Dendritic cell maturation and instruction of $CD4^+$ T cell tolerance *in vitro*

Reifung der dendritischen Zelle und Instruktion der $CD4^+$ T Zell Toleranz *in vitro*

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SUMMARY

Effective T cell immunity was believed to occur by mature DC, whereas tolerogenicity was attributed strictly to immature DC phenotypes. However, intermediate DC maturation stages were identified conditioned by inflammatory mediators like TNF. Furthermore, the T cell tolerance mechanisms are dependent on distinct modes and intensities of co-stimulation. Therefore, in this study it was addressed how distinct DC maturation signatures instruct $CD4^+$ T cell tolerance mechanisms.

DC acquire antigens from apoptotic cells for self-peptide-MHC presentation and functionally adapt presumed tolerogenic DC phenotypes. Here, immature murine bone-marrow derived DC representing both inflammatory and conventional DC subsets adapted a maturation-resistant DC signature upon apoptotic cell recognition but no additional tolerogenic features. Immature DC instruct $CD4^+$ $FoxP3^+$ regulatory T cells in a TGF-β prone micro-environment or generate anergic $CD4^+$ T cells hampered in the TCR-induced proliferation and IL-2 secretion. Secondary stimulation of such anergic $CD4^+$ T cells by immature DC increased primarily IL-10 production and conferred regulatory function. These IL-10^+ regulatory T cells expressed high levels of CTLA-4, which is potently induced by immature DC in particular. Data in this work showed that anergic T cells can be re-programmed to become IL-10^+ regulatory T cells upon ligation of CTLA-4 and CD28 signalling cascades by B7 co-stimulatory ligands on immature DC.

In contrast, semi-mature DC phenotypes conditioned by the inflammatory mediator TNF prevented autoimmune disorders by induction of IL-10^+ Th2 responses as demonstrated previously. Here, it was shown that TNF as an endogenous maturation stimulus and pathogenic *Trypanosoma brucei* variant-specific surface glycoproteins (VSG) induced highly similar DC gene expression signatures which instructed default effector Th2 responses. Repetitive administration of the differentially conditioned semi-mature DC effectively skewed T cell immunity to IL-10^+ Th2 cells, mediating immune deviation and suppression.

Collectively, the data presented in this work provide novel insights how immature and partially mature DC phenotypes generate T cell tolerance mechanisms *in vitro*, which has important implications for the design of effective DC-targeted vaccines. Unravelling the DC maturation signatures is central to the long-standing quest to break tolerance mimicked by malignant tumours or re-establish immune homeostasis in allergic or autoimmune disorders.

ZUSAMMENFASSUNG

Reife DC sind potente Induktoren von T Zell Immunität, wogegen unreife DC Stadien zur Induktion von Immuntoleranz befähigt sind. Zudem sind intermediäre semireife DC Entwicklungsstadien identifiziert worden, wie sie nach Behandlung mit inflammatorischen TNF entstehen. Die bekannten T Zell Toleranzmechanismen sind wiederum abhängig von unterschiedlicher Art und Intensität von Kostimulation. Hier wurde deshalb untersucht wie verschiedene DC Reifungsstadien $CD4^+$ T Zelltoleranz induzieren können.

DC nehmen apoptotisches Zellmaterial auf, was als Antigenquelle zur Präsentation von MHC/Selbstpeptid-Komplexen genutzt wird und tolerogene Funktionen in DC hervorrufen kann. Unsere Ergebnisse zeigten dass aus Knochenmark generierte DC der Maus, die sowohl inflammatorische als auch klassische DC Subtypen darstellen, nach Erkennung apoptotischen Zellmaterials reifungsresistent wurden, jedoch unverändert unreif und keine neuen tolerogenen Funktionen erwarben. Unreife DC induzierten in Gegenwart von TGF-β $CD4^+$ $FoxP3^+$ regulatorische T Zellen und in dessen Abwesenheit anergische $CD4^+$ T Zellen. Wiederholte Stimulation anergischer $CD4^+$ T Zellen durch unreife DC, induzierte deren IL-10 Produktion und regulatorische Eigenschaften. Diese $IL\text{-}10^+$ regulatorischen T Zellen zeigten keine FoxP3 Expression, jedoch verstärkt CTLA-4, insbesondere nach Interaktion mit unreifen DC. Zusammen zeigten die hier erhaltenen Daten, dass das Reprogrammieren anergischer T Zellen zu $IL\text{-}10^+$ regulatorischen T Zellen über CTLA-4 als auch über CD28 Signalkaskaden durch deren B7 Liganden auf der Zelloberfläche unreifer DC gesteuert wird.

Frühere Arbeiten zeigten, dass repetitive Injektion semireifer DC, Autoimmunerkrankungen vorbeugen konnten durch die Induktion $IL\text{-}10^+$ Th2 Antworten. Hier konnte gezeigt werden dass TNF als endogener Reifungsstimulus sowie pathogene *T. brucei* Varianten-spezifische Glykoproteine (VSG) sehr ähnliche semireife DC Reifungsqualitäten hervorrufen. Die entsprechend generierten Th2 Effektor Zellen unterschieden sich lediglich geringfügig in deren Zytokinproduktion. Repetitive Injektionen dieser semireifen DC induzierten ebenfalls $IL\text{-}10^+$ Th2 Differenzierung und effektive Immundeviation *in vivo*.

Insgesamt hat die vorliegende Arbeit wichtige Erkenntnisse ergeben, wie unreife und semireife DC die Generierung unterschiedlicher T Zell Toleranzmechanismen *in vitro* unterstützen. Diese Erkenntnisse sind ein wichtiger Schritt bei der Entwicklung effektiver

DC-basierter Immunvakzinen. Die Definition der verschiedenen DC Reifungsstadien ist von großer Bedeutung bei der Optimierung von Behandlungsverfahren gegen infektiöse Erreger, Krebs oder Autoimmunerkrankungen.

1 INTRODUCTION

1.1 The immune system

1.1.1 Innate immune response

The first protective mechanisms that are initiated rapidly within hours after infections belong to the so-called innate immune response. Even in the absence of invading pathogens, components of the innate immune system actively protect the host from potential danger. Epithelial barriers lining up the skin, gastro-intestinal or respiratory tract function as an efficient defense through mucus secretion, villi motility and production of soluble antimicrobial mediators, etc. Whenever a pathogen manages to cross the epithelial barrier, it will be recognized and attacked though orchestrated actions of the cells of the innate immune system including neutrophils, macrophages, dendritic cells and natural killer (NK) cells assisted by the activation of complement factors, inflammatory reaction, cytokines and others.

Professional phagocytes such as macrophages are well-positioned in the subepithelial regions and initiate inflammation by secreting cytokines like tumor necrosis factor (TNF) and chemokines upon detecting microbial agents or damaged cells. Neutrophils and monocytes are recruited to the site of infection through altered adhesion molecule expression on the adjacent venules and chemokine gradients marking the site of action. Activated neutrophils and macrophages engulf the microbial invader followed by ingestion within phagolysosomes through concerted actions of enzymes and reactive oxygen species (ROS) which, however, might leak out and cause damage to the surrounding host cells. Soluble mediators such as antibodies or complement factors target or opsonize microbial structures for phagocytosis. NK cells detect infected or stressed host cells by combined actions of inhibitory and activation receptors on its surface. Besides destroying infected host cells, NK cells produce IFN-γ which activates phagocytosis function in macrophages. Hence, the innate immune system provides an initial rapid and essential defence strategy to allow the time-demanding but more specialized adaptive immune system to develop.

Cells belonging to the innate immune system recognize a wide array of highly conserved structures on the microbial pathogen, so-called pathogen associated molecular patterns (PAMP) by means of pattern recognition receptors (PRR). Well-studied cell-associated PRR are the Toll-like receptors (TLR), C-type lectins and cytosolic Nod-like receptors (NLR). Microbial PAMP are typically life essential structures which do not exist on the host cells.

Each innate immune cell type expresses an identical set of PRR receptors encoded in the germline DNA and are therefore able to recognize only a limited variety of conserved PAMP structures [1].

1.1.2 Adaptive immune response

Adaptive immunity is a highly specialized defence system dependent on the specific activation of cytokine-secreting T lymphocytes and/or immunoglobulin (Ig) producing B lymphocytes thereby 'adapting' the nature of the immune response to the pathogen invading the host. In contrast to the cells of the innate immune system, each T and B cell clone expresses antigen receptors with a unique specificity generated by the process of somatic gene rearrangements. During development, each lymphocyte recombines various gene segments encoded in the Ig or T cell receptor (TCR) gene loci to a unique antigen receptor gene by the process of V(D)J recombination encompassing variability within the antigen binding domain. The process of random gene segment recombination within each clone creates a virtually infinitive repertoire of antigen specificities or diversities. However, a major drawback of arbitrary gene receptor rearrangements is the potential reactivity towards self-structures and hence, lack of distinction between self and non-self. Immature lymphocytes undergo a stringent selection process in the central lymphoid organs for the maintenance of tolerance to self. The clonal selection theory implies that an individual lymphocyte clone with particular specificity is selected in the secondary lymphoid organs, expands and differentiates to a pool of effector lymphocytes well-equipped to combat the pathogen. Another unique feature of adaptive immunity is the memory cells which survive up to years after initial encounter and eradication of a pathogen able to mount much faster and larger immune responses upon re-exposure. Memory responses form the long-standing principle of vaccination.

Following development in the bone marrow, B cells undergo further maturation in spleen before they home and recirculate to the B cell follicles in the secondary lymphoid organs. Mature B cells express membrane-bound IgM and IgD as B cell receptors (BCR) which recognize whole proteins or complex structures circulating in the lymph or blood or presented as intact antigens by DC in the secondary lymphoid organs. Activation of B cells through detection of cognate antigen leads to proliferation and differentiation to an Ig-producing plasma cell which occurs T cell dependent for most protein antigens or T cell independent for other structures like polysaccharides. Each immunoglobulin consists of two identical heavy

and two identical light chains. Isotype and functionality of an antibody is determined at the carboxy-terminal constant (C) region whereas the amino-terminal variable (V) domain shapes the antigen specificity or epitope binding. During isotype switching, B cells shift expression to another C region adapting the isotype of the antibody and hence, the effector function or nature of the humoral immune response to the pathogen infecting the host.

T lymphocytes finalize their maturation process in the thymus, from where they enter the bloodstream and recirculate through the secondary lymphoid organs scanning antigen-presenting cells (APC) for their cognate antigen. Antigens from the peripheral tissues are concentrated within secondary lymphoid organs for activation of the TCR of T lymphocytes by APC which present captured and processed antigens as peptide stretches within the groove of major histocompatibility complex (MHC) molecules on their surface. Two major T cell subfamilies are defined carrying specialized effector function by the cluster of differentiation (CD) nomenclature: $CD4^+$ helper T cells (Th) recognize peptide-MHC class II complexes and $CD8^+$ cytotoxic T lymphocytes (CTL) scan peptides bound in the MHC class I binding cleft. CTL lyse infected or transformed host cells and produce interferon-γ (IFN-γ) to activate macrophages and other innate cells for eradication of intracellular pathogen reservoirs. T helper cells primarily potentiate the function of other APC or innate cells by production of cytokines and/or ligation of co-stimulatory molecules. The nature and effector function of T cell responses is determined during the conjunction with APC adapting as such the cell-mediated immune response to the type of infection.

Other members of the adaptive immune system include the natural killer T cells (NKT) cells and γδ T cells which express antigen receptors with limited variability due to restricted gene rearrangements and recognize distinct qualities of antigens such as (glyco)lipids presented by MHC class I-like CD1 molecules or phospho-antigens respectively. Both NKT and γδ T cells rapidly secrete cytokines thereby regulating both innate and adaptive immunity [1].

1.2 Dendritic cells

1.2.1 Introduction

In 1973, Steinman and colleagues discovered a novel cell type isolated from the murine spleen adapting particular phenotypes with stellate or dendritic cell shapes and hence, termed it a dendritic cell [2]. DC are highly specialized APC due to their unique features allowing antigen sampling, migration to secondary lymphoid organs and induction of primary immune responses thereby linking innate to adaptive immune responses. DC reside in the peripheral tissues where they continuously scan and sample surrounding tissues for invading pathogens followed by migration, processing and presentation of antigens to the circulating naïve or memory T lymphocytes in the draining lymph nodes. Upon encountering a stimulus, DC undergo a differentiation process termed maturation in which the antigen presentation and T cell activation machinery such as expression of MHC or co-stimulatory molecules is activated [3]. Pathogens are recognized by a variety of PRR such as TLR or C-type lectins vigorously expressed on the DC surface. DC might also sense inflammation through the family of TNF receptors (TNFR) or cytokine receptors [3]. Several subsets of DC have been identified which might determine the nature of the adaptive T cell response [4]. DC develop from hematopoietic stem cells in the bone marrow (conventional DC) or from precursors such as blood monocytes upon inflammation (inflammatory DC) [5]. Next to their immunogenic function, DC are able to induce and maintain immune tolerance during steady-state, a feature believed to originate from the ability to adjust the maturation status upon environmental determinants rather than subset definition [6].

1.2.2 Antigen presentation

DC are optimally equipped for antigen uptake in the periphery: by endocytosis of particulate antigen (phagocytosis), by sampling of soluble material or fluids by pinocytosis and by receptor-mediated endocytosis. The endocytosed antigens are guided through intracellular processing compartments for loading and presentation of the peptides into the groove of MHC proteins on the DC surface. Extracellular antigens are processed and presented on MHC class II (MHC II) proteins to the $CD4^+$ T helper cells, whereas antigens from the cytosolic compartment are loaded in the groove of MHC class I (MHC I) molecules to activate $CD8^+$ CTL [1, 7]. Whereas presentation of peptide-MHC I complexes is attributed to all nucleated

cells, expression of MHC II molecules is restricted to professional APC. Extracellular proteins entering the cell through endocytosis might also reach the MHC I loading machinery for presentation by MHC I complexes on the DC surface, a unique feature termed cross-presentation [8]. Recent reports suggested that peptide loading in the MHC I binding cleft for cross-presentation might occur in organelles or processing routes distinct to the classical MHC I loading machinery [9]. Especially $CD8^+$ conventional DC appeared highly efficient in cross-presentation which might be due to specific receptor expression [10] linked to specialized intrinsic processing features [11]. Beside peptide-MHC presentation for T cell activation, DC are able to present glycolipids loaded in the MHC class I-like CD1d-binding pocket to invariant NKT (iNKT) cells [12].

1.2.3 DC maturation stages

Maturation of DC is a differentiation program in which the antigen uptake skills are reorganized in favour of antigen processing and presentation abilities required for optimal T cell activation [7]. Distinct maturation stages have been described which might functionally link DC and initiation of diverse types of adaptive immune responses (fig 1) [6].

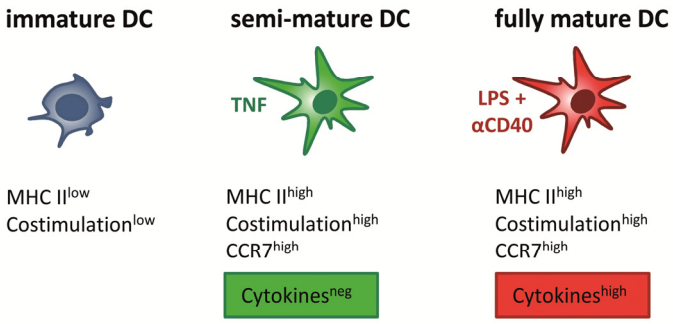


Figure 1. Maturation stages and phenotypes of DC
Immature DC express low amounts of MHC II and co-stimulatory molecules and are highly endocytic. Signalling through PRR- or cytokine receptors re-program DC phenotype and function to become migrating ($CCR7^+$), non-endocytic mature DC specialized for activation of effector T cells. Full maturation, i.e. additional secretion of pro-inflammatory cytokines, is achieved only upon pathogenic stimulation. (TNF = Tumor Necrosis Factor; LPS = Lipopolysaccharide; αCD40 = anti-CD40 antibody)

DC reside in the peripheral tissues in an immature state, i.e. basal expression of MHC and co-stimulatory molecules and no production of pro-inflammatory cytokines. Immature DC function as specialized endocytic cells which continuously sample antigens from the surrounding tissues. In the absence of overt infection, self-antigens such as apoptotic cells or soluble proteins are ingested which lack alarming signals for initiation of the maturation

program in DC. Classically it was assumed that immune tolerance is induced in the absence or limited availability of co-stimulatory molecules on immature DC [13, 14] and hence, for this purpose, several approaches have been developed to keep DC in immature maturation resistant shape [15].

Semi-mature activation stage was introduced to describe an intermediate maturation phenotype in which DC upregulate MHC II and co-stimulatory molecules but fail to secrete pro-inflammatory cytokines [6]. This maturation phenotype can be achieved upon *in vitro* TNF stimulation [16], disruption of E-cadherin cluster interactions (in part through activation of the β-catenin signalling pathway) [17] or inflammatory cytokine conditioning *in vivo* [18]. Recently, our group documented that semi-mature DC signatures occur *in vivo* in the absence of inflammatory conditioning or pathogenic invasion [19]. Semi-mature DC conditioned by inflammatory mediators *in vivo* fail to mount an effective IFN-γ releasing T cell response suggesting that this DC maturation stage might possess anti-inflammatory features. Indeed, repetitive injections of *in vitro* generated semi-mature DC prevented the induction of inflammatory autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) mediated at least in part through IL-10 producing T helper cells [16, 17, 20]. However, TNF conditioned DC fail to control footpad swelling of mice infected with *Leishmania major* parasites indicating that an IL-4/IL-13-prone immune deviating program was initiated [21]. Further research is needed to delineate how this maturation signature contributes to the establishment of T helper cell responses.

Full maturation of DC is achieved upon microbial stimulation, such as bacterial cell wall component lipopolysaccharide (LPS) or other pathogenic derivatives [16, 18]. Fully-mature DC highly express MHC II and co-stimulatory molecules and produce high amounts of cytokines for the induction and polarization of immunogenic inflammatory T cell responses. Although fully mature DC are linked to effective immunity, this DC maturation stage has incorporated negative feedback mechanisms responsive to cytokine or immune cell signalling to shut down or counterbalance overreactive immune responses [22].

1.2.4 DC subsets

One of the hallmarks of DC is their heterogeneity based on anatomical location, phenotype, migratory behaviour, immunological function and generation requirements [23, 24]. DC are identified based on the expression of the integrin CD11c, moderate levels of MHC II expression (already at immature state) and the functional potential to activate naïve T lymphocytes [23].

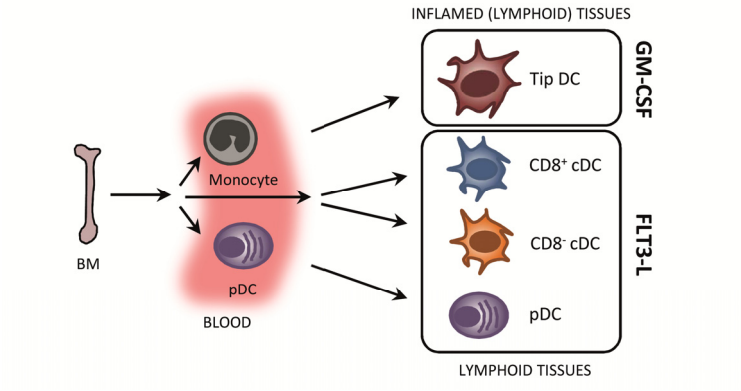


Figure 2. Distinct subsets of DC develop under steady state and inflammatory conditions
Murine BM-cultures supplemented with Flt3-L generate equivalents of splenic $CD8^+$, $CD4^+$ conventional (cDC) and plasmacytoid DC (pDC) which share a common DC precursor *in vivo*. Inflammation leads to development of TNF/iNOS-producing DC (Tip DC) from recruited monocytes which can be generated *in vitro* from murine BM cultures upon addition of GM-CSF. (Flt3-L = fms-like tyrosine kinase 3 ligand; GM-CSF = granulocyte/macrophage colony-stimulating factor; iNOS = inducible nitric oxide synthase)

1.2.4.1 Conventional DC (cDC)

cDC, i.e. DC which show dendritic cell morphology and execute DC functions at an uninfected steady state level (in contrast to DC precursors) [24], develop by means of the cytokine fms-like tyrosine kinase 3 ligand (Flt3-L) [25]. Mice deficient for Flt3-L or its signalling cascade show prominent defects in steady state cDC development but not monocyte-derived DC generation [5, 25, 26]. Based on their migratory behaviour, cDC can be classified into migratory versus lymphoid-tissue-resident DC, i.e. migrate from the peripheral tissues into the draining secondary lymphoid organs (e.g. epidermal Langerhans cells) or remain their lifetime within one lymphoid organ (e.g. murine spleen DC) respectively [24]. The lymphoid-tissue-resident cDC can be further subdivided into cDC subsets based on the expression of CD4 or CD8α surface markers such as $CD8^+CD4^-$ ($CD8^+$) DC, $CD8^-CD4^+$ ($CD4^+$) DC and $CD8^-CD4^-$ DC in murine spleen (fig 2) [23, 24]. Interestingly, murine splenic DC subsets show intrinsic differences in various immunological functions in terms of antigen uptake [10, 11] and apoptotic cell handling [27, 28], cytokine production in steady state [29]

or upon microbial infection [30], antigen-processing and presentation abilities [11] and effector T cell and CTL polarization [4, 31]. Indeed, splenic $CD8^+$ DC subsets appear not only specialized APC for induction of regulatory T cell responses in the steady state [13, 28, 29] but are high-efficient in cross-presentation for the activation of $CD8^+$ CTL responses [11, 31]. The dual role of splenic $CD8^+$ DC in induction of tolerance versus immunity appears to be regulated at the maturation level [11]. Nevertheless, translation of these attractive findings to humans for immunotherapy purpose was hampered by the fact that CD8α is not expressed on human DC. Recently, several groups identified the human equivalent which share multiple functions with its murine $CD8^+$ DC counterpart [32].

1.2.4.2 Inflammatory DC

Monocytes can function as DC precursors and give rise to inflammatory DC, exemplified by the TNF and inducible nitric oxide synthase (iNOS) producing DC (Tip DC) initially identified in *Listeria monocytogenes* infections [24, 33]. *In vitro*, human DC are generated by a common culture method using blood monocytes in addition of granulocyte/macrophage colony-stimulating factor (GM-CSF) with or without IL-4 and murine DC from bone marrow precursors differentiated by GM-CSF with or without IL-4 [34, 35]. It appeared that these long-term established standard protocols for the generation of *in vitro* DC rather differentiate equivalents of the inflammatory Tip DC [36]. Studies with GM-CSF deficient mice indicated that monocyte-derived inflammatory DC are normally not present under steady-state conditions but develop from recruited monocytes at inflammatory cytokine permissive sites [25]. For the study of steady state cDC, researchers optimized an *in vitro* murine bone marrow culture supplemented with the cytokine Flt3-L to generate adequate numbers of splenic DC equivalents of both $CD8^+$ and $CD4^+$ cDC and plasmacytoid DC (fig 2) [37, 38].

1.2.4.3 Plasmacytoid DC (pDC)

pDC appear as DC precursors showing a particular plasma cell-like morphology in quiescent state but differentiate and adapt a DC-like appearance including antigen presentation abilities upon certain microbial stimulation [39, 40]. Development of pDC originates in the bone marrow from hematopoietic precursors and is dependent on Flt3-L [25]. Typical murine pDC markers include B220, Ly6C and intermediate levels of CD11c but no myeloid markers [39].

pDC sense viral nucleic acids by their selective expression of the intracellular TLR-7 and TLR-9 and mount a rapid response by producing large amounts of type I interferons (IFN), such as IFN-α or IFN-β [41]. pDC activation can also be triggered by self-DNA organized in aggregates bound to the anti-microbial peptide LL37 or high-mobility group box protein 1 (HMGB-1) released from activated neutrophils which might lead to severe autoimmune disease [41, 42]. Earlier reports suggested that pDC induce and maintain immune tolerance under steady state conditions by inducing regulatory T cells [43, 44]. How pDC orchestrate their dual role in tolerance versus effective immunity is regulated at the maturation state. In the quiescent state, pDC show only low levels of MHC II and co-stimulatory molecules, whereas high expression of MHC and co-stimulatory molecules is detected upon microbial stimulation [39, 40, 45]. Furthermore, the local microenvironment might condition pDC for induction of tolerogenic features such as partial maturation resistance [46] and/or expression of the tryptophan metabolizing enzyme indoleamine-2,3-dioxygenase (IDO) [44, 47]. Activated pDC upregulate inducible co-stimulatory ligand (ICOS-L) expression conferring IL-10 production and regulatory function in effector T cells [45].

1.3 DC and effector T cell immunity

1.3.1 DC and co-stimulation

According to the two-signal model for effective T cell activation, DC present next to cognate antigen-MHC complexes triggering TCR-activation (signal 1) additional co-stimulatory signals (signal 2) which potentiate and/or regulate TCR signalling cascades. An additional 'signal 3', i.e. a set of cytokines selectively produced by DC upon microbial pathogen recognition drives distinct polarization into distinct modes or effector T cell qualities (fig 3) [48].

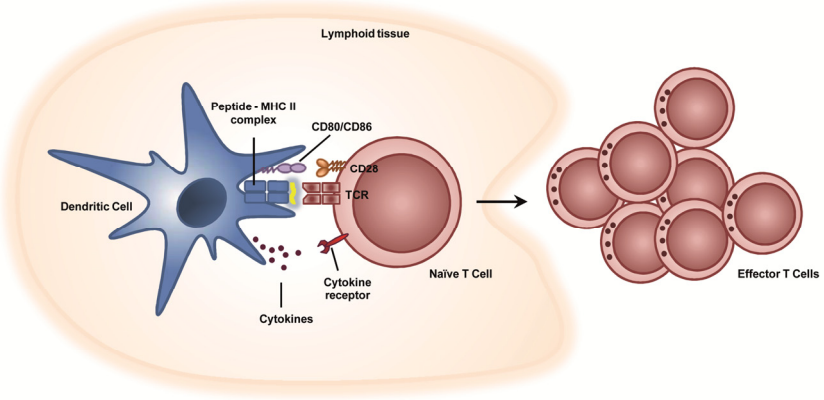


Figure 3. Cognate TCR and co-stimulatory signalling is required for efficient T cell activation
DC present peptide-MHC complexes and additional co-stimulatory proteins to recirculating naïve T cells in the secondary lymphoid organs. Upon recognition of their cognate antigen by the TCR, T lymphocytes receive signals from DC directing proliferation and differentiation into specialized T helper cell subsets.

Co-stimulatory ligands on the APC surface belong to the B7 or TNFR family and interact with the co-stimulatory receptors of the CD28 family on the T cell side. Prominent B7 co-stimulatory family members are CD80 (B7.1) and CD86 (B7.2) expressed on the DC membrane ligating to CD28 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) molecules on the naïve and/or activated T cell surface. Little CD28 is expressed already on the naïve T cell surface whereas CTLA-4 expression is induced and peaks within 48-72 hours in particular after TCR/CD28 ligation [49]. Stoichiometric analysis suggested that CD80 molecules ligate CTLA-4 with higher affinity compared to CD28 at least in part due to their bivalent interaction mode [50].

Earlier reports documented that CD28 lowers the activation threshold for TCR triggering of naïve T cells [51] and hence, amplifies downstream activation of the transcription factors activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor κB

(NFκB) [52, 53]. CD28-mediated activation of the transcription factor network supports proliferation, differentiation and survival of T cells through concerted actions on IL-2 gene transcription and mRNA stabilisation, effector cytokine and chemokine gene transcription, expression of cytokine receptors (e.g. IL-2R) and additional co-stimulatory receptors (e.g. CTLA-4 and CD40 ligand), etc. Signalling through CD28 is initiated at particular amino acid motifs within the cytoplasmic tail to which docking molecules such as phosphatidyl-inositol 3-kinase (PI3K) and Grb2 are recruited [52, 53]. It has been a matter of intense debate whether CD28 supports T cell activation through unique CD28-restricted signalling modulators or simply by amplification of TCR-ligation induced signalling pathways [54]. The mechanism of action of superagonistic anti-CD28 antibodies which deliver mitogenic signals to (naïve) T cells is actually dependent on TCR signalling components [55].

CTLA-4 is a potent negative regulator of T cell activation in general through modulation of the TCR activation threshold. Several inhibitory mechanisms have been proposed: (1) competition for CD80 or CD86 ligand binding, (2) docking of phosphatases at the cytoplasmic tail targeting proximal TCR- and/or CD28 signalling cascades, (3) reduction of TCR microcluster formation and others [52, 56]. Also CTLA-4-dependent stimulatory cascades have been identified thereby increasing T cell motility to reverse the stop signal induced during intense APC-T cell contact and immunologic synapse formation [57]. Recent reports suggested that CTLA-4 inhibits T cell activation by displacing CD28 out of the central supramolecular activation cluster (cSMAC) in the immunological synapse [58]. Hence, CTLA-4 activation lead to a block in cell cycle progression and impaired AP-1, NFAT and NFkB activation. Remarkable about the expression mode of CTLA-4 is its predominant intracellular localisation in the trans-Golgi network as well as in endosomes and lysosomes. Even upon activation only low levels of CTLA-4 can be determined on the T cell surface as CTLA-4 molecules continuously recycle from the surface and are targeted to the lysosomes for degradation or re-expressed on the surface [52].

Other co-stimulatory molecules have been identified such as programmed cell death-1 (PD-1) and inducible T cell co-stimulator (ICOS) which can have both stimulatory and inhibitory effects. Interaction of CD40 ligand (CD40L) and CD27 with their respective ligands CD40 and CD70 on APC surface rather supports potent inflammatory T cell responses.

1.3.2 DC-mediated differentiation into T helper cell subsets:

Although the concept of classical lineage commitment into distinct T helper cell modes has been challenged in part through observations that T cells show substantial functional plasticity in cytokine secretion [59], division of T cell qualities into T helper cell subsets remains a valuable approach to dissect the instructive signals derived from the DC (signal 3) and the molecular requirements needed for subset differentiation (fig 4). Multiple T helper cell subsets or polarization modes have been identified but only the main well-studied T helper cell subsets will be introduced below.

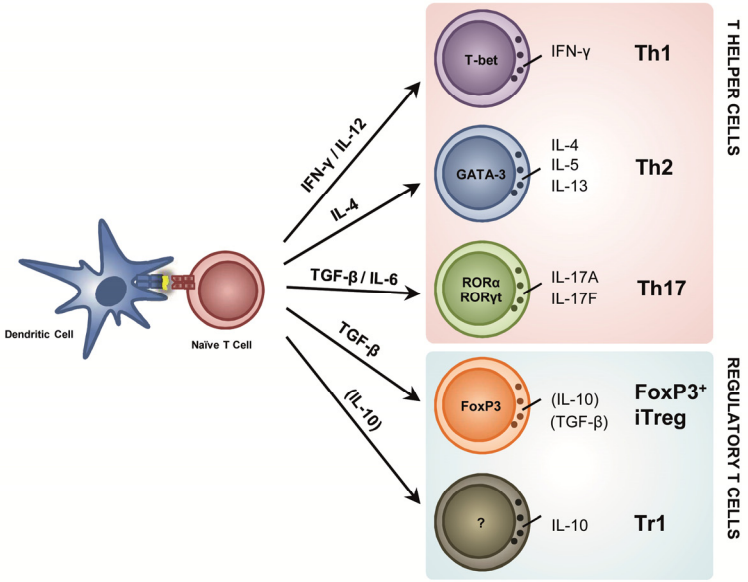


Figure 4. Differentiation of naïve T cells into T cell lineages is directed by permissive cytokines
Cytokines secreted in part by DC induce discrete programming and lineage commitment of naïve T cells into specialized subsets through signal transducer and activator of transcription (STAT)-mediated signalling and upregulation of lineage-specific transcription factors. T helper (Th) cells modulate immune function primarily through secretion of cytokines, whereas regulatory T cells (such as FoxP3^{+} induced regulatory T cells (iTreg) and T regulatory cells 1 (Tr1), *see below*) control immune responses through cell-mediated mechanisms and/or anti-inflammatory cytokine production.

1.3.2.1 T helper 1 (Th1) responses:

IFN-γ producing Th1 cells orchestrate the cellular immunity in that they potentiate function of macrophages and other innate cells for the eradication of intracellular pathogens [60]. T-bet was identified as the "master" transcription factor regulating Th1 cell gene expression and differentiation [61]. Upon TCR-engagement and recruitment of the IFN-γ receptor (IFNGR) machinery, signal transducer and activator of transcription 1 (STAT-1) translocates to the nucleus to induce T-bet expression regulating Th1 cell commitment [62]. After the initial antigen-TCR stimulation phase, IL-12 receptor (IL-12R) signalling is required for the

maintenance and stabilisation of the Th1 phenotype by activating T-bet through STAT-4 signalling [63].

IL-12p70, a heterodimer consisting of a IL-12p40 and a IL-12p35 chain, is a clear driving force for the generation of IFN-γ producing Th1 cells [64, 65]. Other members of the IL-12 cytokine family, such as IL-27, can be produced by DC and have been postulated to contribute to the generation of Th1 responses. Expression of IL-12p35 is usually activated upon ligation of certain TLR by PAMP on DC [66]. Sustained levels of IL-12p70 can also be induced by IFN-γ mediated feedback signalling on DC derived from effector Th1 cells, $CD8^+$ CTL or innate cells such as NK cells. In addition, CD40L ligation by CD40 expressed on effector T cells further enhances the levels of IL-12p70 production by DC and licenses DC for efficient activation of CTL responses [67, 68]. Interestingly, DC can instruct Th1 responses in the absence of IL-12p70 by a CD70-dependent mechanism *in vivo* [69].

1.3.2.2 T helper 2 (Th2) responses:

Th2 regulate B cell immunoglobulin isotype switching and modulate other innate or epithelial cell function through production of IL-4, IL-5, IL-13, IL-9 and others, making them orchestrators of humoral immunity for the eradication of extracellular parasites [60]. GATA-3 was identified as the master regulator of Th2 cell commitment and differentiation [70, 71]. IL-4 receptor (IL-4R) engagement activates GATA-3 expression through STAT-6 mediated signalling. Also IL-2R signalling through STAT-5 might contribute to Th2 cell development [72].

The DC instructive signals necessary for the induction of Th2 responses remained poorly defined. Initially, Th2 responses were believed to be induced by the so-called default or exhaustion pathway, i.e. in the absence of IL-12p70 production [73]. Others defined a peptide dose and/or affinity dependency favouring either Th1 or Th2 effector cell differentiation [74-76]. Differential expression of the Notch ligands Jagged1 and 2 on APC has been identified as a decisive mechanism for the development of Th2 responses [77]. However, Jagged2 expression on DC is not required for Th2 induction *in vivo* [78]. Others identified c-Kit as a crucial Th2 and Th17 inducing signalling axis on DC driving downstream the expression of IL-6 and Jagged2 [79]. Interestingly, IL-6 has been previously identified as a mediator of T helper 2 responses [80]. Several other membrane markers and soluble mediators expressed or

secreted by DC have been described to polarize towards Th2 responses: co-stimulatory molecules such as OX40 [81], ICOS-L [82] or arachidonic acid metabolites such as PGE_2 [83-85]. The undefined role of DC in the instruction of Th2 responses brought its necessity and contribution to Th2 development under debate [86-88]. Indeed, IL-4 driving Th2 commitment via STAT6-GATA3 signalling axis, cannot be produced by DC but can be provided by other innate cells such as basophils, eosinophils and NKT cells although naïve T cells themselves can secrete IL-4 upon TCR-activation [72]. Conditional ablation of DC in mice prevented the induction of asthma features, alum-dependent immune responses and *Schistosoma mansoni* driven Th2 responses *in vivo* further indicating the DC are required for the induction of Th2 responses [89-92].

It seems that helminth-derived secretory products only evoke mild transcriptional programming of DC resulting in immature to partially mature DC phenotypes [93, 94]. Interestingly, also pro-inflammatory cytokines such as TNF or IL-6 [16, 18, 95] or tissue disruption [17] induce a similar partially mature DC phenotype characterized by upregulation of MHC II and co-stimulatory molecules but no production of cytokines. Whether conditioning of DC by endogenous inflammatory mediators or pathogenic signals from parasites trigger the same quality of DC maturation and respective Th2 responses remains to be shown. Insights in partial DC maturation phenotypes will be valuable to understand parasitic immune evasion strategies and the therapeutic potential of Th2 responses in immune deviation settings.

1.3.2.3 T helper 17 (Th17) responses:

Another subset of pro-inflammatory T helper cells was discovered only recently which produces IL-17 (Th17) and develops independent from Th1 or Th2 helper cells [96, 97]. Th17 cells produce additional cytokines such as IL-17F, IL-21 and IL-22 contributing to tissue inflammation and extracellular pathogen clearance, at least in part through neutrophil recruitment and activation [60, 98]. STAT-3 signalling along with the master regulators RORγt and RORα orchestrate Th17 development and differentiation supported by an IL-21-dependent feedback loop [98].

Initial reports demonstrated that Th17 cells develop by means of the joint action of IL-6 and TGF-β [99-101]. Torchinsky and colleagues were the first to show that DC simultaneously produce IL-6 and TGF-β for the development of Th17 cells in response to infected apoptotic

cells through TLR and phosphatidylserine-dependent signalling respectively [102]. Interestingly, DC increased production of IL-23, another member of the IL-12 cytokine family, consisting of an IL-12p40 and the unique IL-12p19 chain, upon TLR and/or C-type lectin receptor engagement [66, 102]. Similar to the DC derived instructive signals for Th1 differentiation, IL-23 might not be required for initial Th17 commitment but rather stabilisation of the Th17 phenotype [98]. A role for IL-1β in Th17 differentiation has also been suggested although it may rather enhance instruction induced by IL-6 and TGF-β [99].

1.4 DC and T cell immune tolerance

1.4.1 Central Tolerance

After successful rearrangement of the TCR, developing T cells undergo a stringent selection process in the thymus in which thymocytes showing high self-reactivity are deleted and not allowed to emigrate into the peripheral T cell pool. In the thymic cortex, developing thymocytes scan self-peptide-MHC complexes on the cortical thymic epithelial cells (cTEC) and only receive survival signals (also termed positive selection) if TCR shows intermediate affinity for peptide-MHC interactions. Thymocytes that fail to express a TCR with basal affinity for the displayed peptide-MHC complexes die by neglect. Too high affinity self-peptide-MHC interactions are selected for apoptotic cell death or clonal deletion during the process of negative selection. Specialized cells for this purpose are medullary thymic epithelial cells (mTEC) which express multiple tissue-restricted antigens under control of the autoimmune regulator AIRE [103, 104]. Intrathymic resident DC or DC migrating from peripheral sites might contribute to clonal deletion by self-peptide-MHC presentation derived from intercellular antigen transfer from the mTEC and/or from peripheral self-antigen pools respectively [105]. Interestingly, autoreactive thymocytes showing high-affinity for self-peptide-MHC complexes might develop into the naturally occurring regulatory T cell lineage [106]. A role for thymic DC in the generation of regulatory T cells has been reported for mice [107] and humans [108], the latter through conditioning with thymic stromal lymphopoietin (TSLP) expressed in Hassall's corpuscles.

1.4.2 Peripheral Tolerance

1.4.2.1 Introduction

Despite effective central tolerance mechanisms operating in the thymus, potential autoreactive T cell clones escape in the periphery and might pose danger to the host from autoimmune reactions to occur. However, several peripheral tolerance mechanisms exist which keep those self-reactive T cell in check, at least in part through orchestrated actions of DC [109, 110]. Indeed, constitutive depletion of DC results in spontaneous autoimmune reactions characterized by activated IFN-γ and IL-17 producing T cells, inflammatory organ infiltrates and elevated immunoglobulin titers [111]. However, these autoimmune phenomena might result from a progressive myeloproliferative disorder rather than a break of peripheral tolerance in the absence of DC indicating that the contribution of DC to establishment and maintenance of peripheral tolerance needs further investigation [112].

1.4.2.2 Mechanisms of peripheral T cell tolerance

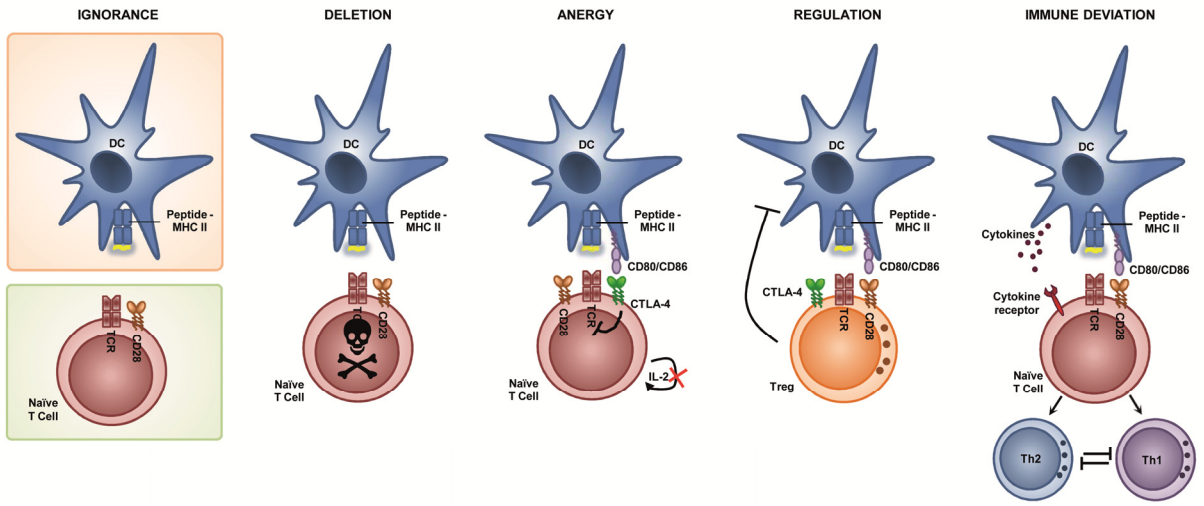


Figure 5. DC and the mechanisms of peripheral T cell tolerance
Self-reactive T cells are kept in check by various peripheral tolerance mechanisms: ignorance of T cells towards their cognate antigen; deletion from the peripheral T cell pool; anergy characterised by defective TCR-induced proliferation and IL-2 cytokine secretion which can be induced by CTLA-4 signalling; regulatory T cells which suppress immune responses through cell-mediated contacts or secretion of soluble mediators and immune deviation into T helper cell lineages showing ineffective helper cell functions. (Treg = regulatory T cell)

1.4.2.2.1 IGNORANCE

If antigens do not reach the blood or secondary lymphoid organs for peptide-MHC presentation by DC, the circulating naïve T cells remain ignorant for the respective antigens and hence, those restricted areas are ignored by (both innate and) adaptive immune systems. Such area include the anterior chamber of the eye [113], the brain protected by the blood-brain barrier [114] and even solid tumours [115] and are termed immunological privileged sites as these specialized sites seemed to be protected from the devastating effect of an inflammatory reaction. However, it becomes increasingly apparent that antigens do leak out into the peripheral lymphoid organs (largely dependent on soluble antigen transport) and that the immune system actively protects these immune-privileged sites [113, 114]. Therefore, a broader definition of immune ignorance might include the failure of T cells typically carrying low-avidity TCR to achieve activation signals as cognate peptide-MHC presentation by DC in the secondary lymphoid organs remains below a certain antigen-dose threshold [116]. Immune ignorance was initially described as a theoretically peripheral tolerance mechanism, but evidence that such a mechanism is actively contributing to the maintenance of tolerance is lacking so far.

1.4.2.2.2 DELETION

The number and size/composition of the mature T cell pool is tightly regulated by means of survival signals acquired through TCR scanning of self-peptide MHC complexes on DC in the secondary lymphoid organs and cytokines belonging to the common gamma chain family, especially IL-7 and IL-15 [117]. T cells which fail to achieve sufficient survival signals undergo apoptosis and are deleted from the peripheral T cell pool. It has been suggested that Fas/Fas-ligand (CD95/CD95-L) dependent interactions control deletion upon TCR engagement and proliferation [118], whereas the pro-apoptotic Bcl-2 homology domain 3 (BH3)-only protein Bim regulates cytokine withdrawal-mediated apoptosis next to thymic negative selection [119, 120]. Abbas and colleagues demonstrated that T cells responding to systemic self-antigens are rendered anergic if deletion was prevented due to absence of Bim indicating that other tolerance mechanisms can compensate for deletion for the maintenance of peripheral immune tolerance towards systemic self-antigens [121]. Nevertheless, Fas/Fas-L interactions are essential to limit excessive T cell expansion and to terminate T cell responses

following optimal T cell priming by so-called activation-induced cell death (AICD) [122-124].

1.4.2.2.3 ANERGY

The term clonal anergy was used to define the functional unresponsive state in $CD4^+$ T lymphocytes (initially characterized in Th1 T cell clones**,** i.e. previously activated T cells) achieved by strong TCR/CD3 engagement (signal 1) in the absence of CD28 co-stimulation *in vitro* (signal 2) [125, 126]. Although a variety of experimental approaches have been used to induce T cell anergy *in vitro*, the most consistent hallmarks are defective IL-2 production and proliferation upon TCR restimulation even in the presence of some extent of co-stimulation [126-128]. The phenotype of clonal T cell anergy could be reversed by addition of exogenous IL-2 as $CD4^+$ T cell clones express high-affinity IL-2 receptors [126]. Reversal of the $CD4^+$ T cell anergic state can also be achieved by exposure to polyclonal mitogens bypassing TCR-signalling such as phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin. However, in contrast to antigen-experienced T cell clones, naïve $CD4^+$ T cells are resistant to anergy induction *in vivo* and *in vitro* upon anti-CD3 TCR ligation in the absence of co-stimulation [129]. Indeed, naïve $CD4^+$ T cells appeared to be also dependent on B7 co-stimulation-driven CTLA-4 engagement for anergy induction [130]. CTLA-4 signalling is obligatory for tolerance induction *in vivo*, also termed adaptive tolerance [126], as judged by studies using CTLA-$4^{-/-}$ T cells or antibody-mediated blocking experiments [131, 132]. The precise role and signalling mechanisms of CTLA-4 for induction of anergy in naïve T cells is still a matter of debate [52]. Earlier reports suggested that CTLA-4 signalling prevents cell cycle progression through regulation of the cyclin-dependent kinase (cdk) inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ [131, 133]. Engagement of CTLA-4 has also been shown to block IL-2 production and IL-2 receptor (IL-2R) expression at least in part through decreased NFAT translocation to the nucleus [133-135]. Conversely, anergy induction in antigen-experienced T cells can be induced by Ca^{2+}/calcineurin-dependent signalling through ionomycin only thereby triggering downstream NFATc2 but not its transcriptional binding partner AP-1 [128, 136, 137]. Rao and colleagues identified a NFAT-dependent transcriptional programme inducing anergy-associated genes which include several E3 ubiquitin ligases such as casitas B-lineage lymphoma B (Cbl-b) and gene related to anergy in lymphocytes (GRAIL) [128, 136]. Members of the E3 ubiquitin ligase family target TCR-signalling molecules for degradation

leading to a defective TCR-signalling and unstable immunological synapse formation thereby crucially contributing to anergy induction *in vitro* and *in vivo* [136, 138-140]. Interestingly, microarray analyses revealed a dominant role for the early growth response genes 2 (Egr-2) and Egr-3 for expression of the anergy-associated transcriptional programme, as identified in clonal anergy and *in vivo* anergy models [141-144].

The question remains which functional role anergic T cells might have since they appear to persist *in vivo* for relatively long periods of time and can potentially reverse their functional unresponsive state [142, 145]. Others reported that anergic T cells acquire the ability to produce anti-inflammatory cytokines such as IL-10 and suppress naïve T cell responses [146, 147]. However, which signalling pathways and/or DC-derived instructive molecules are needed to induce IL-10 and/or regulatory function in anergic T cells remained unknown so far and needs further investigation.

1.4.2.2.4 REGULATION/SUPPRESSION

1.4.2.2.4.1 Introduction

Several specialized regulatory T cell subsets exist which dominantly suppress unwanted self-reactive immune responses or excessive effector T cell responses [148]. Although TCR stimulation is needed to activate suppressive function of regulatory T cells, they can suppress in an antigen-nonspecific manner by so-called bystander suppression. By creating an immunosuppressive environment, regulatory T cells can even promote the differentiation of other suppressor T cells carrying distinct antigen specificities, a concept also termed infectious tolerance [149]. Besides the thymus-derived naturally occurring $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells, several types of adaptive regulatory T cells have been described which typically suppress by cell contact-independent mechanisms (e.g. secretion of IL-10).

1.4.2.2.4.2 Naturally occurring FoxP3⁺ regulatory T cells

Sakaguchi and colleagues discovered a particular subset of $CD4^+$ T cells expressing high levels of the IL-2 receptor α chain (CD25) and showing potent suppressive function essential for the prevention of a severe autoimmune disorder [150]. The discovery of the lineage specific transcription factor forkhead box P3 (FoxP3) necessary for the generation, maintenance and function of regulatory T cells [151, 152] along with the notion that a loss-of-function mutation of the FoxP3 gene in mice (known as "scurfy mice") or humans (immunodysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome) results in a severe, autoaggressive and inflammatory immune disorder [153, 154] facilitated the in-depth study of this unique T cell subset. Regulatory T cells express several other markers such as CD28, CTLA-4 and lymphocyte activation gene 3 (LAG-3) which are, however, also present on activated effector/memory T cells [155]. Initially, regulatory T cells were described as anergic T cells since they fail to proliferate or to produce IL-2 upon TCR stimulation even in the presence of low amounts of co-stimulation [156, 157]. It appeared that regulatory T cells intrinsically repress IL-2 gene expression making them dependent on IL-2 derived from activated effector T cells and/or DC for proliferation and suppressive function [156, 157]. Suppression of responder T cells by regulatory T cells might occur by means of IL-2 cytokine deprivation resulting in starvation-induced apoptotic cell death although this mechanism remains controversial [158, 159]. Others reported inhibition of IL-2 and other cytokine gene transcription in responder T cells as a suppressive mechanism of regulatory T cells [156, 157]. Soluble mediators such as anti-inflammatory IL-10 and/or TGF-β cytokines produced by regulatory T cells might also regulate effector T cell or DC function. Interestingly, several regulatory mechanisms have been described modulating in particular DC maturation and/or function, e.g. expression of the high-affinity MHC II binding and CD4 homologue LAG-3 [158, 159]. Regulatory T cells potently downregulate surface expression of CD80 and CD86 molecules on the DC surface through interaction with CTLA-4 [22, 160]. Recently, it was suggested that CTLA-4 extracts and degrades CD80 and CD86 molecules from the surface of DC by the process of trans-endocytosis [161]. Furthermore, CTLA-4 can induce by means of B7-dependent DC-reverse signalling, production of the regulatory enzyme indoleamine 2,3-dioxygenase (IDO) which cleaves the essential amino acid L-tryptophan into regulatory metabolites [162]. CTLA-4 expressed by regulatory T cells might also target CD80/CD86 molecules upregulated on activated effector T cells resulting in nuclear accumulation of the inducible cAMP early repressor/cAMP response element modulator (ICER/CREM) regulating IL-2 transcription [163].

So-called naturally occurring FoxP3$^+$ regulatory T cells originate in the thymus from T cell precursors whereas regulatory T cells can also be induced from FoxP3$^-$ precursors in the periphery. Beside the dominant role of TGF-β and IL-2 cytokine environment, DC and B7 co-stimulatory molecules contribute to conversion and maintenance of induced regulatory T cells [164, 165]. Development of thymic FoxP3$^+$ regulatory T cells appears to be highly dependent on high-affinity TCR interactions, CD28 signalling and the IL-2 receptor pathway as indicated by dramatic defects in regulatory T cell numbers in the respective knockout mice [166]. A two-step model for the development of thymic regulatory T cells was proposed in which T cell precursors are first committed to the regulatory T cell lineage as cytokine-responsive CD25$^+$ FoxP3$^-$ T cells followed by an IL-2 dependent process for induction of FoxP3 expression and full differentiation into regulatory T cells [167, 168]. The molecular details for the commitment to the FoxP3$^-$ regulatory T cell precursor stage have just begun to be unravelled. Several reports suggested that CD28 signalling might play a crucial role along with the activation of the downstream transcription factor NFκB [169-171]. Hence, it becomes increasingly apparent that other transcription factors or regulatory networks impose regulatory T cell lineage commitment other than FoxP3 which is, however, essential for stabilisation of the regulatory T cell phenotype [172, 173].

1.4.2.2.4.3 Adaptive regulatory T cells

To the adaptive regulatory T cells belong the TGF-β-producing T helper 3 regulatory T cells (Th3), which were initially detected in oral tolerance conditions [174], the T regulatory cells 1 (Tr1) producing IL-10 for suppression [175] and the recently identified IL-35 dependent regulatory T cells (Tr35) [176]. Initially, Tr1 cells were derived from naïve T cells and defined as IL-10 producing T cells which acquired suppressive activity in the presence of IL-10 [146, 175]. Tr1 cells adapted a particular cytokine expression profile distinct from Th1 or Th2 effector T cells [146]. However, ever since, many IL-10 producing regulatory T cells have been described induced under various experimental conditions (reviewed in [175, 177]), even upon chronic stimulation of effector T cells gradually losing cytokine production of IFN-γ or IL-4 [178]. The question remains whether IL-10$^+$ regulatory T cells derived from naïve T cell precursors (Tr1) or chronically stimulated effector T cells (Tr1-like) develop by similar IL-10 and/or co-stimulatory signalling-dependent mechanisms. Recent reports seem to

support the concept of a common signalling pathway triggering IL-10 production in effector T cells for generation of Tr1-like cells [179, 180].

Lack of specific markers hampers the identification of Tr1 cells and of the molecular pathways driving their generation. However, some biological features are consistently reported in the field. Both Tr1 and Tr1-like cells show features of an anergic phenotype [146, 181-186]. The successful use of IL-10 in Tr1 cell generation *in vitro* might be confined to the induction of T cell anergy, rather by modulation of DC or APC maturation than through direct T cell signalling [175, 187, 188]. Indeed, IL-10 is a potent down-modulator of MHC II and co-stimulatory molecule expression on DC [184, 189, 190]. Induction of anergy can occur in absence of co-stimulatory molecules *in vitro*, whereas engagement of CTLA-4 is required for the anergy induction *in vivo* [131, 137]. Interestingly, expression of CTLA-4 on the surface of Tr1 and Tr1-like cells is well-documented [183, 185, 191-193]. Differentiation of Th2 effector cells producing IL-10 is CD28 dependent but inhibited by CTLA-4 [194-196]. Injection of anti-CTLA-4 antibodies in the presence of splenic DC induced $ICOS^+$ regulatory T cells producing both IL-4 and IL-10 [196]. In contrast, triggering CTLA-4 signalling on Th1 cells by CD80 from mature DCs seems to promote IL-10 secretion and Tr1-like differentiation [183]. Thus, CTLA-4 (and CD28) might have opposing functions in Tr1-like generation dependent on the initial differentiation program that shaped the effector T cell, whereas its role for the generation of Tr1 cells remained unexplored so far. Others suggested that expression of the co-stimulatory molecule ICOS is linked to IL-10 secretion by both Tr1 and Tr1-like cells [197, 198] which receive signals through ICOS-L (CD275) on the surface of mature myeloid DC [199] or plasmacytoid DC [45]. Finally, both Tr1 and Tr1-like cells acquire suppressive features and regulate naïve and memory T cell responses through secretion of IL-10 *in vitro* and *in vivo* [146, 189, 198, 200, 201]. Some reports demonstrated that *in vitro* suppression of naïve T cells by Tr1 or Tr1-like cells could not be reversed by neutralization of anti-inflammatory cytokines but by addition of exogenous IL-2 indicating that additional mechanisms such as IL-2 consumption might also contribute [185, 192, 193].

Since adaptive regulatory T cells share a great potential for the treatment of autoimmunity or chronic inflammatory diseases, the molecular signalling pathways contributing to their generation are studied in great detail [175, 202]. Studying the lineage relationship of Tr1 and $FoxP3^+$ regulatory T cells might further elucidate some of the developmental pathways inducing regulatory function in T cells. Both $FoxP3^+$ and $FoxP3^-$ IL-10-producing regulatory T cells are most prevalent in the gut-associated lymphoid tissue (GALT) in steady state

conditions and can develop from FoxP3$^-$ precursors [203]. Where the differentiation program of Tr1 cells diverge from IL-10$^+$ FoxP3$^+$ regulatory T cells remains to be shown, although the availability of TGF-β and/or all-trans retinoic acid produced by DC in the GALT might play a decisive role [204].

1.4.2.2.5 IMMUNE DEVIATION (OR WHY TH1/TH2 RESPONSES CAN BE PROTECTIVE)

The concept of immune deviation is based on the observations that effector T cell polarization into IFN-γ-producing Th1 can be achieved in the presence of IL-12p70 but blocking IL-4, whereas Th2 differentiation is driven by blocking IL-12p70 and/or IFN-γ in the presence of IL-4. During priming, signalling through IFNGR blocks IL-4R recruitment to the immunological synapse (IS), whereas IL-4 signalling excludes IFNGR from the IS and impairs downstream STAT1 translocation [62]. Imprinting of T helper cell differentiation is organized by "master" transcription factors directing cytokine gene expression and epigenetic modifications of the cytokine loci [205]. The fact that T cell polarization is directed into either Th1 or Th2 effector lineages has dramatic implications in the context of clearance of pathogen infection exemplified by the murine *Leishmania major* infection. Resistant C57BL/6 mice mount a protective Th1 response, whereas the susceptible BALB/c strains fail to control parasite replication due to a dominant Th2 polarization. Furthermore, studies with *Trypanosoma brucei* resistant mouse models revealed that mice mount an early IFN-γ response followed by a late cytokine switch to the anti-inflammatory IL-10, IL-13 and IL-4 [206]. This remarkable cytokine shift/immune deviation was also described in other protozoa or helminths infection models such as *Schistosoma mansoni* and *Eccinococcus multilocularis* which seems to protect the host from extensive tissue damage under unrestricted Th1-mediated inflammation [207]. Hence, helminth antigens are extensively studied to define how immune deviation is triggered at the level of DC in terms of maturation phenotype and functional immune modulation [208]. So far, several pathogenic agents have been used to successfully prevent disease in inflammatory autoimmune models such as inflammatory bowel disease (IBD), type 1 diabetes and experimental autoimmune encephalomyelitis (EAE) (reviewed in [209]). Clinical trials using eggs from *Trichuris suis* demonstrated its efficacy for the treatment of patients suffering from IBD [210]. Interestingly, chronic Th2-mediated helminth infections were also able to block inflammatory Th2-driven disorders such as allergic airway inflammation [211]. Hence, it becomes increasingly apparent that helminth-

induced immune deviation mechanisms might be dependent on IL-10 secretion derived from Tr1 or Th2 cells and/or suppression by induced FoxP3$^+$ regulatory T cells [212, 213]. The exact contribution of those regulatory mechanisms to the concept of helminth-driven immune deviation needs further investigation.

1.4.2.3 Antigen acquisition during steady-state for peripheral tolerance:

1.4.2.3.1 APOPTOSIS AND CELL-ASSOCIATED SELF-ANTIGENS

Apoptosis or programmed-cell death is a process in which a cell completely dismantles itself by degrading all essential cellular organelles down to fragmentation of nuclear DNA and is thereby continuously surrounded by intact membrane and organised as blebs for efficient phagocytosis [214]. This intriguing process is orchestrated mainly by members of a family of cysteine proteases called caspases which are activated cell-intrinsically by stimuli causing cellular stress or damage (e.g. irradiation), through the extrinsic pathway by means of death receptor signalling (e.g. FasL) or through the granzyme B pathway released by CTL or NK cells (reviewed in [215]). Cells undergoing apoptosis rapidly send out so-called "eat-me"-signals e.g. flip-flopping phosphatidylserine normally present on the inner leaflet to the outer leaflet of the membrane to ensure engulfment by recruited professional phagocytes such as DC [216]. Membranes of (early) apoptotic debris remain intact and all potential dangerous self-molecules do not leak out in the surrounding environment, therefore it is believed that apoptosis leads to tolerance in contrast to necrosis or accidental cell death where membrane integrity is lost initiating autoimmune inflammatory responses [217-219].

Apoptotic cells might serve as a rich source of self-antigens for peptide-MHC presentation by DC regulating potentially autoreactive T cell responses [220]. Huang et al. reported earlier that DC continuously carry apoptotic remnants of intestinal epithelial cells to the draining lymph nodes [221]. Indeed, DC which captured apoptotic cells *in vivo* were able to present and cross-present cell-associated peptides to the CD4$^+$ or CD8$^+$ T cells respectively [27, 222-225]. Despite being ingested efficiently, apoptotic cells did not induce maturation in DC as no upregulation of MHC II or co-stimulatory molecules could be detected under various experimental conditions [217, 224, 226, 227]. Only human monocyte-derived DC seemed to upregulate the chemokine receptor CCR7 upon ingestion of apoptotic cells *in vitro* [228, 229]. Although the release of anti-inflammatory TGF-β is well-documented for macrophages

capturing apoptotic cells [230], enhanced production of TGF-β by DC in response to apoptotic cells remained controversial [224, 231]. Interestingly, phagocytosis of apoptotic cells seem to block NFκB-dependent activation of DC *in vitro* [232, 233] as a delayed secondary LPS-stimulus failed to induce DC maturation detected by decreased MHC II and co-stimulatory molecule expression and lack of pro-inflammatory cytokine secretion [224, 228, 234-236]. Thus, whereas the immune modulatory effects of apoptotic cells are well-studied, how apoptotic cells per se might trigger tolerogenic signalling in DC for the induction or maintenance of self-tolerance remains elusive so far.

Injection of apoptotic cells *in vivo* has been applied for the induction of transplantation tolerance [227], prevention of EAE [237], induction of diabetic tolerance in NOD mice [238], and others, leading to deletion of both $CD4^+$ and $CD8^+$ antigen-specific T cells and/or generation of $FoxP3^+$ regulatory T cells [227, 239-241]. However, Morelli and colleagues suggested recently that the deficient activation and consecutive deletion of $CD4^+$ effector T cells by immature DC *in vivo* caused only a relative and indirect increase of $FoxP3^+$ regulatory T cells [242]. Furthermore, despite the fact that phagocytosis of apoptotic cells by splenic DC has been reported [28, 224], the role of surrounding macrophages for phagocytosis and maintenance of immune tolerance (at least in part by creating an TGF-β immune modulatory environment) cannot be completely excluded [237, 243, 244]. Hence, whether and how DC conditioned by ingestion of apoptotic cell material instruct $CD4^+$ T helper cells for the development of regulatory T cell responses remains to be shown.

2 MATERIALS & METHODS

2.1 Reagents, Buffers and Media

2.1.1 Chemical reagents

Product	Purchased from
Agarose	Roth (Karlsruhe, Germany)
Alu-Gel-S	Serva Electrophoresis (Heidelberg, Germany)
Ammonium Chloride (10% solution)	Applichem (Darmstadt, Germany)
β-mercaptoethanol	Sigma-Aldrich (Deisenhofen, Germany)
Baby rabbit complement	Biozol (Eching, Germany)
Brefeldin A (from Penicillium brefeldianu)	Sigma-Aldrich (Deisenhofen, Germany)
BSA	Roth (Karlsruhe, Germany)
CFSE (carboxyfluorescein diacetate succinimidyl ester)	Invitrogen (Darmstadt, Germany)
Chloroform	Applichem (Darmstadt, Germany)
Complete Freund's Adjuvant (CFA)	Sigma-Aldrich (Deisenhofen, Germany)
Cytochalasin D	Sigma-Aldrich (Deisenhofen, Germany)
Cytofix fixation buffer	BD biosciences (Heidelberg, Germany)
DAPI (4',6-diamidino-2-phenylindole)	Serva Electrophoresis (Heidelberg, Germany)
DEPC (diethyl pyrocarbonate)	Roth (Karlsruhe, Germany)
Diff-Quick staining set	Medion Diagnostic (Langen, Germany)
DMSO (dimethyl sulfoxide)	Sigma-Aldrich (Deisenhofen, Germany)
dNTPs	Fermentas (St. Leon-Rot, Germany)
EDTA	Applichem (Darmstadt, Germany)
Ethanol	Applichem (Darmstadt, Germany)
Ethidium Bromide	Roth (Karlsruhe, Germany)
FCS (fetal calf serum)	PAA Laboratories (Pasching, Austria)
Fluoromount-G	Serva Electrophoresis (Heidelberg, Germany)
Formaldehyde (37%)	Roth (Karlsruhe, Germany)
Golgistop	BD biosciences (Heidelberg, Germany)
Hydrogen Peroxide (30%)	Applichem (Darmstadt, Germany)
Ionomycin calcium salt (from Streptomyces)	Sigma-Aldrich (Deisenhofen, Germany)
Isopropanol	Applichem (Darmstadt, Germany)
L-Glutamine	PAA Laboratories (Pasching, Austria)
LPS (*E. coli* 0127:B8)	Sigma-Aldrich (Deisenhofen, Germany)
Methanol	Applichem (Darmstadt, Germany)
Mycobacterium tuberculosis H37RA	Difco Laboratories (Detroit, USA)
Paraformaldehyde	Sigma-Aldrich (Deisenhofen, Germany)

Penicillin/Streptomycin	PAA Laboratories (Pasching, Austria)
Pertussis toxin	List Biological Laboratories (Epsom, UK)
Phosflow perm buffer III	BD biosciences (Heidelberg, Germany)
PKH26 cell linker	Sigma-Aldrich (Deisenhofen, Germany)
PMA (Phorbol 12-myristate 13-acetate)	Sigma-Aldrich (Deisenhofen, Germany)
Recombinant human (rh)Flt3-L	Peprotech (Hamburg, Germany)
Saponin (from Quillaja Bark)	Sigma-Aldrich (Deisenhofen, Germany)
Sodium azide (NaN_3)	Roth (Karlsruhe, Germany)
TMB (3,3',5,5'-Tetramethylbenzidine)	eBioscience (Frankfurt, Germany), BD biosciences (Heidelberg, Germany)
TNF	Peprotech (Hamburg, Germany)
TRIzol	Invitrogen (Darmstadt, Germany)
Trypan blue	Sigma-Aldrich (Deisenhofen, Germany)
Tween 20	Applichem (Darmstadt, Germany)

Table 1. List of chemical reagents used throughout experimental set-up

2.1.2 Antigens

Antigens	Purchased from
MOG_{35-55}	synthesized and HPLC purified by R. Volkmer, Charité (Berlin, Germany)
$OVA_{327-339}$	Activotec (Cambridge, UK)
Ovalbumin (Endograde quality)	Hyglos (Regensburg, Germany)
Ovalbumin	Sigma (Deisenhofen, Germany)

Table 2. List of peptides and proteins used as antigens throughout this study

2.1.3 Antibodies

2.1.3.1 Primary antibodies directed against surface or intracellular markers

Antigen	Clone	Isotype	Conjugate	Dilution (fold)	Purchased from
Annexin-V	x	x	FITC or APC_y	20	BD biosciences
Active Caspase-3	polyclonal	Rabbit IgG	PE	20µl/test	BD biosciences
CD3ε chain	145-2C11	Hamster IgG1	unconjugated	5µg/ml	House-made
CD3ε chain	145-2C11	Hamster IgG1	unconjugated	5µg/ml	BioLegend
CD3ε chain	145-2C11	Hamster IgG1	biotinylated or NA/LE	5µg/ml	BD biosciences
CD4	GK1.5	Rat IgG2b	biotinylated or PE	200	BD biosciences
CD4	GK1.5	Rat IgG2a	FITC or APC_y	200	BioLegend
CD4	RM 4-5	Rat IgG2a	APC_y	200	BD biosciences

(Continued)

Antigen	Clone	Isotype	Conjugate	Dilution (fold)	Purchased from
CD8α	53-6.7	Rat IgG2a	PerCP	100	BioLegend
CD8α	53-6.7	Rat IgG2a	biotinylated or APC_y	100	BD biosciences
CD11b	M1/70	Rat IgG2b	FITC or PE or PerCP	100	BD biosciences
CD11b	M1/70	Rat IgG2b	APC_y or PerCP-Cy5.5 or Alexa-647	100	eBioscience
CD11c	HL3	Hamster IgG1	biotinylated or FITC	200	BD biosciences
CD11c	N418	Hamster IgG	APC_y	200	BioLegend
CD11c	N418	Hamster IgG	PE-Cy5.5	100	Caltag
CD25 (IL-2Rα)	7D4	Rat IgM	FITC	100	BD biosciences
CD25 (IL-2Rα)	PC61	Rat IgG1	PE or APC_y	200	BD biosciences
CD28	E18	Mouse IgG2b	unconjugated	x	EXBIO
CD28	E18	Mouse IgG2b	FITC	25	AbD Serotec
CD40	3/23	Rat IgG2a	PE	100	BioLegend
CD40	3/23	Rat IgG2a	FITC or PE	100	BD biosciences
CD45R (B220)	RA-3-6B2	Rat IgG2a	PE or Alexa-647	100	BD biosciences
CD69	H1.2F3	Hamster IgG	PE	100	BD biosciences
CD80 (B7.1)	16-10A1	Hamster IgG2	FITC or PE	100	BD biosciences
CD86 (B7.2)	GL1	Rat IgG2a	FITC or PE	100	BD biosciences
CD86 (B7.2)	GL1	Rat IgG2a	FITC	100	eBioscience
CD117 (c-Kit)	2B8	Rat IgG2b	PE	100	BD biosciences
CD152 (CTLA-4)	UC10-4F10-11	Hamster IgG1	PE	100	BD biosciences
CD152 (CTLA-4)	UC10-4F10-11	Hamster IgG1	unconjugated	x	BioXCell
CD172a (SIRP-α)	P84	Rat IgG2a	FITC	100	BD biosciences
CD197 (CCR7)	4B12	RatIgG2a	biotinylated	50	BioLegend eBioscience
CD223 (LAG3)	eBioC9B7W	Rat IgG1	PE	100	eBioscience
CD273 (PD-L2)	TY25	Rat IgG1	PE	100	BD biosciences
CD274 (PD-L1)	MIH5	Rat IgG2a	PE	100	BD biosciences
CD275 (ICOS-L)	HK-5.3	Rat IgG2a	PE	100	eBioscience
CD279 (PD-1)	J43	Hamster IgG	PE	100	eBioscience
DO11.10 TCR	KJ1-26	Mouse IgG2a	TRI-COLOR	100	Caltag
Foxp3	150D	Mouse IgG1	PE	100	BioLegend
Foxp3	FJK-16s	Rat IgG2a	PE or APC_y	100	eBioscience
I-A/I-E (MHC II)	M5/114.15.2	Rat IgG2b	PE	250	BD biosciences eBioscience
LAP (TGF-β1-associated)	27232	Mouse IgG1	PE	2.5µl/test (1mg/ml)	R&D Systems
Phospho-NFkB p65 (Ser536)	93H1	Rabbit IgG	unconjugated	100	Cell Signaling
Phospho-RelB (Ser552)	polyclonal	Rabbit IgG	unconjugated	100	Cell Signaling
Rel-B	C-19	Rabbit IgG	unconjugated	100	Santa Cruz
Vβ 5.1, 5.2 TCR	MR9-4	Mouse IgG1	biotinylated or FITC	100	BD biosciences

Table 3. List of antibodies directed against surface markers, intracellular markers or transcription factors
NA/LE = no azide low endotoxin (FITC = Fluorescein Isothiocyanate, PE = Phycoerythrin, APC_y = Allophycocyanin, PerCP = Peridinin Chlorophyll Protein Complex)

2.1.3.2 Primary antibodies directed against cytokines

Antigen	Clone	Isotype	Conjugate	Dilution (fold)	Company
IL-2	JES6-5H4	Rat IgG2a	PE	100	BioLegend
IL-4	11B11	Rat IgG1	PE or APC_y	200	BD biosciences
IL-5	TRFK5	Rat IgG1	PE	200	eBioscience
IL-9	RM9A4	Rat IgG1	PE	100	BioLegend
IL-10	JES5-16E3	Rat IgG2b	PE	100	eBioscience
IL-13	eBio13A	Rat IgG1	PE	100	eBioscience
IL-17A	TC11-18H10.1	Rat IgG1	PE	100	BD biosciences
IL-17A	eBio17B7	Rat IgG2a	PerCP-Cy5.5	200	eBioscience
IFN-γ	XMG1.2	Rat IgG1	FITC or PE	200	BD biosciences
IFN-γ	XMG1.2	Rat IgG1	FITC	200	eBioscience

Table 4. List of antibodies directed against cytokines
(FITC = Fluorescein Isothiocyanate, PE = Phycoerythrin, APC_y = Allophycocyanin, PerCP = Peridinin Chlorophyll Protein Complex)

2.1.3.3 Secondary antibodies or reagents

Antigen	Host	Conjugate	Dilution (fold)	Company
F(ab')2 anti mouse IgG (H+L)	donkey	FITC	100	J.I.R.
F(ab')2 anti rat IgG (H+L)	donkey	FITC	100	J.I.R.
F(ab')2 anti rabbit IgG (H+L)	goat	FITC	100	J.I.R.
Streptavidin	x	FITC or Alexa 488 or PE or APC_y or PE-Cy5	300	BD biosciences

Table 5. List of secondary antibodies or conjugates
(FITC = Fluorescein Isothiocyanate, PE = Phycoerythrin, APC_y = Allophycocyanin, PerCP = Peridinin Chlorophyll Protein Complex)

2.1.4 Buffers, media and solutions

For preparation of buffers and solutions, ultrapure Milli-Q water was obtained from Milli-Q water purification systems (Millipore, Schwalbach/Ts, Germany).

Buffers	Composition
PBS buffer (phosphate-buffered saline), pH7.4	0.2g KCl 8.0 g NaCl 0.2 g KH_2PO_4 1.15 g Na_2HPO_4 Fill up to 1l Milli-Q water
RPMI 1640 complete medium	500ml RPMI 1640 (PAA, Paching Austria) 10% heat-inactivated, sterile filtered FCS (PAA) 100U/ml penicillin (PAA) 100µg/ml streptomycin (PAA) 2mM L-glutamin (PAA) 50mM β-mercaptoethanol (Sigma-Adrich)

HL1 complete medium	500ml HL1 (Lonza, Verviers Belgium) 100U/ml penicillin (PAA) 100µg/ml streptomycin (PAA) 2mM L-glutamin (PAA) 50mM β-mercaptoethanol (Sigma-Adrich)
FACS buffer	500ml PBS 0.1% BSA (Roth) 0.1% NaN_3 (Roth)
Fixation buffer (2% Formaldehyde)	35ml PBS 2ml Formaldehyde (37%) (Roth)
Perm buffer	PBS 0.1% BSA (Roth) 0.1% NaN_3 (Roth) 0.5% Saponin (Sigma-Aldrich)
MACS Buffer	PBS 0.5% FCS (PAA) 2mM EDTA (Applichem)
EasySep buffer	2% FCS (PAA) in PBS
ELISA coating buffer *0.2M Sodium Phosphate (pH6.5)*	2.95 g Na_2HPO_4 5.05 g $NaH_2PO_4*2H_2O$ Fill up to 250ml and adjust pH to 6.5
ELISA coating buffer *0.1M Sodium Carbonate (pH9.5)*	2.1 g $NaHCO_3$ 0.89 g Na_2CO_3 Fill up to 250ml and adjust pH to 9.5
ELISA coating buffer *0.05M Sodium Carbonate (pH9.7)*	1.05 g $NaHCO_3$ 1.32 g Na_2CO_3 Fill up to 500ml and adjust pH to 9.7
ELISA coating buffer *0.1M Sodium Carbonate (Isotype asthma)*	4.20 g $NaHCO_3$ Fill up to 500ml and adjust pH to 9.5
ELISA wash buffer	0.05% Tween 20 (Applichem) in PBS
ELISA assay diluent	10% FCS (PAA) in PBS
TBST wash buffer	20mM Tris-HCl (pH 7.6) 150mM NaCl 0.05% Tween 20 (Applichem)
Freezing medium	90% (v/v) FCS (PAA) 10% (v/v) DMSO (Sigma-Aldrich)
DEPC-treated water	Milli-Q water 0.1% (v/v) DEPC (Roth) overnight continuous shaking followed by autoclaving
Tris-acetate-EDTA (TAE) buffer (50x)	242g Tris 57.1ml Acetic acid 100ml (0.5M) EDTA Fill up to 1l DEPC-treated water

Table 6. List of buffers and media

2.2 Laboratory equipment

Instruments	Purchased from
1450 Microbeta counter Wallac-Trilux	PerkinElmer (Rodgau, Germany)
96 Plate washer	Tecan Group (Maennedorf, Switzerland)
Axiostar plus microscope	Carl Zeiss MicroImaging (Goettingen, Germany)
Axiovert 40C inverted microscope	Carl Zeiss MicroImaging (Goettingen, Germany)
Bio-Rad iCycler iQ real time PCR system	Bio-Rad Laboratories (Muenchen, Germany)
CKX31 inverted microscope	Olympus
Confocal microscope system Leica TCS SP2	Leica Microsystems (Wetzlar, Germany)
Confocal microscope: LSM 510 Meta laser scanning system & Axiovert 200M inverted microscope	Carl Zeiss MicroImaging (Goettingen, Germany)
Cytospin Universal centrifuge	Hettich (Tuttlingen, Germany)
FACScan, FACScalibur or LSRII	BD Biosciences (Heidelberg, Germany)
Gelaire BSB 4A Class II Biological Safety Cabinet	Gelaire Flow Laboratories (Meckenheim, Germany)
HeraCell 240 CO_2 Incubator	Thermo Scientific (Langenselbold, Germany)
Heraeus Fresco 21 centrifuge (Heraeus rotor #75003424, max 14800rpm)	Thermo Scientific (Langenselbold, Germany)
Heraeus Megafuge 1.0R Centrifuge (Megafuge rotor #2704, max 5000rpm)	Thermo Scientific (Langenselbold, Germany)
Heraeus Multifuge® 3S+ Centrifuge (Sorvall rotor #75006445, max 4600rpm)	Thermo Scientific (Langenselbold, Germany)
Heraeus Multifuge® 3SR+ Centrifuge (Sorvall rotor #75006445, max 4600rpm)	Thermo Scientific (Langenselbold, Germany)
MSC-Advantage 1.2 Class II Biological Safety Cabinet	Thermo Scientific (Langenselbold, Germany)
MSC-Advantage 1.8 Class II Biological Safety Cabinet	Thermo Scientific (Langenselbold, Germany)
Nanodrop ND-2000 Spectrophotometer (Software idem)	Peqlab Biotechnologie (Erlangen, Germany)
PeqSTAR 96 Universal Gradient	Peqlab Biotechnologie (Erlangen, Germany)
Scotsman AF-80 Ice Flaker	Noras Röntgen & Medizintechnik (Wuerzburg, Germany)
Thermomixer Comfort	Eppendorf (Hamburg, Germany)
Tomtec 96 well harvester	PerkinElmer (Rodgau, Germany)
Ultrospec 2000 spectrophotometer	Pharmacia Biotech
Vmax kinetic microplate reader (Software: SOFTmax® PRO 3.0)	Molecular Devices (Ismaning, Germany)
Wallac Heat Sealer (1295-012)	PerkinElmer (Rodgau, Germany)
Wallac Light Box (1295-013)	PerkinElmer (Rodgau, Germany)

Table 7. List of instruments and equipment

2.3 Mice

C57BL/6 and BALB/c mice were bred in our own animal facilities or purchased from Harlan. OT-II mice (C57BL/6 background, F. Carbone, Melbourne), DO11.10 TCR-transgenic mice (BALB/c background, generated by K. Murphy, New York), IL-$6^{-/-}$ mice (C57BL/6 background, generated and provided by M. Kopf, Zuerich), C57BL/6-Tg(CAG-OVA)916Jen/J mice (here referred to as Act-mOVA mice) (C57BL/6 background, generated by M. Jenkins, Minnesota and kindly provided by A. Beilhack, Wuerzburg), TLR4-mutated C3H/HeJ (JAX mice) and TLR4/MyD88$^{-/-}$ mice (on a 129Sv x C3H/HeN genetic background, originally generated by S. Akira, Osaka and provided by A. Gessner, Erlangen) were all bred under specific pathogen-free conditions. All animal experiments were performed in accordance of the guidelines of the local authorities.

2.4 Primary cellular techniques

2.4.1 Handling of cells

All cell culture handlings were performed in a sterile environment using MSC-Advantage 1.8 or 1.2 Class II biological safety cabinets (Thermo Scientific, Langenselbold, Germany). Centrifugation of cells for cell culture purpose was performed by using a Heraeus Multifuge® 3S+ Centrifuge (Sorvall rotor #75006445, max 4600rpm) at room temperature or a Heraeus Megafuge 1.0R Centrifuge (Megafuge rotor #2704, max 5000rpm) at 4°C (Thermo Scientific). For centrifugation of cells for flow cytometry a Heraeus Multifuge® 3SR+ Centrifuge (Sorvall rotor #75006445, max 4600rpm) was used (Thermo Scientific). Cells were cultured under standard conditions at 37°C and 5% CO_2 in a water-reservoir containing HeraCell 240 CO_2 incubator (Thermo Scientific).

2.4.2 Freezing and thawing of cells

Cells were kept on ice during the whole freezing preparation procedure. Briefly, about 5 to 10 x 10^6 cells were centrifuged by 1000rpm for 10 minutes at 4°C and resulting cell pellet was resuspended in 1ml ice-cold freezing medium and transferred to cryo vials (Greiner Bio-One, Frickenhausen, Germany). Cells were slowly adapted to -80°C using an isopropanol-filled

freezing container (Mr. Frosty, Nalgene Labware, Roskilde, Denmark) before Nunc cryo vials were transferred and stored in liquid nitrogen tanks. For thawing of cells, cryo vials were warmed directly from the liquid nitrogen to 37°C in a water bath by shaking. Then, thawed cells were quickly transferred to a 15ml centrifuge tube (Greiner Bio-One) containing 10 pre-warmed complete RPMI 1640 medium. To remove remaining DMSO, cells were washed once by centrifugation on 1200rpm for 5 minutes, resuspended in 10ml warmed complete RPMI 1640 medium and left overnight in a T25 cell culture flask (Greiner Bio-One) in a 37°C incubator for recovery.

2.4.3 Counting cells

To determine cell concentration, 5 to 10µl of a cell suspension was diluted in a trypan blue solution (Sigma-Aldrich, Deisenhofen, Germany) for visualisation of non-viable cells and counted by using a Neubauer counting chamber (Hartenstein, Wuerzburg, Germany) and a Axiostar plus microscope (with A-Plan objective 10x/0.25 Ph1**,** Carl Zeiss MicroImaging, Goettingen, Germany). Cell concentration was determined using the following formula: [(number live cells large quadrant 1 + number live cells opposing large quadrant 2) \ 2] *dilution *10^4 = cells/ml

2.4.4 Generation of GM-CSF cell supernatant

For the generation of GM-CSF derived DC, culture supernatant was sampled from a murine GM-CSF transfected X63-Ag8.653 myeloma cell line kindly provided by B. Stockinger (London, UK) [245]. Briefly, GM-CSF transfected cell line was thawed according to standard procedure and left for 2 days in a T75 cell culture flask (Greiner Bio-One, Frickenhausen, Germany). Then, 10^7 cells were harvested and transferred to a T182 cell culture flask (Greiner Bio-One) in ca. 90ml complete RPMI 1640 (PAA, Pasching, Austria). After 3-4 days, cell line reached cell growth confluence of ca. 90% in T182 cell culture flask after which culture supernatant was harvested and centrifuged by 1000rpm for 10 minutes. Adherent cells were provided with fresh complete RPMI 1640 (PAA) for 2 days prior to de novo supernatant harvesting. Procedure was repeated 3-4 times before cell line was discarded according to standard GMO-guidelines. Harvested culture supernatant was sterile-filtered with Minisart®

syringe filters (Sartorius Stedim Biotech, Goettingen, Germany) before usage in generation of bone-marrow derived DC.

2.4.5 Isolation of bone marrow (BM) cells

BM-DC were generated as previously described [34]. Briefly, hind limbs were removed from 4 to 12 week-old mice and bones were relieved from surrounding muscle tissue by rubbing with unsterile paper tissues. Then, intact bones were soaked for 1-2min in sterile 10cm petri dishes (#664102, Greiner Bio-One, Frickenhausen, Germany) filled with an ethanol-propanol solution (Terralin® liquid, Schülke & Mayr, Norderstedt, Germany) for disinfection. Remaining alcohol was evaporated by air. A minimal fraction of both ends of the tibiae or femurs was cut by scissors and bone marrow was flushed out with a PBS-filled sterile 10ml tuberculin syringe (Pentaferte, Campli, Italy) using a Neoject® 27G or 0.40mm diameter needle (Dispomed Witt, Gelnhausen, Germany). Bone marrow was washed once by centrifugation by 1000rpm for 10 minutes and clusters of the BM cell suspension was disrupted by vigorous pipetting. About 5 to 7 x 10^7 BM cells could be obtained from one mouse.

2.4.6 Generation of GM-CSF derived BM-DC

At day 0, BM cells were seeded at 3 x 10^6 in sterile 10cm petri dishes (#664102, Greiner Bio-One, Frickenhausen, Germany) containing 10ml complete RPMI 1640 medium supplemented with 10% culture supernatant from a murine GM-CSF transfected X63-Ag8.653 myeloma cell line as described in previous section [245]. At day 3, another 10ml complete RPMI 1640 medium containing 10% GM-CSF culture supernatant was added to the plates. At day 6, BM cells were fed by gently replacing 10ml old RPMI 1640 medium with 10ml fresh complete RPMI 1640 medium containing 10% GM-CSF culture supernatant. At day 7 or 8, cells were used for analysis. Procedure typically yields 60-80% $CD11c^{high}$ expressing cells as determined by flow cytometry.

2.4.7 Generation of Flt3-L cultured BM-DC

Bone marrow precursors were isolated as described in previous section and seeded out at 3 x 10^6 cells per ml in T25 cell culture flasks (Greiner Bio-One, Frickenhausen, Germany). Cells were cultured in 10ml complete RPMI 1640 medium supplemented with 100 to 300ng/ml human recombinant Flt3-L (Peprotech, Hamburg, Germany). No additional medium supplements were added during 8 days of culture. At day 7 or day 8, Flt3-L DC were harvested and replated at 10^6 cells per ml in a 24-well plate (Greiner Bio-One) for maturation or phagocytosis analysis. In some experiments, Flt3-L DC were further purified into subsets by MACS® technology using anti-B220 and anti-CD11b magnetic-bead coupled antibodies and proceeded according to manufacturer's instructions (Miltenyi Biotech, Bergisch-Gladbach, Germany).

2.4.8 Generation of single cell suspension from thymus, spleen and lymph nodes

Organs were isolated from 4-8 week old mice under sterile conditions and transferred in 5cm petri dishes (Greiner Bio-One, Frickenhausen, Germany) containing ice-cold sterile PBS. A single cell suspension was obtained by mashing organs between rough ends of sterile microscopic glass slides (Hartenstein, Wuerzburg, Germany). Then, cell suspension was filtered through a 0.70µm nylon cell strainer (BD Biosciences, Heidelberg, Germany) positioned on a 50ml centrifuge tube (Greiner Bio-One) and washed once by centrifugation on 1000rpm for 10 minutes at 4°C. For spleen cell suspension, an erythrocyte lysis was performed by resuspending cell pellet in a 1:1 solution of PBS and 1.67% ammonium chloride (NH_4Cl) buffer followed by incubation for 5 minutes in 37°C pre-warmed water bath. To remove ammonium chloride, splenocytes were washed by two consecutive rounds of centrifugation for 5 minutes at 1200rpm at 4°C prior to determination of cell count number as described in previous section.

2.4.9 Isolation of $CD4^+$ T cells

For the isolation of $CD4^+$ T cells, lymph nodes and spleens from 8-12 week old C57BL/6, OT-II or DO11.10 TCR-transgenic mice were isolated and a single cell suspension prepared as described in previous section. $CD4^+$ T cells were purified using the Easysep® mouse $CD4^+$

T cell enrichment kit (Stemcell Technologies, Grenoble, France) according to the manufacturer's instruction. Briefly, cells were adjusted to 10^8 cells per ml in PBS containing 2% FCS and supplemented with 5% normal rat serum and transferred to a 5ml BD Falcon® round-bottom tube with cap (#352058, BD Biosciences, Heidelberg, Germany). Then, 50µl of the $CD4^+$ T cell enrichment cocktail consisting of antibodies directed against mouse CD8, CD11b, CD19, CD45R, CD49b and TER119 was added per ml of cells. After 15 minutes of incubation at 4°C, 100µl of the biotin selection cocktail was added directly to the cell suspension and incubated for 15 minutes at 4°C. Magnetic nanoparticles were thoroughly mixed by pipetting and added at 50µl per ml of cells followed by incubation for 15 minutes at 4°C. The total volume of cells was brought up to 2.5ml with sterile PBS supplemented with 2% FCS and the tube was set aside into a pre-cooled purple Easysep® magnet for 5 minutes. Finally, the $CD4^+$ enriched fraction was poured off in a 15ml centrifuge tube (Greiner Bio-One, Frickenhausen, Germany) by inverting the magnet. Procedure of magnetic separation was repeated once to increase purity of enriched $CD4^+$ T cells (more than 90% CD4 positive as determined by flow cytometry analysis).

2.4.10 Depletion of $CD4^+$ $CD25^+$ T cells

A $CD4^+$-enriched T cell population was purified as described in previous section. Depletion of $CD4^+$ $CD25^+$ T cells was performed using the MACS® CD25 microbead kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Briefly, concentration of $CD4^+$ enriched cells was adjusted to 10^7 cells per 100µl with ice-cold MACS buffer and cells were transferred to a 15ml centrifuge tube (Greiner Bio-One, Frickenhausen, Germany) prior to staining with 10µl per 100µl cells of anti-CD25 PE-conjugated antibodies (clone: 7D4; Miltenyi Biotech) for 10 minutes in the dark at 4°C. Cells were washed by adding 3ml of ice-cold MACS buffer followed by centrifugation on 1000rpm for 10 minutes at 4°C. Cell pellet was resuspended to 10^7 cells per 90µl with ice-cold MACS buffer supplemented with 10µl anti-PE microbeads (Miltenyi Biotech) and incubated for 15 minutes in the dark at 4°C. Cells were washed by adding 3ml of ice-cold MACS buffer followed by centrifugation on 1000rpm for 10 minutes at 4°C. A MS magnetic column (Miltenyi Biotech) able to positively select up to 10^7 labelled cells was set into a MiniMACS magnet (Miltenyi Biotech) and rinsed once with 500µl ice-cold MACS buffer. Cells were applied onto the

column in a total volume of 500µl MACS buffer followed by three consecutive washing steps of the MS column. Cells passing through the column were carefully collected, washed once by centrifugation on 1000rpm for 10 minutes at 4°C and cell number determined as described. Positively selected cells were poured off of the column by using the column plunger and cell numbers were determined by trypan blue exclusion. Procedure typically yields more than 90% $CD4^+$ $CD25^-$ enriched cells as determined by flow cytometry.

2.4.11 Generation of apoptotic cells

Thymocytes were isolated from 4-8 week old mice. A single cell suspension was generated as previously described. Cell concentration was adjusted to 10^7 cells per ml in ice-cold complete RPMI 1640 medium and transferred to a cryo vials (Greiner Bio-One, Frickenhausen, Germany) or 15ml centrifuge tubes (Greiner Bio-One) for irradiation in the 'Institut für Medizinische Strahlenkunde und Zellforschung' or Neurology Department respectively. Apoptosis was induced spontaneously by means of survival factor deprivation (indicated as 0Gy) or by 10Gy-irradiation using a Caesa Gammatron (Cs-137; Siemens, Germany) in the 'Institut für Medizinische Strahlenkunde und Zellforschung' of University of Wuerzburg or a Philips RT-250 Unit (Philips Medical systems, Hamburg, Germany) in department of Neurology of the University of Wuerzburg. For apoptosis kinetic studies, a pan-caspase inhibitor (Apoblock; BD Biosciences, Heidelberg, Germany) was added at a final concentration of 40µM prior to transport on ice to irradiation departments. For phagocytosis studies, thymocytes were labelled with PKH26 red fluorescence cell label (Sigma-Aldrich, Deisenhofen, Germany) or CFSE green fluorescence dye (Invitrogen, Darmstadt, Germany) as described in following sections prior to transport and irradiation. Apoptosis was determined using standard flow cytometry techniques as described in flow cytometry section.

2.5 Standard immunological/molecular techniques

2.5.1 Cytokine detection by ELISA

DC culture supernatants were analysed for their cytokine content by commercially available ELISA kits for IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 (BD Biosciences, Heidelberg, Germany), IL-23 (eBioscience, Frankfurt, Germany) and TGF-β1 (Promega, Mannheim, Germany). Supernatants of T cells were analysed for IL-2, IL-4, IFN-γ (BD Biosciences), IL-5, IL-13 and IL-17 (eBioscience). EIA/RIA 96-well Costar® plates (#3690, Corning Life Sciences, Amsterdam, The Netherlands) were used for coating of capture antibodies in respective coating buffer according to manufacturer's instructions. All washing steps were conducted by using an automated 96-plate washer (Tecan Group, Maennedorf, Switzerland) with ELISA wash buffer or TBST wash buffer (for TGF-β1 assay). Samples were stored at -20°C before use in ELISA assay. Acid treatment of samples to convert latent into active form of TGF-β1 was conducted by adding 1.25µl 1N HCL per 50µl sample to reach pH lower than 3 for 15 minutes at room temperature. Samples were neutralized to approximately pH 7.6 upon adding 1.25µl 1N NaOH per 50µl sample. Captured cytokines were detected by cytokine-specific biotinylated detection antibodies and streptavidin-horseradish peroxidase conjugates followed by the substrate 3,3',5,5'-Tetramethylbenzidine (TMB), derived as TMB substrate solution (eBioscience) or freshly prepared by mixing equal volumes of substrate reagent A and substrate reagent B (BD Biosciences). Absorbance was detected at 450nm using a Vmax kinetic microplate reader (Molecular Devices) and analysed by SOFTmax® PRO 3.0 Software (Molecular Devices, Ismaning, Germany).

2.5.2 Flow cytometry

2.5.2.1 Surface staining

Cells were stained in FACS buffer containing ice-cold PBS supplemented with 0.1% BSA and 0.1% sodium azide. To avoid unspecific antibody binding, cells were incubated with supernatant derived from the 2.4G2 hybridoma cell line (anti-Fc-gamma-RII/III; ATCC, Wesel, Germany) or medium supplemented with 10% FCS. Isotype control antibodies were used at the same concentration. Typically, 10^5 to 5 x 10^5 cells were stained in 50µl FACS buffer supplemented with antibodies directed against surface markers in a particular dilution

(see table) for 20-30 minutes at 4°C in the dark. To remove unbound antibodies, cells were washed once by centrifugation on 1200rpm for 5 minutes at 4°C. Samples were stored in 100µl of a 1:1 solution containing 1 part FACS buffer and 1 part 2% Formaldehyde until FACS analysis was performed. Samples were measured at a FACScan or FACScalibur flow cytometer (BD Biosciences, Heidelberg, Germany) provided with CellQuest software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, USA).

2.5.2.2 Intracellular cytokine staining

For intracellular cytokine detection, cells were stained for surface markers as described prior to fixation in a 2% formaldehyde solution during 20-30 minutes at 4°C in the dark. Then, cells were washed with FACS buffer by centrifugation on 1200rpm for 5 minutes at 4°C followed by permeabilisation in Perm Buffer containing 0.5% saponin (from Quillaja Bark; Sigma-Aldrich, Deisenhofen, Germany) during 20 minutes at 4°C in the dark. Staining of intracellular cytokines was performed in 50µl Perm buffer for 30 minutes at 4°C in the dark followed by washing with Perm buffer to remove unbound antibodies and analysis on flow cytometer.

2.5.2.3 Intracellular FoxP3 staining.

Intracellular FoxP3 was stained using the eBioscience® anti-mouse FoxP3 staining set (eBioscience, Frankfurt, Germany) according to the manufacturer's instructions. Briefly, 5-10 x 10^5 cells were stained for surface markers as described and washed by centrifugation. Cell pellet was resuspended in 100µl freshly prepared fixation/permeabilisation buffer for 1 to 2 hours at 4°C in the dark. Then, cells were washed with 200µl freshly prepared permeabilisation buffer (prepared from 10x permeabilisation concentrate in Milli-Q filtered water) followed by centrifugation on 1200rpm for 5 minutes at 4°C. To avoid unspecific binding, cells were stained in 25µl permeabilisation buffer supplemented with 2% mouse serum (Sigma-Aldrich, Deisenhofen, Germany) for 15 minutes at 4°C in the dark prior to addition of 25µl permeabilisation buffer containing 1:50 anti-FoxP3 antibodies for 30 minutes at 4°C. Cells were washed with permeabilisation buffer by centrifugation on 1200rpm for 5 minutes at 4°C and resuspended in FACS buffer for analysis on flow cytometer.

2.5.2.4 Intracellular NFkB transcription factor staining

Staining of (phosphorylated) NFkB transcription factor was performed by using the BD Cytofix fixation buffer and BD Phosflow perm buffer III (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions with minor modifications. Cells were stained for surface markers as described followed by immediate fixation in 100µl ice-cold Cytofix fixation buffer (BD Biosciences) for 30-60 minutes in the dark at 4°C. Cells were washed with normal FACS buffer by centrifugation on 1200rpm for 5 minutes at 4°C. The BD Phosflow perm buffer III (BD Biosciences) was cooled to -20°C for at least 1 hour before use. Then, cell pellet was vortexed to loosen cells and permeabilised by adding 100µl of cooled BD Phosflow perm buffer III (BD Biosciences) under continuous vortexing of cells. Permeabilisation was performed during 30-60 minutes in the dark at 4°C followed by vigorous washing and centrifugation on 1200rpm for 5 minutes at 4°C with normal ice-cold FACS buffer. Cells were stained intracellular in 100µl FACS buffer supplemented with antibodies directed against nuclear transcription factors (see table) during 30 minutes at 4°C in the dark. Cells were washed once by centrifugation and were ready for analysis on flow cytometer.

2.5.2.5 Apoptosis determination

Apoptosis was determined by Annexin-V-FITC (BD Biosciences, Heidelberg, Germany) and 7-AAD (BD Biosciences) staining according to manufacturer's instructions. Briefly, about 5 x 10^5 thymocytes were stained in 50µl 1x Annexin-V binding buffer (BD Biosciences) with Annexin-V-FITC (1:20 dilution) and 7-AAD (1:10 dilution) for 15 minutes in the dark at room temperature. Without washing, 200µl 1x Annexin-V binding buffer (BD Biosciences) was added directly to the cells followed by immediate analysis on FACScan flow cytometer (BD Biosciences). For intracellular active caspase-3 staining, thymocytes were fixed and permeabilised in 250µl Cytofix/Cytoperm buffer (BD Biosciences) for 20-30 minutes at 4°C in the dark followed by repetitive washing in Perm/Wash (BD Biosciences). Then, cells were stained in 100µl Perm/Wash buffer (BD buffer Biosciences) supplemented with anti-active caspase-3-PE (BD Biosciences) for 30 minutes at 4°C followed by washing and analysis on FACScan flow cytometer.

2.5.3 Generation of Fab antibody fragments

Fab antibody fragments were generated using the Pierce® Fab preparation kits (Thermo Scientific, Langenselbold, Germany) according to the manufacturer's instructions. Briefly, 4mg of unconjugated anti-mouse CTLA-4 antibodies (clone UC10-4F10-11 purchased from BioXCell, West-Lebanon, USA) or purified anti-mouse CD28 antibodies (clone E18 [246], purchased from EXBIO, Praha, Czech Republic) were concentrated to a volume of 500µl by using protein ultrafiltration concentrators (Sartorius Stedim Biotech, Goettingen, Germany). Then, antibodies were digested using immobilized papain under continuous mixing during 5 hours at 37°C. Fab antibody fragments were purified using protein A binding capacities. Quality of the generated Fab fragments was routinely tested by gel electrophoresis.

2.5.4 T cell proliferation by [^{3}H]-methyl-thymidine incorporation

Triplicates of 2 x 10^5 OVA-TCR specific T cells suspended in complete RPMI 1640 culture medium were seeded in flat-bottomed 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and restimulated with plate-bound αCD3 antibodies (BD Biosciences, Heidelberg, Germany; BioLegend, San Diego, USA or house-made), plate-bound αCD3 in addition of 5µg/ml αCD28 antibodies added in suspension (BD Biosciences or BioXell, West-Lebanon, USA), or 500U/ml recombinant human IL-2 (Novartis, Basel, Switzerland). Briefly, for generation of plate-bound αCD3 antibodies, 50µl of sterile PBS containing 5µg/ml αCD3 antibodies were transferred to wells of a flat-bottomed 96-well plate (Greiner Bio-One). Plate was coated for 2 hours at 37°C or, alternatively, overnight at 4°C. Then, the remaining solution was discarded and plate was washed once with ice-cold PBS before cell transfer. After 2 to 3 days of cell culture, cells were pulsed with 1µCi/well [^{3}H]-methyl-thymidine (Hartmann Analytic, Braunschweig, Germany) overnight for about 18 hours. Then, plates were harvested onto glass fiber filters (PerkinElmer, Rodgau, Germany) using a Tomtec 96-well harvester (PerkinElmer), sealed in plastic sample bags (PerkinElmer) using a Wallac heat sealer (1295-012; PerkinElmer), filled with Wallac scintillation fluid (PerkinElmer) and counted in 1450 Microbeta counter Wallac-Trilux (PerkinElmer). Counts were acquired with the MicroBeta Workstation software and further analysed with Microsoft Office Excel.

2.5.5 RNA extraction, cDNA synthesis and Real-Time PCR analysis

2.5.5.1 RNA extraction

Total RNA was extracted from DC or T cell lysates using TRIzol® reagent (Invitrogen, Darmstadt, Germany) and performed according to the manufacturer's instructions. Homogenized samples were stored at -80°C before RNA precipitation was performed. Phase separation of homogenized samples was introduced by addition of chloroform followed by centrifugation using a Heraeus Fresco 21 centrifuge (Thermo Scientific, Langenselbold, Germany). Then, RNA was precipitated with isopropyl alcohol. After washing of RNA with 75% ethanol solution, RNA pellet was dissolved in RNase-free 0.1% DEPC-treated water. RNA concentration was determined using spectrophotometry devices (Ultrospec 2000 spectrophotometer, Pharmacia Biotech or Nanodrop ND-2000 spectrophotometer, Peqlab Biotechnologie) following the manufacturer's instructions. Genomic DNA was removed from RNA samples by incubating 10µg extracted RNA with 10u DNase I (Fermentas, St. Leon-Rot, Germany) in the presence of 1x DNase I reaction buffer containing 2.5mM $MgCl_2$ and filled up to a reaction volume of 20µl with DEPC-treated water. Mixture was incubated for 15 minutes at room temperature. Enzyme reaction was inactivated by addition of 1µl of 50mM EDTA followed by heating at 60°C during 10 minutes. Concentration of DNase-treated RNA samples was determined as described. Quality of DNase-treated RNA preparation was routinely checked by gel electrophoresis in which 1µg of DNase-treated RNA was loaded on a 1% agarose gel in TAE-buffer containing 0.5µg/ml Ethidium Bromide run in a gel electrophoresis chamber filled with TAE-buffer. Cleaned-up RNA was stored at -80°C for long-term storage.

2.5.5.2 First-strand cDNA synthesis

cDNA was synthesized using Superscript® III Reverse Transcriptase (Invitrogen, Darmstadt, Germany) or BioScript™ Reverse Transciptase (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. Briefly, 1µg of DNase-treated RNA was added to a 0.2ml polymerase reaction tube (Biozym Scientific, Hessisch Oldendorf, Germany) in the presence of 1µl 0.5µg/µl oligo$(dT)_{18}$ (Fermentas, St. Leon-Rot, Germany) and 1µl 10mM dNTP mix (Fermentas) filled up by sterile deionised water to a volume of 13µl. Mixture was incubated for 5 min at 65°C followed by incubation on ice for at least 1 min. Then, a cDNA synthesis

mix was added containing the following components and mixed in indicated order: 4µl of 5x First-strand buffer containing 15mM $MgCl_2$ (Invitrogen), 1µl 0.1M DTT (Dithiothreitol; Invitrogen), 1µl (or 40u) RNaseOUT™ recombinant RNase Inhibitor (Invitrogen) or RiboLock™ RNase inhibitor (Fermentas) and 1µl (or 200u) Superscript® III Reverse Transcriptase (Invitrogen). Tubes were gently mixed and incubated at 50°C during 60min in a PeqSTAR 96 Universal Gradient PCR cycler (Peqlab Biotechnologie) prior to reaction termination at 70°C for 15min. Alternatively, for first-strand cDNA synthesis according to BioScript™ Reverse Transciptase instructions, 1µl 10mM dNTP mix (Fermentas) was added as a component of the cDNA synthesis mix in addition of 4µl of 5x reaction buffer (Bioline) and 200u BioScript™ Reverse Transciptase (Bioline). cDNA synthesis was performed at 45°C for 60min followed by inactivation at 70°C for 10min. cDNA samples were stored as aliquots at -20°C and used as template for real-time PCR performance.

2.5.5.3 Real-Time polymerase chain reaction (RT-PCR)

Real Time PCR was performed using a iCycler iQ system (Bio-Rad Laboratories, Muenchen, Germany). Quantitative expression of the Notch ligands Jagged1, Jagged2 and Delta4 was determined on cDNA derived from DC using primers previously described [77]. Primers for quantitative analysis of Egr2, Ebi3, Il12a, Gata3, Tbx21 and Nfil3 expression in T cells were synthesized according to previous publications [184, 247, 248]. Real-Time PCR was run for 40 cycles and performed in 25µl volume containing 0.5x Absolute QPCR SYBR Green mix (Thermo Scientific, Langenselbold, Germany) or DreamTaq™ Green PCR Master mix (Fermentas, St. Leon-Rot, Germany), 1µl of 1:10 diluted cDNA sample (prepared as described in previous section) and 0.2µM of each primer. Quantifications of the samples were determined by the ΔΔ cycle threshold (Ct) method. The housekeeping gene β-actin or β-2-microglobulin was used for normalization of the samples as indicated.

Primer	Sequence (5' to 3')
Actb Fwd	GAAGTCCCTCACCCTCCCAA
Actb Rev	GGCATGGACGCGACCA
B2m Fwd	GCTATCCAGAAAACCCCTCAA
B2m Rev	CGGGTGGAACTGTGTTACGT
Delta4 Fwd	AGGTGCCACTTCGGTTACACAG
Delta4 Rev	CAATCACACACTCGTTCCTCTCTTC
Ebi3 Fwd	AGCAGCAGCCTCCTAGCCT
Ebi3 Rev	ACGCCTTCCGGAGGGTC
Egr2 Fwd	GGCGGGAGATGGCATGAT
Egr2 Rev	CCCATGTAAGTGAAGGTCTGGT
Gata3 Fwd	AGAACCGGCCCCTTATCAA
Gata3 Rev	AGTTCGCGCAGGATGTCC
Il12a Fwd	TGGCTACTAGAGAGACTTCTTCCACAA
Il12a Rev	GCACAGGGTCATCATCAAAGAC
Jagged1 Fwd	AGAAGTCAGAGTTCAGAGGCGTCC
Jagged1 Rev	AGTAGAAGGCTGTCACCAAGCAAC
Jagged2 Fwd	AGCCACGGAGCAGTCATTTG
Jagged2 Rev	TCGGATTCCAGAGCAGATAGCG
Nfil3 Fwd	CAGTGCAGGTGACGAACATT
Nfil3 Rev	TTCCACCACACCTGTTTTGA
Tbx21 Fwd	CTAAGCAAGGACGGCGAATG
Tbx21 Rev	CACCAAGACCACATCCACAAA

Table 8. List of primer sequences used for Real-Time PCR.
All primer sequences were synthesized by Eurofins MWG Operon (Ebersberg, Germany) or Sigma-Aldrich (Deisenhofen, Germany) and oligo's were desalted and removed from truncated sequences by the manufacturer. (Fwd = forward primer, Rev = reverse primer)

2.6 Specialised immunological techniques

2.6.1 Maturation of BM-DC

At d7 or d8, BM-derived DC were harvested and re-plated at a density of 10^6 cells per ml in a 24-well plate (non-tissue culture treated; Greiner Bio-One, Frickenhausen, Germany). For maturation analysis by surface marker expression or cytokine production, BM-DC were cultured for 20-24 hours (unless otherwise indicated) in the presence of 500U/ml TNF (PeproTech, Hamburg, Germany), 0.1 µg/ml LPS (*E. coli* 0127:B8; Sigma-Aldrich, Deisenhofen, Germany), 2µg/ml sVSG or mfVSG from clone AnTat1.1 or 2µg/ml sVSG from clone MiTat1.5 (prepared and purified by B. Stijlemans, Brussels, Belgium as described

[249]). For *in vivo* polarization assays, BM-DC were seeded at a density of up to 5 x 10^6 cells per ml, matured for 4-6 hours only with different maturation stimuli and additionally loaded with 40µg/ml MOG_{35-55}-peptide (synthesized and HPLC purified by R. Volkmer, Charité, Berlin, Germany), 10µM OVA-$peptide_{327-339}$ (Activotec, Cambridge, UK) or 50-100µg/ml Endograde OVA protein (Hyglos, Regensburg, Germany) as indicated.

2.6.2 Maturation/Stimulation of BM-DC by apoptotic cells

Thymocytes were isolated and prepared as described in previous sections. After irradiation, thymocytes were immediately added to DC at a 5:1 ratio (thymocytes: DC) directly to the DC petri dishes or 24-well plates (Greiner Bio-One, Frickenhausen, Germany) for 20 to 24 hours unless otherwise indicated. For maturation analysis by surface marker expression or cytokine production, BM-derived DC were harvested at d7 or d8 and re-plated at a density of 10^6 cells per ml in a 24-well plate (Greiner Bio-One) in the presence of treated thymocytes. Addition of 0.1 µg/ml LPS (*E. coli* 0127:B8; Sigma-Aldrich, Deisenhofen, Germany) was performed 4-24 hours after start of thymocytes:DC co-culture as indicated.

2.6.3 Labelling and phagocytosis assay of apoptotic cells and DC

Thymocytes were labelled prior to irradiation with PKH26 red fluorescence cell label (Sigma-Aldrich) or CFSE green fluorescence dye (Invitrogen, Darmstadt, Germany) according to manufacturer's instructions. Briefly, cells were resuspended at 10^7 cells/ml in diluent C and PKH26 dye was added at a final concentration of 2µM. Cells were labelled for 5 minutes at room temperature in a light-protected environment followed by vigorous washing. Alternatively, cells were labelled with CFSE by adjusting to 2 x 10^7 cells/ml in PBS followed by CFSE addition in a final concentration of 3µM. Cells were labelled at room temperature for 10 min in a light-protected environment followed by extensive washing. For phagocytosis kinetic studies, 10^6 labelled irradiated thymocytes were added to 2 x 10^5 DC (5:1 ratio thymocytes: DC) in a sterile 5ml BD Falcon® round-bottom tube (#352058, BD Biosciences, Heidelberg, Germany) in a total volume of 300µl complete RPMI 1640 medium supplemented with 5µM Cytochalasin D (Sigma-Aldrich, Deisenhofen, Germany) when indicated. After 2, 3, 4 or 20 hours of incubation at 4°C or 37°C, cells were stained for surface

markers and stored in FACS buffer supplemented with 2mM EDTA for analysis on flow cytometer. For phagocytosis analysis by confocal microscopy, 10^6 DC were transferred to a 24-well plate (non-tissue culture treated; Greiner Bio-One, Frickenhausen, Germany) to which 2 x 10^6 labelled irradiated thymocytes (2:1 ratio thymocytes: DC) were added in a total volume of 1ml complete RPMI 1640 medium. After 20 hours of incubation at 37°C, cells were harvested and washed in PBS supplemented with 2% FCS. Then, 0.5 x 10^5 total cells were adhered onto poly-L-lysine pre-coated microscopic glass slides (Thermo Scientific, Langenselbold, Germany) for 1 hour at 37°C incubator. Fixation was performed with a 4% freshly prepared paraformaldehyde solution during 20 minutes at room temperature. Microscopic glass slides were washed with PBS, dried and mounted with Fluoromount-G containing DAPI (Serva Electrophoresis, Heidelberg, Germany) prior to analysis on a confocal laser-scanning microscope using a LSM 510 Meta laser scanning system based on an Axiovert 200M inverted microscope (Carl Zeiss MicroImaging, Goettingen, Germany).

2.6.4 *In vitro* regulatory T cell conversion assay

Regulatory T cell assays were set up as described with minor modifications [164]. Briefly, purified $CD4^+$ $CD25^-$ OT-II T cells (2 x 10^4) were cultured with day 8 BM-derived DC (6 x 10^3) matured with various maturation stimuli for 4-6 hours or with irradiated thymocytes for 20-24 hours prior to co-culture and 100ng/ml OVA-peptide$_{327-339}$ (Activotec, Cambridge, UK) in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany). Additional recombinant porcine TGF-β1 (R&D systems, Wiesbaden, Germany) was added to the culture at a concentration of 2ng/ml when indicated. Cultures were analysed on day 5 by flow cytometry staining of surface markers and the transcription factor FoxP3 as described above.

2.6.5 *In vitro* T cell proliferation assay

Purified $CD4^+$ $CD25^-$ OT-II T cells were labelled with CFSE by adjusting to 10^7 cells/ml in PBS followed by CFSE addition in a final concentration of 2µM. Cells were labelled at room temperature for 5-10 min in a light-protected environment followed by extensive washing. Then, 5 x 10^4 CFSE-labelled $CD4^+$ $CD25^-$ OT-II T cells were cultured with 10^5 day 8 BM-derived DC in presence of 100ng/ml OVA-peptide$_{327-339}$ (Activotec, Cambridge, UK) or

20µg/ml Endograde OVA protein (Hyglos, Regensburg, Germany) in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany). DC were matured with maturation stimuli for 4-6 hours or with irradiated thymocytes for 20-24 hours prior to start of co-culture as described in previous sections. Alternatively, 10µg/ml soluble CTLA-4 Ig (sCTLA-4 Ig) (Orencia®, Bristol-Myers Squibb, New York, US) was added to the culture when indicated. Cell division was analysed on flow cytometer 72 hours after start of culture. Division index was calculated as the mean number of divisions among cells, which divided at least once.

2.6.6 *In vitro* T cell priming for anergy induction

$CD4^+$ $CD25^-$ OT-II T cells (4×10^4) were cultured with day 8 BM-derived DC (1.2×10^4) matured with various stimuli for 4-6 hours in the presence of 100ng/ml OVA-peptide$_{327-339}$ (Activotec, Cambridge, UK) in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany). 5-7 days after start of culture, T cells were restimulated as triplicates of 2×10^5 cells with plate-bound αCD3 antibodies (BD Biosciences, Heidelberg, Germany; BioLegend, San Diego, USA or house-made), plate-bound αCD3 in addition of 5µg/ml αCD28 antibodies (BD Biosciences or BioXell, West-Lebanon, USA) or 500U/ml rhIL-2 (Novartis, Basel, Switzerland). 72h after restimulation, supernatant were harvested and analyzed for their cytokine content by ELISA or plates were pulsed with 1µCi/well [^{3}H]-methyl-thymidine (Hartmann Analytic, Braunschweig, Germany) for proliferation analysis. Intracellular cytokine detection was performed by restimulation with 0.01µg/ml PMA (Sigma-Aldrich, Deisenhofen, Germany) and 1µg/ml ionomycin calcium salt (Sigma-Aldrich) in the presence of 0.67µl/ml Golgistop (BD Biosciences) or 5µg/ml Brefeldin A (from Penicillium brefeldianu, Sigma-Aldrich) followed by standard intracellular staining procedures as described in flow cytometry section. Alternatively, 7 days after culture start-up, primed T cells were stimulated a second round by day 8 BM-derived DC in presence of 100ng/ml OVA-peptide$_{327-339}$ (Activotec) in 96-well round-bottom plates (Greiner Bio-One). During restimulation, 10µg/ml sCTLA-4 Ig (Orencia®, Bristol-Myers Squibb, New York, US), 30µg/ml anti-CTLA-4 Fab fragments (clone UC10-4F10-11) or 2.5 µg/ml anti-CD28-Fab fragments (clone E18 [246]) were added to the culture when indicated. After 5 days of restimulation by BM-derived DC, T cells functional assays were performed as described.

2.6.7 Suppression assay

Autologous spleen APC were prepared from spleens of C57BL/6 mice by incubation of culture supernatants containing anti-Thy1.2, anti-CD4 and anti-CD8 antibodies (clone T24131, GK1.5 and 53-6.7 respectively, kindly provided by I. Berberich) during 30 minutes on ice. Labelled cells were removed by incubation with baby rabbit complement (Biozol, Eching, Germany) under continuous shaking at 37°C during 45 minutes followed by removal of cellular debris by pouring suspension through a 0.70µm nylon cell strainer (BD Biosciences, Heidelberg, Germany). Cells were irradiated in the Neurology Department of the University of Wuerzburg for 20Gy using a Philips RT-250 Unit (Philips Medical systems, Hamburg, Germany). Irradiated splenic APC (2×10^5) were transferred as feeder cells to 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany). Then, $CD4^+$ $CD25^-$ T cells were isolated from lymph nodes of C57BL/6 mice as described in previous sections and labelled with CFSE by adjusting cell concentration to 10^7 cells/ml in PBS followed by addition of CFSE in a final concentration of 2µM. Cells were labelled at room temperature for 5-10 minutes in a light-protected environment followed by extensive washing. 5×10^4 CFSE-labelled $CD4^+$ $CD25^-$ T cells were transferred as responder T cells to 96-well round-bottom plates (Greiner Bio-One) in the presence of 1µg/ml purified αCD3 antibodies (BD Biosciences, BioLegend or house-made). To this culture, 10µg/ml αIL-10-receptor antibodies (clone 1B1.2; EXBIO, Praha, Czech Republic) or 5ng/ml rhIL-2 (Novartis, Basel, Switzerland) was supplemented when indicated. Primed OVA-TCR specific T cells or freshly isolated $CD4^+$ $CD25^+$ C57BL/6 T cells were added as suppressors in various ratio's to the responder cells. 72 hours after start of culture, suppression was analysed by CFSE dilution on flow cytometer. Division indices were calculated as the mean number of divisions among cells, which divided at least once.

2.6.8 *In vitro* T cell priming into T helper cell subsets

For T cell differentiation assays, purified $CD4^+CD25^-$ OT-II T cells (5×10^4) were cultured with day 8 BM-derived DC (10^4 to 10^5) and 50nM OVA-peptide$_{327\text{-}339}$ (Activotec, Cambridge, UK) in presence or absence of maturation stimuli during the entire culture period in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany). Cultures were restimulated on day 5 by 0.01µg/ml PMA (Sigma-Aldrich, Deisenhofen, Germany) and 1µg/ml ionomycin

calcium salt (from Streptomyces; Sigma-Aldrich) in the presence of 0.67µl/ml Golgistop (BD Biosciences, Heidelberg, Germany) or 5µg/ml Brefeldin A (from Penicillium brefeldianu, Sigma-Aldrich). Intracellular cytokines were determined by standard intracellular staining procedures described in flow cytometry section.

2.6.9 *In vivo* T cell proliferation and polarization in T helper cell subsets

Total spleens and lymph nodes were isolated from DO11.10 mice and labelled with CFSE (Invitrogen, Darmstadt, Germany) by adjusting cell number to 2 x 10^7 cells/ml in PBS and adding CFSE in a final concentration of 3µM. Cells were labelled at room temperature for 10 min followed by washing. Mice received 10^7 labelled cells injected in the tail vein in addition to 2-2.5 x 10^6 DC matured and loaded with OVA-peptide$_{327-339}$ (Activotec, , Cambridge, UK) as described in previous section. 96h after the final injection, CFSE dilution of splenocytes was analysed by flow cytometry. Division index was calculated as the mean number of divisions among cells, which divided at least once. For in vivo polarization assays, 10^6 purified $CD4^+$ $CD25^-$ OT-II or DO11.10 T cells were injected intravenously followed 24h later by injection of 2-2.5 x 10^6 DC matured and loaded with OVA-peptide$_{327-339}$ (Activotec). Transferred T cells were analysed for their cytokine content by restimulation of splenocytes 6 days after final injection with 10µM OVA-peptide$_{327-339}$ (Activotec) during 72h. Brefeldin A (5µg/ml; Sigma-Aldrich, Deisenhofen, Germany) was added during final 6h of restimulation followed by intracellular cytokine staining as described.

2.7 Animal models for autoimmunity

2.7.1 Experimental autoimmune encephalomyelitis (EAE)

EAE induction was performed as previously described [16]. Briefly, C57BL/6 mice were injected s.c. with 200µg MOG$_{35-55}$ peptide emulsified in Complete Freund's adjuvant (CFA; Sigma-Aldrich, Deisenhofen, Germany) further enriched with 5mg/ml Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, USA) by using Sterican® 23G or 0.60mm diameter needles (Braun, Melsungen, Germany) and a 1ml Injeft®-F syringe (Braun). Additionally, mice were injected with 400ng Pertussis toxin i.p. (List Biological Laboratories, Epsom, UK) at days 0 and 2 of EAE induction by using 1ml tuberculin syringes (Dispomed

Witt, Gelnhausen, Germany) and a Neoject® 27G or 0.40mm diameter needle (Dispomed Witt). Mice were scored daily for clinical disease symptoms according to the following scale: 0, no disease; 1, limp tail weakness; 2, hind limp weakness; 3, hind limp paralysis; 4, hind and fore limp paralysis; 5, moribund or death. DC were injected at days -7, -5 and -3 before EAE induction in the tail vein of mice and for a total of 2-2.5 x 10^6 DC. Thirty days after EAE-induction, spleens were removed for restimulation and seeded out as triplicates of 4 x 10^5 cells per well in complete serum-free HL1 medium in a flat-bottomed 96-well plate (Greiner Bio-One, Frickenhausen, Germany) in the presence of graded concentrations of MOG_{35-55} peptide. After 72h of restimulation, supernatant was harvested and analysed for its cytokine content by ELISA.

2.7.2 Asthma induction

BALB/c mice were sensitized by i.p. injections of 10µg Endograde OVA protein (Hyglos, Regensburg, Germany) mixed in aluminium hydroxide (Alu-Gel-S suspension, Serva Electrophoresis, Heidelberg, Germany) at day 0 and 14 of asthma induction. Mice treated as negative controls received injections of aluminium hydroxide only. DC were injected at day -7, -5 and -3 before asthma induction in the tail vein of mice and for a total of 2-2.5 x 10^6 cells. Then, mice were challenged by intranasal administrations of 100µg OVA protein in 50µl PBS at day 22, 23 and 24 of asthma induction. Six days after the last OVA challenge, mice were lethally anesthetized followed by bleeding of the axillary veins for serum immunoglobulin analysis. Blood was coagulated for 2h at room temperature and centrifuged on 3000g for 5min to recover the serum. Circulating OVA-specific IgG subclasses were determined by ELISA. For this, 96-well plates (#353279; BD Biosciences, Heidelberg, Germany) were coated overnight at 4°C with OVA protein (Sigma-Aldrich, Deisenhofen, Germany; 100µg/ml) in 0.1M $NaHCO_3$ coating buffer. Sera were loaded as serial dilutions in 1% FCS in PBS. OVA-bound antibodies in the sera were detected by horseradish peroxidase-conjugated anti-mouse heavy chain-specific antibodies: anti-mouse IgG1-HRP (AbD Serotec, Düsseldorf, Germany), or IgE-biotin and streptavidin-HRP (BD Biosciences) followed by the substrate tetramethylbenzidine (BD Biosciences). Absorbance was detected at 450nm using an ELISA microplate reader (Vmax; Molecular Devices, Ismaning, Germany). Serum titers were calculated from the serial dilution, which was 1.5-fold increase compared to baseline (optical

density of negative control mice). Broncho-alveolar lavage (BAL) was performed by flushing the lungs through an opening in the trachea with PBS from a syringe. Differential cell count of the BAL was determined by recording total cell amount and spinning cells on microscope glass slides using a Cytospin Universal centrifuge (Hettich, Tuttlingen, Germany). Cytospins were stained with hematoxylin-eosin solution (Diff-Quick staining set; Medion Diagnostic, Langen, Germany) and cells were classified using standard morphologic criteria.

2.8 Statistical analysis

Data are represented as mean data ± SD. Two group comparisons were tested for statistical significance using two-tailed Student's t test and significance accepted if $p < 0.05$. For comparison of more than two groups, statistical significance was analysed with GraphPad Prism software using one-way ANOVA followed by Bonferroni post-testing and significance accepted if $p < 0.05$. Data of EAE and asthma experiments were validated with GraphPad Prism software using Kruskal-Wallis test followed by Dunn's post-test if $p < 0.05$.

3 RESULTS

3.1 Apoptotic cells do not increase tolerance features of DC *in vitro*

3.1.1 Irradiated thymocytes serve as a source of apoptotic self-antigens

Previously our group and others showed that DC induce FoxP3$^+$ regulatory T cells in the steady state by self-antigen presentation *in vivo* [19, 164]. To address whether self-antigens sampled by DC program tolerogenic features in DC, a self-antigen depot was established by inducing apoptosis in primary thymocytes followed by analysis of hallmarks of apoptotic cell death.

Irradiated thymocytes (10Gy) rapidly decreased total cellular volume reflected by means of reduced forward scatter (FSC) but increased cellular granularity indicated by sideward scatter (SSC) (fig 6A, upper row). In addition, irradiated thymocytes showed gradually increased binding to Annexin-V (45.16 ± 8.44 after 5h; Fig 6A, middle row and 6B upper panel) but remained predominately 7-AAD$^-$ up to 7h after irradiation (Fig 6A, middle row and 6B lower panel). Furthermore, irradiated thymocytes showed active caspase-3 activity (fig 6A, bottom row) which could be reverted upon addition of a pan-caspase inhibitor (fig 6A). Thymocytes which were left untreated (0Gy) showed only minor shrunken FSC/SSC profile, Annexin-V binding and caspase-3 activity and underwent apoptosis at a much lower intensity as irradiated counterparts (Fig 6A and B). Both spontaneous and induced apoptosis of thymocytes could be prevented by addition of a pan-caspase inhibitor (Fig 6A). All thymocytes were Annexin-V$^+$ 24h post-irradiation in contrast to non-treated thymocytes which showed only 37.6 ± 4.38 Annexin-V binding capacity (data not shown) after 24h incubation.

Together, thymocytes undergo rapid caspase-dependent apoptosis upon irradiation thereby generating early apoptotic, self-antigen rich material *in vitro.*

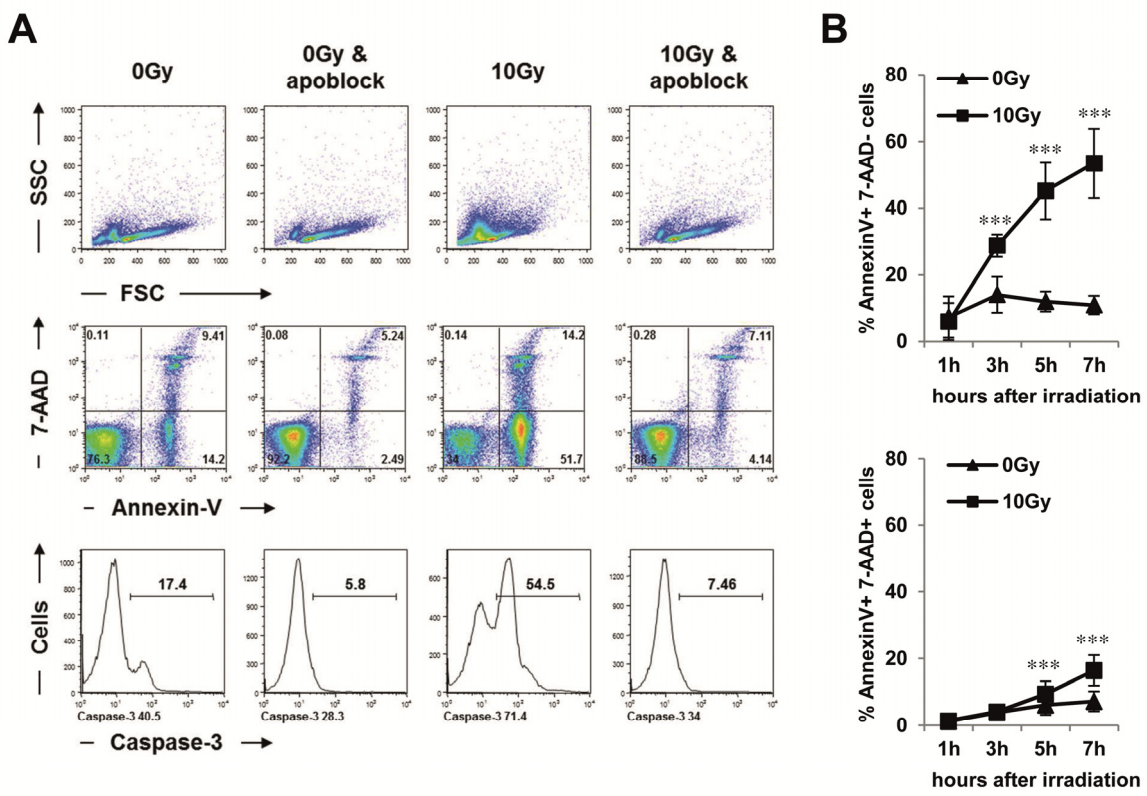


Figure 6. Thymocytes undergo caspase-associated apoptotic cell death
(A) Thymocytes isolated from C57BL/6 mice were irradiated (10Gy) or left untreated (0Gy) in the presence of a pan-caspase inhibitor (apoblock) when indicated and stained 5h after irradiation for apoptosis markers and tested by flow cytometry. The inserted numbers show percentage cells in a particular quadrant or histogram. Cells were not gated to include all cells. One representative experiment from 2 independent experiments is shown. (B) Thymocytes were irradiated (10Gy) or left untreated (0Gy) and stained for apoptosis markers 1 to 7h after irradiation. Figure shows kinetics of early apoptotic (Annexin-V^+ 7-AAD^-; upper panel) and late apoptotic (Annexin-V^+ 7-AAD^+; lower panel) cells as depicted in (A). Results are mean data from up to 7 independent experiments. Statistical significance: *** $p < 0.001$, two-tailed Student's t test

3.1.2 GM-CSF DC internalize cellular material from irradiated thymocytes

To analyse how GM-CSF DC interact with and/or perform phagocytosis of primary thymocytes, GM-CSF BM-derived DC were co-cultured with untreated or irradiated thymocytes and interaction analysed by flow cytometry and confocal microscopy.

Flow cytometry data indicate that DC cluster intensively with untreated or irradiated thymocytes (data not shown) even in the presence of EDTA and cytochalasin D, an inhibitor of actin polymerization (fig 7A). In addition, DC acquire $CFSE^{low}$ cellular material derived from untreated thymocytes (9.36%) or irradiated thymocytes (11.9%) which is reduced to background levels (3.34 or 3.55% respectively) in the presence of cytochalasin D (fig 7A, right panels). Confocal microscopy shows predominately interaction of DC and untreated thymocytes although internalization of thymocyte-derived material in this setting (i.e. at this

timepoint) also occurred (fig 7B, mid panel and insert). After 20h of co-culture, DC almost completely internalized apoptotic irradiated thymocytes as indicated by green remnants in the cytoplasm of DC (fig 7B, right panel and insert).

Taken together, GM-CSF DC show early interactions with primary thymocytes and gain cellular particles thereof irrespective of the apoptotic nature of this cellular source. In addition, these DC were able to internalize the apoptotic irradiated thymocytes efficiently by phagocytosis.

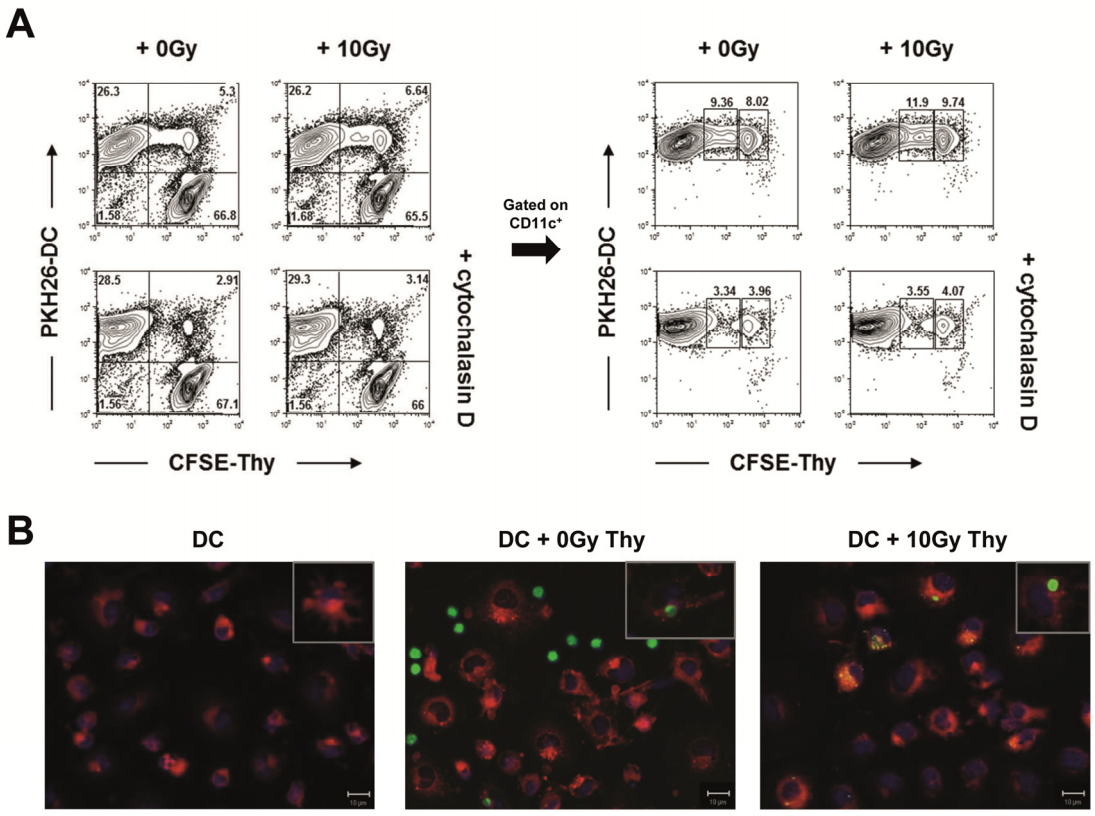


Figure 7. DC ingest particles from irradiated and non-irradiated thymocytes
(A) CFSE-labelled thymocytes were irradiated (10Gy) or left untreated (0Gy) and incubated with PKH26-labelled GM-CSF DC in presence of cytochalasin D when indicated. Interaction and/or phagocytosis by DC were measured by flow cytometry 4h after incubation start. Cells were not gated (left panels) or gated on CD11c^{high} expressing cells (right panels). The inserted numbers show percentage cells in a particular quadrant or gate. One representative experiment from 3 independent experiments is shown. (B) PKH26-labelled (red) GM-CSF DC (left panel) were incubated with CFSE-labelled (green) thymocytes which were left untreated (0Gy; mid panel) or irradiated (10Gy; right panel). Uptake of apoptotic material by DC was studied by confocal microscopy 20h after start of incubation. Nuclei were counterstained with DAPI. Inserts show cellular detail at 1.5x magnification.

3.1.3 Apoptotic cells do not modify surface marker expression of DC but block pro-inflammatory cytokine production

To address whether apoptotic thymocytes influence the maturation phenotype of DC, flow cytometry analysis of various surface marker expression was determined on conditioned GM-CSF DC.

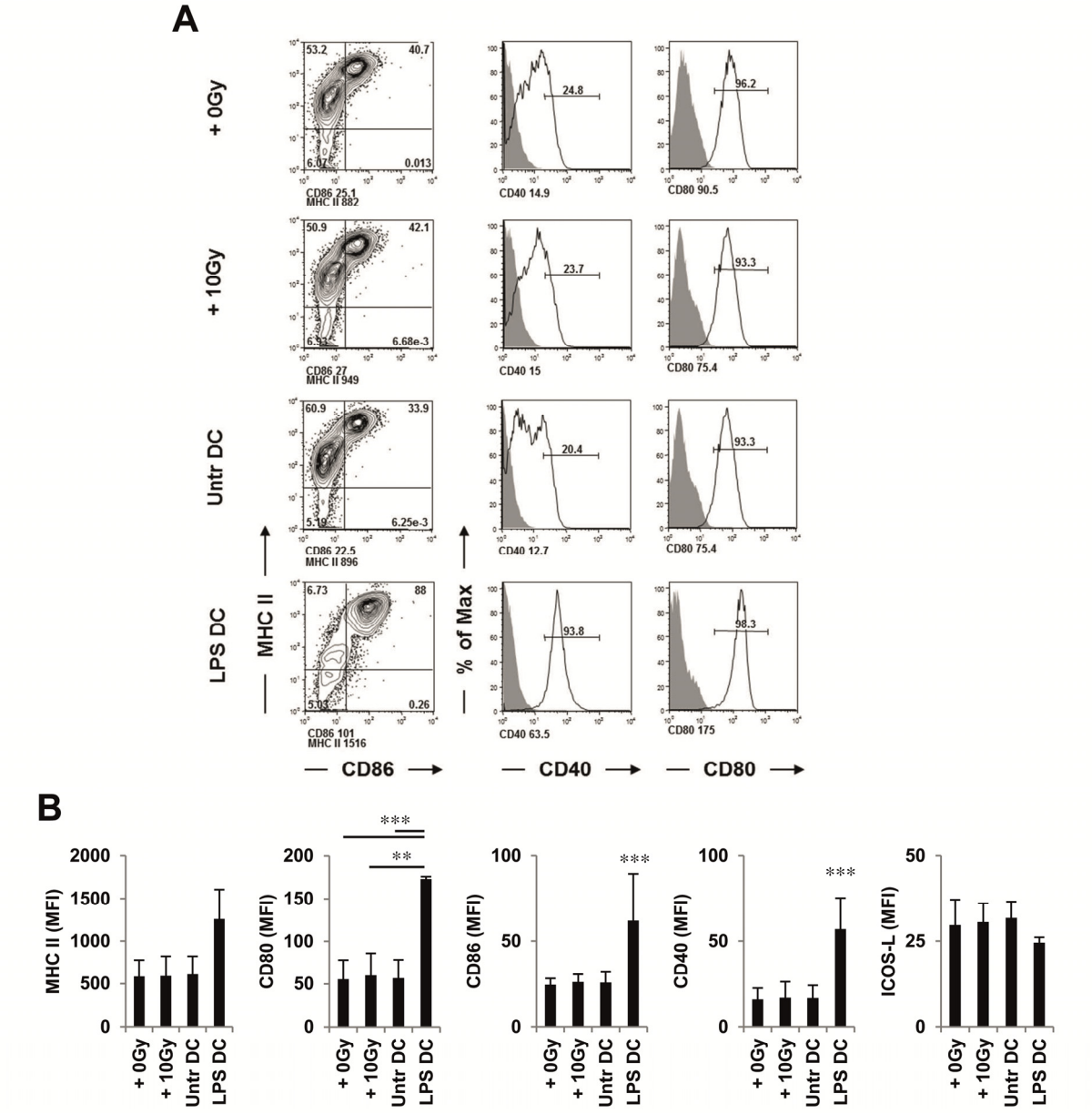


Figure 8. DC do not modify surface marker expression upon ingesting apoptotic cells.
(A) GM-CSF DC were incubated for 24h with irradiated (10Gy) or non-irradiated (0Gy) thymocytes or treated with the indicated stimuli, stained for surface markers expression and tested by flow cytometry. The inserted numbers show percentage cells in a particular quadrant or histogram. The shaded curves in the histogram plots represent isotype controls. Numbers under each plot indicate the MFI. Cells were all gated on CD11c^{high} expressing cells. (Untr = untreated) (B) DC were conditioned and analysed as in (A) but figures show MFI of up to 8 independent experiments. Cells were all gated on CD11c^{high} expressing cells. Statistical significance: ** $p < 0.01$ and *** $p < 0.001$, one-way ANOVA followed by Bonferroni post-test

DC cultured in the presence of untreated or irradiated thymocytes express MHC II, co-stimulatory molecules and the chemokine receptor CCR7 to a similar low extent as untreated DC (fig 8A and B and data not shown). Expression levels of MHC II and the co-stimulatory molecules were markedly below levels detected on LPS-conditioned DC (fig 8A and B), which typically show high surface expression of MHC II and co-stimulatory molecules but hardly modified expression of ICOS-L (fig 8B).

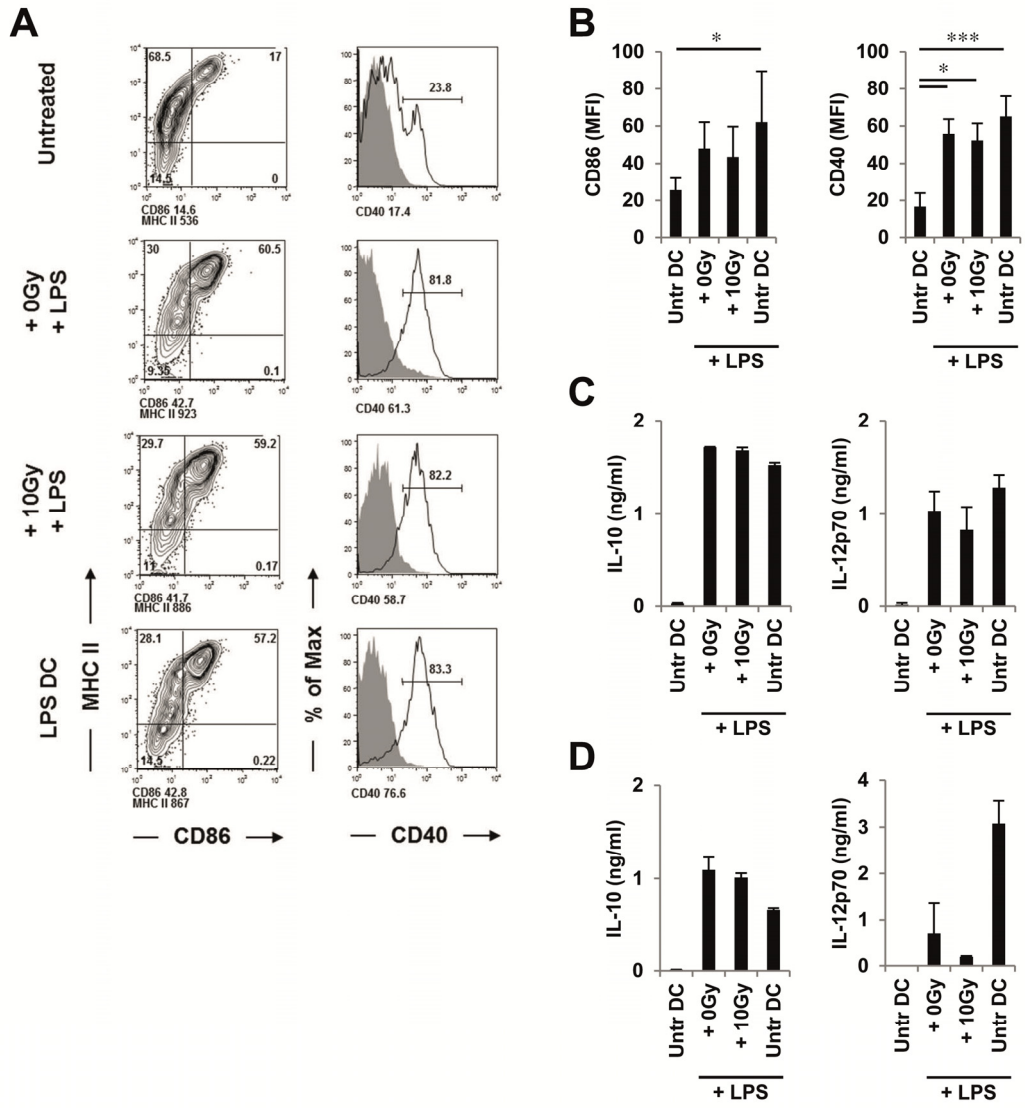


Figure 9. DC show partial maturation block in presence of apoptotic cell material
(A) GM-CSF DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy) for 4h prior to addition of LPS. Maturation of DC was analysed 24h after total incubation time by flow cytometry staining for indicated surface markers. The inserted numbers show percentage cells in a particular quadrant or histogram. The shaded curves in the histograms represent isotype controls. Numbers under each plot indicate MFI. Cells were all gated on $CD11c^{high}$ expressing cells. (B) DC were stimulated and analysed as in (A) but graphs represent MFI of up to 3 independent experiments. (C) Supernatants were harvested after 24h total incubation time and tested for their cytokine content by ELISA. One representative experiment is shown. (D) DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy) for 24h before LPS was added. After 48h total incubation time, supernatants were harvested and cytokine content was analysed by ELISA. One representative experiment is shown. Statistical significance: * $p < 0.05$ and *** $p < 0.001$, one-way ANOVA followed by Bonferroni post-test

Thus, internalization of apoptotic thymocytes does not induce differential expression of MHC II or co-stimulatory molecules on GM-CSF DC suggesting that DC maintain their immature phenotype upon encountering apoptotic self-antigens.

Pre-treatment of DC with thymocytes undergoing spontaneous or irradiation-induced apoptosis for 4h prior to a secondary challenge with LPS modestly decreased expression of CD86 and CD40 on DC (fig 9A and B), lowered secretion of pro-inflammatory IL-12p70 (fig 9C) and elevated anti-inflammatory IL-10 production (fig 9C) although results did not always reach statistical significance. Interestingly, pre-conditioning of DC with spontaneous or irradiation-induced apoptotic thymocytes for 24h completely blocked secretion of IL-12p70 and shifted cytokine production to enhanced IL-10 (fig 9D).

In sum, apoptotic thymocytes condition GM-CSF DC to prevent maturation when DC receive a second delayed TLR stimulus.

3.1.4 Regulatory cytokines are not induced in the presence of apoptotic cells

The role of the regulatory cytokines IL-10 and TGF-β in the induction of regulatory T cell function is well established [146, 164]. Furthermore, the alternative NFκB signalling pathway through RelB in DC might contribute to the development of T cells with regulatory properties [19, 250]. Here, untreated GM-CSF DC hardly expressed TGF-β1-associated latency-associated peptide (LAP) on their surface and expression levels could not be altered in the presence of apoptotic thymocytes or upon stimulation with LPS (fig 10A and B). No or only background levels of active TGF-β1 were detected in the supernatant of DC irrespective of the maturation regimen they received (data not shown). High total TGF-β1 levels were present in DC samples but levels remained unaltered upon stimulation with irradiated thymocytes or with LPS (fig 10C, left panel). The presence of irradiated thymocytes did not induce any IL-10 secretion from DC in contrast to LPS conditioning (fig 10C, right panel). Interestingly, the expression of intracellular RelB or phospho-RelB signalling molecules in DC was not altered upon co-culture with apoptotic thymocytes but DC showed higher RelB activity after LPS conditioning (fig 10D).

In summary, GM-CSF DC failed to induce LAP expression or TGF-β1 secretion *in vitro* irrespective of the presence of apoptotic cell material. The RelB-dependent alternative NFκB

signalling pathway did not play a significant role in the handling of apoptotic thymocytes by GM-CSF DC.

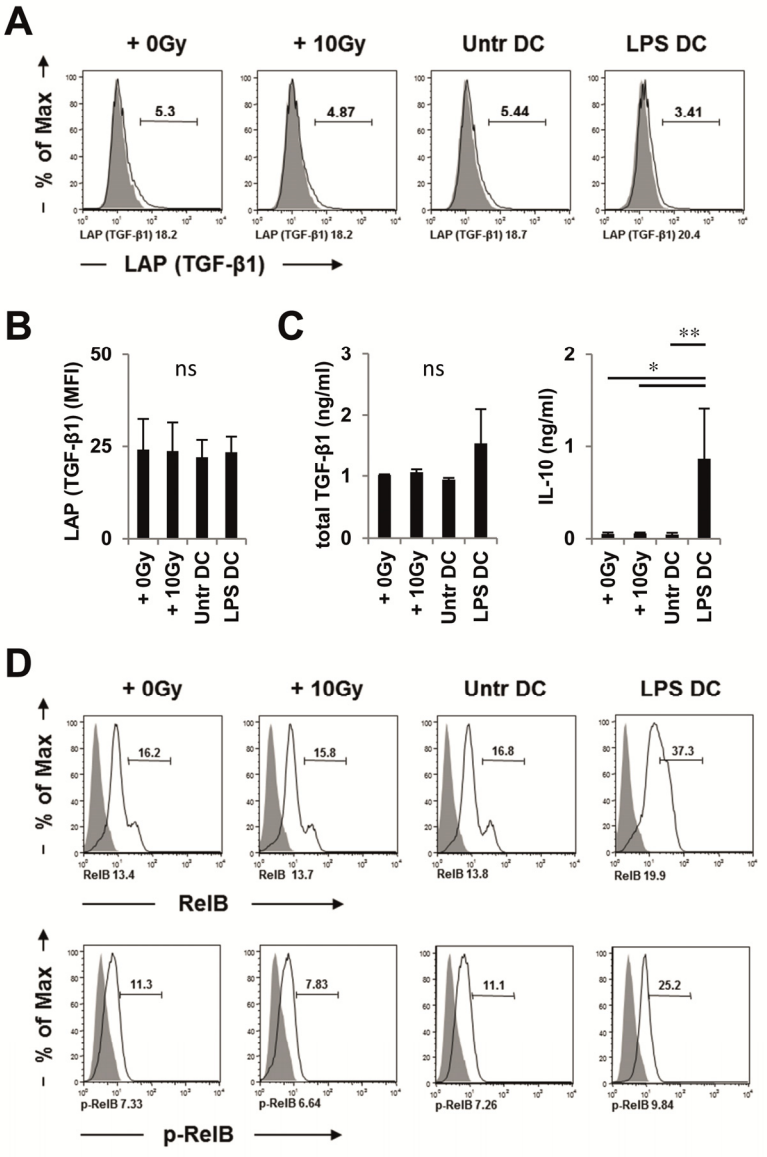


Figure 10. DC do not upregulate regulatory molecules in presence of apoptotic cells
(A) GM-CSF DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy) or DC were stimulated with LPS. Surface expression of TGF-β1-associated LAP was analysed after 24h by flow cytometry. Numbers below each histogram show MFI; numbers within the figures represent percentage cells. Cells were all gated on CD11c^{high} expressing cells. One representative experiment of 2 independent experiments is depicted. (B) as in (A) but graph represents MFI of 2 experiments performed. (C) as in (A) but after 24h, supernatants were harvested and cytokine content analysed by ELISA. Graphs show mean data from up to 3 different experiments. (D) DC were stimulated as in (A) but stained for intracellular RelB and phospho-RelB (p-RelB) 24h after incubation and analysed by flow cytometry. Cells were all gated on CD11c^{high} expressing cells. Shaded curves in histograms show unstained rat serum controls. One representative experiment of 4 is shown. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and ns = not significant, one-way ANOVA followed by Bonferroni post-test

3.1.5 Apoptotic thymocyte conditioning of DC does not favour induction of FoxP3$^+$ regulatory T cells *in vitro*

To address whether phagocytosis of apoptotic thymocytes by GM-CSF DC increased their capacity to induce FoxP3 expression in naïve OVA-TCR specific T cells, their conversion into regulatory T cells after DC co-culture was determined by flow cytometry.

In the absence of exogenous TGF-β, DC induced no FoxP3 expression in naïve CD4$^+$CD25$^-$ T cells independent of the maturation phenotype of the DC (fig 11A, upper panels and 11B).

However, upon addition of exogenous TGF-β, T cells showed high expression of FoxP3 in co-cultures with untreated DC (12.69 ± 2.79% $CD25^+$ $FoxP3^+$ cells) (fig 11A and B). Interestingly, DC did not further increase capacity to induce $FoxP3^+$ T cells upon pre-treatment with irradiated thymocytes (0Gy: 13.46 ± 1.89 and 10Gy: 14.11 ± 3.86 % $CD25^+$ $FoxP3^+$ cells, respectively) (fig 11B). LPS-conditioning induced lower conversion to $FoxP3^+$ regulatory T cells in the presence of exogenous TGF-β compared to untreated DC *in vitro* (fig 11A and B).

Taken together, DC are potent inducers of $FoxP3^+$ regulatory T cells *in vitro* in the presence of exogenously added TGF-β. Pre-conditioning of DC with apoptotic thymocytes did not modify the capacity to convert $FoxP3^+$ regulatory T cells *in vitro*.

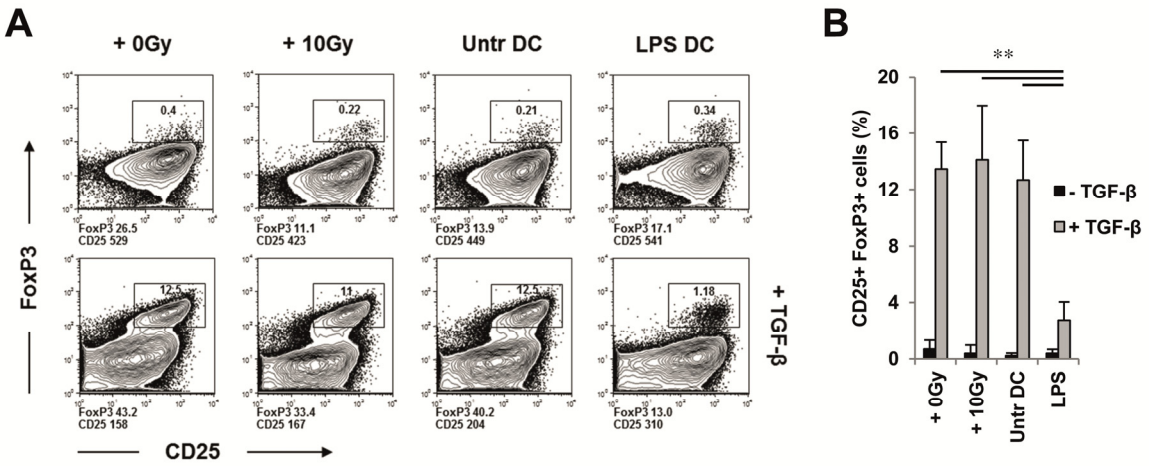


Figure 11. DC do not show increased capacity to induce regulatory T cells in the presence of apoptotic cell material *in vitro*
(A) GM-CSF DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy) or GM-CSF DC were treated with the indicated stimuli for 24h before culture with $CD4^+$ $CD25^-$ OVA-TCR specific T cells. TGF-β was added to the culture when indicated. Induction of $FoxP3^+$ regulatory T cells was analysed 5 days after start of culture by flow cytometry. Numbers in figures represent percentage cells within a particular gate. Numbers under each plot show MFI. Dot plots were all gated on $CD4^+V\beta5^+$ cells. One representative experiment of 7 experiments is shown. (B) as in (A) but graph shows mean percentage $CD25^+FoxP3^+$ cells from 7 independent experiments. Statistical significance: ** $p < 0.01$, one-way ANOVA followed by Bonferroni post-test

3.1.6 Apoptotic thymocytes serve as a source of self-antigens for peptide presentation by DC

To determine whether DC use apoptotic thymocytes as an antigen source *in vitro*, GM-CSF DC were pre-treated with thymocytes derived from wildtype or transgenic mice expressing membrane ovalbumin (OVA) under control of the β-actin promoter (Act-mOVA) prior to culture with CFSE-labelled OVA-TCR specific T cells.

Untreated DC efficiently induced proliferation in T cells in the presence of exogenous OVA protein or peptide as detected by flow cytometry (fig 12A). As expected, LPS conditioning of DC induced more profound proliferation in T cells as indicated by division indices or MFI of CFSE peaks (fig 12A and B). No significant up- or downregulation of CFSE division could be detected when DC were pre-treated with untreated or irradiated thymocytes from wildtype mice prior to culture with CFSE-labelled T cells and in addition of OVA peptide or protein (Fig 12B). Interestingly, DC were able to induce proliferation in T cells in the absence of any exogenously added OVA peptide or protein but solely upon pre-treatment with thymocytes derived from Act-mOVA mice (fig 12A and B). Surprisingly, T cells proliferated equally well if DC were conditioned with Act-mOVA thymocytes which were either left untreated or

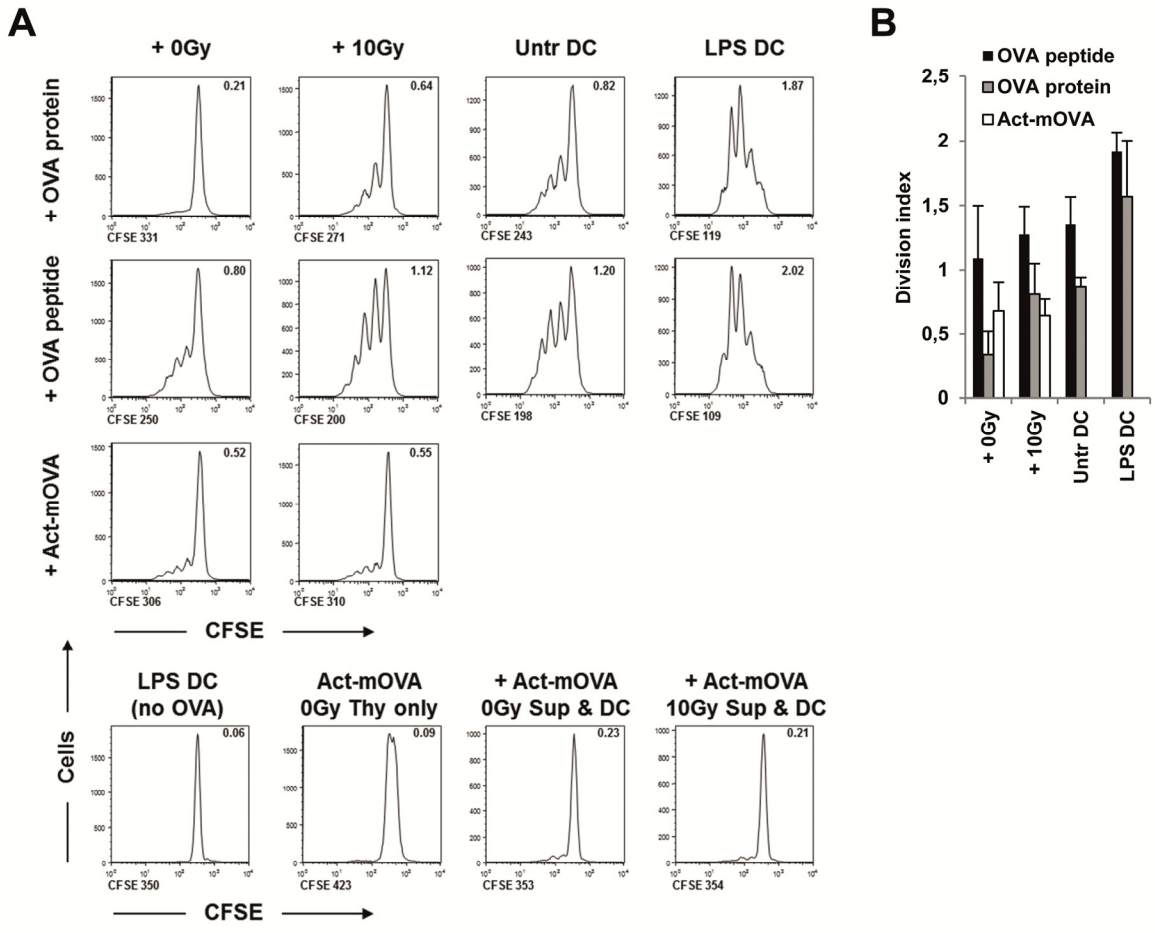


Figure 12. Primary apoptotic and non-irradiated cells serve as self-antigens for peptide presentation by DC
(A) GM-CSF DC were stimulated with the indicated stimuli or incubated with thymocytes isolated from wildtype or Act-mOVA mice which were left untreated (0Gy) or irradiated (10Gy). After 24h, DC were co-cultured with CFSE-labelled OVA-TCR specific T cells in the presence of additional OVA protein or peptide when indicated. Alternatively, thymocytes isolated from Act-mOVA mice which were left untreated (0Gy) or irradiated (10Gy) or supernatants (Sup) thereof sampled during 24h post irradiation were co-cultured with CFSE-labelled OVA TCR-specific T cells in the presence of DC when indicated. 72h after start of co-culture, CFSE dilution was analysed by flow cytometry. Numbers in figures show division index; numbers below each figure represent MFI. (B) as in (A) but graph represent mean division indices from 2 independent experiments performed.

exposed to irradiation (fig 12A and B). Leakage control experiments indicated that T cell division was markedly lower upon addition of supernatant derived from apoptotic Act-mOVA thymocytes compared to thymocytes conditioned DC (fig 12A).
In sum, DC are able to acquire self-antigens for MHC II presentation from primary thymocytes undergoing either spontaneous or irradiation-induced apoptosis.

3.1.7 Flt3-L BM cultures represent steady-state pDC and CD8α⁺-like or CD8α⁻-like cDC which acquire cellular material from primary thymocytes

To determine whether Flt3-L-dependent steady-state conventional DC (cDC) handle apoptotic thymocytes differently from GM-CSF derived DC, BM cultures were supplemented with Flt3-L according to previously established protocols for the *in vitro* generation of steady-state DC equivalents [37, 38].

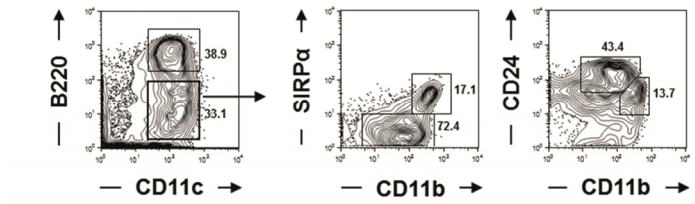


Figure 13. Flt3-L generated BM-DC represent steady-state CD8α⁺-like, CD8α⁻-like and plasmacytoid DC (pDC)
(A) DC were generated in the presence of Flt3-L and stained at day 8 for the indicated surface markers followed by analysis on flow cytometer. Numbers in dot plots represent percentage cells within a particular gate. One representative experiment is depicted.

At day 8, Flt3-L cultured cells appeared much smaller than GM-CSF BM cultures (data not shown and fig 14B) but expressed high levels of CD11c (fig 13). B220 surface marker expression allows division into two major Flt3-L subsets: $CD11c^{hi}/B220^{hi}$ pDC and $CD11c^{hi}/B220^{lo}$ DC cDC (fig 13, left panel). The cDC compartment can be further subdivided by the differential expression of SIRPα, CD11b and CD24 into $CD8\alpha^{+}$-like DC ($SIRP\alpha^{-}$, $CD24^{high}$ and $CD11b^{low}$) or $CD8\alpha^{-}$-like DC ($SIRP\alpha^{+}$, $CD24^{low}$ and $CD11b^{high}$). The $CD8\alpha^{+}$-like DC constitute the majority of BM-derived cDC as described previously [38].
Both $SIRP\alpha^{+}$ and $SIRP\alpha^{-}$ Flt3-L cDC cluster intensively with thymocytes which were either left untreated or exposed to irradiation (fig 14A and B). Analysis of pDC interaction and maturation remained beyond the scope of this study and was not further addressed here. Flt3-L cDC gradually acquire PKH-26^{low} thymocyte-derived material which, similarly to GM-CSF DC, occurred irrespective of thymocyte treatment or radiation exposure (fig 14A and fig 7A,

right panels). Exchange of thymocyte antigens with Flt3-L cDC was inhibited in the presence of cytochalasin D or when cells were incubated at 4°C (fig 14A). Surprisingly, Flt3-L cDC did not internalize cellular material derived from irradiated thymocytes as shown by confocal microscopy (fig 14B, right panel) in contrast to GM-CSF DC which ingested 10Gy-irradiated thymocyte particles efficiently (fig 7B, right panel).

Together, Flt3-L cDC interact intensively with primary thymocytes and gain cellular particles thereof but, in contrast to GM-CSF DC, are not able to perform phagocytosis of apoptotic thymocyte material *in vitro*.

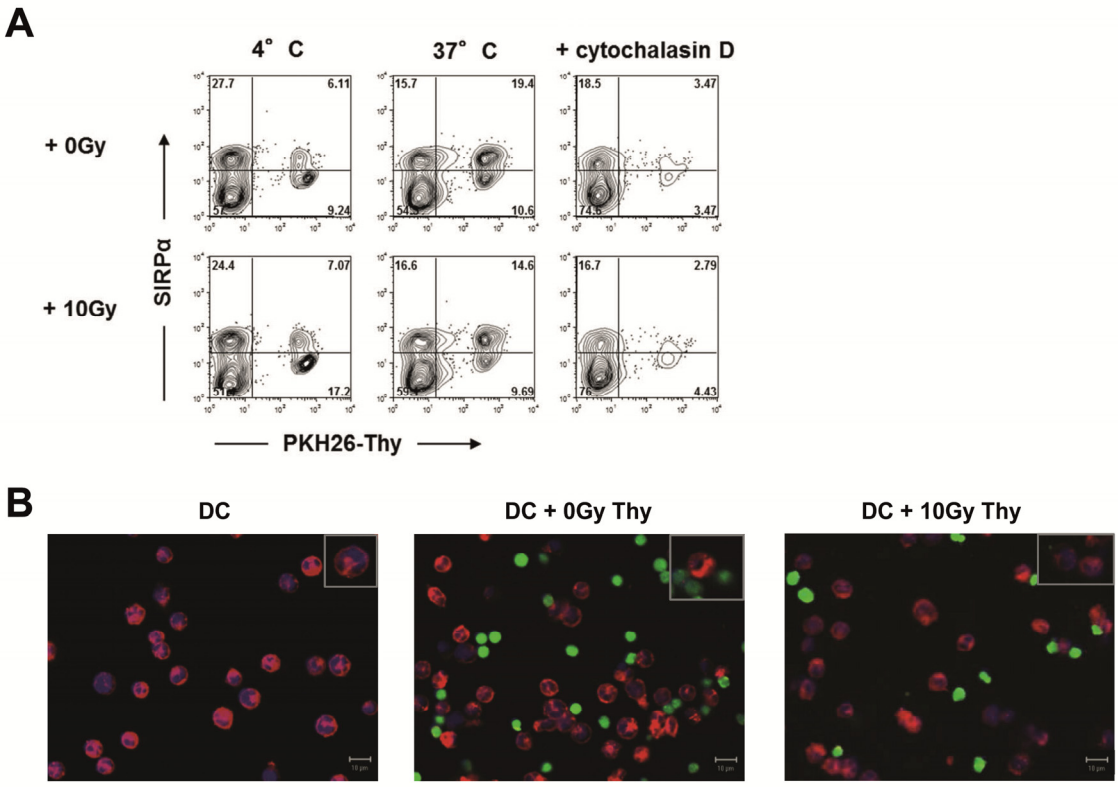


Figure 14. Flt3-L DC adhere to and acquire particles from thymocytes
(A) PKH26-labelled thymocytes were irradiated (10Gy) or left untreated (0Gy) and incubated with bulk Flt3-L DC in presence of cytochalasin D when indicated. Interaction and/or phagocytosis by DC were measured by flow cytometry 3h after incubation start. Contour plots show cells gated on CD11c^{high} and B220^{low} expressing cells. The inserted numbers show percentage cells in a particular quadrant. One representative experiment from 4 independent experiments is shown. (B) B220^{-} PKH26-labelled (red) DC (left panel) were incubated with CFSE-labelled (green) thymocytes which were left untreated (0Gy; mid panel) or irradiated (10Gy; right panel). Uptake of apoptotic material by DC was studied by confocal microscopy 20h after start of incubation. Nuclei were counterstained with DAPI. Inserts show cellular detail at 1.5x magnification.

3.1.8 Apoptotic thymocytes do not induce upregulation of surface marker expression on Flt3-L cDC

Untreated SIRPα$^+$ or SIRPα$^-$ Flt3-L cDC expressed high levels of MHC II but no/low co-stimulatory molecules as detected by flow cytometry (fig 15A). As expected, LPS conditioning of Flt3-L cDC shifted maturation to a fully mature DC profile for both SIRPα$^+$ and SIRPα$^-$ DC indicated by high levels of MHC II and co-stimulatory surface marker expression (fig 15A and B). In the presence of irradiated thymocytes, no significant upregulation of MHC II or co-stimulatory molecules could be detected compared to untreated Flt3-L cDC (fig 15A and B). Furthermore, pre-conditioning of Flt3-L DC with untreated or irradiated thymocytes during 4h decreased secretion of IL-12p70 significantly upon secondary challenge with LPS (fig 15C) as observed previously with GM-CSF DC (fig 9C).
In summary, apoptotic thymocytes do not modify expression of MHC II and co-stimulatory molecules on Flt3-L cDC significantly but inhibit secretion of pro-inflammatory IL-12p70 upon secondary challenge with LPS, similar to what has been observed for GM-CSF DC.

3.1.9 Flt3-L cDC fail to express regulatory cytokines in the presence of apoptotic cells

As Flt3-L cDC did not alter maturation phenotype in the presence of apoptotic cells, the expression of regulatory cytokines was determined by flow cytometry and ELISA.
Similarly to GM-CSF DC, untreated Flt3-L cDC did not show LAP expression on their surface and stimulation with LPS or the presence of apoptotic irradiated thymocytes failed to induce LAP on Flt3-L cDC (fig 16A and B). In contrast to their *in vitro* counterparts, *ex vivo* isolated steady-state CD8α$^+$ and CD8α$^-$ DC showed significant surface expression levels of LAP (fig 16C). Moreover, active TGF-β1 could not be detected in the supernatants of differentially conditioned Flt3-L DC (data not shown), whereas total TGF-β1 levels remained largely unaltered among the various Flt3-L maturation regimens (fig 16D, left panel). Significant production of anti-inflammatory IL-10 by Flt3-L DC was detected upon LPS conditioning only (fig 16D) although at markedly lower levels compared to GM-CSF DC (fig 10C).
Taken together, these data suggest that *in vitro* generated Ftl3-L DC are, similarly to GM-CSF DC, hampered in the production of active TGF-β1 and in surface expression of LAP proteins.

Therefore, no modified expression of TGF-β1 or associated LAP proteins could be detected on DC in the presence of primary apoptotic thymocytes *in vitro*.

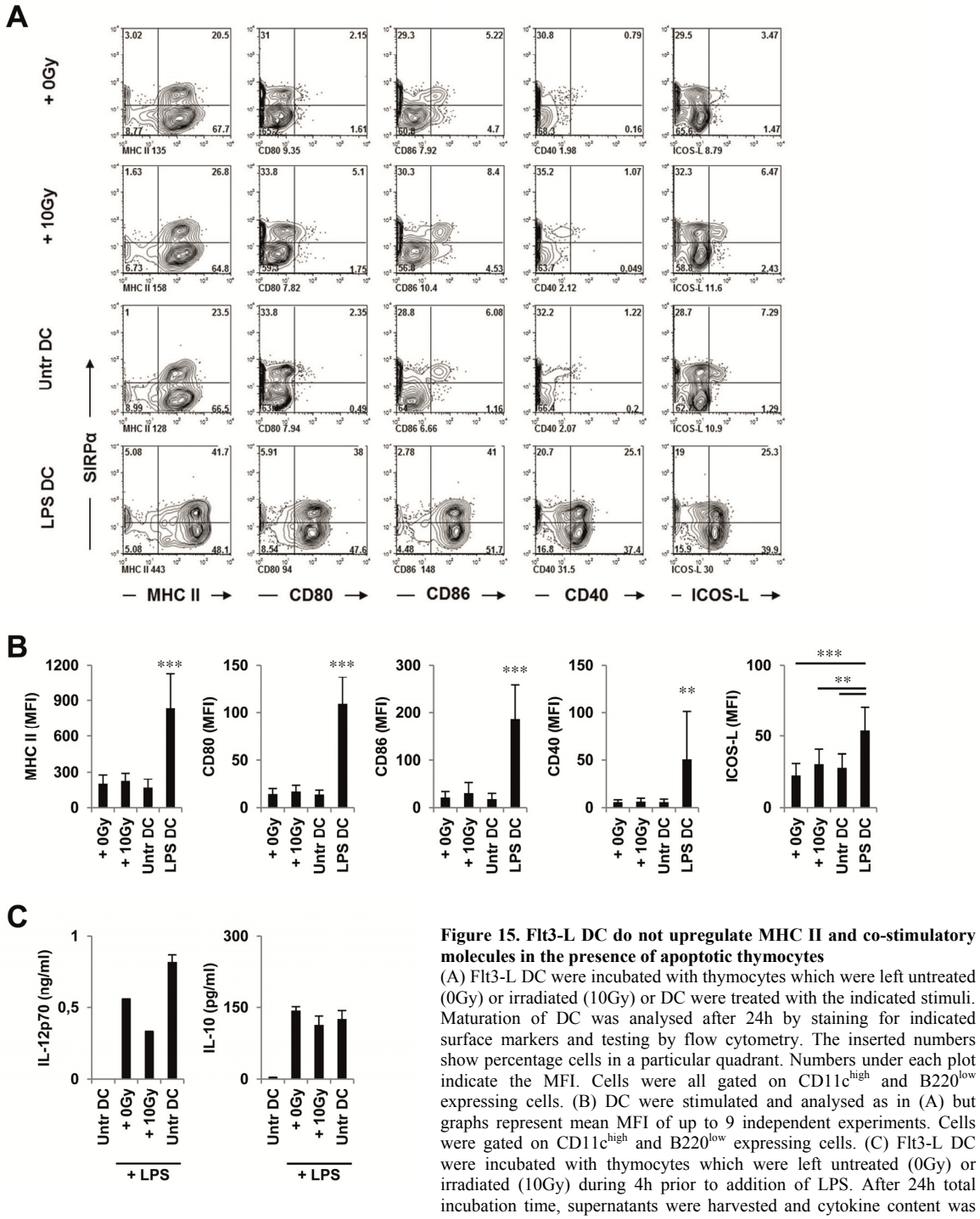


Figure 15. Flt3-L DC do not upregulate MHC II and co-stimulatory molecules in the presence of apoptotic thymocytes
(A) Flt3-L DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy) or DC were treated with the indicated stimuli. Maturation of DC was analysed after 24h by staining for indicated surface markers and testing by flow cytometry. The inserted numbers show percentage cells in a particular quadrant. Numbers under each plot indicate the MFI. Cells were all gated on $CD11c^{high}$ and $B220^{low}$ expressing cells. (B) DC were stimulated and analysed as in (A) but graphs represent mean MFI of up to 9 independent experiments. Cells were gated on $CD11c^{high}$ and $B220^{low}$ expressing cells. (C) Flt3-L DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy) during 4h prior to addition of LPS. After 24h total incubation time, supernatants were harvested and cytokine content was analysed by ELISA. One representative experiment is shown. Statistical significance: ** $p < 0.01$ and *** $p < 0.001$, one-way ANOVA followed by Bonferroni post-test

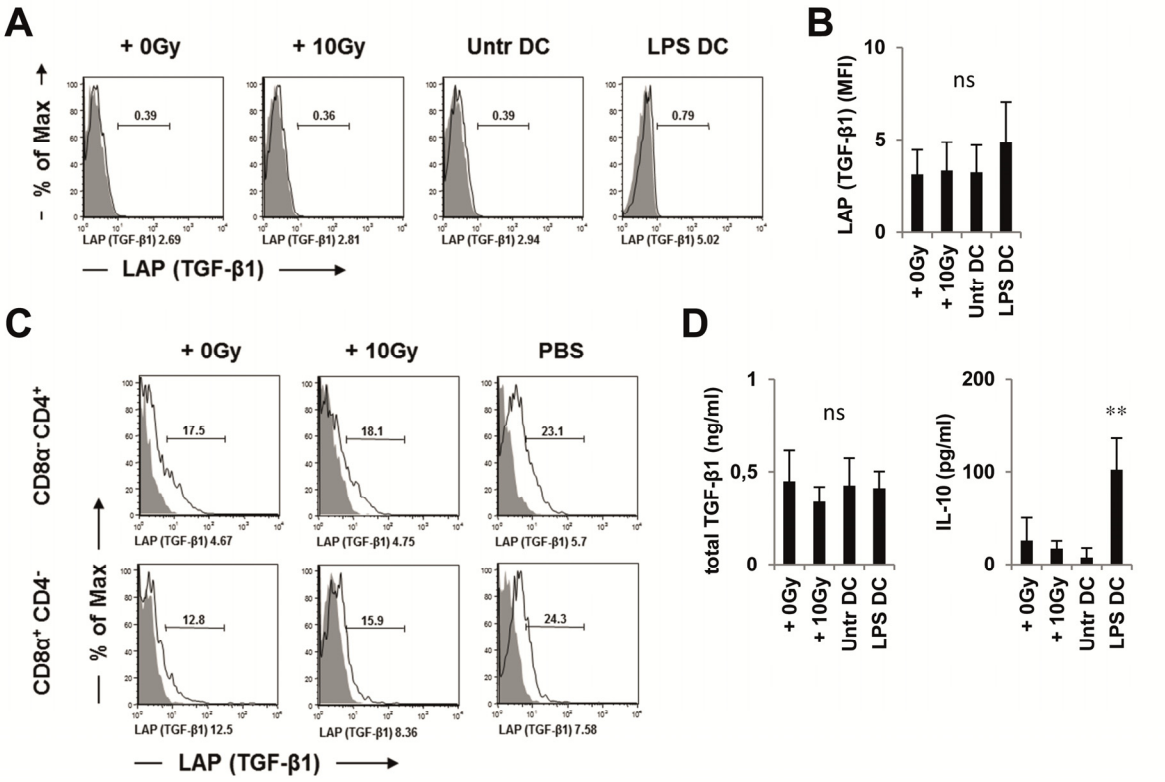


Figure 16. Flt3-L DC fail to induce regulatory molecules in presence of apoptotic cells
(A) Flt3-L DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy) or DC were stimulated with the indicated stimuli. Surface expression of TGF-β1-associated LAP was analysed after 24h by flow cytometry. Numbers below each histogram show MFI; numbers within the figures represent percentage cells. Cells were all are gated on CD11c^{high} and B220^{low} expressing cells. One representative experiment of 7 independent experiments is depicted. (B) as in (A) but graph represent MFI of 7 experiments performed. (C) Mice received one injection of thymocytes which were left untreated (0Gy) or irradiated (10Gy) or mice received PBS. After 20h, splenocytes were isolated and stained for TGF-β1-associated LAP expression followed by analysis on flow cytometer. Cells were gated on CD11c^{high} CD8α^{-} CD4^{+} (upper panels) or CD11c^{high} CD8α^{+} CD4^{-} (lower panels). Numbers below each histogram show MFI and within the figures represent percentage cells. One representative experiment is shown. (D) Flt3-L DC were incubated with thymocytes which were left untreated (0Gy) or irradiated (10Gy). After 24h, supernatants were harvested and cytokine content analysed by ELISA. Graphs show mean data from up to 10 (total TGF-β1) and 4 (IL-10) independent experiments. Statistical significance: ** $p < 0.01$ and ns = not significant, one-way ANOVA followed by Bonferroni post-test

3.1.10 Flt3-L cDC subsets show differential capacity for the induction of regulatory T cells

To determine whether *in vitro* generated Flt3-L DC subsets induce regulatory function in T cells relative to the *in vivo* splenic DC counterparts [29], potentially in response to apoptotic cell conditioning, Flt3-L DC were enriched in a CD8α^{+}- or CD8α^{-}-like fraction before co-culture with OVA-TCR specific T cells.

In the absence of exogenously supplied TGF-β, none of the Flt3-L cDC fractions were able to induce higher levels of FoxP3^{+} CD25^{+} regulatory T cells (fig 17A and C, upper panels) compared to GM-CSF DC cultures (fig 11A). Upon addition of exogenous TGF-β, the enriched CD8α^{+}-like Flt3-L cDC fraction converted more FoxP3^{+} T cells although the induction of FoxP3 expression was regulated independently from the initial DC maturation

stimulus (fig 17A and B). The CD8α$^-$-like DC fraction showed more potent FoxP3$^+$ differentiation from naïve T cell precursors in the presence of supplemented TGF-β compared to the CD8α$^+$-like cDC subset (fig 17C) in which the CD8α$^-$-like DC maturation status modestly influenced FoxP3$^+$ regulatory T cell conversion rate (fig 17D).

Together, both CD8α$^+$- and CD8α$^-$-like Flt3-L DC fractions are dependent on the exogenous supply of TGF-β for the induction of FoxP3$^+$ regulatory T cells *in vitro*. Surprisingly, the FoxP3$^+$ regulatory T cell conversion rate of CD8α$^+$-like DC was independent of the maturation stimulus. The tolerogenic capacity of both GM-CSF and Flt3-L DC treated with apoptotic cells did not increase but their response to subsequent stimuli was impaired.

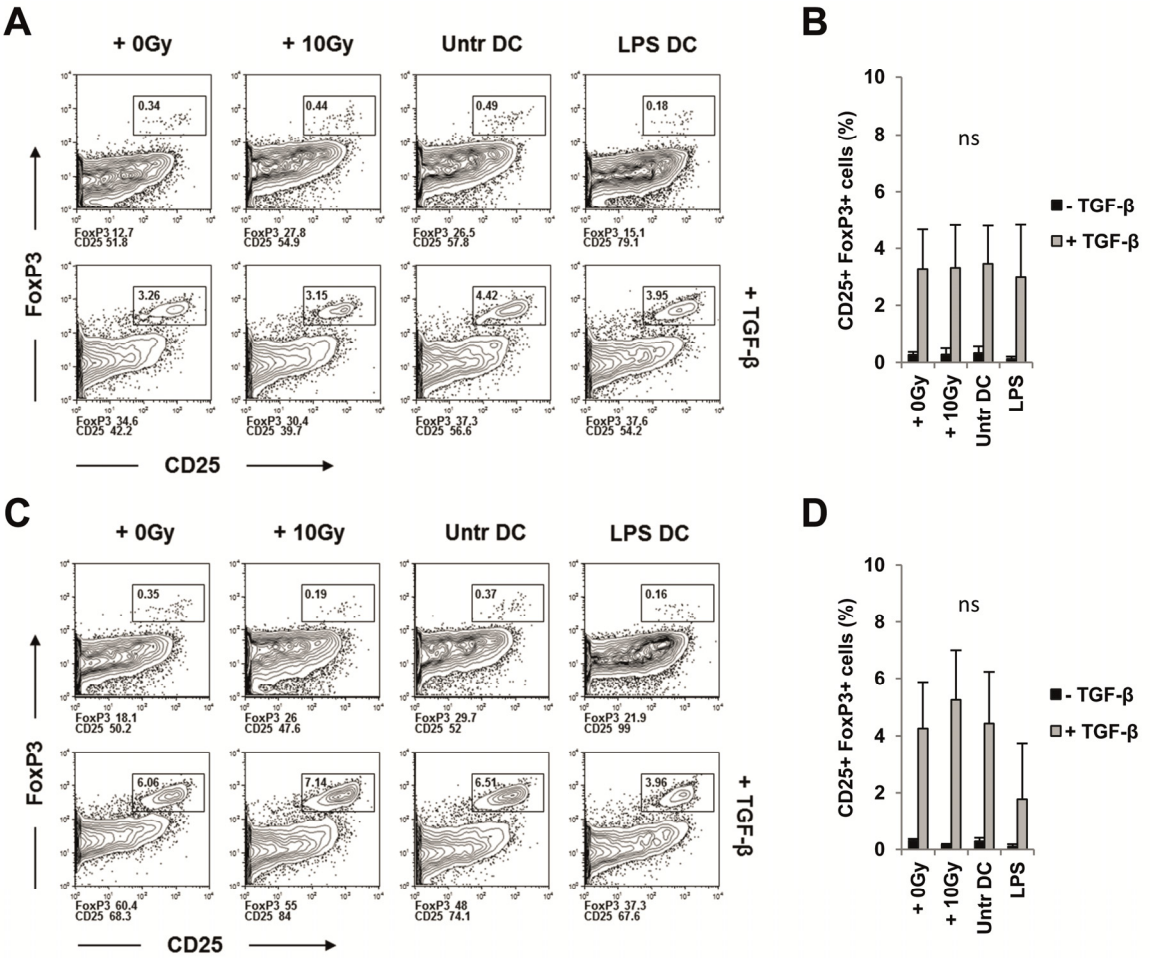


Figure 17. Flt3-L DC do not induce more regulatory T cells in the presence of apoptotic cell material *in vitro*
(A) Flt3-L DC were incubated with thymocytes which were untreated (0Gy) or irradiated (10Gy) for 27h or DC were stimulated with LPS during 4h. Before co-culturing with CD4$^+$ CD25$^-$ OVA-TCR specific T cells, Flt3-L DC were enriched for B220$^-$ CD11b$^-$ expressing cells. TGF-β was added to the culture when indicated. Induction of FoxP3$^+$ regulatory T cells was analysed 5 days after start of culture by flow cytometry. Numbers in figures represent percentage cells within a particular gate. Cells were all gated on CD4$^+$Vb5$^+$ cells. One representative experiment of 3 experiments is shown. (B) as in (A) but graphs show mean data from 3 independent experiments performed. (C) as in (A) but Flt3-L DC were enriched for B220$^-$ CD11b$^+$ expressing cells followed by co-culture with CD4$^+$ CD25$^-$ OVA-TCR specific T cells. (D) as in (C) but graphs show mean data from 3 independent experiments. Cells were all gated on CD4$^+$Vb5$^+$ cells. Statistical significance: ns = not significant, one-way ANOVA followed by Bonferroni post-test

3.2 Immature DC require two steps to convert naïve into anergic IL-10+ regulatory T cells through combined CD28 and CTLA-4 signalling

3.2.1 Immature DC induce anergy in naïve T cells lacking regulatory function

To study whether immature DC direct tolerogenic T cell responses in the absence of exogenously added cytokines, we co-cultured untreated BM-derived GM-CSF DC with naïve $CD4^+CD25^-$ OTII T cells *in vitro*. Naïve T cells differentiated by untreated DC were markedly impaired in proliferation upon TCR or polyclonal restimulation compared to LPS-stimulated controls (fig 18A). Proliferation defects could be restored upon addition of exogenous IL-2 (fig 18A). Furthermore, T cells cultured with untreated DC showed increased expression of the anergy-related transcription factor Egr-2 (fig 18B) and failed to secrete IL-2 cytokines upon TCR-restimulation (fig 19A, right panel). However, upon exposure to mitogens bypassing the TCR such as PMA and ionomycin, T cells differentiated by untreated DC were able to produce IL-2 (fig 19B and C). In contrast to IL-2 expression, naïve T cells differentiated by untreated DC acquired a mixed IFN-γ/IL-13/IL-4 Th0 cytokine profile upon restimulation (fig 19A). The Th0 cytokine signature was confirmed by quantitative real-time PCR as T cells primed by untreated DC failed to induce predominant expression of the transcription factors GATA-3, Tbx21 (T-bet) or NFIL3 (or E4BP4) (fig 19D).

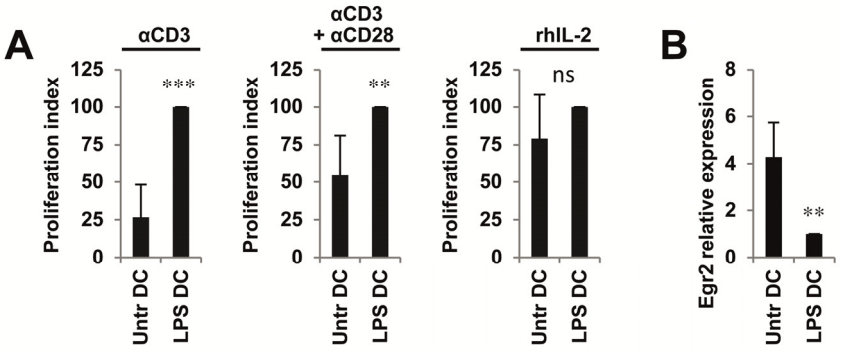


Figure 18. Immature DC induce anergic $CD4^+$ T cells *in vitro*
(A) $CD4^+$ $CD25^-$ OVA-TCR specific T cells were cultured for 7 days with untreated or LPS-stimulated DC in the presence of OVA peptide followed by restimulation on plate-bound αCD3, αCD3/CD28 or rhIL-2. Proliferation was measured by [^{3}H]-methyl-thymidine incorporation 2 to 3 days after restimulation. Graphs show proliferation indices as values normalized to LPS-DC stimulated controls and represent mean data of 6 independent experiments. (B) $CD4^+$ $CD25^-$ OVA-TCR specific T cells were cultured for 7 days with untreated or LPS-stimulated DC in the presence of OVA peptide followed by RNA isolation. Real-Time quantitative PCR for Egr2 was performed on cDNA and expression normalized to β-2-microglobulin levels. Relative expression is represented as levels to LPS-DC stimulated controls. Results shown are mean data of 5 independent experiments. Statistical significance: ** $p < 0.01$, *** $p < 0.001$ and ns = not significant, two-tailed Student's t test

As T cell anergy and suppressive function might be related [126, 146], a suppression assay was performed in which T cells differentiated by untreated or LPS-matured DC were added as

suppressor cells. Anergic T cells generated by one round of stimulation by untreated DC were not able to suppress responder T cells in contrast to $CD4^+CD25^+$ T cells which block proliferation of responder T cells efficiently (fig 20A and B).

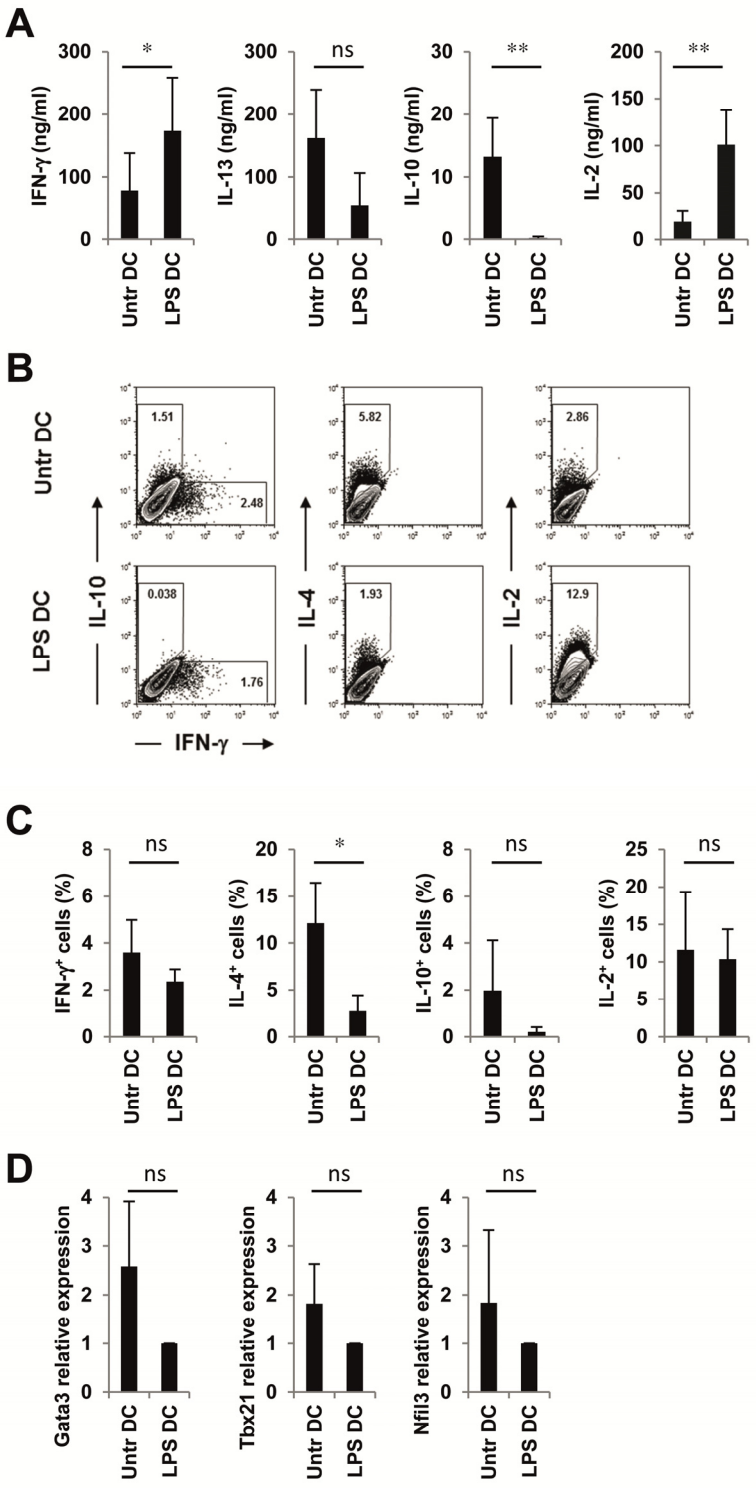


Figure 19. Anergic $CD4^+$ T cells show a Th0-like phenotype but are impaired in IL-2 cytokine secretion
(A) $CD4^+$ $CD25^-$ OVA-TCR specific T cells were cultured for 7 days with untreated or LPS-stimulated DC in the presence of OVA peptide followed by restimulation on plate-bound αCD3 antibodies. After 2 to 3 days, supernatants were harvested and cytokine content analysed by ELISA. Graphs show mean data of up to 5 independent experiments. (B) as in (A) but T cells were restimulated with PMA/ionomycin and intracellular cytokine content determined by flow cytometry. One representative experiment is depicted. Numbers show percentage cells within a particular gate. Cells were all gated on $CD4^+$ expressing cells. (C) as in (B) but graphs show mean data of 3 independent experiments. Cells were all gated on $CD4^+$ expressing cells. (D) $CD4^+$ $CD25^-$ OVA-TCR specific T cells were cultured for 7 days with untreated or LPS-stimulated DC followed by RNA isolation. Real-Time quantitative PCR for Gata3, Tbx21 and Nfil3 was performed on cDNA and expression normalized to β-2-microglobulin levels. Relative expression is represented as levels to LPS-DC stimulated controls. Results shown are mean data of 5 independent experiments. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and ns = not significant, two-tailed Student's t test

Together, immature DC induce anergic Egr2-expressing T cells *in vitro* which fail to produce IL-2 but lack regulatory capacity after one round of stimulation.

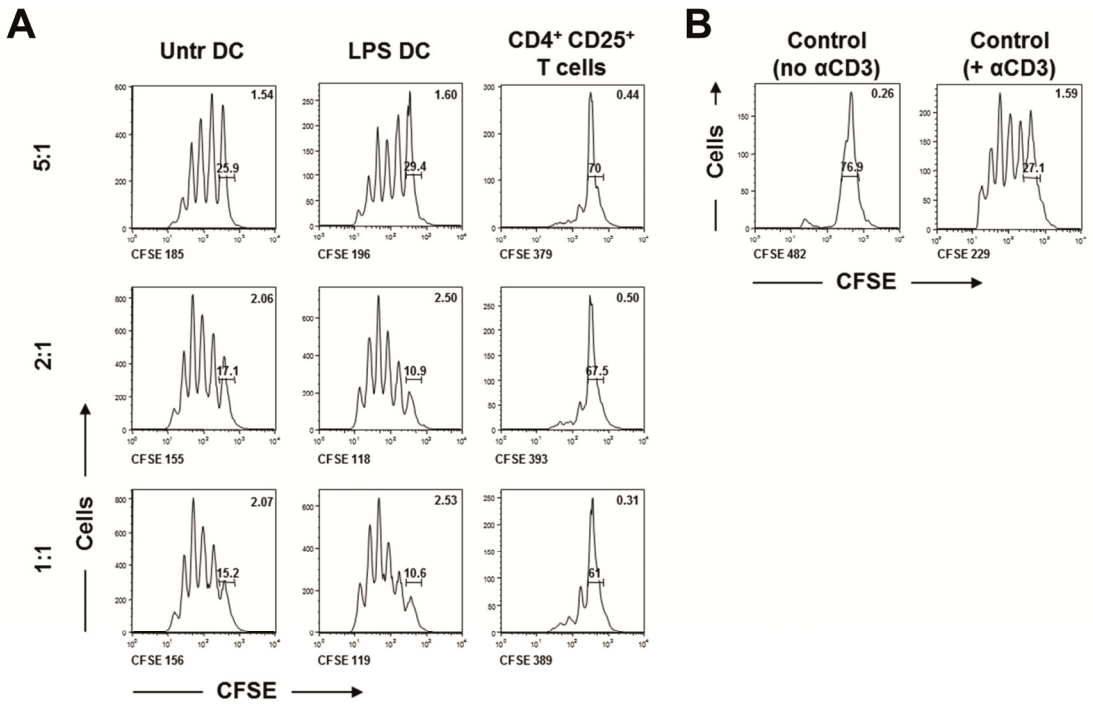


Figure 20. Anergic CD4$^+$ T cells do not show regulatory function
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 7 days with untreated or LPS-stimulated DC in the presence of OVA peptide and were then used as suppressor cells in a suppression assay. After 72h, proliferation of responder T cells was determined by CFSE dilution. Suppressor to responder ratio is indicated next to the Y-axis. Numbers indicated in upper right corner show division indices; numbers above marker gate show percentage undivided cells. MFI of CFSE is depicted under each histogram. One representative experiment of 2 independent experiments is depicted. (B) as in (A) but graphs show proliferation of responder T cells without addition of suppressor cells in absence or presence of αCD3 antibodies.

3.2.2 Repetitive stimulation by immature DC induces IL-10 producing regulatory T cells

Since repetitive TCR signalling has been attributed to the development of IL-10 producing regulatory T cells from anergic or effector T profiles [178, 184], naïve OT-II T cells were stimulated for 2 rounds with untreated DC. Chronically stimulated T cells partially maintained their anergic phenotype as indicated by reduced proliferation upon TCR-restimulation to levels detected after one round of stimulation (fig 21A) which was restored in the presence of exogenous IL-2 (fig 21A) and by high relative expression of the transcription factor Egr-2 (fig 21B). In contrast to LPS-DC stimulated T cells which produced high amounts of IL-2 upon TCR stimulation, T cells kept IL-2 cytokine production low when differentiated by untreated DC (fig 22A). Again, exposure to mitogen cocktail circumventing TCR-signalling restored IL-2 cytokine secretion (fig 22B and C). Surprisingly, untreated DC induced profound

increase in IL-10 cytokine release (16.3 ± 4.1% IL-10^{+}; fig 22C) in T cells compared to one round of stimulation (2.0 ± 2.2% IL-10^{+}; fig 19C).

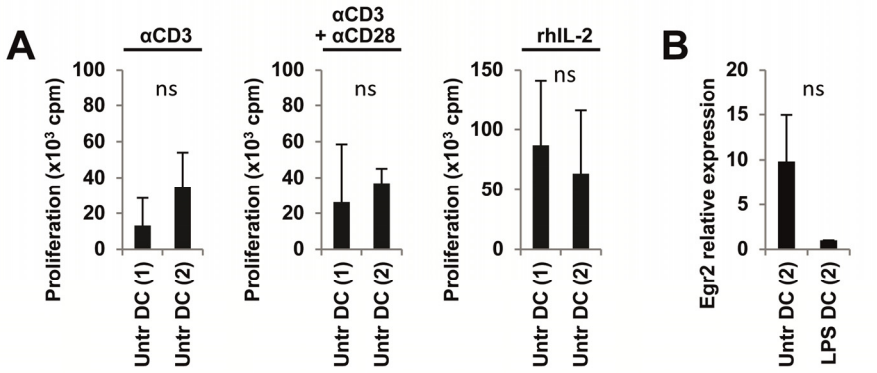


Figure 21. CD4^{+} T cells largely keep the anergic phenotype upon second stimulation with DC
(A) CD4^{+} CD25^{-} OVA-TCR specific T cells were cultured for 1 or 2 rounds with untreated DC in the presence of OVA peptide followed by restimulation on plate-bound αCD3, αCD3/CD28 or rhIL-2. Proliferation was measured by [^{3}H]-methyl-thymidine incorporation. Graphs show proliferation as cpm values and represent mean data of 5 (1 round) and 4 (2 rounds) independent experiments respectively. (B) CD4^{+} CD25^{-} OVA TCR-specific T cells were cultured for 2 rounds with untreated or LPS-stimulated DC in the presence of OVA peptide followed by RNA isolation. Real-Time quantitative PCR for Egr2 was performed on cDNA and expression normalized to β-2-microglobulin levels. Relative expression is represented as levels to LPS-DC stimulated controls. Results shown are mean data of 3 independent experiments. Statistical significance: ns = not significant, two-tailed Student's t test

Furthermore, repetitive stimulation by untreated DC induced additional increase in IL-13 and IL-4 cytokine release upon TCR-restimulation (fig 22A) or exposure to mitogenic cocktail (fig 22C) respectively and in comparison to one round of stimulation as shown previously (fig 19A and 19C). The shifts in IL-10, IL-13 and IL-4 effector cytokine production were further supported by increased expression of transcription factors GATA-3 and NFIL3/E4BP4 (fig 22D). Strikingly, T cells acquired regulatory function upon chronic stimulation by untreated DC only at high suppressor to responder ratio's (fig 23A and B).

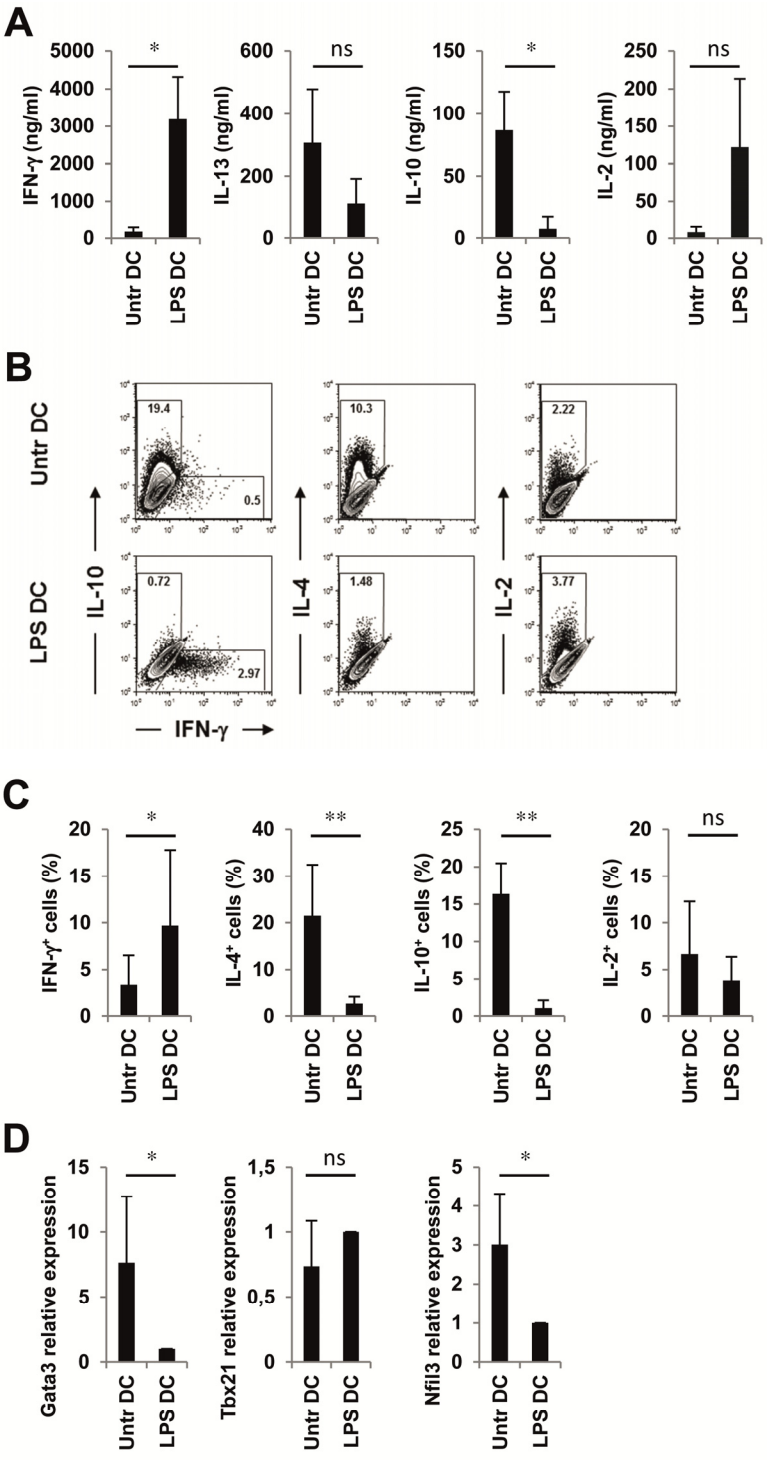


Figure 22. Second stimulation of anergic CD4$^+$ T cells induces IL-10, IL-13 and IL-4 cytokine secretion
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated or LPS-stimulated DC in the presence of OVA peptide followed by restimulation on plate-bound αCD3 antibodies. After 2 to 3 days, supernatants were harvested and cytokine content analysed by ELISA. Graphs show mean data of 3 independent experiments. (B) as in (A) but T cells were restimulated with PMA/ionomycin and intracellular cytokine content determined by flow cytometry. One representative experiment is depicted. Numbers show percentages of cells within a particular gate. Cells were all gated on CD4$^+$ expressing cells. (C) as in (B) but Graphs show mean data of 5 independent experiments. Cells were all gated on CD4$^+$ expressing cells. (D) CD4$^+$ CD25$^-$ OVA TCR-specific T cells were cultured for 2 rounds with untreated or LPS-stimulated DC followed by RNA isolation. Real-Time quantitative PCR for Gata3, Tbx21 and Nfil3 was performed on cDNA and expression normalized to β-2-microglobulin levels. Relative expression is represented as levels to LPS-DC stimulated controls. Results shown are mean data of 5 independent experiments. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and ns = not significant, two-tailed Student's t test

In summary, restimulation of anergic CD4$^+$ T cells with immature DC largely maintains anergic phenotype but induces a striking shift in cytokine balance towards high IL-10 but also IL-4 and IL-13 along with acquisition of suppressive function *in vitro*, being comparable with

Tr1-like IL-10$^+$ regulatory T cells [178], however, not derived from effector but from anergic T cell profiles.

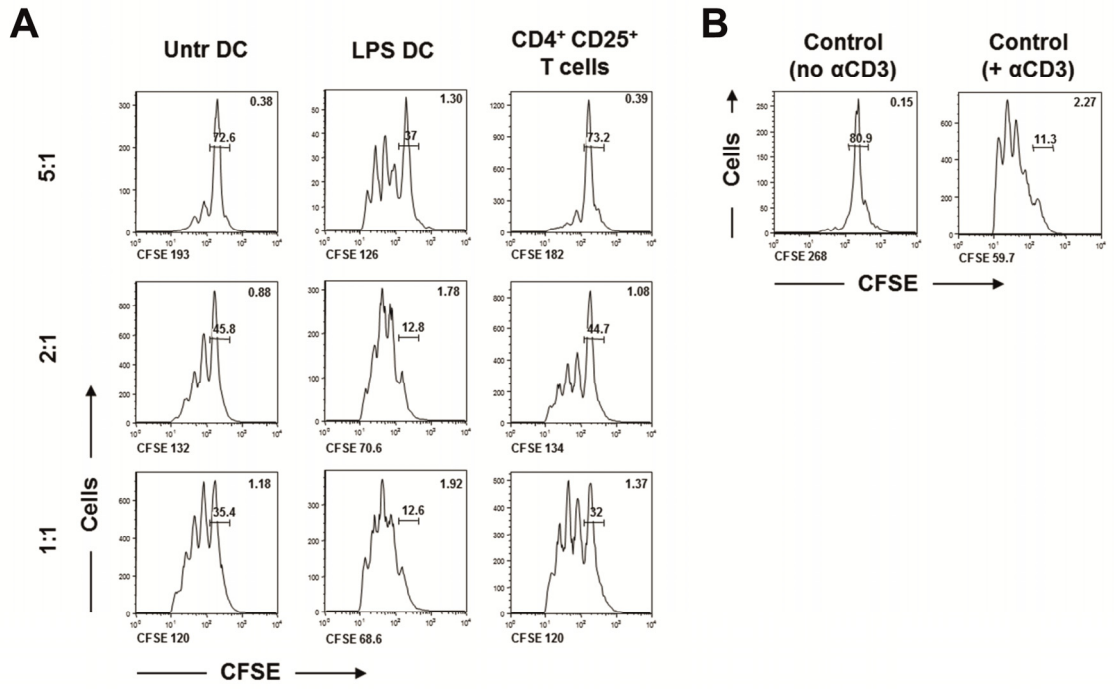


Figure 23. Naïve CD4$^+$ T cells gain regulatory function after two stimulations with immature DC
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated or LPS-stimulated DC in the presence of OVA peptide and were then used in a suppression assay. After 72h, proliferation of responder T cells was determined by CFSE dilution. Suppressor to responder ratio is indicated next to the Y-axis. Numbers indicated in upper right corner show division indices; numbers above marker gate show percentages of undivided cells. MFI of CFSE is depicted under each histogram. One representative experiment of 4 independent experiments is shown. (B) as in (A) but graphs show proliferation of responder T cells without addition of suppressor cells in absence or presence of αCD3 antibodies.

3.2.3 IL-10$^+$ regulatory T cells suppress T cell responses *in vitro* by IL-2 consumption

Earlier reports demonstrated that *in vitro* generated IL-10$^+$ Tr1 cells inhibit naïve T cell proliferation by an IL-10-independent mechanism whereas addition of exogenous IL-2 completely abolished the suppressive function of Tr1 cells *in vitro* [193]. Here, T cells repetitively stimulated by untreated DC showed suppressive function at high suppressor to responder ratio's (fig 23A and 24A, upper row). Addition of αIL-10 receptor antibodies did not abrogate regulatory capacity similarly to freshly isolated CD4$^+$ CD25$^+$ T cells *in vitro* (fig 24A and B, lower graph). Interestingly, addition of exogenous IL-2 partially restored proliferation of naïve responder T cells as seen for CD4$^+$ CD25$^+$ T cells *in vitro* (fig 24A and B, upper panel). Furthermore, real-time PCR analysis revealed that EBI-3, a regulatory subunit of IL-35, is similarly expressed by both untreated and LPS-DC stimulated T cells after

one (fig 25A) or two rounds (fig 25B) of culture, whereas IL-12p35 is expressed predominately by T cells stimulated repetitively by LPS-matured DC only (fig 25B).

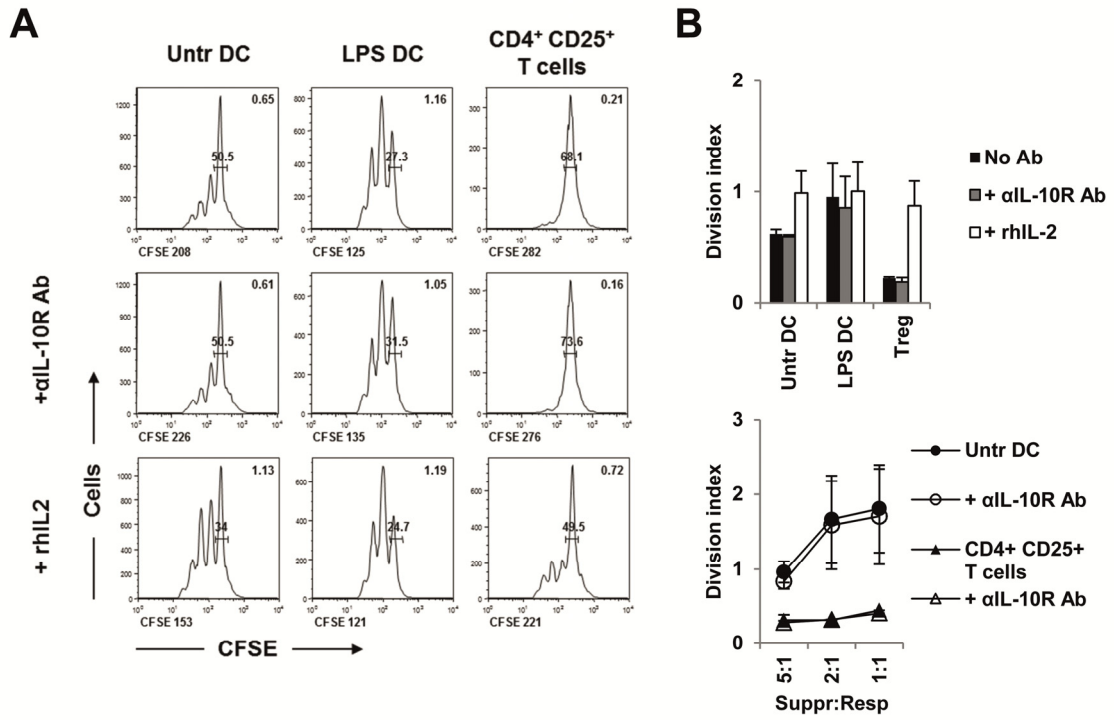


Figure 24. IL-10$^+$ CD4$^+$ regulatory T cells suppress through IL-2 consumption
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated or LPS-stimulated DC and were then used in a suppression assay. αIL-10R antibodies or exogenous rhIL-2 were added to suppression assay when indicated. After 72h, proliferation of responder T cells was determined by CFSE dilution. Numbers indicated in upper right corner show division indices; numbers above marker gate show percentages of undivided cells. MFI of CFSE is depicted under each histogram. (B) as in (A) but graphs show mean division indices of 2 independent experiments performed (upper panel) or mean division indices of 2 independent experiments in the presence of αIL-10R antibodies for various suppressor to responder ratio's (lower panel).

In sum, IL-10$^+$ regulatory T cells developed by repetitive stimulation with untreated DC do not show increased expression of IL-35. Suppression of naïve T cell proliferation occurred independent of IL-10 but through an IL-2 consumption mechanism *in vitro*.

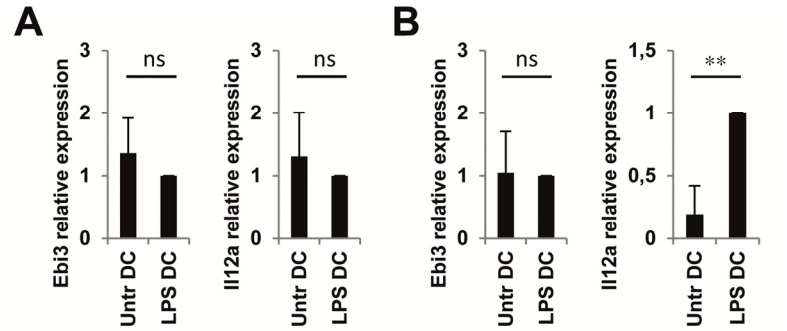


Figure 25. IL-10$^+$ regulatory T cells do not upregulate IL-35 expression
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 7 days in the presence of OVA peptide and untreated or LPS stimulated DC. After 7 days, RNA was isolated followed by cDNA synthesis and real-time quantitative PCR analysis of Ebi3 and Il12a expression was performed. Relative expression is normalized to T cells differentiated by LPS matured DC. Results shown are mean data of 3 independent experiments. (B) as in (A) but CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 consecutive rounds with untreated or LPS matured DC before RNA isolation. Results shown are mean data of 4 independent experiments. Statistical significance: ** $p < 0.01$ and ns = not significant, two-tailed Student's t test

3.2.4 IL-10$^+$ regulatory T cells do not acquire FoxP3, PD-1 or LAG-3 but show a sustained expression of CTLA-4

To define which subset of regulatory T cells is induced upon chronic stimulation of naïve T cells by untreated DC, expression of several regulatory markers was determined. In the absence of exogenously supplied TGF-β, BM-derived DC are unable to convert FoxP3$^+$ regulatory T cells efficiently irrespective of the DC maturation regimen as determined by flow cytometry (fig 26A) and as we showed previously (fig 11). FoxP3 expression was not induced in repetitively stimulated T cells (fig 26B and C) even in the presence of exogenous TGF-β added during the second round of stimulation, although CD25 surface expression decreased under these conditions (fig 26B). Furthermore, T cells differentiated by untreated DC did not upregulate LAG-3 or PD-1 surface marker expression after one or two rounds of differentiation (data not shown and fig 26D).

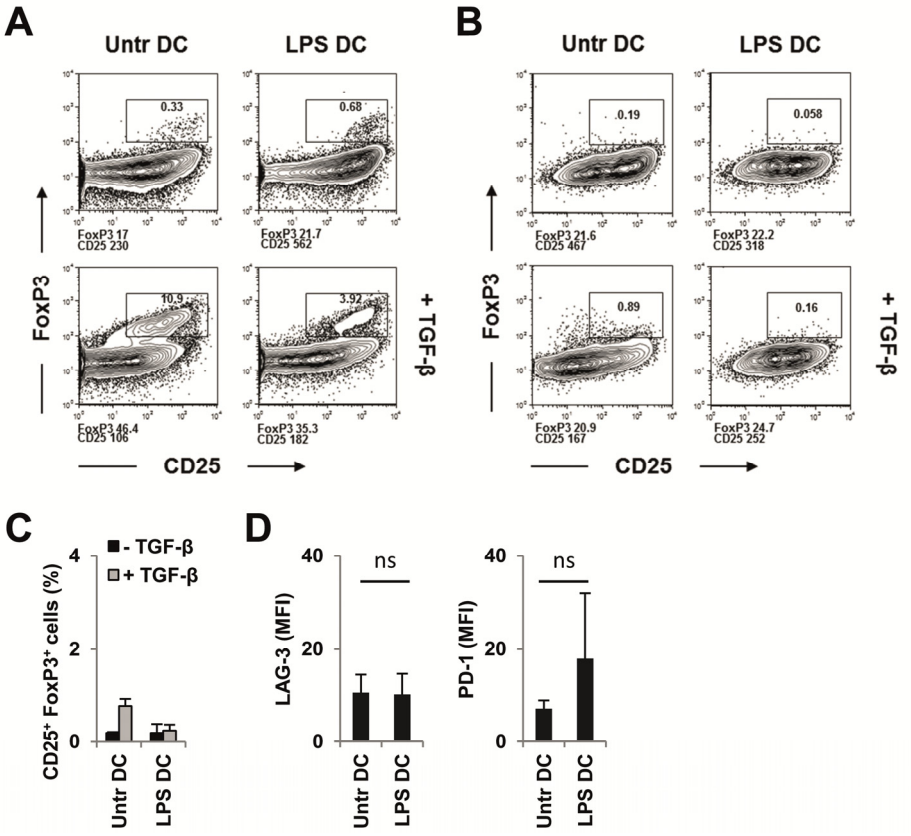


Figure 26. IL-10$^+$ regulatory T cells do not express FoxP3, LAG-3, ICOS or PD-1
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured with untreated or LPS-conditioned DC in presence of exogenous TGF-β when indicated. Induction of FoxP3$^+$ regulatory T cells was analysed 5 to 7 days after start of culture by flow cytometry. Numbers in figures represent percentages of cells within a particular gate. Numbers below figures show MFI values. Plots were all gated on CD4$^+$Vβ5$^+$ cells. One representative experiment of 3 experiments is shown. (B) as in (A) but CD4$^+$ CD25$^-$ OVA-TCR specific T cells were primed for 2 rounds with untreated or LPS-conditioned DC and TGF-β was added to the culture when indicated during the second stimulation only. 5 days after restimulation of T cells, expression of FoxP3 was analysed by flow cytometry. One representative experiment of 2 experiments is depicted. (C) as in (B) but graph shows mean data from 2 independent experiments. Cells were all gated on CD4$^+$Vβ5$^+$ cells. (D) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured with untreated or LPS-stimulated DC for 2 rounds followed by detection of surface marker expression by flow cytometry. Graphs show mean data of 5 (LAG-3) and 3 (PD-1) experiments respectively. Statistical significance: ns = not significant, two-tailed Student's t test

In contrast, high amounts of intracellular CTLA-4 were detected in T cells already after one round of stimulation irrespective of the maturation phenotype of DC (fig 27A and B, left panel). Only under repetitive untreated DC conditions, T cells showed sustained levels of CTLA-4 expression (fig 27C) in contrast to LPS-DC differentiated T cells which downregulated CTLA-4 levels upon restimulation (fig 27C and D, left panel). Although CD25 was predominately expressed on T cells activated by LPS-conditioned DC (fig 27B, right panel), T cells needed only two rounds of stimulation by immature DC to induce elevated levels of CD25 expression (fig 27D, right panel). Remarkably, kinetic studies revealed that untreated DC were superior to LPS-matured DC for the early upregulation of CTLA-4 expression (fig 27E, left panel) in striking contrast to polyclonal, APC-free methods for T cell

activation which largely failed to induce both CTLA-4 and CD25 expression to levels detected under DC conditions (fig 27E).

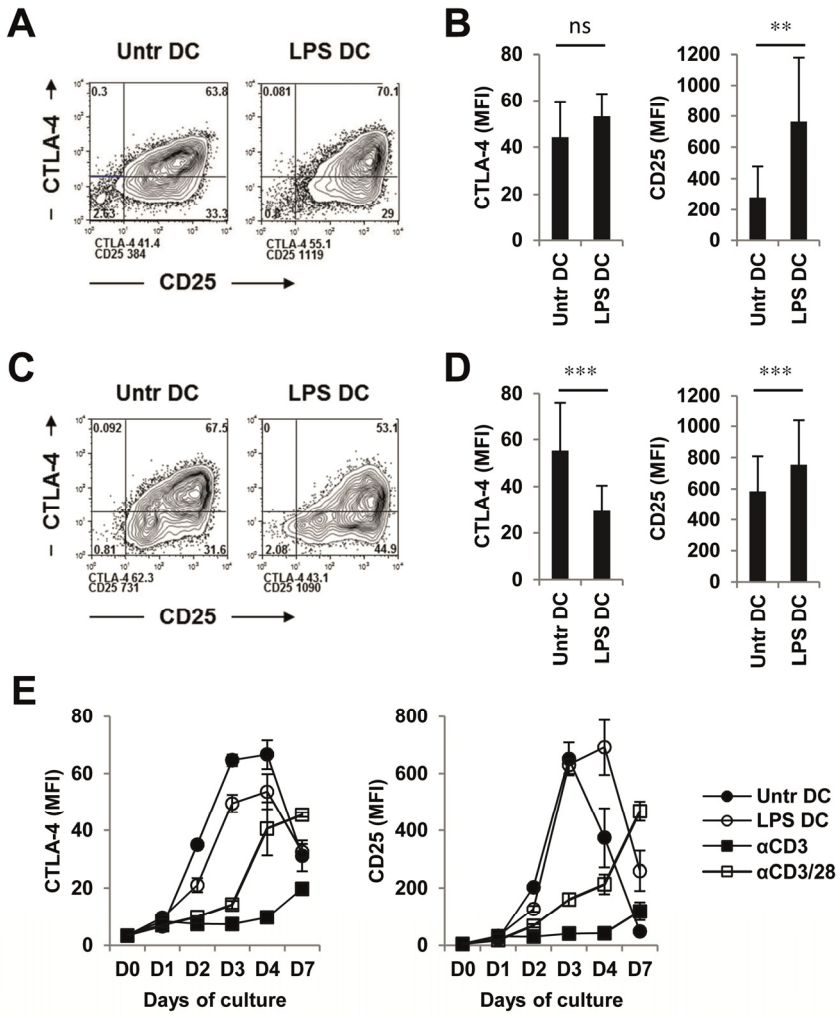


Figure 27. IL-10$^+$ CD4$^+$ regulatory T cells highly express CTLA-4 and CD25
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 5-7 days with untreated or LPS-stimulated DC in the presence of OVA peptide prior to staining for surface markers or intracellular CTLA-4. The inserted numbers in each plot represent percentage cells in a particular quadrant. Numbers below each plot indicate the MFI. Cells were all gated on CD4$^+$ expressing cells. One experiment of 3 independent experiments is shown. (B) as in (A) but graphs show mean data of 3 independent experiments. (C) as in (A) but T cells were cultured for 2 rounds with untreated or LPS-conditioned DC prior to staining for indicated surface markers or intracellular CTLA-4. One experiment of 8 independent experiments is depicted. (D) as in (C) but graph shows mean data of 8 independent experiments. (E) CD4$^+$ OVA-TCR specific T cells were stimulated with untreated or LPS-matured DC in the presence of OVA peptide or alternatively with plate-bound αCD3 or αCD3/CD28 as indicated. Expression of surface CD25 and intracellular CTLA-4 was determined by flow cytometry at the indicated time points. Cells were gated on CD4$^+$ expressing cells. Graphs show mean data of 2 experiments performed. Statistical significance: ** $p < 0.01$, *** $p < 0.001$ and ns = not significant, two-tailed Student's t test

In sum, IL-10$^+$ regulatory T cells generated from anergic T cells by untreated DC do not upregulate FoxP3 expression even in the presence of exogenous TGF-β but are characterized by high levels of CD25 and intracellular CTLA-4.

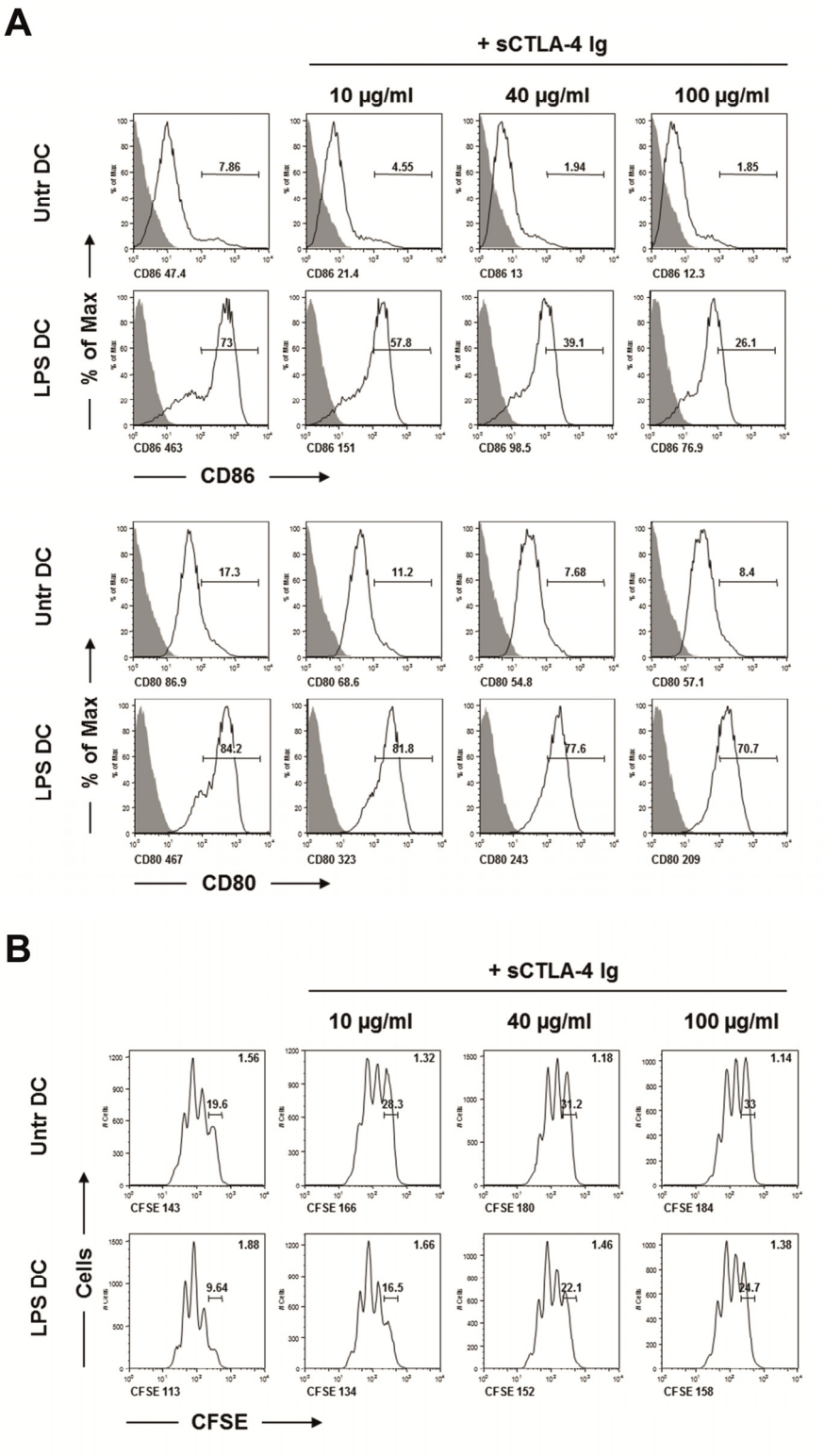


Figure 28. Soluble CTLA-4 Ig decreases co-stimulatory molecule detection on DC
(A) DC were incubated with LPS for 20h or left untreated followed by incubation with indicated concentrations of sCTLA-4 Ig for 1h prior to surface marker staining and testing by flow cytometry. The inserted numbers show percentage cells and the shaded curves in the histograms represent unstained controls. Numbers under each plot indicate the MFI. Cells were all gated on $CD11c^{high}$ expressing cells. One representative experiment of 2 is shown. (B) Untreated or LPS-stimulated DC were co-cultured with CFSE-labelled $CD4^{+}$ $CD25^{-}$ OT-II T cells in the presence of graded concentrations sCTLA-4 Ig. 72h after start of culture, proliferation was measured by CFSE dilution. Numbers in upper corner of plots indicate division indices, numbers above marker gate show percentages of undivided cells. MFI are depicted under each histogram plot. One representative experiment is shown.

3.2.5 Blockade of B7 co-stimulatory markers on DC prevents differentiation from anergic into IL-10$^+$ CD25$^+$ regulatory T cells

So far, the role of B7 co-stimulatory signalling molecules for the differentiation into IL-10$^+$ regulatory T cells remained largely unexplored [251]. Therefore, we added sCTLA-4 Ig in our optimized *in vitro* system during the second round of T cell stimulation to define the necessity of the B7 co-stimulatory signalling network in the switch from anergic to regulatory T cells. Addition of sCTLA-4 Ig decreased detection of CD80 and CD86 surface markers on both untreated and LPS-matured DC by flow cytometry (fig 28A). Proliferation of naïve T cells was slightly reduced under both untreated or LPS-DC conditions in the presence of sCTLA-4 Ig (fig 28B). As we have seen previously in fig 27D, anergic T cells showed elevated expression levels of CD25 and intracellular CTLA-4 upon restimulation with untreated DC. Interestingly, addition of sCTLA-4 Ig during the second round of stimulation with untreated DC reduced expression of CD25 remarkably and modestly affected CTLA-4 expression of T cells (fig 29A and B) back to levels detected after only one round of stimulation with untreated DC (fig 27B). In contrast, no effect on both CD25 and CTLA-4 expression could be detected in the LPS-stimulated T cell cultures upon addition of sCTLA-4 Ig (fig 29A and B).

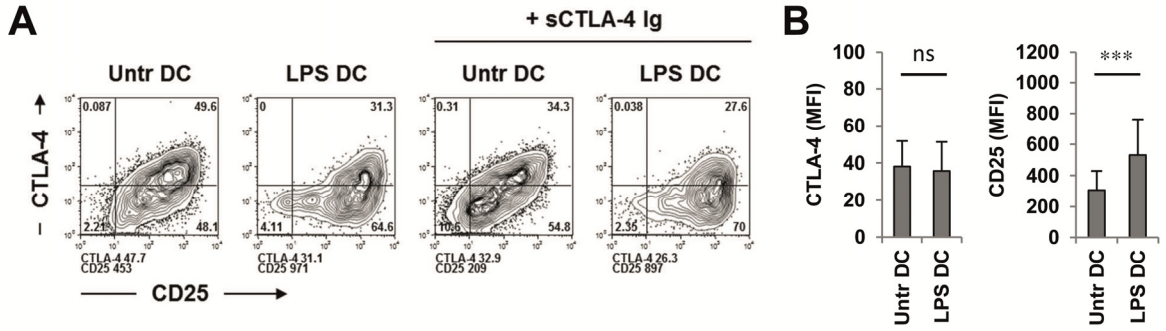


Figure 29. Expression of CD25 and to a lesser extent CTLA-4 of IL-10$^+$ CD4$^+$ regulatory T cells is regulated by DC co-stimulation
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated or LPS-stimulated DC in the presence sCTLA-4 Ig during the second stimulation when indicated. Cells were stained for CD4, CD25 or intracellular CTLA-4 and expression determined by flow cytometry. The inserted numbers in quadrants represent percentages of cells. Numbers below each plot indicate the MFI. Cells were all gated on CD4$^+$ expressing cells. One experiment of 6 independent experiments is shown. (B) as in (A) but graphs show mean data in addition of sCTLA-4 Ig only of up to 6 (CTLA-4) or 11 (CD25) independent experiments respectively. Statistical significance: *** $p < 0.001$ and ns = not significant, two-tailed Student's t test

Furthermore, T cells conditioned by untreated DC showed enhanced secretion of IL-2 in the presence of sCTLA-4 Ig upon restimulation with pharmacological agents bypassing TCR (fig 30A, lower row and B) compared to co-cultures with no additives (fig 30A, upper row). As we have shown above in fig 22C, T cells stimulated in two consecutive rounds with untreated DC increased IL-10 cytokine content to 16.3 ± 4.1% as detected upon exposure to PMA and

ionomycin. Surprisingly, intracellular IL-10 secretion remained unaffected upon B7 ligand blockade (fig 30A and B) as determined by PMA and ionomycin stimulation.

We next addressed whether addition of sCTLA-4 Ig affects the emergence of regulatory function in repetitively stimulated T cells. Blocking B7 co-stimulatory signalling by addition of sCTLA-4 Ig during the second round of culture with untreated DC completely abrogated regulatory function of IL-10$^+$ T cells at both high and low suppressor to responder ratio's (fig 31A and B).

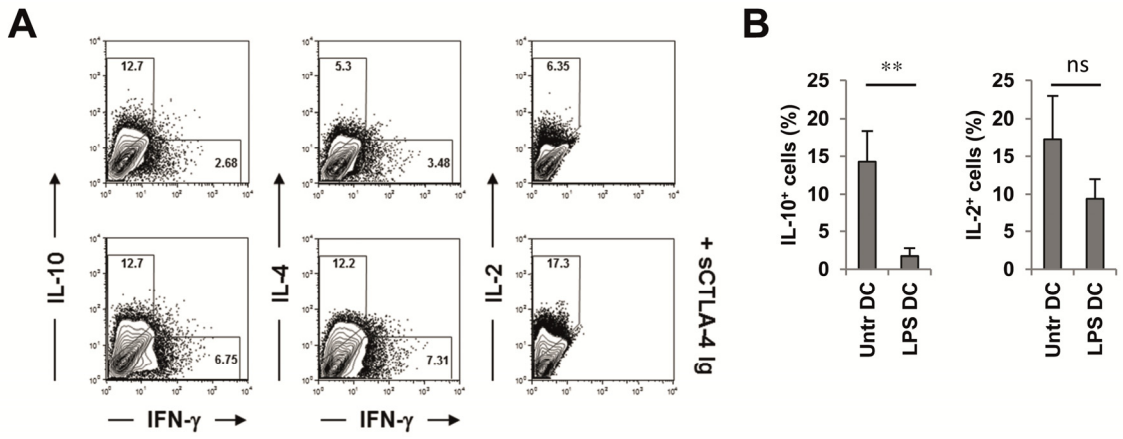


Figure 30. Addition of sCTLA-4 Ig does not impair IL-10 but restores IL-2 secretion of IL-10$^+$ regulatory T cells
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC in the presence sCTLA-4 Ig during the second stimulation when indicated. Primed T cells were restimulated with PMA/ionomycin and intracellular cytokine content determined by flow cytometry. One representative experiment is depicted. Numbers show percentages of cells within a particular gate. Cells were all gated on CD4$^+$ expressing cells. (B) as in (A) but CD4$^+$ CD25$^-$ OVA TCR-specific T cells were cultured for 2 rounds with untreated or LPS-stimulated DC in the presence sCTLA-4 Ig during the second stimulation when indicated. Graphs show mean data in addition of sCTLA-4 Ig only of at least 5 independent experiments. Cells were all gated on CD4$^+$ expressing cells. Statistical significance: ** $p < 0.01$ and ns = not significant, two-tailed Student's t test

Together, B7 co-stimulation is essential for the development of IL-10$^+$ regulatory T cells by untreated DC as it regulates CD25 and CTLA-4 expression, block in IL-2 cytokine release and emergence of regulatory function.

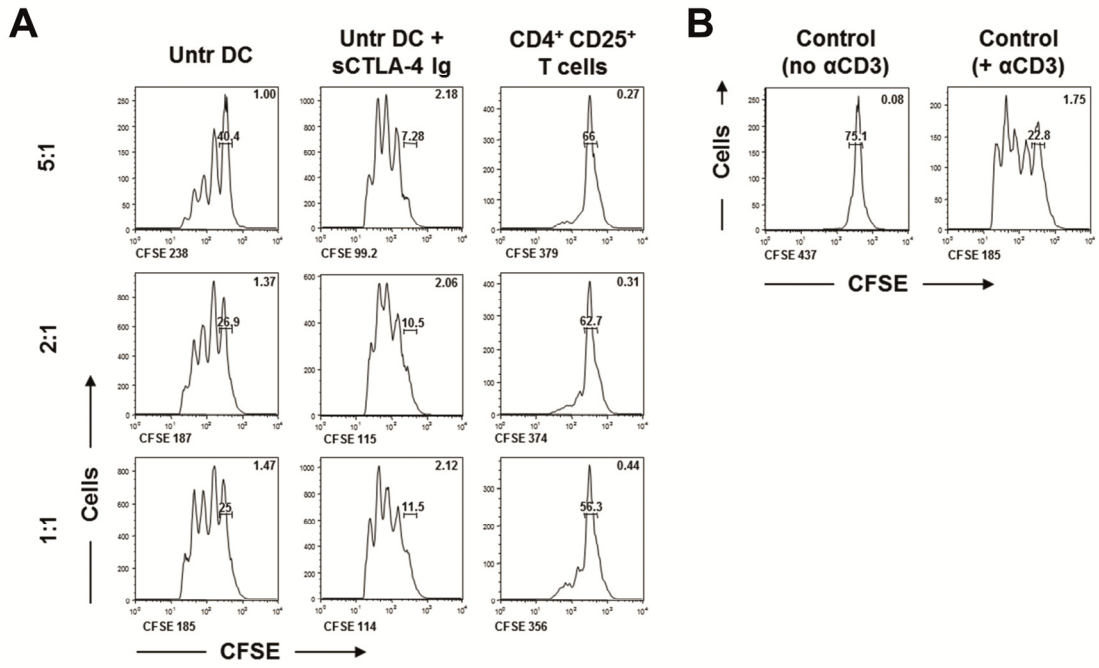


Figure 31. Addition of sCTLA-4 Ig prevents differentiation to CD4$^+$ regulatory T cells
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC in the presence of sCTLA-4 Ig during the second stimulation when indicated and were then used as suppressor cells in a suppression assay. After 72h, proliferation of responder T cells was determined by CFSE dilution. Suppressor to responder ratio is indicated next to the Y-axis. Numbers indicated in upper right corner show division indices; numbers above marker gate show percentages of undivided cells. MFI of CFSE is depicted under each histogram. One representative experiment of 6 independent experiments is shown. (B) as in (A) but graphs show proliferation of responder T cells without addition of suppressor cells in absence or presence of αCD3 antibodies.

3.2.6 IL-10$^+$ regulatory T cells show block in NFATc1/alpha activation

Next we determined the expression of NFAT transcription factors in IL-10$^+$ regulatory T cells generated in our *in vitro* co-culture system in cooperation with Martin Vaeth, Institute of Pathology, University of Wuerzburg. Several members and expression isoforms of the NFAT family of transcription factors have been identified [252], which not only initiate specific effector cytokine gene expression programmes for T cell activation, but also regulate expression of anergy-associated genes such as E3 ubiquitin ligases or Egr-2 [128, 253, 254].
One round of stimulation by mature DC induced profound nuclear translocation of NFATc1 and NFATc2 in striking contrast to T cells differentiated by untreated DC which showed only cytosolic appearance of both NFATc1 and 2 (data not shown). Moreover, inducible NFATc1/alpha expression was only observed in LPS-DC stimulated T cells (fig 32A). Interestingly, restimulation did not affect expression pattern of NFAT transcription factors as T cells derived from untreated DC cultures maintained predominant cytosolic NFATc1 and NFATc2 occurrence and failed to induce NFATc1/alpha expression (fig 32B and data not

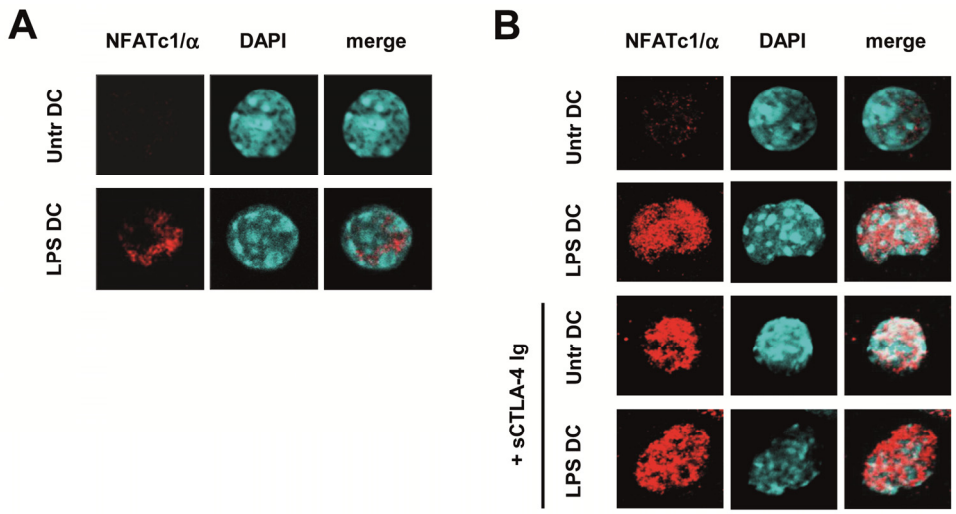


Figure 32. Stimulation of CD4$^+$ T cells with untreated DC fails to induce nuclear NFAT/c1α expression
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 7 days with untreated or LPS-stimulated DC in the presence of OVA peptide. NFATc1/α expression was determined on cytospins and detected by confocal microscopy. One representative experiment is shown. (B) as in (A) but CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC or LPS-stimulated DC in the presence of sCTLA-4 Ig during the second stimulation when indicated. Immunofluorescence staining and confocal analysis were performed by Martin Vaeth, Institute of Pathology, University of Wuerzburg according to methods described elsewhere [255].

shown). Strikingly, addition of sCTLA-4 Ig completely reversed NFAT profile of T cells differentiated by untreated DC shown by nuclear translocation of both NFATc1 and NFATc2 and induced NFATc1/alpha expression similar to the expression pattern observed in LPS-DC activated controls (fig 32B and data not shown). Since FoxP3$^+$ regulatory T cells fail to translocate NFATc1 to the nucleus but show predominant nuclear expression of ICER/CREM, a regulator of IL-2 gene expression [255], we next addressed whether ICER/CREM is expressed in IL-10$^+$ regulatory T cells as generated by two consecutive stimulations of naïve CD4$^+$ T cells with untreated DC. No differential translocation and/or particular expression of ICER/CREM could be detected in untreated versus LPS-DC stimulated T cells (data not shown) suggesting that the IL-2 synthesis block of IL-10$^+$ regulatory T cells as generated here is regulated rather independent from ICER/CREM translocation. Reverse signalling through B7 co-stimulatory ligands on the activated T cell surface might induce ICER/CREM expression [163]. However, T cells showed only modest expression of CD80 or CD86 after one (fig 33B) or two culture rounds with untreated DC (fig 33C) compared to levels detected on LPS-DC stimulated controls (fig 33).

Taken together, IL-10$^+$ regulatory T cells generated by repetitive stimulation of naïve T cells by untreated DC suppress the nuclear translocation of NFATc1 and 2 and block the activation of inducible NFATc1/alpha by a B7 co-stimulatory dependent mechanism.

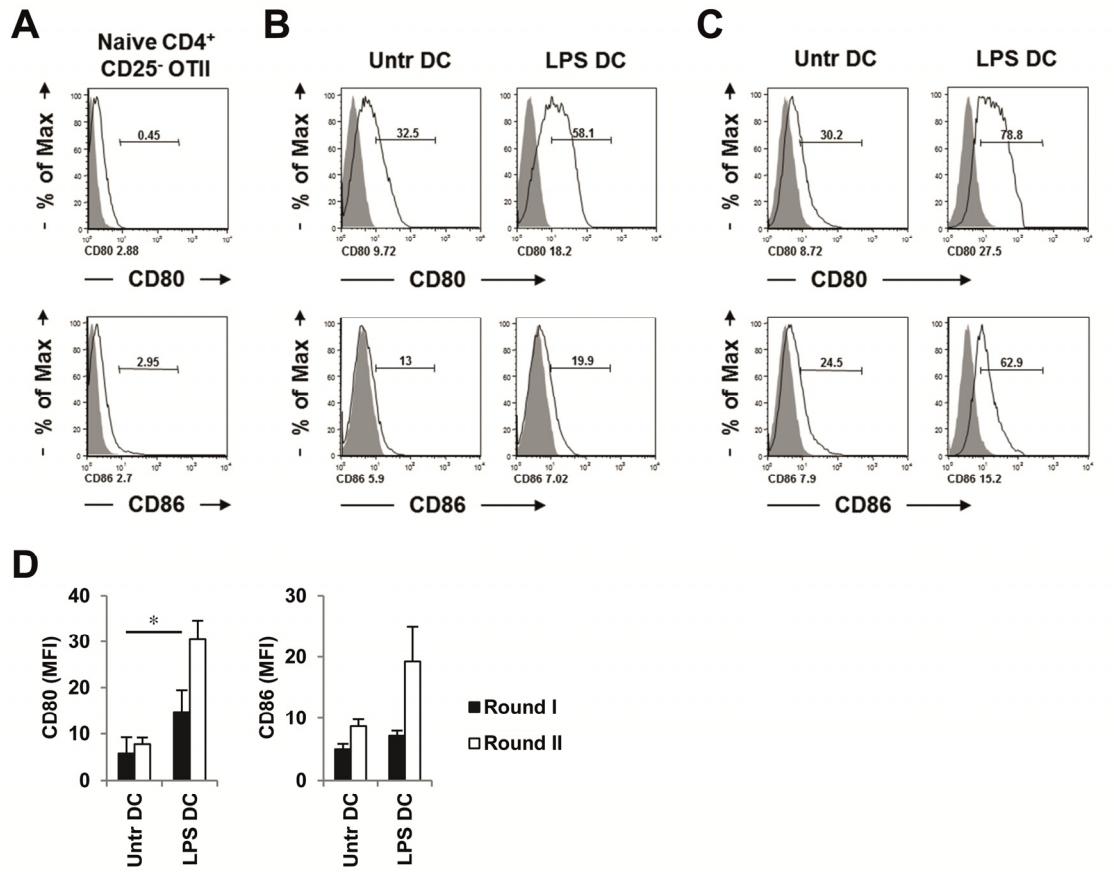


Figure 33. IL-10$^+$ regulatory T cells do not show increased B7 co-stimulatory molecule expression
(A) Naïve CD4$^+$ CD25$^-$ OVA-TCR specific T cells were immediately stained or stimulated for 1 (B) or 2 (C) consecutive rounds with untreated or LPS conditioned DC prior to staining and determination of surface marker expression by flow cytometry. Numbers in histograms show percentages of cells in a particular gate. Numbers under each histogram represent MFI. The shaded curves in the histogram plots show unstained or isotype controls. Cells were gated on CD11c^{neg} and CD4$^+$ expressing cells. One representative experiment is shown. (D) as in (B) for 1 round or (C) for 2 rounds, but graphs show mean data from 3 or 2 independent experiments respectively. Statistical significance: * $p < 0.05$, two-tailed Student's t test

3.2.7 CD28 signalling cascade regulates CD25 expression, blocks IL-2 secretion and imposes suppressive capacity for the development of IL-10+ regulatory T cells

In order to define which B7 counter-receptors regulate the development of IL-10$^+$ regulatory T cells, Fab fragments of αCD28 or αCTLA-4 antibodies were added during the second round of T cell stimulation in our *in vitro* co-culture system. Addition of αCTLA-4 Fab fragments decreased detection of intracellular CTLA-4 on primed T cells by flow cytometry to 42.2% ± 15.6 when added at 30μg/ml (fig 34A and B). Although CD28 is expressed at much lower density on the surface of primed T cells (fig 34C), addition of αCD28 Fab fragments reduced detection of CD28 on activated T cells efficiently (fig 34D). Since CTLA-4 molecules show a continuous intracellular recycling mode [52], αCTLA-4 Fab fragments

were added in excess to the T cell cultures. αCD28 Fab fragments added during the second round of DC/T cell co-cultures significantly downregulated expression of CD25 on primed T cells almost to a similar extent as seen upon addition of sCTLA-4 Ig (fig 35A and B, right panel). In contrast, blocking CTLA-4 signalling further increased CD25 surface levels on T cells compared to untreated conditions (fig 35A and B, right panel) suggesting that CD28 signalling cascades critically upregulate CD25 expression. As shown previously (fig 29A and B), T cells rather decrease levels of intracellular CTLA-4 in the presence of sCTLA-4 Ig (fig 35A and B, left panel). Similar findings were detected in cultures favouring CD28 signalling

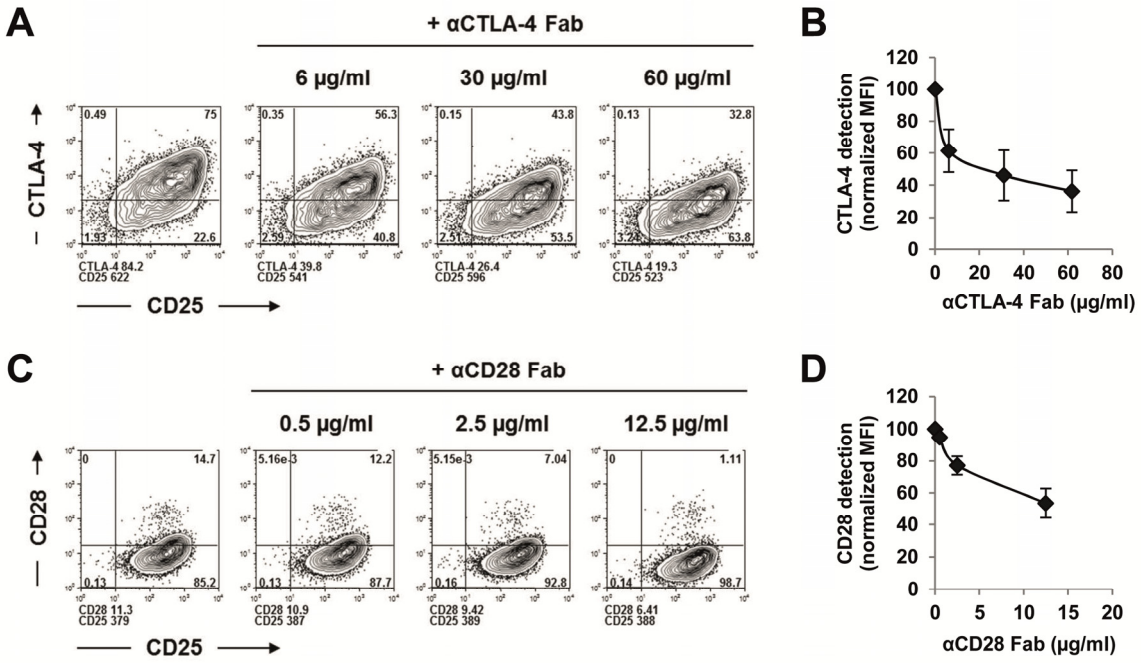


Figure 34. CD28 or CTLA-4 Fab fragments prevent detection of respective signalling molecules on primed T cells
(A) $CD4^+$ $CD25^-$ OVA-TCR specific T cells stimulated for 7 days with untreated or LPS conditioned DC were incubated with αCTLA-4 Fab fragments at indicated concentrations prior to staining of intracellular CTLA-4 and detection by flow cytometry. The inserted numbers within plots represent the percentages of cells. Numbers under each graph show MFI. Cells were gated on $CD4^+$ expressing cells. One representative experiment of 2 experiments is shown. (B) as in (A) but graph shows mean data normalized to values of untreated controls, i.e. in absence of Fab fragments. (C) $CD4^+$ $CD25^-$ OVA-TCR specific T cells were stimulated for 7 days with untreated or LPS conditioned DC followed by flow cytometry staining of surface CD28. Cells were incubated with αCD28 Fab fragments at indicated concentrations prior to staining of surface markers. The inserted numbers within plots represent percentages of cells. Numbers under each graph show MFI values. Cells were gated on $CD4^+$ expressing cells. One representative experiment is shown. (D) as in (C) but graph shows mean data of 2 independent experiments normalized to values of untreated controls.

whereas αCD28 Fab fragments rather upregulated CTLA-4 expression on primed T cells although levels did not always reached statistical significance (fig 35B, left panel). Effector cytokine production of IFN-γ or IL-13 remained largely unaffected by B7 co-stimulatory signalling modulators (fig 36A). Targeting CD28 signalling cascades with Fab fragments seemed to affect IL-10 production slightly (fig 36A) but not under PMA/ionomycin

conditions (fig 36B and C) which we showed previously in fig 30A upon addition of sCTLA-4 Ig. Secretion of IL-2 was enhanced in T cells cultured with sCTLA-4 Ig (fig 36A and C) but unexpectedly also in the presence of αCD28 Fab fragments as determined by TCR-dependent stimulation or by mitogenic agents bypassing TCR triggering (fig 36A and C, respectively). The role of CD28 signalling pathways in suppression of IL-2 synthesis or secretion was further strengthened by the observation that T cells failed to block proliferation of responder T cells if suppressor cells were differentiated in the presence of αCD28 or sCTLA-4 Ig but not αCTLA-4 Fab fragments (fig 37).

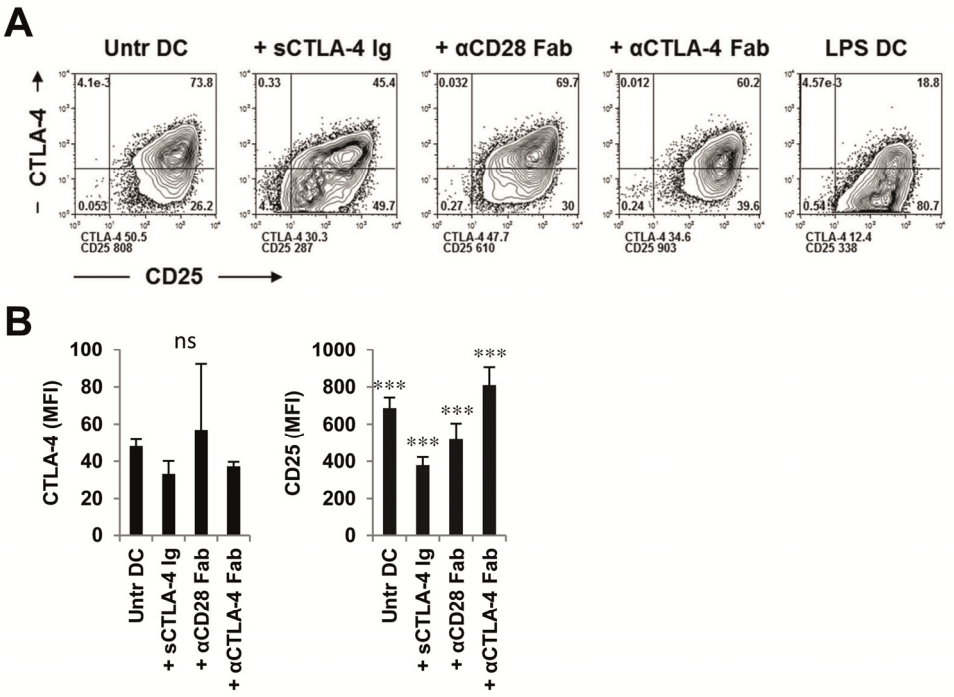


Figure 35. CTLA-4 and CD28 signalling cascades differentially regulate CTLA-4 and CD25 expression on IL-10$^+$ CD4$^+$ regulatory T cells

(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC in the presence of sCTLA-4 Ig, αCD28 Fab or αCTLA-4 Fab fragments during the second stimulation when indicated or with LPS conditioned DC. Surface markers or intracellular CTLA-4 expression was determined by flow cytometry. The inserted numbers in each plot represent percentages of cells in a particular quadrant. Numbers below each plot indicate the MFI values. Cells were all gated on CD4$^+$ expressing cells. One representative experiment is depicted. (B) as in (A) but graphs show mean data of up to 4 independent experiments. Statistical significance: *** $p < 0.001$ and ns = not significant, one-way ANOVA followed by Bonferroni post-test

Targeting B7 co-stimulatory signalling cascades by Fab fragments revealed an unexpected crucial role for CD28 signalling in the upregulation of CD25 surface marker expression, suppression of IL-2 synthesis/release and hence, conversion of regulatory activity of IL-10$^+$ regulatory T cells generated *in vitro* by untreated DC.

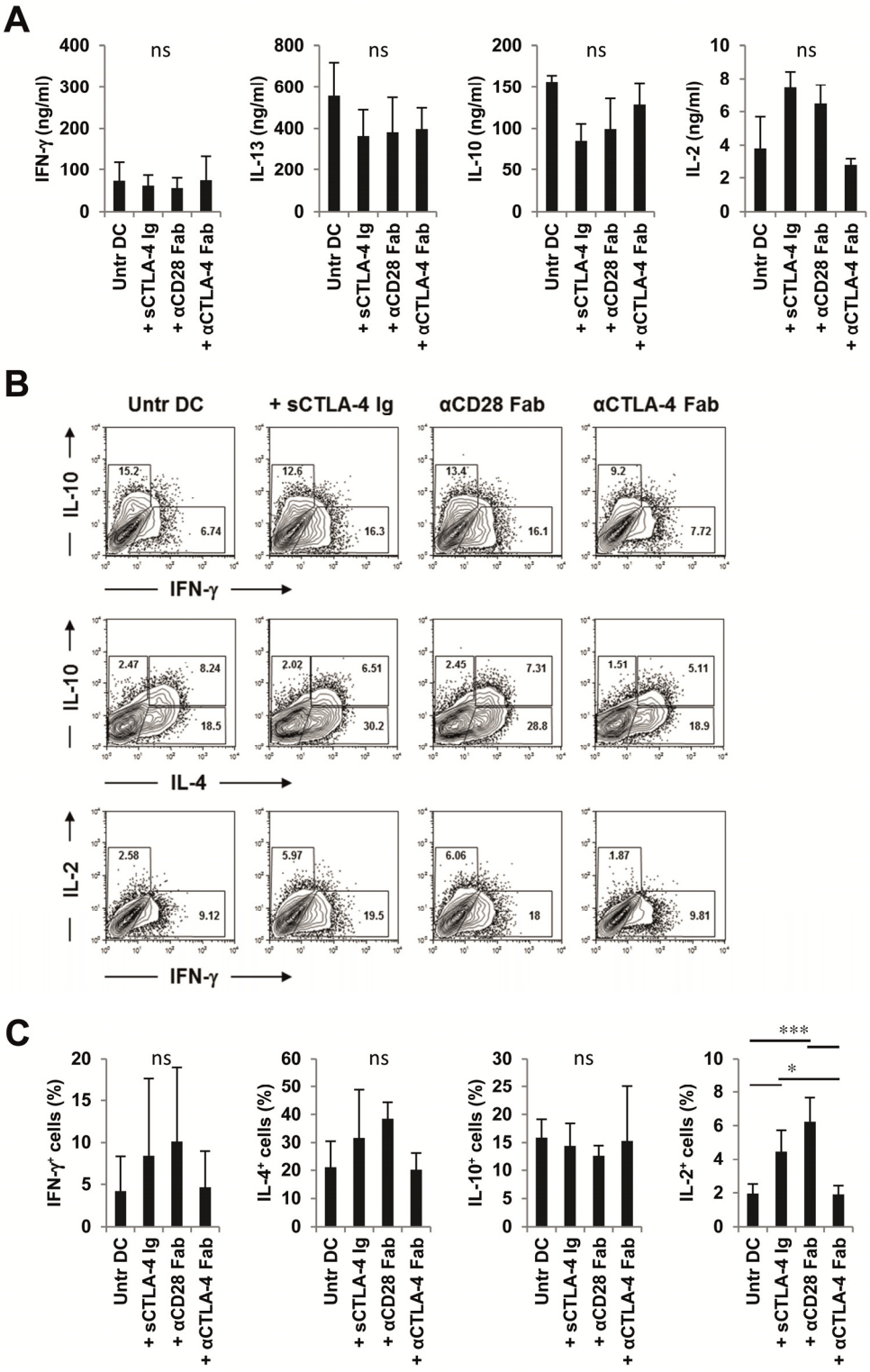


Figure 36. CD28 signalling blocks IL-2 cytokine secretion of IL-10$^+$ CD4$^+$ regulatory T cells
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC in the presence of sCTLA-4 Ig, αCD28 Fab or αCTLA-4 Fab fragments during the second stimulation when indicated. T cells were restimulated on plate-bound αCD3 antibodies for 2 to 3 days followed by supernatants harvesting and cytokine analysis by ELISA. Graphs show mean data of 2 independent experiments. (B) as in (A) but primed T cells were restimulated with PMA/ionomycin and intracellular cytokine content determined by flow cytometry. One representative experiment is depicted. Numbers show percentages of cells within a particular gate. Cells were all gated on CD4$^+$ expressing cells. (C) as in (B) but graphs show mean data of 3 independent experiments. Cells were all gated on CD4$^+$ expressing cells. Statistical significance: * $p < 0.05$, *** $p < 0.001$ and ns = not significant, one-way ANOVA followed by Bonferroni post-test

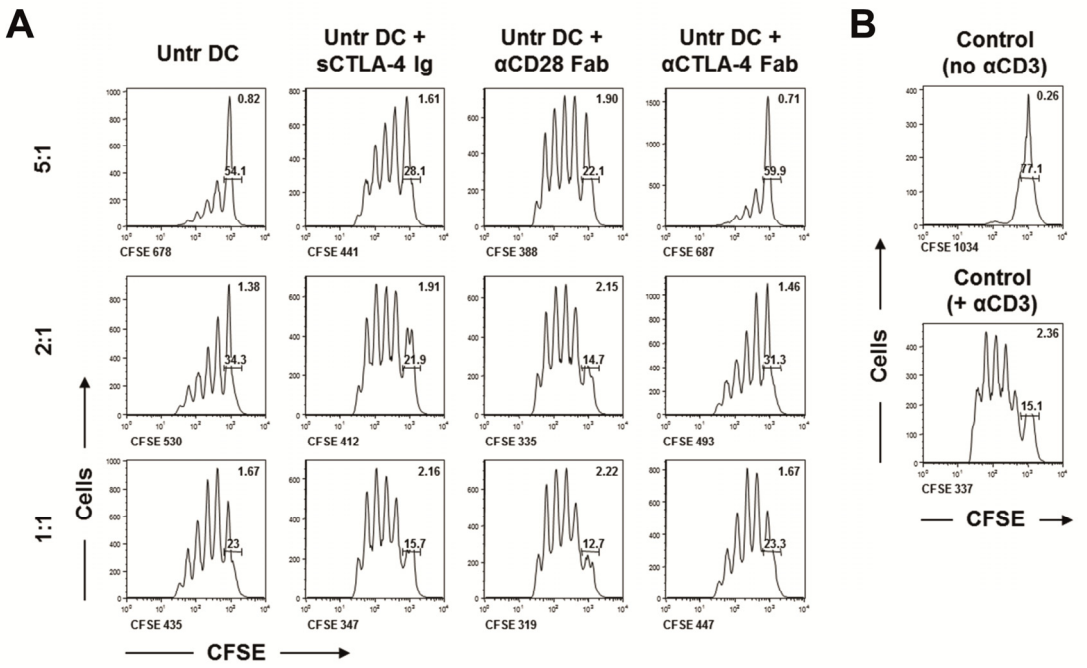


Figure 37. CD28 signalling blockade impairs differentiation into regulatory T cells
(A) $CD4^+$ $CD25^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC in the presence of sCTLA-4 Ig, αCD28 Fab or αCTLA-4 Fab fragments during the second stimulation when indicated. Primed T cells were then used as suppressor cells in a suppression assay. After 72h, proliferation of responder T cells was determined by CFSE dilution. Suppressor to responder ratios are indicated next to the Y-axis. Numbers indicated in upper right corner show division indices; numbers above marker gate show percentages of undivided cells. MFI values of CFSE are depicted under each histogram. One representative experiment of 2 independent experiments is shown. (B) as in (A) but graphs show proliferation of responder T cells without addition of suppressor cells in absence or presence of αCD3 antibodies.

3.2.8 CTLA-4 signalling is required to maintain anergic phenotype of IL-10+ regulatory T cells

The role of CTLA-4 as a negative regulator of T cell activation is well-established [52]. Given the elevated expression of CTLA-4 on $IL\text{-}10^+$ regulatory T cells, we ought to determine how CTLA-4 contributes to the development of $IL\text{-}10^+$ regulatory T cells distinct from the CD28-dependent signalling functions as discussed previously. The anergic phenotype of $IL\text{-}10^+$ regulatory T cells was further deteriorated by blocking B7 co-stimulation with sCTLA-4 Ig as compared to untreated controls (fig 38A). Similar observations were made in the presence of αCD28 Fab fragments thereby favouring CTLA-4 signalling, whereas addition of αCTLA-4 Fab fragments during the DC/T cell co-cultures hardly modified proliferation of TCR-restimulated T cells (fig 38A). Only addition of exogenous IL-2 restored proliferation efficacy of T cells differentiated by untreated DC (fig 38). Interestingly, a trend to hyper-proliferation on exogenous IL-2 could be observed predominately within T cell cultures supplemented with sCTLA-4 Ig (fig 38). Real-time quantitative analysis of Egr-2 transcription factor expression

revealed that disturbing CTLA-4 signalling in primed T cells through addition of αCTLA-4 Fab fragments seemed to decrease Egr-2 relative expression compared to untreated controls (fig 38B). Furthermore, masking B7 co-stimulatory molecules by sCTLA-4 Ig resulted in marked upregulation of Egr-2 expression compared to untreated cultures (fig 38B) suggesting that the CTLA-4 signalling cascade crucially contributes to the maintenance of the anergic phenotype of IL-10$^+$ regulatory T cells. Indeed, confocal analysis of the expression pattern of inducible NFATc1/alpha demonstrated that T cells abrogated NFATc1/alpha expression in addition of αCD28 Fab fragments whereas expression of inducible NFATc1/alpha was dramatically upregulated in the presence αCTLA-4 Fab fragments and hence, when CD28 signal transduction was favoured (fig 39A and B).

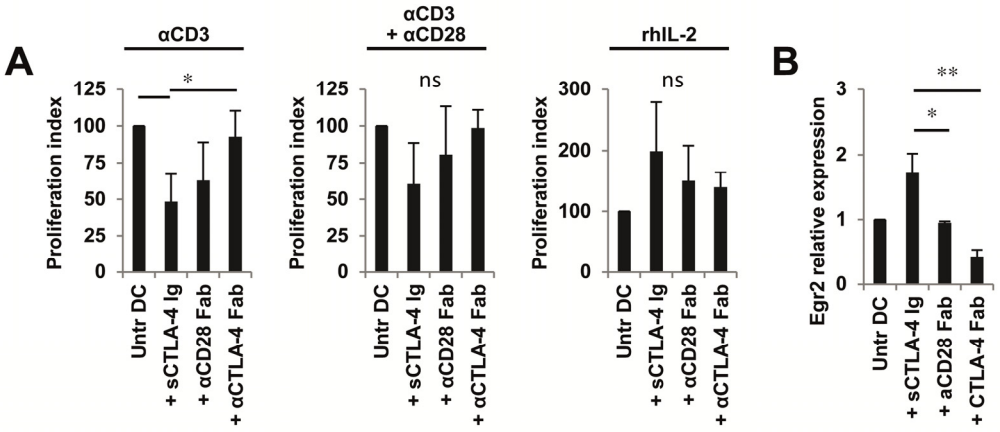


Figure 38. CTLA-4 signalling is required to maintain anergic phenotype of IL-10$^+$ CD4$^+$ regulatory T cells
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC in the presence of sCTLA-4 Ig, αCD28 Fab or αCTLA-4 Fab fragments during the second stimulation followed by restimulation on plate-bound αCD3, αCD3/CD28 or rhIL-2. Proliferation was measured by [^{3}H]-methyl-thymidine incorporation 2 days after restimulation. Graphs show proliferation indices as values normalized to untreated DC controls and represent mean data of 4 independent experiments. (B) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured as described in (A) followed by RNA isolation. Real-Time quantitative PCR for Egr2 was performed on cDNA and expression normalized to β-2-microglobulin levels. Relative expression is shown as levels to untreated DC stimulated controls. Results shown are mean data of 2 independent experiments. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and ns = not significant, one-way ANOVA followed by Bonferroni post-test

In sum, CTLA-4 signalling contributes to the development of IL-10$^+$ regulatory T cells by untreated DC through the maintenance of an anergic phenotype at least in part by the active suppression of inducible NFATc1/alpha expression.

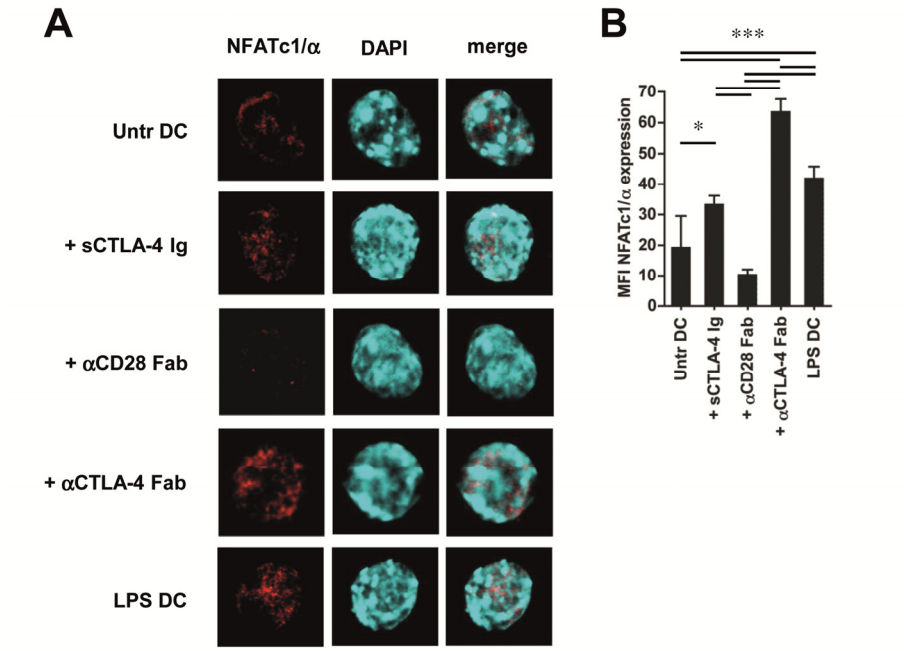


Figure 39. CTLA-4 signalling regulates the hampered NFAT/c1α expression in IL-10$^+$ CD4$^+$ regulatory T cells
(A) CD4$^+$ CD25$^-$ OVA-TCR specific T cells were cultured for 2 rounds with untreated DC in the presence of sCTLA-4 Ig, αCD28 Fab or αCTLA-4 Fab fragments during the second stimulation when indicated or with LPS conditioned DC. NFATc1/α expression was determined on cytospins and detected by confocal microscopy. One representative experiment is shown. (B) as in (A) but graph shows MFI values of at least 15 cells analysed on the confocal microscope. Immunofluorescence staining and confocal analysis performed by Martin Vaeth, Institute of Pathology, University of Wuerzburg according to methods described elsewhere [255]. Statistical significance: * $p < 0.05$, *** $p < 0.001$, one-way ANOVA followed by Bonferroni post-test

3.3 Similar inflammatory dendritic cell maturation signatures induced by TNF or *Trypanosoma brucei* antigens instruct default T helper-2 cell responses

3.3.1 Partial maturation of DC by TNF and *T. brucei*-derived VSG antigens.

To dissect whether pathogenic signals instruct distinct DC maturation phenotypes compared to the inflammatory cytokine TNF, different *Trypanosoma brucei* antigens were compared with TNF and LPS to induce surface marker expression, cytokine secretion and differential expression of Notch ligands on DC. In this study, *T. brucei* components eliciting MyD88-dependent macrophage activation were used, i.e. soluble variant-specific surface glycoproteins (sVSG) and membrane-bound VSG (mfVSG) from the AnTat1.1 strain and sVSG from the *T. brucei* MiTat1.5 strain [256, 257].

All stimuli upregulated the expression of MHC II, CD40, CD80 and CD86 surface markers compared to untreated DC (fig 40A and B). The induction by TNF and *T. brucei* antigens mfVSG and MiTat1.5-derived sVSG was, however, below the expression levels achieved by LPS- or AnTat1.1-derived sVSG conditioned DC (fig 40A and B). Cytokine analysis revealed that TNF conditioned DC do not secrete cytokines or only at very minor levels IL-12p40 or IL-6 (fig 40C) as shown before [16]. The *T. brucei* AnTat1.1-derived mfVSG induced minor amounts of IL-12p40 in DC as compared to TNF-matured DC. Interestingly, MiTat1.5-derived sVSG induced substantial IL-6 cytokine release and some IL-1β. None of the stimuli induced IL-12p70 in contrast to LPS-matured and AnTat1.1-derived sVSG-stimulated DC, which secreted high amounts of all cytokines tested (fig 40C). Furthermore, LPS or AnTat1.1-derived sVSG stimulation of DC showed a higher relative mRNA expression of the Th1-instructive Notch ligand Delta4 and of Jagged1 but downregulated Jagged2 (fig 41). In contrast, the *T.brucei* antigens mfVSG and MiTat1.5-derived sVSG induced high expression of the Th2-associated Jagged2 but showed only low levels of Delta4 and this to a similar extent as TNF stimulation (fig 41).

Together, TNF and the *T. brucei* antigens AnTat1.1-derived mfVSG and MiTat1.5-derived sVSG only partially mature DC as detected by upregulation of surface markers, no or low cytokine production and high relative expression of the Notch ligand Jagged2. In contrast, the AnTat1.1-derived sVSG resembles more LPS-matured DC. Therefore, and within the major

scope of this study, subsequent experiments were conducted with the *T. brucei*-derived mfVSG and MiTat1.5 sVSG antigens.

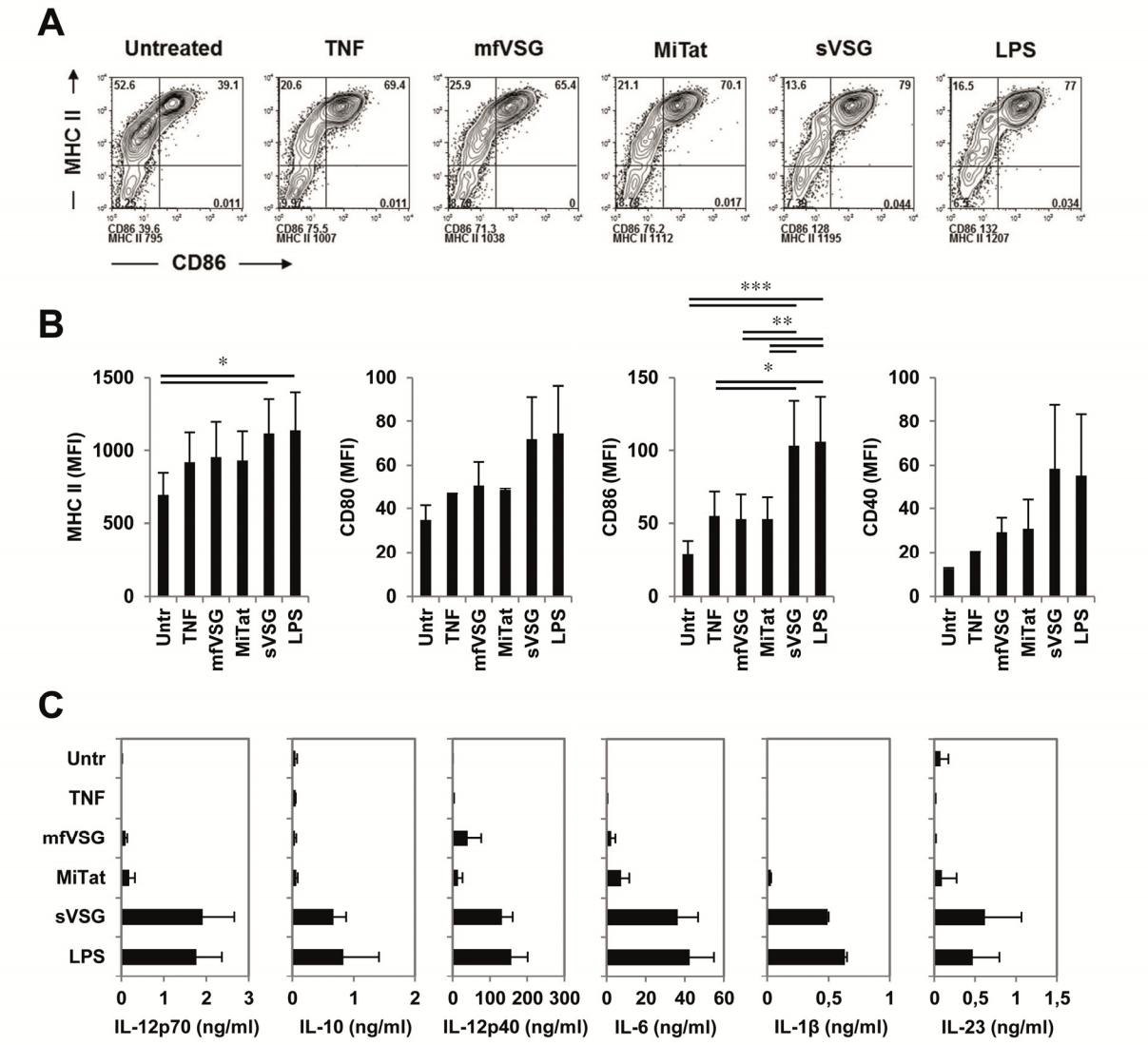


Figure 40. TNF and the *T.brucei* antigens AnTat1.1-derived sVSG and mfVSG, Mitat1.5 sVSG induce upregulation of surface markers expression but different amounts of cytokine production in DC
(A) DC were stimulated for 24h with the indicated stimuli, stained for surface markers and tested by flow cytometry. The inserted numbers show percentages of cells in a particular quadrant. Numbers under each plot indicate the MFI. Cells were gated on CD11c^{high} expressing cells. (B) as in (A) but figures represent mean fluorescence intensities (MFI) of up to 7 independent experiments. (C) Supernatants were harvested and tested for their cytokine content by ELISA. Results are mean data from up to 5 independent experiments. Statistical significance: * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$, one-way ANOVA followed by Bonferroni post-test

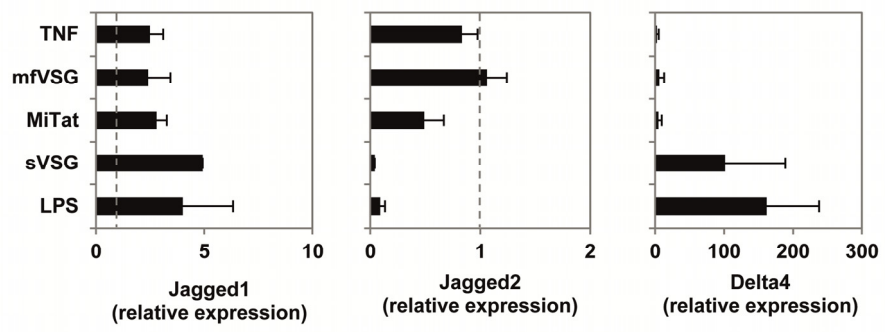


Figure 41. TNF and the *T.brucei* antigens AnTat1.1-derived mfVSG and Mitat1.5 sVSG show relative expression of the Notch ligand Jagged2
DC were matured for 6h with the indicated stimuli followed by RNA isolation. Real-Time quantitative PCR for Jagged1, Jagged2 and Delta4 was performed on cDNA and expression normalized to β-actin levels. Relative expression is represented as levels to untreated DC controls (indicated by dashed line). Results shown are mean data of 2 independent experiments.

3.3.2 Partial DC maturation induced by *T. brucei*-derived VSG antigens is MyD88-dependent.

Next, bone marrow cells were prepared from mice deficient in TLR4 and/or MyD88 adaptor protein signaling to define which pattern recognition receptor cascade is required for the observed partial maturation phenotypes.

DC defective in TLR4 signaling still upregulated MHC II and CD86 upon mfVSG exposure, but largely failed to increase surface markers expression in TLR4/MyD88$^{-/-}$ DC (fig 42). Surprisingly, maturation by MiTat1.5-derived sVSG was almost completely blocked in DC insensitive for TLR4-mediated stimuli and this to a similar extent as LPS-treated DC. In contrast, MHC II and CD86 upregulation remained unimpaired upon TNF conditioning of TLR4 insensitive or TLR4/MyD88$^{-/-}$ DC.

Together, these data indicate that *T.brucei*-derived antigens induce distinct partial maturation stages in DC dependent on MyD88 signaling.

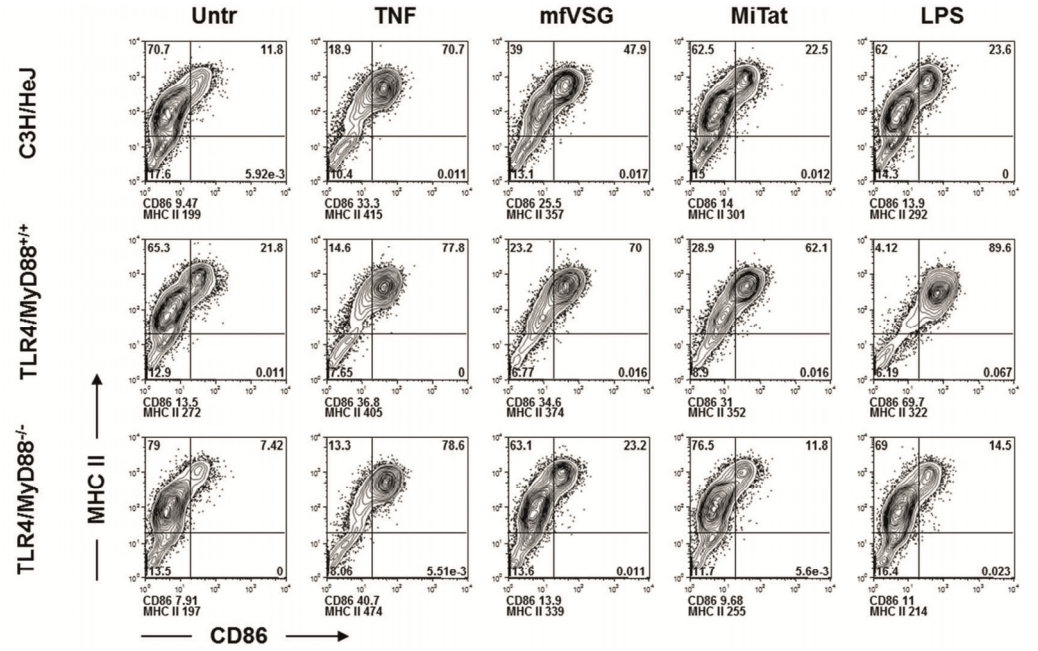


Figure 42. *T.brucei* antigens induce MyD88 dependent maturation of DC
DC generated from C3H/HeJ, TLR4/MyD88$^{+/+}$ and TLR4/MyD88$^{-/-}$ mice were stimulated for 24h with the indicated stimuli, stained for the surface markers MHC II and CD86 and tested by flow cytometry. Numbers in each quadrant represent percentages of cells; numbers under plots show MFI. Cells were gated on CD11c^{high} expressing cells.

3.3.3 DC conditioning by TNF or *T. brucei*-derived VSG antigens share similar gene expression profiles

Since the previous experiments did not reveal major differences in the maturation profiles of TNF-, LPS- or VSG-stimulated DC, microarray analyses were conducted with the differentially stimulated DC to cover a broader spectrum of gene regulations. After 24h DC treatments, cells were prepared for the arrays, performed in collaboration with Vladimir Pavlovic, Institute of Virology and Immunobiology, University of Wuerzburg and analysed by Susanne Kneitz, Microarray Core Unit, Interdisciplinary Center for Clinical Research (IZKF), University of Wuerzburg.

The data indicated that LPS stimulation was much different from that by TNF, mfVSG and MiTat1.5 sVSG and the latter were highly similar to each other and not so different from untreated DC (fig 43A). More detailed analyses of differentially expressed genes indicated that only 175 genes were induced after TNF, 160 with mfVSG, 466 with MiTat1.5 sVSG but 4969 with LPS were changed more than 2-fold over untreated DC (fig 43B). The whole microarray array data are accessible under GEO (www.ncbi.nlm.nih.gov/geo/). These data also indicate that a common inflammatory signature of 24 genes that is shared among all four

conditions (fig 43C). These genes are largely overlapping with those reported by others before as a typical inflammatory pattern for DC [258] and thereby indicate the reliability of the here applied microarray approach. The different intensities of induction between TNF/mfVSG/MiTat1.5 sVSG and LPS further strengthen the semi-mature state of the former group (fig 43C). Remarkably, this common signature is also completely shared among the stimuli TNF, mfVSG and MiTat1.5 sVSG, since no different or additional genes were induced (triple field with 0 genes, fig. 43B). Thus, the semi-mature DC signature was represented by upregulation of CD40, CD72, IL-1α, IL-1β, IL-6, CXCL2, SOCS3, Jagged-1, Pleckstrin-2 (Plek2), serum amyloid 3(Saa3), ladinin (Lad), follistatin, (FST), activin (Inhba) and down-regulation of PGE-receptor 3 (Ptger3), CD62L (Sell) SIGNR2 (CD209c). In contrast, the fully matured DC signature of genes induced by LPS include the common 24 genes, but regulated additional 4498 genes that were not shared with the other stimuli (fig 43B). The exclusive gene signatures induced by TNF alone or the comparisons of mfVSG with MiTat1.5 sVSG were not marked by a strong immunological signature of gene regulations (data not shown).

Taken together, the common signature of DC matured by TNF, mfVSG and MiTat1.5 sVSG induces much less genes than LPS, which are mainly characterized by a common signature of 24 mostly inflammatory genes.

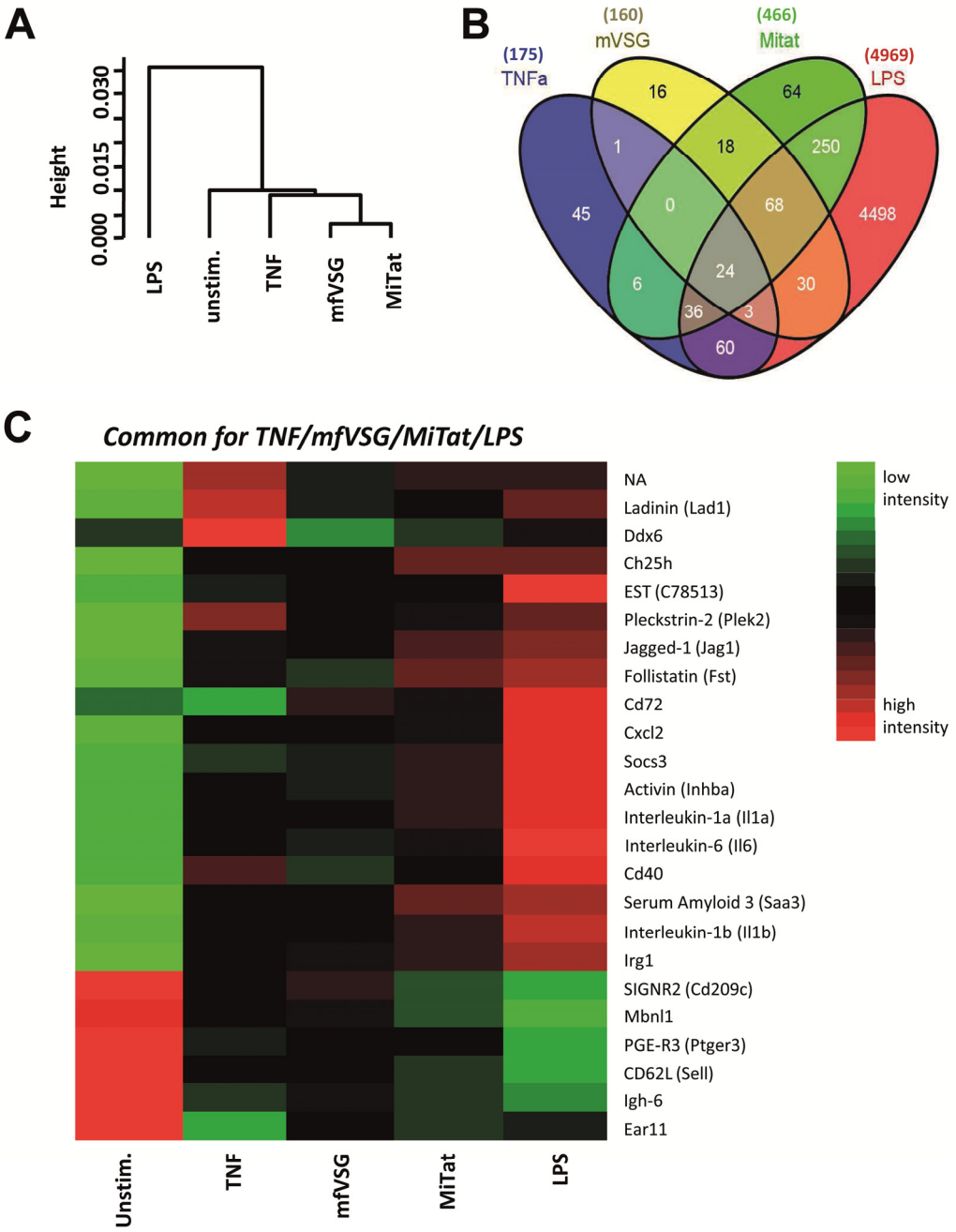


Figure 43. Similar gene expression signatures at partial DC maturation stages
Microarray analysis was performed with BM-DC after treatment for 24h with the indicated stimuli and compared with untreated DC. (A) Hierarchical cluster analysis of gene expression profiles of TNF/mfVSG/MiTat1.5 sVSG stimulated DC as compared with LPS stimulated DC. (B) VENN-diagram indicating the number of differentially regulated genes after the differential treatments. Induction was considered when expression was greater than 2-fold over untreated DC. 24 genes comprising an inflammatory pattern are commonly regulated. (C) Heat map of signal intensities of DC after TNF, mfVSG or MiTat1.5 sVSG stimulation as compared with untreated DCs. Data show single time point values of a single microarray (for Materials and Methods, see [259]). Data set performed in collaboration with Vladimir Pavlovic, Institute of Virology and Immunobiology, University of Wuerzburg for DC preparations and maturation and Susanne Kneitz, Microarray Core Unit, Interdisciplinary Center for Clinical Research (IZKF), University of Wuerzburg for microarray preparations and analysis.

3.3.4 Partial DC maturation stages as induced by TNF or *T. brucei* antigens instruct an IL-4 producing Th cell but no regulatory T cell differentiation pattern

To dissect the importance of the partial DC maturation phenotypes in directing distinct T helper cell differentiation patterns, DC were co-cultured with $CD4^+$ OT-II T cells and the T

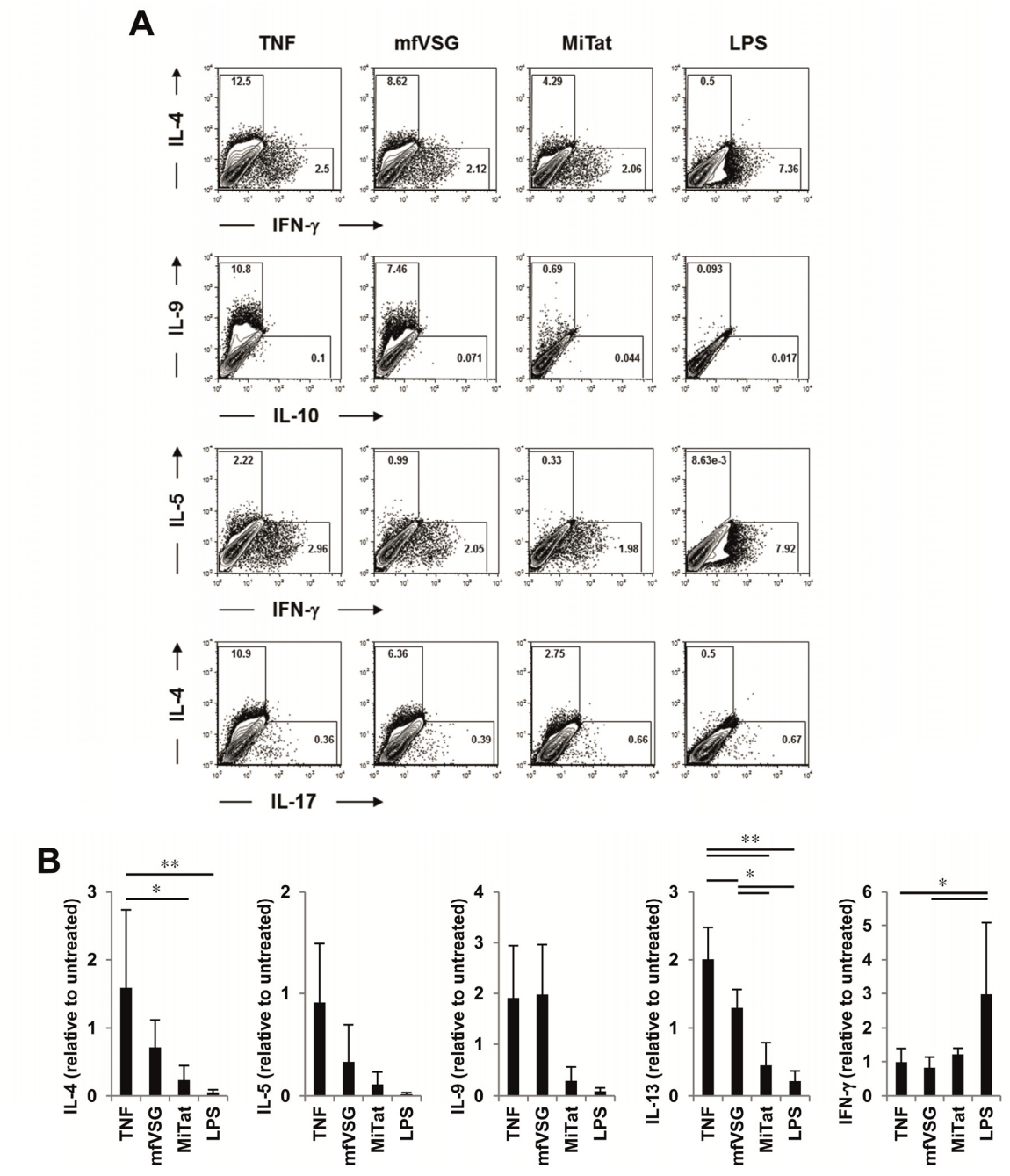


Figure 44. Partially matured DC direct Th2 cell differentiation
(A) DC were matured with the indicated stimuli and co-cultured in the presence of OVA peptide with naïve $CD4^+$ OT-II T cells *in vitro*. At day 5 of culture, T cells were restimulated with PMA and ionomycin and intracellular cytokine levels were determined by flow cytometry. Numbers in gates represent percentage cytokine positive cells gated on CD4 expressing cells. One representative experiment of 4 experiments is shown. (B) Levels of cytokine positive cells as in depicted in (A) but normalized by division to untreated samples. Figures show mean data of up to 4 independent experiments. Statistical significance: * $p<0.05$; ** $p < 0.01$, one-way ANOVA followed by Bonferroni post-test

helper cell profiles were checked by intracellular cytokine staining.

Polarizing by LPS showed a clear shift towards IFN-γ, indicating a Th1 profile. DC maturation with TNF and mfVSG shifted the T cells towards a Th2/Th9 pattern and DC stimulation with MiTat1.5 sVSG heavily reduced Th2 and Th9 but left the Th1 background profile unaltered (fig 44A and B). Furthermore, induction of IL-17 production in T cells was negligible under all conditions (fig 44A and B) and T cells did not produce anti-inflammatory IL-10 after one round of DC stimulation (not shown) and as reported previously [16].

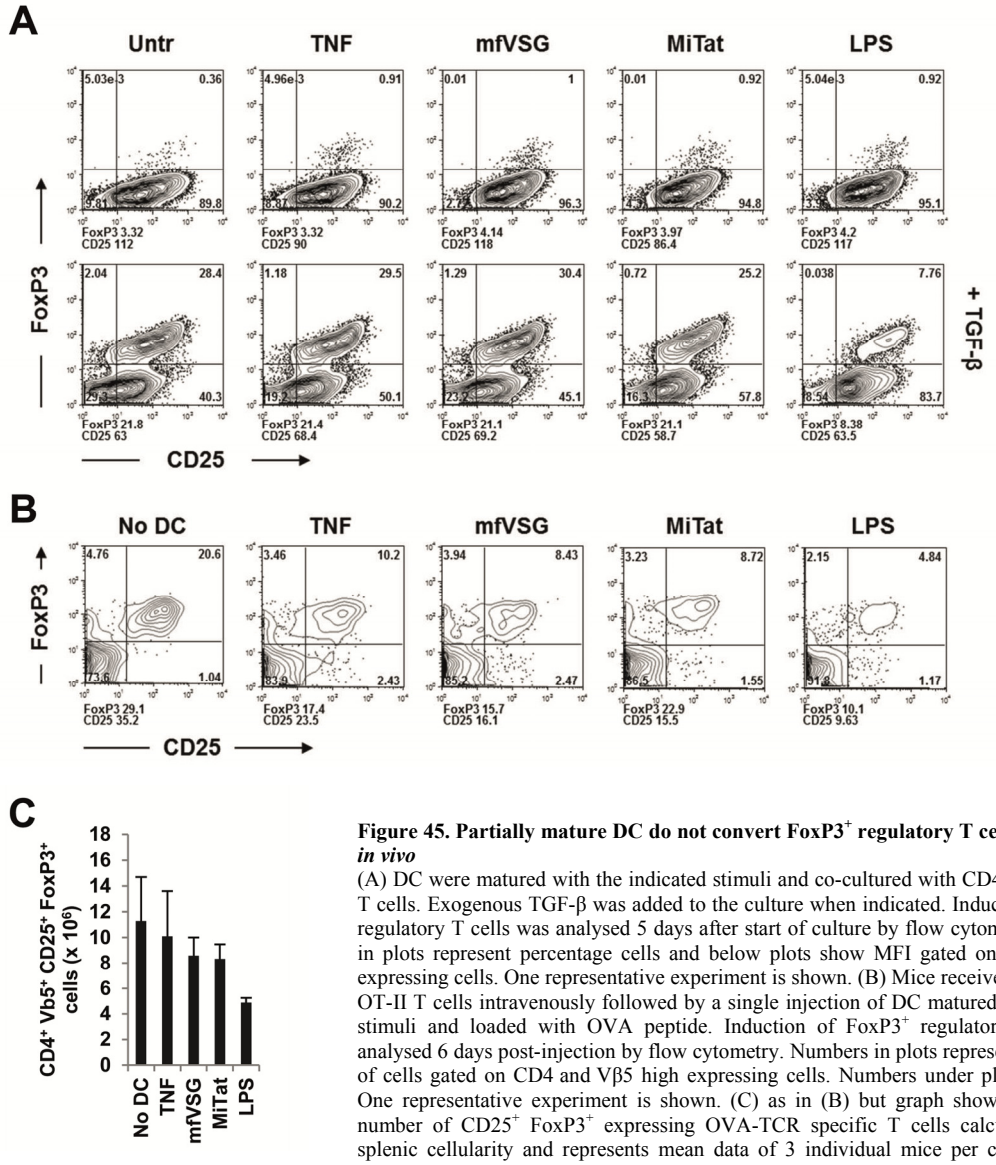


Figure 45. Partially mature DC do not convert FoxP3$^+$ regulatory T cells *in vitro* and *in vivo*

(A) DC were matured with the indicated stimuli and co-cultured with CD4$^+$ CD25$^-$ OT-II T cells. Exogenous TGF-β was added to the culture when indicated. Induction of FoxP3$^+$ regulatory T cells was analysed 5 days after start of culture by flow cytometry. Numbers in plots represent percentage cells and below plots show MFI gated on CD4 and Vβ5 expressing cells. One representative experiment is shown. (B) Mice received CD4$^+$ CD25$^-$ OT-II T cells intravenously followed by a single injection of DC matured with indicated stimuli and loaded with OVA peptide. Induction of FoxP3$^+$ regulatory T cells was analysed 6 days post-injection by flow cytometry. Numbers in plots represent percentages of cells gated on CD4 and Vβ5 high expressing cells. Numbers under plots show MFI. One representative experiment is shown. (C) as in (B) but graph shows absolute cell number of CD25$^+$ FoxP3$^+$ expressing OVA-TCR specific T cells calculated on total splenic cellularity and represents mean data of 3 individual mice per condition. Cells were gated on CD4 and Vβ5 high expressing cells.

BM-derived DC efficiently induce $CD4^+$ $FoxP3^+$ regulatory T cells *in vitro* predominately in the presence of exogenously supplied TGF-β [164]. Indeed, hardly any regulatory T cell generation could be detected in the absence of exogenous TGF-β irrespective of the maturation phenotype of the DC (fig 45A) and as shown previously (fig 11A and 26A). Nevertheless, DC matured with TNF, mfVSG or MiTat1.5 sVSG induced high levels of $FoxP3^+$ regulatory T cells *in vitro* to a similar extent as untreated DC but superior to LPS-conditioned DC when exogenous TGF-β was supplied to the culture, further supporting the observation that DC require TGF-β for the efficient induction of $FoxP3^+$ regulatory T cells *in vitro* as described [164].

In summary, the data described in this study identify Th2 cell differentiation patterns linked to partial DC maturation stages with quantitative differences between pathogen-derived, TLR-dependent VSG antigens and non-TLR dependent TNF stimulation *in vitro*. No induction of $FoxP3^+$ regulatory T cells could be observed by any of the DC signatures in the absence of exogenous TGF-β *in vitro*.

3.3.5 Partially mature DC show similar priming and Th polarizing capacity *in vivo*.

To assess how these DC maturation signatures prime T cell responses *in vivo*, differentially matured and OVA-loaded DC were injected together with OVA-specific TCR-transgenic OT-II T cells i.v. followed by analysis of proliferation and cytokine production of injected T cells. DC matured with TNF, mfVSG or MiTat1.5 sVSG all induced proliferation of CFSE-labelled T cells (fig 46A). The most profound priming in T cells was detected upon injection of LPS-matured DC as determined by flow cytometry (fig 46A) or calculated as the division index (fig 46B). Furthermore, one single injection of DC conditioned with TNF, mfVSG or MiTat1.5 sVSG increased intracellular IL-13 and IL-5 release by *ex vivo* restimulated OVA-TCR specific T cells (fig 46C and D), in contrast to mice which received LPS-matured DC which showed only background levels of IL-13 or IL-5 producing OVA-TCR specific T cells (fig 46C and D). Similar to the *in vitro* findings (fig 44A), a low frequency of IFN-γ-releasing T cells was observed which remained unaltered after one single injection and appeared irrespective of the maturation regimen of injected DC. However, a predominant inflammatory Th1-biased response was detected only upon LPS-matured DC injection only (data not shown and fig 46C and D). Furthermore, injection of DC conditioned with TNF, mfVSG or MiTat1.5

sVSG did not raise the frequency or total cellular amounts of FoxP3$^+$ regulatory T cells among OVA-TCR specific T cells *in vivo* similarly to LPS-matured DC (fig 45B and C) further strengthening the observation that partially mature DC efficiently induce proliferation and priming of (CFSE-labeled) OVA TCR-specific T cells *in vivo*.

Together, DC conditioned by TNF or *T. brucei*-derived VSG antigens induce profound and comparable Th2 cell priming *in vivo*.

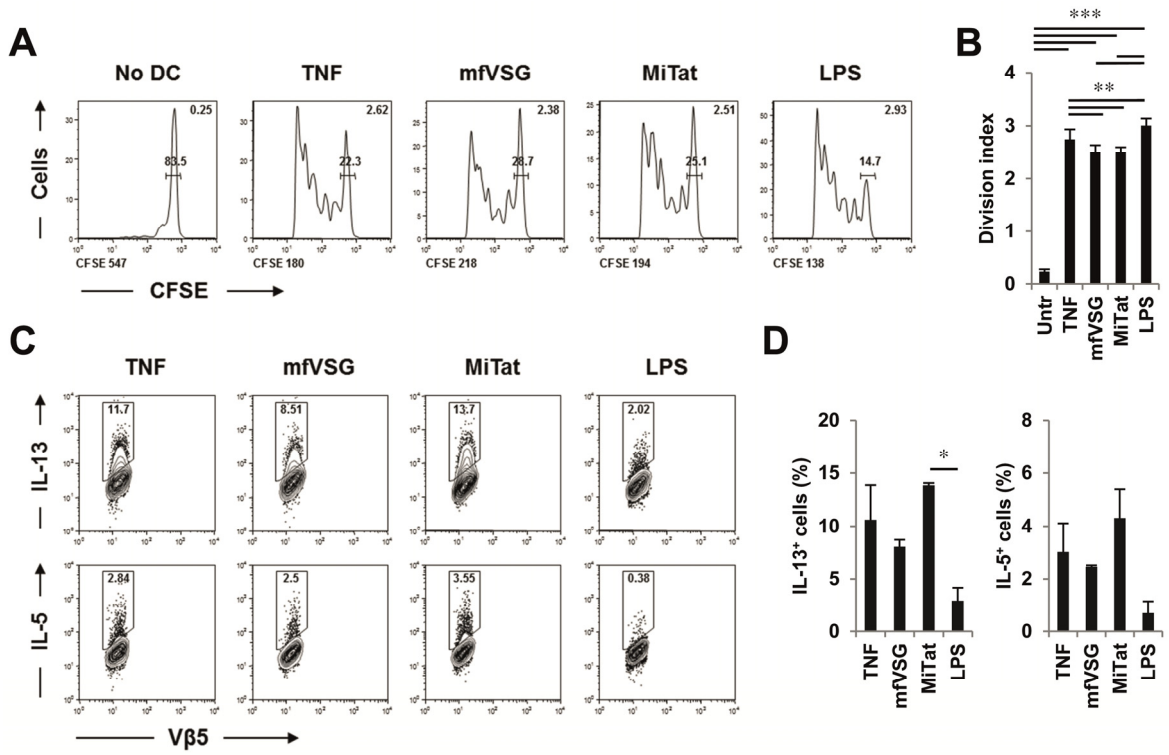


Figure 46. DC conditioned by TNF or *T. brucei* antigens show similar T cell priming capacities *in vivo*
(A) Mice received an intravenous injection of CFSE-labelled DO11.10 T cell suspension followed by DC matured with the indicated stimuli and loaded with OVA peptide. Untreated animals did not receive any DC injections. Histogram plots show CFSE dilution of CFSE$^+$ KJ1-26$^+$ T cells recovered from spleens of mice 4 days after final injection. One representative experiment of 2 experiments is shown. Numbers in upper right corner indicate division indices whereas those below each histogram represent MFI. (B) Level of CFSE dilution calculated as division indices. Figure depicts mean data from 2 independent experiments. (C) Mice received CD4$^+$ CD25$^-$ OT-II T cells intravenously followed by a single injection of DC matured with indicated stimuli and loaded with OVA peptide. Intracellular cytokine content of CD4$^+$ Vβ5$^+$ T cells was determined 6 days post-injection by peptide restimulation of splenocytes. Numbers in plots represent percentages of cells gated on CD4 and Vβ5 expressing cells. One representative experiment is shown. (D) as in (C) but graph represent mean data of up to 3 individual mice per condition. Cells were gated on CD4 and Vβ5 high expressing cells. Statistical significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$, one-way ANOVA followed by Bonferroni post-test

3.3.6 Partially matured DC do not affect asthma in mice

Asthma induced by alum-guided immunization of mice with OVA is a widely used model for a Th2 mediated disease characterized by pro-inflammatory lung infiltrates of eosinophilic granulocytes and a subsequent Th2-dependent production of OVA-specific IgG1 and IgE [260]. Mice subjected to repeated sensitization and antigen challenges showed a profound influx of total cells, in particular eosinophils in the broncho-alveolar lavage (BAL) as a major parameter for asthma (fig 47A). Three repetitive injections of OVA-loaded TNF, mfVSG or MiTat1.5 sVSG matured DC did not change the total cellular influx in the lungs compared to non-injected animals. In addition, the influx of eosinophils, macrophages, neutrophils and lymphocytes was not differentially regulated upon injection of partially matured DC and this independently of the inflammatory versus TLR-dependent quality of the maturation stimuli (fig 47A and data not shown).

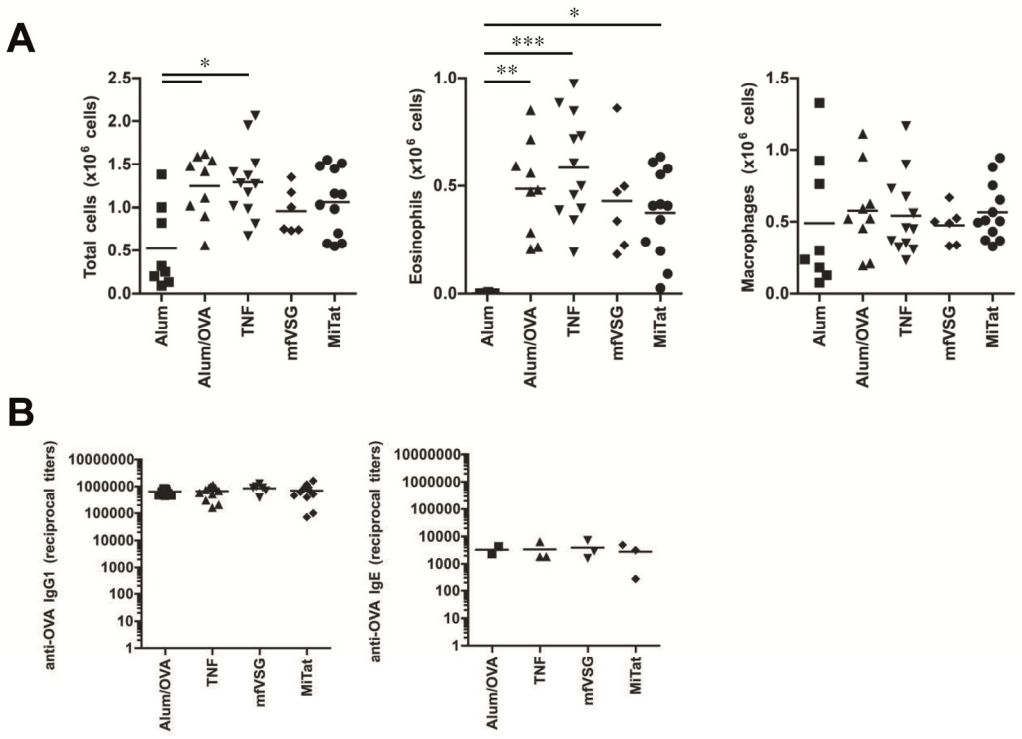


Figure 47. No influence on the allergic asthma model by all types of partially mature DC
(A) Mice received three repetitive injections of DC loaded with OVA protein and matured with the indicated stimuli at day -7, -5 and -3 before asthma induction. Control mice received no DC treatment. Figures show cell numbers recovered from broncho-alveolar lavage (BAL) six days after final antigen challenge. Each dot represents data obtained from one mouse. Results are shown as pooled data of all mice used in up to 4 independent experiments. (B) Graphs show OVA-specific IgG1 or IgE levels in sera of mice as detected by ELISA. Mice received DC treatments as described in (A). Each dot depicts OVA-specific IgG or IgE levels of serum of one mouse used in up to 4 different experiments. Statistical significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$, Kruskal-Wallis followed by Dunn's post-test

OVA-specific Th2-dependent IgG1 and IgE were detected in the serum of mice upon Alum/OVA sensitization and antigen challenges. Surprisingly, no change was detectable at the OVA-specific Ig levels when mice were pre-treated with the differentially matured and OVA-loaded DC (fig. 47B).

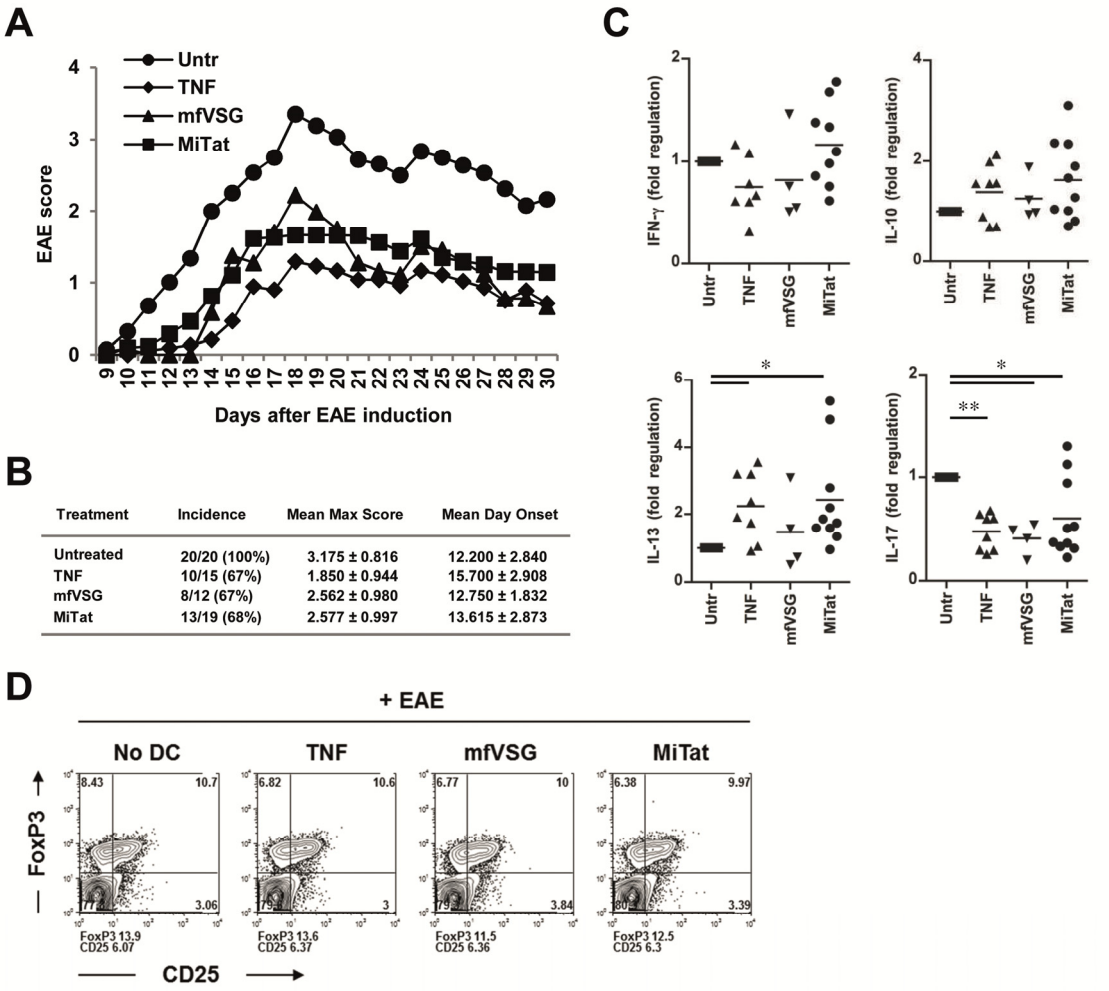
Together, MyD88-dependent *T. brucei*-derived VSG antigens or non-TLR dependent TNF conditioning of DC did not alter subsequent Th2-driven allergic asthma.

3.3.7 Similar protective potential by partially mature DC in the autoimmune model EAE

EAE serves as a common murine model for the early phases of multiple sclerosis, which can be achieved by immunizing mice with the auto-antigen MOG in CFA. Mice develop MOG-reactive pathogenic Th1 and Th17 cells, which then infiltrate into the CNS and cause inflammatory oedema leading to the reversible paralysis symptoms [261]. Previously, it has been shown that repeated injections of DC stimulated with TNF and loaded with MOG-peptide suppressed EAE, partially by creating a Th2/Tr1 cytokine environment including immune deviation and IL-10-mediated suppression [16, 262]. Therefore it was analyzed how the partial DC maturation stages induced by TLR-dependent or independent stimuli would modulate the autoimmune disease EAE.

To detect whether the DC injections ameliorate or worsen the disease, the amount per DC injection was switched from 3-3.5x10^6 cells, which is the fully protective protocol [16, 262, 263] to 2-2.5x10^6 cells, which leads to about 50% reduced clinical score [263]. Three i.v. injections of suboptimal amounts of MOG-loaded TNF-matured DC protected mice partially from EAE as 10 of 15 mice developed clinical symptoms and mice only reached a mean maximum score of 1.850±0.944 (fig 48A and B). Surprisingly, mice, which received three injections of DC matured with the *T. brucei* antigens mfVSG or MiTat1.5 sVSG, were also partially protected from EAE as 8 of 12 and 13 of 19 mice developed signs of EAE, respectively (fig 48A and B).

Together, these data indicate that all partially mature DC protected mice to a similar extent from EAE.



Treatment	Incidence	Mean Max Score	Mean Day Onset
Untreated	20/20 (100%)	3.175 ± 0.816	12.200 ± 2.840
TNF	10/15 (67%)	1.850 ± 0.944	15.700 ± 2.908
mfVSG	8/12 (67%)	2.562 ± 0.980	12.750 ± 1.832
MiTat	13/19 (68%)	2.577 ± 0.997	13.615 ± 2.873

Figure 48. Similar protection efficiency by all types of partially mature DC in the autoimmune model EAE
(A) Mice received three repetitive injections of DC loaded with MOG peptide and matured with the indicated stimuli at day -7, -5 and -3 before EAE induction. Control mice received no DC treatment. Mice were monitored daily for disease score. Results represent the average disease score of all mice used in up to 6 independent experiments (amount of mice indicated in B). (B) Statistical overview showing incidence, mean maximum score and mean day of onset of all EAE experiments performed. Mice were treated with three i.v. injections of MOG-loaded DC stimulated with indicated stimuli before EAE induction. (C) Mice were treated as in (A) but at day 30 after EAE induction, spleens were removed and restimulated with graded concentrations of MOG peptide. Supernatants were analyzed for their cytokine content by ELISA. Graphs show relative cytokine production normalized to the cytokine content of untreated controls. Each dot represents the relative cytokine production of splenocytes of pooled mice for one condition within one experiment restimulated at 20 or 2 µg/ml MOG peptide. (D) as in (A) but at day 30 after EAE induction, spleens were removed and expression of FoxP3 and CD25 determined by flow cytometry staining. Numbers in quadrant represent percentages of cells; numbers below figures show MFI values. Cells were gated on CD4 expressing cells. One representative experiment is shown. Statistical significance: * $p < 0.05$ and ** $p < 0.01$, Kruskal-Wallis followed by Dunn's post-test

3.3.8 DC programmed by TNF or *T. brucei* VSG antigens mount a protective Th2/Tr1 response in EAE

As published previously [262], protection from EAE by TNF matured DC required activation of IL-10^+ IL-13^+ cytokine producing $CD4^+$ Th2/Tr1 cells. IL-4 is also produced but immediately consumed in normal mice and only detectable in IL-4R-deficient mice [262]. It was therefore assessed how the differentially matured DC influenced the T cell cytokine profile of the spleens as detected after MOG peptide restimulation and cytokine analysis.

The cytokine profile of T cells from untreated mice typically consists of high amounts of pro-inflammatory IFN-γ and IL-17 but low amounts of IL-10 and IL-13. In contrast, this pattern becomes inverted in mice which received repetitive injections of TNF matured DC [16, 262]. Under the partially protective conditions the same shifts were observed, although, due to the suboptimal settings, not always reaching statistical significance (fig 48C). Nevertheless, splenocytes from mice injected with DC matured with the VSG significantly down-regulated IL-17 production comparable to the T cell cytokine profile of TNF-DC treated animals. Mice treated with MiTat matured DC, however, were not able to block the non-protective IFN-γ production as TNF-DC treated animals, but in addition, retained high production of the disease-preventing cytokines IL-13 and IL-10 (fig 48C). Moreover, repetitive injections of differentially matured DC did not alter the frequencies of FoxP3-expressing regulatory T cells in spleens of EAE-diseased mice (fig 48D) further suggesting that semi-mature DC regulate EAE by protective mechanisms other than $CD25^+$ $FoxP3^+$ regulatory T cell induction.

In sum, the partial DC maturation stages were all equally effective in creating a protective Th2/Tr1 environment, which was able to block the Th1/Th17-mediated EAE.

4 DISCUSSION

The data provided in this manuscript show that apoptotic cells induce a maturation-resistant state in immature BM-derived DC, representing both inflammatory and conventional DC subsets, but fail to extend the T cell tolerance potential of immature DC *in vitro*. Immature DC trigger B7 co-stimulatory signalling cascades critical for the programming of anergy and suppressive features in $CD4^+$ IL-10^+ regulatory T cells. Partially mature DC signatures induced by conditioning with inflammatory TNF or pathogenic *Trypanosoma brucei* antigens instruct Th2 responses by default which, however, adapt IL-10^+ Th2 cell profiles and immune deviating functions upon repetitive semi-mature DC injections *in vivo*.

4.1 Is apoptosis an essential antigen source for DC peptide presentation?

GM-CSF DC ingested irradiated thymocytes efficiently although untreated thymocyte-derived material also appeared in their cytoplasm. Flt3-L generated cDC acquired thymocyte-derived particles from both untreated and irradiated cells but were not able to engulf the apoptotic remnants *in vitro* as described previously [264]. Both membrane-associated and soluble cytosolic material was captured by DC (indicated by PKH26 or CFSE labelling respectively) heavily dependent on DC metabolism and actin polymerization. Whether cell material was exchanged by apoptotic body secretion or continuous scanning of cells by nibbling remains to be shown. Nevertheless, several reports documented that steady state splenic DC capture and internalize apoptotic cells *in vivo* [27, 224]. The discrepancy between *in vitro* Flt3-L generated DC and steady state splenic DC could be explained by the lack of conditioning from surrounding macrophages and/or complement factors *in vivo* which increases phagocytosis potential [224, 237]. In addition, it remains to be addressed whether particular eat-me receptors or proteins regulating cytoskeleton dynamics are differentially expressed or regulated in BM-derived DC as well.

Despite the fact that primary thymocytes undergo spontaneous apoptosis at a very slow rate presumably by means of survival factor deprivation [265], DC acquired cellular particles from untreated thymocytes within hours after culture start. It cannot be completely ruled out that the captured antigens are derived from early apoptotic cells or living cell material or both.

Proliferation assays showed that DC are able to load and present thymocyte-derived peptides from untreated and irradiated thymocytes in the MHC II binding cleft to naïve T cells with similar efficiency further indicating that DC acquire cellular proteins and/or membrane particles irrespective of the apoptotic or living nature of the cell. Indeed, a recent report from the Hosmalin group showed that DC capture membrane and cytosolic cellular material from living cells both *in vitro* and *in vivo* through vesicle exchange and use the antigens for MHC-peptide cross-presentation [266]. Proteins isolated from living cells are not degraded compared to apoptotic counterparts making loading of MHC I complexes more successful [267], although pre-processing of peptides by apoptotic cells for cross-presentation by DC has also been suggested [268]. Hence, eat-me signals sent out by apoptotic cells might function solely to increase phagocytosis efficiency to prevent leakage of the danger signals in the surrounding tissues but do not seem to broaden the antigen pool or increase antigen-MHC complex presentation compared to living cells although this remains to be formally shown.

4.2 DC and TGF-β production

The data presented in this work indicate that BM-derived DC remained completely inert upon engulfment of apoptotic cell material demonstrated by unaltered levels of MHC II and co-stimulatory molecule expression, absence of alternative NFκB pathway signalling and lack of TGF-β secretion. Secretion of TGF-β by DC independent from apoptotic cell internalization remained controversial [29, 224, 231] but not for macrophages [230, 231]. It has been shown that BM-derived DC abundantly express TGF-β1 mRNA copies [16, 224], but fail to secrete detectable active and/or latent TGF-β1 protein levels and do not show membrane expression of TGF-β associated LAP protein as described in this manuscript. Although secretion of the latent form of TGF-β by BM-derived DC cannot be completely excluded, levels hardly increased compared to serum background levels. Indeed, platelets release their intracellular source of the so-called large latent TGF-β complex (LLC) consisting of the TGF-β dimer covered by LAP proteins and linked to latent TGF-β binding proteins (LTBP) for storage in the extracellular matrix (ECM) into the serum upon clotting [269]. Hence, although *in vitro* a latent TGF-β reservoir is present, the data presented here show that DC are unable to activate the complex for auto- and paracrine signalling purposes. Combined actions of certain matrix metalloproteinases (MMP) and α_v-integrins have been proposed to release the latency-

conferring structures from the active TGF-β dimers. Interestingly, also thrombospondin-1 which is released by apoptotic monocytes and neutrophils blocking DC maturation [234] can mediate these effects [270]. However, a recent publication indicated that rather binding of α_v-integrins and generation of certain contraction forces releases TGF-β out of the LLC although here, attachment to the ECM by LTBP and integrin association with the cytoskeleton is absolutely required [271]. It remains to be investigated whether BM-derived DC lack expression of certain MMP or α_v-integrins to convert TGF-β from the serum reservoir or need additional signalling to activate endogenous TGF-β synthesis and/or secretion pathways. Defective $\alpha_v\beta_8$-integrin expression on DC caused severe wasting autoimmune disease and colitis and splenic DC lacking $\alpha_v\beta_8$-integrins showed impaired TGF-β activity and reduced FoxP3$^+$ regulatory T cells conversion *in vitro* [272]. Furthermore, splenic DC do express membrane bound LAP, as demonstrated in this study, and seemed to produce minor levels of preferably latent TGF-β which increased in response to apoptotic cell engulfment [29, 231]. Most likely environmental conditioning by adjacent cells and/or serum derivatives contribute to the appearance of tolerogenic features in DC *in vivo* (similarly to phagocytosis capacity) as shown previously for gut-derived DC [273] and as suggested for splenic DC [237] but not for BM-derived DC *in vitro*.

4.3 How do apoptotic cells induce suppressive signalling in DC?

Here, apoptotic cells fail to induce surface marker expression and TGF-β cytokine secretion but did suppress additional TLR-mediated DC maturation indicating that *in vivo* environmental conditioning is not essential for the inhibitory signalling effect of apoptotic cell recognition. A central role for Mer tyrosine kinase receptor (MerTK) mediated uptake of apoptotic cells by DC was proposed inducing downstream STAT3 signalling for the blockade of the canonical NFκB pathway [232]. In addition, A20, a ubiquitin editing enzyme downregulating NFκB gene expression by targeting signalling components for degradation critically regulates the NFκB signalling block initiated upon recognition of apoptotic cells [274]. However, how signalling components modulate IL-10 gene expression in DC upon pre-exposure with apoptotic cells remained unexplored so far. Signalling through immunoreceptor tyrosine-based activation motif (ITAM) coupled receptors and integrins such as the apoptotic cell sensors MerTK [232, 275] or β2 integrin receptors (also known as complement receptor

3) [224] can paradoxically transduce inhibitory signals and inhibit other receptor signalling cascades [276]. Interestingly, de novo expression of signalling inhibitor A20 and anti-inflammatory IL-10 and increased STAT3 phosphorylation was detected upon high-avidity ligation of β2 integrin receptors on human macrophages [276]. How ITAMs initiate negative signalling through formation of so-called inhibisomes has only begun to be unravelled [277] and will increase our knowledge how apoptotic cells selectively counter-regulate TLR- and/or inflammatory DC stimulation.

4.4 DC subsets and apoptotic cell response: division of labour or all the same?

In this manuscript, striking differences were observed in GM-CSF versus Flt3-L DC phenotypes in terms of MHC II expression and co-stimulation in particular. The *in vitro* generated Tip DC equivalents already expressed high levels of the co-stimulatory molecules CD80 and ICOS-L at the immature state. In comparison to cDC, GM-CSF DC secreted higher levels of IL-10 upon TLR-maturation and showed phagocytic capacity presumably for engulfment of cellular debris at the site of infection. Of note, both CD80-mediated engagement of CTLA-4 and high ICOS-L expression has been linked to Tr1-like differentiation from IFN-γ producing effector T cells [183, 199]. If no particular TLR- or inflammatory stimulus is encountered as mimicked in our *in vitro* settings, GM-CSF DC performed tolerogenic functions by anergy induction and regulatory T cell conversion if exogenous TGF-β is supplied albeit irrespective of the presence of apoptotic cell material. However, as discussed previously, these unexpected features might be due to the lack of conditioning by the environmental milieu.

Interestingly, Flt3-L cDC subsets expressed moderate levels of MHC II but almost completely lacked expression of co-stimulatory molecules at immature state. Splenic $CD8\alpha^+$ DC are well-equipped to convert de novo $FoxP3^+$ regulatory T cells, whereas the $CD8\alpha^-$ DC preferentially induce $FoxP3^+$ regulatory T cells in the presence of exogenous TGF-β [29]. These features are in agreement with recent findings indicating that regulatory T cell conversion is favoured under weak TCR stimulation and absent to low co-stimulation [164, 278, 279]. Strikingly, it was shown in this study that although both Flt3-L cDC subsets relied

on the exogenous supply of TGF-β, GM-CSF DC were superior in regulatory T cell conversion *in vitro* despite expression of high co-stimulatory molecules. Indeed, in addition of exogenous TGF-β, high co-stimulation provides additional IL-2 derived from proliferating T cells for optimal regulatory T cell conversion [164]. However, maturation into high MHC II and co-stimulatory molecule expressing DC (e.g. upon endotoxin stimulation) will further increase the need for TGF-β to decrease the sensitivity towards strong TCR stimulation for regulatory T cell conversion as indicated here and as suggested previously [278].

Similarly to GM-CSF DC, Flt3-L DC induced proliferation in naïve $CD4^+$ T cells upon recognition of apoptotic cell material but in contrary failed to induce anergy in $CD4^+$ T cells in the absence of TGF-β supplements (unpublished observations and as shown previously [264]). Although these observation might appear artificial due to the lack of environmental conditioning *in vitro* as discussed, several reports documented the occurrence of IFN-γ producing Th1/CTL responses upon apoptotic cell conditioning *in vivo* [264, 280]. Most likely, Flt3-L generated BM-DC induced initial expansion but inefficient activation of $CD4^+$ T cells and/or $CD8^+$ CTL which precedes deletional tolerance as described for splenic DC [11, 13, 227].

Together, GM-CSF and Flt3-L DC differ in their handling of apoptotic cell material *in vitro* which did not appear critical for the maturation resistance or negative signalling induced in DC. A role for DC conditioning from the environmental milieu should not be ignored. Nevertheless, the data presented here favour a model in which apoptotic cells deliver self-antigens to DC but besides maturation paralysis fail to confer particular tolerance features to DC different from those already present in immature DC *in vitro*, i.e. anergy or regulatory T cell induction in the presence of TGF-β. BM-derived DC subsets (GM-CSF versus Flt3-L) show individual characteristics and preferred immune functions but all adjust the maturation phenotype for the induction of tolerance versus immunity.

4.5 IL-4, NFIL3 and GATA-3 and a regulatory T cell phenotype

Here, IL-10$^+$ regulatory T cells increase relative expression of GATA-3 and NFIL3 and secrete in part additional cytokines classically associated with Th2 development but nevertheless develop suppressive features. These phenomena raise the question how these transcription factors influence the generation of an adaptive regulatory T cell phenotype and how related IL-10$^+$ regulatory T cells are to effector Th2 cells.

It has been reported that autocrine IL-10 signalling is required to downregulate IL-4 production capacity in differentiating T cells [200], however, it may not be excluded that some residual IL-4 and/or IL-13 is required to maintain or support generation of IL-10$^+$ regulatory T cells. Ito and colleagues failed to exclude that IL-4 is not required for the ICOS-L mediated generation of IL-10$^+$ regulatory T cells under Th2 conditions [45]. Furthermore, some IL-4 is apparent in the IL-10$^+$ T cell cultures generated under immunosuppressive drugs in the absence of blocking antibodies [200]. Perez et al. demonstrated that dominant CTLA-4 engagement on Th1 cells is critical for the generation of IL-10$^+$ regulatory T cells which next to IL-10 and TGF-β increase secretion of IL-4 [183]. Chronically stimulated Th1 cells start to produce IL-10 and IL-13 (and some IL-5) [180] but this cytokine remained largely undetermined throughout the Tr1 literature. IL-5 production was described in the initial observations delineating the classical phenotype of IL-10$^+$ Tr1 cells [146]. Several reports documented IL-4 mRNA expression by anergic T cells *in vivo* [142, 147], however, anergic cells actually fail to produce effector cytokines presumably controlled by posttranscriptional silencing mechanisms [281]. It should be noted that the stimuli used to induce cytokine secretion for detection purposes (e.g. peptides, peptide-pulsed DC, soluble or plastic coated αCD3 antibodies, mitogens bypassing TCR, etc.) differ in mechanisms and/or intensity to overcome the TCR signalling block associated with anergic T phenotypes and might also differ in the posttranscriptional regulation of effector cytokine transcripts.

Recently, Motomura et al. identified the transcription factor E4BP4/NFIL3 essential for the expression of IL-10 in chronically stimulated Th1, Th2 and regulatory T cells [180]. Interestingly, expression of NFIL3 was also increased in a Ca^{2+}/calcineurin-signalling induced anergic T cells [128]. Identifying the nature of the IL-10$^+$ regulatory T cells generated in this study is further complicated by the observation that next to Th2 cells also naïve CD4$^+$ T cells and FoxP3$^+$ regulatory T cells express detectable GATA-3 [282, 283]. Rudensky and

colleagues suggested earlier that FoxP3$^+$ regulatory T cells adapt their transcriptional program to effectively regulate effector T cell types, exemplified by expression of interferon regulatory factor-4 (IRF4) for regulation of Th2 responses [284]. Loss of GATA-3 in FoxP3$^+$ regulatory T cells resulted in aberrant suppressive function and expression of the FoxP3 transcription factor [283]. Thus, the classical T cell differentiation programs are under debate and considerable plasticity in cytokine production and transcriptional programming can be observed in both effector and regulatory T cells [59]. How the T cell phenotypes irrespective from naïve or effector T cell origin merge into a regulatory signalling network, orchestrating the suppressive features theoretically restricted to FoxP3$^+$ regulatory T cells remains an important question.

4.6 Signalling and suppressive function in IL-10$^+$ versus FoxP3$^+$ regulatory T cells

Immature DC effectively induce FoxP3$^+$ regulatory T cells in the presence of exogenously supplied TGF-β *in vitro* as discussed previously. Instead, naïve T cells are rendered anergic in a TGF-β non-permissive environment and acquire suppressive features upon restimulation with immature DC *in vitro*. Interestingly, repetition of antigen-presentation by DC did not improve conversion efficiency into FoxP3$^+$ regulatory T cells. The failure to convert already primed T cells to the FoxP3$^+$ regulatory T cell lineage despite exogenous TGF-β supply can be attributed to the respective lineage-specific transcription factors (in Th1 or Th2 cells) or autocrine production of IFN-γ and especially IL-4 (under non-polarizing conditions) counterregulating TGF-β induced de novo FoxP3 expression, an effect which can be relieved upon addition of retinoic acid or anti-cytokine directed antibodies [285-287]. However, some similarities in mode of action and acquisition of regulatory function of IL-10 regulatory T cells to FoxP3$^+$ regulatory T cells could be observed and as suggested in earlier reports [193].

FoxP3$^+$ regulatory T cells can suppress responder T cells *in vitro* by inhibiting IL-2 (and other cytokine) gene expression which requires cell contact or at least cellular proximity [156, 157]. Exogenous IL-2 induces proliferation in both responder and FoxP3$^+$ regulatory T cells but fails to reverse the suppression of IL-2 gene transcription in responder T cells [156, 157]. This phenomenon might explain the incomplete reversion of suppressive activity upon exogenous

IL-2 supply as observed in this study. The exact mechanism how FoxP3$^+$ regulatory T cells suppress gene transcription programs in their target cells remained elusive to date. Schmitt and colleagues postulated that FoxP3$^+$ regulatory T cells transmit high levels of their cyclic adenosine monophosphate (cAMP) content through gap junctions to the target cells thereby repressing IL-2 gene expression [288]. Recently, Vaeth et al. demonstrated that increased cAMP in responder T cells correlated with nuclear translocation of the inducible cAMP early repressor (ICER)/cAMP response element modulator (CREM) complex which attenuated not only IL-2 but also inducible NFATc1/alpha gene transcription [255]. Others suggested that the induction of ICER/CREM complexes and attenuation of IL-2 transcription might be propagated through CTLA-4 mediated ligation of B7 molecules on responder T cells [163]. Blocking experiments using anti-CTLA-4 or anti-B7 antibodies did not always relief inhibition by (FoxP3$^+$) regulatory T cells *in vitro*, although these observation might be concentration and Fc-portion dependent [193, 289, 290]. Nevertheless, it cannot be excluded that CTLA-4 regulates responder T cells indirectly by affecting APC function [158]. Whether inhibitory cytokines such as IL-35 can mediate suppression of IL-2 gene transcription needs further investigation [176]. Nevertheless, levels of EBI-3 and IL-12p35 expression remained unchanged within IL-10$^+$ regulatory T cells induced in the *in vitro* co-culture system compared to LPS-stimulated controls indicating that this suppressive mechanisms is rather unlikely.

FoxP3$^+$ regulatory T cells constitutively express CTLA-4, contain elevated levels of cAMP, show enhanced nuclear translocation of ICER/CREM complexes and lack NFATc1 expression [255, 288]. Here, IL-10$^+$ regulatory T cells suppressed the nuclear translocation of NFATc1 and c2 and blocked the activation of NFATc1/alpha by a CTLA-4 dependent mechanism, a feature described in earlier reports [133, 291]. Of note, a central role for NFAT in clonal anergy induction was suggested previously [125, 128], however, the anergic phenotype presented here is more related to those described in *in vivo* anergy models [132, 292]. CTLA-4 maintained the anergic phenotype by blocking cell proliferation upon TCR stimulation and modulating Egr-2 expression levels. The signalling pathways and transmitters downstream of CTLA-4 regulating these effects are under intense investigation. Currently, no signalling link between CTLA-4 and downstream cAMP and ICER/CREM could be established since high cAMP and nuclear ICER/CREM levels were observed under all conditions (unpublished observations). Earlier reports documented that cAMP might induce

CTLA-4 expression in a Ca^{2+}-independent manner [293], but the inverse signalling event remained unexplored to date. Similarly, the NFAT-independent but CTLA-4-regulated expression of Egr-2 is to our knowledge not described previously and needs further investigation. Interestingly, forced expression of Egr-2 increased $p27^{Kip1}$ and $p21^{Cip1}$ cell cycle inhibitors [292], which expression is also regulated upon CTLA-4 signalling [133]. Furthermore, it has been shown that CTLA-4 signalling triggers transcriptional activation of the E3 ubiquitin ligase Cbl-b [294], a mediator known to be transcribed by Egr-2 and Egr-3 transcription factors [141]. Of note, Egr-2 expression has been associated with the formation of IL-10^{+} regulatory T cells *in vivo* [184, 292]. Together, CTLA-4 signalling might play a crucial role in the early (IL-10^{+}) regulatory T cell commitment based on the observations that (1) GM-CSF BM-derived DC widely express CD80 molecules at the immature state, (2) CD80 molecules ligate CTLA-4 by high affinity binding characteristics, (3) untreated DC were unexpectedly high potent inducers of CTLA-4 expression in naïve T cells compared to LPS-matured counterparts and (4) naïve T cells showed anergic features after one single round of stimulation by immature DC (5) NFATc1, c2 and NFATc1/alpha were not nuclear translocated or induced in primed T cells stimulated with immature DC but relative Egr-2 expression was increased already after one round of stimulation.

Anergic cells do not automatically have suppressive capacity [142, 295], and in fact need persistence of antigen to maintain their anergic state [145]. Here we show that acquisition of suppressive function in anergic T cells is dependent on a B7 co-stimulatory engagement with CD28 in the presence of cognate MHC-antigen recognition. It has been postulated that CD28 signalling plays an important role in the thymic generation of $FoxP3^{+}$ regulatory T cells by cell-intrinsic mechanisms [171]. The lack of specific CD28 signalling modulators independent from TCR-induced cascades hampers the identification of the driving force conferring regulatory features on thymic precursors. A role for the NFkB family member c-Rel was proposed for regulatory T cell commitment [169, 170] which could not be confirmed in the IL-10^{+} regulatory T cells generated here (unpublished observations). Instead, the data presented in this manuscript shed new light on CD28 function in that it was absolutely required to effectively block intrinsic IL-2 expression (but enhance IL-2Rα expression) which is opposite to the role of CD28 in naïve or effector T cell activation [53] and which might form the actual basis for regulatory T cell commitment. Decreased IL-2 expression by CD28

signalling might be regulated at the level of epigenetic modifications (e.g. Ikaros), IL-2 gene repression (e.g. Blimp1) or modulation of cytokine mRNA stability and needs further investigation [53]. The novel functions of CD28 as observed in this study might originate from sustained STAT signalling through IL-2R or IL-4R signalling cascades. Not only can CD28 signals promote differentiation of IL-4 producing Th2 primarily through increasing IL-4R sensitivity [296, 297] but exogenous IL-4 can compensate for IL-2 for activation of suppressive activity in FoxP3$^+$ regulatory T cells *in vitro* [157]. Interestingly, IL-2 signalling and even exogenous IL-4 and IL-12 can induce downstream Blimp-1 [298], a well-studied repressor of IL-2 gene transcription in activated effector T cells [299] and activator of IL-10 secretion in FoxP3$^+$ regulatory T cells [300]. Of note, expression of IL-4, IL-10, IL-2Rα chain, Blimp-1 and NFIL3 are induced upon IL-2 signalling in activated T cells [298]. Furthermore, the data presented here also indicated a modest regulation of IL-10 secretion upon CD28 signalling, a factor believed to be critical for generation of Tr1 cells *in vitro* [146, 200] and which induces STAT3 phosphorylation in IL-10$^+$ regulatory T cells generated by repetitive administration of peptides *in vivo* [301]. Hence, whether CD28 signalling potentiates selective STAT signalling in IL-10$^+$ regulatory T cells generated in this study for induction of suppressive function will be subject of further work.

It is tempting to speculate that the suppressive outcome of CD28 signalling as demonstrated here can be substituted by other cascades conferring regulatory function to effector T cells. Indeed, preferential ligation of CTLA-4 by CD80 on IFN-γ$^+$ effector T cells reduced IL-2 and increased IL-10 and TGF-β secretion [183] and is essential to drive TGF-β induced suppressive activity [302]. Nevertheless, Anderson and colleagues showed in an outstanding report that IL-10$^+$ Tr1-like cells differentiated from Th1 cells and created by repetitive intranasal administration of high affinity peptides [184] showed completely absent AP-1 and NFAT and reduced NFκB activation but maintained high levels of phosphorylated STAT3 and STAT5 [292]. *In vivo* differentiated Th17 cells restimulated in the presence of IL-6 and TGF-β acquire a regulatory function able to block EAE through production of IL-10 [303]. Recently, regulatory Th17 cells characterized by expression of CTLA-4, IL-10 but decreased IL-2 were generated *in vivo* which accumulate particularly in the small intestine and were initially driven by APC derived IL-6 [304]. Interestingly, IL-10 production was abrogated in CD4$^+$ T cells stimulated under Th17 polarizing conditions by IL-6 and TGF-β if STAT3 was lacking [305]. Thus, despite distinct effector or naïve T cell origins, regulatory signalling

pathways all seem to merge in orchestrating defective IL-2 secretion but forced expression of IL-10 and CTLA-4, adapting phenotype and function to FoxP3$^+$ regulatory T cells. These insights support the concept that FoxP3$^-$ IL-10$^+$ regulatory T cells, Tr1 and Tr1-like cells might share more phenotypical and functional characteristics than accepted to date [175].

4.7 VSG, TNF and the immune deviation program

Semi-mature DC induced by pro-inflammatory TNF induce IL-4$^+$ Th2 differentiation *in vitro* and *in vivo* [21]. Repetitive injections of TNF matured DC prevented the induction of the autoimmune disease EAE by polarization of IL-10$^+$ IL-13$^+$ CD4$^+$ T cells along with the early activation of CD1d-dependent NKT cells generating a rapid type 2 cytokine environment *in vivo* [16, 262]. These observations supported the concept that TNF-induced semi-mature DC have tolerogenic features at least in part by supporting Th2-immune deviation. These insights prompted us to investigate how the DC maturation signature and respective T helper cell differentiation induced by TNF is related to pathogenic maturation stimuli which have well-studied immune modulatory and evasion strategies *in vivo* [206]. Surprisingly, the data presented in this work show that low concentrations of pathogen-derived TLR stimuli (represented by *T. brucei* antigens mfVSG and MiTat1.5 sVSG) program DC similarly to the inflammatory cytokine TNF for the differentiation towards an inflammatory, semi-mature DC phenotype. These partial DC maturation stages were able to instruct Th2 priming *in vitro* and *in vivo* and induced only quantitative differences in the extent of Th2 differentiation. However, these Th2 profiles did not differ in their intrinsic quality to prevent EAE induction and to affect allergic asthma responses.

4.8 Th2 responses and PRR-signalling

The stimulus LPS triggers DC maturation through TLR4 ligation and directs T helper cell differentiation towards Th1. Less is known which PRR-receptors drive Th2-associated immune responses. Recent reports suggest that house dust mite allergens initiate asthmatic inflammation by signalling through the TLR4 receptor complex in part by LPS contamination [306, 307]. Here, it was shown that *T. brucei* antigen MiTat1.5 sVSG conditioned DC to produce IL-6 and IL-1β, which is dependent on TLR4 and the adaptor molecule MyD88. A

novel TLR4 mediated signaling pathway was identified in which TLR4 stimuli trigger a rapid increase in intracellular cAMP followed by translocation of the transcription factor CREB and IL-6 production [308]. Further investigation is needed to address whether MiTat1.5 sVSG activation of DC is accompanied with an intracellular cAMP rise and CREB transcription factor translocation. The *T. brucei* AnTat1.1 antigen mfVSG triggers activation of DC mainly independent of the TLR4 receptor complex but dependent on the signaling adaptor MyD88. Earlier reports showed that mfVSG triggers macrophage activation through a MyD88-dependent signaling cascade [256, 257]. Heating VSG antigens for 15 minutes at 95°C did not abrogate the DC maturation activity (data not shown) indicating that the glycosyl-inositol-phosphate (GIP) moieties of the GPI-anchor are the DC activating factors as suggested previously for macrophages [257]. In analogy with other parasitic protozoa such as *Leishmania major, Plasmodium falciparum and T. cruzi,* GPI-anchors of *T. brucei* are believed to form the most prominent inflammation and disease-inducing component [309]. Indeed, recent reports showed that the GPI-anchors of *P. falciparum* mainly trigger MyD88-dependent TLR2 and to a lesser extent TLR4 signaling in macrophages [310].

4.9 Semi-mature DC at inflammatory versus steady-state DC level

DC sense different pathogens and respond by upregulating MHC, co-stimulatory molecules as well as cytokine production to mount an appropriate T cell response. However, it appears that DC release substantial amounts of cytokines only upon strong TLR activation [6, 16, 18, 311]. We found that mfVSG and Mitat1.5 sVSG act on DC through MyD88 to mediate maturation but result in a TNF-like inflammation-induced partial maturation profile, leading to Th2 polarization. Although we also detected some IL-9 producing T cells *in vitro*, this was not observed after injection. Since there is ongoing debate whether IL-9 is part of Th2 cells or belonging to an own Th9 subset [312], we did not further address IL-9 in this work. Inflammatory mediators can activate DC also *in vivo*, which are similarly unable to produce IL-6 or IL-12p40 [18, 311, 313]. Spörri and Reis e Sousa have shown that DC activated by inflammatory mediators *in vivo* induced T helper cells but these were unable to support immunoglobulin isotype switching [18]. Similarly, in this study all partially matured DC types were unable to alter IgG1 and IgE levels in the asthma model. Recent reports also suggest that IL-6 by triggering IL-21 secretion in T cells drives the differentiation of Th cells

that acquire the ability to provide B cell help for isotype switching [314]. Here, DC matured with MiTat1.5 sVSG showed substantial production of IL-6, but DC treatment did not modify the isotype switch compared to other maturation stimuli in the allergic asthma model. However, it remains to be determined whether DC conditioned by MiTat1.5 sVSG can induce B-lymphocyte helper T cells in absence of any additional adjuvant activity. The capacity to provide efficient B cell help might further delineate distinct functions of the Th2 subsets induced by inflammatory mediators or TLR-agonists as identified in this study.

In this study the non-pathogen-derived inflammatory stimulus TNF and type 2 pathogen-derived antigens show remarkable similarities for the maturation of BM-derived DC, i.e. the *in vitro* counterpart of the Tip DC [25]. It has been suggested that DC develop by means of the growth factor Flt-3L in the steady state, whereas under inflammation, DC are additionally generated from monocytes by GM-CSF [25]. Initial encounter with a pathogen and hence, initial Th cell polarization will most likely occur solely by the tissue-resident DC or, in case of tsetse fly mediated blood infection with trypanosomes, steady-state DC. Tip DC develop later during infection from recruited monocytes and by GM-CSF secreted from T cells at the site of inflammation. Thus, the BM-DC equivalents to Tip DC might play a decisive role in dampening or modulating the initially mounted Th cell response to effectively eliminate the invading pathogen, a process also referred to as "success-driven" Th cell modulation [315]. The functional difference of inflammatory versus steady-state occurring DC might explain why DC indirectly activated by inflammatory mediators *in vivo* failed to mount Th2 responses, but inflammation drives Th2 differentiation at the Tip DC level [18, 313].

4.10 Mechanism of Th2 induction by DC

The three signal model as initially proposed by Kapsenberg et al. [48] proposes how DC mediate Th cell differentiation: peptide-MHC ligation (signal 1), co-stimulatory signalling (signal 2) and a selective cytokine set initiate the differential Th cell commitment (signal 3). For Th1 polarization, IL-12p70 production by DC is, besides the recently described CD70 dependent pathway [69], a clear signal towards Th1 polarization but signal 3 for Th2 cells remains less clear. Previous reports have shown that the Th2-promoting mediator PGE_2 induces secretion of IL-12p40 in DC thereby inhibiting the production of the Th1-driving

cytokine IL-12p70 [83-85]. It has been proposed that blocking or washing out IL-12p70 production are sufficient to drive the differentiation of Th2 responses by the so-called default or exhaustion pathway [73, 316]. The elimination of IL-12p70 from the context of antigen presentation by mature DC would result in a similar phenotype of inflammatory semi-mature DC as we have generated them here. The differences in the production of low levels of IL-6 or IL-12p40 by DC matured with TNF, mfVSG or MiTat1.5 sVSG do not seem to shift the qualitative Th2 profile but only result in minor quantitatively different amounts of Th2 cells which did not have functional consequences after injection in asthma or EAE models.

Furthermore, the fact that TNF and the mfVSG and Mitat1.5 sVSG regulate only few genes, while LPS regulates the same but almost 5000 genes in addition, argues for predominantly quantitative differences between the two types of DC maturation. However, since these quantitative changes led to qualitatively different Th1 or Th2 polarizations, this may reflect another DC-based aspect of the "strength of signal" theory where peptide titrations and affinities heavily influenced the Th skewing potential [74, 317]. The peptide dose dependency has been shown to be independent of the DC subtype but strong LPS or CpG stimulation clearly shifted towards Th1 [76]. As a mechanism how this could be regulated others proposed that weak T cell stimulation prevents CD40L upregulation, which in turn was required to trigger CD40 on DC for their IL-12 production and Th1 immunity [75]. Thus, weak DC stimulation would then result in a Th2 response, whereas strong DC stimulation, i.e. by DC maturation with LPS or weak maturation but presenting high doses of peptide, would result in a Th1 polarization.

Due to the fact that VSG-mediated semi-maturation of DC is dependent on MyD88 signaling, we may have to consider these Th2-inducing antigens as weak TLR agonists. Others have shown that especially TLR2 triggering of DC can lead to a Th2 priming with or without co-induction of Th17 cells [318, 319] although there are also other results for *Schistosoma* antigens that induce Th2 responses without the involvement of TLR2, TLR4 or MyD88 [320]. This indicates that quantitatively low TLR signalling but also TLR- and MyD88-independent DC maturation can lead to Th2 polarization.

4.11 Semi-mature DC and Th2 responses: inflammation limiting or active immunity?

One would expect that if DC conditioned by TNF or VSG antigens induce preferentially immunogenic Th2 responses, they should increase the severity of asthma symptoms when pulsed with the allergens and injected before disease induction. Alternatively, if these DC prime Th1 responses the disease should ameliorate. We did not test LPS-matured DC in this context. Others have addressed this question before by using CpG-matured BM-DC, which are similar to LPS for instruction of Th1 responses, but without effects on asthma [321]. Lambrecht's group has shown that rather plasmacytoid DC may be able to control asthma [43, 322]. Semi-mature DC prevented the paralysing symptoms in the EAE model by immune deviating towards a Th2/Tr1 protective response, while LPS-matured DC were not protective [16, 262, 323]. However, application of semi-mature DC in Th2-associated asthma model did neither ameliorate nor worsen the disease symptoms, similarly to previous data obtained for the murine *Leishmania major* Th2 infection model [21]. These data suggest that the Th2/Tr1 differentiation as induced by semi-mature DC in Th2 models results in a balance between an intrinsic inflammation-limiting Tr1 response and the active asthma-promoting Th2 response. Interestingly, the upcoming role of such balanced Th2 responses in limiting tissue pathology and inflammation has been discussed previously in several infection models and especially for macrophages [324-326].

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8 ABBREVIATIONS

Actb	β-actin
AICD	Activation-induced cell death
AIRE	Autoimmune regulator
AP-1	Activator protein-1
APC	Antigen presenting cell
APC_v	Allophycocyanin
B2m	β-2-microglobulin
BAL	Broncho-alveolar lavage
BCR	B cell receptor
BH3	Bcl-2 homology domain 3
BM	Bone marrow
BSA	Bovine serum albumin
c	Constant
cAMP	Cyclic adenosine monophosphate
Cbl-b	Casitas B-lineage lymphoma B
CCR7	C-C chemokine receptor 7
CD	Cluster of differentiation
cDC	Conventional DC
cdk	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
Ci	Curie
CIA	Collagen-induced arthritis
CNS	Central nervous system
cSMAC	Central supramolecular activation cluster
Ct	Cycle threshold
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cells
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EBI3	Epstein-Barr virus induced 3
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Egr-2	Early growth response gene 2
EIA/RIA	Enzyme linked immunoassay/Radioimmunoassay
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Flt3-L	Fms-like tyrosine kinase 3 ligand
FoxP3	Forkhead box P3
FSC	Forward scatter
Fwd	Forward
GALT	Gut-associated lymphoid tissue
GATA-3	GATA binding protein 3

GEO	Gene Expression Omnibus
GIP	Glycosyl-inositol-phosphate
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GMO	Genetically modified organism
GRAIL	Gene related to anergy in lymphocytes
Gy	Gray
h	Hours
HMGB-1	High-mobility group box protein 1
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
IBD	Inflammatory bowel disease
ICER/CREM	Inducible cAMP early repressor/cAMP response element modulator
ICOS	Inducible T cell co-stimulator
ICOS-L	ICOS ligand
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IFNGR	IFN-γ receptor
Ig	Immunoglobulin
IL	Interleukin
IL-12R	IL-12 receptor
IL-2R	IL-2 receptor
iNOS	Inducible nitric oxide synthase
IPEX	Immunodysregulation, polyendocrinopathy and enteropathy, X-linked
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
LAG-3	Lymphocyte activation gene 3
LAP	Latency-associated peptide
LLC	Large latent TGF-β complex
LPS	Lipopolysaccharide
LTBP	Latent TGF-β binding proteins
MACS	Magnetic activated cell sorting
MerTK	Mer tyrosine kinase receptor
MFI	Mean fluorescence intensity
mfVSG	Membrane form variant-specific surface glycoproteins
ml	Millilitre
MMP	Matrix metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
mTEC	Medullary thymic epithelial cells
NA/LE	No azide low endotoxin
NFAT	Nuclear factor of activated T cells
NFIL3/E4BP4	nuclear factor, interleukin 3 regulated
NFκB	Nuclear factor κB
NK	Natural killer
NKT	Natural killer T
NLR	Nod-like receptors
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death-1
pDC	Plasmacytoid DC
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein complex
PGE_2	Prostaglandin E2
PI3K	Phosphatidyl-inositol 3-kinase

PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
Rev	Reverse
rh	Recombinant human
RNA	Ribonucleic acid
RNase	Ribonuclease
RORγt	RAR-related orphan receptor gamma t
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (medium)
RT-PCR	Real-time polymerase chain reaction
s.c.	subcutaneous
SA	Streptavidin
sCTLA-4 Ig	Soluble CTLA-4 Ig
SD	Standard deviation
SIRPα	Signal regulatory protein alpha
SSC	Sideward scatter
STAT	Signal transducer and activator of transcription
sVSG	Soluble variant-specific surface glycoproteins
T. brucei	Trypanosoma brucei
TAE	Tris-acetate-EDTA
T-bet / Tbx21	T-box 21
TBST	Tris buffered saline with Tween 20
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
Th1	T helper 1
Th3	T helper 3 regulatory T cells
Tip DC	TNF and iNOS producing DC
TLR	Toll-like receptors
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TNFR	TNF receptors
Tr1	T regulatory cells 1
Tr35	IL-35 dependent regulatory T cells
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
u	Units
v	Variable
V(D)J	Variable – Diversity – Joining
VSG	Variant-specific surface glycoproteins

9 LIST OF PUBLICATIONS

Parts of this work has been published in the following reports:

Pletinckx, K., Stijlemans, B., Pavlovic, V., Laube, R., Brandl, C., Kneitz, S., Beschin, A., De Baetselier, P. and Lutz, M. B., Similar inflammatory dendritic cell maturation signatures induced by TNF or Trypanosoma brucei antigens instruct default T helper-2 cell responses. *Eur J Immunol* 2011. [Epub ahead of print].

Pletinckx, K., Döhler, A., Pavlovic, V. and Lutz, M. B., Role of dendritic cell maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells. *Front Immun* 2011. **2**: 1-16.

11 AFFIDAVIT

I hereby confirm that my thesis entitled "Dendritic cell maturation and instruction of $CD4^+$ T cell tolerance in vitro" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Wuerzburg, 7th November 2011
Place, Date Signature

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die Dissertation "Reifung der dendritischen Zelle und Instruktion der $CD4^+$ T Zell Toleranz in vitro" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt hat.

Wuerzburg, 7 November 2011
Ort, Datum Unterschrift