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Increased differentiation and PRRSV susceptibility of monocytic cells during co-cultivation with immortalized mesenchymal stromal cells

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List of abbreviations

ALCAM	activated leukocyte cell adhesion molecule
BMP	bone morphogenetic protein
COX-2	cyclooxygenase 2
DC	dendritic cells
DMEM	Dulbecco's Modified Minimum Essential Medium
EDTA	ethylene diamine tetra acetic acid
EAV	equine arteritis virus
ERK	extracellular signal-regulated kinases
FSC	forward scatter
FGF-2	fibroblast growth factor 2
GvHD	graft versus host disease
GP	glycoprotein
GM-CSF	granulocyte macrophage colony-stimulating factor
HIV	human immunodeficiency virus
HGF	hepatocyte growth factor
hTERT	human telomerase reverse transcriptase
IFN	interferon
IMEM	Iscover's Minimum Essential Medium
IBMX	3-isobutyl-1-methylxanthine
IDO	indoleamine-pyrrole 2, 3 dioxygenase
iNOS	inducible nitric oxide synthase
Lin	lineage-associated markers
LDV	lactate dehydrogenase-elevating virus
LPS	lipopolysaccharide
LV	Lelystad
M-CSF	macrophage colony stimulating factor
MEK	mitogen-activated protein kinase kinase
MMP13	matrix metallopeptidase 13

MIX	1-isobutyl-3-methylxanthine
MSD	mystery swine disease
NO	nitric oxide
ORF	open reading frame
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
PRRSV	porcine reproductive and respiratory syndrome virus
PEARS	porcine epidemic abortion and respiratory syndrome
PBMC	peripheral blood mononuclear cells
PPAR-γ	peroxisome proliferator-activated receptor gamma
PGE_2	prostaglandin E2
rhCSF1	recombinant human colony-stimulating factor
SSC	side scatter
SD	standard deviation
SV40TL	simian virus 40 large antigen
SHFV	simian hemorrhagic fever virus
SIRS	swine infertility and respiratory syndrome
TNF-α	tumor necrosis factor alpha
TGF-β	transforming growth factor beta
Th	T helper
TCR	T cell receptor
V-CAM	vascular cells adhesion molecule

Chapter 1

Introduction

1.1 Mesenchymal stromal cells

1.1.1 Historical perspective

During the 1960s, Friedenstein and co-workers observed discrete colonies of plastic adherent non-mononuclear, fibroblast-like elongated cells in bone marrow cultures two weeks post-seeding. When these cells were injected underneath the renal capsule of a mouse kidney for a few weeks they were found to initially differentiate into fibrous tissues then to bone and finally to bone containing bone marrow (Friedenstein *et al*., 1966). In the 1970s, Wolf and Trentin described a cell culture model in which differentiation of hematopoietic stem cells can be induced by a spleen or bone marrow microenvironment (Trintin *et al*., 1989). In 1971, Dexter and colleagues found that long-term maintenance of mouse hematopoietic cells in *in vitro* cultures depended on the presence of adherent stromal cells in the culture system as well as a direct contact between hematopoietic stem cells and these stromal cells (Dexter *et al*., 1977). During the 1980s and 1990s, researchers found that the tissue-forming cells described by Friedenstein were multipotent cells that are capable of differentiating into osteoblasts, chondroblasts and adipocytes. In 1999, Mark Pittenger observed these multipotent cells in human bone marrow cells and later on developed techniques to identify these cells by their ability to express certain antigens. In 2008, these multipotent cells were found to be different from endothelial and hematopoietic cells (Delorme *et al*., 2008). Arnold Caplan was the first to name these cells mesenchymal stem cells (Caplan *et al*., 1991). Initially they were called osteogenic differentiation cells by Friedenstein or marrow stromal cells by Maureen Owen, while Paolo Bianco and Pamela Robey called the cells skeletal stem cells because of their ability to give rise to cellular components of the skeleton (Bianco *et al*., 2008). James Dennis suggested that these cells should be called mesenchymal progenitor cells, saying they are bonafide stem cells (Dennis *et al*., 1999). Finally, the international society for cellular therapy proposed the name multipotent mesenchymal stromal cells (Horwitz *et al*., 2005), which can be differentiated into many cell types including osteoblast, adipocytes and chondrocytes and expanded in *vitro.*

Unlike hematopoietic stem cells that were identified at different stages of differentiation using antibodies directed against certain cell surface markers, the mesenchymal stromal cell identification system was established later on using antibodies detecting STRO-1 (Simmons *et al*., 1991). This

cell surface marker is expressed by mesenchymal stromal cells, which can differentiate into chondrocytes, adipocytes and osteocytes and also support the proliferation and survival of hematopoietic cells (Gronthos *et al.*, 1994). Using FACS, mesenchymal stromal cells were separated from other bone marrow cells by their high expression of STRO-1 (Jones *et al*., 2006), which shed more light on the heterogeneity of bone marrow cell populations. When further analyzed, bone marrow STRO-1^{hi} cells were found to be negative for typical hematopoietic cells markers, such as CD34, CD14, CD45 and CD11b. STRO-1^{hi} cells also lack the expression of endothelial cell markers, such as von Willebrand factor (VWF). Also during the time STRO-1 monoclonal antibodies were developed, the monoclonal antibodies SH-2 (CD105), SH-3 and SH-4 (CD73) were produced by Caplan. These mAbs detected antigens on the surface of red bone marrow mesenchymal cells, but not on red bone marrow hematopoietic cells. This allowed the proper classification of bone marrow mesenchymal stromal cells (Hayensworth *et al*., 1992). Other markers such as THY-1 (CD90), which were first identified on mice T-cells, were also found to be highly expressed by fibroblast in the hematopoietic tissues (Raff *et al*., 1971). It was recently recognized as a good marker for mesenchymal cell enrichment (Delorme *et al*., 2008). Furthermore, adhesion molecules, such as CD106 (VCAM-1; vascular cell adhesion molecule) and CD166 (ALCAM; activated leukocyte cell adhesion molecule), which belong to immunoglobulin-domain members of cell adhesion molecules, were also found to be expressed by mesenchymal stromal cells. Finally, in 2006 the International Society of Cellular Therapy set up a minimum of criteria for identifying mesenchymal stromal cells, based on (i) their ability to adhere to cell culture plastic, (ii) expression of specific surface antigens: CD73, CD90, CD44, CD105 and CD55, (iii) absence of CD14, CD11b and CD45, (iv) ability to differentiate into osteocytes, chondrocytes and adipocytes. Initially, bone marrow was the main source of mesenchymal stromal cells, but later it was also derived from adipose tissues, lung tissues, spleen, lymph nodes, umbilical blood and peripheral blood.

1.1.2 Multilineage differentiation

Mesenchymal stromal cells are defined by their ability to differentiate into many cell types including osteocytes, chondrocytes and adipocytes. The following paragraphs describe how to trigger differentiation of mesenchymal cells in these three cell types.

1.1.2.1 Osteogenic differentiation

Osteogenic differentiation of bone marrow mesenchymal stromal cells into osteoclasts is controlled by systemic hormones, such as parathyroid hormone, estrogen or glucocorticoids or by local growth factors belonging to the bone morphogenetic protein (BMP) family, transforming growth factor-beta (TGF-β) and fibroblast growth factor-2 (FGF-2). These factors trigger the activation of specific signaling pathways within the mesenchymal stromal cells causing the modification of several transcription factors leading to differentiation of the cells into osteocytes and not chondrocytes or adipocytes. The major transcription factor responsible for the differentiation of mesenchymal stromal cells into osteocytes is Runt-related transcription factor 2 (Runx-2) (Gaur *et al*., 2005). Differentiation of mesenchymal stromal cells into osteoclasts in *in vitro* cultures is performed in the presence of dexamethasone, ascorbic acid and β-glycerol phosphate (Jaiswal *et al*., 1997). These reagents work at different time points inducing and maintaining osteogenesis. It has been reported that dexamethasone induces the differentiation of mesenchymal stromal cells into both osteocytes and adipocytes. When mesenchymal stromal cells start to differentiate into osteocytes, their morphology changes from spindle-shaped to cuboidal-shaped and mineralization can be observed on the cell membrane with generalized deposition of calcium. The calcium deposit can be demonstrated on the differentiated mesenchymal stromal cells by staining the cells with a solution of alizarin red (see Fig 1).

1.1.2.2 *Chondrogenic differentiation*

Differentiation of mesenchymal stromal cells from different sources (bone marrow, adipose tissue, lungs, spleen and umbilical cord) into chondrocytes has been reported (Studer *et al*., 2012; Barrilleaux et al., 2006). Fibroblast growth factor 2 (FGF-2) plays an important role in the proliferation and differentiation of mesenchymal stromal cells into chondrocytes (Sakaguchi *et al*., 2005). Moreover, it has been reported that serum-free medium enhances the differentiation of mesenchymal stromal cells into chondrocytes (Auletta *et al.,* 2011). *In vitro,* chondrogenic differentiation of bone marrow mesenchymal stromal cells has been studied widely in micromass pellet condition. The initial phase of chondrogenic differentiation of mesenchymal stromal cells favored by this method is characterized by cell condensation, cell-cell and cell-extracellular matrix

interactions. Next, the growth rate increases and the cells start to produce collagen type II, collagen type IX, collagen oligometric matrix protein and matrilin. Finally, the cells take the morphology of chondroblasts (round shape).

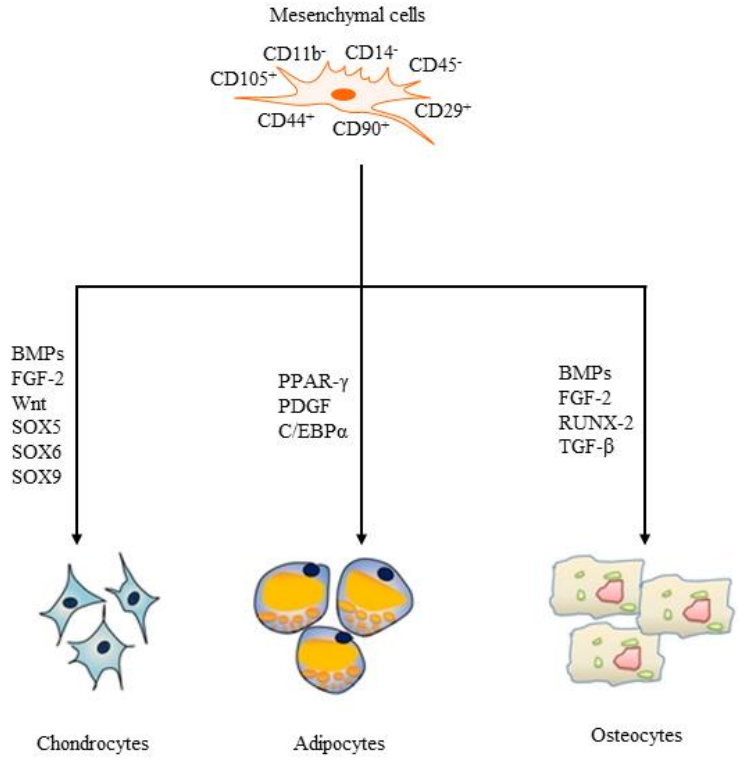

Fig 1: Schematic representation of mesenchymal stromal cell differentiation into chondrocytes, adipocytes and osteocytes. $CD105^+$, $CD90^+$, $CD29^+$ and $CD44^+$ mesenchymal cells are differentiated into chondrocytes by BMPs, FGF-2, Wnt, SOX5, SOX6 and SOX9, into adipocytes by PPAR-γ, PDGF and C/EBPα, and into osteocytes by BMPs, FGF-2, RUNX-2 and TGF-β. Chondrogenic, adipogenic and osteogenic differentiation of the mesenchymal cells are achieved by incubating the mesenchymal cells in chondrogenic, adipogenic and osteogenic differentiation medium for 21 days, 7-14 days and 14 days, respectively, and confirmed by staining with alcian blue, oil red O and alizarin red solution, respectively

Reports indicated that chondrogenic differentiation stages are regulated by BMPs, FGF-2, TGF-β and Wnt (Yang *et al*., 2006; Itoh *et al*., 2008; Leung *et al*., 2011; Torreggiani *et al*., 2012). Major transcription factors controlling stemness properties of cells and chondrogenic differentiation of mesenchymal stromal cells include SOX9, SOX5, SOX6 and Slug (Leung *et al*., 2011; Torreggiani *et al*., 2012) (see Fig 1).

1.1.2.3 *Adipogenic differentiation*

Differentiation of mesenchymal stromal cells into adipocytes is performed in medium supplemented with indomethacin, dexamethasone, isobutylmethylxanthine and insulin. Successful differentiation of mesenchymal stromal cells into adipocytes is confirmed by Oil red O staining which reveals an oil droplet in the cytoplasm of differentiated mesenchymal stromal cells. The initial phase in the differentiation of mesenchymal stromal cells into adipocytes is transformation of mesenchymal stromal cells to pre-adipocytes; these cells have the same morphology as the mesenchymal stromal cells. The final phase is the maturation and full differentiation of the mesenchymal stromal cells into adipocytes. These cells are characterized by an expanded lumen and accumulation of fat droplets in the cytoplasmic space. The adipogenic environment stimulates the activation of ERK1 and ERK2 in the initial phase of the adipogenic differentiation. ERK1 and ERK2 trigger the expression of the transcription factors PPAR-γ and C/EBPα, which are crucial in the terminal stages of adipogenic differentiation of mesenchymal stromal cells (Prusty *et al*., 2002). In addition to dexamethasone, ascorbic acid and β-glycerolphosphate are used to stimulate adipogenic differentiation of mesenchymal stromal cells in *in vitro* cultures; 1-methyl-3-isobutylxanthine (MIX) or 3-isobutyl-1-methylxanthine (IBMX) are used to enhance adipogenic differentiation of mesenchymal stromal cells (Yeh *et al*., 1995) (see Fig 1).

1.1.3 Mesenchymal stromal cells support the viability and proliferation of hematopoietic cells

Hematopoietic cells are pluripotent cells capable of life-long replenishment of the hematopoietic microenvironment with hematopoietic progenitors and all mature blood cells (Zon *et al.,* 2005). A balance is always maintained between the differentiation of hematopoietic cells into specific blood cell lineages and proliferation of undifferentiated hematopoietic cells. Mature white blood cells are typically divided into two separate lineages: the lymphoid and the myeloid lineage. The myeloid lineage includes cells that are morphologically, functionally and phenotypically distinct. The myeloid cell lineage includes granulocytes (neutrophils, eosinophils, basophils) and monocytes. The lymphoid cell lineage comprises T-lymphocytes, B-lymphocytes and natural killer cells. Another cell type with a unique developmental program which can be derived from either myeloid or

lymphoid lineage cells are dendritic cells (Manz *et al*., 2001; Traver *et al*., 2000). Common lymphoid progenitor cells, that can give rise to all lymphoid but not myeloid cell types, and common myeloid progenitor cells that can give rise to all myeloid but not lymphoid cell types, were already successfully isolated and extensively studied (Akashi *et al*., 2000). In mice, the hematopoiesis takes place in a small fraction of the bone marrow. These cells do not express lineage-associated markers (Lin), but are Sca-1 and c-Kit positive (Ikuta and Weissman, 1992). Supporting cells (stromal cells) present in the bone marrow participate in the initiation and maintenance of hematopoiesis.

The mechanism regulating the self-renewal and plasticity of hematopoietic cells is not well understood. However, it has been reported that both intrinsic and environmental cues play an important role in the homeostasis. The hematopoietic microenvironment has been studied *in vitro* by long-term culturing of bone marrow hematopoietic cells on top of stromal cells, in which the survival and proliferation of hematopoietic cells was observed. Stromal cells play a very important role in the survival, maintenance and differentiation of hematopoietic cells through intimate contact with the hematopoietic microenvironment components and through the secretion of cytokines, such as IL-6 and granulocyte macrophage colony stimulating factor (Majumdar *et al*., 1998; Mbalavielle *et al*., 1999).

1.1.4 Immunomodulatory effect of mesenchymal stromal cells

Mesenchymal stromal cells have been found to regulate the immune system modulating immune cell responses in many diseases. Mesenchymal stromal cells have been reported to enhance the survival of lymphocytes, but suppress their proliferation, cytotoxicity and cytokine secretion (Yanez *et al*., 2006; Glennie *et al*., 2005). Moreover, these cells inhibit the secretion of antibodies and co-stimulatory factor by B-cells and the maturation, activation and antigen presentation of dendritic cells. They also inhibit the activation of IL-2 induced NK cells (Spaggiari *et al*., 2006). There are reports suggesting that mesenchymal stromal cells exert a variable immunomodulatory effect on the same immune cells depending on the microenvironment or disease conditions. For instance, mesenchymal stromal cells trigger a decrease in type 1 T helper (Th1) cells in patients with acute graft versus host disease and in patients with an autoimmune disease, such as systemic lupus erythematosus (Le Blanc *et al*., 2004; Rafei *et al*., 2009). Another example is the shift from Th2 to Th1 responses in respiratory allergic inflammatory diseases triggered by bone marrow-derived

mesenchymal stromal cells. Moreover, an inflammatory condition can also improve cell-cell contact leading to an enhanced immunomodulatory response of mesenchymal stromal cells. Mesenchymal stromal cells not only exert their immunomodulatory effect through a contact-dependent mechanism with immune cells, but also through secretions of soluble factors that may affect immune cells.

1.1.4.1 Immunomodulatory effect of mesenchymal stromal cells through the secretion of soluble factors

Several cytokines and other soluble factors expressed by mesenchymal stromal cells have been reported to modulate immune cells, such as hepatocyte growth factor (HGF), transforming growth factor-β1 (TGF-β1), prostaglandin E2 (PGE2), indoleamine-pyrrole 2,3 dioxygenase (IDO), nitric oxide (NO) and interleukin-10 (IL-10). It was observed that some pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), trigger the secretion of some soluble factors by mesenchymal stromal cells, such as IDO, prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX-2), leading to suppression of immune cells (Krampela *et al*., 2006; Ryan *et al*., 2007). Mesenchymal stromal cell secretion of PGE2 was found to suppress T-lymphocyte proliferation, while IDO and NO were found to inhibit both proliferation and function of T-lymphocytes. In addition, IDO also induces allograft tolerance in kidneys (Jarvinen *et al*., 2008; Ge *et al*., 2010). Bone marrow-derived mesenchymal stromal cells were observed to upregulate the secretion of inducible nitric oxide synthase (iNOS; which helps to attenuate a delayed type hypersensitivity reaction) and some chemokines in response to IFN-γ activation. Mesenchymal stromal cells have also been reported to suppress allergic responses in mouse models through the secretion of TNF-β (Ren *et al*., 2008). Other soluble factors, such as galectins, IL-6 and leukemia inhibitory factors, were found to regulate the mesenchymal cell immunomodulatory effect (Najar *et al*., 2010). The exact mechanism used by many other soluble factors and cytokines in the immunomodulatory effect of mesenchymal stromal cells is not well understood.

1.1.4.2 Immunomodulatory effect of mesenchymal stromal cells by cell-cell contact

Many reports indicated that mesenchymal stromal cells modulate immune cells through cell-cell contact. For example, mesenchymal stromal cells derived from bone marrow were found to strongly

decrease the survival and proliferation of T-lymphocytes and increase the proportion of Tregs via a cell-cell contact dependent mechanism (Han *et al*., 2011). IFN-γ-mediated activation of mesenchymal stromal cells could result in the upregulation of adhesive molecules, such as CD44, CD274 and vascular cell adhesive molecule-1, and galectin-1 secretion that support cell-cell contact leading to an enhancement of the immunomodulatory effect of mesenchymal stromal cells (Raicevic *et al*., 2015; Ren *et al*., 2015).

1.1.5 Mesenchymal stromal cells in (pre)clinical studies

The easy isolation and cultivation, ability to differentiate into fat, bone and cartilage and good *in vitro* expansion capacity of mesenchymal stromal cells from different sources led to their use in clinical trials in the treatment of diseases such as diabetes and graft-versus-host diseases (GvHD). For instance, mesenchymal stromal cells function in the modulation and suppression of immune cells allowing them to reduce GvHD thereby making them useful in auto- and allogeneic cell therapy. Mesenchymal stromal cells are recently widely used in clinical trials to study inflammatory and immune disorders. An example of such a trial is the systemic administration of mesenchymal stromal cells to decrease allergen-specific IgE and the Th2-associated cytokines IL-4, IL-5 and IL-13 in bronchial airways, inhibiting respiratory inflammation in mice with chronic or severe asthma (Firinci *et al*., 2011; Lee *et al*., 2011). Mesenchymal stromal cells are found to suppress T-lymphocyte proliferation, inflammatory infiltrates and cytokine expression in experimental models leading to proctitis, colitis, immune thrombocytopenia and autoimmune encephalomyelitis. They were reported to have a great autoimmune suppression effect in an animal model for arthritis. Human mesenchymal stromal cells derived from bone marrow and umbilical cord successfully helped in reconstructing the bone marrow osteoblastic niche and in reversing multi-organ dysfunction. Researchers suggested the use of mesenchymal stromal cells in the treatment of some autoimmune diseases, such as thyroiditis, myasthenia gravis and hearing loss (Zhou *et al*., 2011; Yu *et al.*, 2010).

1.1.6 Mesenchymal stromal cell senescence

Large numbers of mesenchymal stromal cells are sometimes needed for large-scale reconstruction of specific tissues or organs *in vitro*. Mesenchymal stromal cell cultures for long-term research are also needed. Therefore, to have large numbers of mesenchymal stromal cells for large-scale construction of tissues or organs, the cells need to be passaged many times in *in vitro* cultures. However, the growth rate of mesenchymal stromal cells slows down after several passages. The cells flatten and adhere more firmly to the culture plates and sometimes lose their phenotypic characteristics and differentiation potential and finally die (Wagner *et al*., 2008). This phenomenon is referred to as replicative senescence. It is defined as an irreversible state of the cell cycle arrest. During every round of DNA replication, each eukaryotic chromosome becomes shorter at the telomeres because of end-replication errors by the DNA polymerase. Telomerase can elongate the telomeres. Because there is no telomerase activity detected in mesenchymal stromal cells, the telomeres shorten during proliferation. The best way to extend the life span of mesenchymal stromal cells is to immortalize them. Researchers successfully immortalized different cell types (endothelial cells, epithelial cells, mesenchymal stromal cells and fibroblasts) from different sources using different methods, including viral vectors expressing the large T antigen of SV40 (Jha *et al*., 1998; Kirchhoff *et al*., 2004) and human telomerase reverse transcriptase (htert) (Ramirez *et al*., 2004; June et al., 2004; Zimmermann *et al*., 2004).

1.2 Porcine respiratory and reproductive syndrome

1.2.1 Introduction

A pig disease characterized by late term abortion, early farrowing, stillbirth and respiratory problems was reported in the US (Keffaber *et al*., 1989), Canada (Bilodeau *et al*., 1991), Europe (Baron *et al*., 1992) and Asia (Kuwahara *et al*., 1994) in the late 1980s. Due to the lack of knowledge about the disease, many names were assigned to it, including `Mystery swine disease´ (MSD) (Wensvoort *et al*., 1991), blue-eared pig disease (Wensvoort *et al*., 1991), porcine epidemic abortion and respiratory syndrome (PEARS) (Terpstra *et al*., 1991), swine infertility and respiratory syndrome (SIRS) (Benfield *et al*., 1992) and porcine respiratory and reproductive syndrome (PRRS) (Ahl *et al*., 1992). All these names reflect the symptoms of the disease. The infectious agent causing these symptoms was first isolated in 1991 from MSD-affected pigs by a group of researchers at ID-DLO in Lelystad, the Netherlands, (Wensvoort *et al*., 1991). This infectious agent was later characterized and designated as `Lelystad virus` the causative agent of MSD. At around the same time, researchers in the US were able to culture and isolate an American isolate on CL2621 cells, which they later named VR-2332 (Collins *et al*., 1992). During the first international symposium on this disease 'porcine reproductive and respiratory syndrome' (PRRS) and 'PRRS virus (PRRSV)' was accepted as the official name of the disease and the virus, respectively. PRRSV is endemic in swine producing countries worldwide despite a lot of efforts by farmers, veterinarians, researchers and vaccine developers to control the disease. Two PRRSV genotypes have been identified using phylogenetic analysis: the European genotype (type 1), with LV as prototype virus, and the American genotype (type 2), with VR-2332 as prototype (Nelsen *et al*., 1999). Type 1 strains of PRRSV are further subdivided into subtype 1, 2, 3 and 4 (Stadejek *et al.*, 2008). Although these two genotypes differ significantly from one another they are thought to originate from a common ancestor. Originally, genotype 1 was only detected in Europe whereas genotype 2 was restricted to America and Asia. At present, this restriction no longer exists, because both genotype 1 and 2 PRRSV variants are found in Europe, America and Asia (Murtaugh et al 2010; Shi *et al*., 2010). Due to a high mutation rate, PRRSV shows a large variation in virulence, pathogenicity and antigenicity. Some of the isolates are highly pathogenic, while some are either apathogenic or only moderately virulent (Meng *et al*., 2000).

1.2.2 Taxonomy of PRRSV

PRRSV is classified in the order of the *Nidovirales*, family of the *Arteriviridae*, genus *porartevirus* (Snijder *et al*., 1998). Similarities in the genome organization and the relatedness of the proteins involved in RNA replication and transcription are the reasons why viruses are classified within the *Nidovirales* order. The term ´´*nidus*´´ is a latin word which means ´´nest´´ referring to the nested 3´ co-terminal subgenomic-length mRNAs that are generated for expression of the open reading frames (ORFs) downstream of the replicase gene. Beside PRRSV, the *Arteriviridae* family consists of three other members: equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV). Arteriviruses are highly specific in their animal species targeting and may persist in their respective hosts. They have a similar particle morphology, genome organization and replication strategy. Cryo-electron microscopy showed that the particles are spherical ranging from 50 to 70 nm in diameter with a small projection on their surface (Snijder *et al*., 1998). This enveloped positive-stranded RNA group of viruses primarily targets macrophages and endothelial cells. PRRSV has the ability to rapidly evolve, allowing it to undergo huge genetic and antigenic changes over time. This genetic and antigenic variation allows PRRSV to exist in a single individual animal as a cluster of different virus variants. Researchers suggested that PRRSV may exist as a quasispecies as after each round of replication a mixture of virions having different closely related genomes is present.

1.2.3 Genome organization and virion structure

1.2.3.1 *Genome organization*

The genome of PRRSV is a positive-sense, single-stranded RNA approximately 15.1 kb for the European strain and 15.4 kb for the north American strain (Meulenberg *et al*., 1997). The genome contains many ORFs with capped 5´ terminal end and polyadenylated 3´ terminal end (Johnson *et al*., 2011). The 5´ terminal end consists of ORF1a and ORF1b which occupy about 70-80% of the total genome length of PRRSV (Fang *et al*., 2010). Two large polyproteins pp1a and pp1ab which are involved in the generation of a set of non-structural proteins (nsp) in infected cells are encoded

by the so called ´replicase ORFs´ (Brierley *et al*., 1995; den Boon *et al*., 1991). These non-structural proteins are required for PRRSV genome replication and subgenomic mRNA production. Many smaller, overlapping ORFs (ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF6 and ORF7) are involved in encoding the structural proteins (GP_2, E, GP_3, GP_4, GP_5, M and N, respectively) and are located at the 3´ terminal end region of the PRRSV genome (Wu *et al*., 2001; Meulenberg *et al*., 1996; Meulenberg *et al*., 1995; Music *et al*., 2010) (see Fig 3).

1.2.3.2 *Virion structure*

The PRRSV particle consists of an icosahedral nuclear capsid containing the RNA viral genome surrounded by a lipid bilayer of a smooth spherical envelope (Dockland et al., 2010; Dea et al., 1995; Mardassi *et al*., 1994; Wensvoort *et al*., 1992; Spilman *et al*., 2009). The nucleocapsid is composed of nucleocapsid proteins (N) that are highly basic and predominantly disulfide-linked homodimers (Mardassi *et al*., 1996; Wootton *et al*., 2003). The higher organized structure of the PRRSV nucleocapsid is probably due to a non-covalent association of nucleocapsid proteins (N) (Wootton et al., 2003). The other PRRSV structural proteins are virion membrane proteins found enclosed in the envelop of the virion. These proteins are the small envelop protein E, the membrane protein M and the N-glycosylated glycoproteins GP_2, GP_3, GP_4, GP_5 (Mardassi *et al*., 1996). A PRRSV structural protein ORF5a was identified by Jonson et al. (2001) and is assumed to be a membrane protein. Moreover, PRRSV major structural proteins include M, N and GP_5 proteins, while the minor structural proteins of PRRSV are GP_2, E, GP_3, GP_4 (Mardassi *et al*., 1996). The GP5 and M proteins are present in the virion as covalently linked heterodimers because of the disulfide-bonds formed between the ectodomains of these proteins (Dockland *et al*., 2010) (see Fig 2).

1.2.4 Cell tropism

Similar to other members of the *Arteriviridae* family, PRRSV has a tropism for cells of the monocyte⁄macrophage lineage. PRRSV mainly targets and infects subsets of differentiated macrophages residing in the lymphoid tissues, lungs and placenta. Macrophages originate from embryonic precursors which populate the tissues (except the intestine) during embryogenesis.

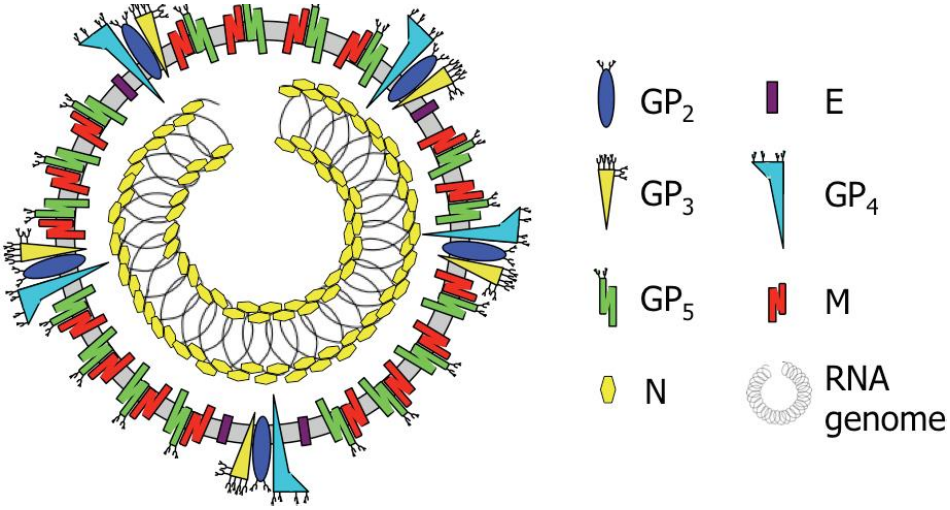

Fig 2: PRRSV virion structure and morphology.
Schematic overview of PRRSV virion structure. The virus particle is made up of a nucleocapsid, which is surrounded by an envelope. The nucleocapsid consists of a viral genome and nucleocapsid proteins. Small envelop protein E, membrane protein M and N-glycosylated glycoproteins GP_2, GP_3, GP_4 and GP_5 are found within the viral envelope. Adapted with the permission of Wander Van Breedam.

These macrophages have a self-renewal ability. During infection, monocytes are attracted to the infected tissues and depending on the local microenvironment may differentiate into new macrophages. These differentiated macrophages are particularly CD163 and sialoadhesin positive cells. Blood monocytes are not susceptible to PRRSV (Duan *et al*., 1997; Teifke *et al*., 2001; Duan *et al*., 1998). Many cell types are used for cultivating and propagation of PRRSV in *in vitro* studies. Primary cultures of porcine alveolar macrophages are widely used to produce the virus because they are the main target of PRRS *in vivo*. The African green monkey kidney cell line MA-104 or cells derived from this cell line are also used to grow some strains (Kim *et al*., 1993; Mengeling *et al*., 1995).

1.2.5 Replication cycle

The ability of PRRSV to successfully enter into its target cell depends on the presence or absence of specific entry mediators (Kreutz *et al*., 1998). Several cell mediators are reported to be involved in PRRSV entry into its target cells. Heparan sulphate was identified in the 1990s. In 1997, Jusa et al. reported the possible use of heparin-like molecules on the surface of Mark-145 cells by PRRSV for entry into these cells. These results were confirmed by incubating PRRSV susceptible cells with

heparin, and later with the virus; this led to a significant decrease in the infection of the cells by PRRSV. Similar results were obtained by incubating the cells with heparinase I, an enzyme responsible for cleaving heparin and heparin sulphate (Delputte *et al*., 2002). When the same experiments were performed with porcine alveolar macrophages similar results were obtained (Delputte *et al*., 2002). Sialoadhesin, also called CD169 or siglec-1, is a member of the sialic acid binding lectin family, called siglecs. This protein is found to be expressed only on differentiated macrophages and is involved in the attachment and internalization processes of PRRSV (Duan *et al*., 1998; Duan *et al*., 1997). Two mAbs 41D3 and 41D5, recognizing sialoadhesin, were generated by Duan *et al*. in 1998. It was found that incubating PRRSV susceptible cells with 41D3 and 41D5 mAbs prevented the attachment of PRRSV to the cells, thereby blocking PRRSV infection. CD163, a type I transmembrane glycoprotein and scavenger receptor is found to be expressed only by cells of the monocyte/macrophage lineage (Sanchez *et al*., 1999; Van den Heuvel *et al*., 1999). Blocking CD163 by incubating macrophages with CD163-specific mAbs clearly reduced PRRSV infection (Van Gorp *et al*., 2008). While siglec-1 and CD163 have been identified as the most important entry mediators for PRRSV (Van Gorp *et al*., 2008; Duan *et al*., 1998; Duan *et al*., 1997), recent research demonstrated that PRRSV also show a tropism for siglec-10, that can replace siglec-1 as receptor mediating PRRSV binding and internalization (Frydas *et al*., 2013; Xie *et al*., 2017). Other entry mediators used by PRRSV for entry into the cells are cellular proteases, such as the aspartic acid protease cathepsin E (Misinzo *et al*., 2008).

Unstable binding of the PRRSV sugars with specific cell entry mediators on the surface of the target cell is the first stage in the infection process. The M/GP_5 complex interacts with heparin sulphate moieties on glycoproteins present on the membrane of the target cells. This weak PRRSV-cell interaction allows the virus to glide on the macrophage surface until it is able to locate its specific receptor sialoadhesin (siglec-1), which mediates the internalization of the virus into the macrophage. Immediately after internalization, PRRSV releases its genome into the cytoplasm of the host cell followed by translation of the ORF1a and ORF1b genes encoding the nonstructural proteins pp1a and pp1ab (Fang *et al*., 2010; Pasternak *et al*., 2006). These two polyproteins undergo extensive proteolytic processes. The pp1a polyprotein is cleaved 9 times by specific proteases producing 10 different nonstructural proteins, while pp1ab is cleaved 3 times yielding 13 nonstructural proteins (Fang *et al*., 2010). A combination of PRRSV RNA-dependent RNA polymerase, RNA helicase, other important enzymes used for the synthesis of viral RNA and other nonstructural protein come

together to form a viral transcription and membrane-associate replication complex that is involved in the replication of the viral genome and synthesis of the nested subgenomic mRNAs (Fang *et al*., 2010; Pasternak *et al*., 2006). Full-length minus strand RNA is synthesized from positive sense genomic RNA in the membrane-associated replication complex. This RNA is used as a template for the synthesis of genomic RNA (Fang *et al*., 2010; Pasternak *et al*., 2006). Many polycistronic subgenomic minus strand RNAs with an identical 3´ and 5´ end terminal leader sequence are generated from the 5´ end terminal region of this genome. These RNAs are used for encoding different structural viral proteins. The GP_2 and E protein are translated from mRNA2 which is functionally bicistronic, while ORF5a and GP_5 proteins are translated from mRNA5. The mRNA3, 4, 5, 6 and 7 are used to generate GP_3, GP_4, GP_5, N and M proteins, respectively (Fang *et al*., 2010; Pasternak *et al*., 2006). After synthesis, all the structural proteins, except nucleocapsid protein N, integrate into the membrane of the endoplasmic reticulum (ER). In the ER, N-linked glycans are added to GP_2, GP_3, GP_4 and GP_5 proteins with the formation of intra- and inter-disulphide bridges in and between the viral envelope. The nucleocapsid protein in the cytosol interacts with the newly synthesized viral genomic RNA strand to form new viral cores. Budding of these virion cores into the lumen of the ER allows them to obtain an envelope containing viral envelop proteins (Faaberg *et al.*, 2008). For the immature virus to become mature it subsequently travels through the lumen of the ER and Golgi apparatus, where the N-linked glycans on the surface of the viral particles undergo further modifications. Finally, the new virus particles are released from the host cell by exocytosis (Faaberg *et al*., 2008) (see Fig 3).

1.2.6 Pathogenesis of PRRSV

The transmission of the virus between infected and non-infected pigs in the herd is through close contact like nose-nose contact or by contact with nasal discharge, faeces and urine. PRRSV can be introduced into a PRRSV-free herd by bringing into the herd infected animals or contaminated fomites like boots, clothes and equipment (Rossow *et al*., 1998). PRRSV can also be transmitted to non-infected pigs by infected pigs via aerosol. PRRSV can travel as an aerosol for up to 9 km. This can be facilitated by low temperature, high humidity and low wind speed (Komijn *et al*., 1991) and is correlated with the pathogenicity of the isolate (Otake *et al*., 2002). In addition, PRRSV spreading through artificial insemination, slurry and arthropod vectors has been reported.

Naturally PRRSV infection begins mainly through inhalation, ingestion and coitus. Experimental infection of pigs with PRRSV can be achieved via intratracheal, intranasal, intramuscular, intravenous, oronasal and intraperitoneal inoculation. Macrophages are the main target of the virus *in vivo.* Especially macrophages from lungs, tonsils, lymph nodes and spleen are very susceptible to the virus. PRRSV infection starts in the macrophages residing at the site of virus uptake and in the draining lymph nodes (Rossow *et al*., 1998). PRRSV can be detected in the blood 12 h to 3 days after infection and persist in the blood for up to 6 weeks after the initial infection with the highest virus titer in the blood between 5 and 14 days after infection. From the blood the virus is able to spread to macrophage subsets in different organs of the body. PRRSV is also found in testicles of infected boars and placenta of pregnant sows, where the virus is capable of crossing the placenta to infect the fetus. This occurs during the third trimester of gestation. It has been reported that PRRSV infected pigs can naturally recover from the disease within 2 to 4 months (Rossow *et al*., 1998).

1.2.7 PRRSV control

Despite many challenges such as genetic variation, vaccination is the most widely used method of preventing and controlling PRRSV infections in pigs (Charerntantanakul *et al*., 2012). But PRRSV control requires much more than vaccination. A systemic approach to PRRS management is necessary to achieve a long-term success. This includes controlling PRRS in breeding herd using for instance the Load-Close-Expose protocol which stabilizes the PRRSV status in a breeding herd before introducing new PRRSV-negative animals. PRRSV can also be controlled by a full depop-repop program (Perez, AM., et al 2015; Corzo, CA., et al., 2010). More recently, researchers started to develop PRRSV-resistant pigs (Burkard *et al*., 2017). This may bring a novel approach to fully control PRRSV.

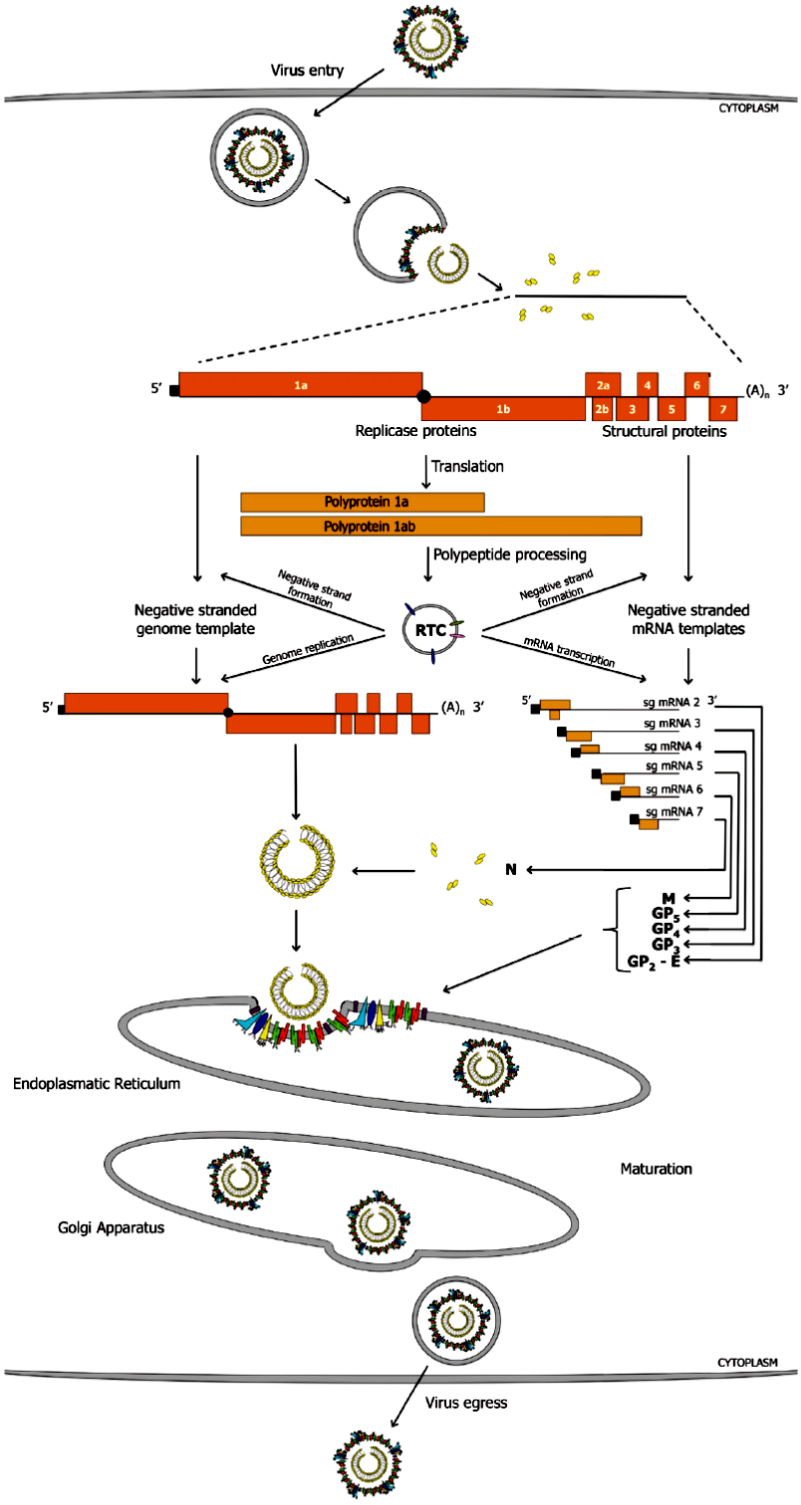

Fig 3: Schematic presentation of PRRSV replication cycle in the host cells.
Adapted with the permission of Wander Van Breedam.

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Chapter 2

Aims

2.1 Aims of the thesis

Mesenchymal stromal cells have been successfully isolated from red bone marrow and several other tissues. These cells can differentiate into many cell types such as chondrocytes, adipocytes and osteocytes. Due to their multi-lineage differentiation capabilities and strong immunomodulatory effect on different types of both innate and adaptive immune cells, mesenchymal cells received considerable attention from researchers as cells with great therapeutic potential. The anti-inflammatory and immunomodulatory effects of mesenchymal stromal cells have been explored for their potential use in the treatment of diseases such as graft-versus-host disease and diabetes. However, keeping mesenchymal stromal cells and their continuous passaging in *in vitro* cultures for a long time is a major challenge because they undergo senescence and die. Therefore, there is an urgent need for establishing continuous mesenchymal stromal cell cultures for studying mesenchymal-immune cell interactions. The development of an *in vitro* model for co-culturing immortalized mesenchymal stromal cells with bone marrow- and blood-derived immune cells will help to better understand mesenchymal-immune cell interactions for therapeutic applications and the effect of mesenchymal stromal cells on host-pathogens interactions, such as PRRSV replication in monocytes/macrophages, easing the way for finding possible treatments for infectious and non-infectious diseases.

The specific aims of this thesis are:

- ✓ To develop a long-term co-culture system for maintenance and differentiation of hematopoietic cells on top of immortalized mesenchymal stromal cells towards the monocyte/macrophages lineage.
- ✓ To establish mesenchymal stromal cell lines from different tissues of pigs for long-term studies such as the differentiation of blood-derived monocytes to siglec-1 expressing macrophages.
- ✓ To explore the effect of mesenchymal cells on pathogen-macrophage interactions by examining the replication kinetics of porcine reproductive respiratory syndrome virus in macrophages co-cultured with immortalized mesenchymal stromal cell

Chapter 3

Long-term culture and differentiation of porcine red bone marrow hematopoietic cells co-cultured with immortalized mesenchymal stromal cells

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3.1 Abstract

Mesenchymal stromal cells are multipotent stromal cells with self-renewal, differentiation and immunomodulatory capabilities. We aimed to develop a co-culture model for differentiating hematopoietic cells on top of immortalized mesenchymal stromal cells for studying interactions between hematopoietic and mesenchymal stromal cells, useful for adequately exploring the therapeutic potential of mesenchymal stromal cells. In this study, we investigated the survival, proliferation and differentiation of porcine red bone marrow hematopoietic cells co-cultured with immortalized porcine bone marrow mesenchymal stromal cells for a period of five weeks. Directly after collection, primary porcine bone marrow mesenchymal stromal cells adhered firmly to the bottom of the culture plates and showed a fibroblast-like appearance, one week after isolation. Upon immortalization, porcine bone marrow mesenchymal stromal cells were continuously proliferating. They were positive for simian virus 40 (SV40) large T antigen and the mesenchymal stromal cell markers CD44 and CD55. Isolated red bone marrow cells were added to these immortalized mesenchymal stromal cells. Five weeks post-seeding, 92±6% of the red bone marrow hematopoietic cells were still alive and their number increased 3-fold during five weekly subpassages on top of the immortalized mesenchymal stromal cells. The red bone marrow hematopoietic cells were originally small and round; later, the cells increased in size. Some of them became elongated, while others remained round. Tiny dendrites appeared attaching hematopoietic cells to the underlying immortalized mesenchymal stromal cells. Furthermore, weekly differential-quick staining of the cells indicated the presence of monoblasts, monocytes, macrophages and lymphocytes in the co-cultures. At three weeks of co-culture flow cytometry analysis showed an increased surface expression of CD172a, CD14, CD163, CD169, CD4 and CD8 up to 37±0.8%, 40±8%, 41±4%, 24±2%, 19±5% and 24±3% of the hematopoietic cells, respectively. In conclusion, continuous mesenchymal stromal cell cultures were successfully established and characterized and they supported the proliferation of red bone marrow hematopoietic cells, which finally differentiated into monocytic cells and $CD4^+$ and $CD8^+$ cells.

3.2 Introduction

The red bone marrow serves as the main source of white blood cells in mammals. Red bone marrow consists of two main cell types, the hematopoietic cells and non-hematopoietic stromal cells. The hematopoietic cells comprise megakaryocytes and erythrocytic, myeloid, and lymphocytic cells. The bone marrow stroma consists of a heterogeneous cell population, which provides mechanical and physiological support to the hematopoietic cells (Summerfield et al., 2001). They include stem cell-like cells that under specific culture conditions can differentiate into osteocytes, chondrocytes and adipocytes. Mesenchymal stromal cells drive the differentiation of hematopoietic cells and are characterized by their ability to adhere to cell culture plates and their expression of specific surface markers such as CD44, CD55, CD73, CD29 and CD90 (Dominici et al., 2006; Wagner et al., 2007; Nesselmann et al., 2008). Leukocyte progenitor cells are used in the treatment of red bone marrow defects either inherited or caused by disease. Red bone marrow defects may also be caused by chemo- or radiotherapy and restored by transplanting auto- or allogenic cells (Zimmermann et al., 2010). There are however limitations regarding the availability of red bone marrow hematopoietic cells for fundamental research. To date, human and mouse red bone marrow is widely used as a source of hematopoietic progenitor cells. However, in man only a little amount of bone marrow can be obtained by biopsy under strict ethical conditions, while in mice, red bone marrow is not always accessible in sufficient quantities, resulting in euthanasia of large numbers of mice (Ingesoll et al., 2010). This is in conflict with the 3R principle of animal welfare. In contrast, pigs are ideal donors as large amounts of hematopoietic cells can be isolated from their bones upon euthanasia. It is therefore interesting to use the pig as a model for a better understanding of how the interactions between the different red bone marrow cell populations control their proliferation and differentiation after co-culture with mesenchymal cells. Some groups reported that cell culture medium supplemented with horse serum, pig serum, and cytokines such as interleukin (IL)-34, IL-4 and macrophage colony stimulating factor (M-CSF) (Chitu et al., 2006; Garceau et al., 2010) enhances the expansion and maturation of red bone marrow hematopoietic cells particularly in humans and mice (Zhang et al., 2008). Other authors reported that red bone marrow cell cultures supplemented with cytokines such as colony stimulating factor 1 (CSF-1) do not support proliferation of

hematopoietic cells (O'Neill et al., 2004). In the present work, we established a technology to generate viable and proliferative cells by co-culturing red bone marrow hematopoietic cells with immortalized mesenchymal stromal cells in a long-term, simple, affordable and effective cell culture system.

3.3 Materials and Methods

3.3.1 Donor animals and culture of red bone marrow mesenchymal stromal cells

Tissues used for these experiments were taken from three 5 to 8-week-old conventional Belgian landrace pigs that were euthanized for other experiments. The euthanasia of the pigs was performed by jugular vein injection of 20% sodium pentobarbital (1 ml/1.5 kg; Kela laboratories, Hoogstraten Belgium). This is in agreement with the requirements of the Local Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University. Femur bones were aseptically removed and immediately placed in ice-cold transport medium containing phosphate buffered saline (PBS; Vel chemicals UBC, Brussels, Belgium), 0.1 mg/mL gentamicin (Invitrogen, Gent, Belgium), 0.1 mg/mL streptomycin (Certa, Eigenbrakel, Belgium), and 100 U/mL penicillin (Continental Pharma, Puurs, Belgium). Later, the bone was cut at both ends and the capita were removed using a sterilized saw. A twenty milliliter syringe with an eighteen-gauge needle was used to flush the red bone marrow with RPMI-1640 supplemented with 0.1 mg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma). Aggregates were disintegrated by pipetting gently and the cell suspension was filtered through a 70 µm cell strainer. The cell suspension was centrifuged at 430 x g at 4°C for 10 minutes and the cell pellet was resuspended in RBC lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA (pH 8)) and incubated on ice for 10 minutes. Later, the cells were washed two times in RPMI supplemented with 1% FCS.

3.3.2 Establishment of primary mesenchymal cell stromal culture

Immediately after isolation, as described above, red bone marrow cells were resuspended in Dulbecco's Minimum Modified Eagle Medium (DMEM) supplemented with 10% FCS, 0.1 mg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and seeded in 24 well plates, kept at 37°C 5% CO_2. Three days post-seeding, half of the medium (500 µl) was replaced with fresh medium. This was repeated every 48 hours for 10 days. At 95% confluency, the fibroblast-like cells were split in 0.25% trypsin.

3.3.3 Immortalization of primary mesenchymal stromal cells

To obtain continuous cell cultures of mesenchymal stromal cells, the primary cells were immortalized with simian virus 40 (SV40) large T antigen. At 50% confluency, a recombinant lentivirus vector containing the sequence encoding SV40LT transforming protein (Applied biological materials Inc., Canada) (1:1) was added to the proliferating primary mesenchymal stromal cells in the presence of polybrene (8 µg/mL, Applied biological material Inc., Canada). Thirty minutes later, supernatant was diluted with complete medium to avoid toxicity and the cells were further incubated overnight. Subsequently, fresh medium was replaced. Seventy-two hours post-transduction, cells were trypsinized with 0.25% trypsin (Sigma-Aldrich) and reseeded at a ratio of 1:3. The cells were monitored every day for their expansion by light microscopy (Olympus).

3.3.4 Immunofluorescence staining of immortalized mesenchymal stromal cells and analysis by confocal microscopy

To confirm that primary mesenchymal stromal cells were successfully immortalized, transduced mesenchymal stromal cells were plated and allowed to attach to a glass cover slide overnight, washed two times with PBS, and fixed with 4% paraformaldehyde at room temperature for 10 minutes. The cells were washed with PBS and permeabilized with Triton X-100 at room temperature for two minutes. Subsequently, the cells were incubated with polyclonal rabbit antibodies against SV40LT (Applied Biological material Inc.) antigen for 1 h at 37°C in the presence of 10% negative goat serum. After two times washing with PBS the cells were further incubated with goat anti-rabbit

IgG-FITC for 1 h at 37°C. The cells were additionally washed two times with PBS. Later, the nuclei were counterstained with Hoechst 33342 for 10 minutes at 37°C. The cells were washed and the cover slide was mounted with glycerin/PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo [2.2.2] octane and analyzed by confocal microscopy (Leica Microsystem DMRBE, Wetzlar, Germany). To confirm the identity of the cells, a phenotypic characterization of the cells was also performed by immunofluorescence staining. Cells seeded on cover slides were washed two times with PBS and fixed with 4% paraformaldehyde at room temperature for 10 minutes. After two washes with PBS, the cells were permeabilized by incubation with Triton X-100 for two minutes at room temperature. The cells were rinsed with PBS and incubated with polyclonal rabbit antibodies against CD44 (ab157107, 1:200, Abcam) and CD55 (ab96680, 1:200, Abcam) containing 10% negative goat serum at 37°C for 1 h. Next, cells were incubated with FITC-conjugated goat anti-rabbit IgG secondary antibodies for 1 h at 37°C. Subsequently, after two washes the nuclei were counterstained with Hoechst 33342 for 10 minutes at 37°C. Finally, the cells were washed two times in PBS and the slide was inverted on a small drop of glycerin/PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo [2.2.2] octane and analyzed by confocal microscopy (Leica Microsystem DMRBE, Wetzlar, Germany).

3.3.5 Isolation of red bone marrow hematopoietic cells and co-culture

Red bone marrow hematopoietic cells were isolated from three different piglets using the same methods described for isolating mesenchymal stromal cells. Immediately after the isolation, red bone marrow hematopoietic cells were resuspended in DMEM supplemented culture medium and seeded on top of the immortalized mesenchymal stromal cells monolayer at the concentration of $2x10^5$ cells/ml. Immortalized mesenchymal stromal cells were seeded at concentration of $1x10^6$ 24 h prior to isolation of hematopoietic cells. The immortalized mesenchymal cells and hematopoietic cells originated from different animals.

3.3.6 Selection of culture medium for co-culturing red bone marrow hematopoietic cells with immortalized mesenchymal stromal cells

To select the optimal culture conditions for the red bone marrow hematopoietic cell survival and proliferation in the presence of mesenchymal stromal cells, different culture media were tested. Immediately after isolation as described above, 1 x 10^6 cells/mL red bone marrow cells were resuspended in four different culture conditions: Dulbecco's Modified Eagle Medium (DMEM), DMEM combined with Ham's F-12 medium (DMEM/F-12) at a ratio of 1:1, Iscove's modified Dulbecco eagle medium (IMDEM; serum-free medium) and RPMI-1640 combined with Minimum Essential Medium (RPMI/MEM) at a ratio 1:1. Each medium except IMDEM was supplemented with 10% FCS. Furthermore, all media were supplemented with 0.1 mg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma). Subsequently, the cells were seeded in 24-well plates containing a confluent monolayer of immortalized mesenchymal stromal cells cultured 24 hours prior to red bone marrow cell isolation. Every three days 500 µl of medium was replaced with fresh medium. Cells were collected from each culture condition every week at a concentration of 1 x10^5 cells/ml. After two washings, cells were resuspended in 400 µl PBS and incubated with propidium iodide (PI) on ice for 10 minutes and analyzed on a FACSCanto flow cytometer (BD bioscience).

3.3.7 Follow-up of the co-culture of red bone marrow hematopoietic cells with immortalized mesenchymal stromal cells

The co-culture was maintained in DMEM supplemented culture medium. Half of the medium (500 µl) was gently replaced twice a week. To passage the co-culture, hematopoietic cells were gently collected in calcium containing phosphate buffered saline and transferred into a 50 ml Falcon tube. Later, the mesenchymal stromal cells were trypsinized with 0.25% trypsin (Sigma-Aldrich) and transferred to a new 50 ml falcon tube. Both cell types were washed in PBS and reseeded in new culture plates at a ratio of 1 red bone marrow hematopoietic cell to 5 mesenchymal stromal cells.

3.3.8 Red bone marrow hematopoietic cell morphology and proliferation analysis

The morphology of the red bone marrow hematopoietic cells was examined on top of immortalized mesenchymal stromal cells by light microscopy and photographed during a period of 5 weeks. To examine the morphology of the red bone marrow hematopoietic cells, a Differential-Quick staining was performed. In the meantime, the proliferation of the red bone marrow hematopoietic stromal cells was assessed. Red bone marrow cells were seeded at a concentration of $1x10^5$ cells/mL as described above on top of immortalized mesenchymal stromal cells in 24-well plate in a DMEM supplemented culture medium. One week post-seeding, red bone marrow hematopoietic cells from three randomly selected wells were gently collected in PBS supplemented with calcium. Red bone marrow hematopoietic cells were centrifuged at 270 x g for 10 minutes and resuspended in fresh culture medium. The cells were diluted 1/10 in PBS. This cell suspension (200 µl) was mixed with 100 µl of 0.4% trypan blue and the cells were counted by light microscopy. The cell suspension was reseeded in 24-well culture plates and used for FACS analysis the following day. Counting was repeated every week for five consecutive weeks.

3.3.9 Flow cytometric analysis

A phenotypic analysis of the red bone marrow hematopoietic cells was performed. Every week, cells were gently collected from the co-culture in calcium containing PBS. Cells at a concentration of 1 x 10^5/mL were washed in PBS containing 5% FCS and 1 mM EDTA, and incubated with one of the following primary antibodies: mouse anti-pig CD172a (DH59B, 1:100, IgG1, VMRD), mouse anti-pig CD169 (41D3, 1:100, IgG1), mouse anti-pig CD163 (2A10/11, 1:100, IgG1, AbD Serotec, Dusseldorf, Germany), mouse anti-pig CD14 (Mil2, 1:100, IgG2b, Karen Haverson Laboratory), mouse anti-pig CD8 (76.2.11, 1:5, IgG2a) and mouse anti-pig CD4 (74.12.4, 1:5 IgG2b). After washing, the cells were incubated with 10% negative goat serum at 4°C for 10 minutes. Subsequently, the cells were incubated with a 1:200 dilution of FITC-labeled goat-anti-mouse IgG (Molecular Probes). Specificity of the staining for different cell markers was demonstrated using irrelevant, isotype-matched controls. Cells were washed twice and resuspended in 400 µl PBS. Prior to analysis on the FACSCanto cytometer (Becton Dickinson Immunocytometry systems) cells were incubated with propidium iodide at 4°C for 10 minutes. All antibody incubations were performed

for 30 minutes in the dark at 4°C. Collection and analysis of data were performed with FACS diva software (BD bioscience).

3.4 Statistical analysis

Statistical significance ($P<0.05$) was calculated using the Friedman test followed by Dunn's multiple comparison test to determine the difference between the cell markers expressed and also the time points. P-values equal to or lower than 0.05 were statistically regarded as significant.

3.5 Results

3.5.1 Immortalization of mesenchymal stromal cell cultures and identification of the cells

Porcine red bone marrow cells were isolated by flushing femur bones in RPMI-1640, yielding approximately 10^8 cells per femur. Two hours post-seeding, the majority of red bone marrow hematopoietic cells were found at the bottom of the flask. Three days after seeding, a large number of elongated fibroblast-like mesenchymal stromal cells adhered to the bottom of the culture plate and started to proliferate. Although mesenchymal stromal cells can stay in co-culture for more than one week without going into senescence, it was difficult to passage the cells more than two times. This adversely affected the survival of the hematopoietic cells. For this reason, the cells were immortalized by transduction with lentivirus vector SV40LT. Confirmation of successful immortalization of the mesenchymal stromal cells was performed by immunofluorescence staining. The mesenchymal stromal cells were found to be uniformly positive for SV40L nuclear antigen (Figure 1). Proliferation and viability of the cells decreased two to three weeks post-transduction after which the cells started to grow faster again. The morphology of the transduced cells was similar to that of primary cells. To confirm that the immortalized cells were mesenchymal stromal cells, immunofluorescence staining was performed to assess the expression of CD44 and CD55. The markers were found to be expressed in almost 100% of the cells (Fig 1).

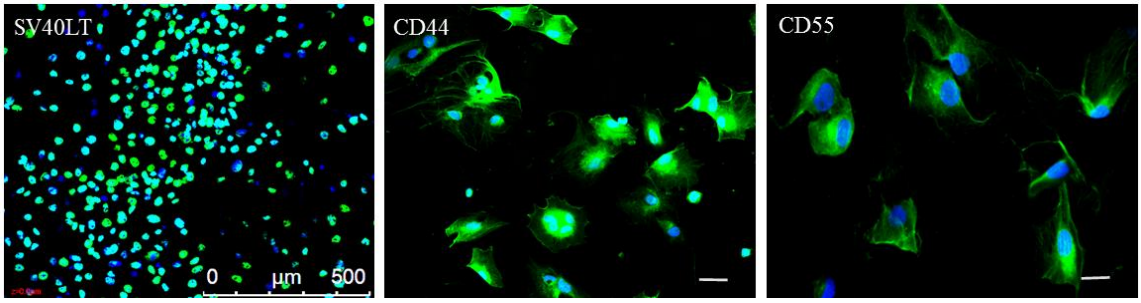

Fig 1. Immunodetection of SV40LT in immortalized mesenchymal stromal cells and characterization of the cells. Immunofluorescence staining was performed to confirm the successful immortalization of mesenchymal cells. The cells were uniformly positive for the large T virus antigen of SV40. Scale bar = 500 µm. Immunofluorescence staining of the immortalized mesenchymal cells was performed to demonstrate the expression of mesenchymal specific markers CD44 and CD55 by these cells. Nuclei were counterstained with Hoechst. Scale bar = 10 µm.

3.5.2 Selection of optimal medium to maintain co-cultures

We next determined the optimal medium to maintain co-cultures of red bone marrow hematopoietic cells and immortalized mesenchymal stromal cells. Among the different culture media tested, DMEM + 10% FCS seemed most suited as the cells still had a viability of more than 90% during 5 weeks of culture. The viability of cells cultured in RPMI-1640/MEM and F-12/DMEM was slightly lower than those in DMEM. Culture in IDMEM resulted in a viability drop of 50% after 3 weeks of culture and 90% after 5 weeks of culture (Fig 2).

3.5.3 Proliferation analysis and identification of red bone marrow hematopoietic cells co-cultured with immortalized mesenchymal stromal cells

To further characterize the cells, both live and stained (Differential-Quick staining) fixed cells were analyzed weekly using light microscopy. The majority of freshly isolated red bone marrow hematopoietic cells were small in size, round with smooth borders and a dark nucleus (Fig 3; row 1, week 0). From three days post seeding most of the red bone marrow hematopoietic cells increased in size, some became elongated, rough, and granular with tiny dendrites (Fig 3; row 1, week 2 and 3). Four to five weeks of culture, the majority of red bone marrow hematopoietic cells clumped together, while some remained single large cells, elongated cells or round cells (Fig 3; row 1, week 4 and 5).

Analysis by a differential-quick staining indicated the presence of granulocytes (blue arrow; note the segmented nucleus), promonocytes (yellow arrow; note the slightly indented nucleus),

lymphocytes (black arrow; note the round nucleus and scanty cytoplasm), and many other cell types in freshly isolated red bone marrow hematopoietic cells (Fig 3; row 2, week 0). The cell types observed during the subpassages for 1-5 weeks resembled monoblasts (red arrow; round to oval nucleus with broad rim of cytoplasm), monocytes (brown arrow; note the bean-shaped nucleus with small amount of cytoplasm), macrophages (green arrow; note the bean-shaped nucleus, the cytoplasm is coarsely granular with small vacuoles) and lymphocytes (black arrows; note the scanty cytoplasm). To check if the porcine red bone marrow hematopoietic cells cultured on top of immortalized mesenchymal stromal cell monolayers were proliferating, the cells were collected every week from the co-culture and upon washing reseeded in new 24-well plates lacking mesenchymal cells.

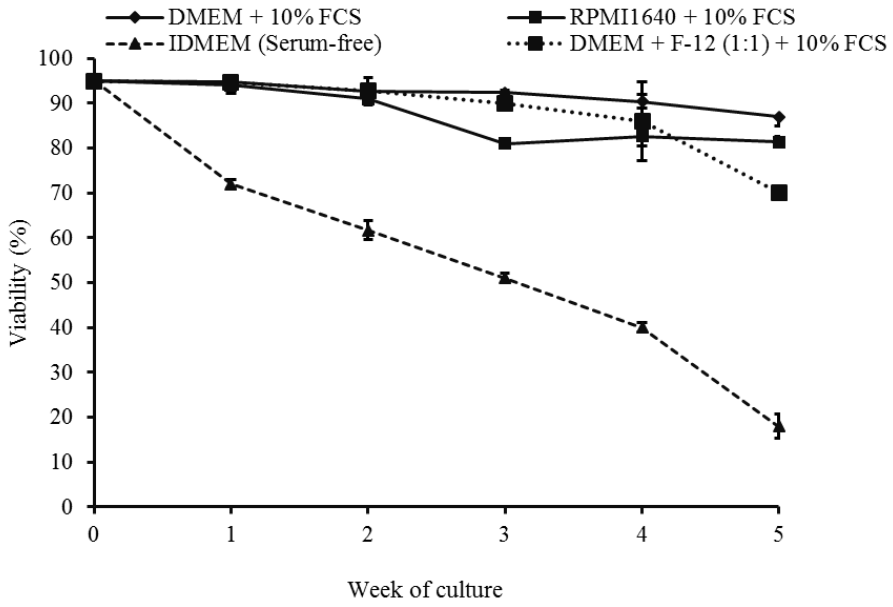

Fig 2. Effect of culture medium on the viability of red bone marrow hematopoietic cells co-cultured with immortalized mesenchymal stromal cells. Red bone marrow hematopoietic cells were co-cultured in DMEM, RPMI, F-12+DMEM all supplemented with 10% FCS and IDMEM, serum free medium. DMEM supplemented with 10% FCS was the best culture condition producing almost 90% of viable cells five week post-seeding. The chart represents the mean and standard deviation of three independent experiments.

After two hours the contaminating fibroblast-like mesenchymal stromal cells were attached to the bottom of the culture plates, while the hematopoietic cells remained in suspension. The non-attached cells were collected and counted by trypan blue exclusion test. The initial number of cells was $2.5x10^4$ /mL. After five weeks of co-culture with immortalized mesenchymal stromal cells, the red

bone marrow hematopoietic cell number increased to $7x10^5$/mL (see Fig 3; row 3). After six weeks of co-culture with immortalized mesenchymal stromal cells, the majority of hematopoietic cells differentiated, therefore their proliferation rate declined. Later on hematopoietic cells completely stopped proliferating.

3.5.4 Phenotypical analysis of differentiating hematopoietic cells

The main populations of red bone marrow hematopoietic cells were categorized based on their light scatter properties and by comparing them with peripheral mononuclear cells (PBMC).

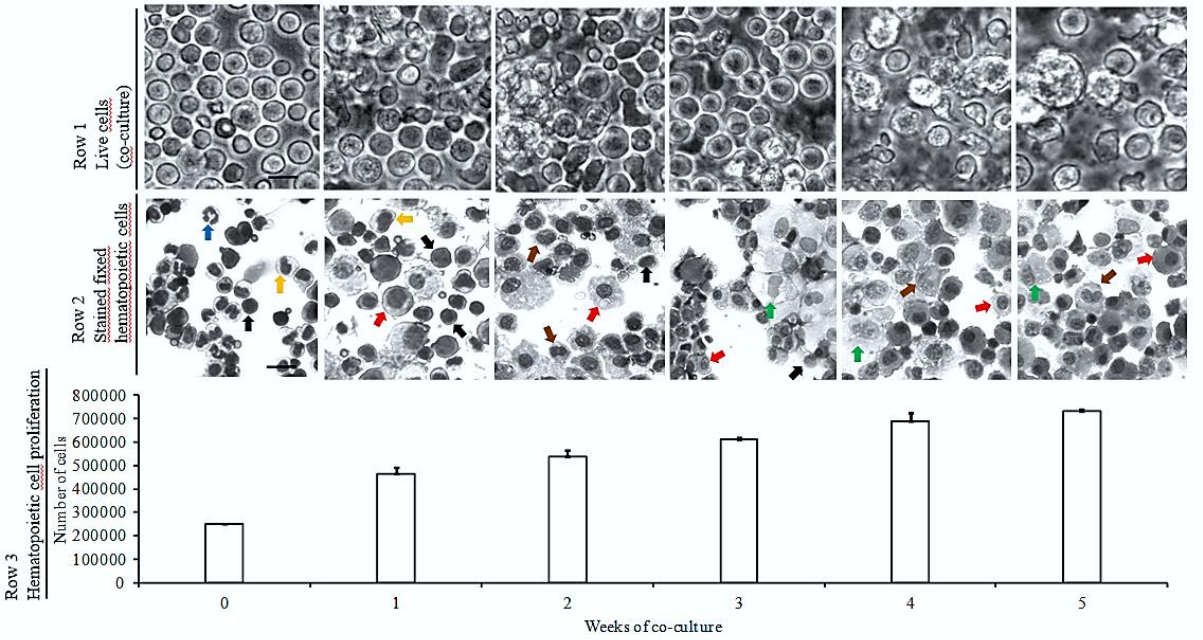

Fig 3. Analysis of the morphology and proliferation of red bone marrow hematopoietic cells co-cultured with immortalized mesenchymal stromal cells. The cell morphology was examined by phase contrast microscopy every week for 5 weeks. At week 0, differential-quick staining of the cells revealed cells with a segmented nucleus (myeloid cells; blue arrow) and cells with a slightly indented nucleus (monocytic cells; yellow arrow). A large number of blast cells (red arrow) and few monocytes (brown arrow), macrophage (green arrow) and lymphocytes (black arrow) were also observed during 1-5 weeks of co-culture (row 2). Proliferation capacity of the bone marrow hematopoietic progenitor cells was assessed. Cells were collected from the cultures, washed and counted every week for five weeks. For the period of the experiments the cells proliferated 3-fold.

To investigate the phenotype of the red bone marrow hematopoietic cells co-cultured with immortalized mesenchymal stromal cells, the expression of the surface markers CD172a, CD14, CD163, CD169, CD4 and CD8 was analyzed, after dead cells, cell debris and doublet cells were excluded. During the measurement, all parameters were kept constant. Generally, the size and granularity of the cells increased from 0 to 5 weeks (results not shown). The percentage of CD172a, CD14, CD163, CD169 CD4 and CD8 positive red bone marrow hematopoietic cells was determined on a fluorescence versus forward scatter dot plot (Figure 4a and 4b). Analysis of flow cytometry data showed that 2.0±3% of freshly isolated red bone marrow hematopoietic cells were positive for CD172a. At week 1, 2, 3, 4 and 5, 5±7%, 22±10%, 37±0.8%, 22±7% and 18±2% of the red bone marrow hematopoietic cells were $CD172a^{+}$, respectively. CD14 was expressed on 5±3% of freshly isolated red bone marrow hematopoietic cells and on 32.0±4%, 34.0±6%, 40.0±8%, 27.0±1% and 39.0±7% of red bone marrow hematopoietic cells co-cultured with mesenchymal stromal cells at 1, 2, 3, 4, and 5 weeks of co-culture, respectively. The percentage of $CD163^{+}$ cells in freshly isolated red bone hematopoietic cells was 1.5±11%, and 7.0±10%, 18.0±2%, 41.0±4%, 12.0±6% and 22.0±9% in red bone marrow hematopoietic cells co-cultured with mesenchymal stromal cells for 1, 2, 3, 4, and 5 weeks, respectively. Two $CD163^{+}$ cell populations were found when red bone marrow hematopoietic cells were co-cultured with mesenchymal stromal cells for two weeks ($CD163^{low}$ and $CD163^{high}$). CD169 was not present on freshly isolated red bone marrow hematopoietic cells and red bone marrow hematopoietic cells co-cultured with mesenchymal stromal cells for one week. However, at 2, 3, 4 and 5 weeks of co-culture 7.0±2%, 24.0±2%, 7.0±4%, and 7.0±0.5% of the red bone marrow hematopoietic cells were CD169 positive. CD4 was also not present on freshly isolated red bone marrow hematopoietic cells while 1.0±14%, 6.0±3%, 23.0±3%, 5.8±1% and 11.0±4% of the red bone marrow hematopoietic cells were positive for CD4 at week 1, 2, 3, 4, and 5, respectively. Among the freshly isolated red bone marrow hematopoietic cells 4.1±4% of the cells expressed CD8. At 1, 2, 3, 4 and 5 weeks of co-culture, 1.8±4%, 4.3±6%, 19.0±5%, 1.5±6% of the red bone marrow hematopoietic cells were positive for CD8 respectively. Three weeks post-seeding in the presence of mesenchymal stromal cells, a significant increase ($p<0.05$) in the percentage of CD163, CD169 and CD8 positive red bone marrow hematopoietic cells was observed when compared to freshly isolated cells and red bone marrow hematopoietic cells. Although statistically, no significant increase was observed in the expression of CD172a,

CD14, and CD4 a trend in increased expression of these markers was also observed when freshly isolated red bone marrow hematopoietic cells were compared with red bone marrow hematopoietic cells co-cultured with mesenchymal stromal cells for 1, 2 and 3 weeks (Fig 4a and 4b).

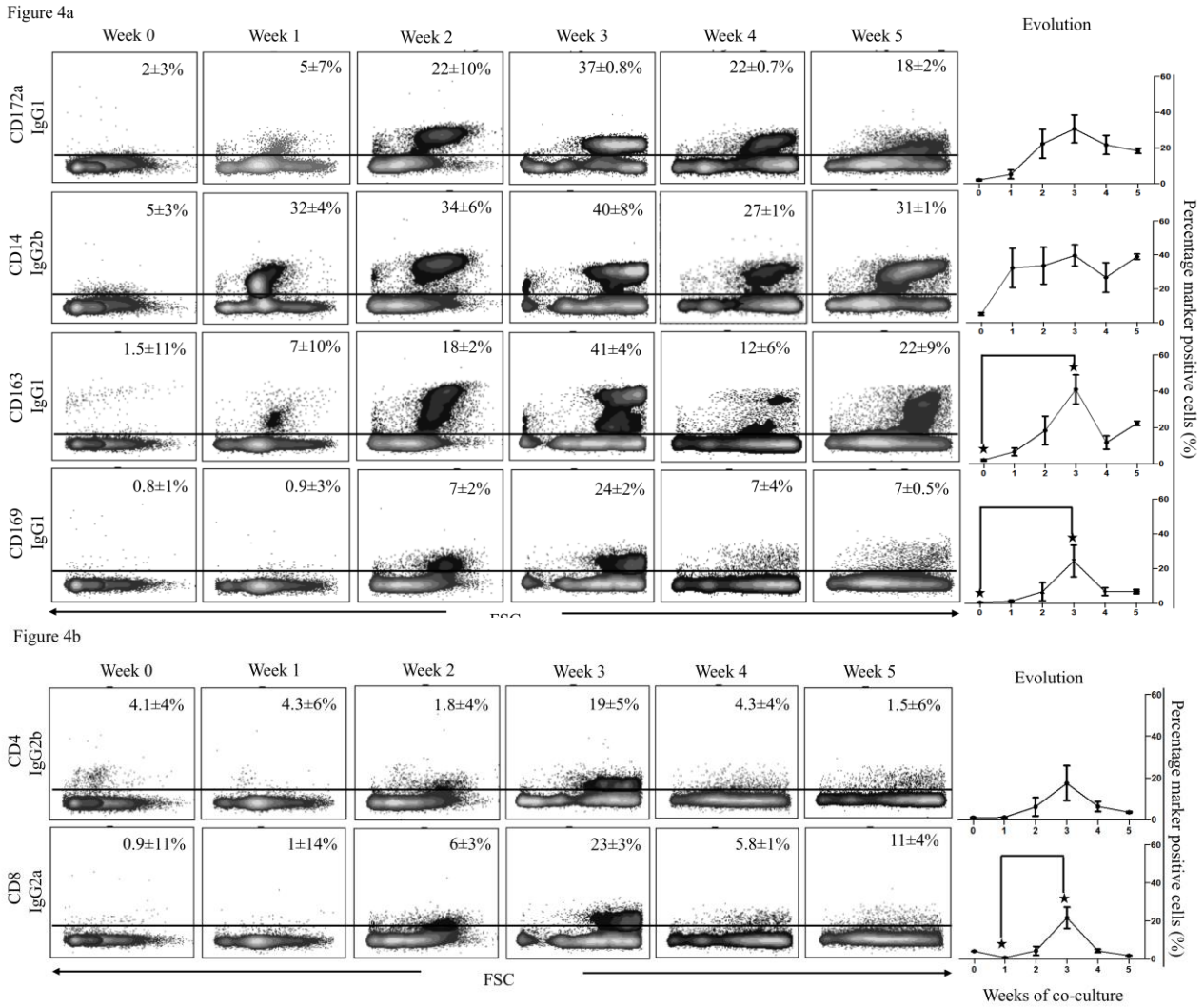

Fig 4. Phenotypical analysis of red bone marrow hematopoietic cells. Cells at concentrations of 1 x 10^5/mL were washed in PBS containing 5% FCS and 1 mM EDTA, and incubated with mouse monoclonal antibodies against porcine CD172a CD14, CD163, CD169, CD4 and CD8. The results of the flow cytometry analysis are shown as fluorescence (y-axis) versus forward scatter (x-axis) dot plot. Chart data from three independent experiments were presented as percentage ± SEM. *: $p<0.05$ as compared to week 0.

3.6 Discussion

In this study, porcine red bone marrow hematopoietic cells were co-cultured with immortalized mesenchymal stromal cells for five weeks. This co-culture led to a 3-fold proliferation and 90±3.2% viable hematopoietic cells. These results are in line with previous findings that human bone marrow derived-mesenchymal stromal cells support the maintenance and proliferation of bone marrow hematopoietic cells in *in vitro* cultures (de Lima et al., 2012; Walenda et al., 2010). That success was attributed to the secretion of some soluble factors and cell-to-cell contacts (Wagner et al., 2007). During the co-culture of red bone marrow hematopoietic cells with immortalized mesenchymal stromal cells, a differentiation of the hematopoietic cells toward monocytes/macrophages (40%) and CD4 and CD8 cells (20-25%) was demonstrated by the use of myelomonocytic and lymphocytic markers (Hsu et al., 2007). Although many groups used a medium supplemented with different cytokines and growth factors to maintain red bone hematopoietic cells in culture, we were able to keep the cells for five weeks in a simple cell culture condition containing DMEM supplemented with 10% fetal calf serum on top of a monolayer of immortalized $CD44^{+}CD55^{+}$ mesenchymal stromal cells. However, six weeks post-seeding hematopoietic cells with immortalized mesenchymal stromal cells, the proliferation rate gradually decreased as more cells differentiated and finally stopped.

CD172a is a pan-myeloid marker and was the first to be detected in porcine myelomonocytic cells. It was the first molecule that was expressed on myeloid cells, and continued to be expressed by these cells as they differentiated into more mature cells (Summerfield et al., 2001). CD172a plays an important role in the control of proliferation, differentiation and activation of myeloid cells (Ezquerra et al., 2008). This marker is detected at high levels on granulocytes, monocytes, macrophages and dendritic cells (van Beek et al., 2005), but compared to monocytes, the levels on DCs and macrophages are low. FACS data from this study indicated an increased expression of CD172a on red bone marrow hematopoietic cells co-cultured with mesenchymal stromal cells for three weeks when compared with freshly isolated red bone marrow hematopoietic cells. The $CD172a^{+}$ cells had a high forward scatter and side scatter (Ezquerra et al., 2008). This is in agreement with the findings that bone marrow cells cultured in the presence of colony stimulating factor 1 (CSF1) increased their expression of CD172a, CD14, CD16 and had higher forward and

side scatter properties with time (maximum of 7 days) (Fairbairn et al., 2011; Kapetanovic et al., 2012). The majority of these CD172a$^+$ cells were observed to be blast cells, monocyte-like macrophages, and vacuolated macrophages (Sladek et al., 2014).

CD14, the co-receptor for Gram-negative lipopolysaccharides (LPS), Gram-positive bacteria peptidoglycans and lipoteichoic acid is expressed at a high level by monocytes and tissue macrophages, but at lower levels by granulocytes. It is used in humans as a marker of monocytes and macrophages (Summerfield et al., 2001; Ezquerra et al., 2008). In humans, CD14 together with CD16 are mostly used as a marker to classify monocytes into different subsets. Mil2 is a pig CD14-specific monoclonal antibody (Thacker *et al*., 2001) characterized by Haverson et al. (1994), while MY-4 is a multispecies reactive monoclonal antibody. Carrasco et al. 2001 and Thacker et al., 2001 assessed and found no difference between MIL2 and MY-4 monoclonal antibodies in terms of detecting the CD14 expression on pig cells. The most unexpected finding of this study was that the percentage of CD14$^+$ red bone marrow hematopoietic cells was higher compared to that of CD172a$^+$ hematopoietic cells throughout the period of the experiment (five weeks), despite the latter being the pan-myeloid marker expressed by every myeloid cell from precursor to matured cells. In addition, CD14 was described as a monocyte marker expressed at a low level during maturation (Steinbach F et al., 1994).

CD163, also called scavenger receptor cysteine-rich type 1 protein, is expressed only by monocytes (subset of monocytes) and macrophages and it is used as a maturation marker for monocytes. It plays a very important role in the infection of macrophages by African swine fever virus and porcine reproductive and respiratory syndrome virus. In this study, CD163$^+$ cells were almost undetectable on freshly isolated red bone marrow hematopoietic cells, which is in agreement with a report from Sanchez et al. (1999). Furthermore, statistical analysis showed a significant increase in the expression of CD163 on red bone marrow hematopoietic cells co-cultured with immortalized mesenchymal stromal cells for three weeks when compared with freshly isolated red bone marrow hematopoietic cells. This is in line with the hypothesis that the expression level of CD163 in blood-derived monocytes increases as the cell matures (Fairbairn et al., 2011; Kapetanovic et al., 2012). It has also been reported by the same authors that culturing porcine bone marrow hematopoietic cells in the presence of rhCSF-1 did not cause increased expression levels of CD163.

Porcine sialoadhesin (CD169; siglec-1) has been identified as a sialic acid-dependent sheep erythrocyte receptor on the bone marrow resident cells of mice. It is a member of the sialic acid

binding immunoglobulin-like lectin family and is involved in the binding and internalization of porcine reproductive and respiratory syndrome virus (Vanderheijden et al., 2003; Crocker et al., 1989; Hartnell et al., 2001). CD169 expression is restricted to specific macrophages residing in lungs, lymphoid tissues, liver and colon (Ezquerra et al., 2008). In the latter study, CD169 was not detected on freshly isolated and one week old porcine red bone marrow hematopoietic cells, which is in agreement with other studies in humans and mice (Duan et al., 1997). It was also reported that culturing freshly isolated porcine monocytes in medium supplemented with FBS or porcine serum causes a very low expression level of CD169 (Crocker et al 1988). We observed a high percentage of $CD169^+$ cells (24±5%; $p<0.05$) in porcine red bone marrow hematopoietic cells, co-cultured with mesenchymal stromal cells for three weeks compared to freshly isolated red bone marrow hematopoietic cells. There were no CD4+ cells detected in freshly isolated and one week old porcine red bone marrow hematopoietic cells. Unlike CD4, $CD8^+$ cells were detected in freshly isolated and one week old porcine red bone marrow hematopoietic cells. These $CD8^+$ red bone marrow hematopoietic cells had a low forward scatter. From two weeks post seeding in the presence of mesenchymal stromal cells, these small $CD4^+$ and $CD8^+$ red bone marrow hematopoietic cells completely disappeared. Most likely these particular cells died out. This is in agreement with a report demonstrating an inhibition of $CD8^+$ and $CD4^+$ T cell proliferation by human bone marrow stromal cells (Vremee et al., 2000).

In conclusion, in this work we successfully established a co-culture model for differentiating porcine red bone marrow hematopoietic cells on top of SV40LT immortalized mesenchymal stromal cells into cells of the monocyte/macrophage lineage and CD4 and CD8 cells. This co-culture model may be used to investigate the mechanism by which mesenchymal stromal cells trigger red bone marrow hematopoietic cell differentiation into cells of the monocyte/macrophage lineage in *in vitro* cultures. Although many research groups studied adhesion molecules in human mesenchymal stromal cells there is little understanding on how porcine mesenchymal stromal cells and red bone hematopoietic cells interact with each other by means of adhesion molecules such as CD44. As the pig is a good model for humans, this co-culture model may be an interesting tool to explore. This will allow the further development of the therapeutic potential of mesenchymal stromal cells in the near future. In addition, the monocytes/macrophages generated by this co-culture will be used to study the replication of porcine reproductive and respiratory syndrome virus in these cells.

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Chapter 4

Immortalized porcine mesenchymal stromal cells derived from nasal mucosa, lungs, lymph nodes, spleen and bone marrow retain their stemness properties and trigger the expression of siglec-1 in co-cultured blood monocytic cells

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4.1 Abstract

Mesenchymal stromal cells have been isolated from different sources. They are multipotent cells capable of differentiating into many different cell types, including osteocytes, chondrocytes and adipocytes. They possess a therapeutic potential in the management of immune disorders and the repair of damaged tissues. In the previous chapter, we showed that the percentage of $CD172a^+$, $CD14^+$, $CD163^+$, $Siglec\text{-}1^+$, $CD4^+$ and $CD8^+$ hematopoietic cells increased when co-cultured with immortalized mesenchymal cells derived from bone marrow. The present work aimed to demonstrate the stemness properties of SV40-immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow and their immunomodulatory effect on blood monocytes. Mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were isolated and successfully immortalized using simian virus 40 large T antigen (SV40LT) and later, co-cultured with blood monocytes, in order to examine their differentiation stage (expression of Siglec-1). Flow cytometric analysis revealed that the five mesenchymal cell lines were positive for mesenchymal stromal cell markers CD105, CD44, CD90 and CD29, but lacked the expression of myeloid cell markers CD16 and CD11b. Growth analysis of the cells demonstrated that bone marrow derived-mesenchymal stromal cells proliferated faster compared with those derived from the other tissues. All five mesenchymal cell lines co-cultured with blood monocytes for 1, 2 and 7 days triggered the expression of siglec-1 in the monocytes. In contrast, no $siglec\text{-}1^+$ cells were observed in monocyte cultures without mesenchymal stromal cell lines. Mesenchymal stromal cells isolated from nasal mucosa, lungs, spleen, lymph nodes and bone marrow were successfully immortalized and these cell lines retained their stemness properties and displayed immunomodulatory effects on blood monocytes.

4.2 Introduction

Mesenchymal stromal cells, also known as mesenchymal stem cells, are multipotent cells derived from the mesoderm during embryonic development (Pittenger *et al*., 1999; Gerson *et al*., 1999). They have been demonstrated by many research groups to be a potential tool in treating cardio-vascular diseases, diabetes and autoimmune diseases, like rheumatoid arthritis as well as in regenerative medicine (Singla *et al*., 2010, Ling *et al*., 2009, Gonzalez-Rey *et al*., 2010). They have immunomodulatory properties, which they effect through many ways, one of which is the secretion of anti-inflammatory factors such as TGF-β (Di Nicola *et al*., 2002). They may inhibit the proliferation of lymphocytes and regulate the differentiation and function of dendritic cells (Nauta *et al*., 2006). Mesenchymal stromal cell co-cultures with macrophages trigger an increase in the expression of IL-10 and decrease the expression of TNF-α and IL-12 (Kim *et al*., 2009). *In vivo* experiments showed the accumulation of macrophages with a regulatory phenotype in inflamed areas upon local infusion of mesenchymal stromal cells. The short life span of primary mesenchymal stromal cells during *in vitro* cultivation prevents their use in long-term experiments (Wagner *et al*., 2008; Bork *et al*., 2010; Stewart *et al*., 2002). Primary mesenchymal stromal cells have a limited number of cellular divisions in cell culture after which they undergo senescence and finally die (Stewart *et al*., 2002; Stewart *et al*., 2006). Because of these limitations, there is an urgent need to establish continuous cell cultures of well-characterized mesenchymal stromal cells for long-term studies. Presently, the most widely used method to immortalize primary cells is by introducing viral genes, such as the gene encoding simian virus 40 large T antigen (Jha *et al*., 1999, Kirchoff *et al*., 2004).

The ability to keep large quantities of mice for repetitive experiments makes it the most widely used animal for studying many human diseases and abnormalities. Many groups conducted research on the potential therapeutic application of mesenchymal stem cells in humans using mice models with successful outcome. However, its small size makes it impossible to collect large amounts of tissues for an experiment. Moreover, results obtained from experiments performed on mice may be difficult to successfully translate to human medicine (Orlie *et al*., 2001).

Alternative large animal models may be developed with pigs, which are more closely related to humans than mice on an anatomical and physiological level (Meurens *et al*., 2012). Large amounts

of tissues can be obtained from pigs to conduct several experiments. Siglec-1, a protein expressed only on macrophages, plays a crucial role in host-pathogen interactions and immune regulation. Pathogens carrying sialic acids can be internalized by siglec-1^+ macrophages (Delputte *et al*., 2011). It mediates the receptor-dependent internalization of PRRSV (Vanderheijden *et al.*, 2003). In the present study, continuous cultures of mesenchymal cells from porcine nasal mucosa, lungs, spleen, lymph nodes and bone marrow were established and used to generate siglec-1^+ macrophages.

4.3 Materials and methods

4.3.1 Cell isolation and cultures

Three pigs were euthanized by injecting sodium pentobarbital (20%, 1ml/1.5 kg; Kela Laboratories, Hoogstraten Belgium) into the jugular vein. The pigs were euthanized for the purpose of other experiments with the approval of Local Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University (Application EC2015⁄04). Nasal mucosa, lungs, spleen and lymph nodes were removed in a sterile way and transferred immediately to a biosafety cabinet. Tissues from these organs were cut into small pieces, transferred into sterile 100 ml bottles containing Dulbecco's Modified Eagle's Medium (DMEM) and incubated at 37°C for 1 h in the presence of 0.5 mg/ml collagenase type IV (Gibco). Next, the cell suspension was filtered using a 70 μm cell strainer and washed two times with PBS. The cells were resuspended in DMEM supplemented with 10% fetal calf serum (FCS; Gibco), 1 mM sodium pyruvate, 1% non-essential amino acid, 0.1 mg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), and 100 U/mL penicillin (Continental Pharma). Cells were seeded in 24-well plates at a concentration of 1 x 10^6 /ml. To deplete the cell cultures of mononuclear leukocytes, half of the medium was replaced every 48 hrs for 1 week. At 80% confluency, spindle and elongated-shaped cells were trypsinized in 0.125% trypsin solution (Sigma-Aldrich) and passaged. Isolation, cultivation, immortalization and characterization of red bone marrow mesenchymal stromal cells were described before (see Chapter 3). To check the epithelial cell contamination in the immortalized mesenchymal cell cultures, at passage 11 the five immortalized mesenchymal stromal cell types were seeded in 24-well plate and allowed to attach to a glass cover slide overnight, washed two times with PBS and fixed with 4%

paraformaldehyde at room temperature for 10 minutes. The cells were washed with PBS and permeabilized with Triton-X 100 at room temperature for two minutes. Next, the cells were incubated with monoclonal mouse anti-human cytokeratin (AE1/AE3, 1:200, IgG1, Dako Denmark A/S) for 1 h at 37°C in the presence of 10% normal goat serum. The cells were washed two times with PBS and later incubated with secondary antibodies goat anti-mouse IgG1-FITC for 1 h at 37°C. The cells were additionally washed two times with PBS and the cover slide was mounted with glycerin/PBS solution (0.9:0.1, v/v) and analyzed by confocal microscopy (Leica Microsystem DMRBE, Wetzlar, Germany). Also to detect the presence of mononuclear cells in the immortalized mesenchymal stromal cell cultures. Cells were fixed with 4% paraformaldehyde for 10 min. at room temperature followed by permeabilization with Triton-X 100 for 2 min. at room temperature. The cells were washed two times with PBS and incubated with rabbit polyclonal antibodies to CD45 (ab10558, 1:100, IgG, abcam) for 1 h at 37°C in the presence of 10% normal goat serum. The cells were washed two times with PBS and further incubated with secondary antibodies goat anti-rabbit IgG-FITC for 1 h at 37°C. After two times washing with PBS, the cover slide was mounted with glycerin/PBS solution (0.9:0.1, v/v) and analyzed by confocal microscopy (Leica Microsystem DMRBE, Wetzlar, Germany). Mesenchymal stromal cells derived from nasal mucosa, lungs, spleen and lymph nodes were monitored by light microscopy to examine their morphology.

4.3.2 Immortalization of primary mesenchymal stromal cells

To establish continuous cultures of mesenchymal stromal cells for long-term studies, cells were immortalized using recombinant lentivirus containing the sequence encoding the simian virus 40 large T antigen (SV40LT) (Applied biological materials Inc., Richmond, BC, Canada). At 50% confluency, cells isolated from nasal mucosa, lungs, spleen and lymph nodes were incubated with an SV40LT gene carrying lentivirus suspension containing polybrene (8 µg/mL, applied biological material Inc., Richmond, BC, Canada). After 30 minutes, the virus was diluted with complete medium to prevent toxicity. Next, the cells were further incubated overnight. After 18 h incubation, the virus suspension was replaced with complete medium, after two times washing. The cells were allowed to proliferate for 2-3 days to 90-100% confluency. Three days post-transduction, cells were trypsinized with 0.125% trypsin (Sigma- Aldrich) and reseeded. The cells were examined every day for proliferation by light microscopy (Olympus). To confirm successful immortalization of

mesenchymal stromal cells, transduced cells were seeded in 24-well plates on glass cover slips and incubated at 37°C, 5% CO_2 for 24-48 h. Cells were washed two times with PBS and fixed with 4% paraformaldehyde for 10 min. at room temperature. Next, cells were additionally washed two times and permeabilized with Triton-X 100 at room temperature for two minutes. Subsequently, the cells were incubated with polyclonal rabbit antibodies against SV40LT (Applied Biological material Inc.) antigen for 1 h at 37°C in the presence of 10% normal goat serum. After two washes with PBS the cells were further incubated with goat anti-rabbit IgG-FITC for 1 h at 37°C. The cells were additionally washed two times with PBS and the cover slide was mounted with glycerin/PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo [2.2.2] octane and analyzed by confocal microscopy (Leica Microsystem DMRBE, Wetzlar, Germany).

4.3.3 Flow cytometry

Immortalized mesenchymal stromal cells were analyzed for the expression of the mesenchymal stromal cell markers CD44, CD29, CD105 and CD90. As a negative control, the expression of the myeloid cell markers CD16 and CD11b were also examined. Immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph node and bone marrow cells were trypsinized with 0.125% trypsin (Sigma-Aldrich) and washed two times in PBS containing 1 mM EDTA-5% FCS. The cells were incubated with the following primary mouse monoclonal antibodies: anti-CD44 (F10-44-2, IgG2a, 1:200, abcam), anti-CD29 (7F10, IgG1, 1:200, THERMO Fischer Scientific Inc), anti-CD105 (MEM-229, IgG2a, 1:200, THERMO Fischer Scientific Inc.), anti-CD90 (3F9, IgG2a, 1:200, Norvus Biological), anti-CD16 (G-7, IgG1, I:100, Antigenix America Inc.) and anti-CD11b (ab8879, IgG1, 1:400, abcam). To demonstrate the specificity of the primary antibodies, 13D12 against gD of PrV (IgG1) and 1C11 against gB of PrV (IgG2a) were used as isotype controls (Nauwynck *et al.,* 1995). The incubation was performed in the presence of normal goat serum for 30 minutes on ice. After two washes, the cells were incubated with appropriate secondary antibodies: Alexa Fluor 647-conjugated goat anti-mouse IgG2a and Alexa Fluor 488-conjugated goat anti-mouse IgG1 for 30 minutes on ice in the dark. Next, the cells were washed two times, resuspended in 100 µl PBS and transferred to 96 well-plates. The measurement of cells and analysis of the results were performed with a cytoFLEX flow cytometer and cytoFlex software respectively (Beckman Coulter, Inc.).

4.3.4 Growth analysis of immortalized mesenchymal stromal cells

To determine the proliferation rate of the immortalized mesenchymal stromal cells, the cells were seeded in six-well plates at 2 x 10^5/ml. Every 24 h, cells were trypisinized and counted during 5 days at 24, 48, 72, 96 and 120 h. The growth analyses were performed in triplicate at each time point.

4.3.5 Cell cycle analysis of immortalized mesenchymal stromal cells

Immortalized mesenchymal stromal cells were trypsinized with 0.125% trypsin solution and washed two times in PBS. The cells were resuspended in ice-cold PBS and their concentrations adjusted to 10^6 cells/ml. One ml of the cell suspension was gently vortexed and added dropwise to 9 ml of 70% ethanol in 15 ml centrifugation tubes. The cells were incubated at 4°C for 2 hours and then centrifuged at 450 x g at 4°C for 10 min. Subsequently, the cells were resuspended in 300 µl propidium iodide (1 mg/ml)/Triton X-100 (0.1%) solution containing 2 mg DNase-free RNase A (Sigma-Aldrich). Later, the cells were incubated at 37°C for 15 minutes in the dark. Next, the cells were transferred to 4°C until measurement. A CytoFLEX flow cytometer (Beckman Coulter, Inc.) was used to acquire and analyze the data.

4.3.6 Osteogenic, chondrogenic and adipogenic differentiation of immortalized mesenchymal stromal cells

Immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were resuspended in complete medium and seeded in 24-well plates at a concentration of 1 x 10^5 cells/ml. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. At 60% confluency, medium was replaced with complete osteogenic differentiation medium (StemPRO® osteogenesis Differentiation Kit; Gibco) and the cells were further incubated. The cell cultures were refed every 3 days. After 5 days of culturing, osteogenic differentiation medium was gently removed and mesenchymal stromal cells were washed two times with PBS. The cells were fixed with 4% parafomaldehyde for 30 minutes at room temperature (RT). The cells were additionally washed two times with PBS and further incubated with 2% alizarin red solution for 3

minutes. After removal of the staining solution, cells were washed two times with PBS and analyzed by light microscopy. Adipogenic differentiation of the mesenchymal cells was performed by culturing mesenchymal stromal cells in adipogenic differentiation medium (StemPRO® Adipogenesis Differentiation Kit; Gibco) for 5 days. To demonstrate the presence of lipid droplets in differentiated mesenchymal stromal cells, adipogenic differentiation medium was removed. Next, the cells were washed two times with PBS and incubated with 4% paraformaldehyde for 30 minutes at room temperature. Later, 200 µl oil red O was added to the cells and incubated for 1 h at room temperature. The cells were washed two times with PBS and analyzed using light microscopy. For chondrogenesis, mesenchymal cells were cultured in chondrogenic differentiation medium (StemPRO® Chondrogenesis Differentiation Kit; Gibco) for 7 days. The cell cultures were refed every 3 days. To confirm the differentiation of mesenchymal stromal cells to chondrocytes, cells were incubated with alcian blue staining solution for 30 minutes at RT. The cells were then washed two times with PBS and analyzed by light microscopy.

4.3.7 Culturing immortalized mesenchymal stromal cells in media supplemented with cytokine-free serum

After detachment, immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were suspended in DMEM supplemented with 10% cytokine-free serum, 0.1 mg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), and100 U/mL penicillin (Continental Pharma). The cells were seeded at 2 x 10^5 cells/ml in 24-well plates and incubated in a humidified environment of 5% CO2 at 37°C. After 3 days of culture the cells were trypsinized using 0.125% trypsin and washed two times. Next, the cells were resuspended in 100 µl PBS in 96-well plates and incubated with 1 mg/ml propidium iodide (PI; Sigma-Aldrich) for 10 minutes on ice in the dark. Afterwards, the viability of the cells was measured using cytoFLEX.

4.3.8 Co-culturing of immortalized mesenchymal stromal cells and blood monocytes

4.3.8.1 *Isolation of blood mononuclear cells*

Blood was taken from three pigs, using 20 ml syringes with 18-gauge needles. The blood was transferred into a sterile 50 ml Falcon tube and diluted 1:1 with ice-cold PBS. Twenty milliliter of diluted blood was gently layered on top of 15 ml Ficoll-paque in a 50 ml tube and centrifuged at 450 x g for 45 min. at RT. The interphase band containing mononuclear cells was gently removed and transferred into a new Falcon tube. After centrifugation, the cells were suspended in lysis buffer and incubated on ice for ten minutes. The cells were washed two times with PBS and resuspended in RPMI/MEM (1:1) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 1% non-essential amino acid, 0.1 mg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), and 100 U/mL penicillin (Continental Pharma). Next, the cells were seeded in 24-well plates on inserts at a concentration of 10^6 cells/ml. The cells were incubated at 37°C, 5% CO_2.

4.3.8.2 *Co-culture of peripheral blood monocytes and immortalized mesenchymal stromal cells*

Eighteen hours post-seeding, non-adherent cells were gently removed by washing. Next, 3 x10^4/ ml immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were added to the monocytic cell cultures in DMEM supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 1% non-essential amino acid, 0.1 mg/mLgentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa), and 100 U/mL penicillin (Continental Pharma). Wells with only monocytic cells were used as controls. The immortalized mesenchymal stromal cell-monocytic cell co-cultures were incubated at 37°C, 5% CO_2. The co-cultures were fixed with 4% paraformaldehyde for 10 minutes at RT at 24 h, 48 h and 1week post-seeding. After permeabilization with 0.1% Triton X-100 at RT for 2 minutes, the cells were incubated with porcine anti-siglec-1 (41D3, IgG1, 1:5) or porcine anti-CD163 (2A10/11, IgG1, 1:200, AbD Serotec, Dusseldorf, Germany) primary antibodies containing normal goat serum for 1 h at 37°C. After two washes, the cells were incubated with goat anti-mouse IgG Alexa-Fluor 594 (A-21125, 1:400, invitrogen) secondary antibodies for 1 h at 37°C. Hoechst 33342 was used to stain the nuclei of the cells. Subsequently, the cells were washed two times with PBS. To demonstrate the specificity of

the primary antibodies used, isotype-matched control monoclonal antibodies against gD of PRV (IgG1, 13D12) were used (Nauwynck *et al.*, 1995). The cells were rinsed with PBS and mounted with glycerin/PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo [2.2.2] octane. Next, the siglec-1 and CD163 positive cells were determined by counting a total of 500 cells per slide using fluorescence microscopy and photographed by confocal microscopy (Leica Microsystem DMRBE, Wetzlar, Germany). Subsequently, ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA) was used to calculate the fluorescence intensity of the siglec-1^+ and $CD163^+$ cells.

4.4 Statistical analysis

Descriptive statistics was used to determine the difference in the percentage and expression of siglec-1 and CD163 by monocytic cells co-cultured with mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and bone marrow.

4.5 Results

4.5.1 Immortalizing mesenchymal stromal cells from different tissues does not affect their morphology

Seventy-two hours after seeding, a variety of cells attached to the culture plates. The cells included mononuclear cells, epithelial-like cells and elongated fibroblast-like cells. Passaging the cells depleted the epithelial-like cells and mononuclear cells, leaving fibroblast-like cells in culture which continued to proliferate. This was confirmed by staining the five immortalized mesenchymal stromal cell types with antibodies against the epithelial cell marker cytokeratin and leukocyte marker CD45. No cytokeratin positive cells were found and no CD45 positive cells were observed in all the five immortalized mesenchymal stromal cell cultures. The mesenchymal stromal cells from nasal mucosa, lungs, spleen and lymph nodes were confirmed to be successfully immortalized by the expression of SV40LT antigens (Fig 1). The morphology of the immortalized stromal cells was similar to that of the primary cells. All five mesenchymal stromal cell lines showed a spindle-shape

morphology (Fig 1). The mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph node and red bone marrow have been passaged 32 times without any problem regarding their morphology and growth characteristics.

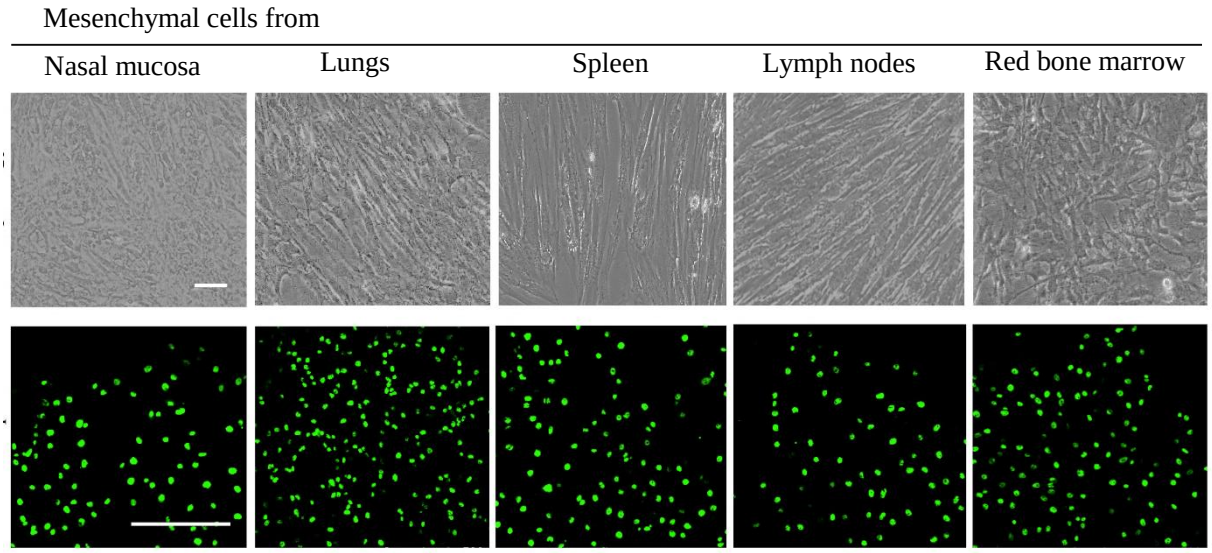

Fig 1. Morphological analysis of the immortalized mesenchymal stromal cells. Top row: the morphology of the immortalized mesenchymal stromal cells. Scale bar == 10 μm. Bottom row: mesenchymal cells expressed nuclear localized SV40TL antigen. Scale bar == 50 μm.

4.5.2 The mesenchymal stromal cell specific marker profile is not affected by immortalization

Immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, lymph nodes, spleen and bone marrow were analyzed for the expression of mesenchymal stromal cell specific markers by flow cytometry. The five mesenchymal stromal cell lines expressed CD105, CD90, CD44 and CD29, but were negative for CD16 and CD11b (Fig 2A, B). The percentages of $CD105^+$, $CD90^+$, $CD44^+$ and $CD29^+$ immortalized mesenchymal stromal cells were 97±3%, 94±4%, 96±3% and 42±9%, respectively, in cells from nasal mucosa; 84±11%, 96±3%, 260 93.8±% and 29±3%, respectively, in cells from lungs; 86±6%, 85±3%, 81±1% and 41±2%, respectively, in cells from spleen; 92±0.5%, 95±0.6%, 91±2% and 34±2%, respectively, in cells from lymph nodes; 70±11%, 82±5%, 70±4% and 34±2%, respectively, in cells from red bone marrow.

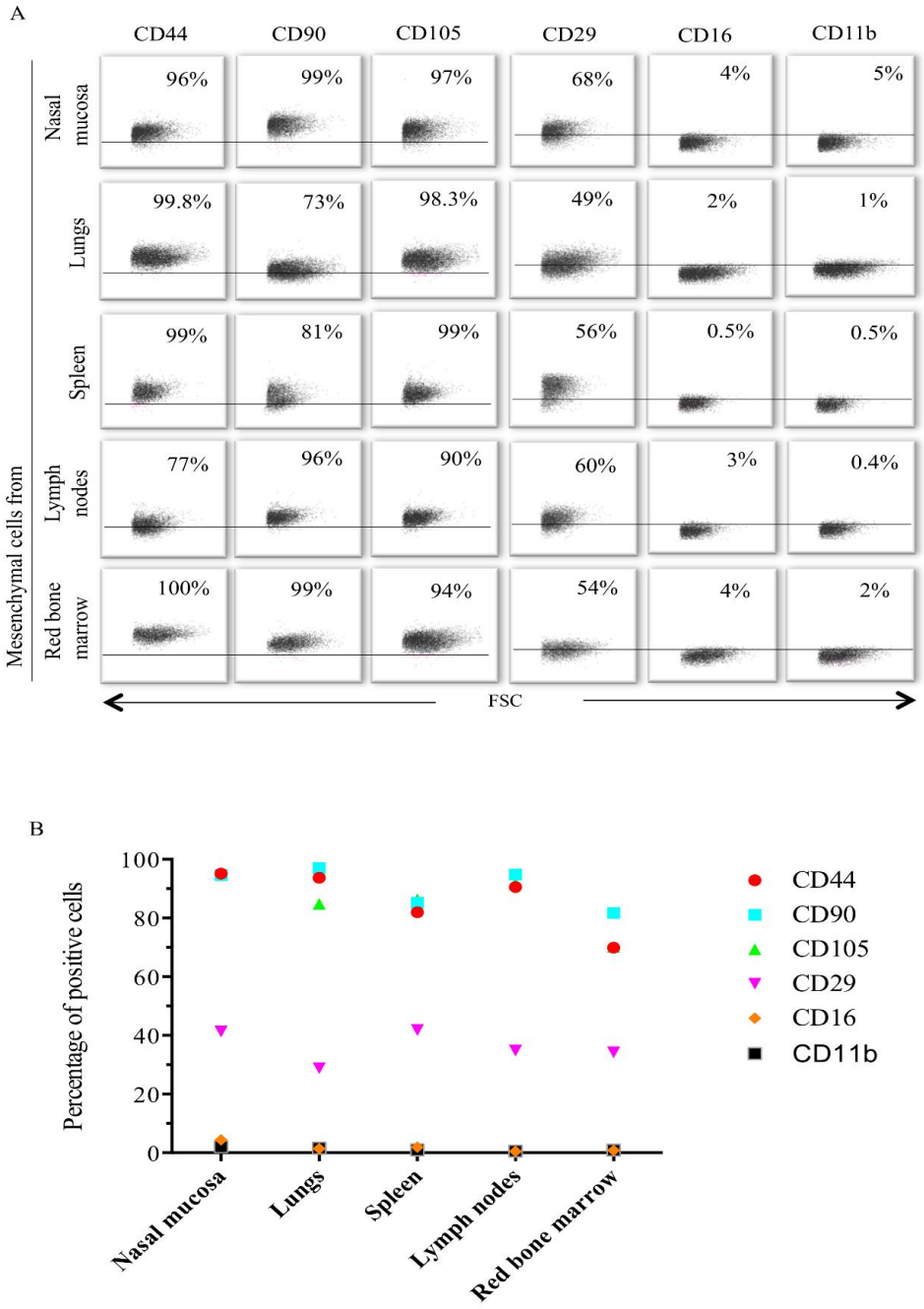

Fig 2. Characterization of immortalized mesenchymal stromal cells by flow cytometry. Immunophenotyping was performed for detection of specific cell markers known to be expressed by mesenchymal stromal cells (CD44, CD105, CD90 and CD29) and non-mesenchymal stromal cell markers (CD16 and CD11b) (A). Three independent experiment (B).

4.5.3 Immortalized mesenchymal stromal cells from different tissues display equal growth curves

The five immortalized mesenchymal stromal cell types showed a similar growth pattern especially at the lag phase of the growth curve (Fig 3).

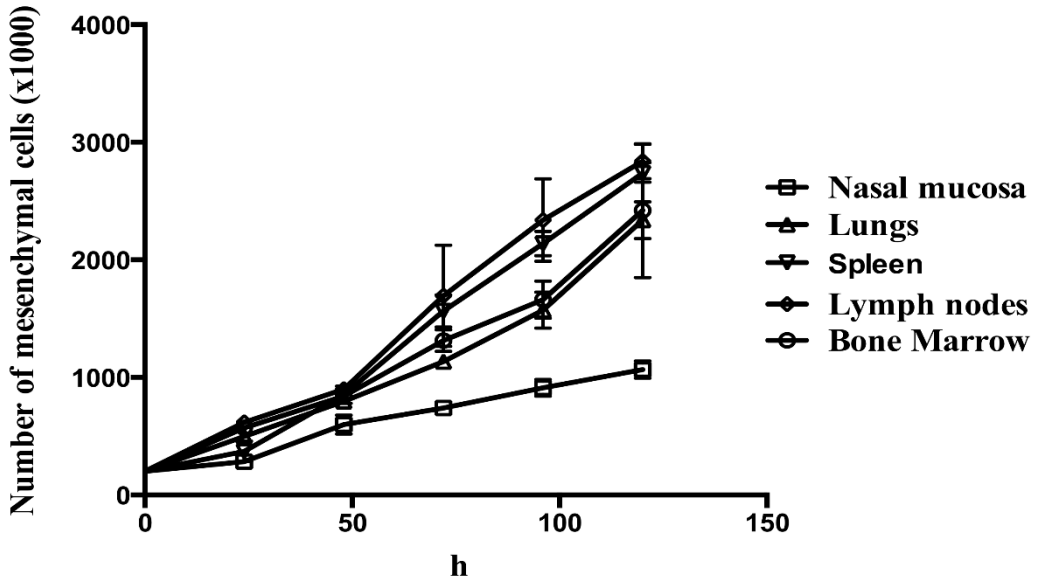

Fig 3. Proliferation analysis of the immortalized mesenchymal stromal cells. Growth pattern of the five immortalized mesenchymal stromal cells. The data are represented as mean +/- SD of three independent experiments.

Five hours after seeding, 90% of the immortalized mesenchymal stromal cells derived from lungs, spleen and lymph nodes attached to the bottom of the culture plates and started to spread. Only 50% of the nasal mucosa mesenchymal stromal cells attached to the plate 5 hours post-seeding. In addition, the mesenchymal stromal cells from the nasal mucosa not only attached slower to the bottom of the culture plate, they also proliferated slower when compared to the other cells.

4.5.4 Replication capabilities of the immortalized mesenchymal stromal cells

Cell cycle analysis was performed to distinguish immortalized mesenchymal stromal cells in different phases of the cell cycle for determining their replicative capabilities. It was observed that the majority of the cells were at the quiescent/preparatory stage (G0/G1 phase) of the cell cycle. More mesenchymal stromal cells were at sub-G1 phase of the cell cycle when originated from nasal

mucosa than from the other tissues. The percentages of nasal mucosa, lung, spleen, lymph node and red bone marrow mesenchymal stromal cells that were at the DNA synthesis stage (S phase) of the cell cycle were 8±0.5%, 7±0.4%, 8±0.6%, 8±0.6%, 4±0.3%, respectively; 6±1%, 8±0.5%, 8±0.1%, 7±0.6%, 8±0.06% were at the DNA duplication and cell division stage (G2/M phase), respectively (Fig 4A, B).

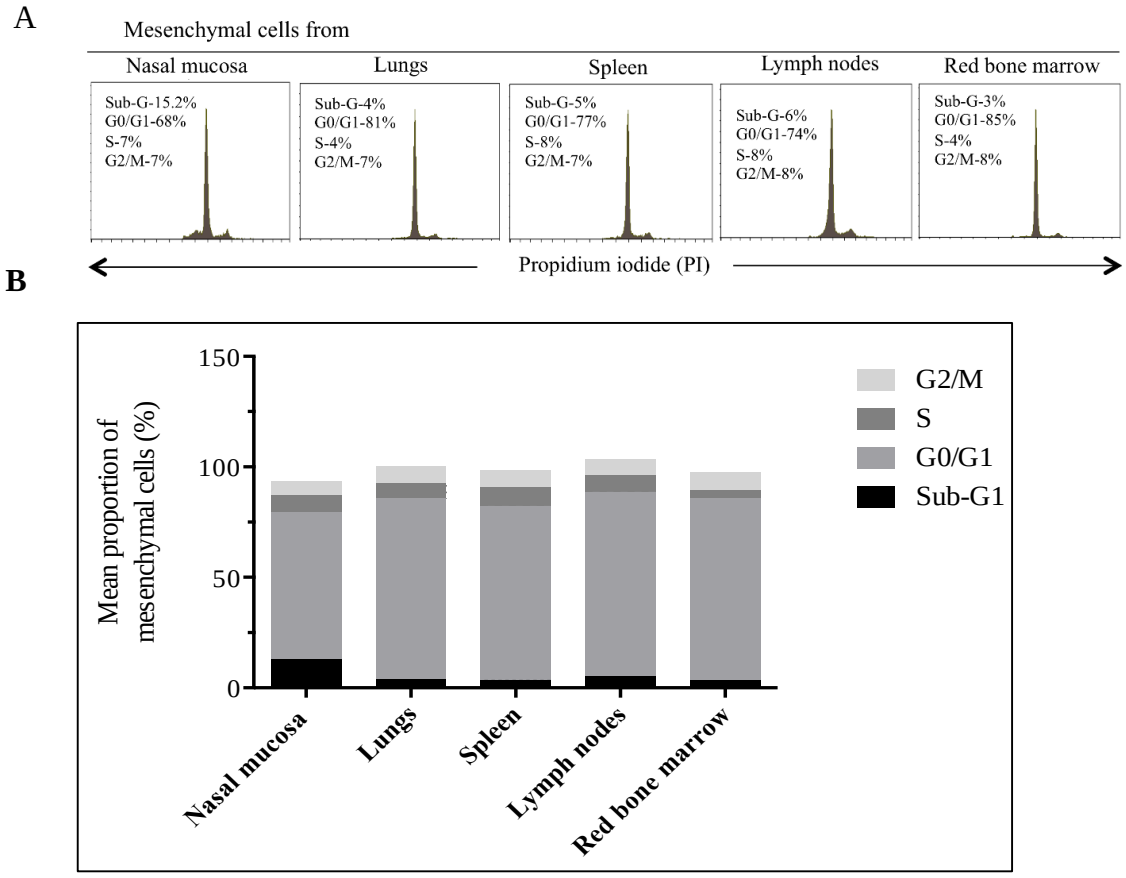

Fig 4. Cell cycle analysis of immortalized mesenchymal stromal cells. Most cells of the five mesenchymal cells were at the G0/G1 phase of cell cycle (A). The data are represented as mean of three independent experiments (B).

4.5.5 Immortalized mesenchymal stromal cells retain the ability to differentiate to osteo-, chondro- and adipocytes

Five days after incubation with osteogenic differentiation medium, the immortalized mesenchymal stromal cells were differentiated into osteoclasts as demonstrated by staining with alizarin red (an

organic compound which binds specifically to calcium ions). Deposits of calcium (red) were observed with the five different mesenchymal stromal cell lines (Fig 5).

100% of the immortalized mesenchymal stromal cells from nasal mucosa, spleen, lymph nodes and red bone marrow and 70-80% of the lung-derived immortalized mesenchymal stromal cells differentiated into osteocytes. After culturing the immortalized mesenchymal stromal cells in adipogenic differentiation medium for 5 days, adipogenesis was demonstrated by staining with oil red O solution. Lipid droplets were observed on all differentiated mesenchymal stromal cells (Fig 5). To confirm the differentiation of mesenchymal stromal cells derived from nasal mucosa, lungs, lymph nodes, spleen and red bone marrow into chondrocytes, immortalized mesenchymal stromal cells were incubated in chondrocyte differentiation medium. After 7 days, the mesenchymal stromal cells showed a dark-blue coloration upon alcian blue staining, indicating that the mesenchymal stromal cells were differentiated into chondrocytes (Fig 5).

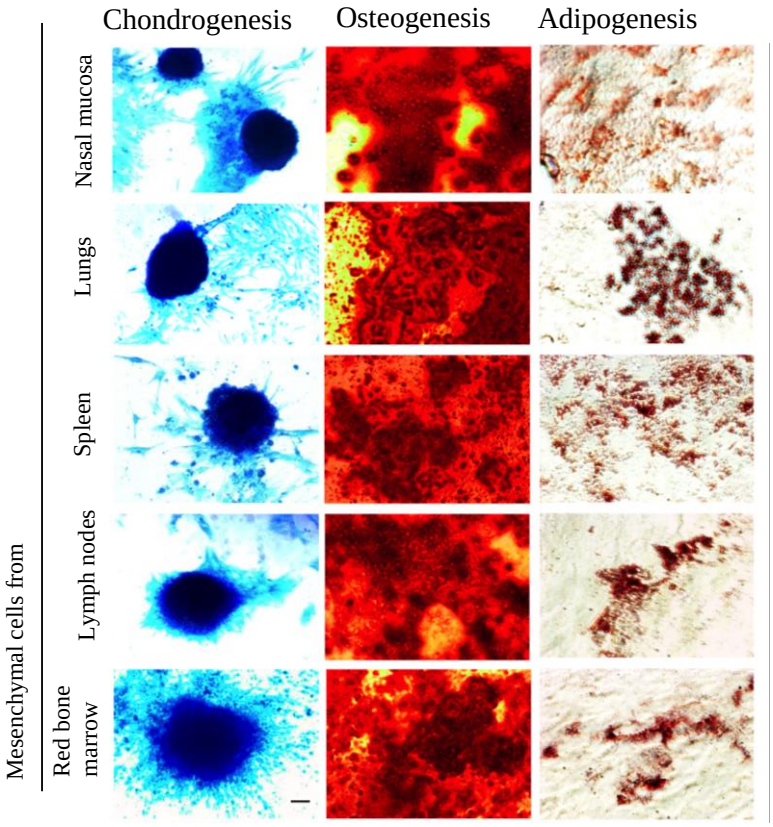

Fig 5. *In vitro* differentiation of immortalized mesenchymal stromal cells. Immortalized mesenchymal cells differentiation into chondrocytes (see dark-blue color-chondrocytes), osteoclast (see red color-calcium deposit), and adipocytes (see lipid droplets in the cells) confirmed by staining with alcian blue, alizarin red and oil red, respectively. Scale bar == 10 μm.

50-60% of the red bone marrow-derived immortalized mesenchymal stromal cells, 70-80% of the nasal mucosa and lymph node-derived immortalized mesenchymal stromal cells and 100% of lung and spleen-derived mesenchymal cells differentiated into chondrocytes after 1 week of culture in chondrogenic differentiation medium.

4.5.6 Viability of immortalized mesenchymal stromal cells cultured in cytokine-free serum

To develop a culture system for accessing the immunomodulatory effect of the mesenchymal cells without interference of cytokines present in the fetal calf serum, immortalized mesenchymal stromal cells were cultured in DMEM supplemented with cytokine-free serum for one week and their viability was determined afterwards. Flow cytometry data analysis indicated 83±1%, 91±8%, 89±12%, 84±2% and 94±3% viable cells in cultures of mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow, respectively, after 7 days of culture (Fig 6).

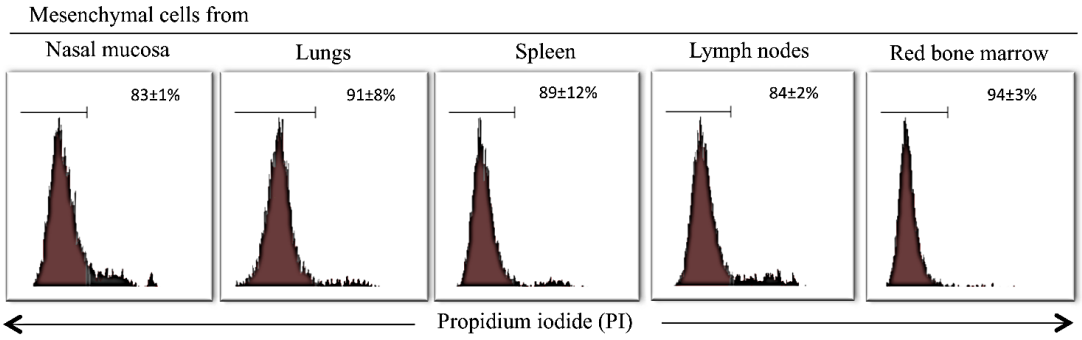

Fig 6. Expansion of immortalized mesenchymal stromal cells in medium supplemented with cytokine-free serum. Viability of immortalized mesenchymal cells derived from nasal mucosa, lungs, spleen, lymph nodes and bone marrow cultured in DMEM supplemented with cytokine-free serum. The data are represent as mean +/- SD of three independent experiments.

4.5.7 Immortalized mesenchymal stromal cells induced the expression of siglec-1 in co-cultured blood monocytes

Nasal mucosa, lung, spleen, lymph node and bone marrow derived immortalized mesenchymal stromal cells were co-cultured with blood monocytes in DMEM supplemented with 10% FCS.

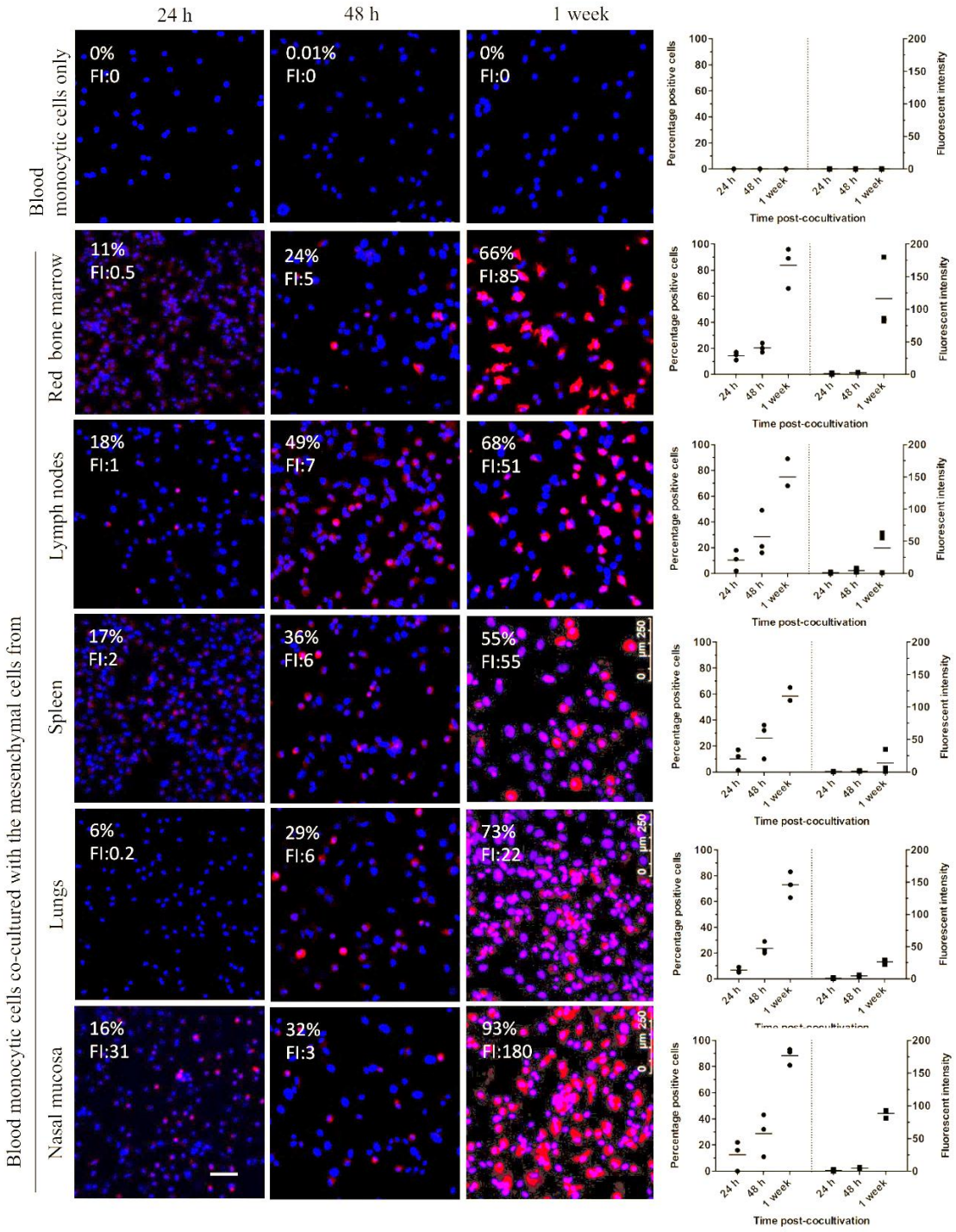

Fig 7. Co-culturing blood monocytic cells with immortalized mesenchymal stromal cells triggers siglec-1 expression. The red staining reveals siglec-1 expression. Percentages in the fluorescent images were for single experiments, while those in the graph were mean percentages of three independent experiments. Each point represents an individual pig (three independent experiments). FI: fluorescent intensity.

An increase in the percentages of siglec-1 positive monocytes and its expression from 24 hours to one week after the start of co-culture was observed: from 16% to 93% (nasal mucosa-derived mesenchymal stromal cells), from 6% to 73% (lung-derived mesenchymal stromal cells), from 17% to 55% (spleen-derived mesenchymal stromal cells), from 18% to 68% (lymph node-derived mesenchymal stromal cells) and from 11% to 66% (red bone marrow derived-mesenchymal stromal cells). Siglec-1 was not expressed in monocytic cells cultured without mesenchymal stromal cells (Fig 7). In contrast to siglec-1, the results indicated some variations in the expression of CD163 especially at 24 h after the start of the co-culture. There was a decrease in the percentages of $CD163^{+}$ blood monocytic cells co-cultured with immortalized spleen, lymph nodes and bone marrow derived-mesenchymal stromal cells when compared with pure blood monocytes (in the absence of mesenchymal stromal cells). Also at 48 h and one week after the start of co-culture a slight decrease in the percentages of $CD163^{+}$ cells was observed when compared with monocytes cultured without mesenchymal stromal cells. Similarly, the fluorescence intensity of $CD163^{+}$ in blood monocytic cells co-cultured with mesenchymal cells decreased when compared with monocytes cultured without mesenchymal cells. Variation among the three individual pigs used in this study was observed (Fig 8).

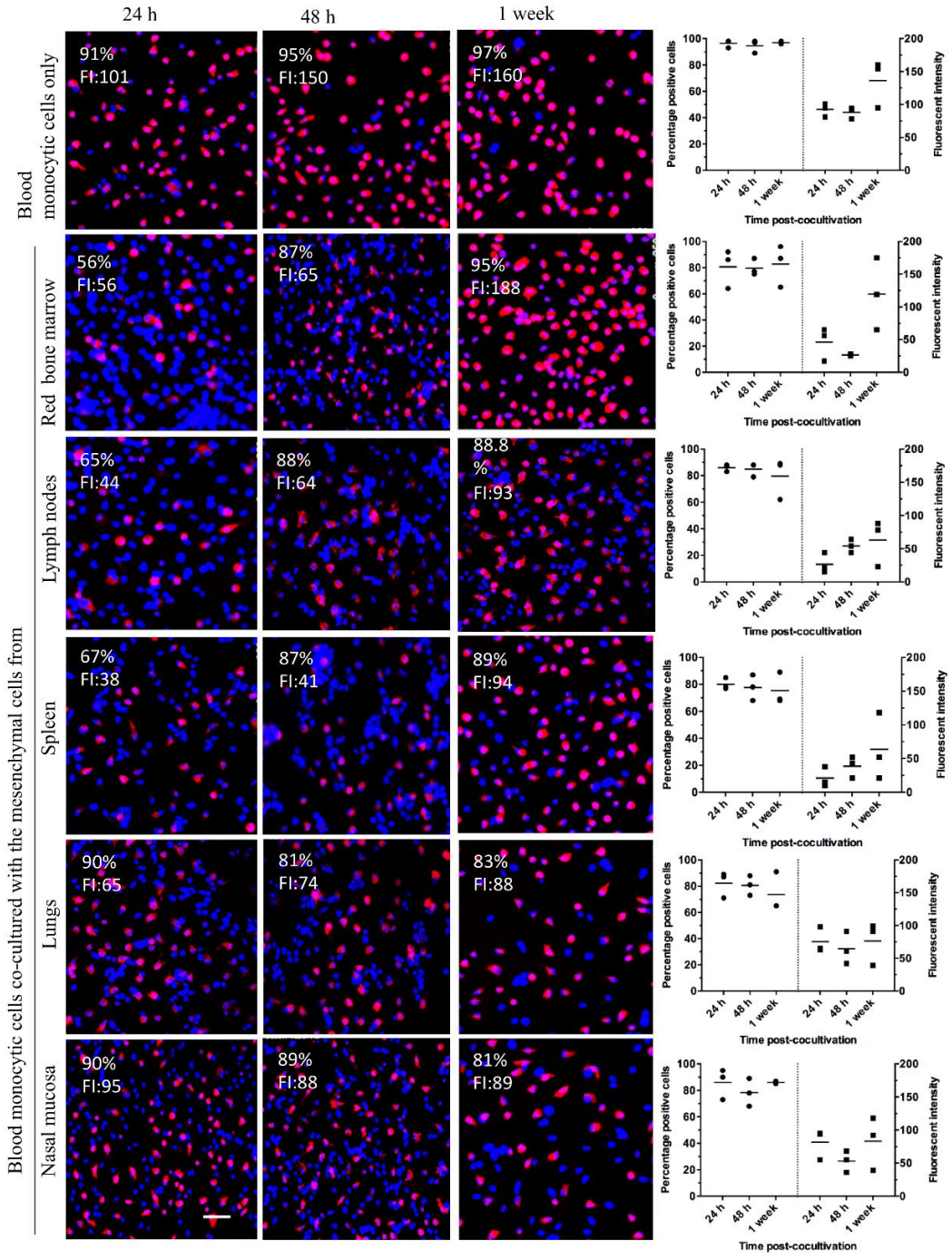

Fig 8. Co-culturing blood monocytic cells with immortalized mesenchymal stromal cells. The red staining reveals CD163 expression. Percentages in the fluorescent images were for single experiments, while those in the graph were mean percentages of three independent experiments. Each point represents an individual pig (three independent experiments). FI: fluorescent intensity.

4.5 Discussion

Mesenchymal stromal cells have already been isolated and characterized from different tissues and organs of humans, mice and other animals (da Silver *et al.,* 2006, DeBari *et al*., 2001; Int´Anker *et al*., 2003; Yang *et al*., 2003). Isolated mesenchymal stromal cells undergo senescence or apoptosis by passaging in *in vitro* cultures. To be able to expand the cells continuously and generate large amounts of mesenchymal cells for long-term research, such as tissue engineering, mesenchymal stromal cells were immortalized using a wide range of methods (Abdallah *et al*., 2006; Lee *et al*., 2013). In this study, mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were successfully isolated, immortalized and characterized. We demonstrated the ability of these cells to trigger the expression of siglec-1 by blood monocytic cells during their co-culture. Results from this work indicated that mesenchymal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow expressed the mesenchymal stromal cell specific markers CD105, CD90 and CD44, but were negative for CD16 and CD11b. Similar results were reported by many research groups for human, mice and porcine mesenchymal stromal cells (Amaral *et al*., 2014, Sung *et al*., 2008; Casado *et al*., 2012). The five immortalized mesenchymal stromal cell lines continued to grow without undergoing senescence after several passages, while the primary mesenchymal stromal cells died after some passages. It was reported that human and guinea pig primary mesenchymal stromal cell growth and proliferation rates decreased gradually after a few passages (Kang *et al*., 2004). A growth curve analysis demonstrated that the five immortalized mesenchymal stromal cell lines had a similar growth pattern, with red bone marrow-derived mesenchymal stromal cells being the strongest grower and nasal mucosa-derived mesenchymal stromal cells being the slowest grower. This was confirmed by cell cycle analysis which showed that a substantial percentage of immortalized nasal mucosa derived mesenchymal stromal cells were in the sub-G phase, which are considered to be dead cells. In this study, the immortalized mesenchymal stromal cells could be differentiated into osteocytes, chondrocytes and adipocytes (Darimont *et al*., 2003, Hung *et al*., 2010, Okomoto *et al*., 2002). Interestingly, all five mesenchymal stromal cell types required only 4 days to differentiate into osteocytes and adipocytes and 7 days to differentiate into chondrocytes after initial culture in osteogenic, adipogenic and chondrogenic differentiation medium, respectively. This indicates that mesenchymal stromal cell

cultures established in this work need only a short time to differentiate into osteoclasts, adipocytes and chondrocytes. Indeed, many reports mentioned that mesenchymal stromal cells of humans and mice origin need two to three weeks to differentiate into osteocytes, adipocytes and chondrocytes after initial exposure to specific differentiation medium (Khatri *et al.,* 2015).

Siglec-1, also called sialoadhesin or CD169, is a member of the sialic acid binding immunoglobulin-like lectin family found to be expressed on differentiated tissue macrophages in lungs, liver, colon, spleen, lymph nodes and bone marrow (Vanderheijden *et al*., 2003; Hua *et al*., 1998). Siglec-1 has been reported to function in cell-cell interactions and in receptor-dependent internalization processes such as the internalization of porcine reproductive and respiratory syndrome virus (PRRSV) (Tse *et al*., 1998). It is not expressed on porcine monocytes.

The majority of research groups that work with human, mice and other animal models mesenchymal stromal cells use T-lymphocytes, B-lymphocytes, NK cells and dendritic cells to assess the immunomodulatory effect of mesenchymal cells with little emphasis given to cells of the monocyte/macrophage lineage (Salmani *et al*., 2008; English *et al*., 2008). Previous findings indicated functional interactions between macrophages and mesenchymal stromal cells leading to phenotypic changes in the macrophages (Anton *et al*., 2012). In the present study, the effect of mesenchymal cells on the differentiation of blood monocytes was examined. SV40LT immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow triggered the expression of the macrophage differentiation marker siglec-1 in co-cultured blood monocytic cells. A higher percentage of siglec-1^{+} monocytic cells was observed when co-cultured with immortalized mesenchymal stromal cells derived from the nasal mucosa.

Conclusions

In conclusion, SV40LT immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were successfully established and differentiated into osteocytes, chondrocytes and adipocytes. Moreover, they were able to activate the expression of siglec-1 on blood monocytes. This method can now be used to establish continuous mesenchymal stem cell lines from different tissues for long-term research such as tissue engineering. It can also

be used to generate large amounts of siglec-1^+ macrophages in a few days in a simple and affordable culture system for use in laboratory and clinical settings.

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Chapter 5

Immortalized mesenchymal stromal cells increase the susceptibility of blood monocytes to PRRSV infection

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Manuscript in preparation

5.1 Abstract

Mesenchymal stromal cells are multipotent, non-hematopoietic cells found in the stroma of bone marrow and in many other tissues. They are capable of differentiating into many cell types including osteoblasts, chondrocytes and adipocytes. Porcine reproductive and respiratory syndrome virus has a restricted tropism for cells of the monocytic lineage. Siglec-1 (sialoadhesin) is a crucial receptor involved in attachment to and internalization of porcine reproductive and respiratory syndrome virus (PRRSV) into macrophages. In the previous chapter we showed that co-culturing blood monocytes with immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow triggers the expression of siglec-1 in blood monocytes. The present work aimed to examine if the latter mesenchymal stromal cells increase the susceptibility of co-cultured blood monocytes for PRRSV infection. Immortalized mesenchymal stromal cells, inoculated with PRRSV (LV (subtype 1) and Lena (subtype 3)), were found to be resistant to PRRSV. When blood monocytes co-cultured with immortalized mesenchymal stromal cells were inoculated with PRRSV (LV (subtype 1) and Lena (subtype 3)) and analyzed by confocal microscopy at 12 hours post-inoculation, up to 7±1%, 18±0.9% and 86±1% of LV-inoculated cells and 33±1%, 45±3% and 88±2% of Lena-inoculated cells were found to be infected at 24 h, 48 h and 72 h of co-cultivation, respectively. In contrast, only 0.8±2-2±1.5% LV-inoculated cells and 1±0.5-3±1% Lena-inoculated cells were observed to be infected in monocyte cultures at 24, 48 and 72 h cultivation and 12 hpi (hours post-inoculation). In conclusion, immortalized mesenchymal stromal cells isolated from nasal mucosa, lungs, spleen, lymph nodes and bone marrow were able to enhance the replication of PRRSV in co-cultured blood monocytes isolated from PBMC.

5.2 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important viral diseases that causes huge economic losses to the pig industry worldwide (Snijder et al., 1998). PRRSV causes reproductive disorders such as late gestation and stillbirth in sows and changes in sperm quality in boars. Porcine reproductive and respiratory syndrome virus (PRRSV) was first reported in America and later in Europe where the infectious agent causing the syndrome was isolated. Two major viral types with strong genetic and antigenic differences were identified: type 1 (European; prototype Lelystad-LV) and type 2 (American; prototype VR2332) (Mardassi *et al.,* 1994). The target cells for PRRSV *in vivo* are differentiated macrophages and porcine primary alveolar macrophages are used to propagate the virus *in vitro*. Sialoadhesin or siglec-1 is expressed only on macrophages and plays an important role in the attachment and internalization of PRRSV into the host cells. Mesenchymal stem cells are multipotent cells capable of differentiating into many cell types, which include osteoblasts, chondrocytes and adipocytes (Pittenger *et al*., 1999; Gerson *et al.,* 1999). They were first identified in red bone marrow cell cultures; later their isolation from various sources was reported. Pre-clinical studies using animal models indicated the therapeutic potential of mesenchymal cells in treating several pathologies such as cardio-vascular diseases, diabetes and autoimmune diseases, as well as in regenerative medicine (Singla *et al*., 2010; Lin *et al.,* 2009; Gonzalez-Rey *et al.,* 2010; Trottier *et al.,* 2008). Mesenchymal stromal cell immunomodulatory properties include modulating different immune cells by secreting anti-inflammatory factors such as TGF-β (Di Nicola *et al.,* 2002) which can inhibit T-cell proliferation. Mesenchymal stromal cells have also been reported to regulate the differentiation and function of dendritic cells (Nauta *et al.,* 2006). Furthermore, it has been observed that co-culturing macrophages with mesenchymal stromal cells increases their IL-10 expression and decreases their TNF-α and IL-12 expression (Kim *et al.,* 2009). Human mesenchymal cells enhance the replication of cytomegalovirus and H1N1 influenza virus by inhibiting the secretion of IFN-γ and proliferation of T-cells responsible for the control of these viruses in *in vitro* studies.

There is a general need for long-term mesenchymal stromal cell cultures for *in vitro* research, which is hindered by the short life span of primary cells in *in vitro* cultures (Wagner *et al.,* 2008; Bork et *al.,* 2010; Wagner *et al.,* 2012). To overcome this limitation, the cells have to be immortalized

(Stewart *et al.*, 2002; 2006). The widely used method to immortalize primary cells is the introduction of viral genes, such as the gene of simian virus 40 encoding large T antigen or the gene encoding a telomerase (Jha *et al.*, 1998; Kirchhoff *et al.*, 2004).
In the present study, we investigated the effect of immortalized mesenchymal stromal cells from porcine nasal mucosa, lungs, spleen, lymph nodes and bone marrow on the susceptibility of blood monocytes to PRRSV.

5.3 Material and methods

5.3.1 Cell isolation and characterization

Isolation, immortalization and characterization of mesenchymal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were fully described in a previous chapters (Garba *et al*., 2017a and Garba *et al*., 2017b). Also the isolation and co-cultures of blood monocytes with the immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were performed as described in chapter 4.

5.3.2 Virus

The PRRSV strains used in this study include the 13^{th} passage of the European subtype 1 Lelystad virus which is a kind gift of G. Wensvoort (Institute of Animal Science and Health, the Netherlands) and the 3^{rd} passage of the European subtype 3 (Lena). Both the Lelystad and Lena strains were propagated on porcine alveolar macrophages (PAM). Supernatant from the cell cultures was harvested and centrifuged at 13 000 x g for 10 min. and filtered through a 0.45 µm cell strainer to remove cellular debris. The virus was diluted in serum-free medium to a final titer of $10^{5.5}$ tissue culture infectious dose with 50% end point ($TCID_{50}$)/mL. Subsequently, virus was stored at -70°C until use.

5.3.3 Virus inoculation of immortalized mesenchymal stromal cells

Before proceeding with the inoculation of co-cultures, immortalized mesenchymal stromal cells from all the five tissues were inoculated with 250 µl of PRRSV strain at a titer of $10^{5.5}$ $TCID_{50}$ /mL for 1 h at 37°C 5% CO_2. Next, the virus suspension was removed from the cells and fresh medium was added to the cells and further incubated. Samples were collected at 24, 48 and 72 hpi.

5.3.4 Virus inoculation of immortalized mesenchymal stromal cell-blood monocyte co-cultures

Twenty-four, 48 and 72 h after the initial co-culture of mesenchymal stromal cells with blood monocytes, the cells were inoculated with 250 µl of PRRSV strain at a titer of $10^{5.5}$ $TCID_{50}$ /mL for 1 h at 37°C 5% CO_2. Next, the virus suspension was removed from the cells and fresh medium was added to the cells and further incubated. Sample collections were performed at 12 hpi. Monocytes that were not co-cultured were inoculated in the same way at 24, 48 and 72 h of culture and collected 12 h later.

5.3.5 Immunofluorescence staining and confocal microscopy

To identify and quantify different PRRSV infected cells in nasal mucosa, lungs, spleen, lymph nodes and red bone marrow derived immortalized mesenchymal stromal cell-blood monocyte co-cultures, a double immunofluorescence staining was performed. The co-cultures were washed two times with PBS and fixed with 4% paraformaldehyde at room temperature followed by permeabilization with Triton X-100 for 2 min at room temperature. After two times washing, cells were incubated with monoclonal antibodies against porcine sialoadhesin (13E2, IgG2a, 1:200) and mAbs against N-protein of PRRSV (41D3, IgG1, 1:5) for 1 h at 37°C. Subsequently, the cells were washed two times with PBS and further incubated with secondary antibodies Alexa Fluor 488-IgG2a and Alexa Fluor 594-IgG1 for 1 h at 37°C. Nuclei of the cells were stained with Hoechst 33342. Subsequently, the cells were washed two times with PBS. To demonstrate the specificity of the primary antibodies used, isotype-matched control monoclonal antibodies against gD of PRV (IgG1, 13D12) and 1C11 against gB of PrV (IgG2a) were used (Nauwynck *et al.*, 1995). The cells were rinsed with PBS and mounted with glycerin/PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo [2.2.2] octane. Sn^{+}

and PRRSV-positive cells were quantified by counting a total of 500 cells per slide using confocal microscopy (Leica Microsystem DMRBE, Wetzlar, Germany).

5.4 Statistical analysis

Two-way Anova was used to calculate the statistical significance ($P<0.05$) of the difference between the number of Lena- and LV-infected cells in blood monocytes co-cultured with mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and bone marrow and in monocytes without mesenchymal stromal cells. P values equal to or lower than 0.05 were regarded as statistically significant.

5.5 Results

Two hours after the addition of the immortalized mesenchymal stromal cells to the monocytic cultures, they started to attach to the bottom of the culture plates and by 24 h post-seeding all the cells were already attached and spread, forming a semi-monolayer while the monocytic cells remained firmly attached to the culture plate.

To confirm the non-permissiveness of mesenchymal cells to PRRSV, the cells were inoculated with the virus using the same titers used to inoculate the co-cultures. Immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph node and red bone marrow were not infected with PRRSV.

The presence of PRRSV infected cells in monocytes inoculated with PRRSV at 0, 24, 48 and 72 post-seeding and at 12 hours post inoculation was determined by immunofluorescence stainings and confocal microscopy. At 0 hours post inoculation, Lena or LV-infected cells were not detected in blood monocytes co-cultured with immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow and in monocytes.

At 24, 48 and 72 h after the co-culture of blood monocytes with mesenchymal stromal cells of different origin and 12 h post LV inoculation, 15-25%, 40-50%, 88-92% of the blood monocytes was Sn^+ and 5-7%, 30-33%, 72-84% of monocytes was $PRRSV^+$, 75-80%, 88-90, 91-98% of the Sn^+ cells was $PRRSV^+$ and 1-5%, 0.6-3%, 0.3-1.2% of the Sn^- cells were $PRRSV^+$. In LV-inoculated monocytes cultured without mesenchymal cells, viral infected cells were not detected at 0 hpi. At 24, 48 and 72 h of cultivation and 12 hpi with LV, 0.8%, 1.5% and 3% respectively, were observed to be $PRRSV^+$ and all of them were found to be Sn^- (Fig 1).

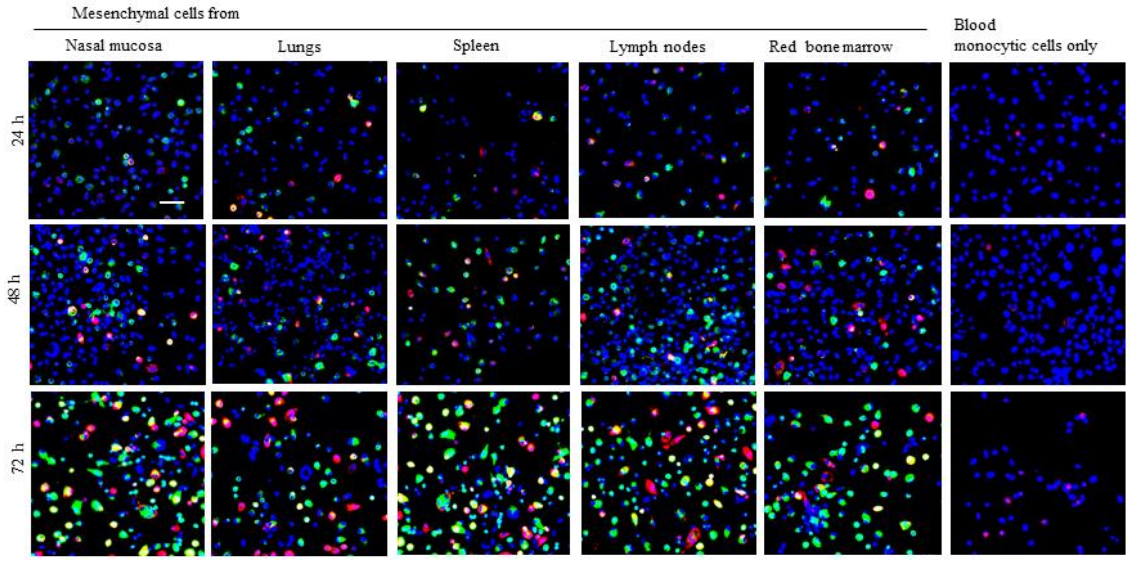
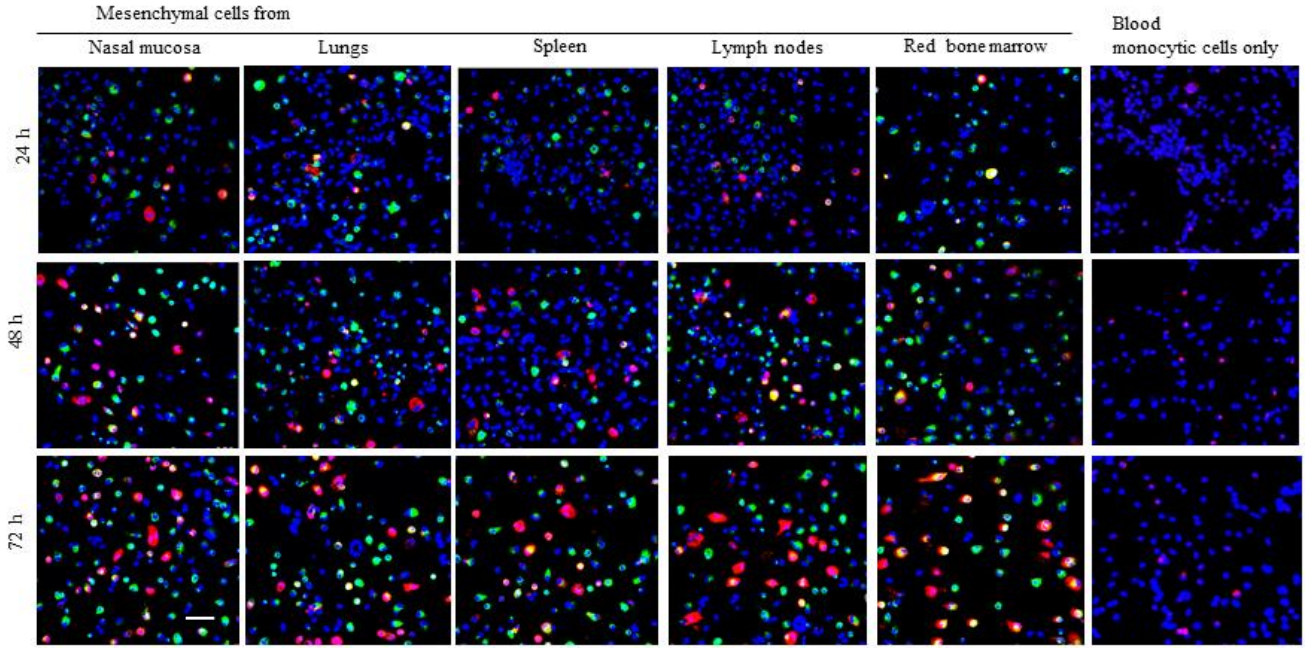


Fig 1. LV infection of pure blood monocytes or blood monocytes co-cultured with immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and bone marrow. The co-cultures were stained for PRRSV N-protein (red) and Sn (green) to quantify and identify ($Sn^{+/-}$) PRRSV infected blood monocytes. Nuclei were counterstained with Hoechst. Scale bar = 10 µm. The $Sn^{+/-}$ PRRSV infected/non-infected blood monocytes were determined by counting a total of 500 cells per slide using fluorescence microscopy. The data are represented as mean of three independent experiments.

At 24, 48 and 72 h after the co-culture of blood monocytes with mesenchymal stromal cells of different origin and 12 h post Lena inoculation, 16-27%, 39-54%, 77-88% of the blood monocytes was Sn^+ and 10-18%, 29-45% 73-88% of the monocytes was $PRRSV^+$; 92-95%, 88-90, 93-99% of the Sn^+ cells was $PRRSV^+$ and 1-3%, 0.6-1%, 0.0-1-0.2% of the Sn^- cells was $PRRSV^+$. In Lena-inoculated monocytes cultured without mesenchymal stromal cells, viral infected cells were not detected at 0 hpi. At 24, 48 and 72 h of cultivation and 12 hpi with Lena, 2%, 2.9% and 6% respectively, were $PRRSV^+$; and all were Sn^- (Fig 2). Based on the work we performed with Lena and LV strain of PRRSV in the present thesis, we observed that Lena strain was more virulent than LV strain. More cells were observed to be floating in cultures infected with Lena than with LV, maybe that was the reason why more Sn^+-infected cells were observed in co-cultures infected with LV compared with co-cultures infected with Lena.



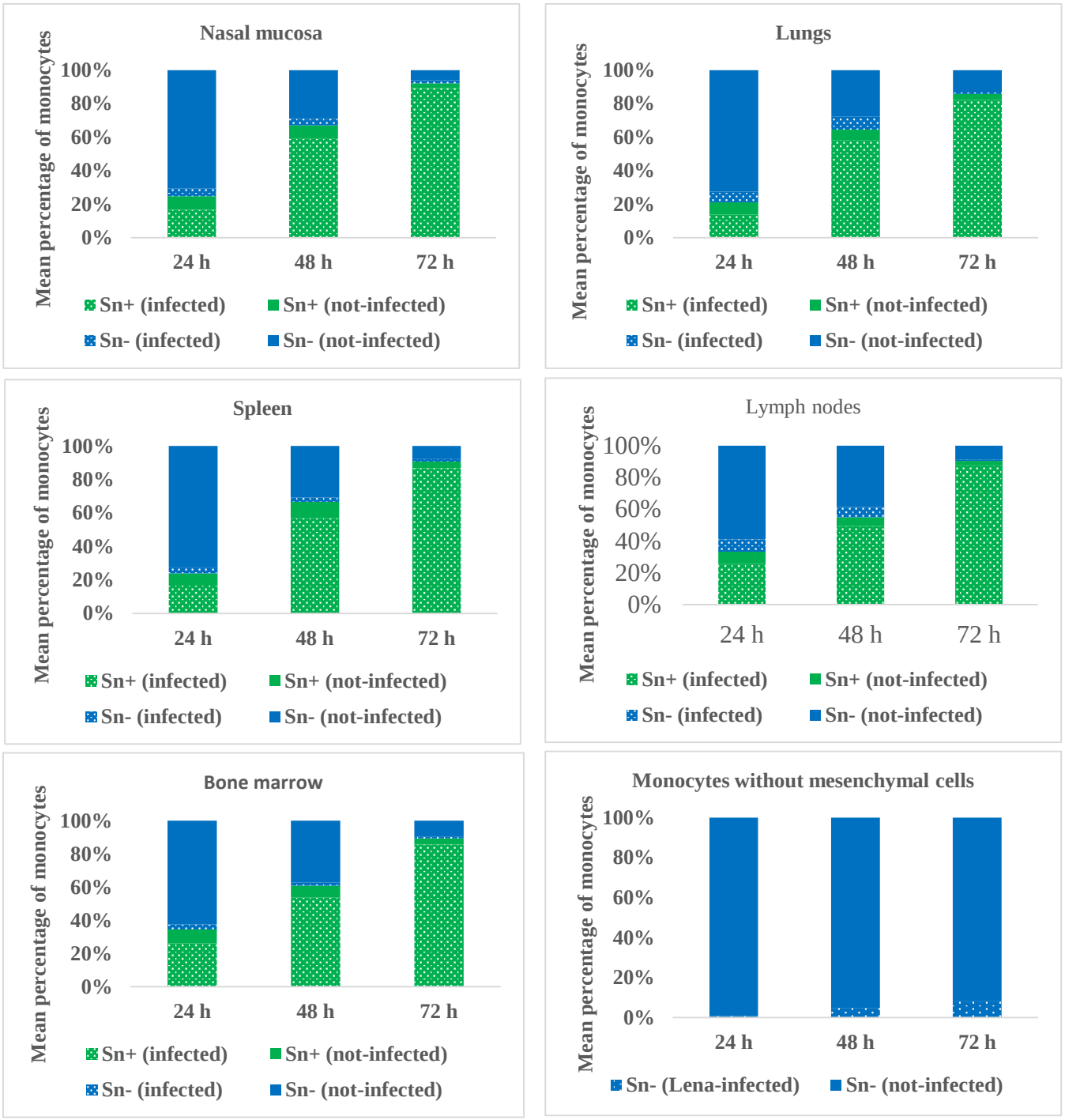


Fig 2. Lena infection of pure blood monocytes and blood monocytes co-cultured with immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and bone marrow. The co-cultures were stained for PRRSV N-protein (red) and Sn (green) to quantify and identify ($Sn^{+/-}$) PRRSV infected blood monocytes. Nuclei were counterstained with Hoechst. Scale bar = 10 µm. The $Sn^{+/-}$ PRRSV infected/non-infected blood monocytes were determined by counting a total of 500 cells per slide using fluorescence microscopy. The data are represented as mean of three independent experiments.

5.6 Discussion

Both *in vivo* and *in vitro*, PRRSV has a very narrow tropism for cells of the monocyte/macrophage lineage. The cellular tropism of this virus depends on the presence or absence of certain important receptors such as siglec-1, siglec-10 and CD163 (Calvert et al 2007; Delputte et al., 2005; Xie *et al*., 2017). PRRSV infects specific differentiated macrophages located in the tonsils, lungs, spleen, lymph nodes, liver, thymus, endometrium and placenta. Many researchers indicated that siglec-1 is an important cellular receptor used by PRRSV for attachment and entry into macrophages (Calvert et al 2007; Delputte et al., 2005). Siglec-10 has recently been identified as an alternative for certain strains. Induction of siglec-1 expression in blood monocytes co-cultured with immortalized mesenchymal stromal cells derived nasal mucosa, lungs, spleen, lymph nodes and red bone marrow was demonstrated in the previous chapter (Garba et al., 2017b), illustrating the ability of mesenchymal cells to modulate and trigger the differentiation of immune cells. In the present work, we demonstrated that porcine immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow strongly increased the susceptibility of blood monocytes to PRRSV. When fresh blood monocytes are inoculated with PRRSV only a low percentage (<0.5%) becomes infected (Delputte *et al*., 2007). Upon culturing of blood monocytes for a few days this percentage slightly increased but remained below 6% (present study). In the present study, the percentage of $PRRSV^+$ blood monocytes was much higher (>73%) when they were co-cultured with immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow. The PRRSV infection in siglec-1 positive monocytes (>75%) was much higher than in siglec-1 negative monocytes (<5%), demonstrating that the PRRSV susceptibility coincides with the siglec-1 expression. Although statistically no significant differences in the percentages of LV and Lena were found, it was observed that Lena infected more monocytes at 24 h and 48 h of co-culture with immortalized mesenchymal stromal cells which may be explained by the higher virulence of this strain compared to LV (Karniychuk *et al*., 2010). Some authors also found differences in replication rates of Lena compared to LV in polarized nasal mucosa explants (Frydas *et al*., 2013). The presence of few Sn^- PRRSV-infected cells in the co-cultures at 24 h, 48 h and 72 h after the initial co-culture and 12 hpi is indicative for the use of a sialic acid binding receptor other than siglec-1, such as siglec-10 for the viral entry (Xie *et al*., 2017). The latter receptor was found to be expressed on a small proportion of monocytes. More work should be

performed on the intriguing world of sialic acid binding proteins and their role in PRRSV infection. In conclusion, immortalized mesenchymal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow enhance the susceptibility of co-cultured monocytes for a PRRSV infection.

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Chapter 6

General discussion

6.1 General discussion

For a long time, adult bone marrow has been described by many research groups as an important source of mesenchymal stromal cells (Friedenstein *et al.,* 1968; Haynesworth *et al*., 1992; Pittenger *et al.,* 1999). Bone marrow mesenchymal stromal cells have been reported to provide mechanical and physiological support and to create a hematopoietic microenvironment (Caplan *et al.,* 1991). Mesenchymal stromal cells have also been found to reside in many other tissues (Wagner *el al.,* 2005; Covas *et al.,* 2008; da silva *et al.,* 2006; Lennon *et al.,* 2006). They are fibroblast-like cells with self-renewing capacity and ability to differentiate into bone, cartilage and fat cells (Caplan *et al.,* 1991). Mesenchymal stromal cells possess an immunomodulatory effect by interacting with a plethora of immune cells leading to phenotypical changes and functional responses (Le Blank *et al.,* 2003). More recently, they are considered to have a therapeutic potential, especially in the treatment of graft-versus-host diseases (Horwitz *et al.,* 2008; Caplan *et al.,* 2009; Jones *et al.,* 2008; Javazone *et al.,* 2004; Le Blanc *et al.,* 2005).

Porcine reproduction and respiratory syndrome virus is described as a rapidly evolving virus with an enormous economic importance for the swine industry all over the world (Dea *et al.,* 1992a; Murtaugh *et al.,* 2010). Genetically, two genotypes of porcine reproductive and respiratory syndrome virus have been identified: type I and type II (Mardassi *et al.,* 1994). PRRSV may cause severe diseases which are characterized by abortion in sows, still- and weak born piglets and is associated with the respiratory disease complex in young piglets (Hill *et al.,* 1990). The virus has a specific tropism for certain subsets of differentiated macrophages in different tissues. The subsets that are targeted depend on the PRRSV variant and is determined by the cellular binding receptors which belong to the siglec superfamily. Each siglec is expressed on a certain macrophage subset. The surrounding mesenchymal stromal cells are believed to be the driving force behind the siglec expression.

Differences in the immunomodulatory effect between mesenchymal stromal cells from different tissues have been recorded (Park *et al.,* 2009; Kyurkchiev *et al.,* 2013; Elman *et al*., 2014). Research groups working with human and murine mesenchymal stromal cells have a restricted access to tissues for isolating mesenchymal cells from different organs or tissues for comparing their differentiation or immunomodulatory capabilities. Either the animal is too small or there are ethical

restrictions to obtain large amounts of tissues from mice and human, respectively. Establishing mesenchymal cell lines from tissues of pigs, an animal closer to man both anatomically and physiologically than mice, will contribute to a remarkable achievement in obtaining a better understanding in the potential of tissue-specific therapeutic applications of mesenchymal stromal cells (Kobayashi *et al*., 2012). Differences in growth rates and induction of monocyte differentiation between immortalized mesenchymal stromal cells isolated from nasal mucosa, lungs, spleen, lymph nodes and bone marrow may help in understanding the immunomodulatory properties of mesenchymal stromal cells in specific tissues and therefore assist in choosing the best mesenchymal cells for studying diseases and malfunctions with regard to a particular tissue. Mesenchymal stromal cells have self-renewal capabilities but no telomerase activities (Kolquist el al 1998). Continuous passaging of mesenchymal stromal cells in *in vitro* cultures leads to accumulation of reactive oxygen species and other cytotoxic agents and subsequent DNA damage and telomere shortening. The latter in turn triggers aging (senescence) and apoptosis of the cells. For long-term *in vitro* experiments with mesenchymal cells such as for tissue engineering, immune modulation and infection experiments, large amounts of mesenchymal cells are needed. For the generation of macrophages by co-culturing with mesenchymal cells for studying PRRSV replication kinetics, mesenchymal cells isolated from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow were successfully immortalized by transducing the cells with the gene encoding the SV40LT antigen. As the presence of non-mesenchymal cells such as epithelial, endothelial and mononuclear cells in mesenchymal stromal cell cultures may interfere with the analysis of the obtained results, they should be absent in the mesenchymal stromal cell cultures. Contamination of mesenchymal cells with epithelial, endothelial cells and mononuclear cells was absent in the mesenchymal stromal cell lines generated in this PhD thesis. For decades, human mesenchymal stromal cells have been used by many researchers to maintain the proliferation of viable monoblasts without inducing their differentiation in long-term *in vitro* co-cultures (Ito *et al.,* 2014). For example, MS5 and HS27 stromal cell lines have been used to maintain viable monoblasts in stromal co-cultures (Klco *et al.,* 2013; van Gosliga *et al.,* 2007). Adding a growth factor, such as IL-3, in combination with stromal cells led to the differentiation of the blast cells during the long-term culture (Ito *et al.,* 2014). This showed that the use of cytokines alone or in combination with mesenchymal cell-progenitor cell co-cultures can promote their differentiation and change their phenotypic properties. Cell-cell contact is crucial in keeping the viability and phenotypic properties of monoblasts in long-term co-cultures with

mesenchymal cells (Ito *et al.,* 2014). Many research groups obtained monoblasts, macrophages and dendritic cells via cytokine stimulation during *in vitro* cultures of monocytes. For example, culturing bone marrow cells isolated from pig sternum in growth medium supplemented with recombinant porcine GM-CSF alone or in combination with recombinant porcine rpTNF-α for 8 days induces the differentiation of these cells to dendritic cells (DC) (Carrasco *et al.,* 2001). Generation of bone marrow derived macrophages from bone marrow cells was also successful in the presence of L929-conditioned medium (Hou *et al.,* 2012), rpGM-CSF and rpIL-4 (Chang *et al.,* 2008) and GM-CSF (Fraile *et al.,* 2012). Cells having similar morphological and functional characteristics as macrophages can be generated by culturing pig bone marrow cells in medium supplemented with horse serum and L929-cell-conditioned medium (Hou *et al.,* 2012). In the present thesis, we were able to maintain hematopoietic cells and differentiate them towards macrophages and $CD4^+$ and $CD8^+$ cells using only mesenchymal stromal cells without cytokines. In agreement with previous studies performed on bone marrow and peripheral blood mononuclear cells (PBMC) cultured in the presence of recombinant human colony-stimulating factor-1 (rhCSF1), our results showed an increase in size (forward scatter) and granularity (side scatter) of hematopoietic cells co-cultured with immortalized mesenchymal stromal cells. Monocyte/macrophage markers CD14, CD172a, CD163 and CD169 and lymphocyte markers CD4 and CD8 were used to analyze the hematopoietic cells during their differentiation. This technology was later used to generate siglec-1 expressing macrophages from blood monocytes for studying the PRRSV kinetics and replication by the use of co-cultures of monocytes with immortalized mesenchymal stromal cells as driving force. Although many cell lines, such as MARC-145 derived from African green monkey (Kim *et al.,* 1993) and porcine kidney epithelial cell (PK-15) expressing the binding and internalization receptors (siglec-1 or siglec-10) together with the disassembly mediator CD163 (Delrue *et al.,* 2010, Xie *et al*., 2017), have been used for propagating PRRSV (Lee et al 2010; Weingartl *et al*., 2002; Kapetanovic *et al.,* 2012). In this context, it is important to find a suitable relevant *in vitro* culture system for generating macrophages to study host-pathogen interactions in order to unravel the many enigmas in the PRRSV pathogenesis. Immortalized mesenchymal stromal cells derived from primary mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph nodes and bone marrow were co-cultured with blood-derived monocytes for one week in a simple culture system containing DMEM supplemented with fetal calf serum without the addition of cytokines. The blood monocytes strongly expressed siglec-1 within two to three days. The ability to use immortalized mesenchymal

stromal cells to generate siglec-1 expressing macrophages from siglec-1 negative blood monocytes is one of the most interesting findings of this work. Siglec-1 becomes expressed in differentiated macrophages which has been suggested to function in regulating immune responses and in receptor mediated binding and internalization of PRRSV (Oetke *et al.*, 2006; van den Berg *et al.*, 1992). Siglec-1 expression in differentiated macrophages residing in tissues such as nasal mucosa, lungs, lymph nodes, spleen and bone marrow and our current findings on induction of this marker on blood monocytes by mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and bone marrow indicates that mesenchymal stromal cells may be involved either directly or indirectly in the pathogenesis of PRRSV *in vivo*. In the present thesis, a high percentage of siglec-1 was induced on hematopoietic cells by immortalized bone marrow-derived mesenchymal stromal cells three weeks after the initial co-culture and on blood-derived monocytes co-cultured with immortalized mesenchymal stromal cells from nasal mucosa, lungs, spleen, lymph node and red bone marrow. In contrast to high percentages of siglec-1 positive cells obtained during our co-cultures, cytokines such as type I or type II IFN, which has been used by many other research groups, induced an expression of siglec-1 in a low number of peripheral mononuclear cells and different subsets of macrophages isolated from humans (York *et al.*, 2007; Rampel *et al.*, 2008), pigs (Delputte *et al.*, 2007) and mice (Chen *et al.*, 2012). The strong immunomodulatory effects of mesenchymal stromal cells and their ability to interact with immune cells may play a role in increasing the susceptibility of co-cultured blood monocytes to PRRSV infection.

The Sn^- PRRSV-infected monocytes in co-cultures and in monocytes cultured without mesenchymal stromal cells may be indicative for the presence of minor subsets of monocytes expressing a marker involved in PRRSV attachment and internalization other than siglec-1. Porcine siglec-10 (Xie *et al.*, 2017) has been reported to be expressed on a minor subset of monocytes and PRRSV may use this siglec in co-cultures and in monocytes without mesenchymal stromal cells cultures in the present study. It is also very well possible that other not yet discovered sialic acid binding molecules may be involved. More work should be performed on this type of molecules on the surface of macrophages.

Expression of CD163 in hematopoietic cells and blood monocytes was also investigated in the present thesis because it is a crucial marker for studying porcine monocyte and macrophage heterogeneity and it is an essential disassembly mediator for PRRSV. The presence of low percentages of $CD163^+$ cells in freshly isolated red bone marrow hematopoietic cells and a clear

increase in the percentage of these cells in hematopoietic cells co-cultured with immortalized mesenchymal stromal cells was observed. Cultivation of red bone marrow hematopoietic cells in the presence of immortalized mesenchymal stromal cells triggered their maturation to monocytic cells, based on their CD163 expression, size (high side scatter) and macrophage morphology. This shows that coculture with mesenchymal cells triggers differentiation of monocytes into macrophages. In another experiment in this thesis, it was found that compared to blood monocytes co-cultured without mesenchymal stromal cells, no differences in the expression level of CD163 was observed on blood monocytes with immortalized mesenchymal stromal cells derived from nasal mucosa, lungs, spleen, lymph nodes and bone marrow. This is in agreement with the reports that the majority of peripheral blood monocytes express CD163 in humans (Sulahian *et al*., 2000; Moniuszko *et al*., 2006). In the present study, the expression of CD163 alone was totally not sufficient for monocytes to become susceptible for PRRSV infection. This demonstrates the need for an additional entry mediator, such as siglecs (Oetke *et al.*, 2006; van den Berg *et al.*, 1992; Vinson *et al.*, 2006; Xie *et al*., 2017). In Fig 1, a hypothetical model of siglec-1 expression upon contact of blood monocytes with mesenchymal stromal cells during extravasation and their increased susceptibility for PRRSV is presented.

One of the limitations of this thesis was the use of only one simple marker (siglec-1) to confirm the differentiation stage of blood monocytes (Gordon *et al*., 2005; Passlick *et al*., 1989; Ziegler-Heitbrock). Human blood monocytes have been divided into three subsets: $CD14^{high}D16^{-}$, known as classical monocytes, $CD14^{+}CD16^{med}$, intermediate monocytes and $CD14^{-}CD16^{high}$, non-classical monocytes. Porcine monocytes have been divided into two subpopulations based on their expression of CD16 and CD163. $CD14^{+}CD163^{+}$ monocytes are similar to human $CD14^{+}CD16^{+}$; they both express high level of TNF-α (Sanchez *et al*., 1999). Although the phenotypic characteristics of porcine $CD169^{+}$ macrophages remains ambiguous, murine $CD169^{+}$ macrophages are further characterized as $CD11b^{+}$, $CD11c^{+}$, MHC-II^{+}, $CD68^{+}$ and $F4/80^{+}$ (Junt *et al*., 2007). The absence of CD169 on blood monocytes has been reported in swine, but recently, a subset of peripheral blood monocytes (CX_3CR1^{+}) in humans has been found to express CD169 (Karasawa *et al*., 2015). Recently, in an effort to characterize monocyte differentiation, research was focused on biomedical and structural changes. For example, receptors of GM-CSF, FLT3, CXCL12, and CCL18 have been shown to be associated with macrophage differentiation (Sanchez-Martin et al., 2011; Schraufstatter *et al*., 2012; Wiktor-Jedrzejczak *et al*., 1994).

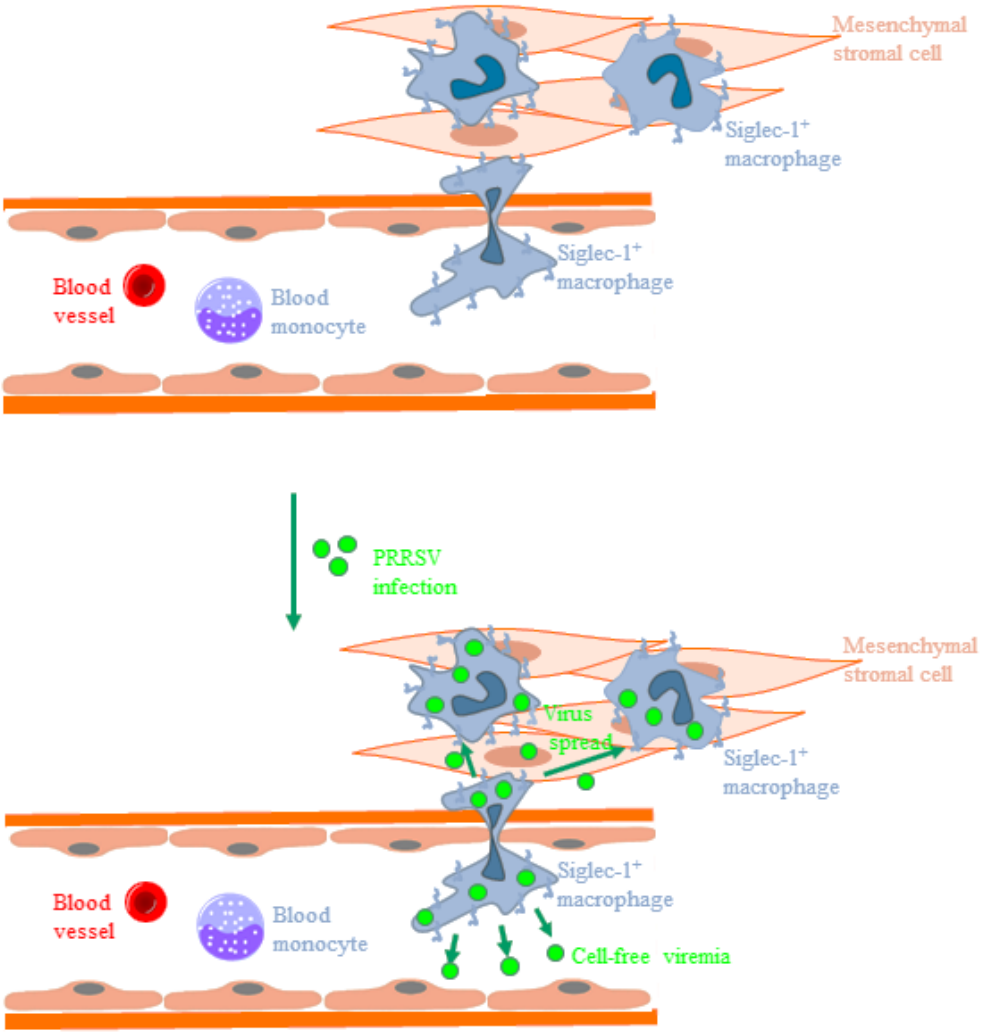

Fig 1. Hypothetical model of siglec-1 expression upon contact of blood monocytes with mesenchymal stromal cells during extravasation (environmental sensing). Mesenchymal stromal cells in tissues from spleen, lymph nodes, lungs and bone marrow, activate extravasating blood monocytes to differentiate and, as a consequence, to express siglec-1 through cell-cell contact. This makes them susceptible to PRRSV.

Genome-wide gene expression profiles associated with monocyte to macrophage differentiation have also been investigated resulting in the identification of several involved pathways and differentiation genes (Hashimoto *et al.*, 1999; Suzuki *et al.*, 2000; Li *et al.*, 2007; Martinez *et al.*, 2006). In the future, it will be interesting to better characterize the transcriptional and phenotypical changes in porcine blood monocytes differentiating into macrophages.

In the present thesis, it was demonstrated that mesenchymal stromal cells isolated from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow may be successfully immortalized by

transducing primary cells with the gene encoding SV40LT antigen. The technique to generate these cell lines from pig tissues can be copied to generate the same cell types from human and other animal tissues. Mesenchymal stromal cells can be used for preclinical applications such as tissue regeneration. Co-cultures of nasal mucosa, lungs, spleen, lymph node and red bone marrow with hematopoietic cells or blood monocytes as established in the present thesis may be used as tissue specific models in studying cross-talk between the two cell types involved. The gained knowledge may be useful in finding possible treatments/managements of immunological and inflammatory disorders such as auto-immune and graft versus host diseases affecting both human and animals. The established co-cultures will also be useful in studying host-pathogen interactions for a better understanding of the pathogenesis of many diseases caused by viruses and bacteria and will be helping in finding effective treatments. Further mesenchymal stromal cells will be useful for the generation of large amounts of differentiated macrophages expressing siglec-1 and CD163 starting from blood monocytes for the mass production of PRRSV stocks.

Future perspectives

Although the findings in this present thesis have undoubtedly increased our understanding of the biology of mesenchymal stromal cells, their immunomodulatory effect and their ability to support the survival of hematopoietic cells and to drive the differentiation of monocytes into siglec-1 differentiated macrophages, several aspects need further investigation. Cumulative data obtained from literature and this thesis indicated the strong immunomodulatory force of mesenchymal stromal cells on immune cells such as hematopoietic cells and blood monocytes. Mesenchymal stromal cells are known to phenotypically change these cells into CD169, CD163, CD14, CD4 and CD8 cells. Data from literature demonstrated that mesenchymal stromal cells, irrespective of their tissue origin, can exert an immunomodulatory effect either through cell-cell contact or by secretion of cytokines such as IL-10 and/or other soluble factors such as prostaglandin E2 (PGE2). An important question that remains to be answered is: what is the precise mechanism used by the immortalized mesenchymal stromal cells established in this present thesis to increase the differentiation of blood monocytes into siglec-1 expressing macrophages. The molecules (cytokines and/or other signaling molecules) produced by these mesenchymal stromal cell lines that are involved in the differentiation and increased susceptibility of blood monocytes to PRRSV may now

be identified and characterized. IL-10, an anti-inflammatory cytokine highly expressed by mesenchymal cells (Ben-Ami *et al*., 2011; Blaber *et al*., 2012) that has been reported to induced a high level expression of CDl63, which is (Philippidis *et al*., 2004) a cell mediator necessary for PRRSV infection, may be a good candidate.

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Summary

Chapter 1 gives a literature review on the characteristics, immunomodulatory effects and stemness properties of porcine mesenchymal cells. Mesenchymal stromal cell-immune cell interactions and immortalization of mesenchymal stromal cells were also introduced in this chapter. Further, an overview on the taxonomy, virion composition, replication cycle and pathogenesis of porcine reproductive and respiratory syndrome virus (PRRSV) is given.

The aims of this thesis are explained **in Chapter 2**.

Mesenchymal stromal cells have been successfully isolated from red bone marrow and several other tissues. These cells can differentiate into many cell types such as chondrocytes, adipocytes and osteocytes. Due to their multi-lineage differentiation capabilities and strong immunomodulatory effect on different types of both innate and adaptive immune cells, mesenchymal stromal cells received considerable attention from researchers as cells with great therapeutic potential. The anti-inflammatory and immunomodulatory effects of mesenchymal stromal cells have been explored for their potential use in the treatment of diseases such as graft-versus-host disease and diabetes. However, keeping mesenchymal stromal cells and their continuous passaging in *in vitro* cultures for a long time is a major challenge because they undergo senescence and die. Therefore, there is an urgent need for establishing continuous mesenchymal stromal cell cultures for studying mesenchymal stromal cell-immune cell interactions. The development of an *in vitro* model for co-culturing immortalized mesenchymal stromal cells with bone marrow- and blood-derived immune cells will help to better understand mesenchymal cell-immune cell interactions for therapeutic applications and the effect of mesenchymal stromal cells on host-pathogens interactions, such as PRRSV replication in monocytes/macrophages, easing the way for finding possible treatments for infectious and non-infectious diseases.

The specific aims of this thesis are:

- ✓ To develop a long-term co-culture system for maintenance and differentiation of hematopoietic cells on top of immortalized mesenchymal stromal cells towards cells of the monocyte/macrophage lineage.

- ✓ To establish mesenchymal stromal cell lines from different tissues of pigs for long-term studies such as the differentiation of blood-derived monocytes into siglec-1 expressing macrophages.
- ✓ To explore the effect of mesenchymal stromal cells on pathogen-macrophage interactions by examining the replication kinetics of porcine reproductive respiratory syndrome virus in macrophages co-cultured with immortalized mesenchymal stromal cells.

In Chapter 3, a successful isolation of mesenchymal stromal cells was achieved by flushing red bone marrow with RPMI. Fibroblast-like cells appeared one week after initial cultivation and were passaged several times. Immortalization of primary mesenchymal stromal cells using a recombinant lentivirus vector containing the sequence encoding SV40LT transforming protein led to the establishment of a mesenchymal cell line from red bone marrow. This was confirmed by the immunodetection of SV40LT antigens in the mesenchymal stromal cells. Immortalized mesenchymal stromal cells were found to be positive for the mesenchymal stromal cell markers CD44 and CD55. After the successful establishment of mesenchymal stromal cell cultures, red bone marrow hematopoietic cells were isolated for mesenchymal stromal cell-hematopoietic cell co-cultures. The long-term maintenance of red bone marrow hematopoietic cells on top of mesenchymal stromal cells was achieved in DMEM supplemented with 10% fetal calf serum without the addition of cytokines. In addition to the 3-fold increase in the number of hematopoietic cells, the immortalized mesenchymal stromal cells were successfully used to trigger the differentiation of hematopoietic cells towards cells having a similar morphology with monocytes/macrophages and lymphocytes. This was confirmed by evaluating the expression of the myelomonocytic markers CD172a, CD14, CD163, CD169 and T-lymphocyte markers CD4 and CD8 in hematopoietic cells co-cultured with immortalized mesenchymal stromal cells using flow cytometry. Five weeks after co-cultivation with immortalized mesenchymal stromal cells, flow cytometry analysis revealed 92±6% viable hematopoietic cells. Three weeks after co-cultivation, flow cytometric analysis showed an increased surface expression of CD172a, CD14, CD163, CD169, CD4 and CD8 up to 37±0.8%, 40±8%, 41±4%, 24±2%, 19±5% and 23±3% of the hematopoietic cells, respectively.

In Chapter 4, primary mesenchymal cells were isolated from nasal mucosa, lungs, spleen and lymph nodes, immortalized and characterized. The obtained immortalized mesenchymal stromal cells were used to examine their effect on the differentiation of immune cells. Growth analysis

revealed that immortalized mesenchymal stromal cells derived from red bone marrow were the strongest growers while immortalized mesenchymal stromal cells derived from nasal mucosa were the slowest growers. When cell cycle analysis was performed using flow cytometry to distinguish immortalized mesenchymal stromal cells in different phases of the cell cycle, the majority of the cells were found in the G0/G1 phase of the cell cycle indicating that the five mesenchymal stromal cell lines replicated normally. More nasal mucosa derived mesenchymal stromal cells were found to be in the sub-G1 phase of the cell cycle compared with the mesenchymal stromal cells from other tissues. Interestingly, immortalization of the mesenchymal stromal cell derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow did not affect their phenotypic characteristics, because they were found to clearly express the mesenchymal stromal cell specific markers CD105, CD90 and CD44 in most of the cells (>80%) and CD29 in 29-42% of the cells. The five cell lines were negative for the myeloid markers CD16 and CD11b. Stemness properties of the five mesenchymal stromal cell lines were not affected by the immortalization processes. This was confirmed by incubating the immortalized mesenchymal stromal cells in osteogenic, adipogenic and chondrogenic differentiation medium. After staining the cells with alizarin red, oil red O and alcian blue staining solutions, calcium deposits, lipid droplets and dark blue cartilage coloration and in the cells were observed, confirming the differentiation of the immortalized mesenchymal stromal cells into osteoclasts, adipocytes and chondrocytes, respectively. The immortalized mesenchymal stromal cells, derived from the five different tissues, were able to trigger the expression of siglec-1 in blood monocytes upon their co-culture with mesenchymal stromal cells.

In Chapter 5, the induction of siglec-1 expression in blood monocytes by immortalized mesenchymal stromal cells from different tissues was used to investigate the susceptibility of co-cultured monocytes for PRRSV infection, because siglec-1 functions as a binding and internalization receptor. The hypothesis that increased expression of siglec-1 corresponds with an increased susceptibility of macrophages to PRRSV was confirmed in this thesis. When immortalized mesenchymal stromal cell-blood monocyte co-cultures were inoculated with PRRSV subtype 1 LV and subtype 3 Lena at 24 h, 48 h and 72 h after co-cultivation and 12 hours post-inoculation, an increase in infection of blood monocytes was observed which corresponded to an increase in siglec-1 expression. Confocal analysis revealed that most of the Lena and LV-infected monocytes were Sn^+; only a few were found to be Sn^-.

Chapter 6 gives a general explanation on the research data generated in the present thesis. The first part of this chapter gives an overview of (i) the past and current findings about the use of mesenchymal stromal cells in the biological world and their immunomodulatory effect and of (ii) porcine reproductive and respiratory syndrome virus (PRRSV). Next the results obtained in this thesis about the ability of immortalized mesenchymal stromal cells to retain their stemness properties, and to drive the differentiation of co-cultured hematopoietic cells and blood monocytes are discussed. Finally, a hypothetical model is forwarded on how mesenchymal stromal cells induces the expression of siglec-1 on blood monocytes making them susceptible for PRRSV.

Several important conclusions can be drawn from this thesis:

- Immortalizing mesenchymal stromal cells from different tissues does not affect their morphology and phenotypic characteristics.
- Immortalized mesenchymal stromal cells from different tissues may differentiate into osteocytes, chondrocytes and adipocytes.
- Immortalized mesenchymal stromal cells from different tissues can direct the differentiation of red bone marrow hematopoietic cells and blood monocytes into macrophages.
- Immortalized mesenchymal stromal cells from different tissues can increase the susceptibility of blood monocytes to PRRSV via upregulation of siglec-1

Samenvatting

In **Hoofdstuk 1** wordt een overzicht gegeven van de karakteristieken, immuno-modulatorische effecten en stamceleigenschappen van porciene mesenchymale cellen. De interacties van mesenchymale cellen met het immuunsysteem en de immortalisatie van mesenchymale cellen worden eveneens beschreven in dit hoofdstuk. Vervolgens handelt dit hoofdstuk over de taxonomie, de structuur van het virion, de replicatiecyclus en de pathogenese van het porcien reproductief en respiratoir syndroom virus (PRRSV).

In **Hoofdstuk 2** worden de doelstellingen van deze thesis toegelicht.

Mesenchymale cellen kunnen succesvol geïsoleerd worden uit het rode beenmerg en verschillende andere weefsels van het varken. Deze cellen zijn in staat te differentiëren tot verschillende celtypes zoals chondrocyten, adipocyten en osteocyten. Wegens hun capaciteit om te differentiëren tot verschillende celtypes en hun sterk immuno-modulatorische effecten, is er reeds geruime tijd een wetenschappelijke interesse in mesenchymale cellen, met het oog op hun potentiële therapeutische toepassingen. Zowel de anti-inflammatoire als immuno-modulatorische effecten van mesenchymale cellen werden reeds uitvoerig onderzocht voor hun gebruik in de behandeling van afweerreacties tegen donorweefsel na transplantatie of diabetes. De korte levensduur van mesenchymale cellen *in vitro* is echter een belangrijke belemmering voor toepassingen. Bijgevolg is er een dringende behoefte aan de ontwikkeling van een continue mesenchymale cellijn om de interacties met immuuncellen te kunnen onderzoeken. Het opzetten van een *in vitro* model voor cocultivatie van geïmmortaliseerde mesenchymale cellen met cellen afkomstig uit beenmerg en bloed zal ons meer inzicht geven in de mesenchymale cel-immuuncel interacties en therapeutische applicaties. Verder zal een dergelijk systeem ons toelaten om de rol van mesenchymale cellen in gastheer-pathogeen interacties te onderzoeken, zoals PRRSV replicatie in macrofagen, wat een belangrijke stap kan zijn in het vinden van mogelijke behandelingen.

De specifieke doelstellingen van deze thesis zijn:

- De ontwikkeling van een lange-termijn cocultuur systeem voor de handhaving en differentiatie van hematopoëtische cellen in monocyten/macrofagen, bovenop geïmmortaliseerde mesenchymale cellen.

- De ontwikkeling van mesenchymale cellijnen afkomstig van verschillende varkensweefsels voor lange-termijn studies zoals de differentiatie van uit het bloed geïsoleerde monocyten in siglec-1 expresserende macrofagen.
- Het onderzoeken van het effect van mesenchymale cellen op PRRSV-macrofaag interacties.

In **Hoofdstuk 3** werden mesenchymale cellen succesvol geïsoleerd door flushing van het rode beenmerg met RPMI-medium. Fibroblast-achtige cellen konden worden opgemerkt na één week cultivatie en deze werden vervolgens meermaals gesubcultiveerd. Daarna, werden primaire mesenchymale cellen geïmmortaliseerd ten einde continue mesenchymale cellijnen te generen van het rode beenmerg. De transformatie van primaire mesenchymale cellen werd verkregen door lentivirale transductie met het simian virus 40 large T (SV40LT) antigen. Succesvolle transformatie werd in een volgende stap aangetoond door middel van immunodetectie van dit SV40LT antigen. De geïmmortaliseerde mesenchymale cellen brachten de mesenchymale merkers CD44 en CD55 merkers tot expressie. Na het bekomen van een mesenchymale cellijn van het rode beenmerg werden ook de hematopoëtische cellen in het rode beenmerg geïsoleerd voor cocultivatie met de mesenchymale cellen. Deze hematopoëtische cellen van het rode beenmerg konden gedurende een lange termijn gecultiveerd worden bovenop de mesenchymale cellen in DMEM-medium aangevuld met 10% foetaal kalf serum (FKS) en zonder toevoeging van cytokines. Cocultivatie met mesenchymale cellen leidde tot een 3-voudige toename van het aantal hematopoëtische cellen. Daarnaast, zetten de mesenchymale cellen de hematopoëtische cellen aan tot differentiatie naar een celtype met een gelijkaardige morfologie als deze van monocyten/macrofagen. Dit werd eveneens bevestigd wanneer de expressie van myelomonocytische merkers CD172a, CD14, CD163, CD169 en T-lymphocyten merkers CD4 en CD8 werd nagegaan in de hematopoëtische cellen die gecocultiveerd werden met de mesenchymale cellen door middel van flowcytometrie. Vijf weken na cocultivatie met geïmmortaliseerde mesenchymale cellen kon door middel van flowcytometrie een vitaliteit aangetoond worden van 92±6%. Na slechts drie weken cocultivatie werd eveneens een toename van de expressie van CD172a, CD14, CD163, CD169, CD4 en CD8 waargenomen tot respectievelijk 37±0.8%, 40±8%, 41±4%, 24±2%, 19±5% en 23±3% van de hematopoëtische cellen.

In Hoofdstuk 4 werden primaire mesenchymale cellen uit de nasale mucosa, de long, de milt en lymfeknopen geïsoleerd, geïmmortaliseerd en gekarakteriseerd. Vervolgens werd de invloed van

deze cellen op de differentiatie van immuuncellen bestudeerd. Analyse van de groei toonde aan dat geïmmortaliseerde mesenchymale cellen afkomstig van het rode beenmerg het snelst groeiden, in tegenstelling tot deze afkomstig van de nasale mucosa die het traagst groeiden. Om geïmmortaliseerde mesenchymale cellen in verschillende fasen van de celcyclus te onderscheiden werd gebruik gemaakt van flowcytometrie, waarbij de meerderheid van de cellen zich in de G0/G1 fase van de celcyclus bevond. Dit toonde aan dat de vijf mesenchymale cellijnen op een normale manier repliceerden. In vergelijking met de mesenchymale cellen van andere weefsels, bevonden relatief meer cellen afgeleid van nasale mucosa zich in de sub-G1 fase van de celcyclus. Opmerkelijk werden de fenotypische eigenschappen van de mesenchymale cellen afkomstig van nasale mucosa, long, milt, lymfeknopen en rode beenmerg niet beïnvloed door immortalisatie aangezien de meeste cellen (>80%) de mesenchymale celspecifieke merkers CD105, CD90 en CD44 en 29-42% de mesenchymale celmerker CD29 tot expressie brachten. De vijf cellijnen waren negatief voor de myeloïde celmerkers CD16 en CD11b. De stamceleigenschappen van de vijf mesenchymale cellijnen werden niet beïnvloed door het immortalisatieproces. Dit werd bevestigd door incubatie van de geïmmortaliseerde mesenchymale cellen in osteogeen, adipogeen en chondrogeen differentiatiemedium. Na kleuring van de cellen met alizarine rood, oil red O en alciaan blauw oplossingen, werden calcium afzettingen, vetdruppels en een donkerblauwe kraakbeenkleuring geobserveerd in de cellen, hetgeen de differentiatie van de geïmmortaliseerde mesenchymale cellen in respectievelijk osteoclasten, adipocyten en chondrocyten bevestigde. De geïmmortaliseerde mesenchymale cellen bekomen uit de vijf verschillende weefsels waren in staat om de expressie van siglec-1 in bloedmonocyten te activeren door co-cultivatie.

In **Hoofdstuk 5** werd de inductie van de siglec-1 expressie in bloedmonocyten door geïmmortaliseerde mesenchymale cellen van verschillende origine gebruikt om de gevoeligheid van gecocultiveerde monocyten voor een PRRSV infectie na te gaan, aangezien siglec-1 fungeert als receptor voor binding en internalisatie. De hypothese dat een verhoogde expressie van siglec-1 gepaard gaat met een verhoogde gevoeligheid van macrofagen voor PRRSV werd bevestigd in deze thesis. Wanneer geïmmortaliseerde mesenchymale cel-bloedmonocyt co-culturen geïnoculeerd werden met PRRSV subtype 1 LV en subtype 3 Lena 24, 48 en 72 uur na co-cultivatie, werd een toegenomen infectie van bloedmonocyten geobserveerd 12 uur post-inoculatie wat overeenkomt met een verhoogde siglec-1 expressie. Confocale analyse toonde aan dat het merendeel van de monocyten geïnfecteerd met Lena en LV Sn^+ waren en enkele Sn^-.

In **Hoofdstuk 6** werden algemene verklaringen gegeven voor de onderzoeksresultaten uit deze thesis. In het eerste deel wordt een overzicht gegeven van (i) eerdere en huidige bevindingen aangaande het gebruik van mesenchymale cellen en hun immunomodulatorisch effect en (ii) het porcien reproductief en respiratoir syndroom virus (PRRSV). Vervolgens wordt het vermogen van geïmmortaliseerde mesenchymale cellen om hun stamcel eigenschappen te behouden en de differentiatie van gecocultiveerde hematopoëtische cellen en bloedmonocyten te dirigeren, bediscussieerd. Finaal wordt een hypothetisch model besproken over hoe mesenchymale stromale cellen de expressie van siglec-1 op bloedmonocyten induceren waardoor ze gevoelig worden voor PRRSV-infectie.

Verschillende belangrijke conclusies kunnen getrokken worden uit deze thesis:

- Immortalisatie van mesenchymale cellen van verschillende weefsels heeft geen invloed op hun morfologische en fenotypische eigenschappen.
- Geïmmortaliseerde mesenchymale cellen van verschillende weefsels zijn in staat om te differentiëren tot osteocyten, chondrocyten en adipocyten.
- Geïmmortaliseerde mesenchymale cellen van verschillende weefsels kunnen de differentiatie van rode beenmerg hematopoëtische cellen en bloedmonocyten sturen in de richting van macrofagen.
- Geïmmortaliseerde mesenchymale cellen van verschillende weefsels kunnen de gevoeligheid van bloedmonocyten voor PRRSV verhogen door opregulatie van siglec-1.

Curriculum vitae

Personalia

Abukar Garba was born in Sokoto, Nigeria on October 3. In 2005, he graduated from Usman Dan Fodio University Sokoto, Nigeria where he obtained a diploma in Veterinary Medicine (Doctor of Veterinary Medicine). After obtaining DVM he worked with the Ministry of Animal Health and Fisheries Development Sokoto State, Nigeria for 6 years (2005-2011). In September 2011, he started his studies at the Vrije Univerteit Brussels, Belgium where he obtained his MSc in molecular Biology on 13th September 2013. In the same month He joined the laboratory of virology, department of immunology, virology and parasitology, Faculty of Veterinary Medicine, Ghent University for his PhD studies. He worked on the interactions of immortalized mesenchymal cells with immune cells and the effect of mesenchymal cells on the susceptibility of blood monocytes to PRRSV.

Academic Qualifications

Ph.D.	2013-2018	Ghent University, Belgium
M.Sc.	2011-2013	Vrije Universiteit Brussel, Belgium
D.V.M	1999-2005	Usmanu Dan Fodiyo University Sokoto, Nigeria

Honors and Awards

1999-2005: Sokoto state Government scholarship award for Veterinary students
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Key Skills

- Primary cells isolations
- Immunofluorescence staining
- Flow cytometry
- Confocal microscopy

Publications

Garba A, Desmarets LMB, Acar DD, Devriendt B, Nauwynck HJ: Immortalized porcine mesenchymal cells derived from nasal mucosa, lungs, lymph nodes, spleen and bone marrow retain their stemness properties and trigger the expression of siglec-1 in co-cultured blood monocytic cells. PLoS ONE 12(10)

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Garba A, Nauwynck HJ: Co-culturing of immortalized mesenchymal cells derived from nasal mucosa, lungs, spleen, lymph nodes and red bone marrow increase the susceptibility of blood monocytes to PRRSV infection (Manuscript in preparation)

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