LSC301 Clinical Biochemistry and Cellular Pathology. Unit 2.

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Part 1. Molecular mechanisms of apoptosis, toxin-induced testicular cell death as a model.

Apoptotic signaling pathways consist of the endoplasmic reticulum pathway, mitochondrial pathway, and death receptor pathway (Xu et al. 2016). As an example, the mitochondrial pathway of death plays a role in the use of the Bcl-2 family of proteins by germ cells (Tripathi et al., 2009). In addition, apoptosis can be triggered by FOXO factors due to the transcription of FasL and the activation of proapoptotic proteins. Fas is one of the known death receptors as are the following four receptors: TNF-R1, DR3, DR4, and DR5. Fas/FasL molecules are involved in the extrinsic pathway of apoptosis used by germ cell signaling (Brown, 2022). In addition, environmental toxins harm the male reproductive function. Therefore, the lack of balance between apoptosis and cell survival induced by environmental factors or disease influences spermatogenesis and can cause azoospermia, oligospermia, or hematospermia (Almeida et al., 2013).

Apoptosis has different functions depending on the developmental phase of the testes. An apoptotic wave is an uncommon event during spermatogenesis, but if this event were to occur, it would be mainly modulated by the Fas-Fas system (Xu et al., 2016). It is crucial to mediate the proportion of germ cells to Sertoli cells, to guarantee male fertility and spermatogenesis. Germ cells express the TNF type I transmembrane receptor protein Fas on their surfaces. The process of apoptosis is then initiated when the FasL binds to the Fas receptor, and consequently, the reaction between the intracellular death domain of Fas and FasR is induced in Sertoli cells (Yao et al., 2009). Apoptosis in the testis is regulated by pathways found in Leydig cells, germ cells, and Sertoli cells. As an example, glucocorticoid induces Leydig cell apoptosis by the activation of the Fas/FasL pathway (Wang & Su, 2018).

Fas ligand pathway

The death receptor pathway consists of several extracellular factors such as apoptotic signals that stimulate apoptotic agents (Ashkenazi and Salvesen, 2014). External triggers induce the production of TNF (tumor necrosis factor). This is a cytokine that promotes apoptosis and

cytotoxicity in different cell types. It is usually involved in the immunoinflammatory response and death by cell signaling (Brown, 2022).

Fas Ligand is part of the TNF group of ligands. It is expressed in the cell membrane as a trimer, whereas Fas receptor (FasR) exists as a monomer (Wang & Su, 2018). Fas protein can be found in several organ tissues, particularly B lymphocytes, peripheral T, mononuclear cells, NK cells, endothelial cells, fibroblasts, and endothelial cells. However, FasL activation is only expressed in phagocytic cells that are part of the immune system, NK cells, activated T cells, and testicular Sertoli cells (Chai et al. 2008). Fas/FasL signaling plays a fundamental role in the death signaling receptor pathway as it is one of the main regulative pathways of apoptosis. For this reason, they have a big impact on the stabilization of apoptosis and proliferation (Lavrik 2014). As an example, T-cell activation induces Fas ligand expression. They are tolerant to Fas-mediated apoptosis at first, but their sensitivity increases the longer T cells are active. In this way, an immoderate immune response is prevented (Brown, 2022).

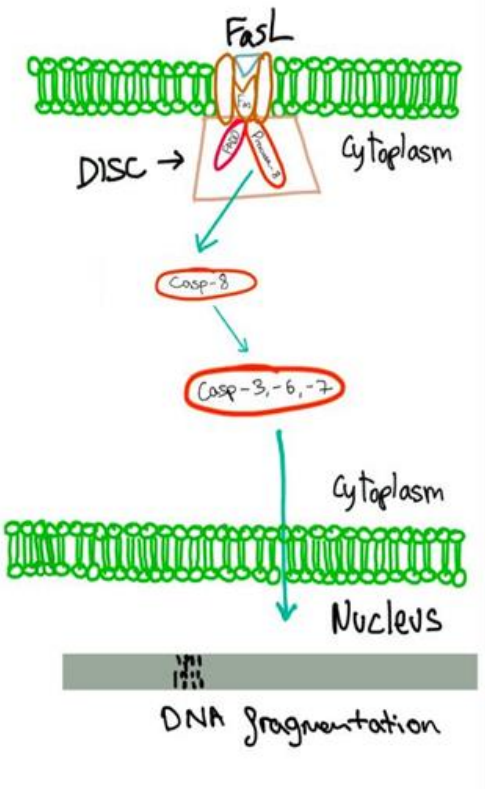

Figure 1: A diagram describing the FasL/Fas pathway.

The Fas/FasL binding occurs when Fas-positive cells contact FasL-positive cells. This induces trimerization of FasR and binds to the death effector domain of the Fas-associated death domain (FADD), the cytoplasmic adapter protein (Wang & Su, 2018). This stimulates the signal to

procaspase 8. This binding forms the death-inducing signalling complex (DISC). Consequently, apoptosis is induced by the trigger of a sequential signaling caspase cascade. All caspases must have an activation process as they are synthesized in cells in the form of inactive zymogens (Brown, 2022). After caspase 8 has been activated, it then triggers the effectors' caspase 3, 6, or 7. This causes the display of many proteins which ultimately leads to apoptosis by DNA fragmentation and damage. Fas/FasL signaling plays a key role in the death signaling receptor pathway as it is one of the main regulative pathways of apoptosis. For this reason, they have a significant impact on the stabilization of apoptosis and proliferation (Lavrik 2014).

Toxins affecting apoptosis in testicular cells

Cells damaged due to environmental or physiological triggers are removed through the phagocytosis process. Reactive oxygen species (ROS) via several cellular pathways such as NADPH oxidases (NOX), xanthine oxidases (XO), cyclooxygenases (COX), lipoxygenases (LOX) and the metabolism of arachidonic acid (AA) can induce apoptosis through different mechanisms. Other factors can induce an increasing of ROS like environmental toxins, inflammatory cytokines, UV and ionizing radiation, quinone compounds, different pharmaceutical agents and chemicals found in tobacco smoke. The p53 apoptosis pathway consists of the nuclear transcription factor p53 that acts as a regulator of apoptotic genes and can be activated by ROS. This pathway can lead to DNA damage and the stimulation of both intrinsic and extracellular apoptotic pathways (Brown, 2022). P53 and FasL can degrade c-FLIP through the signaling pathway ubiquitin-proteasome. Research showed that a higher expression of FasL/Fas stimulates apoptosis of germ cells affecting the seminiferous tubule stages VII-VIII and IX-XII when the cells are adversely affected by environmental toxic injuries (Wang & Su, 2018). In the same way, the absence of the FasL gene results in the lack of germ cells in the testicular seminiferous tubules and only Sertoli cells are present. In addition, a TNF superfamily receptor called TRAIL was constitutively expressed. This demonstrates the importance of FasL in the regulation of germ cells by moderating the levels of the death receptor pathway inhibitor (Wang & Su, 2018).

Exposure to toxins from the embryonic stage raises the risk of anomalous spermatogenesis and can lead to germ cell apoptosis by activating Fas/FasL during adolescence if toxin exposure continues (Traore et al., 2016). Several toxicants-induced germ cell death examples can be used to analyze the behavior of anti- and pro-apoptotic molecules. Different toxins that affect apoptotic pathways will be discussed in the following paragraphs.

Ethane-1,2-dimethanesulfonate (EDS) is a cytotoxic agent that targets Leydig cells and increases testicular Fas and consequently, also increases germ cell apoptosis (Woolveridge et al., 2001). Similarly, an elevated dose of bisphenol A can lead to the activation of the Fas pathway causing

apoptosis of Leydig and germ cells (Tripathi et al., 2009). Bisphenol A is an endocrine disruptor and can be found in the manufacture of plastics. It has been shown to have some repercussions on testicular cells such as DNA and mitochondrial damage, disruption of intercommunication between cells, disruption of tight junctions, and other effects that put at risk male reproductive health (Adegoke et al., 2020). Nitrobenzene (NB) is a testicular toxin that is active even when no Fas is present. Accordingly, studies have shown a higher apoptotic rate in mice exposed to this toxin (Richburg and Nanez, 2003). Similarly, Lindane is a pesticide that has a negative impact on fertility and testicular functions due to its capacity to influence the FasL pathway and the nuclear factor Kappa B (Saradha et al., 2009). NFkB is a critical transcription factor involved in the apoptotic process and can act as both an anti- and pro-apoptotic factor. This family of proteins regulates a significant amount of other cellular processes such as inflammatory response and cellular growth. They are found in the nucleus of several cells like Sertoli cells or B cells (Gilmore, no date).

Hormones can also regulate germ cell survival. After a testosterone injection, the expression of FasL/Fas and the apoptotic signal has been known to increase. On the other hand, a lack of testosterone induces caspase activity and consequently, DNA fragmentation of Sertoli cells occurs (Tripathi et al., 2009). Similarly, estrogen also has an impact on cell death in the testis and spermatogenesis. Germ cells have estrogen receptor-β and -α, and it has been shown that the ones without receptor-α are linked with infertility (Chen et al., 2008). Exposure to endocrine disruptors can affect the estrogenic activity and can cause low fertility, although its impact level remains uncertain (Storgaard et al., 2006). 17β-estradiol exposure stimulates the binding between the Fas ligand and Fas receptor and, as previously mentioned, the caspases cascade is then activated, continuing the apoptotic pathway (Tripathi et al., 2009).

BCL-2 family proteins

2,5-hexanedione exposure stimulates the intrinsic apoptotic pathway by inducing Ca+ to regulate Bcl-xL and Bcl-xS expression. This molecule is a metabolite of the solvent n-hexane, commonly used in industry (Mishra & Shaha, 2005). Overexpression of Bclx and Bcl2 proteins in the testicular germ cells are associated with abnormal spermatogenesis leading to infertility. The first wave of spermatogenesis is influenced by an apoptotic wave to regulate the normal development of spermatogenesis. This is due to the maintenance of a proper balance between survival-promoting proteins and cell death. For this reason, a high level of BAK proteins is expressed in a germinal cell during the first wave (Rodriguez et al., 1997).

There are anti-apoptotic and pro-apoptotic in the BCL-2 family of proteins. Anti-apoptotic proteins have the BH1-4 domain which allows interaction with pro-apoptotic domain 3 BH.

Prosurvival signals regulate the anti-apoptotic expression and can stimulate the inhibition of pro-apoptotic signals. PKB signalling induces BCL2 expression. BAK and BAK proteins are located in the outer membrane of the mitochondria, and they bind to anti-apoptotic BCL2 proteins. If these complexes are dimerised, the outer membrane of the mitochondria starts the process of permeabilization. Consequently, mitochondrial integrity is lost, causing a leak of caspases activator, proteases, and cytochrome C. The calcium leak activates calpains, cathepsins, and other cysteine proteases. Finally, DNA is damaged, the p53 regulates the production of the p53-Upregulated Modulator of Apoptosis (PUMA) and as a result, the damaged mitochondria induce apoptosis (Brown, 2022).

Part 2. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic condition that induces skin rashes, joint pain, and tiredness. It is an autoimmune disease, and consequently, the immune system attacks wrongly healthy body areas. The origin of lupus is not completely understood; however, it can be caused by viral infection, genetic predisposition, childbirth, puberty, menopause, sunlight, and some medicines. In fact, it is more popular between women, Asian and black, than men (NHS, 2020).

There is no existing cure, but symptoms may be improved if prompt treatment is undertaken. It is challenging to diagnose due to its similarity between the symptoms of other conditions and the symptoms shown in this condition. Significant level of antinuclear antibodies in a blood test and the display of common symptoms, means it is likely lupus. These depend on the level of severity of the disease, which can go from mild to severe. Mild lupus targets skin and joint problems and tiredness; moderate severity includes inflammation of several body parts such as skin, joints, liver, heart, lungs, and kidneys; and finally, severe lupus is express through significant inflammation which causes damage to the heart, brain, lungs and can be fatal. The symptoms of these conditions can flare up after treatment and can become worse for some time before they remit. The cause of this fluctuation is unknown. However, some patients experience constant symptoms (NHS, 2020).

The loss of immune tolerance to self-antigen occurs in the clinical onset of SLE. This is due to the interaction of proinflammatory stimuli such as different cytokines and type 1 interferons, other hormonal and immunological factors, environmental precipitants, and genetic predisposition (Fava & Petri, 2019). Numerous genes are associated with a tendency of developing lupus, usually encoding for immunological components such as IRF5, STAT4, CTLA4, BLK, HLA. SLE shows a significant heritability, a genetic susceptibility is indicated by the 11-50% concordance between monozygotic twins and the higher prevalence in families. This suggests that genetics play a significant role in the development of this condition (Generali et al., 2017). Autoimmunity acts by inducing a complicated interplay of apoptotic waste clearance and immune processes besides neutrophil action and interferon pathways among others (Tsokos et al., 2016).

It is known that disruption of both the adaptive and innate immune system is induced by lupus. T cells are compromised, failing to make enough IL-2, and producing an excess of double-negative T lymphocytes. This provides immoderate help to B cells (Katsuyama et al., 2018). Consequently, an excess of B cells reaction induces B cell lymphopenia, which is the outcome of cytokine-mediated apoptosis of CD4 and CD8 T cells, B cells, and dendritic cells observed in sepsis. The

innate immune system plays a key role in recognizing a strong type 1 interferon to identify lupus (Fava & Petri, 2019).

The main targets of the treatments used to deal with lupus are the prevention of organ damage; reduction of the development of several conditions that lupus induces such as accelerated atherosclerosis; the diminution of pain and fatigue; and activity regulation to maintain the lowest degree possible involving immunosuppression by immunomodulators. These can be corticosteroids, biologic, small molecules and cytotoxic-immunosuppressants among others. The drugs that target SLE act as regulators of the immune system without raising the risk of malignancy or infection (Fava & Petri, 2019). Immunomodulators currently in use include hydroxychloroquine, vitamin D, prasterone (dehydroepiandrosterone synthetized), and belimumab. These kinds of therapies are crucial to modify the immune responses linked with lupus without obvious immunosuppression by acting via many cytokines and cellular pathways (Durcan & Petri, 2016).

Main drugs in SLE

Hydroxychloroquine	Inhibition of B and T cell signalling, antigen presentation and NOX signalling. A reduction in the production of pro-inflammatory cytokines occurs by the inhibition of TLR signalling. It also promotes lysosomal activity increasing pH levels and interferes with MHC-antigen binding (Fava & Petri, 2019).
Vitamin D	Immunomodulatory effects due to vitamin D receptor found in several immune cells such as dendritic cells, activated T cells, and monocytes as well as in vasculature, skin, and other tissues (Fava & Petri, 2019). It also has anti-proliferative effect due to the promotion of Th1 (IL-2, TNF- α, IFN- γ) to Th2 polarization as well as Th17 to Treg state. Moreover, it affects the function and development of NKT cells (Fava & Petri, 2019). Furthermore, Vitamin D is an anti-fibrotic molecule. It prevents pro-fibrotic pathways arbitrated by Ras and TGF- β (Fava & Petri, 2019).

Dehydroeipandrosterone	Androgenic steroid that acts on several immunologic pathways and cytokines, therefore it regulates immune function due to its anti-inflammatory effects. Some of these regulator effects have an impact on the modulation of the production of pro-inflammatory cytokine like IL-1, IL-2, TNFa, and IL-6. It has been shown the decrease in IL-10 levels, directly linked with SLE, as it is a B cell stimulatory cytokine (Chang, 2004).
Belimumab	Monoclonal antibody that inhibits the soluble B cells stimulator cytokine. It binds to soluble B cells and prevents the binding with the receptor. For this reason, the binding of soluble B cells to belimumab leads to a decrease in the antiapoptotic proteins production and autoantibody and immunoglobin formation (Dennis, 2011).
Prednisone	Corticosteroid, specifically, a glucocorticoid which reduces inflammation by suppressing the migration of leukocytes and rising capillary permeability. It can also activate several nuclear receptors, and consequently, a prevention of proinflammatory cytokine production (Bergmann et al., 2012).
Cyclophosphamide	An extremely poisonous alkylating agent that diminishes B and T cells and overturns the production of antibodies (Fava & Petri, 2019).
Triamcinolone	Corticosteroid, specifically, a glucocorticoid which shows immunosuppressant and anti-inflammatory activity by preventing the phospholipase A2 enzyme on the phospholipid layer of the cell membrane. This results in membrane failure of leukocyte lysosomes and consequently it inhibits the biosynthesis of leukotrienes and prostaglandins (Hannah et al., 2016). Moreover, triamcinolone prevents leukocyte and macrophage migration to the target area by reversing permeability and vascular dilation. It also shows its anti-inflammatory

	effects via the inhibition of NF-kappa-B and the reduction of IL-8 and IL-6 (Hannah et al., 2016).
Azathioprine	DNA and RNA synthesis inhibitor. In addition, it has a tolerogenic effect due to the inhibition of CD28 mediated signal 2 found in T cells (Fava & Petri, 2019).
Methotrexate	DNA synthesis replication and repair via binding irreversibly to dihydrofolate reductase, reducing the synthesis of purine consequently. Moreover, low doses of methotrexate increase anti-inflammatory adenosine signaling; lower the adhesion of molecules on synovial and endothelial cells; decrease the circulation of pro-inflammatory T-cells; and induce activated lymphocytes apoptosis (Fava & Petri, 2019).
Mycophenolate	This drug gets rid of guanoside nucleotides in B and T cells and inhibits proliferation. It stops monocyte and lymphocyte recruiting to inflamed tissue (Fava & Petri, 2019).
Tacrolimus	T-cell calcineurin inhibitor by forming a complex with protein 12 binding immunophilin FK506. This then prevents the phosphatase activity of calcineurin, and as a result resulting IL-2 transcription is reduced and T cells activated. It also provokes a reduction in the production of IL-4, IL-2, IL-5, TNF-α, and IFN-γ (Hannah et al., 2016).
Rituximab	An anti-CD20 monoclonal antibody that promotes peripheral B cell reduction (Fava & Petri, 2019).
Anifrolumab	Monoclonal antibody that blocks type 1 IFN-α/β/ω receptor, which is the mediator of type 1 interferon signalling. Moreover, this drug was linked with improved C3 and reduced anti-dsDNA titers levels (Fava & Petri, 2019).
Ustekinumab	Monoclonal antibody that blocks IL-23 and IL-12. These are shown to be higher in SLE and consequently, the overstimulation of B cells is induced. Comparably to anifrolumab, ustekinumab enhanced C3 and lowered anti-dsDNA levels (Fava & Petri, 2019).

Baricitinib	Baricitinib is an inhibitor of JAK2 and JAK 1. The Janus kinases are part of the tyrosine kinases family and these mediate intracellular marking of different cytokines involving the JAK-STAT pathway. JAK1 and JAK 2 arbitrate signalling for type 1 interferons, IL-12, IL-6, IFN-γ and IL-23 amongst others (Fava & Petri, 2019).
Atacicept	Protein that prevents the action of B cells via the inhibition of both BLyS and APRIL (Fava & Petri, 2019).

Table 1: A table showing the main drugs used in the treatment of SLE.

Treatment

It is certain that SLE is an autoimmunity disease induced by an immune system disfunction. This can cause immune cell overstimulation, the discharge of proinflammatory cytokines and finally the manifestation of disease. B cells play the main role in clinical manifestations of SLE because they are the mediators of tissue inflammation in lupus. For this reason, most clinical trials and pharmaceutical research have been focused on preventing B cell activity (Dennis, 2011).

A recently marketed drug called belimumab was approved as a treatment of patients with SLE. Belimumab is a biologic molecule that was specifically designed for lupus. It is a monoclonal antibody that binds to soluble B-lymphocyte stimulator (Blys) cytokine, so B cells are not activated but they are led to apoptosis (Figure 1) (Dennis, 2011).

On the other hand, hydroxychloroquine is an antimalarial drug discovered in 1630 but used to treat several rheumatic diseases like SLE since 1940. It negatively mediates different aspects of the adaptive and innate responses such as the prevention of B cell activation with the goal of the reduction of inflammation to help minimize autoimmune diseases (Schrezenmeier & Dörner, 2020).

Mechanism of action

As an overview, the first step of the immunopathogenesis of SLE is the autoantibodies released by autoreactive B cells forming complexes with autoantigens that are circulating around. The complements then bind to these complexes and go to the specific tissues to induce inflammation (Dennis, 2011).

Belimumab is involved in the interaction of BLyS and B cells. B-lymphocyte stimulator protein (BLyS) is part of the TNF family of cytokines, and it is a crucial factor to the survival of B cells. BLyS is expressed and quickly divided by myeloid cells and other immune cells. It is attached to

receptors on the cell surfaces of autoreactive and normal B-lymphocytes, sending them signals to them to keep them living, maturing, and differentiating into autoantibody and antibody-producing cells. The main role of belimumab is to bind to soluble BLyS to stop it from maintain the communication through receptors on autoreactive and normal B lymphocytes. A decrease in the signaling by BLyS induces an increase in the level of cells that go through apoptosis. This affects inflammation as less B cells will survive, so less cells will be able to produce antibodies that binds to antigens so complement bind into those complexes to induce inflammation (Dennis, 2011).

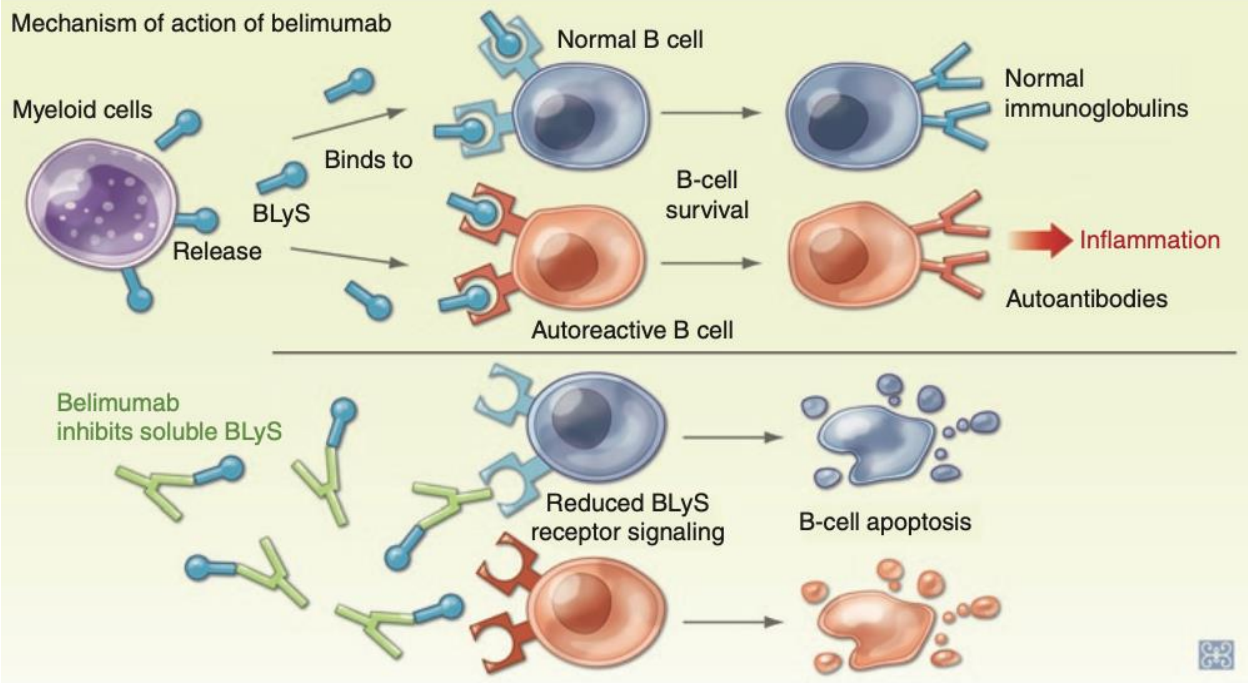

Figure 1: A picture explaining the mechanism of action of belimumab (Dennis, 2011, p.144, figure 1).

On the other hand, hydroxychloroquine influence lysosomal activity, signaling pathways and autophagy. It inhibits TLR signaling by increasing endosomal pH and consequently, it reduces the release of proinflammatory cytokines made by mononuclear cells in the blood such as TNF α, IL-6, and IL-2. Pro-inflammatory cytokines play a role in RA pathogenesis by maintaining chronic inflammatory synovitis, stimulating autoimmunity, and damaging joint tissues. Cytokines like TNF α influence the promotion of B- cell survival and antibody formation, and therefore the process of inflammation is affected. Hydroxychloroquine can prevent T and B cell activation because it also impairs the ability to release of Ca^{+} from the endoplasmic reticulum, inhibiting T cell activation and affecting other Ca^{2+} signaling pathways. The lack of Ca^{2+} also prevents the NFAT production which controls the expression of the gene CD154. The activation of this gene is necessary in B cell activation (Schrezenmeier & Dörner, 2020). Finally, hydroxychloroquine also

influences NOX inhibition which affects pro-inflammatory cytokines and nitric oxide availability, causing a reduction in ROS levels (Nirk et al., 2020).

Health impact

As an immunomodulatory drug, hydroxychloroquine has a good safety profile. The immune system pathways previously mentioned that are affected by this drug and not completely inhibited, stay largely conserved while the patient is undertaken the treatment. Hydroxychloroquine is not associated with risk of infectious conditions or cancer. However, usual side effects include gastrointestinal effects and severe adverse effects can lead to retinopathy. These effects can be reduced by taking the correct drug dosage (Schrezenmeier & Dörner, 2020).

Similarly, belimumab also have common gastrointestinal side effects such as diarrhea and nausea. Nevertheless, due to its immunosuppressive nature, this drug can increase the risk of infections, allergic reactions and hypersensitivity, and mental health problems. Belimumab can also promote progressive multifocal leukoencephalopathy (PML) and cancer. Therefore, it is highly important to use properly this drug and always under medical prescription as a treatment of SLE (Dennis, 2011).

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