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Cell & Gene Therapy

Master 2 option Immunolgy

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Summary

1. Cell therapy

Cell therapy, often referred to as cellular therapy, cell transplantation, or cytotherapy, is a form of treatment in which a patient receives an injection, graft, or implant of live cells to produce a therapeutic effect. It entails either grafting stem cells to repair damaged tissues or transferring T cells that can kill cancer cells through cell-mediated immunity. Cell therapy has its roots in nineteenth-century experiments in which researchers injected animal material into patients to diagnose and treat disease. Even if these efforts had no beneficial results, Further investigations revealed that human cells could help prevent the body from rejecting transplanted organs in the middle of the 20th century. This discovery made successful bone marrow transplantation a common procedure in treating patients with compromised bone marrow due to illness, infection, radiation, or chemotherapy. Researchers have become more interested in stem cell and cell transplantation as a potential novel treatment approach for a variety of disorders, notably degenerative and immunogenic pathologies, in recent years.

Cell therapy also involves replacing damaged or dysfunctional cells with new healthy cells by transferring living cells to a patient. These can be autologous (self-to-self, using cells from the same patient receiving the treatment) or allogeneic (using cells from a donor).

1.1. History of cell therapy

The first human bone marrow transfusion was performed on a patient with aplastic anaemia in 1939. This patient received daily blood transfusions to increase her white blood cell and platelet counts using an intravenous injection of bone marrow. After World War II and the use of the atomic bomb, researchers attempted to find ways to restore bone marrow function in aplasia caused by radiation exposure. In the 1950s, it was proven in a mouse model that secondary bone marrow suppression due to radiation can be overcome by syngeneic marrow transplantation. In 1956, Barnes and colleagues published their experiment on two groups of mice with acute leukemia: both groups were irradiated for anti-leukemia treatment and both were rescued from bone marrow aplasia by bone marrow transplantation. In 1956-1957, doctor Donnall Thomas performed the first successful bone marrow transplant. He administered the marrow of his twin brother to a patient suffering from leukemia, treated by irradiation and chemotherapy. Thanks to this, he was able to reconstitute the patient's hematopoietic system. He then developed the use of bone marrow or hematopoietic stem cell transplantation to treat patients with hematological cancers. This work has made it possible to significantly improve the survival rate of patients thanks to cell therapy, going in some cases from zero to 90%. The work of Donnall Thomas in 1969-1977-1979 allowed him to establish the rules and laws of transplantation, this work was rewarded with the Nobel Prize in 1990.

In 1998 with the development of the first line of human embryonic stem cells, by an American team, or in 1999, researchers discovered that adult stem cells are multipotent, that is to say capable of giving rise to several cell types. Blood stem cells can supply any blood cells. In 2000, the first clinical trial of stem cell transplantation took place aimed at restoring cardiac functions and the first clinical trial in orthopaedic and maxillofacial surgery, for bone reconstructions.

Around these experiments, French legislation authorized research on surplus human embryos in 2004. It was in 2007 that a key discovery changed the perception of human biology. Researchers have successfully reprogrammed an adult human skin cell into a stem cell similar to embryonic stem cells. These cells are called induced pluripotent stem cells (iPS cells). We see the possibility that they could replace embryonic cells in the future. The first creation of induced pluripotent cells was made by the Japanese Yamanaka team.

2. Types of stem cells

Stem cells can be classified according to their developmental potential into 3 main categories (Fig. 1)

2.1. Embryonic stem cells (ESC)

2.1.1. Totipotent Embryonic Stem Cells

Are stem cells capable of differentiating into all embryonic cell types and can give rise to a complete individual. These cells are derived from a fertilized egg.

2.1.2. Pluripotent embryonic stem cells

Are stem cells capable of donating all types of cells in an organism except extra-embryonic tissues. Pluripotent cells are derived from the inner cell mass of the blastocyst and are the origin of the three primary tissues of the embryo: endoderm, ectoderm, mesoderm.

2.2. Somatic Stem Cells (adults, SSC)

2.2.1. Multipotent Stem Cells

Are stem cells capable of donating a large number of stem cells that can give only one type of cell with the ability to self-regenerate. For example, epidermal stem cells can differentiate into keratinocytes of cell types only, but not all. For example, multipotent blood stem cells can donate red blood cells, white blood cells, and platelets.

2.2.2. Oligopotent Stem Cells

Are stem cells that are only capable of generating a few cell types of a given tissue. For example, skin stem cells can give rise to epidermal cells, sebaceous glands, or hair follicles.

2.2.3. Unipotent Stem Cells

A type of stem cell that can give only one type of cell with the ability to self-regenerate. For example, epidermal stem cells can differentiate into keratinocytes only.

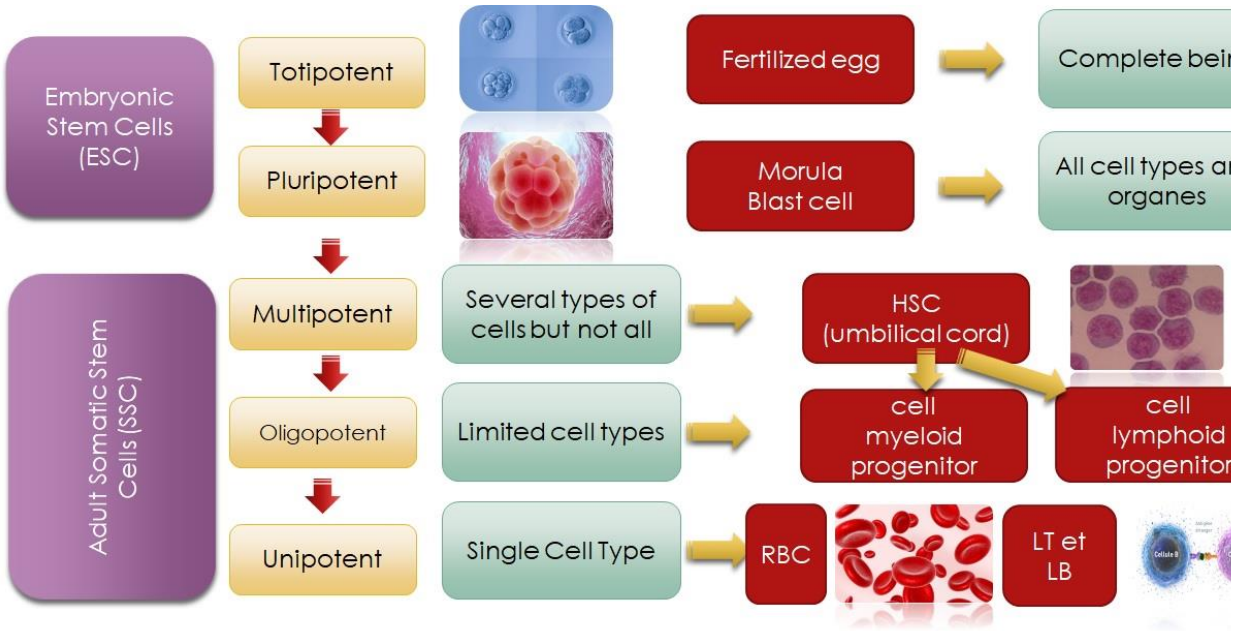


Figure 1: *Different Types of Stem Cells*

2.2.4. Induced pluripotent stem cells (programmed, iPSC /iPS)

Genetically reprogrammed somatic cells are another source of pluripotent stem cells. They are called induced pluripotent stem cells (iPS or iPSCs). iPSCs were generally created by ectopic expression of four transcription factors, which were forced by viral vectors. These transcription factors included c-Myc (cellular homolog of the oncogenic retroviral v-myc), Klf4, Sox2 and Oct3/4 or Sox2, Oct4, Lin28 and Nanog. In order to eliminate the risk of mutation and potential tumorigenicity through viral integration, alternative non-viral reprogramming factors (not carrying aberrant genetic information) were tested. These factors include various cocktails of transcription factors, and various small chemical molecules acting as inhibitors of specific signaling or epigenetic regulators, such as valproic acid or sodium butyrate (histone deacetylase inhibitors), parnate (histone demethylase inhibitor), CHiR99021, a glycogen synthase kinase 3 (GSK-3) inhibitor, or forskolin and D4476 (chemical "substitutes" for Oct4). Even widely used dietary supplements, such as vitamin

C and other antioxidants, have been found to enhance the genomics and epigenomics characteristic of iPSCs, when introduced into reprogramming media.

However, small chemical molecules can also have adverse effects on developing cells and tissues, e.g. valproic acid, which is known to cause serious abnormalities in the embryo and the developing fetus. Other approaches to creating iPSCs have therefore been developed, such as processing cells with small non-coding RNA molecules, called microRNAs (i.e., miRNAs), which play an important role in RNA silencing and post-transcriptional control of gene expression (Singh et al., 2015). Other possible methods include nuclear transfer, transfer of the somatic cell nucleus into an oocyte, or even oocyte parthenogenesis.

2.3. Sources of Stem Cells

The different sources of stem cells can be classified into 4 four main sources depending on the nature of the donors of these cells. **Autologous** stem cells are cells that come from the same person, these cells can be retrieved from the body, grown *in vitro* and injected back into the person. These cells are well tolerated and raise fewer ethical questions. On the other hand, this type of source has a limited supply of cells. **Allogeneic** stem cells are cells that come from another individual of the same species. These cells are available in greater numbers than autologous cells and offer the possibility of standardizing cell transfer processes. However, allogeneic sources raise ethical questions if they originate from embryos and frequently initiate immune reactions (possibility of rejection). Xenogenic stem cells are defined as cells derived from another species, Sources of xenogenic stem cells are vast. Non-human primates, pets, and rodents are promising cell sources for transplantation. Non-human primates that are genetically and physiologically related to humans are the main cellular sources. Xenogenic stem cells are considered to be an easier supply, but they have major drawbacks such as the risk of transmitting a new virus from one species to another and the risk of provoking immune reactions. Immortalized **cell lines** are considered to be the source and source of infinite stem cells. These types of cells present the risk of triggering an immune reaction if the origin of these cells is allogeneic, likewise, they can develop tumors because are immortalized beforehand *in vitro*.

3. Stages of Cell Therapy

Cell therapy can be broken down into several stages. It starts with the collection of cells. These can come from the patient to be treated, these are autografts, they are well tolerated by the patient, they can be multiplied *in vitro* but have a limited supply. A second alternative is to take cells from other sources (allogeneic or xenogeneic). These cells must be amplified by culture so that they multiply and then re-implanted in the patient at the level of the pathological tissue or cell group to replace the diseased cells. A step is carried out to purify the harvested cells to keep only one cell type.

The cells are then modified or treated according to the therapeutic objective to be achieved. At this level, the main challenge is to maintain the viability of the cells during manipulation. The final step is the administration of the cells by surgical intervention and its follow-up.

4. Transplantation

Transplantation is a technique that involves replacing a defective organ, tissue, or cell compartment with a healthy equivalent. Transplantation includes the transplantation of bone marrow, umbilical cord blood or stem cells on the one hand and the transplantation of "solid" organs and tissues on the other. Depending on the nature of the organ donor, transplantation is divided into: autologous transplantation, allogeneic transplantation, allogeneic transplantation (twin) and xenotransplantation.

The first report of successful transplantation dates back to ancient times, in the 6th century BC. In it, an Indian surgeon named Sushruta describes autologous skin grafts from the cheek that allow him to repair severed noses and missing earlobes. His texts detail a method of sterilization and preparation of skin flaps made in such a way as to avoid infection and facilitate tissue revascularization. This effective technique was used by plastic surgeons decades later. The second report dates from the 3rd century. It tells the story of twins Damian and Cosmas, two Arab doctors in Rome, who allegedly amputated the leg of a Justinian deacon and replaced it with the leg of a recently deceased black slave.

The transplant would have been a success and would have allowed the deacon to walk again. However, based on our current knowledge of the mechanisms of transplant rejection, this story seems to be more myth than reality. It was not until the 18th century with John Hunter that an improvement in the grafting technique was observed. Hunter performed tooth transplants from living donors. Although he is not the first to perform this kind of transplant, he has advanced the chances of success through the principles of graft selection. Indeed, it shows that to obtain better graft survival, the donor tooth must be as fresh as possible and as close as possible to the size corresponding to the space present in the recipient. Thanks to this, some dental grafts were maintained for 6 years, an extremely long period for the time. These principles are still used in internal organ transplantation.

In 1912, Alexis Carrel was awarded a Nobel Prize for his improvement of the technical procedures of vessel sutures in whole organ transplantation. Before the middle of the 20th century, surgeons mainly performed autografts and allografts. However, very few of them notice that autologous transplants survive better than allografts. This may be due in part to the many technical failures of early autologous transplants.

4.1. Graft rejection

Rejection occurs when the recipient's immunocompetent cells attack and destroy the transplanted cells. It can occur hyperacutely in the first few hours after transplantation, acutely on the first day of transplantation, or even chronically when the transplant deteriorates over time. Currently, the risk of rejection is very low due to the significant modulation-induced immunosuppression of the receptor and the immunosuppressive therapy administered after transplantation. Factors influencing the occurrence of rejection are lower intensity of pre-transplant conditioning, immunosuppressive agents to prevent rejection, HLA incompatibility, graft T-cell depletion, and prior transfusion that increases recipient cell polymorphisms.

4.1.1. Hyper-acute rejection

Hyperacute rejection occurs only in vascularized grafts. This is due to pre-existing antibodies in the recipient that recognize donor-specific antigens. These donor antigens are usually human leukocyte antigens (HLA). The donor antigen-antibody will pick up the antigen on the surface of the graft's endothelial cells and activate complement. This stimulates the endothelial cells and causes them to release von Willebrand clotting factor. The platelets will then aggregate, causing thrombosis of the blood vessels and leading to necrosis of the graft. In this rejection, immune cells do not infiltrate the transplanted organ. Today, this rejection can often be avoided through HLA compatibility analysis between donor and recipient prior to transplantation.

4.1.2. Acute rejection

Acute rejection is associated with the recognition of donor antigens by the recipient's adaptive immune system. It can be divided into two categories: acute cell rejection (LT-dependent) and acute humoral rejection (LB-dependent). Innate responses will occur first, triggered by tissue stress associated with organ retrieval and ischemia/reperfusion. This stress induces the release of danger signals that activate antigen-presenting cells (APCs). APCs are immune cells characterized by their ability to present Ag to LT. They include monocytes, macrophages, B lymphocytes, and dendritic cells (DCs). This innate response is essential because it determines the development of adaptive responses. Stress also activates the recipient's neutrophils, macrophages, and natural killer (NK) cells, which then infiltrate the graft. These innate cells will contribute to the formation of lesions, which in turn will contribute to the amplification and maintenance of adaptive T responses. During the adaptation phase, the APCs will migrate from the graft to the secondary lymphoid organs, where they will activate the lymphocytes by presenting them with alloantigens. For effective LT stimulation, three activation signals come into play. First signal; TCR will associate with CD3 recognizes the MHC/peptide complex present on APCs. The second signal is that the LTs will recognize co-

stimulation molecules such as CD80, CD86 and CD40 present on the APCs. This interaction will partly allow the change of naïve LTs into LT helper (Th) or regulator LT (Treg). Without this signal, the LTs become anergic or depleted. During the last signal, APCs secrete cytokines necessary for the polarization of the LT helpers to a Th1, Th2, Th17 or Treg profile. This polarization is very important in order to initiate an immune response adapted to the type of danger.

Activation of the LTs is mainly mediated by the recipient's APCs, but the donor APCs present in the graft may also exhibit alloantigen to the LTs. This is because these cells migrate a few hours after reperfusion from the organ to the secondary lymphoid organs and interact with the LTs. However, they are few in number and do not survive long. There are therefore three routes of presentation of donor antigens to lymphocytes. Direct recognition: the donor's APCs have their MHC/peptide complex. Indirect recognition: the recipient's APCs present the alloantigen via their own MHC. Semi-direct recognition: the donor's MHC/peptide complex is presented by the recipient's APCs, following a membrane exchange with the donor's APC

4.1.3. Chronic rejection

Chronic rejection is often characterized by tissue fibrosis leading to functional loss of the graft, or thickening of the intima of the vessels leading to ischemia of the organ. The production of pro-inflammatory cytokines by the recipient's LT cells is one of the causes of fibrosis in the graft. Anti-DSA antibodies are also implicated in chronic rejection. Here, they have an additional role in complement activation by leading to the activation and proliferation of endothelial cells. These cells lead to the differentiation and proliferation of myofibroblasts via the secretion of growth factors and cytokines. Myofibroblasts form an extracellular matrix that, when accumulated and persisted over the long term, leads to tissue fibrosis responsible for the functional loss of the graft. On the other hand, chronic rejection may be the consequence of non-immunological phenomena. This is because certain hormones such as angiotensin II or endothelin I are responsible for the production of an extracellular matrix by endothelial cells, leading to fibrosis. The age of the donor is also correlated with the incidence of chronic rejection. The older the donor, the earlier the senescence of the organ. However, this senescence seems to induce the release of pro-inflammatory cytokines by the organ, leading to its rejection. We can also observe a recurrence of the initial pathology, leading to a deterioration of the transplanted organ, or a deleterious effect of the treatments taken by the patient. For example, cyclosporine A and tacrolimus are nephrotoxic in the long term. As a result, they can lead to graft dysfunction.

4.2. Treatment of rejection

In recent years, improved knowledge of rejection mechanisms, advances in surgical techniques and the induction of immunosuppression have contributed to reducing the occurrence of graft rejection, and in particular acute rejection. Immunosuppression is achieved by a combination of drugs and/or monoclonal antibodies that primarily target lymphocytes, which are major players in rejection (Table 1). They act on lymphocyte activation and proliferation, in part through the inhibition of one of the three LT activation signals provided by APCs. Immunosuppressants (ISs) are a multitude of drugs classified into 5 categories: corticosteroids, calcineurin inhibitors, anti-metabolites, nucleotide synthesis inhibitors, and mTOR inhibitors. Corticosteroids such as Prednisolone bind to glucocorticoid receptors. They decrease the expression of pro-inflammatory genes in target cells, resulting in the decrease of cytokines, chemotactic factors, and adhesion molecules. As a result, there are fewer circulating CD4+ cell cells and fewer macrophages in inflammatory sites. Calcineurin inhibitors (Tacrolimus, cyclosporin A) are lipophilic molecules. As a result of their binding to intracellular immunophilins, chaperone proteins, they have a high affinity for the calcineurin/calmodulin/Ca2+ complex. Normally, stimulation of the T-cell receptor (TCR) results in an increase in free intracellular calcium and the activation of calcineurin. This phosphatase dephosphorylates the transcription factor Nuclear factor of activated T cells (NFAT), leading to the synthesis of IL-2. In the presence of tacrolimus or cyclosporin A, calcineurin can no longer play its phosphatase activity on NFAT. These inhibitors thus abrogate the production of IL-2 and consequently the activation and proliferation of LTs. Mycophenolate Mofetil and anti-metabolites such as Azathioprine block the synthesis of purines (DNA bases A and G). As a result, there is no longer any cell division, leading to a decrease in lymphocyte proliferation. mTOR inhibitors (sirolimus, everolimus and rapamycin) inhibit the activity of the enzyme involved in cell cycle activation following the binding of cytokines to their receptor. Again, cell proliferation is blocked. Monoclonal or polyclonal antibodies, unlike drugs, allow you to specifically target a cell type by attaching to the antibodies that characterize them. Following their binding, these antibodies can lead to the depletion of the target by activation of the complement. For example, anti-CD20 leads to LB depletion and anti-CD52 or ATG (AntiThymocyte Globulins) induces lymphopenia. Some antibodies make the target cell inaccessible to stimulation by blocking particular epitopes. Thus, when anti-CD3 binds to the TCR, it leads to internalization of the CD3/TCR complex. The LTs can no longer receive signal 1 and will not activate. The cells will therefore not proliferate or secrete pro-inflammatory cytokines. The binding of anti-CD3 to activated LTs also induces apoptosis of these cells and allows for a long-term increase in the ratio of Treg to LT effectors. On the other hand, it is possible to inhibit the activation of LTs by preventing their co-stimulation by APCs. For this purpose, CTLA-4Ig is used

to block signal 2 (CD28- CD80/CD86). It is also possible to block the CD40/CD40L signal with Antibodies targeting these 2 molecules. Some antibodies inhibit signal 3 by stopping the binding of cytokines to their receptor.

This is the case of anti-CD25 which binds to the IL-2 receptor. There are also intravenous polyclonal immunoglobulins (IVIg), which are made from a mixture of plasma from many healthy donors. They include a very large repertoire of Acs, making it possible to capture the Non-self ag (pathogenic or foreign). IVIg is used in incompatible transplants into the ABO blood group system in addition to the removal of natural antibodies, thus prolonging graft survival.

Table 1: Examples of immunosuppressive treatments used in transplantation

Class	**Molecule**	**mechanism of Action**
Pharmacological drugs Corticosteroids	Prednisone	Decreased expression of pro-inflammatory genes
	Prednisolone Methylprednisolone	
Inhibition of nucleic acid synthesis	Mycophenolate de mophétil	Inhibition of proliferation
	Mycophenolate de sodium	
Anti-metabolite	Azathioprine	Inhibition of proliferation
mTOR inhibitor	Rapamycin	Inhibition of proliferation
	Everolimus	
Calcineurin inhibitors	Cyclosporine Tacrolimus	Inhibition of lymphocyte activation and proliferation by blocking IL-2 synthesis
	Tacrolimus	
Polyclonal Antibodies Depleting	ATG	Lymphocyte depletion
Mixed	IVIg	Neutralization of cytokines and Ab
		Depletion of immune cells Expansion of Tregs and modulation of DC
		Blocking of activating receptors
Monoclonal Antibodies Depleting	Rituximab (anti-CD20)	Depletion of LBs
	Alemtuzumab (anti-CD52)	Depletion of lymphocytes

Mixed (Blocking activation and depleting)	OKT3 (anti-CD3)	Inhibition of signal 1 + Depletion of 20% of LT
Blocking activation	Belatacept (CTLA-4 Ig)	Inhibition of signal 2
Anti-proliferatives	Basiliximab (anti-CD25)	Inhibition of signal 3

5. Dendritic cells

5.1. Generality

In 1868, a young medical student, Paul Langerhans, described a type of cells characterized by their morphology and specific capacity to initiate a primary immune response by sensitizing naive T lymphocytes. In 1973, Steinman and Cohn identified cells with a particular morphology in lymphoid organs. These cells bear unusual arborescent extensions. They are then called dendritic cells, coming from the Greek Dendreon meaning (tree). These cells are at the interface of innate and adaptive immunity and play a central role in the control of immunity through the capture, transport and presentation of antigens to T lymphocytes. DCs are involved in numerous pathologies involving the immune system: transplantation, and asthma.

Immature DCs reside mainly in peripheral tissues such as skin or mucous membranes. On the one hand, they carry out sampling of their environment thanks to their great capacity for endocytosis. On the other hand, they have receptors recognizing danger signals (PRR) such as molecular patterns associated with pathogens (PAMP) or associated with tissue damage (DAMP). The recognition of danger signals will lead to the maturation of DCs and a transient increase in their phagocytosis capacity, important for Ag capture. A specific signalling cascade is thus engaged, depending on the type of receptor activated by the binding of PAMPs or DAMPs. This makes it possible to arrive at a specific expression profile of DC genes. DCs will also be sensitive to cytokines and chemokines present in the environment. These molecules will allow the recruitment and accumulation of DCs at the inflamed site and their maturation. For example, tissue cells will express CCL20 during inflammation, attracting immature DCs. Therefore, the presence in this chemokine environment will lead to in situ recruitment of DCs, which will capture the Ag and become mature.

5.2. Classification of dendritic cells

The deepening of knowledge on DCs and in particular on their ontogeny and their phenotypic markers is currently the subject of dynamic research. However, recent discoveries have not made it possible to establish a clear classification of DC subpopulations. On the contrary, the sharing of markers between distinct subpopulations and the redefinition of the dendritic population into macrophages have brought some confusion. However, this heterogeneity clearly reflects the diversity necessary within DCs for the establishment of an immune response specific to different types of aggression and the microenvironment.

Currently, DCs are classified according to 2 main classes. The first dimension takes into account the spatial distribution of cells in the body. Migratory DCs are mainly localized in peripheral tissues (skin, lungs, liver, intestines, pancreas, kidneys) until they mature where they will infiltrate the OLS

via the lymphatic network. They are opposed to resident DCs which are not found in peripheral tissues but which circulate in blood vessels or reside in lymphoid tissues. The second dimension is based on both their function, their phenotype and their ontogeny. It therefore separates DCs into 4 subgroups: plasmacytoid DCs (pDCs), conventional DCs (cDCs), Langerhans cells and DCs derived from monocytes (or inflammatory DCs).

5.2.1. Conventional DC (cDC) or classic

Also called myeloid DC or classical DC. These cells are professional APCs, that is to say, they are capable of preparing the Ag and presenting it. In addition, they effectively activate LT via the expression of co-stimulation markers and their production of IL-12 in large quantities. Whether migratory or resident, cDCs are subdivided into 2 groups: cDC1s which develop in a manner dependent on the transcription factors BATF3 and IRF8 and cDC2s whose development is rather dependent on the transcription factor IRF4.

Migratory DCs

Migratory cDCs represent a small proportion of DCs in peripheral tissues (1 to 5% depending on the organ). They capture Ag in peripheral tissues, migrate through lymphatic vessels and infiltrate OLS after becoming mature. Depending on the peripheral tissues from which they come, cDCs have some particularities. For example, cDCs present in the dermis are also called interstitial DCs and are capable of inducing differentiation of LB into plasma cells that secrete IgM immunoglobulin. Furthermore, CD103-CX3CR1+ intestinal mononuclear cells are capable of extending their dendrites between epithelial cells in order to reach the intestinal lumen and capture Ag. They then transfer Ag to CD103+ DC via gap junctions. CD103+ DCs then present Ags to LT, thus initiating an effector response against pathogens or a tolerogenic response to dietary Ags and commensal flora. There are 2 subpopulations of migratory cDCs in mice: cDC1 CD103+ CD11b- and cDC2 CD11b+. These cells are the respective equivalents of CD8α+ and CD11b+ DCs residing in lymphoid tissues. In humans, migratory cDCs are classified according to their expression of CD1a and CD14, cDC1 BDCA3+ CD1a- CD14- Clec9A+ XCR1+ and cDC2 BDCA1+ CD1a+ CD14-. Recent research describes several subpopulations of cDC2, based on the expression of markers CD5, CD14 and CD163. The first group called DC2 includes CD5+ cells. A second set called DC3 brings together CD5- cells: CD163- DCs, 163+ CD14- DCs and inflammatory CD163+ CD14+ DCs. This inflammatory population is increased in patients with systemic lupus erythematosus. Apart from cDC1 and cDC2, there are also CD14+ CD1a-CD11c+ cDCs, sometimes related to macrophages (Fig. 2).

Lymphoid DCs

Resident cDCs are restricted to lymphoid tissues. They represent the majority of DCs in the spleen and thymus but only constitute half of the DCs in the lymph nodes. They are also found in Peyer's patches in the intestine and in humans in the tonsils. In addition to their role in the inflammatory response, these cells participate in the induction of central and peripheral tolerance. Resident cDCs are classified into 2 groups in mice according to their expression of CD11b and CD8α. In humans, the CD8α marker is not present on DCs and therefore it cannot be used as a discriminator of resident cDC subpopulations. However, BDCA3 allows human resident cDCs to be classified into two groups (Fig. 2).

cDC1 BDCA3+ CD11bCD11c+ Clec9A+ XCR1+ (male) These cells analyze the surrounding environment and detect the presence of pathogens or tissue damage. They are therefore located in strategic locations within the lymphoid organs. For example, they are found in the marginal zone of the spleen where they can filter Ag from the blood, or in the subcapsular sinus of the lymph nodes, the arrival site of the afferent lymphatic vessels draining the peripheral tissues. Following the capture of Ag, they will go to meet the LT in the OLS. Due to their lower expression of MHC-II than CD8α-, they will preferentially activate CD8+ LT by cross-presenting. This population can be further subdivided into 2 according to their expression of CD103.

cDC2 BDCA1+ CD11b+ CD11c+ (male) This is the main population of tissue-resident DCs, except in the thymus. They strongly express MHC-II, which gives them a major advantage for the activation of CD4+ LT.

5.2.2. Unconventional (non-classical) DCs

Monocyte-derived DCs (moDCs)

Monocyte-derived DCs (moDCs) are also called “inflammatory DCs.” They originate from the myeloid progenitor and play a vital role in the immune response, because early in infection they tend to provide a pool of APCs that can effectively trigger an adaptive immune response. During inflammation or infection, circulating blood monocytes which are normally known to express receptors for granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and interleukin-4 (IL-4), other differentiation molecules and chemoattractants tend to invade tissues and are attracted to the site of inflammation, where they are then differentiated into moDCs. However, during infection, upon entry of moDCs to the site of infection, moDCs produce TNF-α and inducible nitric oxide synthase (iNOS), which ultimately confers innate protection. Therefore, moDCs are also known as “TNFα, iNOS-producing DCs”, so in terms of processing, presentation and cross-presentation, moDCs are very powerful. Phenotypically,

it is difficult to distinguish moDCs from cDCs because both share similar expression patterns of MHCII, CD11b, and CD11c. However, one indicator of their monocytic background is that moDCs express CD64 and Fc-gamma receptor 1 (FCγR1). Moreover, also functionally, moDCs are similar to cDCs, because both DCs were able to process tissue antigens and eventually migrate via lymphatic vessels to the nearest lymph node, and present the processed antigens to naive T cells. After activation, the lifespan of the two DC subtypes is similar, compared to the residence time of tissue DCs during their immature state. Murine inflammatory DCs retain the monocytic markers Ly6C and CD11b and express CD11c and CX3CR1. In humans, they express numerous markers shared with macrophages (CD11b, CD11c, SIRP-α, CD206) but also markers more specific to DC such as FcεR1. The lifespan of the two DC subtypes is similar, compared to the residence time of tissue DCs during their immature state. Murine inflammatory DCs retain the monocytic markers Ly6C and CD11b and express CD11c and CX3CR1. In humans, they express numerous markers shared with macrophages (CD11b, CD11c, SIRP-α, CD206) but also markers more specific to DC such as FcεR1. The lifespan of the two DC subtypes is similar, compared to the residence time of tissue DCs during their immature state. Murine inflammatory DCs retain the monocytic markers Ly6C and CD11b and express CD11c and CX3CR1. In humans, they express numerous markers shared with macrophages (CD11b, CD11c, SIRP-α, CD206) but also markers more specific to DC such as FcεR1.

Plasmacytoid DCs (pDCs)

pDCs were given their name due to their morphological similarity to plasma cells. Indeed, plasmacytoid dendritic cells (pDCs) are characterized by their tiny, spherical morphology and possess a nucleus that is comparable in size to that of centroblasts. pDCs mostly exist inside tissues, although under certain clinical circumstances (such as autoimmune disorders, infections, and malignancies), they may also be present in peripheral tissues. pDCs may be differentiated from other DCs based on their relatively low expression of the CD11c marker. Mice pDCs have a Ly6c+ B220+ CD11clow CD8α- phenotype and have the ability to generate IFN-α and IL-12. Humans exhibit the BDCA2 marker. Similar to conventional dendritic cells (cDCs), they have the ability to transmit antigens to lymphocytes, although this presentation is less efficient. Nevertheless, plasmacytoid dendritic cells (pDCs) play a crucial role in the initiation of an immune response to viruses via the release of interferons (IFNs). They have the ability to generate a significant amount of type I IFN after detecting viral genetic material via their endosomal PRRs TLR-7 and TLR-9. Activation of these Toll-like receptors (TLRs) and the specific presence of IFN-α stimulate the production of TRAIL by plasmacytoid dendritic cells (pDCs). As a result, they possess cytotoxic properties against infected cells and tumor cells that express TRAIL-R1 or TRAIL-R2. The secretion of type I interferon (IFN) by plasmacytoid dendritic cells (pDCs) facilitates the activation of other cells, including natural killer

(NK) cells. Activation of these Toll-like receptors (TLRs) and the specific presence of IFN-α stimulate the production of TRAIL by plasmacytoid dendritic cells (pDCs). As a result, they possess cytotoxic properties against infected cells and tumor cells that express TRAIL-R1 or TRAIL-R2. The secretion of type I interferon by plasmacytoid dendritic cells (pDCs) facilitates the activation of other cells, including natural killer (NK) cells. Activation of these Toll-like receptors (TLRs) and the specific presence of IFN-α stimulate the production of TRAIL by plasmacytoid dendritic cells (pDCs). As a result, they possess cytotoxic effects on infected cells and tumor cells that express TRAIL-R1 or TRAIL-R2. The secretion of type I interferon (IFN) by plasmacytoid dendritic cells (pDCs) facilitates the activation of other cells, including natural killer (NK) cells.

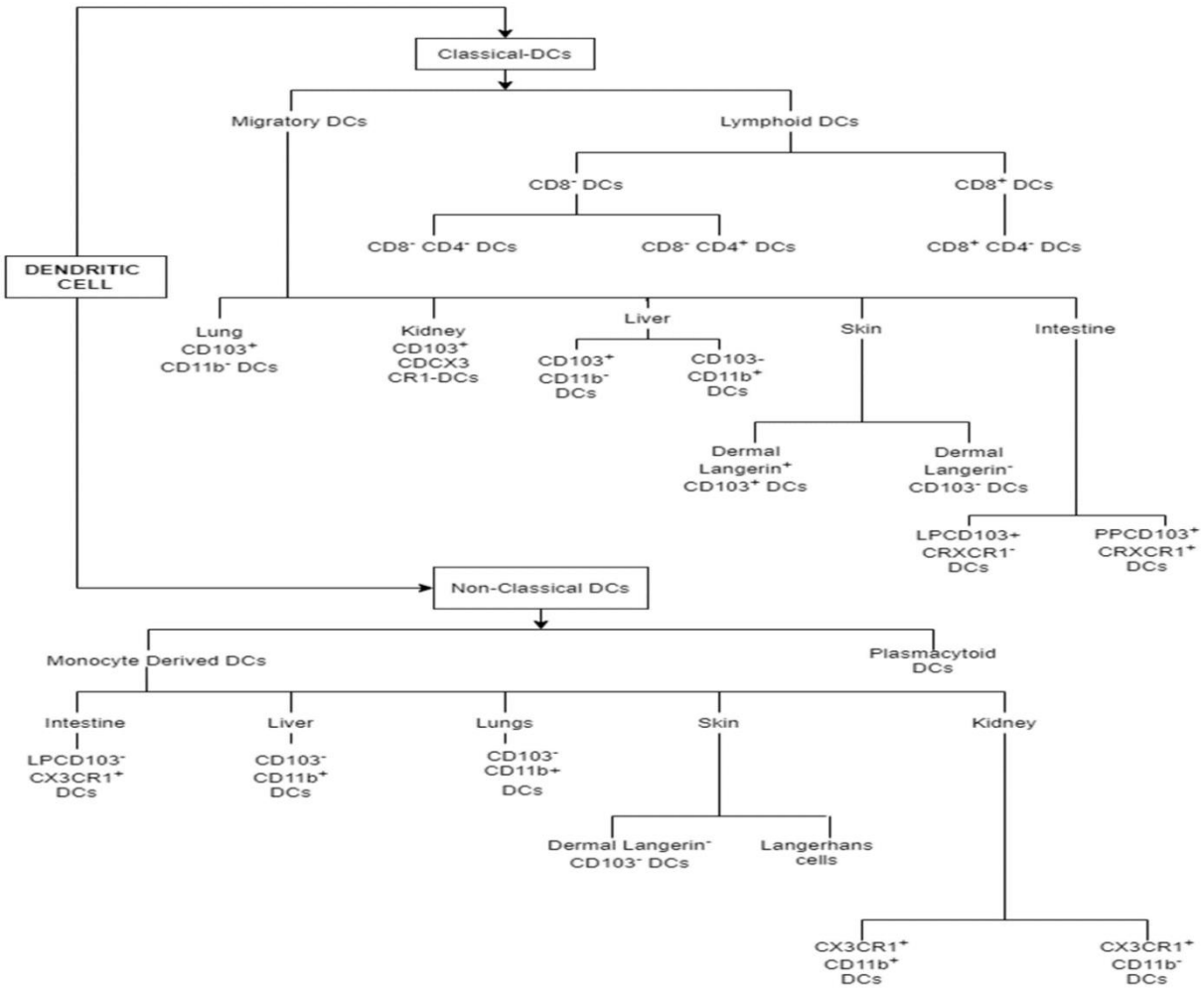


Figure 2 Classification of dendritic cells

Classical-DC: classic dendritic cells, Non-classical DC: non-classical DC.

The Langerhans DCs

Langerhans cells mostly inhabit the epidermis, however they have also been detected in the dermis and mucous membranes. Unlike other dendritic cells (DCs), these cells are formed during the early

stages of development from a hematological precursor originating from the yolk sac. Furthermore, they possess the ability to undergo self-renewal in their current location. These cells may be differentiated from other dendritic cells due to the presence of E-cadherin, epithelial cell adhesion molecules (EpCAM), langerin, and specific organelles known as Birbeck granules on their surface. Murine Langerhans cells have significant expression of CD11b and CD11c, while humans express these molecules to a lesser extent or not at all. These cells have the ability to deliver antigens and activate lymphocytes. They are particularly efficient in polarizing CD4+ lymphocytes and activating cytotoxic CD8+ lymphocytes. The Langerhans cells, which are found mostly in the dermis, seem to be temporarily confined there with the purpose of capturing an antigen and thereafter migrating towards the outer layer of the skin to deliver it. These cells are accountable for the occurrence of touch hypersensitivity.

5.3. Genesis of DC

DC are a rare population of myeloid cells representing 0.3% of blood leukocytes and 1-2% of secondary lymphoid organ (OLS) leukocytes. They are generated from HSCs, multipotent cells which have an unlimited self-renewal capacity (Fig. 3). In adults, DCs are classically generated in the bone marrow during hematopoiesis. Thus, HSCs will generate multipotent progenitors (MPP) which differentiate into common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). These cells are oligopotent and have a more limited self-renewal capacity than HSCs. The CMPs differentiate into common granulocytic and monocytic progenitors (GMP) which will themselves give rise to granulocytes, and on the other hand precursors of macrophages and dendritic cells (MDP). At this stage, the MDPs will enter either into a monocytic lineage with the common monocyte progenitor (CMoP), or into a dendritic lineage with the common dendritic cell precursor (CDP). CDPs can differentiate into pDCs then exit the bone marrow and migrate to OLS via the blood or they can differentiate into DC precursors (pre-DCs) and then migrate to OLS or tissues such as the intestine, liver, lungs and kidney, and become cDCs there. More recently, a parallel pathway was discovered in the DC generation, shorter than this classical pathway. Indeed, like myeloid precursors, lymphoid precursors are capable of giving rise to dendritic cells. For that, CLPs must be under the influence of differentiation factors such as FMS-like tyrosine kinase 3 ligand (Flt3L), Granulocyte-macrophage colony-stimulating factor (GM-CSF) or TNF-α. Monocytes are also capable of differentiating into DCs after their invasion into tissues. To differentiate into moDC, monocytes must be under inflammatory conditions. In humans, monocytes are used as a precursor to generate DCs in vitro. monocytes must be in inflammatory conditions. In humans, monocytes are used as a precursor to generate DCs in vitro. monocytes must be in inflammatory conditions. In humans, monocytes are used as a precursor to generate DCs in vitro.

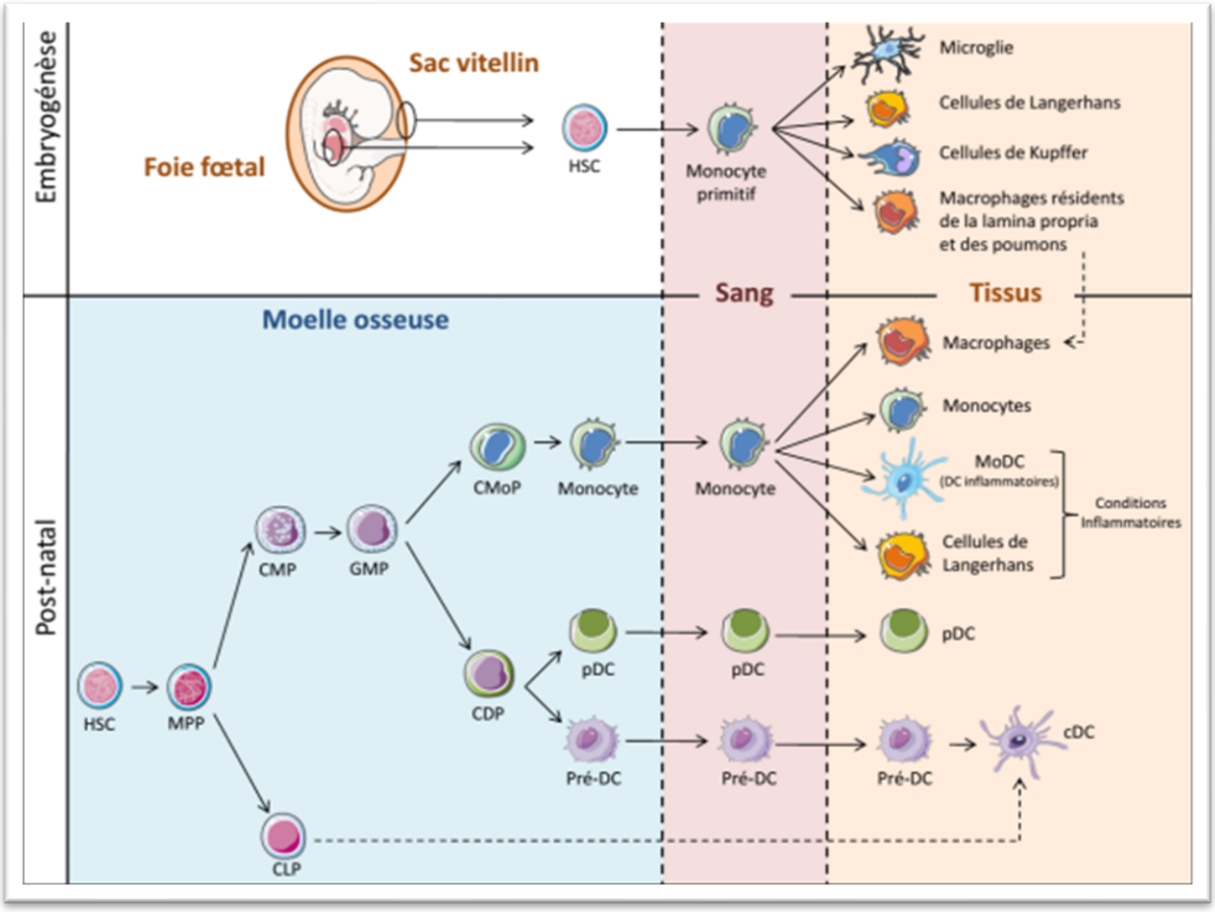


Figure 3 Genesis of dendritic cells.

5.4. DC maturation

The maturation of dendritic cells (DC) leads to significant alterations in the cell, including changes in morphology, the processing of antigens (Ags), their presentation on the cell surface, the release of cytokines, and occasionally the expression of surface molecules required for cell migration and the activation of lymphocytes (LT) in lymph nodes. The maturation of an immature DC might result in two distinct phenotypes, depending on the surrounding environment: an inflammatory phenotype or a tolerogenic phenotype. Therefore, when a pathogen or pro-inflammatory cytokines are present, the DC will undergo differentiation into pro-inflammatory DC. The latter efficiently triggers the activation of LT cells by stimulating their clonal proliferation and boosting their differentiation into effector and memory cells. On the other hand, when there are anti-inflammatory cytokines or when a self-antigen is detected, the dendritic cell (DC) will transform into a tolerogenic DC phenotype (TolDC), which means it will suppress the immune response. Earlier, it was said that juvenile dendritic cells (DCs) had tolerogenic properties, but adult DCs displayed inflammatory characteristics. Recent evidence demonstrates that mature dendritic cells (DCs) have the ability to

induce tolerance. The process of maturation that induces tolerogenic characteristics in dendritic cells results in a gene expression alteration that is as complicated to that found during maturation leading to an immunogenic profile. Furthermore, some procedures for producing TolDC in a laboratory setting include a stage of cell maturation employing pro-inflammatory cytokines. Immature dendritic cells (DCs) were characterised as having tolerogenic properties, but mature DCs were shown to be inflammatory. Recent evidence demonstrates that mature dendritic cells (DCs) have the ability to induce immune tolerance. The process of maturation that induces tolerogenic characteristics in dendritic cells results in a similarly intricate alteration of gene expression as the process of maturation that leads to an immunogenic profile. Furthermore, some procedures for producing TolDC in a laboratory setting include a process of cell maturation employing pro-inflammatory cytokines. Immature dendritic cells (DCs) were characterised as having tolerogenic properties, but mature DCs were shown to be inflammatory. Recent evidence demonstrates that mature dendritic cells (DCs) have the ability to induce tolerance. The process of maturation that induces tolerogenic characteristics in dendritic cells results in a similarly intricate alteration of gene expression as the process of maturation that leads to an immunogenic profile. Furthermore, some techniques for generating TolDC in a laboratory setting include a process of cell maturation employing pro-inflammatory cytokines. The maturation process that induces tolerogenic characteristics in dendritic cells results in a gene expression alteration that is as complicated to that found during maturation resulting to an immunogenic profile. Furthermore, some procedures for producing TolDC in a laboratory setting include a stage of cell maturation employing pro-inflammatory cytokines. The process of maturation that induces tolerogenic characteristics in dendritic cells results in a gene expression alteration that is as complicated to that found during maturation leading to an immunogenic profile. Furthermore, some procedures for producing TolDC in a laboratory setting include a stage of cell maturation employing pro-inflammatory cytokines.

5.5. Activation of DCs

CDCs originate from common DC precursors (CDPs) in the bone marrow and consist of two primary subtypes: the CD8α+ and/or CD103+ cDC1 subtype, and the more diverse CD11b+ cDC2 subtype (Table 1). Two separate kinds of plasmacytoid DCs (pDCs), known as B220+ pDCs, are formed from both PDCs and lymphoid progenitors. Inflammatory situations may stimulate the attraction of blood monocytes that rely on the CC-chemokine receptor 2 (CCR2) and transform into monocyte-derived dendritic cells (moDCs). Distributed control systems (DCs) may also manifest in discrete geographical areas. Tumour lymph node drainage (TDLN) often consists of resident and migratory cDC1 and cDC2 subtypes, along with additional migratory DCs such peripheral tissue-specific cDC subtypes and moDCs. The subpopulations of DCs in human blood, namely CD141+

cDC1s, CD1c+ cDC2, and CD123+ pDC, exhibit a high degree of similarity to mice in terms of transcription and main function. Recent RNA sequencing has revealed new kinds of human dendritic cell (DC) subtypes in blood or tumor-draining lymph nodes (TDLNs). It is worth noting that the surface markers often employed to identify human DC subtypes may not be dependable in various tissue microenvironments. The functional specialisation of DC subtypes is often determined by the specific receptors they express, such as pattern recognition receptors (PRRs) (Table 1). Their ability to initiate T cell activation may also vary, since pDCs have very little capacity to activate naïve T cells, however both human and mouse pDCs can be induced to effectively activate CD8+ T cells. Mouse and human cDC1s, in contrast, possess a remarkable ability to stimulate immune responses against intracellular infections and tumours. This is attributed to their effective processing and cross-presentation of external antigens on MHC class I molecules, which in turn activates CD8+ and CD4+ T cells (Th1). moDCs are mostly produced as a result of inflammation and facilitate the specific development of CD4+ T cells into either Th1 cells, Th2 helper cells, or Th17 cells that produce IL-17. Nevertheless, some human conventional dendritic cells type 2 (cDC2s) have the ability to exhibit markers of monocyte-derived dendritic cells (moDCs), and vice versa.

Resting conventional dendritic cells (cDCs) in their specific habitat, such as the gut, also receive continuous signals such as Flt3L and type I interferon. These signals are partially generated from plasmacytoid cells (PC) (see Figure 4). Activation is initiated by stimuli generated from pathogens, such as TLR agonists or cytokines like IL-1. Within the context of adaptive immunity, conventional dendritic cells (cDCs) located in secondary lymphoid tissues initiate the activation of naïve primary T cells and reinvigorate memory T cells. This process is achieved by the presentation of processed antigens, as well as the provision of co-stimulators and other activation signals. Activated conventional dendritic cells (cDCs) may produce mediators that guide naïve activated or stimulated lymphocytes (LTs) to develop into cytotoxic T lymphocytes (CTLs), T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), or regulatory T cells (Tregs). Activated conventional dendritic cells (cDCs) may also engage in antigen presentation to B cells, therefore triggering a humoral immune response. During innate immunity, active cDCs release VEGF and CXCL2, which have the ability to attract neutrophils. In return, neutrophils provide TNF-α. Natural Killer (NK) cells have the ability to immediately recognise and react to NKG2D ligands that are provided by conventional dendritic cells (cDCs). These cDCs release many cytokines, including IL-12, IL-18, IL-15, and IL-2, which serve as positive signals for the proliferation and cytotoxicity of NK cells. Activated conventional dendritic cells (cDCs) may also transmit signals to cells that are not part of the immune system.

D.C.	**Location**	**Morphology**	**Growth Factor**	**Surface Markers**	**PRRs**	**Function**
pDC	Resident in lymphoid tissues, and are found in blood, lung (Mouse) and tonsil (Human)	Similar to plasma cells	HSC, CDP/ depend on FLT3L/E2-2, IRF7	CD11c-, HLA-DRlow, CD123+, CD303+ (CLEC4C+), CD304+, CCR2+, CXCR3+	TLR7, TLR9, RLR, STING, CLEC12A	* Secretion of type I and type III interferon in cases of acute, chronic or viral infection. * Stimulated to activate CD8+ T cells (cross-presentation). * Involved in the progression of autoimmune diseases. * Play a tolerogenic role (settings poorly described but correlate with poor prognosis in cancer)
cDCs1	Lymphoid tissues and can be found in the blood.	Irregular shape with intensive cytoplasmic extensions. The migratory form is found in peripheral tissues and lymph nodes	HSC, CDP, precDC/ depends on FLT3L, GMCSF/ BATF3, IRF8, BCL-6, ID2, ZBTB46, NFIL3, Notch signalling	CD11clow, HLA-DR+, CD141+a, XCR1+, CLEC9A+, DEC205+	TLR1, TLR3, TLR6, TLR8, TLR10, STING, CLEC12A	* Cellular immunity against tumors and intracellular pathogens. * Th1 cell type immunity CD8+ T cell. * Specialized in cross-presentation. * Produce type I and III interferons and IL-12 (with reduced levels). * Tolerogenic role (poorly described)
cDCs2	Lymphoid tissues can be found in the blood.	Irregular shape with intensive cytoplasmic extensions.	HSC, CDP,preDC/depends on FLT3L, GM-CSF/IRF4, ID2, RBPJ, NOTCH2, KLF4, ZBTB46	CD11c+, HLA-DR+, CD1c+a, CD11b+, CD172a+, CD1a+ (migratory), CD14 and CD5	TLR1– TLR9, RLR, NLR, STING, CLEC4A CLEC6A CLEC7A CLEC10A, CLEC12A	* Induce the activation of Th17 cells and also Th1, Th2 cells,Tregand CD8+. * Activation (cross-presentation) of LT (this depends on the subpopulation of cDCs). * Maintain hemostasis of LTrg-LTh17 in the intestine and lung

MoDCs	Inflamed peripheral tissues, skin, liver and intestines	Context dependent	Monocytes/ Depends mainly on CSF1R, *in vitro*GMCSF and IL-4/MAFB, KLF4, ZBTB46	CD11c+, HLA-DR+, CD1c+, CD11b+, CD14+, CD64+, CD206+, CD209+, CD172a+, CD1a+, CCR2+	Poorly defined	* Studied mainly*in vitro*, the functions depend on the stimulation signals and towards CD8+,Treg, Th1, TH2 and Th17. * Involved in regulatory functions in the skin.

For instance, when CLEC-2 is increased on activated cDCs, it attaches to podoplanin found on lymph nodes and fibroblasts. This attachment enables the stretching of reticular fibroblasts during immunological responses. cDCs further deliver LTβ and IL-1β to reticular fibroblasts and endothelial cells inside lymph nodes. As an ultimate illustration, TGF-β generated during the process of wound healing stimulates the secretion of IL-31 from activated dermal cDC2s, which enhances the susceptibility of itch sensory neurons and triggers an itch response in the injured organism. Various kinds of cDCs may be activated, and their functional characteristics are influenced by variables such as developmental limitations, the type of activation signal, exposure to immunological modulators in the local environment, spatial arrangement, and temporal considerations. It is important to consider that the stimulation of conventional dendritic cells (cDCs) may lead to the development of cDCs that both sustain tolerance and inhibit immunological responses.

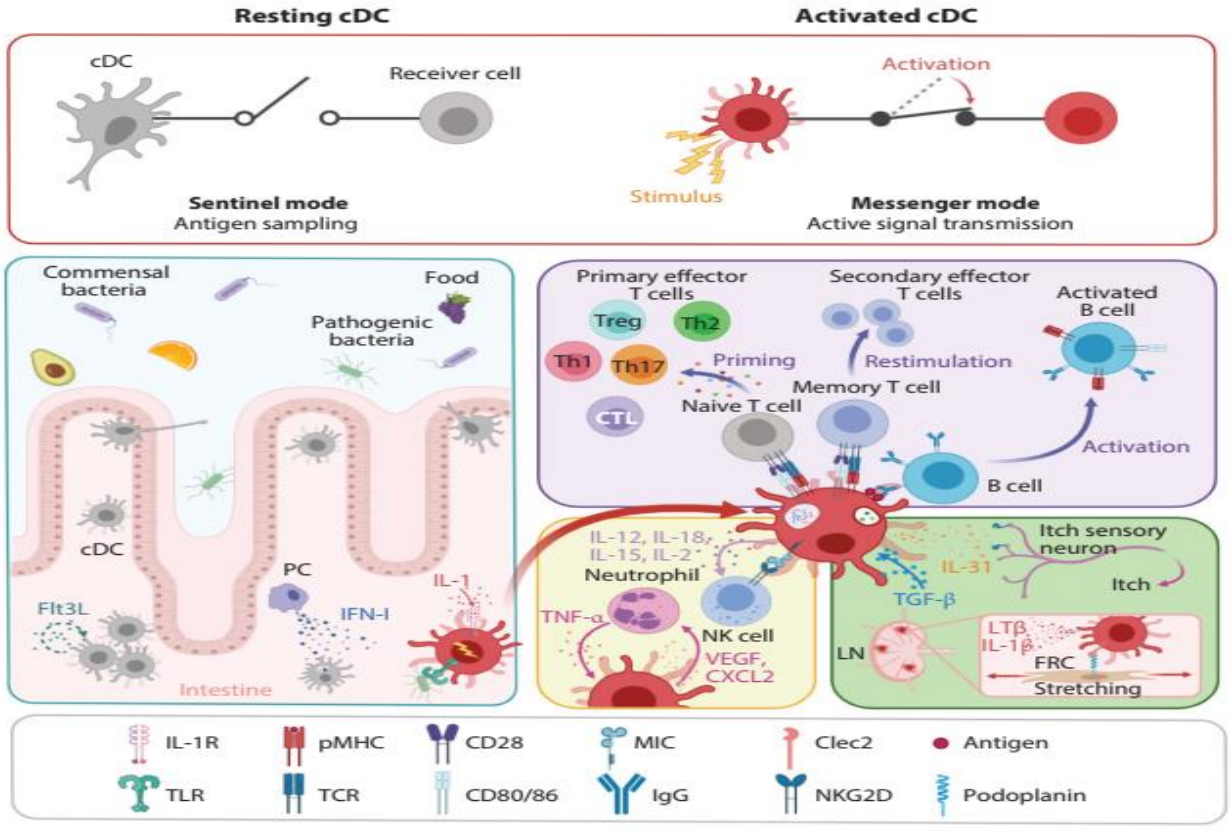


Figure 4. Activation of cDCs.

5.6. Antitumor activity of DCs

5.6.1. Activation of CD4 and TCD8 T cells by DCs

CD8+ T cells play a crucial role in anti-tumor immunity by facilitating the cross-presentation of Tumor-associated Antigens (TAA) by Dendritic Cells (DCs). CDC1s are often linked to improved antigen cross-presentation, leading to more robust CD8+ T cell immunity. Additionally, CDC1s may also contribute to the promotion of Th1 cell polarisation in CD4+ T cells. CDC1s have the ability to deliver tumor-associated antigens (TAAs) to T lymphocytes via a process called cross-presentation. In addition, cDC2s and moDCs have the ability to present antigens, and cDC2s are crucial for initiating immune responses by CD4+ T cells against tumours. In addition, both cDC2 and moDC play a crucial role in directly presenting or cross-presenting AATs after therapy with certain anticancer chemotherapies. CD80 and CD86, which are co-stimulatory molecules found in DCs, regulate the activation or inhibition of T lymphocytes via their interaction with CD28 or the cytotoxic LT4 antigen (CTLA4), respectively. Additional costimulatory pathways play a significant role in the initiation and reactivation of T cells by dendritic cells. T cell effector function relies on cytokines produced by dendritic cells (DCs), such as IL-12 and type I interferons (Fig. 5). IL-12 is primarily

produced by conventional type 1 dendritic cells (cDC1s) and plays a role in activating Th1 cells and priming CD8+ T cells. Additional costimulatory pathways implicated in dendritic cell-mediated T cell initiation and reactivation in cancer immunotherapy are CD40–CD40L, CD137–CD137L, and OX40–OX40L. CD137L, also referred to as 4-1BBL, is present on antigen-presenting cells (APCs) and enhances the activation and viability of CD4+ and CD8+ T cells by interacting with CD137. OX40L found on dendritic cells (DCs) and macrophages also plays a role in T cell survival, therefore supporting the development of immune responses against tumours.

The activity of T cells that carry out their function relies on the release of cytokines from dendritic cells, such as IL-12 and type I interferons (as shown in Figure 5). Monocyte-derived dendritic cells (MoDCs) are capable of producing interleukin-12 (IL-12) in response to immunogenic stimulation. However, it is primarily conventional dendritic cells type 1 (cDC1s) that are responsible for generating IL-12. IL-12 plays a crucial role in activating Th1 cells and priming CD8+ T cells. Both conventional dendritic cell 1 (cDC1) and conventional dendritic cell 2 (cDC2) have the ability to create IL-12 when their TLR-like receptors are stimulated. However, elevated levels of IL-12 in human malignancies are also linked to higher infiltration of cDC14. INF I is now used in clinical practise for the treatment of cancer. Additionally, the identification of nucleic acids via the cGAS-STING pathway has a role in activating dendritic cells and promoting the synthesis of type I interferon, which contributes to the immune response against tumours. Dendritic cells (DCs) are capable of generating chemokines inside the tumour microenvironment (TME) to recruit T lymphocytes. Tumor-infiltrating cDC1s are the primary sources of CXC-chemokine ligand 9 (CXCL9) and CXCL10 inside the tumour microenvironment (TME). Tumour microenvironment (TME) facilitates the recruitment of CD8+ T lymphocytes into the TME. CDC1s facilitate T cell reactivation inside the tumour microenvironment (TME). To summarise, dendritic cells (DCs) have a crucial function in the immune response to tumours. They shape the tumour microenvironment (TME) by releasing soluble substances and also attract, mediate, and activate T lymphocytes that fight against the tumour. These T cells infiltrate the tumour. CDC1s are the primary sources of CXC-chemokine ligand 9 (CXCL9) and CXCL10 in the tumour microenvironment (TME), hence facilitating the attraction of CD8+ T lymphocytes to the TME. CDC1s further facilitate T cell reactivation inside the tumour microenvironment (TME). To summarise, dendritic cells (DCs) have a crucial function in the immune response to tumours. They shape the tumour microenvironment (TME) by releasing soluble substances and also recruit, mediate, and activate T cells that fight against the tumour. Tumor-infiltrating conventional dendritic cells type 1 (cDC1s) are the primary sources of CXC-chemokine ligand 9 (CXCL9) and CXCL10 inside the tumour microenvironment (TME). These chemokines, in turn, facilitate the attraction of CD8+ T lymphocytes into the TME. CDC1s

further facilitate T cell reactivation inside the tumour microenvironment (TME). To summarise, dendritic cells (DCs) have a crucial function in the immune response to tumours. They do this by modifying the tumour microenvironment (TME) via the release of soluble substances, as well as by attracting, facilitating communication between, and preparing antitumor T cells for action.

Cancer cell variations may use dendritic cells (DCs) to facilitate immunological tolerance in response to antitumor immunity. Absence of costimulatory signals during the presentation of antigenic peptides leads to anergy in T8 cells, while excessive receptor engagement may restrict the effector function of T cells. CTLA4, when present on T cells, has a greater affinity for binding with CD80 or CD86 on DCs compared to CD28. This results in a restriction of co-stimulatory signalling and the activation of T cells. The programmed cell death ligands PDL1 and PDL2, present on dendritic cells (DCs) and other cells in the tumour microenvironment (TME), also suppress the growth and cytokine secretion of T cells via programmed cell death. Dendritic cells (DCs) may potentially influence T cell activity by modifying the accessibility of metabolic substrates. L-Tryptophan is necessary for T cell responses and is reduced by its conversion to l-kynurenine by the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) (Fig. 5). IDO1 is upregulated in dendritic cells (DCs) when they detect apoptotic cells or after the interaction between CTLA4 and CD80 or CD86. Significantly, there is a higher level of IDO1 expression in tumor-associated DC60s. The IDO1 produced by DCs inhibits the growth and activity of CD8+ T lymphocytes, natural killer (NK) cells, and plasma cells. Additionally, it has a role in the development of T reg cells. L-Tryptophan is necessary for T cell responses and is reduced by its conversion to l-kynurenine by the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) (Fig. 5). IDO1 is activated in dendritic cells (DCs) when they detect apoptotic cells or when CD80 or CD86 connect to CTLA4. Significantly, there is a higher level of IDO1 expression in DC60s linked with tumours, and the IDO1 produced by DCs inhibits the growth and activity of CD8+ T lymphocytes, natural killer (NK) cells, and plasma cells. Additionally, it has a role in the development of T reg cells. L-Tryptophan is necessary for T cell responses and is reduced by its conversion to l-kynurenine by the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) (Fig. 5). IDO1 is upregulated in dendritic cells (DCs) when they detect apoptotic cells or after the interaction between CTLA4 and CD80 or CD86. Significantly, there is a higher level of IDO1 expression in DC60s linked with tumours. The IDO1 produced by DCs inhibits the growth and activity of CD8+ T lymphocytes, natural killer (NK) cells, and plasma cells. Additionally, it has a role in the development of T reg cells.

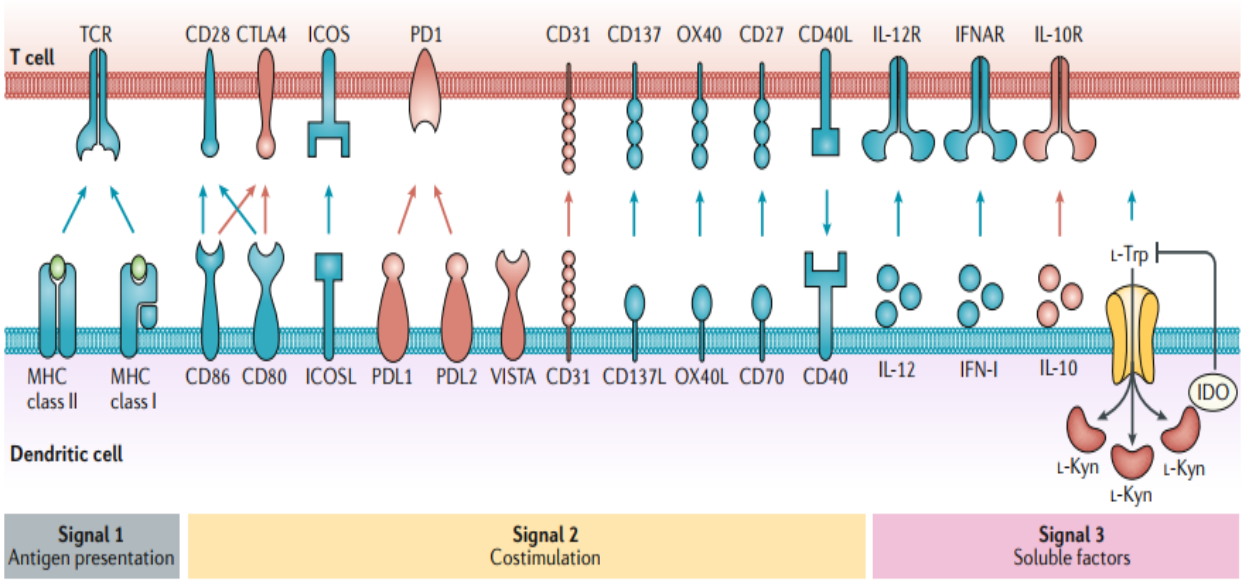


Figure 5. Activation of T lymphocytes by dendritic cells.

5.6.2. Regulation of DC activity by cancer cells

The fundamental components of DC biology that may be modified by tumours are shown. Tumours aim to decrease the anti-tumor immunity mediated by DCs by targeting the key characteristics of DC biology (Fig. 6). (1) Reduced presence of FMS-like ligand tyrosine kinase 3 (FLT3L) in the tumour microenvironment (TME), which might hinder the final maturation of pre-dendritic cells (pre-DCs). Likewise, cytokines (such as IL-6, IL-10, and TGFβ) as well as prostanoids and gangliosides generated from tumours might influence the production of bone marrow and dendritic cells in their original location. (2) Tumours may impede the infiltration of dendritic cells (DCs) by decreasing the expression of DC chemoattractants, such as CC-chemokine ligand 4 (CCL4), or by inhibiting the production of these chemoattractants by other cells, such as NK cells. (3) Tumours evade detection by dendritic cells (DCs) by restricting the secretion of stimulating molecular signals. For instance, the enzyme triple repair exonuclease 1 (TREX1) breaks down ATP alarmin, which in turn hinders the recruitment of moDC and T cells into the tumour microenvironment (TME). (4) Tumours may affect the maturation of dendritic cells (DCs) by directly producing substances that can interfere with the activation of certain pathways. These substances include IL-10, TGFβ, IL-6, or vascular endothelial growth factor (VEGF). Signalling occurs via the process of generating hyperphosphorylation of signal transducer and activator of transcription 3 (STAT3). Tumours may also indirectly impact the development of dendritic cells by secreting CSF1, which attracts tumor-associated macrophages that hinder the maturation of dendritic cells. (5) Tumours hinder the ability of dendritic cells (DCs) to handle, present, and cross-present tumor-associated antigens (TAAs) by promoting the buildup of partially degraded lipids. (6) Tumours modify the metabolism of dendritic

cells (DCs) in order to hinder their activity. This is achieved by promoting the buildup of fatty acids while reducing the availability of nutrients and oxygen. (7) Tumours may weaken the capacity of dendritic cells to survive by affecting parameters including the sensitivity to low oxygen levels and stress in the endoplasmic reticulum. (6) Tumours modify the metabolism of dendritic cells (DCs) in order to hinder their activity. This is achieved by promoting the buildup of fatty acids and reducing the availability of nutrients and oxygen. (7) Tumours may decrease the survival of dendritic cells by affecting variables including hypoxia response and endoplasmic reticulum stress. (6) Tumours modify the metabolism of dendritic cells (DCs) in order to hinder their activity. This is achieved by promoting the buildup of fatty acids and reducing the availability of nutrients and oxygen. (7) Tumours may decrease the survival of dendritic cells by affecting variables including hypoxia response and endoplasmic reticulum stress.

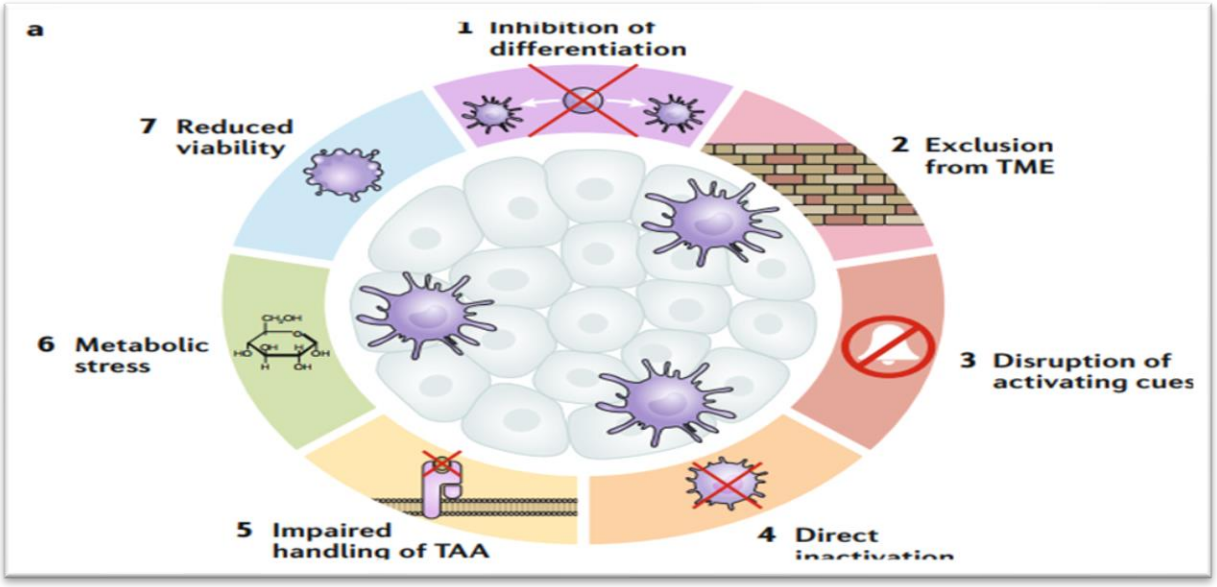


Figure 6 . Modulation of DC activity by cancer cells.

5.6.3. DCs and cancer therapy

Dendritic cells (DCs) have a crucial function in producing potent immune responses against tumours, which are initiated by various cancer treatment approaches. moDCs serve as immune mediators against tumours, activated by chemotherapy and localised radiation. Monocyte-derived dendritic cells (MoDCs) are actively attracted to the tumour microenvironment (TME) in response to the production of certain cell death mediators, including CCl2, CRT, and ATP. Once in the TME, MoDCs play a crucial role in initiating CD8+ lymphocyte responses (see Figure 7). cDC1 has a role in the effects that occur outside of the tumour microenvironment (TME) during radiation. The cGAS-STING pathway is activated by the detection of cytosolic DNA (cytDNA) originating from deceased

cancer cells. cDC1s play a crucial role in immune control during therapy because they have the ability to initiate T cell responses in the immediate area of the tumour and in the lymph nodes that drain the tumour. They also attract T cells to the tumour microenvironment (TME) and modify the TME by producing soluble factors like XCL1, CCL4-5, CXCL9-10, CCR7, FLT3L, INF1, and IL-12. Cell treatment of cancer employs many techniques, including the use of dendritic cells (DCs) and cytokines that stimulate the mobilisation of DCs. Additionally, immunostimulatory adjuvants such as CPG and Poly-I:C, as well as drugs that inhibit the immunosuppressive effects of DCs, are used. The GM-CSF factor directly promotes the differentiation, activation, and migration of dendritic cells. Talimogene laherparepvec (Imlygic, T-VEC) is a modified form of the herpes simplex virus that has been weakened and engineered to produce human GM-CSF. It has been authorised for use in stimulating the body's immune system to fight against tumours and improve the survival rates of individuals with advanced melanoma. Moreover, the use of allogeneic or autologous irradiation tumour cells that have been genetically modified to produce GM-CSF (GVAX vaccines) has shown promising results in preclinical studies involving prostate cancer. Positive findings indicate that the injection of FLT3L enhances tumour immunity by stimulating CD8+ T cells in mouse cancer models. Adjuvants that promote the activation of immunogenic dendritic cells (DCs) are now being extensively researched, especially derivatives of ligands that bind to Toll-like receptors (TLRs) produced by DCs. The intravesical infusion of bacillus Calmette-Guérin (BCG) is linked to a rise in the viability and activation of dendritic cells (DC). The effectiveness of the synthetic TLR3 agonist poly(I:C), which may also activate melanoma-associated differentiation protein 5 (MDA5) and retinoic acid-inducible gene I (RIG-I), has been identified as a possible treatment in cancer immunotherapy. The treatment primarily targets human CD141+ cDC1s because of their high degree of TLR3 expression. Preclinical in vitro studies demonstrate the remarkable effectiveness of poly(I:C) in stimulating dendritic cells (DCs) and causing the creation of pro-inflammatory cytokines. This stimulation also leads to the activation of LTh1 and natural killer (NK) cells, as well as cross-presentation and the generation of anticancer CD8+ T cell responses. Ultimately, this treatment shows promise in treating cancer. Imiquimod, a ligand for TLR7/TLR8, has received approval for local therapy of non-melanoma skin malignancies due to its ability to promote cytotoxicity. TLR7/TLR8 agonists are expected to affect all naturally occurring DC subtypes, stimulating the release of pro-inflammatory cytokines NF-κB and. Unmethylated oligodeoxynucleotides (CpGs) are a diverse set of molecules that may activate human plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) in the body. This activation leads to the stimulation of Th1 cell-mediated immune response and the generation of cancer-specific CD8+ T cell response. An alternative strategy to enhance the function of DCs is to overcome the immunosuppressive actions associated with

malignancy. IDO inhibition is now being investigated in mice and clinical studies. Furthermore, the assessment of STAT3 inhibitors, which have the ability to enhance dendritic cell maturation and immunogenic actions, is now underway. The administration of AATs in living organisms, which may be shown or cross-displayed by naturally occurring DCs, has traditionally considered a promising approach for cancer immunotherapy. The vaccines primarily consist of tumor-associated antigens (TAAs) that are administered as either short or lengthy synthetic peptides, recombinant viruses that express TAAs, or complete tumour lysates. Patients diagnosed with lung cancer or melanoma and exhibiting a significant mutational burden have a heightened response rate to immune checkpoint blockade (ICB), accompanied by an augmentation in dendritic cells (DC), T cells, and CD8+ infiltrates. A strategy often used in cancer treatment is the utilisation of a mix of tumor-associated antigens (TAA) and adjuvants. TAA and/or adjuvants may be included and enclosed inside particulate delivery vehicles, such as single and supramolecular peptide conjugates (e.g., nanofibers, gels, or nanoparticles), liposomes, virosomes, or immunostimulatory complexes. Utilising self-assembling polymer biomaterials or degradable nanoparticles in anticancer treatment has the inherent potential to enhance immunogenic characteristics. Concerning dendritic cells (DCs), nanoparticles of medium size (5-100 nm) have the most efficiency in reaching the lymph node. Adjuvants with a negative charge, such as poly(I:C) and unmethylated CpG oligodeoxynucleotides, may be readily internalised into nanoparticles with a positive charge. It is worth mentioning that nanoparticles with a negative charge, such as the FDA-approved Poly(lactic-co-glycolic acid) (PLGA), have the ability to enhance the maturation of dendritic cells, the cross-presentation of antigens, and the polarisation of Th1 immune response. In addition, TAA-encoded viral vector vaccines are frequently engineered to simultaneously express costimulatory molecules (such as CD80, CD54, and CD58 in the TRICOM vector) or DC activating factors (such as poly(I :C) or GM-CSF). Recently, the FDA approved these vaccines for the treatment of hepatocellular carcinoma and pancreatic cancer. In general, there is still much to be discovered about the most effective antigens, adjuvants, and formulation of cancer vaccines that are based on tumor-associated antigens (TAA) that target dendritic cells (DCs) to stimulate T-cell-mediated immunity against cancer. Directing antigens and adjuvants specifically to dendritic cells (DCs) in the body may improve the immune response against tumours. C-type lectin receptors (CLRs) are expressed in various patterns on dendritic cells (DCs) and have been used as the primary receptors for targeting. Examples of targeting specific types of dendritic cells (DCs) include the use of DEC205, CLEC9A, and langerin to target cDC1s; the use of CLEC4A4 (also known as DCIR2) to target cDC2s; the use of CLEC7A (also known as dectin 1) to target cDC2s and MoDCs; the use of CD209 (also known as DC-SIGN), the mannose receptor (MR) and macrophage galactose-like lectin (MGL) to primarily target cDC2, MoDC and

macrophages; and the use of CLEC12A to target multiple DC subtypes, including cDCs, pDCs, and MoDCs. It is worth mentioning that the antibody-conjugated antigen with adjuvant functioned better than the unconjugated antigen.

The use of Anti-DEC205 antibodies may specifically bind to the MAGEA3 antigen on human MoDCs, therefore inducing CD4+ T cell responses. The fusion of full-length New York oesophagal squamous cell carcinoma 1 (NY-ESO-1) antigen with anti-DEC205 antibodies enhances the activation of CD8+ T cells, in contrast to NY-ESO-1 that is not linked with anti-DEC205 antibodies. A clinical experiment in its first phase showed that the treatment of cancer patients with dermally applied NY-ESO-1, combined with antiDEC205 along with resiquimod and/or poly-ICLC, resulted in the production of antibodies specific to antigens and lymphocytes T. This therapy also led to positive clinical responses without any harmful effects. Human primary monocyte-derived dendritic cells (MoDCs) that were exposed to antigens conjugated with CD209/DC-SIGN, together with adjuvants, induce targeted T cell responses both in vitro and in humanised mice, hence restricting the progression of cancer. Protein antigens produced from blood, namely pDCs, cDC1, and cDC2, may be effectively targeted outside of the body using anti-CLEC12A antibody conjugated to elicit cross-presentation and activation of CD8+ T cells. Furthermore, TAAs may also be linked to non-CLR receptors that are present on DCs or their ligands, such as CD40. For instance, when viral antigen is combined with anti-CD40 and anti-MR antibodies, it efficiently enhances the ability of cDC2s and MoDCs to deliver antigens to other cells outside of the body. Administering oxidised mannan-conjugated MUC1 antigen, which targets the mannose receptor (MR) on dendritic cells (DCs), stimulates the production of specific antibodies and CD8+ T cell responses in breast cancer patients. Delivery of antigens combined with anti-CD40 antibodies may stimulate DCs by binding to CD40 and perhaps improve cross-presentation by reducing the breakdown of antigens in endosomes, but this effect is less specific to DCs in general. Anti-CD40-mediated targeting of the MART1 peptide to MoDCs in vitro is more effective than CLR-targeting antibodies in stimulating CD8+ T cell responses. However, it is less powerful in activating CD4+ T cells. In addition, the introduction of human fusion anti-CD40 papillomavirus antigen to peripheral blood mononuclear cells of cancer patients activates T cells and stimulates CD8+ T cell immunity. This leads to the regulation of cancer development in human CD40 knock-in mice. Moreover, the process of linking anti-CD40 antibodies to adenovirus-based vectors that encode tumor-associated antigens (TAA) is now underway. Intended to precisely target cutaneous dendritic cells. The use of polymer nanoparticles is an appealing strategy due to the potential limitations in the fusion of AAT and adjuvants to these targeted molecules. Human monocyte-derived dendritic cells (MoDCs) effectively internalise poly(lactic-co-glycolic acid) (PLGA) nanoparticles that are coated with anti-DEC205 antibody and loaded with

MART1 peptide. This internalisation leads to an increase in cross-priming activity as compared to exposure to non-targeted nanoparticles. In addition, PLGA nanoparticles coated with anti-CLEC9A and containing a lengthy synthetic peptide GP100 elicit a stronger activation of CD8+ T cells ex vivo in human primary blood CD141+ cDC1, compared to nanoparticles coated with isotype.

DC vaccines against cancer

DC vaccines have undergone extensive investigation for their use in cancer treatment, with more than 200 clinical studies successfully completed so far. This method entails the separation or production and subsequent in vitro amplification of a person's own dendritic cells (DCs), which are then manipulated outside the body and reintroduced into the patients. The studies primarily focused on patients with melanoma, prostate cancer, glioblastoma, or renal cell carcinoma, as these types of cancers are known to have an immunogenic nature. Importantly, these studies showed that vaccination against dendritic cells (DCs) is both clinically safe and effective in stimulating the production of anticancer natural killer (NK) cells, CD8+ T cells, and CD4+ T cell responses. Moreover, given that the majority of the participants were suffering from advanced disease after unsuccessful prior therapies, the notable average overall response rate of 8-15% is commendable. The only APC-based vaccination that has received clinical approval so far is sipuleucel-T (Provenge). This vaccine comprises autologous blood APCs that are loaded with a recombinant fusion protein antigen consisting of prostatic acid phosphatase and the GM-CSF. Evidence demonstrates that it increases the median overall survival of individuals with prostate cancer by roughly 4 months.

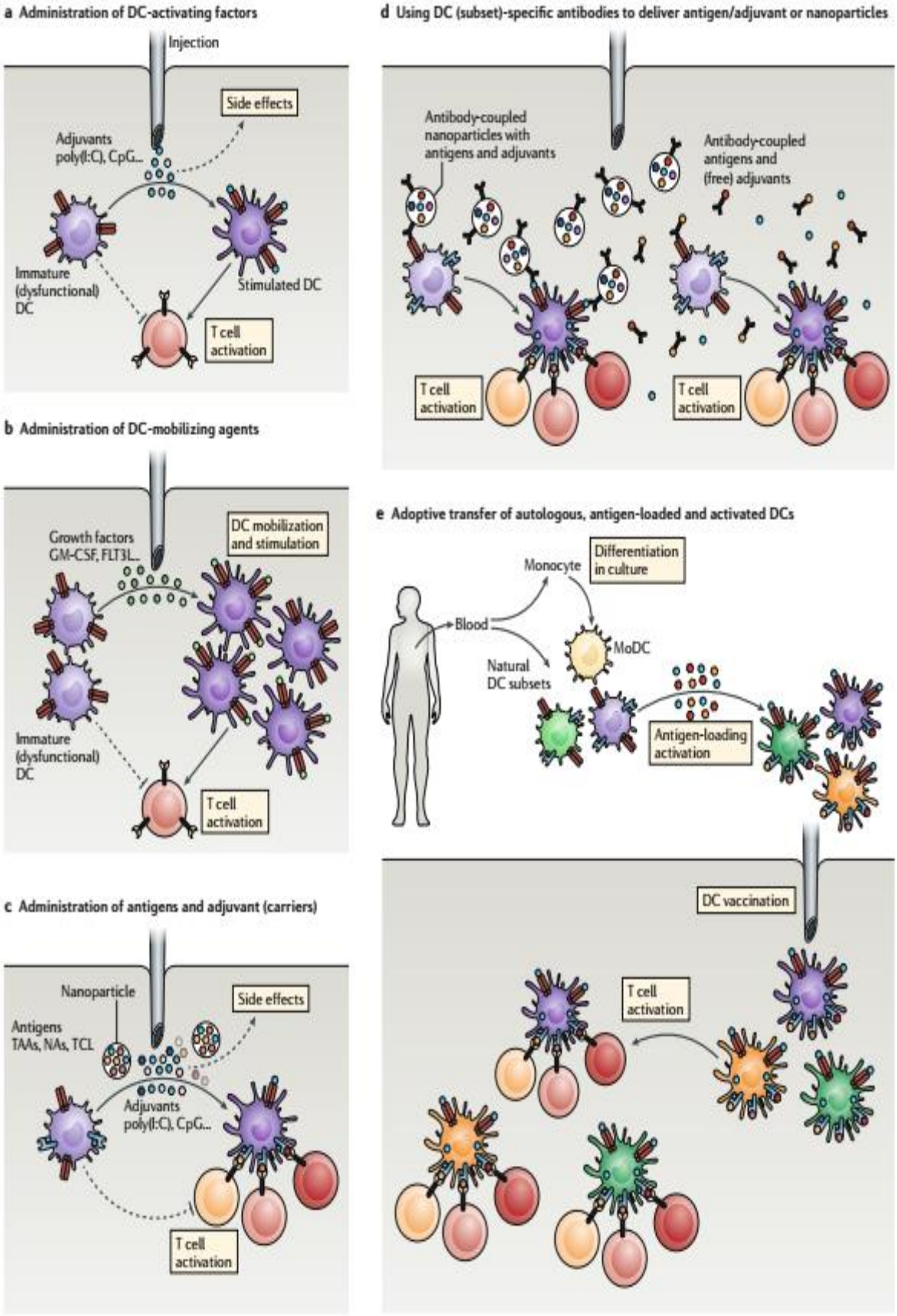


Figure 7. Employing dendritic cells for cancer treatment.

Compound	Features	Effects on DCs and Sys. Immu.
GM-CSF	Essential cytokine on DC development	Mobilization, attraction and maturation of DCs
FLT3L	Essential cytokine on DC development	Mobilization/Expansion of DCs1 and DCs2
TLR4/TLR2 agonists	Synthetic ligands or microbial derivatives of PRR ligands	Activation of DCs2 (release of cytokines), induction of LTCD8+, Prolong the survival of immune cells
TLR3 agonists	Synthetic PRR ligands	Activation of DCs1 (release of cytokines) and direct cytotoxicity on cancer cells, induction of LTh1, LTCD8+ and NK
TLR7/TLR8 agonists	Variable ligands for PRR like Imidazoquinoline	Activation of DCs1 and pDCs (release of cytokines), induction of the antitumor activity of DCs, induction of LTCD8+.
TLR9 agonists	Synthetic PRR ligands, unmethylated CpG oligodeoxynucleotides	Activation of DCs1 and pDCs (release of cytokines), induction of LTh1 and LTCD8+.
IDO inhibitors	Targeted against IDO	Inhibition of L-Trp depletion, intervenes in tolerance with an anergic effect of LT cells
STAT3 inhibitors	Small molecules /Acmonoclonal agents that block the signal via STAT3	Activation of DCs and inhibition of suppression of immune activity ofDCs.

5.7. DC and Tolerence

Recently, there has occurred a change in how dendritic cells (DCs) are understood. They are no longer seen just as initiators of immunological responses but are also seen as important controllers of immunity. This includes their role in both triggering and sustaining tolerance. Progress both comprehending the adaptable characteristics and functional flexibility of DCs, as effectively as their capacity to control its growth and maturity in laboratory settings and living organisms, has laid the foundation for using their natural potential to induce tolerance for therapeutic purposes. This Review aims to consolidate the existing knowledge on the involvement of dendritic cells (DCs) in the process of inducing tolerance, with a specific emphasis on transplantation. Dendritic cells (DCs) are now widely acknowledged as significant regulators of the immune system, playing a vital role in both initiating and maintaining tolerance. Gene-transfer technology has made significant progress in improving the ability of DCs to induce tolerance. DCs may now be genetically modified to produce 'immunosuppressive' molecules, such as IL-10, TGFβ1, or CTLA4-immunoglobulin fusion protein. Additionally, these dendritic cells have the ability to inhibit the activation of NF-κB, hinder the growth of allogeneic T cells, induce and sustain T-cell unresponsiveness, or facilitate the elimination

of antigen-specific T cells by expressing CD95 ligand (CD95L) or TNF-related apoptosis-inducing ligand (TRAIL).

Thus far, there have been no documented instances of successfully developing tolerance across major histocompatibility complex (MHC) barriers solely via the use of genetically engineered dendritic cells (DCs). Nevertheless, the longevity of cardiac allografts may be significantly extended by administering a solitary infusion of donor myeloid DCs that have been treated with NF-κBspecific 'decoy' oligodeoxynucleotides (ODNs) and transduced with a CTLA4–immunoglobulin-fusion protein prior to transplantation. Mouse dendritic cells (DCs) that had their IL-12 synthesis suppressed using RNA interference shown a decrease in their ability to stimulate immune responses and instead facilitated the development of TH2 cells in a particular way related to the antigen.

Dendritic cells (DCs) found in both blood and non-lymphoid tissues exhibit an immature phenotype. These DCs can be increased in number by administering specific growth factors, either individually or in combination. Examples of such growth factors include granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), and FMS-like tyrosine kinase 3 ligand (FLT3L). The administration of donor haematopoietic cell infusions, together with cyclophosphamide and T-cell-depleted donor bone marrow, leads to the expansion of mice DCs. This expansion increases tolerance to donor-specific, MHC-mismatched skin transplants. Allogeneic bone marrow cells that have been increased with FLT3L significantly enhance the presence of hematopoietic cell chimerism, especially in the form of donor dendritic cells. However, this might hinder the beneficial effects of donor bone marrow cells in heart transplant patients who are temporarily immunosuppressed.

Transplanting hematopoietic stem cells from donors who have received G-CSF into people is linked to a reduced occurrence of GVHD. G-CSF enhances the proportion of pDCs in blood stem cell products, while leaving the quantity of myeloid DCs unchanged. After reaching maturity, the pDCs increased by G-CSF stimulate allogeneic TH2-cell reactions, whereas myeloid DCs provoke TH1-cell reactions. In general, the increase of dendritic cells (DCs) caused by growth factors in either the donor or host tissue has shown inconsistent results, resulting in either tolerance or the worsening of rejection, depending on the specific model being studied.

The figure 8 depicts the function of splenic dendritic cells (DCs) in controlling T-cell responses. Following exposure to danger signals or stimuli, immature splenic CD8- DCs migrate to T-cell regions of the spleen, enhancing the expression of MHC and co-stimulatory molecules (CD80/CD86) on their surface, releasing IL-12p70, and improving their capacity to activate naïve or memory T cells. The engagement of CD154 (CD40L) with CD40 on the surface of dendritic cells (DCs) leads to further stimulation and maturation of both populations of DCs. CD8- mature dendritic cells (DCs)

may promote the proliferation of regulatory T cells, which act as a feedback mechanism to suppress the T-cell response. This helps to avoid the breakdown of peripheral tolerance towards self-antigens that are presented alongside foreign antigens by the same mature DC. During the steady state, CD8- and CD8-+ DCs stay inactive after capturing and digesting external antigen. These inactive dendritic cells exhibit minimal quantities of co-stimulatory molecules, resulting in inadequate activation of naïve T cells and either cell death or a state of unresponsiveness. This may potentially lead to the production and/or proliferation of regulatory T cells. The binding of CD80/CD86 on the cell surface to cytotoxic T-lymphocyte antigen 4 increases the production of functional indoleamine 2,3-dioxygenase (IDO), an enzyme that breaks down the important amino acid tryptophan. This depletion of tryptophan inhibits the proliferation of T-cells and leads to the production of metabolites derived from tryptophan that induce T-cell apoptosis. Splenic CD8-+ dendritic cells (DCs) express the functional CD95 ligand (CD95L), which has the ability to induce T-cell apoptosis.

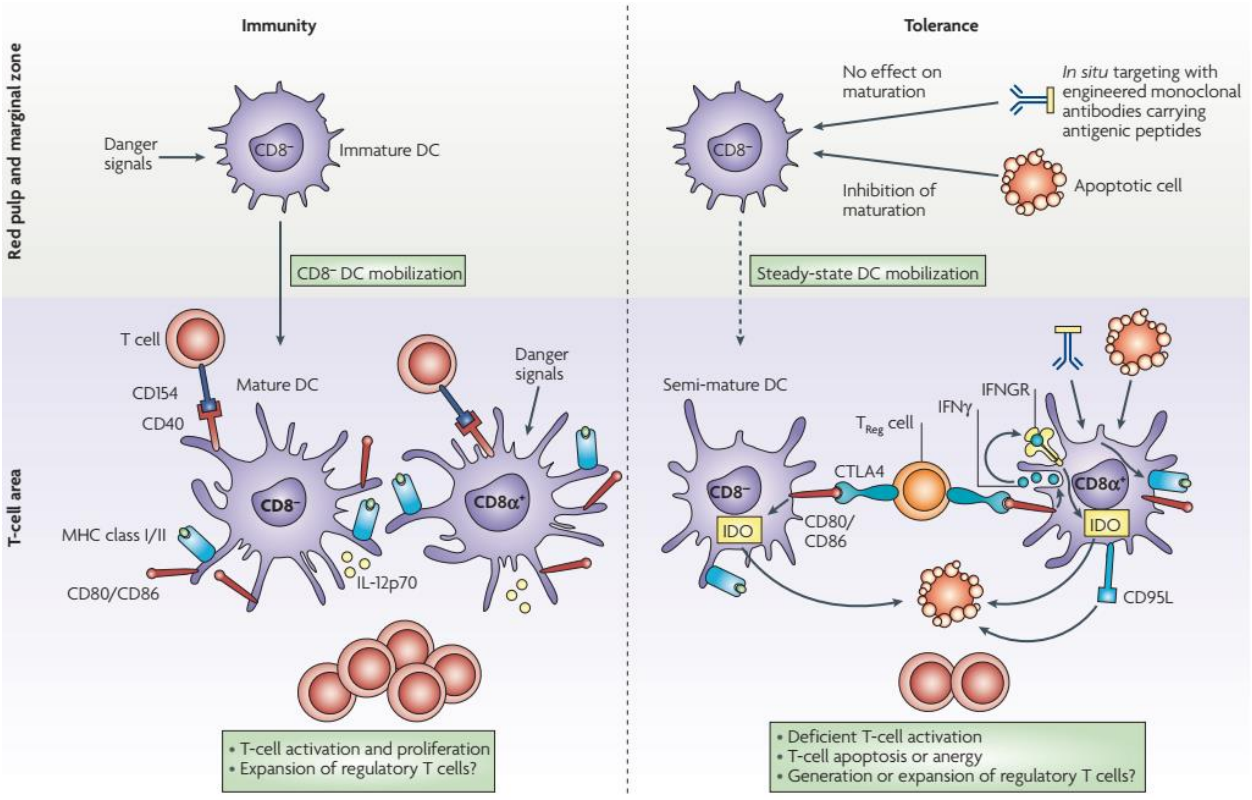


Figure 8 Functions of CD8– (myeloid) and CD8α+ dendritic cells (DCs) in the secondary lymphoid organs of mice are to regulate T-cell immunity and peripheral tolerance.

Mouse dendritic cells (DCs) that express indoleamine 2,3-dioxygenase (IDO) and CD19 have a morphology similar to plasma cells (Fig. 9). These DCs are found in the splenic red pulp and have a mature antigen-presenting cell (APC) phenotype. They also have the ability to stimulate T-cells. In another scenario, IDO+CD19+ DCs have the ability to inhibit the growth of T-cells and induce their death. This is achieved by producing significant quantities of functional IDO in response to the

activation of CD80/CD86 or Toll-like receptor 9 (TLR9) by cytotoxic T-lymphocyte antigen 4 (CTLA4). CTLA4 can exist in a soluble form as the CTLA4-immunoglobulin fusion protein or can be found on the surface of TReg cells. Additionally, the production of IDO can also be triggered by unmethylated CpG-DNA motifs. Plasmacytoid DCs, unlike IDO+CD19+ DCs, are immature DCs found in the T-cell areas of secondary lymphoid organs. They can take on the appearance of mature DCs, produce significant quantities of type I interferons (IFNs), and exhibit T-cell stimulating abilities when exposed to specific viral infections or when stimulated through the interleukin-3 receptor (IL-3R) and CD40 signalling. Conversely, plasmacytoid DCs have the ability to produce substantial quantities of functional IDO when exposed to ligands that activate TLR9 and the CD200R. The initiation of IDO production by IDO+CD19+ dendritic cells (DCs) and plasmacytoid DCs is dependent on the self-secretion of type I interferons (IFNs). Signalling via TLR9 leads to the increase of inducible T-cell co-stimulator ligand (ICOSL) in human developing plasmacytoid DCs. This enables them to create CD4+ regulatory T cells that secrete IL-10. The IFNAR, also known as the type I IFN receptor

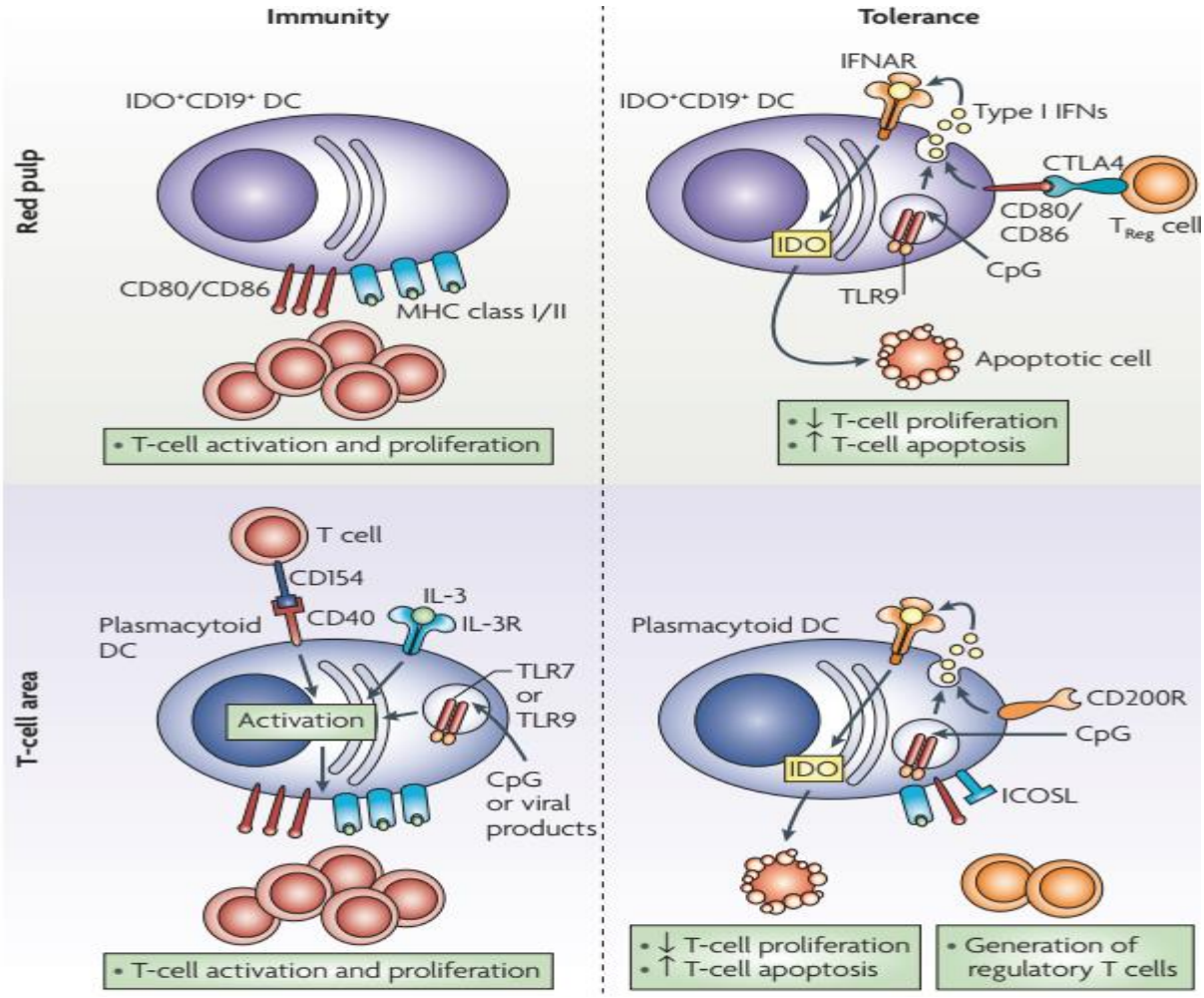


Figure 9 function of IDO+CD19+ dendritic cells (DCs) and plasmacytoid DCs in T-cell immunity and peripheral tolerance

5.7.1. Generation of TolDCs

Tolerogenic dendritic cells (tolDCs) of human origin are mostly generated by growing peripheral blood monocytes in the presence of GM-CSF and IL-4, together with a specific chemical that imparts tolerogenic characteristics. Immature dendritic cells (DCs) are first produced in murine systems by cultivating bone marrow cells with GM-CSF and IL-4. These immature DCs are subsequently transformed into tolerogenic DCs by further culture with a specific chemical that imparts tolerogenic characteristics.

TolDCs have been generated ex vivo from hematopoietic precursors or peripheral blood monocytes using various pharmacological and biological agents. Figure 10 illustrates the primary techniques used for the production of tolerogenic dendritic cells (tolDCs) outside of the body, as well as the typical characteristics shown by tolDCs. Pharmacological substances that are known to stimulate tolerogenic dendritic cells (tolDCs) include vitamin D3, corticosteroid, rapamycin, cyclosporine, tacrolimus, aspirin, atorvastatin, retinoic acid, mycophenolic acid, and minocycline. Vitamin D, dexamethasone, and rapamycin have undergone thorough investigation in both animal models and human subjects to develop therapeutic strategies for preventing transplant rejection and treating autoimmune and chronic inflammatory disorders. Corticosteroids, such as dexamethasone and prednisolone, have been recognised for their ability to reduce inflammation and inhibit the immune system. Dexamethasone stimulates the production of immune cells called tolerogenic macrophages and maintains their ability to induce tolerance for an extended period of time, lasting up to one week, even when dexamethasone is no longer present. Rapamycin is well recognised for its ability to block T cell activation by targeting the serine/threonine protein kinase called mammalian target of rapamycin. Administering rapamycin-generated dendritic cells (DCs) to mouse heart transplantation recipients enhances the longevity of the transplanted organ. This improvement is associated with an augmented generation of Foxp3+ regulatory T cells (Tregs) in the recipient animals.

An inherent limitation of producing tolDCs by the use of these medicines is the cytotoxic impact exerted by these medications. For example, the combination of rapamycin (10 ng/mL), GM-CSF, and IL-4 effectively generates tolDCs from bone marrow cells. Nevertheless, the quantity of CD11c+ cells acquired from rapamycin-conditioned cultures is notably inferior compared to that from rapamycin-unconditioned cultures. Dexamethasone has shown a significant decrease in the recovery of dendritic cells. Minocycline has the distinctive ability to enhance the production of tolDCs from bone marrow cells. Additionally, it stimulates the proliferation of DCs that have been exposed to high doses of rapamycin, vitamin D3, or IL-10, which may be harmful. The tolerogenicity of tolDCs produced in the presence of both minocycline and dexamethasone is either superior or at least

comparable to that of tolDCs generated with either minocycline or dexamethasone alone. TolDCs with powerful tolerogenic qualities may be produced by using combinations of pharmacological substances.

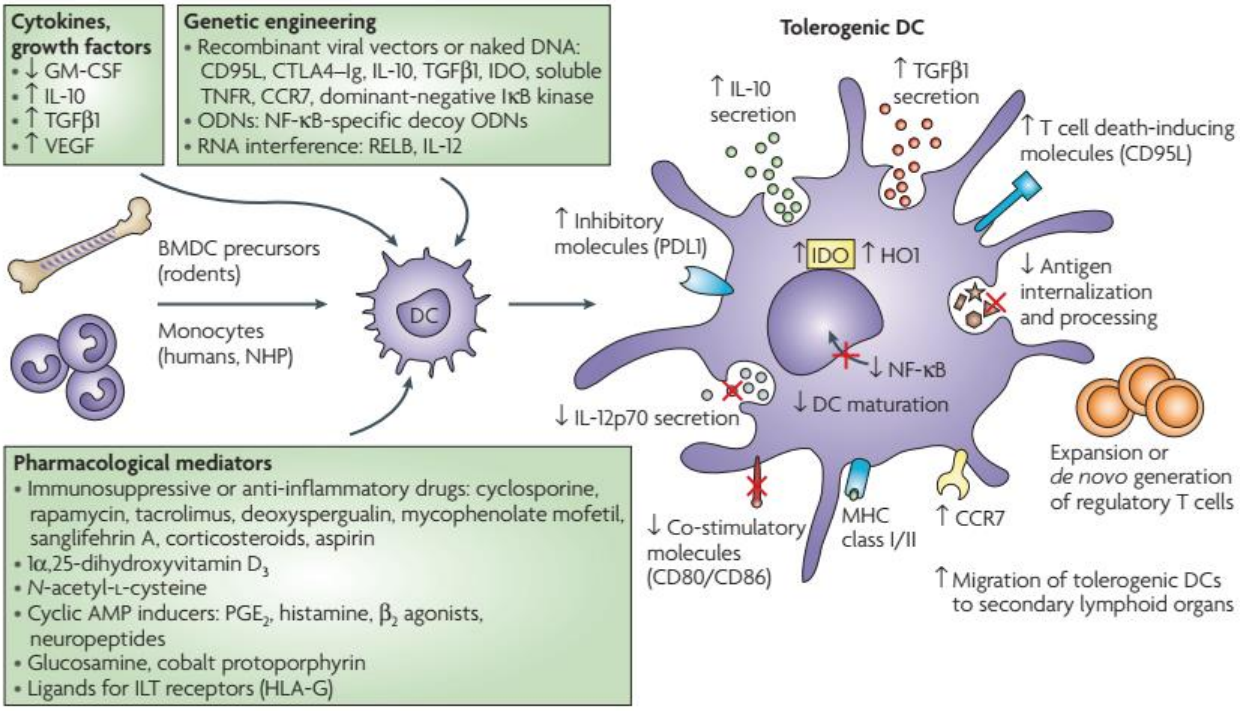


Figure 10 TolDCs generation

5.7.2. TolDC and Chronic diseases

Since the introduction of the first dendritic cell (DC) vaccines in 1995, there has been an increasing demand for DC treatment. This is mostly owing to their proven safety and potential efficacy, as evidenced in both animal models and human studies. Tol-MoDCs have been used in four completed clinical studies for the treatment of autoimmune disorders. In the first experiment, a group of patients with type 1 diabetes who need insulin were divided into two subgroups. Seven patients were administered modified Tol-MoDCs, whereas three patients got unmodified Tol-MoDCs. There were no negative consequences detected. In the second study, a group of patients with rheumatoid arthritis were selected. Specifically, 18 patients who tested positive for HLA got different amounts of Tol-MoDCs. These cells were treated with an NF-κB inhibitor and exposed to citrullinated peptides.

In the third phase, the study specifically targeted individuals with refractory Crohn's disease. A total of 12 patients were either a single dosage or biweekly doses of 2, 5, or 10 million Tol-MoDCs. The majority of patients did not experience any negative effects; however, three individuals discontinued treatment owing to a deterioration of their condition. In the most recent research, conducted on patients with rheumatoid and inflammatory arthritis, 13 individuals were administered either 1, 3, or 10 million cells, while three others were given a saline solution. The therapy was

shown to be both safe and practical, as evidenced by the steady hypertrophy, vascularity, and synovitis seen in all cohorts, including the placebo-treated individuals.

Additional continuing clinical studies are targeting different conditions, such as allergies or multiple sclerosis. A particular experiment is assessing the safety of autologous Tol-MoDCs in patients who are undergoing live donor kidney transplantation and are getting reduced immunosuppression. The Tol-MoDCs have a limited ability to activate allogenic T cells and actively inhibit the growth of activated T cells. Patients are administered Tol-MoDCs intravenously the day before to transplantation, at a dosage of one million cells per kilogramme.

The potential of Tol-MoDC therapy as a therapeutic method for numerous illnesses is becoming more intriguing. However, more clinical studies are necessary to ascertain the optimal dosage, injection circumstances, and accompanying medications for effective treatment.

Figure 11 TolDC and Chronic diseases treatement

TABLE 1 | Completed clinical trials.

Differentiation protocol	Disease	Patients	Cohorts	Biological effect/safety
Tolerogenic monocyte-derived dendritic cells (Tol-MoDC) modified with oligonucleotides (ODN) anti-CD40/80/86	Type 1 diabetes	10	• Unmodified Tol-MoDC • ODN Tol-MoDC	• Increase in B220 B cells in blood • No adverse effects
Tol-MoDC treated with nuclear factor-κB inhibitor and pulsed with citrullinated peptides	Rheumatoid arthritis	18	• Low dose (1 million cells) • High dose (5 millions cells)	• Increase in Treg in blood • Decrease in T-cell response to vimentin 447–455 Cit450 • No adverse effects
Tol-MoDC differentiated with dexamethasone (Dex) and IL-6, TNF-α, IL-1β, and prostaglandin E2	Refractory Crohn's disease	12	• 2, 5 or 10 millions cells • Single dose or biweekly	• Increase in Treg in blood • Decrease in interferon-γ (IFN-γ) in blood • Three patients withdrew due to disease worsening
Tol-MoDC differentiated with Dex and loaded with autologous synovial fluid	Rheumatoid and inflammatory arthritis	13	• 1, 3, 10 millions cells	• No biological effect in blood • No adverse effects

Table. Treatement of some chronic diseases using TolDC

6. Natural Killer cells (NK) Generality

The discovery of NK cells took place early in 1970s when Rolf Kiessling and Ronald Herberman identified them as lymphocyte cells that lacked the characteristics of B and T cells. These cells were shown to possess intrinsic cytotoxicity towards tumour cells. In contrast to T or B lymphocytes, NK cells serve as effectors of the innate immune system and do not need a preceding stimulation or "priming" phase to facilitate their functionalities. Therefore, they serve as an initial barrier that may be promptly activated and have a significant impact on immune surveillance against viral infections and tumour progression. NK cells lack antigen-specific receptors resulting from gene rearrangement, such as the BCR or the TCR. Nevertheless, they possess a diverse array of inhibitory receptors or activators that serve to differentiate between "self" and "non-self" (and/or modified self) and to identify various danger signals. The determination of the response of NK cells is contingent upon the integration of all information received through these receptors. NK cells, although serving as effectors of innate immunity, take a role in the modulation of adaptive immunity by means of cytokine release and their capacity to communicate and engage with other immune cells, including dendritic cells and T lymphocytes. NK cells also perform other tasks that are important in organs hematopoietic stem cell transplantation. NK cells are a kind of lymphocyte characterised by their large size and granular appearance. In humans, they typically constitute a proportion ranging from 2% to 20% of lymphocytes found in the peripheral circulation. The distribution of these cells encompasses a broad range of tissues, and they may be located in several organs, both lymphoid and non-lymphoid in nature. The primary location for their formation is the bone marrow; however, multiple studies have shown that additional organs have the potential to serve as anatomical locations for the growth and maturation of NK cells derived from hematopoietic progenitors.

6.1. NK cell development and maturation

Although it has been noted that the bone marrow serves as a clearly defined location for the formation of NK cells, new research showed that the lymph nodes (LN) may play an important role in of NK cell differentiation. In a recent study, Freud et al. made the observation that LN exhibit a spontaneous and selective enrichment of CD34dimCD45RA+ hematopoietic progenitor cells (HPC). These LN-derived cells have the ability to develop into CD56bright NK cells when exposed to either IL-2 or interleukin-15 (IL-15). Furthermore, it should be noted that CD34dimCD45RA+ HPC has a significantly elevated expression level of adhesion proteins, including alpha4/beta7-integrin and L-selectin. The researchers furthermore provided evidence that CD34dim CD45RA+ HPC make up around 1%, 6%, and 95% of the CD34+ HPC population in bone marrow, blood, and LN, respectively. Moreover, it has been shown that LN CD34dim CD45RA+ HPC are spatially associated

with CD56bright NK cells, namely in the parafollicular region which is characterised by a high concentration of T cells. According to the provided findings, the co-cultivation of LN-activated T cells with autologous LN CD34dim HPC resulted in the development of CD56bright NK cells after a period of 7 days. Furthermore, Fehniger and colleagues demonstrated that the production of IL-2 by T cells inside the body may stimulate CD56bright NK cells to release IFN-γamma, using NK cells' IL-2 receptor high-affinity. Therefore, the observed preferential accumulation of CD34dimCD45RA+ HPC and CD56bright NK cells in the LN compared to the BM or blood indicates that the LN could serve as a location for the formation of NK cells in the living organism. Two different subgroups of human NK cells have been identified based on the cell surface density of CD56. The predominant subset of NK cells in humans is characterised by the CD56dim phenotype, which is associated with elevated expression of CD16 and killer cell immunoglobulin-like receptor (KIR). Conversely, a smaller subset of NK cells, identified as CD56brightCD16dim/neg, exhibit lower cytotoxic activity but increased production of IFN-γ, upon monokine costimulation. The NK cell subpopulation in question also exhibits the expression of c-kit, a molecular marker on the surface of CD34+ HPC cells. The expression of c-kit enhances the IL-2-induced cell growth and induces the excessive production of the bcl-2 protein, which prevents cell death. CD56bright NK cells are the only lymphocytes that naturally express the high-affinity IL-2R. In contrast, CD56dim NK cells only express the intermediate affinity IL-2R and do not have c-kit expression. The aforementioned studies may provide a partial explanation for the differential presence of CD34+ HPC in LN, namely in CD56bright NK cells, as opposed to CD56dim NK cells which exhibit limited proliferation in response to high concentrations of IL-2. The hypothesis has been put up that CD56dim NK cells may be a second phase in the development of CD56bright NK cell subsets, after their identification. The aforementioned evidence indicates that the expression of the c-kit immature cell marker is exclusive to CD56bright NK cells, hence providing support for this idea. Moreover, Romagnani et al. have provided evidence indicating that CD56dim NK cells obtained from peripheral blood have shorter telomeres compared to CD56bright NK cells collected from both peripheral blood and LN. In a similar vein, Ferlazzo et al. have shown the presence of discrete subsets of NK cells, noting that over 95% of CD3-CD56+ cells in the bloodstream exhibit a CD56dimCD16+ phenotype. Conversely, in LN, around 90% of the same cells predominantly display a CD56brightCD16- phenotype. Collectively, these results provide the basis for postulating a potential process by which NK cells might undergo development. Nevertheless, it should be noted that the involvement of the BM in the development of mature CD56+ NK cells, particularly in their quiescent condition, cannot be disregarded. The first phase of NK cell development takes place in the BM and is influenced by stromal cell factors. These factors have a role in driving the differentiation of early hematopoietic

progenitor cells (HPCs) expressing CD34, resulting in the formation of NK cell precursors characterised by the expression of CD34, CD45RA, and alpha4/beta7. The aforementioned cells, which exhibit the presence of cellular adhesion molecules, expeditiously migrate to the LN. Once there, they undergo differentiation into CD56bright NK cells, facilitated by the effect of IL-2 generated by T cells. Subsequently, these CD56bright NK cells have the capacity to further evolve into CD56dim NK cells. The transition from BM to LN appears to play a significant role in the differentiation of NK cells. It has been observed that a greater number of mature CD56bright NK cells develop from peripheral blood CD34+ hematopoietic progenitor cell (HPC) culture compared to BM when IL-2 or IL-15 is present (Figure 12). The present understanding of the developmental phases of NK cells in humans remains limited. The potential for differentiation of human NK cells from CD34+ HPC in vitro allows for the elucidation of several phases of this process. In the current context, several researchers have put forward the hypothesis that NK cells originated from CD34+ HPC. Similar to T- and B-lymphocytes, NK cells exhibit varying patterns of surface marker expression throughout time, which may serve to delineate certain developmental phases. Freud and colleagues have suggested a series of markers, namely CD34, CD117, and CD94, to distinguish between distinct phases of human CD56bright NK cell maturation inside the LN. Their argument is founded upon the following observations: (1) More than 99% of NK cells in LN express CD34, CD117, and/or CD94; (2) CD34 and CD94 are different markers, indicating that NK cells in intermediate stages would initially lose CD34 and then express CD94; and (3) the functional maturity of NK cells (as demonstrated by cytotoxicity and IFN-γ secretion) and the acquisition of surface CD56 occur at a later stage of development. The proposed stages of human CD56bright NK cell development within LN, as determined by this marker panel, are as follows: stage 1 – NK cell progenitor (CD34+CD117-CD94-), stage 2 – Pre-NK cell (CD34+CD117+CD94-), stage 3 – Immature NK cell (CD34-CD117+CD94-), stage 4 – CD56bright NK cell (CD34-CD117+/-CD94+), and stage 5 – CD56dim NK cell (CD34-CD117-CD94+).

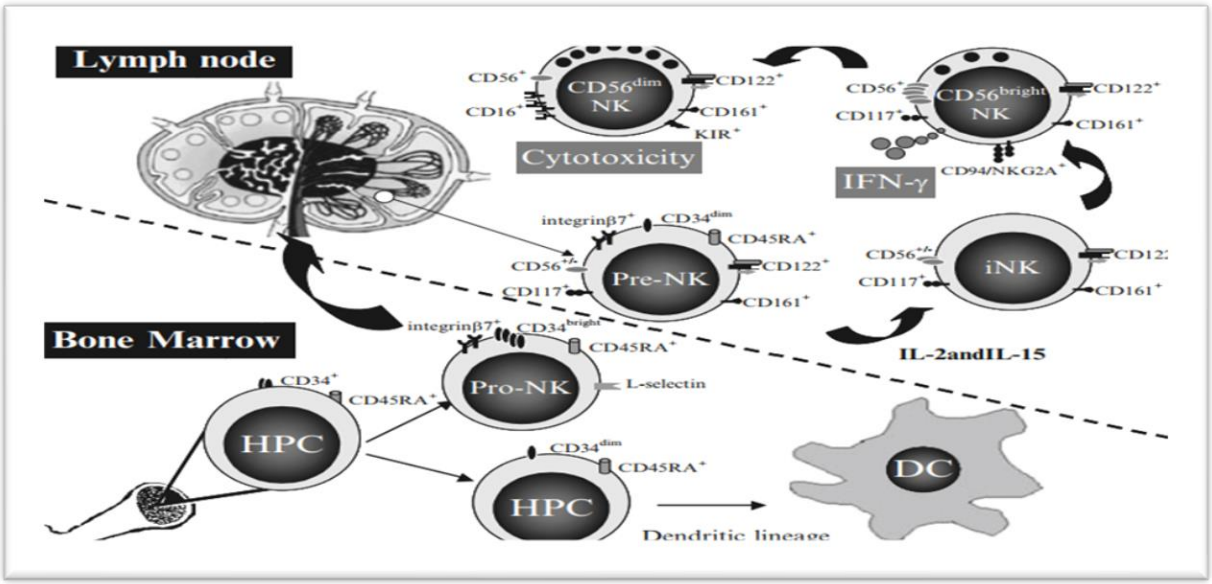


Figure 12. the many phases of in vivo human NK cell ontogeny

According to the proposal made by Freud et al. (47), the figure shown in this study illustrates the many phases of in vivo human NK cell ontogeny. It is widely accepted that the maturation process of NK cells, from hematopoietic stem cells (HSC) generated from the BM, occurs inside the lymph nodes (LN) until reaching their final stages. During the transition from stage 1 to stage 3, NK cells undergo a commitment to the NK cell lineage, resulting in the loss of their ability to grow into T cells or dendritic cells (DCs). During stages 3 to 5, NK cells undergo a process of functional maturation and transitioning. As a result, in the in vivo stage 3, immature NK (iNK) cells are capable of producing growth factors. This ability continues until stage 4. CD56bright NK cells have a propensity to primarily engage in the release of interferon-gamma (IFN-γ), whereas CD56dim NK cells at stage 5 mostly exhibit cytotoxic activity.

The development of early NK cells is contingent upon concurrent response to IL-15. As previously stated, the stromal cell factors KL and FL have the potential to induce the activation of CD34+CD122- progenitor NK cells (pro-NK), resulting in an increase in CD122 expression. This increase is essential for the pro-NK cells to develop into CD34+CD117+ pre-NK cells. Upon stimulation with IL-15, these pre-NK cells may then go through several stages of differentiation. Additionally, it's crucial to acknowledge that not all the cells expressing CD34+ CD117+ CD94- are associated to impaired NK cell development. There is a proposal suggesting that CD34dim human hematopoietic stem cells (HSCs) are dedicated progenitors to plasmacytoid dendritic cells (DCs) that produce type 1 interferon-gamma (IFN-γ). On the other hand, CD34bright cells are believed to be more engaged in the lymphoid lineage, as shown by their expression of CD (2, 7,10), and integrin b7, HLA-DR. Furthermore, Freud and colleagues have made observations indicating that under suitable culture conditions, CD34+CD117+/-CD94- cells have the potential to undergo differentiation into immature T cells. In contrast, it was shown that cells expressing CD34-CD117+/-CD94+/- markers

at stages 3 and 4 did not exhibit the capacity to generate them. The findings presented in this study indicate that the stromal environment and the expression of membrane antigens have a crucial impact in promoting the lineage plasticity of stage 2 cells, leading to their eventual differentiation into NK cells inside the LN. Immature natural killer cells (iNK cells), distinct from early embryonic stages of natural killer cells, lack the ability to create T cells and dendritic cells (DCs) and have undergone commitment to the NK cell lineage. The aforementioned cells exhibit the presence of NK cell antigens such as NKp44, and CD (2, 7, 56, 161) while simultaneously lacking CD10, integrin b7, and HLADR. iNK cells, although being a separate subset of NK cells, presently do not possess the capacity to produce IFN-γ or participate in perforin-dependent cellular cytotoxicity. The researchers provided evidence that CD56+ cells exhibit varying degrees of CD117 expression and may be categorised into two subsets: CD56+CD117high and CD56+CD117low/-. These populations exhibit distinct characteristics as the latter group demonstrates the expression of NKG2D, NKG2A, NKp30, NKp46, and CD94, but the CD56+CD117 high cell population does not possess these markers. It has been demonstrated that CD56+CD117high CD94- cells lack cytotoxic activity, but CD56+CD117low/- CD94+ cells exhibit efficient target cell killing and exhibit elevated expression of FasL and IFN-γ, indicating their differentiation at stage 4. This discrepancy suggests the existence of distinct cell subsets within stage 4 or their presence at a later stage. For the last two decades, there has been a hypothesis suggesting that CD56bright and CD56dim NK cells are distinct phases in the differentiation of NK cells. Additionally, it has been proposed that CD56dim NK cells may originate directly from CD56bright NK cells. Nevertheless, the absence of a method to analyse a complex collection of markers has hindered the availability of evidence supporting this advancement until recently. Freud et al. conducted an assessment of the expression of CD16 and CD94 in cell populations of CD3-CD56+ derived from LN and peripheral blood. Their findings provide support for the hypothesis that the CD56dim cell subset corresponds to the final developmental stage (stage 5) of NK cells. This is evidenced by the observation that CD56bright NK cells in the blood exhibit CD94+CD16+/- expression, whereas CD56dim NK cells in the blood predominantly display CD94+/- CD16+ expression. Furthermore, it has been observed that KIR+ NK cells are predominantly found within the CD56dimCD94+/ -CD16+ subset of cells in both LN and peripheral blood (Fig. 13; Fig. 14).

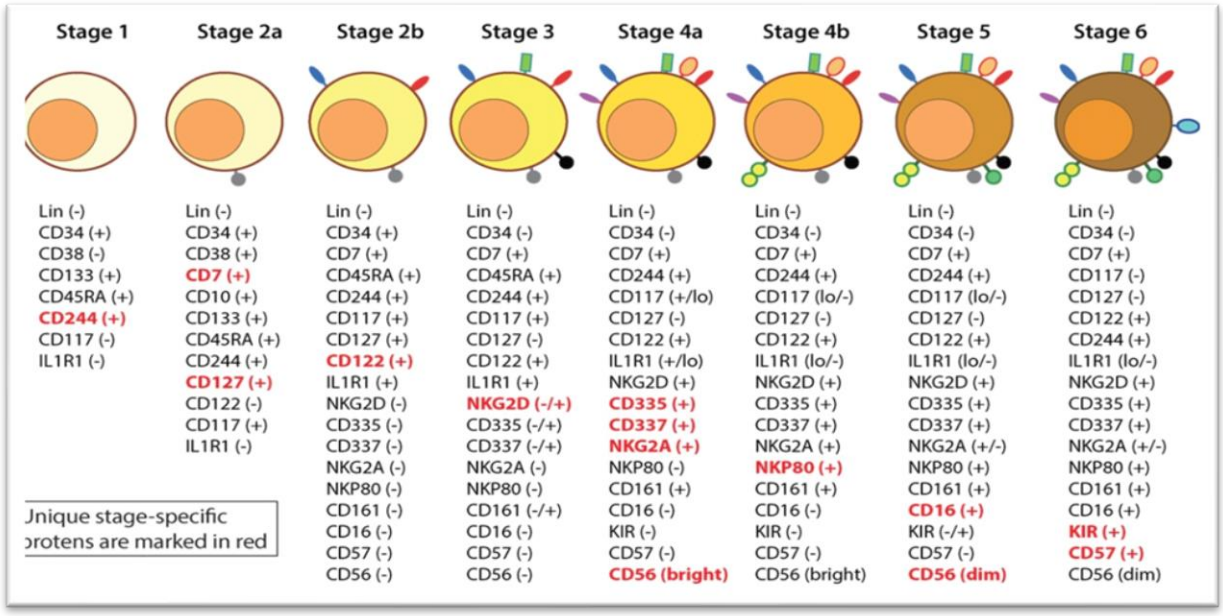


Figure 13 A diagram of NK cell development in humans

Conversely, the CD56brightCD94+CD16+/- subset does not exhibit KIR expression. This finding aligns with existing evidence suggesting that the acquisition of KIR is a relatively late occurrence during the maturation of NK cells, both in laboratory settings and in living organisms. It is noteworthy that the CD56bright NK cells exhibit dominance in the LN, with a median value of 75%. Conversely, the CD56dim subset of NK cells makes up the large amounts of cells in the peripheral blood and spleen, comprising 95% and 85% respectively. In addition, it is worth noting that the CD56dim fractions seen in the spleen and peripheral blood have CD16 expression, while CD56bright NK cells in LN lack CD16 and show limited expression of activation markers such as HLA-DR and CD69. The aforementioned results together indicate that the CD56bright cells exhibit characteristics of immaturity and have the potential to differentiate into CD56dimCD16+ NK cells. This process is believed to occur inside the LN before the CD56dimCD16+ NK cells migrate to the peripheral circulation. It is crucial to acknowledge that although these phases potentially depict a developmental route for NK cells, it remains plausible that some cells within each stage may retain terminal differentiation and perform vital activities in maintaining body homeostasis.

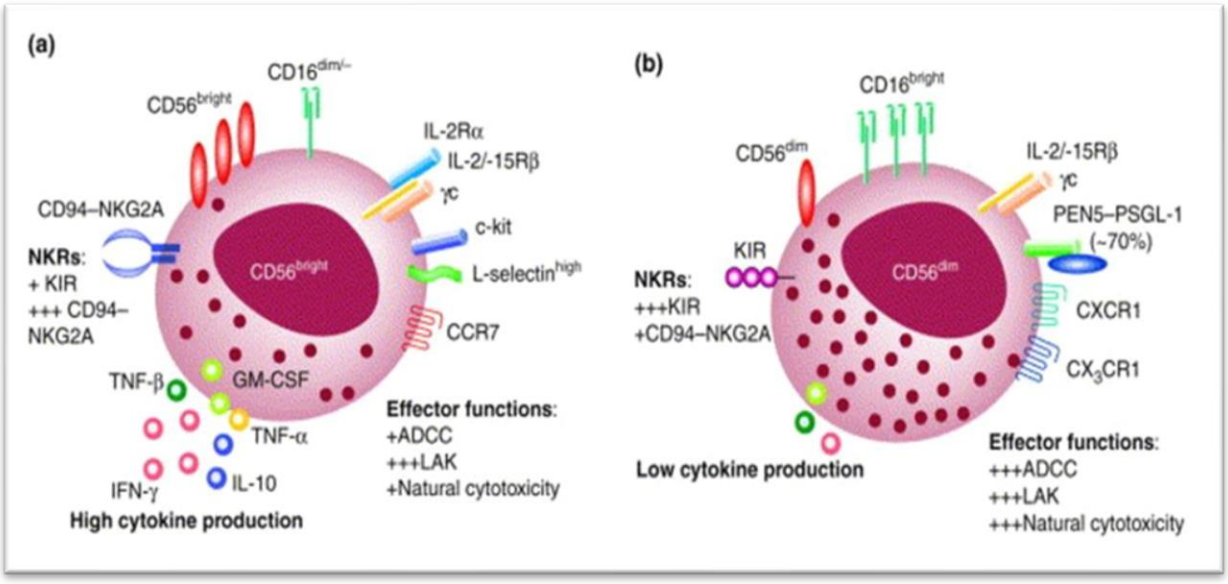


Figure 14 . Phenotype and functions of CD56dim and CD56bright NK cells

6.2. Memory NK cells

It is widely believed that the innate immune compartment lacks particular antigen responses and is incapable of generating memory responses. Nevertheless, despite the current classification of NK cells as components of the innate immune system, fresh experimental evidence has accumulated, indicating their potential to develop into long-lasting memory cells capable of generating a more robust and specific secondary response. Three potential models might potentially result in the development of "memory" NK cells, as seen in Figure 15. 1) The protein m157 is specifically recognised by the murine NK receptor Ly49H in the context of murine cytomegalovirus infection (MCMV). 2) Stimulation by pro-inflammatory cytokines, including IL-12, IL-15, and IL-18, is seen in this context. The creation of a specialised NK cell population referred to as a "memory" NK population, is limited to the liver and exhibits a secondary response unique to antigens. The liver in NK cells elicits a secondary response that is unique to antigens.

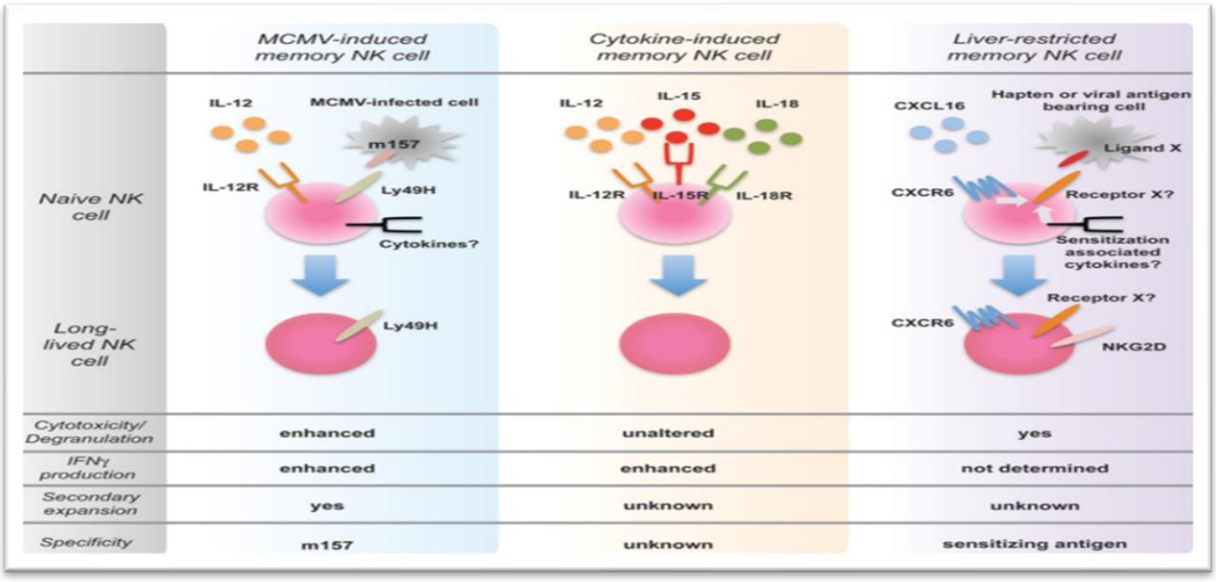


Figure 15. The NK cell: three possible ways to differentiate into a memory cell

The development of memory NK cells in humans is hypothesised to occur, particularly via the involvement of the cytokine pathway. However, except for the functional requirements, there is yet no distinct marker or defining moment that can unequivocally differentiate the population referred to as "memory" in NK cells. The identification of these entities relies on a collection of factors, which include many characteristics such as the contextual circumstances that precipitated their creation. The presence of a "memory" population of NK cells raises various inquiries about the true classification of NK cells within the innate immune system and the fundamental delineation between the innate and adaptive immune systems. In summary, the findings pertaining to the formation of NK cells in humans indicate that commitment to the NK lineage takes place during the transition from stage 1/pro-NK to stage 3/iNK. Furthermore, the process of phenotypic and functional maturation occurs during the transition from stage 3 to stages 4/5/6.

6.3. NK receptors

The absence of a master switch for NK cells, similar to the T cell receptor found in T cells, has been observed. The activation of these entities is a consequence of a series of interactions between activating and inhibitory receptors, as well as immunological checkpoints (Figure 16). Therefore, the activation of receptors facilitates direct interaction with ligands that are overexpressed or newly expressed on altered cells. This interaction enables NK cells to identify and eliminate tumour cells, irrespective of their presence of human leukocyte antigen (HLA)-I. In order to inhibit NK cell-mediated assaults, healthy host cells have elevated amounts of normal major histocompatibility complex class I (MHC-I) molecules. In the context of adult NK cells, it is evident that the inhibitory effects mediated by KIRs are not entirely definitive and may be overridden by strong activating

stimuli. KIRs represent the first set of human receptors that have been thoroughly examined for their specificity towards major histocompatibility complex class 1 (MHC-1) molecules. These receptors possess the unique ability to differentiate between various allelic variations of MHC class 1, hence facilitating the identification of cells that have undergone transformation or infection. While the primary role of KIRs is often associated with inhibition, it is worth noting that a number of activating KIRs have also been discovered. The receptors under consideration are encoded by a total of 14 polymorphic genes, namely KIR2DL1–5, KIR3DL1–3, KIR2DS1–5, and KIR3DS1. Among these genes, seven are classified as inhibitory receptors, while the other seven are categorised as activating receptors. The NK inhibitor receptors CD94/NK group 2 members A and B (NKG2A and NKG2B) have garnered attention as potential targets in immunotherapy, alongside KIRs. The cytoplasmic domains of inhibitory KIRs and NKG2A + B include immunoreceptor tyrosine-based inhibition motifs (ITIMs). When these receptors interact with normal major histocompatibility complex class I (MHC-1) molecules on healthy cells, the ITIMs get phosphorylated by tyrosine kinases belonging to the Src protein family. On the other hand, receptors that are activated include immunoreceptor tyrosine-based activation motifs (ITAMs) inside their cytoplasmic domains. These ITAMs are phosphorylated by Src family kinases upon interaction with neoplastic cells, hence facilitating the transmission of downstream activation signals. The activated NK cells release several key cytokines, including interferon-gamma (IFN-γ), tumour necrosis factor (TNF), granulocyte/monocyte colony-stimulating factor (GM-CSF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL). These cytokines play a crucial role in activating death receptors on target cells and promoting the activation of various immune mediators such as T cells, dendritic cells, macrophages, and neutrophils. In addition, NK cells secrete chemokines, including C–C motif ligand 3 (CCL3; commonly referred to as MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), X–C motif chemokine ligand 1 (XCL1; lymphotactin), and C-X-C motif chemokine ligand 8 (CXCL8; IL-8), which serve to recruit diverse immune cells to the neoplastic tissues. While the majority of activating ligands are typically found on the surface of the target cell membrane, there are some ligands that exist in a soluble form. One example of this phenomenon is the production of platelet-derived growth factor DD by various tumour types. This factor binds to the activating NKp44 receptor located on NK cells, therefore triggering the release of interferon-gamma (IFNγ) and tumor necrosis factor (TNF). The NCRs include a repertoire of highly effective activating receptors that have the ability to engage with ligands that are either attached to the cell membrane or present in a soluble form. Nevertheless, the identity of the majority of tumour-associated ligands belonging to the Natural Cytotoxicity Receptor (NCR) family remains undisclosed. The NKp30, NKp44, NKp46, and NKp80 receptors are expressed in a constitutive manner on both resting and active NK cells. These receptors play a crucial role in the

establishment and functioning of the immunological synapse, as well as in the production of cytokines. Additionally, noteworthy are the retinoic acid-inducible gene-1-like receptors and Toll-like receptors that are responsible for the detection of both double- and single-stranded RNA. Furthermore, the cyclic GMP-AMP synthase and the DNA-dependent activator of IFN-regulatory factors play a crucial role in detecting cytosolic DNA and DNA that is released from tumour cells.

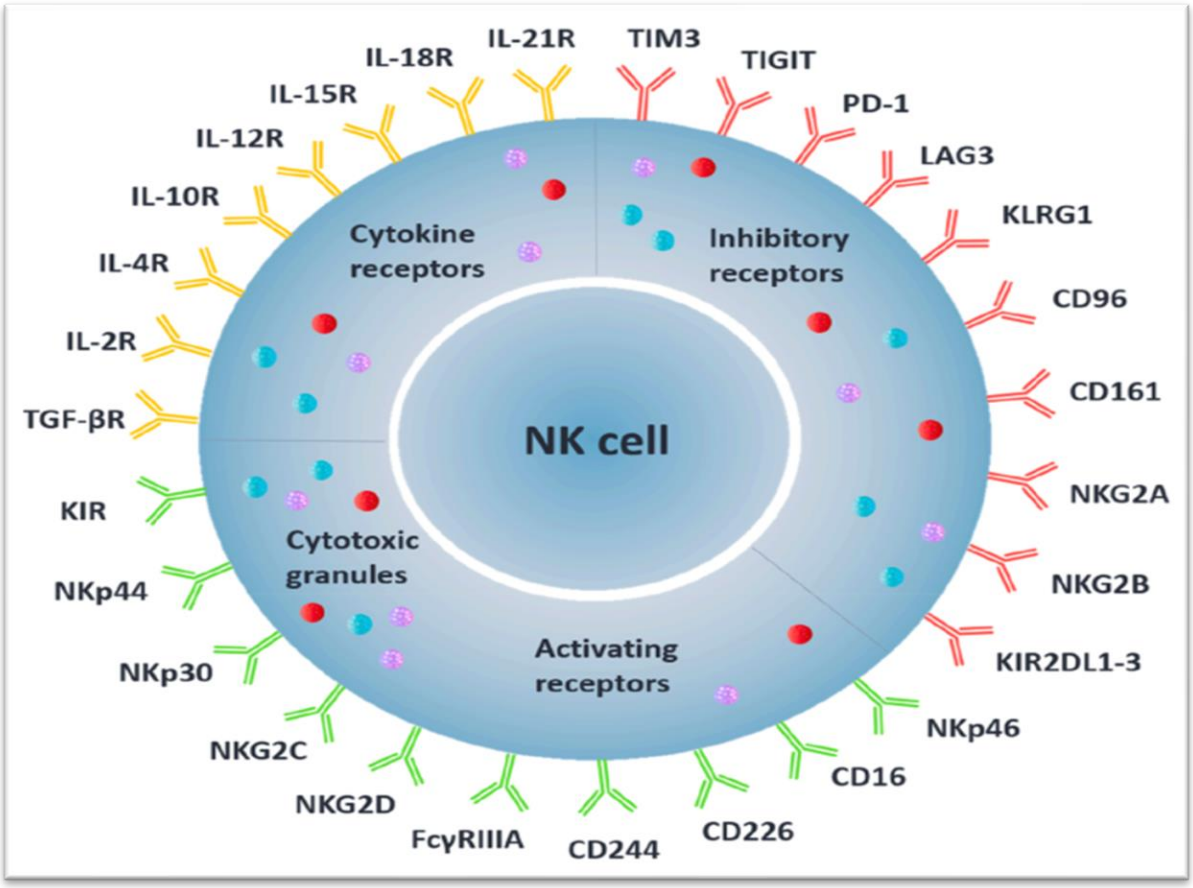


Figure 16. Collections of NK cell inhibitory and activating receptors.

NK cells have a variety of receptors that are involved in both their activation and inhibition. In the context of immune regulation, inhibitory receptors play a crucial role in maintaining self-tolerance. Mature NK cells exhibit the expression of immunological checkpoints, namely inhibitory receptors, as a means of implementing a negative feedback loop to counteract prolonged activation and anergy. The image shown illustrates significant clusters of these receptors. The research of ligands for many receptors is currently ongoing. Receptors that are activated have a role in facilitating both natural cytotoxicity, which involves direct killing, as well as antibody-dependent cell-mediated cytotoxicity. CD: cluster of differentiation molecule; IL: interleukin; FcγRIIIA: fragment crystallizable γ-receptor IIIA; KIR: killer-cell immunoglobulin-like receptors; KLRG1: killer cell lectin-like receptor subfamily G member 1; LAG3: lymphocyte activation gene 3; PD1: programmed cell death protein 1; TIM3: T-cell immunoglobulin domain and mucin domain 3; TGF-βR: transforming growth factor β receptor; TIGIT: T cell immunoreceptor with Ig and ITIM domains.

The effector function of NK cells that has been extensively studied is cytotoxicity. NK cells are capable of targeting several types of cells, including tumour cells, cells infected with viruses, cells

infected with intracellular bacterial pathogens, and, as recently discovered, immature dendritic cells (iDCs). NK cells, in addition to macrophages and several other cell types, exhibit the FcR molecule, which is an activating biochemical receptor responsible for binding to the Fc region of antibodies. This enables NK cells to selectively attack cells that have been targeted by a humoral immune response and to induce cell lysis by antibody-dependent cellular cytotoxicity (ADCC). Multiple activating receptors are present on NK cells, such as CD16, NKG2C, NKG2D, LFA-1, 2B4, and the NCR (NKp30, NKp44, and NKp46). Engagement with these receptors may induce activation and enhance the cytotoxicity of NK cells. Activation of the ITAM receptor triggers reorganisation of the actin cytoskeleton, a process necessary for cellular polarisation and the subsequent release of cytolytic granules containing perforin and granzymes. This activation also leads to the transcription of several genes involved in cytokine and chemokine production. According to recent research, it has been shown that CD16 is involved in the process of degranulation. Furthermore, the interaction between CD16 and LFA-1 has been found to result in effective lysis of target cells. The co-engagement of 2B4 and CD16 has been shown to increase the quantity of degranulating NK cells. Furthermore, this co-engagement has the ability to cause cytotoxicity even in the absence of LFA-1–ICAM-1 contact, (Figure 17).

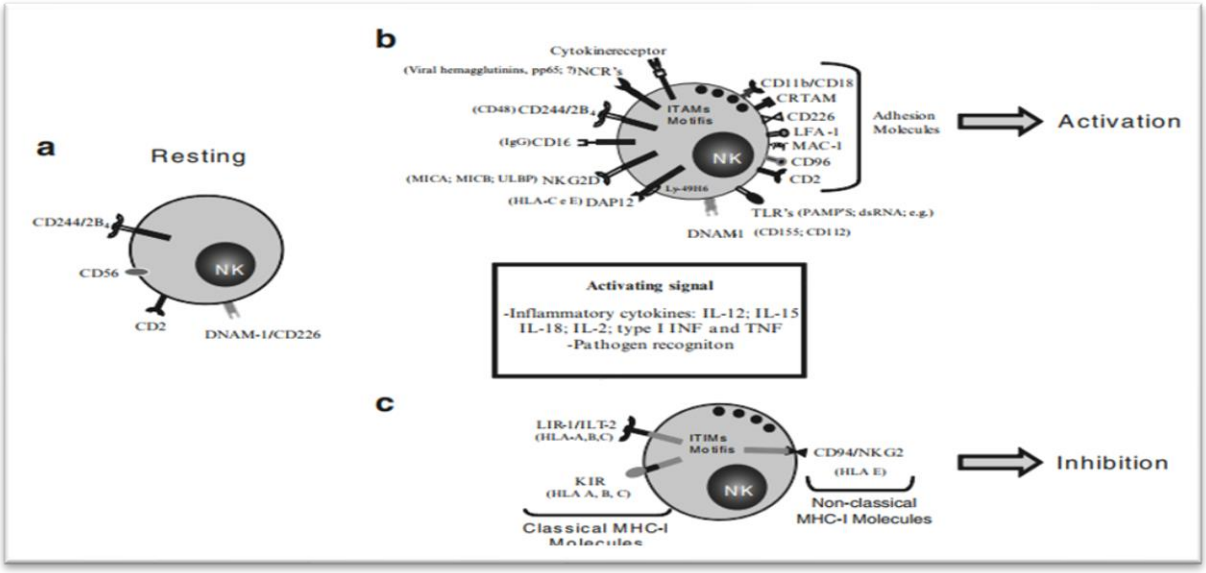


Figure 17. Activation and inhibitory receptors present in human natural killer (NK) cells and their corresponding ligands.

Resting natural killer (NK) cells (a) engage with target cells that display ligands for both (b) activating receptors containing an immunoreceptor tyrosine-based activation motif (ITAM) and (c) inhibitory receptors containing an immunoreceptor tyrosine-based inhibition motif (ITIM).

6.3.1. NK receptor's function

NK cells, being the primary effector cell type in the innate immune response, can eliminate tumour cells and cells infected by viruses during the first stages of infection. The body's limited production of receptors for discerning numerous antigens necessitates the utilisation of the "missing self" and "induced self" mechanisms to identify target cells. This identification process is achieved by carefully regulating the interplay between activating costimulatory signals and inhibitory signals, primarily mediated by functional receptors. The ultimate determination of NK cell activation and functional status is contingent upon the reciprocal exchange of signals between interacting entities. The activation signals include a range of receptors, such as cytokine-binding receptors, integrins, killing receptors (CD16, NKp40, NKp30, and NKp44), receptors that recognise non-self-antigens (Ly49H), and additional receptors (e.g., NKp80, SLAMs, CD18, CD2, and TLR3/9). The activating receptors of NK cells may be categorised into a minimum of three kinds based on their respective ligands: MHC-I specific receptors, MHC-I related receptors, and MHC-I non-related receptors (Table 2). To underscore, Natural Cytotoxic Receptors (NCRs), categorised under the third category, include three distinct molecules (NKp30, NKp44, and NKp46, Table 3). It has been shown that NKp30 can identify B7-H6, a protein expressed on tumour cells. Consequently, NKp30 holds potential as a promising therapeutic avenue for future interventions. The inhibitory signals mostly consist of receptors that recognise major histocompatibility complex class I (MHC-I), including Ly49s, NKG2A, and LLT1, along with some receptors unrelated to MHC-I (refer to Table 2). Furthermore, it is worth noting that inhibitory receptors specific to major histocompatibility complex class I (MHC-I) may be broadly categorised into three distinct categories based on their structure and function. These types include KIRs, killer lectin-like receptors (KLRs), and leukocyte immunoglobulin-like receptors (LILRs).

Table 2. The primary mediators involved in the functioning of NK cells.

Classification	**Mediator**	**Host ligand**
NK cell activator		
MHC-I specific receptor	KIR2DS1,2DS3,3DS5	MHCI
	Ly49c, Ly49i	MHCI
	NKp80	MHCI
	NKG2C, NKG2E	MHCI
MHC-I related receptor	NKG2D	MICA, MICB, ULBPs
MHC-I non-related receptor	DNAM1	Nectin-2, CD155
	NKp46(NCR1)	HSGAGs, CFP
	NKp44(NCR2)	HSGAGs, MLL5, NKp44L, PCNA, BAT3, PDGF-DD, Nidogen-1
	NKp30(NCR3)	HSGAGs, B7-H6, Galectin-3

	Nkp65	Keratinocyte-associated C-type lectin
	LFA-1(αLβ2integrin)	Intercellularcelladhesionmolecule1
	α4integrin	Vascularcelladhesionmolecule1
	CD16	Fc-γ
	CD2	CD581
	TLR3	Microbial constituents, adjuvant
	TLR9	CpG
NK cell inhibitor		
MHC-I specific receptor	KIR3DL1	MHCI
	KIR2DL3,2DL1	MHCI
	NKG2A	MHCI
	KLRB1, LLT1	MHCI
	LILRB1, LILRB2	MHCI
MHC-I non-related receptor	KLRG1	E-, N-, and R-cadherins
	siglec-3,siglec-7,siglec-9	Sialic acid
	CEACAM1	CEACAM1, CEACAM5
	2B4(CD244)	CD48
	IRp60	Phosphatidylserine
	LAIR1	Ep-CAM
	CD96	CD155
	CD73	Antibodies
	PD-1	PD-L1
	TIGIT	CD155
	NKR-P1B	Clr-b
	LAG3	MHC-II

Abbreviations: MHC major histocompatibility complex, KIR killer cell immunoglobulin-like receptor, MIC MHC class I chain-related, ULBP UL16-binding protein 1, DNAM1 DNAX accessory molecule 1, NCR natural cytotoxicity receptor, HS GAGs heparan sulfate glycosaminoglycans, CFP complement factor P, MLL5 mixedlineage leukemia protein-5, PCNA proliferating cell nuclear antigen, BAT3 HLA-B-associated transcript 3, PDGF-DD platelet-derived growth factor-DD, LFA-1 lymphocyte function-associated antigen-1, TLR toll-like receptor, KLR killer cell lectin-like receptor, LLT1 lectin-like transcript 1, LILR leukocyte immunoglobulin-like receptor, Siglec sialic acid-binding immunoglobulin-like lectin, CEACAM carcinoembryonic antigen-related cell-adhesion molecule, IRp60 inhibitory receptor protein 60, LAIR1 leukocyte-associated immunoglobulin-like receptor 1, Ep-CAM epithelial cellular adhesion molecule, PD-1 programmed cell death protein 1, TIGIT T-cell immunoreceptor with Ig and ITIM domains, LAG3 lymphocyte activation gene 3

Table 3. NK receptors and their ligands. BAT3: HLA–B-associated transcript 3, ECTV: ecteromelia virus, VV: vaccinia virus, NDV: Newcastle disease virus

Receptor	**Ligand**	**Effect**
NKp30	B7-H6 (tumor cells)	Activation (cytotoxicity, cytokine secretion)
	BAT3/BAG6 H6 (tumor cells,	Activation (cytotoxicity, secretion of cytokines,
	Dendritic cells (DC))	bidirectional dialogue NK/DC)
	ECTV HA (infected cells)	?
	VV HA (infected cells)	Inhibition
	Fungus: Cryptococcus and Candida, ligand?	Activation (cytotoxicity)

NKp44	21spe-MLL5 (tumor cells)	Activation (cytotoxicity, cytokine secretion)
	Viral hemagglutins (infected cells)	Activation (cytotoxicity, cytokine secretion)
	PCNA (tumor cells)	Inhibition
	BCG, ligand?	Activation
	Trophoblast, ligand?	Activation (cytotoxicity)
	Chondrocyte, ligand?	Activation
	CD4+ T lymphocyte during HIV infection, ligand?	Activation
NKp46	Viral hemagglutins (infected cells)	Activation (cytotoxicity, cytokine secretion)
	Tumor cells, ligand?	Activation (cytotoxicity, cytokine secretion)
	Dendritic cells infected with CMV, ligand?	Activation (cytotoxicity, cytokine secretion)
	Trophoblast, ligand?	Production of chemokines by decidual NK cells

6.3.2. Signaling pathways of KIR

NK express a wide variety of receptors, but the most characteristic receptors are killer cell Immunoglobulin-like Receptors (KIR). The KIRs form a distinct locus with 13 highly similar genes and 2 pseudogenes located on chromosome 19. The different genes were probably created by duplication during evolution. Their organization is complex due to the variability in the number of genes present and the variability of their expression on cells. Four genes, KIR3DL3, KIR3DP1, KIR2DL4 and KIR3DL2, are always present in individuals. Other genes are inherited in haplotypes. KIRs are also polymorphic, which further complicates the system. Some KIRs are inhibitory and have a long intracellular segment, while others are activating and have a short intracellular tail. The type of KIR is indicated in its nomenclature by the penultimate character (L, S, or P; long, short segment, or pseudogene, respectively). Inhibitory KIRs signal through immunoreceptor tyrosine inhibitory motifs (ITIMs), which recruit the phosphatases SHP-1, SHP-2, and SHIP. Activating KIRs do not have a signaling domain, but are associated with an intermediate, DAP12. The DAP12 adapter contains an immunoreceptor tyrosine activating motif (ITAM). ITAMs, when phosphorylated, act as platforms for protein attachment and activation. The KIR2DL4 receptor is an exception and acts as an activating receptor through an adapter, FcεRIγ. KIR2DL4 appears to be involved in the vascularization process during pregnancy rather than in immune reactions. The KIR ligands are human leukocyte antigen (HLA) class I molecules (Table 1). Among these ligands, we find the classic HLA-I (HLA-A, B, C), but also HLA-F and HLA-G which have no known role in antigen presentation. Not all HLAs are recognized by KIRs and some epitopes (C1, C2 and Bw4) have

receptors. The peptides presented by HLA also have an importance in the affinity of KIR-HLA binding; for example, KIR2DS2 can recognize a peptide from the hepatitis C virus presented on HLA-C*01:02. For certain KIRs on the other hand, their ligand still remains to be identified.

Inhibitory pathway

Similar to KIRs, NKG2A is a receptor that pairs with CD94 and whose ligand is the non-classical HLA HLA-E. HLA-E is special because of the type of peptide presented. Classical HLA localization protein sequences bind in the HLA-E cavity. The different peptides modify the affinity of NKG2A for its ligand, creating a gradient of responses to HLA-E. Like the majority of inhibitory receptors, NKG2A has an ITIM domain that recruits the effectors SHP-1 and SHP-2 (Figure 13). The LILRB1 receptor has also been identified as a receptor for HLA class I. Its intracellular portion includes 4 ITIMs to carry out inhibitory signalling. It recognizes HLA-G, HLA-A/B/C and certain viral proteins analogous to HLA, such as UL18 of CMV. This receiver is not entirely redundant with the KIRs, The KLRG1 receptor is also expressed on NK cells and uses an ITIM domain for the transduction of its signalling. It binds to E/N/R-cadherins, and blocks NK cell activity. Its expression increases with the activation or proliferation of NK cells. In addition to having attenuated functions, NK cells that strongly express KLRG1 have decreased survival and limited proliferation.

Activating receptors

Although NK expresses a wide variety of inhibitory receptors, they have a greater variety of activating receptors. The CD16 receptor (FcRγRIII) is only expressed on the CD56DIM NK population with better cytotoxic capacity. It binds the Fc fragment of IgG and can recognize cells opsonized by these antibodies. CD16 is associated with CD3ζ or FcεRIγ receptors, which have ITAM signalling domains (Figure 13). NKRs are an important group of activating receptors. It includes the NKp46, NKp44 and NKp30 receptors. NKp46 and NKp30 associate with the CD3ζ chain or FcεRIγ, while NKp44 associates with DAP12, but also has an ITIM domain on its chain. This ITIM is, however, incapable of recruiting the SHP-1/2 and SHIP molecules, and therefore cannot have inhibitory effects. NCRs recognize molecules from the heparan sulfate family, which can indicate changes in cell status. Components of several pathogens are also recognized by NCRs in general. Receptors also have specific ligands. NKp44 recognizes the NKp44L and PCNA proteins, nuclear proteins whose aberrant surface expression occurs following cellular stress. NKp44 also binds PDGF-DD, a growth factor secreted by many tumours. Tumors can, on the other hand, produce the factor NID1, an inactive ligand of NKp44. The binding of NID1 prevents recognition of PDGF-DD and protects tumours. NKp30 recognizes the ligands B7-H6 and galectin-3, which are overexpressed by several cancers. NKp30 can also recognize BAT3, which can be localized outside the nucleus due to

cellular stress as for NKp44L and PCNA. The CD59 marker does not have a signalling domain, but its binding with the CD2 ligand amplifies the response of the NCRs NKp46 and NKp30. The NKp80 receptor also belongs to the NCR family. NKp80 does not associate with a protein to carry out its signalling, it contains a HemITAM domain in its intracellular portion. Its ligand, AICL, is expressed by cells of myeloid origin, but also on NK cells. The binding of NKp80 with AICL on myeloid cells allows a dialogue between these cells but also contributes to the surveillance of cancers of myeloid origin. Activation of NK cells by IL-12 or IL18 causes expression of AICL, which in turn causes internalization of NKp80 receptors. Another important receptor in NK activation is the NKG2D receptor. Its ligands are MICA/B molecules and ULBP1-6. These ligands are overexpressed in cells under stress. NKG2D pairs with DAP10. The DAP10 protein has an ITT-adjacent signalling site similar to CD28 signalling, which activates the PI3K intermediate. The 2B4 and NTB-A receptors have an ambivalent function. Their activating signalling is dependent on the level of the SAP protein. In the absence of SAP, SHP-1 binds to receptors and their signals become inhibitory. The ligand for 2B4 was identified as CD48, but that for NTB-A remained unknown for a long time. NTB-A forms a homophilic association with NTB-A proteins on target cells. Another activating receptor is DNAM-1. It binds the ligands PVR and Nectin-2, two markers associated with cellular stress. The DNAM-1 signal is initiated by an ITT-adjacent motif as for DAP10. Finally, some receptors have very limited functions under normal conditions. The NKG2C receptor recognizes HLA-E like NKG2A but binds to the adapter protein DAP12 (Figure 18). NKG2C is primarily involved in the response against cytomegalovirus, although its role in the response to cytomegalovirus is not clearly established. The NKG2E receptor is often associated with activating receptors on NK cells. It is very similar to the NKG2C receptor and also recognizes the HLA-E ligand. However, its function is still unknown and it is not expressed on the surface of NK cells due to its cytoplasmic region which retains it to the endoplasmic reticulum. It is therefore unlikely that it serves directly as an activating receptor for NK cells. Adhesion molecules, mainly LFA-1 and LFA-2 (CD2), also play a role in the activation of NK cells. They send the first signal through Erk1/2 and abrogation of their interaction or signaling prevents the cytotoxic functions of NK cells.

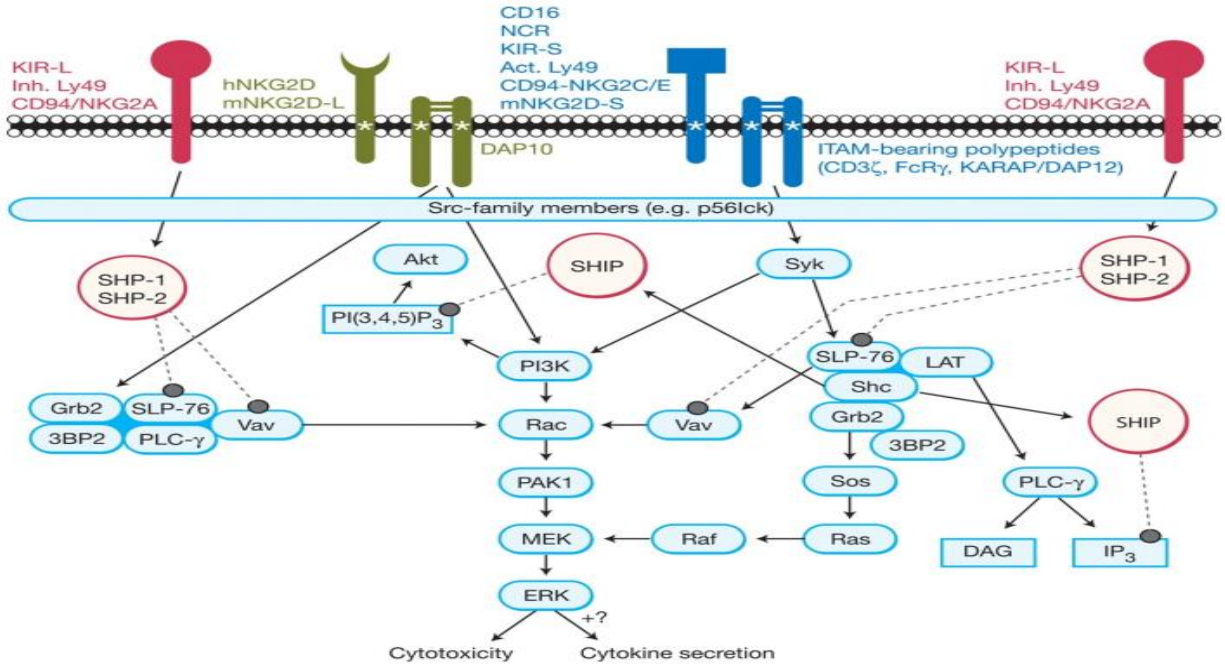


Figure 18. General NK cell signalling

6.3.3. Chimeric antigen receptors (CARs)

For many years, the primary modalities for cancer therapy were surgical removal, chemotherapy, and/or radiation. Nevertheless, in recent years, there has been a growing focus on immunotherapies that use immune checkpoint inhibitors and cellular therapies as potential alternative treatments. In general, the immune system maintains the regular elimination of potentially malignant cells. However, cancer cells possess certain mutations that enable them to evade these systems and collect inside the body. Cancer immunotherapies seek to enhance the patient's immune system in order to facilitate the efficient elimination of cancerous cells. One potential approach to accomplish this objective is the genetic modification of immune cells, namely T cells and more recently, NK cells, to include chimeric antigen receptors (CARs). The development of chimeric antigen receptors (CARs) on T or NK cells enables them to selectively and precisely target cancer cells by recognising particular tumour-associated antigens. The term "Classical" chimeric antigen receptors (CARs) refers to CAR constructs that typically include an extracellular binding domain mostly sourced from a monoclonal antibody fragment, specifically a single-chain variable fragment (scFv). This extracellular domain is then connected to intracellular binding domains taken from the T-cell receptor complex. The activation of T cells by the binding of a tumour antigen by the single-chain variable fragment (scFv) occurs in a way that is not reliant on major histocompatibility complex (MHC) molecules, ultimately resulting in a cytotoxic response. Ongoing advancements in the field of novel chimeric antigen receptor (CAR) designs include the development of modified intracellular co-

stimulatory domains and/or targeting domains. In addition to single-chain variable fragments (scFvs), other molecules such as nanobodies, designed ankyrin repeat proteins (DARPins), ligands, or receptors may be used in later stages. In addition, a class of CARs known as adaptor CARs has been created by the separation of antigen recognition and CAR-mediated immune cell activation. The use of distinct adapter molecules (AMs) that are designed to bind to tumour antigens, in conjunction with chimeric antigen receptor (CAR)-modified immune cells that are engineered to recognise these AMs, enables a treatment that is more accurate and has a limited duration. Hence, it is possible to simultaneously target many antigens, hence allowing for adaptability of the treatment in the event of antigen-loss tumour types. Moreover, this methodology has the potential to suppress the immune response in the event of the emergence of significant adverse consequences. The process of an autologous CAR-T or NK cell treatment involves many sequential stages, as seen in Figure 19. Initially, T or NK cells are extracted from the blood of either the patient or the donor. Following this, cells are genetically modified by introducing chimeric antigen receptor (CAR)-encoding genes, primarily via the use of viral vectors. Chimeric antigen receptor (CAR)-modified immune cells are cultured and increased ex vivo until an adequate quantity is achieved, after which they are then transplanted into the patient's body in an adaptive manner. This therapeutic approach aims to harness the potential of CAR-modified immune cells in combating malignant cells. In most therapeutic contexts, lymphodepletion is often conducted prior to the infusion of CAR-modified immune cells in order to facilitate effective engraftment of the cells. It is noteworthy to acknowledge that CAR-NK cells have the potential to serve as a readily available product, while there is also ongoing progress in the development of allogeneic CAR-T cell therapy.

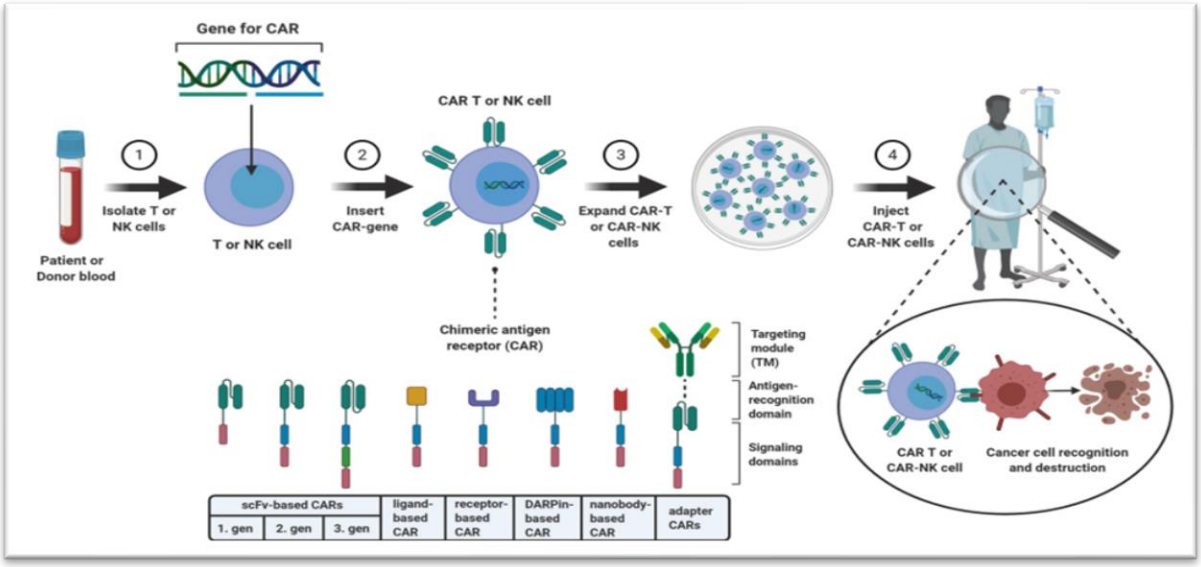


Figure 19. This diagram depicts a schematic representation of a therapeutic approach known as CAR-T or CAR-NK cell treatment.

whereby primary immune cells are used. (1) T or NK cells are extracted from the blood of either the patient or the donor. (2) Following this, cellular modifications are made to enable the expression of chimeric antigen receptors (CARs).

The key aspect of NK-cell engineering is the introduction of genes that determine and enhance the functioning of NK-cells. The CAR is a genetically-engineered recombinant receptor construct consisting of an extracellular single-chain variable fragment (scFv). The svFc is a chimeric protein produced by combining the antigen-binding regions of the light and heavy chains of immunoglobulins from an antibody that can identify a particular target protein without relying on MHC proteins. The scFv is connected to a transmembrane domain by a hinge or spacer peptide, which in turn connects it to the intracellular signalling domain of the CAR construct. The length of the hinge or spacer peptide that connects the extracellular domain to the transmembrane domain may be modified in order to provide the best possible accessibility to the target or epitope, as shown in Figure 15. Up to now, CAR-NK products have used the fundamental structure of CAR T cells. The intracellular region responsible for transmitting the activating signals downstream typically comprises a CD3ζ chain with immunoreceptor tyrosine-based activation motifs (ITAMs). This region is commonly accompanied by co-stimulatory receptor molecules and an interleukin (IL) inducer, which are present in T cells equipped with synthetic chimeric antigen receptors (CARs). At the start of the new century, a very effective group of anti-cancer treatments known as targeted therapies developed. These therapies shown exceptional effectiveness against certain types of blood cancers. The FDA granted approval to the first two CD19 CAR T-cell products, Kymriah and Yescarta, in 2017. These medicines were specifically authorised for the treatment of refractory acute lymphoblastic leukaemia and diffuse large B-cell lymphomas. In 2019, the National Cancer Institute provided further funding to support the investigation of CAR T-cell therapy in the treatment of solid tumours. Nevertheless, CAR T-cell treatment is customised to each patient and has some restrictions, such as the need for HLA matching and the possibility of toxicities including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). Furthermore, the manufacture of an autologous CAR T-cell product is constrained by logistical challenges, high prices, and significant time requirements. Allogeneic NK cells do not need stringent human leukocyte antigen (HLA) matching and are not susceptible to rejection that arises from the identification of self-major histocompatibility complex (MHC). Nevertheless, the longevity of infused allogeneic NK cells may be limited to a certain extent due to the recipient's immune system's alloreactivity. Prior to the transfer of CAR NK-cells, a kind of chemotherapy called lymphodepleting chemotherapy is given. This chemotherapy, often using fludarabine and cyclophosphamide, helps to eliminate lymphoid cells, suppressive regulatory T cells, and MDSC in the tumour microenvironment (TME). The purpose of this chemotherapy is to enhance

the longevity of the infused cells. In order to enhance the viability and proliferation of transplanted CAR-NK cells, interleukin-2 (IL2) is often administered through subcutaneous injection every other day for a duration of two weeks. Due to the absence of any apparent danger of cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), allogeneic chimeric antigen receptor natural killer (CAR-NK) cells may be transplanted without requiring hospitalisation. Allogeneic CARNK cell lines may be cultured in bioreactors, cryopreserved, and stored as numerous doses. They can be readily employed as needed. Furthermore, CAR-NK cells may be generated at a comparatively accelerated rate in comparison to CAR-T cells. A clinical experiment was conducted to evaluate the utility of CAR NK-cell transfer in patients with diffuse large B cell lymphoma, follicular lymphoma, and chronic lymphocytic leukaemia. The CAR construct used in this trial included a CD19–CD28–CD3ζ CAR design, which included an IL15 signalling region.

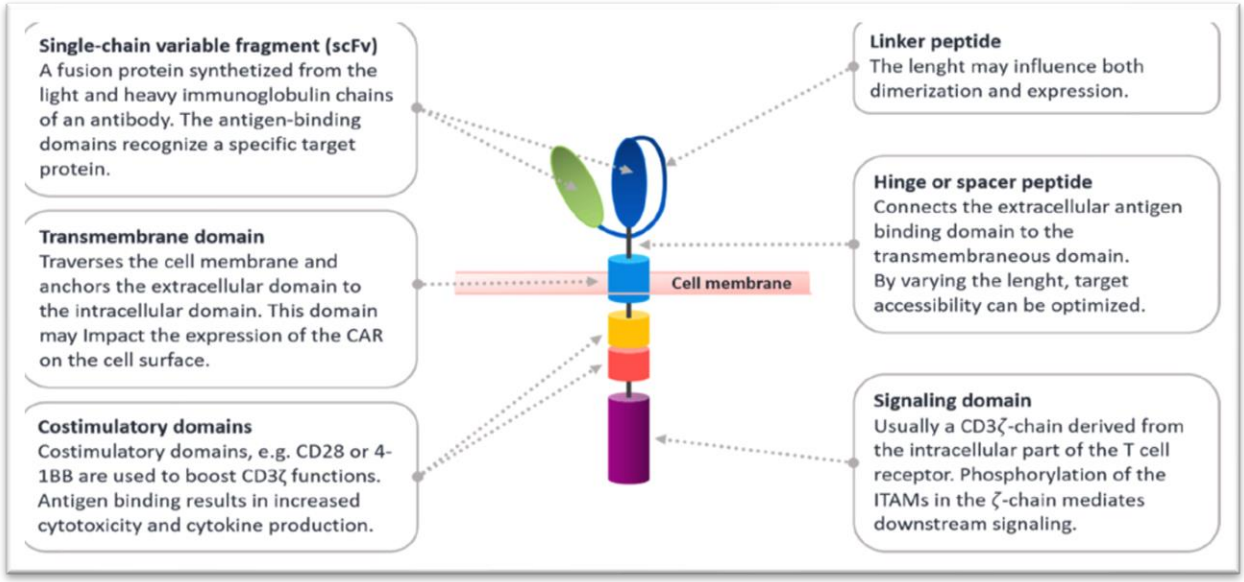


Figure 20 . Structure of Chimeric Antigen Receptor

The incorporation of costimulatory domains into the CAR design played a crucial role in addressing the quick anergy seen in the first production of CAR-T cells, which resulted in a deficiency of cell persistence. The addition of either one (second generation) or two (third generation) costimulatory domains, such as CD28, 4-1BB, or ICOS (inducible T cell co-stimulator/CD278), enhanced the receptors' capacity to stimulate the secretion of cytokines, expansion and proliferation of T cells, thereby facilitating their long-term presence. These are shown graphically in the illustration below, as seen in Figure 16 a. Structures with signalling domains that are more specifically tailored to cell signalling have also been produced for NK cells. Utilising the intracellular domain of 2B4 in conjunction with CD3ζ enables the stimulation of leukemic cell lysis at a level equivalent to that achieved by a second-generation construct. The design has also been used in an in vitro model of

solid tumours, namely primary tumours, yielding outcomes that vary to a greater extent. Multiple receptors use ITAM-mediated signalling. The use of DAP12 has been employed as a substitute for CD3ζ signalling. Although DAP12 only has one ITAM, the scientists noted a more significant impact on target cell destruction compared to CD3ζ. Recent research conducted a comparison of several constructs including segments from CD3ζ, 4-1BB, 2B4, DAP12, and DAP10 (146). Furthermore, the scientists conducted experiments involving the modification of the intracellular component of the receptors, as well as the regions of transmembrane receptors that interact with adapters through this specific area. The transmembrane receptors NKG2D, NKp44, and NKp46 were integrated into chimeric antigen receptors (CARs) (Figure 21. b). The adapters DAP10, DAP12, and CD3ζ/FcεRIγ form associations with their respective channels. The components consisting of portions of NKG2D, 2B4, and CD3ζ exhibited the most favourable reactions in both diffuse and solid tumour models.

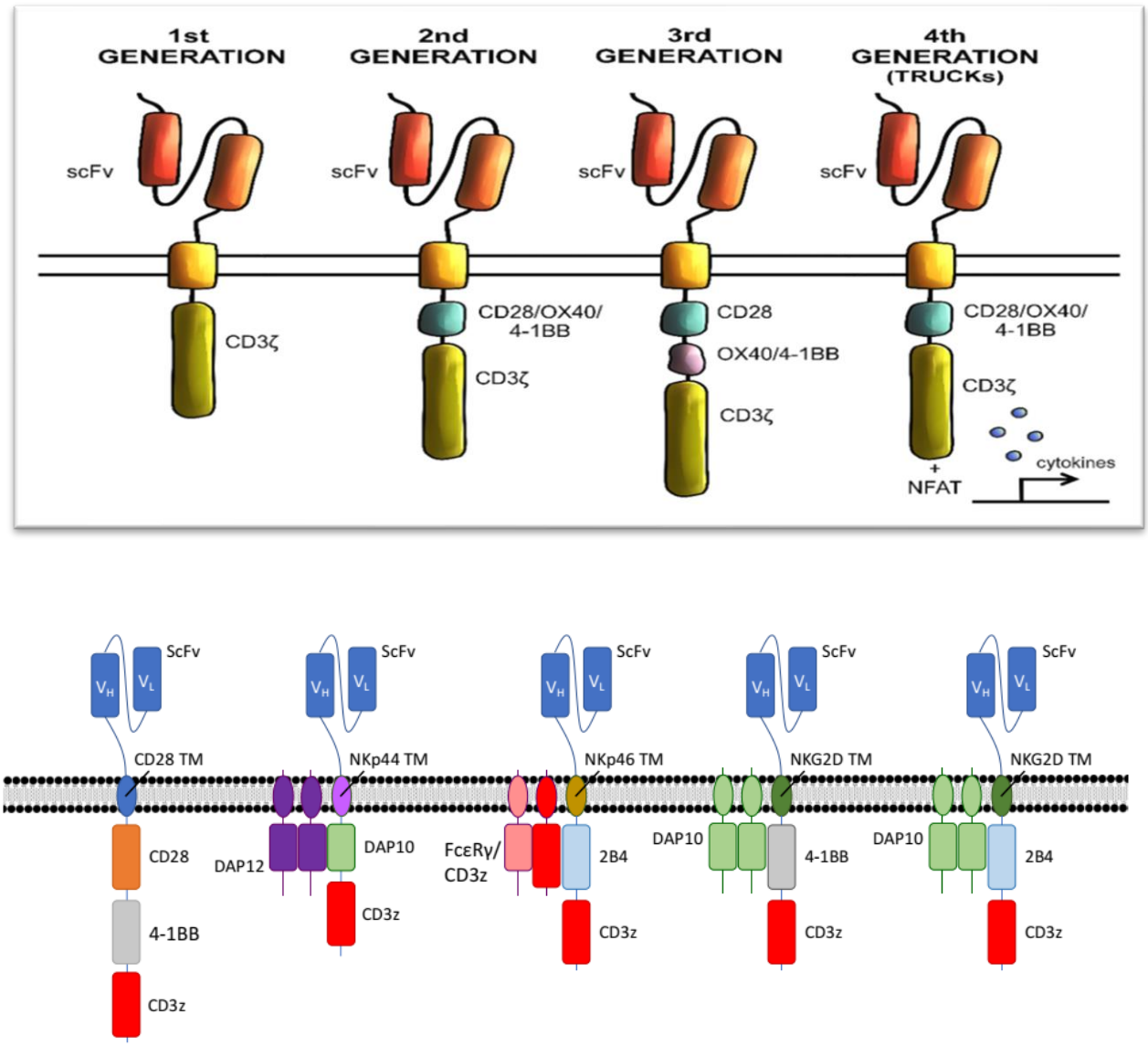


Figure 21. Representation of chimeric receptor constructs

NK cells have a vital function in identifying abnormal cells and contribute to both the immediate and acquired immune responses in the presence of infection and inflammation. They are rapidly recruited and activated, hence enhancing both innate and adaptive immune responses. The absence of NK cells in both mice and humans leads to vulnerability to viral infections and negative clinical consequences. NK cells depend on other stromal cells, such as dendritic cells (DC) and monocytes expressing MICA, to undergo differentiation and trigger effector responses. Additionally, they have the capacity to develop immunological memory, sometimes referred to as "trained immunity." In humans, NK cells have the ability to retain information about past encounters with inflammatory conditions. This results in a transformation of regular NK cells into memory NK (NKm) cells via a process called trans-differentiation. The malfunction of NK and natural killer T (NKm) cells is becoming seen as a crucial occurrence that promotes the growth of tumours and the spread of cancer to distant parts of the body. NK cells depend on the equilibrium between activating and inhibiting receptors to carry out their cytotoxic actions, whereby perforin and the granzyme family of proteins serve as the primary effector molecules. NK cells are inhibited by chemicals generated by tumours, stromal cells that have been taught by tumours, and tumour cells themselves. This ultimately leads to the advancement and multi-step spread of cancer. Tumours have the ability to transform regular NK cells into CD73+ NK cells, which exhibit elevated amounts of checkpoint molecules. This transformation leads to immune evasion. The presence of Th2-polarized iNKT cells in the tumour microenvironment (TME) promotes tumour growth by exerting immunosuppressive effects. Additionally, the persistent exposure to ligands produced on the surface of tumour cells leads to the impairment of NKm cells, which are crucial for long-term anti-tumor immune responses. Stromal cells, including cancer-associated fibroblasts, monocytes, macrophages, and other immune cells, might hinder the cytotoxicity of NK cells. The impaired activity of NK cells after surgery has been recognised as a contributing factor to the spread of cancer cells to other parts of the body, known as metastasis. This may be partially attributed to the disruption of the equilibrium between activating and inhibitory signals. The regulation of metastasis by NK cells depends on the activation of macrophages via dectin-1, specifically involving the plasma membrane tetraspan protein MS4A4A. Lipid buildup is a frequent occurrence in postoperative patients with colorectal cancer, and it hinders the activity of NK cells, hence promoting metastasis. NK cells are seldom seen in metastatic melanoma and mostly consist of TIGI T−CD226− cells, which lack the ability to kill MHC-I-deficient cancerous cells. The metastatic spread of small-cell lung cancer is primarily driven by the lack of NK cells, rather than the absence of CD8+ T cells. Malignant conditions, including as hypertension, diabetes, and obesity, have a role in the development and advancement of tumours. The process of carcinogenesis and cancer development is significantly influenced by the metabolic rivalry between

tumour and stromal cells. Within the tumor-infiltrating stromal (TME) milieu, the functionality of NK cells is hindered by suppressive cytokines and unfavourable metabolic circumstances, such as hypoxia, malnutrition, and aberrant levels of tumor-derived substances like lactate. Obesity is seen as a possible risk factor for breast cancer, since it may lead to increased mental stress and disrupt the functioning of NK cells via the alteration of lipid and glucose metabolic pathways. Gaining insight into how metabolic activity impacts the function of stromal cells that infiltrate tumours, resulting in the advancement of cancer and evasion of the immune system, would provide valuable information for the development of innovative treatments targeting immunometabolic factors. The metabolic abnormalities in the tumour microenvironment (TME) may be modified by several factors, including lipid and glucose metabolic pathways, lipid metabolism, and oxidative phosphorylation (Fig. 22). Elevated levels of cholesterol in the bloodstream and the buildup of cholesterol in NK cells enhance their capacity to combat tumours by promoting the development of lipid rafts in a mouse model with liver tumours. Hypoxia is a frequent characteristic of cancer, and it promotes the production of NK cells via the activation of HIF-1α.

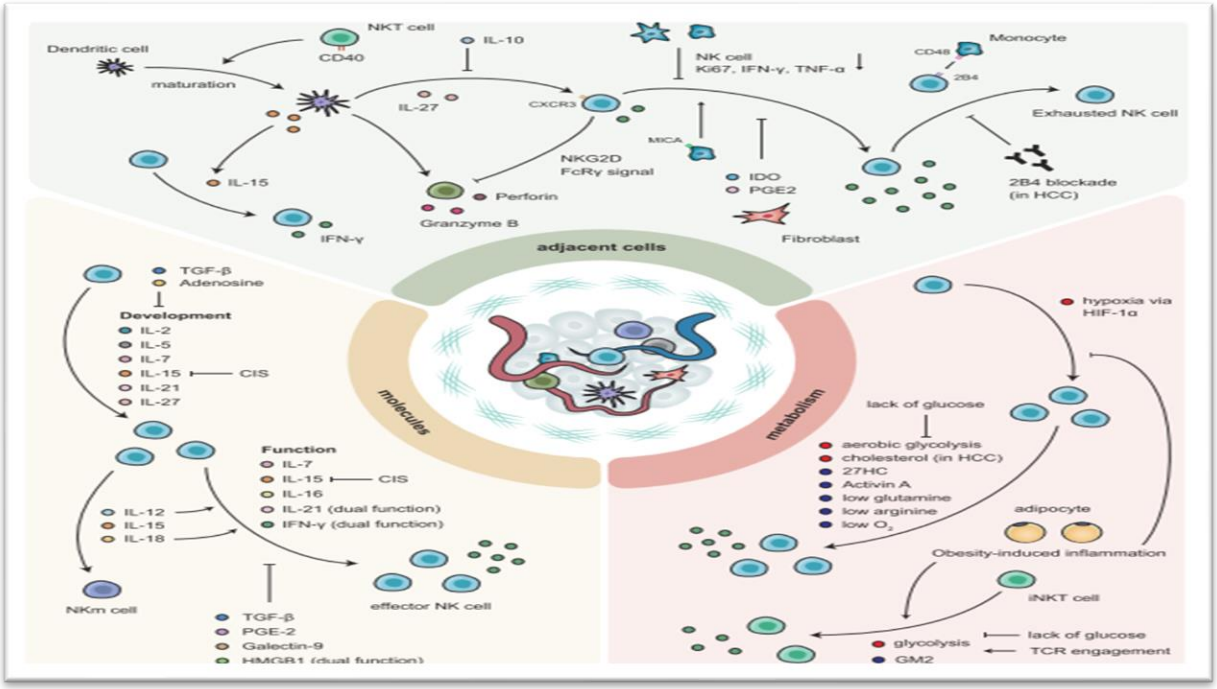


Figure 22. NK cells and microenvironment

NK cells, crucial agents of the innate immune system, have shown promise in the field of cancer treatment. They may be classified into many characteristics and exhibit unique roles, such as iNKT cells, which can be classified into various characteristics and exhibit unique roles. iNKT cells specifically detect glycolipid signals rather than peptides using a semi-invariant TCR. They are limited to recognising glycolipid antigens that are presented by CD1d-related molecules, which are

similar to MHC molecules. These CD1d-related molecules are found in large concentrations, particularly in adipocytes and hepatocytes. Metabolic activity has the potential to modify this process. The use of NK cell-based therapy, either as a standalone treatment or in conjunction with other treatments, has shown positive outcomes, indicating its broad and efficacious application in the treatment of malignancies. The administration of the cytokine supplement IL-15 enhances the growth and capacity of NK cells to kill target cells. Multiple clinical studies have shown the safety of using recombinant human IL-15 (rhIL-15) and its agonist, ALT-803, in various types of tumours, including metastatic lung cancer and post-transplantation patients. The presence of heterodimeric IL-15 may enhance the infiltration of NK cells and CD8+ T cells into the tumour, hence increasing the efficacy of existing immunotherapy. IL-12, IL-15, and IL-18 play a crucial role in the development of NKm cells. When memory-like NK cells are given more IL-12, IL-15, and IL-18, they exhibit improved reactions against acute myeloid leukaemia, both in laboratory settings and in living organisms. The combination of IL-15 and TIGIT inhibition effectively enhances NK cell-mediated immunity. Additional investigation is required to comprehend the impact of intracellular metabolic signals on the viability and activity of iNKT cells, as this might potentially become a focal point of cancer immune-metabolic treatment after CD8+T cells and NK cells. Immune checkpoint drugs, such as cetuximab and PD-1 blockage, have the ability to inhibit NK cells. Following the administration of cetuximab, there is an increased presence of PD-1+ NK cells in the tumour microenvironment (TME) of head and neck cancer patients. This enrichment of PD-1+ NK cells is associated with positive clinical outcomes. Nivolumab, a PD-1 blocker, significantly enhances the activation and activity of NK cells produced by cetuximab in tumours with high levels of PDL1. NK cells exhibiting diminished quantities of T-cell immunoglobulins and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) exhibit elevated levels of cytokine production, degranulation activity, and cytotoxicity. Inhibiting TIGIT might potentially mitigate the phenomenon of NK cell fatigue. CD96, which has the same ligand CD155 as CD226 and TIGIT, exerts a negative regulatory influence on the immunological response by NK cells and serves as a prognostic indicator for worse survival outcomes in individuals with hepatocellular carcinoma. The administration of a CD96 antibody enhances the capacity of NK cells to inhibit the spread of cancer cells to other parts of the body. This effect is significantly amplified when the CD96 antibody is used in combination with anti-CTLA-4, anti-PD-1, or doxorubicin treatment. The concurrent use of CIS inhibition together with CTLA-4 and PD-1 blocking demonstrates enhanced efficacy in suppressing melanoma metastasis, beyond the benefits of each individual therapy alone. Antibodies that specifically target MICA and MICB have the ability to hinder the detection of NK cells and the binding of tumour cells. This inhibition leads to the suppression of tumour development in mice models that possess a fully functional immune

system, as well as in animal models that have been modified to resemble human immune responses. The in vivo efficacy of combination therapy, specifically targeting soluble MIC (MICA and MICB) and PD-L1, surpasses that of monotherapy. Monalizumab, an antibody now utilised in clinical settings, specifically targets NKG2A, which is an inhibitory checkpoint found on NK cells. This antibody has been shown to enhance the activity of NK cells in several preclinical models and also enhances the effectiveness of anti-PD-1 and anti-EGFR therapies. In recent years, NK adoptive treatment and emerging genetic alteration of NK cells have been used to treat certain forms of cancer. Nevertheless, uncertainties persist about the self-renewal capacity of transplanted NK cells, the possibility for NK cells to persist for extended periods of time, and the likelihood of developing NK lymphoma. Chimeric antigen receptors (CARs) show potential in cancer treatment by genetically modifying immune cells to directly attack tumour cells. Kymriah, Yescarta, Actemra, and TriKE are among the CAR-T therapies that have been authorised for the treatment of recurrent and resistant cases of acute lymphoblastic leukaemia, diffuse large B cell lymphoma, and cytokine-releasing storm (CRS). Antibody engineering techniques enhance the ability of NK cells to destroy tumour cells by improving antibody-dependent cell-mediated cytotoxicity (ADCC). This is achieved via the use of bispecific killer cell engager (BiKEs) or trispecific killer engager (TriKEs) antibodies (Fig. 24). These techniques have shown greater capacity for tumour detection and a reduced occurrence of cytokine release syndrome (CRS) in comparison to CAR-T. CAR-NK cells have shown effectiveness and little harm, as evidenced by ongoing clinical studies evaluating their therapeutic potential. Nevertheless, the intricate procedure of manufacturing CAR-NK cells incurs high costs and yields unsatisfactory outcomes when targeting solid tumours. The use of CAR-iPS holds promise as a prospective avenue for advancing anti-tumor immunotherapy. This approach involves cultivating and differentiating CAR-iPS cells in a laboratory setting, which may then be used to directly boost the effectiveness of CAR-NK cells in combating tumours inside the body. The TGF-β molecule and its associated pathway provide promising prospects for the creation of drugs that may combat tumours. The therapeutic manipulation of TGF-β, accomplished by using small-molecule inhibitors and antibodies, is now under investigation in clinical studies. Targeting TGF-β, the TGF-β superfamily, and its downstream pathways in NK cells has potential as a viable approach to improve the effectiveness of existing immunotherapies.

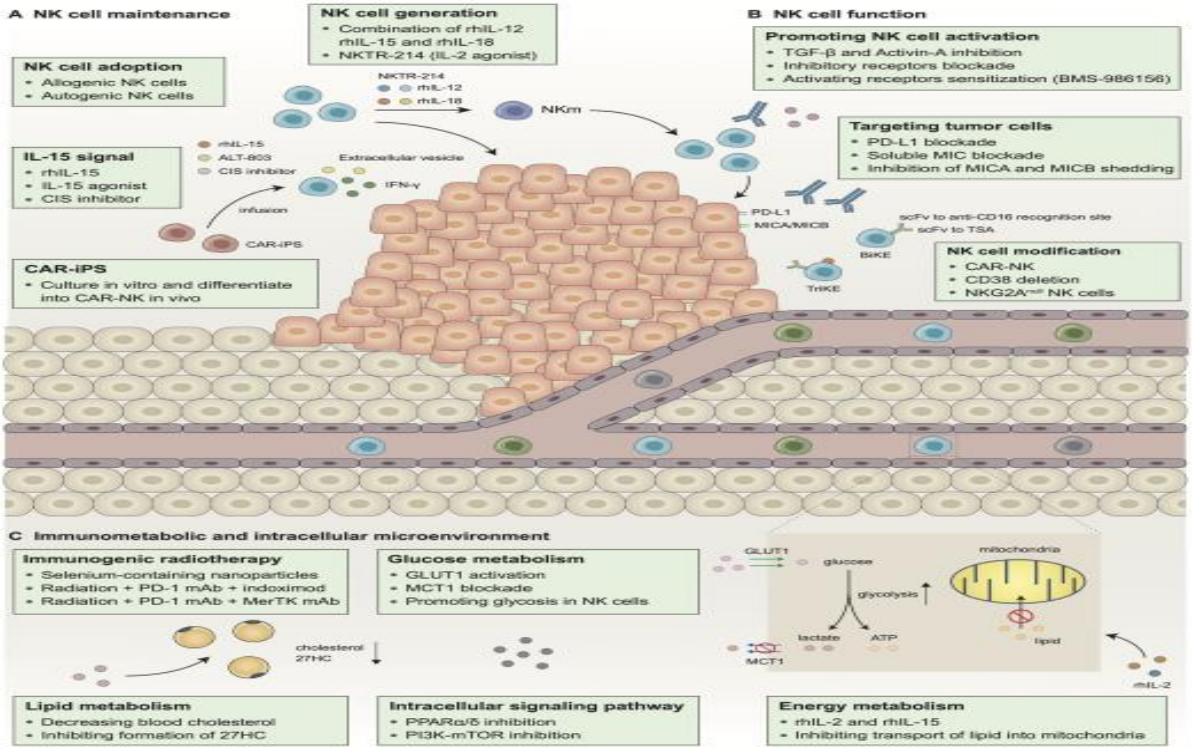


Figure 23. Potential objectives for using NK cells in cancer treatment.

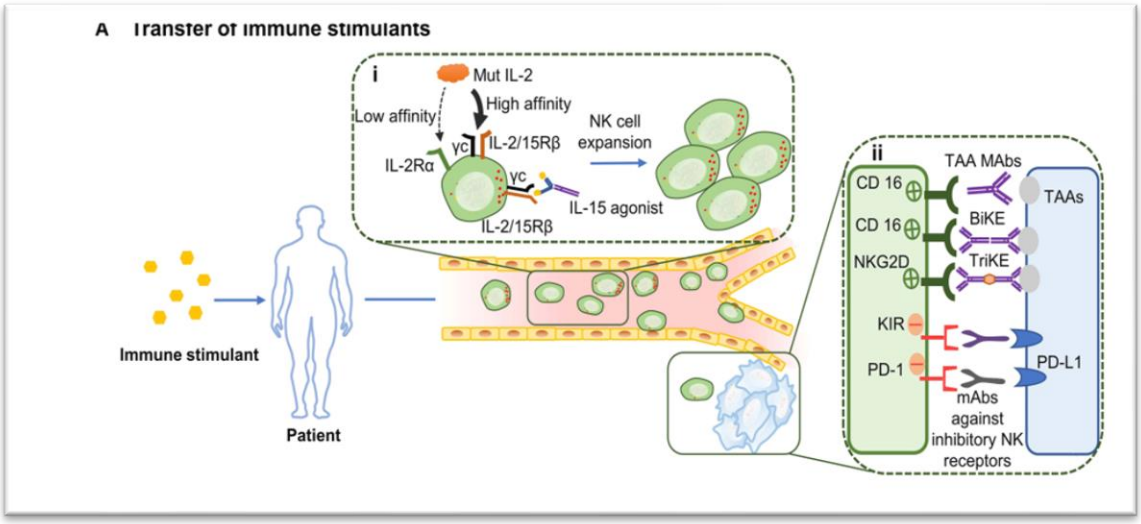


Figure 24. NK cell in cancer therapy

EVs refer to miniature extracellular vesicles characterized by a lipid bilayer membrane, including exosomes, microvesicles, and apoptotic bodies. These vesicles exhibit shared biomarkers and distinct proteins depending on their cellular source. They are seen as possible substitutes for cell-based therapies because they may transport bioactive substances or stimulate signalling pathways in specific cells.

EVs have the ability to passively spread across tissues, which facilitates the merging of EVs with tumour cells. Additionally, they possess the ability to efficiently traverse biological barriers such

as the blood-brain barrier (BBB) and the blood-tumor barrier (BTB), which enhances their storage convenience and accessibility as immediately available "off-the-shelf" resources.

The first investigation on NK EVs revealed that NK EVs obtained from pure NK cell culture had cytotoxic properties against malignant hematologic cell lines, whereas they did not display such effects on solid tumour cells such as breast cancer. The cytotoxic effects of activated NK extracellular vesicles (EVs) on different cancer cell lines indicate that activated NK cells are capable of producing EVs with enhanced immunological activity in comparison to naïve NK cells. NK cells have the ability to induce the death of certain cells via two separate mechanisms: ligand-receptor interactions and fusion of the plasma membranes. Additionally, they demonstrate tumor-specific accumulation and the capacity to traverse the blood-brain barrier (BBB). The processes by which NK extracellular vesicles (EVs) cause cell death may be categorized into three groups: entrance facilitated by perforin and granzyme, engagement mediated by receptor-ligand binding, and action mediated by granulysin. Nevertheless, the precise methods by which NK EVs selectively eliminate tumor cells are not yet fully understood. An acidified microenvironment in solid tumors may facilitate the fusion of exosomes, which might be a potential explanation. It is worthwhile to investigate the potential use of immunotherapy in autoimmune illnesses and its ability to eliminate adverse effects, such as cytokine storms (Fig. 25).

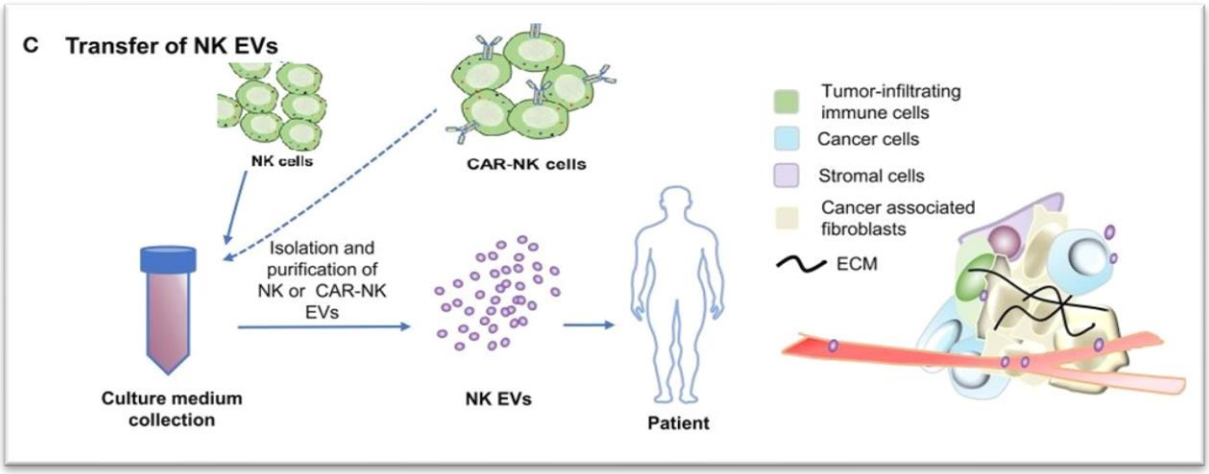


Figure 25. Extra vesicules of NK

Table 3. Strategies of cell therapy using NK cells

Mechanism	Condition	Intervention	Phase
IL-15 signal pathway	Metastatic malignant melanoma, RCC	Recombinanthumaninterleukin-15(rIL-15)	I(first-in human)
	Advancedmetastaticsolidtumor	IL-15bycontinuousinfusion	I
	RefractoryandrelapsedadultTcell leukaemia	IL-15+alemtuzumab(anti-CD52)	I
	Refractory and relapsed chronic lymphocytic leukemia	IL-15+obinutuzumab(anti-CD20)	I

	Hematologic malignancies recurring After transplantation	ALT-803(IL-15superagonist)	I(first-in human)
	Metastatic NSCLC	ALT-803+Nivolumab(anti-PD-1antibody)	Ib
IL-21signal pathway	Relapse/refractory low-grade B-cell LPD	Recombinant human interleukin-21(rIL-21) +Rituximab (anti-CD20 antibody)	I
	Metastatic malignant melanoma, RCC	rIL-21	I
	Stage IV malignant melanoma without prior treatment	rIL-21	IIa
IL-12signal pathway	Metastatic solid tumors	NHS-muIL12 (two IL12 hetero dimers fused To the NHS76 antibody)	I(first-in human)
	Murine mammary /subcutaneous tumors	NHS-muIL12+Avelumab (anti-PD-L1 antibody)	Preclinical models
IL-2 signal pathway	Locally advanced or metastatic solid tumors	NKTR-214(IL-2 pathway agonist)	I/II
	Advanced Solid Tumors (Japanese)	NKTR-214+Nivolumab	I
Anti-KIR antibody	AML in FCR	IPH2101(anti-KIR antibody)	I
	Relapsed/refractory MM	IPH2101	I
	Smol dering MM	IPH2101	II
	Relapsed/Refractory MM null	IPH2101 + lenalidomide (immunomodulatory agent)	I
	AML	Lirilumab(2ndgenerationanti-KIRantibody))	II
	SCCHN	Lirilumab+Nivolumab	II
	Cisplatin-ineligible muscle-invasive bladder cancer	Lirilumab + Nivolumab	Ib
Anti-NKG2A	Advanced gynecologic malignancies	Monalizumab (IPH2201, anti-NKG2A antibody)	I
metastatic microsatellite	metastatic microsatellite-stable colorectal cancer	Monalizumab+durvalumab	First-in human
TNF pathway	Recurrent or metastatic head-neck cancer	Monalizumab+cetuximab	I
	Advanced solid tumors	BMS-986156 (glucocorticoid-induced TNF Receptor-Related Protein Agonist)+/− Nivolumab	I/IIa
Cell adoptive therapy	Caninesarcomas	Radiotherapy+intra-tumoral autologous NK transfer	first-in-dog
	Recurrent medulla blastoma and ependymoma (children)	ex-vivo-expanded NK cells	I
	Metastatic gastrointestinal carcinoma	Adoptive transferred autologous NK cells+ cetuximab	I
	HER2-positive cancers	Adoptive transferred autologous NK cells + trastuzumab	I
	Locallyadvancedcoloncarcinoma	Adoptive transferred autologous NK cells+ chemotherapy	I
	Malignant lymphoma oradvanced	Adoptive transferred allogeneic NK cells	I

	Myeloid leukemia	Adoptively transferred memory-like NK cells Induced by IL-12, IL-15, and IL-18	I (first-in human)
	High-risk AML, MDS, CML	MbIL21 ex vivo -expanded donor-derived NK cells	I
	MDS, AML.	Fludarabine/ cyclophosphamide + total Lymphoid irradiation + adoptive transferred IL2-activated haploidentical NK cells	I
	Older AML patients	Transferred umbilical cord blood CD34 hematopoietic stem + progenitor-derived NK Cells	I(first-in human)
	Non-Hodgkin lymphoma	Haplo identical donor NK cells+rituximab+ IL-2	II
	Myeloma	α-galactosylceramide-loaded monocyte-derived dendritic cells + low-dose lenalidomide (mediate antigen-specific-stimulation of human NK cells)	I
CAR-NK therapy	CD19-positive lymphoid tumors	NK cells expressing anti-CD19 CAR, IL-15 and Inducible caspase 9	I/II

7. Mesenchymal Stem Cells

Alexander Friedenstein was the first to identify colony-forming unit fibroblasts and osteogenic stem cells. Since then, other terminologies have been used and suggested to characterise MSCs. In 1988, Maureen Owen proposed the use of the term "stromal stem cells" to specifically refer to cells that are located in the stromal compartment rather than the hematopoietic compartment. In 1991, Arnold Caplan used the term "mesenchymal stem cells" to emphasise the cells' ability to renew themselves and differentiate into multiple cell types, instead of focusing on their genesis in compartments. Nevertheless, James Dennis posed a challenge to this naming convention by proposing that the cells may perhaps be progenitors instead of stem cells. Consequently, the name "mesenchymal progenitor cells" was suggested. In the year 2000, Paolo Bianco and Pamela Gehron Robey introduced the term "skeletal stem cells" to specifically refer to cells that produce parts of the skeletal system. Just two years later, Yuehua Jiang suggested the term "multipotent adult progenitor cells" (MAPCs) to describe the cells' ability to develop into multiple cell types and their potential as progenitors. In 2006, the International Society for Cell and Gene Therapy (ISCT) introduced the phrase "multipotent mesenchymal stromal cells" since there was no conclusive data showing that MSCs may renew themselves and develop into multiple cell types in living organisms. In 2010, Arnold Caplan proposed that the abbreviation "MSCs" should be interpreted as "medicinal signalling cells" in order to acknowledge that the main therapeutic advantage of MSCs may be ascribed to the release of bioactive substances rather than direct cell substitution. There is a suggestion that all cells expressing MSC markers, which are multipotent, clonal, and fibroblastoid in nature, originate from a single source. However, these cells assume distinct functions throughout embryonic development.

Mesenchymal stem cells (MSCs), also called stromal cells are multipotent adult progenitors that have the capacity for self-renewal and generate several types of mature and functional differentiated cells like: cartilage, bone, muscle, and adipocytes. they are derived from mesodermal progenitors found in both adult and fetal tissues such as umbilical cord blood. In the bone marrow, especially in adults, MSCs form a small population constituting the non-hematopoietic compartment. The “plasticity” and paracrine activity of MSCs are the subject of numerous studies with the aim of developing new therapeutic strategies.

The International Society for Cellular Therapy (ISCT) has established standard criteria for identifying mesenchymal stromal cells (MSCs). MSCs must exhibit flexible adherence and possess the ability to develop into osteoblasts, adipocytes, and chondroblasts. In order to fit the requirements, it is often expected that they possess certain surface antigens while lacking others. Mesenchymal stem cells (MSCs) often have a low level of immunogenicity. They exhibit moderate levels of MHCI expression and little to no expression of MHCII antigens and co-stimulatory components. MHC class

I expression inhibited the NK cell-like behaviour of MSCs, while the absence of costimulatory molecules resulted in T cell exhaustion.

7.2. Sources of MSCs

A significant benefit of MSCs is their wide accessibility. MSCs may be obtained from several sources, including foetal, and adult tissues. These cells have the ability to differentiate into different types of cells when cultured under certain circumstances. The primary sources of mesenchymal stem cells (MSCs) obtained during childbirth are derived from the umbilical cord and placental tissues. The primary sources of MCSs derived from adult tissues are mostly bone marrow and adipose tissue. MSCs derived from various sources have unique properties, referred to as tissue source-associated heterogeneity. MSCs have superior proliferation capacity, longevity, and differentiation ability. We have previously outlined techniques and procedures for producing functional MSCs from pluripotent stem cells (iPSCs), in addition to MSCs obtained from tissue. MSCs were first identified in bone marrow, but further research has shown their presence in several other postnatal organs and tissues, including the brain, kidney, liver, lung, spleen, adipose tissue, muscle, hair follicles, teeth, placenta, and umbilical cord.

MSCs are often classified as mesodermal cells, especially osteoblasts, chondroblasts, adipocytes, or fibroblasts, which are all cells derived from the mesodermic route. Furthermore, they have the capability to induce the development of fully developed cells derived from the three embryonic germ layers by introducing certain in vitro stimuli, such as DNA demethylating agents. Therefore, it is possible to acquire specialised cells from the ectodermal leaf, neuroectodermal, and endodermal. CSMs were traditionally believed to originate from the mesoderm. However, recent evidence has shown that throughout embryonic development, they really arise from many embryonal sources, including the neuroepithelium of the neural crest (Fig.26).

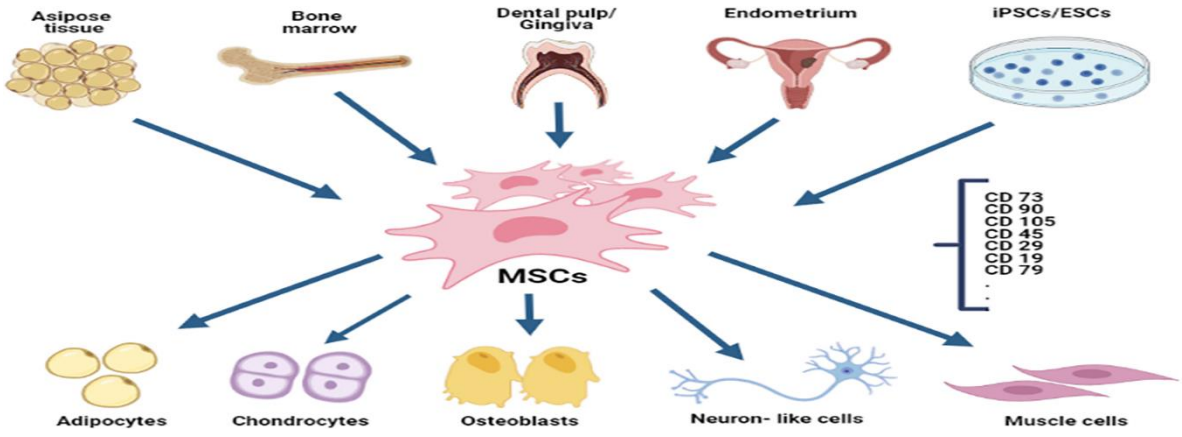


Figure 26. Sources and differentiation of MSCs

In 2005, the International Society for Cellular Therapy (ISCT) established the minimum criteria for defining in vitro human mesenchymal stem cells (MSCs). These criteria are as follows: (a) MSCs must adhere to plastic surfaces and have a fibroblast-like appearance under optimal culture conditions; (b) MSCs must express CD105, CD90, and CD73 surface markers, while not expressing CD34, CD45, CD14, CD19, CD11b, CD79a, and HLA-DR surface markers; and (c) MSCs must have the ability to differentiate into osteoblasts, chondroblasts, and adipocytes in laboratory settings. The purpose of these standards is to differentiate between mesenchymal stem cells and MSCs since they are not the same. Therefore, in addition to their capacity to renew themselves and differentiate into many cell types, MSCs must also exhibit the ability to secrete substances, home to specific locations, and modulate the immune system (Fig.27).

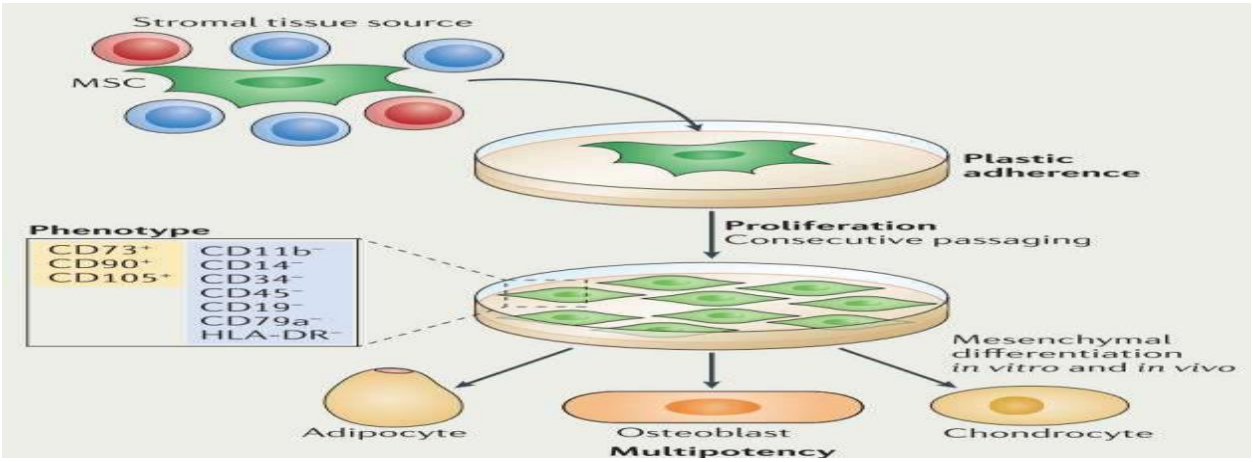


Figure 27. Mesenchymal stem cell characteristics.

7.3. Function of MSCs

7.3.1. Homing and Angiogenesis

Homing refers to the targeted migration of MSCs towards the location of damage, where they release trophic signals. The process consists of three primary stages: chemotaxis towards areas of inflammation, adherence to damaged cells, and infiltration into inflammatory sites by enzymes such as MMPs and TIMPs. MSCs possess distinct characteristics that make them very advantageous for tissue regeneration and repair. These include their capability to transform into other types of cells, their ability to migrate, their potential to promote the formation of new blood vessels, their anti-cell death activity, and their release of biologically active substances.

Mesenchymal stem cells (MSCs) possess functional characteristics that render them well-suited for the purpose of tissue repair and regeneration. These characteristics include their power to transform into various cell types, their ability to travel to damaged tissues, the promotion of new blood vessel formation, their ability to prevent cell death, and their capability to release biologically

active substances. Paracrine signalling has a crucial role in controlling the processes of cell proliferation, antioxidant activity, and differentiation. It involves the recruitment of macrophages and endothelial cells, as well as the activation of resident stem cells, to facilitate tissue healing (Fig. 28).

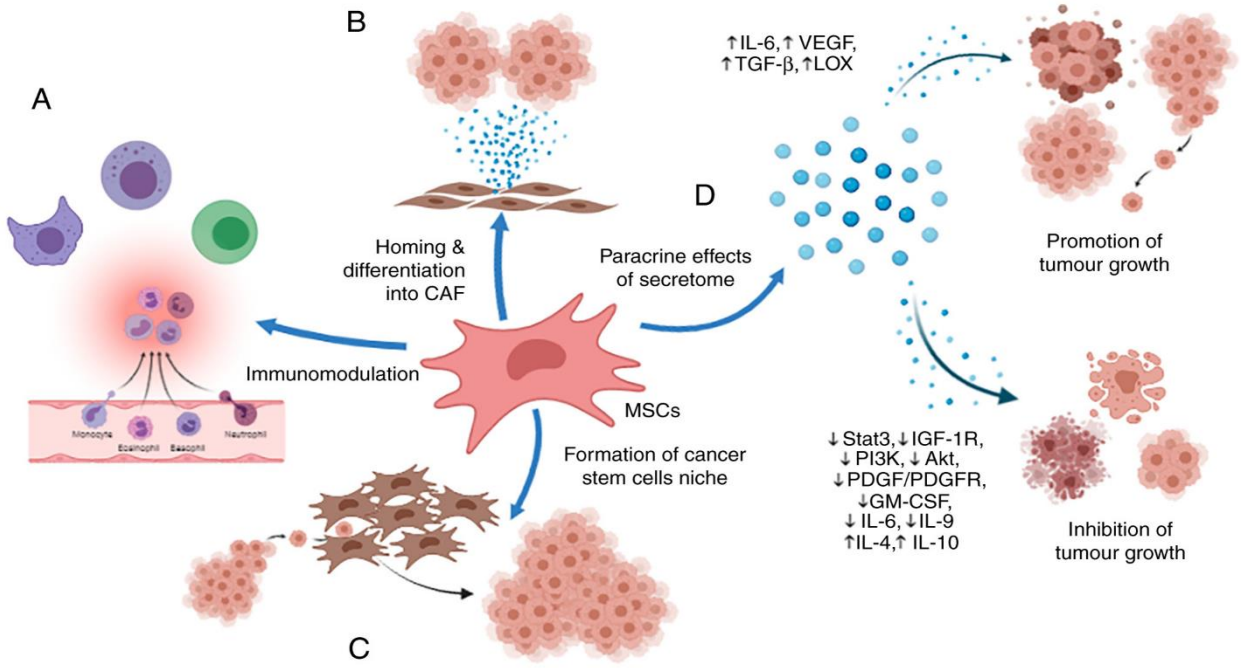


Figure 28. Homing and angiogenesis activity of MSCs.

Angiogenesis is the formation of new blood vessels from pre-existing vessels. MSCs possess substantial angiogenic potency. Restoring a vascular network is crucial for supplying oxygen to the tissue, which is essential for repairing and restoring organ function. The impact is mostly attributed to certain factors that are generated and released by MSCs. The secretome of molecules has been shown to have a significant part in angiogenesis. Some of these molecules include VEGF, FGF-2, angiopoietin-1 (Ang-1), CCL2, Il-6, placenta growth factor (PLGF), and the protein cysteine-rich, angiogenic inducer 61 (Cyr61). Furthermore, adipose tissue-derived MSCs not only outnumber marrow-derived MSCs, but they also possess the highest angiogenic potential and originate from the same progenitors as endothelial cells. Furthermore, research indicates that the substances secreted by MSCs have the ability to maintain and reinforce the existing blood vessels by functioning similarly to pericytes. MSCs seem to influence many phases of angiogenesis, ranging from the attraction of endothelial cells to the consolidation of fully formed blood vessels. The proangiogenic activity of MSCs is a primary mechanism that contributes to the positive benefits found in ischemic diseases. They are commonly utilised in the management of cardiac and renal ischemia, demonstrating a significant impact on both the restoration of glomerular filtration in chronic renal failure models and the ventricular ejection fraction in myocardial infarction models. After Planat-Benard conducted

research in 2004, a phase 1 clinical study was conducted to reestablish a vascular network in individuals suffering from severe limb ischemia.

Mesenchymal stem cells (MSCs) control the immunomodulatory process by releasing substances that disrupt the functioning of the immune system. MSCs demonstrate their immunomodulatory capabilities by engaging in cell-to-cell communication, which results in the activation of monocytes, eosinophils, basophils, and neutrophils. Mesenchymal stem cells (MSCs) are progenitor cells with the ability to migrate, home, and develop into cancer-associated fibroblasts (CAFs). Mesenchymal stem cells (MSCs) may create an environment in the body where cancer stem cells can maintain their capacity to grow and perpetuate the cancerous process. The paracrine actions of the secretome play a role in the dual function of MSCs. As an example, they reduce the levels of Stat3 and IGF1R and elevate the levels of IL4 and IL10 in order to hinder the development of tumours. Simultaneously, they raise the levels of IL6 and VEGF to facilitate the growth of tumours.

7.3.2. Antiapoptotic activity

Scientists from several countries have consistently shown the anti-apoptotic impact of MSCs. Immunohistochemical techniques, such as terminal deoxynucleotidyl transferase dUTP nick end labelling staining, were used in several investigations and showed a significant reduction in the number of cells undergoing programmed cell death in organs that were pre-treated with MSCs and MSC-CM. The amount of Akt was determined in different tests by reverse transcriptase polymerase chain reaction and Western blot analysis. The group treated with MSCs and MSC-CM had a considerable increase in the level of Akt, as shown in both in vivo and in vitro models. Akt's capacity to phosphorylate the BLC-associated cell death promoter (BAD) is responsible for its anti-apoptotic impact. BAD has the capability to create holes in the outer mitochondrial membrane. The development of this hole allows cytochrome c to be released into the cytoplasm, triggering the activation of the caspase cascade, and ultimately resulting in apoptosis. Phosphorylation of BAD causes it to separate, leading to a reduction in its pro-apoptotic capability. The assessment of caspase-3 levels, which serve as a marker for apoptosis, and Ki-67 levels, which serve as a marker for proliferation, was conducted in many investigations. The administration of MSCs resulted in a substantial reduction in caspase-3 levels and an increase in Ki-67 levels. This suggests a decrease in apoptosis and an increase in proliferation. Nevertheless, the ability of MSCs and MSC-CM to prevent cell death by apoptosis is still a subject of debate.

One study has shown that reactive oxygen species are responsible for cisplatin-induced apoptosis, which is a crucial mechanism of cell death. The anti-apoptotic impact of MSCs is most likely due to the process in which the growth factors generated by MSCs reduce oxidative stress by enhancing the activity of antioxidants and restoring normal mitochondrial function. Studies have shown that the

inclusion of Hepatocyte Growth Factor (HGF) enhances cell viability in the context of oxidative stress-induced myocardial infarction. Conversely, there have been reports indicating that some growth factors, such as VEGF, are capable of stimulating Akt activation. Introducing anti-VEGF antibodies decreases the activity of Akt in a laboratory setting and enhances the level of apoptosis. Studies have shown that MSC-CM may stimulate the Akt pathway (via phosphatidylinositol 30-kinase) in the aortic endothelium under hypoxic circumstances, leading to a reduction in apoptosis. Further research is needed to investigate the anti-apoptotic function of MSCs, which presents an appealing field of study for the scientific community.

7.3.3. Immunomodulatory

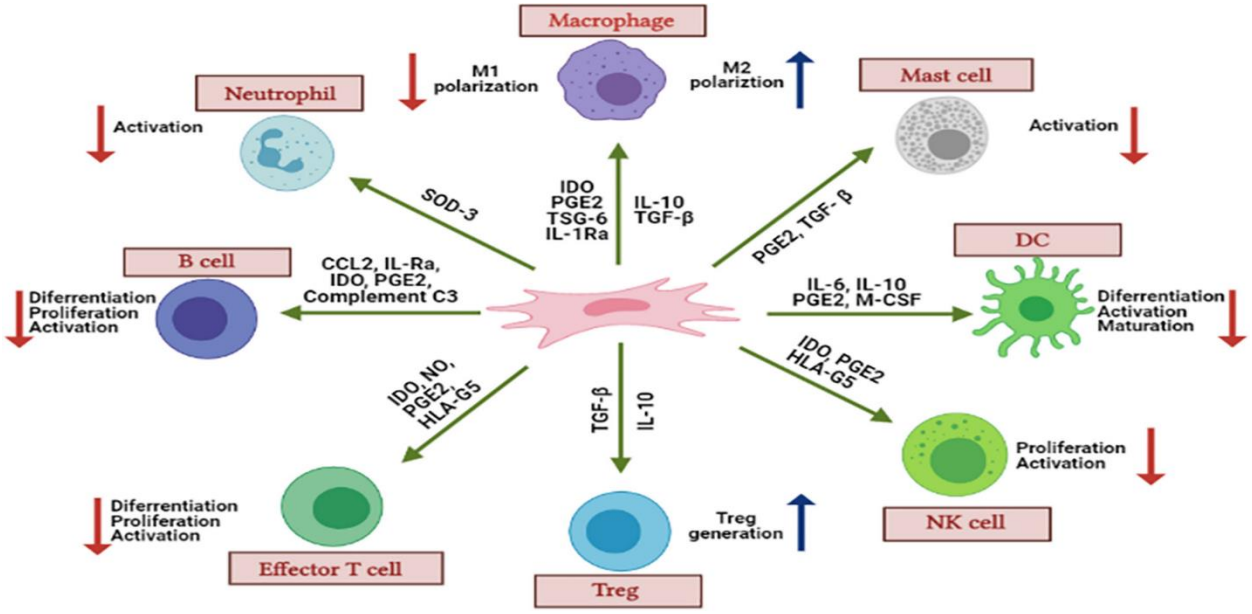
MSCs possess the ability to modulate the immune system, which is a significant characteristic. They may effectively suppress the growth and activity of many immune cells, including B and T lymphocytes, dendritic cells, natural killer cells, monocytes, neutrophils, and macrophages. They have the ability to halt the growth and development of B-cells, hinder the process of changing antibody types, prevent the movement of cells towards chemical signals, enhance the production of IgG antibodies, reduce the secretion of pro-inflammatory cytokines by Th1 cells, promote the secretion of IL-4 by Th2 cells, suppress the proliferation of T cells, promote the formation of regulatory T cells, and reduce the harmful effects of cytotoxic T lymphocytes. Several factors produced by MSCs, such as the enzyme indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), TNF-α-stimulated gene/protein 6 (TSG-6), or transforming growth factor (TGF-β), have been found to play a significant role in suppressing T lymphocytes, promoting the development of dendritic cells (DCs), enhancing the growth of natural killer (NK) cells, and stimulating the production of IL-10 by regulatory T lymphocytes (Figure 28a, 28b).

The host is protected by the anti-inflammatory properties of MSCs, which reduce the intensity of the immunological response to inflammation. Through paracrine signalling, they have the ability to hinder planned cell death, so safeguarding damaged cells and maintaining the functionality of organs. The mediators released by MSCs consist of SDF-1, IGF-1, Nrf2, HIF, HO-1, and VEGF.

The production of angiogenic cytokines, including VEGF, FGF1, FGF2, HGF, Ang-1, Ang-2, and SDF-1, promotes neoangiogenesis in damaged tissues. MSCs release growth factors that induce the multiplication of native stem cells via intricate paracrine and cell-to-cell connections.

MSCs have shown the ability to stimulate the transformation of monocytes/macrophages into an anti-inflammatory/immune regulatory (type 2) state, while also directly preventing their development into the type 1 state and dendritic cells (DCs). Interleukin 1 Receptor Antagonist (IL1-RA) released by MSCs may stimulate the transformation of macrophages into the type 2 phenotype. Monocytes with anti-inflammatory properties release abundant amounts of IL-10 and exhibit reduced levels of

IL-12p70, TNF-a, and IL-17 expression. This effect is facilitated by IL-6 and hepatocyte growth factor (HGF) generated by MSCs. Monocyte-derived IL-10 inhibits monocyte development into dendritic cells (DCs) and redirects monocytes towards an anti-inflammatory subtype that secretes IL-10, thereby establishing a positive-feedback loop. In addition to IL-10, monocytes that have been primed with MCS show elevated expression of MHC class II, CD45R, and CD11b. These monocytes seem to have the ability to inhibit T cell activation, independent of the presence of FoxP3+ Tregs.



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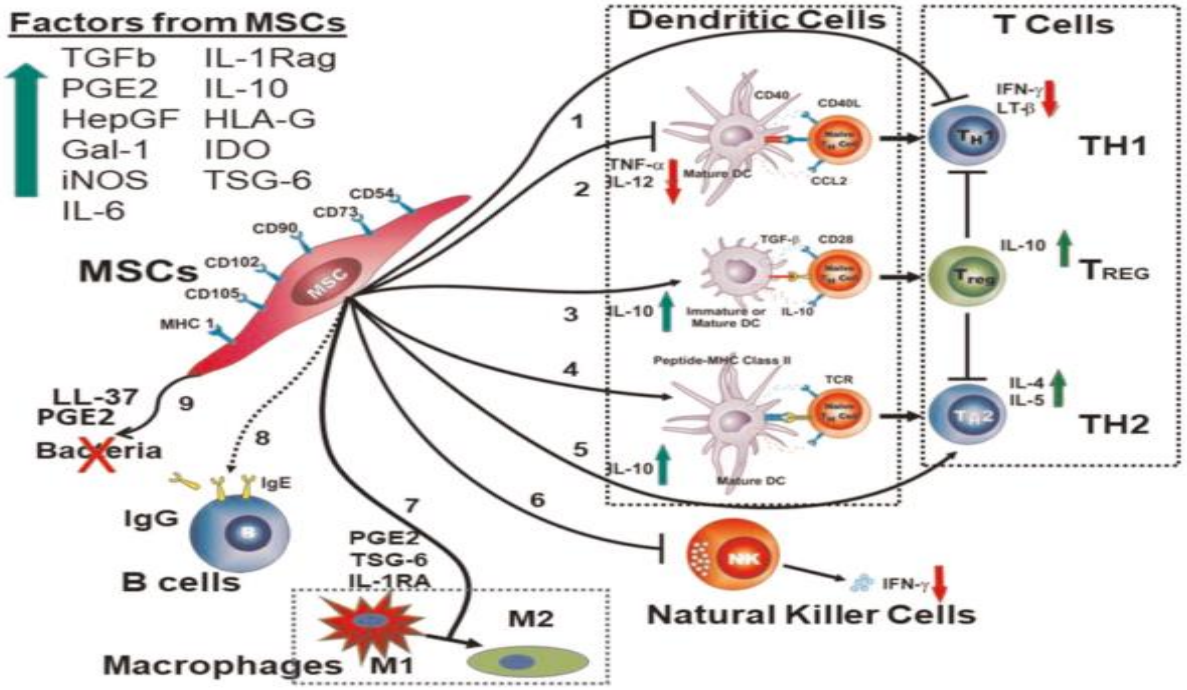

Figure 29. . Immunomodulatory activity of MSCs

The secreted substances from type 2 macrophages stimulate the development of FoxP3+ Tregs from naïve CD4+ T cells, highlighting the significance of soluble molecules in the immunomodulatory effects of MSCs. Monocytes create CCL-18 and release transforming growth factor beta 1 (TGF-β1), which in turn facilitate the development of regulatory T cells (Tregs) driven by monocytes. Macrophages adhere to and subsequently release TGF-β1 while undergoing differentiation into type 2 macrophages, perhaps playing a role in the creation of Tregs produced by MSCs. Neutralising CCL-18 results in a significant decrease in the production of Treg cells produced by MSCs.

MSCs inhibit the movement and development of DCs, reducing their ability to facilitate the growth of CD4+ T cells that target particular antigens and present an MHC class II-peptide complex. Mature type 1 dendritic cells (DCs) exhibit a notable decrease in the production of TNF-α when co-cultured with mesenchymal stem cells (MSCs), whereas anti-inflammatory mature type 2 DCs have an enhanced secretion of IL-10. The Sca-1+CD117−Lin− bone marrow-derived mesenchymal stem cells (MSCs) have shown the ability to produce regulatory dendritic cells (DCs) with immune regulation capabilities from hematopoietic stem cells in mice.

A mechanism for the polarisation of monocytes/macrophages caused by MSCs, which does not need cytokines, has been discovered. Phagocytosed MSCs are mostly located in non-classical Ly6Clow monocytes. Following the process of phagocytosis, monocytes that were prepared in advance were capable of inducing the production of CD4+CD25hi Treg cells in a laboratory setting to a far greater degree than monocytes that were not prepared in advance. The removal of monocytes/macrophages and dendritic cells completely eliminates the capacity of phagocytic cells to limit T cell proliferation in vitro and their immunomodulatory impact in vivo transplant models, highlighting their crucial involvement in MSC-mediated immunomodulation.

Mesenchymal stem cells (MSCs) have a substantial influence on several immune cell populations, such as T cells, B cells, and natural killer cells. They inhibit the growth of T cells in mixed lymphocyte reactions and induce a transition from pro-inflammatory Th1 cells to anti-inflammatory Th2 cells, hence modifying the cytokine profile in favour of anti-inflammatory responses. MSCs also promote the development of regulatory T cells (Tregs), which play a crucial role in maintaining immunological balance by limiting autoimmune reactions. The initiation of CD4+CD25+FoxP3+ regulatory T cells (Tregs) is a fundamental aspect of mesenchymal stem cell (MSC)-mediated immunomodulation and is crucial for promoting tolerance in models of kidney allograft transplantation.

Studies on neutralising TGF-β1 demonstrate that the production of Tregs is influenced by TGF-β1, and MSCs continuously release TGF-β1. Nevertheless, TGF β1 alone is inadequate, since the inclusion of monocytes is necessary for the development of Tregs. When used along with MMF,

MSCs facilitate the direct transformation of IL-17A+ cells into IL-17AnegFoxp3+ Tregs. MSCs also release indoleamine 2,3-dioxygenase (IDO), which is enhanced when stimulated by INF-γ.

MSCs have the ability to directly suppress the growth of alloreactive CD4+ and CD8+ T cells, even without the involvement of other immune cells. This suppression is largely facilitated by galectin-1, which is produced by MSCs. MSCs may inhibit T cell activation and promote permanent T cell hypo-responsiveness and death via the release of PD-L1.

B lymphocytes engage with mesenchymal stem cells (MSCs) and have the ability to diminish the generation of plasmablasts while facilitating the development of regulatory B cells (Bregs). Bregs possess immunosuppressive characteristics, which contribute to the establishment of immunological tolerance. The promotion of Breg formation and IL-10 production by MSCs is dependent on the metabolic activity of the cells. Interleukin-1 receptor antagonist (IL1-RA) released by MSCs hampers the process of B cell development. Additionally, MSCs suppress the growth of B cells when T cells are present.

Natural killer cells (NK cells) exhibit reduced cytotoxicity and cytokine synthesis when co-cultured with MSCs. MSCs have an inhibitory influence on NK cells by the secretion of prostaglandin E2, IDO, TGF-β1, IL-6, and nitric oxide (NO).

7.3.4. Antimicrobial activity

MSCs have inherent bacterial-killing abilities via the release of antimicrobial peptides, including LL-37 and Lipocalin-2, when stimulated by pathogens. These variables are probable causes of bacterial membrane disruption and contribute to the elimination of microorganisms.

7.4. Therapeutic potential of MSCs

7.4.1. Treatment of chronic diseases

MSCs have shown considerable therapeutic promise owing to their distinctive characteristics, including their capacity to integrate into many organs and treat conditions such as cardiovascular, pulmonary, and spinal cord injuries, autoimmune disorders, as well as liver, bone, and cartilage ailments. The effectiveness of MSC treatment relies on their ability to be administered either locally or systemically, depending on their paracrine action. MSC infusion may be administered via three different routes: systemic administration, local/topical/regional delivery, and scaffold/bioengineered construct. The effective dose (ED) necessitates a substantial therapeutic impact, with an agreed-upon dosage of 1×10^6/30 g mouse equivalent to 33×10^6/kg human.

The transplantation of MSCs is deemed safe because of their hypo-immunogenic properties, which makes it an appealing option for therapeutic therapy. MSCs have the potential to be used for local implantation to address specific tissue deficiencies, systemic transplantation to treat widespread and

systemic disorders, and as a means of delivering genes. The therapeutic benefits of MSCs in liver illnesses have shown promise, since they are able to differentiate into hepatocytes and modulate the immune response by releasing trophic factors that impact the activity of NK cells and stellate cells. The use of MSC treatment in liver disease is not only safe, practicable, and successful, but also minimally invasive and devoid of limitations such as donor scarcity, graft rejection, and surgical complications.

MSCs have been investigated for their potential in managing autoimmune illnesses as a safer and more feasible approach to regulate immune disorders. MSCs have been investigated in individuals with graft versus host disease (GvHD), Crohn's disease (CD), multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and type 1 diabetes.

MSCs have shown therapeutic effectiveness in controlling tissue regeneration and repair, inhibiting T cell proliferation in response to antigenic stimuli, and displaying stromal characteristics in GvHD. The immunomodulatory properties of MSCs might potentially improve the development of IBD in Crohn's disease. However, a more efficient approach would include combining the administration of MSCs with antibodies and genetic alteration of autoimmune regulators.

MSCs have shown efficacy in treating systemic lupus erythematosus (SLE), as evidenced by their ability to enhance serological markers, stabilise renal functions, and alleviate SLE symptoms. Nevertheless, Mesenchymal Stem Cells (MSCs) are effective only when given at the onset of the illness, since they trigger the production of cytokines linked to Th17, which exacerbates the clinical manifestations of collagen-induced arthritis (CIA).

In individuals with type 1 diabetes, mesenchymal stem cells (MSCs) have the ability to produce functional populations of pancreatic β-cells. These cells may then replenish the supply of insulin-producing cells that respond to glucose levels. Additionally, MSCs have immunomodulatory properties that counteract autoimmune and improve the rejection of immunological transplantation.

7.4.2. MSCs and Cancer Therapy

Several studies have shown that the method by which MSCs function in cancer treatment is attributed to their capacity to migrate to the site of damage, the paracrine impact of the secretome, and their immunomodulatory capability. The advantages of MSCs are appealing, however, it is crucial to take into account the possible adverse effects and significant risk factors linked to stem cell transplantation (Fig.30).

The therapeutic function of MSCs in cancer treatment is analogous to their involvement in other medical conditions. Tumours release comparable chemical attractants to those released by injured tissues, which trigger the movement of MSCs towards the desired location via the CXCL12 CXCR4 signalling pathway. MSCs have been shown to engage with cancer cells, both directly and indirectly,

influencing the progression of tumours. In addition, mesenchymal stem cells (MSCs) release a range of cytokines and growth factors that modify cellular functions, including cell division, blood vessel formation, cell viability, and immune system regulation, directly impacting cancer development.

Nevertheless, there have been inconsistent findings regarding the opposing tumor-suppressing and tumor-promoting activities of MSCs. The therapeutic function of MSCs in cancer treatment is analogous to their involvement in other medical conditions. Tumours release comparable chemical attractants to those released by injured tissues, which trigger the movement of MSCs to the desired location via the CXCL12 CXCR4 signalling pathway. MSCs have been shown to engage with cancer cells, both directly and indirectly, influencing the progression of tumours. In addition, mesenchymal stem cells (MSCs) release a range of cytokines and growth factors that modify cellular functions, such as cell proliferation (cell cycle), angiogenesis, cell survival, and immunomodulation. These modifications indirectly impact cancer progression. A recent study using transductions lentiviral showed that the MSCs of the human umbilical cord expressing IL-18 inhibit the proliferation and metastasis of breast cancer in mice

Multiple studies have shown that when attracted to tumour locations, the ability of MSCs to develop into various cell types allows them to transform into carcinoma-associated fibroblasts, which directly contribute to the advancement of cancer. MSCs have been documented to stimulate the growth of tumours and the formation of new blood vessels (angiogenesis) by releasing proangiogenic cytokines. They also facilitate the spread of human breast cancer cells to other parts of the body by inducing the cancer cells to produce lysyl oxidase (LOX) from scratch. Furthermore, MSCs regulate the production of regulatory T cells and suppress the function of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), thereby shielding breast cancer cells from immune surveillance. Lastly, MSCs contribute to the metastasis of lung cancer.

Conversely, mesenchymal stem cells (MSCs) enhanced the responsiveness of breast cancer cells to radiation and hindered the advancement of tumours by suppressing the signal transducer and activator of the transcription 3 (Stat3) signalling pathway. A separate investigation discovered that mesenchymal stem cells (MSCs) impeded the formation of liver cancer by releasing paracrine substances that suppressed the insulin-like growth factor 1 receptor (IGF 1R), phosphatidylinositol 3 kinase (PI3K), and Akt signalling pathways.

Prior to the clinical implementation of MSCs for cancer treatment, it is important to exercise care due to the many attributes of MSCs that make them vulnerable to various pathological states in patients. These diseases have the potential to impede the effectiveness of therapeutic processes.

MSCs have been recognised as promising approaches for cancer treatment because of their capacity to move towards tumour locations. Nevertheless, the existing research is inadequate in

providing sufficient evidence for the direct use of these treatments in cancer patients. MSCs may have a substantial impact on mitigating cancer development by enhancing the pharmacological characteristics of anti-cancer medications. Research has shown that the introduction of interferon β (IFN β) into mesenchymal stem cells (MSCs) by genetic modification may effectively reduce tumour development and significantly increase the survival rate of mice, surpassing that of the control group. Administration of cisplatin to a melanoma xenograft mouse model induced a significant degree of death when MSCs transfected with IFN β were used. Tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is a very promising target that specifically triggers programmed cell death (apoptosis) in cancer cells. Studies have shown that TRAIL-modified MSCs have shown anti-tumor activities in several cancer cell lines in a mouse melanoma model.

Several research have investigated the potential of improving the intrinsic therapeutic characteristics of MSCs by genetic manipulation. The primary emphasis of these investigations was on enhancing the migration, adhesion, viability, and decreased cellular ageing of transplanted MSCs. This phenomenon is achieved by introducing a vector containing a genetically engineered cassette into MSCs. The cassette allows for the continuous expression of certain genes or may be regulated using a gene switch. As an example, adipose derived mesenchymal stem cells (AdMSCs) were genetically modified using a retroviral vector to increase the level of CXCR4 expression. The research found that the transduced MSCs exhibited enhanced motility, invasion, and engraftment in the bone marrow upon injection into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice.

MSCs have been genetically modified to increase the expression of integrin-linked kinase (ILK). The research revealed that genetically modified MSCs exhibited a 1.5-fold increase in survival and a 32.3% higher adhesion rate when transplanted into a model of ischemic myocardium. Furthermore, these changed cells demonstrated a retention rate almost four times greater than the control group. Furthermore, it has been observed that BMSCs and AdMSCs have enhanced cell division and specialisation capabilities when genetically modified to excessively express Oct4 and Sox2.

Prior research has demonstrated a correlation between distinct Toll-like receptors (TLRs) and the immunomodulatory characteristics of MSCs. MSC1, which were primed with TLR 4, had a proinflammatory phenotype, while MSC2, which were primed with TLR 3, released immunosuppressive mediators. Directing the differentiation of MSCs into distinct immunomodulatory phenotypes is a very promising approach. MSCs have an impact on tumour development by modulating the immune response. Strategically directing the polarization of MSCs will have advantages for cancer treatment and provide better control over their immunomodulatory abilities.

Research has also examined the possibility of MSCs serving as carriers for oncolytic viruses. For instance, mesenchymal stem cells (MSCs) were used as carriers to transport oncolytic herpes simplex virus to models of human brain melanoma metastases that were cultivated in mice with compromised immune systems as well as animals with fully functional immune systems. Recent research conducted in living organisms investigated the potential of using mesenchymal stem cells (MSCs) obtained from menstrual blood as a carrier for CRAd5/F11 chimaeric oncolytic adenovirus in the treatment of colorectal cancer. A mathematical model has been created to accurately anticipate the effectiveness of MSCs as carriers for biotherapeutic agents in living organisms. This model demonstrates that MSCs are a promising approach that enhances the effectiveness and safety of the therapy.

MSCs may be prepped with anticancer medications for precise administration since they naturally migrate towards the tumour location and have a higher tolerance to treatments that inhibit cell growth and cause cell death. Research has shown that mesenchymal stem cells (MSCs) may develop potent anti-tumor effects by encapsulating and transporting paclitaxel (PTX) via extracellular vesicles. This process results in the production of medicines that exhibit enhanced selectivity for targeting cancer cells. Additional pharmaceuticals, such as doxorubicin and gemcitabine, have also undergone experimentation to assess their effectiveness in stimulating MSCs.

The direct administration of anticancer medications often leads to undesirable side effects, including nausea, vomiting, fatigue, alterations in taste perception, xerostomia, decreased appetite, constipation, and alopecia. Utilising MSCs as carriers for administering therapeutic proteins or anticancer medicines may effectively address this problem. MSCs have the ability to migrate and accumulate specifically at tumour sites, resulting in localised therapeutic effects. This enhances the effectiveness of therapy and minimises the toxicity to the whole body.

Previous studies have shown that pretreating MSCs with pioglitazone may improve the effectiveness of tendon repair. This improvement occurs via the indirect administration of pioglitazone to breast cancer cells, facilitated by the interaction between stem cells and cancer cells. This procedure facilitates the interaction between genetically altered and viable pre-treated stem cells and cancer cells inside the bodies of patients, possibly enhancing the overall treatment outcomes.

Nevertheless, the use of pioglitazone-pretreated MSCs as a method to enhance the overall therapy outcomes is seldom seen. An analogous approach was used, whereby AdMSCs were pre-treated with a peroxisome proliferator-activated receptor gamma (PPARγ) agonist, in order to enhance the regenerative effects in a mouse model of elastase-induced emphysema. IL-6 pretreatment of human umbilical cord-derived mesenchymal stem cells was shown to eliminate the stimulatory impact on the development of gastric cancer cells.

While there are possible advantages, using MSCs for cancer therapies has inherent dangers, including as immediate concerns like inflammatory responses or embolic phenomena, intermediate concerns like graft versus host disease or secondary infection, and long-term concerns like the possibility of tumour progression. Patients administered with MSCs often succumbed to infection, rendering it a precarious therapeutic alternative. Further research is required to gather further data and enhance the therapeutic efficacy of modified MSCs in cancer therapies.

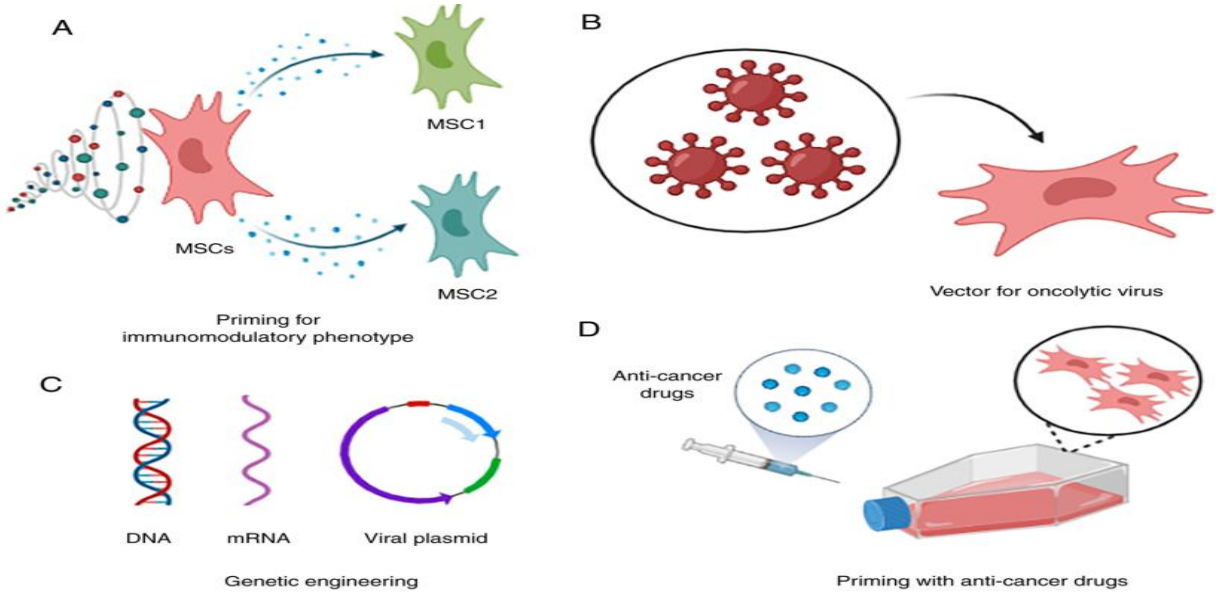

Figure 30. Possible therapeutic approaches of MSCs in the treatment of cancer

Table 4. Treatement of Chronic diseases using MSCs

Source	Clinical research	Research status
BM-MSCs	Diabetes mellitus	A single injection of autologous BM-MSCs can protect and maintain the residual function of pancreatic beta cells, but nonetheless, MSCs cannot replace hypoglycemic drugs. Further research is needed
Stem cells in the islets of adult mice	Diabetes mellitus	It is promising to treat diabetes by transplanting functional islet organoids in the future
MSCs	Diabetes mellitus	Reversing the dedifferentiation of β cells in type 2 diabetic islets and repairing islet function
MSCs	Autoimmune diseases	Apoptosis and metabolic inactivation of MSC have immunomodulatory potential, and regulatory T cells and monocytes play an important role
Remestemcel-L	COVID-19 ARDS	Remestemcel-L has an immunomodulatory effect and has potential applications in the treatment of COVID-19 ARDS
MSCs	Vaccines	Most pathogens have no effective antibodies, which suggest the limitations of the five existing vaccine platforms. The successful establishment of the stem cell vaccine, the sixth vaccine platform, can point out a new direction for future vaccine research and development
MSCs	Cancer	To use MSCs as the carriers of anti-tumor drugs to achieve effective tumor treatment
		Gene-directed enzyme prodrug therapy or suicide gene therapy

Table 4. Treatement of Chronic diseases using MSCs

Source	**Clinical research**	**Research status**
MSCs	Ischemic diseases	The mechanism of MSCs in the treatment of myocardial infarction is not that MSCs migrate to the injured myocardium differentiate into cardiomyocytes but secrete cytokines
MSCs	Anti-aging	YAP plays a key role in maintaining the youthful state of human adult stem cells and may play a role of "de-senescence"
MSCs	OA	A new approach for gene therapy of OA targeting DGCR8
		Exosomes (or similar particles) derived from MSCs may suppress OA development
MSC-derived exosomes	Cancer therapy	MSC-derived exosomes have been suggested as possible cell-free substitutes for intact MSCs
	Gene therapy	
	Drug delivery	
MSCs	POF	The relevant mechanisms need to be further elucidated
BM-MSCs	Organ transplantation	Immune tolerance can be induced by the infusion of autologous BM-MSCs without taking immunosuppressive agents

8. Hematopoietic stem cells (HSC)

Hematopoietic stem cells (HSCs) are multipotent cells that can differentiate into various types of blood cells, such as red blood cells, white blood cells, and platelets. HSCs are essential for maintaining the homeostasis and function of the blood system throughout life. HSCs are also the source of cells for bone marrow transplantation, a lifesaving therapy for patients with blood disorders or cancers. HSCs reside in specialized niches within the bone marrow, where they interact with various factors that regulate their self-renewal, proliferation, and differentiation. Under certain conditions, HSCs can also migrate to other organs, such as the spleen, liver, and thymus. HSCs have been extensively studied for their potential applications in regenerative medicine and gene therapy.

The notion of stem cells originated over a century ago. German scientist F.E.C. Neumann (1834-1918) was the first to propose in the late 19th century that the bone marrow serves as the organ responsible for blood production. Furthermore, he put out a contentious hypothesis at that period suggesting that a stem cell may serve as the origin for all blood lineages. The initial hypothesis about the presence of hematopoietic stem cells (HSCs) has since been confirmed. Colon stem cells have the capacity for self-renewal, enabling them to maintain a consistent population size during an individual's lifespan. In addition, they possess the capacity to generate all mature hematopoietic cells found in circulation by producing progenitor cells that subsequently undergo differentiation into myeloid and lymphoid lineages. Progenitors cannot undergo self-renewal, in contrast to HSCs. Hematopoietic stem cells (HSCs) are responsible for the ongoing and controlled generation of vital blood cells.

HSCs are classified based on the expression of certain cell surface markers that indicate various phases of cellular development. The first cell-surface marker used to concentrate human hematopoietic stem cells (HSCs) was CD34, a molecule that acts as a binding partner for L-selectin and is only present in a tiny fraction of blood or bone marrow cells. Hematopoietic stem cells (HSCs) may be derived from several anatomical sites such as bone marrow, peripheral blood, or umbilical cord blood. The primary distinction in acquiring hematopoietic stem cells (HSCs) from various origins is in the magnitude of cell production. Human umbilical cord blood (HUCB) is a potential source of hematopoietic stem cells (HSCs). However, the amount of HSCs that can be recovered from HUCB is limited, making it suitable primarily for usage in children rather than adults.

Bone marrow is the most effective and practical choice for autologous hematopoietic stem cell (HSC) transplantation, resulting in higher yields. Nevertheless, this technique is associated with pain and potential discomfort. Circulating hematopoietic stem cells (HSCs) are also present in peripheral blood. Recent research suggests that granulocyte-colony stimulating factor (GCSF) may have therapeutic promise in neurological illnesses, including stroke. It has been demonstrated to have

neuroprotective and regenerative properties in experimental stroke models. Recent studies confirm the idea that human hematopoietic stem cells (HSCs) have important regulatory functions in maintaining regarding the maintenance of internal stability and the restoration of the neurological system. For example, the method of delivering a substance across the whole system of an organism transplanting human CD34+ cells into mice that were previously subjected to stroke 48 hours ago leads to neovascularization occurring in the ischemic zone, hence establishing a favourable milieu for the survival of foreign grafts and indigenous stem cells is crucial for brain regeneration. Hematopoietic stem cells (HSCs) may be derived from primary tissue sources, including bone marrow (BM), mobilised peripheral blood (MPB), and umbilical cord blood (UCB). Cellular sources are derived from cultivated cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Producing embryonic stem cells from embryos that are 5-7 days old, the demonstration of induced pluripotent stem cells (iPSCs) derived from adult somatic tissues is also shown. These existing cell lines can be subsequently differentiated into hematopoietic stem cells (HSCs) in a laboratory setting.

Human stem cells, often known as HSCs, play a vital role in the formation of the mature blood system in adults. Due to their multipotency, these cells can specialise and multiply into more specialised progenitors and precursors, giving rise to all fully developed cells from the erythroid, myeloid, and lymphoid lineages. The self-renewal capacity of these cells allows them to continue blood production throughout an organism's life, while also preserving their potential to differentiate into many cell lineages. Embryonic hematopoiesis has been examined in many animal models, mostly focusing on the mouse embryonic model. Adult hematopoietic stem cells (HSCs) are formed throughout the process of embryonic development. The first hematopoietic cells that are created are specialised cells responsible for ensuring enough oxygen supply and safeguarding the growing embryo. The yolk sac (YS) is where hematopoietic synthesis begins. It generates erythroblasts, which are big nucleated erythrocytes, as well as macrophages and megakaryocytes. Specialised structures known as blood islands are formed when a layer of endothelial cells envelops these first blood cells. Maturity cells may be directly detected under a microscope by the use of certain morphological criteria or by analysing surface marker expression using immunohistochemistry. Hematopoietic progenitors may be retroactively detected using a clonogenic assay, which is a short-term in vitro test. Specific criteria may be used to identify and quantify several kinds of lineage-restricted progenitors based on their capacity to produce colonies. Adult hematopoietic stem cells (HSCs) may be extensively purified, in contrast to the first HSCs discovered in the embryo, which have proven to be more challenging to isolate so far. Several markers presently used for the isolation of adult hematopoietic stem cells (HSCs) are not applicable to embryonic HSCs. Furthermore, the expression

of surface markers on HSCs undergoes variations during development and differs across different strains and species. For instance, the SLAM marker CD150, when employed together with the conventional marker combination LSK, enables effective enrichment of HSCs in the adult bone marrow and foetal liver. However, it is not indicative of AGM HSCs. The lineage antibody panel (Lin) is commonly employed to eliminate fully developed cells from the erythroid, lymphoid, and myeloid lineages. However, this method cannot be used to purify embryonic hematopoietic stem cells (HSCs) due to the presence of Mac-1 (CD11b), a marker typically found on macrophages/monocytes in adults, which is also expressed by a subset of AGM HSCs. Hematopoietic stem cells (HSCs) and endothelial cells exhibit several surface markers that indicate their intimate developmental connection. Studying Hematopoietic stem cells (HSCs) is essential for comprehending their ability to repopulate several cell lineages and renew themselves after being transplanted into adult wild-type recipients who have been exposed to radiation. Hematopoietic stem cells (HSCs) are first seen in the aorta-gonad-mesonephros (AGM) area around E10.5. They are specifically found in the aorta, vitelline artery, and umbilical artery. Subsequently, they are also present in other significant hematopoietic locations with extensive blood vessel networks, including the yolk sac (YS), placenta, and foetal liver. Hematopoietic stem cells (HSCs) exhibit distinct behaviour in the embryo as opposed to adult bone marrow. During their development, they traverse many anatomical locations or niches and engage in active self-renewal. The composition of the embryonic milieu that forms subsequent niches is now inadequately defined, but it undeniably affects the balance between hematopoietic stem cell self-renewal and differentiation. Conversely, the HSC niches in the adult bone marrow are well documented. There are now two known kinds of niches: the endosteal niche, where hematopoietic stem cells (HSCs) are in direct touch with the bone surface, and the sinusoidal endothelium, which is connected to the sinusoidal endothelium. The HSC niches with the highest potency are most likely characterised by hypoxia. The distinct and well defined milieu inside the medulla of the niches preserves the majority of hematopoietic stem cells (HSCs) in a state of immaturity and low activity, with their quiescent or slow-cycling condition being a characteristic feature that contributes to their long-term qualities as HSCs. A limited number of hematopoietic stem cells (HSCs) will undergo self-renewal just a few times during their lifespan. Nevertheless, disruption of equilibrium accelerates their ability to regenerate themselves. Mesen chymal stem cells (MDCs), characterised by the presence of the intermediate filament protein nestin, serve a crucial role in preserving the functionality of the hematopoietic stem cell (HSC) niche. The cells generate SDF-1α (CXCL12) and SCF (Stem Cell Factor), which play a crucial role in maintaining HSCs. The precise molecular network architecture that differentiates self-renewing hematopoietic cells from non-self-renewing ones has not been identified in any investigations so far. The bone marrow niches, which are responsible for

maintaining a steady pool of hematopoietic stem cells (HSCs) in adults, have been well characterised. This does not apply to the consecutive niches through which HSCs pass throughout development. Investigations into hematopoietic cells and their surrounding microenvironments throughout development have proven crucial in elucidating the mechanisms behind the generation and expansion of HSCs without compromising their stem cell potential. It is crucial to comprehend the source of their generation in order to further studies on the anatomical source of HSCs.

8.1. Collecting of HSC

8.1.1. Bone Marrow

Haematopoietic stem cells (HSCs) are found near sinusoids, where they are in direct contact with mesenchymal stromal cells expressing the leptin receptor (LEPR) and endothelial cells. These cells are important sources of the stem cell factor (SCF) and CXCL12, which are essential for maintaining HSCs. Around 10% of hematopoietic stem cells (HSCs) are found in close proximity to small-diameter arterioles. These arterioles are also connected to stromal cells that express the LEPR receptor, as well as rare Ng2-CreER+ cells. The Ng2-CreER+ cells express a tamoxifen-activated form of Cre recombinase from the Ng2 gene, which encodes neural-glial antigen 2. It is uncertain whether these Ng2-CreER+ cells are a source of the CXCL12 protein that is necessary for HSC maintenance. Nerve fibres, Schwann cells associated with nerve fibres, megakaryocytes, macrophages, and osteoclasts also play a role in regulating hematopoietic stem cell (HSC) maintenance via various processes. Osteoblasts do not directly control the maintenance of hematopoietic stem cells (HSCs) by any known method. However, they likely indirectly govern HSC maintenance by communicating with other cell types in the bone marrow, such as the cells that make up the blood vessels. Osteoblasts support the survival of a certain group of early lymphoid progenitors by producing small amounts of CXCL12. However, other lymphoid progenitors are found in sinusoidal niches and rely on CXCL12 produced by LEPR+ cells. TGFβ, which stands for transforming growth factor-β, is also involved in this process.

Since the 1970s, bone marrow has been the primary source of hematopoietic stem cells (HCS) utilised for allogenic and autogenic transplantation. It is often administered at the iliac level, or sometimes at the sternum level. Within the bone marrow, there exists a ratio of around 1 in 100,000 cells that are classified as hematopoietic stem cells (HCS). The amount of bone marrow that can be extracted from a donor depends on their weight, with a maximum of 10-20mL per kilogramme of body weight. This corresponds to a range of 600 mL to 1L. Additionally, the concentration of nucleated cells in the extracted bone marrow is typically about 2-3x108 cells per kilogramme of body weight. Typically, the quantity of hematopoietic stem cells (HSCs) obtained is enough for

transplantation. Moreover, the extensive knowledge and expertise gained over 40 years in this approach provide a substantial level of safety when using HSCs derived from bone marrow. The primary drawback of this approach is the need for bone marrow retrieval, which is an uncomfortable procedure that necessitates the use of general anaesthesia for the donor. Furthermore, the received transplant comprises a limited number of T cells, which decreases the occurrence of graft-versus-host disease (GvHD), but prolongs the duration of the transplant process and raises the likelihood of rejection. This indicates that its use has seen a lack of growth or progress in recent years. Minors are legally allowed to utilise HCS only if it is obtained from the bone marrow (Fig. 31).

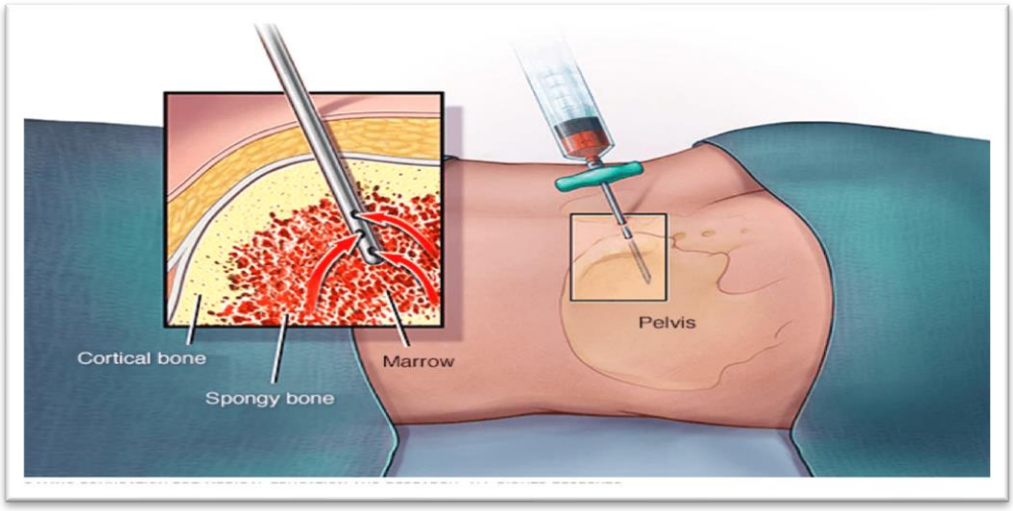

Figure 31. Collecting HSCs from bone morrow (Iliac)

The sternum, often known as the breastbone, is a primary location for hematopoietic stem cells (HSCs). The sternum is a planar bone that constitutes the front portion of the thoracic cage and safeguards the heart and lungs. Hematopoietic stem cells (HSCs) may be obtained from the sternum by the use of a specialised needle, which is passed through the skin and into the bone. This medical operation is referred to as a sternal aspiration, and it is often performed under local anaesthesia. This procedure is reserved for patients over 15 years old and only allows an aspiration of the marrow and not an osteomedullary biopsy. This route only represents 5 to 7% of punctures due to cardiac proximity and greater patient anxiety (Fig. 32).

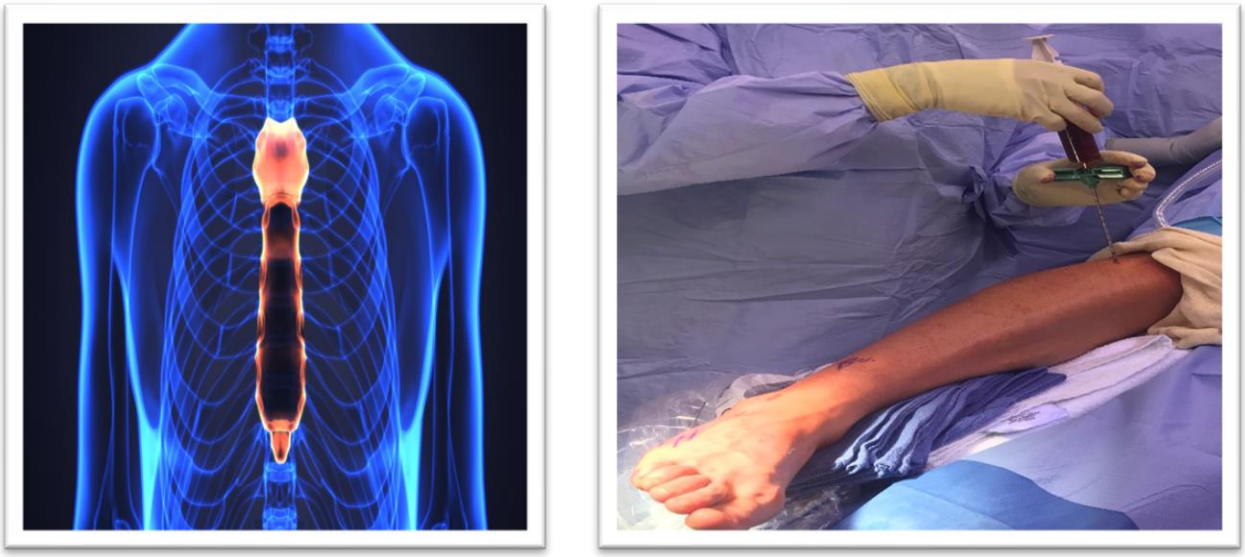

Figure 32. Collecting HSCs from bone morrow (Sternum and Tibia)

8.1.2. Circulating Blood and vascular HSCs' niche

Peripheral blood includes a limited number of hematopoietic stem cells (HSCs) known as peripheral stem cells (PSCs), which are not present in sufficient numbers to be employed directly for therapeutic purposes. In recent years, scientists have shown the feasibility of increasing the quantity of pluripotent stem cells (PSCs) in the circulation by stimulating the movement of hematopoietic stem cells (HSCs) from the bone marrow. This is achieved with the administration of hematopoietic growth agents, such as G-CSF (granulocyte colony-stimulating factor) in France. The injection is administered several days before cell harvesting and yields up to double the amount of pluripotent stem cells (PSC) compared to those obtained from the bone marrow. This source was mostly used for autogenic transplants, but in recent times, it has also been employed for allogeneic transplants simply via the mobilisation of growth factors. The significance of collecting peripheral stem cells (PSCs) lies in the avoidance of general anaesthesia and the ability to get a larger quantity of hematopoietic progenitors. One major drawback of this source is the high presence of T lymphocytes in the graft, leading to an elevated risk and intensity of chronic graft-versus-host disease (GvHD) response. Simultaneously, there is an expedited process of restoring blood and immune functions, resulting in reduced grafting time and fewer instances of rejection.

Quiescent hematopoietic stem cells (HSCs) are found in the tiny arterioles of the endosteal area. These arterioles are surrounded by pericytes that express high amounts of nestin and are positive for NG2. These pericytes, along with sympathetic nerves, work together to support the maintenance and retention of HSCs inside the bone marrow. Nestinbright cells are both dormant and resistant to chemotherapy. Upon activation from a state of inactivity, hematopoietic stem cells (HSCs) are moved

from the NG2+ periarteriolar niche to the Lepr+ perisinusoidal niche. Sinusoidal endothelial cells secrete VEGF, which enhances the survival of hematopoietic stem cells (HSCs) after irradiation. These cells also express tenascin C and produce angiocrine substances, which stimulate the proliferation of HSCs. Due of the significant destruction of nestindim cells caused by chemotherapy, the regeneration process may be triggered by the arterioles. This results in an ongoing interchange between the niches, resulting to continuing proliferation, reconstitution, and hematopoietic regeneration. The tendency of hematopoietic stem cells (HSCs) to migrate and attach to the innermost layer of bone marrow after transplantation is partly facilitated by the synthesis of hyaluronin and the expression of E-selectin by sinusoidal endothelial cells. Additionally, the expression of CaR and Robo4 on HSCs contributes to this process. MPP stands for multipotent progenitor cells (Fig. 33).

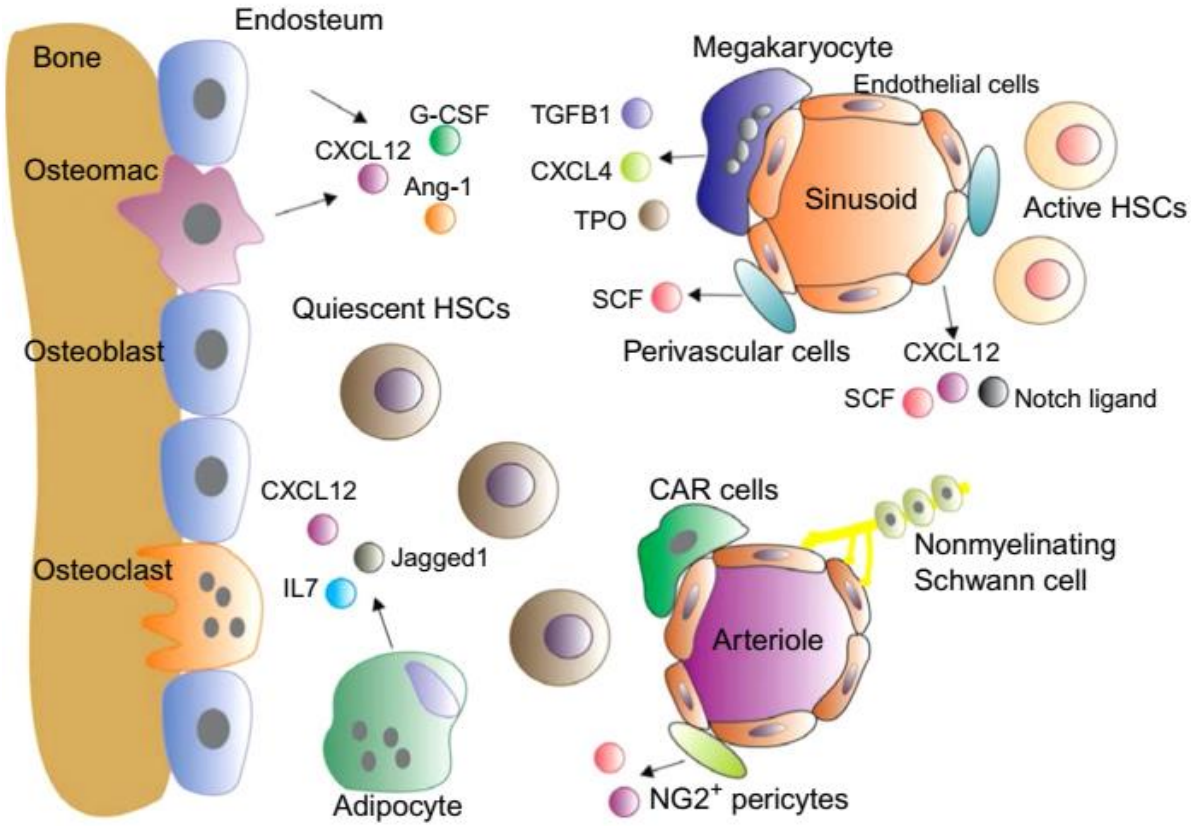

Figure 33. Osteoblaste and vascular niche of hematopoietic stem cells

5.1.3. Umbilical Cord

HSCs in cord blood were identified in 1974 by Knutzon. HSCs are found in a very large proportion but it is only possible to take relatively small volumes, in the order of 80 to 200 mL (Fig. 34). This can only be done after the mother has been informed and authorized. Cord blood aspiration is very easily performed in the delivery room on the umbilical cord clamped after the baby has been

expelled. This technique is quick, painless and without strain for the donor. These HSCs derived from cord blood are mainly used for unrelated allogeneic transplants and, in very rare cases, for family transplants (brother or sister of the patient). These transplants are possible thanks to cord blood banks that cryopreserve HSCs once immunological typing has been performed. The great advantage of this collection route is that cord blood is an overall more tolerant graft than a bone marrow transplant that would be harvested from a child or an adult. This makes it possible to transfuse cord blood grafts that are not completely HLA identical to the sick patient. This is a great advantage since it will be possible to transplant patients who do not have HLA-compatible donors, neither in their siblings nor on the various registries of volunteers for HSC donation. On the other hand, a key characteristic of this type of transplantation is that the grafts have less HSC than for other sources, sometimes making it impossible to transplant to an adult. This is why this therapeutic option was initially used in paediatrics in children since the amount of HSC needed to transplant a 10-kilo child is 5 times less than the amount of cells needed to transplant a large 50-kilo child. Cord blood stem cells are more immature and low in T cells, thus reducing the incidence and severity of observable GVHDs. However, engraftment is slower, and the risk of non-engraftment is increased.

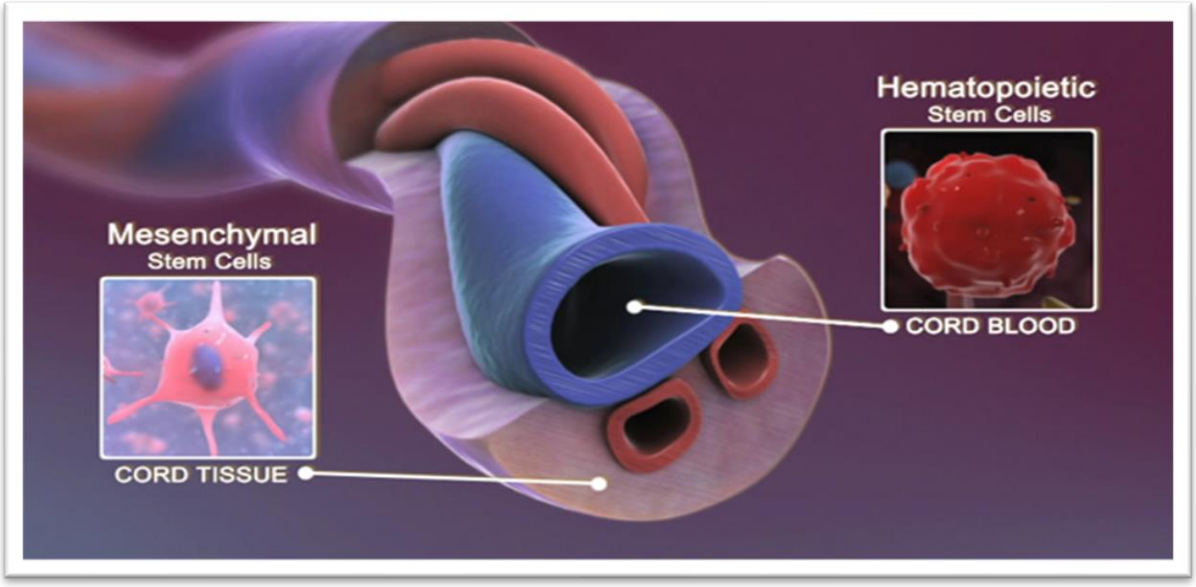

Figure 34. Umbilical cord as source of stem cells

8.2. HSC transplantation Criteria

Transplantation is a potential cancer treatment, and finding a suitable donor is crucial to minimize complications. Human leukocyte antigen matching (HLA) is the process of comparing and matching HLAs, proteins found on the surface of different cells, especially white blood cells (WBCs). The major histocompatibility complex (MHC) is located on the short arm of chromosome 6. Class I

consists of HLA-A, HLA-B, and HLA-C, while Class II consists of HLA-DR, HLA-DP, and HLA-DQ, along with genetic variants of these genes.

An ideal donor is often defined as a totally matched sibling, meaning that both alleles of HLA-A, HLA-B, and HLA-DR are identical to those of the recipient, resulting in a 6-of-6 match. Family members who are fully matched provide the most suitable matches due to the presence of minor HLA antigens that are often not assessed during testing. If the donor and receiver do not have a 6-of-6 match, they are said to be mismatched. The term "haplotypic donor" is used when there is a match of just half the alleles. Unrelated donor searches often aim to find matches that have a compatibility of 10 out of 10 (excluding HLA-DP) or 12 out of 12. For non-related donors, most transplantations consider a full match or a single mismatch to be acceptable, while in some situations a bigger mismatch may be accepted.

Autologous transplantation refers to the process of transferring cells, tissues, or organs from one part of an individual's body to another part of their own body. In general, individuals who are being considered for autologous transplantation do not have any detectable cancerous cells in their blood or bone marrow. The use of autografts results in the lowest rates of treatment-related illness and death, with the primary concern being the recurrence of tumours. This discovery pertains to the lack of a graft-versus-tumour effect, which refers to the immune response of T cells and natural killer cells in the donor graft attacking the tumour.

Donors who are genetically identical twins may have a sibling who is an exact genetic match and may act as a suitable donor. They do not need immunosuppressive medication after transplantation and do not have graft versus host disease (GVHD). However, they have a greater chance of the underlying malignant illness coming back compared to nonidentical sibling donors with comparable HLAs. Survival rates for identical twin and nonidentical sibling transplants are comparable, as although there is a higher risk of leukaemia recurrence with identical twin donors, this is offset by lower rates of death linked to therapy.

The process of nonmyeloablative transplantation involves several types of donors, including compatible, related, unrelated, and haploidentical.

Siblings are often chosen as donors due to the potential inheritance of the same HLA genes on chromosome 6. However, stem cells do not display ABO red blood cell (RBC) antigens, making it difficult to find donors who are related to the patient and have matching characteristics. If the patient's parents possess extremely similar genetic profiles or there has been intermarriage among families, first cousins who are compatible in terms of human leukocyte antigen (HLA) markers are often chosen.

Donors who are not genetically compatible but have some degree of relationship may be considered for transplantation. While most centers mandate a full match at the HLA-A, HLA-B, and HLA-DRB1 sites for a person to be eligible as a transplant donor, some centres may consider using siblings with a single antigen mismatch. However, transplants from these donors have an elevated risk of graft-versus-host disease (GVHD), while the overall survival rate may not exhibit a substantial difference compared to transplants from well matched siblings.

If a suitable donor who is genetically related cannot be found, the process of searching for an unrelated donor is often begun. High-resolution HLA typing of the recipient is necessary, as serologic typing alone cannot guarantee that they have the same HLA genes. This is clinically apparent via the increased susceptibility to graft-versus-host disease (GVHD) in individuals who receive grafts from unrelated donors. DNA-based methods for molecular typing have shown that only 55% of donor and recipient pairings that are serologically identical (meaning they have matching antigens) also have a high level of matching at the molecular level. Patients with a high degree of compatibility tend to experience more favorable results, and most transplantation centres now mandate comprehensive serologic and genetic matching in the class II area before using a donor for a specific transplantation surgery.

Haploidentical donors refer to individuals who are genetically half-matched with the recipient. If there is a lack of well-matched unrelated donor and cord blood options, one may consider using a haploidentical donor. In these instances, the donor and recipient are matched on at least half of their HLA alleles, as indicated by the name. The primary benefit of this source of donors is its abundance, as there are often several persons within a family who might potentially serve as donors, such as parents, siblings, and children.

8.2.1. Autologous transplantation

Currently, HCSs used for autologous transplants come from peripheral blood. The use of HCS from bone marrow has become exceptional. Initially, the patient will receive a so-called conditioning treatment using chemotherapy and/or radiation therapy to reduce the disease to its lowest level. The transplant will then be taken, processed to destroy as many residue malignant cells as possible and then frozen and conditioned to allow storage. Generally, autologous transplantation is performed if the patient can receive high-intensity chemotherapy and/or radiation therapy. Stem cells are taken at the beginning of the protocol to protect them from the adverse effects of chemotherapy and/or radiation therapy. The transplant will then allow him to recover and rebuild his blood and immune system after this intensive treatment.

Malignant hemopathies are 90% indications of autologous HCS transplants. Among these hemopathies, myeloma and non-Hodgkin lymphoma represent the highest number of cases. Then we

find and, in a smaller proportion, Hodgkin's lymphoma and then leukemia. (LAM, LLC, LLA). Autographs can be used for therapeutic intensification or to consolidate treatment in case of relapse. New indications have also emerged in cases of solid tumours in the absence of medulla metastases. The majority of these tumors include breast cancer, neuroblastomas and other tumors of the nervous system, as well as ovarian and bone tumors.

The main advantage of this type of transplant is the total absence of GVH (transplant reaction against host) since the patient receives his own cells. Therefore, the risk of rejection is zero and the recipient generally recovers faster without recourse to anti-rejection immunosuppressive treatments. However, these autographs are not without risks.

The most important is related to chemotherapy and/or radiotherapy treatments that precede the re-injection of the transplant. These high-intensity treatments destroy the patient's immune system in addition to cancer cells. As a result, the risk of infectious complications is very high.

The toxicity of the packaging and the risks associated with recurrence or relapse must also be taken into account. These risks of relapse are mainly related to the absence of GVL effect (transplant reaction to disease) since the donor and recipient are one and the same person. In addition, the transplant that is taken at the time of the disease is very weak may still contain some residue malignant cells. Transplant cleansing techniques exist to get rid of these tumor cells. They can be of two types, either destroying cancer cells or removing only the HCS present in the transplant.

8.2.2. Allogenic transplantation

In an allogeneic transplant or allogeneic transplant, the donor and recipient are two different people. The recipient will undergo chemotherapy and/or radiotherapy as in the autologous protocol to minimize their disease and destroy their bone marrow. They will then be injected with the donor's HSCs, which will replenish the immune system and fight the disease.

From the point of view of histocompatibility, allogeneic transplantation is more complex than autologous transplantation since there is a difference in genetic makeup between the donor and the recipient. They must be as compatible as possible from the point of view of the HLA system so that transplantation can be considered. The recipient's immediate family is most likely to find a donor HLA compatible. If there is insufficient compatibility within the family, doctors will have
use of unrelated donor banks or cord blood banks.
Three types of donors, on a kinship and histocompatibility basis, can be used in this situation.

Genoidentical sibling

The donor is a brother or sister of the patient who carries the same HLA antigens. The graft is said to be genoidentical. As they are not identical twins, there are therefore differences between the donor and the recipient that relate to antigens other than those of the HLA system.

If there are several HLA siblings in a family who are identical to the patient, the donor is chosen according to his or her cytomegalovirus (CMV) status and risk factors for GVHD (sex of the donor, sex difference between the donor and the recipient, number of pregnancies in the donor woman, etc.).

Phenoidentical unrelated donor

The donor is most often not from the family, but a volunteer registered on a donor file, who shares with the patient the same histocompatibility antigens. In this case, either PSCs or HSCs from cord blood are used. These grafts are said to be phenoidentical. In rare cases, these donors may be the father, mother, or other family member.

A non-phenoidentical HLA donor

If no genoidentical family donor or non-apparent donor is compatible, transplants from a non-genoidentical family donor may be considered. This donor is said to be incompatible because it differs from the patient by 1, 2 or 3 antigens of the system HLA. This is called a haploidentical transplant because its HLA antigens are partially identical. Grafts can also be found in the case of a non-familial donor where there are allelic or antigenic incompatibilities on one or more antigens of the HLA system, in number and locations considered potentially compatible with the success of the transplant.

The criteria for allogeneic transplants are broader compared to autologous transplants, with haemopathies being the predominant condition in both cases. The primary indication for both related and unrelated transplants is acute myeloid leukaemia (AML), followed by acute lymphoblastic leukaemia (ALL), non-Hodgkin's lymphomas, myelodysplasia, and myeloma. Allogeneic hematopoietic stem cell (HSC) transplantation may be necessary for several conditions, including solid tumours, constitutional bone marrow aplasia, immunological deficiencies, hemoglobinopathies, or enzymatic deficits of hematopoietic tissue such as Gaucher disease.

There are two main types of allogeneic transplants, myeloablative conditioning transplantation and non-myeloablative conditioning transplantation

8.2.3. Myeloablative conditioning transplantation

Initially, it involves the destruction of the recipient's tumour cells with high-intensity chemotherapy and/or radiotherapy, along with the use of immunosuppressants. Subsequently, healthy stem cells from a donor are transplanted or injected. Within this therapeutic strategy, high-intensity

chemotherapy will eradicate the tumour cells, the patient's hematopoietic system responsible for the disease, and their immune system. Therefore, we have a myelosuppressive and immunosuppressive effect. The patient's immune system is then replaced via the graft with that of the donor, which can combat residual tumour cells. Through this approach, the graft-versus-tumour response is stimulated, but the undesirable effects associated with chemotherapy are more severe, making this procedure less and less used. Myeloablative conditioning graft is indicated for tumours or diseases that affect the bone marrow, such as myelodysplasias, some acute leukaemias, medullary aplasias, and certain myeloproliferative and lymphoproliferative disorders.

8.2.4. Non-myeloablative conditioning transplantation

In this scenario, it circumvents the need for aggressive chemotherapy. Before the transplant, the patient is given immunosuppressive medication to enable him to accept the graft. This therapy works by inhibiting the activity of the recipient's lymphocytes, preventing rejection of the graft. The tolerated graft has antitumor activity against the recipient's cancer cells. Through this approach, the patient is provided with additional resources to combat their sickness, while circumventing the harmful effects of intense chemotherapy. Transplantation with non-myeloablative conditioning is advised for older individuals or those with other health conditions that increase the danger of high-dose chemotherapy. It is also recommended for disorders that progress slowly or have a lazy nature. The use of this packaging has steadily increased during the previous decade. The primary hazards linked to allogeneic transplantation include the toxicity of myeloablative conditioning regimens, the perils of rejection, and graft-versus-host disease (GvHD). The success rate is contingent upon several circumstances, including the HLA compatibility between the donor and recipient, the kind and stage of the illness, and the overall health of the recipient. Consequently, it is very challenging to determine the likelihood of success for an allogeneic transplant.

8.2.5. Advantages and disadvantages of allogeneic transplantation

The primary disadvantage of allogeneic transplantation is the occurrence of graft-versus-host disease (GvHD). As the transplant is obtained from a donor who is not the recipient, it contains T lymphocytes that may identify and eliminate the residual cancerous cells in the patient, among other functions. The transplant recipient will possess the ability to independently combat his illness. Furthermore, the transplanted cells will educate the patient's immune system, which will persist indefinitely, hence reducing the likelihood of rejection in the extended duration.

The disadvantage of this allogeneic approach, similar to autologous transplantation, is the toxicity associated with myeloablative conditioning. Non-myeloablative conditioning is associated with a low

incidence of severe side effects. The transplantation carries the risk of rejection, graft-versus-host disease (GvHD), and infections.

The presence of these problems results in a mortality rate of 15 to 30% after transplantation. The duration of the procedure depends on the patient's age, medical background, and the level of compatibility between the donor and receiver.

8.2.6. Immunosuppressive drugs

The primary post-transplant danger encountered by the recipient in allogeneic transplant situations is the chance of graft rejection. To prevent this, immunosuppressive therapies are performed immediately after the transplantation to reduce the activation and multiplication of T lymphocytes, the cells responsible for rejection. Regrettably, this obstruction lacks specificity and further compromises the recipient's already compromised immune system, resulting from training. Autologous transplants or allogeneic transplants from an identical twin do not need these therapies since the likelihood of graft rejection is nonexistent.

Transplant recipients are required to adhere to a treatment regimen that involves the use of many molecules with distinct mechanisms of action. The specific regimen may vary depending on factors such as the underlying disease, the individual patient, and the duration since the transplant. The survival and quality of life of the recipient are contingent upon these interventions. The medicine combination is tailored based on the specific kind of transplant, the patient's medical state, and their tolerance to the medications.

Presently, there are several categories of immunosuppressants.

Cytokine modulators include the use of calcineurin inhibitors and cytokine activity inhibitors.

Antiproliferatives, often known as antipurines, are substances that inhibit cell proliferation.

Anti-lymphocyte antibodies refer to antibodies that specifically target lymphocytes. In particular, there are antibodies known as anti-interleukin 2 receptor (anti-IL2R) antibodies that are designed to bind to the IL2R receptor in lymphocytes.

Corticosteroids

8.2.7. Syngeneic transplantation

A transplant is referred to be syngenic when the donor of the graft is an identical twin of the recipient. Due to the perfect identity of the HLA systems between the donor and recipient, it may be able to eliminate the need for pre-transplant conditioning, especially for conditions such as bone marrow aplasia and anti-rejection therapy. The infrequency of these transplants is attributed to the scarcity of identical twins within the community.

9. Graft versus host disease (GvHD)

Graft-versus-host (GvH) reaction is absent in autologous transplants due to the absence of cellular incompatibility with the transplant. Conversely, GvH is detected in allographs with a fluctuating occurrence that depends on the source of HCS.

The allogenic transplant involves the administration of T lymphocytes and HSC to an immunosuppressed recipient during the transplant conditioning. This leads to a transplant LT response against the recipient's cells. This leads to the manifestation of symptoms known as graft-versus-host disease or GvHD.

Advancements in understanding the HLA system have shown that the occurrence and intensity of GvH (graft-versus-host) disease increase in proportion to the significance of HLA antigen differences between the donor and recipient. However, in the case of transplants between twins, even when the antigens are completely compatible, half of the patients still have graft-versus-host disease (GvHD).

Without immunosuppressive medications, the majority of HCS allographs are prone to developing HSH, which might aggravate the situation. Presently, this complication continues to be quite prevalent and represents a significant contributor to illness and death in HCS transplantation. Nevertheless, the use of immunosuppressants diminishes the occurrence of HBVs.

GvHD exists in two clinical forms: acute GvH, which occurs during the first 100 days after transplantation, and chronic GvH, which happens beyond the first hundred days of post-transplantation. The 100-day limit is arbitrarily established, although, in reality, acute and chronic HBV may manifest earlier or later.

9.1. Acute GvHD

often occurs during the first month after transplantation and its occurrence is influenced by the degree of compatibility between the donor and the recipient. It manifests about 1 week after an unrelated transplant and roughly 3 weeks after a transplant with an identical genome. This period relates to the time required for transplanted T cells to undergo proliferation and differentiation. Occasionally, HBV might manifest during the first week after transplantation, and the prognosis in such instances is often dire. The occurrence of acute HBV varies significantly, with a mean of 40% in transplants involving genetically identical individuals and 70% in transplants involving unrelated individuals (Fig. 35).

Acute GvH is categorised into several levels of escalating seriousness: grade I, representing the mildest form, to grade IV, indicating the most severe form. Most patients who have grade III or IV GvH succumb to this condition, either directly or indirectly.

9.1.1. Manifestations of acute GvHD

Acute GvHD often affects the skin, liver, and digestive tract.

The diagnosis of acute GvHD is mostly based on differential diagnosis, which involves ruling out the following conditions: toxidermia, viral rash, medication or viral hepatitis, venoocclusive illness, or infectious diarrhoea.

GvH leads to the breakdown of epithelial cells, accompanied by a mild influx of inflammatory cells. This results in the deterioration of the epithelial tissue in the skin and digestive tract, and the death of hepatocytes and the epithelial lining of the bile ducts in the liver. Additional tissues, including as the lungs, heart, kidneys, bladder, conjunctiva, and exocrine glands, may also be affected.

GvHD of the skin

The acute form of HBV often presents as a rash characterised by macules and papules, accompanied by itching and inflammation, which may proceed at different rates. It first makes contact with the palm of the hands and the sole of the feet. Subsequently, it spreads to the torso, including the base of the limbs and eventually encompassing the whole of the legs.

The severity of the condition may range from a mild rash to Lyell syndrome. The mucous membranes may also have an impact. Distinguishing between toxidermia and, less often, infectious erythrodermia may be challenging, and a definitive diagnosis is typically established by histological examination.

Hepatic GvHD

The liver is the organ that experiences the second highest degree of impact, behind the skin. The occurrence of liver injury results in the development of icterus, which may vary in degree. Additionally, there is a steady decrease in cytolysis over time. Additionally, it causes cholestasis in the absence of hepatocellular insufficiency. Necrotic areas form in the liver simultaneously with the damage to the bile ducts. The histological specification of the diagnosis is also required.

Gastrointestinal GvHD

Gastrointestinal symptoms often have a delayed onset in comparison to cutaneous injury. The observed clinical symptoms include watery, greenish, and bloody diarrhoea accompanied with stomach discomfort and vomiting. If there is a low colic injury, digestive haemorrhages and rectal syndrome might ensue. Nevertheless, if the upper portion of the gastrointestinal system is impacted, one may experience symptoms such as loss of appetite, indigestion, queasiness, and regurgitation without the presence of diarrhoea.

9.2. Chronic GvHD

Chronic GvHD often manifests between 100 to 400 days (3 to 13 months) after the transplantation, and its symptoms resemble those of an autoimmune disease. Similar to acute GvHD, the incidence of chronic GvHD varies greatly, with an average of 40% for geno-identical grafts and 60% for unrelated grafts. The occurrence of acute GvHD beforehand is a risk factor for the development of chronic GvHD. There are 3 types of occurrence of GvHD: Either in a progressive onset, or after acute GvHD (Fig. 35). Or in a quiescent manner, developing after acute GvHD without resolution. Chronic GvHD is the primary cause of mortality after allogeneic transplantation, unrelated to any relapse. For GvHD to develop, the graft must include immunologically competent cells, such as mature T lymphocytes. Clinical studies validate experimental data that demonstrate a correlation between the severity of GvHD and the number of donor T cells transfused. Furthermore, there must be HLA differences for tissue antigens to induce the proliferation and differentiation of these T cells. Ultimately, the recipient must be unable to generate an effective response to destroy the transplanted cells.

9.2.1. Manifestations of chronic GvHD

The chronic GvHD has similarities with autoimmune diseases such as scleroderma, systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, and primary biliary cirrhosis.

The most often seen clinical symptoms of chronic GvHD are:

Skin manifestations

The skin is the most often affected organ in chronic GvHD, presenting with hyper- or hypo-pigmented areas, sometimes accompanied by papules. They might manifest as an erythematous eruption with desquamation during sun exposure. Most often, these lesions are pruritic and result in a loss of skin flexibility.

Hepatic manifestations

Hepatic impairment manifests as cytolytic and cholestatic liver damage, which may sometimes lead to the occurrence of jaundice. These disorders may lead to itching. A biopsy and histological analysis are necessary to confirm the diagnosis.

Other signs include oral symptoms such as pain, dryness, gingivitis, mucositis, erythema, ulceration, atrophy, and pigmentation of the lips and teeth.

- Ocular manifestations include dryness, burning feeling, grittiness in the eyes, pain, sensitivity to light, or corneal ulceration.

- Gastrointestinal symptoms such as dysphagia, heartburn, retrosternal pain, mucosal desquamation or ulceration, anorexia, nausea, vomiting, diarrhoea, and weight loss may also be present.

- Pulmonary manifestations characterised by recurrent infections and chronic obstructive pulmonary dysfunction, presenting as a non-productive cough and dyspnea.

- Less common clinical signs also impact the hair, joints, vagina and vulva, as well as the haematological and musculoskeletal systems.

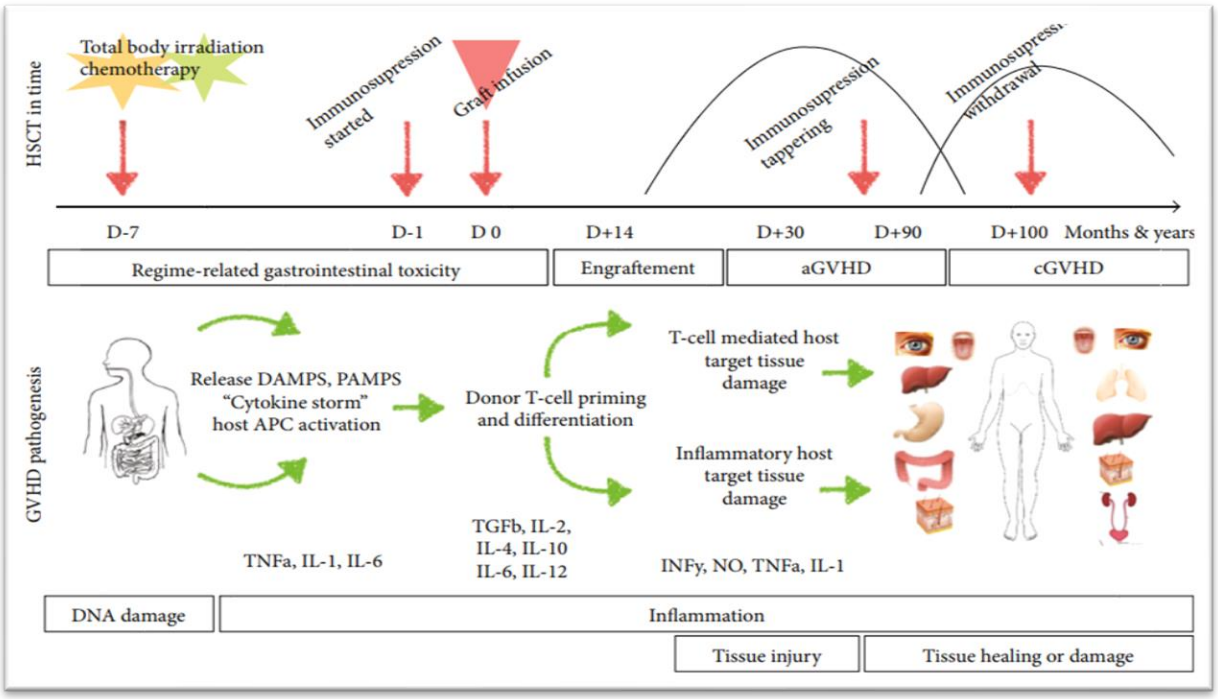

Figure 35. Acute and Chronic GvHD development

10. Regulatory T Cells

Regulatory T cells (Tregs) are a distinct and specialised group of CD4+ T cells that have a crucial function in suppressing excessive immunological responses, preserving peripheral tolerance, and maintaining tissue integrity. Tregs are distinguished by their high IL-2 receptor α-chain CD25 expression and the lineage-specific transcription factor forkhead box protein 3 (FoxP3). The suppressive function of Tregs is dependent on the consistent and elevated expression of FoxP3. This, along with other transcription factors, plays a crucial role in determining the functional programme of Tregs by stimulating the production of particular genes and epigenetic signature throughout their development. The continuous and consistent production of FoxP3 is crucial for maintaining the lineage, since removing Foxp3 from mature Tregs results in the loss of their function and their conversion into other kinds of T helper (Th) cells.

Two main types of Tregs have been identified based on where they come from: central, naturally occurring or thymus derived Tregs (tTreg) and peripheral Tregs (pTreg). T regulatory cells (tTregs) undergo positive selection in the thymus based on their interactions with T cell receptors (TCRs) that are reliant on major histocompatibility complex class II (MHC-II). This selection process leads to the preferential selection of tTregs with a high avidity. Regarding the

On the contrary, peripheral regulatory T cells (pTregs) are derived from conventional CD4+ T cells (Tconv), often in the presence of transforming growth factor-beta (TGF-β) and interleukin-2 (IL-2). These kinds of Tregs have distinct functions in regulating innate and adaptive immune responses to endogenous and exogenous antigens. Following the initial formation, the ongoing maintenance of functional stability and homeostatic proliferation in Tregs need a constant flow of signals. The binding of a similar antigen to the T cell receptor (TCR) triggers the activation of regulatory T cells (Tregs). However, full activation necessitates an additional signal supplied by co-stimulatory molecules such as CD28, ICOS, and/or CD40. When exposed to just the TCR signal, both Tconv and Tregs enter a condition of anergy and become unresponsive. A wide range of co-stimulatory and co-inhibitory receptors and their ligands are involved in the activation process during T-cell receptor (TCR) dependent regulatory T-cell (Treg) activation.

Foxp3, CD25 (IL-2Rα), and CTLA4 are unique markers expressed by CD4+ regulatory T (Treg) cells, which have a vital function in maintaining self-tolerance and preventing autoimmune diseases. These chemicals have specific roles in the survival of T cells and govern crucial biological functions. CD25 and CTLA4 play a role in the suppression mediated by Treg cells by reducing the levels of CD80 and CD86 on antigen-presenting cells (APCs). Meanwhile, Foxp3 is necessary for the increased expression of CD25 and CTLA4.

Although there have been significant breakthroughs in the understanding of Treg biology, no specific markers have been identified that accurately characterise human Tregs. Nevertheless, a preserved non-coding region located inside the Foxp3 gene locus has been identified as the optimal target for authentic human Tregs by the use of gene sequencing methodology. Tregs are present in the thymus during the 13th week of human gestation and they produce the Foxp3 protein, which is a transcription factor belonging to the forkhead/winged helix family. At present, the identification of Treg cells relies on the detection of CD4 and CD25 proteins, as well as the lack of the IL-7R α-chain (CD127), which are recognised as reliable indicators for Treg isolation.

Human Foxp3+ T cells exhibit functional and phenotypic heterogeneity. CD127 is minimally expressed by CD25+ CD4+ regulatory T cells (Tregs), and a subset of Tregs co-express CD4, CD25, and CD127. Nevertheless, the expression of CD127 is reduced in naïve T cells that have been activated by TCR signalling and have upregulated Foxp3 expression. This implies that the percentage of CD4+ CD25+ CD127+ T cells may include additional non-Treg cells that have been triggered and show all of these markers.

Accurate identification of the specific marker proteins on Tregs is crucial for the development of efficacious treatment approaches (Figure 36, Table 5). It is important to differentiate between Foxp3-expressing regulatory T cells (Tregs) and typical Foxp3-expressing T cells. The process of transforming immature CD4 + T cells into either regulatory T cells (Tregs) or effector T cells. The process of choosing naïve CD4 + T cells and natural Tregs takes place in the thymus. Naïve CD4 + T cells can develop into diverse T cell subsets, such as Th1, Th2, and Th17, induced Tregs (iTregs), in the periphery. Each of these subsets has unique immunological activities. The regulation of these differentiation programmes is governed by distinct cytokines. The differentiation of naïve T cells into Th1, Th2, or Th17 cells necessitates the presence of certain transcription factors, namely T-bet and Runx3 for Th1 cells, GATA3 for Th2 cells, and RORγt for Th17 cells. For instance, T-bet is associated with Th1 cells, GATA3 with Th2 cells, RORγt with Th17 cells, and FOXP3 with Tregs. (b) In a healthy person, the immune system maintains regular homeostasis, which allows it to inhibit autoreactive effector T cells and maintain a delicate. balance. Deviant Treg adaptability and deficits in both quantity and function of Treg disrupt immunological balance and lead to autoimmune disorders.

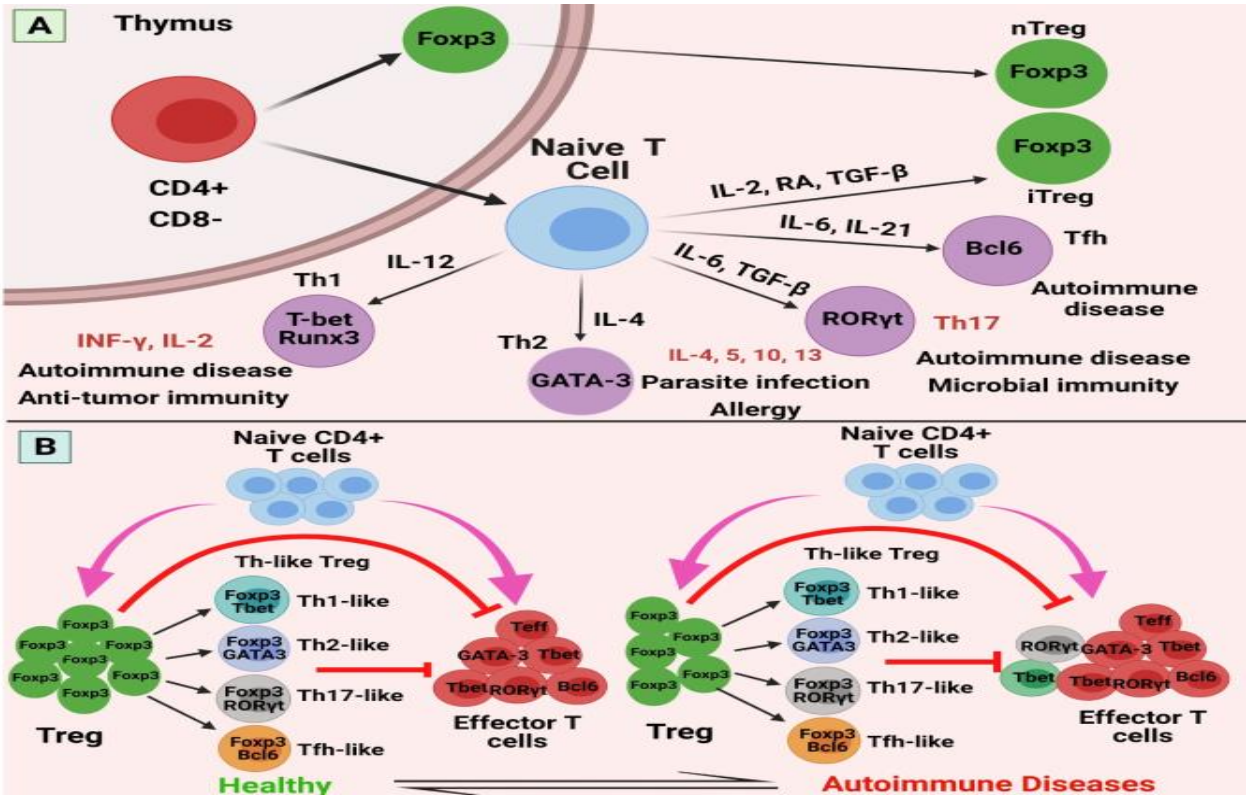

Figure 36. Characterization, phenotypic expression, functional attributes, and equilibrium of Treg cells in the immune response.

(a) Abbreviations: nTreg: natural Treg; iTreg: induced Treg; FOXP3: forkhead Box P3; RA: retinoic acid; CD: cluster of differentiation; IFN: interferon; IL: interleukin; RORγt: retinoid related orphan receptor γ; T-bet: T box transcription factor; TCR: T cell receptor; TGF-β: transforming growth factor-β; Th: T helper cell; GATA3: GATA-binding factor 3; Tfh: follicular helper T cells; Bcl: B-cell lymphoma 2.

Table 5. Essential properties of Treg-cell subtypes perceived in lymphoid tissues

Treg cell subset	Main phenotype	Master regulator	Cytokine produced
Treg1	$CD49b^+$ $LAG3^+$ $CD226^+$ $CD25^+$ $CTLA4^{low}$	FOXP3 negative	IL-10, TGFβ
Th3	LAP^+ $CD69^+$ $CD25^{low}$ $CTLA4^{low}$	FOXP3 negative	IL-10, TGFβ, IL-4
Treg-of-B	$LAG3^+$ $ICOS^+$ $PD1^+$ $GITR^+$ $OX40^+$ $CTLA4^+$	FOXP3 negative	IL-10
tTreg Resting - Fr I	$CD45RA^+$ $CD25^+$ $FOXP3^+$	FOXP3	none
tTreg Activated - Fr II	$CD45RA^-$ $CD25^{high}$ $FOXP3^{high}$	FOXP3	IL-10, TGFβ
pTreg - Fr III	$CD45RA^-$ $CD25^+$ $FOXP3^+$	FOXP3	IL-17, IFNγ, IL-2, IL-10, IL-22, IL-4
Th1-like	$CXCR3^+$	FOXP3, T-bet	IL-10, IFNγ
Th2-like	$CCR4^+$ $CCR6^-$ $CXCR3^-$	FOXP3, GATA3	IL-10
Th17-like	$CXCR3^-$ $CCR6^+$ $CCR4^+$ $CCR10^-$	FOXP3, RORγt	IL-10, IL-17
Th22-like	$CXCR3^-$ $CCR6^+$ $CCR4^+$ $CCR10^+$	FOXP3	IL-10
Tfr	$CXCR5^+$ $PD\text{-}1^+$ $ICOS^+$	FOXP3, BCL6	IL-10

10.1. Importance of regulatory T cells in the immune system

10.1.1. Treg and DCs

The research examines the involvement of PD-L1 and cytokines in the process of Treg induction by DCs. PD-L1 is present on dendritic cells (DCs), however even with the required cytokine signals, they are incapable of stimulating the production of FOXP3+ regulatory T cells (Tregs). Treg proliferation and specialisation are greatly facilitated in the presence of certain cytokines, notably IL-10 and TGF-β. IL-10 is a powerful suppressor of the immune response and has been shown to cause excessive immunological responses to infections, resulting in immunopathology. Tr1 cells, which are functionally analogous to naturally existing Tregs, exert their regulatory role by secreting IL-10. When effector T cells (Teff) are activated by IL-10-treated DCs in a laboratory setting (in vitro), it results in the development of anergy and the production of Tr1 cells. Interleukin-10 (IL-10) treated dendritic cells (DCs) possess strong suppressive capacity and have shown efficacy in experimental interventions for allergic asthma, graft-vs.-host disease, and inflammatory bowel disease.

Dendritic cells (DCs) have also been shown to facilitate tolerance using the immunoregulatory cytokine TGF-β. TGF-β plays a crucial role in the growth and operation of FOXP3+ T cells, and Tregs also promote tolerance by releasing TGF-β. Mice that do not have TGF-β signalling have a lethal lymphoproliferative illness that is comparable to the condition seen in scurfy mice. Dendritic cells (DCs) have been shown to generate FOXP3+ regulatory T cells (Tregs) from FOXP3− progenitor cells when exposed to both exogenous and endogenous TGF-β. Specifically disabling TGF-β receptor signalling in DCs led to increased T cell responses in experimental autoimmune encephalomyelitis (EAE). CD8+CD205+ dendritic cells (DCs) promote the generation of regulatory T cells (Tregs) by producing TGF-β. The capacity of CD8+CD205+ DCs to generate Tregs is eliminated when TGF-β is neutralised using antibodies.

Regulatory T cells (Tregs) may be produced from existing T cells in the peripheral blood of humans or mice via the action of retinoic acid (RA). The suppressive capacity of Tregs is enhanced by incubating them with TGF-β and RA. The capacity of intestinal dendritic cells (DCs) to activate regulatory T cells (Tregs) is lost when they are treated with an antagonist against the retinoic acid (RA) receptor. The precise mechanism by which RA causes the development of tolerogenic DCs is not well understood. Research has shown that the enzyme retinal dehydrogenase 2 (RALDH2), which produces RA, is expressed at high levels by DCs in the mesenteric lymph node (MLN) and Peyer's patches, in comparison to DCs found in other lymphoid organs. Ohoka et al. have shown that rheumatoid arthritis and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulate the production of Aldh1a2 in dendritic cells (DCs) via interacting with the RA receptor and retinoid X receptor complex. This connection leads to the activation of the transcription factor sp1.

Dendritic cells that produce retinoic acid (RA) are crucial for maintaining oral tolerance. T or B cells that are stimulated in the presence of RA undergo a process called "imprinting" which results in the expression of gut-homing receptors. Regulatory T cells (Tregs) have a preference for migrating to the gastrointestinal tract after being treated with retinoic acid (RA). There is a proposal suggesting that RA, which is generated by DCs, enhances the TGF-β-dependent induction of Tregs from naïve T cells by preventing their maturation into inflammatory T cells. The involvement of retinoic acid (RA) in the preservation of oral tolerance is comprehensible due to the continuous exposure of the gut to foreign and commensal antigens. The preservation of tolerance, while maintaining the integrity of the immune system, is of utmost importance.

10.1.2. Treg and Macrophage

Macrophages are vital immune cells that preserve immunological balance by engulfing foreign substances, dead or dying cells, and carry several pathogen recognition receptors (PRRs). They have a crucial impact on the development of the adaptive immune response by presenting antigens to T and B cells and releasing various cytokines. Macrophages may be split into two main categories: proinflammatory M1 macrophages, which develop in the presence of cytokines such as IFN-γ and IL-12, and anti-inflammatory M2 macrophages, which are further categorised depending on cytokine signals.

The interaction between Treg cells and macrophages plays a crucial role in modifying the immunological response of the host. For instance, when macrophages and Tregs are cultured together, it results in a decrease in the expression of HLA-DR on macrophages and a decrease in the production of proinflammatory cytokines when stimulated with LPS. The depletion of regulatory T cells (Tregs) results in an over-activation of macrophages, which ultimately leads to death due to endotoxic shock.

Regulatory macrophages (Mregs) have a notable impact on inflammation and cancer. Specifically, M2c macrophages adopt a regulatory phenotype when exposed to TGF-β and IL-10, two cytokines often linked with Treg activity. Mregs are characterised by their secretion of IL-10, which significantly contributes to their activity. Tregs can guide the development of macrophages towards the M2 regulatory phenotype. Additionally, they may also induce the regulatory phenotype in macrophages by other methods.

Studies have shown that M2 macrophages may stimulate the production of Tregs, whereas Mregs have exhibited therapeutic effectiveness in experimental approaches to treat inflammatory conditions such as allergies and type 1 diabetes. Mregs can stimulate Tregs via the generation of reactive oxygen species (ROS), which may hinder the macrophage-mediated induction of Tregs. In addition, mice with diminished reactive oxygen species (ROS) generation capacity exhibit greater vulnerability to autoimmune disorders in comparison to their wild-type counterparts. Hence, the interaction between

Mreg and Treg is a significant process in maintaining immunological tolerance, which requires more investigation.

10.1.3. Treg and Neutrophiles

Neutrophils play a vital role in initiating the body's natural immune responses and are the most prevalent kind of white blood cells in humans. They express all TLRs except TLR3, which is crucial for modulating both innate and adaptive immune responses. The involvement of neutrophils in modulating immunity has been thoroughly examined, specifically about the interaction between Treg cells and neutrophils, as well as the regulatory function of neutrophils in immune responses.

Neutrophils can adopt distinct characteristics in reaction to their surroundings, such as acquiring anti-tumorigenic (N1) and pro-tumorigenic (N2) phenotypes in mice. TGF-β in the tumour microenvironment leads to the development of N2 neutrophils while inhibiting TGF-β promotes the N1 phenotype. Neutrophils may also display regulatory and immunosuppressive roles in several ways. For example, neutrophil-derived hydrogen peroxide can decrease T cell responses in cancer patients, and they can also suppress T cell responses in sepsis by expressing Mac-1.

Neutrophils have a regulatory role that involves producing cytokines, such as IL-10, which suppresses the immune response of Th17 cells during mycobacterial infection. There is evidence indicating communication between Tregs and neutrophils, and there is considerable interest in understanding the function of Treg/neutrophil interaction in preserving immunological balance. Tregs may stimulate the recruitment of neutrophils by producing CXCL8. Additionally, the production of IL-10 by Tregs can influence the function of neutrophils, and vice versa.

The association between Tregs and neutrophils has been significantly understudied, although it is crucial to acknowledge that the interplay between Tregs and neutrophils is a noteworthy subject of inquiry. Impaired regulatory T cell (Treg) activity has been seen in several autoimmune disorders, and the use of Treg therapy has shown efficacy in their treatment. Richards et al. discovered that defective Treg function is linked to heightened neutrophil activity. They observed that Tregs control inflammation in the skin by suppressing the formation and survival of neutrophils. In addition, a decrease in the number of regulatory T cells (Tregs) in mice causes an excessive activation of neutrophils, leading to death in cases of endotoxic shock.

In addition, Tregs control the viability and function of human and murine neutrophils, and the co-cultivation of Tregs and neutrophils enhances neutrophil death. Further research is required to define the specific function of Treg/neutrophil interaction in autoimmune and inflammatory illnesses, a field of study that is now being actively pursued in our laboratory.

10.1.4. Treg and gamma delta (γδ) t cells

T cells, mostly located in the thymus, possess a T cell receptor (TCR) consisting of αβ chains and are largely present in peripheral lymphoid organs. Nevertheless, there exists a distinct subset known as γδ T cells that originate both inside and outside the thymus, possess TCR consisting of γδ chains, and are plentiful in intra-epithelial compartments. These γδ T cells possess innate-like properties and may be triggered independently of antigen-presenting cells (APCs), therefore classifying them as innate immune cells. In addition, they perform phagocytosis.

γδ T cells have been shown to have both inflammatory and regulatory characteristics, as supported by data. Proinflammatory γδ T cells are categorised based on their secretion of IFN-γ or IL-17 and have been linked to the development of many autoimmune disorders. Conversely, when γδ T cells are stimulated in the presence of TGF-β, it results in the generation of γδ T cells that express FOXP3 and have a suppressive phenotype. The suppressive effect of these regulatory γδ T cells is exerted by the secretion of IL-10 and TGF-β, which decrease the activation and proliferation of T cells.

Tregs, which release cytokines such as IL-10 and TGF-β, can trigger the development of γδ T cells that possess a regulatory phenotype. Recent research has shown that Tregs play a crucial role in maintaining the balance and stability of the intestines by inhibiting the activity of γδ T cells. Moreover, Tregs have shown the ability to control the activity of γδ T cells, suppressing the generation of cytokines by γδ T cells when exposed to M tuberculosis antigen. In paediatric epilepsy characterised by inflammation of the central nervous system, there is a rise in the quantity of γδ T cells, which is accompanied by a decline in the number of Tregs in the epileptogenic lesions.

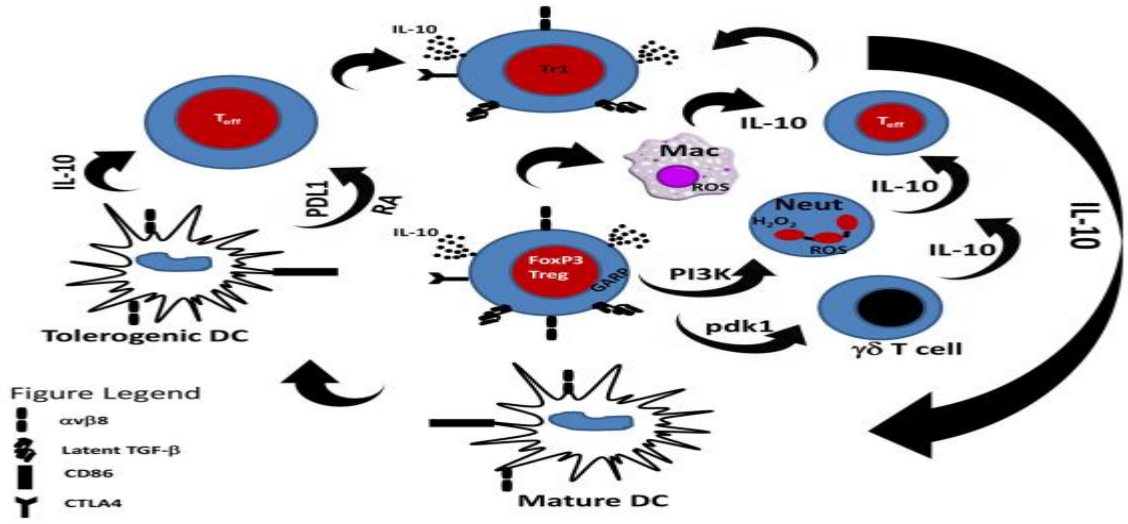

Figure 37. Interactions between regulatory T cells (Tregs) and cells of the innate immune system.

Tregs may influence the behaviour of innate immune cells by releasing IL-10 and TGF-β, which prompts these cells to adopt an anti-inflammatory state. Similarly, when IL-10 is present, innate immune cells can generate Tr1 cells, which can inhibit the response of effector T cells. PDL1 refers to Programmed death-ligand 1, whereas RA stands for Retinoic acid.

10.2. Function of Regulatory T Cells

Treg-mediated immunosuppression encompasses many mechanisms that reduce the immune response, such as the suppression of antigen-presenting cells (APCs) via CTLA4, the consumption of IL-2, and the generation of immunosuppressive cytokines and metabolites. Treg-mediated inhibitory pathways that hinder different immune cells have been categorised into direct channels, where Tregs directly elicit a response in the immune cell, and indirect pathways, in which another cell or molecule is altered, subsequently leading to the inhibition of immune cells. The secretion of diverse cytokines, including IL-10, TGF-β, and IL-35, together with the production of lytic enzymes like granzyme and perforin, leads to the demise of immune cells that are targeted, often by apoptosis. Tregs exert direct suppression on target cells by the expression of CD39/CD73, resulting in a decrease in extracellular ATP levels by generating adenosine and AMP. Tregs also influence alterations in the microenvironment as a result of their heightened CD25 expression.

The processes by which Tregs inhibit immunological responses to preserve homeostasis have been thoroughly researched. Multiple discoveries suggest that immunosuppression arises from the direct interaction between regulatory T cells (Tregs) and effector T and B cells, as well as the release of suppressive cytokines. Tregs inhibit the activation and multiplication of CD4+ T cells via both direct contact and indirect mechanisms. When Tregs (CD4+, CD25+) are cultured along with APCs and other antigen-specific responder T cells (CD4+ and CD8+), in the presence of a particular antigen, it inhibits the proliferation of responder cells and decreases the generation of IL-2. Tregs may impede the antigen-presenting capability of DCs or stimulate the release of suppressive substances, such as indolamine 2,3 dioxygenase (IDO), which is a powerful immunosuppressive enzyme, from DC. The induction of IDO relies on the elevated expression of CTLA4, which is an inhibitory receptor found in Treg cells.

Tregs suppress the capacity of autoreactive B cells to generate harmful autoantibodies, hence eliminating autoreactive B cells. Granzyme B (granular enzyme) and perforin are used to eliminate effector B cells, hence decreasing the formation of autoantibodies. Tregs inhibit the action of NK cells, leading to the advancement of tumours. Tregs directly interact with polymorphs to restrict the invasion of granulocytes by decreasing the production of chemokines that function as chemoattractants. Co-cultivating neutrophils with Tregs in a culture vessel leads to an increase in the production of IL-10 and IL-6, both of which are recognised as having immunosuppressive properties. These results validate the efficacy of human Tregs in avoiding graft-versus-host disease (GVHD) and autoimmune disorders, while also causing a delay in graft rejection.

Tregs have a unique inhibitory effect on monocytes, impeding their process of differentiation, production of cytokines, and expression of antigens. After being co-cultured with Tregs, monocytes

exhibit typical traits of M2 macrophages, including heightened expression of CD206 and CD163, as well as a diminished ability to react to pro-inflammatory signals. Ex vivo expanded regulatory T cells (Tregs) are successful in inducing a shift in monocytes towards a tolerogenic phenotype.

Treg cells regulate immune responses by suppressing the functions of effector T cells (Teff) and antigen-presenting cells (APCs) through various mechanisms (figure 38) , including (i) modulation of dendritic cell (DC) function and prevention of DC maturation by the interaction of CTLA4 and LAG3 expressed by Treg cells and the CD80/86 costimulatory molecules and MHC class II expressed by DC, respectively, leading to IDO generation and inhibition of Teff cell activation; (ii) metabolic disruption, Treg cells can disrupt metabolic roles by the expression of the ectoenzymes CD39/73 allowing adenosine generation and binding of adenosine to the adenosine receptor 2A (A2AR) exposed on Teff cells, or by IL-2 deprivation; (iii) generation and secretion of the antiinflammatory cytokines IL-10, IL-35, and TGF-β that restrain Th1 and Th17 immune responses and the production of IFN-γ and IL-17, respectively; and (iv) direct cytotoxicity, Treg cells can also induce direct killing of effector cells by the release of granzyme A, granzyme B, and perforin, which induce apoptosis in the target cells. Tregs have been seen to directly influence B-cells via the interplay of PD-L1 and PD-1.

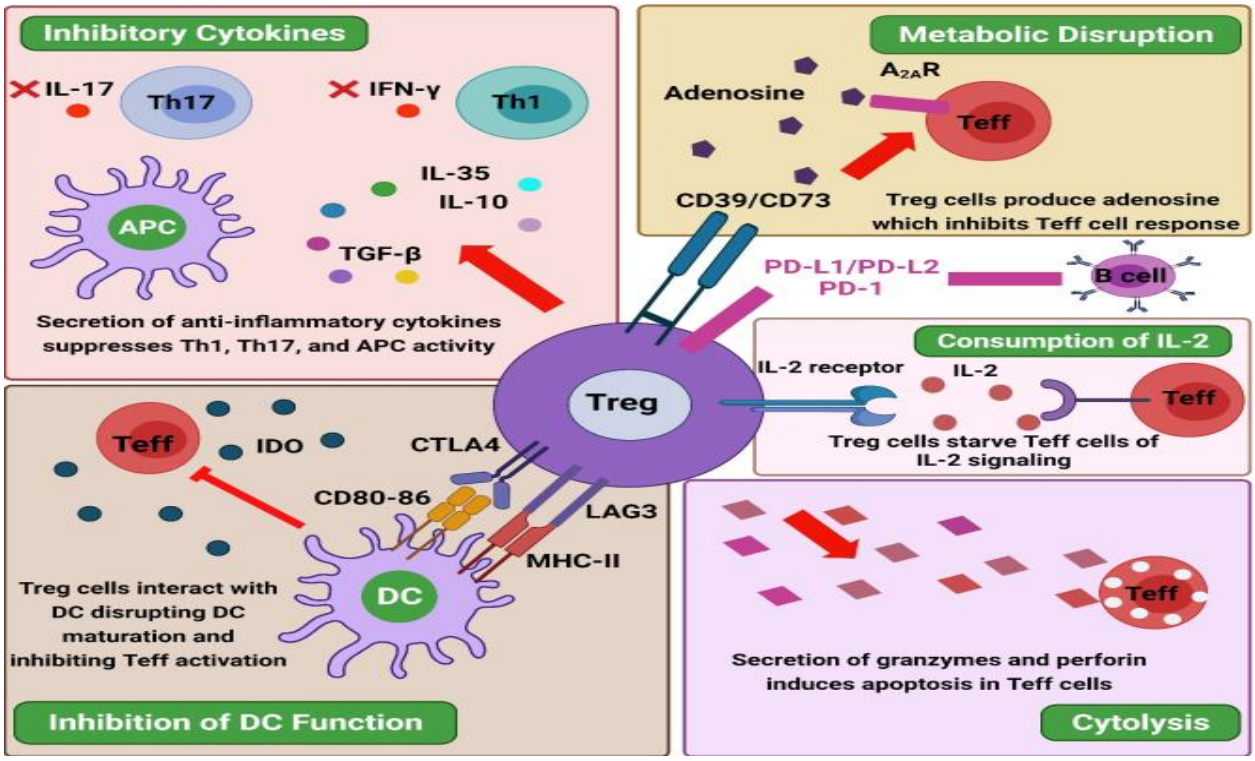

Figure 38. The mechanisms responsible for the immunosuppressive effects produced by regulatory T cells (Tregs).

Abbreviations: APC: antigen-presenting cell; TGF-β: transforming growth factor-β; A2AR: Adenosine receptor 2A; IL: interleukin; IFN: interferon; Teff: effector T cells; Treg: regulatory T cells; CD: cluster differentiation; IDO: indoleamine 2,3-dioxygenase; DC: dendritic cell; CTLA-4: cytotoxic T lymphocyte antigen-4; TGF-β: transforming growth factor-β; LAG3: lymphocyte-activation gene 3; MHC: major histocompatibility complex; PD-1: programmed death-1; PD-Ls: programmed cell death ligands

10.3. Treg and cell therapy

10.3.1. Transferring adaptive regulatory T cells

Adoptive Treg transfer, also known as adoptive cellular treatment (ACT), refers to the customised administration of Tregs that have been identified, expanded, and sometimes genetically modified outside of the body (Fig. 39). Various manufacturing processes exist, all of which include obtaining initial material from the donor, either autologous peripheral blood or an autologous leukapheresis product. From this material, Tregs are identified and cultivated by repeated rounds of stimulation. The production method starts by obtaining peripheral blood, from which CD4+CD25+ lymphocytes are isolated by magnetic enrichment from peripheral blood mononuclear cells (PBMCs). These isolated lymphocytes are then expanded using T-cell receptor (TCR) stimulation in the presence of IL-2 and rapamycin. The resultant polyclonal product is cryopreserved until it is ready for infusion. Alternatively, antigen-specific regulatory T cells (Tregs) may be produced by increasing their numbers in the presence of professional antigen-presenting cells (APCs), often B cells or dendritic cells (DCs). Nevertheless, while the transfer of antigen-specific Tregs that have been adopted may have a greater effect, their precursor frequency is often modest. Expanding an adequate number of cells for therapeutic use may be difficult, especially since extensive growth may reduce the suppressive ability of Treg cells.

10.3.2. IL-2 pathway

IL-2 was first identified as indispensable for effector T cell survival, proliferation, and function before its importance in Treg homeostasis was recognized (Fig. 39). However, the potential for IL-2 to stimulate both effector and regulatory T cells, in addition to its short half-life (under 15 min) necessitating high-dose administration and frequent, occasionally fatal, dose-limiting toxicities curbed early clinical adoption as a therapeutic to stimulate anti-tumour effector populations. However, preferential expansion of Tregs through exploiting IL-2 signalling differences, namely the high affinity (Kd ~ 10-11 M) of the trimeric IL-2 receptor expressed by Tregs, is under active investigation through administering ultra-low dose IL-2, carefully optimized to promote Treg without effector expansion. This approach has been trialled in multiple disease spaces including type 1 diabetes, vasculitis, GvHD, and solid organ transplantation. Ultralow dosing regimens appear to have a tolerable safety profile, in particular concerning the incidence of venous thromboembolism which is especially problematic at higher doses. Nevertheless, the therapeutic window of this approach is very narrow, and the potential to inadvertently expand effector
populations, and thereby worsen the immunopathology, remains significant.

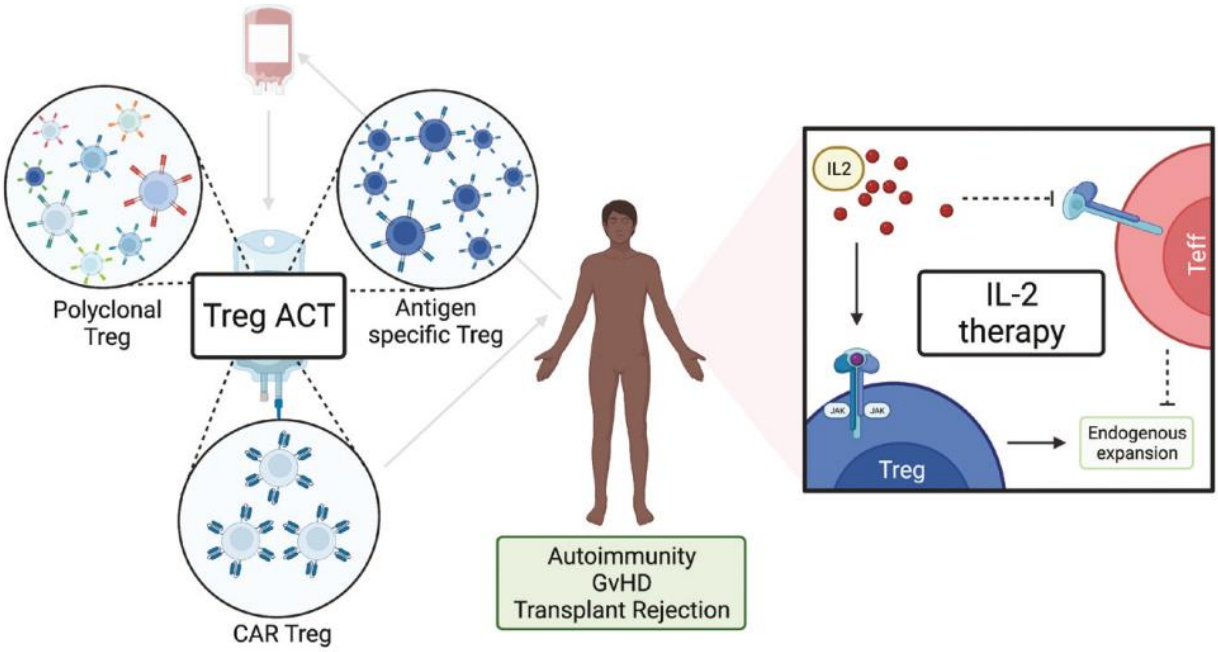

Figure 39. Contemporary methods for Treg treatment.

A comprehensive depiction of the primary Treg therapeutic strategies that have advanced to clinical trials, generated using biorender.com. The left-hand panel depicts the process of adoptive cellular therapy (ACT), which involves obtaining autologous whole blood or a leukapheresis product to manufacture the cell treatment. In general, Treg ACTs may be categorised as follows: polyclonal, which means they have a wide range of TCRs in the product; antigen specific, which means they have a limited range of TCRs; or as Tregs that have been modified with a CAR containing a ScFv targeting a single antigen. The produced product is then reintroduced into the patient donor. The right-hand panel demonstrates the method used to selectively increase the population of naturally occurring regulatory T cells (Treg) in living organisms by administering a little amount of IL-2. In this context, the externally delivered IL-2 is anticipated to selectively activate and increase the population of Treg cells that express the high affinity IL2Rα chain CD25, as opposed to the lower affinity dimeric IL2R produced by effector cells.

10.3.3. Enhancing the selectivity of Tregs using Chimeric Antigen Receptors

The utilisation of Chimeric Antigen Receptor (CAR) technology has resulted in the development of groundbreaking and highly efficient novel treatments for B-cell precursor acute lymphoblastic leukaemia and diffuse large B cell lymphoma. This same technology shows great potential in directing Treg cell therapies with precision and potency towards a known antigenic target. Generally, CARs consist of an external signalling domain, usually a single chain variable fragment (scFv), and an internal signalling domain, often the CD3ζ activation domain of the T cell receptor, which may be enhanced by a co-stimulatory protein like CD28. An extracellular hinge region and transmembrane domain connect them. The presence of the wild-type CD28 co-stimulatory domain is crucial for the formation of CAR-Treg, since the expression of alternative co-stimulatory domains undermines their ability to suppress. Autologous CAR T cell treatments are manufactured by transducing activated autologous T cells with a CAR-encoding vector using either lentiviral or gamma-retroviral methods. Cells that have undergone transduction are multiplied in number and may be either utilised immediately or frozen for later use before being thawed and administered to the patient.

CAR-Tregs provide a pathway to achieve alloantigen-specific tolerance, which has therapeutic implications for several conditions such as type 1 diabetes, inflammatory bowel disease, multiple sclerosis, haemophilia, vitiligo, transplant rejection, and graft-versus-host disease (GvHD). Our primary approach in the field of transplantation has been to take advantage of common mismatches in human leucocyte antigen (HLA) between donors and recipients. We do this by modifying regulatory T cells (Treg) with a chimeric antigen receptor (CAR) that selectively targets the donor's HLA. Thus, CAR-Treg specifically recognise their corresponding antigen only inside the allograft, leading to precise and localised immunosuppression. This method has mostly been confirmed using Tregs that express Chimeric Antigen Receptors (CARs) that target HLAA2, an antigen that is often mismatched between organ transplant donors and recipients. However, only a few number of CARs would be needed to address the majority of possible mismatches. In models of transplant rejection using humanised skin allografts, HLA-A2+ CAR Tregs infiltrate the graft and extend its lifespan compared to polyclonal Tregs. Similarly, CAR Tregs expressing HLA-A2+ molecules effectively inhibit the development of GvHD after being infused with HLA-A2+ human PBMCs. Nevertheless, the capacity of CAR-Tregs to promote tolerance seems to diminish when animals are previously exposed to donor antigens, indicating that their ability to regulate memory responses is restricted, which is a crucial factor to address in translational research (Fig. 40).

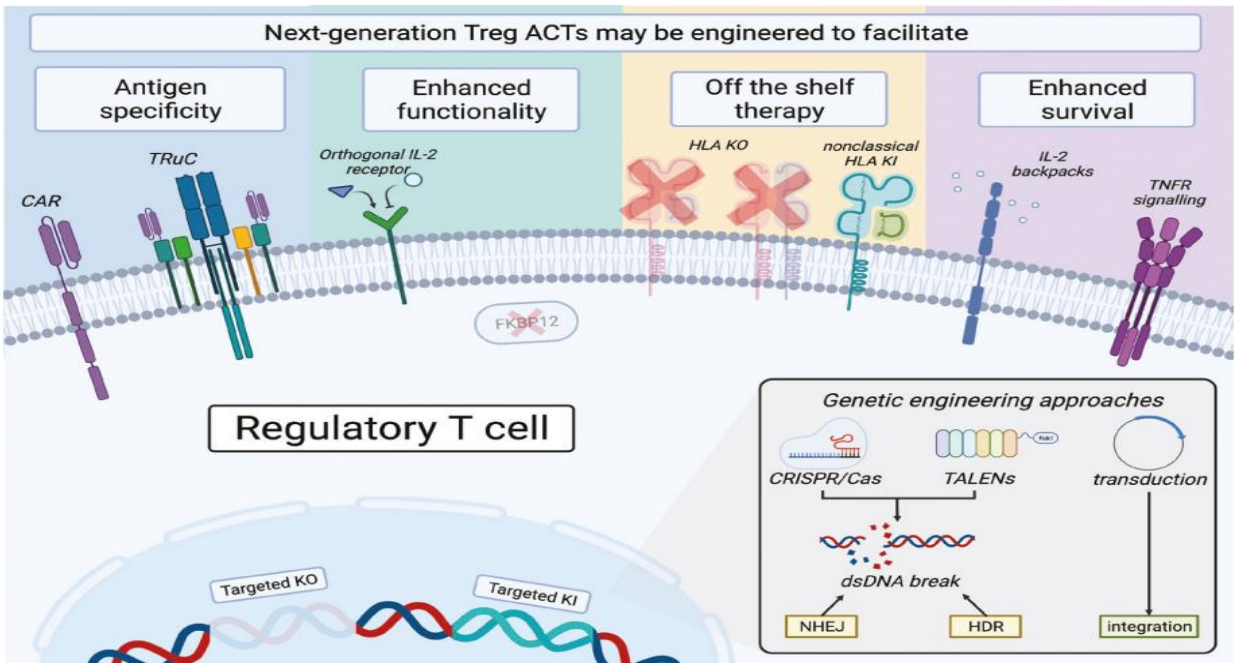

Figure 40. Prospective strategies for Treg treatment.

This is a schematic summary of several experimental procedures that have been used to enhance the effectiveness of Treg cell treatment in pre-clinical studies. The diagram was generated using biorender.com. In general, researchers have studied genetic and bioengineering methods to create specific antigens in polyclonal Treg products. They have also looked into ways to improve the survival and function of Treg cells in the body by modifying their response to important signals for Treg cell balance. Additionally, they have explored the use of Treg products from a different

individual by reducing the expression of HLA on the cell surface, either with or without inducing the expression of other proteins that promote cell survival.

10.4. Using mesenchymal transplantation

‘‘see the section of mesenchymal stem cells’’

10.4.1. Autoimmune hepatitis

The diagnosis of autoimmune hepatitis (AIH) is established by observing elevated liver enzyme levels with the presence of autoantibodies and immune cell infiltration in the liver. The quantity and function of Treg cells in the development of AIH have been a subject of debate. Longhi et al demonstrated a reduction in the number of Treg cells in the peripheral blood of 41 patients compared to control participants. However, other studies have not seen any variations in the frequency of Treg cells in the peripheral blood. According to reports, the presence of IL-2 in liver tissue is often at relatively low levels. As a result, liver tissue does not typically provide the necessary support for the survival and activity of Treg cells. There is a positive correlation between increased frequencies of Treg cells inside the liver and a more favourable response to treatment. Creating a preclinical model for AIH has proven to be a difficult task. Mice lacking AIRE exhibit a condition resembling autoimmune hepatitis, characterised by the presence of autoantibodies and the infiltration of lymphocytes and plasma cells in the liver. The quantity of Treg cells was reduced, and the administration of 8×10^5 polyclonal nonexpanded Treg cells by adoptive cell transfer corrected the histological damage caused by autoimmune hepatitis (AIH) (Fig. 41).

10.4.2. Treatment of Systemic lupus erythematosus with Treg

Systemic lupus erythematosus is a medical condition. Systemic lupus erythematosus (SLE) is an intricate condition characterised by abnormal responses of the body's innate and adaptive immune systems. There is growing evidence suggesting that Treg cells play a significant role, particularly during episodes of illness exacerbation. The administration of ex vivo-expanded Treg cells in mice with autoantibodies slowed the development of renal problems and extended their lifespan. A preliminary investigation including 37 patients diagnosed with systemic lupus erythematosus (SLE) and treated with a 5-day regimen of low-dose interleukin-2 (IL-2) showed positive therapeutic effects. This included an increase in the number of regulatory T cells (Treg cells) and a fall in the scores of the SLE Disease Activity Index, indicating a reduction in disease activity. A recent study documented the administration of 1 x 108 autologous polyclonally expanded Treg cells to a patient with SLE who had active discoid lupus affecting the skin. While the SLE Disease Activity Index score did not change after infusion, the post-infusion biopsy specimens showed a significant decrease in the number of immunological infiltrates (Fig. 41).

10.4.3. Treatment of Inflammatory bowel disease by Treg

Inflammatory bowel disease (IBD) includes both Crohn's disease and ulcerative colitis. The number of Treg cells in the inflamed mucosa of individuals with inflammatory bowel disease only exhibited a little increase when compared to other inflammatory disorders, such as diverticulitis. In addition, T cells generated from mucosal tissue, but not from peripheral blood, exhibit resistance to suppression by Treg cells due to the excessive production of Smad7, which is an inhibitor of TGF-b signalling. CD45RA1 regulatory T cells derived from the peripheral blood of individuals diagnosed with Crohn's disease have the potential for expansion. The Levings group recently shown that Treg cells may be modified to migrate to TH1-inflamed regions by incorporating IFN-g and IL-12 during growth. This modification leads to the development of epigenetically stable CXCR31T-bet1FOXP31 Treg cells. There are many preclinical models available, including dextran sulphate sodium colitis, intrarectal injection of substances like 2,4,6-trinitrobenzene sulfonic acid or oxazolone, and IL-10 mutant mice. The prevailing approach to investigate Treg cell activity in individuals with experimental colitis involves the transplantation of CD4 naïve or Treg cell-depleted T cells into syngeneic immunodeficient severe combined immunodeficiency or Rag2/2 mice. This model demonstrated that type 1 regulatory cells, namely IL–10–producing Treg cells, had more efficacy in avoiding colitis compared to CD41CD25hi T cells. This suggests that IL-10 plays a crucial role in this process (Fig. 41).

10.4.4. Autoimmune hepatitis

The diagnosis of autoimmune hepatitis (AIH) is established by observing elevated liver enzyme levels with the presence of autoantibodies and immune cell infiltration in the liver. The quantity and function of Treg cells in the development of AIH have been a subject of debate. Longhi et al7 demonstrated a reduction in the number of Treg cells in the peripheral blood of 41 patients compared to control participants. However, other studies did not observe any variations in the frequency of Treg cells in the peripheral blood. According to reports, the content of IL-2 in liver tissue is often relatively low. As a result, liver tissue usually does not provide favourable conditions for the survival and functioning of Treg cells. Increased frequencies of intrahepatic Treg cells are associated with a more favourable response to medication. Creating a preclinical model for AIH has proven to be a difficult task. Mice lacking AIRE exhibit a condition resembling autoimmune hepatitis, characterised by the presence of autoantibodies and the infiltration of lymphocytes and plasma cells in the liver. The quantity of Treg cells was reduced, and the administration of 8×10^5 nonexpanded polyclonal Treg cells by adoptive cell transfer corrected the histological damage caused by autoimmune hepatitis (AIH) (Fig. 41).

10.4.5. Pemphigus Vulgaris

Pemphigus vulgaris (PV) is an autoimmune blistering disorder characterised by the presence of IgG autoantibodies that specifically attack the desmosomal adhesion proteins desmoglein (Dsg) 1 and Dsg3. The antibodies specifically bind to the desmosome, leading to acantholysis. Patients with PV have a decrease in the quantity of Treg cells. In the preclinical model, splenocytes obtained from Dsg3-immunized Dsg32/2 mice were transferred into Rag22/2Dsg31/1 mice. The introduction of polyclonal expanded Treg cells, including those derived from Dsg32/2 animals, resulted in a decrease in disease activity and a reduction in the generation of antiDsg3 antibodies. In their study, Schmidt et al. (2016) used a mouse model of pemphigus vulgaris (PV) that was genetically modified to express the HLA-DRB1 04:02 gene. The mice were immunised with human Dsg3 and the researchers demonstrated the crucial involvement of Treg cells in suppressing the T-cell response triggered by Dsg3, as well as the production of antibodies specific to Dsg3 (Fig. 41).

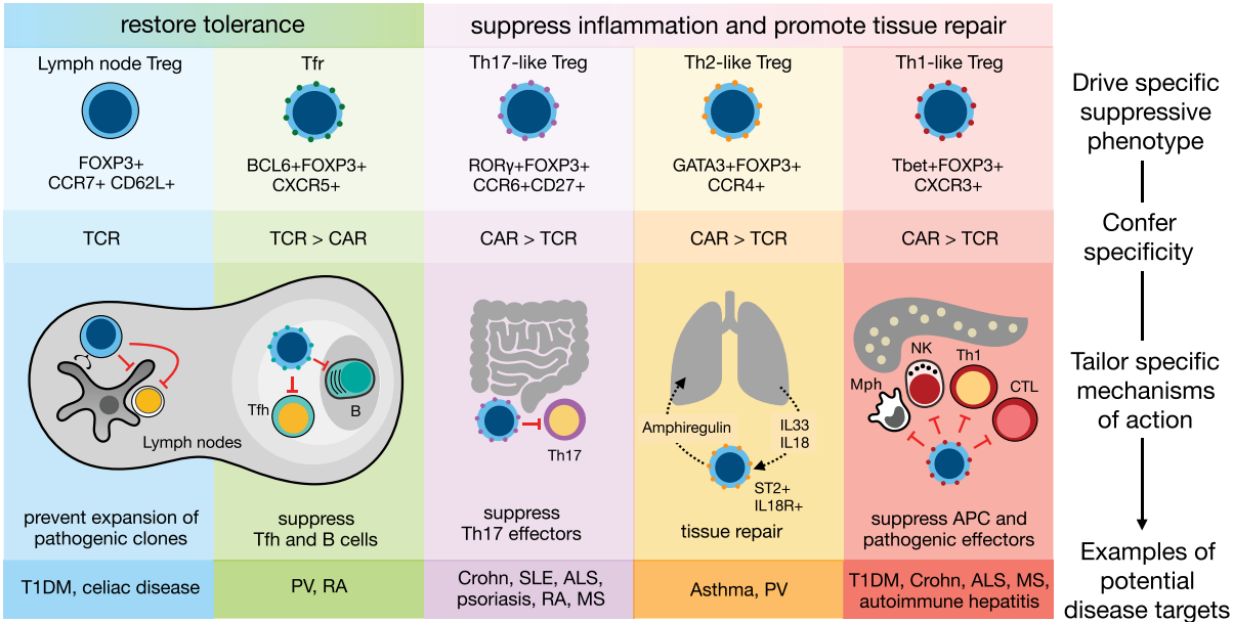

Figure 41. Possible future implementations of Treg cells to selectively target certain tissues and disorders.

Therapeutic Treg cell products can be specialised to inhibit certain subsets of inflammatory T-cells. Treg cells may be modified to specifically migrate to affected tissue by expressing certain antigen receptors. When considering the specificity of antigens, it is important to mention that Treg cells in lymph nodes are likely to use TCRs to target particular antigens, whereas CARs may be more efficient in tissues. Collectively, these strategies may be enough to selectively target certain tissues or organs and effectively manage a range of autoimmune and autoinflammatory disorders. ALS, Amyotrophic lateral sclerosis; APC, antigen-presenting cell; CAR, chimeric antigen receptor; CTL, cytotoxic T cells; Mph, macrophage; MS, multiple sclerosis; NK, natural killer cells; RA, rheumatoid arthritis; T1D, type 1 diabetes mellitus; Tfh, follicular helper T cells; Tfr, follicular regulatory T cells.

10.5. Treg and GvHD

Tregs have a vital function in regulating GVHD by using many strategies, such as cytolysis, release of inhibitory cytokines, disturbance of metabolic processes, and specifically targeting dendritic cells (DCs). Reactive T cells may be eliminated by direct interaction between cells, and they can affect

many target cells, including DCs. The interaction between regulatory T cells (Tregs) and dendritic cells (DCs) plays a fundamental role in the processes that drive Tregs-mediated immunological suppression.

Dendritic cells (DCs) are well recognised as the most potent antigen-presenting cells (APCs) and play a dual function in the development of graft-versus-host disease (GVHD). Tregs express CTLA-4, which inhibits full T-cell activation by preventing the interaction between CD28 and CD80/86. Additionally, they may help in the elimination and breakdown of CD80/86 from DCs using CTLA-4 via trans-endocytosis. Tregs may hinder DC activation and antigen presentation by releasing immunosuppressive cytokines, such as IL-10.

Tregs may impede the maturation of DCs, resulting in their inadequate ability to initiate T cell activation. LKB1 has a significant impact on dendritic cells (DCs) via promoting the expansion of regulatory T cells (Tregs), a finding that has been corroborated by previous research. Granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances the proliferation of CD4+CD8– dendritic cells (DCs), with a particular preference for promoting the growth of regulatory T cells (Tregs). This leads to a reduction in tissue damage in a mouse model of chronic graft-versus-host disease (cGVHD).

The interplay between dendritic cells (DCs) and regulatory T cells (Tregs) plays a crucial role in the regulation of graft-versus-host disease (GVHD). Additional investigation is necessary to enhance our understanding and guide the formulation of novel approaches aimed at preventing GVHD while preserving the graft-versus-leukemia (GVL) effect. Allo-HSCT is recognised as the only treatment that may cure several malignant haematological disorders. However, the immunosuppressive Tregs that manage GVHD by suppressing the early activation of alloreactive T cells may weaken the GVL impact. This, in turn, raises the likelihood of recurrence and infection.

Animal model experiments have shown that Treg treatment may effectively inhibit GVHD while preserving GVL, hence distinguishing between GVHD and GVL. This process may include the suppression of excessive proliferation of donor T cells by Tregs, as well as the reduction of proinflammatory cytokine levels in the blood, without impeding the activation of conventional T cells (Tcons). Nevertheless, few preclinical trials have shown that CD4+ iTregs may partly hinder GVL (graft-versus-leukemia) in a mouse model, resulting in temporary relapse of leukaemia. The combination of CD4+ iTregs and CD8+ iTregs may provide a novel approach to address the issue, since CD8+ iTregs may maintain the GVL effect, while CD4+ iTregs work to reduce the severity of GVHD.

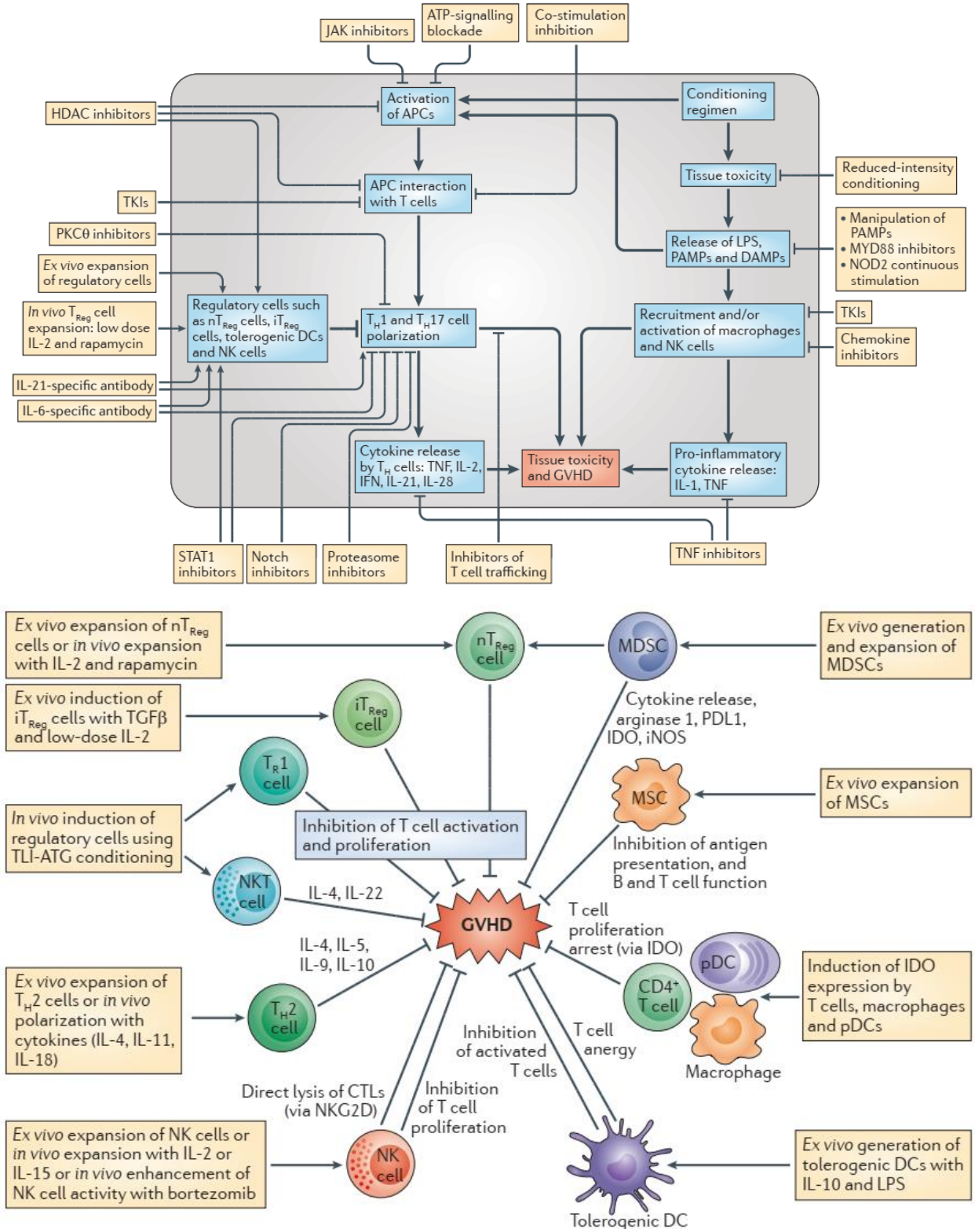

Figure 42. Different pathways using Treg in GvHD treatement

10.6. Treg and Cancer therapy

Tumour immune evasion is primarily facilitated by immunosuppressive regulatory T cells (Tregs). Research efforts are ongoing to enhance cancer immunotherapy by specifically targeting regulatory T cells (Tregs), particularly inside the tumour microenvironment (TME). Recent research has shown

that there is variation and adaptability among regulatory T cells (Tregs) inside tumours. This adds to the intricacy of understanding the function of Tregs in the immune response to tumours and their response to immunotherapy. The varied characteristics and abilities of Tregs inside a tumour may affect how they react to treatment and might provide opportunities to manipulate certain subgroups of Tregs.Treg instability may be triggered by disrupting three primary pathways (Figure 42): (i) T-cell receptor (TCR) signaling/costimulation, (ii) metabolism, and (iii) epigenetic alterations. These pathways function autonomously but can mutually affect one another. The CARD11/Bcl10/MALT1 (CBM) complex plays a crucial role in connecting TCR signalling to the activation of the nuclear factor κB (NF-κB) pathway. This complex supports the development of Treg phenotype, such as the overexpression of CTLA-4, and enhances their activity. Upregulation of the phosphatase and tensin homolog (PTEN) in regulatory T cells (Tregs) functions to suppress the TCR/CD28-driven phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway. This pathway, if not suppressed, would otherwise enhance glycolysis and hinder the metabolic fitness of Tregs. Hence, when the CBM complex or PTEN is inhibited, it results in the destabilisation of Treg function. In addition, the inhibition of CTLA-4 amplifies CD28 signalling and promotes the metabolic shift towards glycolysis, which leads to the destabilisation of regulatory T cells (Tregs). Moreover, studies have shown that agonist anti-OX40 and anti-glucocorticoid-induced tumor-necrosis-factor related protein (GITR) antibodies might cause Treg instability by promoting Treg costimulation. Metabolism: Treg instability can be caused by altering Treg metabolic pathways, shifting from oxidative phosphorylation (OXPHOS) to glycolysis. This can be achieved by inhibiting lactate, such as lactate dehydrogenase (LDH) or monocarboxylate transporter 1 (MCT1), blocking fatty acid uptake with CD36 antibodies, or reducing reactive oxygen species (ROS) with substances like N-acetylcysteine (N-AC). Tregs depend on a distinct epigenetic pattern to maintain their stability and function. This pattern involves various modifications to DNA, histones, and non-histone proteins, which are regulated by specific enzymes known as epigenetic modifiers. These enzymes include EZH2, histone deacetylases (HDACs), and histone acetyltransferases (HATs). These enzymes use metabolic intermediates to facilitate their activities. For instance, methylation is catalysed by S-adenosylmethionine (SAM), acetylation is facilitated by acetylcoenzyme A (acetyl-CoA), and SIRT-mediated histone deacetylation is driven by the redox intermediary NAD+. Therefore, the transition between oxidative phosphorylation (OXPHOS) and glycolysis might influence the function of these enzymes that modulate epigenetic markers in regulatory T cells (Tregs), thereby indirectly influencing their epigenetic profile. Furthermore, the use of drugs to block the activity of EZH2, class-I HDACs, and HATs, namely CREB binding protein (CREBBP)/p300, has been shown to enhance the destabilisation of Treg function. Manipulating the Treg epigenetic profile may influence the

expression of important Treg markers, such as immunological coreceptors on the cell surface, potentially altering TCR signalling.

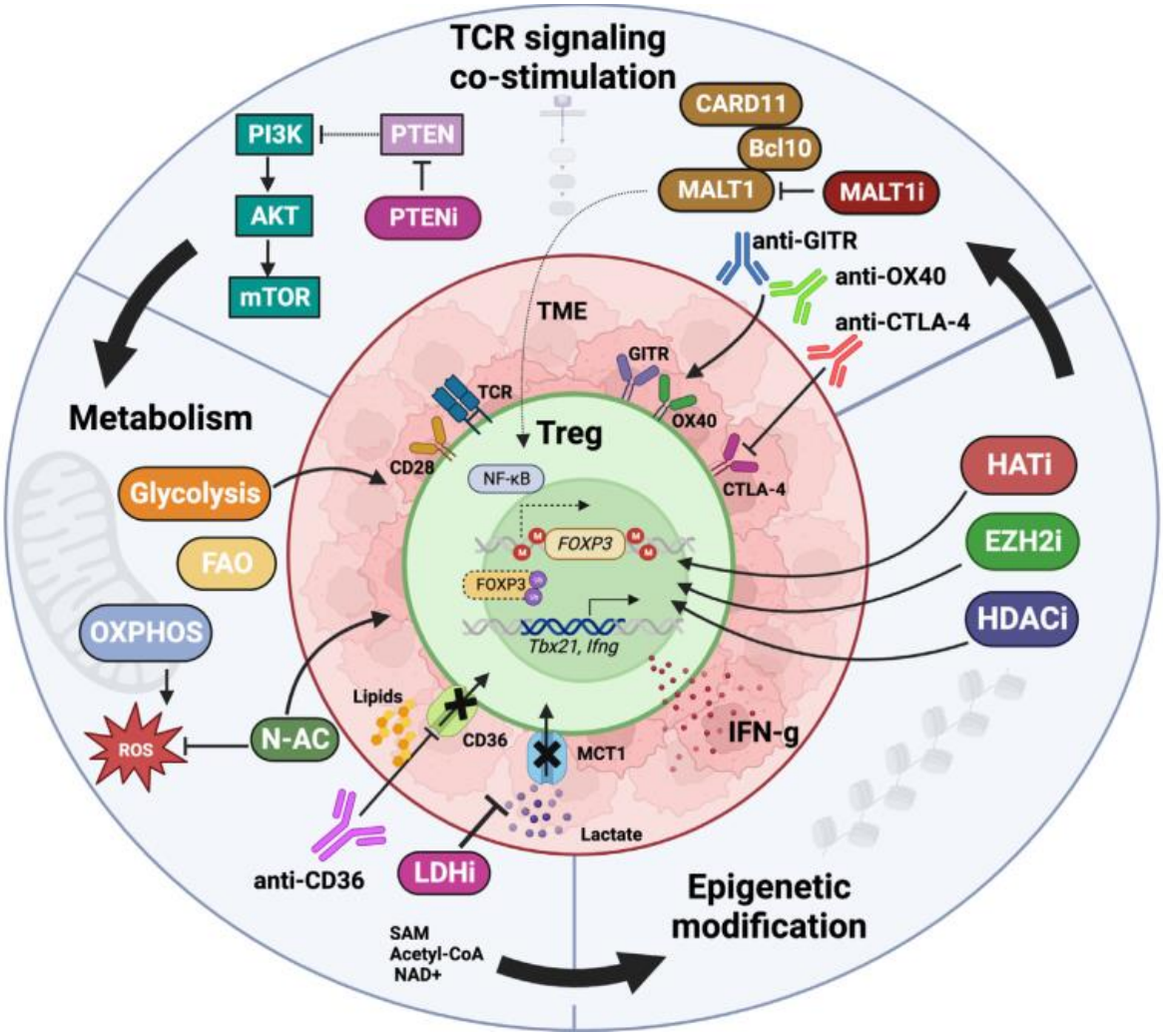

Figure 43. Pathways involved in cancer treatment using Treg Cells

11. Gene therapy

Gene therapy is the introduction of an operating gene, similar to a drug, into the cells of an organism to rectify a genetic aberration (mutation, change) that is the cause of a disease. In a broader sense, the phrase "gene therapy" covers the insertion or modification of DNA or RNA sequences in human cells to treat diseases.

11.1. The different strategies of gene therapy

Gene therapy can be considered using different approaches:

By replacing a deficient gene with its corresponding and functional complementary DNA, the aim is to correct a function defect by providing the missing protein. Some monogenic diseases could be treated this way. For example, cystic fibrosis (dysregulation of the CFTR protein, Cystic Fibrosis Conductance Transmembrane Regulator, due to a mutation), myopathies (mutation of the dystrophin gene for Duchenne muscular dystrophy) or genetic diseases of the immune system (SCID Severe Combined immunodeficiency).

Correction of a point mutation can be used in the case where it is not possible to add a functional gene to replace a defective gene. There is therefore an alternative approach which consists of carrying out a correction of the gene in situ, for example using RNA-DNA hybrid oligonucleotides (chimeraplasts) or single-stranded oligonucleotides.

Inhibition of the functioning of a mutated gene or inactivation of its protein product is used in cases where the provision of a functional gene is not sufficient. Indeed, when a mutation in a gene leads to a gain of function, the symptoms of the disease are linked to the synthesis of an abnormal protein with deleterious effects.

The introduction of a tumour suppressor gene leads to the death of the diseased cell. This technique is carried out by introducing an apoptotic gene or indirectly by sensitizing, for example, cancer cells to drugs: a gene is introduced into the cancer cells which codes for an enzyme activating an inactive drug administered to the patient, this activated drug then destroys the cancer cells.

The addition of a gene to the genome of a cell to produce a therapeutic protein of interest. For example, the expression of growth factors (in the case of cardiovascular diseases: VEGF), coagulation factors (blood factors VIII and IX for haemophilia), interleukins, erythropoietin (for β-thalassemia or anaemia), monoclonal antibodies.

11.2. Victors

In the context of gene therapy, vectors serve as carriers that transport genes into cells to treat diseases.

To claim optimal efficiency, the vector must have various characteristics such as:

- be specific to target cells
- without dissemination of the gene throughout the body
- be resistant to metabolic degradation and/or attacks by the immune system
- have as few side effects as possible
- be able to express sufficiently and in such a way that the expression of the gene of therapeutic interest is as long as necessary

Many methods of gene transfer already exist. They are classified into two categories: transfer by viral vectors, and by chemical or physical approaches qualified as “non-viral”.

11.2.1. Naked plasmid DNA

Plasmid naked DNA is a vector composed of circular DNA carrying a gene encoding a protein of interest. It has the advantage of being poorly immunogenic and therefore allows repeated administrations. It helps generate an essential immune response in the vaccination process. Naked DNA has the advantage of having no limit on the size of the therapeutic gene to be inserted, of being easy to produce and, above all, inexpensive.

However, it is not clear that an injection of DNA allows transfection of cells in vivo. However, the naked DNA injection technique also suffers from many drawbacks. Firstly, it has a low transfection efficiency, in muscle for example, this is generally 1 to 5% of total muscle fibres. On the other hand, transfected cells are limited to cells closest to the naked DNA injection site. Despite the positive results of gene transfer observed in the skin or muscle, the expression levels of the transgene remain low to produce a therapeutic effect, however, this expression remains sufficient for vaccine applications. Other studies have also demonstrated transgene expression after direct injection of naked DNA into numerous organs or tissues: the skin, joints, heart, brain, thyroid, liver, etc. The injected naked DNA is capable of sufficiently expressing an antigenic protein allowing the immune reaction. It is in this capacity that the principle of vaccination is based. Yet naked DNA, as a molecule, does not elicit a specific immune response. It is the presence of plasmids of bacterial sequences rich in unmethylated CpG motifs that promotes inflammatory and immune responses in vivo. The naked DNA vaccine is in the clinical trial phase in the context of different pathologies or diseases such as AIDS, malaria, hepatitis B, as well as various cancers. We thus note various phase I-II and even III clinical trials by direct injection of naked DNA or by ex vivo cell culture in cancer treatments. Very

recently in Brazil, a phase I-II clinical trial was carried out by injection of naked DNA coding for HIV type 1 to prevent infectious risks. A recent study proves that tattooing is the most effective way to administer new DNA vaccines, the use of which was limited due to the poor protection they provided when injected into muscles. Although the injection of naked DNA gives very encouraging results in gene transfer to the skin or muscle, the major problem is the degradation of naked plasmid DNA systemically (intravenous administration). It is degraded very quickly by enzymes and therefore has a very short plasma half-life. It is to increase transfection efficiency (in vitro or in vivo) that the administration of naked DNA is aided by physical techniques. In conclusion, naked plasmid DNA can be used in gene therapy treatments by intramuscular injection, but its applications remain very limited. To improve gene transfer, viral and non-viral vectors have been developed.

11.2.2. Viral vectors

Viruses represent the most evolved natural vectors for the transfer of foreign genetic information into a cell. They are effective tools for gene therapy in terms of intracellular penetration and possibly persistence of genetic material. These are therefore naturally the first vectors to have been used. Moreover, many viruses have been adapted into vectors: the most used are in particular retroviruses, adenoviruses and adenovirus-associated viruses (AAV). The natural viral cycle is divided into two phases, the infection phase and then the replication phase. Infection consists of the introduction of the viral genome into the cell. The expression of viral genes subsequently leads to the formation of new viral particles, this is the replication phase. Most gene therapy protocols have used this type of vector, which allows for easy and efficient transfection. However, wild viruses cannot be used directly for gene therapy experiments. Most of them are pathogenic and dangerous for humans. So, to become a vector, the virus must be modified by genetic engineering. Gene transfer by viral vectors is done as follows: the viruses are rendered incapable of replication by deletion of the sequences responsible for virulence and replication. The expression cassette containing a promoter and the gene of interest is inserted at these deletions (the maximum size of the expression cassette depends on the virus used). However, the virus retains the numerous structures that allow it to infect a cell (envelope proteins, fusogenic proteins, etc.). It therefore allows the production of the protein of therapeutic interest, but without the production of viral particles.

Viral vectors are classified into two large groups: those which integrate their genetic heritage into the DNA of the host cell (lentiviruses and retroviruses) and those which do not integrate it (adenoviral, adenoviral-associated and herpes viruses) (Table 6).

Table 6. Types of viral victor used in gene therapy

	Retrovirus	**Lentivirus**	**Adenovirus**	**AAV**	**HSV**
Virus type	RNA	RNA	dsDNA	ssDNA	dsDNA
Integration	Yes	Yes	No	Yes	No
Ability	≤ 8kb	≤ 8kb	≤ 8kb	≤ 5kb	≤ 40kb
Inflammatory potential	Weak	Weak	Strong	Weak	Strong
Benefits	Ex vivo therapy, low immunogenicity, long-term expression	Infection of quiescent cells, long-term expressions	Transfection efficiency, natural airway tropism, high titer production	Broad tropism, non-inflammatory, non-pathogenic	Large insert capacity, strong tropism for neurons
Limitations	Low in vivo transfection efficiency, production problem, mutational insertion	Pathogen, production problem, mutational insertion	Immunogenic, inflammatory (capsid), medium stability	Low insert capacity, production problem, cytopathic effect	Transient expression in cells (other than neurons), inflammatory, immunogenic

Retroviruses

Retroviruses were the first viruses used in gene transfer for therapeutic purposes. These are viruses of the retroviridae family, with strong oncogenic power. They have an envelope, containing two linear molecules of single-stranded positive RNA. They have a specific enzyme, reverse transcriptase (TR), which allows the conversion of RNA into proviral DNA. The genome of retroviruses contains two regions called Long Terminal Repeat (LTR) separated by the gag, pol, and env genes coding respectively for structural proteins, polymerases and integrases as well as surface glycoproteins. When the retrovirus enters the cell, the viral DNA is transported into the nucleus of the host cell and then randomly inserted into the genome. Indeed, the random integration of the viral genome can cause mutagenesis leading to a cell cycle abnormality, and activate oncogenes as was the case during the treatment of 'bubble children', causing secondary leukaemia in these patients. For chromosomal incorporation to be effective, the retrovirus requires the host cell to replicate, which constitutes a significant disadvantage. However, certain cells such as cardiac myocytes and vascular cells are poorly able to proliferate. They also have the disadvantage of a low capacity for incorporating an exogenous agent (≤ 8 kb) and a lack of cellular specificity. Indeed, envelope proteins are capable of binding to numerous receptors carried by different types of cells. Retroviruses are rapidly inactivated by the complement system when injected systemically. This is why tests carried out in vivo showed reduced transfection efficiency. In contrast, their ex vivo use allows the transfer

of genes into cells such as lymphocytes and hematopoietic stem cells on which considerable clinical experience has been acquired (Fig; 44).

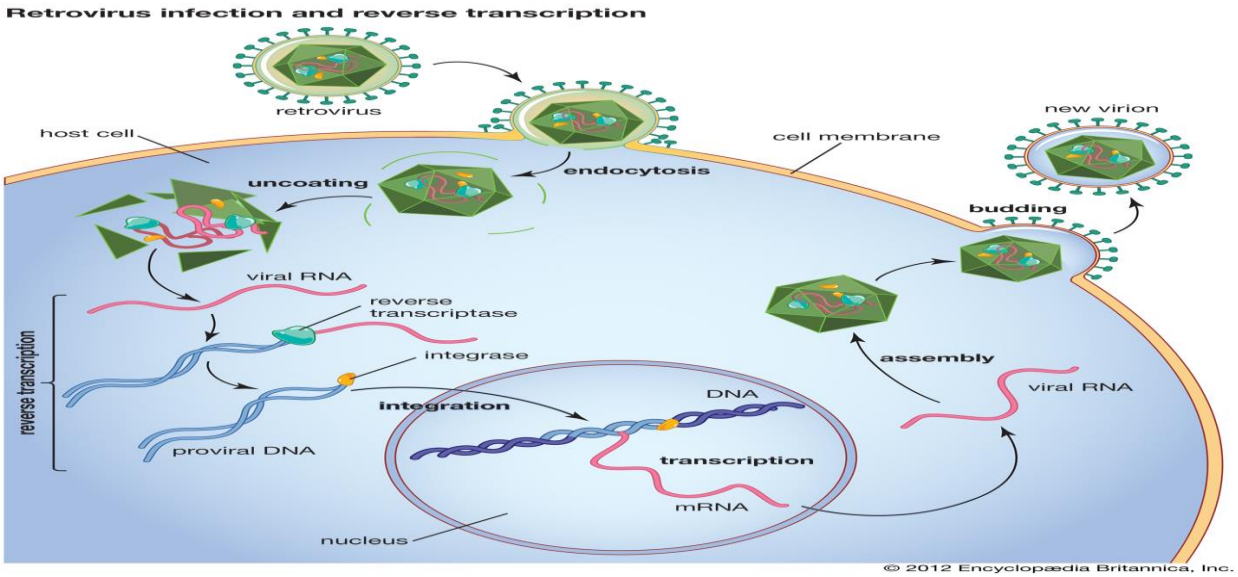

Figure 44. Retrovirus infection and reverse transcription

Among retroviruses, lentiviruses are particularly promising. They have the advantage of integrating into the genome of cells whether they are proliferating or not. Indeed, unlike other retroviruses, lentiviruses possess a nuclear localization signal allowing proviral DNA to penetrate the nucleus in the absence of mitosis. They are single-stranded RNA viruses derived from human immunodeficiency viruses (HIV). The genome of lentiviruses has, in addition to the basic genes of retroviruses, two regulatory genes, tat and rev which are essential for the expression of the genome as well as accessory genes. When the cell is infected, the RNA of the vector which contains the gene of interest is transcribed into DNA using reverse transcriptase. The DNA thus transcribed forms a preintegration complex with the accessory protein Vrp, the integrase enzyme and the protein encoded by the virus's gag gene. This complex contains a nuclear localization signal which allows it to pass the nuclear envelope. In the nucleus, DNA is inserted into the host genome using integrase. Studies have demonstrated the effectiveness of these vectors for direct application in vivo in different organs (brain, muscle, liver, lung), but the risk of insertional mutagenesis remains (Fig.45).

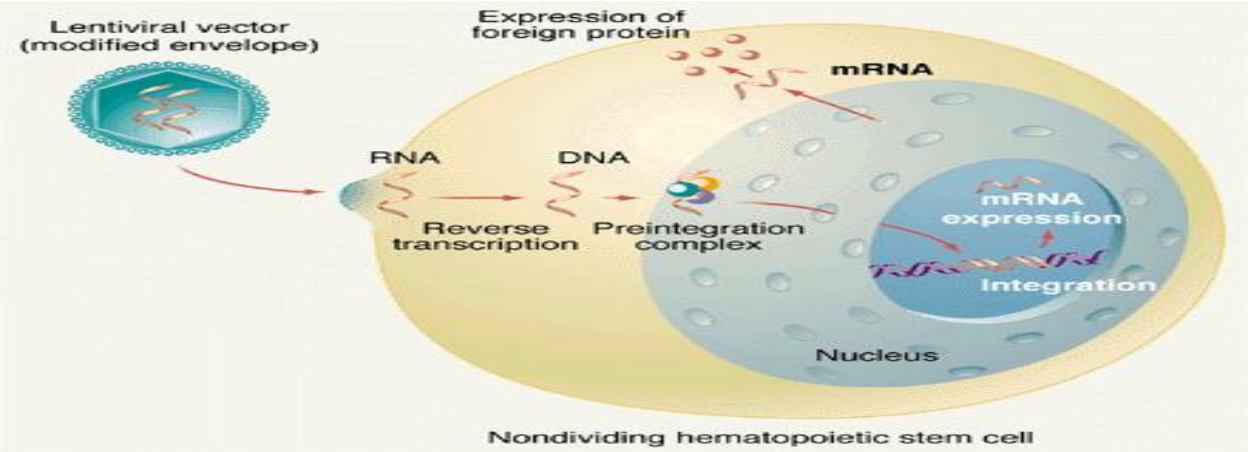

Figure 45. Lentivirus infection and reverse transcription

Adenoviruses

Adenoviruses are viruses from the Adenoviridae family. They are double-stranded linear DNA viruses containing a 36 kb genome limited by inverted terminal repeats (ITR). They are particularly suitable for gene therapy protocols because they replicate very efficiently and are easy to produce. They are responsible for mild infections in humans (conjunctivitis, gastroenteritis, etc.) and are not very pathogenic. The adenovirus infection cycle is divided into two phases: an earlier phase which concerns the entry of the virus into the host cell, the passage of the viral genome to the cell nucleus followed by the transcription of early genes and a later phase. late. After fixation and penetration by endocytosis into the cell, the viral genome migrates towards the nucleus but does not integrate into the genetic heritage of the host, causing only transient expression of the therapeutic gene (which is both an advantage and a disadvantage), but avoiding the risks of mutagenesis. Furthermore, these viruses are capable of infecting many types of quiescent or dividing cells and have a natural tropism for the upper airways (Fig. 46).

The major drawback to the use of these vectors is linked to the strong immunogenic reaction of the host (due to the residual expression of viral proteins), which develops anti-adenovirus antibodies thus preventing repeated administration of these vectors. The other limitation lies in the low capacity for incorporating an exogenous material (≤ 8kb). Recently, new adenoviruses have been developed (Ad.gutless). These are adenoviruses which have been deprived of all the viral genetic sequences, which saved space. These viruses therefore have an incorporation capacity of up to 35kb. But also, the immune response is greatly reduced in vivo. Being devoid of any viral sequence, “gutless” Adenoviruses require the provision of viral proteins by a “helper” virus.

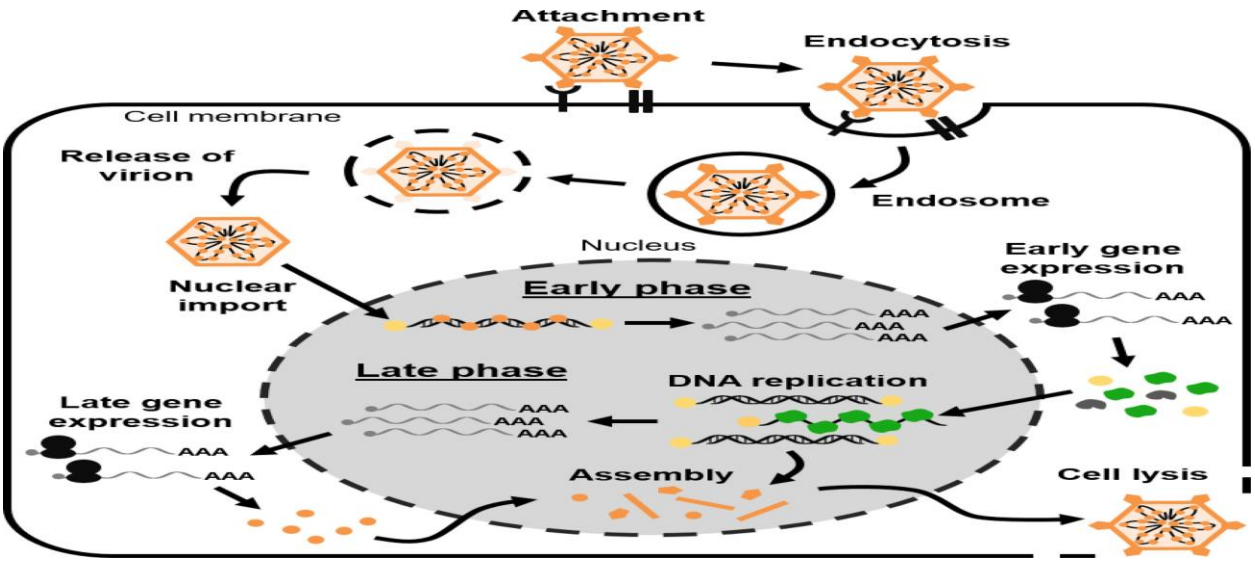

Figure 46. Adenovirus infection and replication

Adenovirus-associated viruses (AAV)

Adeno Adeno-associated virus (AAV) vectors come from the non-enveloped human parvovirus family with a capsid. These are viruses that require another virus (adenovirus or HSV) to complete their replication cycle. They contain two genes that can produce several polypeptides: rep for viral replication and cap for packaging. These two genes are surrounded by ITR (inverted terminal repeat) regions. These are non-pathogenic viruses for humans. The advantage of these viruses is their ability to integrate their genetic heritage into the host cell, allowing prolonged expression of the gene. Furthermore, they are capable of infecting both dividing and quiescent cells. In addition, they have good transfection efficiency in vivo and can transfect different organs (muscles, lungs, liver, etc.). Indeed, in vivo tests concerning treatments for cystic fibrosis, haemophilia and muscular dystrophy have shown expression of the transgene. Although production and purification protocols have been improved over the years, such vectors are difficult to produce in sufficient quantities to constitute clinical batches. They also have a major disadvantage, their storage capacity for exogenous genetic material is limited to 5kb (Fig. 47).

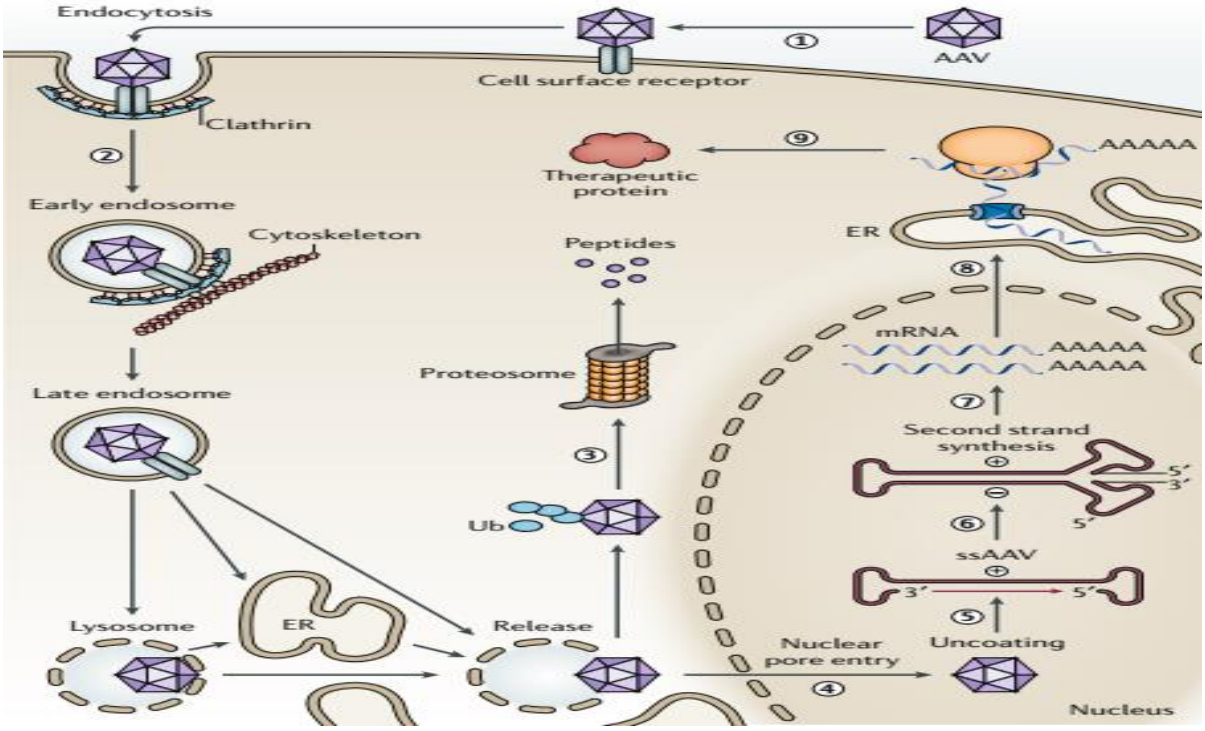

Figure 47. AAV and gene delivery to target cell

Herpes virus simplex (HSV)

Herpes Simplex virus type 1 (HSV-1) is the most commonly used herpes virus in gene therapy. It is a double-stranded linear DNA virus, which infects quiescent neuronal cells. However, it remains not very pathogenic for humans because it is present in 80% of the population. The virus fuses with the membrane of a neuron and is then transported into the nucleus. Indeed, its neurotropism is particularly attractive: HSV vectors offer hope for gene transfer in neuronal diseases such as Parkinson's or Alzheimer's diseases. Amplicons are vectors derived from the HSV-1 virus, identical to the wild HSV-1 virus structurally and immunologically, which contain a plasmid called an amplicon in place of the viral genome (Fig. 48). They have numerous advantages such as the capacity to integrate a transgene of approximately 150kb; to form multiple copies of the transgene in a cell; the ability to infect different cell types including dendritic cells; have limited toxicity due to the low presence of viral genomic sequence. Recent preclinical studies on HSV-1 vectors have made it possible to design vaccines for the treatment of different pathologies. However, efforts still need to be made to produce vaccines in sufficient quantities for the clinical phases. In conclusion, viral vectors have many advantages for gene transfer and their success in gene therapy protocols relies on their significant in vivo transfection potential. However, they suffer from severe disadvantages:

- integration of a large transgene is often very difficult
- certain viruses cause immune reactions

- others may pose toxicity problems or become pathogenic again after mutagenesis or recombination with an infectious parent virus
- finally, their handling and the production of clinical batches are complicated and expensive.

These problems have prompted researchers to develop safer, non-viral gene transfer vectors.

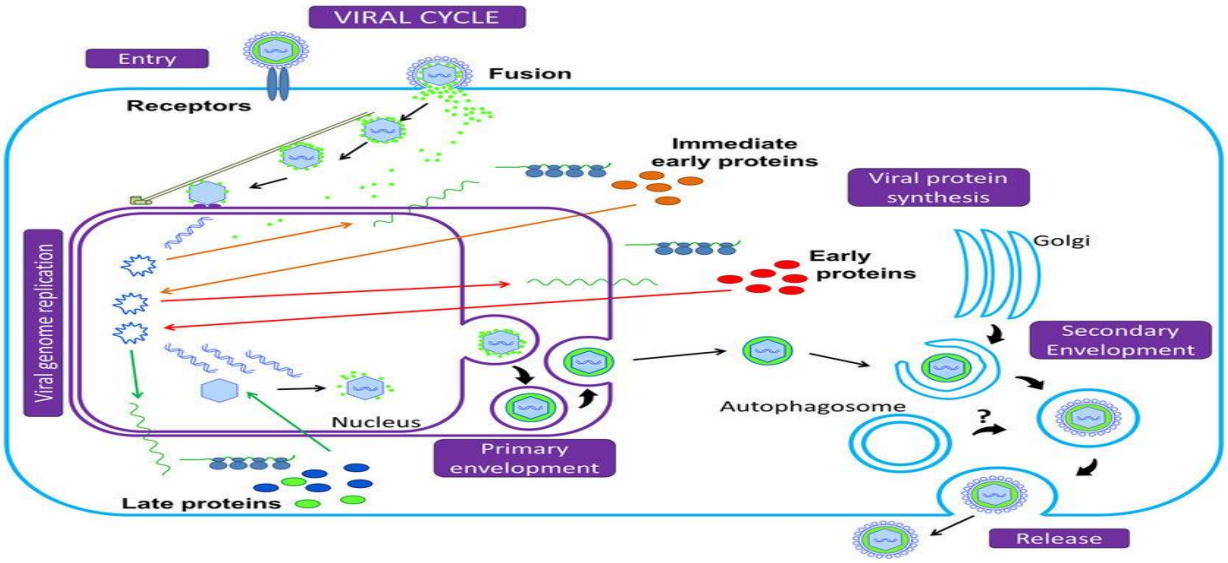

Figure 48. HSV cell' infection

11.2.3. Uses of retrovirus and Lentivirus in treatment of GvHD

Retrovirus and lentivirus are distinct categories of viral vectors employed for gene delivery into cells. Through the utilisation of these vectors, it is possible to genetically alter donor T cells in order to express certain receptors or molecules that can regulate their function and specificity. As an illustration, T cells from a donor can be modified to produce chimeric antigen receptors (CARs) that have the ability to identify antigens associated with tumours and trigger cell death. Alternatively, T lymphocytes from donors can be genetically altered to produce molecules that inhibit their activation or growth when exposed to alloantigens, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) or interleukin-10 (IL-10). These treatments aim to optimise the graft-versus-tumor (GVT) effect while minimising the danger of graft-versus-host disease (GVHD).

Several preclinical and clinical studies have examined the use of retroviral gene therapy in the context of GVHD. In a pre-mouse model of GVHD, researchers demonstrated the ability of retroviral gene transfer of regulatory stem cells (Tregs) to prevent GVHD progression while preserving the graft versus leukemia (GVL) effect.

Multiple preclinical and clinical investigations have demonstrated encouraging outcomes in utilising retrovirus and lentivirus for the alteration of donor T cells in order to prevent and treat GVHD. An example of this is a phase I/II experiment which found that the infusion of donor T cells that had been modified with a retroviral vector containing a suicide gene (inducible caspase 9) following hematopoietic stem cell transplantation (HSCT) was both safe and successful in managing graft-versus-host disease (GVHD) and enhancing the recovery of the immune system. A separate phase I experiment exhibited the feasibility of infusing donor T cells that were genetically modified with a lentiviral vector containing a chimeric antigen receptor (CAR) that targets CD19, following hematopoietic stem cell transplantation (HSCT). This treatment resulted in long-lasting remissions in patients with relapsed or refractory B-cell malignancies, without any significant graft-versus-host disease (GVHD) being detected.

Despite the challenges, the potential of retroviral gene therapy in the treatment of GVHD warrants continued research and development. Future directions in this area could be to refine gene editing technologies such as CRISPR/Cas9 to increase the accuracy and safety of retroviral gene transfer and furthermore, integration strategies such as retroviral gene therapy using immunomodulatory agents may provide synergistic benefits in the management of GVHD

11.2.4. Therapeutic Applications of Adenoviruses

Adenoviruses, which are a type of double-stranded DNA viruses classified under the Adenoviridae family, have been intensively researched due to their wide range of therapeutic uses. Originally acknowledged for their involvement in respiratory diseases, adenoviruses have now become useful instruments in the domains of gene therapy, immunisation, and oncolytic virotherapy. This essay examines the diverse therapeutic uses of adenoviruses, investigating their distinctive characteristics, improvements in vector design, and breakthroughs in clinical applications.

Gene therapy:

Adenoviruses are commonly used in gene therapy because they have a high capacity to effectively transport genetic information to different types of cells. Therapeutic vectors commonly remove the E1 region of the adenoviral genome, which is crucial for viral replication. This deletion makes the vectors unable to replicate, thereby improving their safety. Adenoviruses possess a significant ability to clone and accommodate substantial therapeutic genes, rendering them well-suited for the treatment of several genetic illnesses.

Cystic Fibrosis Gene Therapy:

An important use of gene therapy is in the management of cystic fibrosis, a hereditary condition marked by impaired functioning of the respiratory and digestive systems. Adenoviral vectors have

been employed to transport functional replicas of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to the airway epithelium. Adenoviral gene therapy has shown promise in clinical trials for alleviating symptoms and enhancing lung function in persons with cystic fibrosis.

Cardiovascular gene therapy

has demonstrated potential in using adenoviruses. Clinical trials have investigated the use of these genes for delivering therapeutic effects in diseases including peripheral artery disease and myocardial ischemia. Adenoviral vectors seek to enhance overall heart function by adding genes that encode angiogenic factors, which encourage the creation of blood vessels and promote tissue revascularization.

Immunisation:

Adenoviruses are useful tools for creating vaccines because they can stimulate strong immune responses. Replication-deficient adenoviral vectors are designed to produce certain antigens, rendering them optimal candidates for inducing both humoral and cellular immune reactions.

COVID-19 Vaccine Development:

Given the current worldwide situation, adenoviruses have become increasingly important in the creation of COVID-19 vaccinations. The Oxford-AstraZeneca and Johnson & Johnson's Janssen COVID-19 vaccines employ adenoviral vectors to transport the spike protein gene of the SARS-CoV-2 virus. The efficacy of these vaccines in reducing severe illness and hospitalisation related to COVID-19 has been proven to be high.

Cancer Vaccines:

Adenoviral vectors are now under investigation for their potential use in the creation of cancer vaccines. Through the expression of tumor-associated antigens, these vectors are capable of activating the immune system to identify and initiate an immunological reaction against cancerous cells. Ongoing clinical trials are evaluating the safety and effectiveness of adenovirus-based cancer vaccines in different types of malignancies.

Oncolytic virotherapy:

Oncolytic virotherapy has been made possible by the intrinsic capacity of adenoviruses to specifically reproduce in and destroy cancer cells. Adenoviral vectors are designed to specifically reproduce in cancerous cells while avoiding harm to healthy tissues, making them highly effective tools for treating cancer.

Prostate Cancer Treatment:

Adenoviral vectors have been used to specifically transport therapeutic genes to cancer cells in the case of prostate cancer. As an illustration, clinical experiments have utilised a replication-competent adenovirus that carries the gene for herpes simplex virus thymidine kinase (HSV-TK). Upon the administration of the prodrug ganciclovir, the cancer cells infected with the virus suffer specific and directed cell death.

Glioblastoma Therapy:

Adenoviruses are currently under investigation as a potential treatment for glioblastoma, an extremely aggressive form of brain tumour. Researchers endeavour to enhance the prognosis of patients with this difficult malignancy by either introducing therapeutic genes or utilising the virus's natural ability to destroy cancer cells.

Obstacles and Prospects for the Future:

Although adenoviruses possess significant therapeutic promise, there are still obstacles that need to be overcome. The efficacy of repeated injections may be hindered by immune responses directed towards the viral vector. Scientists are currently investigating methods to reduce these immunological reactions, which involve creating other types of adenoviral vectors, such as those derived from chimpanzees.

Moreover, improvements in vector design, such as the integration of tissue-specific promoters and enhancers, strive to increase the selectivity of adenoviral vectors for certain tissues. Implementing this approach might reduce unintended impacts and enhance the safety characteristics of adenovirus-derived treatments.

Adenoviruses have transitioned from being predominantly recognised as respiratory infections to being potent instruments in the field of therapeutic applications. Due to their ability to be used in gene delivery, immunisation, and oncolytic virotherapy, they are currently leading the way in medical research and clinical application. Adenoviruses have shown great promise in various medical applications, such as addressing genetic problems, battling infectious diseases, and treating cancer, thereby playing a significant role in determining the future of medicine.

To fully harness the therapeutic capabilities of adenoviruses, it is imperative to address the obstacles associated with immune responses and vector design as research advances. The therapeutic scope of adenoviruses is expected to broaden through ongoing clinical trials and innovative methodologies, providing renewed optimism for patients with various medical diseases.

11.2.5. Treatment of Uveitis autoimmune with Adeno Associated virus

Uveitis, a collection of inflammatory disorders that impact the uvea, is a disease that endangers vision and presents substantial difficulties for both ophthalmologists and patients. Conventional therapies for uveitis frequently entail the use of immunosuppressive drugs and corticosteroids, however these methods may be subject to restrictions and adverse reactions. Gene therapy has become a viable approach for generating precise and new treatments for uveitis in recent years. Adeno-Associated Virus (AAV) has become a notable gene delivery technology due to its safety and effectiveness in transporting therapeutic genes. This essay examines the capacity of AAV-mediated gene therapy to treat uveitis.

Uveitis refers to many inflammatory disorders that occur within the eye and specifically impact the iris, ciliary body, and choroid. It may be linked to autoimmune illnesses, infections, or happen spontaneously without a known cause. Inadequate management of chronic inflammation in the uvea can result in problems such as macular edoema, cataracts, glaucoma, and potential vision loss. The current approach to treatment frequently entails the use of corticosteroids, immunosuppressive medications, or biologics, either systemically or locally. However, these treatments can have adverse effects and may not consistently give long-lasting relief in certain instances.

Adeno-Associated Virus (AAV), a virus that does not cause disease and cannot replicate, has become a preferred tool for gene therapy because of its outstanding safety record and its potential to provide long-lasting expression of genes in cells that do not divide, such as those found in the eye. AAV-mediated gene therapy shows potential as a focused and long-lasting method to modify the immune response and reduce inflammation in uveitis.

Gene therapy enables the targeted administration of therapeutic genes to the afflicted tissues, providing a localised and precise treatment approach. AAV vectors can be modified to transport genes that encode anti-inflammatory cytokines or other immunomodulatory substances in the context of uveitis. For instance, the incorporation of genes that produce interleukin-10 (IL-10) or tumour necrosis factor receptor-Fc fusion proteins might effectively inhibit inflammatory reactions in the eye, hence reducing the advancement of uveitis.

When uveitis is linked to autoimmune illnesses, AAV-mediated gene therapy can be developed to specifically address and control abnormal immune reactions. Introducing genes that encode regulatory T cells (Tregs) or other immune checkpoint molecules can facilitate immunological tolerance, hence inhibiting an exaggerated immune response against ocular tissues.

AAV-mediated gene therapy has the benefit of long-lasting and targeted administration of drugs. Conventional therapies frequently include many injections or the use of drugs that affect the entire body, potentially resulting in systemic side effects. AAV vectors can be modified to produce

therapeutic proteins in the eye, so lowering the necessity for repeated treatments and minimising the amount of the drug that enters the bloodstream.

Although the potential of AAV-mediated gene therapy for uveitis is promising, there are various obstacles that must be overcome. The effectiveness of the therapy can be hindered by the immune reaction directed towards the AAV vector. Therefore, researchers are investigating methods to avoid immune detection. Furthermore, it is crucial to prioritise the enhancement of vector design, guarantee accurate tissue targeting, and assess the long-term safety while developing AAV-mediated gene treatments for uveitis.

Multiple preclinical investigations and early-phase clinical trials are currently in progress to assess the safety and effectiveness of AAV-mediated gene therapy for uveitis. The purpose of these studies is to evaluate the practicality of this strategy, detect possible adverse reactions, and improve the therapeutic procedures. As the research advances, it is expected that gene therapy will become a crucial component of the uveitis treatment arsenal, providing personalised and precise solutions for patients.

AAV-mediated gene therapy shows great potential in the field of ophthalmic research for treating uveitis. Researchers and doctors are utilising AAV vectors to deliver therapeutic genes directly to damaged tissues. This approach allows for the exploration of novel methods to modulate immune responses, regulate inflammation, and give long-lasting treatment for patients with uveitis. Despite the existence of obstacles, the continuous progress in vector design, immunomodulation, and targeted drug delivery instill optimism for a future in which gene therapy will have a crucial impact on the management of uveitis. This will lead to improved outcomes and the preservation of vision for individuals affected by this intricate and potentially sight-threatening condition.

12. Poly Cationic vectors (Non-Viral Vectors)

The first non-viral vectors emerged in the 1980s. Currently, the use of these vectors represents around 10% of gene therapy trials. The development of synthetic vectors responds to certain limitations of viral vectors. Firstly, they have less toxicity and do not lead to the activation of an immune reaction. Secondly, they have advantages (Table 7) on a technical level since the size of the genetic material used is not limited, their production is generally simple and finally, these techniques are generally very reproducible.

Table 7: Advantages/Disadvantages of viral and non-viral vectors.

Vectors	**Benefits**	**Disadvantages**
Viral	Efficient DNA transfer Variable size DNA	Immunogen Mutagen integration possibility of recombination Not easy to produce and store
Non-viral	Non-immunogenic Safe. Easy production and storage	Inefficient transfer Weak and random integration into the genome

Just like viral vectors, they must also be suitable for future use in vivo and more particularly in humans. Generally speaking, DNA condensation in stable assemblies is carried out by a simple cooperativity effect using polycationic materials. Synthetic vectors are most often composed of cationic polymers, their charge being conferred by amine groups, which allow them to interact with the phosphate groups of DNA by electrostatic forces. These interactions will lead to the condensation of the DNA into structures of variable size and charge allowing its protection against nucleases by limiting the access of the latter to the DNA. The physicochemical characteristics of these structures depend on the nature of the polymer, its molecular mass and its proportion relative to the DNA. The stability of these complexes is dictated by the nature of the polymer. In general, these tend to aggregate over time or when their concentration increases. Complexes are generally prepared with an excess of polymer which results in a positively charged particle. The charge of these complexes allows interaction with anionic molecules on the cell surface, notably proteoglycans, and their endocytosis. More recently, the use of nanoparticles or microparticles allows the encapsulation of DNA inside a matrix or the adsorption of it on the surface. This gives them relative protection against DNAases.

12.1. Lipoplexes

Since their first use, formulations based on cationic lipids have continued to multiply to currently become the most studied alternative to the use of viral vectors. Cationic lipids form supramolecular assemblies with DNA called lipoplexes by electrostatic interactions which allow very efficient gene transfer. Amphiphilic cationic lipids are made up of 3 domains: a cationic head allowing binding to DNA, one or more hydrophobic chains, and a spacer separating these two elements (Figure 49). In an aqueous solution, the hydrophobic chains associate with each other to form particles (micelles or liposomes). The most stable organization of these amphiphilic molecules is that which makes it possible to minimize the interactions between the hydrophobic components and the water molecules. Many commercial transfection kits, based on the use of this type of vector complex, are available today. The first cationic lipid described is 2,3-Dioleoylloxypropyl-1-trimethyl ammonium bromide (DOTMA). In order to obtain better biodegradability and reduce the toxicity of DOTMA, the ether bond was replaced by an ester bond to obtain 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP). More recently, cationic lipids are complexed with siRNA and tested on breast cells. These include structural analogues 1,2-Dioleoyl-3- trimethylammonium-propane DOTAP, dioctadecylamido-glycylspermine (DOGS or transfectam), N-methyl-4-(dioleyl)methylpyridinium (SAINT-2), cationic lipids containing cholesterol, or lipopolyamines whose cationic head is generally derived from spermine (Figure 6). DNA formulated with cationic lipids, such as DOTMA, and a neutral phospholipid (most commonly dioleoylphosphatidylethanolamine or DOPE), forms lipoplexes capable of transfecting cells with high efficiency in vitro, and in vivo. Since the demonstration of the transfecting efficiency of particles composed of DNA and DOTMA/DOPE, a wide variety of cationic lipids have been synthesized and tested, with the aim of increasing the transfection efficiency in the absence as well as in the presence of serum, reduce the cytotoxicity of formulations and improve the bioavailability of DNA-lipid particles. Different types of self-assembly are possible depending on the lipid concentration, and the temperature but also depending on the geometric shape of the molecules considered (Fig. 50).

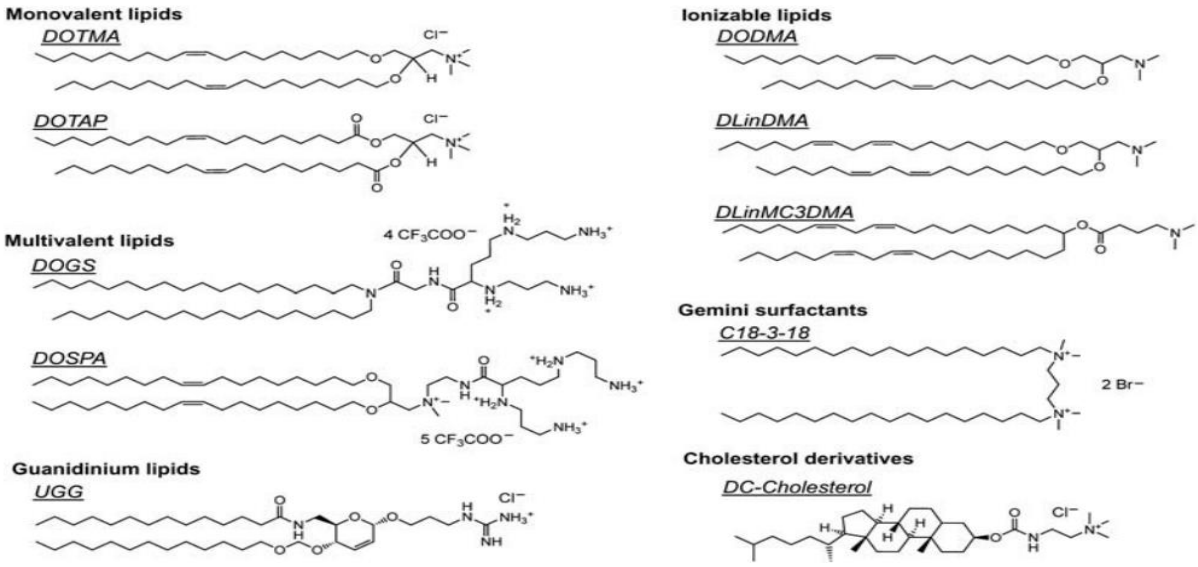

Figure 49. Different forms of lipoplexes used as vectors.

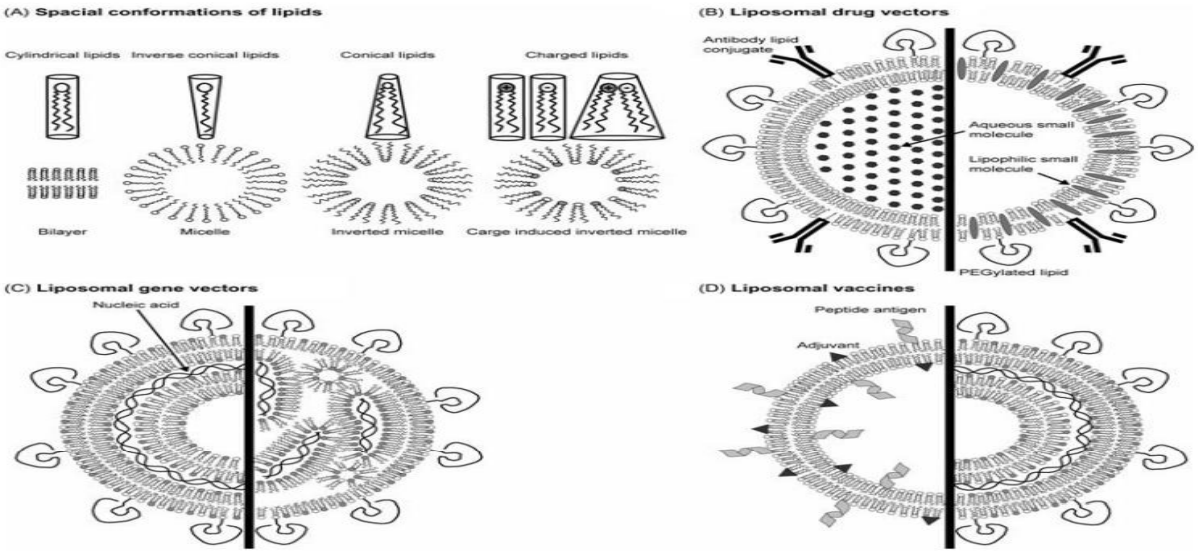

Figure 50. Examples of possible assemblies for amphiphilic molecules in an aqueous solven

Liposomes are the most used structures among those presented in Figure 50. They can be classified according to their size and the number of bilayers. There are multilamellar liposomes (MLV), small unilamellar liposomes (SUV), large unilamellar liposomes (LUV) and giant liposomes (GUV). The simplest method for preparing liposomes is to evaporate the organic solvent in which the lipids are dissolved and then resuspend them in an aqueous solvent. Additionally, like the lipid composition of liposomes, the bilayers can be changed, and the formulations can be customized to obtain desired characteristics (size, surface charge, morphology, etc.).

Although several attempts have been made to use neutral or negatively charged liposomes for gene transfer, the limited efficiency of plasmid DNA encapsulation and consequently low levels of transfection have encouraged researchers to focus on cationic liposomes, capable of complexing and

condensing DNA (Fig. 51). Cationic lipids have already been used to transfect nucleic acids in various *in vivo* gene transfer assays, particularly in the treatment of cancers and cystic fibrosis, but also for anti-inflammatory purposes and for the prevention of viral infections. There are so-called "helper" lipids (DOPE, cholesterol, etc.) to improve the capacity of cationic liposomes to transfect cells. Their "helper" role is to facilitate cytosolic release by destabilizing the endosomal membrane. The choice of "helper" lipids has a major impact on the structure and activity of liposomes. In vitro, studies have shown that liposomes composed of an equimolar mixture of DOPE and cationic lipids can have higher levels of transfection compared to those containing only cationic lipids alone. Cholesterol can be considered a "helper" lipid. Moreover, lipoplexes containing cholesterol showed higher levels of biological activity than lipoplexes formulated with the cationic lipid DOPE, when used in vivo.

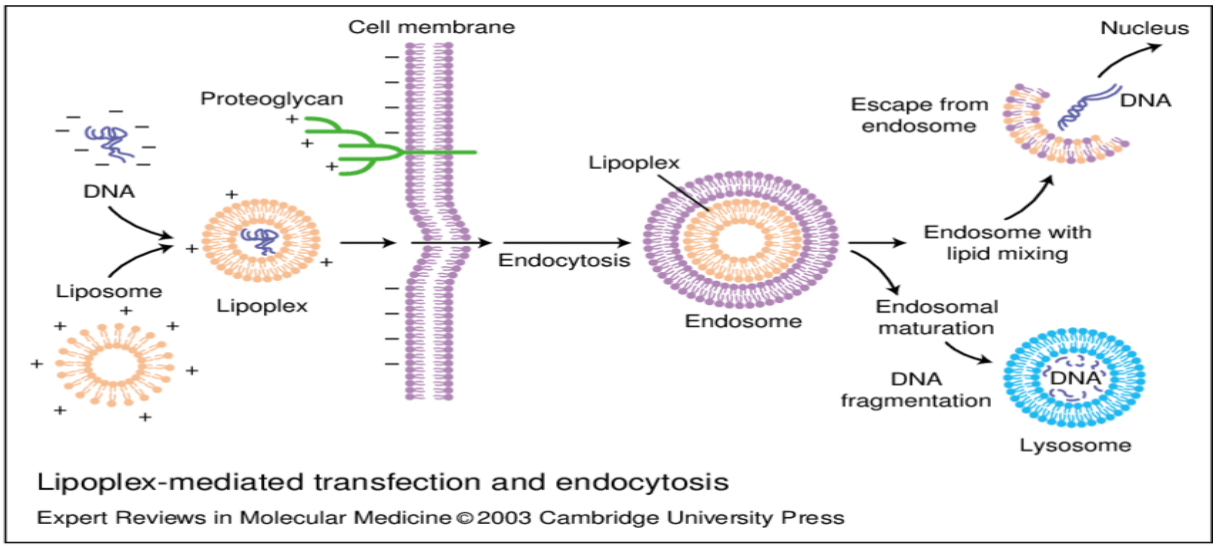

Figure 51. Lipoplex-mediated transfection and endocytosis

12.2. Polyplexes based on cationic polymers

A large number of synthetic polymers efficiently compact DNA, and their use is based on the simple observation of natural events: in eukaryotic cells, the condensation of chromosomal DNA is obtained by the use of proteins rich in lysine and arginine: histones. Likewise, in spermatozoa, it is another protein rich in arginine, and protamine, which allows maximum compaction of the genetic material. Cationic polymers, such as poly(L-lysine) (PLL), protamine, polyethyleneimine (PEI), or cationic dendrimers, associate with DNA through interactions with multiple electrostatics generating a cooperativity process, which generates particles called polyplexes.

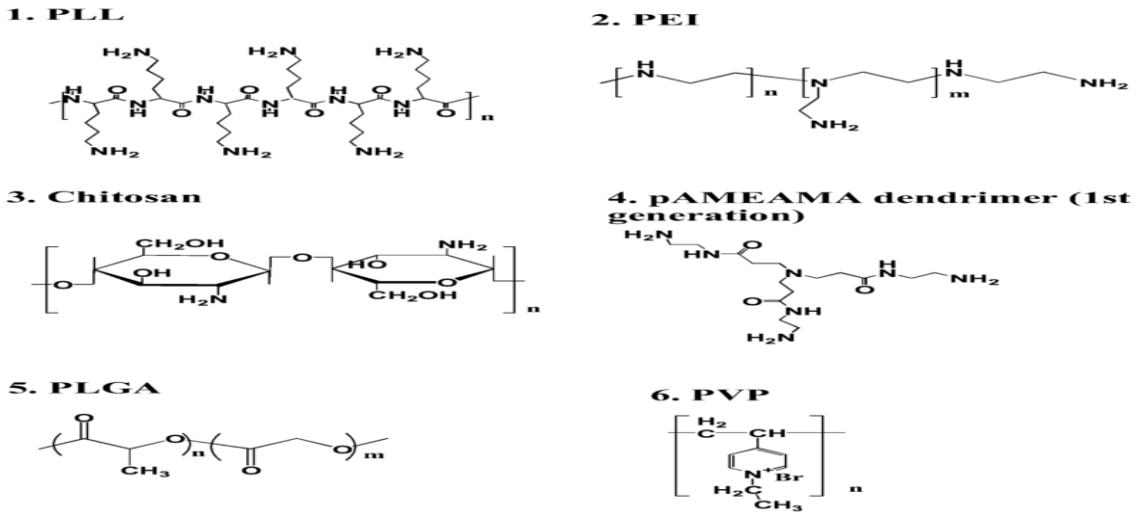

Figure 52. Different polymers used as vectors

12.3. Chitosan

Chitosan is a polysaccharide polymer of natural origin obtained by alkaline deacetylation of chitin extracted from the exoskeleton of crustaceans and insects. Biodegradable and non-toxic, it has low immunogenicity, i.e. three essential properties. It is made up of two subunits, D-glucosamine and N-acetyl-Dglucosamine linked by a glucosidic bond. (Figure 53).

. Chitosan can be characterized by its molar mass but also by its degree of acetylation which corresponds to the percentage of N-acetyl functions. As has been described in numerous reviews, the transfection efficiency of chitosan DNA complexes depends on many factors including, molecular weight, degree of deacetylation, N/P ratio, etc. The molecular weight and degree of deacetylation influence the size and surface potential of chitosan/DNA complexes.

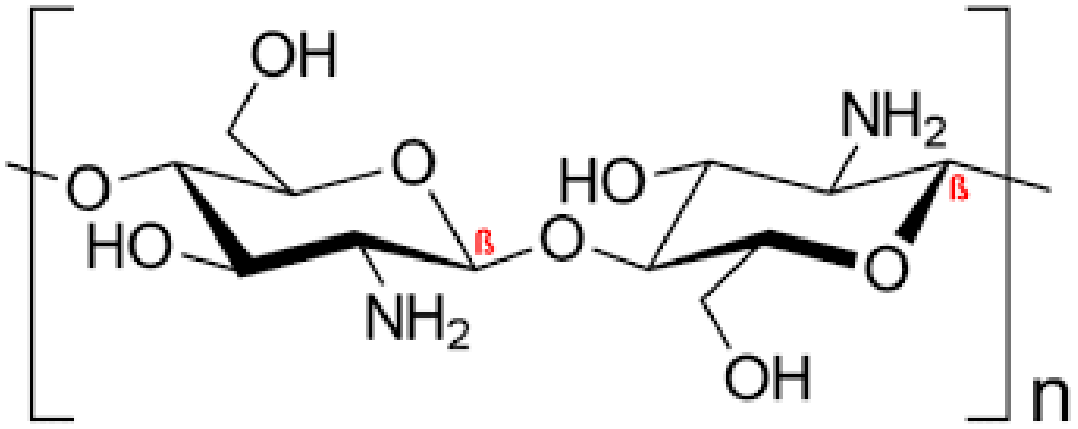

Figure 53. Structure of a chitosan polymer unit

Thus, the complexes have a size varying from 150 to 600 nm, 155 to 181 nm for chitosans of 213 to 48 kDa, beyond for chitosans of lower molecular weights. The same type of evolution is observed as a function of the deacetylation rate for the surface potential of the complexes.

However, no effect on DNA condensation is observed. From a ratio N/P=6, the DNA is completely complexed but the complexes are not very stable over time and we witness a release of the DNA in a few hours in PBS unless the complexes have been crosslinked. beforehand. These complexes exhibit good resistance to nucleases. But in this case, also this is correlated to the molecular weight of chitosan. There is a correlation between the endocytosis of the complexes, the transfection rate, the N/P ratio and the surface potential of the complex.

12.4. Peptides

A certain number of peptides (CPP, Cell Penetrating Peptide) or small proteins (PTD, Protein Transduction Domain), having the property of crossing the cytoplasmic membrane of eukaryotic cells, have been used to vectorize macromolecules including oligonucleotides. CPPs are peptides of 7 to 30 residues, generally divided into two classes: lysine-rich alpha-helical peptides and arginine-rich peptides. Some of these sequences derive from natural proteins such as surface proteins of viruses and bacteria (Table 8).

Table 8: Examples of peptide agents.

Translocation Agents	**Amino acid sequence**	**Origins**
Penetratine	RQIKIWFQNRRMKWKK	Antennapedia
Tat (48-60)	GRKKRRQRRRPPQC	HIV-1
Transportation	GWTLNSAGYLLKINLKALAALAKKIL	Galanin and Mastoparan
Amphiphilic peptide	KALKLALKALKAALKLA	Synthesis
Arg9	RRRRRRRRR	Synthesis

Today there is no consensus regarding the entry process of these CPPs into the cytosol and the nucleus. CPPs could enter the cell by passive mechanisms but also by endocytosis or macropinocytosis. These peptides allow the transport of small molecules but also nanoparticles and the type of associated molecules influences the entry route into the cell. The first CPPs used in transfection come from viral proteins such as haemagglutinin HA2 from the influenza virus, Tat (transcription transactivator) from the acquired immunodeficiency virus, or VP22 from the herpes simplex virus type 1. These peptides cannot complex DNA and are therefore generally associated with another type of transfection vector, cationic lipid or PEI. Very few studies on the transfection of plasmids with CPPs have been reported. Today, we are developing a new class that allows DNA

complexation and translocation within the cell. The first peptide of this type described is KALA (WEAKLAKALAKALAKHLAKALAKALKACEA). It effectively complexes DNA with its lysine and arginine residues and can interact with membranes. Jacobs et al synthesized vectors derived from JTS1 (GLFEALLELLESLWELLLEA) by substituting all glutamic acid residues with lysine or arginine residues. These ppTG1 and ppTG20 peptides are effective for transfection. The lysine residues are distributed on the same side of the alpha helix, which gives the peptide an amphipathic character. As the distance between two NH3+ groups is 7.11 Å, comparable to the distance between two phosphate groups in DNA (6.55 Å), the peptide can interact with DNA without affecting its secondary structure and therefore its activity. fusogenic. This is not the case for other peptides like KALA which assembles in several layers. Furthermore, these are the first peptides described as effective for in vivo transfection. After intravascular injection, the authors observed expression in the lungs. However, their effectiveness is currently 10 times lower than that of DOTAP and cholesterol formulations, but also 40 times lower than PEI under the same conditions. These peptides exist in the form of dendrimers which have an increased transfection capacity comparable to that of synthetic vectors such as Lipofectamine®.

12.4.1. Poly(L-Lysine) PLL

Many authors have been interested in the compacting role that positively charged amino acid polymers can have. Poly(L-lysine) (PLL) is one of the first cationic polymers used for gene transfer. It is a linear polypeptide whose subunit is lysine (Figure 54). It is a degradable polymer, which makes it interesting for in vivo use. PLL is generally characterized by its degree of polymerization or its molecular weight.

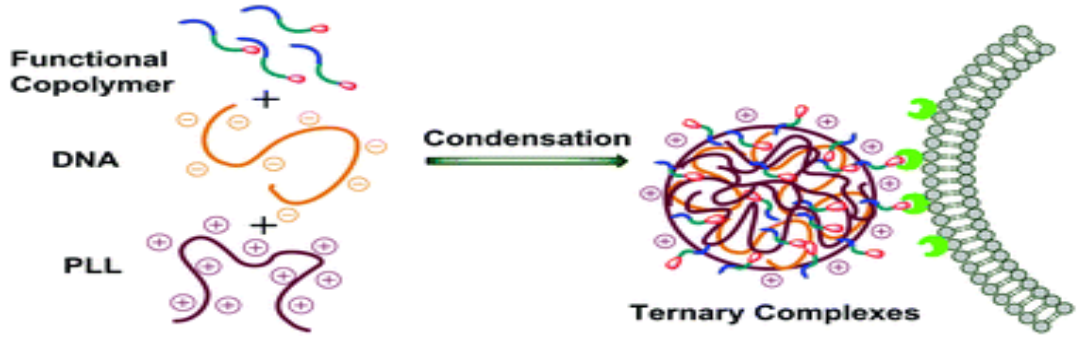

Figure 54. Poly-L-lysine is used as non viral vector.

In the presence of DNA, PLL can form complexes and enter cells. DNA condensation as well as transfection efficiency increases with the molecular weight of the PLL, but in this case, the cytotoxicity of these complexes also increases. This polymer, on the other hand, only has a weak buffering power, preventing effective exit from endosomes. Thus, the use of endosmotic agents such as chloroquine or fusogenic peptides is often necessary to obtain more efficient transfection. Without

modification, PLL/DNA complexes are not good candidates for in vivo transfection. In fact, the complexes aggregate when the salt level reaches a physiological threshold and the fixation of serum proteins leads to their rapid elimination from the circulation. However, various modifications make it possible to circumvent the disadvantages of this polymer. Solubility can be increased by grafting PEG but also contributes to resisting the immune response by extending the half-life of the complex in the blood. In addition, amphiphilic PLLs have lower toxicity and their transfection capacity is 30 times superior to traditional PLLs. The stability of glycosylated PLLs is also greater compared to conventional PLLs and their toxicity is less. Indeed, the addition of histidine residues or imidazole structures makes it possible to increase the transfection efficiency by promoting the escape of endosomes due to the proton pump activity of these residues. Inspired by this polymer, numerous peptide structures have been used such as polyornithine and polyarginine. These different compounds are less toxic and more effective for gene transfer.

12.5. Poly(Ethylenimine)

Polyethylenimine (PEI) is a polycationic compound known for more than fifty years and was previously used for the purification of drinking water, the extraction of minerals, in shampoos, etc. This polymer is a polyamine which goes from 20 to 45% protonation between pH 7 and pH 5 respectively. PEI is a vector with endosmotic power thanks to its “proton sponge” effect. This property allows it to release the polyplex into the cytoplasm without the DNA being degraded by lysosomal enzymes and makes it a good non-viral vector for gene transfer.

Different derivatives of PEI are available on the market (linear PEI of 22 or 25kDa, branched PEI, PEI coupled to biotinylated saccharide residues or to a fluorophore). PEI molecules can take linear or cross-linked (branched) shapes (Fig. 55). Linear and high molecular weight (10kDa) PEI appears to give better transfection rates in vitro and in vivo. It has been used as a transfection vector since 1995 in vitro and in vivo. It is available commercially in the form of transfection reagents using linear PEI (Exgen500®, jetPEI®). PEIs exist in a wide variety of molecular weights ranging from 1000 Da to 1.6 × 103 kDa but the PEIs used in the context of transfection generally have a molecular weight of between 5 and 25 kDa. Indeed, low molecular weights have lower cytotoxicity. Furthermore, studies testing PEIs of different structure and molecular weights have reported no major cytotoxicity at the PEI concentrations used for in vitro and in vivo transfection.

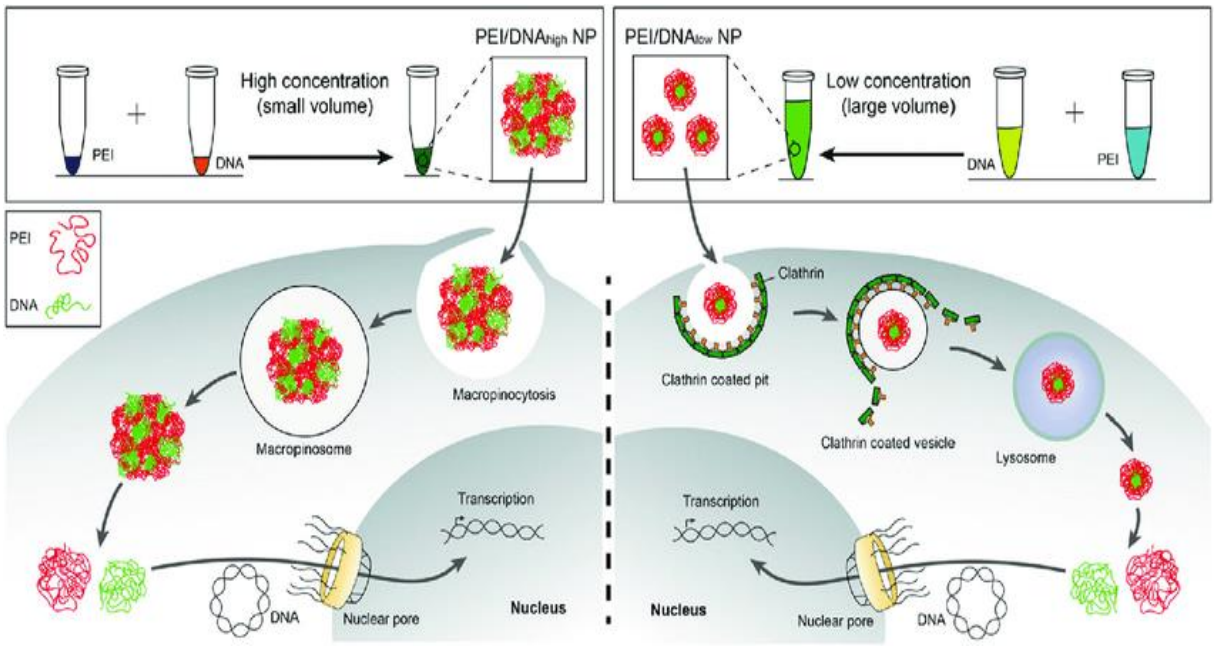

Figure 55. PEI's mechanism for the delivery of therapeutic gene

The transfer efficiency described in the literature varies from one cell line to another but in all cases, the expression levels are generally lower than those obtained with viral vectors. The efficiency of the vector depends on several parameters, that must be taken into account in order to obtain optimal efficiency. Figure 10: Chemical formula of branched and linear PEI.

PEI/DNA complexes have a spherical, globular or rod shape. Complexation occurs mainly by electrostatic interactions. The size of the polyplexes is variable (50 to 100 nm) and depends on external parameters such as the nature of the preparation medium, the nature of the PEI, the N/P ratio, etc. For efficient gene transfer, the overall charge of the PEI/DNA complex must be positive to promote interaction with the negatively charged cell membrane. An essential parameter to take into account when using a PEI derivative is the N/P charge ratio which corresponds to the ratio of the number of amine functions of the PEI (positive charges) to the number of phosphate molecules of the PEI. DNA (negative charges). This N/P ratio must be optimized in order to obtain the best transfection efficiency, for each vector and for each cell type.

During complexation, with N/P greater than 6 a large part of the PEI (86%) remains in the free state. The purification of the complexes leads to the reduction of cytotoxicity but also of the transfection efficiency because the free PEI, endocytosed at the same time as the complexes, would participate in the buffering effect leading to the release of the complexes into the cytoplasm. There is also a link between the size of DNA/PEI complexes and gene transfer efficiency. It seems that small polyplexes (40 nm) are less efficient. Different results were obtained by Bettinger et al. (1999) who achieved better transfection with less bulky DNA/PEI-galactosylated complexes. In vitro, large PEI/DNA particles (<200 nm) transfect cells very efficiently, regardless of the charge ratio used, but

in vivo would pose diffusion problems in biological fluids and tissues, therefore considerably restricting the area to be transfected. As for small particles (>100 nm), they are only effective in transfection if they are strongly positive. Their effectiveness can be increased in vitro:

* By facilitating contact of the complexes with the cells, for example by centrifugation.
* By reducing the volume of medium per well during transfection.
* By improving endocytosis (coupling with a ligand for recognition of complexes by a membrane receptor).
* By optimizing the cytoplasmic release of the gene.

On the other hand, these theoretical data are not necessarily transferable to experimental conditions. The nature of the medium in which the DNA/PEI complexes are prepared determines the stability and size of the polyplexes. The preparation media most often encountered are 5% glucose, 150 mM NaCl, and water. The 22 kDa PEI/DNA polyplexes prepared in 5% glucose have homogeneous sizes between 30 and 80 nm. These same complexes prepared in a 150 mM NaCl saline solution become more voluminous (of the order of a micrometer). Used at a ratio N/P=10, polyplexes based on 25 kDa PEI prepared in a 150 mM NaCl solution make it possible to form polyplexes of slightly larger sizes (50 to 100 nm), than those obtained with 22 kDa PEI in a glucose solution. In vivo in the brain of newborn mice and adult mice, the complexes formed from 22 kDa PEI showed good transfection efficiency and diffusion without apparent toxicity. Injected intravenously into mice at the kidney level, these same complexes (N/P=10, 100 µg of DNA) allow stronger expression of the transgene in different organs such as the myocardium, lungs, liver, spleen and kidneys. In an effort to improve transfection efficiency, modifications have been made to the PEI structure, such as PEGylation. The PEI-PEG copolymer creates a hydrophilicity on the surface which reduces interactions with plasma proteins, therefore protecting the DNA. Other groups have modified the amine function of PEI to improve transfection efficiency. Wang et al in 2002, showed in Jurkat-T cells a reduction in toxicity and an increase in transfection efficiency with cholesterol conjugated to PEI 1000 Da. PEIs do not have high targeting specificity. The latter can be improved by associating with the vector a molecule (glucose, protein, lipid) which will be recognized specifically by its receptor located at the level of the target cells. In a pharyngeal carcinoma line overexpressing proteoglycan receptors, the best transfer results were obtained with tetraglucosylated linear PEI. From another perspective, to mimic the adenoviral cell penetration system, peptides containing the RGD motif (sequence CYGGRGDTP) were grafted onto the 25kDa PEI via disulfide bridges. These peptides are recognized by integrins, receptors expressed on the surface of adherent cells. The PEI-peptide conjugate forms polyplexes of homogeneous size (50 to 100 nm) and allows a transfection efficiency of 10 to 100 times greater than that obtained with the 25 kDa PEI on cells expressing

integrins (Hela and MRC5). However, for in vivo gene transfer, this conjugated system is not very suitable as it also easily aggregates with many blood compounds.

12.6. Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides originating from the enzymatic degradation of starch. The three most common natural cyclodextrins consist of 6, 7 or 8 α-D-glucopyranose units in chair configuration linked together by α-1,4 bonds. They are respectively called α-, β- or γ-cyclodextrins. Their three-dimensional structure appears in the form of a truncated cone outside of which the hydroxyl groups are located. They have a non-polar and therefore hydrophobic internal cavity and a hydrophilic polar external surface (Fig. 56). Cyclodextrins have many properties, the most notable of which is to form inclusion complexes with small hydrophobic molecules. Cyclodextrins are biocompatible and are approved by the FDA as solubilizing agents, or excipients for certain drugs because they protect against enzymatic, physical or chemical degradation. In addition, they increase the membrane permeability of certain drugs.

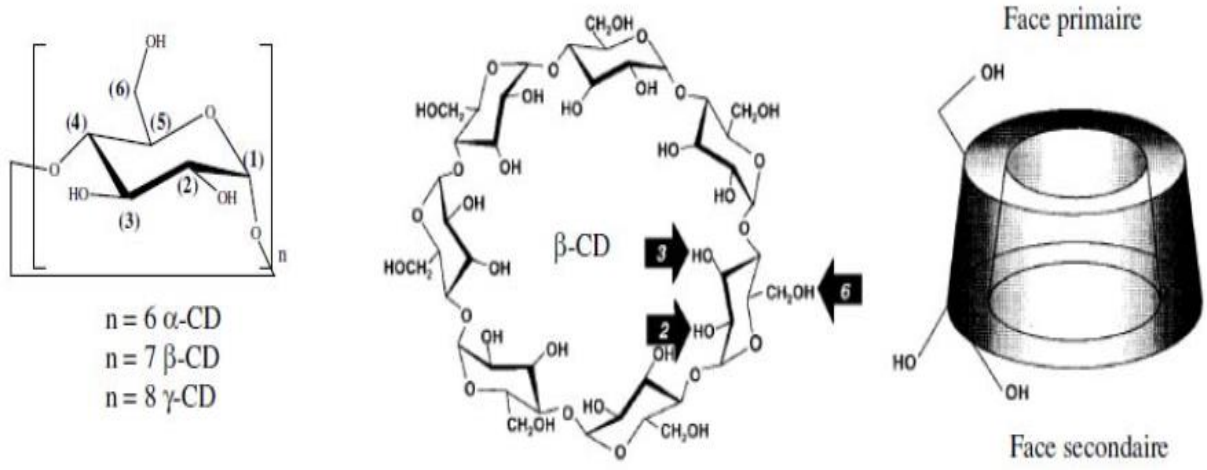

Figure 56. Representation of the chemical structure of natural cyclodextrins.

CDs have low immunogenicity, presenting numerous sites allowing the grafting of functions such as cationic functions capable of interacting with DNA or groups allowing cellular targeting. Neutral CDs can interact with nucleic acids and nucleotides but are unable to complex them. The grafting of amine groups allows the formation of cationic CDs which interact more strongly with nucleic acids. The CDs can therefore be used in transfection. Furthermore, the ability of certain CDs to destabilize membranes promotes the association and handling of oligonucleotides. Derivatives of cationic CDs demonstrated greater nucleotide binding capacity. 2,6-dimethyl-β-cyclodextrin associated with DNA allows transfection 180 times higher than that of naked DNA. Freeman et al. (1996), show that the use of β-cyclodextrin as an adjuvant in a lyophilized formulation of DOTAP and DNA complexes for intratracheal instillation in rats increases the transfection efficiency. Cationic

CDs also interact directly with DNA and form complexes of around a hundred nanometers that are efficient for transfection. Cryan et al. (1996), show that cyclodextrins of the heptakis (6-amino-6-deoxy)-β-cyclodextrin type have a transfection efficiency in the presence of serum equal to that of DOTAP. These complexes have a positive surface charge which allows them to interact with membrane proteoglycans. Transfection by these complexes is increased in the presence of chloroquine, which suggests that their entry into the cytoplasm occurs by endocytosis and not by destabilization of the membrane.

These molecules have also been used by grafting onto cationic polymers or dendrimers to reduce their toxicity. The grafting of CDs to PAMAM dendrimers made it possible to increase the transfection efficiency of these dendrimers in vitro and in vivo. But grafting CDs to cationic polymers provides the opportunity to simply associate another function with the complex. The grafting of low molecular weight PEI 600 Da with 2-hydroxypropyl-gamma-cyclodextrin showed interesting transfection rates in vitro and in vivo, and a reduction in toxicity. Many CD derivatives are synthesized to improve transfection efficiency and reduce cytotoxicity such as amphiphilic polycationic CDs (Fig. 57).

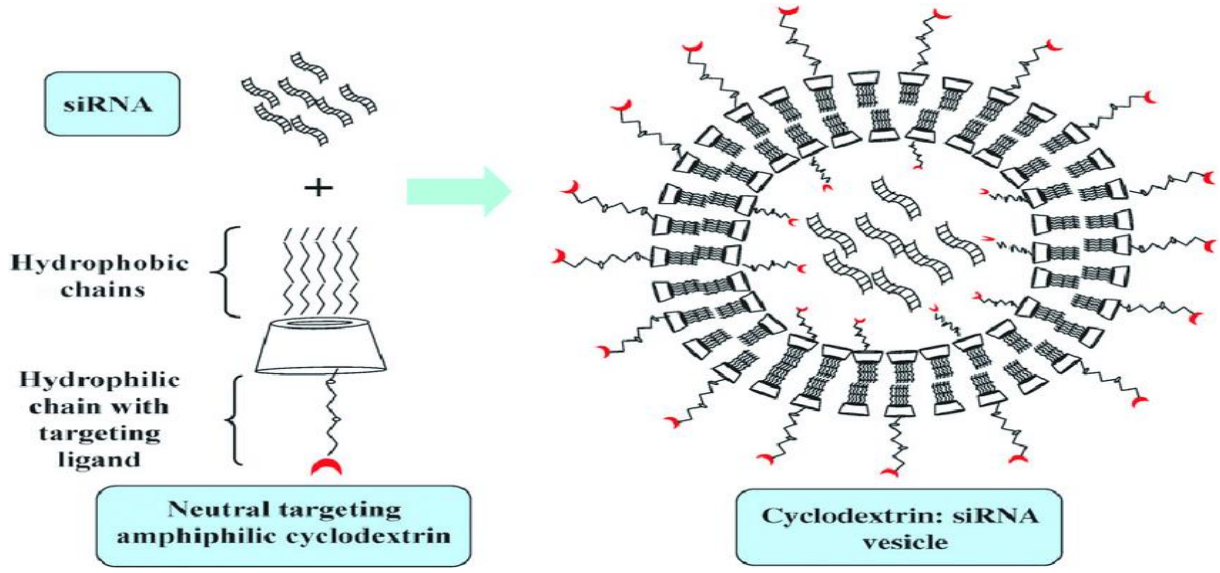

Figure 57.Schematic representation of an amphiphilic polycationic cyclodextrin

12.7. Dendrimers

Dendrimers can be made up of sugars (glycodendrimers), peptides, amidoamines (PAMAM) or even propyleneimine. A dendrimer is a macromolecule made up of monomers which associate in a tree-like process around a central multifunctional core.

Tree construction is carried out by repeating the same sequence of reactions until obtaining, at the end of each reaction cycle, a new generation and an increasing number of identical branches. After a few generations, the dendrimer generally takes a globular spherical shape, highly branched and

multifunctionalized thanks to the numerous terminal functions presented at the periphery. The various ramifications provide internal cavities between them. The complexes can therefore be formed using the cavities formed within the dendrimer or by grafting the molecule to a terminal function. Two classes are used very frequently in transfection due to their chemical properties: poly(amidoamines) and poly(propylene imines) (Fig. 58).

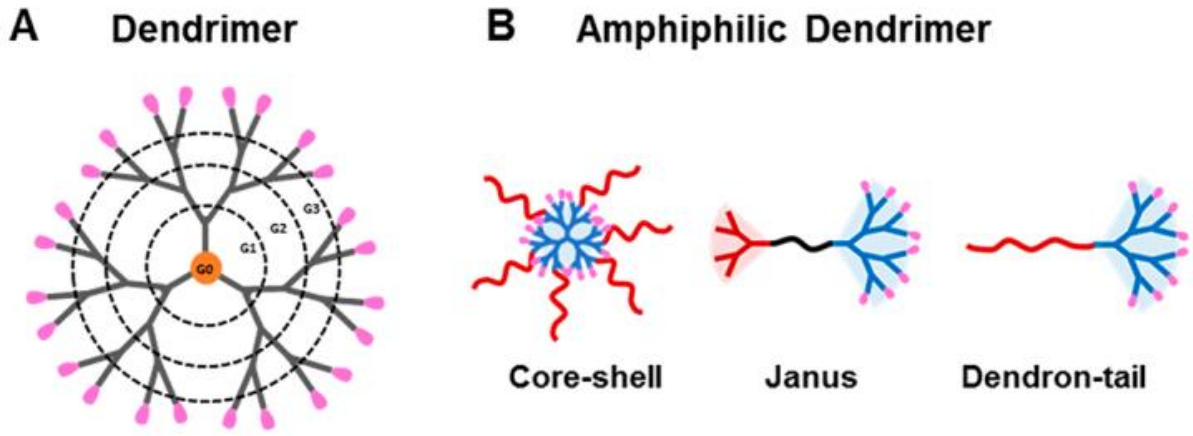

Figure 58. Illustration of dendrimer.

Poly(amidoamines) (PAMAMs) constitute the first family of dendrimers described. These are dendrimers whose monomer is an amidoamine. Their first use in transfection is reported by Haensler and Szoka (1993). It is the presence of numerous secondary and tertiary amine functions that makes them effective for this indication. The terminal amine groups allow interaction with the phosphate groups of DNA. The DNA wraps around the dendrimers to form complexes whose size has been evaluated for 6th-generation polyamidoamines at 50 nm. The type of polyamidoamine used varies the complex but also the transfection efficiency. Thus a 3rd generation dendrimer does not complex the DNA. Beyond the 4th generation and an excess charge, polyamidoamines complex DNA and form particles with a clearly positive charge. The transfection efficiency increases exponentially between the 4th and 10th generations with a plateau at the 8th generation. The formation of complexes is influenced neither by the salt concentration nor by the pH. They therefore have good stability under physiological conditions. Dissociation of the complex only occurs in the presence of an ionic detergent such as SDS. PAMAMs are effective on many cell types. Their effectiveness is 10 to 100 times greater than that of lipids cationic. PAMAMs have a transfection efficiency practically equal to that of PEI, and lower toxicity. Various modifications to the basic structure of the PAMAM compound dendrimer were investigated to improve the transfection efficiency. These modifications take into account different parameters: cytotoxicity, complex formation, binding of the dendrimer to the cell, its release from the endosome, and cell targeting. For example, a PEG group was added with the dendrimer composed of PAMAM in order to improve the transfection efficiency. The use of polyamidoamines partially degraded by hydrolysis in water or in butanol was mentioned by Tang et

al. (1996). Their transfection capacity is greater than that of native dendrimers. This variation is not due to variations in the morphology of the complex, it having the same shape and the same size as the complexes formed with non-degraded dendrimers. It is their greater flexibility which is at the origin of this difference.

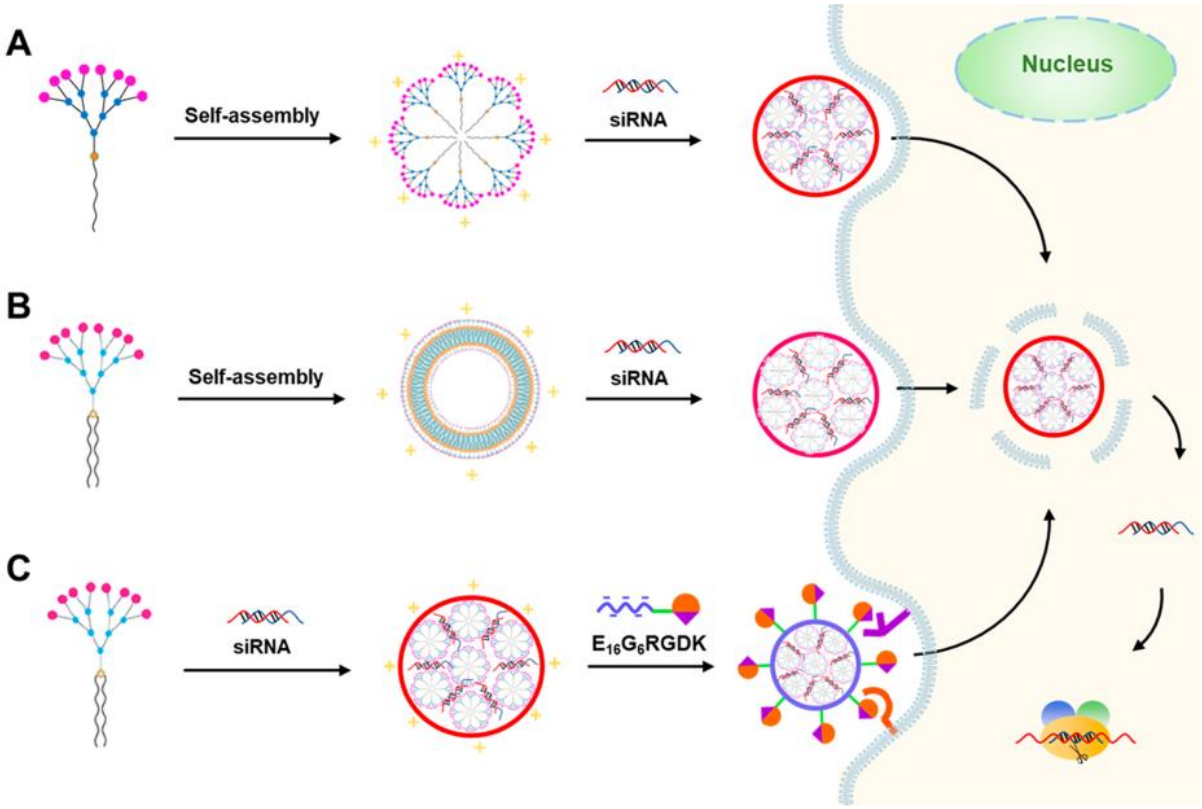

Figure 59. assembly of amphiphilic dendrimers

In fact, the native form is compact and does not allow rearrangement of the molecule. The degraded forms present a developed conformation due to the repulsion between the terminal groups. When complexed with DNA due to the neutralization of terminal charges, PAMAMs have a more compact shape. Within the endosomes, their buffering power will allow the polymer to increase in charge. The quantity of polymer necessary to complex the DNA is therefore less important and a part is released into the medium. These molecules then return to an expanded form. This phenomenon of swelling of the polymers contributes to the swelling of the endosomes which will lead to their bursting and the release of the complexes into the cytoplasm. This is supported by the fact that there is an optimal rate of degradation. Above this rate, the steric hindrance is too great to allow these conformational changes. Below, the molecules are too small to participate in this phenomenon. Their low toxicity makes them an interesting vector for in vivo use. Thus, they present a higher transfection efficiency than the branched PEI of 25 kDa in the context of a study on the transfection of rabbit carotid arteries, but a lower efficiency at the pulmonary level after intratracheal administration. Very recently a team showed interesting results in the context of anti-cancer therapy. In association with a

plasmid, the systemic administration of complexes formed of poly(propylene imine) dendrimers and transferrin is effective in stopping tumour growth and leads to the prolongation of the survival of mice.

12.8. Polymeric Particles

In recent years, one of the main axes of research in galenic pharmacy concerns nano and microparticle vectors of a polymeric nature. More recently, nano- and microparticles have found their place in gene therapy as a tool for intracellular delivery of nucleic acids. Microparticles are systems with a size between 1 and 1000 µm, while nanoparticles are defined as colloidal particles having a size between 10 and 1000 nm. Nanoparticles are particularly interesting in gene transfer due to their small sizes which give them better internalization into the cell. Many particles have been developed as an alternative to the use of liposomes in order to circumvent the stability problems posed by this type of vector such as quantum Dots, gold nanoparticles, metal nanoparticles, polymeric nanoparticles, etc. The advantage of these particulate systems is the protection of the oligonucleotides by encapsulation in the polymer matrix or by adsorption to the surface using a polycationic polymer or a cationic surfactant.

Particles can be formulated with a wide variety of polymers having different properties such as molecular mass, hydrophilic or lipophilic, and their charge. All of these properties influence the final property of the particle. Many polymers can also be used to functionalize the particles for the purpose of cellular targeting or to make the particles stealthy. The main polymers used for the formulation of nanoparticles or microparticles are: copolymers of lactic and glycolic acid, polymethacylates, and poly (ß-amino ester) (PBAE).

* Biodegradable polyesters, copolymers of lactic and glycolic acids (PLGA) and polyl(actic) acid (PLA) are among the most commonly used polymers to encapsulate molecules and biomolecules. The transfection efficiency of these particles is lower than that of liposomes in vitro but the relationship is inverse in vivo. These nanoparticles have the ability to escape from the endo-lysosomal compartment and release the therapeutic molecule into the cytoplasm quickly.

Furthermore, the progressive degradation of these particles also leads to prolonged expression of the transgene in vivo. For example, the expression of the β-galactosidase transgene was detected up to 28 days after intramuscular injection of PLGA particles containing the plasmids in a mouse model.

* Polymethacrylates are cationic polymers that have the ability to condense nucleotides into nanoparticles. Several polymethacrylates such as poly[2-(dimethylamino)ethyl methacrylate] (DMAEMA) and its co-polymers have been used for DNA transfection. A range of polymethacrylates, varying in molecular weight and chemical structure, were evaluated for their potential as gene vectors. Polymethacrylates containing only tertiary amine groups have been shown

to have similar effects to PEI in terms of transfection efficiency while displaying a better biocompatibility profile. Recently a team formulated cationic nanoparticles with commercially available polymethacrylates such as Eudragit® E100 in combination with PLGA, and a cationic surfactant, cetyltrimethyl-ammonium bromide (CTAB). The results showed an in vitro and in vivo transfer efficiency 10 times greater than that of PLGA nanoparticles alone.

* Poly(ß-amino ester) (PABE) are biodegradable cationic polymers of a non-toxic nature. PBAEs are used for their different molecular mass, their terminal chemical function, the size imparted during their complexation with DNA and their N/P ratio. PBAE polyplexes formed with DNA showed similar transfection efficiency to PEI and less toxicity in vitro. PBAE are often used in combination with PLGA to form particles which have shown effectiveness in reducing the size of tumors in mice transfected with the gene encoding luciferase. PBAE/PLGA particles also showed sustained release of the plasmid for 4 days.

13. Physicochemical methods improving transfection

13.1. Physical Methods

Several physical or mechanical methods such as microinjection, DNA gun, sonoporation, laser irradiation, hydrodynamic injection, electroporation or electrotransfer.

13.1.1. Microinjection

Microinjection involves directly injecting genetic material into the cytoplasm or nucleus of a cell using a glass micropipette or syringe, a precision system to control the movement of the micropipette, a microinjector and a microscope. Genetic material is introduced into the cell by hydrostatic pressure. This technique is simple but restrictive since each cell is treated separately. The genetic material of interest is administered into the tissues using a syringe fitted with a needle or by systemic injection from a vessel. Target tissues are muscle, skin, liver, heart muscle and solid tumours. However, the effectiveness is low due to rapid degradation by nucleases in serum and elimination by the mononuclear phagocyte system

13.1.2. Ballistic DNA Cannon

The DNA gun or “gene gun” consists of adsorbing naked DNA around inert metal particles (such as gold or tungsten), and propelling them using a gas into the cell or tissue. This technique was first used in plant cells in 1987. In the early 1990s, it was extended to mammalian cells and tissues. It is based on the principle of delivering heavy metal particles coated with DNA by crossing the target tissue at a certain speed. Sufficient speed is achieved by high voltage electronic discharge, spark

discharge or helium pressure discharge (Fig. 60). The main advantage of the gene gun is the precision of DNA dose delivery. It is most often used in gene therapy research for ovarian cancer.

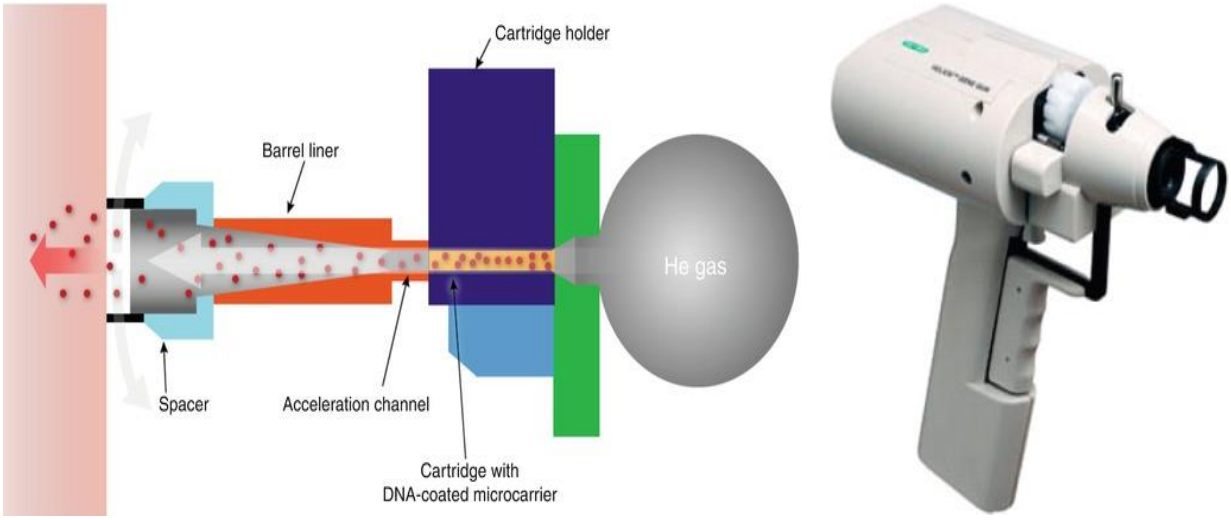

Figure 60. Ballistic DNA Cannon

13.1.3. Electroporation

Electroporation or electrotransfer consists of applying an electric field to the target tissue after injection of naked DNA. Electroporation uses the effect of electrical pulses to destabilize the cell membrane for short periods of time, allowing penetration of the plasmid or other molecules. This technique was previously developed to improve the entry into the cell of poorly permeable molecules or active ingredients. It consists of applying an electric field greater than the capacitance of the membrane causing charges of opposite polarity to align on either side of the cell membrane, thereby forming a potential difference at a specific point on the cell surface (Fig. 61). As a result, the rupture of the membrane forms a pore and allows the molecule to pass through. Formation of the pore occurs in approximately 10 nanoseconds. The membrane pore can be reversible depending on the field strength and pulse.

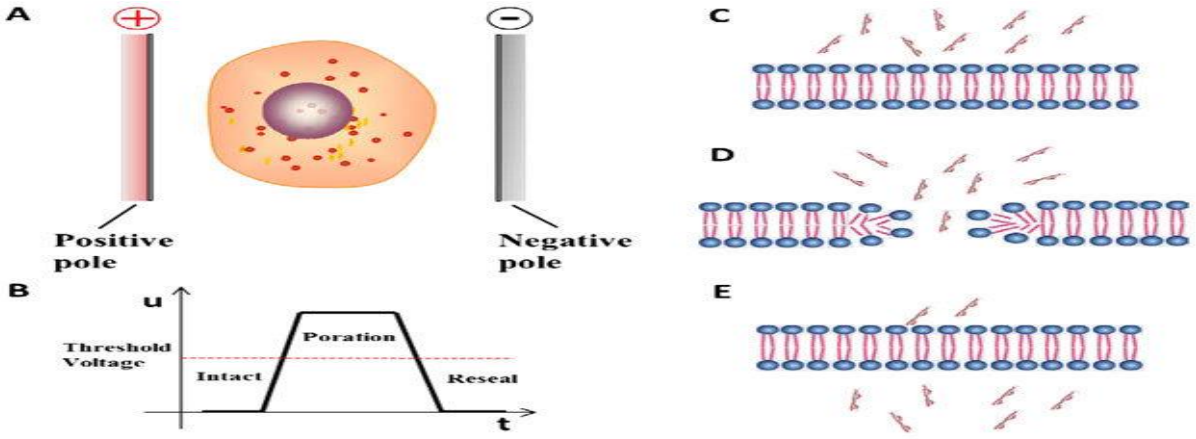

Figure 61. Electroporation technique

If it is reversible, the cells remain viable, otherwise cell death results. Irreversible electroporation is used in cancer treatment to destroy cancer cells. Membrane permeability for gene transfer is controlled by pulse amplitude and duration. The fields currently used are either high-intensity fields (>700V/cm) or low-intensity fields (<700V/cm) with short (microseconds) or long (milliseconds) pulses. The target tissue determines this combination of variables. For example, cancer cells need a low field intensity and a long pulse, while muscle cells need a short pulse and a high field intensity. Electroporation has become a reliable physical method for delivering plasmid DNA. The first study of gene transfer using electroporation was carried out in 1982 by the team of Neumann. Currently, this technique is used at the bedside of patients in the transfer of anti-cancer molecules (such as bleomycin or cisplatin). But also more recently, in the cutaneous and subcutaneous treatment of tumors.

13.1.4. Sonoporation

Sonoporation consists of applying ultrasound which allows the permeabilization of the cell membrane and increases the penetration of molecules. The naked DNA is coinjected with microbubbles (2 to 4 µm) made of proteins, lipids or polymers and filled with air or an inert gas. The genetic material of interest is incorporated into a micro-bubble and administered into the systemic circulation. An external application of ultrasound follows this operation. Ultrasonic waves cavitate the microbubble in the microcirculation of the target tissue, producing biological effects that result in the deposition of the targeted transfection of the therapeutic gene (Fig. 62).

The first result of gene transfer by sonoporation was described in the 1990s. Since then, this technique has been used in a wide variety of tissues including skeletal muscle, solid tumours and neuronal cells.

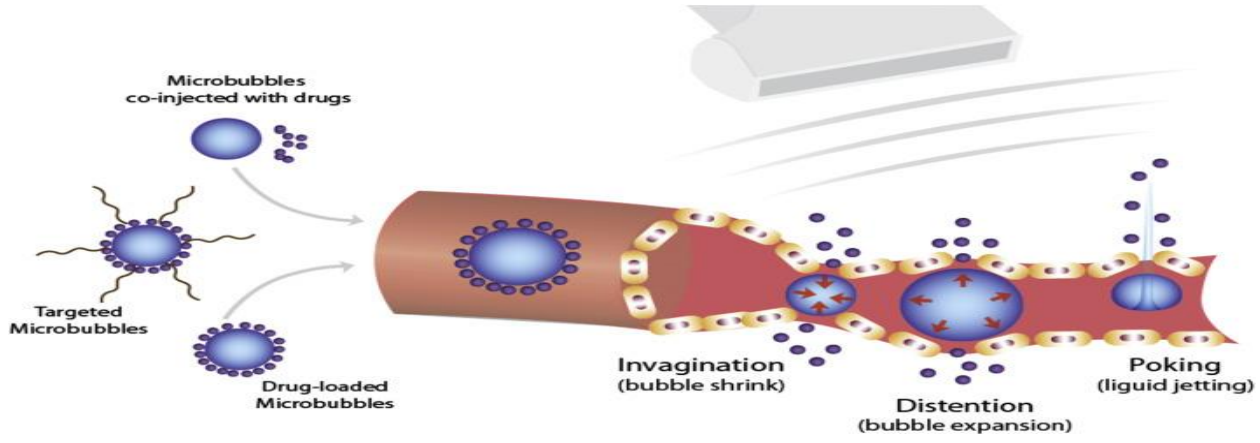

Figure 62. Sonoporation technique

The technique of sonoporation is typically used in the brain, cornea, kidney, peritoneal cavity, muscle and heart tissues.

13.1.5. Photoporation (Laser irradiation)

Laser irradiation is based on the use of a laser source (for example argon laser) or tissues previously exposed to a photosensitizing agent localizing at the membrane level. The mechanism of action is explained by a transient permeabilization of the membrane at the level of the bundle, probably due to a local thermal effect (Fig.63). The photosensitizer is incorporated into the plasma membrane of the target cells. After irradiation at a specific wavelength, the photosensitizer changes from a normal state to a singlet excitation state by absorbing the energy of a photon. The singlet photosensitizer switches very quickly to a triplet excitation state and produces reactive oxygen species, singlet oxygen in particular. Singlet oxygen is an oxidizing agent capable of oxidizing various biomolecules, in particular, to induce damage in the membrane and destroy it. This disruption allows DNA to enter the cell. The first result of gene transfer by laser irradiation was described in 1987. The disadvantage of this technique is its high cost and the heavy infrastructure it requires.

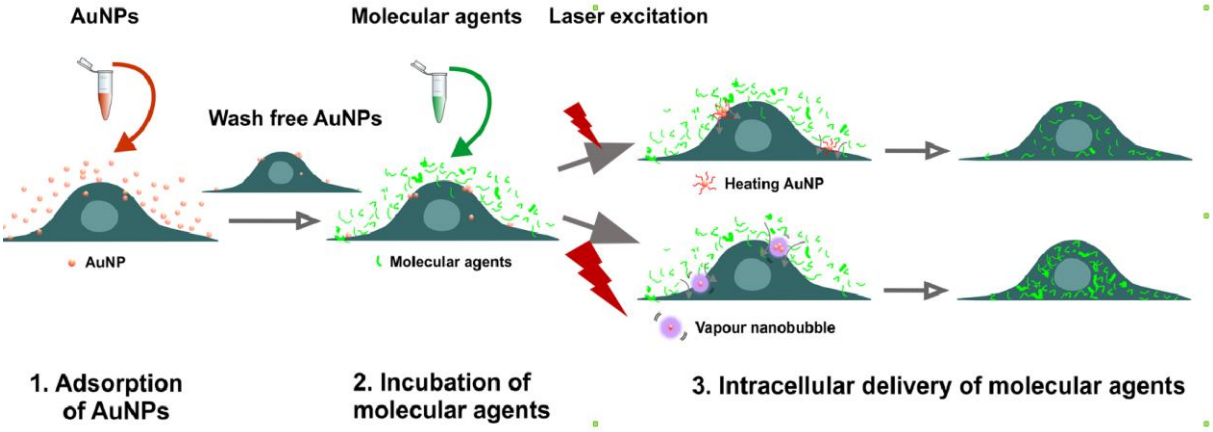

Figure 63. Photoporation gene delivery technique

13.1.6. Magnetofection

The technique is based on the coupling of a therapeutic gene to a magnetic nanoparticle. This complex is introduced into cell culture. The field gradient produced by electromagnets placed under the cell culture increases the sedimentation of the complex and the speed of transfection. In the case in vivo, the therapeutic gene-magnetic particle complex is administered intravenously (Fig. 64). Using powerful, high-gradient external magnets, the complex is captured and held on target. The genetic material is released by enzymatic cleavage of the linker molecule, charge interaction, or matrix degradation. This technique is mainly used in research in vitro to transfect primary cells and cells difficult to transfect by other means.

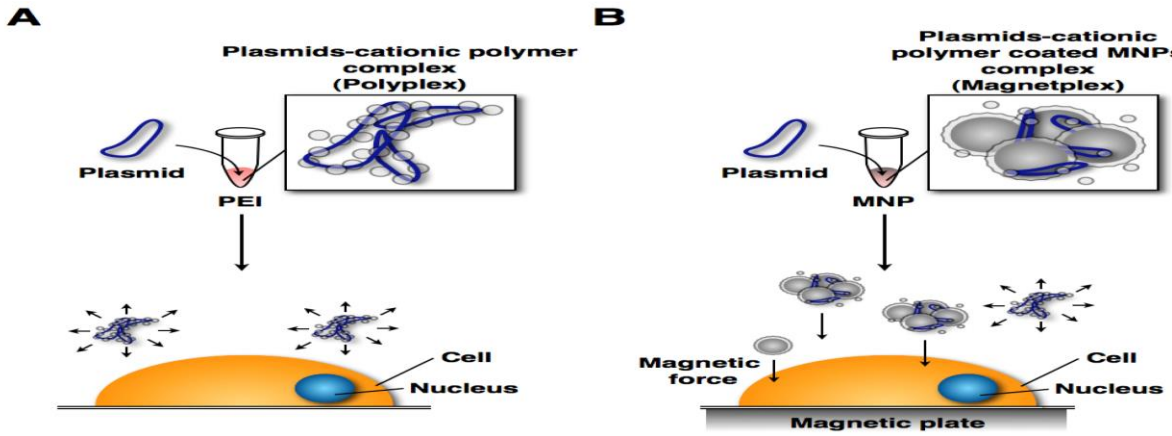

Figure 64. Magnetofection gene delivery.

13.1.7. Hydrodynamic Injection (Hydroporation)

Hydrodynamic injection consists of injecting a large volume of liquid containing DNA either intravenously or intra-arterially which allows penetration of the DNA by mainly targeting hepatocytes (injection into the tail vein of the mouse for targeting the liver), or muscle cells (injection into the arterial system to target muscles). This will create increased permeability of the capillary endothelium and form pores in the plasma membrane surrounding the cells. The therapeutic gene can reach the cell through these pores which then close, thus keeping the genetic material inside the cell. A computer-controlled injection device has recently been developed with the aim of obtaining an efficient, safe and automatically controlled system.

14. SiRNA and VIH Infection

HIV remains a significant worldwide health issue, affecting millions of individuals and resulting in new infections year. The advancement of potent antiretroviral therapy (ART) has considerably enhanced the control of HIV/AIDS. However, obstacles such as drug resistance, enduring adverse effects, and the requirement for lifelong treatment continue to exist. Small interfering RNA (siRNA) is a potential therapeutic strategy for treating HIV infection by selectively targeting crucial viral genes or cellular components necessary for the virus's reproduction process. This essay investigates the potential of siRNA in the context of HIV infection, analysing the fundamental mechanisms, recent advancements, and limitations linked to this unique therapeutic approach.

14.1. An exploration of siRNA and its underlying mechanisms:

siRNA, short for small interfering RNA, is a type of RNA molecules that has a vital function in controlling gene expression. A short, double-stranded RNA molecule, usually measuring 20-25 nucleotides in length. The identification of RNA interference (RNAi), a biological mechanism facilitated by siRNA, has fundamentally transformed the field of molecular biology and provided a new approach for therapeutic treatments.

The RNA interference (RNAi) process commences with the introduction of double-stranded RNA (dsRNA) into the cell. The double-stranded RNA is subsequently subjected to enzymatic cleavage by Dicer, resulting in the generation of tiny fragments known as siRNA. The guide strand of siRNA is integrated into the RNA-induced silencing complex (RISC). The guide strand instructs the RNA-induced silencing complex (RISC) to target messenger RNA (mRNA) molecules that are complementary to it. This targeting might result in the destruction of the mRNA or the prevention of its translation, ultimately resulting to the inhibition of the gene's expression.

14.2. Strategies and Targets for siRNA Targeting HIV:

SiRNA can be specifically tailored to target crucial viral genes or key host cellular components in the context of HIV infection, thereby inhibiting the virus's replication. SiRNA-mediated suppression can potentially target several crucial phases in the HIV life cycle.

Viral Entry and Fusion: siRNA can be specifically engineered to target viral envelope proteins, such as gp120, that play a critical role in facilitating the virus's entry into host cells. Suppressing the production of these proteins can hinder the binding and merging of the virus with the membrane of the host cell.

Reverse Transcription: By targeting the viral reverse transcriptase, an enzyme that converts viral RNA into DNA, we can impede the process of turning the viral genome into a form that integrates into the DNA of the host cell.

Integration: By inhibiting the viral integrase, which is responsible for facilitating the incorporation of viral DNA into the genome of the host cell, the creation of a long-lasting infection can be disrupted.

Transcription and translation: Small interfering RNA (siRNA) can be specifically engineered to bind to viral RNA transcripts, thereby preventing their conversion into viral proteins and decreasing the generation of new viral particles.

Assembly and budding are crucial stages in the life cycle of HIV. Small interfering RNA (siRNA) that specifically targets viral structural proteins, such as Gag and Env, has the ability to disrupt the process of assembling and releasing new virus particles.

14.3. Latest advancements and advancements in clinical practice:

Although the potential of siRNA in combating HIV has been acknowledged for a long time, the process of converting this information into successful therapeutic therapies has been a challenging undertaking. Significant obstacles have been encountered in addressing challenges such as the transportation of siRNA to specific cells, preventing unintended effects on non-target cells, and maintaining long-lasting effectiveness. Nevertheless, recent progress in siRNA technology and delivery technologies has demonstrated potential in tackling these obstacles.

Nanoparticle Delivery Systems: Nanoparticle-based delivery systems have been created to improve the durability and transportation of siRNA. Lipid nanoparticles, polymeric nanoparticles, and other advanced delivery platforms have demonstrated enhanced cellular uptake and precise delivery of siRNA to cells infected with HIV.

Chemical alterations of siRNA: Chemical alterations of siRNA molecules, including as 2'-O-methyl and 2'-fluoro replacements, have been used to augment their durability and diminish off-target consequences. Furthermore, these alterations also enhance the durability of siRNA effectiveness inside cellular environments.

Combination medications: Scientists are investigating the possibility of pairing siRNA with other antiretroviral medicines or immune-modulating medications to provide synergistic results. The objective of these combination techniques is to increase the overall effectiveness of antiviral treatment while reducing the likelihood of the development of resistance.

Clinical Trials: Multiple initial-phase clinical trials have been commenced to assess the safety and effectiveness of siRNA-based treatments for HIV. The main objectives of these trials are to

confirm the practicality of siRNA administration, evaluate the length of time that viral suppression occurs, and monitor any possible negative effects.

14.4. Obstacles and Factors to Take into Account:

Notwithstanding the encouraging progress, there are still various obstacles in the development and implementation of siRNA-based therapeutics for HIV:

Off-Target Effects: The primary issue lies in achieving high specificity in targeting HIV without compromising the functioning of host cells. Non-specific impacts can result in undesired outcomes and negative occurrences.

Delivery to Viral Reservoirs: HIV forms viral reservoirs in specific tissues, including as the central nervous system, where antiretroviral medicines and siRNA may have restricted entry. It is essential to devise delivery systems in order to access these reservoirs, as this is vital for attaining a functional treatment.

Resistance Development: The occurrence of viral mutations has the potential to result in the development of resistance to siRNA therapy. To avoid this risk, it may be beneficial to develop siRNA cocktails or combination medicines that target many targets simultaneously.

immunological Response: The presence of exogenous siRNA can potentially elicit an immunological response. Thorough evaluation of the ability of siRNA to provoke an immune response and implementation of techniques to reduce undesired immunological reactions are crucial.

The utilisation of siRNA as a therapeutic approach against HIV infection signifies a pioneering method in the realm of antiretroviral therapy. The accuracy and particularity of siRNA targeting provide a distinct benefit in fighting the virus at different points in its life cycle. Despite the ongoing obstacles of delivery, off-target effects, and the risk of resistance, recent breakthroughs in siRNA technology and clinical progress offer promising prospects for the creation of novel, efficient, and well-tolerated therapeutics.

As research progresses in understanding the intricacies of siRNA-mediated therapy for HIV, the possibility of achieving long-lasting viral suppression, reducing the need for lifelong treatment, and ultimately working towards a functional cure remains a strong motivation in addressing one of the most significant global health challenges of our era.

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