Year : 2015

Between Immunity And Tolerance: Controlling Immune Responses Employing Tolerogenic Dendritic Cells

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Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine de l'Université de Lausanne

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Lausanne, Février 2015

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Madame	Prof. Isabelle **Decosterd**
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le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Sergio Haller

Master of Science en biologie de l' Université de Zürich

intitulée

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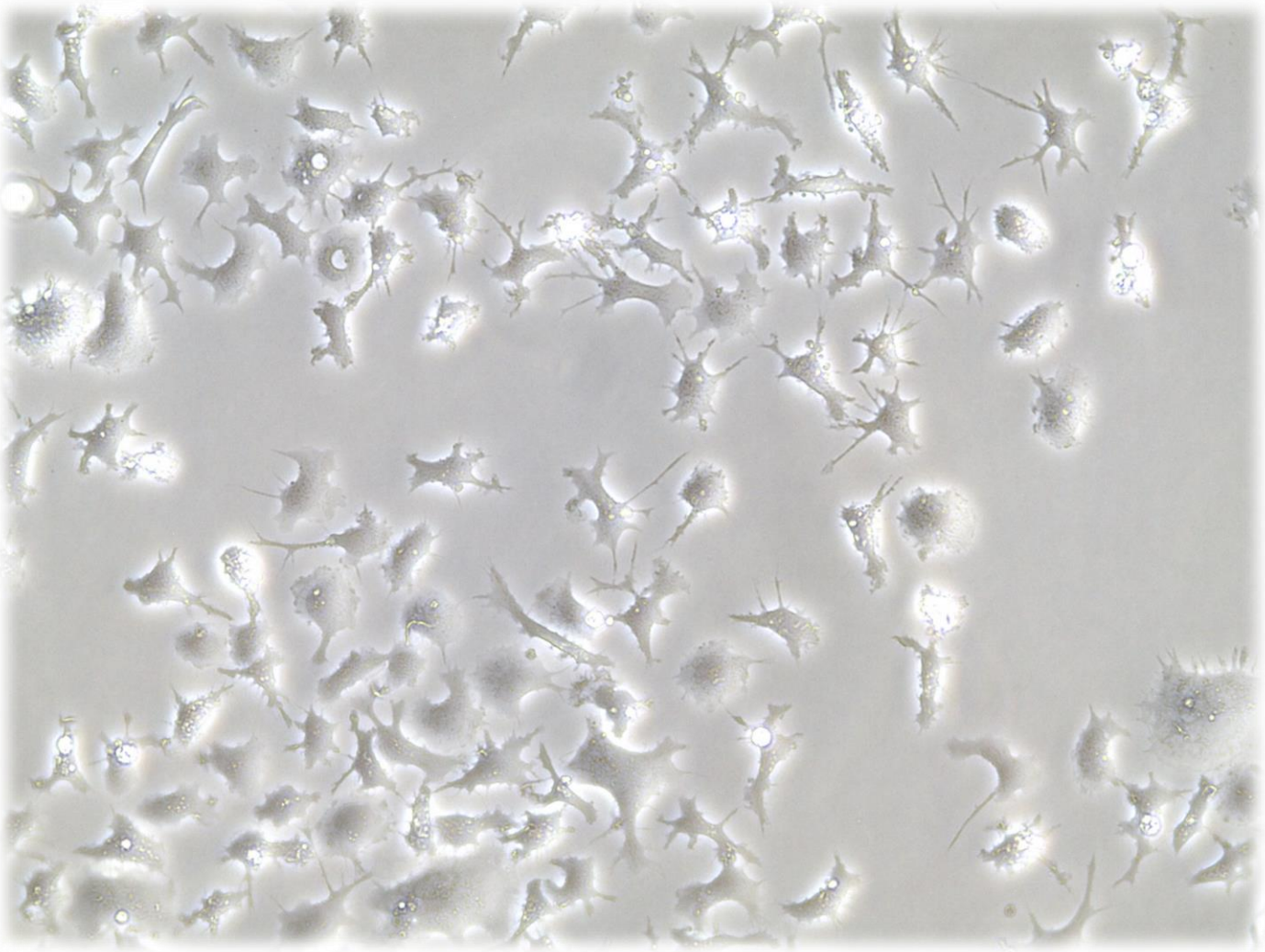
Lausanne, le 9 avril 2015

pour La Doyenne
de la Faculté de Biologie et de Médecine

Prof. Isabelle Decosterd



Für meine Eltern

Résumé Large Public

Les cellules dendritiques sont des cellules du système immunitaire. Dans le corps, les cellules dendritiques circulent continuellement afin de détecter les éléments étrangers. Quand elles reconnaissent une protéine étrangère, c'est-à-dire un antigène, elles l'absorbent, pour ensuite s'activer et subir diverses modifications afin de pouvoir la présenter aux lymphocytes T. Les lymphocytes T sont d'autres cellules du système immunitaire. Elles sont capables de mettre en place une réponse immunitaire adaptive afin de combattre et vaincre une infection. Lors de ce processus, les cellules dendritiques vont interagir avec les lymphocytes T et transmettre les informations nécessaires pour que ces cellules produisent différentes protéines de façon à éliminer le pathogène. En fonction du type de pathogène, les informations échangées entre les cellules dendritiques et les lymphocytes seront différentes de manière à produire la réponse immunitaire la mieux adaptée pour supprimer l'élément infectieux. Les cellules dendritiques ne présentent pas seulement des protéines provenant des pathogènes aux lymphocytes T, mais également des éléments du soi. Les cellules dendritiques doivent alors être capables de différencier les molécules du soi et du non-soi de façon à ne pas induire une réponse auto-immune en présentant un antigène du soi aux lymphocytes T. D'autant plus que lors de leur développement, les lymphocytes T capables de reconnaître le soi sont éliminés. Cependant, ce système n'est pas parfait, en conséquence, certains lymphocytes T auto-réactifs peuvent se trouver dans le corps. C'est pourquoi, il existe d'autres mécanismes en périphérie du site de développement pour inhiber ces lymphocytes T auto-réactifs. Ce sont les mécanismes de tolérance. Les cellules dendritiques détiennent par conséquent une fonction essentielle non seulement dans le but de initier une réponse immunitaire contre des pathogènes mais aussi pour prévenir une réaction contre soi-même.

Dans ce projet de recherche, nous avons travaillé avec des lignées de cellules dendritiques, c'est-à-dire des cellules dendritiques semblables à celles que l'on peut trouver chez l'homme ou les autres animaux vertébrés. Néanmoins, étant immortalisées, elles peuvent être cultivées et manipulées dans in environnement artificiel (*in vitro)*. Nous avons génétiquement modifiés ces lignées cellulaires pour qu'elles expriment des molécules immunosuppressives ainsi que des molécules pro-inflammatoires. Ceci nous permet d'étudier si l'expression de ces molécules génère ou, au contraire, évite une réponse immunitaire. Pour cela, nous avons testé *in vitro* si ces lignées dendritiques sont capables de supprimer ou augmenter l'activation des lymphocytes T. Dans un deuxième temps, nous avons utilisé des modèles murins de tumeurs et de maladies auto-immunes. Nous avons démontré que l'expression des molécules immuno-modulatrices par les cellules dendritique empêchent le développement des maladies auto-immunes. En revanche, en

utilisant les cellules dendritiques pour augmenter la réponse immunitaire, nous avons pu ralentir la croissance des tumeurs.

Pour résumer, nous avons montré que ces lignées de cellules dendritiques peuvent être un outil de recherche précieux. En effet, grâce à ces lignées, nous aurons la possibilité d'étudier les bénéfices de différentes molécules afin de modifier la réponse immunitaire à notre avantage.

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Resumé

Les cellules dendritiques (CD) jouent un rôle central dans l'élaboration des réponses immunitaires adaptatives de par leur capacité à présenter des antigènes, fournir un signal de costimulation et à sécréter des cytokines. En outre, elles sont importantes pour induire et maintenir la tolérance centrale et périphérique en induisant l'anergie, en supprimant les lymphocytes effecteurs ou en induisant des cellules T régulatrices.

Une lignée cellulaire de cellules dendritiques murines CD8α$^+$ (MuTu) a précédemment été dérivée et décrite dans notre laboratoire. Il a été démontré que les cellules de cette lignée MuTu possède les caractéristiques phénotypiques ainsi que fonctionnelles des cellules dendritiques CD8α$^+$ endogènes. En particulier, elles ont maintenu la capacité de la présentation croisée (cross-présentation) des antigènes exogènes aux cellules T CD8$^+$, et l'aptitude à produire de l'interleukine 12 (IL-12) suite à l'activation des récepteurs Toll like. Cette lignée cellulaire constitue une source infinie de cellules dendritiques homogènes et bien définies. Cela nous permet d'étudier le rôle et le potentiel de molécules spécifiques dans l'induction ainsi que dans la régulation des réponses immunitaires par les CDs de manière rationnelle et standardisée.

Dans un premier projet, la lignée de cellules dendritiques MuTu a été génétiquement modifiée afin d'exprimer de façon stable les molécules immunosuppressives interleukine (IL) -10, IL-35 et TGF-β (respectivement IL-10$^+$DC, IL-35$^+$DC ou actTGFβ$^+$DC). Nous avons étudié la capacité de ces cellules dendritiques, potentiellement suppressives ou tolérogènes, à induire une tolérance immunitaire dans le but d'explorer les mécanismes se cachant derrière ce phénomène. L'expression de TGF-β par la lignée dendritique exprimant cette cytokine n'a pas affecté sa propre maturation. Lors de leur 'activation, les IL-10$^+$ et IL-35$^+$DCs présentaient un taux plus faible de CMH (complexe majeur d'histio-compatibilité) classe I et II, ainsi qu'un niveau plus bas de molécules de costimulation. D'autre part, une réduction de la sécrétion de cytokines pro-inflammatoires a été observée.

Lors de la co-culture *in vitro* entre les lymphocytes T CD4$^+$ / CD8$^+$ et les IL-35$^+$ DC, les IL-10$^+$ DC ou les active TGFβ$^+$ DC, la fonction et la prolifération des cellules T se sont trouvées altérées. De plus, les lignées dendritiques produisant de l'IL-35 et/ou du TGF-β actif ont induit un phénotype régulateur sur les cellules T CD4$^+$ *in vitro*, avec ou sans expression de FoxP3. Dans d'autres modèles murins de cancer, la vaccination au moyen de CD exprimant l'IL-35 ou le TGF-β actif a entraîné une croissance plus rapide de la tumeur. Fait intéressant, cette croissance accélérée observée lors de l'injection de IL-35$^+$ DC a pu être retrouvée dans des souris RAG$^{-/-}$, déficientes en cellules T. Curieusement, les vaccins utilisant les CD exprimant l'IL-10 ont plutôt eu l'effet inverse, c'est-à-dire retarder la croissance tumorale. Etonnamment, nous avons trouvé que l'expression d'IL-35 ou l'adjonction d'IL-35 dans le milieu peut prolonger la viabilité des neutrophiles et

augmente la prolifération des cellules endothéliales. Nos résultats indiquent que la cytokine IL-35 pourrait non seulement être un puissant régulateur de la réponse immunitaire adaptative, mais impliquent aussi que l'IL-35 provoque divers effets sur une multitude de cibles cellulaires. Ces capacités font de l'IL-35 une cible prometteuse pour le traitement de maladies auto-immunes ainsi qu'une molécule clef pour améliorer les immunothérapies contre le cancer. En effet, en utilisant les CDs activeTGFβ^{+} nous avons pu inhiber complétement le développement de l'encéphalite auto-immune, alors que avec les IL-35+DC l'on n'a pu que réduire l'incidence et la gravité de la maladie. En outre, il a été démontré que le transfert préventif de IL-35^{+} DC retarde le rejet d'allogreffe cutanée au même niveau que la combinaison de CD exprimant de l'IL-10 et activeTGFβ. Ainsi, l'expression d'un seul type de molécule tolérogène peut être suffisante pour interférer avec l'activation et la fonction adéquate des cellules dendritiques et des lymphocytes T co-cultivées. Les mécanismes de tolérance ne semblent toutefois que partiellement se chevaucher c'est pourquoi l'application d'une combinaison de plusieurs molécules tolérogène pourrait agir en synergie afin de surmonter l' (auto-)immunité.

Dans un deuxième projet, nous avons essayé par deux approches différentes d'augmenter l'immunogénicité des vaccins contre le cancer grâce aux cellules dendritiques. Tout d'abord, la lignée dendritiques MuTu, dérivée de C57BL/6, a été génétiquement modifiée afin d'exprimer la molécule CMH classe I H-2K^{d}. Nous avons émis l'hypothèse que l'expression de CMH classe I haplotype spécifique pour BALB/c (H-2K^{d}) permet à ces CD, une fois injecté dans des souris BALB/c, de stimuler des cellules T CD8^{+} spécifiques contre la tumeur. Effectivement, nous avons pensé qu'un transfert de H-2K^{d+} DC dans des souris BALB/c entraînerait un environnement fortement inflammatoire. Ces cellules pourraient, par conséquent agir en tant qu'adjuvant, ce qui permettrait de lever la suppression immunitaire induite par la tumeur. En utilisant ce que l'on appelle l'approche par vaccination "semi-allogénique", nous pourrions démontrer que l'injection de CD H-2K^{d+} pulsées avec lysat de la tumeur retarde considérablement la croissance tumorale par rapport au vaccin autologue ou allogénique standard. Cependant, nous n'avons pas été en mesure d'élucider les mécanismes cellulaires sous–jacents. Deuxièmement, nous avons généré des lignées dendritiques MuTu exprimant les cytokines pro-inflammatoires IL-2, IL-12 ou IL-15 de façon stable. Nous avons examiné si en combinant vaccination de CD et l'exposition locale aux cytokines pro-inflammatoires, nous pourrions améliorer les réponses T spécifiques contre les cellules tumorales. En effet, nous avons observé une prolifération accrue de cellules T. De plus, celles-ci deviennent activées lorsque cultivées *in vitro* avec des CD exprimant l'IL-12 ou IL-2. Mais nous n'avons malheureusement pas pu observer un impact bénéfique, ou même un effet synergique, sur le développement de la tumeur lorsque l'exposition aux cytokines était combinée avec la vaccination de CD semi-allogéniques.

Abstract

Dendritic cells (DCs) are the most efficient antigen presenting cells, they provide co-stimulation, are able to secrete various proinflammatory cytokines and therefore play a pivotal role in shaping adaptive immune responses. Moreover, they are important for the promotion and maintenance of central and peripheral tolerance through several mechanisms like the induction of anergy or apoptosis in effector T cells or by promoting regulatory T cells.

The murine CD8α$^+$ (MuTu) dendritic cell line was previously derived and described in our laboratory. The MuTu cell line has been shown to maintain phenotypical and functional characteristics of endogenous CD8α$^+$ DCs. They are able to cross-present exogenous antigens to CD8$^+$ T cells and produce interleukin (IL-) 12 upon engagement of Toll like receptors. The cell line constitutes an infinite source of homogenous, phenotypically well-defined dendritic cells. This allows us to investigate the role and potential of specific molecules in the induction as well as regulation of immune responses by DCs in a rational and standardized way.

In a first project the MuTu dendritic cell line was transduced in order to stably express the immunosuppressive molecules IL-10, IL-35 or the active form of TGF-β (termed IL-10$^+$DC, IL-35$^+$DC or actTGFβ$^+$DC). We investigated the capability of these potentially suppressive or tolerogenic dendritic cell lines to induce immune tolerance and explore the mechanisms behind tolerance induction. The expression of TGF-β by the DC line did not affect the phenotype of the DCs itself. In contrast, IL-10$^+$ and IL-35$^+$DCs were found to exhibit lower expression of co-stimulatory molecules and MHC class I and II, as well as reduced secretion of pro-inflammatory cytokines upon activation. *In vitro* co-culture with IL-35$^+$, IL10$^+$ or active TGFβ$^+$ DCs interfered with function and proliferation of CD4$^+$ and CD8$^+$ T cells. Furthermore, IL-35 and active TGF-β expressing DC lines induced regulatory phenotype on CD4$^+$ T cells *in* vitro without or with expression of Foxp3, respectively. In different murine cancer models, vaccination with IL-35 or active TGF–β expressing DCs resulted in faster tumor growth. Interestingly, accelerated tumor growth could be observed when IL-35-expressing DCs were injected into T cell-deficient RAG$^{-/-}$ mice. IL-10expressing DCs however, were found to rather delay tumor growth. Besides the mentioned autocrine effects of IL-35 expression on the DC line itself, we surprisingly observed that the expression of IL-35 or the addition of IL-35 containing medium enhances neutrophil survival and induces proliferation of endothelial cells. Our findings indicate that the cytokine IL-35 might not only be a potent regulator of adaptive immune responses, but it also implies IL-35 to mediate diverse effects on an array of cellular targets. This abilities make IL-35 a promising target molecule not only for the treatment of auto-inflammatory disease but also to improve anti-cancer immunotherapies. Indeed, by applying active TGFβ$^+$ in murine autoimmune encephalitis we were able to completely inhibit the development of the disease, whereas IL-35$^+$DCs reduced disease incidence and severity. Furthermore, the preventive transfer of IL-35$^+$DCs delayed

rejection of transplanted skin to the same extend as the combination of IL-10/actTGF-β expressing DCs. Thus, the expression of a single tolerogenic molecule can be sufficient to interfere with the adequate activation and function of dendritic cells and of co-cultured T lymphocytes. The respective mechanisms of tolerance induction seem to be different for each of the investigated molecule. The application of a combination of multiple tolerogenic molecules might therefore evoke synergistic effects in order to overcome (auto-) immunity.

In a second project we tried to improve the immunogenicity of dendritic cell-based cancer vaccines using two different approaches. First, the C57BL/6 derived MuTu dendritic cell line was genetically modified in order to express the MHC class I molecule H-2K^{d}. We hypothesized that the expression of BALB/c specific MHC class I haplotype (H-2K^{d}) should allow the priming of tumor-specific CD8^{+} T cells by the otherwise allogeneic dendritic cells. At the same time, the transfer of these H-2K^{d+} DCs into BALB/c mice was thought to evoke a strong inflammatory environment that might act as an “adjuvant”, helping to overcome tumor induced immune suppression. Using this so called “semi-allogeneic” vaccination approach, we could demonstrate that the delivery of tumor lysate pulsed H-2K^{d+} DCs significantly delayed tumor growth when compared to autologous or allogeneic vaccination. However, we were not able to coherently elucidate the cellular mechanisms underlying the observed effect. Second, we generated MuTu DC lines which stably express the pro-inflammatory cytokines IL-2, IL-12 or IL-15. We investigated whether the combination of DC vaccination and local delivery of pro-inflammatory cytokines might enhance tumor specific T cell responses. Indeed, we observed an enhanced T cell proliferation and activation when they were cocultured *in vitro* with IL-12 or IL-2-expressing DCs. But unfortunately we could not observe a beneficial or even synergistic impact on tumor development when cytokine delivery was combined with semi-allogeneic DC vaccination.

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I. Introduction

1 General Introduction

1.1 A brief introduction to the immune system

Our body is constantly challenged with infectious pathogens like bacteria, viruses, fungi and other parasites. It further has to cope with stressed and aberrant, potentially tumorigenic cells. These constant threats led to the evolution of a wide variety of molecules, specialized cells and physical structures specifically adapted to detect and protect from infectious pathogens. Together, these components constitute the immune system. In order to elicit functional immune responses while avoiding reactivity to self, the components have to operate in a highly coordinated manner. This is accomplished by a high level of organization and regulation of the different components of the immune system. The immune system can roughly be subdivided in two overlapping and interacting systems: The innate or nonspecific and the adaptive or acquired immunity.

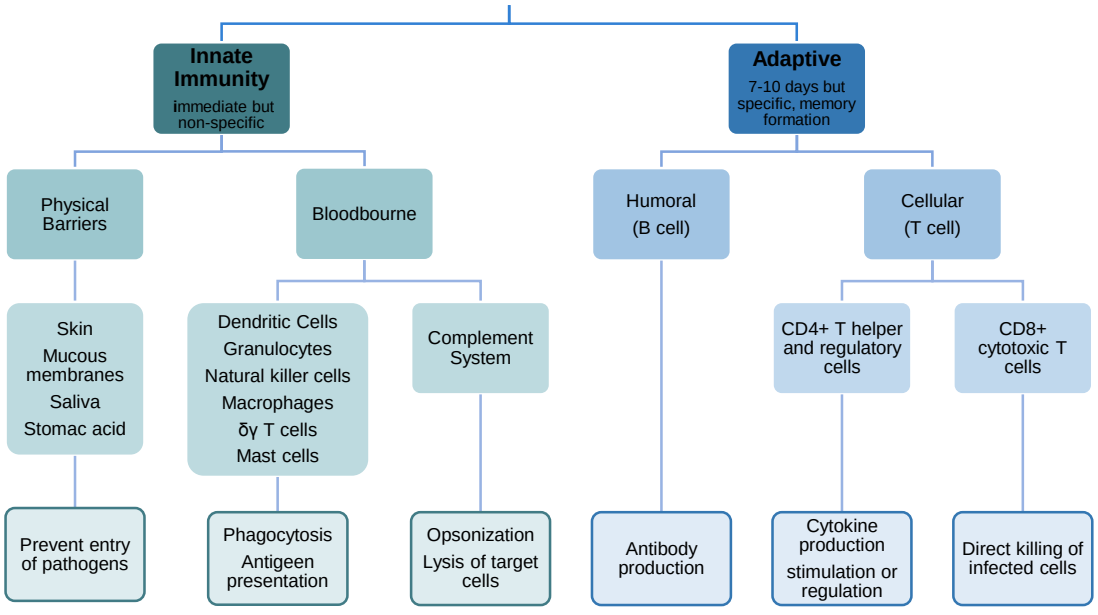


Figure 1 Schematic overview over the components and functions of the innate and adaptive immune system

1.2 Innate immune system

Primitive immune systems co-evolved along with infectious microbes during evolution. Specific mechanisms preventing microbial infections can therefore be found in all multicellular organisms. Transplantation experiments with *porifera* have shown that phagocyte-like cells attack foreign tissue transplants while grafts from the same colony are not rejected. These finding indicating that already evolutionary primitive organisms express molecules that allow them to distinguish between self and foreign [1].

In order to enter the body, microorganisms have to overcome a first line of defense which is composed of the epithelial lining covering the surfaces of all animals. The tight junctions connecting epithelial cells impose a physical barrier. Furthermore, epithelial cells of mucosal surfaces can secrete antimicrobial peptides like defensins and cathelicidins [2].

Second line of innate defense comprises bone-marrow derived phagocytic cells including granulocytes, monocytes, macrophages and dendritic cells. The primary function of these cells is to identify, ingest and destroy microbes as well as to secrete inflammatory cytokines. Innate immune, endothelial and epithelial cells and some other cell types express germline–encoded pattern recognition receptors (PRR). PRRs allow to recognize conserved microbial motifs called pathogen associated molecular pattern (PAMPs) as well as danger associated molecular pattern (DAMPs) which are released by stressed and injured cells. The nature of PAMPs and DAMPs recognized by innate receptors is very diverse and includes proteins such as flagellin, nucleic acid motifs or lipopolysaccharides (LPS). The receptors can be classified into several major groups, including Toll-like receptors (TLR), Nod-like receptors (NLR), C-type lectins (CLR), RIG-I-like helicases (RLH) and others. PRR engagement leads to increased efficiency of intracellular killing, augments the production of antimicrobial agents and facilitate the secretion of pro-inflammatory cytokines. In addition to cell mediated recognition of PAMPs, several soluble PRRs have been described that circulate in blood and extracellular fluids. Engagement of these receptors triggers the activation of proteins of the complement system. Complement activation promotes the killing of microbes either by opsonization or by pore formation and induces inflammatory responses.

Natural killer (NK) cells are derived from a common NK/T lymphocyte precursor but are classified as a part of the innate immunes system because they express only invariant, germline-encoded receptors. The primary function of NK cells is to recognize and kill virus infected, stressed and tumor cells and are a major source for IFN-γ.

In addition to their anti-microbial functions, cells of the innate immune system have also been shown to exert regulatory functions. Several innate cell types, including dendritic cells and innate lymphoid cells, are involved in the maintenance of tolerance against commensal bacteria in the

intestine [3]. Moreover, in pathologic conditions like chronic infection and cancer, M2 alternatively activated macrophages and monocyte or granulocyte derived myeloid derived suppressor cells (MDSCs) have been found to modulate immune responses. The mechanisms by which innate cells suppress immunity will be discussed further below.

1.3 The Adaptive Immune System

Jawed vertebrates have developed an additional, more powerful defense system; the adaptive or acquired immune system. Two defining characteristics distinguish the adaptive from innate immunity: *specificity* and *memory*.

The key players of adaptive immunity are termed B and T lymphocytes. B lymphocytes mediate humoral responses through the secretion of antibodies that opsonize or neutralize pathogens in the blood and other body fluids. T lymphocytes mediated cellular immunity involves the secretion of inflammatory cytokines and the killing of infected or degenerated cells. All lymphocytes are derived from a common lymphoid precursor cell in the bone marrow. While B cells complete their development in the bone marrow, T precursor cells migrate to the thymus where they finalize differentiation.

In contrast to its innate counterpart, the adaptive immune system does not rely on predetermined molecular patterns to recognize pathogens, but is able to respond to a vast variety of not predetermined antigens. That is, it is able to adapt to the nature of the infection. During the development of lymphocytes, recombination activating gene (RAG) mediated, somatic recombination of the receptor gene segments generates clonal T - respectively B cell receptors with a unique antigen specificity. This process is termed V(D)J recombination, according to gene segments that are rearranged. Somatic recombination allows the generation of a receptor repertoire comprising approximately 10^7 to 10^9 distinct antigenic determinants. Thus, every mature T and B lymphocyte is unique in respect to its ability to recognize a specific antigenic determinant or epitope.

Antigen-specific stimulation of T- resp. B cell receptor leads to the activation of multiple signaling cascades, including NFκB and RAS/ERK pathways which result in the clonal expansion and the differentiation into effector cells. The functional outcome of TCR or BCR activation is dependent on different factors like the nature of the antigen, the intensity and duration of antigen engagement, cytokines and signals from costimulatory receptors. Indeed, additional signals are needed to mount functional adaptive immune responses: They are provided in the form of costimulation as well as cytokine signals by antigen presenting cells in the case of inflammation.

This implies that the induction of adaptive immune responses imperatively depends on innate immunity. The final maturation into effector cells takes place in specialized structures within secondary lymphoid organs as lymph nodes or the white pulp of the spleen.

Depending on the nature of the antigen, the costimulatory signal and the cytokine environment, naïve lymphocytes mature into different subsets of effector cells. These subsets can be distinguished by their expression of effector molecules and allow to adapt the immune response to the nature of the infection. $CD4^+$ effector T cells can produce distinct sets of cytokines which allow them to activate distinct effector functions in response to different types of infections. Similarly, activated B cells are able to secrete different classes of antibodies. Each class of these immunoglobulins exhibit specialized functions and are selectively distributed to various body compartments.

The initiation of functional adoptive immune responses are complex and multi-stepped processes: Professional antigen presenting cells have to encounter, process and deliver antigens to the local lymphoid organs where they can induce clonal expansion and differentiation of effector lymphocytes. Protective adaptive immune responses against pathogens encountered for the first time therefore requires several days. Adaptive immune responses in response to subsequent infection with the same pathogen however, have been shown to be mounted more rapid and are more efficient. This effect is associated with the formation of long-lived memory B and T cells during the primary infection. Memory lymphocytes in fact are primarily responsible for immune responses against a recurrent infection [4]. The artificial induction of long-lasting, protective immunity against disease-causing pathogens is the base for the outstanding success of vaccines. Whether memory lymphocytes develop directly upon activation from naïve cells or effector cells become memory cells is not completely resolved [5].

1.3.1 T lymphocytes

T cell progenitors develop from hematopoietic stem cells in the bone marrow but mature into functional T lymphocytes in the thymus. According to the expression of the TCR co-receptors CD4 and CD8, T cells are classified into two populations with different functional properties.

T cell precursor cells entering the thymus do not express any of the coreceptors CD4 or CD8. Positive and negative selection in discrete thymic regions. In contrast to the BCR, the TCR only recognizes peptide antigens presented in the context of self but not foreign MHC molecules. This so-called MHC restriction was discovered by Zinkernagel and Doherty [6]. During the positive selection step in the thymic cortex, $CD4^+$ $CD8^+$ double positive thymocytes recognizing self-

peptides/MHC complex with moderate affinity receive survival signals and are therefore rescued from undergoing apoptosis. Cells that are able to bind to MHC class II differentiate into CD4 single positive T cells while cells recognizing MHC class I molecules give rise to $CD8^+$ lymphocytes. *Autoimmune regulator* (Aire) gene expression by medullary dendritic cells and thymus medullary epithelial cells (mTECs) activates the ectopic transcription of genes specific for various peripheral tissues [7]. CD4 or CD8 single-positive thymocytes relocate from the cortex to the medulla where they interact with the medullary antigen presenting cells. TCRs exhibiting a high avidity for the MHC:self-antigen complex are negatively selected through the induction of apoptosis. Self-reactive T cells with an intermediate affinity for the MHC:self-antigen complex can escape negative selection and leave the Thymus as natural regulatory T cells (nTregs), which will be discussed further below.

Antigen recognition is mediated by the heterodimeric T cell receptor that is composed of two transmembrane protein chains termed α and β chain. Each chain consists of an amino terminal variable and a constant region. Random rearrangement of TCR α and β chain variable regions creates a unique antigen receptor for virtually each developing T cell. This enables T lymphocytes to recognize an enormously diverse range of peptide antigens. In order to induce primary T cells responses, the specific recognition of peptide:MHC class I/II complex by the T cell receptor alone is not sufficient. Two more signals, provided by antigen presenting cells (APCs), are required: So called second signals comprises co-stimulatory receptors as for examples CD80/86 that engage CD28 on the T cells. They promote survival and expansion of the T cells. More recently it was shown that for both, $CD4^+$ and $CD8^+$ T cells, inflammatory cytokines produced by APCs provide a third signal that is needed for optimal proliferation, effector function and to avoid tolerance induction in response to a specific antigen [8].

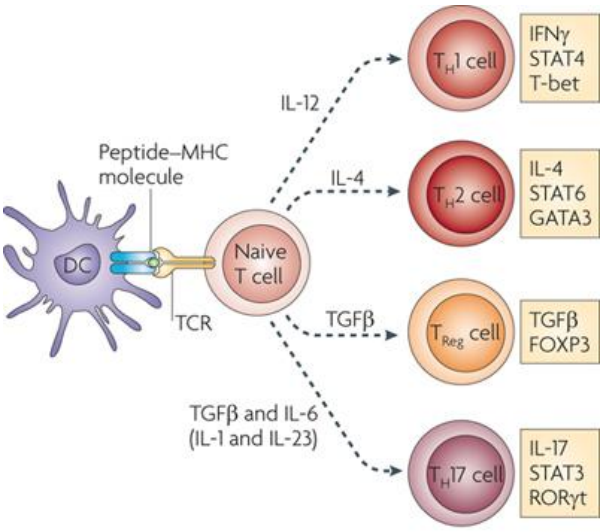


Figure 2 T cell plasticity: Depending on the signals provided by the antigen presenting cells, different T effector phenotypes develop figure adapted from [9]

$CD4^+$ T cells organize cell mediated immunity and regulate antibody secretion by B cells. They recognize exogenous antigens presented by professional antigen presenting cells in the context of MHC class II molecules. $CD4^+$ helper T cells orchestrate cellular immune responses against different classes of pathogens by providing cytokine and other stimulatory signals to various cells of the innate immune system. They further induce Ig class switching in B cells and are required for the activation of cytotoxic functions of $CD8^+$ T cells. Depending on the cytokine environment, naïve $CD4^+$ T cells can differentiate into at least four distinct types of effectors cells termed Th1, Th2, Th17 Tfh and iTr. Th1 help to fight intracellular bacterial and viral infections as well against malignant cells through the secretion of cytokines like IFN-γ, TNFα or CD40L that increase the function of macrophages, NK and dendritic cells. Th2 responses are characterized by the secretion of IL-4 and IL-13. They mediate protection from parasitic infections by the activation of basophils and mast cells and the induction of IgE secretion by plasma cells. Th17 cells are associated with the cytokine IL-17A/E which recruit and activate neutrophils and induces chemokine expression in epithelial cells. Th17 cells therefore are involved in the defense against extracellular bacteria and fungi at mucosal and epithelial barriers. More recent studies have shown that $CD4^+$ T cells themselves are able to exert cytotoxic functions in a MHC class II dependent manner in vivo [10],

$CD8^+$ T lymphocytes are specialized to destroy virus and bacteria infected as well as aberrant cells without affecting healthy tissue. Activation of $CD8^+$ T lymphocytes is initiated by the presentation of cytoplasmic pathogen derived peptides on MHC class I molecules, which are expressed by all nucleated cells. As additional costimulation and/or adequate cytokine stimuli are required to induce cytotoxic functions of $CD8^+$ T cells, the majority of viral infections depend on $CD4^+$ T cell help. Effector $CD4^+$ T cells can fortify the costimulating potential of APCs presenting related antigens and provide the T cell growth factor IL-2. Once activated, $CD8^+$ T cells mature into cytotoxic effector or memory cells. Cytotoxic lymphocytes (CTL) exert their functions by the induction of apoptosis in the target cells. This is achieved by the release of granules containing the cytotoxic molecules perforin and granzymes. While perforin creates pores in the target cell membrane, granzymes induce apoptosis of the target cells. In addition, the expression of the cell surface associated molecule FasL mediates killing of target cells expressing its receptor Fas through the activation of the extrinsic apoptosis pathway. Besides the induction of apoptosis, most CTLs are capable to secrete proinflammatory cytokines like IFN-γ, TNFα and LTα. IFN-γ contributes to host defense by interfering with viral replication, inducing MHC I expression in infected cells and activation of macrophages. TNFα and LTα can further induce target cell apoptosis.

After the successful elimination of a primary infection the predominant proportion of the antigen reactive effector T cell pool dies. However, a small percentage of the cells can persist for years as

so called memory T cells and provide an enhanced protection against recurring infection. $CD4^+$ and $CD8^+$ memory T cells can be divided into three subsets with distinct characteristics. Effector memory T cells (TEM) circulate in the blood and peripheral tissue and are able to mature rapidly into effector cells upon activation. Central memory T cells (TCM) on the other hand express CCR7 and CD62L and can therefore predominantly be found in secondary lymphoid tissue. TCM cells can persist for the rest of the life of an animal but they take longer to mature into effector cells compared to TEM cells. As TEM cells have an abbreviated lifespan or convert into TCM cells, peripheral adaptive immunity is lost over time [11]. Recent studies therefore identified a third memory T cell population termed tissue-resident memory T cells, that contributes to the sustained control of residual or persisting infection in peripheral tissues [12].

1.3.2 B lymphocytes

B lymphocytes mediate humoral immunity by the secretion of large amounts of immunoglobulins called antibodies into the extracellular fluids. Antibody secretion causes the destruction of extracellular pathogens and helps to prevent the dissemination of intracellular infections. B lymphocytes are derived from common lymphocyte progenitor cells and completely undergo their differentiation in the bone marrow. Antigens are recognized by the B cell receptor (BCR) which is a membrane bound immunoglobulin M (IgM) or IgD. The variable, antigen binding region of the immunoglobulins (and therefore also the BCR) undergoes random recombination. This allows the generation of antigen receptors that able to bind specifically to a broad variety of chemical structures like antigenic peptides or pathogen derived molecules such as LPS. Naive B lymphocytes circulate between blood and lymphoid organs where they congregate in B cell follicles and search their environment for antigens. Activation of B cells is induced by antigen recognition and usually requires help from helper T cells. $CD4^+$ T cell help provides a secondary stimulus and cytokine environment Activated B cells differentiate into antibody secreting plasma cells or memory B cells that allow rapid and responses to subsequent antigen encounter.

Five classes or isotypes of antibodies can be distinguished in placental mammals: while the two isotypes IgM and IgD can be found as membrane-bound receptors on naïve B cells, IgG, IgA and IgE are only secreted by plasma cells. The different isotypes differ in the constant region of the Ig heavy chains and have defined roles in humoral immune responses. Antibodies protect against pathogens by different ways: The direct binding to viruses or bacterial products is termed neutralization. This allows to prevent viruses from infecting host cells or the binding of bacterial toxins to their respective receptors thereby inhibiting their biological effects. The coating of larger pathogens like bacteria or parasites by antibodies is not sufficient to stop their replication. The constant region of antibodies can be recognized by specific receptors expressed by phagocytic cells like macrophages or neutrophils. Antibody coating thereby enhances pathogen

uptake and destruction. This process is called opsonization. A third protective mechanism of antibodies is their ability to activate the complement system. Antibodies coating the surface of a pathogen provide a platform to initiate the complement cascade that attracts neutrophils and leads to the formation of membrane pores. Presentation of previously encountered antigens by memory B lymphocytes to their cognate CD4+ T cell leads to a rapid proliferative response of both B and T cells. Antibodies secreted by memory B cells are predominantly of the IgG and IgA isotype and exhibit a much higher affinity than those produced in primary responses due to ongoing somatic hypermutation.

1.4 Immune Tolerance

Innate immune responses are not known to react against self in not inflamed tissue as the innate system has developed to recognize a limited number of molecules that are normally not present in vertebrates. In contrast, the random rearrangement of the respective coding regions create a diverse repertoire of antigen specific B and T cell receptors. This includes receptors recognizing harmless environmental or self – antigens which have to be eliminated or silenced in order to prevent inappropriate or autoimmune responses. Uncontrolled, overshooting immune responses can lead to life threatening conditions as for example sepsis or the development of autoimmune diseases like type II diabetes mellitus or Crohn's disease. Furthermore, the failure to down-regulate inflammatory responses can lead to chronic inflammation which is considered as a critical factor in the initiation and progression of neoplastic disease [13]. Several safety mechanisms have therefore been set in place to eliminate or suppress auto-reactive lymphocytes. Depending on their site of action, the various mechanisms of immune tolerance can be divided into two elemental groups. Central tolerance describes the elimination of potentially auto-reactive lymphocytes during their development in the bone marrow respectively the thymus. As central tolerance alone is not sufficient, different mechanisms in the periphery ensure and help to regulate inflammation.

1.4.1 Central tolerance

The random rearrangement of immunoglobulin heavy and light chains of B cells, respectively the α and β chains of T cell receptors, allows the generation of an enormously diverse receptor repertoire that includes self-antigen specific receptors. Thus, during the development of both B and T cells, several checkpoints ensure the elimination or inactivation of autoreactive lymphocytes. The generation of self-tolerant B and T cells in primary lymphoid organs is called central tolerance.

During their development in the bone marrow, the antigen receptors of immature B cells are tested for reactivity against self-antigens present in their environment. B cells recognizing self-molecules either rearrange the Ig light chain in order to generate a new BCR or they undergo apoptosis [14].

As described above, positive and negative selection in the thymus generates a T cell receptor repertoire that is restricted to self MHC molecules and tolerant against many self-antigens. Deletion of auto-reactive T cells is mediated by bone marrow derived dendritic cells as well as mTECs presenting tissue restricted antigens (TRA) in the context of MHC class I and II molecules. The ectopic expression of TRA is dependent on Aire gene expression. The importance of a functional central tolerance is shown by the observation that mice and humans lacking the transcription factor AIRE have self-reactive T cells in the periphery resulting in the development of severe autoimmunity [[7], reviewed in [15]]. $CD4^+$ thymocytes recognizing self-antigens with an intermediate affinity can induce the transcription of Foxp3 which acts as a master regulator for the development and suppressive function of natural regulatory T cells [16].

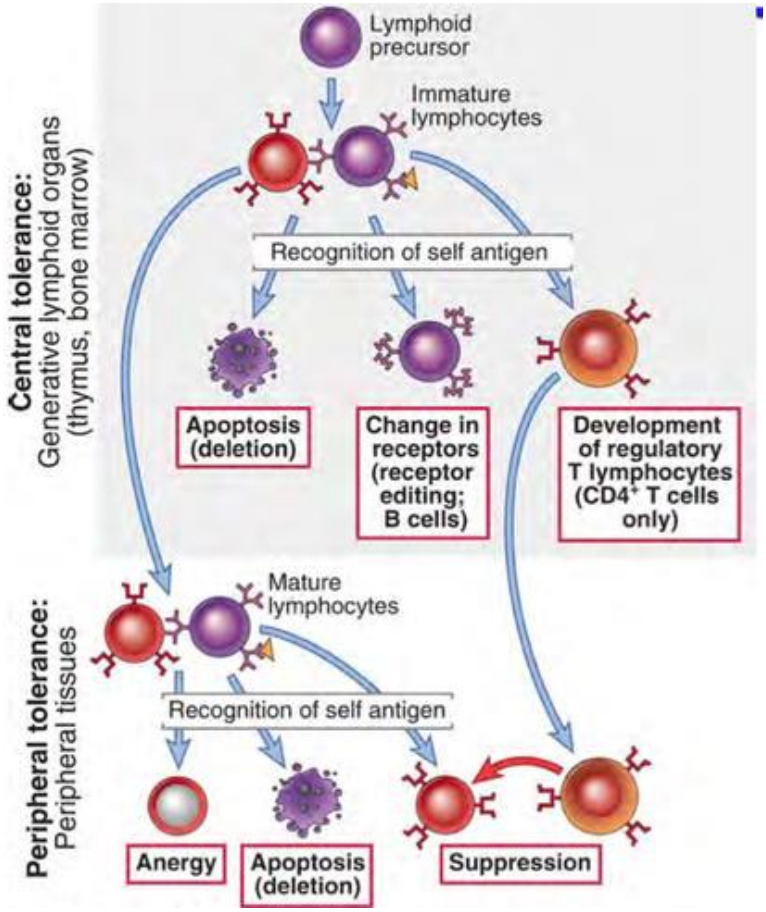


Figure 3 Central and peripheral tolerance mechanisms: Negative selection in the thymus eliminates most of the T and B cells expressing receptors that exhibit high affinity for self-antigens. Lymphocytes escaping negative selection are suppressed or eliminated in the periphery by several different mechanisms. Adapted from [17].

1.4.2 Peripheral or induced tolerance

Adoptive transfer of $CD4^+$ T cells derived from mice without functional negative selection into irradiated, syngeneic host succumb quickly to graft versus host disease. However, the transfer into not irradiated hosts did not lead to overt clinical disease symptoms [18]. These findings indicate the existence of peripheral mechanisms that are capable to regulate self-reactive immunity. In fact, thymic deletion of auto-reactive T cells is not complete. In order to suppress auto-reactive lymphocytes, different mechanisms of peripheral tolerance have developed.

Ignorance: The circulation of naïve T cells is restricted to blood, lymph and secondary lymphoid organs. Thus, they are excluded from non-lymphoid peripheral tissue where the probability to encounter tissue-restricted antigens is much higher. Only activated T cells express the respective adhesion molecules allowing to efficiently enter parenchymal tissue. And only tissue in an inflamed or infected state express the respective ligands that allow efficient extravasation of T lymphocytes.

Anergy: Antigen presenting cells, and especially dendritic cells play an essential role in inducing and maintaining peripheral tolerance. In the absence of inflammatory signals, lymphoid and tissue resident DCs maintain an immature state and present antigens in poorly immunogenic fashion. T cells repetitively encountering these antigens without further immunogenic signals may proliferate initially but are then quickly deleted [19]. Surviving T cells remain unresponsive or anergic to further antigen stimulation, resulting in a systemic antigen-specific tolerance. DCs, macrophages but also nonlymphoid cells can express coinhibitory receptors of the CD28 family like PD-1l., These molecules seem to induce an unresponsive, so called *anergic* state on T cells and have been shown to be essential for the maintenance of T cell homeostasis and self-tolerance [20]. The function of dendritic cells in peripheral tolerance are described below in more detail.

Suppression and apoptosis induction by regulatory T cells: Already early experiments in various animal models suggested the existence of a population of $CD4^+$ T cells that prevents the development of autoimmune diseases (reviewed by [21]). More recent observations revealed a minor population (around 5% of $CD4^+$ T cells in the periphery) of $CD4^+$ $CD25^+$ $Foxp3^+$ T cell population to be able to prevent autoimmunity [22], [23]. Expression of the transcription factor Foxp3 is essential for the generation and function of naturally occurring regulatory T cells (nTreg) as mutation or deletion of Foxp3 gene is associated with the development of autoimmunity in mice and human [24]. Thymocytes expressing a TCR with an avidity for self-antigen that is too high for the induction of conventional $CD4^+$ T cell development but is not high enough to succumb to negative selection can leave the thymus as $CD4^+$ $CD25^+$ $Foxp3^+$ naturally occurring regulatory T cells [16]. The main function of nTregs is to suppress the priming and expansion of effector T

cells. This can be achieved by multiple mechanisms: The IL-2 receptor expressing Treg compete with effector T cells for IL-2 which may lead to the consumption of the cytokine, impairing expansion of effector T cells. nTregs have further shown to produce immunoregulatory cytokines like IL-10, TGF-β or IL-35 which can interfere with target T cell proliferation and function. Finally, through the expression of coinhibitory molecules like cytotoxic T-lymphocyte-associated protein 4 (CTLA4) or neuropilin-1 (Nrp1), nTregs can interfere with APC function. Finally, nTregs have been shown to directly kill antigen presenting cells, but also NK and CTLs in tumor environment by granzyme B and perforin (reviewed in [25]).

Tregs and various other cell types can induce conversion of conventional $CD4^+$ T cells into so called induced regulatory T cells (iTregs). The presentation of self- or foreign antigen by APCs and the presence of certain cytokines can lead to a thymus independent maturation of naïve T cells into Tregs. The conversion of naïve T cells into iTregs is dependent on TCR signaling as well as adequate second and cytokine signals. Especially dendritic cells but also B cells and MDSCs have therefore been shown to induce Treg (reviewed by [26]). In addition, natural Tregs are capable to induce a regulatory phenotype on naïve CD4+ T cells. This conversion has been shown to be dependent on direct cell-cell contact and the regulatory cytokines TGF-β, IL-10 or IL-35 [27], [28]. This mechanism ensures the sustained regulation of effector cells by maintaining and renewing adequate Treg populations and has been termed infectious tolerance [28]. iTregs, contribute to the maintenance of peripheral tolerance mainly by the secretion of anti-inflammatory cytokines, while cell-cell contact dependent mechanisms seem to be less important [29]. Depending on the main effector molecule, two subtypes of iTregs were originally distinguished: Th3 are predominantly found in the oral and intestinal mucosa, They are characterized by the production of TGF-β, which can suppress proliferation of Th1 and Th2 [30]. The cytokine IL-10 leads to the induction of a regulatory population termed type-1 regulatory T cells (Tr1). Regulation by Tr1 cells is dependent mainly on the secretion of IL-10 which antagonizes APC functions and renders T cell anergic [31]. More recently a third iTreg subpopulation was described which is induced and produces IL-35 (iTr35) [32]. A more detailed introduction to suppressive functions of the cytokines IL-10, TGF-β and IL-35 is given below. Recent studies identified even more populations of $Foxp3^+$ Tregs with different gene expression profiles. As it is proposed for effector T cells, Tregs might adopt different functional “programs” depending on differentiation signals or the anatomical location.

There is experimental evidence that many additional cell types may act as regulators of immune responses. These include invariant natural killer T (iNKT) cells that respond to lipid antigens, $CD8^+$ T cells, γδ-T cells, regulatory B-cells and myeloid derived suppressor cells (MDSC) [33]–[35]. Probably the best evidence for a pathophysiological role in immune regulation has been found

for MDSC. They constitute a heterogeneous cell population of myeloid origin and can be classified into $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ monocytic and $CD11b^{+}Ly6C^{lo}Ly6G^{+}$ granulocytic subsets. MDSCs are able to restrain immune responses through a variety of direct and indirect mechanisms. The direct mechanisms comprise the local depletion of the essential amino acids arginine and cysteine mediated by the enzyme arginase1. The degradation of tryptophan and generation of cytotoxic metabolites through indoleamine 2,3-dioxygenase (IDO) or the generation of reactive oxygen and nitrogen species (NOS, resp. ROS) trough NADPH-Oxidase respectively NO synthetase 2. Expression of those enzymes interferes with the proliferation and function of T cells. MDSCs have furthermore been shown to be attracted to tumor environment where they act as tolerogenic antigen presenting cells. In association with the effects of Arg1 or Ido1 expression, MDSCs can induce and expand regulatory T cells [36]. Although MDSCs have mainly been investigated in the context of murine tumor models, their physiological role seems to be to resolve immune responses and promote repair and remodeling of the injured tissue once an infection has been cleared (Reviewed in [33]).

1.5 Cytokines – messengers of the immune system

In a complex system, such as the mammalian immune system, the myriad of involved cells have to be orchestrated in order to get a directed and adequate immune response to a wide diversity of pathogens while maintaining tolerance to self-antigens. This various interactions among the immune, as well as non-immune cells are mediated by a group of proteins collectively termed cytokines. Cytokines are low-molecular weight (glyco-) proteins secreted by leukocytes and various other cells in response to a number of stimuli, like antigen recognition or binding of other cytokines. The ability of these proteins to act in pleiotropic, redundant, synergic and antagonistic ways on their targets permits the restricted number of different cytokines to regulate activity of both, innate and adaptive immunity in a coordinated and interactive way. Binding of cytokines to their respective membrane-bound receptors generally activates specific signal transduction pathways which ultimately induces changes in gene expression of the target cell. Many of these changes result in differentiation and/or proliferation of T and B lymphocytes or activation of effector cells such as macrophages. Both cytokine production as well as cellular responses to them are tightly regulated by different mechanisms and are often self-limited trough the action of inhibitory feedback loops [17].

1.5.1 Interleukin-35

IL-35 is the most recently described member of the interleukine-12 cytokine family. IL-35 is a heterodimeric protein, comprised of the IL12p35α subunit and the Epstein-Barr virus (EBV) - induced gene 3 (ebi3) which is shared with IL-27. IL-35 is thought to signal trough a heterodimeric receptor, consisting of IL-12Rβ2 and IL-6gp130 in T cells or IL-12Rβ2 and IL-27Rα in B cells. Furthermore, signaling via homodimers of either receptor subunits has been proposed [34], [37]. Engagement of the receptor leads to phosphorylation of STAT1 and STAT4 in T cells whereas in B cells phosphorylation of STAT1 and STAT3 have been detected [38]. In contrast to the other members of the IL-12 cytokine family, IL-35 cytokine has been reported to be produced by murine natural regulatory T cells. Ebi3 in fact was shown to be a Foxp3 target gene as the conditional knock-out of Foxp3 resulted in the downregulation of Ebi3 expression in peripheral Tregs. Furthermore, Ebi3 and IL12a deficiency in regulatory T cell has been found to drastically impair the regulatory function of Tregs in vitro and in vivo [39]. Collison et al. were also the first to describe IL-35 to be able to suppress $CD4^+$ T cells proliferation and the induction of a regulatory T cell population that is characterized by the secretion of IL-35 but does not express the classical Treg markers CD25 and Foxp3 [32]. Thus, IL-35 seems to be involved in the negative regulation of T cell responses and mediation of infectious tolerance. Several recent studies indicate IL-35 to be an important mediator of immune tolerance in various experimental immunopathologies and have broadened the range of cells that secrete and/or can be targets of the cytokine. EBI3 but not IL-27p28 deficient mice exhibited increased pathologic features of experimental enterocolitis with increased Th1 and Th17 responses. Ebi3 and IL-12p35 have been found to be transcribed in colonic epithelial cells of healthy mice [40]. Interleukin-35 has been shown to induce IL-10 producing regulatory B cells conferring protection to the development of auto immune uveitis [34]. Mice lacking IL-35 expression in B cells have been shown to reduce susceptibility to *Salmonella* infection but were not able to recover from experimental autoimmune encephalitis (EAE) [38]. Furthermore, the administration of recombinant IL-35 prevented the development of collagen induced arthritis by the induction of IL-10 producing $CD39^+$ regulatory T cells suppressing Th1 and Th17 mediated immunity [41].

In addition to its role in the regulation of autoimmunity, IL-35 has also been found to be exploited by tumor cells to evade immune surveillance. Expression of EBI3, but not IL27p28, has been found in (Epstein-Barr virus-associated) Hodgkin lymphoma, adult T cell leukemia as well as nasopharyngeal carcinoma cells [42], [43]. Ebi3 and IL-12p35 transcription has not only been found in neoplastic but also in tumor associated cells, such as tumor associated dendritic cells [43]. Wang and colleagues were able to detect increased $CD11b^+$ $Gr1^+$ myeloid cell accumulation and neo-genesis of blood vessels in the environment of IL-35 secreting tumors. Despite these

recent insights the biological functions of the cytokine IL-35, especially its effect on cells other than T and B lymphocytes, are poorly understood.

1.5.2 Transforming growth factor β (TGF-β)

In contrast to IL-35, TGF-β has been studied for almost 30 years and its role in immune tolerance is much better understood. In mammals, the TGF-β family consists of the three highly homologous members TGF-β1,2 and 3. Although they fulfill very similar functions on vitro, TGF-β1 is the predominant isoform in mice. The TGF-β genes encode an N-terminal latency-associated peptide (LAP) and the C-terminal mature TGF-β that assembles into homodimers. During the peptide procession, LAP is cleaved but remains noncovalently associated to the mature cytokine, forming the latent cytokine complex. The latent TGF-β complex is not able to engage the TGF-β receptor but allows its storage in the extracellular matrix. In order to become biologically active, TGF-β has to be separated from the LAP. Different peptidases and more recently also mechanical interactions with integrins have been shown to activate latent TGF-β in a context sensitive, well regulated way. Active TGF-β mediates its function mostly through a receptor complex consisting of two type I and two type II TGF-β receptors. Classical TGF-β intracellular signaling involves the phosphorylation of SMAD2/3 which, together with SMAD4, translocate to the nucleus where they act as a transcription factor. In addition to SMAD associated signaling, PI3K, MAP kinase signaling pathways can be activated by TGF-β (reviewed by [44].

Activated effector as well as regulatory T cells are thought to be the principal source for TGF-β. However, while the T cell specific ablation of Tgfb expression is associated with a disordered T cell homeostasis and inflammation, the phenotype is mild compared to mice completely lacking TGF-β expression. This suggests other cells to be important sources for TGF-β. Indeed, a wide array of cells have been found to secrete TGF-β.

Mice lacking TGF-β develop multiorgan inflammation and mostly die embryonically, indicating the importance of the cytokine for immune regulation [45]. T cell specific abrogation of TGF-β signaling results in the spontaneous activation of T cells and lethal multifocal inflammation while crossing TGFb1-/- to MHC-II deficient mice inhibits exaggerated inflammation [46], [47]. These findings indicate T lymphocytes to be the main targets of TGF-β. Indeed TGF-β has been shown to regulate T cell proliferation by suppressing transcription of the T cell growth factor IL-2 [48]. The cytokine has been shown to suppress the transcription of the Th1 master-regulator T-bet as well the Th2 specific transcription factor GATA3 thereby effectively inhibiting the differentiation of functional T helper cells [49]. Similarly, TGF-β has been found to regulate CD8+ T cell proliferation as well as their differentiation into functional CTLs by the downregulation of

effector molecules like Fas ligand [50]. TGF-β has been shown to indirectly suppress T cell responses through the regulation of $Foxp3^+$ Tregs. The cytokine protects thymic nTregs from apoptosis and, at least in vitro, promotes the induction of a regulatory phenotype on naïve $CD4^+$ T cells through the upregulation of Foxp3 [51]. However, the regulation of T cell proliferation differentiation and survival by TGF-β is highly dependent on the inflammatory context, i.e. the presence of costimulatory or cytokine signals. Accordingly, it was shown that TGF-β, together with the pro-inflammatory cytokines IL-6 and IL-21 is crucial for the differentiation of Th17 cells [52].

In addition to the impact on T cell function, TGF-β is an important regulator for many other cell types including B lymphocytes, NK cell, macrophages and dendritic cells. Regarding dendritic cells, TGF-β1 has been found to promote the generation of DCs with an immature phenotype in vitro and to inhibit the expression of MHC class II and costimulatory molecules as well as the secretion of IL-12 cytokine in differentiated DCs [53]. Dendritic cell specific ablation of TGF-βRII resulted in the development of multiorgan inflammation and death of the mice similar to $Tgfb^{-/-}$ mice. These results indicate that, apart from direct effect of TGF-β on the regulation of T cell homeostasis, impaired DC function might contribute to pathogenesis in TGF-β deficient mice [54].

1.5.3 Interleukin-10

Interleukin 10 has pleiotropic immunoregulatory functions and is implicated in the regulation of infectious responses and in the protection from autoimmunity and allergy. Bioactive IL-10 forms a homodimeric molecule that is able to bind to the IL-10 receptor (IL-10R). The Il-10R is a tetrameric complex consisting of two IL-10R1 and two IL-10R2 proteins. Engagement of the IL-10R induces an intracellular signaling involving Jak1/Stat3 phosphorylation which is assumed to be necessary for the inhibitory functions of IL-10 [55]. Interleukin 10 has been shown to be produced by and to affect a wide range of immune cells, including DCs, macrophages, neutrophils, NK and B cells. The cytokine was shown to inhibit the production of proinflammatory cytokines and antigen presentation by activated macrophages. Monocyte derived DC exhibit an immature phenotype when cultured in the presence of IL-10. The cytokine furthermore counteracts chemokine expression implicated to DC, macrophage or neutrophil recruitment during inflammation (reviewed by [56]). Most prominently investigated is the effect of IL-10 on T cells. In *in vitro* experiments, the presence of IL-10 led to proliferative inhibition of $CD4^+$ T cells by reducing IL-2 production [57]. IL-10 deficient mice were shown to develop chronic colitis which is lethal in most cases [58]. Interestingly this chronic inflammation of the gut was ameliorated under germ-free conditions, implicating an important role in the maintenance of intestinal

homeostasis [59]. IL-10 deficient mice were further shown to exhibit dysregulated, detrimental helper T cell effector responses upon infection with various pathogens including *Toxoplasma gondii*, *Leishmania major* or murine cytalomegavirus as well as in autoimmune models like rheumatoid arthritis or EAE (reviewed in [60]). IL-10 secretion substantially contributes to the regulatory function of natural Tregs as shown by the finding that IL-10 deficient Foxp3$^+$ cells develop colitis, in a manner similar to completely IL-10 deficient mice [61]. IL-10 directly inhibits T cell proliferation and reduces IL-2 production [62]. Naïve CD4$^+$ T cells cultured in high concentrations of IL-10 and in the presence of APCs can acquire a regulatory phenotype characterized by IL-10 secretion but do not express Foxp3. This induced regulatory T cell population was termed Tr1 cells and was shown to induce long-lasting T cell anergy [63]. The dependence on APC for the generation of Tr1 cells suggest that IL-10 alone is not sufficient to convert CD4 T cells. Indeed, a IL-10 and immunoglobulin-like transcript 4 (Ilt4) expressing tolerogenic DC subpopulation has been described in mouse and human and was shown to be required for Tr1 induction by IL-10 [64]. More recent work showed that under certain conditions not only Tregs, but also Th1, Th2 and Th17 cells can co-express IL-10 in order to limit infection-driven immunopathology or autoimmunity (reviewed by [60]). In addition to its regulatory functions, IL-10 has also been found to drive proliferation and maintain cytotoxic effector functions of CD8$^+$ T cells [65], [66].

1.6 Dendritic Cells: Bridging and integrating innate and adaptive immune responses

In 1973, Steinman and Cohn described a new population of cells in the spleen with a characteristic dendritic morphology and coined the term “dendritic cells”[67]. Over the past years, further studies showed dendritic cells to constitute a heterogeneous population of lymphoid but also non-lymphoid tissue resident cells. Even though their generation depends on different stimuli, they fulfill different functions and express different surface markers, all DCs share several features which distinguishes them from other antigen presenting cells.

Dendritic cells develop in the bone marrow from hematopoietic stem cell and migrate via lymph or blood stream into the tissues where they terminally differentiate into immature DCs. Immature conventional dendritic cells (cDCs) reside in blood and peripheral tissue and constitutively sample their environment for the presence of infection, stress or injury through phagocytosis and pinocytosis. The ingested antigens are processed and presented to T lymphocytes via classical (MHC class I and II) and non-classical (CD1 family) pathways. Engagement of TLR or other innate receptors by microbial products, necrosis, mechanical trauma or pro-inflammatory cytokines

leads to the maturation of the DCs. This leads to a down-regulation of endocytic activity and a reduced MHC turnover, resulting in retention of peptide-MHC complexes formed at the time of maturation. Furthermore, mature DCs up-regulate expression of co-stimulatory molecules and migrate from the periphery to lymphoid tissue where they interact with T cells in an antigen-specific manner. Cytokine microenvironment and other factors encountered during maturation control the cytokine secretion profile of the DCs. Co-stimulatory signals provided by receptors like B7 family molecules as well the cytokine expression pattern govern the subsequent differentiation of the responding T cells.

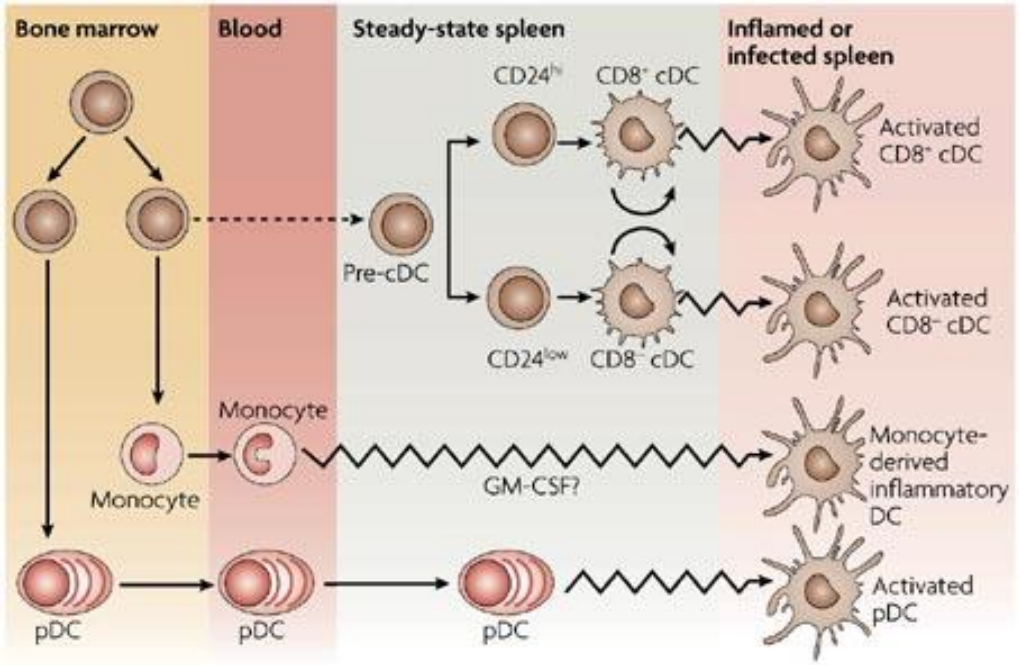


Figure 4 Dendritic cell development and subtypes: Depending on progenitors derived, surface markers, anatomical location and function, a variety of dendritic cells can be discriminated. Taken from [68].

1.6.1 DC subsets and their development in mouse

Dendritic cells, except for the plasmacytoid DC population, are originally derived from a common hematopoietic stem cell (HSC) in the bone marrow. HSCs differentiate progressively into precursor cells that are committed to particular lineages. Due to their functional resemblance to other myeloid cells like macrophages, DCs were initially thought to be mainly derived from common myeloid precursor cells. However, studies conducted by Manz et al. and other groups suggested that all DC subgroups can also be derived from common lymphoid precursor cells and that the important steps of DC lineage commitment are downstream of both myeloid and lymphoid precursors cells [69]. Successive commitment stages include granulocyte-macrophage precursor cells, macrophage/DC progenitors and common dendritic cell precursor localized in

the bone marrow [70]. However, a recent study was not able to determine a common precursor cell restricted to the production of macrophages and lymphoid-resident cDCs [71]. These finding illustrates that DC differentiation seems to be more complex than initially thought and implicate a high degree of developmental flexibility. While all differentiation steps mentioned afore take place in the bone marrow, there is more accordance that the final development of cDCs and pDCs occurs in peripheral tissues. The overall differentiation process is dependent on various cytokines and transcription factors, most of which exert various different functions during hematopoiesis. However, a few molecules are crucial for the generation of dendritic cells. FMS-related tyrosine-kinase 3 ligand (Flt3l) deficient mice have been shown to have only little levels of pDCs and cDCs [72] whereas the addition of Flt3l to bone marrow cells generates a mixture of steady-state pDCs, as well as cDCs [73]. Deficiency of the transcription factors IRF8 as well as Batf3 results in the selective deficiency of $CD8\alpha^+$ DCs while mice lacking IRF4 do not generate $CD4^+$ cDCs [74], [75].

As resumed in table 1 the different dendritic cell subsets can been discriminated according to expression of surface molecules, their functional characteristics as well as their localization. Murine DCs can be classified into three major lineages: plasmacytoid DC (pDC) conventional DC (cDC) and induced DCs.

Plasmacytoid DCs, have a round shape, do not form characteristic dendrites and exhibit a survival time of about 15 days *in vivo*. pDCs circulate through the organism and can be isolated from bone marrow and peripheral tissue. Their main function is to respond to viral infections by the secretion of large quantities of type I interferons. However viral infection has been shown to convert some pDCs into a dendritic form that is able to present antigen and resembles also phenotypically cDCs [76].

Conventional dendritic cells can further be subdivided in migratory and lymphoid resident DCs. Migratory DCs represent the classical text-book dendritic cells. They patrol in peripheral tissues and sample their environment for antigens. Activation of migratory cDCs allows them to enter lymph nodes where they present antigen to T cells and provide costimulation. This class of dendritic cells includes dermal and interstitial DCs as well as Langerhans cells. Lymph node resident DCs remain in the lymphoid organ itself, where they sample blood and lymph for antigens and present them to T cells. Lymphoid resident cDC populations comprise most thymic and splenic DCs. Several subpopulations, which exert different immune functions, can be classified based on the expression of CD4 or CD8α homodimers and. cDCs are generally short-lived, having a turn-over time of about 3 days [77].

So called inflammatory dendritic cells can be found under inflammatory conditions. These cells can be identified as $CD11b^+$ $CD11c^+$ $F4/80^+$ $Ly6C^+$ $MHC\text{-}II^+$. Inflammatory DCs (infDC) are absent from peripheral and lymphoid organs during steady-state conditions but develop in situ from monocytes upon infection with various pathogens or under pathologic autoinflammatory conditions. In contrast to inflammatory monocytes or macrophages, infDC are able to migrate to draining lymph nodes and activate T lymphocytes (reviewed in[78]). Through the in vitro addition of granulocyte-macrophage stimulating factor (GM-CSF) and IL-4 monocytes can acquire a DC like phenotype. Similarly to infDC these in vitro generated DCs are able to process and present antigen to T lymphocytes [79].

Interestingly, several lines of evidence support the notion of division of labor and cross-talk within the DC network. Altogether, DC subsets display differences in their capacity to monitor specific tissue or to circulate, in the expression of PRRs, the production of cytokines, as well as antigen uptake and presentation mechanisms [80], [81].

	Plasmacytoid DC	***Conventional DC***					***inflammatory DC***
		Lymphoid tissue resident			*migratory*		
Phenotype		*$CD4^+CD8^-$*	*$CD4^-CD8^+$*	*$CD4^-$ $CD8^-$*	*Langerhans DC*	*Dermal DC*	
CD11b	-	+	-	-	+	+/-	+
CD11c	*Intermediate*	+	+	+	+	+	+
B220	*Intermediate*	-	-	-	-	-	-
Langerin	-	-	-	-	+	+/-	-
CD4	+	+	-	-	-	-	-
CD8α	-	-	+	-	-	+/-	-
Function	Main Type 1 Interferon producers	Main IL-4 Producers Involved in T_h2 response	Main IL-12 Producers Cross prime CD8+ T Cells $T_h1$response	IL-4, IFNγ Producers Rather involved in Th2 response	Sentinels Carry antigen from epidermis to dLNs $CD8^+$ T cell priming	Sentinels Carry antigen from dermis to dLNs	IL-12, IL-23, IFNγ producers Cross prime CD8+ T Cells
Localization	Blood: DC precursors T cell zones of spleen	Marginal zone of spleen subcapsular sinus of lymph nodes	T cell zone of lymphoid organs thymic cortex	Spleen Thymus	T cell zones of lymphoid organs Epithelia	Skin	Tissue precursors Tcell zone of lymphoid organs

Table 1 Overview over murine dendritic cell subtypes and their principal functions

1.6.2 The CD8α+ dendritic cell subset

Murine conventional dendritic cells (cDC) can broadly be divided into CD8α- and CD8α+ DC subsets. CD8α+ represent about 20% of splenic DCs and about 70% of thymic DCs, whereas no CD8α expressing DCs could be found in non-lymphatic organs. In contrast to CD8+ T cells the CD8 molecules are formed by a homodimer of two α chains. However, no role for CD8 in the development or function of the DC subset has been attributed so far. Transgenic mice lacking CD8 expression actually develop functionally normal DCs of this subset[82]. In contrasts to the mouse, no CD8α+ DC subset was found in humans. However, CD103+ DCs seem to be functional analog to CD8α+ DCs in humans. In order to distinguish from CD8α- cDCs general accepted as CD11c+ CD8α+ MHC-II+ CD205+ (a multilectin) CD24+ Clec9a+ (a C-type lectin) but CD11blo Sirpα- cells. FMS-related tyrosine kinase 3 ligand (FLT3L) mediates the expansion of CD8α+ DCs in peripheral lymphoid organs and mice lacking FLT3l expression developed a markedly reduced amount of this DC subtype [72], [83]. Furthermore development of CD8α+ DCs was shown to be dependent on the transcription factors IRF8 and Batf3 [74], [75]. The cells have a short turnover rate of only 3 days *in vivo*. Lymphoid organ resident DCs therefore must be continuously replaced by precursors from the bone marrow. Bone marrow derived pre-DCs that already express CD11c but not MHC class II migrate via the blood stream and differentiate in spleen and lymph nodes into CD8α+ and CD8α- DCs [84]. Expression of CD24 within the pre-DC population was shown to be associated with the development into CD8α+ DC. However, it remains under debate in which developmental stage this commitment happens.

CD8α+ dendritic cells exhibit a unique cytokine secretion profile which is characterized by their ability to secrete large amounts of IL-12 upon activation. Together with the ability to efficiently cross-present extracellular antigens to CD8+ T cells, CD8α+ DCs are able to mount Th1 and cytotoxic T cell (CTL) responses [85]. Resting CD8α+ DCs on the other hand are described to limit T cell proliferation and are potent inducers of Foxp3+ Tregs through the secretion of TGF-β [86].

1.6.3 Dendritic cell function in peripheral tolerance and autoimmunity

Dendritic cells play a central role in linking and integrating innate and adaptive immune systems, thereby orchestrating functional immune responses. Upon recognition of microbial or tumor associated antigens, migratory as well as lymphoid tissue resident DCs are activated and undergo maturation. They upregulate antigen presentation, provide costimulatory signals and secrete a plethora of inflammatory cytokines. In addition, thymic dendritic cells are involved in the establishment of central tolerance through the (cross-)presentation of self-antigens [87]. In the absence of infection or inflammation, peripheral DCs are characterized by a high level of

endocytosis and pinocytosis but only low expression levels of MHC molecules as well as co-stimulatory receptors [88]. The primary function of these immature DCs is to maintain peripheral tolerance to self-antigens through various mechanisms, which can be grouped into three overlapping mechanistic groups: 1) Low expression of co-stimulatory signals, respectively up-regulation of inhibitory receptors induce T cell anergy [20]. 2) The secretion of cytokines or other soluble factors like IL-10 or transforming growth factor (TGF)-β induce regulatory T lymphocytes [89]. 3) Inhibition of T cell proliferation through secretion of molecules like IDO [90] (Fig.5). While the underlying mechanism for the induction and shaping of adaptive immune responses to infections by DCs is well investigated, their role in the induction and maintenance of peripheral tolerance is much less understood. First evidences for the importance of immature DCs in the induction of self-tolerance were given by Hawiger and colleagues. By targeting antigens to, or expressing them in immature DCs, they could observe proliferation of antigen specific $CD4^+$ T lymphocytes. However, T cell proliferation was not linked to effector cell differentiation. Instead the initial proliferation was not sustained and the remaining antigen specific T cells were unresponsive to re exposure to the antigen [91]. Similarly, applying a Cre/loxP based system that allows the inducible antigen presentation by DCs, Probst and colleagues found that immature DCs induced complete unresponsive $CD8^+$ T cells. In addition, the DC induced tolerance could not be broken by subsequent antigen-specific restimulation of the $CD8^+$ T cells [92]. However, depletion of conventional DCs did not help to shed further light on the role of immature DCs in peripheral tolerance: While a first study found no apparent effect on T cell responses [93], a similar study described development of severe autoimmunity upon DC ablation [94]. More recent, the specific ablation of $CD11c^+$ cells has been shown to enhance the severity of experimental autoimmune encephalitis [95]. The deletion of specific DC subsets like $CD8\alpha^+$ conventional [96] or plasmacytoid [97] DCs led to an increased susceptibility towards viral infections. However, no exacerbated autoimmunity could be observed. Although the absence of dendritic cells by its own might not lead to exacerbated immunity, defects in various molecular mechanisms have been associated with a reduced tolerogenic capability of DCs. Deficiency for the transcription factor Blimp-1, for example, led to an elevated level of MHC II and increased IL-6 secretion by DCs, resulting in development of lupus-like symptoms [98]. STAT3 deficient DCs have been shown to express higher levels of MHC-II as well as co-stimulatory molecules and were unresponsive to IL-10 suppression. STAT3 deficient DCs have further been shown to cause spontaneous inflammation and/or autoimmunity in the gut [99]. Inconsistent with these findings, the deletion of STAT3 did not improve anti-tumor immunogenicity upon prophylactic DC vaccination [100]. The absence of NF-κB1 signaling in DCs has been shown to lead to the production of tumor necrosis factor α (TNFα) by unstimulated DCs. This TNFα secretion led to an increased granzyme B expression by $CD8^+$ T cells causing autoimmune diabetes [101]. CD80/86 expression by

dendritic cells has been shown to be necessary for the maintenance of peripheral regulatory T cells. Interestingly, the depletion of conventional DCs reduced the Treg compartment but did not result in formation of spontaneous autoimmunity [102]. Taken together, dendritic cells seem to hold a central role in the induction and maintenance of peripheral tolerance. Nevertheless, their actual role appears to be ambiguous and complex. While increased or reduced numbers of dendritic cells do not seem to directly cause autoimmunity, genetic deficiencies can indeed lead to aberrant inflammation. This might be due to the redundancy of peripheral tolerogenic mechanisms or might reflect the crucial role of DCs in the induction of T cell responses. However, there is good evidence that dendritic cells are able to promote immune tolerance through various mechanisms and failure of DC function contributes to the development of autoimmune diseases.

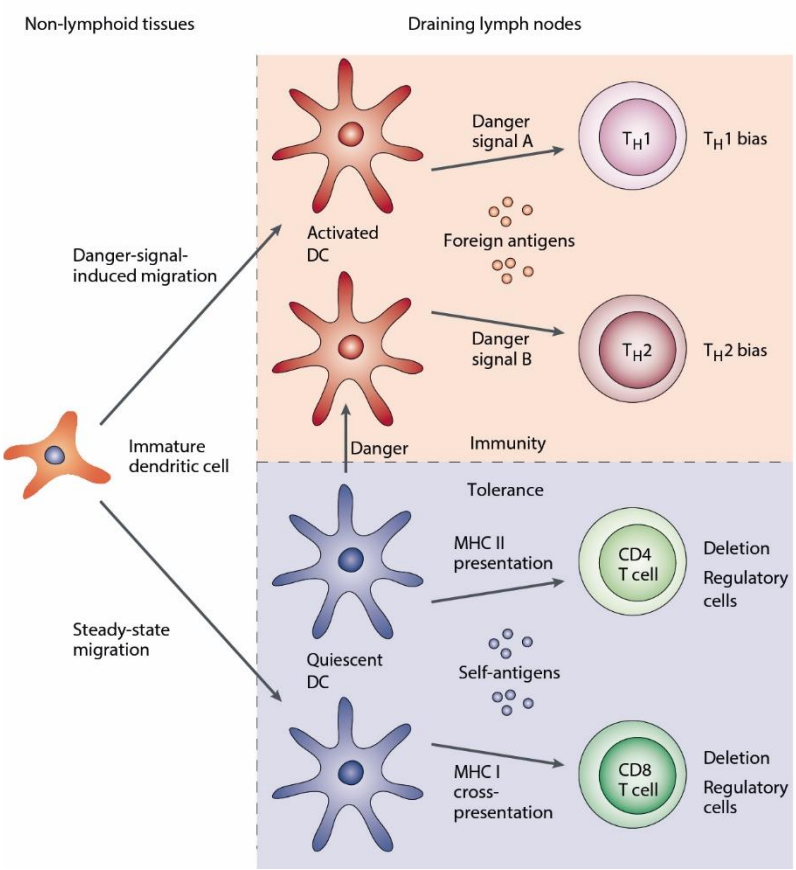


Figure 5 Dendritic cell function between immunity and tolerance: While the presentation of antigens in the context of inflammation leads to the maturation of effector T cells, (self-) antigen presentation in the absence of inflammatory stimuli promotes immunologic tolerance [103].

1.6.4 Autoimmune diseases

Inappropriate activation of T or B cells and imbalanced central and/or peripheral tolerance can lead to chronic immune responses against the body's own tissues. Autoimmune diseases (AID) can affect virtually all tissues and manifest itself by affecting certain organs or systemically involve a particular tissue. AID like type I diabetes, autoimmune thyroiditis or gastritis affect about 5%-8% of individuals in developed countries and shows an increasing prevalence worldwide. Interestingly, almost all AID predominately affect women and tend to cluster in

families and individuals [104]. Together with the observation that autoimmune disease share clinical symptoms, (epi-) genetic factors and can be attributed to aberrant lymphocyte activation, common mechanisms are thought to be involved in disease susceptibility. However, the etiology of autoimmune diseases is still largely unknown. The data accumulated over the last decades indicate the development of AID to be complex and multifactorial. Environmental risk factors like exposure to chemical agents or air pollution but also viral and bacterial infections (trough molecular mimicry) may enhance the development of AID [105]. Current standard treatment approaches against auto-reactive inflammation consist of non-specific and systemic suppression of a broad spectrum of immune cells. This indiscriminate immunosuppression can cause severe adverse effects and toxicity and renders the patient susceptible for infections. In addition, the chronic nature of AID imposes a significant burden on the quality of life of the patient as well as on the utilization of medical care. The need for more specific treatments exhibiting less side effects, but longer-lasting effects is therefore evident.

1.7 Dendritic cell based approaches for treatment of (auto-) immune diseases and cancer

Due to their dual role in inducing and regulating adaptive immune responses, dendritic cells are particularly interesting targets for the development of novel immunotherapeutic approaches. Over the last years, several different strategies have been employed in order to control DC function. Namely for the treatment of cancer as well as of autoimmune diseases or transplantation-related complications. Modification of DCs by immunogenic respectively tolerogenic agents or genetic manipulation have been shown to restore immune tolerance, to inhibit autoimmune pathogenesis or increase immunogenic properties for cancer therapy.

Besides application specific obstacles, several common problems for the successful implementation of DC vaccination have to be taken into consideration:

- Selection of the appropriate DC subtype / differentiation protocol to obtain dendritic cells suitable for a particular task.
- Practical considerations like the nature of the applied antigen, the delivery route of the antigen or the DCs, the frequency of antigen respectively DC transfer may have an critical impact on the efficacy of DC immunotherapy.

Disease model	DC modulation	Effect	Reference
Autoimmune non-obese diabetes (NOD)	Bone marrow derived, NF-κB inhibitor treated DC	Hyporesponsive autoreactive T cells	[106]
Collagen-induced arthritis (CIA)	IL-10 or Dexamethasone stimulated, CII peptide pulsed DC	Th3 skewing (IL-10), respectively induction of hyporesponsive Th1 cells (Dex)	[107]
Experimental autoimmune encephalitis (EAE)	DC targeted MOG expression	PD-1 dependent induction of antigen-specific Treg	[95]
Experimental autoimmune myocarditis (EAM)	Flt3L induced, MyHC-α peptide pulsed CD8α+ DC	Induction of protective Th1 responses	[108]
Skin allograft	Donor derived semi-mature DC	Activation of antigen specific Treg	[109]

Table 2 Examples of beneficial application of tolerogenic DCs in different animal models of autoimmune diseases

1.7.1 Establishment of immune tolerance by dendritic cell-based therapy

Autoimmunity and autoimmune diseases are thought to be the result of a breakdown of central and/or peripheral tolerance mechanisms. Different strategies to generate have been described to create "tolerogenic DCs" (tolDC) with the ability to induce antigen specific T cell anergy or regulatory T cells: Differentiation of monocyte-derived in the presence of anti-inflammatory cytokines like IL-10, or TGF-β, immunosuppressive molecules like vitamin D3. Another approach is the genetic modification of DC in order to express anti-inflammatory molecules like IL-10 or CTLA4, apoptosis inducing molecules such as FasL or inhibit the secretion of pro-inflammatory cytokines like IL-12 (reviewed in [110]). So far the prophylactic and therapeutic application of DCs has predominantly been investigated in animal models and was shown to elicit promising, beneficial effects on several autoimmune and transplantation models (see table 2 for a short summary). Interestingly, the work performed by Fu et al. indicate that so called "semi-mature" DCs may be more efficient for inducing immune tolerance than immature DCs. exhibit an intermediate expression of costimulatory molecules but do not secrete. The elevated expression levels of CD80 and CD86 on semi-mature DCs, have been shown to be optimal for the induction

of regulatory T cells [111], while the lack of pro-inflammatory cytokines might maintain T cell hyporesponsiveness. These findings emphasize the importance of the maturation status on the different tolerogenic functions of dendritic cells.

1.7.2 Dendritic cell-based cancer treatments

The clinical goal of cancer immunotherapy is to elicit innate and/or adaptive immune responses against a weakly immunogenic or immune suppressing neoplasm and thereby providing active or passive immunity. The aim of such cancer vaccines is to break tumor-induced immune suppression and boost tumor associated antigens (TAA) -specific T cell immunity, therefore preventing or at least prolonging the time to recurrence. Especially the expansion of $CD8^+$ T cells recognizing TAA with a high avidity and with high cytolytic activity is regarded as the main target of a cancer vaccine. The formulation of a successful cancer vaccine is dependent on how well defined the TAA are and whether they are shared among tumors in different individuals. Thus, various DC cancer vaccine approaches have been investigated. Classical vaccination approaches use whole tumor cells [112], tumor cell lysates or tumor specific peptides vaccines that can be captured and presented by dendritic cells in vivo [113], [114]. Antigens can also be delivered to DCs in vivo using specific antibodies or viruses (Reviewed by [115]). Protein respectively peptide vaccines are generally poorly immunogenic and the application of a potent adjuvant is crucial to increase the immunogenicity of the vaccines both during the induction of immunity and in the effector phase of the response. Especially (combinations of) TLR agonists have been evaluated (reviewed by [116]). The availability of techniques to expand or generate dendritic cells ex vivo from hematopoietic stem cells or blood monocytes gave rise to new vaccination approaches. Ex vivo culture enables the maturation and antigen pulsing of the DCs in a controllable manner. Furthermore, ex vivo generated or expanded DCs can be genetically modified in order to express immunologically defined molecules as “adjuvants”. Some candidate cytokines like, IFN-α or IL-2 help to boost and maintain immunity, whereas for example IL-12 also specifically drives Th1 responses, which is associated with an improved anti-tumor immunity [117]. Many of these vaccination approaches were shown to evoke tumor specific immune responses and tumor regression in different animal models [112], [118]. In contrast, clinical phase I and II trials (mostly conducted with late-stage patients who display strong immunosuppressive mechanisms), although eliciting measurable tumor-specific immune reaction [114], translated only rarely into clinical benefit. So far only a single DC based cancer vaccine has been approved for clinical application [119].

2 Aim of the project

Dendritic cells play a central role in promoting and regulating adaptive immune responses. Through the expression of specific cytokines we intended to modulate DC maturation or the interactions between DC and T lymphocytes. The application of a homogenous, well-defined dendritic cell line allowed us to investigate the role and the potential of pro-inflammatory as well as tolerogenic molecules in the regulation of immune responses by dendritic cells in a rational and standardized way.

In a first part, the tolerogenic potential of the previously established IL-10 or TGF-β expressing DC lines were evaluated in vivo. Applying the experimental autoimmune encephalitis (EAE) and a skin transplantation model we sought to answer the question whether the transfer of tolerogenic DC lines may help to restore T cell tolerance. In addition, a DC line expressing the recently described immunosuppressive cytokine IL-35 was generated. We were especially interested in the effects of IL-35 on *in vitro* DC maturation and its potential to suppress adaptive as well as innate immune responses *in vivo*.

In a second part, we intended to apply the CD8α$^+$ MuTu DC line as an experimental tumor vaccine and how the immunogenicity of such a vaccination can be increased. We were especially interested whether the provision of allogeneic antigens might augment tumor specific T cell responses. Moreover we addressed the hypothesis that pro-inflammatory cytokines expressed by the transferred dendritic cells might further improve anti-tumor immunity respectively help to reverse the suppressive tumor environment.

3 The CD8α+ MuTu conventional Dendritic Cell line

Although they play a central role in immunity, research on dendritic cells is limited by various restrictions. First of all, the natural scarcity of DCs *in vivo,* but also their functional and anatomical heterogeneity drastically limits the cellular material for experimentation. Isolation of dendritic cells from lymphoid tissue is a labor-intensive procedure yielding only limited numbers of cells. In addition, isolated DCs exhibit an activated phenotype which leads to apoptosis and limits their usability for *in vitro* experiments [120]. Differentiation of so called bone marrow derived DCs (BMDC) by culturing murine bone marrow progenitors with granulocyte-macrophage colony stimulating factor (GMCSF) (and IL-4, depending on the protocol) or Flt3l, has been widely used for *in vitro* research [121], [122]. Generation of BMDCs allows the generation of large quantities of DCs. Nevertheless, the procedure is dependent on the availability of bone marrow or blood derived precursor cells and requires 6-10 days of in vitro differentiation. The resulting cells are short-lived and represent heterogeneous DC populations. Especially GMCSF + IL-4 derived BMDCS are of a mixed, not well characterized phenotype, whereas Flt3l-derived BMDCs are a mixture of cells equivalent to CD8α+ and CD8α- DCs as well as pDCs. Unlike to the T or B cell research fields, only few DC lines have been described and none of them have been thoroughly characterized or is widely accepted [123]–[126]. Three oncogene-driven, immortalized DC lines have been derived from mice: the DC 2.4 [124], the CD8α+ SRDC line [126] and the SVDC line [125]. However, they depend on particular culture conditions or growth factors and may not be equivalent to endogenous DCs. The need for easily cultivable DC lines that retain the biological functions of specific DC subsets was obvious.

3.1 Generation of the CD11c:SV40lgT transgenic mice

In 2005 Steiner et al. generated C57BL/6 mice expressing the SV40 large T oncogene under the control of the CD11c promoter (Fig.6a). Bicistronic expression of eGFP allowed to identify and monitor expression of the transgene. The transgenic mice spontaneously developed pallor and subsequently became rapidly moribund. Correlating with the expression levels of the transgene, median onset of the disease varied between 4 months (Tg line 1) and 13 month (Tg line 2). Diseased mice exhibited formation of solid tumors in spleen and liver which were accompanied by hepatosplenomegalie and drastically decreased hematocrit levels. Thus, CD11c-SV40LgT mice exhibit phenotypic characteristics of human multisystemic histiocytosis and represent an adequate animal model for the disease [127]. Flow cytometric analysis revealed the accumulation

of tumoral, GFP$^+$ cells in spleen, liver and bone marrow. In vitro cultivation of splenocytes from tumor-burdened animals led to the derivation of an easily cultivable cell line thereafter named "MuTu" (for Murine Tumor) line. Further characterization showed the derived cells to express CD11c, CD8α, CD24 and CD205 but no or low expression of B220, CD11b and CD4. Cell surface levels of MHC-II and the co-stimulatory molecules CD40, CD80 and CD86 closely correlated with splenic CD8α$^+$ dendritic cells in steady- as well as in an activated state. The comparison of gene expression as well as proteome profile confirmed the equivalence of the transformed cells to endogenous CD8α$^+$ cDCs. When activated with TLR-ligands in the presence of IL-4, IFN-γ and anti-CD40 stimuli, MuTu DCs secreted high levels of IL-12p40 and p70, which is a particular quality of CD8α$^+$ DCs. The transgenic DCs were also capable to (cross-) present antigens to CD4$^+$ or CD8$^+$ T cells and to induce Th1 and CTL responses (Fig.6b). These findings highlighted that the MuTu DC line not only share phenotypic characteristics but also retained biological function of splenic CD8a$^+$ DCs [128]. In addition, it was shown that MuTu DCs can easily be genetically altered by crossing CD11c-SV40LgT mice to any other transgenic mouse strains. Alternatively, lentiviral transduction of the DC line allowed the stable expression of transgenes. Taken together, the CD11c$^+$ cells derived from the transgenic mice form a transformed cell population that can easily be maintained *in vitro*. The cells phenotypically resemble endogenous CD8α$^+$ cDCs and maintain their functional characteristics. The proliferating cells can be harvested from spleen and liver of transgenic mice and cultured *in vitro* to form a homogenous CD8α$^+$ dendritic cell line, which is stable for more than 60 passages representing an excellent tool for immunologic research.

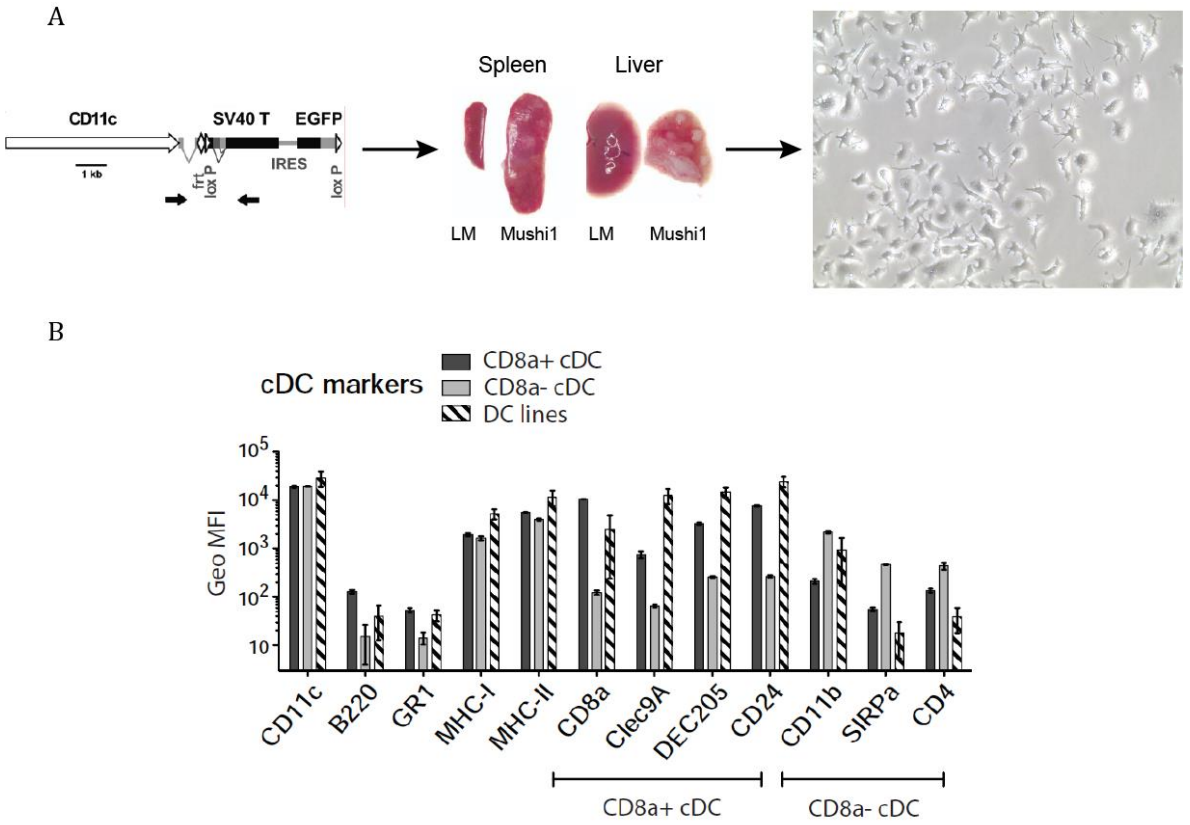


Figure 6 Generation and characterization of the MuTu DC line: A) Transgenic expression of the simian virus 40 (SV40) T antigen under control of the CD11c promoter leads to the formation of tumors in spleen and liver of the transgenic mice. In vitro cultivation of the transformed cells allowed the generation of CD8α+ DC lines. B) Comparison of the derived cell line to endogenic CD8α+ or CD8α- DCs by flow cytometry. The transformed cells were found to resemble splenic CD8α+ conventional dendritic cells and maintain their phenotype, function and maturation capacity. Images adapted from [127], [128]

II. Results

4 Induction of immune tolerance by DC lines secreting tolerogenic molecules

4.1 Generation of tolerogenic dendritic cell lines

4.1.1 Cloning Strategy and generation of transgenic dendritic cell lines

A number of transgenic CD8α⁺ MuTu dendritic cell lines, including the IL-10 and TGF-β expressing lines, were previously generated and described by Anaïs Duval ([129], In preparation) (Fig.7). Briefly, gene coding sequences were amplified from cDNA generated from CpG stimulated CD8α⁺ MuTu dendritic cells (IL-10, Ebi3) or from appropriate plasmids (active and latent form of TGF-β (courtesy from Prof. S. Werner, ETH Zürich), IL-12p35 (courtesy from Prof. B. Becher, University of Zürich). The different coding sequences were cloned into a lentiviral expression (kindly provided by Prof. D. Trono, University of Geneva). Replication deficient lentiviral particles were generated by the transfection of 293T HEK cells. MuTu dendritic cells were transduced using the highest lentiviral concentration not interfering with DC viability. The CD8α⁺ MuTu dendritic cell line was also transduced with the empty lentiviral vector as a control (mockDC). Transgene transcription was assayed by measuring the respective gene product by real-time PCR and western blot (IL-35) or ELISA (IL-10, active TGFβ). The transgene expression exhibited some variation, especially for the IL-35⁺DC line (data not shown). The resulting, stably transduced dendritic cell lines IL-10⁺DC, actTGFβ⁺DC (active form of TGFβ) and IL-35⁺DC were used in the context of this dissertation. Lentiviral transduction itself has previously been shown in our laboratory not to alter the expression of CD8α⁺ DC specific markers CD11c, CD11b, CD8a, CD205, CD24 or Clec9a. In contrast, lentiviral infection of the dendritic cell line was shown to slightly increase expression levels of MHC class II as well as co-stimulatory molecules CD40 and CD80. However, the changes were only minor in comparison to TLR ligand activated DC lines (data not shown and [128]. Transgene expression was shown before to be stable for at least 20 in vitro passages [130]. Nevertheless, the production of the respective transgenic product was assayed before each experiment

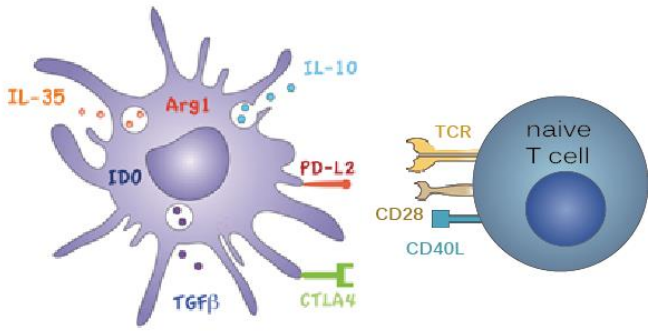


Figure 7 MuTu dendritic cell lines expressing tolerogenic molecules: A number of MuTu DCs were lentivirally transduced in order to express molecules with well-described tolerogenic properties. IL-10, TGF-β and IL-35 expressing lines exhibited most potent suppression of T cells *in vitro* and were therefore further investigated.

4.2 Effect of Interleukin 10 or TGF-β expression on the DC line

As indicated before, the DC lines expressing either the latent or active form of TGF-β as well as IL-10 have been previously generated and described by A. Duval [130]. For the sake of comprehensibility, the main characteristics of the transgenic DC lines will be summarized below.

4.2.1 Interleukin-10 expressing DC lines

IL-10 transduced DC line secreted substantial amounts of IL-10 cytokine as assayed by ELISA. Surprisingly we also found a robust production of TGF-β by IL-10$^+$DCs (Fig.8a). Previous work showed that IL-10$^+$DCs exhibited a reduced ability to upregulate MHC-II and costimulatory receptors CD80 and CD86 upon stimulation with the TLR ligands CpG, pI:C and IFN-γ. Expression of IL-10 furthermore inhibited the release of pro-inflammatory cytokines such as IL-12 or IL-6 upon activation, thus interfering with a functional maturation of the DCs. Ovalbumin peptide pulsed wild-type DCs induced antigen specific proliferation of CD4$^+$ and CD8$^+$ T cells as well as Th1 commitment. In contrast, IL-10$^+$DCs were shown to rather induce Th17 responses as indicated by the reduced IFN-γ and increased IL-17 expression by CD4$^+$ T cells (Fig.8b). However, the proliferative response was comparable to wild-type MuTu DC (wtDC) primed CD4$^+$ T cells. Similarly, ovalbumin peptide pulsed IL-10$^+$DCs did not have an impact on antigen-specific CD8$^+$T cell proliferation but it impaired the secretion of IFN-γ by these cells. The expression of cytotoxic effector molecules granzyme B or perforin was not affected (data not shown).

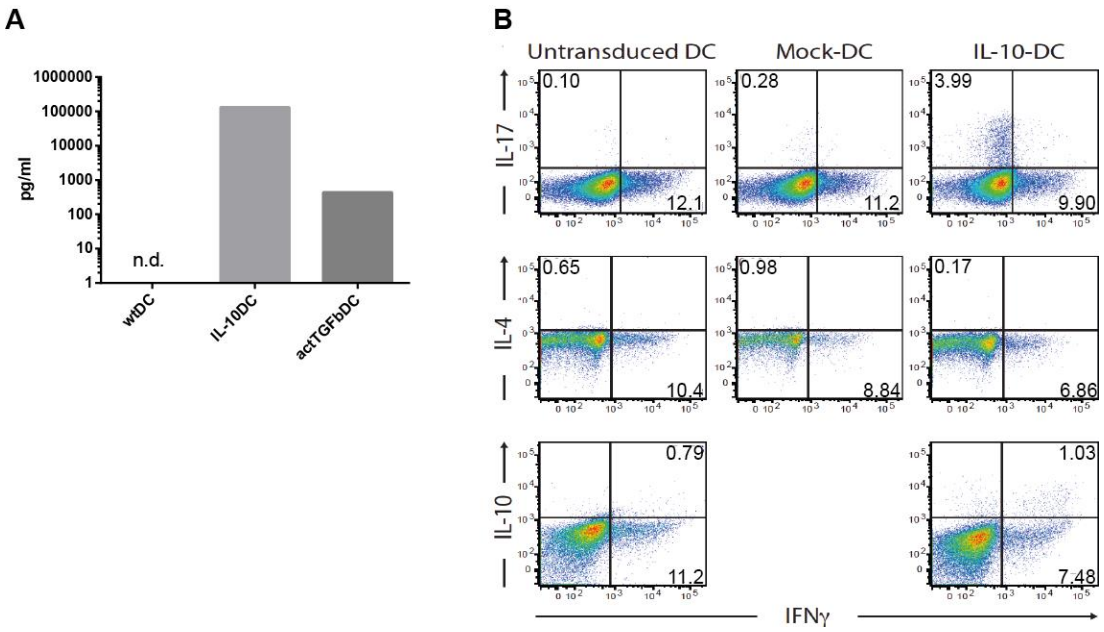


Figure 8 IL-10 expressing DC line impair Th1 commitment of antigen-specific activated CD4⁺ T cells: A) The expression of the immunosuppressive molecule IL-10 was verified by ELISA on culture supernatants collected after 72 hours incubation of the respective DC line. Data represents 1 out of at least 2 independent experiments. n.d. = not detectable. B) OT-II CD4⁺ T cells were cultured for 3 days with the $OVA_{323-339}$ pulsed active TGF-β expressing DC lines. IL-10⁺DCs slightly reduced IFNγ production while increasing the expression of IL-17 by OT-II cells. Data generated by A.Duval.

4.2.2 TGF-β expressing DC lines

As described in the introduction, TGF-β is produced as a latent, biologically not active, protein complex. Separation of the cytokine complex has been shown to be necessary to activate TGFβ, thereby imposing a tight regulation of TGF-β function. In order to avoid these restrictions, we generated a DC line expressing a mutated TGF-β sequence (Cys223Ser; Cys225Ser) that is not able to bind the LAP proteins and thus cannot form the latent complex. Transduction of the wild-type DCs resulted in the stable production of the active form of TGF-β as assayed by ELISA (Fig9a). While TGF-β expression did not affect DC maturation itself, ovalbumin-specific CD4⁺ T cells cultured in the presence of OVA peptide pulsed DCs expressing active TGF-β produced significantly less IFN-γ than T cells primed by wtDCs. Moreover, the constitutive expression of active TGF-β induced a CD25⁺ Foxp3⁺regulatory phenotype on the CD4⁺ T cells (Fig.9b). However, these in vitro induced Tregs were not stable as they completely lost Foxp3 and CD25 expression within six days even after restimulation in the presence of TGFβ. Furthermore, the expression of the active form of TGF-β by the DCs had no effect on the proliferative response of antigen specific CD8⁺ T cells in vitro but potently inhibited the expression of IFN-γ as well as of the cytotoxic effector molecules perforin and granzyme B (data not shown).

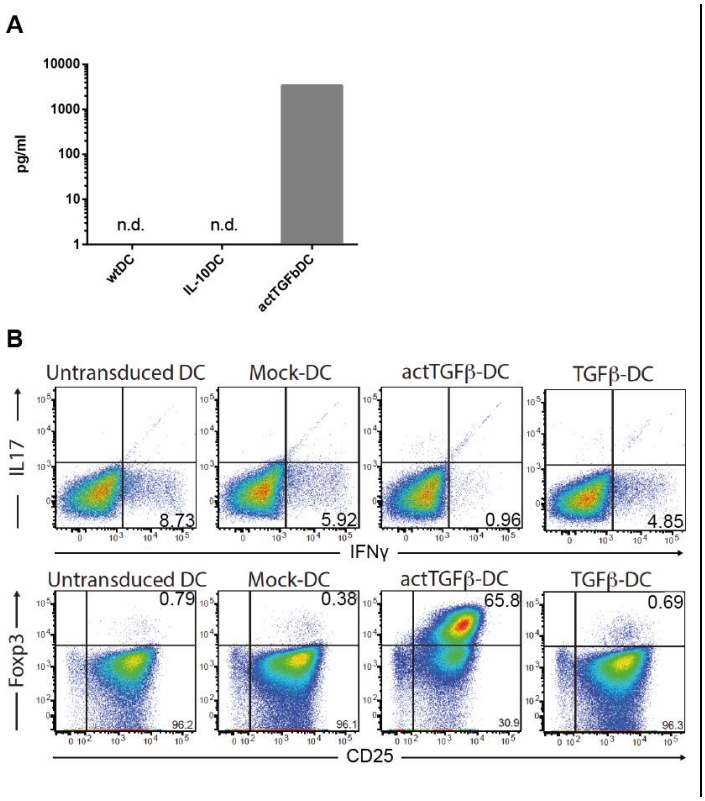


Figure 9 active TGF-β expressing DCs impair Th1 commitment but induce Foxp3⁺ CD25⁺ expression upon the antigen-specific activation of CD4⁺ T cells: A) The expression of the immunosuppressive molecule TGF-β was verified by ELISA on culture supernatants collected after 72 hours incubation of the respective DC line. Data represents 1 out of at least 2 independent experiments. n.d. = not detectable. B) Only a marginal proportion of OT-II cells cultured in the presence of DCs expressing the active but not the latent form of TGF-β produced IFNγ. actTGFβ⁺DC induced a regulatory phenotype on the CD4⁺ T cells as indicated by the increase of CD25 and Foxp3. Data generated by A.Duval.

4.3 Characterization of the Interleukin-35 expressing dendritic cell line

4.3.1 Generation of a constitutively Interleukin-35 expressing DC line

A murine dendritic cell line constitutively expressing a biologically functional, single chain IL-35 construct (Fig.10a) was generated by linking the two IL-35 chains Ebi3 and p35 with a flexible $(Glycine_4Serine)_3$ linker. The short polypeptide linker allows the expression of both cytokine subunits under control of the same promoter while retaining its biologic activity [131]. The native CD8α⁺ MuTu DC line was subsequently transduced using a second generation lentiviral system [132]. Transgene expression was confirmed by real-time PCR detection of IL-12p35 and Ebi3 transcripts (Fig.10c). The single chain protein product was immunoprecipitated and could be detected by western blot in the supernatant and the cell lysate of cultured IL-35⁺DCs (Fig.10b). This transgenic DC line is thereafter referred to as "IL-35⁺DC".

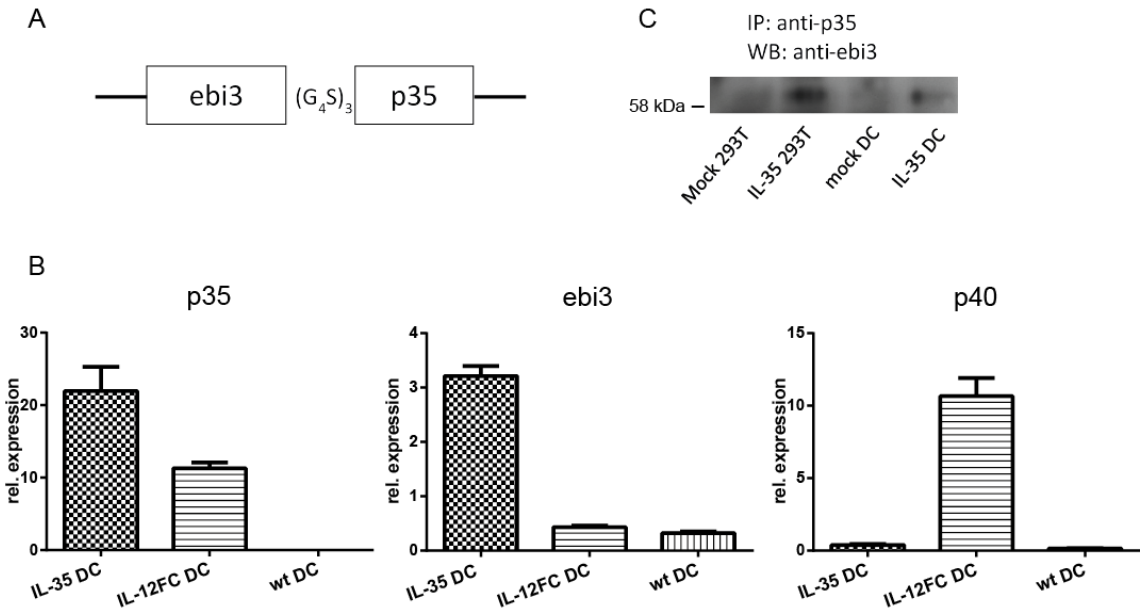


Figure 10 Generation of a constitutively IL-35 expressing CD8α⁺DC line: A) schematic overview of IL-35 expression cassette: The two subunits p35 and ebi3 were amplified from T cell cDNA and introduced into a 2nd generation lentiviral expression vector. A $(Gly_4Ser)_3$ linkerallows the expression as a single-chain construct. Stable expression of the transgene in the CD8α⁺ dendritic cell line was achieved by lentiviral transduction. B) IL-35 subunits p35 and Ebi3 but not IL-12p40 or IL-27p28 were detected in the IL-35 transduced DC line by reverse transcription rtPCR using primers specific for indicated transcript. C) IL-35 protein was precipitated from supernatant of IL-35+DCs as well as transfected 293T HEK cells using p35 specific antibody and detected by Western blot using Ebi3 specific antibodies.

4.3.2 *In vitro* characterization of the Interleukin-35 expressing MuTu DC line

IL-35 has recently been described to affect not only T lymphocytes but also a variety of other cell types: IL-35 was shown to induce regulatory B cells [34]. Furthermore, EBI3 and IL-12p35 have been shown to be expressed in some human cancers [133]. IL-35 expression by murine B16 melanoma and J558 B myeloma have been shown to increase accumulation of myeloid cells and induce angiogenesis in the tumor microenvironment [133]. We therefore investigated whether IL-35 can act on an autocrine manner on the transduced dendritic cells itself.

The IL-35⁺ dendritic cell line was assessed for the expression of a panel of surface markers commonly used to characterize CD8α⁺ DCs by direct comparison to empty vector transduced DCs (mockDC). Flow cytometric analysis revealed significantly reduced expression of CD11c, CD8α and CD205 on IL-35 transduced DC line. The expression of CD11b was drastically increased when compared to empty vector transduced DC lines (Fig.11a). Furthermore, the IL-35 expressing DC line exhibited markedly lower expression of MHC-I and MHC-II as well as co-stimulatory molecules like CD40 and CD86 under steady-state conditions. Activation via TLR 3 ligand (polyI:C), TLR 9 ligand (CpG) and interferon-γ (IFN-γ) led to a markedly increased surface

expression of MHC-II as well as co-stimulatory receptors CD40, CD80 and CD86 on empty vector transduced control DC lines (mockDC). In contrast, the IL-35 secreting DCs were unresponsive to TLR3/9 and IFN-γ stimulation as indicated by the failure to up-regulate any of the analyzed (co-) stimulatory molecules (Fig.11b). Finally, the ability to secrete the pro-inflammatory cytokines IL-12 and IL-6 by the transduced DC lines was assayed. None or basal levels of the two cytokines could be detected in the supernatant of resting cells, activation with the TLR ligands pI:C, CpG as well as IFN-γ upregulated the production of both cytokines. While the levels of IL-6 were comparable to control and IL-35 transduced dendritic cells, IL-12 secretion by IL-35⁺DCs was markedly reduced when comparing to mockDCs (Fig.11c).

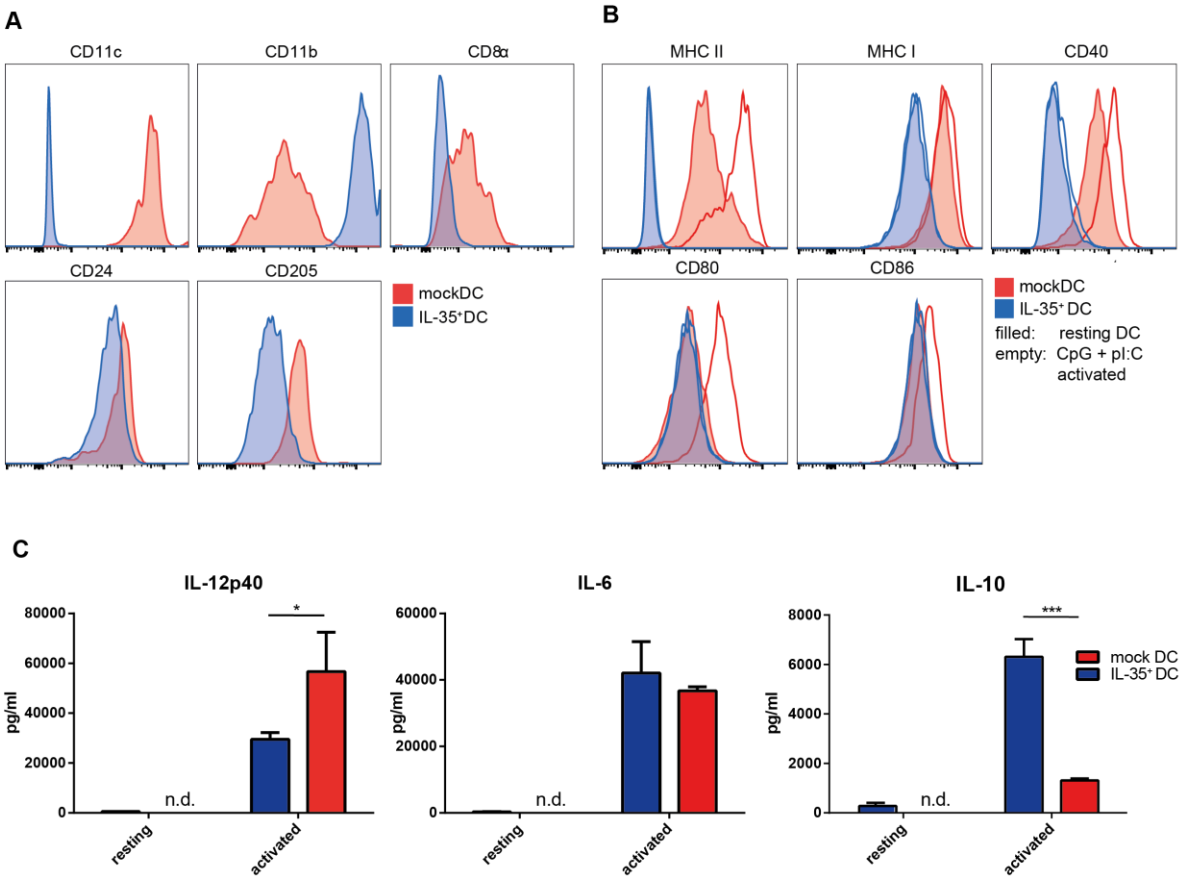


Figure 11 IL35⁺ DCs upregulate CD11b and do not respond to activation stimuli: IL-35 and mock transduced DCs were cultured under same conditions and analyzed by flow cytometry for expression of A) DC specific markers or B) activation markers upon 12h stimulation with 5µg/ml CpG and 1µM pI:C. a) IL-35⁺ DC exhibited a markedly increased expression of CD11b whereas the expression of the CD8α⁺DC specific markers CD11c, CD8α and CD205 was reduced. B) In comparison to mock transduced DCs, IL-35⁺ DCs showed lower expression of MHC-II, MHC-I and CD40 under resting conditions (filled lines). Upon activation (empty lines), IL-35+ DCs did not respond to activation stimuli and were not able to up-regulate MHC as well as costimulatory receptors. Data representative of 4 independent experiments. C) IL-35 or control transduced DCs were seeded and incubated for 3 days. The cells were stimulated for the last 18 hours or not and the secretion of the pro-inflammatory cytokines was quantified by ELISA.

4.3.3 Interleukin-35 reduces the upregulation of MHC class II and costimulatory molecules and induces IDO transcription by CD8α⁺ DCs upon activation

As shown above, IL-35 but not mock transduced dendritic cells exhibited markedly altered phenotypical and functional characteristics (Fig.11). Given the IL-35 induced effects observed on cells of non-lymphoid origin, we wondered whether IL-35 cytokine might directly skew CD8α⁺ DCs towards a rather tolerogenic phenotype. Wild-type MuTu dendritic cells were cultured for four days in the presence of supernatant collected from IL-35 or control transfected 293T cells. Similar to IL-35⁺ DCs, wild -type DCs incubated in the presence of IL-35 conditioned medium expressed higher levels of CD11b, while CD11c expression was down-regulated in comparison (Fig.12a). However, the effect was less pronounced as compared with IL-35 transduced dendritic cells. Upon activation with TLR3 and TLR9 ligands, the up-regulation of MHC-II and co-stimulatory receptors CD40 and CD80 was significantly impaired when wtDC were cultured in IL-35 containing medium whereas we could not see an effect on the expression levels of MHC class I (Fig.12b).

Indolamin-2,3-Dioxygenase (IDO) production by DCs has been shown to efficiently inhibit clonal expansion of T cells depleting the amino acid tryptophan and inducing regulatory phenotype on T cells [134], [135]. CD11b⁺ IDO secreting dendritic cells have recently been proposed to induce systemic immune tolerance [90]. We therefore wondered whether IL-35 induced up-regulation of CD11b on CD8α⁺ DCs is accompanied by IDO expression. As expected, only basal expression levels of Ido1 mRNA were detectable by real-time PCR in wild-type DCs (Fig.12c). IFN-γ stimulation of wild type and mockDCs led to a considerable increase of Ido1 expression that was comparable to Ido1 transduced DCs. Cells that were cultured for 3 days in the presence of IL-35 conditioned medium exhibited a significantly higher Ido1 transcription than cells cultured in the presence of control medium even in the absence of IFN-γ stimulation.

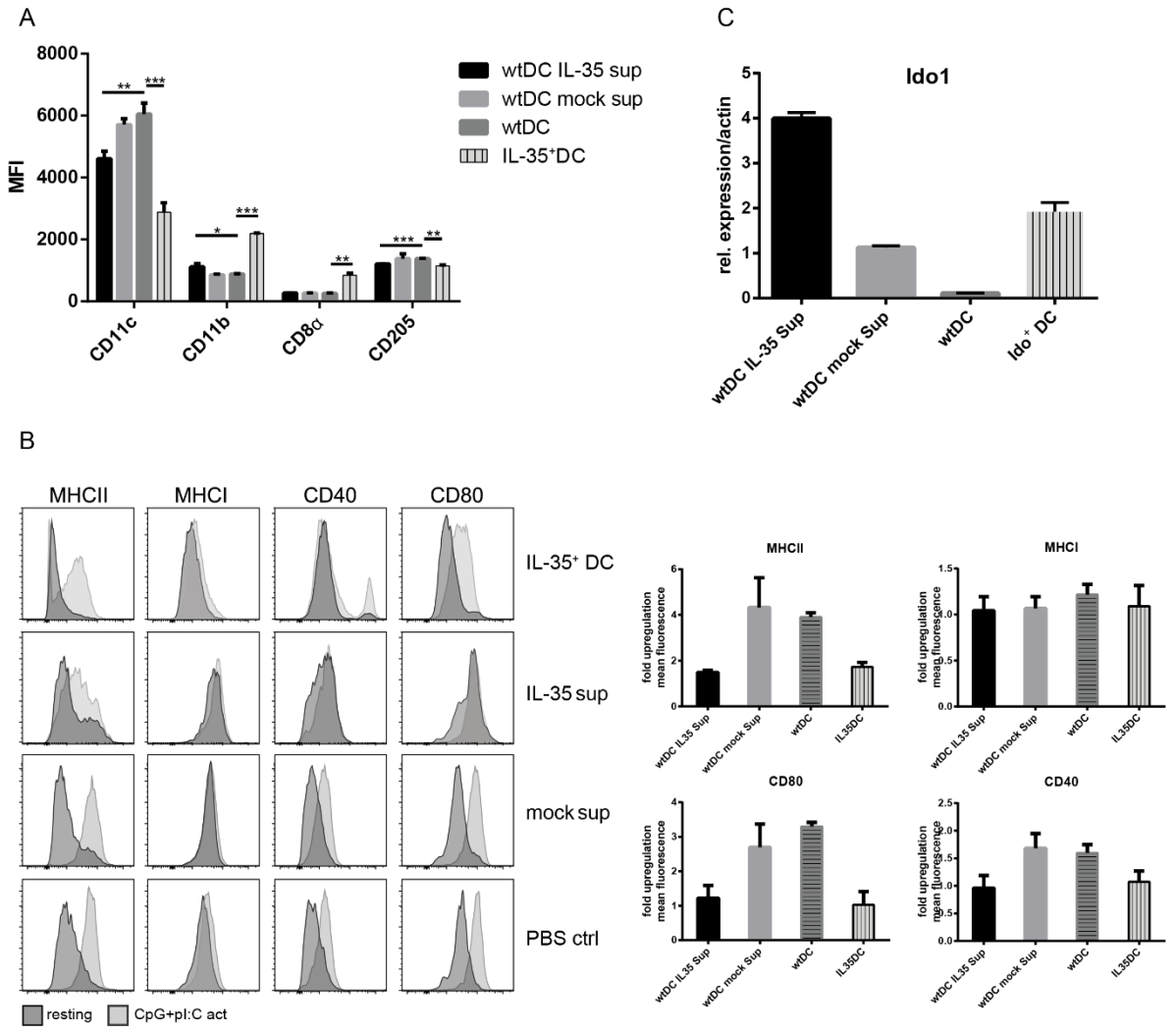


Figure 12 IL-35 alters phenotype of MuTu dendritic cells and induces transcription of Indolamin-2,3-Dioxygenase (Ido1): Wild-type dendritic cell line was cultured for 4 days in presence of filtered and concentrated supernatant of IL-35 or empty vector transfected 293T HEK cells. DCs were analyzed by flow cytometry for expression of A) DC specific markers or B) activation markers upon 6h stimulation with 5µg/ml CpG and 1µM pI:C. Wild-type CD8α+DCs cultured with IL-35 conditioned supernatant but not control supernatant exhibited reduced expression of CD11c as well as CD205 and an increased expression of CD11b. However, the differences were not as distinct as in the IL-35 transduced DC line. B) Activation of wtDCs cultured in IL-35 conditioned medium led to a significant weaker up-regulation of MHC class II as well as co-stimulatory receptors CD40, CD80 and CD86 than in control supernatant cultured dendritic cells. Data shown is representative for 5 independent experiments. Experiments were conducted in triplicates and results are shown as the mean of the ratio of geometric mean activated cells/geometric mean resting cells ±SEM. C) Induction of Indolamin-2,3-Dioxygenase (Ido1) transcription by IL-35 conditioned wtDCs could be detected by reverse transcriptase rtPCR analysis. Graph is representative for two independent experiments. Data was obtained in triplicates and is depicted as mean relative expression ± SD.

A putative heterodimeric receptor for IL-35, composed of the IL-6 receptor family member gp130 and the β-chain of the IL-12 receptor (Il12rb2) has been described for T cells [37]. More recently, B cells were found to express a functional receptor complex comprised of IL-12Rβ2 and IL-27Rα that was able to bind IL-35 [34]. Engagement of IL-35 to the receptor induces downstream

signaling involving formation of STAT1/4 or STAT1/3 heterodimers in T-, respectively B cells [34]. While gp130 is ubiquitously expressed and can consistently be detected on the DC line, we were not able to detected constitutive or induced Il12rβ2 expression by IL-35$^+$ or wild-type DC lines by real time PCR (data not shown).

4.3.4 Interleukin-35$^+$DC inhibit CD4$^+$ and CD8$^+$ T cell proliferation and function *in vitro*

We have previously shown that the MuTu DC line efficiently induces T cell proliferation in MHC-I and MHC-II restricted systems [128]. IL-35 on the other hand, was originally described to inhibit CD4$^+$ T cell proliferation [32], [39]. We therefore tested whether the production of IL-35 by the DC line is capable to affect T cell activation and function. An allogeneic mixed leukocyte reaction (MLR) was performed using variable amounts of mock transduced or IL-35 expressing, C57BL/6 derived DCs as stimulators. Co-culture with mock transduced DCs resulted in a robust proliferation of BALB/c derived CD4$^+$ responder T cells, which was dependent on stimulator cell number. The proliferation of the T cells was accompanied by an increased percentage of activated CD44$^+$CD62l$^-$ T cells and the production of IFN-γ. Target cell proliferation and activation could be severely reduced when IL-35$^+$ DCs were used as activator cells (Fig.13a). In addition to its effect on proliferation, IL-35 is described to induce IL-35 expression in the target T cells. We therefore investigated the transcription of IL-35 related genes in co-cultured CD4$^+$T cells. Analysis of T cell RNA revealed a significant upregulation of p35 and ebi3 but not IL-12p40 or IL-27p28 transcription when cultured with IL-35$^+$DCs but not mockDCs (Fig.13b).

Much less is known about if and how IL-35 can affect proliferation and/or effector functions of CD8$^+$ T lymphocytes. In fact, only one article describes that CD3/CD28 stimulation of human CD8$^+$ T cells can induce IL-35 expression [136]. Another publication described that the presence of IL-35 in an MLR can attenuate the proliferation of CD8$^+$ T cells [137]. In accordance to the latter publication, we found that IL-35$^+$DCs were also able to inhibit proliferation and activation when a MLR was performed using mock transduced or IL-35 expressing, C57BL/6 derived DCs as stimulators and BALB/c CD8$^+$ T lymphocytes as responder cells (Fig 13c). Although IL-35$^+$DCs induced the transcription of significant higher levels of ebi3 and p35 in CD8$^+$ T cells than mock transduced DC, the transcription levels were substantially lower than in CD4$^+$ T cells (Fig.13d).

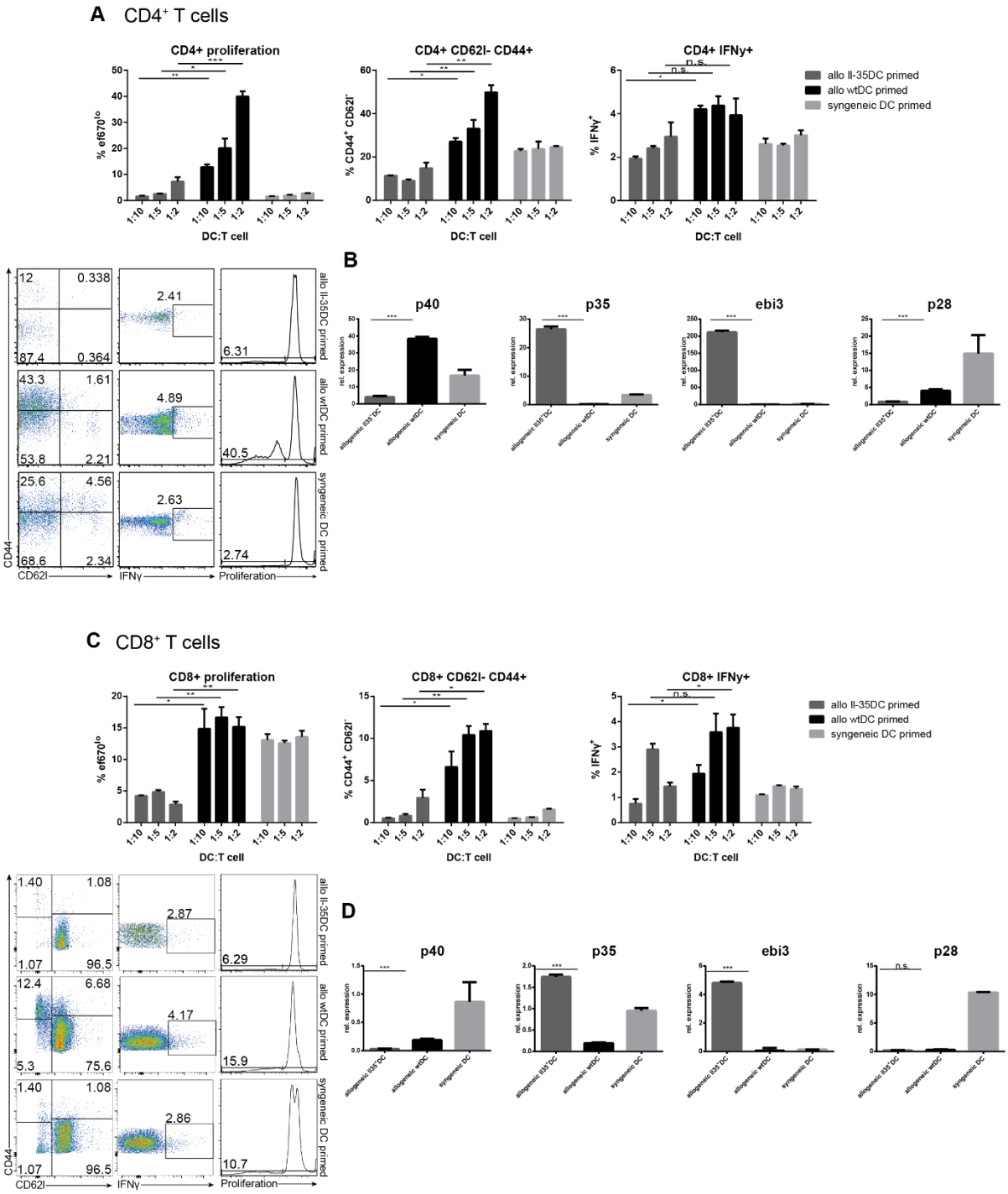


Figure 13 Allogeneic stimulation of BALB/c T cells with C57BL/6 IL-35+ DCs: Naive CD4+ T (A and B) or CD8+ T (C, D) cells were isolated and purified from BALB/c mice and cultured at different ratios with C57Bl/6 derived wild-type or IL-35+ DCs. Proliferation, activation status and transcription of IL-12 cytokine family genes of the T cells were analyzed after 3 days. A) CD4+ T cells proliferated significantly less and exhibited less CD62l- CD44+ activated phenotype when cultured together with IL-35 expressing DCs than with wtDC. Only a slight impairment of CD4+ T cell function as assayed by IFN-γ production could be observed. B) Analysis of T cell cDNA by real-time PCR showed a robust upregulation of IL-35 subunits p35 and Ebi3 but not of IL-12p40 or IL-27p28 transcription. C) CD8+ T cells cocultured with IL-35+ but not wild-type DCs were virtually unable to respond to allogeneic stimulation as indicated by drastically reduced proliferation, activation and IFN-γ production. D) Significant higher p35 and ebi3 transcription were observable in CD8+ T cells. However relative expression levels were considerably lower than in CD4+ T cells. Results representative of 3 (CD4+) respectively 2 (CD8+) independent experiments. Data is depicted as mean ± SD.

In order to exclude the possibility of a contamination of the analyzed T cell RNA by IL-35⁺DCs, we performed PCR reactions on the cDNA samples applying primers that amplify only IL-35⁺DC derived single-chain IL-35 but not the induced endogenous cytokine consisting of two independent protein chains (Fig.14a). While amplification of IL-35⁺DC derived cDNA yielded clear bands of the expected size only a faint PCR product could be detected in one CD8⁺T cell derived cDNA (Fig.14b).

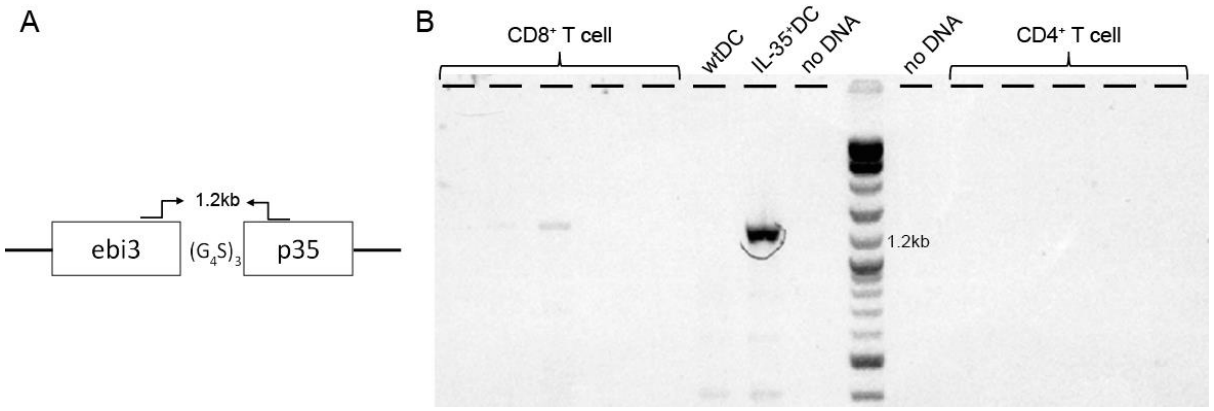


Figure 14 IL-35p35 and ebi3 transcription detected in the MLRs did not stem from single-chain IL-35 as expressed by the transgenic IL-35⁺DC line. A) Schematic overview over the single-chain IL-35 construct as it is expressed by the transgenic IL-35⁺DC line. The two arrows indicate the primer used in the following PCR reaction. B) PCR reaction using single-chain IL-35 specific primers was performed on the same CD4⁺ and CD8⁺ T cell derived cDNA that was applied for rtPCR in Fig.13. Only one of the MLR samples tested exhibited some minor contamination with DC derived IL-35.

As demonstrated above, IL-35⁺DC as well as wild-type DCs cultured in IL-35 conditioned medium exhibited a drastically altered phenotype, including a reduced co-stimulatory potential and the induction of IDO transcription. We therefore considered the possibility that the effects on T cells were not only directly caused by the secreted IL-35, but indirectly through the modulation of the immunogenic potential of the DCs. In order to test this hypothesis we performed an allogeneic MLR as described above and IL-35 cytokine was depleted using an ebi3-specific antibody. Due to the limited availability of the antibodies we could so far only perform a single pilot experiment. As observed in Fig.13, the culture of C57BL/6 IL-35⁺DCs did significantly reduce proliferation (Fig.15a) and IFN-γ expression (Fig.15b) by BALB/c derived CD4⁺ T cells. The addition of anti-p35 or anti-ebi3 antibody did not reverse the inhibitory effect of IL-35 on proliferation or IFN-γ expression by the T lymphocytes. However, although the antibodies are described to neutralize the cytokine, we did not assay the actual efficacy of the neutralization.

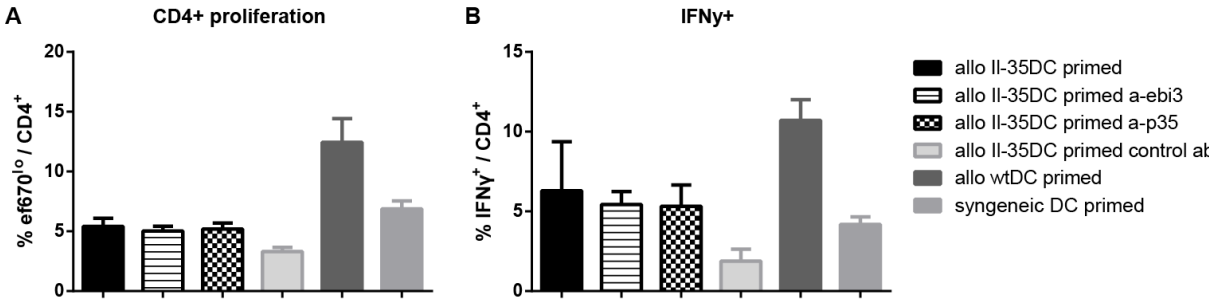


Figure 15 Neutralization of IL-35 cytokine does not restore CD4⁺ T cell proliferation and IFN-γ expression: Allogeneic MLR was performed as described in Fig.13. IL-35 cytokine was neutralized using anti-ebi3 or anti-p35 antibodies. However, the neutralization did not restore CD4⁺ T cell proliferation (A) or (B) IFN-γ expression. The data represents the mean±SD of biological triplicates of one experiment.

In a next step, we investigated the effect of IL-35⁺DC on CD4⁺ T cells proliferation upon *in vitro* stimulation in an antigen-specific manner. Naïve CD4⁺OT-II T cells were cultured for 4 days with IL-35 or wild-type DCs that were pulsed with different concentrations of OT-II specific $OVA_{323-339}$ peptide. OT-II cells primed by wtDCs proliferated consistently and upregulated the activation markers CD25 and CD69 in an $OVA_{323-339}$ peptide concentration dependent manner. Similar to the MLR experiments, the IL-35⁺DCs exhibited a lower immunogenic capacity resulting in a significantly reduced proliferative response and a lower percentage of activated OT-II cells. The IL-35⁺DC induced suppression of OT-II cell proliferation and activation could not be overcome even when high concentrations of $OVA_{323-339}$ peptide were applied. A similar, but slightly weaker effect on OT-II cell proliferation and activation could be observed when the OT-II lymphocytes were cultured with wild-type DCs in IL-35 containing supernatant. High concentrations of $OVA_{323-339}$ peptide could induce some proliferation even in the absence of MuTu DC but did not lead to T cell activation (Fig.16a). Analysis of the OT-II cell derived mRNA revealed a significant upregulation of p35 and ebi3 expression but not IL-12p40, when co-cultured with IL-35⁺DCs. The expression of IL-35 subunits by OT-II cells was comparable to the transcription levels found in IL-35⁺DC. The priming with wtDC induced transcription of the IL-12Rβ2, the receptor chain shared by IL-12 and IL-35 receptors, as well as IFN-γ in an $OVA_{323-339}$ peptide concentration dependent manner. Surprisingly, incubation with IL-35⁺DCs also induced strong IFN-γ expression even in the absence of $OVA_{323-339}$ peptide. In contrast, IL-35⁺DC or the addition of IL-35 containing medium led only to a weak upregulation of IL-12Rβ2 transcription (Fig.16b). As described above, we could not detect any DC derived single-chain IL-35 in the T cell cDNA samples (data not shown).

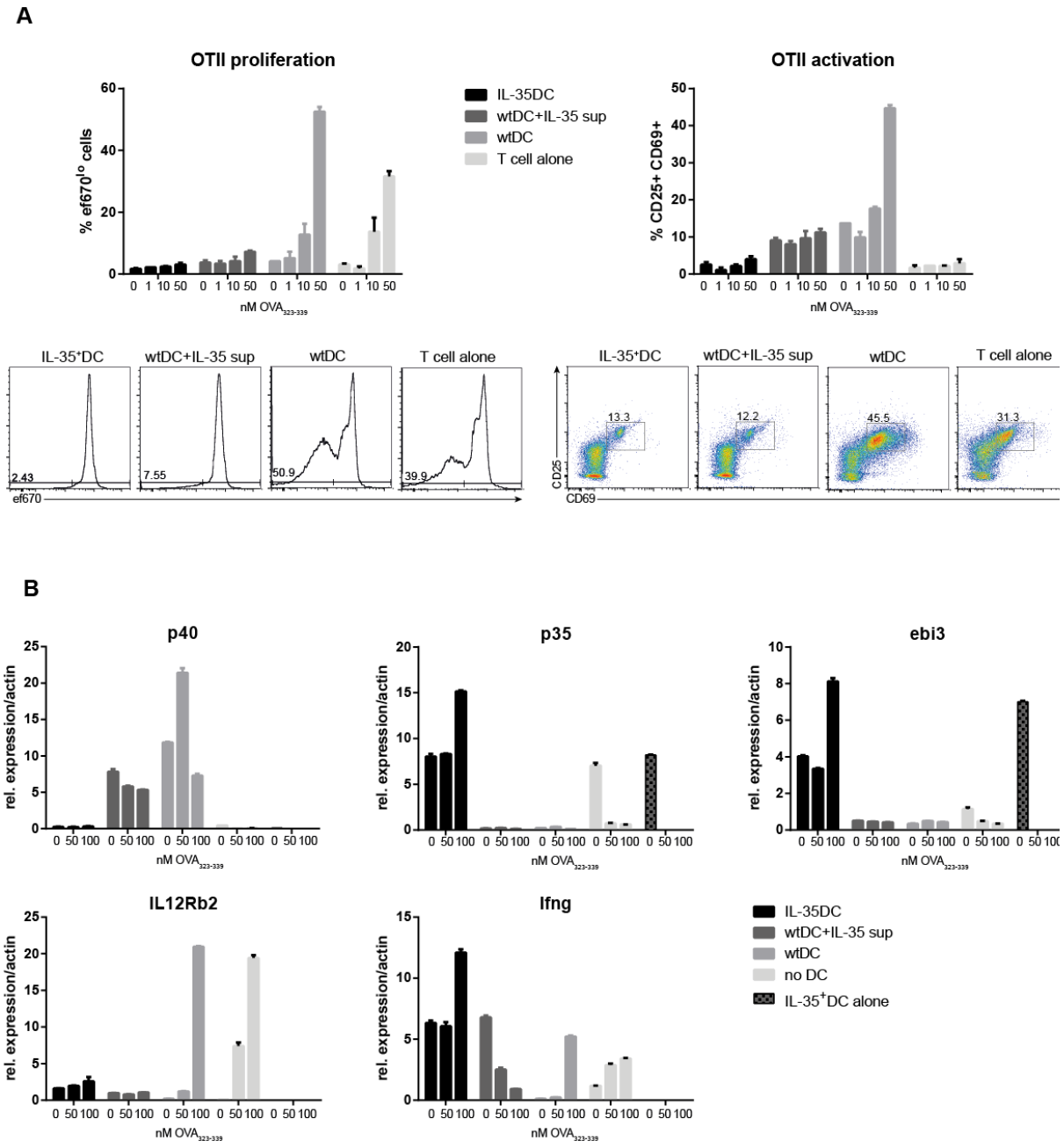


Figure 16 IL-35⁺DCs suppress proliferation and function of OVA peptide specific CD4⁺ T cells in vitro. $5x10^3$ IL-35 transduced or not transduced DCs were seeded in 96-well plate and pulsed for at least 4 hours with the indicated concentrations of $OVA_{323-339}$ peptide before adding $5x10^4$ purified, naive CD4⁺ OT-II T cells. The OT-II cells were harvested after 4 days and analyzed by A) flow cytometry or B) rtPCR. A) Whereas the incubation with wtDC resulted in $OVA_{323-339}$ peptide concentration dependent proliferation and activation of the OT-II cells, the culture with IL-35+DC or in the presence of IL-35 containing medium drastically reduced T cell response. B) real-time PCR analysis of the cDNA generated from the harvested OT-II cells revealed induction of IL-35 subunits p35 and ebi3 only when the T cells were cocultured with IL-35+DCs. While wtDC primed OT-II cells upregulated IL-12Rβ2, the subunit shared by the IL-12 and the IL-35 receptors, only very weak induction of the receptor chain expression was found when cultured in the presence of IL-35.

4.3.5 Interleukin-35⁺DC convert naïve CD4⁺ T cells into a suppressive population

Collison et al. proposed IL-35 cytokine to mediate infectious tolerance by inducing a regulatory phenotype on naïve CD4⁺ T cells [32]. We therefore tested whether CD4⁺ T cells co-cultured with IL-35 expressing DCs or IL-35 containing supernatant acquired a regulatory phenotype. Purified naïve wild-type CD4⁺T cells were co-cultured for 4 days with IL-35⁺ DC or wtDCs. The suppressive capacity of the primed T cells was then assayed by culturing them at different ratios with naïve CD4⁺ responder T cells that were stimulated with anti-CD3 and anti-CD28 antibodies. Proliferation of the responder T cells was analyzed after 3 days by measuring the dilution of the ef670 dye. We could measure a significantly reduced responder cell proliferation when cultured with T cells that were primed in the presence of IL-35⁺DC or wtDC supplemented with IL-2 and TGFβ. Inhibition of the responder T cell proliferation was dependent on the amount of suppressor T cells applied and a maximal suppressive effect was found at low T responder to iTreg ratios. Interestingly, we found the IL-35⁺DC induced Tregs to be more potent suppressors than wtDC+IL-2/TGF-β induced Tregs, both in terms of maximal suppressive capability as well as the minimal ratio of suppressor T cells needed to observe an inhibitory effect. T cells primed in the presence of wild-type dendritic cells did not inhibit responder T cell proliferation at high responder to suppressor ratios. However, at lower ratios a slight suppressive effect could also be observed with wtDC primed T cells. In accordance to literature, IL-35 induced regulatory cells did not express the classical regulatory T cell marker Foxp3, as do Tregs induced in the presence of IL-2 and TGF-β (Fig. 17 and data not shown).

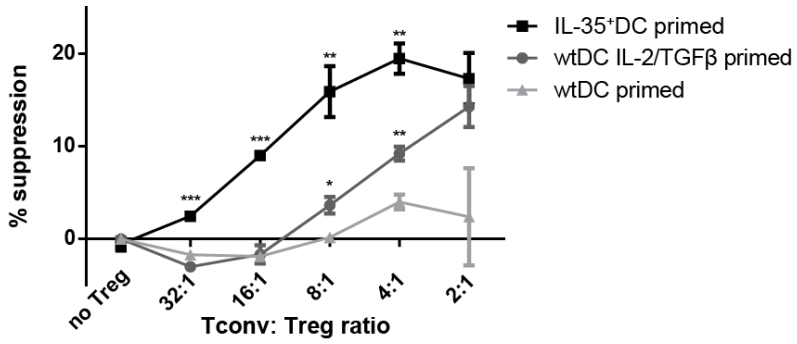


Figure 17 IL-35⁺DC conditioned CD4⁺ T cells acquire regulatory properties: Naïve CD4⁺T cells were primed in the presence of IL-35⁺DCs or wild-type DCs and IL-2/recombinant TGF-β for 4 days. The suppressive capability of the conditioned medium was assayed by measuring the proliferation of naïve target CD4⁺ T cells in the presence of anti-CD3/CD28 stimulation. Results are indicated as mean percent of suppression ± SD. Data representative of 4 independent experiments.

4.3.6 Interleukin-35$^+$DC impair CD4$^+$ and CD8$^+$ T cell responses *in vivo*

In a next step we assayed the potential of IL-35$^+$ DC to interfere with T cell proliferation and function *in vivo*. CD4$^+$ T lymphocytes were purified from CD45.1$^+$ OT-II mice. T cells were co-injected with the respective ovalbumin peptide pulsed, mock transduced or IL-35$^+$DCs into CD45.2$^+$ C57BL/6 mice. The spleens of the injected mice were harvested 4 days later and the lymphocytes isolated by mechanical disruption of the organ. Flow cytometric analysis revealed that the majority of the CD45.1+ OT-II cells did undergo proliferation and exhibited a considerable IFN-γ expression when co-transferred with $OVA_{323-339}$ peptide pulsed mockDCs. Co-transfer of pulsed IL-35$^+$DCs led only to a moderate decrease in the percentage of proliferated cells but the total cell number of retrieved OT-II cells was significantly reduced. In addition, the percentage of IFN-γ expressing OT-II cells was also markedly reduced when co-injected with IL-35$^+$DCs (Fig.18a).

The incubation of IL-35$^+$DC cells with CD8$^+$T affected their function *in vitro*. We therefore used the same experimental approach as described above to investigate possible effects of IL-35$^+$DC on the function of CD8$^+$ T cells *in vivo*. Mock transduced or IL-35$^+$DC were loaded *in vitro* with the ovalbumin derived SIINFEKL peptide at different concentrations and co-injected with purified CD45.1$^+$ CD8$^+$ OT-I T cells. Co-injection of IL-35$^+$DCs led to a drastic reduction of OT-I CD8$^+$ T cells that could be retrieved form the spleen of the respective animals. However, the few remaining OT-I T cells were found to proliferate comparably to OT-I cells isolated from wtDC co-injected animals. The percentage of IFN-γ producing OT-I cells was even significantly higher compared to when wtDCs were co-injected (Fig.18b). Host derived CD45.2$^+$ CD8$^+$ T cell count and IFN-γ expression was found to be similar in all three experimental groups (Fig.18c).

Taken together, the inhibitory effect on T cell proliferation and function *in vitro* and *in vivo* as well as the induction of iTr35 demonstrated that the IL-35 transgenic DC line efficiently interferes with proliferation and impairs the inflammatory functions of CD4$^+$ T cells. The induction of IL-35 subunit transcription and the regulatory potential of co-cultured CD4$^+$ T cells go along with the work published by Collison et al. [32]. Surprisingly, the expression of IL-35 by the MuTu DC line also inhibits proliferation and IFN-γ production by CD8$^+$ T lymphocytes *in vitro* as well as *in vivo.*

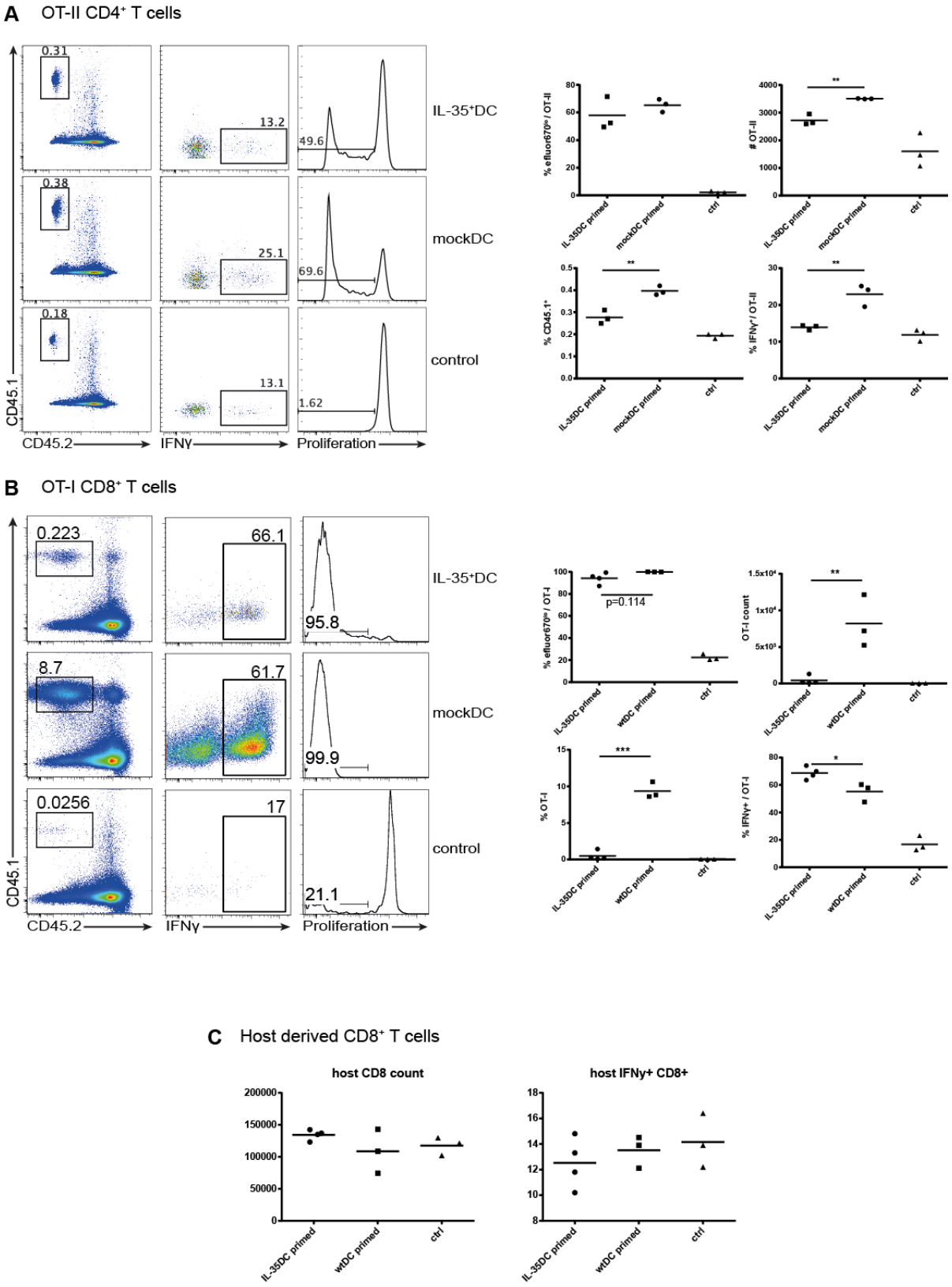


Figure 18 Il-35 expressing dendritic cell line impairs antigen-specific T cell proliferation and function *in vivo*: 2x105 OT-II restricted CD45.1+CD4+ T cells (A) or OT-I restricted CD45.1+CD8+ T cells were co-transferred with 2.5x106 wild-type or IL-35 expressing, peptide pulsed DCs into wild-type C57BL/6 mice (n=3 per experimental group) by intra-venous injection. After 4 days, spleen cells were isolated and T lymphocytes analyzed by flow cytometry. A) Significantly reduced numbers of CD45.1+ CD4+ OT-II T cells could be detected in animals co-injected

with IL-35 secreting DCs when compared to wtDC injected animals. Decreased OT-II count in IL-35+DC co-injected mice was accompanied with a significant reduction in the ability to produce IFN-γ. B) Co-injection of IL-35+DC almost completely abrogated the proliferation of CD45.1+ CD8+ OT-I T cells. However, the few OT-I cells that could be isolated exhibited a significantly increased IFN-γ production. Data shown is representative for 3 independent experiments each.

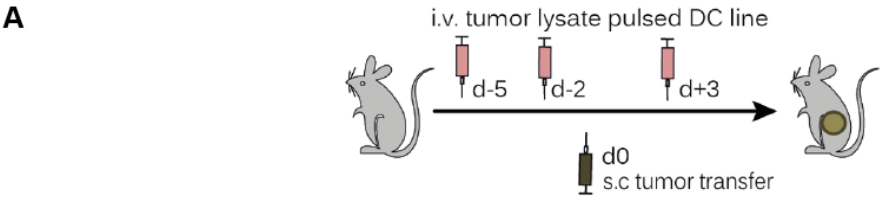
4.4 *In vivo* effects of the interleukin-35 expressing DC line

4.4.1 Vaccination with Interleukin-35+DC line induces tolerance towards tumor growth

In order to determine whether IL-35 secretion by dendritic cells can restrain anti-tumor immune responses and promote tumor growth *in vivo*, we deployed a dendritic cell cancer based vaccination approach. In short, C57BL/6 mice were vaccinated a total of three times with tumor cell lysate pulsed, IFN-γ stimulated, mock transduced or IL-35+ dendritic cells. 5 days after the first vaccination, CMT93 carcinoma cells were transferred subcutaneously into the flank of the mice (Fig. 19a). Non-vaccinated as well as IL-35+ DC vaccinated mice developed palpable tumors within 5 days. Carcinoma growth was characterized by a slow, linear increase in volume until around 10-12 days weeks after tumor inoculation. While the tumors in IL-35+DC vaccinated mice continued to increase in size, the tumor volume in control vaccinated mice decreased and were completely rejected in the majority of animals at the end of the experiment (day 40). Animals that received tumor lysate pulsed mockDC developed palpable tumors within 7 days. However, the tumors failed to grow out but remained stable throughout the course of the experiment or showed even complete remission. Animals vaccinated with IL-35+ DCs showed a progressive growth and tumor volume was significantly higher than tumors of control animals (Fig.19b). Flow cytometric analysis of tumor infiltrating leukocytes 10 days after transfer of tumor cells revealed a markedly decreased accumulation of CD3+ T lymphocytes in IL-35+DC treated mice. While the ratio between CD4+ and CD8+ T cells was comparable in all experimental groups, both CD4+ and CD8+ T cells exhibited a significantly reduced capability to secrete IFN-γ. The production of IFN-γ is associated with anti-tumor immunity promoting Th1 responses. Furthermore, vaccination with IL-35+ dendritic cells furthermore was accompanied with a minor increase of tumor infiltration by CD11b+ cells. Within the CD11b+ cell population, a significantly elevated proportion of Gr1+ myeloid derived suppressor cells MDSCs could be detected when compared to mockDC vaccinated or control animals (Fig.19c).

Similarly, the transfer of $2x10^5$ B16.F0 melanoma cells into non-vaccinated mice led to the development of palpable tumors within 5 days. B16.F0 tumors progressively increased in size to

a volume requiring euthanization in median within 20 days in control mice but only 15 days for mice vaccinated with IL-35^{+}DCs. Mice that received vaccinations with tumor cell lysate pulsed, mock transduced DC exhibited a slight, but not significant delay in tumor growth when compared to control animals. IL-35^{+} DC vaccinated experimental group exhibited progressive tumor growth and bore significant bigger tumors within 13 days after tumor cell transfer (Fig. 19d). Flow cytometric analysis of tumor tissue 12 days after tumor inoculation showed a drastic reduction of tumor infiltration by CD3^{+} T cells in mice that were vaccinated with IL-35^{+}DC. In addition to the reduction of total T cells, infiltrating CD4^{+} and CD8^{+} T cells exhibited a significant reduction in IFN-γ production. Vaccination with control or IL-35^{+}DCs however, did not affect CD11b^{+} myeloid cell infiltration. Within the CD11b^{+} cell population, a tendency towards an increased proportion of Ly6g^{hi} SSC^{int} granulocytic MDSC could be observed (Fig.19e).



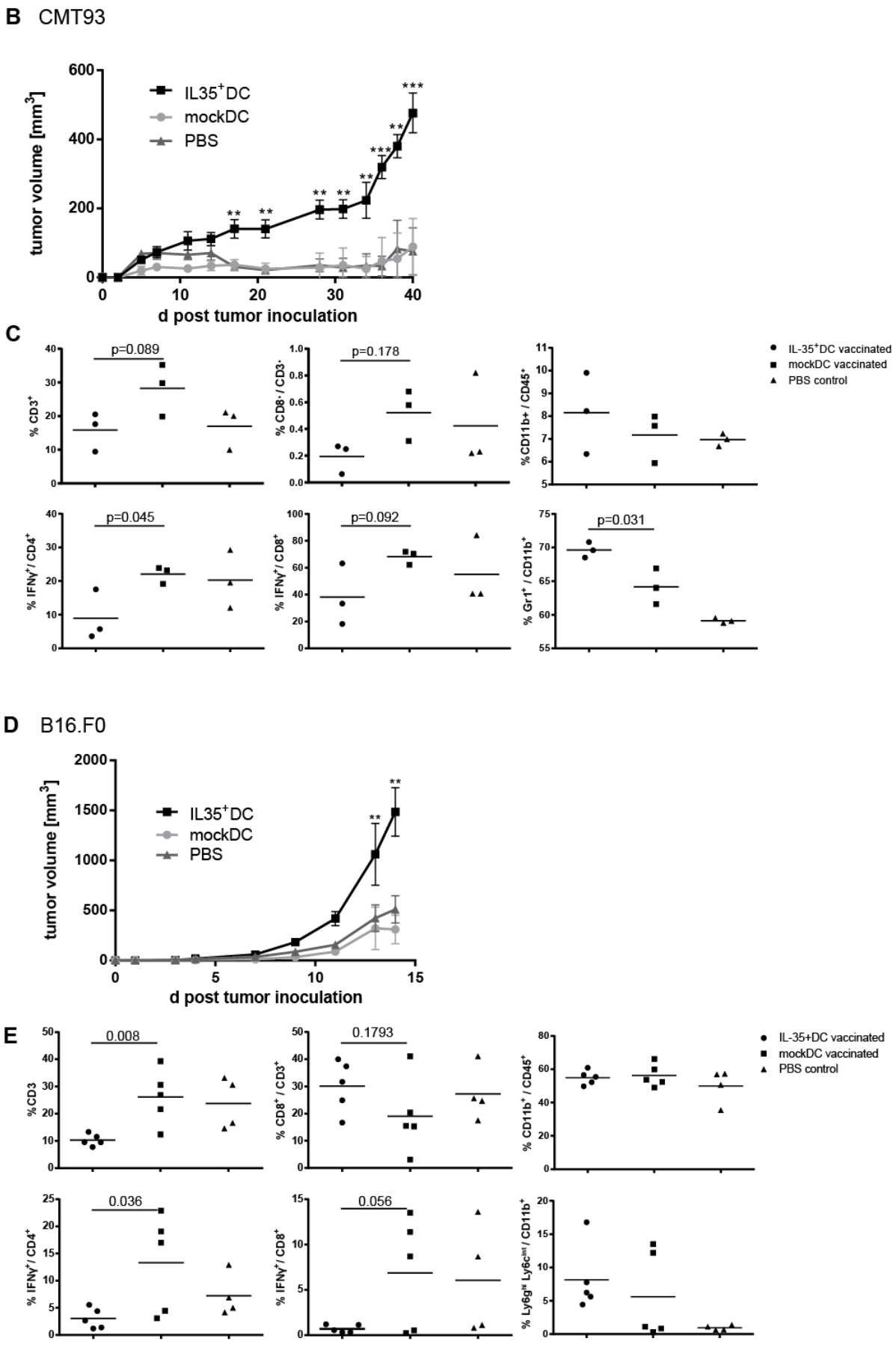

B CMT93
tumor volume [mm3]
IL35+DC
mockDC
PBS
d post tumor inoculation
C
% CD3+
p=0.089
% CD8+ / CD3+
p=0.178
%CD11b+ / CD45+
% IFNγ+ / CD4+
p=0.045
% IFNγ+ / CD8+
p=0.092
% Gr1+ / CD11b+
p=0.031
IL-35+DC vaccinated
mockDC vaccinated
PBS control
D B16.F0
tumor volume [mm3]
IL35+DC
mockDC
PBS
d post tumor inoculation
E
%CD3
0.008
% CD8+ / CD3+
0.1793
% CD11b+ / CD45+
% IFNγ+ / CD4+
0.036
% IFNγ+ / CD8+
0.056
% Ly6gH Ly6cINT / CD11b+
IL-35+DC vaccinated
mockDC vaccinated
PBS control

Figure 19 IL-35 expressing DCs induce tolerance towards tumor growth: A) C57BL/6 mice were subcutaneously vaccinated a total of 3 times with tumor cell lysate pulsed, IFNγ activated 2.5x106 IL-35+DCs, mock transduced DCs or PBS. 1x106 CMT93 carcinoma (B+C) or 2x105 B16.F0 melanoma (D+E) cells were transferred subcutaneously in the same flank as the dendritic cells. B) Mice developed palpable CMT93 tumors within 5 days of transfer. Whereas IL-35+ DC vaccinated mice showed constant tumor growth, tumors in control mice started to shrink between days 10-14 and were rejected in a majority of animals at the end of the experiment. Vaccination with mock transduced DC led to progressive tumor growth within 10 days after tumor cell injection. Results are expressed as the mean of tumor volumes ± SD from 2 pooled experiments (n=4 / experimental group) C) Flow cytometric analysis of tumor infiltrating cells 10 day after tumor inoculation, revealed a reduced infiltration of total CD3+ T cells, and IFN-γ production of both, CD4+ and CD8+ T cells upon vaccination with IL-35+DCs. Reduced T lymphocyte infiltration was accompanied by a significant elevation of CD11b+ Gr1+ myeloid derived suppressor cell infiltration of the tumor. The data shows representative results from one of five independent experiments (n=4/ experimental group). D) Injection of B16.F0 melanoma cells resulted in the development progressively growing tumors that required euthanization in average after in median 20 days in mockDC vaccinated control animals. Mice that received IL-35+DC vaccination, exhibited notably faster tumor growth which required euthanization of the animals in median after 15 days. Results are expressed as the mean of tumor volumes ± SD from one three independent experiments (n=5 / experimental group). E) Analysis of tumor infiltrating cells 12 days after transfer of melanoma cells showed a strong reduction of tumor infiltration by $CD3^+$ lymphocytes. Both, $CD4^+$ and $CD8^+$ T cells were found to produce less IFN-γ. Infiltration by $CD11b^+$ myeloid cells was not altered by vaccination with IL-35+ or mock DCs. However a tendency towards an increased proportion of $CD11b^+$ $Ly6g^{hi}$ SSC^{int} granulocytic MDSC was observable in mice that received IL-35+DC vaccination. The data shows representative results from one of three independent experiments (n=5/ experimental group).

4.4.2 Interleukin-35+ DCs accelerates tumor growth also in the absence of T cells

Recent studies on the function of interleukin-35, as well as our own findings indicate that not only T cells might be able to respond to IL-35 mediated effects. We therefore sought to determine to which extent the accelerated tumor growth in IL-35+ DCs vaccinated mice was dependent on adaptive immune responses. We vaccinated $Rag1^{-/-}$ C57BL/6 mice with IL-35+DC or mockDC and challenged them with CMT93 carcinoma cells as described above. In the absence of B and T lymphocytes, mockDC vaccinated mice developed palpable tumors within the same time frame as in wild type animals (7 days). Further tumor growth was noticeably faster in mockDC vaccinated $RAG1^{-/-}$ than in wild type mice. However, the injection of IL-35 expressing DCs accelerated tumor growth even more, leading to a reduced median survival when compared to mockDC vaccinated $Rag1^{-/-}$ animals (Fig.20a). The injection of transformed MuTu DCs has previously been shown to generate DC tumors within about 4 weeks only in the SV40:CD11c Mushi transgenic or in Rag deficient background [138]. 21 days after the transfer of tumor cells, all IL-35+DC vaccinated but only about 20% of mockDC vaccinated animals mice exhibited a markedly increased spleen and liver size with visible DC tumor nodules (data not shown). Flow cytometric analysis of CMT93 tumor invading cells showed an increased accumulation of $CD11b^+$ myeloid cells and a drastic elevated percentage of $Ly6g^+$ SSC^{int} granulocytic myeloid derived suppressor cells (MDSCs). No overt changes could be detected for $Ly6c^{hi}$ SSC^{lo} monocytic MDSCs or $Ly6c^{lo}$ $Ly6g^{lo}$ SSC^{lo} macrophage populations. A reduced proportion of total $CD11b^+$ myeloid cells was found in the spleen of IL-35+DC vaccinated mice when compared to control animals.

However, the composition of the different CD11b$^+$ populations was not altered (Fig.20b). CD11c GFP$^+$ MuTu DC could be detected at similar percentages in all spleens macroscopically showing DC tumor nodules but not in unaffected organs (data not shown).

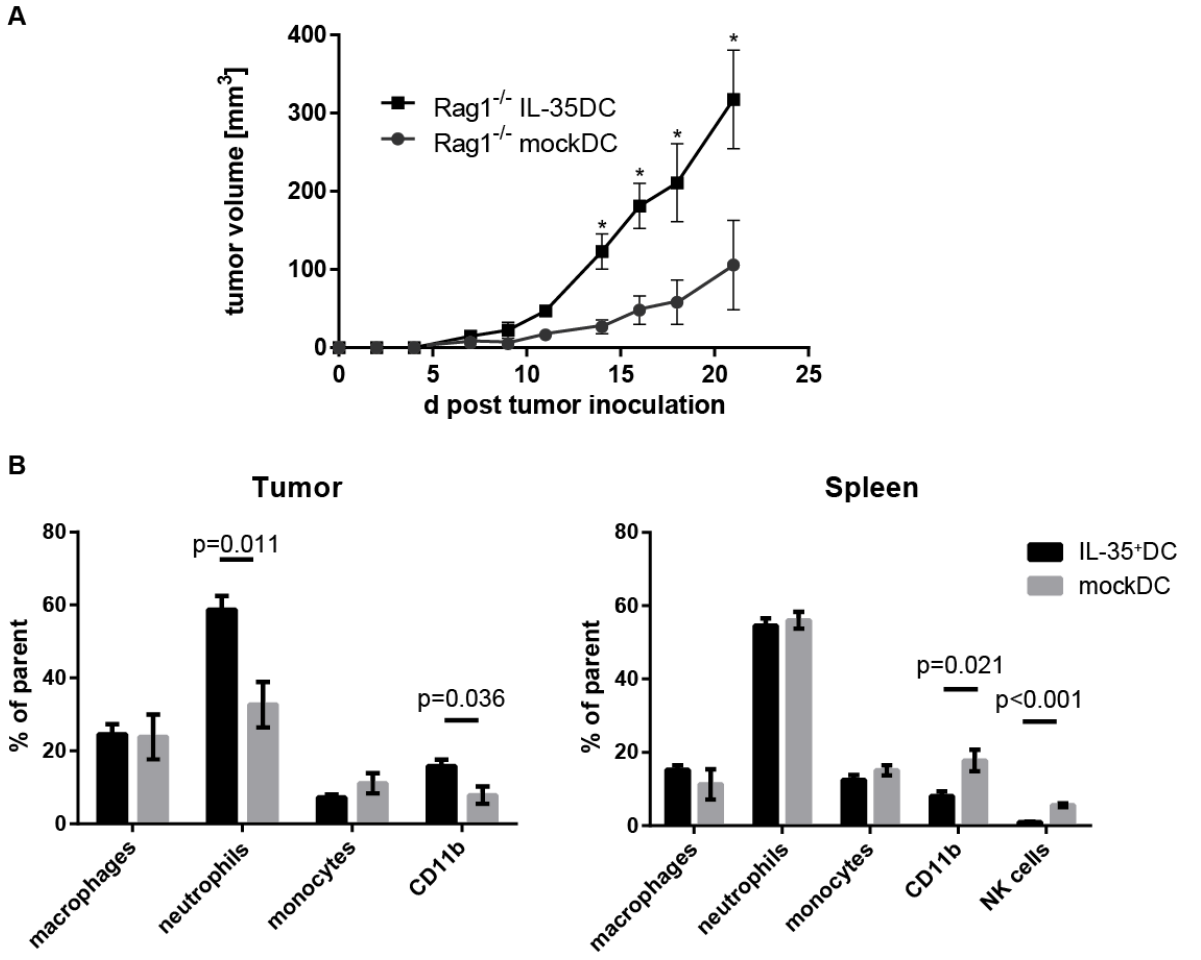


Figure 20 IL-35$^+$DC induce tolerance towards tumor growth also in the absence of T and B cells: A) T- and B lymphocyte deficient Rag1-/- mice were vaccinated a total of 3 times with 2.5x106 tumor cell lysate pulsed IL-35$^+$DCs or mock transduced DCs. Subcutaneous injection of 2x106 CMT93 cells gave rise to palpable tumors within 7 days. IL-35$^+$DC vaccinated mice exhibited a noticeable fester tumor growth, resulting in a significant increased tumor volume within 14 days when compared to control mice. B) Flow cytometric analysis of tumor infiltrating innate cells 21 days after tumor cell transfer revealed a significant increase of total CD11b+ leukocytes and the accumulation of CD11b$^+$Ly6g$^+$SSCint neutrophils in tumors of IL-35$^+$DC vaccinated animals when compared to mockDC vaccination. However, no changes were found for CD11b$^+$ Ly6c^{hi} monocyte or CD11b$^+$ Ly6c$^-$ Ly6g$^-$ macrophage populations. A significant lower percentage of CD11b$^+$ cells was found in the spleens of IL-35$^+$DC vaccinated animals, however the relative proportions of populations within CD11b$^+$ cells was similar in both experimental groups. Results show representative data from one of three independent results, n=4 animals per experimental group. Results are represents as the mean ± SEM.

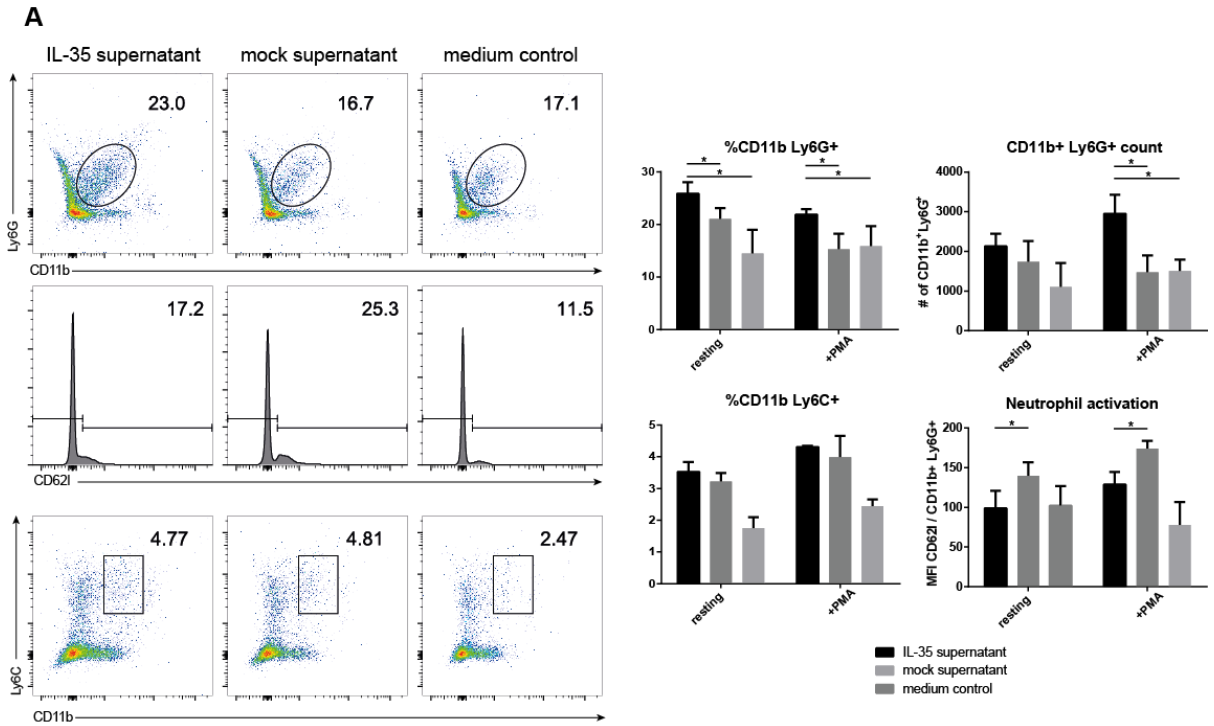
4.5 Investigation of Interleukin-35⁺DC induced effects on cells of non-lymphoid origin

4.5.1 Interleukin-35⁺DC supports neutrophil but not macrophage survival in vitro

To further investigate whether and how IL-35 may affect cells of the innate immune system, we harvested spleen from wild-type C57BL/6 mice. The isolated splenocytes were magnetically depleted from CD3⁺ cells in order to minimize the possibility for T cells to act as a sink for IL-35 and minimize possible indirect effects. The cells were cultured in the presence of supernatant obtained from IL-35 or mock transduced 293T cells for 15 hours. Some of the samples were stimulated for additional 4 hours with the protein kinase C activator PMA and all samples were subsequently analyzed by flow cytometry. (Fig.21a). Addition of mock or IL-35 conditioned supernatant itself seemed to affect activation and survival of the cultured cells. Especially without additional stimulation, the proportions of both Ly6C⁺ and Ly6G⁺ CD11b⁺ myeloid populations were increased. However, the absolute number and proportion of CD11b⁺ Ly6G⁺ neutrophilic granulocytes remaining after overnight incubation was significantly higher in IL-35 containing medium even when compared to mock supernatant treated cells. Furthermore, the addition of IL-35 supernatant led to a reduced expression of CD62L in Ly6G⁺ CD11b⁺ cells which is associated with an activated phenotype in neutrophils. The expression level of CD11b, another marker for neutrophil activation, was comparable within the three experimental groups (data not shown). In contrast, no differences in proportion or cell number for CD11b⁺ Ly6C⁺ monocytes were found. Stimulation of the CD3 depleted splenocytes with PMA, resulted in a noticeable expansion of the total live CD11b⁺ population irrespectively of the medium the cells were incubate with (data not shown). The addition of mock and IL-35 supernatant affected the CD11b⁺ Ly6C⁺ monocytic population similarly. However, the proportion as well as the total cell number of CD11b⁺ Ly6G⁺ neutrophils was augmented only upon incubation with IL-35 containing supernatant. Surprisingly, PMA stimulation slightly increased the expression of CD62L, that is, they exhibited a reduced activation status in the presence of both conditioned media. Nevertheless, the addition of IL-35 activated neutrophils more than mock supernatant, as indicated by a significantly lower expression of CD62L.

Since the previous experiments indicated that IL-35 can influence neutrophils, we sought to investigate the effects of IL-35 on neutrophils during inflammatory conditions *in vivo*. We injected mock transduced or IL-35⁺DCs together with the inflammatory agent thioglycollate into the peritoneal cavity of wild-type C57BL/6 mice. 18h after the second injection we assayed the recruitment of myeloid cells to the site of inflammation as well as for their potential to survive *ex*

vivo. Flow cytometric analysis directly after cell isolation showed the peritoneal exudate cells to consist predominantly of CD11b^{+} Ly6g^{+} SSC^{int} neutrophils (Fig.21b). The percentage of neutrophils was marginally higher in mice injected with IL-35^{+}DCs when compared to mockDC injected animals. The total cell number of neutrophils within the peritoneal exudate was comparable in all three experimental groups. Similarly, the injection of mock or IL-35^{+}DCs did not alter the expression levels of the activation marker CD11b on the CD11b^{+} Ly6G^{+} neutrophil population. Compared to control animals, the transfer of DC led to a reduced proportion of neutrophils that stained positive for the dead cell maker efluor506. The transfer of IL-35^{+}DCs further increased the viability of the recovered CD11b^{+} Ly6G^{+} cells when compared to mockDC injection. A part of the peritoneal exudate cells that were cultured for 18h hours *in vitro* in order to address a potential effect on neutrophil survival by IL-35. Neutrophils constituted an insignificant but reproducible higher relative as well as total proportion of the analyzed cells when IL-35^{+}DC were injected. The transfer of both, IL-35^{+} and mockDCs increased the expression of CD11b respectively reduced the percentage of dead cell marker positive neutrophils. However neutrophils retrieved from IL-35^{+}DC injected mice were significantly brighter for CD11b expression and reproducibly exhibited a higher proportion of viable cells. The same approach was used to investigate the effect of IL-35^{+}DC on inflammatory macrophages. 4 days after thioglycollate injection, peritoneal exudate cells consisted predominantly of CD11b^{+} Ly6c^{-} Ly6g^{-} SSC^{lo} macrophages. As seen before, both the mock or IL-35 transduced DCs slightly altered macrophage frequency and activation when compared to the not injected control group. However, no overt effect on the frequency of the population, expression levels of MHC-II and co-stimulatory molecules or survival could be observed when comparing IL-35^{+}DC to mockDC injected animals (Fig.21c).



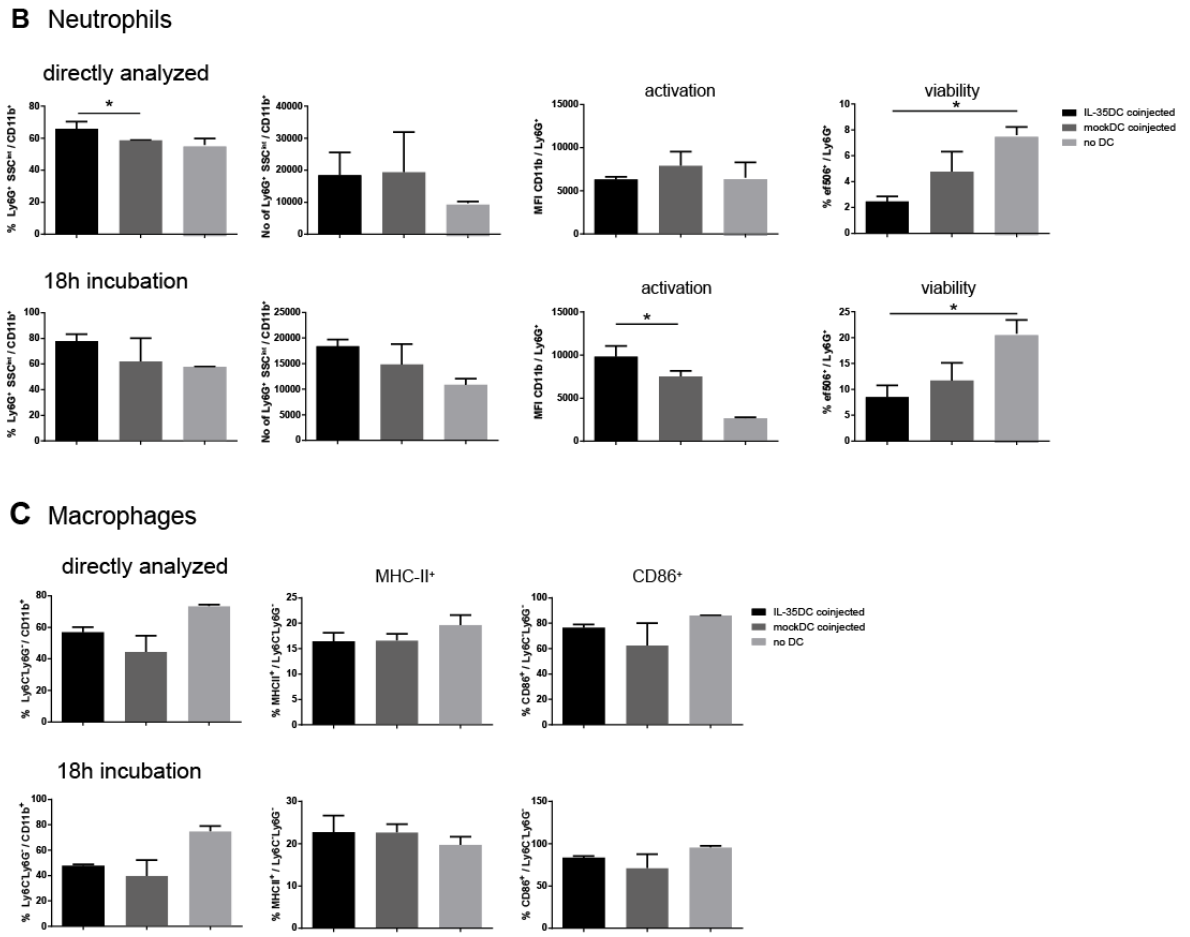


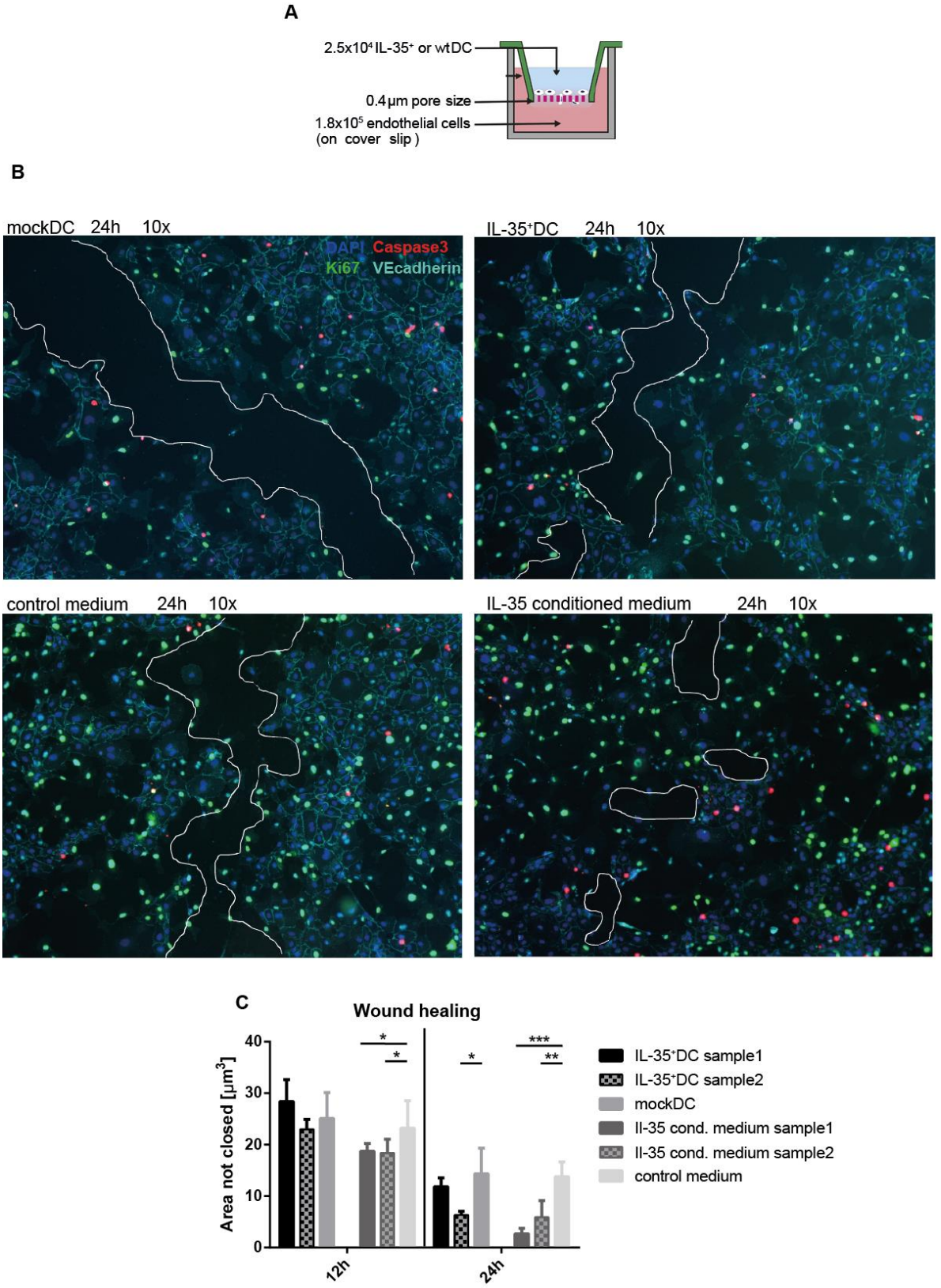
Figure 21 IL-35 supports neutrophil but not macrophage activation and survival: A) Peritoneal exudate cells and spleen were harvested from C57BL/6 mice. Splenic cells were isolated digestion and $CD3^+$ cells were depleted. Peritoneal exudate cells and splenocytes were cultured in the presence of Il-35 conditioned or control medium. After 2 days the cells were harvested and analyzed by flow cytometry. An increased amount of total and activated neutrophils but not monocytes could be detected exhibited when cultured in the presence of IL-35 containing medium. B) $2.5x10^6$ mock transduced or $IL\text{-}35^+$ DCs and 1ml of 4% thioglycollate were intraperitoneal injected into wild-type C57BL/6 mice (n=3 per experimental group).Injections were repeated after 18h and 21h after the first injection peritoneal exudate cells were isolated. The cells were analyzed by flow cytometry either directly or after 18h in vitro culture and were found to consist predominantly of $CD11b^+$ $Ly6g^+$ SSC^{int} neutrophils. $IL\text{-}35^+$DC injected animals exhibited a small but significant increase in neutrophil accumulation and higher viability when compared to control DC injected mice. After 18h of in vitro cultivation, neutrophils derived from $IL\text{-}35^+$DC injected animals exhibited a higher viability and upregulate CD11b, which is described to correlate with neutrophil activation. C) Peritoneal exudate cell isolated 5d after thioglycollate injection consisted predominantly of inflammatory macrophages. The injection of $IL\text{-}35^+$DCs did not affect number or activation state of the isolated macrophages. Results are representative of 3 respectively 2 independent experiments. Data shown as mean±SD.

4.5.2 Interleukin-35 increases the proliferation of endothelial cells *in vitro*

As demonstrated chapter 4.4.2, the vaccination with IL-35 expressing dendritic cells accelerated tumor growth even in RAG deficient mice lacking T and B lymphocytes. Analysis of the tumor infiltrating myeloid cells as well as the *in vitro/ex vivo* experiments described above indicated neutrophils to be a target for IL-35. In two recent publications it was observed that tumor cell derived IL-35 can augment angiogenesis in the tumor environment and thereby promotes tumor

growth [133], [139]. For this reason we were interested whether IL-35 production by DCs does affect proliferation or survival of endothelial cells. In contrast to the aforementioned studies, we decided to address this question in an *in vitro* wound healing assay. HUVEC endothelial cells were plated and once they formed a continuous cell layer, a scratch was inflicted using the tip of a pipette. The endothelial cells were incubated with either supernatant obtained from IL-35 or mock transduced 293T cells. In order to examine whether continuous cytokine production is required, endothelial cells were cultured together with IL-35$^+$DC or mock transduced MuTu DCs plated on transwell inserts. The membrane separating the two chambers had a pore size of 0.4µm avoiding the migration of the DCs into the lower chamber while allowing the diffusion of secreted factors (Fig.22a). The endothelial cells were harvested 12 and 24 hours after the addition of the conditioned supernatant respectively the dendritic cells. The ability to close the injury inflicted by the scratch was assayed by measuring the area not covered by the endothelial cells after the indicated time points (Fig.22b). After 12 hours of co-culture with IL-35$^+$DCs, the endothelial cells did not exhibit any differences in the ability to close the gap when compared with mockDCs incubated cells. The addition of IL-35 conditioned medium on the contrary, led to a markedly reduced average scratch area when compared to control medium. 24 hours after inflicting the scratch, the scratch was mostly closed when IL-35$^+$DCs or IL-35 containing supernatant was added to the endothelial cells. Incubation with control DCs or supernatant exhibited a significantly larger average area not covered by the endothelial cells (Fig.22c).

Immunostaining of the cleaved caspase3 molecule and Ki-67 allowed the detection of apoptotic respectively proliferating cells. 12 hours after the addition of the conditioned medium or the DCs, respectively, the proportion of Ki-67$^+$ endothelial cells tended to be higher when cultured in the presence of IL-35$^+$DCs as compared to those cultured with mockDCs. The addition of IL-35 conditioned supernatant did not enlarge the proportion of proliferating endothelial cells but we could rather observe a substantial increase in the percentage of cleaved caspase3$^+$ apoptotic cells. However, after 24 hours of incubation in the presence of IL-35 conditioned supernatant or IL-35$^+$DCs, the proportion of proliferating endothelial cells was significantly increased when compared to the respective controls. Although capsase-3 expression was quite variable within the different samples, the production of IL-35 by the dendritic cells tended to reduce the fraction of apoptotic endothelial cells when compared to mockDCs (Fig.22d+e).


A
2.5x10⁴ IL-35⁺ or wtDC
0.4µm pore size
1.8x10⁵ endothelial cells
(on cover slip)
B
mockDC 24h 10x
DAPI Caspase3
Ki67 VEcadherin
IL-35⁺DC 24h 10x
control medium 24h 10x
IL-35 conditioned medium 24h 10x
C
Wound healing
Area not closed [µm³]
40
30
20
10
0
12h
24h
*
*
*
***
**
IL-35⁺DC sample1
IL-35⁺DC sample2
mockDC
Il-35 cond. medium sample1
Il-35 cond. medium sample2
control medium

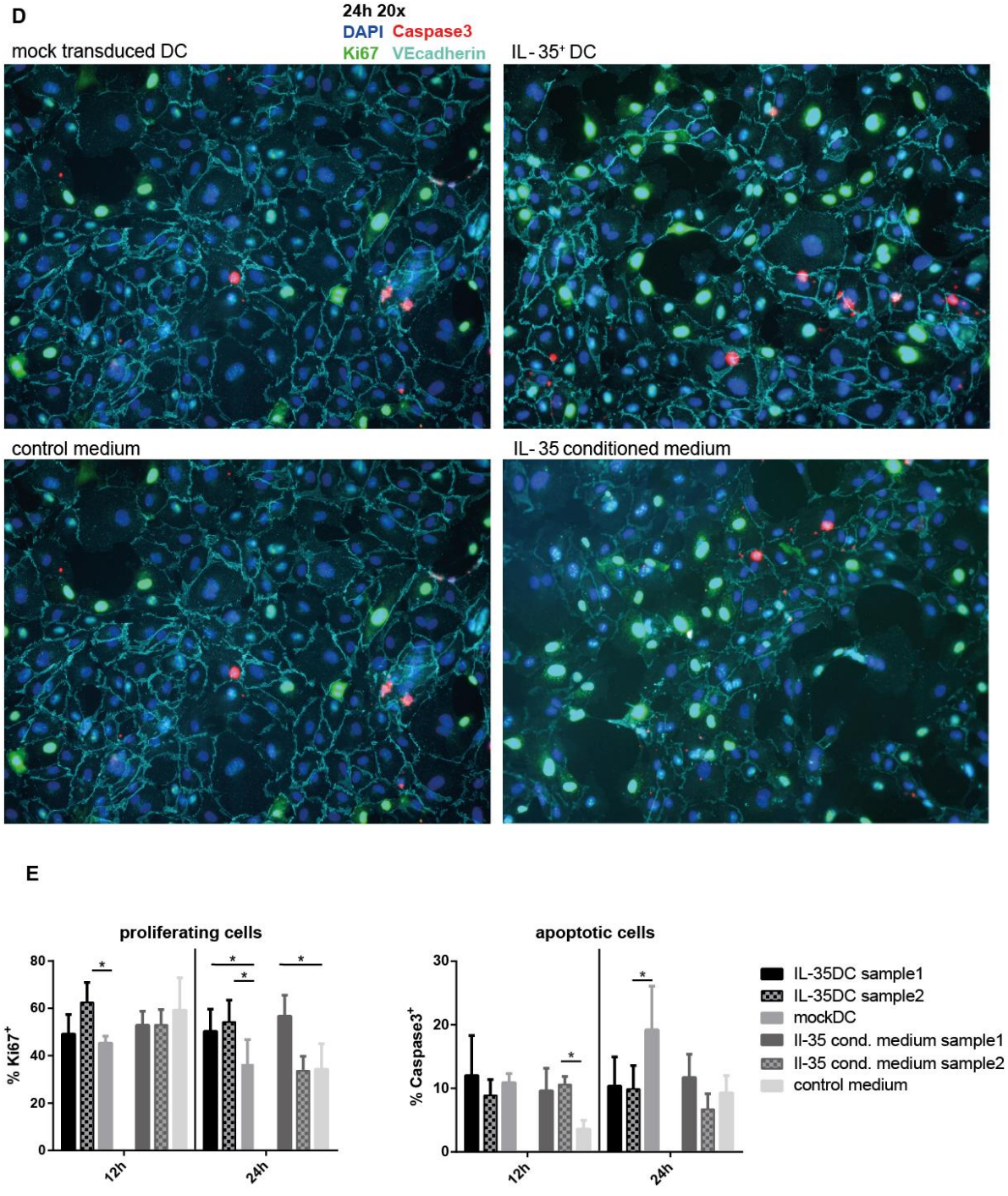


Figure 22 Regulation of proliferation and migration of endothelial cells by IL-35. A) A HUVEC endothelial cell monolayer was scraped using a pipette tip, forming a straight scratch and cultured in the presence of conditioned medium or the respective DC lines plated on the upper chamber of a transwell insert. Endothelial cells were fixed after 12 and 24 hours and histologically analyzed for cell migration, proliferation and apoptosis. B) Representative microscopic views 24 hours after scraping are shown. The white lines indicate the remaining, non-covered scratch area. C) The scratch area was quantified by calculating the mean ± SD of the scratch area of 4 non overlapping sectors. Data indicate improved cell migration after 12 hours when cultured with IL-35 conditioned medium. After 24 hours the remaining scratch area was significantly reduced in both, IL-35+DC and IL-35 conditioned medium cultured endothelial cells. D) Representative microscopic views 24 hours after scraping are shown. Proliferating cells was stained green using the Ki67 specific antibody. The apoptosis-related cleaved caspase 3 was stained in red. E) Proliferation and apoptosis of endothelial cells was quantified by calculating the mean percentage ±SD of Ki67 respectively caspase3 positive cells within 5 randomly selected sectors of the sample.

5 *In vivo* tolerance induction by dendritic cells expressing tolerogenic molecules

5.1 Previous findings on IL-10 and TGF-β expressing DC lines

A total of eight different, well-documented immune suppressive molecules have originally been introduced into the MuTu dendritic cell line and were investigated for their potential to suppress T cell proliferation and effector functions *in vitro*. Some of them, especially the Arginase-1 and Ido1 transduced DC lines, were difficult to maintain under in vitro culture conditions. The constitutive catabolic activity of the expressed molecules probably imposed an inhibitory effect on the DCs itself. Other DC lines did not exert any detectable immunosuppressive effect on T lymphocytes. They were therefore excluded from further investigation. The IL-10$^+$DC line and the DC line constitutively expressing the active form of TGF-β were identified as the most promising candidate DC lines in terms of immune suppressive potential. IL-10 expression by the DCs was shown to impair their capability to respond to activation by TLR-ligands. IL-10$^+$ DCs induced comparable antigen specific CD4$^+$ or CD8$^+$ T cell proliferation but skewed CD4$^+$ T cells towards an IL-17 secreting phenotype. Secretion of active TGF-β by the DC line on the other hand did not influence the expression of MHC and costimulatory molecules by the dendritic cell itself but inhibited Th1 commitment and induced Foxp3$^+$ regulatory T cells.

5.2 Prevention of Experimental Autoimmune Encephalitis by tolerogenic DC lines

Experimental Autoimmune Encephalitis (EAE) is a commonly used murine model for human multiple sclerosis (MS) which is a chronic inflammatory autoimmune disease of the central nervous system (CNS). MS is characterized by the infiltration of the CNS by auto-reactive Th1 and Th17 cells which in turn attract and instruct mature myeloid cells to destroy the CNS parenchyma in a GM-CSF dependent manner (reviewed by [140]). The destruction of myelin producing oligodendrocytes causes demyelation of neuronal axons which results in an impaired transmission of nerve impulses and typically manifests in impaired sensory and motoric functions [141]. While the exact etiology of MS is unknown, the disease susceptibility is associated

with genetic, environmental as well as geographical factors. In one of several proposed models, a so far unknown infectious or environmental trigger causes oligodendrocyte death, which in turn leads to immune dysregulation. While myelin-specific T cells could even be detected in healthy humans, Tregs retrieved from MS patients exhibited a reduced capability to suppress myelin specific effector T cell proliferation [142], [143]. These findings indicate that rather defective immune regulation than de novo priming of auto-reactive T cells is related to MS pathogenesis. In accord, adoptive transfer of myelin specific $CD4^+$ $CD25^+$ regulatory T cells into mice has been shown to protect from EAE development [144].

EAE can be divided into an induction and an effector phase. Immunization with myelin peptides or proteins in complete Freud's adjuvant (CFA) triggers the priming of myelin-specific $CD4^+$ T cells. These T cells cross the blood-brain barrier and generate a pro-inflammatory environment attracting and activating myeloid cells. The resulting demyelation manifests in an impaired neuronal signaling and a progressive but reversible paralysis of the hind limbs. The inflammatory nature of EAE development and the involvement of auto reactive $CD4^+$ T cells make the disease an excellent model for the investigation of immunomodulatory treatment approaches.

5.2.1 Role of DC in EAE development and regulation

DCs are present in the CNS draining cervical lymph nodes and upon inflammation also within the CNS where they efficiently present myelin-derived peptides and provide inflammatory cytokines. The importance of DCs for EAE was shown by the finding that presentation of myelin antigens on MHC-II molecules expressed by non-CNS resident $CD11c^+$ DCs alone is permissive for the initiation of the disease [145]. Furthermore, the depletion of mature DCs by the inhibition of FLT3 signaling correlated with an improved disease progression [146]. Dendritic cells are not only involved in the induction and propagation of EAE but also participated in the regulation. Desphande et al. could demonstrate that at the peak of disease, the CNS residing DCs exhibit an immature phenotype expressing lower levels of MHC class II and costimulatory molecules [147].

5.2.2 Regulation of EAE pathology by TGF-β and IL-10

Both IL-10 and TGF-β have been shown to modulate EAE induction and progression on different levels. TGF-β treated mice exhibited a reduced EAE severity while the neutralization of the cytokine exacerbated the disease [148]. Various mechanisms by which TGF-β ameliorates EAE have been described. Inactivation of TGF-β receptor signaling in $CD11c^+$ DCs caused an increase of EAE severity which was linked to an increase of IFN-γ and IL-17 expressing T cells [149]. The

administration of TGF-β or IL-27 can induce IL-10 secretion by Th1 cells, thereby reducing their encephalitogenic potential [150]. Furthermore, TGF-β induced regulatory T cells have been shown to suppress the induction of EAE [151]. Paradoxically, TGFβRII deficient $CD4^+$ T cells cannot acquire a Th17 phenotype and are not able to initiate EAE, further stressing the multifaceted characteristics of the cytokine [152]

Mice deficient for IL-10 develop more severe EAE, while the over-expression of the cytokine in T or antigen presenting cells protects from the disease [153]. In fact, an increased IL-10 production by several cell types, including astrocytes, NKT, Th1 and Tr1 cells has been shown to correlate with disease remission in human and mice. These findings demonstrate that IL-10 plays a pivotal role in the regulation of autoimmune encephalitis. Therapeutic treatment of EAE by the intracranial injection of IL-10 was shown to downregulate the production of an array of pro-inflammatory cytokines including IL-6, IL-12, GM-CSF, IFN-γ or TNFα. However, depending on the delivery route, timing and the disease model applied, systemic IL-10 delivery has yielded contradictory findings. It is assumed that a continuous delivery of the cytokine in the CNS during the effector phase of EAE is needed to affect EAE pathogenesis. [154].

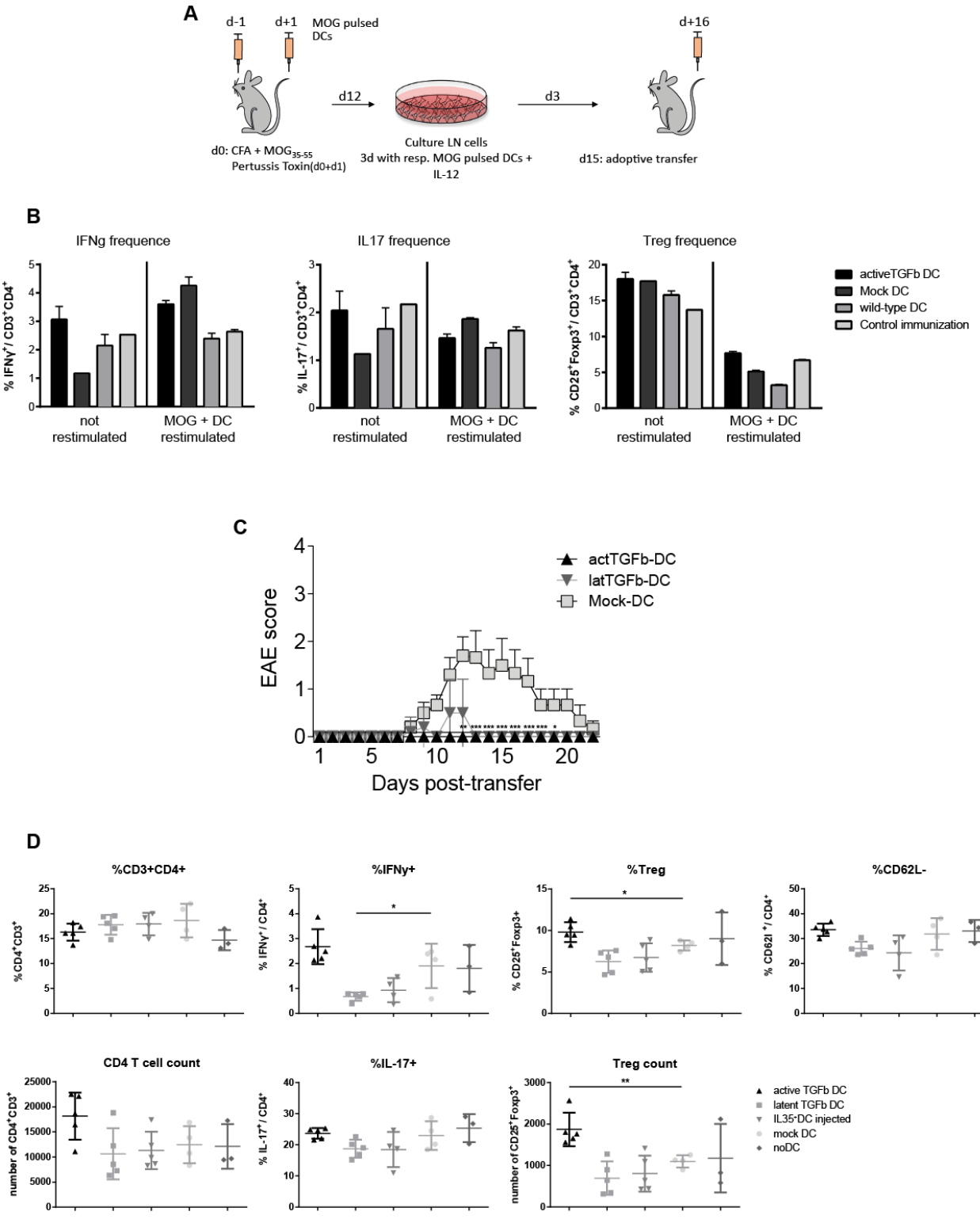
5.2.3 Active TGF-β expressing DCs completely abrogate induction of EAE symptoms

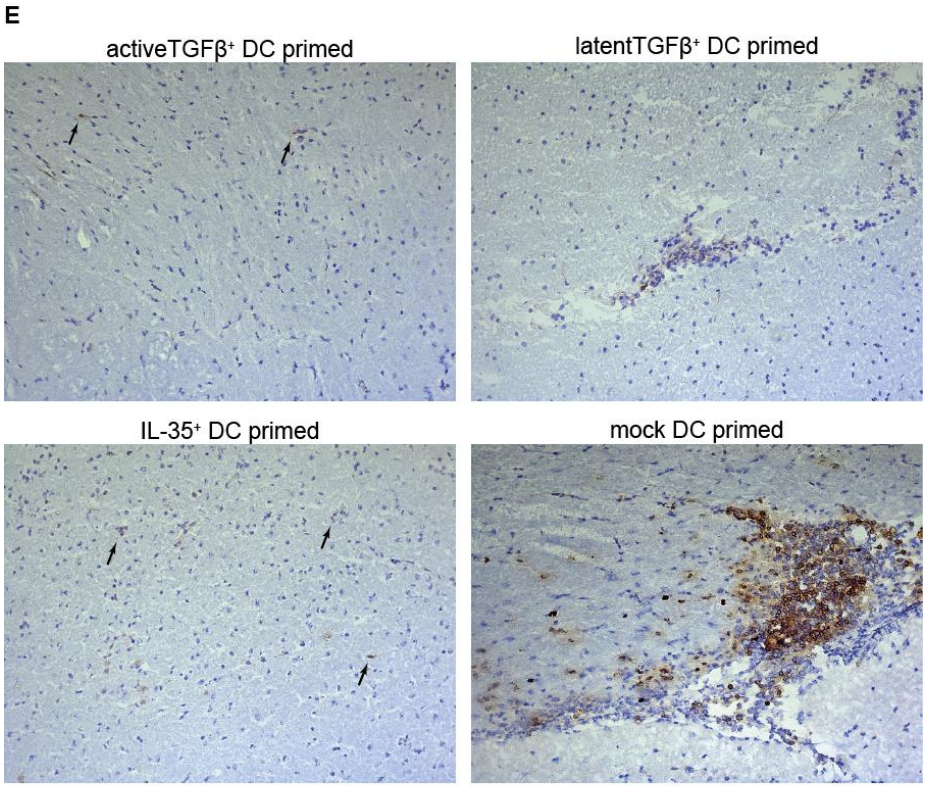
In order to assess the potential of dendritic cell mediated tolerance induction *in vivo*, we compared the potential of IL-10, active TGF-β or IL-35 expressing DC lines to suppress the induction of EAE. While the experiment applying $IL\text{-}10^+$ or $TGF\beta^+$DCs was repeated several times with comparable results, only one experiment using the $IL\text{-}35^+$DCs was performed so far. The results involving $IL\text{-}35^+$DCs have therefore be regarded as preliminary. Due to technical problems inducing EAE using the widely used active induction of EAE and other considerations, we decided to use an adoptive transfer EAE model as it was described by [155]. Briefly, autoreactive T cells were primed in wild-type C57BL/6 mice using the myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide emulsified in complete Freud's Adjuvant. Two injections of Pertussis toxin boost T cell priming and are required to induce reliable disease. In order to study the effect of tolerogenic molecules, the mice received two injections of MOG_{35-55} peptide-pulsed transgenic or control MuTu DCs. 12 days post immunization, lymph node cells of the immunized animals were expanded *in vitro* for 4 days in the presence of the respective MOG_{35-55} peptide pulsed DCs and recombinant IL-12p70. After *in vitro* restimulation the cells were transferred to C57BL/6 recipient mice, which received an additional injection of the respective MOG_{35-55} peptide pulsed DCs (Fig.23a). The adoptive transfer EAE has been described to lead to more severe but also homogenous and reproducible induction of autoimmune CNS inflammation than the generally

used direct induction of EAE. In addition, the *ex vivo* restimulation and expansion of myelin specific T cells allowed to interact with the respective tolerogenic DC line for a prolonged time.

Flow cytometric analysis of the *ex vivo* restimulated lymphocytes revealed no significant differences in regard to Th1/Th17 or Treg differentiation, irrespective of the DC line used for restimulation (Fig.23b). Restimulation of the lymph node cells markedly reduced the frequency of $CD25^{+}Foxp3^{+}$ regulatory T cells in comparison to not restimulated cells, independently of the experimental group. T cells primed and restimulated in the presence of active $TGF\beta^{+}$DCs tended to increase the proportion of Tregs when compared to mock or wild type DC restimulated cells. The presence of $IL\text{-}10^{+}$DCs or IL-35+DCs on the other hand did not affect Th1/Th17 respectively Treg skewing of the T lymphocytes (data not shown). However, we could observe that the formation of proliferative blasts was considerably reduced and fewer cells could be recovered from the respective wells upon restimulation with $IL\text{-}35^{+}$DC but not any other of the DC lines (data not shown).

Mice that received MOG reactive T cells primed in the presence of wild type or mock transduced dendritic cells reproducibly exhibited EAE related disease symptoms within 9 days after T cell transfer. A maximum disease score of 3, corresponding to a paralysis of the hind limbs, could be observed between 12 to 14 days after T cell transfer. The animals subsequently recovered and no disease symptoms could be observed in average 22 days after the transfer of immunogenic T cells. The adoptive transfer of IL-10 expressing DCs led to a disease course that was identical to the control groups (Fig.23c and data not shown). In contrast, none of the mice that received T cells primed and restimulated in the presence of active $TGF\beta^{+}$DCs did show any EAE associated disease symptoms. An intermediate disease progression could be observed in mice that received DCs expressing latent TGF-β or interleukin-35. Animals that were injected with latent TGF-β expressing DCs also exhibited EAE associated symptoms 8 days after T cell transfer but the disease course was less pronounced, reaching a maximal disease score of 1 (reduced tail tonus) and completely recovered within 14 days. Similarly, the presence of $IL\text{-}35^{+}$DCs drastically reduced maximum disease severity to a maximal score of 1 (Fig.23f). Table 3 summarizes the observed clinical scores for all of the investigated tolerogenic DC lines.


A
d-1
d+1
MOG pulsed DCs
d12
d3
d+16
d0: CFA + MOG35-55 Pertussis Toxin(d0+d1)
Culture LN cells 3d with resp. MOG pulsed DCs + IL-12
d15: adoptive transfer
B
IFNg frequence
IL17 frequence
Treg frequence
% IFNγ+/ CD3+CD4+
% IL-17+/ CD3+CD4+
% CD25+Foxp3+/ CD3+CD4+
not restimulated
MOG + DC restimulated
activeTGFb DC
Mock DC
wild-type DC
Control immunization
C
EAE score
Days post-transfer
actTGFb-DC
latTGFb-DC
Mock-DC
D
%CD3+CD4+
%IFNγ+
%Treg
%CD62L-
CD4 T cell count
%IL-17+
Treg count
active TGFb DC
latent TGFb DC
IL35+DC injected
mock DC
noDC



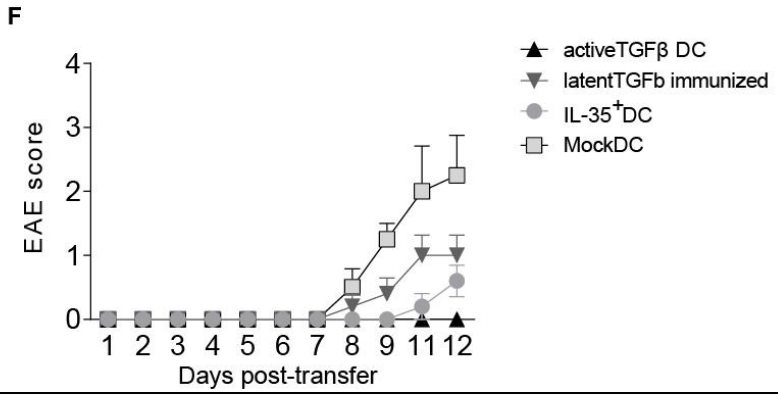


Figure 23 Active and latent TGF-β and IL-35 expressing DCs diminish the development of EAE: A) passive induces adoptive transfer EAE priming of MOG specific T cells in the presence of the respective DC lines in donor mice. Lymph node cells were restimulated in vitro in the presence of the respective DC line and IL-12. The cells were then transferred into recipient mice and the development of EAE associated symptoms was observed. B) Analysis of in vitro restimulated lymph node cells showed only marginal differences between the different experimental groups with a tendency towards increased Foxp3+ Tregs when the cells were cultured with actTGFβ+ DCs C) The development of EAE associated symptoms was observed in control animals but not in actTGFβ+ DC primed animals. D) Analysis of the draining lymph node resident cells 12 days after T cell transfer exhibited significantly increased numbers of Tregs in the actTGFβ+ DC primed group. Data representative for 4 independent experiments. n=4 animals per experimental group Data denoted as mean ± SD. E) Representative microscopic views of the brains of recipient mice 12 days after T cell transfer revealed focal T cell infiltration only in control groups. Indicated are isolated CD3+ cells within the cerebrum. F) Pilot experiment applying IL-35+ DCs resulted in reduced disease severity and yield.

Next we wanted to investigate whether the observed differences in the course of the disease is associated with a phenotypical change of the $CD4^+$ T lymphocytes. We therefore analyzed the infiltration of T cells into the brain as well as of the CNS draining, cervical lymph nodes on day 12 after T cell transfer. At this time point maximal disease severity was generally observed in control animals. Flow cytometric analysis exhibited comparable proportions of $CD4^+$ $CD3^+$ cells in all experimental groups. When looking at total cell numbers, significantly more $CD4^+$ T lymphocytes could be detected in the draining lymph nodes of mice that received actTGF-β expressing dendritic cells when compared to control animals. Interestingly, the transfer of latTGFβ$^+$DCs led to a substantial reduction of IFN-γ expressing $CD4^+$ T cells when compared to mock or actTGFβ$^+$DCs. Although not statistically significant, a similar reduction of IFN-γ expression could be observed upon transfer of IL-35$^+$DCs. The expression of IL-17 by $CD4^+$ T cells was shown to be uniform in all experimental groups. While the transfer of latTGFβ$^+$or IL-35+DC did not affect the $Foxp3^+$ $CD25^+$ $CD4^+$ regulatory T cell population in the draining lymph node, we found that active TGF-β expressing DCs notably augmented Treg percentage as well as total cell number (Fig.23d).

DC line	Incidence	Mean maximal EAE score
wild-type	10/13	2.7
mock	8/16	2.6
active TGFβ	0/13	0
latent TGFβ	4/10	0.6
IL-10	4/5	2.5
IL-35	3/5	0.5

Table 3 Summary EAE scores: The table specifies the pooled mean maximal EAE score and incidence for each of the tolerogenic DC lines investigated.

Histological analysis of the brain revealed the typical focal inflammatory lesions within the myelin rich regions with a preferential perivascular accumulation of $CD3^+$ cells in diseased control animals. No formation of such lesions but only few, isolated T cells could be observed in the actTGF-β as well as in the IL-35 experimental groups (Fig.23e). Due to technical problems we were unfortunately not able to determine the exact phenotype of these infiltrating cells.

6 Induction of tolerance towards allogeneic skin transplants

6.1 Introduction to organ transplantation

Transplantation is the most effective way to replace dysfunctional or failing organs respectively tissues. Autologous transplantation in order to replace, for example burned skin, is widely used and does not impose major difficulties. Allogeneic transplantation on the other hand, requires permanent suppression of the host immune system in order to ensure functional survival of the graft over a maximal period of time. These treatment regimens usually consist of nonspecific immunosuppressive drugs that, besides other secondary effects, increase the risk for life-threatening infections and cancer. In addition, nonspecific immunosuppression is generally not able to ensure graft survival, leading to a gradual loss of its function. Induction of graft specific tolerance, with a minimal impact on the recipients' immune system has therefore long been an aim of transplantation research. T cells were shown to be the major mediators of acute as well as chronic graft rejection [156]. Considering their critical role in the regulation of the adaptive immune response, the application of tolerogenic dendritic cells has been thought to be a feasible approach to specifically delay or even prevent graft rejection and to reduce the dependence on immunosuppressive drugs. The preventive transfer of donor derived immature or semi-mature DCs has been shown to prolong allograft survival, while the transfer of mature DCs lead to acute graft rejection [109]. Various different mechanisms have been evaluated for their potential to generate and retain the tolerogenic phenotype of the applied dendritic cells: Inhibition of the functional maturation of DCs using specific cytokines or drugs. Generation of costimulatory molecules deficient DCs or blockade of the costimulatory receptors using antibodies or small chemical compounds interfering with functional costimulation. Another approach is the generation of DCs expressing immunoregulatory cytokines or molecules inducing apoptosis on the target T cells by genetic modification. Various studies are aiming to induce antigen-specific hyporesponsive T cell responses and/or regulatory T cells (reviewed by [157]).

6.2 Transfer of tolerogenic dendritic cell lines lead to the prolongation of graft survival

The prophylactic transfer of tolerogenic DCs can probably not be directly translated into a clinically relevant treatment approach, for example for autoimmune diseases like multiple sclerosis. We therefore sought to investigate the tolerogenic potential of DCs in a clinically more relevant setting. Tolerogenic DCs have been proposed as a practicable strategy to promote

tolerance towards grafted organs and tissues. Thus, we applied IL-10, IL-35 or activeTGF-β producing DC lines in two different settings of skin allotransplantation. Recipient mice were given intravenous injections of $2.5x10^6$ of the respective tolerogenic or control DC lines two days before and one day after transplantation. Allografting of skin derived from the tail of the respective donor animal was performed as previously described by [158].

6.2.1 Major & multiple minor mismatched

Skin grafts derived from F_1 (C57BL/6 x BALB/c) mice (MHC haplotype b/d) that were grafted onto C56BL/6 mice rapidly developed focal necrosis and were completely rejected within an average of 14 days. Transfer of mock transduced or a mixture of IL-10 and active TGF-β expressing dendritic cells did only marginally affect graft survival (median graft survival for mockDC=15 days, for actTGFβ$^+$/IL-10$^+$DC=16 days) (Fig. 24a). Due to these results we decided to continue only in the multiple minor mismatched system.

6.2.2 Multiple minor antigen mismatch

In a second transplantation model we used mouse strains that are only multiple minor histocompatibility mismatched, while MHC class I and II haplotypes are matched. Thus, 129/Sv (MHC haplotype b) animals received allogeneic skin derived from C57BL/6 (MHC haplotype b) animals. We expected the allograft reaction in this setting to be less aggressive, allowing a possible tolerogenic effect of the transferred dendritic cells to be better observable. As described before, the respective DC lines were transferred one day before and two days after grafting of the skin. Mice that received no or mock transduced DCs completely rejected the grafted skin in median after 15.5 respectively 15 days. The adoptive transfer of DCs expressing IL-10 or active TGF-β did not delay graft rejection. In contrast, the injection of a 1:1 mixture of actTGF-β and IL-10 expressing DCs surprisingly delayed graft rejection drastically to a median graft survival of 20 days. Furthermore, IL-35$^+$DCs alone were also found to significantly delay the rejection of the grafted skin. No long-term survival of the grafted skin could be observed for any of the tested combinations (Fig. 24b).

In a next step we tried to shed light on the cellular mechanisms driving the observed delay in graft rejection. Due to the limited capacity to perform graft experiments, the following experiment has only been performed once and the results have to be considered as preliminary. Mice were bled one day before and seven days after skin transplantation and the peripheral lymphocyte populations were analyzed by flow cytometry. Unfortunately we were not able to establish a clear

connection between the observed differences in graft rejection and the frequency of inflammatory, respectively of regulatory T cells in the peripheral blood of recipient mice. Transfer of a single DC line alone resulted in the reduction of the total $CD3^+$ T cells proportion when compared to the mice that were injected with a mixture of $IL\text{-}10^+$ and $actTGF\beta^+$DCs. This reduction was most pronounced in mice that received $IL\text{-}35^+$DCs. The relative ratios of $CD8^+$ and $CD4^+$ T cells remained comparable in all experimental groups. Surprisingly, the transfer of any of the transgenic DC lines led to a noticeable increase of IFN-γ expressing $CD4^+$ T cells, especially upon the transfer of $IL\text{-}35^+$ or $IL\text{-}10^+$ DCs. $actTGF\beta^+$DCs alone but not in combination with IL-10 expressing DCs were found to significantly impair with the acquisition of a $CD44^+$ $CD62l^-$ activated phenotype on $CD4^+$ T cells. In $CD8^+$ T cells on the other side, the injection of any of the transgenic DC lines was shown to strikingly decrease the expression IFN-γ. Injection of $IL\text{-}10^+$ or $actTGF\beta^+$ alone or in combination reduced the proportion of activated $CD8^+$ T cells similarly when compared to naïve or $IL\text{-}35^+$ treated mice. Finally, the transfer of $actTGF\beta^+$DC alone but not in combination with $IL\text{-}10^+$ DC significantly increased the fraction of $CD25^+$ $Foxp3^+$ $CD4^+$ regulatory T cells found in peripheral blood (Fig. 24c).

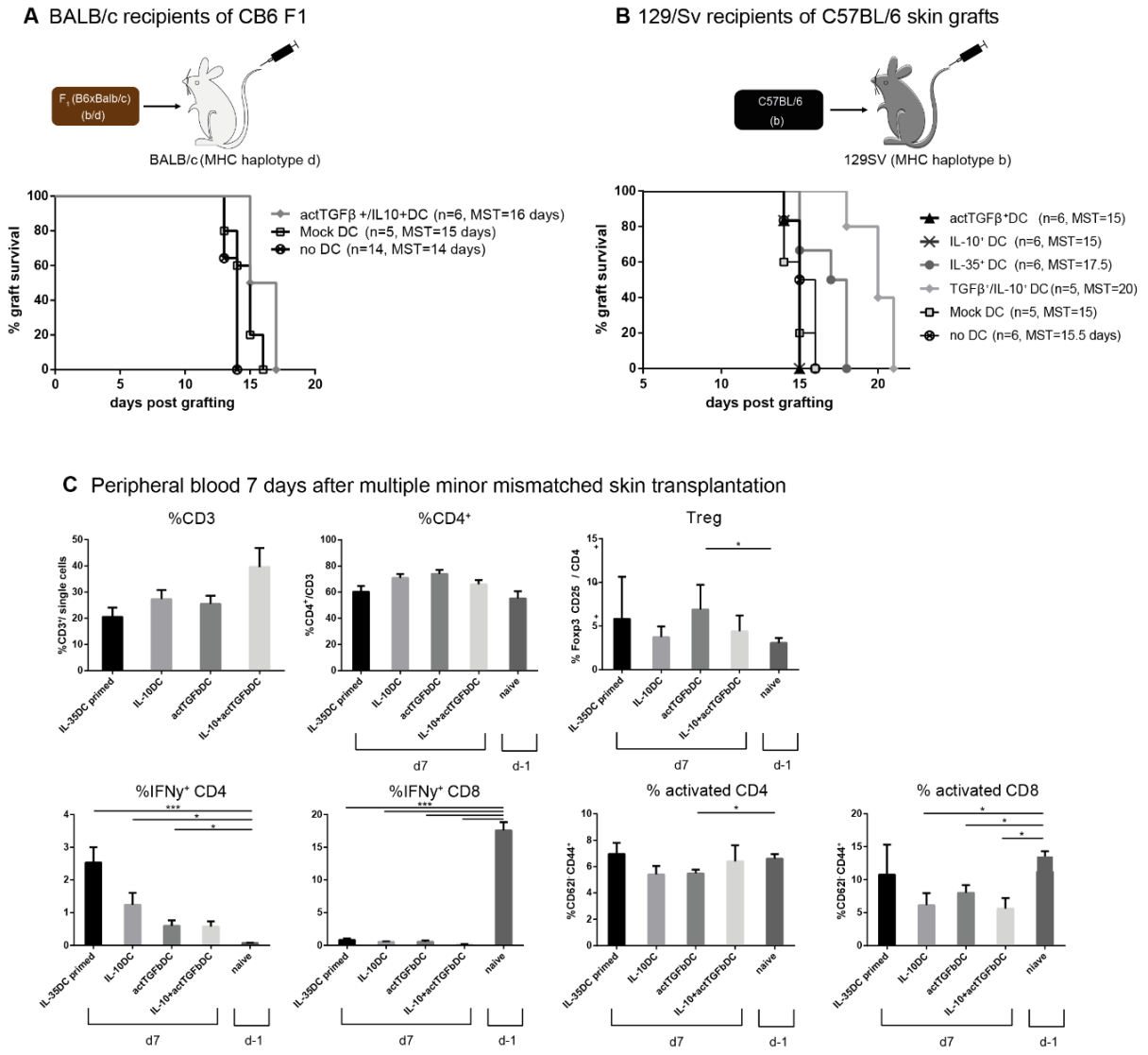


Figure 24 The combination of IL-10 and actTGF-β expressing DCs as well as IL-35+DCs delay allogeneic skin graft rejection: A) No differences in major & multiple minor mismatched allogeneic skin graft survival could be observed with any if the injected DC lines. B) The transfer of IL-35+ or a combination of IL-10+/actTGFβ+DCs prolonged the rejection of the transplanted multiple minor mismatched skin. C) Flow cytometric analysis of peripheral blood lymphocytes 1 day before respectively 7 days after skin transplantation. Data representative of 3 independent experiments. Statistical analysis of the survival curves was performed using Log-Rank test.

7 Augmentation of anti-tumor immunity applying semi-allogeneic DC vaccination

For this second project, we took advantage of the availability of the $CD8^+$ MuTu dendritic cell line and tested its potential as antigen delivery vehicle for cancer vaccination. A vast number of studies applying dendritic cells to induce, respectively reconstitute anti-tumor immunity have been performed. While some of them showed the generation of immunological responses in murine models, only limited clinical impact was observed in most cases [159], [160]. Most of the current DC-based vaccine trials have employed *ex-vivo* maturated, autologous monocyte or $CD34^+$ hematopoietic precursor-derived DC. Depending on the cytokine combinations used for monocyte differentiation, the resulting DC subsets form a diverse population exhibiting a high degree of plasticity and triggering functionally distinct types of immune responses. Kissenpfennig et al. for example demonstrated that different peripheral DC subsets migrate to distinct, contiguous areas within draining LN [161]. Thus, the immune response elicited by current DC vaccination approaches might not be of the quality needed to allow tumor rejection. It is therefore of eminent importance to control the DC subtype(s) applied in cancer vaccines. Cytotoxic lymphocytes are $CD8^+$ effector T cells specialized to eliminate infected or aberrant cells in an antigen specific manner. As $CD8^+$ T cell invasion is correlated with tumor regression and better prognosis in different cancer (models), these cells are regarded potent anti-tumor effectors. Stimulation of naïve $CD8^+$ T cell is dependent on antigen presentation by MHC class I molecules. Cross-presentation provides a direct way for exogenous proteins to be processed and presented on MHC-I molecules on cells not directly infected by microbial pathogens [162]. $CD8\alpha^+$ dendritic cells possess the ability to selectively phagocyte dead cells and efficiently cross-present exogenous antigens on MHC class I receptors. This ability makes them effective activators of $CD8^+$ T cells. $CD8^+$ DCs are furthermore able to produce large amounts of biological active Il-12p70 and therefore promote Th1 type as well as cytotoxic CD8 T-cell responses [85].

Besides the choice of an adequate DC subtype, one of the central challenges of any antitumor DC vaccination is to provide enough costimulation in absence of an acute inflammation. In lack of danger signals, DCs are shown to regulate and maintain peripheral tolerance; they are able to secrete immune-regulatory cytokines as well as they can induce a regulatory phenotype in T lymphocytes. The proper choice of antigen and activation of the cells is therefore of imminent importance to elicit tumor specific immunity.

In order to improve the immunogenicity of the cancer vaccination, a "semi-allogeneic" system was used. Antigen presentation in the absence of sufficient co-stimulation and adequate cytokine signals can even lead to the induction of tolerance against the specific antigen. The antigen presenting cell therefore has to be adequately activated in order to be able to induce pro-inflammatory immune responses. On the other hand, antigens presented by an MHC mismatched cell cannot be recognized by the host T cell receptors due to self-MHC restriction. But it evokes a strong inflammatory response against the foreign MHC molecules. By using an allogeneic dendritic cell expressing MHC class I molecules of the recipient's haplotype as antigen delivery vector, we anticipated to be able to prime tumor antigen specific adaptive immune responses in the context of self-MHC while creating a strong inflammatory environment against the otherwise allogeneic cell (Fig.25). By using this so called "semi-allogeneic" concept we hypothesized to enhance conventional cancer vaccination that are using autologous dendritic cells in various points:

- Due to the high T cell precursor frequency against allogeneic MHC molecule and the possibility of direct recognition of allogeneic MHC class I and II molecules, adaptive immune responses against non-self MHC are generally very strong. On the other side, tumors often induce an immune-suppressing microenvironment leading to weak or even absent $CD4^+$ T-cell help [163]. Thus, by the application of partially allogeneic DCs, an inflammatory environment is created. Through direct and indirect $CD4^+$ T cell responses, the allogeneic dendritic cell-directed inflammation can be exploited to provide potent help for the generation of CTLs against tumor peptides presented on the shared H-2 class I K^d molecules. Indeed, a few reports could show allogeneic DC fused with syngeneic cancer cells to elicit anti-tumor effects superior to syngeneic DCs [164], [165]. Furthermore, it has been shown that CD4 T cell help is as important to mediate and re-establish innate antitumor effectors for example for activation of macrophages and natural killer cells.

- The cell line generated in our lab represents a well-defined, homogenous $CD8\alpha^+$ dendritic cell population which can be produced in theoretically infinite numbers. This facilitates practical realization of experiments and allows us to apply a more standardized vaccine in terms of phenotype and maturation status.

- The cell line is further modifiable, e.g. for overexpression of pro-inflammatory cytokines. This may allow to combine the benefits of site-directed local cytokine therapy and vaccination leading to a more efficient antitumor effect. Further modification of the DC

line enables the local delivery of pro-inflammatory cytokines which might alter the effects of antitumor vaccination.

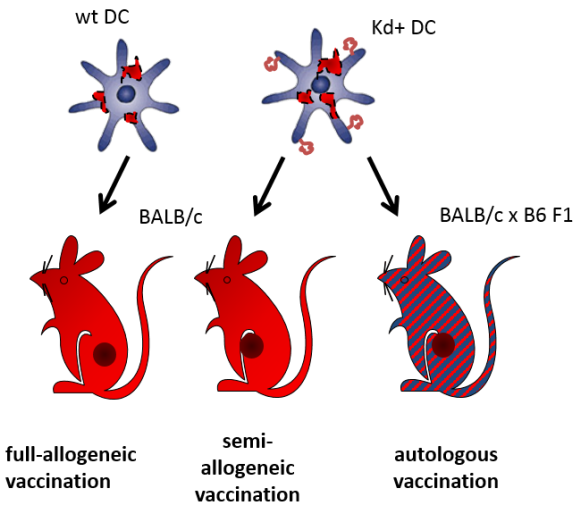


Figure 25 Schematic overview on the "semi-allogeneic" dendritic cell vaccination approach: The C57BL/6 derived MuTu dendritic cell line was modified in order to express the MHC class I molecule H-2K^d. The transfer of allogeneic DCs into BALB/c mice should trigger a strongly inflammatory environment while tumor antigens presented by the autologous H-2K^d molecules may prime a tumor specific cytotoxic response.

7.1 The CT26 colon carcinoma model

In order to elicit allogeneic responses against the DC line, the tumor, respectively its syngeneic host, must not be of C57BL/6 background. For practical reasons a subcutaneously growing tumor is preferred as changes in tumor size can easily be monitored *in vivo*. The tumor cell line that was chosen for this project is CT26, a chemically induced murine colon adenocarcinoma. The cell line established in the 1970ies by repeated intra rectal injections of the carcinogen N-Methyl-N-Nitrosurea (NMU) [166]. It is syngeneic on BALB/c background and has since then been widely used in animal studies. The tumor is an undifferentiated Grade IV carcinoma, exhibiting aggressive growth and spreading. It tends to form metastatic lesions in lung, kidneys, heart, mesentery and diaphragm.

7.2 Establishment and verification of H2K-d$^+$Dendritic cell line

In order to use the dendritic cell line as semi-allogeneic vaccine, the DCs have to express H2-K^d, the MHC class I haplotype expressed by the BALB/c mouse strain presenting the immunodominant antigens in the CT26 tumor model. A lentiviral transfer vector containing the full length MHC-I H2-K^d sequence has previously been created in collaboration with the group of Immanuel Luescher, LICR Lausanne. The wild-type C57BL/6 MuTu DC line was lentivirally transduced and the transduced cells were sorted for the expression of H2- K^d. Purity of the resulting H2-Kd$^+$ population was around 80 percent (Data not shown). The DC line is henceforth called "Kd$^+$DC". The phenotype of the Kd$^+$ dendritic cell line was compared to wild-type MuTu DC

line in steady-state and upon activation with TLR ligands and IFN-γ, showing no significant differences (data not shown).

7.3 Establishment of pulsing protocol

An appropriate protocol for the activation of dendritic cell is one of the key requirements for the generation of effective CTL responses. We decide to pulse the dendritic cells with necrotic tumor cell particles. This approach has the advantage that it may lead to a polyclonal T cell activation and a therefore potentially more vigorous anti-tumor immunity. Indeed, strong CTL responses could be detected even in poorly immunogenic tumor models upon immunization with tumor extract-pulsed DCs [167]. However, the lack of a defined, uniform antigen makes it more difficult to standardize the experimental procedure and different batches of tumor cell lysate might vary in their immunogenicity. In the absence of an appropriate activation, the dendritic cells might fail to induce a beneficial Th1 response or even promote tolerance towards the tumor derived antigens. In a first step, we therefore tried to identify ideal conditions for pulsing and activating the dendritic cells.

Lysate to DC ratio: CT26 tumor cell lysate used for this project was produced by harvesting cells in log phase of their growth curve and lysed by 5 consequent freeze-thaw cycles in liquid nitrogen. In order to titer the CT26 tumor lysate ratios that allow optimal activation and antigen presentation, Kd^+DCs were co-cultured at different proportions of tumor cell lysate or TLR agonists for up to 24h. The expression of MHC class II, the transgenic H2-K^d as well as co-stimulatory molecules CD80, CD86 and CD40 were analyzed by flow cytometry. Activation of DCs applying a high lysate to DC ratio tended to induce DC apoptosis, whereas proportions lower than 1:2 were not efficient in augmenting expression of co-stimulatory molecules (data not shown). A 1:2 ratio of lysate to DCs was therefore applied in all following experiments.

Time: It was previously observed that prolonged activation of the MuTu cell line (and of endogenous $CD8\alpha^+$ DCs) causes induction of apoptosis in a type I interferon dependent manner [128] while a too short activation might not be sufficient for the optimal presentation of antigen and expression of co-stimulatory signals. A time laps experiment was therefore performed to compare the activation status of DCs activated by tumor cell lysate with or without the addition of IFN-γ to DCs activated using the TLR9 agonist CpG and IFN-γ. The addition of CpG tended to

lead to a faster up-regulation of co-stimulatory molecules. However, after 24 hours of activation the tumor lysate activated DCs exhibited elevated levels of MHC class II as well as co-stimulatory molecules that were comparable to CpG induced activation. The addition of IFN-γ resulted in a further up-regulation of surface activation markers, especially MHC-II and CD40 in both, CpG as well as CT26 lysate activated cells. (fig.26a).

In order to provide secondary and tertiary activation stimuli to T lymphocytes, additional inflammatory mediators are normally required. We therefore compared the production of pro-inflammatory cytokines by the KD+DCs upon stimulation with either TLR ligand or tumor cell lysate. IL-12p40 secretion was found to be increased upon addition of CpG or CT26 cell lysate. Again, the kinetics were different with considerable amounts of IL-12p40 produced by CpG stimulated DCs already after 6 hours while cytokine secretion by lysate stimulated DCs could only be detected after 12 hours. Secretion of biological active Il-12p70 heterodimer however could only be detected when the dendritic cells were also supplemented with IFN-γ (fig. 26b).

The preparation of tumor cell lysate is difficult to standardize and different batches of CT26 lysate might vary in their ability to activate DCs. So far, possible differences in tumor lysate efficiency have not been compared. Taken together the results indicate that tumor cell lysate alone seems to be capable to induce DC activation. However, an additional inflammatory stimulus, here provided by IFN-γ, was needed for the full activation of the DC line. More than 12 hours of activation was required to observe a distinctive expression of MHC-II, costimulatory receptors and inflammatory cytokines while activation for more than 24h hours drastically reduces the viability of the DCs. We therefore decided to pulse the Kd^{+}DCs for 18 hours in the following experiments.

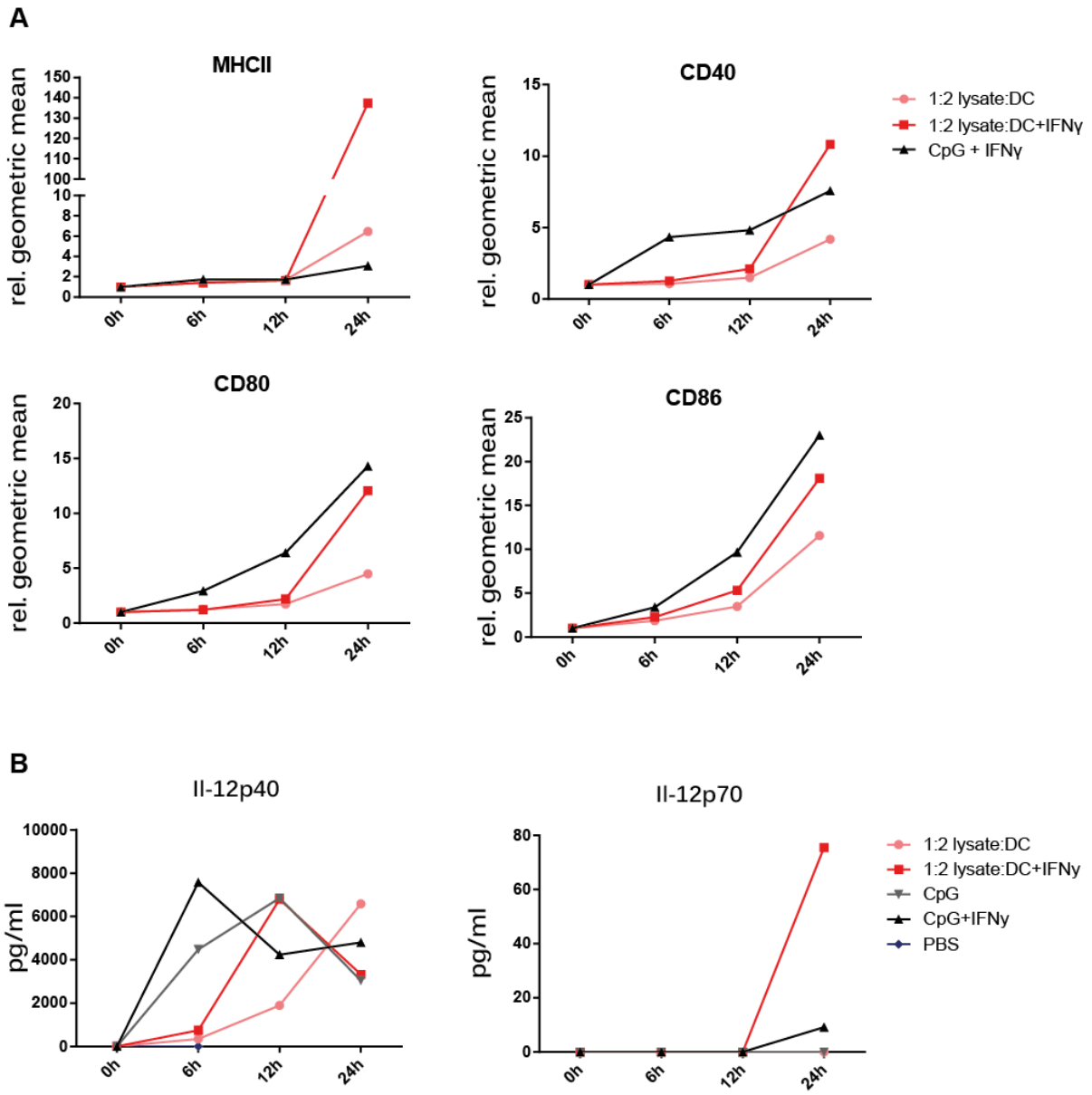


Figure 26 Establishment of a pulsing protocol: In order to determine optimal pulsing and activation conditions, different parameters were examined. A) The expression of MHC class II and co stimulatory molecules and B) the expression of the Th1 associated cytokine IL-12 was measured at different time points after the activation of DCs with CT26 cell lysate +/- IFNγ was compared to CpG and IFNγ activated DCs. Data representative of 2 independent experiments.

7.4 H2-Kd⁺ DC induce CD4⁺ and CD8⁺ T cell proliferation

In the next step we tested whether activated Kd⁺DCs were able to elicit CD8⁺ T cell responses in an *in vitro* assay. Kd⁺DCs were pulsed with CT26 lysate and IFN-γ, CpG and IFN-γ or PBS and irradiated to inhibit their proliferation. BALB/c derived splenocytes were isolated, and co-cultured with the pulsed DCs at different ratios. Proliferation of T lymphocytes was assayed by eFluor670 dilution after 5 days. An increasing proportion of eFluor670^{low}, CD4⁺ and CD8⁺ T cells

could be observed, depending on the DC ratio applied (Fig.27a). Interestingly, the CT26 cell lysate pulsed or CpG activated Kd⁺DC reproducibly led to a noticeable higher percentage of proliferating CD8⁺ T cells at low DC to splenocyte ratios. For CD4⁺ T cells this effect was much more pronounced when the splenocytes were cultured in presence of C26 lysate pulsed DCs.

Analogous to T cell proliferation, an enhanced IFN-γ secretion was measured with increasing Kd⁺DC to splenocytes ratio. Similarly to T cell proliferation, IFN-γ production was found to be considerably higher when the Kd⁺DCs were previously activated with CT26 lysate and IFN-γ. Although only basal amounts of IL-17a could be detected in culture supernatant, IL-17a secretion tended to correlate negatively with DC to splenocytes ratio (Fig. 27b). Moreover, no significant amounts of IL-4 or IL-10 could be measured in culture supernatant (data not shown).

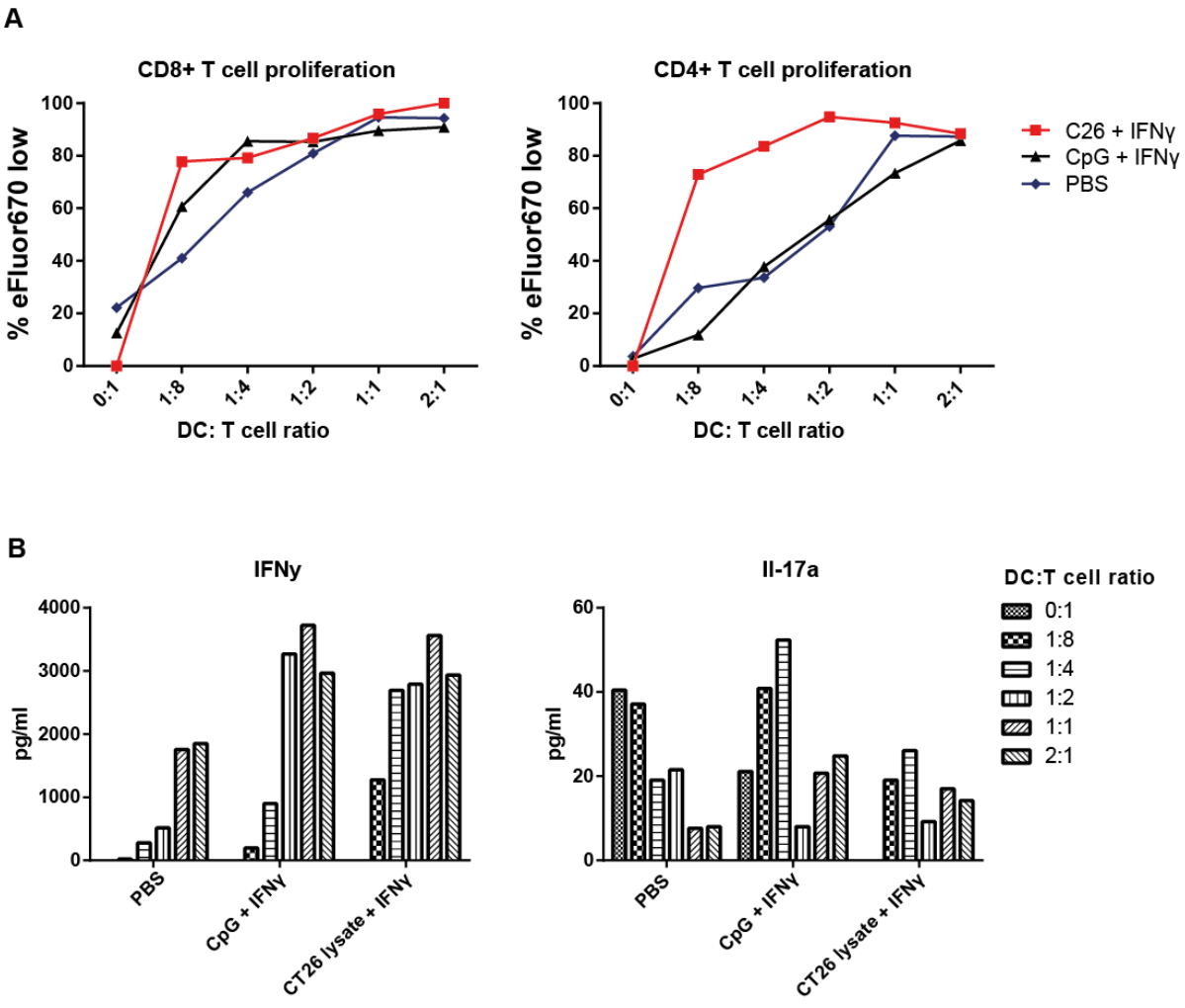


Figure 27 CT26 lysate pulsed *Kd⁺ DC induce* T cell proliferation and Th1 skewing: A) BALB/c derived CD4⁺ or CD8⁺ T cells were cultured at different ratios with CT26 lysate / IFNγ pulsed or CpG/IFNγ stimulated Kd⁺DCs. Whereas the proliferation of CD8+ T cells was comparable, CT26 pulsed Kd⁺DCs induced CD4⁺ T cell proliferation already at lower DC to T cell ratios. B) Cytokine secretion of the cocultured cells was detected by ELISA. CT26 pulsed Kd⁺DCs tended to induce more IFNγ production while reducing IL-17 secretion. No Il-4 and only basal levels of Il-10 could be detected (data not shown). Data representative of 3 independent experiments.

7.5 Vaccination of BALB/c mice with H2-Kd expressing C57BL/6 DC line delays tumor growth

The immunogenic potential of the semi-allogeneic dendritic cell vaccination was assayed in a therapeutic vaccination setting. $5x10^4$ CT26 tumor cells were subcutaneously transferred in the flank of wild-type BALB/c or F_1 (BALB/c x C57BL/6) mice. Three and ten days after tumor inoculation, the mice were vaccinated with $2x10^6$ tumor cell lysate pulsed, activated Kd^+ or wild-type DCs. The development of tumor size was measured regularly. The mice of all experimental groups developed palpable tumors within 8 days. This initial tumor growth was often followed by a short period of contraction that was followed by an anew increase in volume. PBS injected controls mice exhibited a progressive tumor growth leading to a tumor volume that required euthanasia in median 30.5 days after inoculation. A comparable increase of tumor volume was measured for animals that were vaccinated with wtDCs or resting Kd^+DCs. However, the overall better health status of the mice in these experimental groups increased the median survival noticeably (act wtDC: 35 days, resting Kd^+DC: 37.5 days). Vaccination of the mice with CT26 lysate pulsed Kd^+DC led to a markedly slower increase of tumor size when compared to control animals and further increased the median survival to 43 days. Although the observed differences were highly reproducible, they were, especially at later stages, barely statistically significant. This was caused by the considerable variation of tumor volumes within the experimental groups. Surprisingly, we noticed that autologous vaccination of (BALB/c x C57BL/6) F_1 mice with activated Kd^+DCs drastically accelerated tumor growth in a highly reproducible way and reduced the median survival of this experimental group to 27 days (Fig.28a+b).

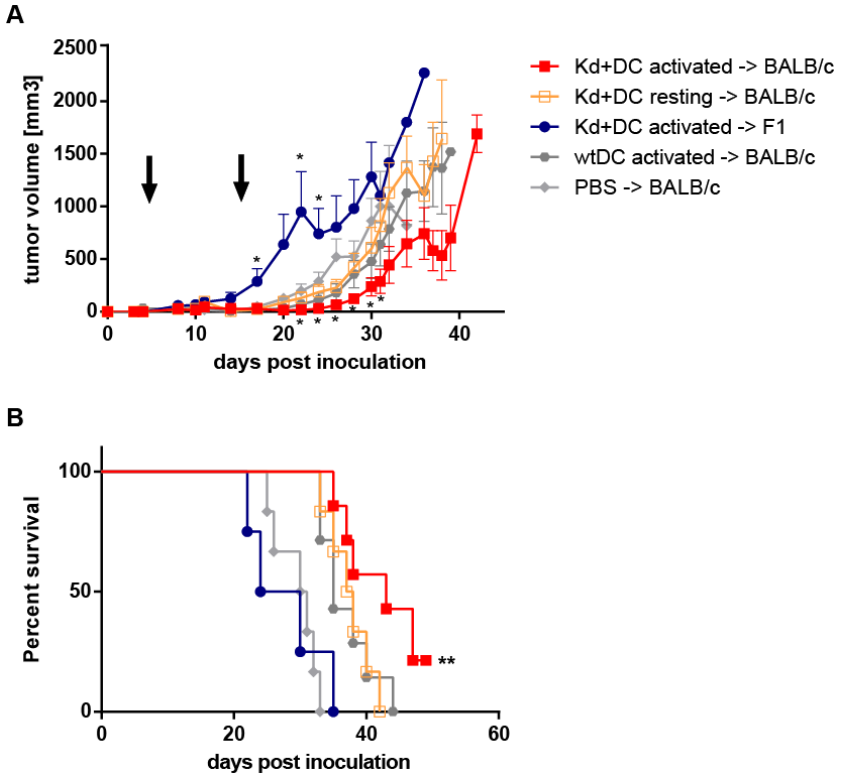
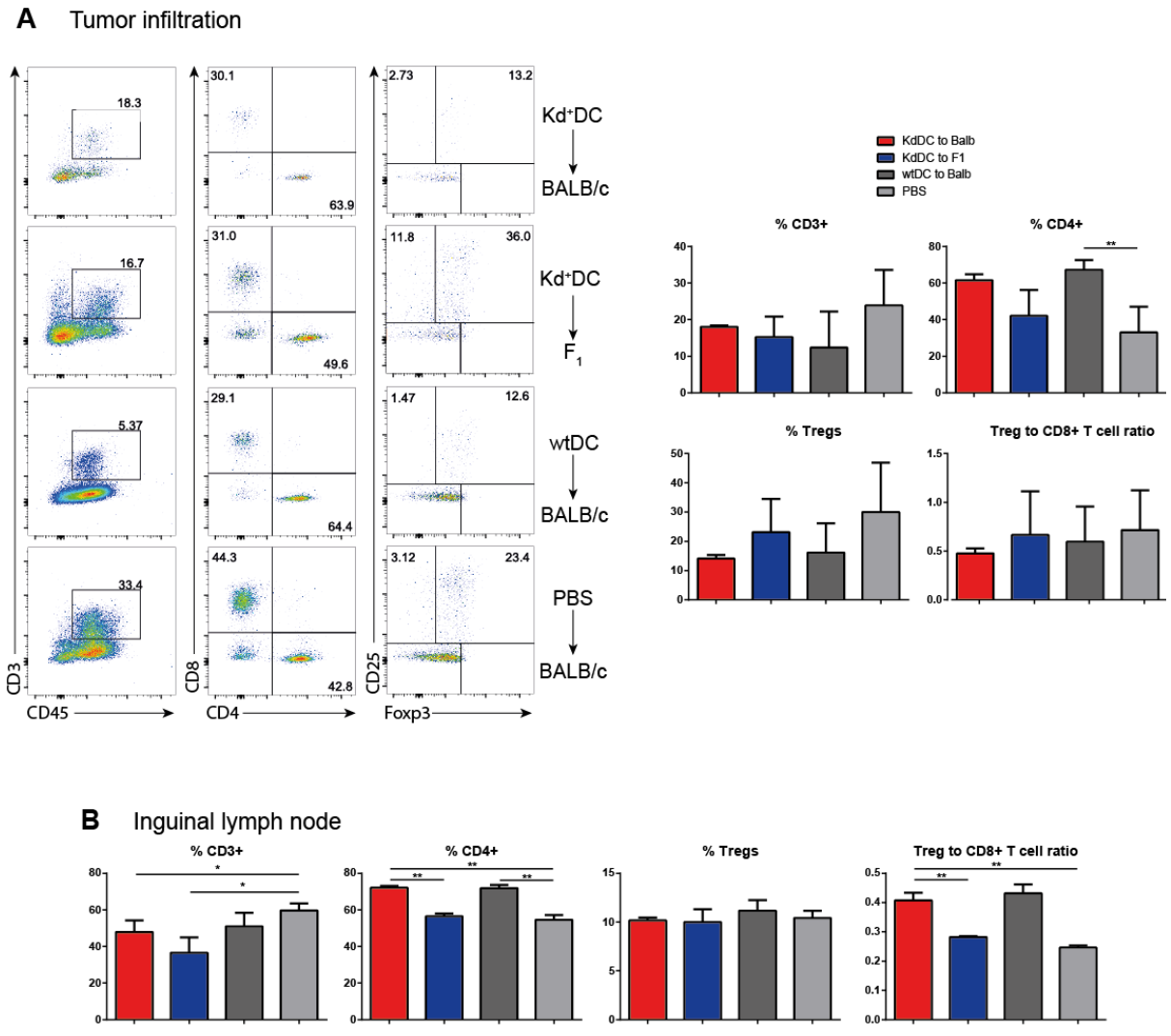


Figure 28 Vaccination of BALB/c mice with H2-K^d expressing C57BL/6 DC line delays tumor growth: CT26 tumor cells were subcutaneously injected into wild-type BALB/c mice. Mice were vaccinated 3 and 10 days later and A) tumor growth and B) survival was followed. Semi-allogeneic vaccination with tumor lysate pulsed, IFNγ activated Kd$^+$DCs reproducibly delayed whereas the autologous transfer into (C57BL/6 x BALB/c) F1 mice surprisingly accelerated tumor growth. Data depicted as mean tumor volume ± SEM. B) Mice were euthanized when the met abort criteria as described in the Material & Methods part. Again, semi-allogeneic vaccination prolonged median survival. Survival curves were compared using Log-Rank test. The graphs are showing pooled results of 2 experiments. A total 6 independent experiments were conducted.

7.6 Semi-allogeneic vaccination did not affect tumor infiltration by CD4$^+$ and CD8$^+$ T cells

As described above, BALB/c or BALB/c x C57BL/6F$_1$ mice were vaccinated with activated, tumor cell lysate pulsed Kd$^+$ or wtDCs. 14 days after tumor cell inoculation, when tumor growth was observable in all experimental groups. The mice were sacrificed and leukocytes were isolated from tumor, tumor draining inguinal lymph nodes, and spleen by collagenase digestion and mechanical disruption. Single cell suspensions were analyzed by flow cytometry for the presence of T lymphocytes as well of a palette of innate myeloid leukocytes. Unfortunately the flow cytometric analysis of the cells yielded partially contradicting results and high inter-experimental discrepancies. The results shown in Fig.29 were chosen from one experiment that was successful from a technical point of view and represents best the observations from the different experiments. When looking at tumor infiltration by total CD45$^+$ hematopoietic cells, we could observe a drastic reduction upon transfer of Kd$^+$DC or wtDC into BALB/c animals. This effect was especially pronounced when C26 lysate pulsed, activated dendritic cells were injected (Fig.29a).

Regarding adaptive infiltration, the vaccination seemed to reduce the recruitment of $CD3^+$ T cells independently of activation status of the DCs and the genotype of the animal. We could further observe a tendency towards a reduced proportion of $CD25^+$ $Foxp3^+$ regulatory T cells and, biologically more relevant, a decreased Treg to $CD8^+$ T cell ratio in the tumors of BALB/c mice that received vaccination. However, the effect was similar when using wild-type or tumor cell lysate pulsed, semi-allogeneic Kd^+DCs. Vaccination of F_1 mice resulted in an infiltration of total $CD4^+$ as well as regulatory T cells that was comparable to not vaccinated control animals. But again, the differences were not statistically significant. Similarly to tumor infiltration, DC injection led to a decrease of total T cell proportion within the tumor draining inguinal lymph node regardless of the genotype of the mice. Semi-allogeneic vaccination but not autologous vaccination did significantly increase the percentage of $CD4^+$ T cells found in the lymph nodes resulting in an increase of the Treg to $CD8^+$ T cell ratio (Fig 29b). When analyzing tumor infiltration by cells of the innate immune compartment, we could not detect any differences in the proportions of $NK1.1^+$ natural killer or $CD11b^+$ cells. However, vaccination with pulsed Kd^+DCs seemed to shift the high $Ly6G^+$ granulocytic infiltration found in control vaccinated animals rather towards an increased invasion by $Ly6C^+$ monocytes or $Ly6G^-Ly6C^-$ cells (Fig 29c). Semi-allogenic or control but not autologous vaccination did significantly reduce the percentage of CD45+ hematopoietic cells found in the spleen of the respective animals. In addition, the transfer of Kd^+ and wild-type DC into BALB/c mice was associated with some minor but statistically significant shifts within the different CD11b+ populations: While the $Ly6G^+$ as well as the $Ly6C^-Ly6G^-$ populations tended to augment, we could detect significantly fewer $Ly6C^+$ monocytes when compared to not vaccinated animals (Fig.29d).


A
Tumor infiltration
18.3
30.1
63.9
2.73
13.2
Kd+DC
BALB/c
16.7
31.0
49.6
11.8
36.0
Kd+DC
F1
5.37
29.1
64.4
1.47
12.6
wtDC
BALB/c
33.4
44.3
42.8
3.12
23.4
PBS
BALB/c
CD3
CD45
CD8
CD4
CD25
Foxp3
KdDC to Balb
KdDC to F1
wtDC to Balb
PBS
% CD3+
% CD4+
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Inguinal lymph node
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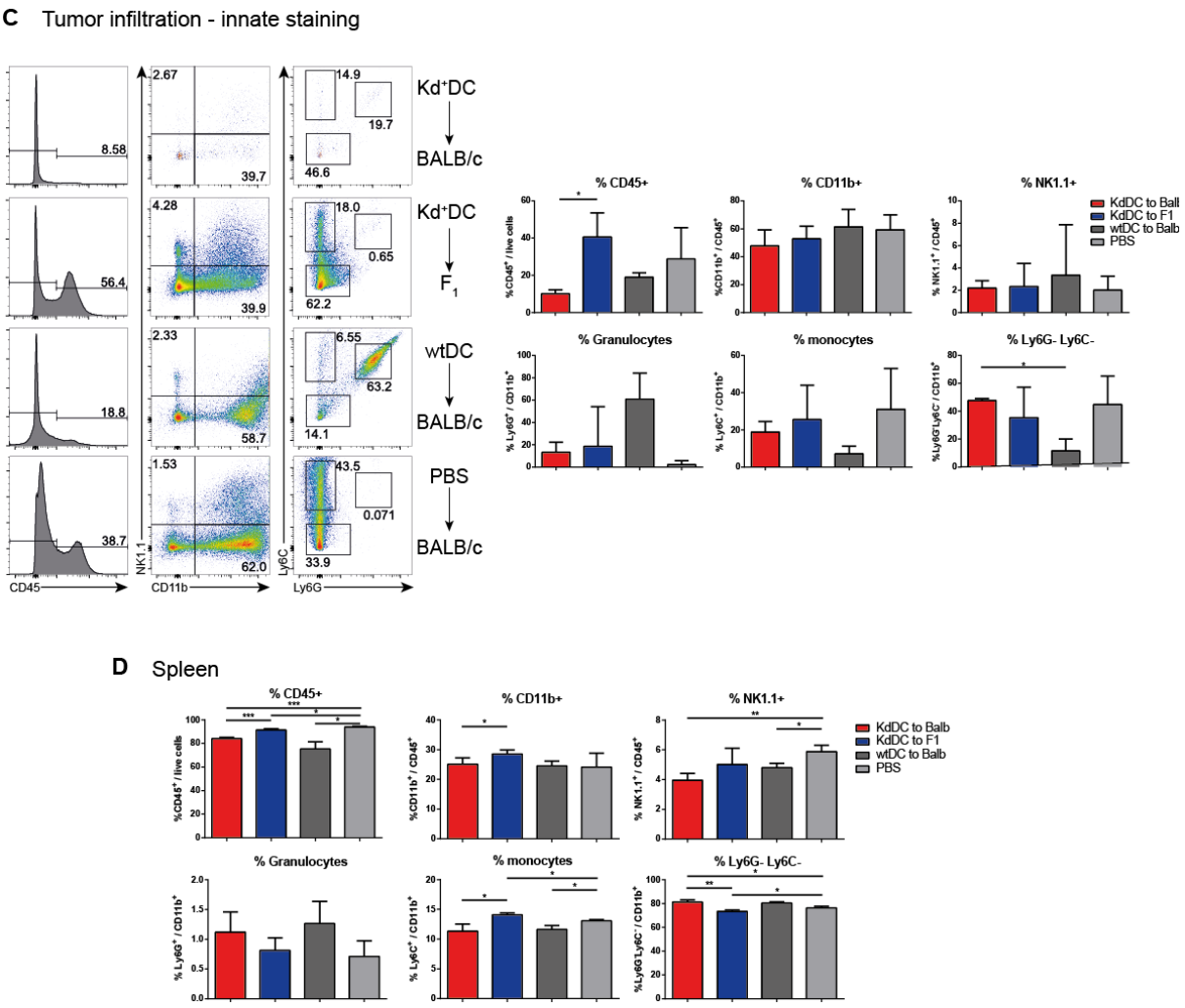


Figure 29 Semi-allogeneic vaccination did not affect tumor infiltration by CD4⁺ and CD8⁺ T cells: CT26 tumor was inoculated and mice were vaccinated as described above. The animals were sacrificed 12 days after CT26 cell injection, when tumor growth was observable in all experimental groups. A+C) Tumor infiltrating, B) tumor draining lymph node or D) spleen resident leukocytes were analyzed by flow cytometry. A+B) No major differences in T cell infiltration and function was observed between the experimental groups. C) An increased infiltration of total CD45⁺ leukocytes could be observed upon autologous vaccination. D) Only minor differences of spleen resident innate cells could be measured between the experimental groups. Results show representative data from 4 independent results, n=4 animals per experimental group. Results are represented as the mean ± SD.

7.7 Enhancing semi-allogeneic vaccination by the application of pro-inflammatory cytokines expressing H2-K^{d+} dendritic cell lines

The application of immunologically defined molecules, in our case pro-inflammatory cytokines, as “adjuvants” has been proposed since the 1990’s (reviewed by [168]). Various cytokines have been described to improve anti-tumor immunity in murine tumor models and clinical studies. Many of the classical candidate cytokines like, IFN-α, IL-2 or IL-12 have been described to promote anti-cancer immune responses (reviewed by [117]). Their pro-inflammatory

characteristics may therefore be combined and exploited to improve the efficacy of cancer vaccination and help to overcome immune suppressive tumor environment.

Three cytokines that have caught special attention for cancer immunotherapy have been selected for this project:

Interleukin-2: Secreted mainly by $CD4^+$ T cells, IL-2 promotes growth, survival and differentiation of antigen-activated T lymphocytes. Responsiveness to this cytokine is controlled by tight regulation of the cognate receptor; functional, high-avidity IL-2R$\alpha\beta\gamma_c$ is found only on activated T cells. Regulatory T cells express IL-2R constantly, even without overt antigen or IL-2 stimulation. It is therefore assumed that the IL-2 has an important role in T_{reg} survival and function *in vivo*. In addition to the effects on T cells, high levels of IL-2 also promote proliferation and differentiation of natural killer cells.

Interleukin-15 shares receptor components of IL-2 and has similar structural characteristics. Thus, the biological activities of the two cytokines overlap. But in contrast to IL-2, IL-15 is expressed only by nonlymphoid cells, including activated monocytes and DCs. A unique characteristic of IL-15 and IL-15Rα is the ability to stimulate neighbor cells via a mechanism known as *trans*-presentation, i.e. the IL-15Rα on the surface of DC or monocytes forms a complex with IL-15 and then presents it in *trans* to neighboring cells. IL-15 induces proliferation of naive and memory $CD8^+$ T cells, promotes primary CTL responses and makes both $CD4^+$ and $CD8^+$ T cells resistant to suppression by Tregs. It is able to maintain central memory $CD8^+$ T cell homeostasis. In addition IL-15 promotes activation of NKs, DCs, monocytes and macrophages [169].

Interleukin-12 acts as a master regulator of the innate and adaptive immune system: The probably most important and characteristic activity is its ability to promote Th1 immune responses: IL-12 stimulates the development of naïve $CD4^+$ T cells as well as resting memory $CD4^+$ T cells into Th1 cells capable of producing large amounts of IFN-γ. It can also act directly by enhancing the lytic activity of NK and cytotoxic $CD8^+$ T lymphocytes [170]. IL-12, together with IFN-γ, inhibits the proliferation of Th2 polarization and therefore regulates the balance between Th1 and Th2 mediated immune responses. There is further evidence that IL-12 is also capable of inhibiting pro-angiogenic factors like vascular endothelial growth factor (VEGF).

7.7.1 Generation of CD8α+ DC lines constitutively expressing pro-inflammatory cytokines

IL-2 and IL-15 were amplified from activated T cell, respectively DC cDNA and cloned into a lentiviral expression vector. For IL-12, a single-chain construct was cloned in the laboratory of Burkhard Becher, Zürich. The two subunits forming IL-12, p35 and p40 are linked by a $(Gly_4Ser)_3$ sequence. In addition, a murine IgG3 Fc tag was added to the single-chain construct in order to increase stability and solubility of the protein product. The H-2Kd+ MuTu DC line was transduced with the respective lentiviral vectors and the expression of the transgene was controlled by rtPCR and ELISA (Fig.30a + b).

In order to assay whether the generated DC lines affect proliferation or function of T cells, OVA peptide specific CD8+ OT-I or CD4+ OT-II cells were stimulated *in vitro* in the presence of the transgenic DCs pulsed with the respective peptide. After 4 days of incubation the T cells were harvested and proliferation as well as activation was analyzed by flow cytometry. As expected, peptide pulsed DCs induced a strong proliferation as well as the CD44+ CD62L- activated phenotype of both OT-I and T-II T cells when compared to T cells cultured with resting DCs. However, both the percentage of proliferated as well as of activated T cells was similar when any of the cytokine transduced DC or the Kd+DC control line was used for T cell simulation (Fig.30c). The secretion of IFN-γ protein by the T cells was measured by ELISA. Incubation with IL-2 expressing DCs reproducibly induced IFN-γ production even in the absence of OVA peptide and led to a further increase of IFN-γ expression by OT-I and OT-II cells when compared to control DCs. IL-12Fc+DC augmented IFN-γ secretion only by CD4+ OT-II cells (Fig30d).

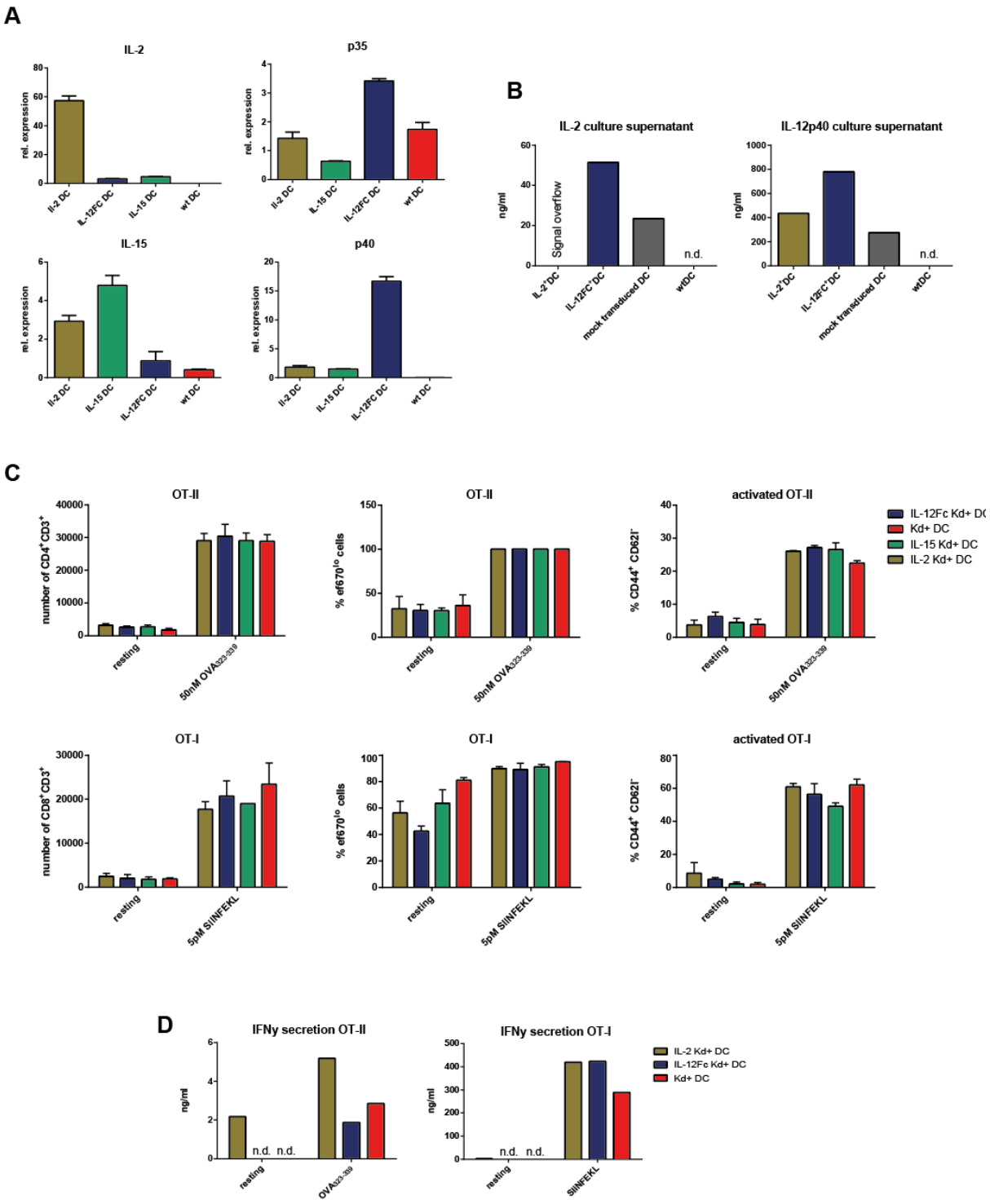


Figure 30: Characterization of proinflammatory cytokine expressing DC lines: The expression of the inflammatory cytokines was verified by A) rtPCR and B) ELISA on supernatants collected after 72 hours incubation of the respective DC line. C) Comparable proliferation and activation of $CD4^+$ OT-II or $CD8^+$ OT-I T cells was measured upon culture with the different DC lines pulsed with the respective peptides. D) Cytokines secretion by the T cell was measured by ELISA on the culture supernatants. The incubation with IL-2 expressing DCs resulted in an increase of IFNγ production by OT-II but not OT-I cells.

7.7.2 Expression of pro-inflammatory cytokines did not improve the efficacy of semi-allogeneic DC cancer vaccination

In a next step we tested whether the expression of proinflammtory cytokines by the DCs might improve inflammatory response towards the tumor in the context of the semi-allogenic vaccination approach. As described before, BALB/c mice were subcutaneously challenged with CT26 tumor cells and were vaccinated two times with the respective activated and CT26 lysate pulsed DC line. Vaccination of the tumor bearing mice with any of the Kd$^+$ dendritic cell lines delayed tumor growth when compared to not vaccinated control animals. However none of the Kd$^+$DCs expressing pro-inflammatory cytokines was able to further significantly delay tumor growth. Only in animals vaccinated with IL-12 expressing Kd$^+$DCs a tendency towards a slower tumor growth and a marginally increased median survival could be observed (Fig.31a+b). Due to the discouraging results using these cytokine transduced DC lines and the contradictory data obtained from the analysis of tumor infiltrating immune cells shown above, we refrained to pursue this part of the project.

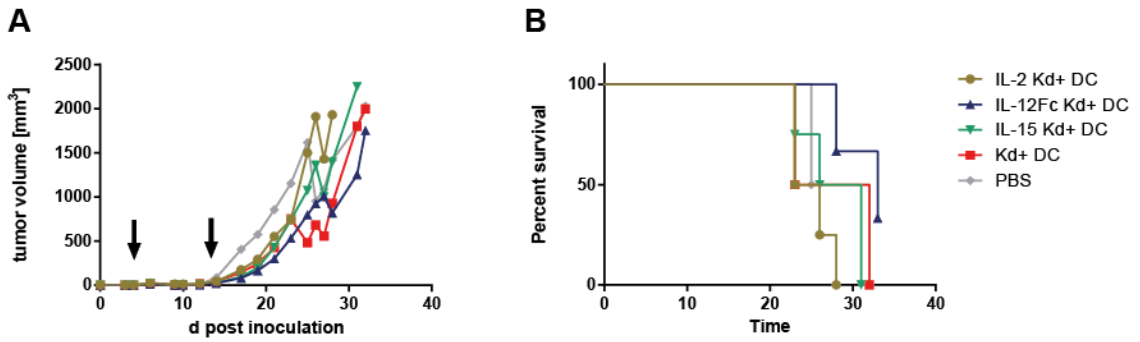


Figure 31 Expression of pro-inflammatory cytokines did not improve the tumor specific response of the semi-allogeneic vaccination CT26 tumor was inoculated and mice were vaccinated with the cytokine expressing Kd$^+$DC lines as described above. No differences in A) tumor growth and B) survival of the mice was measurable when compared to vaccination with control Kd$^+$DC.

III. Discussion

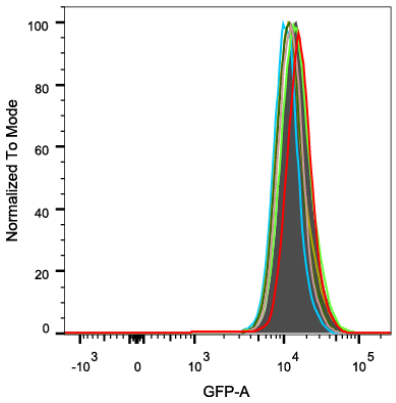
8 Expression of tolerogenic cytokines by the MuTu Dc line

The failure to mount appropriate immune responses against infectious agents or aberrant cells can lead to life-threatening pathologic conditions. On the other side, uncontrolled or chronic immunity against (self-) antigens is associated with the development of a variety of (auto-) immune diseases and can lead to the development of cancer. A high level of organization and regulation is therefore needed to ensure effective functioning of the immune system. Dendritic cells are the most efficient antigen presenting cells, they provide costimulatory, respectively coinhibitory and cytokine signals in order to promote and regulate T cell responses. Due to their central role in determining the outcome and quality of adaptive immune responses, DCs have caught major interest as potential therapeutic tools for the immunotherapy of (auto-) inflammatory disease or cancer.

8.1 Generation of transgenic DC lines

Research on dendritic cells is traditionally limited by the scarcity of the cells *in vivo* and the resulting work and cost intensive isolation. Classical derivation of DCs from bone marrow or monocytic precursor cells is time intensive and results in a less homogenous, at least partially activated DC population. For this study we have taken advantage of the MuTu CD8α$^+$ dendritic cell line that was established and characterized in the laboratory of Prof. Acha-Orbea. The MuTu DC line constitutes a homogenous cell population that corresponds phenotypically as well as functionally to CD8α$^+$ DCs. The DC line can easily be cultured and provides an infinite source for manipulation *in vitro* and *in vivo*. Lentiviral infection of the cells was found to yield high transduction rate and stable expression of the desired transgene without severely affecting DC phenotype [127], [128]. However, the MuTu DC lines were derived from mice expressing the SV40 largeT oncogene and GFP under the control of the CD11c promoter. In retrospect, the use of GFP as marker on the lentiviral expression vectors was therefore not an advantageous decision. While A. Duval was able to sort transduced dendritic cells according to the shift in GFP expression upon lentiviral transduction, we could not detect such an increase of GFP signal when compared to the wild-type DC line (Fig.32). Accordingly we were neither capable to assay efficacy of transduction nor could we sort transgene expressing cells. Although the same amount of lentiviral particles was used to transduce the DCs, we observed variable transgene expression which might explain

some of the variation in the results obtained with IL-35 expressing DCs. As a consequence, a RFP expressing lentiviral transfer vector has currently being used in our laboratory.



Sample Name	Geometric Mean: GFP
IL-2+DC	16174
IL-12FC+ DC	12862
IL-15+ DC	12658
IL-35+ DC new	14979
IL-35+ DC old	13071
H2-Kd+ DC	11280
Wild type DC	15136

Figure 32 Lentiviral transduction of MuTu Dendritic Cell line does not lead to a significantly higher GFP expression: Wild-type MuTu dendritic cells were transduced using different lentiviral particles that allow the stable expression of the gene of interest as well as a GFP marker. The efficacy of the transduction was controlled by measuring the shift in GFP expression since the DC line itself already expresses GFP. In contrast to previous findings we were not able to detect a significant increase in mean fluorescence in any of the lentiviral transduced DCs when compared to not transduced DCs.

Nevertheless, the CD8α+ MuTu DC line has proven to be an invaluable tool for the investigation of various aspects of dendritic cell biology. The field of applications ranges from basic immunologic questions as addressed in this thesis to more molecular questions. For example decipher the molecular mechanisms that regulate cytokine production in response to different TLR and cytokine stimuli (Gupta et al, in preparation).

9 Interleukin 35 expressing dendritic cell line reveals novel functions for IL-35

Analogous to the other members of the IL-12 cytokine family, interleukin 35 is an important regulator of pro- respectively anti-inflammatory immune responses. IL-35 was shown to be a key mediator of Treg function by the suppression of CD4+ T cell proliferation and induction of a regulatory phenotype on naïve CD4+ T cells [39]. Decreased levels of IL-35 have recently been found to be associated with various human (auto-)inflammatory pathologies like systemic lupus erythematosus, COPD or Hepatitis B [171]–[173], On the other hand, IL-35 production by different human tumors has been found and was shown to correlate with a poor prognosis [139]. These findings suggest IL-35 to be a promising target for the treatment of autoimmune diseases

and its inhibition may be beneficial in cancer treatment. However, the biological functions of the cytokine are still poorly understood.

9.1 Interleukin-35 promotes a tolerogenic phenotype on CD8α⁺ DCs

The flow cytometric analysis of the interleukin 35 expressing MuTu DC line revealed remarkably differences in their phenotype when compared to control virus transfected DCs: While the expression of the CD8α⁺ dendritic cell markers CD11c, CD8α and DEC205 was down regulated, IL-35⁺DC prominently upregulated CD11b expression. Activation of the DC lines with TLR ligands led to a marked upregulation of MHC class II as well of co-stimulatory receptors CD40 and CD80/86 by control DCs. In contrast, IL-35 expressing DCs did express lower expression levels of MCH class I and II as well as CD40 already in unstimulated conditions. Activation of the cell line did not cause the upregulation of any of the analyzed molecules. A comparable but less pronounced effect could be observed when wild-type DCs were cultured for 4 days in IL-35 conditioned medium. Considering that neither mock transduced DCs nor DCs cultured in control supernatant exhibited a markedly altered phenotype, we assume it very unlikely that the observed effects were provoked by the lentiviral transduction. Besides the observed immature phenotype, we measured the transcription of the immunosuppressive molecule Ido1 by IL-35 containing supernatant cultured wtDCs. As described, Ido transcription could also be detected in wtDCs activated by IFN-γ and Ido transduced DCs but not in mock supernatant treated DCs. Together, these observations imply a possible direct effect of IL-35 cytokine on the dendritic cells itself. While a large array of mediators have been reported to induce a tolerogenic phenotype on DCs, no such effect has been described for IL-35. In agreement with our observations, tolerogenic dendritic cell populations are generally thought to express low levels of MHC-II and costimulatory molecules. The cells are therefore largely ineffective in activating T cells trough the classical TCR signaling pathways. In addition, tolerogenic, respectively Th2 inducing DCs are described by several studies to have a rather macrophage-like phenotype, including the expression of CD11b [64], [174], [175]. Especially interesting is the finding that a distinct subset of CD11b⁺ dendritic cells that expresses Ido can mediate systemic immune regulation in a model of oral tolerance induction [90].

Taken together, our findings indicate that IL-35 cytokine produced by the transduced DCs or supplemented in the medium directly affects the phenotype of the CD8α⁺ DC line. The inability to

adequately upregulate the expression of MHC II and costimulatory molecules in response to TLR ligand stimulation indicate a possible interference with the immunogenic capabilities of the DC lines. In addition, the production of the regulatory molecule Ido might further contribute to the regulatory functions of IL-35 conditioned DCs. Regarding the drastic effect of IL-35 on phenotype and function of the MuTu DC line, we can hypothesize that IL-35 does not only directly regulate T cell responses but also indirectly via the induction of a tolerogenic phenotype on DCs. Nevertheless, such a direct effect of IL-35 on dendritic cells has yet to be verified. In a pilot experiment we attempted to test the ability of IL-35^{+}DCs to promote T cell proliferation while depleting secreted IL-35 cytokine using an ati-ebi3 antibody. We thereby hoped to be able to distinguish between the direct effects of IL-35 on the T cells and the induced tolerogenic phenotype of the DCs. Unfortunately the experimental setup did not function and has to be improved. In order to be able to response to IL-35 signaling, dendritic cells need to express an IL-35 receptor. Although the exact composition of the receptor is still disputed, there is agreement that IL12rb2 is indispensable for IL-35 signaling. Although IL-12rβ2 transcription was detected in our DC line and is, according to the immgen.org microarray database, moderately expressed in most conventional DC subtypes. We did not address whether the MuTu DCs express a functional IL-35 receptor on protein level. Engagement of IL-35 receptor in B or T cells has been shown to trigger intracellular signaling mediated by the phosphorylation of Stat1/Stat3 respectively Stat1/Stat4 heterodimers [34], [37]. It would therefore be crucial to investigate whether the conditioning of DCs with IL-35 cytokine actually induces Stat signaling. The identification of the target genes induced by IL-35 signaling could give further insight in the effector mechanisms deployed by the cytokine. Lastly, the experiments discussed here were performed on MuTu DC line only. In order to confirm the findings we will repeat the experiment using endogenous CD8α^{+} but also other DCs populations. This will allow us to determine whether the observed effects on MuTu DCs are restricted to the CD8α^{+} DC subtypes or whether it represents a more general mechanism to induce tolerogenic DCs.

9.2 Interleukin-35 expressing DCs mediate T cell tolerance

Studies on IL-35 have been hindered because the stability of commercial available, recombinant IL-35 is rather short. The constitutive expression of IL-35 cytokine by the MuTu dendritic cell lines allowed us to circumvent this problem and to investigate how the cytokine might influence the outcome of DC-T cell interactions. In a first step we therefore sought to assay whether the transgenic expression of the IL-35 construct by DCs can limit the expansion and effector functions of T lymphocytes as it was described by others.

9.2.1 Interleukin-35⁺ DC inhibit CD4⁺ and CD8⁺ T cell proliferation in vitro and in vivo and induce a regulatory phenotype on CD4⁺ T cells.

The contribution of IL-35 to the functions of regulatory T cells was the first attribute of the IL-35 cytokine described in the literature. Its effect on CD4⁺ T lymphocyte function is by now best investigated function. First, the expression of a functional transgene product was verified by in vitro culture of IL-35⁺DCs with CD4⁺ T cells. As expected, IL-35⁺DCs were found to impair expansion of the lymphocytes upon both, polyclonal as well as antigen-specific stimulation. In contrast control DCs induced a robust proliferation. Furthermore, a lower proportion of CD4⁺ T cells exhibited an activated phenotype and tended to produce less IFN-γ when they were primed by IL35⁺DCs. The application of IL-35 expressing DCs in the mixed lymphocyte reaction as well in the OVA peptide specific stimulation setting prompted the transcription of the IL-35 subunits p35 and ebi3 but not the IL-12 related protein p40. Interestingly we found that the addition of IL-35 containing medium to $OVA_{323-339}$ pulsed, wild type DC also led to the inhibition of OT-II CD4⁺ T cells but these cells did not transcribe p35 or ebi3. Furthermore, the expression of IL-35 by DCs converted naïve CD4⁺ T cells into a regulatory population, able to suppress proliferation of other naïve CD4⁺ T cells. The transfer of $OVA_{323-339}$ specific OT-II cells together with peptide pulsed IL-35⁺ DCs reduced the total number of OT-II that could be retrieved when compared to control DCs injected mice. IFN-γ secretion by OT-II was found to be reduced but surprisingly the proportion of proliferating cells within the retrieved OT-II cells was only marginally reduced.

In contrast to the better described responses of CD4⁺ T cells to IL-35, only two studies so far have addressed its effect on CD8⁺ T cells. Guo et al. observed that the expansion of CD8⁺ T cells was significantly inhibited when IL-35 conditioned supernatant was added in a mixed lymphocyte reaction [137]. Moreover, in another study it was shown that anti-CD3/CD28 stimulated CD8⁺ T cells produced comparable amounts of IL-35 cytokine as CD4⁺ T cells[136]. In our hands the polyclonal activation of CD8+ T cells by allogeneic IL-35⁺ DCs did lead to a significantly reduced proliferation, activation and IFN-γ production when compared with control DCs cultured cells. We were able to detect transcription of IL-35 subunits p35 and ebi3 when the primed CD8⁺ T cells were primed by IL-35⁺DC but not with control DCs. However the relative expression of the two subunits was markedly lower than in CD4⁺ T cell primed by IL-35⁺DCs. Comparably, the transfer of OVA specific OT-I CD8⁺ T cells together with peptide pulsed IL-35⁺DC but not control DCs in to C57BL/6 mice almost completely abrogated the expansion of OT-I cells. The remaining OT-I proliferated to the same extend and expressed similar levels of IFN-γ as mockDC injected mice. The endogenous CD8⁺ T cells populations were not affected by the transfer of IL-35⁺DCs. Besides IFN-γ we did not assay a possible effect on other (cytotoxic) effector molecules like perforin or granzyme B nor did we measure the lytic capability of these cells. A suppression of effector

functions by IL-35+DCs can therefore not yet be definitively clarified. Curiously, when similar experiments were conducted by A. Duval using active TGF-β expressing DCs, no suppressive effect on CD8+ T cell responses could be detected. She and other showed that the priming of CD8+ effector responses by DC based vaccines is dependent on endogenous CD11c+ DCs that cross-present the vaccine derived antigen to CD8+ T cells [130], [176]. This was thought to interfere with a possible tolerogenic effect by TGF expressing DCs. based on the available data the discrepancy to the results presented here are difficult to explain. One can speculate that either the production of IL-35 directly imposes suppressive signal that is potent enough to override the activating stimuli by the endogenous APCs. Another possibility is that the IL-35+ DCs might, for some reason, not be able to transfer antigen to the endogenous DCs.

Thus, our data indicate that the IL-35 expressing dendritic cell line is able to exert immune modulation functions on T cells and demonstrate that the expressed transgene is indeed biologically functional. Our results support previous findings that IL-35 does not only impair proliferation and effector functions of CD4+ T cells, but can similarly affect CD8+ T lymphocytes. In contrast to the data published by Guttek and Reinhold, the activation of CD4+ and CD8+ T cells in absence of IL-35 cytokine was not sufficient to induce transcription of the IL-35 related subunits ebi3 and p35. However, CD4+ and CD8+ T cells cultured in the presence of IL-35+DCs or IL-35 containing supernatant did express p35 and ebi3 at least on mRNA level. By now there are also commercial IL-35 ELISA kits available that would allow to assays whether the induction of transcription is translated to protein secretion. The expression of the cytokine by CD4+ T lymphocytes mediates infectious tolerance by suppressing proliferation and inducing a regulatory phenotype on naïve CD4+ T cells termed iTr35 [32]. Our observation that IL-35+DC but not IL-35 containing medium led to the transcription of IL-35 subunits by CD4+ T cells suggest that the engagement of IL-35 alone is not sufficient to generate iTr35 cells. In accordance with the above mentioned Collison paper where T cells were activated with anti-CD3/CD28 in order to acquire suppressive ability. In addition, Guttek and Reinhold showed that immunosuppressants like rapamycin or dexamethasone abrogate the anti-CD3/CD28 activation induced IL-35 production by T cells [136]. Together these findings suggest that IL-35 alone is sufficient to regulate T cell proliferation. But the induction of IL-35 expression by T cells depends on additional activating stimuli. Collison and colleagues described that the addition of IL-10 enhances the conversion of naïve CD4+ T cells into iTr35. While we did find only low IL-10 production by IL-35+DC, the question whether the addition of recombinant IL-10 or the combination with IL-10 expressing DCs would further increase suppressive capability by iTr35 remains open. It might represent a possibility to augment the tolerogenic potential of the IL-35+ DC line. Another aspect of the IL-35+DC induced effects on T cells we did not address is the stability of the induced regulatory phenotype. In the experiments we performed the suppressive

capability of iTr35 cells was assayed directly after 4 days of conditioning by IL-35$^+$DCs. Again, an elegant experiment by Collison et al. showed that in a long-term experiment the transfer of TGF-β induced iTregs was less efficient in delaying disease development of Foxp3$^{-/-}$ mice than iTr35 [32]. Thus, these observations indicate that IL-35 induced regulatory T cells can maintain their suppressive phenotype over a long period.

The observed effects on CD8$^+$T cell raises new questions whether iTr35 can also regulate CD8$^+$ T cell responses and whether CD8$^+$ T lymphocytes can acquire an IL-35 induced regulatory phenotype. TGF-β induced CD8$^+$regulatory T cells have been described but the mechanisms of suppression by these cells are not well defined. According to the literature, the regulatory capability of TGF-β induced CD8$^+$ Tregs seems to be independent from IL-10 and TGF-β (reviewed by [177]). Also in regard to the increasing number of reports indicating a role for IL-35 in the immune evasion by tumors, it would be highly interesting to further investigate the effects of IL-35 cytokine, respectively IL-35$^+$DCs on CD8$^+$T cells.

9.2.2 Interleukin-35$^+$ DC induce tolerance to tumor growth

We further investigated whether the observed suppression of T cell proliferation and function by IL-35$^+$DCs can affect anti-tumor immunity. C57BL/6 mice were vaccinated before and after subcutaneous tumor inoculation with either tumor cell lysate pulsed, IL-35 or mock transduced DCs. In both cancer models we tested, the moderately immunogenic CMT93 colon carcinoma as well as the poorly immunogenic, aggressive B16.F0 melanoma, we observed a significantly increased tumorigenesis upon the transfer of IL-35$^+$DCs when compared to the control groups. Surprisingly, the vaccination by tumor lysate pulsed mockDC did not lead to a delay in tumor growth when compared to not vaccinated mice, which indicates that the vaccination protocol itself might not have promoted anti-tumor immunity. However, IL-35$^+$DCs promoted tumor growth was accompanied by a markedly reduced tumor infiltration by CD3$^+$ T lymphocytes and an impaired IFN-γ production by CD4$^+$ and CD8$^+$ T cells. Interestingly we could observe an accumulation of CD11b$^+$ Gr1$^+$ in both tumor models. When the same experiment was performed using T and B lymphocyte deficient RAG$^{-/-}$ mice, we surprisingly could still observe an accelerated tumor growth upon vaccination with IL-35$^+$DCs. The analysis of tumor infiltrating cells revealed a significantly increased proportion of CD11b$^+$ Ly6G$^+$ SSC^{hi} neutrophilic respectively granulocytic MDSC.

These findings further confirmed the tolerogenic potential of the IL-35 expressing DC line to regulate T cell proliferation and effector function. To our surprise, the induction of tolerance against the malignancy was not only mediated through the regulation of the adaptive anti-tumor

response but also by affecting innate cells. While these tumor experiments were performed, a study was published demonstrating that tumor cells that were genetically modified to express IL-35 enhanced the accumulation of $CD11b^+$ $Gr1^+$ myeloid cells in their environment and led to an increased vascularization [133]. These findings correspond nicely to our results and further reinforce the hypothesis that IL-35 mediated effects are not restricted to T lymphocytes. However, the study by Wang et al. raised a series of new questions. Especially the precise identity of the $CD11b^+$ myeloid cell accumulating in the tumor environment and the mechanisms facilitating tumor growth are of interest. Our data point towards $Ly6G^+$ neutrophils as the IL-35 responding myeloid population, as it is discussed further below in more detail. The depletion of MDSCs using anti-Gr1 antibody or the functional characterization would help to verify the contributions of to the accelerated tumor growth. The use of NOD-SCID mice would further allow to exclude NK cells as IL-35 responder cells. Another open question is whether tumor derived IL-35 acts as an attractant for MDSCs or whether it affects the function or survival of the myeloid cells was. In our experimental system the cytokine was not delivered locally at the site of the lesion, which makes it unlikely that IL-35 exerts direct chemoattractant functions on myeloid cells.

Although the ectopic expression of IL-35 by tumor cell lines was employed in the study by Wang et al., they and several other recent studies have shown that a variety of human tumors express the IL-35 subunits p35 as well as ebi3. In addition, elevated plasma levels of the cytokine can indeed be detected in patients with, for example, non-small lung cancer [178]. The exact mechanism how tumor derived IL-35 supports tumor development is still unclear. Using a human epithelial derived pancreas cancer model, Nicholl et al. demonstrated that tumor-derived IL-35 can act in an autocrine manner and promotes tumor cell proliferation through an increased expression of cyclins B and D while it reduces apoptosis through the expression of anti-apoptotic molecules like Bcl-2 [139]. In contrast, Long et al 2013 claimed that IL-35 expression by several human tumors leads to an induction of cell cycle arrest and increase sensitivity to TNFα induced apoptosis [179]. However, while we did not control for a direct effect of IL-35 expression on tumor growth, none of the just mentioned studies addressed possible effects on tumor associated myeloid cells.

9.3 Interleukin-35 mediates effects on non-lymphoid cells

Over the last 2 years several studies on the effects of IL-35 on tumor cells and B lymphocytes cells were published, demonstrating that the cytokine is not only involved in the regulation of $CD4^+$ T cells responses. However, the possible biological functions of IL-35 on responder cells other than T lymphocytes remains poorly investigated. Only a few studies have addressed the question whether cells of non-lymphoid origin are capable to response to IL-35. The observed induction of a tolerogenic phenotype on $CD8\alpha^+$ MuTu DCs as well as the acceleration of tumor growth upon vaccination with tumor lysate pulsed IL-35^+DCs in T and B cell deficient mice indicated an involvement of cells other than B and T lymphocytes. We therefore expanded our investigations and looked whether IL-35 can affect other cell types.

9.3.1 Interleukin-35 increases neutrophil survival and proliferation

The only indication so far that IL-35 may impose an effect on myeloid cells comes from the already mentioned study by Wang et al. who showed an increased accumulation of CD11b+ $Gr1^+$ myeloid cells in the environment of IL-35 transfected tumors. Since the vaccination with IL-35^+DC in our hands pointed in the same direction, we investigated the effects of IL-35 on myeloid cells *in vitro* under steady-state as well as under inflammatory conditions. The observed accumulation of myeloid cells may have been caused directly by the expression of IL-35 or indirectly through other tumor derived factors. We therefore sought for experimental approaches that exclude such effects.

An increased relative as well as absolute number of $CD11b^+$ $Ly6G^+$ neutrophils could be detected after *in vitro* culture of T cell depleted splenocytes in the presence of IL-35 containing but not control supernatant. Moreover, a higher proportion of the neutrophils exhibited an activated phenotype. On the contrary, $CD11b^+$ $Ly6C^+$ monocytes were not found to be affected by the presence of IL-35. Intraperitoneal injection of the inflammatory agent thioglycollate, together with IL-35^+DCs augmented the number of neutrophils that could be isolated from the peritoneal lavage. A higher percentage of those cells were found to be viable and seemed to be more activated.

Thus, the addition of IL-35 containing culture medium as well as the presence of IL-35+DCs affected the expression of activation markers and survival of $CD11b^+$ $Ly6G^+$ SSC^{int} neutrophils but not of monocytes or macrophages. These results indicate that the observed accumulation of myeloid cells (respectively neutrophils in our experiments) in the tumor environment indeed be a direct effect of IL-35 causing an improved survival of the cells. Depletion of neutrophils for example using Gr-1 antibody should at least partially reverse the tumor supporting effect of IL-

35^{+}DC in our model. Neutrophils are characterized by a short half-live time of only about a few hours (in mice) resulting in a high turnover rate. The life-span of neutrophils can markedly be prolonged by the activation of neutrophils under inflammatory conditions. Especially cytokines as IFN-γ and GM-CSF but also bacterial products and direct interactions with other cells have been shown to activate and prolonging the survival of neutrophils in inflamed tissue. This assures an optimal adaption and to the inflammatory conditions [180]. Another possibility for the increased accumulation of neutrophils would be an enhanced recruitment of the cells. We assume it unlikely that IL-35 directly mediates neutrophil attraction since no such effect has been described for any of the IL-12 family cytokines. It is conceivable that IL-35 might induce the expression of the chemokines CXCL1, CXCL2 or CXCL8, which are the main mediators of neutrophil chemoattraction. However, we did not yet investigate the expression of chemokines by the DCs or the stromal cells that come into question.

Neutrophils are well recognized as critical players during acute infection. They are quickly recruited to an inflammatory site and can eliminate pathogens by various mechanisms. More recently, it has been reported that neutrophils express a variety of molecules as for example the matrix metalloprotease 9 (MMP9) indicating their involvement also in resolving inflammation and promoting tissue repair. Mediate regulation of adaptive immune responses by the production of ROS, arginase 1 or IL-10 which suppress T cell activation and function (reviewed by [181]). In addition, it was shown that Tregs can induce a regulatory phenotype on neutrophils in acute as well as in chronic inflammation [182], [183]. In regard to these finding it can be hypothesized that Treg derived IL-35 might expand neutrophils with anti-inflammatory properties. These neutrophils in turn can contribute to the resolution of inflammation by promoting tissue remodeling and by regulating adaptive immune responses. In the context of malignant disease, tumor cell derived IL-35 might serve to operate the same mechanisms in order to improve the survival and function of tumor associated neutrophils respectively MDSCs. Further investigation is needed to elucidate the effector functions of IL-35 activated neutrophils and to support this hypothesis.

We are aware that more elegant and maybe less ambiguous experimental approaches exist to address the effects of cytokines on different myeloid cell types. Especially neutrophils are described to be not very resistant to manipulations ex vivo [184]. We accordingly tried to separate myeloid populations using different techniques including magnetic separation and density gradient centrifugation. However, these attempts either yielded very low purity, activated the cells or induced death of a large proportion of the isolated cells.

9.3.2 Interleukin-35 increases endothelial cell proliferation

The study by Wang et al. demonstrated that the expression of IL-35 by tumor cells results in the accumulation of $CD11b^+$ $Gr1^+$ myeloid cells in the tumor environment and leads to increased angiogenesis. This could be directly mediated by the IL-35 cytokine or indirectly through the factors secreted by the attracted leukocytes. In order to investigate possible direct effects of the cytokine, we incubated HUVEC endothelial cells either in the presence of IL-35 containing medium or in a transwell assay with $IL\text{-}35^+$DCs. In comparison to control medium respectively control DCs, the addition of IL-35 led to a significantly faster closure of the inflicted scratch. This effect was accompanied by a markedly increased proportion of proliferating cells, especially but not exclusively at the margins of the scratch, as well as a reduction of apoptosis.

These findings indicate that IL-35 might indeed positively affect the proliferation and survival of endothelial cells. We can at this point not discriminate whether the positive effect on endothelial cell growth is directly caused by IL-35 or whether IL-35 induces the expression of endothelial growth factors such as VEGF, which in turn could mediate the observed effects. A recent study by Nicholl et al demonstrated that IL-35 promotes the growth and reduces apoptosis of epithelial derived pancreas cancer cells. IL-35 induced proliferation was shown to be associated with an increase of cyclins B and D as well as the anti-apoptotic molecule Bcl-2 [139]. These results can certainly not directly be translated to non-tumorigenic, endothelial cells. Nevertheless, it demonstrates that IL-35 can exert effects comparable to our observations on cells of non-hematopoietic origin. We are well aware that HUVEC cells are derived from human umbilical cord vein and might therefore not represent an optimal system for our purposes. However, they were the only endothelial cell line that was available to us at that time. In addition, murine and human IL-35 are well conserved from mouse to humans and other murine cytokines of the IL-12 family have been shown to be functional on human cells (personal communication with J. vom Berg, Zürich and according to the company R&D systems). In a next step we want to verify our findings *in vitro* by performing similar scratch assays using the murine endothelial line C166 respectively primary murine endothelial cells. Whether the enhanced endothelial growth translates *in vivo* into an improved vessel formation can be assayed by looking at the vascularization of tumors upon vaccination with $IL\text{-}35^+$DCs as it was performed above. In order to better understand the molecular mechanisms involved in the response to IL-35, it would be interesting to analyze the signaling pathways and target genes targeted in endothelial cells upon IL-35 engagement.

10 Application of tolerogenic DC lines for the of the treatment and prevention of unwanted inflammation

10.1 Induction of tolerance to autoimmune encephalitis

Experimental autoimmune encephalitis, is caused by the infiltration of myelin-specific Th1/Th17 cells that in turn attract and instruct mature myeloid cells to destroy the CNS parenchyma. DCs were shown to be involved in the priming and activation of myelin specific T cells. The modulation of DC function by the expression tolerogenic molecules might therefore represent a promising approach to limit or even prevent EAE.

Since we had some difficulties to elicit robust and reproducible disease course we decided to use an adoptive transfer EAE model as it was described [155] instead of the widely used active induction of EAE. Besides the more reliable EAE induction, the use of the adaptive transfer EAE allowed us to prolong the time the (auto-reactive) T cells can interact with the dendritic cell lines expressing tolerogenic molecules.

10.1.1 Active TGF-β expressing DCs prevent EAE through the induction of Foxp3$^+$ regulatory T cells:

Transfer of actTGF-β DCs alone was sufficient to completely suppress the development of EAE associated symptoms. No reduction of IFN-γ or IL-17 expressing CD4$^+$ T cells could be observed in the CNS draining cervical lymph nodes, while the number of Foxp3$^+$ CD4$^+$ regulatory T cells was significantly increased. Interestingly, only minimal, scattered CNS infiltration by CD3$^+$ T cells was observable in any of the mice. We can therefore assume that the induction of tolerance towards EAE by actTGFβ$^+$DCs was caused by the induction or expansion of Foxp3$^+$ regulatory T. The lack of any CNS T cell infiltration indicates that the regulatory T cells did not suppress effector T cells *in situ* but rather prevented the migration from peripheral lymphoid tissue to the CNS. Since an increased proportion of Foxp3$^+$ Tregs was already detectable during the in vitro restimulation of donor mouse derived lymph node cells, we suppose that the protective Tregs were generated *in vivo* during the priming of CD4$^+$ T cells in the donor mice. Whether the Foxp3$^+$ Tregs were generated by the expansion of nTregs, their elevated survival or the induction of regulatory T cells from naïve CD4$^+$ T cells can only be speculated and would need further investigation. The protective role of regulatory T cells in the prevention of EAE has already been described by different studies: While the depletion of Tregs exacerbates EAE disease, the adaptive transfer of Tregs suppresses CNS infiltration by effector T cells and ameliorates disease. Interestingly, the

injected Tregs did not traffic into the CNS but rather accumulated in spleen and lymph nodes [185], [186].

Mice that were treated with DCs expressing the latent form of TGF-β exhibited an intermediate disease course. The latent complex has to be cleaved in order to become biologically active. Since the *in vitro* restimulation of donor mice derived lymph node cells did not show major differences in the proportions of IFN-γ or IL-17 expressing effector cells nor of Tregs, it appears that latent TGF-β does exert any regulatory functions during the priming phase of adaptive transfer EAE and thus might be cleaved only during the effector phase in the recipient mice.

10.1.2 Interleukin-35 expressing DCs reduce EAE severity

We performed only one experiment applying IL-35$^+$DCs in the adaptive transfer EAE model. The results have therefore to be considered as preliminary. Nevertheless, the significant reduction of disease incidence and severity when compared to the control groups suggest IL-35 to be a promising candidate molecule. This result is in agreement with previous findings by Shen et al who could demonstrate that IL-35 expression by B cells contributes to the recovery from EAE. In their hands B cell-derived IL-35 limited disease by reducing the functional properties of the pathogenic Th1 and Th17 cells [38]. In our hands, Flow cytometric analysis of the ex-vivo restimulated lymph node cells from the immunized donor mice did not affect effector function of the T cells. However, in vitro restimulation in the presence of MOG pulsed IL-35$^+$DC seemed to inhibit the proliferative response of the donor mice derived T cells. Histological analysis revealed a drastically reduced CNS infiltration of CD3$^+$ T cells. Although IL-35 expressing DCs seem to partially inhibit EAE pathogenesis in our model, the preliminary results do not allow to draw conclusions on the cellular mechanisms explaining the observations. The experiment will be repeated in order to verify our findings paying particular attention on the B cell phenotype as suggested by the study mentioned above.

10.1.3 Interleukin-10 expressing DC do not affect EAE development

IL-10 expressing DCs did not affect EAE development in our hands (A. Duval, data not shown). This findings are in discrepancy to the literature showing that IL-10 limits EAE progression by different means. For example have draining lymph node resident, regulatory plasma cells been proposed to be required to limit EAE development in a IL-10 dependent manner [187]. Interestingly, the secretion of IL-10 by regulatory B cells has been shown to be associated with IL-35 expression [38]. In addition, the repetitive stimulation of effector Th1 cells in the presence of TGF-β was found to induce IL-10 production by IFN-γ$^+$ Th1 effector cells. An increased frequency of these double producers was associated with a reduced CNS infiltration and less

severe EAE [150]. Although these observations underline the importance of IL-10 for controlling EAE disease, the expression of IL-10 by MuTu DCs was not sufficient to reduce EAE severity in our hands.

10.2 A combination of IL-10 and activeTGF-β expressing DC lines prolong skin graft survival

Allotransplantation of tissues or organs evokes strong inflammatory responses leading to the rejection of the graft over time. Current treatment mainly consists of broad-range immunosusspression which is non-specific and increases the risk to develop infection and malignancy. The need for new strategies that allow a specific suppression of transplant-directed immunity is therefore obvious. T cells are the main mediators of graft rejection whereas DCs are considered responsible for the induction of graft-directed adaptive immunity [156]. In contrast to autoimmune diseases, transplantation in many cases allows a preventive transfer of (donor-derived) dendritic cells. It therefore can be hypothesized that by the modulation of dendritic cells we may be able to suppress donor-reactive T cells or induce Tregs. Indeed, a recently published meta study showed that the transfer of tolerogenic DC generally prolongs skin allograft survival [157] demonstrating the general feasibility of this approach. The molecular mechanisms underlying DCs for induction of allograft tolerance largely remained poorly understood.

For practical reasons we chose a skin graft model to test our hypothesis. Transplanted skin is generally acutely rejected despite intensive immunosuppressive therapy and thereby represents one of the most immunogenic tissues in allotransplantation. Any observed change in graft survival therefore can be assumed to be the result of a strong modulatory effect. In a full MHC mismatched setting the transfer of a combination of donor derived IL-10 and active TGF-β expressing DCs was not sufficient to delay graft rejection in. It can be assumed that the elicited transplant-directed immune response was too potent to be overcome respectively that a possible tolerogenic effect did not translate into an observable change in graft rejection.

When multiple minor mismatched skin was grafted, the transfer of IL-10 together with activeTGF-β expressing DCs reproducibly delayed rejection of C57BL/6 skin allografts. Surprisingly no effect on graft survival could be observed upon the transfer of only one of the respective DC lines. These results indicate complementary mechanisms that lead to a synergistic tolerogenic effect. In addition, we found that the transfer of IL-35 expressing DCs alone has a minor but statistically significant positive effect on graft survival. In a preliminary experiment, the analysis of peripheral blood lymphocytes harvested 7 days after skin transplantation did not reveal a consistent

mechanism responsible for the synergistic effect. Besides some technical issues it might be possible that blood leukocytes do not reflect the tolerogenic DC induced effects at the site of engraftment. We are planning to repeat the experiment and will analyze lymphocytes infiltrating the graft, within the draining lymph node and the spleen 7-10 days after transplantation.

As opposed to our observations, different studies have shown that the in vivo administration or transfection of TGF-β alone can delay allograft rejection [188], [189]. Experiments by Daley and colleagues have shown that TGF-β signaling is not required for the suppression of graft-reactive effector T cell function but it mainly act through the induction or expansion of Foxp3$^+$ Tregs [190]. IL-10 seems to play a dual role in the context of transplantation: On one side IL-10 inhibits the stimulatory activity of dendritic cells and contributes to graft tolerance in a dose-dependent manner. While high doses of recombinant IL-10 have been shown to reduce allograft survival in rats by the attraction of neutrophils, low doses prolonged graft rejection [191]. Another study showed that the transfer of DCs expressing viral IL-10 does not prolong skin graft survival. In their hands the secreted vIL-10 seemed to inhibit Th2 responses while promoting IFNy production by neutrophils or NK cells [192]. In concordance with our results, a potent synergistic effect was described when combining IL-10 and TGFβ. Several studies have demonstrated that IL-10 and TGF-β can cooperate in order to maximize the potential of each cytokine to induce hyporesponsive T cells respectively Tregs [193], [194]. The mechanisms underlying this synergistic effect are not completely understood. However, it was for example shown that IL-10 upregulates TGF-β receptor expression on effector and memory T cells rendering them susceptible to TGF-β mediated Treg induction [195]. Less is known about the role of IL-35 in transplantation. In fact only two published studies investigated the role of IL-35 in the context of transplantation. Both suggest IL-35 to be important for the expansion of Foxp3$^+$ regulatory T cells: Antibody mediated depletion of IL-35 has been shown to reduce skin graft survival and led to a reduced transplant infiltration by Foxp3$^+$ Tregs [196]. In vivo transfection with an IL-35 expression vector delayed cardiac graft rejection associated with a systemic increase of Treg [137].

So far we have acquired only very limited data record on T cell function and phenotype. We therefore cannot speculate yet why the transfer of IL-10$^+$ or actTGFβ$^+$DC alone did not improve graft viability or why the combination of both DC lines did. Nevertheless, the results indicate that our tolerogenic DC lines represent a useful platform for further improvement of DC-based therapy. In a next step we will therefore investigate whether other combinations of tolerogenic DC lines, for example IL-35$^+$DC together with actTGF-β expressing DCs might also exhibit synergistic effects. We intentionally refrained from using immunosuppressive drugs in the experiments presented here in order to maintain the experimental system as simple and clean as

possible. The combination with initial administration of immunosuppressive drugs might further enhance graft survival by dampening the initial inflammatory response inflicted by the procedure. Finally, we will try to decipher the cellular mechanisms underlying the observed (synergistic) effects as described above.

11 Augmentation of antitumor immunity applying a semi-allogeneic DC line

The application of dendritic cells for cancer immunotherapy has been studied with a great interest. Nevertheless, current DC vaccination strategies are still limited by the low affinity of tumor antigens, not optimal DC maturation and activation protocols and other difficulties. Current vaccination approaches induce tumor specific adaptive immunity in vitro an in rodent models. But they generally fail to translate into a robust, therapeutically relevant response in (pre-) clinical trials. The application of gene-modified DCs, expressing immunostimulatory molecules are therefore thought to enhance the efficacy of DC vaccination by directing antigen delivery and overcoming the immune suppressive environment of the tumor

For this second project we took advantage of the access to the MuTu dendritic cell line. We sought to apply the cell line as a tool to investigate how to improve the immunogenicity of DC based cancer vaccines. The DC line allowed us to investigate in a standardized fashion in terms of DC phenotype and activation status and provided availability infinitive numbers of cells for experimentation.

11.1.1 Semi-allogeneic DC vaccination interferes with CT26 tumor growth

In a first approach to augment immunogenicity of DC cancer vaccines we tested a so called “semi-allogenic” vaccination. The C57BL/ derived MuTu DC line was transduced in order to express the MHC class I molecule H2-K of the BALB/c haplotype H2-d. Tumor bearing BALB/c mice were then vaccinated using this DC line and the effect on tumor growth was observed. We proposed that semi-allogeneic DC-based vaccines may induce an even stronger vaccine-specific immune response than autologous DCs for two reasons: Since an estimated 1–10% of the circulating T cell repertoire is directed against allo-antigens, allogeneic DCs may trigger a broadly reactive T cell response providing $CD4^+$ T cell help for an optimal activation of cytotoxic T lymphocytes. The

expression of the autologous H2-K molecule allows the presentation of tumor antigens in the context of self-MHC, thereby priming a specific cytotoxic T lymphocyte response.

Various parameters like adequate activation and delivery route of the DCs influence the efficacy of a DC based vaccine. By varying dendritic cell to CT26 tumor lysate ratio, duration of activation or the supplementation with stimulatory molecules the conditions that lead to a maximal activation of the Kd^+DCs were determined. Using these conditions for the activation of Kd^+DC, we could observe similar or even more proliferation by cocultured BALB/c derived $CD4^+$ and $CD8^+$ T cells when compared to CpG and IFN-γ activated DCs. The production of IFN-γ but only basal levels of IL-17a upon coculture indicated that the T lymphocytes were probably polarized towards a Th1 phenotype. Due to lack of an adequate H-$2K^d$-restricted tetramer we were unfortunately not able to assay CT26 specific cytotoxic responses elicited by Kd^+DCs primed $CD8^+$ T cells.

Next, the impact of vaccination on tumor growth was assayed by transferring CT26 lysate pulsed Kd^+DC (semi-allogeneic) or wtDC (fully allogeneic) into tumor bearing mice. As expected, semi-allogeneic vaccination reproducibly and statistically significant delayed tumor growth and prolonged survival when compared to not vaccinated or full allogeneic DC vaccinated mice. Surprisingly the autologous vaccination of (BALB/c x C57BL/6) F1 mice with Kd^+DCs did significantly accelerate tumor growth. We expected the DC vaccination to predominately affect the frequency and possibly also the function of $CD8^+$ lymphocytes. However, Flow cytometric analysis of tumor infiltrating, draining Lymph node and spleen resident leukocytes did not allow to correlate the observed slowed tumor growth to a cellular mechanism causing this effect. Whereas in the tumor the frequency of total $CD3^+$ T cells did not significantly change only a minor reduction of the Treg to $CD8^+$ T cell ratio was measured upon transfer of KD^+ DCs into BALB/c animals. Kd^+DC vaccinated F1 mice exhibited a remarkably increased total $CD45^+$ leukocyte infiltration together with an slight increase in Tregs and $CD11b^+$ myeloid cells, which might at least partially explain the accelerated tumor growth. It should be mentioned that the all the organs were harvested at the same time point after tumor inoculation. The observed differences especially of tumor infiltrating cells might therefore rather be caused by the different size and developmental level of the tumor than really representing an effect of the DC vaccination. In addition, some of the variation might has been caused by practical difficulties during the isolation tumor infiltrating cells.

Although we were not able to reveal the cellular mechanisms involved, the observed delay in tumor growth confirmed the applicability of the “semi-allogeneic” DC vaccination approach. Due to its availability and the convenience of genetic modifications, the MuTu DC line a convenient

tool to further investigate how DC based tumor vaccines can be improved. Especially in regard to clinical application, the use of partially mismatched or semi-allogeneic DCs has some major advantages: It can be used as an “off the shelf” treatment, thereby bypassing the need for individually prepared vaccines. This represents a considerable logistic advantage and allows the preparation of large clinical-grade batches of a more standardized DC vaccine in terms of phenotype and maturation status. (Pre) clinical studies applying allogeneic DCs / autologous tumor hybrid cells or partially HLA-matched allogeneic DCs are currently being conducted [197].

11.1.2 Generation of DC lines expressing proinflammatory cytokines

As a second approach to increase the immunogenicity of DC based cancer vaccinations, we generated MuTu dendritic cell lines constitutively expressing the cytokines IL-2, IL-12 or IL-15. We hypothesized that the local delivery of these T cell stimulating cytokines, combined with the semi-allogeneic vaccination, might further promote tumor specific adaptive responses. Although we could clearly detect the expression of the respective transgene by the DCs, *in vitro* proliferation assays did not result in improved $CD4^+$ or $CD8^+$ T cell proliferation or function. Maybe the concentrations of the respective OVA peptides used in the experiments was already sufficient to induce maximal proliferation and activation of T cells covering a potential effect of transgene expression. In order to assay a possible beneficial effect of cytokine delivery, tumor bearing mice were vaccinated with tumor cell lysate pulsed Kd^+DCs expressing any of the cytokines. Unfortunately we could not observe a more potent response on tumor growth or survival of the animals.

The observed results are somewhat surprising, as the proinflammatory properties IL-12 and IL-15 are well documented. Several studies have shown regression of established tumors applying transfected dendritic cells (reviewed by [198]). We can only speculate why the combination of semi-allogeneic vaccination with the delivery of the cytokines did not lead to further delay of tumor growth. On reason might base on the finding that MuTu Dc injected into immunocompetent, autologous hosts has been shown to be rejected within 8 to 13 days [130]. In an allogenic context the DCs are probably rejected even quicker. Thus the timeframe the DCs have to migrate to the tumor draining lymph nodes and can provide interacting T cells with the cytokines may not be sufficient. It might have been better to test a potential beneficial effect first in an autologous vaccination setting. Another possibility is that the intravenous injection route might not allow an adequate delivery of the cytokines. The inflammatory stimulus provided by the cytokine might not be required in the lymphoid organs but rather in the tumor environment in order to support anti-tumor immunity. Indeed, the local, subcutaneous or intratumoral administration of IL-12 or IL-15 expressing DCs has been shown to delay or even prevent tumor development [199], [200]. Only the concomitant, systemic delivery of IL-2 has been shown to enhance the anti-tumor effects

of DC vaccination [201]. It might therefore be interesting to compare different delivery routes of the cytokine expressing DCs.

12 Conclusion & Outlook

In this study we demonstrated the MuTu dendritic cell line represent an excellent tool to investigate how dendritic cells modulate immune responses and how this regulation can be altered according to our requirements. In contrast to endogenous or bone marrow derived DCs, the MuTu cell lines represents a homogenous population that is easily genetically modifiable and is available in infinite amounts without the need for large quantities of mice. These features allow us to define and characterize optimal DC based treatment approaches before applying ex vivo derived dendritic cells.

The results presented here constitute a rather broad overview of how dendritic cells modified to express tolerogenic molecules can influence the outcome of inflammatory responses. The study therefore may be more descriptive than it explains the observations. The cellular and molecular mechanisms underlying several of the observed tolerogenic responses remain unidentified or have been only superficially analyzed. However, the findings raise many new questions. For example on the effector mechanisms of IL-35, on the failure of cytokine expressing DCs for cancer vaccination or the persistence of DC induced tolerance in vivo. Some of these open questions are mentioned in the discussion part of the thesis and should to be addressed experimentally.

12.1 Conclusions tolerogenic DCs

As summarized in table 4, we could demonstrate the tolerogenic potential for several of the investigated molecules. Our result indicate that different mechanisms, such as autocrine effects on the DCs itself, direct inhibition of T cell proliferation or the induction of different Treg populations, are involved in tolerance induction by the DC lines. This finding suggests that the combination of different tolerogenic effector molecules might evoke synergistic effects, multiplying the tolerogenic potential, as it was for example shown in the skin transplantation model.

Active TGF-β as well as IL-35 expressing MuTu DCs have shown to induce immunologic tolerance in both autoimmune encephalitis as well as skin transplantation models. Although the results obtained in this study can certainly not directly be adapted for the clinical applications, they demonstrate the feasibility and potential of the tolerogenic DCs for immunotherapeutic approaches for autoimmune disorders, organ transplantation or in cancerous disease.

Transgene		Effect on T cells	
	DC maturation state	in vitro	in vivo
CTLA-4	Lower CD80/86 in steady state and upon activation, less IL-12, IL-6	Generation of hyporesponsive T cells	induction of tumor tolerance
IL-10	Lower CD80/86 &CD40 upon activation, less IL-12, IL-6	reduced IFN-γ secretion by T cells	delays skin graft rejection with actTGFβ^{+}DCs
Latent TGFβ	No change of activation markers	induction of Tregs and prevention of CTL development	limits EAE, ?
Active TGFβ	No change of activation markers	induction of Tregs and prevention of CTL development	induction of tumor tolerance prevention of EAE development, delays skin graft rejection with IL-10^{+}DCs
IL-35	Lower MHC-II and costimulatory molecules, upregulation of CD11b,	generation of iTr35 cells, moderate inhibition of CTL responses	Seems to limit EAE , delays graft rejection, induces tolerance towards tumor growth, ongoing

Table 4 Overview on the DC lines expressing tolerogenic molecules created in our lab

12.2 Novel cellular targets for IL-35

The results we generated show that IL-35 does not only regulate inflammatory responses of B and T lymphocytes but it can furthermore affect myeloid as well as non-hematopoietic cells. We are, to our knowledge, the first to describe dendritic and stromal endothelial cells as IL-35 targets. Our work therefore adds new insights in the functions of this poorly investigated cytokine. It furthermore underlines the importance for the regulation of inflammatory responses and makes IL-35 a promising target for the therapeutic modulation of immunity. As depicted in Fig.33 we

propose an extended model for the function of IL-35 that takes into consideration our observations. As already mentioned, the functional consequences of IL-35

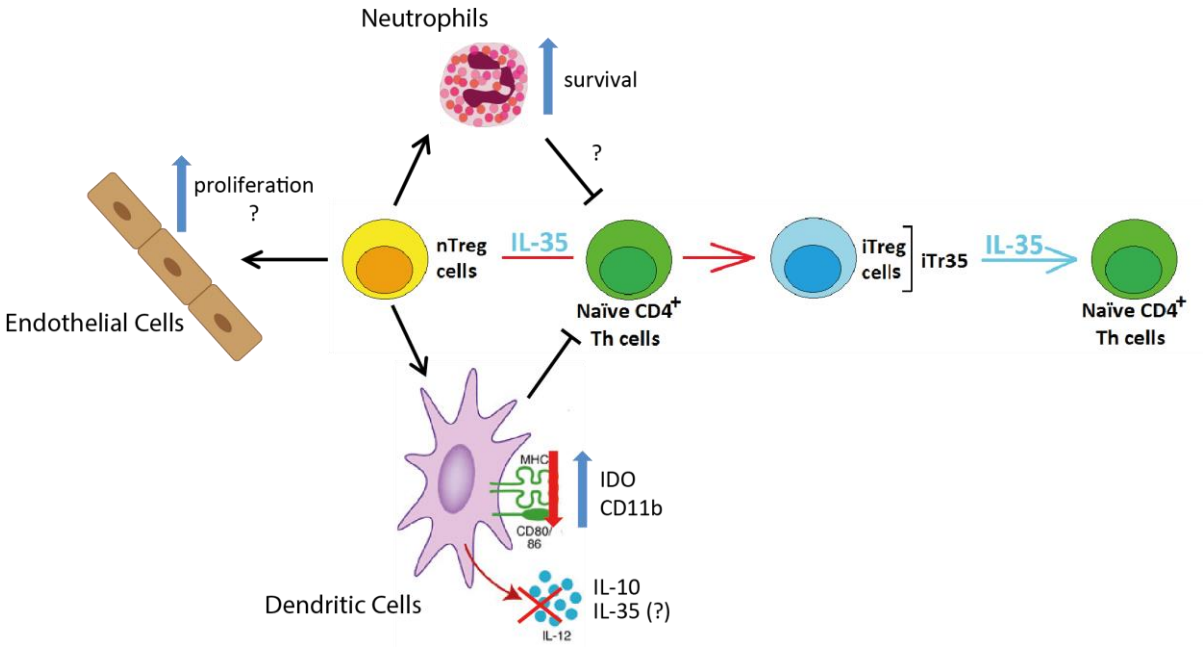


Figure 33 Indirect mediation of immune suppression and propagation of infectious tolerance by IL-35: In addition to the already described effects on T cells, our findings show that non-lymphoid cells can be targets of IL-35.

remain poorly understood and it would be very interesting to elucidate how IL-35 instructed endothelial or granulocytic cells exactly contribute to the regulation of inflammation and to what extend these mechanisms are relevant *in vivo*.

IV. Material & Methods

13 Materials

13.1 Chemicals

Chemical	Supplier	Comment
2-Mercaptoethanol 50mM	Gibco	No. 31350-010
Agarose	Eurogentec	500g, No.83018A
Ammonium Persulfate	BioRad	161-0700
Ampicillin (Amp)	Sigma	5g, No.A9518
Bovine Serum Albumin (BSA)		
Blotto, non-fat dry milk	Santa Cruz Biotech	No. sc-2325
Brefeldin A	Sigma	B7651-5MG
DNase & RNase free ddH_2O	Gibco	10977-035
eFluor 670 cell cell proliferation dye	eBioscience	65-0840
Ethanol 100% (V/V)	Fluka Analytical	02860-2.5L
Fetal calf serum (FCS)	Pan Biotech	P40-37100M
GelRed	Biotium	No.41003
HEPES 1M	Gibco	15630-080
Isopropanol	Fluka Analytical	59310-2.5L-F
Ionomycin	Calbiochem	407950
Methanol	Reactolab	99046
Mitomycin C	Sigma	No.M-0503
Percoll	GE Healthcare	No.17-0891-01
Phosphat Buffered Saline solution (PBS)	MagBil	
phorbol 12-myristate 13-acetate (PMA)	Sigma	P8139
Sodium Pyruvate	Gibco	11360-039
SYBR Safe DNA Stain	Life Technologies	S33102
TEMED	Sigma	T-8133
Thioglycollate Broth	Fluka Analytical	70157-100G
TransIT 293 transfection reagent	Mirus	MIR 2700
TRIzol	Ambion	15596026

Table 5 Chemicals

13.2 Buffers and Solutions

Buffer/Solution	Content/Application	Comment
0.25% Trypsin with EDTA		Gibco, 25200-056
ProtoGel Stacking Buffer	0.5M Tris HCl 0.4% SDS, pH6.8	National Diagnostics, EC-893
1.5M Tris HCl Buffer pH 8.8	PAGE separation buffer	BioRad 161-0798
ProtoGel	30% Acrylamide: 0.8% Bis-Acrylamide Stock solution	National Diagnostics EC-890
40% Acrylamide/Bis Solution, 19:1	For PAGE	BioRad, 161-0144
Dulbecco's Modified Eagles Medium(DMEM) + GlutaMAX		Gibco, 31966-021
ELISA Assay Diluent	10% FCS in PBS	
ELISA Coating buffer	1l dH_20, 8.4g $NaHCO_3$, 3.56g Na_2CO_3, pH 7.0	
ELISA wash buffer	1x PBS, 0.05% Tween20	
Erythrocyte lysis buffer	155mM NH_4Cl, 10mM $KHCO_3$,1mM EDTA at pH 7.3, sterile filtered	
FACS buffer	1x PBS, 2% FCS, 5mM EDTA	
Iscove's Modified Dulbecco's Medium (IMDM) + GlutaMAX		Gibco, Nr. 31980-022
LB powder		AppliChem
Lenee Buffer	20ml Glycerol, 25ml 0.5M Tris pH6.9, 0.02g Bromophenol Blue, 6g SDS, 5ml DTT	
PFA	4% Paraformaldehyde in PBS	
RIPA Buffer		SIGMA, R0278
Roswell Park Memorial Institute Medium 1640 (RPMI)		Gibco, 21875-034
Saponin Cell Permeabilization Buffer	PBS, 3% FCS, 0.5% Saponin	Filtered, stored at 4°C

SDS electrophoresis buffer	15,1mg/ml Tris Base, 72mg/ml Glycine, 5 mg/ml SDS in H2O)	
SOC medium	2% w/v tryptone (20g), 0.5% w/v bacto-yeast extract (5g), 10mM NaCl (0.584g),2.5mM KCl, (0.186g), 10mM MgCl2 (0.952g), 10mM $MgSO_4$, 20mM glucose	Invitrogen, No.15544-034
Spleen digestion buffer	RPMI1640+Glutamax, 10% 1M Hepes, 3% FCS, 1/500 Gentamycin, 1/1000 Collagenase D, 1/25 DNase A at1mg/ml	Always add collagenase and DNase fresh
Tris-acetate-EDTA (TAE) electrophoresis buffer	40mM Tris-acetate, 1mM EDTA	
TE buffer	10mMTris pH7.5, 1mM EDTA	
TENS solution	0.1M NaOH, 0.5% SDS in TE buffer	
WB Transfer Buffer	10% ethanol, 10% 10x Tris/Glycine (30mg/ml Tris, 44 mg/ml Glycine), 80% H2O	
WB Separating gel 10%	5ml 30% Acrylamide/0.8% Bis Acrylamid, 3.75ml TRIS HCL/SDS pH 8.8, 6.25ml dH_2O, 50µl 10%APS, 10 µl TEMED	Enough for two 8-pocket gels
WB Stacking Gel	0.65ml 30% Acrylamide/0.8% Bis Acrylamid, 1.25ml TRIS HCL/SDS pH 6.8, 3.05 ml dH_2O, 25µl 10%APS, 5µl TEMED	Enough for two 8-pocket gels

Table 6 Buffers and Solutions

13.3 Kits and Consumables

Kits/ Consumable	Supplier	Comment
100bp DNA ladder	Invitrogen	No.15628-019
1kb DNA ladder	Invitrogen	No.15615-016
2-Log DNA ladder	NEB	N3200
Cell Strainer 40µm	BD Falcon	No.352350
96 deep well plate	Nunc	No.278743
dNTP Mix	Promega	No.U1515
ECL Prime WB detection reagent	GE Healthcare	RPN2232
Foxp3 staining buffer set	eBioscience	No.00-5523-00
OptEIA Mouse Il-12(p40) ELISA	BD Bioscience	No. 555171
QIAquick PCR Purification Kit	Qiagen	No.28106
QIAprep Spin Maxprep kit	Qiagen	No. 27106
QIAprep Spin Miniprep kit	Qiagen	No. 12163
Wizard SV Gel and PCR Clean-up	Promega	A2982
Protein A/G PLUS Agarose	Santa Cruz	Sc-2003
Rapid DNA ligation Kit	Roche	No. 11 635 379 001
RNeasy RNA extraction Kit	Qiagen	
SYBR FAST qPCR Kit (optimized for use with Roche LightCycler 480)	Kapa Biosystems	KK4610

Table 7 Kits and consumables

13.4 Antibodies

For flow cytometry the antibodies against following molecules were used:

Antibody	Clone	Colors used	Manufacturer
CD11b	M1/70	APC, PE	eBioscience
CD11c	HL3	APC	Biolegend
CD45.1	30-F11	Pe	eBioscience
CD45.2	104	Fitc	eBioscience
CD4	RM4-5	APC, efluor450	eBioscience
CD8α	53-6.7	FITC, APC-Cy7, Pacific Blue	eBioscience
Foxp3	FJK-16a	Pe-Cy5	eBioscience
IFNγ	XMG1.2	Pe	eBioscience
MHC-I (H-2Kb)	AF6-88.5.5.3	APC	eBioscience
MHC-II	M5/114.15.2	PerCp	BioLegend

Pan NK cells (CD49b)	DX5	Fitc	eBioscience
DEC205	205yekta	PerCp-ef710	eBioscience
Clec9A	42D2	PE	eBioscience
GR-1	RB6-8C5	Pe	BioLegend
		PerCP-PeCy5.5	eBioscience
CD40	1C10	APC, Pe	eBioscience
CD80	16-10A1	Brillian Violet 421	BioLegend
CD86	GL1	PeCy7	BioLegend
Ly6C	HK1.4	APC	eBioscience
Ly6G	18A	PerCp-Cy5.5	BioLegend

Table 8 Antibodies for flowcytometric analysis

For immunohistochemistry the following antibodies were used:

Antibody	Clone	Colors used	Manufacturer
CD3			
Cleaved Caspase3			Cell signaling
KI67	B56, mouse IgG1κ		BD pharmigen
VEcadherin	180215, mouse IgG2b		R&D systems

Table 9 Antibodies used for immunohistochemistry

For Immunoprecipitation and Western blot the following antibodies were used:

Antibody	Clone	Manufacturer
Ebi3	V1.4C4.22, V1.4H6.29	Shenondoah Biotech
p35	45806	R&D systems

Table 10 Antibodies used for IP and western blot

13.5 Competent cells

***E.coli* Top10** (Invitrogen): F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ 139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

***E.coli* MAX Efficiency DH5α** (Life Tecchnologies, 18258-012): F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk^-, mk^+) *pho*A *sup*E44 λ$^-$ *thi*$^-$1 *gyr*A96 *rel*A1.

13.6 Enzymes

All restriction enzymes utilized were obtained from New England Biolabs (Ipswich MA, USA) and used according to manufacturer's instructions. All other enzymes are described with the methods they were used in.

Enzymes	Supplier	Comment
Antarctic phosphatase	NEB	M0289
DNase I	Roche	20mg/ml in RPMI Cat.No.104159
Collagenase D	Roche	11088866001
Platinum Taq DNA Polymerase High Fidelity	Invitrogen	
RNase A	Qiagen	Cat. No. 19101
Superscript reverse transcriptase II	Life Technologies	18064-014
T4 ligase	NEB	M202
T4 polymerase	NEB	M0203
Taq DNA polymerase	NEB	Cat.No.M0273

Table 11 Enzymes

13.7 Plasmids

Detailed maps of the vectors are listed in the appendix

Plasmid	Supplier	Comment
pWP-SIN-cPPT-WPRE)-CMV-IRES-GFP	Strategene	2nd generation lentiviral expression plasmid. ≈10350bp, nobody knows where we got it from
pRRLSIN.cPPT.PGK-GFP.WPRE	Trono Lab, EPFL Lausanne	2nd generation lentiviral expression plasmid. Addgene plasmid # 12252, 7384bp
psPAX2	Trono Lab, EPFL Lausanne	2nd generation lentiviral packaging plasmid. Addgene plasmid # 12260. 10703bp
pMD2g	Trono Lab, EPFL Lausanne	VSV-G envelope expressing plasmid. Addgene plasmid # 12259. 5824bp

Table 12 Plasmids

13.8 Software

Software	Supplier	Purpose
AxioVision	Zeiss	Image aqcuisition microscopy
Mendeley Desktop	Mendeley Ltd.	Paper Organization
FACSDiva	BD Biosciences	FACS acquisition /analysis
FlowJo X	Tree Star Inc.	Analysis Facs data
Illustrator CS6	Adobe	
Prism 6	GraphPad	Graphics and statistics
Office 2013	Microsoft	
Photoshop CS6	Adobe	
qBase	Biogazelle	Analysis rtPCR data
VectorNTI 10.3.1	Invitrogen	*In silico* cloning

Table 13 Software

14 Methods

14.1 Generation of transgenic DC lines

14.1.1 Cloning strategy

All primers were designed using the "Primer-BLAST" tool on the NCBI homepage (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?) and ordered from Microsynth, Switzerland.

Oligonucleotide	**Description**	**Sequence 5' -> 3'**
SalIEbi3fw	SalI kozak Ebi3	GTCCG GTCGACGCCACCATGTCCAAGCTGCTCTTC
linkerEbi3rev	ebi3, no stop codon! 15bp of linker	CGAGCCACCGCCTCCGGGCTTATGGGGTGC
linkerfw	26bp linker	GGAGGCGGTGGCTCGGGCGGTGGTGG
SalIp35rev	SalI, p35incl stop codon!	AGCTCGTCGACtcaGGCGGAGCTCAGATA
SalIIl12fw	IL-12Fc cloning	GTCCG GTCGACCTCGCAGCAAAGCAAGAT
FctagSalIrev		CGCTCGTCGACTGTTCTCATTTACCAGGG
BamHIIl2fw	IL-2 cloning	GTCCGGGATCCGCCACCATGTACAGCATGCAGCTC
SalIIl2rev		GGCTCGTCGACTTATTGAGGGCTTGTTGA
BamH1IL15Rafw	Il15ra forward from	TGATAGATCCGCCACCATGGCCTCGCCGCAGCT
Sal1IL15Rarev	reverse	AGCTCGTCGACTTAGGCTCCTGTGTCTTC
	rtPCR primers	
p28rtPCRfw		CATGGCATCACCTCTCTGAC
p28rtPCRrev		AAGGGCCGAAGTGTGGT
p35RTfw		AAATGAAGCTCTGCATCCTGC
p35RTrev		TCACCCTGTTGATGGTCACG
p40RTfw		GGAAGCACGGCAGCAGAATA

p40RTrev		AACTTGAGGGAGAAGTAGGAATGG
ebi3RTfw		ATG TCC AAG CTG CTC TTCCT
ebi3RTrev		AGA GGA GTC CAG GAG CAG TC
IL2RTfw		CCT GAG CAG GAT GGA GAA TTA CA
IL2RTrev		TCC AGA ACA TGC CGC AGA G
IL-15rtfw		AACTGGATAGATGTAAGATATGACC
IL-15rtrev		GGACGTGTTGATGAACATTT
gp130fw		CGTGGGAAAGGAGATGGTTGTG
gp130rev		AGGGTTGTCAGGAGGAAGGCTAAG
il12rb2fw		TGTGGGGTGGAGATCTCAGT
il12rb2rev		TCTCCTTCCTGGACACATGA
IDO1fw		ACT GTG TCC TGG CAA ACT GGA AG
IDO1rev		AAG CTG CGA TTT CCA CCA ATA GAG

Table 14 Primers used for cloning or rtPCR

Genes of interest were amplified by PCR from different templates using the primers indicated in table 14 and Hifi Taq polymerase. IL-12Fc and IL-35p35 were obtained from the expression vector BBV74, a courtesy of Burkhard Becher, University of Zürich. IL-2 cDNA was amplified from cDNA of activated T cells. IL-15 and IL35ebi3 were obtained from cDNA of activated dendritic cells. The cloned sequences were ligated into the lentiviral expression vector. Single E.coli colonies were picked and integration of the transgene was assayed in a colony screening assay. Plasmid DNA was purified using the Plasmid Mega Kit (Qiagen) and sequenced to verify the integrity of the cloned gene sequences. The amplification was carried out with 0.2mM dNTP (Roche), 1x Expand High Fidelity Buffer (Roche), 0.35 Units Platinum High Fidelity Polymerase, 0.5 mM specific primers and the template in a final reaction volume of 20 µl. After initial denaturation at 94°C for 90'', 35 cycles of the following program was done: 30'' at 94°C denaturation, 30'' annealing at the calculated Tm-5°C and extension at 68°C for 1' per kb. Products were finally amplified once at 68°C for 10'. PCR products were loaded in Sea Kem GTG agarose (Lonza) gels and revealed with SYBR Safe DNA gel stain (Lifetechnologies) with the use of an adequate DNA ladder as scale. The amplified DNA fragments were extracted from the agarose gel using the Wizard SV Gel and PCR Clean-up system according to the supplier's instructions.

The gene of interest was inserted into the BamHI/SalI cloning site of the lentiviral expression vector (pWP-SIN-cPPT-WPRE)-CMV-IRES-GFP. For this purpose, BamHI and SalI restrictions

sites were added to the 5' respectively 3' end of the PCR amplified gene of interest. TGF-β as well as IL-35 harbor internal BamH1 restriction sites. This problem was circumvented by adding SalI restriction sites at the 5' and 3' ends.

14.1.2 Ligation and transformation

Lentiviral expression vectors were linearized using the BamH1 and SalI or SalI only. In order to minimize plasmid self-ligation, the expression vectors were dephosphorylated using Antarctic phosphatase and the according buffer (NEB). Reaction was incubated for 15min at 37°C and enzyme was afterwards heat-inactivated for 10min at 65°C. Ligations were performed with 1µl T4 ligase (NEB) and 2µl of the according buffer. Insert and Vector DNA were added in 3:1 and 5:1 molar ratio and dH_2O was added to a total reaction volume of 20µl. Reaction was incubated for 30min at 16°C and enzyme was subsequently heat inactivated for 10min at 65°C. Competent *Escherichia coli* MAX Efficiency DH5α where thawed on ice. 10µl of the ligation mix was added to 50µl bacteria and incubated for 30min on ice. The bacteria were transfected by heat-shocking them for 40sec at 42°C. The cells were placed back on ice for 2min and 150µl SOC medium was subsequently added to each transfection. After incubating one hour at 37°C in the shaking incubator the bacterial suspension was plated on prewarmed agar plates containing the adequate antibiotic and put overnight into a 37°C incubator. On the following day, single colonies were transferred into reaction tubes containing 4ml LB medium and respective antibiotics and were grown over night in the shaking incubator at 37°C.

14.1.3 Isolation of plasmid DNA

Colonies were picked from an agar plate and transferred in to 96-deep well plate (DWP) filled with 200µl LB and adequate antibiotic. The DWP was sealed with punctured Parafilm and incubated at 37°C for 3h on a thermo shaker. Afterwards, 20µl from the cultures were transferred to a PCR plate and 90µl dH_2O was added to each well. Cells were lysed at 95°C for 10 minutes using a PCR-cycler and subsequently centrifuged at 3000g for 5 minutes. 5µl of the supernatant was then taken for PCR. Primers that allow amplification of an insert fragment were chosen and standard PCR was performed. For the detection of correctly ligated clones, presence of PCR amplicons was analyzed by agarose gel electrophoresis. Positive clones were sequenced to verify integrity of the gene of interest. In order to obtain plasmid DNA of high purity for further cloning use, colonies bearing correctly ligated plasmids was extracted using QIAprep Spin Maxiprep Kit and following the manufacturers instruction.

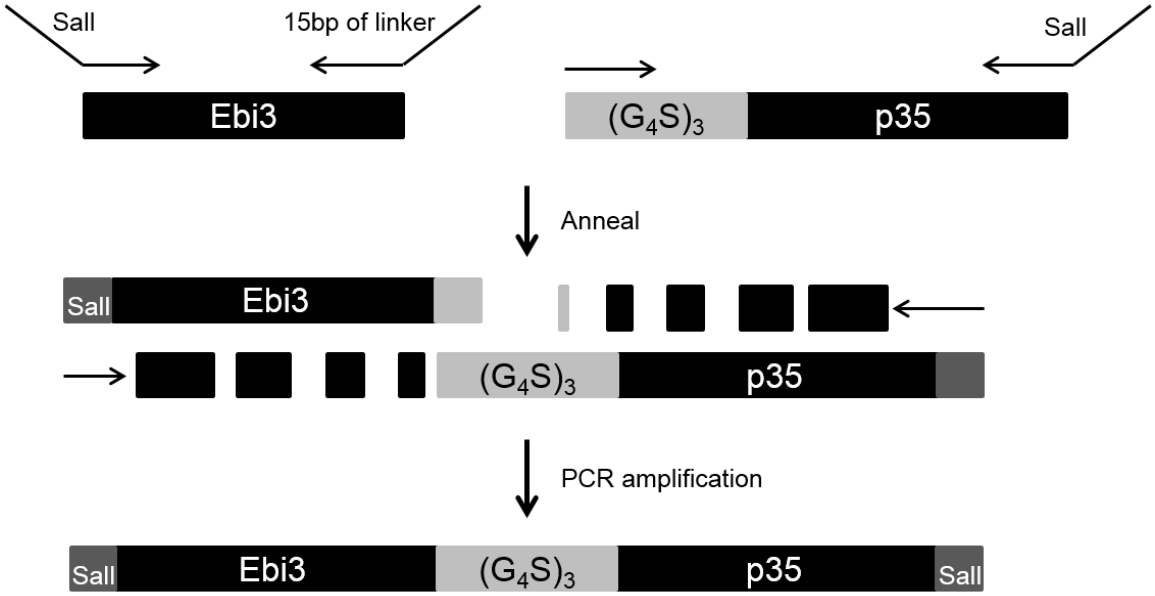


Figure 34 Schematic overview of the cloning of the IL-35 expression vector: Ebi3 and p35 subunits were amplified from T cell cDNA respectively a IL-12 expression plasmid and annealed as indicated. SalI sites were added in order to allow cloning into the lentiviral expression vector.

14.2 Production of Lentiviral Vectors

The second generation lentiviral transduction system applied here consists of 3 different vectors: The expression vectors pWP-SINcPPT-WPRE)-CMV-IRES-GFP or pRRLSIN.cPPT.PGK-GFP.WPRE containing the gene of interest. The envelope vector pMD2G and the packaging psPAX2 provide the genes required for the assembly of replication deficient lentiviral particles.

14.2.1 Transfection

Lentiviral particles were generated in 293T HEK cells. The HEK cells were transfected using the TransIT-293 transfection reagent, adapting the protocol provided by the manufacturer. Briefly, 293T cells were seeded on 10cm culture dishes and inubated until they were ~80% confluent. A TransIT293 reagent: DNA complex was prepared immediately before transfection by mixing 2.5µg of the respective plasmid DNA and 21µl of the transfection reagent in serum-free DMEM medium. The DNA:Transfection reagent complexes were added drop-wise to the cells and rocked gently to distribute the complexes evenly. The cells were incubated overnight and the medium was replaced with DC medium. After another 24h the lentivirus containing supernatant was

harvested and filtered using a 0.45µm PVDF filter (Millex). Supernatants were either directly used for transduction of DCs or aliquoted and stored at -80°C.

14.2.2 Transduction

100'000 MuTu DC line per well of a 6-well plate were plated an incubated overnight. On the following day the media was removed and 1mll of the 293T derived supernatant containing the viral particles was added onto the culture. The medium was replaced with normal DC medium after 24 hours and the efficiency of the transduction was checked by flow cytometry respectively rtPCR.

14.3 Quantification of gene and protein expression

14.3.1 RNA Isolation and Reverse Transcription

RNA isolation for rtPCR was generally performed using the RNeasy Kit (Qiagen) following the instructions of the manufacturer. For other applications, the cells were harvested and lysed directly on cell culture dish by addition of 500µl Trizol and repeated pipetting and 5' incubation at room temperature. Cell lysate was precipitated by addition of 100µl chloroform. Lysate was centrifuged 15' at $16x10^4$ rcf and 250µl of isopropyl alcohol was added to aqueous phase. Precipitate was pelleted by centrifugation for 10' at $16x10^4$ rcf and washed by addition of 500 µl 70% ethanol. Ethanol was completely removed after centrifugation and pellet resuspended in 25µl of RNA secure. RNA was incubated at 60°C for 10' and stored at -70°C. 2µl (200ng/µl) random nonamer primers were added to10µl of RNA and denatured for 5' at 70°C. Primers were allowed to align at 4°C for 10'.4µl of 5x RT buffer, 1µl of 10 µM dNTP, 1µl of 0.5µg/µl BSA, 2µl 100mM DTT and 0.5 RNASin. Mixture was pre-incubated for 10' at room temperature. 0.5µl Superscript reverse transcriptase was added and the reaction incubated for 50' at 42°C. The Enzyme was inactivated at 70°C for 5' and the remaining RNA digested with 1µl RNAse H. The Reaction was purified using a Qiaquick PCR purification kit following the manufacturer's instructions. The resulting purified cDNA was eluted in 30µl ddH_2O and cDNA quantity and purity assayed using a Nanodrop 1000 spectrophotometer (Thermo Scientific). cDNA was only used for rtPCR when ratio of $A_{260}/A_{280}>1.80$.

14.3.2 Semi-quantitative real-time PCR

The relative expression of different genes was assayed using the respective specific primers as depicted in table 14. For each reaction 2 µl of cDNA (2,5ng/µl) with 0.25µl of each primer (10µM) and 2,5µl SYBR Fast Green qPCR Master mix. Reactions were run in technical triplicates on a LightCycler480 rtPCR machine (Roche). A standard protocol was used for most of the analyzed genes: Initial denaturation for 5' at 95°C. 45 cycles of amplification: Denaturation for 10'' at 95°, primer annealing for 10'' at 60°C, expansion at 72°C for 10'' and signal acquisition at 78°C. Followed by acquisition of a melting curve starting at 65°C (60'') and increasing the temperature to 97°C at a rate of 0.11°C/s with 5 signal acquisitioons per second. Relative expression for each gene of interest was calculated using the qBase software and represented as expression normalized to β-actin.

14.3.3 Enzyme linked immunosorbent assay (ELISA)

To compare expression levels of the respective cytokines produced by the dendritc cells, standard enzyme linked immunosorbent assays were performed. $1.5x10^6$ cells were plated and cultivated in fresh medium as described above. Cell supernatant was taken at several time points and immediately frozen at -80°C until further processing. Samples were applied in biological duplicates. ELISA assay was then performed using the respective Kits, following the manufacturer's instructions. Data read out was performed with a microplate reader 560 (BioRad) at 450nm within 30 minutes after stopping of the color reaction.

14.3.4 Immunoprecipitation and western blot

IL-35 protein was first precipitated from cell lysate or IL-35 conditioned cell supernatant using the protein A/G plus beads following the manufacturer's protocol and 2µg anti-IL12p35 antibody per reaction.

Before loading on the SDS-PAGE, equivalent quantity of protein per sample was denatured by incubation in the laemmli buffer (60mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) for 10 minutes at 95°C. Samples were loaded onto a SDS-10% polyacrylamide gel and electrophoresis was run at 120 volts for about 1h in SDS electrophoresis buffer. Gel was blotted onto PVDF membrane (Amersham Pharmacia Biotech) by wet sandwich transfer in WB transfer buffer at 120 volts for 1h-1h30, further verified by Ponceau red (Fluka) staining of the membrane. Membrane was blocked in 1% BSA (Applichem), 5% Blotto (Santa Cruz Biotechnology), 0,1% Tween20-1x PBS (blocking solution) for 1h at RT then

incubated with primary antibodies diluted in the blocking solution for overnight at 4°C. After washing 3 x 10 minutes in 0.1% TBS-Tween20, membrane was incubated in HRP-conjugated secondary antibody for 2h at RT. After washing the membrane 6x for 5minutes, the chemiluminescent peroxidase substrate ECL was added and incubated for 5' in the dark at room temperature-Blotted proteins were visualized by exposure of X-ray film (Kodak) to the membrane.

14.4 Cell culture

All Cells were handled and prepared on a class 2 laminar flow bench under sterile conditions.

293T HEK, CT26, CMT38 and B16 cell lines: were kept in DMEM Glutamax with 10% FCS and 1% Penicillin/Streptomycin) at 37°C in a 5% CO_2 atmosphere. Medium was changed every third day. Before cells reached 90% confluency medium was aspirated and cells washed two times with PBS. Cells were then detached by addition of 0.25% Trypsin. After 3min incubation at 37°C cells were resuspended in 10ml medium and centrifuged for 5 minutes at 360rcf. Cells were split 1:20 into fresh medium. 293T cells were detached by pipetting the cells up and down and directly centrifuged.

Dendritic cell lines: Cells were kept in IMDM Glutamax with 10% FCS, 10mM HEPES, 50µM β-Mercaptoethanol, 50U/ml penicillin and 10µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. When Cells where split when reaching 100% confluency. DCs were harvested by incubation in cell dissociation buffer (10mM HEPES and 5mM EDTA in 1x PBS) and centrifuged for 5 minutes at 320rcf. Cells were re-seeded according to the table below (table XX).

	Surface [cm²]	**Volume [ml]**	**Number of DCs**
96-well	0.32	0.2	20'000
24 well	1.9	0.5	150'000
12 well	3.9	1	300'000
6 well	9.5	2.5	600'000
Culture flask T25	25	6	$1x10^6$
Culture flask T75	75	20	$4x10^6$
Culture flask T150	150	35	$1.2x10^7$

Table 15 Minimal number of MuTu dendritic cells seeded per indicated area

T and Lymph node cells were cultivated in RPMI1640-Glutamax medium supplemented with 10% FCS, 12.5 Hepes, 1mM sodium pyruvate, 0.1mM non-essential amino acids (NEAA), 5µM β-Mercaptoethanol, 50U/ml penicillin and 10µg/ml streptomycin

Granulocytes and Macrophages were cultured in DMEM medium supplemented with 10% FCS and 50U/ml penicillin and 10µg/ml streptomycin.

14.4.1 Cell counting

Cells were counted using an automatic cell counter (CASYcounter, Innovatis), following the manufacturer's instructions.

14.5 Flow cytometry

For surface staining of cells, the cells where washed once in FACS buffer and centrifuged for 10' at 300g. A maximum of $1x10^6$ cells were then resuspended in staining buffer composed of a 1:1 of 24G FC blocking antibody and FACS buffer containing the respective fluorochrome coupled antibodies. After 30' incubation at room temperature cells were washed and directly analyzed.

For intracllular staining of IFNγ, Foxp3 the lymphocytes were restimulated with 50ng/mL phorbol 12-myristate 13-acetate (PMA) and 1µg/mL ionomycin in the presence of 10µg/mL brefeldin a for 4 hours at 37°C, 5%CO_2 prior to surface staining. Cells were then fixed in 4% paraformaldehyde for 30' (or overnight in1% PFA) and permeabilized with saponin buffer for 30'. Cells were washed and stained with the adequate antibodies in a 1:1 mix of 24G and saponin buffer for 30'. Foxp3 was stained using the Foxp3 / transcription factor staining set (eBiosciences) following the manufacturer's instructions.

Data was acquired on LSRII flow cytometers (BD Biosciences) and analyzed with FlowJo X software (Treestar).

14.6 In vitro experiments

14.6.1 Allogeneic Mixed Leukocyte Reaction (MLR)

$1x10^4$, $2x10^4$ or $5x10^4$ wild-type or Il-35DCs were plated on a U-bottom 96 well plate and activated with pI:C, CpG and IFNγ for 4 hours. $CD4^+$ or $CD8^+$ T cells were isolated from wild-type BALB/c lymph nodes and magnetically sorted using the respective MACS kit. Purified T cells were stained with ef670 proliferation dye according to manufacturer's instructions and $1x10^5$ cells per well were added to DCs. After 3 days of incubation T cells were harvested and proliferation and functional phenotype were analyzed by rtPCR and flow cytometry. For depletion of 10µg/ml IL-35 cytokine, anti-p35 (clone 45806, R&D Systems) or anti-ebi3 (clone V1.aC4.22, Shenandoah Biotechnology) were added to the cells at the beginning of the coculture.

14.6.2 Antigen specific OT-I/II proliferation assay

To assess antigen-specific T cell presentation, untransduced or transduced DC were seeded at $5x10^4$ DC per well in a 96-well round-bottom plate one day before. DC were pulsed for 3 hours with the respective specific OVA-peptides: The MHC-II-restricted OVA(323-339) peptide (ISQAVHAAHAEINEAGR) or the MHC-I-restricted OVA(257-264) peptide (SIINFEKL). For MHC-II presentation, naive $CD4^+$ $CD62L^+$T cells were magnetically isolated from lymph nodes of OT-II mice (MACS, Miltenyi). Similarly, for MHCI presentation, $CD8^+$ T cells were magnetically sorted (MACS, Miltenyi) from lymph nodes of OT-I $Rag2^{-/-}$ mice. The T cells were labeled with 5 µM of eFluor670 proliferation dye according to manufacturer's instructions. $1x10^5$ T cells were cocultured with the DCs after washing away the peptide in the case of OT-I pulsed DCs.

4 days later, T cells were nonspecifically restimulated with 10ng/ml of phorbol 12-myristate 13-acetate (PMA), 500 ng/ml of ionomycin and 10 µg/ml of brefeldin A for 4h at 37°. Activation and proliferation was measured by flow cytometry. To analyze cytokine secretion half of the harvested T cells was lysed in Trizol. RNA was extracted by phenol extraction and cDNA synthesized using superscript reverse transcriptase following the protocol provided by the manufacturer. Relative Il12p40, Il12p35, ebi3, Ifng and Il12Rb2 expression was assayed by rtPCR.

14.6.3 iTreg suppression assay

The suppression assay Based on the protocols published by Collison et al. [202]. Shortly, $1x10^5$ IL-35^+DC or wild type DC were plated per well of a24 well plate and incubated overnight. Dendritic cells were activated with CpG, polyI:C, (all 1:1000) for 6 h. In the meantime $CD4^+$ T cells were magnetically isolated from C57BL/6 mice using the naïve $CD4^+$ T cell isolation kit (Miltenyi) and plated at a 10:1 (1x10e6 cells) ratio to wild-type or IL-35^+DCs. 2ng/ml recombinant TGF-β and 10ng/ml IL-2 were added to some of the wells containing wtDCs for generation of $Foxp3^+$ Tregs. Cells were incubate for 4 days. Different amounts of the generated suppressor T cells were seeded with $2.5x10^4$ ef670 proliferation dye stained naïve target T cells. Proliferation of the target cells was assayed by ef670 dilution after 3 days. Percent suppression was calculated using the following formula: $\frac{\%\ proliferated\ Tconv\ alone - \%\ proliferated\ Tconv\ treated\ with\ Treg}{\%proliferated\ T\ conv\ alone} \times 100.$

14.6.4 IL-35^+DC effect on myeloid cells

Spleen were harvested and splenocytes were isolated by mechanical disruption followed by enzymatic digestion in spleen digestion buffer for 20' at 37°C. Collagenase was blocked by adding complete medium. Cellular debris was removed by passing the cell suspension through a 40µm cell strainer. $CD3^+$ cells were magnetically depleted (MACS, Miltenyi) and 1x105 cell were seeded per well of a 96 well plate. The respective conditioned supernatant was added at a final ratio of ¼ to culture medium. 18 hours later the medium was replaced and some of the cells were stimulated for 3 hours with 10ng/ml PMA. Cells were afterwards harvested and analyzed by flow cytometry.

14.6.5 Endothelial cell scratch assay

$1x10^5$ HUVEC endothelial cells were seeded and cultivated for two days on coated cover slips covered with 0.2% gelatin in 24-well plate. A "scratch" was inflicted by scraping the endothelial cell layer using a 200µl pipette tip. Detached cells were washed away with PBA and fresh 1% FBS basal medium was added. The cells were cultured in the presence of either IL-35 conditioned or control medium or $2.5x10^4$ of the respective DC lines seeded on the upper chamber of a transwell insert. The two chambers of the transwell were separated by a membrane with 4µm pore size preventing the transmigration of DCs. After 12, 24 and 36 hours, the HUVEC cells were gently washed in PBS and fixed in 4% PFA for 10 minutes at room temperature. Cells were permeabilized with 0.1% Triton in PBS and stained for KI67, VEcadherin and caspase3.

14.7 In vivo experiments

14.7.1 Mice handling

All experiments were performed in accordance with Swiss federal legislation and had been approved by the Cantonal Veterinary Office. C57BL/6 mice were purchased from Harlan Laboratories. BALB/c mice were purchased from Charles River. The CD11c:SV40lgT, OT-I CD45.1, OT-II CD45.1 and *RAG1*$^{-/-}$ deficient animals were bred and kept in house in a specific pathogen free (SPF) facility under standardized light, temperature and humidity conditions. Maximal 6 adult animals were held together in green line individual ventilated cages (IVC) (Tecniplast). Males were kept together only when littermates. At least 3 age-matched, female animals per experimental group were used for each experiment.

14.7.2 Isolation of spleen, lymph node and tumor cells

Mice were euthanized by cervical translocation. Organs were harvested and shredded using a surgical blade. An adequate amount of lysis buffer was added and incubated for 20' at 25°C for spleen and lymph nodes, or at 37°C for 25' respectively. Samples were passed through a 40µm cell strainer and washed in lysis buffer. For some experiments, erythrocytes were removed by incubation in RBC lysis buffer for 3' at room temperature.

14.7.3 Isolation of peritoneal exudate cells

Mice received an intraperitoneal injection of 1ml 5% thioglycollate using a 21G needle. For neutrophil isolation, the overnight. For macrophage isolation the inflammatory response was allowed to develop for 4 days. Mice were carefully sacrificed. 10 ml sterile PBS was injected through the peritoneal wall using a 21G needle. The peritoneal fluid was harvested after gently massaging the abdomen of the mice. Cells were centrifuged at 200g for 10' and resuspended in neutrophil/macrophage medium.

14.7.4 In vivo antigen-restricted OT-I/II T cell proliferation

The respective DC line was pulsed for 3 hours with MHC-II restricted with either the MHC-II-restricted $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR) or the MHC-I-restricted $OVA_{257-264}$ peptide (SIINFEKL). For MHC-II presentation, naive $CD4^+$ $CD62L^+$T cells were magnetically isolated from lymph nodes of OT-II mice (MACS, Miltenyi). Similarly, for MHCI presentation, $CD8^+$ T cells were

magnetically sorted (MACS, Miltenyi) from lymph nodes of OT-I Rag2$^{-/-}$ mice. The T cells were labeled with 5 µM of eFluor670 proliferation dye according to manufacturer's instructions. 2x10^5 and 2.5x10^6 DCs were mixed and injected intravenously. One day later, the mice received another 2.5x10^6 DC pulsed with the respective OVA peptide. The mice were sacrificed 5 days after the first injection and the spleen and inguinal and brachial lymph nodes were harvested. Single cell suspension of lymphocytes was isolated by mechanical disruption of the organs and passing them through a 40µ cell strainer (Falcon). Proliferation and IFNγ expression of the isolated T cells was analyzed by flow cytometry upon in vitro restimulation with 10ng/ml PMA 500ng/ml ionomycin and 10µg/ml brefeldin A for 4 hours at 37°C.

14.7.5 Induction of adaptive transfer Experimental Autoimmune Encephalitis (EAE)

Adaptive transfer EAE was induced as described by[155]. Shortly, Myelin oligodendrocyte glycoprotein (MOG) reactive T cells were primed in donor C57B/6 mice by two sub-cutaneous injections of MOG_{35-55}-peptide emulsified in complete Freud's Adjuvant (Sigma). Intra peritoneal injection of 200ng Pertussis Toxin (Sigma) was used to boost lymphocyte priming. In order to study the effect of tolerogenic molecules, MOG_{35-55} peptide-pulsed control or transduced DCs were intravenously injected one day before and one day after the immunization. 12 days post immunization, inguinal, brachial and axillary lymph nodes were isolated and by digestion in 1mg/ml Collagenase D (Roche) for 20min at 37°C. Single cells suspension was prepared using a 40µm cell strainer (Falcon). LNC were expanded in vitro for 3 days in the presence of MOG_{35-55} pulsed DCs and 0.5ng/ml recombinant IL-12 (eBiosciences). T cells were then transferred to C57B/6 recipient mice by i.p. injection. Mice were boosted by intra venous injection with MOG_{35-55} pulsed DCs one day after LNC cell transfer. Mice were controlled daily and clinical EAE symptoms were scored: 0 – no obvious changes in motor functions; 1 – decreased tail tonus; 2- abnormal gait (ataxia and/or impaired righting reflex; 3 – partial hind limb paralysis; 4 – complete hind limb paralysis; 5 – complete hind and fore limb paralysis; 6 – moribund or dead.

14.7.6 Induction of tolerance towards tumor growth

B16.F0 melanoma and CMT93 colon carcinoma tumor cell lines were cultured in DMEM+GlutaMAX (GIBCO) medium, supplemented with 8% FCS and 50U/ml of penicillin and 50mg/ml streptomycin (GIBCO). Tumor cell lysate was generated by 5 consecutive freeze/thaw cycles in liquid nitrogen. Lysate was centrifuged at1500g for 5min before use. Mock or IL-35 transduced DCs were pulsed with respective lysate in a 1:4 lysate to DC ratio and 100U/ml recombinant IFNγ (eBioscience). 5 and 3 days before and 3 days after tumor cell transfer, 3x10^6

dendritic cells were subcutaneously injected in the flank. Tumor was induced by s.c. injection of $2x10^5$ B16.F0 or $2x10^6$ CMT93 cells into the same flank. Tumor growth was followed by measuring length and width using a caliper. Tumor volume was measured using the formula $V = \frac{\pi}{6} \cdot 1.58 \cdot (length \cdot width)^{\frac{3}{2}}$ as described by [203].

14.7.7 Skin graft experiments

$2.5x10^6$ of the respective DC lines were intravenously injected into the recipient mice 3 days before and 1 day after skin transplantation. Skin grafting was performed by as described by Golshayan et al. [158]: A longitudinal incision was made on the anterior neck of anaesthetized mice and thymus removed by application of negative pressure through an inserted glass pipette. Full-thickness tail skin was grafted on the flank of recipients. Graft sites were protected under sterile gauze and plaster until day 10, observed daily afterwards and considered rejected when no viable skin remained. Graft survivals between groups were compared using the log-Rank test. No additional immunosuppression was administered.

14.7.8 Tumor experiments

DC were incubated with freeze-thawed tumor lysates at a ratio of 2 tumor cell equivalents to 1 DC (i.e., 2:1) in complete medium. After an 18-hour incubation, the DCs were harvested, washed twice in sterile PBS and resuspended in PBS for further use.

Starting one day after tumor inoculation mice were scored and monitored twice a week and after each vaccination as followed:

Score	Symptoms
0	no visible impairment, normal gait and behavior including explorative behavior in new environment (grid), no steps missed on the grid, no visible or palpable tumor
1	no visible impairment, normal gait and behavior including explorative behavior in new environment (grid), tumor visible, tumor volume ≤ 500mm^3
2	reduced activity, less motile then healthy animals, reduced explorative behavior, signs of discomfort (hunched back position, lack of grooming), tumor volume < 2000mm^3, signs of ulceration (reddening, tendering) of skin on tumor
3	apathia, reduced activity, signs of stress and discomfort, (central) necrosis of tumor, tumor volume ≥ 2000 mm^3

Table 16 Scoring sheet for Tumor experiments

Behavior and condition were observed in the cage without manipulation and on the grid of a cage. Tumor length and width were measured using a caliper. Tumor volume was calculated using the formula Volume $=\frac{\pi}{6} \times 1.58(l \times w)^{\frac{3}{2}}$. Mice were given a clinical score according to the table above. Two weeks after tumor inoculation or as soon as an animal reached a score of 2, the health was checked daily. Mice were sacrificed when they reached a maximal score of 3.

14.8 Statistical Analysis

Unless otherwise stated, statistical analysis was performed applying unpaired, two-sided Student's T – test using Microsoft Excel or Graphpad Prism. Differences were considered significant when * $p<0.05$, ** $p\leq0.005$, *** $p\leq0.001$

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16 Abbreviations

APC	Antigen presenting cells
BCR	B Cell Receptor
BMDC	Bone Marrow Derived DC
CFA	Complete Freud's Adjuvant
CD	Cluster of differentiation
CHO	Chinese hamster ovary
CNS	Central nervous system
CpG	CpG oligodeoxynucleotides
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EAE	Experimental Autoimmue Encephalitis
FACS	Fluorescence-activated cell sorting
GM-CSF	Granulocyte/macrophage colony stimulating factor
i.p.	Intraperitoneal
i.v.	Intravenous
ICS	Intra cellular staining
IDO	Indolamin-2,3-Dioxygenase
IFN-γ	Interferon γ
IL	Interleukin
int	intermediate
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MDSC	Myeloid derived suppressor cell
MLR	Mixed Lymphocyte Reaction
MS	Multiple Sclerosis
NK	Natural Killer Cell
PAMP	Pattern Associated Molecular Pattern
p.i.	Post injection
pI:C	Polyinosinic:polycytidylic acid
pfu	Plaque forming unit
PRR	Pattern Recognition Receptor
RAG	Recombination-activating gene
s.c.	subcutaneous
TAA	Tumor associated antigen
TAM	Tumor associated macrophages
TCR	T cell receptor
TGF-β	Transforming growth factor β
Tk	Thymidine kinase
TLR	Toll-like receptor
Treg	Regulatory T cells

17 Appendix

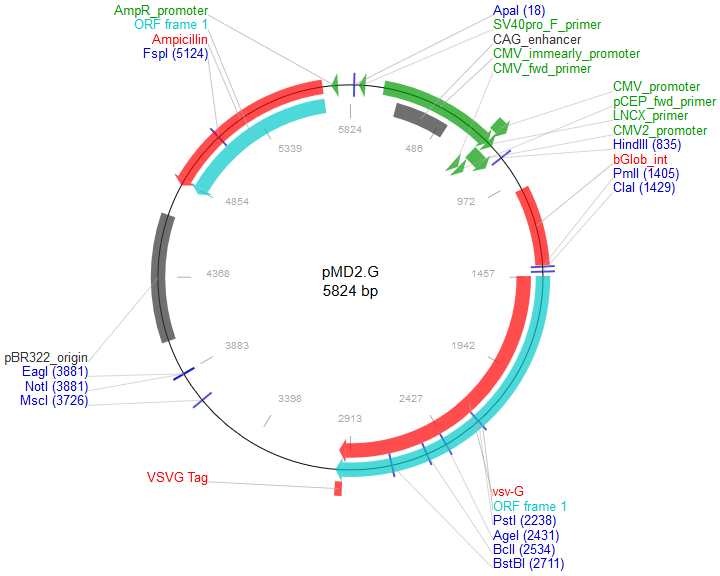

Figure 35 pMD2g VSV-G envelope expression vector

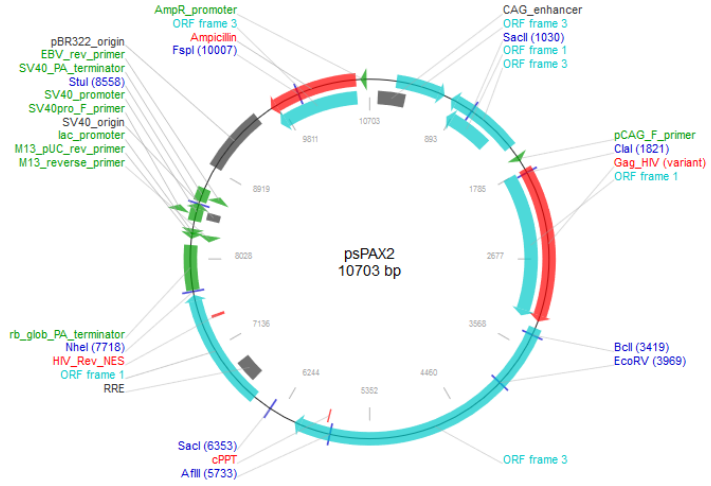

Figure 36 psPAX2 lentiviral packaging plasmid

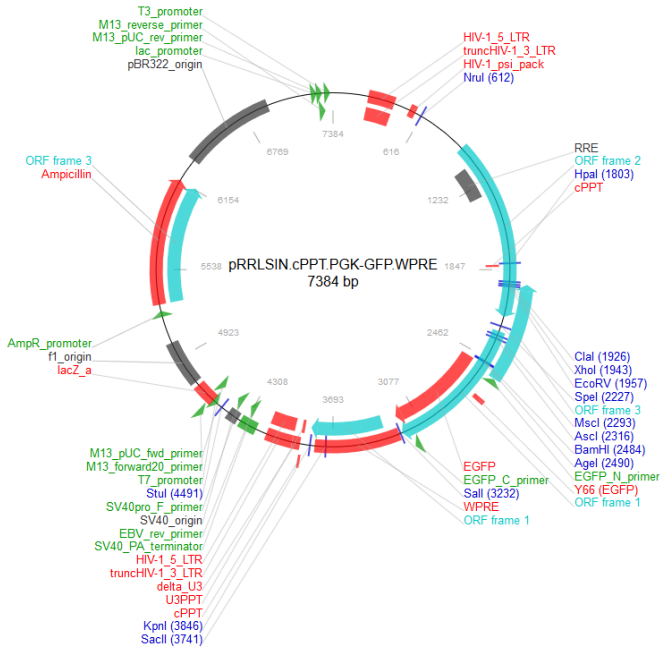

Figure 37 pRRLSIN.cPPT.PGK-GFP.WPRE lentiviral expression vector

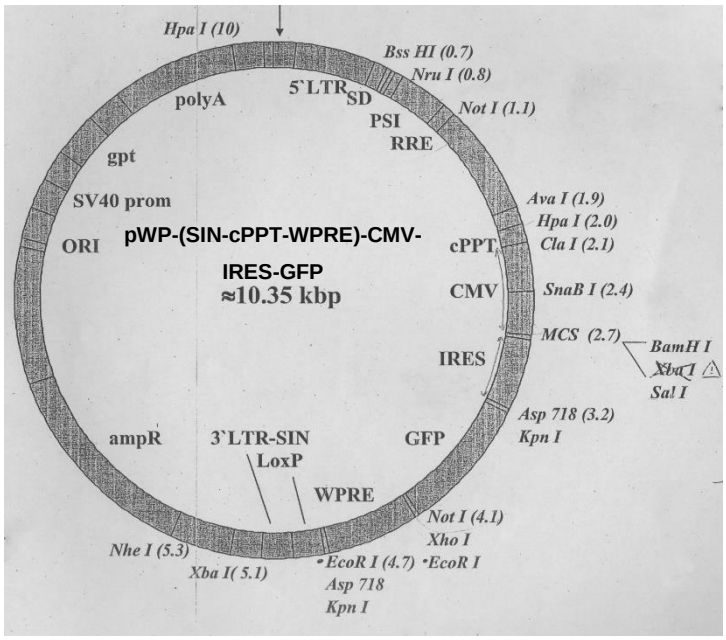

Figure 38 pWP-(SIN-cPPT-WPRE)-CMV-IRES-GFP lentiviral expression vector

18 Acknowledgements

So, it is finally done. The work of 4.5 years is brought on paper. During this time a lot of people contributed in some way or the other to the projects. For that I would like to express my gratitude. Special thanks go to:

First of all Hans Acha-Orbea, for giving me the opportunity to work, study and learn in your lab. From the start you gave me the freedom to initiate and pursue the projects I was interested in. You let me acquire the craft of a scientist in an independent, explorative manner but also supported me with ideas from your enormous knowledge whenever it was needed.

The members of the thesis committee for spending time, for the discussions and the valuable input you provided.

Anaïs, who started the project on the tolerogenic DC lines. Without the solid foundation you laid it wouldn't have been possible to obtain all this interesting and promising data.

Christoph, Devika, Matteo and Mathias for your friendship, the stimulating conversations on the balcony, your practical support during experimentation, for correcting my poor writing, and a zillion other things you helped me with.

Vanessa, Christine and Muriel for your assistance and help performing experiments. It is valuable to hand over experiments to you and to know it will be well done and the results can be trusted.

All the other actual and former members of the Acha lab for your help and for making the lab a place I really like to go every day.

All the people in the institute and at the different facilities. Especially Francoise you always has good council on basically everything a PhD student needs to know. Rosa and Maria for taking care of the mice. Danny and Steffi for your precious help and advice with everything flow cytometry related.

All my friends in Lausanne who made the last 4.5 years an amazing time! Be it in the bars, on the slopes, at the lake or at somebodies dinner table.

A special thank goes to all the mice that spent and let their lives at the institute in the course of the thesis. Without their involuntary sacrifices this thesis could not have been made. I really hope the findings made at their expense will one day justify their sufferings.

Svende, you help me to find the balance, give me strength whenever needed and keep me on the ground while letting me dream.

Last but not least my parents. Without your support and engagement I would have never been able to reach this point. You made this possible, for which I am infinitively grateful.