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Abstract

Interleukin-35 (IL-35) is a novel member of the IL-12 family and consists of the subunits Epstein Barr virus induced gene 3 (EBI3) and p35, which it shares with IL-27 and IL-12, respectively. The classical IL-12 family members (IL-12, IL-23, IL-27) are produced by antigen presenting cells, in particular dendritic cells (DCs), and regulate T cell function. Here we demonstrate that human IL-35, produced by IL-35^{+} regulatory T cells, is an inhibitory factor for DCs but not T cells. Induction of proliferation of T cells with allogeneic DCs was reduced when we added graded amounts of recombinant IL-35. This effect could be observed with immature DCs as well as with LPS-stimulated DCs. In contrast, IL-35 did not diminish the proliferation of purified peripheral blood T cells as well as naïve T cells isolated from cord blood upon activation with anti-CD3, CD3/CD28 or CD3/CD63 mAbs. We further demonstrate an inhibitory effect of IL-35 on the proliferation of the epithelial HeLa Ohio cell line, but not on other cell lines. Moreover, pretreatment of DCs with IL-35 inhibited their T cell stimulatory capacity as well. The cytokine profile of these cells activated with LPS illustrates a diminished IL-10 and IL-12p40 release and a slight increase in IL-6, IL-1β and TNFα secretion in comparison to untreated DCs. The surface marker profile of DCs remains unaffected after IL-35 treatment. We exhibit that an IL-35 fusionprotein binds to DCs and HeLa Ohio cells but not to peripheral T cells and all other tested cell lines. The expression of the subunits of IL-35 (EBI3 and p35) is up-regulated in T cells stimulated with human rhinovirus treated DCs (R-DCs) and in T cells stimulated with DCs which were activated with IFNα. The CD2 expression level on T cells co-cultured with R-DCs is lower than on T cells cultivated with DCs. We found out that about 1.6% of total T cells in the peripheral blood are CD2$^{-/low}$. Thus, IL-35 differs from the other members of the IL-12 family in cause of acting on DCs and not on T cells.

Zusammenfassung

Interleukin-35 (IL-35) ist ein neues Mitglied der IL-12 Familie und besteht aus den Untereinheiten Epstein Barr virus induced gene 3 (EBI3) und p35. EBI3 ist außerdem ein Bestandteil von IL-27 und p35 fungiert in dem Zytokin IL-12 als Untereinheit. Die klassischen Mitglieder der IL-12 Familie (IL-12, IL-23, IL-27) werden von Antigen präsentierenden Zellen, wie zum Beispiel dendritische Zellen (DCs), produziert und regulieren T Zell Funktionen. Hier identifizieren wir das von IL-35^{+} regulatorischen T Zellen produzierte humane IL-35 als inhibitorischen Faktor für DCs jedoch nicht für T Zellen. Die Induktion der T Zell – Proliferation eingeleitet durch allogene DCs war reduziert als verschiedene Mengen des rekombinanten IL-35 hinzugefügt wurden. Dieser Effekt wurde mit unreifen DCs genauso wie mit LPS – stimulierten DCs erreicht. Nach einer Aktivierung von T-Zellen mittels Antikörpern gegen CD3, CD3/CD28 oder CD3/CD63 konnte die Proliferation von gereinigten peripheren Blut-T Zellen sowie von naiven T Zellen des Nabelschnurbluts durch IL-35 nicht gehemmt werden. Weiters zeigen wir einen hemmenden Effekt von IL-35 auf die Proliferation der HeLa Ohio Zelllinie, epitheliale Zellen aus einem humanen zervikalen Karzinom. Eine vorangehende Behandlung von DCs mit IL-35 (IL-35-DCs genannt) führte auch zu einer inhibierten Kapazität T Zellen zu stimulieren. Das Zytokin-Profil jener IL-35-DCs, die mit LPS aktiviert wurden, zeigt eine verringerte IL-10 und IL-12p40 Produktion und einen leichten Anstieg in der IL-6, IL-1β und der TNFα Sekretion im Vergleich zu unbehandelten DCs. Das Oberflächen-Marker-Profil der DCs weist keine Veränderung nach der IL-35 Behandlung auf. Wir konnten außerdem feststellen, dass ein IL-35 Fusionsprotein an DCs und HeLa Ohio Zellen bindet jedoch nicht an periphere T Zellen und alle anderen getesteten Zelllinien. IL-35 wird von regulatorischen T Zellen, die mit humanen Rhinovirus aktivierten DCs ko-kultiviert wurden, produziert. Hier demonstrieren wir eine Hochregulierung der beiden IL-35 Untereinheiten, EBI3 und p35, in T Zellen nach einer R-DCs Stimulierung. Auch T Zellen, die mit IFNα aktivierten DCs stimuliert wurden, zeigen eine Steigerung der EBI3 und

p35 Expression. Wir konnten eine Verringerung der CD2 Expression auf jenen T Zellen feststellen, die mit R-DCs kultiviert wurden. Es wird eine $CD2^{low/-}/CD3^{+}$ T Zell Population in mononukleären Zellen von frischem Blut gezeigt, die zu circa 1,6% der T Zellen in verschiedenen Spendern vorkommt. Zusammenfassend unterscheidet sich IL-35 von den anderen Mitgliedern der IL-12 Familie, weil es auf DCs aber nicht auf T Zellen wirkt.

Abbreviations

APC	antigen presenting cell
ATCC	American type culture collection
BSA	bovine serum albumin
CCR	chemokine receptor
CD	cluster of differentiation
cpm	counts per minute
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DMSO	Dimethylsulfoxide
EBI3	Epstein Barr virus induced gene 3
ER	endoplasmic reticulum
FASL	FAS ligand
FITC	fluorescin-5-isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
HRV	human rhinovirus
IFN	interferon
Ig	immunoglobulin
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
iNKT	invariant natural killer T cell
JAK-STAT	Janus activated kinase–signal transducer and activator of transcription
kDa	kilo Dalton
LC	Langerhans cell
LPS	lipopolysaccharide

mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MFI	mean fluorescence intensity
MHC	major histocompatbility complex
MLR	mixed leucocyte reaction
MV	mean value
MNC	mononuclear cells
moDCs	monocyte derived dendritic cells
MyD88	myeloid differentiation factor 88
NK	natural killer cells
OG	oregon green
PBMNC	peripheral blood mononuclear cells
PE	phycoerythin
PBS	phosphate buffered saline
R-DC	human rhinovirus activated dendritic cells
SD	standard deviation
TAP	transporters associated with antigen processing
TCID	tissue culture infectious dosis
TCR	T cell receptor
TGF	transforming growth factor
TH	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
T regs	regulatory T cells
ZYM	Zymosan

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1 INTRODUCTION

1.1 Cytokines

The name cytokine derived from Greek and means “to set cells in motion“. Cytokines are 8 to 30 kDa intracellular signalling peptides acting either in an autocrine, paracrine or endocrine way (1). They are secreted by cells of the innate and adaptive immune system in response to diverse antigens or as response to former distributed cytokines. The cytokines bind to receptors on target cells which activate intracellular signalling pathways (2).

Cytokines are divided into two related groups, the type I and the type II hematopoietic cytokine/receptor families, and an IL-1/Toll-like family group (3). The type I cytokine family consists of 34 human cytokine receptor chains and 27 ligands. These cytokines, mostly interleukins (IL), function as immunomodulators and share several structural motifs (3,4). The receptors of this family share an extracellular fibronectin-like domain with 4 cysteines in the membrane-distal region and a membrane-proximal WSXWS motif (5). Most of these cytokines are monomers with a conserved 4-fold α-helical core structure (4,6).

The type II cytokine family differs from the type I cytokine family in 4 cysteine residues in the membrane-proximal region and lacks the WSXWS motif in the receptors (7,8). Distinct subunits of the receptors can heterodimerize and form complexes (8). This family includes interferons and some related cytokines such as IL-10 and IL-19 (9).

The third group of cytokines, the IL-1/Toll-like family includes only two interleukins, IL-1 and IL-18, and the Toll-like receptors (TLRs) (10).

1.2 Interleukin-12 (IL-12) family

The IL-12 cytokine family, which is also linked to the IL-6 cytokine superfamily, consists of IL-12, IL-23 and IL-27 and the recently described IL-35 (11). The members are heterodimeric cytokines that share ligand and receptor subunits, and are important actors in innate and adaptive immunity (12). IL-12 was first described in 1989 (13) as the first type I cytokine having a heterodimeric structure. They are composed of an α subunit with helical structure and a β subunit. IL-12 family members often share one of the subunits (3). All of these cytokines exhibit a response to microbial and host immune stimuli and have immunomodulatory functions on T cells promoting their expansion and differentiation and inducing interferon-γ (IFNγ) production (14,15). Signal transduction occurs via Janus activated kinase–signal transducer and activator of transcription (JAK-STAT) pathways (14).

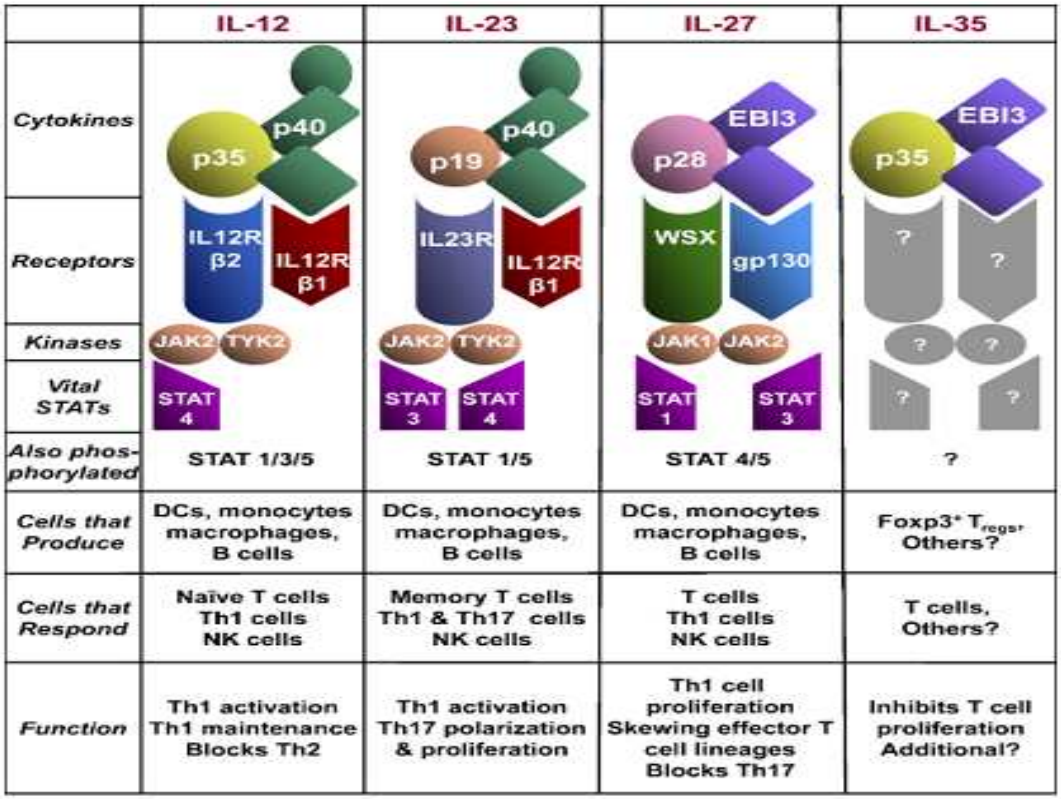


Figure 1: IL-12 cytokine family (Collison and Vignali, 2008, Immunol Rev, 226:248-62)

1.3 The classical IL-12 family members

Interleukin-12 (IL-12)

IL-12 comprises the subunits p35 (α) and p40 (β) (16). The p40 subunit shows similarities to cytokine receptors and the p35 subunit is related to IL-6 and Granulocyte-Colony Stimulating Factor (G-CSF). The expression level of p40 is higher than needed for the heterodimer formation of p40/p35 (17,18). IL-12 is induced in a T cell independent way by microbial products via TLRs and in a T cell dependent manner through CD40-CD40L interaction (15). It is produced by monocytes, macrophages, dendritic cells, neutrophils and B cells. Inhibition of IL-12 production occurs via release of IL-10, IL-11, IL-13 and type 1 interferons (IFNs) (19). IL-12 induces IFNγ release and enhances the cytolytic activity in natural killer (NK) cells and T cells. Furthermore IFNγ positively regulates IL-12 production. In dendritic cells (DCs) IL-12 is induced by previously produced IL-12. The IL-12 receptor, composed of the subunits IL12Rβ2 and IL12Rβ1, is expressed on Th1 cells, NK cells, naive T cells and DCs (15,20,21). Interaction of IL-12p40 occurs via the IL-12Rβ1 and IL-12p35 associates with IL-12Rβ2 (15). $CD4^+$ T cells are able to develop into Th1 or Th2 cells according to the cytokines they encounter. IFNγ producing Th1 cell differentiation and cell-mediated immunity is mainly promoted by IL-12, while the presence of IL-4 drives Th2 cell differentiation (15,22,23).

Interleukin-23 (IL-23)

IL-23 is composed of the subunits p40 and p19, which are linked by a disulfide bridge, and is produced by monocytes, macrophages, DCs, B cells and developing Th17 cells (24,25). Induction of p19 production in monocyte derived DCs occurs via CD40 stimulation or TLR2 agonists, such as peptidoglycan (26). IL-23 induces Th1 activation and Th17 polarization and proliferation, which is blocked by IL-27 (4,27). IFNγ and IL-12 production is increased in DCs, when treated with IL-23 (28). In a mouse model the IL-23 receptor was also found in $CD4^+CD45RB^{low}$

memory T cells and Th17 cells. IL-23 leads to IL-17 production in activated $CD4^+$ T cells (29). The IL-23 receptor comprises the subunits IL23R and IL12Rβ1. IL-23R has an extracellular N-terminal immunoglobulin (Ig) –like domain and two cytokine receptor domains similar to the IL-12 receptor subunit IL-12Rβ2. Human memory and activated T cells and NK cell lines express the IL-23 receptor, as well as monocytes, macrophages and myeloid DCs express it but only at low levels (30).

Interleukin-27 (IL-27)

IL-27 consists of a p28 subunit and an EBI3 subunit. This cytokine is produced by monocytes, macrophages, DCs, when stimulated via pattern recognition receptors, and B cells. It mediates Th1 cell proliferation by inducing IL-12Rβ2 in $CD4^+$ T cells (31). It synergizes with IL-12 and promotes IFNγ production. By inducing the production of IL-1 and TNFα in monocytes and mast cells and additionally IL-18 and IL-12 in monocytes IL-27 plays a role in innate immunity. Cytokine production is suppressed by IL-27 in activated T cells (32). IL-2 production is limited by IL-27 and also IL-12 to ensure Th1 differentiation (31,33). IL-27 blocks IL-23 induced Th17 polarization and proliferation and inhibits the differentiation of Th17 cells (27). B cell differentiation and immunoglobulin production is also influenced by IL-27 by inducing only the proliferation of activated naive B cells but not memory B cells (31,34). The activity of NK cells is augmented by IL-27. This is independent of IFNγ production (31,35). In monocytes, IL-27 mediates the increase of the major histocompatibility complex class I and II (MHC I/II) expression as well as the expression of transporter associated with antigen processing-1 (TAP-1) and β2 microglobulin (36). Therefore IL-27 may enhance antigen presentation in these cells. The IL-27 receptor is composed of the subunits WSX-1 and gp130 (4).

1.4 Interleukin-35 (IL-35)

Recently, IL-35, a new cytokine, belonging to the IL-12 cytokine family, was described (11,37). EBI3, which also serves as a subunit of IL-27, and p35, being a part of IL-12 as well, are the described subunits of IL-35. Initially the interaction of EBI3 and p35 was described in human placental trophoblasts (11,38).

IL-35 has been shown to expand murine $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs), if T cells are cultivated under strong inductive conditions with polyclonal T cell receptor (TCR) activation and the presence of co-stimulatory signals. Due to its suppressive function on the proliferation of murine $CD4^+CD25^-$ effector T cells and the inhibiting effect on the differentiation of Th17 cells and IL-17 production, IL-35 is assumed to have anti-inflammatory activity (37). $CD4^+CD25^+FoxP3^+$ Tregs express EBI-3 and IL-12α/p35 at higher levels then $CD4^+$ effector T cells. In a mouse model IL-35 is produced by Tregs and contributes to their suppressive function. It was also described that EBI3 is a downstream target of FoxP3, a transcription factor that is required for Treg development and function. $EBI3^{-/-}$ and $p35^{-/-}$ deficient Tregs have significantly reduced regulatory activity in vitro and fail to control homeostatic proliferation (39). The suppressive function of murine Tregs and the production of IL-35 and IL-10 to a lesser degree in Tregs increases significantly when they are in contact with conventional T cells (40).

It has been described that human $CD4^+CD25^+FoxP3^+$ Tregs do not constitutively express IL-35. The analysis of IL-35 expression was performed by using only EBI3 detection (41). IL-35 does not contribute to the suppressive function of FoxP3 induced human Tregs (42). Recently we could demonstrate that dendritic cells, activated with human rhinovirus (R-DCs), induce FoxP3 independent T regs that release an inhibitory factor (43). Human rhinovirus causes the common cold, which is one of the most frequent infections worldwide. HRV primarily infect the ciliated

epithelial cells of the nose (44). These human T regs induced by R-DCs act independent of IL-10, TGF-β and IFNα. The T regs produce and release IL-35 and this factor contributes to their inhibitory function. The induction of IL-35 is reliant on B7-H1 and Sialoadhesin interactions with their ligands, as presented in Figure 2 (43).

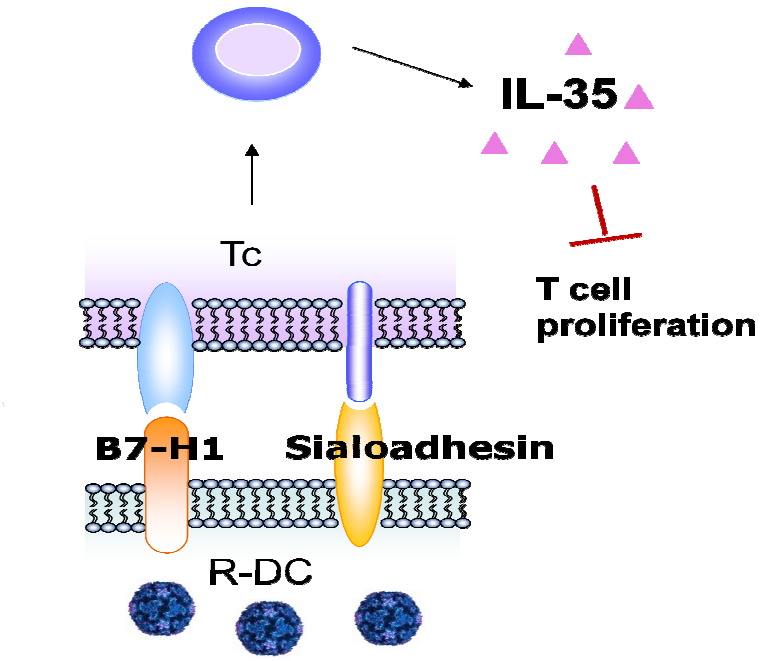


Figure 2: Rhinovirus activated dendritic cells induce B7-H1 and sialoadhesin dependent IL-35 production by T cells. The released IL-35 inhibits T cell proliferation. (Seyerl et al., 2009, Eur J Immunol, 40:321-29)

As mentioned before EBI3 is part of the newly described IL-35 and also IL-27. Primarily EBI3 was described as a molecule expressed in B lymphocytes infected with Epstein Barr virus (45). EBI3 is a homologue to the p40 subunit of IL-12 and encodes a 34 kDa glycosylated protein (46). Its expression was discovered in lymphoid blasts of reactive lymphoid organs, in the light zone of germinal centres, where B cells are associated to $CD3^+$ T cells, and a splice variant of EBI3 was described to be located in the spleen, liver and kidney (27). It was also demonstrated that EBI3 is expressed throughout pregnancy by syncytiotrophoblasts and extravillous

trophoblasts and the levels of EBI3 are strongly up-regulated in sera from pregnant women (47). Intestinal epithelial cells, as initial APCs, constitutively express EBI3, comparable to p35, and the expression level is up-regulated through IL-1α or TNFα addition. In contrast, the p35 level is also increased in the presence of IFNγ (48). It is proposed that free, latent EBI3 homodimer produced by antigen presenting cells (APCs) acts as an antagonist of IL-27 and therefore prevents any further IL-27 stimulation of naive T cells (46). It was demonstrated that the lack of EBI3 in mice has no influence on the number of B cells and T cells but causes a reduced number of invariant natural killer T cells (iNKT). EBI3 deficient mice show a decrease of IL-4 production and thus a diminished Th2 rate. They express higher levels of IL-17, RORγt, a Th17 transcription factor, and IL-22. It is suggested that EBI3 is a negative regulator of Th17 development (49,50). Sensitisation of $EBI3^{-/-}$ mice with ovalbumin leads to a less severe airway hyper-responsiveness, decreased numbers and degranulation of eosinophils and a significantly reduced number of $VCAM\text{-}1^{+}$ cells in the lungs than in wild type mice. Inhibition of EBI3 expression in DCs of the lung after ovalbumin challenge leads to an increased release of IL-10 and IL-12 while not expressing IFNα in CD11c+ cells (51). Sauer et al. demonstrated that targeting EBI3 in mice leads to a T-bet mediated antitumor $CD8^{+}$ T cell responses in the lung (52). EBI3 deficient mice that were infected with *Leishmania major* show approximately threefold increases lesion volume and a greater parasite number in dermal lesions compared wild types. They indicate increased IL-4, IL-10 and IL-23 and decreased IFNγ levels (53). For an acute myeloid leukemia cell line it was described that IL-18 induces, beside Th1 signature parameters such as T-bet and IFNγ, an up-regulation of EBI3 in the KG1 cell line (54). EBI3 possibly plays a role in atherogenesis, because it was proposed that in atheromatous lesions, smooth muscle cells express EBI3, as well as p28 and p35, which is up-regulated as response to proinflammatory factors like TNFα and IFNγ (55). IFNβ is described to inhibit the expression of p35 and to enhance that of EBI3 in immature human DCs (56). EBI3 is strongly

induced through stimulation of TLR2, TLR4 and TLR9 in primary DCs (57). For EBI3 induction the myeloid differentiation factor 88 (MyD88), NFkappaB and PU.1, which binds to the promoter region, are required as well (57).

1.5 Dendritic cells

Dendritic cells (DCs) were first described in human skin as cutaneous nerve cells by Paul Langerhans (58,59) and later discovered in mouse spleen (59,60). DCs are bone marrow derived leukocytes (61) and can be classified into two types including myeloid DCs and plasmacytoid DCs. Myeloid DCs can be further divided into blood monocyte derived DCs (moDCs), dermal or interstitial DCs and Langerhans cells (LCs) (59). DCs are APCs and are able to initiate and modulate immune response. They efficiently stimulate B cells and T cells.

The precursor of moDCs is the $CD14^+$ monocyte in the peripheral blood. Monocytes differentiate after IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) addition into $CD14^-$, $CD11c^+$, $CD83^+$ and HLA-DR^{bright} DCs (59,62,63). Dermal or interstitial DCs and LCs are not located in the blood but reside in places in the body where the immune system first of all has to act against infectious compounds. Thus, LCs localize to epithelial surfaces of skin and mucosa and the dermal or interstitial DCs localize to the subepithelial tissues of the dermis in skin and the interstitia of solid organs (59). Plasmacytoid DCs can be identified as lineage negative, HLA-DR^{bright}, BDCA-2^+, BDCA-4^+ and $CD123^{bright}$ cells. They can be fully activated via IL-3 and CD40 ligand (CD40L) or microbial product stimulation (59).

DC maturation

Upon activation, DCs migrate to lymphoid tissues such as the spleen and the lymph nodes. The cells lose their adhesiveness for epithelia and start to express the chemokine receptor CCR7, which is specific for chemokines produced in the T cell zones of lymph nodes. DC maturation

is mediated through proinflammatory cytokines such as TNF-α, and by TLR- and CD40L-dependent signals (62,64), which upregulate the costimulatory molecules CD80 and CD83, that are transported together with major histocompatibility complex (MHC) class II molecules to the cell surface where they remain associated within membrane microdomains (65). After that maturation is completed they attract T and B lymphocytes by releasing chemokines (2,66). Immature DCs or DCs that present self-antigens induce tolerance in T cells whereas mature DCs or DCs that present foreign antigens induce immunity and naive T cell proliferation in vitro. The phenotype of mature DCs is associated with an increased expression of MHC and T cell co-stimulatory molecules such as CD80 and CD86, high capacity to present antigens captured at the time of receiving the maturation stimulus, but incapacity to process and present subsequently encountered antigens (67).

Antigen processing and presentation

Antigens are captured by immature DCs via phagocytosis, endocytosis, pinocytosis and specific receptors, such as mannose receptor or TLRs (59). After antigen capturing these proteins are processed into peptides capable of binding to MHC molecules (2). DCs get activated by antigen uptake and processing and through cytokines that serve also as response to innate immunity. After activation the ability to capture antigens rapidly declines (66).

There are two different pathways of antigen processing in APCs. Antigens that are presented by MHC class II are infiltrated into the cells and processed in the endocytotic pathway. Internalized antigens are located in the endosomes. These endosomes fuse with lysosomes where the antigens are degraded into small peptides. MHC class II molecules are synthesized in the endoplasmic reticulum (ER) and transported to endosomes with an associated protein called the invariant chain, which occupies the peptide-binding sides of the newly synthesized class II molecules. The invariant chain is removed from MHC class II molecules

and antigenic peptides are then able to bind the available peptide binding clefts of the MHC II molecules. MHC class II molecules are stabilized by the bound peptides and the stable peptide-class II complexes are delivered to the surface of the DC, where they are displayed for recognition by $CD4^+$ T cells (2).

MHC class I molecules present peptides that derive from cytosolic proteins. Foreign cytosolic antigens may be the products of viruses or other intracellular microbes. These cytosolic proteins get proteolysed into peptides by the proteasome. The generated peptides are translocated by the TAP transporter into the ER, where they bind to newly synthesized MHC class I molecules. The peptide – MHC class I complex is delivered to the surface of the APC where the antigen is presented to $CD8^+$ T cells (2,66). All nucleated cells have MHC I but only APCs express MHC II.

IL-10 and DCs

IL-10 acts as an inhibitor of DCs and is thus involved in the control of innate immune reactions and cell-mediated immunity. It suppresses the production of IL-12 by DCs and therefore the production of IFNγ. It also inhibits the expression of co-stimulators and MHC class II molecules on these cells, which causes an inhibition of T cell activation and proliferation (2). IL-10 is expressed, secreted and differentially regulated by a variety of cell types, including T cells, monocytes/macrophages, DCs, and epithelial cells, usually after an activation stimulus. Functionally, IL-10 acts on a wide variety of cells, including B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, DCs, keratinocytes and endothelial cells (68,69).

1.6 T cells

The precursors of T cells arise in the bone marrow and migrate into the thymus. T cells recognize the antigen presented by an APC and as response to that can either destroy the cells that present the antigen or release cytokines to activate other cells or fall in anergy, because of recognizing self-antigen. Naive T cells are inexperienced T cells that have

yet to see their cognate antigen and get activated. TCR engagement in an inflammatory cytokine milieu induces the cell to undergo numerous changes, including chromatin remodelling and DNA methylation modifications, leading to the induction or repression of transcription factors in differentiated cell subsets (70,71). This in turn results in the differential expression of cell-surface receptors and cytokines. T cells are further divided into two types: $CD4^+$ T cells or Th cells provide “help” to B cells and other T cells in directing B- and T cell responses and $CD8^+$ T cells or cytotoxic T cells owing to their high expression of IFNγ and granzymes. Th cells have important roles in combating infections and cancers and dysregulation of their function can lead to chronic inflammatory diseases (71).

1.7 T cell activation

T cells can recognize the antigens presented by DCs when they contact these cells. The area where these two cell types establish the contact is termed the immunological synapse, where the T cell receptor (TCR) interacts with the MHC-peptide complex and co-stimulatory molecules aggregate (72). The ability of T cells to clonally expand depends on the strength of the signals received by TCR MHC-peptide complex interaction (signal 1) and co-stimulatory molecules (signal 2) (73). The amount of signal that T cells receive is dependent on the level of peptide-MHC complexes, the level of co-stimulatory molecules that amplify the signaling process, and the stability of the synapse (74). For an efficient T cell activation the signaling via the TCR requires the interaction of co-stimulatory molecules, like CD28 with CD80 or CD86. Activated T cells produce IL-2 which causes a rapid T cell proliferation and can act in an autocrine and paracrine way. A third signal is acquired for T cell differentiation, which is elicited by DCs through cytokine secretion. T cells that receive a short TCR signaling in the absence of IL-12 proliferate, but cannot differentiate into effector cells. T cells that receive a prolonged TCR signal in presence of IL-12 differentiate into Th1 cells and those in presence of IL-4 differentiate into Th2 cells. Th1 and Th2 cells acquire the

capacity to migrate to inflamed non-lymphoid tissues to execute their effector functions (74).

1.8 Immunological Synapse

The immunological synapse is formed between a T cell and an APC, e.g. the DC, via the TCR and peptide – MHC complex. The mature immunological synapse is defined by a specific pattern of receptor segregation with a central cluster of TCRs surrounded by a ring of integrin family adhesion molecules (75,76). After contact between T cell and DC is established, three different physical states can be achieved, all of which have an impact on contact duration, receptor engagement and downstream signalling (77).

The development of the immunological synapse is characterized by 5 phases. The first phase of immunological synapse formation occurs when moving T cells recognize peptide–MHC complexes, leading to a transient arrest in migration. The second phase is the most important phase for signal induction and is therefore crucial for determining the outcome of the T cell–APC interaction. Within the first few seconds of contact, calcium signalling, followed by recruitment of signalling molecules is observed (77). At the same time, the TCR, CD4 and other receptors (such as CD28) move to the contact zone, where the dynamic signalling platform is being assembled (77,78). In the third phase a mature immunological synapse forms at the cell–cell junction within 5–30 minutes of continuous T cell–APC interaction. Maturation is defined by molecular segregation, which leads to a central zone that contains TCR and CD3 and, at least temporarily, CD28, CD2, CD4 (transient), CD45 (transient) and cytotoxic T-lymphocyte antigen 4 (CTLA-4). In the fourth phase, after sustained signalling, the TCR is internalized from the central zone into cytoplasmic vesicles, thereby limiting its availability at the interaction plane. Immunological synapse resolution occurs when the T cell and the APC separate in the fifth and last phase (77).

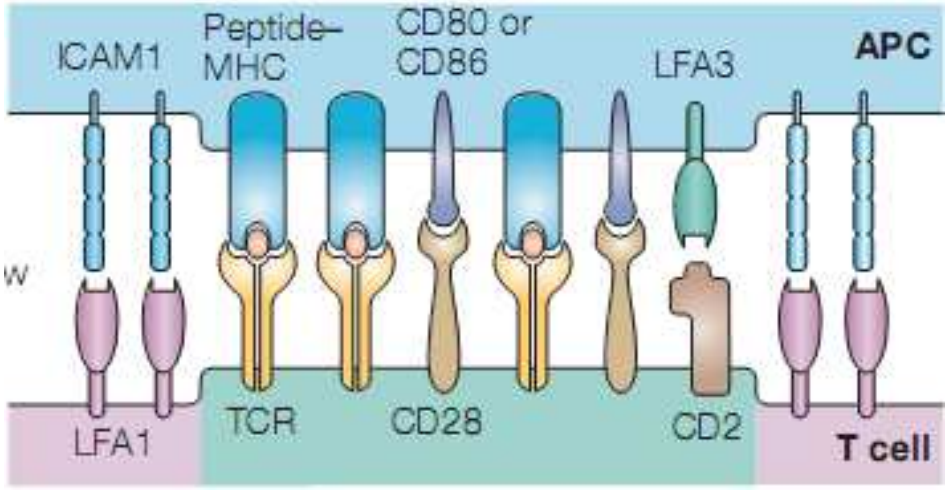


Figure 3: Immunological synapse (Friedl P. et al., 2005, *Nat Rev Immunol,* 5:532-45)

1.9 T cell subsets

$CD4^+$ T cells / T helper cells

$CD4^+$ T cells play a central role in immune protection. This is accomplished by their capacity to help B cells to produce antibodies, to induce macrophages to develop enhanced microbicidal activity, to recruit neutrophils, eosinophils, and basophils to sites of infection and inflammation, and, through their production of cytokines and chemokines. Several types of the $CD4^+$ T cell population represent alternative patterns of differentiation. Naive $CD4^+$ T cells can differentiate into distinct populations by the pattern of signals they receive during their initial interaction with antigen. These populations are Th1, Th2, Th17, induced regulatory T (iTreg) cells, and the recently described Th9 and Th22 cells. Th1 cells are regarded as critical for immunity to intracellular microorganisms and Th2 cells for immunity to many extracellular pathogens (79,80).

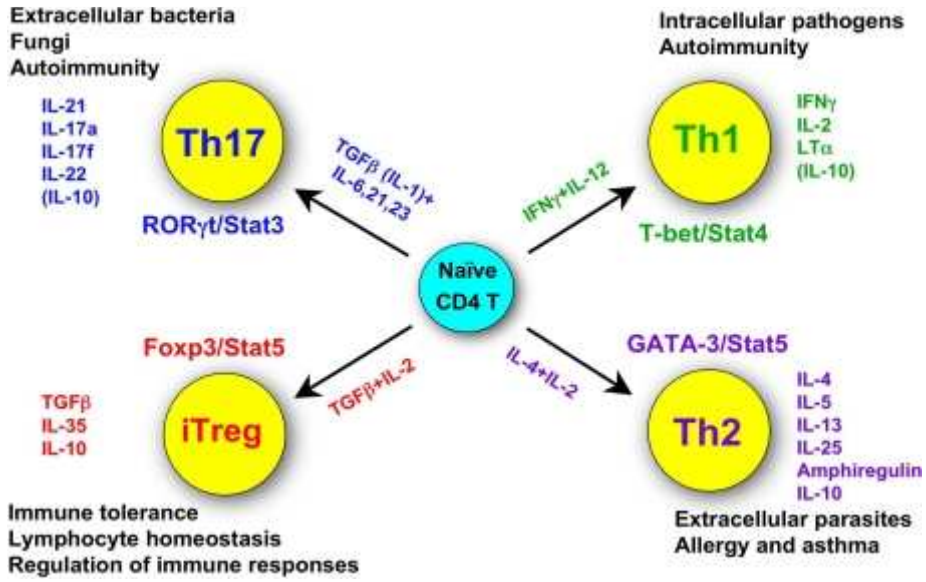


Figure 4: CD4+ T cell subsets (Zhu, J. and Paul, W.E., 2008, Blood, 112:1557-69)

Th1 cells mediate immune responses against intracellular pathogens (79). In humans, they play a particularly important role in resistance to mycobacterial infections. Th1 cells are also responsible for the induction of some autoimmune diseases (80). They produce IFNγ, important in activating macrophages to increase their microbicidal activity (81), lymphotoxin α (LTα), a marker for the disease progression in multiple sclerosis patients (82), and IL-2, important for $CD4^+$ T cell memory (80). Th1 cells express the IL-12 receptor (IL-12R) as well as the IL-18 receptor (IL-18R). The subunit IL-12R β1 is constitutively expressed on naive $CD4^+$ T cells and increases in Th1 cells (83) and IL-12R β2 expression is induced by TCR activation and then maintained by IL-12 and IFNγ stimulation (84). Although IL-18 is not involved in the differentiation of Th1 cells, it can synergize with IL-12 inducing IFNγ, implying that IL-18 plays an important role in Th1 responses (80,85). The main transcription factor for Th1 cells is T-bet, which is Stat1 and then IFNγ induced and up-regulated during Th1 differentiation. Th1 polarization and proliferation can be induced by the IL-12 secretion of APCs as a result of their activation. This also promotes Th1 cell differentiation by acting on both NK cells and

T cells. Collaboration between IFNγ and IL-12 induces full Th1 differentiation (80,86).

Th2 cells mediate host defense against extracellular parasites including helminths (79). They are important for the induction and persistence of asthma and other allergic diseases. Th2 cells produce the cytokines IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin (80). These cells express several surface markers including the IL-4 receptor (IL-4R). During differentiation the subunit of this receptor IL-4Rα is up-regulated by IL-4. T1/ST2 (IL-33Rα) is known as the most important surface marker for Th2 cells (87) and belongs to the IL-1R superfamily, which also includes IL-18Rα (80). The most important transcription factor for Th2 differentiation is GATA-3, which is induced by IL-4 activated Stat6 (88). In vitro Th2 differentiation requires IL-4 and IL-2 (89). If IL-4 is provided exogenously GATA-3 expression is induced by Stat6 activation, but if IL-4 is not provided it can be produced by naive CD4+ T cells in limited amounts after TCR-mediated GATA-3 transcription and IL-2-mediated Stat5 activation (90). The collaboration of Stat5 and GATA-3 is responsible for full Th2 differentiation in vitro (91).

Th17 cells mediate immune responses against extracellular bacteria and fungi by releasing IL-17 which recruits and activates neutrophils (92). They participate in the induction of many organ-specific autoimmune diseases. Th17 cells produce IL-17a, IL-17f, which are genetically linked and often co-expressed, IL-21 and IL-22. IL-17a has an important role in inducing inflammatory responses, because it is able to stimulate inflammatory cytokines such as IL-6. Th17 cells also secrete IL-21, a stimulatory factor for Th17 differentiation, which also acts on $CD8^+$ T cells, B cells, NK cells and DCs (93). Th17 cells express high levels of IL-23R (25). The most important transcription factor of Th17 cells is RORγt (94). Stat3, the major signal transducer for IL-6, IL-21 and IL-23, is indispensable for IL-17 production and deletion of Stat3 results in the loss of IL-17 producing cells (80,95). A combination of IL-6, induced by TLR signalling, and TGF-β

induces Th17 differentiation, IL-21 production and the expression of IL-23R and RORγt (96,80). IL-6 can be replaced by IL-21 for inducing RORγt and IL-17 expression (25). IL-23, initially proposed as the differentiation factor for Th17 cells, fails to induce Th17 differentiation from murine naive $CD4^+$ T cells but is critical for Th17 cell survival and/or for maintaining their function. Therefore, Th17 cell differentiation consists of 3 stages: a differentiation stage, based on TGFβ and IL-6; an amplification stage, mediated by IL-21; and a stabilization stage due to IL-23. Importantly, all three cytokines, IL-6, IL-21, and IL-23, activate Stat3 (80).

Regulatory T cells

T regs are very important for maintaining self-tolerance and regulating immune responses (97). T regs can be divided into two groups, first, the natural occurring $CD4^+CD25^+$ T regs, which express the transcription factor FoxP3 their whole living already in the thymus and, second, the inducible T regs, in which FoxP3 is induced in the periphery. Additionally, two subsets of inducible T regs with regulatory and suppressive function were described, Tr1 cells and Th3 cells, believed to suppress immune responses through the production of IL-10 and TGF-β (98,99). Natural T regs are described to inhibit the development of autoimmune disease elicited by T regs depletion and to suppress the proliferation of antigen-stimulated naive T cells in vitro (100). T regs produce and release several cytokines such as TGF-β, IL-10 or IL-35. It was suggested that T regs act in cell-contact dependent manner, because they fail to suppress proliferation of responder T cells, when they were separated through a semi-permeable membrane and the neutralisation of IL-10 and TGF-β does not avoid the in vitro suppression (100,101,102). TGF-β is believed to act as a suppressive mediator in a membrane-bound form (103) and activates Smad3 while TCR stimulation induces NFAT activation, both promote FoxP3 expression. FoxP3 expression is also induced by Stat5 activation, what is mediated by IL-2 (80).

Beside this, TGF-β together with the mode of antigen-presentation also initiates the differentiation of inducible T regs from $CD4^+$ T cells (80). Tr1 cells can be generated by addition of IL-10 and they further produce high levels of IL-10. They do not express FoxP3 and are able to develop in the absence of natural T regs. But Tr1 cells inhibit T cell proliferation with comparable efficiency to natural T regs. Th3 cells were found after orally tolerizing mice with low antigen doses. These cells were described to produce TGF-β (99).

$CD8^+$ T cells

$CD8^+$ T cells are key players in mediating immunity to intracellular virus pathogens and tumors. $CD8^+$ T cells recognize antigen in the context of MHC class I inducing $CD8^+$ T cell activation, proliferation and differentiation into effector cytotoxic T lymphocytes (CTLs); this is known as the primary response (104). The help of $CD4^+$ T cells is essential for CTL priming and confers a key feature of immune memory (105). After the primary response, most effector $CD8^+$ T cells die, but some rest as long-lived memory cells. Memory cells are able to respond with enhanced efficacy to a second challenge with the same antigen (secondary response) in the absence of T cell help (104,105). The $CD8^+$ T cells that are not helped by $CD4^+$ T cells can mediate effector functions such as cytotoxicity and cytokine secretion upon re-stimulation, but do not undergo a second round of clonal expansion (105,106,107).

The effector functions of $CD8^+$ T cells are presented by killing their targets (infected cells) via the release of cytoplasmic granules that comprise membrane pore forming proteins. The killing occurs via two mechanisms that require direct contact between the effector and target cells. In the first pathway, cytoplasmic granule toxins and granzymes (family of structurally related serine proteases) are secreted by exocytosis and together induce apoptosis of the target cell (108,109). The granule-exocytosis pathway activates efficiently cell-death pathways that operate through the activation of apoptotic cysteine proteases (caspases), but it also leads to cell death

in the absence of activated caspases (109,110,111).The second pathway involves the engagement and aggregation of target-cell death receptors, such as FAS, by their cognate ligands, such as FAS ligand (FASL), on the killer-cell membrane, which results in classical caspase-dependent apoptosis (109,112).

$CD2^{-/low}$ T cells

In 1988 a subset of T cells lacking CD2 expression or only expressing CD2 in very low amounts was identified. CD2 was thought to be expressed on all T cells just like CD3. These $CD2^{-/low}CD3^{+}$ T cells were first described in fetal human spleen and thymus (113), and in a subset of $CD3^{+}$TCR-γ/δ^{+} peripheral blood T cells from one individual (114). Also $CD2^{-/low}CD3^{+}$ TCR-α/β^{+} peripheral blood T cells were isolated and these cells can be induced to proliferate, produce IL-2 and display cytotoxic effector function (115). These cells show a mostly CD4/CD8 double negative, $CD3^{+}TCR^{-}$ immature phenotype. They produce the type 2 cytokines IL-13, IL-4 and IL-5, but not IFNγ or IL-10, and upon culture with IL-12 and TCR-mediated stimuli, differentiate to functionally mature IFN-γ^{+} cells (116). This population is previously described to represent <0,1% of the T cells in freshly isolated lymphocytes. It was described that peripheral $CD2^{-/low}$ T cells are possibly early differentiated T cells that may undergo extrathymic maturation, and potentially contribute to maintain the peripheral naive T cell pool (117). But the function of these cells is unclear.

2 AIM OF THIS STUDY

The cytokines of the IL-12 family have similar structures and similar functions. They are produced by APCs, especially DCs, and act mainly on T cells in different kind of ways. The recently described IL-35 differs from the other cytokines of the IL-12 family. In the mouse system IL-35 is promoted to have anti-inflammatory function and is produced by $CD4^+CD25^+FoxP3^+$ regulatory T cells (T regs) and contributes to their suppressive function (39). In the human system the characterisation of IL-35 is not comparable with that in mice, because IL-35 is not constitutively expressed by T regs (41). We recently could demonstrate that IL-35 is produced by T cells that were in contact with human rhinovirus activated DCs (R-DCs) (43). So at this time point the role of IL-35 is not entirely clear. What we want to find out in this study is if IL-35 does act on DCs, on T cells or on both cell types and how it affects these cell types.

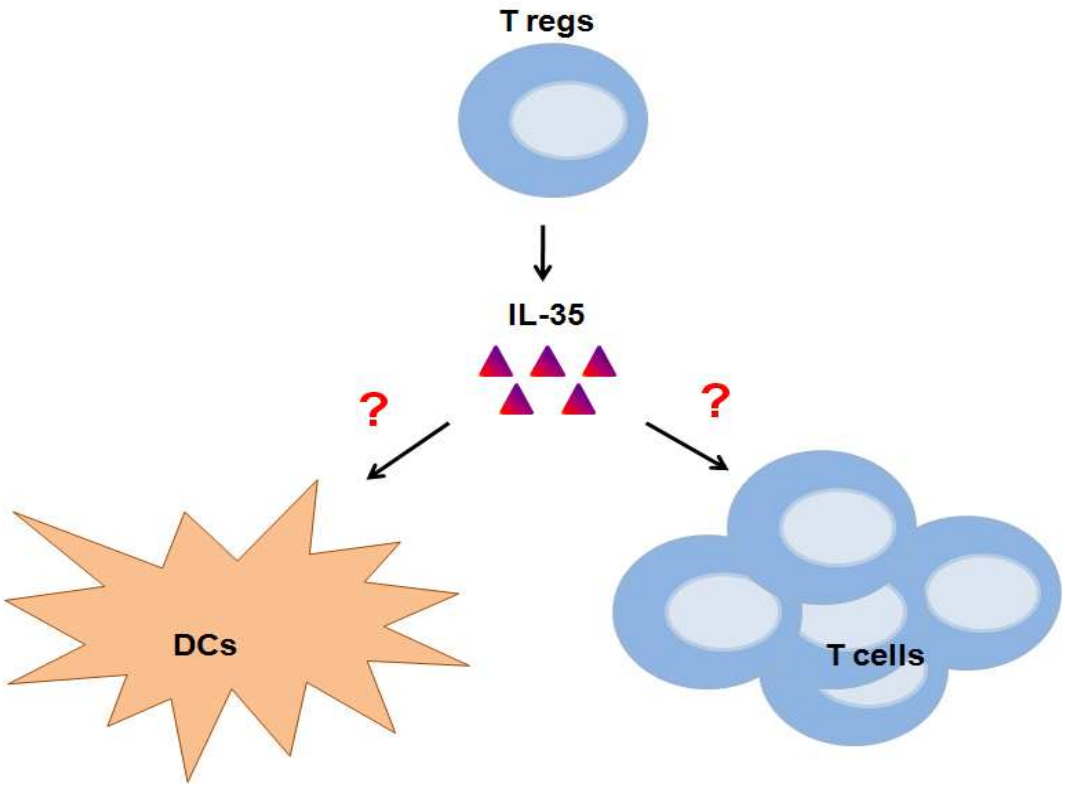


Figure 5: Scheme of this aim

3 MATERIAL AND METHODS

3.1 Reagents and chemicals

3.1.1 List of mouse anti-human mAbs

Specificity	Clone	Isotype	Source
T cell stimulation			
CD3	OKT3	IgG2a	Jansen-Cilag, Vienna
CD28	15E8	IgG1	Caltag Laboratories, Burlingame, CA
CD63	CD63-11C9	IgG3	Otto Majdic, Institute of Immunology, Vienna
Staining			
Calf intestine alkaline phosphatase	Viap-2D5	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD1a	VIT6B	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD2	TS2/18	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD3	Ucht1	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD14	VIM13	IgM	Otto Majdic, Institute of Immunology, Vienna
CD40	G28-4	IgG1	American Type Culture Collection (ATCC), Rockville, Tenesse
CD80	7-480	IgG1	Otto Majdic, Institute of Immunology, Vienna
CD83	HB15e	IgG1	BD, New Jersey, NJ
CD86	BU63	IgG1	Caltag-Medsystems Ltd , Buckingham
MHC I	W6/32	IgG2a	ATCC, Rockville, Tenesse
MHC II – HLA-DR	L243	IgG2a	Otto Majdic, Institute of Immunology, Vienna

B7-H1	5-272	IgG1	Otto Majdic, Institute of Immunology, Vienna
B7-H3	13-I/241	IgG1	Otto Majdic, Institute of Immunology, Vienna
Siglec-1	7-239	IgG1	Otto Majdic, Institute of Immunology, Vienna
S100-A4	8-9	IgG1	Otto Majdic, Institute of Immunology, Vienna
Mxa	383-7D4	IgG1	Otto Majdic, Institute of Immunology, Vienna
p35	27537	IgG1	R&D Systems Inc., Minneapolis, MN
EBI3	polyclonal		Abnova, Taipeh, Taiwan

3.1.2 List of recombinant cytokines

Cytokine	**Trade name**	**Source**
IL-35:Fc	522-140-C010	Alexis Biochemicals, San Diego, CA
Human recombinant IL-10	217-IL	R&D Systems Inc., Minneapolis, MN

3.1.3 List of stimuli

Stimulus	**Concentration**	**Source**
HRV14	5 $TCID_{50}$	Joachim Seipelt
IFNα	100 U/ml	PBL Biomedical Laboratories, Piscataway, NJ
LPS	1 µg/ml	Sigma-Aldrich
polyIC	20 µg/ml	Sigma-Aldrich
Zymosan A, *S. cerivisiae*	30 µg/ml	Sigma-Aldrich

3.1.4 List of cell lines

Cell line	Cell type	Source
HeLa Ohio	Human cervix carcinoma	ATCC
A431	Human epidermoid carcinoma	ATCC
SCC4	Human squamous cell carcinoma (tongue)	ATCC
SCC9	Human squamous cell carcinoma (tongue)	ATCC
Jurkat	Human T cell leukemia	ATCC
Daudi	Human Burkitt´s lymphoma (EBV^+)	ATCC
Otma	EBV transformed B cells	Otto Majdic, Institute of Immunology, Vienna

3.2 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMNCs) were isolated from fresh blood (Buffy coats, Red Cross Vienna) from healthy donors. Blood was diluted with RPMI1640 medium (Gibco Ltd., Paisley, Scotland) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) and 10 U/ml heparin (EBEWE Pharma, Unterach, Austria). The diluted blood was then overlaid above 15 ml Ficoll-Paque (Axis-Shield PoC AS, Oslo, Norway) to a final volume of 50 ml. For density gradient centrifugation the tubes were centrifuged at 900 g for 30 min at 25℃ without brake. In the Ficoll gradient cells are separated due to their different density. The pellet contains mainly erythrocytes and leukocytes, whereas the white interphase contains PBMNCs consisting of lymphocytes and monocytes and the upper phase contains serum. The interphase with the PBMNCs was then collected into fresh tubes and washed twice with MACS buffer (1% PBS supplemented with human serum albumin (Aventis Behring, Vienna, Austria) and 5 mM EDTA (Gibco Ltd., Paisley, Scotland).

3.3 Magnetic-activated cell sorting (MACS)

Magnetic-activated cell sorting (MACS) is a method for the selective enrichment or depletion of cells which express a cell type specific surface protein. Therefore cells can be labelled with biotin coupled antibodies directed against this specific molecule. These labelled cells can be targeted with a secondary antibody coupled to magnetic beads directed against the biotin residues on the primary antibody. Thus specific cells labelled with the magnetic beads in a suspension can be selectively retained in a column when a magnetic field is delivered. In this strong magnetic field the cells labelled with paramagnetic beads stack to the iron mesh and were retained while non-labelled cells passed through. Retained cells were eluted by removing the column from the magnetic field.

3.4 Monocyte isolation

For the isolation of monocytes up to 1 x 10^9 PBMNCs were incubated with 15 µg/ml of biotinylated anti-CD14 (VIM13, MEM18). Therefore PBMNCs were resuspended in 750 µl MACS buffer and incubated with 250 µl biotinylated antibodies for 15 min at 4 °C. To remove unbound antibodies the cells were washed twice with MACS buffer and again resuspended in 750 µl buffer. Then 250 µl streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the suspension and incubated for 15 min at 4°C. In the meantime a CS column (Miltenyi Biotec), which can be loaded with a maximum of 10^9 cells, was placed onto a VarioMACS apparatus and equilibrated with 40 ml MACS buffer.

Monocytes were positively enriched. The PBMNCs incubated with the CD14 mAb and the streptavidin MicroBeads were loaded onto the CS column. $CD14^+$ monocytes remained in the column, $CD14^-$ cells were washed out with 40 ml MACS buffer. The flow through was collected as monocyte negative fraction containing T cells, B cells and NK cells (fraction 1).

The column was removed from the magnetic field and the MACS buffer was added in 10ml portions alternating through the side syringe or loaded on the top of the column. After each addition step the column was put

back into the magnet and about 10ml were let to run through. These steps were repeated until a volume of 50ml had passed through (fraction 2).
For monocytes collection the column was removed from the magnet and MACS buffer was added several times into the reservoir. After each step the liquid was taken off through the lateral valve using a syringe. These steps were repeated until the collected volume came to 40ml (fraction 3). The third fraction was centrifuged for 5´ at 900g, resuspended and the number of monocytes was determined.
The purity (> 95%) of monocytes was controlled by immunofluorescence analysis. Double-staining with directly labeled Antibodies using CD4-PE/CD3-FITC; CD8-PE/CD3-FITC, CD19-PE/CD3-FITC, CD56-PE/CD3-FITC, HLA-DR-PE/CD3-FITC, CD14-PE/HLA-DR-FITC.

3.5 T cell isolation

To isolate T cells from up to 1 x 10^9 PBMNCs the MACS technique was used. T cells were isolated by collecting the flow through of PBMNCs depleted by using different mAbs at a concentration of 10 µg/ml containing anti-CD14 (MEM 18) for collecting monocytes, anti-CD16 (3G8) for binding granulocytes and NK cells, anti-CD19 for B cells (BU 12) and anti-CD33 (4D3) for monocytes, thrombocytes and myeloid progenitors (negative selection). Freshly isolated PBMNCs were resuspended in 750 µl MACS buffer and incubated with 250 µl biotinylated antibodies for 15 min at 4 ℃.
To remove unbound antibodies the cells were washed twice with MACS buffer and again resuspended in 750 µl buffer. Then 250 µl streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the suspension and incubated for 15 min at 4℃. In the meantime a CS column (Miltenyi Biotec), which can be loaded with a maximum of 1x10^9 cells, was placed onto a VarioMACS apparatus and equilibrated with 40 ml MACS buffer. PBMNCs were loaded onto the column, T cells were eluted and washed until a volume of 50 ml was reached. The collected T cells were centrifuged for 5´ at 900g, resuspended and the number of cells was determined.

The purity (> 95%) of T cells was controlled by immunofluorescence analysis. Double-staining with directly labeled Antibodies using CD4-PE/CD3-FITC; CD8-PE/CD3-FITC, CD19-PE/CD3-FITC, CD56-PE/CD3-FITC, HLA-DR-PE/CD3-FITC, CD14-PE/HLA-DR-FITC.

3.6 Generation of monocyte derived DCs

DCs were generated by culturing purified monocytes for 6 days with a combination of 50 ng/ml GM-CSF and 100 U/ml IL-4 in RPMI1640 (Gibco Ltd., Paisley, Scotland) supplemented with 10% characterized FCS (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich). Maturation of DCs was initiated by adding 1 µg/ml LPS (Sigma Aldrich, St. Louis, MO) for 24 h.

3.7 Generation of human rhinovirus treated DCs (R-DCs)

For the generation of R-DCs monocyte derived DCs, cultured at a density of $1x10^6$ cells/ml, were treated with human rhinovirus 14 (HRV14) at a dose of 5 $TCID_{50}$ for 48 hours.

3.8 Generation of IL-35 pre-treated DCs (IL-35-DCs)

Purified monocytes were cultured for 3 days with a combination of 50 ng/ml GM-CSF and 100 U/ml IL-4 in RPMI1640 (Gibco Ltd., Paisley, Scotland) supplemented with 10% characterized FCS (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich). Then human IL-35 (Alexis Biochemicals, San Diego, CA) at a concentration of 2 ng/ml or human recombinant IL-10 (R&D Systems Inc., Minneapolis, MN) at a concentration of 20 ng/ml was added and further cultured for 3 days to obtain IL-35 or IL-10 pre-treated DCs. Maturation of DCs was initiated by adding 1 µg/ml LPS (Sigma Aldrich, St. Louis, MO) for 24 h.

3.9 Freezing and thawing of cells

Mammalian cells can be stored in liquid nitrogen for prolonged periods of time with minimal loss of viability in subsequent cell culture. For that purpose, cells were centrifuged, counted, resuspended in freezing medium

(RPMI1640 supplemented with 25% FCS and 10% DMSO (Sigma)) and adjusted to a cell number ranging from 10^7 – 5 x 10^7 cells/ml. 1 ml aliquots were filled into cryotubes (Nalgen Nunc International, Roskilde, Denmark) and kept overnight at - 80℃ in a freezing box fill ed with isopropanol before being transferred to liquid nitrogen.

Cryotubes were thawed in 37℃ warm water bath. A re st frozen media was left to avoid overheating, that would thaw on ice. When all frozen media was dissolved droplets of cell culture medium were added and then cells were washed two times with RPMI1640 plus 10% FCS (Sigma-Aldrich), L-glutamine, penicillin, streptomycin. Thawed cells were counted and cultured in medium.

3.10 Cell Culture conditions

For cell culture RPMI supplemented with 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine (NBK, Novartis Research Institute, Vienna), 100 U/ml penicillin and 100 µg/ml streptamycin (NBK, Novartis Research Institute, Vienna) was used. Cells were cultured at 37℃ with 5% CO2.

3.11 DC and T cell co-culture

T cells were co-cultured with DCs or R-DCs in a cell ratio ranging from 5:1 to 10:1 for 3 days. The supernatants of the co-cultures were collected and added to a MLR or to a T cell proliferation assay. The cells of the co-cultures were analysed by FACS.

3.12 Mixed Leukocyte Reaction (MLR)

In an allo – MLR lymphocytes from a potential donor are mixed with stimulator cells from a potential recipient, i.e. APCs like DCs. If the two cell populations are not compatible in their MHC, which means they are allogeneic, proliferation of T cells occurs.

1 x 10^5 responder T cells were cultivated together with 1 x 10^4 DCs and 100 µl of DC/T cell or R-DC/T cell supernatant or 10 ng/ml and 1 ng/ml human IL-35 (Alexis Biochemicals, San Diego, CA) in RPMI1640 (10%

FCS (Sigma-Aldrich), L-glutamine, penicillin, streptomycin) for 5 days in a final volume of 250 µl under standardized cell culture conditions (37℃, 5% CO_2, humidified atmosphere).

For the MLR with IL-35-DCs, 1 x 10^5 responder T cells were cultivated together with 1 x 10^4 DCs, IL-35-DCs or IL-10-DCs with or without LPS in RPMI1640 (10% FCS (Sigma-Aldrich), L-glutamine, penicillin, streptomycin) for 5 days in a final volume of 250 µl under standardized cell culture conditions (37℃, 5% CO_2, humidified atmosphere).

Each experiment was performed in triplicates in 96-well plates (Costar, Sigma Aldrich). On day five 25 µl of 1:20 diluted methyl-3[H]thymidine (1 µCi/well) (Perkin Elmer, Waltham, MA) was added to the cells and incubated for 18 h. The cells were lysed, their DNA collected onto filter-plates (Perkin Elmer, Wellesley, MA) and after adding 25 µl of Microscint scintillation mix (Packard, Meriden, Connecticut) in each well radioactive emissions quantified on a Packard microplate scintillation counter.

3.13 T cell proliferation assays

T cells were either mock treated or stimulated with anti-CD3 (OKT3), anti-CD3/CD28 (OKT3/15E3) and anti-CD3/CD63 (OKT3/11C9) mAbs at a concentration of 2 µg/ml for 3 days. Experiments were performed in 96-well cell culture plates. The plates were coated with a volume of 30 µl of antibody solution over night at 4℃ or for about 2 hours at 37℃. Then the antibody solution was removed and the plates were washed twice with PBS. 1x10^5 T cells in RPMI-1640 medium supplemented with 10% FCS were added into each well. Proliferation of T cells was monitored by measuring (methyl-3H)-TdR (Perkin Elmer, Waltham, MA) incorporation on day 4 of culture. Cells were harvested 18 hours later, and radioactivity was determined on a microplate scintillation counter (Perkin Elmer, Wellesley, MA). Assays were performed in triplicates.

3.14 Staining of surface proteins

Membrane staining with unconjugated mAb:

Unspecific binding of mAbs to Fcγ-receptors was blocked by incubation of cells with human immunoglobin Beriglobin (Aventis Behring GmbH, Vienna). As isotype control VIAP, a calf intestine alkaline phosphatase-specific antibody was applied. For secondary labelling Oregon Green-conjugated goat anti mouse antibody was used. Dead cells were excluded from analysis by staining with propidiumiodide.

• PBS/BSA: 1x PBS + 1% BSA

• Beriglobin (final concentration 20 mg/ml) diluted 1:8 in PBS/BSA (1% v/v BSA) (Aventis Behring GmbH, Vienna)

• Primary antibody: 20 µg/ml

• Secondary antibody: Oregon Green-conjugated goat-anti-mouse IgG 20 µg/ml in PBS/BSA (Molecular Probes; Eugene, Oregon)

First the cell suspension (5 x 10^5 /assay) was centrifuged for 5 minutes at 300g and the pellet was resuspended with 50 µl Beriglobin/assay and kept 10 minutes on ice. Then 20 µl of the antibody were prepared in Micronic-tubes and 50 µl of the cell suspension was added, mixed and incubated 30 minutes at 4℃. Each assay was washed twice with PB S/BSA (resuspended in PBS/BSA, centrifuged (5 minutes at 300g), the supernatant was discarded). 20 µl Oregon Green were added to the cells, and again incubated for 30 minutes at 4℃. Each ass ay was washed twice with PBS/BSA and the cells were resuspended in 50 µl FACS fluid (Becton Dickinson, Franklin Lakes, NJ). The tubes were kept on ice until they were analyzed by flow cytometry using a FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA)

Membrane staining with conjugated mAb:

The cell suspension (5 x 10^5 /assay) was centrifuged 5 minutes at 300g and the pellet was resuspended with 50 µl Beriglobin/assay and kept 10 minutes on ice. 20 µl of the respective antibody was prepared in Micronic-tubes and 50 µl of the cell suspension was added, mixed and incubated 30 minutes at 4℃. Each assay was washed twice with PBS/BSA (resuspended in PBS/BSA, centrifuged (5 minutes at 300g), the supernatant was discarded) and the cells were resuspended in 50 µl FACS fluid (Becton Dickinson, Franklin Lakes, NJ). The tubes were kept on ice until they were analyzed by flow-cytometry.

3.15 Cytokine binding assay

Membrane staining with Fc fragment of immunoglobulin conjugated cytokines:

Unspecific binding of cytokines to Fcγ-receptors was blocked by incubation of cells with mouse anti-human VIAP, a calf intestine alkaline phosphatase-specific antibody. As negative control human immunoglobulin Beriglobin (Aventis Behring GmbH, Vienna) was applied. For secondary labelling phycoerythrin-conjugated $F(ab)_2$ fragment goat-anti human IgG, Fcγ specific antibody was used.

- PBS/BSA: 1x PBS + 1% BSA
- VIAP: final concentration 20 µg/ml in PBS/BSA
- Cytokines: 20 µg/ml
- Secondary antibody: phycoerythrin-conjugated $F(ab)_2$ fragment goat-anti human, Fcγ specific IgG 20 µg/ml in PBS/BSA (Jackson ImmunoResearch)

The cell suspension (5 x 10^5 /assay) was centrifuged for 5 minutes at 300g and the pellet was resuspended with 50 µl VIAP/assay and kept 20 minutes on room temperature. Then 10 µl of cytokines were prepared in Micronic-tubes and 50 µl of the cell suspension was added, mixed and incubated 30 minutes at 4℃. Each assay was washed twice with

PBS/BSA (resuspended in PBS/BSA, centrifuged (5 minutes at 300g), the supernatant was discarded). 20 µl of Fcγ specific IgG were added to the cells, and again incubated for 30 minutes at 4℃. E ach assay was washed twice with PBS/BSA (resuspended in PBS/BSA, centrifuged (5 minutes at 300g), the supernatant was discarded). The cells were resuspended in 50 µl FACS fluid (Becton Dickinson, Franklin Lakes, NJ) and the tubes were kept on ice until they were analyzed by flow cytometry using a FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA)

3.16 Immunofluorescence of cytoplasmic proteins

Cells of the DC and T cell co-culture were harvested and washed with PBS/BSA. The pellet was resuspended in 50µl Beriglobin/assay and 50µl fixation medium (Bio Research GmbH, An der Grub, Kaumberg, Austria) and incubated for 20 minutes at RT. Each assay was washed once with PBS/BSA (resuspended in PBS/BSA, centrifuged (5 minutes at 300g), the supernatant was discarded). 50 µl permeabilisation medium (Bio Research GmbH) and 20 µl mAb were added and incubated 20 minutes at RT. The cells were washed twice with PBS/BSA. 20 µl Oregon Green and 50 µl permeabilisation medium were added to the cells, and again incubated for 20 minutes at RT. The cells were washed twice with PBS/BSA. The samples were prepared for immunofluorescence analysis by addition of 50µl of FACS fluid (Becton Dickinson, Franklin Lakes, NJ). The tubes were kept on ice until they were analyzed by flow cytometry using a FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA).

3.17 Flow cytometric analysis

The FACS technique permits the isolation of different live cell populations via their different surface molecules, stained with specific fluorescent antibodies. In a flow cytometer, single cells move past the excitation source and the light hitting the cells is either scattered or absorbed and then re-emitted (fluorescence). This scattered or re-emitted light is collected by a detector. The rate of scattered light depends on cell size and complexity. The forward scatter determines the diffraction of light and

depends on the volume of cells. The sideward scatter determines the refraction of light and depends on granularity, size and structure of the nucleus and the rate of vesicles of cells.

3.18 Preparation of protein samples

0.1 µg or 1 µg of the recombinant IL-35 were mixed with 75 µl 2x Laemmli buffer containing 50 µl β-mercaptoethanol / ml (both BIORAD, Hercules, CA). Samples were frozen in liquid nitrogen and transferred to -80ºC for long term storage.

The samples were sonicated (15 s) and denatured (5min., 95ºC) before SDS-PAGE.

3.19 SDS-polyacrylamide electrophoresis (SDS-Page)

SDS Polyacrylamide Electrophoresis allows the separation of proteins according to their size. SDS has a very hydrophobic end and a highly charged part. Under denaturing conditions SDS masks the charged residues of proteins. The protein size was determined by using a protein standard of defined molecular weight.

The gel caster (Hoefer Scientific Instruments, San Francisco, CA) was prepared by assembling ethanol-cleaned glass / aluminium oxide plates and spacers. The running gel was prepared, cast up to 2 cm below the upper edge of the glass plate and covered with 96% ethanol (20 min.). After removing residual ethanol, the stacking gel was added and a comb carefully placed on top (20 min.). After successful polymerization the chambers in the gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) were filled with 1x running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). Samples (10-12 µl) were loaded along with 5 µl SeeBlue Plus2 prestained marker as protein standard (Invitrogen GmbH, Lofer, Austria). Empty slots were filled with Laemmli buffer. Gels were run constant at 60 V until the running gel was reached. Then the voltage was set to 120 V till proper separation.

Running gel (10%, for 2 gels):

4.8 ml aqua dest.
3 ml Tris HCl 1.5 M pH 8.8
4 ml Rotiphorese Gel 30 solution (Roth, Karlsruhe, Germany)
100 µl 10% SDS
100 µl 10% APS
8 µl TEMED

Stacking gel (4%, for 2 gels):

2 ml aqua dest.
840 µl Tris HCl 0.5 M pH 6.8
400 µl Rotiphorese Gel 30 solution (Roth, Karlsruhe, Germany)
35 µl 10% SDS
35 µl 10% APS
4 µl TEMED

3.20 Western Blot analysis

Blotting buffer (1x):

100 ml stock (25mM Tris, 192mM glycine)
100 ml methanol
800 ml aqua dest.

PBS-T (wash solution):

1x PBS + 0,05% Tween-20

Blocking solution:

5% milk in PBS-T

After electrophoresis, SDS – gels were separated from the glass plates and marked at one end. The PVDF membrane (Immobilon-P, 0.45 µm, Millipore, Billerica, MA) and filterplates (blotting paper 703, VWR, Darmstadt, Germany) were cut to fit the size of the gel. The PVDF membrane was shaken with methanol (1 min.) and equilibrated together

with the gel in blotting buffer (5 min.). Finally the sandwich was assembled by putting membrane and gel between 3 buffer soaked filter papers at each side. The proteins were transferred onto the membrane at a constant voltage (15 V, 1 h) using the Semi-Phor blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA).

To prevent antibodies from unspecific binding to the membrane, it was shaken with blocking solution (1 h, 4ºC). Incubation with the primary mouse anti-human EBI3 antibody were carried out in a 50 ml falcon tube (Sarstedt, Nümbrecht, Germany) with a minimum of 4 ml antibody solution diluted in blocking solution (7 rpm, overnight, 4ºC). After washing (3x 10 min.) membranes were incubated with the secondary HRP conjugated anti-mouse-Ig antibody dilution (1:10000 in blocking buffer) in 50 ml falcon tube (7 rpm, overnight, 4ºC). The membranes were washed 3 times for 10 min.

Enhanced chemiluminescence (ECL) was used for the detection of proteins. Therefore membranes were incubated with ECL solution (20 ml 1 M Tris pH 8.8, 500 µl p-coumaric acid 340 mg in 26 ml DMSO, 1 ml Luminol 2.26 g in 51 ml DMSO, 180 ml aqua dest.) mixed with 3 µl/ml 3% hydrogen peroxide for 1 min., placed between two plastic overhead sheets, covered with a Kodak XAR film (Sigma-Aldrich, St. Louise, MO) and placed into a film cassette. After exposure from 1 s up to 1 h specific bands were visible on the film where light had been emitted during HRP enzyme activity after developing the film in a developing machine.

3.21 Quantification of cytokines via LUMINEX^{100}

The Flourokine MAP system, using LUMINEX xMAP technology (R&D Systems Inc., Minneapolis, MN), provides a tool to simultaneously measure multiple cytokines in a single sample. Analyte-specific antibodies are pre-coated onto colour-coded beads. Standards and samples are pipetted into the wells and analytes of interest are bound by the immobilized antibodies. After washing away any unbound substances, labelled antibodies specific to the analytes of interest are added. Following a wash to remove any unbound labelled antibodies, the beads are resuspended in buffer and read using the LUMINEX^{100} analyser. Cell culture - supernatants remaining from various experiments (DC incubated with or without human recombinant IL-35) were measured via the Luminex^{100} System (R&D Systems Inc., Minneapolis, MN) for their content of IL-10, IL-6, IL-1β, IL-12 p40, IL-12p70 and TNFα.

3.22 Cell culture of tumor cell lines

Cells of the Hela Ohio, A431, Jurkat, Daudi and Otma cell lines were cultured in RPMI1640 (Gibco Ltd., Paisley, Scotland) supplemented with 10% characterized FCS (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich). The SCC cell line was cultured in DMEM (Gibco Ltd., Paisley, Scotland) supplemented with 10% characterized FCS (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich), non-essential amino acids, 5 µg/ml Insulin (Sigma Aldrich) and 10 µg/ml Transferrin (Sigma Aldrich). Cells were cultured at 37℃ with 5% CO2.

3.23 Buffers and media

RPMI medium: Gibco Ltd., Paisley, Scotland

Heparin medium:

500ml RPMI 1640 medium supplemented with 1% glutamine and 2.5 ml antibiotic solution, i.e. (prepared penicillin/streptomycin mixture stored at –20℃). Add 1 ml heparin (EBEWE Pharma, Unterach, Au stria) of the stock solution.

DMEM medium: Gibco Ltd., Paisley, Scotland

Freezing medium:

RPMI 1640 supplemented with 25% FCS and 10% DMSO (Sigma)

MACS-buffer (stored at 0℃):

1000 ml 1x PBS def. + 25 ml HSA (stock: 20%, Centeon, Vienna) + 10 ml EDTA (stock: 0.5 M); sterile filtered

10x PBS stock solution:

5.8 g KH2PO4 16.6 g Na2HPO4 2H2O 72 g NaCl Solubilise the salts in aquabidest (=ddH2O), fill up to 10 litres and adjust pH to 7.2

PBS/BSA stock solution (20%):

100 g BSA 10 g NaN3 dissolved in 500 ml PBS buffer. For the PBS/BSA wash buffer, prepare a 1:20 dilution with PBS buffer.

4 RESULTS

4.1 Human IL-35 fusionprotein can be detected by an anti-EBI3 mAb

We have described recently that human rhinovirus (HRV) activated DCs, named R-DCs, induce suppressor function in T cells and the production of IL-35 by these cells. The supernatant of this R-DC/T cell co-culture reduces the proliferation of T cells that were activated via DCs (43). To test the function of IL-35 alone we investigated in a human IL-35 fusionprotein. It carries an Fc fragment, named IL-35:Fc. First of all we determined if an anti-EBI3 mAb recognizes this protein in a western blot to prove its IL-35 identity. Therefore samples with 0.1 µg or 1 µg of IL-35:Fc were generated and analysed with the anti-EBI3 mAb or with anti-VIAP mAb, a IgG1 isotype control, as negative control.

As shown in Figure 6 the human IL-35 was detected with the anti-EBI3 mAb. Therefore it can be assumed that the protein concerns IL-35.

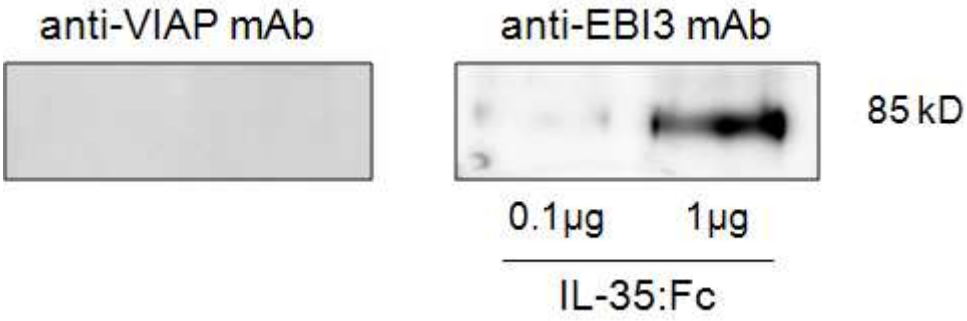


Figure 6: Anti-EBI3 mAb detects IL-35:Fc

Samples of 0.1 µg or 1 µg IL-35:Fc (right) were generated for SDS-PAGE. Specific signals for EBI3 were detected by western blot analysis.

4.2 Human recombinant IL-35 suppresses peripheral blood T cell proliferation induced by dendritic cells

Because of our recent findings, we investigated in the effects of the human recombinant IL-35 on T cell proliferation. Therefore we co-cultured peripheral blood T cells with monocyte derived DCs and graded amounts of IL-35 for 5 to 6 days. The DCs were either immature or LPS activated (mDCs). We observed a suppression of T cell proliferation induced by immature as well as mature DCs that is most effective at a concentration of 1 ng/ml IL-35. The titration curve is depicted in Figure 7.

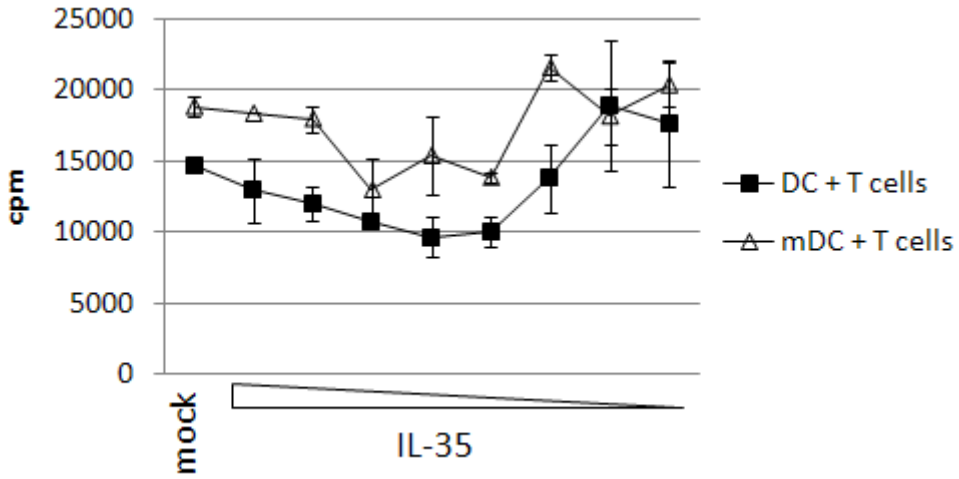


Figure 7: IL-35 suppresses T cell proliferation most effective at a concentration of 1 ng/ml

DCs ($1x10^4$ cells) were co-cultured with peripheral blood T cells (1×10^5) and graded amounts of human IL-35 from 100 ng/ml to 0.01 pg/ml for 5 days. Black filled triangles show immature DCs; white triangles show LPS activated DCs (24h). Proliferation of T cells was monitored on day 5 culture by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18h later. The results of one representative experiment of 3 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

4.3 The suppression of T cell proliferation through IL-35 is comparable to the supernatant of a R-DC/T cell co-culture

We further investigated in the suppressive function of IL-35. Therefore we compared the supernatant of a R-DC/T cell co-culture with the human recombinant IL-35. To generate R-DCs monocyte derived dendritic cells were first incubated with HRV14 for 48 hours. Then R-DCs or untreated DCs were co-cultured with T cells in a 1:5 to 1:10 ratio for 1 day. The supernatants of these co-cultures were harvested and tested for their inhibitory effect in a MLR. To test whether IL-35 alone also has suppressor function, human IL-35 was added to the MLR. Therefore 1 x 10^4 DCs were mixed with 1 x 10^5 T cells and the supernatant of the DC/T cell co-culture or the R-DC/T cell co-culture (at a volume of 100 µl) or 1 ng/ml human IL-35 was added. We tested isolated peripheral blood T cells and naive T cells from cord blood for their proliferation capacity after DC stimulation.

Human IL-35 suppresses proliferation of peripheral blood T cells after DC activation almost as efficient as the supernatant of a R-DC/T cell co-culture, which was previously described (Fig. 8A). In contrast, naive T cell proliferation was not inhibited by IL-35 or the supernatant of R-DC/T cell co-culture (Fig. 8B). The supernatant of a DC/T cell co-culture has no effect on T cell proliferation. The same effect of inhibition by IL-35 and the R-DC/T cell co-culture supernatant can be shown when T cells were activated with LPS treated mature DCs (mDCs) as depicted in Figure 8C.

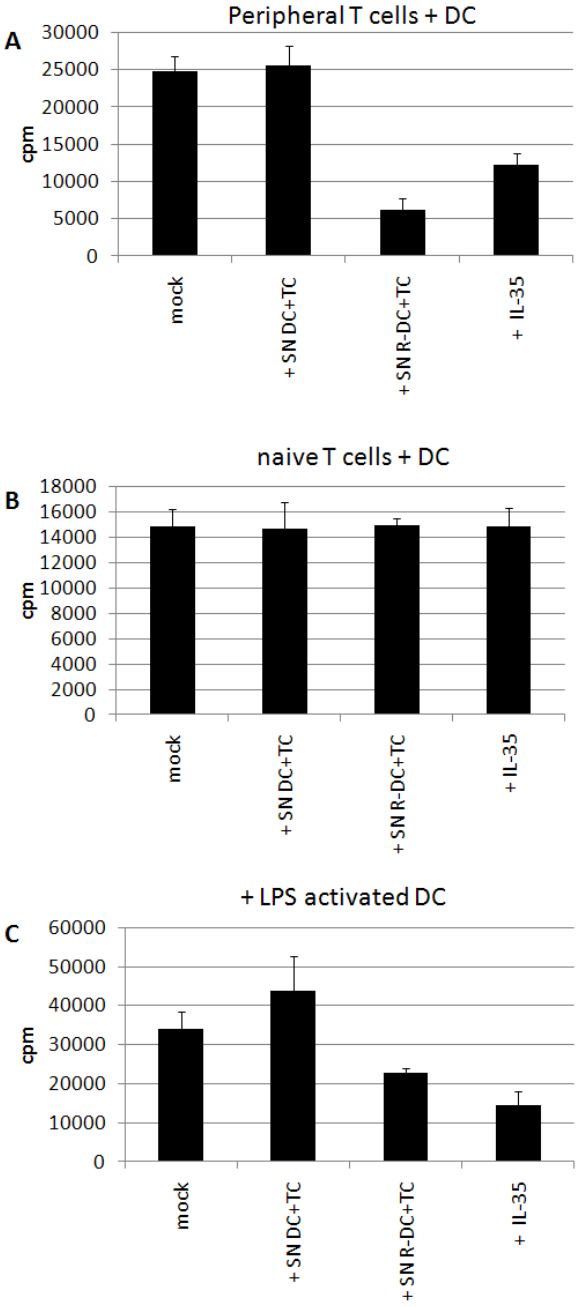


Figure 8: IL-35 inhibits DC dependent T cell proliferation

DCs ($1x10^4$ cells) were co-cultured with A) peripheral blood T cells or B) naive T cells of cord blood ($1x10^5$ cells) and the supernatants of DC/ T cell, R-DC/T cell co-culture or 1 ng/ml of human IL-35 for 5 days. C) DCs were activated for 24h with LPS and then co-cultured with peripheral blood T cells for 5 days. Proliferation of T cells was monitored on day 5 culture by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18h later. The results of one representative experiment of 3 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

4.4 Human recombinant IL-35 does not affect T cell proliferation in absence of APCs

To determine if T cell proliferation induced by an APC-free or DC-free, alternative stimulation can also be inhibited through human IL-35, we used different monoclonal antibodies for T cell activation. TCR signalling is initiated through the binding of the CD3 mAb (OKT3) to the ζ-chain of the CD3 molecule expressed on mature T cells. T cells deliver signal 1 by binding of OKT3 and get activated. Signal 2, induced by co-stimulation, is provided by the binding of anti-CD28 and anti-CD63 mAbs. The co-stimulatory molecules CD28 and CD63 are expressed on T cells and induce a stronger T cell activation signal when they interact with their ligands on APCs. Human peripheral T cells were activated with plate-bound mAbs against CD3, CD3 combined with CD28 and CD3 in combination with CD63. Different concentrations of human IL-35 were added and the cells were cultured for 3 days.

As shown in Figure 9 human IL-35 does not affect T cell proliferation after anti-CD3, anti-CD3/CD28 or anti-CD3/CD63 stimulation. There was neither an increase nor a decrease in comparison to the untreated T cells. Even high doses of IL-35 did not reveal differences in T cell proliferation.

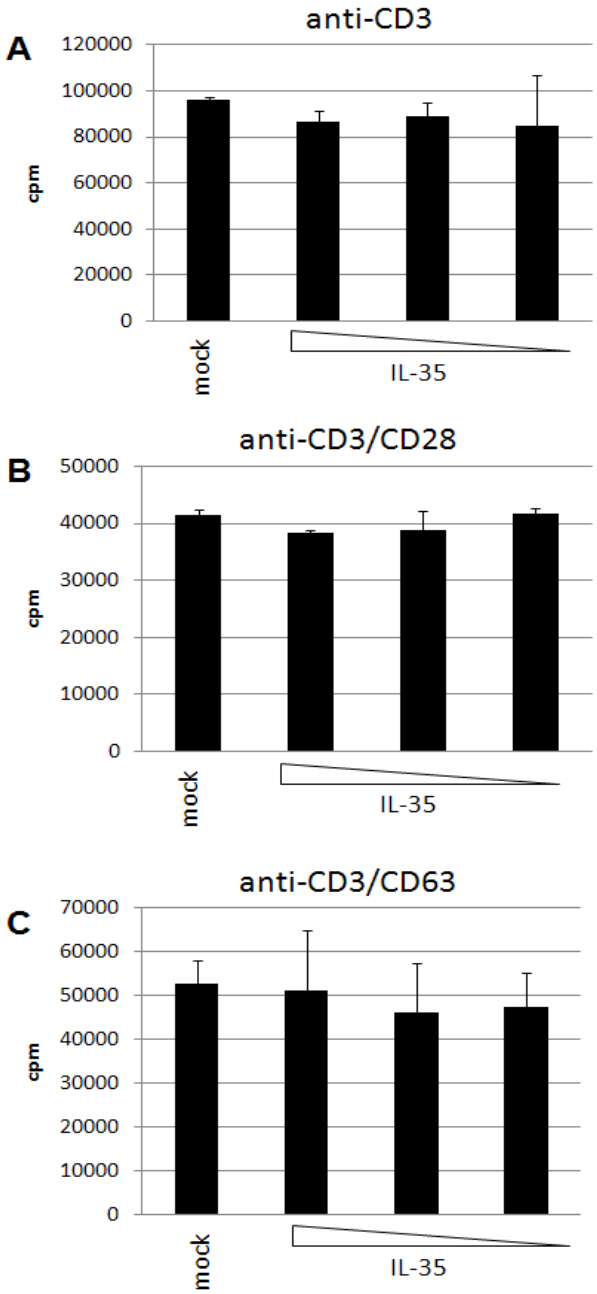


Figure 9: DC independent T cell proliferation cannot be suppressed by IL-35

T cells were stimulated with plate-bound A) CD3, B) CD3/CD28 and C) CD3/CD63 mAb or mock stimulation for 3 days and cultured in the presence of 0.1, 1 and 10 ng/ml of human IL-35. Proliferation of T cells was monitored on day 5 of culture by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18 h later. The results of one representative experiment of 3 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

4.5 DCs pretreated with human recombinant IL-35 have a reduced T cell stimulatory capacity

As shown before T cell proliferation can be inhibited by human IL-35, when DCs serve as stimulators. But in a DC-free environment, activated through binding of anti-CD3, anti-CD3/CD28 and anti-CD3/CD63 mAbs, T cells treated with IL-35 proliferate the same way as the untreated cells do. So we investigated whether IL-35 does affect DCs or T cells. We tested the direct effect of IL-35 on DCs. Therefore monocytes were cultivated with IL-4 and GM-CSF for three days, not yet reaching the DC status. We added IL-35 or IL-10, for comparison, and continued culturing them for another three days. After these six days we got DCs, further termed IL-35-DCs or IL-10-DCs. We also generated untreated DCs, which serve as control. On day 6 the immature DCs were activated with LPS to get mature DCs (Fig. 9). After 24 hours the cells were harvested and supernatants were collected. We performed a MLR with these DCs as stimulators and analysed the cells for their surface marker profile. Cytokines typical for DCs were measured via LUMINEX[100]. The workflow of this DC generation is depicted in Figure 10.

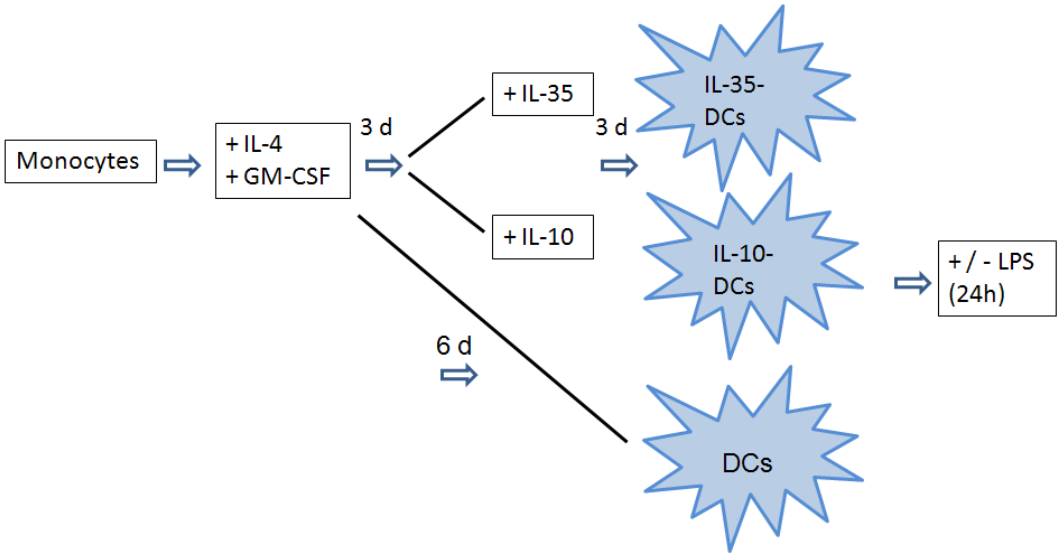


Figure 10: Scheme of IL-35-DCs generation

Monocytes were cultivated with IL-4 and GM-CSF for 3 days, IL-35 or IL-10 was added. After 3 days DCs were activated with LPS for 24 hours.

4.5.1 IL-35-DCs have a reduced capacity to activate T cells

We investigated whether these IL-35 pretreated DCs (IL-35-DCs) have reduced T cell stimulatory capacity. To compare the effect with a known DC suppressor cytokine, we also generated IL-10 pretreated DCs (IL-10-DCs). IL-10 is described to inhibit DCs by reducing IL-12 and IFNγ release (2).

DCs, IL-35-DCs and IL-10-DCs, either immature or LPS activated, were co-cultured with T cells for 5 days. We observed that DCs treated with IL-35 have a reduced capacity to stimulate T cells. Immature IL-35-DCs show a similar suppression of T cell proliferation as IL-10 treated cells (Fig.11A). Compared to the co-culture of untreated DCs and T cells with addition of recombinant IL-35 the T cell stimulatory capacity of the IL-35-DCs is even more reduced (Fig. 8). When IL-35-DCs were activated with LPS they lose their ability to inhibit T cell proliferation. This effect cannot be compared with matured IL-10-DCs, because they maintain their inhibitory character (Fig. 11B).

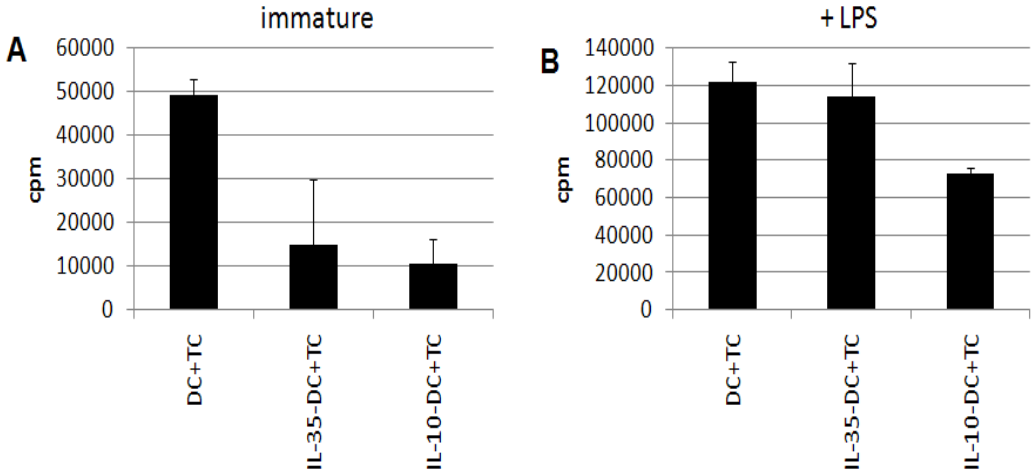


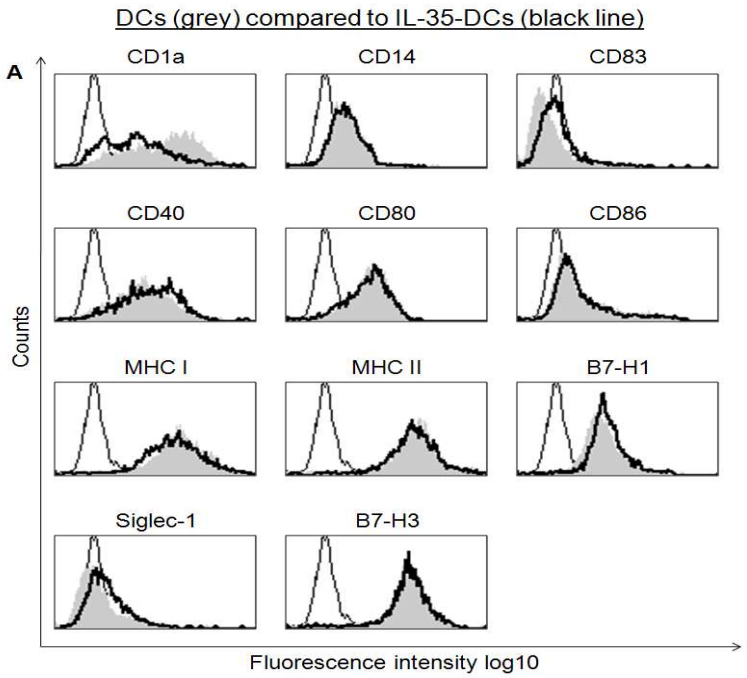
Figure 11: Immature IL-35-DCs inhibit T cell proliferation nearly as efficient as IL-10 treated DCs

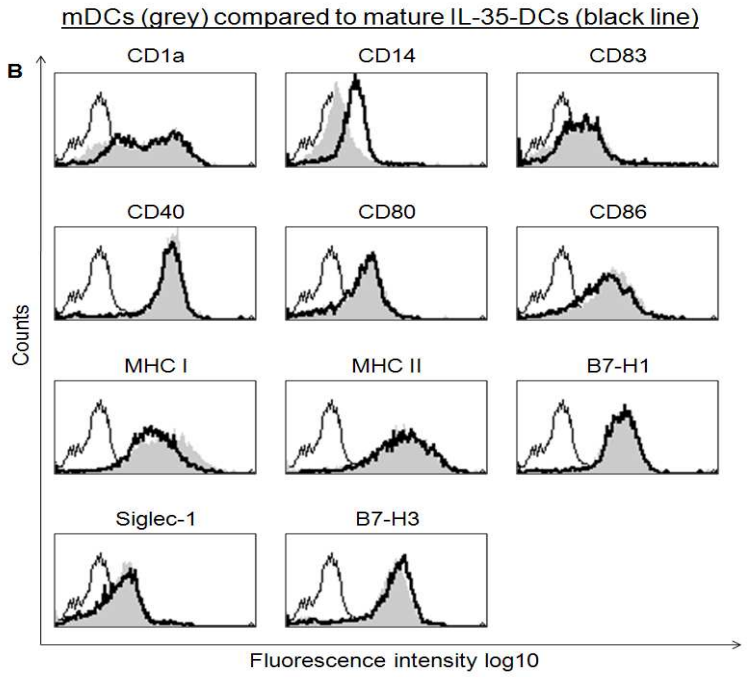
DCs, IL-35-DCs or IL-10-DCs (1 x 10^4 cells), immature (A) or LPS-activated (B) were co-cultured with peripheral blood T cells (1 x 10^5 cells) for 5 days. Proliferation of T cells was monitored on day 5 culture by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18 h later. The results of one representative experiment of 6 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

4.5.2 Cell surface marker profile of IL-35-DCs

Typical surface molecules, such as CD83 or MHC II, expressed on DCs get up-regulated when they are activated. In the presence of inhibitory factors like IL-10 the expression level of these DC markers decreases in immature cells (2). Therefore we analysed the expression levels of CD1a, CD14, CD40, CD80, CD83, CD86, MHC I, MHC II, B7-H1, B7-H3 and Siglec-1 on DCs, IL-35-DCs or IL-10-DCs. CD1a and CD14 served as controls for the verification that monocytes changed to DCs, because CD1a is expressed on DCs and CD14 is expressed on monocytes and its expression decreases on DCs. The surface markers CD40, CD80, CD83, CD86, MHC I, MHC II, B7-H1, B7-H3 and Siglec-1 are higher expressed when DCs get mature. IL-10 decreases the expression of co-stimulatory molecules, like CD80 and CD86, and MHC on DCs (2). The expression levels were measured by flow cytometry.

As shown in Figure 12A, the expression of all tested surface markers remained unchanged when DCs were incubated with IL-35. LPS activation leads to a general up-regulation of the DC markers. Comparison with LPS-activated IL-35-DCs shows that the markers CD83, CD40, CD80, CD86, MHC I, MHC II, B7-H1, Siglec-1 and B7-H3 are expressed at the same level. CD14 expression was unaffected at the majority of the FACS staining (Fig. 12B). In IL-10 pretreated DCs compared to untreated DCs the expression levels of the surface markers remain mostly unaffected (Fig. 12C). When IL-10-DCs were activated with LPS the cells persist immature, because the expression of the surface molecules in comparison to mature DCs is diminished (Fig. 12D). It seems that maturation cannot be achieved when DCs were cultured with IL-10 during their development.


DCs (grey) compared to IL-35-DCs (black line)
A
CD1a
CD14
CD83
CD40
CD80
CD86
MHC I
MHC II
B7-H1
Siglec-1
B7-H3
Counts
Fluorescence intensity log10


mDCs (grey) compared to mature IL-35-DCs (black line)
B
CD1a
CD14
CD83
CD40
CD80
CD86
MHC I
MHC II
B7-H1
Siglec-1
B7-H3
Counts
Fluorescence intensity log10

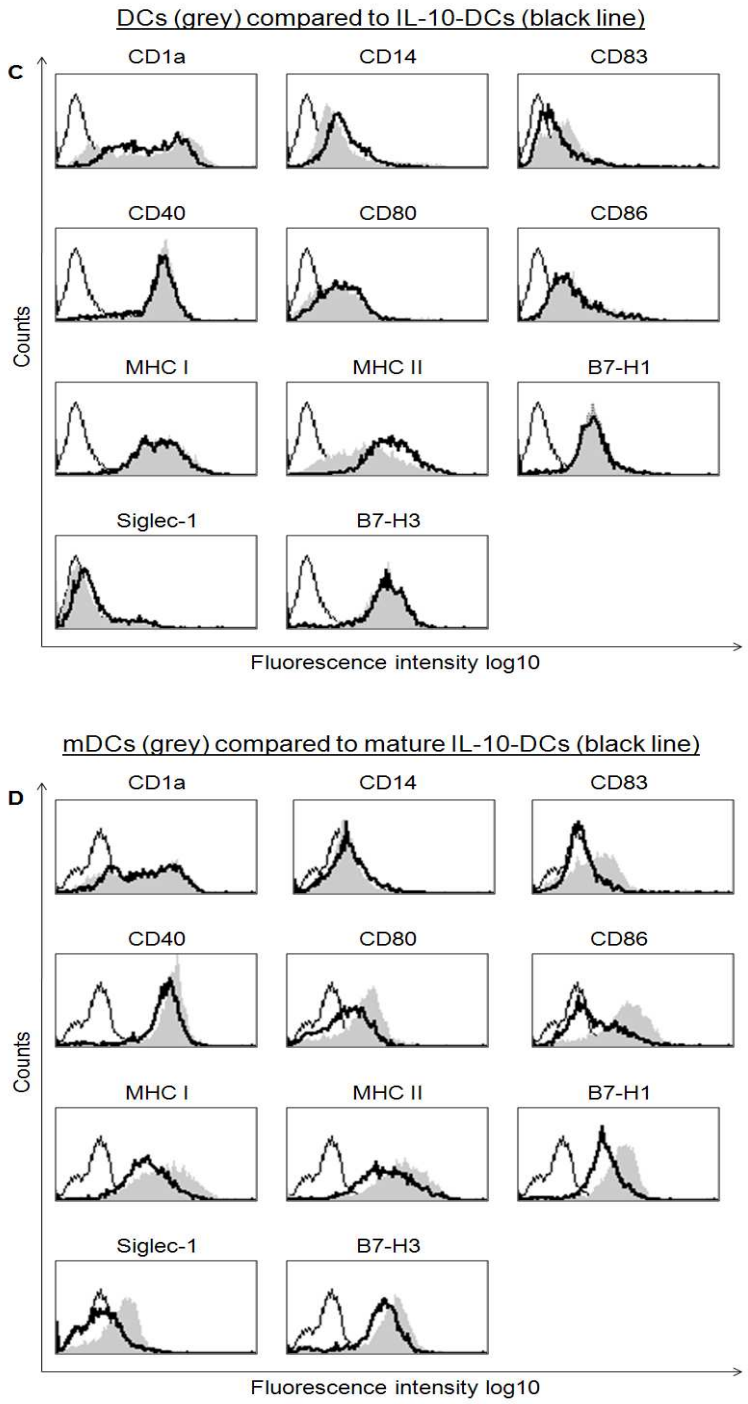


Figure 12: Surface marker profile of DCs compared with IL-35-DCs

Immature DCs and IL-35-DCs (A), DCs and IL-35-DCs stimulated with LPS for 24h (B), immature DCs and IL-10-DCs (C), DCs and IL-10-DCs stimulated with LPS for 24h (D) were stained with the mAbs against CD1a, CD14, CD83, CD40, CD80, CD86, MHC I, MHC II, B7-H1, Siglec-1 and B7-H3 and analysed by FACS. Black dotted line: Isotype control; grey filled histogram: DCs or mDCs, black line: DCs/mDCs treated with IL-35 or IL-10. FACS histogram plots of 1 representative experiment out of 3 are shown.

4.5.3 Cytokine profile of IL-35 treated DCs

We further analysed the effect of IL-35 on cytokine secretion by DCs. After addition of IL-35 or IL-10 to DCs and activation with LPS, we harvested the supernatants and determined the levels of secreted cytokines. We measured IL-10, IL-1β, IL-6, IFNγ, TNFα and IL-12p40, one subunit of the IL-12 cytokine. For IL-10-DCs we expected a decreased IL-12p40, IL-6 and TNFα production compared to untreated DCs. We were interested if IL-35-DCs also have a diminished pro-inflammatory cytokine secretion. Therefore we collected the supernatants of DCs, IL-35-DCs and IL-10-DCs, immature and LPS activated, and analysed them via LUMINEX^{100}.

There is no release of cytokines detected in the supernatants of immature DCs, because the release starts when DCs were activated. Pretreatment of DCs with IL-35 or IL-10 does not affect the secretion. Only IL-10 is detectable in the supernatant IL-10-DCs due to the fact that IL-10 was added during IL-10-DCs generation. The same effect occurs after LPS activation. Figure 13 shows that IL-10 release by mature DCs is reduced after IL-35 treatment and also IL-12p40 is suppressed although to a lesser degree. But the secretion of IL-6 and TNFα by LPS activated IL-35-DCs increased slightly, whereas the supernatants of IL-10-DCs show a down-regulation of these cytokines compared to untreated DCs.

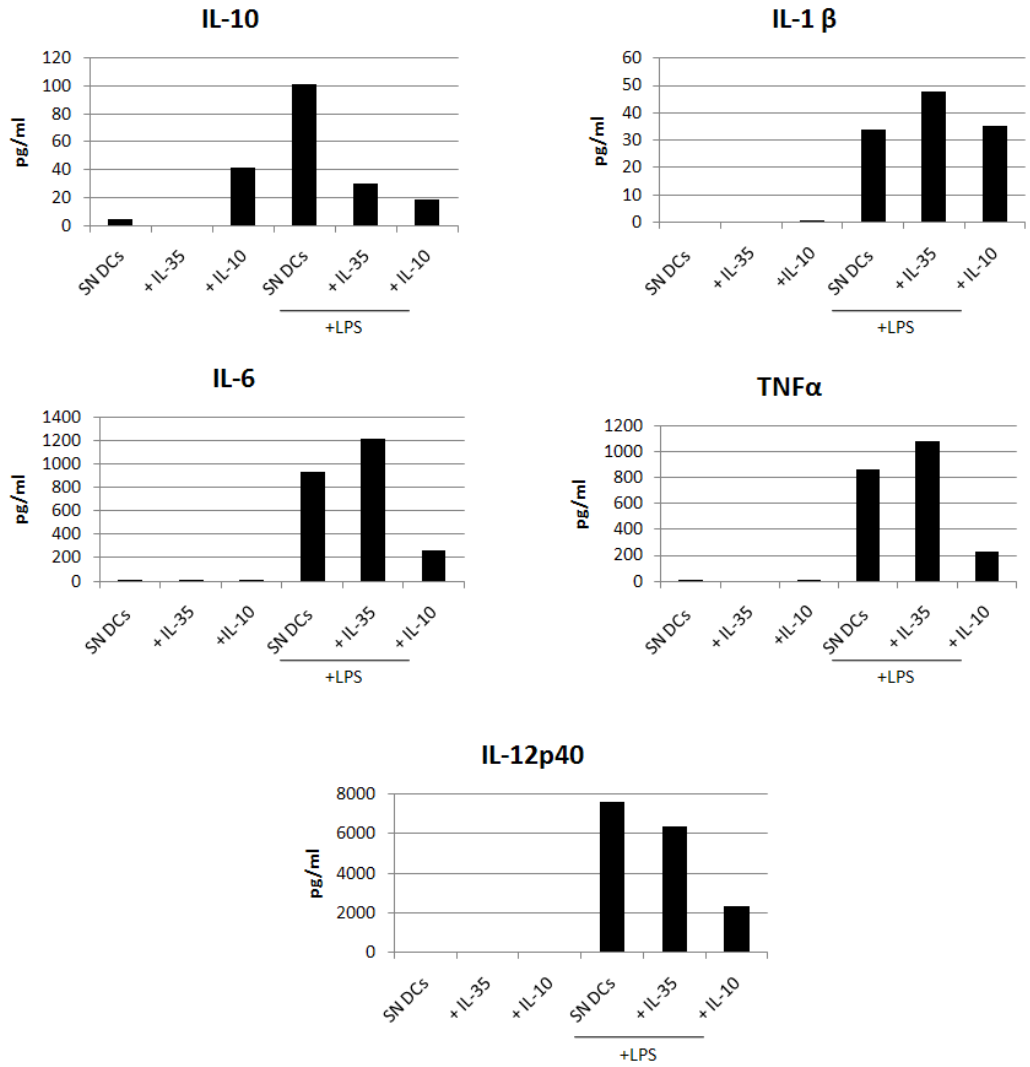


Figure 13: Cytokine profile of the supernatants of DCs, IL-35-DCs and IL-10-DCs

The supernatants of the DCs were harvested after IL-35 or IL-10 addition and LPS activation. The cytokines IL-10, IL-1β, IL-6, TNFα and IL-12p40 were measured via LUMINEX[100]. The results of one representative experiment of 3 independent experiments are shown.

4.6 Human IL-35 fusionprotein binds to DCs but not to peripheral blood T cells

Because of the assumption that IL-35 acts on DCs by inhibiting their T cell stimulatory capacity, we were interested if IL-35 can also bind to DCs and/or T cells. Therefore we used a human IL-35 fusionprotein fused to a Fc fragment, termed IL-35:Fc. We were able to detect its binding via a secondary mAb that binds to the Fc part of the fusionprotein. We blocked the Fcγ receptors of the cells with a mouse IgG mAb, and then incubated them with IL-35:Fc. For detection of cells that bind IL-35 a secondary phycoerythrin-conjugated $F(ab)_2$ fragment goat-anti human, Fcγ specific, IgG was added and the cells were analysed via FACS. Human immunoglobulin (IgG), termed Beriglobin, served as negative control. A CTLA-4 fusionprotein fused to the Fc fragment also served as a control, because its receptors, CD80 and CD86, are expressed on DCs (118). We analysed the binding ability of IL-35:Fc to monocytes, DCs, mature DCs (mDCs) activated with LPS and peripheral blood T cells via flow cytometry.

Results presented in Figure 14 show that IL-35 binds to DCs to the same extent as CTLA-4 does. The expression of the unknown IL-35 receptor seems to increase on DCs when they were activated with LPS. These findings were observed in two experiments out of five, so clearly more tests have to be performed. Monocytes neither bind CTLA-4 nor IL-35. A binding of IL-35 to T cells was also not detected. The ability of the IL-35:Fc to bind DCs goes in accordance with the found reduction of the T cell stimulatory capacity of DCs.

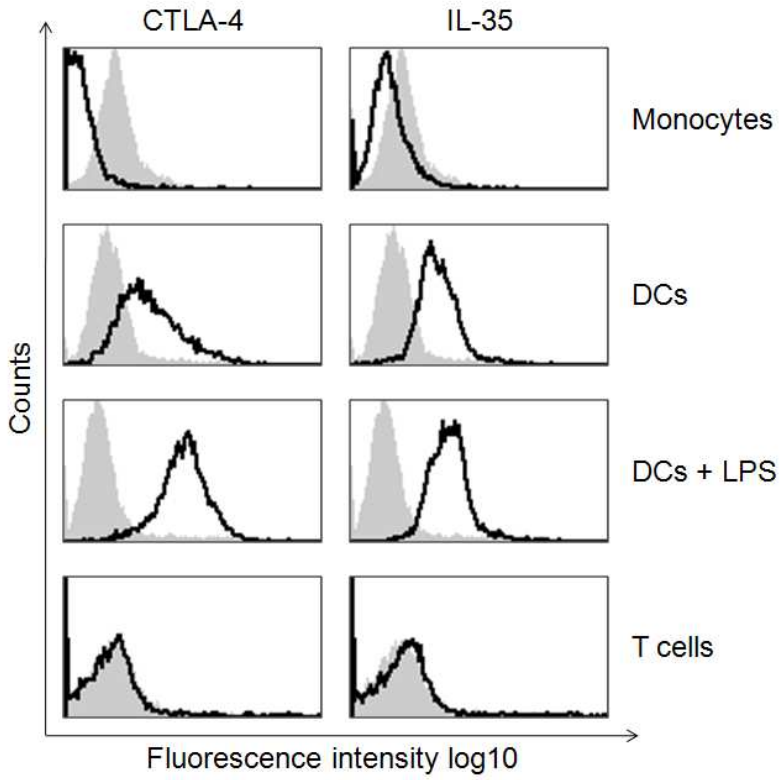


Figure 14: Binding analysis of IL-35:Fc

Monocytes were incubated with IL-4 and GM-CSF for 6 days to obtain DCs, T cells were purified from blood. The cells were blocked with human IgG, VIAP, and incubated with CTLA-4:Fc (left) or IL-35:Fc (right). Beriglobin serves as negative control (grey histogram). The black line shows CTLA-4 or IL-35. The binding was detected with a phycoerythrin-conjugated $F(ab)_2$ fragment goat-anti human, Fcγ specific, IgG via FACS. The results of one experiment out of 2 positive ones from 5 independent experiments are shown.

4.7 Human IL-35:Fc binds to HeLa Ohio cells but not to other cell lines

Because of the binding ability of IL-35:Fc to DCs, we wondered how specific this effect would be. Therefore we tested whether the IL-35:Fc can bind other cell types. We analysed epithelial (HeLa Ohio, A431 and squamous cell carcinomas (SCC), and hematopoietic (Jurkat and Otma) cell lines. The binding analysis shows that IL-35:Fc binds to HeLa Ohio cells but not to the other tested cell lines, as shown in Figure 15.

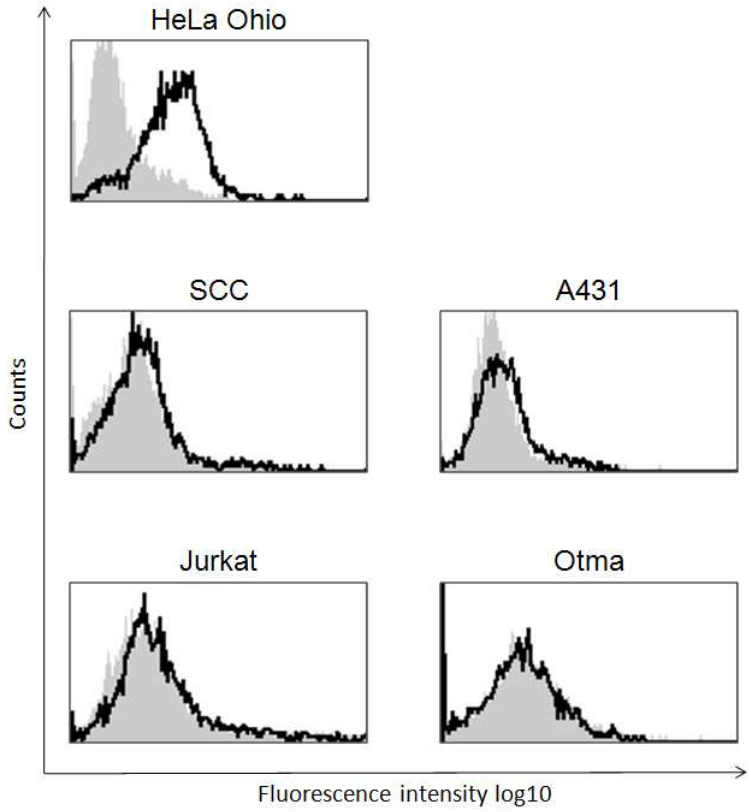


Figure 15: Binding analysis of IL-35:Fc fusionprotein to the utilized cell lines

The cells were blocked with human IgG, VIAP, and incubated with IL-35:Fc. Beriglobin served as negative control (grey histogram). The black line shows IL-35. The binding was detected with a phycoerythrin-conjugated $F(ab)_2$ fragment goat-anti human, Fcγ specific, IgG via FACS.

4.8 Human recombinant IL-35 suppresses cell growth of the tumor cell line HeLa Ohio

HeLa was the first human epithelial cancer cell line established in long-term culture. It was derived from an adenocarcinoma of the cervix in 1951 from cancer cells (119). The cells have a hypertriploid chromosome number, specific numerical deviations and 20 clonally abnormal chromosomes (119). The genome of HeLa cells is characterized by multiple copies of the human papilloma virus type 18 (HPV18) integrated at specific sites (120). HeLa cells are adherent cells and sustain contact

inhibition *in vitro*. The HeLa Ohio cells are derivative of the parent HeLa line.

To examine if the binding ability of IL-35 to HeLa Ohio cells goes in accordance to its inhibitory capacity we were interested if IL-35 has the capacity to suppress the cell growth of tumor cell lines. First we added human IL-35 to the HeLa Ohio cell line and measured their proliferation rate via thymidine incorporation.

We observed that IL-35 is able to inhibit the proliferation of HeLa Ohio cells (Fig. 16).

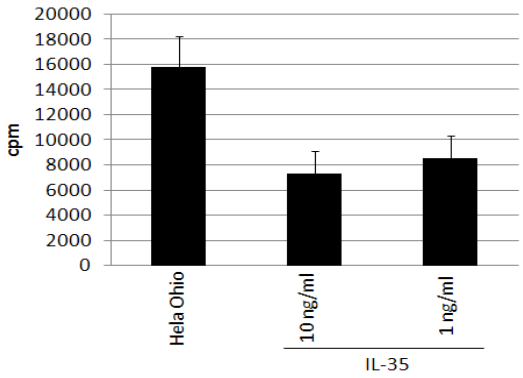


Figure 16: Cell growth inhibition of HeLa Ohio cells by IL-35

1 x 10^5 HeLa Ohio cells were cultured for 24 h with 10 ng/ml or 1 ng/ml human IL-35. Proliferation of HeLa Ohio cells was monitored by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18h later. The results of one representative experiment of 3 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

Because of this finding we were interested whether IL-35 is a common tumor suppressor or this effect is HeLa specific. We tested the cell number of HeLa cells, the time point and the concentration of IL-35 to get an efficient tumor cell growth inhibition. We used 2.5 x 10^4, 5 x 10^4 and 7.5 x 10^4 cells and let them grow for 24, 48 and 72 hours. Titrated IL-35 from 1 µg/ml to 0.1 ng/ml was added to each approach. After 24, 48 or 72 hours we added H3 thymidine and harvested the cells 18 hours later. Figure 17 shows the 48 hours time point with 5 x 10^4 HeLa Ohio cells. For further

experiments we decided to use 5 x 10^4 cells of each cell line and 10 ng/ml and/or 1 ng/ml human IL-35.

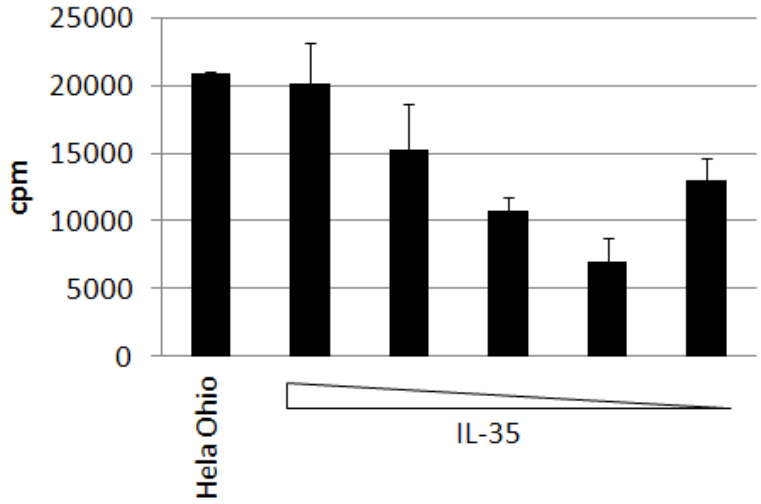


Figure 17: Titration of IL-35 for cell growth inhibition in HeLa Ohio cells

5 x 10^4 HeLa Ohio cells were co-cultured with 1 µg/ml to 0.1 ng/ml human IL-35 for 48h. Proliferation of HeLa Ohio cells was monitored by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18h later. The results of one representative experiment of 3 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

4.8.1 Other epithelial cell lines show no suppressed proliferation after addition of IL-35

Because of the suppressive effect of IL-35 on HeLa Ohio cells, we were interested if this cytokine is also able to inhibit the proliferation of other epithelial cell lines. We tested different squamous cell carcinomas (SCC) from the tongue, termed SCC4 and SCC9, and an epithelial cell line of the skin (A431).

5 x 10^4 cells of each cell line were cultured with 10 ng/ml or 1 ng/ml for 48 hours. Human IL-35 does not affect cell growth of these cells (Fig. 18).

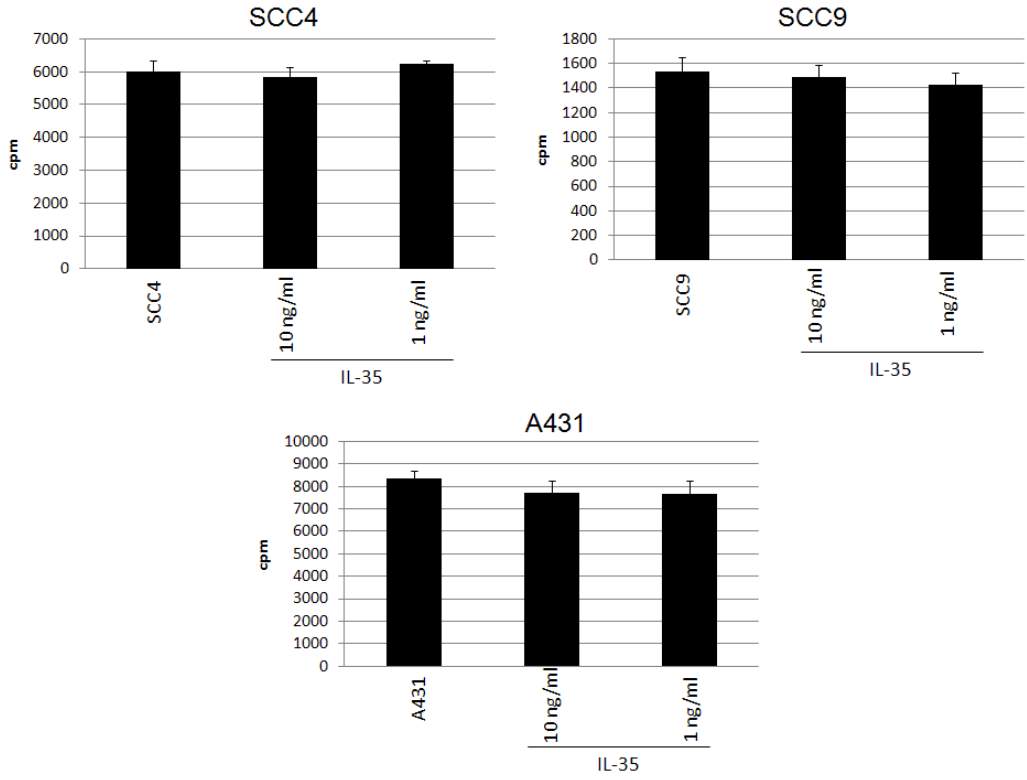


Figure 18: IL-35 does not suppress the proliferation of other epithelial tumor cell lines

$5x10^4$ cells of the cell lines SCC4, SCC9 and A431 were co-cultured with 10ng/ml or 1ng/ml human IL-35 for 48h. Proliferation of the cells was monitored by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18h later. The results of one representative experiment of 3 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

4.8.2 IL-35 does not suppress cell growth of hematopoietic cell lines

To determine if IL-35 has an effect on hematopoietic cell lines we tested three different cell lines. Jurkat cells, derived from human T cell leukaemia, Daudi cells, derived from Burkitts lymphoma and transformed with EBV, and Otma cells, EBV-transformed B cells, were cultivated with 10 ng/ml or 1 ng/ml human recombinant IL-35 for 2 days. The proliferation of all three hematopoietic cell lines used cannot be suppressed with human IL-35 (Fig. 19).

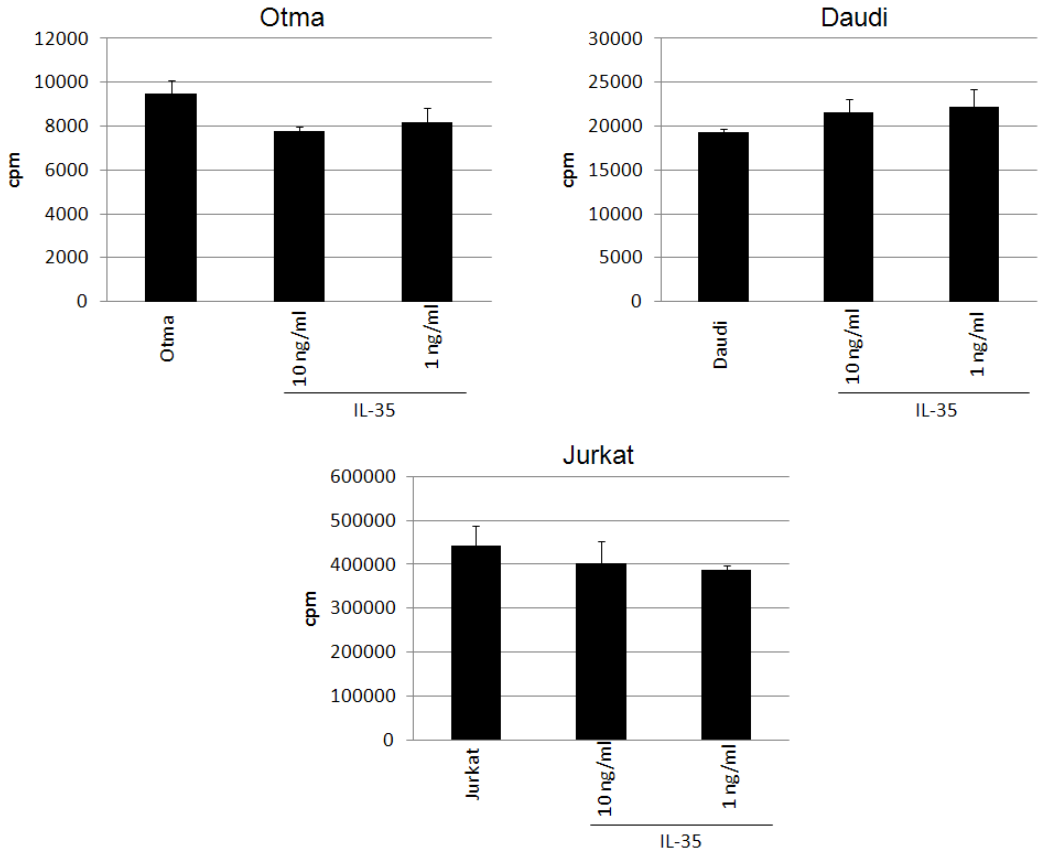


Figure 19: IL-35 does not suppress the proliferation of various hematopoietic cell lines

5 x 10^4 cells of Otma, Daudi and Jurkat were co-cultured with 10ng/ml or 1ng/ml human IL-35 for 48h. Proliferation of the cells was monitored by adding [methyl-3H]TdR followed by measuring [methyl-3H]TdR incorporation 18h later. The results of one representative experiment of 3 independent experiments are shown. Mean values of triplicate determinations ± standard deviation are shown.

4.9 EBI3 is up-regulated on T cells co-cultured with human rhinovirus or IFNα treated DCs

We recently demonstrated that IL-35 is produced by regulatory T cells induced by human rhinovirus (HRV14) activated DCs (R-DCs). We could also show that EBI3, a subunit of IL-35, is up-regulated in T cells co-cultured with these R-DCs (43). Because of these findings we were interested if EBI3 is also up-regulated in T cells when they were co-cultured with other types of DCs. Therefore we stimulated monocyte

derived DCs for 3 days with HRV14, Lipopolysaccharide (LPS), Zymosan (ZYM), IFNα and poly-IC RNA. The differently activated DCs and, as control, immature DCs were co-cultured with T cells for 1 day. The cells were harvested and stained intracellulary for p35 and EBI3. The staining was analysed via FACS with a special attention on the T cells.

Anti-S100-A4 mAb, a calcium-binding protein, and anti-Mxa mAb, an IFNα/β inducible protein, served as controls. The VIAP antibody serves as isotype control.

As depicted in Figure 20 all T cells co-cultured with different types of DCs show S100-A4 expression. P35, a subunit of IL-35 and IL-12, is described to be constitutively expressed in all T cells, which is the case, and is up-regulated in T cells co-cultured with virus treated DCs (R-DCs) and in T cells co-cultured with IFNα treated DCs. EBI3 is highly expressed on the R-DC treated T cells and the IFNα treated T cells. In all other types of activated T cells EBI3, which serves as a subunit for IL-35 and IL-27, is only expressed at very low levels. The positive control for virus infected and also for IFNα treated cells, Mxa, is highly expressed in those T cells as well.

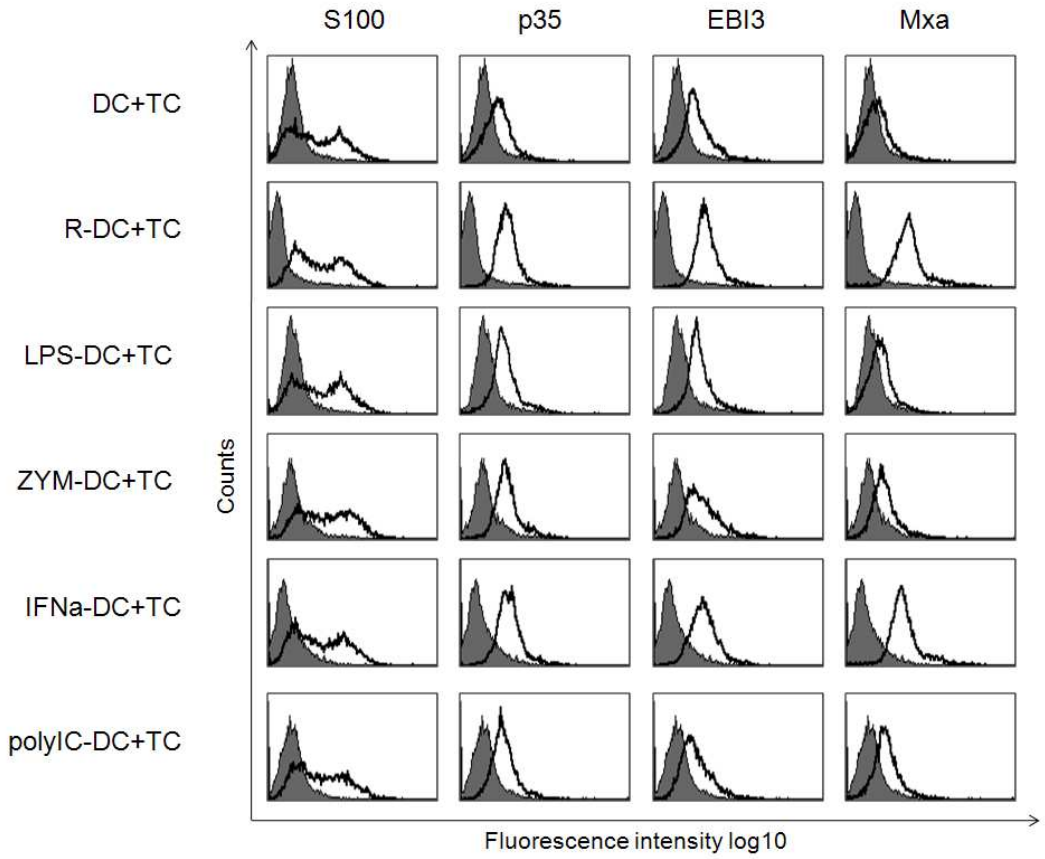


Figure 20: EBI3 and p35 are up-regulated in T cells co-cultured with R-DCs or IFNα treated DCs

Immature DCs and DCs activated with HRV14, LPS, Zymosan, IFNα and poly IC (from top) for 3 days were co-cultured with T cells from a different donor for 1 day and then stained intracellulary with mAbs against S100, p35, EBI3 and Mxa (from left) and analysed by FACS. Cells were gated on T cells. Grey histogram: Isotype control; black line: specific mAb. FACS histogram plots of 1 representative experiment out of 3 are shown.

4.10 T cells co-cultured with R-DCs show a diminished CD2 expression

In the FACS analysis of differently activated DCs in combination with T cells we were also interested in various surface markers of T cells. We observed no effect at the expression levels of the most markers. But when T cells were co-cultured with R-DCs we observed that CD2 expression declines compared to a DC/T cell co-culture. CD2 is a 50-55 kDa

glycoprotein and is expressed on T cells, like CD3, and is also expressed on NK cells. It binds to CD58, which is expressed on APCs. CD2 forms loose association with the TCR complex and the CD2-CD58 interaction initiates T cell/ APC contact. As described before we co-cultured DCs or R-DCs with T cells for 24 hours and then stained all cells on the surface with an anti-CD2 mAb. The staining was analysed via FACS with a special attention on the T cells.

We observed that the expression level of CD2 is down-regulated on T cells when they were co-cultured with R-DCs but not with immature DCs (Fig. 21).

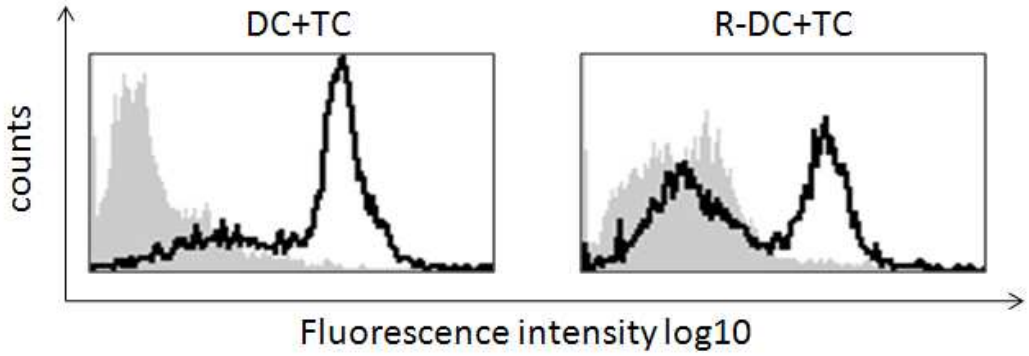


Figure 21: CD2 expression is down-regulated in T cells co-cultured with R-DCs

DCs and R-DCs were co-cultured with T cells for 1 day and then stained for CD2. The black line shows the isotype control and the red line shows the CD2 expression. MFI accords to the MFI of CD2 minus the isotype control. FACS histogram plots of 1 representative experiment out of 3 are shown.

4.11 $CD2^{-/low}/CD3^{+}$ T cell subpopulation in different donors

We found out that, after the stimulation with R-DCs, the T cell population shows a decrease in the CD2 expression. T cells always express CD3 on their surface. Such a subpopulation of $CD2^{-/low}/CD3^{+}$ T cells was first identified in fetal human T cells of the spleen and thymus in 1988. These cells proliferate well and produce IL-2 when treated with anti-CD3 mAbs

(113,115). We were interested in which amounts these $CD2^{-/low}/CD3^{+}$ T cells are represented in different donors. Therefore we isolated the mononuclear cells from fresh blood from various donors and analysed their CD2 and CD3 expression via FACS. The number of this subpopulation is low and varies from donor to donor (MV 0.6% +/- 0.5 SD of PBMNCs). The percentage of $CD2^{-/low}$ cells in $CD3^{+}$ T cells is about MV 1.6% +/- 1.2 SD. $CD2^{-/low}/CD3^{+}$ T cells are represented in the range of 0.5% to 3.9%. The stainings of PBMNCs gated on lymphocytes of two representative donors are depicted in Figure 22 and the percentages of $CD2^{-/low}$ T cells in PBMNCs or T cells are shown in Table 1.

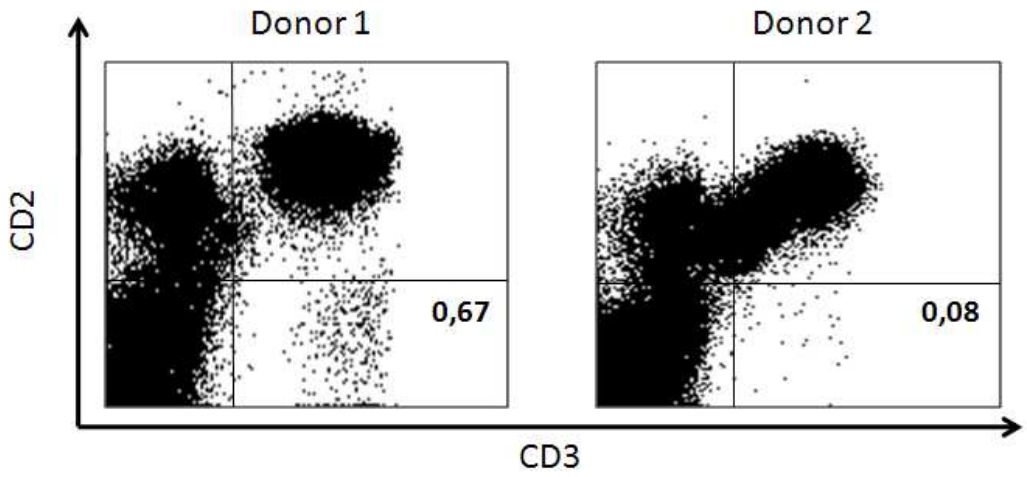


Figure 22: Surface expression of CD2 and CD3 in mononuclear cells

PBMNCs were isolated from fresh blood and stained with FITC labelled anti-CD3 and with PE labelled anti-CD2 mAbs (VIAP served as isotype control). FACS dot plots of 2 representative donors out of 7 are shown.

Donors	1	2	3	4	5	6	7	MV	SD
PBMNCs	0,38	0,14	0,58	0,61	0,06	1,57	0,68	0,6	0,5
CD3+T cells	1	0,9	2,2	1,3	0,5	3,9	1,1	1,6	1,2

Table 1: Percentage of $CD2^{-/low}$ T cells

Percentage of $CD2^{-/low}$ T cells out of PBMNCs (upper row) or CD3+ T cells (lower row) are shown.

5 DISCUSSION

IL-35 is the most recently described cytokine of the IL-12 family (37,38) (39). It is known to have anti-inflammatory activity in the mouse system, because it is produced by $CD4^+CD25^+FoxP3^+$ T regs and the inhibition of Th17 polarization. In mice IL-35 contributes to the suppressive function of T regs (37,39). Controversially, in humans IL-35 is not constitutively expressed in natural T regs and it does not contribute to the suppressive function of FoxP3 induced T regs (41,42). Our recent findings demonstrate that IL-35 producing T regs can be induced by interaction of DCs with T cells via B7-H1 and Sialoadhesin, which are up-regulated when DCs get in contact with human rhinovirus (43). What we know about IL-35 is that it is produced by T regs, but it is not clear which role IL-35 plays in the immune response, how it influences the inhibition of T cell proliferation and which cell type interacts with this cytokine.

We have previously shown that the supernatant of a R-DC/T cell co-culture, containing IL-35, inhibits T cell proliferation (43). We wanted to find out if a human recombinant IL-35 alone inhibits T cell proliferation as efficient. We observed that T cells get activated via allogeneic DCs show reduced proliferation in the presence of IL-35. This reduction is comparable with the inhibition capacity of the supernatant of the R-DC/ T cell co-culture. The same effect was obtained with LPS activated DCs, although mature DCs have an increased competence to stimulate T cells. IL-35 alone is able to suppress the proliferation of T cells. But without DCs as stimuli, when T cells were activated alternatively with anti-CD3, anti-CD3/CD28 and anti-CD3/CD63 mAbs, IL-35 was no longer capable to reduce the growth of T cells. It seems that IL-35 only practices its inhibitory function on T cell stimulation in presence of DCs. Naive T cells from cord blood cannot be inhibited by IL-35, neither through DC activation nor through TCR and co-stimulatory molecule stimulation. IL-35 is not a

typical cytokine of the IL-12 family, because it is produced by Tregs not by APCs. In this study we found out that IL-35 is a inhibitory cytokine which acts on DCs. This differs from all other described IL-12 family members, which affect T cells.

So DCs play an important role in IL-35 function. To determine how IL-35 influences DCs we performed an assay where they were directly affected by IL-35 as it was added in the development phase of DCs from monocytes. IL-10 served as a comparable cytokine because of its described suppressor functions. Pretreated DCs, either through IL-35 (IL-35-DCs) or through IL-10 (IL-10-DCs) also have a diminished capacity to induce T cell proliferation. The quantity of the suppressive effect of IL-10-DCs and IL-35-DCs is comparable. This effect was not detectable when the DCs were matured with LPS. So IL-35 reduces the T cell stimulatory capacity of DCs in their developmental phase. But LPS seems to abrogate this effect and activated IL-35-DCs are able to induce T cell proliferation as efficient as mature DCs.

The development of DCs from monocytes seems to be unaffected by IL-35, because the cells look like DCs after the 6 day co-culture. After 6 days the cells, co-cultured without cytokines or with IL-35 or IL-10, display extensions and loose their round shape. Also the surface markers correspond to DCs, because IL-35-DCs express CD1a but no CD14 as untreated DCs do. IL-10-DCs show no difference in their surface marker expression compared to untreated DCs as previously described (121). The surface marker profile of IL-35-DCs behaves similar, because they remain expressed on the same level as on immature DCs. After LPS addition the surface markers are up-regulated. In presence of IL-10 the anticipated increase of the surface molecule expression is missed. As already described we also observed that IL-10-DCs cannot be matured through LPS activation (121). In contrast, the maturation of DCs is not affected by IL-35, because the expression of CD83, CD40, CD 80, CD86, MHC I, MHC II, B7-H1, B7-H3 and Siglec-1 remains at the same level as in

untreated mature DCs. This effect is not equivalent with IL-10-DCs and serves as a possible explanation why mature IL-35-DCs induce T cell proliferation as efficient as mature DCs.

For the detection of typical DC cytokines the cells have to be activated, because cytokine secretion increases with maturation. All measured cytokines are not detectable in the supernatants of immature DCs, IL-35-DCs and IL-10-DCs. Therefore no difference in their cytokine release can be observed. The supernatant of immature IL-10-DCs contains IL-10, what could be explained by IL-10 pretreatment. But the possibility of IL-10 induction has to be tested by performing more experiments. LPS strongly induces the release of pro-inflammatory cytokines, so IL-10, IL-1β, IL-6, IL-12 and TNFα are up-regulated with maturation. Compared to untreated mature DCs, IL-35-DCs show a slight increase in IL-1β, IL-6 and TNFα secretion upon stimulation with LPS. This differentiates from LPS activated IL-10-DCs, because they suppress cytokine release of IL-6 and TNFα as well. But in case of IL-12p40 the supernatant of IL-35-DCs exhibits a slight down-regulation, whereas IL-10-DCs strongly diminish IL-12p40 detection. Interestingly, this inhibitory cytokine, IL-35, down-regulates IL-10 secretion in DCs. But this could explain why the suppressive effect of IL-35-DCs is missed in matured cells. It also designates a T cell response that keeps off Th1 and the up-regulation of IL-6 could be an indication for Th17, because IL-6 in combination with TGFβ are essential for Th17 cell differentiation (80).

It was described that the inhibition of cytokines by IL-10 is not responsible for the impaired stimulatory capacity of DCs. But the alteration of surface molecule expression after IL-10 treatment and the modified DC – T cell contact plays a crucial role (121). The mechanism for the reduction of the T cell stimulatory capacity of IL-35-DCs is not yet known. It is not explainable through changes in their surface molecules expression and their cytokine profile. In LPS activated IL-35-DCs the suppressor function is lost, what could be explained through the slight up-regulation of pro-

inflammatory cytokines and the decrease of IL-10, as a suppressor cytokine. To get more information about the mechanism more studies have to be performed.

Today the receptor for IL-35 is not known. In binding assays we analysed if IL-35 is able to bind to various cell types. We observed that IL-35 interacts with DCs and LPS activated DCs but not with T cells and monocytes. So it is possible that the receptor of IL-35 is only expressed on DCs. Collison and Vignali proposed that the receptor chains of other members of the IL-12 family build this receptor. The IL-12Rβ2 chain binds to the p35 subunit, which also exists in IL-12, and therefore it is a feasible candidate. IL-27, which carries EBI3 as well, requires both receptor subunits, gp130 and WSX-1, for signalling. It is possible that both chains are part of the IL-35 receptor or only one of them (11). Gp130 is also part of the IL-6 receptor and associates with other receptor components to generate different receptors (122). All of the three possible receptor chains are expressed in T cells and in DCs (123,124,125,126), but IL-12Rβ2 is not expressed on monocytes (127). Therefore none of these chains or only one of them may be involved in the IL-35 receptor formation. It is also possible that new, unknown subunits build this receptor alone or maybe with one or two chains of the proposed ones. Table 1 shows on which cell types IL-35 binds in our study and which receptor subunits are expressed on these cells. In Figure 23 the possible chains of the IL-35 receptor candidates are depicted.

Cell type	IL-35 binding	IL-12Rβ2 expression	WSX and gp130 expression
Monocytes	-	-	+
DCs	+	+	+
mDCs	+	+	+
T cells	-	+	+

Table 2: Summary of IL-35 binding assays compared to the known receptor subunits of the described IL-35 subunits

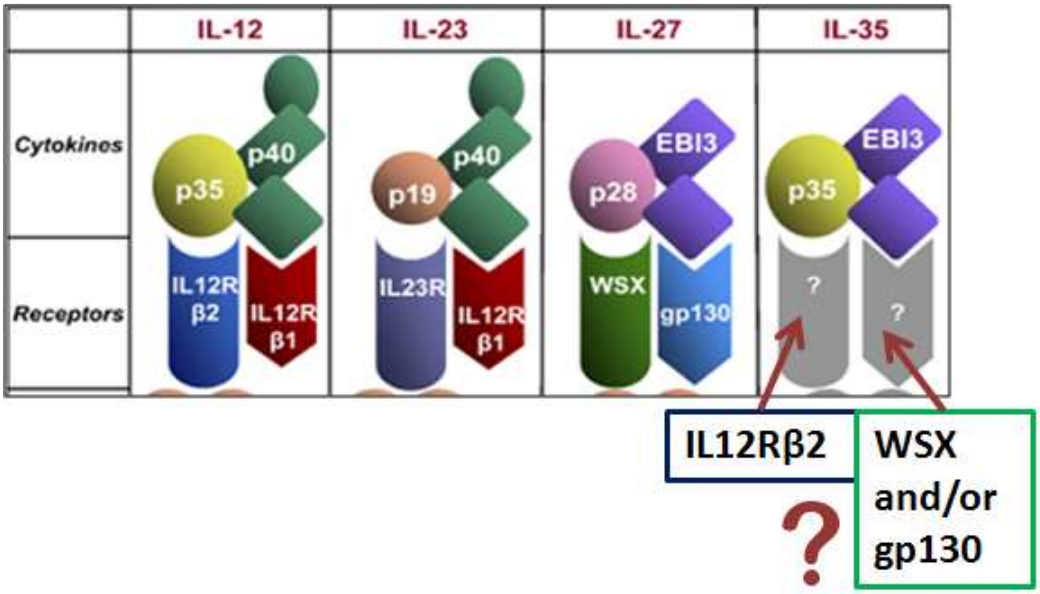


Figure 23: Possible subunits for the IL-35 receptor (detail of Collison and Vignali, 2008, Immunol Rev)

We demonstrated that IL-35 is able to inhibit tumor cell growth of the HeLa Ohio cell line. Interestingly the capacity of IL-35 to suppress HeLa Ohio proliferation increases from 1 µg/ml to 1 ng/ml and then decelerates. So the amount of IL-35, which corresponds to cell growth inhibition, is very important. Other epithelial cell lines could not be suppressed by IL-35. Also the tested hematopoietic cell lines show no difference in their proliferation when IL-35 was added. The binding assays demonstrate that IL-35 only binds to HeLa Ohio cells but not to the other tested cell lines. So there is probably no cell growth inhibition in the other epithelial and hematopoietic cell lines because of the lack of specific receptors for the interaction with IL-35. To conclude these findings we can say that IL-35 does not serve as a common tumor suppressor, because the inhibitory capacity seems to depend on cells that carry the receptor or not.

IL-35 is produced by regulatory T cells induced by R-DCs. That was for example demonstrated by the up-regulation of EBI3 in T cells co-cultured with these R-DCs (43). Because of these facts we were interested if EBI3 is also up-regulated in T cells co-cultured with differently activated DCs.

We found out that T cells co-cultivated with IFNα activated DCs also express EBI3 in a higher level, like T cells that were activated with R-DCs. All the other types of differently stimulated T cells show no influence in EBI3 expression. IFNα is produced in high levels when cells are in contact with virus. Viral components are detected by several receptors, such as TLR3, Mda-5 and Rig-I, what causes signal transduction for type I IFN production (128). When DCs are activated with HRV, IFNα is also induced. In combination with these facts the EBI3 up-regulation may be associated with viral infection. Mxa, an intracellular factor induced by type I IFNs, is expressed on T cells co-cultured with R-DCs and on T cells co-cultured with IFNα activated DCs (129). The flow cytometry results show that p35 is expressed in all types of differently stimulated T cells in low doses and gets more in cause of DCs plus HRV or DCs plus IFNα. IL-35, consistent of p35 and EBI3, is produced by R-DC induced regulatory T cells, what explains the up-regulation of the p35 and EBI3 expression compared to T cells co-cultured with untreated DCs. Maybe IL-35 is also a product of T cells that were activated with IFNα-DCs.

The FACS analysis of different surface molecules on T cells that were co-cultured with R-DCs or immature DCs suggests that the CD2 expression decreases when T cells were activated via R-DCs. So a population of $CD2^{-/low}$ T cells appears. $CD2^{-/low}/CD3^{+}$ T cells were first identified in fetal human T cells of the spleen and thymus in 1988 (113). We know that IL-35 is produced by T regs that were induced by R-DCs, but the special type of these cells is not yet described. These R-DCs co-cultured T cells have a decreased CD2 expression. That combination raises the question if these $CD2^{-/low}/CD3^{+}$ T cells are possible IL-35 producers. So we wanted to know if we can detect such a subpopulation in the blood of healthy donors via flow cytometry. In mononuclear cells these $CD2^{-/low}/CD3^{+}$ T cells are not easy to detect. But we also observed that the number of this subpopulation varies from donor to donor without R-DC treatment. Maybe the frequency of this subpopulation in different donors correlates with

infectious diseases the persons currently have or had. But to answer these questions more studies have to be performed.

To sum up our findings we can say that IL-35 produced by T regs acts on DCs and binds to them. IL-35 is also for the reduction of the T cell stimulatory capacity of DCs. The subpopulation of T regs which release IL-35 is not yet known, but a possibility lies in the $CD2^{-/low}/CD3^{+}$ T cell population. Figure 24 demonstrates a hypothesis of the functional effects of IL-35.

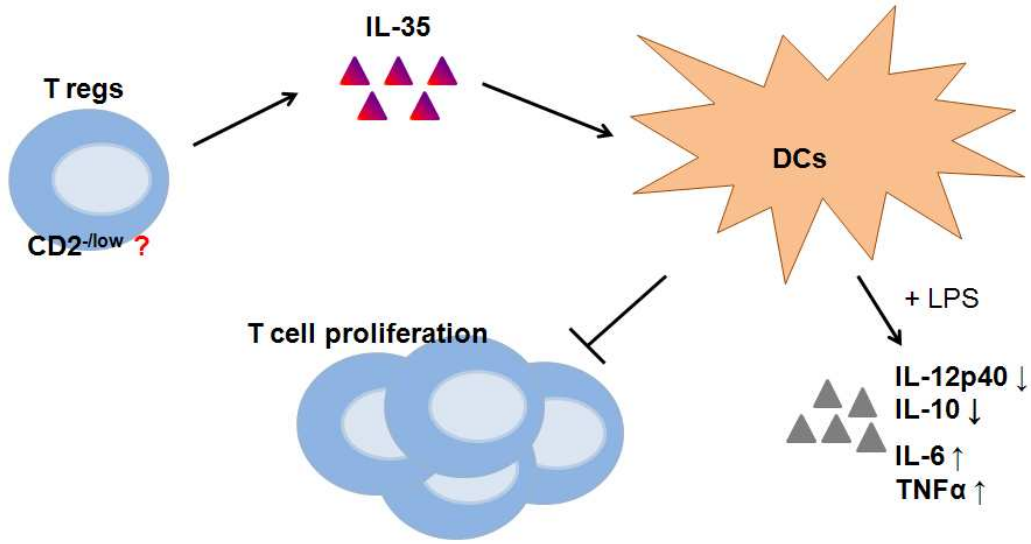


Figure 24: Summary

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7 Curriculum vitae

Personality

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