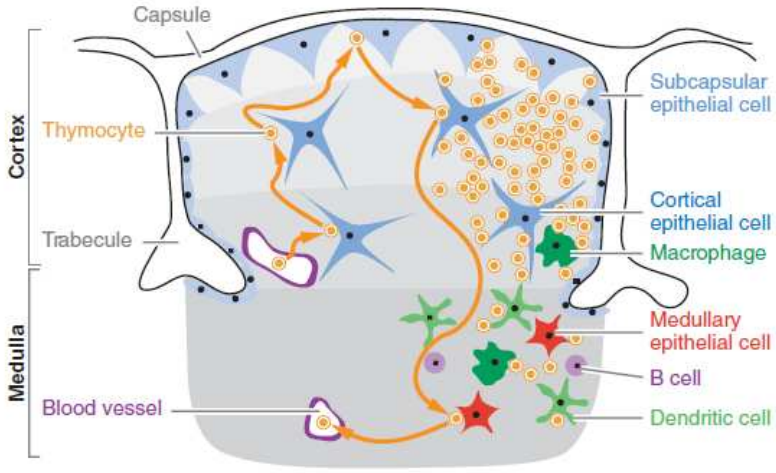
INDEX

INTRODUCTION

I PART:

The immune system is delicately balanced between self-antigen-driven tolerance and pathogen-driven immunity. The primary function of thymus is to continuously generate a diverse population of T cells that can elicit adaptive immune responses against invading pathogens, while promoting self-tolerance [1]. The thymus is a rather vulnerable organ as many factors, including environmental insults, aging, genetic composition, virus infection, irradiation, and anti-cancer drug treatments, can all irreversibly compromise its function [2, 3]. Impaired immune surveillance consequent to thymic dysfunction leads to diseases ranging from autoimmunity to immunodeficiency and malignancy [4]. The thymus provides the essential microenvironment for T-cell development and maturation. It's organized into two morphologically and functionally distinct compartments: the cortex and the medulla, which house two distinct populations of thymic epithelial cells (TECs): the cortical TECs (cTECs) and the medullary TECs (mTECs) [5-6]. Other thymic stromal cells (TSCs) include thymic fibroblasts, endothelial cells, as well as antigen presenting cells like macrophages and dendritic cells. Together, this network of thymic cells provides both homing signals for the immigration of lymphocyte progenitors originated from the bone marrow (BM), and trophic factors necessary for the differentiation and maturation of thymocytes [7]. The cTECs and mTECs have been well documented to be critical in these regulated processes participating in positive and negative T-cell selection, respectively [8].



Cellular composition of thymus. The major cell types and the sequential cell-cell interactions along the migratory route of developing thymocytes are depicted. The different APCs are color-coded. mTECs, highlighted in red, play an essential role in self-tolerance induction toward tissue-restricted self-antigens. Shaded areas depict functionally distinct stratified microenvironments as recently proposed.

The programme of T cell development involves sequential interactions with distinct thymic stromal cells that form specialized microenvironments in the thymus. Thymic stromal cells are heterogeneous, consisting of epithelial and mesenchymal cells, endothelial cells, as well as bone marrow-derived macrophages and dendritic cells [9]. Of these cell types, thymic cortical and medullary epithelial cells have been shown to provide essential signals at multiple stages of thymocyte development. For example, MHC class II + epithelial cells in the thymic cortex are known to be important in αβT cell receptor-mediated positive selection [10, 11], while medullary epithelial cells, in addition to dendritic cells, are important in ensuring tolerance to self antigens [12, 13]. The epithelial component of the thymus arises from the out-budding of endoderm in the third pharyngeal pouch that begins around day 10-11 of gestation. Recent advances have led to a better understanding of the genetic mechanisms regulating thymic epithelial cell development, with the identification of a role for a number of genes including FoxN1, Pax9, Hoxa3 and Eya1 [14]. However, despite these advances, the developmental stages and events that lead to formation of the distinct cortical and medullary epithelial subsets, required to regulate Tcell development, are still not fully understood [15].

T cell development in the human thymus

In the human thymus, CD34+CD1a− precursor cells enter at the cortico-medullary junction. From this area, T cell precursors migrate towards the cortical region where proliferation and differentiation are initiated by interactions with the thymic stroma. The distinct stages of T cell development are defined by the sequential expression of cell-surface antigens [16]. Expression of CD1a marks commitment to the T cell lineage since at this developmental stage the cells start to rearrange their TCR genes. Next, CD4 is upregulated on $CD4^+$ immature single positive (ISP) cells, that can still develop into TCRαβ or TCRγδ T cells. $CD4^+$ ISP cells which have succesfully rearranged a TCR-β gene are selected for further differentiation in a process referred to as β-selection. At this checkpoint the TCR-β chain dimerizes with a pre-TCR-α chain and signaling via this complex results in proliferation and survival of the cells [17]. Cells that have failed rearrangement at one locus will attempt to rearrange the β-gene at the other allele, but when still unsuccessful these cells do not receive proliferative and survival signals and will eventually die. Notably, β-selection in human does not seem to be restricted to the $CD4^+$ ISP stage, but likely continues in the transition from the $CD4^+$ ISP to the $CD4^+CD8^+$ double-positive (DP) stage [18,19]. During the subsequent stages in T cell development, which are defined by the expression of CD8α, referred to as early $CD4^+CD8\alpha^+$ double-positive (EDP) cells, and CD8β (CD4+CD8α+β+ DP), TCR-α rearrangements are initiated. $CD4^+CD8^+$ DP cells which acquire a functional TCRαβ on their cell surface are subjected to positive selection involving interaction with MHC molecules that are expressed on the cortical epithelial cells [20]. Low affinity interactions of the TCRs with self-peptide-MHC complexes result in positive selection of the T cells, whereas T cells are negatively selected when high-affinity receptors for self-peptide-MHC complexes are expressed. Most DP thymocytes however do not interact at all with MHC molecules and will die by neglect. Positively selected T cells further differentiate into either $CD4^+$ or $CD8^+$ single-positive (SP) T cells, before they leave the thymus to seed the periphery as naıve T cells.

Homeostasis of epithelial tissues is usually maintained by continuous self-renewal of epithelial stem cells. Although it remains controversial whether the common stem cells for both cTECs and mTECs reside in the thymus, an emerging The primary function of thymus is continuously to generate a diverse population of T cells that can elicit adaptive immune responses against invading pathogens, while promoting self-tolerance [1] amount of data support the existence of thymic epithelial progenitor/stem cells (TEPCs/TESCs) [21-25]. Significant progress has been made to identify and characterize TEPCs using lineage track analysis and clonal assays in recent years. However, the phenotypic characteristics of thymic epithelial progenitors and whether or not they persist in the thymus at later stages remains controversial. In this regard, the antibody Mts24 has been used to define heterogeneity within thymic epithelium, with equal proportions of $Mts24^{+}$ and $Mts24^{-}$ thymic epithelial cells being reported as early as embryonic day 12 of gestation, a stage where bipotent epithelial progenitors are known to be present [26]. Moreover, the ability to give rise to functional thymic tissue containing both cortical and medullary epithelial lineages has been thought to be restricted to this Mts24+ subset [27, 28].

The polymorphic function of the autoimmune regulator AIRE has intrigued immunologists for decades**.** Classically the AIRE deficiency is associated to the breaking of central T tolerance being involved in the clonal deletion or inactivation of semimature self-reactive thymocytes and in the transcriptional control of many tissue-restricted self-antigen genes (TSAs) in the thymus [29, 30]. The broad range of tissue-specific genes that are ectopically expressed in the thymus, and the seminal studies of Aire knockouts highlight the importance of central selection mechanisms in establishing and maintaining a T cell repertoire that is tolerant towards peripheral tissues [31]. As a putative transcription factor, Aire was shown to promote the thymic/ectopic expression of a number of peripheral tissue antigens (PTAs), such as Mucin-6 (stomach specific) and interphotoreceptor retinoid-binding protein (Irbp, eye specific), which facilitate self/non-self distinction. For all these reasons, classically, Aire

deficiency is associated to the breaking of central T tolerance being promoting the clonal deletion or inactivation of semimature self-reactive thymocytes. In particular the disruption of thymic expression of the single tissue-specific self-molecule Insulin in mTECs is sufficient to induce anti-insulin autoimmunity resulting in the pathologic damage of β-cells even in the presence of disease-resistant alleles of MHC molecules [32].

Expression of tissue specific Antigens (TSA) on the mTECs are crucial for establishing central tolerance. Epitopes derived from TSAs were presented to the developing thymocytes either directly by mTECs, or indirectly by thymic antigen presenting cells (APCs) via cross-presentation. Thymocytes expressing TCRs with high affinity to TSAs are eliminated in the thymus by negative selection. Autoreactive T cells that escape the negative selection can cause autoimmunity in the peripheral organs. Thymus-specific deletion of TSA(s) results in an autoimmune reaction in the target organ(s) (Table 1-1/1-2, Fig.1) The autoimmune regulator Aire is mostly expressed in a small proportion of mTECs able to impose tolerance on the large repertoire of differentiating thymocytes. However, the origin and the development of Aire^{+} mTECs remain poorly understood as well as the fundamental regulation systems that govern their cell lineage determination. The essential role of tissue specific antigen (TSA) expression in mTECs in mediating central tolerance has been well established. It was previously shown that mice with mTEC specific insulin deletion develop autoimmune diabetes within 3 weeks postnatal.

Target antigen	Expressing organ	Manifestation	Reference
α-MyHC	Heart	- Myocarditis	Lv, et al. J Clin Invest. 2011; 121(4)
insulin	Pancreas	- Insulitis - Early onset of autoimmune diabetes	Fan, et al. EMBO J. 2009; 28(18) (Figure A)
IRBP	Eye	- Uveitis	DeVoss, et al. J Exp Med. 2006; 203(12)

Table 1-1. Examples of deletion of a single tissue specific antigen leading to organ-specific autoimmunity.

Target gene	Manifestation	Reference
Traf6	- Defects in mTEC maturation - Autoimmune hepatitis	Bonito, et al. J Clin Invest. 2013; 123(8)
FoxN1	- Fewer naïve T cells - Reduced CD8+ T cell response to infection - Increased lung injury	Guo, et al. PLoS One. 2012; 7(4)

Table 1-2. Examples of genes that affect mTEC development.

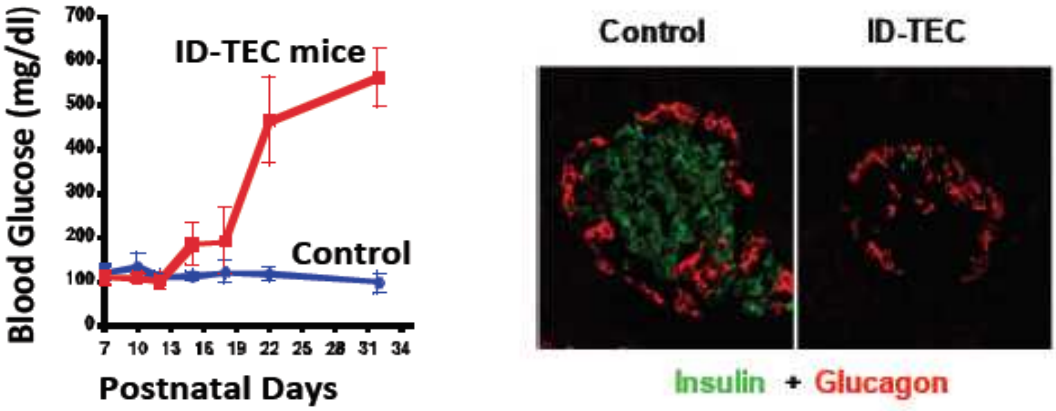


Figure 1: **mTEC-specific deletion induces autoimmune diabetes.** Aire-Cre system was used to specifically delete insulin gene in mTECs (insulin-deleted mTEC are the IDTEC mouse model). Blood Glucose level representation in control and ID-TEC mice. Islet specific. Histological images of day 23 postnatal pancreatic islets.

However, recent evidences attribute different and emerging roles to Aire's action especially in mTEC development biology. Matsumoto et al. proposed two contrasting models to explain when and how Aire controls the differentiation program of mTECs required for establishment of self-tolerance. This critical issue arises from the outcome of the loss of Aire and TSAs during thymic organogenesis. Briefly, the first theory assume that Aire induce the interruption of mTEC maturation and only the absence of Aire would prevent apoptosis revealing the full program of mTEC terminal differentiation ending with a globular cytokeratin 5-cytokeratin 8-cytokeratin phenotype. Regarding model 2, lack of Aire results in defective accomplishment of the differentiation program, with the cells remaining at the pre-mature stage just before terminal differentiation. These ''Aire-less'' mTECs have a globular cell shape and lack the transcriptional activity for Aire-dependent PTA genes. Currently, different opinions seems to favor the second model (Promotion of the mTEC differentiation program by Aire) over the first one (Interruption of the mTEC differentiation program by Aire). Nevertheless, the origin

and the development of Aire$^+$ medullary thymic epithelial cells (mTECs) remain poorly understood as well as the fundamental regulation systems that governs their cell lineage determination. Recent evidences confirmed the existence of a Aire-mTEC subset of precursors in the fetal thymus indicating that the Aire$^+$ phenotype is a downstream product in the mTEC lineage. On the other hand their self-renewal capability seems transient according to the profound decline in number of TECs with ageing and the physiological process of thymic involution.

While numerous efforts have been made to correct thymic defects, manipulating the thymus, either *in vitro* or *in vivo*, proves to be challenging. This is mainly attributed to the unique architecture of the thymic stroma that is essential for the maturation, survival and function of TECs. Unlike epithelial cells of other visceral organs, which form a two-dimensional (2-D) sheet-like structure on the basement membrane to create borders within and between organs [33], TECs form a sponge-like three dimensional (3-D) network that is essential for their function [34]. TECs cultured on irradiated 3T3 feeders (a 2-D environment) are unable to support T-cell differentiation from lymphocyte progenitors, but start to express markers of terminally differentiated epithelial cells [35]. Recently, TEC stem cells derived from early embryos were shown to differentiate into skin cells when cultured in 2-D environment [36]. Indeed, the expression of key genes for the specification and proliferation of TECs (e.g. FoxN1, DLL-4, CLL-22 and Tbata) are shown to be dependent on the 3-D organization of the thymic stroma, further indicating that the unique microenvironment of the thymus is essential to maintain the unique property of TECs to support T lymphopoiesis [37]. Over the years, substantial progress has been made to reproduce the thymic microenvironment. Matrigel and other collagen-based synthetic matrices were shown to be able to support limited differentiation of lymphocyte progenitors into T-cells [38]. TECs cultured in artificial 3-D matrix are viable and can partially support thymocyte development. Recently, Kyewski and colleagues developed a co-culture system, in which mTECs were layered on top of a 3-D

artificial matrix embedded with human skin-derived dermal fibroblasts. Under such conditions, mTECs can retain some of their key features (e.g. expression of FoxN1, Aire and tissue-specific antigens) [39]. In a similar approach, Chung et al. mixed TECs and thymic mesenchyme, both isolated from postnatal human thymi, with $CD34^+$ cells from cord blood to form implantable thymic units [40]. The thymic microenvironments of these thymic reaggregates can support thymopoiesis *in vitro* and are able to generate a complex T-cell repertoire when transplanted in NOD.scid gamma (NSG) humanized mice *in vivo.* However, to date, none of these approaches has been able to fully recapitulate the function of a thymus. Recently, significant advances have been made in "cell-scaffold" technology [41]. This groundbreaking technology uses a detergent-perfusion based approach that allows the clearance of the cellular constituent of almost any organ of any scale, while retaining its original 3-D architecture and extracellular matrix (ECM) components [42, 43]. Repopulating the decellularized natural scaffolds with tissue-residing mature cells or progenitor/stem cells can promote its recellularization, and partially recover organ function [44]. To date, these "cell scaffolds" have been primarily applied to manufacture and implant relatively simple organs, such as tissue engineered vascular grafts and skin, with some success. Regeneration of complex organs such as liver, heart, lung, and kidney has also been attempted in animal models [43, 45-48]. While limited, encouraging functional regeneration of the engineered organs was observed. Furthermore, a successful clinical implantation of reconstructed decellularized trachea underlines the clinical potential of this technology [49]. Here, we show that thymus organoids reconstructed with the "cell-scaffold" technology can support thymopoiesis *in vivo* to establish both humoral and cellular adaptive immunity athymic nude mice. Additionally, they also induce central immune tolerance to allo-skin grafts.

INTRODUCTION

II PART

Stem cells and their immunosuppressive properties

Multipotent MSCs are adult mesenchymal, non-hematopoietic stem cells with self-renewing capability. Because they share the expression of some pluripotency genes, such as the transcription factors NANOG and Sox2, MSCs utilize self renewal pathways similar to those in embryonic stem cells [50]. The International Society of Cellular Therapy has defined MSCs as a heterogeneous stem cell population, which is characterized by (i) the adherence to plastic under standard culture conditions; (ii) a fibroblast-like morphology; (iii) the potential to differentiate into three main tissues (osteoblast, chondrocytes, and adipocytes); (iv) the lack of expression of the hematopoietic markers CD11b, CD14, CD34, CD19 or CD79a, CD45, HLA-DR and the vascular marker CD31 [51, 52]; (v) the expression of CD13, CD44, CD54, CD73, CD90, CD105, CD166, and Stro-1 [53] (Fig.2). Furthermore, MSCs have been shown to secrete a large number of growth factors, cytokines and chemokines, which allow their migration and expansion, exert immunomodulatory activities, modulate angiogenesis and apoptosis, and support the differentiation and engraftment of HSCs. With the advancing knowledge of MSC biology, these criteria require some adjustment depending on the tissue, patient health, and source of MSCs [54].

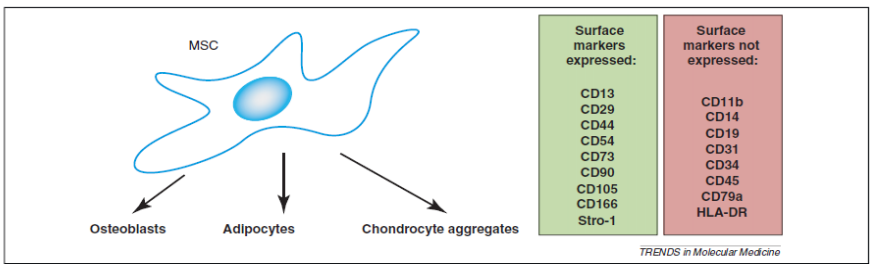


Figure 2. **Main features of mesenchymal stem cells (MSCs).** MSCs can be isolated from many tissues, including bone marrow, peripheral blood, adipose tissue, pancreas, cord blood, amniotic fluid and placenta. The International Society for Cell Therapy (ISCT) uses three minimal standard criteria to identify MSCs: (i) adherence to plastic; (ii) expression of the cell surface molecules CD13, CD29, CD44, CD54, CD73, CD90, CD105, CD166 and Stro-1 as well as a lack of expression of the hematopoietic cell surface molecules CD14, CD19, CD34, CD45 and HLA-DR; and (iii) in vitro differentiation of MSCs under specific culture conditions into osteoblasts, adipocytes or chondrocytes.

Expression of immunomodulatory molecules by MSCs

A number of both surface and intracellular molecules are required to mount a proper immune response or induce immune suppression [55, 56]. Whereas adhesion molecules and the major histocompatibility complex (MHC) antigens are involved in the interaction with immune cells, particularly T cells, co-stimulatory molecules and/or the Fas ligand/Fas receptor interaction (FasL/FasR) are important for T cell activation and/or effector function [57]. MSCs exert an immune tolerant phenotype which is characterized by low levels of MHC class I surface antigens and the lack of MHC class II antigens [58], FasL and the co-stimulatory molecules B7-1, B7-2, CD40 or CD40L [59]. With the exception of ICAM1, which is only expressed upon induction, the adhesion molecules are constitutively expressed in MSCs. Once adherent to endothelial cells via adhesion molecules, MSCs migrate to the injured tissues [23]. MSCs also express the Toll-like receptors (TLRs) 2, 3, 4, 7 and 9 at the protein level, which affect the immunomodulatory properties of MSCs. Recent results demonstrated that MSCs are activated by TLR ligands, leading to the modulation of proliferation, differentiation, migration, survival, and immunosuppressive capacity [60]. The mechanisms for the immunosuppressive potential of MSCs are not fully understood. Cell-cell contact dependent mechanisms and the release of soluble immune modulators such as indoleamine 2,3 dioxygenase (IDO), prostaglandin E2 (PGE-2) or nitric oxide (NO) upon activation in response to immune cells seems to be necessary. Some of these immune modulators are downstream of signal pathways triggered by TLRs, leading to the hypothesis that TLR ligands may induce the production of anti-inflammatory mediators in MSCs and result in an immunosuppressive phenotype [60]. In addition to these primarily immunostimulatory molecules, MSCs have been shown to constitutively express the non-classical HLA-G antigens and the co-inhibitory molecules B7-H1 (PD-L1) and B7-H4, both of which negatively interfere with immune responses [61]. Under physiological conditions, constitutive HLA-G expression is found in immune-privileged organs (e.g. testis, ovary and fetal cells)

and is associated with tolerogenic properties via the interaction with inhibitory receptors on dendritic cells (DCs), natural killer (NK) cells and T cells.

Immune regulation by MSCs depends on different factors

Numerous biologically active factors are secreted by MSCs, in particular growth factors, cytokines, chemokines and hormones, all of which exert paracrine effects on neighboring (immune) cells and allow homing, migration, and their attachment to injured cells . MSCs express a variety of chemokine receptors, such as CCR1, CCR4, CCR7, CXCR5 and CCR10, which might be involved in migration of MSCs into injured tissues along a chemokine gradient. These include biologically active factors secreted by MSCs such as interleukin (IL)-6, the leukemia inhibitory factor (LIF), the stem cell factor (SCF), Jagged 1 and angiomotin (Ang)1. In addition, immunosuppressive factors such as IL-10, the transforming growth factor (TGF-β), the vascular endothelial growth factor (VEGF), soluble HLA-G (sHLA-G), the hepatocyte growth factor (HGF), IDO, NO and PGE-2 can also be secreted by MSCs, creating an immunosuppressive environment [62] (Fig.3).

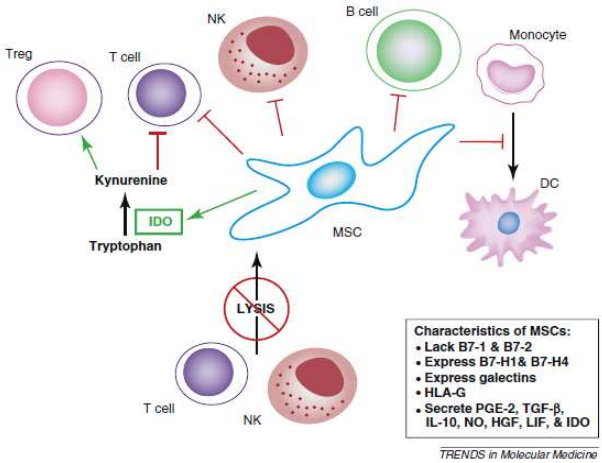


Figure 3. **Immunosuppressive properties of mesenchymal stem cells (MSCs).** MSCs inhibit proliferation, cytokine production and/or the cytotoxicity of T cells, natural killer (NK) cells and B cells. They also block differentiation of monocytes into antigen-presenting dendritic cells (DCs) by preventing expression of co-stimulatory molecules B7-1 and B7-2, inducing expression of inhibitory molecules B7-H1, B7-H4 and HLA-G, and secreting soluble HLA-G5, prostaglandin E-2 (PGE-2), transforming growth factor β (TGF-β), interleukin 10 (IL-10), nitric oxide (NO), hepatocyte growth factor (HGF) and indolamin-2,3-dioxygenase (IDO). IDO converts tryptophan to kynurenine, which leads to T cell inhibition and activation of immunosuppressive regulatory T cells (Tregs). However, these immunomodulatory properties depend on the experimental conditions, such as the cultivation conditions, and activation of target and effector cells, the suppressive microenvironment of the MSCs, treatment of MSCs with preprocessed antigens or transfection with mRNA of specific antigens as well as the ratio of target-to-effector cells.

These immunosuppressive effects could be mitigated by specific inhibitors, such as inhibitors of prostaglandin synthesis. In addition, MSCs alter the cytokine secretion profile of DCs, naive T cells, T helper (Th) 1 and Th 2 cells, as well as NK cells by suppressing the secretion of proinflammatory cytokines, such as interferon (IFN-γ), IL-1β and tumor necrosis factor (TNF-α), and increasing anti-inflammatory cytokines such as IL-10 [62], resulting in a more tolerant MSC phenotype.

Immunomodulatory properties of MSCs

In addition to their differentiation potential, MSCs exhibit pleiotropic immune regulatory activities both *in vitro* and *in vivo*, which are mediated by complex mechanisms that inhibit the function of different immune cell subpopulations of the innate and adaptive immunity. These include professional antigen presenting cells (APCs) such as B cells [63], DC and macrophages [64], as well as different effector cells such as NK cells [63], $CD8^+$ cytotoxic lymphocytes (CTL), regulatory T cells (Tregs) [65] and unconventional T cells (NKT and γδ cells). It is worth noting that the *in vitro* results could not be directly translated into the *in vivo* situation because there are differences between MSCs *in vitro* and *in vivo*. These differences might have implications on the immune stimulatory and/or inhibitory activity of immune cells. For example, *in vitro* expanded MSCs lack expression of MHC class II and B7-H2 molecules, whereas both markers are present on MSCs *in vivo* [64].

T lymphocytes and regulatory T cells

In general, the proliferation of T lymphocytes is induced in response to anti-CD3- and anti-CD28-specific antibodies or alloantigens. By contrast, MSCs inhibit T cell proliferation induced by mitogens, CD3 and CD28 antibodies as well as by allogeneic antigens, as determined by in vitro mixed lymphocyte reactions and transplantation of MSCs across MHC barriers. Thus, suppression of T cell proliferation did not require MHC restriction. The

expression of activation markers such as CD25, CD3[8] is reduced in lymphocytes by MSCs exerting similar effects on naıve and memory $CD4^+$ and $CD8^+$ T cells. Upon co-cultivation of MSCs with peripheral blood mononuclear cells (PBMCs) CCL1 and sHLA-G were induced, leading to a shift from allogeneic T cell responses to the secretion of a Th2 cytokine profile and inhibition of cytotoxic T cell-mediated lysis of allogeneic cells. In addition, other inhibitory factors secreted by MSCs upon interaction with immune effector cells, such as PGE-2, IDO and TGF-β, negatively interfere with T cell activation and function. Moreover, MSCs have been shown to modulate immune responses by the de novo induction and expansion of $CD4^+CD25^+FoxP3^+$ and $CD8^+$ regulatory T cells (Tregs) [66], which are responsible for inhibiting allogeneic lymphocyte proliferation. The MSC-mediated induction of Tregs is caused not only by direct cell contact between MSCs and $CD4^+$ T cells but also by the secretion of PGE-2 and TGF-β1. Finally, a functional role for the constitutive expression of HLA-G and the co-inhibitory molecule B7-H4 on MSCs has been reported, their constitutive expressions reduces an immunosuppressive effect on T cell activation, proliferation, and/or NK and T cell-mediated cytotoxicity. The inhibition of HLA-G or B7-H4 by respective blocking antibodies significantly increased lymphocyte proliferation, suggesting that both molecules are involved in the immunosuppressive properties of MSCs [53]. Although different mechanisms involved in the immunosuppressive activity of MSCs on T cells have been elucidated, there still remain several questions to be answered, in particular regarding the high concentration of MSCs required for mounting these immunosuppressive effects.

Induction of T Lymphocyte Anergy by MSCs

Naive T cells must receive two signals in order to be activated upon antigenic stimulation. The first signal results from the interaction between the T-cell receptor and the MHC molecules while the second signal is the costimulatory signal which results from the

interaction between CD28 and B7 molecules. In the absence of costimulatory signal, the T cells become anergic, i.e., they cannot proliferate or secrete IL-2 upon antigenic stimulation. However, the addition of IL-2 can abrogate anergy. Human and mouse MSCs do not express the costimulatory molecules CD40, CD80, and CD86, but they can render T lymphocytes anergic [67]. Nevertheless, the removal of mouse MSC from T cell co-culture could restore the production of IFN-γ but failed to reverse the proliferation of T cells despite the addition of exogenous IL-2 [68]. MSCs were found to arrest T cells in the G0/G1 phase of the cell cycle. But this form of T-cell unresponsiveness, which is triggered by MSCs, is not the classical form of anergy since it is not reversed by exogenous IL-2, although T cells express IL-2 receptor; thus indicating that MSCs induce in a state of division known as “tolerance arrest” in T cells [68]. The lack of expression of costimulatory molecules by MSCs and their ability to induce anergy in T lymphocytes supports the use of MSCs as therapy in regenerative medicine.

Induction of T Lymphocyte Apoptosis by MSCs

Several studies contradict each other as to whether MSCs inhibit the proliferation of lymphocytes by apoptotic mechanisms or not. Human MSCs inhibit the proliferation of T cells by inducing apoptosis in T cells via a mechanism involved IDO and IFN-γ [69]. A recent study demonstrated that fetal liver MSCs can inhibit T cell proliferation by inducing apoptosis in T cells through a HLA-G independent mechanism [70]. In contrast, other studies have reported that human Bone Marrow SCs (BMSCs) and pluripotent MSCs (pMSCs) inhibit the proliferation of T cells by a non-apoptotic mechanism since the proliferation of T cells efficiently resumed when restimulated with cellular or humoral activators in the absence of MSCs [63, 71]. The nonapoptotic mechanism mediating the immunosuppressive effect of MSCs on lymphocytes was confirmed by others [63, 71, 72].

Limbal Stem Cells

The junction of the cornea and conjunctiva is known as the limbus. It's now extensively used for ocular surface resurfacing in patients with limbal stem cell deficiency (LSCD).The cornea is located in the anterior part of the eye and acts as a protective barrier to the interior structures. It consists of three major layers, which are derived from different germ layers (Fig.4). The epithelial layer of the cornea develops from ectoderm, whereas the stroma and endothelium are mesenchymal in origin.

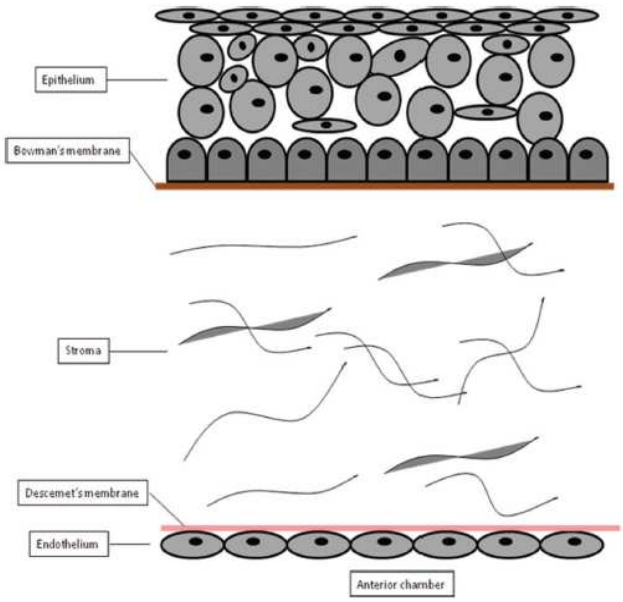


Figure 4. Diagrammatic representation of human cornea showing the three main layers: epithelium, stroma, and endothelium. The Bowman's membrane is an acellular layer that lies in the anterior stroma, just beneath the basement membrane of the epithelium. The Descemet's membrane is the basement membrane of the endothelium.

There are six to eight cell layers of the epithelium, which lies on the Bowman's membrane. The majority of the corneal thickness is contributed by the stromal layer, whereas the endothelial monolayer sits on the Descemet's membrane, making up the most posterior part of the cornea. The cornea is devoid of blood vessels and is assumed to be protective of immune rejection of transplanted grafts, a condition termed "**corneal immune privilege"** [73]. Its avascularity implies a lack of angiogenic factors or the possibility that it may secrete antiangiogenic factors. Vascularization evokes an immune response and has implications for graft allorejection. In addition, the absence of corneal lymphatics prevents the channeling of

antigen-presenting cells to the regional lymph nodes, thus not allowing alloantigen-specific T cells to be activated. Activated T cells travel to the graft bed and initiate the crucial process of graft rejection. However, the relative ease of topical steroid application on the cornea and the immune tolerance in the anterior chamber also contribute to the relative success rate of corneal transplantation [74].

The limbus is a highly specialized region of the eye hosting a well-recognized population of epithelial stem cells (LESCs), which continuously renew the corneal surface [75]. The limbal niche is characterized by stromal invaginations that provide anatomical and functional dimensions to maintain “stemness,” protect stem cells from traumatic and environmental insults, allow epithelial-mesenchymal interactions, and supply access to chemical signals that diffuse from the rich underlying vascular network. A critical advantage of limbal cells is that they are easily accessible with a well-established and minimally invasive procedure. LESCs have been widely characterized [76] and investigated for their differentiation potential, which seems to be restricted, corneal fate. However, human ocular stem cell research has been mainly focused on the tissue-specific differentiation that may be of clinical significance in the context of eye diseases, as demonstrated by their clinical use in ocular surface reconstruction There is recent evidence that the limbal niche also hosts stromal fibroblast-like stem cells (f-LSCs), with apparent multilineage transdifferentiation potential [77]. Pheno-type of f-LSCs is reportedly characterized by variable expression of several stem cells markers, which are distinct from those described for LESCs. However, the lack of agreement on specific molecular hallmarks for the identification of the pluripotent subpopulation among the limbal stromal cells has so far limited the investigation of their differentiation potential to a few studies. We previously described a subpopulation of f-LSCs characterized by robust proliferative capacity, stable expression of several pluripotent stem cell markers, self-renewal ability and capability to generate pancreatic endocrine cells [78]. It also has recently documented that stem cells obtained from limbal murine adult tissues possess

immunoregulatory properties and inhibit proinflammatory immune reactions [79]. Here we have explored the immunosuppressive properties of the stromal stem cells niche isolated from the human limbus.

Etiopathogenesis of autoimmune thyroid disease

Autoimmune thyroid disease (AITD) comprises diseases including Hashimoto's thyroiditis (HT) and Graves' disease (GD), both characterized by reactivity to autoantigens causing, respectively, inflammatory destruction and autoimmune stimulation of the thyroid-stimulating hormone receptor. AITD is the most common thyroid disease and the leading form of autoimmune disease. These organ specific autoimmune disorders include infiltration of the thyroid by lymphocytes which are auto-reactive to thyroid antigens, presence of circulating thyroid autoantibodies, immunological overlap with other autoimmune diseases, a story of familiar occurrence, mainly in females. It occurs due to loss of tolerance to autoantigens thyroid peroxidase (TPO), thyroglobulin (Tg), thyroid stimulating hormone receptor (TSH-R) which leads to the infiltration of the gland. T cells in chronic autoimmune thyroiditis (cAIT) induce apoptosis in thyroid follicular cells and cause destruction of the gland. Presences of TPO antibodies are common in HT and GD, while Tg has been reported as an independent predictor of thyroid malignancy. According to American Thyroid Association large numbers of patients develop thyroid nodules by the age of 60, although majority of these nodules are benign. The Whickham study recorded 9.3% of women and 1.2% of men to have serum TSH concentrations > 10 mIU/l. Presence of TPO antibodies was significantly associated with thyroid failure with increasing age, mainly in women [80]. Antigen-presenting cells (APCs) (macrophage, dendritic cells) belonging to major histocompatibility complex (MHC) class II, especially dendritic cells, accumulate within the thyroid gland and present specific thyroid antigens to lymphocytes, which leads to activation and proliferation of auto-reactive B and T lymphocytes. Thus, activated antigen-specific T-helper $CD4^+$ lymphocytes induce the

formation of cytotoxic $CD8^+$ T cells, and activate B cells, which produce autoantibodies. The destruction of thyroid parenchyma is due to gland infiltration by cytotoxic T cells [81]. Cytokines are small proteins play an important role in autoimmunity, by stimulating B and T cells. Various cytokines IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL- 14, TNF-α and IFN-γ are found in thyroid follicular cells which enhance inflammatory response with nitric oxide (NO) and prostaglandins.

It has been proposed that MSCs can contribute to the control of inflammatory diseases, as has been demonstrated by the MSC-mediated attenuation of inflammation in myocarditis (14), rheumatoid arthritis (15), and experimental autoimmune diseases (16, 17). Here we proposed, for the first time, an *in vitro* co-culture system to investigate the immunoinhibitory effect played by f-LSCs on PBMCs of HT patients. For their immunosuppressive properties, f-LSCs can prevent the inappropriate activation of T lymphocytes and generate a tolerogenic environment to stop the immune response thus contributing to the maintenance of immune homeostasis.