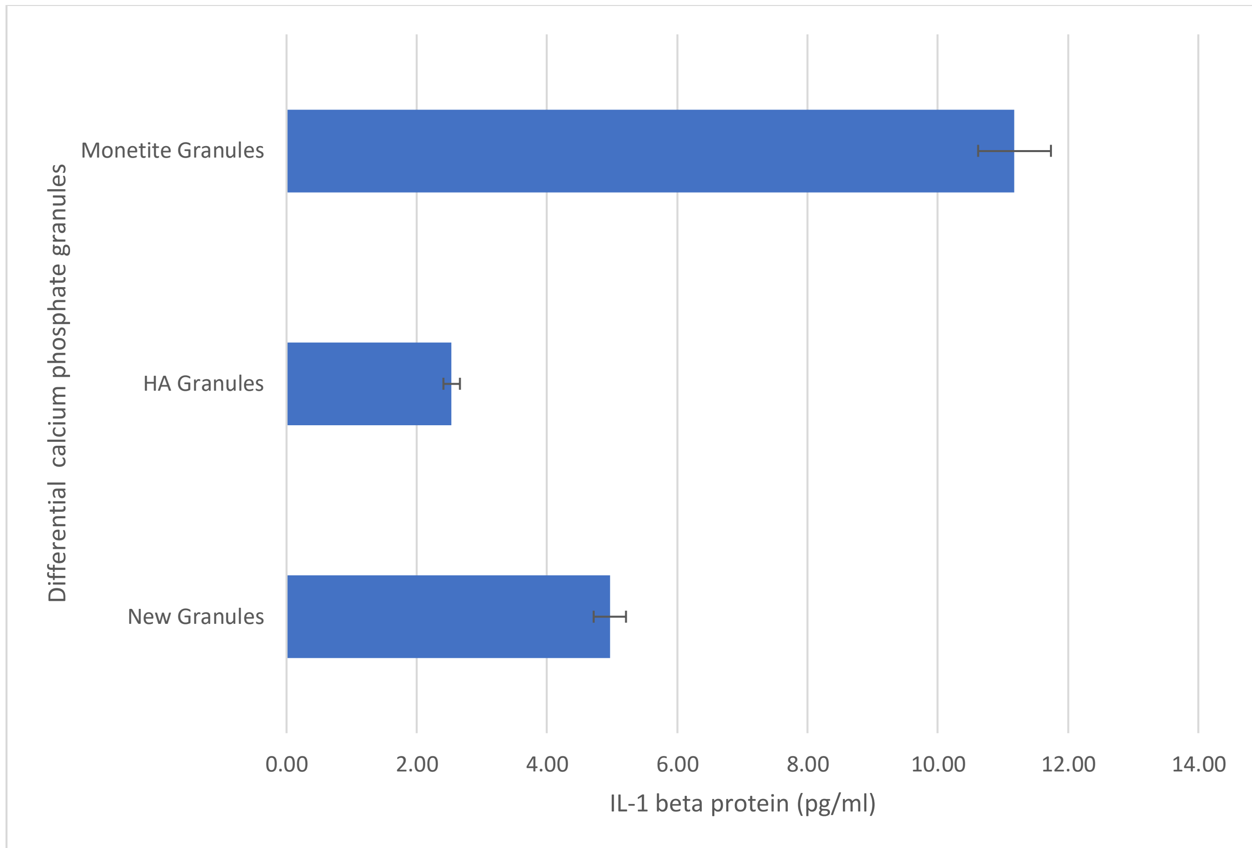

Figure 2.17 THP-1 Human Monocytes IL-1 beta concentrations when co-culture with different calcium phosphate granules at 24hrs minuses cells culturing alone. 1) THP-1 linage human monocytes culture with different biomaterials over 24hrs, and the media is extracted and stored in the freezer for 2 weeks prior to the ELISA assay. 2) X-axis shows the calculated IL-1 beta protein concentrations using the standard curve came with the kit mentioned earlier, unit is pg/ml. 3) Y-axis is the different calcium phosphate granules used to co-culture with THP-1 monocytes. 4) The readings had already subtracted IL-1 beta protein concentrations from THP-1 monocytes culturing alone, only the exceed part is left in the graph.

Figure 2.17 shows the IL-1 beta protein ELISA for THP-1 human monocytes IL-1 concentrations when co-culture with different calcium phosphate granules at 24hrs. Monocytes co-culture with monetite granules has the highest exceeded concentrations of IL-1 beta proteins at close to 11pg/ml, while new granules show the second highest exceeded IL-1 beta proteins concentrations at close to 5pg/ml. and the HA granules only exceed the material free control cell by close to 2.5pg/ml.

Human monocytes co-culture with monetite granules again has the highest concentrations of IL-1 beta released at 24hr.

New Granules (pyrite phosphate):

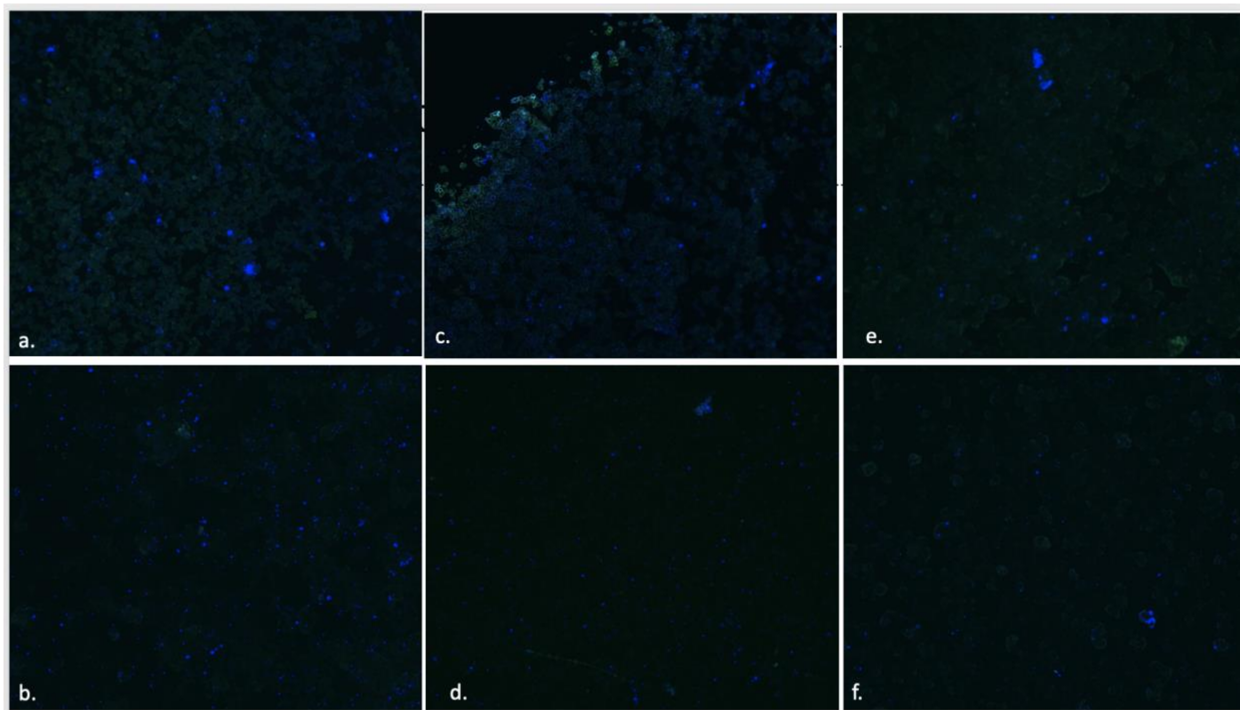

Figure 2.18Pyrite phosphate granules with leukocytes. a) New granules (pyrite phosphate) co-culture with human macrophages 24hrs stained with Edu and Hoechst, 5X magnification. b) New granules (pyrite phosphate) co-culture with human THP-1 monocytes 24hrs stained with Edu and Hoechst, 5X magnification. c) New granules (pyrite phosphate) co-culture with human macrophages 48hrs stained with Edu and Hoechst, 5X magnification. d) New granules (pyrite phosphate) co-culture with THP-1 monocytes 48hrs stained with Edu and Hoechst, 5X magnification. e) New granules (pyrite phosphate) co-culture with macrophages 5days(120hrs) stained with Edu and Hoechst, 5X magnification. f)New granules (pyrite phosphate) co-culture with THP-1 monocytes 5days(120hrs) stained with Edu and Hoechst, 5X magnification.

Figure 2.18 shows the new granules co-culture with human THP-1 monocytes and macrophages. The image reveals a prominent autofluorescence that makes it extremely challenging to differentiate between cells, crystals, and granules. The pyrite phosphate granules have a similar diameter to the cells, and it made it difficult to do any leukocytes identification or quantifications.

However, it was observed that some cell attachment to the pyrite phosphate granules to the limited extend. At 24hrs co-culture with human macrophages, some phagocytic activities were observed, but it was incredibly challenging to identify if these fluorescence signals are crystals or cells phagocytosis the small pyrite phosphate granules. For the further experiment, there will be need to develop a systematic technique to stain cells, count cells, trypsinize cells, identify cells, and quantify cells when co-culture with small size granules such as pyrite phosphate granules.

Negative Control:

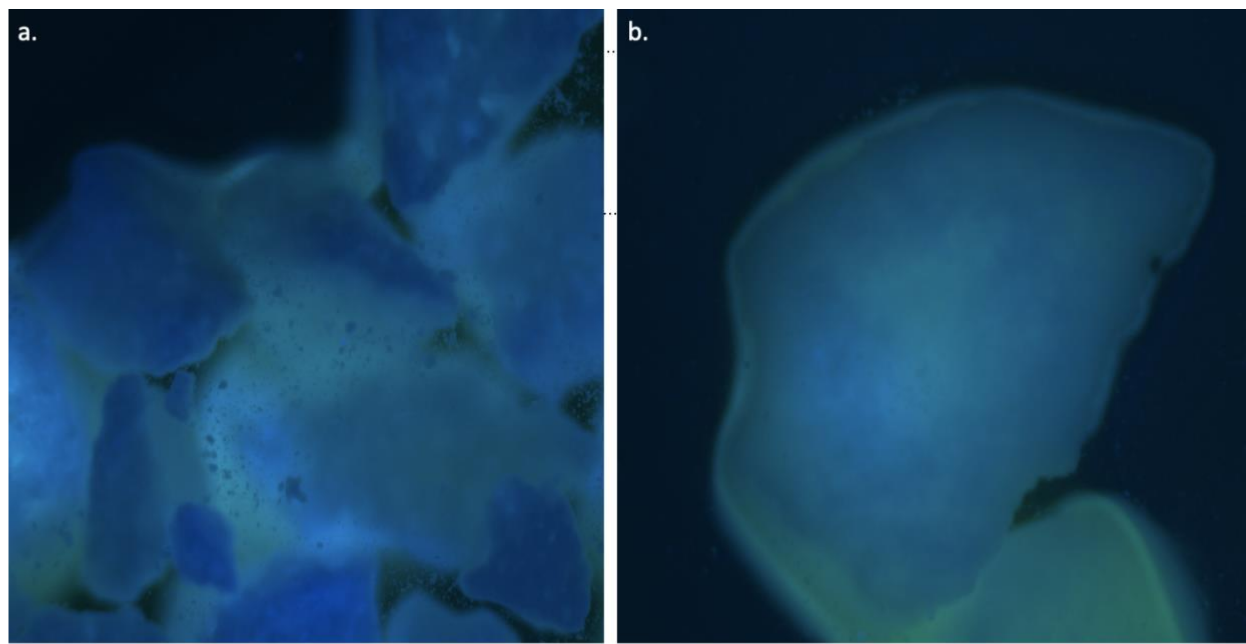

Figure 2.19 Negative controls. a) Monetite granules co-culture with RPMI-1640 media alone (- control) for 5 days at 5X magnification b)HA granules co-culture with RPMI-1640 media alone (- control) for 5 days at 5X magnification

In order to validate all the experiments, a negative control is always needed. In these sets of experiments, a negative control was carried out to make sure that the blue stain attached to the granules are cells instead of random bacterial infection or crystals. As mentioned earlier, granules itself can have autofluorescence. Figure 2.18 shows the negative control in this study, no cells were observed on any of the negative control, but the monetite and HA granules showed autofluorescence in the Live-Dead Assay.

Chapter 6: Discussion:

6.1summary of objectives:

Calcium phosphate materials have been widely used as bone and teeth implants, coating scaffold to deliver drugs, repopulation of blood vessels and nerves and a lot more. When calcium phosphate biomaterials are used to repair the bone and the body, the initial cell colonization is indeed monocytes and macrophages from the peripheral blood. The primary goal of this experiment is to develop the reproducible 3D microenvironment in vitro using rat peripheral blood leukocytes cells, human THP-1 linage monocytes and macrophages culture with calcium phosphate granules. When implanting calcium phosphate biomaterials inside the body, there are macrophages and other leukocytes colonized inside these materials. It is important to investigate whether these peripheral blood leukocytes could attach, proliferate, and differentiate into the desired WBC cell types to the monetite and HA granules, which are the main source of instant cells colonization after the implantation.

6.2 Summary of findings:

Washing cells with PBS, as well as washing all the biomaterial granules with PBS at least three times to reach a neutralized pH prior to the 3D cell culture is crucial for proper leukocytes attachment, growth and even differentiation. When the local acidity is too high, biomaterials' ability to grow cells is hindered. Acidic biomaterials can negatively impact the rats' and human's WBC growth and viability.[161] Although facing numerous challenges, I was able to find the stable 3D in vitro culture parameters for leukocytes co-culture with biomaterials' granules. I was also able to produce the reproducible quantitative result of both rat and human leukocytes attach and grow on the calcium phosphate monetite and HA granules. Both human and rat leukocytes are able to attach to the monetite and HA granules very well and proliferate better compared to cells growing alone. However, monetite granules provide the best 3D microenvironment for rat leukocytes, and THP-1 monocytes to survive and proliferate as they have the highest MTT absorbance OD=590nm readings, hence the highest cell viability and proliferations compared to cells culture with HA granules and cells growing alone. Monetite granules also enhances human macrophage proliferation and perhaps differentiation. Nonetheless, HA granules provide the best microenvironment for human macrophage proliferation at 48hrs, as it has the highest ELISA IL-1 beta protein concentrations as well as the highest cell counts using Image J cells batch processing.

6.3 Interpretation:

Washing both cells and granules with PBS is an important leukocytes cell culture parameter to generate the reproducible in-vitro cell culture. 3D cell culture microenvironment is an overly complex system that requires a thorough understanding of the cell growing mechanism. There are a lot of factors that play major roles in cell culture. The current cell culture parameter is 37°C, 5% CO_2, 95% humidity and make sure the cells are not under hypoxia, monitor cell densities to avoid overcrowd of the cells, as well as to use 10%FBS, 1% anti-fungal RPMI-1640 media. The lab station should be properly sterilized, and limited incubator sharing with others. The cells and the granules should also be washed with PBS multiple times ensure the neutral pH for proper cell attachment, especially phagocytic cells such as monocytes and macrophages. This finding is fundamental to create the pH sensitive biomaterial coating drug delivery system. As for the granules and all the tools, lab equipment used to handle cells and granules should be autoclaved to avoid any contamination. Contamination is a big problem for all cell culture and could significantly disturb the overall result. Both rat leukocytes and human monocytes, macrophages could attach to the granules very well in 24hrs, thus we can confirm that leukocytes attach to biomaterials in a rapid manner. Calcium phosphate biomaterials will attract rat leukocytes, human monocytes, and macrophages attachment. Quantitively, monetite and HA granules promote both rat and human leukocytes' cells proliferation and increase cytokine release as well as cell numbers in vitro. In addition to macrophages differentiating into osteoclast to reshape bones and teeth, macrophages also play a significant role in phagocytic activities that contributes to normal bone and wound healing as well as muscle regeneration. Inadequate transition from M1 pro-inflammation state to M2 anti-inflammation and healing state leads to osteoporosis and delayed healing and regenerations. It is also deducted that these biomaterials can promote monocytes to osteoclast, dendritic cells, and macrophages differentiation, as well as macrophages M1 to M2 transition, because more cells are observed, and more cytokines are being produced. By promoting endogenous healing and regeneration, optimizing the ratio of pro-and anti-inflammatory macrophages responses can help tissue and biomaterial engineering strategies.

6.4 Limitations:

There are other complications occurred that had massive impact on the results of cell culture, such as bacterial and fungal infections happened in the lab, which was resolved by cleaning the

incubator and changed the filter completely. The observation of cells was also challenging because monetite granules, HA granules, pyrite phosphate granules themself have autofluorescence and are non-transparent 3D structure. It makes it impossible to observe the granules and its 3D structure under the normal light microscope because light cannot pass through. As well as it was particularly challenging to observe under the non-inverted fluorescence microscope. This problem was resolved at the beginning of the second year with the modern technology of ECO PRO® microscope, which can observe the plate directly and gave the possibility to characterize granules directly on the plate with fluorescence. The data transmission, photo capturing and data acquiring were also much easier and faster with the new microscope. Cell culture conditions and reproducibility was a big challenge as well. Especially with the rat leukocytes separated from the peripheral blood using Ficoll density centrifugation. There were complications such as only a small number of cells were collected, centrifugation did not work, machine broken. Sometimes when the centrifugation did not work well, the peripheral blood collected from the rat was wasted and a lot of time was wasted as well. That is the reason to use the commercial linage human THP-1 cells for the human leukocytes' in vitro experiments. Handling with the small granules was also very demanding. Time and extra care are needed to practice handling and transferring granules as they became very sticky after they made contact with the cells and the media. Granules are also very fragile and easily broken apart when any force is applied, which will result in more crystals produced. It is ideal to avoid transferring or touching the granules after they had been in contact with the cells, PBS, or media in order to get the best result. In addition, there are other difficulties associated with the cell culture, for example, the sterility of the 3D microenvironment, pH of the cell culture, pH of the granules, culturing parameter such as oxygen level, different FBS level, culturing chamber specifications, cell densities, biological variation, viability were problems need be solved regarding the cell culture. In the end, I was able to conquer these challenges to produce the reproducible cell culture and reproducible result. Cell number was another big challenge in my experiment. Initially, when I stained rat leukocytes trypsinized from the granules with H&E, there were extremely limited number of cells observed. It is also due to the fact that only a small amount of peripheral blood could be collected from rats due to the practical limitations that every time the rat size is different, thus blood volume collected sometimes is small, as well as the ethical limitations that collecting rat peripheral blood need to respect the according animal rights ethical protocols. The small sample size made it extremely difficult to get reproducible data. The best way

to culture both rat and human leukocytes with biomaterials is to wash the cells and the autoclaved granules thoroughly with PBS at least 3 times to ensure the neutral pH and sterility. The washing step reduces the cell cytotoxicity, which increases the cell numbers, viability, and the ability to attach to the biomaterials in the in vitro 3D microenvironment rats and human leukocytes' conditions. Quantification of the cell culture was merely impossible in the beginning as only the limited viable cell numbers was observed. However, this was resolved with the repeatedly washing step and switched to linage cells. A large number of cells was cultured and attached to the biomaterials. Furthermore, more literature review was done, and the experiment was redesigned with the addition of MTT and ELISA assay in order to get the quantification result.

6.5 Future Recommendation:

After successfully create the reproducible 3D rat leukocytes culture with different biomaterials, the small rat blood volume and small cell number can be substituted with the larger volume of human blood. Human blood can be easily collected but ethical considerations need to take place. It is important to do this experiment again with human peripheral blood to verify if they have the same result as the linage cells. In addition, for the future experiment extension, more time and effort need to be put in to repopulate the human monocytes and macrophage and co-culture them with pyrite phosphate, as well as to develop a better systematic technique to stain cells, visualize cells, count cells, differ cells from the autofluorescence pyrite phosphate granules. It is also needed to develop a better immunohistochemistry approach to be able to stain the granules directly and acquire a more comprehensive quantitative data. It is expected to identify exactly what cells attach to the granules in order to see if the granules are selective to bind to certain types of leukocytes. It is yet to conclude if certain types of biomaterials promote a certain type of leukocytes differentiation. In addition, if we can positively identify if monetite or HA materials promotes certain types of monocytes differentiation, especially with an interest on M1, M2 macrophages. These biomaterials can potentially be used for drug delivery coating or implants to combat osteoporosis, as well as to promote wound and bone healing, muscle, tissue, and vessel regenerations for a speedy injury repair. One of my lab partners also work on the in-vivo experiments, where to implant monetite and HA granules onto rat's skull to observe if these biomaterials can improve the skull's wound healing. In addition, repeatedly micro-CT scans were performed to examine these rats' vessels and nerves' repopulation.

6.6 Conclusion:

Successfully generate reproducible in-vitro 3D cell culture can have a wide variety of applications. Thorough understanding of the 3D microenvironment can help Increase the in-vitro cell culture cell survival rate and proliferation rate, which can used to design the bioreactors with the best culture conditions to generate a large number of viable cells. This can benefit patients who require cell therapies such as CAR-M, CAR-T cell therapies. Washing cells and the biomaterials with PBS to neutralized pH is crucial in designing any 3D in vitro cell microenvironment. Rat leukocytes, human THP-1 linage monocytes and macrophages all have shown a very rapid attachment to the calcium phosphate granules. In addition, an increase in cells' proliferation and cytokine release are also observed when these leukocytes co-culture with monetite and HA granules. It is further expected that calcium phosphate materials can promote distinct types of leukocytes differentiation, but better immunohistochemistry staining procedure, as well as better identification of leukocytes, and quantification method need to be developed to validate this finding in the future. More importantly, switching from using human linage cells to leukocytes separated from healthy and unhealthy human peripheral blood in 3D in-vitro cell culturing is also required for the future study.

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15. Author links open overlay panelKrishnamoorthi Sumaiya a, a, b, c, AbstractMacrophage migration inhibitory factor (MIF) is a proinflammatory cytokine encoded within a functionally polymorphic genetic locus. MIF was initially recognized as a cytokine generated by activated T cells, Kleemann, R., Kerschbaumer, R. J., Karsten, E., Jung, H., Jose, S., Jankauskas, S. S., Jager, B., Ito, T., Israelson, A., Hare, A. A., Gu, R., Farag, A. G. A., El-Mahdy, R. I., Delaloye, J., … Calandra, T. (2021, October 19). *Macrophage migration inhibitory factor (MIF): A multifaceted cytokine regulated by genetic and physiological strategies*. Pharmacology & Therapeutics. Retrieved March 28, 2023, from https://www.sciencedirect.com/science/article/abs/pii/S0163725821002266
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