# Exploring the function of IL-10, BTLA and DCs as regulators of immune responses

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# Abbreviations

| | |
|---|---|
| Ab | antibody |
| ALT | alanine transaminase |
| APC | antigen presenting cell or allophycocyanin |
| approx. | approximately |
| BAC | bacterial artificial chromosome |
| Bio | biotinylated |
| β-ME | β-mercaptoethanol |
| bp | base pair |
| BMDCs | bone marrow derived dendritic cells |
| BSA | bovine serum albumin |
| °C | temperature in celsius degrees |
| CD | cluster of differentiation |
| CFA | Complete Freund's Adjuvant |
| CFSE | carboxyfluorescein diacetate succinimidyl ester |
| cDNA | complementary DNA |
| CNS | central nervous system |
| cpm | counts per minute |
| Cre | site-specific recombinase (causes recombination) |
| d | day/s |
| d.p.i | days post immunization |
| DC | dendritic cell |
| DMEM | Dulbecco's modified Eagle medium |
| DN | double negative |
| DNA | desoxyribonucleic acid |
| dNTP | desoxynucleotide triphosphate |
| DP | double positive |
| DTA | Diphtheria toxin A |
| DTT | dithiothritole |
| DTR | Diphtheria toxin receptor |
| Dtx | Diphtheria toxin |
| EAE | experimental autoimmune encephalomyelitis |
| EDTA | ethylene-diaminetetraacetic acid |
| ELISA | enzyme-linked immuno-sorbent assay |
| ES | embryonic stem |
| EtOH | ethanol |
| FACS | fluorescence activated cell sorting |
| FCS | fetal calf serum |
| Fig. | Figure |
| FITC | fluorescein isothiocyanate |
| Flp | site-specific recombinase, product of yeast *FLP1*-gene |
| FoxP3 | forkhead box protein 3 |
| FRT | Flp recombination target |

| | |
|---|---|
| gr | gram |
| hr | hour/s |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid |
| iDTR | inducible Diphtheria toxin receptor |
| i.p. | intraperitoneally |
| i.v. | intravenously |
| ICS | intracellular staining |
| IFN-γ | interferon-γ |
| Ig | immunoglobulin |
| IL | interleukin |
| kb | kilobase pair |
| l | liter |
| LN | lymph node/s |
| *loxP* | recognition sequence for Cre (locus of X-ing over of phage P1) |
| LPS | lipopolysaccharide |
| Ly6C | lymphocyte antigen 6 complex, locus C |
| M | molar |
| MACS | magnetic activated cell sorter |
| MFI | mean fluorescence intensity |
| $MgCl_2$ | magnesium chloride |
| MHC | major histocompatibility complex |
| min | minute |
| ml | milliliter |
| mM | millimolar |
| MOG | myelin oligodendrocyte glycoprotein |
| mRNA | messenger RNA |
| MS | multiple sclerosis |
| MΦ | macrophage/s |
| NaCl | sodium chloride |
| n | nano |
| NaOH | sodium hydroxide |
| neo | neomycin resistance gene |
| ng | nanogram |
| o/n | over night |
| OD | optical density |
| pDC | Plasmacytoid dendritic cell |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PE | phycoerythrine |
| Ptx | Pertussis toxin |
| RAG | recombinase activating gene |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RT | room temperature |
| sec | seconds |
| SA | streptavidine |

| | |
|---|---|
| s.c. | subcutaneously |
| sc | spinal cord |
| SDS | sodium dodecyl sulfate |
| SN | supernatant |
| SP | single positive |
| SSC | sodium chloride/sodium citrate buffer |
| TAE | Tris-acetic acid-EDTA buffer |
| TAM | Tamoxifen |
| Taq | polymerase from *Thermus aquaticus* |
| TCR | T cell receptor |
| TE | Tris-EDTA buffer |
| TEC | thymic epithelial cell |
| tg | transgenic |
| TGF-β | transforming growth factor-β |
| Th | helper T cells |
| $T_{eff}$ | effector T cells |
| Tregs | regulatory T cells |
| Tris | 2-amino-2-(hydroxymethyl-)1,3-propandiole |
| U | units |
| UV | ultraviolett |
| V | volts |
| vs | versus |
| v/v | volume per volume |
| w/v | weight per volume |
| WT | wild type |
| µg | microgram |
| µl | microliter |
| µM | micromolar |
| 3’ | three prime end of DNA sequences |
| 5’ | five prime end of DNA sequences |

# 1 INTRODUCTION

Immune homeostasis depends on the existence of equilibrium between responses that control infection and tumour growth, and reciprocal responses that prevent inflammation and autoimmune diseases. Protection against infection is fundamental to the survival of all multicellular organisms and is mediated by the immune system, which has evolved both innate and adoptive mechanisms to deal with invading microorganisms. The effector mechanisms used by the host to control infection include production of proinflammatory cytokines and chemokines, recruitment of inflammatory cells to the site of infection and activation of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, which lyse infected host cells. Although these responses help to eliminate or slow the spread of the pathogen, if they are not tightly controlled, they can result in severe inflammation and collateral tissue damage (Artavanis-Tsakonas et al., 2003).

As the cells and molecules of the immune system that respond to pathogen-derived antigens might also respond to self-antigens, autoimmune disease can result if this reactivity is not tightly controlled (von Herrath and Harrison, 2003). Therefore, inflammation and immune response to pathogens are regulated by various host suppressor mechanisms, including the production of anti-inflammatory cytokines, co-inhibitory signals and regulatory cells.

$CD4^+$ T cells, also known as T helper (Th) cells, play an important role in orchestrating adaptive immune responses to various infectious agents. They are also involved in the induction of autoimmune and allergic diseases. Upon T cell receptor (TCR)-mediated cell activation, naïve CD4 T cells can differentiate into at least four major lineages, Th1, Th2, Th17 and iTreg cells, which participate in different types of immune responses. Networks of cytokines and transcription factors are critical for determining $CD4^+$ T cell fates and effector cytokine production. The major determinant for Th cell differentiation is the cytokine milieu

at the time of antigen encounter, although the nature of cognate antigen and its affinity to the TCR as well as the available co-stimulants, many of which regulate initial cytokine production, can influence Th cell fate.

Interleukin 10 (IL-10) is a cytokine that modulates both innate and adoptive immunity, primarily by exerting anti-inflammatory effects. IL-10 is produced by a variety of cell types of both innate and adoptive origin. During many infections, $CD4^+$ T cells produce both IFNγ and IL-10, as the IL-10 produced by effector Th1 cells helps limit the collateral damage caused by exaggerated inflammation. However, this control may limit the effectiveness of the immune response, resulting in a failure to fully eliminate pathogens.

The specific function of an individual's immune system in different physiological and pathological setting is regulated by the action of opposing factors or systems. Common examples are the polarization of T helper cells into Th1 and Th2 subsets, and the balance between effector T-cell ($T_{eff}$) activation and regulatory T-cell ($T_{reg}$) activation. At the molecular level, co-stimulatory members of the B7 and TNF superfamilies can have both stimulatory and inhibitory effect on T-cell activation.

Co-signalling molecules are cell-surface glycoproteins that can direct, modulate and fine-tune T-cell receptor signals. On the basis of their functional outcome, co-signalling molecules can be divided into co-stimulators and co-inhibitors, which promote or suppress T cell activation, respectively. By expression at the appropriate time and location, co-signalling molecules control the priming, growth, differentiation and functional maturation of a T cell response.

Traditionally, co-signalling molecules were termed as receptors or ligands to distinguish the direction of signal transmission. However this nomenclature is entirely operational and does not reflect the intrinsic nature of these molecules. A ligand could be either a co-stimulator or co-inhibitor, depending on the specific receptor it interacts with: for example, the binding of CD80/CD86 to CD28 transmit a co-stimulatory signal, whereas the binding of CD80/CD86 to CTLA4 transmit a co-

inhibitory signal. Moreover, a ligand could become a receptor (reverse signalling) as it receives a co-inhibitory or co-stimulatory signal, a process that had been described for CTLA4, PD-1 and BTLA (Dong et al., 2003; Fallarino et al., 2003; Grohmann et al., 2002; Nguyen et al., 2002).

Dendritic cells (DCs) are considered as the most potent antigen presenting cells (APCs) and as such, play a central role in the orchestration of the various forms of immunity and tolerance. Their immune-regulatory role relies mainly on the ligation of specific receptors that initiate and modulate DC maturation, resulting in the development of functionally different effector DC subsets that selectively promote Th-1, Th-2, Th-17 or Treg cell responses.

This thesis focuses on different aspects of immune regulation, both at the cellular and molecular levels. More specifically, this work concentrates on the importance of Interleukin-10, B and T Lymphocyte Attenuator (BTLA), and dendritic cells in respect to immune regulation, with special emphasis on autoimmunity.

## 1.1 Interleukin-10

Interleukin-10 (IL-10) is a cytokine that modulates both innate and adaptive immunity, primarily by exerting anti-inflammatory effects. Originally identified as a Th2 cell derived factor (Fiorentino et al., 1989), IL-10 was later found to be secreted by a variety of haemopoietic cell types including activated macrophages (de Waal Malefyt et al., 1991; Maeda et al., 1995), dendritic cells (Anderton et al., 2002; Iwasaki and Kelsall, 1999; Khanna et al., 2000; McGuirk et al., 2002; Mizoguchi et al., 2002; O'Garra et al., 1992; Stumbles et al., 1998), B cells and mast cells (Masuda et al., 2002). IL-10 is also produced by regulatory T cells (Tregs), Th1 cells (O'Garra and Vieira, 2007; Trinchieri, 2007), and Th17 cells (Awasthi et al., 2007; Fitzgerald et al., 2007; McGeachy et al., 2007; Stumhofer et al., 2007) as well as Th9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008). In addition, keratinocytes can be induced to secrete IL-10 by contact allergen or UV irradiation (Pinto et al., 2006).

### 1.1.1 IL-10 protein, gene and expression

IL-10 protein is a homodimer composed of two interpenetrating polypeptide chains, similar to interferon gamma (IFNγ) (Syto et al., 1998; Walter and Nagabhushan, 1995). IL-10 open reading frames (ORF) encode a secreted protein of 178 amino acids with rather a well-conserved sequence of about 73% homology shared by human and mice.

The murine IL-10 (mIL-10) gene is encoded in five exons, located on chromosome 1 (Kim et al., 1992). Activation of IL-10 gene expression results in a 1.4 kb mRNA, which can be regulated by the transcription factors Sp1 and Sp3 (Tone et al., 2000) as well as at the posttranscriptional levels (Powell et al., 2000), indicating that the IL-10 gene is transcribed to some degree constitutively and subject to control by alteration of posttranscriptional RNA degradation mechanisms.

It has been suggested that Toll Like Receptor (TLR)-2 agonists are specialized in inducing IL-10 expression by antigen presenting cells (Agrawal et al., 2003; Dillon et al., 2004; Hu et al., 2006; Netea et al., 2004). IL-10 production is also induced by TLR4, TLR9 and TLR3 ligands (Boonstra et al., 2006). Following TLR stimulation,

activation of extracellular signal-regulated kinases (ERKs) modulates IL-10 expression (Agrawal et al., 2006; Dillon et al., 2004; Kaiser et al., 2009; Yi et al., 2002). In addition, IL-10 synthesis can be induced by other non-TLR receptor, such as DC-SIGN or dectin-1 alone or in combination with ligation of CD40 or Fc receptors (FcRs) (Edwards et al., 2002; Geijtenbeek et al., 2003; Gerber and Mosser, 2001; Rogers et al., 2005). All T cell subsets can produce IL-10 under different condition. Th1 cell that produces both IFNγ and IL-10 can be generated when inducing T cell proliferation, though strong TCR triggering by high antigen dose and endogenous IL-12 are essential (Saraiva et al., 2009). IL-10 production by Th1 cells is STAT4 and ERK dependent (Saraiva et al., 2009). In Th2 cells, IL-10 production is regulated by the hallmark Th2 signalling pathways and transcription factors: IL-4, STAT6 and GATA3 (Chang et al., 2007; Zhu et al., 2004). IL-10 production by Th17 seems to occur in a STAT3, and in some cases, STAT1 dependent manner (Stumhofer et al., 2007; Xu et al., 2009) thus, IL-10 production by the different Th subsets requires Th-specific signalling and transcription factors, though all IL-10 producing Th subsets depend on ERK signalling (Saraiva et al., 2009). IL-10 expression is also highly associated with the suppression capacity of regulatory T and B cells (Asseman et al., 1999; DiLillo et al., 2010; Powrie et al., 1994) as well as the recently identified Th-9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008).

### 1.1.2 IL-10 receptor and signaling

IL-10 receptor is a type two cytokine receptor of the interferon receptor (IFNR) family, consist of two IL-10Rα and two IL-10Rβ subunits. IL-10Rα (also known as IL-10R1) is the ligand-binding subunit and is expressed on most hematopoietic cells, although generally at a low level of few hundreds per cell (Carson et al., 1995; Ho et al., 1993; Tan et al., 1993). Following activation, the expression of the IL-10Rα subunit on T cells is down-regulated both at the mRNA and protein levels (Tan et al., 1993), whereas its expression on monocytes is upregulated. IL-10Rα has also been reported to be expressed on non-hematopoietic cells such as fibroblasts, epidermal cells, keratinocytes placental cytotrophoblasts

and colonic epithelium, although the expression appeared to be induced rather then constitutive. IL-10Rβ (also known as IL-10R2) is the accessory subunit that is responsible for the IL-10/IL-10R signalling. IL-10Rβ contributes little to the IL-10-binding affinity and is primarily manifesting the recruitment of a Jak kinase into the signalling complex (Kotenko et al., 1997; Spencer et al., 1998). IL-10Rβ is shared by several additional cytokines of the IL-10 superfamily, IL-22, IL-26, IL-28 and IL-29 and is constitutively expressed on most cells and tissue (Commins et al., 2008; Gibbs and Pennica, 1997; Lutfalla et al., 1993). This expression pattern is not further regulated by activation, thereby any stimulus delivered by the IL-10Rα subunit should be sufficient to render most cells in becoming responsive to IL-10.

### 1.1.3 IL-10R signal transduction

IL-10 main signalling pathway is that of the Janus Kinase (JAK) / Signal Transduction and Activation of Transcription (STAT) pathway. IL-10/IL-10R interaction engages the tyrosine kinase Jak1 and Tyrosine Kinase 2 (Tyk2) which are associated with the IL-10Rα and IL-10Rβ respectively. Following their recruitment, Jak1 and Tyk2 are phosphorylated and activate the transcription factors STAT1 and STAT3 (Wehinger et al., 1996). IL-10 is also involved in other signalling pathways such as the NFκB pathway and AP-1 signalling. IL-10 inhibits NFκB signalling in at least two distinct ways: by inhibiting activation of IκB kinase and by inhibiting NFκB DNA binding activity (Wang and Baldwin, 1998). In $CD8^+$ T cells IL-10 can activate AP-1 and NFκB (Hurme et al., 1994). This is in line with the ability of IL-10 to promote differentiation, proliferation and cytotoxic activity of $CD8^+$ T cells (Groux et al., 1998; Santin et al., 2000).

### 1.1.4 IL-10 Function

#### 1.1.4.1 Antigen presenting cells

IL-10 inhibits T cell activation in an indirect fashion by modulating the function of the accessory antigen presenting cells. IL-10 regulate a variety of APC activation signals such as cytokine production, both CC and CXC chemokine

expression, MHC-II expression and the expression of co-stimulatory molecules such as IL-12, CD80 and CD86 on these myeloid origin cells (Bogdan et al., 1991; Ding et al., 1993; Fiorentino et al., 1991; Murphy et al., 1994). IL-10 also inhibits DC maturation from monocyte precursors (Allavena et al., 1998; Buelens et al., 1997). In humans, IL-10 exhibits similar suppressive effects by modulating the function and cytokine production of monocytes, macrophages and DCs. Collectively, these observations indicate that IL-10 induces differentiation of myeloid cells that limits ongoing immune responses and inflammation.

#### 1.1.4.2 T cells

IL-10 strongly inhibits cytokine production and proliferation of $CD4^+$ T cells via its regulation effects on APC function. However, IL-10 can also directly affect $CD4^+$ T cells function and inhibit IL-12 and TNF production, depending on the activation conditions (de Waal Malefyt et al., 1993; Taga et al., 1993). The activation of T cells in the presence of IL-10 can induce anergy, which cannot be reversed by additional exogenous IL-2 or stimulation with anti-CD3/CD28 (Groux et al., 1996). IL-10 mediated anergy can induce the generation of regulatory T cells which produces high levels of IL-10 and can suppress antigen-specific responses both *in-vivo* and *in-vitro* (Asseman et al., 1999; Groux et al., 1997; Tanchot et al., 1998). In contrast, IL-10 stimulates $CD8^+$ T cells, induces their recruitment, cytotoxic activity and proliferation (Groux et al., 1998; Jinquan et al., 1999; Santin et al., 2000). IL-10 is also expressed by Th1 cells (Anderson et al., 2007; Assenmacher et al., 1994; Del Prete et al., 1993; Gerosa et al., 1996; Jankovic et al., 2007; Pohl-Koppe et al., 1998). Under inflammatory conditions, Th17 cells may also produce IL-10 (Li and Flavell, 2008; Stumhofer et al., 2007). Recently, a new subset of T helper cells that produces IL-9 (Th9) has been reported to coproduce IL-9 and IL-10 (Dardalhon et al., 2008; Veldhoen et al., 2008). Although Th17 and Th9 cells produce IL-10, it is not fully known yet what role thus IL-10 serve.

### 1.1.5 IL-10 in infectious disease

Pathogen-derived products activate macrophages and DCs through pattern recognition receptors (PPR), which in turn trigger the expression of cytokine and other factors (Medzhitov, 2007). IL-10 is a key player in controlling the balance between pathology and protection.

Activation of an innate response to intracellular bacteria, fungi and protozoa infection involves their recognition by TLRs and other lectin type receptors such as DC-SING and dectin-1, found on dendritic cells and other innate cells such as macrophages, neutrophils and granulocytes. Following TLR ligation, signalling cascades are activated, mainly through the adaptor molecules MyD88 and TRIF, leading to the production of IL-10 and other pro-inflammatory cytokines (Boonstra et al., 2006). TLR signalling through MyD88 activates mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NFκB) (Akira and Takeda, 2004). This in turn leads to the production of numerous cytokines, in particularly IL-12, IL-18, TNF and IL-1, which stimulate NK cells to produce high levels of IFNγ. This NK-produced IFNγ induces macrophage phagocytosis, production of nitric oxide (NO), reactive oxygen species (ROS) and stimulates cell infiltration. Under these conditions, naïve T cells preferentially differentiate into Th1 cells that can further fight against the infection with greater specificity. IL-10 negatively regulates many of these processes including IL-1, IL-12 IL-18 and TNF synthesis by macrophages, granulocytes and dendritic cells, production of NO and ROS by macrophages and alike, upregulation of MHC-II and co-stimulatory molecules on antigen presenting cells, secretion of IFNγ by the NK cells and activation and differentiation of naïve T cells into effector Th1 cells.

In chronic non-healing lymphocytic choriomeningitis virus (LCMV) infection, $CD8^-$ DCs produces high amounts of IL-10. *In-vivo* blockade of the IL-10R by neutralizing antibody or removal of IL-10 by genetic means restored T cell function, eliminated viral infection and allowed the generation of antiviral memory T-cell response (Brooks et al., 2006; Ejrnaes et al., 2006).

IL-10-deficient mice (IL-$10^{-/-}$) exhibit elaborated Th1 responses and spontaneously develop inflammatory bowel disease (IBD) as a result of an unbalanced response to environmental antigens (Kuhn et al., 1993). These mice also exhibit higher susceptibility to immunopathology upon parasitic and bacterial infection (Deckert et al., 2001; Gazzinelli et al., 1996; Kullberg et al., 1998; Namangala et al., 2001) or lipopolysaccharide (LPS) exposure (Berg et al., 1995a), as well as enhanced contact hypersensitivity reactions and irritant responses of the skin (Berg et al., 1995b). IL-10 producing Th1 cells have been isolated from the bronchoalveolar lavage of patients with active pulmonary tuberculosis (Gerosa et al., 1999). IL-10-producing Th1 cells were also present in mice infected with *Toxoplasma gondii* or *Leishmania major* (Anderson et al., 2007; Jankovic et al., 2002; Shaw et al., 2006). These IL-10-producing Th1 cells, rather than IL-10 producing Th2, Treg or innate cells, were shown to be important for the regulation of these infections (Anderson et al., 2007; Jankovic et al., 2007).

### 1.1.6 IL-10 in autoimmunity

IL-10 plays a central role in peripheral tolerance and in inhibiting autoimmune responses. In chronic autoimmune diseases such as diabetes, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA), pathogenicity has been attributed to Th1 cells due to their production of cytokines such as TNF, lymphotoxin and IFNγ (Liblau et al., 1995; O'Garra et al., 1997; Powrie and Coffman, 1993). On the other hand, in both IBD and experimental autoimmune encephalomyelitis (EAE), regulatory $CD4^+$ T cells can produce high levels of IL-10 that inhibits the proliferation and differentiation of naïve T cells to become effector Th1 cells and thereby restricting disease pathology (Groux et al., 1997; Groux and Powrie, 1999; Powrie et al., 1996; Roers et al., 2004). IL-10 deficient mice develop spontaneous IBD as well as an enhanced pathology during EAE, due to the loss of the suppressive activity of regulatory T cells (Asseman et al., 1999; Berg et al., 1996; Kuhn et al., 1993; Roers et al., 2004). Several B cell subsets, which produce IL-10, have been shown to have regulatory function during distinct autoimmune diseases. IL-10-producing B cell subsets include IL-10-producing peripheral B cells, $CD5^+$ B1a

cells (O'Garra et al., 1992; O'Garra et al., 1990), $CD1d^{+}$ marginal zone B cells, transitional-2-marginal zone precursor B cells (Harris et al., 2000) and the recently identified splenic $CD1d^{hi}CD5^{+}CD19^{hi}$ B cells (Yanaba et al., 2008). These $CD1d^{hi}CD5^{+}$ B cells (termed B10 cells) exclusively produce high levels of IL-10 and negatively regulate inflammation and autoimmunity (Yanaba et al., 2009). These B10 cells are antigen specific and been shown to control T cell activation and inflammatory response through IL-10 production (Matsushita et al., 2008; Yanaba et al., 2008). During IBD in $TCR\alpha^{-/-}$ mice, B cells isolated from inflamed mesenteric lymph nodes (MLN) produced high levels of IL-10 transcript and had regulatory properties (Mizoguchi et al., 2002). In EAE, when B cell depletion approach was taken, different B cell subsets were shown to play different role. B10 cell depletion seven days prior to active immunization results in increased CNS infiltration (of both Th1 and Th17) and exacerbates disease while B cell depletion 14 days after immunization ameliorate disease progression. Disease exacerbation was attribute to the loss of IL-10 producing regulatory B10 cells (Matsushita et al., 2008). In collagen-induced arthritis (CIA), splenic B cells activated with collagen and agonistic CD40 produced high levels of IL-10 and when adoptively transferred, prevented arthritis development (Mauri et al., 2003). Splenic B cells from lupus-prone mice were found to secrete IL-10 when cultured with CpG oligodeoxynucleotides, while B cells from DBA/1 mice did not (Fife and Bluestone, 2008). Thus, although primarily exhibiting regulatory functions, IL-10 can also serve as an effector cytokine, contributing to disease pathology.

### 1.1.7 IL-10 and experimental autoimmune encephalomyelitis

Early studies had shown that spontaneous recovery of EAE is accompanied with IL-10 producing Th2 cells (Issazadeh et al., 1995; Kennedy et al., 1992; Khoury et al., 1992). During EAE relapse, the detectable IL-10 levels were very low, suggesting that endogenous IL-10 could regulate CNS pathology (Issazadeh et al., 1996). IL-10 production can be induced by IFNβ, which is used as a treatment in multiple sclerosis (MS) (Navikas et al., 1995). Furthermore, IL-10 deficient mice develop a more severe EAE with higher incidence (Bettelli et al., 1998; Segal et al.,

1998). When using anti-IL-10 neutralizing antibodies, EAE relapse severity was increased (Crisi et al., 1995) whereas treating mice with recombinant IL-10 (rIL-10) resulted in contradictory outcomes: upon active immunization, systemic administration of rIL-10 partially inhibited EAE development and/or progression, though only if IL-10 was given from the very beginning (Nagelkerken et al., 1997; Rott et al., 1994). The site of, the timing and the root of IL-10 administration also appear to have an effect on disease outcome (Croxford et al., 1998; Shaw et al., 1997). Other reports, however, showed that IL-10 could fully inhibit EAE (Bettelli et al., 1998; Cua et al., 1999; Cua et al., 2001; Mathisen et al., 1997). B cell-derived IL-10 was shown to play a key role in EAE as bone marrow chimeric mice reconstituted with IL-10 deficient B cells failed to control disease recovery (Fillatreau et al., 2002). The depletion of B cells prior to MOG immunization by anti CD20 antibody exacerbates disease. Adoptive transfer of B10 cells, but no other B cell subsets, normalized EAE whereas adoptive transfer of IL-10 deficient B10 cells failed to normalize disease severity (Matsushita et al., 2008).

## 1.2 B and T Lymphocyte Attenuator

The response of immune cells, particularly T cells and APCs, is dictated by the balance between negative signals from co-inhibitory receptors and positive signals from co-stimulatory receptors (Bretscher, 1999). Receptors and their cognate ligands on T cells and APC are vital for delivering stimulatory or inhibitory signals that enable immune cells to remain inactive or to respond effectively to numerous environmental stimuli (Gimmi et al., 1993; Greenwald et al., 2005; Ravetch and Lanier, 2000). The CD28/B7 superfamily and tumor necrosis factor (TNF)/TNF-receptor (TNFR) superfamily contains many of these molecules and were thought to be distinct functioning modules (Cai and Freeman, 2009).

The co-inhibitory molecules of the CD28 superfamily comprise immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Leibson, 2004), recruit phosphatases, such as Src-homology protein tyrosine phosphatase-2 (SHP-2)

(Leibson, 2004; Salmond and Alexander, 2006), and negatively regulate in a manner distinct from the apoptosis-inducing TNFR molecules, by modulating signaling cascades induced through the co-stimulatory and antigen receptors.

B and T Lymphocyte Attenuator (BTLA) is a CD28 superfamily cell surface protein (Han et al., 2004; Watanabe et al., 2003) found on activated T cells, as well as B cells, natural killer (NK) cells, NK-T cells, dendritic cells and macrophages (Han et al., 2004; Hurchla et al., 2005). BTLA signaling can suppress a T cell response (Han et al., 2004; Watanabe et al., 2003), which is confirmed by hyper-reactivity of BTLA-deficient cells (Han et al., 2004) and evidence of enhanced *in vivo* immune responses in BTLA-deficient animals (Watanabe et al., 2003). BTLA considered being a co-inhibitory molecule throughout its interaction with herpes virus entry mediator (HVEM), also known as tumor necrosis factor receptor superfamily member 14 (TNFRSF14), a TNF superfamily member (Cheung et al., 2005; Sedy et al., 2005). HVEM can also interact with other co-inhibitory molecule such as CD160 (Cai et al., 2008) or co-stimulatory molecules such as lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT) and lymphotoxin-alpha (LT$\alpha$) (Mauri et al., 1998). The signal following BTLA/HVEM interaction can be transmitted in *cis* or *trans* fusion, as both BTLA and HVEM are expressed on both lymphocytes and APCs.

### 1.2.1 Co-receptors of the CD28 and TNFR superfamilies

Recent studies have highlighted the structural requirements for T cell co-stimulation. The molecular architecture of a given receptor-ligand pair defines its signal transduction pathway (Schwartz et al., 2002). The two main co-receptor families are the CD28/B7 superfamily and the TNF/TNFR superfamily. Generally, it has been accepted that the receptor-ligand pair are of the same superfamilies, with CD28 co-receptors binding B7 ligands and TNFRs binding TNF-ligands. Co-stimulatory members of the CD28/B7 superfamily includes CD28 and inducible co-stimulator (ICOS) that binds CD80/86 and ICOS ligand, respectively, whereas the CD28/B7 co-inhibitory molecules includes cytotoxic T lymphocyte-associated

antigen 4 (CTLA-4), program death 1 (PD-1) and BTLA that binds CD80/86, PD-L1/PD-L2 and HVEM, respectively. The co-stimulatory members of the TNFR superfamily includes 4-1BB, OX40, CD27, CD30, CD40 and HVEM. The respective TNF-ligands are 4-1BBL, OX40L, CD70, CD40L and LIGHT or LTα (Greenwald et al., 2005).

BTLA and CD160, two CD28 superfamily members, are a rare exception of direct interaction between the two superfamilies. Crystal structure of the different CD28 superfamily receptor members CD28, CTLA-4 and PD-1, revealed that each of this protein contain an extracellular immunoglobulin variable (IgV) domain (Evans et al., 2005; Metzler et al., 1997; Zhang et al., 2004b). Similarly, the extracellular domains of the B7 ligands are composed of Ig domains. The binding of these CD28-B7 receptor-ligand pair requires the close proximity of the two Ig domains in order to form the needed compact interface (Schwartz et al., 2001; Stamper et al., 2001). In contrast, the TNF-TNFR pairs are formed by very different structural elements and interact in a distinctive manner. These proteins are homotrimeric proteins containing multi cysteine-rich domains (CRDs), which are crucial for the receptor-ligand binding and signal transduction (Bodmer et al., 2002) therefore structurally, the binding of CD28 superfamily BTLA and/or CD160 to HVEM was unexpected.

### 1.2.2 BTLA structure, expression and signaling

BTLA is a type I transmembrane glycoprotein with an extracellular IgV, a transmembrane region, a cytoplasmic region and a signal sequence (Gavrieli et al., 2003). The BTLA protein is a monomer that lacks the membrane proximal cysteine needed for it dimerization (Compaan et al., 2005; Greenwald et al., 2005). The crystal structure of BTLA revealed a distinct binding surface, that forms a single receptor-ligand complex (Compaan et al., 2005). Full-length BTLA (fl-BTLA) cytoplasmic region contains two ITIMs, which are associated with the phosphotases SHP-1 and SHP-2 (Gavrieli et al., 2003). In addition to the major fl-BTLA isoform, two other BTLA alternative spliced variant (sBTLA) exists. One isoform is lacking the Ig domain while the other isoform lacks the transmembrane region (Han et al., 2004; Watanabe et al., 2003). BTLA expression varies depending on cell type,

differentiation and activation statues. During thymocytes development, BTLA is expressed starting from the transition phase of $CD4^-CD8^-$ double negative (DN) into the $CD4^+CD8^+$ double positive (DP) stage, with slightly higher expression on $CD4^+$ single positive (SP) in comparison to $CD8^+$ SP T cells (Krieg et al., 2005). BTLA deficient mice however, show no signs of abnormalities in thymic development or peripheral T cell subpopulations (Han et al., 2004; Watanabe et al., 2003). Naïve T cells constitutively expressed BTLA, though to a very low level (Han et al., 2004) whereas activated T cells show increasing expression levels of BTLA (Compaan et al., 2005; Gavrieli et al., 2003; Krieg et al., 2005; Watanabe et al., 2003). BTLA is abundant on activated Th1 cells, and its expression is reduced during the contraction phase. During the memory phase, Th1 cells but not Th2 cells, further upregulate BTLA expression (Gavrieli et al., 2003; Loyet et al., 2005). BTLA is expressed at low levels by B cells in the bone marrow (BM) during the pro- and pre-B cell stage, somewhat higher levels on immature B cell and is constitutively and highly expressed on resting peripheral B cells (Hurchla et al., 2005). However, BTLA deficient B cells showed only slight augment responses to anti-IgM stimulation and no difference in response to LPS (Watanabe et al., 2003). BTLA deficient mice also exhibit a mild increased level of specific IgG1, IgG2a and IgG2b upon immunization with nitrophenol (NP)-conjugated limpet hemocyanin (NP-KLH), indicating a role for BTLA in T cell-dependent antibody (Ab) response (Watanabe et al., 2003). Moreover, BTLA deficient mice immunized with NP-ficoll show modest enhancement of IgG3, which is primarily associated with T cell independent response (Hurchla et al., 2005). Bone marrow derived DCs (BMDCs) following LPS stimulation showed elevated BTLA expression levels (Han et al., 2004). BTLA may also play a role in DC homeostasis (De Trez et al., 2008), as BTLA deficient mice show increased amounts of $CD8^-$ conventional DCs (Kabashima et al., 2005).

Since BTLA contains an extracellular Ig domain, its counter-receptor or ligand was also predicted to have an Ig domain as other CD28-B7 receptor-ligand pair. Early studies suggested that the orphan B7 molecule B7x is the missing ligand (Watanabe et al., 2003; Zang et al., 2003), however the screening of a retroviral

spleen cDNA expression library identified HVEM as being BTLA's counter-receptor (Gonzalez et al., 2005; Sedy et al., 2005).

HVEM was first identified as a receptor for herpes simplex virus type-1 (HSV-1) glycoprotein-D (gD) (Montgomery et al., 1996). Later on, two other TNF-ligands, LIGHT and LTα, had been shown to bind HVEM (Mauri et al., 1998). Recently another molecule, CD160, was added to the growing list of HVEM-ligands (Cai et al., 2008). HVEM is a type-1 transmembrane glycoprotein, which is expressed on the cell surface as a monomer. Following the binding to its trimeric ligands, HVEM is predicted to form a 3:3 complex (Gonzalez et al., 2005; Mauri et al., 1998). HVEM extracellular domain consists of four CRDs (Bodmer et al., 2002) and its binding regions are distinct for each of its ligands. While LIGHT homology domain interacts with HVEM CRD2 and CRD3 and LTα homology domain interacts with CRD3 (Sarrias et al., 2000), gD, BTLA and CD160 binds the membrane distal CRD1 of HVEM (Connolly et al., 2002; Sedy et al., 2005; Whitbeck et al., 2001). BTLA and gD bind to an overlapping site on HVEM (Cheung et al., 2005). However, whereas BTLA-Ig completely blocked the binding of CD160 to HVEM, CD160-Ig only partially blocked BTLA-HVEM interaction (Cai et al., 2008), indicating that the two binding sites are not fully identical.

## 1.3 Dendritic cells

Experimental autoimmune encephalomyelitis (EAE) is a T cell mediated autoimmune disease of the central nervous system (CNS) and is widely used as a multiple sclerosis model, with which it shares many clinical, immunological and pathological characteristics (Gold et al., 2006). EAE is induced by priming and expanding $CD4^+$ T cells using CNS-derived myelin antigens, which are normally sequestered behind the blood-brain barrier. It is not known exactly which APCs are responsible for activating these autoreactive T cells. Dendritic cells are considered prototypic APCs (Banchereau and Steinman, 1998; Thery and Amigorena, 2001). In

absence of inflammation or infection, most DCs in peripheral tissues and lymphoid organs have a resting, immature phenotype, characterized by high endocytic capacity and low surface expression of MHC and co-stimulatory molecules. However, upon interaction with microbial ligands, pro-inflammatory cytokines or CD40 ligand, DCs rapidly acquire an activated phenotype and become proficient in activating naïve T cells (Steinman et al., 2003).

DCs are sparse in a healthy CNS and primarily found in vessel-rich areas, such as the meninges and choroid plexus (Greter et al., 2005). CNS inflammation induced by an autoimmune response or infection is accompanied by entry of DCs into the CNS (Matyszak and Perry, 1996; Serafini et al., 2000). These CNS DCs, but not resident microglia or infiltrating macrophages, are the most efficient APCs in driving the reactivation of transferred myelin-specific $CD4^+$ T cells (Greter et al., 2005).

In addition to their pro-inflammatory role, DCs also promote immune homeostasis by inducing and maintaining peripheral T cell tolerance (Steinman and Nussenzweig, 2002). Further to the elimination of self-reactive T cells in the thymus, tolerance is maintained in the periphery through clonal deletion, induction of anergy and differentiation of regulatory T cells. In contrast to the naturally occurring Tregs (nTreg) developing in the thymus, induced Tregs (iTreg) are generated in the periphery and DCs appear to play an essential role in their development and maintenance (Darrasse-Jeze et al., 2009; Kretschmer et al., 2005; Yamazaki et al., 2008).

Recently, it has been established that animals that constitutively lack classical $CD11c^{hi}$ DCs display unimpaired thymic negative selection but that the absence of DCs triggers a progressive myeloproliferative disorder, driven by elevated Fms-related tyrosine kinase 3 ligand (Flt3L) levels (Birnberg et al., 2008). As a result of this disorder, DC-less mice develop signs of lymphocyte hyper-activation. Moreover, Ohnmacht et al. reported that animals lacking DCs succumb to a spontaneous fatal auto-inflammatory disorder (Ohnmacht et al., 2009). In line with the above, a recent report uncovered a direct correlation between DC numbers and Tregs as part of a feedback control mechanism (Darrasse-Jeze et al., 2009).

# 2 MATERIALS AND METHODS

## 2.1 Chemicals and biological material

Chemicals were purchased from Sigma-Aldrich (Steinheim), Fluka Chemie (Deisenhofen), Merck (Darmstadt) or AppliChem (Darmstadt) unless stated otherwise. Solutions were prepared with double distilled water (dd$H_2O$). Bacterial media were autoclaved prior to use. Sterility of solutions and chemicals used in cell culture was maintained by working under a sterile hood (Heraeus, Germany).

| **Name of chemical** | **Supplier** |
|---|---|
| β-Mercaptoethanol (β-ME) | Fluka Chemie GmbH, Switzerland |
| Acetone | Merck, Darmstadt |
| Agar | Gibco Life Technologies GmbH, Karlsruhe |
| Agarose, electrophoresis grade | AppliChem, Darmstadt |
| Ampicillin | Sigma-Aldrich, Steinheim |
| L-Arabinose | Sigma-Aldrich, Steinheim |
| Bovine serum albumin (BSA) | Sigma-Aldrich, Steinheim |
| Calcium chloride | Sigma-Aldrich, Steinheim |
| Chloroform | Merck, Darmstadt |
| 2'-Deoxyguanosine Monohydrate | AppliChem, Darmstadt |
| Diethylpyrocarbonate (DEPC) | AppliChem, Darmstadt |
| Dextran sulfate | AppliChem, Darmstadt |
| Dithiothreitol (DTT) | Boehringer Mannheim GmbH, Mannheim |
| Dimethylsulfoxide (DMSO) | Merck, Darmstadt |
| dNTPs | Pharmacia Biotech, USA |

| | |
|---|---|
| Ethylendiamine tetraacetate (EDTA) | Fluka Chemie GmbH, Switzerland |
| Ethanol, abs. | AppliChem, Darmstadt |
| Ethidium bromide | Sigma-Aldrich, Steinheim |
| Fetal calf serum (FCS) | Boehringer Mannheim GmbH, Mannheim |
| Ficoll 400 | Amersham Pharmacia, Freiburg |
| Glacial acetic acid | Fluka Chemie GmbH, Switzerland |
| Hydrochloric acid (37 %) | Merck, Darmstadt |
| Isopropanol | AppliChem, Darmstadt |
| Magnesium chloride | Sigma-Aldrich, Steinheim |
| Magnesium chloride (for PCR) | Gibco Life Technologies GmbH, Karlsruhe |
| Mineral oil | Sigma-Aldrich, Steinheim |
| Phenol | Sigma-Aldrich, Steinheim |
| Potassium acetate | Fluka Chemie GmbH, Switzerland |
| Potassium chloride | Merck, Darmstadt |
| Proteinase K | Roche, Switzerland |
| Salmon sperm DNA | Biomol, Hamburg |
| Sodium azide | Fluka Chemie GmbH, Switzerland |
| Sodium chloride | AppliChem, Darmstadt |
| Sodium citrate | Fluka Chemie GmbH, Switzerland |
| Sodium dodecyl sulfate | AppliChem, Darmstadt |
| Sodium hydrogencarbonate | Fluka Chemie GmbH, Switzerland |
| Sodium hydroxide | Fluka Chemie GmbH, Switzerland |
| Tris base | Fluka Chemie GmbH, Switzerland |
| Tris/ HCl | AppliChem, Darmstadt |

**Table 1: Chemicals**

## 2.2 Molecular biology

### 2.2.1 Competent cells and isolation of plasmid DNA

Competent *Escherichia coli* DH5α cells were prepared according to the protocol of Inoue *et al*. (Inoue et al., 1990) and used in heat shock transformations of plasmid DNA. DNA ligation was performed with the Takara DNA ligation Kit (Takara, Japan) according to the manufacturer's instructions. Plasmid DNA was isolated from transformed *Escherichia coli* DH5α bacteria with an alkaline lysis method (QIAGEN, Hilden, Germany). Plasmid DNA of higher purity was obtained with QIAGEN columns (QIAGEN, Hilden, Germany) following the supplier's instruction.

### 2.2.2 Isolation of genomic DNA from ES cells and mouse organs

Cells were lysed over night at 56°C in lysis buffer (10 mM Tris-HCl, pH 8; 10 mM EDTA; 150 mM NaCl; 0.2% (w/v) SDS; 400 mg/ml proteinase K). Subsequently, DNA was precipitated from the solution by the addition of an equal volume of isopropanol. DNA was pelleted by centrifugation, washed in 70% (v/v) EtOH and resuspended in TE-buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA). From ES cell clones, grown in 96-well tissue culture dishes, DNA was extracted and prepared according to the protocol of Pasparakis and Kollias. Mouse tissue was incubated over night at 56°C in lysis buffer (0.1 M Tris-HCl, pH 8.5; 5 mM EDTA; 0.2% (w/v) SDS; 0.2 M NaCl; 600 mg/ml proteinase K). Undissolved debris was pelleted and the supernatant was mixed with an equal volume of isopropanol to precipitate the DNA. The DNA was washed in 70% (v/v) EtOH, dried at RT and resuspended in TE-buffer.

### 2.2.3 RT-PCR and quantitative real-time PCR

RNA from isolated cellular subsets was prepared using the RNeasy mini kit (QIAGEN, Hilden, Germany) or Trizol (Invitrogen) according to the manufacturer's protocol. DNA was removed by DNAseI digestion (Promega, Mannheim, Germany). cDNA synthesis of 5 µg total RNA was performed as described in superscript II protocol (GIBCO, Karlsruhe, Germany). cDNA was subsequently used for RT- and Real-Time PCR. Expression was normalized to that of GAPDH expression. Quantitative Real-Time PCR was performed using primers from QIAGEN as described on their homepage https://www1.qiagen.com/GeneGlobe/Default.aspx.

### 2.2.4 Agarose gel electrophoresis and DNA gel extraction

Separation of DNA fragments by size was achieved by electrophoresis in agarose gels (0.8% - 2% (w/v); 1x TAE (Sambrook, 1989); 0.5 mg/ml ethidium bromide). DNA fragments were recovered from agarose gel slices with either the QIAEX II or the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions.

### 2.2.5 DNA sequencing

DNA was sequenced using the 'Big Dye termination Cycle Sequencing Kit' (Applied Biosystems, Foster City, USA), which is a PCR-based modification of the original Sanger protocol (lexSanger et al., 1977). The fluorescently labeled DNA fragments were separated and analysed with the ABI373A and ABI377 systems (Applied Biosystems, Foster City, USA).

### 2.2.6 Quantification of DNA

The concentration of nucleic acids was determined by measuring the absorption of the sample at 260 nm and 280 nm, respectively, in a spectrophotometer. An $OD_{260}$ of 1 corresponds to approximately 50 µg/ml for double stranded DNA or 40 µg/ml for RNA and single stranded DNA. Purity of nucleic acids was estimated by the ratio $OD_{260}/OD_{280}$, with 1.8 and 2.0 optimal for DNA and RNA, respectively. Alternatively, the DNA was electrophorated in an agarose gel, and the concentration was evaluated from the band intensity in comparison with a standard.

### 2.2.7 Polymerase Chain Reaction (PCR)

PCR (Mullis and Faloona, 1987) was used to screen mice and ES cells for the presence of targeted alleles or transgenes and to amplify fragments for sequencing (primers shown in Table 2 and 4). Reactions were performed in either Hybaid machines (MWG-Biotech, Ebersberg, Germany) or Triothermocyclers (Biometra, Göttingen, Germany). Genotyping of mice and ES cells was generally performed in a total volume of 50 µl in the following reaction mix: 50 pmol of each primer, 1.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (homemade), 250 mM dNTPs, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 ng template DNA. Amplification started with denaturation for 4 min at 94 °C followed by 30-35 cycles of 94 °C for 30 sec, 52-65 °C for 30 sec, 72 °C for 30 sec and a final extension step at 72 °C for 10 min.

| Name of primer | Sequence (5´-3´) | $T_{Ann.}$ °C |
|---|---|---|
| Cre3 | TCC AAT TTA CTG ACC GTA CAC | 58 |
| Cre7 | TCA GCT ACA CCA GAG ACG G | 58 |
| CD4cre (CD4L6) | CCC AAC CAA CAA GAG CTC | 59 |
| CD4cre (Cre6) | CCC AGA AAT GCC AGA TTA CG | 59 |

| | | |
|---|---|---|
| CD19c | AAC CAG TCA ACA CCC TTC C | 57 |
| CD19d2 | CCA GAC TAG ATA CAG ACC AGG A | 57 |
| RosaFA | AAA GTC GCT CTG AGT TGT TAT | 58 |
| RosaRA | GGA GCG GGA GAA ATG GAT ATG | 58 |
| SpliAcB | CAT CAA GGA AAC CCT GGA CTA CTG | 58 |
| LysM 1 | CTT GGG CTG CCA GAA TTT CTC | 60 |
| LysM 2 | TTA CAG TCG GCC AGG CTG AC | 60 |
| Cre8 | CCC AGA AAT GCC AGA TTA CG | 60 |
| CD11c-Cre (boris) s | ACT TGG CAG CTG TCT CCA AG | 63 |
| CD11c-Cre (boris) as | GCG AAC ATC TTC AGG TTC TG | 63 |
| IL-10 (MCO2) | CCA GCA TAG AGA GCT TGC ATT ACA | 58 |
| IL-10 (IL-10EX2) | GAG TCG GTT AGC AGT ATG TTG TCC AG | 58 |

To amplify cDNA fragments for cloning or sequencing, the High Fidelity Master Kit with proofreading activity was used (Roche, Mannheim, Germany) with the primers shown in Table 2. PCR of transgenic Cre lines was performed as published.

### 2.2.8 Southern blot analysis

Digestion of 5-15 mg DNA was performed overnight with 50-100 U of the appropriate restriction enzyme. Subsequently, the DNA fragments were separated by agarose gel electrophoresis and transferred onto Hybond™-N+ (Amersham, Illinois, USA) or GeneScreenPlus (Dupont, Wilmington, USA) nylon membranes by an alkaline capillary transfer according to the method of Chomczynski and Qasba (1984). Membranes were baked at 80°C for 20 min to fix the DNA, equilibrated in 2x SSC and then prehybridised at 65 °C for 4 h in hybridisation solution (1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulfate, 50 mM Tris-HCl pH 7.5, 250 µg/ml sonicated salmon sperm DNA). The radioactively labeled probe was added to the

hybridization solution and allowed to hybridize at 65 °C for 10 h in a rotating cylinder.

Aliquots of 50 ng DNA of the above probes were radioactively labeled with 2.5 µCi [$^{a32}$P]dATP (Amersham, Braunschweig, Germany) using the Ladderman™ Labeling Kit (Takara, Japan), based on the principle of random primed oligolabeling (Feinberg and Vogelstein, 1984). Unincorporated radiolabeled nucleotides were removed with MicroSpin™ S-200HR columns to reduce background during hybridisation (Pharmacia, Freiburg, Germany). The probe was denatured at 100°C for 5 min, cooled on ice, and then added to the hybridization solution. After hybridisation, stringent washes were initially performed twice in 2x SSC / 0.1 % (w/v) SDS and then followed by washes in 1x SSC / 0.1 % (w/v) SDS and 0.5x SSC / 0.1 % (w/v) SDS, if necessary. All washes were performed at 65 °C under gentle shaking for 30 min. After each wash, the filter was monitored with a Geiger counter and the washes were stopped when specific signals reached 50 to 200 cps. Then, the filter was sealed in a plastic bag and exposed to X-ray film (Kodak XAR-5 or BioMAX MS; Eastman Kodak) at -80°C. Films were developed in an automatic developer or alternatively, filters were exposed to a phosphoimager screen (Fuji, Fuji, Japan) and analysed on a Bio-Imaging Analyser (Fuji Bias 1000; Fuji, Japan).

## 2.3 Cell biology

### 2.3.1 Embryonic stem cell culture

All gene-targeting experiments were performed in C57BL/6 (Bruce-4) ES cells. Culturing and transfection of ES cells was performed according to standard laboratory protocols. To maintain the pluripotency of the ES cells, the latter were cultured in ES cell medium in the presence of leukaemia inhibiting factor (LIF) on a layer of embryonic feeder (EF) cells. The ES cell medium (DMEM, 15% (v/v) FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 x non essential amino acids, 1:1000 diluted LIF supernatant, 0.1 mM 2-β-mercaptoethanol) contained FCS that had been tested to promote ES cell growth and to prevent *in vitro* differentiation (Gibco,

Karlsruhe, Germany). ES and EF cells were grown in tissue culture dishes (Falcon, Bedford, USA) and kept at 37°C under humid atmosphere with 10% $CO_2$. EF cells in DMEM supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, were never passaged more than three times and then mitotically inactivated by mitomycin C treatment (10 mg/ml for 2 h), before seeding with ES cells. ES cell colony growth was stopped before they became confluent. Colonies were washed twice with PBS and then treated shortly with trypsin (0.05 % (w/v), 0.02 % (v/v) EDTA in PBS; Gibco, Karlsruhe, Germany) at 37°C, until the cells detached from the dish. The cell suspension was then used for passaging, transfection or freezing. ES cells were frozen in 90% (v/v) FCS, 10% (v/v) DMSO at -80°C and later transferred into liquid nitrogen for long-term storage. For transfection, 1 x $10^7$ ES cells were mixed with 30 to 40 mg DNA in 800 ml transfection buffer (RPMI w/o phenol red, Gibco, Karlsruhe, Germany) and electroporated at 23°C (500 mF, 240 V). After 5 minutes of incubation, ES cells were transferred onto an embryonic feeder layer and 48 h later placed under G418 (300 mg/ml, 71% active) selection. On approximately day 10 after transfection, resistant colonies were picked and split into EF-containing 96-well tissue culture dishes for expansion.

### 2.3.2 Tat-Cre protein (HTNC) treatment

2x$10^5$ ES cells were plated in a well of a 6-well culture dish with MEFs, 5 h prior to HTNC treatment. The medium was removed and replaced with DMEM without FCS/PBS, 1:1, 4-0.25 µM HTNC from a 50% (v/v) glycerol stock solution of 180 µM HTNC for 20 h (Peitz et al., 2002). Thereafter, the cells were cultured as described above. The deletion efficiency at 2 µM HTNC was around 50%.

### 2.3.3 EL-4 cell line culture and electroporation

1x$10^7$ EL-4 cells (mouse T-lymphocyte lymphoma cell line) were plated in a 75cm culture flask with T cell medium (RPMI, 10% (v/v) FCS, 1 mM sodium pyruvate, 1

mM HEPES, 2 mM L-glutamine, 1 x non essential amino acids, 0.1 mM β-ME,). Three days later the cells were split and $10^6$ cells were first washed in ice-cold ($Mg^{2+}$, $Ca^{2+}$ free) PBS and resuspend in 100µl buffer R. 8µg of plasmid DNA was mixed with the cells and the cells were then electroporated according to the manufacture instruction (Puqlab). Thereafter, the cells were cultured in 6 well plates.

### 2.3.4 Preparation of Mouse Embryonic Fibroblasts (MEFs)

Females of the desired genotype were mated with males of the desired genotype. Mating was detected by a daily plug check. Thirteen and a half days to fourteen and a half days after mating, the pregnant mice were sacrificed by cervical dislocation. The embryos and uterine horns were separated from the abdomen, carefully detached from the animal and placed in a dish with PBS where the liver and as many red blood cells as possible were removed. The tissue was minced in a dish containing Trypsin/EDTA. The minced embryos were placed in an incubator for 10 min and then all the contents of the dish were transferred into a 50 ml conical tube. The tube was centrifuged at 3.500 rpm for 10 min. The pellet was resuspended in MEF medium supplemented with Pen/Strep and plated on 10 cm plates. After 24 h, the medium was changed to remove cellular debris. Confluent plates were either expanded or frozen.

### 2.3.5 Preparation of cells from lymphoid organs

Thymus, spleen, and lymph nodes were aseptically removed from mice and then passed through a sterile sieve to obtain single cell suspensions. Bones were flushed with medium (DMEM, 10% (v/v) FCS, 2 mM L-glutamine) to extract bone marrow cells. Erythrocytes were lysed from spleen and thymus preparations in 140 mM $NH_4Cl$, 17 mM Tris-HCl pH 7.65 for 2 min. Blood from the tail vein was collected in a tube with heparin (Liquemin, Roche, Mannheim, Germany) and then layered on top of 7% (w/v) Ficoll 400 (Pharmacia, Freiburg, Germany). After 300 g centrifugation

at 23°C for 8 min, lymphocytes were recovered from the interphase of the gradients and resuspended in DMEM, 10% (v/v) FCS, 2 mM L-glutamine and kept on ice.

### 2.3.6 Culture of ex vivo lymphocytes

Spleen and LN were aseptically removed from mice and then passed through a sterile sieve. Erythrocytes were lysed for 2 min by $NH_4Cl$ (140 mM $NH_4Cl$, 17 mM Tris-HCl pH 7.65). Lymphocytes were kept in RPMI 1640 (supplemented with 10% (v/v) FCS (decomplemented), 1 mM sodium pyruvate, 2 mM L-glutamine, 1x non-essential amino acids, 0.1 mM 2-β-mercaptoethanol, and 10 mM HEPES (Gibco), supplemented with the indicated compounds, e.g. MOGp35-55, in the indicated concentration for 3-5 days at 37°C.

### 2.3.7 Cell counting

Viable cells were assessed using the trypan blue dye exclusion test and counted using a Neubauer chamber (Assistent, Sondheim, Germany). To this end, an aliquot of the cell suspension was diluted with physiological trypan blue solution (Gibco, Long Island, NY, USA). Dead cells are stained blue whereas live cells cannot take up the dye due to their intact membrane. After counting 16 single quadrants, the counted cell number (N) was multiplied by the dilution factor (V) and the 'chamber factor' ($10_4$) resulting in the number of live cells per ml ($N \times V \times 10_4$ = cell number /ml).

### 2.3.8 Adoptive T cell transfer and CFSE labeling

Freshly isolated $CD4^+$ T cells were washed twice with PBS, pH 7.4 and then incubated with 0.5mM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, Oregon, USA) for eight minutes as described previously (Lyons and Parish, 1994). To stop the staining reaction, RPMI 1640 (Gibco, Long Island, NY, USA) was supplemented with a further 10% FCS. The cells were then

washed twice in RPMI and resuspended in the appropriate volume of PBS or T cell medium. For proliferation studies, enriched CD4 2D2 transgenic (Thy1.1$^+$) T cell suspension were CFSE-labeled, counted and 7x10$^6$ cells injected intravenously in PBS into MOG/CFA-immunized (Thy1.2$^+$) recipient mice. The mice were sacrificed five days after T cell transfer and cells were analyzed by flow cytometry.

### 2.3.9 Flow Cytometry

Single cell suspensions were prepared from all tested organs. Red blood cells from BM and spleen were lysed in cell suspension with Tris-ammoniumchloride, pH 7.2. Cells ($10^6$ per sample) were surface stained in 30 ml PBS, 0.1% (w/v) BSA, 0.01% (w/v) $NaN_3$ with combinations of fluoresceine isothiocyanate (FITC), phycoerythrine (PE) and Cy-Chrome™ (Cyc), APC or bio-conjugated mAbs for 20 min on ice. Stainings with biotinylated mAbs were followed by a secondary staining step with Streptavidin-Cychrome™ (Pharmingen). After staining, the samples were washed and resuspended in PBS-BSA-$NaN_3$. Stained cells were analysed on a FACSCalibur and data were evaluated using CellQuest software (Becton Dickinson, Mountain View, USA). Dead cells were labeled with 7AAD and excluded from the analysis. Monoclonal antibodies, listed in Table 1, were either homemade (C. Uthoff-Hachenberg, B. Hampel, Institute for Genetics, Cologne, Germany) or purchased from Pharmingen (San Diego , USA).

| Specificity | Clone | Supplier |
|---|---|---|
| CD4 | GK.1.5/4 | Pharmingen |
| CD4 | GK.1.5 | eBioscience |
| CD8α | 53-6.7 | Pharmingen |
| CD8α | 53-6.7 | eBioscience |
| CD8α | 53-6.7 | eBioscience |
| CD11b | M1/70 | eBioscience |
| CD11c | HL3 | Pharmingen |
| CD19 | 1D3 | Pharmingen |

| | | |
|---|---|---|
| CD25 (IL-2Ra) | 7D4 | Pharmingen |
| CD44 | KM114 | Pharmingen |
| CD45.1/Ly5.1 | A20 | Pharmingen |
| CD45.2/Ly5.2 | 104 | eBioscience |
| CD62L (L-Selectin) | MEL-14 | Pharmingen |
| CD69 | H1.2F3 | Pharmingen |
| CD90.2/Thy1.2 | 53-2.1 | eBioscience |
| CD90.1/Thy1.1 | HIS-51 | eBioscience |
| CD103 | M290 | Pharmingen |
| CD207 | eBioL31 | eBioscience |
| B220/CD45R | RA3-6B2 | Pharmingen |
| BTLA | 8F4 | eBioscience |
| Foxp3 | FJK-16s | eBioscience |
| GR1 (Ly6c) | AL-21 | Pharmingen |
| IFN-γ | MXG1.2 | Pharmingen |
| IL-17A | TC11-18H10 | Pharmingen |
| IL-17F | 12-7471-80 | eBioscience |
| MHC II | M5/114 | Pharmingen |
| PDCA1 | eBio927 | eBioscience |
| PD-L1 | MIh5 | eBioscience |
| PD-L2 | 122 | eBioscience |
| PD-1 | J43 | eBioscience |
| Seglec-H | eBio440 | eBioscience |
| TCRb | H57-597 | Pharmingen |
| Vβ-11 | RR3-15 | Pharmingen |

**Table 2: List of antibodies used for flow cytometry**

### 2.3.10 Flow Cytometry and Intracellular Cytokine Staining (ICS)

Antibodies specific for mouse IL-17A (TC11-18H10.1, BD), IFN-γ (XMG1.2, BD) and Foxp3 (FJK-16s; eBioscience) were used for intracellular cytokine staining.

Intracellular cytokine analysis was performed after stimulation of T cells for 4 hours with phorbol 12-myristate 13-acetate (PMA, 100ng/ml), Ionomycin (200ng/ml) and Brefeldin A (1µg/ml). Commercially available kit was used for intracellular cytokine staining in accordance with the manufacturer's protocol (BD Biosciences). Samples were acquired using distinct BD flow cytometers (FACS-Scan, FACS Calibur or FACS Canto II) Analysis was performed by Cellquest (BD) or by FlowJo (Tree Star Inc. Ashland, OR, USA) when data from Canto-acquired samples were analyzed.

### 2.3.11 Magnetic cell sorting and FACS sorting

Specific cell populations were either sorted or depleted from a heterogenous cell suspension by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Cell populations were labeled with antibody-coupled microbeads (10 µl beads, 90 µl PBS-BSA-$N_3$ per $10^7$ cells) and separated on LS or LD MACS columns in a magnetic field (Miltenyi et al., 1990). For cell sorting, the desired cells (B cells, T cells or DCs) were enriched by MACS technology and then stained with antibodies against various cell surface markers. The cells of individual subset were then sorted using a dual laser FACStar (Becton Dickinson, Franklin Lakes, USA). The purity of isolated populations was subsequently tested by FACS analysis: MACS-isolated cells were normally >85% pure and sorted cell subpopulations were >95% pure. FACS sorting was performed with the help of Magdalena Brkic (III med. Klinik/TVZ. Mainz, Germany).

### 2.3.12 Isolation of CNS infiltrates

CNS infiltrates were isolated as previously described (Greter et al., 2005) Briefly, mice were perfused using ice cold PBS. Brain and spinal cord were removed and incubated in PBS containing collagenase type II (2mg/ml, Gibco) and DNAse (20 Units, Sigma). The tissue was then homogenized loaded on a 30/37/70 percent percoll gradient for enrichment of CNS infiltrates.

### 2.3.13 Isolation of splenic DCs

Mice were sacrificed using Isofluoran, spleens were removed and incubated with Collagenase D ((2mg/ml) Roche Diagnostic, IN, USA) for 30 minutes. Thereafter, for single cell suspension, spleens were mashed through a 70µm cell strainer (BD Biosciences). DCs were labeled with CD11c beads and enriched by MACS columns.

### 2.3.14 Induction of CD11c-CreER$_T$ activity in vivo

Cre activity of CD11c-CreER$_T$ mice was induced *in vivo* by a sequential i.p. injections with 100 µl containing 2 mg of tamoxifen (TAM; ICN Biomedicals Inc., Aurora, OH, USA). TAM was suspended in 96% EtOH, 9 volumes of olive oil (ICN Biomedicals Inc., Aurora, OH, USA) were added, and TAM was dissolved at 37°C.

### 2.3.15 In vitro 2D2-iTreg differentiation and analysis

1.5x10$^5$ MACS-purified naïve CD4$^+$ 2D2 transgenic T cells were co-cultured with 5x10$^4$ splenic APCs, 20µg/ml of MOG peptide and TGFβ at the indicated concentration. For intracellular Foxp3 detection, cells were stained with a commercially available kit (eBioscience).

### 2.3.16 T$_{eff}$ and iTreg *in vitro* Differentiation

2x10$^5$ MACS-purified naïve CD4$^+$ T cells were stimulated with anti CD3 and anti CD28 mAb (3µg/ml and 6ng/ml, respectively). In combination with the following conditions: For iTreg differentiation, TGFβ was added (4ng/ml). For the differentiation of Th1 cells, IL-12 (10ng/ml), IFNγ (10µg/ml), and anti IL-4 (10µg/ml) were added. For the differentiation of Th2 cells, IL-2 (10ng/ml), IL-4 (50ng/ml) and anti IFNγ (10µg/ml) were added, whereas for Th-17 differentiation, TGFβ (4ng/ml) and IL-6 (10ng/ml) were used. Cells were analyzed for the presents of the desired population three to five days post initial differentiation conditions. Intracellular cytokine analysis was performed after stimulation of T cells for 4 hours

with phorbol 12-myristate 13-acetate (PMA, 100ng/ml), Ionomycin (200ng/ml) and Brefeldin A (1µg/ml). Commercially available kit was used for intracellular cytokine staining in accordance with the manufacturer's protocol (BD Biosciences).

## 2.4 *In vivo* depletion of CD25+ cells

Endogenous $CD25_+$ cells were depleted from mice two days before induction of EAE by i.p. injection with 1 mg of anti-CD25 (PC61; rat IgG1; lab-made). Control mice received an i.p. injection of 1 mg of HRPN (rat IgG1). Confirmation of $CD25_+$ cell depletion by PC61 was determined staining peripheral blood of all mice two or three days after treatment with an Ab that recognizes a different epitope of CD25 (7D4), and it resulted in upwards of 90% $CD25_+$ cell depletion.

## 2.5 *In vivo* depletion of Dendritic cells

In order to achieve *in vivo* DC-depletion, mice were injected intraperitoneally with 25ng DT per gram of body weight. DC-depletion during EAE was carried out following repetitive DT injections. Generally, DT was applied every third day. Specific injection regimes are described for individual experiments. To achieve *in vivo* pDC-depletion, mice were subjected to a single injection of 0.5mg anti-mPDC1 (Fischer, 2004) followed by three consecutive injections of 0.25mg anti-mPDC-1 every second day. For the purpose of pDC-depletion during EAE, mice were deprived of pDCs, DCs or the combination of both by applying anti-mPDC1 and DT on days six, four and one prior to EAE induction. For *in vitro* depletion of DCs, C56BL/6 mice were sacrificed using Isofluoran. The spleen was removed and a single cell suspension was performed. Using MACS depletion columns, total splenocytes were then depleted of either T cells (Thy1.2 beads) or the combination of both T cells and DCs (CD11c beads) in accordance with the manufacturer's instructions. The depletion efficiency was higher then 95% for both T cells and DCs.

## 2.6 Tissue preparation for immunohistochemistry

Mice were sacrificed using Isofluoran. Histology was performed as previously described (Mildner et al., 2009). In brief, the spinal cords were removed and fixed in 4% buffered formalin and were then dissected and embedded in paraffin before being stained with luxol fast blue (LFB) to assess the degree of demyelination, MAC-3 (BD Pharmingen) for macrophages/microglia, CD3 for T cells (Serotec, Düsseldorf, Germany), B220 for B-cells (BD Pharmingen) and amyloid precursor protein ((APP) Chemicon, Temecula, CA. USA).

### 2.6.1 Flow-Cytomix

Multiple cytokine and chemokine levels were detected using FloxCytomixTM technology (BenderMedsystems, Vienna) according to the manufacturers instructions.

### 2.6.2 ELISA

Detection of cytokines (IFNγ, IL-10 and IL-17A) was performed with ELISA (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

## 2.7 Mouse Experiments

### 2.7.1 Induction and assessment of EAE

$MOG_{35-55}$ peptide (amino acid sequence MEVGWYRSPFSRVVHLYRNGK) was obtained from Research Genetics (Huntsville, Alabama, USA). Active EAE was induced by immunization with $MOG_{35–55}$ peptide (500µg/ml) emulsified in CFA (Difco Laboratories, Detroit, Michigan, USA), which was supplemented with

5.5mg/ml of heat-inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories). The emulsion was administered as a 100µl subcutaneous injection into the tail base. Mice also received 200ng of Pertussis toxin (Sigma Aldrich, Steinheim, Germany) intraperitoneally on the day of immunization and two days later. A clinical assessment of EAE was performed daily according to the following criteria: 0 = no disease; 1 = decreased tail tone; 2 = abnormal gait (ataxia) and/or impaired righting reflex (hind limb weakness or partial paralysis); 3 = partial hind limb paralysis; 4 = complete hind limb paralysis; 5 = hind limb paralysis with partial fore limb paralysis; 6 = moribund or dead.

### 2.7.2 Induction and assessment of Colitis

$10^6$ MACS-purified $CD4^+$ $CD25^-$ T cells were transferred i.v. into recipient RAG-1 deficient mice. Thereafter, the mice were monitored weekly for changes in their body weight as well as signs of Colitis as assessed by mini-endoscopy. A clinical assessment of gut inflammation was performed weekly according to the following criteria: no disease; loss of gut epithelium translucency; fibrin secretion; feces solidly and tumour development. Final score was calculated as the sum of all criteria, with a given values of 0-3 for every criteria- reaching a maximal score of 15.

### 2.7.3 MCMV and MHV Infection

8 to 10 weeks old mice were infected subcutaneaous at the footpad, with $10^5$ PFU of the virus mCMV-WT (Strain Smith; ATCC VR-1399). Mice were injected i.p. with 50 PFU of MHV A59, representing a low dose infection with maximal liver disease around day 5 comparable with the kinetics of systemic infection as described previously (Cervantes-Barragan et al., 2007). MHV A59 was generated from a molecularly cloned cDNA based on the Albany strain of MHV A59 and propagated on L929 cells (Coley et al., 2005).

## 2.8 Statistics

Values are typically represented as mean ± SEM (standard error of mean). Statistical significance was assessed using 2-tailed Student's *t*-test. p-values < 0.05 were regarded significant, displayed by '*' in the figures (* = p-value < 0.05; ** = p-value < 0.005).

## 2.9 Mice

C57BL/6 and RAG-1$^{-/-}$ mice were obtained from Charles River or Jackson Laboratories. The following strains have been previously described: CD11c-Cre (Caton et al., 2007), DTA (Brockschnieder et al., 2004), CD11c-CreER$_T$ (Probst et al., 2003), liMOG (Frommer et al., 2008), iDTR (Buch et al., 2005), PD-1$^{-/-}$ (Nishimura et al., 1999), 2D2 (Bettelli et al., 2003), CD4-Cre (Wolfer et al., 2001), CD19-Cre (Rickert et al., 1997), LysM-Cre (Clausen et al., 1999) and Dereg (Lahl et al., 2007). All mice were generated in the C57BL/6 genetic background or bred for at least 10 generations to this background, housed in specific pathogen-free conditions and used in accordance with the guidelines of the Central Animal Facility Institution of the University of Mainz.

# 3 Results

## 3.1 Interleukin-10

In order to assure that the IL-10 deletion occurs specifically in the desired cellular subset and not in other cell types, different Cre recombinase expressing mouse lines were used. To confirm IL-10 deficiency specifically in T cells, $IL10^{f/f}$ mice were crossed to the CD4-Cre mouse line (hereon termed CD4/IL10 mice), whereas IL-10 deficiency specifically in B cells was achieved by crossing the $IL10^{f/f}$ mice with CD19-Cre mouse line (hereon termed CD19/IL10 mice). Similarly, crossing of the $IL10^{f/f}$ mice with CD11c-Cre mouse line (hereon termed CD11c/IL10 mice) was used for IL-10 deletion specifically in DC's and the cross of $IL\text{-}10^{f/f}$ mice with a LysM-Cre mouse line (hereon termed LysM/IL10 mice) resulted in macrophage/neutrophil-IL-10 specific deletion.

In order to confirm the specificity of the cellular source of IL-10, CD19/IL10, CD4/IL10 or $IL10^{f/f}$-derived B and T cells were MACS-purified or FACS-sorted and cultured under different stimulating conditions. ELISA was used to determine the amount of secreted IL-10 protein found in the supernatant of the cultured cells. 60 hours post LPS stimulation, B cells from either $IL10^{f/f}$ or CD4/IL10 mice produced substantial amount of IL-10 whereas CD19/IL10-derived B cells produced very little IL-10 (Fig.1a). Similar results could be obtained with T cells: 60 hours post Th2 stimulating conditions (anti-CD3/28 combined with rIL-2 and rIL-4), T cells from either $IL10^{f/f}$ or CD19/IL10 mice produced substantial amounts of IL-10 whereas T cells from CD4/IL10 mice produced hardly any IL-10 (Fig.1b). The amount of IL-10 production by both $IL10^{f/f}$ and CD19/IL10 derived T cells was even higher when measured five days post stimulation. Yet, CD4/IL10 derived T cell were still unable to produce IL-10 (Fig.1c). Taken together, these data confirm the inactivation of the IL-10 gene depending on the different Cre lines used, as previously reported (Roers et al., 2004). Although low, the detectable amount of IL-10 produced by LPS stimulated CD19/IL10 B cells or anti CD3/28 stimulated CD4/IL10 T cells is most likely to come from other cell types, as both B and T cells were enriched either by

positive selection using MACS columns or by FACS sorting, and this did not result in hundred percent purity. More over, it has been shown that both CD19-Cre and CD4-Cre are not active in hundred percent of B or T cells, respectively, therefore it is possible that the detectable low levels of IL-10 are produced by these cells in which the deletion did not occur.

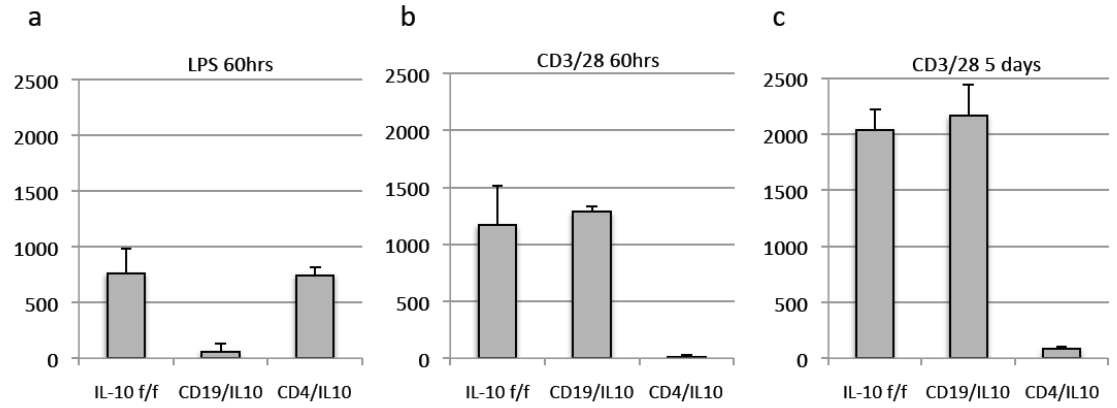


**Figure 1. Specific deletion of the IL-10 encoding gene**

FACS-sorted B and T cells from CD19/IL10, CD4/IL10 or $IL10^{f/f}$ mice were stimulated by LPS (a) or anti-CD3/28 plus rIL-2 and rIL-4 (b and c). ELISA was used to measure IL-10 production [pg/ml] 60 hours or five days post stimulation (a and b or c, respectively). Data is representative of two independent experiments, of both MACS-purified and FACS-sorted cells, with minimum of two mice per group and quadruplicate wells of each stimulation condition.

IL-10 is regarded predominantly as an anti-inflammatory cytokine. IL-10 deficient mice develop spontaneous IBD with severe gut inflammation (Kuhn et al., 1993). It has been shown that during IBD, $CD4^+$ T cells derived, and more specifically Treg derived IL-10, is the main source of protecting cytokine against an unbalanced response to environmental antigens (Asseman et al., 1999; Roers et al., 2004; Rubtsov et al., 2008). The IL-10 deficient mice also exhibit increased susceptibility to immunopathology upon parasitic and bacterial infections or LPS exposure (Berg et al., 1995a; Deckert et al., 2001; Namangala et al., 2001; Yap et al., 1998) as well as the development of aggravate autoimmune diseases (Segal et al., 1998), in particularly EAE, though not in a spontaneous manner (Bettelli et al., 1998). IL-10 producing Th1 cells were first reported in patients with active

pulmonary tuberculosis (Gerosa et al., 1999) and later on were also identified in parasite-infected animals (Jankovic et al., 2007; Jankovic et al., 2002; Shaw et al., 2006). IL-10 producing Th1 and Th17 cells were also identified in both the peripheral lymphoid organs and in the CNS of EAE mice (Fitzgerald et al., 2007; McGeachy et al., 2007; Stumhofer et al., 2007). The fact that Th1 cells produce IL-10 is rather surprising as IL-10 is an inhibitor of Th1 cell cytokine production through it suppressive action on DCs and macrophages.

As IL-10 deficient mice developed a vigorous clinical disease course when afflicted with EAE, with higher incidence in comparison with IL-10 proficient mice, it became an interest to find out the cellular source of IL-10 controlling disease progression. B cell-derived IL-10 has been reported to be important in controlling the disease retraction phase (Fillatreau et al., 2002; Matsushita et al., 2008). Indeed, MOG immunized CD19/IL10 mice did not show the same EAE recovery curve as littermate control $IL10^{f/f}$ mice. In fact, these mice completely failed to recover from EAE while the control group showed a slow, though consistent, recovery (Fig. 2a). As IL-10 production by macrophages and DCs is associated with inhibition of T cell activation and function, CD11c/IL10 or LysM/IL10 mice were MOG-immunized and EAE progression was monitored daily. LysM/IL10 mice developed enhanced EAE in comparison with the control $IL10^{f/f}$ mice (Fig. 2b). Although the clinical score of the LysM/IL10 mice were just mildly stronger then the control group, it is in accordance with the common assumption that IL-10 inhibits APC maturation, antigen presentation and thereby the activation of T cells. DCs are considered as proteotypic APCs and thereby the lack of IL-10 specifically in DCs was expected to have a profound effect on T cell activation, even stronger than that of macrophages. Therefore the observation that CD11c/IL10 mice develop similar disease as IL-10 proficient DC mice was unexpected (Fig. 2c).

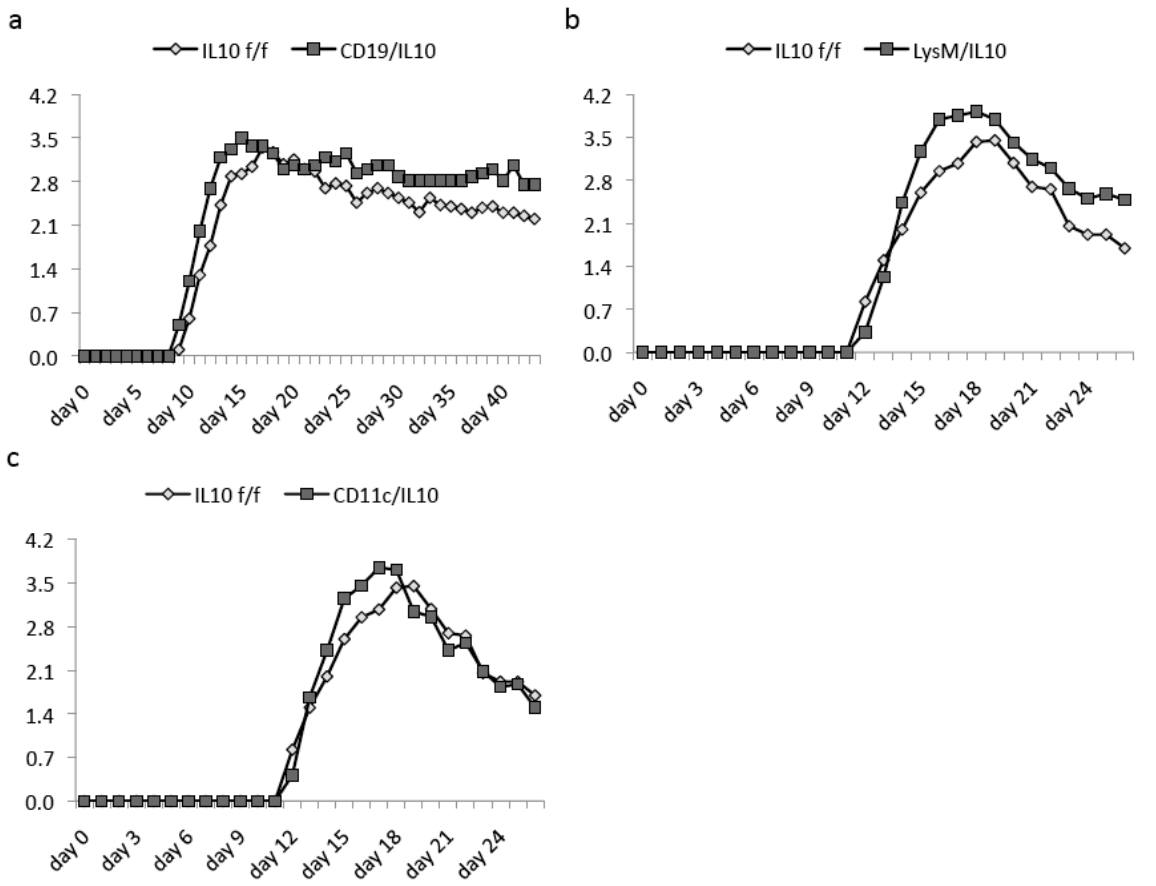


**Figure 2. IL-10 deficiency by different cell types results in altered EAE**

IL-10 deficient mice specifically in B cells (a), macrophages (b) or dendritic cells (c) were monitored daily for disease progression following MOG/CFA immunization. Data is representative of three (a and b) or four (c) independent experiments, with a minimum of five mice per group.

Several studies have shown that during EAE, effector Th1 and Th17 cells can co-express IL-10 and that IL-10 producing Treg are important in controlling the disease. Since IL-10 deficient mice develop vigorous EAE and EAE is predominantly considered as a $CD4^+$ T cell mediated CNS autoimmune disease, the common concept at present suggests that T cell-derived IL-10 is predominantly protective against EAE and that T cells are an important cellular source of IL-10 production during EAE. In order to test the role of T cell-derived IL-10 during EAE, CD4/IL10 and littermate $IL10^{f/f}$ mice were subjected to MOG immunization. Although disease onset appeared not to be effected, to our surprise T cell-specific IL-10-deficient mice (CD4/IL10) developed milder disease in comparison with littermate control

($IL10^{f/f}$) mice. This moderate EAE was highly significant throughout the whole experimental period (Fig. 3a). When comparing the individual maximal score of the different mice, the CD4/IL10 mice exhibited a significant lower score then the control littermate $IL10^{f/f}$ mice (Fig. 3b). Furthermore, a comprehensive comparison of EAE incidence uncovered that while disease incidence in control $IL10^{f/f}$ mice was ranging from 90-100% in all experiments and more than 95% overall, CD4/IL10 mice had far lower incidence, ranging between 40-90% in all experiments and around 75% overall (Table 1). To further examine disease pathology, mice were sacrificed and analyzed at different time points. Lymph nodes, spleen and CNS-single cells suspension were prepared from individual mice, and CNS infiltrates as well as peripheral Tregs were assessed by FACS analysis. During both the early phases of disease onset and at the peak of disease, CD4/IL10 mice exhibited higher percentages of peripheral $Foxp3^+$ Tregs in their draining lymph nodes (Fig. 3c). At the peak of disease, the CD4/IL10 mice had dramatically lower percentages of CNS infiltrating $CD4^+$ T cells in comparison with the $IL10^{f/f}$ mice (Fig. 3d top panel), however within these infiltrating $CD4^+$ T cells, the ratio of Th1 or Th17 effector cells as well as the suppressive Tregs was similar in both groups of mice (Fig. 3d middle and lower panels). Furthermore, immunohistochemistry analysis of spinal cord and brain of individual mice by days 29-32 post EAE induction, corroborates our previous CNS infiltration FACS analysis, as it revealed similar degree of demyelination, CNS infiltrating macrophages and B cells and significantly lower percentage of T cell infiltration (Fig. 3e-g).

Table I

| Mouse strain | $IL10^{f/+}$ | $CD4/IL10^{f/+}$ | $IL10^{f/f}$ | $CD4/IL10^{f/f}$ |
|---|---|---|---|---|
| number of mice | 9 | 5 | 67 | 71 |
| EAE incidence | 100 % | 100 % | 95.5 % | 77.5 % |
| EAE onset (dpi) | 10.8 | 11.0 | 9.9 | 10.4 |
| Average max. score | 3.9 | 4.1 | 3.4 | 2.6 |

Figure 3

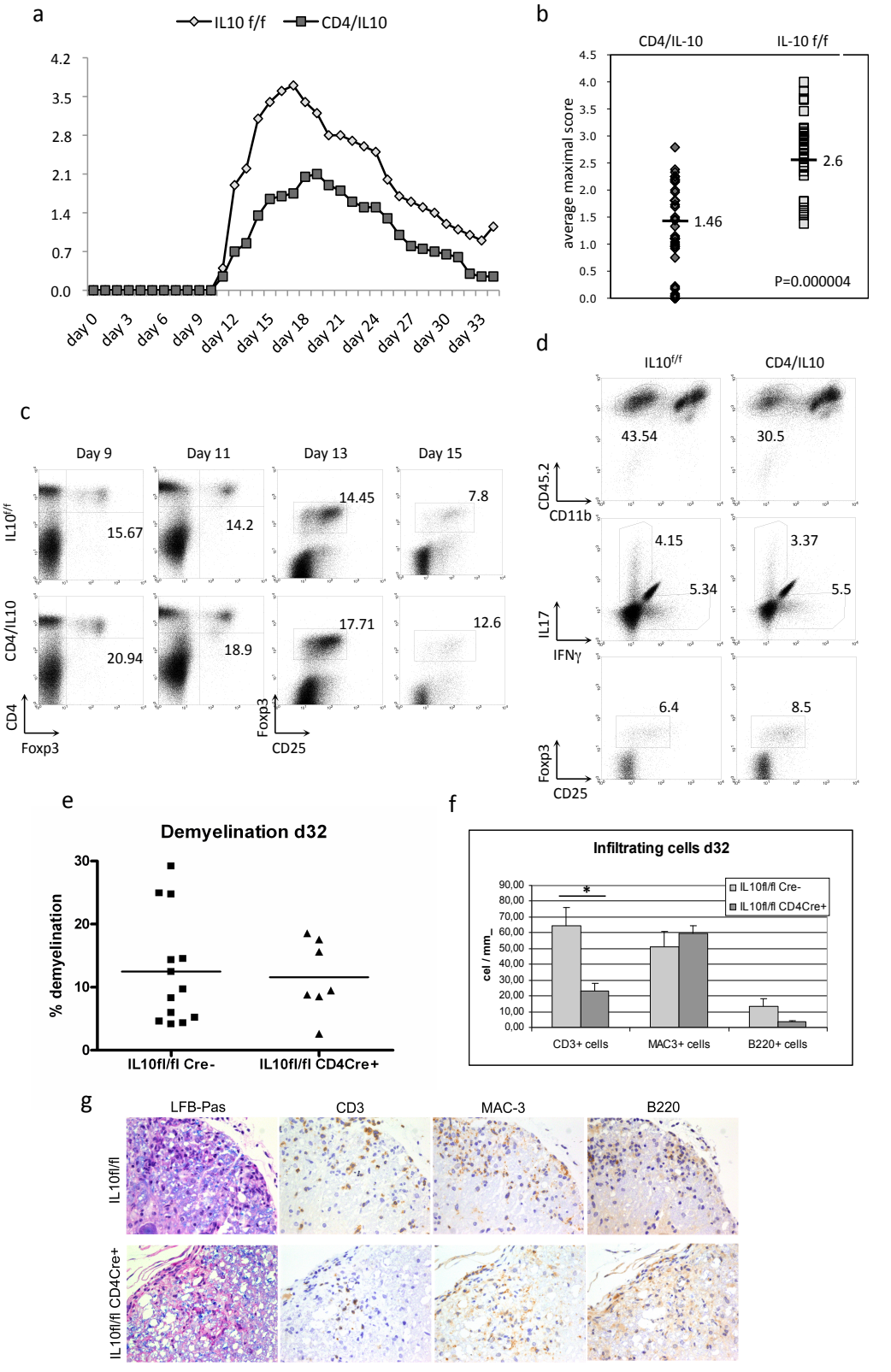
a
IL10 f/f
CD4/IL10
day 0
day 3
day 6
day 9
day 12
day 15
day 18
day 21
day 24
day 27
day 30
day 33
b
CD4/IL-10
IL-10 f/f
average maximal score
1.46
2.6
P=0.000004
c
Day 9
Day 11
Day 13
Day 15
IL10f/f
CD4/IL10
15.67
14.2
14.45
7.8
20.94
18.9
17.71
12.6
CD4
Foxp3
Foxp3
CD25
d
IL10f/f
CD4/IL10
43.54
30.5
CD45.2
CD11b
4.15
3.37
5.34
5.5
IL17
IFNγ
6.4
8.5
Foxp3
CD25
e
Demyelination d32
% demyelination
IL10fl/fl Cre-
IL10fl/fl CD4Cre+
f
Infiltrating cells d32
IL10fl/fl Cre-
IL10fl/fl CD4Cre+
cel / mm_
CD3+ cells
MAC3+ cells
B220+ cells
g
LFB-Pas
CD3
MAC-3
B220
IL10fl/fl
IL10fl/fl CD4Cre+

**Figure 3. T cell-specific IL10-deficient mice develop a moderate EAE**

A summarizing graph of three individual experiments (a) and the individual maximal clinical score as calculated based on the day of onset (b), demonstrating significantly moderate disease in the CD4/IL10 mice. Representative FACS-analysis of Foxp3 ICS nine to fifteen dpi (days post immunization) revealing elevated levels of peripheral Tregs in the dLN of CD4/IL10 mice (shown as percent of total T cells) (c). FACS-analysis of CNS infiltration at day 13 post EAE induction: percentages of total T cell infiltration (upper panel), percentages of $CD4^+/Foxp3^+$ cells (middle panel) and percentages of IL17 or IFNγ producing $CD4^+$ cells (d). Data is representative of a minimum of three mice per group (c and d). Quantification of CNS demyelination and cell infiltration (e and f, respectively) as well as histological analysis of CNS infiltration 32 dpi (g). Quantification represents the analysis of a single experiment (of two) with $n \geq 18$ mice per group.

As most CD4/IL10 mice develop spontaneous colitis with visible sings of rectal prolapses by the age of three to four months (Table 2), different experimental approaches were taken to assure that the moderate EAE observed in these mice is not secondary to the ongoing gut autoimmune response. First, an analysis of colon destruction in MOG immunized mice revealed that while control $IL10^{f/f}$ mice show no signs of colon inflammation, all CD4/IL10 mice developed strong sings of colon pathology, regardless of whether the mice were very sick or completely resistant to EAE (Fig. 4a). In a second experiment, in order to verify the possible connection between the spontaneous gut autoimmunity and CNS autoimmunity, DSS-colitis was first induced in wild type C57BL/6 mice and two weeks later the mice were immunized with MOG to induce EAE. A comparison between MOG-immunized wild type mice and mice that first had colitis and then immunized with MOG revealed no major differences in EAE incidence, onset or severity (Fig. 4b). Previously it has been reported that under germfree or specific-pathogen-free (SPF) conditions, IL-10 deficient mice show resistance to the development of spontaneous enterocolitis (Kuhn et al., 1993). Therefore mice were treated with a strong antibiotic cocktail to restrict the compassion of the gut microflora. To assure the integrity of the treatment, the antibiotic was given in the drinking water already during pregnancy (so the embryos were exposed to the antibiotic through their entire embryonic developmental stage) and the newborn mice were kept treated throughout their entire lives. A comparison of EAE severity of these antibiotic treated mice uncovered once again that the CD4/IL10 mice developed a weaker disease than antibiotic-

treated littermate control IL10$^{f/f}$ mice (Fig. 4c). Taken together, these experiments point out that the partial resistance and milder EAE observed in the CD4/IL10 mice, most likely does not result from the early development of a spontaneous gut autoimmune syndrome.

The mammalian genome contains cryptic or pseudo *loxP* sites, whose sequence can considerably differ from the consensus *loxP* site. Yet, these cryptic/pseudo *loxP* can serve as functional recognition sites for Cre. Although Cre affinity for these cryptic recognition sites is much lower than for the concurrence *loxP* sites, DNA damage could arise from Cre-mediated recombination between cryptic *loxP* sites (Thyagarajan et al., 2000). As some Cre recombinase had been reported to have potential toxicity effects (Schmidt-Supprian and Rajewsky, 2007), different Cre positive or Cre negative mouse lines were subjected to MOG induced EAE. The course of disease in both IL10$^{f/+}$ and CD4/IL10$^{f/+}$ mice (as well as wild type C57BL/6, CD4-Cre$^{+/-}$ mice or CD11c-Cre$^{+/-}$, data not shown) was similar (Fig. 4d), indicating that the reduced disease observed in the CD4/IL10 mice is not due to a toxic effect driven by the CD4-Cre itself.

Table II (* ≥ 5 month old)

| Mouse strain | IL10$^{f/+}$ | CD4/IL10$^{f/+}$ | IL10$^{f/f}$ | CD4/IL10$^{f/f}$ | CD11c/IL10$^{f/f}$ | CD19/IL10$^{f/f}$ | LysM/IL10$^{f/f}$ |
|---|---|---|---|---|---|---|---|
| 3.5 month old | 25 | 28 | 424 | 308 | 63 | 44 | 30 |
| Rectal prolepses | 0 % | 0 % | 0.7 % | 78 % | 6.4 % | 0 % | 0 % |
| ≥8 month old | 4* | 7* | 92 | 41 | 5 | 8 | 17 |
| Rectal prolepses | 0 % | 0 % | 3.3 % | 92.7 % | 80 % | 0 % | 0 % |

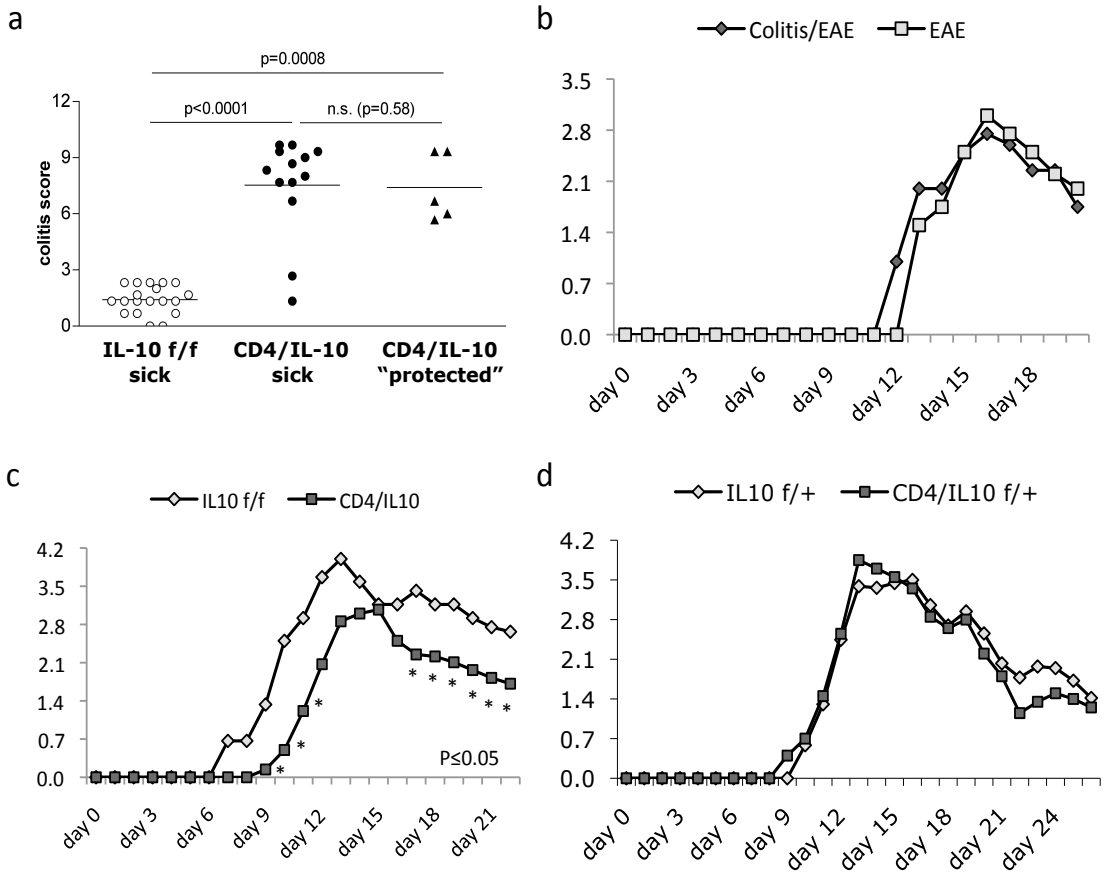


**Figure 4. Disease severity in the CD4/IL10 mice is not affected by the pre-existence of spontaneous colitis**

Quantification of gut inflammation in the different mice 32 days post induction (dpi) (all mice were MOG-immunized: "sick"= maximal clinical score ≥1; "protected"= maximal clinical score ≤1)(a). Assessment of EAE in wild type C57/BL6 mice as compared with wild type C57/BL6 mice pre-treated with DSS for a period of two weeks (n=15 mice per group)(b). Assessment of EAE in antibiotic-treated CD4/IL10 (n=7) as compared with $IL10^{f/f}$ littermate mice (n=5)(c). Assessment of EAE in $CD4/IL10^{f/+}$ (n=5) as compared with $IL10^{f/+}$ littermate mice (n=9)(d).

After we observed that EAE development in the CD4/IL10 mice was accompanied with elevated levels of peripheral Tregs, we speculated that the rising numbers of Tregs is the main reason for the milder disease. In order to check whether the increased percentages of Tregs resulted from the MOG immunization (and therefore are antigen specific) or result as a consequence of the immunization per se, mice were immunized with CFA/PBS emulsion without additional MOG or any other antigen. Ten days post immunization, analysis of the peripheral $CD4^+$ T

cell compartment have illustrated that the CD4/IL10 mice contain increased percentages of both Foxp3$^+$ Tregs as well as effector IL-17 and IFNγ producing T cells (Fig. 5a). However, the percentages of total CD4$^+$ T cells were once again declined (Fig. 5b). Previously, Tregs had been shown to control EAE induction, progression and recovery (Kohm et al., 2002). Moreover, Treg suppression was shown to be IL-10 dependent as adoptively transferred IL-10 deficient Tregs failed to suppress ongoing EAE whereas the transfer of IL-10 proficient Tregs did (Zhang et al., 2004a). As the moderate EAE observed in the CD4/IL10 mice was accompanied by increased percentages of both peripheral and CNS-infiltrating Tregs (Fig. 3c,d), different experiments were designed to check whether (a) IL-10 plays a role in the differentiation of Tregs, and (b) whether in our model, IL-10 deficient Tregs plays a role in suppressing EAE. Using a reporter transgenic mouse line that contains DTR-GFP elements under the control of the Foxp3 promoter (DEREG mice), it become possible to delete DTx-sensitive cells (Tregs) by repetitive DTx injections. Following DTx injections, naïve CD4$^+$ T cells were FACS-sorted (CD4$^+$CD62L$^{hi}$CD44$^{lo}$GFP$^-$; purity of 98%) and stimulated *in-vitro* under conditions supporting iTreg differentiation. The differentiating iTregs were exposed to increasing concentrations of either recombinant IL-10 (rIL10) or neutralizing IL-10 antibodies (anti-IL10) and *bona fide* iTregs were FACS-analyzed based on the expression of CD4$^+$GFP$^+$. iTreg differentiation under the different conditions demonstrated that by adding rIL10 the percentage of GFP$^+$ Tregs was declining, while the additional anti-IL10 neutralizing antibody had the opposite effect, manifesting increasing percentages of GFP$^+$ Tregs (Fig. 5c). Similar results were obtained using wild type sorted naïve T cells that have been cultured under iTreg differentiating conditions and stained for Foxp3 expression (not shown). However, these results were rather mild and only partially reproducible in both DEREG and wild type sorted naïve CD4$^+$ T cells.

Next, in order to investigate whether the increased percentage of Tregs found in the CD4/IL10 mice are responsible for the reduced EAE pathology, the different mice were injected with either anti-CD25 depleting antibody (PC61, rat IgG1) or a

mock antibody (rat IgG1) and MOG-immunized two days later. Twenty-four hours after PC61 treatment, evaluation of the presence of $CD4^+CD25^+$ T cells in peripheral blood revealed that the depletion efficiency reached about seventy-five percent as compared with mock-treated animals. Following depletion and immunization, mice were monitored daily for disease progression. Consequently of Tregs depletion by PC61 treatment, control $IL10^{f/f}$ mice developed aggravated disease, although with similar incidence and onset as the mock-treated mice. However, the treatment of CD4/IL10 mice with PC61 resulted in full restoration of the clinical score, bringing disease severity to similar extent as that of PC61 treated $IL10^{f/f}$ mice (Fig. 5d). Yet, the percentage of disease incidence were still lower than those observed in the control mice, with some CD4/IL10 mice still exhibiting "full resistance" to EAE induction in both the PC61 and mock treated groups (table 3), implying that the increased percentage of Tregs is not the sole reason for the reduced clinical score.

Table III

| Mouse strain | $IL10^{f/f}$ mock | $IL10^{f/f}$ PC61 | CD4/IL10 mock | CD4/IL10 PC61 |
|---|---|---|---|---|
| EAE incidence | 90 % | 90 % | 61 % | 78 % |
| Average day of onset | 10.9 | 11.2 | 11.4 | 11.1 |
| Average max score | 2.8 | 3.8 | 1.9 | 3.3 |

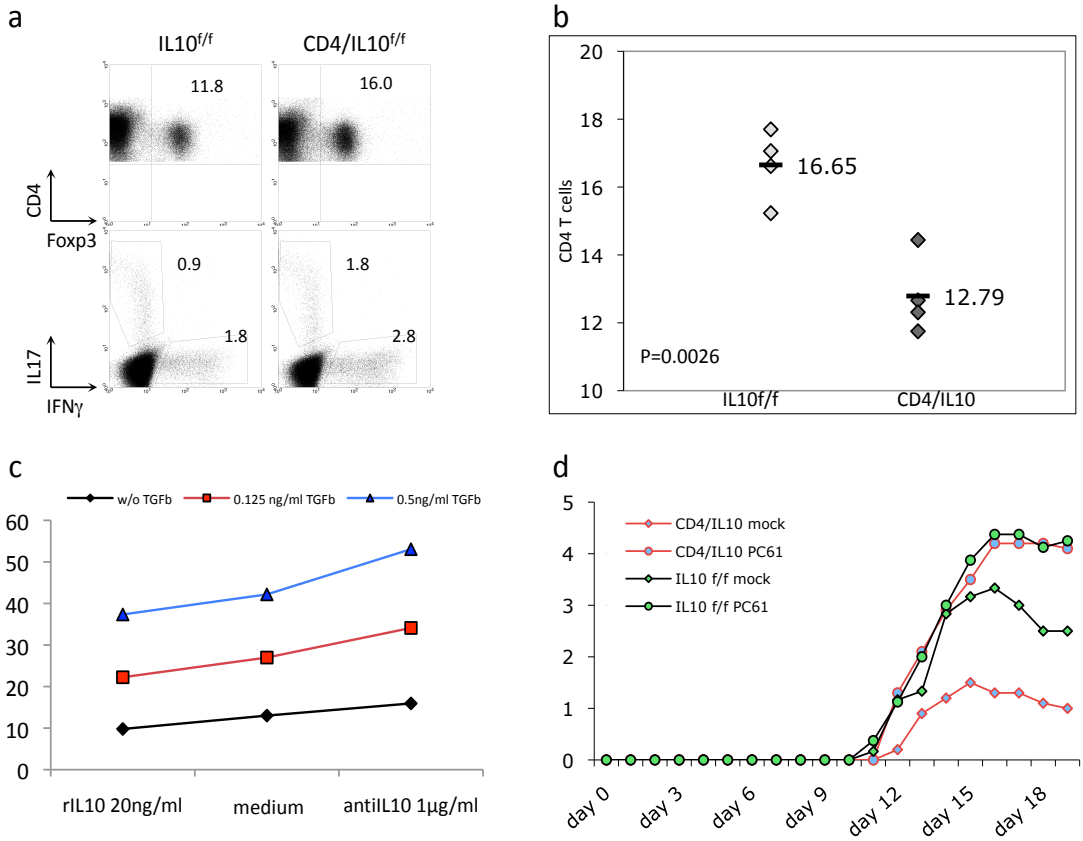


**Figure 5. The lack of IL-10 can support Treg differentiation and accumulation**

Percentages of Th1, Th17 and Treg cell differentiation 10 days after CFA/PBS immunization (n=2 mice per group)(a). Percentages of CD4+ T cells in dLN (of total cell) nine days after CFA/PBS immunization (n=5 mice per group)(b). In-vitro iTreg differentiation of FACS-sorted naïve CD4+ T cells with anti IL-10, medium or rIL-10 (one experiment of four, Dereg mice n=3, triplicate wells per stimulation)(c). EAE severity is fully restored following Treg-depletion (one representative experiment of four, n=5 mice per group)(d).

In spite of the milder EAE observed in the CD4/IL10 mice, the ratio of CNS-infiltrating Th1 and Th17 cells was comparable between both groups when immunized with MOG, or even higher when immunized with CFA alone (Fig. 3d and 5a, respectively), indicating that the CD4/IL10 derived T cells do not share an intrinsic impediment in their differentiation properties. To test whether the weaker EAE results from discrepancy of IL-10 deficient T cells in responding to antigen stimulation, a MOG recall assay was performed. In order to evaluate the difference observed in the different mice, total splenocytes isolated from MOG-immunized IL10$^{f/f}$ sick, CD4/IL10 sick or CD4/IL10 "protected" mice were either re-challenged

with MOG peptide (20μg/ml) or left in medium alone. Measuring thymidine incorporation seventy-two hours post MOG re-challenge unveil that CD4/IL10 derived splenocytes exhibit reduced proliferation in comparison with $IL10^{f/f}$ derived splenocytes. This reduced proliferation was significant in both groups of CD4/IL10 splenocytes, regardless whether the mice were "fully protected" from EAE or got sick. However, the proliferation capacity of both sick and "protected" CD4/IL10 mice was found to be similar, with both groups showing reduced proliferation as compared with $IL10^{f/f}$ mice (Fig. 6a). Culturing of these cells in medium alone resulted in a very poor proliferation that was similar in all groups (not shown). In another experiment, mice were sacrificed ten days post MOG immunization and MACS purified $CD4^+$ T cells were re-challenged with MOG-pulsed BMDC (5μg/ml MOG) supplemented with rIL-2 and rIL-12, for further support T cell survival. Similarly to the pervious experiment, five days after MOG re-challenge, the CD4/IL10 derived $CD4^+$ T cells showed reduced percentages of blasting cells as determined by flow cytometry (Fig. 6b, black). However, a comparison of total live cells between the two genotypes clearly showed a reduced percentage of live cells in the CD4/IL10 mice (Fig. 6b, red). The reduced proliferation in response to the MOG stimulation could result from a lower number of MOG-specific T cells in the CD4/IL10 mice. Alternatively, CD4/IL10 derived T cells might respond differently to stimuli than $IL10^{f/f}$ derived T cells, as indicated by the reduced percentage of live cells after MOG restimulation. To further investigate this, MACS purified $CD4^+$ T cells isolated from MOG immunized mice were re-challenged by MOG-pulsed BMDCs (1μg/ml). Forty-eight hours post stimulation, assessment of cell activation and viability revealed that although the CD4/IL10 derived T cells exhibit higher percent of activated cells (Fig. 6c, black), overall the percentage of live $CD4^+$ T cells was lower than those of the $IL10^{f/f}$ derived T cells (Fig. 6c, red). Suggesting that (a) IL-10 deficient T cells are able to respond to the MOG stimuli, and (b) that the outcome of this stimuli results in reduced survival. For further examination, isolated splenocytes of MOG-immunized mice were stimulated with 5μg/ml Concanavalin A (ConA) and seventy-two hours post stimulation, cell proliferation was measured by thymidine incorporation. In accordance with the previous results, CD4/IL10 ConA

stimulation also manifested a reduced proliferation as compared with the $IL10^{f/f}$ derived splenocytes (Fig. 6d). As ConA is a general stimulus for T cell activation, this reduced proliferation illustrates that the IL-10 deficient T cells potentially respond differently than wild type T cells to any stimuli. To further test this, MACS purified $CD4^+$ T cells were isolated from naïve young CD4/IL10 or $IL10^{f/f}$ mice and stimulated with anti CD3/CD28. Subsequently, 24 hours post stimulation a FACS analysis revealed that the control $CD4^+$ T cells had significantly higher percentage of blasting cells than IL-10 deficient $CD4^+$ T cells (Fig. 6e and f), confirming once more that the CD4/IL10 derived $CD4^+$ T cells respond less also to a general stimulus. Taken together, these data demonstrate that IL-10 deficient T cell manifest inferior response than IL-10 proficient T cells to both antigen specific and general stimulation as well as to antigen re-challenge.

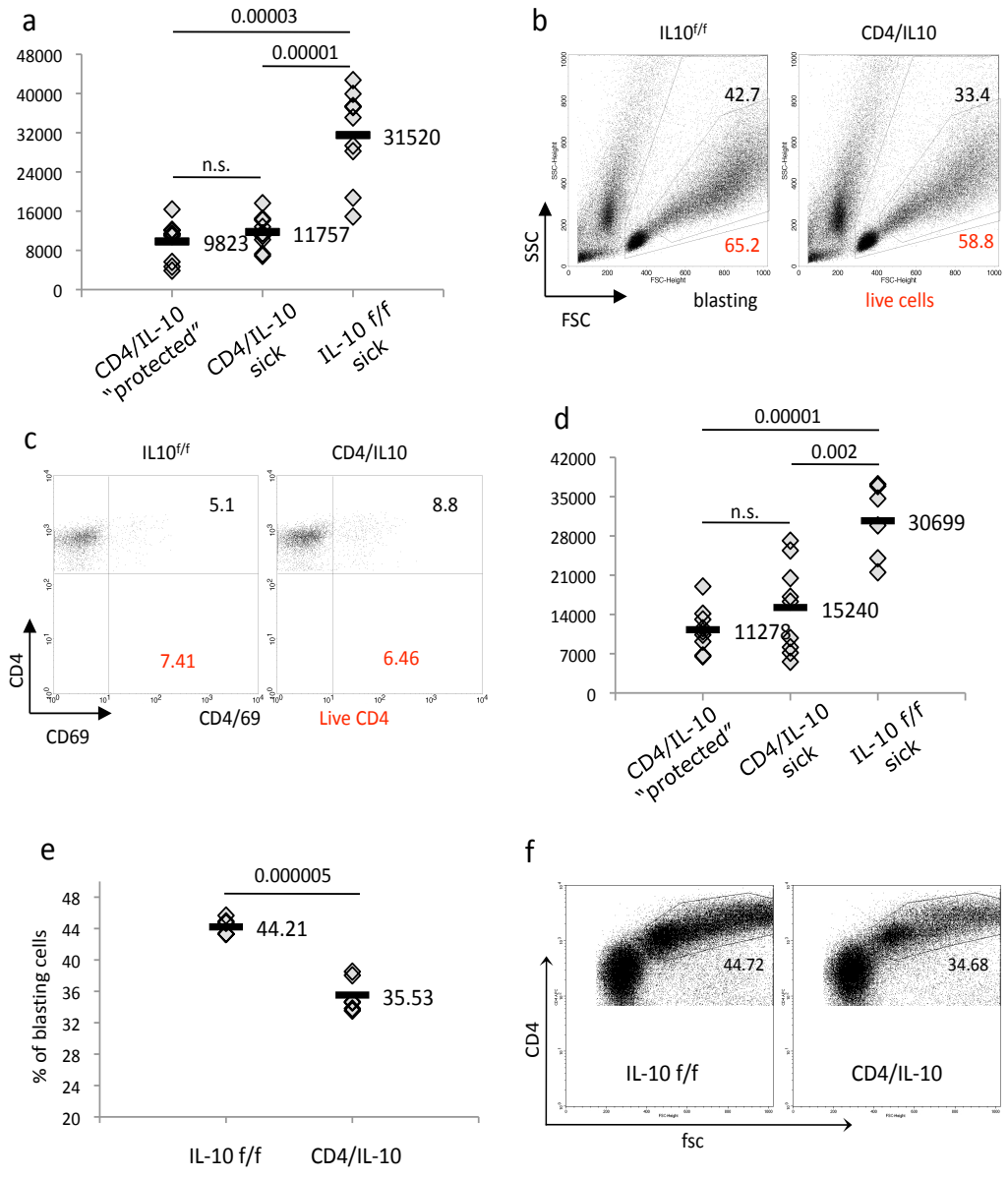


**Figure 6. IL10-deficient T cells exhibit reduce proliferation following antigen-specific and unspecific stimulation**

$H^3$ incorporation (cpm) of MOG-rechallenged (20µg/ml) splenocytes, isolated form EAE mice (n=3 mice per group) and cultured for seventy-two hours (a). MACS-purified $CD4^+$ T cells, isolated from EAE mice ten dpi, were co-cultured with MOG (5µg/ml)-pulsed BMDC for five days. Shown are percentages of live and blasting cells (b). Co-culture of MACS-purified $CD4^+$ T cells (isolated from EAE mice thirty-four dpi) with MOG (1µg/ml)-pulsed BMDC. 48 hours post MOG-rechallenged, cells were FACS-analyzed for their activation and viability status (c). $H^3$ incorporation (cpm) following ConA stimulation (5µg/ml) of total splenocytes isolated form EAE mice (triplicate wells, n=3 mice per group) and cultured for seventy-two hours (d). MACS-purified $CD4^+$ T cells, isolated from naive mice (triplicate wells, n=2 mice per group) were triggered by anti CD3/28 stimuli. The percentages of

blasting cells were determined by FACS-analysis twenty-four hours post stimulation (e and f).

As a whole, the finding that Tregs depletion did not result in full restoration of EAE incidence (Table 3) and that following stimulation T cells deficient of IL-10 exhibit reduced viability (Fig. 6), suggested that IL-10 may play an essential role in T cell survival following their activation. To this end, naïve mice were analyzed for their steady state immune compartment. Examination of three-month old naïve mice has shown no significant differences in the percentages of naïve $CD4^+$ T cells. However, at the age of six-month the CD4/IL10 mice manifest significantly higher percentages of naïve $CD4^+$ T cells (Fig. 7a). Interestingly, the percentages of Tregs found in the draining LN of three-month old CD4/IL10 mice were significantly higher than those found in littermate control $IL10^{f/f}$ mice (Fig. 7b). As most Tregs fall into the $CD62L^{hi}CD44^{lo}CD4^+$ population, they can be mistaken as naïve $CD4^+$ T cells unless further defined by the presence of Treg hallmark transcription factor Foxp3. Moreover, Tregs have been shown to be less susceptible to activation induced cell death (AICD) (Frommer and Waisman, 2010). Since activated IL-10-deficient T cells exhibit reduced survival, we speculated that the increased percentages of naïve T cells found in aged CD4/IL10 mice directly results from activation induced cell death (AICD). Indeed, analysis of T/B cells ratio in the draining LN of naive mice confirmed just that, showing higher percentage of B cells and lower percentages of T cells in the CD4/IL10 mice as compared with control littermate $IL10^{f/f}$ mice. However within the T cell compartment, the CD4/CD8 ratio was not dramatically affected, expressing similar percentages of CD4 and CD8 T cells, with slightly reduced CD4 compartment (Fig. 7c).

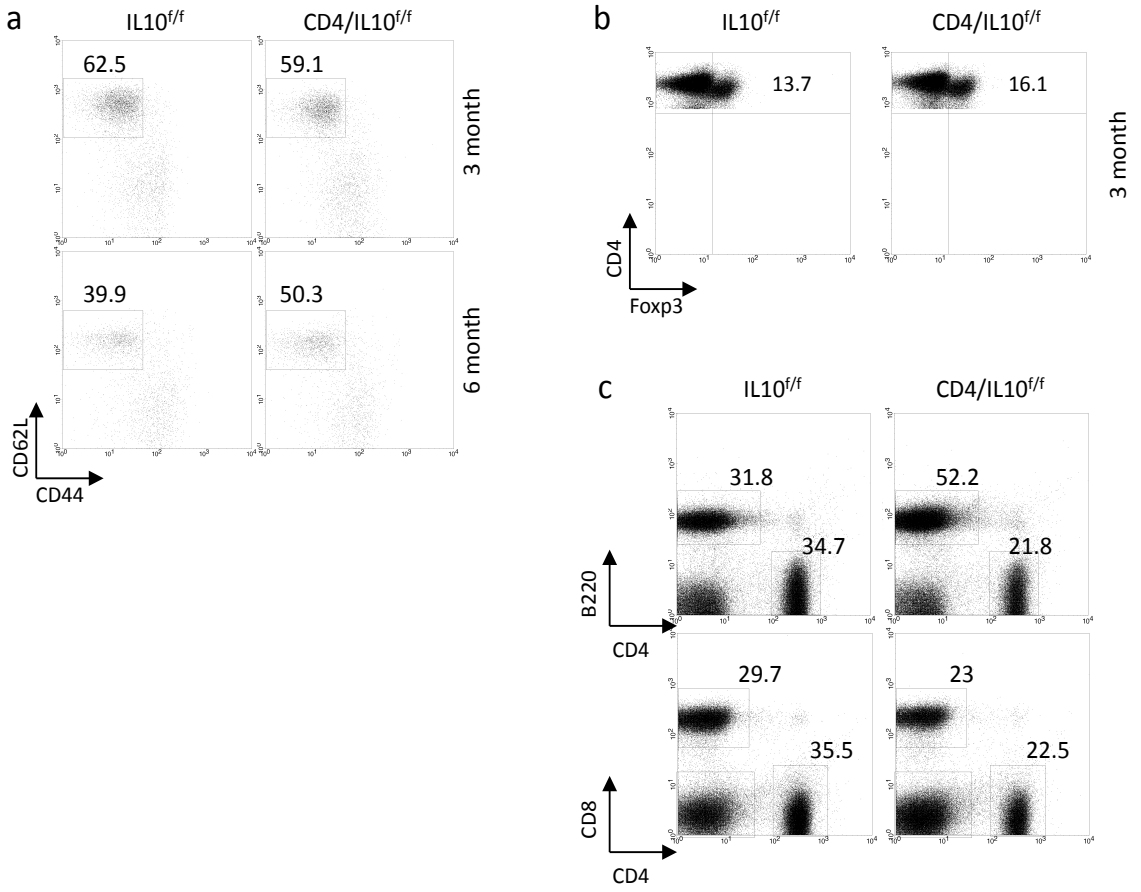


**Figure 7. Naïve CD4/IL10 mice contain reduced percentages of CD4⁺ T cell, elevated percentages of Tregs and naïve CD4⁺ T cells**

Percentages of peripheral naïve CD4⁺ T cells in naïve mice (three month n=2 mice per group, six month n=4 mice per group)(a). Percentages of Tregs (dLN) isolated form three-month old, naive mice (n=2 mice per group)(b). B/T cell-ratio and CD4/CD8 T cell ratio (dLN) in naive mice (CD4/IL10 n=5, $IL10^{f/f}$ n=4 mice per group)(c).

Recently it has been shown that the development of IL-10 producing Th1 cells required high TCR ligation, sustained ERK phosphorylation and IL-12-induced STAT4 activation. Repeated TCR triggering led to enhanced IL-10 production, and continued IL-12 and high-dose TCR signalling were required for the development and maintenance of IL-10-producing Th1 cells. Whereas low antigen-dose, which results in a weak TCR triggering, and transient ERK activation favoured the development of IL-4 and IL-10 producing Th2 cells (Saraiva et al., 2009). High antigen-dose and/or strong TCR-signalling are known to induce anergy and even

AICD (Green and Scott, 1994). During thymic development, thymocytes are undergoing a strong selection process that is dependent on the principle of TCR engagement. TCR engagement that is too strong or too weak will lead to thymocyte elimination (Sprent and Webb, 1995). As our data imply an intrinsic defect in T cell survival following TCR engagement (as seen by the reduced percentages of total T cells and elevated levels of naïve T cells in ageing mice as well as a poor primary and secondary T cell response), MACS-purified naïve $CD4^+$ T cells were stimulated in vitro with anti CD3 and anti CD28. Twenty-four hours post stimulation cells were analyzed for the percentages of blasting (activated) cells as well as for the percentages of dying cells. Based on the expression of the cell-death marker 7AAD and the early apoptotic marker Annexin-V, it was possible to distinguish between live cells, cells that are undergoing apoptosis and dead cells. A FACS analysis revealed that following TCR-engagement, IL10-dificient T cells undergo rapid apoptosis as compared with IL10-proficient T cells (Fig. 8a). This profound cell death was accompanied with reduced percentages (and absolute cell numbers) of blasting IL10-dificient T cells (Fig. 8b). Next, $IL10^{f/f}$ or CD4/IL10-derived $CD4^+$ T cells were stimulated using different antigen-doses and twenty-four hours post stimulation, the percentages of apoptotic T cells were determined by FACS analysis. High concentration of anti-CD3 stimulation (1µg/ml) resulted in significantly higher percentages of apoptotic CD4/IL10-derived T cells, as compared with the control $IL10^{f/f}$-derived T cells, whereas lower antigen doses did not show a significant difference (Fig. 8c, red). A comparison of the effect of different antigen-doses on AICD revealed that T cell stimulation with high to intermediate antigen-doses resulted in significant enhancement of dying cells, irrespectively of their genotype (Fig. 8c, 1, 0.1µg). Yet, while low antigen-dose (0.01µg/ml) had no obvious effect on AICD of IL10-proficient T cell, the same stimulation conditions manifested significantly higher percent of apoptotic IL10-deficient T cells (Fig. 8c, 0.01µg). Similar results were obtained when the different T cells were stimulated with ConA (Fig. 8d). Consequently of ConA stimulation, high antigen-dose (10µg/ml) resulted in significantly higher percentage of apoptotic T cells, regardless of their genotype, as compared with unstimulated cells (Fig. 8d, 10µg), whereas lower antigen-doses

did not manifest any significant differences (Fig. 8d, 1, 0.1μg). Moreover, different concentrations of ConA stimulation (10 and 0.1μg/ml) resulted in significantly higher percentages of apoptotic IL10-deficient T cells, as compared with the control IL10-proficient T cells (Fig. 8d, red).

The accumulating *in-vivo* and *in vitro* data obtained from the above experiments as well as from recent literature, suggested that diminutive amount of IL-10 might be necessary for controlling T cells survival following their TCR ligation. We speculate that IL-10 works in an autologous fashion, were IL-10 secretion by T cells signals back to the same T cell or other T cells that are found in close proximity. This autocrine (and possibly paracrine) feedback loop is vital for maintaining T cell viability, allowing appropriate T cell response. When T cells activation occurs in an environment that is insufficient of IL-10, the outcomes result in AICD. Although there are many other cellular sources able of producing IL-10, it appears that the timing of IL-10 production (and perhaps the location) can play a critical role in defining T cell activation vs. elimination. In order to assess whether autocrine IL-10 signalling is necessary to assure proper T cell response, naïve T cells isolated from CD4/IL10 or $IL10^{f/f}$ mice were stimulated with anti-CD3/28 mAb alone, anti-CD3/28 mAb plus recombinant IL-10 (rIL10) or anti-CD3/28 mAb plus neutralizing IL-10 (anti-IL10). Cells were then FACS-analyzed for the presence of early apoptotic cells vs. live cells based on the expression of the early apoptosis and cell death markers, Annexin-V and 7AAD. Forty-eight hours post stimulation, the addition of exogenous rIL10 results in twenty-five percent reduction of dying ($Annexin\text{-}V^{+}/7AAD^{-}$) IL10-proficient T cells as compared with the same cells stimulated with anti CD3/28 mAb alone. However, the effect of rIL10 on the IL10-deficient T cells was profoundly greater, reaching near fifty percent reduction in cell death of the apoptotic-susceptible IL10-deficient T cells as compared with the percentages of the same cells stimulated with anti-CD3/28 mAb alone (Fig. 8e, middle). In line with the above, while the effect of anti-CD3/28 stimulation in combination with additional anti-IL10 had a mild negative effect on IL10-deficient T cell survival, the same stimulation had the complete opposite effect on IL10-proficient T cells, resulting in more than forty percent increase in cell death as compared with the percentages of

the same cells stimulated with anti-CD3/28 mAb alone (Fig. 8e, right). Collectively, these results demonstrate that additional exogenous rIL10 to freshly activated IL10-deficient T cells can reverse AICD, whereas removal of IL10 by anti-IL10 results in enhanced AICD of freshly activated wild type T cells.

Currently, ERK and STAT3 are regarded as indispensible for IL-10 signalling in all IL-10-responsive cells (Saraiva and O'Garra, 2010) however other IL-10 signalling pathways exist. IL-10 inhibits NFκB activation in response to different stimuli by inhibiting the activation of IκB kinase-β, similar to salicylate (Yin et al., 1998) and by inhibiting NFκB DNA binding activity (Schottelius et al., 1999). Depending on the different cell type, IL-10/IL-10R signalling pathway involves different transcription factors. These transcription factors can modulate many different pathways involving cell proliferation and activation, cell survival, cytokine production, inflammation, apoptosis (and more). Therefore interference in IL-10 signalling cascade might result in direct abrogation of one or more of the outcome pathways mentioned above. The increased apoptosis observed in the IL10-deficient T cells suggested a possible interference in pathway(s) regulating cells survival and/or apoptosis. Indeed, by comparing apoptosis-related gene-expression in T cells derived from either CD4/IL10 or littermate control $IL10^{f/f}$ mice, we were able to identify elevated levels of several known pro-apoptotic genes and decreased levels of other anti-apoptotic genes (Fig. 8f).

Figure 8

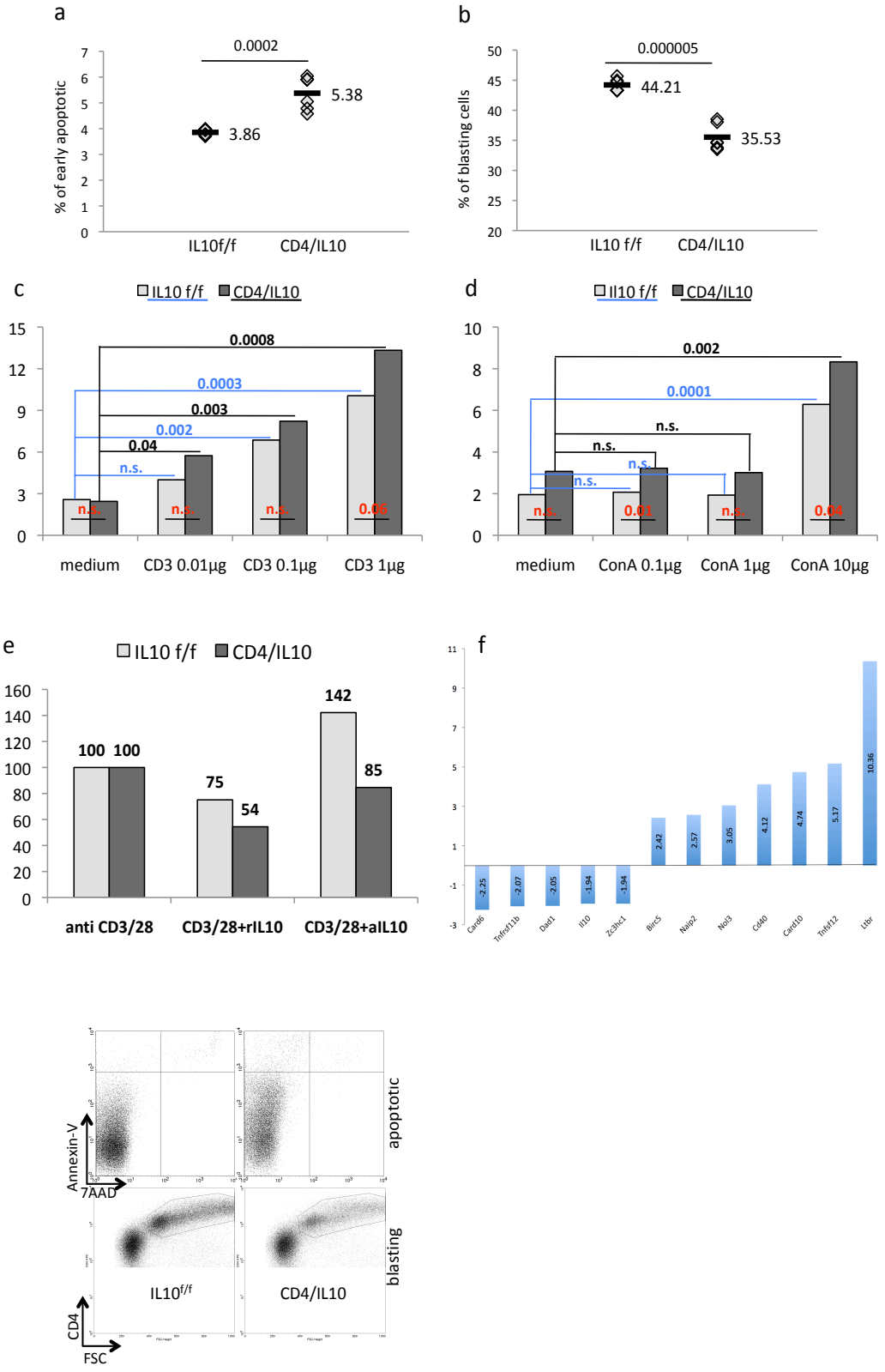
a
0.0002
5.38
3.86
% of early apoptotic
IL10f/f
CD4/IL10
b
0.000005
44.21
35.53
% of blasting cells
IL10 f/f
CD4/IL10
c
IL10 f/f
CD4/IL10
0.0008
0.0003
0.003
0.002
0.04
n.s.
medium
CD3 0.01µg
CD3 0.1µg
CD3 1µg
d
Il10 f/f
CD4/IL10
0.002
0.0001
n.s.
n.s.
n.s.
0.01
medium
ConA 0.1µg
ConA 1µg
ConA 10µg
e
IL10 f/f
CD4/IL10
100 100
75
54
142
85
anti CD3/28
CD3/28+rIL10
CD3/28+aIL10
f
-2.25
-2.07
-2.05
-1.94
-1.94
2.42
2.57
3.05
4.12
4.74
5.17
10.36
Card6
Tnfrsf11b
Dat1
Il10
Zc3hc1
Birc5
Naip2
Nol3
Cd40
Card10
Tnfrf12
Ltbr
Annexin-V
7AAD
apoptotic
blasting
IL10f/f
CD4/IL10
CD4
FSC

**Figure 8. The lack of IL-10 renders CD4$^+$ T cells susceptible to AICD**

MACS-purified naïve CD4$^+$ T cells were stimulated with anti CD3/28 and 24 hours post stimulation, the ratio of apoptotic and blasting cells was determined by FACS-analysis (triplicate wells, n=2 mice per group)(a and b). MACS-purified naïve CD4$^+$ T cells were stimulated with different concentrations of anti CD3 or ConA. 24 hours post stimulation cell death was determined by FACS-analysis (n=3 mice per group)(c and d). Cell death of purified naïve CD4$^+$ T cells 48 hours post stimulation with anti CD3/28 alone (set as 100%), anti CD3/28 supplemented with rIL-10 or anti CD3/28 supplemented with anti IL-10 (one representative experiment of four, triplicate wells, n=2 mice per group)(e). Gene-array analysis of apoptotic-related genes in freshly isolated CD4/IL10 or IL10$^{f/f}$-derived T cells (n=3 mice per group)(f).

## 3.2 BTLA

### 3.2.1 Generation of BTLA over expressing mouse

In order to generate a conditional BTLA over expressing mouse, a loxP-flanked STOP cassette followed by the full-length BTLA (fl-BTLA) cDNA and an Interal Ribosome Entry Site (IRES)-EGFP reporter cassette were inserted into the Rosa26 locus under the control of the chicken β-actin (CAG) promoter. The ROSA-CAGs-STOP-eGFP vector was a kind gift from Dr. Thomas F. Wunderlich (Institute for Genetics, University of Cologne, Germany). Using this vector it was possible to clone a PCR-amplified BTLA open reading frame (ORF) from freshly activated T cells into a unique AscI restriction site located downstream of the CAG promoter and a LoxP-flanked transcriptional STOP cassette. The BTLA ORF was amplified using primers tipped with a rare-cutting restriction site, AscI. An AscI site is located between the 3' LoxP site and the 5' frt site in the ROSA-CAGs-STOP-eGFP vector. Thus, the PCR product can be amplified using primers tipped with AscI restriction sites, and further digested using AscI. The digested product can then be cloned into the empty vector linearized with AscI. This vector can be further linearized using SgfI, another rare-cutting enzyme (Fig. 9).

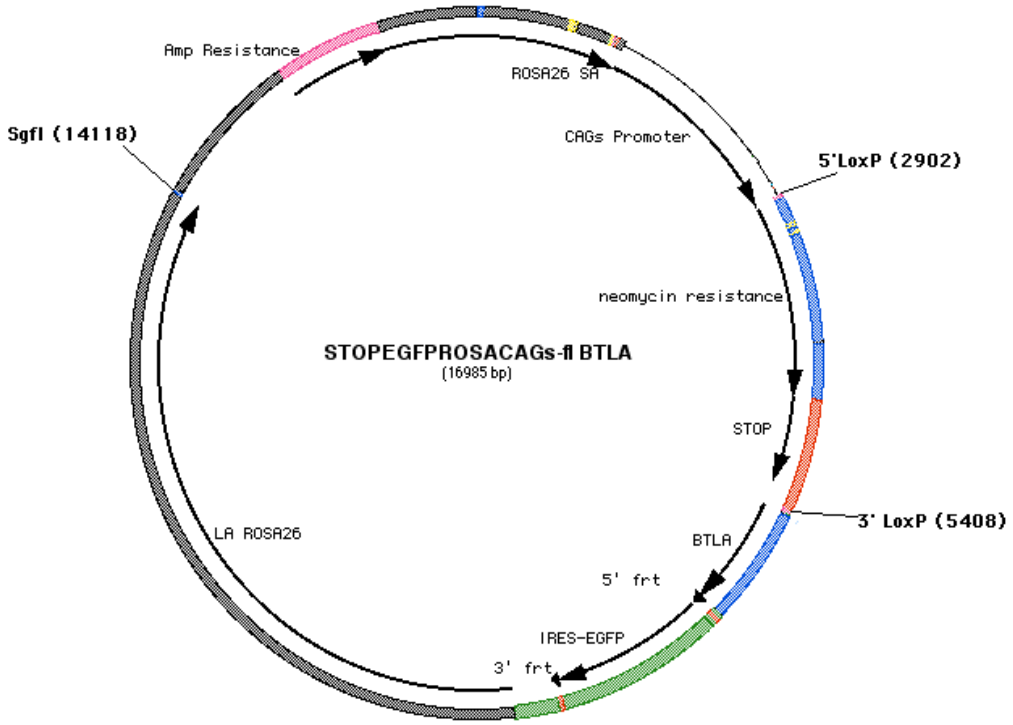


**Figure 9. Schema of the ROSA-CAGs-STOP-BTLA-eGFP targeting vector**

Short and long arms of homology to direct homologous recombination to the ROSA26 locus flank the fl-BTLA construct. A neomycin-resistance cassette and a transcriptional STOR cassette are situated between LoxP sites. Thus, the upstream CAGs promoter is held distal to the fl-BTLA cDNA. After a Cre-mediated recombination, both the neomycin resistance and transcriptional STOP cassette are excised, permitting expression of fl-BTLA and eGFP under the control of the CAG promoter.

The complete targeting ROSA-CAGs-STOP-BTLA-eGFP vector was then confirmed by digestion as well as sequence analysis. The linearized vector was then used to target embryonic stem (ES) cells. Bruce4 ES cells were thawed and cultured on monolayer mouse embryonic fibroblast (MEF) "feeder" cells. Following a single passage, $1x10^7$ ES cells were electroporated with the linearized targeting vector. After a two days recovery phase, ES medium supplemented with G418 was added. ES colonies are sensitive to the G418 selection and will die unless they contain the neomycin resistance cassette found in the targeting vector, therefore only colonies bearing an integration of the targeting vector would survive the G418 selection. Colonies that had survived the G418 for ten days were picked and reintroduced into ES medium without the selection reagent. Growth in G418-containing medium

confirms an integration of the targeting vector into the electroporated ES cells genome. However, the insertion of the neomycin resistance cassette allows the survival of both random and homologues recombinant clones. To confirm homologues recombinant of the targeting vector into the ROSA26 locus, Southern blot analysis was performed on stem cells found to be resistance to the G418 selection. A 900 bp external probe (termed "Orkin") was used to hybridize digested genomic ES DNA. Digestion of genomic DNA and successfully targeted genomic DNA results in bands of 15.6kb and 7.7kb, respectively, when hybridized with the Orkin probe (Fig. 10b, left). Upon Cre-mediated recombination the stop cassette is excised, resulting in a 10.5kb band (Fig. 10b, right). Of ~200 colonies picked, 19 homologues recombinant clones were identified. By performing a second Southern blot analysis on DNA extract from the expanded 19 clones, 13 clones were reconfirmed positive as homologues recombinants. The clones harboring a homologues recombination between the targeting vector and the ROSA26 locus were then selected as potential candidate for blastocyst injection. However, prior to injection, the construct was tested *in vitro* for the expression of BTLA and eGFP. Soluble Tat-Cre protein (Peitz et al., 2002) was added to trypsinized homologues recombinant ES cells of the selected clones and the treated cells were assayed for their GFP expression. While ES cells that had been treated with Tat-Cre became $GFP^+$, the same cells cultured with medium alone were negative for GFP expression (Fig. 10c). In order to confirm BTLA overexpression, a murine cell line, named EL-4 was transfected with either the targeting vector alone or in combination with a PGK-Cre vector. 48 hrs post transfection, the cells were harvested and analyzed for the expression of GFP and BTLA. While EL-4 cells that have been transfected with the targeting vector alone did not express elevated levels of BTLA and were $GFP^-$, EL-4 cells co-transfected with both the targeting vector and the PGK-Cre-containing vector became $GFP^+$ and expressed high levels of BTLA (Fig. 10d). Depending on GFP expression levels after Tat-Cre treatment, different positive clones were then used for multiple injections into BALB/c blastocysts. Of these injections, several chimeras were born, ranging 50-100% chimerism. These chimeras were crossed to C57BL/6 mice in order to

achieve germline transmission. Subsequent to germline transmission, the transgenic allele was identified in ~50% of young mice and showed no preference for gender, as expected by Mendelian autosomal inheritance ratios. Cre-expression is necessary to induce both BTLA and eGFP expression in this strain. Prior to crossing to well-established Cre lines available in our laboratory, $BTLA^{f/+}$ mice were intercrossed to achieve homozygosity of the $BTLA^{f/+}$ allele. $BTLA^{f/f}$ mice were identified and kept as a homozygous strain in the SPF facility in Mainz. The $BTLA^{f/f}$ mouse strain shows no signs of abnormalities or sterility, as these mice are healthy, ageing and breeding normally. By crossing the $BTLA^{f/f}$ mouse strain to a tissue-specific or ubiquitous Cre recombinase-expressing mouse line, we were able to elucidate the role of BTLA in selected cellular subsets of both the innate and adaptive immune response.

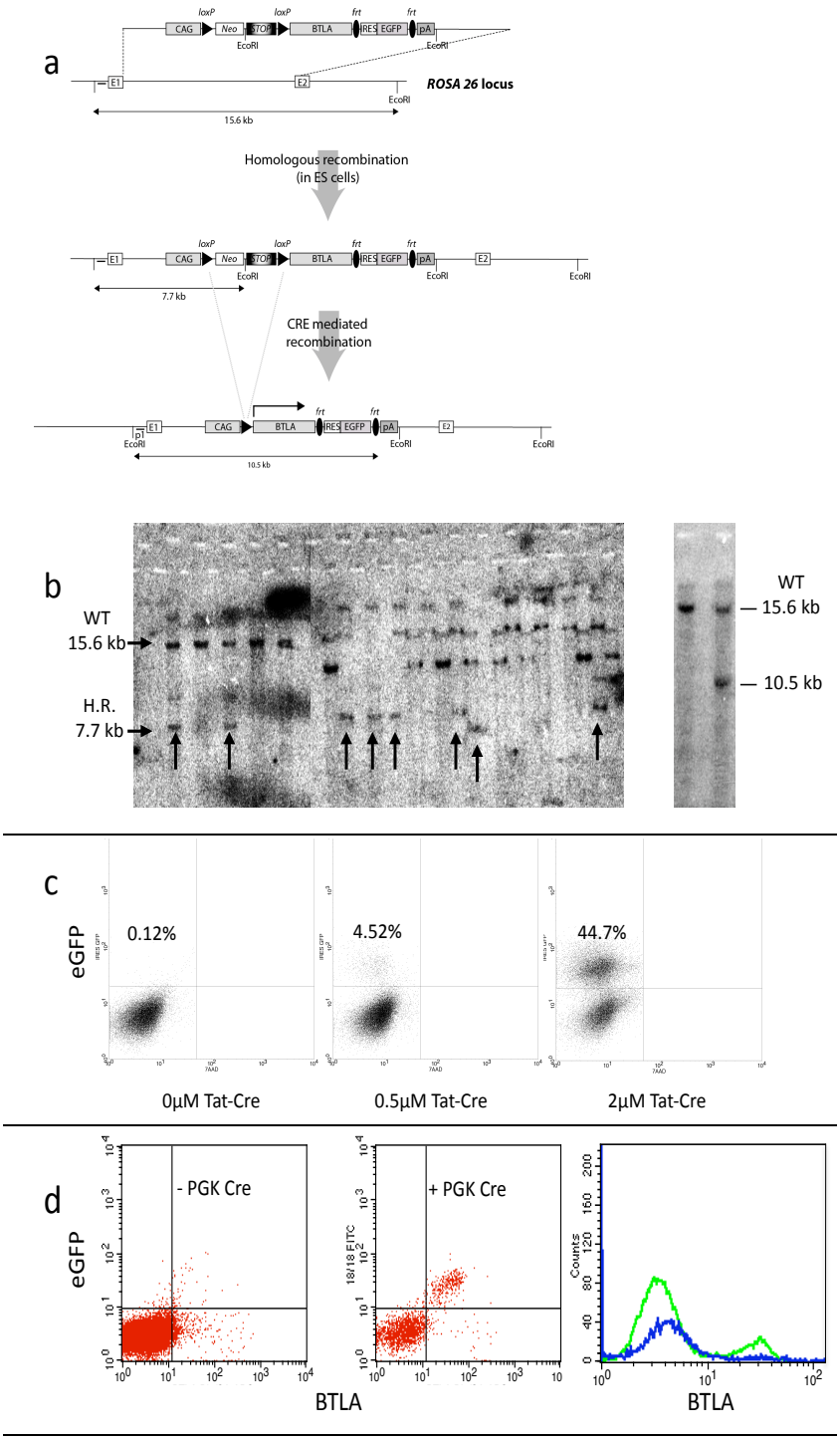


**Figure 10. Schema of fl-BTLA targeting construct**

A LoxP-flanked neomycin resistance (Neo) cassette and transcriptional STOP cassette lie downstream of the chicken β-actin (CAG) promoter. After Cre mediated recombination, both the Neo and the STOP cassettes are excised, allowing the expression of fl-BTLA cDNA. An IRES-eGFP element results in simultaneous eGFP and fl-BTLA co-expression. This construct was used to target the wild type (wt) ROSA26 locus (a). EcoRI digestion yields a

wt band of 15.6kb. Successful targeting introduces another EcoRI site, and successful homologous recombination (h.r.) between the fl-BTLA targeting vector and the ROSA26 locus gives a band of 7.7kb. Homologous recombined colonies were subjected to reconfirmation Southern blot analysis. Arrows indicate several positive clones containing the 7.7kb h.r. fl-BTLA band (b). The positive identified clones were treated with a transducible TAT-Cre protein and subsequently FACS-analyzed for the expression of eGFP (c). Similarly, EL-4 cells were transfected with either the targeting vector alone (left) or in combination with a PGK-Cre vector (middle). 48 hrs post transfection, the cells were harvested and analyzed for the expression of GFP and BTLA (d).

Since BTLA can be expressed by most hematopoietic cells, and it is not yet fully understood whether its interaction with HVEM result in signaling through HVEM, BTLA or bidirectional manner, as well as the possibility of signaling in a *cis* or *trans* fashion, several experiments were needed to be performed in order to address these issues.

### 3.2.2 BTLA over expression by dendritic cells

Dendritic cells are considered as prototypic APCs in the initiation of immune responses. By crossing the $BTLA^{f/f}$ mice with a DC specific CD11c Cre recombinase-expressing mouse line (here on named CD11c/BTLA), all conventional DCs over express BTLA.

It has been suggested that BTLA has an effect on DC maturation and activation (Han et al., 2004). Indeed, *in vitro* cultured CD11c/BTLA-derived BMDCs exhibit an immature phenotype as compared to BMDCs cultured derived from $BTLA^{f/+}$ littermates (Fig. 11). Both CD11c/BTLA and $BTLA^{f/+}$ BMDCs expressed comparable levels of the integrin alpha x chain- CD11c (Fig. 11a). This is of importance, as CD11c promoter is responsible for the Cre expression and as a result, BTLA over expression. As expected, CD11c/BTLA BMDCs expressed elevated levels of BTLA and become GFP positive (Fig. 11 b and c). When examining the expression pattern of MHC-II and co-stimulatory molecules on BMDCs derived from the different genotypes, a significant difference was found with CD11c/BTLA BMDCs displaying reduced levels of MHC-II, CD80, CD86, and CD40 (Fig. 11 d-g). During their maturation, BMDC up regulate the expression of these molecules, thereby the

observed differences indicate for the CD11c/BTLA BMDC immature phenotype. However, upon overnight stimulation with LPS these maturation differences were lost and both BTLA$^{f/+}$ and CD11c/BTLA BMDCs displayed comparable levels of MHC-II, CD80 and CD86 (Fig. 11h).

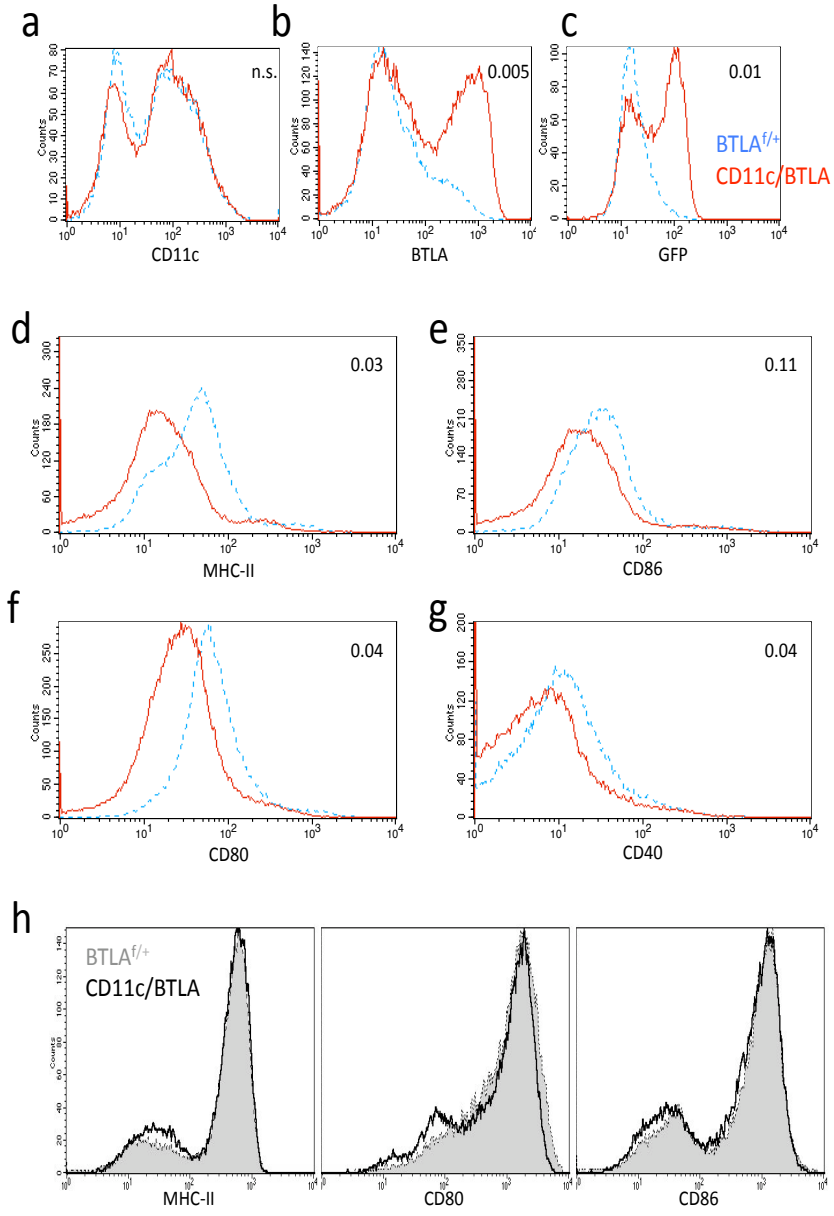


**Figure 11. BTLA overexpression by BMDCs results in reduced maturation**

Histogram overlay FACS-analysis of eight days cultured CD11c/BTLA-derived and BTLA$^{f/+}$-derived BMDCs for the expression of CD11c (a), BTLA (b), eGFP (c), MHC-II (d) as well as the co-receptors CD86, CD80 or CD40 (e-g). Histogram overlay analysis of nine days cultured BMDCs for the expression of the co-receptors MHC-II, CD80 or CD86 following o/n LPS-maturation (h).

In order to assess whether CD11c/BTLA DCs are immunosuppressive as predicted by the literature and by the immature phenotype observed in BMDCs (Fig. 3), wild type and TCR-transgenic T cell were co-cultured with $BTLA^{f/+}$ or CD11c/BTLA BMDCs. TCR transgenic T cells were stimulated by BMDCs pre-pulsed with specific peptides whereas wild type T cells were activated using soluble anti CD3 mAb. As predicted, the activation of wild type $CD4^+$ T cell by CD11c/BTLA BMDCs and anti CD3 resulted in reduced proliferation (Fig. 12a, left). Moreover, CD11c/BTLA BMDCs pre-pulsed with OVA 323-339 peptide recognized by OT-II TCR transgenic $CD4^+$ T cells also exhibit reduced proliferation as compared with the proliferation of the same T cells primed by $BTLA^{f/+}$ BMDCs (Fig. 12a, middle). However, CD11c/BTLA BMDCs pre-pulsed with $MOG_{35-55}$ peptide, recognized by 2D2 TCR transgenic $CD4^+$ T cells did not show a significant difference (Fig. 12a right). Unlike the activation of wild type $CD4^+$ T cells, activation of wild type $CD8^+$ T cells by CD11c/BTLA BMDCs plus anti CD3 did not result in reduced proliferation (Fig. 12b, left). Nevertheless, the co-culture of CD11c/BTLA BMDCs pre-pulsed with the Ad5 $E1a_{324-343}$ peptide or $OVA_{257-264}$ peptide, recognized by ST40 or OT-I TCR transgenic $CD8^+$ T cells, respectively, resulted in significant enhanced proliferation (Fig. 12b, middle and right, respectively). These results were somewhat unexpected- while CD11c/BTLA BMDCs suppressed the activation and proliferation of both wild type and TCR transgenic $CD4^+$ T cells, they did not appear to have the same suppressive affect on wild type $CD8^+$ T cells. Not only that they were unable to suppress the activation of wild type $CD8^+$ T cell, the co-culture of CD11c/BTLA BMDCs with TCR transgenic $CD8^+$ T cells resulted in enhanced proliferation. These *in vitro* experiments suggest that:

a) BTLA might differentially regulate $CD4^+$ and $CD8^+$ T cells responses.

b) BTLA might differentially regulate T cells activation depending on the route of stimulation.

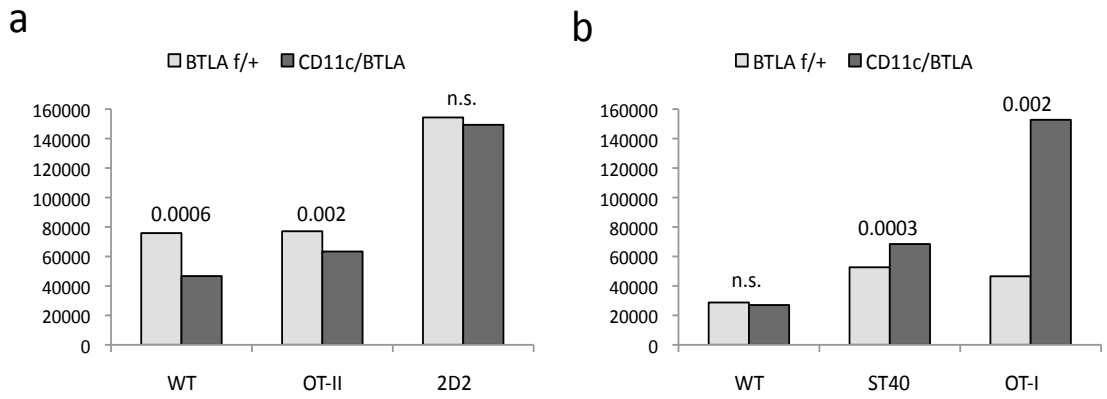


**Figure 12. T cell proliferation following their activation by different BMDCs**

Thymidine incorporation in $CD4^+$ T cells (a) or $CD8^+$ T cells (b) 48 hours post stimulation by either $BTLA^{f/+}$ or CD11c/BTLA-derived BMDCs. Shown average values of triplicate wells and minimum of two mice per group.

Although BTLA-HVEM crosstalk had been suggested to play a role in different DC-subsets differentiation, maturation and distribution (De Trez et al., 2008; Kabashima et al., 2005), when analyzing *ex vivo* DCs of either $BTLA^{f/+}$ or CD11c/BTLA mice, no obvious differences could be found in terms of DC subsets ($CD8^+$, $CD8^-$ or pDCs) or maturation (CD80, CD86 and MHC-II). Furthermore, a thorough examination of naïve mice did not reveal significant differences in T cell activation or in percentage of Tregs (data not shown).

BTLA has been suggested to play a role during EAE, an animal model of CNS autoimmune disease, as BTLA deficient mice developed a stronger disease (Watanabe et al., 2003). This is in line with the reduced $CD4^+$ T cell proliferation by CD11c/BTLA BMDCs, as $CD4^+$ T cells have been shown to be the main effector and regulatory cells during EAE (Baron et al., 1993; Park et al., 2005). To address the questions whether a) the reduced autoimmunity observed in the BTLA-deficient mice is due to the lack of BTLA on DCs and b) can BTLA expression on DC also inhibit T cell activation *in-vivo* (either directly by acting as a ligand for HVEM expressed on T cells or indirectly by inhibiting DC maturation and/or antigen presentation), CD11c/BTLA and littermates control $BTLA^{f/+}$ mice were immunized with MOG/CFA to induce EAE. Following active immunization, both CD11c/BTLA

and BTLA$^{f/+}$ mice developed comparable disease pathology, without obvious significant differences in disease incidence, onset, intensity or recovery (Fig. 13).

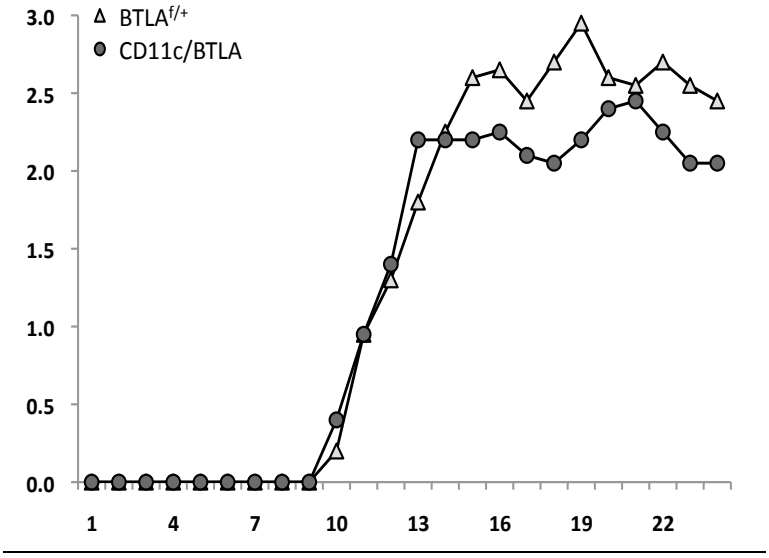


**Figure 13. CD11c/BTLA mice develop normal EAE**

EAE was induced in CD11c/BTLA and BTLA$^{f/+}$ littermate mice by MOG/CFA immunization. Disease progression was monitored daily. Shown a representative experiment. Disease score represent the daily average values of each group of mice (BTLA$^{f/+}$ n=13; CD11c/BTLA n=8).

The *in vitro* proliferation experiments uncover unexpected enhanced CD8$^+$ T cell proliferation (Fig. 12b), suggesting that CD4$^+$ and CD8$^+$ T cells may respond differently to BTLA expression by DCs. In order to test whether BTLA-overexpressing DCs can also induce a stronger CD8$^+$ T cells response *in-vivo*, CD11c/BTLA and littermate control BTLA$^{f/+}$ mice were infected with different viruses, including Mouse Cytomegalovirus (MCMV), Mouse Hepatitis Virus (MHV) and Lymphocytic Choriomeningitis Virus (LCMV). Analysis of acute phase-MCMV infected mice, confirmed that also *in-vivo* the developed CD8$^+$ immune response is stronger when BTLA is over expressed by DCs. When incubating day seven-MCMV infected-CD11c/BTLA or BTLA$^{f/+}$ total splenocytes with MCMV-specific peptides, the CD11c/BTLA splenocytes exhibit higher percentages of IFNγ producing CD8$^+$ T cells in response to specific MCMV epitopes that are normally highly expressed during the acute phase (Munks et al., 2006) (Fig. 14).

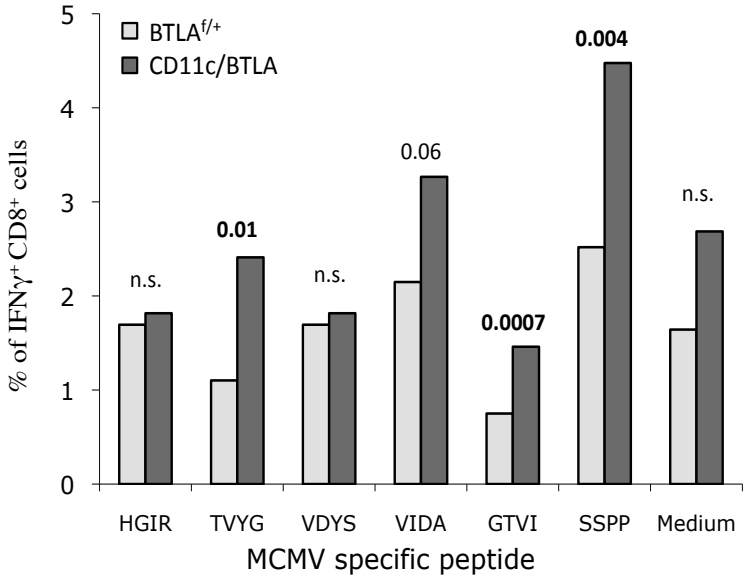


**Figure 14. CD11c/BTLA mice display enhanced MCMV-specific CD8+ T cell immune response**

IFNγ ICS of total splenocytes ($1x10^6$) isolated from $BTLA^{f/+}$ (n=5) or CD11c/BTLA (n=5) littermate mice seven days post MCMV infection. Prior to the ICS, cells have been incubated in the presence of MCMV-specific epitopes for six hours. Date represent one experiment of two.

In another model of viral infection, CD11c/BTLA and littermate control $BTLA^{f/+}$ mice were infected with MHV (Fig. 15). Examination of viral titers five days post infection showed major differences between the two groups, with livers of the CD11c/BTLA mice harboring significant lower number of MHV plaque-forming unit (PFU) (Fig. 15a). Alanine transaminase (ALT) is a transaminase enzyme normally found in the liver and blood. Elevated levels of ALT often suggest the existence of a pathologic problem such as viral hepatitis or other liver-damaging agent. In line with the lower PFU values found in the MHV-infected CD11c/BTLA mice, these mice also showed dramatically lower measurement of ALT values in their peripheral blood as compared with those detected in the $BTLA^{f/+}$ mice (Fig. 15b). MHV is a positive-strand RNA virus of the *Coronaviridae* family and its natural host is the mouse. MHV strain A59 (MHV-A59) is both hepatotropic and neurotropic and can infect hepatocytes, macrophages, conventional DCs (cDCs), and pDCs. Although

virus replication is controlled by pDC-mediated alpha IFN (IFN-α) production during the early phase of infection, acute viral hepatitis and markedly elevated serum ALT levels become apparent by day 5 post infection as result of activated natural killer (NK) cells against virus-infected hepatocytes. When examining MHV-infected mice for NK-infiltration, it became apparent that the CD11c/BTLA mice have significantly reduced levels of liver-specific NK-infiltration (Fig. 15c). This was not a result of general NK-reduction in these mice, as the total percentages of NK cells were not affected, as seen by spleen-specific NK-infiltration (Fig. 15d). In line with the above, examination of MHV-specific $CD8^+$ T cell-infiltration revealed reduced liver-specific, but not spleen, infiltrating MHV-specific $CD8^+$ T cells in the CD11c/BTLA as compared with $BTLA^{f/+}$ mice (Fig. 15e and f). A histological analysis of MHV-infected mice liver sections, stained with Haematoxylin Eosin (H&E), exhibit robust liver-pathology in the control $BTLA^{f/+}$ mice compared to the minor liver damage observed in the CD11c/BTLA mice (Fig. 15g).

Together, the date obtained from both MCMV-infection and MHV-infection experiments as well as our previous *in vitro* assays, demonstrate that BTLA overexpression by DCs results in enhanced $CD8^+$ T cell response followed by a better anti viral immune response and a reduced pathology.

Figure 15

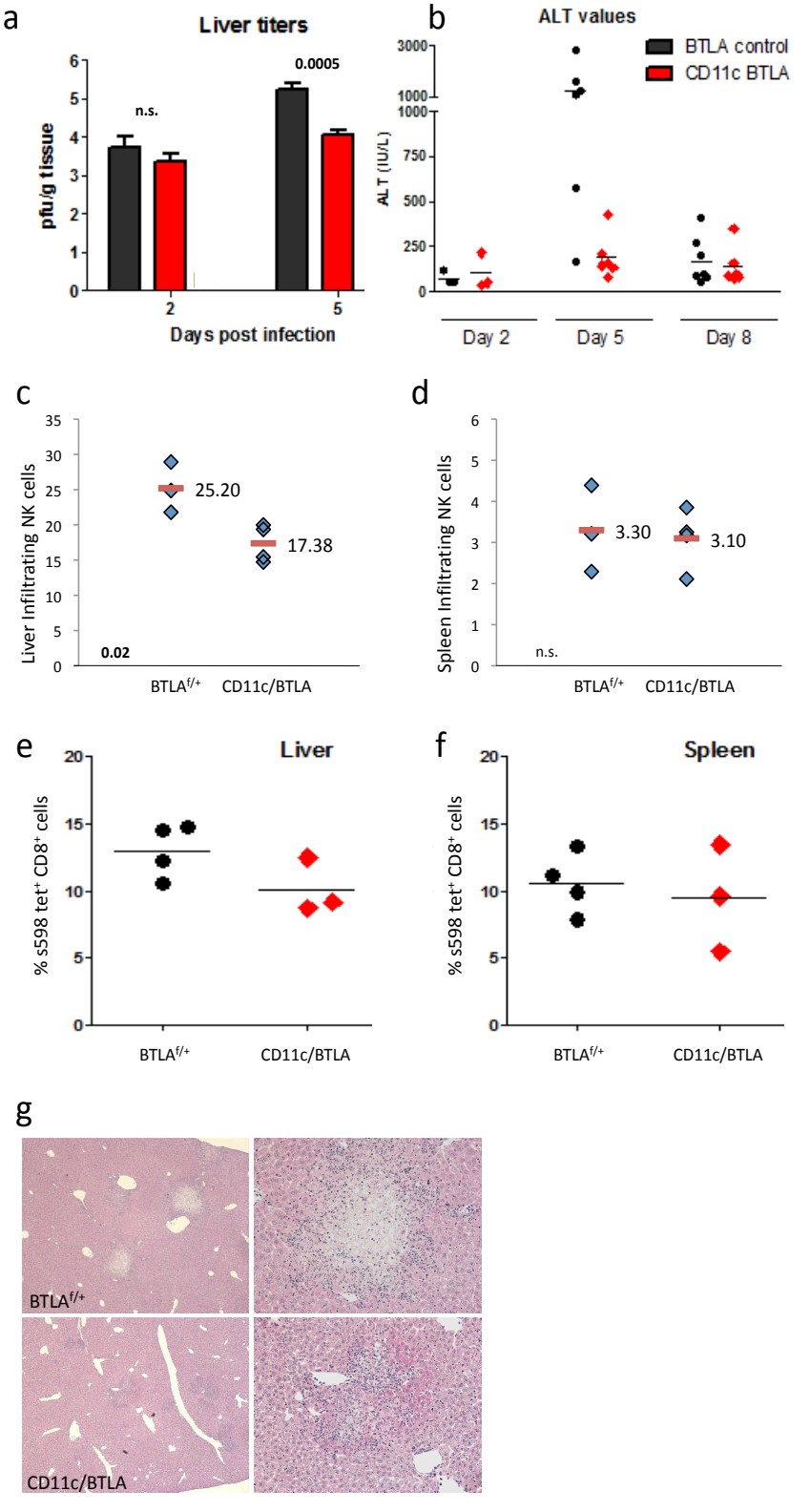
a
Liver titers
pfu/g tissue
n.s.
0.0005
Days post infection
b
ALT values
BTLA control
CD11c BTLA
ALT (IU/L)
Day 2
Day 5
Day 8
c
Liver Infiltrating NK cells
25.20
17.38
0.02
BTLA f/+
CD11c/BTLA
d
Spleen Infiltrating NK cells
3.30
3.10
n.s.
BTLA f/+
CD11c/BTLA
e
Liver
% s598 tet+ CD8+ cells
BTLA f/+
CD11c/BTLA
f
Spleen
% s598 tet+ CD8+ cells
BTLA f/+
CD11c/BTLA
g
BTLA f/+
CD11c/BTLA

**Figure 15. CD11c/BTLA mice display reduced Liver pathology following MHV infection**

Liver MHV-viral titers in MHV-infected CD11c/BTLA or $BTLA^{f/+}$ littermate mice (a) as well as ALT values detected in the peripheral blood of these mice two, five or eight days post infection (b). Shown one representative experiment of four independent experiments (n≥5 mice per group). NK cell-infiltration into the liver (c) and spleens (d) of the MHV-infected CD11c/BTLA or $BTLA^{f/+}$ littermate mice, five days post MHV infection. Shown one representative experiment of three (n≥3 mice per group). Percentages of MHV-specific $CD8^+$ T cell infiltrating the liver (e) or spleens (f) of CD11c/BTLA and $BTLA^{f/+}$ mice, five days post MHV infection. Shown one representative experiment of three (n≥3 mice per group). H&E staining of liver section derived from MHV-infected CD11c/BTLA or $BTLA^{f/+}$ mice, five days post infection (g). Shown one representative liver-section of two independent experiments (n≥5 mice per group).

### 3.2.3 BTLA over expression by T cells

During thymic development, T cell express low levels of BTLA, however once the T cells had left the thymus, the circulating naïve T cells express intermediate levels of BTLA. Shortly after their activation, BTLA expression is further up regulated, whereas fully activated effector T cells down modulate BTLA expression (Han et al., 2004; Hurchla et al., 2005; Watanabe et al., 2003).

By crossing the $BTLA^{f/f}$ mice to a T cell specific CD4 Cre recombinase-expressing mouse line (here on referred as CD4/BTLA), all T cells over express BTLA starting from the thymic CD4/CD8 DP stage (Fig. 16a). Once a Cre excised the stop cassette, the ubiquitously active Rosa26 locus-derived BTLA expression is sustained throughout the entire life of the cell. Analysis of T cell development revealed minor, though consistent, differences in T cell thymic development. CD4/BTLA thymocytes show increase percentage of CD4/CD8 DP as well as CD8 SP thymocytes and a decrease percentage of CD4 SP thymocytes as compared with $BTLA^{f/+}$ thymocytes (Fig. 16b). When further gating on $TCR\beta^{+/hi}$ thymocytes, these differences become even more profound. We found a significant decrease in percentages of mature BTLA overexpressing CD4 SP thymocytes and increase of DP in comparison with $BTLA^{f/+}$ thymocytes. However, although exhibiting slightly increased percentages of CD8 SP, this difference did not reach significance (Fig. 16c). We also observed that PD-1 expression on the developing CD4 or CD8 SP

CD4/BTLA thymocytes was reduced by 50% as compared with littermates control SP thymocytes (Fig. 16d).

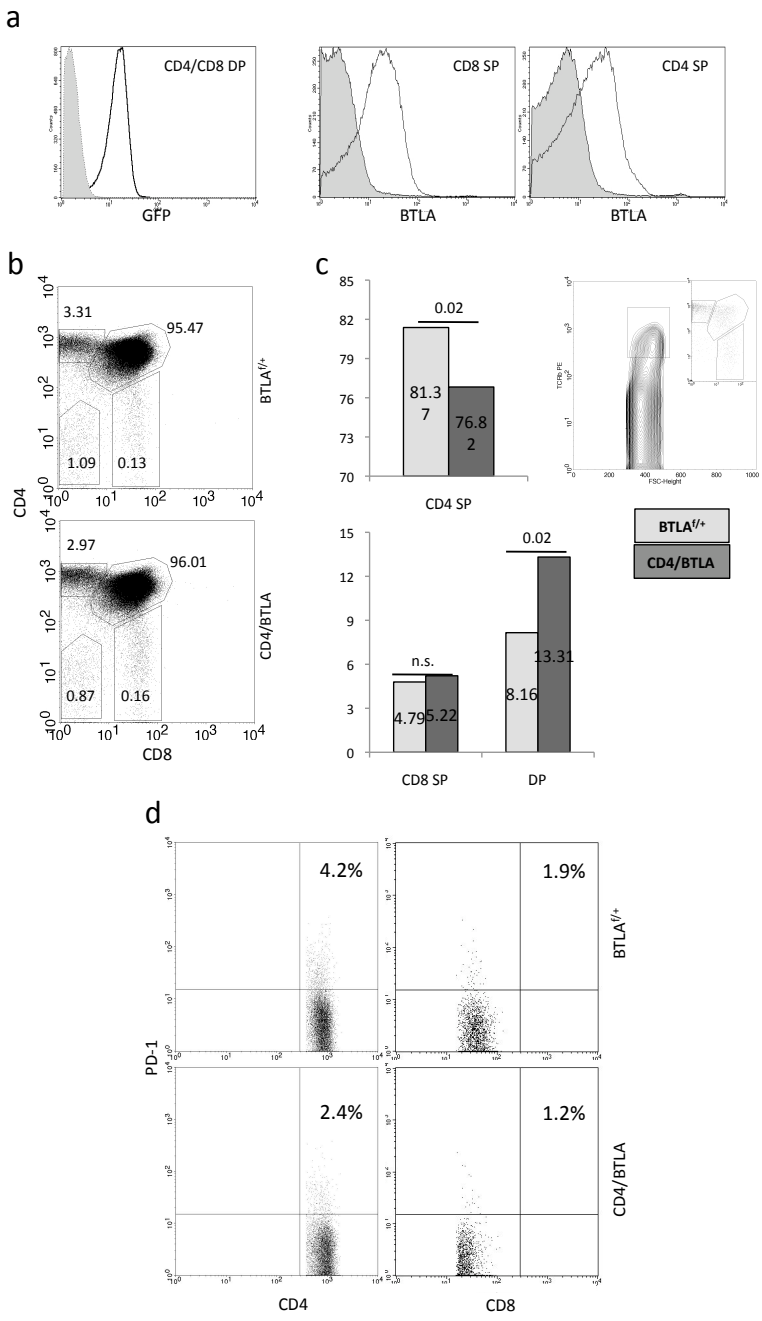


**Figure 16. CD4/BTLA-derived thymic development**

Analysis of BTLA expression during thymic T cell development, demonstrating BTLA overexpression starting from the DN stage (a). FACS-analysis comparing thymocytes development (b) percentages of TCRβ$^{+/hi}$ thymocyte (c) and PD-1 expression on SP thymocytes (d) isolated from naïve, age-matched CD4/BTLA or BTLA$^{f/+}$ littermate mice. Shown FACS analysis representative of four independent experiments (n≥5 mice per group).

Similar to the minor thymic changes, examination of peripheral T cell subsets and distribution, both in spleen and draining lymph nodes (dLN), revealed a slight reduction in percentages of splenic T cells, and a decreased $CD4^+$ T cell (and/or increased $CD8^+$ T cell ratio) in both spleen and dLN (Fig. 17a). $Foxp3^+$ is a hallmark transcription factor, controlling the differentiation and survival of regulatory T cells (Treg) (Fontenot et al., 2003; Fontenot et al., 2005; Hori et al., 2003; Khattri et al., 2003). Intracellular staining of $Foxp3^+$ uncovered increasing numbers of Tregs in both spleen and LN of naive CD4/BTLA mice as compared with littermates $BTLA^{f/+}$ mice (Fig. 17b). However, comparison of dLN composition for naïve and activated CD4 T cells showed no difference in $CD62L^{hi}$ naïve CD4 T cells between the CD4/BTLA and littermate control $BTLA^{f/+}$ mice (Fig. 17c). Once they departure the thymus, naïve peripheral T cells express intermediate levels of BTLA that further increases following their activation. During the effector stage BTLA is down modulated, exhibiting lower expression levels then those found on naïve cells (Fig. 17d upper panel). Naive CD4/BTLA T cells exhibit elevated levels of BTLA to begin with. Following their activation BTLA expression increase even further and even though BTLA expression is down regulated during the effector stage, it remains higher then the endogenous levels found on naïve cells (Fig. 17d lower panel). CD69, a common marker for defining activated cells, is up regulated on activated T cells and remains high for several days. Similarly to the control $BTLA^{f/+}$ T cells, CD4/BTLA T cells up regulate CD69 upon activation. However, unlike $BTLA^{f/+}$ T cells, CD69 expression on CD4/BTLA T cells is only short lasting and is down modulated within the first 48 hrs (Fig.17e). PD-1 expression on resting T cells is thought to be low, whereas following T cell activation PD-1 expression is up regulated (Waisman and Yogev, 2009). Interestingly, naïve CD4/BTLA mice expressed reduced PD-1 levels on mature resting peripheral T cells as compared with littermate control $BTLA^{f/+}$ mice (Fig. 17f). The endogenous levels of BTLA expression on $CD4^+$ and $CD8^+$ T cells is slightly different, with wild type $CD8^+$ T cells expressing lower levels of BTLA then $CD4^+$ T cells. Similarly, although "forced" to over express BTLA, CD4/BTLA derived $CD8^+$ T cells also express lower levels of BTLA as compared with $CD4^+$ T cells derived from the same mice (Fig. 17g).

Figure 17

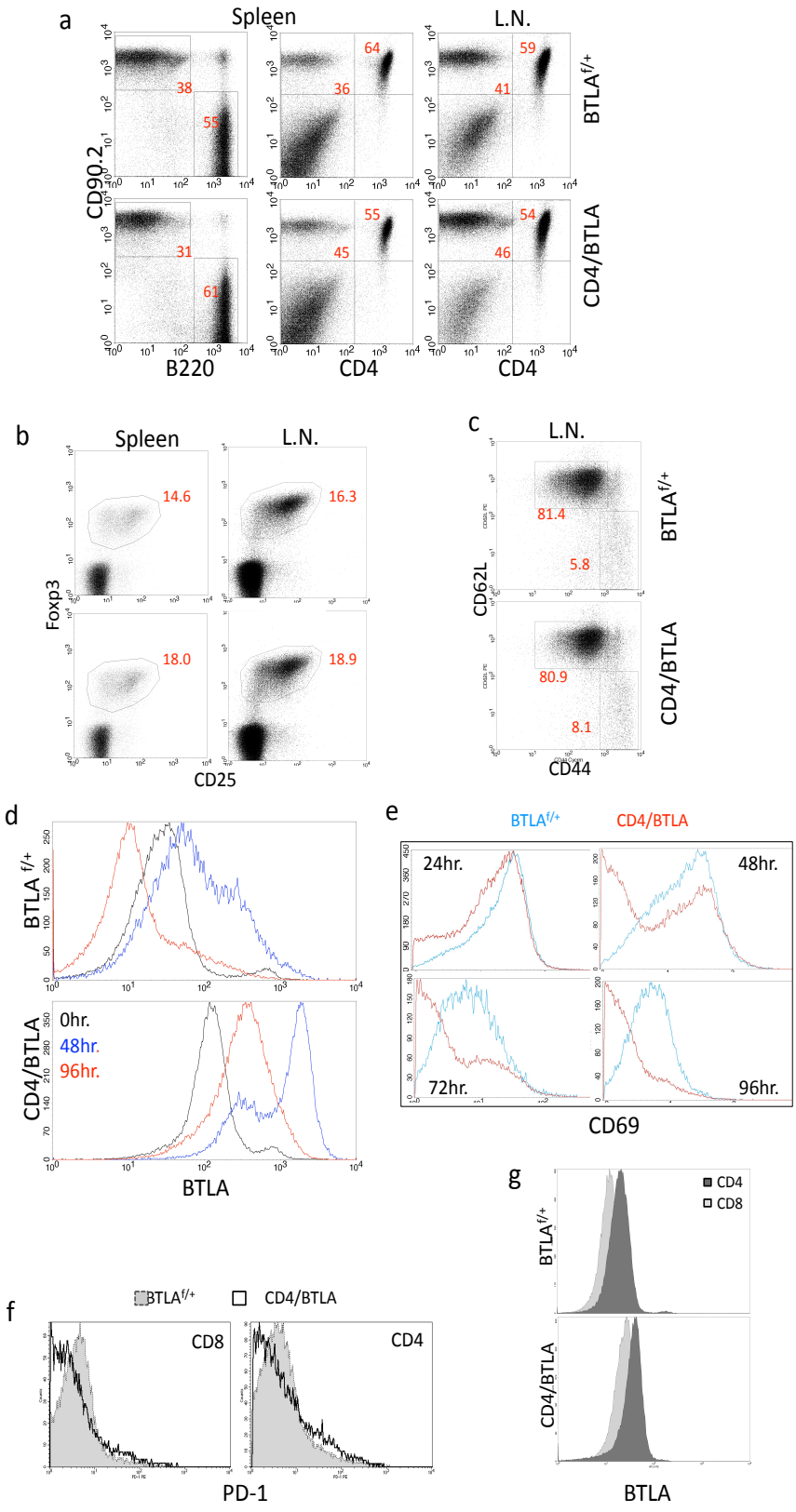
a
Spleen
L.N.
CD90.2
B220
CD4
CD4
BTLA f/+
CD4/BTLA
38
55
64
36
59
41
31
61
55
45
54
46
b
Spleen
L.N.
Foxp3
CD25
14.6
16.3
18.0
18.9
c
L.N.
CD62L
CD44
BTLA f/+
CD4/BTLA
81.4
5.8
80.9
8.1
d
BTLA f/+
CD4/BTLA
0hr.
48hr.
96hr.
BTLA
e
BTLA f/+
CD4/BTLA
24hr.
48hr.
72hr.
96hr.
CD69
f
BTLA f/+
CD4/BTLA
CD8
CD4
PD-1
g
CD4
CD8
BTLA f/+
CD4/BTLA
BTLA

**Figure 17. Distribution of peripheral CD4/BTLA-derived lymphocytes**

Analysis of steady state, naïve mice (a-c): splenic B/T cell ratio (a, right) and CD4/CD8 T cell ratio (a, middle) as well as dLN CD4/CD8 T cell ratio (a, left). CD4/CD8 T cell ratio is calculated as percentage of total CD90.2$^+$ cells. Foxp3 ICS of peripheral lymphocytes (b). Comparison of dLN naïve/effector CD4$^+$ T cell ratio (c), (b and c; shown dot-plots as pre-gated on CD4$^+$ T cells). BTLA (d) and CD69 (e) expression at different time points following an in-vitro activation of MACS-purified CD4$^+$CD25$^-$ T cells by anti CD3/28. PD-1 expression on resting peripheral T cells (f). Basal level of BTLA expression by peripheral CD4$^+$ and CD8$^+$ T cells, isolated from dLN of naïve young mice (g). Data represent minimum of three independent experiments with n≥5 mice per group.

Our *in vitro* DC/T cells co-culture experiment (Fig. 12) and the examination of thymocytes development (Fig. 16) as well as that of peripheral T cell subsets (Fig. 17) revealed some discrepancy between CD4$^+$ and CD8$^+$ T cells, suggesting that CD8$^+$ T cells might be less sensitive to the effect of BTLA inhibition. Indeed, it has been reported that CD8$^+$ T cells are less sensitive to the inhibitory effect of BTLA, as agonistic anti BTLA mAb was able to suppress CD4$^+$ T cell proliferation, but failed to suppress CD8$^+$ T cell proliferation (Krieg et al., 2005).

To test the functionality of BTLA in suppressing T cell activation and proliferation, T cells were stimulated *in vitro* under different conditions. MACS purified wild type C57BL/6 CD4$^+$ T cells were CFSE labeled, plated in a 48 well plate that either been pre-coated with anti CD3 or not, and stimulated with either IgG isotype control mAb or anti BTLA mAb. Surface expression of CD69 and CFSE dilution were measured every twenty-four hours to determine cell activation and proliferation. CD69 up regulation indicates that under both stimuli condition- anti CD3 alone or in combination with anti BTLA, T cell activation can be achieved. In contrast, incubation of the same cells without the anti CD3 stimuli did not induce any modulation of CD69 (Fig. 18a, left). Although the stimulated T cells up regulated CD69 under both conditions, T cell activation by anti CD3 alone induced proliferation as expected, while the same cells activated in the presence of anti CD3 plus anti BTLA barely proliferated- as seen by the CFSE dilution (Fig. 18a, right).

To test whether BTLA over expression by T cells can affect their differentiation properties, MACS purified naïve CD4$^+$ T cells were subjected to different polarizing stimulation condition that are preferentially necessary for Th1, Th17 or iTreg

differentiation. BTLA over expression by T cells did not block T cell commitment in becoming effector T helper or regulatory T cells, as both CD4/BTLA and $BTLA^{f/+}$ T cells could fully differentiate into IFNγ producing Th1 cells, IL-17 producing Th17 cells or $Foxp3^{+}$ iTregs (Fig. 18 b-d, respectively). Moreover, the degree of which the T cells have differentiated under those polarizing condition was similar for both CD4/BTLA and $BTLA^{f/+}$ T cells (Fig. 18 b-d). The comprehensive analysis of steady state T cell compartment as well as *in-vitro* activated T cells, uncovered slightly reduced $CD4^{+}$ T cell population (both thymic and peripheral), reduced activation, and increased percentage of peripheral Treg. These findings go hand in hand with the previous publications proposing BTLA as a negative regulator of immune responses.

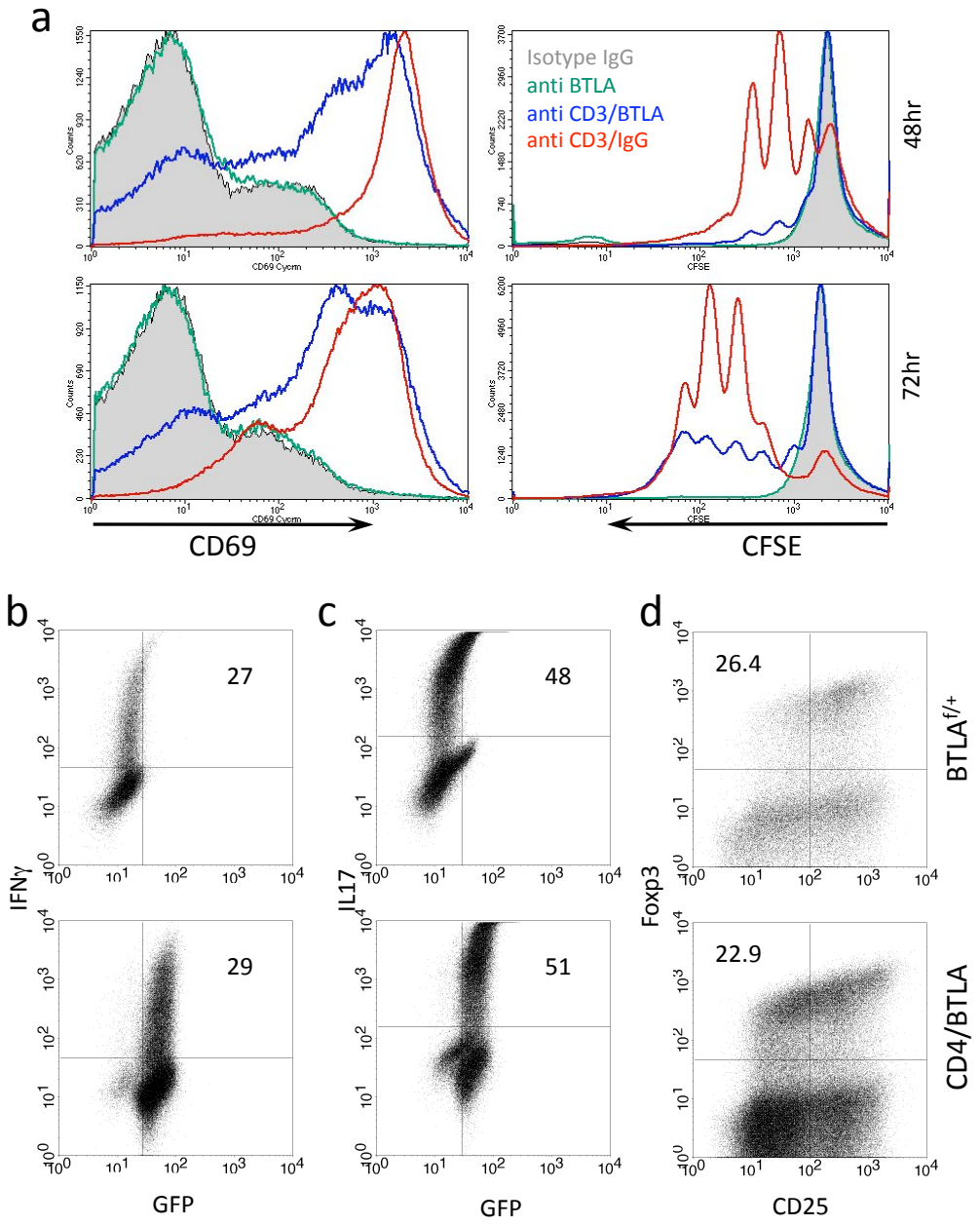


**Figure 18. BTLA signalling effects in vitro T cell activation, proliferation and differentiation**

CFSE labelled MACS-purified wild type CD4+ T cells were activated by anti CD3 (blue and red lines) or left in culture without stimulation (gray and green lines). The same cells were treated in parallel with either anti BTLA (green and blue lines) or with isotype IgG antibody (gray and red). CD69 expression and CFSE dilutions were measured 48 and 72 hours post initial stimulation (a). MACS-purified CD4/BTLA and $BTLA^{f/+}$-derived CD4+ T cells cultured under different conditions supporting Th1 (b), Th17 (c) and iTreg differentiation (d).

Although BTLA was described as a suppressive molecule, BTLA over expression by DCs (CD11c/BTLA mice) did not manifest an obvious difference in $CD4^+$ T cell activation and proliferation *in vivo* (Fig. 13). Therefore it became an interest to check whether BTLA over expression on T cells can inhibit T cell activation. When CD4/BTLA or littermates control $BTLA^{f/+}$ mice were subjected to MOG/CFA immunization, the CD4/BTLA mice developed a milder course of EAE. This mild disease was not associated with a lower incidence or delayed onset of EAE. Moreover, the reduced clinical score was observed only in the CD4/BTLA group while both control groups, littermates $BTLA^{f/+}$ mice and mice harboring CD4-Cre alone developed stronger though similar clinical symptoms (Fig. 19a). Indicating that the reduced disease severity was not an outcome of Cre toxicity or a secondary effect as result of the targeting construct integration, but rather a direct effect of BTLA signaling. When comparing CNS composition, we observed a twenty percent reduction in absolute number of CNS infiltrating T cells in the CD4/BTLA mice as compared with littermate control $BTLA^{f/+}$ mice (Fig. 19b). Further examination of the different subsets of infiltrating T cells into the inflamed CNS revealed no difference in the ratio of infiltrating effector Th1 or Th17 cells as well as peripheral $Foxp3^+$ regulatory T cells (Fig. 19 c-e, respectively). Once again, this data indicates that BTLA overexpression by T cells does not effect $CD4^+$ T cell differentiation but rather inhibits their activation, both *in vitro* and *in vivo*.

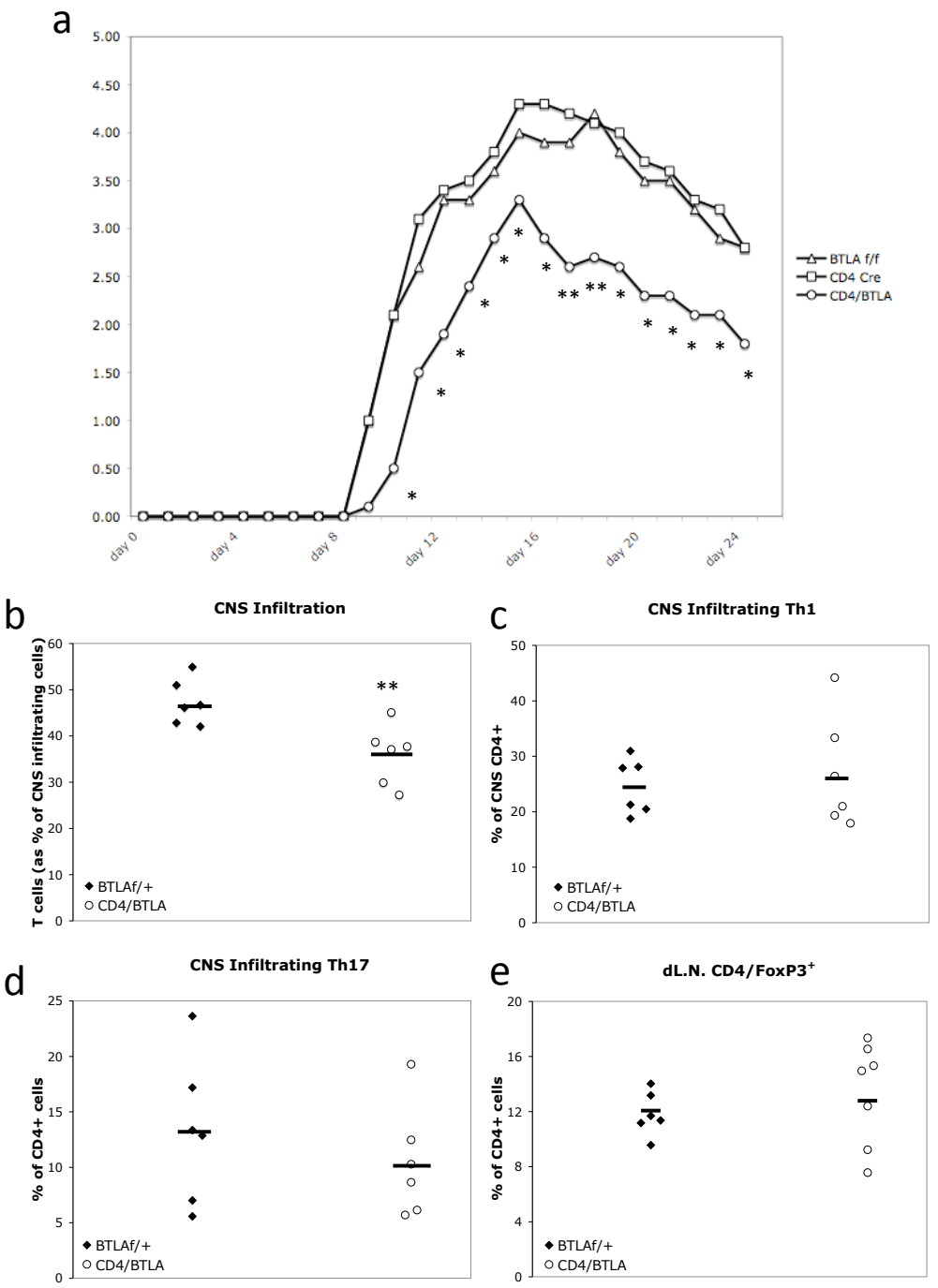


**Figure 19. CD4/BTLA mice develop a mild CD4 T cell-mediated autoimmune response**

EAE was induced in BTLA$^{f/+}$ and CD4/BTLA littermate mice as well as CD4-Cre$^{+/-}$ mice by MOG/CFA immunization. Disease progression was monitored daily. Shown one representative experiment (a). Disease score represent the daily average values of each group of mice (n=5 per genotype). CNS-infiltrating cells were isolated by day 13 using a percoll gradient. Percentage of CNS infiltrating T cells (b) as well as ICS of the different infiltrating Th1 (c), Th17 (d) and Tregs (e) are shown. CNS infiltration represents n≥6 BTLA$^{f/+}$ and n≥6 CD4/BTLA.

In T cell transfer model of Colitis (Powrie et al., 1994), another model of $CD4^+$ T cell-mediated autoimmune disease, Rag-$1^{-/-}$ host mice repopulated with $CD4^+/CD25^-$ $BTLA^{f/+}$ T cells start loosing body weight within ten days after T cell transfer (Fig. 20a) and developed clinical symptoms of gut inflammation as revealed by mini-endoscopy (Fig. 20 b and c). Consequently, the severe gut inflammation led to increased morbidity incidents (Fig. 20d). Unlike the Rag-$1^{-/-}$ mice that received $BTLA^{f/+}$-derived $CD4^+/CD25^-$ T cells, Rag-$1^{-/-}$ mice receiving CD4/BTLA-derived $CD4^+/CD25^-$ T cells did not show signs of weight loss, gut inflammation or morbidity (Fig. 20 a-d). In this model of transferred colitis, Tregs been shown to be essential in controlling gut inflammation (Groux et al., 1997; Groux and Powrie, 1999). Five weeks post T cell transfer, the percentage of $Foxp3^+$ Treg cells extracted from the mesenteric lymph nodes (mLN) were comparable in both sets of mice (Fig. 20e). It is possible that CD4/BTLA T cells do not survive as good as $BTLA^{+/-}$ T cells when transferred into Rag-$1^{-/-}$ host mice. To further investigate whether $CD4^+$ T cells overexpressing BTLA are subjected to proliferation disadvantage in a lymphopenic environment, $CD90.1^+$ wild type and $CD90.2^+$ CD4/BTLA $CD4^+/CD25^-$ T cells had been co-transferred intravenously into Rag-$1^{-/-}$ mice at a starting ratio of 1:1. When checking for survival/proliferation capacity of the different T cells in the rag-$1^{-/-}$ lymphopenic environment, it became clear that the CD4/BTLA $CD4^+$ T cells has the "upper hand" and proliferate up to five times more then the wild type $CD4^+$ T cells within just two to three weeks (Fig. 20f).

Figure 20

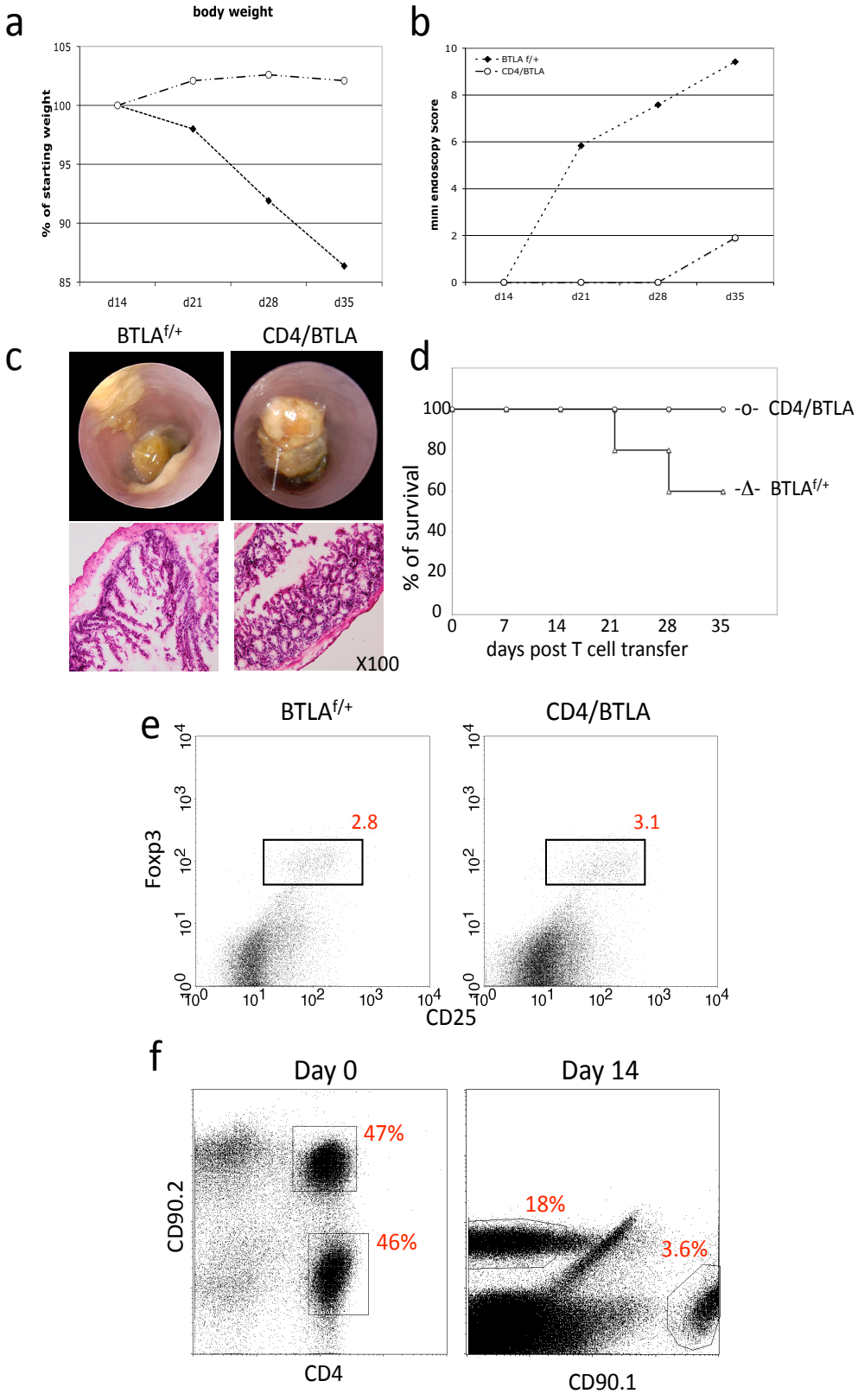
a
body weight
% of starting weight
105
100
95
90
85
d14
d21
d28
d35
b
BTLA f/+
CD4/BTLA
mini endoscopy score
10
8
6
4
2
0
d14
d21
d28
d35
c
BTLA^f/+
CD4/BTLA
X100
d
% of survival
100
80
60
40
20
0
0
7
14
21
28
35
days post T cell transfer
-o- CD4/BTLA
-Δ- BTLA^f/+
e
BTLA^f/+
CD4/BTLA
Foxp3
CD25
2.8
3.1
f
Day 0
Day 14
CD90.2
CD4
CD90.1
47%
46%
18%
3.6%

**Figure 20. Naïve BTLA-overexpressing T cells are incapable of inducing Colitis when transferred into Rag-1$^{-/-}$ mice**

1x10$^{6}$ MACS-purified naïve CD4$^{+}$/CD25$^{-}$ T cell were injected i.v. into Rag-1$^{-/-}$ mice in order to induce Colitis. Following T cell transfer, mice were monitored weekly for alteration in body weight (a), gut pathology (b and c) and survival (d). Shown data of one representative experiment out of four, with n≥7 mice per group. Foxp3 ICS of total cells isolated from the mLN five weeks after T cell transfer (e). Foxp3$^{+}$ cells are calculated as percentages of CD4$^{+}$ T cells, n=5 mice per group. Co-transfer of MACS-purified CD4$^{+}$CD25$^{-}$ T cells isolated from CD90.1$^{+}$ C57BL/6 mice or CD90.2$^{+}$ CD4/BTLA mice into Rag-1$^{-/-}$ mice (f). Starting ratio 1:1, shown cell ratio in dLN 14 days after T cell transfer.

## 3.3 Dendritic cells

In order to elucidate the role of DCs in maintaining and controlling autoimmune CNS inflammation, we utilized our previously described iDTR transgenic mouse model, which allows the ablation of DCs in combination with a Cre-recombinase gene under the CD11c promoter (Buch et al., 2005; Caton et al., 2007). Depletion of DCs in CD11c/iDTR mice is achieved by injection of diphtheria toxin (DT). Following repetitive DT injections, we reached a prolonged reduction of more than 94% of the DCs in the spleen and approximately 80% in the lymph nodes (LNs) (Fig. 21a) including both $CD8\alpha^+$ and $CD8\alpha^-$ DCs (Fig. 21b), whereas plasmacytoid DCs (pDCs) essentially remained unaffected (Fig. 21c). Additionally, staining of epidermal sheets for MHC class II showed effective depletion of Langerhans cells (Fig. 22). Importantly, even after repetitive DT injections, none of the animals died (data not shown), contrasting with a previously described model for DC ablation (Jung et al., 2002).

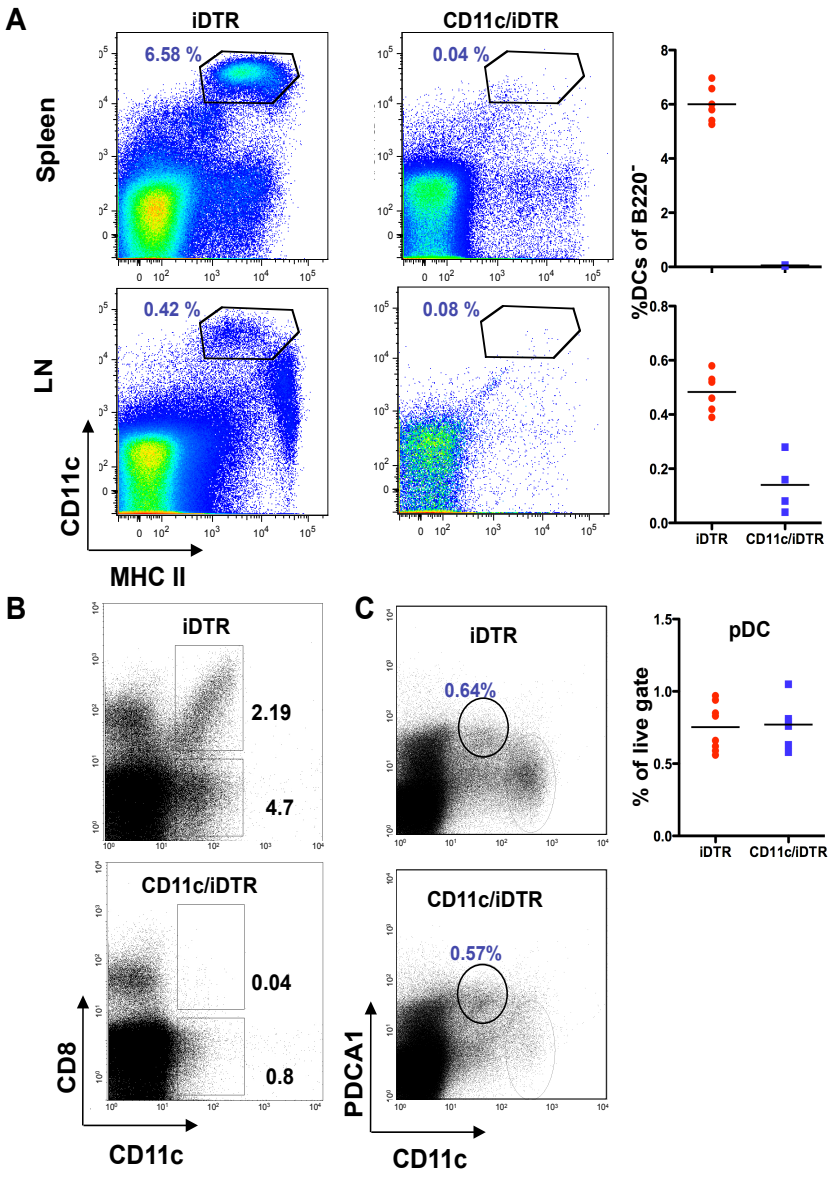


**Figure 21. Depletion of different DC subsets in the iDTR and DTA systems. (A)** CD11c/iDTR (n=4) or iDTR (n=6) littermate mice were subjected to three DT injections. Twenty-four hours after the final DT injection, mice were sacrificed and the efficiency of DC ablation ($CD11c^{hi}$) in spleen and LNs was assessed by flow cytometry, which are shown as dot-plots (on the left) or quantitative in a graph (on the right). **(B)** Depletion of $CD8^+$ DCs in the iDTR system. CD11c/iDTR (n=4) or iDTR (n=6) littermate mice were subjected to a single DT injection and depletion of $CD11c^+CD8^+$ cells was analyzed thirty-six hours later by flow cytometry. The abundance of splenic $CD11c^+CD8^+$ cells was reduced by 98%, whereas $CD11c^+CD8^-$ cells were depleted by 83%. **(C)** DCs and not pDCs are depleted in the CD11c/iDTR system. DT was administered for 8 consecutive days and, 48 hours after the last injection, splenic pDC populations were FACS-analyzed by CD11c and PDCA1 staining. Total live-gated cells are shown on the left. On the right is a summary of the percentages of pDCs from iDTR (n=8) as compared to CD11c/iDTR (n=6) mice treated with DT.

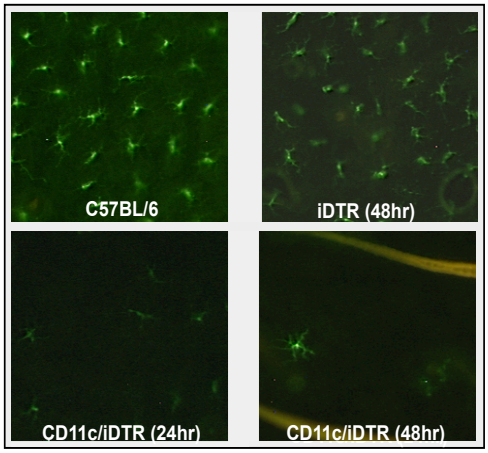


**Figure 22. Depletion of Langerhans cells in iDTR mice.** The depletion of epidermis-resident dendritic (Langerhans) cells in DT-treated CD11c/iDTR mice was assessed by fluorescence-immunohistochemical staining for MHC class II. Data are representative of three or more independent experiments with no less then five mice per group.

Surprisingly, when EAE was induced in DC-depleted CD11c/iDTR mice using $MOG_{35-55}$ peptide in complete Freund's adjuvant (CFA), disease severity increased rather than diminished in comparison to control littermates (iDTR or CD11c-Cre single transgenic mice injected with DT) (Fig. 23a). This difference became even more apparent and significant in experiments where control mice developed only mild EAE (Fig. 23b). When we analyzed the inflammatory CNS infiltrates, we found no major differences in $CD4^+$ T cell frequencies in the DC-depleted mice compared with control mice (CD11c/iDTR: 23.0% compared with iDTR: 21.1%). An analysis of DC reappearance (spleen and LNs) revealed that three days after the last DT treatment, DCs could already be detected. Seventy-two hours after a single DT injection, DCs were back up to 24% of the DC levels in the control group. Due to adverse toxic side effects when using CFA, PT and DT together (Meyer Zu Hörste et al., 2010), which impaired our scoring, we had to discontinue DT injections by day 6 after EAE induction. As a result, the amounts of cDCs in the CNS were similar in both sets of mice 14 days after EAE induction (CD11c/iDTR: 8.8%. and iDTR: 10.7%). In addition, the ablation of DCs before and after immunization through repetitive DT injections resulted in a slightly enhanced EAE score for the CD11c/iDTR mice when compared with littermate control mice (Fig. 23c).

To circumvent the inherent problem when using the DT in combination with active immunization, we used a second system in which DCs are continuously ablated by crossing the CD11c-Cre mice to Rosa-DTA mice (Birnberg et al., 2008; Brockschnieder et al., 2004). In these animals, termed CD11c/DTA, the diphtheria toxin A (DTA) cDNA is expressed specifically in DCs, which leads to constitutive ablation of $CD11c^+$ cells as of birth. Consequently, these mice lacked DCs both in the peripheral priming phase and in the effector phase in the CNS. Interestingly, we found that these mice exhibited somewhat higher clinical scores than the control littermates upon immunization with the $MOG_{35-55}$ peptide (Fig. 23d). The clinical EAE scores of the CD11c/DTA mice tie in very well with the histological analyses, showing similar degrees of demyelination, amyloid precursor protein (APP) deposition, and infiltration/activation of macrophages/microglia as well as T and B cells in DC-proficient mice when compared with DC-less mice (Fig. 23e).

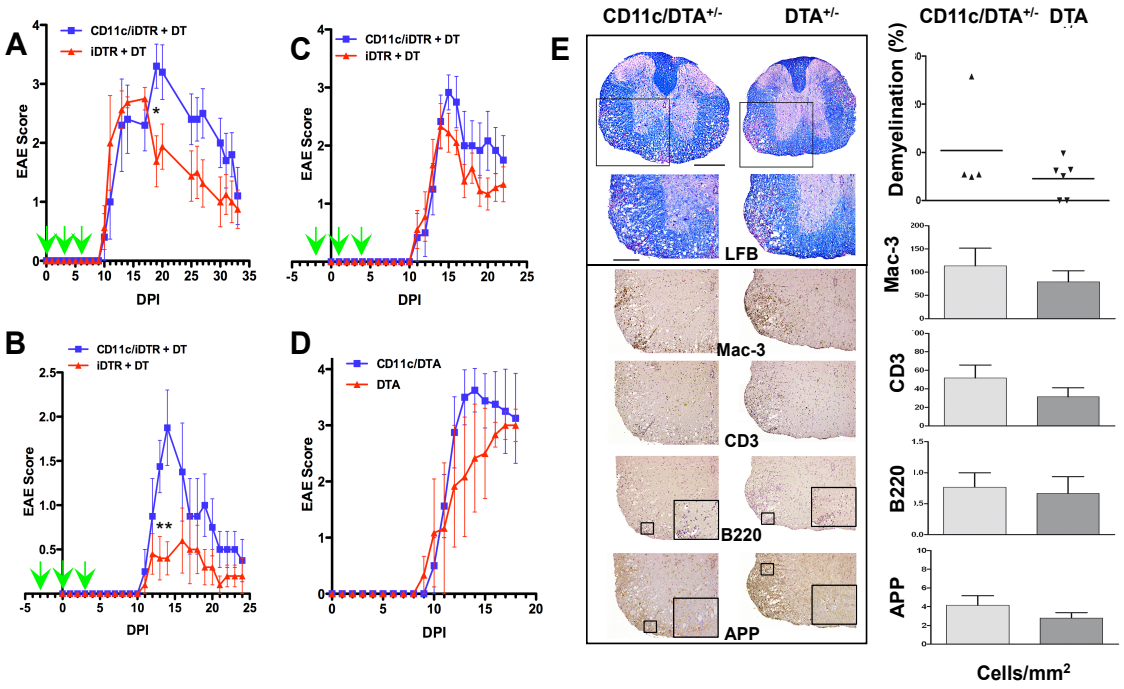


**Figure 23. Absence of DCs is permissive to EAE induction and enhances EAE severity. (A-C)** EAE was induced by $MOG_{35-55}$/CFA immunization and mice were subjected to DT injection (green arrows) at days 0, 3 and 6 (A), -3, 0, and 3 (B) or -2, 1 and 4 (C) of EAE induction (A-C, 5-8 mice per group). **(D)** EAE progression in DTA compared with CD11c/DTA mice, which lack DCs from birth. **(E)** Immunohistological analysis of spinal cord taken from EAE mice (as shown in D). Luxol fast blue (LFB) staining for demyelination as well as specific antibody staining of infiltrating macrophages, T cells, B-cells and amyloid precursor protein (APP) were performed. Demyelination and cell numbers are quantitatively represented in bar charts showing $CD11c/DTA^{+/-}$ (n=4) compared with $DTA^{+/-}$ (n=6) mice. Data are representative of two or more independent experiments. (*, significance, $p<0.05$)

Plasmacytoid DCs (pDCs) can replace the priming function of DCs in lymph nodes after ablation of classical DCs and were shown to contribute to EAE development (Sapoznikov et al., 2007), (Isaksson et al., 2009). Since pDCs were only partially affected in the CD11c/iDTR (Fig. 21c) and CD11c/DTA mice (Birnberg et al., 2008), we combined the CD11c/iDTR system with a depleting antibody specific to pDCs (anti-mPDCA-1) (Fischer, 2004). When we injected the mice with the antibody, they were devoid of pDCs and DCs (Fig. 24a) but still developed EAE similar to mice, which were only lacking DCs (Fig. 24b).

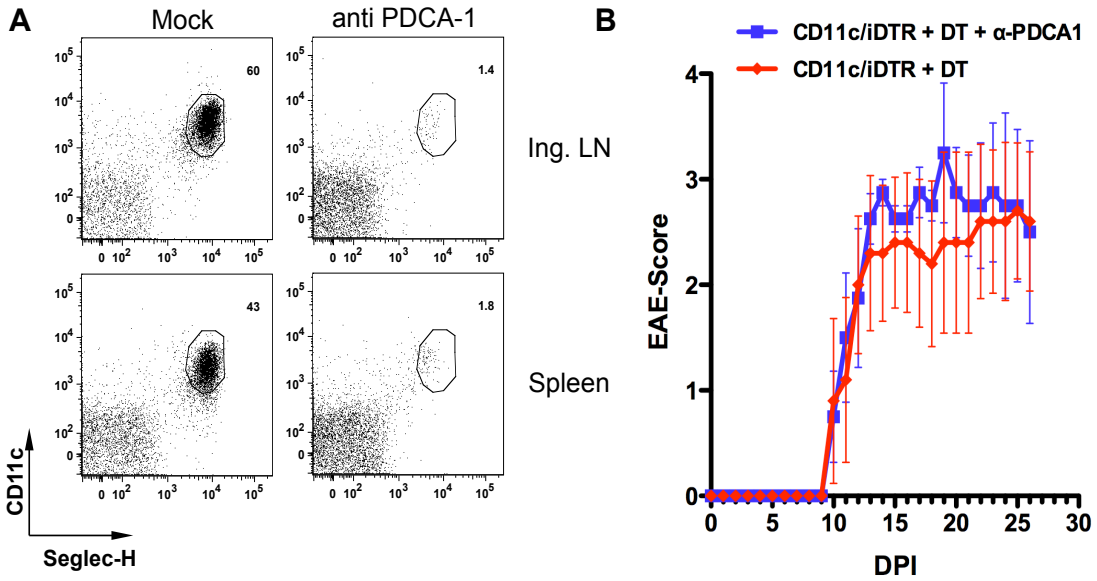


**Figure 24. Depletion of plasmacytoid DCs does not attenuate EAE severity in the absence of DCs. (A)** pDCs were depleted using an anti-PDCA1 monoclonal antibody as well as the respective isotype control antibody. Depletion reached more then 95% in both spleen and LNs as monitored by flow cytometry (gated on $B220^{+}/Ly6c^{+}$, n=3 mice per group). (**B)** EAE progression in CD11c/iDTR mice after DC depletion by DT or depletion of both DC and pDC subsets (n=5 mice per group). Data are representative of two independent experiments with no less then five mice per group.

Furthermore, we analyzed CNS infiltration and cell composition by flow cytometry at day 16 after EAE induction (Fig. 25). We found that leukocytes, $CD4^{+}$ T cell infiltration and Th1/Th17 composition in the CD11c/DTA mice were comparable to control littermates (Figs. 25 a and b). As expected, the numbers of $CD11c^{+}$ cells in the CNS of diseased CD11c/DTA mice were significantly reduced, albeit they were not fully absent. The Treg numbers in the CNS of these animals were lower, although not to a significant extent (Fig. 25c).

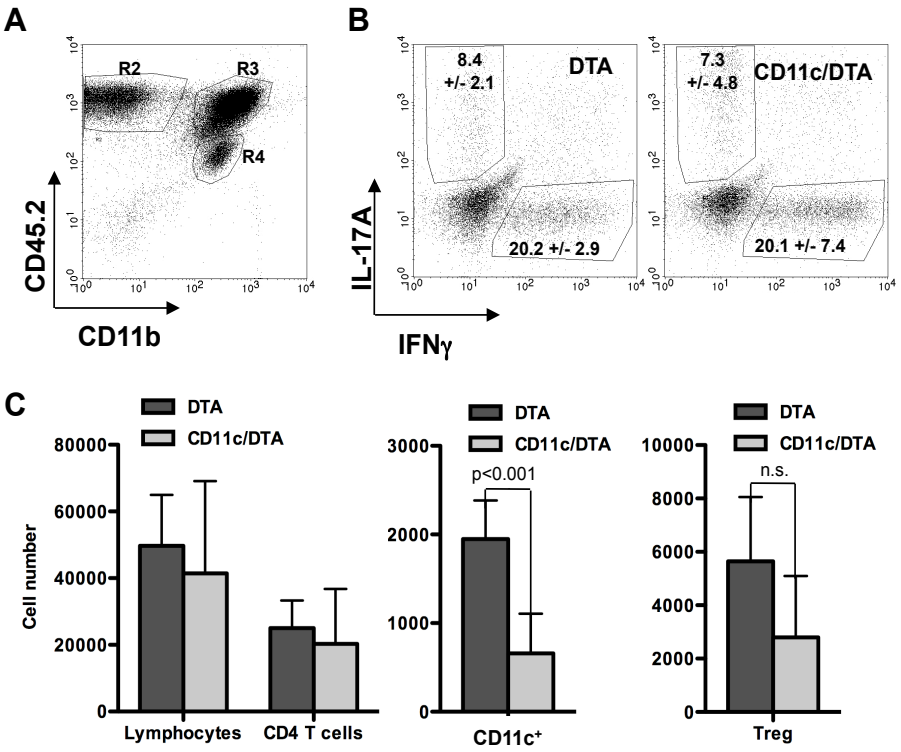


**Figure 25. Normal CNS infiltration in DC-less CD11c/DTA animals.** Bone marrow (BM) chimeric CD11c/DTA→C57BL/6 (n=5) or DTA→C57BL/6 (n=5) control animals were immunized for EAE and CNS infiltration was analyzed 16 days later. These mice were used in this specific experiment due to low numbers of CD11c/DTA mice in our colony, but behaved like the CD11c/DTA mice in all experiments. (**A)** Representative dot plot showing the gating strategy of lymphocytes (R2) compared with macrophage/DC (R3) and microglia (R4) cells. (**B)** CNS infiltrates were restimulated with PMA/Ionomycin and Brefeldin A, surface stained for CD4 and stained intracellularly for IFNγ and IL-17A. (**C)** Total CNS infiltrates numbers for different cell populations.

The observation that active EAE was of greater severity in mice with drastically reduced numbers of DCs suggests that a physiological number of steady state DCs is crucial for the induction and/or maintenance of self-tolerance, whereas either drastically reduced numbers of mature DCs or other APCs types may still be sufficient to induce full-scale EAE upon immunization with self-antigen plus adjuvant. To analyze this further, we used a system in which the relevant MOG peptide is presented in an inducible manner by DCs. We therefore crossed the tamoxifen (TAM)-inducible CD11c-CreER$_T$ mice (Probst et al., 2003) to the IiMOG

mice (Frommer et al., 2008), resulting in animals in which the $MOG_{35-55}$ peptide is expressed as a CLIP replacement peptide upon injection with TAM ($iDC^{MOG}$ mice). As seen in Fig. 26a, TAM-treated $iDC^{MOG}$ mice were completely resistant to EAE induction when DCs were forced to express the MOG peptide following TAM injection, confirming previous studies that showed that steady-state DCs induce tolerance (Bonifaz et al., 2002), (Jiang et al., 1995), (Probst et al., 2003).

To elucidate the molecular mechanism of DC-induced tolerance, we transferred $Thy1.1^+$ T cell receptor transgenic $MOG_{35-55}$-specific, CFSE-labeled, 2D2 $CD4^+$ T cells into $Thy1.2^+$ $iDC^{MOG}$ mice and analyzed the grafted cells for proliferation and upregulation of tolerance-associated cell surface molecules. Although the 2D2 cells proliferated when injected into TAM-treated $iDC^{MOG}$ mice (Fig. 26b), we found a vast upregulation of PD-1 and a moderate elevation of BTLA on these cells when compared to 2D2 T cells transferred to control mice (Fig. 26c).

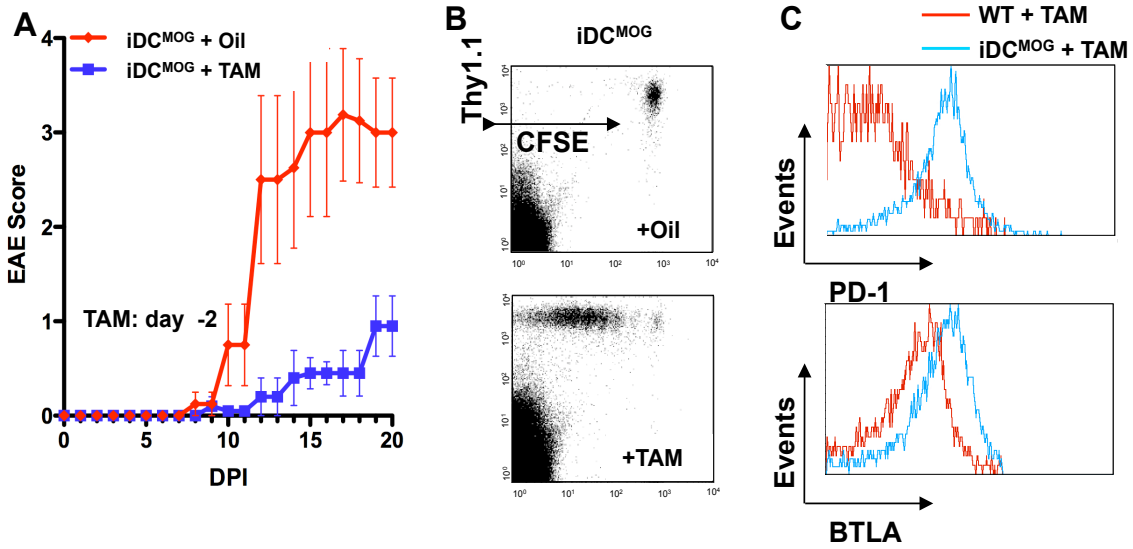


**Figure 26. Antigen presentation by DCs induces tolerance to EAE and expression of co-inhibitory molecules on T cells. (A)** EAE was actively induced and tamoxifen (TAM) was injected two days before EAE induction. $iDC^{MOG}$ + TAM mice develop significantly less severe EAE compared to WT mice or $iDC^{MOG}$ mice treated with the carrier oil alone. (**B & C)** Naïve CFSE-labeled $Thy1.1^+$ 2D2 $CD4^+$ T cells were adoptively transferred to $Thy1.2^+$ WT or $iDC^{MOG}$ mice. Four days after transfer, mice were injected with TAM and five days later, cells were monitored for proliferation as depicted by loss of CFSE of Thy1.1 positive cells or for the expression of co-inhibitory molecules. Shown are $Thy1.1^+$ gated cells. Data are representative of three or more independent experiments with no less then five mice per group.

As PD-1 expression is strongly associated with tolerance (Fife and Bluestone, 2008; Probst et al., 2005; Waisman and Yogev, 2009) and BTLA is a negative co-stimulatory molecule (Han et al., 2004; Sedy et al., 2005; Watanabe et al., 2003), we tested whether the expression of these molecules upon immunization would be affected by DC ablation. To this end, we transferred Thy1.1$^+$ CFSE-labeled 2D2 CD4$^+$ T cells into MOG peptide immunized, DT-treated Thy1.2$^+$ CD11c/iDTR or CD11c/DTA mice (as well as their control littermates) and analyzed cell proliferation and surface expression of BTLA and PD-1 5 days later. Remarkably, 2D2 T cell proliferation was enhanced in mice in which DCs were conditionally depleted or missing from birth (Figs. 27 a and b, respectively). Importantly, we found the upregulation of PD-1 and BTLA only on 2D2 T cells transferred to DC-proficient animals (Figs. 27 c and d). Since the proliferation of 2D2 cells was even stronger in the absence of DCs, the upregulated PD-1 and BTLA expression is not a mere sign of activation, but indeed a clear indication of a specific interaction of DCs with CD4$^+$ T cells which leads to the upregulation of these tolerance-associated molecules.

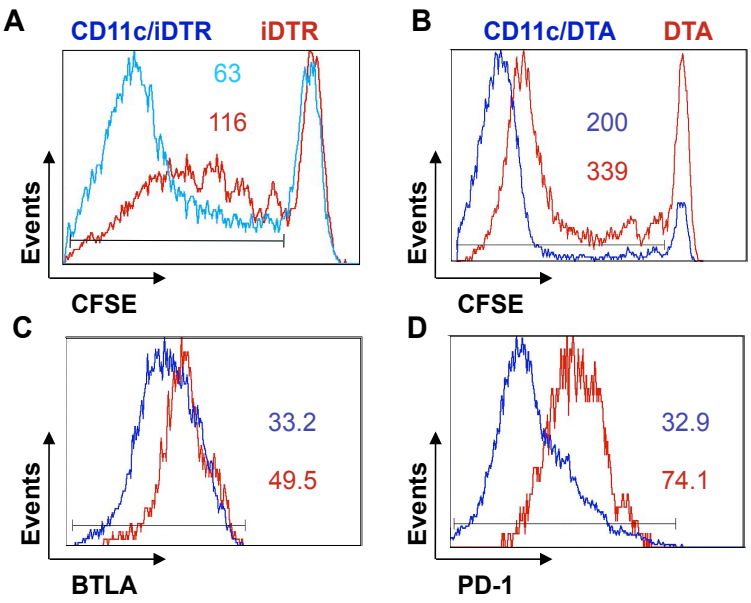


**Figure 27. Proliferation of 2D2 transgenic T cells is enhanced in the absence of DCs *in vivo*. (A & B)** Naïve CFSE-labeled 2D2 Thy1.1$^{+}$ T cells were transferred intravenously into MOG/CFA-immunized iDTR compared to CD11c/iDTR, or DTA$^{+/-}$ compared to CD11c/DTA$^{+/-}$ littermates (all recipients mice were Thy1.2$^{+}$). Five days later, cells were isolated from the draining LN and proliferation was assessed by flow cytometry. (**C & D)** Surface expression levels of the inhibitory molecules BTLA (C) or PD-1 (D) on the transferred Thy1.1$^{+}$ CD4$^{+}$ 2D2 T cells. The different genotypes are indicated in the legend. Data are representative of three independent experiments with no less then three mice per group.

Since our data indicated that the tolerogenic effect of DCs is present in the priming phase (first week after EAE induction), we analyzed the differentiation of CD4$^{+}$ T cells 8 days after immunization of CD11c/DTA mice. As seen in Fig. 5, despite the fact that the experimental group of mice lacked DCs (Fig. 28a), we did not find discrepancies in Th1 or Th17 differentiation in the draining LNs at this point (Fig. 28b). However, we observed a significantly lower number of Foxp3$^{+}$ T cells in spleens of DC-depleted animals (Fig. 28c). Given that this finding may explain the higher degree of EAE severity in DC-less mice, we analyzed Tregs in a third system which has been successfully used in the past to deplete DCs, *i.e.*, CD11c-DTR mice (Jung et al., 2002). Bone Marrow (BM) chimeras of such animals allow DCs to be efficiently depleted over a long period (Zaft et al., 2005). We therefore analyzed Tregs from these BM chimeras (CD11c-DTR BM into lethal irradiated WT mice) after EAE induction on day 12 of EAE and, in line with previous experiments, we found a significant reduction in the number of Tregs in the spleen but not in the LNs of these

mice (Fig. 28d, left). Moreover, we observed that Tregs in these animals expressed significantly lower Foxp3 levels than those from the control BM chimeric EAE mice (Fig. 28d, middle). Non-immunized, DT-treated BM chimera showed comparable levels of Tregs (Fig. 28d right). Accordingly mRNA levels for both Foxp3 and TGFβ were strongly reduced in spleens of immunized DC-depleted BM-chimeric animals (Fig. 28e). To assess the induction of bona fide Treg by DCs *in vivo*, $CD4^{+}CD45.1^{+}CD62L^{hi}GFP^{-}$ T cells were isolated from mice that express eGFP under the control of the Foxp3 promoter (Korn et al., 2007) and transferred into either $CD45.2^{+}$ WT or CD11c-DTR bone marrow reconstituted mice. Prior to T cells transfer, the bone marrow chimeric mice were subjected to DT injection to achieve DC depletion. Twenty-four hours post T cell transfer the mice were immunized and seven days later the newly generated iTreg were assessed by FACS analysis, based on the expression of CD45.1 and Foxp3 (Fig 28f). In line with the previous *in vitro* data, we found that iTregs differentiated much more efficiently when DCs were present. Demonstrating that de novo Treg differentiation is indeed disturbed in absence of DCs during immunization.

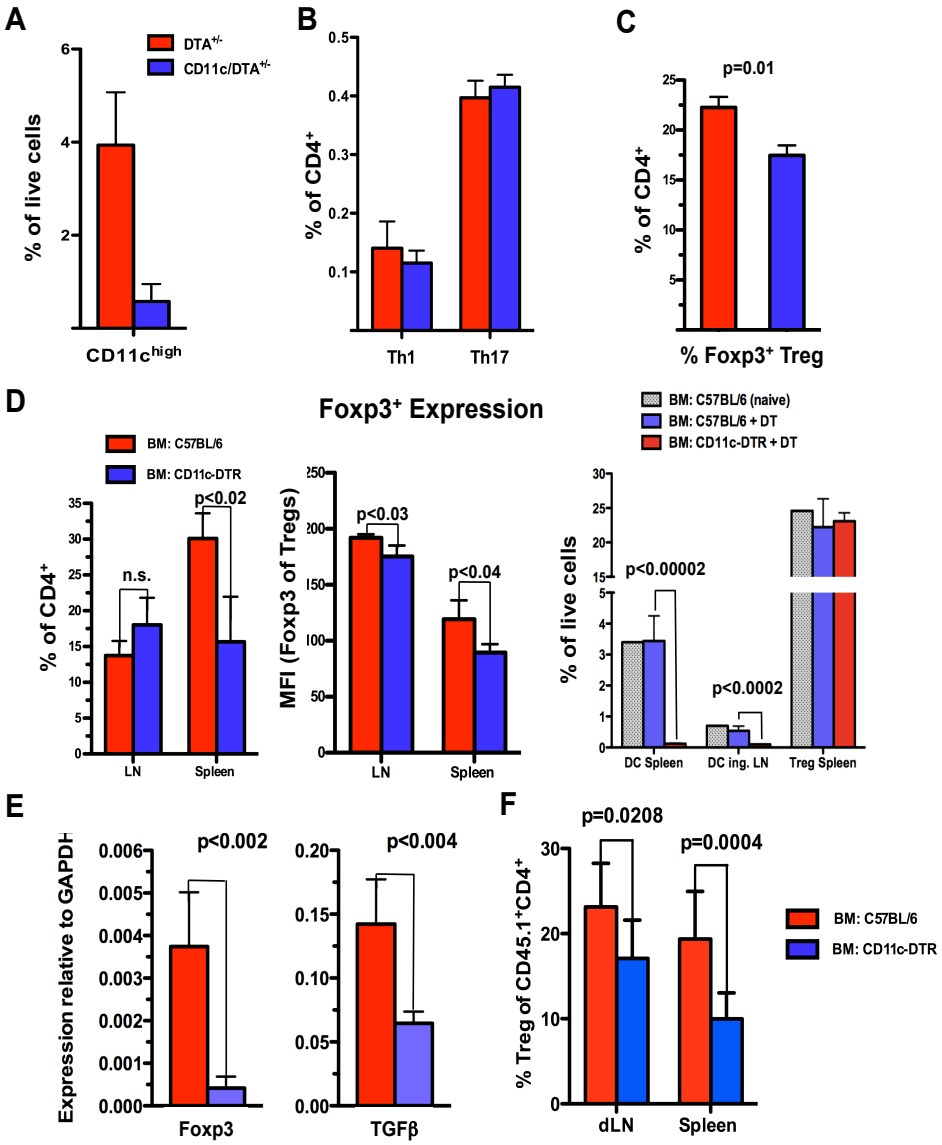


**Figure 28. Immunization of mice lacking DCs results in reduced Treg number. (A-C)** The indicated mice were immunized with $MOG_{35-55}$/CFA and analyzed eight days later for the presence of DCs, Foxp3$^+$, IL-17A and IFNγ-producing T cells. (**A)** Analysis showing the absence of splenic DCs ($CD11c^{hi}$) in the DTA system. (**B)** Percentages of IFNγ-producing or IL-17A-producing $CD4^+$ cells (designated Th1 and Th17, respectively) in the draining LN as determined by flow cytometric analysis. (**C)** Percentages of $CD4^+$ $Foxp3^+$ regulatory T cells (Treg) in the spleen of MOG/CFA-immunized mice as determined by flow cytometric analysis. ($DTA^{+/-}$, n= 3; $CD11c/DTA^{+/-}$, n=2). (**D**) Treg percentage in spleens or LNs of immunized BM-chimeric CD11c-DTR mice on day twelve of EAE (BM: C57BL/6, n= 4; BM: CD11c-DTR, n=3) (left). Foxp3 expression as measured by mean fluorescence intensity (MFI) in Tregs of LNs and spleens of the latter mice (middle). Treg percentage in spleens or LNs of DT-treated BM-chimeric CD11c-DTR or C57BL/6 mice as well as percentage of DCs in the spleen and LNs (BM: C57BL/6, n= 5; BM: CD11c-DTR, n=5) (right). (**E)** Real-time mRNA analysis of splenocytes from DT-treated BM chimeric animals 5 days after immunization (BM: C57BL/6, n= 5; BM: CD11c-DTR, n=4). Data are representative of three or more independent experiments. **(F)** MACS purified $CD45.1^+$ naïve CD4 T cells were transferred into $CD45.2^+$ BM chimeric mice previously injected with DT (BM: C57BL/6, n=9; BM: CD11c-DTR, n=9). DT was applied every second day, starting from day -6 until day +4 post MOG immunization.

Recently, it been reported that granulocyte/macrophage colony-stimulating factor (GM-CSF)-dependent radiosensitive CD103$^{+}$ CD207$^{+}$ dermal DCs are crucial for EAE induction (King et al., 2010). These cells are MHC class II$^{high}$ and CD11c high to intermediate and are present in both the dermis and in the draining lymph nodes. Our lymph node staining show near complete absence of MHC$^{high}$/CD11c$^{+}$ cells in DC-less mice (Fig. 21a). A careful examination of a skin (including both epidermis and dermis) from the CD11c-DTR BM chimeras, revealed nearly complete loss of these dermal CD103$^{+}$ CD207$^{+}$ DCs without influencing the host-derive radio-resistant epidermal Langerhans cells (Fig. 29).

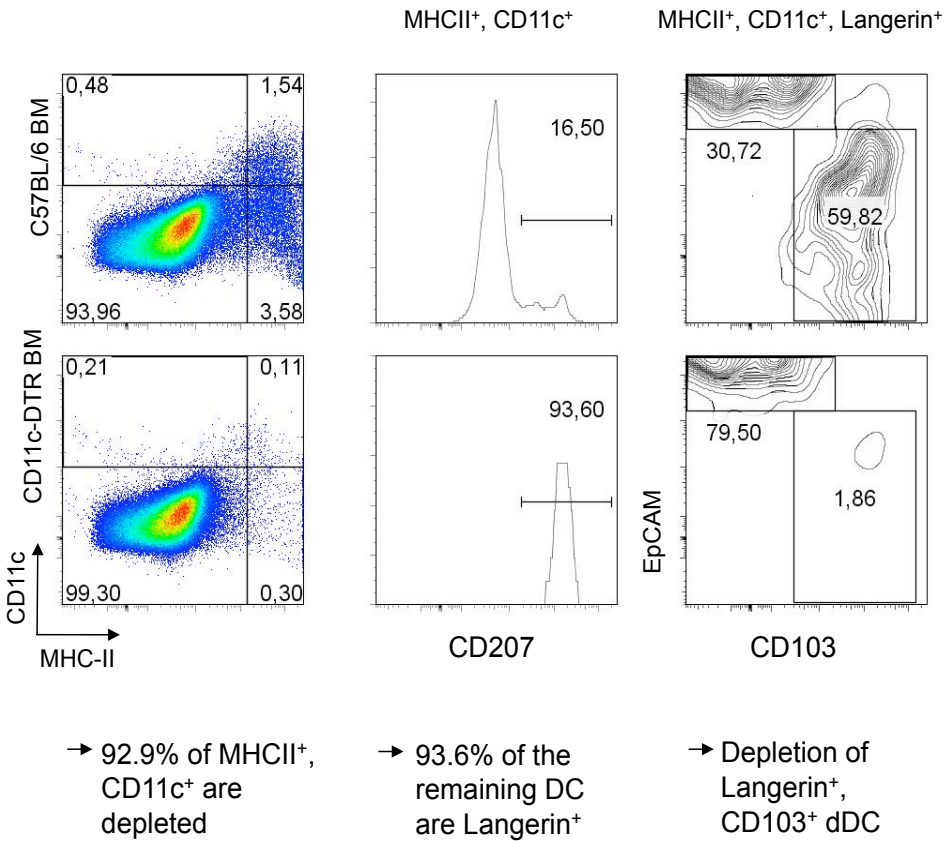


**Figure 29. DT-injection of CD11c-DTR BM chimera results in ablation of dermal CD103$^{+}$CD207$^{+}$ DC.** C57BL/6→C57BL/6 (n=5) or CD11c-DTR→C57BL/6 BM (n=5) chimeras were continuously treated with DT (same injection regime as used in the EAE experiment, i.e. DT was administrated every second day) and analyzed by flow cytometry on day six to determine the presence of epidermal Langerhans cells (host derived) and dermal DCs (donor derived).

Naïve CD11c/DTA mice harbor normal numbers of Treg cells (Birnberg et al., 2008). We assumed that the lower percentage and total number of Tregs found in immunized CD11c/DTA and CD11c-DTR animals may be due to insufficient induction of Tregs shown previously after EAE induction (Korn et al., 2008; Wang et al.). To test this hypothesis, we co-incubated naïve 2D2 $CD4^+$ T cells with WT splenic APCs, which were *in vitro* depleted of DCs using MACS beads, together with the MOG peptide and increasing concentrations of TGFβ. In agreement with our finding of lower Treg numbers in DC-depleted immunized mice, *in vitro* DC-depleted APCs were considerably less efficient in inducing $Foxp3^+$ Tregs compared to DC-containing APCs (Figs. 30 a and b). Not only were the Treg numbers reduced, we also found similar as *in vivo* a decreased level of the Foxp3 protein expressed in the Tregs, which had differentiated in the absence of DCs (Fig. 30c).

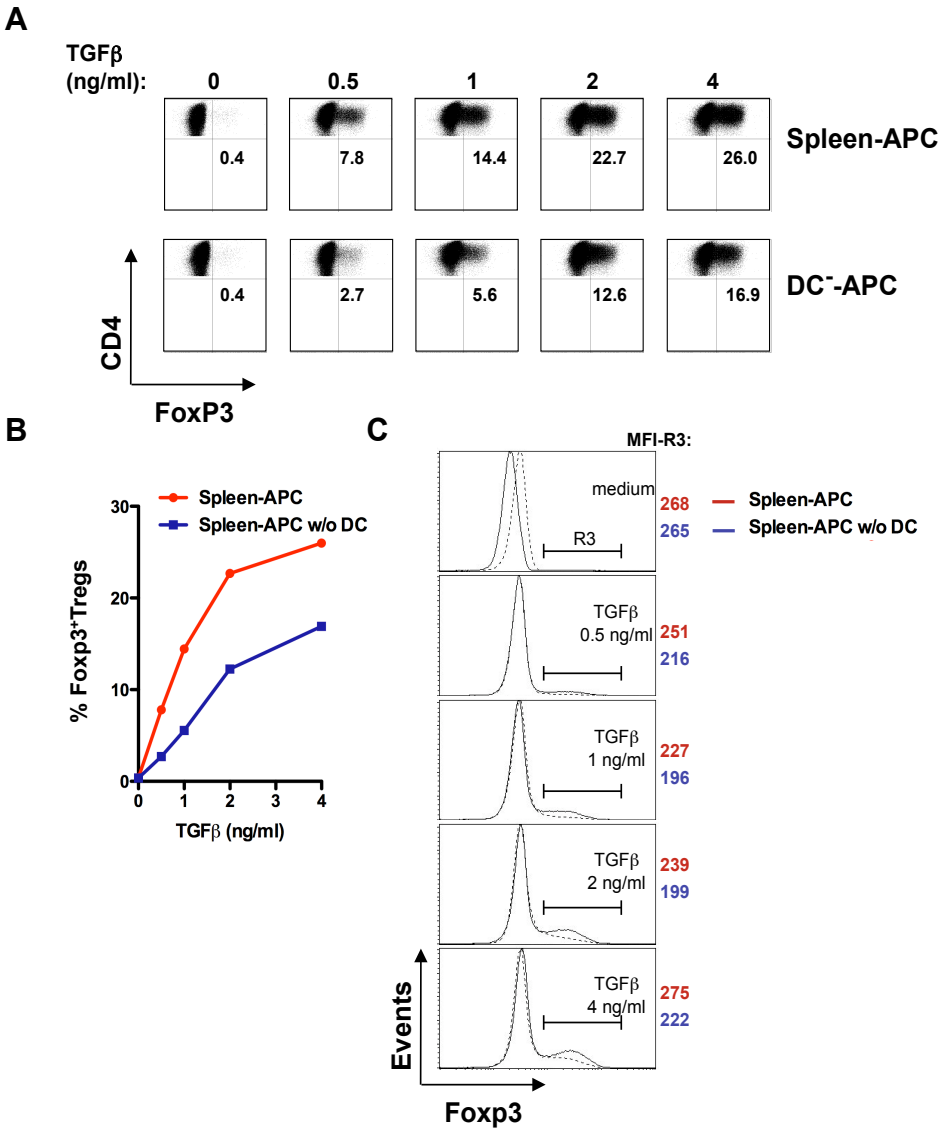


**Figure 30. Impaired induced Treg differentiation in the absence of DCs. (A & B)** MACS-purified naive CD4$^+$ 2D2 transgenic T cells were differentiated using TGFβ at indicated concentrations in the presence of $MOG_{35-55}$ and co-culture with either WT total splenic APCs or WT splenic APCs depleted of DCs, using MACS beads. Cells were FACS-analyzed by CD4 and Foxp3 staining. Percentages of CD4$^+$Foxp3$^+$ cells are indicated. **(B)** Percentages of Foxp3$^+$ of total live CD4$^+$ cells are summarized in the diagram. **(C)** Differences in the mean fluorescence intensity (MFI) of CD4$^+$Foxp3$^+$ T cells are shown by histograms. The respective MFI values of cells in the marked region (R3) are indicated by color. Data are representative of four independent experiments.

As PD-1 signaling was recently demonstrated to favor the development of iTregs (Francisco et al., 2009; Wang et al.), we hypothesized that the higher PD-1 expression by T cells in the presence of DCs may favor iTreg development in DC-sufficient mice leading to reduced EAE scores compared to the DC-ablated mice. We

therefore tested whether PD-1 expression on T cells was necessary for DC-mediated Treg induction in the presence of TGFβ. Therefore, we used naïve $CD4^+$ T cells from 2D2/PD-$1^{-/-}$ mice and performed a similar experiment as described in Fig. 30. Interestingly, we found that PD-1-deficient T cells did not respond to the presence of DCs with higher iTreg differentiation, whereas T cells from a PD-1 sufficient background did (Fig. 31a). To assess the functional implication of the lower expression of the PD-1 ligands, we tested whether recombinant PD-1 ligands were able to restore the iTreg inducing deficits in DC-depleted APCs. Indeed, the combination of both PD-L1 and PD-L2 - when added as recombinant proteins linked to Fc - fully complemented the lacking DCs in inducing regulatory T cells in the presence of TGFβ (Fig. 31b). In line with these experiments, we found that DCs are a major population with PD-L1 expression, which is lost upon depletion of DCs in BM-chimeric CD11c-DTR mice and the other DC-less models (Fig. 32). These findings suggest that DCs induce Treg cells via a PD-1-dependent mechanism, and thereby contribute to tolerance.

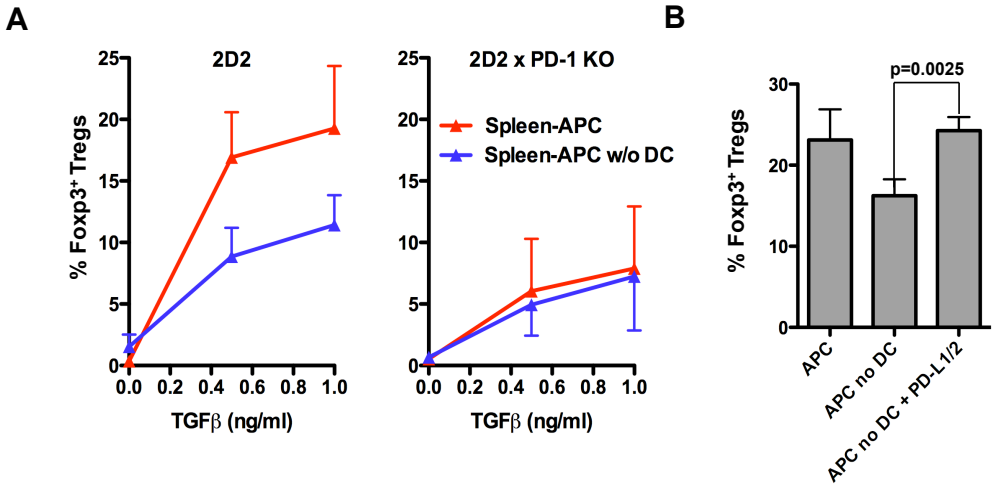


**Figure 31. PD-1 expression on T cells is required for induced Treg differentiation by DCs. (A)** MACS-purified naïve $CD4^+$ T cells from either 2D2 or 2D2/PD-$1^{-/-}$ transgenic mice were differentiated using TGFβ at the indicated concentrations in the presence of $MOG_{35-55}$ and co-culture with WT T cell-depleted total splenic APCs or APCs additionally depleted for DCs. (**B)** Recombinant PD-L1 and PD-L2 Fc-fusion proteins complement the lack of DCs for iTreg development *in vitro*. Naïve $CD4^+$ 2D2 T cells were cultured under an iTreg polarizing condition (TGFβ 1ng/ml) together with total splenic APCs, DC-depleted splenic APCs or DC-depleted splenic APCs supplemented with recombinant PD-L1 (5µg/ml) and PD-L2 (10µg/ml). Data are representative of three independent experiments.

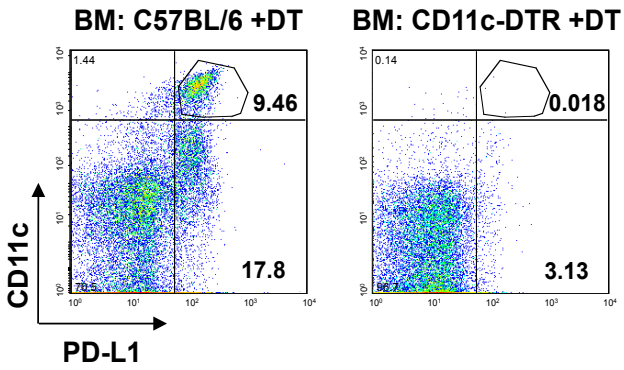


**Figure 32. PD-L1 is highly expressed on DCs.** C57BL/6→C57BL/6 or CD11c-DTR→C57BL/6 BM chimeras were continuously treated with DT, immunized with MOG/CFA and analyzed by flow cytometry on day twelve after EAE induction to determine the expression of PD-L1 on DCs (dot-plot shows cells gated for $CD19^{-}CD90.2^{-}$cells).

# 4 DISCUSSION

## 4.1 Interleukin-10

IL-10 is an important immunoregulatory cytokine with multiple biologic effects on different cell types. It is regarded predominantly as an anti-inflammatory cytokine, inhibiting APC maturation and activation, as well as directly inhibiting T cell activation through its effect on CD28 and ICOS. All T cell subsets have been shown to produce IL-10 in a process that requires ERK phosphorylation and depends on the different STATs needed for the differentiation of the different Th-lineages. IL-10-deficient mice exhibit higher susceptibility to immunopathology upon parasitic and bacterial infection as well as enhanced contact hypersensitivity reactions and irritant responses of the skin. These mice display complex Th1 responses and develop spontaneous IBD as well as enhanced pathology during EAE. Having said that, it is not yet clear which of the different IL-10 producing cellular subsets are responsible for the enhanced EAE pathology. By crossing different Cre recombinase mouse lines with the conditionally flanked IL-$10^{f/f}$ mice, we were able to delete IL-10 encoding gene in specific cellular subsets and study the role of IL-10 during EAE in a cell specific manner.

To our surprise, we found that upon MOG-immunization mice lacking IL-10 specifically in T cells developed significantly milder clinical symptoms which is accompanied by a lower disease incidence, while other mice lacking IL-10 specifically in macrophages, dendritic cells or B cells, developed a normal or even exacerbated disease. The milder EAE observed in the CD4/IL10 mice co-exist with comparable levels of demyelination as well as CNS-infiltration of B cell and macrophages. However, both the absolute cell numbers and percentages of CNS-infiltrating T cells were found to be significantly reduced. Further analysis have shown that within the CNS-infiltrating T cells, the distribution of the effector and regulatory $CD4^+$ T cells was normal while in the periphery, the CD4/IL10 mice had higher percentages of Tregs. The observation that CD4/IL10 mice develop an attenuated disease was rather unexpected as the IL-10 deficient mice develop

stronger EAE. However, the vigorous EAE in the IL-10 deficient mice could result from the lack of IL-10 in different cellular subsets, as the loss of IL-10 in B cells and, though to a lesser extent in macrophages, result in prolonged and enhanced pathology.

As the CD4/IL10 mice develop spontaneous colitis, it was important to further confirm that the reduced CNS-autoimmune response is not secondary to the pre-existing spontaneous gut associated autoimmunity. Indeed, by using several different approaches we were able to show that the pre-existence of one autoimmune response does not prevent the development of another autoimmune response.

As the CD4/IL10 mice exhibit increased percentages of peripheral Tregs upon immunization, we investigated whether IL-10 is necessary for Treg differentiation and whether Treg deletion can fully restore EAE severity. Our data suggest that IL-10 can play a negative role in iTreg differentiation (at least *in vitro*). However, although these results were rather mild and only partially reproducible, they are complying with the poor response obtained by the commercially available rIL10 or anti-IL10.

Despite the fact that Treg depletion by anti-CD25 could fully restore disease severity, EAE incidences was still lower in the CD4/IL10 mice than in the control $IL10^{f/f}$ littermate mice, suggesting that the elevated percentage of Tregs found in the CD4/IL10 mice were only partially responsible for the milder disease.

When examining the different mice for CNS infiltration following MOG immunization, we found comparable ratios of effector Th1 and Th17 as well as Tregs within the infiltrating $CD4^+$ T cells, indicating that the loss of IL-10 does not interfere with the ability of naïve cells to differentiate into the effector or regulatory subsets. Yet, the reduced percentages of peripheral and CNS-infiltrating $CD4^+$ T cells following immunization and the weaker rechallenge response, suggests that these cells do not respond to antigen-challenge as good as wild type $CD4^+$ T cells. Similarly, a general activation by anti-CD3 results in reduced percentages of activated cells. Indeed we were able to show that upon stimulation (both primary and memory) these cells undergo extend AICD. In line with that, we were able to

show that although the percentages of T cells decline in ageing CD4/IL10 mice, the ratio of naïve T cells is increased, indicating once again that these cells share an intrinsic problem with their activation. Finally, a PCR array analysis revealed upregulation of several known pro-apoptotic genes and down-modulation of several other genes known for their anti-apoptotic properties.

Recently, $CD4^+$ T cells that express T-bet and secrete large amounts of IFNγ were found to be the source of IL-10 that regulates the immune response against *T. gondii and L. major* (Anderson et al., 2007; Jankovic et al., 2007). In addition, these cells were shown to be negative for CD25 and Foxp3 expression and can therefore be identified as *bona fide* Th-1 cells that were induced in response to *T. gondii* infection. Importantly, IL-10 produced by the very same cells that produce IFNγ to fight the pathogen is crucial for the survival of the host. IL-10 was rapidly, though transiently, induced only in activated antigen-specific $CD4^+$ T cells, whereas IFNγ production was induced with similar kinetics in either activated or resting T cells. This suggests that the production of IL-10 by antigen-specific Th-1 cells is a normal stage in their differentiation program, which is reached after full activation, in order to moderate or inhibit the inflammatory response.

As a whole and in accordance with the role of IL-10 in regulating Th-1 inflammatory responses, this work suggests a new role for IL-10 in modulating a T cell-dependent immune response. We hypothesise that an autocrine IL-10 feedback loop is needed for controlling $CD4^+$ T cell fate following their TCR engagement. We speculate that this autocrine IL-10 signal is mandatory for controlling the strength, and therefore the outcome, of the TCR-signal and thereby responsible for fine-tuning a T cell survival/response.

## 4.2 B and T Lymphocyte Attenuator

BTLA as been described by different groups as a co-inhibitory molecule, regulating the activation and implementation of an immune response. However, as

BTLA and HVEM are believed to be able of transmitting a signal in a *cis* and a *trans* fashion as well as being expressed on the same cells and/or on opposite cells, the complexity of the receptor-ligand pair interaction as well as their cellular expression pattern could not have been properly investigated when using the fully deficient $BTLA^{ko}$ mice. By using the newly generated conditional BTLA over expression mice we were able to further study the function of BTLA in a cell specific manner.

BTLA overexpression by DCs: When analyzing BMDCs derived from either CD11c/BTLA or littermate $BTLA^{f/+}$ mice, we observed that CD11c/BTLA BMDCs exhibit an immature phenotype, though upon LPS-stimulation these BMDCs upregulate both co-stimulatory molecules as well as MHC-II expression to similar levels as control BMDCs (Fig. 11). Our co-culture experiment demonstrated that, at least *in vitro*, $CD4^+$ T cell stimulation by BTLA over expressing DCs results in reduced proliferation (Fig 12a). Nevertheless, after MOG/CFA immunization of CD11c/BTLA and $BTLA^{f/+}$ mice, we did not find a significant difference in disease between the animal groups (Fig. 13). The lack of difference in disease severity could be explained by different means, one of which is that the CFA used for the MOG emulsion contains LPS, which in term can trigger TLR4 signaling and cause the maturation of DCs. As EAE is a $CD4^+$ T cell driven autoimmune disease, another possible explanation is that under these conditions BTLA expression by DCs acts as a receptor rather than a ligand, transmitting the signal back to the DCs themselves rather than affecting the T cells by signaling through HVEM expressed on T cells themselves. Surprisingly however, while the co-culture of CD11c/BTLA-derived BMDCs with $CD4^+$ T cells inhibited the latter proliferation (Fig. 12a), the consequence of BMDCs co-culture with $CD8^+$ T cells had the opposite effect, resulting in enhanced proliferation (Fig. 12b). Similar to BTLA effect on CD8 T cells activation *in-vitro*, *in-vivo* infection of mice with different viral models result in a stronger anti viral $CD8^+$ T cell immune response (Fig. 14 and 15). Indeed it has been reported that $CD8^+$ T cells are less sensitive to the inhibitory effect of BTLA, as agonistic anti BTLA mAb was able to suppress $CD4^+$ T cell proliferation but failed to suppress $CD8^+$ T cell proliferation (Krieg et al., 2005).

BTLA overexpression by T cells: In accordance with the *in vitro* DC/T cell co-culture experiment (Fig. 12), the examination of thymocytes development (Fig. 16) and peripheral T cell subsets (Fig. 17) revealed some differences between $CD4^+$ and $CD8^+$ T cells in response to BTLA expression, suggesting once more that $CD8^+$ T cells might respond differently to BTLA than $CD4^+$ T cells.

Previously, it has been shown that BTLA is expressed on thymocytes as early as the DP stage (Han et al., 2004). The analysis of CD4/BTLA thymic development uncovered a role for BTLA in CD4 lineage choice (fig. 16 a-c). During thymic development, the strength/intensity and duration of TCR signaling is believed to direct lineage choices, with strong and/or long-lasting signals promoting CD4-lineage choice whereas weak and/or short-lasting signals promoting CD8-lineage choice (Bosselut, 2004). GATA3 has been shown to be essential for $CD4^+$ T cell lineage commitment (Hernandez-Hoyos et al., 2003; Pai et al., 2003) and different studies using mature T cells have shown that many factors regulate *gata3* gene expression, including co-stimulatory molecules (Murphy and Reiner, 2002). As $CD4^+$ T cells express higher endogenous levels of BTLA than $CD8^+$ T cells (Fig. 17g), it is tempting to speculate that a) the negative co-stimulatory function of BTLA may influence *gata3* expression thereby directly influencing CD4-lineage differentiation, and b) BTLA co-inhibitory function might be sufficient for attenuating the stimuli strengths/duration needed for CD4 SP thymocyte development, resulting in fewer CD4 SP thymocytes. Activated BTLA have been reported to associate with SHP1 and SHP2 (Han et al., 2004; Watanabe et al., 2003). These phosphatases have been implicated both in directly down-regulating proximal TCR signaling, and in inhibiting phosphatidylinositol 3-kinase (PI3K) activation and subsequent protein kinase B (PKB) activation (Gavrieli and Murphy, 2006; Gavrieli et al., 2003). Suggesting once more that BTLA may directly regulate thymocyte development by controlling TCR-signal, yet BTLA deficient mice have been reported to have normal B and T cell development in their bone marrow and thymus, respectively (Han et al., 2004; Watanabe et al., 2003). Although the importance of CD28 and other co-signals during thymic development is still subjected to debate, the importance of second co-stimulatory or co-inhibitory signals needed for the activation of mature peripheral T

cells is well established. Therefore the selective thymic development disadvantage shown in the CD4/BTLA mice is most likely further enhanced in the periphery, as we observed a lower T/B cell ratio as well as CD4/CD8 T cell ratio (Fig. 17a).

The Improved $CD8^+$ T cell response observed in the *in vitro* activation experiments (Fig. 12b) or in response to the different viral infection (Fig. 14 and 15), might be explained by the nature of T cell activation. That is, while $CD4^+$ T cell activation is believed to occur following multiple and relatively prolonged interaction with an APC, and requires a strong stimulus, $CD8^+$ T cell activation requires a relatively short and fewer APC-interactions, and a weaker stimulus. CD11c/BTLA-derived DCs can therefore attenuate the TCR signal strength and/or the duration of DC/T cell interaction and by doing so improving the $CD8^+$ T cell response.

Many chronic viral infections are associated with functional impairment of antigen-specific $CD8^+$ T cells. In recent years, PD-1 has been heavily linked with viral specific $CD8^+$ T cell "exhaustion" (Barber et al., 2006; Jin et al., 2010). PD-1 expression has been shown to be upregulated on exhausted LCMV-specific $CD8^+$ T cells and blocking of PD-1/PD-L1 interaction *in vivo* could fully reverse $CD8^+$ T cell exhaustion, resulting in enhanced viral-specific $CD8^+$ T cell response (Barber et al., 2006). Therefore the observation that BTLA-overexpressing T cells down modulate PD-1 expression (Fig. 16d and 17f) could serve as a possible explanation for the improved $CD8^+$ T cell response.

The *in vitro* activation of $CD4^+$ T cells by soluble CD3/28 resulted in up regulation of CD69 that was rapidly down modulated on CD4/BTLA-derived T cells as compared with $BTLA^{f/+}$-derived T cells (Fig. 17e, 24 and 48 hours). In another experiment, naïve wild type $CD4^+$ T cells stimulated with plate-bound anti CD3 sustained CD69 expression even after seventy-two hours. Unlike the soluble CD3/28 used in the previous experiment, the stimulation with anti BTLA mAb in addition to the plate-bound CD3 stimulus had only a minor effect on CD69 expression (Fig. 18a). The different CD69 expression following $CD4^+$ T cell stimulation could result from the usage of anti CD28 in the first experiment – where BTLA might directly regulate CD28 downstream signals. Alternatively it could simply reflect the signal strength,

as a stimulus mediated by a cross-linked Ab. is known to be stronger than the one transmitted by a soluble one.

*Iv vitro*, BTLA overexpression did not seem to affect the differentiation of naïve $CD4^+$ T cells as both CD4/BTLA and $BTLA^{f/+}$ T cells equally differentiated when stimulated by soluble CD3/28 together with specific Th-1, Th-17 or iTreg supporting conditions (Fig. 18b). Whereas *in vivo*, when EAE was induced in CD4/BTLA or littermate control $BTLA^{f/+}$ mice, the CD4/BTLA mice developed a weaker disease that resulted from reduced $CD4^+$ T cell activation and/or CNS-infiltration (Fig. 19). Similarly to the EAE experiment, CD4/BTLA mice also failed to induce colitis when transferred into Rag-$1^{-/-}$ mice (Fig. 20 a-d). The latent colitis did not result from elevated numbers of Tregs (Fig. 20 e-f) nor was it due to survival disability of the transferred CD4/BTLA T cells, as the latter proliferated better in lymphopenic environment. This enhanced proliferation should have resulted in enhanced pathology, as the number of transferred cell into Rag-$1^{-/-}$ host mice can determine disease onset and severity. Given that the percentage of Treg was similar in the MLN it is unlikely that the difference observed in colitis susceptibility is due to the protective role of Treg.

Taken together, this work while examining the effects of BTLA-signaling on different cell types has demonstrated that an immune response can be modified based on the cellular source that expresses BTLA. In order for BTLA to regulate $CD4^+$ T cell-mediated autoimmune responses such as EAE or colitis, BTLA needed to be expressed on the T cells themselves, whereas BTLA expression on dendritic cells did not effect on $CD4^+$ T cell responses but rather resulted in enhanced $CD8^+$ T cell response both *in vitro* and *in vivo*.

## 4.3 Dendritic cells

This work demonstrate that induction of EAE through immunization with $MOG_{35-55}$ in CFA is possible in mice that possess less then 5% of the normal number of DCs. Remarkably, DC-less mice developed more severe EAE compared to mice

with normal DC numbers, suggesting that during immunization DCs reduce the responsiveness of $CD4^+$ T cells to self-antigens, including those sequestered in the CNS. We identified PD-1-dependent induction of $MOG_{35-55}$-specific Treg by antigen-presenting DCs as a crucial mechanism underlying reduced T cell responsiveness and susceptibility to EAE. Furthermore, our results suggest that either the number of mature DCs required for priming is extremely low or that other APC types, most likely $CD11c^-$, are sufficient for EAE induction.

CD11c positive DCs are *bona fide* APCs inducing CD4 and CD8 T cell responses upon immunization (Sapoznikov et al., 2007) and have been implicated in thymic negative selection (Brocker et al., 1997). However, recent studies using DC-less mice showed that both thymic and peripheral T cell repertoire is not affected by the loss of DCs (Birnberg et al., 2008). Nevertheless, these experiments confirmed the importance of splenic and thymic DCs for inducing mixed lymphocyte alloreactions and the formation of antiviral T cell immune responses (Birnberg et al., 2008). Indeed, ovalbumin-specific T cells did not react to systemic antigen in spleens upon depletion of DCs but, surprisingly, these T cells reacted strongly in the LNs to subcutaneous immunizations with antigen, despite a lack of DCs (Birnberg et al., 2008). This DC-independent T cell activation in the LNs may explain the efficient MOG peptide-specific T cell activation in DC-less mice reported in this paper. Our experiments, in which both DCs and pDCs were depleted, imply the possibility that cells other than DCs act as primary APCs in the LNs when DCs are absent. B-cells and macrophages are both potential candidates. In the case of B-cells, it has been shown that they are not mandatory for EAE induction with the MOG peptide, as B-cell-deficient animals develop a severe chronic form of EAE (Fillatreau et al., 2002; Wolf et al., 1996). These data either point to a strong redundancy between the distinct DC populations or the presence of another (so far) undiscovered $CD11c^-$ APC being the main priming cell type in EAE. Formally, however, we cannot exclude that the very low residual numbers of DCs are sufficient to prime T cells in LNs.

A recent paper showed that $CD103^+$ CD207 (langerin)$^+$ dermal DCs are crucial for EAE induction (King et al., 2010). These cells are MHC class $II^{high}$ and CD11c high to intermediate and are present in both the dermis and in the draining lymph nodes.

Our lymph node staining show near complete absence of $MHC^{high}/CD11c^{+}$ cells in DC-less mice. Moreover, a careful examination of a skin (including both epidermis and dermis) from the CD11c-DTR BM chimeras, revealed nearly complete loss of these dermal $CD103^{+}$ $CD207^{+}$ DCs without influencing the host-derive radio-resistant epidermal Langerhans cells. The disparity of EAE development in the two models could be explained by the concurrent depletion of tolerogenic steady state DCs in our system, which may lower the threshold for EAE induction and therefore circumvent the need for $CD103^{+}$ $CD207^{+}$ dermal DCs in EAE induction. In line with this, our DC-less CD11c/iDTR mice showed an absence of $CD8^{+}$ DCs, which are most notably implicated in tolerance induction (Yamazaki et al., 2008).

Similarly, as previously described (Birnberg et al., 2008), we found enlargements of the spleen and LNs in aged CD11c/DTA mice (not shown), but not a strong autoimmune lymphoproliferative infiltration with a fatal autoimmune syndrome, as reported by others (Ohnmacht et al., 2009). This distinct outcome may be explained by different hygienic housing conditions, as our data point to a strong role of DCs in the formation of adaptive Tregs, which may be crucial in controlling the immune response against viral or bacterial infections. Naïve, non-immunized CD11c/DTA mice harbor an unimpaired thymic Treg compartment and similar numbers of splenic Tregs as WT animals. Here we show that upon immunization, CD11c/DTA as well as DT treated BM chimeric CD11c-DTR mice display a reduced number of Tregs in the spleen in comparison to WT animals. We think that this finding indicate a role of DCs in the induction of regulatory T cells. This is in line with previous findings that immunization induces a substantial amount of novel antigen-specific Tregs (Korn et al., 2008; Kretschmer et al., 2005; Wang et al., 2010). Indeed, when we transferred naïve T cells before immunization we observed a strong reduction of iTreg differentiation in the absence of DCs. Our *in vitro* Treg induction experiments and *in vivo* analysis of Foxp3 expression fully support this role of DCs, since naïve T cells differentiated considerably better into $Foxp3^{+}$ cells in the presence of DCs. The iTregs that developed in the presence of DCs also expressed higher Foxp3 levels than those that developed in the absence of DCs. High

expression of Foxp3 is shown to promote a full Treg program and -an enhanced suppression profile by Tregs (Williams and Rudensky, 2007) Together, these data suggest that Tregs depend on DCs for full phenotypic and functional development.

When we transferred naïve antigen-specific TCR transgenic T cells to immunized CD11c/DTA mice, we discovered that they did not upregulate PD-1 expression when compared with cells transferred to control mice, in spite of a strong and even enhanced proliferative response. These findings suggest that DCs are involved in the programming of gene expression in T cells in a unique manner, *i.e.*, not shared by other APCs. Although we do not fully understand the precise mechanism by which DCs induce tolerance, prime candidates are distinct co-stimulatory pathways or cytokine milieus in the presence of DCs. In light of data reported by Siffrin et al. (unpublished), signaling via CD80/CD86 may be an important feature of DC priming leading to PD-1 upregulation. Upon PD-1 cell surface expression, signaling via PD-L1 and PD-L2 is enhanced, leading to a primary tolerogenic program and differentiation of antigen-specific T cells to $Foxp3^{+}$ regulatory cells. We found that DCs express high levels of PD-L1, indicating that one major PD-L1 expressing population vanishes upon depletion of DCs in immunized animals. A recent publication (Amarnath et al., 2010) shows in a xenogenic transfer system that the transfer of human DCs, which were pre-incubated with Tregs, induced the upregulation of PD-1 on T-effector cells. Due to the fact that this depended on PD-L1 being expressed on these tolerogenic DCs, it is conceivable that PD-L1 signaling in a tolerogenic positive feedback loop leads to PD-1 upregulation on T cells.

Here, we demonstrate that EAE can be induced in the absence of classical DCs. Furthermore our data confirm that DCs are important tolerance mediators for CD4 T cell responses. Given that significant numbers of $PD\text{-}L1^{high}$ DCs are found in the spleen, we favor the following scenario: T cells are first primed by DCs or other cells in the draining LNs. After priming, T cells migrate to the spleen and are modulated there by DCs. Depending on the initial priming strengths, this second phase of modulation dictates whether immunity or tolerance prevails.

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# 6 Summary

This thesis focuses on different aspects of immune regulation, both at the cellular and molecular levels. More specifically, this work concentrates on the importance of Interleukin-10, B and T Lymphocyte Attenuator (BTLA), and dendritic cells in respect to immune regulation, with special emphasis on autoimmunity.

In this thesis, we show that the cellular source of IL10 production can dramatically influence the outcome of an autoimmune response. We show that T cell-derived IL10 plays an important role in controlling the viability of recently activated T cells, allowing them to become fully functional T effector cells. T cell-specific IL10-deficient mice failed to induce EAE when immunized with MOG peptide. Furthermore, when re-challenged with MOG or other stimuli, these T cells exhibited increased apoptosis rates.

Here we report for the first time the generation of a novel mouse model that allows the conditional over-expression of BTLA. We show that BTLA can negatively regulate $CD4^+$ T cells responses, when expressed by the T cells themselves. BTLA over-expression by $CD8^+$ T cells or dendritic cells, however, resulted in enhanced viral clearance.

In this study, we show that depletion of DCs, either early on from birth or later in adulthood, does not prevent EAE induction, but instead leads to a lower state of tolerance and stronger immune response. We also show that DCs are responsible for the upregulation of PD-1 on antigen-specific T cells and subsequently induce the formation of Tregs during immune responses.

# 7 Zusammenfassung

Diese Arbeit behandelt verschiedene Aspekte der Immunregulation auf zellulärer und molekularer Ebene. Dabei liegt der Fokus auf der Rolle des Cytokins Interleukin 10, des Proteins BTLA (B and T Lymphocyte Attenuator) als auch der Funktion von dendritischen Zellen in der Regulation der Immunantwort und im speziellen der Autoimmunantwort.

In der vorliegenden Arbeit zeigen wir, dass IL10 einen großen Einfluss auf den Ausgang einer Autoimmunantwort hat. T-Zell-sekretiertes IL10 reguliert die Viabilität kürzlich aktivierter T Zellen und ermöglicht deren Entwicklung zu funktionalen Effektor T Zellen. Mäuse, deren T Zellen kein IL10 produzieren können zeigten sich resistent gegen EAE (Experimentelle autoimmune Enzephalomyelitis, ein Modell für Multiple Sklerose) nach einer Immunisierung mit dem MOG-Peptid. Nach erneuter Verabreichung des MOG-Peptids oder eines anderen Stimulus, zeigten die T Zellen eine erhöhte Apoptoserate.
In dieser Arbeit wird ebenfalls ein neues Mausmodell vorgestellt, dass eine konditionale Überexpression des Proteins BTLA ermöglicht. Wir zeigen, dass BTLA einen negativen autoregulatorischen Einfluss auf die CD4 T-Zellantwort hat. BTLA-Überexpression in CD8 T Zellen oder denritischen Zellen führte zu einer beschleunigten Virusbekämpfung (viral clearance).

Des Weiteren zeigen wir, dass die Depletion von dendritischen Zellen von Geburt an oder auch zu einem späteren Zeitpunkt EAE nicht verhindert, sondern sogar eine niedrigere Toleranz und stärkere Immunantwort verursacht. Wie liefern weitere Beweise dafür, dass dendritische Zellen die PD-1 Expression auf antigen-spezifischen T-Zellen induzieren und somit die Bildung regulatorischer T Zellen während einer Immunantwort herbeiführen.

# 8 Acknowledgements

# 9 Lebenslauf

# 10 Publications

**Smad7 in T cells drives T helper 1 responses in multiple sclerosis and experimental autoimmune encephalomyelitis.**
Kleiter I, Song J, Lukas D, Hasan M, Neumann B, Croxford AL, Pedré X, Hövelmeyer N, Yogev N, Mildner A, Prinz M, Wiese E, Reifenberg K, Bittner S, Wiendl H, Steinman L, Becker C, Bogdahn U, Neurath MF, Steinbrecher A, Waisman A.
Brain. 2010 Apr;133(Pt 4):1067-81. Epub 2010 Mar 30

**Nonredundant roles for B cell-derived IL-10 in immune counter-regulation.**
Madan R, Demircik F, Surianarayanan S, Allen JL, Divanovic S, Trompette A, Yogev N, Gu Y, Khodoun M, Hildeman D, Boespflug N, Fogolin MB, Gröbe L, Greweling M, Finkelman FD, Cardin R, Mohrs M, Müller W, Waisman A, Roers A, Karp CL.
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**A transgenic mouse model of inducible macrophage depletion: effects of diphtheria toxin-driven lysozyme M-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes.**
Goren I, Allmann N, Yogev N, Schürmann C, Linke A, Holdener M, Waisman A, Pfeilschifter J, Frank S.
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Waisman A, Yogev N.
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Frommer F, Heinen TJ, Wunderlich FT, Yogev N, Buch T, Roers A, Bettelli E, Müller W, Anderton SM, Waisman A.
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Vallon-Eberhard A, Landsman L, Yogev N, Verrier B, Jung S.
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Kang TB, Ben-Moshe T, Varfolomeev EE, Pewzner-Jung Y, Yogev N, Jurewicz A, Waisman A, Brenner O, Haffner R, Gustafsson E, Ramakrishnan P, Lapidot T, Wallach D.
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# 11 Erklärung