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mRNA NEOANTIGEN VACCINES AND THE CD8 T CELL RESPONSE TO INFECTIONS

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ABSTRACT

Neoantigens are novel proteins usually generated by tumor mutations, that are recognized by the immune system as non-self. Infections can also lead to the generation of neoantigens, resulting in a strong response from CD8⁺ cytotoxic cells. CD8⁺ immune cells are specialized in recognizing intracellular foreign antigens (as occurs in viral infections) and eliminating infected cells. In addition to having a lytic function, CD8⁺ cells and their cellular subpopulations also maintain immune homeostasis. During infection, almost all microbial antigens are neoantigens that are generated through specific genetic mutations recognized as foreign by the immune system. RNA neoantigen vaccines were developed following the creation of mRNA vaccines. Neoantigens are identified through genome sequencing, and the mRNA vaccine is designed by synthesizing an mRNA encoding the neoantigen. Neoantigen vaccines elicit an immune response that protects the body from these new proteins.

KEYWORDS: mRNA, neoantigen, vaccine, CD-8 T, tumor, infection, immune response

INTRODUCTION

Neoantigens are altered proteins that appear as a result of genetic mutations in tumor cells or cells infected by microorganisms. These novel antigens are recognized as non-self by the immune system (1). Neoantigens derive from mutated or abnormal proteins produced only by diseased cells, not by healthy cells. The immune system selectively attacks cells that express neoantigens.

These concepts can also be applied to viral, bacterial, and/or parasitic infections, where a strong CD8⁺ T cell response is required. CD8⁺ lymphocytes are very important in infections because many viral and bacterial pathogens (such as *Mycobacterium tuberculosis*), replicate within the host cell (2). In these cases, the antibodies (Abs) produced by B cells are insufficient to fight the infection, and therefore, cytotoxic CD8⁺ cells are needed to kill the infected cells.

Cytotoxic CD8⁺ cells are immune cells specialized in recognizing and destroying cells infected by viruses or intracellular bacteria (3). In addition to their cell-lysis function, CD8⁺ cells also participate in immune homeostasis (4). They are a family of cells that includes several subtypes: T central memory cells (Tcm), which are COR7⁺ and CD62L⁺ and reside in lymph nodes and multiply rapidly; effector memory (Tem) or tissue memory T cells such as CCR7⁻ and CD62L⁻ present in peripheral tissues; and tissue resident memory cells (Trm) CD69⁺ and CE103⁺ which do not circulate but are stable in tissues (5). Another subpopulation of CD8⁺ cells is exhausted T cells (Tex) present in chronic infections and tumors (6). They express PD-1⁺, TNM-3⁺, and LAG-3⁺, and their function is mildly cytotoxic. The cytotoxic

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regulatory T (Treg) subtypes that express CD8⁺ are less well known than CD4⁺ Tregs which express CD25⁺, FoxP3⁺, and CTLA-4⁺ (7). Tregs function to suppress excessive immune responses, as occurs in autoimmunity. Virtual memory (VM) CD8⁺ T cells possess immune memory without exposure to antigen (8). They are generated by cytochemical signals such as IL-5 and IL-4, while stem cell-like memory (SCM) CD8⁺ T cells are subtypes with a very long memory capable of self-renewal and a phenotype similar to naive T cells.

DISCUSSION

By inducing the intracellular expression of antigens, mRNA vaccines are well-suited to stimulating major histocompatibility complex (MHC)-I and therefore CD8⁺ cells (9). Neoantigens are newly formed antigens that are not self-antigens and can arise from mutations, infectious agents, or recognition. In infectious states, almost all microbial antigens are neoantigens that form following specific genetic mutations, particularly in tumor cells (10). These mutations can be recognized as foreign by the body and thus activate an immune response.

RNA neoantigen vaccines are derived from mRNA vaccines and are tailored to induce an immune response against tumor neoantigens (tumor-specific mutations are not present in healthy tissue) (11). Neoantigen identification is done by sequencing the tumor genome and comparing it with the patient's healthy DNA. Neoantigens are selected based on their immunogenicity and ability to bind to the patient's MHC-I and II molecules (1).

The mRNA vaccine is designed by synthesizing an mRNA encoding the selected neoantigens. The mRNA is optimized and enclosed in lipid nanoparticles (LNPs) to protect it and facilitate cell entry. Once ready, the vaccine is administered to the patient, where the LNPs fuse with the cell membrane, releasing the mRNA into the host cell's cytoplasm (12). The mRNA is then translated into antigenic peptides by antigen-presenting cells (APCs) such as macrophages and dendritic cells. Peptides enter the endoplasmic reticulum, loaded onto MHC-I, and are presented to cytotoxic CD8⁺ T lymphocytes, with some neoantigens binding to MHC-II and activating CD4⁺ helper T cells. CD8⁺ T lymphocytes then recognize and destroy tumor cells expressing the neoantigen (13).

Using mRNA neoantigen vaccines presents a lower risk of autoimmunity than other antigens (14). RNA neoantigen vaccines result in effective immune stimulation and tumor cell recognition, resulting in a strong CD8⁺ T cell response. Activated CD4⁺ cells release cytokines that enhance the CD8⁺ response and stimulate B lymphocytes (15). Neoantigens can induce immunological memory against tumor neoantigens.

CD8⁺ immune cells play an important role in recognizing and eliminating infected cells or tumor cells. They recognize antigens present on target cells via MHC-I (16). The mechanisms of destruction of cells expressing neoantigens form the basis of immunotherapy against tumor cells. Neoantigens play an important role in infections where viruses and intracellular bacteria induce new proteins in the host cell (17). These proteins are detected by the immune system, which recognizes them as foreign neoantigens and initiates a specific immune response. In particular, bacterial infections, which cause genetic mutations or alterations in the host cell, can potentially generate neoantigens that stimulate the immune system (17). Although there are some differences, the basic mechanism of an immune response is similar in both tumors and microbial infections, as these reactions tend to eliminate the pathogen, which in the case of tumors, is the tumor cell (18). In infections, the immune system acts to eliminate the microorganism with a rapid and strong response, while in tumors, neoantigens are generated through pathological cellular evolution that makes their elimination difficult, as tumor cells can evade the immune system (19).

CONCLUSIONS

Neoantigens can be formed either through tumor mutations or through intracellular infection. CD8⁺ cells and their subtypes work by eliminating cells infected by intracellular microorganisms. Vaccines against neoantigens help protect the body from these new protein formations.

Conflict of interest

The authors declare that they have no conflict of interest.

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PARASITIC INFECTIONS ACTIVATE TLR4, CD3, IFN- γ , IL-22, AND IL-13: INVOLVEMENT IN INFLAMMATION

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ABSTRACT

Parasites such as protozoa and helminths can infect living things, benefiting at the expense of the host. They are fought by both the innate and adaptive immune responses. The innate response is activated by the binding of the parasite to the Toll-like receptor (TLR)-4 which can recognize specific parts of the parasite. Parasites interact with TLR-4, activating it and inducing an anti-inflammatory immune response, which helps the parasite survive. TLR-4 recognizes pathogen-associated molecular patterns (PAMPs), triggering an innate immune response and the production of pro-inflammatory cytokines such as interferon-gamma (IFN- γ) that help fight the infection. TLRs and the CD3 complex are key components of the immune system in parasitic infections. CD3 is important for signaling from the T-cell receptor (TCR) to the T cell after antigen recognition. In bacterial, viral or parasitic infections, the cytokine IL-22 plays an essential role by helping to maintain the integrity of barrier tissues attacked by microorganisms, particularly in organs such as the intestine, lungs and skin. IL-22 reduces the expression of IL-13-induced M2 markers in macrophages and regulates macrophage Th2 polarization. Activation of the IL-22 receptor (IL-22R) leads to the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT)-3 and 5, which is important for regulating responses to infection-induced cellular stress. IL-13 also plays a key role in immune responses, particularly in parasitic infections by helminth and protozoan parasites. IL-13 is mainly generated by helper T cells and is crucial in mucosal defence against helminths. After a parasitic infection, IL-13 promotes tissue repair and has a fibrotic wound healing effect. The activation of TLR-4, the CD3 complex, and the cytokines IFN- γ , IL-22, IL-13, is fundamental for the immune response against parasites.

KEYWORDS: *Parasite, infection, immune response, Toll-like receptor, inflammation, cytokine*

INTRODUCTION

Parasites include protozoa, helminths, and other organisms that live on or within a host, and benefit at the expense of them (1). The immune system responds to parasitic infections using various pathways related to both innate and adaptive or acquired immunity (2).

The adaptive immune system, mediated by T cells, plays a crucial role in activating specific immune responses to eliminate or kill the parasite and protect against future infections (3). Parasitic infections can have profound effects on T cells, which play a central role in adaptive immunity (4).

However, the immune response varies depending on the type of parasite. For example, helminths can trigger a Th2 immune response, with the release of several cytokines, including IL-13 (5), while intracellular parasites such as

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Plasmodium malariae can activate a Th1 response involving interferon-gamma (IFN- γ) (6). Parasites display highly antigenic molecules on their surface (7) while the outer membrane of Gram-negative bacteria is characterized by the presence of lipopolysaccharide (LPS) that binds Toll-like receptor (TLR) 4, which can recognize specific components of parasites, and activates the innate immune response (8). However, parasites have developed sophisticated mechanisms to evade or manipulate host immune responses, including interactions with TLRs (9).

DISCUSSION

TLR-4 is often associated with bacterial infections, and in parasitic infections, it can help recognize certain components of the parasite and activate an immune response (10). TLR-4 is a pattern recognition receptor (PRR) found on infectious agents such as bacteria, viruses, fungi, and parasites. When TLR-4 recognizes pathogen-associated molecular patterns (PAMPs), it triggers an immune response that helps fight the infection (11). TLR-4 is a pattern recognition receptor (PRR) found on immune cells such as macrophages and dendritic cells (12). Activation of TLR-4 leads to the production of pro-inflammatory cytokines such as IFN- γ , which enhances parasite clearance (13). Helminths can modulate the host immune system by interacting with TLR-4 (14).

Parasites can release molecules that activate TLR-4, which can lead to a regulatory or anti-inflammatory immune response, which helps the parasite survive within the host (15). For example, the blood fluke *Schistosoma mansoni* releases lipids that can bind to TLR-4, triggering immune responses that dampen inflammation and allow the parasite to persist (16). Additionally, Leishmania species, which cause leishmaniasis, are recognized by the immune system through various TLRs, including TLR-4 (17). Parasite surface molecules, such as lipophosphoglycan (LPG), can interact with TLR-4 to modulate the host immune response (18). Evidence suggests that Leishmania can suppress TLR-4-mediated responses, evading the immune system (19).

Parasites often manipulate TLR signaling to promote chronic infection by inducing anti-inflammatory responses, altering the cytokine milieu, or blocking full activation of the immune system (20). For example, helminths often shift the host immune response from a pro-inflammatory Th1 or Th17 response to a more anti-inflammatory Th2 response, thereby avoiding strong immune attacks (21). Similarly, some parasites reduce TLR-4 signaling to avoid triggering a potent immune response (22).

TLRs and the CD3 complex are critical components of the immune system, and both play distinct but complementary roles in the response to parasitic infections (23). The CD3 complex consists of several proteins such as CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ , and is essential for transmitting the signal from the TCR to the T cell after it recognizes an antigen presented by the major histocompatibility complex (MHC) on antigen-presenting cells (APCs) (24).

CD3 is a protein complex present on the surface of T cells and is essential for activating the specific response of T cells by antigen, which forms the basis of adaptive immunity (25). CD3 is part of the T-cell receptor (TCR) complex, which is essential for activating T cells. All T cells express CD3 cells, which help transmit signals into the T cell once the TCR binds to an antigen. In parasitic infections, the number and function of CD3 $^{+}$ T cells is critical to mounting an effective immune response (26). In particular, CD4 $^{+}$ helper T cells guide other immune cells, while CD8 $^{+}$ cytotoxic T cells kill infected cells.

The CD3 receptor binds the anti-CD3 monoclonal antibody to the membrane of T cells. This practice is used in immunotherapy to suppress the immune response in some immune disorders such as autoimmune diseases or to prevent transplant rejection. Anti-CD3 T cells damp the immune response, increasing the risk of infection (27). Anti-CD3 therapy inhibits T lymphocytes, which are essential for fighting infections. Therefore, immunosuppression with anti-CD3 increases susceptibility to infections by parasites and viruses such as cytomegalovirus, herpes simplex, and Epstein-Barr virus. In addition, anti-CD3 treatment can cause cytokine release syndrome, a serious side effect in which a strong immune response leads to fever, inflammation, and increased risk of infection (28).

Bacterial, viral, fungal, or parasitic infections trigger immune responses in which the CD3 receptor and cytokines such as IL-22 play essential roles (29). IL-22 helps maintain the integrity of barrier tissues attacked by microorganisms, while CD3 supports the adaptive immune response to directly fight the infection (30). IL-22 is a signaling cytokine involved in immune responses, particularly in mucosal immunity (e.g., skin, gut, respiratory tract). IL-22 helps protect tissues from damage during infection by promoting tissue repair and the production of antimicrobial peptides (30). It is essential for the defense against bacterial and parasitic infections, particularly in organs such as the gut, lungs, and skin (31).

IL-22 does not act directly on immune cells, but targets epithelial cells, promoting barrier function and defense against pathogens by inducing the secretion of immune molecules. Furthermore, IL-22 reduces the expression of M2 markers such as CD163 and CD200R induced by IL-13 in macrophages (32). IL-22 $^{+}$ cells regulate Th2 polarization of macrophages during *Schistosoma* infection and negatively influence liver fibrosis by acting directly on hepatic stellate cells, which are pericytes found in the perisinusoidal space, a small area between the sinusoids and the hepatocytes of the

liver (called the space of Disse) (33). Engagement of the IL-22 receptor (IL-22R) leads to the activation of several downstream signaling pathways, most notably the phosphorylation of p38 MAPK, STAT3, and STAT5 (34).

IL-22 binds to its receptor IL-22R1 and IL-10R2 to activate the p38 MAPK kinase, a biochemical cascade that regulates cellular stress responses, inflammation, and cytokine production (35). IL-22 signals primarily through the JAK/STAT pathway, where binding of IL-22 to IL-22R activates the receptor-associated Janus kinases JAK1 and Tyk2 (36). These kinases phosphorylate STAT3, which subsequently dimerizes and translocates to the nucleus (37). This reaction induces the transcription of target genes involved in inflammation, survival, and tissue repair (38). Binding of IL-22 to its receptor IL-22R can also lead (with less intensity than the previous reaction) to the phosphorylation of STAT5. Phosphorylated STAT5 may have overlapping or distinct roles in immune regulation. Activated IL-22R triggers the phosphorylation of p38 MAPK, STAT3, and STAT5, influencing processes such as inflammation, cell survival, tissue homeostasis, and immune responses. These pathways are critical in several diseases, including parasitic infections.

IL-22 and IL-13 are cytokines that play key roles in immune responses, especially in parasitic infections involving helminths and protozoan parasites (39). Their roles in immune defense against parasites and inflammation are distinct but complementary.

IL-13 is a Th2-type cytokine, meaning it is typically produced by T helper 2 (Th2) cells, a subset of CD4⁺ T cells (40). It is involved in regulating effective immune responses against extracellular parasites, particularly helminths. IL-13 plays a key role in the body's response to infection and is produced primarily by type 2 T helper (Th2) cells, which are involved in orchestrating immune responses against extracellular pathogens, including parasites. IL-13 is an important cytokine in mucosal defense and protection against external insults, including infections that infect tissues such as the gut, skin, and lungs (41). This cytokine promotes mucus production by goblet cells in the intestinal lining, helping to expel the infectious agent. During helminth infection, IL-13 not only promotes tissue repair but can also mediate a fibrotic wound healing effect by stimulating collagen production in fibroblasts (42). In addition, IL-13 induces smooth muscle hypercontractility in the intestine, promoting expulsion of worms with the help of increased peristalsis (43).

CONCLUSIONS

Parasitic infections activate several immune and inflammatory processes involving the TLR-4 and CD3 receptors, which, once activated, lead to a biochemical pathway with release of cytokines such as IFN- γ , IL-22, and IL-13. However, despite the remarkable progress in understanding the processes described above, the precise nature of the immune response to infections and its regulatory mechanisms remain unclear.

Conflict of interest

The authors declare that they have no conflict of interest.

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CALCIUM ION SIGNALS ARE FUNDAMENTAL IN VIRAL INFECTION

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ABSTRACT

Calcium ions (Ca^{2+}) are important intracellular signaling messengers that allow cells to communicate and regulate various biological functions. In resting cells, Ca^{2+} is low and then increases after of various types of stimulus. After stimulation, Ca^{2+} returns to the initial resting state. Fura-2 fluorescence is one of the main methods for measuring the concentration of Ca^{2+} levels in the cytosol. Cellular Ca^{2+} is mainly found in the endoplasmic reticulum (ER), nucleus, Golgi apparatus and lysosomes. In viral infections, the virus uses the host cell to replicate, exploiting its metabolism. Infectious states alter cellular Ca^{2+} homeostasis in host cells to facilitate the entry and replication of microorganism. The entry of the virus into the cell is mediated by Ca^{2+} which regulates the replication of new virions. Viruses can influence mitochondrial energy production and induce ER stress with Ca^{2+} release. Rising Ca^{2+} levels activate protein kinase C (PKC), mediate viral replication, and act on pattern recognition receptors (PRRs) and Toll-like receptors (TLRs), resulting in activation of NF- κ B and interferon (IFN). Modulation of viral Ca^{2+} can inhibit cell apoptosis and regulate autophagy for viral survival. During an infection, Ca^{2+} in the ER can mediate inflammation and cytokine release. Pharmacological inhibition of Ca^{2+} has antiviral effects, while kinase inhibitors act on viral replication.

KEYWORDS: *Virus, infection, calcium ion, intracellular signaling, immune response*

INTRODUCTION

Intrinsic and extrinsic stimuli on cells cause a biological response mediated by calcium ion (Ca^{2+}) signals which serve as important cellular messengers (1). Normally, cells in the resting state have cytoplasmic Ca^{2+} basal levels of approximately 100 nM that changes upon cell activation (Ca^{2+} signals typically reach peak levels of 0.5–1 μM) (2).

External stimuli on the cell can be of various types: hormones, neurotransmitters, growth factors, antibodies, mechanical or electrical signals, temperature, pH, or osmotic changes, cytotoxic reagents, or microbial invasion (3). Stimuli that influence Ca^{2+} signaling can also be intrinsic to the cell, as occurs within cardiac myocytes (4) and developing neurons (5). Typically, these stimuli lead to an increase in Ca^{2+} concentration and modification of the cellular response. At the end of the stimulation, the Ca^{2+} concentration returns to the resting state (6). The Ca^{2+} levels in the cytosol also depend on the intensity of the stimulus, its concentration and duration (7).

A widely used method to monitor the Ca^{2+} values in the cell is fluorescence using Fura-2 (8). After stimulation, the release of Ca^{2+} from intracellular organelles and/or the influx of Ca^{2+} across the plasma membrane triggers signals that specifically activate or deactivate cellular processes. Ca^{2+} is mostly concentrated in the endoplasmic reticulum (ER),

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nucleus, Golgi apparatus, and lysosomes (9). Viruses need the host cell to replicate, exploiting its metabolic apparatus and using its own genetic information.

DISCUSSION

During viral infections, Ca^{2+} signaling plays an important role in both biochemical and immunological reactions (10). In these infections, Ca^{2+} homeostasis in host cells is altered, facilitating viral entry, replication, and assembly. Thus, a change in intracellular Ca^{2+} fluxes causes a biochemical reaction followed by a biological response.

In viral infections, the entry of the virus into the cell is mediated by Ca^{2+} with fusion of the virus to the host cell membrane (11). In addition, endocytosis is also used by viruses to penetrate the cell (12). Viruses use Ca^{2+} -dependent enzymes such as kinases and phosphatases to regulate their replication (13). Ca^{2+} influences cytoskeletal reorganization, vesicular trafficking, and budding of new virions (14).

Many viruses, for example hepatitis viruses, induce ER stress, an effect that leads to the release of Ca^{2+} from the ER via inositol 1,4,5-triphosphate receptors (IP3R) (15). Many microorganisms, including viruses, manipulate mitochondrial Ca^{2+} levels, influencing energy metabolism and apoptosis (16). These effects mediate immune responses and the production of reactive oxygen species (ROS) (17). Ca^{2+} activated by protein kinase C (PKC), is involved in viral replication and influences pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) (18). In addition, activation of TLRs involves NF- κ B and interferon (IFN) regulatory factors, regulating the antiviral response (19).

Many viruses modulate Ca^{2+} to inhibit apoptosis, enhance replication, and spread the infection (20). Another function of Ca^{2+} is to regulate autophagy, an effect that some viruses exploit to survive (21). Many viral proteins modulate Ca^{2+} signaling by interacting with host Ca^{2+} channels to manipulate host homeostasis (22). These proteins increase intracellular Ca^{2+} , influencing neuronal function and inflammation (23). During viral infection, Ca^{2+} stores in the ER are manipulated to promote inflammation and cytokine release (24).

Ca^{2+} channel blockers, such as verapamil and nifedipine, produce antiviral effects, and kinase inhibitors inhibit viral replication by modulating Ca^{2+} signaling (25). ER stress modulators can also limit viral replication (26).

CONCLUSIONS

Ca^{2+} fluxes are an important factor in viral infections, as they impact viral replication, the immune response, and host cell survival. These important observations may be useful to improve the understanding of the relationship between Ca^{2+} and viruses, but future studies will be needed to address new therapeutic challenges regarding this topic.

Conflict of interest

The authors declare that they have no conflict of interest.

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CLINICAL USE OF AEROSOL THERAPY IN INFECTIOUS RESPIRATORY DISEASES

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ABSTRACT

Inhalation therapy has been used since ancient times and today the use of aerosol is a method that uses the dispersion of liquid or solid particles that can be breathed. Aerosol therapy is very common in the treatment of many respiratory diseases and its use is effective in treating respiratory infections, as the selected drug is injected directly into the target organ. With aerosol therapy, drugs directly reach the site of infection in the lungs through inhalation using devices such as nebulizers, which are commonly pneumatic or ultrasonic, although today new generation devices with double Venturi effect are used which allow the particles to be aerosolized much more quickly. The effect of aerosol therapy depends on how the particles are deposited and how they interact with cells and molecules in the respiratory system. In the lungs, the drug interacts with respiratory epithelial cells, bronchial smooth muscle cells, and immune cells, which absorb it through passive diffusion or transporters, modulating mucus secretion and the production of inflammatory mediators.

KEYWORDS: *Aerosol therapy, respiratory disease, infection, inhalation, respiratory tract*

INTRODUCTION

Inhalation therapy originated in ancient times when active or presumed active ingredients were added to boiling water and then inhaled to obtain a beneficial effect on health (1). Aerosol refers to the fine dispersion of liquid or solid particles (dispersed phase) in a gas suitable for breathing (dispersing phase). The first aerosol system of the modern era was conceived in 1978 (2). Since then, aerosol therapy has undergone considerable diffusion and has assumed scientific and therapeutic validity for the treatment of numerous pathologies, including infectious ones of the oral cavity (3).

In the treatment of respiratory diseases, the inhalation route represents the most rational method for the administration of drugs, presenting undoubted advantages. It allows a selective direct action of the drug in the target organ without having to pass through the bloodstream, achieving the same effect with a lower dosage than that required by oral or parenteral therapy, with a consequent reduced possibility of side effects (4). Additionally, aerosol therapy has a greater speed of action, is non-invasive, and allows the use of active drugs only if administered by aerosol (for example topical steroids).

Aerosol therapy is also indicated in the treatment of respiratory infections (5), with the inhalation route representing the most effective method for the administration of drugs because it allows a direct and selective action of the drug in the target organ, achieving the same effect with a lower dosage than that required by oral or parenteral therapy and thus

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reducing any side effects of the drugs (4). The administration of drugs directly into the lungs through inhalation is achieved by using devices such as nebulizers, metered dose inhalers, or dry powder inhalers (6). With this method, the drug is administered directly to the site of infection, ensuring better therapeutic efficacy and minimizing side effects on the entire organism (3).

The identification of the most suitable device for the individual patient is a crucial point for achieving the therapeutic objective and it must derive from a considered choice evaluating numerous factors (7). The multiple possibilities of choice are advantageous, but at the same time, can create doubts and uncertainties among both patients and doctors (8). Several studies have highlighted the inadequate knowledge of medical personnel regarding the use of inhalation instruments (9,10).

DISCUSSION

Aerosol therapy is a valid therapeutic method in the treatment of upper airway conditions such as rhinitis, rhinopharyngitis, rhino-tympanitis, rhino-sinusitis, and laryngotracheitis (11) (Table I). Among the respiratory diseases mentioned above, asthma plays a primary role as one of the major causes of morbidity and mortality in the world and has significantly increased in recent years (12). Numerous drugs can be administered via aerosols, such as beta-2 agonists, topical steroids, anticholinergics, chromones, antibiotics, ribavirin, and vaccines (13).

Table I. *Inhalation therapy is indicated in pathologies characterized by airway obstruction and wheezing. The diseases indicated in the table are the most common ones treated by aerosol therapy.*

▪ Asthma	▪ Bronchiolitis
▪ Cystic fibrosis	▪ Acute laryngitis
▪ Bronchodysplasia or chronic lung disease	▪ Recurrent bronchospasm during infections, especially viral ones: asthmatic or asthmatic bronchitis

For therapeutic purposes, for the inhaled drug to reach all areas of the respiratory tract, the inhaled particles must have a median mass diameter between 0.5 and 5 μm (14). The median mass diameter is the diameter that divides the total mass of the aerosol into two equal parts, one of which is composed of particles with a diameter smaller than that indicated and the other with a larger diameter, referred to as the spherical particle of unit density and having the same sedimentation speed (15). Larger particles may settle at the level of the nose-oropharyngeal tract and be swallowed (6). On the other hand, particles that are too small may not settle at the level of the lower airways and be exhaled or may carry a modest quantity of drug, which is why in this case the therapeutic efficacy will be reduced (16).

Nebulizers can be distinguished in two types: pneumatic and ultrasonic. Pneumatic nebulizers exploit the Venturi effect, in that the liquid to be nebulized is pushed by a compressed gas towards a narrow orifice called a Venturi narrow (17). The air expands and determines a negative pressure and the fragmentation of the liquid into droplets with a diameter of 15-500 μm (7). Only even smaller particles will be able to pass and will be inhaled thanks to the presence of a deflector placed on the orifice. The most recent instruments consist of a device with a double Venturi effect that allows the particles to be aerosolized much more quickly, reducing the delivery time and improving compliance (18).

Ultrasonic nebulizers produce the aerosol by vibrating a piezoelectric crystal (19). These vibrations of the crystal are transmitted to the drug in solution where waves are formed, and droplets fall from the crest of the waves and are released as an aerosol (20). In pneumatic nebulizers, a "whisker" stops the droplets that are too large from falling back into the reservoir and this liquid is subsequently re-nebulized (21). Nebulizers can be used both with a mask or with a mouthpiece, however the mouthpiece is preferable because it allows double the amount of drug to reach the airways.

Nebulizers can be used at any age without difficulty because they do not require a respiratory procedure other than a physiological one. The use of nebulizers is indicated in patients who have poor cooperation and in all those subjects who, due to their incapacity or because they have an acute pathology in progress (for example, asthma attack), are unable to use other devices (22). They are also useful in cases where it is necessary to administer high doses of the drug.

Aerosol devices are machines with poor performance (23). In fact, only 10% of the nebulized product is available to the patient (output) and that only a part of this output will be pharmacologically available in relation to the type of machine used, the type of patient to be treated, and the type of drug used. There are several elements to consider when choosing a good nebulizer and among these, particularly important are the volume of the particles to be nebulized (granulometry), the age of the patient and his/her compliance, the pathology, and the cost (24).

Drug particles introduced through aerosol therapy are deposited in the respiratory tract, interacting with respiratory epithelial cells, bronchial smooth muscle cells, and immune cells. The drug is absorbed through passive diffusion or transporters, modulating mucus secretion and the production of inflammatory mediators.

The patient's disease conditions the choice of nebulizer. Cystic fibrosis patients need devices that nebulize particles of 0.5-3 μm to better reach the alveoli (25). The amount of drug to be put in the nebulizer vial must be at least 3 ml for the pneumatic nebulizer, and 5 ml for the ultrasonic nebulizer. Lower amounts of liquid are not effectively nebulized with an increase in osmolarity and possible paradoxical bronchoconstriction, while higher amounts lead to an increase in delivery time with excessive cooling of the nebulizer and reflex blocking of the respiratory act.

Traditional nebulizers are inefficient because they deliver the drug both in the inspiratory and expiratory phases and during the respiratory pause. To reduce drug waste during expiration, new nebulizers have been designed, some with a plastic bag that acts as a reservoir, others "improved" (enhanced) or activated (actuated) by breathing (26). Breath-enhanced nebulizers, through the open-vent system, allow an increase in output during inspiration compared to that generated during expiration, with a consequent reduction in drug loss during expiration (27). Drug loss during the expiratory phase can be eliminated if the nebuliser is activated only during the inspiratory phase, as happens with breath-actuated nebulisers. New "smart" nebulisers combine the technology of the perforated vibrating membrane with that of adaptive aerosol delivery (AAD) (28).

The most surprising and revolutionary innovation is the presence of a chip that records and transmits data via the internet, allowing the doctor to examine and monitor the patient's performance in real-time (29). From a clinical point of view, they have numerous advantages: lower residual volume of the drug, greater precision of the dose delivered as they adapt to the patient's breathing without wasting the drug during expiration, greater adherence to therapy thanks to the presence of a visual, sound, or vibratory signal that informs the patient of the correct and complete execution of the maneuver. These are silent, portable, and rechargeable devices that allow, through a single platform, the administration of multiple drugs. The first studies have highlighted the possible use of the AAD System for the administration of α -1-antitrypsin in patients with cystic fibrosis, with an evident saving of time and high levels of compliance (30).

Prescribing the most appropriate device can significantly improve the management and prognosis of respiratory diseases such as asthma. All types of aerosol devices have advantages and disadvantages, and the choice must be evaluated on a case-by-case basis. A summary of key points to evaluate for a reasoned choice is shown in table II (Table II).

Table II. *Factors to consider when choosing an inhaler.*

▪ Clinical picture and patient characteristics	▪ Possibility of using a single type of device
▪ Patient ability to use the device correctly	▪ Cost of therapy
▪ Availability of the drug-device combination	▪ Patient preferences

CONCLUSIONS

Aerosol therapy is an important and evolving tool in the management of respiratory infectious diseases and plays a critical role in the treatment. It involves the administration of medication directly into the lungs via inhalation, using devices such as nebulizers. This method offers targeted and effective treatment options. Ongoing research is aimed at improving its applications and effectiveness.

Conflict of interest

The authors declare that they have no conflict of interest.

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PHOSPHATIDYLCHOLINE INFLUENCES MEMBRANE PERMEABILITY AND PATHOGEN PENETRATION

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ABSTRACT

Phosphatidylcholine (PC) is an important membrane phospholipid involved in microorganism-host interactions. Choline is a precursor to acetylcholine, which is a precursor molecule to PC. PC is an essential nutrient that has structural, functional, and cell membrane integrity roles. It plays a role in the interaction between microorganisms and host cells, influencing the course of infection. It acts as a barrier and influences cell membrane permeability and the penetration of microorganisms. Lipid mediators such as lysophosphatidylcholine (LPC) participate in the inflammatory process by causing complement activation and the recruitment of neutrophils and macrophages to the site of infection with the production of pro-inflammatory cytokines, although some species of LPC or PC may also have anti-inflammatory activity. LPC inhibitors reduce the pathogenic effect of microorganisms, and PC act as an anti-inflammatory.

KEYWORDS: *Phosphatidylcholine, phospholipid, lipid mediator, infection, pathogen*

INTRODUCTION

Phosphatidylcholine (PC) belongs to an important class of membrane phospholipids involved in pathogen-host interactions (1). PC is a phospholipid that contains choline as a functional group. Structurally, PC consists of a polar head (choline linked to a phosphate group) and two hydrophobic fatty acid tails (2). Choline is a precursor molecule of PC, which is an essential nutrient involved in numerous brain functions (3). One of its main roles is serving as a precursor to acetylcholine, an essential neurotransmitter for cognitive function (4).

Genes involved in PC biosynthesis (such as *pcs*, *pmtA*, *choA/B*) are used to study lipid transcriptional regulation (5). PC plays structural and functional roles, influencing the fluidity, curvature, and integrity of the cell membrane (6). Interactions between microorganisms and host cells are mediated by PC metabolism, which is influenced by viruses, bacteria, and parasites to promote infection (7) (Table I). PC is a major phospholipid in cell membranes and plasma lipoproteins and is composed of glycerol, fatty acids, choline, and a phosphate group, and its degradation products are implicated in inflammatory reactions (8,9). PC can be synthesized *de novo* through the CDP-choline pathway (or Kennedy pathway), which is the main synthesis pathway, or through PC methylation (10).

Table I. *Phosphatidylcholine (PC) in microorganisms.*

Characteristics:	<ul style="list-style-type: none"> - Bacteria, Archaea, Unicellular eukaryotes (yeasts, protozoa) - Very abundant - Presence of PC: In ~12% of species (mainly pathogens and symbionts) - Type of lipid bond: fatty acid esters with glycerol - Esters of fatty acids with glycerol - Biosynthetic pathways: CDP–choline (Kennedy pathway)
Main functions:	<ul style="list-style-type: none"> - Adhesion, virulence, host mimicry, membrane stability - Thermal stability, environmental adaptation - Membrane integrity, secretion, division, metabolism Lipid
Molecular role:	<ul style="list-style-type: none"> - Bacterial-host interaction, regulation of secretion systems, extreme adaptation (high temperature, pH, salinity), regulation of vesicle trafficking and endoplasmic reticulum stress

DISCUSSION

PC plays a key role in the host barrier and signalling in infections, influencing both cell membrane permeability and microorganism penetration (11). Lipid mediators such as lysophosphatidylcholine (LPC) mediate the inflammatory response by attracting immune cells such as neutrophils and macrophages to the site of infection (12). Complement, which participates in the immune response, is also activated in the inflammatory response (13).

Many pathogens exploit PC by degrading it to promote infection. Bacteria such as *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* produce phospholipases C or D that degrade PC, damaging host membranes and obtaining choline (14). Other bacteria such as *Legionella pneumophila* and *Helicobacter pylori* modify PC to evade immune recognition (15). Some viruses, such as influenza virus, hepatitis C virus, and SARS-CoV-2, also act on PC-rich membranes to create replication vacuoles (16). Differential membrane PC formation can influence the assembly and release of viral particles (17). In pathogen-host interactions, PC improves the ability of pathogenic bacteria to infect eukaryotic cells, and mutations in PC synthesis pathways are exploited to modulate immune responses or lipid production (18) (Table II). In yeast, PC synthesis pathways are exemplary models for the study of lipid metabolism and gene regulation (19,20).

Table II. *Biological roles of phosphatidylcholine (PC).*

In <i>bacteria</i> :	<ul style="list-style-type: none"> - Essential for secretion, division and lipid metabolism - Promotes interaction with eukaryotic cells - Present in pathogens or symbionts (e.g., Brucella, Rhizobium) - Helps camouflage the host membrane - Regulates protein stability and secretion
In <i>eukaryotes</i> :	<ul style="list-style-type: none"> - Major membrane phospholipid - Maintains fluidity and asymmetry of the bilayer

Parasites such as *Leishmania* and *Trypanosoma* alter PC metabolism to survive macrophage phagocytosis (21). LPC is produced by PC through phospholipase A₂, a molecule implicated in the inflammatory process mediated by cytokines, including IL-1 and TNF. These cytokines activate endothelial cells and leukocytes to produce inflammatory molecules (22). However, some species of LPC or PC may also have anti-inflammatory activity and limit tissue damage (23). This depends on the degree and type of infection. Therapy with phospholipase inhibitors can reduce bacterial virulence, while PC supplementation can help stabilize membranes and reduce inflammation (24).

CONCLUSIONS

The metabolic targets of some PC synthesis pathways are essential for viral or bacterial replication and therefore can be used as potential drug targets. PC improves the ability of pathogenic bacteria to infect eukaryotic cells, and its alteration leads to endoplasmic reticulum stress and mitochondrial dysfunction. PC dysregulation is treated with phospholipase inhibitors, PC supplementation, and specific metabolic targets for PC synthesis. Exploring the evolution of metabolic pathways in microorganisms is important, and analyzing PC-deficient mutants helps to understand the role of phospholipids in membrane function.

Conflict of interest

The author declares that they have no conflict of interest.

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TUMOR CELLS TRANSFECTED WITH THE β -CHEMOKINE GENE CCL16 (LEC) ARE MORE VULNERABLE TO INFECTION

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ABSTRACT

This study targets the chemokine CCL16 and its gene, LEC, which belongs to the β -chemokine family, also known as CC chemokines. The specific location of the LEC gene has not yet been precisely determined. In macrophages, LEC is encoded by two mRNAs, is influenced by IL-10, and plays an interesting biological role during the early stages of the inflammatory process. Recombinant LEC protein has demonstrated a dose-dependent chemoattractive capacity towards non-activated human monocytes and the human THP-1 monocyte line. Here we report that subcutaneous injection of cells obtained from a murine mammary adenocarcinoma (TSA) engineered to produce LECs leads to the development of an inflammatory response, resulting in hyperplasia of the lymph nodes draining the inoculation area. Our results demonstrate that LEC is a chemokine with high proinflammatory activity and LEC production is upregulated by IL-10.

KEYWORDS: *CCL16, LEC, chemokine, tumor cell, gene, IL-10, inflammation*

INTRODUCTION

Transfection is a useful method in gene therapy that introduces exogenous genetic material (transgene) into recipient cells (1). Transfection is used to introduce foreign DNA or RNA into cells, allowing for the expression of specific genes (2). It can be transient or stable if the transfected DNA is kept in the cytoplasm for a limited period or integrated into the cellular genome. However, transfected tumor cells can be more susceptible to infections for various reasons (3). Transfection can modify the expression of genes involved in the immune response, and the transfected tumor cells may become more susceptible to viral, bacterial or fungal infections (4). Some transfections are designed to knock down or knock out specific genes, including those that might be important in immune responses.

There are 3 subclasses of chemokines: α -chemokines (or CXC chemokines), β -chemokines (or CC chemokines), and CX3C chemokines. CXC chemokines have the first 2 cysteine residues separated by a different amino acid residue (5), while CC chemokines have the first 2 cysteine residues adjacent. CX3C chemokines are encoded by a single gene located on chromosome 16q13 (6) (Table I).

Table I. Organization of Human CXC and CC genes.

<i>Family</i>	<i>Cytokine</i>	<i>Human Chromosome</i>
CXC	Platelet factor 4	4(q12-----q21)
	Platelet basic protein	?
	Y IP-10	4(q12)
	Gro/MGSA	4(q13-----q21)
	NAP-1IL-8	4(q12-----q21)
	ENA-78	?
CC	MIP-1a	17(q11-----q21)
	MIP-1b	17(q11-----q21)
	I-309	17(q12)
	JE/MCP-1	17(q11.2-----q12)
	RANTES	17(q11.2-----q12)
	HC-14	?

At least 27 distinct members of the CC subgroup have been reported in mammals, with CC chemokine ligands (CCL) designated from 1 to 28 (CCL10 is equivalent to CCL9). The CC chemokine group includes MIP-1 α , MIP-1 β , MCP-1, RANTES, C10, and I309. CC chemokines are primarily represented by MCP-1, which acts on monocytes, lymphocytes, and eosinophils by binding to the CCR2 or CCR3 receptor (7). Based on the chemokine class, chemokines are distinguished between CXC2, 3, 4, and 5 receptors, which bind CXC chemokines, and CCR1 and CCR9 receptors, which bind C-C chemokines. XCR1 receptors bind leptin (a C-C chemokine), while CX3CR1 receptors bind fractalkine or neurotactin (8) (Table II, Fig.1).

Table II. Summary of the known chemokine receptors and some of their known human ligands.

<i>CHEMOKINE RECEPTORS</i>	<i>HUMAN CHEMOKINE LIGANDS</i>
CXCR1	IL-8, GCP-2
CXCR2	IL-8, GCP-2, Gro α , Gro β , Gro γ , ENA-78
CXCR3	MIG, IP-10, I-TAC
CXCR4	SDF-1/PBSF
CXCR5	BLC/BCA-1
CCR1	MIP-1 α , MIP-1 β , RANTES, HCCC-1,2,3
CCR2	MCP-1, MCP-2, MCP-3, MCP-4
CCR3	Eotaxin-1, Eotaxin-2, MCP-3
CCR4	TARC, MDC, MIP-1 α , RANTES
CCR5	MIP-1 α , MIP-1 β , RANTES
CCR6	MIP-3 α /LARC
CCR7	MIP-3 β , ELC, 6CKINE/LC
CCR8	I-309
CCR9	TECK
XCR1	LIMPHOTACTIN
CX3CR1	FRACTALKINE/NEUROTACTIN

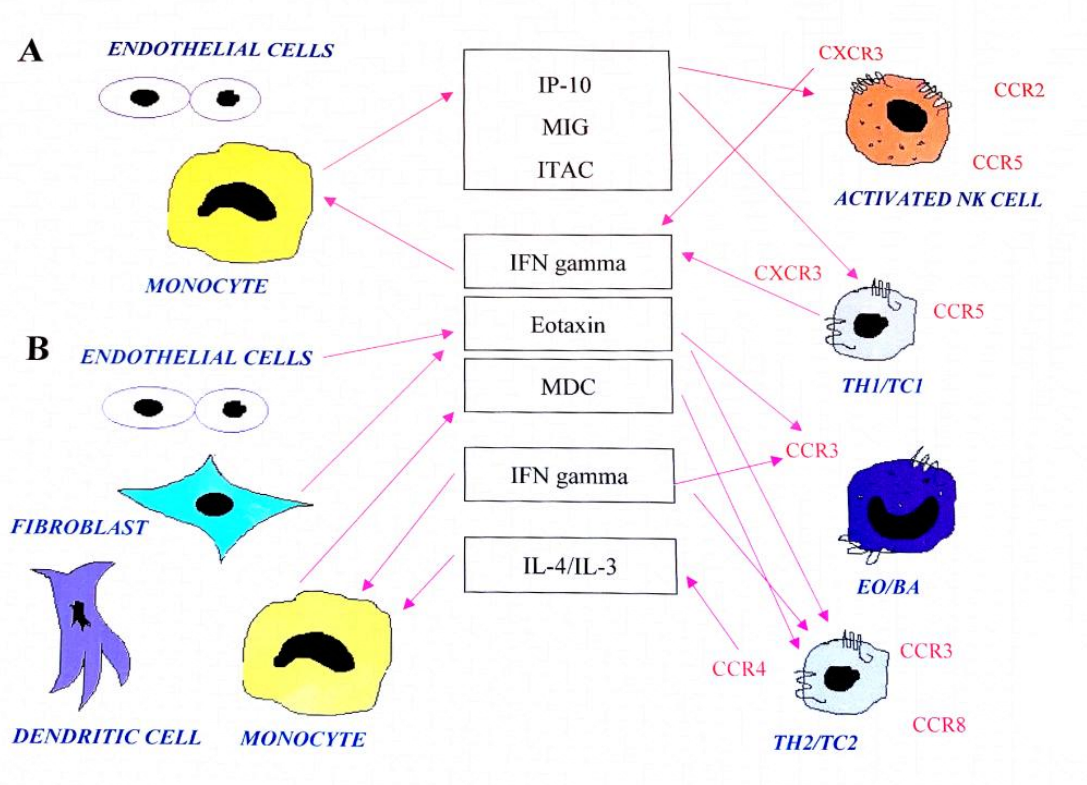


Fig. 1. Endothelial cells, monocytes, fibroblasts, and dendritic cells produce cytokines and chemokines that influence the activity of immune cells (Th1, Th2, basophils, and natural killer cells) and vice versa.

CCL16 is a ligand of the C-C chemokine family that is involved in immune and inflammatory responses (9). CCL16 can attract different types of immune cells (including monocytes and lymphocytes) to sites of infection, where they mount an immune response against the microorganisms (10). CCL16 binds to specific receptors on immune cells, guiding them to the site of infection. During the infectious phase, CCL16 expression can increase, allowing the recruitment of immune cells to fight pathogens and inflammation. However, it is important to understand the mechanism by which CCL16 intervenes in infections. This could contribute to the development of new therapeutic strategies against infectious and inflammatory processes.

Our study aimed to analyse the role of the chemokine CCL16, which has been reported as an unmapped LEC gene that resides in a chemokine-encoded gene cluster located on chromosome 17 (17q11.2) and is considered to be a chemoattractant for lymphocytes and monocytes. The protein encoded by the LEC gene is composed of a 120-amino acid chain with a 20-amino acid primer and a carboxy-terminal end containing a putative N-linked glycosylation site (11).

CCL16 is encoded by two mRNAs; one is 1.5 Kb long and is detected in activated monocytes and stabilized (or upregulated) by the interleukin IL-10, while the other is 0.5 Kb long and whose expression is unaffected by IL-10 (12). The effect of IL-10 in enhancing LEC mRNA transcription is unique, as IL-10 generally downregulates the expression of all other cytokines. This suggests that LEC plays an interesting biological role during the early stages of the inflammatory process. In some studies, a virtually ubiquitous transcription of the 0.5 Kb LEC mRNA has been found (13).

Some biological characteristics of LEC have been tested *in vitro* and mirror the properties of other chemokines of the C-C subclass. In particular, the recombinant protein CCL16 has demonstrated a dose-dependent chemoattractive capacity towards non-activated human monocytes and the human monocyte cell line THP-1, with a peak response at a concentration of 1 µg/ml (14). The observed activity is certainly chemotactic and not chemokinetic. Furthermore, CCL16 induces a flux of Ca²⁺ ions in THP-1 monocytes. Ca²⁺ ion flux in response to CCL16 is observed at chemokine concentrations as low as 10⁻⁹ mol/L and is dose-dependent, with a maximal response of 10⁻⁶ mol/L (15). Ca²⁺ ion flux is reduced by prior incubation of THP-1 monocytes with RANTES.

In this study, we demonstrate how subcutaneous injection of cells derived from a murine mammary adenocarcinoma (TSA) engineered to produce LECs leads to the development of a complex inflammatory response that causes hyperplasia of the lymph nodes draining the injection site.

MATERIALS AND METHODS

Tumor Cells

The TSA cell line was derived from an aggressive, poorly immunogenic, moderately differentiated mammary adenocarcinoma that arose spontaneously in female BALB/c mice. Parental TSA-pc cells express major histocompatibility complex class I (MHC-I) molecules, but not MHC class II (MHC-II) molecules, and secrete G- and GM-CSF, TGF- β , basic fibroblast growth factor (β FGF), and VEGF, but not LEC. TSA-pc cells were grown to a cell density of 6×10^4 /100 mM in the culture dish and were transfected with the LEC gene using Lipofectamine reagent (Life Technologies, Rockville, MD). The clone derived from the TSA-LEC transfection, seeded at a concentration of 1×10^4 /ml, was cultured at 60-70 ng of LEC/1 ml for 48 hours.

Mice

7-week-old BALB-cAnCr female mice (Charles River Laboratories, Calco, Italy) were treated by injecting 0.2 ml of a cell suspension containing 1×10^6 TSA-pc or TSA-LEC cells into the left flank.

Morphological analysis

For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin-eosin or Giemsa. For electron microscopic analysis, samples were fixed in glutaraldehyde in 2.5% cacodylate buffer, fixed in osmium tetroxide, and embedded in EPON 812. Ultrathin sections were then stained with uranyl acetate-lead citrate. For immunohistochemical investigation, acetone-fixed cryostat sections were incubated for 30 minutes with antibodies to CD4, CD8-a (Sera Laboratories International Ltd, West Sussex, UK), MAC-1 (anti CD11b/CD18) anti-Mac-3, 1a (Behringer Mannheim, Milan Italy), polymorphonuclear leukocytes (RB6-8C5) anti-endothelial cells (mEc-13,324 or anti-CD31 (provided by Istituto Negri Nord, Italy), dendritic cells (DCs) (NLDC 145, DEC 205, Cederlane Laboratories, Ontario, Canada), anti-TNF, anti-IFN γ (provided by Dr. S Landolfo, University of Turin Italy), anti-MCP-1, anti-CD11b (integrin (Mach-1 alpha chain) and anti-CD61 (beta-3 integrin chain), collagen type 4 (Chemicon, Temecula, California), anti-lamin (Becton Dickinson, Bedford, Massachusetts), anti-VEGF, anti-FGF β and anti-PDGF (Santa Cruz Biotechnology, Inc., Santa Cruz, California), anti-IP-10, anti-Exodus-2/SLC (secondary lymphoid tissue chemokine) and anti-RANTES (Peprotech, Inc., Rocky Hill, NJ), anti-MIG (R&D Systems GmbH, Wiesbaden, Nordenstadt, Germany) and anti-MIP-2.

After washing, the sections were stimulated for 30 minutes with biotinylated goat anti-rat, goat anti-hamster, goat anti-rabbit, or horse anti-goat immunoglobulins. Unbound antibodies were removed by repeated washing, and the sections were then incubated with an avidin/biotin alkaline phosphatase complex. Quantitative studies were then performed in a blinded fashion on three or more samples from different animals, examining ten random fields for each sample. Antibody-positive CD31 microvessels and cells were counted under a microscope (10 fields with a 40x objective, 0.18 mm² per field). The expression of cytokines and adhesion molecules on cryostat sections tested with the corresponding antibody was classified as absent (-), weak (+/-), moderate (+), diffuse (++) and strong (+++).

Chemokine production

To obtain macrophages, the peritoneal cavity of BALB-c mice was washed several times with 5 ml of RPMI 1640 containing 10 U/ml heparin. Differential cell counts, performed on the supernatant treated with Diff Quick, showed that the cell population obtained with repeated washings consisted of approximately 55–65% macrophages. To increase macrophage counts, 1 ml of this suspension, containing 8×10^6 cells, was plated in 24 wells and incubated at 37°C for 2 hours. Non-adherent cells were removed by vigorous washing with RPMI. Adherent cells (macrophages) were incubated in a final volume of 1 ml of RPMI with 10% FBS, in the presence or absence of 1, 10, and 100 ng/ml of recombinant LEC (Peprotech). The plates were incubated for 72 hours at 37°C, and the amount of MCP-1, MIP-2, MIP-1 α , and RANTES released was determined by ELISA using commercial kits (R&D System Inc., Minneapolis, MN). The results shown here are representative of at least four independent experiments.

Antibody screening

7-30 days after the first injection of TSA-LEC, sera were collected from groups of 5 mice. Normal sera were instead collected from 5 untreated animals. TSA-pc cells from *in vitro* cultures were washed twice with cold HBSS with 2% BSA and 0.05% sodium azide and tested against immune serum or normal serum in a 1:10 dilution with HBSS-azide-BSA. Subsequently, the following antibodies were used: FITC-labelled goat anti-mouse immunoglobulin (Technogenetics, Milan, Italy), FITC-labeled rat anti-mouse immunoglobulins of the G1, G2 α Ig, and G3 classes. All steps ended with a

30-minute incubation at 4°C and were separated by 2 washes with cold HBSS-azide BSA. The labelled cells were then analyzed with a FACS analysis cytofluorimeter (Becton Dickinson, Mountain View, CA). In each experiment, 10⁴ cells were analyzed.

RESULTS

Histology and immunohistochemistry

Seven days after inoculation, numerous macrophages, lymphocytes, granulocytes, and DCs were observed in BALB/c mice, both among the TSA-LEC cell aggregates and at the periphery of the resulting mass (Fig.2b, Table III). Giemsa staining and immunohistochemistry with anti-TNF antibodies demonstrated the presence of basophils and mast cells with numerous metachromatic granules in the cytoplasm. This inflammatory infiltrate was associated with the marked expression of adhesion molecules (ICAM-1, ELAM-1, and VUCAM-1) on the endothelium of numerous vessels (Fig.2d,2e). The numerous capillaries formed an extensive network between the TSA-LEC cells (Fig.2b, Table III). The lamina, an essential component of the basement membrane, was arranged linearly and without interruptions around some capillaries, but appeared inhomogeneous and fibrillar around micro vessels originating from sprouting. Staining with antibodies against type 4 collagen showed a distribution pattern very similar to that found for the lamina. These characteristics were very frequent in the growth area of TSA-LEC cells, while they were only occasionally present in the tumor mass consisting of TSA-pc (Fig.2a).

The expression of the proangiogenic factors VEGF and FGFβ and the antiangiogenic chemokines MIG and IP10 was almost superimposable in the growth areas of TSA-pc and TSA-LEC. In the latter, a different expression of PDGF was observed near the areas rich in macrophages and endothelial cells (Table III). In the LEC-secreting cell inoculation area, locally recruited leukocytes showed a massive expression of pro-inflammatory cytokines, primarily TNF and IFN-γ. The chemokine RANTES was sparsely present, while MIP-2 and MCP-1 were highly expressed (Fig.2b, Table III).

Histological and immunohistochemical analysis demonstrated hyperplasia of both areas (cortical and paracortical) and of the lymph node draining the LEC-secreting cell area, while the marginal sinus appeared dilated and filled with mononuclear cells. In the cortical area, several secondary follicles with large germinal centers were observed. Hyperplasia of the lymph node draining the TSA-pc area was much less pronounced and mainly confined to the paracortical area. A particular characteristic was the massive infiltrate of DCs among the T lymphocytes in the paracortical area of the lymph node draining the TSA-LEC inoculation area (Fig.2a,2c). In this lymph node, the expression of Exodus 2/SLC in the high endothelial venules (HEVs) and surrounding cells was much more evident than in the lymph node draining the TSA-pc inoculum. MCP-1 expression was strong and localized mainly in the mantle zone and the luminous zone of the germinal centers of the lymph nodes draining the TSA-LEC cells.

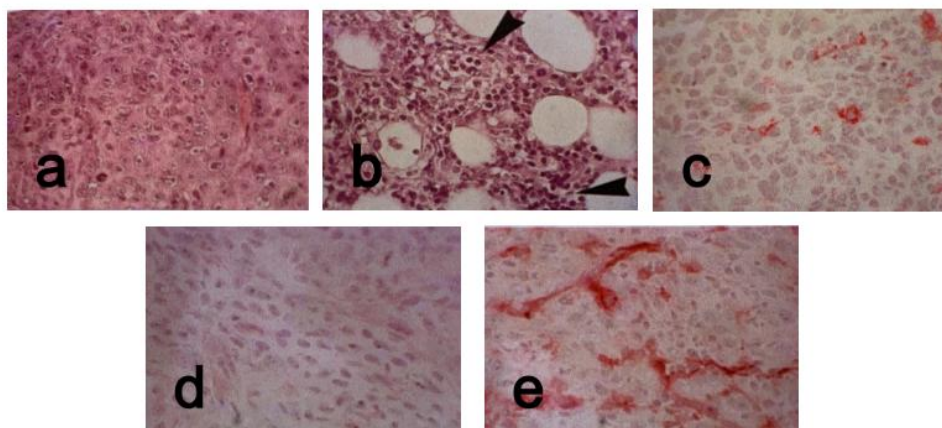


Fig. 2. Figures (2a) and (2c) show dendritic cell (DC) infiltration among lymphocytes in the paracortical area of the lymph node draining the TSA-LEC inoculation site. Exodus 2/SLC expression in the high endothelial venules (HEVs) and surrounding cells is much more pronounced than in the lymph node draining the TSA-pc inoculum. MCP-1 is mainly localized in the mantle zone and the luminous zone of the germinal centers of the lymph nodes draining the TSA-LEC cells. (2b) TSA-LEC cell aggregates containing macrophages, lymphocytes, granulocytes, and DCs. (2d,2e) Inflammatory infiltrate associated with the marked expression of adhesion molecules on the endothelium of numerous vessels.

Table III. Histochemical and immunohistochemical analysis of the TSA-pc and TSA-LEC cell injection area 7 days after challenge.

Immune cells, cytokines and chemokines	TSA-pc			TSA-LEC		
DCs	12.7	+/-	2.4 ^a	28,0	+/-	5,1*
Basophils/mast cells	1.3	+/-	0.5	6,8	+/-	1,6*
Neutrophils	9.0	+/-	3.5	52,2	+/-	6,2*
Macrophages	38.5	+/-	8.2	87,2	+/-	12,0*
CD8+ lymphocytes	5.1	+/-	2,2	70,5	+/-	8,4*
CD4+ lymphocytes	2,0	+/-	1,4	67,7	+/-	8,6*
Microvessel count	25,7	+/-	4,9	29,1	+/-	6,0
BFGF		+ ^b			+	
VEGF		+			+	
PDGF		+			++	
MIP-2		+/-			++	
MCP-1		-			++	
IP-10		+			+	
MIG		+			+	
RANTES		-			+/-	
TNF		+			+++	
IFN γ		-			++	

a. Cell and microvessel counts were performed at $\times 400$ in a 0,180 mm² field on 10 randomly chosen field/sample. Results are means +/- DS of positive cells or vessels/field evaluated on cryostat sections by immunohistochemistry and/or Giemsa-stained sections (basophils/mast cells).

b. The expression of angiogenic factor and chemokines was classed as absent (-), scarcely (+/-), moderately (+), frequently (++), or strongly (+++) present on cryostat sections tested with corresponding Abs. Values significantly different ($p < 0,005$) from corresponding value in TSA-pc.

Ultrastructural examination

The immunohistochemical demonstration of a rich cellular infiltrate in the growth area of TSA-LEC was supported by ultrastructural images showing the presence of DCs and macrophages in proximity to or in direct contact with the numerous lymphocytes present among the cellular aggregates of TSA-LEC. The basophilic and mast cell infiltrate was present in foci. Signs of degranulation were also characteristic. The newly formed capillaries in TSA-LEC consisted of endothelial cells covered with rolling or adherent lymphocytes. Their lumen was sometimes interrupted or obstructed by thrombi composed of platelets and leukocyte aggregates. Fragmentation of the lamina was frequent and associated with neoangiogenesis, recognizable by the presence of numerous buds.

Chemokine production by recombinant LEC-stimulated macrophages in vitro

Numerous reactive cells, including macrophages, showed expression of various chemokines. Confirmation of these immunohistochemical findings was obtained by assessing the presence of RANTES, MCP-1, MIP-1 α , and MIP-2 in the supernatant of peritoneal macrophages after 72-hour culture with or without recombinant (rLEC) supplementation at 1, 10, and 100 ng/ml. The secretion of RANTES, MCP-1, and MCP-2 increased 6.35-fold and 1.2- to 1.7-fold, respectively, in the presence of rLEC, while the secretion of MIP-1 α was unchanged.

Anti-TSA-pc antibody production

The presence of specific anti-TSA-pc antibodies was assessed by flow cytometry in the sera of normal mice and mice inoculated with TSA-LEC. Anti-TSA-pc antibodies were present as early as 7 days after inoculation of TSA-LEC cells. At 30 days post-inoculation, the antibody level was even higher (98% positive cells). These antibodies belonged to the IgG1 and IgG2 immunoglobulin subclasses.

DISCUSSION

Using an experimental model in which engineered tumor cells act as micropumps that ensure the massive presence of a given factor in their growth microenvironment, it is possible to study the activity of cytokines and chemokines *in vivo* (16). The activity of the LEC gene has not yet been well defined (14). The use of TSA cells engineered with the LEC gene is able to release high amounts of this chemokine and has led to the conclusion that LEC has a strong pro-inflammatory activity that does not appear to be directly related to its chemotactic activity as demonstrated *in vitro* on DCs and the myeloid cell line THP-1 (17). The chemotactic activity demonstrated *in vitro* occurred only at high concentrations of 1 µg per ml and cannot be considered representative of *in vivo* activity.

Our studies suggest that the recruitment of such diverse leukocyte subpopulations into the cellular microenvironment of TSA-LECs results from the cascade of other factors, all induced by LECs. However, the recruitment of monocyte-macrophages appears to play a major role. Human monocyte-macrophages express CCR1 and CCR8, identified as two receptors for LECs (17). Similar receptors also appear to be expressed by murine monocyte-macrophages, as they respond to LECs by producing RANTES, MCP-1, and MCP-2, as demonstrated *in vitro* and *in vivo* (18).

MCP-1 is the major chemotactic activating factor for monocytes-macrophages, mast cells, and basophils (19). It promotes the recruitment of T lymphocytes and DCs (20). Indeed, the latter cell populations are more representative in the response after TSA-LEC inoculation. Ultrastructural data have also demonstrated that basophils actively participate in the inflammatory process induced by TSA-LEC by releasing their granules (21). MIP-2, also known as GRO/KC, is the murine functional analogue of IL-8 and is chemotactic for neutrophils and T lymphocytes (22). Chemokines produced by macrophages can recruit T lymphocytes and DCs to the site of TSA-LEC injection. Macrophages and granulocytes are also attracted to the site of TSA-LEC injection into nu/nu nude mice.

The chemotaxis exerted by LEC secretion is mediated by LEC-induced production of MCP-1 and MIP-2 on macrophages themselves, while it does not appear to depend on the expression of other pro-inflammatory cytokines or endothelial adhesion molecules (23). Chemokines produced by macrophages can recruit T lymphocytes and DCs to the TSA-LEC injection site. Macrophages and granulocytes are also attracted to the site by TSA-LEC cells injected into nu/nu nude mice. The chemotaxis exerted by LEC secretion is mediated by LEC-induced production of MCP-1 and MIP-2 on macrophages themselves, while it does not appear to depend on the expression of other pro-inflammatory cytokines or endothelial adhesion molecules. Both of these, detected in the TSA-LEC growth site in normal mice, are induced by T lymphocytes, which play a primary role in the cascade of LEC-induced inflammatory events (24).

LEC-activated inflammation is associated with angiogenesis, as demonstrated by vascular sprouting and increased micro vessel numbers in the TSA-LEC-induced microenvironment (25). Immunohistochemical data indicate that the angiogenic mediators TNF, PDGF, and MIP-2 are expressed to a greater extent than IP10 and MIG. Comparison with the TSA-pc model could lead to an underestimation of the angiogenic potential of TSA-LEC cells, since TSA-pc cells produce VEGF and FGFβ (26). The LEC-induced reaction is accompanied by hyperplasia of the lymph node draining the inoculation area, which shows a marked expression of Exodus 2/SLC in the paracortical area and in the numerous high endothelial venules, whose volume is densely populated by lymphoid cells.

Physiologically, Exodus 2/SLC is involved in the migration of DCs from the skin to the draining lymph nodes. It may therefore be active on DCs at an intermediate stage of maturation, immediately after antigen uptake on their way to secondary lymphoid organs. Exodus-2/SLC is also chemotactic for T and B cells, and for natural killer (NK) cells (27). Numerous secondary follicles characterized by extensive cortical germinal centers represent the morphological background of the significant humoral response (28). MCP-1 expression in the mantle zone and germinal centers indicates an ongoing B cell response directed at TH2 lymphocytes (29). However, the presence of IgG2a and IgG2b antibodies in serum suggests that the reaction triggered by LEC is not only TH2-type, but also TH1-type (30). However, the fact that the antibody response was so immediate and intense after TSA-LEC inoculation highlights that the interrelationships between antigen-presenting cells (in particular DCs) and lymphocytes are particularly facilitated. This is particularly true in the inoculation area, where macrophages are directly or indirectly attracted and where DCs and T lymphocytes are induced into a close interaction regulated by LECs, through a stabilization of the bonds between the CCR1 and CCR8 receptors (31).

The cytokine cascade found in untreated animals, but absent in nu-nu mice, indicates that these interactions result in intense CCR1-mediated T cell activation (32). The increased adhesion of antigen-presenting cells and T cells does not prevent their drainage towards the local lymph node, where the intense traffic found at the level of the marginal sinus and high endothelial venules, and the hyperplasia of the cortical and paracortical areas, strengthen the influx of immune cells capable of triggering an efficient immune response through the production of specific antibodies.

Tumor cells transfected with a β-chemokine gene (such as CCL16) undergo changes in the microenvironment that can affect their survival. β-chemokines can modulate T-cell-mediated immunity against pathogenic microorganisms,

particularly viruses. When a tumor cell expresses CCL16, it attracts immune cells into the tumor, which recognize and destroy the tumor cell. This can create a vulnerable environment for pathogens to more easily express their infectious power. Therefore, tumor cells transfected with β -chemokine genes become more visible and susceptible to attack by the immune system, which can indirectly influence infections and antiviral responses.

CONCLUSIONS

In this work, it is important to emphasize that our results demonstrate that LEC is a chemokine with high pro-inflammatory activity and LEC production has been reported to be upregulated by IL-10, which is an immune inhibitor. These findings suggest that alterations in LEC production may be a key feature of some human inflammatory disorders characterized by the presence of elevated IL-10 levels, such as inflammatory bowel disease, microbial infections, and rheumatoid arthritis.

Conflict of interest

The authors declare that they have no conflict of interest.

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BUTYRATE IS A SHORT-CHAIN FATTY ACID THAT IS BIOLOGICALLY ACTIVE IN THE GUT MICROBIOME AND IN ULCERATIVE COLITIS

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ABSTRACT

Short-chain fatty acids (SCFAs) have approximately 1-6 carbon atoms, and this category includes butyrate, a molecule that provides energy to various tissues, including the intestinal lining. Butyrate plays an important role in human health by regulating the immune system, reducing inflammation, and improving cell binding to insulin, and additionally, in ulcerative colitis. The intestinal microbiota produces a significant amount of butyrate from specific bacteria, which plays a beneficial role in the colon. In the intestine, butyrate produces energy, strengthens the intestinal barrier, and has anti-inflammatory and antitumor effects by inducing apoptosis. Dysbiosis reduces the amount of butyrate in the intestine, leading to increased tissue permeability, vulnerability to infections, and neurological disorders. Butyrate is metabolized in the mitochondria and is an important source of energy. It stimulates the expression of tight junction proteins that are important for the occlusion and narrowing of the intercellular spaces of the endothelium. Butyrate inhibits pro-inflammatory cytokines through the inhibition of NF- κ B. An alteration of the intestinal microbiota is a risk factor for potentially lethal ulcerative colitis. Treatment with anti-inflammatory drugs and anti-inflammatory cytokines is useful.

KEYWORDS: *Butyrate, short-chain fatty acid, SCFA, ulcerative colitis, microbiome*

INTRODUCTION

Bacteria produce short-chain fatty acids (SCFAs) that support various pathophysiological processes (1). Most fatty acids consist of 16-18 carbons, but SCFAs have 1-6 carbons. Among these molecules is butyrate, which provides energy to various tissues, including the epithelial cells lining the intestine (2). Butyrate reduces inflammation and improves the cells' ability to capture insulin and plays an important role in human health and in some intestinal diseases, such as ulcerative colitis (3).

The human body produces a good amount of butyrate through its microbes (4). Butyrate is produced mostly by the gut microbiota and is derived from the bacterial flora of the colon, where it plays beneficial roles for both the colon and systemic health (5). Butyrate is produced by specific bacteria, such as *Aceratherium prausnitzii*, *Enterobacterium rectal*, and *Roseburia spp* (6,7). These bacteria ferment soluble fiber and resistant starch to produce SCFAs, including acetate, propionate, and butyrate (8).

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DISCUSSION

Butyrate plays a key role in maintaining colon health (9). At the intestinal level, butyrate is a primary energy source for colonocytes, strengthens the intestinal barrier, and reduces colon inflammation (9). Butyrate also has an anti-tumor effect by inducing apoptosis in tumor cells (10). Systemic effects include its action on metabolism, affecting insulin production, and its interaction with the gut-brain axis (11).

In dysbiosis, the microbiota is altered, resulting in a reduction of butyrate-producing bacteria (12) (Fig.1). Butyrate deficiency causes increased intestinal permeability, increased susceptibility to inflammation, and increased neurological and metabolic disorders (13). A diet rich in soluble fiber, starches, or probiotics can promote the production of butyrate (14).

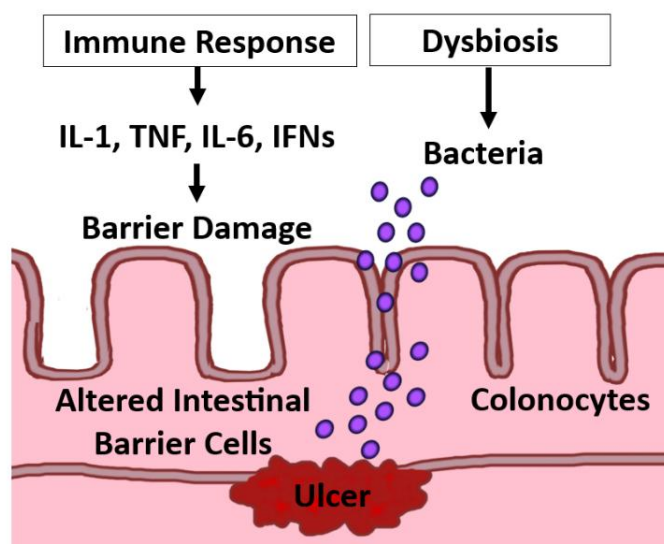


Fig. 1. Pro-inflammatory cytokines such as IL-1, TNF, IL-6, and IFNs are produced during the immune response, increasing intestinal permeability and disrupting the tight junctions between epithelial cells. In dysbiosis, some pathogenic or opportunistic bacteria produce pro-inflammatory metabolites and further activate the immune system. This leads to increased inflammation and damage to colonocytes.

Butyrate is the most important source of energy for colonic epithelial cells (15). It is metabolized in the mitochondria to produce ATP (16). Butyrate stimulates the expression of tight junction proteins such as *claudin*, *occludin*, and *zoulin* (17), which is important for the occluding and narrow tight junctions that form a barrier regulating the flow of liquid substances in the spaces between the cells of epithelial and endothelial tissues, preventing the leakage of molecules while maintaining cell polarity (18). *Zoulin* induces the synthesis of protective mucin by goblet cells (19).

Butyrate inhibits NF- κ B, a crucial transcription factor for the production of inflammatory cytokines such as TNF, IL-6, and IL-1 β (20). These reactions are important for controlling intestinal inflammation that occurs in certain diseases such as Crohn's disease and ulcerative colitis (21) (Fig.2).

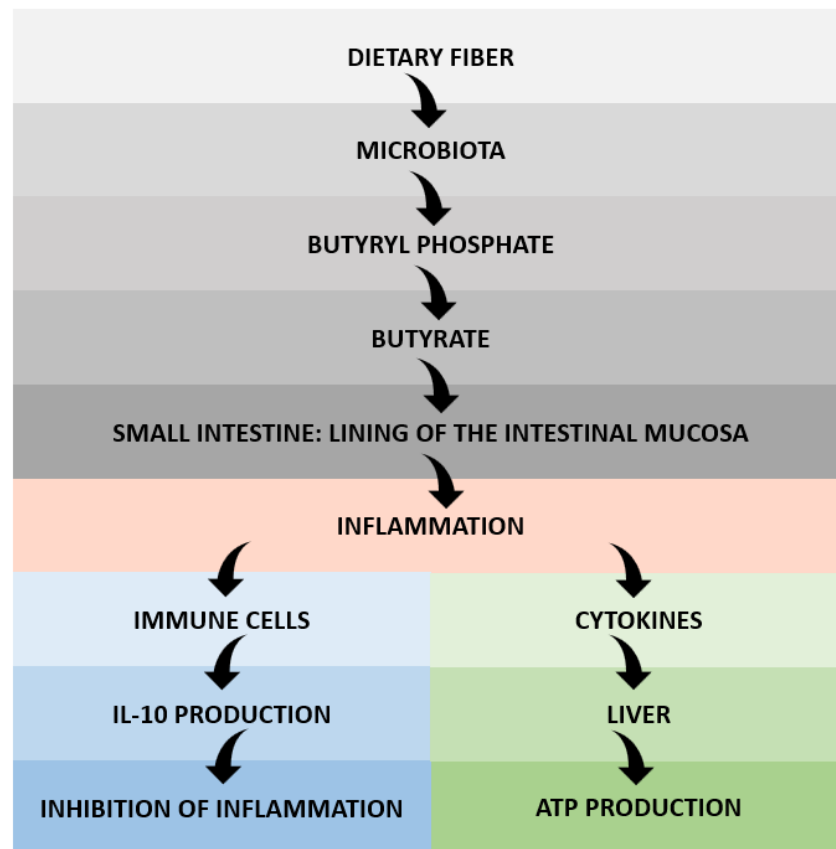


Fig. 2. Dietary fiber is used by the gut microbiota, which produces metabolites with anti-inflammatory effects such as butyrate. Butyrate helps maintain the intestinal barrier and produce the anti-inflammatory cytokine IL-10. The liver produces ATP in response to immune activation and the production of pro-inflammatory cytokines.

Ulcerative colitis is a chronic inflammatory bowel disease that primarily affects the colon and rectum (22). In this disease, inflammation affects the intestinal mucosa, often resulting in bleeding ulcers (23). It differs from Crohn's disease, which can affect any part of the gastrointestinal tract and even the deeper layers of the intestinal wall (24).

Ulcerative colitis affects the colon and rectum, where it begins and spreads steadily upward (25). It is characterized by inflammation affecting only the superficial layer of the mucosa (26). Symptoms include chronic abdominal diarrhea, fecal urgency, weight loss, and, in severe cases, fever (27). The cause of ulcerative colitis is unknown, although it is often attributed to a dysfunction of the immune system, which can overwork the intestinal flora (28). Genetic and environmental factors, such as diet, pollution, and excessive use of antibiotics, are also implicated (29). Furthermore, alterations in the intestinal microbiota may also be involved (30).

People with ulcerative colitis may experience colon cancer, erythematous arthritis, uveitis, liver dysfunction, anaemia, and potentially life-threatening colon dilation (31). Treatment is performed with local nonsteroidal anti-inflammatory drugs, corticosteroids, immunosuppressants, biologics (anti-TNF, anti-IL1 receptor antagonist), and probiotics (30). Additionally, the anti-inflammatory cytokines IL-37 and IL-38 are under consideration for treating ulcerative colitis and other chronic inflammatory diseases. In severe cases, surgical removal of the colon may be necessary (32). A high-fiber diet can increase the production of butyrate, which is important in ulcerative colitis (33). Butyrate can also be administered topically in enemas for therapeutic effects (34).

CONCLUSIONS

Butyrate is a SCFA that provides energy to various tissues, including the intestinal mucosa. Butyrate improves cellular binding to insulin and reduces inflammation. The gut microbiota generates butyrate from bacteria, supporting the physiological functioning of the colon. Butyrate is metabolized by mitochondria and reduced by dysbiosis, resulting in vulnerability to infections and neurological disorders. Butyrate inhibits inflammatory cytokines through the NF- κ B

pathway and can be used in combination with other anti-inflammatory drugs to alleviate chronic inflammatory diseases, such as ulcerative colitis.

Conflict of interest

The authors declare that they have no conflict of interest.

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