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Lymphocytes

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and **Hasan Basri İla**

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Meet the editors

Dr Erman Salih İstifli received his Ph.D. from the Biology Department of Cukurova University, Institute of Science and Letter. In his doctoral study, Dr İstifli focused on the elucidation of the genotoxic and cytotoxic effects of a commonly used anticancer agent (antifolate) on human lymphocytes. Dr İstifli, during his period of doctoral research, joined the molecular cytogenetics group at the Max Planck Institute for Molecular Genetics in Berlin, Germany, and there he focused on investigating the molecular cytogenetic causes of some human rare diseases. During these studies, he contributed experimentally to the identification of four candidate genes (GRIA2, GLRB, NPY1R, and NPY5R) responsible for intelligence and obesity. He was assigned as an expert and rapporteur on eight candidate projects in the Marie-Sklodowska Curie-Actions Innovative Training Networks in 2016. He is a published author of several articles in journals covered by the SCI and SCI-E, and has manuscripts in other refereed scientific journals. He currently serves as a referee in several journals covered by the SCI and SCI-E. His studies mainly fall into the field of genetic toxicology.

Prof. Dr. Hasan Basri İla received his Ph.D. from Biology Department of Çukurova University, Insitute of Science and Letter. Prof. Dr. Hasan Basri İla, during his doctoral study, investigated the effects of a commonly used antibiotic on chromosome aberration and micronucleus formation by in vivo tests. Dr. İla has several publications in the internationally indexed (SCI, SCI-E) journals. He actively took responsibility in 17 national projects as a researcher and/or project leader. He has been actively giving lectures on biology, cytology, genetics, evolution, organelle genetics and cancer genetics. He has numerous poster and/or oral scientific presentations in several international conferences. Dr. Ila's articles have been cited 442 times.

Contents

Preface

Lymphocytes are one of the white blood cell subtypes in the immune system of vertebrates. These cells include natural killer cells (cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity), and B cells (for humoral, antibody-driven adaptive immunity). These are the main types of cells found in the "lymph" and are therefore called "lymphocytes". Furthermore, although we emphasize “lymphocytes”, the immune elements in vertebrates, there is also an immune system in invertebrate organisms that has evolved over approximately 500 million years, which appears to be a precursor form of the immune system of vertebrates.

Lymphocytes have been the main research object of the medical and toxicological sciences for the last six decades. In addition to important roles they play in the immune system, lymphocytes have been the primary test material often employed in pure research-focused studies that provide important contributions to our knowledge of biochemistry, molecular biology and genomics. To give a prominent example, the majority of DNA used for sequencing of the human genome was isolated from lymphocytes of male donor blood.

Today, the use of lymphocytes for research purposes is increasing, and the variety of tests performed on these cells are also diversifying each day. Lymphocyte activation assays, fluorescence-based assays for high-throughput screening of small molecules, cytotoxic T lymphocyte assays, cell proliferation assays, blastogenesis assays for T lymphocytes for identifying immunomodulatory drugs of lymphocyte extravasation and sequencing and genotoxicity assays are among the tests that lymphocytes are frequently used for. As can be readily understood, lymphocytes serve in different ways in terms of understanding the cell structure, unknown features of the immune system, genome and chromosomes.

It is clear that lymphocytes will continue to serve as experimental material in the most important areas of science in the near future. Although it is impossible to follow simultaneously the growing body of knowledge in this area, this book was aimed to present the most pertinent findings and reviews related to the response of the human lymphoid system to various occupational and environmental substances, effects of beta glucan on the radiation protective and immunopotentiating effect of lymphocytes, effects of microgravity on lymphocytes, signaling pathways in the development of pre-B cells, B lymphocyte development, immunotherapeutic approaches against hematologic malignancies and solid tumors as well as the Raman microspectroscopy in lymphocyte studies.

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Chapter 1

Alteration of Various Lymphocytes by Particulate and Fibrous Substances

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Additional information is available at the end of the chapter

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Abstract

Various occupational and environmental substances alter the cellular and molecular function of the human lymphoid system. For example, silicosis patients who have been chronically exposed to silica particles often complicate with autoimmune diseases such as rheumatoid arthritis and systemic sclerosis. From our investigations, silica particles affect CD4+ responder T cells and regulatory T cells (Tregs), which results in the disruption of autoimmunity. Asbestos fibers are a type of mineral silicate, and patients exposed to asbestos fibers revealed cancers such as mesothelioma and lung cancer. In these cases, asbestos fibers may reduce antitumor immunity. Our results investigating the effect of asbestos on cytotoxic T lymphocyte, natural killer (NK) cells, CD4+ cells, and Tregs revealed a reduction in antitumor immunity. To date, the effects of silica and asbestos on Th17 cells and antigen-presenting cells such as dendritic cells and macrophages remain unclear. Based on these findings, it will be possible to generate earlier detection methods to identify the occurrence of immune alterations in silicosis as well as the appearance of a decreased antitumor immunity in asbestos-exposed populations. Additionally, research efforts should also be directed at discovering and identifying physiological substances from foods, plants, and other sources that can restore the immune status in people exposed to particulate and fibrous substances.

Keywords: silica, asbestos, responder T cell, regulatory T cell, cytotoxic T lymphocyte, NK cell

1. Introduction

There are a variety of lymphocytes including T cells, B cells, and natural killer (NK) cells [1–4]. Additionally, there are other smaller populations of lymphocytes such as natural killer T (NKT) cells. T cells are further divided depending on their surface molecules as well as function and cytokine production in CD4+ and CD8+ T cells. CD8+ cells are designated as cytotoxic T lymphocytes (CTLs), which express T cell receptors (TcRs) and recognize a specific antigen. TcRs on CTLs can bind to the complex of the class I major histocompatibility complex (MHC) molecule and antigen. Thereafter, CTLs can destroy the cells using granzymes and perforins as the attacking molecules. CD4+ T cells include various subpopulations [1–4]. Although CD4+ cells are referred to as T helper (Th) cells, depending on the kind of stimulation, cytokine circumstances surrounding Th cells, naïve Th cells are skewed to Th1, Th2, Th17, and Treg (regulatory T) cells. Th1 and Th2 cells are balanced subpopulations. The proliferation of Th1 cells is triggered by interleukin (IL)-12 and produces IL-2 and interferon (IFN)-γ. The key transcription factors are T-bet and the signal transducer and activator of transcription 4 (STAT4). Th1 cells act against intracellular bacteria by activating macrophages. On the other hand, Th2 cells are activated by IL-4 and IL-2, and results in the secretion of IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 cytokines. The key transcription factors for Th2 are SATA6 and GATA3. IL-4 produced by Th2 cells acts as a positive feedback to restimulate Th2 cells and stimulate B cells to produce immunoglobulin (Ig) E. Therefore, Th2 cells play an important role in the context of allergies and hypersensitivity such as atopic dermatitis and bronchial asthma [1–4].

Additionally, Th17 and Treg cells are also balanced according to the skewing cytokine circumstances surrounding Th naïve cells. Initially, transforming growth factor (TGF)-β stimulates naïve Th cells to express two transcription factors, RORγt and forkhead box (Fox) P3. Although IL-6 together with TGF-β causes activation of the STAT3 signaling pathway to suppress FoxP3, IL-2 together with TGF-β facilitates the movement of cells toward the Th17 phenotype. Tregs produce IL-10, TGF-β and IL-35 and act to inhibit effector T cell reactions against foreign as well as non-self/self-antigens. On the other hand, Th17 cells produce IL-17, IL-21 and IL-22 and play an important role in pathogen clearance at the mucosal surface and are implicated to play a role in autoimmune and inflammatory disorders [1–4].

B cells act in humoral immunity to produce Ig/antibodies to attack foreign antigens. Additionally, B cells express B cell receptors (BcRs), which allow for the binding of B cells to a specific antigen in the process involving innate immunity [1–4].

NK cells also act in innate immunity by quickly attacking virally infected cells, as well as tumor cells, after recognition. NK cells do not need MHC presentation. These cells can kill virally infected cells and tumor cells *via* lytic reactions and apoptosis by releasing granzymes and perforins [1–4].

When the immune system recognizes the presence of foreign substances such as bacteria and viruses, as well as transformed cells derived from self, the human immune system works to attack these non-self substances and facilitates the curtailment of alterations in the human body such as various symptoms resulting from bacterial and viral infections and cancers.

These reactions are physiological. Additionally, certain alterations of the immune system may recognize self-antigens and subsequently cause various autoimmune diseases [1–4].

2. Occupational and environmental substances that affect the human immune system

There are many environmental and occupational substances which induce alterations of the immune system. For example, isocyanate latex, cement including chromium, and whitening agents including persulfates and other substances cause occupational hypersensitivity (allergy). Sometimes, patients exposed to these reagents reveal respiratory asthma and contact dermatitis. However, these allergic reactions may be categorized as a range of physiological reactions against foreign antigens [5–11]. On the other hand, some occupational and environmental substances cause autoimmune diseases. For example, vinyl chloride, silica dust, and chemicals including trichloroethene and epoxy resins can cause systemic sclerosis, one of the typical generalized autoimmune diseases that occur with certain frequencies in exposed populations. Unlike occupational allergies, autoimmune diseases caused by occupational and environmental substances seem to be the result of disruption of the human immune system caused by these substances [12–15]. Additionally, there are many cancers caused by occupational and environmental substances such as lung cancers due to asbestos, tobacco smoke, certain metals including arsenic, chromium, nickel and beryllium, bladder cancer caused by benzidine, β-naphthylamine and other aromatic hydrocarbons, and others [16–22]. Of course, there are many mechanisms involved in the genesis of these occupational cancers when triggered by certain substances, such as DNA damage caused by reactive oxygen species (ROS), formation of DNA adducts, and others. However, it is also possible that certain substances that cause cancers may also affect the human immune system by reducing antitumor immunity. If so, it is understandable that a relatively long latency period may exist prior to the occurrence of occupational cancers following initial exposure to carcinogenic agents.

Taken together, it is important to assess how these occupational and environmental substances alter the human immune system. Unlike standardized toxicological investigations, people or workers are usually exposed to these substances in low-dose, chronic, and continuous ways. Thus, it is important to assess the cellular and molecular alterations in immune cells derived from exposed populations, as well as to establish continuous and low-dose exposure models using human lymphoid cells exposed *in vitro* to substances that may cause alterations in autoimmunity or act as carcinogens.

From this viewpoint, we have been investigating the immunological effects of silica particles or asbestos fibers since certain silica-exposed populations such as silicosis patients (SIL) are often complicated with various autoimmune diseases such as rheumatoid arthritis (known as Caplan syndrome) [23], systemic sclerosis (known as Erasmus syndrome) [24], systemic lupus erythematosus (SLE) [25], and antineutrophil cytoplasmic antibody (ANCA)-related vasculitis/glomerulonephritis [26–29]. Asbestos fibers are a type of mineral

silicate, while silica is particulate in nature. Additionally, asbestos can cause cancers such as malignant mesothelioma (MM) and lung cancer [30–33]. Silica particles can act to disrupt the regulation of autoimmune tolerance while asbestos fibers can facilitate a decline in antitumor immunity.

3. Silica and disruption of lymphoid cells

Silica exposure causes disruption of autoimmune tolerance. Thus, silicosis patients are often complicated with autoimmune diseases [34, 35].

When considering the effects of B cells and plasma cells, various autoantibodies have been detected in sera derived from silicosis patients. For example, some autoantibodies (AAB) are typically detected in autoimmune diseases such as anti-nuclear antibodies (ANA), anti-topoisomerase I (Sck-70) AAB [36], and anti-CENP-B (centromere) AAB [37]. In addition to these typical AABs, we found anticaspase-8 AAB [38, 39], anti-Fas-AAB [40], and antidesmoglein AAB [41], which is usually detected in skin bullous autoimmune diseases such as pemphigus vulgaris. However, we have not investigated alterations of B cells caused by silica exposure. The production of various AABs from B cells/plasma cells may be caused by alterations of B cells or be dependent on T cells which produce AABs to B cells.

What about effector T cells? There are many alterations that have been detected in effector T cells. CD4+ responder T cells showed an increase in activation markers and an excess of survival markers. For the former, the expression of CD69, a typical early activation cell surface marker for T cells, increased at 5–10 days when peripheral blood mononuclear cells (PBMCs) derived from healthy donors (HD) were cultured *in vitro* with silica particles [42]. Additionally, soluble IL-2 receptor (sIL-2R) levels in serum derived from silicosis patients showed an increasing tendency compared with those of HD. Then, if we set 1, 2, and 3 as sequential numbering for HD, silicosis, and SSc for autoimmune disruption, this number and the level of serum sIL-2R in these three categories showed a significant positive correlation [43]. Although sIL-2R is considered as a tumor marker for T cell acute lymphoblastic leukemia and T cell malignant lymphoma, the elevation of serum sIL-2R was recently detected in various autoimmune diseases. This increase in serum sIL-2R is the evidence of chronic activation of T cells in certain pathological situations such as autoimmune diseases. Thus, silicosis is also considered as a condition whereby peripheral T cells are activated chronically, and the degree of activation was higher than that in HD, although less than that in autoimmune diseases such as SSc. Moreover, programmed death-1 (PD-1) gene expression in peripheral CD4+ cells derived from silicosis patients was significantly higher than that in HD [44]. Although PD-1 is one of the most important molecules that act in the immune checkpoint system, PD-1 is also a marker of T cell activation. Thus, upregulation of PD-1 in T cells derived from silicosis patients also indicates that T cells in silicosis are chronically activated (due to long-term and continuous exposure to silica particles in the body, such as lung fields and related lymph nodes).

Considering survival factors, we found that serum from silicosis patients showed significantly higher levels of soluble Fas (sFas) compared with HD and similar levels with SLE patients [45].

Additionally, since sFas is a product due to alternative splicing of the Fas gene, losing 63 bp of the transmembrane domain, mRNA expression of wild-type membrane Fas and sFas transcripts were examined. As a result, the ratio of wild-type Fas message divided by soluble-type alternatively spliced message decreased in PBMCs from silicosis patients compared with HD [46]. These findings indicated that T cells in silicosis patients are protected against Fas-ligand-induced apoptosis by increasing the binding of Fas ligand and sFas at extracellular spaces, thereby resulting in the extended survival of T cells in silicosis patients. Additionally, the scenario of Fas, sFas, and Fas ligand was employed for tumor necrosis factor-related apoptosis-inducing ligand (Trail) receptor, decoy receptor 3 (DcR3), and Trail. DcR3 mRNA expression levels in PBMCs derived from silicosis patients were higher compared with HD [47]. Furthermore, serum DcR3 levels were higher in silicosis patients compared with HD. Hence, in both Fas and Trail systems, T cells in silicosis patients were protected from apoptosis. Taken together, T cells in silicosis patients are chronically activated as well as continuously protected from apoptosis, thereby resulting in the circulation of longer surviving T cells in the peripheral blood of silicosis patients. If so, rare self-antigen acting T cell clones may also survive longer and be chronically activated. Thus, antigen (non-self or self)-activated T cells increase their volume in silicosis patients.

What about Tregs? It is known that Fas/CD95 expression on the cell surface of Tregs is one marker of Treg activation. Thereafter, Tregs proceed to apoptosis when its role is ceased. Thus, we examined Fas expression in peripheral Treg (CD4+ and FoxP3+) as well as CD4 + FoxP3- T cells derived from HD and silicosis patients. As a result, Fas expression was significantly higher in Tregs from silicosis patients compared with HD [44, 48]. Of course, CD4 + FoxP3- T cells did not express sufficient amounts of Fas in HD or silicosis patients. Then, PBMCs derived from HD or silicosis patients were cultured with agonistic antibody. Tregs from silicosis patients showed earlier and higher apoptosis compared with HD because of a greater expression of Fas. Thereafter, PBMCs from HD were cultured with silica particles for 4 days. As a result, CD4 + FoxP3+ cell levels were significantly reduced. However, CD25+ FoxP3- cell levels were not altered, translating Tregs were reduced, and initial CD4 + CD25- cells were activated to reveal CD25 as an activation marker.

Taking together the results of responder T cells and Tregs, an imbalance between responder CD4 cells and Tregs was found, in that the increase of responder T cell levels increased, while Treg cell levels decreased. This tendency is well known in the area of dysregulation of autoimmunity which leads to the occurrence of autoimmune diseases. Thus, silica exposure affects the immune system to create an imbalance between responder T cells and Tregs and sets the foundation for the appearance of autoimmune diseases in silicosis patients.

What about Th17? Unfortunately, we have not investigated the Th17 status in silicosis patients. It was reported that Th17/I-17 is involved in silicosis to facilitate the progression of lung fibrosis found in silicosis [49, 50]. As mentioned earlier, Tregs in silicosis seem to progress toward apoptosis. This may induce an increase in Th17 levels in the peripheral blood of silicosis patients and consequently make these patients more susceptible to autoimmune diseases. Thus, further investigation of Th17 in silicosis is necessary from the viewpoint of efforts to delineate the early processes involved in the disruption of autoimmunity.

4. Asbestos and antitumor immunity

As mentioned earlier, carcinogenic factors among occupational and environmental substances may facilitate a decline in antitumor immunity. We chose asbestos since it is a mineral silicate and possesses the potential to disrupt human lymphocytes in such a way as to make the human body prone to autoimmune diseases.

For the CTLs, we used a mixed lymphocyte reaction (MLR) to assess the asbestos fibers with respect to clonal expansion of the cells examined. The use of chrysotile asbestos resulted in a decreased differentiation and proliferation of CD8+ cells and a decreased production of cytotoxic granules such as granzyme B and perforin [51–53]. These findings were confirmed using peripheral blood CD8+ cells derived from MM patients considered to have a history of exposure to asbestos even though the patients did not remember the exposure. Interestingly, the status of intracellular perforin expression in CD8+ cells from patients with pleural plaque (PP) differed from that of HD and MM patients [51–53]. Perforin expression in CD8+ cells increased somewhat in PP patients. These findings indicated that immunological alterations, especially in CTLs, differed depending on the disease status when examining PP and MM patients exposed to asbestos. This may be dependent on the occurrence of cancer in the body [51–53].

What about NK cells? We tried to expose a human NK cell line, freshly isolated peripheral NK cells derived from HD, as well as NK cells from PP and MM patients to asbestos fibers. The killing activity was reduced in a cell line model subjected to continuous exposure to asbestos, *ex vivo* activated and expanded freshly isolated NK cells cultured with asbestos fibers, and NK cells derived from patients. Additionally, the most correlated marker of killing activity was the cell surface expression level of NKp46 [54–58]. NKp46 is an NK cell activating receptor that belongs to the natural cytotoxicity receptor (NCR) family. In addition to a reduction in NKp46 expression, phosphorylation was induced of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) which are involved in the mitogen-activated protein kinase (MAP-K) signaling pathway [54–58]. Moreover, degranulation to produce cytotoxic molecules such as granzyme and perforin was also suppressed by asbestos exposure. These results were obtained from a cell line model, an *ex vivo* culture model, and from the investigation of freshly isolated NK cells derived from PP and MM patients exposed to asbestos [54–58].

Next, the continuous and low-dose exposure of human CD4+ T cells to asbestos fibers was investigated. We employed the human T cell leukemia virus (HTLV)-1 immortalized human polyclonal T cell line MT-2 as the cell line model, an *ex vivo* clonal expansion model using freshly isolated peripheral blood CD4+ cells derived from HD, and peripheral CD4+ cells derived from PP and MM patients exposed to asbestos. As a result, all models showed that continuous and low-dose exposure to asbestos induced a decline in chemokine receptor CXCR3, a $G\alpha_i$ protein-coupled receptor of the CXC chemokine receptor family [59, 60]. The reduction in CXCR3 resulted in diminished trafficking of T cells which produce IFNγ to attack tumor cells. In addition to this, CD4+ T cells exposed to asbestos showed potential to produce IFNγ. Both findings suggest a reduction in antitumor immunity [59, 60].

The MT-2 cell line was reported to possess Treg functionality. Thus, Treg suppressive functionality in MT-2 cell sublines continuously exposed to asbestos fibers was compared with the original MT-2 cell line not exposed to asbestos. As a result, sublines showed enhanced suppressive function by cell-cell contact in addition to excess production of typical soluble factors such as IL-10 and TGFβ [61]. In addition to these enhanced functions in MT-2 (a model of Tregs) caused by continuous exposure to asbestos, these sublines showed decreased levels of FoxO1 transcription factor [62]. FoxO1 regulates cell cycle progression in a negative fashion by inhibiting various cyclins as well as in a positive fashion by regulating many cyclin-dependent kinase inhibitors (CDK-Is) such as Cip1/p21, Kip1/p27, Kip2/p57, and CDKN2A to 2D such as p16, p15, p18, and p19. As a result of decreased levels of FoxO1, MT-2 sublines showed an enhanced expression of cyclins, especially cyclin D1, and a reduced expression of CDK-Is. Additionally, cell cycle progression was also enhanced compared with the original MT-2 line not exposed to asbestos [63]. Thus, both function and proliferation were enhanced with continuous and low-dose exposure of Tregs to asbestos, which suggest that the antitumor immunity controlled by Tregs was reduced [61–63].

Taken together, continuous and low-dose exposure to asbestos caused a reduction in antitumor immunity in CTLs, NK cells, CD4+ T cells, and Tregs [64–66]. Thus, it could be considered that people subjected to continuous, low-dose exposure to asbestos are susceptible to the onset of cancers because of a gradual reduction in antitumor immunity. Thereafter, certain localized areas such as lung fields or the pleural cavity may become the locus where fibers remain in the body and chronic stimulations may occur at these locations on the basis of reduced antitumor immunity [64–66]. This may represent the mechanism by which asbestos-induced cancers occur in the long term, after a latency period of approximately 30–40 years, following initial exposure to asbestos fibers.

5. Conclusion

All findings described in this chapter are summarized in **Figure 1**. In this review, the effects of silica [67–69] and asbestos [64–66] are introduced and discussed. However, people are exposed to many more potentially hazardous occupational and environmental substances, such as various materials in air pollutants and a variety of metals in work environments. Thus, any immunological alterations induced in cells by these and many other substances should be investigated utilizing the methods described earlier to further our understanding of immune responses and cancer.

Additionally, as described earlier, the identification of certain physiologically active materials among various foods, plants, and other sources, which modify and repair the immune state following disruption by various occupational and environmental substances such as silica and asbestos, should lead to the establishment of effective preventive and treatment measures. So far, we have examined immune-neutralizing effects of some extracts from bamboo and some carbohydrates made from starch such as corn. However, we have not obtained enough results yet. In the future, these approaches should be continued to find some substances.

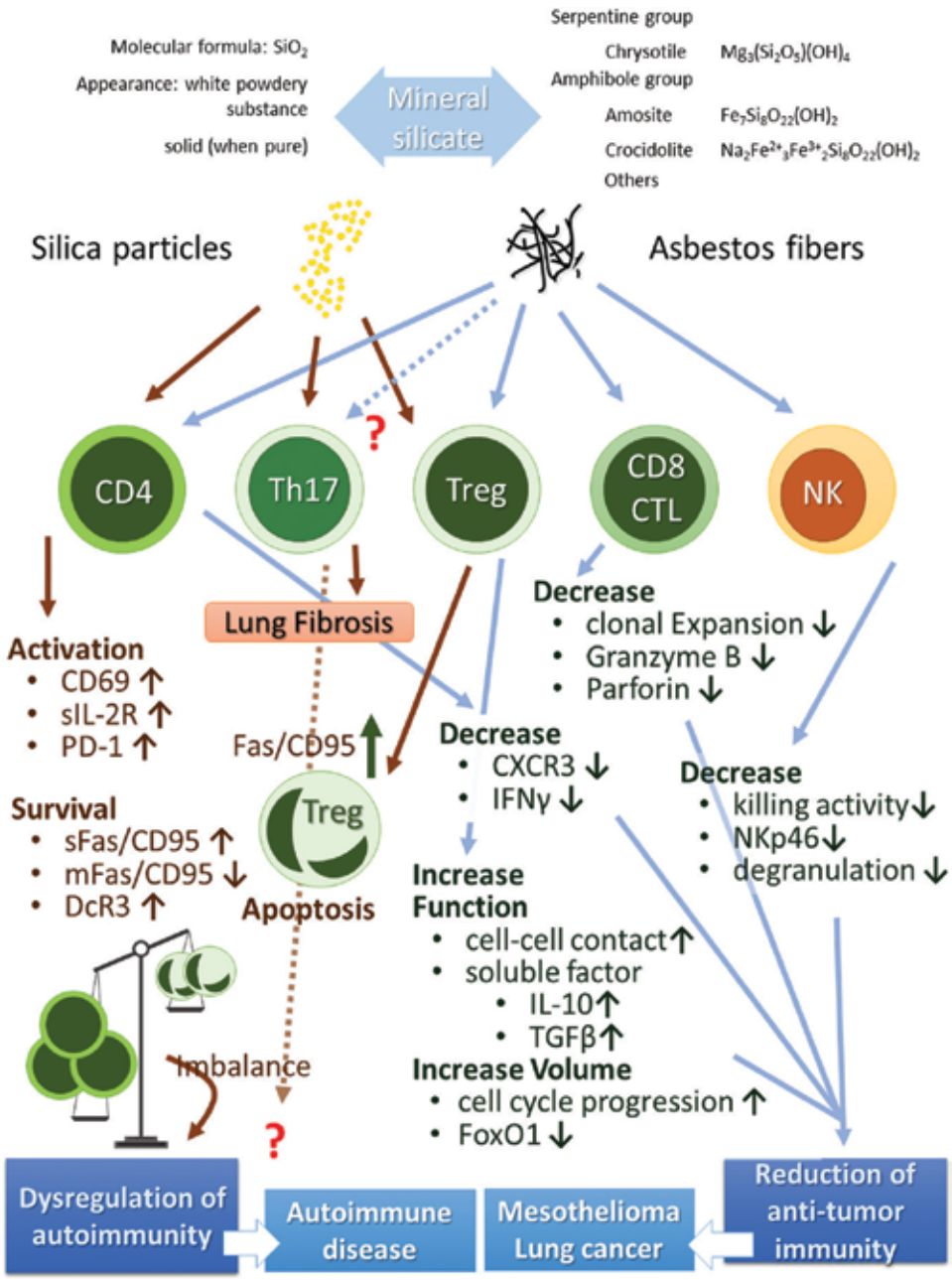

Figure 1. Summary of effects of silica and asbestos on various lymphocytes. Silica exposure induces the disruption of autoimmunity resulting in frequent complications such as autoimmune diseases in silicosis patients. Additionally, asbestos exposure causes a reduction in antitumor immunity in CD4+ T cells, Tregs, CTLs, and NK cells. These result in the onset of cancers such as mesothelioma and lung cancer in the long term after a latency period following initial exposure.

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Conflicts of interest

All authors declare that there are no conflicts of interest.

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Chapter 2

Lymphocyte Signaling and Function in Altered Physiological Environments

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Additional information is available at the end of the chapter

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Abstract

The immune system is the body's defense against infectious organisms and other invaders. It is our immune system that keeps us healthy as we drift through a sea of pathogens. Healthy immune function depends on meticulous regulation of lymphocyte activation. Previous studies have shown unfavorable effects of μg on several physiological systems, including a significant reduction of the adaptive immune response. Lymphocyte movement through interstitium is critically important for the immune response. Thus, the activation of lymphocytes depends on various factors such as cell-to-cell contact due to temporary contact, permanent aggregation or by the uptake of soluble factors such as interleukin 1. Microgravity induced loss of lymphocyte locomotory activity, along with diminished lymphocyte activation, can be counteracted by nutritional supplements such as nucleotides. A study conducted by Andreazzoli et al., proposes that the knowledge of cellular and molecular mechanisms of gravity and its influence on T cells is required for creating the provision of therapeutic and possible preventive targets to keep the bone and immune systems of astronauts fully functional during long-term space missions, in addition to aiding regular people with immune deficiencies. When an immune system is compromised it can lead to various infections as well as cancerous growths. Discovering the ins and outs of the lymphocyte regulatory pathways can account for controlling and studying medicinal treatments for all forms or immune disorders. Therefore, studying both the long-term and short-term effects of microgravity is of great significance, as it has an invalidation nature that affects how the regulators of the immune system are readily able to function.

Keywords: lymphocyte, immunity, inflammation

1. Introduction

The study of lymphocytes in microgravity has been conducted by various researchers to investigate how the immune system is altered due to the change in the gravitational pull [1–3]. Immune system adaptation during spaceflight is a concern in space medicine. Decreased circulating leukocytes observed during and after space flight infer suppressed immune responses and susceptibility to infection. Research has provided sound studies that outline the correlation between extended exposure to microgravity and inhibited activation of lymphocytes. One of the major concerns from the effects of microgravity is the potential for astronauts to be afflicted with opportunistic infections that can only be acquired through a seriously compromised immune system [4]. The microgravity aspect of the space environment has been simulated on Earth to study adverse biological effects in astronauts. It is well known that immune cell function is severely suppressed in microgravity, which renders the cells of the immune system an ideal model organism to investigate the influence of gravity on the cellular and molecular level. In lymphocytes, microgravity affected the protein kinase C [5]. The various factors in the space environment that contribute to immune dysregulation during and post-spaceflight include exposure to microgravity, stress, deconditioning (the reduced physical activity and shift of fluids), and radiation exposure. The body's primary immune defense heavily relies on immune cell distribution and function, which can clearly be influenced by a combination or synergy of any of the factors described above that exist in the space environment. Lymphocytes are a type of white blood cell critical for adaptive immune responses. Of the blood cell types, lymphocytes are the most sensitive to ionizing radiation exposure.

During flight, the activation of cultured human lymphocytes is depressed to less than 3% of the ground controls when exposed to Concanavalin A [4]. When human lymphocytes are exposed to microgravity in a rapidly rotating clinostat, Concanavalin A stimulated T cell activation is depressed [6]. The functionality and activation of lymphocytes is altered and compromised due to space flight. Microgravity and radiation exposure in space suppress human immune systems but, some countermeasures to rescue immune systems in microgravity have been discovered. With the emphasis of space travel being centered on finding various areas to support substantial human life outside of earth, it is imperative that we find ways to supplement a safe journey to another planet.

2. Lymphocyte activation

Earthbound living organisms, including cells, are affected by two new environmental conditions known as microgravity and cosmic radiations when introduced to space. In several experiments dedicated to space missions and simulated microgravity, evidence has shown that microgravity causes a dramatic depression of the mitogenic in vitro activation of T lymphocytes [7]. Lymphocyte activation is dependent upon cell-to-cell contact established in three ways, temporary contacts, permanent aggregation or the uptake of soluble factors such as interleukin 1. Granted, in microgravity, it has been suggested that cell-to-cell interactions are less probable or even impossible.

Studies finding the reduction of lymphocyte activation during exposure to microgravity corroborates with the observations of Soviet and U.S. astronauts during the pre-Shuttle era [5]. The data also reflects the more recent data collected from 41 crew members of shuttle flights (Taylor et al., 1986) who each had declining immune functions to activate lymphocytes upon their return to earth. Additionally, recent studies are deriving similar results of a functional association between the cytoskeletal protein spectrin and PKCβ in the cytoplasm of lymphocytes [8]. PKCβ has a critical role in lymphocyte activation-related signaling, which could be disturbed by disorganization in the cytoskeleton due to gravity, resulting in a disturbed localization of signaling molecules. Results of experiments done by Dr. Chang and her colleagues indicate that microgravity was the causative factor for impaired T cell activation during spaceflight by inhibiting transactivation of key immediate early genes. [9].

Microgravity plays a major role in activating the self-limiting gene expression through miR-21. In microgravity the miR-21 will maturate and accumulates causing the inhibition of translation of the target genes, thus suppressing normal immune responses that would occur at ground level [10]. The heat map found in Hughes-Fulford et al. study shows the expression of 17 significant gene targets of miR-21 from three donors that regulated differently after 1.5 h of activation. They followed the activation under normal gravity and microgravity conditions on board the ISS ($P \leq 0.05$) (**Figure 1**). For some genes there were multiple gene probe sets targeted to different

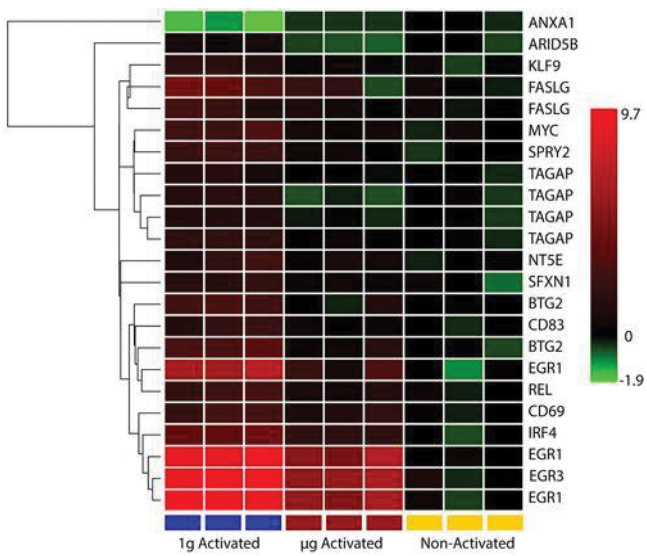

Figure 1. Heat map of 17 significant genes. Courtesy: [10]. Heat map of predicted miR-21 target genes showing differential regulation of T-cell activation after 1.5 h in microgravity (mg) and normal gravity (1 g). The Pearson's centered metric with centroid linkage was used to enforce the grouping of the genes. Rows are comprised of the gene probes while the columns indicate the expression profiles of the donors. Using log2 normalized expression values, the scale of the heat map ranges from green (indicating downregulation) to red (indicating upregulation). The blue-labeled columns illustrate normal gravity-activated samples, in the red labeled columns microgravity-activated samples are illustrated, and yellow-labeled columns illustrate microgravity-nonactivated T cells. Seventeen unique miR-21 gene targets are depicted with differential expression in normal gravity and microgravity condition (P # 0.05; FASLG P # 0.06). Even with considerable variability in nonactivated samples and some variability in microgravity activated donors the gene expression in normal gravity-activated samples become significantly more uniform after 1.5 h of activation.

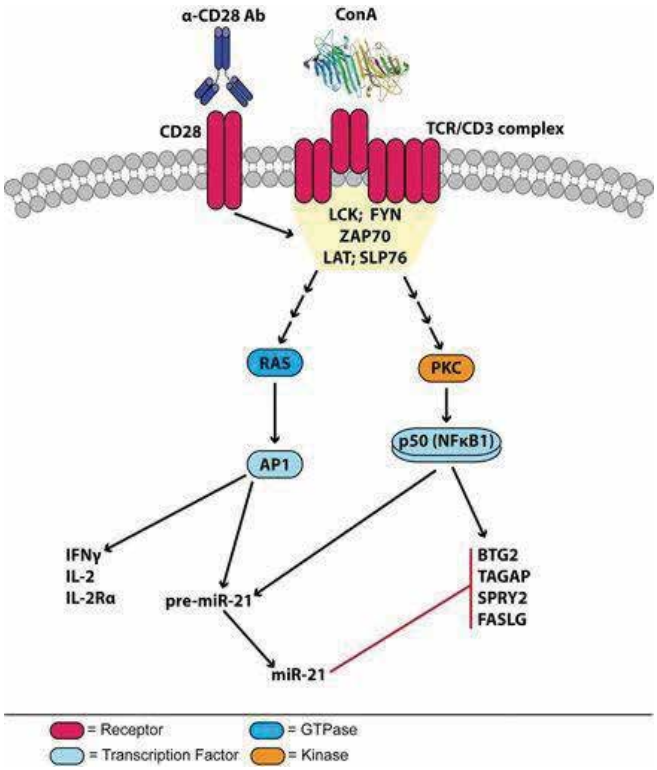

Figure 2. Hypothesized pathways of expression and downregulation. Courtesy: [10]. Postulated actions of miR-21 and gene expression of early T-cell activation. miR-21 is known to downregulate AP1 (28), additionally the presence of its seed sequence in 39-UTR of target genes, it will also downregulate BTG2, TAGAP, SPRY2, and FASLG as miR-21 expression increases after 24 h of activation. These results suggest that miR-21 projects a self-limiting mechanism to regulate T-cell activation. It is possible that miR-21 provides a mechanism of self-limited induction regulating T-cell activation.

regions. Three specific genes with multiple probe sets include FASLG, TAGAP, and EGR1, each showing the same trends of significant inhibition of gene expression under different gravities. Due to their uniformity in their activation profile of normal gravity, there is evidence that after activation with concanavalin A/anti-CD28 all three donors were stimulated in similar genes and pathways. In the microgravity flown samples gene expression was low and less uniformity than the activated normal gravity samples. Consequently, some cases produced a microgravity profile almost equivalent to nonactivated samples, illustrating the suppression of gene expression in all three donors. The onboard normal gravity-activated, microgravity-activated, and non-treated control conditions varied distinctly in their profiles across the 17 predicted miR-21 gene targets, due to this, early activation gene expression could be pinpointed for each condition.

In the study by Martinez et al., they compared the conditions of microgravity spaceflight, random positioning machine (RPM) and rotating wall vessel (RWV) during gene activation of iI2 and iI2r in the genes of mouse splenocyte. They were able to confirm two early activated T-cells, ligp1 and Slamf1 in which they confirmed activation of these genes for the ground samples and suppression of the mouse's immune function in the other conditions **Figures 2** and **3**.

Figure 3. Rotatory cell culture system. Courtesy: OIPL lab, Texas Southern University. The RCCS was originally developed in NASA's Johnson Space Center in order to simulate the microgravity conditions of space. It is structured is based on the principle of clinorotation, which is defined as the nullification of gravity's force by slowing the rotation around one or two axes. NASA's clinostat is a single axis device known as the rotating wall vessel (RWV), the RCCS is the commercial version of this device. The NASA rotating bioreactor simulates microgravity by gently moving the growth medium while growing cells are kept in suspension by a constant "free fall" effect.

In the study of Bradley et al., they investigated how T lymphocytes recognized the antigen presenting cells like the dendritic cells which invokes T cell proliferation driven by interleukin-2 (IL-2) and other cytokines. With this activation these cells are able to function in order to kill invaders and tumors, in retrospect, during spaceflight the production of cytokines is reduced along with reduced proliferations and effector functions, symbolizing the control that gravitational forces have over the immunity function. The researchers then propose that this may be the leading cause for the opportunity for cases of viral reactivation events and opportunistic infections associated with astronauts of numerous missions. In conclusion they found that over exposed culture of T cells in SMG resulted in increased expression of the inhibitory receptor, CTLA-4. Blockade of CTLA-4 interaction with DC ligands resulted in improved T cell IL-2 production [4].

In the study of the microgravity effects of embryotic growth, Shinde et al. found that their morphological changes in the EBs size and shape observed by clinorotation as compared to 1 g control EBs. The number of total EBs formed under clinorotation was less than 1 g indicating that there were no significant differences between proliferation in microgravity conditions and ground conditions. They were able to determine that the major effect of microgravity was the suppressive conditions of cardiomyogenic [11].

3. Lymphocyte locomotion

In order to foster efficient human exploration of space, various studies have been conducted to understand the fundamental role of gravity in development and function of biological organisms The effects of microgravity can be attributed to numerous physical phenomena relevant

to biological research; this included but is not limited to hydrostatic pressure in fluid-filled vesicles, sedimentation of organelles, and buoyancy-driven convection of flow and heat [12]. These physical phenomena can in turn, directly and indirectly, affect cellular morphology, metabolism, locomotion, secretion of extracellular matrix and soluble signals, and assembly into functional tissues [12]. In order to start locomotion on a surface or also transmigration through the epithelium, a resting lymphocyte changes its round shape into a polarized one. This involves a reorganization of the cytoskeletal network with a collapse of the vimentin system [13]. In long-term space travel, the crewmembers are exposed to various amounts of microgravity and radiation that invoke potential hazards to the immune system. The activation of T cells is a critical point in the immune responses. In both microgravity and modeled microgravity (MMG) the receptor-mediated signaling is inhibited which is followed by diminished DNA synthesis in the peripheral blood lymphocytes, which in turn diminish lymphocyte locomotion through type I collagen [14]. Lymphocyte motility through interstitium is critically important for the immune response. It is investigated *in vitro* using collagen as a model matrix. Motile morphology not only includes the constriction and longitudinal contraction shape changes, but also includes clustering [15]. Clustering is random and preceded the motile morphology, which in turn preceded locomotion. Two phases of locomotory activity have been investigated: (a) native motility in lymphoid cell populations, and (b) induced motility resulting from incubation of lymphocytes with matrix, polyclonal activators, and cytokines. Much of the characterization of lymphocyte translocation has been performed on the latter model.

Previous studies showed that modeled microgravity (MMG/RCCS) and Space Shuttle Missions STS-54 and STS-56) microgravity (MG) inhibit human lymphocyte locomotion [16]. Modeled microgravity also suppressed polyclonal and antigen-specific lymphocyte activation [17]. By applying video microscopy and digital scanning, we observed, both in lymphocyte samples from the RCCS and from the Shuttle missions, changes in cell shape, suggestive of a decreased ability to polarize.

The loss of locomotory activity in microgravity, along with decreased activation, suggests that tumor allografts may not be rejected in spaceflight, since the absence of locomotion portends poor invasion of the grafted tissue. Interestingly, activation of lymphocytes prior to exposure to analog microgravity partially or completely abrogated the inhibitory effect of microgravity on lymphocyte motility. Our data suggest that the loss of locomotory function and T cell activation is probably due to lesion(s) in transmembrane signaling possibly involving calcium independent PKC isoforms such as delta and epsilon [6, 14, 18, 19]. Gene expression data from our laboratory on normal and activated peripheral blood lymphocytes cultured in modeled microgravity indicate that selective genes involved in inflammatory processes are affected by changes in gravity. Locomotion can be recovered by nutritional supplements such as nucleotides [1, 2, 20] (Ward et al., 2007).

4. TH1 to TH2 transition in microgravity

Mature CD4+ helper T lymphocytes have been categorized into two major functional phenotypes, TH1 and TH2. Cytokine data from crew members on short- and long-duration

spaceflight showed a significant reduction of IFN-γ and IL-2 [21]. In the 45-day head down bed rest (HDBR) study designed to mimic a real space flight, they found similar decreases of IFN-γ after activation of peripheral blood T cells with anti-CD3 and anti-CD28. Additionally, there was a significant reduction of IL-17A suggesting a weakened T helper (Th1) along with Th17 types of responses. Defective T cell activation or cytokine expression, increased Th2 type cytokine production [22]. Various documentations of decreased Th-1 cytokines production have been assessed repeatedly in astronauts (Taylor et al., 1997). In most flight there is decrease in the IFNg:IL-10 ratio and a Th1:Th2 shift during space flight (Curcian et al., 2008). Th1:Th2 shift can most likely be seen in support of cell-mediated immunity during space flight (Taylor and Janney, 1992; [8]) yet unaltered humoral immunity during space flight (Fuchs and Medvedev, 1993; Stowe et al., 1993).

5. Countermeasures to rescue immune systems in microgravity

Astronauts are exposed to radiation and microgravity during space missions, which causes harm to their immune systems and other negative health effects. Previous studies report that, benzofuran-2-carboxylic acid and its derivatives (KMEG) may provide protection from radiation and restore normal immune function [23] **Tables 1** and **2**.

Since lymphocyte locomotion is inhibited due to gravity, direct activation of protein kinase C (PKC) bypassing cell surface events using the phorbol ester PMA rescues MMG-inhibited lymphocyte activation and locomotion. [14]. Microgravity exposure changed expression of 78 lymphocyte genes. Subsequent treatment with KMEG induced upregulation of six genes namely; CDCA 7, RAB 17, RPSA, YME1L-1, LY96, and AIF1 all involved in T cell early response, lymphocyte activation, growth and proliferation, and the downregulation of five genes namely; ANKHD1, AVP, TINAG L1, CDK5RA 2, and FYN all involved in tumor

Upregulated genes	Fold increase	Functions
RAB17	2.949	Plays an important role in the regulation of membrane trafficking
CDCA7	4.537	Early response gene mediating C-*myc*-related proliferation
RPSA	1.780	Required for the assembly and/or stability of the 40S ribosomal subunit
YME1L-1	1.559	Ensures cell proliferation, promotes anti-apoptotic activity and protects mitochondria from the accumulation of oxidatively damaged membrane proteins
LY96	1.482	Cooperates with TLR4 in the innate immune response to bacterial lipopolysaccharide (LPS)
AIF1	1.338	Promotes the proliferation of T-lymphocytes

The information on the functions of genes described was obtained from the Gene Card website (http://www.genecards.org/).

Table 1. Genomic analysis of significantly upregulated T cell proliferation genes in spaceflight KMEG-treated lymphocytes (μg LT) compared to ground KMEG-treated lymphocytes (1 *g* LT).

Downregulated genes	Fold decrease	Function
ANKHD1/ANKHD1-EIF4EBP3	−4.86	Isoform 2 may possess an anti-apoptotic effect and protect cells during normal cell survival through its regulation of caspases.
AVP	−3.016	Neurophysin 2 specifically binds vasopressin; acts as a negative regulator of innate immunity by inhibiting TLR2/TRL4 associated pattern recognition and pro-inflammatory cytokine production.
TINAGL1	−2.815	Maybe implicated in the adrenocortical zonation and in mechanisms for repressing the CYP11B1. This is a non-catalytic peptidase C1 family protein.
CDK5RAP2	−2.489	Potential regulator of CDK5 activity via its interaction with CDK5R1.
FYN	−2.442	Non-receptor tyrosine protein kinase that plays a role in many biological processes including regulation of cell growth, survival and cell adhesion.

The information on the functions of genes described was obtained from the Gene Card website (http://www.genecards.org/).

Table 2. Genomic analysis of significantly downregulated T cell proliferation genes in spaceflight KMEG-treated lymphocytes (μg LT) compared to ground KMEG-treated lymphocytes (1 g LT).

progression and metastasis. These findings suggest KMEG diminishes microgravity induced immune dysfunction, advancing development of countermeasures to lessen the risks faced by the crew members [18].

Various countermeasure studies have been conducted to find different ways to rehabilitate the compromised immune system. The compromised immune system can lead to various infections as well as cancerous growths. Also discovering the ins and outs of the lymphocyte regulatory pathways can account for controlling and studying medicinal treatments for all forms or immune disorders [3].

Tauber et al. [24] suggests that with all the viable information we have at our hands there is still an unanswered question as to how or by which mechanisms can the cell sense gravity to be affected. It is this question that causes them to propose that this mechanism is most important to explore because alleviating this unknown sensor may be the defying force to creating uninterrupted biological strife in space exploration. They introduce a popular model which enforces the idea that the cytoskeleton is the force that is affected and alarms the cell though unnatural tensions that there is a shift in the atmosphere, thus creating shock to the immune system.

The study done by Bradley et al. [4], further supports this notion to underlining the inhibitory pathways will be a viable countermeasure to restore T cell responsiveness in astronauts during long-term spaceflight or those living in microgravity environment following stimulations. If there is a way to predict the interruption of a pathway or even mimic the mechanism of a pathway to jump start healthy immune functions, test can immediately be conducted to indicate where and when these artificial mechanisms need to be administered to imitate the natural performance of the immune responses.

6. Conclusion

To conclude, it is known that microgravity causes immune system modifications. Factors in the space environment contributing to immune dysregulation during and post-spaceflight include exposure to microgravity, stress, deconditioning and radiation. When human lymphocytes are exposed to microgravity in a rapidly rotating clinostat, concanavalin A stimulated T cell activation is depressed. Lymphocyte locomotion through type I collagen is diminished through the inhibition of receptor-mediated signaling in microgravity. Decreased production of Th-1 cytokines was documented repeatedly in astronauts and hence the defects in T cell activation or cytokine expression, increased Th2 type cytokine production. Although crew members and astronauts have a certain risk on their immune system due to their exposure to microgravity, some countermeasures to rescue their immune systems in microgravity have been discovered. Since lymphocyte locomotion is inhibited due to gravity, direct activation of protein kinase C bypassing cell surface events using the phorbol ester PMA rescues MMG-inhibited lymphocyte activation and locomotion. Previous studies also report that, benzofuran-2-carboxylic acid and its derivatives (KMEG) provide protection from radiation and restore normal immune function.

It is very important to study both the long-term and short-term effects of microgravity as it has a suppressant nature that affects how the regulators of the immune system are readily able to function. Throughout the various pieces of literature, it is having been found that the healthy immune function depends on precise regulation of lymphocyte activation [3]. The access that these studies provide will not only help astronauts who have long-term side effects from space travel, it will also amplify the creation for solutions to repairing the compromised immune systems of people who are already on Earth. Each piece of research looks into the how lymphocytes are activated and then potentially suppressed due to their exposure to the gravitational pull of microgravity. In a study done by Thiel et al. [25] they suggest that long-term in vitro studies should be taken into consideration to have supporting detailed and prolonged data analysis in hopes to identify and understand adaptation mechanisms of the immune system in altered gravity. Additionally, Andreazzoli et al. [19], proposes that extensive knowledge of cellular and molecular mechanisms of gravity and its influence on T cells is an invaluable requirement for the provision of therapeutic or preventive targets to keep the bone and immune systems of astronauts fully functional during long-term space missions as well as regular people with immune deficiencies.

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Chapter 3

Signaling Pathway for the Development of Pre-B Cells

Tatsuaki Tsuruyama

Additional information is available at the end of the chapter

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Abstract

Pre-B cells represent the immature stage of the B cell lineage and express genes for the pre-B cell receptor (preBCR). PreBCR consists of lambda 5 and VpreB and its expression elicits a rearrangement of the immunoglobulin heavy chain prior to rearrangement of the immunoglobulin light chain. The lambda 5 and VpreB form a surrogate light chain, which is a premature type of light chain immunoglobulin. PreBCR may cooperate or interact with the IL-7 receptor, which contributes to pre-B cell development. The preBCR distal signaling pathway recruits several adaptor proteins and protein kinases. This review aims to illustrate the framework of the signaling pathway that contributes to B cell lineage development and reconsiders the relationship between the preBCR and IL-7 receptors.

Keywords: pre-B cell receptor, IL-7 receptor, ZFP521, adaptor protein, rearrangement of immunoglobulin

1. Introduction

B cell lymphocytes develop from common lymphocyte progenitors (CLPs) to form mature B cells, plasma cells, or memory B cells in close association with immunoglobulin genes (*Ig*) rearrangement. *Ig* rearrangement (IgR) status is a phenotypic marker of immature B cell lineage. IgR occurs with the stepwise rearrangement of the V, D, and J segments of the immunoglobulin heavy chain (*IgH*) and immunoglobulin light chain (*IgL*) gene chain in the bone marrow. PreBCR is transiently expressed by developing precursor B cells.

The surrogate light chain is tentatively expressed on the membrane surface, followed by expression of mature *IgL*. Surrogate light and heavy chains form preBCR, involving the adaptor proteins CD79a and CD79b (alternatively termed as *Igα* and *Igβ*). It is not clear how preBCR reacts with external derived antigens and contributes to the formation of mature B cell receptors (BCR). To understand the role of preBCR, it is necessary to elucidate the preBCR distal signaling

pathway. It is well known that many key kinases participate in the BCR signaling pathway, such as spleen tyrosine kinase (Syk), bruton's tyrosine kinase (Btk), B lymphocyte kinase (Blk), and a transcriptional repressor Blimp (B lymphocyte-induced maturation protein). BP-1 (CD249) is another key player in the immature B cell lineage. In this article, the preBCR signaling pathway will be reviewed in reference to the BCR pathway in mice.

The B cell developmental stage has been extensively investigated using surface markers for the appropriate preBCR antibodies. The best known classification for immature phenotype markers is the Hardy's classification fraction (Fr) [1]. These markers range from Fr. A–D (Fr. A: B220+, IgM-, BP-1-, CD43+, and CD24-; Fr. B: B220+, IgM-, BP-1-, CD43+, and CD24+; Fr. C: B220+, IgM-, BP-1+, CD43+, and CD24+; Fr. C′: B220+, IgM-, BP-1+, CD43+, and $CD24^{high}$; and Fr. D: B220+, IgM-, BP-1-, CD43-, and CD24+) with ongoing *IgH* recombination [2–4].

2. Signal cascade in pre-B cells

2.1. IL-7 and IL-7R signaling cascade

IL-7 and IL-7R play crucial roles in B cell development that function in parallel with *Ig* recombination. IL-7R is a heterodimer of the α chain (CD 127) and the common γ chain (γ_c chain). IL-7Rα chains are primarily expressed in thymocytes, dendritic cells, mature T cells, and monocytes. This molecule is also expressed on the surface of pre-B cell lineages, but not on mature B cell lineages. In T cells and pre-B cells, the signal cascade via IL-7R recruits Janus kinases (JAKs), activates the signal transducer and activator of transcription (Stat) by phosphorylation. Among JAKs, Jak2 lacks Src homology binding domains (SH2/SH3) and JAK homology domains (JH1-JH7). The carboxy-terminal JH domain contains full kinase function (JH1), while the JH2 domain has significantly lower kinase activity than the JH1 domain. JAKs are recruited when IL-7 binds to IL-7R, leading to IL-7R auto-phosphorylation. Phosphorylated tyrosine residues of JAKs bind Stats with an SH2 domain [5] and Stat is phosphorylated by JAK. Phosphorylated STAT forms a dimer and translocate to the nucleus. Thereafter, the dimeric STAT binds to the promoter region containing palindromic gamma interferon activation site (GAS) elements that are positioned upstream of Bcl-xL, Cyclin D1, pim1, c-myc, and other genes inducing proliferation [6]. Suppressor of cytokine signaling (SOCS) controls this cascade in a feedback manner, because the *Socs* gene promoter has the GAS element. The SOCS family negatively controls by binding to JAKs. For example, the SOCS3 protein can bind to JAK2 kinase and inhibit its activity [7].

JAK2 mutations have been implicated in myeloid proliferative disorders such as chronic myeloid leukemia [8] polycythemia vera [9], primary myelofibrosis, essential thrombocythemia, and pre-B or lymphoblastic mature B ALL [10]. In these disorders, a change of valine to phenylalanine at the 617 position (V617F) in JAK2 induces higher sensitivity to cytokines such as erythropoietin, thrombopoietin, and IL-7. In therapies targeting JAK2-STAT5 inhibition, *JAK2* mutations are observed in the poor prognostic *BCR-ABL1*-like subtypes of pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Momelotinib and ruxolitinib were therapeutically effective for ALL. In the development of the lymphoblastic cells, the JAK-STAT pathway plays an important role [10].

IL-7R expression is also controlled in a developmental stage dependent manner. In parallel with *Ig* heavy chain rearrangement in pre-B cells (termed large pre-B or pre-B I), IL-7R expression augments and gradually decreases with expression of the surrogate light chain, consisting of VpreB and lambda5. Accordingly, the enhanced effect of the IL-7R signaling cascade is limited to the large pre-B or pre-B I stage. Accordingly, the pre-B ALL phenotype may depend on the IL-7R pathway. Thus, the activated pathway may determine the tumor phenotype. In the bone marrow, stromal cells actively produce IL-7 and promote the proliferation of bone marrow pre-B cells. Cytokine dependency is also observed in T cell leukemia/lymphoma and Human T-cell Leukemia/lymphoma Type 1 (HTLV-I)-induced adult T cell leukemia/lymphoma. When the pre-B cells develop immature-B cells, such dependency on the IL-7 and IL-7R pathway is lost and immature-B cells are recruited to extra-bone marrow area. In contrast, the excess IL-7R expression on the tumor progenitor cells may be activated by lower IL-7 in the peripheral environment, which may result in tumor development. However, the effect of IL-7 on human and mouse pre-B cells are different for proliferation and there have been reports that IL-7 may not be an essential factor in human cells.

Further, the relationship between IL-7R and Jak-STAT during *Ig* rearrangement remains controversial. In a previous study, MLV-integration based Stat5 activation was observed in pre-B cell lymphomas in an inbred strain of SL/Kh mice [9]. However, the dependency of proliferation on IL-7R is a part of lymphomagenesis, because the genetic background or microsatellite instability unique to this strain is also required [18]. For this reason, Stat5 activation is not sufficient for pre-B cell lymphoma or ALL [6].

2.2. IL-7R signaling cascade and Ig rearrangement

There has been discussion regarding the relationship between IL-7R signal cascades RAG1/2 (recombination activating gene 1/2) that mediate *Ig* rearrangement. In fact, it is not clear how transcription factors mediate transcriptional repression of Rag. Recombination activating implies that this gene is involved in immunoglobulin V-D-J recombination. The cleavage activity of RAG1 requires RAG2 as a partner. The RAG-1/2 complex nicks the Recombination Signal Sequence (RSS) that flanks the V, D, and J regions.

In the RAG1/RAG2 complex, RAG1 has the most catalytic activity while RAG2 provides a binding scaffold for the tight association with DNA [11]. There has been a controversial discussion in previous reports that the IL-7R pathway may enhance or suppress recombination. To explain the opposing effect of IL-7, it acts on *Ig* rearrangement in a dose-dependent manner. RSSs consists of three elements, heptamer sequences, spacer sequences, and nonamer sequences that flanks the V, D, and J sequences in the *IgH* and the V and J sequences in the *IgL* region. Spacer sequences are either 12 base pairs or 23 base pairs long and are located between the heptamer and nonamer sequences. Heptamer sequences are CACAGTG and nonamers are usually ACAAAAACC. After rearrangement, RSSs are spliced out of the final *Ig* mRNA. When B cells undergo *IgH* rearrangement, IL-7R expression increases, suggesting that IL-7R may be associated with the rearrangement. However, the downstream molecule Stat5 may contribute to suppression of the rearrangement. As a candidate for the suppression effect, Ebf1 expression is promoted by Stat5, suggesting a link between the negative regulations of Rag transcription [12].

Using model mice with Stat5 high expression, the *IgH* rearrangement was repressed. A schematic representation of immunoglobulin loci is illustrated below (**Figures 1** and **2**).

2.3. PreBCR and surrogate light chain

PreBCR is expressed on a large pre-B cell after or during *IgH* rearrangement. This contains a surrogate light chain consisting of VpreB and lambda 5. In addition, CD79a, CD79b, and the Ig muH chain participate in the formation of preBCR. Although it is not clear how antigens or ligands stimulate preBCR, signaling through preBCR controls allelic exclusion. One of the *IgH* locus rearrangements is inactive, promotes proliferation, and induces differentiation to small pre-B cells. *IgL* rearrangement follows this expression of preBCR. *IgL* rearrangement proceeds after the expression of preBCR in small pre-B cells. CD79a and CD79b contain tyrosine-rich ITAMs that can recruit the cytosolic SRC homology 2 (SH2)-domain-containing SYK following phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs). One of the pan-B cell markers, CD19 and CD45, interact with the preBCR to serve as regulators of positive signaling. In contrast, negative signaling and the regulatory mechanisms of preBCR have not been sufficiently addressed, Therefore, preBCR signaling has not been elucidated. It is possible that immunoreceptor tyrosine-based inhibitory motifs (ITIMs) function to recruit cytosolic

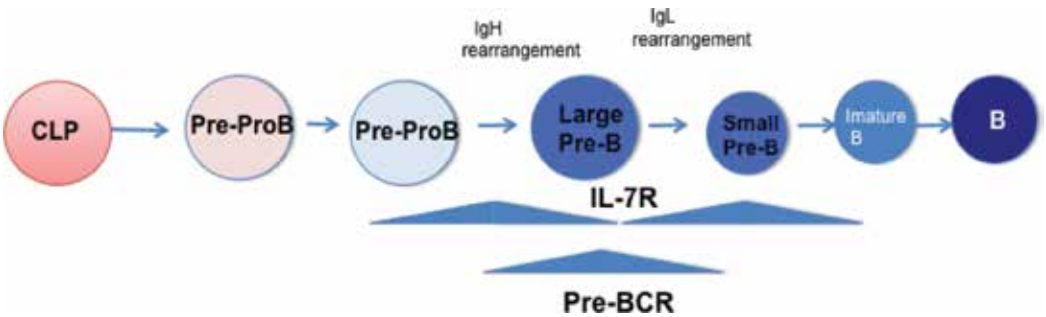

Figure 1. A scheme of B cell differentiation and immunoglobulin rearrangement.

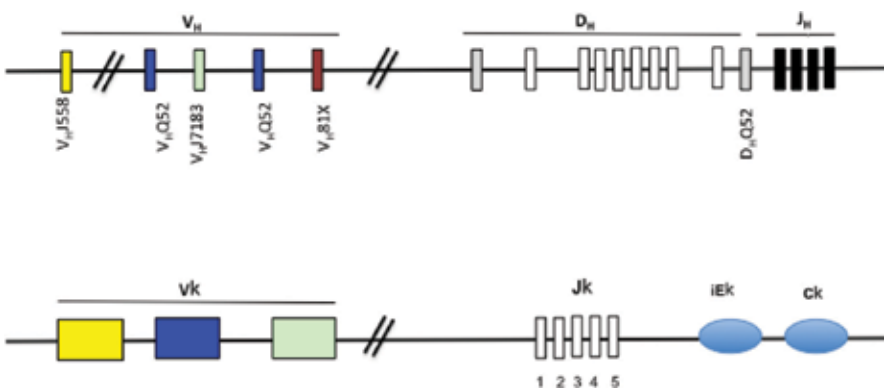

Figure 2. Immunoglobulin gene loci. VH, variable segment; D, diverse segment, J, junction segment. Vk, variable segment of kappa light chain.

protein tyrosine phosphatases (PTPs) such as SH2-domain-containing PTP 1 (SHP1). PIK3K is another adaptor protein of the CD79a and CD79b heterodimer. The RAS-ERK cascade and AKT-FOXO cascade may induce a positive signal inducing proliferation and BCl6, a marker of diffuse large B cell lymphoma/leukemia that may induce B cell lineage proliferation.

SYK can associate with other protein kinases, LYN and Slp65 /BLK or BLNK, that recruit BTK in a phosphorylation dependent manner. BTK associates with SLP-65, also known as BLNK or BASH, another important linker protein. BLNK suppresses Pre-B cell leukemogenesis through JAK3 inhibition [13–15]. BLNK KO mice developed pre-B cells or ALL in an experimental study. BLNK is part of a signaling complex involving Grb-2 and Vav, prior to arrangement of the cytoskeleton. BLNK, BTK, and PLCgamma 2 may form a complex that promotes IRF4 expression linked to *IgL* rearrangement that suppresses or downregulates the preBCR and IL-7R signal cascades simultaneously. In this way, the transition of *IgH* rearrangement to *IgL* rearrangement is controlled by the orchestration of various kinases and adaptor proteins. BLNK is involved in switching cell fate from proliferation to differentiation [16]. Additionally, BLNK recruits active H-Ras to the BCR complex, which is essential for sustained BCR surface expression and for the signal leading to functional ERK activation [17], potentially resulting in B-cell proliferation. Upregulation of *BLNK* may be a consequence of the negative-feedback mechanism and this upregulation results in tumor suppression. BLNK knockdown resulted in downregulation of *BP-1*, a pre-B cell marker [18]. Mice deficient in Slp65 /Blnk spontaneously develop pre-B cell leukemia [13], originating from pro-B cells with V(H)-to D-J(H) recombination. Nevertheless, *IgH* rearrangement was restricted to V(H)14-1 and V(H)14-2, V(H)14 *IgH* chains did not provide increased proliferative signals. PreBCR specificity did not contribute to oncogenic transformation.

Ikaros is required for the differentiation of large pre-B to small pre-B cells and is also required for the down-regulation of the preBCR, Igκ germline transcription, and *IgL* chain recombination. The Ikaros family are regulators of B-cell development by DNA-binding. Ikaros functions as a tumor suppressor in pre-B ALL [19] by controlling BCR-ABL1 kinase signaling from SRC kinase-activation to BLNK [20] or c-Myc expression [21]. Interestingly, BCR-ABL1 induces aberrant splicing of Ikaros in pre-B ALL [22]. The loss of Ikaros DNA-binding function leads to the progression of acute lymphoblastic leukemia [23]. Recently, MLL1 is found to be a regulator of preBCR signaling [24].

2.4. Orchestration of the preBCR and IL-7R signal cascades

In developing B cells, the IL-7R and preBCR synergize or act exclusively to induce proliferation [25]. However, preBCR is also critical to control differentiation through suppression of c-Myc function in large preB cells [26]. PreBCR is thus timely expressed in the transition of large to small preB cells [27]. PreBCR signaling does not affect interactions between the intronic enhancer and V (kappa) genes in proB cells. The kappa enhancers interact with the V (kappa) region already in proB cells. PreBCR signaling induces accessibility through functional redistribution of long-range chromatin interactions within the V(kappa) region [28]. *ZFP521* expression during cell growth is attenuated by the addition of IL-7 [16, 29]. Stimulation of preBCR modulated the growth of *ZFP521*-overexpressing cells [25]. IL-7 and preBCR control the development of preB cells into mature B cells [27, 30]. When IL-7R expression is gradually attenuated during the late stage of large preB cell development, preBCR signaling replaces the dominant pathway. B-cell

development is controlled in a stepwise manner where the first stage of development is completed before the subsequent stages are initiated.

There has been discussion as to whether preBCR functions as a tumor suppressor in the all cases of human acute lymphoblastic leukemia lymphoma (ALL). A distinct subset of human ALL is sensitive to preBCR [31]. The effects of preBCR stimulation were attenuated by the addition of IL-7 [16, 29]. Although both pathways are orchestrated during B-cell development [27], they are linked with immunoglobulin rearrangement [32]. During the development of pro-B cells into pre-B cells, IL-7 signaling is a major mediator with IL-7R expressed at high levels. In contrast, during the development of pre-B cells into mature B cells, preBCR signaling may be the dominant pathway after IL-7R expression is attenuated. Thereafter, mature BCR replaces preBCR. B-cell development is controlled in a stepwise manner in which the first stage of development is completed before the subsequent stages are initiated. In summary, the relationship between preBCR and the IL-7R cascade is complicated and forms an interactive network. The outcome of pre-B cell stimulation is difficult to predict in terms of proliferation or development. This network may be a necessary checkpoint for the developmental stage in a dose-dependent of IL-7 and other stimuli (**Figure 3**).

2.5. Other candidate cascades in pre-B cells

We previously reported that MLV insertion into the signal transducer and activator of transcription factor 5 (*Stat5*), Homeodomain-interacting protein kinase 2 (*Hipk2*), and Flt3-interacting zinc finger protein 1 (*Fiz1*) in the pre-B cell lymphoma genome. These genes encode proteins

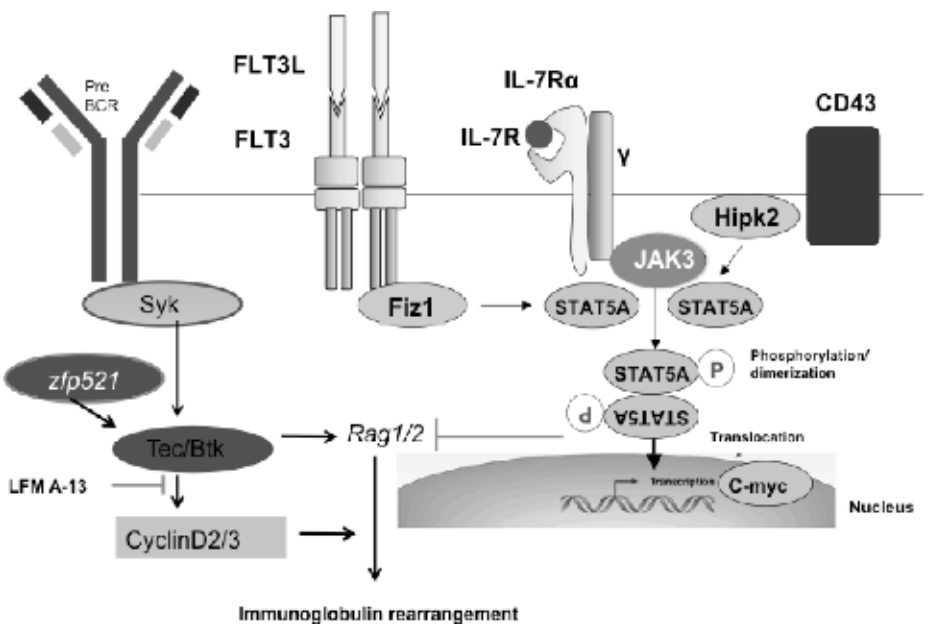

Figure 3. The interactive responsiveness to stimulation through preBCR, IL-7, and other signaling cascades. In this scheme, bank 1 is selected for the modulator that is controlled by zfp521.

that are involved in pre-B cell-specific molecular signaling pathways such as IL-7R, CD43, and Fms-like tyrosine kinase 3 (FLT3). The dysregulation of this preBCR signal is responsible for pre-B cell lymphomagenesis [33]. ZFP521 contributed to mouse and human pre-B-cell lymphomagenesis (i.e., human B-cell lymphoblastic lymphoma). Pre-B cell proliferation depended on the activation of preBCR signaling molecules, which were upregulated by ZFP521.

2.6. Pre-B cell and acute lymphoblastic neoplasia in mouse

To date, pre-B cell lines from acute lymphoid tumors require strict cell culture conditions and consistent time course research. Using these cell line, IL-7 supply is critical, but IL-7 and v-Ha-ras expression are not individually sufficient to induce tumorigenicity. Their co-expression yields highly tumorigenic pre-B lymphoid cell lines [34].

On the other hand, as an experimental model, SL/Kh is known to be useful for tumorigenesis of pre-B cells and signaling pathways. This strain has two copies of AKV endogenous MLV and other retrovirus-derived fragments [3]. The expressed viral vector infects the host B cell progenitors and retroviral elements, such as promoters and enhancers promote *Stat5, c-myc, ZFP521, N-myc*, and other oncogenes [3, 6, 18, 35–37]. For this, this strain serves as an appropriate model for analysis of interaction between these molecules and their related signal pathways.

2.7. Role of *ZFP521* during lymphoid differentiation

The mechanisms by which preBCR-related genes are controlled are not sufficiently understood relative to the mature B cell receptor. *ZFP521* has been recently recognized as an important gene in pre-B cell lymphomagenesis. When *ZFP521* is upregulated, BTK, BLNK, and BANK1 are involved in the preBCR signaling pathway and are comprehensively upregulated. ZFP521 contributes to the upregulation of *Ccnd3* and *Ccnd2*, enhancing the cell cycle and inducing proliferation. In a previous study, preBCR also activates the Ras-MEK-extracellular signal-regulated kinase (ERK) pathway, cell cycle exit, and light chain recombination by silencing *Ccnd3* [38]. *Ccnd3* gene expression is probably responsible for the growth of pre-B cells [39]. *BTK1* and *BANK1* are downstream of preBCR or BCR is controlled by *ZFP521* and upregulation of *BTK1* and *BANK1* contributes to pre-B cell proliferation.

BANK1 is a modulator of the pre-B cell signaling pathway disrupted by IL-7R signaling that interacts with phospholipase gamma2 [40]. Overexpression of *BANK1* enhances BCR-induced calcium mobilization. Another lymphocytic associated kinase, LYN, associates with BANK1. LYN is activated with catalyst tyrosine phosphorylation of IP_3R (Inositol 1,4,5-trisphosphate receptor).

BTK is a useful diagnostic marker for Hodgkin's and B-cell non-Hodgkin's lymphoma [41]. BTK-dependent pathways are involved in maintaining the malignant phenotype in B-cell lymphomas and leukemias [31, 42–44]. Anti-apoptosis signaling in various B-cell malignancies requires BTK-dependent signals from the B-cell antigen receptor. A distinct subset of human ALL is selectively sensitive to preBCR antagonists, such as those employed for ibrutinib therapy for B cell malignancy [31, 42–44] In contrast, several reports suggested that BTK acts

as a tumor suppressor in the majority of human ALL cases [31, 41]. The BTK-dependent pathway is controlled in an expression dependent manner. Additionally, overexpressed BTK affects the survival or selection of B cells during the development of malignancies [45] and contributes to malignant transformation.

In humans, fusion of the *Pax5* exon 7 to *ZNF521* exon 4 has been observed in pre-B cell acute lymphocytic leukemia by genome-wide analysis of genetic alterations [46]. Importantly, this breakpoint is located near the conserved integration target sequence in human *ZNF521*, suggesting that the locus is active in pre-B cells as in *Pax5*, which is essential for pre-B cell development. Thus, we can conclude that aberrant release of the *ZFP521* gene control leads to pre-B cell lymphomagenesis through activation of pre-B cell-specific molecular signaling pathways. Moreover, c-Jun expression was observed in lymphoma tissues exhibiting *ZFP521* overexpression, suggesting that c-Jun is associated with lymphomagenesis [25].

MLV insertion into *ZFP521* gene was observed in the lymphomas with its insertion to *Sat5, Hipk2,* or *Fiz1* genes. These target tgenes may interact in the development of pre-B lymphoma. The dysregulation of this preBCR signal is responsible for pre-B cell lymphomagenesis [33]. *ZFP521* contributes to mouse and human pre-B cell lymphomagenesis (i.e., human B-cell lymphoblastic lymphoma). Pre-B cell proliferation depends on activation of preBCR signaling molecules including BANK1, which are upregulated by *ZFP521*.

ZFP521 is involved in tumorigenesis in pre-B cell lymphoblastic lymphoma through upregulation of preBCR signaling molecules, interfering with the IL-7R signaling pathway. *ZFP521* also mediates the expression of *Ccnd3*, *c-jun*, and other cell cycle-related genes. Therefore, these data suggested that *ZFP521* might be a promising target for targeted molecular therapy for ALL or B-precursor lymphoma.

3. Conclusion

The early stage of B cell differentiation is characterized by immunoglobulin rearrangement. Rearrangement is controlled by both the enzyme RAG 1/2, and IL-7R and preBCR signaling pathways. These two pathways sometimes function cooperatively, sometimes antagonistically and seem to support the timing of immunoglobulin gene rearrangement.

4. Materials

4.1. SL/Kh strain

SL/Kh is an inbred mouse strain that shares the AKV1 pro-virus with the AKR strain, which is susceptible to T cell leukemia/lymphoma [3]. This strain has been developed over 30 years through brother-sister mating and acquired susceptibility to MLV-mediated pre-B lymphoma [47]. AKV was mapped as the endogenous *ecotropic murine virus 11* (*Emv11*) onto chromosome 7 [48, 49]. In this strain, the pro- to pre-B cells expand in the bone marrow in a polyclonal manner before monoclonal expansion [18]. Afterwards, more than one copy of the proviral

genome is acquired during the development of lymphomagenesis. More than 90% of these mice spontaneously develop sIgM pro- or pre-B lymphomas, positive for preBCR, by 6 months of age. MLV genomes were integrated into Stat5, c-Myc, ZFP521, and other oncogenes in the lymphoma cell genome. Upregulation in expression of Stat5 and ZFP521 are not sufficient, as pre-B cell lymphomagenesis and a strain-dependent background are required [50, 51].

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Conflicts of interest

The authors have declared no conflict of interest.

Appendices and nomenclature

MLV	murine leukemia retrovirus
ZFP521	zinc finger protein 521
Stat5a	signal transducer and activator of transcription

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Chapter 4

Understanding B Lymphocyte Development: A Long Way to Go

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Additional information is available at the end of the chapter

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Abstract

B lymphocytes play a significant role in both antigen-dependent and antigen-independent pathways. Although significant progress has been made in context of delineating the pathways that lead to their development, maturity and differentiation, their detailed mechanisms are yet to be dissected out completely. This chapter is aimed towards summarising the knowledge that has been gained till date and identifying the areas that need to be addressed in future research work. Overall, the chapter is planned in a sequential way to guide the reader through the processes of B cell development and the various latest findings that have improved our understanding of this vital physiological system.

Keywords: B lymphocyte, bone marrow, marginal zone, antigen, spleen, germinal centre

1. Introduction

B lymphocytes play vital role in maintaining the normal immunologic functions of the body. Their functions range from producing antibodies to presenting antigens. They are also involved with productions of several regulatory cytokines, such as IL-2, IL-4, IL-6, IL-10, IL-12, TGF-β1, TNF and IFN-γ. Each of these functions are further fine tuned by the fact that they are dependent upon several factors, including the B lymphocyte subsets, their location and the type of stimuli that is encountered by the specific B cell subset in that particular environment. Understanding all these components of B cell development is not only required for getting a better picture of B cell biology, but is also necessary for understanding various immunologic anomalies that lead to disorders. They are also important towards generating effective B cell based therapies. This chapter begins with explanation of the overall pathway

of B cell development and the organs that are involved in its various stages. This is followed by discussion on the role of gene rearrangements in the entire process. Subsequently, the role of various transcription factors has been addressed.

2. Steps in B lymphocyte developmental pathway

2.1. Bone marrow-dependent stages

During foetal life, B cells are generated in the foetal liver. Subsequently in the adults, they are produced by differentiation of haematopoietic cells (HSCs) in the bone marrow [1]. Most of the stages of B lymphocyte development take place in this primary lymphoid organ. The pluripotent HSCs gradually differentiate into progenitors, which have increasingly lower potency. Initially, they form a population of cells that are known as multipotent progenitors (MPPs). These progenitors, in turn, give rise to two main progenitor populations: common granulocyte/megakaryocyte/granulocyte progenitor (CFU-GEMM) and early lymphoid progenitor (ELP). CFU-GEMMs subsequently develop into cells that have either myeloid or erythroid potential. On the other hand, cells with lymphoid potential arise from ELPs. Thus, CFU-GEMMs are the primary source of those elements of blood that are non-lymphoid in nature, whereas the lymphoid elements originate from ELPs. Two major precursors arise from the ELPs, common lymphocyte progenitor (CLP) and early T-lineage precursor (ETP). Both Pre-NK cells and Pre-B cells develop from CLPs, which eventually give rise to NK cells and B cells, respectively. T cells derive from thymocytes, which are generated by differentiation of the ETPs. The CLPs give rise to early Pro-B cells first. They mature to form the late Pro-B cells, which eventually develop into Pre-B cells. Immature B cells arise from these Pre-B cells and they leave the bone marrow to enter into the secondary lymphoid organs. Subsequent stages of B cell development primarily continue in the spleen. During this entire period of maturation, the various B cell subpopulations are found to migrate within the bone marrow, keeping in touch with the stromal cells. Initially, the progenitor cells having highest potency lie in the endosteum. This region is located near the inner surface of the bone. As the B cell progenitors mature to give rise to cell types that have less potency and are more committed towards the B cell fate, they start migrating towards the central sinus of the bone marrow cavity. During this entire process, these maturing cell populations are reported to remain in contact with the reticular stromal cells, which are believed to provide indispensable signals for migration and maturation. This process continues till the developing cell reaches the stage of Immature B cell [2–4].

2.2. Role of bone marrow cell populations in B cell development

In addition to HSCs, B lymphocytes (in various stages of development) and plasma cells (PCs), the bone marrow consists of specialised cells that have multiple roles in various stages of this developmental process. Together they form the “niches” that are vital for normal functioning of the different systems associated with the bone marrow. The most important components of these “niches” are stromal cells and regulatory T cells. Out of these, the stromal cells form an

extensive network of non-lymphoid connective tissue in the bone marrow [5, 6]. They serve dual functions of forming adhesive contacts with developing lymphocytes and providing cytokines/chemokines/growth factors to them as per requirement. In context of maintenance of plasma cells, earlier reports have indicated that they are provided with survival signals such as CXCL13, IL-6, APRIL and BLγS by the stromal cells [7]. In addition, it has been found recently that the regulatory T cells present in the bone marrow play vital role in maintaining plasma cell pool [8]. The dendritic cells (DCs), present as perivascular clusters in the bone marrow, have also been reported to provide signals that are vital for B lymphocytes [9]. Megakaryocytes [10], eosinophils [11] as well as basophils [12] resident in the bone marrow have also been found to play role in maintaining plasma cell pools.

2.3. Spleen-dependent stages

The immature B cell undergoes final stages of development in the spleen to form mature B cells. The spleen primarily consists of red pulp, white pulp and marginal zone. The red pulp is made up of large, blood-filled sinuses and serves as the blood-filtering system of the spleen. In addition, the splenic macrophages play important role in recycling of iron. The white pulp is organised in line with the lymph nodes and consists of lymphoid sheaths having distinct B-cell and T-cell compartments. The marginal zone is a layer of highly specialised cells that surrounds the white pulp [13]. It plays a very important role in immunity because those haematopoietic cells that remain in circulation (as part of the surveillance mechanism) need to be able to migrate through blood and lymphatic systems continuously. The marginal zone plays a vital role in this process due to its strategic location in the spleen. It has been observed that G-protein linked receptors are involved in the signalling process that is responsible for active transport of B- and T-lymphocytes to-and-from the white pulp [14]. The specialised cells that constitute the marginal zone include two subsets of macrophages, the marginal-zone macrophages and the marginal-zone metallophilic macrophages. The first subset is present as an outer ring and express SIGNR1 (a C-type lectin) [15–17] and MARCO (a type I scavenger receptor) [18]. The second subset is present as an inner ring, lies closer to the white pulp and expresses SIGLEC-I (an adhesion molecule) [19]. A specialised B-cell population, known as marginal zone B cell, and DCs are located in between these two rings of macrophages [20, 21]. **Figure 1** shows the major cell populations that are generated in the bone marrow and peripheral lymphoid organs during the process of B cell development.

It has also been reported that the antigens are encountered by the mature B cells in the lymphoid follicles. This process is aided by T cells present in the germinal centres. All the subsequent stages of B cell development, including generation of various Ig isotypes, class switching and somatic hypermutation, contribute towards diversification of the antibody repertoire [22].

On activation by cognate antigen, the activated B cell can either differentiate into antibody-secreting plasma cells/plasma blasts or get recruited into a specialised region known as the germinal centre (GC). Those activated B cells that enter the GC subsequently undergo several rounds of proliferation, class-switching and affinity maturation. Thereafter, the GC B cells that have completed these steps successfully give rise to either long-lived plasma cells or

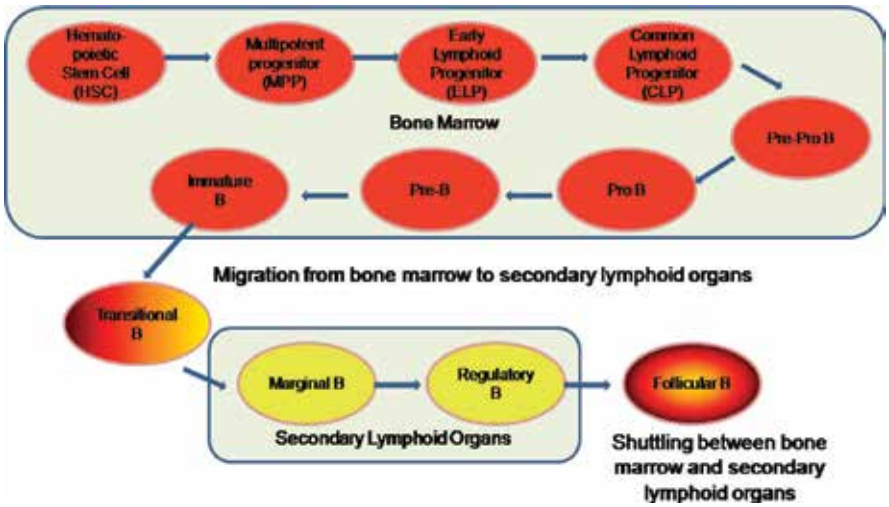

Figure 1. Major stages of B cell development.

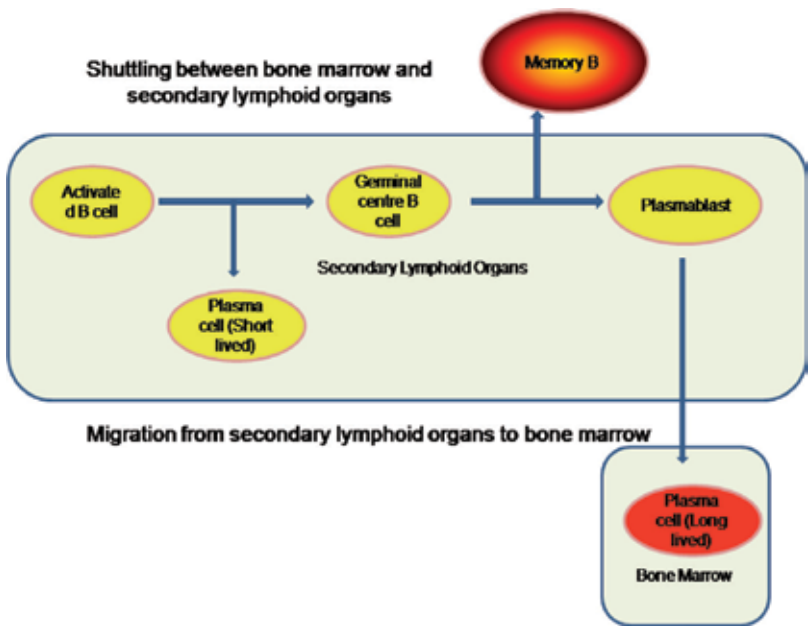

Figure 2. Major stages after activation of B cells.

memory B cells [23]. When the B cells undergo these changes in the GC, their transcriptional repertoire also undergoes huge transformation [24]. **Figure 2** shows the various stages that are developed after activation of B cells.

3. B cell subsets

In general, three subsets of B cells derive from naive B cells, B-1 B cells, follicular B cells and marginal zone (MZ) B cells. Furthermore, B-1B cells form two subsets, B-1a and B-1 b B cells [25]. All the subsets can be clearly identified on the basis of their surface markers. These surface markers can also be used to identify their progenitor populations. **Table 1** shows the various stages of B cell development and the most prominent cell surface markers that are used to identify them. Each one of these subsets maintains distinct location(s) and function(s). All these subsets of B cells produce functionally important antibodies. However, they vary in huge terms in reference to their origin and function [26–28].

3.1. B-1 B cells

B-1 B cells have not been successfully studied in mammals, including humans [29]. Thus, most of the findings are based on studies performed in mice. The most interesting finding from these studies is that although the progenitors of B-1 a and B-1 b cells are distinct, they are found to occupy the same locations, namely the pleural and peritoneal cavities. In addition, it has been observed that the environment offered by these cavities plays a significant role in

Developmental stage	Mouse cell surface marker		Human cell surface marker	
	Positive	Negative	Positive	Negative
Pre-Pro B	CD43, B220, Flt3, CD93, CXCR4, IL7R	CD19, cKit^{low}	CD34, CD38, CD10	cKit^{low}
Pro B	CD19, CD43, B220, IL7R	Flt3, ckit^{low}	CD19, CD34, CD38, CD10, CD24, IL7/3R	ckit^{low}
Pre B	CD19, B220, IL7R, CD24, Siglec-G	CD43^{low}, ckit	CD19, CD10, CD20, CD24, CD38, IL3/4/7R	CD34, ckit
Immature B	CD19, CD43, B220, CD24	CD43, CD23	CD19, CD10, CD20, CD21, CD40, IL4R, CD24^{high}, CD38^{high}	ckit, CD27, IL7R
Transitional B	B220, CD19, CD24, CD93, CD21^{var}, CD23^{var}		CD5, CD21, CD19, CD20, CD24^{high}, CD38^{high}	CD27, CD10^{low}
Marginal Zone B	B220, CD9, CD1d, CD21^{high}, CD35^{high}	CD93, CD23	CD19, CD20, CD1c, CD21^{high}, CD27^{var}	
Regulatory B	CD19, CD5, CD24, TIM, CD1d^{high}	CD62L, CD93^{var}	CD19, CD5, CD21, CD1d^{high}, CD24^{high}	CD27^{var}
Follicular B	B220, CD19, CD23, CD38, CD22	CD93, CD1d^{low}, CD21/35^{low}	CD19, CD20, CD21, CD24, CD23, CD22	CD27, CD10, CD24^{low}, CD38^{low}
Activated B	B220, Flt3, CD27, CD80, MHC II^{high}	CXCR4, CD138	CD19, CD20, CD27, CD25, CD69, CD30, CD135	
Germinal Center B	CD19, CD20, Siglec2, GL7, CD37	CD93, CD38^{low}	CD19, CD10, CD23, CD27, CD20, CD38^{high}	CD24^{low}
Plasmablast	CD138, CD19, MHC II, CXCR4	Flt3, B220^{low}	CD19, MHC II, CD269, CD38^{high}, CD27^{high}	CD138, CD20
Plasma cell (short lived)	CD138, CD93, CXCR4^{high}	CD19, B220^{low}, CD38^{low}, MHC II^{low}	CD138, CD269, CD27, CXCR4, CD38^{high}	CD20, CD19^{low}, MHC II^{low}
Plasma cell (long lived)	CD138, CXCR4^{high}	CD19, B220^{low}, CD38^{low}, MHC II	CD138, CD269, CD27, CXCR4, CD38^{high}	CD20, CD19^{low}, MHC II
Memory B	B220, CD38^{var}, CD80^{var}, CD62L^{var}, CD95^{low}		CD19, CD40, CD20, CXCR4/5/7, CD27^{var}	CD38, CD23^{low}

Table 1. Most prominent cell surface markers used to identify various stages of B cell development.

shaping the functional characteristics of these B cell subsets. The milieu of these cavities also influences the functional characteristics of B-2 cells that reside there, although in low numbers [30, 31].

3.2. Follicular B cells

Several studies have shown that the naive, mature peripheral follicular B cells reside in two main niches during their circulation/recirculation through the bone marrow. Out of these, the "follicular niche" present in the spleen/lymph nodes/Peyer's patches is the main site that is occupied by these cells. These "follicular sites" are thought to play important role in those immune responses against protein antigens, which are T cell-dependent [25].

In addition, some follicular B cells have also been reported to home in the bone marrow [32]. The site of their homing has been termed as "perisinusoidal niche" and consists of a part of population of the same circulating follicular B cells that are found in the secondary lymphoid organs mentioned earlier. Interestingly, the follicular B cells residing in the bone marrow are involved in T cell-independent immune responses against microbial pathogens harboured by blood [33].

3.3. Marginal zone (MZ) B cells

MZ B cells mainly home near the marginal sinus of the spleen. This process is facilitated by the molecules SIP 1 and SIP 3, which are receptors for sphingosine-1-phosphate [34–36]. These cells are mainly involved in T cell-independent immune responses against blood-borne microbes [34]. It has also been reported that these MZ B cells can transport pathogens from the marginal sinus to the splenic follicles, sites where the follicular B cells reside [21, 37]. Moreover, a few *in vitro* studies have shown that these MZ B cells might also be contributing towards T cell-dependent pathways of antigen recognition and subsequent immune responses. Some reports have demonstrated that they may have better capability than follicular B cells in context of activation of T cells [38, 39].

4. Contributions of various players towards B cell development and selection

4.1. Pathways involved in B cell maturation stages in bone marrow

As mentioned earlier, B cell development starts from haematopoietic stem cells (HSCs) in the bone marrow and continues either at the same site or in the peripheral sites. Two cellular pathways are believed to be involved in formation of mature B cells from T2-like cells, one in the bone marrow, and the other in the peripheral sites [25]. Thus, the population of mature B cells present in the bone marrow is heterogeneous in nature. It has been observed that one population of these cells is characterised by $sIgM^{high}$ IgD^{low} $CD23^{-}$, and do not respond either to BLγS/BAFF or multivalent antigens. In contrast, another population of these cells is $sIgM^{high}$

IgD^{high} $CD23^{+}$, and responds to both BLγS/BAFF and multivalent antigens [32, 40]. The exact mechanisms that are responsible for this differential developmental pathways and their significance is yet to be understood completely.

4.2. Pathways involved in B cell maturation at the peripheral tissues

Once immature B cells form, they generally migrate to the peripheral sites. These immature B cells have very short half-lives and on engagement with BCRs, they tend to undergo apoptosis instead of proliferation [41, 42]. The immature B cells are also referred to as "transitional B cells" and can be further subdivided into three subsets; T1, T2 and T3 [41, 43, 44]. CD93/AA4 (the B-lineage precursor marker) is expressed in all of these "transitional B cell" subsets. However, they display differential expression of IgM and CD23 on their surfaces that is exploited to identify them. T1 cells are characterised by IgM^{high} $CD23^{-}$, T2 cells by IgM^{high} $CD23^{+}$ and T3 cells by IgM^{low} $CD23^{-}$ [41]. All data till date suggest that T2-like cells give rise to mature B cells, either in the bone marrow or in the peripheral sites.

4.3. Tolerance and B cell development in peripheral sites

Tolerance to self-reactive antigens can take place by any of the existing three mechanisms: deletion, editing or anergy. In spite of significant number of studies addressing this aspect, it is not yet clear whether this tolerance is achieved by negative selection of self-reactive B cell clones or failure of positive selection. Several studies have suggested that local levels of BAFF may influence this decision [45, 46].

In humans, majority (around 55–75%) of immature B cells have been found to be self-reactive [47], indicating that clonal deletion may serve as one of the main mechanisms of elimination of these self-reactive cell populations. However, no such experimental data is available from mice. In addition, receptor editing has also been found to contribute towards elimination of such self-reactive immature B cells. The process of anergy, although a bit controversial, has emerged as the third mechanism involved in removal of self-reactive B cells [48].

4.4. Pathways involved in B cell maturation stages in the spleen

Spleen is the main site for positive selection of non-self reactive B cell clones. It has been reported that survival of the peripheral B cell populations is dependent upon continuous signalling through B Cell Receptor (BCR). It holds true for both populations, follicular B cells as well as marginal zone (MZ) B cells [49, 50]. Although a wide variety of V-gene segments are expressed by both follicular and MZ B cell populations, it has been observed that in IgH transgenic mice, those immature B cells that are specific for phosphorylcholine give rise to MZ B cells [51]. In another study, it has been observed that deletion of RAG2 in adult mice results in selective retention of MZ B cells over follicular B cells [52].

In addition to BCR, Notch2 signalling plays a significant role in MZ B cell development [53, 54]. Notch2 is a member of Notch family of receptors and ligands [55]. Interaction between Notch2 and DL1 has been found to be responsible for developing a unique MZB cell niche [54]. Moreover,

interaction of Notch2 with NF-κB pathway component p50 helps in maintaining MZ B cell pool, without affecting the pool of follicular B cells [56]. The NF-κB pathway may also work in synergy with the BAFF-BAFF-R pathway, both in context of survival of follicular B cells and generation of MZ B cells [57]. In addition, studies have indicated that BCR signalling and Notch2 signalling may work synergistically for development of MZ B cells [58].

5. Gene rearrangements during B lymphocyte development

The antibody responses demonstrated by the various subsets of B cells depend upon formation of the fully functional antibody having the required specificity. Each such fully functional antibody is constituted by two light chains (IgL) and two heavy chains (IgH). Each light chain, in turn, consists of variable (V), joining (J) and constant (C) domains. On the other hand, each heavy chain consists of variable (V), diversity (D), joining (J) and constant (C) domains. As mentioned earlier, mature B cells arise by stepwise development from Hematopoietic Stem Cells (HSCs). The genes encoding various domains of the heavy and light chains of the antibody also get progressively rearranged during these developmental phases [59, 60]. Finally, a specific group of genes encoding the various regions of the antibody get expressed [61, 62]. This remarkable process gives rise to the huge repertoire of antibodies, having infinite types of antigen specificities, from a limited pool of gene fragments encoding the various V(D)J domains. This process is known as V(D)J recombination, and is the hallmark of adaptive immunity [63]. The process of gene rearrangement begins in the heavy chain loci of the earliest progenitor cells that get fully committed to B cell lineage. Initially, μ gene rearrangement starts. If DH to JH recombination is successful, VH to DJH recombination follows. Formation of a productive μ gene on any one of the alleles results in expression of a functional immunoglobulin heavy chain μ (Igμ protein) on the cell surface and differentiation of the cell into precursor B cell (pre-B cell) [64, 65]. Once a successful recombination results in formation of a functional heavy chain, further rearrangements in remaining heavy chain loci cease. This newly formed functional heavy chain is stabilised by pairing with an invariant light chain. This heterodimer forms the pre-B-cell receptor (pre-BCR) in association with the signal transducing units, Igα and Igβ [66, 67]. In the next step, rearrangement in the immunoglobulin κ (Igκ) light chain gene locus begins for generating a successful recombination of its V and J fragments [68, 69]. This step of κ light chain gene rearrangement is stimulated by the newly formed Igμ heavy chain [70]. In case this rearrangement does not succeed, rearrangement begins in the immunoglobulin λ (Igλ) light chain gene locus for recombination of its V and J fragments to give rise to a functional λ light chain [71, 72]. If light chain gene rearrangement is successful, the light and heavy chains combine to form the functional IgM antibody molecule. This antibody is membrane bound and is expressed on surface of the B cell. Only those B cells which express a functional BCR as well as a functional IgM on their surface, progress to give rise to mature B cells.

The process of V(D)J recombination is never perfect. This imperfection allows development of diversity in the antibody structure. However, this imperfection also leads to formation of non-productive recombinations, which can be as high as two-thirds of the total number of recombinations. Several studies have shown that these non-productive rearrangements can be rescued by VH replacement [73–77]. Each of these gene segments is flanked by conserved sequences, known as Recombination Signal Sequences (RSS). The RSSs consist of a heptamer

(having the sequence CACTGTG) and a nonamer (having the sequence (GGTTTTTGT). They are separated by a spacer, which has a length of either 12- or 23-bp [78]. V(D)J recombination follows 12/23 rule. This rule refers to the fact that recombination is preferred between those gene segments that are flanked by RSSs of different spacer lengths. These RSSs are substrates for enzymatic activity of the products of recombination-activating genes (RAG-1 and RAG-2) [79–82]. This entire enzyme-substrate system is under tight regulation of systems that operate in tissue-specific and stage-specific manner. This ensures that they work in accordance with the lymphocyte developmental pathway [83].

6. Regulation of B lymphocyte developmental pathway: role of transcription factors and epigenetics

Transcription factors (TFs) play vital role during the entire process of B cell development and maturation from HSCs [84–86]. The entire process involves multiple changes both at the levels of transcriptional state as well as chromatin structure. They include at least four broad areas: (a) developing a localised chromatin state that will be favourable for gene activation through priming the enhancers in lineage-specific manner [87, 88]; (b) expressing TFs in lineage-specific manner [89]; (c) interaction between the various TF networks resulting in formation of extremely complex and fine-tuned regulatory networks that can activate various gene networks in lineage-specific manner [90]; and (d) activating TFs repression mechanisms to prevent development of alternative cell fates so that lineage-specific decisions and commitments do not get altered [91, 92].

During embryogenesis, three SOXF factors (SOX7, SOX17 and SOX18) play regulatory role in development of haematopoietic system [93]. Out of them, SOX17 is directly involved in the process of expansion of foetal HSCs [94]. A very recent study has shown that SOX7 promotes formation and proliferation of early blood progenitors; and blocks lineage commitment and formation of B lymphocytes [95].

The adult haematopoietic system is established by the coordinated functioning of three TFs, c-myb, acute myeloid leukaemia (AML)-1 and SCL-Tal [96]. Another TF MEF2C is present in very high levels in CLPs and B lymphocytes in the bone marrow [97]. Recently it has been reported that this TF protects B cell progenitors and helps in their survival by enhancing expression of the factors that are involved in DNA repair and recombination [98]. Arid3a and Arid3b, members of the ARID (AT-rich interaction domain) family of TFs, are required for B cell development. However, HSC development can take place independent of Arid3b [99]. Three main TFs, E2A [100–102], Ikaros [103, 104] and PU.1 [105, 106] specify B lineage commitments in the progenitor populations of HSCs and MPPs. As a result, LMPPs are generated from them. The effects of some of these TFs work in dose-dependent manner. For example, levels of the TF PU.1 in the MPPs determine whether they will progress towards myeloid or B lymphoid lineage [107–109]. EBF (early B-cell factor) 1 is a known TF that plays vital role in B cell differentiation. In CLPs, E2A has been shown to regulate expression of this TF [110, 111]. In turn, EBF-1 acts in coordination with the TFs E2A and Foxo1 to regulate expression of Pax5 gene, which plays extremely important role in B cell development [112].

Thus it seems that a regulatory network of several TFs determines and regulates lineage commitment in a recurrent manner [113]. Other significant contributors to this regulatory network include Gfi1 [114, 115], members of NF-κB family [116–118], members of interferon regulatory factors (IRF-4/Pip and IRF-8/ICSBP) [119], T-bet [120], E47 [121], Krüppel-like factor 3 (Klf3) [122] and Fli-1 [123].

Epigenetic control plays a vital role in the entire process of lineage commitment and downstream B cell development. Tet (10–11 translocation family) enzymes, known to oxidise 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), are important regulators of somatic cell differentiation. Recent studies have indicated that at least two Tet enzymes, Tet2 and Tet3 are involved in the process of tissue-specific methylation of DNA essential for B cell differentiation [124]. Other studies have indicated the role of high mobility group (HMG) proteins, in particular HMGN, in the process of activation of naive B cells. This group of proteins was reported to act on the chromatin regulatory sites of the resting B cells [125]. Interestingly, the role of various TFs in regulating chromatin accessibility is also being observed. For example, a very recent study on EBF-1 has revealed that its C-terminal domain (CTD) is essential for gaining access to those regions of the genome that are least co-occupied by other TFs. This allows those regions of untouched chromatin to become accessible for structural modulations, such as demethylation. Subsequently, changes leading to B cell fate take place [126]. Earlier studies had shown that Ebf-1 deficient cells get stalled at the point where B cell lineage commitment gets implemented [127, 128]. Gain- and loss-of-function studies on EBF-1 have also shown that this TF upregulates genes that promote B lineage commitment and downregulates those genes that lead to commitment towards non-B lineages [129, 130], thus further confirming the regulatory role played by this TF.

7. Conclusions

B lymphocytes are key players in the immune regulation system. Thus, any alteration in their development or functioning is manifested in the form of a diseased state. Complete understanding of the pathways and the underlying molecular mechanisms of B cell development/functioning is vital as it may lead towards generating new medical interventions. Although we are yet to obtain a clear understanding of the intricacies that govern development of a specific a B cell type, a huge number of studies have contributed towards getting a better picture. Further research is needed for gaining better insight into these processes.

Conflict of interest

None declared.

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Chapter 5

Prognostic and Therapeutic Implications of Lymphocytes in Hematological Disorders and Solid Malignancies

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Additional information is available at the end of the chapter

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Abstract

An efficient and specific cytotoxic immune response against a tumor requires a complex, rapidly evolving interaction between various immune cell types in the adaptive and innate immune system. This pliable interplay is a relentless process that has been concisely organized in three different phases: elimination, equilibrium, and escape. The identification of key immune players and molecules involved in this interplay has been crucial for the introduction of reliable prognostic factors and effective therapeutic protocols against cancers. In this chapter, we aim to depict the roles of these distinct immune cell subsets, summarize the prognostic value of immune cells in different cancer types, and discuss briefly the principles of different immunotherapeutic approaches against hematologic malignancies and solid tumors.

Keywords: lymphocytes, prognostic, therapeutic, implications, hematological, solid, malignancy

1. Introduction

Lymphocytes are a diverse population of cells that participate in both innate and adaptive immunity. There are three broad classes of lymphocyte—B cells, T cells and NK cells—and these have different developmental pathways, life span, preferred areas of settlement within the lymphoid organs, surface structure, molecular markers, and function. For many years, the cornerstones of cancer treatment have been surgery, chemotherapy, and radiation, and

more recently targeted therapies. Although these approaches have contributed to improved outcomes, most malignancies still carry a poor prognosis. Recently, cancer immunotherapy is the most exciting advancement in cancer therapy. In the following chapter, four main aspects will be addressed;

i. Immune cell types involved in tumor recognition and rejection.

ii. Role of different immune cells in hematological malignancies; prognostic and therapeutic implications.

iii. Prognostic value of infiltrating immune cells in solid tumors.

iv. Immunotherapeutic modalities for solid tumors.

2. Immune cell types involved in tumor recognition and rejection

An efficient and specific cytotoxic immune response against a tumor requires a complex, rapidly evolving interaction between various immune cell types in the adaptive and innate immune system. These cells include [1]: (i) CD8+ lymphocytes and Th1/Th2 subclasses of CD4+ T lymphocytes, traditionally referred to as cytotoxic T cells and helper T cells. They initiate the distinction between self and non-self-antigens, through recognition at the "immune synapse", (ii) natural killer (NK) cells, characteristically, do not require antigen presentation by the major histocompatibility complex (MHC) for cytotoxic activity. Like T cells, NK cells express numerous inhibitory molecules, e.g. various killer immunoglobulin-like receptor (KIR) subtypes [2], (iii) additional cell types, such as FoxP3+ CD25+ CD4+ T regulatory (Treg) and myeloid derived suppressor cells (MDSCs) largely inhibit cytotoxic T lymphocyte activity [3] and (iv) macrophages differentiate into at least 2 different phenotypes: M1 macrophages, which release interferon (IFN) gamma and are responsible for phagocytosis, and M2 macrophages, which release cytokines such as IL-4, IL-10, transforming growth factor beta (TGF-beta), and dampen inflammatory responses and foster tolerance [4]. The "*immune synapse*" is the ability of T lymphocytes to distinguish self- versus non-self-antigens, which are presented by antigen-presenting cells (APCs) such as dendritic cells. Overall, the cytotoxic activity of a CD8+ T cell is regulated by the presence and spatial orientation of a set of stimulatory and inhibitory receptors whose expression is regulated by a myriad of cytokines. Together, this configuration is often referred to as the "immune synapse" [1]. For efficient activation of a naïve CD8+ T cell, its T cell receptor (TCR) must bind to a peptide presented by the MHC in the presence of a second set of costimulatory signals. This interaction leads to CD3 intracellular signaling that causes secretion of pro-inflammatory cytokines such as IL-12 and IFN gamma. In the absence of a costimulatory signal, a state of peripheral tolerance to the antigen "anergy" develops [5]. The most important costimulatory signal in naïve T cells is CD28, which binds to B7-1 and B7-2 (CD80/86) on the APC. This costimulatory process is tightly regulated by both "agonist" molecules (e.g., GITR, OX40, ICOS) and inhibitory signals on both the APC and T cells, often collectively referred to as "immune checkpoint" molecules. Examples of co-inhibitory or "immune checkpoint" molecules include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death-1 (PD-1), TIM3, and LAG3. Chronic recognition of an

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antigen may lead to feedback inhibition of effector T cell function, resulting in a phenotype termed "exhaustion" [6].

2.1. Tumor evasion of immune surveillance

The theory of 'immunoediting,' developed by Dunn et al. [7], emphasizes the dual role of the immune system in tumor progression, defining a very fine dynamic interplay between immune and malignant cells, which is characterized by three different phases: elimination, equilibrium, and escape (**Figure 1**). (i) *The elimination phase* consists of innate and adaptive

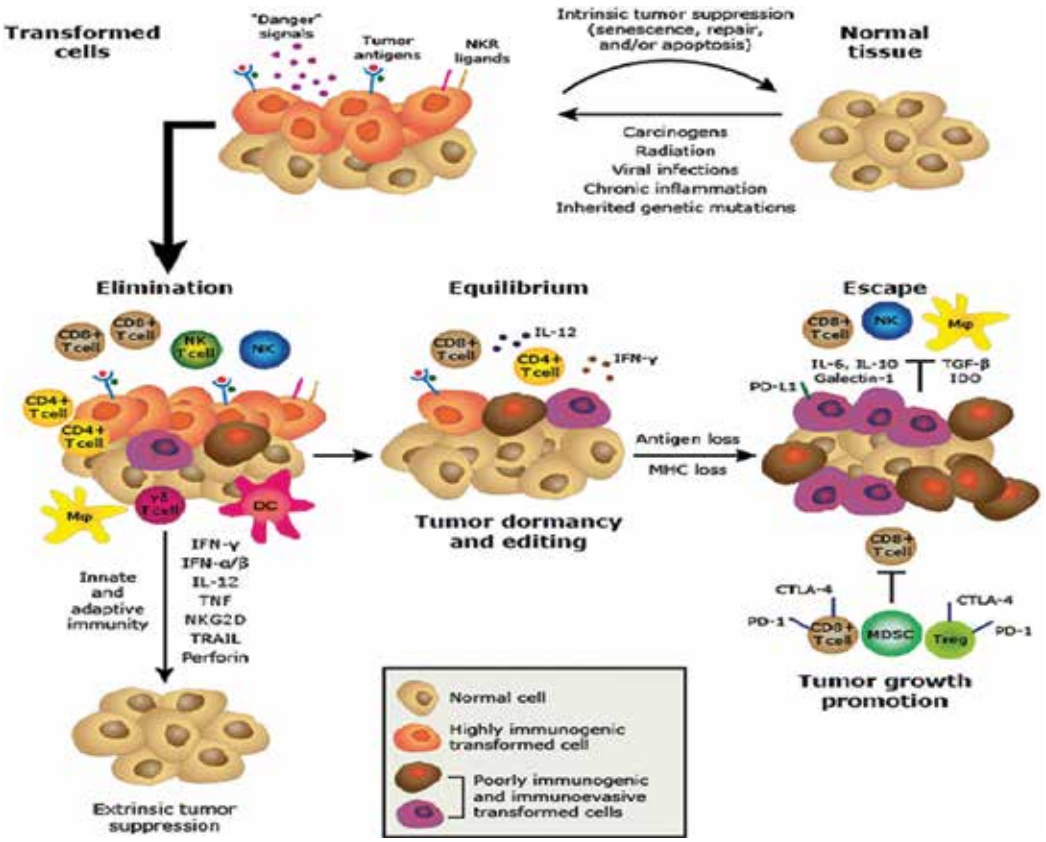

Figure 1. Cancer immunoediting. Cancer immunoediting is an extrinsic tumor suppressor mechanism that engages only after cellular transformation has occurred and intrinsic tumor suppressor mechanisms have failed. Cancer immunoediting consists of 3 sequential phases: elimination, equilibrium, and escape. In the elimination phase, innate and adaptive immunity work together to destroy developing tumors long before they become clinically apparent. If, however, a rare cancer cell variant is not destroyed in the elimination phase, it may then enter the equilibrium phase, in which its outgrowth is prevented by immunologic mechanisms. Equilibrium is a function of adaptive immunity only, where T cells, IL-12, and IFN-γ are required to maintain tumor cells in a state of functional dormancy, whereas NK cells and molecules that participate in the recognition or effector function of cells of innate immunity are not required. Editing of tumor immunogenicity occurs in the equilibrium phase. Equilibrium may also represent an end stage of the cancer immunoediting process, where outgrowth of occult cancers is restrained for the lifetime of the host. However, as a consequence of constant immune selection pressure placed on genetically unstable tumor cells held in equilibrium, tumor cells may then enter the escape phase, in which their outgrowth is no longer blocked by immunity. These tumor cells emerge to cause clinically apparent disease.

immune responses to specific tumor-associated antigens and is characterized by T, B, and NK cell effector function, which is mediated by cytokines such as IFN alpha, IFN gamma, and IL-12. (ii) *The equilibrium phase* is a balance between immune-mediated destruction by the adaptive immune system (e.g. activated CD4+ and CD8+ T cells) and persistence of rare malignant clones. (iii) *Immunologic escape* describes the phase where malignant clones have acquired the ability to evade the adaptive immune system.

3. Role of different immune cells in hematological malignancies; prognostic and therapeutic implications

3.1. Dendritic cells

Dendritic cells (DCs) are bone-marrow-derived immune cells which have a critical role in the initiation and modulation of the adaptive immune response. They support the innate immune response independently from T cells. Besides functioning as the most effective APCs within the immune system, DCs can induce tolerance in the central and peripheral lymphoid organs. Therefore, they act as suppressors rather than stimulators of the immune response. DCs can capture antigens from viable or damaged tumor cells and present the processed peptides to T-cells to prompt the generation and maintenance of an effective tumor-specific T-cell response [8]. It has been clearly established that DCs are pivotal in regulating the delicate balance between immunity and tolerance, so representing the linkage between the innate and adaptive immune responses [9].

3.1.1. Dendritic cells and cancer

DCs are crucial arbiters of the host immune response against tumors since they can regulate effector cells of innate immunity such as NK cells and NK-T cells. DCs also play a fundamental role in orchestrating adaptive immune response as APCs able to cross-present tumor-associated antigens (TAA) to CD4 and CD8 T lymphocytes in regional LNs [10]. The potential cytotoxic activity of killer DCs (KDCs) is dependent on exogenous activation signals such as IFN-α, IFN-γ, CD40L or viruses [11]. KDCs promote tumor cell death by a broad array of mechanisms including TNF-a, Fas Ligand, and TNF-related apoptosis-inducing ligand (TRAIL) dependent versus independent pathways [12]. Therefore, it is not surprising that infiltration from DCs within the primary tumor masses has been correlated with a significantly prolonged survival of patients and a reduced incidence of metastatic disease with favorable prognostic features in different types of malignant tumors [13]. On the other hand, tumor cells can deregulate DCs function by different mechanisms; including antigen down-regulation and secretion of cytokines and other factors (IL6, IL10, Vascular Endothelial Growth Factors [VEGF], Transforming Growth Factors beta [TGF-β]). These mechanisms can together adjust the immunostimulating or immunosuppressive functional plasticity of DCs [14]. Cancer microenvironment can prompt the acquisition of tolerogenic and the immunosuppressive functions of DCs turning them into a regulatory state (reg DCs (which hamper the adaptive immune response against some tumor antigens) [15]. The immuno-suppressive activity of reg DCs can be exerted through a variety of enzymatic mechanisms. Reg DCs can secrete immunosuppressive cytokines such as TGF-β and IL-10 which impair the effector immune response

[15]. Moreover, reg DCs express inhibitory molecules such as programmed cell death 1 ligand 1(PDL-1) and PDL-2, which represent a further pathway for the inhibition of an effective antitumor immunity. Overall, these data underline how malignancies can modulate the pathobiology of DCs favoring their maturation into pathways associated with an immunosuppressive function [16]. Immune dysregulation is a fundamental aspect in the pathogenesis of bone marrow (BM) failure in patients with myelodysplastic syndromes (MDS), a group of heterogeneous clonal hematopoietic disorder characterized by ineffective hematopoiesis, resulting in various degrees of peripheral blood cytopenias [16]. A remarkable genetic alteration of circulating DCs was also demonstrated in acute myeloid leukemia (AML) patients [17]. The recovery of DCs in the BM was investigated in AML patients that were mostly treated with chemotherapy. DCs were absent or at very low levels at diagnosis as compared with the average DC values obtained at complete remission (CR) after chemotherapy [18]. The occurrence and distribution of peripheral blood-derived DCs in mature lymphomas showed that in non-Hodgkin's lymphoma (NHL), DCs were reduced in frequencies and altered in functions as compared to normal and reactive LNs [19]. In chronic lymphocytic leukemia (CLL), a significant reduction in DCs proportions was demonstrated in comparison with healthy subjects, suggesting that DC development is significantly affected by the leukemic process and may contribute to immune dysregulation [20]. Finally, in the setting of an autologous hematopoietic stem cell transplantation (HSCT) for relapsed or refractory diffuse large B-cell non-Hodgkin lymphoma (DLBC NHL), improved overall survival (OS) was correlated with the higher blood pre-transplant and post-transplant levels of DC frequencies [21].

3.2. Natural killer cells

The biology of natural killer (NK) cells is complex. They have powerful cytotoxic activity, however, their activity may be eluded by the tumor microenvironment. Evading NK-cell responses to tumors may occur through immunosubversion, immunoediting or immunoselection of poorly immunogenic tumor cells and interference with tumor infiltration. Tumor cells, together with tumor-associated fibroblasts and tumor-induced aberrant immune cells (e.g. tolerogenic or suppressive macrophages, DCs and T cells) can interfere with NK-cell activation pathways or the receptor array that regulate NK-cell activation and antitumor activity. Therefore, the definition of tumor microenvironment-related immunosuppressive factors, and the identification of new classes of tissue-residing NK-like innate lymphoid cells, may represent attractive insights toward effective NK-cell-based therapies [22] (**Figure 2**).

NK cells are the first subset of lymphocytes to reconstitute after HSCT and likely play an important role in offering protection against relapse in the early months after transplant. In contrast to T cells, NK cells do not cause graft-versus-host disease (GVHD) in the allogeneic setting; indeed, a number of preclinical studies suggest that they may even protect against GVHD by targeting the recipient's dendritic cells [23]. Importantly, the role of NK cells in immune monitoring of tumors is mainly due to their non-human leukocyte antigen (HLA) restricted effect, as the absence or abnormal expression of HLA molecules induces NK-cell cytotoxicity; the so-called "missing self" hypothesis [24]. NK cells play a major role in innate defenses and are also thought to be part of the immunosurveillance against tumors. They express an array of surface receptors that mediate NK cell function. However, NK cell activation and induction of cytolytic activity and cytokine production depends on another important checkpoint, namely the expression on target cells of ligands recognized by activating NK receptors [25] (**Figure 2**).

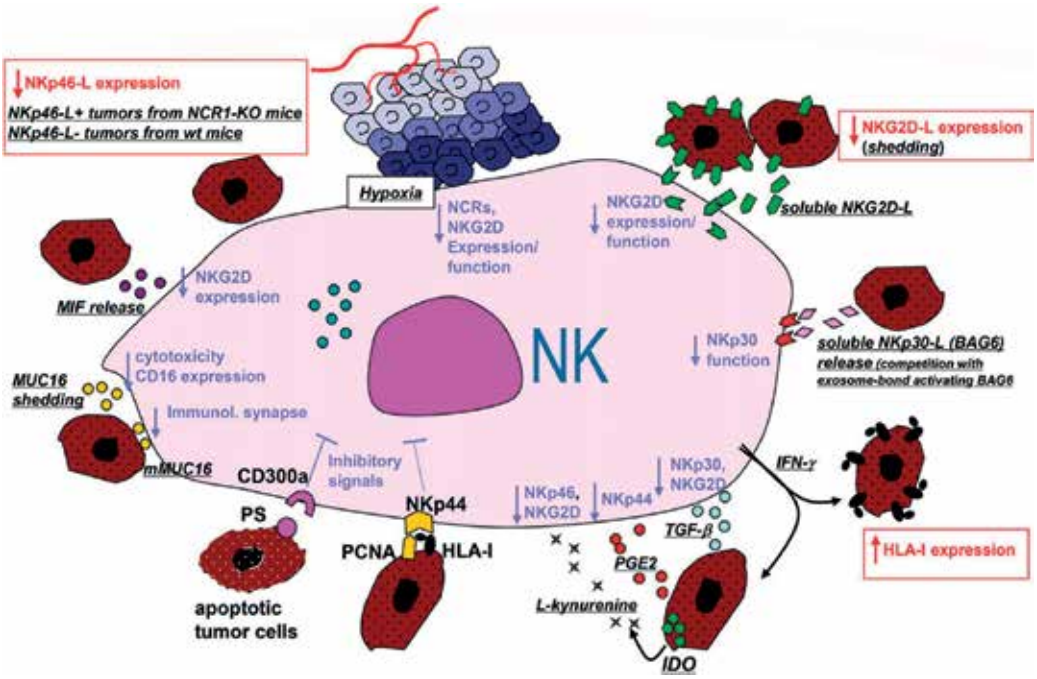

Figure 2. Mechanisms of tumor evasion from the NK-cell attack. The tumor microenvironment can suppress NK cells in different ways (indicated in blue font). Tumor cells (in brown) can induce downregulation of different activating NK-cell receptors and of NK-cell functions by different mechanisms including: the release/expression of macrophage migration inhibitory factor (MEF), MUC16, TGT-β, PGE2, IDO; the chronic engagement of NKG2D by soluble or cell surface-expressed ligands (NKG2D-L); the release of soluble NKp30- L competing for NKp30 with the activating exosome-bound NKp30-L. In addition, apoptotic tumor cells can expose phosphatidylserine (PS) at the outer cell surface. PS can induce inhibitory signals through the engagement of CD300a inhibitory receptor. Unconventional inhibitory signals can also be induced through the engagement of NKp44 by proliferating cell nuclear antigen (PCNA) associated with HLA-I molecules. The pressure of NK cells may favor the editing/selection of tumor cells expressing NK-resistant phenotypes (indicated in red font). The shedding of NKG2D-L, besides inducing NKG2D downmodulation on NK cells, also results in a reduced NKG2D-L expression on target cells and consequent reduced susceptibility to NK-cell-mediated cytotoxicity. Finally, NK cells, through IFN-γ production, can induce HLA-I expression increase on tumor cells.

Hematological malignancies are cancers that affect blood, BM, and LNs. Notably, a major difference from solid tumors, is that hematological malignancies arise from the immune system itself. The majority of adult lymphoid malignancies originate from mature B cells, while a minor proportion arises from mature T cells. Therefore, the role of immune pathways in these tumors is complex [26]. Constant immune surveillance is expected, since cells of the immune system and those of malignancy are in constant contact with each other within the hematopoietic system. Moreover, since the cellular origins of malignancy are those of the immune system, these malignant cells are considered immunostimulatory by their nature [26].

3.2.1. How do hematological malignancies escape from NK cell innate immune surveillance?

Interestingly, tumor-cells develop various escape mechanisms to NK cell surveillance and contribute to the dysfunction of NK-cell cytotoxicity [23]. Defects in NK-cell cytotoxicity have been observed in all hematological malignancies. There are general mechanisms for escape of hematological malignancies from NK cell immunity, which are common to all

immune-effector cells, and they include saturation of the immune system by rapid growth of the tumor and inaccessibility of the tumor because of deficient vascularization [26]. In addition to these general mechanisms, hematological malignancies may adopt NK cells effectors' quantitative deficiency [27]. However, in most hematological malignancies, qualitative impairment of the capacity of NK cytotoxic seems to be more important for tumor escape than quantitative defects. These qualitative defects could be achieved through increased inhibition of NK cell cytotoxicity [28], impaired activation of NK cells [29], impaired differentiation signaling of NK cells [26], and through certain cytokines [23].

3.2.2. Therapeutic approaches utilizing NK cells in hematological malignancies

The cure of high-risk leukemias in the haploidentical HSCT setting is considered the most important clinical application utilizing NK cells. In this scenario, NK cells originated from hematopoietic stem cells of HLA-haploidentical donors may express Killer Immunoglobulin-like receptors (KIRs) that are mismatched with the HLA class I alleles of the recipient. Thus, NK cells are allowed to kill leukemia blasts residual after the conditioning regimen, while sparing normal cells. Another promising approach is based on the use of anti-KIR blocking monoclonal antibodies, rendering alloreactive any KIR+NK cells [28].

3.2.3. Autologous NK cell immunotherapy

Many clinical trials initially explored the possibility of expanding and enhancing the anti-tumor activity of the native lymphocytes of patients in vivo simply by giving the patients high-dose interleukin-2 (IL-2). The use of high-dose IL-2 led to enormous expansion of NK cells in vivo and enhanced in vitro lytic activity against NK-resistant cell lines. Many researches had utilized ex vivo activated/expanded autologous NK cells along with intravenous or subcutaneous low-dose IL-2 [30]. Despite it was better tolerated, responses remained suboptimal, likely due to IL-2-induced expansion of regulatory T cells (T regs), which inhibit NK cell proliferation and function and/or due to the inhibition of autologous NK cells by the self-HLA molecules on the tumor cells. Due to these limitations, the use of allogeneic NK cells was the next logical step for scientists to explore [30].

3.2.4. Allogeneic NK cell immunotherapy in the setting of HSCT

Allogeneic HSCT creates a unique condition for NK cell alloreactivity by virtue of the "missing-self" phenomenon. As the KIR genes (on chromosome 19q13.4) and the HLA genes (on chromosome 6p21) segregate independently, a donor–recipient pair can be HLA-matched and KIR-mismatched simultaneously [31]. In fact, only 25% of the HLA-matched sibling donor/recipient pairs are KIR identical, while the probability of an HLA-matched unrelated donor (MUD)/recipient pair to be KIR identical is virtually zero [32].

3.3. Other immunotherapeutic approaches in hematologic malignancies

3.3.1. Biology of immune inhibitory molecules and the story of check point inhibitors

For proper T cell activation, two separate signals are required. The first signal is mediated by antigen-dependent T cell receptor (TCR) binding to the MHC molecule of an APC. However,

the second signal is antigen-independent, co-stimulatory, or co-inhibitory signal delivered by the APCs, which modulates TCR signaling and determines the fate of T cells. There are several costimulatory or coinhibitory molecules on T cells with their respective ligands that are collectively known as B7-CD28 family. The prototypical co-stimulatory molecule is CD28 on resting naïve T cells, which induces interleukin-2 (IL-2) production, cell-cycle progression, and clonal expansion is constitutively expressed in resting naïve T cells. Without these co-stimulatory second signals, T cells fall into a state of anergy. Inversely, cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a co-inhibitory receptor on T cells that induces T cell tolerance [33]. CTLA-4 exerts its effect when it is present on the cell surface of CD4+ and CD8+ T lymphocytes, where it has higher affinity for the costimulatory receptors CD80 and CD86 (B7-1 and B7-2) on antigen-presenting cells (APCs) than the T cell costimulatory receptor CD28. The expression of CTLA-4 is upregulated by the degree of T cell receptor (TCR) activation and cytokines such as IL-12 and IFN gamma, forming a feedback inhibition loop on activated T effector cells. As a result, CTLA-4 can be broadly considered a physiologic "brake" on the CD4+ and CD8+ T cell activation that is triggered by APCs [33]. Additional second signal molecules include programmed death-1 (PD-1), T cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), lymphocyte activation gene-3 (LAG-3), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), or B- and T-lymphocyte attenuator (BTLA) [34].

3.3.1.1. PD-1 and PD ligand 1/2

The programmed cell death 1 (PD-1) receptor is expressed on activated T cells, B cells, macrophages, Tregs, and NK cells. Binding of PD-1 to its B7 family of ligands, programmed death ligand 1 (PDL1 or B7-H1) or PD-L2 (B7-DC) results in suppression of proliferation and immune response of T cells. Activation of PD-1/PD-L1 signaling serves as a principal mechanism by which tumors evade antigen-specific T-cell immunologic responses. Antibody blockade of PD-1 or PD-L1 reverses this process and enhances antitumor immune activity [35] (**Figure 3**). Many cancers exploit inhibitory molecules such as PD-1, CTLA-4, LAG-3, or TIM-3 to escape immune surveillance. Supporting evidence confirms that functions of these molecules are dysregulated in lymphoid neoplasms, myelodysplastic syndrome, plasma cell myeloma, and AML. Clinical trials had observed that PD-1 blockade is an attractive way to reinstate the host's immune function in lymphoid neoplasms, particularly in classical Hodgkin lymphoma. Serum level of soluble PD-L1 measured by enzyme linked immunosorbent assay (ELISA) can be a potential predictive biomarker in patients with refractory relapsed lymphomas or leukemias [36, 37]. Based upon prolonged overall survival in phase III trials and durable responses in phase II studies, antibodies inhibiting PD-1 (e.g. pembrolizumab, nivolumab) and PD-L1 (e.g. atezolizumab, avelumab, durvalumab) have been approved [40]. However, as the number of PD-1/PD-L1 inhibitors undergoing development is expected to rise in the foreseeable future, several important points (e.g. predictive biomarkers, mechanisms of resistance and hyperprogressors, immune-related adverse events, optimal treatment duration, treatment beyond progression, and response after prior PD-1/PD-L1 blockade), need to be taken into consideration in order to optimize the anticancer potential of this class of agents [38, 39].

3.3.2. Manipulating T cells

Adoptive T cell transfer broadly refers to the practice of manipulating patient-specific T cells ex vivo to make them more reactive to specific antigens.

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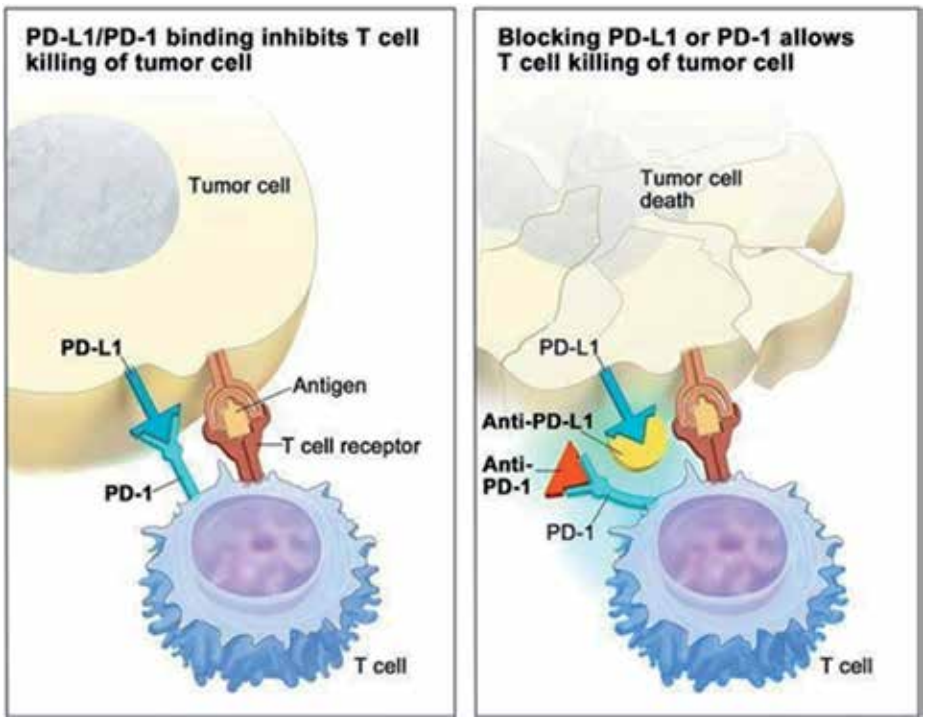

Figure 3. Programmed-death 1 receptor (PD-1, CD279). Programmed-death 1 receptor (PD-1) is one of the crucial molecules that turns down the activation of the immune response. The introduction of PD-1/PD-L1 inhibitors has a great revolution in patients with haematological malignancies.

3.3.2.1. Chimeric antigen receptors

One promising approach to cancer immunotherapy entails genetically engineering a patient's T cells to express chimeric antigen receptors (CARs) that can recognize and attack tumor cells. Upon expression by a T cell, CARs confer antigen specificity determined by the targeting domain [40]. Interestingly, in contrast to conventional T cell receptors (TCRs), which recognize antigens in a MHC-dependent manner, CARs can redirect the effector functions of a T cell toward any protein or non-protein target that is expressed on the cell surface. Therefore, this strategy avoids the need for antigen processing and presentation by the target cell and can be applicable to non-classical T cell targets like carbohydrates [41]. CAR T cells have been studied extensively in hematologic malignancies, and the final goal is to induce durable immunity against disease progression without severe adverse effects. Clinical trials targeting CD19, the pan-B cell antigen, have shown remarkable success in B cell acute lymphoblastic leukemia (B-ALL) and pre-B-cell ALL [42]. Trials in patients with CLL have also shown promising results [43]. A suicide system has been developed to eliminate gene-modified T cells when they display unwanted toxicities, such as the thymidine kinase gene of the herpes simplex virus. In order for that therapy to become routinely used, automation and robotic culture technologies should be performed during the manufacturing process instead of manual cell culture technologies. Whether this treatment option will replace HSCT or be used as a bridge to HSCT in the near future is still a debateful question [44].

3.3.3. Monoclonal TCRs

Another approach to increase effector T cell function against a particular antigen is engineering a soluble TCR (CD8) to recognize a particular antigen target and fusing this to the variable fragment that recognizes an effector target, such as CD3. The ability to engineer a TCR rather than an antibody fragment can lead to higher affinity for a given peptide chain and allow for targeting of intracellular peptide fragments. This approach must be engineered using a specific MHC class 1 molecule, but complications have occurred through TCR cross-recognition of other antigens [45].

4. Prognostic value of infiltrating immune cells in solid tumors

It was observed that the 'pro-inflammatory' tumor microenvironment and infiltrating CD8-expressing T lymphocytes were associated with improved clinical outcomes in a broad range of tumor types. On the other hand, the inhibitory function of other immune cells (e.g. regulatory T cells (Tregs), and myeloid-derived suppressor cells) play a major role in disrupting the capacity for the immune control of cancers, thus was associated with worse outcomes [46]. Herein, tumor-specific prognostic values of infiltrating immune cells in some solid cancers are mentioned:

4.1. Colorectal cancer (CRC)

Several large studies of CRC have shown that tumor lymphocytic reaction and T-cell subpopulations are significant prognostic biomarkers, even after adjusting for stage, lymph node count, and well-established prognostic biomarkers [47]. In a meta-analysis of nine trials, the pooled hazard ratio (HR) confirmed an OS benefit for patients with prominent TILs compared with those without, with a HR of 0.59 ($P < 0.001$) and a HR for cancer-specific survival of 0.40 ($P < 0.001$), respectively [48]. However, despite these convincing data, the host immune response is still not yet considered to represent a "standard" prognostic indicator for clinical use in CRC. This may be attributed to the need for a "standard immunoscore" to quantify the in situ immune infiltrate as a novel instrument for classification of CRC [49, 50].

4.2. Breast cancer (BC)

The presence of TILs was associated with improved prognosis in human epidermal growth factor receptor 2 (HER2) positive and triple negative breast cancers (TNBC), but not in luminal subtypes [46]. For luminal B/HER 2 negative BC treated with neoadjuvant chemotherapy (NC), high intratumoral $CD8^+$ TIL expression was significantly predictive of pCR post-NC, and an independent prognostic factor for improved OS [51]. In contrast to CRC, a meta-analysis of 25 published studies comprising over 22,000 patients with BC, failed to show that immune infiltrates are associated with OS, but did find such an association in TNBC (HR: 0.79). CD8-expressing lymphocytes were associated with improved disease-free survival (DFS; HR: 0.69) and breast cancer-specific survival (HR: 0.78) in the overall population, whereas the FOXP3-expressing lymphocytes were associated with worse DFS (HR: 1.47) and OS (HR: 1.50, $P = 0.004$), respectively [52].

4.3. Melanoma

Checkpoint inhibitors were first approved in melanoma after a long history of interest in the immune response to these tumors after observation of spontaneous responses [53]. Overall, there is a large body of evidence documenting the prognostic value of the immune infiltrate in melanoma [54].

4.4. Nonsmall cell lung cancer (NSCLC)

In a meta-analysis of 29 trials with over 86,000 patients, high levels of CD8-expressing cells infiltrating the tumor or in the tumor stroma of NSCLC specimens were associated with better OS, compared with tumors without lymphocytes present. CD3 expression also demonstrated similar findings. Presence of intratumoral CD4-expressing cells between the tumor cells resulted in improved OS. FOXP3-expressing T cells in the tumor stroma had association with worse progression-free and OS [55].

4.5. Renal cell carcinoma (RCC)

There is contradictory evidence regarding the role of the immune cell infiltrate in RCC. Several studies have demonstrated a worse outcome in patients with a neutrophilic, and/or lymphocytic infiltrate [56].

4.6. Hepatocellular carcinoma (HCC)

The importance of FOXP3 in both the development and prognosis of HCC, was demonstrated in 2 large meta-analyses [57, 58]. Gabrielson et al. [59] applied the Galon Immunoscore [49] to HCC and confirmed its prognostic value, where CD3 and CD8 cell densities predicted recurrence with ORs of 5.8 and 3.9, respectively. On the other hand, PDL1 staining positively correlated with high CD3 and CD8 density and predicted a lower rate of recurrence [59].

5. Immunotherapy for solid tumors

A number of therapeutic approaches are being studied to liberate the immune system and control malignancy. In the following section, we will discuss briefly the principles of these approaches and their applications in clinical oncology. These approaches include T cells (checkpoint inhibitors, agonism of costimulatory receptors), cytokines, manipulation of T cells, oncolytic viruses, therapies directed at other cell types, and vaccines.

5.1. T cells

5.1.1. Checkpoint inhibitors

5.1.1.1. PD-1 and PD ligand 1/2

As mentioned above, the programmed cell death 1 (PD-1) receptor is expressed on activated T cells, B cells, macrophages, regulatory T cells (Tregs), and natural killer (NK) cells.

Antibody blockade of PD-1 or PD-L1 reverses this process and enhances antitumor immune activity [35]. Currently, the FDA has approved PD-1/PD-L1 inhibitors for the treatment of nine cancer types [38].

5.1.1.2. CTLA-4

As mentioned above, CTLA-4 is a co-inhibitory receptor on T cells that induces T cell tolerance [33]. CTLA-4 exerts its effect when it is present on the cell surface of CD4+ and CD8+ T lymphocytes, where it has higher affinity for the costimulatory receptors CD80 and CD86 (B7-1 and B7-2) on APCs than the T cell costimulatory receptor CD28. The anti-CTLA-4 antibody ipilimumab was the first immune checkpoint inhibitor to be approved based upon its ability to prolong survival in patients with metastatic melanoma [60].

5.1.2. Agonism of costimulatory receptors

Multiple costimulatory receptors are involved in the immune response to tumors, and hence are potential targets for cancer immunotherapy. Examples include; 4-1BB (CD137) [61] and inducible T cell co-stimulator (ICOS) [62].

5.1.3. Combination of immune checkpoint blockade

Based on the results of checkpoint inhibitors as monotherapy, multiple clinical trials are currently investigating combinations of various checkpoint inhibitors. Examples include; concurrent CTLA-4 and PD-1 blockade, ipilimumab plus nivolumab, and nivolumab plus ipilimumab [63].

5.2. Cytokines

Initial approaches to immunotherapy had utilized the numerous downstream effects of cytokines and other substances that influence immune cell activity. Examples include;

5.2.1. Interleukin (IL)-2

Interleukin (IL)-2 was initially discovered as T cell growth factor. IL-2 has pleiotropic effects on both cytotoxic T cell function as well as Treg cell maintenance. The effects partially depend upon the dose and timing of IL-2 administration [64]. High-dose IL-2 achieved durable objective responses in a minority of patients with melanoma and RCC.

5.2.2. Interferon (IFN) alfa-2b

Interferon (IFN) alfa-2b promotes Th1-mediated effector cell responses such as IL-12 secretion via STAT-1 and STAT-2-mediated downstream signaling events. IFN alfa has been used as adjuvant treatment of high-risk melanoma, although its long-term impact on OS is controversial [65].

5.2.3. Bacillus Calmette-Guerin (BCG)

Bacillus Calmette-Guerin (BCG), derived from attenuated mycobacterium bovis, induces a robust inflammatory response when injected in the bladder and is used for the treatment and secondary prevention of superficial bladder cancer [66].

5.3. Manipulating T cells

Adoptive T cell transfer broadly refers to the practice of manipulating patient-specific T cells ex vivo to make them more reactive to specific antigens.

5.3.1. Chimeric antigen receptors (CAR)

As mentioned above, CAR T cells have been studied extensively in hematologic malignancies. However, their success in solid tumors has been limited due to many reasons, including: (i) the lack of a unique tumor-associated antigen (TAA) in most cancers; (ii) inefficient trafficking of CAR T cells to tumor sites; (iii) the inability of *ex vivo* expanded CAR T cells to persist and proliferate following adoptive transfer; (iv) heterogeneous expression of the targeted antigen (s) leading to outgrowth of antigen-negative tumor variants; (v) the presence of immunosuppressive molecules and cells; (vi) the lack of survival and growth factors (e.g., IL-2); and (vii) the metabolically hostile tumor microenvironment [67].

5.3.2. Ex vivo expansion of tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) represent an immune cell population that recognizes tumor antigen but may have developed an exhausted phenotype due to the tumor microenvironment. Ex vivo expansion of TILs utilizes freshly resected tumor tissue to extract TILs and co-culture with IL-2 to stimulate in vitro TIL expansion. Prior to reinfusion of expanded TILs, the patient receives non-myeloablative chemotherapy regimens such as cyclophosphamide or total body irradiation, which functions to deplete inhibitory Treg cells and other lymphocytes in the patient to improve the rate of in vivo expansion of the stimulated TILs. The in-vitro-stimulated TILs, largely comprised of CD8+ and to a lesser extent CD4+ T lymphocytes, are then reintroduced into patients at high doses, together with HD IL-2, where they can recognize specific tumor antigens in a microenvironment that is now less prone to induce tolerance [68].

5.3.3. CD3-directed therapies

5.3.3.1. Bispecific T cell engagers

Theoretically, bispecific T cell engager antibodies (BiTEs) function as linkers between T cells and specific target antigens in an MHC-subtype independent manner. BiTEs consist of tandem single-chain variable fragments (scFv), targeting CD3 on T cells and a tumor associated antigen, respectively. Thus, T cells are recruited to tumor cells irrespective of T-cell receptor specificity, antigen presentation, and costimulation. BiTE-mediated T-cell–tumor cell interaction triggers the formation of immunological synapses, which ultimately results

in tumor-specific cell lysis and release of TH1 effector cytokines [69]. For treatment of non-hematologic malignancies, BiTEs targeting CEA (NCT02291614), EpCAM (NCT00635596), and PSMA (NCT01723475) are in clinical development. However, engaging these antigens may cause on-target off-tumor toxicities and successful BiTE therapy of solid tumors has not been reported so far [70].

5.4. Oncolytic viruses

Oncolytic viruses mediate antitumor effects in several ways. Viruses can be engineered to efficiently infect cancer cells preferentially over normal cells, to promote presentation of tumor-associated antigens, to activate "danger signals" that promote a less immune-tolerant tumor microenvironment, and to serve as transduction vehicles for expression of immune modulatory cytokines [71]. The agent furthest along in clinical development is talimogene laherparepvec (T-VEC), which utilizes an attenuated herpes simplex virus 1 virus to overexpress granulocyte macrophage colony-stimulating factor (GM-CSF), which promotes DC-mediated antigen presentation [72]. Numerous other virus backbones are under clinical or preclinical investigation, including adenovirus, reovirus, Newcastle disease virus, and others [73].

5.5. Oncolytic virus plus checkpoint inhibition

Injection of oncolytic viruses may synergize with checkpoint inhibitors by increasing CD8+ T cell infiltration and IFN gamma signaling as well as upregulating PD-L1 in the microenvironment [74].

5.6. Therapies directed at other cell types in the tumor microenvironment

Cell types other than tumor-specific and circulating T cells contribute to an effective versus a suppressed immune response, and thus represent additional targets for immunotherapy beyond T cells.

5.6.1. NK cells

As discussed previously, in the section of hematologic malignancies.

5.6.2. Macrophages

The presence of intratumoral macrophages can portend a poor prognosis. Although an oversimplification, the general categorization of macrophages into classically activated phenotype (M1) and alternatively activated (M2) suggests that in the context of malignancy, M2 macrophages play a pro-tumoral role due to their involvement in immunosuppression, angiogenesis, and tumor cell activation [75]. Intratumoral macrophages are largely recruited by C-C chemokine ligand 2 (CCL2) or colony-stimulating factor 1 (CSF-1), and pre-clinical and clinical data have focused on targeting the CSF-1/CSF-1 receptor axis. The antitumor impact of CSF-1 receptor (CSF-1R) inhibition in pre-clinical models varies, but there are promising data in combination with other modalities such as chemotherapy, radiation therapy, angiogenic inhibitors, adoptive cell transfer, as well as when used in conjunction with CTLA-4 and PD-1 blockade in the challenging setting of pancreatic adenocarcinoma [76].

5.6.3. IDO

Indoleamine 2,3-dioxygenase 1 (IDO1) catalyzes the rate-limiting step in the conversion from the essential amino acid L-tryptophan (Trp) into L-kynurenine (Kyn). IDO1 expression by tumors can promote evasion of immune surveillance by suppressing T cell function and impairing immune surveillance [77].

5.7. Vaccines

There is a long history of attempting to utilize the adaptive immune recognition of a cancer-related antigen to effect antitumor responses. A simplistic way to view vaccine development method is that varying types of antigens, administration schedules, and accompanying immune adjuvants can influence an adaptive immune response. Antigen choices range from simple peptides, which are easy to administer but affect a narrow antigen spectrum and are often restricted by specific HLA class 1 molecule expression that allows efficient antigen presentation, to whole cell preparations that offer a broader range of antigens but are more costly and time-consuming to prepare [78]. The only currently approved vaccine-based therapy for advanced cancer is sipuleucel-T, which is an autologous dendritic-cell preparation engineered to target prostatic acid phosphatase (PAP) that demonstrated an overall survival benefit in men with castrate-resistant prostate adenocarcinoma [79]. Given the increasing understanding of the importance of immune recognition of multiple patient-specific, tumor-specific antigens, efforts are ongoing to explore the use of individualized pooled antigens. This suggests that patient-specific vaccination approaches may be feasible, particularly in immunogenic tumors such as melanoma, NSCLC, mismatch-repair deficient CRC, and bladder carcinoma [80].

6. Conclusions

The immune response against a tumor requires a complex, rapidly evolving interaction between various immune cell types in the adaptive and innate immune system. The identification of key immune players and molecules involved in this interplay has been crucial for the introduction of reliable prognostic factors and effective therapeutic protocols against cancers. Tumor-infiltrating lymphocytes have been identified as important prognostic factors in many solid tumors. A number of therapeutic approaches are being studied to liberate the immune system and control malignancy. Checkpoint inhibition has already become a primary treatment modality for patients with a broad diversity of cancers, resulting in significantly prolonged survival in some patients. Trials exploring other malignancies and a wide variety of immunotherapy combinations are in progress and should improve these results.

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Conflict of interest

The authors had no conflict(s) of interest for this work.

List of abbreviations

APC	antigen presenting cell
AML	acute myeloid leukemia
BM	bone marrow
B-ALL	B cell acute lymphoblastic leukemia
BC	breast cancer
BiTEs	bispecific T cell engager antibodies
BTLA	B- and T-lymphocyte attenuator
CARs	chimeric antigen receptors
CCL2	C-C chemokine ligand 2
CD	cluster of differentiation
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CRC	colorectal cancer
CSF-1	colony-stimulating factor 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DFS	disease-free survival
DLBC	diffuse large B-cell
ELISA	enzyme linked immunosorbent assay
FOXP3	forkhead box P3
GM-CSF	granulocyte macrophage colony-stimulating factor
GVHD	graft-versus-host disease
HER2	human epidermal growth factor receptor 2
HLA	human leukocyte antigen
HR	hazard ratio
HSCT	hematopoietic stem cell transplantation

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HPV	human papilloma virus
ICOS	inducible T cell co-stimulator
IDO1	Indoleamine 2,3-dioxygenase 1
IFN	interferon
IL	interleukin
KDCs	killer DC
KIR	killer immunoglobulin-like receptor
LAG-3	lymphocyte activation gene-3
LN	lymph node
MDS	myelodysplastic syndrome
MDSCs	myeloid derived suppressor cells
MHC	major histocompatibility complex
NC	neoadjuvant chemotherapy
NHL	non-Hodgkin's lymphoma
NK	natural killer
NSCLC	non-small cell lung cancer
OS	overall survival
PAP	prostatic acid phosphatase
PD-1	programmed cell death-1
PDL-1	programmed cell death 1 ligand 1
scFv	single-chain variable fragments
TAA	tumor-associated antigens
TIM-3	T cell immunoglobulin and mucin domain-containing protein-3
TCR	T cell receptor
TGF-B	transforming growth factor beta
TILs	tumor-infiltrating lymphocytes
TNBC	triple negative breast cancer
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cells
VEGF	Vascular Endothelial Growth Factor
VISTA	V-domain Ig suppressor of T cell activation

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Chapter 6

Lymphocytes Studied by Raman Microspectroscopy

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Abstract

The Raman spectroscopy detects the interaction of the incident light with the electrons in the illuminated molecule. The use of Raman spectroscopy to investigate biological molecular structures and the recognition of their particular functional groups have been growing rapidly, and nowadays the use of Raman spectroscopy has expanded toward the cellular level. The activation of lymphocytes occurs when they are exposed to viruses or other foreign antigens. We have observed that Raman spectroscopy can be used to screen the activation of lymphocytes during viral infection. We have indicated the bands that reveal differences between activated and intact cells. The most important marker of the lymphocyte activation process is the prominent 521 cm^{-1} disulfide band which marks the immunoglobulin formation. The blood from the patients with viral infections, e.g., mononucleosis, and from healthy volunteers was obtained by venipuncture during hospitalization in the University Hospital in Kraków.

Keywords: Raman microspectroscopy, viral infection, activation of lymphocytes

1. Introduction

The proper functioning of the immune system results from close cooperation of the mechanisms of nonspecific (innate) immunity and adaptive immunity (acquired) [1]. Both mechanisms operate by direct contact between the cells and through interactions in which cytokines or chemical mediators participate.

The basic task of the immune system is to detect the hazard in the form of foreign antigens, infectious agents, or their own altered cells that have been infected or transformed, for example, by cancer. To distinguish between self and foreign antigens, the host organism engages specific and nonspecific response cells showing the presence of appropriate receptors. Nonspecific response receptors recognize a very wide range of structure characteristics for infectious agents that are not produced in higher organisms. These are pathogen-associated molecular patterns (PAMP) or microbe-associated molecular patterns (MAMP). The specific response receptors behave differently. They recognize highly specific antigen fragments, single epitopes. They are B cell receptors (BCR) and antibodies and also T cell receptors (TCR) of T lymphocytes.

Lymphocytes are totally responsible for specific immunological recognition of pathogens [2]. They initiate a specific immune response. All cells derived from myeloid stem cells but T lymphocytes then develop in the thymus, while B cells develop in the bone marrow. Each B cell is programmed to express on the surface of receptors specific for a particular antigen. Antibodies neutralize foreign factors due to specific binding to antigens [3].

Advances in microspectroscopic techniques have contributed to a new insight of the molecular environment for lymphocyte activation [4]. The explanation of the immunological synapse function, which influences the B cell acquisition of membrane-attached antigens, was explained with the use of three-dimensional confocal microscopy (3D-CM) [5]. Infrared microspectroscopy and Raman microspectroscopy are new methods that give the opportunity to observe human cells, tissues at the molecular level, and give the opportunity to examine physiological and pathological changes [6–17]. Revealing even small spectral differences between distinct regions of a cell, vibrational spectroscopy provides considerable potential as a rapid screening method [8, 18].

Raman spectroscopy, with an important contribution of resonance Raman phenomenon by using certain chromophores, can be applied to resolve certain chemical species and can be considered as a diagnostic method [6, 19].

Raman spectroscopy is able to give some insight into the functioning of the immune system. Some worth mentioning guidelines in B and T cell lymphocytes differentiation is described in the Hobro et al. work [20], although the authors conclude that the misclassification rate is relatively high. Being aware that the immune response may be very complex, it is of interest to establish how a virus infection and single lymphocyte cell activation by Raman spectroscopy are visible.

2. Blood samples

We have studied two types of viral diseases, influenza and infectious mononucleosis.

The healthy volunteers constitute the reference group. The infectious patients were treated in the Department of Infectious Diseases, Jagiellonian University Hospital in Kraków.

The blood was deposited as a film to measure. The cells were allowed to settle for about 10 minutes prior to measurement.

The studies were conducted in accordance with the guidelines for good clinical practice (GCP) according to the ethical principles for medical research involving human subjects (Declaration of Helsinki). The study was approved by the local bioethical committee.

3. Single cell measurements by Raman spectroscopy

Raman spectra were collected using a Renishaw inVia spectrometer, working in confocal mode, connected to a Leica microscope. A 785-nm HP NIR (high-power near IR) diode laser and a 514.5-nm Ar^+ laser were used to excite the samples. The laser beam was focused by a 100-fold magnifying glass, the high-class Leica objective for standard applications, with a large numerical aperture (NA = 0.9). The laser power was kept low, ca. 1–3 mW at the sample, to ensure a minimum invasion of cells.

During the point mapping experiments, the sample was shifted on a motorized stage (Prior Scientific) at fixed intervals within the defined area. For the mapping experiments, the factory-supplied software was used (Renishaw, WiRE v. 2.0 and 3.4). The Raman images are based on Raman intensities.

Principal component analysis (PCA) was applied to determine the variance between the Raman spectra of a single lymphocyte of a patient with identified early stage of infectious mononucleosis and healthy donors. PCA was performed in the whole spectral region with the Unscrambler X software packages (v. 10.3, CAMO Software, Oslo, Norway). The Raman spectra were smoothed using a Savitzky-Golay smoothing algorithm (13 smoothing points), baseline corrected and unit vector normalized.

The first principal component, PC-1, contains the highest percentage of variation, which indicates the direction of the maximum variation in the dataset.

4. Raman spectra of naive lymphocytes

Lymphocytes carry out immune surveillance, that is, they constantly monitor tissues for the presence of foreign antigens [21]. The human body continuously produces new cells. They circulate between blood and lymph, not only by lymphatic organs but also to a lesser extent by other body tissues [22]. It takes around 24 hours to complete the circulation. Circulating lymphocytes are a group composed of small, long-living cells. The continuous movement of these lymphoid cells means that they are always available for immune defense [3].

The circulatory route of naive lymphocytes T and B is different from that of the memory and effector lymphocytes. In the pool of circulating lymphocytes, the majority of cells are immune memory T cells [21]. The B lymphocytes account for only 15% of the total pool of circulating lymphocytes. Their circulatory pathways are still not well understood.

Cellular processes may be monitored using Raman spectroscopy exploiting the chemical specificity of using the high polarizability of certain functional groups that build molecular cell systems [22–24]. No staining is necessary to observe the diversity of areas inside the cell [25]. The

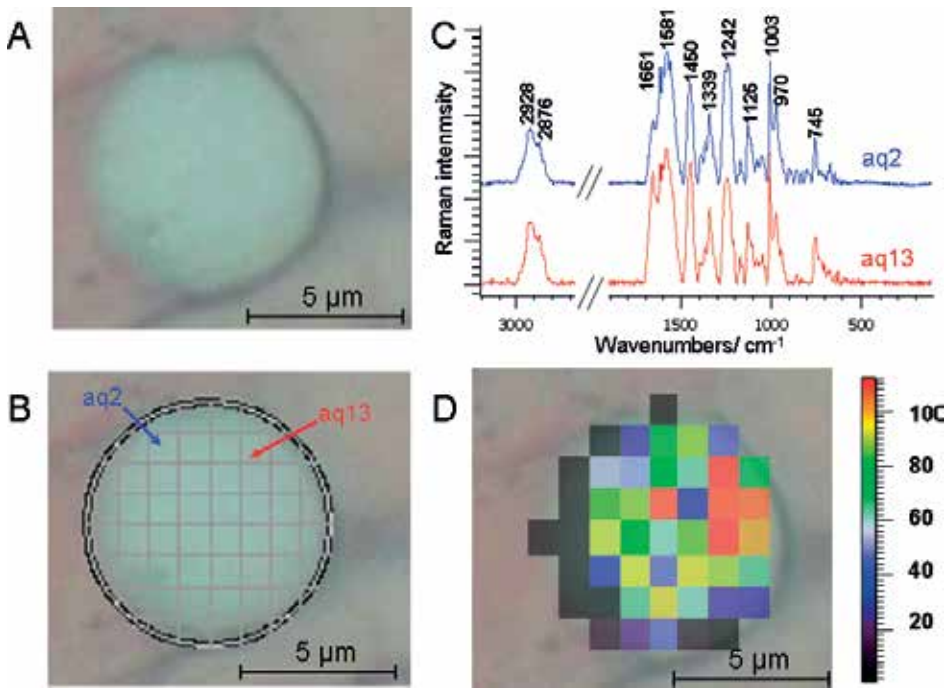

Figure 1. The single, naive human lymphocyte. (A) A photomicrograph (reflected light, objective 100×); (B) with the marked region over which the map experiment was performed; (C) Raman spectra of the sample area including the cell membrane (aq3) and the center of the cell (aq13); and (D) distribution of proteins in the lymphocyte (1660 cm^{-1} marker band), excitation laser line 785 nm.

Raman bands [cm^{-1}]				**Assignment [7–18, 25, 33, 34, 38, 41]**
785 nm	**785 nm**	**785 nm**	**514.5 nm**	
Naïve	**Activated (influenza)**	**Activated (EBV)**	**Activated (EBV)**	
2926	2926	2933	2937 (aq13) 2932 (aq6)	CH sym str of CH_3 methyl group
2875	2875	2889	2878 (aq13) 2874 (aq6)	CH asym. str of CH_2 methylene group
1662 (aq2) ↓ 1662 (aq13) ↑	1657 (aq41) ↑ 1657 (aq3) ↓	1661	1657 (aq13) 1664 (aq6)	Amide I
1617			1617	Tyr
1581	1581	1578	1581	G, A, nucleic acids, Trp, Phe
1450	1450 (aq41) ↑	1450	1452 (aq6)	CH_2 deformation modes, proteins and lipids
1339	1340	1342		A, CH deformations, nucleic acids and proteins
1243	1246	1254	1241	A, Amide III
1123	1125	1132		CN str
—	1100	1100		PO_2^- phosphate backbone vibration (DNA marker)

Raman bands [cm^{-1}]				Assignment [7–18, 25, 33, 34, 38, 41]
785 nm	785 nm	785 nm	514.5 nm	
Naïve	Activated (influenza)	Activated (EBV)	Activated (EBV)	
1031	1031	1035		Phe, C—H in-plane stretching
1003	1003	1005	1005	Phe, ring breathing mode
970	970			CC, skeletal mode
855/835	855 (aq41) ↑	855		Tyr, buried/ exposed
751 (aq2)	740 (aq41)	754 (aq5)↑		T-ring breathing, nucleic acids, Trp
747 (aq13)	752 (aq3)			
620 (aq13)	620 (aq3)	621 (aq5)↑		C—S stretching mode, Phe, His
	617 (aq41)	610 (aq8)		
587 (aq13)				S—S disulfide bridge, Trp
521	521 (aq41) ↑	521	520 (aq13)	V_L domain S—S bridge

A, adenine; G, guanine; T, thymine; Phe, phenylalanine; Tyr, tyrosine, Trp, tryptophane; His, histidine.

Table 1. Assignments of significant Raman bands observed for human lymphocyte (785 and 514.5 nm excitation laser line).

membrane of the cell (e.g., aq2) clearly differs from the center (e.g., aq13) (**Figure 1C** and **D**); this variation is related to the lower presence of protein in the cell membrane, estimated by the intensity of the amide I band. The middle area is relatively homogeneous due to the distribution of proteins, which indicates fully mature cell (**Figure 1D**). The significant Raman bands observed for human lymphocytes and their assignments are collected in **Table 1**.

5. Immunity against viruses

Viruses are usually small compared to other organisms that cause infection; their metabolism is closely dependent on the host's metabolism [26]. That is why viruses are not capable of replicating themselves. Therefore, the key process in viral infection is intracellular replication, which can even lead to the death of infected cells.

Antibodies prevent the penetration of the virus into an uninfected cell and thus limit some viral infections that spread with the blood.

6. Influenza and its etiology

Influenza is an acute illness of the respiratory system caused by influenza viruses that belong to the *Orthomyxoviridae* family. They are divided into three types A, B, and C on the basis of antigenic features of nucleoprotein and matrix protein antigens [27].

The host's response to flu infection is a complex system of dependencies between humoral immunity, local antibody production, cellular immunity, and other mechanisms. The presence

of antibodies confirming the response to the infection is established in biochemical tests, the most important of which is hemagglutination inhibition (HI) test.

7. Infectious mononucleosis and its etiology

EBV virus induces an infectious mononucleosis characterized by heterophilic antibodies, fever, sore throat lymphadenopathy, and atypical lymphocytosis [28]. The virus belongs to the family *Herpesviridae*. The viral genome is a double-stranded DNA with a linear system, surrounded by a nucleocapsid with an icosahedral symmetry; on the outside there is a capsule containing glycoprotein.

More than 90% of adults report the presence of antibodies to EBV infection. An EBV is transmitted with saliva. The infection of B lymphocytes present in the tonsils crypts may take place directly; the next stage is blood virus spreading. There is observed lyphoid tissue growth due to EBV infected B lymphocytes proliferation and the accompanying T-cell reaction.

8. Raman spectrum of activated lymphocytes in response to the influenza virus

Once naive lymphocytes have been exposed, they are activated. This is an important element in the humoral immune response of the body. Free antibodies present in the blood, in the lymph, in all body fluids, and in secretions are involved in this process [1].

They are produced by B cells, initially functioning as APC, which, due to cooperation with Th2 lymphocytes, are transformed into clones of plasma cells producing antigen-specific antibodies [29]. Although antibodies do not destroy infectious agents but they bind epitopes in a specific way, so they are able to initiate effector mechanisms and eliminate the pathogen from the host organism.

That is why LeBien and Tedder commented that the discovery of B cells did not result from cell identification, but rather the identification of a protein, that is, immunoglobulin or antibody [30].

As a response to increased lymphocyte activation, a significant change in their shape and behavior is detected (compare **Figure 1A** and **Figure 2A**). Some of the stimulated B lymphocytes become active in the production of antibodies against foreign antigens, and they are transformed into plasma cells (compare **Figure 1D** and **Figure 2D**). This process allows the immune system to recognize infectious agents and prepare responses to them.

The spectroscopic marker of the lymphocyte activation process is a prominent 521 cm^{-1} disulfide band which marks the formation of the immunoglobulin (**Figure 2C**, aq41).

Immunoglobulin, initially present in the cytoplasm and then bound to the surface, is the main feature of B lymphocytes. They are used to identify specific antigens [26]. Immunoglobulins,

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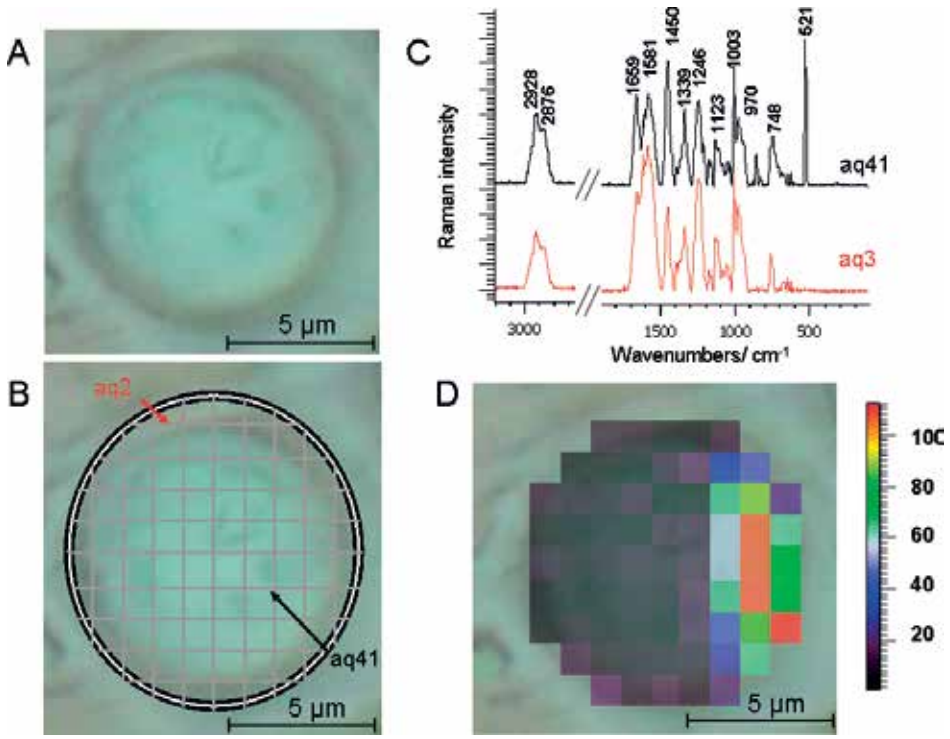

Figure 2. Activated B lymphocyte in response to the influenza virus. (A) A photomicrograph (reflected light, objective 100×); (B) with the marked region over which the map experiment was performed; (C) Raman spectra from an exemplary area including nonactivated (aq2) and from the activated region of the cell (aq41); and (D) distribution of immunoglobulins in the lymphocyte (map created from 521 cm^{-1} marker band), excitation laser line 785 nm.

regardless of function, have a similar molecular structure as well as basically identical mechanism of reacting with antigen [21]. They are all built from the same basic units, light (L) and heavy (H) polypeptide chains [31]. The basic immunoglobulin unit is a tetramer consisting of two identical heavy chains and two light chains. The two heavy chains are linked to each other by disulfide bonds, in so-called hinge region, and each heavy chain is linked to a light chain by a disulfide bond (**Figure 3**). The disulfide bond is a structural feature of many significant biological molecules, because it provides additional stability to a protein molecule. Therefore, the structural characteristics of the CS—SC dihedral angle and the structure of the entire molecule are important [32].

Raman spectroscopy is the best spectroscopic technique to monitor the disulfide bonds and the structure and conformation of a protein [33, 34]. Compounds containing disulfide bonds usually show well-defined bands in the Raman spectra that arise from C—S and S-S stretching modes that are also sensitive to the CS—SC dihedral angle [32]. Additionally, they appear in a region of the Raman spectrum that is relatively free from other intense bands. The experimental Raman data can be correlated with X-ray data and the results obtained from calculations [35–38].

The spatial structure within the N-terminal section of the H and L chains makes it extremely important [21]. It is now known that one antibody molecule may bind to two or more

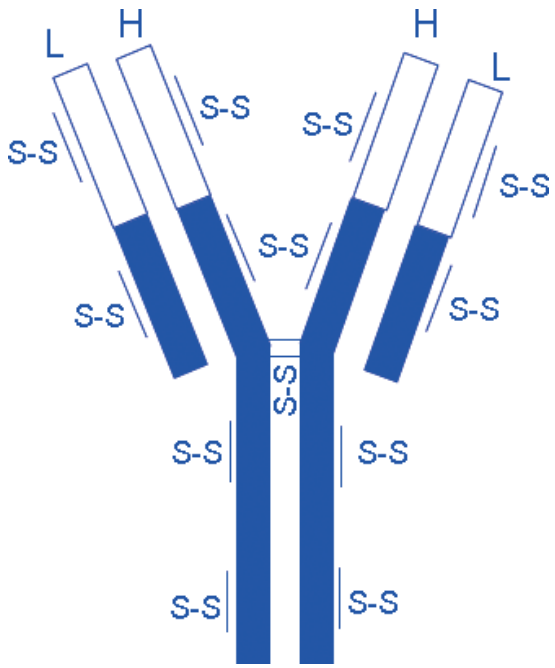

Figure 3. Diagram of the molecular structure of the immunoglobulin molecule.

completely different chemically determinants, which indicates that the anti-determinant does not directly recognize the specific chemical configuration of the antigen, but its overall spatial shape. Therefore, the role of Raman spectroscopy, which makes it possible to determine the structure, seems to be very promising.

Activation of lymphocytes, marked by the appearance of an immunoglobulin, also manifests itself as changes in the cell content. The peak of ca. 1130 cm^{-1} (protein C—N str.) loses its intensity as well as 1100 cm^{-1} phosphate backbone vibration, indicating DNA concentration [9]. These spectral changes signify the lymphocyte evolution toward the plasma cell, which has a small eccentric nucleus, and most of the cell is filled by cytoplasm and well-developed rough endoplasmic reticulum (RER) where immunoglobulin synthesis takes place.

This is in agreement with the presence of intense lipid Raman bands at 1450 cm^{-1} in plasmocytes (because of RER).

Another important Raman band, amide I (C═O stretching and N—H bending vibrations) at 1662 cm^{-1}, is more intensive (**Table 1**). This position indicates that immunoglobulin in the intact form is predominantly composed of antiparallel β-sheet structure [33]. The protein composition seems to be different in activated form as amide I band intensity is slightly shifted toward lower wave numbers (**Table 1**).

A further characteristic band, which occurs as well in naive lymphocytes as in the activated form, is the 850 cm^{-1} peak due to the exposed tyrosine residues in the proteins (**Table 1**) [33]. Tyrosine is an important amino acid in signal transduction and regulating cellular activity (by tyrosine kinase) [39].

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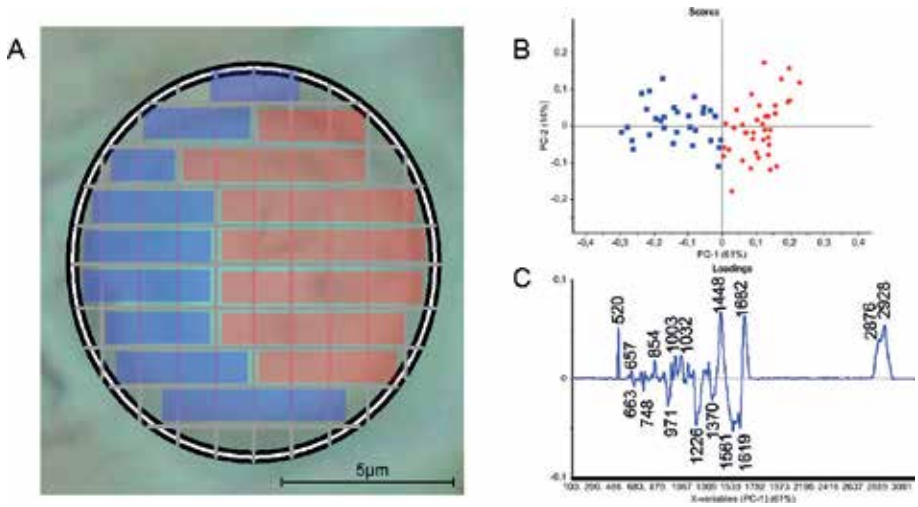

Figure 4. The results of PCA applied to Raman spectra of activated (red dots) and nonactivated (blue dots) B lymphocyte regions in the spectral range 3200–100 cm^{-1} showing (A) a photomicrograph (reflected light, objective 100×) with marked activated (red) and non-activated (blue) regions; (B) scores plot for PC-1 and PC-2 (C) loadings plot for PC-1, excitation laser line 785 nm.

The principal component analysis (PCA) was applied to distinguish two areas of the B cell, classified as activated, and the one in which the presence of an immunoglobulin is not yet manifested (**Figure 4**). The PCA used to reduce the large amount of spectral information contained in the Raman spectra into a few principal component parameters. The activated area is characterized by PC-1 up to 61%.

Lymphocyte activation is defined by Raman bands:

- 520 cm^{-1} (disulfide band)
- 657 cm^{-1} (Tyr)
- 854 cm^{-1} (Tyr, exposed)
- 1003 cm^{-1} and 1032 cm^{-1}(Phe)
- 1448 cm^{-1} (CH_2 def., lipids)
- 1662 cm^{-1} (amide I, β-sheet conf.)
- 2876 cm^{-1} (CH asym str, in CH_2)
- 2928 cm^{-1} (CH sym str, in CH_3)

The following bands are specified for the nonactivated lymphocyte area:

- 663 cm^{-1} (CS str)
- 748 (T, nucleic acids)

- 971 cm^{-1} (CC, structurally sensitive mode)
- 1226 cm^{-1} (CH_2 def.)
- 1370 cm^{-1} (T, A, G, nucleic acids, Trp, Phe, CH_2, and CH_3 def.)
- 1561 cm^{-1} (amide II, His)
- 1619 cm^{-1} (Tyr)

9. Raman spectrum of activated lymphocytes in response to the EBV virus infection

The B cell activated in EBV infection and distribution of immunoglobulin is presented in **Figure 5** (785 nm laser line) and **Figure 6** (514.5 nm laser line). The marker for cell activation is the 521 cm^{-1} immunoglobulin band. The plasma cell shown in **Figure 5** reveals relatively uniformly activated cell. The position of the immunoglobulin marker indicates that this band characterizes the disulfide bridge in the domain of the V_L light chain (**Table 1**). Similar results were received for immunoglobulin in activated lymphocytes in influenza.

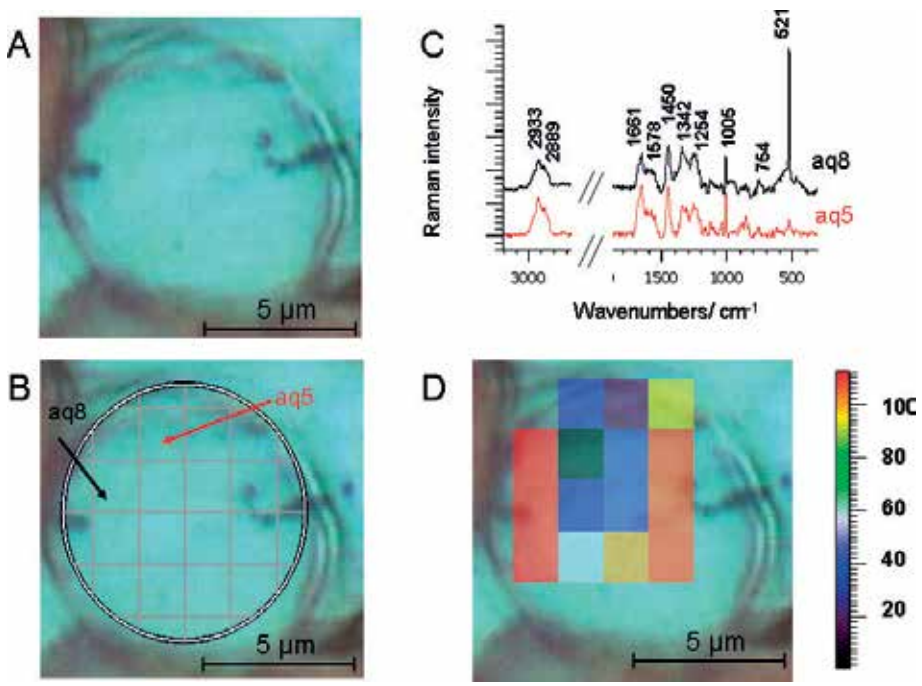

Figure 5. Activated B lymphocyte in response to the EBV virus. (A) A photomicrograph (reflected light, objective 100×); (B) with the marked region over which the map experiment was performed; (C) Raman spectra from an exemplary area including the nonactivated (aq5) and from the activated regions of the cell (aq8); and (D) distribution of immunoglobulins in the lymphocyte (map created from 521 cm^{-1} marker band), excitation laser line 785 nm.

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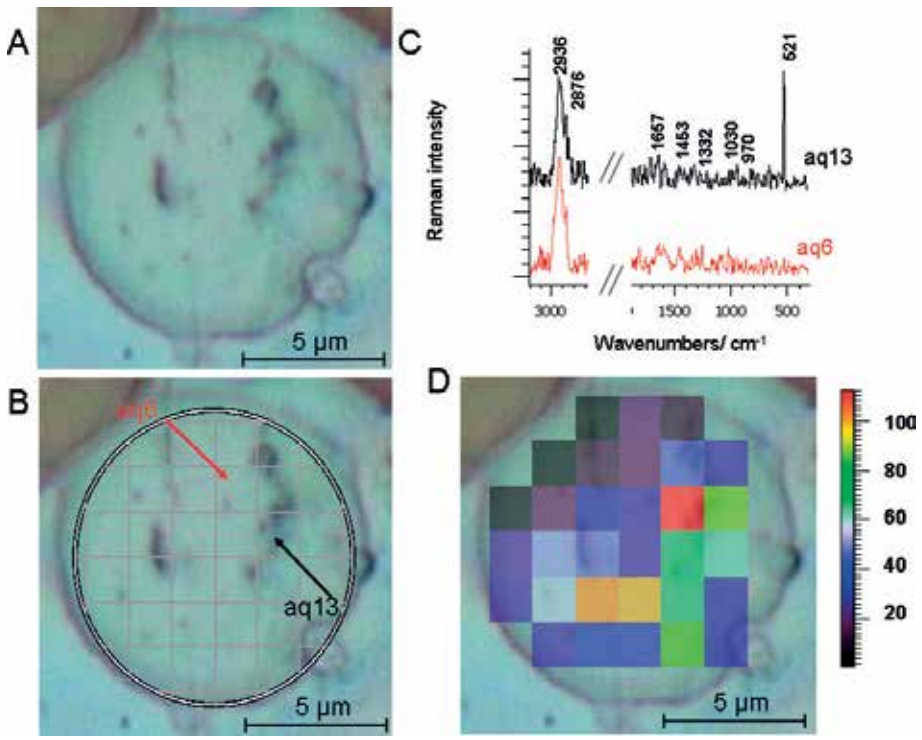

Figure 6. Activated B lymphocyte in response to the EBV virus. (A) A photomicrograph (reflected light, objective 100×); (B) with the marked region over which the map experiment was performed; (C) Raman spectra from an exemplary area including the nonactivated (aq6) and from the activated regions of the cell (aq13); and (D) distribution of immunoglobulins in the lymphocyte (map created from 521 cm^{-1} marker band), excitation laser line 514.5 nm.

The PCA model was built based on spectra recorded for lymphocyte B for the two research groups, at the beginning of hospitalization due to EBV infection and after receiving medical tests indicative of recovery (**Figure 7A**) [40]. The PCA score plots show a separation based on differences in the lymphocytes' content for these two research groups.

Maxima bands in the loadings plot (**Figure 7B**) at wave number values of 3175 and 2891 cm^{-1} are assigned to vibrations of the CH and antisymmetric C—H stretching vibrations of methyl and methylene groups which originate from lipids in the EBV-activated cells. The shifts observed in the protein modes at 1638 and 1673 cm^{-1} in the loading plot for EBV activated and not activated B cells, respectively, are possibly indicative of a difference in protein secondary structure between the two experimental groups. The positive loading at 750 cm^{-1} correlated with positive scores along PC-4 indicates predominance of nucleic acids in activated lymphocytes. It must be noted that the fourth principal component, PC-4 that separates classified groups, counts only 1% variation for spectral dataset analyzed in the whole range. When a narrower range is analyzed, an important component becomes PC-1 that sums 37% of variation (**Figure 7C**). Perhaps, this indicates the specificity of EBV pathogenesis and the formation of latent form in the B cell.

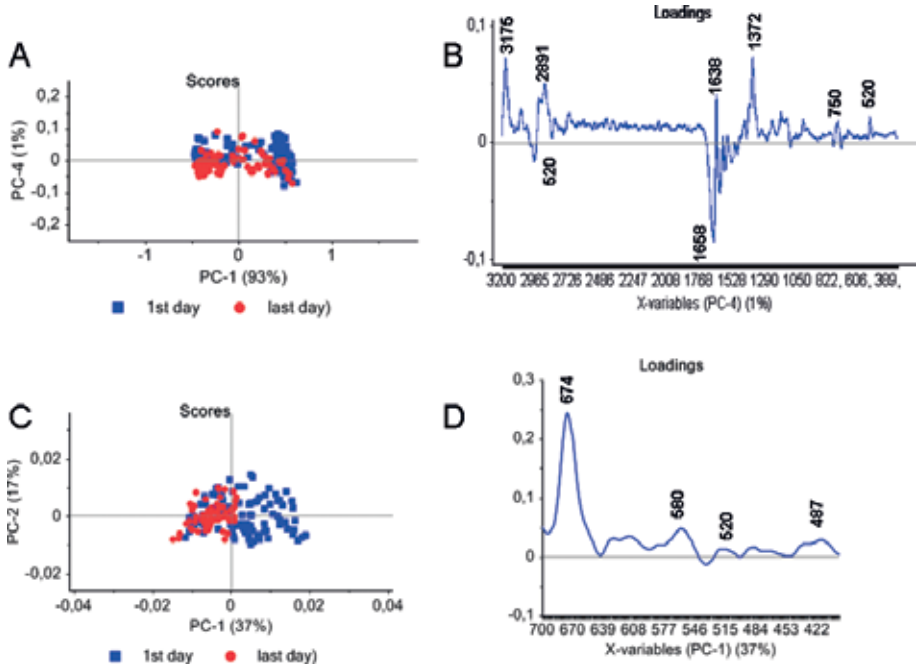

Figure 7. The results of PCA applied to Raman spectra at the beginning of hospitalization due to infectious mononucleosis (blue dots) and after the symptoms of infection have stopped (red dots), (A) in the spectral range 3200–300 cm^{-1} showing scores plot PC-1 vs. PC-4 and (B) loadings plot for PC-4 and (C) in the spectral range 700–400 cm^{-1} showing scores plot PC-1 vs. PC-2 and (D) loadings plot for PC-1, excitation laser line 514.5 nm.

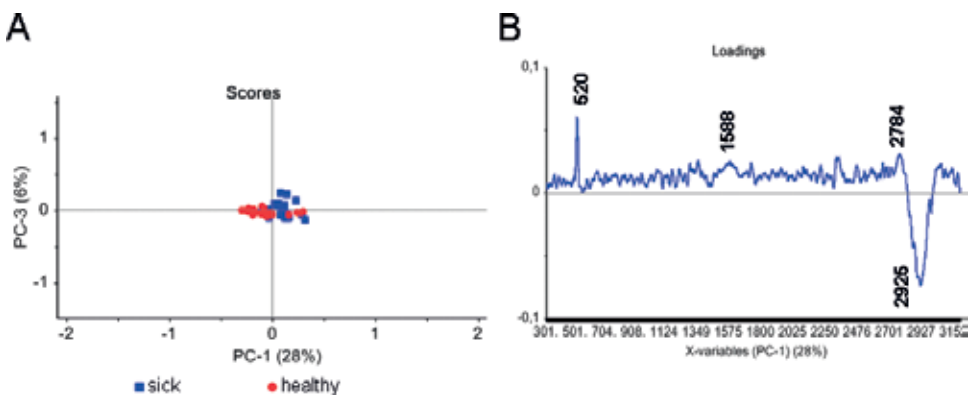

Figure 8. The results of PCA applied to Raman spectra of naïve (red dots) and activated B lymphocytes in response to the EBV virus (blue dots) in the spectral range 3200–300 cm^{-1} showing (A) scores plot for PC-1 and PC-3 and (B) loadings plot for PC-1, excitation laser line 514.5 nm. The magnetic labeling was used to separate B cells from the whole blood (Miltenyi Biotec, Germany).

Figure 8 presents the results of PCA model of Raman spectra of naïve and activated B lymphocytes in response to the EBV virus in the spectral range 3200–300 cm^{-1}. The examined cells were separated from the whole blood using the magnetic B lymphocyte labeling system.

10. Conclusions

In the case of flu infection, the presence of antibodies in the serum can be confirmed by hemagglutination inhibition (HI) test, in complement fixation (CF) or neutralization reaction, and also using ELISA, the enzyme immunoassay [1]. Using these biochemical methods, antibodies are recorded only after 1 week. Raman spectroscopy gives this information, at the level of a single cell, immediately when single cell is activated.

Raman spectroscopy allows to identify a B lymphocyte. Once stimulated by binding to a foreign antigen, for example, virus, a lymphocyte is getting ready to multiply into a clone of identical cells.

On the other hand, it allows immediately to determine that B lymphocyte was in contact with the virus by the appearance of the 521 cm^{-1} marker band.

Antibody molecule may not directly recognize the specific chemical configuration of the antigen, but its overall spatial shape; therefore, the role of Raman spectroscopy, which makes possible to determine the structure, seems to be very promising.

The character of considered viral infections is different. In Raman spectra, we can observe it in PCA. The fact that only less than 40% for EBV is separated, between ill and control recovery groups, could be due to a latent form of EBV virus. In influenza the observations were different—there were about 60% PC factor-separating data.

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Chapter 7

Radiation Protective and Immunopotentiating Effect of Lymphocytes by β-Glucan

Yeun-Hwa Gu

Additional information is available at the end of the chapter

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Abstract

Various intractable diseases have been cured by the development of many new medicines. However, cancer is still a major cause of death. We focused on β-glucan which heat treated Enterococcus faecalis in human intestine, and investigated antioxidant activity, antitumor activity, immune response, etc. In this study, we studied the immunostimulatory effect of β-glucan and its application as a radioprotective agent. As a result, β-D-glucan is known to possess antioxidant activity. Its action is thought to be due to radical scavenger reaction. In our study, β-glucan increased the amount of killer cells and suppressor T cells. Therefore, administration of β-glucan is thought to have an anticancer action. In the future, we hypothesize that β-glucan could be used as an immunopotentiating agent which is effective for the prevention of cancer. From now on, further research will be necessary on the search for cancer cell receptors and to conduct fundamental research on the synergistic effects of radiation therapy. β-glucan showed strong antitumor activity against two kinds of solid carcinoma. Taken altogether, this strongly suggests that β-glucan enhances innate functions of macrophages and NK cells, and as a result, secondarily enhances the immune reaction and suppresses tumor growth.

Keywords: radioprotection, β-glucan, antitumor activity, immunoresponse, IFN-γ, NK cells, TNF-α, lymphocytes

1. Introduction

Previously, various intractable diseases have been cured by the development of many new or alternative medicines approaches. Although there is considerable progress in the development of anticancer drugs, cancer is still the leading cause of death in developed countries. Carcinogenesis induced by various environmental factors has different mechanisms.

Depending on the type of cancer, the therapeutic effect varies and individual differences are also variably large. Therefore, early detection, early treatment and early treatment are urgent [1].

The primary cause of death in Japan is malignant tumors. The incidence of malignant tumors is increasing year by year and one out of three patients die from malignant tumors [2]. Japanese longevity is considered a lifestyle such as ingestion of a lot of fish, ingestion of enzyme foodstuffs and diligence [3, 4]. In any case, malignant tumors have to be destroyed and, at present, there are three main forms of treatment that directly kill the lesion or malignant cells [5].

These three major forms of therapy have severe mental and physical burdens as well as the side effects and weakening of the immune system are also serious problems. In addition, there is no possibility of cessation of treatment, and the motivation for life is also considerably reduced [6–9].

Therefore, research on treatments that exclude the burden on the body as much as possible, such as surgery using a robot and immunotherapy, is underway in recent cancer treatment methods [10, 11].

At the same time, research has also been made to improve disease from the root cause from the viewpoint of oriental medicine. However, it seems that the three major therapies will continue to establish immovable status in the treatment of malignant tumors, and side effects caused by them as well as the reduction of immunity are main problems [3, 12–14].

Radiotherapy is especially important because it is a noninvasive and function-preserving method. And, if the side effects on normal tissues related to treatment can be overcome, it would be one of the most important methods in the future. When hematopoietic tissue is affected by radiation, bacterial and viral infections due to immune system weakness, genetic changes due to cell necrosis and DNA damage, mutations, and cancer occur as side effects [15–17].

For that reason, various radioprotective agents have also been studied. In fact, a radiation protection agent called WR-2721 was developed at the Water Reed Army Hospital in the United States and clinical trials were conducted, but it is no longer commonly used due to side effects such as vomiting and rash [18–20].

Therefore, it is urgent to develop a radiation protection agent with fewer side effects, and approaches focusing on natural substances, which are said to have relatively few side effects.

In recent years, approaches for reducing the side effects of anticancer drugs with natural substances in Japan and Korea are prosperous, among which propolis is drawing attention as a natural anticancer agent and an immunopotentiating agent [19, 21].

Various medicinal ingredients and physiological effects have been reported on this substance. As components thereof, carbohydrates, amino acids, minerals, terpenoids, amino acids and the like are contained and components contained therein are changed by the extraction method.

In the case of general alcohol extraction, numerous lipophilic components such as flavonoids, which are said to be effective ingredients, are extracted, but in the case of the water extraction method devised by Suzuki et al. the levels of flavonoids decrease. However, there are reports that water extraction has higher antitumor effect than alcohol extraction. Antitumor effects are reported for β-(1-3) D glucan, β-(1-6) D glucan [22, 23].

Based on the above information, in this study, we examined the protective effect of β-glucan on lymphocytes exposed to radiation as well as its ability in reducing side effects caused by radiation therapy [24–27]. In case of radiation therapy, it is applied when the radiation sensitivity is high under the condition that the cancer condition is local.

We also examined the effect on tumor growth when combined with radiation therapy. Therefore, this study aims to propose β-glucan as the first step as a new radioprotective agent that can be used at the time of radiotherapy and to contribute data for future development.

2. Materials and methods

2.1. Animals

Mice were purchased from a Japanese SLC company and adjusted to the experimental conditions of this study for 1 week prior to the start of the experiment. The mouse used in this study is a male C3H/Hej mouse. In order to synchronize the purchased mouse, the light was adjusted (12 L:12 D) and kept. Food and water were allowed *ad libitum*.

2.2. Test material

Enteroccous Faecalis 2001®, a glucan product, composed of yeast extract, dextrin and gelatin was supplied by Nihon BRM Co. Ltd. (Tokyo, Japan) and β-1,3 glucan as an active ingredient was contained at the ratio of 6.5 mg/g of the product. *Enteroccous Faecalis 2001*® is hereafter described as β-glucan throughout this paper. Glucan was suspended in physiological saline at concentrations of 2, 4 and 8% (w/v).

2.3. Radio-protective effect

Each mouse was intraperitoneally injected with β-glucan at a dose of 200, 400, 2800 mg/kg/day at 1 day intervals for 2 weeks. The sham control group of mice received an equal volume of normal saline. After the final injection, the mice were irradiated with 2 Gy of X-ray. Whole body irradiation was performed with a dose of 8 Gy (dose rate 1.12 Gy/min) using an X-ray irradiation device (MG226/4.5, Phillips, Inc., Tokyo). Body weight and number of surviving animals were monitored daily.

2.4. Antitumor effect

SSC-7 carcinoma cells were inoculated subcutaneously (5×10^5) into the right femur of mice.

After the average long diameter of the tumors reached 5 mm, mice were intraperitoneally injected with β-glucan suspended in physiological saline at a dose of 200, 400 and 800 mg/kg/day for 5 consecutive days. Control mice received an equal volume of normal saline. At the given time point, the major and minor axes of the tumor were measured with a caliper and the tumor volume was calculated by the following equation; $V = (\pi/6)\ ab^2$, a: major axis length, b: minor axis length.

2.5. Leukocyte and lymphocyte counts

The β-glucan suspended in physiological saline was intraperitoneally injected into mice at doses of 200, 400, 800 mg/kg. Control mice received an equal volume of normal saline. After the injection, in order to measure the number of white blood cells and lymphocytes using an automated hemocytometer (Celltac-α, MEK-6318, Nihon kouden Co. Ltd., Tokyo), a blood sample was transferred from the tail vein into a heparinized tube.

2.6. NK cell activity

The β-glucan suspended in physiological saline was intraperitoneally injected at a dose of 400 and 800 mg/kg for 2 weeks at intervals of 1 day. Vehicle control mice received an equal volume of normal saline. Spleen cells were prepared to measure NK cell mediated cytotoxicity by ^{51}C-release from labeled AC-1 cells (2×10^4 cells) after the final injection. Briefly, 51Cr labeled YAC-1 cells (2×10^4 cells) were added to various dilutions of splenocyte suspensions in flat bottom microplates. The mixture was incubated for 4 h at 37°C in a CO_2-incubator. The radioactivity released to the supernatant was counted with a γ-counter and the magnitude of cell lysis calculated based on the average radioactivity of the control group was defined as NK cell activity.

2.7. Statistical analysis

Significance of the difference in each parameter among groups was assessed by Tukey's multiple comparison tests following analysis of variance. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Radio-protective effect and leukocyte and lymphocyte counts

The survival of irradiated mice is summarized in **Figures 1–5**. All of the animals in the irradiated control group died from the 5th day to the 7th day following irradiation. Glucan injected intraperitoneally prolonged the survival of mice 80% on the 7th day at the doses of 200 mg/kg.

The number of leukocytes increased with time at least up to 24 h after each repeated dose of glucan in a dose-dependent manner. The lymphocyte counts also showed a similar tendency to increase as in the leukocyte counts.

3.2. Antitumor effect

Figure 6 shows anticancer effect of various β-glucan concentrations. The tumor of the control group grew with time, whereas glucan injected intraperitoneally made tumor growth delay significantly in a dose-dependent manner **Figure 3**.

3.3. NK activity

Figure 7 shows NK activity of various β-glucan concentrations. NK cell activities in mice are shown in **Figure 7**. The NK activity increased significantly about twofold to threefold after each repeated dose of glucan (400 and 800 mg/kg).

3.4. Discussion

In the present study, we have found that β-glucan showed antioxidant activity. Previously, Ekaterini et al. have reported that β-glucan shows antioxidant activity [28], suggesting that β-glucan may be responsible for the activity that we found in our study. β-Glucan is well known for its radioprotective and antitumor effect *in vivo* [29–32], and these effects were reproduced in this study.

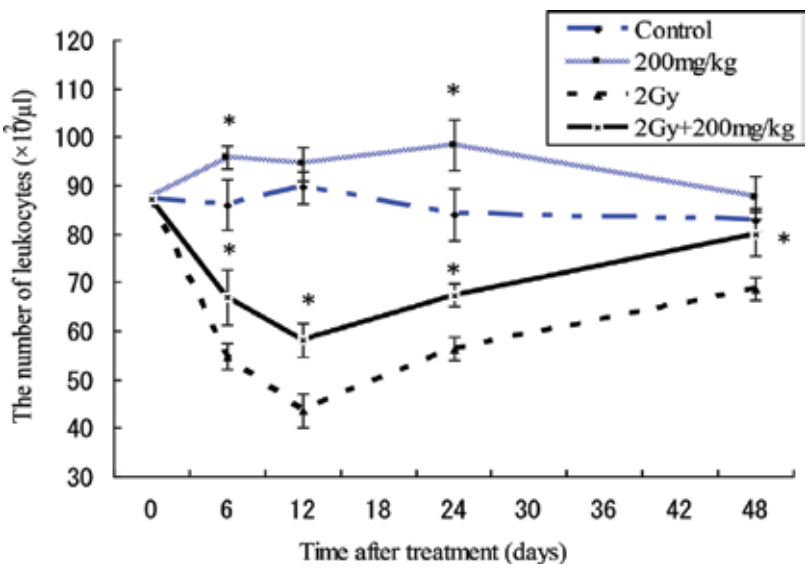

Figure 1. Leukocyte counts on different days after irradiation in mice of different groups. The number of leukocyte was calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation. *Statistically significant ($p < 0.05$) from the control group.

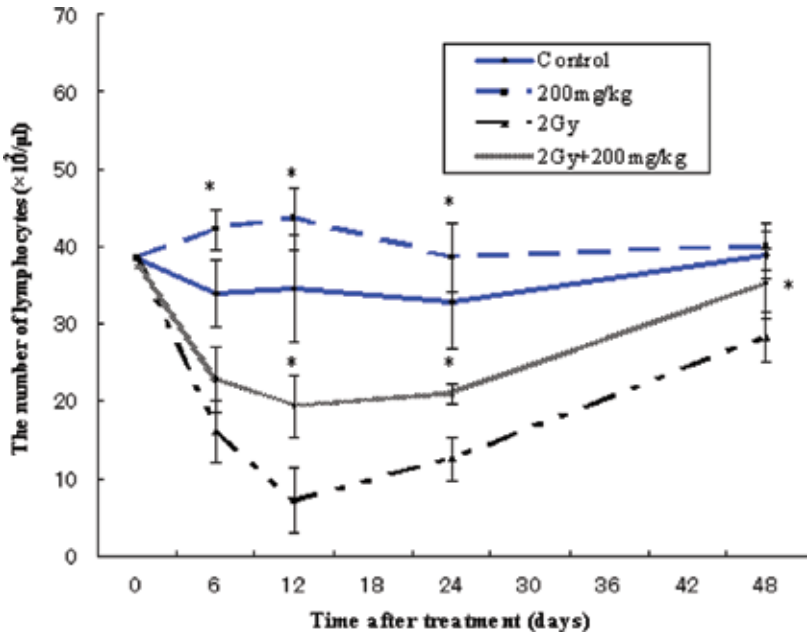

Figure 2. Lymphocyte counts on different days after irradiation in mice of different groups. The number of lymphocyte was calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation. *Statistically significant ($p < 0.05$) from the control group.

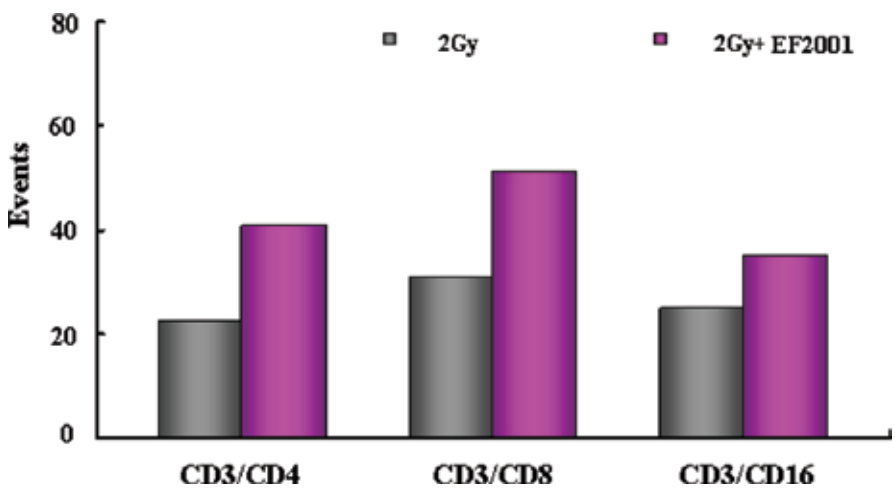

Figure 3. The increased percentage of CD4+, CD8+ and CD16+ T-lymphocytes in PBLs compared to the experimental data baselines of the groups. The unit is in percentage (%). Significantly different from *$p < 0.05$ control group vs. β-glucan groups by Dunnett's test.

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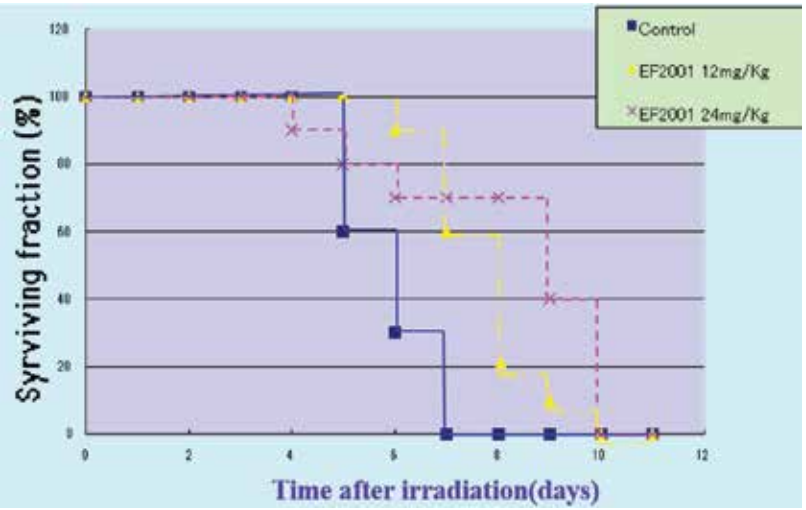

Figure 4. Survival after irradiation. Surviving fraction was increased after injection of β-glucan.

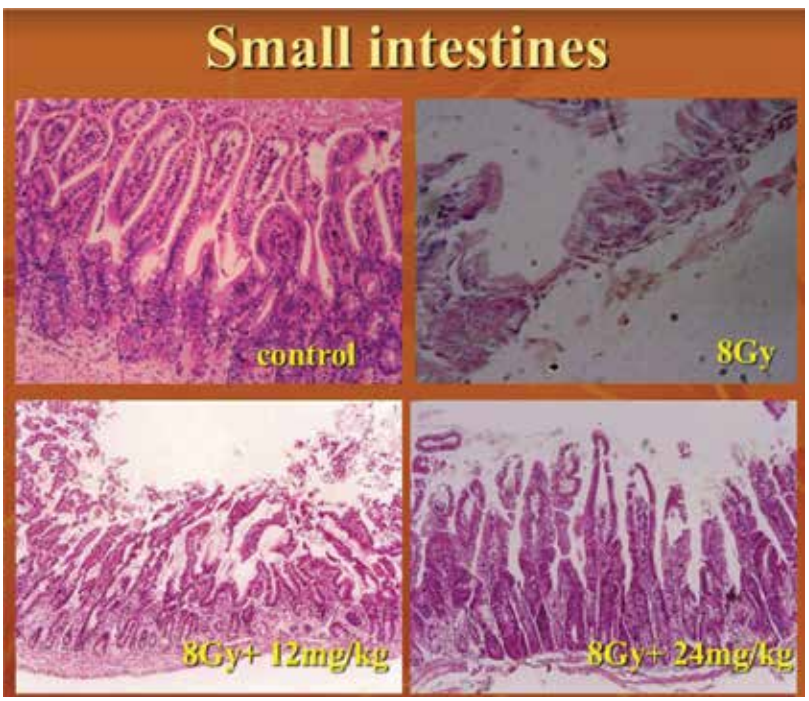

Figure 5. Radiation protection effect of mucosal damage of small intestine to β-glucan of both doses of 12 and 24 mg/kg concentration.

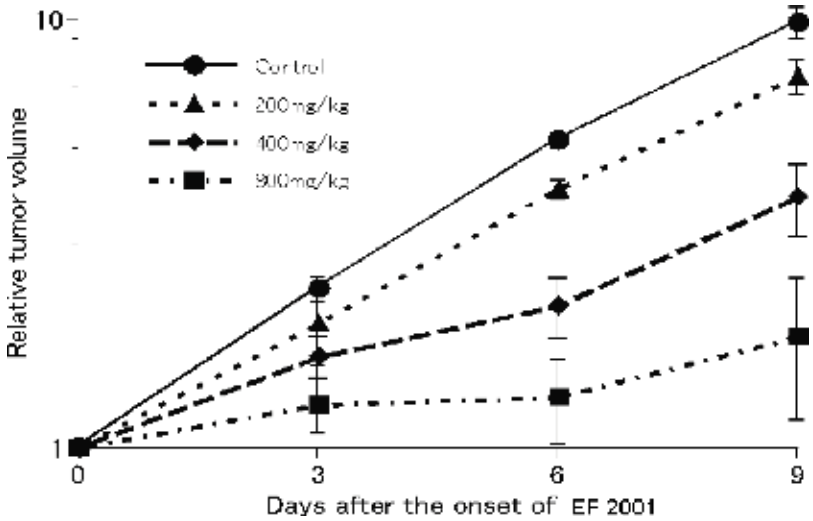

Figure 6. Effect of β-glucan on the tumor growth in mice inoculated with SSC-7 carcinoma cells. Groups of 10 mice were subjected to different treatment. Results represent means ± S.D. *Statistically significant ($p < 0.05$) from the control group.

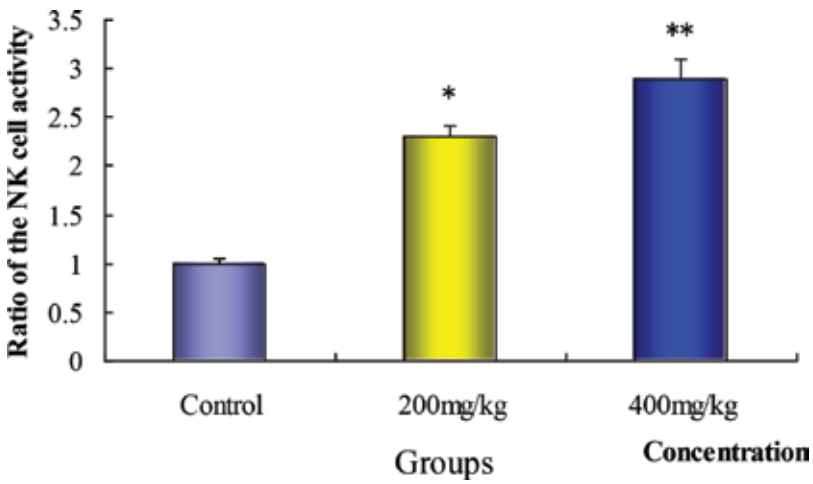

Figure 7. Repeated dose effect of β-glucan on the NK activity in mice. Groups of 10 mice each were subjected to each treatment. Results represent means ± S.D. *Statistically significant ($p < 0.05$) from the control group. **Statistically significant ($p < 0.01$) from the control group.

To confirm the elucidation mechanism by which β-glucan exerts these effects, the number of white blood cells and lymphocytes was monitored as a hematopoietic effect. Furthermore, NK cell activity was measured as an immunological parameter. The results of these parameters demonstrated that the radioprotective effect of β-glucan is mediated, at least in part, by hematopoietic effects in irradiated mice. This is because the leukocyte and lymphocyte counts

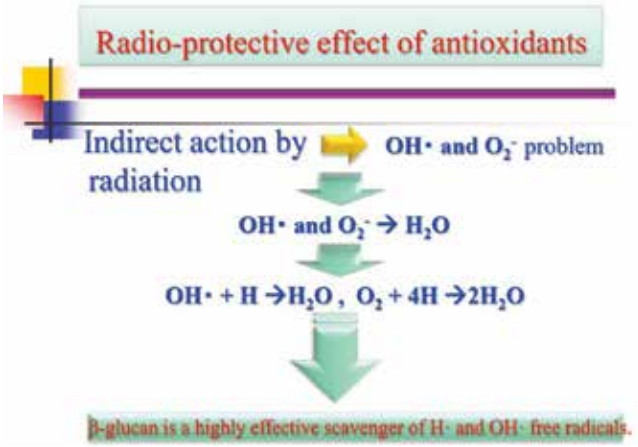

Figure 8. This slide shows a mechanism of radiation protection effect of various β-glucan concentrations.

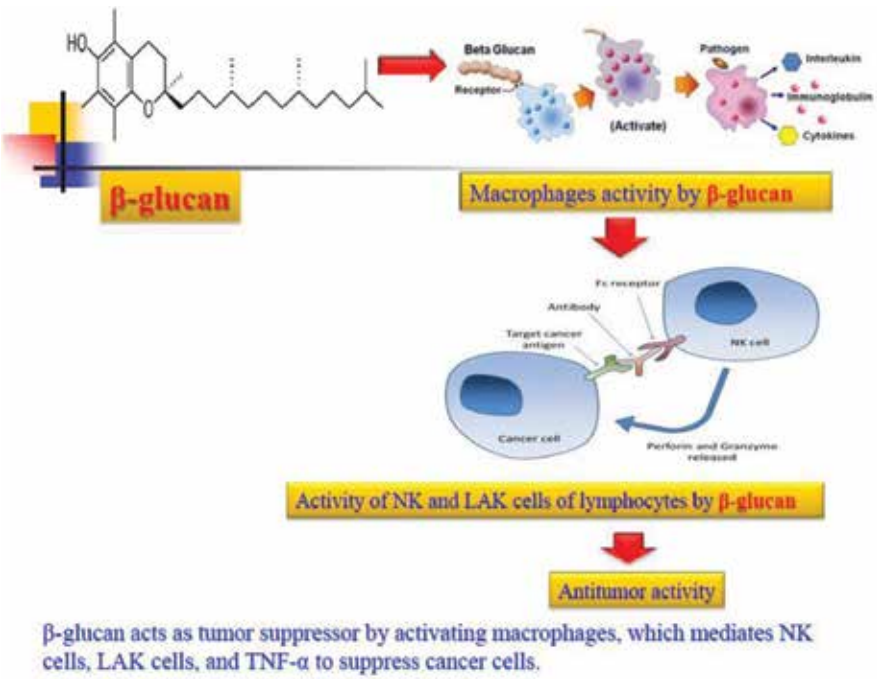

Figure 9. This slide demonstrates a mechanism of activity of NK and LAK cells of lymphocytes by various β-glucan concentrations.

increased with a single administration of β-glucan. In addition, the enhanced immunological activity as seen by the increase in NK cell activity by β-glucan appears to play a role in the prevention of secondary infections associated with radiation.

Natural killer (NK) and lymphokine-activated killer (LAK) cells are well known to be associated with cytotoxic effects on various kinds of tumor cells [33, 34]. The reason why the immature immune response of young mice was activated to the same level as observed in adult mice is that the various components in the sample used in this experiment most probably activated IL-2 and IL-6 of the immune system. IFN-γ, cytokines and the like are multifunctional factors showing antiviral effect, inhibition of cell proliferation, antitumor effect, macrophage activation, enhancement of NK cell activation, regulation of immune response, and differentiation induction.

Specifically, high levels of IFN-γ were measured in mice bearing the S-180 carcinoma, after administration of β-glucan [35–38].

IL-4 is mainly associated with a class switch of IgM to IgG. IFN-γ and IL-4 are related to differentiation of Th1 and TH2, respectively, and these cytokines suppress each other [37–39].

When antitumor action was examined using two kinds of sarcoma (Ehrlich solid carcinoma and Sarcoma-180 carcinoma), tumor-suppressive ratios after treatment with β-glucan was 83%. When Sarcoma-180 solid carcinoma was used, tumor-suppressive ratios was 60%. Thus, β-glucan showed strong antitumor activity against two kinds of solid carcinoma [40, 41].

Therefore, there is the possibility that after treatment with β-glucan, in which no antioxidant activity is found, the balance between NF-κB and I-κB is lost, and the apparent tumor-suppressive ratio is low due to suppression of apoptosis of tumor cells. Thus, it is possible that β-glucan restores the balance between NF-κB and I-κB, and induces apoptosis and tumor suppression secondarily [42].

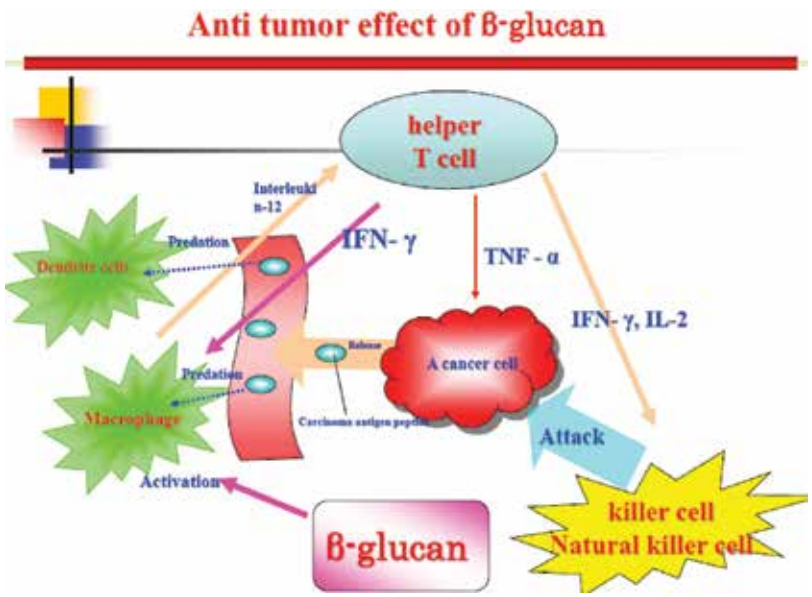

Figure 10. This slide shows a mechanism of activity of antitumor effect by β-glucan.

Therefore, increased activity of NK by β-glucan contributes most probably to attenuated tumor growth in tumor-bearing mice. Based on these data, glucan is expected to be a promising agent for the treatment of cancer patients receiving radiotherapy (**Figures 8–10**).

4. Conclusion

In this study we have evaluated the anticancer properties of β-glucan using a mouse model. β-glucan was found to upregulate CD8+ lymphocytes and leukocytes as well as reducing tumor volume and mass. We believe two phenomena may be correlated but further research is required to elucidate the exact role of β-glucan. Nonetheless, it may be possible to suggest that β-glucan could be used as a potential anticancer agent. β-Glucan was also found to improve recovery rate of lymphocytes and leukocytes following irradiation. We believe this may be due to antioxidant properties of β-glucan suggesting its possible use as an alternative radioprotective agent with reduced side effects.

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Although all the features of the immune system have not been fully resolved yet, the knowledge we have gained from studies on lymphocytes, the basic elements of the immune system, is quite lucid. For this reason, the significance of lymphocytes (the cells that are the source of most of the information we have obtained about the human genome, the negative effects of drugs on the genetic system, the development and behavior of immune system, antigen-antibody association, cytotoxic adaptive immunity, antibody-driven adaptive immunity, cancer and autoimmune diseases) is clear. Studies on lymphocytes will not only help us develop tools to combat human diseases more effectively in the future, but will also help us understand how evolution shapes the immune system in living organisms.

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