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Characterization of human IL-35^{+} regulatory T cells induced by Rhinovirus-treated dendritic cells

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Verfasserin / Verfasser:	Maria Seyerl
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Betreuerin / Betreuer:	Ao. Univ.-Prof. Dr. Johannes Stöckl

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Content

Abbreviations

APC	antigen presenting cell
ATCC	American type culture collection
BSA	bovine serum albumine
CD	cluster of differentiation
CTL	cytotoxic T cell
cDNA	complementary DNA
cpm	counts per minute
DC	dendritic cell
EBI3	EBV-induced gene 3
EBV	Epstein Barr Virus
FITC	fluorescin-5-isothiocyanate
Foxp3	forkhead box P3
GM-CSF	granulocyte-macrophage colony stimulating factor
HRP	horse radish peroxidase
HRV	Human Rhinovirus
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Iono	ionomycin
JAK	Janus kinase
kDa	kilo-Dalton
LDL	Low density lipoprotein
mAb	monoclonal antibody
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MV	Mean value
MW	molecular weight

OG	oregon green
PBMNC	peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	phycoerythrin
PMA	phorbol myristate acetate
SD	standard deviation
SDS-PAGE	sodiumdodecylsulfate polyacrylamide gelelectrophoresis
SN	supernatant
STAT	signal transducer and activator of transcription
TCID	Tissue culture infectious dose
TCR	T cell receptor
TGF-ß	transforming growth factor beta
Th	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
qPCR	quantitative PCR

Summary

IL-35 is a heterodimer of EBV-induced gene 3 (EBI3) and of the p35 subunit of IL-12, which has recently been identified as an inhibitory cytokine produced by natural regulatory T cells in mice, but not in humans. Here we demonstrate that dendritic cells (DCs) activated by human rhinoviruses (R-DCs) induce IL-35 production and release in human T cells.

The first finding of this thesis was that R-DCs induce a suppressor function in cocultured T cells. This suppressor function was induced in $CD4^+$ and $CD8^+$ T cells derived from human peripheral blood but not in naïve T cells from cord blood. It was further observed that the cell culture supernatant (SN) of peripheral blood T cells (both $CD4^+$ and $CD8^+$) cocultured with R-DCs had an inhibitory effect on T cells. Therefore the inhibition was not cell-cell contact dependent and we focused on soluble factors. When we blocked the classical inhibitory cytokines including IL-10, TGF-ß or IFN-α with antibodies against the respective factors, the SN was still inhibitory. Therefore the inhibition was not caused by the release of these cytokines. Via qPCR, flow cytometry and immuno-precipitation it was shown that, R-DC-induced regulatory T cells produced and released IL-35. When we blocked the inhibitory SN with Abs against either subunit of IL-35, the inhibitory effect was gone. Hence IL-35 was responsible for the inhibitory effect of the SN. In addition, the p35 depleted SN was no longer inhibitory. The induction of IL-35 producing T cells ($IL\text{-}35^+$ regulatory T cells) by R-DCs did not correlate with an increased Foxp3 expression, indicating that R-DCs do not induce classical regulatory T cells.

In order to determine which structures on R-DCs influence the generation of $IL\text{-}35^+$ regulatory T cells, blocking antibodies against R-DCs inhibitory surface molecules B7-H1 (CD274) and sialoadhesin (CD169) were added. Most importantly we observed that, when we blocked B7-H1 and sialoadhesin on the R-DC side with specific mAb against both receptors, the induction of IL-35 was prevented. Thus, the combinatorial signal delivered by R-DCs to T cells via B7-H1 and sialoadhesin is crucial for the induction of human $IL\text{-}35^+$ regulatory T

cells, defining a new route of T-cell instruction.

These results demonstrate a novel pathway and its components for the induction of immune-inhibitory T cells and show for the first time that IL-35 is immune-inhibitory in humans. The findings of this thesis contribute to a better understanding of immune evasion strategies of human rhinoviruses (HRV) the major cause of the common cold.

Zusammenfassung

IL-35 ist ein Heterodimer, bestehend aus EBV-induziertem Gen 3 (EBI3) und p35, einer der beiden Untereinheiten von IL-12. Dieses Zytokin wurde kürzlich als inhibitorisch beschrieben und wird von den natürlich regulatorischen T-Zellen der Maus, jedoch nicht von denen des Menschen produziert. In dieser Dissertation wird gezeigt, dass Dendritische Zellen, die mit humanem Rhinovirus (HRV) aktiviert sind (R-DZs), die Produktion und Freisetzung von IL-35 in humanen T-Zellen induzieren.

Werden T-Zellen mit R-DZs kokultiviert, wird in den T-Zellen eine supprimierende Funktion auf andere T-Zellen beobachtet. Sowohl in $CD4^+$ als auch in $CD8^+$ T-Zellen des peripheren Blutes, jedoch nicht in aus humanem Nabelschnurblut gewonnenen naiven T-Zellen, konnte diese inhibierende Funktion durch R-DZs induziert werden. Weiters wird gezeigt, dass der Zellkulturüberstand von T-Zellen des peripheren Blutes ($CD4^+$ als auch $CD8^+$ T-Zellen), die mit R-DZs kokultiviert wurden, inhibierend ist. Diese Beobachtung lässt den Schluss zu, dass die Effekte nicht durch Zell-Zell-Kontakt, sondern durch lösliche Faktoren hervorgerufen wurden. Die klassischen immun-inhibitorischen Zytokine IL-10, TGF-ß oder IFN-α waren jedoch nicht für diesen Effekt verantwortlich, da ein Blockieren dieser Faktoren durch Antikörper nichts an der hemmenden Eigenschaft des Zellkulturüberstandes änderte. Wir konnten mittels qPCR, Durchflusszytometrie und Immun-Präzipitation zeigen, dass IL-35 von mit R-DZs kokultivierten T-Zellen produziert und freigesetzt wird. Ein Blockieren der beiden IL-35 Untereinheiten (EBI3 und p35) mit Antikörpern gegen diese Strukturen konnte die Hemmung aufheben. Damit wird gezeigt, dass IL-35 für die inhibitorische Eigenschaft des Zellkulturüberstandes verantwortlich ist. Depletion von IL-35 aus dem zuvor hemmenden Zellkulturüberstand führte ebenso zum Verlust dieser Funktion. Die Induktion dieser IL-35 produzierenden T-Zellen ($IL\text{-}35^+$ T-Zellen) durch R-DZs zeigte keine Korrelation mit erhöhter Foxp3 Expression. Dies könnte ein Hinweis darauf sein, dass keine klassischen regulatorischen T-Zellen induziert werden.

Durch das Hochregulieren der inhibitorischen Oberflächenmoleküle B7-H1 (CD274) und Sialoadhesin (CD169) sind R-DZs schlechte T-Zell-Stimulatoren. Als wir diese beiden Oberflächenmoleküle mit Antikörpern, die gegen diese Strukturen gerichtet sind, blockierten, verhinderten wir die Induktion von IL-35. Wir schließen daraus, dass ein kombinatorisches Signal der R-DZs, vermittelt durch B7-H1 und Sialoadhesin, an die T-Zellen für die Induktion der IL-35$^+$ T-Zellen verantwortlich ist.

Unsere Ergebnisse beschreiben einen neuen Weg und seine Bausteine in der Induktion von immun-inhibitorischen T-Zellen. IL-35 wird zum ersten Mal als inhibitorisches Zytokin im Humansystem nachgewiesen. Weiters bekommen wir Einblick in die Strategien von HRV, dem Haupterreger des Schnupfens, dem Immunsystem zu entgehen.

Introduction

One of the main functions of the immune system is the control of infection [1]. The contact with a pathogen requires a strong and efficient response of the immune system to prevent harm for the organism. Yet, potent immune responses can be accompanied by severe side-effects with immune-pathology as a final result. Thus, anti-pathogen responses need to be controlled adequately. There is increasing evidence that suppressor or regulatory T cells (Tregs) are critically involved in the control of immunity to infection. In turn, recent studies suggest that pathogens actively provoke the generation of Tregs thereby harnessing these regulatory cells to evade the immune system.

T Lymphocytes (T cells)

T cells are main effectors in the adaptive immune response. Furthermore they serve as crucial effector cells through antigen specific cytotoxic activity and the production of soluble mediators (cytokines). The T cells antigen receptors are membrane molecules distinct from but structurally related to antibodies. T cells have a restricted specificity for antigens; they recognize mainly peptide antigens attached to host proteins that are encoded by genes in the MHC and that are expressed on the surface of other cells [2].

T cells consist of functionally distinct populations, the best defined of which are helper T cells, which express the CD4 co-receptor on their surface and are restricted to MHC class II, and cytotoxic T cells (CTLs), which are CD8 positive and recognize MHC class I. In response to antigenic stimulation helper T cells secrete proteins called cytokines. Their function is to stimulate the proliferation and differentiation of the T cells as well as of other cells, including B cells, macrophages, and other leukocytes. CTLs kill cells that present foreign antigens, such as cells infected by viruses or other intracellular microbes.

The initiation and development of adaptive immune responses requires antigens to be captured and displayed to specific T cells. The cells that serve

this role are called antigen presenting cells (APCs). The most highly specialized APCs are dendritic cells (DC). DCs capture antigens that enter from the external environment, transport these antigens to lymphoid organs, and present the antigens to naïve T cells to initiate immune responses. APCs also include mononuclear phagocytes and B cells, but also mesenchymal cells such as fibroblasts. All of these cells are capable of expressing MHC class II molecules and co-stimulatory molecules [2].

T cell subsets

$CD4^+$ T cells – T helper cells

Helper T cells usually express CD4 and recognize peptides displayed by MHC class II molecules. In 1986 Mosmann and Coffman reported that distinct CD4 T cell subtypes, termed T helper type 1 (Th1) and T helper type 2 (Th2) T cells, could be distinguished on the basis of cytokine secretion and function [3]. The Th1/Th2 model provided a useful and relatively simple explanation for our understanding of the mechanisms of immunity to infection. The original hypothesis was that interferon-gamma (IFN-gamma) secreting CD4 cells mediated protective immunity to intracellular pathogens. In contrast, CD4 T cells that secreted IL-4, IL-5, IL-10 and IL-13 were considered to be the main helper T cells providing help for B cell production of antibody and mediated protective immunity to extracellular pathogens. Over the years the picture became more complicated and also more subsets of T helper cells arised.

Th1 and Th2

After being activated, naive $CD4^+$ T cells differentiate into functional subsets called Th1 and Th2 cells, based on their production of IFN-gamma and IL-4, respectively [4]. Th1 and Th2 subsets develop from the same precursors, and the pattern of differentiation is determined by stimuli present early during immune response [2]. The decision to develop into Th1 or Th2 effectors

appears to be dependent on cytokines these naïve T cells are exposed to during primary activation. IL-12, produced by activated macrophages and DCs, is considered the principal Th1-inducing cytokine, while IL-4 appears to be the important cytokine for induction of a Th2 response [5]. IL-12 enhances expression of the transcription factor T-bet and STAT-4 and promotes development of Th1 cells, whereas IL-4 enhances expression of GATA-3 and STAT-6 and promotes development of Th2 cells. Although the differentiation and function of these Th subsets has been intensively studied, little is known about the contribution of cell surface receptors to these differentiation events, other than those for soluble cytokines, such as IL-12 or IL-4 [6]. The Notch signaling pathway is an evolutionarily highly conserved mechanism for cell-to-cell communication, important for many types of cell fate determinations [7]. APCs use the Notch pathway to instruct T cell differentiation. Strikingly, of the two Notch ligand families, Delta1 induces Th1, while Jagged induces the alternate Th2 fate. Expression of these different Notch ligands on APC is induced by Th1- or Th2-promoting stimuli [8]. Delta1 interaction with Notch3 on $CD4^+$ T cells transduces signals, promoting development toward the Th1 phenotype [6].

A given Th cell subset is characterized by its cytokine-secretion profile which is intimately associated to the effector functions. Th1 cells mediate cellular immunity against intracellular bacteria and viruses by secreting cytokines such as IFN-gamma and tumor necrosis factor-α (TNF-α). Th2 cells regulate humoral immunity and immunity against extracellular parasites by producing IL-4, IL-5 and IL-13 [9]. Th1 cells can mediate macrophage activation and delayed type hypersensitivity, which are collectively termed cell-mediated immune responses [10]. Whereas Th2 T cells promote secretion of IgG1 and IgE by B cells and promote immediate-type hypersensitivity reactions, collectively termed humoral immunity [11].

Th17, Th9 and Th22

The Th17 cells, are defined via their ability to produce IL-17 and develope via cytokine signals distinct from, and antagonized by, products of the Th1 and Th2 lineages [12]. Th17 cells are a distinct linage from Th1 or Th2 cells [13]. Th17 cells produce IL-17A, IL-17F, and IL-22 and play important roles in the clearance of extracellular bacteria and fungi, especially at mucosal surfaces. Th17 cell differentiation requires retinoidrelated orphan receptor (ROR-γt). This transcription factor is induced by TGF-ß in combination with the proinflammatory cytokines IL-6, IL-21 and IL-23, all of which activate STAT-3. T-bet and GATA3, which are involved in the development of Th1 and Th2 cells, inhibit the differentiation of Th17 cells.

The cytokine IL-9 has largely been regarded as a Th2 cytokine that makes multifocal contributions to allergic disease. Recent data suggest that under certain conditions a distinct population of IL-9-producing 'Th9' helper T cells can arise. This new subset of the T helper population is different from Th2 and produces IL-9 in large quantities. TGF-ß ‘reprograms’ the differentiation of Th2 cells and promotes this subset [14, 15].

IL-22 was described originally as a cytokine characteristic of fully differentiated Th17 cells [16]. Recently, a subset of human skin-homing memory T cells was shown to produce IL-22, but not IL-17 or IFNγ. Differentiation of IL-22 producing T cells, named Th22 cells, could be promoted by stimulation of naive T cells in the presence of IL-6 and TNF-α or by the presence of plasmacytoid DCs. The human Th22 cell population coexpresses the CCR6 chemokine receptor and the skin-homing receptors CCR4 and CCR10. This led to hypotheses that these cells may be important in skin homeostasis and pathology [17-19].

CD8$^+$ T cells – Cytotoxic T cells

CD8$^+$ T cells mediate their effector functions through production of cytokines such as IFN-gamma and TNF-α and/or by cytolytic mechanisms. Their major effector function is to recognize and kill host cells infected with viruses or other intracellular microbes. Such responses are important in preventing or maintaining control against disease in a variety of intracellular infections and perhaps also against certain tumors [20]. CTLs usually express CD8 and recognize microbial peptides displayed by class I MHC molecules. CTL killing of infected cells involves the release of cytoplasmic granules whose contents include membrane pore forming proteins and enzymes. CTLs kill their cellular targets by either of two mechanisms that require direct contact between the effector and target cells. In the first pathway, cytoplasmic granule toxins - predominantly a membrane-disrupting protein known as perforin and a family of structurally related serine proteases (granzymes) with various substrate specificities are secreted by exocytosis. This induces apoptosis of the target cell. The granule-exocytosis pathway powerfully activates 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. The second pathway involves the engagement and aggregation of target T cell death receptors, such as Fas (CD95), by their cognate ligands, such as Fas ligand (FasL), on the killer-cell membrane, which results in classical caspase-dependent apoptosis [21].

T cell activation

One of the central processes inducing and regulating immune function involves T cells establishing cell–cell contacts with APCs, such as B cells and DCs [22, 23].T cells express a clonal antigen specific receptor, the T cell receptor (TCR). T cells bind to an MHC-antigen complex on an APC via TCR. In this way, the cell recognizes the antigen it has been designed for. The ability of naive T cells

to clonally expand and acquire effector functions depends on the strength of signals received by the TCR and by an array of co-stimulatory receptors [24]. The T cell recognizes both peptides associated with MHC molecules on the APC and also other molecules in a complex structure known as the immunological synapse [25]. The development and maintenance of an adaptive immune response is mediated by the trafficking of T cells through different tissues and organs and the subsequent interactions of these cells with other immune cells [26].

T cell activation requires two signals

For the full activation of T cells, the cells need to receive two distinct signals. The specificity of T cell responses is controlled by the antigen-specific TCR but the recognition of the antigen in form of the peptide-MHC complexes expressed by the APC (=SIGNAL 1) is not sufficient for optimal activation. Full T cell activation is generally achieved only in the presence of additional receptor ligand interactions so called costimulatory signals (=SIGNAL 2). In the absence of costimulatory signals lymphocytes fail to respond efficiently to antigenic stimulation and are rendered anergic. The interaction between T cells and the APC during antigen recognition creates a unique physical site, the immunological synapse, where specific ligands and costimulatory molecules trigger and sustain the T cell activation process [23]. This so called co-stimulation is crucial to the development of an effective immune response.

Co-stimulation can simply promote more efficient engagement of TCR molecules to enhance the initial activation. It can also provide additional signals to promote cell division, augment cell survival, or induce effector functions such as cytokine secretion or cytotoxicity in the process of T cell response [27, 28]. The best described costimulatory molecules on the APC side are B7.1 (CD80) and B7.2 (CD86), that signal is received via CD28 on the T cell side. The CD28 molecule on T cells delivers a costimulatory signal upon engaging either of its ligands. There is a constantly growing number of other co-stimulatory molecules being described even though their role or mechanism of action is not always

clear.

The immunological synapse

The immunological synapse is a complex cellular structure that forms at the interface of a T cell and a cell that expresses the appropriate peptide–MHC complexes [27]. The immunological synapse consists of a central cluster of T cell molecules surrounded by a ring of adhesion molecules [29]. TCR molecules, CD2 and other molecules on the T cell side, such as CD28 and the peptide-MHC-complex and B7.1/B7.2 on the APC side are enriched in a central supramolecular activation cluster (cSMAC). This is surrounded by a peripheral ring (pSMAC) where molecules such as LFA-1 and CD45 are expressed [30]. The stable immunological synapse results from a polarized interaction between a T cell and an APC. It frequently lasts 30 minutes or longer and is defined by recruitment and segregation of cell-surface receptors and adaptor proteins at the cell–cell junction, and is responsible for sustained transmission of intracellular signals. The development of the immunological synapse comprises five phases:

1. cellular scanning, contact acquisition and adhesive arrest;
2. early immunological-synapse assembly and signalling;
3. maturation and receptor segregation;
4. TCR internalization; and
5. immunological–synapse dissolution [23, 31].

Progression through all of these phases requires sustained peptide–MHC–TCR interaction, sustained membrane-proximal signalling and an intact T cell cytoskeleton. Although the processes that regulate the first four phases have been studied in detail, the processes that control immunological-synapse resolution and T cell detachment are not as clear [32].

Following the initiation of T cell activation the process of T cell response involves a series of complex events. After recognition of the antigen MHC complex on the APC, T cells commit to programmed expansion in the first few

hours of activation. Subsequently T cells go on to differentiate into effector cells, which can interact with B cells in the germinal center and migrate out of the lymphoid organs carrying out their effector functions in the peripheral tissue. Although the majority of T cells are short lived, some antigen experienced cells remain as long lived memory cells.

Regulatory T Cells

Besides soluble mediators that control T cell homeostasis and thereby autoimmunity there is a need for a cellular authority to regulate both the naïve and memory T cell pool together with possible self reactive T cell populations that escaped central tolerance mechanisms. These subpopulations of cells have been termed regulatory or suppressor T cells (Treg). Tregs are crucial players in the induction of peripheral tolerance to self and foreign antigens [33, 34]. Tregs have essential roles in maintenance of immune homeostasis. They regulate effector T cell responses and thus prevent their potentially pathogenic effects through a variety of mechanisms [35].

The first experiments in which such cells have been suggested, even though these cells were CD8 positive, were done as early as 1969 [36]. The field of Treg was re-discovered by the observations of Sakaguchi et al. in 1995. It was observed that depletion of the minor population of $CD4^+$ T cells that coexpress CD25, from a population of normal adult $CD4^+$ T cells, generated a population of cells that induced a spectrum of autoimmune diseases when transferred to an immunocompromised recipient. Cotransfer of the $CD25^+$ cells prevented the development of autoimmunity [37].

In the last decade regulatory T cell populations have been recognized as one of the key players in controlling T cell homeostasis, thereby maintaining the quantitative and qualitative dimensions of the T cell pools. Besides the important roles in controlling autoimmunity, evidence has also emerged that regulatory T cells control immune responses to pathogens like bacteria, viruses or fungi.

Today regulatory T cells can be subdivided in two main categories. First the naturally occurring regulatory T cells, that are generated in the thymus, namely $CD4^+CD25^+$ regulatory T cells [38], and secondly: the inducible regulatory T cells, namely Tr1, Th3 and inducible $CD4^+$ $CD25^+$Treg subsets, that arise in the periphery. [34, 39, 40]. Both share some distinct properties that make them different compared to naïve and memory T cells. Upon TCR engagement of peptide complexed to MHC II these cells become hyporesponsive and do not proliferate like naïve or memory T cells [33]. Furthermore regulatory T cells are able to suppress the proliferation of activated responder T cells in an antigen independent manner although their activation itself seems to depend on antigen specificity [35].

Natural occurring Tregs consist of peripheral $CD4^+$ T cells, and are characterized by the constitutive expression of CD25 and the transcription factor Foxp3 [34]. These Tregs inhibit effector T cells responses via so far unknown mechanism, involving cell-cell contact. Inducible Tregs develop from mature T cell populations under certain conditions f.i. upon stimulation with tolerogenic DCs or by IL-10 treatment [41, 42]. Inducible Tregs primarily act via soluble mediators and typically produce high levels of immune-suppressive cytokines (IL-10 and/or TGF-β). The suppressive function of human inducible Tregs seems to be Foxp3-independent [43, 44].

Tregs not only control autoimmunity but also immunity to pathogens, this makes them potentially dangerous. On the one hand, in many infectious diseases, immune responses can result in collateral damage of host tissue. Therefore immune regulatory mechanisms are essential to control immuno-pathology. On the other hand, pathogens can exploit these regulatory mechanisms causing a sort of immune subversion, thereby prolonging pathogen survival and chronic infection of the host [45, 46].

Inducible regulatory T cells

Virtually any responder T cell can be induced to get a regulatory phenotype. The factors important for this induction are so far not fully understood but it is clear that the cytokine milieu plays a crucial role. T cells are in general very plastic and even fully developed effector T cells can by “re-programmed” to another fate [47]. IL-10 and TGF-ß two cytokines with suppressive capacity are known to be produced during the late stages of an immune response. Furthermore cultivation of T cells in the presence of type I IFNs and these two cytokines induces regulatory T cells [48]. The role of tolerizing DCs is also under investigation. So far it is not clear whether APC to T cell contact could be required but DCs are prominent cytokine producers and might be indirectly involved in the induction of regulatory T cells. Others propose that Tregs are capable to induce regulatory T cells via cell to cell contact [49, 50]. Suppression by induced regulatory T cells is mainly mediated via soluble factors. They require antigen specific activation but exert their suppressive function in an antigen unspecific way.

Type 1 regulatory T cells (Tr1)

T regulatory type 1 (Tr1) cells are defined by their specific cytokine production profile. This includes the secretion of high levels of IL-10 and medium levels of transforming growth factor-ß (TGF-ß). Secondly they are defined by their ability to suppress antigen-specific effector T cell responses via a cytokine-dependent mechanism [48].

In contrast to naturally occurring $CD4^+CD25^+$ Tregs, that emerge directly from the thymus, Tr1 cells are induced by antigen stimulation via an IL-10-dependent process in the periphery. The full biological characterization of Tr1 cells has been difficult by the lack of specific marker molecules [39]. Tr1 cells regulate immune responses through the secretion of the immunosuppressive cytokines IL-10 and TGF-ß. It is clear that Tr1 cells play a key role in regulating adaptive immune responses in both, mice and humans. They have a low proliferative

capacity when activated via the TCR which can be overcome by IL-15 [51]. Furthermore they suppress immunoglobulin production by B cells and the antigen presenting capacity of monocytes and DCs [52]. All these effects described can be reverted by the addition of neutralizing antibodies against IL-10 and TGF-ß [44]. Although antigen specific Tr1 cells need to be activated via the TCR in order to exert their suppressive function, once activated, Tr1 cells can mediate bystander suppressive activity against other antigens [39].

Tr1 cells can be generated in vitro and in vivo upon priming of naive T cells with antigen in the presence of IL-10. Specialized IL-10-producing DCs, such as those in an immature state or those modulated by tolerogenic stimuli, play a key role in inducing these cells. For example, repetitive stimulation of naive cord blood $CD4^+$ T cells with allogeneic immature DCs (iDCs) results in the differentiation of IL-10-producing Tregs that suppress T cell responses via a cell-contact [53]. A large body of evidence suggests that not only iDCs but also specialized subsets of tolerogenic DCs may prime Tr1 cells [54]. Tolerogenic DCs can be induced either by biological or by pharmacological agents. For example, DCs treated with immunomodulatory cytokines such as IL-10 [55], TGF-ß [56], IFN-α [57], or TNF-α [58] become tolerogenic and induce the differentiation of Tr1 [54].

T helper 3 cells (Th3)

Th3 cells have been identified during the investigation of oral tolerance [59], which is an active process and a continuous immunologic event, driven by exogenous antigen that has been orally administered. It is induced in the gut-associated lymphoid tissues including Peyer's patches and lymph nodes. For the induction of Th3 cells the cytokine microenvironment is very crucial. High levels of IL-4, IL-10 and TGF-ß produced by naïve T cells, mucosal cells and APCs can induce the differentiation of Th3 cells [60]. They exert their suppressive function via the production of high levels of TGF-ß and medium levels of IL-10 [61]. Just like Tr1 cells they need antigen specific activation whereas suppression is antigen unspecific.

Adaptive Foxp3$^+$CD4$^+$CD25$^+$ regulatory T cells

This quite recently described population, the adaptive Foxp3$^+$CD4$^+$CD25$^+$ regulatory T cells develops outside the thymus. They arise probably under subimmunogenic antigen presentation, during chronic inflammation, and during normal homeostasis of the gut. These Tregs are essential in mucosal immune tolerance and in the control of severe chronic allergic inflammation, and most likely are one of the main barriers to the eradication of tumours. The adaptive Foxp3$^+$CD4$^+$CD25$^+$ Treg cell repertoire is drawn from naïve conventional CD4$^+$ T cells, whereas naturally occurring Treg cells are selected by high-avidity interactions in the thymus. The full extent of differences and similarities between iTreg and naturally occurring Treg cells not entirely defined at the moment [40].

Naturally occurring regulatory T cells (Tregs)

The last population I am going to describe is the one that received most of the scientific attention in the field of T cells with regulatory properties, the Tregs or nTregs. Tregs constitute approximately 5-10% of the peripheral naive T cell repertoire [62] and have emerged as the dominant T cell population governing peripheral self tolerance [38]. TCR stimulation is required to induce suppressor function which is exerted in an antigen non-specific (bystander) fashion. Tregs themselves are hyporesponsive upon activation via the TCR and start to proliferate only when exogenous IL-2 is added to the culture. Many models have been proposed concerning the development of Tregs which takes place in the thymus. Natural CD4$^+$CD25$^+$ Tregs are selected in the thymus and thus have predefined antigen specificity [62]. CD4$^+$CD25$^+$ Tregs are identified on the basis of cell surface markers and expression of Foxp3.

The mode of action of Tregs is not fully understood yet. It is accepted that suppression is mediated via a cell – cell contact dependent mechanism as was proved by transwell experiments [63]. The role of suppressive cytokines, which are the suppressive mediators of inducible regulatory T cells, is a controversial issue. In 2001 Nakamura et al. suggested that TGF-ß was the mediator of

suppression not in its soluble form but bound to the cell membrane of Tregs [64]. They and others showed that Tregs express TGF-ß on their cell surface, that antibodies directed against this cytokine abolished suppression of responder T cells and that membrane bound TGF-ß on Tregs can only exert suppression as long as it is biological active. Other in vitro studies do not suggest necessity for cytokines, because Tregs still suppress responder T cells when blocking antibodies against IL-10 and TGF-ß are added to the media [33, 63, 65].

It is also suggested that Tregs are capable to induce a suppressive phenotype in $CD25^-$ responder T cells – termed infectious tolerance [50] – which become inducible Tr1 or Th3 cells.

There are several key molecules whose deficiency or functional alteration affects the generation or function of Tregs. Although it is known that there are essential molecules for Treg function, markers proposed for this cell type are also known to be expressed on activated T cells, like the IL-2Rα chain (CD25). Other surface markers are CD62L, CD58, GITR, CD122, CD45R0 and MHC II [65]. In vitro model systems have identified a long list of molecules and processes that contribute to Tregs suppressive activities, but it remains unclear whether any of the conclusions drawn from these studies shed light on how Tregs function in vivo [35].

Foxp3 – The master regulator of Tregs

As mentioned it has been shown that naturally occurring Tregs highly express the transcription factor Foxp3. Although it seems in mice to be uniquely expressed in Tregs in humans Foxp3 expression is also observed in activated T cells [43].

Foxp3 first gained attention for its importance in Tregs after identification of natural mutations in the gene encoding Foxp3. These cause a fatal autoimmune disorder known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) in humans [66] and the scurfy phenotype in Foxp3 knock out mice

[67]. Patients who suffer from this disease have massive lymphoproliferation, diabetes mellitus, thyroiditis, eczema, enteropathy and allergies already early in life.

Foxp3 belongs to a family of transcription factors that is characterized by a winged helix-forkhead DNA-binding domain called forkhead box (Fox). High homology exists between human, mouse and rat. So far little is known about biochemical pathways by which Foxp3 might act although it is predicted to be a transcriptional repressor [68]. It was reported that transgenic expression of Foxp3 inhibits endogenous cytokine expression which is driven by NFAT and NFκB in conventional T cells [69]. Foxp3 itself is thought to be regulated by the oestrogen promoter on the transcriptional level. It has a direct binding site in the promoter region and furthermore there are reports that TGF-ß can induce Foxp3 expression through the activation of the SMAD signalling cascade [70]. There is a growing number of publications that show that decreased Foxp3 expression correlates with decreased Treg function, especially in the murine system. To sum up; Foxp3 plays a central role in Treg development and function.

Role of Foxp3 in human $CD4^+CD25^+$ Tregs

It is now well established that human and mouse natural occurring Tregs express high levels of Foxp3 constitutively. However, in contrast to results described in murine cells, in humans it is still controversial whether Foxp3 overexpression in naive $CD4^+$ T cells is sufficient to confer a regulatory function [43]. Allan et al. show that retroviral mediated ectopic expression of Foxp3 in $CD4^+CD25^-$ T cells does not result in a strong and stable suppressive capacity equivalent to that of natural $CD4^+CD25^+$ Tregs [71], whereas Sakaguchi and coworkers reported high suppressive activity after Foxp3 gene transfer [72].

Foxp3 is a faithful marker for Tregs in the mouse, and both necessary and sufficient for their development and function. Initial work in human systems supported this conclusion, with Foxp3 being found only in $CD4^+CD25^+$ T cells

with in vitro suppressor activity [48]. However, Walker et al. showed that TCR stimulation of $CD4^+CD25^-Foxp3^-$ T cells led to the induction of Foxp3 expression in those cells that also upregulated CD25 [73].

In human cells, Foxp3 is also expressed by activated non-suppressive $CD4^+CD25^-$ effector T cells [71] suggesting that in addition to this transcription factor other components may be required for optimal suppressor activity [68]. Upon activation, human effector $CD4^+CD25^-$ T cells and also Tr1 cells can express Foxp3, albeit transiently. The role of transient Foxp3 expression in Teff cells is poorly understood but it is becoming clear that it does not correlate with suppressor function (Figure 1) [43]. The initial studies indicating that Foxp3 was exclusively expressed in human naturally occurring Treg have been disputed. In human $CD4^+$ Teff cells, induction of Foxp3 expression upon activation occurs in dividing cells and does not interfere with proliferation and cytokine production of these cells [74].

Human and mouse Tr1 cells generated in vitro do not constitutively express Foxp3, but upon activation can up-regulate this transcription factor to levels similar to those observed in activated Teff cells [75].

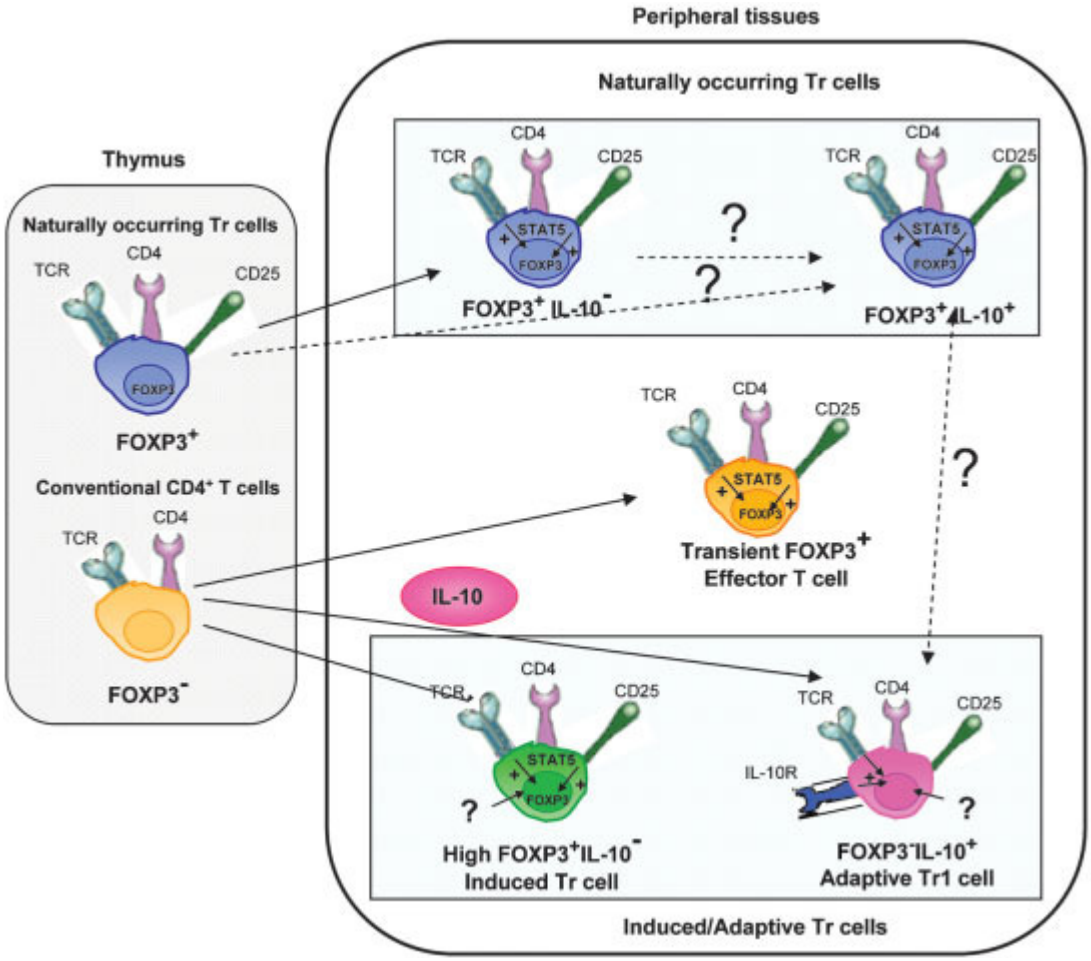


Figure 1. Human Teff cells and regulatory T cells; a complicated picture

In the thymus, naturally occurring CD4⁺Foxp3⁺ Treg and conventional CD4⁺Foxp3⁻ cells are generated. In the periphery, naturally occurring Tregs maintain high sÄtable expression of Foxp3. It is still a question whether naturally occurring Tregs can become IL-10-producing Tregs. Conventional CD4⁺ T cells, after TCR engagement, can either transiently up-regulate Foxp3 and become effector T cells or stably express Foxp3 and become induced Treg. Moreover, conventional CD4⁺ T cells upon encounter with the antigen in the presence of IL-10 become adaptive Tr1 cells, which do not stably express Foxp3 [43].

Dendritic cells

A short history

In 1868, dendritic cells (DCs) were discovered in human skin by Paul Langerhans [76]. These cells were named Langerhans cells (LCs) after their discoverer who, due to their dendrites, regarded them as neurons. Steinman and Cohn identified DCs in peripheral lymphoid organs of the mouse in 1973 [77]. Also Katz et al. reported that skin epidermal LCs derive from cells that originate in the bone marrow [78]. These and many other studies inspired active research in the 1980s that defined many of the structures and fuctions of LCs and DCs. Their role as the key cell type in the regulation of an adaptive immune response was defined.

Role of DCs

Efficient host defense depends on a concerted action of antigen nonspecific innate and antigen specific adaptive immunity. The innate immune system rapidly recognizes antigens by shared patterns. Adaptive immunity is distinguished by the capability to rearrange immunoglobulin genes and to establish immunological memory. DCs link these two parts by capturing and presenting antigens resulting in the initiation of adaptive immunity. DCs are the "sentinels" of the immune system are distributed in most tissues, where they reside as immature cells with high phagocytotic capacity [79].
Following antigen uptake DCs can mature and migrate to lymphoid organs. Maturation can be induced by several danger signals for example bacterial LPS, CpG and viral double stranded (ds) RNA. In the secondary lymphoid organs, mainly the draining lymph node, DCs interact with antigen-specific T cells initializing an immune response. DCs are the only APC able to stimulate naive T cells and to induce primary immune responses and are therefore also called professional APCs [80, 81].

DCs are less defined by phenotypic markers than by their morphology and their function. The term “dendritic cell” refers to their extraordinary shape, which is marked by large cell membrane processes or veils [77]. Immature DCs are well equipped to capture antigen. They apply several mechanisms like macropinocytosis, receptor-mediated endocytosis via C-type lectin receptors (mannose receptor, DEC 205), or Fc-gamma receptor types I (CD64), or II (CD32) or phagocytosis of particles like apoptotic or necrotic cell fragments, viruses or bacteria [81].

Several factors including pathogen-derived signals like LPS, CpG and dsRNA, cytokines and T cell derived signals can induce DC maturation [79]. During infections, DCs are activated by stimulatory signals from invading pathogens, called pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) [82]. Classical PAMPs are for example LPS, an integral part of the cell wall of Gram-negative bacteria, which is recognized by Toll-like receptor (TLR)-4, and lipopeptides from bacterial cell walls and dsRNA, signaling through TLR-2 and TLR-3, respectively [83, 84].

Maturation of DCs goes along with far-reaching changes converting them from cells specialized on antigen uptake to cells specialized on antigen presentation. Costimulatory and adhesion molecules like CD40, CD54, CD58, CD80 and CD86 are upregulated and MHC II, which is already synthesized, is redistributed from intracellular compartments to the plasma membrane. The capability to endocytose declines and cytoskeleton reorganization permits a higher motility [80, 81]. Chemokines guide the mature DCs via the afferent lymph to the T cell area of the draining lymph nodes where additional maturation signals like CD40L and RANKL are provided by T cells.

Antigen presentation

DCs encounter naive T cells in the T cell areas of secondary lymphoid organs where they stimulate their proliferation, their differentiation or their deletion [85]. In the area of contact between T cells and DCs adhesion molecules, like

integrins ß1and ß2, CD2 and ICAM-3 on the T cell and ICAM-1, ICAM-2, LFA-3 and DC-SIGN on the APC, are arranged around the center of the synapse: This center is made up of the TCR and costimulatory molecules [30]. Adhesion molecules like the ICAM-3-binding lectin DC-SIGN, which is specifically expressed on monocyte-derived DCs, are responsible for the initial contact and for the maintenance of interaction [86]. It is supposed that the duration of TCR stimulation together with polarizing cytokines determines the direction of the response. T cells, which receive a short TCR stimulation in the absence of IL-12 proliferate, but do not differentiate into effector cells. A secondary antigenic stimulation triggers their differentiation [87]. Once a naive T cell is activated it can stimulate DCs via CD40L to increase the expression of the costimulatory molecules CD80 and CD86 and to release cytokines.

By sampling different self-antigens and presenting those to T cells, DCs are suggested to play also a critical role in the induction of peripheral tolerance. Thereby DCs are proposed to be involved in the prevention of autoimmune diseases. The state of DC maturation seems to be an important aspect: Immature, resting DCs are relatively poor stimulators of T cell proliferation and can induce in context of auto-antigens regulatory T cells. Naive $CD4^+$ T cells when repetitively stimulated with allogeneic iDCs were shown to differentiate into non-expanding regulatory T cells in vitro [53].

Dendritic cells and pathogens

The major entry sites for pathogens are the epithelial surfaces of the mucosa and the skin. Following antigen deposition DCs are rapidly recruited (within 1 hour) to mucosal surfaces [88]. In the epithelium DCs can get into contact with pathogens or their components by different antigen-uptake mechanisms, or they get infected themselves. The recognition of microorganisms occurs by means of receptors for pathogen-invariable structures like TLRs. Beside the regular MHC class I and II presentation pathways, cross-presentation plays an essential role in immune responses to pathogens. Cross-presentation enables the induction of

CTL responses to pathogens that do not infect DCs and it allows CTL responses even to pathogens that trigger apoptotic death of DCs, inhibit maturation or otherwise impair DC function [89].

DC migration from the periphery to lymph nodes serves to activate T cells, but it can also be exploited by pathogens. For example HIV-1 binds to the DC-specific lectin DC-SIGN (CD209). The infectious particle is transported on the DC surface to the lymph nodes, where it infects $CD4^+$ T cells in trans [90]. There are many mechanisms for pathogens to evade host immune responses. Microorganisms impair the recognition of infected cells, the acquisition and processing of antigens or the maturation of DCs. Poxviruses and Herpesviruses impair the recruitment of DCs by secreting chemokine-receptor homologs that bind locally produced chemokines. Some viruses try to circumvent antigen-presentation by destruction of DCs: Canarypox and vaccinia virus infect DCs and induce apoptosis. Herpesvirus and Vacciniavirus can inhibit DC maturation. Herpesvirus also impairs DC migration to lymph nodes by inhibition of CCR-7 upregulation [89]. Likewise, pathogens are able to alter the interaction between DCs and T cells. For instance, following measles virus infection DCs mature, but are not able to stimulate T cells. The measles virus partly impairs CD40L-dependent activation of DCs and downregulates the production of IL-12, responsible for the differentiation of $CD4^+$ T cells into Th1 cells [91]. Also Human rhinovirus induces the downregulation of IL12 production by monocytes, but also leads to the release of the immunosuppressive cytokine IL-10 that may contribute to the disturbed cellular immune response during HRV infections [92]. But more about HRV in the next chapter.

Human Rhinovirus (HRV)

HRV overview

Rhinovirus is a genus of positive, single-stranded RNA viruses belonging to the family of *Picornaviridae*. The HRV genome consists of one plus strand RNA molecule of about 7200 bases. The *Picornaviridae* include small (greek: pico) RNA-containing viruses without envelope. Other members of the *Picornaviridae* family are for example the enteroviruses, such as poliovirus and Coxsackie viruses. The whole *Picornaviridae* family consists of 5 genera: hepato-, entero-, cardio-, aptho- and rhinoviruses [93].
Thus far, there are >130 rhinovirus serotypes identified [94]. HRV serotypes are phylogenetically subdivided into three groups; HRVA, HRVB and HRVC, based on sequence comparison [93, 95]. Rhinoviruses can also be further divided, depending on receptor utilization, into major group Rhinoviruses, which bind to ICAM-1 (Intercellular adhesion molecule 1) and minor group Rhinoviruses binding LDL-R (Low Density Lipoprotein Receptor) and related receptors [96, 97]. More than 90% of Rhinoviruses belong to the major group [98].

HRV structure

HRV are non-enveloped viruses with an icosahedral capsid enclosing a single-stranded, positive-sense RNA genome that is translated after cell entry [98]. The three-dimensional structure of rhinoviruses was examined via X-ray cristallography [99]. The naked icosahedral capsids measure approximately 20-30 nm. The rhinovirus capsid, which protects the central RNA core, is composed of 60 copies (protomers) of each of 4 structural proteins. Virus protein 1 (VP1), VP2, and VP3 are located on the capsid surface and are responsible for its antigenic diversity. The fourth protein, VP4, is located inside the virus and anchors the RNA core to the viral capsid [96]. The VP1 protein is the most surface-exposed one of the rhinovirus capsid proteins, and it contains

a number of major epitopes recognized by neutralizing antibodies [100].
Each of the twelve 5-fold axis of HRV is encircled by a 20 Å deep and narrow canyon. This depression is the receptor binding site. HRV receptors were predicted as small molecules fitting into the depression and binding to conserved residues, while antibodies were suggested to be hindered to enter the canyon and permitted HRV to escape host T cell immune response. This suggestion was called the canyon hypothesis [101]. But later X-ray structures proved that antibodies exist, binding to conserved residues within the canyon [102].

HRV - The major cause of common cold in humans

The common cold with its characteristic symptoms is one of the most frequent and familiar illnesses in humans. The majority of colds is caused by HRV, by one of over 100 serotypes. In normal individuals HRV causes only mild symptoms like sneezing, nasal congestion, cough, nasal obstruction, sore throat, rhinorrhea and headache. Colds partly result in sinus infections and otitis media. In patients with asthma or cystic fibrose a HRV infection can be harmful by worsening symptoms [103]. An HRV infection normally starts by self-inoculation with the virus by hand into the nose or into the eyes, from where the virus comes via the lacrimal duct into the nose [104].
The optimal replication temperature of HRV is at 33°C. Due to this, HRV was supposed to infect only the upper respiratory tract. But it was shown that HRV is also capable to infect the lower airways, maybe contributing to exacerbations of asthma, pneumonia, bronchitis and cystic fibrose [105].

ICAM-1 (CD54)

The intercellular adhesion molecule 1 (ICAM-1) is a single-span, membrane-anchored glycoprotein, a member of the immunoglobulin superfamily and composed of five immunglobulin domains. ICAM-1 has important functions in

cell-cell adhesion, in inflammation and immune responses. It is expressed on endothelium and functions in leukocyte binding to the blood vessel wall and in transendothelial emigration at inflammatory sites. ICAM-1 can be induced on many cell types like connective tissue cells, monocytes and antigen-presenting DCs, or up-regulated, like on B cells in germinal centers or epithelial cells. Among the cells, which constitutively express ICAM-1 are epithelial cells of the nasal mucosa. ICAM-1 is important in adhesion of leukocytes and endothelial leukocyte migration and accumulation through binding to its cellular ligands LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) [106]. It is also an essential co-stimulatory receptor on APCs. Mice deficient in ICAM-1 fail to recruit leukocytes to inflamed tissues [107].

ICAM-1 is the major group rhinovirus receptor, it serves >90% of rhinovirus serotypes, whereas some of the remaining minor group rhinoviruses bind members of the low-density lipoprotein receptor family [108]. Rhinovirus attachment to ICAM-1 initiates entry into the host cell with insertion of the virus genome and thus infects the cell [109].

Additionally ICAM-1 is exploited from several other pathogens. A-type coxsackieviruses like HRV, use the binding to ICAM-1 for triggering RNA release into the host cell. Plasmodium falciparum binds endothelial cells via ICAM-1 and invades; HIV-1 uses ICAM-1 as a coreceptor [110, 111].

Infection cycle

Following attachment to ICAM-1 the virus releases its RNA and bound VP4 into the cytosol of the host cell. Receptor binding of major group viruses leads to conformational changes of the capsid, which facilitate together with a low pH uncoating within endosomes and release of viral RNA [112]. These changes would result in a higher affinity binding between ICAM-1 and HRV through a temperature-sensitive distortion of VP1. This movement occurs only at temperatures > 30°, correlating with body temperature. VP1 would act as a hinge inducing a destabilisation of the capsid and opening a channel at the 5

fold axis for release of RNA [111, 113].

The plus strand RNA is released into the host cytosol, where it directly acts as mRNA. Translation is initiated by binding of ribosomes to the IRES (internal ribosomal entry site). The RNA is translated by polysomes, carrying up to 40 ribosomes. The produced polyprotein has to be processed to obtain the mature proteins. The first cleavage is done by the protease 2A and leads to the liberation of the capsid protein precursor P1 from the rest of the molecule. Protease 3C performs the following cleavage steps [98]. Replication of new viral RNA starts with the production of complementary minus strand RNA to incoming plus strand RNA by a RNA-dependent RNA polymerase. The minus strands serve as templates for the synthesis of new plus strands.

The crucial step for the start of virion assembly is the cleavage of the precursor protein P1, which forms immature protomers consisting of the tightly assembled proteins VP0, 3, 1. Increasing concentrations of immature (5S) protomers trigger the further association of pentamers and the packaging of plus-strand VPg RNA into the developing particles. The resulting 150S provirions are not yet infectious. A final maturation step by auto-catalytically cleavage of VP0 in the mature protomer proteins VP2 and 4 leads to infectious virions. The mature virions are released by cell lysis [98]. Assembled mature virions are released from the cell after 8–10 h by cell lysis of epithelial cells.

Immune response to HRV

The typical common cold symptoms were originally considered to be produced by HRV induced destruction of the nasal mucosa. This assumption was disproved by examination of nasal epithelium in biopsy specimen from people with natural cold. Surprisingly the nasal epithelium was shown to be intact. The only distinguishing feature during common cold was the increased number of polymorphonuclear neutrophils invading into the submucosa and epithelium [114]. HRV replicates only in a small number of mucosal cells and is only slightly inducing cytopathic effects [115]. The conclusion was that the cold

symptoms were induced by the host response itself. The viral infection seems to trigger an inflammatory cascade [116].

HRV infection of primary epithelial cells and epithelial cell lines is accompanied by a release of inflammatory mediators in vivo and in vitro. In particular pro-inflammatory cytokines including IL-1ß, TNF-α, IL-8, IL-6 and IL-11 and the vasoactive peptides bradykinin and lysyl bradykinin [117]. In addition, chemokines such as Rantes, MCP-1 and IP-10 as well as the angiogenic factor VEGF have been found in nasal secretions of patients with colds [117]. As a result, it is now considered that common cold symptoms result from an inflammatory 'cytokine disease' of the host in response to the virus and not from the virus itself. Attracted by these mediators, inflammatory leukocytes, granulocytes, DCs and monocytes are found to migrate to the site of HRV infection [118]. Especially neutrophil infiltration into the submucosa and epithelium was observed during the common cold and may be caused by IL-8, which is a potent chemoattractant and mediator for the invasion of neutrophils [119].

Antibodies against HRV are detectable in the serum 5-7 days after onset of illness. The rise in serum antibodies, mostly IgG, goes along with the rise of antibodies in secretions, mostly IgA. The antibody response has no role in recovery from infection, because it starts when the symptoms already cease [120].

HRV also stimulates nonspecific activation of lymphocytes through a monocyte-dependent mechanism, supposed to enhance airway inflammations involved in the exacerbations of asthma [121]. In certain in vitro experiments immunosuppressive effects of HRV are seen. These would correlate with the fact that HRV infections often promote secondary bacterial infections due to a hindered immune response. High concentrations of HRV were shown to inhibit the T cell responses to memory-antigens and also the responses of cytotoxic T cells to alloantigens, but not responses to mitogens, IL-2 or natural killer cell function [122]. HRV binding to monocytes was shown to induce the immunosuppressive cytokine IL-10 and to inhibit the activating cytokine IL-12

and results in inhibition of antigen-specific T cell responses [92].

HRV immune evasion

A virus's success is directly dependent on its ability to mount an effective anti immune response within the host. As a consequence of this, successful viruses have developed a broad range of mechanisms to manipulate, modulate or shut off key functions of the host immune system [123, 124].
Rapid replication and antigenic variation are critical immune evasion mechanisms of small RNA viruses [124]. Therefore the large number of serotypes combined with a high mutation-rate might be part of the success of HRV infections. However, the intrinsic antigen-variations of HRV do not explain why, despite recruitment of leukocytes, appropriate immune responses appear to be dysregulated or hindered in the respiratory tract upon HRV infection. Also HRV bind to but do not infect or damage leukocytes [92,121].

Modulation of leukocyte interactions

An efficient anti-immune response strategy of certain viruses is the selection of an immunologically relevant cellular receptor. As mentioned, the vast majority of HRV uses the cell surface receptor ICAM-1 to bind to and infect epithelial cells. ICAM-1 is an essential co-stimulatory receptor on APCs and ICAM-1-deficient APCs have a strongly impaired capacity to induce T cell responses [125]. Thus, HRV can directly interact with leukocytes and since the binding site of HRV on ICAM-1 overlaps with the binding site of LFA-1, it is intriguing that binding of HRV to leukocytes might have profound functional consequences. This was demonstrated; ICAM-1 expressed on the surface of human fibroblasts functions as a receptor for HRV as well as a receptor for LFA-1 bearing human T cells, which could be inhibited by an antibody to ICAM-1 [126]. Interestingly, binding of HRV to fibroblasts inhibited the subsequent adhesion of T cells. HRV16, a major group HRV, significantly inhibited T cell proliferation induced by antigen but not proliferation secondary to mitogens, IL-2, or an irradiated allogeneic T

cell line. Noninfectious (UV-irradiated) HRV had similar effects. Inhibition of T cell proliferation was dependent on HRV binding to ICAM-1 on monocytes, indicating that the virus interferes with lymphocyte activation indirectly through effects on antigen-presenting cells [122].

HRV is a natural ligand for ICAM-1 with special binding and consequently signaling features. One HRV capsid has 60 potential binding sites located in depressions of the viral protein coat, the so called canyons [113]. Due to the multivalency of binding sites on HRV particles, this allows high avidity interactions with their receptors. Therefore, HRV can be considered to resemble a perfect cross link. It was investigated by our lab, whether HRV binding to ICAM-1 might influence the adhesiveness of leukocytes [127]. Engagement of ICAM-1 with HRV14 on human mononuclear phagocytes induces homotypic clustering of monocytes and monocyte-derived DC (but not in lymphocytes) which was found to critically involve homophilic PECAM-1 interactions and is mediated via LFA-1/ICAM-3.

Data from another study from our lab suggest that HRV may polarize monocyte differentiation towards a 'Th-3 phenotype'. HRV14 efficiently inhibit antigen-specific T cell responses. These inhibitory effects were found to result from a particular spectrum of cytokines released by mononuclear phagocytes upon HRV14 interaction. Most prominent among them is IL-10, which is a well-established immunosuppressive cytokine, which is strongly induced by HRV14 [92].

Immune Evasion by Targeting DC

DCs are the most potent APCs and play a central role in the generation of primary T cell responses and the maintenance of immunity. Because of their importance in initiating antiviral immune responses, DCs are ideal targets for viral immune evasion strategies. Several viruses are known to modulate DC function and thus impair antiviral T cell responses. The result can be a transient or prolonged suppression of the immune response, often associated with secondary microbial infections or the initiation of a persistent infection. A

particularly efficient trick of viruses, e.g. measles, vaccinia virus, CMV or HIV, is the targeting of the co-stimulatory function of DC. Thereby the virus is able to prevent pathogen specific immunity (i.e. ignorance) and, even more effective, is able to induce pathogen-specific tolerance, when DC present antigens in the absence of costimulatory signals [124, 128].

HRV14 has an immune modulatory effect on monocyte-derived DC

We have recently demonstrated that upregulation of inhibitory accessory molecules on DC represents another efficient viral strategy to turn off antigen-specific immune responses. HRV can blunt adaptive immune responses through the induction of a novel DC activation program. Cocultivation of DCs with HRV-14 (R-DCs) induces the expression of inhibitory receptors B7-H1 (PD-1L, CD274) and sialoadhesin (Sn, Siglec-1, CD169), a sialic acid binding lectin, on the R-DCs surface, without affecting the expression of stimulatory receptors such as CD80 or CD86. HRV14 fails to mature DC, also R-DCs have a diminished T cell stimulatory capacity. The consequence of this altered accessory repertoire on R-DCs is that cocultured T cells acquire a deep anergic state [123, 129].

Since monocytes are the precursor cells of our DCs, we expected that IL-10 production induced upon co-culture of DC with HRV14 was involved in this process. Although small amounts of IL-10 are produced by R-DC, neutralizing antibodies against IL-10 failed to reverse the inhibitory effect of HRV14. Thus, HRV seemingly modulates the accessory function of mononuclear phagocytes and DC through different mechanisms. Importantly, HRV14 effects were not detectable upon addition of the virus to the MLR cultures or due to a brief pretreatment of DC with HRV14 but required a co-culture of at least 24 h. During this time, HRV14 obviously modulates the co-stimulatory repertoire of DC in a special way. We could indeed identify two of such receptors on R-DC, which seem to deliver inhibitory signals to T cells: B7-H1 and sialoadhesin [129].

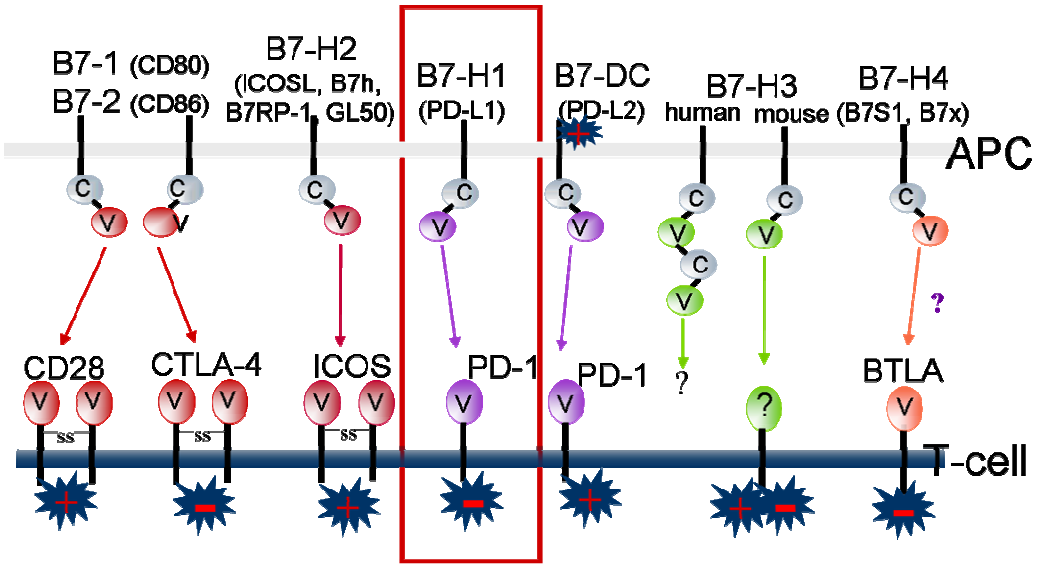


Figure 2. The B7 family

B7-H1, a member of the B7 molecule family, is a well-defined accessory molecule with inhibitory effects through its receptor PD-1 on the T cell side (Figure 2) [130]. B7-H1 is critically involved in the induction and maintenance of T cell anergy induced by IL-10- treated DC [131]. Yet, blocking of B7-H1 was not sufficient to revert the deep anergic state in T cells triggered by R-DC. Since other inhibitory accessory molecules on DC are not characterized so far, it was necessary to search for such candidate structures on R-DC. S.Kirchberger could identify sialoadhesin as that inhibitory accessory molecule on R-DC (Figure 3) [129].

Sialoadhesin is a member of sialic acid binding lectin family of I-type lectins which preferentially binds to sialylated carbohydrate structures (e.g. NeuAca2,3-Gal) [132]. Sialoadhesin is a frequently used marker for macrophages because it is not expressed on monocytes, lymphocytes and DC. Our finding that sialoadhesin is strongly induced on DCs upon HRV14 treatment after 1–2 days correlates with the appearance of the inhibitory phenotype of R-DC. CD43 has been recently described as ligand for Sn on T cells [133].

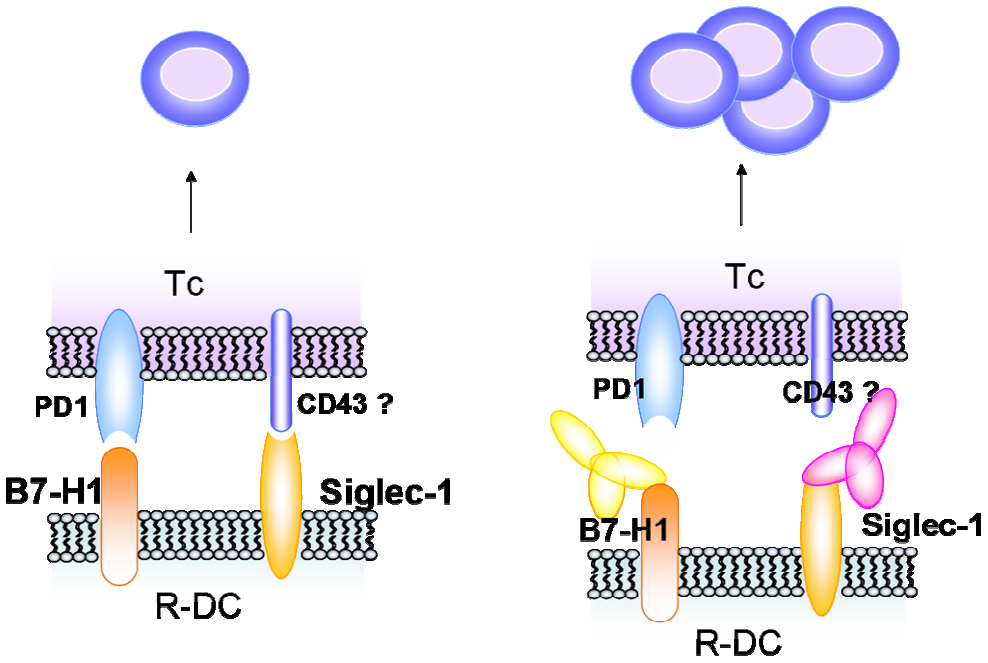


Figure 3. HRV14 inhibits the accessory function of DCs

In a previous study of our lab it was found by using an antibody-based screen, that B7-H1, a recently described inhibitory member of the B7 family and Sialoadhesin, a lectin which belongs to the Siglec family, are strongly upregulated on R-DC. These molecules are also involved in the weak T stimulatory function of R-DC, as blocking of these molecules by antibodies restored T cell proliferation. Adapted from Kirchberger et al [129].

Now, we decided to further examine the function of these R-DC treated T cells. What we already knew was that the consequence of this altered accessory repertoire on R-DC is that co-cultured T cells acquire a deep anergic state.

The IL-12 cytokine family

Cytokines are the major mediators of host defense, they regulate communication between APC, lymphocytes and other host cells in the course of inflammation, infection and antigen specific immune responses. Cytokines are autocrine and paracrine signalling peptides which essentially act between immune cells temporally and spatially [134]. Most of them are immediately released into the surrounding interstitial fluid upon biosynthesis [135], modulate the functional activities of individual cells and tissues, thereby mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Cytokine expression is normally transient and can be regulated at all levels of gene expression [136]. Multiple cytokines often have overlapping ubiquitous biological activities so that their physiologic significance as normal regulators is often difficult to assess [137]. This is explained in part by the sharing of common receptor subunits among members of the cytokine family. A single cytokine may elicit reactions under certain circumstances, which are the reverse of those seen under other circumstances. Various factors can influence the effects of a certain cytokine like: the type, the duration, the growth state of the responder cell, the type of neighbouring cells or the combination of other cytokines present at the same time together with the temporal sequence of several cytokines acting on the same target cell [134]. Regulation of this system is of great importance and for example several cytokines are inhibited by other cytokines or soluble factors [138]. A number of viruses exploit these facts to evade immune responses of the host.

The interleukin- 12 (IL-12) cytokine family remains one of the most important ones and includes IL-12, IL-23, IL-27, and the recently identified IL-35. IL-12 cytokine family members differ from other type I cytokine superfamily members, because they are heterodimeric complexes.

IL-12

Interleukin-12 is a 70 kD heterodimer consisting of two subunits, designate p40 and p35 and signals through a heterodimeric receptor. The p40 subunit shows similarities to cytokine receptors and the p35 subunit is related to Granulocyte-Colony Stimulating Factor and IL-6. For IL-12 signaling, the IL-12p40 subunit interacts primarily with IL-12Rb1, while p35 binds to IL-12Rb2 [139]. IL-12 is mainly produced by activated APCs (monocytes, macrophages,DCs, neutrophils and B cells) and has been shown to play a role in differentiation and expansion of Th1 responses [140]. Beside its effects on Th1 responses it was shown that IL-12 is required for the generation of CTLs [141, 142]. Inhibition of IL-12 production occurs via the release of IL-10, IL-11, IL-13 and type 1 interferons (IFNs) [143].

IL-23

In 2000, p19, was identified on the basis of its homology with IL-6 and the p35 chain [144]. This protein was shown to associate with the p40 chain to form another heterodimeric cytokine known as IL-23. IL-23, like IL-12, is a pro-inflammatory cytokine that induces IFN-gamma production and promotes the Th1 response. IL-23 participates in a positive feedback loop promoting IL-12 production by DCs to enhance p19 transcription and further IL-23 production. However, in the absence of IL-23, IFN-gamma production and Th1 differentiation are normal [145], indicating that while IL-12 and IL-23 can synergize for maximal Th1 induction, IL-23 is not absolutely required for Th1 differentiation. IL-23 also has a role in promoting natral killer (NK) cell activity, in that IL-23 synergizes with IL-12, IL-18, and IL-2 to promote IFN-gamma production by NK cells [146]. A Th1 independent function of IL-23 has also been elucidated. Unlike the other IL-12 family cytokines, IL-23 promotes the survival of Th17 cells induced by IL-6 and transforming growth factor ß (TGF-ß) [147]. In fact, exposure to IL-23 and IL-1 is necessary for full effector differentiation and function of Th17 cells [148]. The IL-23 receptor is composed of IL-12Rb1 and

the novel receptor chain IL-23R [146].

IL-27

IL-27 comprises the p28 and EBI3 (Epstein–Barr virus-induced gene 3) chains and displays homology with the p35 and p40 chains, respectively [149]. Following the discovery of IL-27, initial reports indicated that IL-27, in combination with IL-12, enhanced the production of IFN-gamma by naive T cells and NK cells by promoting expression of T-bet and IL-12Rb2 [150]. This observation suggests that IL-27 sensitizes T cells to the effects of IL-12 and is critical for the early events leading to Th1 cell polarization. However, more recently, of immunomodulatory functions have been ascribed to IL-27 as reviewed by Stumhofer and Hunter [151]. It is indicated that IL-27 can inhibit Th1- driven infections, limit Th2 activity toward *Leishmania major* and block the development of Th17 and TGF-ß driven induced Treg formation [152]. Like IL-12, IL-27 can directly inhibit secretion of IL-4 and antagonize IL-2 production, hence limiting Th2 cell differentiation and effector cell function [153]. IL-27 signals through a heterodimeric pairing of gp130 and the previously orphaned receptor chain WSX-1 [147, 154].

IL-35

Quite recently a new member of the IL-12 family, called IL-35, was described. IL-35 is a heterodimeric cytokine consisting of EBV-induced gene 3 (EBI3) and the p35 subunit of IL-12 and has a size of 78 kDa [155]. The discovery of this cytokine further confirms that many pairings of IL-12 family proteins are possible, as EBI3 binds to p35.

EBI3 is a homologue to IL-12p40, and was discovered in EBV (Epstein Barr virus) infected B lymphocytes [156]. EBI3 is also a part of IL-27. The EBI3 protein has a size of 34 kDa. While p35 is constitutively expressed in most tissues, EBI3 is mainly expressed in hematopoetic cells. Its expression was

discovered in lymphoid blasts of reactive lymphoid organs, in the light zone of germinal centers, where B lymphocytes are associated to T cells. A splice variant of EBI3 was described to be located in the spleen, liver and kidney [157]. 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 [158]. Intestinal epithelial cells (as initial APCs) constitutively express EBI3 (comparable to p35) and the expression level is up-regulated through IL-1α or TNF-α addition. In contrast, the p35 level is also increased in the presence of IFN-gamma [159]. It is proposed that free, latent EBI3 homodimer produced by APCs acts as an antagonist of IL-27 and therefore prevents any further IL-27 stimulation of naive T cells [149].

Collison et al. demonstrated that the EBI3–p35 heterodimeric complex is constitutively produced by mouse regulatory T cells and not effector T cells. In particular EBI3 is strongly overexpressed in Tregs. In this study, *EBI3–/–* and *p35–/–* regulatory T cells had reduced regulatory activity both *in vitro* and *in vivo*, which indicated that IL-35 is required for the suppressive activity of regulatory T cells, at least in mice [160]. IL-35 has been shown to expand murine $CD4^+CD25^+Foxp3^+$ regulatory T cells, if T cells are cultivated under strong inductive conditions with polyclonal T cell receptor activation and the presence of costimulatory signals [155]. IL-35 is required for the suppressive activity of regulatory T cells, and EBI3 is a downstream target of Foxp3, a transcription factor that is required for Treg development and function [160-162]. IL-35 also downregulates the production of IL-17 under Th17-inducing conditions and was shown to attenuate collagen-induced arthritis [155]. In the same paper IL-35 was also shown to suppress the proliferation of $CD4^+CD25^-$ murine effector T cells, but expand the proliferation of CD4+CD25+ Tregs.

IL-35 inhibited clinical manifestation of collagen-induced arthritis or could cease further disease exacerbation upon initiation of IL-35 treatment [155]. Exogenous IL-35 treatments suppressed Th1 and Th17 cells and promoted CD39 expression by $CD4^+$ T cells. Sorted $CD25^-CD39^+CD4^+$ T cells from IL-35-treated

mice produced IL-10 and, upon adoptive transfer, were sufficiently potent to inhibit subsequent development of inflammation in mice with collagen-induced arthritis. IL-35 stimulation of $CD39^+$ regulatory T cells confers protection against collagen II-induced arthritis via the production of IL-10 [163].

In contrast to all the murine data, human $CD4^+CD25^+Foxp3^+$ regulatory T cells do not constitutively express IL-35 and induction of Foxp3 does neither upregulate EBI3 nor p35 mRNA. IL-35 does not contribute to the suppressive function of Foxp3 induced human Tregs [164, 165]. Nevertheless EBI3 transcripts are found in activated effector CD3 positive T cells and p35 is described to be constitutively expressed in various T cell subsets. [164, 165].

In addition, previous work from 1997 indicated that EBI3 and p35 are highly expressed in human placental trophoblasts, as shown by co-precipitation experiments. These findings suggest that, IL-35 may be an immunomodulator at the feto-maternal border [166]. Whether this effect is due to Treg mediated suppression of maternal responses to the fetus or due to the suppressive capacity of other cell types that might secrete IL-35 remains to be determined.

Although many of the biological effects of IL- 35 are still being elucidated, it is clear that IL-35 directly suppresses Teff cell proliferation in vitro, in an APC free culture. Moreover, loss of IL-35 expression results in reduced in vivo suppressive capacity of Tregs [160]. How IL-35 can suppress in vivo Th17 development and ameliorate collagen-induced arthritis [155] also requires further study. Also the receptor of IL-35 and the following signalling cascade remain to be explored.

In summary, IL-35 is a novel inhibitory cytokine that is produced by Tregs and contributes to their suppressive activity. In contrast to all other known IL-12 family members, which are not expressed by T cells, IL-35 is secreted by Treg cells. It functions in an inhibitory, rather than an immunostimulatory or proinflammatory, manner. Synonymous with differential expression, the IL-12 cytokine family also exhibits distinct functional differences. While IL-12, IL-23 and IL-27 share the common feature of inducing IFN-gamma production and promoting Th1 cell differentiation and proliferation, they act differentially on

subsets of T cells and with different kinetics. In contrast, IL-35 appears to function solely in an anti-inflammatory fashion by inhibiting T cell proliferation and perhaps other parameters [160].

The biological effects of IL-12, IL-23, IL-27 and may be also IL-35 are mediated through their interaction with their receptor chains that bind to and induce phosphorylation of the Janus kinase (Jak) family proteins [146]. Jak proteins then phosphorylate tyrosine residues on the intracellular domains of the receptor chains, which act as docking sites for various members of the signal transducer and activator of transcription (STAT) family. Phosphorylated STAT proteins form intramolecular complexes that translocate into the nucleus and bind DNA to initiate the gene expression [2].

All four members of the family are heterodimeric cytokines, composed of an alpha chain (p19, p28, or p35) and a beta chain (p40 or EBI3), and (may) signal through unique pairings of five receptor chains (IL-12Rb1, IL-12Rb2, IL-23R, gp130, and WSX-1) as also shown on Figure 4.

Macrophages, DCs and monocytes can produce IL-12, IL-23 and IL-27 following exposure to PAMPs that act through TLRs [167], this is not the case for IL-35, because it is described to be released by T cells (at least in mice). Due to the fact that APCs produce both IL-12 and IL-27, it is possible that IL-35 is can also be produced by a subset of APCs, because APCs are equiped to produce both subunits of IL-35 (Figure 4).

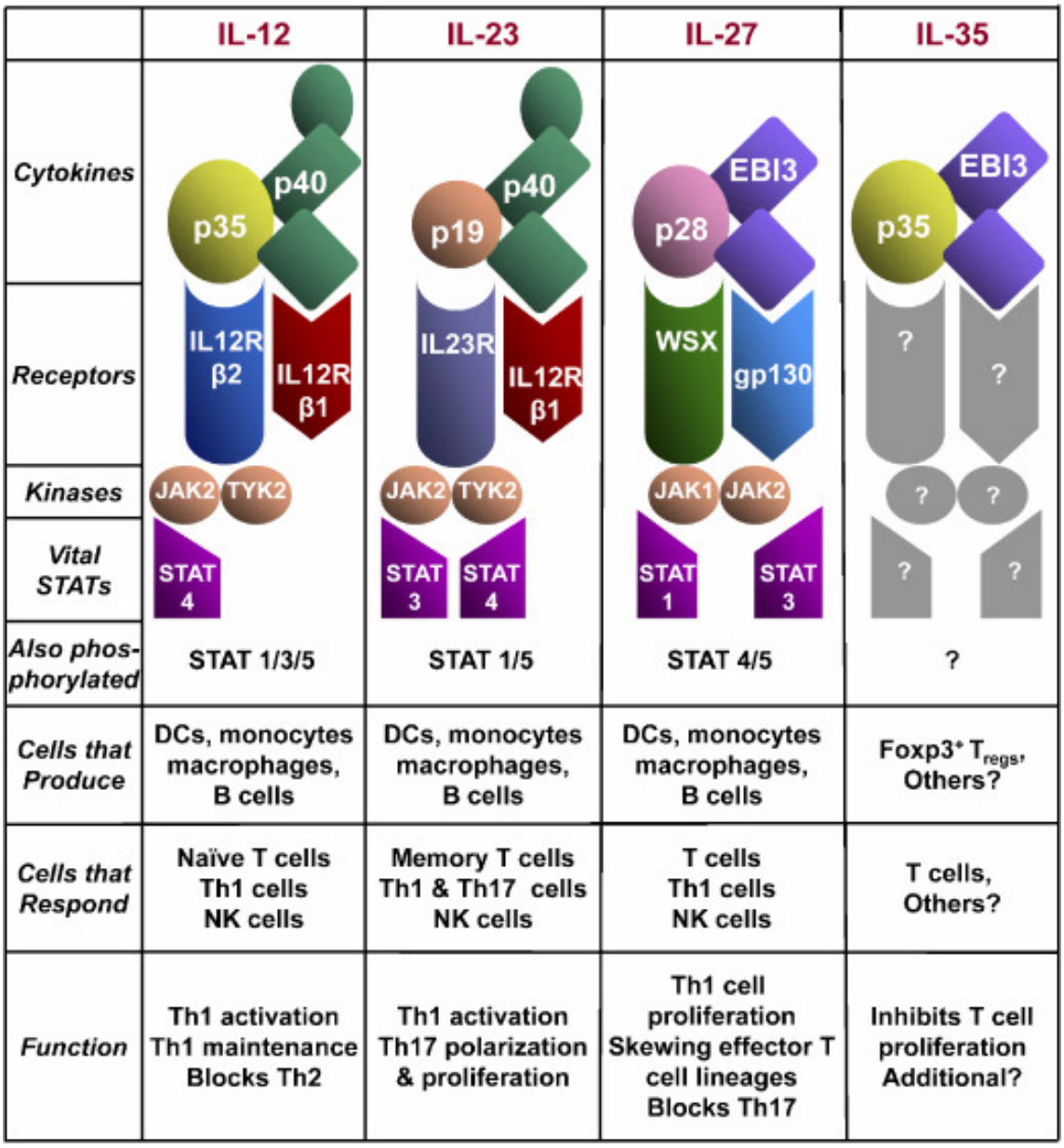


Figure 4. The IL-12 cytokine family

The interleukin- 12 (IL-12) cytokine family is of great importance and includes IL-12, IL-23, IL-27 and the recently identified IL-35. All four are heterodimeric cytokines, composed of an α chain (p19, p28, or p35) and a ß chain (p40 or Ebi3), and signal through unique pairings of five receptor chains (IL-12Rb1, IL-12Rb2, IL-23R, gp130 and WSX-1); adapted from [161].

Aim

Human rhinoviruses (HRV) are the major cause of common cold in humans and are known to circumvent adaptive immune responses. Rhinovirus treated dendritic cells (R-DCs) have a strongly diminished T cell stimulatory capacity. T cells cocultured with R-DCs acquire a deep anergic state. Anergy is a hallmark of regulatory T cells that is why, in this study, we intended to explore if T cells co-cultured with R-DCs, also gain regulatory function.

And if so, to identify the factor(s) responsible for the inhibitory nature of R-DC treated human T cells.

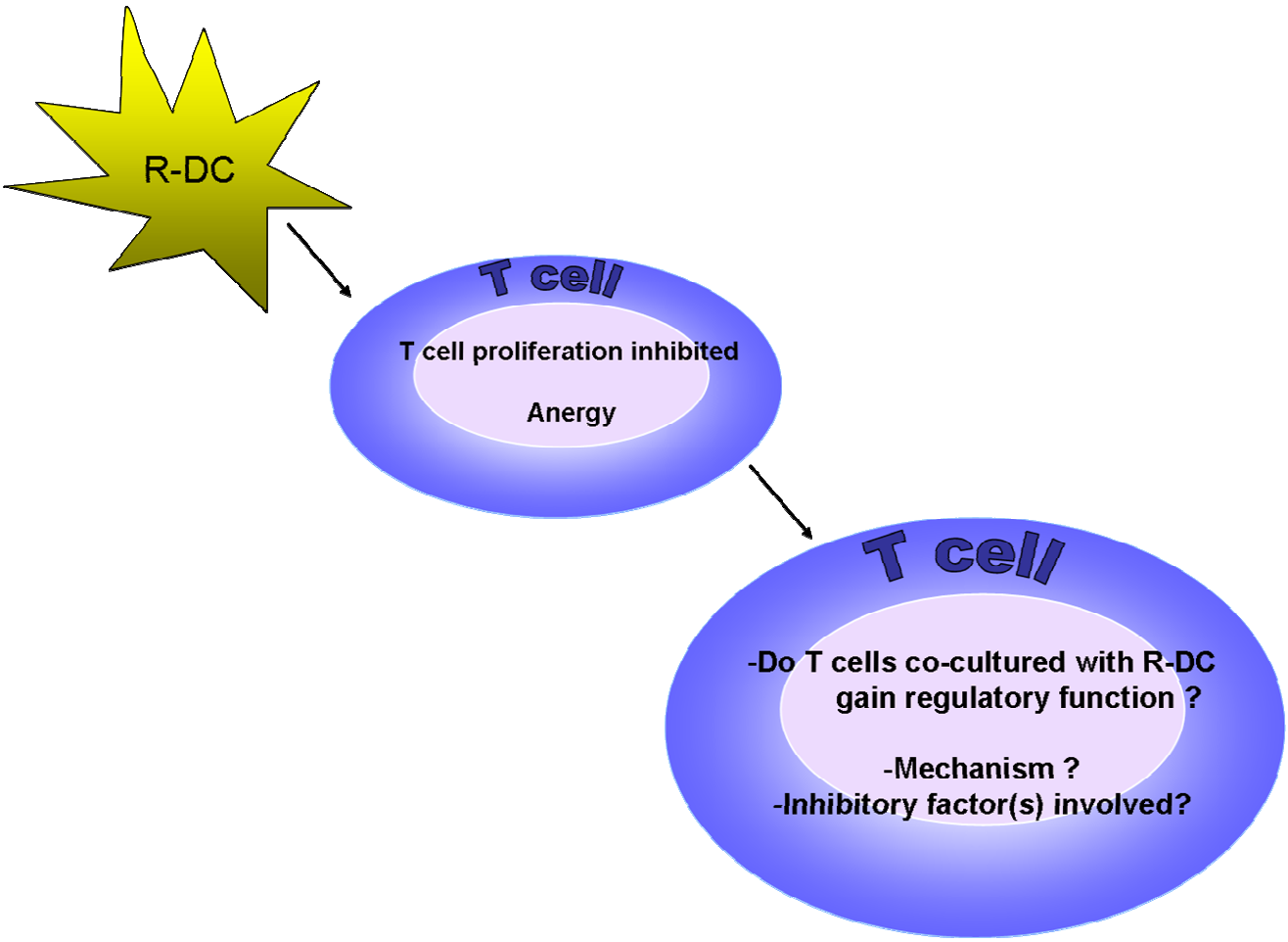


Figure 5. Aim of study

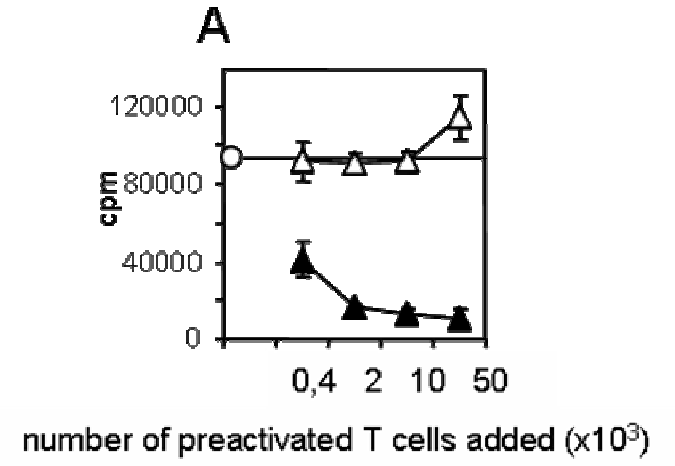
Results

Dendritic cells treated with human rhinovirus induce regulatory T cells

Our laboratory has a longstanding interest in human rhinoviruses (HRV) and their interaction with immune cells. Kirchberger et al. recently found out that HRV is able to subvert the T cell stimulatory function of human dendritic cells (DCs). Cocultured T cells acquire a deep anergic state [129]. The first aim of this study was to investigate if T cells stimulated with HRV treated DCs (R-DCs) in addition to the already described anergy, also gain regulatory function. For that matter T cells were co-cultured with R-DCs and these T cells, after irradiation, added to an MLR.

The results presented in Figure 6A demonstrate that addition of T cells, which were prestimulated with R-DCs, strongly inhibited T cell proliferation induced by untreated DCs in an MLR. Such an effect was not seen when T cells primed with untreated DCs were used.

Prior fixation of R-DCs-induced Treg-cells with formaldehyde, reverted the inhibitory function as depicted in Figure 6B. Addition of the inhibitor WIN 52035-2 at the time of transfer, which specifically blocks HRV binding to its cellular receptor ICAM-1 [168], did not remove the suppressive effect. This indicates that viral transfer is not involved in the inhibitory response (Figure 6C). Depletion of R-DCs from the coculture with T cells did not alter the results, the purified prestimulated T cells showed eminent inhibitory effects when added to an MLR. We conclude that T cells prestimulated with R-DCs are responsible for the inhibitory effect observed.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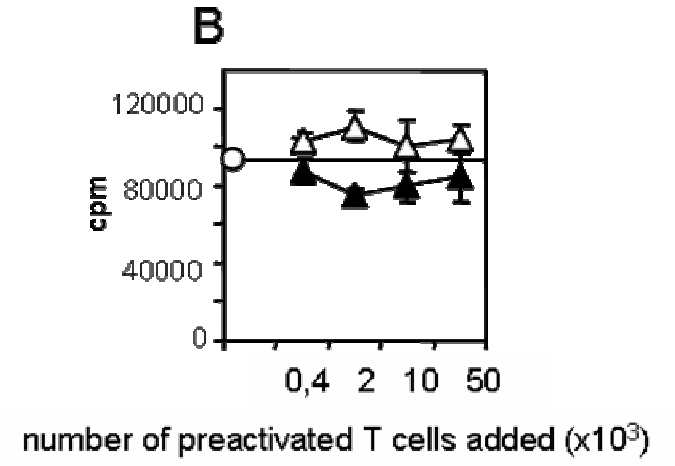
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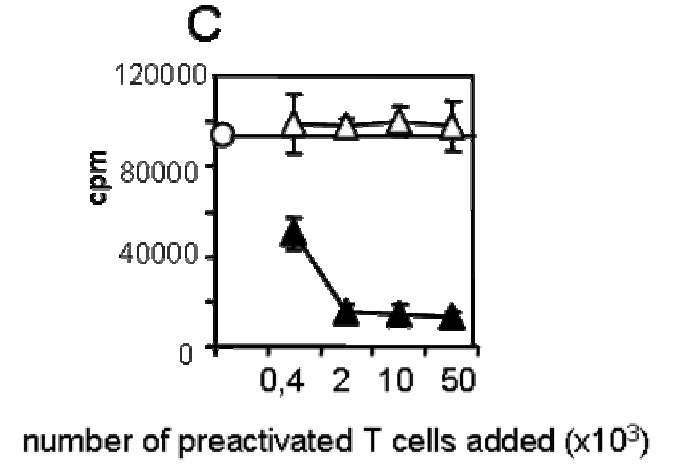
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Figure 6. R-DCs induce Treg-cells

(A) Human purified T cells (1x10^5) were stimulated with allogeneic DCs (1x10^4) alone (○) or in the presence of graded numbers of irradiated T cells, which had been preactivated with HRV-14 treated DCs (R-DCs, ▲) or mock treated DCs (∆). The Figure shows the result of one representative out of four independent experiments expressed as MV ± SD of triplicate determinations.

(B) As in A, but the preactivated T cells (▲ and ∆) were fixed with formaldehyde (1%) before addition to the T cell proliferation assay. The Figure shows the result of one representative out of four independent experiments expressed as MV ± SD of triplicate determinations.

(C) As in A, but in the presence of WIN52035-2 (5 µg/ml), an inhibitor of HRV-receptor interaction. The Figure shows the result of one representative out of four independent experiments expressed as MV ± SD of triplicate determinations.

R-DCs induced regulatory T cells are not correlated with a change in Foxp3 expression

Foxp3 is a forkhead family transcription factor very importantly involved in the development of regulatory T cells (Tregs), therefore we evaluated its levels in R-DCs induced Tregs. Analysis of Foxp3 expression via quantitative PCR (qPCR) in CD25- T cells revealed that induction of Foxp3 does not differ between DC and R-DC stimulated T cells. This indicates that the suppressive capacity of R-DC stimulated T cells is not directly correlated with an increased induction of Foxp3. Also we have observed that Foxp3 levels drop again after 48 h and 96 h in T cells stimulated with R-DC as well as DC (Figure 7). Such activation-induced transient Foxp3 expression in human T cells has been frequently observed before [43, 74, 75] and is not necessarily correlated with regulatory activity [43, 165, 169].

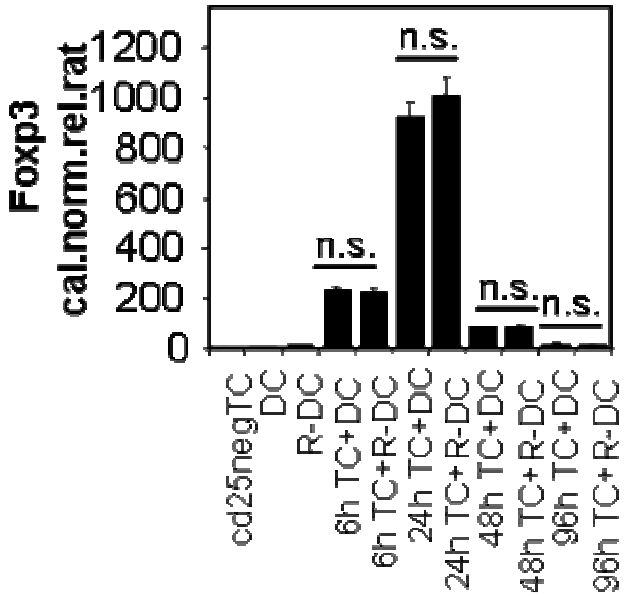


Figure 7. R-DC stimulated T cells are not correlated with an increased induction of Foxp3

CD25⁻ T cells were cocultured with DCs or R-DCs for the indicated timepoints and Foxp3 expression was evaluated via qPCR. The results of one representative experiment out of four independent experiments are shown. MV of duplicate determinations ± SD are shown; n.s. not significant.

R-DCs induced peripheral blood regulatory T cells release an inhibitory factor

In order to analyze whether the observed inhibitory effect was mediated through one ore more soluble factors, we added the supernatant (SN) of the R-DCs-induced Tregs to T cell/DC cocultures. The SN of R-DCs alone, as described by us before, does not lead to diminished T cell proliferation [129].

The inhibitory effect was however found in the SN of R-DCs-induced Tregs which is shown in Figure 8A, were the SN was added to an MLR. This indicates that a compound in the SN and not cell-cell contact establishes the inhibition of T cell proliferation.

To further define the nature of the T cells releasing this factor(s) we looked at $CD4^+$ and $CD8^+$ T cells separately. Either purified $CD4^+$ or $CD8^+$ peripheral blood T cells were cocultured with R-DCs and the SN of both, $CD4^+$ as well as $CD8^+$ T cells contained this suppressive factor (Figure 8B and C), because the SN of both again showed a strong T cell inhibitory capacity.

This factor was not released by naïve T cells isolated from human cord blood, since the SN of naïve T cells cocultured with R-DCs was not inhibitory, as shown in Figure 8D.

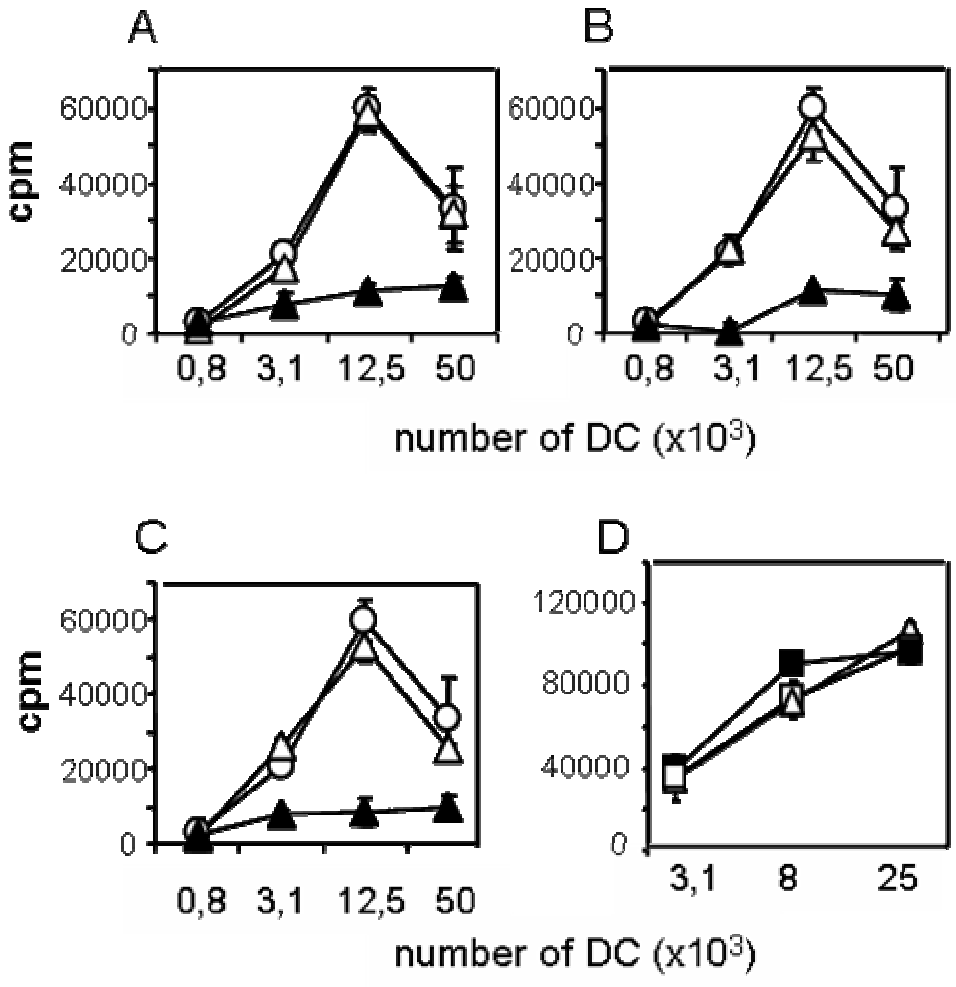


Figure 8. R-DCs induced Treg-cells release an inhibitory factor

(A) Purified peripheral blood T cells were stimulated with DCs or R-DCs for 3 d.

The cell culture SN was harvested and examined in an allogeneic T cell DC coculture. DCs-triggered allogeneic T cell proliferation alone (○) or plus 100 µl SN from T cells preactivated with R-DCs (▲) or SN of T cells preactivated with mock-treated DCs (∆) is shown. The Figure shows the results of one representative experiment out of four independent experiments. MV of triplicate determinations ± SD are shown.
(B) As in A, but the SN was derived from purified $CD4^+$ T cells (▲ and ∆). The Figure shows the results of one representative experiment out of four independent experiments.
(C) As in A, but the SN was derived from purified $CD8^+$ T cells (▲ and ∆). The Figure shows the results of one representative experiment out of four independent experiments.
(D) Purified naïve T cells isolated from human cord blood were stimulated with DCs or R-DCs for 3 d. The cell culture SN was harvested and examined in an allogeneic T cell DC coculture. DCs-triggered PB T cell proliferation alone (∆) or plus 100 µl SN from naïve T cells preactivated with R-DCs (■) or the SN of naïve T cells preactivated with mock-treated DCs (□) is shown. The Figure shows the results of one representative experiment out of four independent experiments. MV of triplicate determinations ± SD are shown.

R-DCs do not inhibit proliferation of naïve T cells

We than decided to take a closer look on naïve T cells isolated from human cord blood. These T cells, when cocultured with R-DCs did not show reduced proliferation (Figure 9B). This stands in contrast to the finding that in the coculture of peripheral blood T cells and R-DCs, T cell proliferation is impaired as described by Kirchberger et al. [129] and what we also reproduced in Figure 9A. Thus, R-DCs mediated inhibition is specific for $CD4^+$ and $CD8^+$ effector T cells but not for naïve T cells isolated from human cord blood.

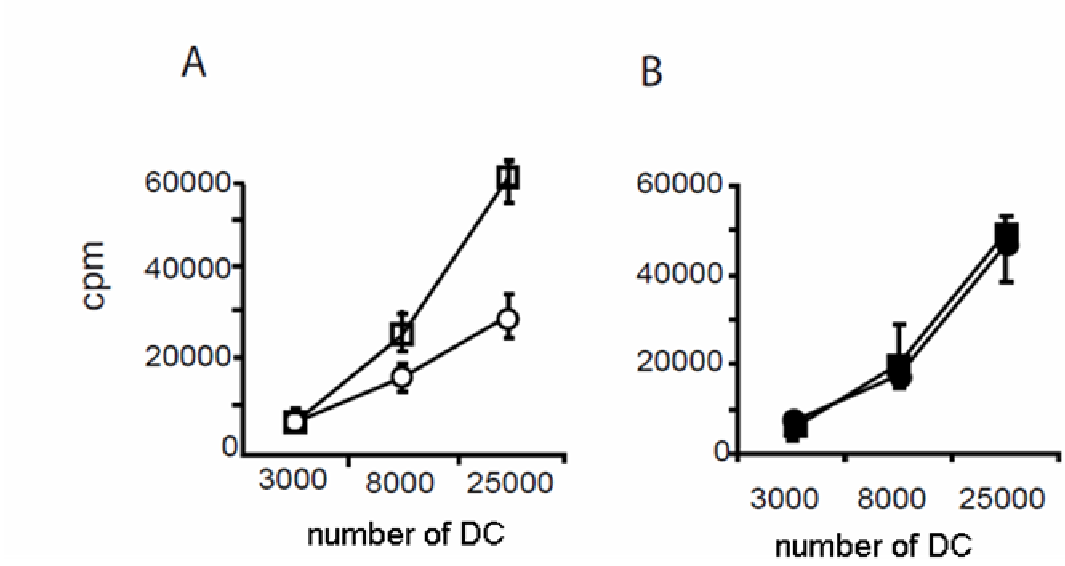


Figure 9. R-DCs do not inhibit naïve T cells

(A) Purified peripheral blood T cells were stimulated with graded numbers of allogeneic DCs inoculated with HRV-14 (○) for 24 h, or mock-treated DCs (□).The results of one representative experiment out of four independent experiments are shown. MV of triplicate determinations ± SD are shown.

(B) Purified naïve T cells isolated from human cord blood were stimulated with graded numbers of allogeneic DCs inoculated with HRV-14 (●) for 24 h, or mock-treated DCs (■). The results of one representative experiment out of three independent experiments are shown. MV of triplicate determinations ± SD are shown.

R-DCs induced human regulatory T cells act independent of IL-10, TGF-ß and IFN-α

One group of T cells with suppressor function are inducible Tregs. These cells can develop from mature T cell populations under certain conditions, e.g. upon stimulation with tolerogenic DCs. They act via release of soluble factors such as IL-10, a well established inhibitory molecule. IL-10 and TGF-ß, two cytokines

with suppressive capacity, can be released by inducible Tregs. Furthermore cultivation of T cells in the presence of type I IFNs and these two cytokines induces regulatory T cells [48].

In order to elucidate if the found inhibitory effect was mediated through IL-10 or other prominent inhibitory factors, we added the SN of our R-DCs-induced Treg-cells to an MLR and investigated whether the inhibitory effect was reversible with neutralizing antibodies to IL-10, TGF-ß or IFN-α [44, 170].

The inhibitory quality of the SN of R-DCs-induced Treg-cells was not reversible with mAbs against IL-10, IFN-α and TGF-β as depicted in Figure 10.

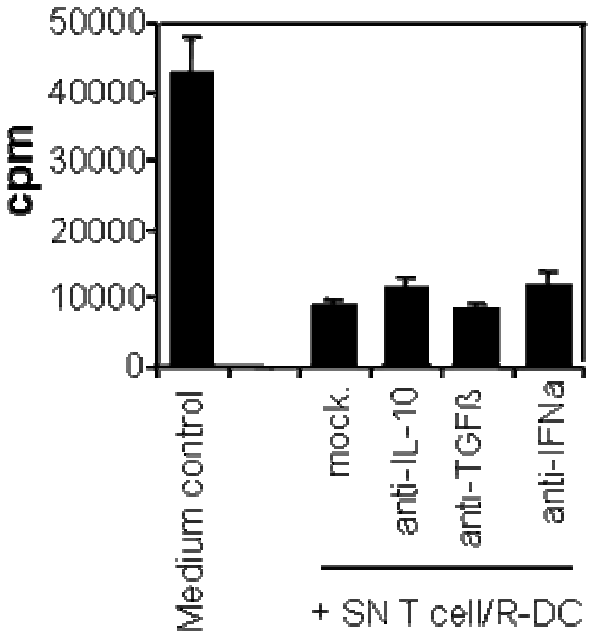


Figure 10. The released inhibitory factor is not IL-10, TGF-ß or IFN-α

Purified T cells were stimulated with DCs or R-DCs for 3 d. The cell culture SN was harvested and preincubated with neutralizing mAbs against IL-10, TGF-β and IFN-α (10 µg/ml) before examination in an allogeneic T cell proliferation response induced by DCs. Data are representative of 3 independent experiments. MV of triplicate determinations ± SD are shown.

The utilized mAbs against IL-10, TGF-β and IFN-α have neutralizing capacity

To demonstrate that the utilized anti-cytokine Abs for Figure 10 were indeed neutralizing the following experiment was performed. First the inhibitory effect of IL-10, TGF-β and IFN-α on a T cell/DC coculture (MLR) was established and then the reversibility of this effect by adding the neutralizing respective antibodies was tested, this is depicted in Figure 11.

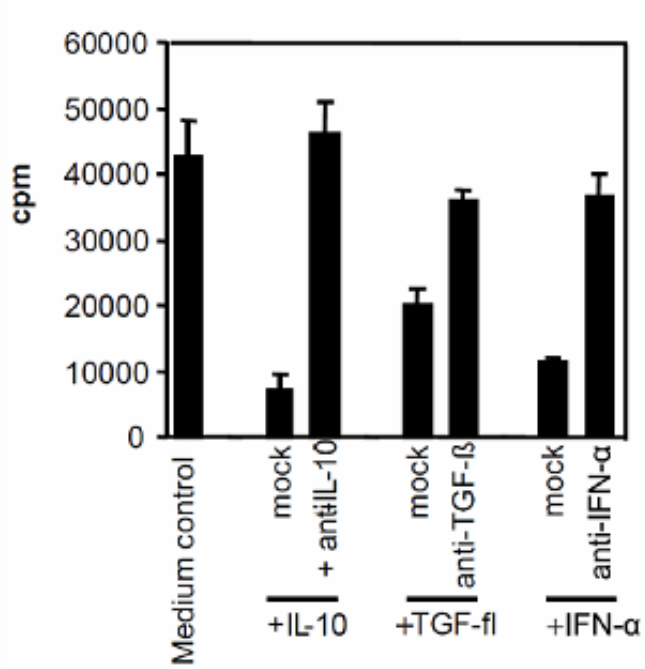


Figure 11. The utilized mAbs have neutralizing capacity

Proliferation of purified peripheral blood T cells ($1x10^5$) was induced with allogeneic DCs ($1x10^4$). IL-10, TGF-β or IFN-α (5 ng/ml) was added to the DCs/T cell coculture either alone or in combination with neutralizing mAbs (10 µg/ml) against the respective factor. The results of one representative experiment out of three independent experiments are shown. MV of triplicate determinations ± SD are shown.

The released inhibitory factor is ≥ 50 kDa

To determine the size range in which the inhibitory factor could be expected size fractionation of the T cell/R-DC SN was performed. It revealed that the inhibitory factor is found in the >50 kDa fraction and not in the <50 kDa molecular weight range (Figure 12).

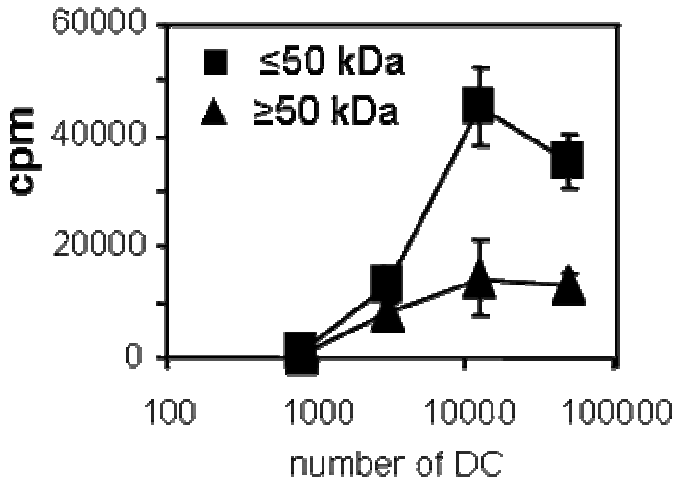


Figure 12. The released inhibitory factor is ≥ 50 kDa

Purified T cells were stimulated with DCs or R-DCs for 3 d. The cell culture SN was harvested and size-fractionated. The inhibitory effect of the SN was found in the fraction ≥50 kDa (▲) and not in the fraction ≤50 kDa (■). Data are representative of 2 independent experiments. The results are expressed as MV ± SD of triplicate determinations.

R-DCs induce IL-35 expression in human T cells

The observation that the inhibitory factor is expected to be >50 kDa lead us to investigate the recently described IL-35. IL-35 is a heterodimeric cytokine consisting of EBI3 (EBV-induced gene 3) and the p35 subunit of IL-12. It was

described to have inhibitory function, a molecular size of 78 kDa and to be produced by T cells [155].

We observed that T cells cultured with R-DCs, showed elevated levels of EBI3 and p35 mRNA, as shown by qPCR in Figure 13A. But there were no changes in the p28 levels, p28 forms together with EBI3 IL-27. Intracellular stainings showed that EBI3 was also upregulated at the protein level in peripheral blood T cells stimulated with R-DCs in comparison to T cells cocultured with DCs (Figure 13B, left column). In naïve T cells stimulated with R-DCs we did not observe an upregulation of EBI3 (Figure 13B, right column). P35 is constitutively expressed in DCs or R-DCs stimulated peripheral blood T cells or naïve T cells as shown in Figure 12B. This is in accordance to previous findings showing that p35 is constitutively expressed in various types of human T cells [164].

In order to find out whether R-DCs induced inhibitory T cells release IL-35, we co-immunopreciptated the cytokine out of SNs of T cells and R-DCs or DCs co-cultures, the supernatants were pooled from 6 donors. The cytokine was captured with an anti-p35 mAb coated on anti-mouse beads and than visualized with an anti-EBI3 Ab on the western blot membrane. As shown in Figure 13C, R-DCs treated T cells release eminently more IL-35 as the DCs stimulated T cells.

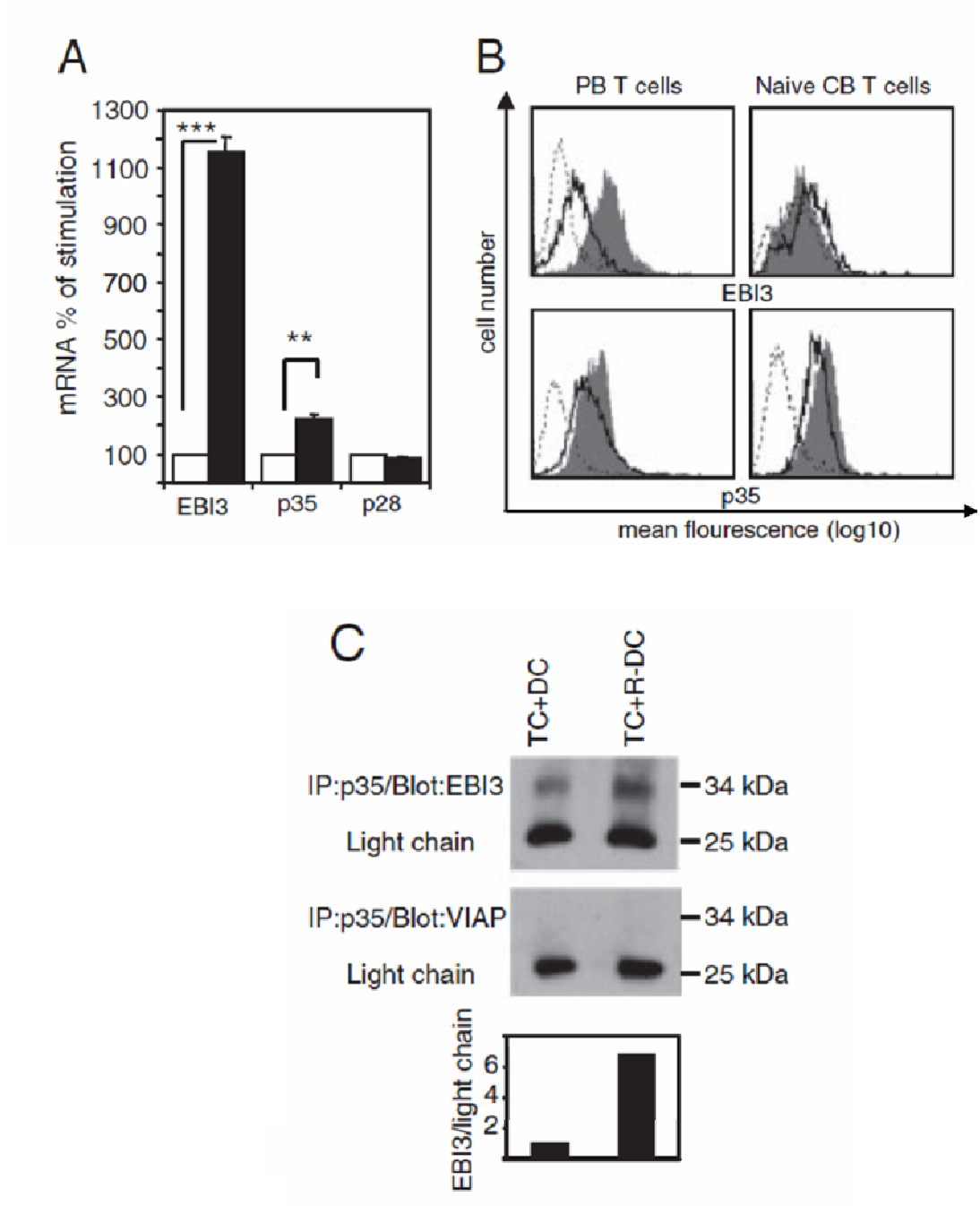


Figure 13. R-DCs induce IL-35 expression, production and release in human T cells

(A) T cells were cocultured with R-DCs (black bars) or DCs (white bars) in a 10:1 ratio for 24 h and the production of EBI3, p35 and p28 was measured at the mRNA level via qPCR. One representative experiment out of three is

shown; MV ± SD are calculated from duplicates.
(B) EBI3 and p35 production in peripheral blood (PB) T cells or naïve T cells isolated from human cord blood (CB) cocultured with DCs (open histogram) or R-DCs (filled histogram) for 24 h was detected by intracellular staining. The dotted line shows the isotype control mAb VIAP. The T cells gate is shown. Data are representative of three independent experiments.
(C) Cell culture SN of T cells stimulated with DCs or R-DCs for 24-72 h were pooled from 6 independent experiments and immunoprecipitated with an antibody against p35. These samples were immunoblotted with an EBI3 antibody (upper blot) or a control Ab: lower blot). As a loading control, the light chain of the p35 mAb used for precipitation, detected with sheep-anti–mouse-HRP (SaM), was used. Blots were further analyzed densiometrically using Image J software. Data are presented as ratio of EBI3 protein to light chain. This experiment was performed three times and is representative of a pool of 6 cell donors; $p < 0.01$ (**) and $p < 0.001$ (***).

R-DCs induced inhibitory T cells do not release IL-12 and IL-27

We wanted to control further, that no other cytokine of the IL-12 family was detected by our system. We excluded that IL-12 (which also has a p35 part) and IL-27 (sharing EBI3) were released by our R-DCs induced inhibitory T cells.
First the anti-p35-mAb coated beads used for immuno-precipitation (previous Figure) of IL-35 out of the T cell/R-DC SN were analyzed via flow cytometry and show clear reactivity with the EBI3 antibody, confirming the release of IL-35, as depicted in Figure 14A. Only weak reactivity is observed with the respective beads precipitating out of the T cell/DC SN (Figure 14A). Moreover, you can see in Figure 14B only weak reactivity of the beads with anti-p40 mAb (IL-12) and no reactivity with anti-IL-27 mAb. This proves that T cells stimulated with R-DCs do not release the IL-35 relatives: IL-12 and IL-27.

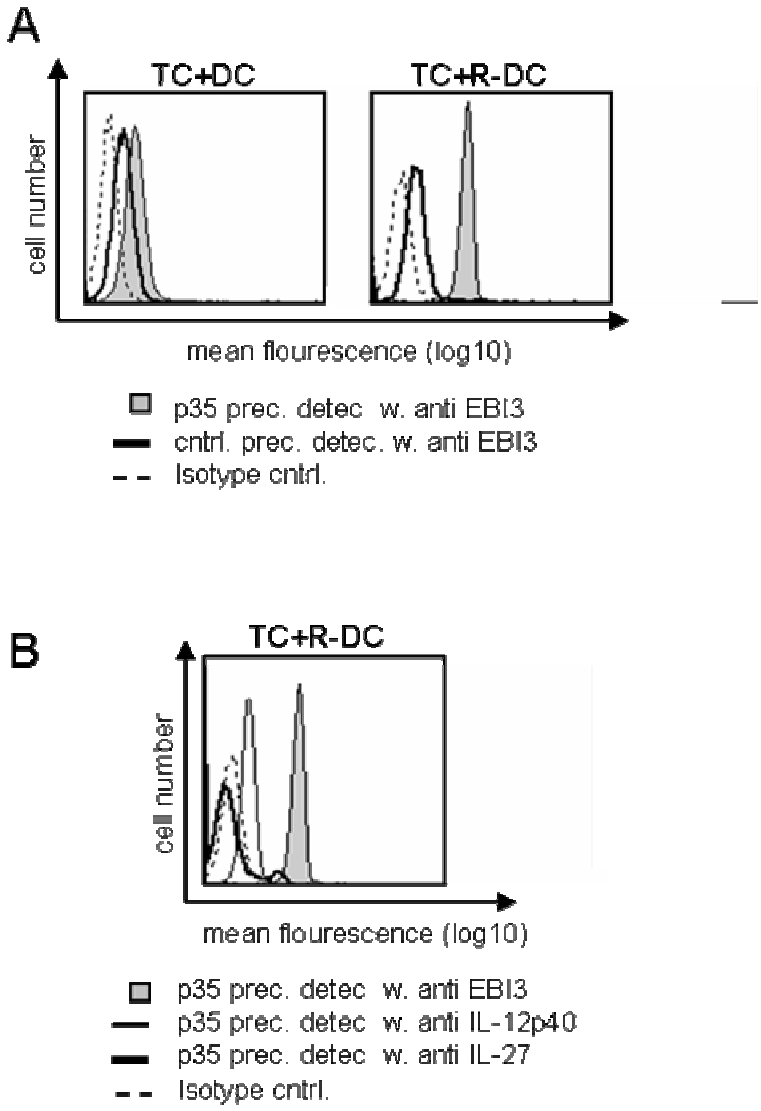
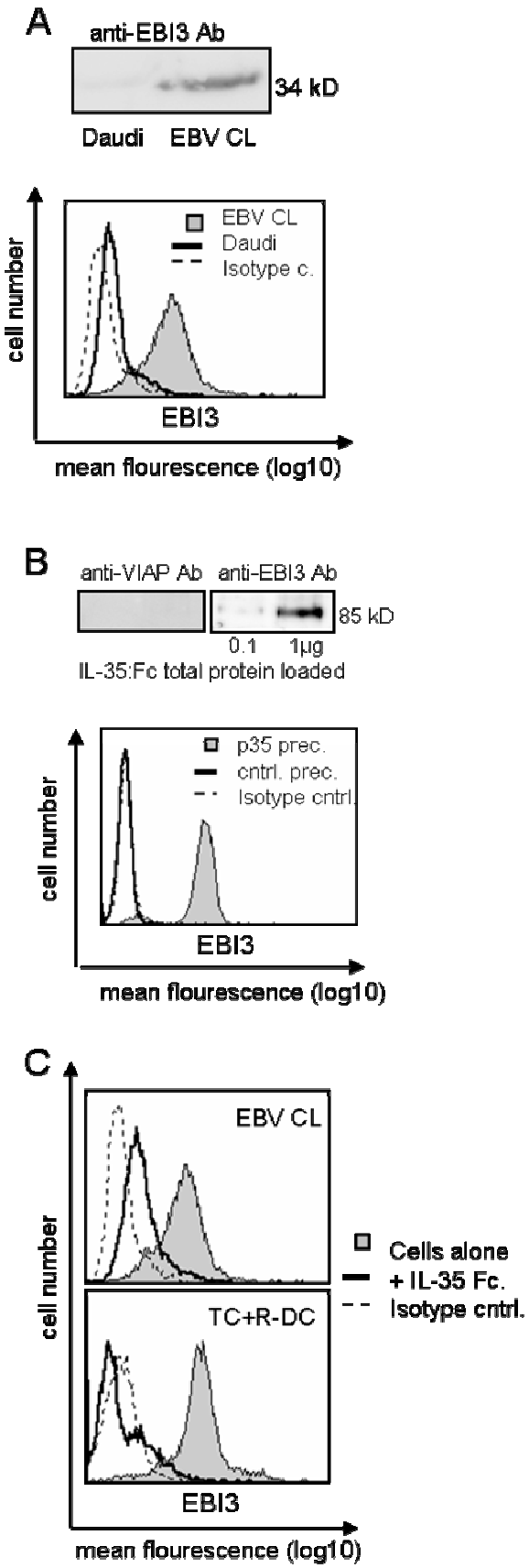


Figure 14. R-DCs induced inhibitory T cells do not release IL-12 and IL-27

(A) Cell culture SN of T cells stimulated with DCs or R-DCs for 24-72 h were pooled from 6 independent experiments and immunoprecipitated with an antibody against p35. The anti-p35 coated beads left after precipitation were analyzed via flow cytometry. IL-35 was detected with an anti-EBI3 Ab. This experiment was performed once and is representative of a pool of 6 cell donors. (B) As in A but the IL-35 loaded anti-p35 coated beads were stained with anti-IL-12p40 and anti-IL-27 mAb. This experiment was performed once and is representative of a pool of 6 cell donors.

The utilized antibody against EBI3 is specific for human EBI3 and IL-35

EBI3 is a 34 kDa glycoprotein identified in B lymphocytes following EBV infection, it is homologous to the p40 subunit of IL-12. EBI3 gene expression is restricted to specific cell types (hematopoietic cells) and highly inducible.
Results presented in Figure 15 show that the polyclonal anti-EBI3 Ab that was used throughout the study is specific for EBI3. It detects a band of correct size (34 kDa) in an EBV transfected cell line. These cells also could be stained with the anti EBI3 Ab (Figure 15A below). Also the Ab reacts with purchased recombinant IL-35:Fc in western blot analysis (also at the predicted protein size specified by distributer) as well as in flow cytometry (Figure 15B).
Finally addition of recombinant IL-35:Fc to the EBV transfected cell line as well as to T cells deriving from a T cell/R-DC coculture, blocks the binding of the EBI3 antibody as shown in Figure 15C.


A
anti-EBI3 Ab
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Daudi
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cell number
EBI3
mean flourescence (log10)
B
anti-VIAP Ab
anti-EBI3 Ab
85 kD
0.1
1µg
IL-35:Fc total protein loaded
p35 prec.
cntrl. prec.
Isotype cntrl.
cell number
EBI3
mean flourescence (log10)
C
EBV CL
TC+R-DC
Cells alone
+ IL-35 Fc.
Isotype cntrl.
cell number
EBI3
mean flourescence (log10)

Figure 15. The utilized antibody against EBI3 binds human EBI3 and IL-35
(A) The Daudi B cell line and an EBV transformed B cell line were analyzed via western blot and flow cytometry for their EBI3 expression. The results of one representative experiment out of three independent experiments are shown.
(B) Purchased human IL-35:Fc was loaded on a gel and detected via western blot with the polyclonal anti-EBI3 Ab. Further a solution of IL-35:Fc (1 µg/ml) in medium was immunoprecipitated with anti-p35 coated beads or control Ab coated beads. The beads were analyzed via flow cytometry and IL-35:Fc was detected with an EBI3 antibody. The results of one representative experiment out of three independent experiments are shown.
(C) IL-35:Fc (1 µg/ml) was added to the EBV cell line (upper blot) or a TC/R-DC coculture (lower blot) before staining. EBI3 expression was determined via intracellular staining. The results of one representative experiment out of three independent experiments are shown.

PMA/Ionomycin activated T cells cocultured with R-DC express elevated levels of EBI3 and p35

To test if additional T cell activation could alter the IL-35 release, we activated the T cells cocultured with DC or R-DC with PMA/Ionomycin for 12 h. T cells previously activated with R-DC additionally stimulated with PMA/Ionomycin showed a slight increase in p35 and EBI3 detected via flow cytometry, as shown in Figure 16. This is in comparison with the same cells activated only with R-DC. Resting PB T cells or T cells prestimulated with DC did not express IL-35 subunits upon PMA/Ionomycin stimulation.

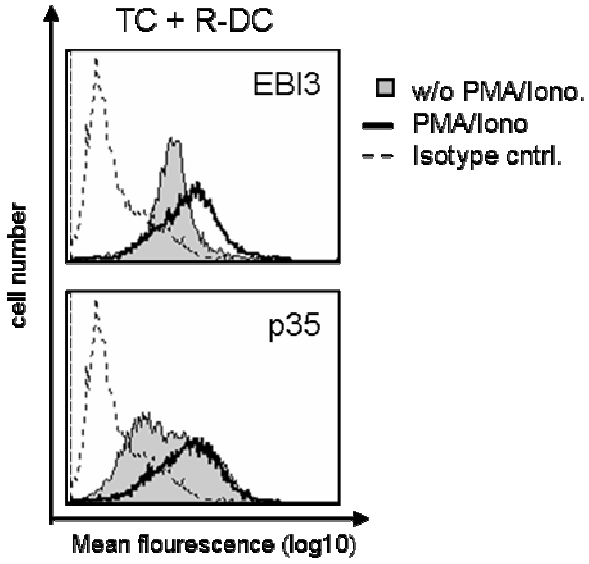


Figure 16. T cells cocultured with R-DC express elevated levels of EBI3 and p35 upon PMA/Ionomycin stimulation

T cells cocultured with R-DCs for 3 days where activated with PMA/Ionomycin for 12 h, or left untreated. EBI3 and p35 expression detected by intracellular staining was analyzed via flow cytometry. The dotted line shows the isotype control mAb VIAP. One representative experiment out of three is shown.

IL-35 contributes to the inhibitory function of R-DCs induced regulatory T cells

As R-DCs-treated T cells display a regulatory phenotype and release IL-35, the following experiments were designed to examine whether the observed effects were mediated by this cytokine.

We added the inhibitory SN of the R-DCs-induced T_{reg}-cells to an allogeneic MLR together with a polyclonal antibody to EBI3 or a mAb against p35. We could show that the inhibitory effect of the SN from T cells was abolished and proliferation restored. Figure 17A and B illustrate that antibodies directed against both subunits were able to neutralize the inhibitory capacity of the T cell/R-DC SN.

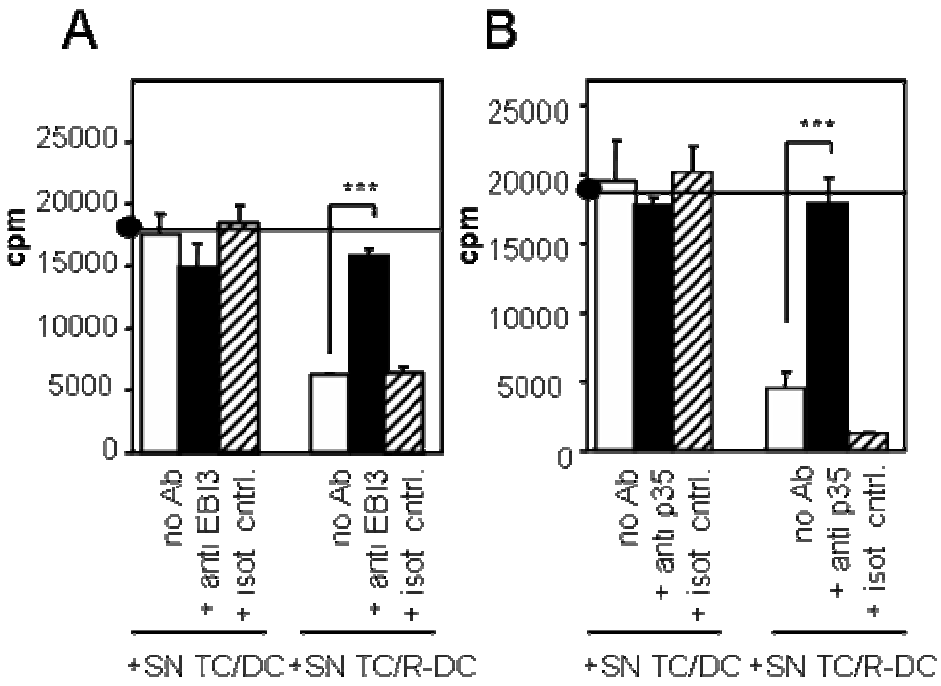


Figure 17. IL-35 is responsible for the inhibitory effect

(A) Purified T cells were stimulated with DCs or R-DCs for 2 days. The cell culture SN was harvested and examined in an allogeneic T cell DC coculture. The SN was preincubated with a neutralizing polyclonal Ab against EBI3 (10 µg/ml / black bars) or a polyclonal isotype control (10 µg/ml / striped bars) or mock treated (white bars). T cell proliferation with allogeneic DCs ($1x10^4$) alone is depicted as a black circle. Data are representative of 5 independent experiments. MV of triplicate determinations ± SD are shown.

(B) As in A with a neutralizing mAb against p35 (10 µg/ml / black bars) or an isotype control mAb VIAP (10 µg/ml / striped bars) or mock treated (white bars). Data are representative of 3 independent experiments. MV of triplicate determinations ± SD are shown.

Neutralizing IL-12 or IL-27 Abs do not alter the inhibitory function of the SN of the R-DCs-induced regulatory T cells

We added the inhibitory SN of the R-DCs-induced T_{reg}-cells to an allogeneic MLR together with antibodies against IL-12p40 or IL-27. In comparison to the previous Figure these Abs did not alter the inhibitory function of the SN (Figure 18A and B).

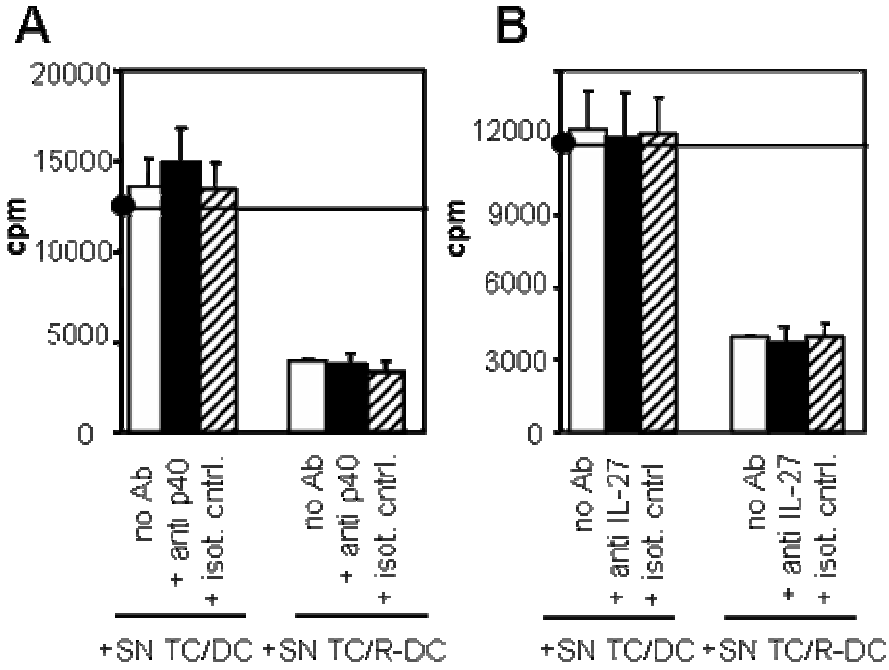


Figure 18. Neutralizing IL-12 or IL-27 Abs do not alter the inhibitory function of the SN

(A) Purified T cells were stimulated with DCs or R-DCs for 2 days. The cell culture SN was harvested and examined in an allogeneic T cell DC coculture. The SN was preincubated with a mAb against IL-12p40 (10 µg/ml / black bars) or an isotype control mAb VIAP (10 µg/ml / striped bars) or mock treated (white bars). Data are representative of 3 independent experiments. MV of triplicate

determinations ± SD are shown.
(B) As in A with a mAb against IL-27 (10 µg/ml / black bars) or an isotype control mAb VIAP (10 µg/ml / striped bars) or mock treated (white bars). Data are representative of 3 independent experiments. MV of triplicate determinations ± SD are shown.

The IL-35 depleted SN is no longer inhibitory

We were able to precipitate IL-35 out of the SN of the T cell/R-DC coculture (as shown in Figure 13 and 14). Next we used the p35-depleted SN in an MLR. This SN was no longer inhibitory as depicted in Figure 19, whereas the T cell/R-DC SN, precipitated with a control Ab or mock treated, was still inhibitory. Thus the inhibitory effect of R-DCs induced Tregs is mediated by IL-35.

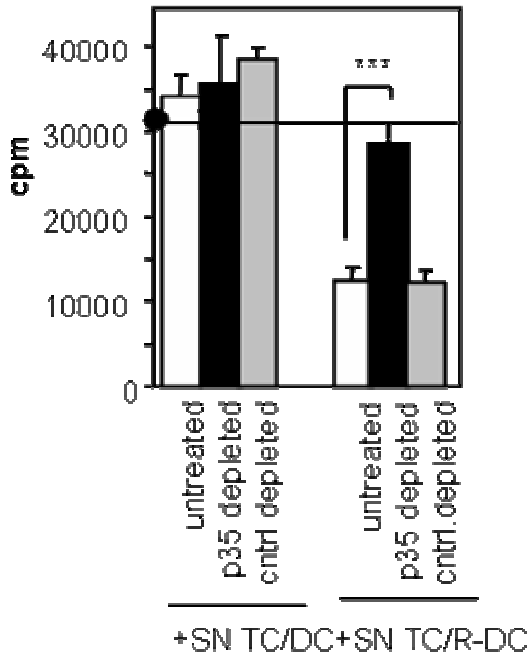


Figure 19. IL-35 depleted SN is no longer inhibitory
The p35 depleted or with control Ab depleted SN left after precipitation (black and grey bars) were examined in an allogeneic T cell DC coculture in

comparison with the non depleted ones from the same donor pool (white bars). T cell proliferation with allogeneic DCs (1x10^4) alone is depicted as a black circle. Data are representative of three independent experiments. MV of triplicate determinations ± SD are shown;

The same SN of a T cell/R-DC coculture, depleted with anti-IL-12p40 or anti-IL-27 Abs was still inhibitory, when tested in an MLR (Figure 20A and B). We can thereby further exclude an involvement of these cytokines in the observed effects.

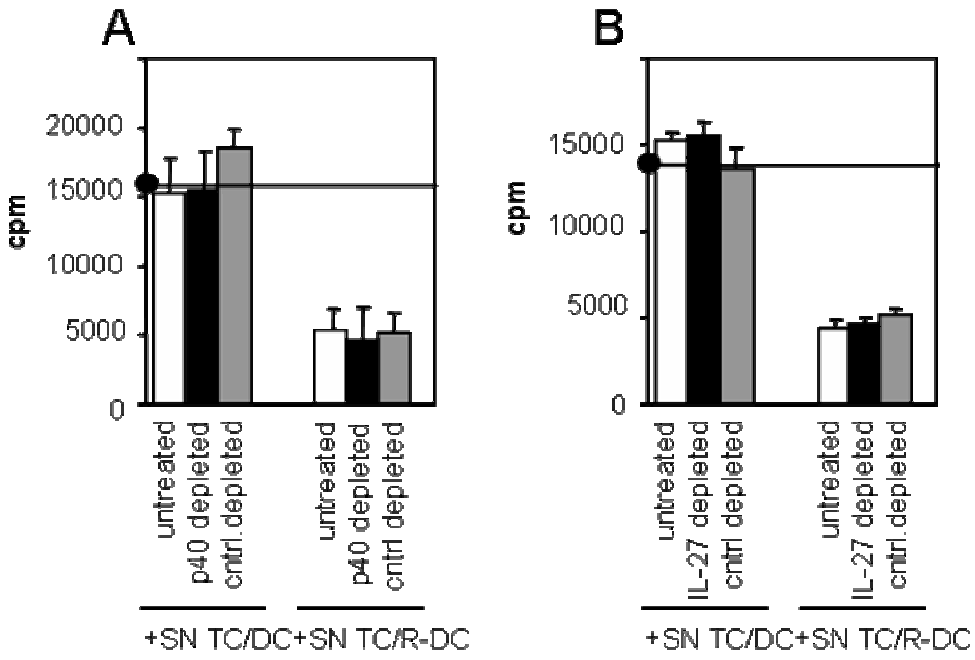


Figure 20. IL-12 as well al Il-27 depleted SN is still inhibitory

(A) Purified T cells were stimulated with DCs or R-DCs for 2 days. The cell culture SN was IL-12p40 depleted or with control Ab depleted and than examined in an allogeneic T cell DC coculture. Data are representative of three independent experiments. MV of triplicate determinations ± SD are shown.

(B) as in A but the SN was IL-27 depleted or with control Ab depleted. Data are representative of three independent experiments. MV of triplicate determinations ± SD are shown. p<0.001 (***).

Next we checked as a control for the above results, if we were able to precipitate recombinant IL-12 with the utilized anti-p40 mAb. As you can see in Figure 21, this is the case. The mAb was used in Figure 20 to check if removal of possible IL-12 present in the inhibitory SN could change the outcome of the experiment.

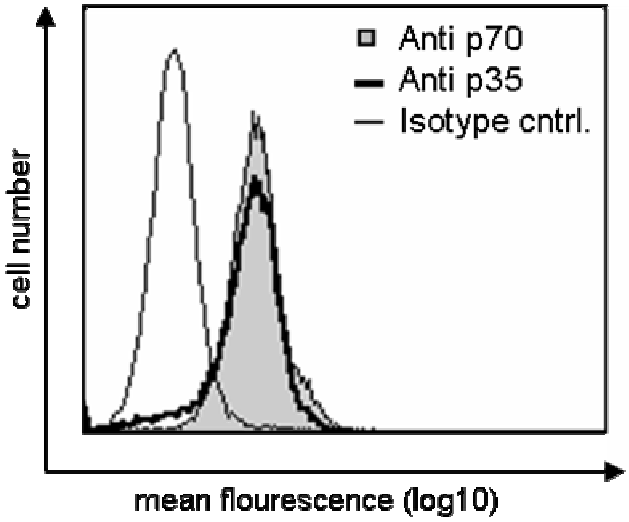


Figure 21. The utilized antibody against IL-12p40 can precipitate recombinant human IL-12

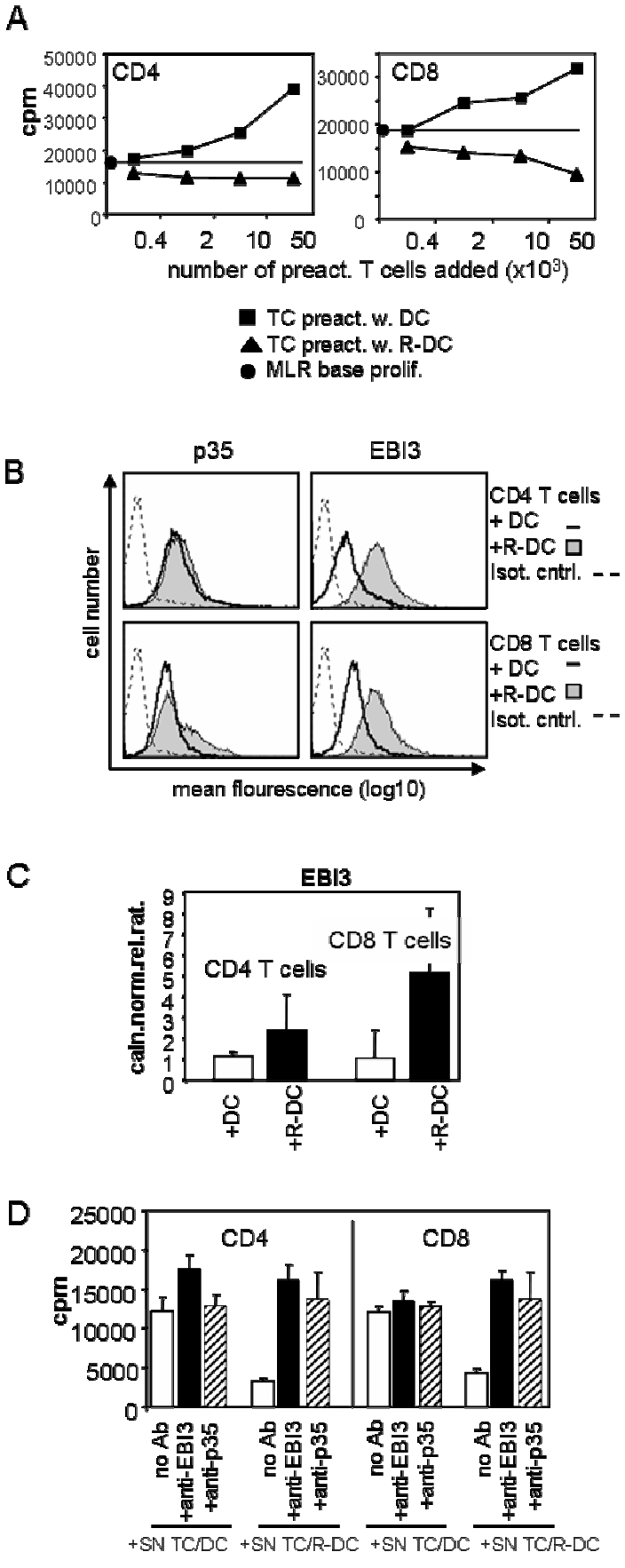
A solution of recombinant human IL-12 (1 µg/ml) in medium was immunoprecipitated with anti-p40 coated beads. The beads were analyzed via flow cytometry and IL-12 was detected with a p35 and p70 antibody, or isotype control VIAP. The results of one representative experiment out of two independent experiments are shown.

CD4$^+$ as well as CD8$^+$ T cells produce EBI3 and p35 upon stimulation with R-DC

There are two major subsets of T cells that differ in effector function, MHC restriction and accessory molecule usage, namely the CD4 (T helper cells) and the CD8 (cytotoxic T cell) positive subset of T cells.

We repeated some of the so far performed experiments with purified CD4$^+$ and CD8$^+$ T cells, to clarify if we could produce the observed effects with each cell type. We have already shown in Fgure 8, that both T cell subsets are able to produce the inhibitory SN.

Both subsets gain regulatory function upon stimulation with R-DC (Figure 22A), express EBI3 and p35 as shown by flow cytometry (Figure 22B) and qPCR (Figure 22 C). Also the inhibitory effect of the SN can be reverted by antibodies against IL-35 (EBI3 and p35; Figure 22D). This indicates that both subsets can produce IL-35.


A
CD4
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0.4 2 10 50
0.4 2 10 50
number of preact. T cells added (x10³)
TC preact. w. DC
TC preact. w. R-DC
MLR base prolif.
B
p35
EBI3
cell number
CD4 T cells
+ DC
+R-DC
Isot. cntrl.
CD8 T cells
+ DC
+R-DC
Isot. cntrl.
mean flourescence (log10)
C
EBI3
caln.norm.rel.rat.
CD8 T cells
CD4 T cells
+DC
+R-DC
+DC
+R-DC
D
CD4
CD8
cpm
25000
20000
15000
10000
5000
0
no Ab
+anti-EBI3
+anti-p35
+SN TC/DC
+SN TC/R-DC
+SN TC/DC
+SN TC/R-DC

Figure 22. CD4$^+$ and CD8$^+$ T cells produce EBI3 and p35 upon stimulation with R-DC

(A) Human purified T cells (1x10^5) were stimulated with allogeneic DCs (1x10^4) alone (●) or in the presence of graded numbers of CD4$^+$ or CD8$^+$ T cells, which had been preactivated with R-DCs (▲) or mock treated DCs (■). The Figure shows the result of one representative out of three independent experiments.

(B) EBI3 and p35 production in peripheral blood CD4$^+$ or CD8$^+$ T cells cocultured with DCs (open histogram) or R-DCs (filled histogram) for 24 h was detected by intracellular staining. The dotted line shows the isotype control mAb VIAP. Data are representative of three independent experiments.

(C) CD4$^+$ or CD8$^+$ T cells were cocultured with R-DCs (black bars) or DCs (white bars) in a 10:1 ratio for 24 h and the production of EBI3 was measured at the mRNA level via qPCR. One representative experiment out of three is shown, MV ± SD are calculated from duplicates.

(D) CD4$^+$ or CD8$^+$ Purified T cells were stimulated with DCs or R-DCs for 2 days. The cell culture SN was harvested and examined in an allogeneic MLR. The SN was preincubated with an Ab against EBI3 (10 µg/ml / black bars) or a mAb against p35 (10 µg/ml striped bars) or mock treated (white bars). Data are representative of 5 independent experiments. MV of triplicate determinations ± SD are shown.

B7-H1 and sialoadhesin on R-DCs induce the production of IL-35

We have recently found that R-DC work via B7-H1 and sialoadhesin. Blocking of the accessory molecules B7-H1 and sialoadhesin on R-DCs with specific mAbs against both receptors reverted the inhibitory phenotype of R-DCs [129]. Neutralizing antibodies to B7-H1 and sialoadhesin were added to the T cell/R-DCs coculture. The production of EBI3 and therefore the production of IL-35 could be effectively blocked by a combination of the two mAbs as presented in Figure 23. P35 expression did not change considerably with addition of the neutralizing antibodies (Figure 23A right column).

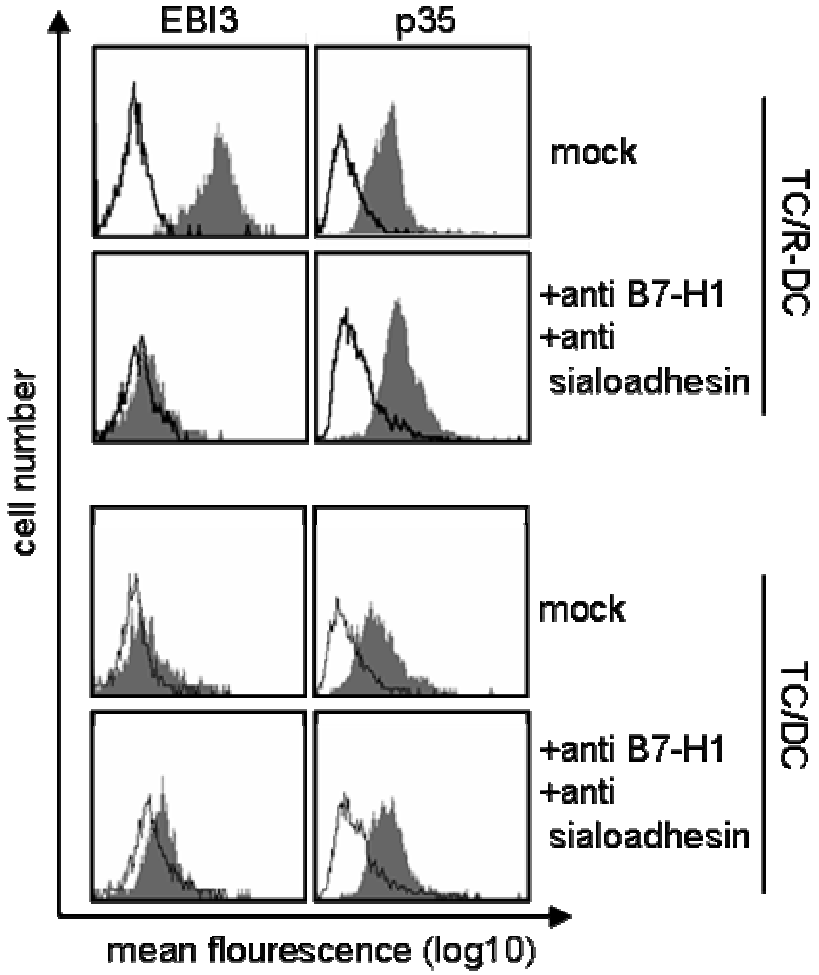

Figure 23. Blocking of B7-H1 and Sialoadhesin on R-DCs prevents the production of IL-35

T cells were cocultured with R-DCs or DCs in a 10:1 ratio for 24 h in the presence of blocking antibodies (10 µg/ml) to B7-H1 (5-272) and sialoadhesin (7-239), or mock treated. EBI3 and p35 production was evaluated by intracellular staining (filled histograms). The black line shows the isotype control mAb VIAP. The T cell gate is shown in the histograms. Data are representative of two independent experiments.

The neutralizing antibodies were added also to a T cell/R-DC coculture and the cell culture SN of these cells (without Abs) was able to inhibit T cell proliferation. The antibodies alone partially reverted the inhibitory effect. By using a combination of both antibodies, proliferation could be restored, this is shown in Figure 24.

Thus, the cell surface structures sialoadhesin and B7-H1 are involved in the induction of the IL-35^{+}-Treg-cells.

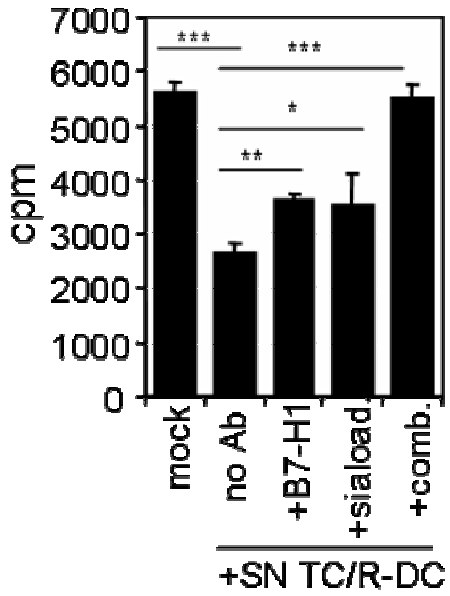

Figure 24. Blocking of B7-H1 and Sialoadhesin circumvents the inhibitory quality of the IL-35^{+} regulatory T cells

Purified T cells were stimulated with R-DCs for 2 days in the presence of blocking mAbs (10 µg/ml) to B7-H1 (5-272), sialoadhesin (7-239), both mAbs or mock treated. The cell culture SN was harvested and examined in the allogeneic T cell proliferation response induced by DCs. The Figure shows the results of one representative experiment of two independent experiments. MV of triplicate determinations ± SD are shown; $p < 0.05$ (*), $p < 0.01$ (**) and $p<0.001$ (***).

Discussion

We demonstrate in this study that IL-35 production and release is induced in human peripheral blood $CD4^+$ and $CD8^+$ T cells, upon B7-H1 and sialoadhesin co-stimulation provided by DCs. Such IL-35^+-T cells are potent Tregs, which, in contrast to IL-10-driven type-1 regulatory T cells (Tr1), do not suppress T cell responses via IL-10 and/or TGF-β [44, 39], but via IL-35. Similar to the Tr1 situation, also in our setting the supernatant of the IL-35^+-T cells alone is sufficient to inhibit DC driven T cell proliferation. Several pieces of evidence support the conclusion that the R-DCs induced Tregs act through IL-35. Neutralization with anti-EBI3 and anti-p35 antibodies as well as depletion of IL-35 removed the inhibitory effect of the SN of Tregs. Naïve T cells from cord blood, which do not produce IL-35 upon stimulation with R-DCs, lack suppressor function. Thus, induction of IL-35 represents a novel activation program in human T cells in response to viral infection.

EBV-induced gene 3 (EBI3) is a member of the IL-12 family that was first identified in B lymphocytes based on its induction following EBV infection. It codes for a 34 kDa-secreted glycoprotein homologous to the p40 subunit of IL-12. Recent studies have shown that EBI3 can dimerize with IL-12 p35 and EBI3/p35 was called IL-35 [155, 160]. Data presented in Figure 17 and 19 demonstrate that IL-35 and not the other related cytokines IL-27 or even IL-12 are responsible for the inhibitory effect of the supernatant, that was observed in our study.

The first critical step in how HRV alters DC function is binding to its cellular receptor ICAM-1 (CD54). To exclude that HRV is transferred from the R-DC to the T cells we added the specific inhibitor WIN 52035-2 to the co-culture after the R-DC generation. WIN 52035-2 blocks HRV binding to ICAM-1 [168]. Even though this reagent was present the R-DCs were still able to introduce the regulatory phenotype in the T cells, showing that viral transfer and infection of T cells with HRV does not play a role in IL-35 generation. This data is given in Figure 6C.

The data of this study demonstrate for the first time that IL-35 is a potent regulatory cytokine also in the human immune system. This works through a combinatorial signal delivered from DCs to T cells via the accessory molecules B7-H1 and sialoadhesin. This signal is critical for the induction of human IL-35$^+$ regulatory T cells (Figure 25).

As we have already shown before HRV inhibit the accessory function of DCs by inducing sialoadhesin and B7-H1 expression [129]. This shows that DCs not only efficiently stimulate T cell activation, but can also regulate T cell responses. B7-H1, a member of the B7-molecule family, is a well-defined accessory molecule with inhibitory effects through its receptor PD-1 on the T cell side [130, 171]. We and other groups have recently demonstrated that B7-H1 is critically involved in the induction and maintenance of T cell anergy [131]. There is abundant evidence that different viruses abuse B7-H1 to turn-off effector T cell responses [172-174]. The findings of this study imply that B7-H1-mediated inhibition of T cell responses is at least in part due to its property to contribute to the induction of IL-35 production. Yet, B7-H1 alone was not sufficient to induce IL-35, but required co-signaling via sialoadhesin. Sialoadhesin is a member of sialic acid binding lectin family of I-type lectins, which preferentially bind to sialylated carbohydrate structures (e.g. NeuAcα2,3-Gal) [132] and CD43 has been recently described as a ligand for sialoadhesin on T cells [133]. Sialoadhesin is a frequently used marker for macrophages because it is typically not expressed on monocytes, lymphocytes and DCs. Yet, type-I IFNs have been recently reported to up-regulate sialoadhesin on monocytes [133, 175-177] but also on DCs (C. Schrauf, unpublished observation). Thus, sensing of viral infections by DCs leads to the up-regulation of the inhibitory receptor pair B7-H1 and sialoadhesin, this is critical for the induction of IL-35$^+$ Treg-cells.

The IL-12 cytokine family, which is related to the IL-6 cytokine family, is of great importance and includes IL-12, IL-23, IL-27 and the newest member IL-35. All four are heterodimeric cytokines, composed of an alpha chain (p19, p28, or

p35) and a beta chain (p40 or Ebi3). IL-12, IL-23 and IL-27 signal through unique pairings of five receptor chains (IL-12Rb1, IL-12Rb2, IL-23R, gp130 and WSX-1) [161]. The potential receptor or receptors for IL-35 have not been defined so far, neither for the murine nor the human system. But one can strongly speculate that they could consist of the already identified receptors for other members of the IL-12 family. All other IL-12 family members signal through dimeric receptors followed by intracellular signaling using the phosphorylation of JAK, followed by STAT family member activation [167]. IL-12, was first described in 1991 [178] and provided the first insight into the extensive chain sharing that has become a characteristic of this cytokine family. Over 10 years ago, a second ß chain, EBI3, was identified. The p35 chain of IL-12 signals through the IL-12-receptor ß2 chain and the EBI3 part of IL-27 either signals via gp130 (CD130, IL-6 receptor ß) or via WSX-1 (IL-27R, TCCR) [161]. The p35 subunit of IL-12 binds to IL-12-receptor ß2 chain therefore this may be a chain of the putative IL-35 receptor. IL-27 signaling requires WSX-1 as well as gp130; however, direct binding studies to determine which subunit of IL-27 interacts with the individual receptor chains have not been performed yet. Therefore, it is possible that either WSX-1 or gp130 may be members of the IL-35 receptor. Whether IL-35 signals through a dimeric receptor or not and whether this receptor is composed of already known modules has not been defined yet, also the nature of the signalling cascade it initiates remains to be determined.

Although chain as well as receptor sharing is important in the IL-12 cytokine family, there is still also the possibility that IL-35 binds to a completely unknown receptor. A hint for this would be that all the other members of the IL-12 family have rather immune-stimulatory effects, in contrast to IL-35. They activate different subsets of T cells and are produced by APCs, mainly DCs, following their activation via the recognition of pathogen specific patterns [161]. IL-35 for that matter is produced by T cells and seems to have opposing effects; it acts on T cells in an immune-inhibitory manner. While IL-12, IL-23, and IL-27 share the common feature of inducing IFN-gamma production and promoting Th1 cell

differentiation and proliferation, IL-35 appears to function in an anti-inflammatory fashion by inhibiting T cell proliferation and perhaps other parameters [155, 160].

Understanding the unique features of IL-12, IL-23, IL-27, and IL-35 and potential synergy and/or competition between the members of the IL-12 cytokine family is of great importance, and many further studies in this direction are necessary. Significant insight is likely to be gained from the determination of the IL-35 receptor and its expression pattern.

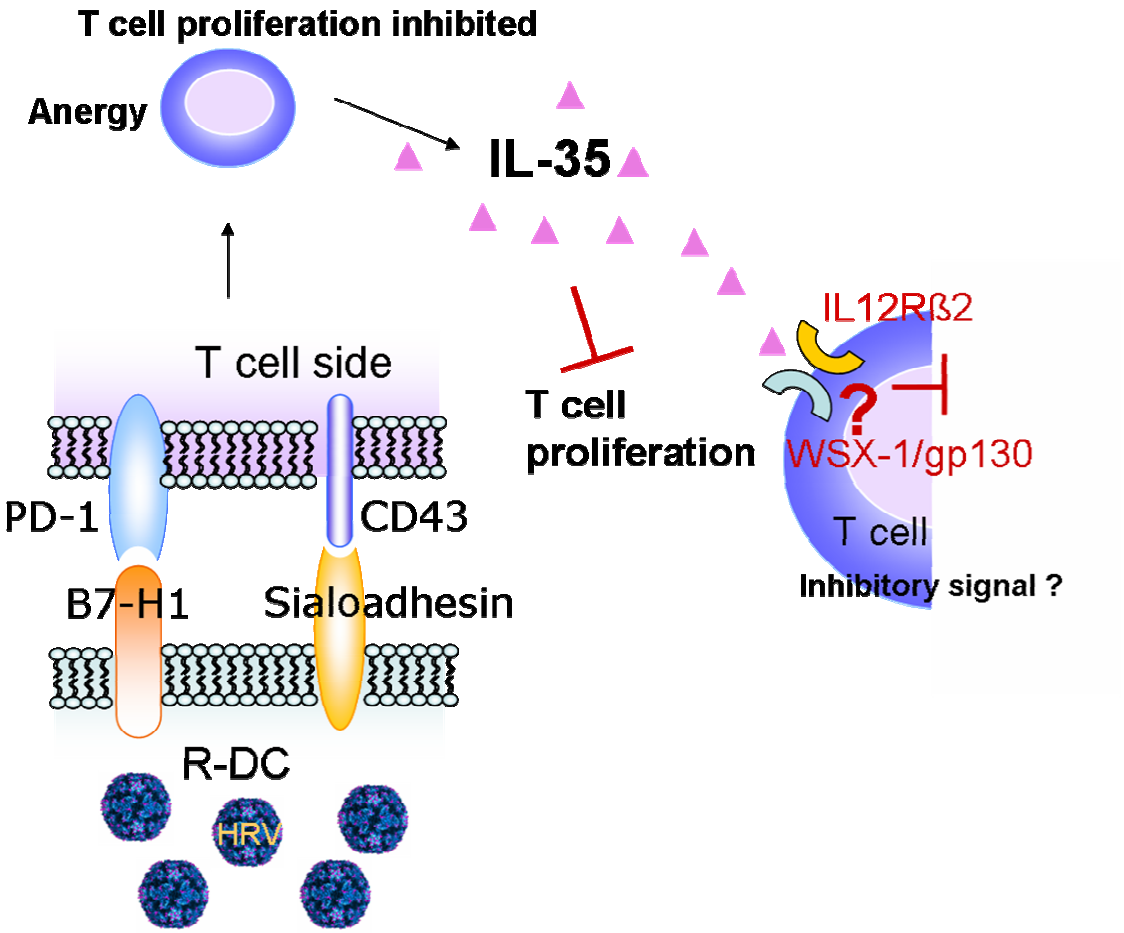

Figure 25. Summary

Upregulation of the surface structures B7-H1 and sialoadhesin on HRV treated DCs leads to the induction of IL-35 producing T cells. IL-35 then has an inhibitory effect on other T cells, via so far undefined mechanisms.

IL-35 is composed of two chains, which it shares with two other heterodimeric cytokines. IL-35 is required for maximal regulatory function in vivo in the mouse as Tregs deficient in either chain are unable to control homeostatic T cell expansion and inflammatoty bowel disease (IBD) [160]. Given that IL-35 has been described more recently, we still have a limited understanding of its biological activity, which has also been complicated by the lack of appropriate tools for its functional dissection. At the moment for example there is no ELISA or even Ab commercially available that detects IL-35. Recombinant IL-35 as well has proven particularly challenging to generate and purify according to the review of Vignalli and Bettini [179]. They speculate that the apparent poor stability of IL-35 might underlie important physiological features of this cytokine, such as limited potency over short-range. Alternatively, DCs that secrete IL-12 or IL-27 may be precluded from generating IL-35, because of preferential pairing of IL-12 and IL-27. It is clearly important to determine the bioactivity of IL-35. Whether IL-35 can inhibit all Th subsets and other cellular populations, such as B cells, macrophages or DCs, or has more selective targets also remains to be determined.

The in vivo association between EBI3 and p35 was originally evidenced in human placental extracts, the group describing this was able to co-precipitate EBI3 and p35 [166], similar as in this study. EBI3 and p35 are highly expressed in placental trophoblasts, which suggests that IL-35 may be an immunomodulator at the feto-maternal border. Trophoblasts have been thought to be key players in maternal tolerance to the fetus, and that they accomplish that in part by cytokine production [180]. They are at the frontier between the placenta and the potentially immunologically hostile environment of the uterus. IL-35 seems to be of importance in situations were immuno-suppression is needed, like during pregnancy. Already in this early study from 1997 [166] is was speculated that IL-35 (although at that time it was calles EBI3/p35 heterodimer) is likely to antagonize the biological effects of IL-12. What this early studies also showed is that EBI3 can be expressed at high levels not only

in placental trophoblast cells but also in activated DCs, human B lymphoblast cells, tonsils and spleen [158, 166].

More recent studies demonstrated that IL-35 is constitutively expressed by mouse $CD4^+CD25^+Foxp3^+$ Tregs [155, 160]. In the murine system IL-35 is a downstream target of Foxp3 [160]. Transcripts coding for EBI3 and p35 were observed to be constitutively coexpressed by mouse Tregs and EBI3/p35 heterodimer was coprecipitated from the cell culture supernatant of these cells. In addition, in vitro and in vivo studies suggested that the expression of IL-35 by mouse Tregs contributed to their suppressive function [161]. EBI3 alone is not immunosuppressive [155]. Recombinant mouse IL-35 was shown to inhibit the proliferation of mouse effector T cells in vitro. In another recent study, a single chain mouse IL-35-Fc fusion protein was able to enhance the proliferation of mouse Treg-cells, while inhibiting the development of Th17 cells [155].

However, human $CD4^+CD25^+Foxp3^+$ regulatory T cells do not constitutively express IL-35 and induction of Foxp3 does neither upregulate EBI3 nor p35 mRNA in human T cells. Nevertheless EBI3 transcripts are found in activated effector CD3 positive T cells and p35 is described to be constitutively expressed in various T cell subsets. [164, 165]. These findings do not contradict ours; the IL-35 producing T cell population that we describe does not have the $CD4^+CD25^+Foxp3^+$ natural regulatory T cell phenotype, but is a new, so far un-described adaptive T cell subset. Its regulatory phenotype is induced by R-DCs.

One interesting feature of R-DCs is that the expression of most costimulatory molecules analyzed was not affected on their surface [129]. For instance, CD40, CD58, CD80, and CD86 expression was similar on R-DCs and on untreated DCs. Likewise, expression of MHC class I and class II molecules, was not down-regulated on R-DC. Thus, the clearly reduced T cell stimulatory capacity of R-DC did not correlate with a reduced ability to deliver signal 1 and 2 to T cells. Knowing this, we could speculate that R-DC co-cultured T cells (despite their anergy) could actually be activated in a way by the R-DCs, to obtain their IL-35 producing phenotype. This fits well to the findings of Bardel et al. [164], that EBI3 expression in humans is found in the activated T cell population. Also

conventional $CD4^+CD25^+Foxp3^+$ natural Treg-cells need to be activated via their TCR to become fully potent suppressors [35].
We observe transient Foxp3 expression in T cells stimulated with R-DC as well as DC. Such temporal activation-induced Foxp3 expression in human T cells has been described before for effector T cells as well as for Tr1 cells and is not obligatory correlated with regulatory function, whereas natural $CD4^+CD25^+$ regulatory T cells show constitutive high Foxp3 expression [43, 68]. The sole use of Foxp3 expression to determine if a human T cell has regulatory function has been questioned, and this molecule no longer seems to be the master regulator for Tregs in humans. Foxp3 expression is for instance not a prerequisite for Tr1 cells, and we can show here that it is also seems not to be necessary for IL-35 producing T cells.

The expression of EBI3 and p35 is up-regulated in T cells stimulated with R-DCs and also in T cells stimulated with DCs which were activated with IFN-α (diplma thesis S.Zeiner), suggesting that other viral stimuli could also trigger IL-35. Also, since induction of B7-H1 and sialoadhesin expression on DC seems to be induced by many other viruses as well, it is intriguing to suggest that induction of $IL\text{-}35^+$ Treg-cells is a general theme in viral infections.
Because of their importance in initiating antiviral immune responses, DCs represent an ideal target for viral immune-evasion strategies. Several viruses are known to modulate DC function and thus impair antiviral T cell responses [181]. We have discovered a novel pathway of immune-regulation by analyzing the impact of HRV on DCs. HRV are specialized pathogens and only infect humans with all the well-known symptoms of a cold. HRV infection is probably the most frequent human infectious disease, which indicates that the host/HRV relationship is highly evolved. HRV utilizes a variety of tricks to blunt our immune-system [123] and induction of $IL\text{-}35^+$ Treg-cells may represent another prominent immune-evasion mechanism.

Material and Methods

Antibodies

The following murine mAbs were generated in our laboratory: negative control antibody VIAP (against calf intestine alkaline phosphatase), 5-272 (B7-H1), 7-239 (CD169, sialoadhesin), VIT6b (CD1a). The polyclonal murine antibodies against EBI3 and GNAI2 (guanine nucleotide binding protein, alpha inhibiting activity polypeptide 2; used as isotype control for EBI3) were purchased from Abnova Corporation (Taipei, Taiwan).
The monoclonal antibodies against p35 (clone27537), IL-12p40 (mAb609), IL-12p70 (mAb611), IL-27 (mAb25261) and TGF-β (mAb240) were purchased from R&D Systems Inc. (Minneapolis, MN). The neutralizing polyclonal anti–IL-10 antibody (PAL-hIL10) was obtained from Strathmann Biotech (Hannover, Germany). MAb EB-I against IFN-α was kindly provided by G. Adolf (Boehringer Ingelheim).

Cells

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMCs were isolated from buffy coats obtained from the Red Cross in Austria. PBMNC were separated from whole blood of healthy donors by density gradient centrifugation using Ficoll-Paque (GE Healthcare, London, UK). Blood was diluted 1:2 to 1:3 with heparin-medium. For density gradient centrifugation 15 ml of Ficoll-Paque were prepared in 50 ml tubes and a layer of heparinized blood was carefully pipetted onto. The cells were spun 30 minutes at 900 g without brake. After centrifugation granulocytes and erythrocytes gathered at the bottom of the tube, peripheral blood mononuclear cells in the interphase. The white interphase ring was transferred into a new tube and spun down (5 minutes at 900 g). The supernatant was discarded. The cells were washed twice with

MACS-buffer: The pellet was resuspend in a few ml of buffer, filled up, spun down. The supernatant was discarded, and the cells again resuspend. The number of PBMCs was determined and the cells were used for monocyte and T cell isolation.

Magnetic cell sorting (MACS)

Magnetic cell sorting (MACS) is a method for the selective enrichment or depletion of cells which express a surface protein distinct for their cell type. Therefore cells can be labelled with antibodies directed against the specific molecule which have been coupled to biotin. These labelled cells can be targeted with magnetic beads that contain a secondary antibody directed against the biotin residues on the primary antibody. Therefore specific cells in a mixture can be selectively retained in a magnetic column.
The mAbs we used were labelled with biotin. These biotinylated antibodies recognizing cell-type specific surface molecules were mixed with the cells to be separated. Then the cells were washed twice with MACS-buffer. In a second incubation step paramagnetic beads (50 nm in diameter) were coupled to the cells. The cells were applied onto a separation column placed in a strong permanent magnet. 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.

Isolation of monocytes and T cells from peripheral blood

Freshly isolated PBMCs were resuspended in 750 µl MACS buffer and incubated with 250 µl biotinylated antibodies for 15 minutes at 4 °C. To remove unbound antibodies the cells were washed with MACS buffer and again resuspended in 750 µl buffer. Then 250 µl anti-Streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the suspension

and incubated for 15 minutes at 4°C. In the mean time a CS column (Miltenyi Biotec) was placed onto a VarioMACS apparatus and equilibrated with 40 ml MACS buffer. Afterwards the labelled PBMCs were loaded onto the column and for monocyte enrichment washed with 40 ml MACS buffer. The flow through was collected as monocyte negative fraction. The column was further washed 4 times with 10 ml MACS buffer and monocytes were collected by removal of the column from the magnet and aspiration of the retained cells from the side valve using a syringe.

Monocytes were positively selected by magnetic cell sorting from PBMCs. For the selection we used the monocyte marker CD14 (mAb: VIM13: 15 µg/ml & MEM18: 15 µg/ml) produced in our laboratory. For the isolation of monocytes up to 1 x 10^9 PBMCs were incubated with 250 µl of biotinylated CD14 and positively enriched (positive selection).

T cells were isolated by collecting the flowthrough of PBMCs depleted by using an antibody cocktail containing anti-CD14 (monocytes), anti-CD16 (monocytes, NK–cells), anti-CD19 (B cells), anti-CD36 (monocytes, thrombocytes), anti-CD56 (NK-cells) and anti-CD123 (progenitor cells, megakaryocytes, granulocytes, negative selection). For T cells the flowthrough was collected, washed with MACS buffer and counted. 20 µl of this solution were pipetted into a tube for cell counting with the coulter. Before measuring the cell number two drops of Zap-Oglobin® (Beckman Coulter, Miami, FL) are added to remove residual erythrocytes.

The purity of monocytes and T cells was controlled by immunofluorescence analysis. Double-staining with directly labelled Antibodies using: CD14-PE/CD45-FITC; CD19-PE/CD3-FITC, CD8-PE/CD4-FITC, HLA-DR-PE/CD3-FITC, CD56-PE/CD3-FITC, CD14-PE/HLA-DR-FITC.

Isolation of naïve T cells from human cord blood

Naive T cells were isolated from cord blood as described above. Cord blood samples from healthy donors were collected during healthy full-term deliveries.

Approval was obtained from the Medical University of Vienna institutional review board for these studies. Cord blood T cells used in this study were $CD45RA^+$ (92 ±3%) and $CD45RO^-$.

Cell Culture conditions

For cell culture RPMI 1640 (Gibco Ltd., Paisley, Scotland) supplemented with 10% FCS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptamycin (Sigma-Aldrich) was used. Cells were cultured at 37°C with 5% CO2.

Freezing and thawing of cells

Mammalian cells can be stored in liquid nitrogen for prolonged periods of time with minimal loss of viability. For that purpose, cells were spun down, counted and resuspended in freezing medium to a concentration between 10^7 – 5 x 10^7 cells/ml. 1 ml aliquots were filled into cryotubes (Nalge Nunc International, Roskilde, Denmark) and kept overnight at - 80°C in a freezing box filled with isopropanol before being transferred to liquid nitrogen.
Cryotubes were thawed in a 37 °C warm water bath until all frozen media was dissolved. Cells were washed two times with RPMI Media (Gibco Ltd., Paisley, Scotland) resuspended in RPMI 10% FCS supplemented L-glutamine, penicillin and streptamycin and further cultured.

Generation of monocyte-derived DC

Monocyte-derived immature dendritic cells were generated by culturing peripheral blood $CD14^+$ monocytes for 7 days in the presence of 50 ng/ml GM-CSF (Novartis Research Institute, Vienna) and 100 U/ml IL-4 (Novartis Research Institute, Vienna).

Preparation and purification of Rhinoviruses – Generation of R-DC

HRV-14 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and routinely grown in suspension cultures of HeLa cells (strain Ohio; Flow Laboratories, McLean, Virginia, USA). Cells were cultivated in S-MEM medium (Joklik modification; catalog no. 22300-107; Life Technologies Inc., Rockville, Maryland, USA) supplemented with 7% heat-inactivated horse serum (catalog number 01051-M; Diagnostic Products GesmbLT, Wiener Neudorf, Austria), 1% penicillin/streptomycin, 1% glutamine, 1% pluronic F68 (catalog no. P1300; Sigma Chemical Co., St. Louis, Missouri, USA), and 1% nonessential amino acids.
To obtain HRV-14 treated DCs (R-DC), immature DCs were cultured with 1 $TCID_{50}$ (50% tissue culture infectious dose) HRV-14 for 2 days.

Allogeneic Mixed Leukocyte Reaction (Allo-MLR)

Lymphocytes from one donor are mixed with stimulator cells from another. If the two cell populations are not compatible in their MHC, which means they are allogeneic, proliferation of T cells occurs. In our experiments we measured the capacity of T cells preactivated with HRV-treated and untreated DCs to induce T cell proliferation in an MLR. We also investigated the influence of different supernatants (SN) on T cell proliferation in an MLR and also added specific antibodies wherever mentioned. The expansion of T cells was determined by measuring the methyl-3H-thymidine incorporation into the DNA.
Stimulator cells (DC) from a different donor as the T cells used were cultivated as described before. On day 7 the DCs were harvested: spun down for 5 minutes at 300 g, the supernatant was discarded, the cells were resuspend and counted. Finally the cell number was adjusted to $1x10^6$ cells/ml. In most assays the concentration of stimulator cells used was $1x10^4$ cells/well.
Frozen T cells were thawed as described before. There cell number was adjusted to $1x10^6$ cells/ml. For the MLR, allogeneic, purified T cells ($1x10^5$) were

incubated with DCs. The experiments were performed in a 96 well U-bottom cell culture plate plates (Packard, Meriden, Connecticut), in triplets. Generally we added 100 µl of T cells to each well i.e. 100.000 cells/well. The MLR-assay was incubated for 5 days at 37°C 5% CO2 .

For Figure 6 pre-activated T cells were harvested, irradiated (30 Gy, 137Cs source) and tested for their suppressive function, for Figure 9 pre-activated T cells were not irradiated. For that purposes, increasing numbers of pre-activated T cells were added to an allogeneic MLR with a fixed number of T cells ($1x10^5$) and DCs ($1x10^4$).

To quantify the extend of the T cell proliferation we measured the incorporation of methyl-3H-thymidine into the DNA. On the fourth day of cultivation 25µl of methyl-3H-thymidine 1mCi/ml (Perkin Elmer, Waltham, MA) were added to each well. 18 hours later (incubation at 37°C 5% CO2) the cells were harvested, by transferring the radioactively labelled DNA to Unifilter 96 plates using a Plate Harvester. The filter plates (Milipore Corp., Bedford, MA) were dried for 1hour at 37°C, an a sticker was pressed to the bottom side, 25 µl of Microscint scintillation mix (Packard, Meriden, Connecticut) were added in each well and the plate was covered by an adhesive seal. Radioactivity was determined using a microplate scintillation counter (Packard).

The HRV-blocking reagent WIN 52035-2 [168] was a kind gift from the Sterling-Winthrop Research Institute (Rensselaer, NY) and was used at a final concentration of 5 µg/ml, to exclude the possibility of viral transfer.

All the antibodies used in the allo-MLRs for neutralization were diluted with cell culture medium to a concentration of 10 µg/ml. To prove that neutralization worked, recombinant human IL-10, IFN-α, TGF-β were used at 5 ng/ml. IL-10, TGF-β and IL-12 were purchased from R&D Systems Inc. (Minneapolis, MN). IFN-α (isoform 2c) was purchased from Boehringer Ingelheim (Vienna, Austria).

T cell – DC / supernatant generation

To examine the suppressor activity of the SN of R-DCs-induced Treg-cells, T cells were added to R-DCs or DCs in a 10:1 or 5:1 / T cell to DC ratio. These SNs were harvested after 1-3 days of coculture and 100 µl/well were added to different MLRs. Or the SN was used for other purposes, like precipitation.
Centricon YM-50 filters (Millipore) were used for size fractionation of the SN. The fraction containing molecules > 50 kDa was compared to the fraction containing molecules < 50 kDa in an allogeneic MLR.
The T cells of the coculture were also investigated by intracellular staining or analyzed via qPCR (both described below).

Flow cytometry

Flow cytometry can be used to examine diverse properties of cells including the relative size, relative granularity and relative fluorescence intensity. Cells are transported in a fluid stream to a laser beam. To accomplish that single cells are passing through the beam, a principle related to laminar flow is applied. The sample is injected into a stream of sheath fluid. Cells within this stream are accelerated and are focused to the center, a process called hydrodynamic focussing.
The incident laser light is scattered by the cells and detected in different angles. Forward scatter light (FSC) is measured just of the axis of the incident beam by a photo diode and gives information about the size of a particle. Side scatter light (SSC) is collected by a photo-multiplier at approximately 90° of the laser beam axis and is proportional to the granularity.
The argon ion laser used emits light at 488 nm, a wavelength matching with the absorption spectrum of a range of fluorescent dyes. These fluorochromes can be excited by the laser, which means that an electron is raised to a higher state of energy. After returning to ground state a photon is emitted and fluorescence can be detected after passing a system of lenses and filters. The intensities of

different fluorochromes can be analysed at once, provided that their absorption maximae are not to close to each other.

Immunofluorescence of cytoplasmic proteins

Cells were incubated for 12 h in presence of monensin (10 µM, Sigma-Aldrich) that impairs further secretion of proteins, were mentioned also PMA and Ionomycin (both Sigma-Aldrich) was added (both 100 nM). 50 µl of cell suspension were mixed with 100 µl fixation medium (Bio Research GmbH, An der Grub, Kaumberg, Austria) and incubated for 20 minutes at room temperature. 100 µl permeabilisation medium (Bio Research GmbH, An der Grub) and 20 µl mAb were mixed and incubated 20 minutes at room temperature.
The cells were washed with PBS def; spun for 5 minutes at 300 g, the supernatant was discarded. 50 µl permeabilisation medium and 20 µl of Oregon Green-conjugated goat-anti mouse Ig Abs from Invitrogen (Carlsbad, CA) mixed and incubated for 20 minutes at room temperature.
The cells were washed twice with PBS def., spun for 5 minutes at 300 g. Samples were prepared for immunofluorescence analysis by addition of 50 µl of sheath fluid. Flow cytometric analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Immunoprecipitation, cytokine depletion and Western blotting

Preparation of Dyna-beads

500 µl of sheep anti-mouse IgG coupled Dynabeads (Dynal, Oslo, Norway) with 2,8 µm diameter were washed twice with PBS def 1x using the Dynal magnet. The beads were mixed with 100 µl of murine antibody and incubated gently rotating for 1h. The antibody loaded beads were washed three times to get rid of unbound antibody.

Immunoprecipitation and cytokine depletion

For immunoprecipitation mAbs p35, p40 or IL-27 (all R&D Systems Inc.) or mAb VIAP (isotype control) was loaded onto $7x10^7$ sheep anti-mouse IgG coupled Dynabeads (Dynal, Oslo, Norway) with 2,8 µm diameter as described in detail above. Then the Ab coupled beads were incubated with the pooled cell-culture SNs (of a T cell/DC or T cell/R-DC coculture from six independent donors) for 12 h at 4°C on a rotator. The beads were spun down and the SN of the beads was considered depleted of p35, p40 or IL-27 and tested in an MLR.
The beads themselves were washed three times with cold PBS def 1x using the Dynal magnet and a part of the beads ($2x10^6$) was analyzed via flow cytometry. Therefore beads were incubated for 30 minutes at 4°C with unconjugated Abs against EBI3, IL-12p40, IL-27 or isotype control. After washing, Oregon Green-conjugated goat anti–mouse-Ig from Invitrogen was used as a second-step reagent. Flow cytometric analysis was performed using a FACScalibur flow cytometer (BD Biosciences).
Concerning the rest of the beads; bound protein was eluted with reducing sample buffer (100-200 µl / Biorad, Richmond, CA) by boiling for 5 minutes at 95°C and the beads were removed using the magnet. For further identification, proteins were separated via SDS-PAGE and western blotting was performed.

SDS-PAGE and Western Blot

SDS-PAGE

SDS Polyacrylamide Electrophoresis is a useful system to separate proteins according to their size. SDS is a powerful detergent, which has a very hydrophobic end (the lipid like dodecyl part) and highly charged part (the sulfate group). The dodecyl part interacts with hydrophobic amino acids in proteins. Since the 3D structure of most proteins depends on interactions between hydrophobic amino acids in their core, the detergent destroys 3D structures,

transforming what were globular proteins into linear molecules now coated with negatively charged SDS groups masking the actual charge of proteins. After boiling in SDS proteins therefore become elongated with negative charges arrayed down them, so they will move towards a positive electrode more or less fast according to their respective size. The reason why β-mercaptoethanol is usually included in the sample buffer is to cleave disulfide bonds within or between molecules, allowing molecules to adopt an extended monomeric form.
Cell Lysates (f.i. the DAUDI cell line) or precipitated proteins or human IL35:Fc from Alexis Biochemicals (San Diego, CA - 1 µg/ml) were mixed with reducing Laemmli sample buffer (Biorad, Richmond, CA) containing 5% ß-mercaptoethanol (Biorad) and boiled before at 95 °C for 5 min. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels using the Hoefer Mighty Small system (Amersham, Little Chalfont, UK). As running buffer 25 mM Tris base, 192 mM glycine, 0,1% SDS was used.
As protein standard SeeBlue Plus2 prestained marker (Invitrogen) was used.

Polyacrylamid Gel preparation

Reagent	**10% Separation gel**	**2% Stacking gel**
30% Acrylamide-solution	4 ml	440 µl
H_2O	4,8 ml	2 ml
1,5 M Tris HCl pH 8,8	3 ml	-----
0,5 M Tris HCl pH 6,8	------	840 µl
10% SDS-solution	100 µl	35 µl
Ammoniumpersulfate (10%)	100 µl	35 µl
TEMED	8 µl	4 µl

Western Blotting

Western Blotting allows to determine, with a specific primary antibody, the relative amounts of the protein present in different samples. Separated samples

are transferred to a membrane for detection. The membrane is incubated with a generic protein to bind to any remaining sticky places on the membrane and therefore blocking unspecific antibody adherence on the membrane itself. A primary antibody is then added to the solution which is able to bind to its specific protein. A secondary antibody-enzyme conjugate, which recognizes the primary antibody is added to find locations where the primary antibody has bound.

Proteins that were separated by SDS-PAGE were blotted onto Immobilon-P PVDF membranes (Millipore, Billerica, MA) using the Hoefer Semiphor TE77 system (Amersham, Buckinghamshire, UK) for 1 hour at 15 V. As blotting buffer 25 mM Tris base, 192 mM glycine, 5% Methanol was used. Membranes were blocked with 5% dry milk/0,1% Tween20 and incubated with primary antibody (1 µg/ml) in the same solution. Bound mAbs were detected using HRP-conjugated goat antibodies to mouse Ig (used at 1:10.000; Dako) and chemiluminescence detection (Western Lightning; Perkin-Elmer, Wellesley, MA). Signals were detected on Kodak Biomax XAR films (Sigma-Aldrich) and quantified using the ImageJ 1.32 software (National Institutes of Health, Bethesda, MD).

RNA-Isolation and cDNA preparation

RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO). Up to 1 x 10^7 cells/ml were resuspended in 1 ml TRI reagent and incubated for 5 minutes (optional: -20°C until need) at room temperature. To remove protein 100 µl Chloroform were added, vortexed and incubated again for 5 minutes at room temperature. After centrifugation for 15 minutes at 13 000 rpm at 4°C the aqueous phase was transferred in new tube and 300 µl isopropanol were added. Samples were vortexed, incubated and centrifuged again at 13 000 rpm at 4°C for 10 minutes. The pellet was washed in 1 ml 75 % ethanol. The dried pellet was resuspended in A.dest (~10 µl) and the concentration was measured at OD260. The ratio 260/280 should be over 1,5.

2 µg of total RNA was reverse transcribed with MuLV-RT (Fermentas,

Burlington, Canada) using Oligo (dT)18 primers, according to the manufacturer's protocol. 2 µg RNA were mixed with 0,5 µg Oligo (dT)18 primers and A.dest was added up to 11 µl, incubated for 5 minutes at 70°C and chilled on ice. To each reaction 4 µl of 10 x M-MuLV reverse transcriptase buffer, 1mM 4 dNTP mix, 20 U ribonuclease inhibitor, 200 U of RevertAid H Minus M-MuLV reverse transcriptase (all Fermentas) and A.dest to a final volume of 20 µl were added. The mixture was incubated for 5 minutes at 37°C, followed by incubation for 60 minutes at 42°C and 10 minutes at 70°C. Finally cDNA was diluted 1:3 to use it for qPCR. cDNA was stored at -20°C until use.

Quantitative PCR (qPCR)

Quantitative (Real–Time) PCR (qPCR) was performed by the Mx3005P QPCR system (Stratagene, Cedar Creek, TX) using SYBR Green I (Fermentas) detection according to the manufacturers protocol. In all assays, cDNA was amplified using a standard program (2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 sec at 95°C / 15 sec at 60°C / 30 sec at 72°C). G3PDH was used as a housekeeping gene.

The following primers were used:

hFoxp3 forward:	5`-GAA ACA GCA CAT TCC CAG AGT TC-3`
hFoxp3 reverse:	5`-ATG GCC CAG CGG ATG AG-3`
hEBI3 forward:	5`-TCT GAG ATC TCT GCC CGC CCT GCA GTG GAA GG-3`
hEBI3 reverse:	5`-CTT GAG ATC TGC CCA GGC TCA TTG TGG CAG TG-3`
hIL-12 p35 forward:	5`-TTT GCG GCC GCA CCT CCC CGT GGC CAC TCC AG-3`
hIL-12 p35 reverse:	5`-TTT GCG GCC GCA TTC AGA TAG CTC ATC ACT CT-3`
hp28 forward:	5`-GCG GAA TCT CAC CTG CCA-3`
hp28 reverse:	5`-GGA AAC ATC AGG GAG CTG CTC-3`

G3PDH forward: 5`-CGACCACTTT GTCAAGCTCA-3`
G3PDH reverse: 5`-AGGGGAGATT CAGTGTGGTG-3`

Cell lines

The following cell lines were used in this study: the EBV-transformed lymphoblastoid B cell line (EBV CL) OTMA was generated in our laboratory [182]. The Daudi cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA).

Statistical analysis

Statistical analysis was performed using a 2-tailed Student's t test using unpaired nonparametric test (Mann-Whitney). Significance is represented as $p < 0.05$ (*), $p < 0.01$ (**) and $p<0.001$ (***), n.s. not significant.

List of chemicals and reagents

Reagent	final conc.	source
Beriglobin	20 mg/ml (in PBS/BSA)	Aventis Behring, Vienna, Austria
PMA	100 nM	Sigma, St. Louis, MO
Ionomycin	1 µM	Sigma, St. Louis, MO
GM-CSF	50 ng/ml	Novartis Research Institute, Vienna
IL-4	100U/ml	Novartis Research Institute, Vienna
Human IL35:Fc		Alexis Biochemicals, San Diego, CA
methyl-3H-thymidine	1mCi/ml	Perkin Elmer, Waltham, MA
Monensin	10 µM	Sigma, St. Louis, MO

TRI Reagent (Trisol)	undiluted	Sigma, St. Louis, MO
FIX Solution	undiluted	An der Grub, Kaumberg, Austria
PERM Sulution	undiluted	An der Grub, Kaumberg, Austria
IFN-α	5 ng/ml	Boehringer Ingelheim Vienna, Austria
IL-10	5 ng/ml	R&D Systems Inc. Minneapolis, MN
TGF-β	5 ng/ml	R&D Systems Inc. Minneapolis, MN
IL-12		R&D Systems Inc. Minneapolis, MN
WIN 52035	5 µg/ml	Sterling-Winthrop Research Institute , Rensselaer, NY

Buffers and media

Heparin media:
Use 500 ml RPMI 1640 medium. Add 100 U/ml penicillin + 100 µg/ml streptamycin and 2mM L-glutamine (stored at –20°C, Sigma-Aldrich)). Add 10 U/ml Heparin (stock: 5000 U/ml, Baxter, Vienna).

Freezing medium:
RPMI 1640 supplemented with 25% FCS and 10% DMSO

MACS-buffer (stored at 0°C):
1000 ml 1x PBS def. + 25 ml Human Serum Albumin (stock: 20%, Centeon, Vienna) + 10 ml EDTA (stock: 0,5 M); filtrated sterile

10x PBS stock solution:
5.8 g KH_2PO_4 16.6 g Na_2HPO_4 $2H_2O$ 72 g NaCl Solubilise the salts in aquabidest (=ddH_2O), fill up to 10 litres and adjust pH to 7.2

PBS/BSA stock solution (20%):
100 g BSA 10 g NaN_3 Dissolve in 500 ml PBS buffer.
For the PBS/BSA wash buffer, prepare a 1:20 dilution with PBS buffer.

Solutions for western blot:

Running buffer (4x):
Tris 12 g
SDS 4 g
Glycin 57,6 g
up to 1000 ml ddH_2O

Blotting Buffer:
250ml of 4x running buffer
200 ml methanol
up to 1000 ml ddH_2O
Can be scaled down to 800 ml total volume.
Degas before use!!!

PBST (PBST 0.5%):
PBS with 0.5 % Tween 20

Dry milk solution
5% dry milk powder dissolved in PBST buffer.

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Curriculum Vitae

Name	Maria Seyerl
Date of birth	3. April.1983
Place of birth	Freistadt, Austria
Academic Degree	Mag. rer. Nat.
Citizenship	Austria
Residence	A-1160 Wien
Address (University)	Institute of Immunology Medical University of Vienna A-1090 Wien, Borschkegasse 8a
e-mail	maria.seyerl@meduniwien.ac.at

Education

1989-1993	elementary school VS Sandl
1993-1997	secondary school, HS Sandl
1997-2001	secondary school BG/BRG Freistadt
June 2001	Matura passed with excellent success
WS2001-WS2006	Study of Biology – Genetics and Microbiology at the University of Vienna, Austria
2005-2006	Diploma Thesis at the Institute of Immunology, Medical University of Vienna, Austria Title: Regulation of T-cell activation by oxidized Phospholipids
March 2007	Graduation
since April 2007	PhD thesis at the Institute of Immunology, Medical University Vienna

Spoken languages	German	native speaker
	English	fluently
	Spanish	basic knowledge

Membership	Austrian Society for Allergology and Immunology - OEGAI
Poster Presentations	Pirquet symposium, Vienna, December 2006 Annual meeting ÖGAI, Alpbach, December 2007 Joint annual meeting of Immunology (OEGAI, DGFI), September 2008 ECI (2nd European Congress of Immunology) Berlin, Germany, September 2009
Oral Presentations	4th. PHD Symposium, General Hospital Vienna, May 2008 World Immune regulation meeting III, Davos, Switzerland, March 2009 Karl Landsteiner Meeting, Salzburg, November 2009 FEBS & EFIS Workshop:"Inflammatory Diseases and Immune Response: Basic Aspects, Novel Approaches and Experimental Models" September 2010, Vienna
Awards	EFIS travel grant (2009) OEGAI travel grant (2009)

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