Lentivirus-mediated Knockdown of Galectin-10 and Foxp3 in Human Immune Cells

Dissertation

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INTRODUCTION

Immune cells in peripheral blood provide immune protection of every cell type of the body. Skin and mucosal epithelia lining airway and gut are the first defence against invading pathogens. They keep pathogens out by preventing their adherence and secretion of antimicrobial enzymes and peptides. Pathogens overcome these barriers immediately encounter tissue macrophages equipped with surface receptors that can bind these pathogens and induce phagocytosis. Pathogens binding to macrophage receptors initiate the release of cytokines and small lipid mediators. This leads to an inflammatory response which causes accumulation of phagocytic neutrophils and macrophages at site of infection. Thereby, phagocytic neutrophils and macrophages ingest and destroy the invading pathogens. This represents one mechanism that underlies the innate immune response of the body. Other important innate immune cells are dendritic cells (DCs), monocytes, eosinophils and natural killer (NK) cells [1, 2].

There are conserved patterns of molecular structure which are shared by several pathogens but not present on the body's own cells (i.e. all Gram-negative bacteria express lipopolysaccharide [LPS]) [3]. These patterns are recognized by specific receptors expressed by cells of the innate immune system known as pattern recognition receptors (PRRs). One critical function of the innate immune system is to alert the adaptive immune response by the action of specialized antigen presenting cells (APCs: macrophages, B cells and DCs) that present pathogen-derived peptides on their surface via major histocompatibility complex molecules (MHC) [4]. APCs translate and transfer information from the periphery to adaptive immune system, allowing a systemic response to a localized threat. DCs are the most effective APCs because of their unique capacity to activate naïve T cells. DCs displaying pathogen-derived peptide on the cell surface leave the infected site and migrate to the nearest lymph node where they activate antigen-specific T cells. Thereby, T cells do not directly recognize the whole pathogen, but are specifically activated by DCs which present molecular fragments via MHC molecules at the cell surface [4].

Exogenous antigens presented through MHC-class-II are recognized by $CD4^+$ T cells. $CD4^+$ T cells often represent the first lymphocyte subset activated during an adaptive immune response. $CD4^+$ T cells play both modulatory and effector roles during an immune response. They act primarily by secreting soluble factors (cytokines) that are able to exert direct antimicrobial properties and affect the behavior of other immune cells. In most cases, $CD4^+$ T cells help other immune cells to perform their task and are therefore referred to as T helper cells (Th). However, the activation of $CD4^+$ Th cells

requires at least two signals. Complex formation between the T cell receptor (TCR) and MHC peptide expressed on the surface of DC provides signal one [5].

Activation of T cell is accomplished by receiving signal two, the co-stimulatory signal. The B7 family ligands (CD80/CD86) expressed on the surface of DCs are the most potent co-stimulatory molecules that interact with CD28 on the T cell surface [6]. Other molecules such as CD2, OX40, ligands for ICOS and CD40 can serve as co-stimulatory receptors of T cell activation [7].

Several sub-populations of Th cell have been identified based upon the type of cytokines they secrete and differing abilities to help other subsets of immune cells. The Th1 cells secret mainly interferon gamma (IFN-γ), a cytokine known to limit pathogen survival and spreading. It is also known to promote the differentiation of cytolytic cells ($CD8^+$ T cells) that are able to destroy cells infected with intracellular pathogens. Therefore, Th1 cells are important for inducing immune responses involved in the clearance of intracellular pathogens. The Th2 cells produce cytokines (Interleukins [IL], IL-4, IL-5, IL-13) particularly important at activating innate cells (eosinophils and mast cells) which are often involved in the immune response to large extracellular parasites. The Th17 produce IL-17 as a marker cytokine and are apparently highly pathogenic and have a role in antimicrobial defence and might contribute in immunity against extracellular bacteria by activating neutrophils. Regulatory T cells (Tregs) control and counteract excessive immune responses [4, 5].

MHC-class-I restricted presentation of antigens results in the stimulation of $CD8^+$ T cells. $CD8^+$ T cells can directly lyse target cells by means of perforin and granzymes. They enable $CD8^+$ T cells specifically to kill cells displaying pathogen-derived peptides presented by MHC-class-I-molecules, for example a virus infected cell [8].

In the lymphnode, the activated T cells inturn activate B cells to secret antibody [9]. Effector T cells and antibody molecules return to the circulation, they leave the circulation again at the site of infection, where inflammatory mediators have induced changes in the blood vessel endothelium. $CD4^+$ T cells activate macrophages to become more cytotoxic, while antibody recruits complement to lyse bacteria directly and to opsonize them enhancing their uptake by phagocytes. In case of viral infection, activated $CD8^+$ T cells kill any infected cells present. This represents the mechanism that underlies the adaptive immune response of the body [10, 11].

All innate and adaptive immune cells of the body originate from hematopoietic stem cells (HSC). HSC are characterized by two important properties. They have the ability to renew themselves and to produce cells that give rise to all different types of blood cells [12]. HSC are found in bone marrow and peripheral blood [13, 14]. Supplying a potent stimulator such as granulocyte-colony stimulating factor (G-CSF) induces the

migration of HSC from the bone marrow into the blood [14]. Another important source of HSC is the blood of umbilical cord. Since its recognition as a rich source of HSC, the use of these cells has grown quickly [15].

Studies have revealed the presence of two kinds of HSC. HSC which are capable of self-renewal and restoring the hematopoietic system over some months and for the entire life span. They are considered to be long-term stem cells. Short-term progenitor or precursor cells can immediately regenerate all the different types of blood cells but can not renew themselves over the long term [16].

Identification and isolation of HSC represent critical challenges. HSC with long-term replicating ability are rare. However, most attempts to identify HSC utilized the high proliferative response of such cells *in vitro* in the presence of hematopoietic cytokines. These attempts revealed CD34 to be a potent cell surface marker [17]. Short-term HSC differentiate into lymphoid and myeloid progenitors, the two major classes of progenitors for most lineages of blood cells [18, 19]. Common myeloid progenitors give rise to either megakaryocyte/erythrocyte or granulocyte/macrophage progenitors [19]. Lymphoid progenitors differentiate into T cells, B cells, and NK cells [20]. The mechanisms and pathways that lead to their differentiation are still being investigated**.**

In order to produce functionally competent T cells, HSC develop lymphoid multipotent progenitors (LMPPs) and early lymphoid progenitors (ELPs) in the bone marrow. LMPPs and ELPs migrate from the bone marrow to enter through an anatomically distinct organ, namely, the thymus. Once they reach the thymus, the T cell progenitors undergo a series of defined differentiation steps that occur in specific zones of the thymus [21].

In the thymus, populations of CD4 and CD8 double negative (DN) thymocytes were identified as precursors of T cells. They differentiate into $CD8^+CD4^+$ double positive (DP) cells which in turn differentiate into mature T cells. DN thymocytes are divided into four populations (1-4) according to the surface expression of CD25 and CD44 [22, 23].

The most immature population DN1 is $CD25^-CD44^+$ and has multi-lineage potential including the three types of lymphocytes (T cell, B cell and NK cell) and myeloid cell in addition to DC [24-26]. The next differentiation stage DN2 has $CD25^+CD44^+$ surface phenotype and can differentiate to T cell, NK cell and DC but it loses the B cell potential [27]. The third developmental stage DN3 has the phenotype of $CD25^+CD44^-$ and is followed by DN4 which is $CD25^-CD44^-$. Both DN3 and DN4 are committed to T cell lineage only. An extensive cell proliferation takes place in DN1 and DN2 stages to expand the number of cells that will rearrange the T cell receptor β-chain (TCRβ) to ensure the clonal diversification of TCRβ [28]. This is followed by the expression of recombination activating genes (RAG-1 and RAG-2) at DN2 and DN3 stages to induce the

rearrangement of TCR loci (β,γ,δ) [29, 30]. T cell lineage commitment occurs at the DN3 stage coincidently with the initiation of V(D)J rearrangement of the TCR β-chain, TCR γ-chain and TCR δ-chain genes. Cells that rearrange TCRγ and TCRδ diverge from αβ lineage and differentiate into γδ mature T cells [31].

These TCR complexes are then subjected to positive and negative selection processes to screen for their ability to interact with self-peptide/major histocompatibility complex molecules (spMHC) expressed on the thymic stromal cells. RAG genes are expressed in DP thymocytes until the αβTCR complex is able to engage intrathymic spMHC complexes with a sufficient affinity to transduce survival signal [32-35]. These initial signals lead to the termination of RAG gene expression which prevents any further TCRα gene rearrangements and fixes the TCR specificity [34, 36, 37]. The cells that fail to express αβTCR or express TCRs with too low affinity to spMHC ligands do not receive any TCR survival signals and undergo apoptosis (death by neglect) [38-42]. Eventually, only DP thymocytes with intermediate affinity for intrathymic spMHC ligands receive survival signals and can differentiate into single positive (SP) CD4 or CD8 mature T cells. This process is known as positive selection [39, 43, 44].

In contrast, DP thymocytes with autoreactive potential because of the high affinity of their TCR for intrathymic spMHC ligands receive death signals and undergo apoptosis. This process is referred to as negative selection or central tolerance [45-48].

Central tolerance is the clonal deletion of autoreactive T cells during their development in the thymus. It provides an important mechanism to prevent autoaggressive immune reactions in the periphery [49]. However, clonal deletion is not perfect and potentially autoaggressive T cells do escape into the periphery [50]. Different mechanisms have been purposed to maintain the tolerance in the periphery. Among which, active suppression by Tregs moved into the forefront of maintaining the peripheral tolerance.

Thymus-derived Tregs (tTregs) develop in the thymus as a distinct cell lineage predestined to suppress immune responses and prevent autoimmunity [51, 52]. They are mainly characterized by the high constitutive surface expression of the alpha subunit of the IL-2 receptor (CD25) so they are $CD4^+CD25^+$ T cells [53]. Prevention of tTregs development in mice few days after birth by thymectomy triggers the development of autoimmune and inflammatory diseases like gastritis, thyroiditis, type 1 diabetes and inflammatory bowel disease [53, 54]. The potential of Tregs to prevent pathological immune responses in autoimmune diseases, graft-versus-host disease and allergy was shown in many investigations [54-56]. These observations demonstrated that autoaggressive T cells exist in normal mice, and that Tregs are capable of suppressing these pathogenic cells.

In human, tTregs were detected in the thymus of human fetus as early as 13 weeks of gestation together with the first mature T cells [57]. They constitute approximately 5-10% of $CD4^+$ T cells in the periphery [52]. *In vitro* studies showed that these cells can not be induced to proliferate upon polyclonal activation using anti-CD3 monoclonal antibody (mAb) [58, 59]. The anergic state of Tregs can be partially reversed by high amounts of IL-2 [58]. It was shown that Tregs are able to suppress proliferation and cytokine production when co-cultured with $CD4^+CD25^-$ T effector cells after activation in a dose- and cell-contact dependent, but cytokine-independent manner [58-62].

A significant advance in more precisely defining the Treg population occurred when the transcription factor Foxp3 was identified as being necessary for Treg development [63]. Foxp3 is a member of the forkhead/winged-helix family of transcription factors [64]. Mutations in the murine FOXP3 gene lead to inability to regulate T cells properly and their hyperproliferation [64]. Consequently, these mice develop fatal severe lymphoproliferative autoimmune phenotype, known as *scurfy*, resulting in lethality in males 16-25 days after birth [64]. Exogenous expression of Foxp3 was capable of rescuing the mice from *scurfy* phenotype and demonstrated that a mutation in FOXP3 was responsible for the *scurfy* phenotype [64-66]. Mutations in the human FOXP3 gene are characterized by an aggressive autoimmunity known as IPEX syndrome (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) which is attributed to the absence of functional Tregs leading to a complete loss of Treg derived suppressive activity [67-70].

In mice, Foxp3 is an exclusive marker for Tregs [71, 72]. Foxp3 is not induced in conventional $CD4^+CD25^-$ T effector cells upon TCR-dependent stimulation. Ectopic expression of Foxp3 onto conventional $CD4^+CD25^-$ T effector cells confers a regulatory phenotype with suppressor function. Thus, Foxp3 serves as a master regulator for both the development and function of tTregs in mice [73, 74].

In humans, because of the high expression level of Foxp3 in tTregs compared to $CD4^+CD25^-$ T effector cells, Foxp3 was thought to be an exclusive marker for tTregs [75, 76]. However, in contrast to murine T cells, Foxp3 is transiently up-regulated after TCR-dependent stimulation in conventional human $CD4^+$ T effector cells [66, 77, 78]. Foxp3 expression was shown to be transient in nonsuppressive T cell population, while it is stably expressed in Tregs that do display suppressive function [79]. This suggests that Foxp3 is rather an activation marker in $CD4^+CD25^-$ T effector cells in humans and can not be used as a lineage specific marker to define Tregs [66, 77, 78]. Moreover, Foxp3 is expressed not only in Tregs but also in epithelial cells from various organs [80]. In addition to its role as an essential transcription factor in Tregs, the FOXP3 gene is an epithelial cell-intrinsic tumor suppressor for breast, prostate cancers and cutaneous melanoma [80-82].

Isolation of Tregs based upon the expression of Foxp3 is not possible as it is expressed intracellularly [72]. Another important constitutive, long lasting membrane molecule expressed on activated Tregs is cytotoxic T-lymphocyte antigen-4 (CTLA-4) [83, 84]. However, CTLA-4 is also expressed on the surface of activated effector T cells [85]. *In vivo* studies showed that CTLA-4 has a negative regulatory effect on T cells by down regulating IL-2 production [83, 85]. Nevertheless, the function of CTLA-4 for the suppressive activity of murine $CD4^+CD25^+$ cells *in vitro* remains controversial. While few studies in humans and mice showed that blocking of CTLA-4 with monoclonal antibodies did not reverse the anergic and suppressive properties of Tregs [58, 59], another study showed that suppression by murine Tregs was abrogated by the addition of huge amounts of antibodies against CTLA-4 [84].

In vitro studies of murine and human tTregs showed that activation of tTregs via their TCR is required for a cell contact-dependent mechanism of suppression. Therefore, signalling through the TCR of tTregs leads to induction of cell surface molecules directly associated with their suppressor function [67]. It was shown that stimulated Tregs express high and persistent amounts of membrane-bound transforming growth factor-β (TGF-β) on the cell surface [86]. Although it was strongly suggested that Tregs exert suppression by a cell-cell interaction involving cell surface TGF-β [86], another study showed that TGF-β-deficient tTregs are suppressive as wild type tTregs [87].

TGF-β is normally found in the latent form associated with latency-associated peptide (LAP) which prevents its activity. TGF-β can be secreted in a small latent form associated with LAP, or this complex can further associate with latent-TGF-β binding protein (LTBP) to produce a large latent form for deposition onto the extracellular matrix. Small latent TGF-β found to be expressed on the membrane of activated Tregs together with a member of the leucine-rich repeat family of proteins that has been termed GARP (glycoprotein A repetitions predominant). It was showm that GARP binds and anchors latent TGF-β and function as its cell surface receptor. It was shown also that GARP/LAP complex does contribute to Treg mediated suppression *in vitro*. However, mutations or deletions in GARP would only affect membrane bound TGF-β and not the secreted pool [88-90].

Another possibility for the activation of Tregs is through anti-CD4 antibodies. Tregs activated by anti-CD4 antibodies were able to suppress the proliferation of $CD4^+$ and $CD8^+$ T cells [91]. Moreover, gp120 (HIV surface envelope glycoprotein subunit) was found to activate Tregs by binding and signalling through CD4 receptor [92].

Stimulated Tregs show accumulation of cyclic adenosine monophosphate (cAMP) in their cytosol [92]. This second messenger is known to be a potent inhibitor of proliferation and IL-2 synthesis in T cells. cAMP increment in $CD4^+CD25^-$ T effector cells

upon co-activation with Tregs proved the presence of communication via cell contact–dependent gap junction. This suggests that cAMP is crucial for Treg–mediated suppression and traverses membranes via gap junctions [93, 94].

Besides tTreg, there are distinct subsets of secondary suppressor T cells include Th3 and Tr1 cells [95-98]. As these cells are differentiated in the periphery, they are referred to as peripherally derived Tregs (pTregs) [51]. Th3 and Tr1 cells produce high amounts of IL-10 and/or TGF-β. The suppressive mechanism of Th3 and Tr1 cells is mediated by these immunosuppressive cytokines and therefore cell contact-independent [95, 97, 98]. IL-10-producing Tr1 cells are *in vitro*–induced Tregs (iTregs) which are generated by differentiation of resting $CD4^+$ T cells in the presence of IL-10, either by repetitive stimulation of cord blood-derived naive $CD4^+$ T cells with immature monocyte-derived DCs or by activation with anti-CD3 and anti-CD46 mAb [83, 95, 99, 100]. TGF-β-secreting Th3 cells are also iTregs by culture of resting $CD4^+$ T cells with TGF-β, IL-10 and anti-IL-12 while IL-4 seems to be the key factor for their differentiation [98, 101, 102]. Th3 cells produce variable amounts of TGF-β and IL-10. However, TGF-β was identified as being essential for contact-independent suppression by induced Th3 suppressor cells [97, 103]. Although the relationship between Treg, Tr1 and Th3 suppressive T cell populations is unclear, Tr1 and Th3 cells appear to be distinguishable based on their cytokine profiles, mode of action and different α/β–integrins expression. The integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ are homing receptors for cellular migration of T lymphocytes to inflamed tissues and to mucosal sites, respectively [97, 104, 105]. The $\alpha_4\beta_7^+$ Tregs induce suppressor cells that produce high amount of IL-10 (Tr1-like) while the $\alpha_4\beta_1^+$ Tregs induce TGF-β-producing suppressor cells (Th3-like) [97]. Thereby activated tTregs can directly suppress T cells by a primary cell-contact-dependent local suppression. The suppressed induced Tregs can mediate a secondary cell-contact-independent suppression based on Tr1 and Th3 like cells via the soluble factors IL-10 and TGF-β [97, 103, 106]. This infectious suppressive activity, represented by induction of suppressive properties in conventional $CD4^+$ T effector cells by tTregs underlies the phenomenon of infectious tolerance [103].

Clearly, elucidating the mechanisms underlying Tregs suppressive function is of utmost importance and gained a significant progress over the last few years. However, there are much remained to be determined and many questions still unanswered. Among which, are there more unknown mechanisms and/or molecules that mediate Tregs suppression and functions? Can any of these unknown molecules represent more specific markers for the characterization and isolation of Tregs?

To date, there is no unique marker exclusively expressed for Tregs. A novel protein known as galectin-10 or Charcot-Leydin crystal (CLC) which has never been described in lymphocytes so far has been identified in Tregs [107]. The CLC protein which

is nearly absent in resting and activated $CD4^+CD25^-$ T effector cells belongs to galectins family [107].

Galectins are β-galactoside-binding animal lectins. Members of galectin family are characterized by sharing conserved amino acid sequences in the carbohydrate-recognition domain (CRD) and their affinity for β-galactosides [108, 109]. There are 15 members which have been identified in mammals. The galectins family is composed of one-CRD type (galectin-1, 2, 5, 7, 10, 11, 13, 14, and 15), which are monomers or homodimers, two-CRD type (galectin-4, 6, 8, 9, and 12), which contains two distinct but homologous CRDs in a single polypeptide chain and a chimeric type (galectin-3), which contains a nonlectin part made of proline- and glycine rich short tandem repeats connected to a CRD. Thus, they can form ordered arrays of lectin-carbohydrates lattices [110].

The secretory pathway for galectin family proteins are yet undefined. They are known to be localized in the cytoplasm and were detected in the extracellular space. However, under certain conditions, they can be transported into the nucleus or associated with intracellular vesicles [108]. Investigations showed that galectins do not have specific individual receptors, but each can bind to a set of cell-surface glycoproteins containing suitable oligosaccharides through lectin–carbohydrate interactions [110].

Furthermore, studies have demonstrated that galectins can function inside the cell in a fashion that is independent of their carbohydrate-binding activities. Galectin-3, in particular, has been shown to bind to a number of intracellular proteins known to participate in intracellular signalling pathways via protein-protein interactions [108].

CLC protein was first described in Paris in 1853 by Charcot and Robin in the post-mortem spleen and heart blood of a leukaemia patient. Nineteen years later, Leyden described the same protein in the sputum of asthmatics ending by its nomination as Charcot-Leyden crystal protein [111]. It was observed as distinctive hexagonal bipyramidal crystals in tissues, body fluids and secretions as hallmarks of allergic inflammation involving eosinophils and basophils [112]. CLC protein is a major constituent in eosinophils comprising about 7-10% of total cellular protein and localized to cytosolic, nuclear and primary granular sites [111, 113]. However, it was also identified in granules of human blood basophils and in basophils that participate in a variety of inflammatory tissue reactions [114, 115]. The facts that eosinophils and basophils contain large quantities of a lysophospholipase (LPLase) enzyme and that eosinophils express 3-8 fold more LPLase activity than other granulocytes suggested a LPLase activity for the CLC protein [112, 116].

However, the CLC protein was shown to possess an extremely low specific activity as a LPLase compared with the enzymatic activities of other known mammalian and prokaryotic LPLases [112].

Moreover, the lack of significant amino acid sequence similarities of CLC protein to any other lipolytic enzymes including LPLase have argued against its role as an eosinophil LPLase [117]. In contrast, CLC protein had sequence similarities to members of the family of S-type or S-Lac (soluble lactose-binding) and IgE-binding animal lectins [117], now known as galectins [118, 119].

Determination of the x-ray crystallographic structure of CLC protein showed that its overall tertiary fold was highly similar to that of the prototype galectins [111]. The structure of CLC protein provided details of a CRD with both similarities to and differences from other members of the galectin family. Taking together, the observation of weak affinity for lactosamine-containing sugars [111, 120], with the highly conserved gene structure comparable with other galectins [121], resulted in CLC protein being designated galectin-10 [122].

Eosinophil galectin-10 is formed by a small (16.5 kDa) hydrophobic polypeptide CLC protein comprising 142 amino acids [117,123]. The protein sequence contains seven out of the twelve conserved residues compromising the CRD but it shares many other residues with the mammalian galectins [117]. It is most similar to galectins 3 and 4 (25-30% overall identity) and least similar to galectins 1 and 2 (15-20% overall identity) [111].

As other galectins, galectin-10 is found as an abundant cytosolic protein and lacks a secretion signal peptide, but under certain conditions might be released extracellularly [124-126]. Regulated non-classical secretion has been demonstrated for galectin-10 protein [127, 128]. The protein is found in the nucleus of both eosinophils and basophils [115, 129, 130]. Although its function is still elusive, it has been proposed that it might plays a biological role pertained to the eosinophils and basophils function in inflammatory allergic reactions.

The ability of galectin-10 to form hexagonal bipyramidal crystals is perhaps the most ambiguous of its physicochemical properties [111]. Although the protein has been reported to exhibit weak lysophospholipase (LPLase) activity [116], enzymatic assays have demonstrated that it loses LPLase activity irreversibly upon crystallization [111, 113]. A study showed that purified soluble galectin-10 protein from AML14.3D10 cell lysates did not possess any significant LPLase activity[113].

Maintaining the mysterious image, so far no any natural carbohydrate ligand is known for this protein, its functional role has to remain speculative. One crystallographic structure study has reported mannose to bind CLC protein in a manner very different from other related galectins that have been shown to bind lactosamine [113].

The change in the topology and chemical nature of the CRD might be attributed to the partial conservation residues involved in carbohydrate binding [113]. Although it was shown that the crystalline protein binds mannose, however, the natural glycoconjugate ligand of galectin-10 has not yet been identified.

Using differential proteomics, an unexpected finding revealed the presence of galectin-10 protein in Tregs compared with $CD4^+CD25^-$ T effector cells [107]. Interestingly, the same study has identified 3 isoforms (A,B,C) of galectin-10 expressed in Tregs, which differ in their isoelectric point and molecular weight. However, the different isoforms described are not yet characterized. Moreover, it was demonstrated that the constitutively expressed novel marker is not only essential for the anergic property of Tregs but even for their suppressive property when co-cultured with $CD4^+CD25^-$ T effector cells [107]. However, the fact that galectin-10 protein remains in the nucleus and cytoplasm and is not apparently secreted or released from either resting or activated Tregs suggested a strictly intracellular function of this protein [107, 131].

The fact that there is no known structural rodent counterpart to the human galectin-10 renders it as specific protein marker for humans. However, the presence of very few numbers of Tregs in human peripheral blood makes the identification and isolation of Tregs for galectin-10 investigation more challenging and time-consuming. Human eosinophils could not provide a better solution for studying galectin-10. That is because eosinophils require tedious purification of small numbers of short-lived cells from large volumes of blood [132].

Therefore, *in vitro* differentiation of eosinophils from HSC provides a promising approach to obtain a stable galectin-10 expressing population in a larger scale. The differentiation of eosinophils from HSC was investigated in many studies [133, 134]. It was shown that galectin-10 is the second most abundantly expressed gene at the mRNA level in both developing eosinophil progenitors from umbilical cord blood [135] and mature eosinophils [136]. However, more investigations on galectin-10 protein expression in eosinophils derived from HSC are still required.

An alternative approach is to search for a tumor cell line which exhibits an advanced eosinophilic phenotype. The search which was crowned by finding a tumor cell line called AML14.3D10 derived from a human myeloid leukemic cell line. The AML14 human myeloid leukemic cell line was established in 1992 from a 68-year-old man who was diagnosed with acute myeloid leukaemia [132]. Freshly isolated peripheral blood blast cells were cultured for a prolonged period in a cocktail of cytokines, including IL-3, granulocyte macrophage-colony-stimulating factor (GM-CSF) and IL-5. After several weeks in culture, a population of cells emerged that exhibited phenotypic characteristics of advanced eosinophilic differentiation [132]. When the AML14 cell line was subcloned by

limiting dilution, a number of clones of varying proliferative and differentiative capacity were obtained [137]. A subclone that both proliferated vigorously and maintained an advanced eosinophilic phenotype grew out of the D10 well of the third 96-well plate. The subclone was selected for further study and designated AML14.3D10 [132]. The subclone maintained an advanced eosinophil phenotype and proliferated with a double speed in the absence of cytokine supplementation [137]. The cells can be easily maintained in a simple culture medium. They represent a continuous, rapidly proliferative source of eosinophil-like cells that can be grown to enormous numbers for investigations [132]. In fact, it is not clear what molecular mechanisms are responsible for the unusual ability of AML14.3D10 to maintain eosinophilic differentiation [132]. The AML14.3D10 cell line produces huge amounts of eosinophil granule proteins including major basic protein, eosinophil derived neurotoxin, eosinophil cationic protein, eosinophil peroxidase and galectin-10 [132].

Aim of the Work

The study presented an approach to transduce $CD34^+$ HSC with lentiviral particles encoding GFP and/or shRNA against galectin-10 or Foxp3. The investigations done intended to test the capability of lentiviral particles encoding shRNA to knock down galectin-10 in AML14.3D10 cells and eosinophils differentiated from $CD34^+$ HSC and Foxp3 in $CD4^+$ T cells *in vitro*. The experiments done intended to study the impact of galectin-10 and Foxp3 knockdown *in vitro* before proceeding into *in vivo* experiments in humanized mice.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory equipments

Instrument	Supplier
Analytical scales	Scout Pro SPU601 (Sartorius AG)
Autoclave	Varioklav Steam sterilizer (H+P Labortechnik GmbH)
Bench-top shaking incubator	Thermo Fisher Scientific
Camera system for microscope	3 CCD color videocameraA/15014 (AVT Horn), Spot insight camera (model #3.1.0; Diagnostic instruments Inc., Sterling Heights)
Centrifuges	Sorvall RT 6000D, Multifuge 3L-R, Biofuge primo R (Heraues), Sorvall RC5C Plus (Du Pont Instruments)
Clean bench	HERAsafe HS12 (LaminarAir® Heraeus)
Counting chamber	Neubauer counting chamber improved (VWR)
Depletion magnet	DYNAL MPC-6, 50 (Dynal)
DNA-electrophoresis-chamber	BioRad
ELISA-photometer	Immunoreader NJ-2000 (Intermed, Nunc)
FACSCalibur (Fluorescence activated cell sorting)	FACScan (Becton-Dickinson) CellQuestPro™ SoftwareV3.3 1994 -1999
Fluorescence reader	SpectraFlourreader (TECAM)
Heating block DRI-BLOCK BD 2D	Techne
Incubator	Hera Cell 240 (Heraus)
Irradiator	Gammacell 2000 (Isgaard medical)
Magnetic cell separator	VarioMACS-Separator (Milteny-Biotech)
Microscope	Leitz SM-LUC, Axiovert 135 (Carl Zeiss)
Microwave	Severin 800 (Sundern)
Minicoldlab	2023 Minicold Lab (LKB)
Nano-Drop spectrometer	NanoDrop 2000 (PeqLab Biotechnologie GmbH)
PCR-Cycler	i-Cycler (Biorad) Light-Cycler (Roche)
pH-meter	CG 841 (Schott AG)
Refrigerated incubator shaker (Innova 4230)	Artisan Scientific

Sample mixer	Dynal sampel-Mixer (Dynal)
Shaker	Polymax 2040 (Heidolph)
Thermomixer	Thermomixer compact (Eppendorf AG)
Ultracentrifuge	Kontron Centrikon T-1170 (Laborgerätebörse)
Vortexer	Vortex Genix2^{TM} (Bender & Hobein AG)

2.1.2 Chemicals

Chemical	Supplier
Agarose NEEO Ultra Quality	Roth
Aqua destilled water 1 Liter	B.Braun, Sigma
37% Formaldehyde	Roth
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Sigma
Calcium Cloride ($CaCl_2$)	Merck
Dimethylsulfoxide (DMSO)	Sigma
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma
Ethylene di-amine tetra-acetic acid (EDTA)	AppliChem
Ethanol	Roth
Ethidium bromide	Sigma
Fibronectin	Roche
L-glutamine (GlutaMAX^{TM})	Invitrogen
Hydrocloric acid (HCl)	Merck
Isopropanol	Merck
Penicillin Streptomcin 100 ml	Invitrogen
Propidium iodide (PI)	Sigma
Protamine sulfate	Sigma
Sandoglobulin	CSL Behring
Sodium biocarbonate	Merck
Sodium butyrate	Sigma
Sodium chloride (NaCl)	Roth
Sodium dihydrogen phosphate dihydrate	Merck
Sodium Hydroxide (NaOH)	Merck
Sodium pyruvate	Biochrom AG
tris(Hydroxymethyl)aminomethan (Tris)	Roth

Trypsin-EDTA	PAA
Tween-20	Merck

2.1.3 Plastic and glass wares

Material(s)	Supplier
Cell culture flasks (75 cm^2)	TPP
Counting chamber Neubauer Improved	VWR
Cover slips	Menzel
Cryotubes (2 ml)	Nunc
Eppendorf tubes (0.5,1.5,2 ml)	Sarstedt
FACS plates	Greiner
FACS tubes	BD Pharmingen™
Falcon sterile centrifuge tubes (15 and 50 ml)	Greiner
Glass pipettes (10, 25)	Greiner
Parafilm	VWR
Pasteur pipettes	Brand
Pipette tips (10 µl, 200 µl and 1000 µl)	Greiner
Sterile filters (0.22 µm and 0.45 µm)	Millipore
sterile Pipettes (2 ml, 5 ml, 10 ml, 25 ml)	BD Pharmingen™
Syringes (5,10 and 20 ml)	BD Pharmingen™
Tissue culture dishes (10 cm)	Greiner Bio-One
Tissue culture plates (6, 12, 24, 96 well)	Costar
Ultracentrifuges tubes	Beranek

2.1.4 Buffers, media and solutions

Buffer or Solution	Ingredients, Features and Supplier
Annealing buffer (5 x)	0.5 M Tris (pH 7.4-7.5) 0.35 M $MgCl_2$
Bovine serum albumin BSA (10%)	10% (m/v) BSA (Sigma) dH_2O, Sterile-filter and store at 4°C
CFSE(CFDA-SE)	5,6-Carboxyflouresceine diacetate succinimidyl ester (5-(6)-CFDASE;CFSE) purchased from Molecular Probes Europe BV, diluted to 2 mM and aliquots stored at -20°C,

	and used for marking T cells
DMEM (Dulbecco's Modified Eagle Medium)	Life technologies (#41966029)
DNA Ladder	New England Biolabs
DNA loading buffer (6 x)	0.05% bromophenol blue (Serva) 0.05% xylene (Merck) 15% Ficoll Type 400(PAA) dH_2O
Dynalbeads depleting media	MEM + 2% HSA (Alburex® 20, CSL Behring)
eFlour®670 cell labeling	eBioscience
FACS fixation solution	2% Paraformaldehyde in PBS
FACS-buffer	1x PBS 0.5% HSA 1 mM EDTA 10 µg/ml Sandoglobulin (CSL Behring), (store at 4°C)
FACS Staining Buffer (Galectin-10 FACS Staining)	1x PBS 3% FCS 0.5% Tween-20
FCS (Fetal Calf Serum)	FCS charge A01129-377 (PAA Laboratories #A15-649). Inactivating the complement components of the serum for 30 m by heating at 56°C for 30 m and then aliquots of 100 ml were stored at –20°C
Freezing Medium	HSA (Human Serum Albumin) (Alburex® 20, CSL Behring) 20% DMSO (Dimethylsulfoxide) (Sigma)
Ficoll (Bicoll Separating Solution)	Biocoll Separating Solution; density: 1.077 (Biochrome, #L-6115) Lymphocyte Separating Medium; density (LSM):1.077 (PAA, J#15-004)
2x HBS	281 mM NaCl 100 mM HEPES 1.5 mM Na_2HPO_4,pH= 7.12 sterile filtered through 0.45 filter and aliquots were stored at –20°C
MACS Buffer	1x PBS

	0.5% HSA
	3 mM EDTA (AppliChem)
MEM (Minimal Essential Medium)	For 5 liters media
	49 g MEM (Life technologies)
	4.77 g/l HEPES (Biochrom AG)
	2.2 g/l Sodiumbicarbonate (Merck)
	3.6 ml/l Mercaptoethanol (Serva, #28625)
PBS (Phosphate buffered saline)	80.4 g/l Sodium chloride (Roth)
Stock solution (10x)	15.6 g/l Sodium dihydrogen phosphate dihydrate (Merck)
	adjusted with 10 N sodium hydroxide to 6.6 then autoclaved
PBS/EDTA	1x PBS
	1 mM EDTA (AppliChem)
PBS/EDTA/Liquemin	1x PBS
	1 mM EDTA (AppliChem)
	0.2% Liquemin N2500 (Roche)
RPMI 1640 (Roswell Park Memorial Institute)	without L-Glutamine, Bio*Whittaker (#12-167F)
RPMI 1640	For 10 liters media
	102 g RPMI 1640 (Life technologies)
	3.6 g/l HEPES (Biochrom AG)
	10 ml/l Penicillin-Streptomycin (Biochrom AG)
	10 ml/l Sodium pyruvate (Biochrom AG)
	2 g/l Sodium bicarbonate (Merck)
	10 ml/l Non-essential amino acid (Biochrom AG)
	3.5 ml/l Mercaptoethanol (Serva, #28625)
	100 µl/l Indomethacine
	pH = 7.2
STE Buffer	0.1 M NaCl
	10 mM Tris buffer
	1 mM EDTA
	20% sucrose
TAE (50x)	1220 g TRIS-HCl
	285.5 g acetic acid
	500ml 0.5M EDTA

	5L dH_2O pH = 8.0
Trypan Blue Solution	Trypan blue stained dead cells while living cells with intact cell membrane were not stained, with this solution the number of living cells in a cell suspension can be determined. Trypan blue solution (0.4%)(Sigma, #T8154)
X-Vivo-15	Lonza Verviers (#BE04-418Q)

2.1.5 Commercially available kits

Kit	Supplier
Block-iT™ U6 RNAi Entry Vector kit	#K4944-00 and K4945-00 Life technologies
Cell Fixation/Permeabilization Kits for Intracellular Cytokine Analysis	#554715 BD
FastStart Taq DNA Polymerase, dNTPack	#04738314001 Roche
Foxp3 Fixation/Permeabilization Concentrate and Diluent, Permeabilization Buffer (10X)	#5521-00, #88333-56 eBioscience
HIV-1 p24 Antigen ELISA	#0801111 ZeptoMatrix Corporation
Qiagen gel extraction kit	#28704 Qiagen
Qiagen Plasmid Maxi kit	#12163 Qiagen
Qiagen Plasmid Mini kit	#12125 Qiagen

2.1.6 Enzymes and markers

Enzyme or Marker	Supplier
100bp-Standard marker	New England Biolabs
1kb-Standard marker	New England Biolabs
Restriction enzymes	New England Biolabs
RNase (DNase free)	Roche

2.1.7 Oligonucleotides

All oligonucleotides were purchased from Life Technologies

Name	Primer Sequence (5' to 3')
pENTR/U6 Top	AGCTATCGATCTGGATCCGGTACCAAG
pENTR/U6 Bottom	AGCTGAATTCGCTGCCAGGAAACAGC
shgalectin-10 Top	CACCCTACTGGTTCTACTGTGACAACTCGAGTTGTCACAGTAGAACCAGTAG
shgalectin-10 Bottom	AAAACTACTGGTTCTACTGTGACAACTCGAGTTGTCACAGTAGAACCAGTAG
shFoxp3 Top	CACCGCATGTTTGCCTTCTTCAGAAAGAGCTTGTTCTGAAGAAGGCAAACATGC
shFoxp3 Bottom	AAAAGCATGTTTGCCTTCTTCAGAACAAGCTCTTTCTGAAGAAGGCAAACATGC
shnon-target Top	CACCCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG
shnon-target Bottom	AAAACAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG

2.1.8 Plasmids

Name	Discription
pCDH-CMV-MCS-EF1-copGFP	Gene transfer monocistronic vector optimizied for high expression of GFP with EF1 promoter
pCDH-U6-shFoxp3-EF1-copGFP	Gene transfer bicistronic vector optimizied for high expression of GFP with EF1 promoter and shRNA against Foxp3 with U6 promoter
pCDH-U6-shgal-10-EF1-copGFP	Gene transfer bicistronic vector optimizied for high expression of GFP with EF1 promoter and shRNA against galectin-10 with U6 promoter
pCDH-U6-shnon-target-EF1-copGFP	Gene transfer bicistronic vector optimizied for high expression of GFP with EF1 promoter and non targeting shRNA with U6 promoter that will activate RISC and RNAi pathway, but does not target any human or mouse genes. This allows for examination of the effects of shRNA transduction on gene expression.

pCMVΔ8.91	Packaging vector for the expression of Gag/Pol genes with CMV promoter
pENTR™/U6	Linearized vector in which the ds oligo encoding the shRNA of interest cloned to generate an entry clone containing the U6 RNAi cassette (human U6 promoter + ds oligo + Pol III terminator)
pLK0.1-puro	Gene transfer monocistronic vector for expression of shRNA against galectin-10 with U6 promoter (Sigma-Aldrich)
pMD.G	Envelope vector for the expression of vesicular stomatitis virus G (VSV-G) protein with CMV promoter

2.1.9 Media

2.1.9.1 Cell culture

Cell culture medium RPMI 1640 and DMEM were purchased from Gibco BRL. For eukaryotic cell lines culture, RPMI and DMEM were supplemented with:

10% (v/v)	Fetal calf serum (FBS) (heat inactivated, 56°C, 30 m)	Pan Biotech GmbH
1% (v/v)	L-Glutamine	PAA
1% (v/v)	Penicillin/Streptomycin	PAA

For AML14.3D10 cell line RPMI was supplemented with

10% (v/v)	Fetal calf serum (FBS) (heat inactivated, 56°C, 30 m)	Pan Biotech GmbH
2% (v/v)	L-Glutamine	PAA
1% (v/v)	Penicillin/Streptomycin	PAA
1% (v/v)	Non-essential amino acids	Bio Whittaker
1% (v/v)	Sodium pyruvate	Bio Whittaker

2.1.9.2 Bacteria culture

Media were autoclaved for 20 m at 121°C at a steam pressure of 180 to 200 kPa. Agar-containing growth medium was cooled down to 55°C before the addition of antibiotics.

Medium	Ingredients, Features and Supplier(s)
Luria Broth (LB) medium	20g LB (Invitrogen) 1000 ml H_2O 1 ml ampicillin, store at 4°C
LB medium (Agar)	20g LB (Invitrogen) 1000 ml H_2O 40 µg/ml Ampicilin, Store plates at 4°C

2.1.10 Cell lines

Cell Line	Description	Medium/Application
AML14.3D10 [132]	Human acute myeloid leukemia	see 2.1.9.1 Cell Culture / Galectin-10 expression
HEK 293T [138]	Human embryonic kidney	DMEM / Viral particles production
Raji [139]	Human B lymphocyte	DMEM / Determination of transduction unit/ml
Karpas-299 [140]	Human T-cell lymphoma	RPMI / Foxp3 expression

2.1.11 Antibodies

Primary Antibodies	Marker	Type (clone)	Supplier
Mouse anti-human-Foxp3	PE (phycoerythrine)/ Allophycocyanin (APC)	Monoclonal, isotype IgG1, clone: MAR4	eBioscience
Mouse anti-human galectin-10	-	Monoclonal, isotype IgG1, clone: B-F42	Diaclone Immunology Products
Mouse anti-human CD25	FITC (Fluorescein Isothiocyanate)/ PE/ phycoerythrine cyanine dye Cy5™ (PE-Cy5)	Monoclonal, isotype IgG1, clone: M-A251	BD Pharmingen™
Mouse anti-human CD33	PE	Monoclonal, isotype IgG1, clone: WM53	BD Pharmingen™
Mouse anti-human CD34	PE	Monoclonal, isotype IgG1,	BD Pharmingen™

		clone: 563	
Mouse anti-human CD40L(CD154)	PE	Monoclonal, isotype IgG1, clone: TRAP1	BD Pharmingen™
Mouse anti-human CD54	-	Monoclonal, isotype IgG1, clone: 28/CD54	BD Pharmingen™
Mouse anti-human CD80	-	Monoclonal, isotype IgG1, clone: L307.4	BD Pharmingen™
Mouse anti-human CD86	-	Monoclonal, isotype IgG1, clone: 2331(FUN-1)	BD Pharmingen™
Mouse anti-human CD123	PE	Monoclonal, isotype IgG1, clone: 9F5	BD Pharmingen™
Mouse anti-human HLA-class I	-	Monoclonal, isotype IgG1, clone: MEM-147	ImmunoTools
Mouse anti-human HLA-DR	PE	Monoclonal, isotype IgG2a, clone: G46-6	BD Pharmingen™

Isotype Antibodies	**Type (clone)**	**Supplier**
Mouse IgG1	MOPC-21	BD Pharmingen™
Mouse IgG2a	MOPC-173	BD Pharmingen™
Rat IgG2a	R35-95	BD Pharmingen™

Secondary Antibodies	**Marker**	**Supplier**
Donkey anti-mouse IgG	PE	Dianova
Donkey anti-rat IgG	PE	Dianova
Rat anti-mouse IgG2a+b	PE	BD Pharmingen™
Mouse anti-rat	PE	BD Pharmingen™

IgG2a

Antibodies	Type (clone)	Supplier
Mouse anti-human CD3	Monoclonal, isotype IgG1k, clone: OKT-3	-
Mouse anti-human CD28	Monoclonal, isotype IgG1k, clone: CD28.2	BD Pharmingen™

Antibodies for Cell Separation

Antibodies	Type (clone)	Supplier
CD4	Monoclonal, Isotype IgG1, clone:SK3	Miltenyi Biotec
CD25	Monoclonal, Isotype IgG21	Miltenyi Biotec
CD34	Monoclonal, Isotype IgG1, clone AC136	Miltenyi Biotec

Dynabeads for depletion	Supplier
CD8	Life Technologies
CD14	Life Technologies
CD19	Life Technologies
CD25	Life Technologies

2.1.12 Cytokines

Cytokine	Supplier
FLT3L	#308-FK-005 (R & D Systems GmbH)
GM-CSF	Sargramostim
IL-2	#AI-96E0816 (Chiron-Behring)
IL-3	#203-IL-010 (R & D Systems GmbH)
IL-5	#205-IL-005 (R & D Systems GmbH)
IL-6	#206-IL-010 (R & D Systems GmbH)
IL-7	#207-IL-005 (R & D Systems GmbH)
SCF	University Medical Center – Universitätsmedizin Mainz
TPO	University Medical Center – Universitätsmedizin Mainz
TGF-β	#240-B (R & D Systems GmbH)

2.1.13 Bacteria

Escherichia coli (E. coli), TOP10 strain, was provided by Invitrogen. The genotype of *E. coli* TOP10 is: F^- mcrA Δ(*mmr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 ΔlacX74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*K *rps*L (Str^R) *end*A1 *nup*G.

2.2 Molecular biology methods

2.2.1 DNA digestion with restriction enzymes

All DNA restrictions were performed using commercially available restriction endonucleases according to the supplier‘s instructions. All reactions were performed in a total volume of 25 µl for 1-3 h at 37°C or the optimal temperature for the restriction enzyme according to the manufacturer instructions. In case of double digestion an optimal buffer was used according to the supplier‘s instructions.

2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis allows the separation of linear DNA fragments according to their molecular weight. To prepare the gels, the corresponding amount of agarose was dissolved in 1x TAE buffer (1:100 w/v) using a microwave oven. Ethidium bromide (0.1 µg/µl) was added before casting the gel to intercalate into- and visualize the DNA strands under UV light. After polymerization, gels were loaded onto a tray in an electrophoresis chamber and covered in 1x TAE buffer and then loaded with the DNA samples. DNA samples were mixed with 6x loading dye before loading. DNA ladder was used as a marker. DNA fragments were separated by applying 80-120 V for 25-120 m and then visualized on a trans-illuminator and documented or extracted from the gel.

2.2.3 Isolation of DNA fragments from agarose gel

Qiagen gel extraction kit was used according to the manufacturer‘s instructions to isolate DNA fragments from agarose gels. Briefly, after gel separation, gel piece containing the DNA fragment of interest was transferred into a 1.5 ml micro tube. After melting the piece at 50ºC with a high-salt supplied buffer, it was transferred into micro spin cups containing a surface-modified silica membrane to bind the DNA. DNA was eluted from the membrane with water.

2.2.4 Production of pENTR™/U6 vector containing ds oligo encoding the shRNA of interest

Block-iT™ U6 RNAi Entry Vector kit was used according to the manufacturer‘s instructions. Briefly, complementary DNA oligos (top and bottom) were annealed to generate a ds oligo which was cloned into linearized pENTR™/U6 vector using T4 DNA

ligase and the ligation mixture was transformed into one shot® TOP10 chemically competent *E. coli*.

2.2.5 Transformation of *Escherichia coli* with plasmid DNA

Transformation of *E. coli* was the method of choice to amplify plasmid DNA through cellular replication. A microtube containing 50 µl *E. coli* TOP 10 bacteria was thawed on ice and approximately 100 ng DNA or 10 µl of a ligation reaction was added into the bacteria and mixed. Bacterial cells were then incubated on ice for 30 m and subsequently heat shocked for 45 s at 42°C and then transferred back into ice for 30 s. After that, 500 µl of pre-warmed (37°C) LB medium was added to the bacteria followed by incubation at 37°C for 45 m with shaking. 70 µl of the bacteria suspension was applied to LB plates supplemented with ampicillin and incubated at 37°C over-night. Plates were checked for the presence of colonies in the following day and stored at 4°C until required for DNA mini preparation culture inoculations.

2.2.6 Mini preparation of plasmid DNA

Single bacterial colonies were picked from transformation plates and inoculated into 15 ml Falcon tubes containing 3 ml of LB medium supplemented with Ampicillin. Bacterial cultures were incubated overnight in a bench-top shaking incubator at 37°C with vigorous shaking. One and a half ml of starter cultures were used for plasmid preparation, the remainder was stored at 4°C until required for DNA maxi preparation culture inoculation. The Qiagen miniprep kit was used to isolate plasmid DNA from transformed bacteria according to the manufacturer‘s instructions. This kit procedure relies on a modified alkaline/SDS method to prepare cleared lysates from bacteria. After neutralization, the cleared lysate was applied directly onto a Qiagen column to have the plasmid DNA bound to an adsorption matrix. A washing step was performed to remove RNA, proteins and all other impurities. The purified plasmid DNA was eluted from the spin column with water.

2.2.7 Maxi preparation of plasmid DNA

To isolate large amounts of DNA, 250 ml LB medium, supplemented with ampicillin, was inoculated with transformed bacteria and cultivated over night at 37°C in 1500 ml glass flasks. Preparation of plasmid DNA from transformed bacteria was performed using Qiagen plasmid maxi kit according to the manufacturer‘s instructions. This kit employs an anion exchange resin where plasmid DNA selectively binds under low salt and pH conditions. RNA, proteins, metabolites, and other low molecular weight impurities were removed with a medium salt buffer, and pure plasmid DNA was eluted

with a high salt buffer. The DNA was concentrated and desalted with isopropanol precipitation and collected by centrifugation.

2.2.8 Culture conditions and preservation of *E. coli*

After transformation, plasmid-containing *E.coli* bacteria were cultured in an ampicillin supplemented LB-medium (100 µg/ml). Single clone colonies were produced by plating *E. coli* bacteria on LB-agar at 37°C for 12 to 16 h. Agar plates were stored at 4°C and used for inoculation of mini and maxi preparations for several weeks. For cryo stock preparation, *E. coli* was cultured over-night in a 37°C shaking incubator. 500 µl of the culture was mixed with 500 µl 7% DMSO in H_2O and stored at -80°C. Fresh cultures were inoculated by scratching a small amount of bacteria out of the cryo-tube and transferring it into LB-ampicillin medium.

2.2.9 Sequencing of DNA preparations

DNA sequencing was performed at GENterprise GENOMICS in Mainz. For this purpose, 0.5 µg plasmid (conc.:50-500 ng/µl) and 10 pmol/µl sequencing primer in 5-15 µl total volume were prepared and transferred to the sequencing facility.

2.2.10 Polymerase Chain Reaction (PCR)

PCR is a standard method to amplify specific DNA sequences. The amplified fragments can be used for further molecular biological applications. DNA polymerase amplifies a DNA template in the presence of appropriate buffers, oligonucleotides (primers), deoxynucleotides and cycling conditions in an exponential manner. A typical PCR cycle comprises a denaturizing step at 95°C, leading to the dissociation of double stranded template followed by a hybridization step that allows primer annealing to complementary sequences on a single stranded template. The hybridization temperature depends on G/C-A/T content in primers. It can be calculated roughly according to the Wallace rule TH = 4x(G+C) + 2x(A+T) - 5. After hybridization, DNA elongation takes place at an optimum temperature for DNA-polymerase. Repeating this cycle leads to an exponential amplification of template DNA.

2.2.10.1 Standard PCR

FastStart Taq DNA Polymerase, dNTPack kit was used for perfoming the PCR. All reactions were performed using 0.5 µg template genomic DNA (0.5 µl), 10x PCR buffer (5 µl), 10 mM dNTPs (10 µl), (5 µl) of each 10 pmol/µl oligonucleotide primer pairs and 0.5 µl FastStart Taq DNA polymerase (5 U/µl) per reaction in a total volume of 50 µl in distilled H_2O. Cycling conditions for the target sequences are described in the following table.

Cycling step	Cycles	Time	Temparature
1- Initial denaturation	1	4 m	95ºC
2- Denaturation	35	30 s	95ºC
3- Annealing	35	30 s	50ºC
4- Elongation	35	90 s	72ºC
5- Final extension	1	7 m	72ºC
6- Cooling		Unlimited time	4ºC

2.2.11 Cloning of shRNA into monocistronic GFP gene transfer vector to obtain GFP+shRNA bicistronic vectors

Top and bottom oligonucleotides of shgalectin-10, shFoxp3 and shnon-target primers were annealed to generate double-stranded oligonucleotides (see 2.2.4). The shnon-target (shNT sequence) does not target any gene. This allows for examination of the effects of shRNA transduction on gene expression. Integrity of ds oligonucleotides was verified using 4% agarose gel electrophoresis where detectable higher and lower molecular weight bands representing annealed ds olgionucleotides and unannealed single stranded oligonucleotides respectively were seen. The ds oligonucleotides were cloned in the linearized pENTR™/U6 vector. Products of ligation reaction were transformed into one shot® TOP10 chemically competent *E.coli*. Three colonies from each ligation reaction were selected and cultured to obtain reasonable quantity of vectors to perform sequencing. Using M13 Reverse Primers, sequencing results showed successful cloning of the ds oligonucleotide inserts into pENTR™/U6 vectors.

PCR from pENTR™/U6 vectors for the U6 promoters including the shRNA inserts were carried out using pENTR/U6 top and bottom primers. PCR products were verified using 1% agarose gel electrophoresis and showed successful amplification of shRNA with their U6 promoters. PCR products were cut and purified from the gel and subjected to digestion by EcoRI and claI restriction enzymes. To ensure successful digestion of PCR products, pCDH-CMV-MCS-EF1-copGFP vector was digested with the same enzymes under the same conditions and the digestion products were verified using 1% gel electrophoresis.

Vector backbone and PCR digested products were cut and purified from the gel. Ligation for each PCR digested product with pCDH-CMV-MCS-EF1-copGFP vector backbone was carried out. The products of ligation reactions were transformed into one shot® TOP10 chemically competent *E.coli*. Three colonies from each ligation reaction were selected and cultured to obtain reasonable quantity of vectors to perform sequencing.

Sequencing results using pENTR/U6 bottom primer showed successful cloning of the shRNA genes with their U6 promoters to obtain pCDH-U6-shRNA-EF1-copGFP bicistronic vectors.

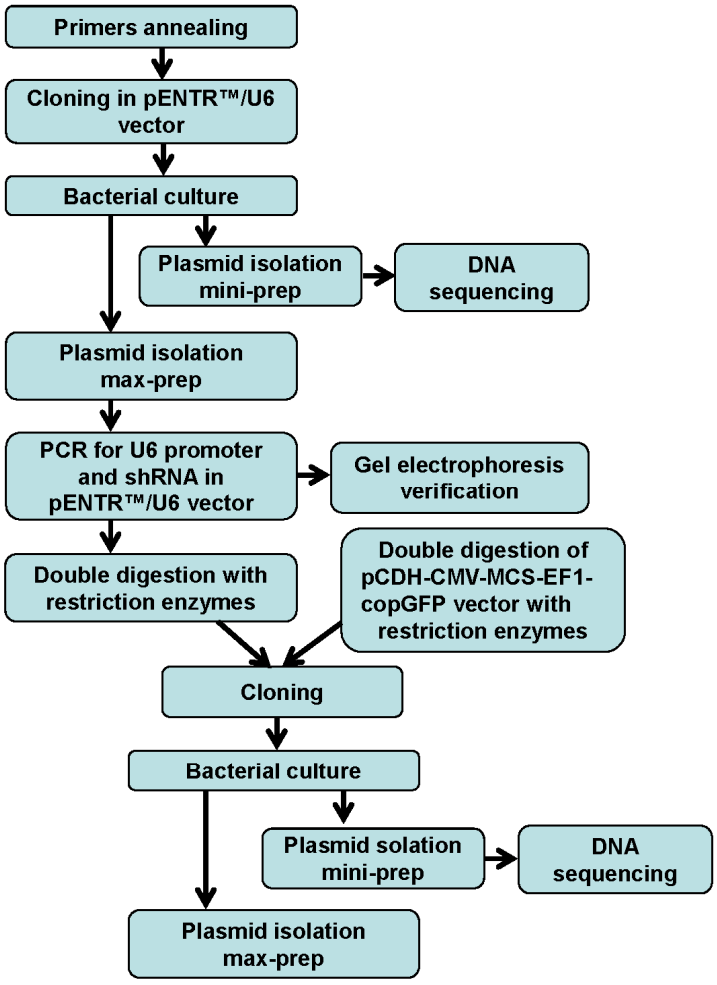

Fig. 2.1: Schematic representation for the main molecular biology steps done to obtain GFP+shRNA bicistronic gene transfer vectors

2.3 Cell culture methods

2.3.1 Isolation of peripheral blood mononuclear cells (PBMC) by Ficoll-Hypaque gradient centrifugation

Heparinized blood was placed into 100 ml sterile conical flask using a sterile pipet, the flask was filled upto 100 ml with PBS/EDTA/Liquemin. 15 ml Ficoll-Hypaque solution added to 50 ml tube and blood/PBS mixture slowly layered on the tube wall to maintain Ficoll-Hypaque/blood interface, it is helpful to hold the centrifuge tube at a 45º angle. Centrifuation for 30 m at 350 xg at 24°C, with no brake was carried out and using a sterile pipet, the upper layer that contains plasma and most of platelets were removed and

mononuclear cell layer was transferred to another centrifuge tube. Cells were washed by adding excess PBS/EDTA and centrifuged for 6 m, 350 xg at 4°C. Supernatant was removed, cells were resuspended in PBS/EDTA and washing step was repeated to remove most of remaining platelets. After the final washing step, supernatant was removed and cell pellet was resuspended in X-Vivo-15 and counted to determine viability by trypan blue exclusion.

2.3.2 Isolation and stimulation of human T cell populations

$CD4^+$ T cells and $CD25^+$ Tregs were isolated from buffy coats of healthy volunteers as described before [103, 141]. Briefly, $CD25^+$ cells were separated using limited amounts of $CD25^+$ microbeads resulting in CD25-high cells. Afterward, undesired cell populations were depleted using $CD14^+$ (1 bead/cell), $CD8^+$ (2 beads/cell), and $CD19^+$ (2 beads/cell) Dynabeads according to the manufacturer's protocol resulting in a purity of $CD4^+CD25^{high}$ T cells greater than 95%. $CD4^+$ T cells were isolated using $CD4^+$ microbeads, afterward, $CD25^+$ T cells were depleted with CD25-Dynabeads (0.5 bead/cell) according to the manufacturer's protocol. For polyclonal activation of T cells, 0.5 µg/ml anti-CD3 mAb (OKT-3) and 1 µg/mL anti-CD28 mAb were used. In T cell proliferation assays, 5×10^5 T cells were stimulated with 0.5 µg/ml anti-CD3 (OKT-3) in the presence of irradiated PBMC in 1:2 (Tregs:PBMC) and 1:1:2 (Tregs:$CD4^+$ T cells:PBMC) ratios in 24 well-plates [58, 141].

2.3.3 Cell proliferation dye labeling

T cells were washed with warm PBS to remove serum. For CFSE labeling, cells labeled with 0.5 µM of CFSE for 10 m at 37°C. Reaction was stopped with warm X-Vivo-15 +10% HSA, cells were washed with warm X-Vivo-15. For eFluor®670, cells labeled with 1 µM of eFluor®670 for 10 m at 37°C. Reaction was stopped by incubating cells with cold X-Vivo-15 +10% HSA for 5 m on ice, cells were washed with X-Vivo-15 +10% HSA 3 times, and resuspended in X-Vivo-15. For labeling with both dyes, cells were counted and seeded according to required concentration. Proliferation was controlled by flow cytometry at different time points.

2.3.4 Collection and isolation of $CD34^+$ hematopoietic stem cells (HSC) from cord blood

Cord blood samples were obtained after informed written consent from the obstetric department of the University Hospital Mainz with approval by the local ethical committee of the University Medical Center Mainz. $CD34^+$ HSC cells were purified by immunomagnetic positive selection using immunomagnetic bead system. More than 95%

of the enriched cells were $CD34^+$ cells, as shown by flow cytometric analysis. $CD34^+$ HSC cells were used either immediately or after storage in liquid nitrogen.

2.3.5 Freezing and thawing of cells

For long-term storage, cells were kept in liquid nitrogen. Cultured suspension cells were counted. Adherent cells were trypsinized, diluted in excess of complete culture medium and counted. Cell suspensions were then transferred into a Falcon tube and pelleted with centrifugation for 5 m at 350 xg. Supernatants were discarded and cell pellets were re-suspended in 500 µl HSA and transferred into cryotube containing 500 µl freezing medium and stored at -80°C overnight and then transferred into liquid nitrogen containers.

To restore frozen cells in cryotubes, cells were thawed rapidly in a water bath at 37°C. Cell suspension was immediately transferred into a 50 ml falcon tube containing 2-3 ml HSA, the tube was filled with cold RPMI and centrifuged for 6 m at 350 xg. The supernatant was discarded and the cell pellet was re-suspended in cold RPMI then centrifuged for 6 m at 350 xg. The supernatant was discarded and cell pellet was re-suspended in suitable media and seeded into appropriate cell culture flasks. 24 h later, fresh medium was added in case of resuspended cells or medium was exchanged in case of adherent cells.

2.3.6 Cell counting by means of 'counting chamber'

Cells were counted using Neubauer cell counting chamber. 10 µl of cell suspension was diluted with 1x trypan blue dye which stains dead cells so that living cells could be differentiated and counted. Cell suspension (10 µl) was introduced into space between a glass cover and a counting chamber surface. Chamber contains 9 big squares (each contains 9 small squares). Cells in two corner big squares were counted and cell count was calculated by the following formula:

Cell no. per ml = average cell no. per 1 big square x dilution factor x volume factor (10^4)

2.3.7 Cultivation of cell lines

HEK 293T were cultivated in complete DMEM medium at $5x10^5$ cells/ml and incubated at 37°C, 5% CO_2 and saturated water atmosphere and were passaged twice a week when they were grown to 80-90% confluency. For passaging, cells were washed once with PBS and then detached using Trypsin-EDTA which was added to cover the entire monolayer. After 4 m incubation time, cells were washed from the flask bottom using DMEM complete medium and carefully resuspended. Cultures were split into new flasks containing fresh medium at a ratio of 1:10 to 1:15 depending on their growth rate. Cells after passage 15 were not used or if they showed slow growth rate. AML14.3D10,

Karpas-299 and Raji cells are resuspended cells. It was critical to prevent AML14.3D10 cells from exceeding a density greater than 1 x 10^6/ml. For all cell lines, growth rate was always controlled and split whenever required.

2.3.8 Transient transfection of HEK 293T cells and production of lentiviral particles

Recombinant viral particles were produced through transfection of HEK 293T cells with three plasmids to generate replication deficient viruses. Calcium phosphate method which is based on slow mixing of HEPES buffered saline (HBS) containing sodium phosphate with calcium chloride which leads to the formation of a calcium phosphate-DNA precipitate that is taken up by the cells through endocytosis [142] (Figure 2.2).

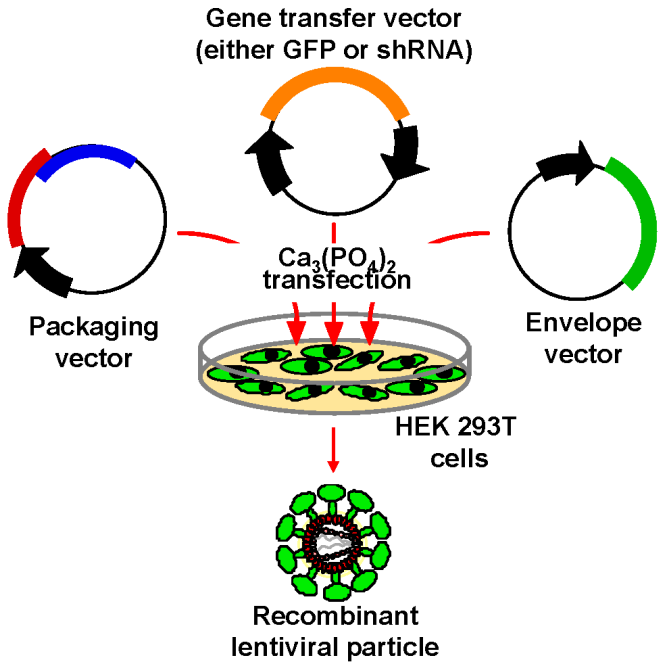

Fig. 2.2: The packaging procedure for lentiviral particles

The structural and replication genes necessary to produce viral particles are separated into three plasmids. Lentiviral particles are packaged in producer cell line such as HEK 293T cells. Upon co-transfection of the plasmids using $Ca_3(PO_4)_2$ method, all required sequences are available to produce and package a viral particle containing the transgene of interest [143].

24 h prior to transfection 5x10^6 HEK 293T cells in 7 ml DMEM medium were seeded into T75 flask. On the next day, medium was exchanged with 10 ml complete DMEM medium. All plasmids were highly purified using Qiagen Plasmid Maxi kit and adjusted to 1 µg/µl concentration. Transfection master mix was prepared in 50 ml rounded bottom tube for one flask and contained

Ingredients	Volume (µl)
1- pMDG	4.5
2- pCMVΔR8.91	8.5
3- Gene Transfer vector	13
4- 2.5 M $CaCl_2$	50
5- dH_2O	424

500 µl of 2x HBS was added drop-wise to the mixture in 50 ml rounded bottom tube under vortexing. Subsequently, final 1 ml mixture was added to the culture flask. After 17 h medium was exchanged with 10 ml fresh DMEM medium. Medium containing viral particles (supernatant) was harvested 48 and 72 h after transfection. Collected supernatants were sterile filtered through a 0.45 µm membrane and ultracentrifuged.

2.3.9 Ultracentrifugation of lentiviral supernatants

Sterile filtered supernatant was ultracentrifuged at 4°C for 3.5 hr at high speed or frozen at -80°C till ultracentrifugation was carried on. Ultracentrifugation tubes were filled with 10 ml lentiviral supernatant and 1.5 ml STE buffer (mainly 20% sucrose) and accurately balanced before ultracentrifugation. After that, lentiviral particles stuck on the bottom of ultracentrifuge tubes were recovered by good resuspending in 150 µl PBS after getting rid of supernatant, Aliquots of 30 µl lentiviral vector stocks were stored at -80°C till use.

2.3.10 Titration of lentiviral supernatants

Infectivity of the lentiviral particles produced is expressed in transduction unit per ml (TU/ml). The titer of produced lentiviral particles (TU/ml) is determined by titration of 30 µl aliquot of ultracentrifuged lentiviral particle stock in 270 µl media (1:10 dilution, Figure 2.3).

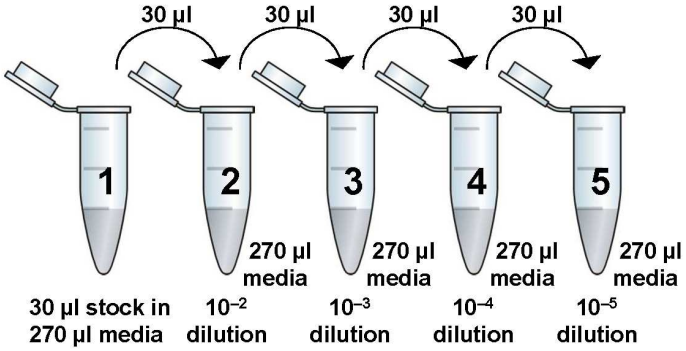

Fig. 2.3: Titration of ultracentrifuged lentiviral particles stock by 1:10 serial fold dilution
Titrating ultracentrifuged lentiviral particles by 1:10 serial fold dilution in order to determine the infectivity of viral particles by transduction of 250 µl of particles from each tube into $1x10^5$ cells in 50 µl media.

Cell line used for determination of titer was either Raji cells or AML14.3D10. In 6 wells of 48 well plate, $1x10^5$ cells in 50 µl suitable media were seeded in each well, 250 µl from each tube of titered dilutions of lentiviral particles were added to each well, 250 µl of medium without particles was added to one well as control. Transduction was carried out by centrifugation under 860 xg for 60 m at 32ºC, after 4 h from centrifugation, media were filled up to 1 ml. After 48 h from transduction, cells were subjected to analysis of GFP expression by flow cytometry. GFP expression of cells between 2% and 20% reflects the tranduction of one viral particle per cell.

The transducing unit/ml was calculated by the general formula

Transducing unit (TU/ml) = F × D / V (ml)

F = (percentage of GFP positive cells × number of cells at the time of transduction)/100, D = fold dilution of vector sample used for transduction and V = volume (ml) of diluted vector sample added into each well for transduction.

Example for determination of TU/ml in Raji and AML14.3D10 cells:

For Raji cells, F = (percentage of GFP-positive cells (4%) × number of transduced cells ($1x10^5$))/100, D = fold dilution of vector sample used for transduction(10^4) and V = volume (ml) of diluted vector sample added into each well for transduction (0.25 ml). TU/ml in Raji cells = 1.6×10^8, TU/ml in AML14.3D10 = (($17x10^5$/100) x 100) / 0.25 = 6.8×10^6.

2.3.11 Preparation of fibronectin coated plates

Fibronectin coated well plates were used for transduction of primary immune cells through bringing lentiviral particles and target cells to close proximity which leads to easier infection of target cells. Fibronectin was provided as a lyophilized powder (1 mg) which was dissolved in 1 ml sterile distilled water without agitation.

Solution concentration was diluted with X-Vivo-15 (1:200 dilution) to a final concentration of 5 ng/µl with a final volume of 250 µl for each well used. Coating was done one day before the transduction and coated plate kept at 37°C incubator overnight. Before transduction, supernatant of fibronectin was sucked leaving non-visible layer of fibronectin on well surface.

2.3.12 Transduction of tumor cell lines and primary immue cells

Cells were transduced by addition of certain volume of lentiviral particles and filling the medium up to 300 µl, addition of 3 µl protamine sulphate (4 µg/ml) and centrifugation under 860 xg for 60 m at 32ºC, after 2-4 h from centrifugation, medium were filled up to 1 ml.

2.3.12.1 Transduction of Raji and AML14.3D10 tumor cell lines

Cells were thawed according to thawing protocol (see 2.3.2 Freezing and thawing of cells). Cell condition was controlled regularly by microscopy. Cells in good condition were counted, plated in 48 well plate at $1x10^6$ cells/ml and subjected to the previous transduction protocol. Re-transduction was repeated when needed.

2.3.12.2 Transduction of $CD4^+$ T effector cells and PBMC

After their isolation, $5x10^5$ $CD4^+$ T effector cells and PBMC were plated on fibronectin coated plates and polyclonally stimulated with 0.5 µg/ml anti-CD3 mAb (OKT-3) and 1 µg/ml anti-CD28 mAb with/without TGF-β in order to increase Foxp3 expression and 0.5 µg/ml anti-CD3 mAb in case of PBMC. Cell condition was controlled by microscopy. Once started to proliferate, cells were tranduced by lentiviral particles.

2.3.12.3 Transduction of $CD4^+CD25^+$ Tregs

After their isolation, $5x10^5$ $CD4^+CD25^+$ Tregs were plated (no fibronection) with IL-2 (100 IU/ml) and IL-7 (1 ng/ml). Cell condition was controlled by microscopy. After 3 days in culture, cells were transduced by lentiviral particles.

2.3.12.4 Transduction of $CD34^+$ HSC

After their isolation, $5x10^5$ $CD34^+$ HSC were plated on fibronection coated plate with SCF (50 ng/ml), FLT3L (50 ng/ml), TPO (20 ng/ml) and IL-6 (10 ng/ml) or SCF, GM-CSF and IL-3 (50 ng/ml) from each. Cell condition was controlled by microscopy. Once started to proliferate, cells were tranduced by lentiviral particles. Re-transduction was repeated when needed.

2.3.13 Differentiation of CD34$^+$ HSC into myeloid cells

Introduction of SCF, GM-CSF and IL-3 (50 ng/ml) into CD34$^+$ HSC culture directed their differentiation into common myeloid progenitors (CMP). CMP quickly developed into granulocyte/moncyte progenitors. Supplementing the culture with IL-5 (50 ng/ml) from day 6 on directed their differentiation and maturation into eosinophils. Keeping the culture under the same conditions ended with a heterogeneous culture containing different myeloid cells i.e. neutrohils, eosinophils and macrophages [133, 134, 144] (Figure 2.4).

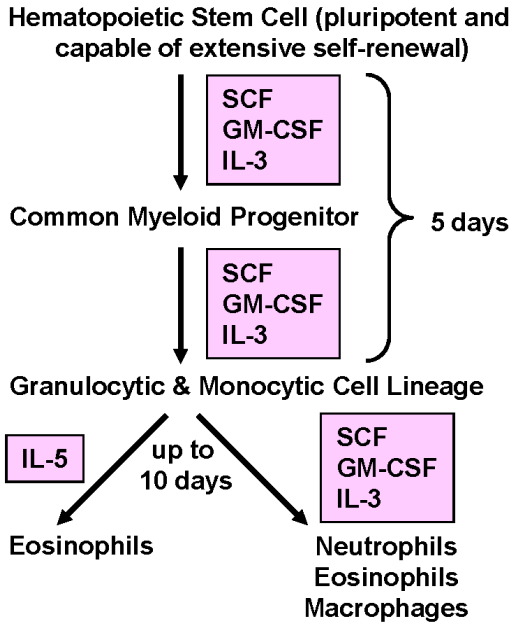

Fig. 2.4: Schematic diagram showing the steps of differentiation of CD34$^+$ HSC into different myeloid cell lineages

2.3.14 Cytochemical analysis

Cells were cytocentrifuged onto glass slides and were stained by Pappenheim and Giemsa staining as described before [133, 145]. To detect eosinophil granules, cytospin preparations of cells were air-dried. Slides were fixed in 4% paraformaldehyde for 15 m at room temperature and analyzed by microscopy.

2.3.15 Microscopy

Pictures were taken by Spot insight Camera mounted over an Axiovert 135 microscope using different objectives. Immunefluorescence confocal laser scanning microscopy (CLSM) analysis was carried out with Zeiss Laser Scanning System LSM 710 at Prof. Susanne Strand laboratory of the University Hospital Mainz.

2.3.16 Preparation of cultured cells for flow cytometry

Cultured cells were transferred from culture plate to FACS plate, minimum number of cells required for staining of each sample was 1 x 10^5 cells. Cells were washed once with FACS buffer, centrifuged at 350 xg for 6 m at 4°C, supernatant was discarded. Cells were stained for 20 m at 4°C with optimal dilution for each mAb. Cells were washed again in FACS buffer, resuspended with 150 µl FACS buffer pro sample, transferred into FACS tubes and analyzed by flow cytometry.

2.3.17 Galectin-10 staining for flow cytometry

5x10^5 cells were fixed in 1 ml PBS with 1% paraformaldehyde and 0.05% Tween-20 overnight at 4°C. Cells were washed with FACS buffer (PBS 1x, 3% fetal calf serum, 0.5% Tween-20) and incubated with mouse anti-human galectin-10 mAb (clone B-F42) with isotype control, washed with FACS buffer and incubated with anti-mouse IgG PE conjugated Ab. After washing in FACS buffer, cells were subjected to FACSCalibur for immunofluorescence measurements with CELLQuest software and the data were analyzed with FlowJo software.

2.3.18 Foxp3 staining for flow cytometry

Cells were fixed and permeabilized using a Fixation/Permeabilization kit according to the manufacturer's protocol. Cells were stained by anti-Foxp3 PE mAb (clone 259D) with isotype control. Cells were subjected to FACSCalibur for immunofluorescence measurements with CELLQuest software and the data were analyzed with FlowJo software.

2.3.19 Analysis of apoptosis by propidium iodide (PI) and 7-Aminoactinomycin D (7-AAD) stainings

Propidium iodide (PI) is a fluorogenic compound that binds stoichiometrically to nucleic acids so that fluorescence emission is proportional to the DNA content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid (sub-G_1) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channel [146]. Appropriate pretested concentration of PI solution was mixed with cell samples directly before analysis with flow cytometer. Like PI, 7-Aminoactinomycin D (7-AAD) is a fluorescent chemical compound which intercalates in double stranded DNA, with a high affinity for GC-rich regions, however it is useful to detects viable cells prior to lysis or fixation [147].

2.4 Flow cytometry

Flow cytometry is a powerful technique for the analysis of multiple parameters of individual cells within heterogeneous populations. The flow cytometer performs this analysis by passing thousands of cells per second through a laser beam and capturing the light that emerges from each cell as it passes through. The heart of the system is where the laser and the sample intersect and the optics collects the resulting scatter and fluorescence. The data gathered can be analyzed statistically by flow cytometry software to report cellular characteristics such as size, granularity and phenotype. The sample stream containing the cells is injected into a flowing stream of sheath fluid. The sample stream becomes compressed to roughly one cell in diameter in a process which is called hydrodynamic focusing. Cytometer detects cells between 1 and 15 μm in diameter. As a cell passes through the laser, it refracts or scatters light at all angles. Forward scatter (FSC), or low-angle light scatter, is the amount of light that is scattered in the forward direction as laser light strikes the cell. Because small cells produce a small amount of forward scatter and large cells produce a large amount of forward scatter, the magnitude of forward scatter is roughly proportional to the size of the cell, and this data can be used to quantify that parameter. However, a cell traveling through laser beam will scatter light at larger angles, for example to the side (sideward scatter:SSC), and is caused by granularity and structural complexity inside the cell. The forward and sideward scattered light is received by separate detector which translates the intensity into a voltage pulse.

One of the most common ways to study cellular characteristics using flow cytometry involves the use of fluorescent molecules such as fluorophore-labeled antibodies. The labeled antibody is added to the cell sample. The antibody then binds to a specific molecule on the cell surface or inside the cell. Finally, when laser light of the right wavelength strikes the fluorophore, fluorescence occurred by excitation of the fluorophore to a higher energy level followed by the return of that fluorophore to its ground state with the emission of light. The emitted light has a specific wavelength, and a specific color and its energy is dependent on the energy level to which the fluorophore is excited.

The fluorescent light coming from labeled cells as they pass through the laser is directed through a series of filters and mirrors, so that particular wavelength ranges are delivered to the appropriate detectors. The fluorescent light is directed to the appropriate detector is translated into a voltage pulse proportional to the amount of fluorescence emitted. All of the voltage pulses are recorded and can be presented graphically. Therefore, a fluorescent signal could be detected by the flow cytometer.

All measurements were carried out on FACS Calibur which is equipped by a blue argon laser and CELLQuest software for data acquisition. The data were analyzed with FlowJo software. Green Fluorescent Protein (GFP) was excited at 488 nanometers with a

peak emission at 509 nanometers and is detected in the fluorescence channel-1 (FL-1) detector on the FACSCalibur. As GFP was endogenously expressed in cells transduced with lentiviral particles, untreated cells were used as negative control (comparable to isotype control staining). The gating strategy used to analyze GFP-expressing cells based on PI staining of both untreated and transduced cells in order to exclude dead cells which might fluorescence in GFP channel. Dead cells displayed a broad peak in FL-3 channel due to intercalation of the stain with their double stranded nucleic acid and became fluorescent. The stain was excluded by viable cells as it could not penetrate through cell membrane and led to a narrow peak. By gating viable cells, life gate is defined (Figure 2.5). All life gate dot plots were shown in red colour or otherwise mentioned. Therefore, GFP could be analyzed by displaying life gate dot plots and setting a quadrant to discriminate between negative and positive GFP-expressing cells and to quantify the percentage of GFP-expressing cells.

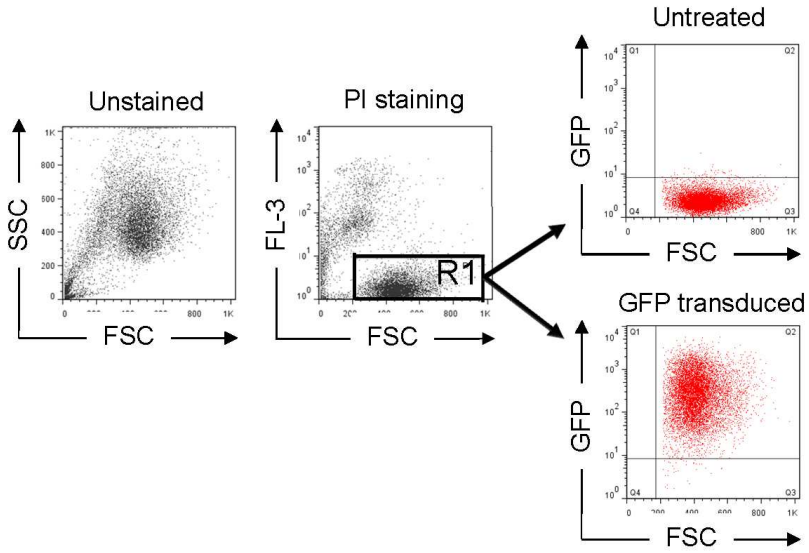

Fig. 2.5: Example for gating strategy showing how to gate viable cells for GFP analysis
Forward scatter (FSC) and sideward scatter (SCC) dot plot shows viable and dead cells with different sizes and granularities. Staining cells with propidium iodide (PI) differentiated between dead cells with high peak from viable cells with low peak in the red fluorescence channel (FL-3). By gating viable cells, life gate was defined (R1). Quadrants were set in life gate dot plots (shown in red colour) to differentiate between negative and positive GFP-expressing cells.

The gating strategy used to analyze galectin-10- and Foxp3-expressing cells based on 7-AAD staining of both untreated and transduced cells prior to their fixation in order to exclude dead cells (Figure 2.6:A). Cells in gated region (R1) were displayed in dot plots to analyze galectin-10 or Foxp3 expression. Isotype control staining was always carried out within each experiment to define region with no positive expressing-cells (R2).

Alternatively, quadrants were set in case of galectin-10 analysis. Thereby, tested cells showing positive expression could be analyzed and quantified in comparison to cells in R2 region of isotype control dot plot. Gating strategy used to analyze cell proliferation based on staining the cells with proliferation dye (eFluor®670 or CFSE) after their isolation and gating a region where the cells were supposed to proliferate (Figure 2.6:B).

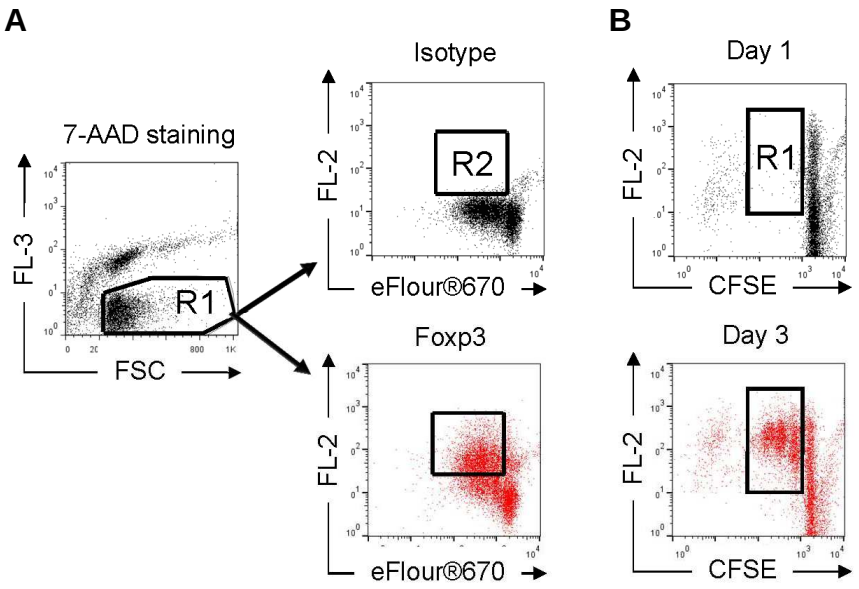

Fig. 2.6: Examples for gating strategy showing how to gate cells for Foxp3 analysis and cell proliferation

A) Staining of cells with 7-Aminoactinomycin D (7-AAD) prior to their fixation for Foxp3 staining differentiated between dead and viable cells. Cells with lower peak in FL-3 channel were gated (R1) and displayed in new dot plot for Foxp3 analysis. Isotype control staining enabled a new region (R2) with no positive expressing-cells to be defined (Dot plot shown in black colour). Foxp3 expression could be analyzed and quantified by plotting the same region (R2) within the tested sample dot plot (Dot plot shown in red colour). B) Staining of cells with CFSE enabled the proliferation of cells to be traced upon analysis of R1 region. Dot plots showing CFSE staining of cells at day 1 and day 3 are shown in black and red colours respectively.

Contour plot represents an alternative way to analyze the same data as in dot plot. The frequency of cells at each point in the contour plot enables precise localization of peaks that distinguish different cell subsets (Figure 2.7:A). Histogram plot displays a single measurement parameter (relative fluorescence intensity) on the x-axis and the number of events (cell count) on the y-axis. It was critical to carry out isotype control staining to set a baseline control peak to overlay with positive histogram peaks coming from positively-expressing cells for the marker of interest (Figure 2.7:B).

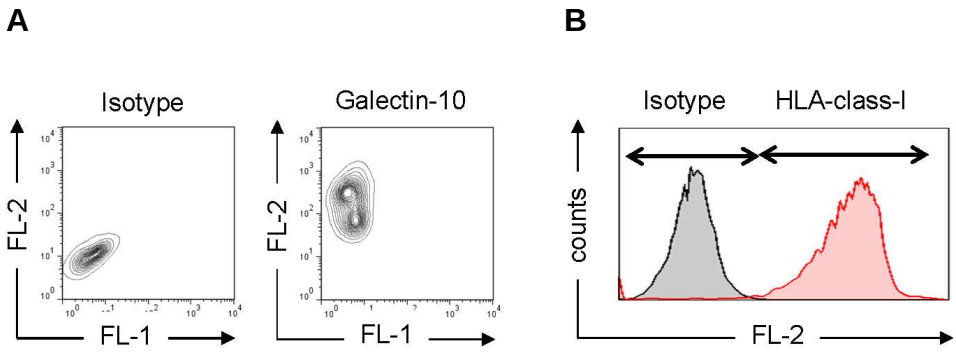

Fig. 2.7: Contour and histogram plots represent different alternatives of data analysis

A) Staining of eosinophils differentiated from stem cells with galectin-10 Ab. Data representation in contour plot demonstrates the presence of high and low galectin-10-expressing subsets with their precise localization. B) Overlay of histogram plots shows positive expression of HLA-class-I molecules in eosinophils differentiated from stem cells represented by red histogram peak in comparison to isotype control staining of cells represented by grey histogram peak.

RESULTS

Maintenance of tolerance to self is a function of Tregs which suppress inadequate and autoaggressive immune responses [52]. Galectin-10 and Foxp3 are key molecules which participate in the suppressive mechanisms of Tregs [76, 107]. Galectin-10 is one of the oldest known human-specific proteins which were not found in any other species [111]. Foxp3 is an activation marker which is expressed in human $CD4^+CD25^+$ Tregs and activated $CD4^+CD25^-$ T effector cells [65, 79]. Due to the need to highlight the significance of both Treg-markers in a functional humanized *in vivo* model, this study aimed to transduce human $CD34^+$ HSC with lentiviral particles encoding specific shRNA against galectin-10 or Foxp3. The use of shRNA lentiviral technique was critical to mediate a long-term knockdown of targeted molecules during the humanization process. The transduced $CD34^+$ HSC could be used afterwards to reconstitute immunodeficient mice [148]. Tracking human cell populations in all murine organs would open the possibility to investigate the significance of Tregs-associated molecules *in vivo*.

3.1 Production of GFP monocistronic lentiviral particles

Prior to production of lentiviral particles encoding specific shRNA against galectin-10 or Foxp3, procedures for production of lentiviral particles should be established. Production of lentiviral particles encoding GFP as a reporter transgene (GFP monocistronic lentiviral particles) was essential to follow up this aim. GFP expression within transduced cells served as a proof-of-concept that a gene encoded by lentiviral particles would integrate into the genome. Moreover, GFP expression enabled the infectivity of the produced lentiviral particles to be determined. The infectivity of lentiviral particles was determined by titration of produced particles pursued by their transduction into Ragi and AML14.3D10 cells.

3.2 Determination of transduction unit per ml in Raji and AML14.3D10 cells

Determination of the viral titer enabled the multiplicity of infection (MOI) of viral particles used in each experiment to be calculated. MOI reflects the ratio of transducing viral particles (TU) to the number of cells being transduced. MOI should be the same for all groups transduced by lentiviral particles within one experiment. Through keeping the same of number of cells within each group in every experiment, pre-determination of transduction unit per ml (TU/ml) for lentiviral particles after their production was important to define the MOI used.

The titer of produced lentiviral particles was determined by serial dilution using 1:10 fold dilution steps. These different dilutions of lentiviral particles were used to transduce Raji and AML14.3D10 cells. After 48 h from transduction, cells were subjected

to flow cytometric analysis of GFP expression. Viable cells expressing GFP between 2% and 20% reflects the tranduction of one viral particle per cell. Therefore, the TU/ml of produced lentiviral particles could be determined (Figure 3.1).

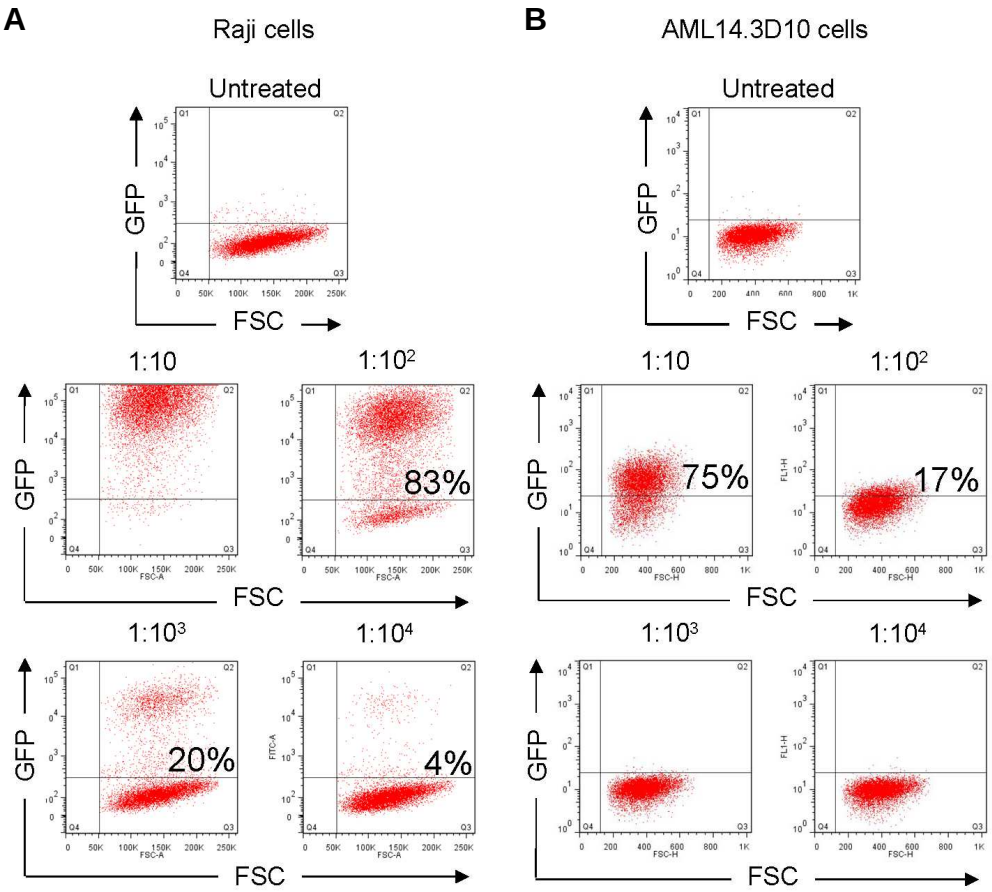

Fig. 3.1: Transduction of Raji and AML14.3D10 cells with different serial dilutions of GFP monocistronic lentiviral particles

1:10 fold dilution steps of produced lentiviral particles were used to determine TU/ml. Different dilutions of lentiviral particles were used to transduce Raji and AML14.3D10 cells. After 48 h from transduction, cells were subjected to flow cytometric analysis. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour. A) Raji cells. B) AML14.3D10 cells.

3.3 Transduction of tumor cell lines and primary immune cells with GFP monocistronic lentiviral particles

The positive outcome of initial lentiviral transduction experiments was the basis to establish further protocols in order to transduce specific cell lines of interest and primary immune cells. Production of large amount of GFP monocistronic lentiviral particles allowed the analysis of transduction efficiency for each cell type. Due to their significant

importance for expression of galectin-10 and Foxp3, tumor cell lines like galectin-10 expressing AML14.3D10 and Foxp3 expressing Karpas-299 as well as primary immune cells like human PBMC, $CD4^+$ T cells and $CD25^+$ regulatory T cells (Tregs) were transduced with GFP monocistronic lentiviral particles.

3.3.1 Transduction of AML14.3D10 and Karpas-299 cell lines

Galectin-10 expressing AML14.3D10 and Foxp3 expressing Karpas-299 cells are tumor cell lines which continually proliferate in culture [132, 140]. Targeting these cells with lentiviral particles made it possible to demonstrate the efficiency of GFP gene transfer using lentiviral particles. Thus, both cell lines were transduced with GFP monocistronic lentiviral particles. After 48 h from transduction, GFP expression of 93% and 39% were obtained respectively (Figure 3.2:A,C).

In order to study the stability of transferred GFP gene in transduced cells, the MOI of lentiviral particles used to transduce AML14.3D10 cells was increased. After 2 days from transduction, an increment of GFP expression by nearly all of the cells was achieved. GFP-expressing cells were kept in culture upto 1 year with regular splitting twice per week. After 1 year in culture, nearly all cells showed stable GFP expression assuming a stable integration of GFP gene into the genome of AML14.3D10 cells. This provides evidence that lentiviral particles are efficient gene transfer vehicles for human tumor cell lines such as AML14.D10 cells (Figure 3.2:B).

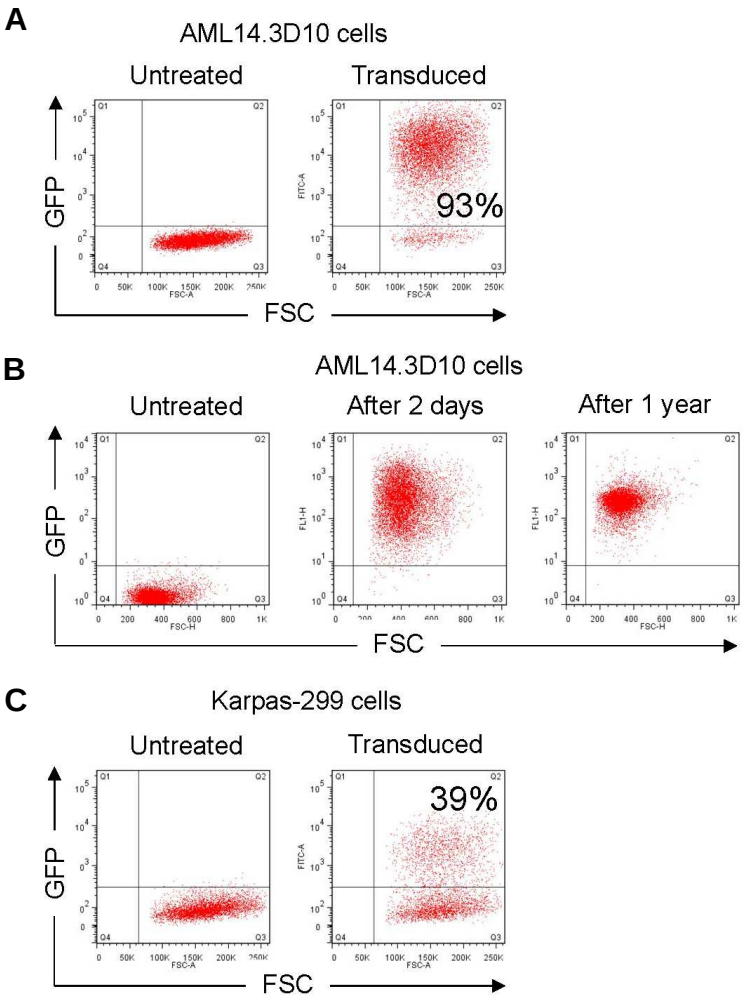

Fig. 3.2: Transduction of AML14.3D10 and Karpas-299 cells with GFP monocistronic lentiviral particles and stable GFP expression in AML14.3D10

A) AML14.3D10 cells were transduced with GFP monocistronic lentiviral particles (MOI:10.2). After 48 h from transduction, cells were subjected to flow cytometric analysis of GFP expression. B) AML14.3D10 cells were transduced with GFP lentiviral particles (MOI:24). After 48 h from transduction, cells were subjected to flow cytometric analysis of GFP expression. Nearly all cells showed GFP expression. Cells were kept in culture with regular splitting whenever needed (2x per week). After 1 year in culture, cells were subjected again to flow cytometric analysis of GFP expression. C) Karpas-299 cells were transduced with GFP monocistronic lentiviral particles (MOI:10.2). After 48 h from transduction, cells were subjected to flow cytometric analysis of GFP expression. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour.

3.3.2 Transduction of PBMC, $CD4^+$ T cells and $CD4^+CD25^+$ Tregs

To investigate the efficiency of transduction of lentiviral particles in primary immune cells, human PBMC, $CD4^+$ T cells (non-depleted $CD25^+$) and $CD4^+CD25^+$ Tregs were transduced by GFP monocistronic lentiviral particles. Activation of primary cells is crucial for successful transduction by lentiviral particles. For that reason, polyclonal stimulation of both PBMC with anti-CD3 mAb and $CD4^+$ T cells with anti-CD3 and anti-CD28 mAb were carried out. Therefore, engagement of TCR and costimulatory molecules (e.g CD28) triggers the signal transduction cascades that ultimately results in cell proliferation. Proliferation serves to ensure the transition of cells from the resting phase of cell cycle to G_1b phase. This step is crucial for successful transduction by lentiviral particles [149]. After 24 h from stimulation, cells were transduced with GFP monocistronic lentiviral particles. Flow cytometric analysis after 3 days from transduction demonstrated that only 43% of PBMC were positive for GFP. $CD4^+$ T cells showed 98% of GFP expression after 2 days from transduction (Figure 3.3).

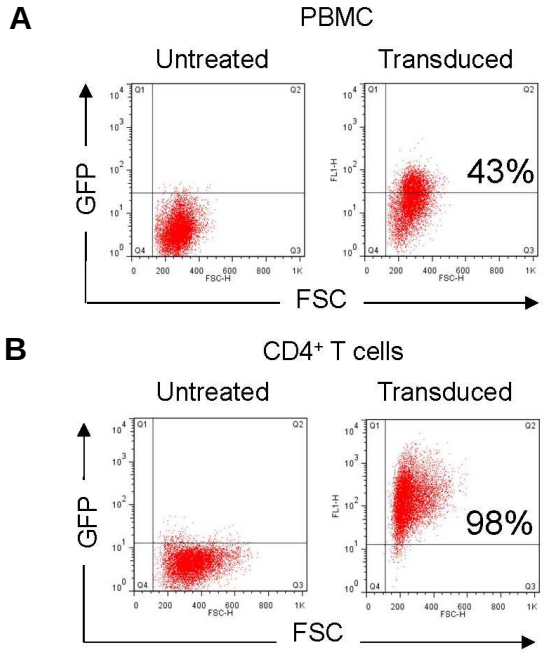

Fig. 3.3: Transduction of primary immune cells with GFP monocistronic lentiviral particles
A) GFP expression in human PBMC polyclonally stimulated with anti-CD3 mAb (1 µg/ml). After 24 h, cells were transduced by GFP monocistronic lentiviral particles (MOI:0.36). 72 h later, PBMC were subjected to flow cytometric analysis of GFP expression. B) GFP expression in human $CD4^+$ T cells (non-depleted $CD25^+$) polyclonally stimulated with anti-CD3 (0.5 µg/ml) and anti-CD28 (1 µg/ml) mAb. After 24 h, cells were transduced with GFP monocistronic lentiviral particles (MOI:0.72). After 48 h from transduction, cells were subjected to flow cytometric analysis of GFP expression. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour.

$CD4^+CD25^+$ Tregs express both galectin-10 and Foxp3. This made $CD4^+CD25^+$ Tregs an interesting target for transduction by lentiviral particles. However, $CD4^+CD25^+$ Tregs are non-proliferating cells. Therefore, establishment of suitable protocol to transduce non-proliferating $CD4^+$ T cells with lentiviral particls was considered as a prerequisite for transduction of anergic non-proliferating $CD4^+CD25^+$ Tregs.

Although lentiviral particles were shown to transduce various types of non-proliferating cells [150, 151], resting T lymphocytes turned out to be refractory to gene transfer with lentiviral particles [152]. Nevertheless, exposing resting T cells to cytokines such as IL-2 and IL-7 induced them to enter in G_1b phase of cell cycle. In G_1b phase, cells were rendered permissive to transduction by lentiviral particles without starting to proliferate [152].

The protocol was primarily tested with $CD4^+$ T cells (non-depleted $CD25^+$) in absence of TCR stimulation in order to avoid strong activation and proliferation. Cells were kept for 3 days in culture including IL-2 and IL-7 before being transduced with GFP lentiviral particles. After 48 h from transduction, nearly all cells were expressing GFP (Figure 3.4:A).

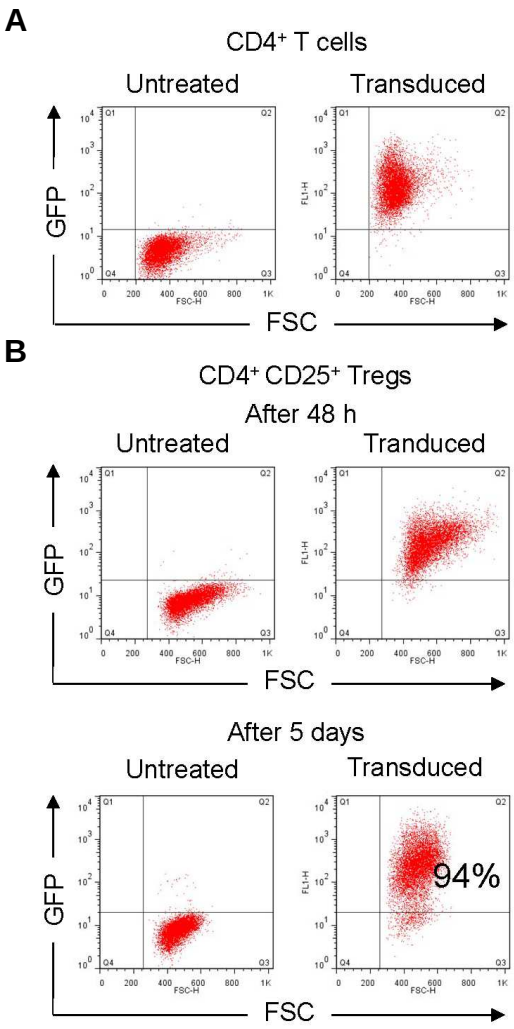

Fig. 3.4: Transduction of CD4$^+$ T cells with GFP monocistronic lentiviral particles without stimulation

A) CD4$^+$ T cells (non-depleted CD25$^+$) were cultured for 3 days in presence of IL-2 (100 IU/ml) and IL-7 (1 ng/ml). After 3 days, cells were transduced with GFP lentiviral particles (MOI:0.24). After 48 h from transduction, cells were subjected to flow cytometric analysis of GFP expression. B) CD4$^+$CD25$^+$ Tregs were cultured for 3 days in presence of IL-2 (100 IU/ml) and IL-7 (1 ng/ml). After 3 days, cells were transduced with GFP lentiviral particles (MOI:0.24). After 48 h and 5 days from transduction, cells were subjected to flow cytometric analysis of GFP expression. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour.

This preliminary result encouraged us to go further and transduce CD4$^+$CD25$^+$ Tregs. CD24$^+$CD25$^+$ Tregs which express Foxp3 without TCR stimulation and are known to be anergic *in vitro* [60] were subjected to the same transduction protocol. 48 h after transduction, majority of cells showed GFP expression. Keeping the cells in culture for 3 more days, GFP expression reached 94% (Figure 3.4:B).

In conclusion, we were able to establish protocols which enabled us to transduce tumor cell lines like galectin-10 expressing AML14.3D10 and Foxp3 expressing Karpas-299 successfully. Transduction of proliferating primary immune cells like human PBMC and $CD4^+$ T cells was also possible. However, transduction of anergic $CD4^+CD25^+$ Tregs required a restricted transduction protocol in order to gain a stable transduction by GFP monocistronic lentiviral particles. Sustained expression of GFP upto 1 year in AML14.3D10 cell line emphasized the establishment of transduction procedure by lentiviral particles.

Based upon these results, production of lentiviral particles encoding shRNA against galectin-10 and Foxp3 was carried out. Transducing cell populations expressing galectin-10 and Foxp3 with these particles was extremely crucial. This provided evidence for production of functional shRNA lentiviral particles against galectin-10 or Foxp3 upon their specific knockdown. Therefore, the same particles could be used for transduction of $CD34^+$ HSC. The next part highlights cell populations expressing galectin-10.

3.4 Galectin-10 expression in AML14.3D10 cells and human PBMC

AML14.3D10 is a tumor cell line which carries an advanced eosinophilic phenotype. Hence, they produce huge amounts of eosinophilic granules including galectin-10. Staining of galectin-10 in AML14.3D10 cells demonstrated its consistent expression (Figure 3.5:B).

Staining of galectin-10 in human PBMC showed an expression percentage ranging between 3-4% of galectin-10-positive cells. Back-gating these cells revealed galectin-10 expression within lymphocyte and granulocyte regions (Figure 3.5:A bottom row). These results are consistent with literature, where galectin-10 expression is restricted to human $CD4^+CD25^+$ Tregs and granulocytes [107, 108, 110].

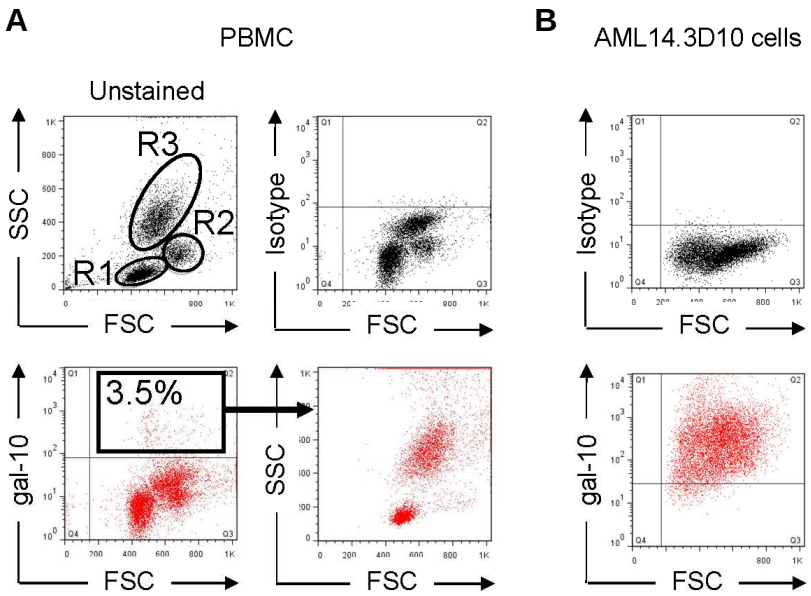

Fig. 3.5: Galectin-10 expression in human PBMC and AML14.3D10 cells

A) Galectin-10 staining of human PBMC showed 3.5% positive cells. These cells coincide within lymphocyte and granulocyte regions. Lymphocytes are situated in R1 region, monocytes in R2 region and granulocytes in R3 region. Galectin-10 expression was analyzed by setting quadrants to differentiate between galectin-10 positive and negative cells. Dot plot showing isotype control staining is shown in black colour. Dot plots of galectin-10 staining and back-gating of PBMC are shown in red colour. B) Staining of galectin-10 in AML14.3D10 cells showed a constitutive expression of the molecule in AML14.3D10 cells. Galectin-10 expression was analyzed by setting quadrants to differentiate between galectin-10 positive and negative cells. Dot plot of isotype control staining is shown in black colour and dot plot of galectin-10 staining is shown in red colour.

Reconstituting immunodeficient mice with HSC to generate so called humanized mice [148] was established in our group. However, knocking down of galectin-10 in humanized mice compromised a more challenging enterprise. This could be achieved by transduction of HSC with shRNA lentiviral particles against galectin-10 prior to reconstitution. Consequently, the next step was to stain galectin-10 in $CD34^+$ HSC gained from freshly isolated cord blood. $CD34^+$ HSC did not show any galectin-10 expression suggesting that galectin-10 is starting to be expressed in differentiated cell populations (Figure 3.8:A).

This thesis directed us to pay more attention to differentiate HSC into lymphocyte or granulocyte population potentially expressing galectin-10. Known as the only lymphocyte population expressing galectin-10, *in vitro* differentiation of HSC into $CD4^+CD25^+$ Tregs is not possible without thymic selection processes. Therefore, differentiation of HSC into granulocytes expressing galectin-10 was the remaining alternative to carry out the *in vitro* galectin-10 knockdown experiments.

3.5 Galectin-10 expression in myeloid cells derived from CD34$^+$ HSC

CD34$^+$ HSC were freshly isolated from cord blood. Introduction of SCF, GM-CSF and IL-3 into the HSC culture mediated the development of common myeloid progenitor cells (CMP) from HSC. After 5 days in culture, cytokines were replaced with IL-5. IL-5 stimulated CMP to develop into eosinophils while attaining their maturation to a final stage within 10 days (IL-5 cytokine protocol). However, keeping cells in culture supplemented with SCF, GM-CSF and IL-3 induced the production of neutrophils, eosinophils and macrophages (3 cytokines protocol).

Characteristic changes in morphology of CD34$^+$ HSC were detected in culture. At day 2, the small rounded stem cells were elongated in shape and started to proliferate (Figure 3.6:A). Increase in number and size of cells was clearly observed within the following days. Granulation of cells was also detectable from day 9 onwards (Figure 3.6:B). Difference in size and granularity between cells cultured by the IL-5 cytokine protocol and the 3 cytokines protocol was demonstrated by forward and sideward scatter upon flow cytometric analysis (Figure 3.6:C).

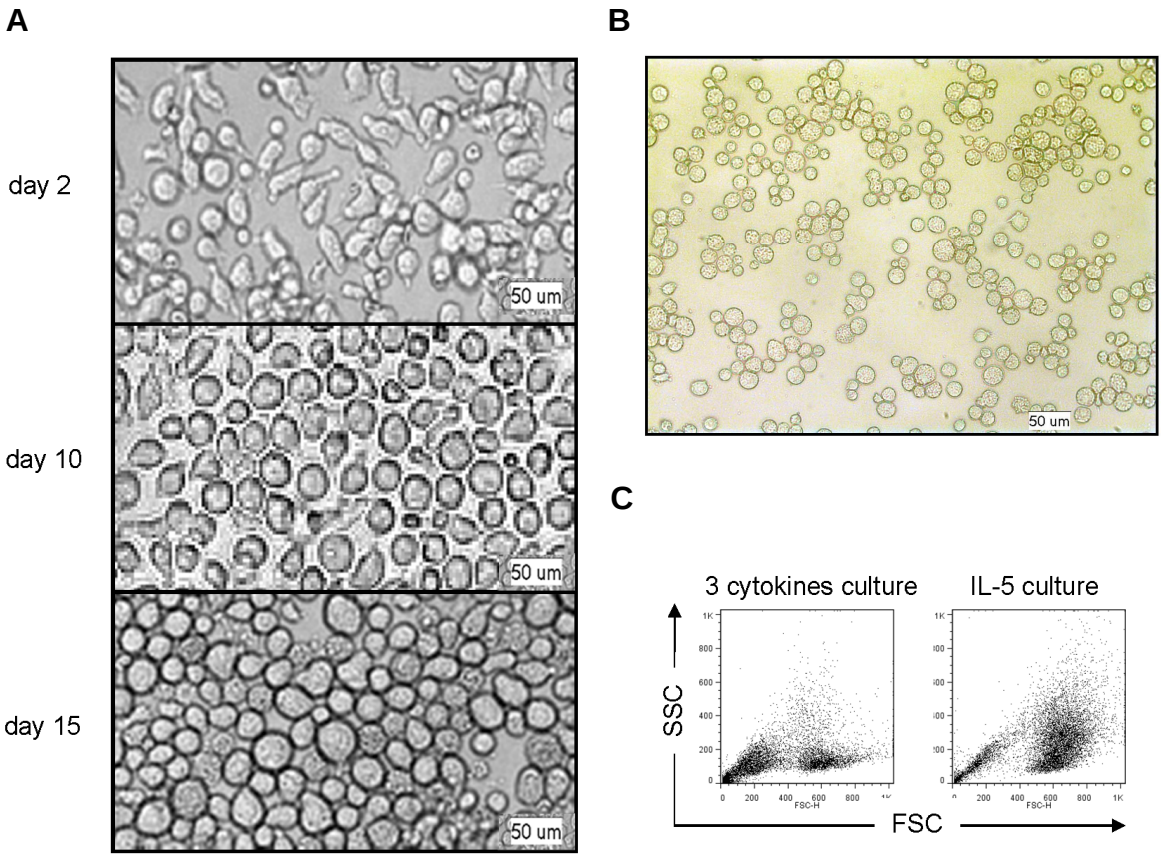

Fig. 3.6: Morphological and flow cytometric analyses of myeloid cells derived from CD34$^+$ HSC

A) Microscopic pictures showing the main steps of differentiaton of CD34$^+$ HSC into eosinophils at different time intervals: SCF, GM-CSF and IL-3 (50 ng/ml) of each cytokine were introduced into CD34$^+$ HSC culture upon their isolation. Cytokine milieau was changed to IL-5 (50 ng/ml) from day 6 (IL-5 cytokine protocol). B) Microscopic picture from cytospin preparations of eosinophils at day 15 using IL-5 cytokine protocol. C) Flow cytometric analysis showed the difference in size (FSC) and granularity (SSC) of cells cultured by the 3 cytokines protocol and cells cultured by IL-5 cytokine protocol at day 15.

Due to its significance in identifying different types of granulocytes and other blood cells, Pappenheim staining was carried out on cells produced by 3 cytokines protocol. Staining demonstrated the presence of neutrophils, eosinophils and macrophages assuming a heterogenouse culture upon differentiation of CD34$^+$ HSC (Figure 3.7:A). Giemsa staining of cells produced by IL-5 protocol showed a high quality staining of chromatin. Nuclei of stained cells matched the typical bi-lobed nuclei of eosinophils (Figure 3.7:B).

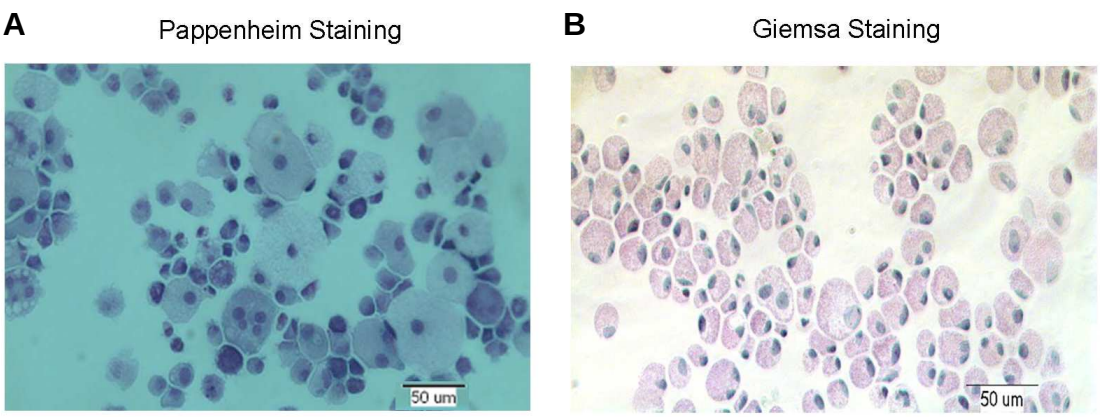

Fig. 3.7: Microscopic pictures of myeloid cells derived from CD34$^+$ HSC

A) Pappenheim staining (May-Grünwald Giemsa staining) of myeloid cells derived from CD34$^+$ HSC: Upon their isolation, CD34$^+$ HSC were subjected to the 3 cytokines protocol then stained with Pappenheim stain at day 15. B) Giemsa staining of eosinophils derived from CD34$^+$ HSC: Upon their isolation, CD34$^+$ HSC were subjected to IL-5 cytokine protocol then stained with Giemsa stain at day 15.

To investigate the expression of galectin-10 in myeloid cells derived from CD34$^+$ HSC by flow cytometry, galectin-10 staining was carried out in cultures undergo both protocols (3 cytokines and IL-5 cytokine protocols). Cells from both cultures showed galectin-10 expression (Figure 3.8:C,D). However, eosinophils from culture obtained by IL-5 cytokine protocol showed higher galectin-10 expression in comparison to myeloid cells obtained by 3 cytokines protocol. AML14.3D10 cells were used as positive control for galectin-10 expression (Figure 3.8:B).

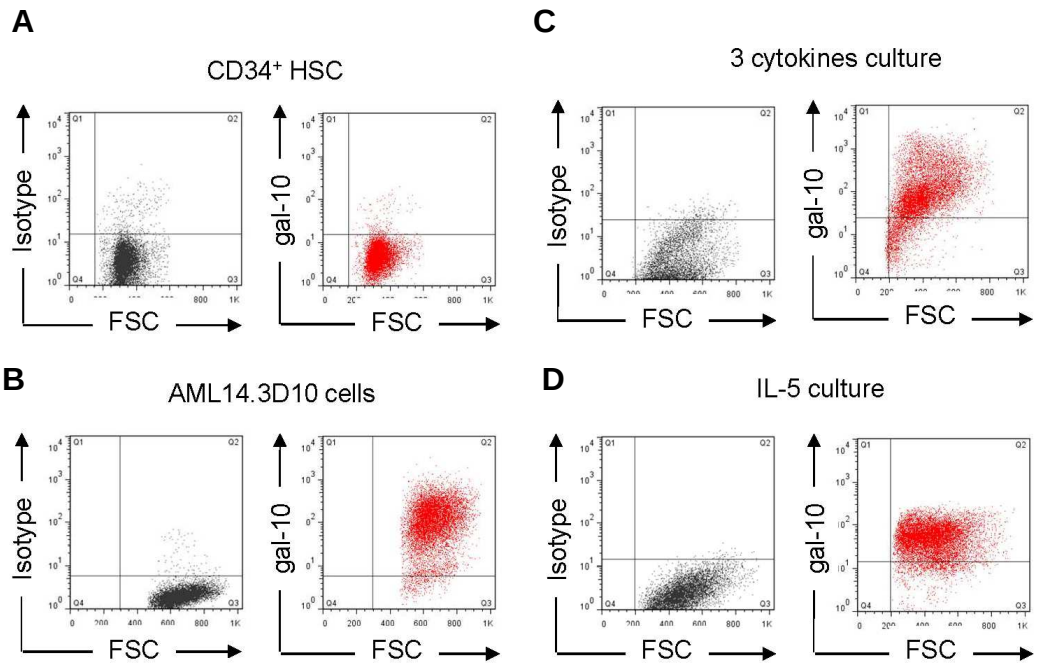

Fig. 3.8: Galectin-10 analysis in CD34$^+$ HSC and myeloid cells derived from CD34$^+$ HSC

CD34$^+$ HSC and myeloid cells derived from CD34$^+$ HSC were subjected to galectin-10 staining and were analyzed by flow cytometry. Galectin-10 expression in each cell type was analyzed by setting a quadrant to differentiate between galectin-10 positive and negative cells. Dot plots of isotype control staining of each cell type is shown in black colour while dot plots of galectin-10 staining is shown in red colour. A) Freshly isolated CD34$^+$ HSC. B) AML14.3D10 cells used as positive control. C) Myeloid cells derived from CD34$^+$ HSC using 3 cytokines protocol. D) Eosinophils derived from CD34$^+$ HSC using IL-5 cytokine protocol.

In conclusion, it was possible to differentiate CD34$^+$ HSC into galectin-10-expressing eosinophils *in vitro*. These results provided a significant step in the way to inaugurate galectin-10 knockdown in humanized mice.

Galectin-10-expressing eosinophils derived from CD34$^+$ HSC appeared to be a stable and accessible galectin-10-expressing resource. This made them an attractive target to test the efficiency of shgalectin-10 (shgal-10) monocistronic lentiviral particles with the galectin-10-expressing AML14.3D10 tumor cell line. Functional knockdown of galectin-10 in stem cells derived cells afford the possibility to study the impact of galectin-10 knockdown *in vitro* before proceeding into *in vivo* studies.

Nevertheless, the thought of generating cells expressing shRNA against galectin-10 together with GFP to facilitate their sorting afterwards guided us to the idea of designing lentiviral particles containing both GFP and shRNA against galectin-10 (GFP+shgal-10 bicistronic lentiviral particles). Therefore, it was important to test the influence of GFP on expression level of galectin-10 and furthermore on its knockdown in both AML14.3D10 cells and eosinophils derived from CD34$^+$ HSC.

3.6 Influence of GFP on galectin-10 expression and knockdown in AML14.3D10 cells and eosinophils

Aim of the next experiments was to test whether GFP expression would affect the endogenous galectin-10 expression in AML14.3D10 cells and eosinophils derived from $CD34^+$ HSC. Therefore, first step was to transduce both cell types with GFP monocistronic lentiviral particles then to detect galectin-10 expression in comparison to untreated cells. Second step was to test galectin-10 knockdown possibility within the GFP positive cells in both cell types.

3.6.1 Galectin-10 expression and knockdown in AML14.3D10 cells transduced with GFP monocistronic lentiviral particles

shgal-10 monocistronic lentiviral particles were produced. AML14.3D10 cells were transduced with GFP monocistronic lentiviral particles and nearly all cells showed GFP expression after 2 days from transduction (Figure 3.2:B). Nearly all cells which were still expressing GFP after 5 months in culture were re-transduced with shgal-10 monocistronic lentiviral particles. Galectin-10 and GFP expression was measured after 14 days from the second transduction. Endogenous expression of galectin-10 was not affected by the GFP expression (Figure 3.9: third row). Moreover, 60% reduction of galectin-10 high expressing subset was observed upon re-transduction with shgal-10 monocistronic lentiviral particles (Figure 3.9: fourth row). Galectin-10 knockdown was specific since it did not influence the GFP expression. (Figure 3.9: first column).

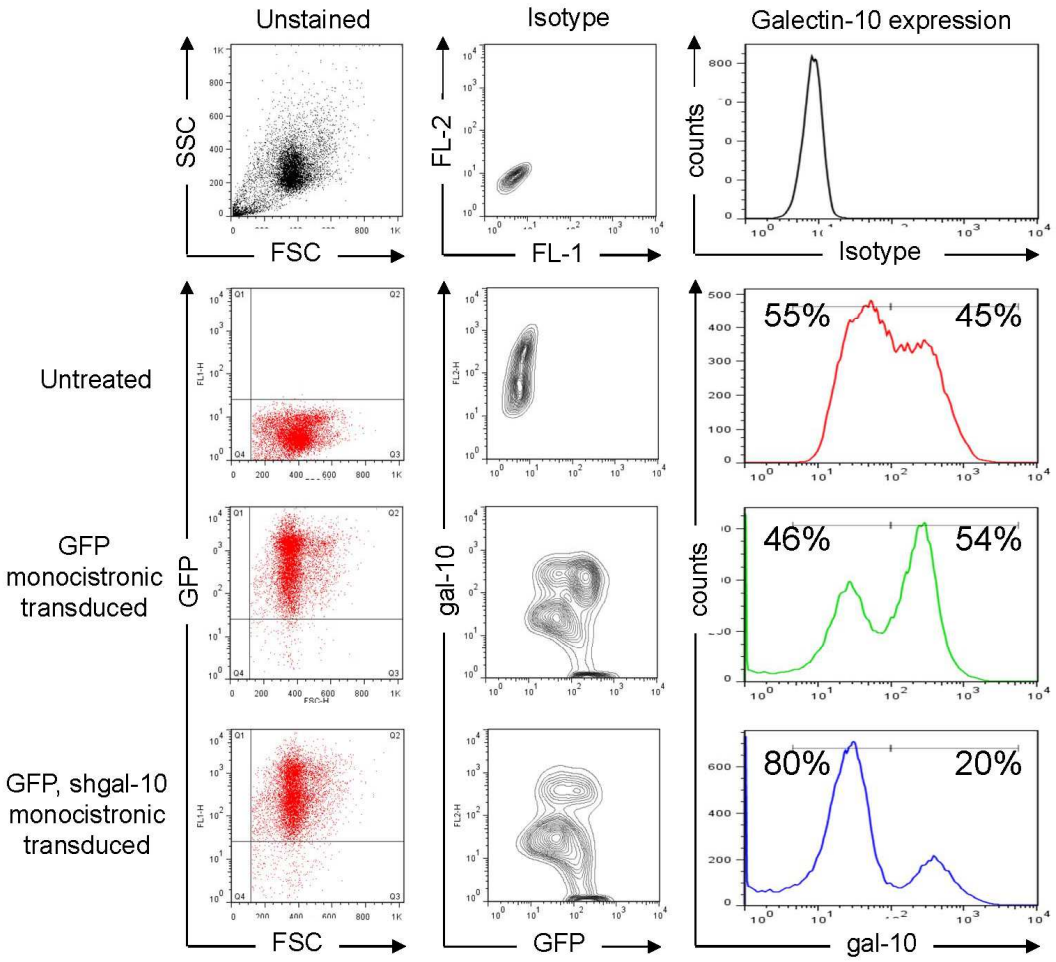

Fig. 3.9: GFP and galectin-10 expression in AML14.3D10 cells after transduction by GFP and shgal-10 monocistronic lentiviral particles

AML14.3D10 cells transduced with GFP monocistronic lentiviral particles (MOI:24) were re-transduced with shgal-10 monocistronic lentiviral particles after 5 months from primary transduction (MOI:24). 14 days from second transduction, cells were subjected to flow cytometric analysis. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour. Contour plots of GFP versus galectin-10 expression are shown in black colour. Histogram plot of cells stained with isotype control is shown in black line. Histogram blots of galectin-10 expression in untreated cells, cells transduced with GFP particles and cells transduced with GFP and shgal-10 monocistronic particles are shown in red, green and blue lines respectively.

3.6.2 Galectin-10 expression and knockdown in eosinophils derived from CD34$^+$ HSC co-transduced with GFP monocistronic lentiviral particles

Cord blood is one possible source of CD34$^+$ HSC. However, a major limitation for the usage of CD34$^+$ HSC from cord blood is their small number. Therefore, expansion of HSC *in vitro* is extremely useful. Studies have demonstrated that cord blood long-term repopulating stem cells can be expanded *ex vivo* in presence of SCF, FLT3L, TPO and IL-6 cytokines [153]. Moreover, it was shown that CD34$^+$ HSC cells that are in G_1 or G_2/M phase are more effectively transduced by lentiviral particles than those in G_0 [154]. For these reasons, freshly isolated CD34$^+$ HSC were cultured in X-Vivo-15 supplemented with SCF, FLT3L, TPO and IL-6. After 2 days in culture, cells were transduced with GFP monocistronic lentiviral particles. Thereafter, cells were transferred into medium supplemented with SCF, GM-CSF and IL-3 and in order to differentiate them into eosinophils with IL-5. Next day, transduction was repeated with the same MOI of GFP monocistronic lentiviral particles in order to improve the transduction efficiency.

Starting from day 2 after second transduction, cells were subjected to flow cytometric analysis and analysis was repeated frequently every 2 days till day 8. For analysis, 49% of transduced cells were viable in comparison to 91% of cells in the untreated group. This assumes that transduction procedure significantly affected the viability of transduced cells.

GFP expression reached 70% after 2 days from transduction. Cells showed maximum expression of GFP at day 4 with 88%, 57% at day 6 and 52% at day 8 (Figure 3.10:A). Day 6 was taken randomly to investigate the galectin-10 expression in GFP transduced cells. 43% in the transduced group were viable at day 6 in comparison to 50% in the untreated group. Surprisingly, galectin-10 expression was detected after 7 days from starting the differentiation of CD34$^+$ HSC. This showed that CD34$^+$ HSC transduced with GFP monocistronic lentiviral particles were able to co-express galectin-10 simultaneously with GFP in eosinophils derived from CD34$^+$ HSC (Figure 3.10:B).

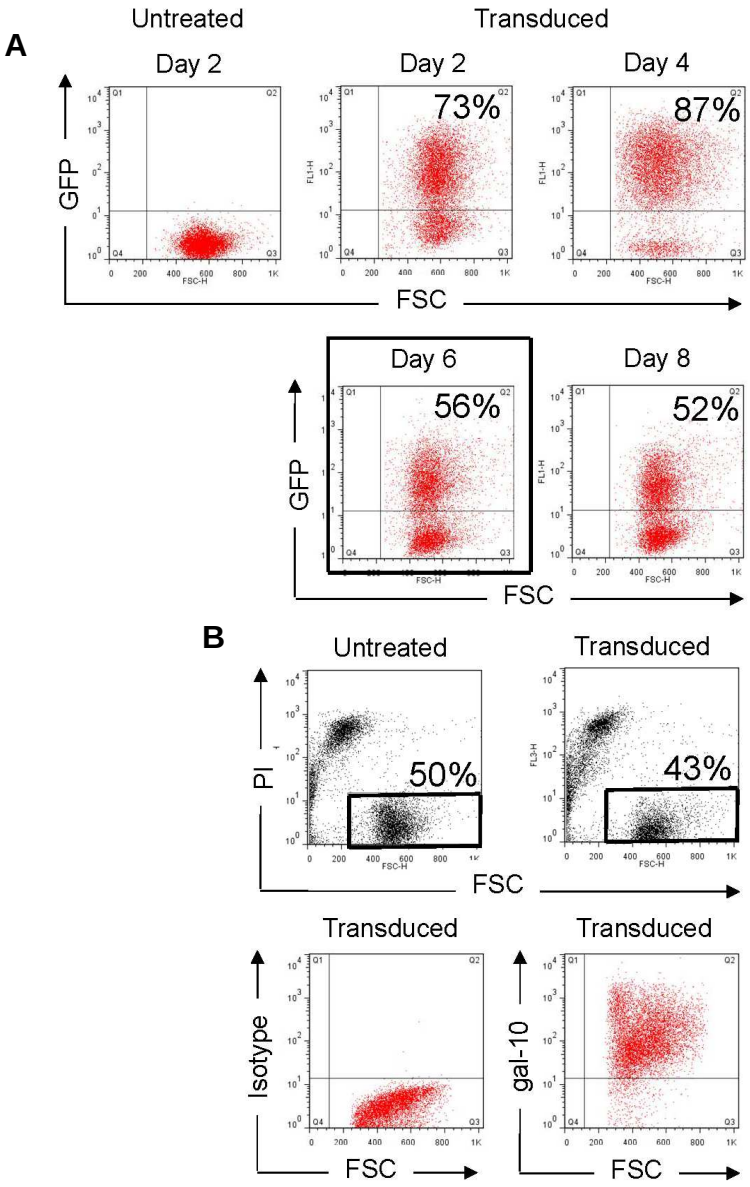

Fig. 3.10: GFP and galectin-10 expression in eosinophils derived from CD34$^+$ HSC after transduction with GFP monocistronic lentiviral particles

A) Freshly isolated CD34$^+$ HSC were cultured in X-Vivo-15 containing SCF (50 ng/ml), FLT3L (50 ng/ml), TPO (20 ng/ml) and IL-6 (10 ng/ml). After 2 days in culture, cells were transduced with GFP monocistronic lentiviral particles (MOI:38.3). After transduction, cells were subjected to IL-5 cytokine protocol to differentiate them into eosinophils. Next day, transduction with GFP monocistronic lentiviral particles was repeated (MOI:38.3). Cells were subjected to flow cytometric analysis after 2,4, 6 and 8 days from second transduction. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour. B) Propidium iodide and galectin-10 stainings of eosinophils derived from CD34$^+$ HSC after 6 days from second transduction. PI stain differentiated between viable and dead cells. A gate was set in viable cells region to quantify them. Galectin-10 expression was analyzed by setting quadrants to distinguish between positive and negative galectin-10 cells. Dot plot of isotype control staining is shown in black colour while dot plot of galectin-10 expression is shown in red colour.

To test whether GFP expression would affect galectin-10 knockdown in eosinophils derived from $CD34^+$ HSC, previous experiment was repeated with exception that cells were co-transduced with GFP and galectin-10 monocistronic lentiviral particles at the same time point. Transduction was carried out 3 times consequently in order to increase the GFP expression and knockdown of galectin-10. 8 days after the last transduction, cells were subjected to flow cytometric analyses for expression of GFP and galectin-10. Co-transduced cells showed 62% GFP expression (Figure 3.11:A). Galectin-10 knockdown reached about 50% in galectin-10-high population (Figure 3.11:B). These results reveal that GFP expression does not interfere with galectin-10 synthesis or its knockdown pathways.

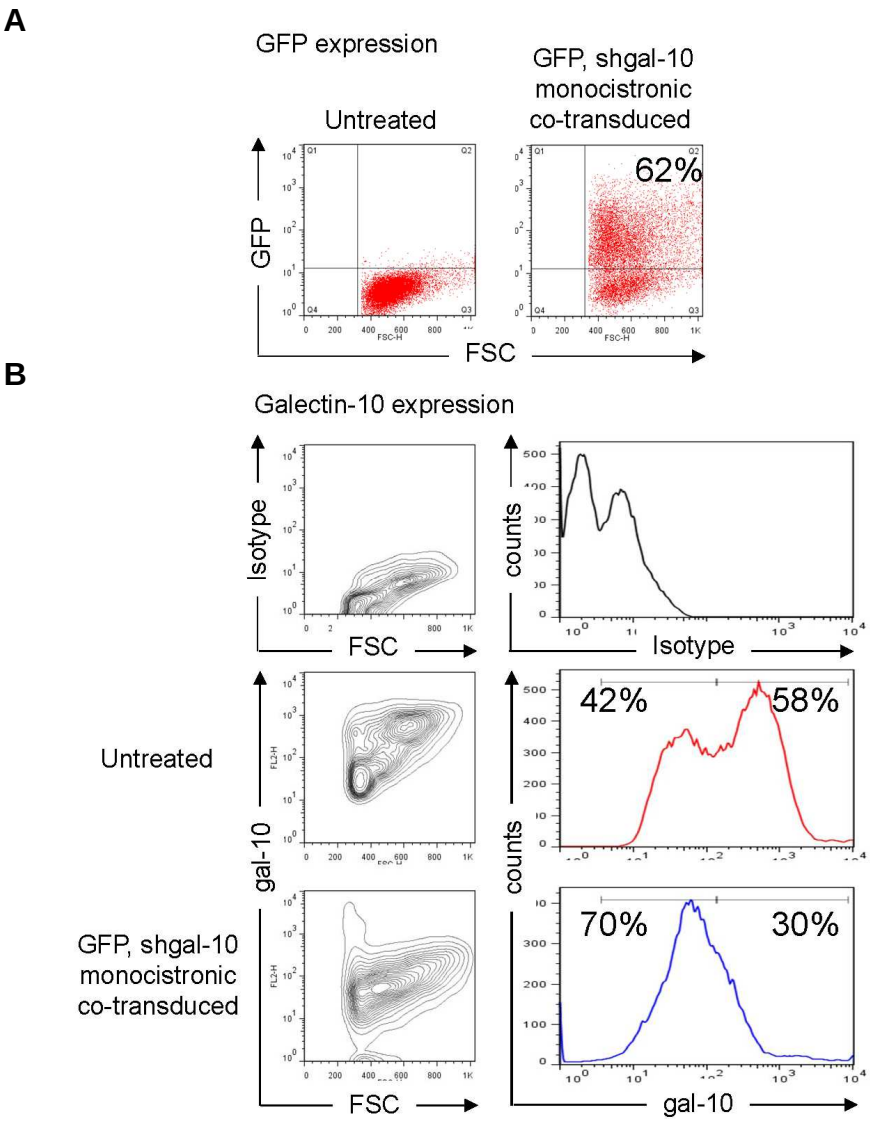

Fig. 3.11: GFP and galectin-10 expression in eosinophils derived from CD34$^+$ HSC after co-transduction with GFP and galectin-10 monocistronic lentiviral particles

Freshly isolated CD34$^+$ HSC were cultured in X-Vivo-15 supplemented with SCF (50 ng/ml), FLT3L (50 ng/ml), TPO (20 ng/ml) and IL-6 (10 ng/ml). After 3 days in culture, cells were co-transduced with GFP and shgal-10 monocistronic lentiviral particles (MOI:38.3). After transduction, cells were subjected to IL-5 cytokine protocol in order to differentiate them into eosinophils. Cells were re-transduced after 4 and 6 days from isolation. 8 days after last transduction, cells were subjected to flow cytometric analysis: A) GFP expression was analyzed by setting quadrants to distinguish between positive and negative GFP-expressing cells. Life gate dot plots are shown in red colour. B) Contour plots of isotype control staining and galectin-10 expression in untreated cells and cells co-transduced with GFP and shgal-10 monocistronic particles are shown in black colour. Histogram plot of cells stained with isotype control is shown in black line. Histogram blots of galectin-10 expression in untreated cells and cells co-transduced with GFP and shgal-10 monocistronic particles are shown in red and blue lines respectively.

In the light of above results, it was concluded that GFP expression neither interfere with expression level of galectin-10 nor with its knockdown in both AML14.3D10 cells and eosinophils derived from $CD34^+$ HSC. These results paved the way for the design of bicistronic gene transfer vector to be used for the production of GFP+shgal-10 bicistronic lentiviral particles.

3.7 Cloning of shRNA into monocistronic GFP gene transfer vector

shRNA sequences were successfully cloned in pENTR™/U6 vectors (for more details see Materials and Methods). PCR from pENTR™/U6 vectors for the U6 promoters with the shRNA inserts were carried out using pENTR/U6 top and bottom primers. PCR products were verified using 1% agarose gel electrophoresis and showed successful amplification of the shRNA sequences with their U6 promoters which appeared at ~500bp (Figure 3.12:A). PCR products were cut and purified from the gel and subjected to digestion by EcoRI and ClaI restriction enzymes. To ensure successful digestion of PCR products, GFP monocistronic gene transfer vector was digested with the same enzymes under the same conditions and digestion products were verified using 1% agarose gel electrophoresis. While digestion of PCR products appeared at the same DNA band size as before digestion (~500bp), digestion of GFP monocistronic gene transfer vector resulted into 2 bands (Figure 3.12:B), small DNA product of CMV gene appeared at ~375bp and large DNA product of vector backbone appeared at ~7000bp. In conclusion, bicistronic gene transfer vectors containing GFP with shgal-10, GFP with shFoxp3 and GFP with non targeting shRNA sequences were obtained. Each of 3 gene transfer vectors were used separately with envelope and packaging vectors to produce GFP+shgal-10, GFP+shFoxp3 and GFP+shnon-target (GFP+shNT) bicistronic lentiviral particles.

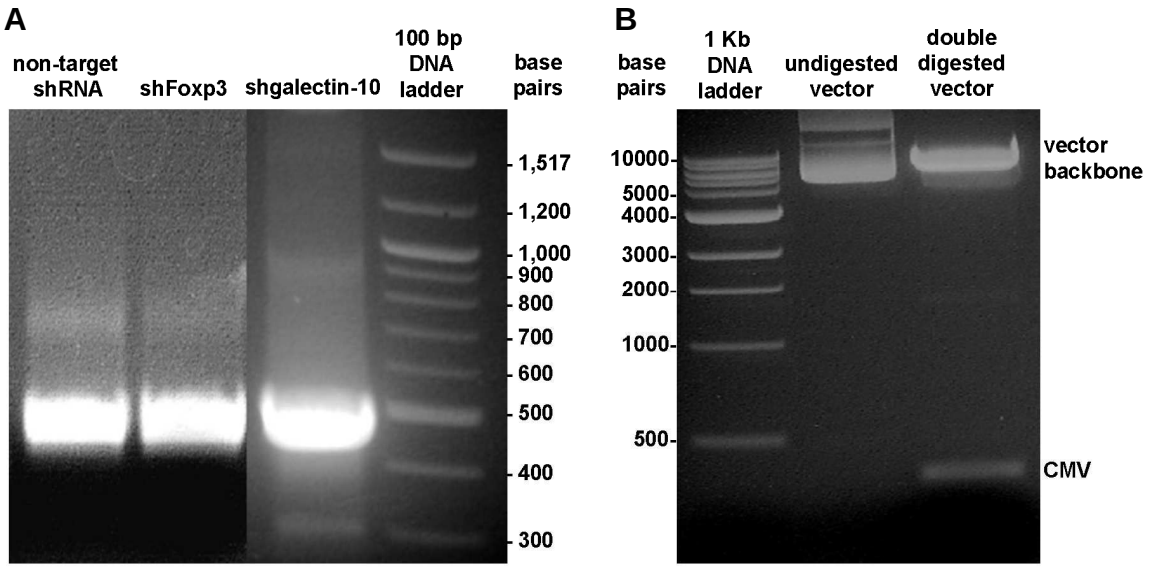

Fig. 3.12: DNA agarose gel pictures of PCR and double digestion products

A) DNA agarose gel picture of PCR products of non-target shRNA, shFoxp3 and shgalectin-10 sequences. Sequences were amplified from their corresponding pENTR™/U6 vectors showing appearance of products at ~500bp. B) DNA agarose gel picture upon digestion of GFP monocistronic gene transfer vector with EcoRI and ClaI restriction enzymes.

Consequently, transduction of AML14.3D10 and $CD34^+$ HSC with GFP+shgal-10 bicistronic lentiviral particles was carried out.

3.8 Transduction of AML14.3D10 cells and $CD34^+$ HSC with bicistronic lentiviral particles

AML14.3D10 cells were subjected to detailed phenotypic characterization for their cell surface markers. This characterization allowed us to follow up any change might occur in the expression profile of any surface marker upon galectin-10 knockdown later. However, this change could be ascribed to activation of shRNA pathway rather than a direct impact of galectin-10 knockdown. Thus, transduction by GFP+shNT lentiviral particles which do not target any human gene but activates the shRNA pathway turned out to be an important control to carry out such experiment. Thereby, any change in surface marker expression due to galectin-10 knockdown could be determind in a specific manner.

3.8.1 Transduction of AML14.3D10 cells with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles

AML14.3D10 cells were transduced with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles. Untreated AML14.3D10 cells served as control. GFP expression started to be measured after 2 days from transduction while galectin-10 expression was

detected after 13 days together with GFP expression. Photos made by fluorescence confocal laser scanning microscopy (CLSM) after 13 days from transduction showed successful transduction of cells with GFP bicistronic lentiviral particles. There was no detectable change in morphology in comparison to the untreated group (Figure 3.13:A). In order to localize GFP expression inside the cells, a stack of two-dimensional images were taken and showed the localization of GFP in every cytosolic part and cell surface but was not detectable inside the organelles (Figure 3.13:B). Galectin-10 staining showed no influence in the galectin-10 expression after transduction with GFP+shNT lentiviral particles compared to untreated cells. However, significant knockdown of galectin-10 was detectable in the group transduced with GFP+shgal-10 bicistronic lentiviral particles but not in to the two other groups (Figure 3.14).

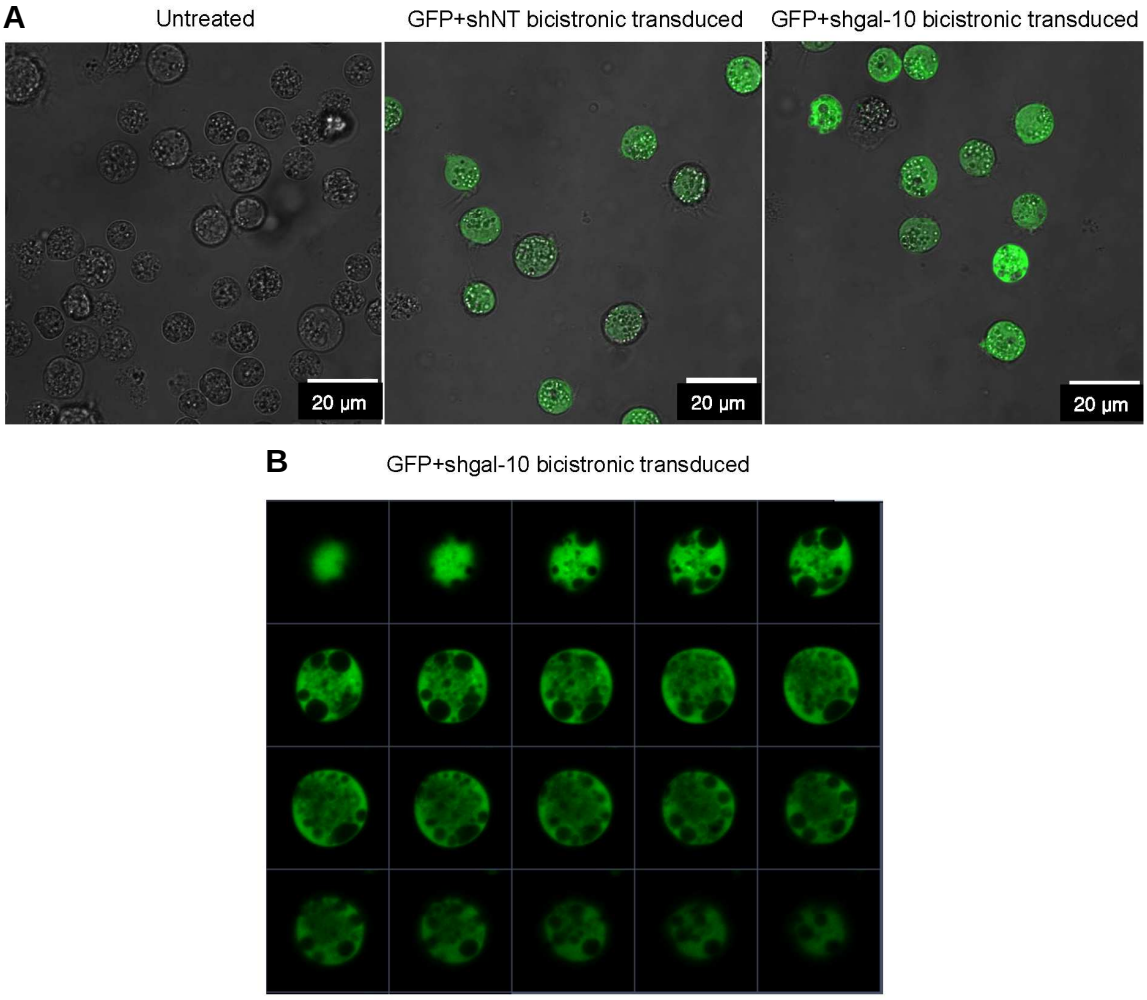

Fig. 3.13: Morphology of AML14.3D10 cells transduced with bicistronic lentiviral particles

A) AML14.3D10 cells were transduced with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles (MOI:96.2). 13 days after transduction cells were subjected to morphological analysis by fluorscence confocal laser scanning microscopy. B) Stack of 2-dimensional images in a cell transduced by GFP+shgal-10 bicistronic lentiviral particles 13 days after transduction by GFP+shgal-10 bicistronic lentiviral particles.

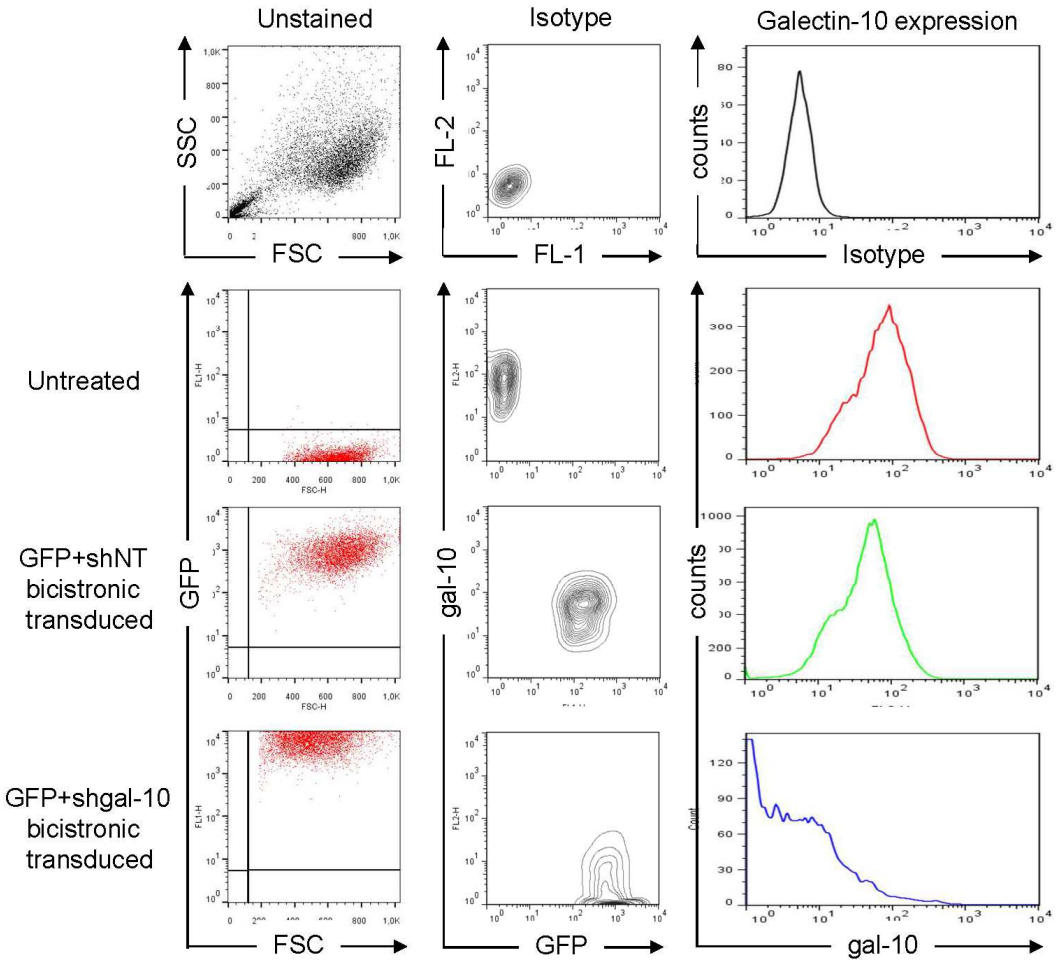

Fig. 3.14: GFP and galectin-10 expression in AML14.3D10 cells after transduction with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles

AML14.3D10 cells were transduced with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles (MOI:96.2). 13 days after transduction cells were subjected to flow cytometric analysis. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour. Contour plots of GFP versus galectin-10 expression are shown in black colour. Histogram plot of cells stained with isotype control is shown in black line. Histogram blots of galectin-10 expression in untreated cells, cells transduced with GFP+shNT particles and cells transduced with GFP+shgal-10 are shown in red, green and blue lines respectively.

In order to study the influence of galectin-10 knockdown on phenotype of AML14.3D10 cells, expression profiles of a wide range of cell surface markers were determined. Among the tested eosinophilic markers were CD33, CD54, CD11b, CD11c, CD9, CD123 and CD29. These markers are adhesion molecules which are involved in cell-cell and cell-substrate interactions or in release of granular proteins. They are commonly expressed in eosinophils and AML14.3D10 as a tumor cell line which exhibits phenotypic characteristics of advanced eosinophilic differentiation. Results showed that

majority of AML14.3D10 cells in the untreated group exhibited high expression level of CD33 and HLA-class-I molecules. Part of cells was positive to CD54, CD80 and CD86. However, there was no signficant change in expression profiles of any tested marker between untreated group and groups transduced with lentiviral particles (Figure 3.15).

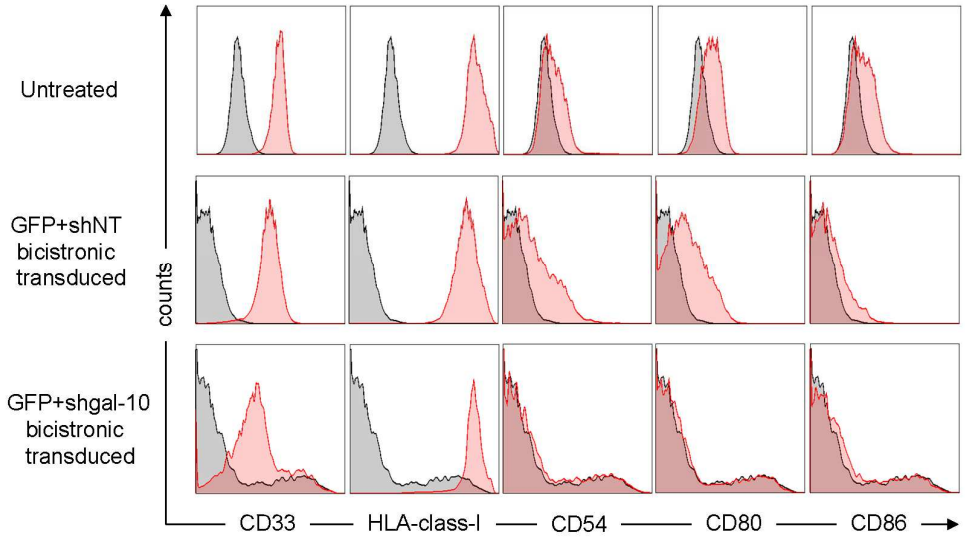

Fig. 3.15: AML14.3D10 cell surface markers after transduction with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles

AML14.3D10 cells were transduced with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles (MOI:96.2). 13 days after transduction cells were subjected to flow cytometric analysis of surface markers. Histogram plots of cells showing expression of CD33, HLA-class-I, CD54, CD80 and CD86 (shown in red colour) overlaid to histogram plots of cells stained with isotype control (shown in grey colour).

3.8.2 Transduction of $CD34^+$ HSC with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles

$CD34^+$ HSC were freshly isolated from cord blood, divided into control, GFP+shNT and GFP+shgal-10 groups and cultured according to IL-5 cytokine protocol to differentiate them into eosinophils. At day 5, cells were transduced with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles. 48 h later, transduction was repeated and cells were kept in culture till day 15. GFP and galectin-10 expression together with the characterized cell surface receptor markers were detected at days 15 and 16. At day 15, galectin-10 staining of the three groups showed nearly complete knockdown of the galectin-10-high subset in the group transduced with GFP+shgal-10 bicistronic lentiviral particles (Figure 3.16:third column). Nearly all cells showed GFP expression in the transduced groups (Figure 3.16:first column). A small difference in galectin-10-high and -low subsets between the untreated cells and cells transduced with GFP+shNT bicistronic lentiviral particles was detectable (Figure 3.16:third column).

There was no significant change in expression level of CD123, CD54, HLA-class-I molecules and CD86 in all groups at day 16 (Figure 3.17). Moreover, galectin-10 knockdown or GFP expression did not trigger any characteristic change in morphology of eosinophils compared to untreated eosinophils as shown by Giemsa staining (Figure 3.18).

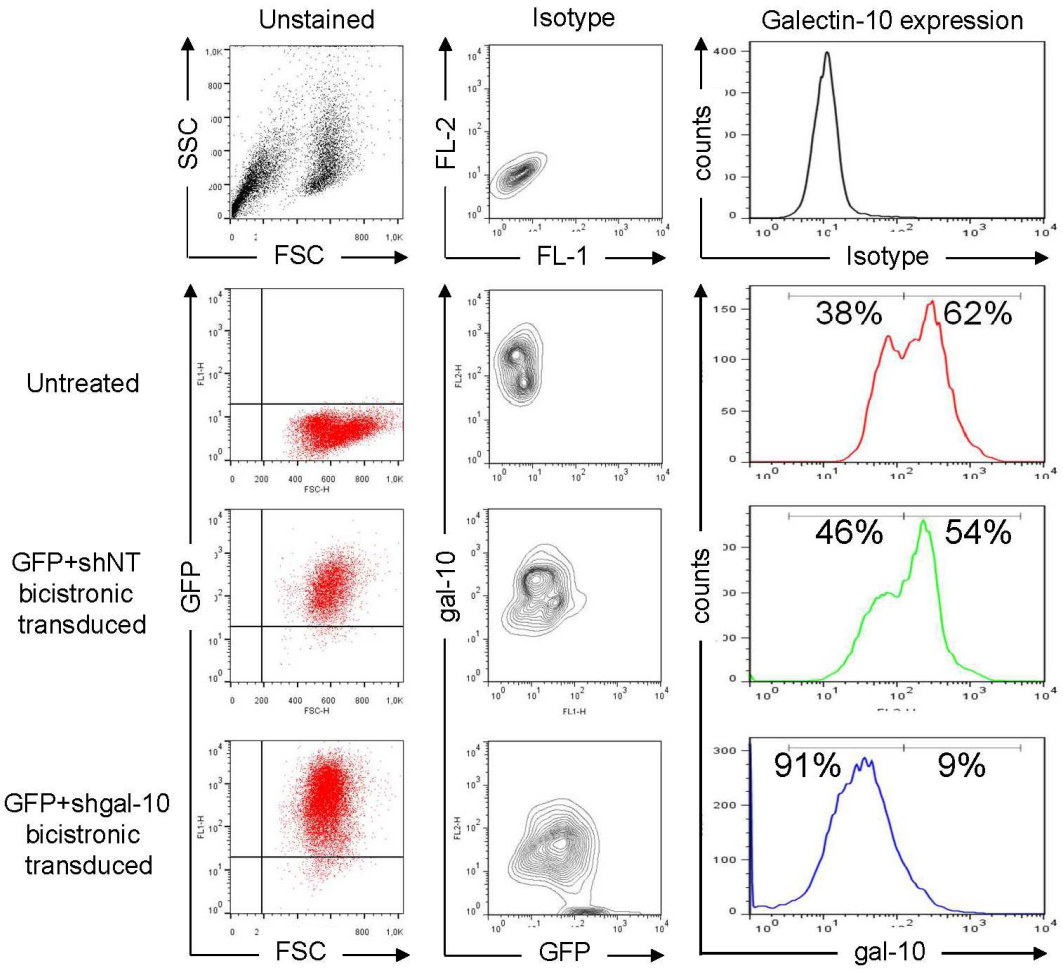

Fig. 3.16: GFP and galectin-10 expression in eosinophils derived from CD34$^+$ HSC after transduction with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles

Freshly isolated CD34$^+$ HSC were cultured by IL-5 cytokine protocol to differentiate them into eosinophils. At day 5, cells were transduced with GFP+shNT and GFP+shgal-10 lentiviral particles or left untreated (MOI:36). 48 h later, transduction was repeated (MOI:16). At day 15, cells were subjected to flow cytometric analysis. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. All life gate dot plots are shown in red colour. Contour plots of GFP versus galectin-10 expression are shown in black colour. Histogram plot of cells stained with isotype control is shown in black line. Histogram blots of cells showing galectin-10 expression in untreated cells, cells transduced with GFP+shNT particles and cells transduced with GFP+shgal-10 are shown in red, green and blue lines respectively.

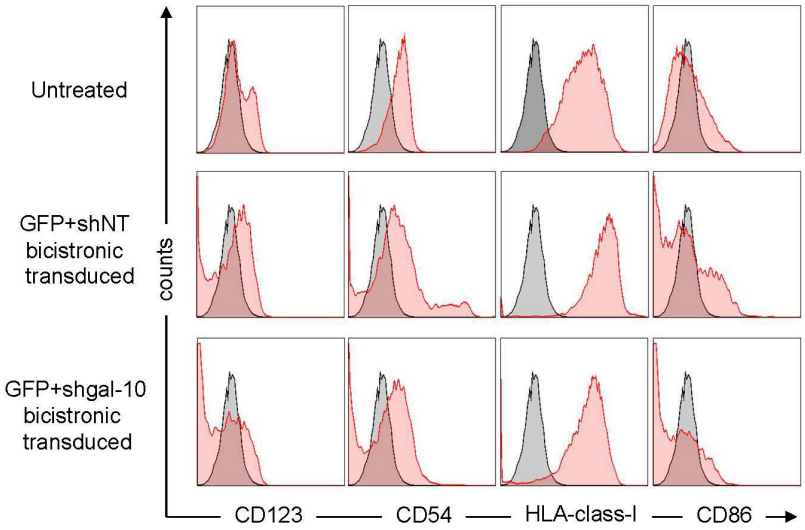

Fig. 3.17: Surface markers of eosinophils derived from CD34$^+$ HSC after transduction with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles

Freshly isolated CD34$^+$ HSC were cultured by IL-5 cytokine protocol to differentiate them into eosinophils. At day 5, cells were transduced with GFP+shNT and GFP+shgal-10 lentiviral particles or left untreated (MOI:36). 48 h later, transduction was repeated (MOI:16). At day 15, eosinophils were subjected to flow cytometric analysis of surface markers. Histogram plots of cells showing expression of CD123, CD54, HLA class-I molecules and CD86 (shown in red colour) overlaid to histogram plots of cells stained with isotype control (shown in grey colour).

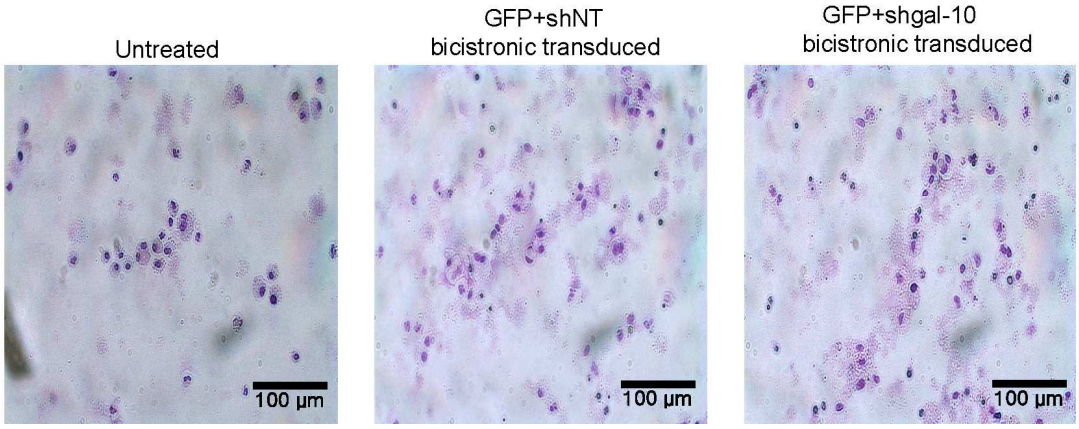

Fig. 3.18: Microscopic pictures of eosinophils derived from CD34$^+$ HSC after transduction with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles

Giemsa staining of eosinophils derived from CD34$^+$ HSC transduced with GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles at day 15.

In conclusion, AML14.3D10 cells and eosinophils derived from CD34$^+$ HSC transduced by GFP+shNT lentiviral particle expressed GFP without any alteration regarding galectin-10 expression. Both cell types transduced by GFP+shgal-10 bicistronic lentiviral particles expressed GFP with significant knockdown in galectin-10 expression.

Therefore, both GFP+shNT and GFP+shgal-10 bicistronic lentiviral particles were shown to function properly and could be used for transduction of $CD34^+$ HSC prior to injection into immunodeficient mice. Moreover, GFP could be used as a reliable cell marker to identify all transduced cells regardless of their galectin-10 expression. Nevertheless, galectin-10 knockdown has neither changed the morphology nor the expression profile of surface markers in both AML14.3D10 and eosinophils derived from $CD34^+$ HSC *in vitro*.

As the impact of galectin-10 knockdown in hematopoietic cells of humanized mice is unpredictable and was not shown in any study before, it was important to compare the outcoming results of this experiment with results coming from a well studied molecule in which the impact of its deletion in human cells is well characterized. Foxp3 was the molecule of choice as it is shown to be constantly expressed in Tregs and mutations in its gene in humans lead to aggressive autoimmunity known as IPEX syndrome [68, 70]. Bicistronic GFP+shFoxp3 gene transfer vector was successfully cloned in a similar way as shown for GFP+shgal-10 and GFP+shNT. Bicistronic gene transfer vector was used with envelope and packaging vectors to produce GFP+shFoxp3 bicistronic lentiviral particles.

The next experiment was done to test whether the produced GFP+shFoxp3 bicistronic lentiviral particles were functional in knocking down Foxp3. The fact that GFP expression does not interefere with Foxp3 expression or its knockdown was shown in other studies [155, 156] and there was no need for further investigation.

3.9 Transduction of $CD4^+$ T cells with bicistronic lentiviral particles

Although Foxp3 knockdown in $CD4^+CD25^+$ Tregs is a crucial goal, transduction of the anergic $CD4^+CD25^+$ Tregs by lentiviral particles is more challenging than transduction of proliferating cells. Therefore, knocking down of induced Foxp3 in $CD4^+CD25^-$ T effector cells after polyclonal activation could be a good measure for production of functional GFP+shFoxp3 bicistronic lentiviral particles.

3.9.1 Transduction of $CD4^+CD25^-$ T effector cells with bicistronic lentiviral particles

$CD4^+$ T cells were depleted from $CD25^+$ Tregs, labeled with proliferation dye and polyclonally stimulated with anti-CD3 and anti-CD28 mAb supplemented with TGF-β to increase Foxp3 expression [157]. Cells were divided into 3 groups, one transduced with GFP+shNT and one with GFP+shFoxp3 bicistronic lentiviral particles while the third group left untreated to serve as control. 3 days after transduction, GFP and Foxp3 expression was determined by flow cytometry. Cells transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles showed GFP expression of of 87% and 96% respectively (Figure 3.19:first column). Untreated cells, cells transduced with GFP+shNT and

GFP+shFoxp3 bicistronic lentiviral particles showed Foxp3 expression of 43%, 31% and 4% respectively. Labeling cells with eFluor®670 proliferation dye allowed cell proliferation to be followed. Proliferation of of 66%, 70% and 72% was obtained respectively (Figure 3.19:third column). There were no detectable differences in expression profiles regarding CD25, CD40L, MHC-class-II molecules and CD69 between untreated and transduced cells (Figure 3.20). These surface molecules are known to be expressed upon $CD4^+$ T activation. While CD25 was highly expressed assuming successful activation of $CD4^+$ T effector cells, MHC-class-II molecules, CD69 and CD40L were not upregulated at that time point.

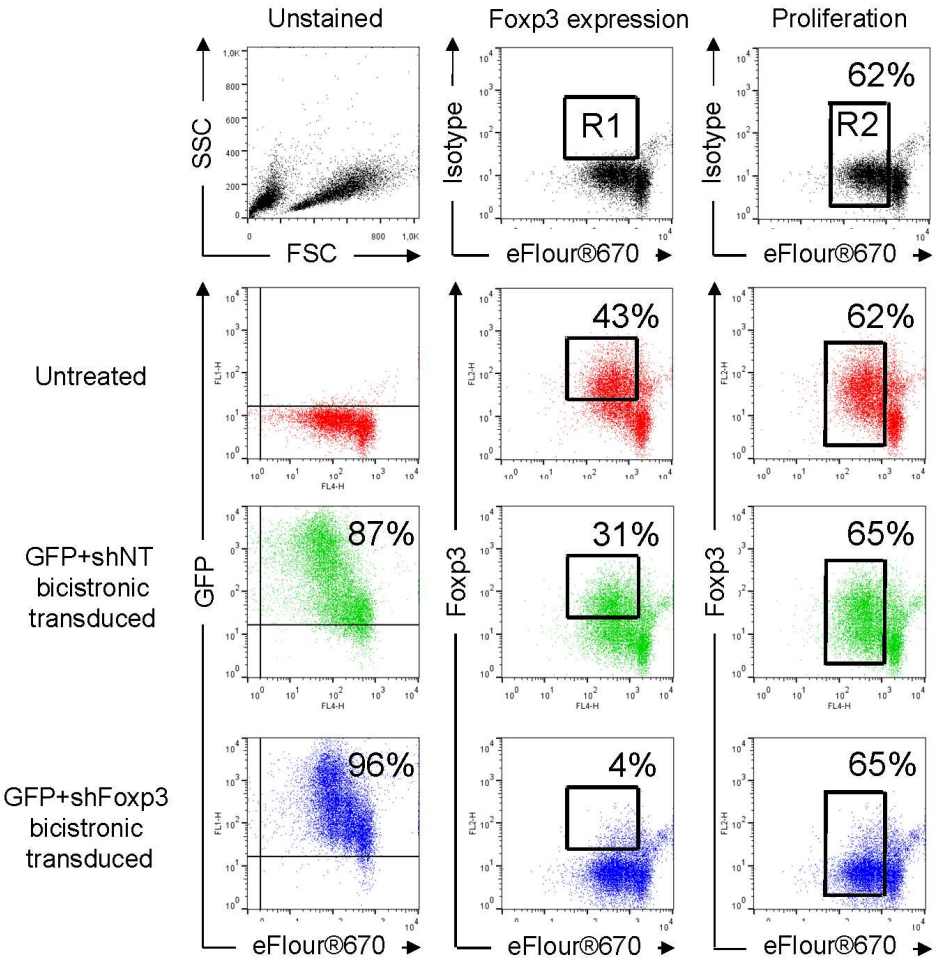

Fig. 3.19: GFP and Foxp3 expression and proliferation of CD4$^+$ T effector cells after transduction with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles

CD4$^+$ T cells were depleted from CD25$^+$ Tregs, labeled with eFluor®670 proliferation dye, stimulated with 0.5 µg/ml anti-CD3 and 1 µg/ml anti-CD28 mAb supplemented with 1 ng/ml TGF-β. After 24 h in culture, cells were transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles respectively (MOI:3.6). After 3 days from transduction, cells were subjected to flow cytometric analyses of GFP, Foxp3 expression and proliferation. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. Two gates were set in dot plot representing isotype control staining, R1 to analyze Foxp3 expression and R2 to analyze cells proliferation. Dot plot of cells stained with isotype control staining is shown in black colour. Dot blots of Foxp3 expression in untreated cells, cells transduced with GFP+shNT particles and cells transduced with GFP+shFoxp3 are shown in red, green and blue colours respectively.

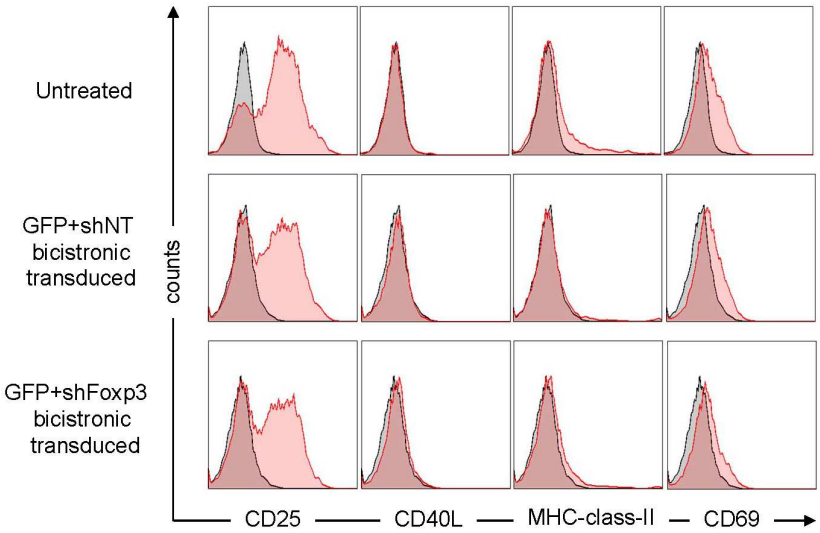

Fig. 3.20: Expression of CD4$^+$CD425$^-$ T effector cell surface molecules after transduction with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles

CD4$^+$ T cells were depleted from CD25$^+$ Tregs, labeled with eFluor®670 proliferation dye, stimulated with 0.5 µg/ml anti-CD3 and 1 µg/ml anti-CD28 mAb supplemented with 1 ng/ml TGF-β. After 24 h in culture, cells were transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles respectively (MOI:3.6). After 3 days from transduction, cells were subjected to flow cytometric analysis of surface molecules. Histogram plots of cells showing expression of CD25, CD40L, MHC-class-II and CD69 (shown in red colour) overlaid to histogram plots of cells stained with isotype control (shown in grey colour).

In conclusion, produced bicistronic lentiviral particles GFP+shNT and GFP+shFoxp3 were shown to function in CD4$^+$CD25$^-$ T effector cells. Foxp3 knockdown in CD4$^+$CD25$^-$ T effector cells did not influence their ability to proliferate. Moreover, Foxp3 knockdown did not trigger any change in surface molecules such as CD25, CD40L, MHC-class-II-molecules or CD69.

3.9.2 Transduction of CD4$^+$CD25$^+$ Tregs with bicistronic lentiviral particles

In order to knock down Foxp3 in CD4$^+$CD25$^+$ Tregs using lentiviral particles, CD4$^+$CD25$^+$ Tregs were cultured for 3 days in presence of IL-7 before being transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles respectively. After 24 h from transduction, untreated and transduced CD4$^+$CD25$^+$ Tregs were divided into three parts. First part of untreated and transduced CD4$^+$CD25$^+$ Tregs was kept in IL-7 without co-culturing (single cultures) in order to analyze their GFP and Foxp3 expression. Second part was co-cultured in presence of anti-CD3 mAb and irradiated PBMC. Third part was co-cultured with previously eFluor®670 labeled and CD25$^+$ depleted CD4$^+$ T effector cells in 1:1 ratio in presence of anti-CD3 mAb and irradiated PBMC. 4 days after transduction,

$CD4^+CD25^+$ Tregs transduced with GFP+shNT and GFP+shFoxp3 lentiviral particles showed 92% and 80% GFP expression in single cultures respectively (Figure 3.21:A). However, there was no detectable Foxp3 knockdown in cells transduced with GFP+shFoxp3 particles in comparison to Foxp3 expression in cells transduced with GFP+shNT particles or untreated cells (Figure 3.21:B).

Activation of $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T effector cells in co-cultures was carried out by anti-CD3 mAb and irradiated PBMC. $CD4^+CD25^+$ Tregs showed GFP expression in both cells transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles measured at day 4 after transduction (Figure 3.22:first column). Moreover, there were no alterations in suppressive function of $CD4^+CD25^+$ Tregs as a result of transduction was observed (Figure 3.22:second column).

In conclusion, Foxp3 knockdown by lentiviral transduction in non-proliferative $CD4^+CD25^+$ Tregs was not successful so far. Therefore, impact of Foxp3 knockdown on anergic and suppressive function of $CD4^+CD25^+$ Tregs could not be investigated.

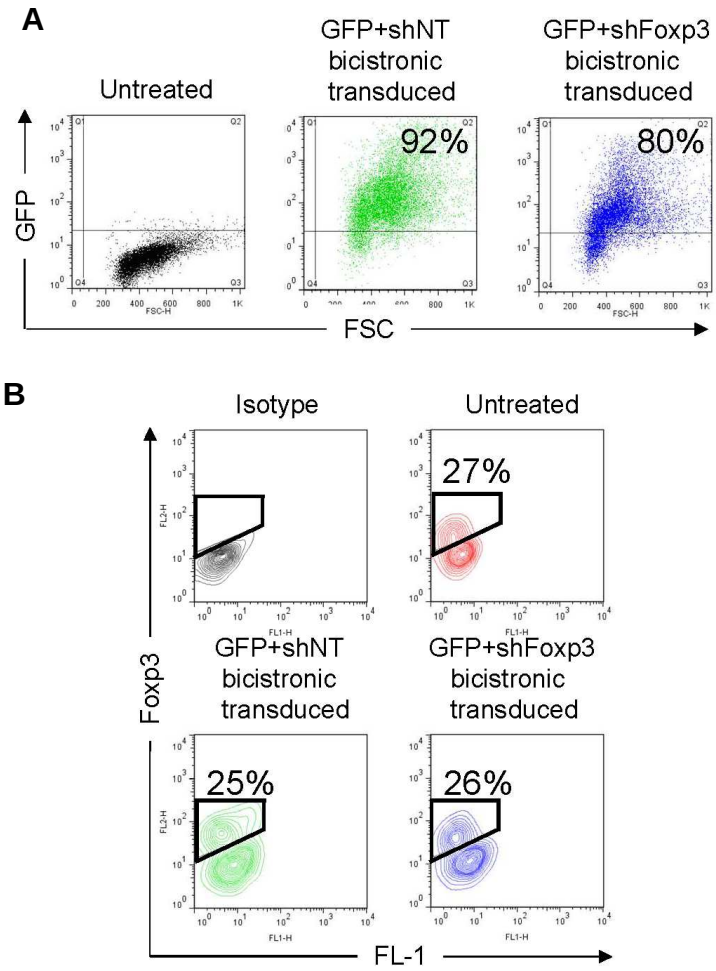

Fig. 3.21: GFP and Foxp3 expression in CD4$^+$CD25$^+$ Tregs transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles

CD4$^+$CD25$^+$ Tregs were isolated, divided into three groups and cultured for 3 days in presence of 1 ng/ml IL-7. After 3 days in culture, two groups were transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles respectively (MOI:3). After 4 days from transduction, CD4$^+$CD25$^+$ Tregs were subjected to flow cytometric analysis. A) GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. Dot plots of untreated cells, cells transduced with GFP+shNT particles and cells transduced with GFP+shFoxp3 particles are shown in black, green and blue colours respectively. B) Foxp3 expression was analyzed by setting a gate in contour plot representing isotype control staining. Contour plot of cells stained with isotype control is shown in black colour. Contour plots of of untreated cells, cells transduced with GFP+shNT and cells transduced with GFP+shFoxp3 are shown in red, green and blue colours respectively

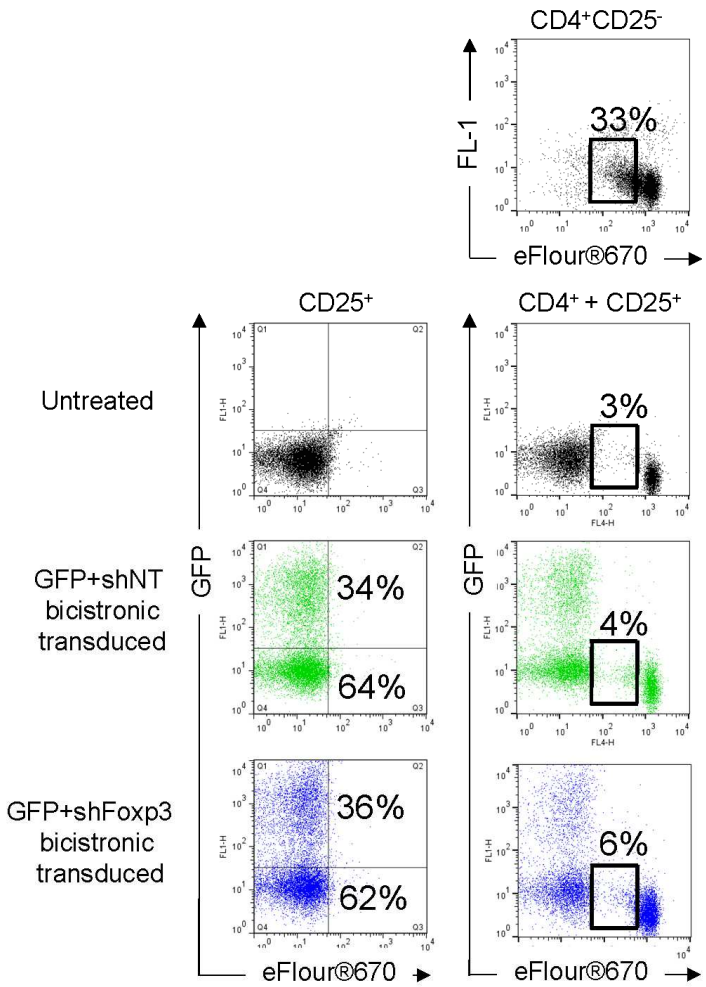

Fig. 3.22: GFP expression in CD4$^+$CD25$^+$ Tregs transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles and proliferation of CD4$^+$CD25$^-$ T effector cells co-cultured with CD4$^+$CD25$^+$ Tregs pre-transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles

CD4$^+$CD25$^+$ Tregs were isolated, divided into three groups and cultured for 3 days in presence of 1 ng/ml IL-7. After 3 days in culture, two groups were transduced with GFP+shNT and GFP+shFoxp3 bicistronic lentiviral particles respectively (MOI:3). After 24 h from transduction, freshly CD4$^+$ T effector cells were isolated, depleted from CD25$^+$ Tregs, labeled with eFluor®670 and co-cultured with the transduced CD4$^+$CD25$^+$ Tregs in 1:1 ratio in presence of 0.5 µg/ml anti-CD3 mAb and irradiated PBMC. Parts of the transduced CD4$^+$CD25$^+$ Tregs were kept without co-culturing with CD4$^+$CD25$^-$ T effector cells (First column). After 4 days from transduction, CD4$^+$CD25$^+$ Tregs were subjected to flow cytometric analysis of GFP expression and proliferation. GFP expression was analyzed by setting quadrants in life gate dot plots to distinguish between positive and negative GFP-expressing cells. Dot plots of untreated cells, cells transduced with GFP+shNT particles and cells transduced with GFP+shFoxp3 particles are shown in black, green and blue colours respectively. Capability of CD4$^+$CD25$^-$ T effector cells to proliferate was tested by culturing the untreated cells in presence of 0.5 µg/ml anti-CD3 mAb and irradiated PBMC (First dot plot in second column). A gate was set to analyze the proliferation CD4$^+$CD25$^-$ T effector cells.

DISCUSSION

The ultimate goal of the study was to knock down galectin-10 and Foxp3 *in vivo* in order to investigate the significance of both Treg-markers. The methodology intended to achieve this goal utilizing shRNA lentiviral technique and humanized mice. The privilege of shRNA lentiviral technique is to combine the ability of the viral particles to transduce and integrate their encoded shRNA genes into the host genome, together with the ability of the shRNA transcribed from the integrated genes to evoke an enduring knockdown of the specific mRNA [158-160]. Humanization of mice is carried out by reconstitution of immunodeficient mice with human $CD34^+$ HSC [148] pre-transduced by lentiviral particles encoding galectin-10- or Foxp3-specific shRNA.

Since human immunodeficiency virus type 1 (HIV-1), the best characterized of the lentiviruses [161], has emerged as a powerful tool for transgene delivery and lentiviral particles derived from HIV-1 were widely used to transduce a broad variety of dividing and nondividing cells [150, 151, 162]. The general strategy used to produce lentiviral particles has been delineated to eliminate all non essential genes from the HIV-1 genome [161]. Also, to separate the transgene to be delivered, in our study shRNA and/or green fluorescent protein (GFP) gene, with sequences required for RNA production and packaging in a plasmid called gene transfer plasmid from sequences code for structural proteins and enzymes in another plasmid called packaging plasmid [161]. Structural proteins are required for particle production while reverse transcriptase and integrase enzymes are packaged into the viral particle and are required upon subsequent transduction [161]. HIV-1 envelope protein controls the key process of viral entry into the cell by determining viral tropism and facilitating the membrane fusion process that allows invasion of the viral genome [163]. gp120, surface envelope glycoprotein subunit of HIV-1, binds to CD4 and a coreceptor (a seven-transmembrane protein of the chemokine receptor family), which are present on susceptible cells such as T lymphocytes and macrophages [163]. A variety of envelope glycoproteins have the ability to associate with the HIV-1 viral membrane, a process that is referred to as pseudotyping. A common used envelope protein is the vesicular stomatitis virus protein G (VSV-G) which confers the ability of HIV-1 based lentiviral particles to transduce a broad range of cell types and is encoded by a third plasmid called envelope plasmid [161, 162]. For that reason, envelope plasmid encoded VSV-G protein was always used in all our experiments to produce lentiviral particles.

In order to establish the procedures for lentiviral particles production, the calcium phosphate transfection method of Graham and van der Eb as one of the most cost-effective and reproducible transfection methods, was used to transfect HEK 293T cells

[138, 142]. Upon subsequent harvesting and concentrating the viral preparations, the titer of the produced lentiviral particles was tested in order to determine their efficiency. Many methods have been described to evaluate lentiviral particle titers [162]. Among which are p24 antigen ELISA, assessment of the reverse transcriptase activity and determination of the genomic RNA concentration in vector preparations by semi-quantitative northern blotting, dot blot analysis or RT-qPCR [162]. p24 protein is a part of the capsid around the RNA genome and the pool that is quantified in the ELISA assay includes a variable amount of free p24 and p24 that originates from non-functional viral particles. Similarly, RNA titers will also assess defective particles, whereas the RT-assay just demonstrates RT activity [162]. Therefore, these techniques overestimate the functional particles titer and are considered as non-functional titration methods [162, 164]. Ideally, a lentiviral particle would contain both a reporter gene such as GFP or an antibiotic resistance gene and the shRNA silencing cassette, both encoded in the gene transfer vector [161]. Hence, transduction of cells following serial dilution of the lentiviral particles and subsequent evaluation of reporter protein activity provides a more accurate and functional technique to quantify functional particles [162]. For this reason, production of lentiviral particles possessing GFP reporter transgene followed by transduction experiments of these GFP monocistronic lentiviral particles were taken over to establish our primary experimental settings. Determination of efficiency of the produced particles through analyzing the GFP expression in the transduced cells by flow cytometry as a most widespread and straightforward technique was carried out after 48 h from the transduction in order to quantify lentiviral titers.

It was critical to begin testing the produced particles in a cell line which demonstrated high capability of transduction by lentiviral particles. Based upon that fact, a continual proliferating tumor cell line of B lymphocyte lineage known as Raji, was the first cell line of choice to determine the TU/ml. A study showed that lentiviral transduction efficiency reached more than 90% in Raji cells [165]. Another study demonstrated a constant level of 17–30% GFP-positive Raji cells over a period of 19 days in order to verify gene expression stability mediated by lentiviral particles [166]. Raji cells showed successful transduction upon serial dilution of GFP monocistronic lentiviral particles. This assumed the production of functional lentiviral particles. GFP expression of cells between 2% and 20% reflects the transduction of one viral particle per cell. Therefore, TU/ml of produced lentiviral particles could be determined. Consequently, MOI which reflects the ratio of transducing viral particles (TU) to the number of cells being transduced in each experiment could be determined. The theory behind MOI is to introduce one viral particle to every cell present in the culture. However, more than one viral particle may transduce the same cell leaving a percentage of cells untreated. Using a higher MOI to ensure that

every cell is transduced could overstep this problem. Increasing the MOI could be achieved either by using more transducing viral particles (increase TU) or reducing the number of cells to be transduced.

Discrepancies in transduction efficiencies of different cell lines using the same volume and titer of vector were previously observed [167]. A study which recognized differences in gene transfer efficiencies between different cell lines suggested a role for VSV-G receptors in this variation [167]. The receptors for VSV-G protein are known to be phospholipids such as phosphatidylcholine (PC) and phosphatidylserine (PS), which are displaying highly dynamic movements. Biosynthesis and degradation of membrane phospholipids are dependent on cell type and cell cycle and/or metabolic activity of individual cells [167]. The study argued for doubling the number of target binding sites for lentiviral particles as well as allowing a period with more favorable conditions (DNA synthesis) for integration during the intermitotic period of division. Another study showed that PS is not the cell surface receptor for VSV-G, although it may be involved in a postbinding step of virus entry [168]. Our result showed that transduction of both continual proliferating galectin-10 expressing AML14.3D10 and Foxp3 expressing Karpas-299 tumor cell lines with GFP lentiviral particles showed a higher GFP expression in AML14.3D10 (93%) cells than in Karpas-299 (39%) cells. Inspite of the ability of the VSV-G to mediate virus entry into all cell types tested to date [168], the reason for different transduction efficiencies of VSV-G pseudotyped HIV-1 based lentiviral particles within different cell types is not fully understood and considered as one of the most overlooked variables in lentiviral transduction [167]. Due to their significance for the expression of galectin-10 and their efficient transduction in comparison to Karpas-299 cells, AML14.3D10 cells were the cells of choice for determination of TU/ml in all further produced lentiviral particles.

Additionally, transduction of AML14.3D10 cells proved that lentiviral particles are efficient gene transfer vehicle by integrating their viral genes into the genome of transduced cells. GFP expression after one year from transduction of AML14.3D10 cells supports that idea and excludes the probability of pseudotransduction. Pseudotransduction might occur due to direct transfer of GFP protein present in lentiviral supernatant to transduced target cells [169].

Several studies have shown the capacity of HIV-1 based lentiviral particles to transduce various types of dividing and nondividing cells [150-152, 162, 170]. Transduction of nondividing cells with lentiviral particles compromises an important privilege for the usage of lentiviral particles over gamma-retroviruses. Several resting cell types such as retinal cells, pancreatic islets, cells of the central nervous system or progenitor and differentiatied hematopoietic cells have shown promising results upon

transduction with HIV-1 based lentiviral particles [152, 170]. A significant drawback associated with the use of gamma-retroviruses is their inability to transduce non-proliferating cells, since retroviral integration is dependent on target cell mitosis [152].

However, early progenitor HSC in G_0, monocytes and resting T lymphocytes are refractory to gene transfer with lentiviral particles [152]. HSC in G_0 represent the most primitive, uncommitted truly quiescent hematopoietic progenitors and in the absence of cytokines, a study observed the block in transduction of HSC in G_0 operated near to the initiation of reverse transcription of viral cDNA while unlikely the block was at the level of virus binding and entry [171]. However, once HSC had been cultured in presence of stimulating cytokines for 48 h, transduction rates markedly become higher. By sorting the cells prior to transduction, it was found that cells in either G_1 or $S/G_2/M$ were up to 10-fold more transducible than cells in G_0 [171]. This assumes that transduction of human HSC with HIV-1 based lentiviral particles is cell cycle dependent [171]. Other studies showed efficient transduction of HSC, after a short incubation with lentiviral particles, in absence of cytokine stimulation. Transduced human $CD34^+$ cells were capable of stable, long-term reconstitution of Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice [172]. Nevertheless, a significant enhancement in gene transfer in presence of a combination of early-acting cytokines IL-6, SCF, TPO and Flt3L were observed [172]. A study showed that transduction enhancement by cytokines was not dependent on cell cycle progression [172]. However, restriction of lentiviral particles to transduce HSC occurred at a postentry step by a proteolytic proteasome complex. Cytokine stimulation rapidly and substantially downregulated proteasome activity while inhibition of the proteasome during transduction dramatically enhanced HSC gene transfer [172]. This assumes according to the absence or presence of cytokines, HSC gene transfer can be tuned to limit the average level of vector integration or to maximize transduction and transgene expression levels [172]. Another important role for the early acting combination of cytokines demonstrated by their ability to mediate a significant *ex vivo* expansion of human HSC capable of repopulating NOD/SCID mice [153, 173]. SCF and Flt3L have been used as key cytokines for HSC expansion, because c-Kit and Flk2/Flt3, tyrosine kinase receptors for SCF and Flt3L, respectively, were shown to transduce signals crucial for HSC development [153]. TPO, a ligand for c-Mpl, has also been shown to stimulate the expansion of primitive hematopoietic cells. Gp130 signal activated by a complex of IL-6 and soluble IL-6 receptor (IL-6/sIL-6R) synergizes with c-Kit or Flk2/Flt3 signal to expand multipotential hematopoietic progenitor cells [153]. In conclusion, the cytokine requirements and cell cycle status of HSC for lentiviral transduction remain controversial.

Our result showed that it was possible to transduce $CD34^+$ HSC with lentiviral particles upon their stimulation for 2-3 days with the early acting cytokines or with SCF,

GM-CSF and IL-3. SCF, GM-CSF and IL-3 are important to differentiate HSC into myeloid progenitor cells which terminally differentiate into eosinophils upon supplying IL-5 to the medium [133].

Previous studies showed possible differentiation of eosinophils from HSC [133, 134]. The significance of such differentiation for this study based on the fact that galectin-10 is the major constituent in eosinophils comprising about 7-10% of total cellular protein [111]. Moreover, galectin-10 was shown to be found in Tregs and essential for their anergic and suppressive properties when co-cultured with $CD4^+CD25^-$ T effector cells [107]. However, *in vitro* differentiation of $CD34^+$gal-10^- HSC into $CD25^+$gal-10^+ Tregs is not possible without thymic selection processes. Therefore, differentiation of $CD34^+$gal-10^- HSC into granulocytes expressing galectin-10, specifically eosinophils, was the remaining alternative to achieve this goal.

GM-CSF and IL-3 induce a significant increase in eosinophil precursors. However, they induce the differentiation into neutrophils and macrophages under the same conditions [144]. Receptors for GM-CSF, IL-3, and IL-5 consist of a ligand-specific α subunit (GMRα, IL-3Rα, and IL-5Rα respectively) and a common β-subunit (β_C). The α-subunit is the major ligand-binding subunit and on its own does not seem to transduce any of the biological activities ascribed to GM-CSF, IL-3 and IL-5 [174]. The β_C-subunit, on the other hand, converts the ligand-bound α subunit to a high affinity state and is important for the signaling process [174]. Three signal pathways at least are known to be activated by the three cytokines, the JAK/STAT pathway, the ras/MAP kinase pathway, and the PI 3-kinase pathway [174]. These signal pathways emanating from β_C were shown to be important for cell proliferation, survival and differentiation of myeloid cells [174]. IL-5 is specific and a late-acting factor for eosinophils production, survival, differentiation and granule release of eosinophils and participates in allergic reactions and anti-parasite responses [144].

The validation of finally differentiated eosinophils from $CD34^+$ HSC *in vitro* was examined by histological analysis showing typical morphology in agreement with previous study [133]. Another study showed that galectin-10 is one of the most abundantly expressed proteins at the mRNA level in developing eosinophil progenitors from umbilical cord blood [117]. However, our result provided a premier investigation for detection of galectin-10 on protein level in eosinophils differentiated from $CD34^+$gal-10^- HSC from umbilical cord blood. Moreover, instead of tedious purification of small numbers of short-lived eosinophils from peripheral blood [132], our result showed that eosinophils derived from $CD34^+$ HSC are long living stable galectin-10 expressing cells. They could be used to test the efficiency of lentiviral particles encoding shRNA against galectin-10 upon

transduction of $CD34^+$ HSC and differentiating them into galectin expressing eosinophils together with the constitutively galectin-10 expressing tumor cell line AML14.3D10.

Nearly all of both AML14.3D10 cells and eosinophils derived from $CD34^+$ HSC showed homogeneous galectin-10 expression. However, both cells showed two subsets of galectin-10, a high and a low expressing subset. The GFP expression in the majority of AML14.3D10 cells apparently did not influence the ensemble expression of galectin-10. A slight increase in galectin-10 high subset was recognized, the effect which could be attributed to the equal frequency of integration of the GFP gene throughout the AML14.3D10 cell genome. This frequent integration might upregulate nearby genes including the galectin-10 gene. Previous studies showed that integration may cause tumors by upregulating cellular oncogenes adjacent to the integration site [175, 176]. Although GFP expression in eosinophils differentiated from $CD34^+$ HSC was not detectable in majority of cells, nevertheless, nearly all cells including GFP-expressing cells showed homogeneous galectin-10 expression. The discrepancy in GFP expression and transduction efficiency of $CD34^+$ HSC we showed in comparison to other studies could be attributed to many reasons including differences in purity of stem cells, lentiviral particles construct, MOI and transduction protocol used or in handling of cells after transduction [154, 170-172, 177].

Galectin-10 knockdown in both AML14.3D10 cells and eosinophils derived from $CD34^+$ HSC provided a compelling evidence for production of functional lentiviral particles encoding shRNA against galectin-10. Galectin-10 knockdown in both cell types of cells was not shown in any study. Additionally, we showed that GFP expression did not interfere with the knockdown pathway of galectin-10 in both types of cells. 60% and 50% reductions in the high galectin-10 subset in AML14.3D10 and eosinophils respectively were compensated by the same magnitude of increments in low galectin-10 subset. These results showed that shRNA encoded by lentiviral particles is targeting galectin-10 high subset only and design of bicistronic gene transfer vector encoding GFP and shRNA against galectin-10 to be used for production of GFP+shgal-10 bicistronic lentiviral particles could be a useful approach.

Together with designing of GFP and shgal-10 bicistronic lentiviral vector, bicistronic vector encoding GFP and non-target shRNA (shNT) vector was designed in order to produce GFP+shNT bicistronic lentiviral particles. GFP+shNT lentiviral particles provided a useful negative control that activated RNA-induced silencing complex (RISC) pathway, but did not target any other investigated genes. This allowed examination of transduction effects of a short-hairpin on expression of gene of interest (galectin-10 and Foxp3). Cells transduced with these particles provided a useful reference for interpretation of the knockdown results [178].

AML14.3D10 cells and $CD34^+$ HSC transduced by GFP+shNT lentiviral particles expressed GFP without any change in relative galectin-10 expression in comparison to untreated cells. This result confirmed the previous one that GFP expression did not influence endogenous expression of galectin-10. However, a slight reduction in the high galectin-10 expressing subset within the eosinophils differentiated from $CD34^+$ HSC (54%) transduced with GFP+shNT lentiviral particles compared to the untreated cells (62%) was recognized. This reduction could be attributed to the activation of RISC pathway by non-target shRNA. Nevertheless, a significant knockdown of high galectin-10 expressing subset within eosinophils differentiated from $CD34^+$ HSC transduced with GFP+shgal-10 lentiviral particles was detected. This increase in galectin-10 knockdown using bicistronic lentiviral particles is attributed to increase in the MOI compared to previous experiments done with monocistronic lentiviral particles. Increasing the MOI led to increase in GFP expression and a significant galectin-10 knockdown.

It was important to investigate the consequence of galectin-10 knockdown on the phenotype of AML14.3D10 cells and eosinophils derived from $CD34^+$ HSC as it was not shown before. No morphological alterations were detected upon galectin-10 knockdown in both cell types. However, it can not be excluded that some morphological changes which are below our detection limits occurred upon galectin-10 knockdown. Automatic detection of granules and statistical analysis of their spatial locations in different cell groups were proposed in one study and provided an advanced technique in morphological cell image analysis [179]. To detect any change in phenotype on surface marker level upon galectin-10 knockdown, surface receptor profiles of eosinophils derived from $CD34^+$ HSC were determined as shown in previous studies [134, 180]. The same receptors were tested in AML14.3D10 cells as tumor cell line which exhibits an advanced eosinophilic phenotype [132].

CD54 is an intercellular adhesion molecule known as ICAM-1, the ligand for LFA-1 (leukocyte function-associated antigen-1) and the highest density of ICAM-1 is in the endothelium, its presence influences LFA-1 dependent adhesion of leukocytes to endothelial cells and immune functions involving cell to cell contact [181, 182]. CD54 expression on AML14.3D10 cells and eosinophils derived from $CD34^+$ HSC is in agreement with previous study which argued for adhesion-mediated cross-talk between eosinophils and LFA-1-positive cells. This cross-talk is influenced by stimulation of eosinophils with selected inflammatory cytokines and demonstrating a role for IL-3, IL-5, and GM-CSF in regulation of ICAM-1 expression in human cells [183]. Another interesting marker is the adhesion glycoprotein CD33 which was found to be highly expressed on AML14.3D10 cells with a low expression level in eosinophils. CD33 functions as a sialic acid-dependent cell adhesion molecule whose functions and binding properties are

unknown [184]. CD11b, CD11c, CD9 and CD29 are also adhesion molecules which are involved in cell-cell and cell-substrate interactions [185-189]. CD11b and CD11c associate with integrin β2 and function as receptors for ICAM-1 [187, 189]. CD123 the α-chain of the IL-3 receptor (IL-3Rα) plays an important role in hematopoietic cell growth and differentiation [144, 174, 190]. In both cell types, there was no significant change in the expression profiles of any tested surface marker upon galectin-10 knockdown. This result is in agreement with previous studies showing that galectin-10 is present intracellulary in eosinophils, basophils and Tregs [107, 108, 110], and supports the postulation that galectins do not have specific individual cellular receptors, but each can bind to a set of cell-surface glycoproteins containing suitable oligosaccharides through lectin–carbohydrate interactions [108].

Another important molecule which is expressed in Tregs and was a crucial target for shRNA lentiviral transduction was Foxp3. While galectin-10 was shown to be expressed in eosinophils and basophils [111], Foxp3 is transiently expressed in activated $CD4^+CD25^-$ T effector cells [77, 78].

However, studies showed that resting T lymphocytes are refractory to gene transfer by lentiviral particles [152, 191]. Anti-CD3/CD28 stimulation of quiescent $CD4^+$ T cells is important for successful infection by lentivirus like HIV-1 [149]. This study demonstrated that activation of $CD4^+$ T cells with anti-CD3 mAb alone resulted in cell cycle progression into only G_1a and incomplete viral reverse transcription. However, transition of cells into the G_1b phase of the cell cycle was required to efficiently complete reverse transcription process and was achieved by co-stimulation of the CD28 receptor [149]. Based upon that fact, we polyclonally stimulated $CD4^+$ T cells with anti-CD3 mAb and anti-CD28 mAb as optimal stimuli for co-stimulation signal or by interaction with CD80/CD86 ligands on APCs. A step which was crucial for successful transduction of activated $CD4^+$ T cells by GFP+shFoxp3 and GFP+shNT lentiviral particles. Consequently, TCR-dependent stimulation of human $CD4^+CD25^-$ effector T cells *in vitro* leads to transient up-regulation of Foxp3 [77, 78]. A recent study showed that Foxp3-deficient T effector cells proliferate more and produce more cytokines than wild type T effector cells and have differential expression of 264 genes [192]. Our result, in contrast, showed that there was no detectable difference on the prolilferative capacity or surface marker expressions upon Foxp3 knockdown. The reason for this discrepancy in results is still not clear and should be a matter for more investigations. However, the same study showed that no difference was found in expression of CTLA-4 or CD25 between control or siFoxp3 transduced T effector cells.

Therefore, activation of resting T cells by anti-CD3/CD28 mAb causing G_0 to G_1b transition of cell cycle is required to relieve the block in the reverse transcription process

[149]. This could render the cell permissive to transduction with lentiviral particles but consequently triggers cell division and proliferation. Moreover, TCR stimulation of T cells alters their half life and immune competence and leads to enrichment in activated memory cells associated with loss of naïve T cell subsets and a skewed TCR repertoire [149, 193-196]. Alternatively, several studies suggested the exposure of T cells to cytokines such as IL-2 and IL-7 that do not trigger cell division but render the cells permissive to transduction with lentiviral particles in absence of TCR stimulation [149, 196-198]. While IL-2 was shown to be necessary for development and homeostasis of Tregs [149, 199, 200], IL-7 is known to be a master regulator of T cell survival and homeostatic proliferation [152, 201-203]. In agreement with previous studies, our results showed that exposure of freshly isolated Tregs to IL-2 and/or IL-7 alone rendered them permissive to lentiviral transduction in the absence of TCR activation. It is assumed that cytokine-treated Tregs moved out of G_0 into the G_1b phase of cell cycle, the phase in which cells are susceptible to lentiviral transduction, but did not start to proliferate [149, 152, 196-198]. Thereby, partial activation of resting T cells is sufficient for gene transfer by lentiviral particles without allowing for DNA synthesis or mitosis. Although cells transduced with GFP+shNT and GFP+shFoxp3 lentiviral particles showed GFP expression assuming successful transduction of lentiviral particles into the cells, no detectable knockdown of Foxp3 in $CD4^+CD25^+$ Tregs after transduction with GFP+shFoxp3 lentiviral particles was recognized. However, as siRNA technique silences the target molecule on RNA level [159, 178], RT-PCR is strongly recommended and provided a suitable method to detect the impact of shRNA knockdown on the mRNA level. A study showed possible transduction of $CD4^+CD25^+$ Tregs with lentiviral particles upon activation of cells with soluble anti-CD3 mAb, IL-2 and autologous irradiated APC for 18 h [204]. Transduction efficiency ranged from 30% to 85% with significant down regulation of Foxp3 obtained in siFoxp3 transduced cells in comparison to mock transduced Tregs. Expression of CTLA4 was reduced in siFoxp3 transduced cells. Moreover, Foxp3 knockdown in Tregs was sufficient to reverse the anergic phenotype and suppressive potential evidenced by clear reduction in suppression and proliferative response. Therefore, it is recommended to try the same protocol used in this study to knock down Foxp3 in Tregs. Moreover, it is also recommended to measure Foxp3 expression at least after 12 days from activation as performed in the study showing Foxp3 knockdown in Tregs. Regardless from Foxp3 knockdown in $CD4^+CD25^+$ Tregs, our result showed that IL-7 did not affect the suppressive function of Tregs after 7 days in culture. This result is in agreement with previous study which reported that IL-7 do not modulate the phenotype and functional characteristics of the transduced T cells and induces a much more restricted number of cell divisions compared to a TCR mediated signal [204].

Although Foxp3 knockdown utilitzing shRNA lentiviral transduction was not detectable in $CD4^+CD25^+$ Tregs, the knockdown of Foxp3 in $CD4^+CD25^-$ T cells displayed a crucial evidence for the production of functional shFoxp3 lentiviral particles.

Therefore, both shRNA against galectin-10 and Foxp3 could be used to highlight the significance of these molecules in a functional humanized *in vivo* model.

For the next *in vivo* experiments, it is strongly recommended to use bicistronic lentiviral particles encoding shRNA against galectin-10 or Foxp3 and GFP. The main advantage from using GFP bicistronic lentiviral particles is to sort the GFP expressing $CD34^+$ HSC after short time from transduction. This sorting allows reconstitution of immunodeficient mice with GFP^+CD34^+ HSC and their differentiation into hematopoietic cells showing galectin-10 or Foxp3 knockdown immune cells *in vivo*. Also, GFP expression will facilitate detection of all human immune cells upon following the humanization process in peripheral blood and different organs in all treated groups. It is also recommended to decrease the number of transduction times into one or two times maximum, reduction in transduction efficiency resulting from this could be compensated by increasing the MOI. It is critical to avoid prolonged stimulation of cells with cytokines as this could increase the risk of altering their characteristic biological properties and reduce their long-term *in vivo* repopulation ability.

SUMMARY

The maintenance of tolerance to self is a function of $CD4^+CD25^+$ regulatory T cells ($CD4^+CD25^+$ Tregs). $CD4^+CD25^+$ Tregs suppress inadequate and autoaggressive immune responses. Galectin-10 and Foxp3 are important proteins that participate in suppressive mechanisms of Tregs. Galectin-10 is one of the oldest known human-specific proteins which is not found in any other species. Foxp3 is a transcription factor expressed in human $CD4^+CD25^+$ Tregs and in activated $CD4^+CD25^-$ T effector cells. siRNA-mediated knockdown of this intracellular soluble protein abolishes the suppressive function of human $CD4^+CD25^+$ Tregs.

This work presents *in vitro* investigations carried out preceding the risky endeavour to knockdown galectin-10 and/or Foxp3 in humanized mice. It was possible to establish the procedures for production of lentiviral particles, which proved to be an efficient vehicle for gene transfer into human stem cells and different tumor and immune cells. This result was indicated by long term expression of GFP in AML14.3D10 tumor cells transduced by lentiviral particles encoding GFP. Moreover, it was possible to generate lentiviral particles encoding shRNA against galectin-10. The generated particles were shown to be functional and knocked down galectin-10 in AML14.3D10 cells which constitutively express galectin-10. Our study presented a premier investigation for detection of galectin-10 on protein level in eosinophils differentiated from human $CD34^+$ hematopoietic stem cells (HSC). Significance of this stable galectin-10 expression *in vitro* evoked from being an accessible alternative for the impossible *in vitro* differentiation of $CD4^+CD25^+$ Tregs from $CD34^+$ HSC. Incorporation of GFP gene within the lentiviral particles encoding shRNA against galectin-10 was a crucial step in order to mark the cells displaying galectin-10 knockdown. The novel bicistronic lentiviral particles turned out to be functional in both eosinophils differentiated from $CD34^+$ HSC and AML14.3D10 cells which exhibit an advanced eosinophilic phenotype. Finally, utilizing bicistronic lentiviral particles encoding GFP and shRNA against Foxp3, it was possible to knock down Foxp3 in $CD4^+CD25^-$ T effector cells referring to the production of functional lentiviral particles.

LIST OF ABBREVIATIONS

A	Adenosine
7-AAD	7-Aminoactinomycin D
Ab	Antibody
AML	Acute myeloid leukaemia
APC	Allophycocyanin
APCs	Antigen presenting cells
BSA	Bovine serum albumin
β_C	common β-subunit
bp	Base pairs
C	Cytosine
^oC	Degree Celsius
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
$Ca_3(PO_4)_2$	Calcium phosphate
CD	Cluster of differentiation
CFSE	5,6-Carboxyflouresceine diacetate succinimidyl ester
CLC	Charcot-Leydin crystal
CLSM	Confocal laser scanning microscopy
CMP	Common myeloid progenitors
Conc.	Concentration
CRD	Carbohydrate-recognition domain
CMV	Cytomegalovirus immediately early promoter
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DCs	Dendritic cells
DMEM	Dulbecco‘s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
ds	Double stranded
E. coli	*Escherichia coli*
EDTA	Ethylene di-amine tetra-acetic acid
EF1	Elongation factor 1
ELISA	Enzyme-linked immunosorbent assay

ELPs	Early lymphoid progenitors
FACS	Fluorescence activated cell sorter
Fig	Figure
FITC	Fluorescein Isothiocyanate
FCS	Fetal calf serum
FL	Fluorescence channel
FLT3L	Fms-like tyrosine kinase 3 ligand
FOXP3	Forkhead box P3
FSC	Forward scatter
G	Guanine
$G_{(number)}$	Gap
gal-10	Galectin-10
GARP	Glycoprotein A repetitions predominant
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony-stimulating factor
h	Hour
HEK	Human embryonic kidney
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV-1	Human immunodeficiency virus type 1
HSA	Human serum albumin
HSC	Haematopoietic stem cells
IFN-γ	Interferon gamma
ICAM-1	Intercellular adhesion molecule 1
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
iTregs	*in vitro*-induced Tregs
JAK	Janus Kinase
LA	Luria Broth
LAP	latency-associated peptide
LFA-1	Leukocyte function-associated antigen 1
LMPPs	Lymphoid multipotent progenitors
LPS	Lipopolysaccharide
LTBP	latent-TGF-β binding protein
LPLase	Lysophospholipase
M	Mitosis

m	Minute
mAb	Monoclonal antibody
MACS	Magnetic activated cell separation
MAP	Mitogen activated protein
MEM	Minimal essential medium
MHC	Major histocompatability complex
MOI	Multiplicity of infection
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NK	Natural killer
No.	Number
NOD	Non-obese diabetic
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
Oligo	Oligonucleotides
p	Plasmid
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phycoerythrine
PE-Cy5	Phycoerythrine cyanine dye Cy5
PI	Propidium iodide
PI 3-kinase	Phosphatidylinositol 3-kinase
Pol III	RNA polymerase III
PRPs	Pattern recognition receptors
PS	Phosphatidylserine
pTregs	peripherally derived Tregs
R	Receptor
R(number)	Region
RAG	Recombination activating gene
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
RT-qPCR	Real time quantitative polymerase chain reaction

S	Synthesis
s	Second
SCID	Severe combined immnodeficiency
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
shNT	shnon-target
shRNA	Short hairpin RNA
sIL-6R	Soluble interleukin-6 receptor
SRC	Severe combined immnodeficiency (*scid*) mouse-repopulationg cells
SP	Single positive
spMHC	self-peptide/major histocompatibility complex
SSC	Side scatter
STAT	Signal transducer and activator of transcription
T	Thymine
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TH	Hybridization temperature
Th	T helper
TPO	Thrombopoietin
Tregs	Regulatory T cells
Tris	Tris(Hydroxymethyl)aminomethane
tTregs	Thymus-derived Tregs
TU	Transducing unit
UV	Ultraviolet
V	Voltage
VSV-G	Vesicular stomatitis virus protein G
w/v	Weight per volume

LIST OF FIGURES

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CURRICULUM VITAE

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Education and Academic Summary

- 2013: **Ph.D** in **Immunology, University Medical Center** of the **Johannes Gutenberg-University Mainz, Department of Dermatology**, Mainz, Germany.
- 2007: **M.Sc** in **Molecular Bioengineering, International Master Program of Dresden University of Technology (Bioinnovation Zentrum and Max Plank Institute of Molecular Cell Biology and Genetics/ Dresden**). Dresden University of Technology, Dresden, Germany.
- 2002: **B.Sc** in **Pharmaceutical Sciences,** Faculty of Pharmacy, Cairo University, Cairo, Egypt.
- 1997: General Secondary School Certificate, Giza, Egypt

Work and Research Experience

- 2013: Did my doctoral's thesis in immunology at the laboratory of HD Dr. Helmut Jonuleit research group in Department of Dermatology, **University Medical Center** of the **Johannes Gutenberg-University** in **Mainz**.
- 2007: Did my master's thesis which is entitled "Regulation of I_{to} current in **human's heart** by the accessory subunit MiRP1 using *Xenopus laevis* oocytes as an expression system", at the **Institute of Pharmacology and Toxicology** in **Medical Theoretical Center** (**MTZ**) of **Carl Gustav Carus Medical Faculty** of TU-Dresden**,** under supervision of Prof. Erich Wettwer.
- 2005 – 2008: Worked as student assistant at the **Institute of Pharmacology and Toxicology** of **Carl Gustav Carus Medical Faculty**.
- 2005: Worked in the Institute of "**Physiology I"** at **Eberhard Karls University** in Tübingen at the laboratory of Prof. Christoph Böhmer.
- 2003 – 2004: **Quality Control Analyst** at the **National Organization for Drug Control and Research** in Cairo, Egypt.