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„Regulator of G Protein Signaling 16 (RGS16) Protein, a putative immune checkpoint expressed in dendritic cells“

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1. Introduction

1.1 Dendritic cells

The immune system is commonly divided in two parts, the innate and adaptive immunity. The innate immune response is an early defense mechanism, which is immediately activated upon pathogen recognition and represents an unspecific immune response. Different cell types, including macrophages, natural killer cells and neutrophils are involved as well as the complement system. On the contrary, the adaptive immunity is acquired and modified over an entire life-span and works in an antigen-specific manner mediated primarily by B and T lymphocytes. B cells provide an immune response based on the release of antigen-specific antibodies. T cells on the other hand, have a wide range of functions. Some subsets control B lymphocytes, others recognize and destroy infected cells and other subtypes support phagocytic cells to destroy pathogens¹.

However, the adaptive immune response heavily relies on activation by the innate immune system, specifically on professional antigen-presenting cells (APCs). The dendritic cells (DCs) are considered to be the most important mediators of immune responses. Their main task is to take up, process and present antigens on their surface to stimulate cells of the adaptive immune system².

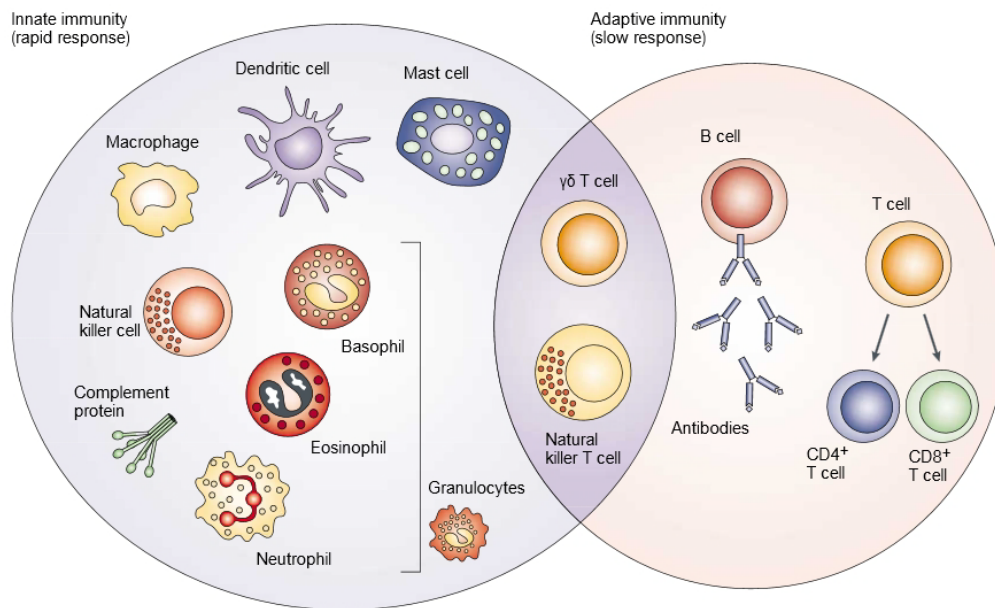


Figure 1.1: The cells of the innate and adaptive immune system. The innate immune response is considered to be the first line of defense and reacts quickly when facing pathogens. The adaptive immunity takes longer to be initiated, but provides a highly-specific response and memory effect to enhance the response further upon repeated challenge with the same antigen. Natural killer T cells and $\gamma\delta$ T cells are thought to be part of both immune responses³.

DCs express a high number of different receptors such as toll-like receptors (TLRs) or cytoplasmic receptors. Therefore, they are considered to be the most versatile sensors of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs). They are able to stimulate cells of the adaptive immune system by presenting antigens via major-histocompatibility complex (MHC) molecules amplified by co-stimulatory molecules, illustrating their importance in the initiation of adaptive immune responses. In their immature state the DCs scan their environment and take up antigens, but they are not able to efficiently present them on the surface. In their mature stage, DCs were shown to have a highly decreased ability to take up antigens, but with exceptional capacity to display antigen-peptides on the surface and stimulate T cells^{4,5}.

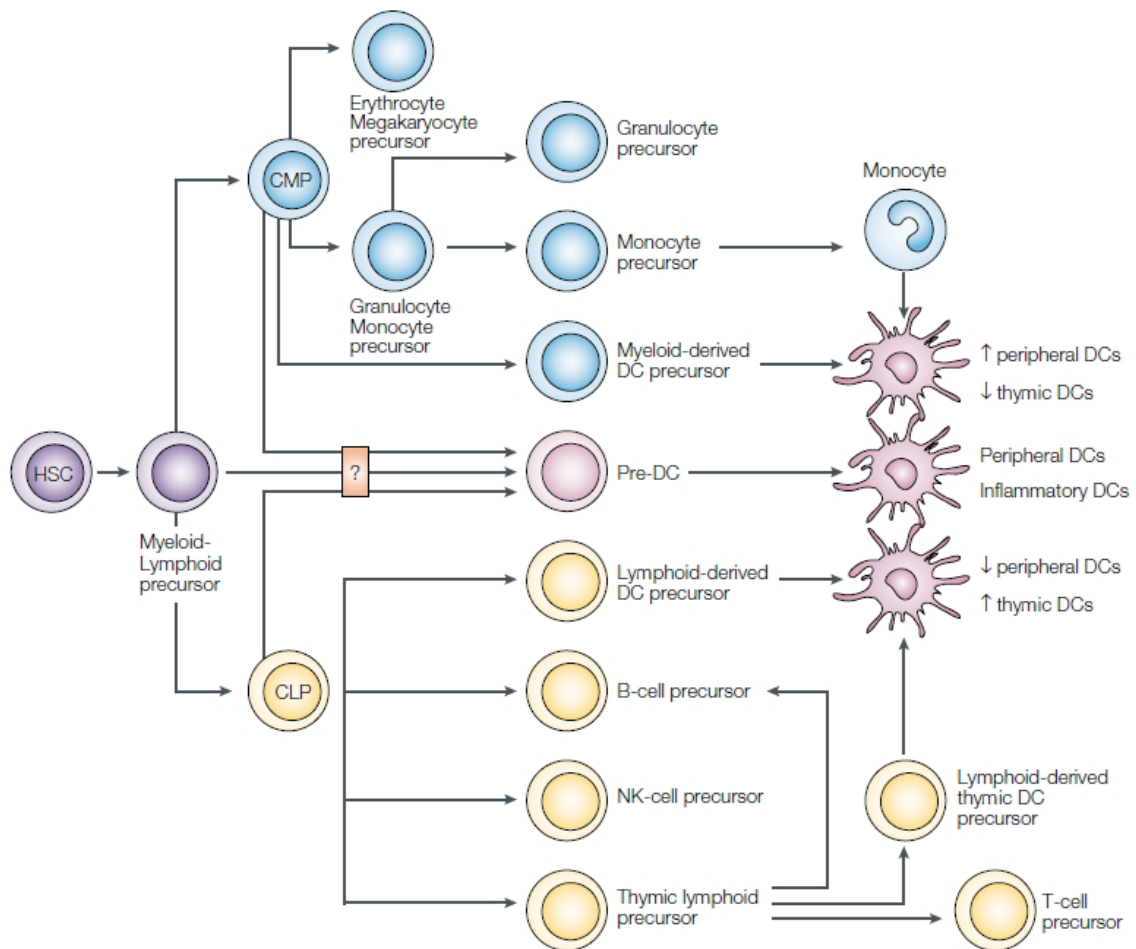


Figure 1.2: DCs develop from precursor cells of lymphoid and myeloid origin. Myeloid-derived DCs are derived from the common myeloid progenitors (CMP) expressing fms-related tyrosine kinases 3 (FLT3⁺). Lymphoid-derived DCs develop from common lymphoid progenitor (CLP) in the bone-marrow or from thymic lymphoid precursors. HSC, haematopoietic stem cells; NK, natural killer⁶

Originally, it was believed that DCs originated exclusively from myeloid precursors. However, nowadays it is generally known that DCs also develop from lymphoid origin as displayed in figure 1.2⁶⁻⁸. In the last two decades it has been established that DCs consist of a diverse spectrum of subpopulations, which revealed a significant variation in the expression and function of its innate receptors⁹⁻¹¹.

1.1.1 DC Maturation

DCs are located in the majority of tissues throughout the body, as for example in the blood (in their immature state) or in the skin, where they are called Langerhans cells. In the immature state their ongoing task is to capture and phagocytose antigens. Once they encounter an activation stimulus, they take it up and process it for subsequent presentation on the surface. Consequently, the maturation process is initiated¹.

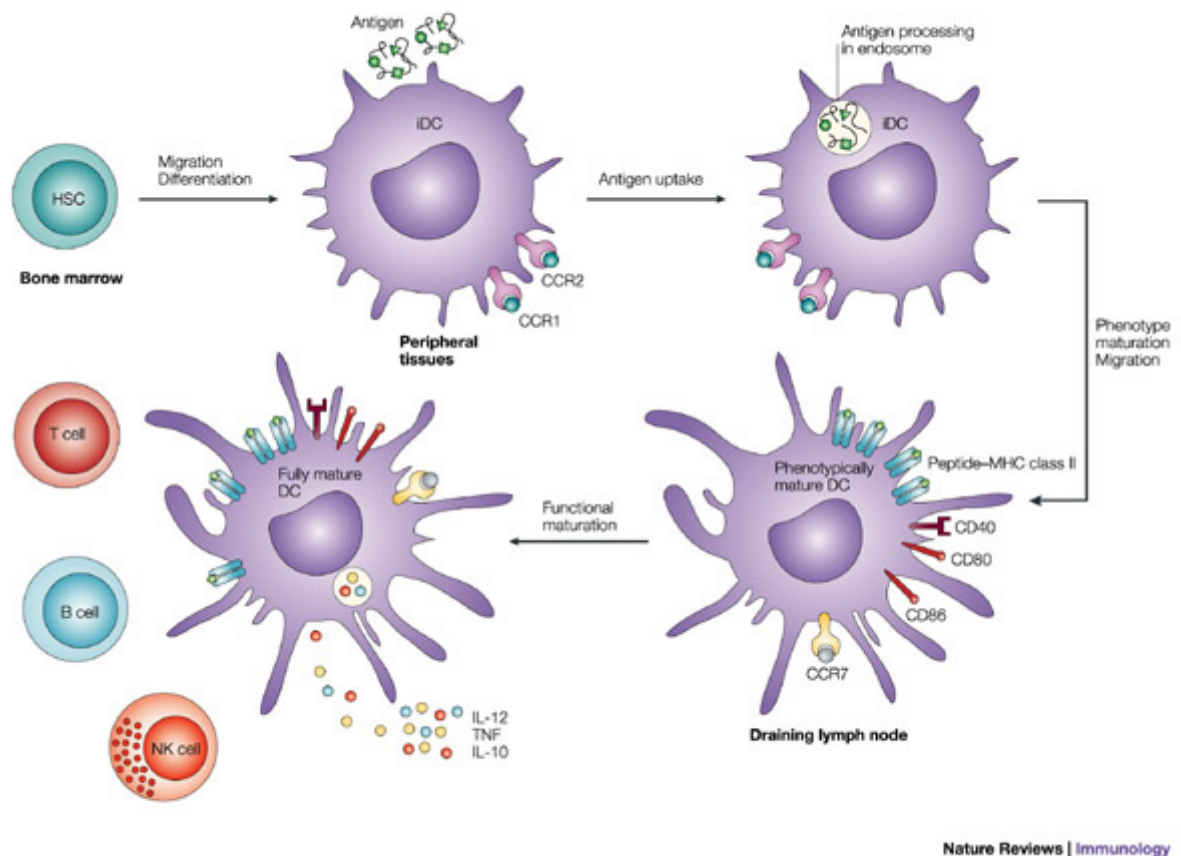


Figure 1.3: DC maturation and migration to the lymph nodes. Upon antigen uptake DCs are activated and the maturation process is initiated. They start to migrate into the lymph node through the lymphatic vessel system. Furthermore, they activate and prime T lymphocytes specific for the uptaken antigen¹².

The DCs downregulate their ability to take up antigens and upregulate several surface molecules, such as MHC II-peptide complexes or co-stimulatory molecules CD80 and CD86, which vastly enhance their stimulatory capacity to T cells. Furthermore, by upregulating CCR7, they start to migrate through the lymphatic system to regional lymph nodes. In the secondary lymph organs they are capable of attracting and activating CD4⁺ and CD8⁺ T cells^{2,13,14}.

1.1.2 Immune activation and regulation

As previously stated, when DCs receive an activation stimulus, they migrate to the draining lymph nodes and stimulate T, B and NK cells. The DCs can distinguish between the incredible diversity of antigen stimuli and as a result induce different immune responses¹².

To this day, one of the most prominent models that explain the interaction between DC and T cells is the Signal model. Initially, a T cell binds to the antigen-presenting MHC molecule with its specific TCR, defined as 'Signal 1'. Then, 'Signal 2' is referred to as 'co-stimulation', since it is administered by co-stimulatory molecules as CD80 and CD86 which bind to CD28 on the T cell surface. Both signals are required for full activation of the T cells. Additionally, the DCs release different cytokines depending on the antigen stimulus they originally received. It is referred to as 'Signal 3' and determines the polarization into the different types of effector cells, such as T helper cells or cytotoxic T lymphocytes (CTLs) (see figure 1.3)¹⁵⁻¹⁷.

Exogenous pathogens, including bacteria and parasites are presented via MHC class II molecules, which lead to an interaction with CD4⁺ T cells, also called helper T cells. So far, several subtypes of CD4⁺ T cells have been discovered, e.g. T helper type 1, 2 or 17 (T_H1, T_H2, T_H17), regulatory T cells (T_{reg})^{18,19}.

Intracellular antigens, e.g. tumor cells or virus-infected cells, are displayed by MHC class I molecules, which are present on the surface of nearly all cells in the body, and activate CTLs. Moreover, the ability of certain antigen-presenting cells to take up, process and present extracellular antigens through MHC class I molecules is called cross-presentation and is important for immunity against viruses which e.g. impair DC function and tumors. This process is also essential to induce cytotoxic immunity by vaccination. When virus- or tumor-specific effector CTLs detect an antigen, which is displayed on the surface, the TCR binds to the MHC class I molecule complex and destroys the cell. However, naïve CTLs need to be stimulated by professional APCs first, usually DCs, to efficiently exert their cytotoxic function. The antigen-presentation by other cells then enhances the CTL response^{20,21}.

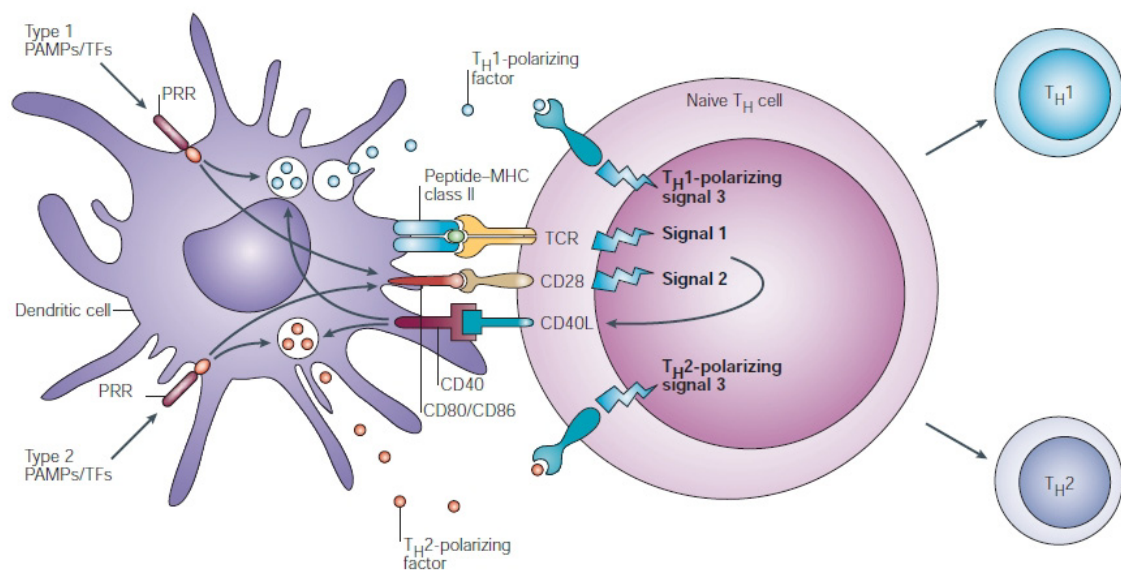


Figure 1.4: Dendritic cells activate T cells. Following antigen-presentation by the DCs, specific TCRs bind to the MHC-antigen complex and the T cell is stimulated with the antigen-specific Signal 1. Signal 2 comes from co-stimulatory molecules such as CD80/CD86 on the DC surface, which triggers CD28 on the T cell surface. Both signals are required for a full activation of the T cell. Additionally, varying with the antigen-signal, the DCs release cytokines, which polarizes the T cell response in a certain direction, lately referred to as Signal 3¹⁶.

However, DCs are not only important in activating adaptive immune responses. It has been found, that DCs also play a crucial role in the maintenance of tolerance. In the absence of maturation stimuli they can tolerize CD4⁺ and CD8⁺ T cells. Immature DCs

continuously take up antigens. If it is a self-antigen, they present them in absence of co-stimulatory molecules and this leads to inactivation of naïve T cells through anergy, deletion or stimulation for development into regulatory T cells (T_{regs}). Furthermore DCs are able to induce tolerance by engaging CD80/CD86 to the cytotoxic T-lymphocyte antigen 4 (CTLA4), a negative regulator of T cell activation^{22,23}. Therefore, 'Signal 2' cannot be used as a marker for immunogenicity, since it also has a function in tolerance induction. This is an important mechanism that eliminates autoreactive T cells in the periphery, which escaped thymic deletion and would be activated by DCs presenting self-antigens. Hence, DCs in the steady state contribute to the control of autoimmunity^{24,25}.

1.2 Exosomes

1.2.1 Biogenesis & Secretion

Exosomes are small vesicles released by a large number of mammalian cells. They are present in many and mayhaps all biological fluids such as plasma, urine, saliva, bronchoalveolar lavage (BAL) and cerebrospinal fluid (CSF)^{26–28}. These small vesicles range between 30 and 120 nm in size, and are therefore too small to be visualized by photon microscopy or directly measured by flow cytometry²⁹. Exosomes originate from multivesicular bodies (MVBs), which is shown by the presence of late endosome components including Alix, TSG101 or Tetraspanins such as CD9, CD63 and CD81^{27,30}.

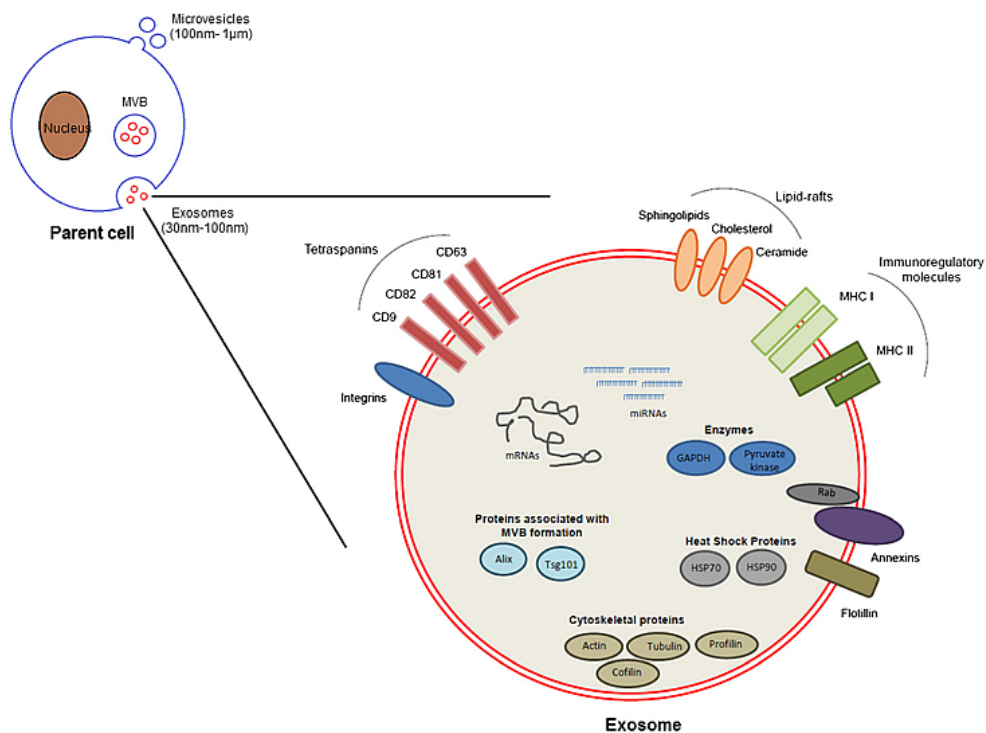


Figure 1.5: Exosome composition. Exosomes are capable of transporting a variety of cellular components such as viruses, mRNAs, miRNAs, DNA and proteins depending on various factors including the type and state of the original cell. The exosome membrane contains several proteins involved in antigen presentation (MHC I, MHC II), targeting and adhesion (integrins, tetraspanins), as well as membrane trafficking (annexins, rab proteins)³¹.

Cells use their endosomal system to release exosomes. Also, two membrane inversion steps are necessary, which allow exosomes to present their transmembrane cargo to the plasma membrane in the same orientation relative to the cytoplasm^{30,32}. The first inversion is named endocytic internalization, where the plasma membrane invaginates before budding off. Then, at the early endosome, depending on several specific protein interactions, the cargo is either recycled or the early endosomes mature into late endosomes where the second membrane inversion occurs³³. The intraluminal vesicles bud off into the lumen of the late endosome, allowing the uptake of cytoplasmic material. Regulated by endocytic proteins the exosomes are formed in the MVBs and loaded with cargo. In a majority of cells MVBs are destined to fuse with lysosomes to ensure the degradation of their content. However, MVBs with specific surface molecules, such as CD63, LAMP1/2 or e.g. MHC class II in APCs, prevent the

degradation. Furthermore, these late endosome MVBs fuse with the plasma membrane and release exosomes into the extracellular space^{34,35}.

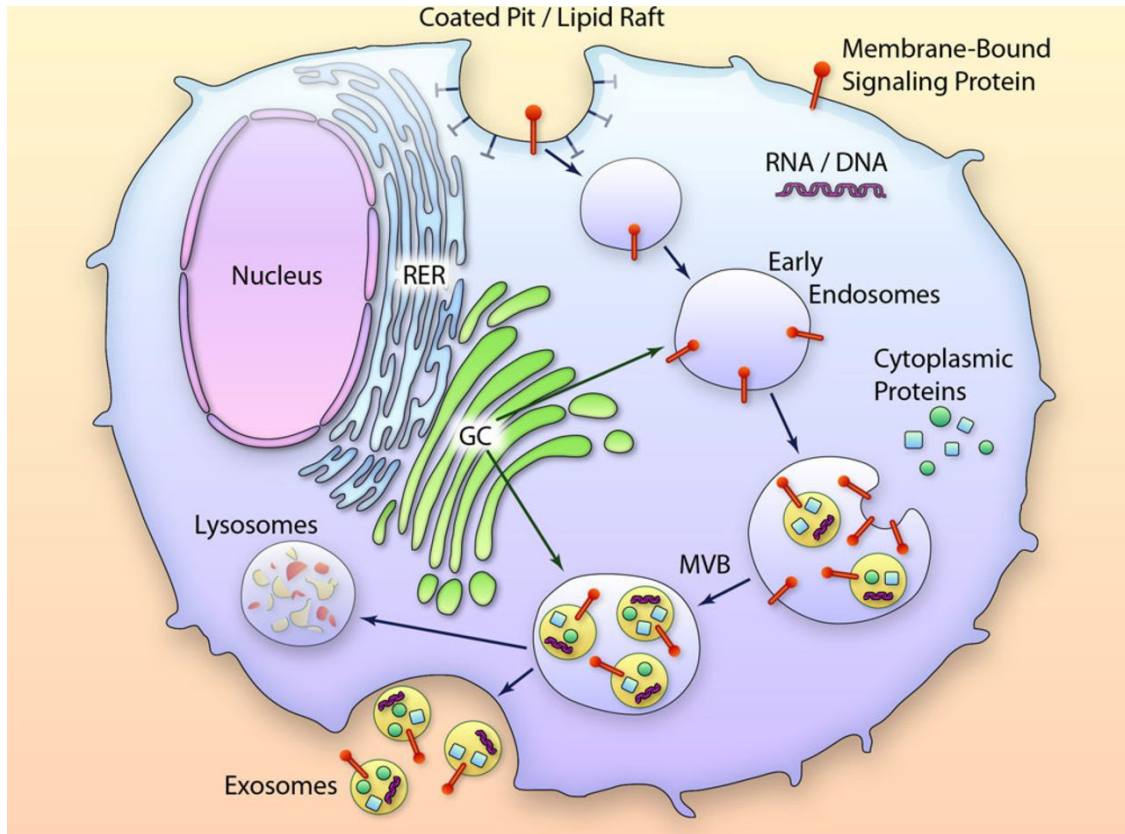


Figure 1.6: The biogenesis of exosomes. Exosomes are generated in late endosomes and proteins from coated pits, proteins from the cytoplasm, RER and GC as well as mRNA, miRNA and DNA. By fusion with the plasma membrane the late endosomal compartment releases exosomes to the extracellular space. MVB, multivesicular body; GC, Golgi complex; rough endoplasmic reticulum³⁶.

The functionality of exosomes is highly dependent on their ability to interact with the recipient cells and deliver their cargo into the cell. Exosomes can either be taken up by cells close to the originating cell, in some range within the tissue or may travel through systemic circulation to reach distant cells. The specificity of exosomes to bind their target cells is likely to be determined by adhesion molecules^{37,38}. As an example, T cells were shown to be capable to recruit DC-derived exosomes containing MHC class II molecules³⁹. Moreover, differences in the exosome-specific compilation of tetraspanin complexes influence the selection of the recipient cell, which has been shown *in vitro*

and *in vivo*. This effect is modulated by associated proteins including other adhesion molecules^{38,40}.

However, numerous studies that exist today suggest that the specificity of targeting recipient cells by exosomes is dependent on surface ligands present on both³³.

Once exosomes reach their target cell they bind to its plasma membrane. They either dissociate and fuse directly with the plasma membrane or are endocytosed by the cell⁴¹. When internalized by endocytic pathways, exosomes can fuse with the endosomal delimiting membrane and release their cargo or may also be taken up for degradation by lysosomes. The endocytic pathway is assumed to be much more common than the plasma membrane fusion process^{37,42}.

1.2.1 Exosomes in the immune system

In 1996, exosomes were firstly mentioned in the context of immune responses. It was discovered that Epstein-Barr virus-transformed B lymphocytes were able to secrete exosomes presenting antigens on MHC class II molecules. Additionally it was shown that these exosomes were able to display the antigens to specific T cells suggesting a role in adaptive immunity⁴³. Since then, the potential role of exosomes as additional mediators regulating adaptive immune responses has led to a huge number of publications and still provides a very intriguing field with several suggestions of a potential use of exosomes as therapeutic agents, e.g. in cancer patients⁴⁴.

Exosomes are capable of stimulating primed CD4+ and CD8+ T cell including memory T cells^{39,43,45,46}. Exosomes can also activate naïve T lymphocytes, but not directly. They have to be taken up by DCs, which subsequently present the antigen from the exosomes. Interestingly, the DCs capturing the antigen-presenting exosomes often do not have the right MHC molecules themselves and are therefore able to present the antigen on their surface^{46,47}.

Be that as it may, just a few years ago it was shown that DC-derived exosomes are capable of activating CD8⁺ T cells from OT-I mice *in vitro*. Additionally, the DCs were shown to be able to use exosomes as source of antigen, stimulating CD8⁺ T cells by loading and presenting the peptide onto their own endogenous MHC molecules⁴⁸. Exosomes from matured DCs revealed higher efficiency inducing T cell activation *in vitro* and effector T cell activation *in vivo* in comparison to exosomes derived from immature DCs^{41,49}. Furthermore it was shown that mature DC-derived exosomes have a higher impact on the antibody response⁵⁰. However, exosomes released from immature DCs or DCs that were treated with immunosuppressive agents were shown to promote tolerogenic immunity, which would provide a potential way of treatment in the context of autoimmune diseases^{51,52}.

In addition, exosomes are also capable of carrying antigens from their originating cell. DCs can capture the exosomes and degrade the antigen in their machinery to associate them with MHC molecule for presentation to T lymphocytes. This effect was shown by using exosomes from cells infected with different pathogens e.g. macrophages infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis*, as well as cytomegalovirus-infected endothelial cells. Exosomes from these cells were able to induce distinct CD4 and CD8 T cell responses^{53,54}.

To summarize, the currently proposed role of exosomes in adaptive immunity is to spread antigens or MHC-antigen complexes to enhance the DC mediated immunity or to directly interact with primed T lymphocytes boosting the immune response.

1.2.1 Exosomes & tumors

The development of cancer is a complex, multistep process and the importance of exosomes is accentuated by their abundance in biological fluids of cancer patients compared to healthy people⁵⁵. Intriguingly, exosomes have been reported to cause anti- and protumorigenic effects. On the one hand cancer cell-derived exosomes are

able to enhance the anti-tumor immunity by transporting tumor antigens to DCs and therefore induce immune responses^{56,57}. It has been shown in mice that exosomes, from heat-stressed tumor cells or cells expressing inflammatory cytokines, are capable of inducing anti-tumor immune responses^{58,59}.

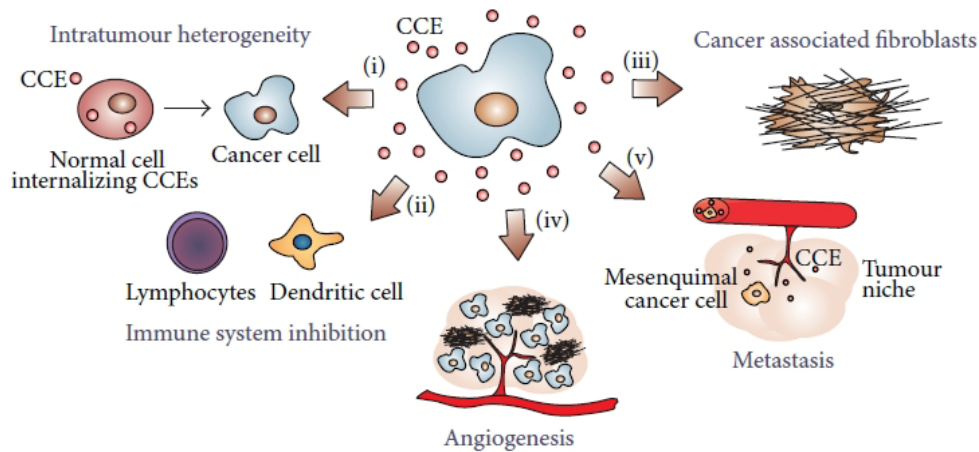


Figure 1.7: Protumoric effects of cancer-derived exosomes influencing the tumor microenvironment. Normal cells take up the cancer-cell derived exosomes (CCE) and become cancer cells (i). Exosomes inhibit the immune response (ii), support differentiation of fibroblasts into cancer associated fibroblasts (iii) and facilitate metastatic expansion by preparing premetastatic niches at distant locations. Furthermore, they enhance the angiogenesis in the microenvironment, to sustain the increased demand of oxygen (iv)⁵⁶.

On the other hand however, tumor-derived exosomes enhance tumorigenesis on several levels. They modulate the tumor microenvironment to generate a metastatic niche to ensure growth and expansion^{60,61}. They facilitate evading and attenuating immune responses^{62,63} and enhance the invasion ability to normal cells^{64,65}. It has been shown *in vitro* that tumor-derived exosomes may contain immunosuppressive molecules which decrease proliferation of CD4 and CD8 T cells^{66–68}, natural killer cells^{69,70} and enhance the proliferation of immune-suppressive cells such as regulatory T cells and myeloid cells^{71,72}. When a tumor grows so quickly, that it outgrows its blood supply, partial regions of the tumor are deprived of oxygen. In this case, tumors secrete exosomes with enhanced angiogenic and metastatic potential to accelerate the angiogenesis and create a more favorable environment for metastasis^{73,74}.

The contradictory immune effects are currently explained by the heterogeneous nature of exosome populations depending on the cells of origin and their state when releasing the exosomes. In the future, this field of research will receive increasing attention, which could potentially provide therapies against several diseases including cancer and autoimmune disorders.

1.3 G protein coupled receptor signaling & Regulator of G protein signaling proteins

1.3.1 G protein coupled receptor signaling

G protein coupled receptors (GPCRs) represent one of the largest families of proteins. They consist of seven transmembrane helices, an extracellular amino-terminal domain and an intracellular carboxyl-terminus and provide the largest source of targets for pharmaceutical drug application. Due to their great diversity GPCRs are able to detect an incredibly large spectrum of extracellular stimuli. They are not only part of several physiological processes such as the senses of vision, smell and taste, but are also involved in the regulation of mood and behavior. Moreover, they play a role in chemokine signaling regulating immune responses and inflammation. In its inactive state a GPCR is bound to a heterotrimeric G protein. The heterotrimeric G proteins administer the signal transduction between the membrane-bound receptor and the intracellular effector molecules^{75,76}.

In its inactive form the guanine exchanging factor (GEF) domain is bound to the equally inactive α subunit of a heterotrimeric G protein, consisting of an α , β and γ subunit. When an agonist binds to the extracellular or transmembrane domains of a GPCR, conformational changes are initiated and the activation signal is transmitted through the transmembrane domains. The GEF domain activates the G protein by promoting the replacement of a GDP molecule for a GTP at the α subunit. The G protein dissociates from the GPCR and furthermore detaches from the $\beta\gamma$ -dimer. As a result,

the free G_{α} -GTP and $G_{\beta\gamma}$ complexes interact with further effector molecules and therefore administer the signal transduction between the membrane-bound receptor and the intracellular enzymatic effectors. The α subunit possesses a slow intrinsic GTPase activity. Hence, the inactive trimeric G protein form is slowly regenerated^{77,78}.

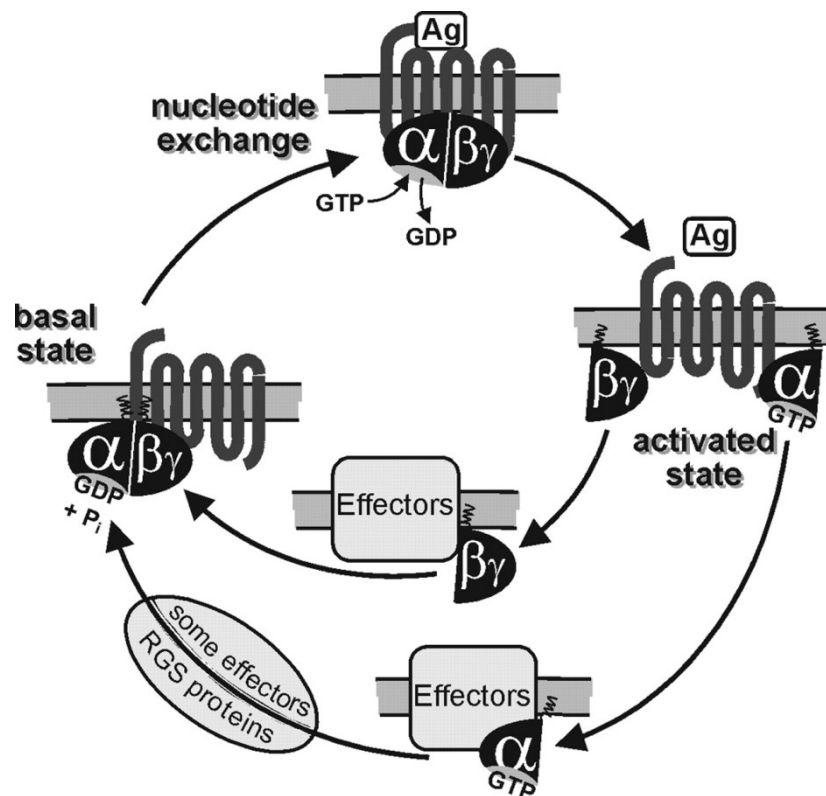


Figure 1.8: GPCR activation cycle. In the inactive state the α subunit forms a trimer with the $\beta\gamma$ subunit. Upon ligand binding, the transmembrane receptor undergoes conformational changes, which activate the GEF activity and stimulates the exchange of GDP with GTP on the α subunit. Next, the G_{α} and $\beta\gamma$ subunit complex dissociate from each other and activate downstream signaling pathways. The intrinsic hydrolysis activity of G_{α} is strongly enhanced by the stimulation of RGS protein. G_{α} and the $\beta\gamma$ subunit go back to their inactive state as a trimer bound to the GPCR⁷⁸.

Since, the intrinsic GTPase activity from G_{α} is very low, there is an additional regulation process to conduct the G protein back into its inactive form. GTPase-activating proteins (GAPs) bind to the active G_{α} and enhance its inherent GTPase activity drastically, which leads to a rapid shut down of the G protein mediated signaling. The regulator of G protein signaling protein (RGS) is the antagonist to the GEF domain and provides an important part of G protein mediated signaling regulation^{79–81}.

1.3.2 Regulator of G protein signaling proteins

As mentioned before, RGS proteins act as GAPs terminating GPCR-mediated signaling and are able to accelerate the GTPase activity up to 1000-fold (Posner 1999). Common, for all members of the RGS protein family, is the RGS box with approximately 130 amino acids. This region is highly conserved and crucial for the GAP activity. The RGS domain alone is capable of binding G_{α} subunits and accelerating their intrinsic GTP hydrolysis activity^{82,83}. Each RGS protein has different motifs flanking the box sequence, which determines its specificity for protein-protein interactions⁸¹. Furthermore RGS proteins were described to almost exclusively interact with α subunits of the G_q and $G_{i/o}$ family⁸⁴. Many RGS proteins have additional domains (as can be seen in figure 1.5) that allow interactions with binding partners for a variety of roles including several non-GAP functions. The non-canonical functions of RGS proteins are a rising topic and will reveal interesting new insights into the biology of RGS proteins in^{85–87}.

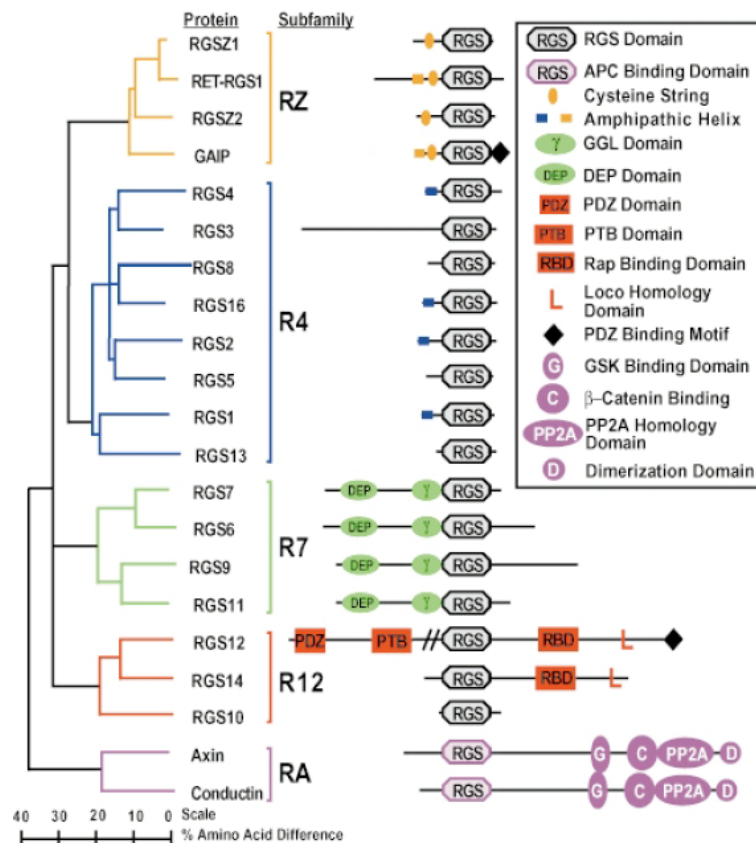


Figure 1.9: Classification of mammalian RGS proteins. The cladogram is constructed from amino acid sequences within the RGS domain and defines the four subfamilies RZ, R4, R7 and R12. The scale displays the approximate amino acid identities, which are calculated as 100% minus the horizontal distance to the next branch point. Mostly RGS proteins from one subfamily are very similar in the regions flanking the RGS box. APC, adenomatous polyposis coli; GGL, Gy-like; DEP, PDZ and PTB, protein interaction domains; PP2A, protein phosphatase 2A⁸⁸.

Today, 20 mammalian RGS proteins are known, they are divided into four subfamilies, RZ, R4, R7, and R12. The subfamily RA is added to the classification, due to sequence similarity, although no GAP or G_{α} binding activity has been shown yet. The classification is based on the sequence homology within their RGS box⁸⁸.

The first group, R4 mainly consists of small RGS proteins with relatively short N- and C-termini flanking the RGS box (with the exception of RGS3). The N-terminus presents a membrane targeting signal, which is crucial for localization near the membrane and activated G_{α} proteins⁸⁹. Although the R4 RGS proteins lack additional motifs, several members of this subfamily were shown to execute functions beyond their GAP activity⁹⁰. RGS2 has been suggested to be a part in the regulation of cell differentiation

by administering tubulin dynamics. Furthermore, there is evidence showing that RGS4 may play a role in the inhibition of G_q -mediated calcium signaling⁹¹. Also, RGS16 has been found to be involved in the regulation of PI3K signaling⁹².

The members of the R7 family of RGS proteins are the highly conserved RGS6, 7, 9 and 11. They are mainly expressed in the nervous system⁹³. All four have a G protein γ like (GGL) domain, which is able to recruit $G_{\beta 5}$ and is crucial for the expression and stability of all R7 RGS proteins⁹⁴. Furthermore, they have a Disheveled, Egl-10, Pleckstrin (DEP) domain, which is important for protein-protein interactions as well as anchoring the R7 RGS proteins to the membrane and is located near the N-terminus^{84,95}.

The R12 family is comprised of three RGS proteins, RGS10, RGS12 and RGS14. RGS12 and RGS14 both have additional functional motifs such as a Loco Homology (GoLoco) motif, a phosphotyrosine-binding (PTB) domain and a PDZ binding motif⁹⁶. The PTB domain is able to regulate calcium channel-based signaling⁹⁷ and the amino-terminal PDZ domain is reportedly associated with either the IL-8 receptor or its own C-terminal PDF binding motif⁹⁸. The GoLoco motif prevents the nucleotide exchange when bound to $G_{\alpha i}$ and therefore inhibits the G protein activation⁹⁹.

1.4. Regulator of G protein signaling 16 (RGS16) protein

1.4.1 Preliminary experiments identifying RGS16 as potential immunosuppressive target

Based on data for MAPK-activated protein kinase 2 (MK2) in macrophages a huge preliminary DNA microarray experiment was performed by Dohnal and his group. The human bone marrow-derived DCs were stimulated with LPS and analyzed for differences in expression patterns. Several genes known to be involved in immune

regulation such as IL-10 were upregulated after 24 hours, including Regulator of G protein signaling 16 (RGS16) (see figure 1.9)¹⁰⁰.

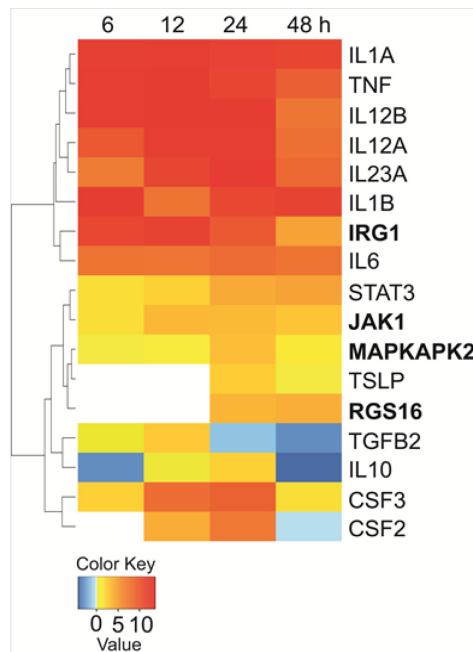


Figure 1.10: RGS16 was identified as an immunosuppressive candidate in DCs. A DNA microarray analysis from human monocyte-derived DCs revealed an upregulation of RGS16 after 24 hours of LPS stimulation, which coincides with the upregulation of several genes involved in immune regulation such as IL-10. The highlighted genes are currently under investigation in our research group¹⁰⁰.

To confirm the immunosuppressive function of the new targets of interest, functional assays were performed in which DCs were silenced by siRNA knockdown for a target of interest and then co-cultured with T lymphocytes. This experiment revealed that DCs silenced for RGS16 had a higher capacity to stimulate proliferation in CD4+ and CD8+ T cells. Indoleamine 2,3-dioxygenase (IDO) and Interferon-regulating factor 4 (IRF4) were used as control genes, since they are known repressors of T cell stimulation by DCs^{101,102}.

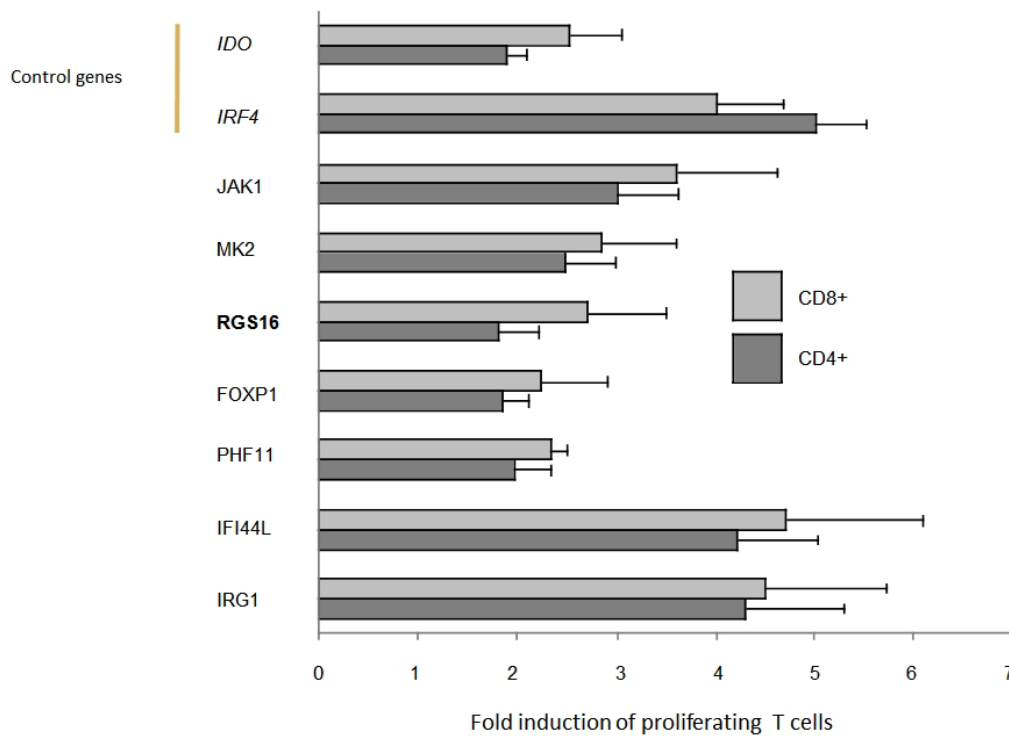


Figure 1.11: Co-culture of T cells and DCs. Previous to the co-culture the DCs were silenced for one gene individually by siRNA knockdown. The co-culture revealed higher proliferation capacity for CD4+ and CD8+ T cells for RGS16 and also other genes. In the figure the ratio of absolute numbers of proliferating T cells cultured with silenced DCs vs. control silenced DCs (treated with non-targeting siRNA) is shown. IDO and IRF4 are used as positive controls (unpublished data)

Therefore, RGS16 was chosen to be the investigated concerning its potential impact in the regulation of immune responses mediated by DCs. An additional part of this study, alongside from *in vitro* studies, was the breeding for a CD11c-cell specific RGS16 knockout, which has been initiated with mice from Jax laboratories. The breeding strategies and the genotyping results of the first wave of offspring are elucidated in detail in 1.5 ‘Generation of a RGS16-specific knockout mouse’.

1.4.2 Regulator of G protein signaling 16 protein

Regulator of G protein signaling 16 (RGS16) protein is a small member of the R4 subfamily of RGS-proteins. RGS16 is encoded by five exons, consists of 202 amino-acids

and does not feature large motifs outside of the RGS box aside from an amphipathic helix near the N-terminus (see RGS16 in figure 1.8). As it is for all the other RGS proteins, the main known function of RGS16 is to act as a GAP, terminating the signal transduction of G protein mediated signaling. RGS16 specifically binds to G_i and G_q proteins^{103,104}. Moreover, it is known that for full maturation into its stable form, all post-translational modifications are required^{105–107}.

Initially RGS16 was cloned from the retina^{108,109}, but today it is known that high expression of RGS16 is found in the brain, heart, liver and lungs¹¹⁰, since it has been shown that RGS16 has a significant impact on the circadian rhythm, liver metabolism and immune cell migration^{111,112}, which has been investigated in knockout or knockdown mice^{113–115}.

1.4.2 RGS16 in the immune system

GPCRs are described to be mediators of a huge variety of physiological processes and consequently are linked to many diseases. Approximately half of the pharmaceuticals targeted GPCR molecules in 2002¹¹⁶.

Except for the resting macrophages, RGS16 is expressed by most immune cell subsets¹¹⁷. It has been shown that RGS16 was expressed in human peripheral blood mononuclear cells (PBMCs) upon stimulation with TLR agonists. Furthermore, it was revealed that monocyte-derived DCs express RGS16 upon engagement of TLR3 and TLR4^{118,119}.

Several RGS proteins were shown to attenuate the extracellular signal-regulated kinase (ERK) group of mitogen-activated protein kinases^{120,121}. RGS16 was shown to attenuate platelet-activating factor-induced p38 MAPK activation together with RGS1, indicating distinct modulatory specificities of different RGS proteins in MAPK signaling¹²².

However, RGS16 not only modulates certain MAPK pathways, but is also involved in the regulation of the PI3K/Akt pathway⁹².

RGS16 seems to be playing a crucial role in the regulation of pro-inflammatory cytokine-secretion. Monocyte-derived DCs stimulated with a TLR2 agonist led to the production of IL1 β , IL-6, IL-8 and TNF α . Overexpression of RGS16 was shown to significantly downregulate the secretion of these cytokines. These results were confirmed by experiments with monocytes silenced for RGS16, since they revealed increased production of inflammatory cytokines¹²³. Similar results were demonstrated in the lung, where RGS16 appears to be an important factor in the regulation of Th₂/Th₁₇ inflammatory responses. It is indicated that RGS16 directly controls differentiated Th/effector T cell migration patterns, but is not involved in the regulation of trafficking quiescent, naïve T lymphocytes. RGS16 is upregulated in Th₁, Th₂ and Th₁₇ compared to naïve CD4 T cells and the expression of RGS16 inversely correlated with the extent of migration and chemotaxis (Shankar 2012). It also has been suggested that RGS16 may generally be used to inhibit chemotactic responses of not only CD4+, but also CD8+ T cells to e.g. IL-8 or CCL5¹²⁴.

Intriguingly both, Shankar *et al.* and Boudinot *et al.* found increased levels of IL-10 despite knockout/-down of RGS16^{114,123}. IL-10 is considered to be a regulatory cytokine controlling inflammation and therefore it is suggested that the role of RGS16 is thought to be part of a complex feedback loop, regulated by multiple control loops in order to attenuate inflammation¹²³.

Furthermore, RGS16 is involved in the CXCR4/CXCL12 chemokine axis and has been described in several cell types such as B cells, T cells and megakaryocytes. It inhibits the G protein-coupled chemokine mediated signaling via CXCR4 and CCR4 which leads to a restriction in T cell migration^{114,125}. In germinal centers (GCs), follicular DCs (FDCs) upregulate CXCR4 to stimulate germinal center T lymphocyte trafficking towards its ligand CXCL12. However, the FDCs also upregulate RGS16 and RGS13 to inhibit the T cell and B cell migration, since premature migration would lead to inefficient responses to thymus dependent antigens¹²⁶. RGS16 is also involved in a mechanism, where IL-17 promotes the formation of spontaneous GCs and is therefore driving autoimmune

responses¹²⁷. In megakaryocytes, RGS16 is a negative regulator of SDF1-CXCR4 mediated migration, mitogen-activated-protein-kinase (MAPK) and protein kinase B (AKT) activation and therefore regulates their maturation¹²⁸.

1.4.3 RGS16 & cancer

In general, uncontrolled growth of cells through decreased apoptosis and increased proliferation describes a cancer. Moreover, tumors are capable of invading nearby tissues and metastasize to distant tissues and organs. In the last years, several reviews portrayed the role of GPCR signaling in tumors and their involvement in proliferation, evasion of apoptotic signals and immune responses as well as invasion and progression through metastasis^{129–131}. Multiple RGS proteins have been discovered to be differently expressed in a variety of cancers. The most noteworthy changes include the significant downregulation of RGS2 in androgen-independent prostate cancer¹³² and upregulation of RGS5 in hepatocellular carcinoma and in the vasculature of renal cell carcinoma^{133,134}.

To this day, there are several different tumors that have known deviations concerning their RGS16 expression. RGS16 was described to be overexpressed in dub(1q) positive acute lymphoblastic leukemia and Burkitt lymphoma¹³⁵. Moreover, RGS16 has been investigated heavily in the context of colorectal cancer. Human gastrointestinal cell lines were silenced for RGS16 and in 17 of these 22 the silencing led to a proliferative suppression. Also, 124 paired cases of colorectal cancer were analyzed for RGS16 and patients from the high expression group revealed poorer overall survivor. Therefore, RGS16 was proposed as a potential marker for patient prognosis of colorectal cancer¹³⁶. Overexpression of RGS16 was also shown to play a role in pineal parenchymal tumors¹³⁷.

However, significant changes in expression of RGS16 in tumors do not always occur upwards. Lymph node metastases are one of the most prominent adverse prognostic

markers for pancreatic cancer. Interestingly, RGS16 was significantly lower expressed in pancreatic cancer patients with lymph node metastasis compared to patients without metastasis, alongside from FBJ murine osteosarcoma viral oncogene homolog B (FosB). Both genes were also associated with poorer survival rates and were therefore suggested as prognostic markers for pancreatic cancer with lymph node metastasis¹³⁸.

Furthermore, RGS16 is involved in the regulation process of Phosphatidylinositol 3-kinase. It was shown, that RGS16 mitigated PI3K signaling by sequestering the p85 α subunit from its signaling complexes. The resistance of breast cancer cells to chemotherapies such as tyrosine kinase inhibitors (TKIs) has been linked to persistent PI3K activity and analysis of 222 primary breast cancers revealed RGS16 mutations and reduced RGS16 protein levels in 50 percent of the cases. The results of further investigations led to the indication that the loss of RGS16 enhances PI3K signaling in some breast tumors and hereby promotes proliferation and evasion of TKI treatment⁹².

In conclusion, RGS16 seems to be involved in growth and expansion processes of many tumors and it appears to be part of complex feedback and regulation loops, since its expression varies with the type and location of the malignancy.

1.5 Generation of RGS16-specific knockout mice

An additional part of this study was the establishment of a RGS16-specific knockout mouse. The goal was to create mice with a RGS16-specific knockout in DCs to further elucidate the role of RGS16 in immune regulation and in the tumor microenvironment. For this purpose we acquired heterozygous B6.129X1(Cg)-Rgs16tm1Tmw/J mice from Jax Laboratories. The mice come from a C57BL/6 and SJL background, were backcrossed with C57BL/6 mice for 10 generations and carry a knockout gene of RGS16 on one allele. As can be seen in figure 1.12 the construct contains FRT and loxp sites

(tm1a). This construct is a knockout-first allele, RGS16 is out of frame and therefore homozygous mice would represent a systemic knockout. The breeding was supervised by Dr. Harald Höger from the Medical University of Vienna in Himberg. To determine the genotype, we analyzed the tail tips from the offspring in our lab.

Knockout-first allele: Promoter-driven selection cassette

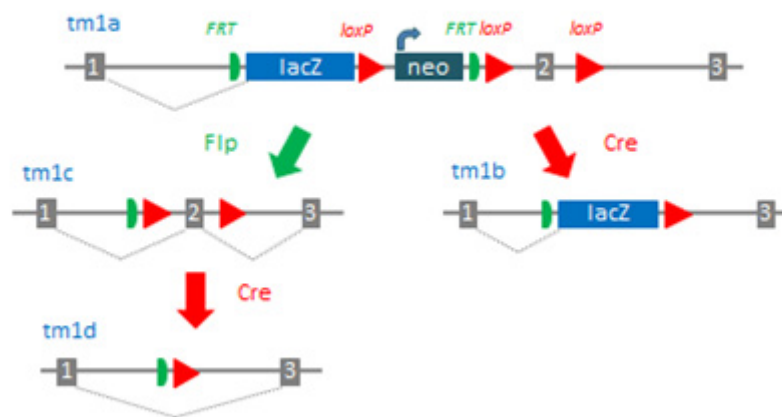


Figure 1.12: Allele construct of tm1a mouse and possible mating strategies with Flp or Cre mice. The mice acquired from Jax Laboratories (tm1a) carry a knockout first construct of RGS16 which contains frt and loxP sites for site-specific deletions through Cre-lox- and FRT/Flp-recombination.

We initiated three different breeding strategies. The first two strategies create systemic RGS16 knockout mice and the third one leads to a DC-specific RGS16 knockout.

To determine the genotype of the offspring from the breeding pairs we performed PCRs with DNA isolated from the tailtips of the offspring mice in our lab. The primer pairs were designed by Dr. Emilio Casanova from the Ludwig Boltzmann Institute for Cancer Research (see table 2.15).

3.2.1 RGS16 x RGS16

The first breeding strategy is aiming for a mouse with the initial construct on both alleles (see figure 1.13). Since, RGS16 is out of frame in the tm1a construct,

homozygous offspring give rise to a systemic RGS16 knockout mouse. Therefore, heterozygous RGS16 knockout mice from Jax Laboratories are mated with each other and DCs from homozygous offspring can be used for *in vitro* studies.

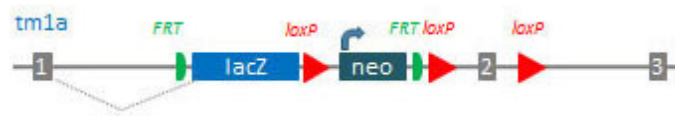


Figure 1.13: Allele construct of RGS16tm1 mouse.

3.2.2 RGS16 x Cre

The second breeding strategy generates a systemic knockout of RGS16 without the neomycin cassette from the initial construct. First the tm1a mice are paired with CMV-Cre mice to exert a site-specific deletion of the loxp-flanked sequence by the Cre recombinase. Therefore, the exon and the neomycin cassette are removed from the allele. Heterozygous mice with the tm1b construct are then mated with each other to obtain homozygous mice leading to a systemic RGS16 knockout. The DCs can be used for investigations *in vitro*.

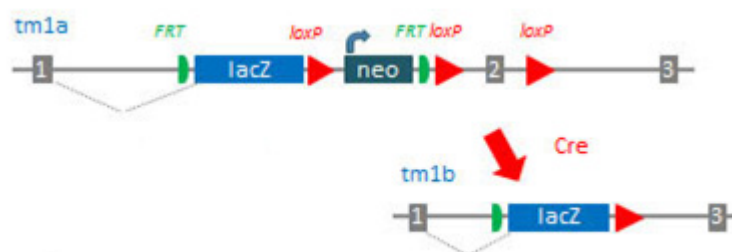


Figure 1.14: Mating strategy to obtain systemic RGS16 knockout mice after two breeding steps. Tm1a mice paired with CMV-Cre mice will lead to a cutout of the exon and the neomycin cassette. After this step, two heterozygous tm1b mice would be mated and the homozygous offspring could be used for several experiments.

3.2.3 RGS16 x GTRosa

The third breeding strategy aims to create a DC-specific RGS16 knockout. The tm1a mice are mated with Flp delete mice. The Flp mediated recombination leads to the deletion of the FRT-site flanked sequence. In the tm1c mice the RGS16 gene is back in frame. Heterozygous tm1c mice are paired with each other, and the homozygous offspring is mated with CD11c-Cre mice, to delete the loxp-site flanked sequence, including the exon of RGS16 in DCs. Since, the offspring will again be heterozygous, one additional breeding step is necessary to create a homozygous DC-specific RGS16 knockout mouse.

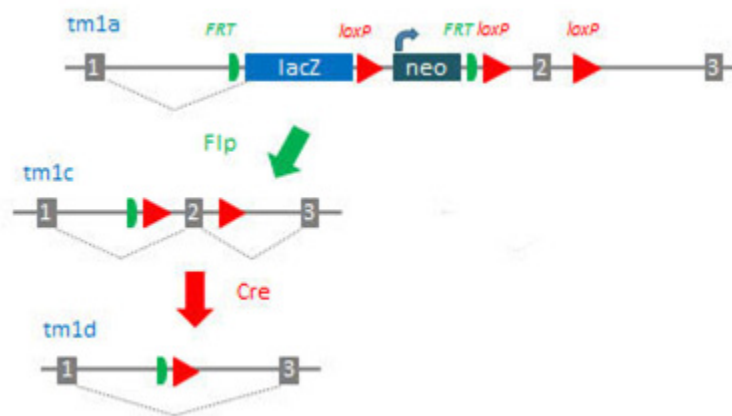


Figure 1.15: Mating strategy to obtain a DC-specific RGS16 knockout mouse. After FLP-mediated deletion the tm1c mice would be mated to breed homozygous mice. Then these mice will be paired with CD11c Cre mice to remove the loxp-flanked sequences in DCs. Therefore RGS16 will be knocked out in DCs.

1.6 Aims of the study

Based on preliminary data in human DCs we suggest an immunosuppressive role for RGS16 in DC-mediated immunity and regarding the general lack of data on RGS16 in DCs, the main aim of this master thesis was to investigate RGS16 concerning its expression on protein and RNA levels and its influence in DC-mediated immunity. Since, RGS16 was described to be heterogeneously regulated in different tumors, depending on the location and type of malignancy, another goal of this master thesis was to investigate its expression patterns in the established mouse tumor models of our group.

Additionally, the breeding of a CD11c positive cell-specific RGS16 knockout mouse for future experiments was initiated and played a supplementary role in this master thesis.

2. Material & Methods

2.1 Cell Culture

2.1.1 Generation of dendritic cells from murine bone marrow

Prepare complete Medium

To a 500 mL bottle of Iscove's Modified Dulbecco's Medium (IMDM - GIBCO by Life Technologies, Paisley, UK), 10% fetal calf serum (FCS - PAA Clone, PAA, Pasching, Austria), 10 mL L⁻¹ penicillin/streptomycin 100x (PAA, Pasching, Austria), 10 mL L⁻¹ MEM non-essential amino acids 100x (GIBCO by Life Technologies, Paisley, UK) and 0.0002% β-mercaptoethanol (Sigma-Aldrich, Vienna, Austria) were added. Further on, it will be referred to as 'medium'

Set up bone marrow cell plates

Wild type C57BL/6N Mice were used for all the experiments. They were sacrificed by cervical dislocation. Their femur and tibia were rinsed with medium to isolate their bone marrow cells. The cells were centrifuged with 460g at 4°C for 7 minutes, and then incubated in erylisis buffer for 2 minutes on ice to lyse the erythrocytes. After another centrifugation step and resuspension in medium the bone marrow cells were plated at a density of $0.3-0.5 \times 10^6 \text{ cm}^{-2} \text{ mL}^{-1}$ with 50 mL medium. To each plate, 1500 U / mL recombinant murine GM-CSF and 50 U / mL recombinant murine IL-4 were added. The cells were cultivated for 6 days on 37°C and 5 % CO₂.

Table 2.1: Erylisis buffer composition

Concentration	Substance	Purchased from
0.15 M	NH ₄ Cl	Roth, Karlsruhe, Germany
10 mM	KHCO ₃	Merck, Darmstadt, Germany
0.1 mM	Na ₂ EDTA	Roth, Karlsruhe, Germany

On the days 3 and 5 of cultivation, 30 mL of medium were removed and newly added including the cytokines GM-CSF and IL-4.

2.1.2 DC Maturation

Harvest immature dendritic cells

First 1mL of the supernatant was taken from the surface of a plate from each mouse and frozen at -20°C for further analysis. Then, 30 mL of medium were carefully removed from the surface. The remaining cell solution was harvested into falcons and then centrifugated with 460g at 4°C for 7 minutes. The pellet was resuspended in 10 mL medium. To count the number of immature dendritic cells, 10 µL of the cell suspension were put in TruCount™ tubes (BD Biosciences, San Jose, USA) with 90 µL, previously added, medium. The cells were dyed with 5 µL of a mix of antibodies for Fluorescence-activated cell sorting FACS analysis (referred to as 'DC Mix' in table 2.2). After 15minutes of incubation in the dark the samples were analyzed with flow cytometry and the cell numbers were determined via FlowJo V10. Also, all dotblots were done with FlowJo.

Table 2.2: Antibody-mixes for FACS Analysis

Mix	Antibody
DC Mix	α -CD80 PE-Cy5
	α -CD83 eFluor [®] 660
	α -CD86 PE
	α -MHC I FITC
	α -MHC II PerCP-eFluor [®] 710
	α -CD11b APC-eFluor [®] 780
	α -CD11c PE-Cy7
MLR Mix	α -CD3e APC
	α -CD8a APC-eFluor [®] 780
	α -CD25 PE Cy7
Tumor Mix	α -CD11c PE-Cy7
	α -MHC II PerCP-eFluor [®] 710
	α -CD45

Stimulation of dendritic cells

Immature dendritic cells were plated at a density of 1×10^6 cells cm^{-2} mL^{-1} . Table 2.3 shows the surface of each plate. For quality controls or experiment-specific stimulation different ligands were used to stimulate the dendritic cells (see Table 2.4). The plates were then cultivated at 37°C for a specific time, e.g. 4, 6 or 24hours.

Table 2.3: Different TLR-Ligands for stimulation of immature dendritic cells

Stimulant	Stock	$\mu\text{L/mL}$ used
LPS	10 $\mu\text{g/mL}$	10 ($\triangleq$ 100ng/mL)
R848	1mM	1 ($\triangleq$ 1 μM)
Poly I:C	2mg/mL	1 ($\triangleq$ 2 $\mu\text{g/mL}$)
IFNγ	10 $\mu\text{g/mL}$	2 (20ng/mL)

Harvest mature dendritic cells

First 1 mL of supernatant was taken from the plates and frozen at -20°C for further analysis. The stimulated dendritic cells are adherent to the ground of the plate, therefore they had to be scraped off and then transferred into a tube. They were centrifuged with 460g at 4°C for 7 minutes and resuspended with medium. To count the cells and check the maturation, cells were dyed with the FACS 'DC mix' in Trucount™ tubes and measured with Flow Cytometry. Again, FlowJo was used for analysis.

2.1.3 RNA interference

Silencing RNA (siRNA) was used to shut down mRNA expression of specific targets. RGS16 and IRG1 were silenced in dendritic cells with the following protocol.

First the immature dendritic cells had to be harvested, counted and adjusted to $1.3 \times 10^6 \text{ mL}^{-1}$. Then, the transfection reagents were prepared (see table 2.4).

Table 2.4: Solutions for dendritic cell silencing assay

Solution	Composition
A	585.6 μL + 14.4 μL DharmaFECT
B	588 μL OptiMEM + target siRNA
C	588 μL + NTC siRNA

Each reagent was incubated on room temperature for 5 minutes before reagent A was added to reagent B and C. Then, this solution was incubated on room temperature for 20 minutes. 4.8 million immature dendritic cells were seeded in transfection plates (6 cm diameter, 21.29 cm^2) and the transfection reagents were added carefully to the plates. The plates were incubated for 15 hours at 37°C. On the next day the DCs were stimulated with LPS (for RGS16 silencing) or IFN γ (for IRG1-silencing). After 4 hours of

stimulation the medium was changed and the plates were furthermore incubated for 20 more hours at 37°C.

2.1.4 Mixed Leucocyte Reaction

CD8+ T cell isolation

From the spleen of OT-I (C57BL/6-Tg(TcraTcrb 1100Mjb/Ctrl) transgenic mice a single cell suspension was prepared. First the spleen was smashed through a 70 µm cell strainer. After erylisis (as described in 2.1.1 'Set up bone marrow cell plates') CD8a+ T cells were isolated by negative selection using MACS® T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) in the MACS® separation system of LS columns with MACS® magnets according to the instructions of the manufacturer. Then, the cells were centrifuged at 460g for 7 minutes at 4°C and resuspended in supplemented IMDM medium.

CFSE labelling of T cells

Isolated CD8+ T cells were adjusted to 1×10^7 cells mL⁻¹ in Dulbecco's PBS (PAA, Pasching, Austria) containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA). Next, the cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Lofer, Austria). For this, they were kept at 37°C and 5% CO₂ for 10 minutes. Subsequently the volume was doubled by adding FCS and incubated for 5 minutes at room temperature. At the end the cells were washed by adding supplemented IMDM up to the volume of 10mL, a centrifugation step (460g, 7 minutes, 4°C) and resuspension in the same medium.

Co-culture of DCs and T cells

Before the co-culture was set up, the DCs were stimulated for 4 hours with LPS as described in 2.1.2. After harvesting different cell numbers of DCs were added to the

96-well round bottom plate (see Table 2.5). Thereafter, isolated and CFSE-labelled CD8+ T cells were added. The co-culture was incubated at 37°C for three days.

Table 2.5: Scheme of different co-culture setups.

Ratio	DC number	T cell number	Total volume
1:2	25 000	50 000	200 µL
1:5	10 000	50 000	200 µL
1:10	5 000	50 000	200 µL

Proliferation and CFSE signal intensity, as well as cell numbers were determined using the TruCount™ system.

2.1.5 Isolation of exosomes from DC supernatants

The Total Exosome Isolation Reagent (from cell culture media) from Life Tech (Carlsbad, USA) was used to isolate small vesicles from dendritic cell supernatants. First the supernatant samples had to be centrifuged for 30 minutes at 2000g to remove cells and debris. Then, 0.5 volume of isolation reagent was added followed an over-night incubation at 4°. On the next day, the samples were centrifuged at 10000g for one hour at 4°C. The pellet was resuspended in resuspension buffer from the Total Exosome RNA & Protein Isolation Kit (Life Tech, Carlsbad, USA). Isolated exosomes were either kept at 4°C for short-term storage or frozen at -20°C for long-term storage.

For Western Blot application 5 µL of the samples were mixed with 5 µL 2x SDS buffer and heated at 95°C for 10 minutes.

2.1.7 Reverse Transcription Real-Time Polymerase-Chain-Reaction (RTqPCR)

Sample preparation

The cells were centrifugated with 460g for 7 minutes at 4°C, the supernatant was discarded, the cell pellet was resuspended in 350 µL RLT Plus Buffer, unless a sample contained more than 5 million cells, then 600 µL of the buffer was used. For every mL of RLT Plus Buffer, 10 µL of β-mercapto-ethanol was added to the buffer.

Total DNA/RNA purification

The RNA and DNA from the sample were isolated using the QIAGEN AllPrep DNA/RNA Mini Kit. One after another, Ethanol, RW1 buffer and RPE buffer were added with centrifugation steps in between. At the end the RNA was eluted with 30-50 µL of RNase-free water. The isolation of the genomic DNA started with adding 500 µL of AW1 buffer, followed by AW2 buffer and EB buffer to elute the DNA. Again, there were centrifugation steps in between. Then, the DNA/RNA yield was measured on the NanoDrop®1000 (Thermo Fischer Scientific, Wilmington, U.S.A.). All samples were stored at -80°C.

Reverse Transcription – generating cDNA

First 10 µL of isolated RNA was mixed with 10 µL of the master mix for cDNA preparation.

Table 2.6: Components of the master mix for generating cDNA.

Component	Volume in µL for 1 sample
Nuclease free water	3.2
10x RT Buffer	2
10x RT Random Primers	2

25x dNTP Mix (100mM)	0.8
RNase Inhibitor	1
MultiScribe reverse Transcriptase	1

Then, the samples were put into a thermal cycler and the program from Table 2.7 was carried out.

Table 2.7: Program for reverse transcription.

Steps	Temperature (°C)	Duration (minutes)
Step 1	25	10
Step 2	37	120
Step 3	85	5
Step 4	4	∞

The samples were kept at 4°C for a short period of time. For longer storage they were frozen at -20°C

Real time PCR

The qPCR was performed for the target gene (RGS16) and the housekeeping gene (β 2M).

Table 2.8: Components from the 2x TM buffer

Component	Volume for one 96-well plate (μL)
Water	622.5
Magnesium chloride	350
10x TM buffer	250
dNTPs 100mM	15
Gold Tag Polymerase	12.5
Total	1250

For each gene a separate qPCR mix was prepared including the 2x TM buffer, the gene-specific probe (20x TM assay) and RNase-free water.

Table 2.93: Components from the gene-specific qPCR mix

Component	Volume for one sample (μL)
20x TM assay	1
2x TM buffer	10
RNase-free water	7
Total	18

Per sample 18 μL of qPCR mix and 2 μL of cDNA were mixed together. Depending on its concentration the cDNA was eventually diluted in ratios of 1:1 or 1:10 with RNase-free water. When the amount of isolated RNA was low, the prepared cDNA was used undiluted. After pipetting the plate was sealed, centrifuged and put into a 7500 Real-Time PCR System. The program that was used is shown in Table 2.10.

Table 2.104: Parameters for the program used for qPCR analysis

Step	Temperature (°C)	Duration (seconds)
Hold	50°C	120
Hold	95°C	600
45 Cycles	95°C	15
	60°C	60

2.2 In vivo experiments

2.2.1 B16F10 melanoma cell line

The mouse melanoma cell line B16F10 was used for the *in vivo* tumor experiments. The cell line was maintained in DMEM High Glucose (4.5 g L⁻¹) with stable glutamine (PAA, Pasching, Austria) supplemented with 10% FCS (PAA Clone, PAA, Pasching, Austria) and 10 mL L-1 penicillin/streptomycin 100x (PAA, Pasching, Austria) and cultured at 37°C.

2.2.2 Tumor inoculation

To inoculate the cells, they were washed and detached from the culture flask using accutase (PAA, Pasching, Austria). 5×10^5 B16F10 cells were resuspended in a volume of 100 μ L PBS (Dulbecco's, PAA, Pasching, Austria) and injected subcutaneously in the back, on the left and right side lateral to the lumbar spine of C57BL/6N mice. For inoculation, the mice were anesthetized and shaved. Furthermore, the eyes were covered with Oleovit eye salve (Fresenius Kabi, Graz, Austria). Every two days, the mice were checked for their tumor growth.

2.2.3 Tumor isolation

After approximately two weeks, the tumors either started to become necrotic on their surface or reached the previously defined maximum size (~1.5 cm diameter), and therefore we decided to sacrifice the mice and isolate the tumors to proceed in the experiment. The mice were sacrificed by cervical dislocation. The spleen and the tumors were isolated from the mice and cut into small pieces. Then, the tissue

material was separately digested in collagenase IV (Roche Diagnostics, Mannheim, Germany) for 1 to 2 hours at 37°C (5 mL / tumor, 3 mL / spleen). The suspensions were rinsed through a 70 µm cell strainer centrifuged with 460g for 7 minutes at 4°C and resuspended in 1 mL medium. Then 1µl of each antibody from the 'Tumor Mix' (see table 2.2) was added and the suspensions were incubated in the dark for 20 minutes. Then the CD45⁺MHCII⁺CD11c⁺ and CD45⁻ cells were isolated from all the samples via FACS Cell Sorting. The samples were then centrifuged with 460g for 7 minutes at 4°C, the supernatant was discarded and the cells resuspended in 350 µL of RLT Plus Buffer for RNA isolation and RTqPCR.

2.3 Protein Detection

2.3.1 Immunoblotting

Sample Preparation for Immunoblotting

450,000 cells were transferred into a new tube. They were centrifuged with 460g at 4°C for 7 minutes. The pellet was resuspended in 7.5 µL 1xPBS and 7.5 µL 2xSDS loading dye to have 30,000cells per µL. Then, the cells were incubated at 95°C for 10 minutes and directly stored at -20°C.

Table 2.11: 2x SDS loading buffer composition

Concentration	Substance	purchased from
0.25M	TRIS (pH 6.8)	AppliChem, Darmstadt, Germany
3%	SDS	Roth, Karlsruhe, Germany
6%	β-mercaptoethanol	Sigma-Aldrich, Vienna, Austria
20%	Glycerol	Sigma-Aldrich, Vienna, Austria
0.005%	Bromophenol blue	US Biochemical Corp, Cleveland, USA

SDS-Polyacrylamid Gel Electrophoresis (SDS-PAGE)

For protein separation 5 µL of sample were loaded onto a gel, either a self-made 12.5% gel or a Mini-PROTEAN® prest 4-15% gradient gel (Bio-Rad Laboratories, Hercules, USA). As marker 5 µL of a PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific, Rockford, USA) was loaded onto a separate slot. The gels were run at 20 mA for a few minutes, followed by approximately 40 minutes at 40-50 mA in 1x running buffer. 10x Running buffer was distilled 1:10 with distilled water (see Table 2.12).

Table 2.12: 10x Running buffer composition

Concentration	Substance	Purchased from
0.25 M	TRIS	AppliChem, Darmstadt, Germany
1.92 M	Glycine	ROTH, Karlsruhe, Germany
1%	SDS	ROTH, Karlsruhe, Germany

Western Blot

Proteins were blotted onto Whatman™ Protran nitrocellulose membranes (GE Healthcare, Dasse, Germany) at 300mA for 1.5h in 1x transfer buffer (see Table 2.13). The transfer of the proteins was confirmed by unspecific Ponceau staining (Sigma-Aldrich, Vienna, Austria). Then, the membrane was unstained with regular dH₂O.

The primary antibody was diluted in 0.5x Blocking Reagent in 0.1% TBS-T. The membrane was incubated for either one hour on room temperature or over-night on 4°C

Table 2.13: Composition of 1x transfer buffer and 1x TBS

Buffer	Concentration	Substance	Purchased from
1 x Transfer Buffer	25 mM	TRIS	AppliChem, Darmstadt, Germany
	187 mM	Glycine	ROTH, Karlsruhe, Germany
	20 %	Methanol	ROTH, Karlsruhe, Germany
1 x TBS	50 mM	TRIS	AppliChem, Darmstadt, Germany
	150 mM	NaCl	ROTH, Karlsruhe, Germany

Secondary Antibody

The secondary antibody is an anti-rabbit horseradish peroxidase conjugated antibody diluted 1:10000. The solution is made of 9.5mL 0.1% TBS-T, 500 μ L 10x blocking solution and 1 μ L of the anti-rabbit antibody.

Then, the membrane has to be washed 3 times with 0.1% TBS-T for 7-10 minutes and then once with 1x TBS for 10 minutes.

Developing Film

SuperSignal West Pico (normal sensitivity for GAPDH control) or SuperSignal West Femto (ultrasensitive, for the target antibodies) Substrate components are diluted 1:1 with 1.5mL each. Then, the solution is incubated on room temperature for 5 minutes. The exposure time of the film to the membrane may vary depending on the amount of protein in the samples and ranges from 30seconds to an hour for the dendritic cell supernatant western blots.

2.4 Genotyping

2.4.1 Determination of Mouse Genotype

Tail Tip Lysis

To each tail tip, 22.5 μL lysis buffer and 2.5 μL proteinase K (10mg/mL stock) were added and heated at 55°C for 3 hours. Afterwards the samples were boiled at 99°C for 10 minutes and 150 μL of water were added.

DNA Precipitation

0.1 volume of 2M NaCl and 3 volumes of ice-cold 96% EtOH (which is stored at -80°C) were added to the tail tips. The solution was kept at -20°C for two hours, centrifuged, washed with 70% EtOH and then air-dried over-night. On the next day the DNA was resuspended in distilled water and its concentration was measured with NanoDrop®.

Polymerase Chain Reaction (PCR)

For each PCR assay a master mix was prepared before adding the DNA.

Table 2.14: Components for PCR

Component	Volume (μL)	Final concentration
10x PCR Puffer	2	1 x
MgCl ₂ (25mM)	1.6	2 mM
Forward Primer (20 μM)	0.2	0.2 μM
Reverse Primer (20 μM)	0.2	0.2 μM

dNTPs (2.5mM)	1.6	0.2mM
Gold Tag Polymerase	0.2	1 Unit
ddH₂O	12.2	
Total Volume	18	

For each tail tip, 2 µL of DNA was added to 18 µL of master mix.

Table 2.15: List of primers for genotype characterization from offspring. WT, construct present in wild-type form; KO, construct with complete/partial deletion of sequences; Flox, construct with complete. FW, Forward Primer; RP, Reverse Primer; WT, wild-type; KO, knockout

PCR - determination wt / heterozygous / homozygous - tm1a mice		
FP	5' CCAGCACTCGGCTCCGGCCTTGACTC	WT = 298 bp
RP	5' CTTTGTTCCTCTACTTTCTTGCTGAG	KO = 356 bp
PCR for determination of FLP-mediated deletion in tm1c mice		
FP	5' GTTGTGCTATAAAGACCCCAAAGTG	WT = 127 bp
RP	5' GACACTTGACCATTCCTCAAGGCC	KO = 300 bp
PCR for control of exon deletion		
FP	5' GGAACGCGGGGAAACGGGGACAGCCGGG	Flox = 1.8 kbp
RP	5' CTTTGTTCCTCTACTTTCTTGCTGAG	KO = 373 bp
PCR for determination of loxp-mediated deletion in tm1b mice		
FP	5'GTGTCAAAAATAATAAACCAGGCAGG	KO = 400 bp
RP	5'GCCGGTCGCTACCATTACAGTTGGTC	
3 oligos PCR - Genotyping of final KO (after Flp, Cre-mediated deletion)		
FP	5'GTGTCAAAAATAATAAACCAGGCAGG	KO = 450 bp
FP #2	5' CCAGCACTCGGCTCCGGCCTTGACTC	Flox = 302 bp
RP	5' CTTTGTTCCTCTACTTTCTTGCTGAG	

Gel Electrophoresis

First, a 1% agarose gel was prepared. Then ethidiumbromide was added and the gel was cured on room temperature. 2 µL of loading dye was added to 10 µL sample, pipetted onto the gel and run at 120 Volts. The picture of the gel was taken with GelDoc IQ Program on a Gel Doc XR+ System (Bio-Rad Laboratories, Hercules, USA).

3. Results

3.1 Characterization of RGS16

3.1.1 RGS16 protein and mRNA expression in bone marrow derived DCs stimulated with TLR-ligands

The first part of this study was devoted to investigate RGS16 protein expression in bone marrow derived DCs in their immature state as well as upon binding of different toll-like receptor (TLR) agonists.

For protein expression profiling we chose the TLR ligands LPS, inducing signaling through TLR4, Resiquimod (also referred to as R848), a TLR7/8 ligand and poly I:C as a TLR3 ligand to activate bone-marrow derived DCs. We harvested the activated DCs at different time points between 4 and 48 hours after activation and analyzed them by immunoblot analysis. We also tested RGS16 expression by harvesting DCs from the bone marrow differentiation culture before adding the stimulant.

RGS16 was already detected in immature DCs. Up to 24 hours the amount of detected protein was consistent, with a clear decrease at the latest time point when DCs were stimulated with LPS or R848. Both delivered comparable results. Poly I:C stimulation led to an earlier decrease compared to the other TLR stimulants (figure 3.1).

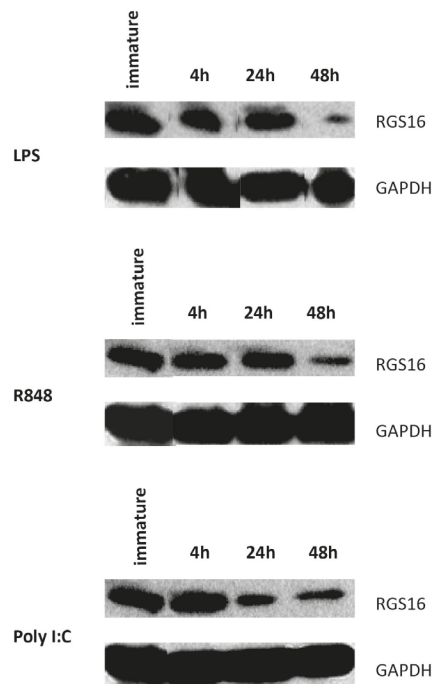


Figure 3.1: RGS16 protein in DCs activated with different TLR agonists. Bone marrow derived DCs were stimulated with LPS, R848 and poly I:C for 4, 24 and 48 hours. The cells were harvested at the given time points and 150 000 cells per lane were applied for immunoblot analysis. After incubation with the primary antibodies specific for RGS16 or GAPDH as a loading control, the proteins were visualized with a HRP-conjugated secondary antibody.

RGS16 protein was detected at the size of 26 kDa and the GAPDH control at 36 kDa. Both proteins appeared at the expected sizes according to calculated molecular weight.

DC quality control measurement with flow cytometry illustrated the maturation of the DCs, shown by the upregulation of the surface markers CD80, CD83, CD86, and MHC class I and II after 4 and 24 hours. The quality control also revealed a large amount of dead cells at the latest time point (see figure 3.2).

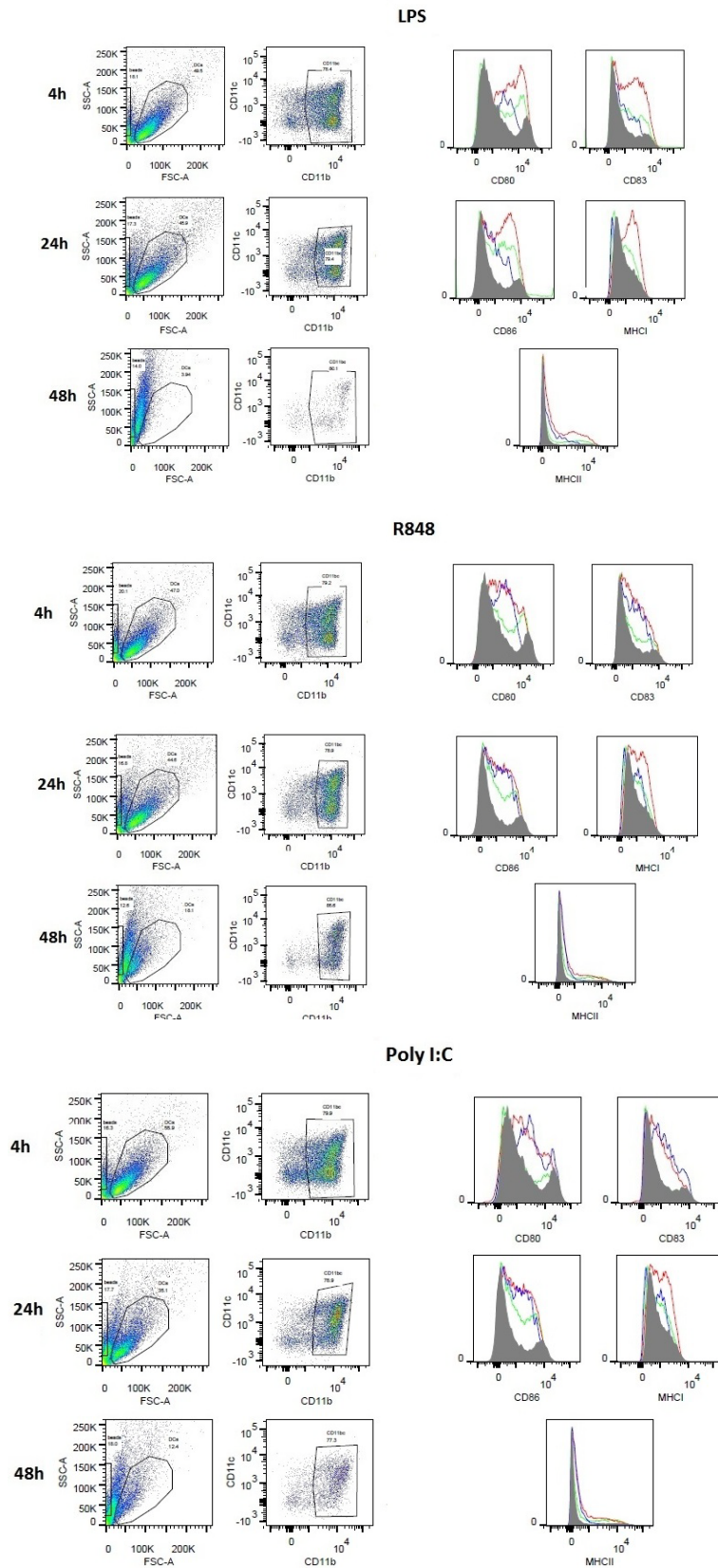


Figure 3.2: Analysis of DC phenotype and surface marker expression of bone marrow-derived DCs with flow cytometry. The bone marrow-derived DCs used for immunoblotting in figure 3.1 were analyzed by flow cytometry as a quality control. The DCs were stained with antibodies specific for CD80, CD83, CD86, MHC class I and II. First the DCs were selected according to phenotype, and then CD11b/CD11c double

positive cells were selected to count the bone marrow-derived DCs. The histograms depict the expression of single surface markers over the time of stimulation. The grey area represents the DC culture before adding the TLR-stimulant. The green line represents the time point of 4 hours after activation, the red line illustrates the surface marker expression after 24 hours of stimulation, and the blue line depicts the latest time point, 48 hours after adding the TLR ligand. FSC-A, Forward Size Scatter, characterizes the size of the cells; SSC-A, Side Size Scatter, depicts the granularity of the cells.

The next part of this study served to elucidate the expression kinetics of RGS16 on mRNA level. The DCs were stimulated with LPS and R848 for 4 and 24 hours. At the time points, DCs were harvested in order to perform quantitative PCR (qPCR).

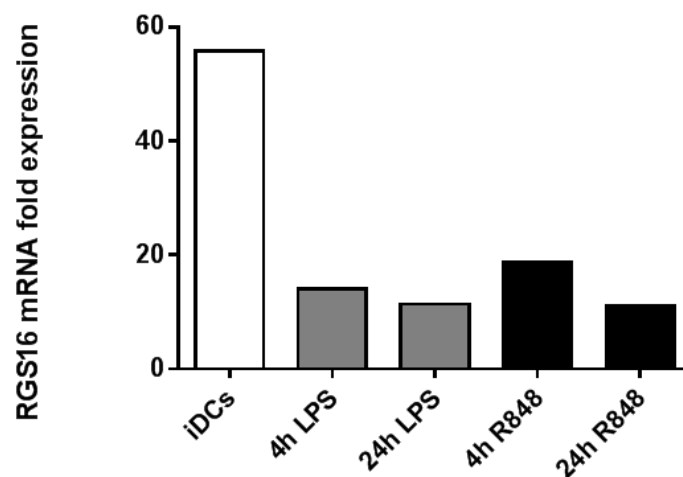


Figure 3.3: RGS16 mRNA expression in bone marrow-derived DCs after TLR mediated stimulation. The bone marrow-derived DCs were stimulated with LPS and R848 for 4 and 24 hours. The cells were harvested at the time points in order to perform qPCR. The samples were applied in duplicates and RGS16 mRNA expression was normalized to β 2M.

In the immature DCs the RGS16 mRNA expression is almost 3-fold higher than in the TLR stimulated DCs. After 4 hours of stimulation for both, LPS or R848, the levels of RGS16 are highly decreased and this effect is even stronger in the 24 hour cultures (see figure 3.3).

In summary both, the protein and mRNA expression kinetics revealed high levels of RGS16 in immature bone marrow derived DCs. While the amount of protein takes

longer to decrease, the mRNA levels quickly decline with the addition of the TLR ligands within 4 hours.

3.1.2 RGS16 Protein in the supernatant of DC cultures after LPS stimulation

The focus of these experiments was to identify potential RGS16 protein in the supernatant of DC cultures, which have been stimulated via TLR4. The DCs were activated with LPS and cultured for 4, 24 and 48 hours.

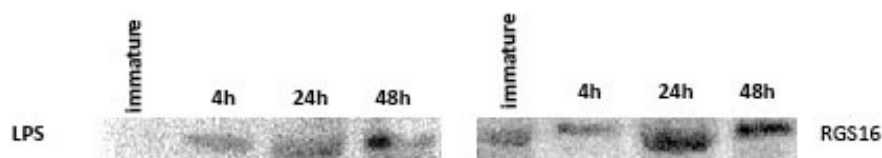


Figure 3.4: RGS16 protein released into the supernatant of bone marrow-derived DC-cultures. Bone marrow-derived DCs were activated with LPS for 4-48 hours. The supernatants were harvested at the given time points and analyzed by immunoblotting. The figure shows samples from two different mice. After incubation with the primary antibodies specific for RGS16 or GAPDH as a loading control, the proteins were visualized with a HRP-conjugated secondary antibody.

As can be seen in figure 3.4 the immunoblot analysis revealed that RGS16 was present in the supernatant of stimulated DC cultures, while immature DCs showed little to no amount of protein. The second mouse already showed a slight presence of protein from cultures with immature DCs. However, the amount of total protein increased during the time of LPS-stimulation.

Interestingly the detected extracellular protein was much larger than the intracellular protein. The bands from the supernatant samples were detected at approximately 55 kDa.

This experiment was repeated with different time points to investigate if the increase of protein in the supernatant occurred due to higher numbers of dying cells or if the protein amount increases independently of this effect.

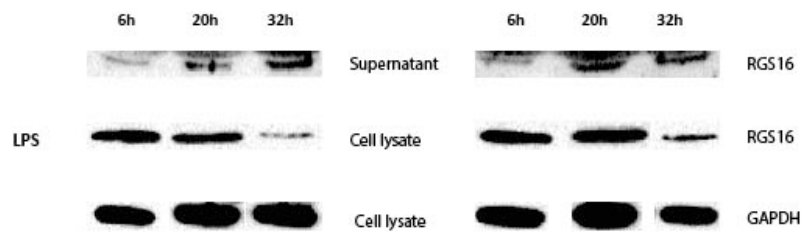


Figure 2.5: RGS16 protein released into the supernatant compared to intracellular expression in bone marrow-derived DCs. Bone marrow-derived DCs were activated with LPS for 6-32 hours. The cells and supernatants were harvested at the given time points and analyzed by immunoblotting. The figure shows samples from two different mice. After incubation with the primary antibodies specific for RGS16 or GAPDH as a loading control, the proteins were visualized with a HRP-conjugated secondary antibody.

As previously observed, the protein levels were elevated with the time of stimulation. After 6 hours minor levels were detectable, which strongly increased with time (see figure 3.5). Again, RGS16 protein derived from DC lysates was detected at 26 kDa. In the supernatant we observed a band at approximately 55 kDa.

The quality control of the DCs revealed less dying of DCs at the latest time point of 32 hours after activation compared to the flow cytometry results of the DC cultures which have been stimulated for 48 hours (see 3.6 compared to 3.2).

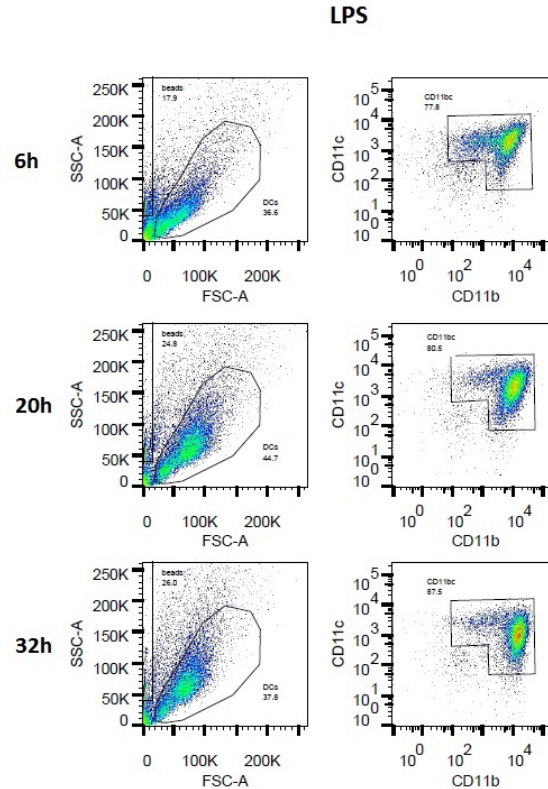


Figure 3.6: Analysis of bone marrow-derived DC phenotype and surface marker expression with flow cytometry. The bone marrow-derived DCs used for immunoblotting in figure 3.5 were analyzed by flow cytometry as a quality control. The bone marrow-derived DCs were selected according to phenotype, and then CD11b/CD11c double positive cells were selected to count the bone marrow-derived DCs. FSC-A, Forward Size Scatter, characterizes the size of the cells; SSC-A, Side Size Scatter, depicts the granularity of the cells.

In summary, the intracellular RGS16 protein decreased with time and was detected at the calculated amino acid-sequence-based size of 26 kDa. In contrast we detected significantly elevated levels of a 55 kDa protein in the supernatant of LPS stimulated DCs, suggesting putative RGS16 protein modified on post translational level.

3.1.3 RGS16 in DC supernatant-derived exosome isolations

DCs are well known to secrete exosomes to mediate a variety of functions e.g. to regulate other immune cells. Since, RGS16 was shown to be secreted into the supernatant, we investigated a potential transport by exosomes. We used an isolation

kit specific for cell-culture media and analyzed these exosome isolations by immunoblotting.

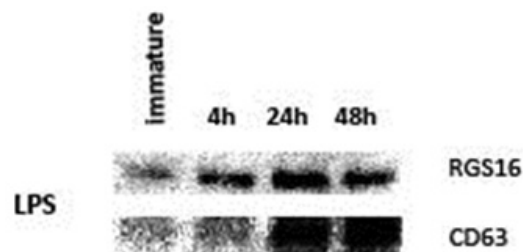


Figure 3.7: RGS16 protein in exosome isolations from bone marrow-derived DC supernatant taken after LPS stimulation. Bone marrow-derived DCs were activated with LPS for 4-48 hours. The supernatants were harvested at the given time points and were used for exosome isolation. After incubation with the primary antibodies specific for RGS16 or CD63 as a loading control, the proteins were visualized with a HRP-conjugated secondary antibody.

We were able to detect RGS16 in exosome samples, which increased with the time of stimulation (see figure 3.7). Similar to the kinetic of the supernatant study putative RGS16 protein at a detected size of around 55 kDa increased with ongoing LPS stimulation. CD63 was detectable in the exosome isolations at the height of 50 kDa. CD63, also called TSPAN30, is a member of the tetraspanin protein family and, alongside from CD9 and CD81, generally used as exosome marker^{29,139}. The kinetic showed a similar result to the one of RGS16. While, the exosome isolations of the immature DCs revealed low levels of protein, the expression is highly increased upon LPS stimulation.

3.1.4 RGS16 mRNA expression

In this experiment, we wanted to compare the RNA levels of RGS16 between bone-marrow derived DCs and splenic DCs. Both have been stimulated with LPS.

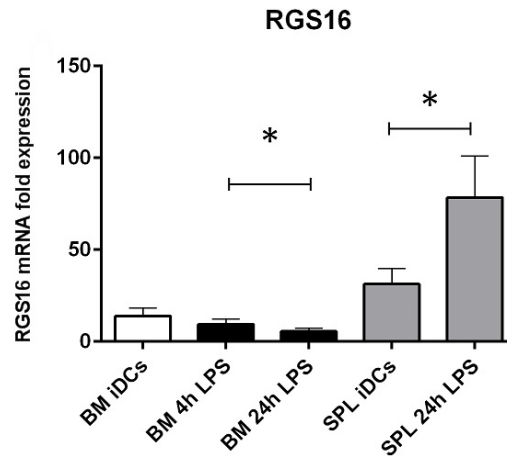


Figure 3.8: RGS16 mRNA expression in bone-marrow derived and splenic DCs. The bone-marrow derived DCs from three different mice were stimulated with LPS for 4 and 24 hours, the splenic DCs were stimulated for 24 hours. The cells were harvested at the given time points in order to perform a qPCR. The samples were applied in duplicates and RGS16 mRNA expression was normalized to $\beta 2M$. Mean $\pm$ standard deviation is shown. *) $p < 0.05$.

In the bone marrow-derived DCs we could again observe the decrease in RGS16 mRNA expression. The difference between the two latter time points of the bone marrow-derived DC cultures revealed statistical significance ($p = 0.0402$). Furthermore, the increase in RGS16 mRNA expression after 24 hours of LPS stimulation was also significant ($p = 0.0355$).

The main observation was that the RGS16 mRNA expression profile from the splenic DCs differs from the one of the bone-marrow derived DCs. In contrast to the bone-marrow derived DCs, which showed a decreasing RGS16 expression with LPS stimulation, the mRNA levels in the splenic DCs were generally higher and the 24 hours culture had a significantly stronger expression than the immature DCs (see figure 3.8).

3.1.5 RGS16 inhibits CD8⁺ T cell proliferation *in vitro*

In this study we wanted to investigate the impact of RGS16 in DCs concerning their ability to activate CD8⁺ effector T cells. Therefore we silenced DCs for RGS16, loaded them with the MHC class I OVA (257-264) peptide (SIINFEKL) and stimulated them with LPS for 4 hours. For the co-culture, CD8⁺ T cells were isolated from OT-I mice spleens, which carry a transgenic T cell receptor (TCR) that recognizes specifically the SIINFEKL peptide. Since we wanted to measure the proliferation of the CD8⁺ T cells, they were labeled with Carboxyfluorescein succinimidyl ester (CFSE). Then, the DCs silenced for RGS16 and the control DCs were both co-cultured with the same set of labeled CD8⁺ T cells for three days.

Three different DC:T cell ratios were chosen for each condition and each condition was performed in triplicates. Additionally a condition where only T cells were present was applied as a negative control. After three days, the cells were harvested and stained for CD3, CD8 and CD25 (see 'MLR mix' table 2.2) for flow cytometry analysis. To measure the numbers of proliferated CD8⁺ T cells, the activated cells were analyzed regarding their declining CFSE fluorescent signal intensity.

There was a big discrepancy between the three conditions concerning the proliferation. The higher the number of DCs used for the culture the lower was the proliferation. It has to be noted that while the number of DCs added was different, the overall volume of the culture was the same in all three conditions.

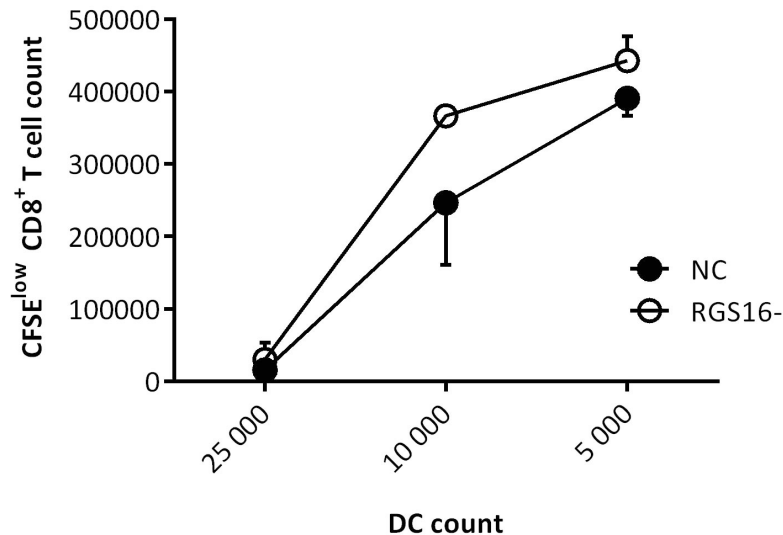


Figure 3.9: Proliferation of CD8⁺ T cells upon co-cultivation with bone marrow-derived DCs silenced for RGS16. Bone marrow-derived DCs of three mice were silenced, loaded with the SIINFEKL peptide and stimulated with LPS. Then, the bone marrow-derived DCs were put in co-culture with CD8⁺ T cells, isolated from OT-I mice. The T cells were harvested after 3 days of co-culture and their proliferation was measured as absolute numbers of cells with low CFSE fluorescence signal intensity. Different numbers of DCs were used with a constant number of T cells (50 000). RGS16-, co-culture with DCs silenced for RGS16; NC, co-culture with DCs treated with a non-targeting control silencing RNA.

DCs silenced for RGS16 showed an enhanced capacity of inducing proliferation of the CD8⁺ T cells. The biggest increase in proliferation between silenced DCs and DCs treated with non-targeting siRNA was observed in the cultures with 10 000 DCs, but the absolute numbers of T cells peaked in the co-culture with the fewest DCs. With 25 000 DCs present in the well, the T cells displayed almost no proliferation (see figure 3.9). The wells where only T cells were present did not show any proliferation (data not shown).

All in all we observed a positive effect concerning T cell proliferation when RGS16 was silenced, suggesting an immunosuppressive role for RGS16 in DC mediated immunity. To ensure, that the observed effects were due to the lack of RGS16 in the co-culture, we used supernatants of the harvested DCs for immunoblotting to control the DC silencing.



Figure 3.10: RGS16 protein released into the supernatant of bone marrow-derived DC-cultures silenced for RGS16. Bone marrow-derived DCs from three different mice were silenced for RGS16 and activated with LPS for 4 hours. The supernatants were harvested at the given time points and analyzed by immunoblotting. After incubation with the primary antibodies specific for RGS16, the proteins were visualized with a HRP-conjugated secondary antibody. +, DCs silenced with non-targeting control siRNA; -, DCs silenced with siRNA targeting RGS16

DCs silenced for RGS16 revealed less protein expression than the negative control, although the silencing did not shut down RGS16 completely (see figure 3.10). The quality control of the DCs did not reveal any notable differences between the DCs silenced for RGS16 and the DCs silenced with the non-targeting control concerning cell death.

3.1.6 B16F10 melanoma expresses RGS16 *in vivo*

In the literature RGS16 was described to be differentially expressed in several tumors and tumor cell lines. In most cases it was shown to be upregulated, while only a few cancers revealed down-regulation of RGS16. Therefore, we wanted to investigate the expression of RGS16 in mice upon injection of the B16F10 melanoma cell line, which is regularly used by our group and widely established as a tumor model.

For this purpose, we injected 5×10^5 B16F10 cells subcutaneously in the back on both sides of the spine of C57BL/6 wt mice. The mice were sacrificed in a timeframe of 13 to 17 days after injection. The tumors and spleens were isolated, digested and stained with antibodies specific for CD45, MHC class II and CD11c flow cytometry cell sorting.

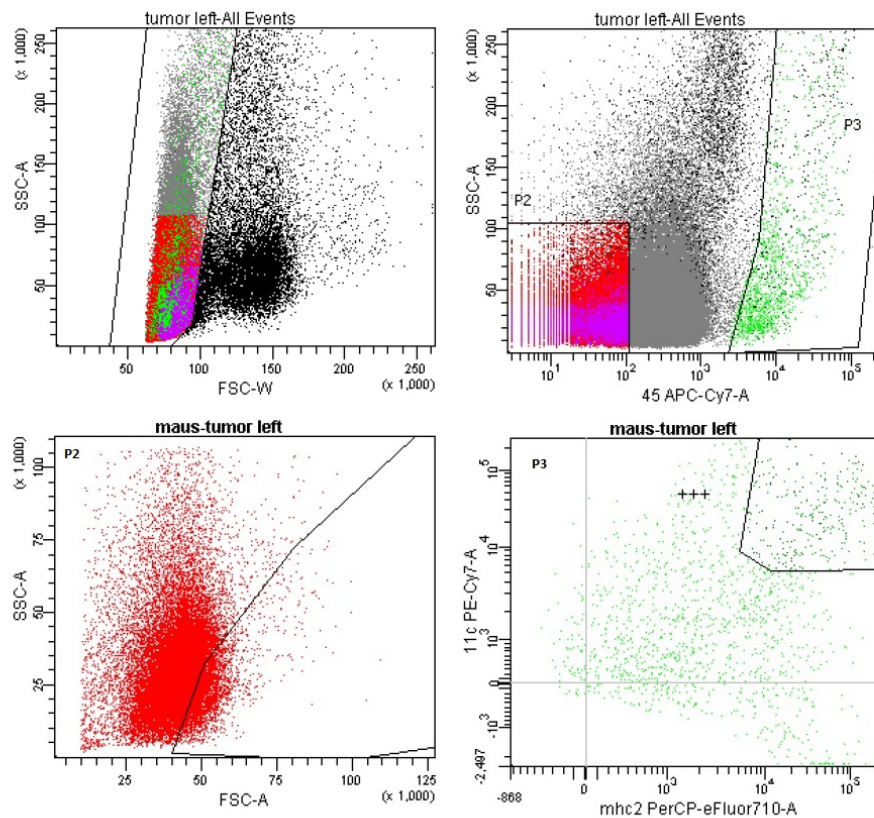


Figure 3.11: Sorting cells of the tumor samples with flow cytometry. B16F10 tumors were isolated, digested and stained with antibodies specific for CD45, MHC class II and CD11c for flow cytometry cell sorting (see 2.9). The tumor-resident DCs were sorted by gating on the cells positive for CD45 and then selecting the MHCII/CD11c double positive cells. The tumor cells were also sorted, by selecting the CD45 negative cells from the tumors. One representative sorting procedure of a tumor, which was isolated on day 15 after tumor inoculation, is shown. FSC-W, Forward Size Scatter, characterizes the size of the cells; SSC-A, Side Size Scatter, depicts the granularity of the cells; 45-APC-Cy7-A, represents the expression of CD45 on the surface of the cells; 11cPE-Cy7-A, portrays the expression of CD11c on the cell surface; mhc2PerCP-eFluor710-A, illustrates the expression of MHC class II molecules on the surface of the cells.

We separated tumor-resident DCs from tumor cells according to CD45, MHCII and CD11c expression. CD45 negative cells were additionally sorted in order to select for pure tumor cells without leukocyte infiltrates (see figure 3.11). Accordingly, splenic DCs were isolated by selecting for CD45, MHCII and CD11c positive cells. However, there was a significantly higher amount of DCs isolated from the spleen (see figure 3.12).

After flow cytometry sorting the cells were harvested in order to perform qPCR. The expression of RGS16 was normalized to the housekeeping gene $\beta 2M$.

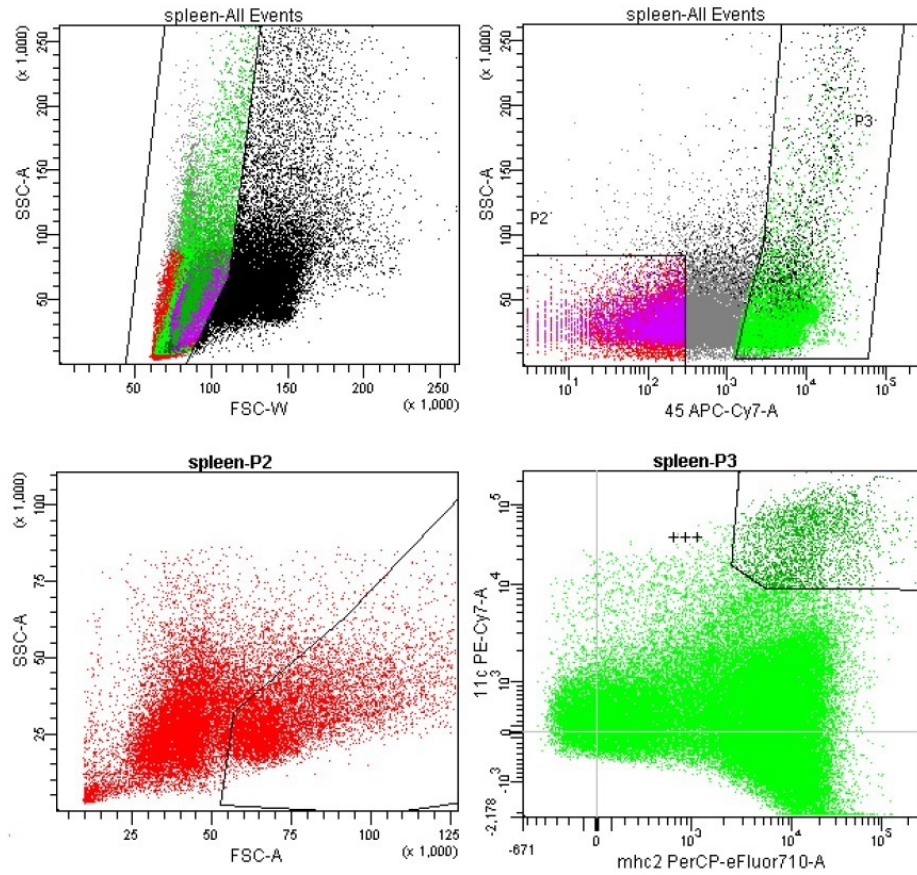


Figure 3.12: Sorting cells from isolated spleens with flow cytometry. The spleens were isolated, digested and stained with antibodies specific for CD45, MHC class II and CD11c for flow cytometry cell sorting (see 2.9). The splenic DCs were sorted by gating on the cells positive for CD45 and then selecting the MHCII/CD11c double positive cells. One representative sorting procedure of a spleen, which was isolated on day 15 after tumor inoculation, is shown. FSC-W, Forward Size Scatter, characterizes the size of the cells; SSC-A, Side Size Scatter, depicts the granularity of the cells; 45-APC-Cy7-A, represents the expression of CD45 on the surface of the cells; 11cPE-Cy7-A, portrays the expression of CD11c on the cell surface; mhc2PerCP-eFluor710-A, illustrates the expression of MHC class II molecules on the surface of the cells.

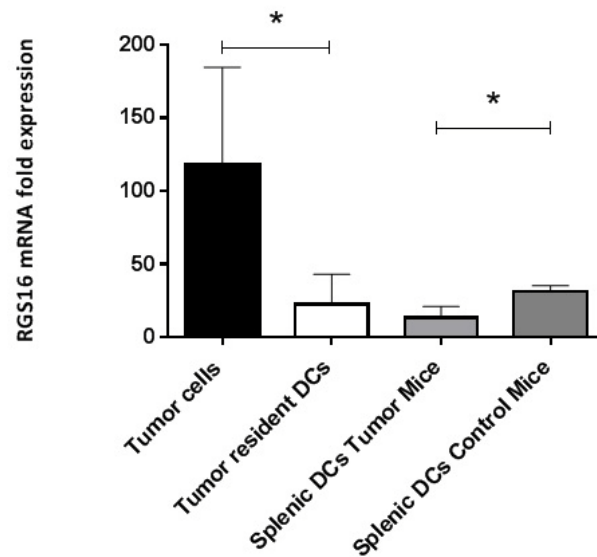


Figure 3.13: RGS16 mRNA expression in B16F10 melanoma, tumor-resident DCs and splenic DCs. After flow cytometry sorting the cells were harvested in order to perform qPCR. The samples were applied in duplicates and RGS16 mRNA expression was normalized to β 2M. Tumor cells, CD45 negative cells isolated from the tumors; Tumor-resident DCs, cells positive for CD45, MHC class II and CD11c isolated from the tumor; Splenic DCs Tumor Mice, cells positive for CD45, MHC class II and CD11c isolated from the spleen of mice bearing B16F10 tumors; Splenic DCs Control mice, cells positive for CD45, MHC class II and CD11c isolated from the spleen of mice without tumors. Mean $\pm$ standard deviation is shown. *) $p < 0.05$.

In the tumor cells RGS16 expression was significantly higher compared to the tumor-resident and splenic DCs. The tumor-resident DCs showed a slightly higher expression than the respective splenic DCs (see figure 3.13). However the splenic DCs from the control mice showed higher levels of RGS16. The difference in RGS16 mRNA expression between the tumor cells compared to tumor-resident DCs was highly significant ($p = 0.024$), as well as the difference between the splenic DCs from mice bearing tumors and the control mice ($p = 0.016$).

3.2 Generation of RGS16-specific knockout mice

3.2.1 RGS16 x RGS16

The mice from Jax laboratories carried the RGS16 construct on one allele (see figure 1.12). Since, homozygous offspring of these mice result in a systemic knockout of RGS16 the heterozygous tm1a mice were mated with each other. In order to determine the genotype of the offspring the isolated DNA from the tailtip was analyzed by 2nd loxp-PCR (see table 2.15). The upper band illustrates the allele with the inserted construct, the lower band the wild-type allele.

In total, 58 offspring mice were analyzed. Approximately two third of the mice were heterozygous, the rest were wild-type mice. We obtained no homozygous offspring.

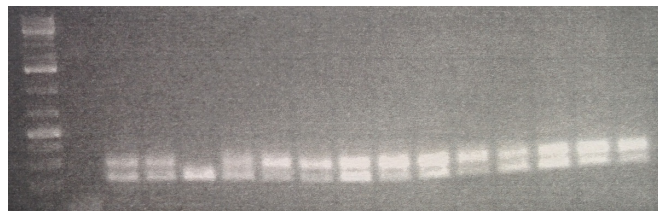


Figure 3.13: Agarose-Gel with samples from RGS16 x RGS16 offspring. The 2nd loxp-PCR was performed with all offspring mice from the RGS16 x RGS16 matings in order to determine the genotype of the offspring. One representative of the PCR analysis on an agarose-gel is shown. The upper band shows the allele with the inserted construct, the lower band the wild-type allele.

3.2.2 RGS16 x Cre

In order to obtain a homozygous genotype without the exon and the neomycin cassette, heterozygous mice with the RGS16 knockout construct were crossed with Cre-CMV mice in order to cut out the loxp-flanked sequence. The DNA from the tailtips

was analyzed via PCR and the offspring with a heterozygous genotype was selected for further mating to obtain a homozygous genotype in the next generation. Offspring revealing a band on the agarose-gel were heterozygous, while the lack of a band displayed a wild-type mouse.

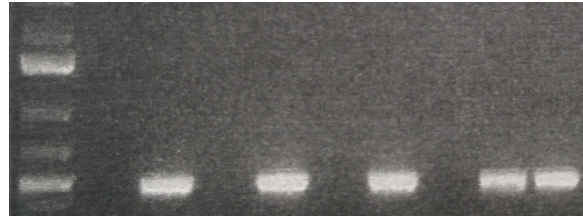


Figure 3.14: Agarose-Gel with samples from RGS16 x Cre offspring. The PCR for determination of loxp-mediated deletion in tm1b mice (see table 2.15) was performed with all offspring mice from the RGS16 x Cre-CMV matings in order to determine which offspring mice were heterozygous (see figure 1.14). One representative of the PCR analysis is shown. The lanes with one band illustrate the offspring mice with a heterozygous genotype, which were selected for the next breeding step, the lanes without bands are wild-types.

3.2.3 RGS16 x GTRosa

The heterozygous RGS16 knockout mice were crossed with heterozygous GTRosa Flp delete mice to obtain the tm1c mice, where the lacZ gene and the neomycin cassette were removed from the allele (see figure 1.15). In this case, the RGS16 gene was back in frame. After mating two heterozygous mice with each other, the homozygous offspring were mated with CD11c Cre mice to create a RGS16-specific knockout in DCs.

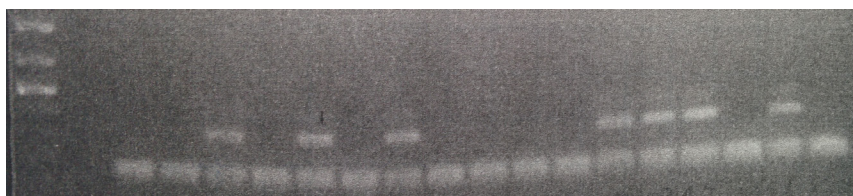


Figure 3.15: Agarose-Gel with samples from RGS16 x GTRosa offspring. The PCR for determination of FLP-mediated deletion in tm1c mice (see table 2.15) was performed with all offspring mice from the RGS16 x GTRosa matings in order to determine the heterozygous offspring (see figure 1.15). One representative of the PCR analysis is shown. The lanes with two bands represent heterozygous offspring, the lanes with one band illustrate wild-type mice.

4. Discussion

The focus of our research group is to investigate potential immunosuppressors in DCs, to gain a better understanding of the regulatory processes of DC-mediated immune responses in order to enhance anti-tumoral immunity. In preliminary experiments several immunosuppressive candidate genes were discovered, of which a few are currently under investigation. RGS16 was chosen to be the target of interest for this study. The general aim of this master thesis was to characterize RGS16 in DCs concerning the RNA and protein expression, its impact on immune regulation and investigate the expression of RGS16 in the tumor cell line *in vivo* in mice.

4.1 RGS16 protein expression

Regarding the lack of literature, concerning data about RGS16 in DCs, we started by stimulating murine bone-marrow DCs with different TLR-ligands for 4, 24 and 48 hours and looked at the protein kinetics by immunoblotting. RGS16 was present after activation independently of the stimulant, but the amount of protein started to decrease after prolonged activation with all three TLR-ligands. Exposure to LPS, and R848 (also called resiquimod), led to a very constant protein expression, with a clear drop off after 48 hours, while bone marrow-derived DCs stimulated with poly I:C, showed already a mild decrease of RGS16 protein after 24 hours (see figure 3.1). A possibility for the decrease, that should be considered, is that cells could simply be dying due to exhaustion caused by the prolonged exposure to the activation stimuli. As a loading control, the housekeeping gene GAPDH was chosen and it was expressed very equally in all samples. However, this does not give any insights into the state of the cells. Therefore, we analyzed the bone marrow-derived DCs by flow cytometry in order to check their phenotype and also their maturation by looking at the abundance of surface molecules. The bone marrow-derived DCs were activated, since several surface molecules including the co-stimulatory molecules CD80 and CD86 as well as

MHC class I and II molecules were upregulated upon stimulation. However, the analysis also revealed a large amount of dead cells especially at the latest time point of 48 hours. Additionally, the aforementioned surface molecules were down-regulated after 48 hours, indicating towards the assumption of dying cells (see figure 3.2). Therefore, the steady GAPDH levels of the immunoblotting could be misleading, since a certain amount of GAPDH protein could originate from dying cells.

In conclusion we demonstrated that the RGS16 expression was upheld for a certain amount of time by stimulation via TLR3 (poly I:C), TLR4 (LPS), TLR7 and TLR8 (R848). It should also be noted that RGS16 protein was already expressed in the murine immature bone marrow-derived DCs, which differs to findings of Shi et al., which demonstrated that RGS16 protein is induced upon engagement of TLR3 and TLR4 through¹¹⁸.

To further investigate the decrease of RGS16 we decided to continue solemnly with LPS stimulation, since we observed very similar effect for all ligands. We changed the duration of stimulation to 6, 20 and 32 hours to obtain a better picture of the progression the cells go through. The immunoblotting revealed similar results as can be seen in figure 3.5. The GAPDH control was evenly expressed in all samples as well as intracellular RGS16 in the early time points. After 32 hours of stimulation an obvious decrease in amount of protein could be observed, while the GAPDH control did not differ compared to previous time points. Interestingly, the quality control of the DCs revealed much lesser dying of the cells after 32 hours than after 48 hours of stimulation (see figure 3.6). Therefore, it could be possible that the expression of RGS16 protein is indeed down regulated and might not be influenced as much by cell death as initially assumed. Also, the widespread cell death among the cultures appears to take place in the time-window between 32 and 48 hours after activation.

However, an unexpected finding came up in these experiments. The antibody for RGS16, which was used for the cell lysate immunoblotting, was employed on bone marrow-derived DC supernatant samples from the previous experiments and detected increasing amounts of RGS16 protein with prolonged time of stimulation. Interestingly,

the kinetic of the extracellular RGS16 protein was inverted to the kinetic of the intracellular polypeptide (see figure 3.4 and 3.5). Additionally, the protein discovered in the supernatant of the DCs was much larger, approximately 2-fold in size. It can be excluded, that the discrepancy in size is caused by the formation of macromolecular complexes, e.g. dimers, since the samples are denaturized by incubation with SDS previous to the application onto the gel. The increase in size could eventually be explained by posttranslational modifications, and this matter will be addressed by analyzing isolated protein from DC cultures as well as from DC culture supernatants by mass spectrometry. However, no other protein of the RGS family has been described to be found outside of cells or specifically for DCs.

Be that as it may, it can be argued, that the high abundance of RGS16 protein, especially at the latest time point (48 hours) could stem from the dying cells, which release their content to the supernatant. However, there were only little to no amount of intracellular RGS16 detected on the immunoblotting membranes from the supernatant samples, independently of the time point.

We went on to further investigate these findings by performing exosome isolations from the supernatant samples and analyzing them via immunoblotting. The exosome isolations revealed very similar kinetics compared to the results from the supernatant experiments (see figure 3.7). The highest amount of protein is found in the sample with the longest period of stimulation. The presence of exosomes in the isolations was confirmed by detection of CD63, often referred to as TSPAN30, a surface protein of the tetraspanin family, which are generally used as exosome markers²⁷. Although CD63 was positive, we could not detect CD81 within these samples. These results probably point towards the heterogeneous nature of exosomes³⁰. Further investigations will be necessary to reveal detailed characteristics of the exosomes, e.g. analyzing the surface molecules from immature DCs and over time after activation. The expression of the exosomal surface molecules could be explored by binding them to beads for flow cytometry analysis, but also functional assays, e.g. co-cultures with T cells could give further insights in their function. Since RGS16 is predominantly regulated upon

exosome release, it would be interesting to additionally investigate the exosome regulation process in DCs.

Furthermore, it has to be noted that it cannot be concluded for a certainty that the detected RGS16 protein is originating from the exosomes. The isolation kit used for this experiment excludes apoptotic bodies and other cell particles from the samples, but they may contain other microvesicles and free protein. Therefore it is equally possible, that the extracellular RGS16 protein might be isolated alongside and not in the exosomes or transported in other microvesicles. According to the supplier the amount of free protein is insignificant and should not be detectable via immunoblotting. However, further investigations are necessary to ensure the exosomal transport of RGS16 protein. Moreover, the current situation of nomenclature in the field of microvesicles and exosomes is anything but unified and a somehow less strict application to the terminology has developed in recent years, classifying the small vesicles by their method of isolation, size or morphology as well as by their physical and biochemical features³³. Therefore, literature as well as assays 'specific' for exosomes or other microvesicles and the consequent results have to be handled carefully.

However, if RGS16 is shown to be transported via exosomes, it would demonstrate an interesting, additional way of DCs to regulate immune responses. Moreover, other candidates could potentially be secreted by DCs.

4.2 RGS16 mRNA expression in dendritic cells upon stimulation with TLR-ligands

After revealing the kinetics on protein levels, bone marrow-derived DCs were stimulated with LPS to investigate the changes in mRNA expression of RGS16 after stimulation with LPS. In a preliminary experiment bone marrow-derived DCs from one mouse were stimulated with LPS or R848. Interestingly, the expression of RGS16 was

approximately 3-fold higher in the immature DCs, than in the activated cells (see figure 3.5). Furthermore the experiment was conducted with more mice in a different setting. DCs from splenic origin were added to the investigation and all the DCs were activated with LPS. These experiments confirmed the high RGS16 mRNA expression in immature DCs while showing a decrease with prolonged stimulation in the bone marrow-derived DCs. However, the splenic immature DCs exhibited higher levels of RGS16 and LPS-stimulation led to a further increase (see figure 3.6).

Interestingly, the data from the bone marrow-derived DCs does not match with the mRNA expression kinetics from the early experiments in human monocyte-derived DCs. RGS16 was shown to be upregulated with time upon LPS stimulation in human¹⁴⁰. This observation has to be further investigated, since it could be possible that RGS16 is regulated on different levels in humans and mice, although it might have the same function. Also, the stimulation of murine splenic DCs with LPS revealed an upregulation of RGS16 after 24 hours of activation. Therefore, the regulation of RGS16 might be different depending on the host and the origin of the DC.

4.3 Silencing of RGS16 enhances CD8+ T cell proliferation

Previous data from human bone marrow-derived DCs suggested an immunosuppressive function for RGS16 in DCs (see 1.10). Furthermore, Boudinot et al. (2015) reported that RGS16 seems to play a crucial role in the regulation of pro-inflammatory cytokine-secretion in human monocytes¹²³. Therefore, this part of the study depicts an important point for the general goal of our research group, since we aim to enhance the pro-inflammatory capacities of DCs in order to elicit more efficient immune responses against tumors. The numeric increase of activated CTLs can be viewed as an indicator for an effective immune response against tumors, since their main function consists of killing tumor cells.

We performed a co-culture experiment in order to determine the impact of RGS16 in DCs on the proliferation of CD8+ T cells *in vitro*. For this purpose we silenced DCs for RGS16 before setting up the co-culture. In summary, the main conclusion of this part of the study was that the silencing of RGS16 in DCs led to a higher proliferation of CD8+ T cells. The effect could be observed in the co-culture setup of DC: T cell ratios of 1:5 and 1:10 (see figure 3.7).

The co-culture experiment was set up in different ratios to investigate the induction of CD8+ T cell proliferation in the presence of different densities of DCs (see table 2.5). For all ratios the number of T cells was constant, while the number of DCs varied. Moreover, the volume of the culture was equal for all wells, therefore the density of DCs decreased drastically from the 1:2 to the 1:10 DC:T cell ratio. In the 1:2 DC:T cell ratio, there was almost no proliferation, though it represented the setup with the highest density of DCs. Previous experiments have revealed that a high density of dendritic cells can have a negative effect on T cell proliferation. The other ratios displayed significantly more proliferation, which points towards a better environment in culture. Previous experiments have also indicated, that the cell culture conditions, most importantly the number of added DCs, impact the stimulatory effect from DCs to T cells *in vitro*^{140,141}. It is important to note, that the 1:10 DC:T cell ratio is an almost optimal setting for T cell priming. Obviously, this would not be an ideal setup for the investigation of an immunosuppressive molecule *in vitro*, but it is simultaneously important to demonstrate the overall capacity of the DCs to induce T cell proliferation.

The biggest difference, between the co-culture setup with silenced and non-silenced bone marrow-derived DCs, was observed in the 1:5 DC:T cell ratio. Bone marrow-derived DCs, which were silenced for RGS16 revealed a higher capacity to stimulate T cell proliferation. Although the overall induction of proliferation was higher in the 1:10 DC:T cell ratio, the difference between silenced DCs and control DCs was smaller than in the 1:5 DC:T cell ratio, which is probably explained by the aforementioned effect, since repressive molecules might not have as much impact as they presumably have due to the ideal conditions for T cell priming. Furthermore, these results need to be

confirmed with knockout DCs, since the siRNA was not able to completely shut down the RGS16 expression.

In regard to the previously discussed exosome data, the indication of an immunosuppressive role for RGS16 is highly intriguing. Therefore it is of interest to investigate the impact of RGS16 in exosomes, since it already has been shown that they are able to regulate immune responses^{39,43,45,46,48,51,52}.

4.4 RGS16 is highly expressed in B16F10 melanoma *in vivo*

RGS16 has been described in several different tumor cell lines and cancer tissues. It has been shown to be overexpressed in a variety of tumors, often correlating with inferior chances of survival^{135–137}, while also being linked to poorer prognosis when downregulated, e.g. in pancreatic cancer¹³⁸. Therefore we wanted to investigate the expression pattern of RGS16 in the B16F10 melanoma cell line, which was already established as a tumor model in our group and has been used for investigations concerning other immunosuppressive candidate genes.

Our experiments revealed a significantly higher expression of RGS16 in the tumor cells compared to the tumor-derived DCs. Furthermore, the RGS16 levels were even lower in the splenic DCs from the mice bearing tumors, while the splenic DCs from the untreated control mice revealed a higher expression than the other DC samples. The high expression of RGS16 in the tumor cells arose as an interesting finding, since there is not much literature that links RGS16 directly to melanoma cell lines or skin cancer. RGS16 has been linked to the regulation of PI3K, which has been connected to melanoma⁹². Also, RGS16 has been suggested to inhibit Akt, by inhibiting its phosphorylation. Overexpression of Akt was indicated to lead to malignant melanomas^{142,143}. Interestingly, the expression of RGS16 in the DCs from the mice bearing tumors, regardless whether from the tumor microenvironment or from the spleen was lower than in the control mice suggesting a systemic effect on the RGS16

expression. Essentially, it would equally stand to reason that a tumor present in the organism would lead to an upregulation of immunosuppressive molecules in DCs, including RGS16, in order to inhibit immune responses. Therefore the low levels of RGS16 in DCs would have to be examined.

With this in mind, it is worth noting that tumors are well known to release exosomes to influence their surrounding (see figure 1.7)^{56,57,144}. Potentially, tumors could be releasing exosomes with RGS16 or other immunosuppressive molecules in order to aid their progression in the organism.

Further investigations will address the function behind RGS16 in these types of malignancy and in the tumor microenvironment, which should help to understand the processes in which it might be involved, either as GAP in GPCR signaling or in a non-canonical function.

4.5 Generation of a CD11c-specific RGS16 knockout mouse

An additional part of the study was the generation of a RGS16 knockout mouse. Two strategies aimed for systemic RGS16 knockouts, while a third one targeted a RGS16-specific knockout in CD11c positive cells. The breeding was executed by Prof. Höger at the breeding station in Himberg, Austria. The three breeding strategies are elucidated in chapter 1.5.

Interestingly the first breeding strategy (see 3.2.1), to obtain a homozygous systemic RGS16 knockout mouse including the original construct with the lacZ gene and neomycin cassette, revealed only heterozygous and wild-type offspring, although more than 50 mice were genotyped. Also, the second breeding strategy to create a homozygous RGS16 knockout mouse, including only the lacZ gene, did not divulge any homozygous offspring (see 3.2.2).

The lack of homozygous mice, despite the high number of offspring gave us the notion, that there might be selection pressure *in utero*. Initially, the neomycin cassette was assumed to cause problems, a sentiment supported by Prof. Emilio Casanova, who designed the primers for the entire RGS16 genotyping. Therefore, the second breeding strategy was initiated in parallel, in case of such difficulties. However, since the second breeding strategy produced no homozygous mice too, the possibility of lethality of a systemic RGS16 knockout was suggested.

Nonetheless, the group of Dr. Kirk Druey has shown that a systemic knockout of RGS16 in C57BL/6 mice is not a lethal condition. Obviously, the generation of their RGS16 knockout mouse differed to our approach, since Shankar et al. made the knockout mouse themselves, but they have been able to work with their systemic RGS16 knockout mouse¹¹⁴.

The third breeding strategy could not be finished during this study, but will be continued until a CD11c specific knockout of RGS16 is achieved.

5. Conclusion & Outlook

After being identified as an interesting target with immunosuppressive potential in the preliminary experiments by our group, RGS16 was selected to be the topic of this master thesis. During this study, we revealed not only that RGS16 might potentially be secreted from DCs, but is eventually transported by exosomes. Moreover, our observations suggest an immunosuppressive effect in DC-mediated immunity. Together, these results could indicate an additional way of DCs regulating immune responses. Furthermore, we discovered that RGS16 was highly expressed in B16F10 melanoma cells compared to DCs in the tumor microenvironment or in the spleen. Therefore RGS16 might potentially be used by tumors to escape immune surveillance.

For the future, the expression levels of additional tumor cell lines e.g. glioblastoma and osteosarcoma will be investigated. Furthermore, knocking out RGS16 in these tumor cell lines would allow studying the impact of RGS16 on the tumor microenvironment *in vivo*. Also the release and the function of exosomes containing RGS16 will be addressed in detail, as well as their potential immunosuppressive capacity, e.g. in functional *in vitro* assays.

6. Literature

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Abstract

Dendritic cells (DCs) are important mediators of immune responses. They are not only pivotal for the activation of the adaptive immunity, but also play a crucial role in regulating these immune responses in order to prevent tissue damage or auto-immunity.

Based on data from human monocyte-derived DCs we detected several targets with potential immunosuppressive capacity. One of the targets we are currently investigating is the regulator of G-protein signaling protein 16 (RGS16), which was chosen as the topic for this master thesis. Aside from its function as a transduction inhibitor in G-Protein coupled receptor signaling, not much is known about RGS16 in DCs. RGS16 protein is not only present in DCs, we also found it in the supernatant of LPS-stimulated bone-marrow-derived DCs from mice. We have indications that it is transported via exosomes where it might exert immune-regulatory functions on other immune cells, such as T-cells. Furthermore, DCs silenced for RGS16 expression were able to induce higher proliferation in CD8⁺ T-cell populations compared to control DCs. As we are interested in immune-regulatory mechanisms in the tumor microenvironment we inoculated B16F10 melanoma into wild-type mice. In the splenic DCs from mice bearing tumors, the RGS16 expression was even lower than in the tumor-resident DCs. However in the tumor cells, RGS16 expression was almost 10-fold higher. The role of RGS16 in immune regulation has yet to be determined, including its function outside of DCs, as well as its potential transport via exosomes. Its role in the tumor microenvironment is yet not described and seems to differ depending on the malignancy. Therefore, more different tumors will have to be analyzed.

In conclusion, RGS16 seems to play an immunosuppressive role in DC mediated immunity. Also, it is highly expressed in a melanoma cell line *in vivo*, which goes along with most of the known literature about RGS16 in tumors, although its role in the tumor microenvironment has yet to be investigated. Furthermore, the transport of RGS16 in exosomes could represent an intriguing mechanism with which DCs regulate immune responses or tumors aid themselves to evade anti-tumor immunity.

Zusammenfassung

Dendritische Zellen (DCs) sind die wichtigsten Mediatoren zwischen angeborenem und adaptivem Immunsystem. Sie können spezifische Immunantworten induzieren, sowie diese Prozesse regulieren um exzessive Entzündungsreaktionen mit folgenden Gewebeschäden oder Autoimmunität zu verhindern. Erste Experimente mit aus menschlichen Monozyten generierten DCs zeigten mehrere potentielle Targets mit immunsuppressiver Kapazität. Einer dieser anti-inflammatorischen Faktoren wurde für diese Diplomarbeit ausgewählt, das ‚Regulator of G protein signaling 16‘ (RGS16) Protein. Abgesehen von seiner Funktion der Inhibition der Signaltransduktion von G-protein-gekoppelten Rezeptoren ist nicht viel über RGS16 in DCs bekannt. Wir konnten RGS16 nicht nur in, aus dem Knochenmark von Mäusen, generierten DCs nachweisen, sondern ebenso im Überstand dieser Zellkulturen. Ferner, detektierten wir RGS16 in Exosom-Isolationen, welche aus den DC Überständen hergestellt wurden. Dies könnte einen zusätzlichen Mechanismus darstellen, mit dem DCs andere Immunzellen wie z.B. T-Zellen regulieren. Weiters konnte gezeigt werden, dass RGS16 silencing in DCs zu einer höheren Proliferation in CD8⁺ T-Zellen führte. Nachdem uns die immunregulatorischen Mechanismen im Tumormikromilieu interessieren, inokulierten wir B16F10 Melanomzellen in wild-typ Mäuse und analysierten die mRNA expression von RGS16 via Real-Time PCR. DCs, isoliert aus Milzen tumor-tragender Mäuse, zeigten eine niedrigere Expression als DCs welche aus dem Tumorgewebe selbst entnommen wurden. Auffallend war jedoch die etwa zehnmal so hohe Expression in den Tumorzellen.

Zusammenfassend scheint RGS16 eine immunsuppressive Funktion in der DC-vermittelten Immunität einzunehmen. Die hohe Expression in der Melanomzelllinie geht mit zahlreichen Ergebnissen der Literatur einher, wobei die genaue Rolle im Tumormikromilieu in Zukunft untersucht werden sollte. Ferner könnte der Transport von RGS16 in Exosomen einen interessanten neuen Mechanismus zur Regulierung von Immunantworten darstellen, von welcher Tumore gleichfalls Gebrauch machen könnten um sich vor dem Immunsystem zu schützen.

Curriculum Vitae

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