UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Fisica e Astronomia "Galileo Galilei"

Corso di Laurea Triennale in Fisica

Tesi di Laurea

Characterization of the role of reactive oxygen species in lymphocyte function: a possible implication in the effect of microgravity on the immune system

Caratterizzazione del ruolo delle specie reattive all'ossigeno nella funzione dei linfociti: una possibile implicazione nell'effetto della microgravità sul sistema immunitario

Relatore

Prof. Cinzia Sada

Correlatori

Prof. Denis Martinvalet

Laureanda

Clara Guizzetti

1216633

Anno Accademico 2022/2023

Abstract

Environmental or extracellular exposure to reactive oxygen species (ROS) is particularly detrimental to immune cell effector functions. Extracellular ROS exposure dampens Natural Killer cells, CD8+ and CD4+ T cell functions, which provide responses against tumor cells or virus-infected cells, while enhancing regulatory T cell function and increasing the immunologic tolerance against antigens. Interestingly, similar immunomodulation has been observed during short- and long-term exposure to microgravity (µG). In fact, immunosuppression during spaceflight has been recognized since the Apollo missions and remains a major health risk for astronauts. It is possible that both exposure to ROS and µG could share common mechanisms of immunosuppression. Therefore, the aim of this thesis is to investigate how lymphocytes sense environmental ROS as a first step to dissect the molecular mechanisms involved in this redox immunosuppression. This could lead to a better understanding of the impact of µG on immunity and open the door for the design of preventive measures to improve the health of astronauts for future long spaceflight. Statistical data analysis will be applied, and a physical model investigated.

Sommario

L'esposizione ambientale o extracellulare alle specie reattive dell'ossigeno (ROS) è particolarmente dannosa per le funzioni effettrici delle cellule immunitarie. L'esposizione extracellulare alle ROS smorza le funzioni delle cellule Natural Killer, CD8+ e CD4+, che forniscono risposte nell'eliminazione di cellule tumorali o infettate da virus, mentre innalzano la funzione regolatrice dei linfociti T, aumentando la tolleranza immunologica contro gli antigeni. È interessante notare che analoghe immunomodulazioni sono state osservate durante l'esposizione a lungo e breve termine alla microgravità (µG). Infatti l'immunosoppressione durante il volo spaziale è stata riconosciuta fin dalle missioni Apollo e rimane un rischio importante per la salute degli astronauti. È possibile che l'esposizione alle ROS e ai µG condividano meccanismi comuni di immunosoppressione. Pertanto, l'obiettivo di questa tesi è quello di studiare come i linfociti percepiscono le ROS ambientali, come primo passo per analizzare i meccanismi molecolari coinvolti in questa immunosoppressione redox. Ciò potrebbe portare a una migliore comprensione dell'impatto dei µG sull'immunità e aprire la strada alla progettazione di misure preventive per migliorare la salute degli astronauti in vista di futuri voli spaziali di lunga durata. Verrà applicata un'analisi statistica dei dati e verrà studiato un modello fisico.

Contents

Introduction

Multicellular organisms are exposed to several harmful invaders, collectively called *pathogens*. They defend themselves from these attacks using a sophisticated defense system called *immune system*. Encounter with a pathogen initiates an immune response which relies on both the *innate* and the *adaptive immune system*. Both arms of the immune system collaborate closely to avoid infection caused by millions of pathogens that the human body encounters daily. The immune responses are carried out by quite different elements from proteins and cells, to entire tissues and organs.

We will focus on a particular family of cells, called *lymphocytes*, that play a key role in the human immune response to pathogens and cancer cells. Lymphocytes are white blood cells that can be divided into three classes: *T cells*, *B cells* and *natural killer cells*.

We analyzed particularly the behavior of *cytotoxic T lymphocytes* (CTL) and *natural killer cells* (NK cells). The first is a specific class of T-cells belonging to the adaptive immune system, the second instead is part of the innate one. Among all their tasks, these cells can recognize an infected cell and cause its death by *apoptosis*: the process of programmed cell death.

Interestingly, exposure to microenvironmental ROS severely alters the mechanism used by both CT cells and NK cells to attack and kill their target cells. ROS are a large class of highly reactive chemicals both radical and non-radical forms resulting from the partial reduction of diatomic oxygen (O_2). In normal conditions, they are an essential part to the metabolism of oxygen and contribute to many physiological processes. However, when produced in excess, high concentrations of ROS activate the cellular antioxidant system to protect the cells from oxidative lethal damages. When the cellular antioxidant machinery is overwhelmed, this situation generates a state of oxidative stress in the cell that leads to extensive oxidative damage and eventually cell death.

To investigate how cytotoxic lymphocytes sense environmental reactive oxygen species, we first describe the main mechanisms underlying the action of the immune system and then we will analyze its link with ROS.

Chapter 1

The Immune System

The innate and adaptive immune systems are allied in defeating the same enemy, but these systems are radically different from one another. First, innate immune responses are sometimes referred to as *nonspecific* responses, because they don't act selectively against one pathogen. On the contrary, the adaptive immune system is highly specific to the given pathogen activating it.
Every encounter with an invader is called *immunization*. Not all immunizations trigger the immune response in the same way: in particular, during the first immunization (*primary immune response*) the innate responses are immediate but adaptive ones are much slower, taking days or even weeks to develop. Due to *immunological memory*, the following encounters differ from the first one: they are characterized by a shorter lag time and more efficient response.

1.1 The Innate Immune System

Innate immunity protects us against invaders from birth and is the first mechanism that detects them. A big challenge for the immune system is to recognize and destroy only the pathogens and, for this reason, it has to find a way to discriminate the self from the non-self and to avoid self-harming. To do so, the innate immune system recognizes particular molecules, called *pathogen-associated* or *microbe-associated immunostimulants*, that are common to wide varieties of pathogen families but absent on the host cells. A relevant example comes from the translation initiation in bacteria, which is characterized by formylated methionine[1], that differs from eukaryotic translation initiation, which involves only regular methionine. This difference allows our body to recognize peptides, containing formylmethionine, as bacterially originated [1]. These different microbe-associated immunostimulants can often be found in repeating patterns, called *pathogen-associated molecular patterns (PAMPs)*, that are identified by families of *pattern recognition receptor*, such as *Toll-like receptor family* or *NOD proteins* (1.1.3). We are now introducing the three major ways in which the innate system operates.

1.1.1 Epithelial Surfaces and Mucus Layers

The first line of response involves anatomic barriers, both physical and chemical, in order to keep the largest possible number of foreign microorganisms outside the human body. Physical

[1]Formylmethionine is an essential amino acid derived from adding a formyl group (an organic compound, also known as aldehyde, containing a functional group R-CH=O) to normal amino acid methionine.

barriers consist in the skin and other epithelial surfaces, which are strengthened by a mucus layer and normal flora. Normal flora provides additional protection by limiting the resources available to pathogenic entities due to the fact that they compete for the same ecological niche [1].
On the other hand, mucus is a complex dilute aqueous viscoelastic secretion containing water, electrolytes, lipids, and proteins, most of these consist in mucin [2] and other glycoproteins, which are proteins that contain oligosaccharide chains attached by a covalent bond to its amino acid side chains. The mucus layer also contains defensins [3], antimicrobial polypeptides (12-50 amino acids long) positively charged, that manage to enter the arterial membranes and induce the formation of pores with consequent death by lysis of the pathogen. When microorganisms breach the physical barricades, the second and the third line are triggered.

1.1.2 Cell-intrinsic responses

The second line consists of cell-intrinsic responses by which an individual cell recognizes that it has been infected and takes measures to kill or cripple the invader [4]. An effective defensive mechanism is the degradation of the virus double-stranded RNA (dsRNA) by the host cell. Indeed, the microbe-associated immunostimulants on the surface of bacteria and parasites do not occur on the surface of viruses, so the cell uses some element of the viral genome as a wake-up call, precisely dsRNA [5].
Double-stranded (ds)RNA is generated in host cells during the replication of most viruses. The innate immune system recognizes dsRNA as a PAMP: the host cell detects the presence of dsRNA, it starts a program that occurs in two steps. First, the cell degrades the virus's dsRNA into small fragments (about 21–25 nucleotide pairs in length), using the enzyme *Dicer*. These double-stranded fragments bind to any single-stranded RNA (ssRNA) in the host cell that has the same sequence. The second step consists of the dsRNA inducing the host cell to produce and secrete two cytokines, *interferon-α* (IFNα) and *interferon-β* (IFNβ), the binding of the interferons to related cell-surface receptors leads to the production of more than 300 gene products, including a large number of *cytokines* [5].
Cytokine is a general name for a family of small secreted proteins released by cells. More precisely, this group includes *lymphokine*, cytokines made by lymphocytes, which we will discuss later, *monokine* by monocytes, *chemokine*, cytokines with chemotactic activities, and *interleukin*, cytokines made by one leukocyte and acting on other leukocytes. Cytokines may act on the cells that secrete them, in an autocrine action, on nearby cells with a paracrine action, or in some instances on distant cells, with endocrine action [6].
During this process, the cell is affected by all cytokines. In particular, interferon presence activates a *latent ribonuclease* and a *protein kinase*. The ribonuclease is a particular type of nuclease[2], which is responsible for cleaving ssRNA nonspecifically, a process that leads to the destruction of both degraded bacterial ssRNA and ssRNA from the host cell bound to it.
The protein kinase selectively modifies other proteins by covalently adding phosphates to them, in a process termed phosphorylation. In this case, it changes the protein synthesis initiation factor eIF-2, inhibiting protein synthesis.
Therefore, the cell tries to resolve the infection by turning off all protein synthesis and by

[2]Nucleases are a family of enzymes capable of breaking the phosphodiester bonds between nucleotides of nucleic acids

destroying most of its RNA. If this mechanism fails the cell commits suicide by apoptosis to prevent viral replication and spreading [5].

Apoptosis
Apoptosis is the most common and best-understood type of programmed cell death. It is not just an essential weapon in the fight against pathogens, but is a crucial part of our growth: it kills unwanted cells during body formation, sculpting hands and feet during embryonic development or it regulates cell death in the developing nervous system [7].
In this thesis work, cells that undergo this process has been recognized using the following procedure. We can certainly identify them thanks to their shrinking, which is followed by cell *pyknosis*. Pyknosis is the most characteristic feature of apoptosis and consists of the irreversible condensation of chromatin in the nucleus, a complex of DNA and protein that package long DNA molecules into more compact ones. Therefore the first visible manifestations are that the cell stars become smaller in size, the cytoplasm is getting denser and the organelles are tightly packed. Next, an extensive plasma membrane blebbing occurs, followed by *karyorrhexis*, which is a destructive fragmentation of the nucleus, and separation of cell fragments into apoptotic bodies[3] [8].
The pathways that lead to apoptosis are very different and they depend on the stimulus that initiates it, but in all of them, the key effector molecules are *caspases*. They belong to a family of proteases, that have a cysteine at their active site. Proteases are enzymes that catalyze the hydrolysis of the peptidic bounds between amino acids to break down proteins into smaller polypeptides or single amino acids. Given the fact that unregulate caspase activity would be lethal for the cell, these enzymes are expressed as an inactive precursor called *procaspase*. This precursor needs to be activated by cleavage at specific aspartic acids[4] to change into active caspase. The more active caspase, the more active caspase will be able to cleavage the procaspase. This is the reason why the mechanism that is involved in apoptosis is called caspase cascade. Once activated caspase will cleave various cellular substrates leading to the demise of the cells [7] [9].

1.1.3 Phagocytic cells

Another innate defense relies on the *phagocytes*. Phagocytes can be classified as professional or non-professional depending on how effective they are at phagocytosis. The main difference between professional and non-professional phagocytes is that professional phagocytes have *pattern recognition receptors* (PRR), on their surfaces. One of the most important receptor families is the Toll-like receptor (TLR) family. Humans have at least 10 TLRs, due to the fact that different ligands activate different TRLs.
They are single-pass type I transmembrane proteins and most of them are located on cell surfaces. They act as an alarm system by binding the PAMPs that reveal the presence of the pathogen. This bind activates a variety of signaling pathways that promote the inflammatory response by inducing the expression of numerous genes, including gene encoding for inflammatory cytokines, chemokines, antimicrobial molecules, and *major histocompatibility complex (MHC)* [10], a group of genes that code for *class I and II MHC proteins*. These are two crucial

[3] cytoplasm with tightly packed organelles with or without a nuclear fragment
[4] an α-amino acid that is used in the biosynthesis of proteins

classes of proteins that share the task of presenting peptides on the cell surface for recognition by both T-cells and NK cells [11], which we will discuss later.
A second family of PRR is called *NOD proteins*, they work similarly to TLRs but recognize a distinct set of ligands.

The two main phagocytes are *macrophages* and *neutrophils*.
Macrophages are long-lived cells, that phagocytose bacteria and secrete both pro-inflammatory and antimicrobial mediators. They form from *monocytes*, which themselves derive from the bone marrow. Monocytes are a type of blood cells that circulate through the blood for one to three days before migrating into tissues, where they differentiate into macrophages or *dendritic cells*, a type of antigen-presenting cell that plays a key role in linking the innate and adaptive immunity. Macrophages are abundant in areas where infections are likely to arise.
Neutrophils are, on the contrary, short-lived cells. They are granulocytes, easily identified by their granular cytoplasm and lobulated nuclei. They are not present in healthy tissues, but they are abundant in blood and rapidly recruited at the site of infection upon macrophage activation and production of pro-inflammatory cytokines [12].

When a professional phagocyte binds a receptor that mediates the phagocytosis, the *actin polymerization* is induced at the site of the pathogen attachment. An actin protein is a globular multi-functional protein. It acts as the monomeric subunit of two types of filaments in cells: microfilaments, one of the three major components of the cytoskeleton, and thin filaments. On the other hand, actin polymerization is a reversible process, in which monomers both associate with and dissociate from the opposite ends of the actin filaments. This process allows the phagocyte plasma membrane to surround the pathogen and engulf it in a large membrane-enclosed phagosome.
When macrophages or neutrophils engulf pathogens, they deploy an impressive arsenal to kill them. The location of the phagocyte's weaponry is readily visible in the light or electron microscope as dense membrane-enclosed organelles called *granules*.
These granules are specialized lysosomal derivatives[5] fuse with the phagosomes, delivering enzymes that can degrade the pathogen's cell wall and proteins. The granules also contain defensins, the antimicrobial peptides that makeup about 15% of the total protein in neutrophils. In addition, the phagocyte assembles *NADPH oxidase complexes* on the phagolysosomal membrane, which catalyzes the production of highly toxic oxygen-derived compounds. Even at the end of the attack, the triumphant phagocytes are not useless. The contact with pathogens put the phagocyte in a state of high alert, in which not only is it more effective at phagocytosing and killing pathogens, but it also releases cytokines to attract more white blood cells to the site of infection [12].

1.1.4 NK cells

Other essential elements of the third pathway of the innate immune response, and one of the main subject matter of this thesis, are *Natural Killer Cells*. They are large ($4\mu m$ diameter)

[5] A lysosome is a membrane-bound organelle found in many animal cells. They are spherical vesicles that contain hydrolytic enzymes that can break down many kinds of biomolecules.

cytotoxic lymphocytes that represent 5–20% of all circulating lymphocytes in the human body.

They display a large, dark-staining nucleus, and in their cytoplasm, separated by a bi-layer membrane, some lytic or cytotoxic granules can be found. These granules are specialized *secretory lysosome* and they contain perforin, granzymes, Fas ligand, granulysin, a small antimicrobial peptide [14]. When the NK cells interact with their target cells, both NK cells and target cells form effector-target cell conjugates which are separated by a small space called immunological synapse. Perforin, a multi-domain and pore-forming protein, is released in synaptic clef from where it inserts, and forms pores in the target cells membrane, to facilitate the entry of granzymes into the target cell. Granzymes are serine proteases[6] which activate caspase molecules leading to the induction of apoptosis of target cells in a process that we will soon discuss.

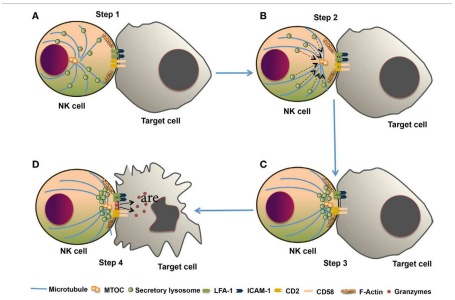


Figure 1.1: The cytotoxic response of natural killer (NK) cells [13]

Another important factors, in the Nks play, are the membrane proteins. LAMP-1 is a particular lysosomal-associated membrane protein, widely used as a marker to follow NK-cell degranulation. Cell surface expression of LAMP-1 serves as a ligand for selectins[7] and for this reason, they help mediate cell-cell adhesion.

Before initiating the cytotoxic response, the NK must first be activated, in other words, we need the NK to understand that the cytotoxic pathway has to start. The Tyrosine phosphorylation of substrates by Src-family tyrosine kinases (SFK) represents the first step in NK and T cell activation. Among all members of this family, *Lck* is central to signal transduction downstream of the TCR and NK cell-activating receptors. After receptor-ligand interaction, Lck is recruited close to the immunological synapse as it interacts with the co-receptors such as CD4 or CD8 in T cells and CD137 or NK1.1 in NK cells. The knockdown of Lck resulted in significant reductions in NKG2D and, CD137-mediated cytotoxicity and cytokine production by NK cells, but no change in the cytokine production mediated by IL-12 and IL-18 stimulation. This suggests that Lck playing a dominant role downstream of some, but not all activating receptors [15]. Once activated, there are at least two different processes that the NK uses to kill the target cell. The first implies the use of the death receptor pathway as the result of binding between the death receptor protein Fas/CD95 on the target cell and the Fas ligand on the NK cells. The second mechanism involves the cytotoxic granule pathway [16]. We will focus on the cytotoxic granules pathway in this thesis work as it the main pathway used by the cytotoxic lymphocyte

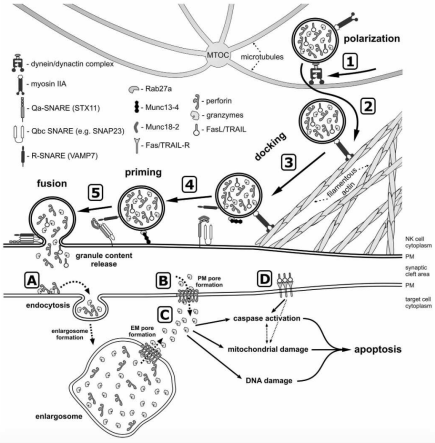


Figure 1.2: the exocytosis process [14]

[6]enzymes that cleave peptide bonds in proteins

[7]family of cell adhesion molecules

to kill their target. There are four main events that characterize this pathway:
1) The process begins with the formation of *immunological synapse*[8] followed by a reorganization of the *actin cytoskeleton*, which is a dynamic network made up of actin polymers and associated actin-binding proteins. This element in combination with the other parts of the cytoskeleton, including intermediate filaments and microtubules, is responsible in maintaining the cell structure, axonal growth and cell migration [17];
2) In the meantime the polarization of the microtubule organizing center (MTOC) occurs. MTOC is a protein complex from which the microtubules emerge. This step is fundamental for granules' movement around the NKs body. Indeed, we can think of microtubles as the rail on which that granules can travel in order to reach the immunological synapse;
3) As far as the MTOC assumes the right position, the granules start migrating and reach the immunological synapse. This entire process, which concerns the granules migration, is known as *granule polarization*;
4) In the fourth stage of NK cell attack, the actin network, which is coating the cyotplasmic side of the presynaptic membrane (of the NK cell membrane engage in the conjugate formation), reorganizes to allow the arriving of cytotoxic granules to contact the NK cell membrane. Once this contact happens, the fusion of cytotoxic granules with the presynaptic membrane of the NK cells begins, a process called *exocitosys*. Therefore, perforin and granzyme, are released in the synaptic cleft from where perforin facilitate granzyme entry into the target cells cytosol as described earlier [13][14]. Although, perforin alone is not sufficient to induce target cell death, but it remains essential for both NK and CTLs killing ability. Indeed, perforin-deficient mice demonstrated profound T cell dyscrasia despite quantitatively normal T cell development and a normal thymus but severe incapacities of NK effector cells [16]. The other factors that contribute to immune cell-killing ability are granzymes: the key to apoptosis initiation. Five different granzymes have been described in humans: granzymes A, B, H, K and M. We will focus on Granzyme A and B as they are the most abundantly expressed and best characterized.
Granzyme A induced cell death is mainly characterized by the generation of single-stranded DNA nicks and does not result in the activation of caspases. This process requires the loss of mitochondrial inner membrane potential and the release of reactive oxygen species (ROS) [18].
Granzyme B is the most abundant granzyme, and it induces apoptosis indirectly through a mitochondrial pathway: to induce mitochondrial changes, granzyme B cleaves the BH3-only pro-apoptotic protein Bid[9]. This cleavage results in the activation of pro-apoptotic proteins that trigger the mitochondrial outer membrane permeabilization. In addition to Bid, granzyme B can induce *cytochrome c* release, which is crucial in caspase activation and apoptosome[10] formation. [18] [20].
As said before, both NK cells and cytotoxic T cells recognize a special class of proteins located on the cell surface, called *major histocompatibility (MHC) class I proteins*. The mechanism that these two elements establish, NKs and T-cells, is complementary and very effective. Cytotoxic T cells recognize peptide fragments of viral protein bound to these MHC proteins on the surface of the infected cells. However, many viruses have managed to avoid the expression of such proteins in order not to be traced by cytotoxic T lymphocytes. Here the NK cells come into action by monitoring the level of all the MHC proteins on the surface of all host cells and by attacking only cells expressing low levels. This process goes under the name of *Missing Self*

[8]The immunological synapse consists in a junction between the infected cell and the NK cell
[9]proteins that are effectors of canonical mitochondrial apoptosis [19]
[10]a large quaternary protein structure, that can recruit and activate some caspase

Hypothesis and rule our understanding of how NKs operate. With this scheme, an infected cell is recognized by T cells if it has a large number of MHC proteins, while it is recognized by NK if it tries to evade the T cells by reducing the expression of class I MHC proteins. In this thesis, we will use some target cells that show a deficiency of MHC proteins in order to trigger the Missing Self Mechanism.

1.2 The Adaptive Immune System

Adaptive immune responses are carried out by white blood cells, called *lymphocytes*. White blood cells are a cellular component of the blood that lacks hemoglobin. They have a nucleus, in contrast to other blood cells, and they are capable of motility. These cells allow the adaptive response to be highly specific by distinguishing between very similar *antigens*: from proteins that differ in only one amino acid to two optical isomers of the same molecule.
We refer by the name of antigen to any substance capable of raising an adaptive immune response. On their surface, we can identify parts called *antigenic determinants* or *epitopes* that bind to receptors on lymphocytes.

Lymphocytes are concentrated in lymphoid organs, such as the thymus, lymph nodes, spleen and appendix. There are about $2 \cdot 10^{12}$ lymphocytes in the human body and they develop from the same *common lymphoid progenitor cells*, which in turn derives from *hemopoietic stem cells*. The homopoietic stem cells which reside in bone marrow, can give rise to all of the blood cells: red blood cells, white blood cells and platelets. The bone marrow and the thymus are the *primary lymphoid organs* because they are sites where lymphocytes develop and differentiate from precursor cells. An astonishing fact is that as soon as the lymphocyte develops in a primary lymphoid tissues, they becomes committed to reacting with a particular antigen before ever being exposed to such antigen. Most lymphocytes remain in this primary lymphoid organs, others mature and migrate via the blood to the *secondary lymphoid organs*, to be activated by foreign antigens. Once there, the binding of the antigen to the receptors activates the lymphocyte, causing it to proliferate, thereby producing many more cells with the same receptor in a process called *clonal expansion*. The encounter with antigen also causes the cells to differentiate into *effector cells*. An antigen therefore selectively stimulates those cells that express complementary antigen-specific receptors and are thus already committed to responding to it. This is called the *clonal selection theory* [21]. Lymphocytes occur in different stages of maturation. They can be found as *naive cells*, *effector cells* or *memory cells*. The naive stage is the one that precedes the encounter with the antigen. The antigen causes it to proliferate and differentiate into effector cells, which perform the adaptive response. Not all naive cells develop into effector cells, some differentiate into memory cells, which are more easily turned into effector cells during the next or subsequent encounter with the same antigen. The peculiarity of these memory cells is that they also persist for a lifetime, unlike effector cells that die in a few days or weeks after the resolution of the infection. There are three classes of lymphocytes: *B cells*, *T cells* and *NK cells*. We have already described the NK cells role within the innate immune response. The remaining B and T cells carry on the adaptive immune responses. B cells perform *antibody responses* although T cells perform *T-cell-mediated immune responses*. We will focus on the T-cell-mediated immune responses.

1.2.1 T cell-mediated immune responses

T cells develop in the *thymus*, the organ from which they owe their name. Unlike B cells, T cells monitor and eliminate pathogens that are inside the host cell, that would otherwise be invisible. They are produced and endure in a naive state throughout their lives unless an *antigen-presenting cell (APCs)* crosses their path. Indeed, T cell recognize antigens that have been partially degraded and that are presented on MHC proteins.
Now, let proceed and explore the mechanisms by which T cells recognize and bind MHC proteins. The T cell receptor called (TCRs) is never secreted, but express at the surface of the T lymphocytes, is directly involve it the T cell antigen recognition process. The TCRs consist of an $\alpha\beta$ heterodimer disulfide-linked polypeptide chains, containing two Ig-like domains. Each monomer contains one variable and one constant portion. The naïve T cells recognize the antigenic peptide presented by the MHC molecules on the surface of an antigen presenting cells (APC). APCs are unique in their ability to activate the naïve T lymphocytes thanks to the co-stimulation they also provide. The engagement of the APC PRR by the pathogen- associated molecular patterns (PAMPs) leads to their activation, a critical step in for their maturation which is essential for their ability to activate the naïve T cells. Once activated, the naïve T cells experience clonal expansion and differentiate into effector T cells which interact directly with infected host cell, using the same mechanism. This interaction results in either the killing of the target cells or a signal in some way. After the activation, some of the T cells migrate via blood to the site of infection (where the dendritic cell-antigen encounter occurred), while others remain in the lymphoid organ [22].

Dendritic cells (DCs) are among the most important APCs located in tissues throughout the body. They display a large variety of PRRs, including TLRs and NOD proteins. When they encounter a pathogen on their way, these receptors engagement with their cognate antigens to activate the DCs and enable them to phagocytose the invading pathogens. The DCs partially digest proteins of the engulfed pathogens into peptide fragments, which then bind to MHC proteins that carry them to the surface for presentation to the T cells.

The encounter between the DCs and the antigen may happen either at the infection sites or directly in the secondary lymphoid organs. For this reason, dendritic cells are called *migratory APCs*. In addition to MHC proteins, dendritic cells display on their surface two additional molecules: *co-stimulatory proteins*, which bind to complementary receptors on the T cell surface; *cell-cell adhesion molecules*, which maintain the binding with the T cell for long enough to ensure proper T cell activation, a process that takes hours [23] . T cells are grouped into a series of subsets based on their function: *cytotoxic T cells*, *helper T cells* and *regulatory T cells*.

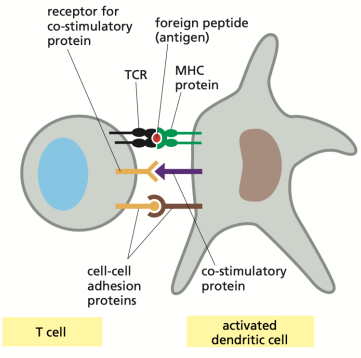


Figure 1.3: Link between dendritic cell and T cell [23]

Once activated, **cytotoxic T cells** kill any target cell harboring the same antigen. T cell first recognizes and then binds the MHC protein on the surface of the infected cell. After the binding, the T cell reorganizes its cytoskeleton and internal structure with the aim of redirecting its killing apparatus against the target cell as we have described for the

NK cells. The centrosome[11] in the T cell is positioned at the point of cell-cell contact. Like NK cells, CTL can kill their target either through the death receptor pathway or the cytotoxic granule pathway, the later being the most proficient as described for the NK cells.
Helper T cells produce cytokines to initiate immune responses from other white blood cells. They also activated macrophages to destroy any intracellular pathogens and can also stimulate dendritic cells to maintain an activated state. The last major type of T cells is the **regulatory T cell**, these cells modulate the activity of other lymphocytes and inhibit autoimmune processes. In these ways, they prevent adaptive immune responses from damaging host tissues [24].

MHC proteins As said before, cytotoxic T cells and helper T cells are initially activated in peripheral lymphoid organs through the recognition of MHC peptide complexes on the surface of the antigen-presenting cells. The MHC molecules are glycoproteins encoded in the major histocompatibility complex loci: a cluster of genes located on chromosome 6. Humans possesse at least 12 genes encoding these proteins. Two separate properties of the MHC make it difficult for pathogens to evade immune responses. There are two main classes of MHC proteins: class I MHC proteins and class II MHC proteins. The MHC is polygenic with several different MHC class I and MHC class II genes. Every individual possesses a set of MHC molecules with different ranges of peptide-binding specificities. Second, the MHC is highly polymorphic. There are multiple variants of each gene within the population. The MHC genes are, in fact, the most polymorphic genes known to date [25].
MHC class I and class II share a similar 3D structure. They are both transmembrane heterodimers[12] with extracellular N-terminal domains. At the end of these molecules, we can find a single *peptide-binding site* facing away from the plasma membrane. The binding region consists of a deep groove formed by protein domains.
Class I MHC proteins are assembled from transmembrane α-chains, folded in three extracellular domains (α_1, α_2, α_3) and a small extracellular protein called β_2-microglobulin.

Class II MHC proteins consist of two Ig-like domains close to the membrane (α_2, β_2), and two variable N-terminal domains farthest from the membrane (α_1, β_1). Because of the different conformation, these two types of proteins can bind different peptides. Class I MHC antigen binding groove narrows at both ends, so it can accommodate peptides about 8-10 amino acids long antigenic peptide. Class II MHC proteins groove does not narrow so it can accommodate longer antigenic peptides, usually 12-20 amino acids long [11].
A typical peptide binds in the groove of a class I MHC protein is an extended conformation. The antigenic peptide amino and carboxy-terminal groups bind to invariant amino acids located at the extremities of the MHC antigen-binding groove. Some amino acid side chains of the peptide bind to the variable

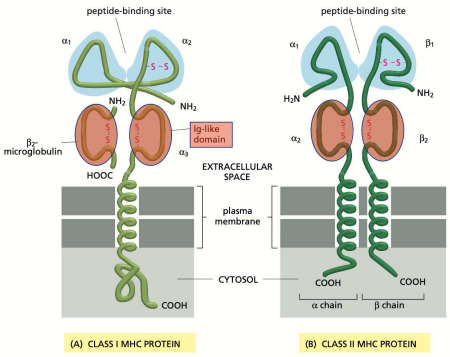


Figure 1.4: Class I and II MHC protein structure[23]

[11]The centrosome is an organelle that serves as the main microtubule organizing center (MTOC)
[12]a protein composed of two polypeptide chains differing in composition in the order, number, or kind of their amino acid residues

amino acids of the MHC protein distributed along the groove, while other side chains point outward, in a position to be recognized by TCRs on cytotoxic T cells [23]. The binding of TCRs to peptide–MHC complexes on an APC or target cell is usually too weak by itself to mediate a functional interaction between the two cells. Indeed, as mentioned before, T cells normally require *accessory receptors* to help stabilize the interaction by increasing the overall strength of the cell–cell adhesion. Accessory receptors cooperate in activating the T cell by generating their own intracellular signals. *CD4* and *CD8* are both single-pass transmembrane proteins with extracellular Ig-like domains. CD4 is expressed on the surface of both helper and regulatory T cells, instead of CD8 is found on cytotoxic T cell surfaces.
Both of them act as co-receptors for their T cells, but they recognize different types of MHC proteins by binding the invariant part of the peptide chain, far away from the binding groove. CD4 binds to class II MHC proteins and CD8 binds to class I MHC proteins.
The cytoplasmic (intracellular) tail of the CD4 and CD8 proteins is associated with a member of the Src family, *Lck*, which participates in the activation of the T cell likewise in the NK activation. Due to the fact that CD4 and CD8 are attracted to the immunological synapse during antigen recognition, Lck are brought into proximity with a TCR that is recognizing antigen (in the process that we have called receptor clustering), where it kick-starts the signaling cascade [26].

Chapter 2

ROS

Proofs of how intricate the study of the human body is can be found in the *oxygen paradox*: aerobic eukaryotes are organisms that cannot survive without oxygen, nevertheless, oxygen is naturally dangerous to their survival. The catch with oxygen lies in its electronic configuration. Each oxygen atom (O) has one unpaired electron in its exterior valence shell and molecular oxygen (O_2) has two unpaired electrons. This makes atomic oxygen a free radical and molecular oxygen a (free) bi-radical [27]. Reactive oxygen species (ROS) are generated due to partial reduction of O_2 or as a result of the transfer of energy to O_2. The ROS family mainly comprises of singlet oxygen $^1O_2$, hydrogen peroxide H_2O_2, superoxide $O_2^{\bullet -}$ and hydroxyl radical $OH^{\bullet}$ [28] [29].
Some of these molecules contain unpaired electrons and thus belong to *oxygen free radicals*, any chemical species capable of independent existence that contains one or more unpaired electrons belong to a free radicals class. Superoxide anion $O^{2-\bullet}$, hydroxyl radical and nitric oxide NO belong to this group. In contrast, ROS like hydrogen peroxide, hypochlorous acid HOCl and peroxynitrite $ONOO^-$ do not contain unpaired electrons, but they are still powerful oxidizing agents.
Cellular redox homeostasis is the essential process involved in ensuring the balance between reducing and oxidizing reactions within cells and regulates a plethora of biological responses and events. This is a highly dynamic machinery, based on a highly responsive system that senses changes and realigns quickly the metabolic activities in order to restore redox homeostasis [30]. Our physiology strongly depends on the presence of ROS. These molecules are determinants in countless processes that occur in the human body daily.
We can start by mentioning the oxidative burst: one of the first lines of defense against environmental pathogens, or the fact that the production of ROS regulates the initiation of inflammatory response [27]. But their action is not only confined to the immune system, indeed ROS are involved in signal transduction, cell migration, differentiation and proliferation, vasoconstriction, inflammation, senescence aging and cell death.
An important field in which we can appreciate the role of ROS is *cell signaling*. Cell signaling is the ability of a cell to detect, process, and transmit signals and changes in its environment and to generate an appropriate physiological response upon information processing.
This process typically involves three major steps. It starts with the binding of the extracellular ligand to its cognate receptor protein on their surface, this is the case in which cytokines, secreted by professional macrophages, enhance an antibody response. When a signaling molecule binds to its receptor, it alters its shape or activity. This activated receptor in turn results in the activation of one or more intracellular signaling pathways involving a series of signaling

proteins. These pathways involve intracellular signaling proteins that spread the signal from the surface of the cell to the appropriate intracellular target. Lastly a protein lies, it is called *effector proteins* and starts a cellular response [29].
In this context, ROS can cause redox modulation, by modifying the balance in the redox status that is characterized by alterations in cellular dynamics (*redox modulation*) of signaling proteins. By using this type of modulation ROS is strictly involved in the proliferation and differentiation of cells either directly or indirectly by modulating the redox status of the cell component and by regulating the vital transcription factors associated with cellular proliferation and differentiation [31]. We have observed the significance of this element within the human system; however, it also poses a threat to our well-being. If not properly controlled, they can enhance an oxidative process that results in unstable molecules with a deficit in electrons. Therefore, the alarming side of ROS presence in our system lies in their redundancy. Their excess is called *oxidative stress* and it can lead to irreversible modifications, such as extensive protein oxidation and lipid peroxidation, causing oxidative damage, cellular degeneration, and even functional decline. The brain, with its high oxygen consumption, is highly susceptible to oxidative stress and this stress has serious implications for central nervous system (CNS) functioning. Indeed, evidence of increased brain oxidative damage has been reported for neurodegenerative diseases, including Alzheimer disease, Parkinson's disease, amyotrophic lateral sclerosis, cerebrovascular disorders, and psychiatric disorders[32]. This evidence goes hand in hand with the fact that ROS contributes to many cell death pathways. ROS are in fact a common determinant of all forms of cell death [33].

Given this danger, even though the essential role of the ROS, the human body developed an antioxidant response system. The main actors in this process are molecules called *antioxidants*. This compounds delays, prevents, or removes oxidative damage by directly scavenging ROS or indirectly inhibiting ROS production. Antioxidants can be classified as enzymatic or non-enzymatic antioxidants. Catalase, superoxide dismutase, glutathione peroxidase and thioredoxin enzyme system are enzymatic antioxidants. Ascorbate, fibrin, glutathione, melatonin, and some vitamins (A, C, E, and K) are non-enzymatic antioxidants. For example, superoxide dismutase catalyze the dismutation[1] of superoxide to hydrogen peroxide, while catalases catalyze the conversion of hydrogen peroxide to water and oxygen [34].
Glutathione peroxidases and *reductase* are two enzymes that respectively catalyze the oxidation and the reduction of glutathione. *Glutathione* is a peptide derived from three amino acids joined by two peptide bonds. This molecule can be found in reduced (GSH) or oxidized (GSSG) forms.
We can easily see that each time the GSH is used against ROS, it is oxidized into GSSG, which has to be recycled to its reduced form to be used again. This chain process goes under the name of *glutathione metabolism* [35]. Another chain process, that is a key point in this analysis, is the *thioredoxin system*. The thioredoxin (Trx) system, which is composed of NADPH, thioredoxin reductase (TrxR), and thioredoxin [36]. Trx exerts its antioxidant effects primarily by acting as an electron donor for *peroxiredoxins* (Prx) [37]. Peroxiredoxins is reduced by thioredoxin after reducing hydrogen peroxide (H_2O_2) in the following cycle:

$$\begin{aligned} \text{Prx(reduced)} + \text{H}_2\text{O}_2 &\longrightarrow \text{Prx(oxidized)} + 2\,\text{H}_2\text{O} \\ \text{Prx(oxidized)} + \text{Trx(reduced)} &\longrightarrow \text{Prx(reduced)} + \text{Trx(oxidized)} \end{aligned}$$

[1] a reaction in which oxidized and reduced forms of a chemical species are produced simultaneously

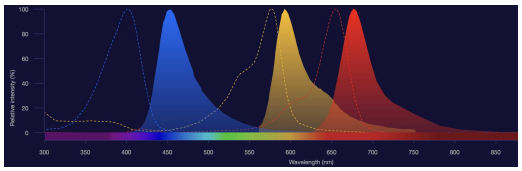
These reactive oxygen species are produced in both extracellular and intracellular states, but we will focus on how the extracellular ones affect our immune system.

Chapter 3

Experiment

3.1 The populations

The experimental setup involves two different types of populations: NK cells as effectors, and target cells. The 721221 target cells are *multiple myeloma* cells, a type of malignant plasma cells. These cells are ideal for this investigation since they do not express MHC class I proteins making them very good targets for NK cells. To test our hypothesis that the redox status of the target cell directly affects the ability of the effector cell to kill, we used the 721221 parental cells as control and generated oxidized 721221. In the oxidized cell population, redox homeostasis is dysregulated due to a disruption of the gene encoding for thioredoxin reductase 2. This modification leads to an inability to properly remove ROS.
During the acquisition, the behavior of all these populations is analyzed as they interact within the same field. To determine whether a cell belongs to one population rather than another, all the cells are stained with different dyes. The spectrum of these dyes is given below:



	Excitation (nm)	Emission (nm)
Lysotracker Deep Red	647	668
Cell Trace Violet	405	450
Calcein Red-Orange	577	590

Lysotracker Deep Red is a cell-permeable dye that stains the cytotoxic granule due to their acidic pH and was used to label the effector NK cells. CellTrace Calcein Red-Orange and CellTrace Violet are cell-permeant dyes that are used to stain respectively oxidized and control target cells. This differential labeling allows us to discern and differentiate between the cell-producing ROS from control target cells.

3.2 The lab apparatus

To follow the effector and target cell interactions, we used fluorescence live microscopy imaging. As indicated earlier, each cell type was separately labeled before being mixed and incubated in the live imaging chamber. The acquisition process is divided into two phases. In the first scan, the laser is configured to excite the violet dye, the deep red dye, and the bright field. During the second part of the acquisition, the focus shifts to the red-orange dye. The inclusion of the

bright field acquisition is essential for visualizing the cell body and improving the ability to distinguish the occupied area of each cell.

Let us now proceed to provide a detailed description of the various components comprising the optical path. It is crucial to distinguish between the excitation path, responsible for the illumination of the specimen, and the emission path, which involves the detection of emitted light:
1) The electromagnetic radiation originates from a laser system, comprising a collimated mercury lamp that serves as the illumination source. The laser light is characterized by monochromaticity and coherence, set to match the specific requirements of each dye utilized in the experiment;

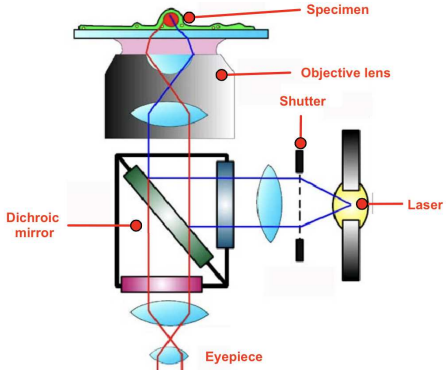


Figure 3.1: A diagram illustrating the operation of an inverted confocal microscope

2) Shutters are employed to block the light source from illuminating the specimen between camera exposures and are particularly important when imaging delicate fluorescent specimens as a means of minimizing *photobleaching* (the decreasing in the intensity of the dyes due to long illumination) and *phototoxicity* (death of the cell caused by the illumination);
3) A *dichroic mirror* is placed between the signal collection region and the chamber. It works as a beamsplitter but without a large loss in the intensity of either beam. It reflects light with wavelengths shorter than a specific threshold while transmitting light with longer wavelengths. As a result, the light originating from the primary source is reflected and directed through the objective lens onto the sample. Simultaneously, the longer-wavelength light emitted by the fluorescent specimen passes through both the objective lens and the dichroic mirror. This selective reflection and transmission mechanism allows our eyes to perceive only the emitted light from the fluorescent dye, filtering out the scattered light from the laser source. Additional filters are strategically placed along the optical path to further isolate and select the appropriate wavelengths for detection;
4) The objective lens is where the confocal part comes into play. It focuses the light onto a single, confocal point within the specimen. The objective lens is regarded as the core component of the microscope, as it primarily governs the formation of the optical image and establishes the resolution limits of the system;
5) Then, the emitted light travels back through the objective lens and before it reaches the detector, it passes through a spatial pinhole, that allows only the in-focus emitted light to pass through, while blocking the out-of-focus light;
6) Subsequently, the light that traverses the pinhole is captured by a photomultiplier tube (PMT), which transforms the incoming light into an electrical signal[38];
Moreover, as we can see, a peculiarity of this microscope is that it is set in an *inverted* configuration. Unlike a normal microscope where the objective lens is positioned below the stage, an inverted microscope has its objective lens situated above the stage. This unique inverted configuration allows for convenient observation and manipulation of samples.

3.3 Results

In the context of this thesis project, a total of five acquisitions were conducted. Each acquisition session had a duration of approximately 150 minutes and images were captured every 3 minutes. During each data collection, all three populations were simultaneously present in the same chamber. This approach allowed for the comprehensive observation and analysis of the interactions and dynamics among these populations over an extended period.
To proceed with the acquisition in a condition compatible with cell survival of the cells, a specialized setup was employed to maintain the condition in the chamber at a constant temperature of 37°C. This temperature control system played a vital role in ensuring the accuracy and reliability of the experimental results by preserving the integrity and functionality of the cells throughout the duration of the acquisitions. In addition to temperature regulation, another crucial aspect for maintaining the well-being of the cells was the control of carbon dioxide (CO_2) levels. This was achieved through the utilization of a buffer solution, in which the cells were suspended during the experimental procedures. During the data analysis process, several assumptions were made to establish criteria for identifying relevant events. Firstly, only NK cells that exhibited granules in at least one of the acquired time points were considered as effectors. This criterion was employed to identify NK cells that were potentially involved in cellular interactions. Additionally, the relevance of an event was determined based on the presence of both a granule-presenting effector and a properly stained target cell. In other words, an event was considered significant when there was evidence of NK cells with granules, indicating their potential cytotoxic activity, along with appropriately labeled target cells, that we can therefore recognize. Throughout the experiments, the ratio between the control population (CTR) and the oxidized population (ROS) in the observed field displayed minor variations. However, these fluctuations did not deviate mostly from an approximately constant value of 1. This indicates that, on average, the ratio between the CTR and ROS populations remained relatively stable across the different experiments.

Ex.	CTR numerosity	ROS numerosity
field 1	24	29
field 2	37	30
field 3	19	7
field 4	10	13
field 5	38	33

It is important to acknowledge that the absence of granules or dyes in certain cells can be attributed to the focal planes at which the image is acquired. In other words, some cells may not have their fluorescence detected during the experiment because they are located on a different z-plane compared to the majority of the cells. This discrepancy in focal planes can result in the underrepresentation of fluorescence signals from specific cells, leading to the absence of detectable granules or dyes. Additionally, during the analysis, events in which the cell body of the effector is not visible in the bright field are excluded.

3.3.1 Performance

In the analysis of the effector's behavior during the killing process of the target, two key aspects are considered: the number of contacts between the effector and the target (referred to as the *number of contacts*) and the time required for the effector's granules to become polarized (referred to as *polarization time*). These parameters are investigated to provide valuable insights into the nature of the effector's interaction with the target throughout the killing process.

Number of contacts

During each acquisition, the number of contacts between the effector and the target is calculated individually for the control population and the oxidized target population. As the size of these populations can vary between experiments, it is necessary to normalize the number of contacts to enable a fair comparison across different experiments and account for any differences in population numerosity. To achieve this, the number of contacts is divided by the number of cells observed in each respective population during the specific acquisition being analyzed. This normalization process ensures that the contact numbers are adjusted based on the population size for that given experiment. By dividing the number of contacts by the number of cells, the resulting value represents the average number of contacts per cell within each population for that given acquisition. After normalization of the number of contacts, the following key statistical measures are estimated for the normalized values. The mean, standard deviation, and standard deviation of the mean. The individual data item is exposed with the standard deviation.

	non normalized contact		normalized contact	
Ex.	CTR	ROS	CTR	ROS
sample 1	16	8	0.7 ± 0.4	0.3 ± 0.2
sample 2	37	9	1.0 ± 0.4	0.3 ± 0.2
sample 3	7	1	0.4 ± 0.4	0.1 ± 0.2
sample 4	16	9	1.6 ± 0.4	0.7 ± 0.2
sample 5	42	12	1.1 ± 0.4	0.5 ± 0.2

The mean number of contacts per cell is:

$$n_{CTR} \pm \sigma_{n_{CTR}} = 0.9 \pm 0.2 \tag{3.1}$$

$$n_{ROS} \pm \sigma_{n_{ROS}} = 0.39 \pm 0.09 \tag{3.2}$$

On average, the control cells exhibit approximately 0.9 contacts with effector cells. However, the oxidized target cells have approximately 0.39 contacts, less than half the value observed for the control target cells. This stark contrast in the number of contacts between effector cells and the two types of target cells strongly suggests a notable difference in their interaction dynamics during the killing process. This higher average number of contacts per cell observed in the control target cells population indicates a stronger or more frequent interaction between the control target and the effector cells. This suggests that the effector cell preferentially attacks the control non-oxidized target compared to the oxidized one. When graphing the number of contacts as a function of time, the contrasting behavior between the control population and the oxidized target cells becomes increasingly evident. The observed trend can be seen as follows: With regard to the control population, at the initial time points, the histogram shows a

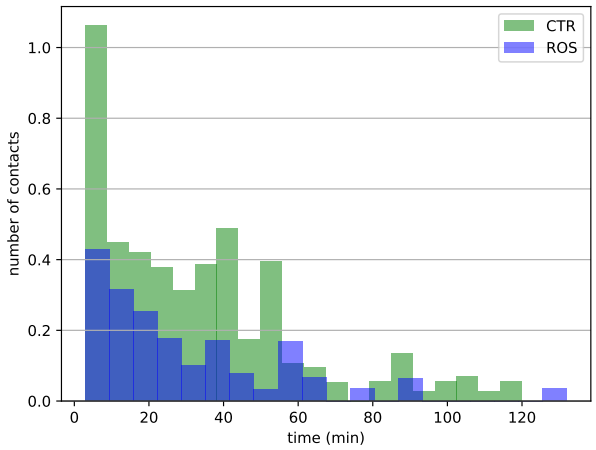


Figure 3.2: Histogram representing normalized the time point at which contact occurs

relatively larger number of contacts with the target. This indicates a more active interaction between the population and the NK population. As time progresses, the distribution gradually shifts towards lower contact numbers. The decrease in the number of contacts per cell as the killing process continues potentially indicates a reduced availability of viable targets or a saturation effect, wherein the effector cells receive fewer signals to attack the control target cells. In contrast to the control population, the histogram for the oxidized target population exhibits a lower frequency of cells in the entire time range. This indicates a lower efficiency of the effector cells in establishing contact with the oxidized targets.
Overall, both the histograms show a relatively higher peak or frequency of effector: target contacts although at different amplitude at early time points, followed by a gradual decline in contact numbers over time. In the histogram analysis of the number of contacts, each data point is assigned a weight that reflects the inverse of the number of cells observed for the specific population in the given experiment. This weighting scheme ensures that the histogram accurately represents the relative contribution of each data point based on the population numerosity. Once again, by incorporating these weights into the histogram analysis, we can accurately represent the contact distribution, accounting for variations in population sizes and ensuring that the histogram reflects the true contact behavior.

Polarization time

The polarization time is a crucial parameter that provides insights into the effectiveness of the contacts to activate the effector cells to kill the target cells. As said before, polarization time refers to the time required for the granules within the effector cells to move to the synapse. The reason why this step is critical in the killing mechanism of NK cells is that it involves the reorganization of cellular components, such as the MTOC (Microtubule Organizing Center), and subsequently microtubules, towards the immunological synapse. This reorganization prepares the NK cells for the release of cytotoxic granules. The data from each acquisition were treated as repeated measures and thereafter averaged. The mean ($\bar{t}$), the standard deviation (σ_t) and the standard deviation of the mean ($\sigma_{\bar{t}}$) were calculated:

	CTR population	ROS population
$\bar{t} \pm \sigma_{\bar{t}}$ (min)	10 ± 1	29 ± 6
σ_t (min)	10	30

The results show a shorter polarization time, this indicates a more efficient and rapid response of the effector in targeting the control cells and initiating the second part of the killing process. To determine if the two average values obtained from the control population and the oxidized target population are statistically representative of different populations, a T-test was conducted. Before conducting the T-test, it is necessary to calculate the degrees of freedom. By calculating this parameter, we can determine the appropriate critical value from the T-distribution and assess the statistical significance of the observed difference between the two average values. The degrees of freedom ($d.f.$) for an independent samples T-test can be calculated using the following formula:

$$d.f. = \frac{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)^2}{\frac{\left(\frac{s_1^2}{n_1}\right)^2}{n_1 - 1} + \frac{\left(\frac{s_2^2}{n_2}\right)^2}{n_2 - 1}} = 19 \tag{3.3}$$

where s_1 and s_2 are the standard deviations of the two samples, while n_1 and n_2 are the size of the samples. To calculate the p-value for the T-test, we assume the null hypothesis (H_0) to be that there is no difference between the population means. In this case, the null hypothesis is stated as $\mu_1 - \mu_2 = 0$, where μ_1 represents the mean of the control population and μ_2 represents the mean of the oxidized target population. The p-value can be calculated using the following formula:

$$p = \frac{(\overline{x}_1 - \overline{x}_2) - (\mu_1 - \mu_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} = -2.99 \tag{3.4}$$

This result provides evidence that we can reject the hypothesis that they came from the same distribution with a confidence level of 99.5%, given the fact that the p-value obtained is above the critical value $\alpha = |2.878|$. The test conducted considers a two-tailed T-distribution. This choice is appropriate because we are interested in detecting any significant difference between the means of the two populations, regardless of the direction.

3.3.2 Influences

Let's delve into examining how the presence of one population impacts the interaction between the effector and the other population. To investigate this, we can compare the interaction parameters between the two scenarios. When both populations are present simultaneously in the same field area and when only one population is present in that area. This analysis allows us to assess whether there are any synergistic or inhibitory effects when the two populations coexist. Specifically, our investigation focused on examining whether the presence of target cells from the other population has an impact on the polarization time of the effector cells. To do so, we compared two scenarios where the effector cells are exposed to the same target cells they are attacking (intra-population scenario) with that where they are also exposed also to the other (inter-population scenario). This analysis is of great significance as it reflects the realistic scenario encountered in tumor microenvironment. Indeed, in the microenvironment of tumoral lesions, effector cells are expected to face the coexistence of both healthy cells and tumoral cells from different grades and ROS content.

To gain a better understanding of this aspect, we organized the collected data in a systematic manner. We employed a visualization approach by plotting the polarization time as a function of the number of nearby cells from the other population.

To determine the proximity of two cells, a criterion based on the distance between their centers was employed. Specifically, two cells were considered close together if the distance between their centers was less than one and a half times the diameter of a single cell. This fixed distance criterion provides a standardized measure for determining proximity between cells. By using a threshold of one and a half times the diameter of a single cell (approximately $8\mu m$), it ensures that cells are classified as adjacent only when they are in relatively close physical proximity to each other. In the provided figure, the concept of proximity is specifically applied in relation to the target cell, which is represented by the green cell. The two oxidized cells, represented by the blue cells, are not considered in this proximity analysis as their centers fall outside the circle. The following trend has been found separately for attacks toward the oxidized target and CTR target: The graph revealed a significant correlation between the number of nearby cells from the opposite population and the effector cell polarization time observed during the killing process. Firstly, the presence of oxidized cells hampers the effector cell polarization of granules when attacking a control target cell. In this case, the polarization time almost doubles when we consider a control cell with two nearby oxidized cells. In fact, there is a relative increase of approximately 1.6. Conversely, when examining the effector's action on oxidized cells specifically, a distinct observation emerges.

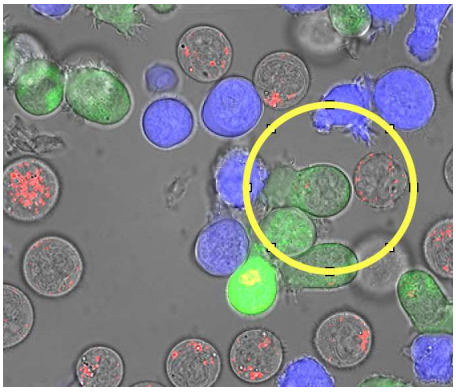


Figure 3.3: Example of how the proximity of two cells is computed

The effector cell polarization time when attacking an isolated oxidized cell appears to be largely delayed compared to a situation in which the oxidized cell is near one or more control cells. Indeed, we observe a relative decrease of approximately 0.6. This analysis also enables us to calculate the polarization time specifically for the control population without any nearby ROS cells, as well as for the oxidized cells:

$$\bar{t}_{CTR} \pm \sigma_{CTR} = (6.1 \pm 0.9) min \tag{3.5}$$

$$\bar{t}_{ROS} \pm \sigma_{ROS} = (50 \pm 10) min \tag{3.6}$$

To further investigate the relationship between various factors and polarization time, an additional analysis was carried out. This analysis aimed to analyze the potential correlation between two key parameters: the total number of cells within each population and the total number of cells present in the field, and how they are linked with the polarization time. The following results were obtained:

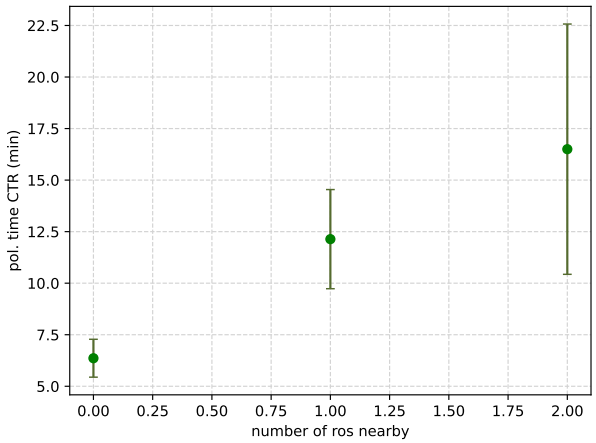


Figure 3.4: Connection between the polarization time of NK granules, during the killing of a control cell, and the number of nearby oxidized cells

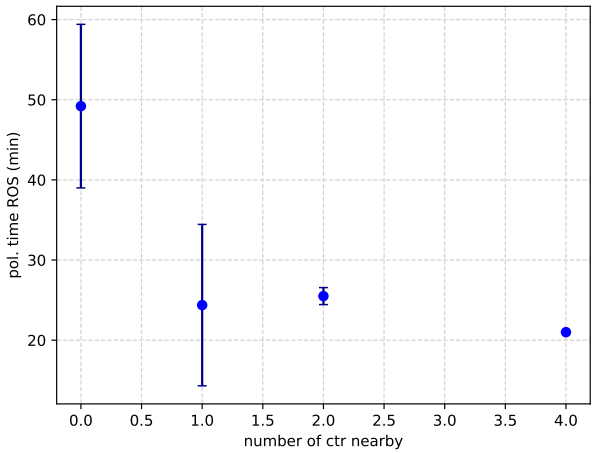


Figure 3.5: Connection between the polarization time of NK granules, during the killing of an oxidized cell, and the number of nearby control cells

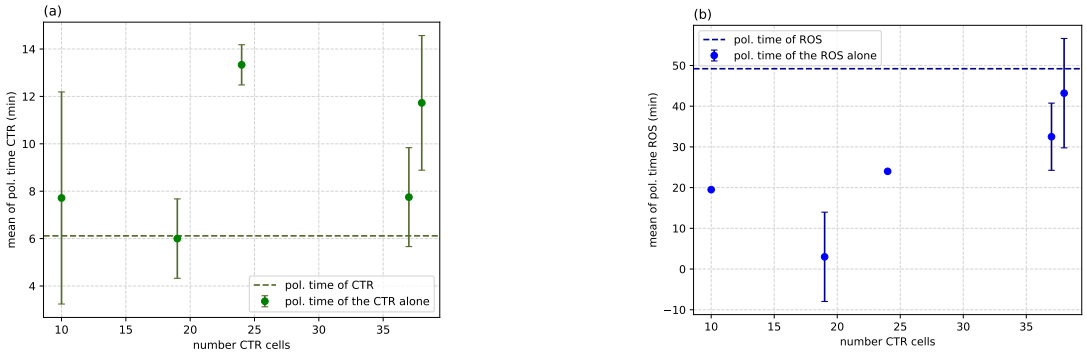


Figure 3.6: Variation of the polarization time during an attack as a function of the number of control cells in the field. (**a**) Polarization toward CTR cells. (**b**) Polarization toward ROS cells.

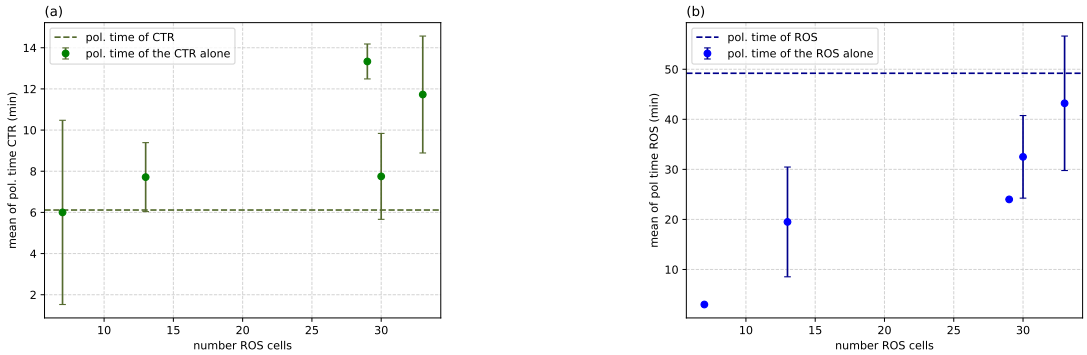


Figure 3.7: Variation of the polarization time during an attack as a function of the number of oxidized cells in the field. (**a**) Polarization toward CTR cells. (**b**) Polarization toward ROS cells.

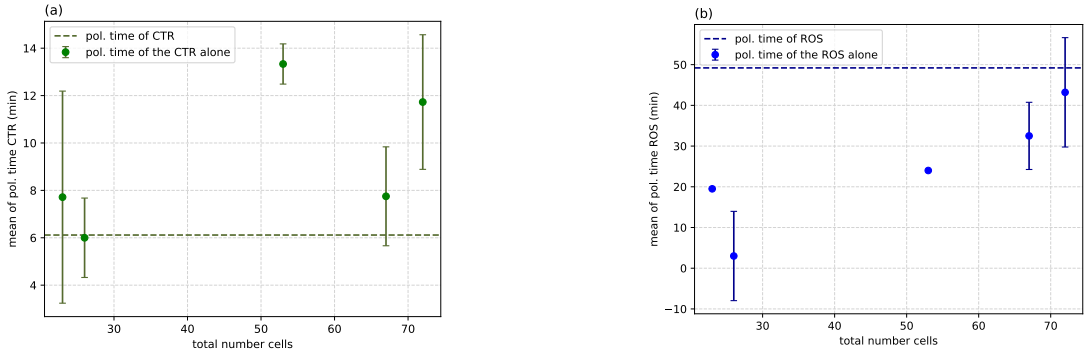


Figure 3.8: Variation of the polarization time during an attack as a function of the total number of cells in the field. (**a**) Polarization toward CTR cells. (**b**) Polarization toward ROS cells.

We can see how the data fluctuate in the range of numerosity of cells taken into consideration. These fluctuations indicate that the cell density within a population might indeed have a significant impact on the polarization dynamics observed during the killing process. Indeed, the variation is systematically deviating from the "alone" value. The majority of the collected data pertaining to the control population consistently demonstrated polarization times that exceeded the polarization time observed in the absence of any nearby reactive oxygen species (ROS) cells. On the contrary, the data related to the oxidized cells exhibited an opposite behavior: the data are located under the polarization time for ROS alone and they tend to reach this minimal result while increasing the number of CTR nearby.
The pattern regarding the control population (CTR) is not as clear and conclusive as the pattern observed with the oxidized cells. Indeed, we can see an almost steady enhancement in the polarization time from the minimum value of about 6 minutes by about 600%. Conversely, the data pertaining to the control population exhibit fluctuations or inconsistencies. We can also see a consistent increase of about 117% from the minimum value but the fluctuation of the data makes it challenging to establish a definitive correlation between the number of nearby control cells and the polarization time. To gain a better understanding of this uncertainty and to determine the extent of the observed fluctuations, additional analysis is required. Further investigations could involve expanding the dataset by collecting more data points or conducting multiple experiment replicates.

Chapter 4

Conclusion

In this thesis work, we study the interaction between Natural Killer cells and oxidized cells or control cells, when exposed simultaneously to these two types of target cells.
Our immune system, acting through the Natural Killer, is confronted with reactive oxygen species (ROS) in a multitude of circumstances. One of the main situations in which we can find this interaction is tumors. Indeed, this analysis concern a type of cancerous cells as targets, particularly multiple myeloma cells. We have analyzed the correlations between either targets and NK separately and NK with both populations together. To do so, we considered the number of contacts and polarization time two critical parameters defining the contact of the immune effector cells and their targets. We found out that the presence of environmental ROS significantly affects the reactivity and speed of action of NK.
We have further attempted to provide a better understanding of these interactions in a more physiological setting by assessing the impact of the coexistence of different types of targets. Within the cancerous lesions, the coexistence of healthy cells and tumoral cells with different grades and ROS burden creates a unique and challenging environment for the immune effector cells. Our findings reveal that in the absence of nearby control cells, the polarization process is significantly delayed, an effect that is alleviated when control cells are present. Conversely, the polarization of effectors towards control cells consistently increases in the presence of nearby oxidized target cells. Finally, it is crucial to consider the limitations and exclusions implemented during the analysis when interpreting the results. Factors such as potential variations in focal planes and the exclusion of events with non-visible effector cell bodies can influence the overall depiction and analysis of cellular interactions. Therefore, in future experiments, it is essential to meticulously examine and analyze these factors to mitigate potential biases and ensure the reliability and validity of our findings.

Bibliography

[1] John Wilson and Tim Hunt. "Molecular biology of the cell: the problems book". In: Fifth ed. 2008. Chap. Human Cells Recognize Conserved Features of Pathogens, p. 1526. ISBN: 978-0-8153-4110-9.

[2] Sandra Pinzón Martín, Peter H. Seeberger, and Daniel Varón Silva. "Mucins and Pathogenic Mucin-Like Molecules Are Immunomodulators During Infection and Targets for Diagnostics and Vaccines". In: 7 (2019). ISSN: 2296-2646. DOI: 10.3389/fchem.2019.00710. URL: https://www.frontiersin.org/article/10.3389/fchem.2019.00710/full.

[3] Tomas Ganz. "Defensins: antimicrobial peptides of innate immunity". In: 3 (2003), pp. 710–720. ISSN: 1474-1733, 1474-1741. DOI: 10.1038/nri1180. URL: http://www.nature.com/articles/nri1180.

[4] John Wilson and Tim Hunt. "Molecular biology of the cell: the problems book". In: Fifth ed. 2008. Chap. Barriers to infection and the innate immune system. ISBN: 978-0-8153-4110-9.

[5] John Wilson and Tim Hunt. "Molecular biology of the cell: the problems book". In: Fifth ed. 2008. Chap. Virus-Infected Cells Take Drastic Measures to Prevent Viral Replication, p. 1534. ISBN: 978-0-8153-4110-9.

[6] Jun-Ming Zhang and Jianxiong An. "Cytokines, Inflammation and Pain". In: 45 (2007). DOI: 10.1097/AIA.0b013e318034194e. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2785020/.

[7] John Wilson and Tim Hunt. "Molecular biology of the cell: the problems book". In: Fifth ed. 2008. Chap. Apoptosis. ISBN: 978-0-8153-4110-9.

[8] Susan Elmore. "Apoptosis: A Review of Programmed Cell Death". In: 35 (2007), pp. 495–516. ISSN: 0192-6233. DOI: 10.1080/01926230701320337. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2117903/.

[9] Guy S. Salvesen Kelly M. Boatright. *Mechanisms of caspase activation*. DOI: 10.1016/j.ceb.2003.10.009.

[10] *Toll-like receptors | Elsevier Enhanced Reader*. en. DOI: 10.1016/j.cub.2011.05.039. (Visited on 12/19/2022).

[11] Marek Wieczorek et al. "Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation". In: 8 (2017). DOI: 10.3389/fimmu.2017.00292.

[12] John Wilson and Tim Hunt. "Molecular biology of the cell: the problems book". In: Fifth ed. 2008. Chap. Phagocytic Cells Seek, Engulf, and Destroy Pathogens, p. 1532. ISBN: 978-0-8153-4110-9.

[13] Sourav Paul and Girdhari Lal. "The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy". In: 8 (2017). DOI: 10.3389/fimmu.2017.01124. URL: http://journal.frontiersin.org/article/10.3389/fimmu.2017.01124/full.

[14] Konrad Krzewski and John Coligan. "Human NK cell lytic granules and regulation of their exocytosis". In: 3 (2012). ISSN: 1664-3224. URL: https://www.frontiersin.org/articles/10.3389/fimmu.2012.00335.

[15] Kamalakannan Rajasekaran et al. "Signaling in Effector Lymphocytes: Insights toward Safer Immunotherapy". In: 7 (2016).

[16] J. A. Trapani. "Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein, perforin, and the serine protease, granzyme B". In: 25 (1995).

[17] Sally Robertson. "What is the Actin Cytoskeleton?" In: ().

[18] Michael Bots and Jan Paul Medema. "Granzymes at a glance". In: 119.24 (2006). DOI: 10.1242/jcs.03239. URL: https://doi.org/10.1242/jcs.03239.

[19] Elena Lomonosova and G. Chinnadurai. "BH3-only proteins in apoptosis and beyond: an overview". In: 27 (2008). DOI: 10.1038/onc.2009.39. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2928556/.

[20] Joseph A Trapani and Vivien R Sutton. "Granzyme B: pro-apoptotic, antiviral and antitumor functions". In: 15 (Oct. 2003). DOI: 10.1016/S0952-7915(03)00107-9. URL: https://www.sciencedirect.com/science/article/pii/S0952791503001079.

[21] John Wilson and Tim Hunt. "Molecular biology of the cell: the problems book". In: Fifth ed. 2008. Chap. Lymphocytes and the cellular basis of adaptive immunity. ISBN: 978-0-8153-4110-9.

[22] Stephen J. Gaudino and Pawan Kumar. "Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis". In: 10 (2019). DOI: 10.3389/fimmu.2019.00360. URL: https://www.frontiersin.org/article/10.3389/fimmu.2019.00360/full.

[23] John Wilson and Tim Hunt. "Molecular biology of the cell: the problems book". In: Fifth ed. 2008. Chap. T cells and MHC proteins. ISBN: 978-0-8153-4110-9.

[24] Ryan S. Sauls, Cassidy McCausland, and Bryce N. Taylor. *Histology, T-Cell Lymphocyte*. 2022. URL: https://www.ncbi.nlm.nih.gov/books/NBK535433/.

[25] Jr Charles A Janeway et al. "The major histocompatibility complex and its functions". In: (2001). URL: https://www.ncbi.nlm.nih.gov/books/NBK27156/.

[26] Nicholas R.J. Gascoigne et al. "Co-Receptors and Recognition of Self at the Immunological Synapse". In: 340 (2010). DOI: 10.1007/978-3-642-03858-7_9. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5788015/.

[27] Sanaa K. Bardaweel et al. "Reactive Oxygen Species: the Dual Role in Physiological and Pathological Conditions of the Human Body". In: 50 (2018). DOI: 10.5152/eurasianjmed.2018.17397. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6263229/.

[28] "An Introduction to Reactive Oxygen Species - Measurement of ROS in Cells". In: ().

[29] Robert Li, Zhenquan Jia, and Michael A. Trush. "Defining ROS in Biology and Medicine". In: 1 (2016). DOI: 10.20455/ros.2016.803. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5921829/.

[30] Kristell Le Gal, Edward E. Schmidt, and Volkan I. Sayin. "Cellular Redox Homeostasis". In: 10 (2021). DOI: 10.3390/antiox10091377. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8469889/.

[31] Abinaya Manivannan, Prabhakaran Soundararajan, and Byoung Ryong Jeong. "Role of Reactive Oxygen Species Signaling in Cell Proliferation and Differentiation". In: *Reactive Oxygen Species in Plants*. 2017. DOI: 10.1002/9781119324928.ch17. URL: https://onlinelibrary.wiley.com/doi/abs/10.1002/9781119324928.ch17.

[32] Samina Salim. "Oxidative Stress and the Central Nervous System". In: 360.1 (2017). DOI: 10.1124/jpet.116.237503. URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5193071/.

[33] Yun Chen et al. "Distinct Types of Cell Death and the Implication in Diabetic Cardiomyopathy". In: 11 (2020). URL: https://www.frontiersin.org/articles/10.3389/fphar.2020.00042.

[34] *Members of Antioxidant Machinery and Their Functions | IntechOpen*. URL: https://www.intechopen.com/chapters/49498#.

[35] Guoyao Wu et al. "Glutathione Metabolism and Its Implications for Health". In: 134 (2004). DOI: 10.1093/jn/134.3.489. URL: https://www.sciencedirect.com/science/article/pii/S0022316623026639.

[36] Arne Holmgren Jun Lu. *The thioredoxin antioxidant system | Elsevier Enhanced Reader*. en. DOI: 10.1016/j.freeradbiomed.2013.07.036.

[37] *Thiols: Role in Oxidative Stress-Related Disorders | IntechOpen*. URL: https://www.intechopen.com/chapters/75841.

[38] "Confocal Microscopy / Denis Semwogerere, Eric R. Weeks". In: *Encyclopedia of Biomaterials and Biomedical Engineering*. 2nd ed. 2008.