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The Immunostimulatory Potential of Mesenchymal Stem Cells for formation of Tertiary Lymphoid Structures in Lupus Nephritis

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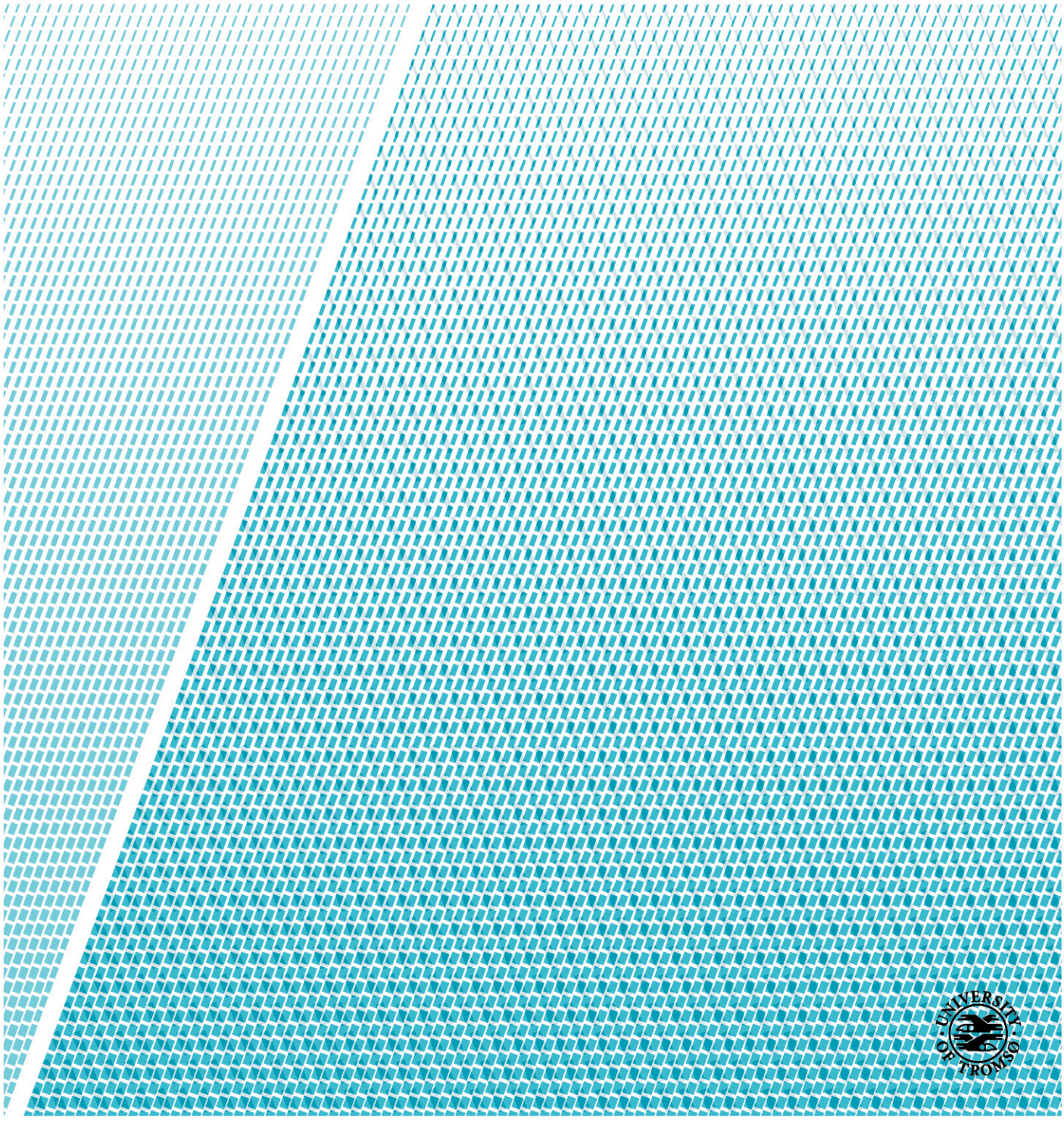
Master's thesis in Biomedicine (MBI-3911), May 2017



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Aud-Malin Karlsson Hovd
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Abstract

Formation of tertiary lymphoid structures (TLS) occurs in tissues targeted by chronic inflammatory processes, such as infection and autoimmunity. In systemic lupus erythematosus (SLE), TLS have been observed in the kidneys of lupus-prone mice and in kidney biopsies of SLE patients with Lupus Nephritis (LN). Here the role of tissue-specific mesenchymal stem cells (MSCs) as lymphoid tissue organizer cells on the activation of CD4+ T cells from three groups of donors; Healthy, SLE patients and LN patients were investigated.

Human MSCs were stimulated with the pro-inflammatory cytokines TNF-α and IL-1β to resemble an inflammatory condition. $CD4^+$ T cells isolated from PBMC were co-cultured with stimulated and non-stimulated MSCs at 1:1 and 1:100 ratios (MSCs:$CD4^+$ T cells) or seeded alone as a control. The AlamarBlue® proliferation assay was performed on $CD4^+$ T cells at day zero and at day 5, 7 and 10 after co-culture. Flow cytometric analyses were conducted on $CD4^+$ T cells at day zero and day 10 to analyse the Th1, Th2, Th9, Th17, Th22, and Th1/17 subsets before and after co-culturing with MSCs. To detect MSCs within TLS in kidneys of lupus-prone (NZBxNZW) F1 mice confocal imaging was used.

After stimulation a significant increase in the expression of CCL19, VCAM1, ICAM1, TNF-α, and IL-1β were observed in MSCs. For all groups $CD4^+$ T cells co-cultured with stimulated MSCs and non-stimulated MSCs at 1:100 ratio proliferated significantly more at day 10 compared to day zero and $CD4^+$ T cells cultured. $CD4^+$ T cells co-cultured with stimulated MSCs at 1:100 ratio proliferated significantly more than co-cultured with non-stimulated MSCs at day 10 in healthy and SLE groups, but not in the LN group. No difference in cell proliferation at 1:1 ratio was detected. An increase in Th2 and Th17 subsets were observed in the healthy group at day 10 when co-cultured with stimulated MSCs at 1:100 ratio compared to day zero and $CD4^+$ T cells alone at day 10. MSC-like cells were detected within the pelvic wall of the kidneys and within the developed TLS.

Our data suggest that tissue-specific MSCs could have pivotal roles in accelerating early inflammatory processes and initiating the formation of TLS in chronic inflammatory condition.

Abbreviations

ACR	American College of the rheumatology	***ICAM-1***	Intercellular Adhesion Molecule 1
AIR	Annual incidence rate	***IFN***	Interferon
ANAs	Antinuclear antibodies	***Ig***	Immunoglobulin
BCR	B cell receptor	***IL***	Interleukin
CCR	C-C chemokine receptor	***LN***	Lupus nephritis
CD	Cluster of differentiation	***LT***	Lymphotoxin
CTL	Cytotoxic CD8+ T cells	***MALTs***	Mucosal associated lymphoid tissues
CXCR	C-X-C motif chemokine receptors	***MHC***	Major histocompability complex
DCs	Dendritic cells	***MSCs***	Mesenchymal stem cells
dsDNA	Double stranded deoxyribonucleic acid	***SCA-1***	Stem cell antigen-1
ECM	Extra cellular matrix	***SLE***	Systemic lupus erythematous
FcR	Fragment-crystallize receptor	***SLEDAI***	SLE disease activity index
FDCs	Follicular dendritic cells	***SLOs***	Secondary lymphoid organs
FoxP3	Forkhead box P3	***TCR***	T cell receptor
FRCs	Fibroblastic reticular cells	***TGF***	Transforming growth factor
GC	Germinal centre	***Th cells***	CD4+ T helper cells
HEV s	High endothelial venules	***TLR***	Toll like receptor
HMLE	Human mammary epithelial cells	***TLSs***	Tertiary lymphoid structures
HSCs	Hematopoietic Stem cells	***TNF***	Tumour necrosis factor
HUV-EC-C	Human Umbilical Vein Endothelial Cells	***VCAM-1***	Vascular cell adhesion protein 1
ICs	Immune complexes	***PBMC***	Peripheral blood mononuclear cells

1 Introduction

1.1 Immune system, tolerance and autoimmunity

The immune system is a creation of the evolution as a defence against the potential danger pathogenic infections generate. The immune system can roughly be divided into two main compartments the innate and the adaptive immune system, where the "communication" between these parts plays an important role in the development and control of the disease progression. The innate immune system involves most cell types in the body and mediates a general protection against infection, and can for example mediate an activation of the adaptive immune system through antigen presenting cells such as dendritic cells (DCs) and macrophages.

The specificity of the immune system, the power of which a single pathogen are recognized and eliminated, lies within the adaptive immune system. The effector cells of the adaptive immune system are the B and T cells with their respectively B cell receptor (BCR) and T cell receptor (TCR) [1]. With the two processes of selective somatic genome modification; V(D)J recombination of both TCR and BCR in primary lymphoid tissues and somatic hypermutation of the BCR in peripheral lymphoid tissue, receptors with a enormous diversity can be produced [1]. Interesting 20-50% of the V(D)J recombined BCR and TCR can in the theory bind with high affinity and react to a self-antigen, but only 3-8% of the world population is affected by autoimmune diseases [1].

One of the reasons why the percentages of the developing autoimmune diseases in the world population are so low compared to the percentages of autoreactive BCR and TCR, is the tolerance mechanisms involved in the developing lymphocytes and controlling of lymphocytes [1]. There are two main types of tolerance mechanisms acting on the developing lymphocyte: central and peripheral tolerance [2], illustrated in **Figure 1.1** and further explained in section ***1.4 Lymphoid organs*.**

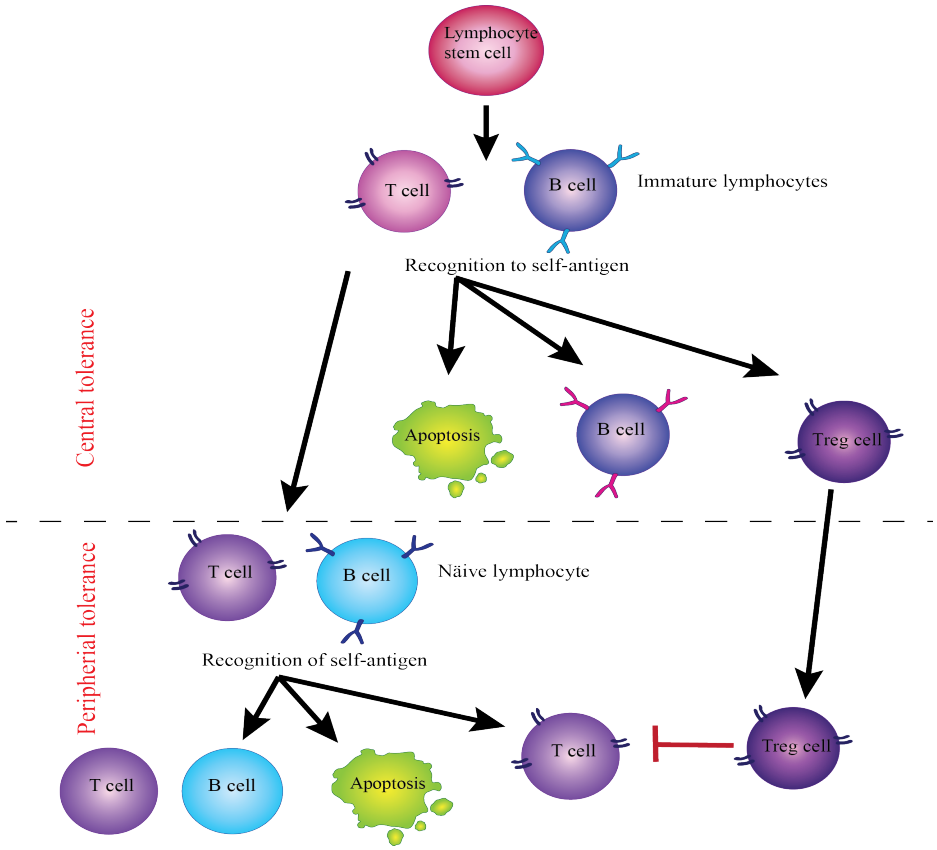


Figure 1.1: Tolerance mechanisms for controlling and removing of autoreactive lymphocytes.
In central tolerance an autoreactive lymphocyte can be removed during their development in either the thymus or in the bone marrow. There are three main mechanisms for central tolerance; BCR change in autoreactive B cells, development of Treg cells or that the autoreactive lymphocyte dies via apoptosis. In peripheral tolerance the autoreactive T or B cell can either be functional inactive in a process known as anergy, die via apoptosis or of autoreactive T cells be controlled by Treg cells. Modified from ref [2]

Autoimmunity arise when an organisms immune system start to produce an immune reaction against its own cells, tissues and/or organs [3]. The knowledge about the existence of autoimmune diseases has been known for over 100 years with more than 80 human diseases being investigated, but the underlying initiation mechanisms for why autoimmunity exists are still a mystery among scientists [3]. It is clear that there are three main phases of an autoimmune disease; initiation, propagation and resolution (**Figure 1.2**), which all can be linked to a deficiency in the immune regulatory mechanisms [4].

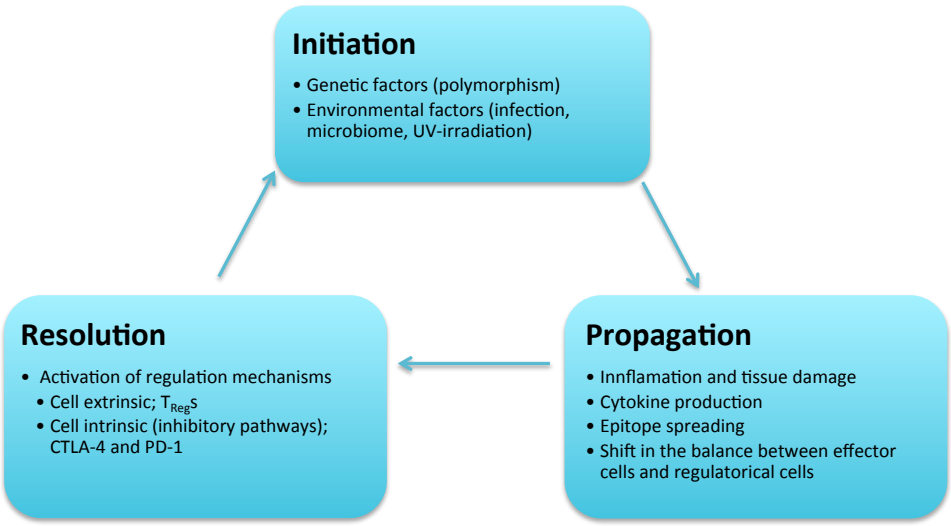


Figure 1.2: Common disease progression in autoimmune diseases. Normally when the patients are in the resolution phase, a relapse occurs and acute inflammation with tissue damage are observed [4].

1.2 Systemic Lupus Erythematous

Systemic Lupus Erythematous (SLE) is an autoimmune rheumatic disorder that can affect multiple organs systems, which is a result after a loss of immunological tolerance and immune response against self-antigens [5, 6]. The diagnosis of SLE follows the 1997 updated version of the 1982 revised criteria by the American College of the rheumatology (ACR) [7, 8] where it is required that the patient have the presence of four out of eleven criteria before the diagnosis SLE is set [6, 7]. In addition to the ACR classifications the severity of SLE is assessed by the *SLE disease activity index* (SLEDAI), which is a scoring system that includes 24 clinical and laboratory variables that are weighted differently according to how life threatening the manifestation is [9, 10]. From these criteria it is obvious that this disease may affect some if not most of the vital organs and tissues of the body, implying it is crucial that the diagnostic tools and therapeutic agents are further developed to improve the life and health quality of the patient.

It is common knowledge that one of the major risk factor for SLE is gender, observed by that at least 9 out of 10 patients are women [6, 11, 12]. The incidence of SLE is varying worldwide depending on the geographic and ethnicity. Studies from United Kingdom (UK) and North America observed that Afro-Americans and Asians had a higher risk to develop SLE than other ethnical groups [13]. In Scandinavia the total annual incidence rate (AIR) is lower, between 2.35-3.5/100 000 [5, 10, 13, 14], compared to countries with a much wider ethnicity spectrum such at United Kingdom (total ACI; 4.7-4.9/100 000)[15, 16] and USA (Total ACI; 5.5-7.22/100 000) [17, 18]. In the study by Lerang and colleagues from 2012 the

prevalence of SLE in Norway were calculated to be in total 51.8/100 000, where the prevalence for women were 91.0 and for men 10.7 [13]. At approximately the same time Lerang published her data, Hermansen and colleagues calculated the prevalence of SLE in Denmark. Interesting the prevalence from Denmark were lower than the prevalence in Norway with a total prevalence of 45.2/100 000, 79.6/100 000 for women and 10.1/100 000 for men [5].

1.2.1 Pathology SLE

The one and exact factor for the disease development in SLE is sadly still unknown, making it difficult to predict, diagnose and treat. In **Figure 1.3** some of the more common factors that might stimulate disease development are illustrated, but it is wise to be aware that development of the disease often are caused by a mix of several factors [19, 20]. **Figure 1.3** also illustrates some of the most common immunological effects and which organ that are associated with SLE. One of the most central immunological disturbance in SLE is the production of autoantibodies, which is an important contributor in the pathogenicity and diagnostic of the disease. Antinuclear antibodies (ANAs), which are found in 90 to 95% of SLE patients [21], are antibodies that can recognize and bind to components in the cell nucleus, such as DNA, RNA, nuclear protein, and the protein-nucleic acid complexes nucleosome and spliceosome [21, 22]. In SLE there are several factors that could contribute to the development of autoantibodies, but the deficiency of removal of apoptotic cell debris might be one of the leading mechanisms [23].

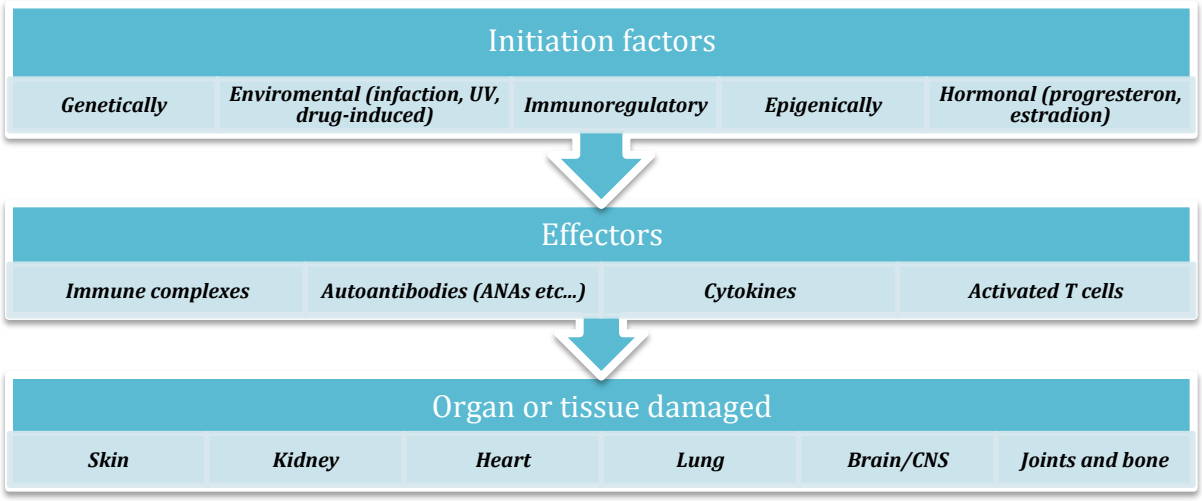


Figure 1.3: Overview of some of the pathogenically hallmarks of SLE. This illustration was inspired by "Figure 1" in the review article "*Mechanisms of disease - Systemic Lupus Erythematous*" published in 2011 by G.C Tsokos.

1.2.2 Lupus nephritis

Lupus nephritis (LN) is the kidney manifestation of the SLE autoimmune disease and is one of the more severe manifestations of SLE [21]. 25% to 60% of the patients with SLE are affected by this renal manifestation and this occurs often during the first year of disease course [10]. Classification of LN follows the classification system provided by the International Society of Nephrology and the Renal Pathology Society from 2003 (ISN/RPS 2003) [24]. This criteria system is based on the glomerular changes in LN patients, from when immune complexes deposits (ICs) in the glomerular to when sever scaring occurs and the function of the glomeruli are lost and proteinuria is observed.

The pathology of LN (**Figure 1.4**) is characterized by deposition of immune complexes (IC) in the glomeruli, which will lead to an inflammatory cascade with activation of Fc receptors (FcR) and Toll-like receptors (TLRs) on the cells in the glomeruli and the tubulointerstitium [20, 21, 25-27]. Activation of these receptors will stimulate the production of proinflammatory cytokines such as IL-1, IL6, TNFα and monocyte chemoattractant protein 1 (MCP-1), which again will contribute to the recruitment of immune cells [26, 28]. In addition to the production of proinflammatory cytokines, the cells in the kidney will also start the production of extracellular matrix (ECM) compounds [29]. These ECM compounds promote the scaring formation in for example the glomeruli and causes glomerulosclerosis, leading to organ failure and proteinuria [29].

Deposition of ICs in the glomeruli might also stimulate mesangial cells to proliferate and expand the mesangial matrix, leading to a reduced flow of filtrating in the glomerular capillaries and thereby eventually seal the capillary lumen [30]. The fenestrated endothelial cells in the glomeruli will also be activated and start to express adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, when the ICs are deposited in the glomeruli [25, 31, 32]. These adhesions molecules are important for the recruitment and infiltration of immune cells to the subendothelium and mesangium [25, 32]. The deposition of ICs in glomeruli and with the infiltration of immune cells and expanding mesangium, add a huge stress on the specialized glomerular epithelial cells; the podocytes. One of their main responses is the loss of their characteristic pattern of foot processes in a process called effacement in podocyte foot processes [33]. In a study by Wang et al. from 2014, a positive correlation between the widths of the foot processes and the level of proteinuria was observed [34]. These findings might be explained with that the foot processes are important for the filtration processes in the glomeruli, and when they are damaged the filtration will be affected and interrupted in a way leading to proteinuria [33].

Infiltrating leukocytes are also associated with formation of tertiary lymphoid structures (TLS) with active germinal centres (GCs), which will promote B cell differentiation into antibody secreting plasma cells and stimulate a local production of autoantibodies [26]. Chang and colleagues published in 2011 the first article where they describe how infiltrating immune cells are capable organize into B-T cell aggregates and GCs in lupus patients with nephritis [35]. From this study, they found that there was a correlation between the B-T cell aggregate and GCs formation and the IC deposition in the tubular basement membrane [35].

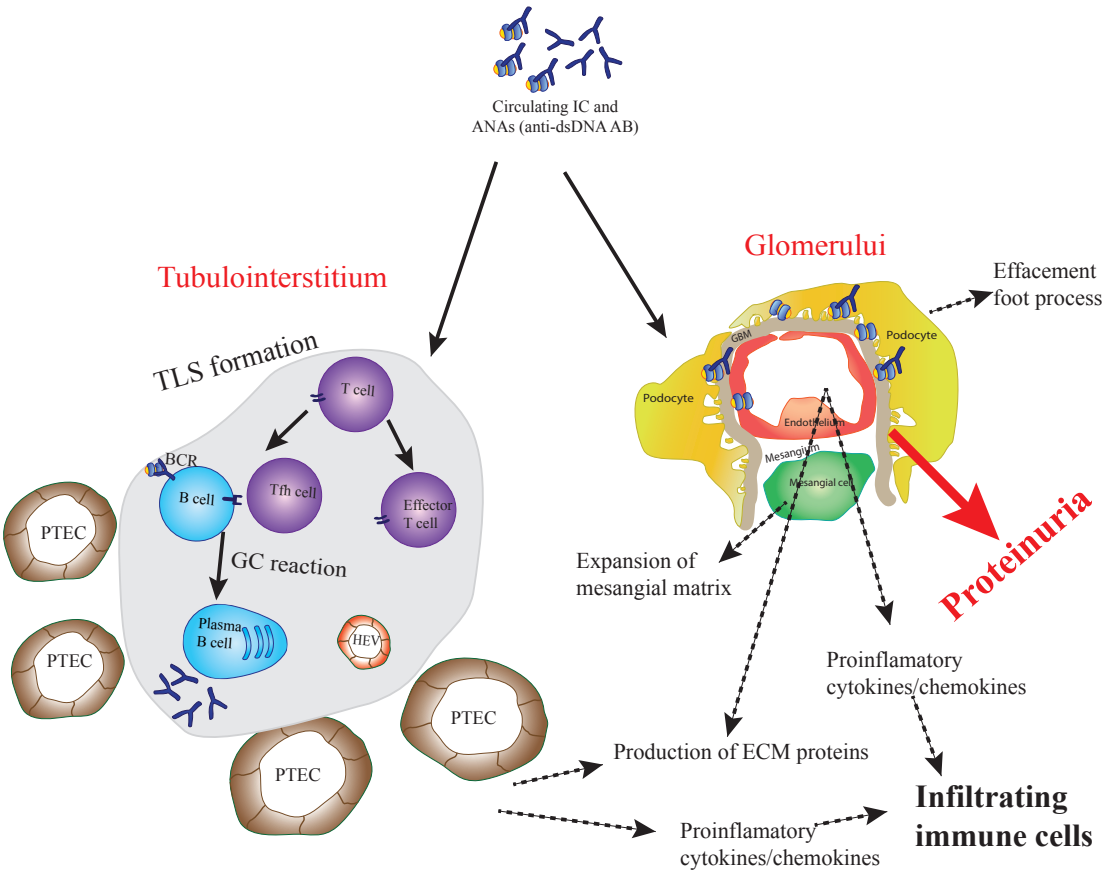


Figure 1.4: Some of the possible outcome when circulating immune complexes and ANAs are deposited in the kidney. When ICs are deposited in the glomeruli or in the tubulointerstitium they will activate the cells in the tissue to produce proinflammatory cytokines and chemokines, which will recruit immune cells to the site of inflammation. The kidney cells will also have an increased production of ECM components leading to fibrosis. TLSs have been observed in kidneys of LN patients and in murine models of LN. The end outcome of the damaged kidney is eventually kidney failure with proteinuria. Based on ref [20, 26, 29, 34, 35]

1.3 Immune cells

The soldiers of the immune system are the immune cells, which are composing both the innate and adaptive immune system (**Figure 1.5**). The innate immune system could be viewed as the first line defence and the cells in this part of the immune system are the first one to react to a pathogen exposure [36]. The phagocytic cells, such as neutrophils and macrophages; cytotoxic natural killer cells and granulocytes will carry out the effector functions of the innate immune responses [36, 37]. The adaptive immune responses will immediate an antigen-specific defence with the development of the (long lived) antigen-specific lymphocytes; B and T cells [36, 37]. Antigen-presenting cells, which include cells from the innate immune system such as macrophages and DCs, are important in the activation and priming of the antigen-specificity of the adaptive immune system. In this study the T cells from the adaptive immune system are in focus and will therefore be discussed further.

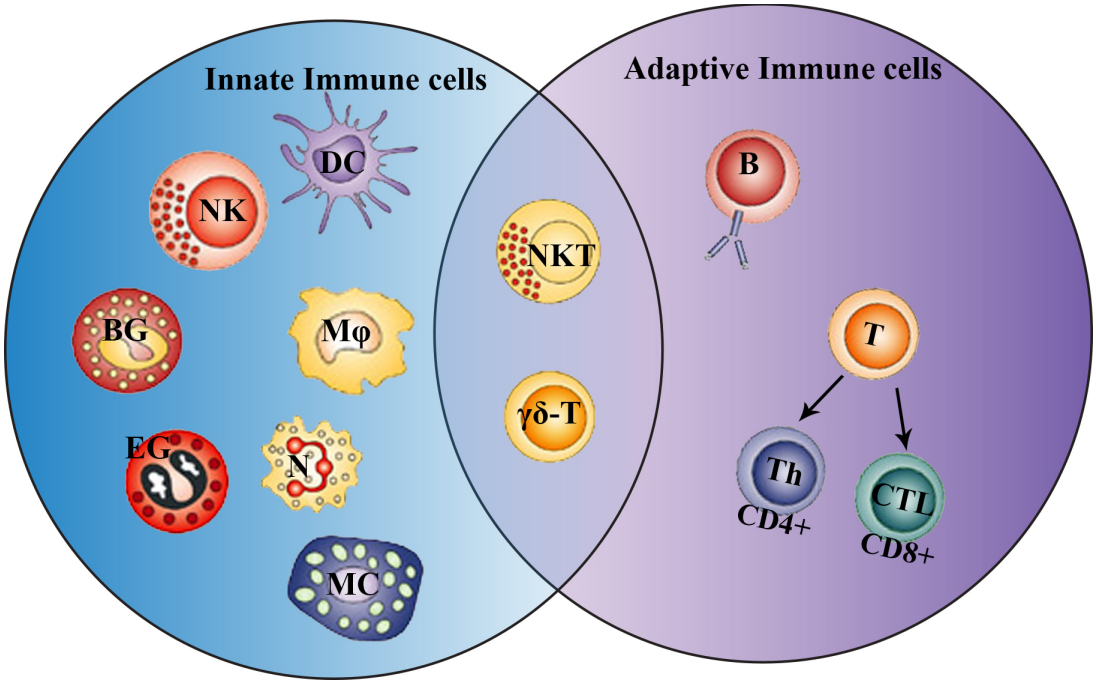


Figure 1.5: Immune cells of the innate and adaptive immune system. The innate immune system consist of the dendritic cells, the granulocytes (basophils, eosinophils, neutrophils), macrophages, NK cells and mast cells, while the adaptive immune system consists of the antibody producing B cell, the CD4+ and CD8+ T ecIls. γδ T cells and NK-T cells cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity. B; B cells, BG; basophil granulocyte, DC; Dendritic cells, EG; eosinophil granulocyte, MC; Mast cell, Mφ; Macrophage, NK; natural killer cells, NKT; natural killer T cells, T; T cells, CD4+Th; CD4+ T helper cells, CD8+ CTL; CD8+ cytotoxic T cells, γδ-T; gamma delta T cells. Figure modified from Figure 1 by Dranoff [37].

1.3.1 T cells and their main linages

T cells are lymphocytes that play an important role in cell-mediated immunity, and are characterized with their expression of their T cell receptor (TCR). These cells develop in the bone marrow and are primary matured in the thymus as discussed in the **Section 1.4.1**. The T cells are broadly divided into the two main linages, αβ and γδ, based on how their TCR are composed of [38]. The first combination of the TCR consist have α and β chains, and most of the T cells belong to this class. TCR of both the sublinage of CD4+ T helper cells (Th cells) and the cytotoxic CD8+ T cells (CTL) consist of α and β chains. These cells will recognize antigens presented on the MHC molecule to either assist other cells in immunological effector functions (Th Cells), or to kill infected cells and cancer cells (CTL). In the αβ linage of T cells consists also of the Natural killer T (NKT) cells, which have both phenotypic and functional characteristics found in both conventional NK cells and αβ-T cells [39]. The NKT cells have the ability to recognize lipid antigens presented by the CD1d-molecule cell types present in the intestine and liver, and could thereby contribute to the immune responses in the digestive system for both promoting health and disease [39].

The second main linage of T cells, the γδ T cells, show several innate cell-like features that permit early activation and recognition of conserved non-peptide ligands presented by stressed cells [40]. Interesting these γδ T cells are mainly located in mucosal tissues and on epithelial surfaces, such as the gut mucosa, skin, lungs and uterus, where they migrate early in their development [40]. The main functions of the γδ T cells varies from a protective immunity against extracellular and intracellular pathogens, tumour surveillance, modulation of both innate and adaptive immune reactions to tissue healing and regulation of the function of a physiological organ [40].

1.3.2 The family of CD4+ Th cells

The CD4+Th cells are further divided in to subsets based on the differentiation of naïve CD4+ T cells, which depends on the antigen, the strength of the TCR signal and the cytokines in the environment [41] (**Figure 1.6**). In 1986 Mosman and Coffman distinguished Th1 and Th2 subsets form each other based on the cytokine profile of these two subsets [42]. The main functions of Th1 cells are involved in cell mediated inflammation, defence against intracellular pathogens and in delayed-type hypersensitivity reactions [43]. The Th1 cells are known for their production of the characteristic cytokines: IL-2 and IFNγ, but they can also produce other cytokines such as TNFα and LTα [43]. The T cells of the Th2 subset are

involved in humoral-mediated immunity and their main function are to defend the host against extracellular pathogens, but unfortunately the Th2 subset are also associated with allergy, eczema and asthma [43, 44]. The characteristic cytokine profile of the Th2 subset consists of IL-4, IL-5 and IL-13, as well as IL-9 ad IL-10. IL-4 is a cytokine with several functions for other lymphocytes and for cells from the innate immune system. The IL-4 cytokine promote activation in macrophages and monocytes, stimulate development and maturation of dendritic cells, and for plasma cell differentiation and antibody isotype switching to IgG1 and IgE [43].

After the discovery of Th1 and Th2 subsets of CD4+T cells, several subsets of CD4+ T cells have been classified such as Th9, Th17 and Th22 cells, T regulatory cells (Treg) and follicular helper T cells (Tfh) [43]. The Th17 subset is characterized by its expression of IL-17, primarily IL-17A and IL-17F, in addition to their expression of TNFα, IL-6, IL-22, IL-21 and IL-26 [43, 45]. The main function of the Th17 subset involves the host defence against extracellular bacteria, fungi and viruses [43, 45], where these cells stimulate production of antimicrobial peptides, increase the barrier function of epithelial cells and lead to recruitment of neutrophils and monocytes to the site of inflammation [46]. The Th9 subset of the CD4+ T cell repertoire are one of the main producers of IL-9, which will stimulate inflammation by promoting the growth of leukocytes such as mast cells and the secretion of chemokines that will stimulate the recruitment of more immune cells to the site [47]. In addition to their production of IL-9, the Th9 cells can also produce IL-10, which is an anti-inflammatory cytokine and indicating that Th9 cells might perform immune regulatory mechanisms [43]. The newest member to the Th subset is the Th22 cell, which has several similarities to the Th17 cell with the production of IL-22 [43]. The IL-22 is a member of the IL-10 family, indicating that the role of Th22 cells in host defence acts on non-immune cells and promote enhancement of innate immunity and tissue regeneration [43, 48].

The linage of Treg cells are a subset of specialized T cells that execute suppressive functions of other effector T cells and could be seen as a “police” or a “break” of the immune system. Their main task is to control and supress overactive immune cells [43]. Some examples where they have an important role are: their suppression of allergy; in the establishing of tolerance to organ grafts and to prevent graft-versus-host disease; and to promote feto-maternal tolerance in pregnant women [49]. αβTCR linage of Treg cells are characterized by their transcription factor FoxP3 and can be developed in the thymus (nTregs), as a result of central tolerance, or they could be induced via post-thymic maturation

(iTreg) that often are characterized as CD4+CD25+FoxP3+ [43]. The Tregs uses multiple methods to perform their effector functions: one through modulation of APCs and thereby indirectly supress T effector function, or by directly suppress T and B cells [50]. Treg cells can secrete of anti-inflammatory cytokines (IL-10 and TGFβ), which will inhibit the proinflammatory effector functions of lymphocytes and APCs [50]. Treg cells are also capable with expression of inhibitory receptors (CTLA-4) [43], which can down regulate the expression of MHC-II molecule and the costimulatory molecules CD80/CD86 and CD28 on the APC [43, 49]. The Treg can induce apoptosis in T cells or APCs through cell-to-cell contact by a granzyme or perforin mechanism, or via the stimulation of tryptophan catabolism in APCs through indoleamine 2,3-dioxygenas (IDO) that produce the T cell toxic compound kynurenines [49].

The follicular helper T cells (Tfh) are a specialized subset of T cells whose main task is to provide B cell help in the GCs together with follicular dendritic cells (FDCs) [51]. Through their expression of surface molecules and chemokines such as; CD40L, IL-21, IL-4, PD-1, and BAFF, they will regulate the B cell survival and proliferation, participate in the initiation of somatic hypermutation and differentiation of B cells into plasma B cells and memory B cells [51]. The Tfh can also induce apoptosis via Fas-FasL interactions in B cells that fail to present cognate antigen [51].

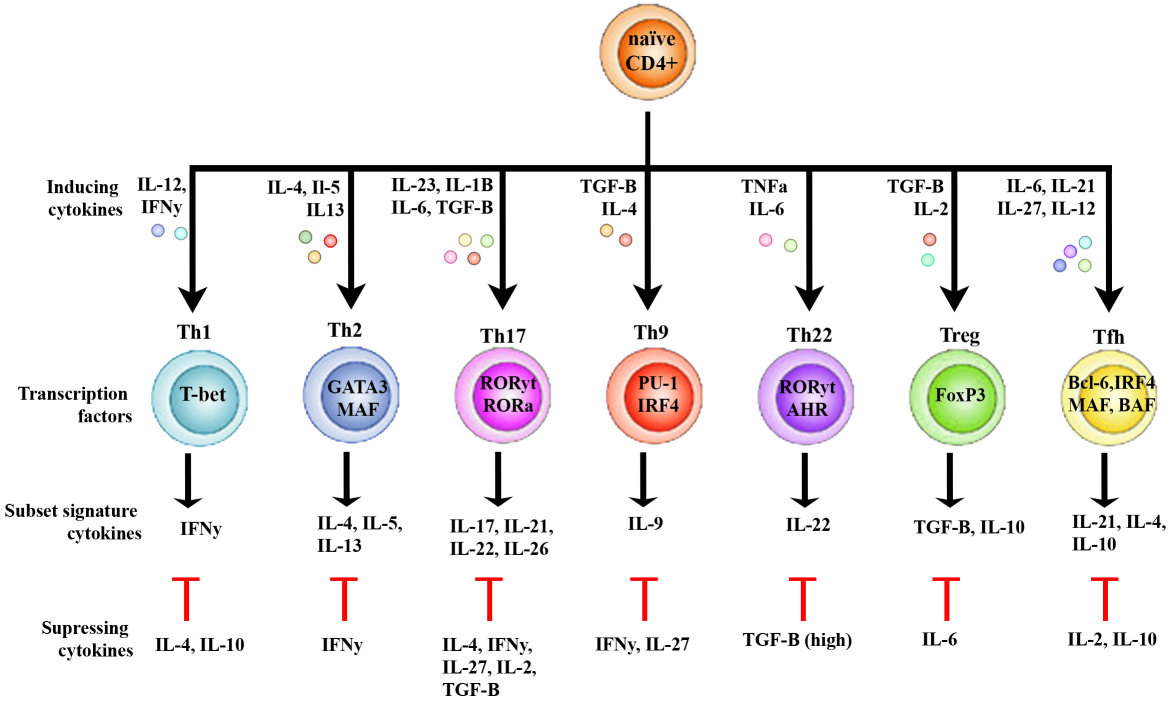


Figure 1.6: The CD4+ Th subsets; their inducing cytokines, their expression of transcription factor, and their main production of chemokines. The chemokines and transcription factors included are only some of the factors that will determine the fate of the naïve CD4+ T cell. Information from Table 1 by Tangye et.al (2013) [48]

1.3.3 CD4+ T helper cells and their role in lupus

It is observed that T cells from patients have abnormal phenotypes and functions [52], which can lead to exaggeration of TCR response to a stimuli and the T cells get activated [19, 53]. The CD4+ T helper cells are important in the production of autoantibodies and tissue inflammation, and they have a strong connection to the pathogenicity of SLE and LN [54] (**Figure 1.7**). All the cytokines produced by Th1 and Th2 are important for the pathogenicity of SLE and lupus prone mice that are IFNγ$^{-/-}$ and IL-4 $^{-/-}$ have reduced lymphadenopathy and end-organ disease compared to the cytokine sufficient control group [55]. In murine models of SLE, deficiency in the IFNγ and IL-4 have shown to be important for the pathology of the disease [43, 56].

Today it is clear that other subsets of T cells, both within the Th family and in other subset of T cells, are of high importance in the contribution to the immune disturbance in SLE [6, 57]. An increase in the IL-17-levels in the blood and tissues (kidney, skin) from lupus patients have been observed [54], indicating that the Th17 cells are involved in the pathogenicity of lupus. From murine models IL-17 have shown to promote spontaneous formation of ectopic GCs, stimulate loss of B-cell tolerance and maturation of B cells into plasma cells, in addition to induce autoantibody production in these B cells [43, 54, 56]. IL -17 have also been associated with infiltration of NK cells and neutrophils, and an increased IFNγ production by NK cells, CTLs and Th1 cells has been detected in patients with nephritis [56]. Circulating follicular helper-like CD4+ T (cTfh-like) cells have been observed to be associated whit the disease activity in SLE patients were, which can indicate that the regulation of the maturation of naïve B cell might be disturbed and promote the development of ectopic GCs [58, 59]. The development of the different Th effector-subsets and the Tfh are under control of Treg cells [49]. If the balance between the effector and regulatory cells is disrupted, the chances of developing autoimmune diseases are increased [49]. Impaired functions and reduced numbers of Treg cells in patients with lupus have been reported, and are linked to the disease progression in SLE (reviewed in [60]). In 2005, Hatashi and colleagues found depletion of Treg cells in murine models resulted in increased titers of ANAs and an early development of glomerulonephritis compared to the control [61]. These results support the importance of Treg in the control of effector functions of the immune system.

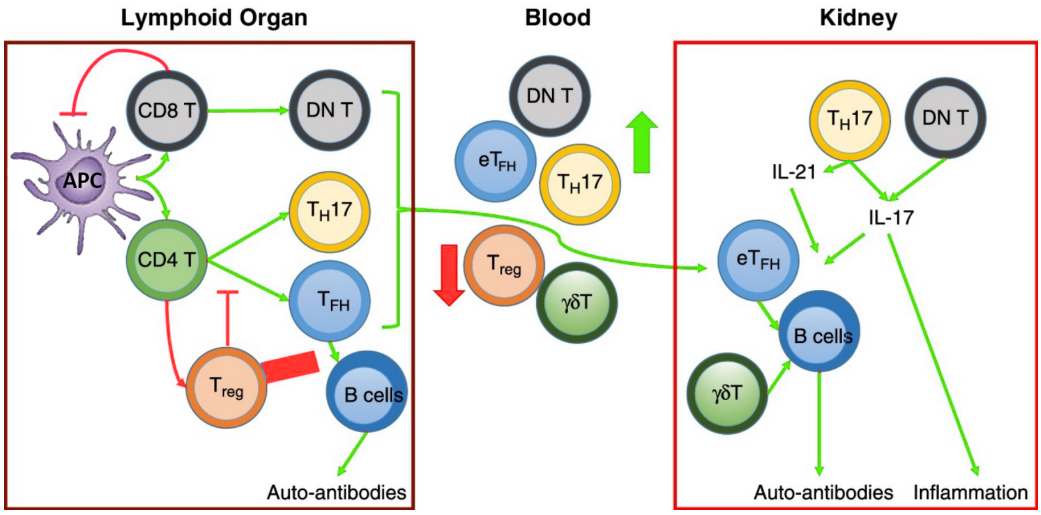


Figure 1.7: Dysregulation of T cell function and subset population in SLE pathogenesis. Reduced effector functions of the Treg and the CD8+ CTL stimulate an increase in pro-inflammatory Th and Tfh subsets, which infiltrate tissues, enhance the inflammation processes, and stimulate autoantibody production. DN-T cells are also observed to contribute to the disease pathology through their production of IL-17. Green arrows indicating upregulated pathway and red arrows indicating down regulated pathway. Modified from figure 1 from Suárez-Fueyo et.al (2016) [54]

1.4 Lymphoid organs

Even though good defences against infections are scattered in tissues throughout the body, the optimized structures to create pathogen specific lymphocytes include the lymphoid organs. These organs are important for the production and activation of "combat approved" lymphocytes, which during normal conditions are not self-reactive. The lymphoid organs can be divided into three main categories; primary, secondary and tertiary lymphoid organs or tissues, and their main function and development will be discussed in this section.

1.4.1 Primary lymphoid organs

Primary lymphoid organs are defined as organs, or compartments within organs, where hematopoietic progenitor cells differentiate into an abundance of immune cells capable of performing effector functions [62]. There are three main types of primary lymphoid organs: the bone marrow, the thymus and the fetal liver, which will follow a programmed development during embryogenesis [62]. In addition to produce immune cells with effector function, the primary lymphoid organs also hold the site of central tolerance mechanisms (**Figure 1.1**).

In the adults the bone marrow harbour the source of self-renewing stem cells such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells, in addition to various progenitor cells that have started their pathway in differentiation

and specialization into specific cell types [62, 63]. One of the main functions of the bone marrow is to produce erythrocytes, platelets and leukocytes such as neutrophils, monocytes and mast cells, just to mention some of the common leukocytes. The bone marrow is also the site of B cell maturation, where IgM producing B cells are developed before they are distributed into the blood. The central tolerance of B cell maturation occurs in the bone marrow, where a combination of positive and negative signals from the BCR and co-receptors together with signals from survival factors (i.e. BAFF) will determine the fate of the B cell [2, 64]. The three outcomes of these signals are the survival and activation of the developing B cell, the clonal deletion by apoptosis or the B cell will reach an inactivated anergic phase. Self-reactive B cells can undergo receptor editing, where an additional light chain VJ recombination and new Ig light chain production occurs, in the hope of changing the BCR specificity not self-reactive. If the outcome of the receptor editing still creates a self-reactive B cell, the B cell dies via apoptosis [64].

The thymus is the main location where lymphocyte progenitors cell undergo a multistep maturation, differentiation, expansion and selection program to become either a naïve CD4+ or CD8+ T cell [62], which can be activated in secondary lymphoid organs and mediate cellular immunity. In this maturation program of T cells, there are some essential checkpoints that the developing T cell has to pass before it is released into the circulation as mature naïve CD4+ or CD8+ T cell. T progenitor cells (thymocytes) entry the thymus in the corticomedullary junction and start their journey through out the thymus [65]. At this stage the thymocyte lack the expression of the TCR, CD4 and CD8, and are termed double-negative (DN) thymocytes. In the cortex of the thymus these DN thymocytes goes through four stages of differentiation and simultaneously starts to express their pre-TCR molecule [66]. When the thymocyte manages to successfully express the pre-TCR, the thymocyte will proliferate and become double positive (DP) thymocytes with their expression of CD4 and CD8 [62]. Then the TCR on the DP thymocytes will interact with peptide-MHC complexes that are expressed by cortex stromal cells, such as cortical thymic epithelial cells (cTEC) and DCs in the cortex. Low-avidity interactions will induce the DP thymocytes to receive signals for survival and differentiation into single positive (SP) thymocytes [65]. The next event in the developing thymocyte is the central tolerance, which occurs in the medulla of the thymus. Here the autoimmune regulator (AIRE) expressing medullary thymic epithelial cells (mTEC) will interact with the SP thymocytes through peptide-MHC complexes, and recognize thymocytes which binds to strongly to the complex [65]. These self-reactive thymocytes will either fate death by apoptosis or be stimulated to become FoxP3+ nTreg cells [65]. Sphignosine-1-

phosphate receptor 1(S1P1) expressing mature T cell, which have overcome all the developing checkpoints in the thymus, will be attracted to the circulation where they can be activated and become effector T cells [65].

1.4.2 Secondary lymphoid tissues

Lymph nodes, spleen, and mucosa associated lymphoid tissues (MALTs), such as payer's patches (PPs) and tonsils, are common known as secondary lymphoid organs (SLOs) located statically within the lymph and blood [62, 67, 68]. The organization of the immune cells share several similarities

The lymph node is a highly organized organ that is surrounded by a capsule, and the three main compartments are the cortex, the paracortex and the medulla [62, 69]. The cortex contains B cells, macrophages and follicular dendritic cells (FDCs) arranged into primary B cell follicles, where the chemokine CXCL13 produced by the FDCs plays the dominant role in the position of the B cells via the interaction with CXCR5 [62]. In the primary follicles B cells immunity are mediated by FDCs [70]. These FDCs can present antigens in form of immune complexes that are bound via Fc and complement receptor, and thereby stimulate B cells through the BCR receptor and promoting germinal centre formation [70]. The T cell zone located in the paracortex are mainly composted of T cells, DCs and fibroblastic reticular cells (FRCs), where the chemokines CCL19, CCL21 and CXCL12 are important for the organization and recruitments of T cells and DCs [69]. It is in the T cell zone naïve T cells are activated by antigen-presenting cells APCs, which in most cases are mature DCs. In between the cortex and paracortex are the location of the secondary B cell follicles and germinal centres (GCs), where Tfh cells are involved in somatic hypermutation and isotope switch of the B cells Ig-molecule [71]. The most inner layer of the lymph node, the medulla, consists of the medullary cords that are separated by the lymph filled spaced of the medullary sinuses [69]. In the lymphatic sinuses, the filtration of lymph from afferent lymph vessels occurs, before the compounds from the lymph flows to the B cell area in the cortex or via the subscapular sinuses and out of the lymph node via the efferent lymphatic vessel [71]. The afferent and the efferent lymphatic vessels lined with lymphatic endothelial cells, together with the high endothelial venules (HEVs), complete the vasculation of the lymph node [71].

B and T cells will, together with blood antigens, enter the lymph node via HEVs in a process known as the leukocyte adhesion cascade [62]. The HEVs in the lymph node express a special selectin ligand called pheripheral LN adressin (PNAd) on their luminal surface,

which will interact with the L-selectin (CD62L) on B and T cells and initiate the rolling cascade. The chemokines located at the HEVs; CCL19, CCL21, CXCL12 and CXCL13, are important for guiding and select the B and T cell via their chemokine receptors (CCR7, CXCR4 and CXCR5) into the lymph node. These chemokines will activate the chemokine-triggered adhesion to the HEVs, which involves activation of the $\alpha 1\beta 2$ integrin to ICAM1/ICAM2 on the HEVs. [62]

Immunological properties

The main functions of the SLOs are to filter the blood and lymph to trap and concentrate foreign antigens in addition to attract antigen-presenting cells (APCs), which have brought in antigens from surrounding tissues, to initiate an adaptive immune response with activation of naïve lymphocytes [62, 63, 67, 72]. Second important functions of the SLOs are their capacity to execute mechanisms for peripheral tolerance. There are several ways that the tolerance mechanisms are archived, and are involved with several cell types inside the SLOs. The essential parts in the decision of the fate of the naïve lymphocytes lies in the presence or absence of antigen, co-stimulation, cell interaction and/or chemokines/cytokine [69].

In the classical activation of naïve T cells, the naïve lymphocyte needs two types of signal to be fully activated. The first signal is depended of the TCR:MHC interaction between the T cell and the APCs, while the second signal is provided to the T cell by the APC and it is composed of costimulatory molecules such as CD80/CD86 and chemokines [73]. Usually the APC that are involved in this process are mature DCs, which have migrated from surrounding tissues after they have been activated by a "danger signal" via their pattern recognition receptors (PRRs) and captured antigens. In for example the lymph node, there are several immature DCs that have not been activated but can present antigens on their MHC-II molecules [74]. If the MHC-II molecule on this immature DC interacts with the TCR on a naïve CD4+ T cell, the T cell either dies or become anergic since the immature DCs can't provide with the important second signal with the expression of costimulatory molecules [74, 75].

Initially stromal cells were thought to be mainly involved in supporting of the structure of SLO [69, 75]. However, later research has found that they can also serve an important immunological function in the regulation of the adaptive immune system [69, 75]. These stromal cells, especially the fibroblastic reticular cells (FRCs), can express peripheral tissue-restricted antigen (PTAs), such as proteins associated with pancreas, eye, intestine,

thyroid, skin, CNS and liver, in a similar fashion as the mTECs does in the thymus [76]. The PTA expression has shown to induce anergy and subsequent elimination of CD8+ T cells, and might be a method to increase the odds for eliminating rare, self-reactive T cells [76].

Development of secondary lymphoid organs

The development of the SLOs is a pre-programmed process and occur either during the embryogenesis or early in the post-natal period [68]. The SLO development requires the interaction between the mesenchymal stromal cell expressing the lymphotoxin-β receptor (LTβR) and the hematopoietic lymphoid tissue inducer (LTi) cell, which express the lymphotoxin-α1β2 (LTα1β2) complex [70, 72]. LTα1β2 is a heterotrimeric complex belonging to the TNF superfamily, it is composed of the membrane-bound LTβ2 and the soluble LTα1 and by binding to its receptor LTβR will initiate a signalling cascade necessary for the developing lymphoid organ and the interaction is necessary for the maintenance of the organized structure [77].

The LTi cells are derived from the family of type 3 innate lymphoid cells (ILC3) and are characterized by their expression of being ID2+RORγt+CD4+ [78, 79]. The development of the LTi cells from the ILC3 is strictly depended on their expression of the transcription factors RORγt and the ID2 and the TNF family ligand-receptor pair RANKL-RANK/TNFRSF11A [79], where the RORγt expression are controlled by maternal retinoic acid [78]. **Figure 1.8** illustrates the main events that occur during SLO development. Before the interaction between the LTo cells and the LTi cells occurs, the LTi cells need to be clustered in a LTα1β2/LTβR independent manner [72, 80].

The chemokine CXCL13 produced by the mesenchymal stromal cells are important for the initial clustering of LTi cells by binding to the CXCR5 on these cells [81], and activation of the CXCR5 results in increased levels of LTαβ on their cell surface [79]. In mice with deficient CXCL13 and CXCR5 signalling have an insufficient development of peripheral lymph nodes [81] and the white pulp in spleen [82]. The production of the CXCL13 chemokine is under the control of retinoic acid from neurons, observed by retinoic acid producing neurons are located near to CXCL13 expressing stromal cells near the branching site of blood vessels [81]. Retinoic acid will control the gene expression of *CXCL13* by binding to DNA-binding RA receptors (i.e. RARβ), which then will bind to the retinoic-acid-responsive elements (RARE) in the CXCL13 gene and induce the CXCL13 production [81].

The increased expression of LTαβ on the LTi cells promote the LTα1β2/LTβR interaction between the LTi and the mesenchymal stromal cells, and results in maturation of the mesenchymal stromal cell into lymphoid tissue organizer cell (LTo). The LTo are characterized with their expression of the adhesion molecules (VCAM-1, ICAM-1 and MADCAM-1) and their increased production of the homeostatic chemokines (CXCL13, CCL19 and CCL21) [72, 79, 80]. The LTo cells will also produce interleukin-7 (IL7), which together with the homeostatic chemokine upregulation lead to a positive feedback loop that will result in an increased recruitment of LTi cells [80]. The LTo cell will also contribute to the incorporation of lymphatic endothelial cells (LECs) by expressing VEGF-C [62]. LTo cells secreting potent vascular growth factors will stimulate LTβR expression on endothelial cells, which are important for the differentiation into HEVs [83]. In addition the LTβR signalling is necessary for maintenance and homeostasis of this HEV network in the developing SLO [83]. The LTo will later in the SLO development differentiate into the non-haematopoietic stromal cell types such as FDCs and FRCs [80]. In development of PPs in the small intestine, a second distinct population of CD4-CD45+ ILRα- CD11c+ lymphoid tissue induction (LTin) cells are involved in the stromal activation in the developing organ [80].

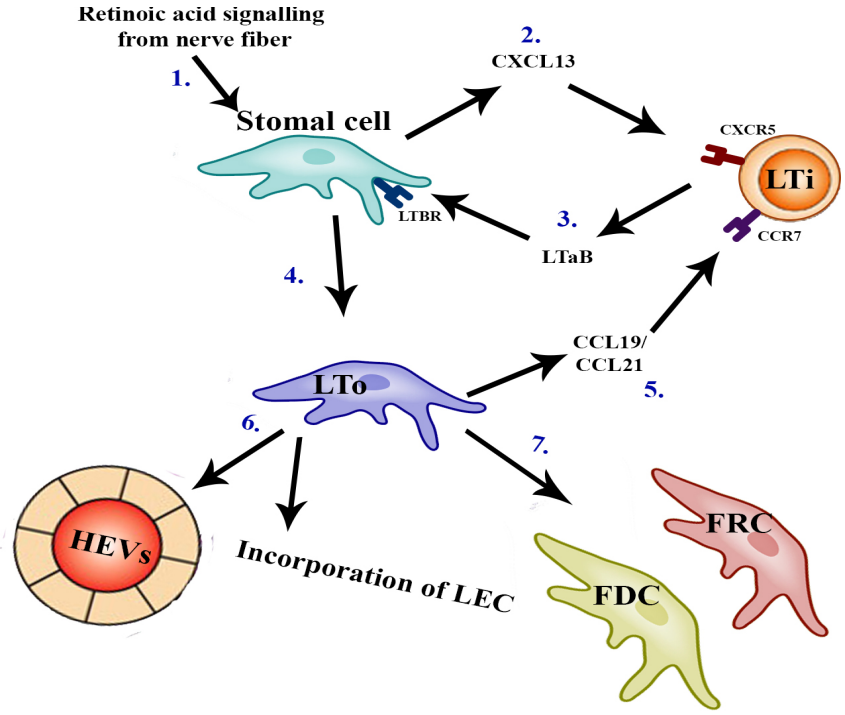


Figure 1.8: The first events in the developing SLO.1. Retinoic acid from nerve cell will stimulate mesenchymal stromal cell to produce CXCL13, which will interact on the CXCR5 receptor on a LTi cell (2.). The LTi cell will respond with production of LTαβ (3.), which will interact with the LTβR on the stromal cell and stimulate differentiation into LTo cell (4.). The LTo cell will produce a positive feedback and recruit more LTi cells to the site by producing CCL19 and CCL21 (5.). The LTo cell will also contribute the formation of HEVs and incorporation of lymphatic endothelial cells (LECs) to the structure (6.). In the later stages of the developing SLO, the LTo cell will further differentiate into FRCs and FDCs (7.)

1.4.3 Tertiary lymphoid structures

Tertiary lymphoid structures (TLS) are ectopic accumulation of lymphoid, myeloid and stromal cells, which occur after birth and are often observed as a response to an environmental stimuli and/or the transition from an acute to a chronic inflammation [62, 79]. TLSs are observed in a large variety of diseases ranging from several autoimmune diseases [84], cancer [85], infections [86], to transplant graft rejections [86]. How the TLS is organized varies between the site of inflammation, organ affected and individual variance between each patient. The degree of organization ranges from oligocellular accumulation of B and T cells to more sophisticated structures similar to SLO with distinct B and T cell area, active GCs, PNAd+ or MAdCAM-1+ HEVs, FRCs and FDCs [62]. The TLS formation is a reversible process, observed with that they can decrease in size after the removal of the initiating stimulus or after therapeutic intervention, and thus making TLSs to be a quite plastic lymphoid organ [67].

The development of TLS, or the neogenesis of the TLS, shows several similarities to the SLO development regarding the chemokine/cytokine and adhesion molecule expression patterns [79]. One of the most dominant similarities is the expression of homeostatic chemokines (CXCL13, CCL19, CCL21 and CXCL12) and signalling through LTα1β2/LTβR leading to the positive feedback loop that guide the recruitment and organization of lymphocytes [87]. The more striking differences between the SLO and TLS development are that the TLS development does not require fetal derived CD4+CD3-RORγ+Id2+ LTi cells and inflammatory events are triggers for the TLS development, while the SLO development is depended of fetal derived LTi and is pre-programmed during embryogenesis [87]. The questions then arise regarding which cell type can function as an LTi cell candidate in the developing TLS. Infiltrated immune cells, such as DCs, macrophages, Th17 cells and γδ T cells could function as an LTi-like cell and express LTα1β2 [77, 86]. Recent studies have shown that the cytokine IL-17, which are produced by Th17 and γδ T cells, are important in the initiation of TLS structures [77]. The cells that can act as an LTo cell candidate in the developing TLS are the stromal cells (from a mesenchymal progenitor cell) in the inflamed tissue; the fibroblasts, myofibroblasts and the perivascular cells, which under stimulation by proinflammatory cytokines can express LTβR and produce compounds for recruitments of LTi-like cells leading to the formation of TLS [88]

Tertiary lymphoid and diseases

What outcomes, both beneficial and detrimental, are associated with TLS formation in a host? To answer this question the case studied has to be defined, since there are both pros and cons with this ectopic lymphoid tissue development. In viral infections, such as in acute influenza virus infection, bronchial-associated lymphoid tissue (iBALT) formation in the lower respiratory tract is observed and induces a host protective role against the infection [89].

On the opposite site of the scale, the development of TLS might be the cause of the induction or exacerbating of an autoimmune reactions and thereby is associated the detrimental effects [80]. Activation of autoreactive B cells in TLS is not so strictly regulated compared to the activation of B cells in the SLOs, increasing the risk of differentiation and expansion of these autoreactive B cells and promote a local production of autoantibodies in the inflamed tissue [90]. TLSs have been associated with several autoimmune diseases and where these structures develop are related to where the immune system usually attack in the disease [89]. For example TLSs have been observed in the joints and lung of patients suffering of rheumatoid arthritis [91], in the salivary glands in Sjögren's syndrome [92], in the pancreas in diabetes [93], and in the kidney of SLE patients [35]. TLS formation in patients might also affect how they are responding to the therapy, making it challenging to treat this patient group [90].

1.5 Stem cells

Stem cells are characterized with their differentiation potential to become multiple mature cell types and their ability to self-renewal, which are important to replenish the stem cell pool [94, 95]. The ability to differentiate into various cell types is described with the potency of the cell. The more cell types a stem cell can be the ancestor of, the higher is its potency, which can range from a totipotent (ie. Zygote) to unipotent (ie. spermatogonial stem cells) [94]. The stem cells can broadly be divided into two main categories; the embryo stem cells and the adult somatic stem cells, also known as the nonembryonic stem cells [96]. The embryo stem cells are isolated from the inner mass of a blastocyst and are derived from the totipotent zygote [94, 96]. The embryotic stem cells, categorized as pluripotent stem cells, have the ability to become the ancestor of all the cells in a developing fetus and some of the extra embryonic cells such as cells in the placenta [94]. The adult somatic stem cells can be found in adults and children, but also in the infants, placenta and the umbilical cord blood [96]. These adult stem cells are known to be multipotent, meaning that they have the capacity to generate the mature cell type of their tissue origin, but will not differentiate into unrelated linages under normal physiological circumstances [94]. The bone marrow harbours two types of adult somatic stem cells; the hematopoietic stem cell (HSCs) and the mesenchymal stem cell (MSCs) [96]. In this study MSCs are in focus, and their functions and role in medicine are further discussed.

1.5.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are nonhematopoietic stromal cells that have the potential to differentiate into tissues of mesenchymal origin such as bone, cartilage, adipose, connective tissue, smooth muscle and hematopoietic supporting stroma [97, 98]. Isolation of MSCs have been successfully performed from various tissues such as bone marrow, adipose tissue, nervous tissue, placenta, menstrual blood and dental pulps [99, 100]. A challenge in the field of the study of MSCs have been the lack of one uniform specific marker, but the MSCs cells do express patterns of surface markers [99, 100]. **Table 1.1**, present some of the common surface molecules that MSCs are expressing [100], but the MSCs should also lack the expression of CD45, CD34, CD14, CD11b, CD79a, CD19 and HLA-DR (MHC-II) surface molecule [101].

Table 1.1: Some of the surface molecules expressed by MSCs, table modified from Xie 2015 [100]

Surface molecules expressed by MSCs	Other cell types expressing the surface molecule
Stro-1	Endothelial cells
Sca-1	HSCs, cancer stem cells
CD13	Cancer stem cells, myeloid cells
CD29	Neural stem cells, cancer stem cells
CD44	T cells, cancer stem cells
CD73	Endothelial cells, lymphocytes
CD90 (Thy-1)	T cells
CD105 (Endoglin)	HSCs, endothelial cells, macrophages
CD106 (VCAM-1)	Endothelial cells
CD146 (MCAM)	T cells, pericytes, endothelial cells
CD166 (ALCAM)	Epithelial cells
CD271 (LNGFR)	Neural stem cells, cancer stem cells
Nestin	Endothelial progenitor cells, endothelial cells, fibroblasts
PDGFR-α (CD140a)	Fibroblasts, smooth muscle cells
Leptin-R	Adipocytes

In addition to their ability to differentiate into several cell types, the MSCs exhibit the immuno regulatory capacity of immune cells [102]. This characteristics have made these cells interesting in the development for treatment of immune-mediated disorders [102]. The immune phenotype of the MSCs is considered as non-immunogenic, characterised as MHC I+, MHC II-, CD40-, CD80- and CD86-, and transplantation into an allogeneic host may not lead to an allogeneic response [97, 102]. MSCs activated in a milieu with high levels of IFNγ, TNF-α, IL-1α and IL-1β have shown to stimulate the immunosuppressive mechanisms of MSCs, which can supress the effector functions of macrophages, neutrophils, NK cells, DCs, T cells and B cells (**Figure 1.9**) [103, 104]. Some of the secreted compounds that MSCs use in their function in immunosupressive mechanisms are IL-10, TGF-β, nitric oxide (NO), catabolites of IDO activity (i.e. kynurenine), Tumor necrosis factor-inducible gene 6 protein (TSG6), and prostaglandin E2 (PGE2) [103, 104]. These compounds will stimulate the differentiation of M2 macrophages from monocytes, which are important for tissue repair and have anti-inflammatory properties with its enhanced production of IL-10 and TGF-β [104]. In addition the MSCs will also stimulate the recruitment of monocytes and macrophages to the site, through their production of CCL2, CCL3 and CCL12, thus enhance the differentiation of more M2 macrophages [104]. With their production of catabolites of the IDO activity and PGE2, the MSCs will both supresses T cell proliferation (arresting in the G0/G1 phase of cell cycle) and favour the iTreg differentiation [104, 105]. MSCs can induce a cytokine profile shift in Th1-Th2 balance towards the Th2 subset of CD4+ T cells [106].

Environments with weak inflammation have paradoxically shown to stimulate MSCs and enhance immune responses by stimulating T effector cell function and differentiation of the proinflammatory M1 macrophage [103, 104]. In an early phase of inflammation or during chronically inflammation, the proinflammatory activities of MSCs can be beneficial in creating a proper immune response (**Figure 1.9**) [104]. When MSCs are exposed to low levels of the proinflammatory cytokines, such as TNFα and IFNγ, they can produce the chemokines CXCL9, CXCL10 and CXCL11 [104]. This response is observed to occur in mice, when NO production is insufficient [107]. One theory of how MSC can be polarized toward proinflammatory or anti-inflammatory phenotypes is through their activation of TLRs, this process is common known as "licensing" [108]. TLR-4 and TLR-2 are toll like receptors that will recognize components of the bacteria wall: the lipopolysaccharide (LPS)-layer from gram-negative bacteria for TLR-4 activation and lipoproteins from gram-positive bacteria for TLR-2 activation [104, 108, 109]. Priming of TLR-4 or TLR-2 priming has shown to promote the proinflammatory properties of MSCs, which will start to produce of proinflammatory cytokines such as IL-6 and IL-8 [104, 108, 109]. Contrary, activation of TLR-3, by virus dsRNA, have shown to promote the anti-inflammatory properties of the MSCs [108].

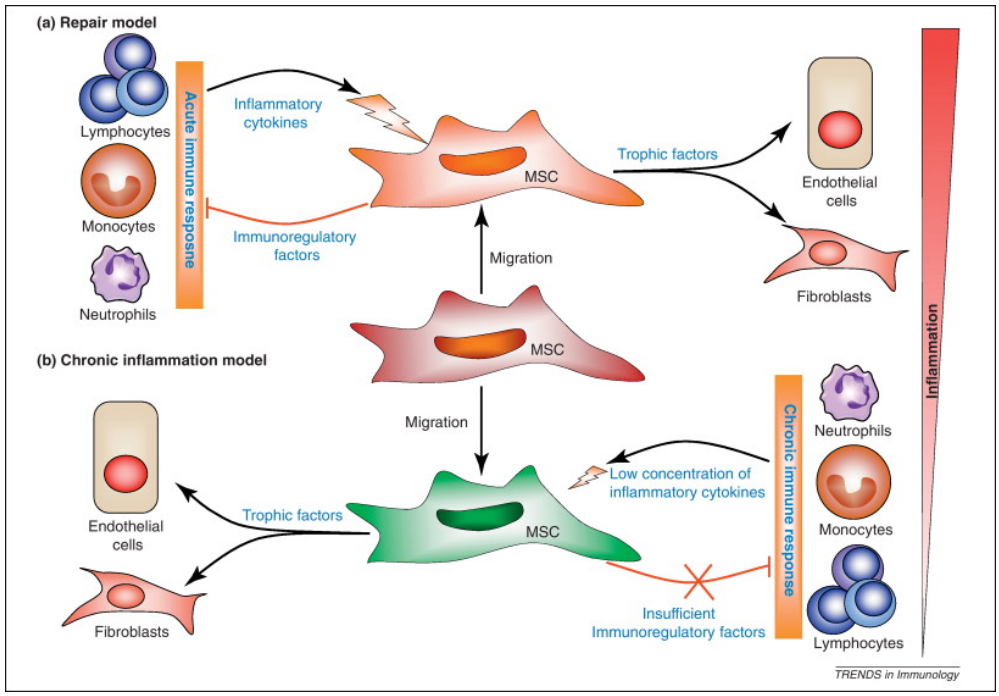


Figure 1.9: Role of MSCs in tissue repair and chronic inflammation. Recent studies on MSC-mediated immunoregulation suggest that MSCs are recruited to sites of tissue damage and activated by local inflammatory cytokines produced by activated immune cells. Depending on the types of immune responses (acute vs. chronic inflammation), MSCs may either attenuate the inflammatory response and lead to repair of the damaged tissue, or maintain a persistent chronic inflammatory response, leading to fibrosis and deformation of tissue architecture. Reused with permission from Shi et al. [110]

1.5.2 Mesenchymal stem cells in treatment of SLE

With their immunosuppressive properties the MSCs have been studied as a candidate in therapy for autoimmune diseases such as SLE [111]. In a small pilot study from 2010 (n=15), administration of MSCs had a positive effect in improvement of the disease [112]. After one year only two of the patients had relapse of proteinuria, while the rest had a significantly decrease in disease activity and an improvement of the levels of Treg cells [112]. In an article published in 2013 by Wang et.al, promising results were reported in patients with severe SLE (n=87) treated with MSCs derived from the bone marrow and the umbilical cord [113]. After 4 years of studying the clinical effects of transplantation, 50% of the treated patents had entered clinical remission, although 23% had suffered from disease relapse [113]. In a smaller study from the same research group published in 2014, 40 patients with active SLE got intravenously transplantation with umbilical cord MSCs on day 0 and 7. After one year 32,5% of the patients reported major clinical response to the treatment and 27,5% reported a partial clinical response, while 17,5% patients suffered with disease relapse [114]. In this study an improvement of the CD4+FoxP3+ Treg cell levels were observed 3 month after transplantation, in addition the urinary protein levels were decreased [114]. In a small pilot study from 2010 (n=15), administration of MSCs had a positive effect in improvement of the disease [112]. After one year only two of the patients had relapse of proteinuria, while the rest had a significantly decrease in disease activity and an improvement of the levels of Treg cells [112].

In murine models of SLE, MSCs treatments have also reported in promising effects with suppression of immune reactions and disease recovery [115-117]. In the study published by Ma et.al [116], reported that MRL/lpr mice treated with murine derived MSCs had an increased probability of surviving compared with the untreated control group. They could also reported that the treated mice had smaller spleens than control animals, with fewer activated Th1, Th2, B cells and plasma cells, in addition to a decreased production of anti-dsDNA autoantibodies and proteinuria [116]. The treatment of MSCs in the murine model NZBW-F1, on the other hand, was shown to increase the severity of disease and stimulate anti-dsDNA autoantibody production [118]. After treatment the mice had increased levels of plasma cells in the bone marrow, increased levels of deposited glomerular immune complexes and sever proteinuria compared to the untreated mice [118].

1.6 Aim of study

MSCs and their immunosuppressive potential have made them a excellent candidate for cell immune-therapy [111]. In the trials where SLE patients were treated with allogeneic MSCs indicated promising results such as decrease in disease severity and recovery from proteinuria, but there were several cases that experienced disease relapse after a short period of time [112-114, 119]. In murine models of SLE, MSCs treatment showed also a promising effect with suppression of immune reactions and disease recovery [115-117]. Other publications report that MSCs treatment had no effect on the clinical symptoms, such as proteinuria or anti-dsDNA antibody levels [118, 120]. These conflicting observations indicate the need for more investigation within the field of MSCs and their immunoregulatory properties, to evaluate the risk with treatment and make suggestions for improvement. Therefore the aim of this study was as follow:

- To evaluate the immunostimulatory potential of MSCs in inflammatory environment.
- To examine the effects of MSCs on proliferation and differentiation of CD4+ T cells, and how the MSC:CD4+ T cell ratio will influence their immunoregulatory effects.
- To investigate if there were any difference in the T cell response between normal healthy donor, SLE patients with and without LN.
- Determine if MSCs could play the role of LTo cells in initiating and developing of TLS

Figure 1.10 illustrates the workflow of this study

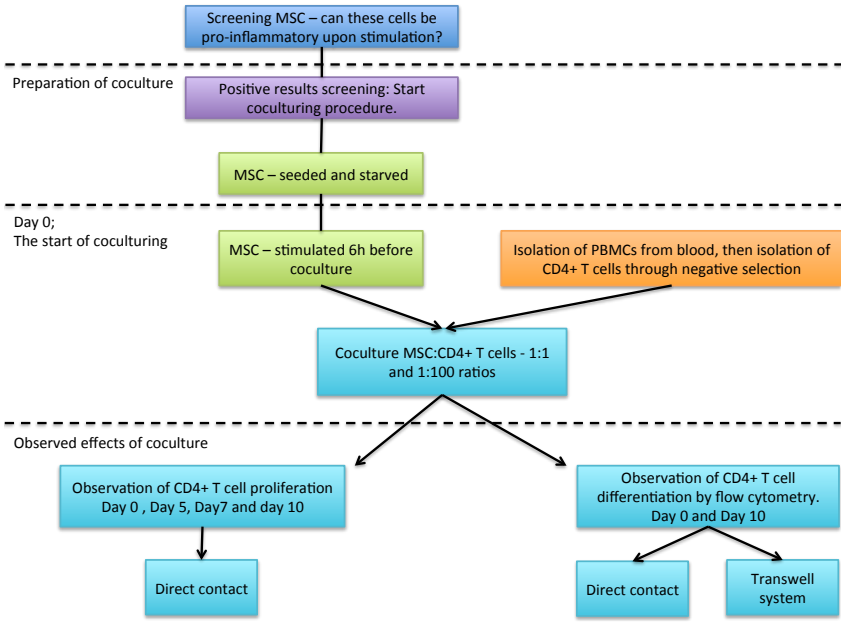


Figure 1.10: Workflow; first the immunostimulatory potential of MSCs were evaluated in a screening before their effect on proliferation and differentiation were examined in a coculture between MSCs CD4+ T cells isolated from Healthy blood donors, SLE patients and LN patients.

2 Materials and methods

2.1 Cell work

In this project three different cell lines were used; human mesenchymal stem cells (MSC, PCS-500-012, lot no: 0216, ATCC Manassas, US), human umbilical vein endothelial cells (HUV-EC-C, CRL-1730TM, lot no: 3822271, ATCC) and human mammary epithelial cells (HMLE). The HMLE cell line was a kind gift from Robert Weinberg (Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology). The cell lines were cultured with their appropriate media as described in **Table 2.1**. In this study, the following passage number was used; passage number 9 for MSC, passage number 18 for HUV-EC-C and the passage number 18 for the HMLE cells.

Table 2.1: Culture media for the cell lines

Cell line	Basal media	Supplemented with
MSC	Mesenchymal stem cell basal medium (PCS-500-030, ATCC)	Mesenchymal stem cell growth kit – for bone marrow derived MSCs (PCS-500-041, ATCC). Containing L-alanyl-L-Glutamine, FBS, rh GF-1, rhFGF-b and 1% penicillin-streptomycin (Sigma-Aldrich)
HMLE	1:1 mixture of MEGM™ Mammary Epithelial Cell Growth Medium (Lonza) and DMEM/F12 (11320033, Thermo Fisher)	10ng/mL EGF 0.5 µg/mL hydrocortisone 0,01mg/mL insulin and 1% penicillin-streptomycin (Sigma-Aldrich)
HUV-EC-C	F-12K Kaighn´s Modification of Ham´s F12 media (30-2004, ATTC)	0,1mg/mL of sodium heparin (H3393-199KU, Sigma Aldich), 0,03% endothelial cell growth supplement (ECGS, E2759, Sigma Aldrich), 1% penicillin-streptomycin (P0781-100mL, Sigma-Aldrich) and 10% Fetal bovine serum (FBS, 7524, Sigma-Aldrich)

2.1.1 Cell culturing

2.1.1.1 Maintenance of cell culture and cell counting

All procedures were performed under laminar flow hood, and all equipment were sterilized with 70% ethanol before starting the experiments. The different cell lines were grown in the appropriate medium in cell culture incubators at 37°C with 5 % CO2, 95 % air and 100 % humidity. Cells were grown until they reached 80% confluency.

Cells passaging

Cell culture medium was aspirated, and cells were washed once with 1×PBS (Dulbecco's phosphate buffered saline, D8537-500ML, Sigma-Aldrich Corporation, US). Pre-warmed 0.25%Trypsin-0.04% EDTA solution (T4049-100mL, Sigma-Aldrich Corporation, US) was added to cells in the culture flask. After 5 minutes incubation at 37°C (until 80% of cells were rounded up and detached), an equal volume of pre-warmed growth medium containing 20% FBS (7524, Sigma-Aldrich Corporation, US) were added to the flask to neutralize the trypsin. Cells were gently homogenized by pipetting up and down to get a single cell suspension. Cells were centrifuged at 250 x g for 5 minutes, and the supernatant was aspirated. The cell pellet was resuspended with the appropriate amount of medium and transferred to a new flask and incubated at 37°C inside a 5% CO_2 humidified incubator. It was possible to calculate the cell count (**Table 2.2**) of the resuspended cells before they are transferred to the flask.

Table 2.2 Equations for calculations of cell count and cell solutions with wanted cell numbers

Eq. 1. Cell count	$\frac{\boldsymbol{Sum\ cells\ counted\ in\ n\ large\ squares\ in\ Burker's\ chamber}}{\boldsymbol{nr\ of\ squares\ counted}} \boldsymbol{x10^4}$
Eq. 2. *Calculation of total cell nr in solution*	$(Cell\ count)\ x\ (volume\ in\ cell\ suspension)$
Eq. 3. *Volume cell suspension in the seeding solution*	$\frac{nr\ of\ cells\ needed}{cell\ count} = \frac{(nr\ of\ wells)\ x\ (nr\ cells\ pr\ well)}{cell\ count}$
Eq. 4. *Volume media in the seeding solution*	$Total\ volume\ needed - volume\ cell\ suspension$

2.1.1.2 Cryopreservation

When cells reached logarithmic growth phase (80% confluency), the culture medium was changed with fresh growth medium 24 hours before freezing. Cells were collected as describe in the **Section 2.1.1.1 Maintenance of cell culture and cell counting**. The cell pellet was resuspended in complete medium (**Table 2.1**) containing 20% FBS and 10% dimethyl sulfoxide (DMSO, D2650-100mL, Sigma-Aldrich Corporation, US) at a cell density of 10^4-10^6. The cryovials stored at -80^{o}C for 24 hours and transferred into liquid nitrogen for long-term preservation.

2.1.1.3 Thawing of cell lines

A cryovial of cells was obtained from the liquid nitrogen storage tank, and quickly thawed in a 37^{o}C incubator for 3-5 minutes. As soon as the cells were completely thawed, they were transferred to a 50 mL conical tube containing an appropriate volume of pre-warmed medium inside a laminar flow hood. The cells were resuspended gently and transferred to the new flask and incubated at 37°C inside a 5% CO2 humidified incubator. Next day, the medium of the cell culture was aspirated and cells were washed once with pre-warmed 1xPBS and an appropriate volume of pre-warmed complete media was added to the flask.

2.1.2 Stimulation of MSCs and HUV-EC-C for co-culture

Cells were seeded based on different experiments set up (**Table 2.3**) and allowed to grow for 24 hours. Then, cells were washed once with 1xPBS and starved with complete MSCs basal medium (ATCC, **Table 2.1**) containing 3% FBS for 18 hours. Next, cells were washed once with PBS and stimulated with 8ng/mL TNFα (210-TA-005, R&D systems, US) and IL-1β (201-LB-005, R&D systems, US) in complete media containing 0.1% FBS for 6 hours. The stimulation setups were based on a previous study, which has been established in our group (RNA and molecular pathology research group, IMB, UiT). For all the different setups, separate control replicates were included, where cells were starved but not stimulated.

Table 2.3 Cell number of the MSCs and HUV-EC-C seeded out for the co-culture assays and the cell number of HMLE and HUV-EC-C seeded out as control cell lines.

		Number of cells seeded out in each well	Type of plate used	Volume in each well
Coculture assays – MSCs : T cells **Or** **HUV-EC-C : T cells**	***Proliferation assay***	2000	24well plate (PLATE TYPE)	½ mL
	"Flow assay"	5000	24well plate (PLATE TYPE)	½ mL
	Trans well – migration assay	8000	6 well plate (PLATE TYPE)	2mL directly in well 1mL in insert
HMLE cells		100 000	24well plate (PLATE TYPE)	½ mL
HUV-EC-C cells		100 000	24well plate (PLATE TYPE)	½ mL

2.1.3 Stimulation of HMLE and HUV-EC-C cell lines in time series

HMLE and HUV-EC-C cell lines as controls were stimulated with 8ng/mL TNFα and IL-1β according to the same set up as for the MSCs (Section 2.1.2) at the different time points; 1, 3, 6, 12, 24, 48 and 72 hours. HMLE cells were not starved before stimulation since complete medium did not contain FBS. After each time point, cells were washed and harvested with TRIsure (**Section 2.8**) for further analysing.

2.2 *Patients in this study*

This study was approved by the Regional Committees for Medical and Healthy Research Ethics (REC; Norway, 2015/1315) .Ten patients, eighth women and two men, whit a diagnosis of SLE according to the criteria of the American College of Rheumatology were studied, and **Table 2.4** present the main clinical data of these patients. Five of the patients were also diagnosed with lupus nephritis (LN), which was classified according to the World Health Organization. Patient samples were collected in 6mL sodium heparin blood tubes. The normal controls in this study were healthy blood donors that had donated blood for the blood bank at the Univeristy Hospital of North Norway (UNN), and blood form these donors were collected and stored as buffy coat. Employers of the UNN performed the blood sampling.

2.3 *Peripheral blood mononuclear cells (PBMCs) isolation*

Buffy coat or blood obtained from the blood bank (UNN) was diluted with 1xPBS + 2%FBS (1:1). Diluted buffy coat or blood was layered gently (to minimize mixing) on top of Lymphoprep (1114545, STEMCELL Technologies, Canada) and centrifuged at 800 rpm for 30 min at 15 - 25°C. Peripheral blood mononuclear cells (PBMCs) layer (grey layer, **Figure 2.1**) were collected carefully without disrupting plasma and erythrocyte/granulocyte layers. PBMCs were resuspended in 1xPBS and centrifuged at 1200 rpm for 15 minutes. The supernatant was aspirated and the pellet was resuspended in appropriate working buffer containing 1xPBS pH 7.2, 0.5% bovine serum albumin (BSA) and 2 mM EDTA (**Table 2.5**) for further procedures.

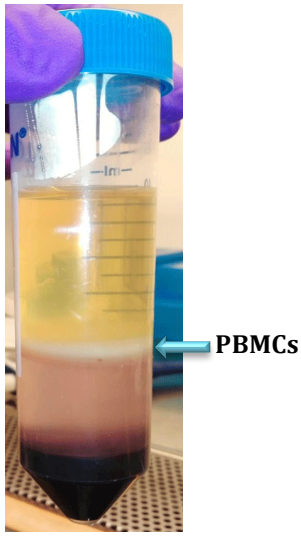


Figure 2.1: Density separation by Lymphoprep, where the marked grey/white layer consists of PBMCs.

Table 2.4: Patient information

Patient	Sex.	Age	Disease duration	Main diagnosis	Main clinical symptoms	Other clinical diagnosis	Immun-ological disruption	Nephritis stage/ duration	Current therapy
P1	M.	53	6md.	SLE	Hem(Ly), Se (Pl), Diskoid ANA, Im	RP, Lymph	anti-dsDNA, LAC, low C3	-	Met, Pred, Pla
P2	F.	33	6y.	LN	Rash, Ou, Ar, N, ANA, Im	RP	RNP, anti-dsDNA AB	Class 2 (6y) Class 4 (3y)	CC, Pred, Ren, Pla, CF
P3	F.	38	1y.	LN	N, ANA, Hem(Ly), Im,	CAC, IRI PCOS, APS, latent TB, SSS	ACA-B2-IgA, ACA-IgA, ACA-IgM, anti-RNP, anti-SSA, anti-SSB, anti-Sm	Class 3A (1y) Class 5 (?)	SM, CC, Pla, Albyl-E
P4	M.	58	5y.	SLE	Rash, Ph, Hem-Le, **Ar,** ANA,	Sicca, pre-OP	SSA, SSB,	-	Pla, Ale, CF
P5	F.	36	17y.	SLE	Er, Rash, Hem-Le, Ar, ANA,	RP, SICCA	dsDNA, anti-sm, Comp, ACA-IgM, ACA-IgG, ACA-B2-IgA	-	Albyl-E, Imu, Pla, Pred, CF
P6	F.	20	12y	SLE	Diskoid, rash, Ou, Ar, ANA, Im, Hem (Ly), CNS?	Alopecia	Anti-SSA,	-	Pla, Imu, Lev
P7	F.	73	1y.	LN	Ph, Rash, Hem(An), N, Im, Ar?	TC (-89), PT, HBP,BCC-RE, Hip-OA, Epilepsy (-02)	Anti-Nucleus	Class 2 (6md)	Euro lupus protokoll. SM, Sen, Pred,
P8	F.	64	28y	LN	N, Im	APS	anti-DNA, , anti-smD, low C3,	Class 2 (16y)	Hyd, Pred
P9	F.	61	31y	LN	Ar, Discoid, N, Hem(Ly)	APS	dsDNA, SSA, low C3/C4 (normal at time of test)	Class 2B 16y(LN)	Kio, Pla, Pred, CC, Ben,
P10	F.	82	42y.	SLE	Diskoid, Ar, CNS, Im, ANA	HypoT4, DM, Endo-PC, OP	Anti-SSA	-	Imu, Pred

Abbreviations Table 3.3: ***F.***; female, ***M.***; Male, ***Y.***;year, ***md.***; months, ***LN***; Lupus nephritis, ***SLE***; Systemic lupus erytheomaus, **Main clinical symptoms**: ***ANA***; ANA-positive screening, ***Ar***; Arthritis, ***CNS***; CNS affection (epilepsy and/or psychosis); ***Discoid***; Discoid Lupus erythematous (skin affections), ***Hem (An, Le, Ly, Tr);*** Haematological manifestations (haemolytic anaemia, leukopenia/lymfopenia, thrombocytopenia), ***Im***; Immunological disruption, ***N;*** Nephropathy (protenuria >0,5g/day and/or), ***Ou***; oral ulcers, ***Ph***; Photosensitivity, ***Rash***; Malar rash/Butterfly rash, ***Se (Pl, Pe, Ed)***; Serositis (Pleuritt, perkaritt, edokarditt). **Other clinical diagnosis:** ***APS***; antiphospholipid antibody syndrome, ***BCC-RE***; Basal cell carcinoma right eye, ***CAC***; Chronic angular cheilitis, ***DM***; Diabetes mellitus, ***Endo-PC***; Endocrine Pancreatic cancer. ***HBP***; Hypertension, ***HIP-OA***; Hip osteoarthritis, ***HypoT4***; Hypothyroidism, ***Iri***; iridocyclitis, ***Lymph***; Lymphoma, ***OP***; osteoporosis, ***PCOS***; Polycystic ovary syndrome, ***pre-OP***; Osteopenia, ***PT***; Partial thyroidectomy, ***RP***; Raynaud's phenomenon, ***SICCA***; Sicca symptoms, ***SS***; Sjögrens syndrome, ***SSS***; Secondary Sjögrens Syndrome, ***TB***; Tuberculosis, ***TC***; Thyroid cancer. **Immunological disruption:** ***anti-*** indicating antibodies against the compound are found in screening, ***ACA-B2-IgA***; anti-B2-glykoprotein IgA, ***ACA-IgA***; Anti-cardiolipin antibody IgA, ***ACA-IgG***; Anti-cardiolipin antibody IgG, ***ACA-IgM***; Anti-cardiolipin antibody IgM, ***Comp***; Complement activation, ***LAC***; lupus anticoagulant. **Current therapy:** ***Alby-E;*** Alby-E, ***Ale***; Alendronat, ***Bav***; Bactrim, ***Ben***; Benlysta, ***CC***; CellCept, ***CF***; Calcigran Forte, ***Hiz***; Hizentra, ***Hyd;*** hydroxycloroquine, ***Ig***; tilskudd immuno-globuliner, ***Imu***; Imurel, ***Kio***; Kiovig, ***Lev***; Levaxin, ***MabT***; MabThera, ***Met***; Methotrexate, ***Mety***; Metylrednisolon, ***Pla***; Plaquenil, ***Pred***; Prednisolon, ***Pro***; Prograf, ***Ren***; Renitec, ***San***; Sandimmun, ***Sen***; Sendoxan, ***SM***; Solu-Medrol

2.4 CD4+T cells isolation

Cell numbers of mononuclear cells obtained from peripheral blood was determined, and cell suspension was centrifuged at 300×g for 10 minutes. The supernatant was aspirated and the cell pellet was resuspended in 40 µL of working buffer (containing PBS, pH 7.2, 0.5% BSA, 2mM EDTA) per 10^7 total cells. Next, 10 µL of CD4+ T Cell Biotin-Antibody Cocktail (130-096-533, Miltenyi Biotec, Germany) per 10^7 total cells was added and incubated at 4 °C for 5 minutes. Then, 30 µL of the working buffer and 20 µL of the CD4+ T Cell MicroBead Cocktail (130-096-533, Miltenyi Biotec) per 10^7cells were added. The cell suspensions were mixed well and incubated for 10 minutes at 4°C. LS MACS Columns (130-042-401, Miltenyi Biotec) were placed in QuadroMACS (Miltenyi Biotec) magnetic separator and rinsed with 3 mL of working buffer. Cell suspensions were applied into the columns and flow-through containing unlabelled cells (representing the enriched CD4+ T cells) were collected. Through this method, non-target cells were magnetically labelled with the CD4+ T Cell Biotin-Antibody Cocktail and CD4+ T Cell MicroBead Cocktail, and CD4+ T Cell were obtained by negative selection.

Table 2.5: CD4+ Working Buffer recipe; PBS pH7.2, 0.5% BSA and 2mM EDTA

400mL buffer	0,5M EDTA	20mL 10% bovine serum albumin (BSA)	Dulbecco's Phosphate Buffered Saline (1xPBS)
	16 mL	20 mL	364 mL

2.5 CD4+T cells co-culture

The collected CD4+ T cells were centrifuged at 1200rpm for 10minutes at room temperature, and resuspended in the appropriate media. Cell suspensions with the different cell numbers for different experiments were prepared (see **Table 2.3 and Table 2.6**). The stimulated and non-stimulated MSCs and HUV-EC-C cells (see **Section 2.1.2**) were washed two times with 1xPBS to avoid dead cells and pre-added pro-inflammatory cytokines. Next, CD4+ T cells were added based on experiments set up (**Table 2.6**) to each well with complete medium. For the trans well system, MSCs or HUV-EC-C were seeded in wells, and CD4+ T cells were added into the inserts (to avoid cells contact).

Table 2.6: Cell numbers in the experiment

		Cell number					
	Assay type	Proliferation assay ½ mL in each well		Flow measurement ½ mL in each well		Trans well system 1 ½ mL in well + 1 ½ mL in insert	
	Cell ratio	**1:1**	**1:100**	**1:1**	**1:100**	**1:1**	**1:100**
Cell type	*MSC or HUV-EC-C*	2000	2000	5000	5000	8000	8000
	CD4+ T-cell	2000	200 000	5000	500 000	8000	800 000

2.6 Proliferation assay

To perform a proliferation assay AlamarBlue® (ThermoFisher) was used according to the manufacturer's instructions. At days zero, 5, 7, and 10, the proliferation of CD4+T cells co-culture with MSCs and CD4+T cells alone were measured. After transferring the CD4+T cells to a new flat clear bottom 24 wells plate (Corning Inc, US), 50µL of AlamarBlue (10% of media total volume) was added directly to the wells. Cells were incubated for three hours at 37°C with 5% CO2. The fluorescence of reduced AlamarBlue® in the supernatant was measured (excitation at λ=540 nm; emission at λ=590 nm) with a CLARIOstar® (BMG LABTECHq).

2.7 Fluorescence-activated cell sorting (FACS) of the $CD4^+$ T cell subset

The immunofluorescence staining of the $CD4^+$ T cells was performed according to the manufacturer's instructions. Cells were stained with antibodies against CD4, CCR4, CCR6, CCR10 and CXCR3 (**Table 2.7**). Cell suspensions were centrifuged at 300×g for 10 minutes, and the supernatants were completely aspirated. The cell pellet was resuspended in 100µL of working buffer (containing PBS, pH 7.2, 0.5% BSA, and 2 mM EDTA). 5µL of the antibodies were added and incubated for 10 minutes in the dark at 4 °C. Then, cells were washed by adding 1 mL working buffer and were centrifuged at 300×g for 10 minutes. The cell pellet was resuspended in 100µL MACSQuant running buffer (130-0922-747, Miltenyi Biotec) and 5µL of Propodium iodide (130-093-233, Miltenyi Biotec) was added shortly before running the samples on the FACSARIA III (BD Biosciences).

2.7.1 Compensation for flow cytometry

The compensation was performed according to the manufacturer's instructions (Miltenyi Biotec). For each fluorochrome-conjugated human and mouse Igκ antibody a separate tube was prepared, containing 100µL of the MACSQuant Running Buffer. Next, 10 µL of intended Igκ (human or mouse) fluorochrome-conjugated antibodies were added to the specified sample tube. A full drop of the MACS Comp Beads – anti-human or anti-mouse Igκ (Miltenyi Biotec) and a full drop of the MACS Comp Beads – Blank (Miltenyi Biotec) were added to each tube and incubated for 10 minutes in the dark at room temperature (21 °C) and tubes were shaken during incubation. Samples were diluted by adding 1mL of the MACSQuant Running Buffer. In the end, they were preceded on FACSARIA III to optimally compensate the fluorescence spillover from fluorochrome-conjugated antibodies α.

Table 2.7: Flow cytometry analysis antibodies and reagents.

Antibody	Cat.no	Fluorophore marked	Excitation wavelength	Emission wavelength	Compensation kit used
Anti-CD4-antibody	130-106-655	VivoGreen™	405nm	520nm	MACS® comp bead kit anti-mouse IgK
Anti-CD194(CCR4)-antibody	130-103-813	APC	561/635nm	660nm	MACS® comp bead kit anti-human IgK
Anti-CD196(CCR6)-antibody	130-107-142	PE-Vivo-615	488/561nm	615nm	MACS® comp bead kit anti-human IgK
Anti-CCR10-antibody	130-104-822	PE	488/561nm	578nm	MACS® comp bead kit anti-human IgK
Anti-CD183(CXCR3)-antibody	130-101-381	PE-Vivo-770™	488/561nm	775nm	MACS® comp bead kit anti-human IgK

2.8 RNA isolation

Adherent cells were washed two times with PBS and 500µL TRIsure (BIO-38033 Bioline, London, UK) was added to the cells directly. For the non-adherent cells (Th subsets), cells were centrifuged at 1000xg for 10 minutes at 4^{o}C and the supernatant was discarded and 150µL TRIsure was added to the cells pellet. Both adherent and non-adherent cells were incubated 5 minutes at room temperature after adding TRIsure, and samples were collected and stored at -80^{o}C.

Further procedure of the RNA extraction was continued with phase separation by adding 0.2 volumes of chloroform (32211-1L, Sigma Aldrich) to each sample, and kept on ice for 20 minutes and shook every two minutes. Samples were centrifuged at 9000xg for 20 minutes at 4^{o}C, and the upper aqueous phase were collected and transferred to clean DNA-low-bind tubes (Eppendorf, Sigma-Aldrich). The RNA was precipitated by adding 1 volume of isopropanol and incubated overnight at -20^{o}C to have better participation. Next day, the RNA was pelleted down by centrifugation at 15000xg for 1h at 4^{o}C. The supernatants were discarded and the RNA pellets were washed two times with 1mL ice-cold 80% ethanol. Ethanol was discarded and RNA pellets were briefly air dried and dissolved in 10 µl RNase free water (L0015, Merck Millipore). The RNA extraction of the HUV-EC-C cells from the time series set up (**Section 2.1.3**) was performed by using the kit Direct-zol^{TM} RNA MiniPrep (R2052, Zymo Research) based on manufacture instruction. RNA quantity and quality were determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific, US).

2.9 cDNA synthesis

High Capacity cDNA Reverse Transcription kit (4368813, Applied Biosystems® by Life Technologies, US) was used to synthesize cDNA. For HMLE and HUV-EC-C cell lines, 10µL RNA sample (containing 150ng RNA in total) was used. The 2X Master mix was prepared based on manufacture instruction (see **Table 2.8)**, then 10µl of The 2X Master Mix was added to each sample. Samples were briefly vortexed to mix solution and centrifuged to eliminate air bubbles. The samples were transferred to the Mastercycler gradient (5331-01627, Eppendorf, Germany), which was programmed for the optimal conditions for reverse transcription according to the manufacturer (**Table 2.9**). In the end, samples were diluted 1:10 in RNase-free water and stored at -20^{o}C. Two negative controls were also prepared, one minus template and one minus reverse transcriptase enzyme to evaluate procedure accuracy.

Table 2.8: 2x Reverse Transcription Master Mix. The volumes are given for 1 reaction and were multiplied with the total number of samples that was run each time the cDNA synthesis was performed.

Components	10x RT Buffer	10x RT Random primers	25x dNTP (100mM)	Multiscrible^{TM} reverse transcriptase	RNase-free H_2O	Total volume
Reagents for each sample	2,0µL	2,0µL	0,8µL	1,0µL	4,2µL	10,0µL
RT negative control	2,0µL	2,0µL	0,8µL	0 µL	5,2µL	10,0µL

Table 2.9: Settings for the Therminal cycler used in the cDNA synthesis. ***The total reaction volume was 20µL and the synthesis was performed in 0.5mL DNA loBind eppendorf tubes***

	Step 1	Step 2	Step 3	Step 4
Temperature (^{O}C)	25	37	85	4
Time (min)	10	120	5	oo

2.10 Real time quantitative PCR (qPCR)

The mRNA expressions of genes (**Table 2.10**) were analysed by qPCR. The master mix containing 5 µL of TaqMan® Fast Universal Master Mix (4352042, Applied Biosystems), 0.5 µL of primer probe and 2 µL of RNase-free water were prepared for each reaction. 2.5µl of cDNA and 7.5µL of the master mix (in total 10 µl) were pipetted to 96 wells plate (4346906, Applied Biosystems) and the plate was sealed (MicroAmp optical adhesive film, 4311971, Applied Biosystems) before centrifuged briefly at 10800xg for one minute. The plate was run on the LightCycler® Analysing machine (Roche Holding AG, Basel, Switzerland) with the settings as described in **Table 2.11.**

Table 2.10: TaqMan primer probes used to analyse the gene expression by qPCR. Applied Biosystems by Thermo Fisher are the provider of the primer-probes.

Gene	Cat.nr	Gene	Cat.nr
CCL19	HS00171149	*LTβR*	HS00158922
CCL20	HS00989654	*PDPN*	HS00366766
CXCL13	HS00757930	*TBP*	HS00427621
ICAM-1	HS00164932	*TNFα*	HS00174128
IL-1β	HS00174097	*VCAM-1*	HS01003372

Table 2.11: Settings for the running of qPCR in the LightCycler® Analysing machine (96system)

Program		Temperature	Duration	Ramp
Preincubation		95°C	600sec	4,4
2-step amplification – 40 cycles	*Step 1* *Step 2*	95°C 60°C	15sec 60sec	
Cooling		37°C	30sec	

2.11 Immunofluorescence staining and confocal microscopy

2.11.1 Immunofluorescence staining of a tissue section

Immunofluorescence staining was performed on kidney cryosections (5µm in thickness) from (NZBxNZW) F1 mice (**Table 2.12** for description of mice). Sections were dried at room temperature for 30 minutes and then fixed for 5 minutes in 4% paraformaldehyde. Sections were washed two times in 1xPBS for 5 minutes each and incubated with blocking serum containing 1xPBS with 10% donkey serum (AB7475, Abcam, UK) for 30 minutes. The sections were incubated with primary antibodies cocktail (**Table 2.13**) for 30 minutes and washed two times by dipping in 1xPBS for 5 minutes each. Secondary antibodies plus PerCP-CyTM 5.5 mouse-anti-mouse CD45 cocktail containing fluorophore-conjugated antibodies was prepared by mixing the antibodies as described in **Table 2.14**. The sections were incubated with the secondary antibody cocktail for 30 minutes at dark and were washed two times by deeding in 1xPBS for 5 minutes each. The sections were carefully dried and mounted by using 20µL Mowiol (**Table 2.15**). For each sample, a negative control was prepared through the same procedure minus primarily antibodies and PerCP-CyTM 5.5 mouse-anti-mouse CD45 conjugated.

Table 2.12: Information regaring the disease progression in (NZBW)-F1 mice studied

Mice	Age (week)	Proteinuria	TLS
Young	6	1	Negative
Anti-dsDNA Ab positive	30	1	Positive
Proteinuric	27	4	Positive

2.11.2 Laser-scanning confocal microscopy

Laser-scanning confocal microscopy was performed on LSM780 AxioObserver (Carl Zeiss, Oberkochen, Germany) by using the ZEN 2012 (black edition) software. Alexa Flour 405, Alexa Flour 488, and Alexa Flour 594 were excited at 405, 488 and 561 nm and they were emitted at 440, 522 and 609 nm respectively. PerCP-CyTM 5.5 CD45.2 conjugated was excited at 405 nm and was emitted at 435 nm. Two different objectives i plan-Apochromat 63×/1.4 Oil DIC M27 and i plan-Apochromat 20×/0.8 M27 were used. The same instrument setting was used for all samples.

Table 2.13: Primary antibodies and their dilutions in the primary antibody cocktail.

Antibody against	Dilution in primary antibody cocktail	Host	Cat. nr	Manufacture
NESTIN	1:100	Rabbit	AB7659	Abcam, UK
SCA1	1:50	Rat	AB25195	Abcam, UK
PDGFRα	1:100	Goat	AF1062	R&D Systems, US

Table 2.14: Fluorophore conjugated primary and secondary antibodies and their dilutions in the secondary antibody cocktail. For the primary antibody negative samples, the antibody against CD45 was excluded from the cocktail solution.

Antibody ID	Dilution in secondary antibody cocktail	Fluorophore conjugated	Cat. nr	Manufacture
Donkey-anti-Rabbit	1:1000	Alexa Fluor 405	AB175651	Abcam, UK
Donkey-anti-Rat	1:1000	Alexa Fluor 594	A-21209	Thermo Fisher Scientific, US
Donkey-anti-goat	1:1000	Alexa Fluor 488	A-11055	Thermo Fisher Scientific, US
PerCP-Cy™ 5.5 mouse-anti-mouse CD45	1:50	PerCP-CyTM	552950	BD Biosciences, US

Table 2.15: Mowiol Mounting Medium

Reagent	Mowiol 4-88 (CALBIOCHEM code: 475904)	Glycerol	dH_2O	0.2 M Tris pH 8.5
	2.4g	6 g	6mL	12mL

2.12 Statistic analysis

2.12.1 Statistical analysing of data generated by the qPCR; Stimulation time series of HUV-EC-C and HMLE cell lines

The raw data of the gene of interest from the qPCR, given as threshold cycle number (Ct), was first converted into fold changes in Microsoft Excel 2011 (version 14.7.2). This was performed as following; the Ct mean of the reference gene (TBP) were subtracted from the Ct mean of the gene of interest, creating the normalized ΔCt value. The ΔCt values were further corrected by the average ΔCt values of the non-stimulated cultured cells at each time point, resulting in the ΔΔCt value. The fold change was then calculated by $2^{(-_{\Delta\Delta}Cq)}$. For the non-stimulated cells, the ΔCt values were also normalized against the ΔCt of the 0h cells, this to observe any changes from the start of the stimulation.

Statistical analyses of the differences between the mRNA expressions of the investigated genes were performed in Graph Pad Prism 7 (version 7.0b). Missing values were handled with the average and standard error of each time point within the two groups (stimulated and non-stimulated/control) were calculated together with the number of measured values in the actual time point. To find statistical significant effects of the cytokine stimulation and the time of stimulation, two-way ANOVA was performed on the datasets. Further Sidak's multiple comparison tests was used to compare the fold change at a given time point between the stimulated group and control group, and Dunnett's multiple comparison test was used to compare the changes from 0h in fold changes within a group.

2.12.2 Statistical analysing of data generated on proliferation and differentiation of T cells in coculture

Figure 2.2 present the grouping system over the data produced of T cells cocultured with MSCs. CD4+ T cells isolated from the main groups: healthy (n=5), SLE (n=5) and LN (n=5), were cultured under three main conditions: alone (T cell), in coculture with stimulated and non-stimulated MSCs. In addition the effects of the different ratio of MSCs on CD4+ T cells were evaluated, with T cells either cocultured in a low ratio (1:100, MSCs:CD4+Tcells) or high ratio (1:1, MSCs:CD4+Tcells). The changes in T cell proliferation were examined by the Alamar Blue proliferation assay, where the T cell proliferation was measured as optical density (OD) at day 0, day 5, day 7 and day 10. To analyse the statistical significant effects provided by the time and coculture two-way ANOVA and the post-hoc analyses of Dunnett's and Tukey's multiple comparison tests were performed. Flow cytometry technique was used to measure the CD4+ T cells differentiation to different Th cells subsets in coculture with MSCs at day 10. The CD4+ T cells differentiation was examined to be either contact depended or not by applying a Transwell system, which prohibited direct contact between T cells and MSCs ($n_{healthy}$=3, n_{sick}=3). As control cell line, the same experiment was performed by HUV-EC-C cells (endothelial cells) to compare with MSCs (n=3). Statistical analyses of the differences between the proliferations and differentiation of the cocultured T cells were performed in Graph Pad Prism 7 (version 7.0b). Missing values were handled with the average and standard error of each time point within the tree culture conditions (T cells alone, cocultured with stimulated MSCs, cocultured with non-stimulated MSCs).

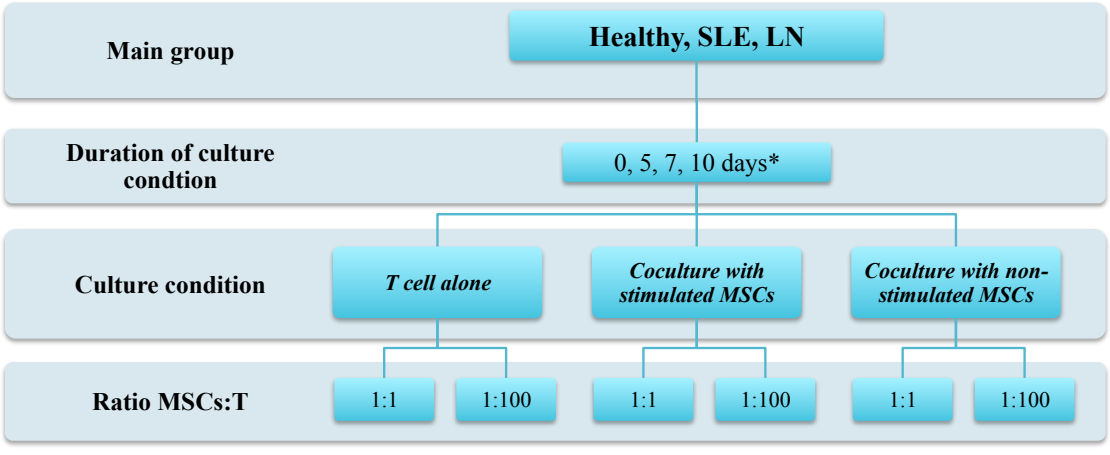


Figure 2.2: Overview over the grouping system that includes the main groups the T cells were isolated from, which culture condition and ratio with MSCs the T cells were cultured in. For the culture condition "T cells alone" the 1:1 and 1:100 cell ratio refer to the matching T cell number as for the cocultured T cells. *Duration of coculture* indicate for when T cells were harvested for measurement. For the proliferation assay, the T cell proliferation was measured at before culturing (day 0) and after five, seven and ten days after start of culturing. The measurement of Th subset with flow cytometry occurred only before culturing (day0) and after ten days in culture with MSCs.

3 Results

3.1 Gene expression of control cell lines

To study under which inflammatory conditions MSCs could function as a stimulator of the immune system, a screening were performed. In this screening MSCs were stimulated with various combination of cytokines at different time points, to examine the optimal conditions for these cells. By combining low concentration (8ng/mL) of the proinflammatory cytokines TNFα and IL-1β, the immune-stimulatory characteristics of the MSCs were more potent MSCs significantly increased the expression of TNFα, IL-1β, CCL-19, ICAM-1 and VCAM-1 as presented in **Figure 3.1**. CCL19, ICAM-1 and VCAM-1 had their peak in mRNA expression after 6hour with stimulation, therefore this time with stimulation was used in the coculture between MSCs and CD4+ T cells. In the first phases of screening, when only one cytokine were used: the LTβR and PDPN were expressed for both stimulated and non-stimulated MSCs with relatively high Ct values, but the expressions were not affected by the stimulation (data not shown). (My co-supervisor performed this screening before I started with my master project).

The cell lines, HMLE and HUV-EC-C, were cultured in 24 well plates and stimulated with a combination of 8ng/mL TNFα and IL-1β. The cells were stimulated for 0, 1, 3, 6, 12, 24, 48 and 72 hours before they were harvested.

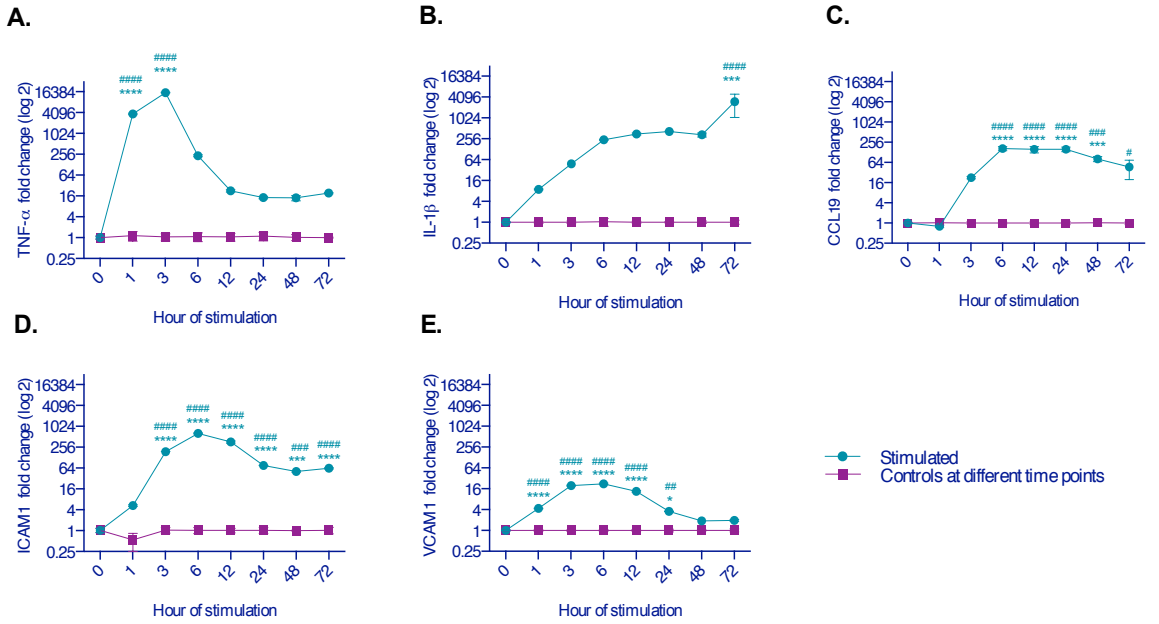


Figure 3.1: Expression of A.] TNFα, B.] IL-1β, C.] CCL19, D.] ICAM-1 and E.] VCAM-1 in MSC stimulated by 8ng/mL TNFα and IL-1β for 6h, 12h, 24h, 48h and 72h. Data reported as mean with SEM. Two-way ANOVA was performed with the post-hoc analysis of Sidak's multiple comparison test (to compare between the groups at given time point – marked with *) and Dunnett's multiple comparison test (to compare within a group towards time point 0h – marked with #). Only statistical significant values are included in the figures and marked with either "*" or "#" where: */# equals a p-value <0.05, **/## equals a p-value <0.01, *** /### equals a p-value <0.001 and ****/#### equals a p-value <0.0001. This unpublished result is a part of a pilot study performed in the research group RAMP by my co-supervisor.

3.1.1 Stimulation of HMLE

An increase in the mRNA expression of IL-1β, TNFα and ICAM-1, represented as fold changes, were observed in HMLE cells upon cytokine stimulation with 8ng/mL of TNFα and IL-1β (**Figure 3.2**), but they did not express CCL19, CXCL13 and VCAM-1 as no mRNA were detected by qPCR (data not shown). The results of the statistical analysis of the fold changes by two-way ANOVA are shown in **Table 3.1** for HMLE cells. Two-way ANOVA was conducted to examine both the individually and combined effects of stimulation and time on the fold change of the mRNA expression of the gene of interest in the HMLE cells (**Table 3.1**). Stimulation, time and the interaction statistical significantly influenced the expression of IL-1B and TNFα in HMLE cells, while the expression of ICAM-1 was affected by stimulation alone inducing a statistical significant effect.

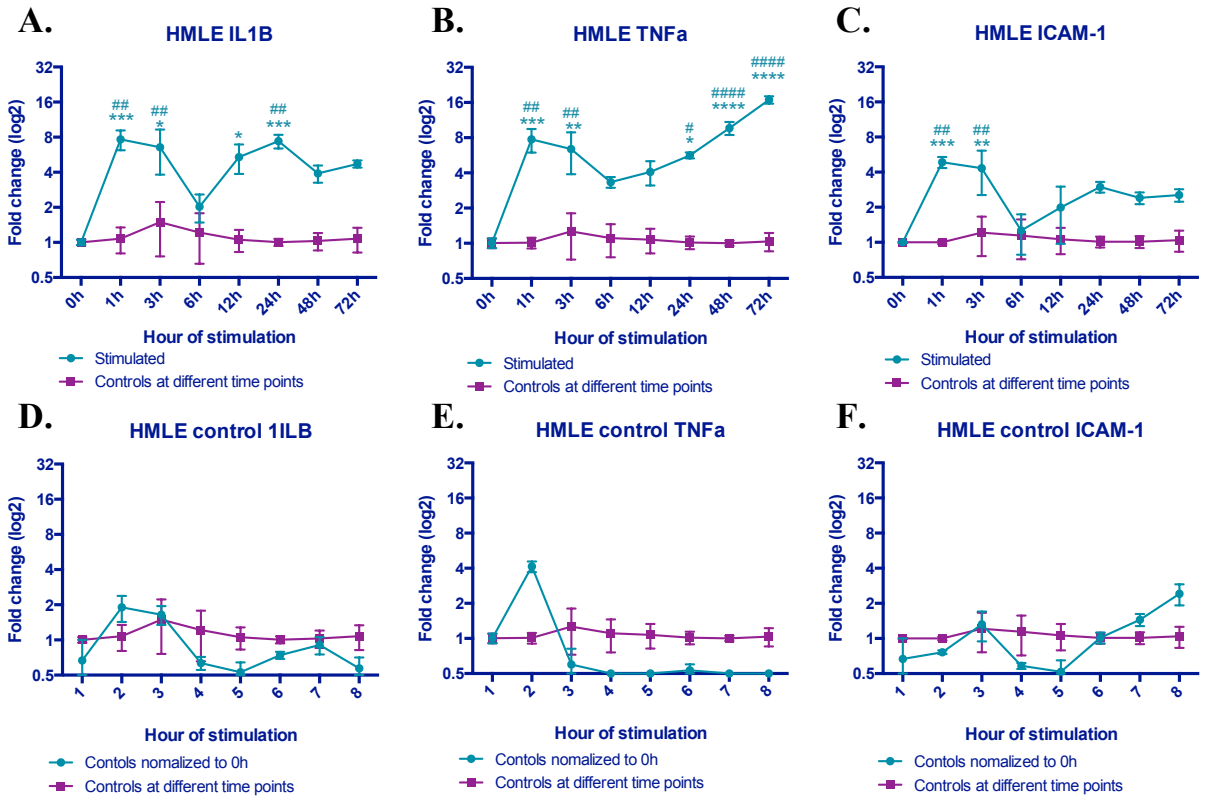


Figure 3.2: mRNA expression of IL-1β(A), TNFα(B) and ICAM-1(C) in HMLE cells stimulated by 8ng/mL of TNFα and IL-1β and harvested after 0h, 1h, 3h, 6h, 12h, 24h, 48h and 72h after stimulation. Two-way ANOVA was performed with the post-hoc analysis of Sidak's multiple comparison test (to compare between the groups at given time point – marked with *) and Dunnett's multiple comparison test (to compare within a group towards time point 0h – marked with #). Data reported as mean with SEM, only statistical significant values are reported. **A.] mRNA expression of IL-1β:** Sidak's multiple comparison test: 1h (p=0.0006), 3h(p=0.0107), 12h(p=0.0388) and 24h (0.0009). Dunnett's multiple comparison test: 1h (p=0.0016), 3h(p=0.0093) and 24h (p=0.0025). **B.] mRNA expression of TNFα** Sidak's multiple comparison test:1h (p=0.0003), 3h(p=0.0072), 24h(p=0.0182), 48h(p<0.0001) and after 72h(p<0.0001). Dunnett's multiple comparison test: 1h (p=0.0010), 3h(p=0.0094), 24h (p=0.0299), 48h (p<0.0001) and 72h (p<0.0001). **C.] mRNA expression of ICAM-1**: Sidak's multiple comparison test:1h (p=0.0026) and 3h(p=0.0118). Dunnett's multiple comparison test: 1h (p=0.0023), 3h(p=0.0099) **D-F] Controls normalized to the 0h fold change for the expression of IL-1β (D), TNFα (E) and ICAM-1 (F).**

Performing the post-hoc analyses, Sidak's multiple comparison test and Dunnett's multiple comparison test, after the 2-way ANOVA test gave the opportunity to compare the expression of the gene of interest in the stimulated cells at each time point compared to the non-stimulated control cells and to the 0h control. The expression of IL-1β had an immediately increase upon stimulation with the fold change around 8 at the 1h time point, a bit less at 3h, but still significant. At the 6h time expression decreased and the difference became statistical insignificant with the non-stimulated expression with a fold change around 2 (**Figure 3.2.A**). At the 12h time point, the expression increased in stimulated cells and it was statistically significant compared non-stimulated group with a fold change 5 in the stimulated group (p=0.0388). Increase in expression was continued until the 24h time point, but at the 48h time point, no statistical differences was observed compared to the 0h time point with a fold change of 2.884. The expression pattern of TNFα in the stimulated cells showed a statistically significant increase after one-hour stimulation (fold change of 7.735) and it decreased slightly at the 6h and 12h time point. The expression started to increase and became statistically significant at 24h time point with a fold change around 6 (**Figure 3.2.B**). The ICAM-1 expression (**Figure 3.2.C**) had an immediate statistically significant increase after one hour with a fold change of 4.872 and a decrease at the 6h time point (1.263). The ICAM-1 expression was slightly increased at the 12h time point (fold change: 1.990), but it was not statistically significant.

Table 3.1: Two-way ANOVA HMLE result table

	HUV-EC-C IL-1β	**HUV-EC-C TNFα**	**HUV-EC-C ICAM-1**
Interaction (Time*stim.)	F(7,29)=2.591 p= 0,0332 (*)	F(7, 28) = 11,00 p<0.0001 (****)	F(7, 29) = 2,264 p=0.0574 **(ns)**
Time	F(7,29)=2.708 p=0.0274 (*)	F(7, 28) = 10,91 p<0.0001 (****)	F(7, 29) = 2,336 p=0.0509 **(ns)**
Stimulation	F(1, 29) =49.42 p<0.0001 (****)	F(1, 28) = 124,3 p<0.0001 (****)	F(1, 29) = 25,62 p<0.0001 (****)

3.1.2 HUV-EC-C cells

The HUV-EC-C cells stimulated with 8ng/mL of TNFα and IL-1β showed an increased in the mRNA expression as fold changes of IL-1β, TNFα, ICAM-1 and VCAM-1 as presented in **Figure 3.3.** The expression of LTβR in HUV-EC-C cells was not affected by the TNFα and IL-1β stimulation (**Figure 3.3.E),** but the Ct-values for LTβR for both stimulated and non-stimulated HUV-EC-C cells were relatively high compared to the reference gene (see appendix, **Fig. A**) indicating a relatively high and unchanged expression. The results of the statistical analysis of the fold changes by two-way ANOVA are shown in **Table 3.2**. No mRNA expression of CCL19, CCL21, CXCL13 and PDPN were observed when HUV-EC-C cell line was stimulated by 8ng/mL IL-1β and TNFα.

A two-way ANOVA performed on the HUV-EC-C cells, showed that the individual effects of time, stimulation and the combined effect would give a statistically significant increase in mRNA expression of IL-1β, TNFα, ICAM-1, and VCAM-1 ($p<0.0001$) (**Table 3.2**). The expression of LTβR was influenced by the stimulation alone ($F(7,32)=7,092$, $p=0,0120$), but neither the time alone nor the interaction between time and stimulation gave any statistically significant effect.

The Sidak's multiple comparison test showed a statistical significant increase of mRNA expression of IL-1β in the stimulated cells after 3h compared to the control cells (non-stimulated) with an fold change around 52 ($p=0.0221$). The increase in IL-1β expression continues with an approximate 415 fold change after 72 hours (**Figure 3.2.A**). Comparing the mRNA expression of TNFα between the stimulated and control cells, and the expression of TNFα within the stimulated cells, showed that there were only statistically significant differences between the stimulated and control cells at 3h, 6h and 12h time points (**Figure 3.2.B)**. The peak of TNFα mRNA expression occurred after 3hours with a fold change around 40 ($p<0.0001$), and expression decreased and became statistical insignificant at 24 hours with a fold change of 6,606 compared to the non-stimulated cells ($p=0.4226$) and the 0h time point ($p=0.2837$)

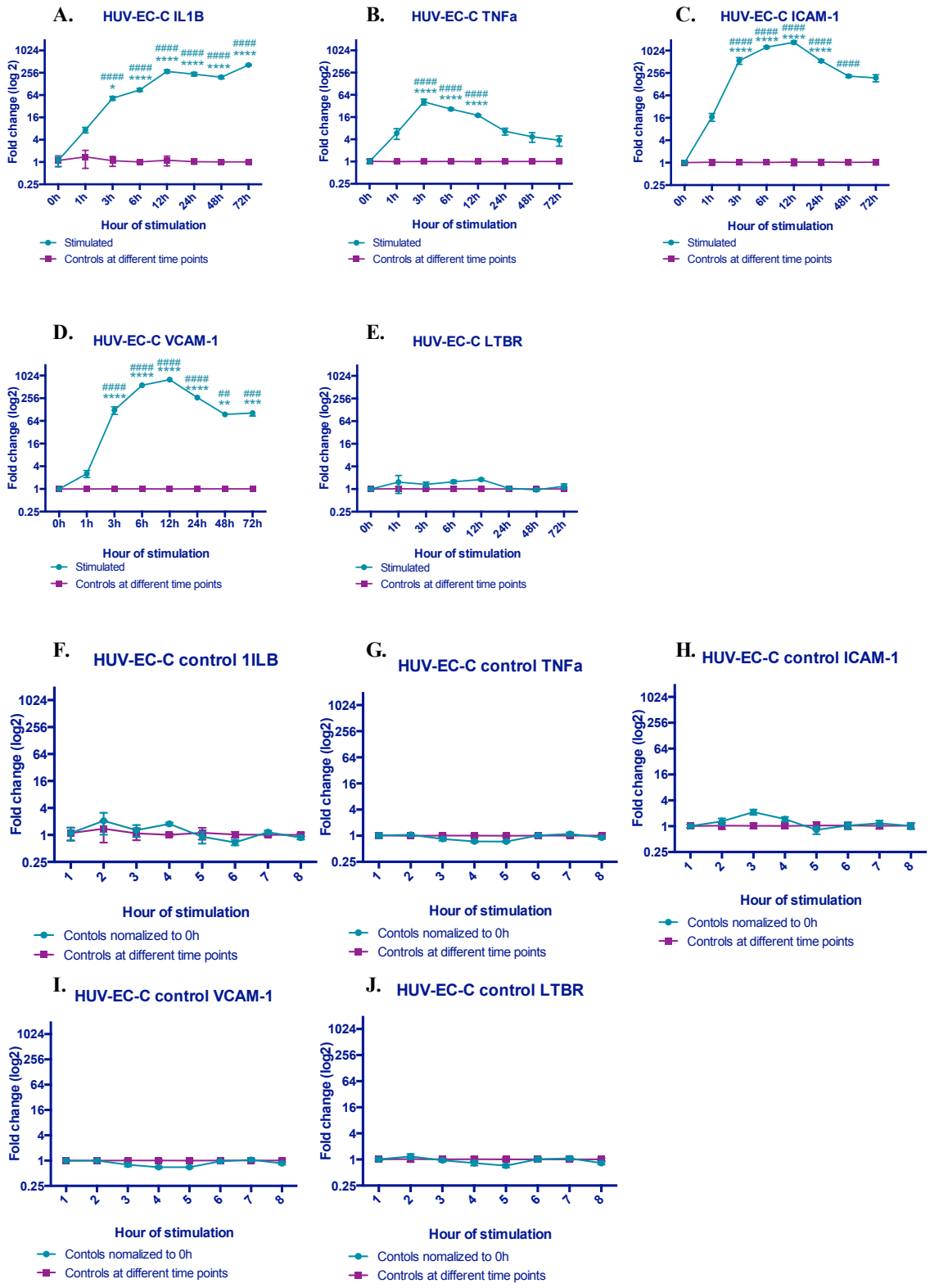


Figure 3.3: mRNA expression of IL-1β (A), TNFα (B), ICAM-1 (C), VCAM-1 (D) and LTβR (E) given as fold changes (mean with SEM) in HUV-EC-C cells stimulated by 8ng/mL of TNFα and IL-1β and harvested after 0, 1, 3, 6, 12, 24, 48 and 72hours (h. Two-way ANOVA was performed with the post-hoc analysis of Sidak's multiple comparison test (to compare between the groups at given time point – marked with *) and Dunnett's multiple comparison test (to compare within a group towards time point 0h – marked with #). Data reported as mean with SEM, only statistical significant values are reported. **A.] mRNA expression of IL-1β:** Sidak's multiple comparison test: 3h ($p=0.031$), 6h($p<0.0001$), 12h($p<0.0001$), 24h($p<0.0001$), 48h($p<0.0001$), and 72h($p<0.0001$). Dunnett's multiple comparison test: 3h ($p=0.0221$), 6h ($p<0.0001$), 12h ($p<0.0001$), 24h ($p<0.0001$), 48h($p<0.0001$), and 72h($p<0.0001$), **B.] mRNA expression of TNFα** Sidak's

multiple comparison test: 3h ($p<0.0001$), 6h($p<0.0001$) and 12h($p<0.0001$). Dunnett's multiple comparison test: 3h ($p=0.0001$), 6h ($p<0.0001$), 12h ($p<0.0001$), **C.] mRNA expression of ICAM-1**: Sidak's multiple comparison test: 3h ($p<0.0001$), 6h($p<0.0001$), 12h($p<0.0001$) and 24h ($p<0.0001$). Dunnett's multiple comparison test: 3h ($p<0.0001$), 6h ($p<0.0001$), 12h ($p<0.0001$), 24h ($p<0.0001$) and 48h($p=0.0384$) **D.] mRNA expression of VCAM-1:** Sidak's multiple comparison test: 3h ($p=0.031$), 6h($p<0.0001$), 12h($p<0.0001$), 24h($p<0.0001$), 48h($p=0.002$), and 72h($p=0.007$). Dunnett's multiple comparison test: 3h ($p<0.0001$), 6h ($p<0.0001$), 12h ($p<0.0001$), 24h ($p<0.0001$), 48h($p=0.0015$), and 72h($p=0.0006$), **D.] mRNA expression of LTβR:** Neither the Sidak's nor Dunnett's multiple comparison test gave a statistical significant changes in the mRNA expression of LTβR when stimulated with TNFα and IL-1β. **F-J] Controls normalized to the 0h fold change for the expression of IL-1β(F), TNFα(G), ICAM-1(H), VCAM-1(I) and LTβR (J).**

The mRNA expressions of ICAM-1 and VCAM-1 (**Figure 3.2.C & D**) upon stimulation of HUV-EC-C cells showed a massive increase after 3hours with and fold change of 548 and 123.7 respectively. The peak in expression observed after 12 hours and was followed by a decrease at the 24h, 48h, and 72h time points There was not detected any statistical significant change in mRNA expression of LTβR in HUV-EC-C cells after stimulation in different time points (the Sidak's and Dunnett's multiple comparison tests on the stimulated and control group were performed) (**Figure 3.2.E**).

Table 3.2: Results of the two-way ANOVA of the mRNA expression given as fold changes of IL-1β, TNFα, ICAM-1, VCAM-1 and LTβR in HUV-EC-C cell lines stimulated with 8ng/mL IL-1β and TNFα.

	HUV-EC-C IL-1β	HUV-EC-C TNFα	HUV-EC-C ICAM-1	HUV-EC-C VCAM-1	HUV-EC-C LTβR
Interaction (Time*stim.)	F(7,32) = 78,63 p<0,0001 (****)	F(7,32) = 23,04 p<0,0001 (****)	F(7,32) = 71,24 p<0,0001 (****)	F(7,32) = 159 p<0,0001 (****)	F(7,32) = 0,9405 p=0,4897 (ns)
Time	F(7,32) = 78,50 p<0,0001 (****)	F(7,32) = 23,03 p<0,0001 (****)	F(7,32) = 71,24 p<0,0001 (****)	F(7,32) = 159 p<0,0001 (****)	F(7,32) = 0,9752 p=0,4659 (ns)
Stimulation	F(1,32) = 727 p<0,0001 (****)	F(1,32) = 143 p<0,0001 (****)	F(1,32) = 465,2 p<0,0001 (****)	F(1,32) = 904,4 p<0,0001 (****)	F(1,32) =7,092 p=0,0120 (*)

3.2 Effects of MSC on the CD4+Tcells differentiation and proliferation

CD4+ T cells isolated from the main groups: healthy (n=5), SLE (n=5) and LN (n=5), were cultured under three main conditions: alone (Tcell), in coculture with stimulated and non-stimulated MSCs. In addition the effects of the different ratio of MSCs on CD4+ T cells were evaluated, with MSCs cells either cocultured in a high ratio (1:100, MSCs:CD4+Tcells) or low ratio (1:1, MSCs:CD4+Tcells). The changes in T cell proliferation were examined by the Alamar Blue proliferation assay, where the T cell proliferation was measured as optical density (OD) at day 0, day 5, day 7 and day 10. Flow cytometry technique was used to measure the CD4+ T cells differentiation to different Th cells subsets in coculture with MSCs at day 10. As control cell line, the same experiment was performed by HUV-EC-C cells (endothelial cells) to compare with MSCs (n=3).

3.2.1 The positive effects of stimulated MSCs on CD4+ T cell proliferation.

To find the effect of MSCs on the proliferation of CD4+ T cells isolated from healthy blood donors, SLE- and LN patients, a proliferation assay was performed. **Figure 3.4** present the data related to the proliferation of T cells from three groups (Healthy, SLE, and LN), cocultured under the three conditions: T cell alone, in coculture with stimulated MSCs and coculture with non-stimulated MSCs. Two-way ANOVA was performed, which gave an examination over the effects provided by both time and coculture individually and combined had on the proliferation (**Table 3.3**). First, the post-hoc analysis with Tukey's multiple comparison test was applied to examine statistical differences between the culture conditions of the CD4+ T cells, then Dunnett's multiple comparison test was used to test if there were any statistical significant changes in proliferation compared to the day 0.

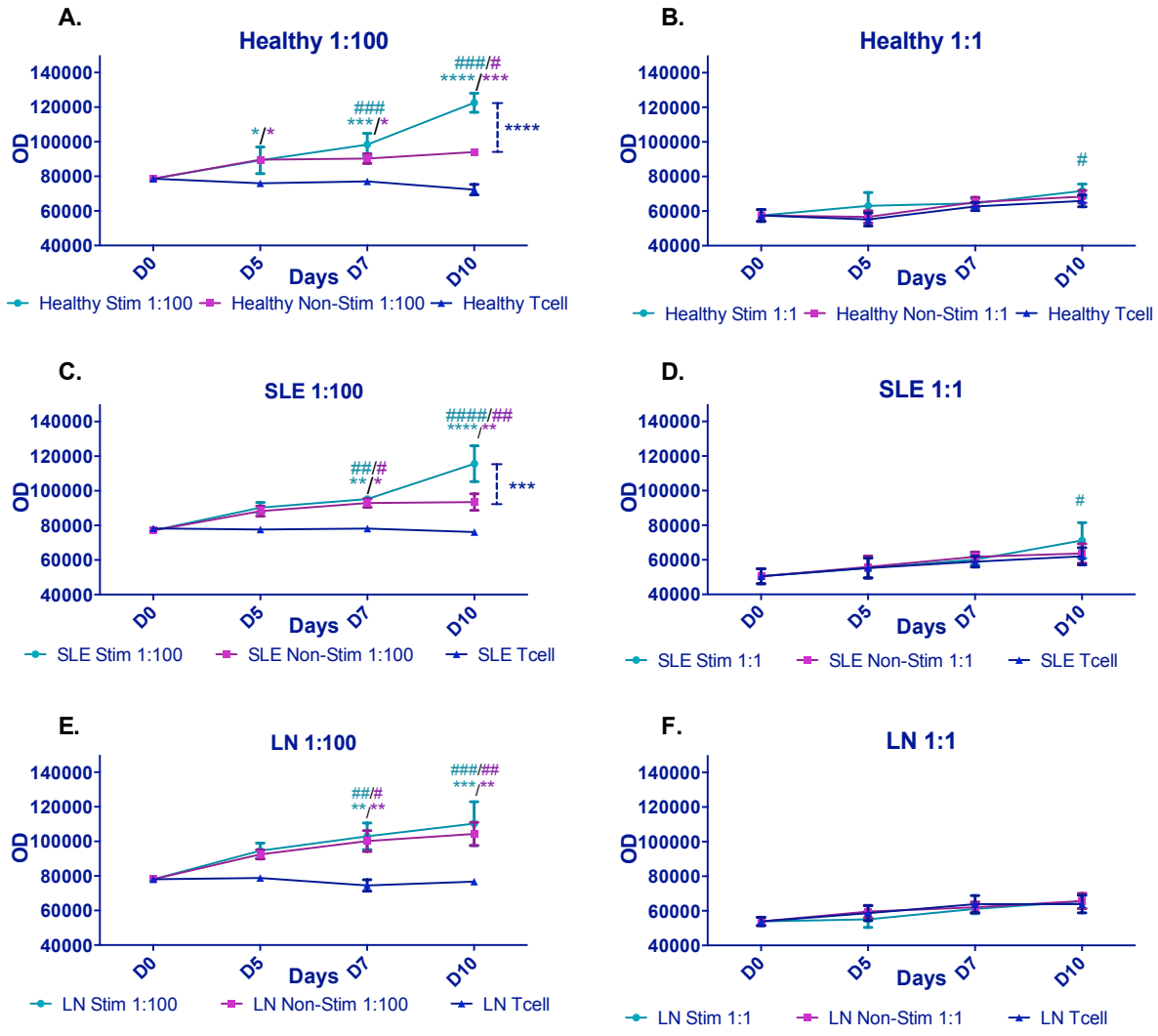


Figure 3.4: Proliferation of CD4+ T cells isolated from healthy blood donors (A, D), LN patients (B, E) and SLE patients (C, F) in coculture with MSC at 1:100 or 1:1 ratio. The T cells were either cultured alone (Tcell – dark blue) or in coculture with MSC, which were either stimulated (Stim – turquoise) or not stimulated (Non-stim, pink) before coculture. Two way ANOVA and the post-hoc analyses Dunnett's (#) and Tukey's (*) multiple comparison tests were performed, to test for differences between the proliferation at the different days to D0 within one group and to test for differences between the different group at a given time point respectively. Data reported as mean with SEM. Only statistical significant values are included in the figures and marked with either "*" or "#" where: */# equals a p-value <0.05, **/## equals a p-value <0.01, *** /### equals a p-value <0.001 and ****/#### equals a p-value <0.0001. In addition the statistical significant values are colour coded, where turquoise marks and pink marks are for cocultured with simulated MSCs and non-stimulated MSCs respectively. Blue marks indicate comparison of T cell proliferation between T cells in coculture with stimulated MSCs and non-stimulated MSCs. See appendix **Table B** to **Table D** for adjusted p-values. OD; optical density.

MSC cocultured with high ratio MSCs:CD4+ T cells (1:100 ratio), had positive effects on the T cell proliferation. This was observed with an increased T cell proliferation when the T cells were in coculture with MSCs compared to when they were cultured alone (**Figure 3.4.A/C/E**). Statistical analysis with the two-way ANOVA showed that both coculture and time individually and combined had a positive effect on the T cell proliferation (**Table 3.3**), when the T cells were cocultured with high ratio MSCs:CD4+ T cells (1:100 ratio).

Table 3.3: ANOVA table for the two-way ANOVA performed on the T cell proliferation from the three main groups

	1:1			1:100		
	Healthy	**SLE**	**LN**	**Healthy**	**SLE**	**LN**
Interaction	F(6,48)=0.274 p=0.9465 **(ns)**	F(6.45)=0.2188 p=0.9688 **(ns)**	F(6.48)=0.1397 p=0.9902 **(ns)**	F(6,48)=9.086 p<0.0001 **(****)**	F(6.45)=5.229 p<0.0001 **(****)**	F(6.48)=2.305 p<0.0001 **(****)**
Time	F(3,48)=5.443 p=0.0027 **(**)**	F(3.45)=4.348 p=0.0090 **(**)**	F(3.48)=4.911 p=0.0047 **(**)**	F(3,48)=12.5 p<0.0001 **(****)**	F(3.45)=10.99 p<0.0001 **(****)**	F(3.48)=6.921 p<0.0001 **(****)**
Coculture	F(2,48)=0.995 p=0.3772 **(ns)**	F(2.45)=0.2274 p=0.7975 **(ns)**	F(2.48)=0.1612 p=0.8516 **(ns)**	F(2,48)=34.48 p<0.0001 **(****)**	F(2.45)=19.97 p<0.0001 **(****)**	F(2.48)=15.6 p<0.0001 **(****)**

The results from the post-hoc analyses gave that T cells isolated from healthy donors and cocultured in high ratio (1:100) with MSC had after five days a statistical significant increase when compared to T cells cultured alone (**Figure 3.4.A**). The proliferation of these healthy T cells in coculture continued to increase, and at day 7, in coculture, there was also a statistical significant difference between the proliferation compare to the day 0. At the day 10, the proliferations difference between T cells cocultured with stimulated MSCs and non-stimulated MSCs were observed, and it was statically significant ($p < 0.0001$).

The effects of the MSCs were similar to the T cells isolated from SLE patients (1:100) as for the effects observed with the T cells isolated from the healthy blood donors (**Figure 3.4.C**). T cells at day 7 proliferated statistically significant in the cocultured compared to T cells cultured alone, and at day 10 there were a statistical significant increase in proliferation of T cells cocultured with stimulated MSCs compared to the T cells cocultured with non-stimulated MSCs ($p=0.0003$). The proliferation of the T cells isolated from the LN patients was increased when cocultured with a high MSCs: CD4+ T cells ratio (1:100) (**Figure 3.4.E**), but we did not observed statistical significant difference between the T cells cocultured with stimulated and non-stimulated MSCs at the day 10 (ratio 1:100).

When MSCs were cocultured with a low number of T cells (1:1 ratio), only time had a statistical significant influence on the T cell proliferation for all three groups (T cells cultured alone, T cells cocultured with stimulated and non-stimulated MSCs) at day 10 (**Table 3.3**). As showed in **Figure 3.4.B/D/F,** the proliferation of the T cells isolated from healthy (**B**), SLE patients (**D**) and LN patients (**F**), had no statistical significant differences by the post-hoc analyses between the three culture conditions for the T cells at 1:1 ratio.

T cells proliferation related to stimulated-MSCs:CD4+ T-cells 1:100 ratio for all individuals in SLE and LN groups were plotted (**Figure 3.5**). In the SLE group, all patients until day 7 showed same proliferation pattern, but at day 10, they showed different

proliferation pattern (some higher and some lower) (**Figure 3.5.A**). We did not see the same pattern in LN group and there were a larger variance between the patients (**Figure 3.5.B)**

T cells cultured alone (both 1.1 and 1:100 ratios) from the three main groups plotted together, and all 15 patients from three groups showed the same proliferation pattern and no statically significant differences were observed between the groups (**Figure 3.6**).

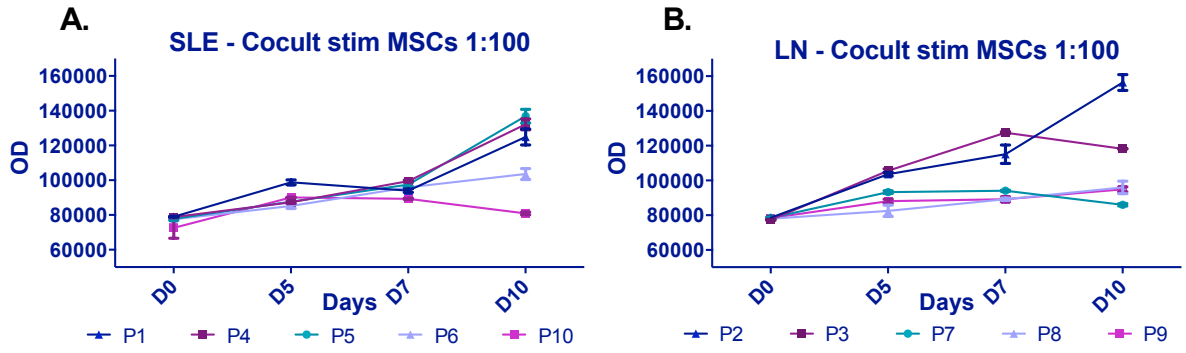


Figure 3.5: Proliferation assay of CD4+ T cells isolated from SLE (A.) and LN (B.) patients cocultured with stimulated MSCs

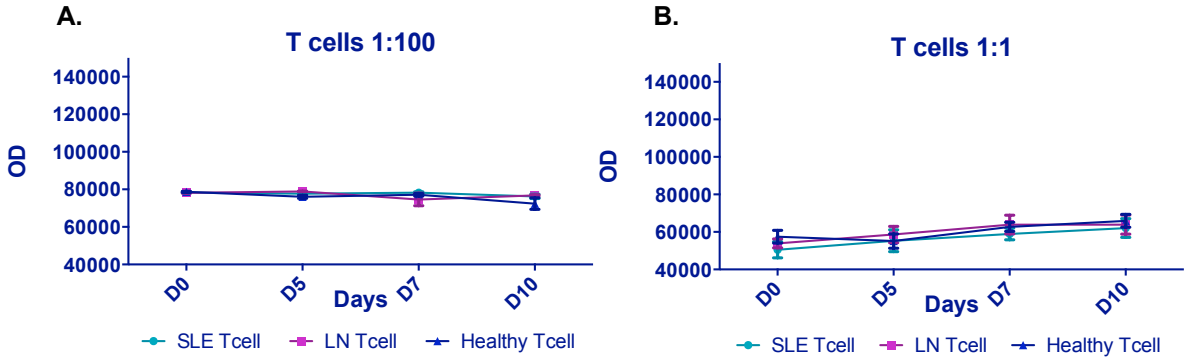


Figure 3.6: Proliferation assay of CD4+ T cells isolated from healthy blood donors, LN patients and SLE patients and cultured alone. A.] T cell cultured alone for the 1:100 coculture setup and B.] T cells cultured alone for the 1:1 coculture setup.

3.2.2 Effects of HUV-EC-C cells on the proliferation of the CD4+ T cells from healthy blood donors.

To examine the effect of HUV-EC-C cells on T cell proliferation, CD4+ T cells were isolated from healthy individuals and cocultured with a low ratio of HUV-EC-C cells: CD4+ T cells (1:1 ratio) and a high ratio of HUV-EC-C cells:CD4+ T cells (1:100 ratio). The results from the two-way ANOVA analysis showed that both coculture and time individually and combined have statistical significant effects on the proliferation of the T cells cocultured in a low ratio (1:100) (**Table 3.4**). When the T cells are cocultured in 1:1 ratio with HUV-

EC-C cells, only time will have a statistical significant effect on the T cell proliferation (**Table 3.4**).

The post hoc analyses of the proliferation of the T cells showed that the coculture in 1:100 ratio were increased from day 5 when compared to the T cells cultured alone (**Figure 3.7.A**). We did not observe a statically significant difference in T cells proliferation cocultured with stimulated HUV-EC-C cells compered to non-stimulated at day 10. When the T cells were cultured at 1:1 ratio, no statistically significant differences was observed by the post-hoc analyses between three culture conditions (**Figure 3.7.B**), but there were statistical significant differences between the day 0 and day 10 for all culture conditions of T cells.

Table 3.4: Two-way ANOVA of the proliferation assay of CD4+ T cells in coculture with HUV-EC-C.

	HUV-EC-C 1:1	**HUV-EC-C 1:100**
Interaction (time*coculture)	F (6, 24) = 0,1080 p = 0,9947**(ns)**	F (6, 24) = 15,10 p < 0.0001 (****)
Time	F (3, 24) = 14,54 p < 0.001 (****)	F (3, 24) = 53,99 p < 0.0001 (****)
Coculture	F (2, 24) = 0,2329 p = 0,7940 **(ns)**	F (2, 24) = 60,35 p < 0.0001 (****)

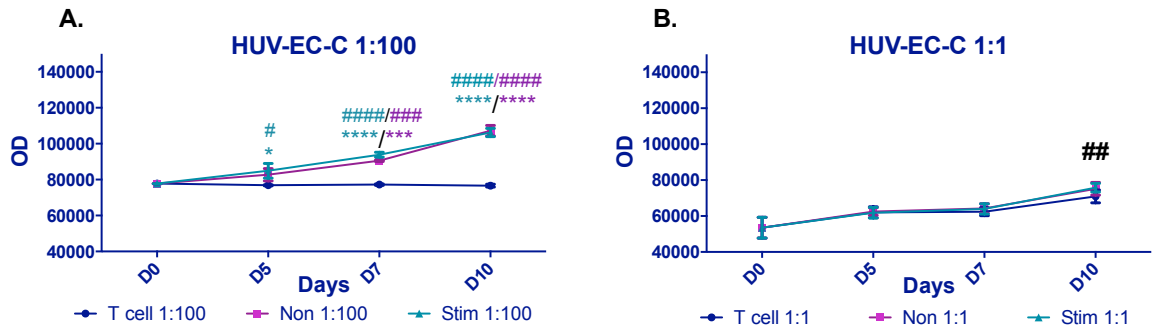


Figure 3.7 Proliferation of CD4+ T cells isolated from healthy blood donors in coculture with HUV-EC-C in 1:00 (A) and 1:1 (B) ratios. The T cells where either cultured alone (T cell – dark blue) or in coculture with HUV-EC-C, which were either stimulated with IL-1β and TNFα (Stim – turquoise) or non-stimulated (non-Stim – pink)**.** Two way ANOVA and the post-hoc analyses Dunnett's (#) and Tukey's (*) multiple comparison tests were performed, to test for differences between the proliferation at the different days to D0 within one group and to test for differences between the different group at a given time point respectively.. Data reported as mean with SEM. Only statistical significant values are included in the figures and marked with either "*" or "#" where: */# equals a p-value <0.05, **/## equals a p-value <0.01, *** /### equals a p-value <0.001 and ****/#### equals a p-value <0.0001. In addition the statistical significant values are colour coded, where turquoise marks and pink marks are for cocultured with simulated MSCs and non-stimulated MSCs respectively. See appendix **Table E** for adjusted p-values. OD; optical density.

3.2.3 Flow cytometric analysis of CD4+ Th subsets

Figure 3.7 present the positive compensation of the gating system, which was applied in this study. Single cells were identified by FSC-A vs. FSC-H blots (**Figure 3.8.A**). After gating on live T cells (PI negative cells, **Figure 3.8.B**), CD4+Th subsets were identified by CD4+T cells vs. SSC-A blot (vio-green positive, **Figure 3.8.C**). The CD4+T cells were analysed for CXCR3 and CCR4 expression (**Figure 3.8.D**). The CXCR3 positive cells from **Figure 3.8.D** were gated and further analysed with their expression of CCR6 and CCR10 (**Figure 3.8.E**), which will determine if the cells belonged to the Th1 or Th17 subpopulation. From **Figure 3.8.D**, where expression CXCR3 and CCR4 were plotted against each other, CCR4 positive cells were gated and further analysed in **Figure 3.8.F** to distinguish Th2 cells from the Th1/Th17 population. The CD4+ cells were also analysed for CCR4 and CCR6 expression to distinguish Th9 cells (**Figure 3.8.G**). Th22 cells were detected by CXCR3 vs. CCR10 plot (**Figure 3.8.H**).

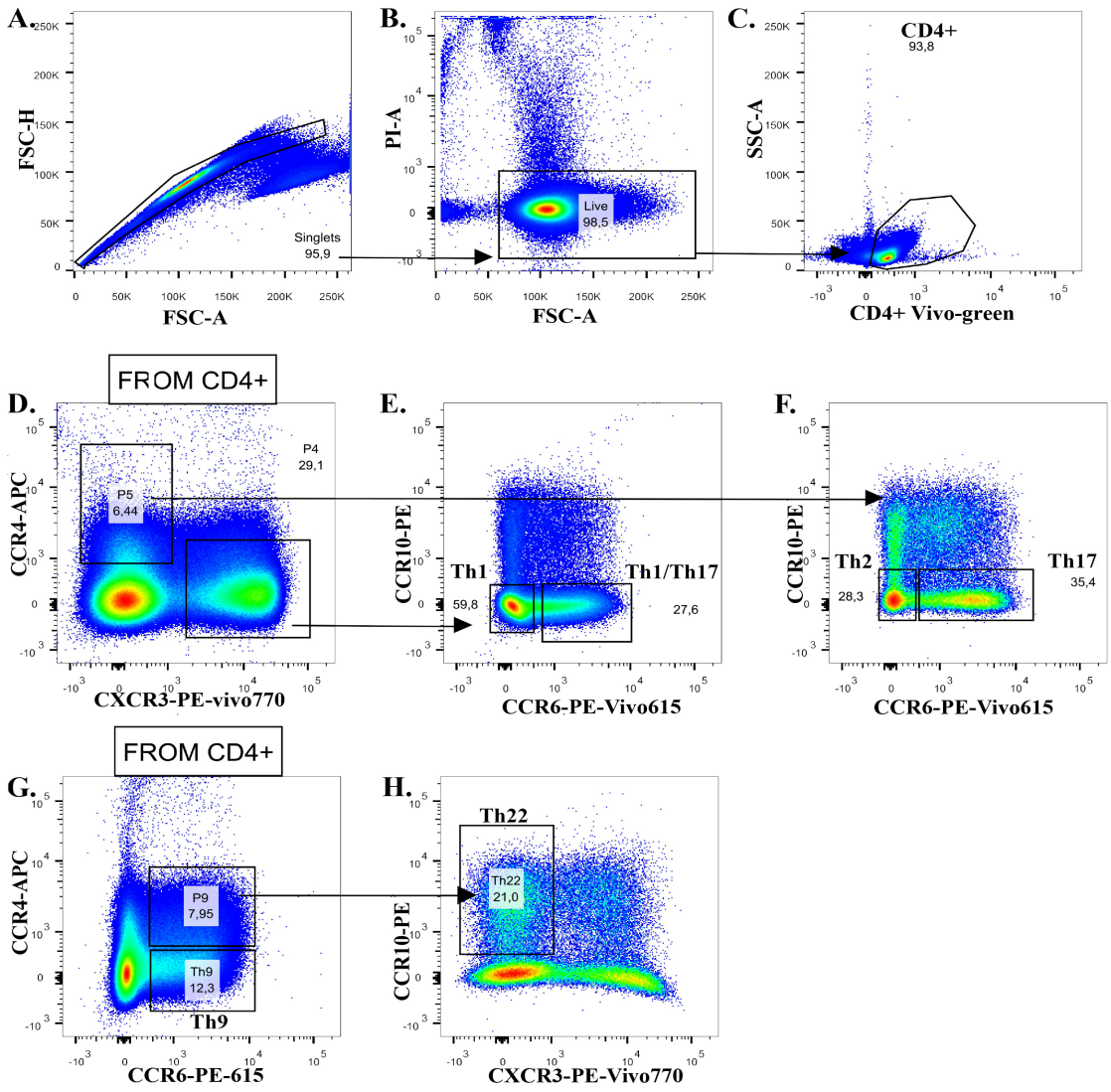


Figure 3.8: Positive control gating strategy for CD4+ Th subsets population.

3.2.4 CD4+ T cell differentiation in coculture with MSCs

Flow cytometry was performed to examine the CD4+ T cell differentiation in the coculture with MSCs. The percentage of the Th1, Th2, Th17, TH17/TH1, Th9 and the Th22 subsets of healthy donors, SLE and LN patients are presented in **Figure 3.9**. Statistical analyses were determined by using one-way ANOVA (**Table 3.5**) and the Sidak's multiple comparison test as post hoc analysis. The different culture set up for the CD4+ T cells were only statistical significant for the T cells isolated form healthy group for Th2 and Th17 subsets, when CD4+ T cell were cocultured with stimulated MSCs a 1:100 ratio at day 10 compared to the day 0 and CD4+ T cells cultured alone at day 10 at 1:100 ratio (**Figure 3.9.B & C**). For the rest of the Th subsets in all three groups, only non-statistical significant trends were observed. For example, the Th2 subset showed a trend for the SLE group with increase level at day 10 for cocultured with stimulated MSCs at 1:100 ratio compared to the day 0 and CD4+ T cells cultured alone at day 10 at 1:100 ratio.

To examine the variation in the Th subsets between the healthy, SLE and LN group, the levels of the various subsets at day 0 were compared between the groups. One-way ANOVA showed that there were only statistical significant differences for Th1 and Th9 subsets at day 0 in the SLE group compared with the healthy group. Both subsets were lower in the SLE group compared to the healthy group (**Figure 3.10.A & E**). For the LN group, a trend with higher levels of Th2 and Th17 were observed, but the differences were not significant (**Figure 3.10.B & C**).

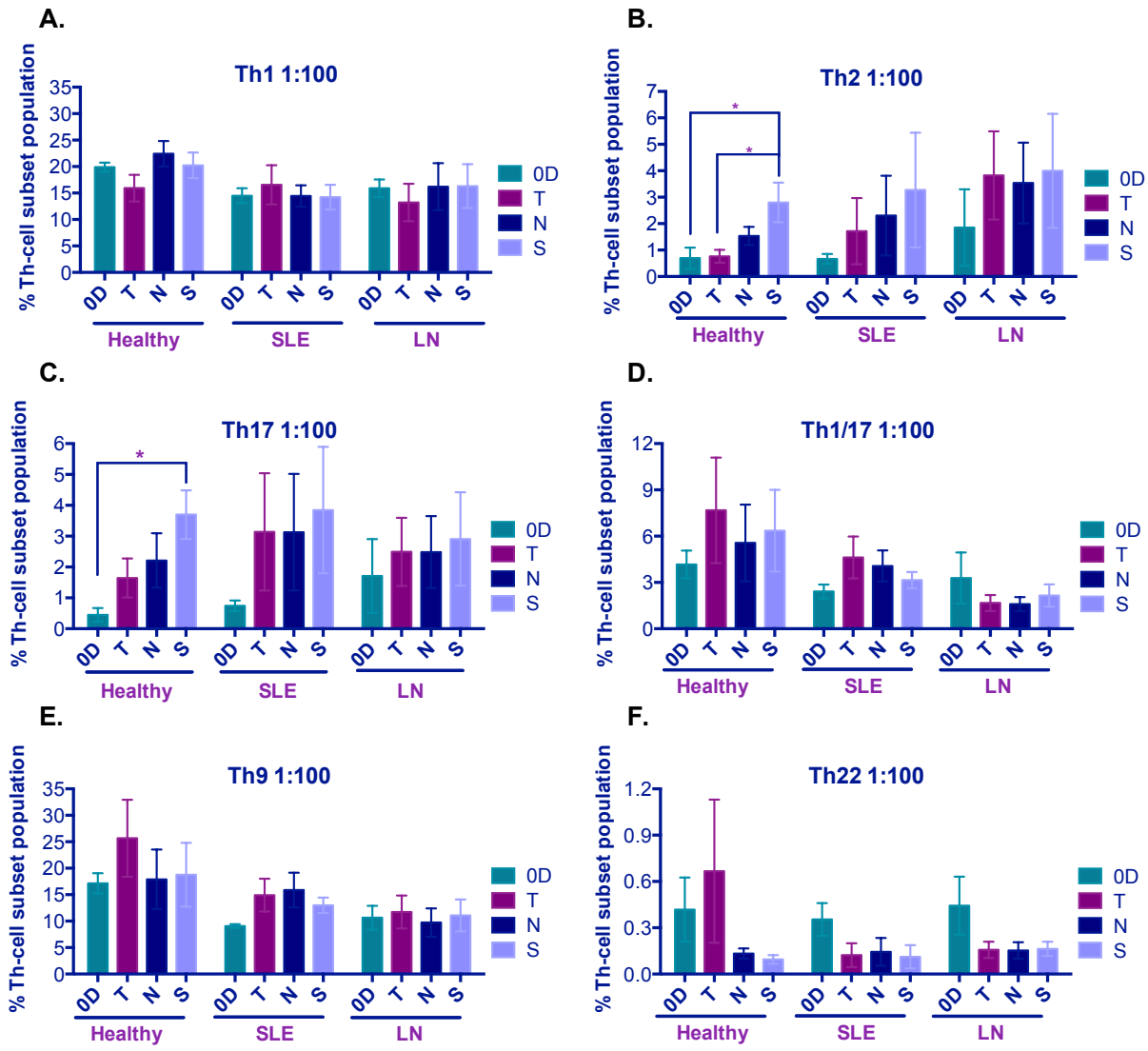


Figure 3.9: Th subsets (from healthy donors, and SLE and LN patients) differentiation in coculture with MSCs. Abbreviations; T cells from day 0 (D0), T cells grown alone for ten days (T), T cells grown in coculture with MSCs pre-stimulated for 6hours with TNFα and IL-1β(S), T cells grown in coculture with non-stimulated MSCs (N). Data are presented as mean with SEM. One-way ANOVA and the post-hoc analysis Sidak's multiple comparison tests were performed on the Th-cell subsets within each group (Healthy, SLE or LN)**. A.] Th1 subset; B.] Th2 subset;** statistical significant differences observed for the healthy group between the T cells at day 0 (**D0**) and T cells from coculture with pre-stimulated MSC (**S**) (p=0.0373), and between T cells cultured alone at day 10 (**DT**) and T cells from coculture with pre-stimulated MSC (**S**) (p=0.0462). **C.] Th17 subset;** statistical significant differences occurred only for the healthy group between the T cells at day 0 (**D0**) and T cells from coculture with pre-stimulated MSC (**S**) (p=0.0224). **D.] Th1/Th17 subset; E.] Th9 subset; F.] Th22 subset.**

Table 3.5: One-way ANOVA results of the changes in Th subset populations upon coculture with stimulated or non-stimulated MSCs.

	Healthy 1:100	SLE 1:100	LN 1:100
Th1	F(3, 16)=1.575 p=0.2345 **(ns)**	F(3, 16)=0.1878 p=0.9031 **(ns)**	F(3, 16)=0,1653 p=0,9182 **(ns)**
Th2	F(3, 16)=4.259 p=0.0217 **(*)**	F(3, 16)=0.5540 p=0.6528 **(ns)**	F(3, 16)=0.3277 p=0.8054 **(ns)**
Th9	F(3, 16)=0.4930 p=0.6922 **(ns)**	F(3, 16)=1,611 p=0.2261 **(ns)**	F(3, 16)=0.08958 p=0.9647 **(ns)**
Th17	F(3, 16)=3.955 p=0.0276 **(*)**	F(3, 16)=0.6463 p=0.5965 **(ns)**	F(3, 16)=0.1580 p=0.9230 **(ns)**
Th22	F(3, 16)=1.103 p=0.3766 **(ns)**	F(3, 16)=1.707 p=0.2057 **(ns)**	F(3, 16)=1.915 p=0.1679 **(ns)**
TH1/Th17	F(3, 16)=0.3350 p=0.8002 **(ns)**	F(3, 16)=1.142 p=0.3624 **(ns)**	F(3, 16)=0.6476 p=0.5958 **(ns)**

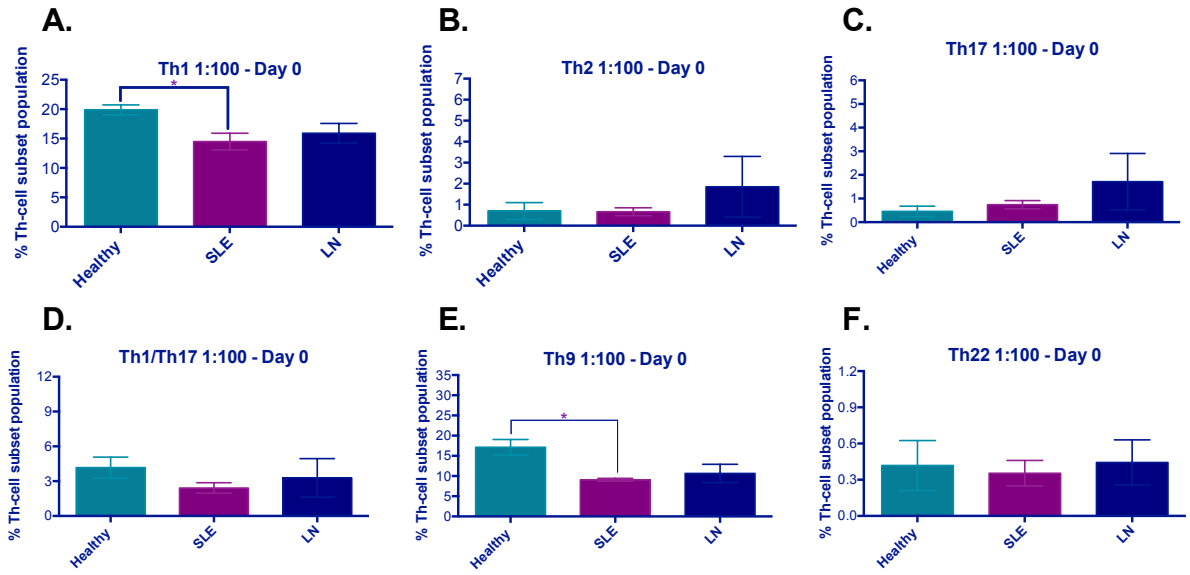


Figure 3.10: Th subsets from healthy donors, and SLE and LN patients at day 0. One way ANOVA and the post-hoc analysis Sidak's multiple comparison tests were performed on the Th-cell subsets within each group (Healthy, SLE or LN) Statistical significant values are marked with **"*" where: * equals a p-value <0.05, ** equals a p-value <0.01 and *** equals a p-value <0.001.A.] Th1 subset;** there were significant statistical differences between the levels of Th1 from healthy blood donors and Th1 from SLE patients (p=0.0449). **B.] Th2 subset; C.] Th17 subset; D.] Th1/Th17 subset, E.] Th9 subset**; there were significant statistical differences between the levels of Th1 from healthy blood donors and Th1 from SLE patients (p=0.0183). **F.] Th22 subset.**

To determine if the differentiation of Th subsets were depended on direct contact with MSCs or not, a Transwell system were used (**Figure 3.11**). The results showed that Th2 and Th17 subsets differentiation were contact dependent since no statistically significant changes were observed at 1:100 ratios, especially in the healthy group (**Figure 3.11**). Further, the different Th subset populations in Transwell system were compared with T cells in direct contact with MSCs at 1:100 ratios (**Figure 3.12**).

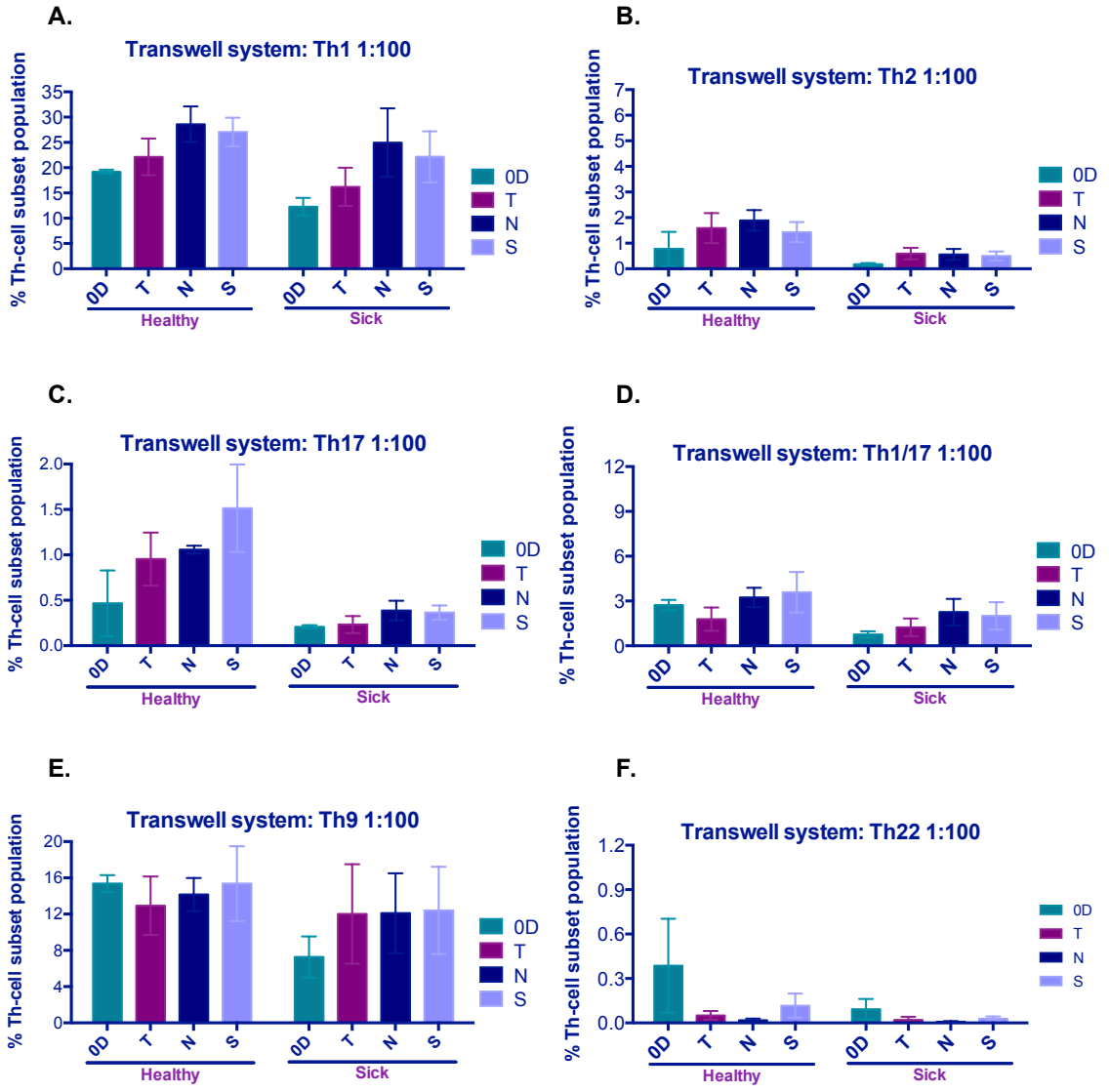


Figure 3.11: Th subset differentiation in T cells obtained from health blood donors (n=3) and patients (n SLE=1, n LN= 2), when Transwell system were applied to the culture condition. T cells from day 0 (D0), T cells grown alone for ten days (T), T cells grown in coculture with MSCs pre-stimulated for 6hours with TNFα and IL-1β(S), T cells grown in coculture with non-stimulated MSCs (N). Data are presented as mean with SEM. Only statistical significant values are included in the figures and marked with either "*", where: *equals a p-value <0.05, **equals a p-value <0.01, *** /equals a p-value <0.001 and **** equals a p-value <0.0001**. A.] Th1 subset, B.] Th2 subset, C.] Th17 subset, D.]Th1/Th17 subset, E.]Th9 Subset, F.] Th22 subset**

The one-way ANOVA and the Sidak's multiple comparisons between Th2 and Th17 subsets from direct contact compared to the Transwell at 1:100 ratio, showed only significantly lower Th17 subset at day 10 for the T cells cultured alone in the Transwell system compared to the Th17 subset in the direct contact in SLE group at 1:100 ratio. In the Healthy group, Th2 and Th17 populations from cocultured with stimulated MSCs at 1:100 ratio were higher, but not statically significant, in direct contact compared to Transwell system. In the SLE and LN groups, Th2 and Th17 populations in all three different setups in direct contact (cocultured with stimulated MSCs, cocultured with non-stimulated, and T cells cultured alone at 1:100 ratio) were higher compared to Transwell system, but it were not statically significant.

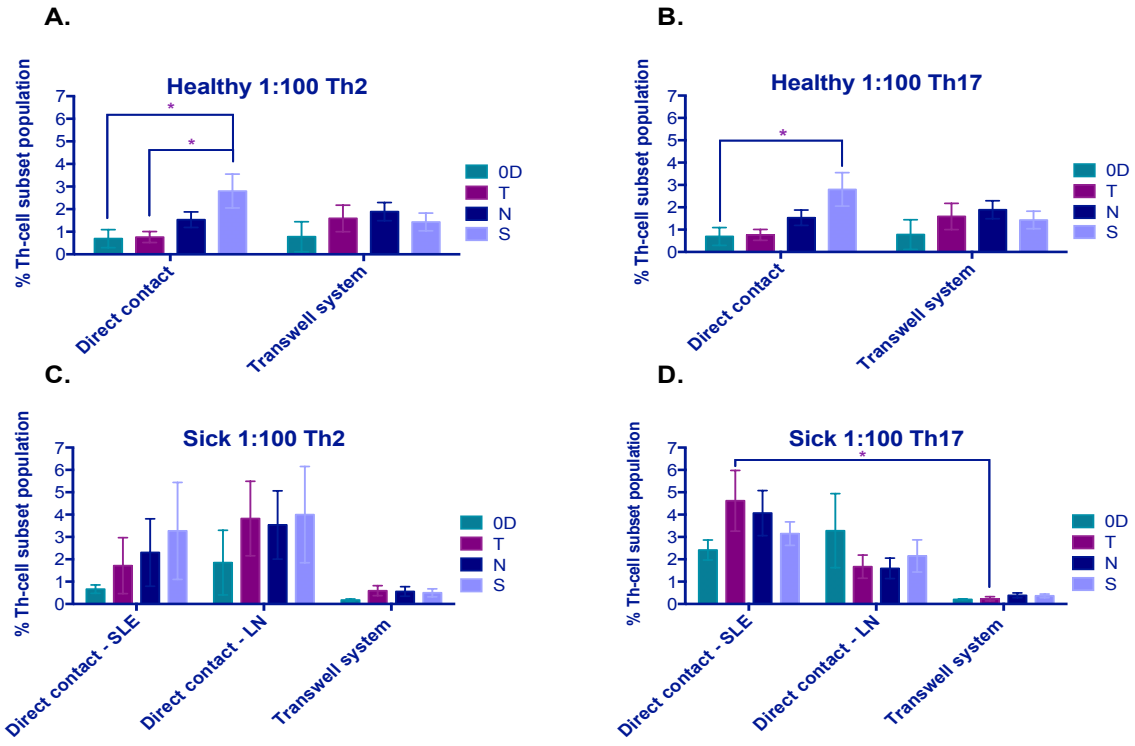


Figure 3.12: Comparison of the Th subsets when the T cells are cocultured with MSCs. Data is presented as mean with SEM. One-way ANOVA and the post-hoc analysis Sidak's multiple comparison test were performed on the Th-cell subsets, which CD4+ T cells were cocultured in direct contact with MSCs or in Transwell system. In the post-hoc analysis following comparison were performed; T cells cocultured in direct contact with stimulated MSCs compared with T cells cocultured with stimulated MSCs in Transwell system (light blue); T cells cocultured in direct contact with non-stimulated MSCs compared with T cells cocultured with non-stimulated MSCs in Transwell system (dark blue); T cells cultured alone for the direct contact setup compared with T cells cultured alone for the Transwell system (purple). **A.] and B.] present the changes in respectively Th2 and Th17 subset populations, when T cells from healthy blood donors were cocultured in direct contact or in Transwell system with MSCs.** The marked statistical significant with an increase levels of Th2 and Th17 population, when the T cells from healthy blood donors were cocultured in direct contact with MSCs, were based on the calculation in **Figure 3.8**. **C.] and D.]Changes in Th2 and Th17 subset populations for when T cells from patients are cultured with MSCs, either in direct contact or in Transwell system.** One-way ANOVA and Sidak's multiple comparison test showed statistical reduction in Th17 population for T cells cultured alone in the Transwell system, compared to the T cells from SLE patients and cultured without the Transwell system (p= 0.0218). **Abbreviations: T cells from day 0 (D0), T cells grown alone for ten days (T), T cells grown in coculture with MSCs pre-stimulated for 6hours with TNFα and IL1β(S), T cells grown in coculture with non-stimulated MSCs (N).** Only statistical significant values are included in the figures and marked with either "*", where: *equals a p-value <0.05, **equals a p-value <0.01, *** /equals a p-value <0.001 and **** equals a p-value <0.0001

3.2.5 CD4+ T cell differentiation in coculture with HUV-EC-C cells

The same experiment was performed with HUV-EC-C cells to examine if HUV-EC-C cells also could induce differentiation of CD4+ T cell isolated from healthy individuals (n=3) or not (**Figure 3.13**). One-way ANOVA analysis showed that only the coculture between T cells with HUV-EC-C cells could only have a statistical significant effect on the Th1/Th17 subset population ($F(3,8)=13.73, p=0.0016$), but not for the remaining CD4+ subset population. Th1/17 subset increased significantly at day 10 in all three different setups (cocultured with stimulated HUV-EC-C, cocultured with non-stimulated, T cells cultured alone at 1:100 ratio) compared to day 0, but there was not a significant difference between cocultured with stimulated HUV-EC-C compared to T cells cultured alone at 1:100 ratio.

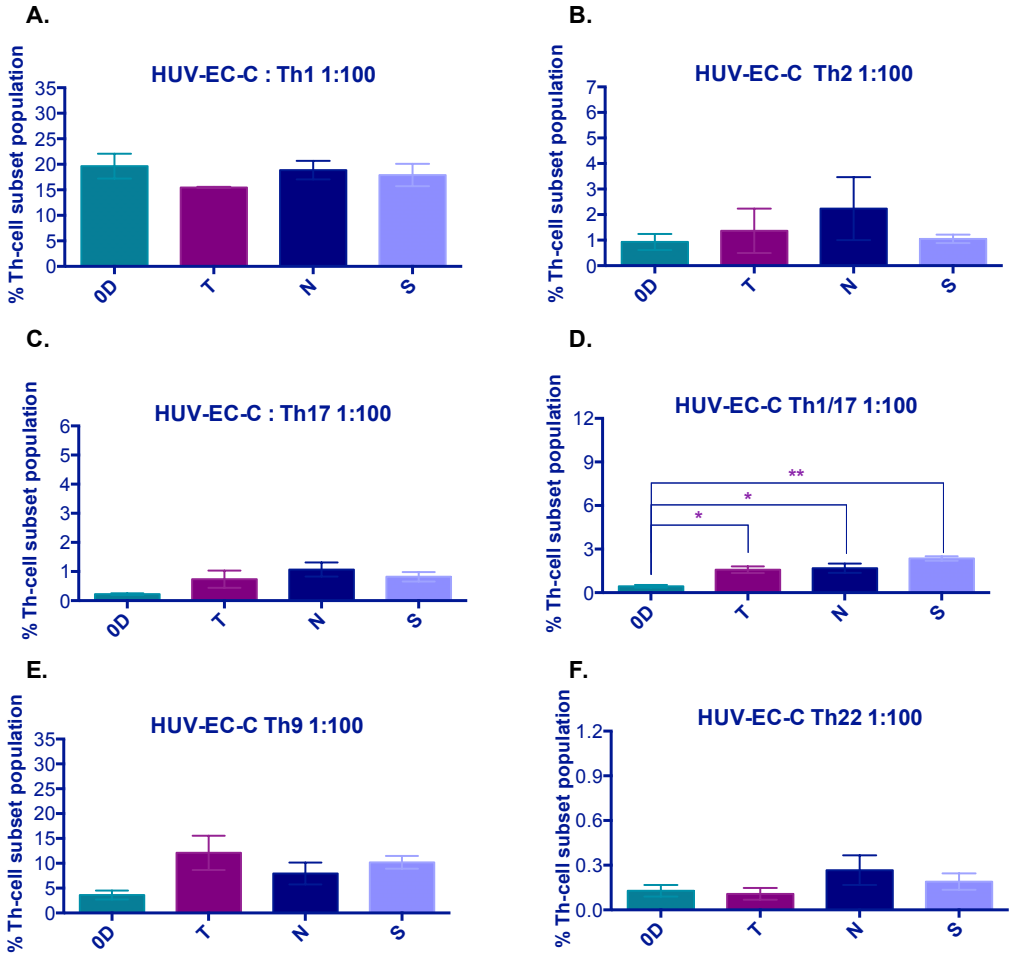


Figure 3.13: Th subsets healthy donor in coculture with HUV-EC-C cell line, which were either pre-stimulated with TNFα and IL-1β (S) or were non-stimulated (N). T cells from day 0 (D0) and T cells grown alone for ten days (T). One way ANOVA and the post-hoc analysis Sidak's multiple comparison tests were performed on the Th-cell subsets, and statistical significant values are marked with "*" where: * equals a p-value <0.05, ** equals a p-value <0.01 and *** equals a p-value <0.001**. A.] Th1 subset, B.] Th2 subset, C.] Th17 subset, D.] Th1/Th17 subset;** Statistical significant differences between D0 to T (p=), D0 to N (p=) and between D0 and S (p=)**, E.] Th9 subset**, **F.] Th22 subset**

3.2.6 Flow analysis on T cells cocultured in 1:1 ratio of MSCs

CD4+ T cells viability among all three groups (Healthy, SLE and LN) reduce sharply at day 10 in 1:1 ratio (MSCs:CD4+ T cells) coculture set up compared to 1:100 (**Table 3.6**). Due to the low number of live CD4+ T cells, we observed dramatically small cell population, few cells, (**Figure 3.14**) in different Th subsets selected populations. In some of the patients, only one or two cells could (e.g., Th17 in the Healthy group, **Table 3.6**) change the population percentage significantly. Hence, it is not possible to evaluate reliably the CD4+ T cells differentiation at 1:1 ratio.

Table 3.6: Cell count and the population percentage of different CD4+ T cells subsets after cocultured with non-stimulated MSCs at 1:1 ratio.

		Healthy	SLE	LN
Live	%	11.4	2.27	14.3
	#	1674	302	1923
Th1	%	28.1	0	7.3
	#	406	0	123
Th2	%	0.42	0	2.31
	#	6	0	39
Th17	%	0.21	0	2.02
	#	3	0	34
Th9	%	22.8	40.6	12.7
	#	329	54	214
Th1-17	%	4.64	6.77	1.6
	#	67	9	27
Th22	%	0	1.5	0
	#	0	2	0

% Cell population percentages, # cell count

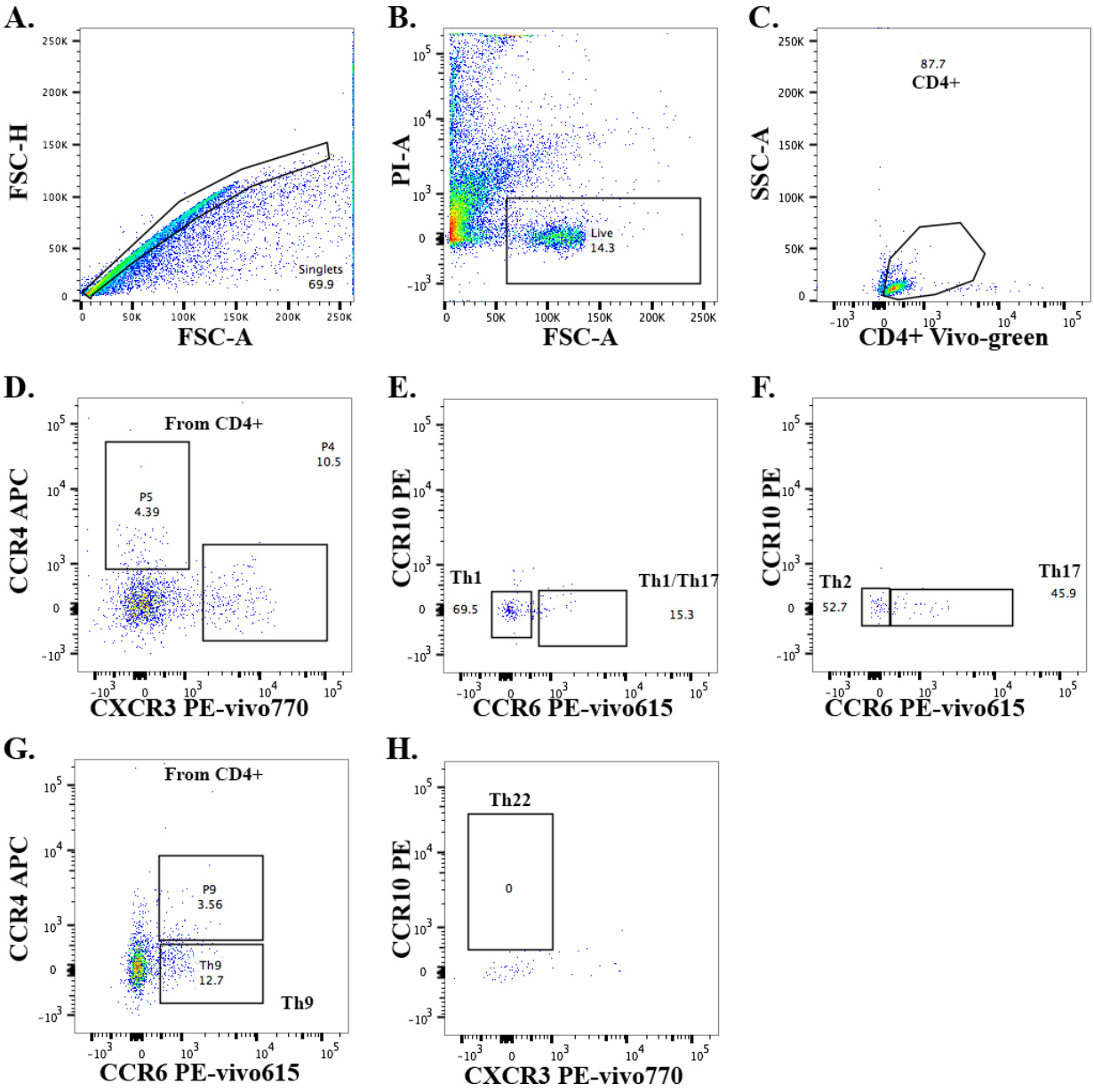


Figure 3.14: CD4+ T cells from SLE patient (P1) cocultured with non-stimulated MSCs at 1:1 ratio.

3.3 Confocal of murine kidney

Tissue specific MSC-like cells were detected in murine kidneys (NZBW-F1), which have been stained with immunofluorescence marked antibodies against PDGFR-α, Nestin, SCA-1 and CD45 (**Figure 3.15**). The MSC-like cells were characterized by their expression of PDGFR-α, Nestin and SCA-1 and lacking the expression of CD45. There were endothelial cells in the veins and arteries and some of the tubular cells, which were positive for Nestin and SCA-1, and negative for PDGFR-α and CD45. MSC-like cells were mostly located in the pelvic wall within the kidney from the young mice, and CD45+ tissue resident immune cells were observed at the edge of the pelvic wall (**Figure 3.15.A**). The antibody positive and protenuric mice had developed TLS in the pelvic wall, relatively larger in protenuric mice (**Figure 3.15 B & C**). In TLS, MSC-like cells were observed mostly in the pelvic wall and in between the CD45+ immune cells (**Figure 3.15.B & C**). Higher magnification of Region of Interest (ROI) are shown in **Figure 3.16** combined with signal intensity indicators, and signal intensity graphs presented in **Figure 3.17**. The signal intensity indicators were located in two distinct areas, area 1 (higher frequency of MSC-like cells) and area 2 (higher frequency of CD45+ immune cells) (**Figure 3.16 A6, B6 & C6**).

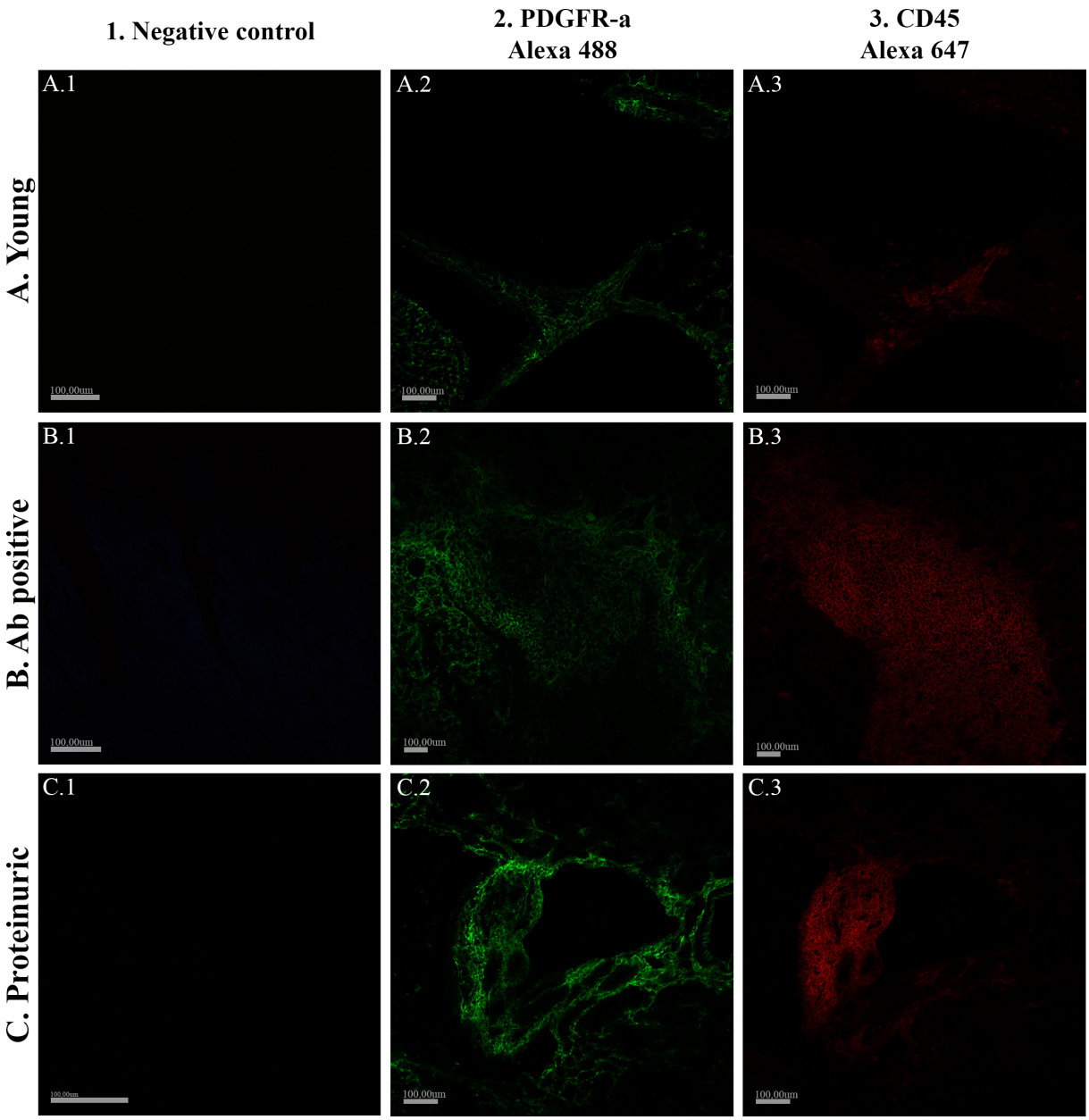
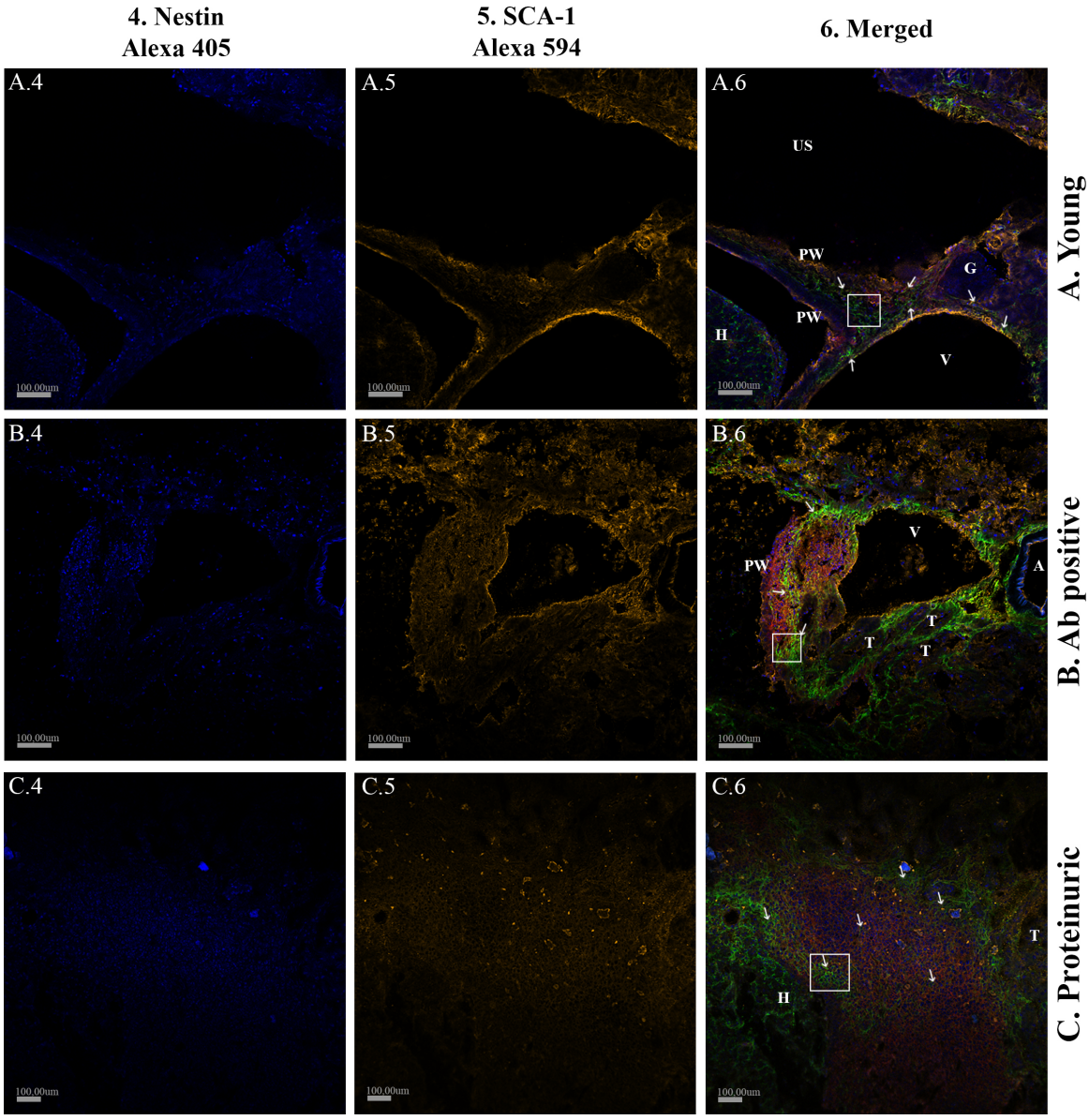


Figure 3.15: IF of NZBW-F1 murine kidney from A.] Young mouse (6weeks old, Ab negative proteinuria negative), B.] Antibody positive mouse (30 weeks old, Ab positive, proteinuria negative), and C.] Protenuric mouse (27 weeks old, Ab positive, proteinuria), stained with antibodies against (2.) PDGFR-α, (3.) CD45, (4.) Nestin and (5) SCA-1. The arrows in the merged images (6.) are pointing on areas containing MSC-like cells (PDGFR-α, Nestin+, SCA-1+, CD45-). The boxes in the merged images are further presented in Figure. 3.X**. Abbreviations**: A; artery, Ab; dsDNA antibody G; Glomeruli, H; Hilum, PW; pelvic wall, T; tubule, US; urinary space, V; vein.


4. Nestin
Alexa 405
5. SCA-1
Alexa 594
6. Merged
A.4
A.5
A.6
US
PW
PW
G
H
V
A. Young
B.4
B.5
B.6
V
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A
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B. Ab positive
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C. Proteinuric
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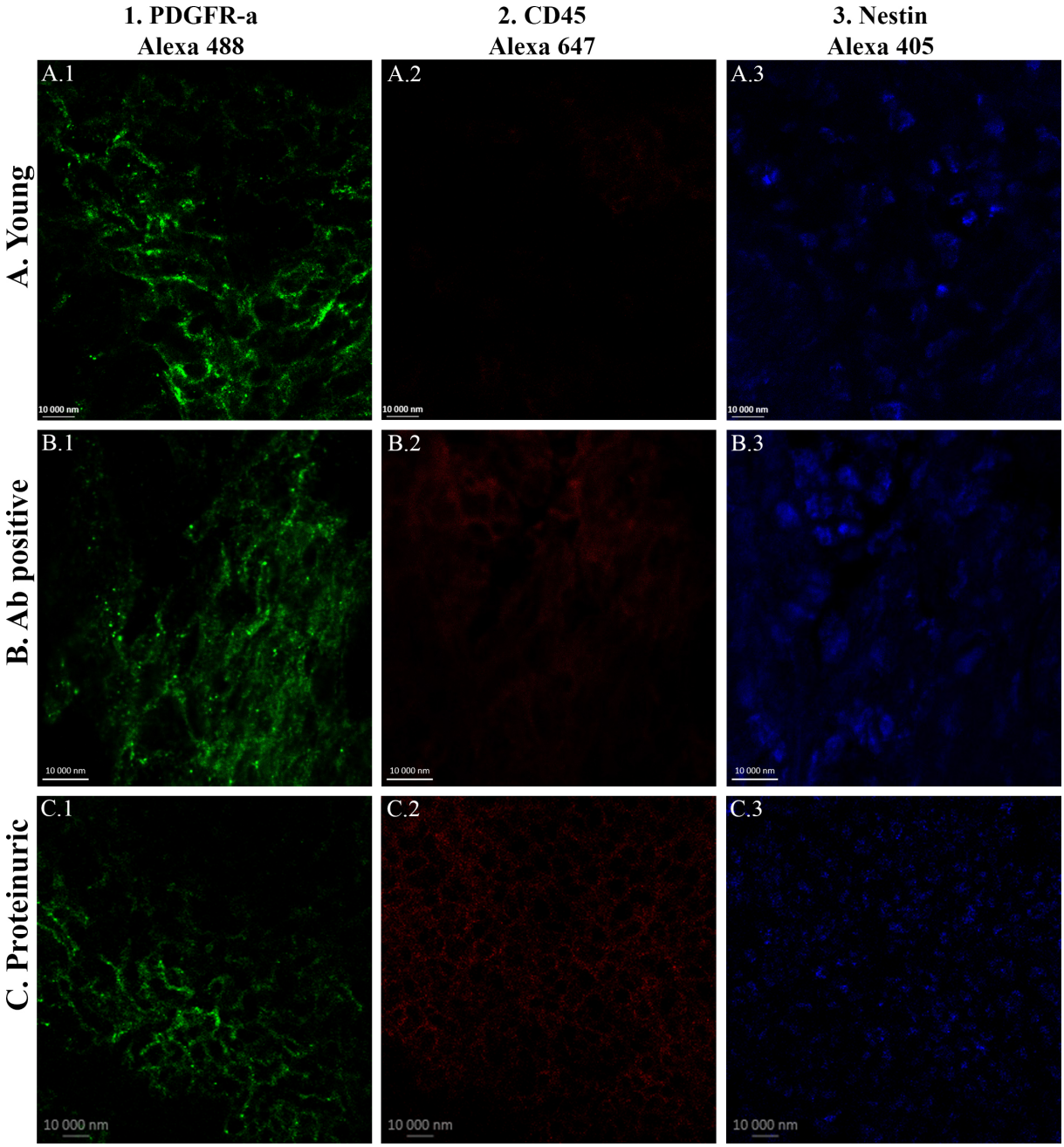
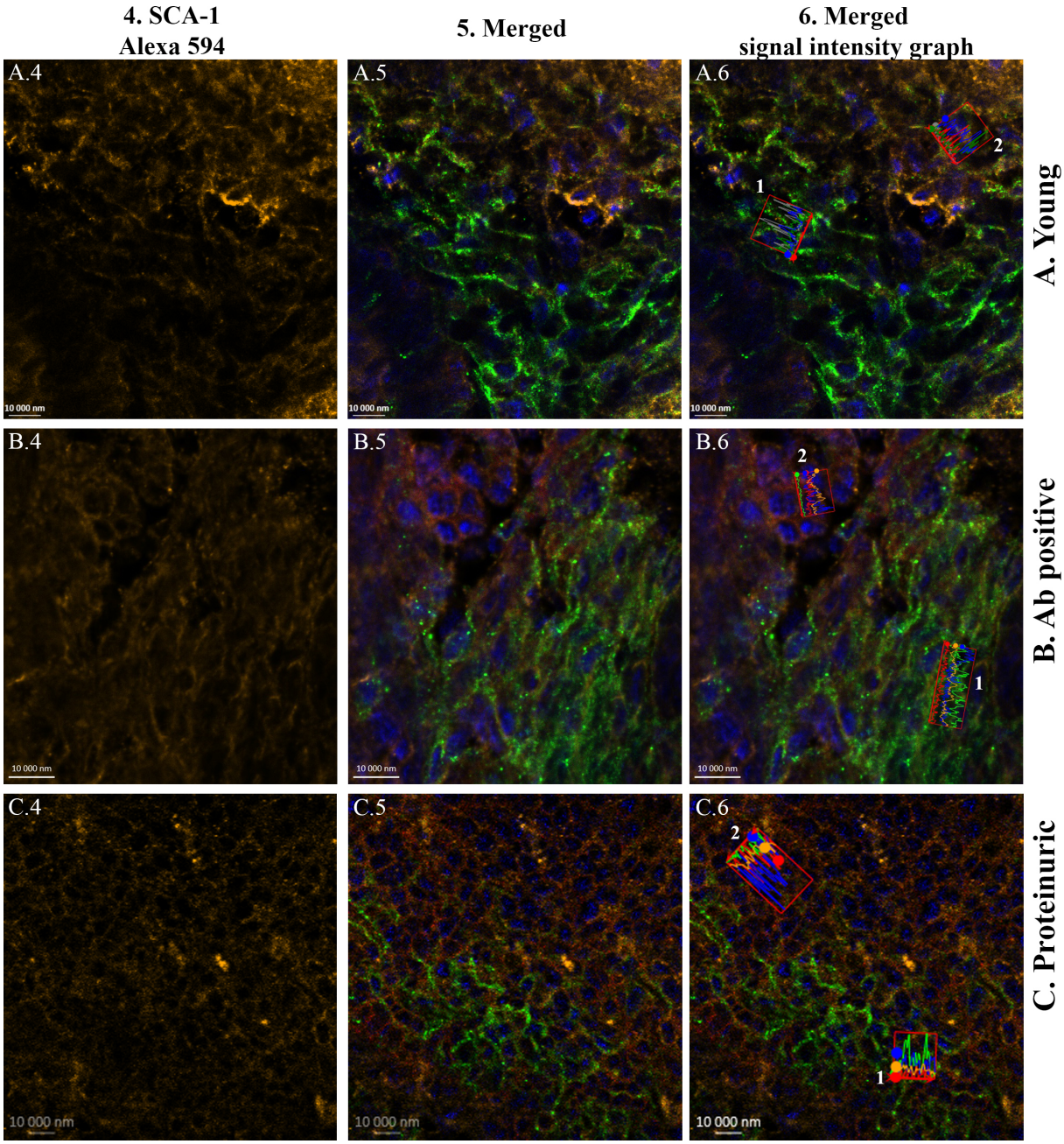


Figure 3.16: Magnification of the images from the boxes in "Figure 3.X; IF of NZBW-F1 murine kidney". Kidney from A.] young mouse (6weeks old, Ab negative, proteinuria negative), B.] Antibody positive mouse (30 weeks old, Ab positive, proteinuria negative), and C.] Protenuric mouse (27 weeks old, Ab positive, proteinuria), stained with antibodies against (1.) PDGFR-α, (2.) CD45, (3.) Nestin and (4.) SCA-1. In the merged images with the signal intensity graphs (6.), the numbers 1 and 2 are referring to areas with MSC-like cells (1, PDGFR-α+, Nestin+, SCA-1+) and areas without these cells (2, low PDGFRα- and high CD45 signals).


4. SCA-1
Alexa 594
5. Merged
6. Merged
signal intensity graph
A.4
A.5
A.6
1
2
A. Young
B.4
B.5
B.6
2
1
B. Ab positive
C.4
C.5
C.6
2
1
C. Proteinuric
10 000 nm

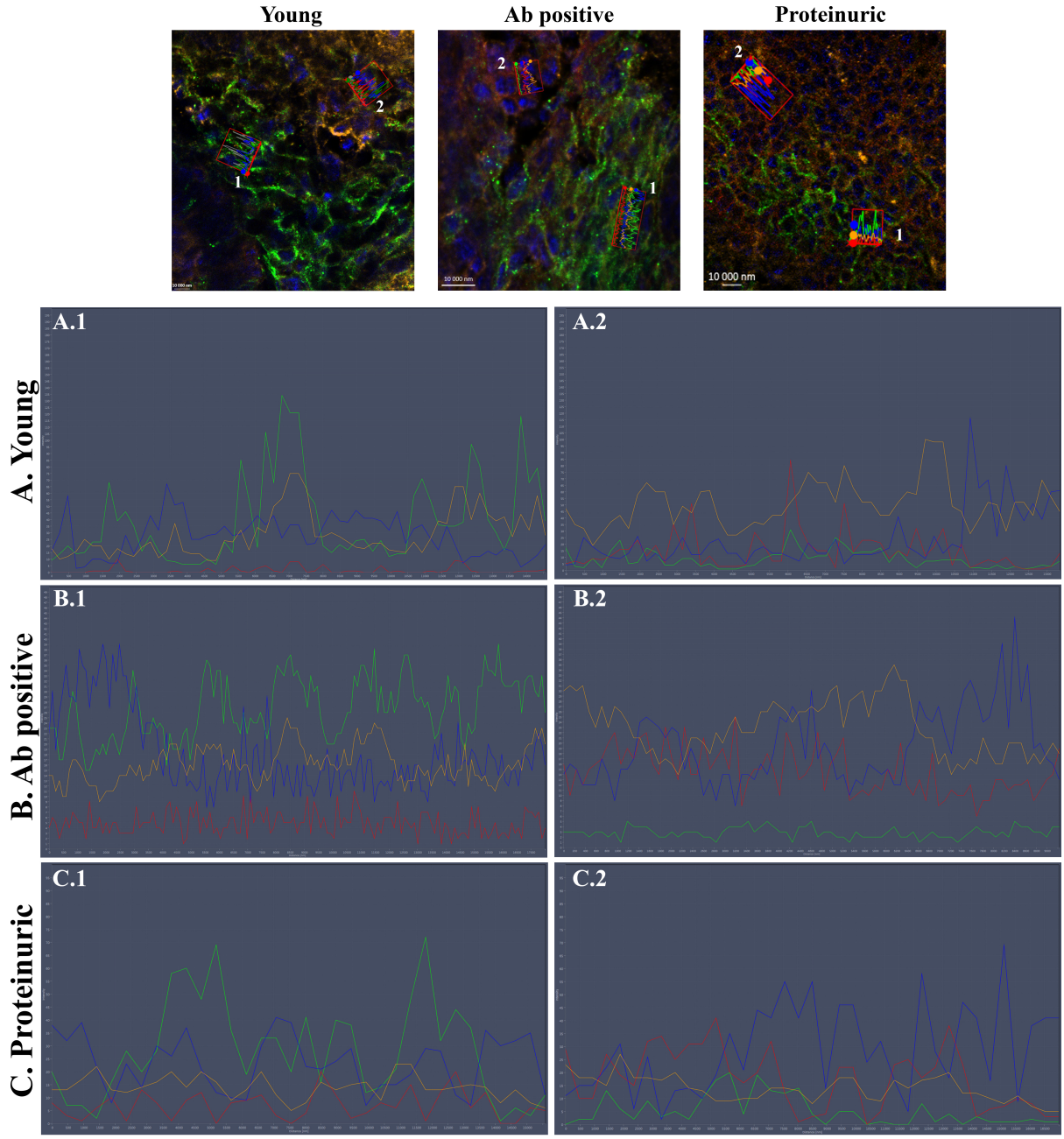


Figure 3.17: Signal intensity graphs over the areas with areas marked with signal intensity graphs in Figure 3.16. A.1, B.1 and C.1 are referring to the areas with MSC-like cells, where A.2, B.2 and C.2 are referring to the areas without these cells.

4 Discussion

In the present study we evaluated the immunostimulatory potential of MSCs and their effects on proliferation and differentiation of CD4+T cells. We also observed if there were any differences in the CD4+ T cell response in normal healthy blood donors and in SLE patients with and without nephritis. Based on these observations we investigated if MSCs could be a potential candidate as LTo cells in developing TLS.

4.1 The immune-stimulatory potential of MSCs and their effects on CD4+ T cells in coculture

Previous studies have assessed that MSCs can have proinflammatory properties if the pattern recognition receptors TLR-2 or TLR-4 are activated [104, 107, 109]. In our study we validated the immunostimulatory potential of these cells during inflammation, through resemble the conditions at the site of inflammation. By applying a screening of MSCs with different cytokines for various time lengths, we found the immunostimulatory potential of these cells were most potent in a low inflammation environment. The MSCs cultured with a low concentration (8ng/mL) of TNFα and IL-1β for a short period of time (6hours) presented inflammatory characteristics, with their enhanced production of proinflammatory cytokines, expression of adhesion molecules and an increased production of chemokines like CCL19, which is known for T cell recruitment. CCL19 is a chemokine, which is important for the recruitment and organization of T cells in an developing lymphoid tissue [86].

From the stimulation of MSCs with low concentration of proinflammatory cytokines, we showed that these cells could initiate an early inflammatory cascade by the production of cytokines and chemokines. The next step in this study was to assess their effect in a coculture with CD4+ T cells isolated from healthy individuals, SLE patients and LN patients. The results from our study showed that MSCs cocultured in a low (1:1) CD4+ T cell ratio did not have any stimulatory influence on the T cell proliferation. While MSCs cocultured in a high (1:100) CD4+ T cell ratio, had an enhanced effect on proliferation and differentiation of T cells.

Najar et al. [106], showed the importance of the MSCs:T cells ratio in coculture and how this could influence the MSCs' immunoregulatory potential. They cocultured MSCs for five days in a relatively low ratio with T cells (1:4 and 1:8), and observed inhibitory effect of MSCs on the mitogenic T cell proliferation (the authors did not specified clearly what kind of T cells, CD4+ or CD8, they were using) [106]. Their results also imply that the T cell

proliferation will be stimulated by MSCs, if the MSCs were occulted with T cells in a high MSC:T cell ratio (1:80) [106]. The study by Le Blanc et al. supports this theory, which the ratio between MSCs and T cells in culture will influence the observed results of T cell proliferation [121]. By stimulating peripheral blood leukocytes in a mixed leukocyte reaction (MLR) before coculture they showed MCSs in a low MSC:Tcell were inhibiting the MLR, while MSCs in a high MSC:T cell ratio had the opposite effect and were enhancing the MLR [121]. Bocelli-Tyndal and colleagues also support the possible outcome of that MSCs can be immunostimulatory at specific conditions [122]. In their study, they stimulated PBMCs, total T cell population and the T cell subset (CD4+ and CD8+) with IL-2, IL-7 or IL-15, then cocultured with MSCs derived from the bone marrow. The PBMCs and T cells were cocultured from a low ratio (1:2) to a middle high ratio (1:50) for one week before the proliferation was measured. The PBMCs and T cells stimulated with IL-2 and IL-15 before cocultured and cocultured with MSCs in a 1:50 ratio (MSCs:PBMC/Tcell), had an increased proliferation compared to PBMC or T cells were cultured alone. However, in their study, there were no effects on proliferation when T cells were not stimulated before cocultured with MSC. In addition they did not stimulate the MSC before coculture with T cells.

The results from our study showed that MSCs cocultured in a low (1:1) CD4+ T cell ratio did surprisingly not have any effect on the T cell proliferation. In the results published by other research groups, the low MSC:T cell ratio will promote the immuno-suppressive potential of MSCs and the T cell proliferation will be inhibited [106, 121, 122]. In these reported studies the authors did not include how the time and the stimulation alone will affect the proliferation of activated T cells, they did this by exclude the data from the day 0. By excluding those data, the accuracy of their results is open for questions.

MSCs cocultured in a high ratio of CD4+ T cell, promoted the immunostimulatory potential of the MSCs and an increased proliferation of CD4+ T cells were observed for all three groups (healthy, SLE, LN). By stimulate the MSCs before coculture we tried to resemble an early inflammation environment to observe if this treatment would influence the observed effects on the T cell proliferation. Stimulation of MSCs before coculture enhanced the proliferation of CD4+ T cells isolated from healthy blood donors and SLE patients without nephritis, compared with the proliferation of CD4+ T cells in coculture with non-stimulated MSCs. For the CD4+ T cells isolated from the LN patients: the T cells proliferation for the CD4+ T cells cocultured with non-stimulated MSCs showed a similar enhanced proliferation, compared to CD4+ T cells cultured alone. This proliferation response of the CD4+ T cells from the LN patients can be linked to the possibility that these cells could

be more activated in patient's body, based on disease activity level, compared to the CD4+ T cells isolated from healthy blood donors and SLE patients. We did not observe any differences in T cell proliferation between the three groups (healthy, SLE, LN), when the CD4+ T cells were cultured alone. These observations indicate that the observed proliferations are initiated by MSCs in the coculture, and are not a result of other factors.

By comparing the Th-subsets from the three groups (healthy, SLE, LN) at day 0, a trend with higher levels of Th2 and Th17 subset were observed for the LN group. Th17 and their characteristic cytokine IL-17 are found to be increased in SLE patients, and even more during a SLE flare [123]. In the article by Saber and colleagues [124]: an correlation between Th17 cells levels in blood and the concentration of IL-17 in urine can be linked to nephritis in SLE patients and are good markers for disease activity in LN. In lupus nephritis, autoantibody deposition in the kidney have a major pathological role where they will promote the inflammation cascade within for example the glomeruli and contribute to development of proteinuria. Often, the deposited autoantibodies are from the subclasses of IgG (IgG1, IgG3, IgG4), IgE and IgA [29, 125, 126], indicating that Th2 cells are involved in the activation of antibody producing plasma B cells in lupus.

By studying how the MSCs will influence the proliferation of the CD4+ T cells isolated from the three groups (healthy, SLE, LN), it is possible to assume the MSCs perform their immunostimulatory potential and stimulate the CD4+ T cells when they are cocultured in a high T cell ratio. The next step was to study if this interaction between MSCs and CD4+ T cells could influence the Th cell population by stimulating differentiation into one or several Th subsets. MSCs, which were stimulated with cytokines to resemble the inflammatory condition, were capable to induce Th2 and Th17 differentiation in the healthy group in the MSC: CD4+ T cell at ratio of 1:100 with direct contact. In addition by separating the CD4+ T cell from the MSCs, we could observe that the shift in CD4+ Th subset were contact depended, as crosstalk through Transwell system were not sufficient to initiate the differentiation. Duffy et al. [127] reported in 2011 that MSCs could inhibit Th17 differentiation of CD4+ T cells in a low MSC:T cell ratio. Similar results were presented by Carrion and colleagues, where they had cocultured MSCs with T cells under Th1, Th17 or Treg polarizing conditions in 1:10 MSC:T cell ratio [111]. Controversially Rozenberg et al. [128] reported that MSCs could stimulate Th17 response in an coculture with PBMCs, where increased levels of IL-17 were observed when activated PBMCs were cocultured with MSCs for 3 days (1:10, MSC:PBMC ratio). In a study by Darlington et al. [129] medium from MSCs cultures, where the cells were either stimulated with 5pg/mL IL-1β or kept non-

stimulated, had the potential to supress Th1 cells and simultaneously stimulate Th17 cell responses. These findings from Rozenberg and Darlington support the hypothesis regarding the immunostimulatory properties of MSC. Under the specific conditions, MSCs can shift the CD4+ T cell population and promote Th17 subset response. Thereby, MSCs can promote an inflammation cascade and contribute to increase the severity of the disease, since the effector mechanisms of the Th17 subset are involved in several autoimmune diseases [130].

Batten et al. [131] reported that in a coculture with a low MSC:PBMC ratio, MSCs suppressed subsequent CD4+ T cell proliferative responses to allogeneic PBMCs in a contact-dependent manner [131]. Interestingly they also reported an increased Th2 response in the coculture, indicating that MSCs can promote Th2 differentiation [131]. Increased levels of Th2 in the blood have also been observed after MSCs transplantation in animal models of autoimmune diseases [132, 133]. In the animal model of multiple sclerosis, mice treated with MSCs transplantation could recover from limb paralysis [132]. This observed neurological improvement was associated with reduced infiltration of CD45+ leukocytes in the brain and spinal cord. The levels of Th1 and Th17 cells and their associated cytokines were reduced, while the IL-4 producing Th2 cells were elevated [132]. In the animal model of type 1 diabetes, Fiorina and colleagues analysed the Th1 and Th2 cytokine patterns in immune cells isolated from the spleen of mice treated with MSCs for 4weeks [133]. They found that the treatment affected the Th effector response between Th1 and Th2 cells, observed with increased IL-4 and IL-10 and thereby a shift toward a Th2 profile [133]. Interestingly, none of these studies could report an increase in both the Th2 and Th17 profile simultaneously, as we observed in our study. One of the reasons might be because they were focusing on either the Th1/Th2 balance or Th1/Th17 balance, and not the changes in the entire CD4+ T cell population. Nerveless, in both of the cases, when either Th17 or Th2 effector functions were increased, the Th1 effector functions were decreased [128, 129, 132, 133].

In our study we observed individually differences on the proliferation within the patient groups. This individually differences will interrupt the observation and overview over the trends that are reported in each patient group. Each patient holds his or her own unique disease progression and treatment, which is reflected into our results. Ideally, it would have been advantageous to increase the patient number in each group, for example from n=5 to n=10, to correct for those observed individual differences and to be able to give stronger statistical observations for the patients as two distinct populations.

From the screening of MSCs with low concentrations of the proinflammatory cytokines IL-1β and TNFα and from the coculture between T cells isolated from three groups

and MSCs, we have examined the immunostimulatory potential of MSCs. They have the capacity of stimulate CD4+ T cell proliferation and initiate CD4+ T cell differentiation into Th2 and Th17 subset. We believe, it is important to consider our results before administrating MSCs to SLE and LN patients as cell therapy, since the MSC:T cell ratio and the local inflammatory environment can lead MScs to initiate and stimulate the immune response more severe.

4.2 MSC in the role as a LTo cell in developing of TLS

In the theory of TLS development, stromal cells with mesenchymal origin have been hypothesized to play the role as LTo cells. From the confocal microscopy of the kidney from the young mice, we observed that MSC-like cells were located in the pelvic wall before TLS formation start. In the TLS observed in the kidneys of the antibody positive mice and from the protenuric mice, the MSC-like cells were located in the pelvic wall surrounding the TLS structure and also in between the CD45+ immune cells. These observations indicate that MSC-like cells are involved in the TLS formation and function. By merging this information with the observations from the coculturing with CD4+ T cells in a 1:100 ratio, it is possible to suggest that MSCs can play the role as LTo cells and initiates the TLS formation.

From the literature the mesenchymal stem cells are assumed to initiate the formation of SLO in a developing embryo [70, 88]. The formation of SLO and TLS share several patterns and mechanisms, and one can assume that the mesenchymal stem cells cells also are important in the developed TLS [88]. In our study a possible connection between TLS formation and the immunostimulatory properties of MSCs, can start with the CCL19 production. MSCs were able to produce CCL19 upon stimulation with low concentrations of the proinflammatory cytokines IL-1β and TNFα for a short period of time. This cytokine is necessary for recruitment of T and is involved in homing of T cells and DCs to lymphoid tissues [134], indicating that MSCs can be involved in the first phases of recruiting T cells to the site of inflammation. When the ratio between MSC and T cells get sufficient enough, the MSCs will stimulate the T cells to proliferate and initiate the T cell differentiation into inflammatory Th2 and Th17 subset. The Th17 will produce IL-17 and this cytokine has been shown to promote expression of CXCL13, CCL19 and CCL21 in tissue stromal cells, which are involved in the initial formation of lymphoid organs and are important in the maintenance of the structure [62, 135]. From the developing SLO, the first stages in lymphoid development were involved in recruiting of LTi cells to the site and were not depended on LTα1β2/LTβR

signalling [87]. In the developing TLS in the murine kidney, a crosstalk between the IL-17 producing T cell and the MSC-like cell could then initiate the LTα1β2/LTβR signalling between the MSC-like cell and the CD4+ T cell and then promote initiating TLS formation and development. The formation of the Th2 subset of CD4+ Th cells by MSC cells, in a tissue can promote the developing of IgG producing B cells and induce a local production of antibodies that are involved in renal manifestations of lupus. In fact Th2 cells have shown to become Tfh-like cells in response, like what we see in helminths infections with persistence of pathogen/antigen conditions [136].

4.3 Epithelial and endothelial cells immunostimulatory properties

In our study stimulation of the HMLE and HUV-EC-C cell linages were used as control for the observed effects with stimulation of the MSC cells. By stimulating these two cell types, HMLE as epithelial cells and HUV-EC-C as endothelial cells, we were able to study whether low concentration of cytokines could have an effect on two normal cell types within a tissue. Ideally, it would have been optimally to use epithelial and endothelial cells derived directly from the kidney, for example epithelial cells from the pelvic wall or proximal tubule cells. However in this study the use of HMLE and HUV-EC-C cells were (as mention) as control cell line, in the comparison of the effects observed by the MSCs.

The HUV-EC-C cells had a large increase in their expression of the adhesion molecules VCAM-1 and ICAM-1 upon stimulation, which is a common response in activated endothelium [137-139]. Activated endothelium will increase their expression of adhesion molecules to promote leukocyte rolling and transmigration and are important for the recruitment and infiltration of immune cells into an inflamed tissue [139]. Both the HUV-EC-C and the HMLE cells had an increase in their production of the pro-inflammatory cytokines IL-1β and TNFα, which was an expected result of positive-feedback reaction to enhance the inflammation response initiated by mentioned proinflammatory cytokine [137, 139].

With their unchanged expression of LTβR upon stimulation with low concentrations of proinflammatory cytokines, the HUV-EC-C cell can be a potential candidate as progenitor cell for HEV development. Activation of LTβR on endothelial cells are important for development of HEVs in SLOs [83], predicting that they can become HEVs in a developing TLS. The protein podoplanin (PDPN) is frequently expressed in lymphoid tissues and are assumed to be important for the integrity of HEVs [140, 141]. By evaluating the expression of PDPN in stimulated HUV-EC-C cells, we can observe if stimulation of these endothelial cells

can initiate the differentiation into the HEVs or other lymphoid tissue cells such as lymphatic endothelial cells. Since HUV-EC-C cells were not capable to express PDPN upon stimulation of low concentration IL-1β and TNFα, we can conclude that these cells can't differentiate into lymphoid tissue cells upon stimulation of low concentration of just IL-1β and TNF-α. To be able to differentiate into HEVs, endothelial cells need activation through their LTβR and archive growth factors from an LTo cell [83].

By using the HUV-EC-C cell line as a control for the coculture, we assessed the possible outcome of if endothelial cells in an inflamed tissue also could promote the changes in Th cells subsets population. From our results, the HUV-EC-C cells are capable to stimulate proliferation of healthy T cells, but will not influence the changes of the CD4+ Th subset population. In the stimulation of HUV-EC-C cells we observed that these cells were capable of producing high level of the proinflammatory cytokines IL-1β and TNFα, in addition to high levels of the adhesion molecules ICAM-1 and VCAM-1. These compounds might be one of the factors that could explain the proliferation of T cells. Endothelial cells are one of the first cells to interact with microbial components in the circulation and tissue damage, and will provide important signals for early activation of immune cells [142]. IFNγ stimulation of endothelial cells has been shown to promote production of compounds of the TNF family, which was observed with increased cytokine production and proliferation of CD4+ and CD8+ memory T cells [143]. In the study by Wheway et al., microparticles shed from activated endothelial cells were capable of stimulating both CD4+ and CD8+ T cells [144], which could also be one explanation of why we can observe increased CD4+ T cell proliferation in the coculture with HUV-EC-C cells. If HUV-EC-C cells could differentiate into HEVs in a developing TLS, they will also harbour an important immunostimulatory potential by regulating the recruitment of immune cells through their production of the chemokines CCL19, CCL21, CXCL12 and CXCL13 [62]. However, in our study we aimed to observe the effects of MSCs. The reason why we choose to work with HUV-EC-C and not HMLE as control for the coculture: is because of their immunological important properties as a messenger in an early inflammation cascade [142].

5 Conclusion

Our study indicates that the MSCs are capable to trigger an inflammation reaction, when they are exposed to an environment with low concentrations of proinflammatory cytokines. These cells are capable to stimulate T cell proliferation and differentiation when they are cocultured with a high ratio T cell (1:100), and thereby promote the development of TLS as an LTo cell. These factors are important in the consideration of treatment of lupus patients with MSCs, and this might be one of the factors that will determine if some of the cases of transplantation went wrong and a relapse occurred. Further research in the fields of TLS formation and the immunoregulatory potential of MSCs are needed to assess the possible outcome and safety with MSC transplantation.

6 Future directions

Further research has to be done to fill in gaps in our understanding of the immunostimulatory properties of MSCs and their role in TLS development. From the coculture between MSC and CD4+ T cells, an interesting approach to understand how these cells interacts will be by analysing and studying the changes in mRNA expression culturing has on the MSCs and the CD4+ T cells. "*How will the 1:1 and 1:100 MSC-T cell ratio influence this mRNA expression?*" is a question, which is longing for an answer. In our hypothesis an increased activation of LTβR and markers for FDC and FRC differentiation will support that the MSCs can function as an LTo cell.

Another approach is to study the media from the coculture for soluble proteins and cytokines. Are there any differences in the compensations of the media collected from the coculture with CD4+T cells in 1:1 or 1:100 ratios, and will stimulation of MSCs before coculture influence this compensation of the media? Can we transfer the media from the MSC:T cell coculture to cultured B cells, and how will this influence the B cell response?

A third possible approach in the study of the TLS development is to analyse the mRNA expression of the HUV-EC-C cells from the CD4+T cell coculture. Can these endothelial cells begin their journey towards HEV differentiation when in coculture with T cells? Or under which condition are needed for HEV development?

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Appendix

Additional Data

Table A: ANOVA table for the statistical two-way ANOVA analysis performed on the fold changes of the genes of interest for MSC (screening)

	MSC IL-1β	MSC TNFα	MSC ICAM-1	MSC VCAM-1	MSC CCL19
Interaction (Time*stim.)	F (7, 22) = 3,363 P=0,0136 (*)	F (7, 26) =957,5 p<0,0001 (****)	F (7, 22) =533,6 p<0,0001 (****)	F (7, 24)=169,1 p<0,0001 (****)	F (7, 22) = 20,11 p<0,0001 (****)
Time	F (7, 22) = 3,363 p<0,0136 (*)	F (7, 26) =957,5 p<0,0001 (****)	F (7, 22) =534,1 p<0,0001 (****)	F (7, 24)=169,2 p<0,0001 (****)	F (7, 22) = 20,10 p<0,0001 (****)
Stimulation	F (1, 22) = 8,045 p<0,0096 (**)	F (1, 26) =1550p<0,0001 (****)	F (1, 22) = 2553 p<0,0001 (****)	F (1, 24)=1021 p<0,0001 (****)	F (1, 22) = 189,8 p<0,0001 (****)

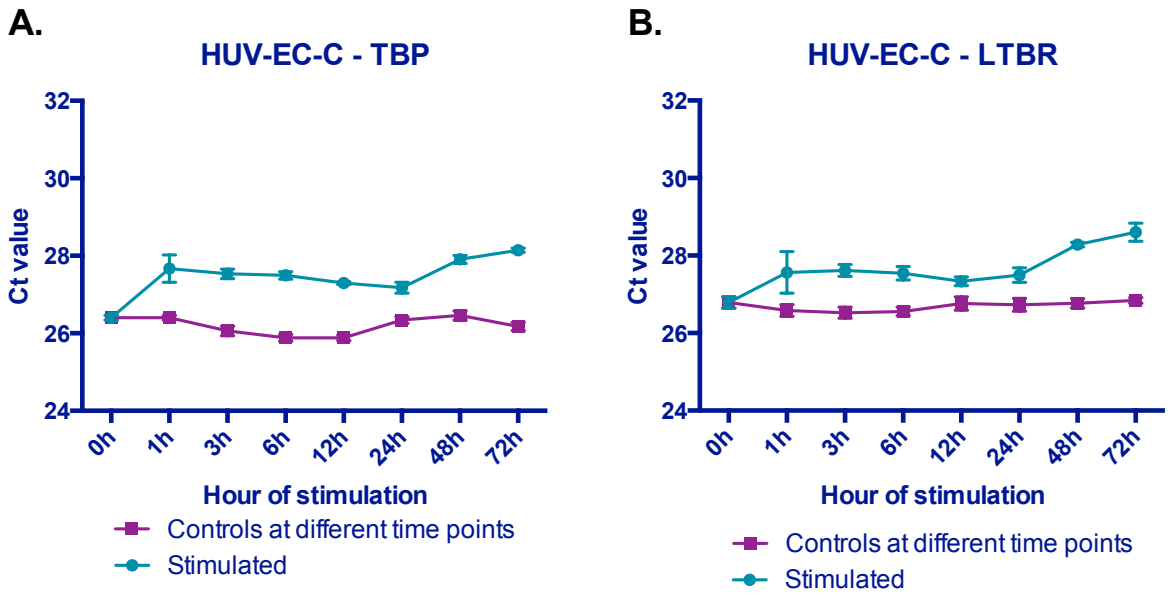

Fig. A Ct-values for (A) TBP and (B) LTβR for both stimulated and non-stimulated HUV-EC-C cells (stimulated with 8ng/mL TNFα and IL-1β)

Table B: Adjusted p-values from the post-hoc analyses of the two-way ANOVA performed on T cell proliferation. T cells isolated from healthy blood donors and cocultured with MSCs.

Healthy		1:1			1:100		
Post-hoc analysis	Time in coculture	5 days	7 days	10 days	5 days	7 days	10 days
Tuckey's multiple comparison test	S VS N	0,4789	0,9923	0,8207	0,9964	0,2623	<0,0001
	S VS T	0,3323	0,9404	0,5555	0,0335	0,0004	<0,0001
	N VS T	0,9615	0,8935	0,899	0,0275	0,0342	0,0003
Dunnett's multiple comparison test	S	0,6147	0,4369	0,0354	0,1087	0,001	0,0001
	N	0,9977	0,367	0,1345	0,0916	0,07	0,0113
	T	0,9547	0,6601	0,3008	0,9196	0,9814	0,4802

Abbrevations: T: T cells cultured alone; S: T cells in coculture with stimulated MSCs; N: T cells in coculture with non-stimulated MSCs.

Table C: Adjusted p-values from the post-hoc analyses of the two-way ANOVA performed on T cell proliferation. T cells isolated from SLE patients and cocultured with MSCs.

SLE		1:1			1:100		
Post-hoc analysis	Time in coculture	5 days	7 days	10 days	5 days	7 days	10 days
Tuckey's multiple comparison test	S VS N	0,997	0,9737	0,5747	0,9374	0,9055	0,0003
	S VS T	>0,9999	0,9859	0,4411	0,0897	0,0064	<0,0001
	N VS T	0,9971	0,9235	0,9732	0,1763	0,0198	0,0053
Dunnett's multiple comparison test	S	0,8748	0,4282	0,0205	0,0582	0,0035	0,0001
	N	0,8323	0,3059	0,1928	0,1267	0,0115	0,0087
	T	0,8739	0,5315	0,2851	0,9986	0,9999	0,9572

Abbrevations: T: T cells cultured alone; S: T cells in coculture with stimulated MSCs; N: T cells in coculture with non-stimulated MSCs.

Table D: Adjusted p-values from the post-hoc analyses of the two-way ANOVA performed on T cell proliferation. T cells isolated from LN patients and cocultured with MSCs.

LN		1:1			1:100		
Post-hoc analysis	Time in coculture	5 days	7 days	10 days	5 days	7 days	10 days
Tuckey's multiple comparison test	S VS N	0,6862	0,9838	0,9983	0,9583	0,9273	0,708
	S VS T	0,7957	0,8643	0,9593	0,1036	0,0013	<0,0001
	N VS T	0,9814	0,9372	0,9418	0,1781	0,0038	0,0018
Dunnett's multiple comparison test	S	0,9923	0,4141	0,0993	0,0863	0,0052	0,0003
	N	0,5915	0,3182	0,0882	0,1533	0,0143	0,0031
	T	0,7182	0,1738	0,1726	0,9993	0,9339	0,9952

Abbrevations: T: T cells cultured alone; S: T cells in coculture with stimulated MSCs; N: T cells in coculture with non-stimulated MSCs.

Table E: Adjusted p-values from the post-hoc analyses of the two-way ANOVA performed on T cell proliferation. T cells isolated from healthy blood donors and cocultured with HUV-EC-C.

HUV-EC-C		1:1			1:100		
Post-hoc analysis	Time in coculture	5 days	7 days	10 days	5 days	7 days	10 days
Tuckey's multiple comparison test	S VS N	0,6862	0,9838	0,9983	0,9583	0,9273	0,708
	S VS T	0,7957	0,8643	0,9593	0,0192	<0,0001	<0,0001
	N VS T	0,9814	0,9372	0,9418	0,1781	0,0002	<0,0001
Dunnett's multiple comparison test	S	0,9923	0,4141	0,00014	0,0412	<0,0001	<0,0001
	N	0,5915	0,3182	0,0011	0,1533	0,0003	<0,0001
	T	0,7182	0,1738	0,0097	0,9993	0,9339	0,9952

Abbrevations: T: T cells cultured alone; S: T cells in coculture with stimulated HUV-EC-C cells; N: T cells in coculture with non-stimulated HUV-EC-C cells.

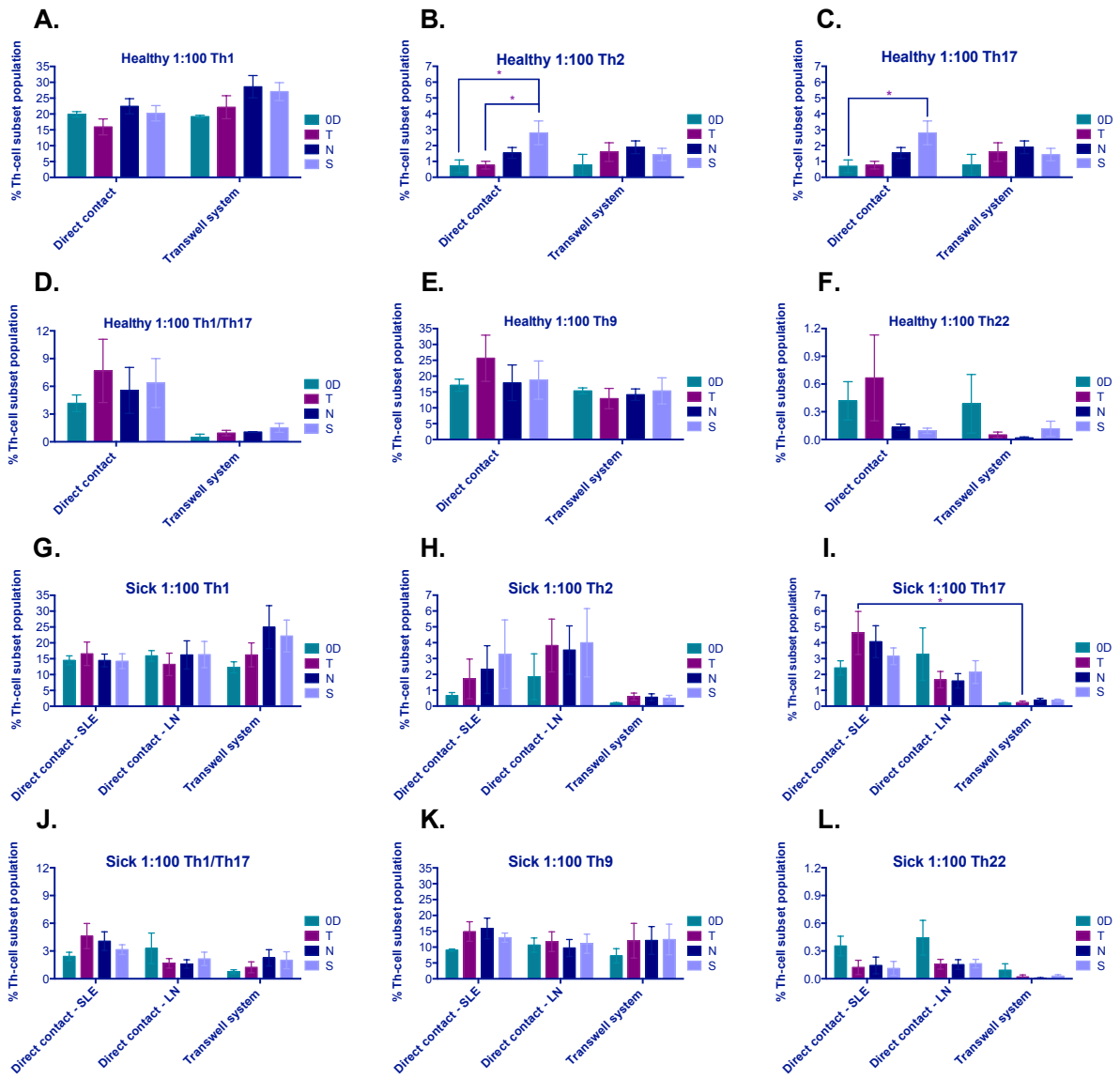

Fig. B: Comparison of the Th subsets when the T cells are cocultured with MSCs in direct contact or in Transwell system. Data is presented as mean with SEM.