Regulatory T Cell-mediated Suppression of Th9 Cell Development and Effector Function

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Table of Contents

1 Introduction

The immune system protects the organism against invading pathogens like bacteria, viruses, fungi and parasites but also against threads coming from degenerated cells like cancer. At the same time, the immune system fulfills the important function to maintain self-tolerance, thereby preventing autoimmunity.

Pathogens penetrating the first physical barrier, the skin or the mucosal surfaces are attacked by innate immune cells. Varieties of innate immune cells patrol in nearly all tissue environments and form the second barrier of defense. These cells are able to identify evolutionary conserved structures specific for pathogens. This recognition *via* germline encoded pattern recognition receptors (PAMP), like toll-like receptors (TLR), leads to the activation of the innate immune cells, enabling these cells to combat the intruders immediately at the side of entry.

Professional antigen presenting cells (APC), like dendritic cells, phagocytize the pathogens and initiate an adaptive immune response. After activation, dendritic cells can migrate to the lymph nodes, where they activate the adaptive immune system by presentation of pathogenic antigens. Pathogen-specific activation of the adaptive immune system, allows a precise and customized response towards nearly all types of pathogens.[1,2] The adaptive immune system can be divided into two major subclasses, the humoral und cell-mediated immune response.[3]

The cellular immune reaction is important for clearance of intracellular pathogens and tumor control. The cellular immune response is mainly mediated by cytolytic CD8-positive ($CD8^+$ cluster of differentiation) T cells. These cells are able to lyse target cells by different mechanisms.[4,5] The humoral immunity is mediated by B cells and CD4 positive ($CD4^+$) T cells. With the help of $CD4^+$ T helper cells (Th), B cells are able to produce highly antigen-specific antibodies crucial for the clearance of extracellular pathogens. The cell mediated as well as the humoral immune responses are able to establish an immunological memory through the generation of long-living B and T cells. This memory facilitates a more efficient and quicker response to second encounter of the same pathogen.

Although the immune system is crucial for the survival of the organism, its deregulation or malfunction can cause harm. Regulatory T cells (Tregs) are responsible for self-tolerance leading to prevention of autoimmunity, thereby protecting the tissue against undesired reactions of the immune system.

T cells play a crucial role and are vital for the complex function of the adaptive and innate immune system. $CD4^+$ and $CD8^+$ T cell compartments can be further itemized in different subclasses. The subsets of $CD4^+$ T helper cells are further described below, showing a variety of functions in immune reactions, as well as in pathological diseases.

1.1 T helper cell subsets

First identification of two different T helper cell subsets from known $CD4^+$ T cell clones was in the 1980s. The discovery of Mossman and Coffmann led to the Th1/Th2 paradigm, dividing the $CD4^+$ T helper cells in two different compartments with distinct functions.[6] Th1 cells are crucial for cellular immunity, while Th2 cells are important for the humoral response.[7] T helper cell subsets are defined by a lineage-specific master transcription factor and secreted signature cytokines. To stabilize the key features of each lineage and ensure lineage commitment a positive feedback loop is known for many T helper cell subsets. These findings led to the idea that T helper cell commitment is lineage restricted and terminal. In contrast to that, newest discoveries showed lineage plasticity among different T helper cell subsets in particular situations. The flexibility is at the moment under investigation, making a revision of the stable T helper cell lineage commitment necessary.

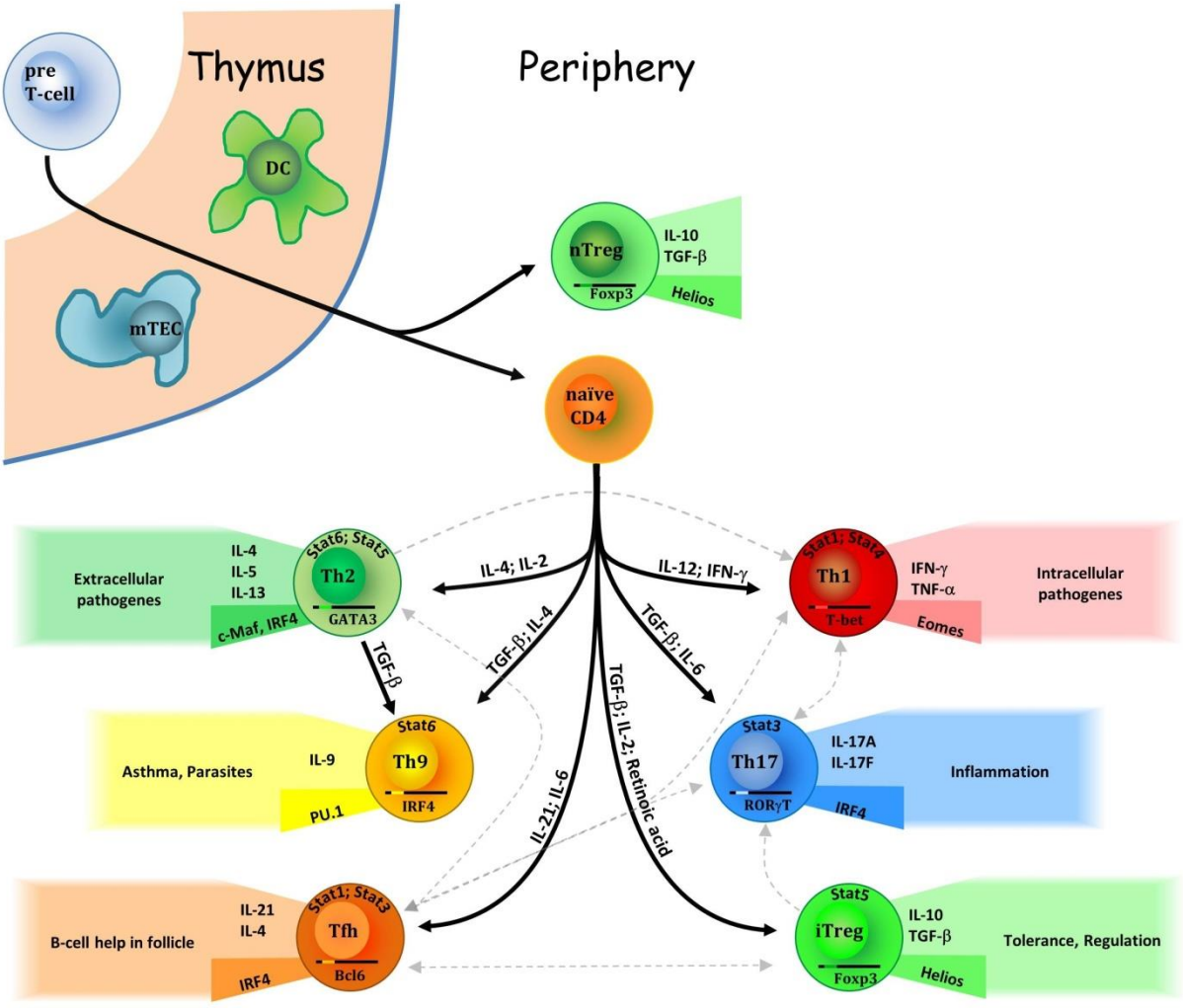

Figure 1 Development and function of T helper cells

Commitment points during the life cycle of a T cell. After activation naïve $CD4^+$ T cells commit to a lineage due to cytokine milieu. After signaling through distinct receptors, specific transcription factors are expressed. The secreted cytokines are part of the immunological function indicated on the site. T helper cell plasticity is indicated by thin grey lines. Adapted from O'Shea.[8]

1.1.1 Th1 cells: Helpers of the CD8 mediated cellular immune response

First experiments showed that Th1 cells mainly produce Interferon γ (IFN-γ) and Interleukin (IL)-2 after stimulation with antigen or Concanavalin A (ConA). These cytokines were believed to be Th1 specific, but later only IFN-γ remained as an exclusive Th1 cytokine.[6] IL-12 secreted by phagocytic cells primes antigen activated naïve $CD4^+$ T cells for the Th1 lineage. IL-12 signals are indispensable for the development of the Th1 cells and the production of IFN-γ.[9,10]

To stabilize Th1 commitment, IFN-γ itself affects the expression pattern of the T cells in an autocrine manner.[11] The binding of IFN-γ to the corresponding receptor leads to the enhanced transcription of Th1 related genes [12–15]. One of the most prominent targets is the master transcription factor of the Th1 lineage called *T-box transcription factor TBX21* (T-bet).[11]

T-bet is not only related to IFN-γ production, it also influences the repression of Th2 associated cytokines.[16–18] The deficiency of T-bet predestinates T cells to commit to the Th2 lineage.[19] The physical interaction with the master regulators of nearly all T helper cell subsets brings T-bet into focus of T helper cell fate decisions.

T helper cell subsets have established positive feedback loops to minimize the differentiation into another subset and thereby ensure stability of the lineage commitment. T-bet is able to induce the β-chain of the IL-12 receptor, enabling predefinition towards the Th1 lineage.[11] Additionally, the release of IFN-γ leads to an increase of IL-12p40 and IL-12p35 transcription in Lipopolysaccharide (LPS) stimulated monocytes, indirectly promoting Th1 commitment.[20] Both mechanisms secure the decision towards Th1 cells. A feedback loop from T-bet on its own expression is not observed.[11]

1.1.2 Th2 cells: Helpers of the humoral immune response

The activation of the humoral immune response enables the clearance of extracellular pathogens *via* an antibody response. Activated Th2 cells support this process by costimulation of B cells. The main cytokines secreted by Th2 cells are IL-4, IL-5 and IL-13.[6,7,21–23] Newest results indicate that the former Th2 restricted IL-10 and IL-9 are not solely produced by this T helper subset.[6,7,24,25]

The crucial cytokines for the Th2 development are IL-4 and IL-2.[26–28] They are important for cytokine production, T cell proliferation and lineage commitment. The production of IL-4 thereby leads to a positive feedback loop similar to the IL-12 dependent one for Th1 cells.

The major transcription factor defining Th2 commitment is GATA binding protein 3 (GATA3).[29] Lineage stability by GATA3 is controlled by a negative influence on Th1 differentation.[30,31] GATA3 is the factor strongly controlling the IL-6, IL-13, and IL-5 transcription, by binding to the promoter region of the corresponding gene.[29,31–33] In the

control of IL-4 expression, GATA3 only has the role of a distal enhancer and is not crucial for the expression.[34] Two additional minor transcription factors could be identified who are not able to drive Th2 differentiation by themselves. V-maf musculoaponeurotic fibro sarcoma oncogene homolog (c-Maf) and Interferon regulatory factor 4 (IRF4) are minor transcription factors for Th2 cells.[29,35,36] c-Maf and IRF4 physically synergize with nuclear factor of activated T cells (NFAT) to induce the *Il4* promotor.[36,37] c-Maf is able to bind the *Il4* promoter *via* a half-Maf recognition elements (half-MARE) site, while there is no direct effect of either transcription factor on IL-5 or IL-13.[36,38]

1.1.3 Th17: Regulators for inflammation

Naïve $CD4^+$ T cells cultured with low amounts of transforming growth factor β (TGF-β) and IL-6 develop in an IL-17 producing phenotype.[39–41] The Th17 phenotype is mainly controlled by the transcription factor retinoid-related orphan receptor gamma (RORγT).[42] It has been demonstrated that Th17 cells play important roles in inflammation and experimental autoimmune encephalmyeltis.[43]

1.1.4 Tfh: Helpers of B cells

A T helper cell subset found in the B cell follicle are the follicular T helper cells (Tfh).[44–46] These cells are characterized by the master regulator B cell lymphoma 6 protein (Bcl-6) and the surface marker C-X-C chemokine receptor type 5 (CXCR5). Their function to produce IL-21 is important to regulate the germinal center reaction and B cell response.[47–50]

1.1.5 Th9 cells: An IL-9 producing T helper cell subset

After the identification of the Th1 and Th2 cells, other cytokines connected to $CD4^+$ T helper cells were discovered. One of these cytokines is the T cell growth factor P40. This cytokine is produced by a T cell clone and is different from the known growth factors IL-2 and IL-4.[51,52] It was found that this growth factor P40 is identical with another factor called the T cell growth factor III (TCGFIII) and is not able to stimulate the proliferation of naïve $CD4^+$ T cells.[53] The finding that the mast cell growth-enhancing activity factor (MEA) is identical with the P40 growth factor led to the denotation as IL-9.[54]

The expression and release of IL-9 by activated $CD4^+$ T cells after T cell receptor (TCR) dependent stimulation is mainly driven by the local cytokine milieu. *In vitro* studies showed that under the influence of TGF-β, stimulated $CD4^+$ T cells produce significant amounts of IL-9. Albeit not being able to induce IL-9 on its own, IL-4 is able to enhance IL-9 secretion under the stimulus of TGF-β.[55] The differentiation of the IL-9 producing phenotype depends on the presence of IL-2.[55] IL-4 signaling *via* the signal transducer and activator of transcription (Stat)6 pathway reduces the TGF-β induced expression of forkhead box P3 (Foxp3), preventing the induction of Tregs.[56] IL-9 released by T helper cells was associated

with the Th2 subset for a longtime, although a different regulation of IL-9 compared to the other known Th2 cytokines like IL-4, IL-5 and IL-13 was observed.

Elegant studies by Stockinger and colleagues showed for the first time, that under any known $CD4^+$ T helper cell differentiation condition, IL-9 is not coproduced with the classical Th2, Th1 and Th17 cytokines.[57] The same group described that TGF-β converts the Th2 subset to an IL-9 generating phenotype while losing the expression pattern of classical Th2 cells, with the exception of IL-10. This IL-9 producing subset is called `Th9` cells.

Other ways of Th9 induction were discovered, unveiling that the signal from TGF-β is indispensable, while the IL-4 signal can be substituted.[58] It was demonstrated that IL-1α produced by APCs, can serve as a secondary signal during Th9 differentiation *in vitro* and *in vivo*.[24] Further studies showed that also IL-1β, IL-18 and IL-33 are able to compensate for the IL-4 signal.[58,59] The augmentation of IL-9 production is also promoted by IL-25, Type I Interferons (IFN-α, IFN-β) and IL-21.[60,61]

Because all of the major T helper cell subsets are characterized by specific expression of transcription factors like T-bet for Th1, GATA3 for Th2, the discovery of the master regulator for the Th9 subset is critical for the definition of a discrete T helper cell lineage.

IRF4 is able to bind to the *Il9* promoter. It also upregulates IL-9 in a concentration dependent manner in Th9 differentiated $CD4^+$ T cells.[62] Another transcription factor important for the Th9 phenotype is the E-twenty six (ETS) family transcription factor PU.1.[63] It presumably modulates the histone modification at the *Il9* promoter and may be involved in the downregulation of Th2 connected cytokines by direct interaction with the Th2 transcription factor GATA3.[64] It remains elusive how *Il10* transcription is regulated since it was shown that Th9 cells have the capacity to produce IL-9 as well as IL-10.[56]

During early stages of Th9 differentiation GATA3 has a pivotal role, while other transcription factors were not found.[56,57,65]

1.1.5.1 Effects of IL-9 on T cells

IL-9 shows growth stimulating capacities for mast cells and T cells. These are presumably transmitted through the common γ-chain that is shared with other growth stimulating factor receptors like IL-2, -4, -7, -15 and -21. So far IL-9 shows direct effects on $CD4^+$ T cells.[66–69] It could be demonstrated that IL-9 is able to substitute IL-6 during Th17 differentiation, although not being able to induce the same amounts of IL-17.[70] The role of IL-9 in the induction of proinflammatory T cells is one side of its effects. In contrast to these proinflammatory effects, IL-9 also has an enhancing effect on the suppressive capacity of naturally occurring regulatory T cells *via* the IL-9 receptor in *in vitro* proliferation assays. These results being confirmed *in vivo* by systemic blockade of IL-9 *via* antibody or IL-9 receptor deficient mice.[70,71]

1.2 Th9 cells and IL-9 in health and disease

1.2.1 IL-9 in different autoimmune diseases

IL-9 producing cells, especially IL-9 producing T cells, are connected to a variety of different autoimmune diseases, especially asthma. Although IL-9 producing T cells were not observed in many human disease patterns, there are implications from mouse models showing their potency for different clinical aspects.

To prove that Th9 cells exhibit proinflammatory properties and discriminate them from IL-10 producing Tregs, a colitis model showed that Th9 cells were able to induce a mild colitis when transferred into recipient mice. Their proinflammatory capacities were ascertained by a higher colitis score when transferred together with effector T cells.[56]

A more prominent role of IL-9 was shown in the experimental autoimmune encephalomyelitis (EAE) model, the mouse model of multiple sclerosis.[72] The role of IL-9 in the EAE model seems to be ambivalent. On the one hand, blockade of IL-9 *via* antibody ameliorates EAE while on the other hand *Il9r* (IL-9-receptor)- deficient mice show enhanced EAE symptoms due to reduced suppressive activity of Tregs.[70,73,74] It seems that these ambivalent effects are mainly due to a direct or indirect recruitment of Tregs, Th17 and other inflammatory immune cells to the central nervous system (CNS).

IL-9 producing cells seem to play a prominent role in worm infections as demonstrated by expulsion of the *Trichuris muris*.[75,76] The modern lifestyle and health care in Western countries may play a role in the rising incidence of overreacting immune responses like allergy and especially asthmatic diseases. It is believed that the reduced burden of parasitic worms promote the development of allergic diseases, especially allergic asthma.[77]

1.2.2 IL-9 and asthma

The first evidence that IL-9 is one of the mediators of allergic asthma was found in human sibling studies where high serum IgE is co-inherated with bronchial hyperresponsiveness, identifying chromosome 5q31-33 that carries the *Il9* locus as the genetic background.[78] Due to the fact that different Th2 associated cytokines are located in humans on this chromosome, a specific candidate could not be determined. Genes that are located on the 5q31-33 in humans are split on three different chromosomes in mice. Genetic studies in mice identified the *Il9* locus as a gene that is associated with bronchial responses.[79]

The overexpression of IL-9 under the Clara-cell 10 promoter in mice, inducing an expression only in lung epithelial cells, leads to allergic-like symptoms without prior sensitization. The symptoms are comparable to asthmatic symptoms including eosinophilia, lymphocyte infiltration and mast cell hyperplasia. Furthermore, higher mucus production as well as airway remodeling is observed. The most striking feature is an increased airway hyperresponsiveness reaction (AHR) towards bronchi contracting metacholine.[80] Similar

symptoms can be observed in *Il9*-transgenic mice that systemically overexpress *Il9*.[81] Overexpression of other cytokines that are associated with Th2 cells show no difference in AHR in the case of IL-4 or symptoms that are not common with the human disease in the case of IL-13.[82,83]

The observations in *Il9*-overexpressing mice can be dissected and clearly linked to the function of IL-9. Not only is IL-9 a mast cell growth factor, the recruitment of those to the site of inflammation is also partly controlled by IL-9.[73,80,84]

Another innate immune cells involved in asthma pathogenesis and displaying infiltration in asthmatic tissue are eosinophils. Not only does the IL-9 signaling increase survival of eosinophils by upregulation of the IL-5 receptor, enhanced recruitment to the site of IL-9 production may contribute to higher eosinophil numbers in affected lungs.[85,86]

A burden to asthmatic patients is the increased mucus in the lungs. This fluid consists of different, highly glycosylated mucin proteins released mainly by the lung goblet cells. As described above, *Il9*-transgenic mice have a higher mucus load. The same effect could be induced by intratracheal application of IL-9 into the lungs of healthy mice, demonstrating direct effects of IL-9 on the mucus production of lung epithelial cells.[80,87]

In vitro depletion of IL-9 in allergenic bronchi alveolar lavage fluid was able to inhibit higher mucus production in lung epithelial cells. An effect that could not be observed with IL-5 or IL-13.[88] Other studies concluded that in fact IL-9 only leads to IL-13 release of mast cells, which by itself induces goblet cell hyperplasia and mucin.[89]

A major cause of asthmatic attacks in allergic patients is the release of cytokines by mast cells after crosslinking of membrane bound IgE. This specific crosslinking is only possible after induction of an IgE antibody response against the allergen. IL-9 is also able to enhance this B cell mediated response by augmenting the antibody production in an IL-4 restricted manner.[81,90,91]

As outlined, IL-9 plays a crucial role in the severity of asthmatic diseases. However, some publications described an IL-9 independent generation of allergic asthma. No differences in any of the major clinical indicators were observed in *Il9* deficient mice, suggesting that the major Th2 cytokines can compensate for IL-9 deficiency.[92] Nevertheless, these results do not stand in contrast to the role of Th9 and IL-9 in asthma since adoptive transfer of *in vitro* generated Th2 and Th9 cells showed different asthmatic characteristics in recipient mice. Notably, only the Th9-mediated asthma was reduced by the application of an IL-9 neutralizing antibody while Th2 receiving mice remained unchanged.[62] Asthmatic diseases have a broad range of clinical course which might be due to the participation of different subsets of T helper cells. One striking feature is that Th9 cells seem to mediate the disease at an early stage. Investigations of an IL-9 fate-reporter model, where cells are marked with green fluorescent protein (GFP) that expressed IL-9 during their lifespan, revealed an

expression of IL-9 in $CD4^+$ T cells only at an early time point in inflammatory responses in the lungs.[93]

Since IL-9 is involved in the pathogenesis of acute asthma, new treatment procedures are being tested. The application of IL-9 neutralizing antibody leads to the reduction of the asthma associated symptoms. Due to the pleiotropic effects of IL-9 towards different asthma involved cell types, the development of a fully humanized antibody promises positive results, through influencing different asthma targets.[94,95] The benefits of targeting IL-9 above other cytokines that play a role in asthma, like IL-4 and IL-13, is that the systemic administration of anti-IL-9 does not interfere with the basic immune pathways. The targeting of Th2 cytokines like IL-4 or IL-13 may interfere with the Th2-mediated humoral response. The fully humanized antibody against IL-9 called MEDI-528 passed the Phase I study in healthy volunteers that ensured safe application of the antibody without major side effects.[96] Until now seven clinical trials investigating positive effects of MEDI-528 on the asthmatic conditions of patients show clinical relevance in moderate asthmatic patients.[97,98]

1.2.3 IL-9 in tumor immunology

Not much is known about the influence of IL-9 on tumors. In this line, anti- as well as pro-tumorigenic effects of IL-9 on different tumor cells have been observed. Earlier it was described that IL-9 promotes growth of thymic lymphomas.[99] These discoveries showed that IL-9 has tumor promoting activities most possibly due to the ability of IL-9 to promote T cell growth. Additionally, in humans, IL-9 expression was observed in different Hodgkin lymphomas and large cell anaplastic lymphoma.[100] T cell lymphomas do not only grow more extensively when IL-9 is present, IL-9 receptor expression on tumor cells could also be directly shown in mice and humans, indicating a direct link between IL-9 and tumor growth.[101,102]

Beside these tumor-promoting features of IL-9, newest results indicate a role of IL-9 in anti-tumor responses against different tumor types. The effect of IL-9 in melanoma immunity is not directly mediated by the $CD4^+$ T helper cells. Th9 cells play a crucial role in tumor response by attracting mast cells, dendritic cells and cytotoxic $CD8^+$ T cells *via* IL-9 which is indispensable for tumor reduction.[103,104] Blocking of IL-9 by the application of IL-9 antibody greatly diminished the tumor response of wild-type mice, while the transfer of Th9 effector cells in recombination activating gene 2 (*Rag2*) deficient mice enhanced their survival compared to transfer of other T helper subsets, underlining their crucial role in melanoma response.

In the overall function of immune system, Th9 cells have a protective role during the expulsion of parasites and in the rejection of melanoma tumors. Overreacting Th9 cells on the contrary, are also associated with the pathogenesis of autoimmune diseases like acute asthma.

1.3 Regulatory T cells

1.3.1 Naturally occurring regulatory T cells (nTregs): Guardians of tolerance

It was discovered that mice, thymectomized on day 3 after birth, develop autoimmune disease symptoms later on.[105] This effect was later attributed to Tregs. During the maturation of T cells in the thymus, T cells can commit towards the Treg subset. Tregs ensure tolerance towards self-antigens, thereby preventing autoimmunity. The discovery of $CD45RB^{low}CD4^{+}$ T cells, showing the ability to inhibit autoimmunity, allowed a Treg subset to be defined for the first time.[106] Today, the commonly used surface marker for Treg identification in mice is CD25, the α-chain of the IL-2 receptor. Depletion of $CD4^{+}$ $CD25^{+}$ T cells *in vivo* leads to the development of an autoimmune phenotype.[107] Since nTreg depletion leads to similar symptoms as the scurfy disease in mice and immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans, a link to the *Foxp3* gene was discovered. The Foxp3 transcription factor is the master regulator of Tregs.[108–111] The direct connection of Foxp3 to autoimmunity enabled the discovery of the decisive role of Foxp3 for Treg function and lineage stability.[112–115] Foxp3 not only shows enhancing or activating effects when binding to its target sites, but also represses other genes.[116,117]

One point of commitment towards the regulatory lineage is in the thymus. nTregs are selected *via* a single TCR encounter, against potentially high affinity cognate antigens.[118,119] The selection process in the thymus is complex and will not be discussed. *In vivo* models of Treg development showed that they consist of a different TCR repertoire than do normal $CD4^{+}$ T cells.[120] This different TCR repertoire is due to the fact that nTregs are selected towards self-antigens. This selection is mediated by thymic medullary epithelial cells (mTECs), expressing autoimmune regulator (AIRE), and thymic dendritic cells.[121,122] Both cell types are able to present antigens that are normally not expressed in the thymus ensuring self-tolerance to organ specific antigens.

1.3.2 Induced regulatory T cells (iTregs): Mediators of peripheral tolerance

Although the maturation of T cells in the thymus ensures tolerance *via* negative selection and nTreg induction, a selection against food antigens or other non-harming antigens is not possible. To assure acceptance to these kinds of antigens, the formation of induced Tregs in the periphery is necessary and can be mediated *via* different mechanisms.

Mucosal dendritic cells are able to induce the regulatory phenotype in peripheral T cells. This lineage progression towards iTregs can also be mediated *in vitro via* TGF-β and retinoic acid or TGF-β and IL-2.[123–126] Another way to induce iTregs *in vivo* is the ongoing application of low dose antigen.[127,128] It seems that iTregs do not differ from nTregs in their capability to inhibit targets and only show a discrepancy in methylation of the *Foxp3* promoter, affecting lineage stability.[129]

1.3.3 Mechanism of suppression: A variety of possibilities

Tregs, whether of nTreg or iTreg origin, use the same pathways to regulate different steps of the immune response. The arsenal can be divided into three major groups: contact dependent, soluble factors and deprivation of cytokines.[130,131] To investigate the suppressive capacity of Tregs *in vitro*, a model system was established, using APC as costimulus and a TCR signal induced *via* antigen or anti-CD3 antibody. The proliferation of normal $CD4^+$ responder T cells is followed *via* H^3-Thymidine or carboxyfluorescein succinimidyl ester (CFSE).[132,133] Most of the mechanisms descripted in the following are replaceable and can be substituted by other inhibitory pathways.

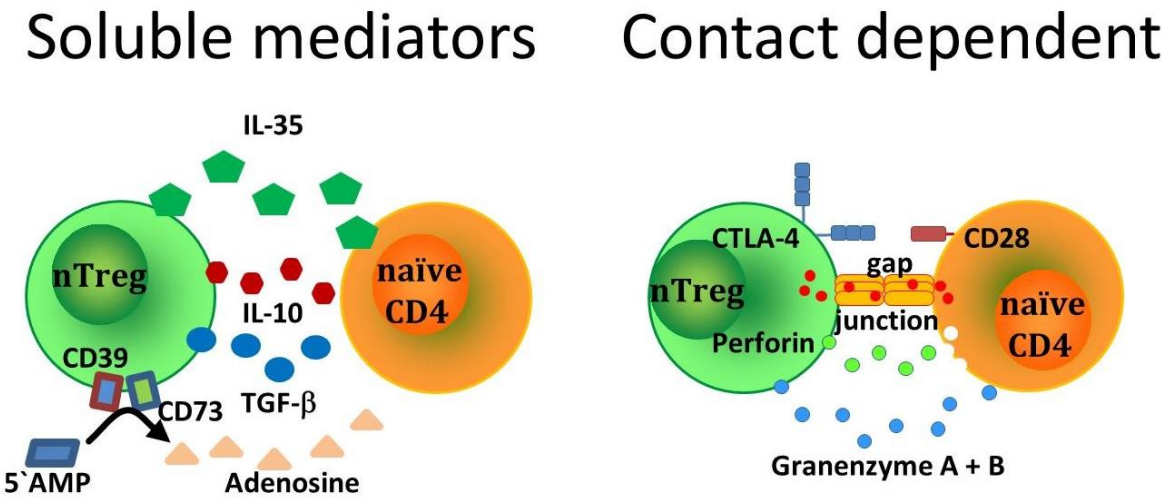

Figure 2 Mechanisms of suppression
Different systems of suppression are described below. The suppressive mechanisms are separated for contact dependent and contact independent pathways.

1.3.3.1 Contact-dependent mechanisms

The fact that the suppression of Tregs is contact dependent was shown by using a transwell assay or displaying the inefficacy of supernatant to suppress proliferation of responder T cells. In concordance with that, deficiency of different soluble repressive factors or neutralizing antibodies have little effect on the capacity of Tregs to interfere with T cell activation.[132,133]

One of the first mechanisms discovered, was the suppression *via* cytotoxic T-lymphocyte antigen-4 (CTLA-4). The treatment of mice after T cell transfer with blocking anti-CTLA-4 antibody led to the development of intestinal inflammation even with co-transfer of Tregs.[134,135] The impact of this regulation affects different targets in at least two independent ways. One is directly aiming on the responder T cells *via* the interaction of B7-1 or B7-2 with CTLA-4. The other way is by activating dendritic cells to express of indoleamine 2,3-dioxygenase (IDO), consuming the essential amino acid trypthophan.[136–138]

The lymphocyte activation gene-3 (LAG-3) surface protein that is upregulated on activated Tregs shows only marginal contribution to Treg-mediated suppression. The CD4 homolog LAG-3 interacts with the major histocompatibility complex II (MHCII) proteins on the surface of dendritic cells and interferes with their function.[139,140]

Death of autoimmune reactive cells in an antigen specific manner is a mechanism that can be initiated by different pathways. It can be transmitted by the Fas ligand signaling.[141] The lysis of target cells can also be initiated by granzyme B.[142,143] Other reports showed a function for pore forming perforin and granzyme A.[144] The specific lysis of autoimmune reactive cells influences the overall Treg suppressive capacities only marginally.

TGF-β as a suppressive factor targets responder cells in two ways. The protein TGF-β itself is presented on the surface of Tregs in a latent form and seems to be able to inhibit target cells.[145] The source of soluble TGF-β in an *in vivo* suppression setting is not necessarily the Treg itself, but also bystander cells can secrete the cytokine.[146] Soluble TGF-β enhances the inhibitory capacity of Tregs *in vitro* as *in vivo.*[147–149]

The direct transport of 3'-5'-cyclic adenosine monophosphate (cAMP) from Tregs *via* gap junctions into the target cell is another contact dependent inhibition pathway. The rise of the intracellular cAMP level in responder T cells mediates the repression.[150,151]

1.3.3.2 Soluble suppressive factors

Likewise, inhibition of T cells is mainly mediated by direct contact. The cytokine milieu itself still influences this suppression. The three major soluble factors IL-10, soluble TGF-β and adenosine are responsible for the contact independent suppression of normal T cells.

Soluble TGF-β, as mentioned above, can influence the *in vivo* response of Tregs in different model system.[145–149]

Although IL-10 is not solely produced by Tregs, *in vivo* experiments were convincingly showing induction of autoimmunity when anti-IL-10 antibody was applied to healthy mice.[152–156] The IL-10 connected regulation of inflammation seems to be most important in the gut associated lymphoid organs.[153]

Experiments with adenosine monophosphate (AMP) showed that Tregs were able to suppress T cell proliferation in an AMP dependent manner.[157] The mechanism behind this regulation is *via* two proteins on the Treg surface. Ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5`-nucleotidase (CD73) in cooperation are able to convert AMP into Adenosine.[158] The soluble Adenosine binds to the adenosine A2A receptor on the target T cell, leading to an inhibition of T cell activation by increasing the intracellular cAMP levels.[159]

The newest addition to the orchestra of suppressive cytokines is IL-35. Epstein-Barr-virus induced gene 3 (Ebi3), the β-chain of IL-27, and IL-12a form the IL-35. Deletion of either of these genes leads to a partial abrogation of Treg-mediated suppression.[160]

1.3.3.3 Deprivation of growth factors

Tregs express the alpha chain of IL-2 receptor constitutively during their lifetime.[107,161] They are not able to produce IL-2 and use foreign produced IL-2 to survive in their suppressive phenotype.[162] Normal effector T cells are able to produce IL-2 after activation and the binding of IL-2 to its receptor enhances the proliferation in an autocrine manner. This positive feedback loop is interrupted by Tregs as they consume IL-2 earlier than the effector T cell, thereby inhibiting effector T cell responses.[163,164]

The deprivation of the essential amino acid tryptophan by dendritic cells *via* the expression of IDO was mentioned earlier. The turnover of tryptophan leads to a reduced ability to generate protein, reducing the ability of T cells to respond to activation signals.[137]

1.3.4 Newest insight in Treg-mediated suppression

New mouse models led to further insight in the function of Treg. Tregs controlling a Th1 response express the transcription factor T-bet and secrete low amounts of IFN-γ, while retaining their suppressive capacities and Foxp3 expression. The combination of both transcription factors enables Tregs to express similar homing marker and signaling receptors as differentiated Th1 cells, leading to a specific inhibition of an ongoing Th1 response at the site of inflammation.[165] These expression patterns, of different master transcription factors in combination with Foxp3, were observed for most of the known T helper cell subsets, regulating all kinds of different immune responses.[166,167]

1.4 Aim of the project

Recently, it was shown in a preclinical model of asthma that the transfer and subsequent antigen-specific activation of Th9 cells leads to the development of IL-9-induced asthmatic symptoms. Concerning Tregs it was found in several studies that this suppressive T cell population can ameliorate such asthma symptoms. Therefore, the suppressive potency of Tregs for the development and effector function of Th9 cells was a part of the focus of this doctoral thesis. To that end, the influence of Tregs was analyzed *in vitro* at the transcriptional and translational level *via* co-culture assays. Finally, these studies were complemented with the aid of two Th9-dependent preclinical animal models.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

All chemicals were supplied by either Roth (Roth, Karlsruhe, Germany) or Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA).

Table 1 Chemicals used for molecular methods

Supplement/Media/chemical	Supplier
SA-HPO	Roche, Indianapolis, USA
Dynabeads M-450 Epoxy	Invitrogen Dynal AS, Oslo, Norway
Dynabeads Mouse panB (B220)	Invitrogen Dynal AS, Oslo, Norway
Dynabeads Mouse CD8 (Lyt-2)	Invitrogen Dynal AS, Oslo, Norway
Streptavidin – (SA-) micro beads	Miltenyi Biotec, Bergisch Gladbach, Germany
SYBR-Green	Invitrogen Life Technologies, Carlsbad, USA
Reverse transcriptase	Thermo Fisher Scientific, Waltham, USA

All plastics were supplied by Greiner Bio-One (Greiner Bio-One, Kremsmünster, Austria), VWR (VWR International, Radnor, USA) and Eppendorf (Eppendorf AG, Hamburg, Germany).

2.1.2 Buffers and Solutions

Buffer and solutions for molecular biology methods

DEPC-H_2O (Diethylpyrocarbonat)

0.1 % DEPC solved in H_2O over night

Followed by autoclaving

Total RNA Isolation (TRI) Reagent

TRI-Reagent was supplied by Ambion Life Technologies, Carlsbad, USA.

75 % Ethanol in DEPC-H_2O

Solution was prepared using 99 % ethanol p.a. diluted with DEPC-H_2O to 75 %. The solution was stored at -20 °C and used at that temperature.

Glycogen

For RNA preparation, the glycogen solution was prepared using 20 µg/mL glycogen in DEPC-H_2O and incubated for three days at 37 °C. The solution was stored at -20 °C.

dNTPs (2'-Desoxynucleosid-5'-Triphosphate)

The 10 mM stock solution of the combined nucleotides was prepared by solving them in H_2O and storing at -20 °C.

Hexanucleotides primers (N_6 primer)

The primers were used at a concentration of 6 ng/mL in H_2O and stored at -20 °C.

Oligo$(dT)_{18}$ primers

The primers were used at 100 ng/mL according to the supplier's manual (Thermo Fisher Scientific, Waltham, USA) and stored at -20 °C.

PBS 10x (phosphate buffered saline)

1.4 M NaCl

0.1 M NaH_2PO_4

set to pH 6.6 with 10 N NaOH

70 % Ethanol

70 mL 98 % Ethanol p.a.

30 mL DEPC-H_2O

FACS-Flow

1x PBS

Trypan blue staining solution

The isotonic staining solution was prepared by mixing solution 1 in a 5:1 ratio with solution 2.

Solution 1: 0.2 % Trypan Blue in VE-H_2O

Solution 2: 4.25 % NaCl in VE-H_2O

Buffer and solutions for enzyme-linked immunosorbent assay (ELISA)

Coating buffer

0.1 M Na_2HPO_4 pH 9.2

Blocking buffer

1x PBS

+ 0.5 % bovine serum albumin (BSA)

Washing buffer

1x PBS

+ 0.1 % Tween 20

Citrate buffer

40 mM citric acid

set pH 4.4

Buffer and solutions for cell isolation and cultivation

Cell cultivation medium substrate was supplied by PAN Biotech (Aidenbach, Germany). Before use all solutions listed below were filtered with a 0.2 µM filter and stored at 4 °C.

Supplier's list for cell culture substances

Table 2 Chemicals used in cell culture

Component	Supplier
FCS	Vitromex (Vilshofen, Germany)
MEM substrate	PAN Biotech (Aidenbach, Germany)
Sodium Pyruvate	Serva Electrophoresis GmbH (Heidelberg, Germany)
Penicillin/streptomycin	Serva Electrophoresis GmbH (Heidelberg, Germany)
IMEM substrate	PAN Biotech (Aidenbach, Germany)
HEPES	BioWest (Nuaillé, France)

Disodium ethylenediamine tetracetic acid (EDTA)

EDTA was used in a 0.5 M stock solution. The pH was adjusted to pH 8.0 with 10 N NaOH and was autoclaved before being used as supplement.

Fetal calf serum (FCS)

Serum was incubated for 35 min at 56 °C to inactivate the complement system and stored at 4 °C. Before supplementing the media, the FCS was centrifuged for 15 min at 600 xg to remove particles.

Gey's solution

8.29 g/L NH_4Cl

1.0 g/L $KHCO_3$

0.037 g/L EDTA

ad 1 L H_2O

GM-Buffer

1x PBS

+ 0.5 % BSA

+ 0.01 % NaN_3

5 mM EDTA

PBS + fetal calf serum (FCS)

1x PBS

+ 10 % FCS

Minimal essential medium MEM

10.58 g MEM substrate

4.77 g HEPES

1 % Penicillin/Streptomycin

0.05 mM 2-Mercaptoethanol

ad 1 L H_2O

MEM + 2 % FCS

MEM

+ 2 % FCS

Iscove's modified Dulbeccos's medium (IMDM)

17.67 g IMDM substrate

3.02 g $NaHCO_3$

1 % Penicillin/Streptomycin

0.05 mM 2-Mercaptoethanol

Phenol red as indicator

Test medium (TM + 5 % FCS)

IMDM

+ 5 % FCS

+ 1 % L-Glutamine

+ 1 % Sodium pyruvate

+ 1 % Penicillin/Streptomycin

50 µM 2-mercaptoethanol

Test medium (TM + 10 % FCS)

IMDM
+ 10 % FCS
+ 1 % L-Glutamine
+ 1 % Sodium pyruvate
+ 1 % Penicillin/Streptomycin
50 µM 2-mercaptoethanol

Th1 differentiation medium d1

3000 U/mL IL-12
100 U/mL Proleukin (recombinant, human IL-2)
10 µg/mL anti-IL-4 (11B11)

Treg medium

100 ng/mL mrIL-2
in TM + 5 % FCS

Th2 differentiation medium d1

1000 U/mL IL-4
100 U/mL IL-6
20 µg/mL anti-IFN-γ (XMG1.2.3)
in TM + 5 % FCS

Th9 differentiation medium d1

300 U/mL mrIL-4
5 µg/mL anti-IFN-γ (XMG1.2.3)
5 µg/mL anti-IL-6 (D6906B4.M)
4 ng/mL porcine TGF-β
in TM + 5 % FCS

Th2 medium d3

200 U/mL mrIL-4
100 U/mL IL-6
100 U/mL Proleukin (recombinant, human IL-2)
in TM + 5 % FCS

Th9 medium d3

300 U/mL mrIL-4

100 U/mL Proleukin (recombinant, human IL-2)

1 ng/mL porcine TGF-β

in TM + 5 % FCS

Dendritic cell medium

2 % GM-CSF

in TM + 5 % FCS

2.1.3 Additive cytokines for cell culture

Cytokines used for cell culture and cell differentiation were also used as standard for specific ELISA. The cytokines were affinity purified, if not indicated otherwise, in house.

Murine recombinant Interleukin 2 (mr-IL-2)

mr-IL-2 was purified from supernatant of IL-2 cDNA transfected myeloma X63Ag.653 cells.[168] These cells were kindly provided by Dr. F. Melchers. mr-IL-2 from the supernatant was purified by Dr. E. Schmitt.

Murine recombinant Interleukin 4 (mr-IL-4)

mr-IL-4 was purified from the supernatant of IL-4 cDNA transfected myeloma X63Ag8-653 cells.[168] The recombinant IL-4 was purified from Dr. E. Schmitt *via* affinity chromatography. One unit is defined as concentration that leads to a half-maximum rise in proliferation rates of cell line F4/4K.6.

Proleukin

Proleukin was purified in house.

Porcine Transforming Growth Factor Beta (TGF-β)

TGF-β was supplied by R&D Systems (Minneapolis, USA). It was reconstituted in 4 mM HCl with 0.1 % BSA and stored at -70 °C. Activity was measured according to the supplier's manual.

Murine recombinant Interleukin 6 (mrIL-6)

The mr-IL-6 was supplied by R&D System (Minneapolis, USA). It was reconstituted in PBS according to the supplier's manual and stored at -70 °C.

Granulocyte macrophage colony-stimulating factor (GM-CSF)

GM-CSF was used from supernatant of GM-SCF cDNA transfected myeloma X63Ag8-653 cells.[168]

2.1.4 Antibodies

Antibodies used for FACS analysis

Table 3 Antibodies used in FACS analysis

Antibody (Clone)	Conjugation	Supplier/Reference
CD90.1 (HIS51)	eFlour450	eBioscience (San Diego, USA)
CD25 (3C7)	Alexa 488	eBioscience (San Diego, USA)
CD44 (IM7)	PE	Biolegend (San Diego, USA)
CD62L (MEL-14)	APC	eBioscience (San Diego, USA)
IL-4 (11B11)	PE	eBioscience (San Diego, USA)
IL-9 (RM9A4)	APC, PE	Biolegend (San Diego, USA)
Isotype IL-4 (MPOC-21)	PE	Biolegend (San Diego, USA)
Isotype IL-9 (RTK2071)	APC, PE	Biolegend (San Diego, USA)
B220 (RA3-6B2)	APC	eBioscience (San Diego, USA)
MHCII	PacificBlue	Biolegend (San Diego, USA)

Compensation (BD™ CompBead) and drop delay beads (BD™ Accudrop Fluorescent Beads) were supplied by BD Bioscience (New Jersey, USA).

Antibodies used for ELISA analysis

Table 4 Antibodies used for ELISA

Antibody (Clone)	Conjugation	Supplier/Reference
IL-4 (BVD4-1D11)		Provided by A. O'Garra (MRC, UK) [169]
IL-4 (BVD6-24G2)	bio	Provided by A. O'Garra (MRC, UK) [169]
IL-9 (229.4)		Fusion of rat spleen cells immunized with mIL-9[55]
IL-9 (C12)	bio	Provided by J. van Snick (LICR, Belgium) [55]

IL-10 (JES5-2A5)		BD Biosciences (New Jersey, USA)
IL-10 (JES5-16E3)	bio	BD Biosciences (New Jersey, USA)

Antibodies used for cell culture

Table 5 Antibodies used for cell culture

Antibody (Clone)	Conjugation	Supplier/Reference
mCD4 (H129.19)	bio	acquired by the American Type Culture Collection (ATCC TIB 207)[170]
mCD25 (7D4)	bio	BD Biosciences (New Jersey, USA)[171]
mCD3ε (145-2C11)		Provided by J. Bluestone (UCSF, USA)[172]
mCD28 (37.51)		Provided by J.P. Allison through G. Leclercq (University of Gent, Belgium)[173]
mIFN-γ (XMG1.2)		Provided by A. O'Garra (MRC, UK)[7]
mIL-6 (D6906B4.M)		Provided by J. van Snick (LICR, Belgium) [174]
mIL-4 (11B11)		Provided by W. Paul (National Institutes of Health, Bethesda, MD)[175]

2.1.5 Oligonucleotides

Table 6 Oligonucleotides used for qRT-PCR

Gene	Sequence (5' - 3')
Plscr1 for.	5'- AGC AAG CAA ACT GAG GCT CCC - 3'
Plscr1 rev.	5'- TGG GGT ATG GGG TAA GCA GCA - 3'
Gpr83 for.	5'- GGC ATG TGT CAT GTC AGT CGC T - 3'
Gpr83 rev.	5'- GGC AGA TGG CAT GTG GCA GA - 3'
Phb for.	5'- GGA AAG TTC GGC CTG GCG TT - 3'
Phb rev.	5'- GGT CGA GAG CGG CAG TCA AA - 3'
Ier3 for.	5'- CAC CGA AAA CGC AGC CGA AG - 3'

Ier3 rev.	5'- AAA TGG GCT CAG GTG TCA CGG - 3'
Id1 for.	5'- TGC AGC TGG AGC TGA ACT CG - 3'
Id1 rev.	5'- AAG ATG CGA TCG TCG GCT GG - 3'
Lif for.	5'- GGG GAG CCG TTT CCC AAC AA - 3'
Lif rev.	5'- GCG CTC AGG TAT GCG ACC AT - 3'
Nod1 for.	5'- GCA GCA GCT GGA GGA TGC TT - 3'
Nod1 rev.	5'- CTG GGC GTA GCA CAG CAT GA - 3'
Samd9l for.	5'- CAG GCA GCC AAG CAC ACT CT- 3'
Samd9l rev.	5'- TTG CCT TCA GCC TGG CCA TC - 3'
Pyhin1 for.	5'- AAC AGC ATC CAG CAG TGC CC - 3'
Pyhin1 rev.	5'- CCA CGG TGG CAT GGA ACA TCT - 3'
Cxcr4 for.	5'- AGC AGC GTT CTC ATC CTG GC - 3'
Cxcr4 rev.	5'- CTG GCT GAC GTC GGC AAA GA - 3'
Scg5 for.	5'- CGG CTC ACC AGG CCA TGA AT - 3'
Scg5 rev.	5'- GGG TCT GGG TAG CCT TGA TCC T - 3'
Itgae for.	5'- TCC TGG ATG GCT CAG GAA GCA - 3'
Itgae rev.	5'- AAC TTT GGC AAG GGA GGC GTT - 3'
Bcl6 for.	5'- TGT CAT CGT GGT GAG CCG TG - 3'
Bcl6 rev.	5'- CCA GGA GGA TGC AAA ACC CCT C - 3'
Clec2i for.	5'- ATG CCA GAT TGC TTG GAG ACA GG - 3'
Clec2i rev.	5'- CCT CCA GTT CCA AGG GGT CCA T - 3'
Il18r1 for.	5'- TGG GAC ACG GTA CAA CAT CAC CA - 3'
Il18r1 rev.	5'- GCT GTC CTC TTT CCT GAT GCT CC - 3'
Hgprt for.	5'- GTT GGA TAC AGG CCA GAC TTT GTT G - 3'
Hgprt rev.	5'- GAG GGT AGG CTG GCC TAT AGG CT - 3'
IRF4 for.	5'- GCC CAA CAA GCT AGA AAG - 3'
IRF4 rev.	5'- TCT CTG AGG GTC TGG AAA CT - 3'
IL-9 for.	5'- CTG ATG ATT GTA CCA CAC CGT GC - 3'
IL-9 rev.	5'- GCC TTT GCA TCT CTG TCT TCT GG - 3'
IRF8 for.	5'- GCA ACG CGG TGG TGT GCA AG - 3'
IRF8 rev.	5'- GCA TCA GGG AGC CAG CAC CG - 3'
T-bet for.	5'- CAA CAA CCC CTT TGC CAA AG - 3'
T-bet rev.	5'- TCC CCC AAG CAG TTG ACA GT - 3'
GATA3 for.	5'- GAC ATC CTG CGC GAA CTG TCA G - 3'
GATA3 rev.	5'- GCG GTG ACC ATG CTG GAA GGG - 3'
cMaf for.	5'- GGC ATG TGT CAT GTC AGT CGC T - 3'

cMaf rev.	5'- GGC AGA TGG CAT GTG GCA GA - 3'
PU.1 for.	5'- GCG ATG GAG AAA GCC ATA GCG ATC - 3'
PU.1 rev.	5'- GGG CTG GGG ACA AGG TTT GAT AAG - 3'

2.1.6 Technical equipment

Table 7 Technical equipment used for experiments

Equipment	Supplier
CO_2 incubator	Sanyo
FACS Cytometer LSRII	BD Biosciences, New Jersey, USA
Cell sorter ARIA	BD Biosciences, New Jersey, USA
Light cycler MyIQ iCycler	Bio-Rad, Hercules, USA
Luminometer	Turner Design, Sunnyvale, USA
MACS magnets	Miltenyi, Bergisch Gladbach, Germany
Nucleofector	Lonza Group Ltd, Basel, Switzerland
Pipetboy	Integra Biosciences, Fernwald, Germany
Clean bench	Heraeus, Hanau, Germany
Megafuge 1.0R	Heraeus, Hanau, Germany
Biofuge pico	Heraeus, Hanau, Germany
Pipettes 1 mL 200 µl 100 µl 10 µL	Eppendorf, Hamburg, Germany
Neubauer counting chamber	Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany

2.2 Mice

Mice, with an age of 6 to 10 weeks, were used for the experiments. All animal procedures were in accordance with the guidelines of the Central Animal Facility Institution of the University of Mainz. The animals were kept under pathogen-free conditions in a circadian rhythm with water and food *ad libitum*.

BALB/cJ

The animals were on the genetic BALB background and bred in the Central Animal Facility Institution of the University of Mainz.

BALB/c x Ova-tg Thy1.1

BALB/cJ x Ova-tg Thy1.1 mice were on the genetic BALB background and bred in the animal facility of the Institute for Immunology of the University Medical Center Mainz. The

Thymocyte differentiation antigen 1 (Thy1) is amongst others constitutively expressed on all mouse T cells. The Thy1.1 strain is characterized by a specific mutation leading to an amino acid exchange of Arginine to Glutamine on position 108. This mutation allows distinguishing Thy1.1 from Thy1.2, which represents the wild-type Thy1 expressed in most mice strains. The αβ T cell receptor of these mice recognized the chicken Ovalbumin (Ova) 323-339.

BALB/c DO11.10 (C.Cg-Tg(DO11.10)10Dlo/J)

The BALB/c DO11.10 mice were kindly provided by D.Y Loh (Nippon Roche Research Center, Kamakura-shi, Japan) through M.Kopf (Swiss Federal Institute of Technology, Zurich, Switzerland). The mice have a αβ T cell receptor that is able to recognize the chicken Ova 323-339. The animals were on the genetic BALB background and bred in the animal facility of the Institute for Immunology of the University Medical Center Mainz.[176]

BALB/c Rag2 -/-

BALB/c Rag2 deficient mice were bred in the Central Animal Facility Institution of the University of Mainz under special pathogen free conditions. Due to a mutation in the recombination activating gene 2 (*Rag2*) the VDJ recombination is impaired, resulting in the loss of adaptive immune cells.[177]

C57BL/6

The animals were on the genetic B6 background and bred in the Central Animal Facility Institution of the University of Mainz.

C57BL/6 Rag1-/-

C57BL/6 Rag1 deficient mice were bred in the Central Animal Facility Institution of the University of Mainz under special pathogen-free conditions. Due to a mutation in the *Rag1* gene, the VDJ recombination is impaired, resulting in the loss of adaptive immune cells.[178]

C57BL/6 OT-II

The C57BL/6 OT-II mice have a transgenic T cell receptor on CD4 T cells. This receptor recognizes the Ova peptide 323-339. The animals were kindly provided by the group of Prof. Grabbe (Hautklinik, Mainz, Germany) or TRON gGMBH (Mainz, Germany).[179]

2.3 Methods

2.3.1 Cell culture methods

2.3.1.1 Determination of living cell counts

The trypan blue staining dye was used to distinguish living from dead cells. Due to the ability of the dye to trespass the membrane of dead cells, the dead cells appeared blue, while living cells remained colorless. The cells were diluted in physiological trypan blue staining solution and counted under a light microscope in a Neubauer counting chamber. The total cell number was determined by multiplication of the mean counted cell number of at least three large squares with the chamber factor, the dilution factor in the staining dye, and the volume.

$$N(total) = cellnumber \times chamber\ factor\ \times volume\ \times dilution$$

2.3.1.2 T cells isolation and culture methods

2.3.1.2.1 Isolation of $CD4^+$ T cells

The T cells used for the experiments were isolated from splenocytes of 6 to 10 week old mice. All steps were performed under sterile conditions and with chilled media. The mice were sacrificed by cervical dislocation, the spleens were removed, placed in a 45 µm cell strainer (BD Biosciences, New Jersey, USA), and covered with MEM + 2 % FCS. The tissue was dissociated with a blunt end of a syringe. To recover most cells all equipment was washed thoroughly afterwards with MEM + 2 % FCS. Large tissue fragments were allowed to sink to the bottom by gravity and then the supernatant was collected. After centrifugation at 200 xg for 5 min at 4 °C, the pellet was resuspended in prewarmed Gey's solution (1 mL per spleen). Incubation for 2 min led to the lysis of red blood cells. The lysis was stopped by addition of 3 mL of MEM + 2 % FCS. Cells were counted (2.3.1.2.1) and centrifuged by 200 xg for 5 min at 4 °C, before being adjusted to $1x10^8$ cells/mL in cold GM-Buffer. The biotinylated anti-CD4 antibody (H129.19) was added to a final concentration of 0.5 µg/mL. The cells were then incubated for 10 min at 4 °C. To remove unbound antibody, the cells were washed two times with GM-Buffer by centrifugation at 200 xg for 5 min at 4 °C and adjusted to their desired volume. Streptavidin (SA) micro beads (Miltenyii Biotec GmbH, Bergisch Gladbach, Germany) were added in a 1:40 dilution. After 10 min of incubation the cells were separated *via* a preequilibrated LS column (Miltenyii Biotec GmbH, Bergisch Gladbach, Germany). This column was magnetized with a strong magnet leading to the recovery of micro bead labeled cells in the column. Undesired cells were not marked with the antibody and magnetic beads and therefore were able to pass through the column. The column was used according to the supplier's manual. Shortly, cells were applied to the

column in 5 mL GM-Buffer. Following two washing steps with GM-Buffer unlabeled cells were removed. The column was removed from the magnetic field and flushed with 5 mL of GM-Buffer to harvest the labeled cells. The whole magnetic isolation procedure was repeated a second time to increase purity. The isolated $CD4^+$ T cells were then ready to use with purity of more than 95 %, controlled by flow cytometry.

2.3.1.2.2 Isolation of $CD4^+$ $CD25^-$ T cells

A single cell solution was prepared and adjusted as described in 2.3.1.2.1. To remove the $CD25^+$ cells, the cells were labeled with a biotinylated anti-CD25 antibody (PC61) (0.5 µg/mL) for 10 min at 4 °C. Unbound antibody was removed by washing the cells twice with GM-Buffer, by centrifugation at 200 xg for 5 min at 4 °C. An Incubation with 1:40 diluted SA micro beads (Miltenyii Biotec GmbH, Bergisch Gladbach, Germany) for 10 min at 4 °C labeled undesired $CD25^+$ cells. In 5 mL GM-Buffer, the cell suspension was loaded on the magnetic LS column according to the supplier's manual (Miltenyii Biotec GmbH, Bergisch Gladbach, Germany). The flow-through was collected and the column was washed once. $CD25^+$ cells were removed from the suspension and unmarked $CD25^-$ cells remained in the flow through. The enrichment of $CD4^+$ T cells, described in 2.3.1.2.1, followed to isolate $CD4^+$ $CD25^-$ T cells. The purity of $CD4^+$ $CD25^-$ T cells was controlled by flow cytometry and usually more than 95 %.

2.3.1.2.3 Isolation of $CD4^+$ $CD25^+$ nTregs

A single cell suspension was prepared from murine spleens as described in 2.3.1.2.1 and labeled with biotinylated anti-CD25 antibody (7D4) in a final concentration of 1.25 µg/mL. The cells were incubated in GM-Buffer for 20 min at 4 °C and subsequently washed twice with GM-Buffer by centrifugation at 200 xg for 5 min at 4 °C to remove unbound antibody. Streptavidin-Phycoerythrin (SA-PE) (Dianova GmbH, Hamburg, Germany) was added at 2.5 µg/mL for 15 min at 4 °C. After two further washing steps with GM-Buffer by centrifugation at 200 xg for 5 min at 4 °C, the anti-PE micro beads (Miltenyii Biotec GmbH, Bergisch Gladbach, Germany) were added using 10 µL per $1x10^8$ cells in 1 mL GM-buffer. The beads were incubated with the cells for 10 min at 4 °C and 10 min on a shaker at room temperature, before being applied to the pre-equilibrated magnetized LS column according to the supplier's manual (Miltenyii Biotec GmbH, Bergisch Gladbach, Germany). The cells were applied in 5 mL GM-Buffer and the column was washed two times with 5 mL GM-Buffer. To receive higher purity, the eluted cells were again administered to another LS column and the procedure was repeated.

To reduce the contamination with other $CD25^+$ cells, Dynabeads against macrophages (M-450), B cells (B220) and $CD8^+$ T cells (Lyt-2) (Invitrogen Dynal AS, Oslo, Norway) were added and incubated on a shaker for 20 min in 1 mL PBS + 10 % FCS. Half the volume calculated for B220 Dynabeads was used for CD8 and M-450 Dynabeads.

$$Vol(B220) = \frac{total\ cell \times 3}{10 \times 2} \times 8 \div 4 \times 10^8 \times 1000$$

The micro beads were removed with a magnet and the cells were washed once with 5 mL PBS + 10 % FCS to increase the yield of $CD4^+$ $CD25^+$ T cells.
The isolated $CD4^+$ $CD25^+$ T cells were counted and ready to use for the experiments. The purity of Tregs was controlled by flow cytometry analysis of Foxp3 positive cells since Foxp3 expression is used as a marker for Tregs, and was above 98 %.

2.3.1.2.4 Isolation of $CD4^+$ $CD44^{low}CD62L^{high}$ T cells (naïve T cells)

Despite being able to remove activated $CD4^+$ T cells by depleting $CD25^+$ cells, immune responses in the mice produce other $CD4^+$ T cells like $CD4^+$ memory T cells. Memory T cells are characterized by a specific expression pattern of the surface molecules like CD44 and CD62L.
$CD4^+$ T cells were isolated according to 2.3.1.2.1 and 2.3.1.2.2. To remove memory T cells, $CD4^+$ cells were labeled with anti-CD62L (1:400), anti-CD44 (1:400) and anti-CD25 (1:200) antibodies in GM-buffer for 20 min at 4 °C. The cells were washed with buffer by centrifugation at 200 xg for 5 min at 4 °C to remove unbound antibody. The labeling enabled the identification of $CD25^-$ $CD62L^{high}$ $CD44^{low}$ T cells, which were sorted with the BD FACSAria™ II (BD Biosciences, New Jersey, USA) with a maximal flow rate of 2.4. Sorting parameters were set to Purity001 to minimize cross contamination by other cells. The exemplary selection chart is shown in Figure 3.

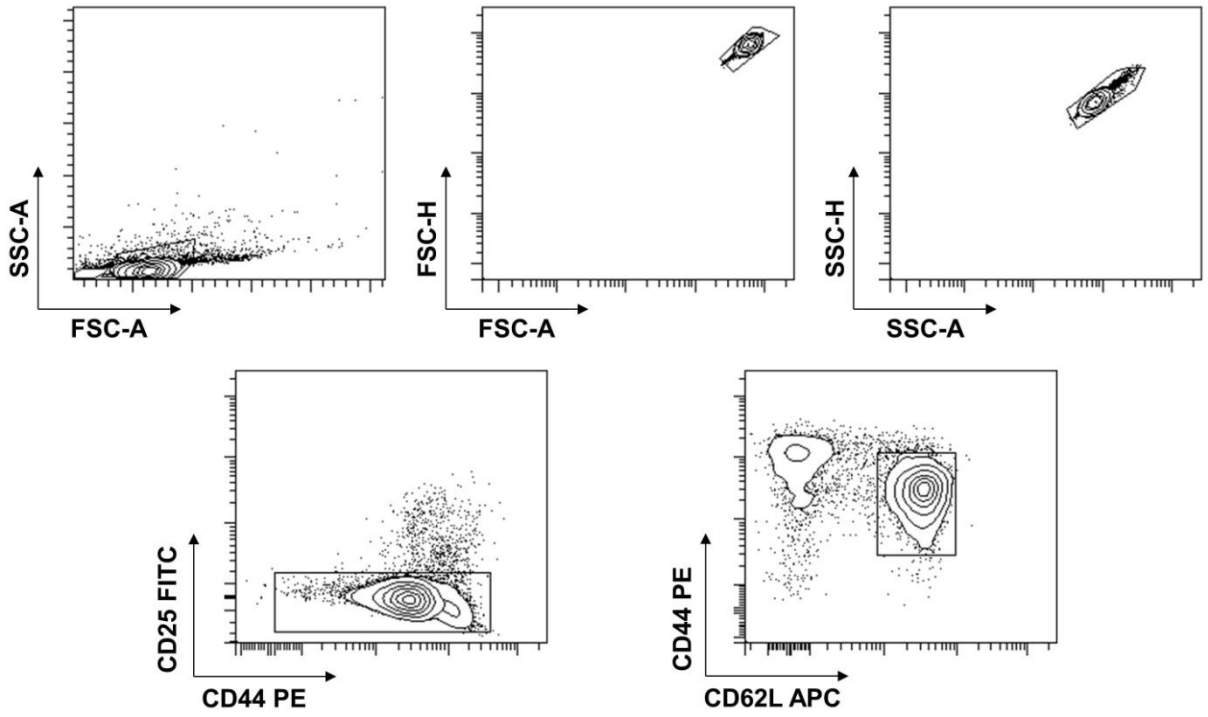

Figure 3 Sorting layout for naïve CD4$^+$ T cells

Cells were pre-gated on single cells *via* comparison of area versus height of FSC and SSC. The single cells appear on the bisecting line. Gates from FSC and SSC plots were intersected to remove any cell aggregations. Negative gaiting on CD25 led to depletion of nTregs and activated T cells. The naïve T cells compartment is defined by CD62L^{high} CD44^{low} cells.

2.3.1.2.5 Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) staining of T cells

The labeling is based on intracellular esterases, which remove the acetate group of CFDA-SE. The resulting succinimidyl ester is covalently joined to the amino group of proteins. This staining enables tracking cell division of labeled cells, due to the fact that the proteins, together with the covalently bound dye are passed to the daughter cells in equal amounts, dividing the fluorescence intensity for both daughter cells by two.

Isolated T cells were washed two times at 200 xg for 5 min at 4 °C with PBS to remove free proteins. The cells were adjusted to 1x10^7 cells/mL in PBS and diluted 1:2 in a fresh prepared CFDA-SE staining solution (5 µM CFDA-SE in PBS). After incubation for 4 min at 37 °C, the labeling reaction was terminated by the addition of excessive protein solution (TM + 10 % FCS). After washing two times at 200 xg for 5 min at 4 °C with TM + 10 % FCS the cells appeared yellow and were ready to use.

2.3.1.2.6 Coating of cell culture plates

To activate naïve CD4$^+$ T cells, two signals are indispensable: The T cell receptor and a costimulatory signal. To activate the T cells *in vitro*, mimicry of these signals is necessary.

The antibody against CD3ε (145-2C11), that mimics the TCR signal was bound to the cell culture plate for 30 min at 37 °C at 4 µg/mL in PBS. To remove unbound antibody the plates were washed two times with PBS. The anti-CD28 antibody (37.51) provided the costimulatory signal and was applied to the cell culture plate at 4 µg/mL in PBS for 30 min at 37 °C, followed by two washing steps with PBS. At all steps, drying of the cell culture plate was avoided. The crosslinking of CD3 and CD28 *via* the plate-bound antibodies provided the signals similar to those in immunological synapse, leading to activation of T cells.

2.3.1.2.7 Differentiation of T cells

The freshly isolated T cells (see 2.3.1.2.1) were spun onto the pre-coated cell culture dish (see 2.3.1.2.6) at 40 xg for 2 min. To differentiate T cells in one of the T helper subsets, the cells were cultured under specific conditions to ensure commitment to the distinct lineage. On day three, the cells were removed from the anti-CD3/anti-CD28 plate and diluted into T helper cell medium described in 2.1.2. The cells were cultured in TM + 5 % FCS and defined cytokines at 37 °C and 5 % CO_2. On day five, the cells were fully differentiated and ready to use for experiments.

2.3.1.2.8 Cultivation and preactivation of regulatory T cells

Isolated Tregs (see 2.3.1.2.3) were spun on anti-CD3/anti-CD28 coated cell culture dishes at 40 xg for 2 min (see 2.3.1.2.6) and cultured in Treg medium (see 2.1.2). The cells were fed on day one with 500 µL prewarmed Treg medium. On day two, the cells were removed from the coated plate and were used as preactivated Tregs in the experiments. Otherwise, the cells were removed from the coated plate on day three and then further cultivated till day five.

2.3.1.2.9 Intracellular cell staining

The Investigation of surface expression patterns of cells is easily possible. However, to determine its interior composition, the plasma membrane has to be passed. Therefore, the intracellular staining was done after fixation of the intracellular components *via* crosslinking. Following permeabilization, the interior was accessible for antibodies. The fixation and permeabilization were done with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, USA) according to the supplier's manual.

2.3.1.2.10 Restimulation of T cells for intracellular cytokine staining

The production of cytokines in the supernatant cannot directly be linked to a specific expression profile of a cell. These limitations are overcome by the intracellular cytokine

staining. To accumulate cytokines in the cell to be detectable in flow cytometry, the release of cytokines *via* the Trans-Golgi network was stopped with the antibiotic Monensin (Biolegend, San Diego, USA). During the incubation with Monensin, the cells were stimulated with a strong stimulus of 1 µM Ionomycin (Iono) and 20 ng/mL Phorbol 12-myristate 13-acetate (PMA). The cytokines accumulated inside the cells and were be stained with specific antibodies. The cells were cultured according to the manufacturer's manual for 4-5 h with the antibiotic, following normal surface (2.3.1.4), intracellular stainings (2.3.1.2.6) and analyzed *via* flow cytometry (2.3.1.4).

2.3.1.2.11 Transfection of murine T cells using electroporation

To investigate the role of different proteins during T cell development and commitment, it is inevitable to determine their role in *ex vivo* T cells. While most cell lines are more resistant to stress signals, T cells easily undergo apoptosis. T cells were cultured for two days on anti-CD3/anti-CD28 coated plates. This stimulus was removed 12-15 h before transfection to minimize the cell death of transfected cells.

The cells were then harvested, the medium removed, and stored for later cultivation. The T cells were washed once with pre-warmed PBS by centrifugation at 200 xg for 5 min at room temperature. The transfection *via* electroporation was performed according to the supplier's manual "Mouse T Cell Nucleofector® Kit" (Lonza Group Ltd, Basel, Switzerland). After resting 4 h in recovery media provided by the supplier at 37 °C and 5 % CO_2, the transfected T cells were cultured in their earlier medium and used for further experiments.

2.3.1.2.12 Suppression assay for regulatory T cells

To analyze the suppressive capacities of Tregs, a proliferation assay was used. Normal stimulation of Tregs co-cultured with responder $CD4^+$ $CD25^-$ T cells *via* anti-CD3/anti-CD28 antibodies would overcome Treg-mediated suppression of responder T cells due to the strong stimulation. To avoid these limitations and to test the functionality of Tregs, a different co-culture setting was established, using different read out markers like proliferation or cytokine production of responder T cells. [132,133]

Freshly isolated $CD4^+$ T cells labeled with CFDA-SE (see 2.3.1.2.1) were co-cultured with costimulatory A20 cells, providing CD28 stimulation. The primary stimulus for the T cell activation was provided by 3 µg/mL soluble anti-CD3 antibody (145-2C11). Tregs can be subsequently diluted to investigate their suppressive capacities. The number of divisions of the responder $CD4^+$ T cells can be followed by FACS analysis using CFDA-SE staining (see 2.3.1.2.1).

Beside the proliferation, Interleukin production of responder T cells can be used as a factor to determine the suppressive abilities of Tregs. Thus, the supernatant of the co-culture was analyzed for cytokine content on day three with cytokine specific ELISA.

In the experiment, T helper cells and Tregs were co-cultured under T helper skewing conditions in equal ratios to generate suppressed T helper cells. To compare normal T helper cell development, the $CD4^+$ T cells were cultured in the absence of Tregs.

The assay was also used to investigate the impact of Tregs on the suppression of fully committed T cells subsets. A similar setup was used, except that differentiated T helper cells on day 5 and Tregs on day 2 were used for restimulation.

Dendritic cells, as generated in 2.3.1.5, can also substitute A20 cells. Dendritic cells as professional antigen presenting cells provided the TCR signal *via* peptide loaded MHCII and the costimulatory signal by CD80/86. To activate dendritic cells 100 ng/mL LPS was added to the co-culture assay.

2.3.1.3 Cultivation of cell lines

2.3.1.3.1 Cultivation of B16F10 OVA melanoma cells

The B16 cells are a melanoma cell line which grows adherent under normal conditions.[180] The model antigen Ova and selection marker G418 was transfected. To generate a homogenous stable transfected cell line the antibiotic G418 (0.5 µg/mL) was added to the culture allowing only transfected cells to grow. The cells were cultured with TM + 5 % FCS and G418 0.5 mg/mL at 37 °C and 5 % CO_2. For experiments, single cells suspensions were used. Therefore cells were incubated in 0.25 % (w/v) Trypsin/EDTA (Gibco Life Technologies, Carlsbad, USA) for 3 min. After adding TM + 5 % FCS, the cells were washed by centrifugation at 200 xg for 5 min at 4 °C and diluted to desired cell concentrations.

2.3.1.3.2 Cultivation of the A20 cell line

The A20 cell line is a B cell lymphoma cell line that has costimulatory properties for T cell activation.[181] The cells were cultured in TM + 5 % FCS at 37 °C and 5 % CO_2 and diluted when necessary.

2.3.1.3.3 Treatment of A20 cells with MitomycinC

To use A20 cells as a costimulatory signal in co-culture assays, an inactivation of cell proliferation is necessary to prevent effects on the culturing system. A20 cells were treated with the cytostatic drug MitomycinC (Sigma-Aldrich, St. Louis, USA) to inhibit replication. MitomycinC is able to intercalate into the DNA, leading to a covalent binding of both DNA

strands and thereby inactivating cell division. A20 cells were incubated with 60 µg/mL MitomycinC in TM + 5 % FCS for 30 min at 37 °C. The cells were washed five times with MEM + 2 % FCS by centrifugation at 200 xg for 5 min at 4 °C to remove any remaining MitomycinC that would negatively influence T cell culture. In the co-culture experiments, one tenth of used T cells numbers were added from the A20 cells to provide the costimulatory signal.

2.3.1.4 Fluorescence activated cell sorting (FACS) analysis

Flow cytometry allows distinguishing between different cell types according to their protein expression and cell properties. At first, different cell types can be discriminated *via* their shape and intracellular composition. Forward scattered light from the laser enables to compare the cell size of the analyzed cells while the light scattered for 90° allows drawing conclusions on their intracellular structure and granularity. The expression of specific proteins can be assessed with specific labeled antibodies.

Cells were stained with specific antibodies for surface proteins in GM-Buffer for 20 min at 4 °C in the dark. To remove unbound antibodies, the cells were washed twice by centrifugation with GM-Buffer at 200 xg for 5 min at 4 °C. Then the cells were resuspended in GM-Buffer for FACS analysis. The FACS analysis was done with the BD FACS DIVA software (BD Biosciences, New Jersey, USA) and the BD LSRII (BD Biosciences, New Jersey, USA).

2.3.1.5 Generation of bone marrow derived dendritic cells (BMDCs)

BMDCs were generated according to a current protocol, described in detail below.[182]

BMDCs were differentiated from progenitors that are present in the bone marrow. The bone marrow was isolated from the lower leg and thighbone from 6 to 12 week old mice. The bones were prepared free from flesh and opened. The bone marrow was rinsed using a 0.55 x 25 mm syringe and PBS. To generate a single cell suspension, the cells were repeatedly aspired by the syringe. To remove residual red blood cells, the single bone marrow cell solution was incubated for 2 min with Gey's solution and the lysis was stopped by adding three parts of MEM + 2 % FCS. The cells were washed once with MEM + 2 % FCS using centrifugation at 200 xg for 5 min at 4 °C. Afterwards, the cells were set to $4x10^6$ cells/mL in DC medium (TM + 5 % FCS supplemented with 2 % GM-CSF). 1 mL was plated in 4 mL DC medium in adhesive cell culture plates. Although the cytokines in the DC medium promote dendritic cell formation, other cell types like macrophages can contaminate the culture. To remove contaminating cells, the plates were gently agitated on day two. As macrophages are not able to attach strongly to the cell culture plate, they detach

during shaking and can be removed together with the supernatant. The supernatant was discarded and new pre-warmed DC medium was added. On day four the medium was changed again. The cells were harvested on day 6 and were ready to use for the experiments. As BMDCs express both CD11c and MHCII on their cell surface the purity of the cells was checked by CD11c MHCII expression analysis *via* flow cytometry. The purity was around 85 to 90 percent.

2.3.2 Molecular biological methods

2.3.2.1 Messenger ribonucleic acid (mRNA) purification and reverse transcription

To investigate the expression profile of cells, their total mRNA was isolated. The cells were harvested, lysed in TRI Reagent (Ambion Life Technologies, Carlsbad, USA) (1 mL per $1x10^6$ cells), and frozen immediately at -20 °C. After defrosting, the samples were incubated at room temperature for 10 min before 200 µL chloroform/isoamyl alcohol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added. Through extensive mixing, the sample a higher yield of RNA can be reached. Subsequently, the samples were incubated at room temperature for 10 min and then centrifuged for 15 min at 12500 xg and 4 °C. The mRNA dissolved in the aqueous phase was collected and equal amounts of 2-propanol was added. The mRNA precipitated after the addition of 1.5 µL glycogen (20 µg/mL) and was pelletized during centrifugation for 15 min at 12500 xg and 4 °C. The pellet was washed twice with 75 % ethanol in DEPC water with centrifugation for 10 min at 12500 xg and 4 °C. Ethanol was completely removed by vaporization. The full removal of ethanol is necessary to ensure that further reactions were not compromised. The pellet was resolved in DEPC water and RNA concentration was measured by NanoDrop.

For the reverse transcription reaction, 500 ng mRNA was used. The reaction was set up according to the supplier's manual (Thermo Fisher Scientific, Waltham, USA). N_6-hexamers and polydT primers were used for the transcription at 42 °C for 1 h. N_6-hexamer primers initiate the reverse transcription at random initiation points of the transcript according to the random binding site of the hexanucleotides. In contrast polydT primers bind to polyA tails of eukaryotic mRNA and initiate the reaction from this point. This defined starting point may not be able to cover the whole mRNA. To benefit from both techniques, both primers were used to generate a cDNA, covering of the whole mRNA transcriptom.

2.3.2.2 Quantitative real-time polymerase chain reaction (qRT-PCR)

To analyze the expression pattern in target cells, the qRT-PCR is able to specifically monitor expression levels of genes of interest. After generation of the cDNA from mRNA, described

in 2.3.2.1, the qRT-PCR enables to compare the amount of cDNA between different samples and thereby determine the expression of mRNA.

The measurement of DNA content is possible due to a fluorochrome, which is only able to emit light when intercalated in double stranded DNA. After each DNA duplication, the light emission was measured. The first significant signal emerges, called the cycle threshold (cT) value, when the emitted light is higher than the background emission. Due to exponential amplification in the first phase of the PCR, the cT value gives information about the amount of expressed mRNA in the sample. Since it is equally expressed in all cell types under the conditions used, to correct for total amount of cDNA the house keeping gene *hypoxanthine-guanine phosphoribosyltransferase* (HGPRT) was measured. The expression of target genes were compared to corresponding HGPRT values, correcting for different amounts of cDNA. The used length of amplification is around 75-200 base pairs (bp). This reduces the false data due to poor amplification efficiencies of long amplicons.

The samples were prepared in triplicates according to the supplier's manual of "SYBR Green for iCycler" (Invitrogen Life technologies, Carlsbad, USA). The PCR cycles were according to the supplier's manual with annealing temperature of 57 °C. Melting curves were generated at the end of the run. For the detection of light emission, the MyIQ iCycler (Bio-Rad, Hercules, USA) was used. The data analysis was performed with Bio-Rad iQ5 Standard Version 2.0 (Bio-Rad, Hercules, USA). Samples with no melting curve were removed from the analysis and cT values in triplicates were not allowed to be more than one cycle apart.

2.3.2.3 Enzyme-linked immunosorbent assay (ELISA)

A technique to measure the concentration of cytokines in the supernatant of cells is the ELISA. To capture soluble proteins a specific antibody is bound to the plate *via* adsorption. After incubation of the plate with capture antibody in coating buffer for 1 h at 37 °C, the plate was washed three times with washing buffer and incubated for 30 min at 37 °C with blocking buffer to block any remaining binding sites. The cell supernatant was diluted 1:1 in the first row and further titrated at the same ratio for each row following. After capturing the cytokine of interest by the antibody during incubation for 1 h at 37 °C the supernatant was removed and followed by three washing steps with washing buffer. The secondary biotinylated detection antibody, that binds to a different epitope than the capture antibody was applied for 1 h at 37 °C. The detection antibody was marked with biotin and linked to streptavidin coupled horseradish peroxidase *via* SA-HPO (Roche, Indianapolis, USA) for 30 min at 37 °C. The measurement of bound cytokine was achieved by the oxidization of a substrate that changes its spectrometric properties. This oxidization was catalyzed by the peroxidase using 0.03 % H_2O_2 as oxygen supplier. The table below shows concentrations for the used antibodies and detection substrates. Total amounts of cytokines were determined by using a known standard for each plate.

Table 8 Antibodies used for ELISA and detection substrate

Cytokine of interest	Capture Antibody Clone (concentration)	Detection Antibody Clone (concentration)	Detection substrate
IL-9	229.4 (3 µg/mL)	C12 (1 µg/mL)	ABTS
IL-4	BVD4-1D11 (1 µg/mL)	BVD6-24G2 (0.6 µg/mL)	ABTS
IL-10	JES5-2A5 (4 µg/mL)	JES5-16E3 (2 µg/mL)	TMB

2.3.2.4 Western-Blot analysis

The changes of protein expression during T cell activation and suppression by Tregs were followed by Western-blot analysis.

Isolated T cells were washed once with PBS to remove soluble proteins by centrifugation for 5 min with 200 xg at 4 °C and then lysed in 7 M urea buffer ($1x10^6$ cells per 50 µL). To completely disintegrate cellular organization, the cells were incubated for 5 min in an ultrasonic bath. The cellular DNA was removed by centrifugation of the cell lysate at 12500 xg for 15 min at 4 °C. The proteins were collected in the supernatant while large debris and cellular DNA pelletized. To dissociate secondary protein structures, the protein suspension was heated for 3 min at 95 °C with 4x loading buffer containing sodium dodecyl sulfate (SDS). The samples were loaded on the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and separated at 120 V in SDS-Buffer according to electrophoretic mobility.

To transfer the separated proteins to the polyvinylidene difluoride-membrane (PVDF-membrane) Immobilon (Millipore, Billerica, USA) the gel was stacked in a semi-dry blotter and 50 mA were applied for 4 h (setting described in Figure 4). The PVDF membrane was pre-incubated with methanol to ensure protein attachment, while the chromatography papers as liquid reservoir were soaked with blotting buffer.

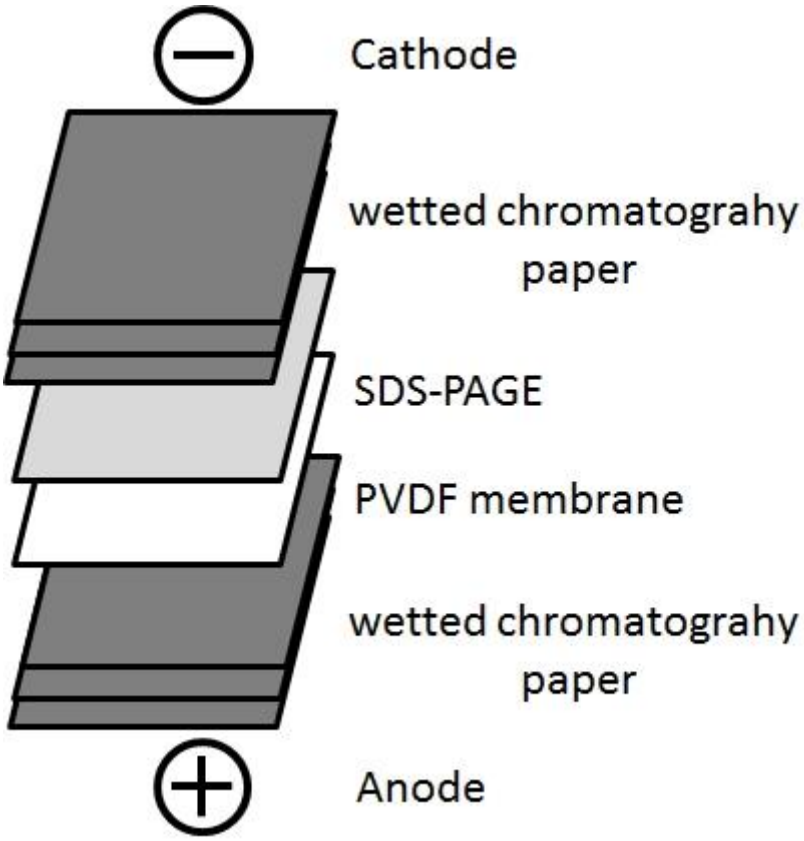

Figure 4 Schematic Western-Blot setup

Efficient transfer was controlled by protein ladder transmission to the PVDF membrane. Subsequently, the membrane was blocked with 5 % milk powder in TBST for 1 h at room temperature. After washing the membrane three times with TBST for 5 min, the primary antibody was incubated over night at 4 °C in TBST + 0.5 % BSA. To remove unbound primary antibody, the membrane was washed three times with TBST. The secondary antibody coupled to HRP was incubated for 1 h at room temperature in TBST with 5 % milk powder.

Residual tween that might interfere with HRP function, was removed by excessive washing for three times with TBS. Detection of light emitted by coupled HRP was performed using ChemiDoc XRS (Bio-Rad, Hercules, USA), Quantity One 1-D Analysis Software (Bio-Rad, Hercules, USA) and luminescence solution SuperSignal West Femto Maximum sensitivity substrate (Thermo Fisher Scientific, Waltham, USA).

To control protein loading, the blot was incubated with an anti-Actin antibody coupled to HRP for 1 h in TBST + 5 % milk powder and subsequently washed three times with TBS. The signal was detected using the same solutions and specifications as before.

Table 9 Antibodies used for Western-Blot analysis

Antibody	Dilution	Supplier
anti-IRF4 (M-17)	1:1000	Santa Cruz biotechnology, inc; Dallas, USA
donkey anti-goat IgG-HRP	1:5000	Santa Cruz biotechnology, inc; Dallas, USA
anti-Actin HRP	1:40000	Sigma-Aldrich, St. Louis, USA

2.3.2.5 Luciferase activity assay

To determine the influence of transcription factors on protein expression, the luciferase activity assay is commonly used. The promoter region of a gene, was cloned in front of a luciferase coding sequence. Due to this construct, the transcription factors that influence the promoter now can either directly enhance or attenuate luciferase transcription. As an internal control of transfection efficiency and cell viability a different luciferase construct was co-transfected into the target cells. The T cells were transfected as described in 2.3.1.2.11 with 2.5 µg of each DNA and 300 ng of *Renilla* luciferase (*Renilla reniformis*), as control luciferase per transfection. After transfecting of the constructs containing the transcription factors in pcDNA3.1+ vector (Invitrogen Life technologies, Carlsbad, USA) and the reporter constructs, the T cells were rested for 4 h before being restimulated with plate bound anti-CD3/anti-CD28 for another 4 h in their prior harvested medium.

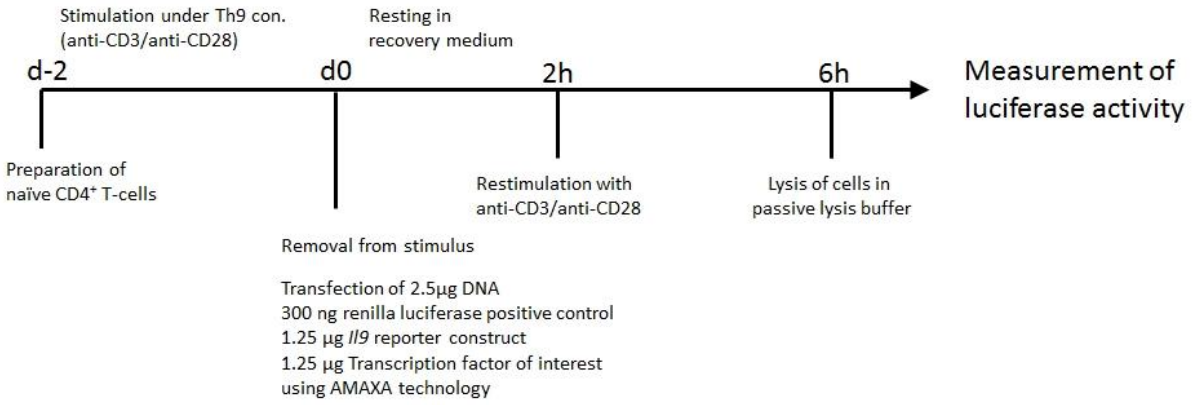

Figure 5 Timeline of a reporter gene assay

Naïve $CD4^+$ T cells are stimulated under Th9 skewing conditions with anti-CD3/anti-CD28 for 2 d. The T cells were transfected with DNA and subsequently rested for 2 h. The T cells were lysed with passive lysis buffer and measured with Dual-Luciferase® Reporter Assay.

For measurement of luciferase activity, the cells were harvested, washed with PBS by centrifugation at 200 xg for 5 min and 4 °C and lysed in lysis buffer (Promega, Fitchburg, USA). The luciferase activity was then measured according to the supplier's manual of the Dual-Luciferase® Reporter Assay System (Promega, Fitchburg, USA) with TD-20/20

Luminometer (Turner Design, Sunnyvale, USA). The luminescence signal form the *Photinus* luciferase (*Photinus pyralis*) is controlled through the promoter region of interest and influenced by the co-transfected transcription factors. The first signal was then quenched and the internal control signal from the *Renilla* luciferase (*Renilla reniformis*) was measured. The specific signal was reassessed afterwards with help of the internal control, to directly quantify the influence of the transcription factors on the promoter region.

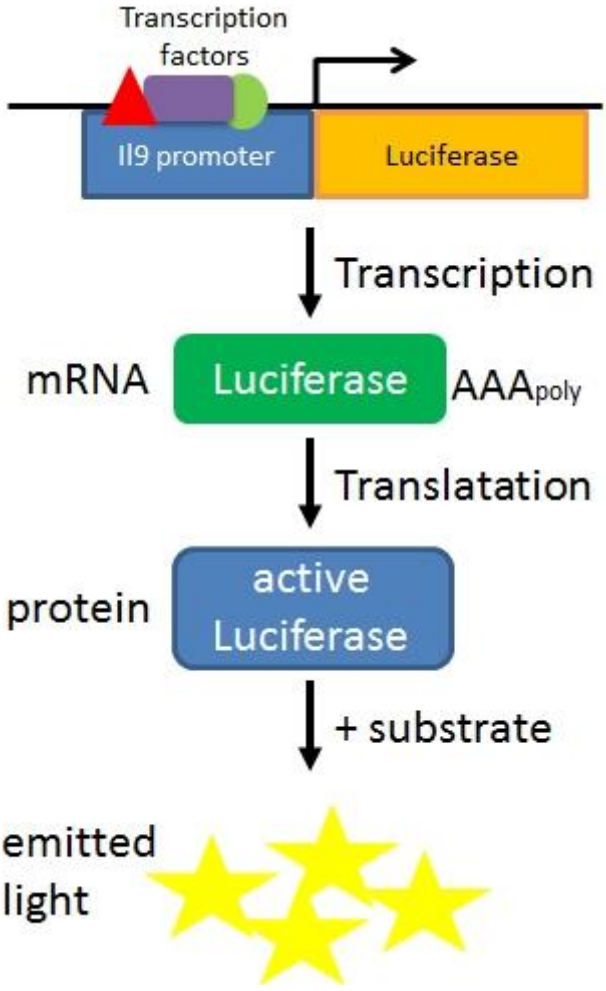

Figure 6 Schematic workflow of a reporter gene assay
Luciferase transcription is regulated by transcription factors binding to the promoter region. Measurement of emitted light allowed a conclusion on negative or positive influences of transcription factors. Figure changed after.[183]

2.3.2.6 Next generation sequencing (NGS)

To investigate the mRNA expression pattern of a cell, NGS can be used. This method is well fitted to get a broad overview of expressed genes in target cells. The mRNA transcriptome allows drawing conclusions on the status and expression pattern of the cells. The flexibility of mRNA transcription can thereby be investigated. Neither the post-transcriptional regulation of protein expression is detectable with NGS, nor are posttranslational modifications.

Despite these restrictions, NGS remains a powerful tool to investigate cell differentiation and expression patterns. The advantage is that *via* NGS the whole transcriptome can be sequenced, liberating from the need to pre-define the target genes as necessary in qRT-PCR or northern-blot analysis. Also, western-blot analysis requires existence of suitable antibodies and pre-defined targets. NGS is comparable to mass spectrometry analysis, although NGS is more sensitive to different genes, while only mass spectrometry can identify post-transcriptional and -translational modifications.

2.3.2.6.1 TruSeq RNA sample preparation

The mRNA analyzed *via* next generation sequencing was prepared as described in 2.3.2.1. To ensure that the quality of the mRNA sample is sufficient for library preparation, the mRNA was solved in distilled water (Gibco Life Technologies, Carlsbad, USA). The quality of the RNA sample was determined with RNA Nano Chip (Agilent Technologies, Santa Clara, USA) using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The quality of the RNA samples was therefore determined by the RNA integrity number (RIN). The RIN value of mRNA used for NGS sample preparation was above 8.5.

The synthesis of the NGS samples were performed according to the supplier's manual, TruSeq RNA Sample Prep v2 LS Protocol (Illumina, San Diego, USA).

In short, total RNA was purified and fragmented to a fragment size of around 300 bp. Then the first strand of the cDNA was synthesized before the second strand was subsequently synthesized. To avoid ligation of cDNA during the adaptor ligation, the 3' terminus was adenylated with a single Adenine. This adenylation enabled the ligation of adapters that identified the samples according to their adapter sequence and permitted the identification of the different cDNA when measured parallel. Finally, the cDNA amplified by PCR was checked for quality.

The validation of the cDNA library was done using High Sensitivity DNA Chip (Agilent Technologies, Santa Clara, USA) in combination with the Bioanalyzer 2100. The average size of the prepared library DNA was below 500 bp.

2.3.2.6.2 Concentration determination of NGS library

To measure DNA concentration, the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, USA) was used. Further accuracy of concentration was done with the KAPA Library Quant Illumina Kit (KAPA Biosystems, Woburn, USA). The prepared cDNA library was diluted to the required concentration and applied to the sequencing Kit according to the supplier's manual.

2.3.2.6.3 NGS run performance and data analysis

The sequencing run was performed on MiSeq (Illumina, San Diego, USA) using the MiSeqv2 Reagent Tray (Illumina, San Diego, USA). The data analysis was performed with Illumina Sequencing Analysis Viewer (Illumina, San Diego, USA) and Microsoft Excel 2010 (Microsoft Cooperation, Redmond, USA).

2.3.3 *In vivo* mouse models

2.3.3.1 Reconstitution of mice with T cells

T cells were injected *intra venous* to reconstitute Rag deficient mice. The direct injection of T cells in the blood stream facilitates reconstitution of the whole body and immunological organs with the adoptive transferred cells.

The T cells were washed once with PBS and adjusted to the desired cell number in PBS before being injected with a 27-gauge needle in a total volume of 200 µL into the tail vein. To widen the tail vein and facilitate injection, the mice were heated up under an infrared lamp and fixed in an injection chamber.

2.3.3.2 *In vivo* asthma model

In vivo models are useful to analyze complex questions. *In vitro* assays described in this thesis are focused on the suppression of Th9 cells by Tregs. The *in vivo* experiments are associated to all T cell subsets. Because Th2 cells are closely linked to Th9 cells, a Th2 *in vivo* model is of interest. A well-known Th2 model is the acute asthma model and can also be applied for Th9 cells.

Under normal circumstances acute asthma is induced by antigens from various sources like pollen or house dust mite. Antigen specific T cells are essential for the initiation of the asthmatic disease. In order to generate a model with a defined antigen that induces the asthmatic response, T cells from DO11.10 mice were used. This strain possesses a transgenic T cell receptor present on all T cells, recognizing the amino acids 323-339 of the chicken Ova. To avoid bystander reaction from host T or B cells, Rag2 deficient mice of the same background are reconstituted with T cells. The enzyme Rag2 is essential for the rearrangement of the variable chain of the B and T cell receptor. Deficiency of Rag2 enzyme leads to an arrested lymphocyte development and absence in the periphery. The reconstitution of Rag2 deficient mice with differentiated T cells of DO11.10 origin allowed the study of T helper cell suppression by Tregs. The acute asthma can be induced by the Ova avoiding unspecific disease induction. The experimental setup is described in Figure 7.

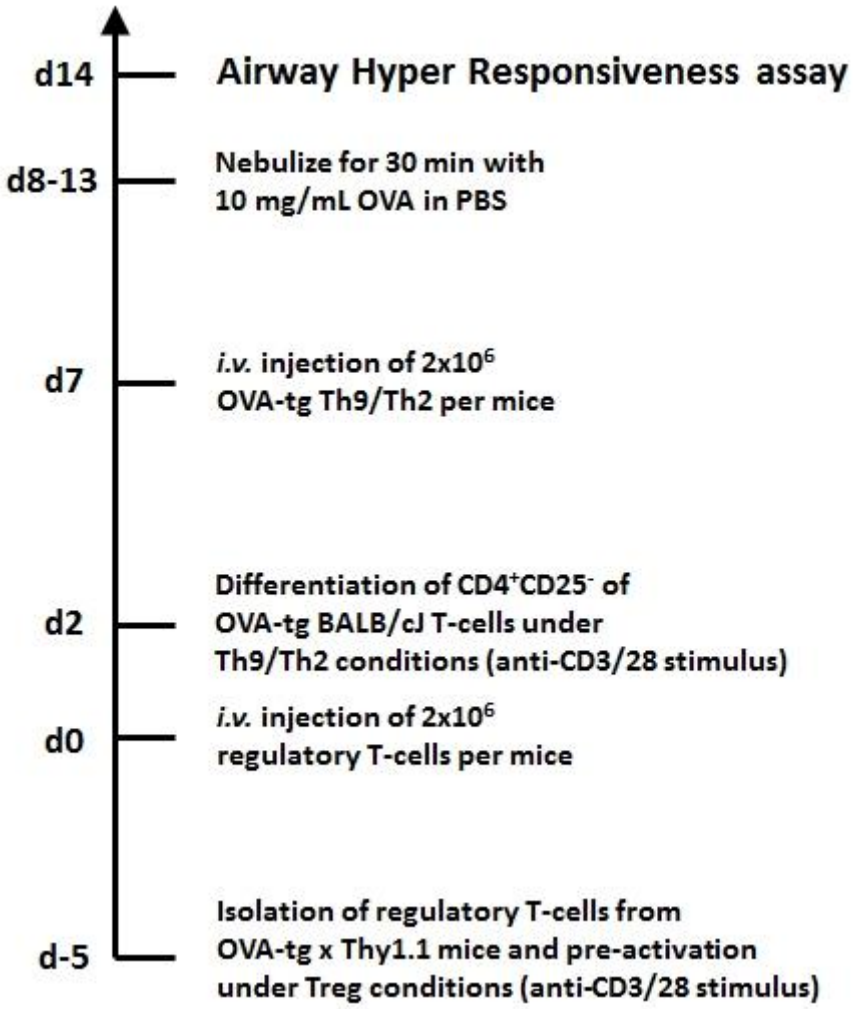

Figure 7 Flow chart of asthma experiments

Flow chart indicating the time points of isolation and injection of T cells during an acute asthma experiment.

2.3.3.2.1 Treatment of mice with nebulized Ovalbumin (Ova)

To initiate the asthmatic disease and induce inflammation of the lung tissue after reconstitution of the mice with helper and regulatory T cells, mice were challenged with 10 mg/mL Ova grade V in PBS. The mice were treated for 20 min in a chamber allowing consequent exposure to the nebulized Ova using a nebulizer and nebulizer chamber (NE-U17, Omron, Hoofdorp, The Netherlands). The airflow and nebulizing volume was set to stage 4. The provocation *via* the lung induces the asthmatic disease by recruitment of Ova specific T cells to the lung tissue. Their effector function and release of cytokines induces the typical asthmatic symptoms like airway remodeling, high mucus production, and immune cell infiltration.

2.3.3.2.2 Measurement of airway hypersensitivity

The model of allergic asthma in mice does reflect the human disease in different ways. Airway Hypersensitivity Reaction (AHR), mucus production, cell infiltration and airway remodeling are main characteristics of the human disease as well as of the murine model.

Lung infiltrating cells were identified by analysis of the bronchi alveo fluid as described in 2.3.3.2.3.

The measurement of airway remodeling and AHR was done by invasive measurement methods. The mice were narcotized and intra-tracheally intubated, to enable the measurement of lung function and airway resistance. The measurement of the compliance allows for drawing conclusions on the lung remodeling. The experiments were performed in close collaboration with the group of AG Buhl/Reuter of the 3rd Medical Department of the University Medical Center Mainz. For closer description of the experiments and measurement parameters, see the dissertation of Nina Dezhad " Suppression des allergischen Asthmas durch regulatorische T-Zellen - Mechanismen und potenzielle therapeutische Ansätze " where the technical equipment and settings were used.[184]

2.3.3.2.3 Bronchoalveolar lavage (BAL)

The infiltration of inflammatory cells into the lung of asthmatic patients is one of the key features of the disease. The mouse model of acute asthma reflects this effect in challenged mice. While under steady state conditions mainly macrophages are present in the lung, the inflammation of the lung does lead to an increase of inflammatory cells like neutrophils, lymphocytes, and eosinophils. The model used in this setting does induce an antigen specific T cell response followed by invasion of different types of innate immune cells. To investigate the composition of infiltrating cell types, the lavage was performed.

The lethal anesthetized mice were intubated in the trachea. 1 mL of PBS was slowly applied *via* a syringe in the lung. The liquid was carefully regained to around 70 to 80 %. Cells were centrifuged at 4 °C and 150 xg for 10 min and the supernatant was frozen until the cytokine milieu was determined *via* ELISA analysis. The cells were set to 200 µL and applied to cytospin analysis (2.3.3.2.4).

2.3.3.2.4 Preparation of cytospin

To identify the different cell types present in inflamed lungs of asthmatic mice, cytospin evaluation was used.

Cells harvested using BAL procedures were spun via a cytofuge on a microscopic slide.

100 µL of cells were placed in a funnel above the slide and then spun at 100 xg at RT for 10 min. The slide was dried at room temperature overnight.

2.3.3.2.5 Hematoxylin-eosin (H&E) staining of cytospins

To differentiate various cell types in the BAL fluid, the H&E staining was used. According to their physical properties, the cells can be distinguished in the light microscope. The Hematoxylin is used in the staining solution as haemalum. Its properties enable it to bind to acidic structures like the nucleus. To distinguish the surrounding cytoplasm, basic structures like proteins are stained with the acidic dye eosin. A combination of those two dyes allows an easy differentiation of various cell types in the BAL fluid due to their morphological properties.

The staining procedure is performed with the Microscopy Hemacolor® (IVD)-Kit (Merck, Darmstadt, Germany). The cells were fixed on the slide *via* a fixation solution. Then the cells were stained with the eosin solution and after that stained with the Hematoxylin. Remaining dye was removed by gently washing with buffer solution to Weise.

Macrophages, eosinophils, neutrophils, and lymphocyte were counted for at least 200 cells per slide. Percentages of total cells were calculated later.

2.3.3.3 Growth tumor model of B16 melanoma

Another possibility to follow the function of Th9 cells *in vivo* was recently proposed by the group of Kupper.[103] They used a model of the melanoma cell line B16F10, showing in convincing fashion, that Th9 cells are able to reduce tumor growth in an IL-9 dependent manner. The tumor killing effect is not directly driven by IL-9 or the T cells. Moreover, the recruitment of mast cells to the tumor site by tumor specific T cells is proposed to be the underlying mechanism.

In order to test the regulation of Th9 development and effector function, B16 tumor expressing the complete Ova as a model antigen was used. The transfer of OT-II T cells, expressing a transgenic T cell receptor for the Ova peptide 323-339 allowed mimicking an antigen specific T cell tumor response. To avoid bystander T cell activation from endogenous T cells, Rag1 deficient animals of the same background were used. These are similar to the asthma experiments that were described in 2.3.3.2.

2.3.3.3.1 Primary tumor transplantation of B16 melanoma

The single cell suspension of B16F10Ova cells was prepared according to 2.3.1.2.11. The cells were prepared to $2x10^6$ cells/mL in PBS. The mice were anesthetized with 200 µL Ketamin/Rompun (Ratiopharm, Ulm, Germany/Bayer Healthcare, Leverkusen, Germany) *intra peritoneal* (i.p.) per mice before the flank was shaved and $2x10^5$ tumor cells were injected *sub cutaneous* (s.c.).

2.3.3.3.2 Measurement of tumor growth

To follow the growth of the tumor, the mice were controlled three times a week. The area of tumor growth was determined by measuring two orthogonal axes. For time of measurement, mice were anesthetized with Forene (Abott, Abott Park, USA).

2.3.3.3.3 Antigen specific restimulation of responder T cells

The *ex vivo* stimulation of responder T cells is useful to define their cytokine profile and identify the underlying mechanisms of tumor repression. Mice were sacrificed at the end of the experiment on day 19 and the inguinal draining lymph node was dissected. A single cell suspension was prepared and washed twice with PBS. The cells were counted and set to $1x10^6$ cells/mL before being stimulated with 2.5 µg/mL Ovalbumin peptide 323-339, and to increase costimulatory signals of present dendritic cells, also with 100 ng/mL LPS.

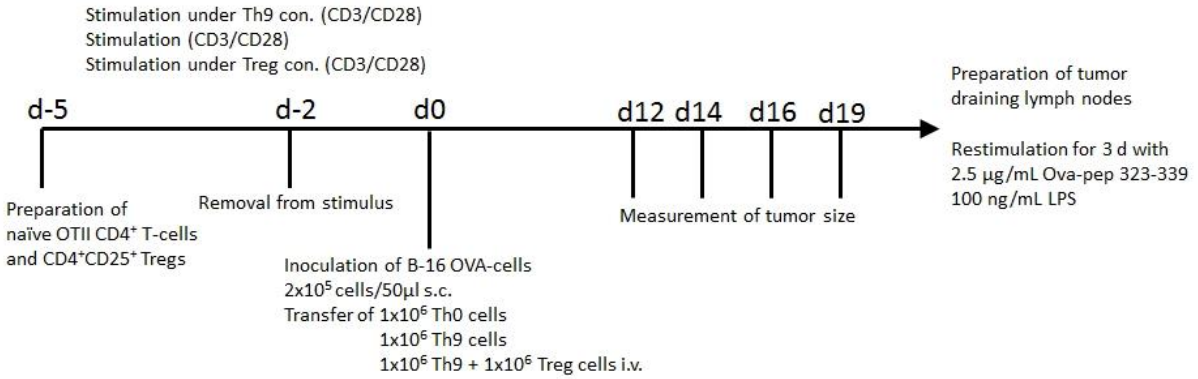

Figure 8 Timeline for B16 melanoma model

Naïve $CD4^+$ T cells and Tregs form OT-II mice were stimulated under described conditions and on day five injected i.v. into Rag1 deficient mice. At the same time, the B16 tumor expressing the Ova was inoculated s.c. in the flank. After appearance of the tumor, the tumor size was measured.

2.3.4 Statistical analysis

The statistical analysis was performed using different software. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, USA) was used for 1way ANOVA and 2way ANOVA, both times Bonferroni posttest were performed. TTests were performed using Microsoft Excel 2010 (Microsoft Cooperation, Redmond, USA).

3 Results

Tregs play a pivotal role by dampening ongoing immune responses, thereby circumventing overwhelming immune reactions like autoimmunity and allergy. It has been demonstrated that one mechanism of Treg-mediated immune suppression is to prevent the differentiation from naïve T cells towards Th2, but also towards the Th1 phenotype.[185] Since Th9 cells are able to develop from Th2 origin under the influence of TGF-β, it is tempting to speculate that Tregs may utilize comparable suppressive mechanisms.[57]

The fact that Tregs are able to express the IL-9 receptor after activation may influence their ability to inhibit the formation of an IL-9 producing T helper subset from naïve T cells.[70] To clarify if Tregs are able to inhibit IL-9 mediated immune responses, the suppression of *in vitro* differentiation of Th9 cells from naïve T cells precursors was investigated.

3.1 Inhibition of Th9 cell development by regulatory T cells

To study the effect of Tregs on Th9 development, an *in vitro* suppression assay was performed as described in 2.3.1.2.12. Naïve T cells, so called responder T cells, were stimulated with anti-CD3 (αCD3) crosslinking antibody for the primary signal while costimulation was provided by A20 cells. To ensure the development of responder T cells into the Th9 lineage the isolated T cells were cultured with activating stimuli for three days in the presence of Th9 polarizing cytokines IL-4 and TGF-β. In response to the activating stimuli the $CD4^+$ T cells commit to the Th9 lineage, characterized by IL-9 production and the expression of the transcription factors IRF4 and PU.1. Through the same activating stimulus, the present Tregs also get activated and may suppress the surrounding responder cells.

The suppressive capacity of Tregs was determined by using different ratios of responder T cells to Tregs. By comparison of IL-9 production of normal T cells with T cell co-cultured with Tregs, the influence of Treg suppression of Th9 development was analyzed. The amount of IL-9 in the supernatant was measured on day three by specific ELISA.

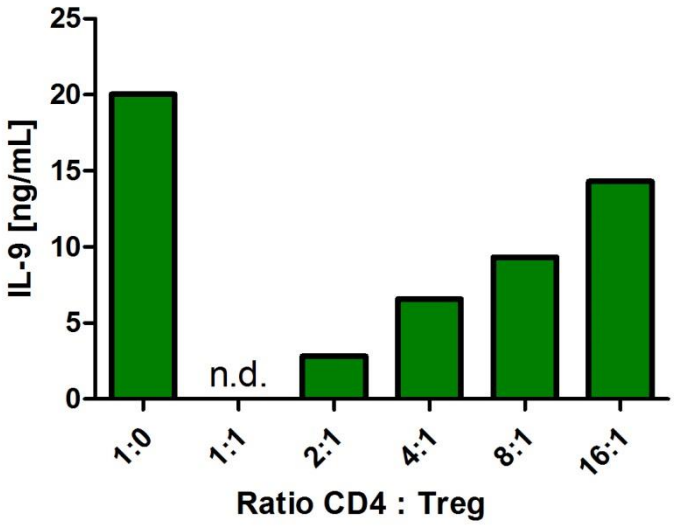

Figure 9 Inhibition of Th9 cell-derived IL-9 by Tregs

Tregs are able to inhibit the IL-9 production of developing Th9 cells in a ratio dependent manner. $CD4^+$ $CD25^-$ T cells were stimulated with MitomycinC treated A20 cells (2.3.1.3.3) and 3 µg/mL anti-CD3 under Th9 skewing conditions as described in 2.3.1.2.12. $CD4^+$ $CD25^+$ nTregs were co-cultured in a cell ratio dependent manner. The suppressive capacity of Tregs was assessed by production of IL-9 on day three *via* specific ELISA. One of three representative experiments is shown.

The production of IL-9 from Th9 cells is suppressed by Tregs in a cell ratio dependent manner. At equal cell ratios, Treg are able to completely abrogate IL-9 production, while at lower ratio (8:1 or 16:1) Tregs are able to inhibit the IL-9 production of T helper cells to around 50 % to 25 % (Figure 9). This shows that Tregs are able to suppress responder T cells and are not able to produce detectable amounts of IL-9 by themselves. This finding could be confirmed by mRNA analysis (see chapter 3.1.2).

Since Th9 cells are not only reported to produce IL-9, but are also capable of producing IL-10, the supernatants of naïve $CD4^+$ T cells in the absence or presence of Tregs at 1:1 ratio were tested for IL-10 production on day three after activation under Th9 skewing conditions (Figure 10).[57]

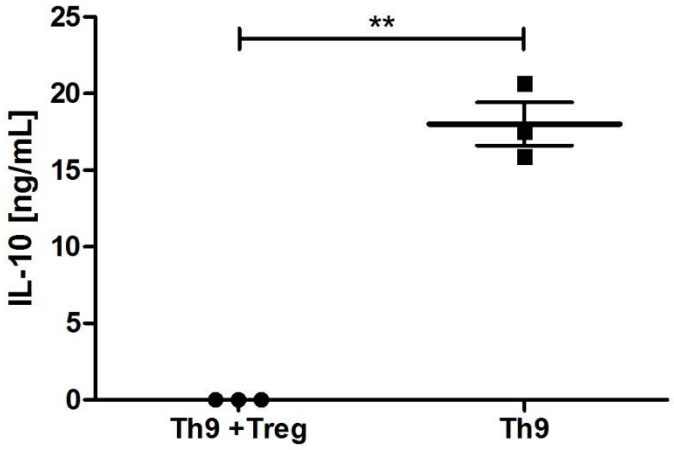

Figure 10 IL-10 production of Th9 cells in presence or absence of regulatory T cells
Tregs are able to inhibit the IL-10 production of developing Th9 cells in equal ratios. $CD4^+$ $CD25^-$ T cells, with or without Tregs in 1:1 ratio, were stimulated with MitomycinC treated A20 cells (2.3.1.3.3) and 3 µg/mL anti-CD3 under Th9 skewing conditions as described in 2.3.1.2.12. IL-10 production was assessed on day three by a specific ELISA. Mean and standard deviation, analyzed by TTest p=0.0030.

Tregs can completely inhibit the production of IL-10 by Th9 cells in the supernatant (Figure 10). Although Tregs are reported to release IL-10 after activation, Treg-derived IL-10 is not detected under co-culture conditions. This might be due to the fact that IL-10 binds to its receptor and is consequently consumed.

As described in chapter 1.3.3, Tregs are able to inhibit a T cell response at different developmental stages under certain conditions. One stage is the suppression of proliferation of responder T cells. The consumption of the growth cytokine IL-2 by Tregs or the active inhibition by released cytokines like IL-10 can prevent T cell proliferation by inhibition of APC activation.

To clearly show that Tregs are capable of directly downregulating the production of IL-9 and not only reduce proliferation rate of responder T cells, a proliferation assay was performed (2.3.1.2.12). The division rate of responder T cells was analyzed *via* CFSE dilution assay (Figure 11).

Naïve $CD4^+$ T cells were labeled with CFDA-SE and then used in a co-culture experiment with or without Tregs in an equal cell ratio. The proliferation of responder T cells is depicted by CFSE dilution on day three (Figure 11).

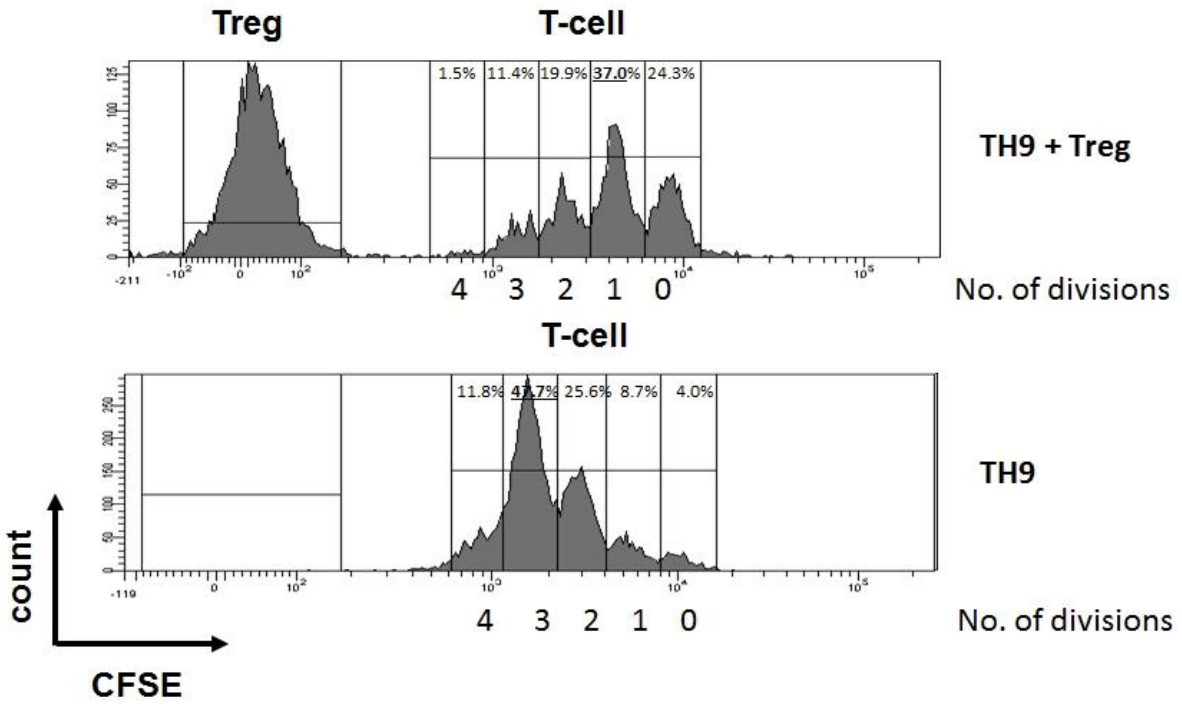

Figure 11 Suppression of T cell proliferation by regulatory T cells

Tregs reduce the proliferation of Th9 cells to some extent in co-culture experiments. $CD4^+$ $CD25^-$ responder T cells were isolated and labeled with CFDA-SE prior to stimulation to track cell proliferation (2.3.1.2.1). T cells were co-cultured with or without Tregs in equal ratio and stimulated with MitomycinC treated A20 cells (2.3.1.3.3) and 3 µg/mL anti-CD3 under Th9 skewing conditions as described in 2.3.1.2.12. CFSE staining was measured on day three by FACS analysis.

Suppressed responder T cells are still able to proliferate to some extent (Figure 11) when co-cultured with equal numbers of Tregs, despite not being able to produce IL-9 on day three (Figure 9). However, the suppressed Th9 cells do not achieve the proliferation rate of activated T cells cultured alone. While 75 % of normal developing Th9 cells divide two or three times, around 60 % of Th9 cells in the co-culture with Tregs only divide once or twice, while 25 % do not divide (Figure 11). The reminiscent proliferation of responder T cells from co-culture might be due to exogenous IL-4. Tregs appear as $CFSE^{negative}$ in the analysis.

3.1.1 Analysis of suppressive mechanisms

The experiments above clearly showed that Tregs are able to inhibit Th9 commitment in a cell ratio dependent manner (Figure 9). Today there are several mechanisms described in the literature about how Tregs inhibit responder T cells. Yet, the question of whether there is a master suppression/regulation pathway exerted by Tregs to suppress T helper cell commitment remains elusive. The known suppressive pathways can be divided into three major groups: soluble factors, surface molecules, and direct cell-to-cell contact.

To determine the contribution of CTLA-4 and IL-10 to Treg-mediated suppression, the specific blocking antibodies were added to the suppression assays. For blocking the CTLA-4 mediated signal, an anti-CTLA-4 antibody (anti-CTLA-4) was added and the inhibitory signal

of IL-10 was blocked by using an anti-IL-10 receptor antibody (anti-IL-10R). Suppressive capacity of Tregs was assessed on day three by measuring IL-9 in the supernatant (Figure 12).

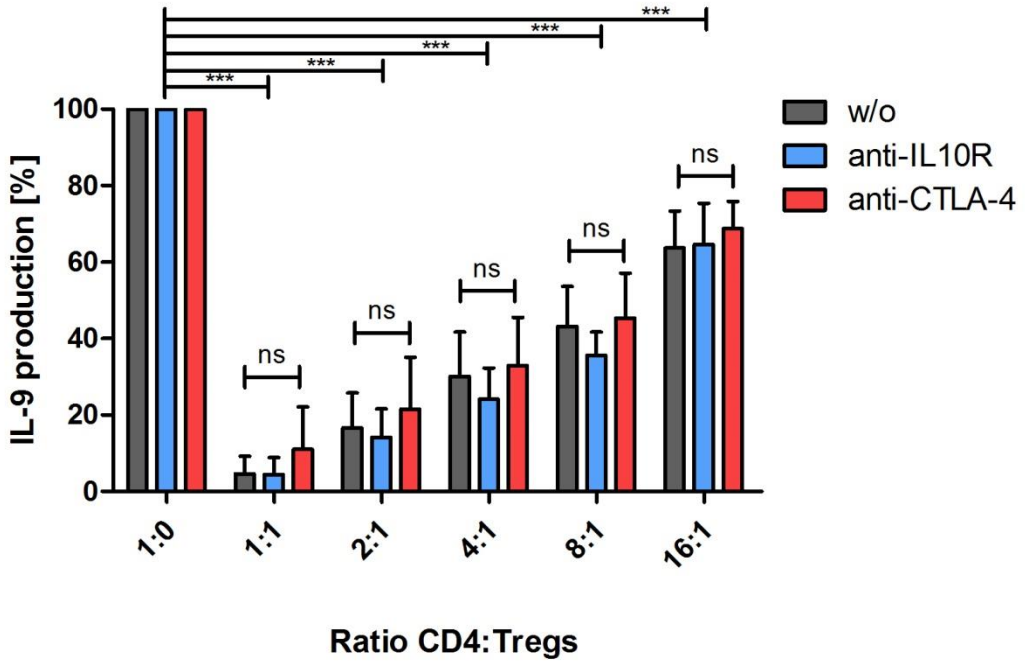

Figure 12 Contribution of IL-10 and CTLA-4 to Treg-mediated suppression

Tregs are able to inhibit the IL-9 production of developing Th9 cells in a cell ratio dependent manner. Neither blockade of IL-10 nor CTLA-4 signaling resulted in a reduction of the suppressive capacity of Tregs. $CD4^+$ $CD25^-$ T cells co-cultured with or without Tregs in different ratios, were stimulated with MitomycinC treated A20 cells (2.3.1.3.3) and 3 µg/mL anti-CD3 under Th9 skewing conditions as described in 2.3.1.2.12. IL-9 in the supernatant was measured on day three by specific ELISA. The IL-10 signaling pathway was blocked by the administration of an IL-10 receptor antibody (2 µg/mL), the CTLA-4 pathway by a CTLA-4 antibody (10 µg/mL). Mean and standard deviation, analyzed by 2way ANOVA Ratio p<0.0001; treatment p=0.4847. Shown are Bonferroni comparison tests.

No statistically significant differences in the IL-9 production can be observed when one of the blocking antibodies was added to the co-culture experiments. The abrogation of the IL-10 and CTLA-4 suppression pathway does not influence the suppressive capacities of Tregs (Figure 12). However, the substitution of either mechanism by the other cannot be excluded. In addition, a different mechanism could be essential (Figure 2).

Another direct mechanism of T cell suppression is the intracellular rise of cAMP in the responder T cells. This can be achieved by two mechanisms, binding of adenosine to its receptor on the cell surface and subsequent production of intracellular cAMP or by direct transport of cAMP from Tregs to the target cell *via* gap junctions.[150,158] The cAMP is most probably passively transported by the concentration gradient from $cAMP^{high}$ Tregs to $cAMP^{low}$ responder T cells.

To simulate the suppression of cAMP, dibutyryl cyclic adenosine monophosphate (dbcAMP) was used to mimic a rising intracellular cAMP level in responder T cells that can be observed in suppressed T cells. The non-degradable cAMP derivate dbcAMP is able to activate the same suppressive signaling cascades as Treg-derived cAMP. Naïve $CD4^+$ T cells were stimulated with anti-CD3 and A20 cells according to chapter 2.3.1.2.12. dbcAMP was added in different concentrations to mimic cAMP-mediated suppression by Tregs (Figure 13).

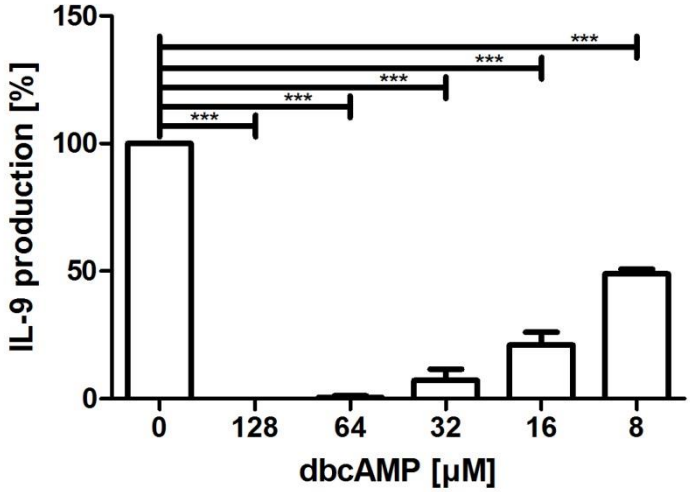

Figure 13 Suppression of Th9 cell commitment *via* dbcAMP

The cAMP derivate dbcAMP is able to inhibit the production of IL-9 in responder T cells. $CD4^+$ $CD25^-$ T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3 under Th9 skewing conditions as described in 2.3.1.2.12. Different concentrations of dbcAMP were added to mimic the effect of rising cAMP levels in the target cells. IL-9 in the supernatant was measured on day 3 by specific ELISA. Mean and standard deviation, analyzed by 1way ANOVA $p<0.0001$. Shown are Bonferroni comparison tests.

The production of IL-9 is reduced by high concentration of intracellular cAMP. The production of IL-9 was reduced by 50 % in low concentrations of 8 µM dbcAMP, while high concentration of 128 µM and 64 µM totally abrogated IL-9 production (Figure 13). Additional experiments with other cAMP elevating agents like Forskolin also showed an inhibition of IL-9 production, supporting these results (data not shown). It seems that cAMP is one of the key components being able to inhibit Th9 commitment. Other mechanism by which IL-9 production could be restricted in suppressed T cells, were not further investigated.

3.1.2 Suppression of Th9 development on the transcriptional level

To explore the suppression of IL-9 production on a transcriptional level the mRNA levels of suppressed Th9 cells were compared to normal developing Th9 cells. The molecular basis of the IL-9 suppression is either the interference of Tregs with *Il9* transcription or IL-9 release. In combination it can be investigated if Tregs are able to produce IL-9 on their own, or consume IL-9 of Th9 origin *via* the IL-9 receptor during the co-culture period, reducing the

amount of detectable IL-9 in the supernatant. The analysis of *Il9* mRNA would clearly identify the producers of IL-9.

Since the direct identification of IL-9 producers *via* FACS analysis was not possible, the focus shifted towards the study of the transcriptional level. To distinguish between regulatory and responder T cells, the $CD4^+$ responder T cells were labeled with the fluorescent dye CDFA-SE or were isolated from Ova-tg Thy1.1 origin and thereby identifiable through the expression of Thy1.1 (CD90.1). Due to the lack of mice, both sorting methods were used in the experiments. After cell sorting, as shown for CFSE in Figure 14, cells were analyzed for their expression of *Il9* mRNA.

Naïve responder T cells were co-cultured with Tregs in an equal ratio as described in 2.3.1.2.12.

In order to remove costimulatory A20 cells and to be able to differentiate between $CD4^+$ T cells and Tregs, the cells were labeled with specific antibodies. A20 cells were marked with anti-B220 and responder T cells were identified by using either anti-CD90.1 or CFSE. The process of Th9 commitment on mRNA level over time was monitored by sorting cells on three subsequent days after stimulation.

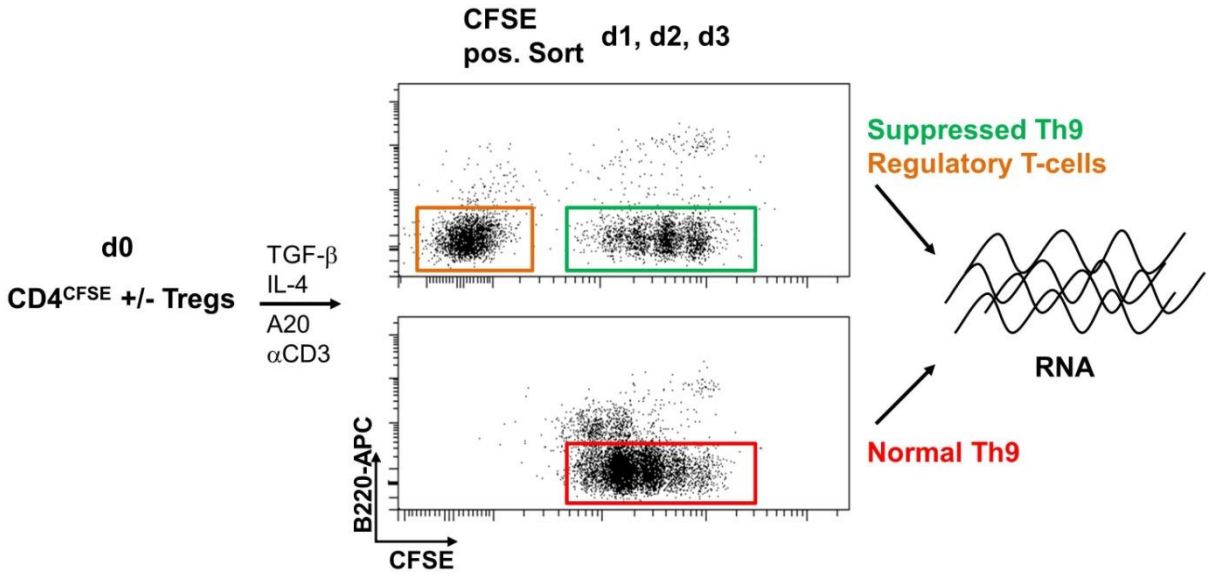

Figure 14 Sorting pattern of suppressed and normal Th9 cells

Isolated $CD4^+$ $CD25^-$ T cells and $CD4^+$ $CD25^+$ Tregs were co-cultured in an equal ratio according to 2.3.1.2.12 under Th9 skewing conditions. T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3. For separation, the $CD4^+$ T cells were labeled according to 2.3.1.2.1 with CDFA-SE or are of Thy1.1 origin, to separate Tregs from normal $CD4^+$ T cells. Before sorting, A20 cells were labeled with B220 antibody. If responder T cells were of Thy1.1 origin, those cells were labeled with anti-CD90.1 antibody. The cells were sorted according to 2.3.1.4 and subsequent mRNA was prepared as described in 2.3.2.1.

In a first experiment the *Il9* expression was analyzed on a transcriptional level in Th9 cells (Figure 15A). Tregs do not express any detectable amounts of *Il9* mRNA during any stages of co-culture (data not shown).

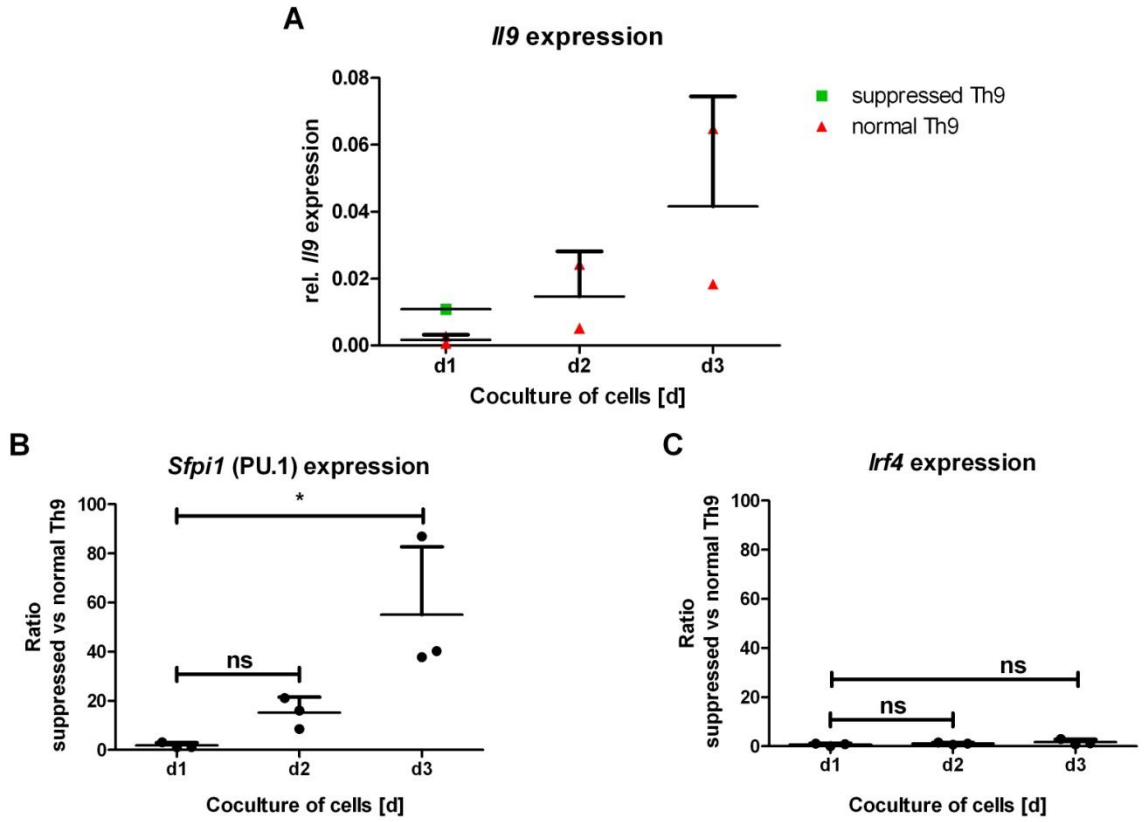

Figure 15 Th9 commitment in suppressed and normal developing Th9 cells

The comparison of suppressed Th9 cells to normal developing Th9 cells showed nearly no *Il9* expression in suppressed T cells (A). The comparative expression of Th9 transcription factors *Irf4* (C) and spleen focus forming virus pro-viral integration oncogene *Sfpi1* (PU.1) (B) in suppressed and normal developing Th9 cells showed an increase of *Sfpi1* (PU.1) expression in suppressed Th9 cells (B), while *Irf4* mRNA levels (C) remained unchanged. mRNA levels of suppressed and normal Th9 cells were compared to investigate the effect of suppression on known IL-9 activating transcription factors. Naïve $CD4^+$ T cells were labeled with CFDA-SE and cultured with or without equal numbers of Tregs for 3 days under Th9 skewing conditions. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3. At the given time the responder cells were sorted according to CFSE staining as displayed in Figure 14. mRNA was prepared according to 2.3.2.1 and analyzed by qRT-PCR for expression of target genes (2.3.2.2).
Mean and standard deviation, analyzed by 1way ANOVA p=0.0177 (B) and p=0.3775 (C). Shown are Bonferroni comparison tests.

As shown in Figure 15, the expression of *Il9* was detected in normal developing Th9 cells, while there was only minor expression of *Il9* at early time points in Th9 cells during co-culture with Tregs. These remnants of *Il9* expression at day one in co-culture are completely absent on day two and three (Figure 15A).

The absence of *Il9* mRNA in suppressed Th9 cells indicates that the transcription of the *Il9* gene is already suppressed. To test whether the regulation of *Il9* transcription is taking place on the promoter level, it is necessary analyze at the expression of transcription factors that are capable to bind to the *Il9* promoter. The regulation of IL-9 production in Th9 cells is reported to be regulated by two main transcription factors, namely IRF4 and PU.1.[62,63]

Additionally, these two factors play a pivotal role in Th9 development and lineage commitment. The expression ratio of suppressed versus normal developing Th9 cells for *Irf4* (Figure 15C) showed no significant changes, while *Sfpi1* (PU.1) (Figure 15B) showed a 50 fold increased expression in the suppressed Th9 cells co-cultured with Tregs.

To investigate the effect on protein level Western Blot experiment comparing suppressed and normal developing Th9 cells were performed. T cells were separated at indicated time points by cell sorting and subsequently lysed. IRF4 levels were measured by Western Blot analysis (Figure 16). Unfortunately, the expression of PU.1 could not be followed due to the lack of a suitable antibody.

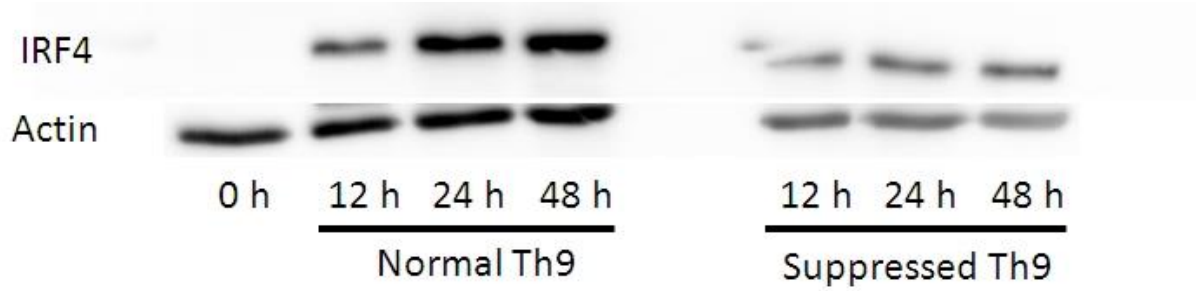

Figure 16 Western Blot analysis of IRF4 in developing Th9 cells

IRF4 showed no differences in protein levels when normal and suppressed Th9 cells were compared. Naïve $CD4^+$ T cells of Thy 1.1 origin were cultured with or without equal numbers Tregs for 3 days under Th9 skewing conditions. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3. Responder $CD4^+$ T cells from co-culture (2.3.1.2.12) were separated at indicated time points by cell sorting according to Thy1.1 expression (Figure 14) and lysed. Equal amounts of cell lysates were subjected to western blot analysis. Actin was used as loading control. Shown is one example out of three independent experiments.

The Western Blot analysis of IRF4 (Figure 16) confirmed that the amount of IRF4 is not altered in the presence of Tregs.

To investigate whether the co-culture with Tregs specifically inhibited the formation of Th9 cells by promoting the development into other T helper cell subsets, different subset specific transcription factors were analyzed, namely *Tbx21* (T-bet) for Th1, *Gata3* for Th2 and *Maf* (c-Maf) for IL-10 producing T cells.

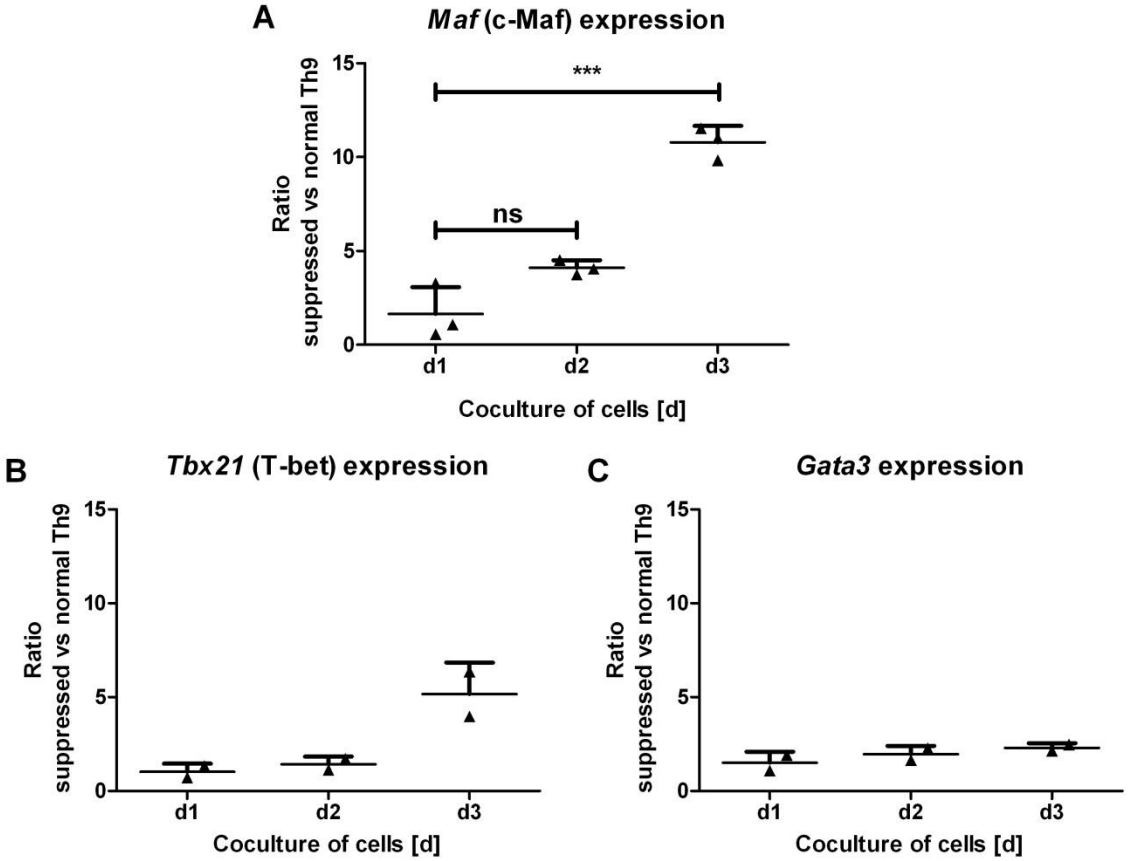

Figure 17 Expression pattern of transcription factors in suppressed versus normal Th9 cells

Comparative expression analysis of different T helper subsets defining transcription factors like *Maf* (c-Maf) (A), *Tbx21* (T-bet) (B), and *Gata3* (C) in suppressed and normal developing Th9 cells were performed. Only a co-culture dependent rise in *Maf* (c-Maf) expression was observed over time in suppressed Th9 cells. Other transcription factors remained unchanged during the first days of culture. *Tbx21* (T-bet) showed an increase only on day three, while *Gata3* remained unchanged. Naïve $CD4^+$ T cells were labeled with CFDA-SE and cultured with or without equal numbers of Tregs for 3 days under Th9 skewing conditions. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3 (2.3.1.2.12) and sorted at indicated time points according to CFSE staining as displayed in Figure 14. mRNA was prepared according to 2.3.2.1 and analyzed by qRT-PCR for expression of target genes 2.3.2.2. Mean and standard deviation, analyzed by 1way ANOVA p=0.0083 (A); p=0.0485 (B) and p=0.3387 (C). Shown are Bonferroni comparison tests.

Noteworthy, is that the major transcription factors for the Th1 or Th2 lineage were only slightly differently expressed upon co-culture with Tregs. *Tbx21* (T-bet) as transcription factor for Th1 showed (Figure 17B) only a five-fold increase on day three while remaining unchanged for the first two days. *Gata3* as transcription factor for Th2 cells showed (Figure 17C) only a two-fold increase in suppressed Th9 cells above the normal developing Th9 cells.

In contrast to *Tbx21* (T-bet) and *Gata3,* the minor Th2 transcription factor *Maf* (c-Maf) showed a drastic upregulation in expression when Tregs are present (Figure 17A). *Maf*

(c-Maf) mRNA expression is upregulated in suppressed Th9 cells, while it is being expressed to lower extent during normal Th9 development. The major targets of c-Maf in T cell development is the regulation of IL-4 during Th2 cell development. Therefore, c-Maf could be influencing the IL-4 production of suppressed Th9 cells, but no expression of *Il4* was found in suppressed Th9 cells. Other reported targets influenced by c-Maf like *Il10*, *Il21*, *Bcl2*, and *Il13* were investigated, but neither of them showed significant changes in expression (data not shown). One target of c-Maf might be directly IL-9 since it could be demonstrated that overexpression of c-Maf downregulated IL-9 production in Th9 cells.[65]

3.1.3 Transcriptome analysis of Th9 development in presence and absence of Tregs

The approach used in 3.1.2 is only capable of determining already known target genes, as specific primers must be designed. Therefore, the target genes have to be preselected based on current knowledge about Th9 development or other T cells subsets. To identify yet unknown genes that play a role in Th9 development and suppression, next generation sequencing (NGS) experiments were conducted.

In order to examine the transcriptome of Th9 cells differentiated in the presence and absence of Tregs, mRNA sequencing by NGS was performed. A suitable time point for NGS sample preparation was determined by a prior analysis on *Il9* gene expression. The criteria in these analyses were a detectable robust *Il9* expression and a hardly detectable IL-9 production in the supernatant. To determine this time point, cytokine analysis was performed by ELISA and qRT-PCR was conducted to measure *Il9* gene expression.

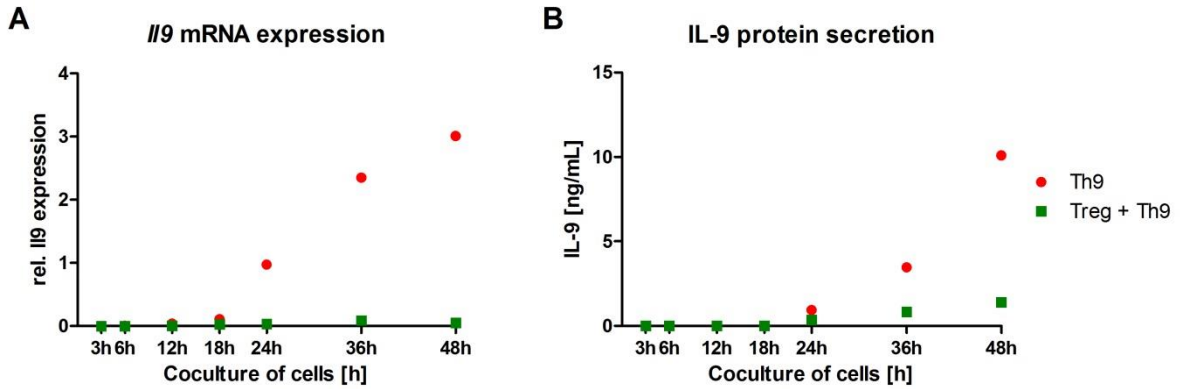

Figure 18 *Il9* expression and secretion of normal developing and suppressed Th9 cells
***Il9* expression started at 18 h after stimulation while cytokine release was detected six hours later. Naïve CD4$^+$ T cells were cultured with or without equal numbers of regulatory T cells for 3 days under Th9 skewing conditions. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3 (2.3.1.2.12). Whole batches were harvested and analyzed at the indicated time points for *Il9* expression by qRT-PCR and cytokine release by ELISA. (Displayed is one preliminary experiment.)**

The expression of IL-9 on mRNA and protein level in the co-culture experiment is time dependent (Figure 18). *Il9* gene expression in normal developing Th9 cells was first detectable at 18 h, while release of IL-9 in the supernatant was first detectable after 24 h (Figure 18A+B, red circle). The mRNA expression of *Il9* in the co-culture of T cells with Tregs was nearly abolished and only minor amounts of IL-9 were detectable during co-culture (Figure 18A+B, green boxes).

The fact that obvious differences in *Il9* mRNA expression between normal developing Th9 cells and Th9 cells in co-culture with Tregs were detected at 24 h led to the assumption that genes regulating the suppression in T cells should be highly expressed at these early time points of T cell commitment. After stimulating the naïve $CD4^+$ T cells with MitomycinC treated A20 cells and anti-CD3 (3 µg/mL), either alone or in the presence of equal numbers of Tregs for 24 h, as described in 2.3.1.2.12 the cells were separated by FACS sorting according to their Thy1.1 (CD90.1) expression, discriminating $Thy1.2^+$ Tregs from $Thy1.1^+$ responder T cells (see Figure 14). The isolated cells were used for mRNA isolation and following NGS probe preparation.

3.1.4 Comparative analysis of gene expression in the presence and absence of Tregs (NGS)

Comparable NGS analysis was performed from suppressed and normal developing Th9 cells at 24 h after stimulation. As a transcribed reference gene for data validation, *Il9* was chosen as one known direct target.

As cut off points were chosen with an RPKM (Reads per kilo base per million) value of three in at least one of the samples and a fold change value in expression of at least four. The RPKM value was used to compute for the mRNA abundance in a certain sample. A RPKM value of 0.5 for a mRNA could not be detected by qRT-PCR while a value of 12 was easily measurable. Therefore, a value of 3 was selected as basis for competitive analysis, as it could also be detected in qRT-PCR.

Furthermore, IL-9 in the supernatant was checked at 48 h and 72 h to confirm Th9 development. IL-9 was only measured when T cells were cultured in the absence of Tregs, demonstrating the suppressive capacity of the Tregs used (data not shown).

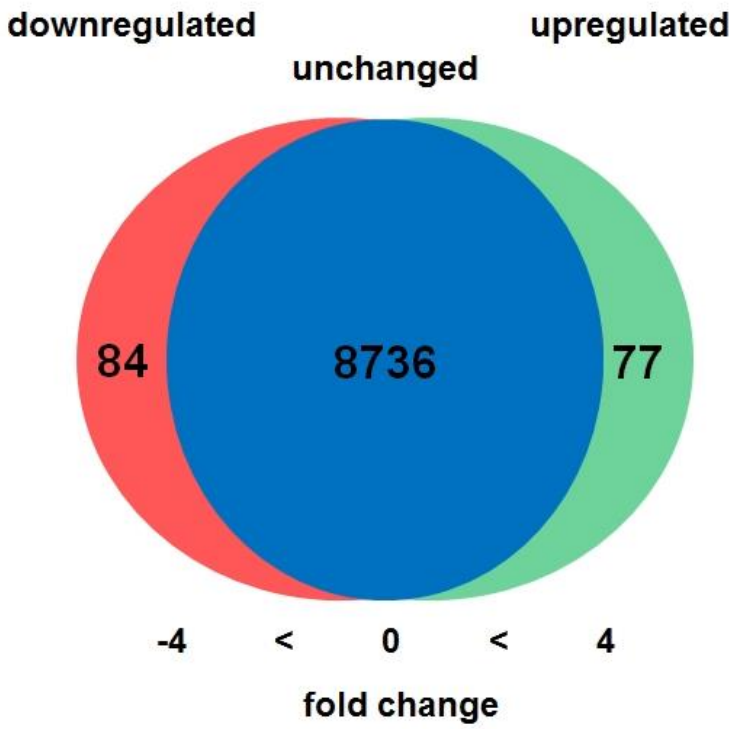

Figure 19 Comparative transcriptome analyses

8736 transcribed genes remained unchanged, 84 were downregulated in developing Th9 cells by the presence of Tregs, while 77 were upregulated. Naïve CD4$^+$ T cells of Thy 1.1 origin were cultured with or without equal numbers of Tregs under Th9 skewing conditions. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3 (2.3.1.2.12). Cells were co-cultured for 24 h (2.3.1.2.12) before being sorted according to CD90.1 similar to Figure 14. cDNA library was prepared according to 2.3.2.6. Transcribed genes were selected with a RPKM value of three. Genes transcribed were designated to either population if a fold change of more than four was on hand.

The next generation sequencing identified 8.897 transcribed genes that were assigned during competitive analysis and passed the criteria (Figure 19). For the majority of transcribed genes, no differences were found in the expression detected as a fold change value of less than four. 84 transcribed genes showed reduce expression in developing Th9 cells in the presence of Tregs when compared to normal developing Th9 cells. 77 transcribed genes were upregulated in responder T cells through the presences of Tregs.

3.1.4.1 Transcribed genes up- or downregulated in the presence of Tregs

The differentially expressed genes were grouped into three different sections, 1. cell cycle connected, 2. connected to immunological functions and 3. unknown function. The assignment to the category occurred according to current literature.

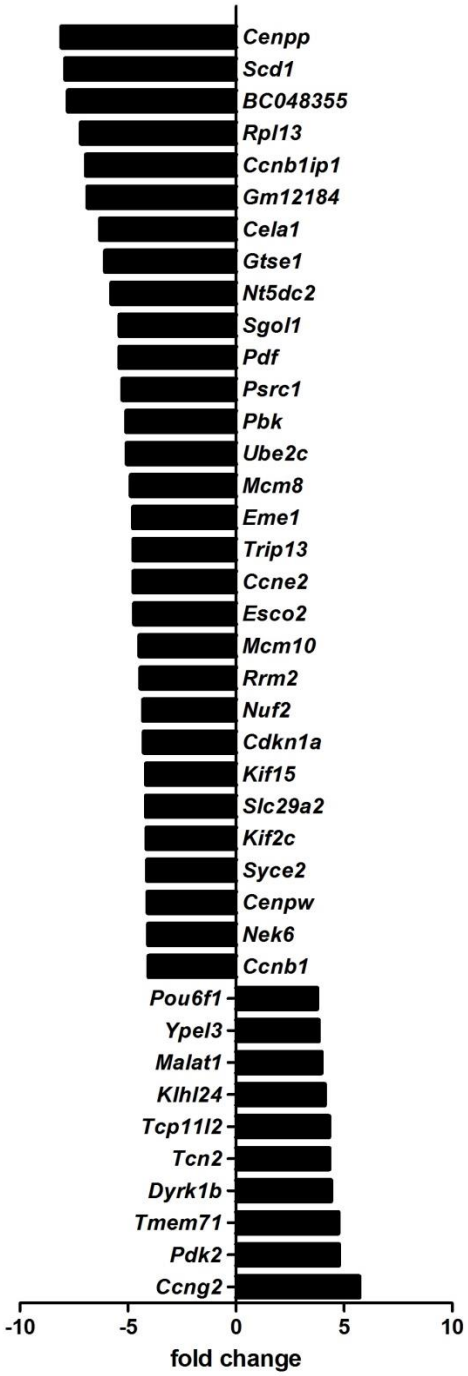

Figure 20 Transcribed genes connected to cell cycle arrest or progression

Analysis of transcriptome comparing suppressed to normal developing Th9 cells. Naïve CD4$^+$ T cells of Thy 1.1 origin were cultured with or without equal numbers of Tregs under Th9 skewing conditions. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3 (2.3.1.2.12). Cells were co-cultured for 24 h (2.3.1.2.12) before being sorted according to CD90.1 similar to Figure 14. cDNA library was prepared according to 2.3.2.6. Transcribed genes were illustrated with a RPKM value of three and a minimal fold change of four.

The analysis revealed, that most of the transcribed genes, that are connected to progression of cell cycle are upregulated in normal developing Th9 cells. This shows that the Th9 cells already entered the proliferation state while genes that are highly expressed in suppressed Th9 cells are connected to cell cycle arrest.

Not only were targets of the cell cycle arrest and progression affected, but also genes connected to immunological functions were expressed differently. Since no protein is known to mediate the suppressive properties of Tregs in target cells, identification of the targeted signaling cascade proved to be difficult. Transcribed genes that were upregulated either in normal developing Th9 cells or in suppressed Th9 cells are shown in Figure 21.

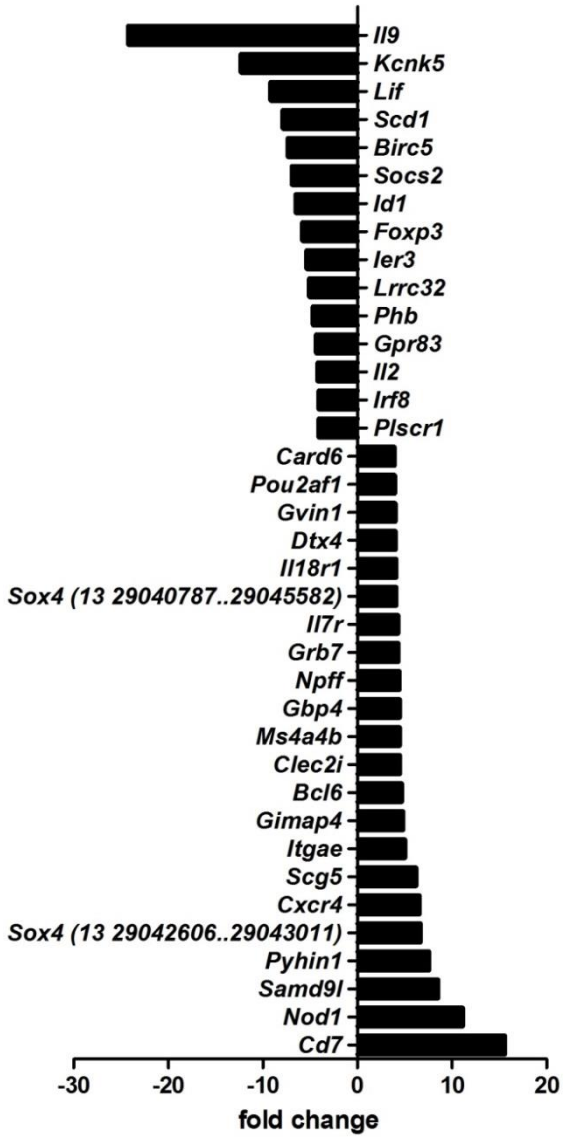

Figure 21 Transcribed genes connected to immunological functions

Analysis of transcriptome comparing suppressed to normal developing Th9 cells with genes of known immunological functions. Naïve CD4$^+$ T cells of Thy 1.1 origin were cultured with or without equal numbers of Tregs under Th9 skewing conditions. Cells were co-cultured for 24 h (2.3.1.2.12) before being sorted according to CD90.1 similar to Figure 14. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3 (2.3.1.2.12). cDNA library was prepared according to 2.3.2.6. Transcribed genes were selected with a RPKM value of three and a minimal fold change of four.

Beside the transcribed genes with known functions, the NGS analysis also revealed some transcribed genes whose function was not yet clear. These genes are not described here and there was no further analysis due to the lack of suitable biochemical tools.

3.1.4.2 Validation of transcribed genes upregulated in the presence of Tregs

In order to validate the genes differently expressed in suppressed Th9 cells, qRT-PCR analysis was performed for most of the genes known to regulate immunological functions. mRNA was prepared as described in 3.1.2. Of the tested 16 transcribed genes only five showed different regulation.

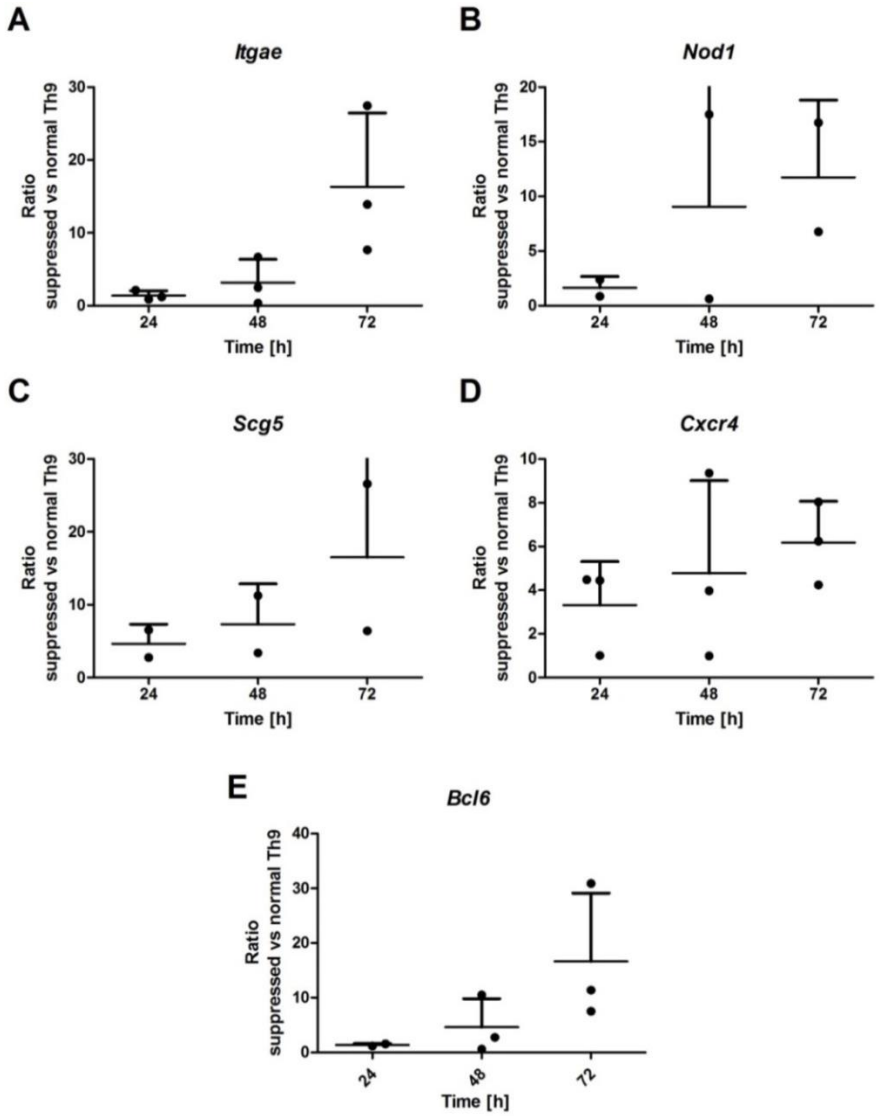

Figure 22 Validation of NGS target genes *via* qRT-PCR

To validate the transcribed genes with immunological functions discovered by NGS analysis qRT-PCR analysis was performed. Naïve CD4$^+$ T cells of Thy 1.1 origin were cultured with or without equal numbers of Tregs under Th9 skewing conditions. The T cells were stimulated with MitomycinC treated A20 cells and 3 µg/mL anti-CD3 (2.3.1.2.12). Responder T cells were sorted according to CD90.1 expression at indicated time points as in Figure 14. cDNA was prepared and qRT-PCR performed. Only *Itgae* (A), *Nod1* (B), *Scg5* (C), *Cxcr4* (D) and *Bcl6* (E) were found to be expressed differently over time. Other transcribed genes showed no difference (data not shown). Shown are mean and standard deviation.

Further characterization of their expression profile over time was performed by qRT-PCR to investigate the role of the transcribed genes found by NGS analysis. Most of the genes showed no consequent differential expression over the three subsequent days of co-culture.

Only Integrin, Alpha E (*Itgae)*, Nucleotide-binding Oligomerization domain containing 1 (*Nod1)*, Secretogranin V (*Scg5)*, Chemokine (C-X-C motif) receptor 4 (*Cxcr4)*, and *Bcl6* were differently expressed when comparing normal developing to suppressed Th9 cells. *Itgae*, *Scg5*, and *Nod1* showed a ten-fold upregulation in transcribtion in suppressed Th9 cells on day three, rising constantly from day one (Figure 22A,B,C). *Cxcr4* only showed a five-fold upregulation in transcription in suppressed Th9 cells with subsequent overexpression over three days of analysis (Figure 22D). The transcription factor *Bcl6* showed a fifteen-fold upregulation in transcription in suppressed Th9 cells on day three with significant rise from day one (Figure 22E).

A reporter gene assay was used to identify the effects of transcription factors on the *Il9* promoter activity in activated T cells. Through this assay, the effects of different transcription factors on the *Il9* promoter were analyzed. Thereby direct or indirect effects on the *Il9* expression could be determined.

The transcription factors IRF4 and PU.1 are already known to play a role in IL-9 the regulation of *Il9* gene expression T cells. By binding directly to the *Il9* promoter region these transcription factors are believed to trans-activate the *Il9* locus. c-Maf which was found in highly expressed on day three in suppressed Th9 cells was investigated for its effect on IL-9 production. It is known that c-Maf can regulate IL-9 production, since its overexpression in Th9 cells reduces the IL-9 production by half.[65] To investigate if this effect is due to direct binding to the promoter region or an indirect effect, reporter gene assay experiments were performed. An interaction of c-Maf with the *Il9* promoter region is likely due to a half-MARE binding side located -370 bp upstream of *Il9* transcriptional start site, shown in Figure 23. Schematic workflow and timeline are depicted in Figure 5 and Figure 6.

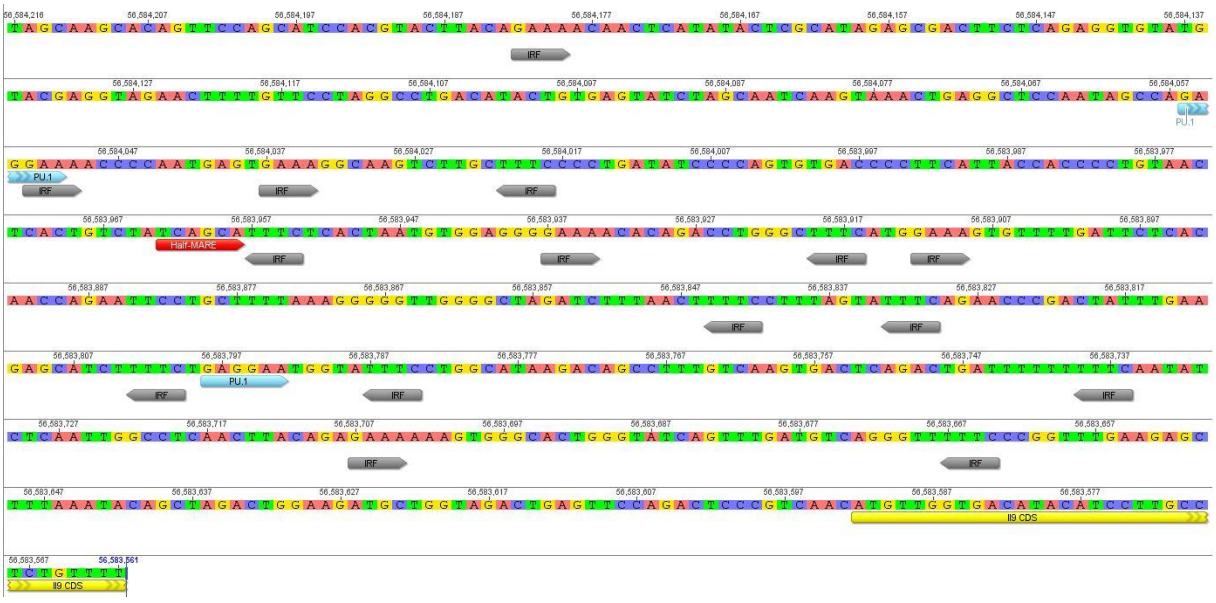

Figure 23 *Il9* promoter region -610 to +32 bp

Promoter sequence upstream of the *Il9* gene. Binding sites for IRF (grey), PU.1 (light blue), and c-Maf half-MARE (red) are annotated. GeneiousR6 (Biomatters, New Zealand) showing the IL-9 promoter NC_000079.6 (56482527-56482200)

To check whether c-Maf has a modulatory effect on the regulation of *Il9* expression a reporter construct of the *Il9* promoter was co-transfected with the transcription factors of interest in developing Th9 cells on day two. If transcription factors influence the *Il9* promoter, the expression of luciferase will change according to activation or downregulation. After transfection and restimulation for 4 h, T cells were harvested and lysed before luciferase activity was measured. The transcription of luciferase is controlled by the *Il9* promoter and thereby influenced directly by the binding of the different transcription factors. Therefore, luciferase activity allows for the drawing conclusions on the influence of transcription factors on the *Il9* promoter activity.

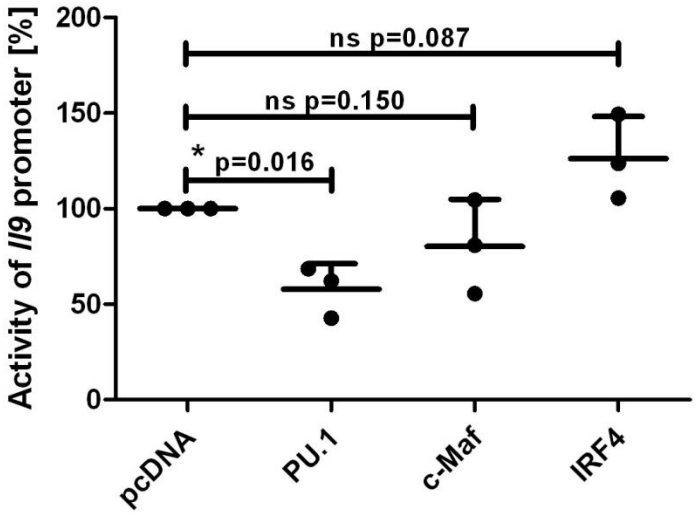

Figure 24 Reporter gene assay for the transcription factors

PU.1 and c-Maf are able to downregulate *Il9* promoter activity, while IRF4 has positive effects. T cells were stimulated for 2 d with plate-bound anti-CD3/anti-CD28 (4 µg/mL/4 µg/mL) under Th9 skewing conditions, then harvested, and transfected with equal amounts of *Il9* reporter construct and the indicated transcription factors (2.3.1.2.11). The cells rested for 4 h in supplied medium and were then restimulated with anti-CD3/anti-CD28 for 4 h before the cells were lysed and luciferase activity was measured (2.3.2.5). Mean and standard deviation were analyzed by TTest. Shown are TTest values.

Overexpression of IRF4 in activated Th9 cells on day two led to increased activation of the *Il9* promoter as described in the literature by 25 %. The overexpression of PU.1 and c-Maf resulted in a downregulation of *Il9* promoter activity of 40 % and 20 % respectively.

3.2 Minimal inhibition of Th9 effector cells by regulatory T cells

3.2.1 *In vitro* analysis of Treg-mediated Th9 suppression

As indicated by the present results, Th9 development can be inhibited early during T helper cell commitment by Tregs. Nevertheless, the question of whether terminal differentiated Th9 cells are also suppressed by Tregs remained elusive. To answer that question, suppressive effects of Tregs on fully committed Th9 cells were tested in a proliferation assay.

Fully committed T helper cells are stable under steady-state conditions *in vivo* as well as *in vitro*. The commitment is reinforced by the secreted cytokines that can act in a positive feedback loop. Small changes in the cytokine milieu or present pathogens are able to change the commitment towards a T helper cell subset. This plasticity has been more and more investigated in recent years.

It is known that the profile of fully established Th9 cells is relatively robust to exterior influences in investigated models, although not many experiments were conducted concerning Th9 plasticity. Those cells seem to be extremely suitable to investigate the impact of Tregs on the effector function.

To identify the influence of Treg on Th9 effector function co-culture experiments were performed. Since freshly isolated Tregs only have limited suppressive capacities, it was investigated if this relatively weak suppression is able to inhibit Th9 effector function. Freshly isolated Tregs (fresh Tregs) as well as two-days-preactivated Tregs (pre Tregs) were co-cultured with fully differentiated Th9 cells. The cells were restimulated with 3 µg/mL anti-CD3 and MitomycinC treated A20 cells (2.3.1.3.3) according to chapter 2.3.1.2.12.

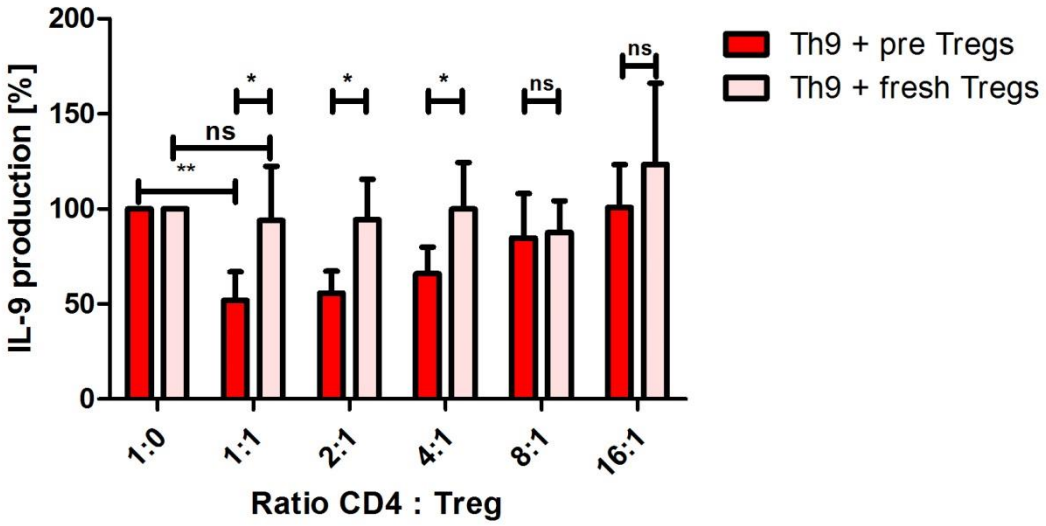

Figure 25 Comparison of suppressive capacities of regulatory T cells

Only preactivated Tregs showed suppressive capacity during co-culture, while freshly isolated Tregs have no suppressive effect on Th9 cell effector function. Freshly isolated Tregs and two day preactivated Tregs were tested for their capability to inhibit IL-9 production in fully differentiated Th9 cells. Naïve $CD4^+$ T cells were cultured for 5 days under Th9 skewing conditions before restimulation with anti-CD3 (3 µg/mL) and MitomycinC treated A20 cells for two days. A suppression assay was performed (2.3.1.2.12). IL-9 production was assessed using specific IL-9 ELISA. Mean and standard deviation were analyzed by 2way ANOVA ratio p=0.0006 and Treg activation status p=0.0059. Shown are TTest values.

The suppression of fully committed Th9 cells is only possible by preactivated Tregs (Figure 25). In an equal cell ratio preactivated Tregs are able to inhibit effector function of Th9 cells to around 50 %, with decreasing suppressive capacities in lower cell ratios. Freshly isolated

Treg cells were not capable of inhibiting the IL-9 release of Th9 cells, even in equal ratio. The fact that freshly isolated Tregs were not capable of inhibiting the release of IL-9 is in accordance with experiments from Treg suppression of Th2 effector function.[186]

To examine why the suppression of the effector function was only down to 50 %, isolation and activation of procedures of Tregs from animals were tested. To ensure that activation and isolation of Treg cells from animals did not affect the suppressive capacity of Tregs, their ability to inhibit Th2 function was tested. The Th2 subset is closely related to the Th9 subset and thus seems optimal for testing the suppressive capacity.

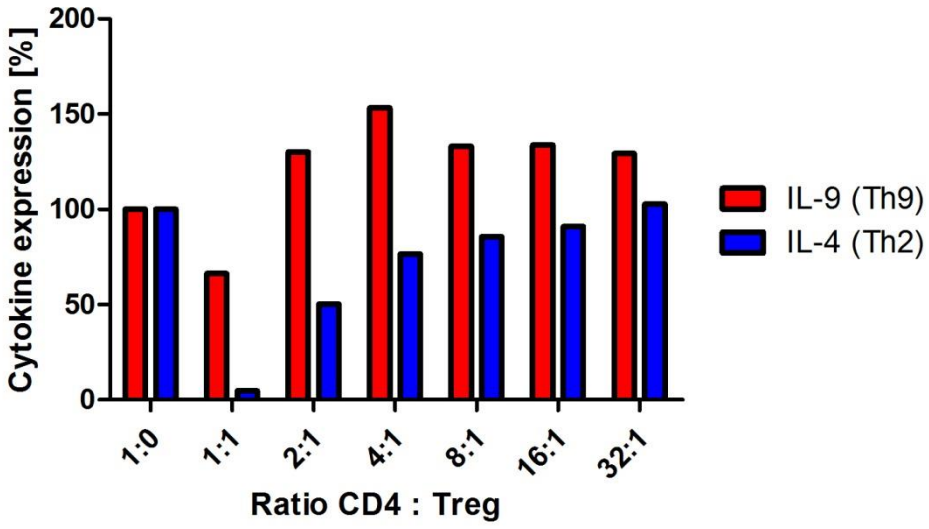

Figure 26 Inhibition of effector function of Th2 and Th9 cells

Preactivated Tregs were tested for their capability to inhibit IL-9 and IL-4 production in fully differentiated Th9 and Th2 cells. Naïve $CD4^+$ T cells were cultured for 5 days under Th9 or Th2 skewing conditions. T cell were stimulated for 3 days with plate-bound anti-CD3/anti-CD28 (4 µg/mL/4 µg/mL) and then rested for two days, before being restimulated with MitomycinC treated A20 cells and anti-CD3 (3 µg/mL) for two days. A suppression assay was performed (2.3.1.2.12). Cytokine production was assessed employing specific ELISA. The signature cytokine IL-4 of Th2 cells release was strongly suppressed while IL-9 of Th9 cells was less susceptible to suppression. Shown are preliminary data.

The capacity of Tregs to suppress the effector function was not compromised by isolation or preactivation since their ability to completely suppress the effector function of Th2 cells was not impaired. In an equal ratio Tregs fully abrogated the IL-4-producing properties of Th2 cells, while Th9 cells were only affected to a maximum of 50 %. In lower ratios the suppression of Th9 cells was totally absent.

To prove that A20 cells and anti-CD3 do not influence suppression of effector function, a more reliable and suitable model that better reflects the situation *in vivo* was used. Bone marrow derived dendritic cells (BMDC) as antigen presenting cells were generated. These BMDCs were able to better mimic the process of restimulation *in vivo* during for example asthmatic diseases.

BDMCs were generated as described in 2.3.1.5 loaded with Ova peptide ($Ovalbumin_{323-339}$ 2.5 µg/mL) and activated with LPS (100 ng/mL). These BMDCS were used as antigen presenting cells, substituting for anti-CD3 and A20 cells. Naïve Ova-TCR transgenic $CD4^+$ T cells were isolated from DO11.10 mice and differentiated under Th9 skewing conditions for 5 days, while Tregs were isolated and preactivated for two days. Cells were co-cultured for two days and suppression of cytokines IL-4 and IL-9 was assessed by specific ELISA.

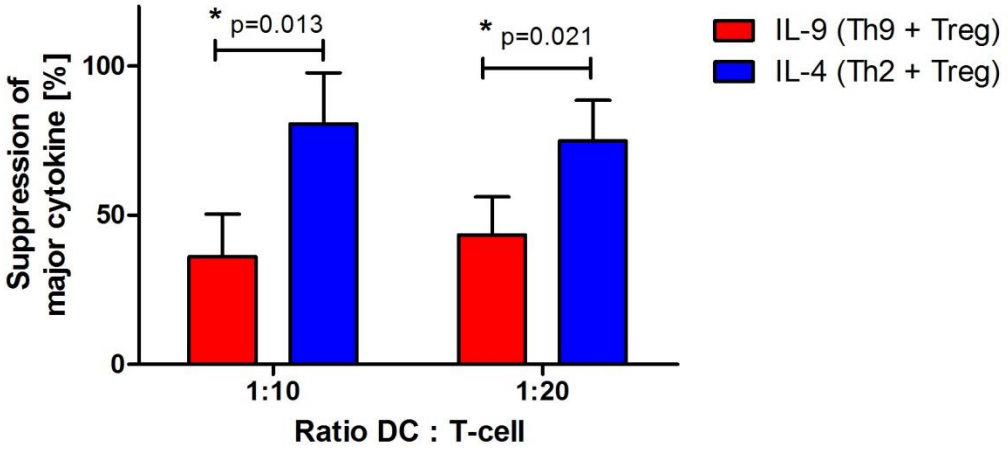

Figure 27 Suppression of effector cytokines during restimulation with BMDCs

Tregs were able to nearly abolish IL-4 production in Th2 cells. IL-9 production in Th9 cells was only marginally affected by the presence of Tregs. T cells cultured without Tregs were not suppressed. BMDCs generated as described in 2.3.1.5 were used to investigate the inhibition of cytokine release. T cells isolated from DO11.10 mice were stimulated under Th2 or Th9 skewing conditions with plate-bound anti-CD3/anti-CD28 (4 µg/mL/4 µg/mL). On day 5, fully committed T helper cells were co-cultured with or without an equal ratio of preactivated Tregs at different ratios of dendritic cells. To stimulate the T cells, Ova peptide (2.5 µg/mL) recognized by the transgenic TCR was added and LPS (100 ng/mL) to activate the dendritic cells. The suppressive effect was measured by the release of cytokines IL-9 and IL-4 *via* specific ELISA. Normal developing cells were unsuppressed, while absence of cytokines indicated full suppression. Shown are mean and standard deviation of three independent experiments, analyzed by TTest. Shown are TTest values.

The suppression of effector cytokines by Tregs is possible when the T cells are stimulated in an antigen specific manner. As shown in Figure 26 the suppression of IL-4 is possible in Th2 cells. The suppression of Th2 cells is around 70 to 80 %. IL-9 production of Th9 cells, on the contrary, is only marginally suppressed by around 40 %.

The suppressive mechanisms underlying these results are of multiple origins. To investigate, if a master mechanism underlies the suppression of effector cytokines, antibodies were used to block IL-10 receptor signaling and the CTLA-4 pathway. Both antibodies, when added to the co-culture of Tregs and Th9 cells, showed no increase in IL-9 production (Data not shown).

To study the role of direct cell-to-cell contact *via* gap junctions between Tregs and responder Th9 cells, dbcAMP was used to mimic the rise in intracellular cAMP levels as observed in this context.

Naïve $CD4^+$ T cells were isolated and stimulated with anti-CD3/anti-CD28 for 5 days under Th9 skewing conditions. Th9 cells were then restimulated with anti-CD3 (3 µg/mL) and MitomycinC treated A20 cells in the presence of varying concentrations of dbcAMP. IL-9 production was measured on day two in the supernatant with specific ELISA.

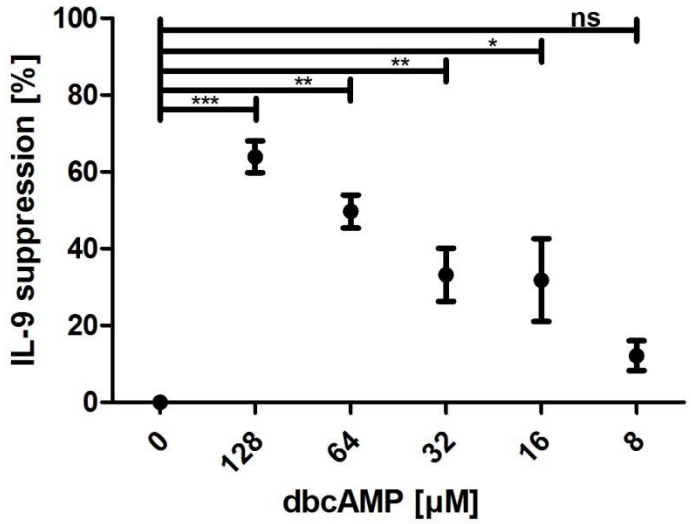

Figure 28 dbcAMP suppression of Th9 effector function

High concentration of dbcAMP are not able to completely inhibit IL-9 effector function, while low levels show nearly no inhibition of IL-9 production. T cells cultured for 5 d under Th9 skewing conditions were restimulated with MitomycinC treated A20 cells (2.3.1.3.3) and anti-CD3 (3 µg/mL) for 2 d in the presence of the indicated concentrations of dbcAMP. The co-culture with dbcAMP mimics the rise of internal cAMP levels due to direct transport from Tregs *via* gap junctions. IL-9 production was assessed using specific IL-9 ELISA and percentage of suppression was calculated to untreated Th9 cells, reflecting zero percent suppression. Mean and standard deviation were analyzed by 1way ANOVA ratio p=0.0006 and Treg activation status p<0.0001. Shown are Bonferroni tests.

Since the release of IL-9 from Th9 cells is only marginally affected in co-culture with Tregs, the mimicry of the suppressive mechanism *via* high intracellular cAMP levels show that the IL-9 release is also only partially blocked by about 60 % (Figure 28). It can be assumed that the highest concentration (128 µM) of dbcAMP is not physiological, but lower concentrations show even further diminished IL-9 suppression. In contrast to that, suppression of IL-4 in Th2 cells at high concentration of dbcAMP is complete.

The question of which of the suppressive mechanisms was not functional could not be entirely answered due to their complexity and possible redundancy. Further experiments are needed to dissect the exact signaling cascade.

3.2.2 Suppression of Th9 effector cells on the transcriptional level

To examine the Treg induced alteration in the expression profile of fully committed Th9 cells; mRNA of normal and suppressed Th9 cells was analyzed for the expression of different transcription factors on the second day of restimulation. Naïve $CD4^+$ T cells that were cultured for 5 days under Th9 skewing conditions were subsequently co-cultured with Tregs that had been preactivated for two days. Cells were stimulated with MitomycinC treated A20 cells and soluble anti-CD3 (3 µg/mL). On the second day of restimulation Th9 cells were sorted according to CD90.1 or CFSE labeling. Their total RNA was prepared and the major transcription factors *Irf4*, *Sfpi1* (PU.1) and *Maf* (c-Maf) were checked by qRT-PCR.

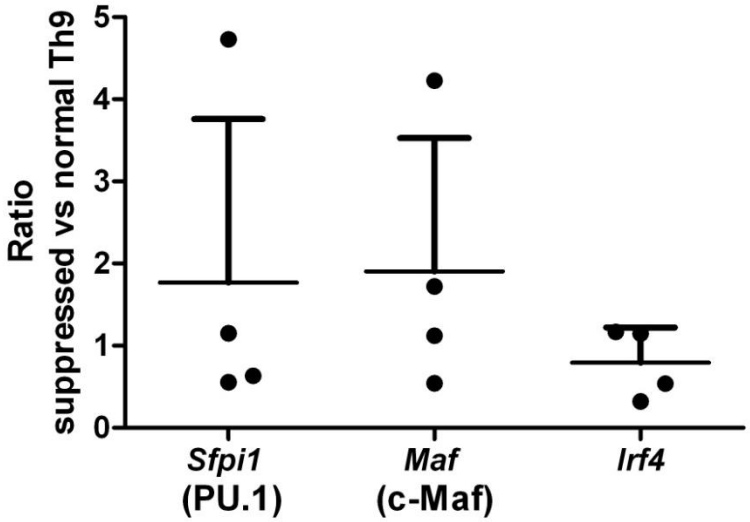

Figure 29 Major Th9 transcription factors during suppressed restimulation

No changes in the expression level of Th9 transcription factors occurred during restimulation of Th9 cells in the presence of Tregs. Naïve $CD4^+$ T cells were cultured under Th9 skewing conditions for 5 days before being co-cultured in equal ratio with two days preactivated Tregs accordingly. Cells in co-culture were restimulated with MitomycinC treated A20 cells and anti-CD3 (3 µg/mL). On day two of restimulation, Th9 cells were sorted according to CD90.1 or CFSE staining as illustrated in Figure 14. cDNA was prepared and *Sfpi1* (PU.1), *Maf* (c-Maf) and *Irf4* mRNA expression were checked with qRT-PCR. Ratio was calculated with suppressed divided by normal Th9 according to 2.3.2.2 and ratio calculated. Mean and standard deviation are shown.

The expression level of the two main transcriptions factors of Th9 cells remains unchanged in co-culture with Tregs. Neither *Irf4* nor *Sfpi1* (PU.1) showed great differences in transcription when Th9 cells were restimulated with or without Tregs. Although *Maf* (c-Maf) transcription is upregulated by developing Th9 cells that are suppressed by Tregs, a change in the transcriptional level during co-culture of Th9 cells with preTregs could not be detected. Since the IL-9 release by Th9 cells is only marginally affected by Tregs, the investigation of further expression patterns was foregone.

The possible production of IL-9 by Tregs, as published by Eller et al. could be excluded.[71] Tregs sorted from the same cultures as Th9 cells were analyzed for their *Il9* gene expression, but showed no expression of *Il9* mRNA. (Data not shown)

3.2.3 Analysis of Treg-mediated Th9 suppression *in vivo*

Different approaches were used to investigate the control of effector function of Th9 cells by Tregs *in vitro* with inconsistent and unreliable results. The effector function of Th9 cells could only be partially suppressed, while in the related Th2 subset IL-4 production was strongly controlled. To investigate the role of Th9 cells *in vivo*, different models are used in current literature. As described in 2.3.3.2, the acute asthma model in Rag2 deficient mice is able to give insight in T cell function during allergic asthma. The attraction of immune cells as well as increased airway resistance towards the bronchia contractual metacholine are signs of the disease progression.

Another possibility to measure effector function of Th9 cells is analyzing the efficiency of tumor immunity to melanoma tumors. Using these tumor models, in Rag1 deficient mice, enables the examination of Th9 effector function. The attraction of mast cells by T cell produced IL-9 leads to slower tumor growth over time and enhanced survival.[103] Also, the enhanced attraction of dendritic cells and $CD8^+$ cytotoxic T cells *via* Ccl20 released by Th9 activated epithelial cells is controlling tumor progression, but not in this Rag1 model.[104]

Tumor specific Th9 cells are able to attract and activate mast cells in an IL-9 dependent manner, inducing degranulation of mast cells leading to lysis of tumor cells.

These two models are suitable to investigate the effect of Tregs on IL-9 production of Th9 cells, since the parameters used to evaluate the T cell function are directly connected to IL-9.

3.2.3.1 Th9 effector function in acute asthma

As described in the literature and in the studies to define the role of the transcription factors during Th9 commitment, the acute asthma model is used to determine the function of Th9 cells *in vivo*.[62] The used model used is described in detail in 2.3.3.2 and a workflow is described in Figure 7.

T helper cells from DO11.10 mice were differentiated *in vitro* for 5 days under Th9 or Th2 skewing conditions and, stimulated with plate-bound anti-CD3/anti-CD28 (4 µg/mL/4 µg/mL). Afterwards, the T cells were injected into Rag2 deficient mice, lacking B and T cells. To investigate the role of Tregs on T helper cell suppression, one week before T helper cell injection, equal numbers of Tregs of Ova-TCR transgenic Thy1.1 origin were injected *intra venous* in the mice to re-populate the immune system. Those Tregs were stimulated *in vitro* for 5 days under Treg conditions with plate-bound anti-CD3/anti-CD28 (4 µg/mL/4 µg/mL). In order to determine the effect of Tregs and assure their functionality *in vivo*, Th2 cells were used as control.

After injection of T helper cells, the mice were challenged for 6 subsequent days with complete Ova in PBS *via* the lung to induce the acute asthma. The challenge for the mice was done by nebulizing Ova according to chapter 2.3.3.2.1. After the last challenge the mice rested for 24h before their lung function was assessed *via* the challenge with metacholine

according to 2.3.3.2.2. Infiltration of immune cells was characterized *via* H&E staining of the cytospins, according to 2.3.3.2.3, 2.3.3.2.4, and 2.3.3.2.5.

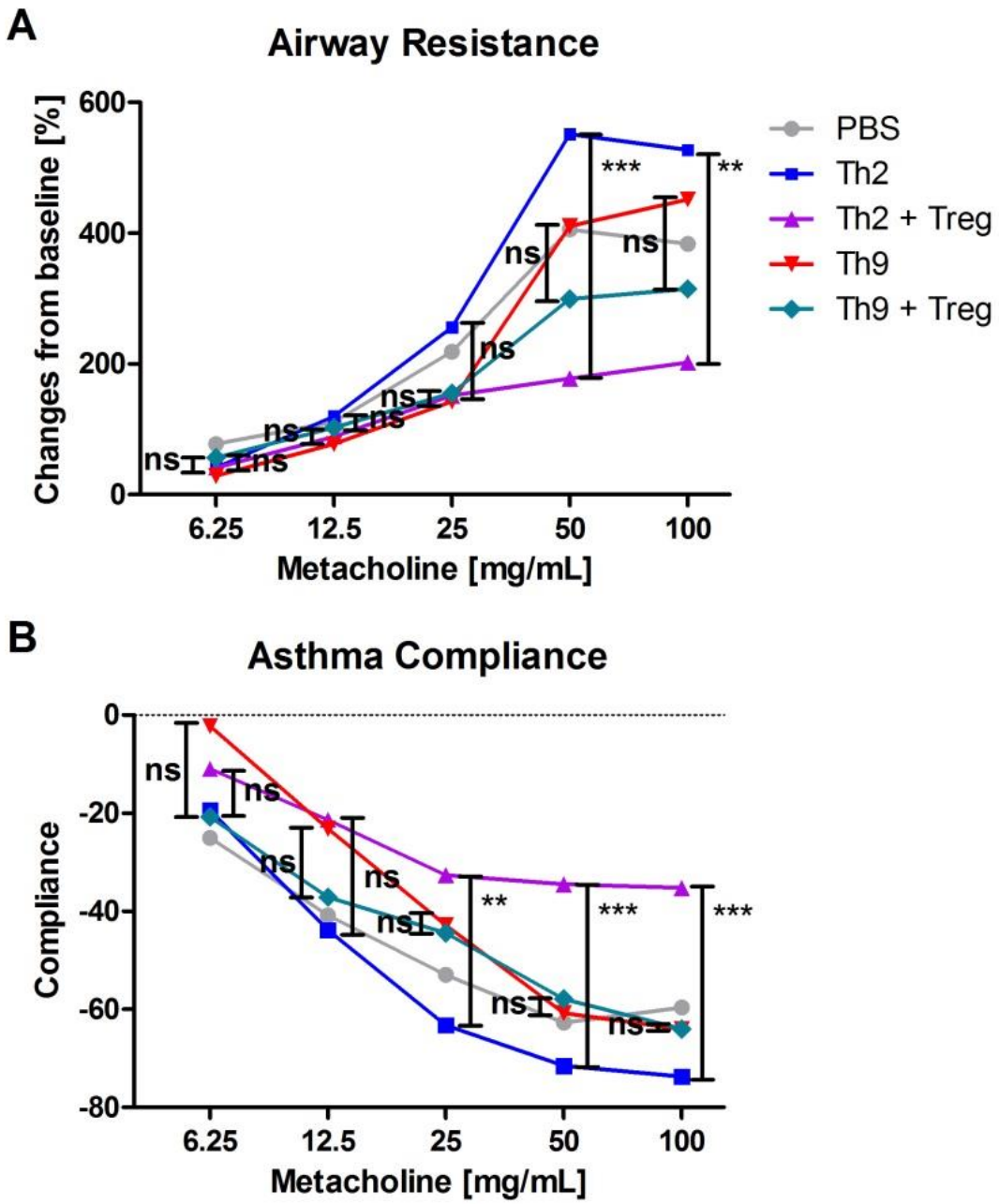

Figure 30 Suppression of Th2 and Th9 cells in acute asthma

To investigate the suppressive capacities of Tregs on Th2 and Th9 cells *in vivo*, Rag2 deficient mice were injected with Tregs one week prior to the injection of Th2 or Th9 cells, each 1x10^6 cells per mice. After injection of T helper cells, mice were challenged with Ova (10 mg/mL) for 6 subsequent days prior to measurement of lung function. Airway resistance was measured by challenging mice with different concentrations of metacholine. The whole procedure was performed according to 2.3.3.2. The Airway resistance (A) shows function of the lung, while Compliance (B) is indicating lung remodeling. Th2 cells show decreased asthma symptoms when Tregs are present. The function of Th9 cells is hardly diminished by Tregs. Mean and standard deviation, analyzed by 2way ANOVA transferred T cells p=0.0020 (A); p<0.0001 (B) and metacholine dose p<0.0001 (A); p<0.0001 (B). Shown are Bonferroni tests.

The suppression of Tregs in the model of acute asthma is detectable mainly for Th2 cells. In high concentrations of metacholine, the suppression of Th2 cells by Tregs is distinct when compared to mice that solely received Th2 cells (see Figure 30 A+B). It is known that Th2 cells are able to induce the asthmatic symptoms of AHR and lung remodeling. The suppression of Th9 cells by Tregs is clearly diminished when compared to the suppression of Th2 cells (see Figure 30). Although Th9 cells have similar capabilities compared to Th2 cells for the induction of asthmatic symptoms, it is not known if their course of disease is comparable. The Th9-evoked airway resistance as well as the lung remodeling is hardly diminished when Tregs are present while Th2 cells are clearly suppressed. This data is comparable to the *in vitro* suppression of both cell types (3.2.1).

Another indicator for disease progression in acute asthma is the infiltration of innate immune cells causing the asthma symptoms. This infiltration is induced by the release of cytokines from T cells since only those cells are able to respond to the allergen in an antigen specific manner.

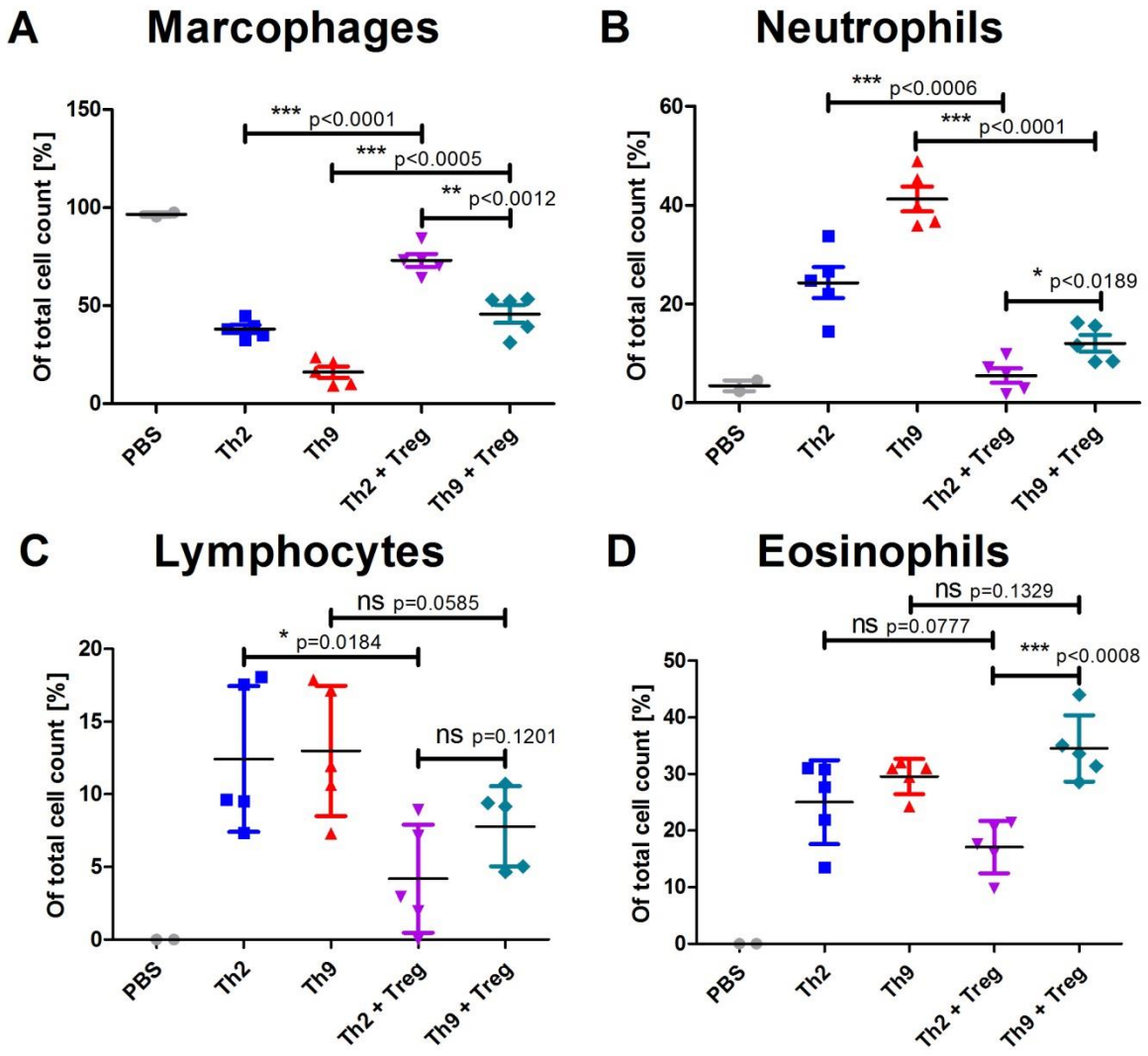

Figure 31 Infiltration of cell types in the lung during acute asthma

The infiltration of immune cells in the lung is a sign of acute asthma. The analysis of different cell types was done with H&E staining of cytospins from BAL fluid. In healthy mice only macrophages (A) were present in the lung, while other cell types were attracted by antigen presence, chemokines and cytokines as for lymphocytes (A), or only cytokines and chemokines as for neutrophils (B), eosinophils (D), or macrophages (A). The infiltration with lymphocytes into the lung was observable (C) as was a reduction of infiltration due to the presence of Tregs. Neutrophils (B) and eosinophils (D) were only attracted when T cells are present. The invasion of neutrophils was reduced by Treg presence for both T helper cell subsets. Eosinophil numbers were only marginally reduced for Th2 cells, while cell numbers remain unchanged for Th9 cells. Mean and standard deviation were analyzed by TTest. Shown are TTest values.

The infiltration of the lung tissue by innate immune cells was enhanced by antigen specific T cells. The reconstitution of mice was ascertained by the detection of lymphocytes in the BAL fluid in all mice except for mice that were not reconstituted as shown in Figure 31 C. While under steady state conditions only macrophages are present in the lung, reconstituted mice showed infiltration of different cells types as depicted in Figure 31. The infiltration of all

cell types into the lung is inhibited by Tregs. Only macrophages levels are higher since those are present in healthy mice (Figure 31 A). As shown in Figure 31 D, the number of eosinophils present when Tregs inhibit Th9 cells is not affected. The attraction of eosinophils into the lung *via* IL-9 was not interfered by Tregs, confirming the *in vitro* data.

3.2.3.2 Th9 effector function in B16 melanoma immunity

The role of Th9 cells in tumor immunity is described in detail in 1.2.3. The role of IL-9 produced by Th9 cells is clearly linked to tumor growth suppression. It has been shown that this inhibitory role is indirect *via* the recruitment of mast cells to the tumor environment.[103] Additionally, the attraction of $CD8^+$ cytotoxic T cells in a Ccr6/Ccl20 dependent fashion is a way of Th9 cells to reduce tumor growth.[104] Ccl20 released by epithelia cells, induced by Th9 cells, leads to the attraction of lymphocytes and dendritic cells that are important for tumor control. Both anti-tumor responses are mediated *via* indirect mechanisms of Th9 cells since those cells show no direct cytotoxicity.

Since Tregs are present in the tumor site, protecting it against rejection by the immune system, the role of Th9 cell suppression *via* Tregs was evaluated. Rag2 deficient mice were reconstituted with Ova-TCR transgenic Th9 cells or undifferentiated T cells with or without Ova-TCR transgenic Tregs. T cells and Tregs were isolated and stimulated with plate-bound anti-CD3/anti-CD28 (4 µg/mL/4 µg/mL) under Treg or non-polarizing conditions, or under Th9 skewing conditions for 5 days. The Ova expressing B16F10 melanoma was inoculated on the same day as T cell were transferred *sub cutaneous*. The tumor size was measured over time and mice were sacrificed at the end of the experiment. The draining lymph node was isolated and restimulated. The experimental setup is explained in detail in chapter 2.3.3.3 and workflow described in Figure 8.

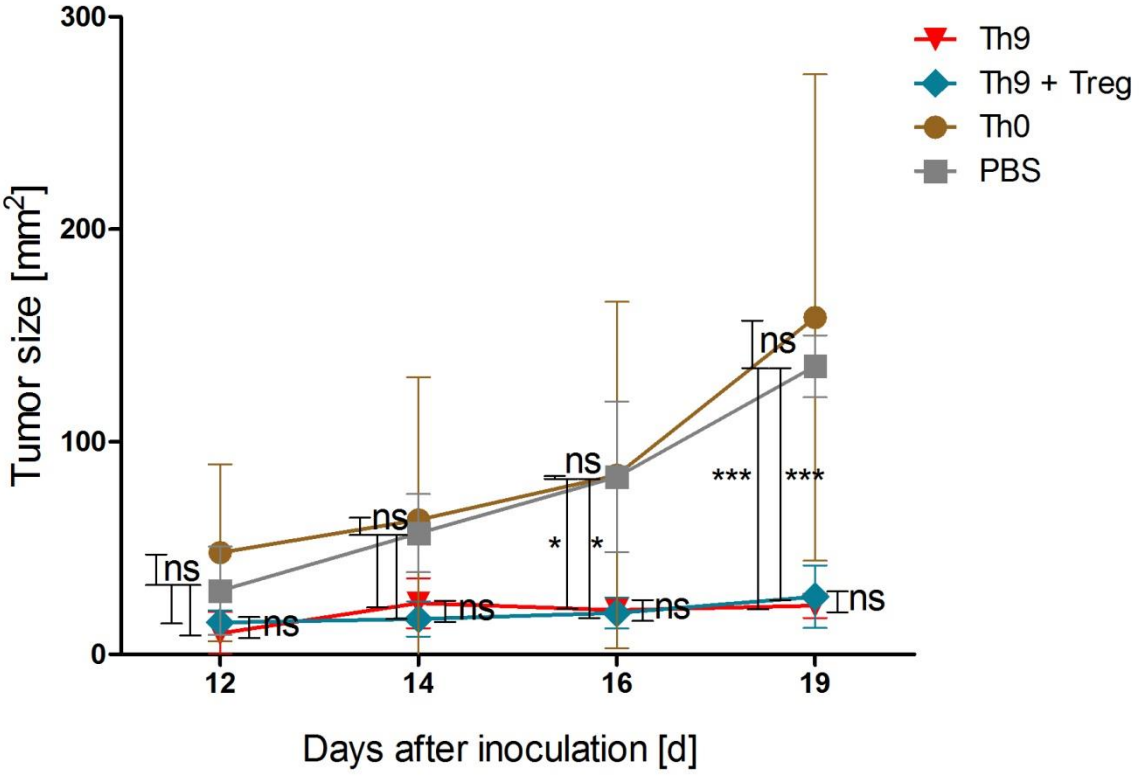

Figure 32 Suppression of Th9 function in melanoma rejection model

Rag1 deficient mice were reconstituted with Ova-TCR transgenic CD4$^+$ T cells of OT-II mice origin. T cells were cultured under Th9 skewing conditions or without cytokines (Th0) for 5 days before 1x10^6 cells were injected with or without equal numbers of OT-II Tregs also stimulated for 5 days under Treg conditions. On the same day 2x10^5 Ova expressing B16F10 melanoma cells were injected s.c. in the flank. Tumor size was measured over the following days. Mice were sacrificed on day 19 of the experiment and the draining lymph nodes isolated and restimulated. Th9 cells showed, even in the presence of Tregs, a reduction in tumor burden. Undifferentiated T cells showed no effect. Mean and standard deviation were analyzed by 2way ANOVA tumor size over time p<0.0001 and transferred T cells p<0.0001. Shown are Bonferroni tests.

The reconstitution of Rag1 deficient mice with Th9 cells is essential for the reduction of tumor size over time. The presence of undifferentiated T cells (Th0) in mice does not lead to tumor reduction compared to untreated Rag1 mice.

Since the phenotype of Th9 cells is essential for melanoma reduction, the suppressive capacities of Tregs on Th9 cells were assessed *in vivo*. The co-transfer of Tregs does not impair the functionality of normal Th9 cells. The co-transfer of Th9 cells together with Tregs does lead to normalization of tumor growth compared to undifferentiated Th0 cells. Tregs do not affect the Th9 tumor immunity.

4 Discussion

Since the identification of the first two different T helper cell subsets, Th1 and Th2 by Mossman and Coffman, additional subsets have been identified. The description of IL-9 producing T cells after stimulation was a first hint for a new distinct T helper subset in the early 90s.[51–54] In recent years this T helper cell subset was a focus in T helper cell research. It was shown that the redifferentiation of Th2 cells under the influence of TGF-β also leads to IL-9 producing T cells.[56] This and the ability to directly identify IL-9 producers *in vitro* that do not co-express Th2 signature cytokines like IL-4 *via* specific antibody staining, led to the description of a new T helper cell subset distinct from Th2 cells. The former Th2-related cytokine IL-9 could then be designated to the so called Th9 cells.[57] The final conclusion that Th9 cells are an IL-9 producing T helper cell subset was shown by two independent groups, identifying the pivotal role of the transcription factors IRF4 and PU.1 in Th9 development.[62,63] One functional key aspect of Th9 cells is the differentiation and expansion of mast cells in different organs by the secretion of IL-9.[54,187] There are strong hints that in asthmatic patients IL-9 producing T cells are key players of the disease, leading to infiltration of immune cells into the lung.[86,87,94,95] By contrast, in melanoma Th9 cells have a protective role by attracting mast cells, dendritic cells and $CD8^+$ T cells that control tumor growth.[103,104] Since in asthma and in cancer the immune system is deregulated, the interaction between Th9 cells and Tregs is of crucial interest.

4.1 Suppression of Th9 development by Tregs

4.1.1 *In vitro* suppression of Th9 cell development by Tregs

The ability of Tregs to inhibit IL-9 release during Th9 development was shown in Figure 9, as was already demonstrated for other T helper cell subsets.[185,186] This ratio dependence can also be expected *in vivo*, although lower frequencies of Tregs might be required to inhibit T cell commitment *in vivo*.[188] It has been reported that Th9 cells are able to produce IL-10 after stimulation under Th9 skewing conditions. Although ratio dependence could not be demonstrated, Tregs are able to abolish IL-10 release when being present in equal numbers (Figure 10).[56] This data demonstrate that the Treg-mediated suppression during the development of Th9 cells is not specific for a certain cytokine, rather it affects several or all effector components. The same observations were previously made in Th1 and Th2 cells.[185,186]

4.1.2 Mechanisms of suppression

The observation that Tregs are able to inhibit T helper cell commitment was described earlier for Th1 and Th2 cells.[186] Activated T cells release high amounts of IL-2 to induce their

proliferation after stimulation in an autocrine manner. The expression of IL-2, as well as upregulation of the IL-2 receptor complex (IL-2Rα,β,γ), leads to the formation of a positive feedback loop causing extensive T cell proliferation. To inhibit IL-2-mediated T cell proliferation at early stages, Tregs constantly express low amount of the IL-2 receptor α-chain to reduce the amount of available IL-2.[133,161,162] Proliferation experiments (Figure 11) revealed that responder T cells are still able to proliferate in the presence of Tregs. Although IL-2 deprivation is a common suppression mechanism of Tregs and *Il2* mRNA expression is reduced after 24 h as shown by NGS analysis (Figure 21 A), the impact on Th9 cell proliferation seems to be mild. This mild effect on cell proliferation might be explained by residual IL-2 that is not absorbed by Tregs. Also IL-4 that is also present in the Th9 culture conditions can compensate for the missing IL-2.[189]

IL-2 deprivation is not the only known suppressive mechanism of Tregs.[163] By blocking different suppressive mechanisms neither the inhibition of IL-10 nor CTLA-4 could abolish Treg-mediated suppression of Th9 development (Figure 12).[135,154] Only rising levels of cAMP in target cells either mediated by direct transport *via* gap junction or *via* CD73 and CD39 could inhibit Th9 commitment.[150,151,158] To induce intracellular rise of cAMP, the cAMP analog dbcAMP was used (Figure 13) and able to inhibit Th9 commitment. This result suggests that the rise of intracellular cAMP in target cells induced by Tregs is one of the key inhibitory mechanisms in Th9 cell suppression. Probably the cAMP is directly transferred to the target cells *via* gap junctions. The suppression *via* the A2A Adenosine receptor is rather unlikely since its expression is not detectable until day 4 after T cell activation.[158] Suppression by cAMP could possibly be mediated on transcriptional level by inducible cAMP early repressor (ICER). ICER competes for the same DNA binding sites as the transcription factor cyclic AMP response element binding protein (CREB). Through the cAMP-mediated expression of ICER, CREB is no longer able to recruit CREB binding proteins to the DNA and thereby induce the transcription of target genes, like the T cell growth factor IL-2.[150,190,191] To test this hypothesis, T cells from ICER deficient mice could be used in co-culture experiments. T cell activation should no longer be suppressed *via* Tregs through cAMP-mediated signaling and differentiation towards the Th9 lineage should be possible.

4.1.3 Treg-induced transcriptional changes in developing Th9 cells

On the one hand T helper cell subsets are defined by their signature cytokines and on the other hand by subset-specific master transcription factors. To check whether Tregs inhibit Th9 development by altering the expression profile of Th9 cells, mRNA levels of *Irf4* and *Sfpil1* (PU.1) in responder T cells were analyzed by qRT-PCR. *Irf4* mRNA expression in suppressed Th9 cells did not differ in any way from those in normal developing Th9 cells (Figure 15C), while *Sfpi1* mRNA (PU.1) expression (Figure 15B) revealed a drastic increase in *Sfpi1* (PU.1) levels of around 50 fold in suppressed Th9 cells. Unchanged IRF4 levels

could be confirmed by Western Blot analysis (Figure 16). The unchanged level of IRF4 suggests that Th9 development requires further transcription factors in addition to IRF4. This is supported by the fact that IRF4 in combination with other transcription factors is an important regulator of different T helper cell subsets, e.g. IRF4 in combination with GATA3 drives Th2 development, while IRF4 in combination with RORγT drives Th17 development.[35,192] This suggests that the role of IRF4 is dependent on the subset-specific transcription factors also expressed during T helper cell differentiation, as shown for Th17 cells.[193–195]

The other transcription factor important for IL-9 production during restimulation is ETS-family transcription factor PU.1. However the group of Kaplan did not include indications for the role of PU.1 during Th9 priming.[63] Other publications by the same group showed epigenetic effects of PU.1 in IL-9 regulation by changing the accessibility of the *Il9* gene through recruitment of histone modifying enzymes, but not during Th9 development.[196]

The expression of *Maf* (c-Maf) was upregulated in the presence of Tregs (Figure 17A) and increased over time when compared to normal developing Th9 cells. The increase of c-Maf in responder T cells is known to upregulate IL-10 production in T cells, by directly binding to the *Il10* promoter.[197] While no IL-10 was determined in the co-culture experiments using ELISA (Figure 10), the *Il10* promoter could be negatively regulated by an unknown factor or because other factors needed for its activation are not present. Additionally, no *Il10* mRNA expression was found in preliminary experiments in suppressed Th9 cells. Although c-Maf might not be crucial for Th9 development, it might have a potential function in IL-9 regulation.

To identify additional transcription factors important for Th9 development or Treg-mediated suppression of Th9 development, transcriptome analysis using NGS was performed of developing Th9 cells co-cultured with or without Tregs.

4.1.4 *In silico* analysis of Treg-mediated suppression of Th9 development using Next Generation Sequencing (NGS)

NGS allows the analysis of the whole transcriptome of a given cell at a distinct time point by sequencing and mapping transcribed mRNA. Although regulation *via* phosphorylation or epigenetic mechanisms cannot be detected, much information can be gained using this approach. This *in silico* analysis focused on the change in the transcriptome of naïve T cells cultured alone or in the presence of Tregs under Th9 skewing conditions, thus revealing the impact of Tregs on Th9 development. A suitable time point for this analysis was found to be 24 h after T cell activation, because this ensured *Il9* mRNA expression being detectable in normal developing Th9 cells while it can be expected that early transcribed genes regulating IL-9 suppression were still expressed.

Confirming the separation protocol for responder T cells, suppressed developing Th9 cells show less *Foxp3* expression than normal developing Th9 cells. The expression of *Foxp3*

upregulated in the Th population that developed under Th9 skewing conditions is probably due to co-induction of iTregs because of the presence of TGF-β.[125] The results confirm that virtually no contaminating Tregs from the Treg-Th9-co-culture were present during NGS analysis. As expected, the *Il9* mRNA levels were downregulated in suppressed Th9 cells, while being strongly expressed in normal developing Th9 cells.

Overall, nearly 9000 transcribed genes were detected in the NGS analysis (Figure 19). 161 met the criteria for differential expression, 84 were downregulated in developing Th9 cells from co-culture with Tregs; while 77 were upregulated when compared to normal developing Th9 cells. The transcribed genes were categorized into three groups according to their potential function: cell proliferation (Figure 20), immunological function (Figure 21), and unknown function.

4.1.4.1 Tregs suppress the expression of cell cycle genes in developing Th9 cells

Suppressed Th9 cells from Treg co-culture showed lower expression of genes that are associated with cell cycle progression than normal developing Th9 cells. This is not very surprising since Tregs in general are able to inhibit activation and proliferation of their target cells. Although the proliferation of suppressed Th9 cells was found to be only marginally affected, the underlying changes in the transcriptome seem to be more dramatic. It has to be considered that the results were obtained from co-culture without precedent synchronization leading to a rather complex pattern of gene expression. Below some examples of differently transcribed genes are given. Overall, it can be concluded that transcribed genes important for cell proliferation are downregulated in suppressed Th9 cells, while transcription of genes associated with cell cycle arrest are upregulated. The transcription of the *centromere protein P* (*Cenpp*) is downregulated in suppressed developing Th9 cells. It is part of a complex that is crucial for the assembly of the kinetochore and thereby plays a role in mitotic progression.[198,199] Other downregulated genes are important for membrane production like *stearoyl-coenzyme A desaturase 1* (*Scd1*) or nucleotide production like *ribonucleotide reductase M2* (*Rrm2)*.[200,201]

By contrast, a few transcribed genes that are negatively associated with progression of the cell cycle were found to be upregulated (Figure 20B). Genes like, *cyclin G2* (*Ccng2*) and *dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1b* (*Dyrk1b*) are known to inhibit cell cycle progression directly.[202–204] Thus, the genes negatively associated with cell proliferation that are expressed in suppressed T cells exhibit direct as well as indirect repressive effects for cell mitosis.

4.1.4.2 Tregs lead to the differential expression of genes in developing Th9 cells connected to immunological functions

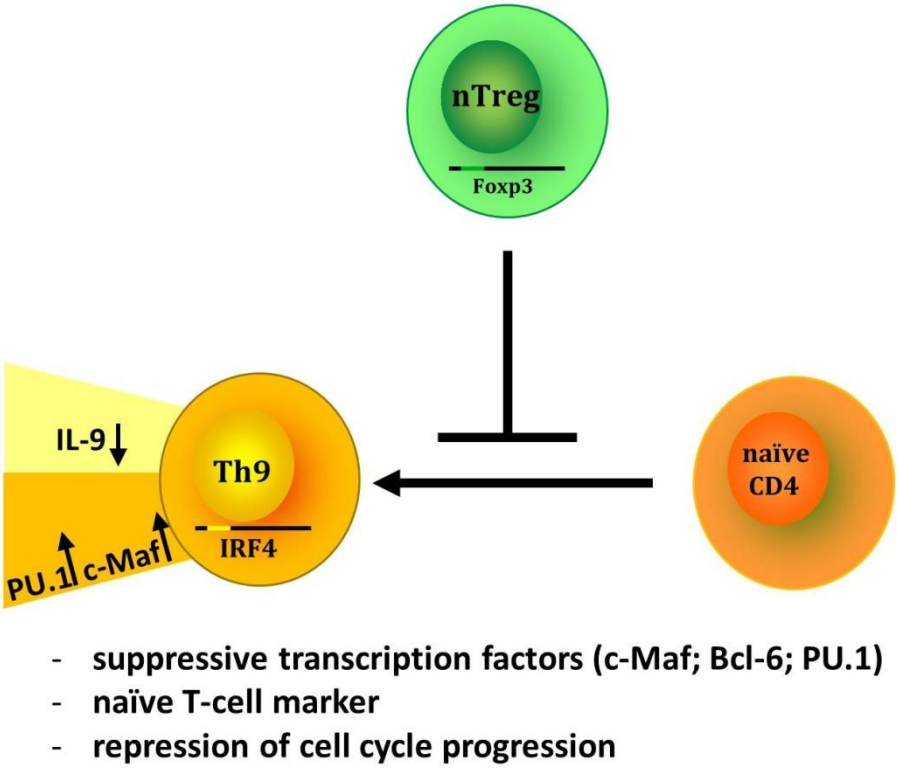

Figure 33 Suppression of Th9 development
Tregs are able to suppress naïve CD4$^+$ T cell differentiation in Th9 cells, possibly by the induction of PU.1 and c-Maf expression. IRF4 levels remain unaltered while IL-9 production by T cells is abrogated.

The expression of some genes that were found by NGS analysis to be suppressed in developing Th9 cells from co-culture with Tregs were validated by qRT-PCR. Unvalidated candidates like *Il2*, *Il7 receptor* and *Pyhin1* have plausible function in Treg-mediated suppression. Especially *Il9* expression was reduced in agreement with previous results. *Il2* expression was downregulated four fold in developing Th9 cells isolated from Treg co-cultures. This reduction might be responsible for the repression of IL-9 expression, since it has been shown that IL-9 production is dependent on endogenous IL-2.[55] *Il2* expression might be reduced due to high levels of transferred cAMP to responder T cells from Tregs *via* gap junctions.[150] Inside the target cell cAMP can abrogate T cell activation either by ICER induction as described earlier or abrogate T cell activation *via* the activation of the Protein Kinase A (PKA) after cAMP binding. The catalytic subunit of PKA is able to phosphorylate the C-terminal Src kinase (Csk). Csk subsequently inhibits the lymphocyte-specific protein tyrosine kinase (Lck), thereby abrogating the signaling cascade after T cell activation *via* the T cell receptor. (reviewed by Taskén)[205]

Another gene found to be upregulated in the presence of Tregs was the *Il7 receptor*. The IL-7 receptor was found to be preferentially expressed in naïve T cells and being downregulated

in T cells after activation via the TCR.[206,207] Therefore, it seems that at the time of investigation, at least some of the T cells from co-culture with Tregs retained their naïve T cell profile when compared to normal developing Th9 cells. A gene with a largely unknown function seems to be potentially interesting. *Pyrin And HIN Domain Family, Member 1* (*Pyhin1)* expression, which was upregulated in the presence of Tregs, was shown to be linked to asthmatic diseases when mutated in individuals of African descent and its gene family is believed to play a role in autoimmunity.[208] Thereby *Pyhin1* seems to link asthmatic diseases to IL-9 deregulation.

Five genes from those found by NGS analysis could be identified on the transcriptional level by qRT-PCR to be upregulated in T cells co-cultured with Tregs during the three subsequent days of co-culture, namely *Bcl6*, *Itgae*, *Nod1*, *Scg5* and *Cxcr4*.

It was demonstrated for Th2 cells that Bcl-6 is able to inhibit the development of this T helper cell lineage. Bcl-6 is able to bind to the *Il4* promoter with a specificity that blocks Stat6 from binding to the promoter. Thereby Bcl-6 is able to inhibit the IL-4 positive feedback loop.[209] Also Bcl-6 can directly inhibit IRF4 positive regulation of transcription by altering IRF4 binding to target sequences or recruitment of other suppressive factors to the target promoter, as shown for B cells.[210] The upregulation of *Itgae* expression also known as CD103 in suppressed Th9 is interesting, since it is associated with the homing of T cells into the gut associated lymphoid organs.[211,212] A functional role of suppressed Th9 cells might be homing into different organs. Due to the anti-inflammatory milieu in the lymphoid organs of the gut, the T helper subset stability may be attenuated leading to re- and transdifferentiation.[213] Meanwhile, this plasticity has been described for all T helper subsets (Figure 1).[8] This possible rediffentiation of Th9 cells into iTreg *via* a strong anti-inflammatory milieu might further contribute to tolerance induction.[214] The identification of *Nod1* in suppressed Th9 cells might be associated with Treg-induced apoptosis, since Nod1 is known to induce apoptosis in a caspase-9-dependent manner in T cells.[215]

In order to gain further insight into the complex interaction of transcription factors that were found to bind to the *Il9* promoter, reporter gene assays have been performed. IRF4 overexpression in this reporter gene assay showed an upregulation of *Il9* promoter activity, while c-Maf overexpression minimally and PU.1 overexpression strongly suppressed *Il9* promoter activity (Figure 24). The downregulation of IL-9 expression through c-Maf has already been reported by Goswami in a cell based model. It was shown that Th9 cells overexpressing c-Maf produced significant lower amounts of IL-9, confirming the results from the reporter gene assay.[65] Although the combined influence of the transcription factors on *Il9* promoter has not been investigated in this thesis, interactions of the different transcription factors have been described. It has been shown that IRF4 and PU.1 interact with each other in B cells leading to repressed *immunoglobulin* λ expression.[216] Their combined binding to

DNA was also shown *in silico*.[217] Other interactions associated with transcription factors identified in developing Th9 cells from co-culture with Tregs were described earlier. c-Maf, IRF4 and NFATc2 are also able to synergistically enhance *Il4* expression. PU.1 and Bcl-6 interaction have repressive function during gene expression in B cells, while c-Maf and Bcl-6 positively enhance Tfh differentiation.[37,218,219] One can assume that the composition of the *Il9* promoter complex changes: In the presence of Tregs a complex that represses *Il9* transcription is formed, while without Tregs a complex that promotes gene transcription is present. Some of the transcription factors might be present in both, while others are only expressed in specific transcriptional states.

4.2 Inefficient suppression of Th9 effector function by Tregs

Freshly isolated $CD4^+$ T cells need several days to proliferate and produce cytokines after primary activation. By contrast, established Th9 cells produce cytokines with a comparatively rapid kinetics. Therefore it was questionable whether freshly isolated Treg cells, although activated together with Th9 cells, were able to inhibit the cytokine production of Th9 cells. To get a first impression on the susceptibility of Th9 cells to Treg-mediated suppression, freshly isolated and preactivated Tregs were used in a suppression assay. It was published with regard to Th2 cells that preactivated Tregs are able to control their effector function, while freshly isolated Tregs were unable to suppress Th2 cells.[186] Concerning Th9 cells, freshly isolated Tregs, which are known to have a comparatively low suppressive potency could not inhibit IL-9 production, whereas preactivated Tregs were able to suppress the production of IL-9 by around 50 % (Figure 25).[133] Hence, it was concluded that Th9 cells are to some extent resistant to Treg-mediated suppression (Figure 34). In an antigen specific setting with BMDCs being more comparable to the *in vivo* system, the IL-9 release of Th9 cells was also reduced by 50 % in the presence of equal numbers of Tregs (Figure 27A). In contrast, the production of IL-4 by Th2 effector cells was completely suppressed (Figure 27B), demonstrating the functionality of the Tregs.

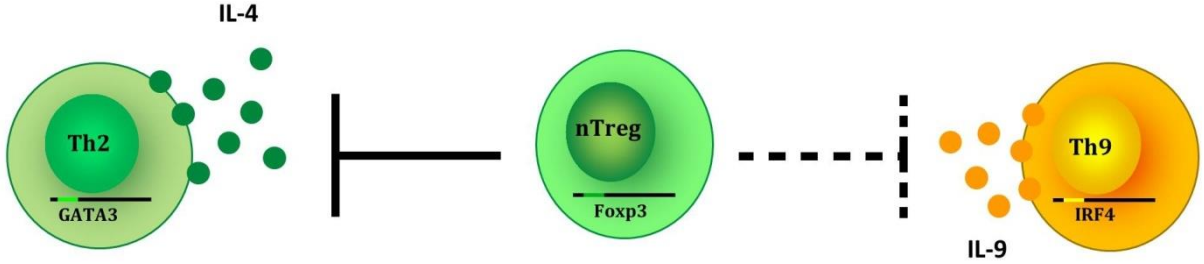

Figure 34 Suppression of T helper cell effector function
Tregs are only partially able to suppress the effector function of Th9 cells. cAMP mediated suppression seems to be disrupted. On the contrary Th2 effector function could be blocked *in vitro* as well as *in vivo.*

To test which suppressive mechanisms were responsible for this partial inhibition of Th9 cells, a suppression assay was done with the blockade of IL-10R signaling and CTLA-4 signaling but showed no changes in IL-9 production (data not shown). The presence of the cAMP analog dbcAMP during restimulation of Th9 cells could only reduce the IL-9 production by around 50 % at high concentrations (Figure 28), while in freshly isolated $CD4^+$ T cells this concentration can totally abrogate T cell-derived IL-2 production. Obviously the key mechanism of cAMP-mediated suppression is functionally impaired in Th9 cells (Figure 28). The impaired suppressive mechanisms in Th9 cells might be the result of an insufficient suppressive influence of ICER as a mediator of cAMP induced suppression, that was described earlier. Since suppression *via* ICER is mediated by competition for DNA binding sites with CREM, higher amounts of CREM reported in fully committed Th9 cells might be a reason for poor cAMP suppressive function *via* ICER.[220] The finding that the transcriptional profile of established Th9 cells with regard to IRF4, PU.1 and c-Maf was not altered by Tregs also supports the observation that Tregs are not able to interfere with basic Th9 effector functions (Figure 29).

4.2.1 No suppression of Th9 cells *in vivo*

The suppressive potency of Tregs for Th9 effector functions was further analyzed in two murine *in vivo* models: experimental asthma and melanoma tumor. Concerning the acute asthma model, the functionality of Th9 effector cells *in vivo* was not significantly compromised by Tregs. Acute asthma caused by Th2 cells was significantly reduced by Tregs shown by lower airway resistance and lower compliance (Figure 30A+B). These results demonstrate that the Tregs used had suppressive capacities. This is in agreement with the finding that remodeling of asthmatic lung tissue can be reduced by Tregs, when Th2 cells induce the disease.[185] Nevertheless when Th9 cells are mediators of the disease, the progression is not ameliorated in the presence of Tregs. Although Tregs reduced the number of lymphocytes in the lung of mice with Th9-induced asthma, they could not inhibit typical symptoms of asthma as in the case of asthma caused by Th2 cells (Figure 31). The inability of Tregs to suppress Th9 cells might also be a result of the fact that cAMP can enhance IL-9 production. It was shown that through elevated cAMP levels the activated PKA is able to repress the functionality of the glycogen synthase kinase (GSK)–3β, thus preventing the export of NFATc2 from the nucleus and thereby promoting the binding of NFATc2 to the *Il9* promoter. PKA is also able to induce higher expression of PU.1.[221] This might stabilize the Th9 subset and induce higher IL-9 production in the presence of Tregs, thus at least partially compensating their suppressive influence.

While IL-9 in asthma has a pathological role, IL-9 derived from Th9 cells has been proven protective in melanoma.[78,80,81,103,104] Tumor antigen specific Th9 cells in melanoma models are believed to attract mast cells, dendritic cells and $CD8^+$ T cells to the tumor *via* IL-9

production and Tregs might impair this protective role.[54,187] However, in analogy to the murine model of experimental asthma the protective effects of Th9 cells were found not to be inhibited by Tregs (Figure 32). Obviously a partial reduction of Th9-derived IL-9 could not prevent IL-9-mediated tumor immunity.

In summary, this thesis demonstrates that Tregs are potent inhibitors of Th9 development while IL-9 production of established Th9 cells was only partially suppressed. Correspondingly, IL-9-derived pathologic and protective effects of Th9 cells *in vivo* could not be inhibited by Tregs. Nevertheless it can be expected that Tregs can indirectly suppress the function of Th9 cells in the course of a chronic disease by inhibiting the differentiation of Th9 cells from naïve $CD4^+$ T cells.

5 Abstract

T helper (Th) 9 cells are an important subpopulation of the $CD4^+$ T helper cells. Due to their ability to secrete Interleukin-(IL-)9, Th9 cells essentially contribute to the expulsion of parasitic helminths from the intestinal tract but they play also an immunopathological role in the course of asthma. Recently, a beneficial function of Th9 cells in anti-tumor immune responses was published. In a murine melanoma tumor model Th9 cells were shown to enhance the anti-melanoma immune response *via* the recruitment of $CD8^+$ T cells, dendritic cells and mast cells. In contrast to Th9 effector cells regulatory T cells (Tregs) are able to control an immune response with the aid of different suppressive mechanisms. Based on their ability to suppress an immune response Tregs are believed to be beneficial in asthma by diminishing excessive allergic reactions. However, concerning cancer they can have a detrimental function because Tregs inhibit an effective anti-tumor immune reaction. Thus, the analysis of Th9 suppression by Tregs is of central importance concerning the development of therapeutic strategies for the treatment of cancer and allergic diseases and was therefore the main objective of this PhD thesis.

In general it could be demonstrated that the development of Th9 cells can be inhibited by Tregs *in vitro*. The production of the lineage-specific cytokine IL-9 by developing Th9 cells was completely suppressed at a Treg/Th9 ratio of 1:1 on the transcriptional (qRT-PCR) as well as on the translational level (ELISA). In contrast, the expression of IRF4 that was found to strongly promote Th9 development was not reduced in the presence of Tregs, suggesting that IRF4 requires additional transcription factors to induce the differentiation of Th9 cells. In order to identify such factors, which regulate Th9 development and therefore represent potential targets for Treg-mediated suppressive mechanisms, a transcriptome analysis using “next-generation sequencing” was performed. The expression of some genes which were found to be up- or downregulated in Th9 cells in the presence of Tregs was validated with qRT-PCR. Time limitations prevented a detailed functional analysis of these candidate genes. Nevertheless, the analysis of the suppressive mechanisms revealed that Tregs probably suppress Th9 cells *via* the increase of the intracellular cAMP concentration. In contrast, IL-9 production by differentiated Th9 cells was only marginally affected by Tregs *in vitro* and *in vivo* analysis (asthma, melanoma model). Hence, Tregs represent very effective inhibitors of Th9 development whereas they have only a minimal suppressive influence on differentiated Th9 cells.

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7 List of abbreviations

AHR	airway hyperresponsivness reaction
AIRE	autoimmune regulator
APC	antigen presenting cell
BAL	bronchoalveolar lavage
Bcl6	B cell lymphoma 6 protein
BMDC	bone marrow derived dendritic cells
BSA	bovine serum albumin
cAMP	3'-5'-cyclic adenosine monophosphate
Ccng2	cyclin G2
CD	cluster of differentiation
Cdkn1a	cyclin-dependent kinase inhibitor 1a
cDNA	complementary deoxyribonucleic acid
Cenpp	centromere protein P
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
c-Maf	V-maf musculoaponeurotic fibrosarcoma oncogene homolog
CNS	central nervous system
ConA	concanavalin A
CTLA-4	cytotoxic T-lymphocyte antigen-4
CXCR	C-X-C chemokine receptor type
dbcAMP	dibutyryl cyclic adenosine monophosphate
DC	dendritic cell
dNTP	2'-Desoxynucleosid-5'-Triphosphate
Dyrk1b	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1b
EAE	experimental autoimmune encephalomyelitis
Ebi3	Epstein-Barr-virus induced gene 3
EDTA	disodium ethylenediamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
eomes	eomesodermin
ETS	E-twenty six
FACS	Fluorescent activated cell sorting

FCS	fetal calf serum
Foxp3	forkhead box P3
GATA3	GATA binding protein 3
GFP	green fluorescent protein
GM-Buffer	great MACS buffer
GM-CSF	granulocyte macrophage colony-stimulating factor
H&E	hematoxylin-eosin
Half-MARE	half-Maf recognition elements
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horse reddish peroxidase
ICER	inducible cAMP early repressor
Ier3	immediate early response 3
IFN-γ	interferon γ
IgE	immunoglobulin E
IL10R	interleukin-10 receptor
IMEM	Iscove's modified Dulbeccos's medium
Iono	Ionomycin
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF4	interferon regulatory factor 4
ISO	indoleamine 2,3-dioxygenase
Itgae	integrin, alpha E
iTreg	induced regulatory T cells
LAG-3	lymphocyte activation gene-3
LPS	lipopolysaccharides
MDSC	myeloid-derived suppressor cells
MEA	mast cell growth-enhancing activity factor
MEM	minimal essential medium
MHCII	major histocompatibility complex II
mRNA	messenger ribonucleic acid
mTEC	medullary epithelial cells
NFAT	nuclear factor of activated T cells
NGS	next generation sequencing

Nod1	nucleotide-binding oligomerization domain-containing protein 1
ns	not significant
nTreg	naturally occurring regulatory T cells
Ova	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAMP	pattern recognition receptors
PBS	phosphate buffered saline
PE	phycoerythrin
PKA	protein kinase A
PMA	phorbol 12 myristate 13 acetate
Pou2af1	POU class 2 associating factor 1
Pou6f1	POU class 6 homebox
PVDF	polyvinylidene difluoride
qRT-PCR	quantitave real-time polymerase chain reaction
Rag	recombination activating gene
RIN	RNA integrity number
RNA	ribonucleic acid
RORγt	retinoid-related orphan receptor gamma
RPKM	reads per kilo base per million
Rrm2	ribonucleotide reductase M2
Runx1	runt-related transcription factor 1
SA	streptavidin
SA-HPO	streptavidin coupled horseradish peroxidase
Scd1	stearoyl-coenzyme A desaturase 1
Scg5	secretogranin
SDS	sodium dodecyl sulfat
SFPI1	spleen focus forming virus proviral integration oncogene 1
Socs2	suppressor of cytokine signaling 2
Sox4	sex determining region Y box 4
Stat	signal transducer and activation of transcription
T-bet	T-box transcription factor TBX21

TCGFIII	T cell growth factor III
TCR	T cell receptor
Tfh	follicular T helper cells
TGF-β	transforming growth factor beta
Th	T helper cells
Thy1	thymocyte differentiation antigen 1
TLR	toll-like receptors
TM	testmedium
Tregs	regulatory T cells

8 Curriculum vitae

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9 Danksagung

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10 Erklärung

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