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Influence of toll-like receptor ligands on the generation and function of regulatory T cells

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"There are only two ways to live your life:

One is as though nothing is a miracle. The other is as though everything is a miracle." (Albert Einstein)

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ABBREVIATIONS

α	anti
α	alpha
ABTS	2,2´-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
AhR	aryl hydrocarbon receptor
ANA	anti-nuclear antibodies
AP-1	adaptor-protein 1
APC	antigen-presenting cell
APC	allophycocyanin
ATRA	all-trans retinoic acid
Bcl 6	B cell lymphoma 6 protein
BCR	B cell receptor
bp	base pair
BSA	bovine serum albumin
°C	centigrade
Ca	calcium
CD	cluster of determination
cDC	conventional dendritic cell
cDNA	complementary DNA
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLR	c-type lectin receptors
c-Maf	avian musculoaponeurotic fibrosarcoma v-maf
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DNMT	DNA methyltransferase

Dotap	N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propan methylsulfat
ds	double stranded
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveoretinitis
EDTA	ethylenediaminetetraacetic acid
e.g.	for example (exempli gratia)
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium monoazide
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
Flt3-L	FMS-like tyrosine kinase 3 ligand
FL-DC	Bone marrow derived DCs generated with Flt3-L
Foxp3	Forkhead box P3
GATA-3 (gene: *gata3*)	GATA binding protein 3
GC	germinal center
GITR	glucocorticoid-induced TNFR-related protein
gp130	glycoprotein 130
h	hour
HCl	hydrogen chloride
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
H_2O	dihydrogen monoxide
HRP	horseradish peroxidise
IC	immune complex
IFN	interferon
Ig	immunoglobulin

IκB	inhibitor of NF-κB
IKK	inhibitor of κB kinase
IL	interleukin
IL-1R	interleukin 1 receptor
i.p.	Intraperitoneal
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
iTreg	induced regulatory T cell
i.v.	intravenous
kb	kilobase
KO	knockout
LL37	cathelicidin anti-microbial peptide LL37
LN	lymph node
LRR	leucine rich repeat
LPS	lipopolysaccharide
M	molar
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinases
MD-2	lymphocyte antigen 96
MFI	mean fluorescence intensity
µg	microgram
mg	milligram
Mg	magnesium
MHC	major histocampatibility complex
min	minute
ml	millilitre
mLN	mesenteric lymph node
mM	millimolar
MS	multiple sclerosis
MyD88	myeloid differentiation primary response gene 88

n	number of replicates
N	normal
NaOH	sodium hydroxide solution
NAP	NAK-associated protein 1
NEAA	non essential amino acids
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κB
ng	nanogram
NK	natural killer cell
NLR	NOD-like receptors
nm	nanometer
nt	nucleotide
nTreg	natural regulatory T cell
ODN	oligodeoxynucleotide
OVA	ovalbumin
P	phosphorylated
p	peptide
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	R-phycoerythrin
PE-Cy5	R-phycoerythrin-cyanine dye 5
PerCP-Cy5.5	Peridinin chlorophyll protein-cyanine dye 5.5
PFA	Paraform aldehyde
PI	propidium iodide
Pristane	2,6,10,14-Tetramethylpentadecane
PRR	pattern recognition receptor
qRT-PCR	quantitative RT-PCR

R	receptor
RA	rheumatoid arthritis
RIP 1	receptor interacting protein 1
RLR	retinoic acid inducible (RIG)-I-like receptors
RNA	ribonucleic acid
RORγt (gene: *rorc*)	RAR-related orphan receptor γt
rpm	revolutions per minute
RT	room temperature
s	soluble
sec	seconds
SD	standard deviation
SDS	sodium dodecyl sulphate
SINTBAD	similar to NAP1 TBK1 adaptor
SLE	systemic lupus erythematosus
Sm	Smith protein
snRNP	small nuclear ribonucleoprotein
ss	single stranded
STAT	signal transducer and activator of transcription
strep	Streptavidin
TAE	Tris-acetate-EDTA
TAB	TAK1-associated protein
TAK1	TGF-β activated kinase 1
T-bet (gene: *tbx21*)	T-box transcription factor 21
TBK	TANK binding kinase
TCR	T cell receptor
T_{FH}	T follicular helper cell
TGF-β	transforming growth factor beta
TI	T cell independent
TIR	Toll/IL-1R homology domain
TIRAP (Mal)	TIR domain containing adaptor protein
T_H	T helper cell
TLR	Toll-like receptor

TMB	3,3´,5,5´-Tetramythylbenzidine
TNF	Tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF related adaptor molecule
Treg	regulatory T cell
Tresp	responder T cell
TRIF	TIR domain containing adaptor inducing IFN-β
U	units
Ub	ubiquitin
Ubc13	E2 ubiquitin conjugating enzyme 13
Uev1A	ubiquitin-conjugating enzyme E2 variant 1A
UNC93B1	revolutions per minute
v/v	volume per volume
w/o	without
wt	wildtype
w/v	weight per volume

1 INTRODUCTION

The immune system is a complex defense system consisting of different organs, cell populations and molecules interacting with each other to defend the host against pathogens, for example viruses and bacteria. It is divided into the innate and the adaptive immune response and these two arms of the immune system interact with each other to ensure an effective defense. While the innate immune system is a conserved system and the first line of defense against invading pathogens, the adaptive immune system consisting of T and B cells comes into operation at a later time-point and starts a highly pathogen-specific attack against the invasion and generates long-lasting immunological memory.
Secondary to the effective defense against pathogens it is also necessary that the immune response is under tight control to prevent the development of autoimmune diseases. However, these mechanisms are not always effective and so autoimmune diseases such as systemic lupus erythematosus (SLE) can develop.

1.1 Dendritic Cells

1.1.1 The Role of Dendritic Cells in the Immune System

Dendritic cells (DC) are an important part of the immune system because they hold a key position between the innate and adaptive immune system. They represent a distinct lineage of mononuclear phagocytes and are highly specialised in antigen presentation and initiation but also control of immunity. While one major responsibility of DCs is the induction and regulation of highly pathogen-specific adaptive immune responses they also hold a central role in the development of immunological memory and tolerance. The group of DCs is divided into conventional DCs (cDC) and plasmacytoid DCs (pDC) (Geissman et al, 2010). PDCs circulate through blood and lymphoid tissues and reside in the bone marrow and all peripheral organs. They can function as antigen-presenting cells (APC) to control T cell responses but their main role is to respond to viral infection with a massive production of type I Interferon (IFN) which sets neighbouring cells in an antiviral status and protects them against infection (Geissman et al, 2010). There is also an upregulation of other

proinflammatory cytokines like tumor necrosis factor (TNF-) α, Interleukin (IL-) 6 and IL-12 (Krug et al, 2001). This response is possible through the expression of toll-like receptors (TLR) 7 and 9 which recognize viral RNA and DNA (Hornung et al, 2002). The combined effect of the cytokines produced by pDCs ultimately leads to an increase in the cytotoxic activitiy of natural killer (NK) cells, promotion of plasma cell differentiation, increased antibody production and the recruitment of activated T cells and macrophages to the site of inflammation (Avalos et al, 2010; Blanco et al, 2008; Colonna et al, 2004; Kadowaki et al, 2002). Studies show that beside the beneficial effect of type I IFN against viral infection there are several systemic autoimmune diseases like SLE which display a prominent type I IFN gene signature within peripheral blood mononuclear cells (PBMC) and this may be important in their pathogenesis (Eloranta et al, 2009; Hagberg et al, 2011, Savarese et al, 2006).

Conventional DCs are highly specialized APCs and can produce cytokines like IL-6 and IL-12 among others upon activation. They are further divided into migratory and blood-derived or resident DCs. Migratory DCs reside in peripheral tissues in an immature state which is characterized through low expression of major histocompatibility complex (MHC) class I and II and T cell co-stimulatory molecules on the cell surface. They are specialized for sampling the environment for foreign antigens. They are also equipped with a series of receptors for pathogen-assaciated molecular patterns (PAMP) e.g. TLRs as well as cytokine and chemokine receptors. Signals through these receptors trigger DC migration towards the T and B cell zones of secondary lymphoid organs although migration happens independently from pathogens and TLR signalling. There they develop a mature state which is defined by high expression levels of MHC and co-stimulatory molecules and present antigens captured in the periphery on their surface to T cells (Geissmann et al, 2010; Turnbull et al, 2001; Villadangos et al, 2007).

Resident DCs which constitute 50% of all lymph node (LN) DCs and all DCs in thymus and spleen are ideally located to monitor the blood for infection. They develop in the lymphoid organs from bone-marrow precursors without trafficking through peripheral tissues first and exhibit an immature phenotype. Therefore they maintain their immature phenotype their entire lifespan under steady state conditions. In response to pathogen-associated stimuli or endogenous inflammatory molecules released from damaged tissues the resident DCs acquire a mature phenotype characterized by expression of high levels of costimulatory and MHC class I and MHC class II molecules (Villadangos et al, 2007).

In addition to the high capacity of DCs to capture, process and present antigens on their surface, a prerequisite for T cell priming, they also have an important role in establishing tolerance against self antigens. In the thymus they present self antigens to developing thymocytes which leads to negative selection of CD4+ and CD8+ thymocytes (Proietto et al, 2008). Furthermore, thymic DCs contribute to the induction of regulatory T cells (Treg). Although the elimination of autoreactive T cells in the thymus is very efficient some T cells still escape thymic selection and enter the periphery. There they are held in check by peripheral tolerance mechanisms which are also primarily elicited by DCs (Proietto et al, 2008; Villadangos et al, 2007).

1.1.2 Dendritic Cell Subsets in the Murine Spleen

Table 1: DC subtypes in the murine spleen

Nomenclature	CD8α+ DCs	CD8α- DCs	pDC
Phenotype	CD11c+ Class II MHC high CD8α+ DEC205+ CD11b low/- CD4- CD86+ CD40+ B220- 120G8-	CD11c+ Class II MHC high CD8α- DEC205- CD11b+ CD4- or CD4+ CD86+ CD40+ B220- 120G8-	CD11c low Class II MHC low CD8α+ or CD8α- DEC205+ or DEC205- CD11b- CD4+ CD86 low CD40 low B220+ 120G8+
Localization in the spleen	T cell zones	Marginal zones of spleen, Move to T cell zones, when activated	Marginal zones of spleen, T cell zones
Function			
Antigen capture	+	+	+
Antigen processing	++	++	+
TLR expression	TLR1+, 2+, 3++, 4+, 5-, 6+, 7-, 8+, 9+	TLR1+, 2+, 3-, 4+, 5+, 6+, 7+, 8+, 9+	TLR1+, 2+, 3-, 4-, 5-, 6+, 7+, 8+, 9+
IL-6 secretion	+	+	+
IFN-α secretion	+	-	++++
IL-12p70 secretion	++++	+/-	-
CD4+ T cell priming	++	++++	+
CD8+ T cell priming	++++	+	++
Cross-presentation	++++	+/-	+/-
CTL priming	++	+++	?
Tolerance	+++	+/-	+

IFN: Interferon; CTL: cytotoxic T lymphocyte

(Data from Pulendran, 2004; Villadangos and Young, 2008)

In the murine spleen pDCs and cDCs reside. All murine DCs express CD11c, although pDCs express CD11c to a lower extent than cDCs. Through the expression of different cell surface markers different subsets of DCs can be distinguished. PDCs on the one hand can be identified through the expression of for example 120G8 and B220 and can be either CD8+ or CD8- (Karsunky et al, 2005). On the other hand cDCs in the spleen can be divided into CD8+ CD4- CD11b- DCs, CD8- CD4+ CD11b+ DCs and CD8- CD4- CD11b+ DCs. The DC subtypes differ in the expression of cell surface markers, TLRs, cytokine secretion and their intrinsic ability to capture and process antigens (see Table 1) (Liu, 2001; Pulendran, 2004; Shortman et al, 2002; Villadangos et al, 2007).

1.2 B Cells

B and T cells together form the adaptive immune system which is highly specialized in specific antigen recognition and is set in motion when the innate immune system is not sufficient to clear the infection. T cells are responsible for cell-mediated immunity while B cells take responsibility for the humoral immune response.
B cells derive from the bone-marrow and as mature B cells recirculate regularly through secondary lymphoid organs in search of signs of infection. On their surface they carry cell-surface immunoglobulin, the B cell receptor (BCR), which is specific for antigen. After antigen encounter, B cells initially congregate at the boundary between B cell follicles and T cell areas in search of T cell help. Encounters with T cells and engagement of CD40/CD40L are a prerequisite for initial B cell proliferation and germinal center (GC) formation. In GCs activated B cells undergo clonal expansion, class switch recombination, affinity maturation and B cells differentiate into memory or effector B cells. Effector B cells differentiate into plasma cells and produce specific antibodies (Fillatreau, 2011; Gatto and Brink, 2010; Gonzales et al, 2011; Kurosaki, 2011).

Since B cells also express non-specific pattern recognition receptors (PRR), especially TLRs, they can also be activated in a T cell-independent (TI) manner. In this case intrinsic TLRs cooperate with adaptive immunoglobulin (Ig) receptors to achieve a rapid humoral immunity and preserve long-term memory (Cerutti et al, 2011; Green and Marshak-Rothstein, 2011). The TI pathway is especially important in the activation of autoreactive B cells in the

periphery. These cells have escaped both selection checkpoints in bone-marrow and spleen and are responsive to self antigen. Although they often express low affinity receptors and are therefore ignorant B cells, it is possible to activate them through the combination of a weak BCR signal and signals from additional receptors like TLRs. This leads to autoantibody production and ultimately autoimmune disease (Gonzales et al, 2011; Green and Marshak-Rothstein, 2011).
In addition to the production of antibodies B cells are also able to act as APCs to T cells and they secrete proinflammatory cytokines like IL-6 (Dienz and Rincon, 2009; Gatto and Brink, 2010).

1.3 T Cells

T cells are responsible for the highly specific cell-mediated immune response and contribute to long-lived immunological protection through the rise of memory T cells. Depending on the T cell receptor (TCR) chains T cells are divided into two distinct lineages: γ:δ T cells and α:β T cells. In this manuscript, the term T cell refers to α:β T cells only.
Although T cell progenitors originate in the bone marrow like B cells, all important stages of their development and selection occur in the thymus. T cell precursors pass through various stages marked by changes in the status of TCR genes and TCR expression and also by changes in the expression of other surface proteins like the CD3 complex and the co-receptor proteins CD4 and CD8 until they emerge as either CD4 or CD8 single positive T cell into the periphery (Borowski et al, 2002). Although the selection processes for TCRs are effective, central tolerance has its limitations and it is possible for T cells with autoreactive TCRs to enter the periphery. Under normal conditions these cells are under control of peripheral tolerance mechanisms like anergy or are even eliminated (Steinman and Nussenzweig 2002). However, these mechanisms can also fail and autoreactive T cells play an important role in the development of autoimmune diseases.

Both CD4+ and CD8+ T cells consist of different subsets and have different functions in the defense against pathogens. On the one hand, CD8+ T cells recognize peptides bound to MHC class I molecules. These peptides originate from intracellular cytosolic proteins and can be self-antigens, in which case CD8+ T cells are not activated due to tolerance mechanisms, or foreign mainly due to viral infections. Upon antigen recognition, the CD8+ T cells proliferate rapidly and differentiate into short-lived effector cytotoxic T lymphocytes (CTLs), which

have the ability to kill the infected target cell in a perforin and granzyme dependent manner, or they differentiate into induced Tregs or long-lived CD8+ memory T cells (Cui and Kaech, 2010; Pomié et al, 2008).

On the other hand, CD4+ T cells recognize peptides bound to MHC class II molecules. MHC class II molecules present peptides derived from extracellular proteins which are endocytosed, and processed (Lippolis et al, 2002). After antigen recognition and depending on the surrounding cytokine milieu, activated naïve CD4+ T cells differentiate into different T helper subsets with different responsibilities (see 1.3.1). Therefore, CD4+ T cells play a central role in the function of the immune system by orchestrating immune responses against a wide variety of pathogens, by providing help to other immune cells like B cells and CD8+ T cells and by regulating immune responses to adjust the magnitude and to prevent autoimmunity. In addition to that they are important mediators of immunologic memory (Ahlers and Belyakov, 2010; Zhu et al, 2010).

1.3.1 CD4+ T helper Cell Lineages

After activation of naïve CD4+ T cells through the TCR complex and co-stimulatory molecules the T cells can differentiate into different T helper cell lineages depending on the cytokine milieu and the intensity of the activation signals. All T helper cell lineages have a share in the smooth operation of an adequate immune response although they cover different domains. They can be discriminated by the cytokines they produce and the transcription factors they specifically express and which determine the T cell fate. At the moment there are five distinct T helper cell lineages identified in mice that contribute to immune defense and inflammation – T_H1, T_H2, T_H9, T_H17 and T follicular helper cells (T_{FH}). In addition to that there are three T helper cell lineages that have regulatory and immunosuppressive functions and contribute to tolerance. These are called T_H3, Tr1 and forkhead box P3 (Foxp3)+ Tregs. (Coghill et al, 2011; Corsini et al, 2011; Curotto de Lafaille and Lafaille, 2009; Jäger and Kuchroo, 2010). Figure 1 shows an overview of all known T helper cell lineages in the mouse, the main cytokines responsible for the differentiation of T cells into a distinct lineage and the respective signature transcription factors, the roles they play in the immune system and the main cytokines they produce.

The first T helper cell lineages that were identified were T_H1 and T_H2 cells. T_H1 cells promote the cellular responses that mediate immune defense against intracellular bacteria, viruses and tumors, and they are associated with activation of the M1 macrophage phenotype, support induction of CD8+ CTLs, memory formation and NK cell lysis. As effector cytokine they secrete mainly IFN-γ, but also TNF-α and lymphotoxin. The differentiation of T_H1 cells depends on signal strength and proinflammatory cytokines, such as IL-12 and type I IFNs. These cytokines trigger signal transducer and activator of transcription (STAT) 4 activation which leads to the expression T-box transcription factor 21 (T-bet). T-bet is the T_H1 cell-specific transcription factor controlling IFN-γ production as well as conferring higher IL-12 responsiveness, thus stabilizing the T_H1 phenotype (Ahlers and Belyakov, 2010; Zhu et al, 2009). Activated T_H1 cells up-regulate CD40L which triggers CD40 signalling in DCs to further enhance the production of inflammatory cytokines, especially IL-12, and upregulation of co-stimulatory molecules. This step is central to the initiation of cell-mediated immune response (Kapsenberg, 2003; Murugaiyan et al, 2007; Ruedl et al, 2000). Detrimental effects of T_H1 responses happen when systemic activation promotes pathological consequences and autoimmune disease (Ahlers and Belyakov, 2010).

T_H2 cells are generated by activation via the TCR and IL-4 receptor. Through binding of the cytokine IL-4 to its receptor the transcription factor STAT6 is phosphorylated (pSTAT). pSTAT6 plays a critical role in the induction of the T_H2 signature transcription factor GATA binding protein 3 (GATA3) which ultimately leads to the expression of the T_H2 specific cytokines IL-4, IL-5 and IL-13 while simultaneously downregulating STAT4 and IL-12-receptor-β3 (IL-12Rβ3). T_H2 cells are important for clearing extracellular organisms like parasites and helminthes and providing B cell help for the production of antibodies. However, the involvement of T_H2 cells can also have detrimental effects for the host because it was shown that they are critically involved in eosinophilic inflammation and IgE production in allergic reactions and asthma. In addition to that, in the systemic autoimmune disease SLE T_H2 cells provide help for autoreactive B cells in autoantibody production (Jäger and Kuchroo, 2011; Murphy and Reiner, 2002; Zhu et al, 2009).

T_H9 cells are also involved in the expansion of B cells, in helminth infections, asthma and allergy and were long considered an IL-9 producing subset of T_H2 cells. Now, because they do not express the transcription factor GATA3, they are considered an independent T helper cell lineage although a signature transcription factor is not found yet. T_H9 cells differentiate when IL-4 in combination with transforming growth factor (TGF)-β is present in the

environment. Fully differentiated they predominantly produce IL-9 and IL-10. In experiments it was shown, that T_H9 cells can induce the development of colitis, induce peripheral neuritis and experimental autoimmune encephalomyelitis (EAE) (Dardalhon et al, 2008; Jäger et al, 2009).

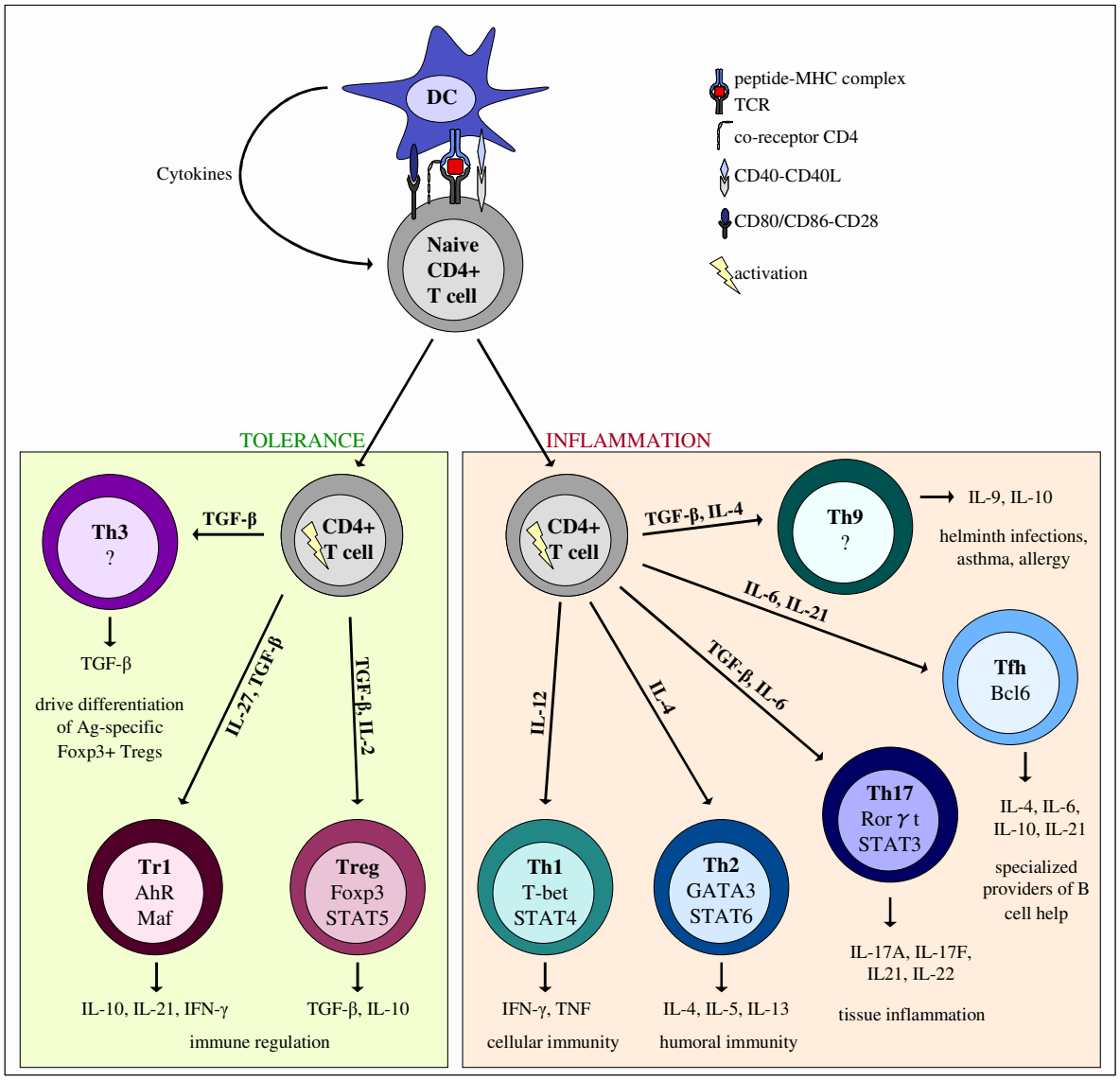

Figure 1: Differentiation of T helper cell subsets
The activation and differentiation process of a naïve CD4+ T cell to effector T helper cell requires three signals that are provided by mature DCs. The first signal is provided through the interaction of the TCR with the peptide-MHC class II complex on the DC cell surface. Interaction of the co-receptor CD4 with the β_2 domain of the same MHC class II molecule stabilizes TCR binding and boosts the activation signal. The second signal is provided through the interaction of co-stimulatory molecules on T cells and DCs like CD28-CD80/CD86 or CD40L-CD40 which is necessary for the T cell to not become anergic. Signal number 3, provided by different cytokines or cytokine combinations, determines the polarization of the naïve T cell depending on the cytokine milieu. Each T helper cell lineage can be identified by the expression of signature transcription factors which determine the T cell fate.

T_{FH} cells are important for the formation and maintenance of GCs. There they regulate germinal center B cell differentiation into plasma cells and memory B cells. For this reason

they produce mainly IL-4, IL-6, IL-10 and IL-21 which are all cytokines that induce and enhance plasma cell differentiation. T_{FH} cell development is driven by the transcriptional repressor B cell lymphoma 6 protein (Bcl6), though the exact mechanism is not understood yet (Coghill et al, 2011; Crotty, 2011).

The last T helper cell lineage important for immunity known to date is the T_H17 cell lineage. These cells produce mainly IL-17A and F, but also IL-21 and IL-22, and are characterized by the expression of the transcription factors RAR-related orphan receptor (ROR-) γt and STAT3. It was found that the minimal requirements for the differentiation of murine T_H17 cells are a combination of the cytokines TGF-β and IL-6 which promote RORγt and STAT3 expression. The differentiation process is supported by IL-1 and IL-21 while IL-23 supports T_H17 cell expansion and maintenance (Chung et al, 2009; Coghill et al, 2011; Dong, 2011; Veldhoen et al, 2006). T_H17 cells play an important role during immune responses against extracellular bacteria and fungi and in tissue inflammation. They are also linked to several autoimmune diseases like multiple sclerosis (MS), rheumatoid arthritis (RA) and SLE (Apostolidis et al, 2011; Bettelli et al, 2008; Siffrin et al, 2010).

Tr1 cells, T_H3 cells and induced Foxp3+ Tregs can also be generated from naïve CD4+ T cells depending on the cytokine milieu and are all known to promote tolerance. Both Tr1 and T_H3 cells are Foxp3- (Curotto de Lafaille and Lafaille, 2009) and need TGF-β for their differentiation. For T_H3 cell differentiation, TGF-β alone is enough and mature T_H3 cells produce TGF-β themselves to suppress immune responses and support the differentiation of antigen-specific Foxp3+ Tregs (Carrier et al, 2007).
Tr1 cells also need IL-27 in addition to TGF-β to successfully differentiate and develop full suppressive function. IL-27, which is produced by tolerogenic DCs, induces three different pathways during Tr1 cell differentiation. First, IL-27 promotes T-bet expression resulting in IFN-γ secretion which leads to an enhanced IL-27 expression by DCs. Second, IL-27 activates the expression of the transcription factors avian musculoaponeurotic fibrosarcoma v-maf (c-Maf) and the aryl hydrocarbon receptor (AhR) which in turn drive together the expression of IL-21 (maintaining c-Maf and AhR expression) and IL-10. Third, IL-27 induced AhR alone or together with an unknown cofactor leads to Granzyme-B expression. IL-10 and Granzyme-B are both essential for the suppressive function of Tr1 cell: IL-10 acts in a cell contact-independent manner by suppressing proliferation, Granzyme-B mediates the cell contact-

dependent suppressive activity (Curotto de Lafaille and Lafaille, 2009; Peterson, 2012; Pot et al, 2011).
The induced CD4+Foxp3+ Tregs will be discussed in detail under point 1.3.2

1.3.2 Natural and Induced CD4+Foxp3+ Tregs

In the murine system CD4+ Tregs are primarily characterized by the expression of Foxp3, a member of the forkhead/winged-helix family of transcription factors. Foxp3 acts as a "master" regulator for the development of Tregs and their suppressive function. Mutation or deficiency in the *foxp3* gene causes severe autoimmune disorders through uncontrolled cytokine secretion and proliferation of CD4+ T lymphocytes and other cells. Thus, the suppressive and immunomodulatory function of CD4+Foxp3+ Tregs is crucial to reduce inflammation induced by pathogens and to prevent autoimmunity (Lal and Bromberg, 2009; Peterson, 2012).
The complete peripheral Foxp3+ Treg pool consists of two populations which differ in their developmental pathways. The first population are the so called natural CD4+Foxp3+ Tregs (= nTreg) who originate from the thymus and are important for central and peripheral tolerance mechanisms. They develop from self-reactive T cells after MHC class II dependent TCR activation that result in high-avidity selection and are able to suppress other self-reactive T cells in the periphery which escaped negative selection in the thymus. Thymic DCs and thymic epithelial stromal cells play an important role in the successful generation of nTregs (Curotto de Lafaille and Lafaille, 2009; Maggi et al, 2005; Proietto et al, 2008).

The second population is generated in the periphery from antigen-specific naïve CD4+ T cells and these cells are called induced CD4+Foxp3+ Tregs (= iTregs). The differentiation into iTregs takes place when naïve CD4+ T cells are activated via the TCR and co-stimulatory molecules after antigen exposure and in the presence of TGF-β and IL-2 with TGF-β being the driving factor (Curotto de Lafaille and Lafaille, 2009; Yao et al, 2007). Both IL-2 and TGF-β signalling pathways induce Foxp3 expression, TGF-β via phosphorylation of the intracellular proteins Smad2 and Smad3 which enables translocation into the nucleus and induction of Foxp3 transcription (Maruyama et al, 2011; Yoshimura et al, 2010) and IL-2 via activation of the Janus kinase Jak3 and subsequent phosphorylation of STAT5. STAT5 is both crucial for Foxp3 expression and Treg cell maintenance because it directly binds to the *foxp3*

gene (Yao et al, 2007). Furthermore, TGF-β inhibits the expression of DNA methyltransferases which is important for Foxp3 expression (Lal et al, 2009).
Although these two Treg populations develop in different locations, they have a very similar phenotype as both express the Treg signature surface molecules CD25, cytotoxic T-lymphocyte-associated antigen (CTLA)-4, glucocorticoid-induced TNFR-related protein (GITR) (Maruyama et al, 2011) and therefore it is difficult to discriminate the populations from each other. But despite their similar phenotype the two populations differ in their functional stability as nTregs are far better in retaining Foxp3 expression and hence Treg phenotype and function under a proinflammatory environment than iTregs (Lal and Bromberg, 2009; Yao et al, 2007; Zhou (1) et al, 2009). This phenomenon can be attributed to the epigenetic regulation of Foxp3 in the two populations. Since methylation of CpG residues respresses Foxp3 expression, complete demethylation of the *foxp3* gene is necessary for stable Foxp3 expression. It was shown that nTregs and iTregs differ in the methylation of CpG dinucleotides: they are completely demethylated in nTregs but not in iTregs rendering Foxp3 expression less unstable (Lal and Bromberg, 2009).

1.4 Toll-like Receptors

PRRs are a part of the innate immune system and mediate the initial sensing of an infection since they are able to recognize defined PAMPs to initiate and regulate innate and adaptive immune responses. There is also evidence that PRRs are able to recognize damage-associated molecular patterns (DAMPs) that are endogenous molecules released from damaged cells. At the moment four different PRR families have been identified: the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the NOD-like receptors (NLRs) (Kawai and Akira, 2009; Takeuchi and Akira, 2010).

1.4.1 Structure, Localization and Ligands of Toll-like Receptors

The family of TLRs is one of the best characterized PRR families at the moment. TLRs are widely expressed by both professional immune cells like DCs and other cell types like endothelial cells and fibroblasts (see Table 2).

Table 2: Toll-like receptors and their ligands

TLR	LOCALIZATION	LIGAND	ORIGIN OF LIGAND	MAJOR CELL TYPES
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria	Macrophages, cDC, neutrophil, mast cells, T and B cells, endo/epithelial cells, keratinocytes, pDC
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self	Macrophages, cDC, neutrophil, mast cells, T and B cells, endo/epithelial cells, keratinocytes, pDC
TLR3	Endolysosome	dsRNA	Virus	cDC, macrophages, T cells, NK cells, keratinocytes, endo/epithelial cells, neurons
TLR4	Plasma membrane	LPS	Bacteria, viruses, self	Macrophages, cDC, neutrophil, mast cells, eosinophil, T cells, endo/epithelial cells, keratinocytes
TLR5	Plasma membrane	Flagellin	Bacteria	Monocytes, cDC, iEC, T cells, NK cells, endo/epithelial cells, keratinocytes
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, Viruses	Monocytes, mast cells, cDC, neutrophil, T and B cells, endo/epithelial cells, keratinocytes, pDC
TLR7	Endolysosome	ssRNA	Virus, bacteria, self	pDC, neutrophil, eosinophil, NK cells, T and B cells, endothelial cells, cDC
hTLR8	Endolysosome	ssRNA	Virus, bacteria, self	Monocytes, cDC, mast cells, neutrophil, NK cells, endothelial cells, pDC, cDC
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self	pDC, NK cells, eosinophil, neutrophil, T and B cells, endo/epithelial cells, keratinocytes, cDC
TLR10	Endolysosome	Unknown	Unknown	Macrophages, T and B cells, endo/epithelial cells
mTLR11	Plasma membrane	Profilin-like molecule	Protozoa	Epithelial cells, DCs, macrophages

hTLR: human TLR; m: mouseTLR; LPS: lipopolysaccharide; ds: double-strand; ss: single-strand
Data pooled from Cai et al, 2009; Chang, 2010; Kawai and Akira, 2009; Pulendran, 2004; Takeuchi and Akira, 2010

All TLRs belong to the type I transmembrane glycoprotein receptor family and together with the Interleukin-1 receptor (IL-1R) family they form the IL-1R/TLR superfamily in which all

members share a common Toll/IL-1R (TIR) homology domain. The TIR domain represents the cytosolic portion of the TLR and is responsible for the recruitment of adaptor molecules and initiating the downstream signalling cascade. Recognition of the respective TLR-ligand is performed by 16 – 28 leucine rich repeat (LRR) domains at the N-terminal end of the TLR. (Bell et al, 2003; Chang, 2010; Kawai and Akira, 2010; O´Neill and Bowie, 2007; Takeuchi and Akira, 2010; Yamamoto et al, 2004).

Currently ten different TLRs have been identified in humans and 13 in mice although the ligands for TLR10, TLR12 and TLR13 are not identified yet and therefore are not discussed here. The rest of the TLRs can be roughly divided into two subgroups depending on their localization in the cell and their ligands. The fist group consists of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 which are all expressed on the cell surface and recognize mainly microbial membrane components like lipids, lipoproteins and proteins. TLR3, TLR7, TLR8 and TLR9 form the second group. These TLRs recognize microbial nucleic acids and are found in intracellular vesicles like the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes. A detailed list of the ligands and the localization of the TLRs 1 to 11 can be found in Table 2 (Chang, 2010; Kawai and Akira, 2010; Shi et al, 2011; Takeuchi and Akira, 2010). Starting from now, only TLRs 1 to 9 are discussed.

1.4.2 Toll-like Receptor Signalling Pathways

TLRs occur as homodimers like TLR3, TLR7 and TLR9 or as heterodimers like TLR2, which dimerizes either with TLR1 or TLR6, and TLR4, which forms a complex with MD2 (lymphocyte antigen 96). After ligand binding TLRs are assumed to undergo symmetrical dimerization and a conformational change which brings the two TIR domains into closer proximity and makes recruitment of adaptor molecules for signal transmission possible (O´Neill and Bowie, 2007).

All of the adaptor molecules for TLR signalling contain a TIR domain and are called myeloid differentiation primary response gene (MyD) 88, TIR domain containing adaptor protein (TIRAP (Mal)), TIR domain containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). TRAM and TIRAP function as sorting adaptors for recruiting TRIF to TLR4 and MyD88 to TLR2.

TLR3 activates TRIF-dependent signalling pathways which lead to both type I IFN and inflammatory cytokines. Only TLR4 uses both the MyD88- and the TRIF-dependent pathways (Kawai and Akira, 2010; Lee and Kim, 2007).

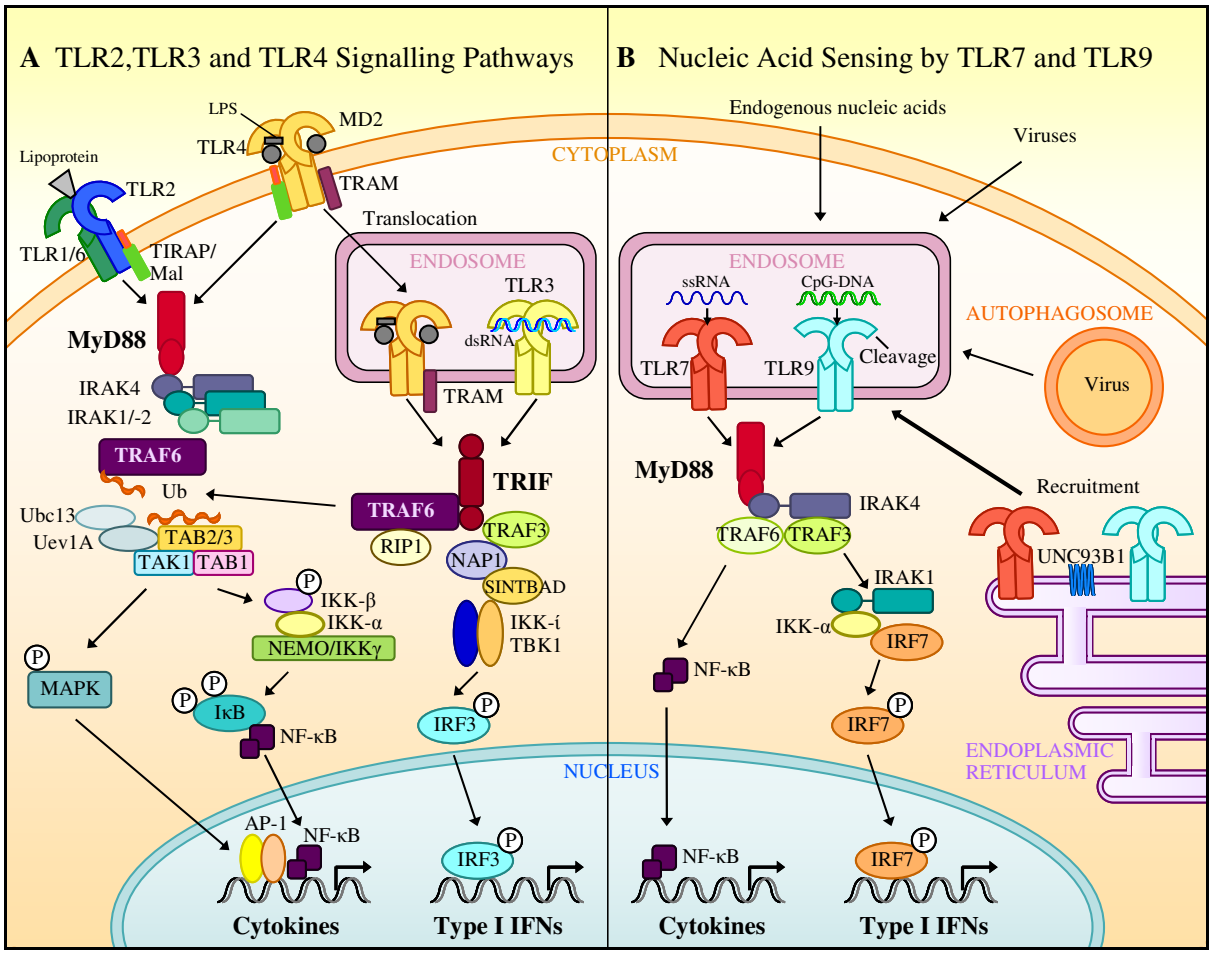

Figure 2: TLR signalling pathways (adapted from Takeuchi and Akira, 2010)

A) Cell surface TLRs (TLR1, 2, 4 and 6) recognize their respective ligands on the cell surface. Stimulation leads to the recruitment of MyD88 and TIRAP to the TLR and complex formation with IRAKs and TRAF6. TRAF6 acts as an E3 ubiquitin ligase and catalyzes formation of a K63-linked polyubiquitin chain on TRAF6 itself and generation of an unconjugated poliubiquitin chain with an E2 ubiquitin ligase complex of Ubc13 and Uev1A. Ubiquination leads to the activation of a complex of TAK1, TAB1 and TAB2/3 which results in the phosphorylation of NEMO, activation of an IKK complex and phosphorylation of IκB. Phosphorylated IκB is degraded so that NF-κB is free to translocate to the nucleus and drive expression of cytokine genes. TAK1 also activates MAP kinase cascades leading to the activation of AP-1 and induction of other cytokine genes. LPS, the ligand for TLR4, induces translocation of the TLR4-MD2 complex together with TRAM to the endosome. TLR3 resides also in the endosome. Both TLR 3 and 4 activate TRIF-dependent signaling which activates NF-κB via TRAF6 and RIP1, and IRF3 via NAP1, SINTBAD and TBK1/IKK-*i*. Activation of NF-κB and IRF3 results in the expression of proinflammatory cytokine genes and type I IFN genes.

B) TLR7 and TLR9 are located in the ER. Upon virus infection or stimulation with their respective ligand, ssRNA for TLR7 and CpG DNA for TLR9, TLR7 and TLR9 traffick from the ER to the endolysosome with the help of UNC93B1. In addition, TLR9 undergoes cleavage by proteases present in the endolysosome. A complex of MyD88, IRAK-4, TRAF6, TRAF3, IRAK-1, IKK-α and IRF7 is recruited to the TLR. IRF7 is phosphorylated and translocates to the nucleus where it activates type I IFN genes. NF-κB is activated via TRAF6 and induces proinflammatory cytokine genes. There are different routes how TLR7 and TLR9 ligands reach the endolysosome. Viruses that have entered the cytoplasm are engulfed by autophagosomes and deliver viral nucleic acids to the endolysosome. Furthermore, endogenous nucleic acids may reach TLR7 and TLR9 through binding to various cell surface.

The inflammatory cytokines produced after TLR signalling via MyD88 are predominantly TNF, IL-1, IL-6 and IL-12 which orchestrate the inflammatory response by regulating cell death, vascular endothelial permeability, recruitment of blood cells to inflamed tissues and induction of acute phase proteins (Takeuchi and Akira, 2010). In addition to that, IL-6 and IL-12 are key cytokines in the differentiation of T_H17 and T_H1 cells, respectively, as described above (1.3.1).

Nucleic acid sensing by TLR7 and TLR9 and the subsequent signalling differs to the other TLRs insofar as they both use MyD88 as adaptor molecule to induce type I IFN via IRF7 in addition to inflammatory cytokines (Kawai and Akira, 2010; Takeuchi and Akira, 2010). A more detailed overview of TLR signalling pathways is found in Figure 2.

1.4.3 The Role of Toll-like Receptors in Diseases

Recognition of PAMPs by TLRs is important for a successful host defense against pathogens. But it is also possible that TLR ligands lead to excess responses which causes lethal septic shock syndrome, e.g. high levels of LPS recognized by TLR4 can lead to multiorgan failure in the host (Takeuchi and Akira, 2010). Furthermore, TLRs play a role in the pathogenesis of Alzheimer`s and stroke (Carty and Bowie, 2011).

In addition to that, some TLRs, especially TLR3, TLR7 and TLR9, are also capable to recognize endogenous nucleic acids. Under physiological conditions, TLR3, TLR7 and TLR9 do not encounter self-nucleic acids from damaged cells because they are not located on the cell surface and the nucleic acids are rapidly degraded. But it is possible for various cell proteins to interact with self-nucleic acids and the resulting nucleic acid-protein complexes are endocytosed allowing activation of endosomal TLRs by self nucleic acids. Two examples for nucleic acid-protein complexes are autoantibodies recognizing self-DNA and self-RNA that bind to FcγRIIa and are internalised. Furthermore, the antimicrobial peptide LL37 associates with endogenous DNA. Recognition of self-nucleic acids and activation via TLR3, TLR7 and TLR9 leads to the production of autoantibodies, inflammatory cytokines and type I IFN which causes inflammation and consequently may lead to autoimmune diseases such as multiple sclerosis or SLE. (Green and Marshak-Rothstein, 2011; Ewald and Barton, 2011; Takeuchi and Akira, 2010).

1.5 Systemic Lupus Erythematosus

An example for a severe autoimmune disease where activation of TLRs by endogenous ligands plays an important role in disease onset is SLE. SLE is an autoimmune disease which is characterized by inflammation in several organs including the skin, kidneys, heart, lungs and the brain. It develops as the result of a combination of genetic and environmental factors. Genetic factors are e.g. defects in tolerance mechanisms, apoptosis and the clearance of apoptotic material which are the cause for uncontrolled hyperactivity of self-reactive B and T cells and accumulation of potential self-antigens and TLR ligands like nucleic acids and their associated proteins.

But the onset of SLE is most likely triggered by environmental factors that include sterile injury and infections because these inflammatory events also lead to B cell activation and antibody production and further increase the amount of cell debris. (Apostolidis et al, 2011; Eloranta et al, 2009; Green and Marshak-Rothstein, 2011; Savarese et al, 2006).
The significant event in SLE development is the production of autoantibodies by self-reactive B cells against nuclear components like Smith (Sm) proteins or small nuclear ribonuleoproteins (snRNP) and DNA and these are called anti-nuclear antibodies (ANAs).

Normally, self-reactive B cells which escaped negative selection often have low-affinity BCRs for self-antigens. Therefore, signals are too weak to elicit a B cell response. This is overcome by simultaneous signals from TLRs, in case of SLE especially from the nucleic acid binding TLR7 and TLR9, and autoreactive antibodies against nuclear antigens are produced (Green and Marshak-Rothstein, 2011; Savarese et al, 2006). This is the first step in driving a self-amplifying cycle of immune reactions leading to full-blown SLE (see Figure 3):

The production of ANAs leads to the formation of immune complexes (ICs) containing endogenous TLR7- and TLR9-ligands (self RNA and self DNA) which deposit in many organs and tissues and cause inflammation through TLR7 and TLR9 activation in B cells and DCs and subsequent type I IFN and IL-6 production. In turn, type I IFN leads to DC differentiation and maturation, render cells more susceptible to apoptosis, promotes TLR7 expression and responses to TLR7-ligands in B cells, promotes B cell survival and differentiation, antibody class switching and increases BCR signalling (Eloranta et al, 2009; Green and Marshak-Rothstein, 2011; Savarese et al, 2006).

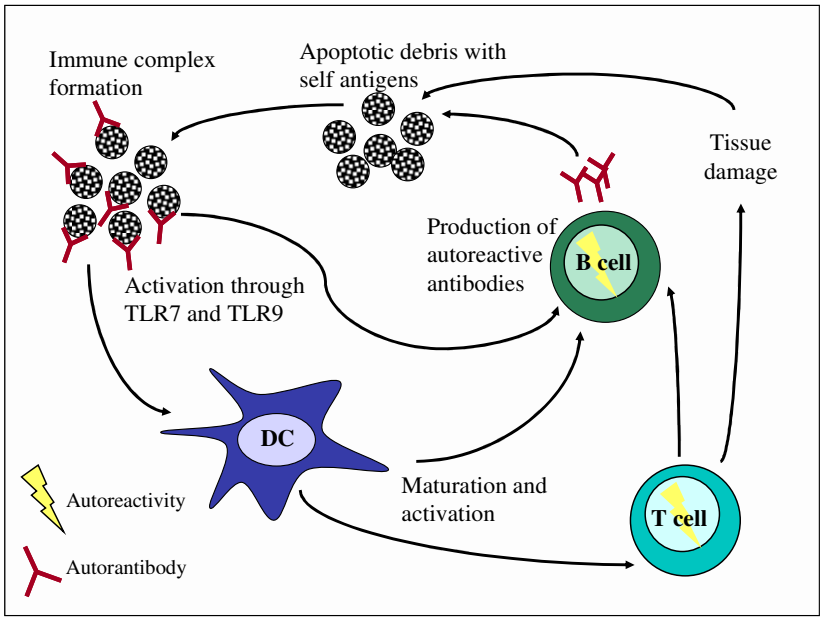

Figure 3: Model of SLE development
In SLE a defect in the clearance of apoptotic material leads to the accumulation of apoptotic debris and subsequently to the presentation of self antigens and to the formation of autoimmune complexes. TLR7- and TLR9-ligands present within these complexes activate dendritic cells (DC). Through a breakdown in peripheral tolerance autoreactive T and B cells are activated and are the cause of further inflammation, tissue damage and organ failure.

This leads to increased tissue damage and autoreactive antibody production. IL-6 also plays a critical role in causing B cell hyperactivitiy, but also in the differentiation of plasma cells and self-reactive effector T cells, especially T_H17 cells (Apostolidis et al, 2011; Chun et al, 2007; Richards et al, 1998). Furthermore, IL-6 has an impact on the suppressive effect of Tregs on self-reactive naïve T cells by rendering them unresponsive to Treg-mediated suppression (Sakaguchi, 2005) leading to tolerance breakdown and uncontrolled activity of self-reactive B and T cells.

2 AIMS OF THE STUDY

During infections or autoimmune diseases like SLE exogenous and endogenous ligands for TLRs are present and directly activate DCs and B cells and thus support the generation of effector T and B lymphocytes. To this end, the immunosuppressive function of Tregs must be overcome to generate an effective antiviral or autoreactive adaptive immune response.
It was shown that not only B cells and DCs express TLRs, but also both naïve CD4+ T cells and CD4+Foxp3+ Tregs can express TLRs. There was evidence that TLR signalling may directly or indirectly regulate the immunosuppressive function of Tregs in immune responses and may shift the balance between proinflammatory T helper cells and Foxp3+ Tregs (Caramalho et al, 2003; Chang, 2010; Liu and Zhao, 2007).
So the first aim of this study was to examine the influence of the engagement of TLR4, TLR7 and TLR9 on the differentiation of *de novo* generated Tregs and whether the effect is directly mediated through the TLRs expressed by naïve T cells or if a mediator like DCs or B cells is required to influence Treg generation.
After establishing if and how TLR4, TLR7 and TLR9 influence the generation of Tregs, the second aim was to study the stability of Foxp3 expression and the efficiency of the suppressive function of Tregs which were generated under the influence of an inflammatory setting.

3 MATERIAL

3.1 Equipment

INSTRUMENT	COMPANY
Agarose Gel electrophoresis system	BioRad, Munich, Germany
Analytical balance	Ohaus, Pine Brook, USA
Balance	Ohaus, Pine Brook, USA
Centrifuge Biofuge Fresco	Heraues, Hanau, Germany
Centrifuge 5415 D	Eppendorf, Hamburg, Germany
Centrifuge 5430 R	Eppendorf, Hamburg, Germany
Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Electrophoresis power supply PowerPac 300	BioRad, Munich, Germany
FACSCalibur flow cytometer	BD Biosciences, Heidelberg, Germany
FACSAria flow cytometer and Cell Sorter	BD Biosciences, Heidelberg, Germany
Freezer -20°C	Siemens, Munich, Germany
Freezer -80°C	Liebherr, Bulle, Switzerland
	Kendro, Langenselbold, Germany
Fridge	Liebherr, Bulle, Switzerland
Gallios flow cytometer	Beckman Coulter, Fullerton, USA
Gel documentation system Gel Doc XR	BioRad, Munich, Germany
Ice Machine	Ziegra, Isernhagen, Germany
Incubator Hera Cell 240	Heraeus, Hanau, Germany
Infra-red Lamp 808 (100 W)	Efbe-Schott, Bad Blankenburg, Germany
Laminar flow Hera Safe	Kendro, Langenselbold, Germany
MACS Multistand	Miltenyi Biotec, Bergisch-Gladbach, Germany
MACS Midi Magnet (for LS Columns)	Miltenyi Biotec, Bergisch-Gladbach, Germany
MACS Mini Magnet (for MS Columns)	Miltenyi Biotec, Bergisch-Gladbach, Germany
Magnetic Stirrer	Heidolph, Schwabach, Germany
Microwave	Siemens, Munich, Germany

Microscope Optech IB	Exacta Optech, Munich, Germany
MoFlo cell sorter	Beckman Coulter, Fullerton, Germany
Multichannel pipette Calibra 852	Socorex, Ecublens, Switzerland
Multipipette Plus	Eppendorf, Hamburg, Germany
Multiscan EX Microplate Photometer	Thermo Labsystems, Langenselbold, Germany
Nanodrop ND-100 Spectrophotometer	Peqlab, Erlangen, Germany
Neubauer Counting Chamber	Roth, Karlsruhe, Germany
Nitrogen freezing tank MVE 6000	MVE, Marietta, USA
PCR Cycler Mastercycler	Eppendorf, Hamburg, Germany
PCR Cycler C1000 Thermal Cycler	BioRad, Munich, Germany
pH-Meter	WTW, Weilheim, Germany
Pipetboy acu	Integra Biosciences, Fernwald, Germany
Pipettes	Gilson, Middleton, USA
Spectrophotometer SmartSpec	BioRad, Munich, Germany
StepOne Plus Real-Time PCR Systems	Applied Biosystems, Carlsbad, USA
Thermomixer	Eppendorf, Hamburg, Germany
VortexGenie 2	Scientific Industries, Bohemia, USA
Water Bath	GFL, Burgwedel, Germany

3.2 Software

SOFTWARE	COMPANY
Ascent Software for Multiscan EX Microplate Photometer	Thermo Labsystems, Langenselbold, Germany
CellQuest	BD Biosciences, Heidelberg, Germany
FlowJo	Treestar, Ashland, USA
Kaluza	Beckman Coulter, Fullerton, USA
Microsoft Office	Microsoft, Redmond, USA
StepOne Software	Applied Biosystems, Carlsbad, USA

3.3 Consumables

CONSUMABLE	COMPANY
Aspiration pipette	BD Falcon, Franklin Lakes, USA Sarstedt, Nümbrecht, Germany
BD Micro-Fine+ U-100 Insulin (0,5 ml, 30G)	BD Medical, Le Pont de Claix Cedex, France
BD Plastipak 1ml Sub-Q (26G x ½)	BD Medical, Franklin Lakes, USA
BD Microtainer SST Tubes	BD, Franklin Lakes, USA
Bottle Top Filter	Corning, Lowell, USA
Cell Culture Plates (6-Well, 96-Well-round bottom, 96-Well-flat bottom plates)	BD Falcon, Franklin Lakes, USA
Cell Strainer 100 µm Nylon	BD Falcon, Franklin Lakes, USA
Combitips	Eppendorf, Hamburg, Germany
Conical tubes (15 ml, 50 ml)	Greiner Bio-One, Frickenhausen, Germany
Filcons 50 µm, Syringe-Type (non sterile and sterile)	Günter Keul GmbH, Steinfurt, Germany
Filter Tips	Greiner Bio-One, Frickenhausen, Germany
MACS Separation Columns (LS and MS Columns)	Miltenyi Biotec, Bergisch-Gladbach, Germany
MACS Pre-Separation Filter (30 µm)	Miltenyi Biotec, Bergisch-Gladbach, Germany
Maxi-Sorp ELISA Plate	Nunc, Wiesbaden, Germany
MicroAmp Fast Optical 96-Well Reaction Plate (0.1 ml)	Applied Biosystems, Carlsbad, USA
Microcentrifuge tubes (0.5 ml, 1.5 ml)	Sarstedt, Nümbrecht, Germany
Needles (23G x 1¼, 27G x ¾)	Braun, Melsungen, Germany
Parafilm	Roth, Karlsruhe, Germany
PCR tubes	Biozym, Hessisch-Oldendorf, Germany
Safe lock microcentrifuge tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Serological pipettes	Sarstedt, Nümbrecht, Germany
Syringe Filter (0,2 µm)	Corning, Lowell, USA

Syringe Luer Lok sterile (5ml, 10 ml)	BD Falcon, Franklin Lakes, USA
Tissue Culture Inserts 8 Well Strip (0,2 µm Anopore Membrane)	Nunc, Wiesbaden, Germany

3.4 Cells

Naïve T cells, B cells and dendritic cells were freshly isolated from murine spleens or mesenterial lymph nodes of C57BL/6 mice or TLR7 -/- mice. FL-DCs were generated from bone-marrow cells.

3.5 Reagents

3.5.1 Cell Culture Reagents

REAGENT	COMPANY
DMSO	Sigma-Aldrich, St. Louis, USA
EDTA	Fluka, Seelze, GErmany
Fetal Bovine Serum (Sera Plus)	PAN, Aidenbach, Germany
FBS low IgG	PAA, Cölbe, Germany
Flag Flt3-ligand	Purified from CHO supernatant
Glutamax 100x (200mM)	Gibco, Karlsruhe, Germany
HEPES	Gibco, Karlsruhe, Germany
β-Mercaptoethanol (14,3 M)	Sigma-Aldrich, St. Louis, USA
NEAA 100x	PAA, Cölbe, Germany
PBS (Ca^{2+}- and Mg^{2+}-free)	PAA, Cölbe, Germany
Penicillin-Streptomycin	PAA, Cölbe, Germany
RPMI 1640	PromoCell, Heidelberg, Germany
	Gibco, Karlsruhe, Germany
Sodium pyruvate (100 mM)	PAA, Cölbe, Germany

3.5.2 Chemicals and Reagents

REAGENT	COMPANY
ABTS (50 mg/ml)	Roche, Basel, Switzerland
Acetic Acid (AcOH), 100%	Roth, Karlsruhe, Germany
Agarose	Biozym, Hessisch-Oldendorf, Germany
BSA fraction V pH = 7,0	Serva, Heidelberg, Germany
Bromophenol blue	Sigma-Aldrich, St. Louis, Germany
carboxyfluorescein diacetate succinimidyl ester (CFSE)	Invitrogen, Karlsruhe, Germany
Citric acid	Roth, Karlsruhe, Germany
Chloroform	Merck, Darmstadt, Germany
Direct PCR Lysis Reagent	Peqlab, Erlangen, Germany
DOTAP	Roth, Karlsruhe, Germany
Ethanol absolute	J.F. Baker, Deventer, Netherlands
Ethidium monoazide bromide (EMA)	Sigma-Aldrich, St. Louis, USA
GolgiPlug	BD Biosciences, Franklin Lakes, USA
GolgiStop	BD Biosciences, Franklin Lakes, USA
H_2SO_4 (2 N)	Roth, Karlsruhe, Germany
HCl (1 N)	Merck, Darmstadt, Germany
Isofluran (Forene 100% v/v)	Abbott, Wiesbaden-Denkenheim, Germany
Paraformaldehyde (PFA)	Merck, Darmstadt, Germany
PBS powder (Ca^{2+}- and Mg^{2+}-free)	Invitrogen, Karlsruhe, Germany
Pristane (2,6,10,14-tetramethylpentadecan)	Sigma-Aldrich, St. Louis, USA
Propanol	J.T. Baker, Deventer, Netherlands
Propidium iodide (1mg/ml)	Sigma-Aldrich, St. Louis, USA
Proteinase K (20 mg/ml)	Roth, Karlsruhe, Germany
Saponin	Roth, Karlsruhe, Germany
Sodium azide	Sigma-Aldrich, St. Louis, USA
Sodium chloride	Fluka, Seelze, Germany
Sodium dodecyl sulphate (SDS)	Fluka, Seelze, Germany
Streptavidin-HRP	Amersham GE Healthcare, Piscataway, USA

TMB substrate	Ebioscience (NatuTec), Frankfurt, Germany
Tris-HCl	Roth, Karlsruhe, Germany
Trypan Blue	Invitrogen, Karlsruhe, Germany
Tween 20	Sigma-Aldrich, St. Louis, USA

3.6 Antibodies, Cytokines and Stimuli

3.6.1 Cell Surface Antibodies for Flow Cytometry

ANTIBODY SPECIFICITY (CLONE)	CONJUGATE	COMPANY
CD3ε (145-2C11)	APC	BD Biosciences, Franklin Lakes, USA
CD4 (L3T4)	FITC PE AlexaFluor647 PE-Cy5 PerCP-Cy5.5	BD Biosciences, Franklin Lakes, USA Caltag Laboratories, Hamburg, Germany Ebioscience (NatuTec), Frankfurt, Germany
CD8α (53-6.7)	PE	BD Biosciences, Franklin Lakes, USA
CD11b (M1/70)	FITC PE	BD Biosciences, Franklin Lakes, USA
CD11c (N418)	APC PE-Cy7	Ebioscience (NatuTec), Frankfurt, Germany
CD19 (1D3)	FITC	BD Biosciences, Franklin Lakes, USA
CD25 (7D4) (PC61.5) (eBio7D4)	FITC PE Biotin	BD Biosciences, Franklin Lakes, USA Ebioscience (NatuTec), Frankfurt, Germany
CD45R (B220)	PE APC	Southern Biotec, Birmingham, USA BD Biosciences, Franklin Lakes, USA
CD62L (MEL-14)	PE APC Biotin	BD Biosciences, Franklin Lakes, USA BD Biosciences, Franklin Lakes, USA

CD69 (H1.3F3)	PE Biotin	BD Biosciences, Franklin Lakes, USA
CD86 (GL-1)	PE	BD Biosciences, Franklin Lakes, USA
CD103 (M290)	PE	BD Biosciences, Franklin Lakes, USA
CTLA-4 (UC10-4B9)	APC Biotin	Ebioscience (NatuTec), Frankfurt, Germany
Folate Receptor 4 (eBio12A5)	APC	Ebioscience (NatuTed), Frankfurt Germany
120G8	FITC	Purified from 120G8 hybridoma supernatant
DO11.10 TCR (KJI-26)	PE APC Biotin	Ebioscience (NatuTec), Frankfurt, Germany Caltag Laboratories, Hamburg, Germany
Fc-Block	Unlabeled	Hb197 hybridoma supernatant
CD16/CD32 (93)	Unlabeled	Ebioscience (NatuTec), Frankfurt, Germany

All antibodies for Flow Cytometry were diluted 1:200, except for anti-CD11b-PE which was used in a dilution of 1:400 and Fc-Block was diluted 1:2.

3.6.2 Intracellular Antibodies for Flow Cytometry

ANTIBODY SPECIFICITY (CLONE)	COLOUR	COMPANY
Foxp3 (FJK-16s)	PE APC AlexaFluor700	Ebioscience (NatuTec), Frankfurt, Germany
CTLA-4 (UC10-4B9)	APC Biotin	Ebioscience (NatuTec), Frankfurt, Germany
IFN-γ (XMG 1.2)	FITC PE	Ebioscience (NatuTec), Frankfurt, Germany BD Biosciences, Franklin Lakes, USA
IL-4 (11B11)	PE	BD Biosciences, Franklin Lakes, USA
IL-10 (JES5-16E3)	PE	BD Biosciences, Franklin Lakes, USA

IL-17 (eBio17E7)	APC	Ebioscience (NatuTec), Frankfurt, Germany

All antibodies for Flow Cytrometry were diluted 1:200, except for the anti-IL-17 antibody which was diluted 1:100.

3.6.3 Neutralizing Antibodies for Cell Culture and *in vivo* Experiments

ANTIBODY SPECIFICITY (CLONE)	COMPANY
IFN-γ (XMG 1.2) IL-4 (11B11) IL-6 (MP5-20F3) IL-6Rα (D7715AD)	Ebioscience (NatuTec), Frankfurt, Germany
IL-6 (MP5-20F3) sgp130Fc	Gift from Prof. Dr. Rose-John, Christian-Albrechts-University Kiel

3.6.4 Antibodies for Cell Stimulation

ANTIBODY SPECIFICITY (CLONE)	COMPANY
CD3ε (145-2C11) CD28 (37.51)	Ebioscience (NatuTec), Frankfurt, Germany

3.6.5 Matched Antibody Pairs for ELISA

ANTIBODY SPECIFICTIY (CLONE)	COMPANY
IL-6 capture (MP5-20F3) Biotin IL-6 detection (MP5-32C11)	BD Biosciences, Franklin Lakes, USA
IL-12 (p40/p70) capture (C15.6) Biotin IL-12 (p40/p70) detection (C17.8)	BD Biosciences, Franklin Lakes, USA

3.6.6 Cytokines

CYTOKINE	COMPANY
Recombinant murine IL-2 (cell culture)	Promokine, Heidelberg, Germany
Recombinant murine IL-6 (cell culture and ELISA)	BD Biosciences, Franklin Lakes, USA
Recombinant murine IL-12 p40 (ELISA)	BD Biosciences, Franklin Lakes, USA
Recombinant human TGF-β2	Peprotech, Hamburg, Germany

3.6.7 Cell Stimuli

STIMULUS	COMPANY
S-27609 (TLR7)	3M Pharmaceuticals, St.Paul, USA
R848 (TLR7)	Invivogen, San Diego, USA
CL-075 (TLR7)	Invivogen, San Diego, USA
U1snRNP (TLR7)	Gift from Berthold Kastner, Max-Planck-Insitute for Biophysical Chemistry, Göttingen, Germany
CpG 1668 (TLR9)	Synthesized by Eurofins MWG Operon, Ebersberg, Germany
LPS from *E. coli* strain 0111:B4 (TLR4)	Sigma-Aldrich, St. Louis, USA
OVA peptide$_{323-339}$	GenScript, Piscataway, USA
OVA protein	Hyglos, Regensburg, Germany

3.7 Reagents for Molecular Biology

REAGENT	COMPANY
DNA ladder (100 bp and 1 kb)	NEB, Frankfurt, Germany
DNase I (deoxyribonuclease I)	Fermentas, St. Leon-Rot, Germany
dNTP (10 mM)	Invitrogen, Karlsruhe, Germany
DTT (0.1 M)	Invitrogen, Karlsruhe, GErmany

EDTA (25 mM)	Fermentas, St. Leon-Rot, Germany
Ethanol (100%) for Molecular Biology	Merck, Darmstadt, Germany
Ethidiumbromide (10 mg/ml)	Invitrogen, Karlsruhe, Germany
GoTaq	Promega, Mannheim, Germany
H_2O ultra pure, DNase + RNase free	Gibco, Karlsruhe, Germany
$MgCl_2$ (50 mM)	Invitrogen, Karlsruhe, Germany
Superscript II Reverse Polymerase	Invitrogen, Karlsruhe, Germany
Oligo $(dT)_{15-18}$ Primer	Promega, Mannheim, Germany
10x Taq Buffer	Invitrogen, Karlsruhe, Germany
TagMan Gene Expression Master Mix	Applied Biosystems, Carlsbad, USA
Taq Polymerase (5 U/µl)	Invitrogen, Karlsruhe, Germany

3.7.1 PCR Primers

GENE OF INTEREST	PRIMER SEQUENCE	FRAGMENT LENGTH
gfp	P162: 5´-GCG AGG GCG ATG CCA CCT ACG GCA-3´ P163: 5´-GGG TGT TCT GCT GGT AGT GGT CGG-3´	450 bp
tlr7	wt TLR7 (AKM128): 5´-ACG TGA TTG TGG CGG TCA GAG GAT AAC-3´ Extra-TLR7 (AKM 129): 5´-CCA GAT ACA TCG CCT ACC TAC TAG ACC-3´ Neo 1500 (AKM 130): 5´-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3´	wt: 1200 bp (AKM128/129) TLR7 -/-: 1200 bp (AKM129/ 130)

All PCR primers were synthesied by Eurofins MWG Operon, Ebersberg, Germany and were used for PCR in a concentration of 10 µM.

3.7.2 TaqMan® Gene Expression Assays (Applied Biosystems)

GENE SYMBOL	ASSAY ID	AMPLICON LENGTH
foxp3	Mm01351178_g1	72
gata3	Mm00484683_m1	57
hprt1	Mm01324427_m1	108
il-6 (IL-6)	Mm00446190_m1	78
il-10 (IL-10)	Mm01288386_m1	79
il-17a (IL-17a)	Mm00439619_m1	91
il-23 (IL-23)	Mm00518984_m1	61
torc (Rorγt)	Mm01261022_m1	54
tbx21 (T-bet)	Mm00450960_m1	69

Applied Biosystems, Carlsbad, USA

3.8 Cell Culture Media

MEDIUM	SUPPLEMENTS
DC-medium: RPMI 1640 (PromoCell)	10% FBS 1% Penicillin/Streptomycin 1% Non essential amino acids 1% Sodium pyruvate 1% Glutamax 50 µM β-Mercaptoethanol
2x DC-medium: RPMI 1640 (PromoCell)	20% FBS 2% Penicillin/Streptomycin 2% Non essential amino acids 2% Sodium pyruvate 2% Glutamax 100 µM β-Mercaptoethanol

3.9 Buffer and Solutions

PBS used for all buffers and solutions was Ca^{2+} and Mg^{2+} free.

BUFFER	RECIPE
MACS Buffer	PBS 2% FBS 2 mM EDTA
FACS Buffer	PBS 2% FBS
FACS Sort Buffer	PBS 2% FBS 0.5 mM EDTA
ELISA (IL-6 and IL-12 p40)	
Coating Buffer	PBS
Blocking Buffer	PBS 10% FBS
Dilution Buffer	PBS 10% FBS 0.05% Tween 20
Washing Buffer	PBS 0.05% Tween 20
Intracellular Cytokine Staining	
Fixation Buffer	PBS 2% PFA

Permeabilisation Buffer (Saponin-Buffer)	PBS 0.5% Saponin 5% FBS 10 mM Hepes 0.02% NaN3
50x TAE-Buffer	2 M Tris 100 mM EDTA (0.5 M) pH 8,0 5.71 % (v/v) glacial acetic acid in H_2O pH 8.5 with 1 N NaOH

SOLUTION	RECIPE
ELISA Substrate solution	0.1 M Citric acid pH = 4 1 mg/ml ABTS 1:1000 H_2O_2 (30%)
ELISA Stop solution	1% SDS

3.10 Kits

METHOD	KIT	COMPANY
ELISA	Ready-Set-Go IL-4 Ready-Set-Go IL-17 Duo Set mouse IFN-γ Duo Set mouse IL-10	Ebioscience (NatuTec), Frankfurt, Germany R&D System, Minneapolis, USA
FACS	Foxp3 Staining Kit	Ebioscience (NatuTec), Frankfurt, Germany
RNA Isolation	RNeasy Mini Kit Trizol Reagent	Qiagen, Hilden, Germany Invitrogen, Karlsruhe, Germany
Cell Isolation	CD4+ T cell Isolation Kit, mouse CD11c+ MicroBeads CD45R(B220) MicroBeads	Miltenyi Biotec, Bergisch-Gladbach, Germany

3.11 Mouse strains

MOUSE STRAINS	
C57BL/6	from Harlan Winkelmann, Borchen, Germany bred in Animal facility
Balb/c	from Harlan Winkelmann, Borchen, Germany bred in Animal facility
TLR7 -/-	Animal facility
DEREG 23.2	Gift from Tim Sparwasser, bred in animal facility
DEREG 23.2 x TLR7 -/-	Animal facility
DO11.10/Rag2 -/-	Animal facility
CD45.1 congenic mice	Animal facility
OT-II/Rag2 -/- DEREG	Animal facility

4 METHODS

4.1 Animal Care and Experiments

WT C57BL/6 and Balb/c mice were purchased from Harlan Winkelmann (Borchen, Germany) or bred in the animal facility of the Klinikum rechts der Isar under specific pathogen free conditions. Knockout and transgenic mouse strains were also bred in the animal facility: TLR7 -/- (originally a gift from Prof. Dr. Shizuo Akira), TLR9 -/-, DEREG 23.2 (Lahl et al, 2007), OT-II/Rag2 -/- DEREG and CD45.1 congenic mice were all on the C57BL/6 background and DO11.10/Rag2 -/- mice were on the Balb/c background. Experiments were performed in accordance with the German animal care and ethics legislation and had been approved by the local government authorities.

4.1.1 Generation of DEREG 23.2 x TLR7 -/- mice

DEREG 23.2 reporter mice express enhanced green fluorescent protein (eGFP) in Tregs under the control of the Foxp3 promotor (Lahl et al, 2007) and therefore it is possible to distinguish Tregs during FACS analysis without intracellular staining. In order to be able to isolate Tregs by FACS sorting from WT and TLR7 -/- mice DEREG 23.2 were crossed with TLR7 -/- mice to generate a TLR7 -/- Treg reporter mouse strain.
First of all we crossed a female TLR7 -/- with a male DEREG 23.2 and the offspring were analyzed concerning GFP and TLR7 with specific PCRs. Since the gene encoding for TLR7 is located on the X chromosome all male progeny were TLR7 knockout while females were heterozygous for TLR7. Next, TLR7 -/0 GFP+ males were crossed with TLR7 +/- GFP+ females to obtain TLR7 -/- GFP+ females. Unresponsiveness to TLR7 activation was then checked by stimulating FL-DCs from WT, TLR7 -/- and DEREG 23.2 x TLR7 -/- with the TLR7 ligand S-27609 and as control CpG1668 was used.
Offspring of DEREG 23.2 x TLR7 -/- were constantly checked for TLR7 und GFP expression by PCR analysis (see below).

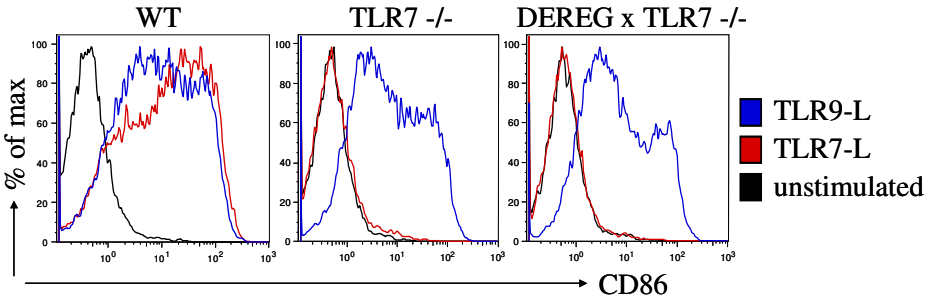

Figure 4: Stimulation of FL-DC with Imiquimod (S-27609) or CpG 1668
$2x10^5$ FL-DCs generated from bone marrow cells isolated from wt, TLR7-/- and Dereg 23.2 x TLR7-/- mice were stimulated with 3 µM S-27609 (TLR7-L) or 0.5 µM CpG 1668 (TLR9-L) on day 7 of culture in a 96-well plate. After two days cells were stained for BST2, CD11c and CD86. Histogram Plots indicate CD86 expression compared to an unstimulated control.

4.1.2 Short-term pristane experiments

Intraperitoneal injection of the mineral oil 2, 6, 10, 14-Tetramethylpentadecane (pristane) is known to cause a lupus like syndrome in mice after 3-6 months, but the reaction of the immune system in the peritoneal cavity starts immediately after injection (Lee et al, 2008) and might have an influence on the Treg compartment.
8-12 week old mice (WT C57BL/6, TLR7 -/-, DEREG 23.2, DEREG 23.2 x TLR7 -/-) were injected intraperitoneally (i.p.) with 0.5 ml of PBS or pristane with a BD Micro-Fine+ U-100 Insulin or BD Plastipak 1ml Sub-Q needle. After eight days mice were sacrificed.
First, 3 –8 ml of ice cold PBS or RPMI1640 was injected with a 27 G x ¾ needle into the peritoneum and was recovered with a 23 G x 1¾ needle after massaging the stomach to loosen peritoneal cells. The cell suspension was kept on ice until further handling.
Then blood was taken directly from the heart and one half was used to make serum in BD Microtainer SST Tubes.
For fluorescence activated cell sorter (FACS) analysis cells were stained with fluorescently labelled antibodies. Serum and the supernatant of the peritoneal lavage were stored at -20°C for detection of cytokines by enzyme-linked immunosorbent assay (ELISA).

4.1.3 T cell transfer and in vivo induction of regulatory T cells

Naïve CD4+ CD25- T cells were isolated (see 3.2.4) from the spleens of DO11.10/Rag2 -/- mice and stained with 5µM CFSE in PBS/0.1% BSA for 10 min at 37°C. $2\text{-}4x10^6$ cells were resupended in PBS and injected intravenously (i.v.) into the tail vein of Balb/c mice. After 24

h mice were immunized i.v. with 5µg OVA peptide$_{323-339}$ mixed with 30 µg TLR7 ligand R848. For neutralization of IL-6 anti-IL-6 antibody (500 µg) or soluble gp130Fc (sgp130) (50 µg or 200 µg) of was injected i.p. at the time of T cells transfer.
Three hours after pOVA and R848 injection mice were bled and 2 –3 drops were collected in BD Microtainer SST Tubes and centrifuged to prepare serum. Serum was stored at -20°C.
Four days after immunization mice were sacrificed, cells were isolated and pooled from spleen, mesenteric and inguinal LNs and were stained for CD4, DO11.10-TCR (KJI-26) and Foxp3.

4.2 Cell Preparation and Culture

4.2.1 Preparation of spleens and mesenteric lymph nodes

Spleens or mesenteric LNs were removed from euthanized mice and incubated in 3 ml RPMI 1640 (Gibco) supplemented with 500 µg/ml collagenase D and 100 µg/ml DNase 1 at 37°C and 5% CO_2 in a cell incubator for 30 min.
Then spleens or mesenteric LNs were passed through a 100 µm nylon strainer. If cells were used directly for FACS analysis the cell suspension was incubated in Red Blood Cell Lysis Buffer for 5 min at RT, if cells were used for cell separation using magnetic-activated cell sorting (MACS) technology (see below) erythrocytes were not lysed to treat DCs with care.

4.2.2 Cell Separation by MACS technology

MACS technology stands for magnetic-activated cell sorting where magnetic MicroBeads are fused directly to antibodies against cell surface molecules or antibodies recognizing biotin or fluorescent dyes. By labelling cells with magnetic beads these cells can be collected in a column which resides in a strong magnetic field. This makes it possible to separate cell populations and there are two different ways to isolate the desired population:
Positive separation (DCs, B cells) means that the desired population is in the magnetic field of the column and all other cells flow through, but can be used for further separation steps whereas in a negative separation (naïve T cells) all unwanted cell populations are labelled and the untouched desired population flows through. Since the rest of the cells are already labelled

there are no further separation steps possible, so it is important to finish all positive separation steps before negative separation.
The following order for separation of splenocytes was applied: First, DCs then B cells were positively selected and at the end naïve T cells were negatively selected.

4.2.2.1 Isolation of DCs

Isolation of DCs from LN cells with CD11c MicroBeads was performed according to the manufacturer`s instructions, isolation of DCs from splenocytes with CD11c MicroBeads was performed according to the manufacturer`s instructions with some modifications.
First, pooled splenocytes from 3 spleens were resuspended in 800 µl MACS Buffer, 100 µl of Fc-Block were added and the suspension was incubated for 10 min at RT. 100 µl of CD11c MicroBeads were added and the cells were incubated for another 15 min at 4°C. Then cells were washed, suspended in 5 ml MACS buffer and 3 ml were passed through a LS Separation Column, followed by a washing step with 3 ml MACS Buffer. After that the rest of the cells were put on the column and the column was washed once with 5 ml MACS Buffer. The flow-through containing B and T cells was collected for further isolation steps and the cells were counted. After removing the LS column from the magnetic field, CD11c+ DCs were recovered with 5 ml MACS Buffer.
To improve the purity up to 90-98%, the isolated DCs were passed through a MS column according to the manufacturer´s instruction.
After purification some cells were stained with the following antibodies for FACS analysis: anti-CD11c to check the purity of the isolation in general in combination with anti-CD11b and anti-B220 or anti-120G8 and anti-CD8α to identify the different DC subpopulations.

4.2.2.2 Isolation of B cells

B cells were positively separated with CD45R (B220) MicroBeads according to the manufacturer´s instructions from the flow-through of the DC isolation. Purity of the B cells was over 95% and was controlled by staining of cells with antiB220 and antiCD19.

4.2.2.3 Isolation of naïve T cells

To isolate naïve CD4+CD25- T cells the CD4+ T cell Isolation Kit supplemented with 8 µg/ml biotinylated anti-CD25 antibody was used according to the manufacturer´s instructions but with only half the amount of the recommended Biotin antibody cocktail and antiBiotin MicroBeads. The purity of CD3+CD4+CD25- naïve T cells was between 85-95%.

4.2.3 Cell counting

The cell number was determined by using a Neubauer counting chamber. Dead cells were excluded by diluting the cell suspension 1:2 with Trypan Blue. Using a microscope, the number of cells in 2 big squares consisting each of 16 smaller squares was counted. The cell concentration was assessed by the following calculation:

$$\frac{\text{cell concentration}}{\text{ml}} = \frac{\text{cell count}}{2} \times \text{dilution factor} \times 10^4$$

4.2.4 Co-culture setup

In co-culture experiments naïve T cells were incubated with DCs or B cells under Foxp3 inducing conditions (costimulation, IL-2 and TGF-β in the coculture medium titrated to an optimal result of Foxp3+ T cells) to test the effects of different TLR ligands on the generation of induced Tregs. The percentage of CD4+Foxp3+ T cells cultured under optimal conditions ranged between 50-90% after four days of culture.

4.2.4.1 Treg induction with antigen-unspecific stimulation

The appropriate number of wells of a 96-well-u-bottom plate were coated with 50 µl PBS containing 5 µg/ml anti-CD3 antibodies and incubated for 2 hours at 37°C. Immediately before adding the cells the antibody solution was completely aspirated from each well. Cells for coculture were isolated from WT C57BL/6, TLR7 -/-, DEREG 23.2 or OT-II/Rag2 -/- DEREG mice. The following culure conditions were used: T cells alone with 5 µg/ml

soluble anti-CD28 antibody, T cells and DCs or T cells and B cells at a ratio of 2:1 T cells to APCs (4-**$8x10^4$** T cells/well) unless indicated otherwise.
All cultures were kept in DC medium (200 µl/well) supplemented with 200 U/ml IL-2, 3-5 ng/ml TGF-β and with or without different TLR-ligands at the following optimal concentrations if not indicated otherwise:
The TLR7 stimulating Imidazoquinoline compound S-27609 (TLR7-L) was used at the final concentrations of 3 µM and R848 was used at 5 µg/ml. CL-075, a thiazoloquinolone, was also used at 5 µg/ml. The TLR9 ligand ODN CpG 1668 (TLR9-L) had the final concentration of 0.5 µM and TLR4 stimulating LPS (TLR4-L) was used at 100 ng/ml.

To stimulate TLR7 with the endogenous ligand U1snRNP which is also present in SLE autoantigens U1snRNP was complexed to the cationic lipid DOTAP for intracellular delivery. Briefly, 50 µg/ml DOTAP and 160 µg/ml U1snRNP were incubated in 50 µl PBS for 15 min at RT and then 50 µl 2x DC-Medium + 400 U/ml IL-2 + 10 ng/ml TGF-β were added. The final concentrations of DOTAP and U1snRNP in the coculture were 12.5 µg/ml and 40 µg/ml respectively.

T cells with or without DCs or B cells were cultured up to 5 days at 37°C in a cell incubator containing 5% CO_2. At the indicated time points cells were analyzed and/or used for further experiments such as re-stimulation, RNA-isolation or FACS sorting followed by suppression assays or RNA-isolation. The cell-free supernatants were collected for ELISA and stored at -20°C.

4.2.4.2 Treg induction with antigen-specific stimulation

For antigen-specific stimulation co-cultures were performed with $4x10^4$ DCs isolated from WT Balb/c mice and $8x10^4$ T cells isolated from DO11.10/Rag -/- TCR transgenic mice in the presence of 1 mg/ml OVA protein and TGF-β/IL-2 as described above. TLR7-ligand S-27609 was used at the same concentration as mentioned above.

4.2.5 Restimulation of T cells

After stimulation CD4+ T cells secrete cytokines. Which cytokine they secrete depends on the T helper cell type they differentiated into. Through the measurement of these "signature" cytokines it is possible to conclude which T helper cell type arose in the coculture. In order to get a strong cytokine response T cells had to be re-stimulated. This is achieved by PMA and Ionomycin stimulation. Cytokines were either measured by ELISA in the supernatants of the re-stimulated cells or by FACS analysis after intracellular staining.
For FACS analysis cells were stimulated for 6 hours in a 96-well round bottom plate with DC-Medium containing PMA and Ionomycin. GolgiPlug and GolgiStop were added according to the manufacturer´s instructions for the last 4-5 hours to prevent cytokine secretion.
If cytokines were to be detected in the supernatant by ELISA, cells were counted and $1.5x10^5$ cells in 150 µl DC-medium were used per stimulation in a 96-well flat bottom plate. Then cells were re-stimulated PMA + Ionomycin for 18-24 hours. Supernatants were collected after centrifugation of the plate and stored at -20°C.

4.2.6 Suppression Assay

First, naïve T cells from DEREG 23.2 or OT-II/Rag2 -/- DEREG mice were co-cultured with DCs under Treg inducing conditions with or without the TLR7 ligand S-27609 for two or four days. The co-culture result was controlled by FACS analysis.
Then, iTregs were sorted from the coculture by gating on the CD4+ GFPhigh CD25high population. Purity of the sorted iTregs was detected by intracellular Foxp3 staining.
Meanwhile, naïve CD4+ responder T cells (Tresp) were isolated from CD45.1 congenic mice and labelled with 0.5 µM CFSE for 5 min at 37°C.
Tresp alone or with iTregs were cultured in DC Medium supplemented with soluble 5 µg/ml anti-CD3 and anti-CD28 antibodies in a 96-well round bottom plate for four days. Different ratios of Tresp : iTreg were used to follow the suppressive capacity of a decreasing number of iTregs. The ratios employed were dependent on the resulting cell number of the iTregs after FACS sorting.

4.2.7 Generation and stimulation of FL-DCs

Bone marrow cells were flushed out from the femora and tibiae of C57BL/6 WT, TLR7 -/- and DEREG 23.2 x TLR7 -/- mice with a 27G needle filled with RPMI1640. Cells were spun down at 1500 rpm for 5 min at 4°C and were incubated in Red Cell Lysis Buffer for 5 min at RT. Cells were washed, counted and adjusted to a concentration of $1.5x10^6$/ml in DC-Medium. Flt3-ligand (Flt3-L) which generates a mixed culture of cDCs and pDCs was added to a final concentration of 20 ng/ml. Each well of a 6 well plate was filled with 3 ml of the cell suspension. The cells were cultured for 7 days in a cell incubator at 37°C and 5% CO_2 without disturbance.
On day 7 cells were recovered from each wellby pipetting and the pooled cell suspension was counted. After centrifugation cells were adjusted to a concentration of $1x10^6$ cells/ml in DC-Medium. $2x10^5$ cells/200 µl were stimulated with 3 µM S-27609 or 0.5 µM CpG 1668 for 24 h and then stained with antiCD86-PE. Activation of cells was controlled by FACS-Analysis.

4.3 Immunological Methods

4.3.1 Enzyme-linked Immunosorbant Assay (ELISA)

The concentrations of different cytokines in the supernatants or in the serum can be measured by performing specific sandwich ELISAs.
Detection of IL-4, IL-10, IL-17A and IFN-γ was performed by using kits according to the manufacturers instructions.
IL-6 and IL-12p40 ELISA were performed by coating Maxi-Sorp ELISA Plates with the capture antibody (dilution IL-6 capture 1:250, IL-12 p40/70 capture 1:2000) in 50 µl coating buffer and incubation for 3h at RT. Then the plates were washed 3 times and incubated with 200 µl Blocking Buffer for 1h at RT. The blocking buffer was aspirated from each well and 50 µl of the 1:2 standard dilution series (range from 8000 pg/ml - 0 pg/ml) and the diluted samples (IL-6 ELISA: sample dilution factor 1:4; IL-12p40 ELISA: sample dilution factor 1:10) were added and incubated overnight at 4°C. After washing four times the detection antibody was added (dilution IL-6 detection 1:250, IL-12 p40/p70 detection 1:1500 in 50 µl dilution buffer) and the plates were incubated for 2h at RT, followed by washing 4 times. Streptavidin-HRP was diluted 1:3000, 50 µl was added to each well and incubated for 1h at

RT. The plate was washed 4 times and each well was filled with 100 µl of the substrate solution and incubated for 10-15 min at RT. To stop the reaction 100 µl stop solution was added to each well.
The optical density was measured by the ELISA reader at 405 nm. The resulting standard curve was assessed by point-to-point analysis and the respective cytokine concentrations in the samples were calculated accordingly. The detection limit of the ELISAs lay in the range of 15-30 ng/ml.

4.3.2 Flow Cytometry

Cells can be labelled with fluorescent dyes or fluorophores coupled to monoclonal antibodies. Fluorescent dyes like CFSE or PI stain cells independently of their cell type but can be used to detect cell proliferation or cell death. By contrast specific antibodies recognize only specific epitopes on the surface or inside cells. By choosing antibodies binding to cell type specific surface molecules it is possible to distinguish different populations and also to characterize their activation status.
Fluorescently labelled cells are detected by flow cytometry (FACS) where single cells pass through laser beams and can be distinguished according to their size (forward scatter = FSC), granularity (side scatter = SSC) and fluorescence (FL1 – 10). Analysis was performed on a FACS Calibur (4 colours) or Gallios (up to 10 coulours) flow cytometer.
It is also possible to separate different cell populations by flow cytometry and sorting. This was done on a FACS Aria or MoFlo cell sorter.

4.3.2.1 Surface antibody staining

Before staining, cells were washed once in FACS buffer. All centrifugation steps were performed at 1500 rpm for 5 min at 4°C. After this washing step up to $1x10^6$ cells were suspended in 50 µl staining solution which consisted of 25 µl FACS buffer, 25 µl Fc-Block supernatant and the antibodies in a final dilution of 1:200. The cells were incubated for 15 min at 4°C in the dark und then washed 3 times in 200 µl FACS buffer. For FACS analysis cells were suspended in 100 – 200 µl FACS buffer and immediately before analysis PI was added in a final concentration of 1.5 – 3 µg/ml to distinguish life and dead cells.

4.3.2.2 Intracellular staining

It is also possible to stain for molecules which are inside the cells. To this end cells have to be fixed and permeabilised to get access to the intracellular epitopes which are then recognized by the antibody.
First, cells are stained for surface molecules following the surface staining protocol (see above) with the exception that EMA instead of PI was added to the staining solution to distinguish life and dead cells. Then cells are fixed and permeabilised with the particular buffers (see protocols for cytokine and Foxp3 staining below) and stained with the specific antibodies.

4.3.2.3 EMA staining

Since cells are permeabilised for intracellular staining it is not possible to use PI to distinguish life and dead cells. Therefore EMA was used which, like PI, diffuses into dead cells and intercalates into DNA. But unlike PI, EMA covalently binds to the DNA upon exposure to light and can not be washed out or diffuse into former life cells after permeabilisation.
EMA was dissolved in DMSO at a concentration of 5 mg/ml and stored at -20°C. It was found that the optimal concentration for EMA staining was 5 µg/ml.
All steps had to be performed in the dark and EMA staining was carried out simultaneously with the surface antibody staining (see above). After the incubation time cells were brought near a light source for 15 min and then the cells were washed 3 times. EMA is detected in the FL-3 channel.

4.3.2.4 Cytokine staining

After the 3 washing steps cells were suspended in fixation buffer for 20 min at RT. After fixation the cells are smaller and harder to spin down. Therefore all centrifugation steps following fixation were performed at 2000 rpm for 5 min. After fixation cells were centrifuged and incubated in permeabilisation buffer for 20 min at RT. Then cells were suspended in permeabilisation buffer containing antibodies against different cytokines. Cells

were incubated for 20 min in the dark at RT and washed times 3 times with permeabilisation buffer. At last cells were suspended in FACS Buffer.

4.3.2.5 Foxp3 staining

Staining of the intranuclear transcription factor Foxp3 was carried out with the Foxp3 staining kit from ebioscience which contains its own fixation and permeabilisation buffer system and an antibody against Foxp3. Staining was performed according to the manufacturer´s instructions with the one exception that the antibody incubation time was reduced to 15-20 min. Centrifugation was performed at 1600 rpm for 4 min after fixation.

4.3.2.6 CFSE staining

CFSE is a fluorescent dye which enters the cytoplasm of cells and binds covalently to intracellular proteins. It can be used to monitor lymphocyte proliferation *in vitro* and *in vivo* due to the progressive halfing of CFSE fluorescence within daughter cells after each cell division. Since CFSE is toxic for cells at high concentrations staining must be performed under optimal conditions. Staining time, staining buffer, cell concentration and CFSE concentration play a role for the optimal staining procedure.
It was found that CFSE staining for *in vitro* culture of CD4+ T cells was optimal using the following protocol: T cells were washed once in FACS buffer and then suspended in FACS buffer at a concentration of $2x10^6$ cells/ml in a 50 ml conical tube. All centrifugation steps were performed at 1500 rpm for 5 min. CFSE was diluted to a concentration of 1 µM in FACS buffer in the same volume as the cell suspension so that after mixture of CFSE and cells the cell concentration was $1x10^6$ cells/ml and CFSE had a final concentration of 0.5 µM. CFSE dilution and cell suspension were mixed very fast by pipetting up and down and then cells were incubated at 37°C in a water bath. After 5 min the reaction was stopped immediately by adding the same volume of pure FBS. Then RPMI 1640 (Gibco) 10% FBS was added ad 50 ml. Cells were centrifuged, washed in 10 ml RPMI 1640 10% FBS and again centrifuged. After this the CD4+ T cells were suspended in DC-Medium.
For use of CFSE staining *in vivo* another staining protocol was applied since a higher CFSE intensity had to be achieved. Therefore $1x10^7$ T cells/ml were stained using 5 µM CFSE in

PBS/0.1% BSA for 10 min at 37°C. After staining cells were washed one time in PBS and then solved at a concentration of 1-2x10^7 T cells/ml in PBS for i.v. injection.

4.3.2.7 Cell sorting

After cell surface staining of DEREG 23.2 or OT-II Rag2 -/- DDEREG cells from the coculture with CD25-PE and CD4-PECy5 and washing, cells were suspended in FACS sort buffer at a concentration of max 10^6 cells/ml and passed through a 50 µm Filcon filter before cell sorting. PI was not added to the cell suspension. Sorted cells were collected in a Gold Tip Tube containing pure FBS after sorting.

DEREG 23.2 Tregs express eGFP under the control of the Foxp3 promotor. So it is possible to distinguish Tregs induced in the coculture from other T helper cell types by GFP expression and separate them. But as shown in Figure 5, GFP expression does not completely overlap with Foxp3 expression in cultured cells. Since Foxp3can only be stained intracellulary it had to be determined prior to sorting which population is CD25+ GFP+ Foxp3+.

Therefore, it was investigated first by intracellular Foxp3 staining on which cells the gate has to be set for future sortings to achieve a high purity of Foxp3+ T cells. The CD25high GFPhigh population was selected which was most likely to contain a very high percentage of Foxp3+ T cells (see Figure 5, red squares).

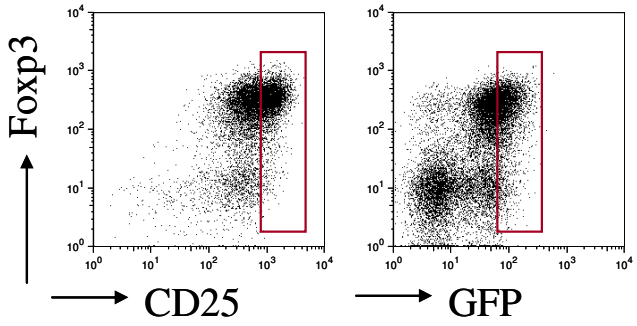

Figure 5: Determiantion of Foxp3+ T cell population for cell sorting
Isolated Dereg 23.2 splenic naive CD4+ T cells were incubated under Treg inducing conditions (TGF-ß, IL-2). On day 4, cells were stained for CD4, CD25, Foxp3. A fraction of cells also expressed GFP. Red squares indicate that the cells which are CD25high and GFP high are most likely to also express Foxp3.

4.4 Molecular Biology

4.4.1 Tail Lysis and PCR for Genotyping

To determine the genotype of DEREG 23.2 mice and TLR7 -/- mice, a 0.5 – 0.6 cm piece of the tail was cut off and incubated in 200 µl Direct PCR Tail Lysis Reagent supplemented with 2 mg/ml Proteinase K for 3-16 hours at 55°C and 1200 rpm in a Thermomixer. Then the solution was incubated at 85°C for 45 min, followed by centrifugation for 10 sec. This solution could be directly used for PCR Analysis. The PCR-Mix and program for GFP and TLR7 can be viewed in the tables below.

PCR-Mix:

GFP		TLR7 WT		TLR7 -/-	
Mastermix:		A dest	18.25 µl	A dest	18.25 µl
		10x Taq Buffer	2.5 µl	10x Taq Buffer	2.5 µl
GoTaq	10 µl	$MgCl_2$	1.5 µl	$MgCl_2$	1.5 µl
P162	0.5 µl	dNTP	0.5 µl	dNTP	0.5 µl
P163	0.5 µl	AKM128	0.5 µl	AKM129	0.5 µl
A dest	7 µl	AKM129	0.5 µl	AKM130	0.5 µl
		Taq	0.25 µl	Taq	0.25 µl
18 µl mastermix					
+ 2 µl DNA		+ 1 µl DNA		+ 1 µl DNA	

PCR-Program:

GFP		TLR7 WT		TLR7 -/-	
94°C	5 min	94°C	3 min	94°C	3 min
94°C	1 min	94°C	30 sec	94°C	30 sec
62°C	1 min 32x	67°C	30 sec 35x	64°C	1 min 35x
72°C	1 min	72°C	1.5 min	72°C	1.5 min

72°C 10 min 4°C ∞	72°C 7 min 4°C ∞	72°C 7 min 4°C ∞

The PCR products were loaded on a 1% agarose gel + ethidium bromide in the case of the GFP-PCR and on a 1.5% agarose gel + ethidium bromide in the case of the TLR-PCRs for gelelectrophoresis. The gels were run at 130V for about 45 min.

4.4.2 RNA Isolation and cDNA Synthesis

RNA was isolated from suspension cells using Trizol Reagent (> $1x10^7$ cells) or the RNeasy Mini Kit (< $1x10^7$ cells) according to the manufacturer´s protocol. After the purification steps RNA was dissolved in 30 µl ultra pure H_2O. Directly after purification 1 µl DNase I (=1U) from Fermentas was added to digest genomic DNA. Digestion was performed at 37°C for 15 min. The reaction was stopped by adding 1 µl EDTA from Fermentas and incubation at 65°C for 10 min. RNA yield and integrity was measured by a Nanodrop Spektrophotometer. RNA was stored at -80°C.
In order to make cDNA the SuperScript II Reverse Transcriptase was used on 10 µl of the RNA according to the manufacturer´s instructions. Briefly, 200 Units of SuperScript II Reverse Transcriptase (= 1 µl) were added to the mastermix already containing RNA (= 19µl) and incubated 50 min at 42°C.

4.4.3 Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) is used to amplify and quantify specific DNA sequences. The procedure follows the general principle of PCR, but its key feature is that the amplified DNA is detected over time as the reaction progresses. In case of the TaqMan® Gene Expression Assay from Applied Biosystems which contains two unlabeled primers and a dye-labeled probe a specific PCR product is detected during PCR cycles through the

fluorogenic probe which binds to the specific PCR product. qRT-PCR was performed on the StepOne Plus Real-Time PCR System.
Here, qRT-PCR was used to compare the relative mRNA expression, normalized to the house-keeping gene HPRT, of different target genes –the transcription factors Foxp3, GATA3, T-bet and RORγt and the cytokines IL-6, IL-10, IL-17a and IL-23 – in coculture T cells and sorted iTregs from different coculture conditions and time points.
The relative mRNA expression was calculated according to the following formulas:

$$\Delta C_T = C_T \text{ (target gene)} - C_T \text{ (HPRT)}$$
$$\Delta\Delta C_T = \Delta C_T \text{ (control)} - \Delta C_T \text{ (sample)}$$
$$\text{Relative mRNA expression} = 2^{-\Delta\Delta Ct}$$

4.5 Statistical analysis

The results are shown as arithmetic mean of two or more independent experiments +/- the standard deviation to show how far a particular result disperses around the average. The standard deviation is calculated according to the following formula:

$$\sqrt{\frac{\Sigma (x - \bar{x})^2}{n}}$$

x = sample mean
n = sample size

The results of different experimental groups or conditions were compared using an unpaired, two-sided Student's t test to see if there was a difference between the groups. If the result was $p \leq 0.05$ the difference was assumed as significant and marked by an asterisk (*).

In the event of using results for multiple comparisons (see Figures 6, 10 and 16) the level of significance was adjusted by applying the Bonferroni correction according to the following formula:

$$p_B \leq \frac{0.05}{\text{number of comparisons}}$$

Results from the Student´s t tests were compared to the new level of significance p_B and significant differences were marked with an asterisk (*).

5 RESULTS

Naive CD4+ T cells are able to differentiate into induced CD25+Foxp3+ Tregs (=iTregs) and contribute to peripheral tolerance. The immune response to virus infections or the systemic autoimmune response in SLE in which endogenous TLR7- and TLR9-ligands are critically involved might interfere with this pathway and lead to an impaired generation and function of iTregs thus contributing to the loss of peripheral tolerance.

5.1 Influence of TLR-ligands on the generation of induced regulatory T cells

5.1.1 TLR7- and TLR9-activated DCs reduce the generation of induced regulatory T cells

To address the question if TLR-ligands, especially TLR7- and TLR9-ligands, influence iTreg generation and if any effect was direct via TLR engagement on the T cells themselves or indirect via other TLR bearing immune cells initially an *in vitro* culture model was used. In this model freshly isolated splenic naïve CD4+ T cells were cultured under Foxp3+ Treg-polarizing conditions (co-stimulation of TCR, IL-2 and TGF-β) with or without splenic DCs in a ratio of 2:1 and with or without TLR7-ligand S-27609 (TLR7-L), TLR9-ligand CpG 1668 (TLR9-L) or TLR4-ligand LPS (TLR4-L). In the cultures without DCs anti-CD28 antibody was used to achieve co-stimulation. After 4 days of culture, the supernatant was collected for ELISA and one part of the cells was used for restimulation with PMA/Ionomycin for another 24 hours while the other part was stained for CD4, CD25 and Foxp3 for FACS analysis to determine the percentage of Foxp3+ T cells (iTregs).
Figure 6A and B show the FACS results. As shown in figure 6A left column, culturing CD4+ naïve T cells alone with the respective TLR-ligands has no effect on the generation of iTregs since the percentage of Foxp3+ T cells stayed the same in all conditions. Adding DCs or DCs plus TLR4-L ($p = 0.06$) to the culture (figure 6A right column) also had no major effect on the generation of Foxp3+ T cells. By contrast, the addition of both TLR7-L ($p = 0.001$) and TLR9-L ($p = 0.001$) significantly reduced the percentage of Foxp3+ T cells in the DC/T cell co-culture to about one half compared to control without TLR-ligands. Figure 6B which

depicts the FACS plots for one representative experiment also shows that the mean fluorescence intensity (MFI) of the Foxp3 signal is reduced under these conditions reflecting lower Foxp3 expression levels in these iTregs.

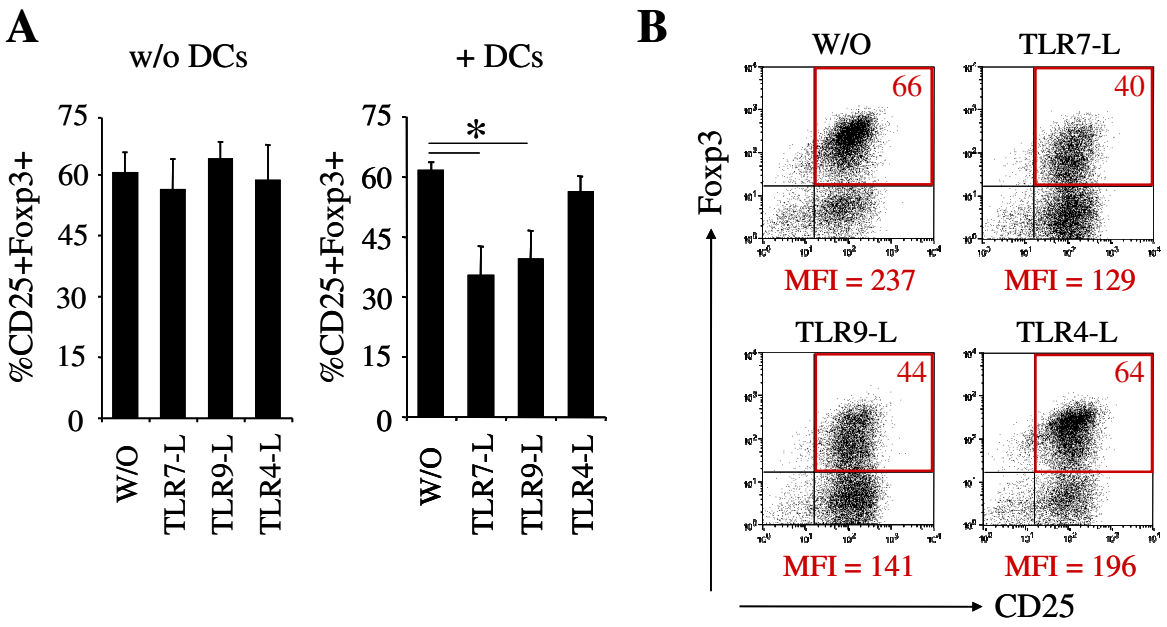

Figure 6: Inhibition of Treg induction in DC/T cell co-cultures by TLR7- and TLR9-ligands
Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with or without DCs and with or without (W/O) ligands for TLR7 (S-27609 = TLR7-L), TLR9 (CpG1668 = TLR9-L) and TLR4 (LPS = TLR4-L) for 4 days. (A) shows the mean values ± SD of 5 independent experiments of the percentage of CD25+Foxp3+ T cells in the different conditions (level of significance $p_B \leq 0.017$). (B) Results of the FACS-analysis of one representative experiment of 5 DC/T cell co-cultures are shown.

In the next step, a titration of DC numbers in the DC/T cell co-cultures was performed (Figure 7A). Again LPS had no effect on the percentage of Foxp3+ T cells, but TLR7-ligand S-27609 and TLR9-ligand CpG1668 reduced the percentage of Foxp3 expressing cells. This reduction directly correlated with the number of DCs in the co-cultures.

In the second step, experiments were performed to answer the question if the T cells become responsive to TLR-ligands through interaction with activated DCs thus leading to lower percentages of Foxp3+ T cells. Therefore, naïve CD4+ T cells and DCs from WT C57BL/6 or TLR7-/- mice were co-cultured at different compositions with or without S-27609 or CpG1668. WT T cells were cultured with WT DCs as control to see a reduction in the percentage of Foxp3+ T cells in the presence of TLR7- and TLR9-ligands (see Figure 7B). The second condition contained TLR7-/- T cells and TLR7-/- DCs. Stimulation of TLR7 did not lead to a reduction since the cells lack TLR7, but stimulation of TLR9 still led to a reduction in the percentage of Foxp3+ T cells. The next condition contained TLR7-/- T cells

and WT DCs. Both stimulation of TLR7 and TLR9 led to a reduction in the percentage of iTregs. And in the last condition, WT T cells were cultured with TLR7-/- DCs where TLR7 stimulation had little effect while TLR9 led to a clear reduction of Foxp3+ T cells.

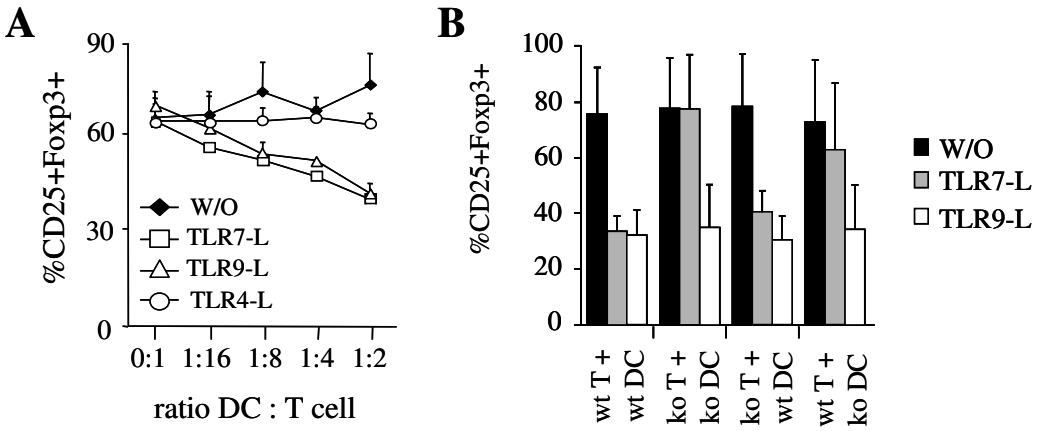

Figure 7: Reduction in the percentage of Foxp3+ T cells is dependent on DCs
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with DCs in different ratios and with or without (W/O) ligands for TLR7 (S-27609 = TLR7-L), TLR9 (CpG1668 = TLR9-L) and TLR4 (LPS = TLR4-L) for four days. Results show the mean value ± SD of two independent experiments. (B) Naïve splenic CD4+CD25- T lymphocytes from WT C57BL/6 mice (= wt) or TLR7-/- (ko) were cultured under Treg-polarizing conditions either with WT 57BL/6 or TLR7-/- DCs for 4 days with or without (=W/O) a ligand for TLR7 or TLR9. Mean values ± SD represent results from 4 independent experiments.

The results show that the reduction in the percentage of Foxp3+ T cells in response to TLR7-ligand is dependent on the presence of TLR7 in DCs and does not require TLR7 expression in T cells.

5.1.2 Cytokines in the DC/T cell co-cultures

Cytokines direct differentiation of naïve T cells into one of the different effector T helper cell lineages or into Tregs. Since cytokines produced by DCs after TLR activation as well as cytokines produced by T cells have an influence on the differentiation pathway of naïve T cells the concentration of the cytokines IL-6, IL-12p40, IL-17A, IFN-γ, IL-4, IL-10 and IL-23 were determined in the supernatants of 4 day DC/T cell co-cultures with or without TLR ligands. The results for all cytokines except IL-23 which was below detection level are displayed in Figure 8 A. The results show that IL-6 and IL-12 concentrations which are cytokines mainly produced by DCs were elevated 10 – 20 fold after TLR7 and TLR9 stimulation compared to the control without TLR stimulation, but IL-6 was only slightly

increased and there was no difference in IL-12 concentrations after TLR4 activation by LPS. IL-17A levels were found to be increased in all conditions with TLR ligands whereas IFN-γ was not induced by TLR activation but rather a reduction was observed in response to TLR7 stimulation. There were no differences in IL-4 and IL-10 levels compared to control.

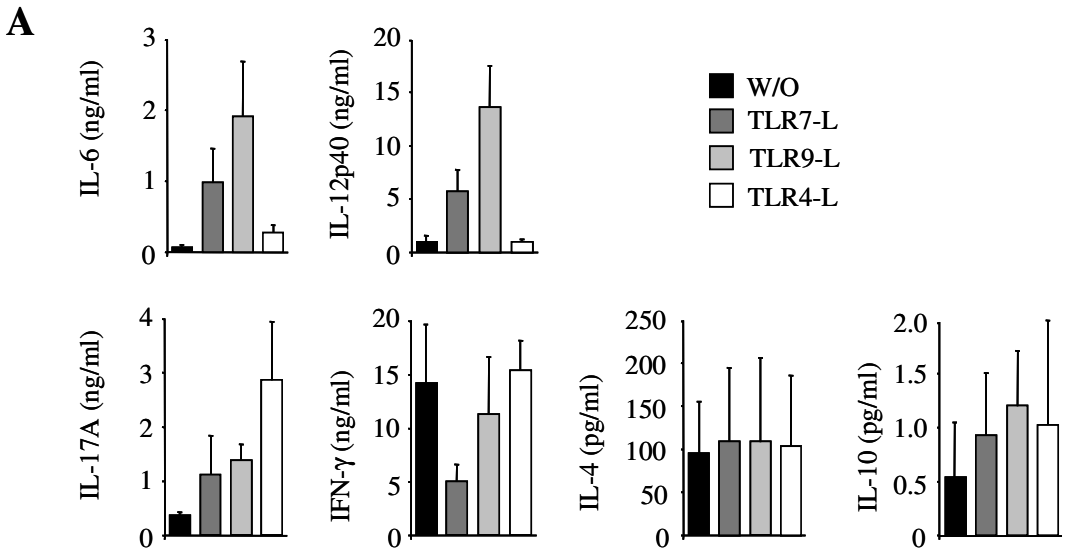

Figure 8: Cytokines in the DC/T cell co-culture and after restimulation
Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with or without DCs and with or without (W/O) ligands for TLR7 (S-27609 = TLR7-L), TLR9 (CpG1668 = TLR9-L) and TLR4 (LPS = TLR4-L) for 4 days. The supernatants of DC/T cell co-cultures were collected on day 4 of the co-culture and cytokines were measured by ELISA. The mean values ± SD (n = 4) is shown.

Expression of different co-stimulatory molecules on the DC in different co-culture conditions was determined by FACS. Since survival of freshly isolated splenic DCs is poor in culture (approximately 20% life DCs after 24 hours), expression of co-stimulatory molecules was measured after one day and not at the end of the co-culture (4 days). CD86 is shown as an example and the results are depicted in Figure 9.

Since isolated DCs can mature spontaneously in culture and are activated by contact with T cells high expression of CD86 was found even in the condition without TLR ligands and there was no difference to CD86 expression levels in TLR7- and TLR9-activated DCs. But DCs activated with LPS expressed slightly higher levels of CD86 on their surface compared to the other conditions. Thus, DCs were efficiently activated in the presence of TLR4-ligand despite production of lower levels of cytokines

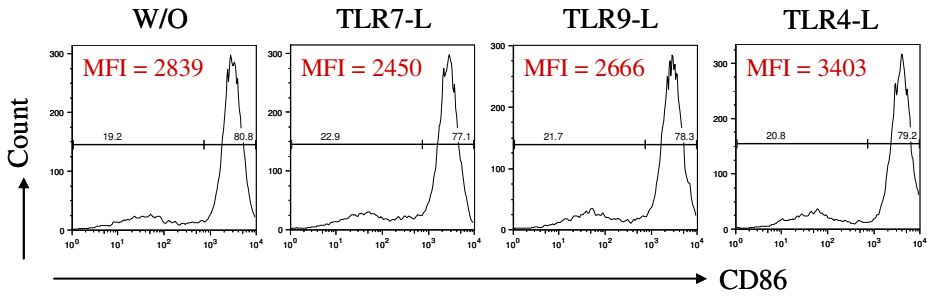

Figure 9: CD86 expression on DCs in the co-culture
DCs were isolated from the spleen of C57BL/6 mice and co-cultured with naïve splenic CD4+ T cells under Treg polarizing conditions with or without (= W/O) TLR ligands; S-27609 = TLR7-L, CpG1668 = TLR9-L and LPS = TLR4-L. After 24 hours, cells were harvested and analysed for CD86 expression on the cell surface by FACS analysis. One representative of 3 experiments is shown.

This result also demonstrates that it is not likely that the reduced Treg induction is dependent on TLR-ligand induced costimulatory molecule expression on DCs because unstimulated DCs expressed high levels of costimulatory molecules but had no effect on Treg induction.

5.1.3 Influence of B cells and other DC subtypes on the generation of induced regulatory T cells

Before investigating further which factors are responsible for the reduction in the percentage of iTregs in the co-culture the question came up if this effect was singular for TLR7 or TLR9 activated splenic DCs or if the effect can also be conveyed by other TLR expressing cell types or DCs with different properties. Therefore naïve splenic CD4+ T cells were cultured together with B cells or DCs isolated from mesenteric LNs with or without TLR ligands and their influence on the generation of Foxp3+ T cells was determined.
The results for the co-culture of naïve CD4+ T cells with B cells and with or without ligands of TLR7, TLR9 and TLR4 are similar to the results gathered for the co-culture of T cells with splenic DCs (see Figure 10 A): TLR4 stimulation with LPS had no effect on the percentage of Foxp3+ T cells ($p = 0.15$), but the percentage was reduced significantly when the B cells were stimulated with TLR7 ligand S-27609 ($p = 0.003$) or TLR9-ligand CpG1668 ($p = 0.006$). The reduction of the MFI of the Foxp3 signal in Foxp3+ T cells in the co-cultures containing TLR7- or TLR9-ligands was also seen.
Since TLR7 is critically involved in the development of SLE as seen in mouse models for SLE (Lee et al, 2008; Savarese et al, 2008) further experiments focused on the comparison

between co-cultures with or without TLR7-ligands. Using TLR7-ligand S-27609 in co-cultures of CD4+ T cells and mesenteric LN DCs again led to a lower percentage of Foxp3+ T cells. This shows that not only splenic DCs are able to reduce Treg induction after TLR7 stimulation, but also DCs from other compartments like the mesenteric LNs which have other properties (see discussion) as well as B cells.

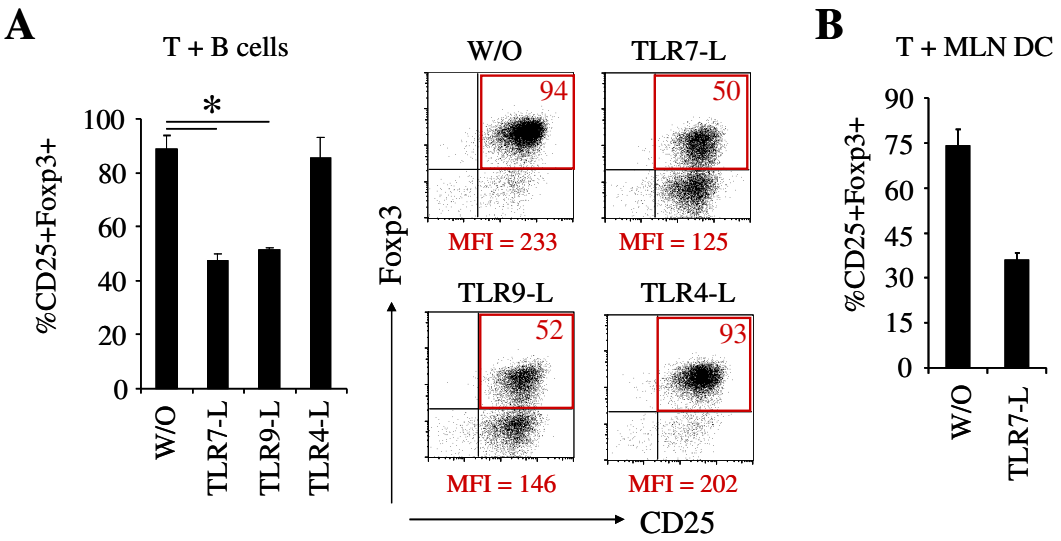

Figure 10: Influence of B cells and mesenteric LN DCs on the generation of Foxp3+ T cells
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with or without splenic B cells and with or without (W/O) ligands for TLR7 (S-27609 = TLR7-L), TLR9 (CpG1668 = TLR9-L) and TLR4 (LPS = TLR4-L) for 4 days. Left part shows the mean values ± SD of 3 independent experiments (level of significance $p_B \leq 0.017$). Right part shows the results of one representative experiment. (B) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with or without DCs isolated from mesenteric lymph nodes (MLN DC) and with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days. Mean values ± SD of two independent experiments are shown.

5.1.4 Antigen-presenting DCs reduce induction of regulatory T cells after TLR7 stimulation

DCs can not only be activated through TLRs to produce cytokines and express co-stimulatory molecules, but they are also professional antigen-presenting cells and prime antigen-specific T cells. Until now all experiments were executed using plate-bound anti-CD3 antibody to activate TCR signalling, but there was also the question if TLR7 in addition to antigen-specific stimulation by DCs had the same effect.

In order to be able to validly compare results between antigen-unspecific and antigen-specific stimulations, experiments with soluble antiCD3 antibody were conducted first. As can be seen in Figure 11 A the results are comparable to experiments with plate-bound antiCD3 antibody and the percentage of Foxp3+ T cells was reduced in the presence of TLR7-ligand S-27609 compared to the control. Since the results were comparable, further experiments with antigen-unspecific stimulation were therefore again conducted with plate-bound antiCD3 antibody.

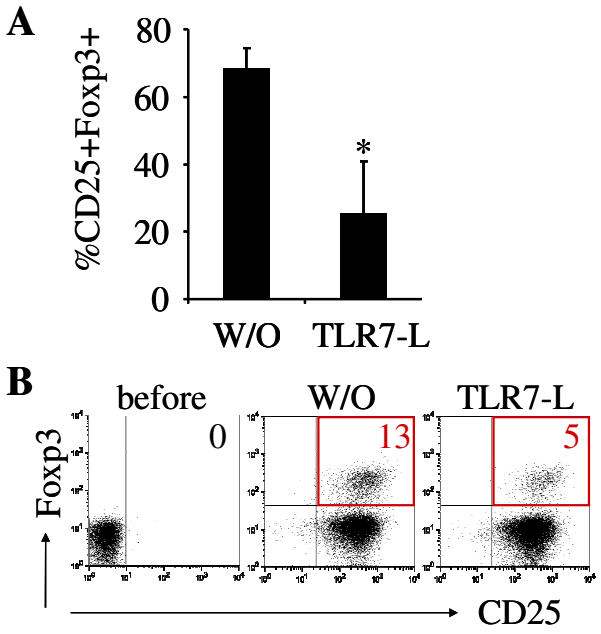

Figure 11: Reduction in the percentage of Foxp3+ T cells by antigen-presenting DCs
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs and soluble anti-CD3 antibody with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days. Mean values ± SD of 3 independent experiments is shown. (B) DO11.10/Rag2 -/- splenic T cells were cultured with Balb/c splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) and 1 mg/ml OVA for 4 days under Treg-polarizing conditions. One of 3 representative experiments are shown.

For antigen-specific stimulations naïve splenic CD4+ T cells from DO11.10/Rag2 -/- mice and splenic DCs from WT Balb/c mice were used. DO11.10 Rag2 -/- T cells express the same transgenic DO11.10 TCR specific for ovalbumin (OVA) peptide$_{323-339}$ (Murphy et al, 1990). Thus, they have the advantage that they are all truly naïve and lack Foxp3 expression. Therefore, Tregs generated in culture are truly *de novo* induced and not expanded from preexisting Tregs. T cells and DCs were cultured under Treg-polarizing conditions (IL-2 and TGF-β) with or without TLR7-ligand S-27609. For antigen presentation by DCs 1 mg/ml OVA protein was added to the culture. After 4 days cells were analysed by FACS. Although the percentage of Foxp3 expressing cells was low compared to experiments with antigen-unspecific stimulations the same difference in Foxp3+ T cell percentages was found between the control without TLR ligand and the condition in which the TLR7 ligand was present. This means that iTregs are induced in the presence of antigen-presenting DCs under Treg-polarizing conditions but simultaneous TLR7 activation impairs this process.

5.1.5 Synthetic and endogenous TLR7-ligands impair regulatory T cell induction

In all experiments so far the TLR7 agonist S-27607 was used which is an Imiquimod-related synthetic compound (Smorlesi et al, 2005). To exclude the possibility that the reduction in the percentage of Foxp3+ expressing T cells was limited to this TLR7-ligand alone, several commercially available synthetic TLR7 agonists like CL-075 and R848 were tested in the co-cultures. As can be seen in Figure 12 A, all synthetic TLR7 ligands had the same decreasing effect on the percentage of iTregs.

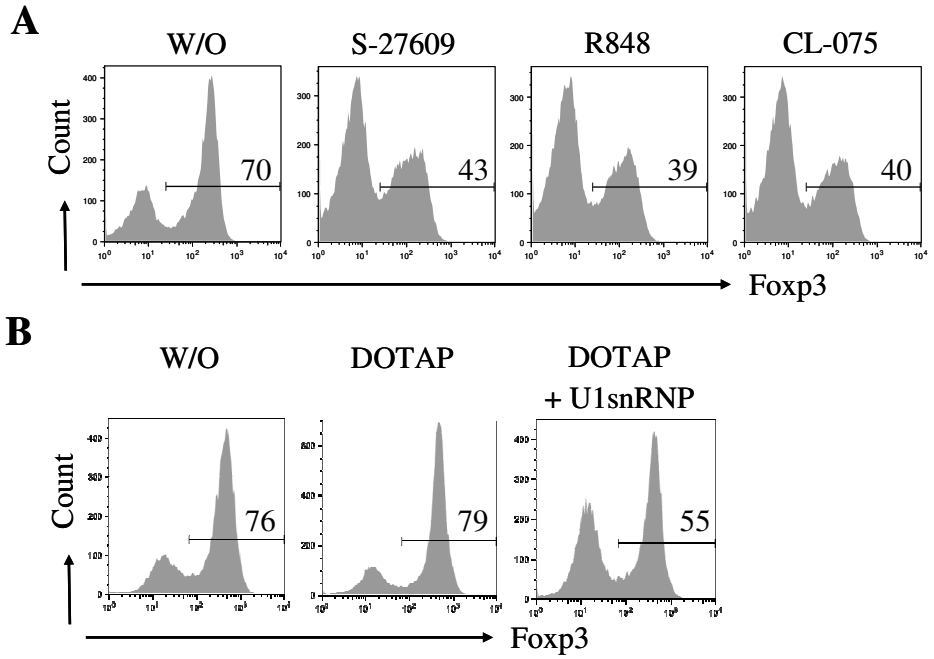

Figure 12: Synthetic and endogenous TLR7-ligands reduce the percentage of Foxp3+ T cells
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with or without splenic DCs and with or without (W/O) the synthetic TLR7-ligands S-27609, R848 or CL-075 for 4 days. One of 2 representative experiments is shown. (B) CD4+CD25- T cells were cultured under Treg polarizing conditions in the presence of splenic DCs with DOTAP alone or U1snRNP complexed with DOTAP. Foxp3 expression was assessed after 4 days. The percentages of Foxp3+ T cells of one out of 3 experiments are shown in the histograms.

Furthermore, experiments were conducted using an endogenous TLR7-ligand were conducted. As endogenous TLR7-ligand U1snRNP was used which is part of the mammalian spliceosome and consists of the unique small nuclear RNA molecule U1-snRNA, 3 specific associated proteins and the 7 core proteins called Sm proteins. In SLE, autoantibodies can be found against different parts of U1snRNP and the U1-snRNA part of U1snRNP itself acts as

an endogenous ligand of TLR7 leading to DC maturation, cytokine production and lymphocyte activation (Kattah et al, 2010). U1snRNP can be purified from cell nuclear extracts and, complexed to a cationic lipid, can be used to stimulate DCs via TLR7 (Savarese et al, 2006).
As a cationic lipid the transfection reagent DOTAP was chosen and U1snRNP was complexed to DOTAP prior to use in the DC/T cell co-culture. DOTAP alone was used in the co-culture as control. Figure 12 B shows that DOTAP alone had no effect on the generation of Foxp3+ T cells, but a complex consisting of DOTAP and the TLR7-ligand U1snRNP led to a reduction in the percentage of Foxp3+ T cells. Therefore, activation by endogenous TLR-ligands might not only directly stimulate immune cells but might also affect Treg induction.

5.1.6 TLR7 activated DCs lead to increased T_H17 cell differentiation

From the cytokine data (see 5.1.2 and Figure 8) the notion was that activation of DCs through TLR7 and the resulting cytokine response, namely increased production of IL-6, might favour T_H17 differentiation since TGF-β combined with IL-6 are the key cytokines to drive the T_H17 cell generation. T_H17 cell differentiation instead of Treg induction might be the cause for the reduced percentage of Foxp3+ T cell in the co-culture. To confirm this idea, after 4 days of co-culture in the presence or absence of S-27609 T cells were restimulated for 6 hours with PMA/Ionomycin and then cells were stained intracellularly for different cytokines (IL-17, IFN-γ, IL-10 and IL-4). In addition to that, co-culture cells were used for RNA isolation followed by qRT-PCR to determine the relative mRNA expression of genes known as signature transcription factors for different T helper cell lineages (*rorc* for T_H17, *tbx21* for T_H1, *gata3* for T_H2 and *foxp3* for Tregs) and different cytokine genes (*il-10, il-17a, il-23* and *il-6*).
The results for the intracellular cytokine stainings are shown in Figure 13 A and indeed a 2.5 – 9 fold increase in IL-17-producing T cells was observed when a TLR7 ligand was present in the co-culture, but the overall percentage of these cells stayed below 1% of all CD4+ T cells, so it is unlikely that the lower percentage of Foxp3+ T cells was entirely due to differentiation into T_H17 cell instead of Tregs. Also a 6- fold increased expression of *rorc* (RORγt) compared to control and a five fold increased expression of IL-17, but no increase in IL-23 (Figure 13 B) was observed.
The gene for the T_H1 signature transcription factor T-bet (*tbx21*) and the gene encoding IL-10 were not differently expressed compared to control and expression of gata3 could not be

detected at all. Thus, it is unlikely that T_H1, T_H2 or IL-10 producing T cells differentiate in favour of iTregs and thus lead to the reduction in the percentage of Foxp3+ T cells in co-cultures with TLR7-ligands.

But an increase in IL-6 expression was found on the mRNA level compared to control. This is either due to remaining IL-6 producing DCs in the co-culture which upregulated IL-6 expression after TLR7 activation although splenic DC survival in cultures is very poor after 2 days. Or it could be due to T cells which have the ability to produce IL-6 themselves, for example T_H17 cells (Korn et al, 2009). Foxp3 mRNA expression was downregulated by about one half to one third compared to control correlating with the lower percentage of iTregs and lower expression level of Foxp3 in the co-cultures containing TLR7-ligand.

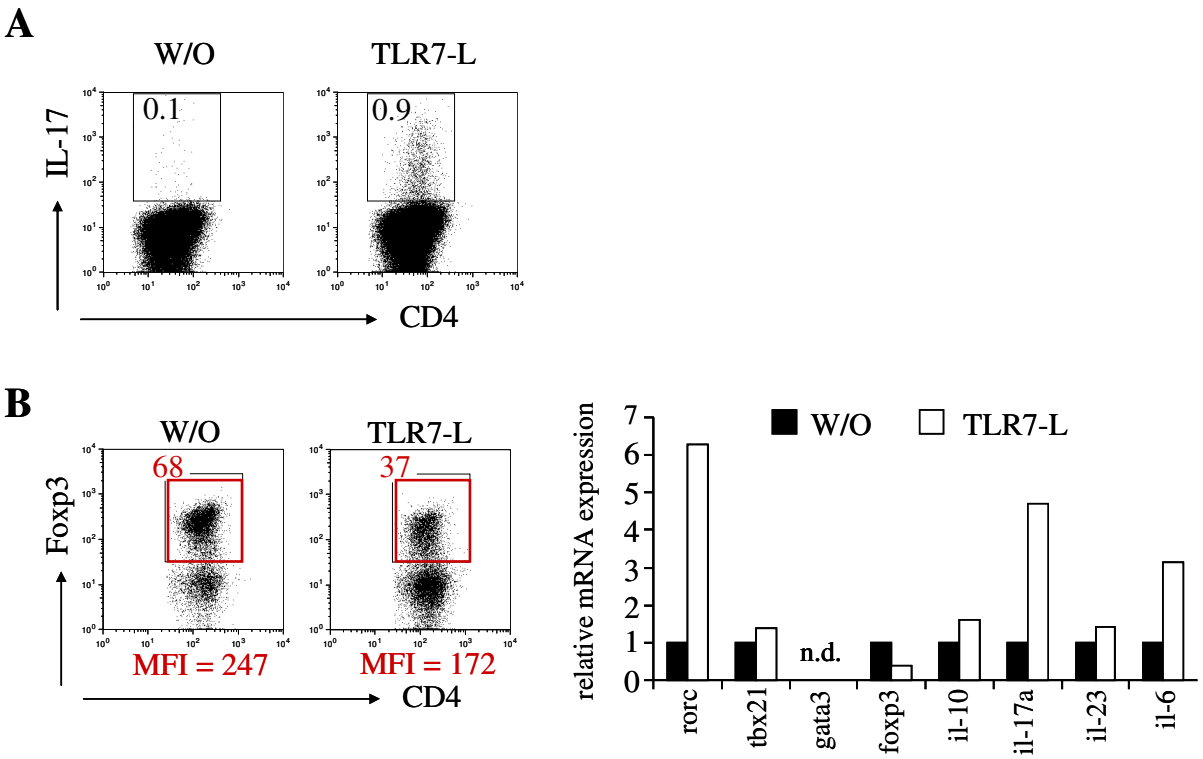

Figure 13: Intracellular staining and qRT-PCR of T cells generated in the co-cultures
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with or without splenic DCs and with or without (W/O) TLR7-ligand S-27609 (=TLR7-L) for 4 days and then restimulated for 6 hours with PMA/Ionomycin. Cells were fixed and stained intracellularly for IL-17a production. One representative of 3 experiments is shown. (B) Naïve splenic CD4+CD25- T lymphocytes from Dereg 23.2 mice were cultured under Treg-polarizing conditions with or without splenic DCs and with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days. Then RNA was isolated and relative mRNA expression of Foxp3, RORγt, T-bet, GATA3, IL-17, IL-10, IL-23 and IL-6 was determined by quantitative RT-PCR using Taqman primers and probes (right column). Some cells were retained for FACS-analysis (left column). (n.d. = not detected)

5.1.7 IL-6 accumulation in co-cultures over time

Three times higher IL-6 expression on the mRNA level was reproducibly detected in co-cultures with TLR-ligands compared to control after 4 days (see Figure 13 B). This was surprising because in the DC stainings described under 5.1.2 and Figure 9 it was observed by means of PI staining that after one day only 20-30% of DCs were still alive and after two days only 10-15%. Since there is also the possibility of T cells to acquire the ability to produce IL-6 the concentration of IL-6 was followed in the co-cultures over several days by taking supernatants every day and measuring the IL-6 concentration.
As can be viewed in Figure 14, the IL-6 concentration increased every day in the supernatant over the course of five days. Interestingly, when comparing the changes in concentration between day 1 and day 3 (Δ 208 pg/ml) and between day 3 and day 5 (Δ 464 pg/ml), it is obvious that the IL-6 concentration rises much faster after three days than in the beginning. Considering the fact that after two days almost all DCs are dead and more so after three or four days it seems unlikely that such an increase can be attributed to IL-6 production by DCs. The more likely explanation in this circumstance would be that IL-6 is also produced by T cells which gained this ability during the co-culture.

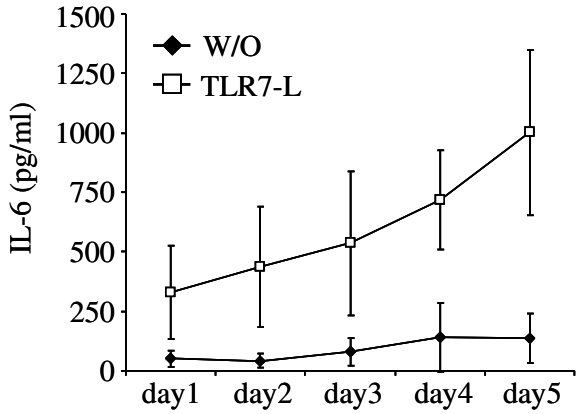

Figure 14: IL-6 concentration in the supernatant over time
Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for one to 5 days. Supernatant was taken every day and IL-6 concentration in the supernatant was measured by ELISA. Mean value ± SD of 3 independent experiments is shown.

5.2 Reduced regulatory T cell induction is mediated by IL-6

In the next step our experiments were designed to investigate what factor or factors lead to the lower percentage of Foxp3+ T cells in cultures with DCs and TLR7-ligands. There were 3 possibilities: soluble factors like cytokines produced after TLR7 stimulation which lead to the

reduction, a cell contact-dependent mechanism or a combination of both. Since no differences in the expression of co-stimulatory molecules on DCs could be detected between the control and TLR7 stimulated DCs (see Figure 9), but different cytokine profiles were observed, our focus lay on the possibility that the reduction is due to a soluble factor.

Therefore, first experiments were conducted with cell component free supernatant from DCs which were stimulated with TLR7-ligand for 48 hours. This supernatant was used in different dilutions either on cultures of TLR7-/- naïve CD4+ T cells alone or on co-cultures of TLR7-/- T cells and DCs (TLR7-/- cells were used to avoid direct effects of the TLR7-ligand present in the supernatant). Figure 15 A shows a reduction in the percentage of Foxp3+ T cells in both conditions and this reduction correlated with the concentration of the supernatant. The effect of the DC-supernatant on the T cell culture shows that there is no need for cell contact between T cells and DCs to convey the effect of TLR7 stimulation. To confirm this finding we conducted transwell experiments where DCs and T cells were separated from each other by a membrane which is permeable for soluble factors. After four days, no differences in the outcome between normal DC/T cell co-cultures and transwell co-cultures were detected (Figure 15 B). Therefore, it was concluded that the reduction in Foxp3+ T cells is mediated by a soluble factor.

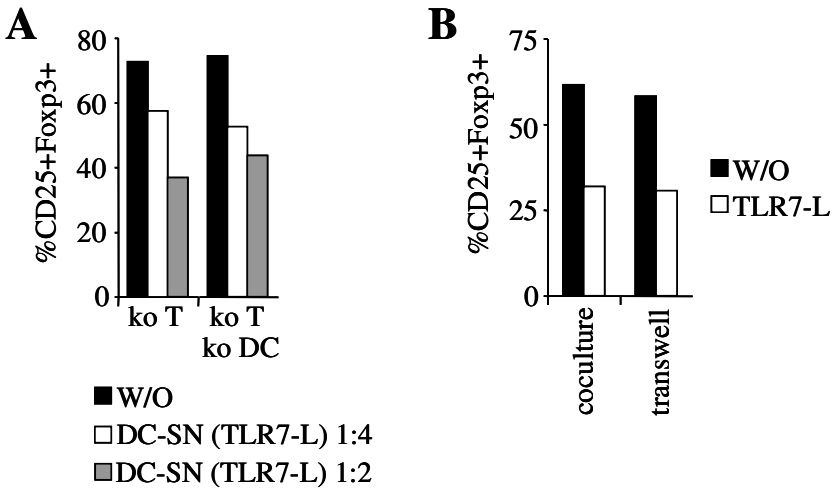

Figure 15: Reduction of Foxp3+ T cells is mediated by soluble factor
(A) $8x10^4$ C57BL/6 DCs were stimulated with TLR7-ligand S-27609 (=TLR7-L) for 48 hours and then the supernatant (=DC-SN) was collected and filtered. TLR7 -/- (= ko) T lymphocytes with or without TLR7 -/- DCs were cultured unter Treg-polarizing conditions with or without DC supernatant in different dilutions. The percentage of iTregs was measured after 4 days. One of 3 representative experiments is shown. (B) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions together with (= coculture) splenic DCs or separated from DCs by a transwell insert (= transwell) with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days. One of 2 experiments is shown.

To identify the responsible soluble factor neutralizing experiments were executed. Several factors which might contribute to the reduction in Foxp3 expression came into consideration. First of all, there were the two cytokines IL-6 and IL-12 because they were elevated in co-cultures with TLR7- or TLR9-ligands, but not in co-cultures with TLR4-ligand LPS which also had no effect on the percentage of Foxp3+ T cells. According to the mRNA and cytokine staining data, IL-12, which is the driving factor in T_H1 differentiation, did not seem to have an effect on rising T_H1 differentiation in the co-culture whereas elevated T_H17 differentiation was observed.

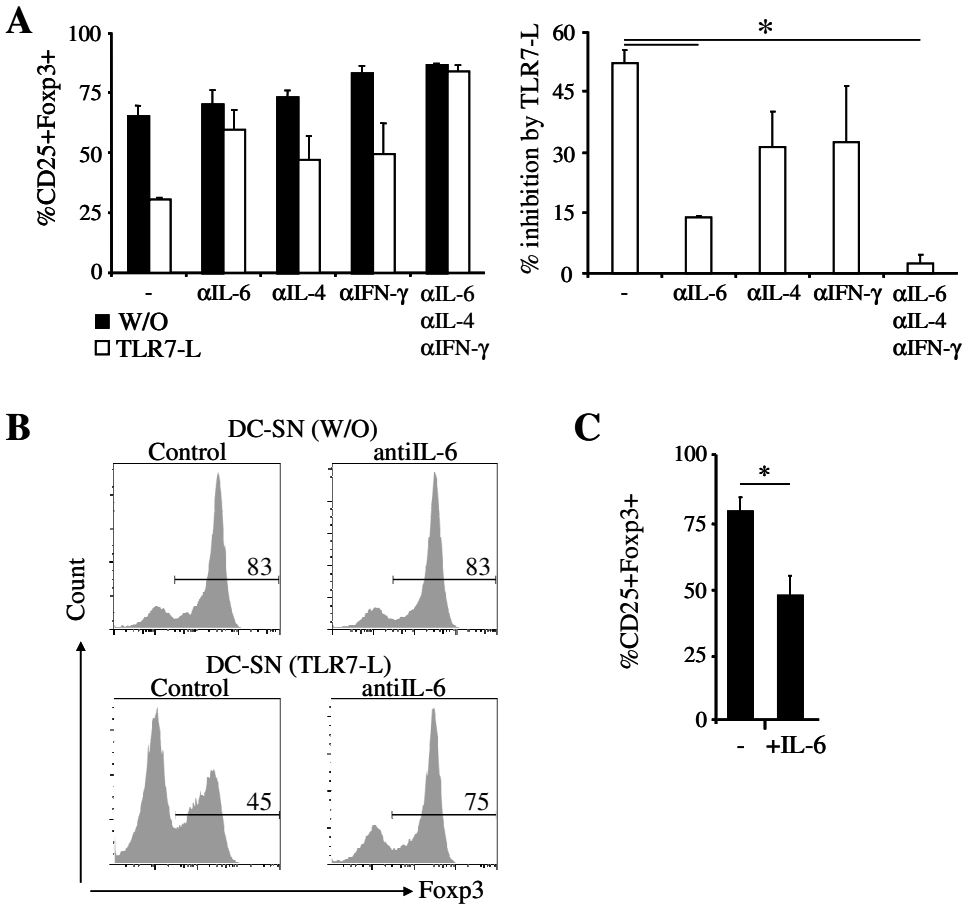

Figure 16: IL-6 as the driving factor behind the reduction in the percentage of Foxp3+ T cells
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) and neutralizing antibodies against IL-6 (5 µg/ml antiIL-6 + 2 µg/ml antiIL6Rα), IL-4 (2 µg/ml antiIL-4) or IFN-γ (2 µg/ml antiIFN-γ). The percentage of iTregs was measured after 4 days (left column) and the percentage of the inhibiton by theTLR7-ligand was calculated for all conditions (right column). Mean values ± SD of 3 experiments are shown (level of significance $p_B \leq 0.0125$). (B) Supernatants of $8x10^4$ DCs which were stimulated for 48 hours with or without (= W/O) TLR7-ligand S-27609 (= TLR7-L) was pre-incubated with antibodies against IL-6 (= antiIL-6) for 1 hour prior to use in the culture of TLR7 -/- T lymphocytes. IL-6 in control supernatant (= Control) was not neutralized. The supernatant was diluted 1:2 in the co-culture. One of 2 experiments is shown. (C) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with or without (-) recombinant IL-6 (3 ng/ml) in the presence of splenic DCs for 4 days. The mean values ± SD (n=3) of the percentage of iTregs is shown.

So between these two cytokines the focus was set on neutralizing IL-6 as it was also reported to interfere with the generation of Foxp3+ T cells (Bettelli et al, 2006). Besides IL-6, the cytokines IFN-γ and IL-4 were neutralized since these cytokines were found in the co-culture and were also reported to inhibit Foxp3 expression in an autocrine manner (Wei et al, 2007).

The results in Figure 16 A (right column) represent percent inhibiton by the TLR7-ligand. They show that blocking IL-6 by adding neutralizing antibodies against both IL-6 and the IL-6 receptor α-chain (IL-6Rα) at the beginning of the co-culture greatly reduced the inhibitory effect on TGF-β induced Foxp3 expression mediated by TLR7-ligand, because instead of a reduction of 52.1±3.3% without neutralizing antibodies we observed only a reduction 13.9±0.3% after neutralizing IL-6 ($p = 0.00008$).

Neutralizing antibodies against IL-4 (31.8±8.8% versus 52.1±3.3% inhibition, $p = 0.04$) and IFN-γ (32.5±13.9 versus 52.1±3.3% inhibition, $p = 0.12$) had only minor effects in reversing the inhibitory effect compared to the neutralization of IL-6. The strongest reduction of the inhibitory effect mediated by TLR7-ligand was achieved by simultaneously neutralizing IL-6, IL-4 and IFN-γ (2.4±2.3% versus 52.1±3.3% inhibition, $p = 0.00006$). Nonetheless, it was clear that IL-6 was the driving factor behind the reduction in the percentage of iTregs with only a minor contribution by IL-4 and IFN-γ.

To further support this interpretation, IL-6 was neutralized in the supernatant of DCs stimulated with TLR7 prior to use in the culture of naïve TLR7 -/- CD4+ T cells (Figure 16 B). Supernatant of unstimulated DCs with or without neutralizing antibodies against IL-6 had no effect on Treg induction whereas the supernatant of TLR7-ligand stimulated DCs reduced the percentage of Foxp3+ T cells by about 54%. This reduction was inhibited to about 10% by prior neutralization of IL-6 in the supernatant. This result is in concordance with the effect of neutralizing IL-6 directly in the co-culture.

Since the neutralization experiments strongly suggested IL-6 as the driving factor of the inhibitory effect mediated by the TLR7-ligand, recombinant IL-6 was added to the culture of naïve CD4+ T cells. The addition of recombinant IL-6 reduced the percentage of Foxp3+ T cells in the culture as effectively as the stimulation of DCs with a TLR7-ligand (see Figure 16 C) confirming IL-6 as the driving factor in the inhibition of iTreg induction.

5.3 Phenotypic changes in iTregs meditated by TLR7-stimulated DCs

The addition of TLR7-ligands to DC/T cell cultures reduced the percentage of Foxp3+ T cells and the MFI of the Foxp3 signal in iTregs which hinted to an impaired Treg function as Foxp3 expression is essential for the suppressive function of Tregs. But also several Treg signature surface molecules are reported to be important for full Treg function, like CTLA-4 and CD103. CTLA-4 was found to interact with CD80 and CD86 on DCs and seems to be involved in the suppression of proliferation (Shevach, 2009). CD103 is reported to be expressed by effector/memory type Tregs and by iTregs. The CD103+ Tregs are highly potent in immunosuppression and represent "inflammation-seeking" Tregs *in vivo* (Huehn et al, 2004; Zhao et al, 2008). Changes in the expression of CTLA-4 and CD103 might also lead to a change in the suppressive capacity of iTregs.

To address the question if the addition of TLR7-ligands to DC/T cell co-culture change the expression of these two molecules in Tregs, the T cells were stained after 4 days of co-culture with or without TLR7-ligand S-27609 for CD4, CD25, Foxp3 and CTLA-4 or CD103.

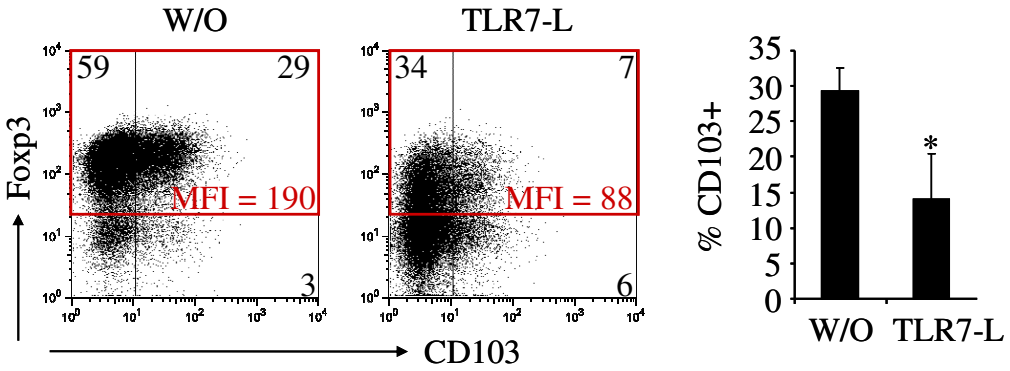

Figure 17: Reduction in the percentage of CD103+Foxp3+ T cells
Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions in the presence of splenic DCs and with or without (= W/O) TLR7-ligand S-27609 (= TLR7-L) for four days. FACS plots show the results of Foxp3 and CD103 staining, column on the right show the mean value ± SD of the percentage of Foxp3+CD103+ Tregs (n = 3).

No differences in the percentage of CTLA-4+Foxp3+ Tregs could be detected. In both conditions, over 95% of Foxp3+ T cells were are also CTLA-4+ (data not shown). In contrast to this finding, only about one third of Foxp3+ T cells also expressed CD103 on the cell surface in the condition without TLR-ligand. Addition of the TLR7-ligand reduces the

CD103+Foxp3+ T cell fraction by 50% (see Figure 17). The reduced CD103+ iTreg fraction might lead to a reduced suppressive capacity of the whole iTreg population.

5.4 Influence of TLR7-ligands on Tregs in vivo

5.4.1 Tregs in the pristane-induced mouse model of SLE

Determining that the activation of TLR7 in DCs or B cells leads to a reduced percentage in Foxp3+ T cells mediated through IL-6, the effect of TLR7 activation on Tregs *in vivo* was also of interest. To this end, the pristane-induced mouse model of SLE was employed. Injection of the mineral oil pristane into the peritoneal cavity leads to inflammation, apoptosis of peritoneal cells and in the end to a lupus-like disease including autoantibody production and immune complex deposition in the kidney leading to glomerulonephritis after around 6 months (Conti et al, 2011; Lech et al, 2010; Perry et al, 2011; Savarese et al, 2008). It was reported that TLR7 plays an important role in disease development since anti-snRNP antibody production and immune complex nephritis was completely dependent on TLR7 expression (Savarese et al, 2008). It was also shown that TLR7 activation is essential for the increase in immature monocytes in the peritoneal cavity and the early IFN-I production only days after pristane injection (Lee et al, 2008). For our experiments, the focus was laid on this early TLR7-dependent response to pristane injection as it stands to reason that the breakdown of peripheral tolerance which leads to autoantibody production and hyperactivity of T and B cells critical at the beginning of disease development.

In order to investigate TLR7-dependent differences in the Treg compartment shortly after pristane injection the composition of CD4+ T cells in the peritoneal-lavage of C57BL/6 wt and TLR7 -/-mice or of DEREG 23.2 and DEREG 23.2 x TLR7 -/- mice was compared 8 days after injection. In addition to that, the IL-6 concentration was measured in the peritoneal-lavage fluid and in the serum since the reduction in the percentage of Foxp3+ T cells *in vitro* was mediated by IL-6.
First the IL-6 concentration was compared in the peritoneal-lavage between WT and TLR7-/- mice. In the peritoneal-lavage from WT mice injected with pristane the IL-6 concentration was increased 30-fold compared to the PBS control. Elevated IL-6 levels were also found in

the peritoneal-lavage of TLR7-/- mice injected with pristane but the IL-6 concentration was reduced by half compared to WT.

In the FACS-analysis, a small increase in the percentage of Foxp3+ T cells in the CD4+ T cell pool was detected after pristane injection. Despite the reduction of IL-6 levels in the peritoneal-lavage fluid in TLR7-/- versus WT mice, no differences in the percentage of Foxp3+ were detected when comparing WT to TLR7-/- mice. Also no differences could be found in the percentage of Foxp3+CD103+ T cells as both in WT and TLR7-/-mice a 3.5-fold increase in this population was found compared to the PBS control. It has to be taken into consideration that nTregs may be preferentially recruited to the peritoneal cavity after pristane injection in contrast to our *in vitro* studies where *de novo* Treg induction from naïve T cells was investigated.

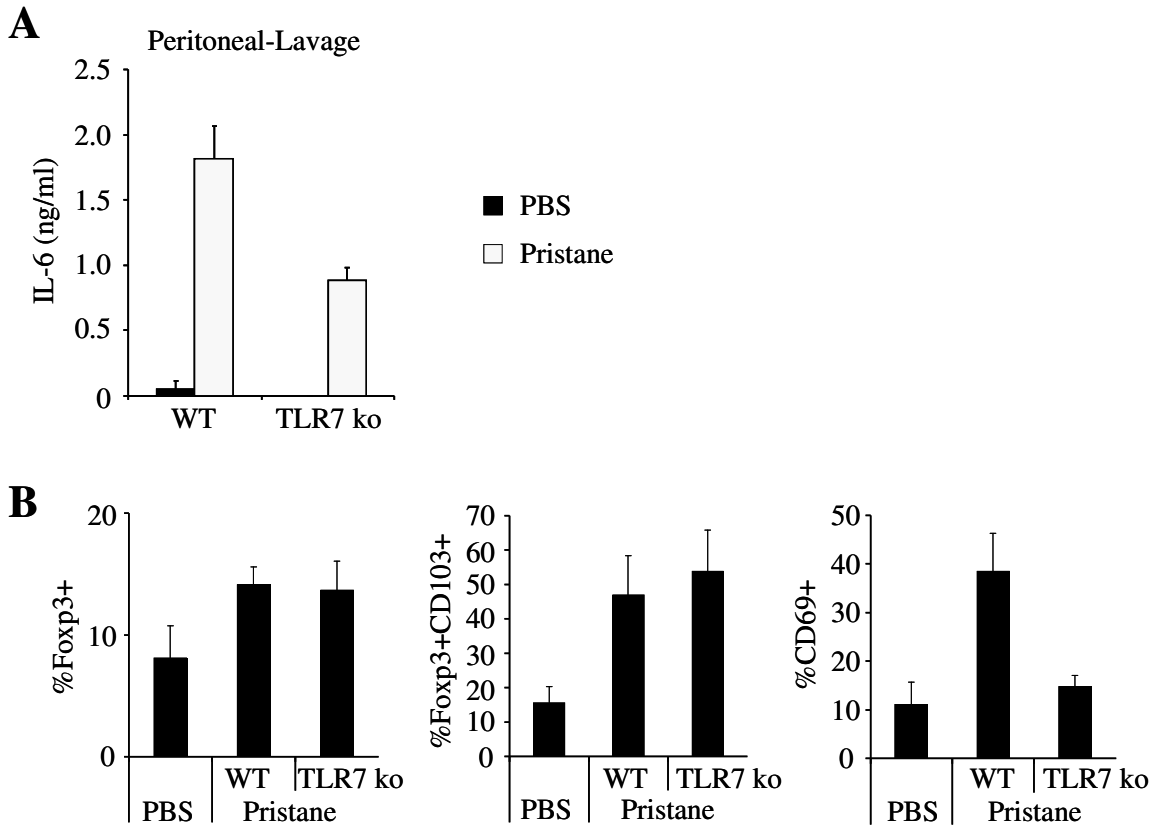

Figure 18: IL-6 levels and Foxp3+ T cells in the pristane-induced mouse model of SLE
(A) C57BL/6 WT or TLR7-/- (= TLR ko) mice were injected *i.p.* with 0.5 ml PBS or pristane and were sacrificed on day 8 after injection. Peritoneal-lavage was conducted with 3 ml ice cold RPMI1640. Blood was drawn from the heart. Cell-free peritoneal-lavage fluid was used to perform IL-6 ELISA. Mean values ± SD from two different mice are shown. (B) Wt C57BL/6 (=WT) or TLR7-/- (= TLR ko) mice were injected *i.p.* with 0.5 ml PBS or pristane and were sacrificed on day 8 after injection. Cells from the peritoneal-lavage were stained for CD4, CD25, Foxp3, CD103 and CD69 for FACS-analysis. CD4+ T cells were gated and the percentages of subpopulations were investigated. Mean values ± SD from n = 3 mice for each condition is shown.

The only difference between wt and TLR7 -/- mice which could be detected in this experiment besides different IL-6 levels were the percentages of CD4+CD69+ T cells. While the percentage of CD69+ T cells in the CD4+ T cell population was increased about 4-fold in pristane-treated wt mice compared to PBS control, no difference existed between the control and TLR7 -/- mice. CD69 is reported to be induced transiently upon lymphocyte activation and is expressed in chronic inflammatory infiltrates and at sites of active immune responses *in vivo* (Sancho et al, 2005). The fact that the percentage of CD4+CD69+ T cells is not increased in TLR7 -/- points towards reduced lymphocyte activation in response to pristane when TLR7 expression is missing.

5.4.2 Influence of TLR7-ligands on the *de novo* generation of iTregs *in vivo*

Due to lack of differences in the Treg compartment between wt and TLR7 -/- mice during the induction phase of the disease further experiments using the pristane-induced mouse model for SLE were abandoned. Instead the focus concentrated on a different experimental setup to specifically investigate the effect of TLR7-ligands on the *de novo* generation of Tregs *in vivo*. After transferring truly naïve OVA-specific T cells isolated from DO11.10/Rag2 -/- mice into wt Balb/c mice, it is possible to induce Foxp3 expression in these T cells by injecting OVA peptide. The effect of TLR activation on the generation of Foxp3+ T cells can be studied by simultaneously injecting the respective TLR-ligand.

Using this protocol CD25 expression was induced in around 15% and Foxp3 expression in around 5% of the transferred T cells in the unstimulated conditions. Simultaneous injection of the TLR7-ligand R848 significantly reduced both the percentages of CD25+ and Foxp3+ T cells (Figure 19 A + B).
Moreover, elevated IL-6 levels were detected after co-administration of OVA peptide and TLR7-ligand R848. Therefore, the question was addressed if *in vivo* neutralization of IL-6 reduces the observed inhibition of the *de novo* generation of Foxp3+ T cells.
Since binding of IL-6 to its specific membrane-bound IL-6 receptor (IL-6R) is a prerequisite for the activation of the signal-transducing receptor glycoprotein 130 (gp130) an anti-IL-6 antibody was used for neutralization. But IL-6 can also bind to a soluble form of the IL-6R and initiate IL-6 trans-signalling. This can be prevented by the soluble form of gp130

(sgp130), so sgp130 was used as well (Tenhumberg et al, 2008). To get an idea if IL-6 neutralization was successful IL-6 ELISA of mouse serum was performed.

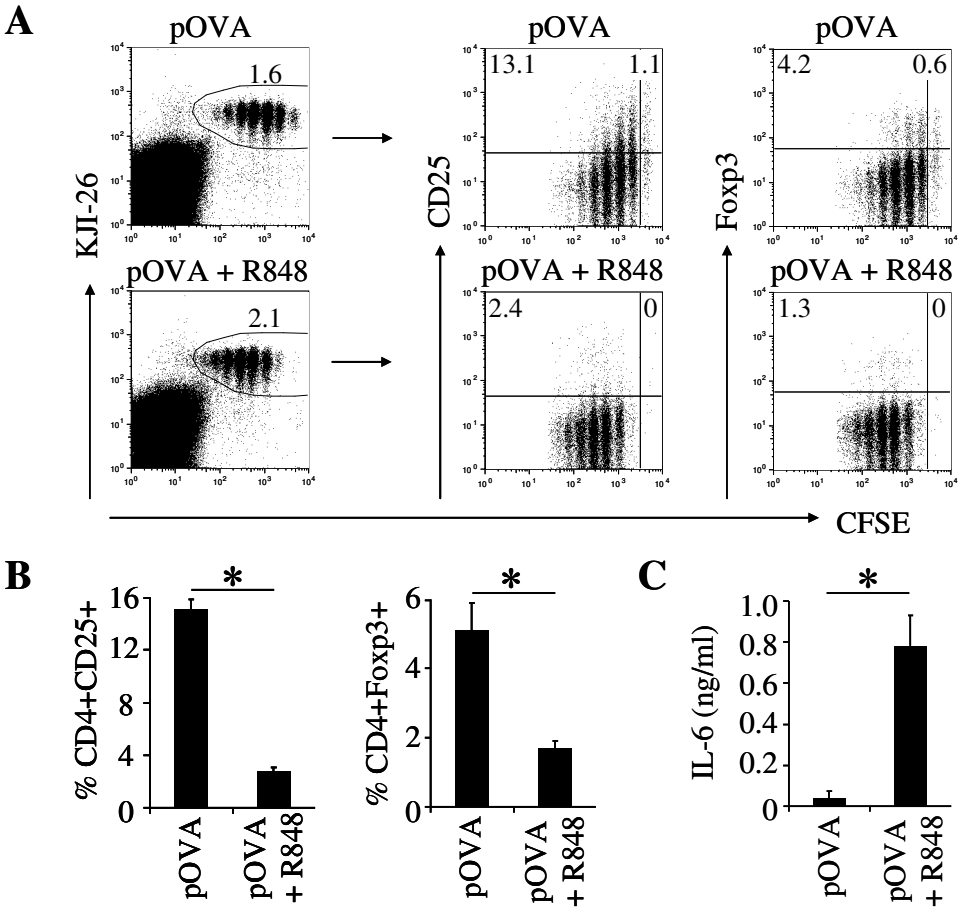

Figure 19: Inhibtion of the *de novo* generation of Foxp3+ T cells by TLR7-ligand *in vivo*
(A – C) CFSE-labeled naïve T cells from DO11.10/Rag2 -/- mice were transferred i.v. into Balb/c mice. After 24 hours mice were immunized i.v. with 5 µg OVA peptide without (= pOVA) or with (= pOVA+R848) 30 µg of TLR7-ligand R848. (A+B) On day 4 after immunization Foxp3 expression in the transferred T cells (stained by KJI-26 antibody) in cells pooled from spleen and LNs was assessed by FACS analysis. (A) One representative experiment is shown. (B) Mean values ± SD of n = 3 mice is shown. (C) Mice were bled from the cheek 3 hours after immunization to measure the IL-6 concentration in the serum. Mean values ± SD of n = 3 mice is shown.

The results are shown in Figure 20 A. Again, the *de novo* generation of Foxp3+ T cells was inhibited by simultaneous injection of OVA peptide and R848 compared to OVA peptide alone. But neither neutralization with antiIL-6 antibody nor the use of sgp130 had any effect to reverse the inhibitory influence of TLR7-ligand R848. The IL-6 data (Figure 20 B) showed that neutralization with anti-IL-6 antibody was not completely successful and with sgp130 even higher concentrations of IL-6 in the serum could be detected. Thus, remaining IL-6 after treatment with neutralizing anti-IL-6 antibody might still be enough to exert an effect. In

addition, IL-6 may not be the sole factor involved in the inhibition of the *de novo* generation of Foxp3+ T cells *in vivo*.

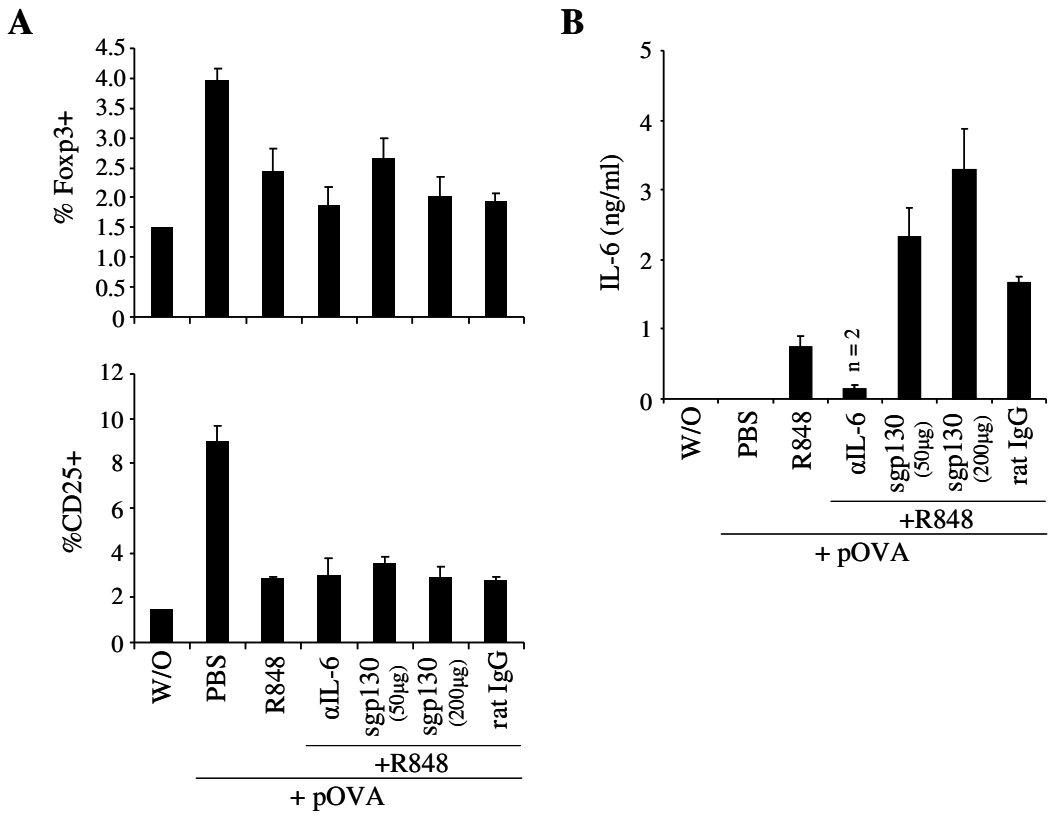

Figure 20: *In vivo* neutralization of IL-6
(A+B) CFSE-labeled naïve T cells from DO11.10 Rag2 -/- mice were transferred i.v. into Balb/c mice together with either 500 µg antiIL-6 antibody (= αIL-6) or 50µg or 200 µg of sgp130, 500 µg rat IgG was used as control. After 24 hours mice were immunized i.v. with 5 µg OVA peptide without (= pOVA) or with (= pOVA+R848) 30 µg of TLR7-ligand R848. (A) On day 4 after immunization Foxp3 expression in the transferred T cells (stained by KJI-26 antibody) pooled from spleen and LNs was assessed by FACS analysis. Mean values ± SD (n = 3 mice) of the percentage of Foxp3+ T cells (upper column) or of CD25+ T cells (lower column) is shown. (B) Mice were bled from the cheek 3 hours after immunization to measure the IL-6 concentration in the serum. Mean values ± SD (n = 3 mice) is shown.

5.5 Instability of Foxp3 expression

Korn et al. reported that IL-6 is extremely potent in inhibiting TGF-β induced Foxp3 expression and at the same time drives T_H17 differentiation (Korn et al, 2008). Although our data coincide with these findings as IL-6 was the key factor in reducing the percentage of Foxp3+ T cells, a reduction of "only" about 50% compared to control was observed which means that still around 30-50% of all T cells were Foxp3+. This suggests that quite a few T cells become Foxp3 expressing T cells albeit the potent inhibiting effect described of IL-6.

The first thought was that in DC/T cell co-cultures stimulated with TLR7-ligands IL-6 must be produced first, so it might be possible that Foxp3+ T cells were generated in the co-culture before IL-6 could exert its inhibitory effect.
For a start, this possibility was excluded since there was no obvious difference in the reduction of the percentage in Foxp3+ T cells between TLR7-stimulated co-cultures and cultures with recombinant IL-6 (around 50% reduction in both conditions) where IL-6 is present from the beginning and therefore able to exert its inhibitory effect immediately (see Figures 6 and 16 respectively).

So to further investigate the question why the inhibitory effect of IL-6 was not more drastic Foxp3 expression was analysed every day for five days in total in TLR7-stimulated co-cultures or in co-cultures with recombinant IL-6. Surprisingly, in the TLR7-stimulated co-cultures no difference in the percentage of Foxp3+ T cells and in the MFI of Foxp3+ T cells was detected until day 3 (Figure 21 A). So the initial Foxp3 induction was not affected. Not until day 4 a significantly lower percentage of Foxp3+ T cells and a reduced MFI were observed and the percentage of Foxp3+ T cells further decreased between day 4 and day 5. The results with recombinant IL-6 in the co-culture were similar (Figure 21 B), albeit not as pronounced as the results from TLR7-stimulated co-cultures. Still, an initial rise in the percentage of Foxp3+ T cells and the MFI of Foxp3+ T cells was detected which peaked on day 3 and then went down again. The difference to TLR7-stimulated co-cultures was that on all time points, except day 3, the percentage of Foxp3+ T cells was significantly lower with IL-6 than in co-cultures without. The MFI was the same on day 1 and 2 and then diverged.

Therefore it seems that there are two different decreasing effects on the percentage of Foxp3+ T cells in the condition with IL-6. The first seems to happen straight at the beginning and inhibits TGF-β induced Foxp3 expression. This leads to lower percentages of Foxp3+ T cells compared to control from the start. This effect is absent in TLR7-stimulated co-cultures maybe due to delayed IL-6 production. The second effect takes hold around day 3 and leads to a decrease in the percentage of iTregs. This decrease can have several reasons. One, the Foxp3+ T cells have a survival disadvantage and die. Two, Foxp3- cells proliferate faster and therefore outnumber the Foxp3+ T cells after several days. Three, Foxp3+ T cells loose Foxp3 expression and become effector T cells.

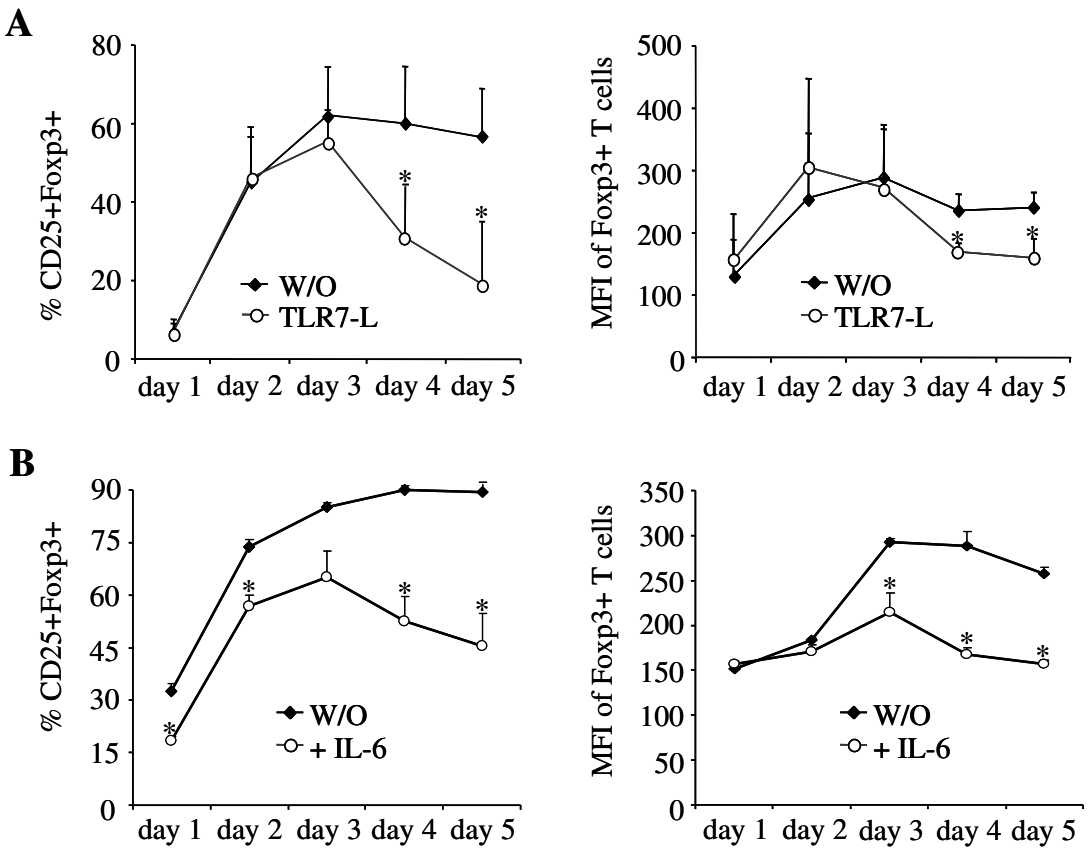

Figure 21: Foxp3 expression in TLR7-stimulated co-cultures or co-cultures with IL-6
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs and with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for one to 5 days. The percentage of Foxp3+ T cells (left) and the MFI of Foxp3+ T cells (right) were measured by FACS analysis. Mean values ± SD of 4 experiments are shown. (B) Naïve splenic CD4+CD25- T cells from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs and with or without (W/O) 1 ng/ml recombinant IL-6 (= + IL-6) for one to 5 days. The percentage of Foxp3+ T cells (left) and the MFI of Foxp3+ T cells (right) were measured by FACS analysis. Mean values ± SD of 3 experiments are shown.

5.5.1 Survival and Proliferation of iTregs

Options one and two that survival and proliferation of Foxp3+ T cells differ from Foxp3- T cells in the condition with TLR7-ligand were addressed first.

To this end, the amount of dead Foxp3+ T cells in the co-culture was analysed by EMA staining. The results of the EMA staining (Figure 22 A) show that the overall amount of dead cells in both conditions was comparable (34% in TLR7-stimulated co-cultures versus 37% without). There were also no differences in the percentage of dead cells within the Foxp3+ T cell fraction between the conditions. So the addition of TLR7-ligands does not lead to increased cell death and Foxp3+ T cells do not disappear from the TLR7-stimulated co-culture due to a survival disadvantage.

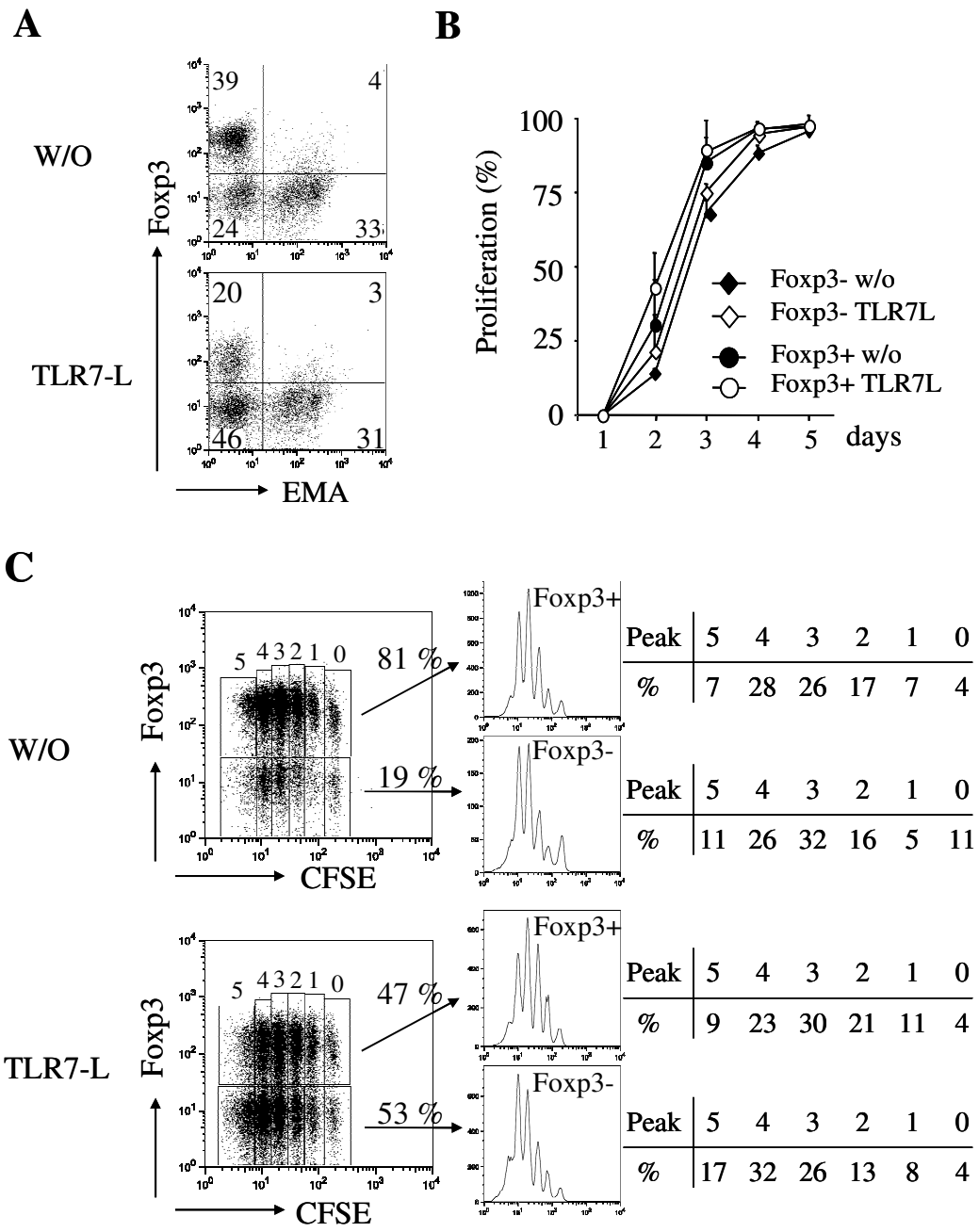

Figure 22: Survival and proliferation of T cells in the co-culture
(A) Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs and with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days. Cells were stained with EMA to determine dead cells. One of five experiments is shown. (B+C) CFSE stained naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs and with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for one to 5 days. Proliferation and Foxp3 expression was measured at indicated time points. Proliferation was measured as the percentage of cells which show CFSE dilution. (B) Mean values ± SD (n=3) of proliferated cells. (C) Representative results of CFSE dilution in correlation with Foxp3 expression are shown in dot plots. Peak by peak analysis on day 5 shows the percentage of cells in each division peak. Unstimulated control defined the gate between undivided cells and cells which have undergone one division.

Proliferation of T cells was measured as the percentage of cells which showed CFSE dilution and cells were divided into Foxp3+ and Foxp3- T cells (Figure 22 B). All cells slightly faster with the addition of TLR-ligand, but this had no adverse effect on the proliferation of Foxp3+ T cells. In both conditions, Foxp3+ T cells proliferated even faster than Foxp3- T cells, so that Foxp3- T cells do not have a proliferation advantage.

To confirm that the extent of the proliferation stayed the same between the two conditions, the CFSE dilution was analysed peak by peak on day 5 (Figure 22 C). This analysis revealed no significant differences between the two conditions as Foxp3+ T cells proliferate to the same extent in the presence or absence of TLR7-ligand.

5.5.2 Loss of Foxp3 expression and reprogramming of iTreg cells

As no differences were detected in survival and proliferation of Foxp3+ T cells the possibility was investigated that induction of IL-6 in the TLR7-stimulated co-culture leads to a delayed active downregulation of Foxp3 in iTregs and so to a lower percentage of Foxp3+ T cells. The first evidence hinting towards a lower Foxp3 expression was the significant reduction in the MFI of Foxp3+ T cells in conditions with TLR7 stimulation compared to control (MFI = 165 ± 19 vs MFI = 225 ± 7 respectively; $p < 0.01$) as can also be seen in Figure 21.

To investigate if Foxp3 expression was reduced in iTregs from TLR7-stimulated co-cultures naïve CD4+ T cells and DCs were isolated from the spleens of Dereg 23.2 mice. These mice express eGFP under the control of the *foxp3* gene locus (Lahl et al, 2007) which means that Foxp3+ T cells can be detected by eGFP expression without intracellular staining. This makes it possible to sort life iTregs from the co-cultures and use them for further analysis.
To determine and compare expression levels of Foxp3, RORγt, IL-17 and IL-6 on the mRNA level in iTregs from both conditions, co-cultures were set up and iTregs were sorted from co-cultures on day 4. Foxp3+ T cells (iTregs) were around 95% pure (see Figure 23 A).

From the sorted iTregs RNA was isolated, transcribed to cDNA and qRT-PCR was performed. The results for the relative mRNA expression of Foxp3, RORγt, IL-17 and IL-6 are shown in Figure 23 B.

The results clearly show that iTregs derived from the TLR7-stimulated co-culture have 50% reduced Foxp3 mRNA expression while at the same time the T_H17 signature genes RORγt and IL-17 are upregulated 7-fold and 25-fold, respectively. This means that not only iTregs loose Foxp3 expression but they are also reprogrammed to become T_H17 cells given the time. IL-6 mRNA expression is upregulated in these iTregs which would explain the increased accumulation of IL-6 in the co-cultures between days 3 and day 5 (Figure 14) and that the continuous accumulation of IL-6 protein in the co-culture over time is at least in part due to IL-6 producing T cells.

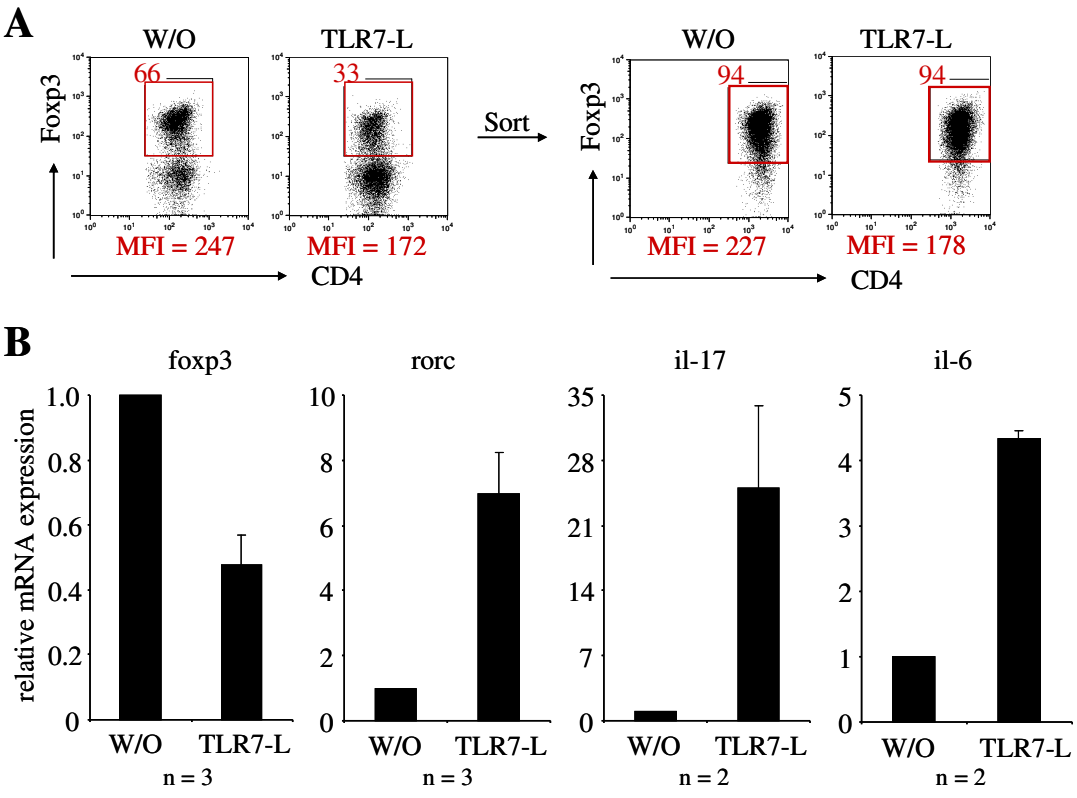

Figure 23: Downredulation of Foxp3 and upregulation of RORγt, IL-17 and IL-6
Naïve splenic CD4+CD25- T lymphocytes from Dereg 23.2 were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days and then CD4+ GFPhigh CD25high iTregs were sorted. (A) The percentages and MFI of Foxp3+ T cells before and after FACS sort were determined by FACS anlysis. One FACS sort is shown. (B) RNA from sorted iTregs was isolated and relative mRNA expression of Foxp3, RORγt, IL-17 and IL-6 was determined by quantitative RT-PCR using Taqman primers and probes (TaqMan® Gene Expression Assays, Applied Biosystems). (Mean values ± SD)

Since a slightly faster proliferation of T cells was detected in the condition with TLR7-ligand, it was determined if the total cell count between the two conditions differs. A higher number of cells in the TLR7-stimulated condition might lead to a faster depletion of factors required

for Foxp3 induction and maintenance and thus enhance the loss of Foxp3 expression. Therefore, the cells in the co-culture were counted after 4 days of co-culture and the results were compared (Figure 24).

There was no difference in total cell numbers between the two conditions after 4 days of co-culture. Therefore, it was also clear that reduced percentages in Foxp3+ T cells reflected reduced total cell numbers of Foxp3+ T cells in the TLR7-stimulated co-culture.

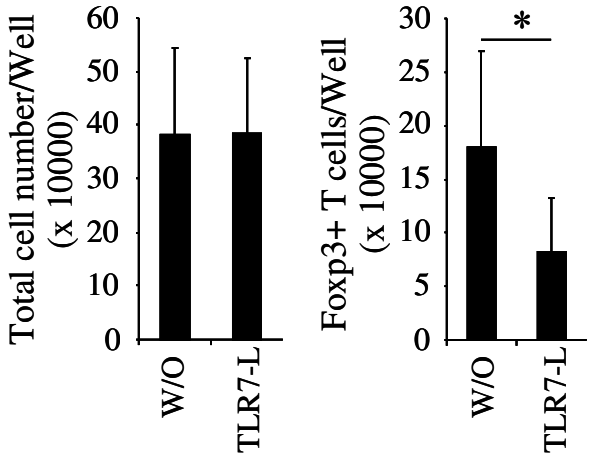

Figure 24: Total and Foxp3+ T cell number on co-cultures with or wothout TLR7-stimulation
Naïve splenic CD4+CD25- T lymphocytes from C57BL/6 mice were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days and total cells were counted. The percentages of Foxp3+ T cells were determined by FACS analysis and cell numbers of Foxp3+ T cells was determined from the total cell count. Mean values ± SD of 4 experiments is shown.

To further ensure that downregulation of Foxp3 was not due to a limitation of Foxp3 inducing factors, co-cultures were supplemented with either TGF-β or IL-2 alone or both every two days and Foxp3 expression was followed over time but no significant differences could be detected in the outcome. This indicates that not only are there no limitations due to consumption of these factors, but also that an abundance of TGF-β and/or IL-2 are not sufficient to rescue Foxp3 expression in the presence of IL-6 induced by TLR7 stimulation.

5.5.3 The cytokine environment determines Foxp3 expression

The data shown in Figure 21 reveal that the initial Foxp3 induction until around day 3 was not impaired since percentages and MFI of Foxp3+ T cells did not differ in the presence or

absence of TLR7-ligand. This means that the reduction in Foxp3+ T cells mediated by IL-6 is delayed in taking effect. Therefore it seems that until day 2 or day 3 iTregs from both conditions are similar and while continuous and mounting exposure to IL-6 then leads to Foxp3 downregulation in TLR7-stimulated co-cultures, Foxp3 expression stays stable in co-cultures without TLR7. The question here is if exposure to IL-6 at one time is enough to "prime" T cells to loose Foxp3 expression irrespective of the cytokine environment at a later time point or if continuous IL-6 exposure is necessary to downregulate Foxp3.

To address this question an experiment was conducted (see Figure 25 A) where two different co-cultures were set up on the same day. One with naïve CD4+ T cells and DCs from DEREG 23.2 mice with or without TLR7-ligand and the other with naïve CD4+ T cells and DCs from CD45.1 congenic mice with or without TLR7-ligand or recombinant IL-6. DEREG 23.2 CD4+ GFPhigh CD25high T cells were sorted from co-cultures on day 2 when percentages and MFI of Foxp3+ T cells did not differ between the conditions (see data from co-culture before sort on day 2, Figure 25 A). The sorted populations were > 95% pure concerning GFP and CD25, and 75 – 80% also expressed Foxp3 (data after Sort, Figure 25 A middle). The sorted iTregs from both conditions were equally distributed among all co-culture conditions with CD45.1 cells .

On day 4, unmanipulated co-cultures of DEREG 23.2 and CD45.1 cells were checked if they showed a lower percentage and MFI of Foxp3+ T cells when TLR7 or IL-6 was present. This was the case (Figure 25 A, at the right). Then the fate of the sorted iTregs in co-cultures with CD45.1 DCs and T cells (Figure 25 B) was investigated. Since DEREG 23.2 T cells express the marker CD45.2 this makes it possible to discern them from CD45.1+ T cells.
Both the sorted iTregs from the condition without TLR7-ligand (=W/O) as well as the sorted iTregs from the condition with TLR7-ligand (=TLR7-L) maintained a stable Foxp3+ population in the co-culture with CD45.1 DCs and T cells without TLR7-ligand since input and output percentage of Foxp3+ T cells was similar. In co-cultures with the TLR7-ligand both sorted iTreg populations had lower percentages of Foxp3+ T cells, reduced by 43% in iTregs (W/O) and 57% in iTregs (TLR7-L), compared to those in co-cultures without the TLR7-ligand. This was further exacerbated when the sorted iTregs were cultured in CD45.1 co-cultures with recombinant IL-6. Then the reduction of the percentage of Foxp3+ T cells amounted to about 80% reduction.

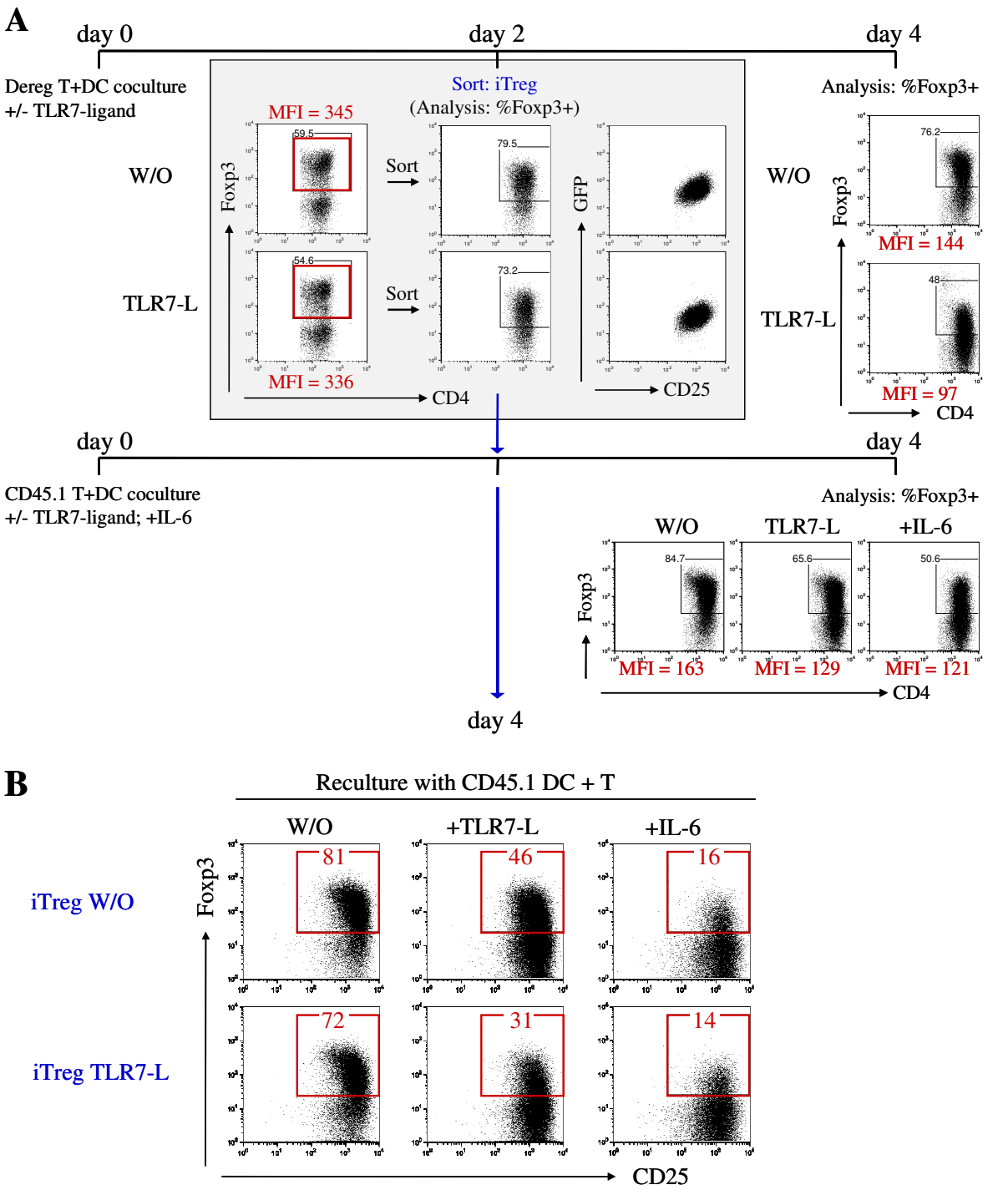

Figure 25: Reculture of sorted DEREG 23.2 iTregs in CD45.1 co-culture
Naïve splenic CD4+CD25- T cells from DEREG 23.2 mice were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 2 or 4 days. Naïve splenic CD4+CD25- T cells from CD45.1 congenic mice were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) or IL-6 (1 ng/ml) for 4 days. On day 2 DEREG 23.2 CD4+ GFPhigh CD25high iTregs were sorted and recultured in CD45.1 co-cultures until day 4. (A) shows the time line of the experiment and all intermediate results (DEREG 23.2 co-culture day 2 and day 4, FACS sort and CD45.1. co-culture day 4) of one out of two experiments. (B) Results of reculture of sorted DEREG 23.2 iTreg on day 4. Results of one representative of 2 experiments are shown.

The results obtained from the reculture experiment show that iTregs from co-cultures with TLR7-stimulation were not yet primed to loose Foxp3 expression after two days as they kept Foxp3 expression in CD45.1 co-cultures without TLR7-ligand. On the other hand, iTregs sorted from co-cultures without TLR7-ligand did not maintain stable Foxp3 expression in co-cultures where IL-6 was present.
From these results the conclusions were drawn that to induce loss of Foxp3 expression in iTreg a prolonged IL-6 exposure is needed or that IL-6 exerts its effect at a certain time point and not before. Foxp3 expression remains stable if the influence of IL-6 is removed in favour of Treg-polarizing conditions, at least at the time point chosen. On the other hand, iTregs generated without IL-6 influence are not stable in surroundings where IL-6 is present and loose Foxp3 expression.

5.6 Impaired function of induced regulatory T cells

From prior experiments it was clear that in the presence of TLR7-ligand Foxp3 is downregulated in iTregs after 4 days of co-culture leading to lower Foxp3+ T cell numbers and a lower Foxp3 expression. The percentage of CD103+Foxp3+ T cells was also found to be significantly lower in co-cultures with TLR7-stimulation. Since Foxp3 expression is reported to be essential for full functionality of Tregs (Williams and Rudensky, 2007) and the CD103+ Treg population is reported to be highly potent in suppression (Huehn et al, 2004), the suppressive activity of iTregs from co-cultures with or without the TLR7-ligand S-27609 was investigated. Also comparisons were made between iTregs after 2 and 4 days of co-culture, because there were no differences in the expression of Foxp3 between co-cultures on day 2.

Therefore the co-cultures were set up with either DEREG 23.2 or truly naïve OT-II/Rag2 -/- DEREG CD4+ T cells. On day 2 and day 4 iTregs (CD4+ GFPhigh CD25high) were sorted from the co-culture (purity 95 – 98% Foxp3+) and used together with CFSE-labelled naïve CD4+ responder T cells (Tresp) from CD45.1 mice in suppression assays to determine their suppressive activity. The results of the suppression assays are outlined in Figure 26.

Figure 26 A shows a comparison of the suppressive activity of DEREG 23.2 iTregs sorted at day 2 and day 4 of the co-culture. Sorting iTregs from co-cultures on day 2 revealed that iTregs from both conditions are equally potent in suppressing Tresp proliferation. So initially,

TLR7-stimulation and the resulting IL-6 in the co-culture do not affect iTreg functionality, most likely because Foxp3 expression is not yet affected.

In contrast to that, when iTregs were sorted on day 4 the iTregs originating from co-cultures with TLR7-stimulation were less potent to suppress Tresp proliferation. The same was true when truly naïve T cells from OT-II/Rag2 -/- DEREG mice were used. Since the iTregs that were generated in the condition with TLR7-stimulation expressed lower levels of Foxp3 mRNA (see Figure 23 B) and protein this finding coincides with the reports that downregulation of Foxp3 expression leads to loss of suppressive functionality and acquisition of proinflammatory effector functions (Mellor and Munn, 2011).

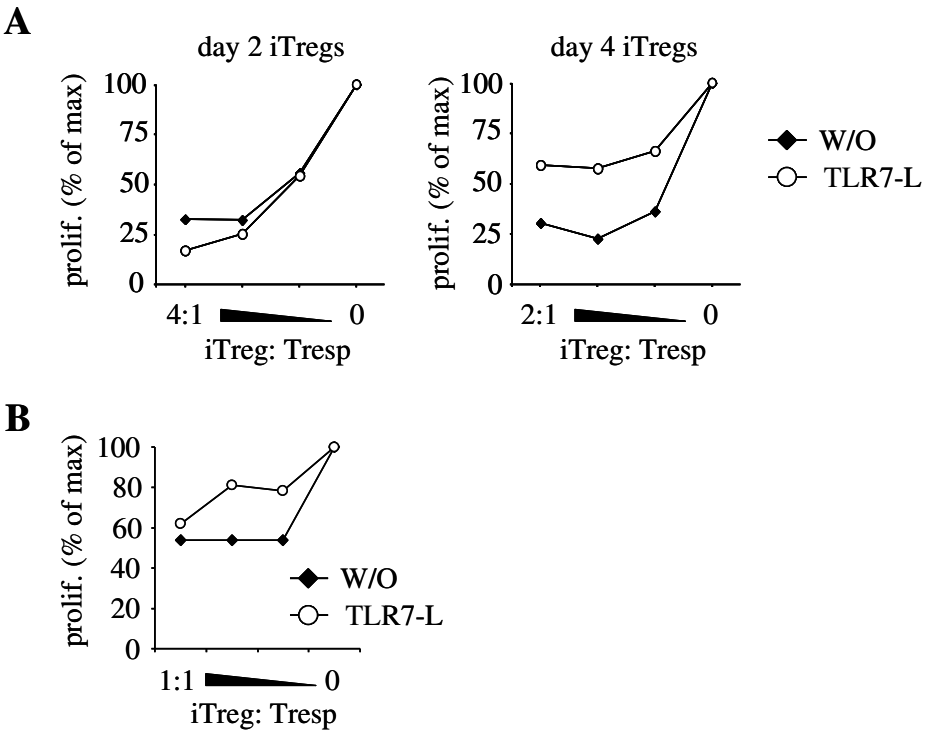

Figure 26: Suppressive activity of iTregs at different time points
(A) Naïve splenic CD4+CD25- T cells from DEREG 23.2 mice were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 2 or 4 days. Then CD4+ GFPhigh CD25high iTregs were sorted (iTreg) and used in suppression assays in decreasing ratios with CFSE stained naïve CD4+ T cells from CD45.1 congenic mice (Tresp). Proliferation of Tresp alone, measured by CFSE dilution, was set to 100% proliferation. Results of one representative experiment out of 2 are shown. (B) Truly naïve T cells from OT-II/Rag 2-/- DEREG mice were cultured under Treg-polarizing conditions with C57BL/6 wt splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days. Then CD4+ GFPhigh CD25high iTregs were sorted (iTreg) and used in suppression assays in decreasing ratios with CFSE stained naïve CD4+ T cells from CD45.1 congenic mice (Tresp). Proliferation of Tresp alone, measured by CFSE dilution, was set to 100% proliferation. Results of one experiment out of 2 are shown.

In one of the suppression assays, at lower iTreg:Tresp ratios proliferation of Tresp was even enhanced by the presence of iTregs from co-cultures with TLR7-stimulation although

suppressive activity at the highest ratio was nearly as potent as the suppression by iTregs from co-cultures without TLR7-ligand (Figure 27 A). After observing this effect, the percentage of eGFP expressing iTregs after the suppression assay was evaluated. The result is shown in Figure 27 B.

The percentage of eGFP expressing T cells remained quite stable during the suppression assay within iTregs sorted from co-cultures without TLR7-stimulation (88% output versus 98% input) whereas the percentage of iTregs from the condition with TLR7-ligand maintaining eGFP expression was very low (26% output versus 96% input) at the end of the suppression assay. It can be speculated that in this experiment enough iTregs remained at high iTreg:Tresp ratios to exert potent suppression, probably even suppress former iTregs which gained effector functions, whereas at lower ratios the remaining iTreg were not enough to suppress proliferation of Tresp and then the former iTregs which have lost Foxp3 expression during the assay might even enhance proliferation.

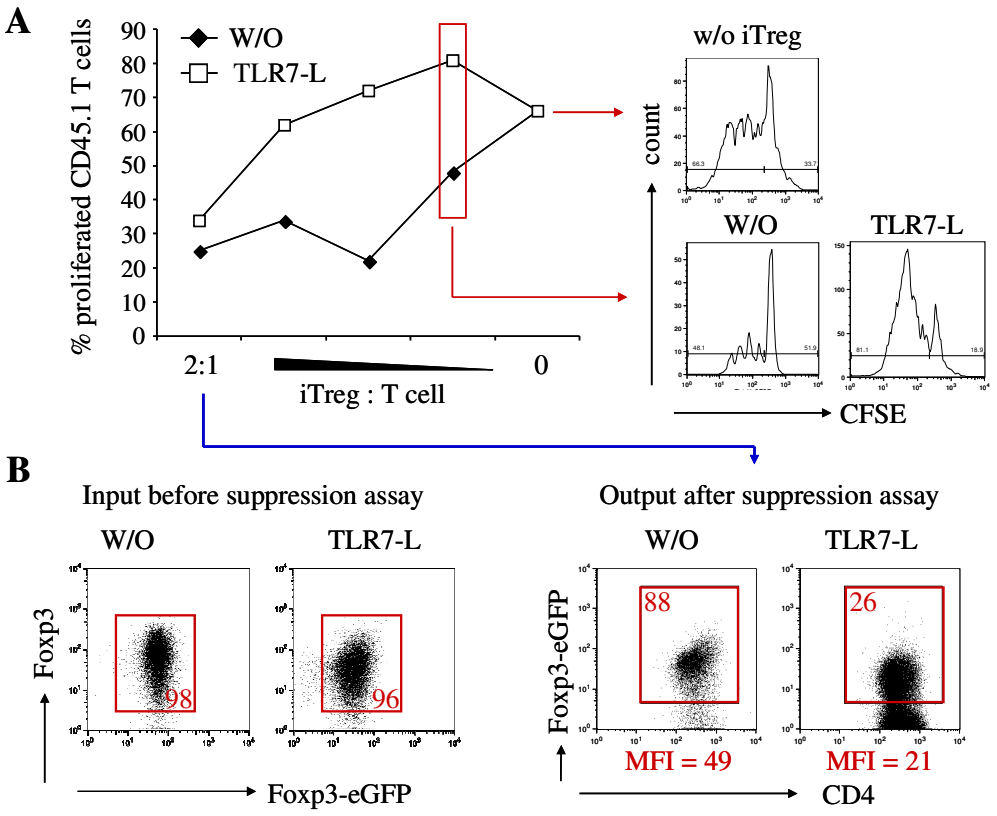

Figure 27: Enhanced proliferation of Tresp at lower iTreg:Tresp ratios
(A) Naïve splenic CD4+CD25- T cells from Dereg 23.2 mice were cultured under Treg-polarizing conditions with splenic DCs with or without (W/O) TLR7-ligand S-27609 (= TLR7-L) for 4 days. Then CD4+ GFPhigh CD25high iTregs were sorted (iTreg) and used in suppression assays in decreasing ratios with CFSE stained naïve CD4+ T cells from CD45.1 congenic mice (Tresp). Proliferation of Tresp was measured by CFSE dilution. Results of one experiment are shown. (B) At day 4 of the suppression assay Foxp3-driven eGFP expression was measured in CD25.2+ T cells by flow cytometry. Data of one representative experiment out of 2 is shown.

After this finding, the iTreg output in other suppression assays was also re-evaluated to see if there was a difference. It was observed that the percentage of T cells maintaining eGFP expression was generally lower when the sorted iTregs derived from co-cultures with TLR7-stimulation although the difference between input and output was not as pronounced as in the experiment shown in Figure 27 B. Thus, it is likely that the impaired suppressive activity is not only due to a lower Foxp3 expression in the sorted iTregs but also correlates with further downregulation of Foxp3 expression during the suppression assay and lower iTreg numbers.

6 DISCUSSION

After Foxp3 expressing CD4+ T cells were found to be a distinct T helper cell lineage they were termed Tregs because they have the ability to regulate the immune system in order to prevent excessive and harmful immune responses to infections and to suppress the development of autoimmune diseases. But in contrast to the T helper cell paradigm that states that commitment of T helper cells to distinct lineages after cytokine polarization involves stable gene expression programs (Zhou L, Chong and Littman, 2009) Tregs, especially iTregs, are not stable under certain circumstances and loss of Foxp3 expression leads to the acquisition of proinflammatory effector functions. It was also shown that Treg induction can be inhibited by several proinflammatory cytokines including IL-6, IL-4 and IFN-γ (Korn et al, 2009; Hall et al, 2008). These findings make sense insofar as it is obligatory for effective immune responses to overcome the immunomodulatory function of Tregs but it would be detrimental in the development of autoimmune diseases such as SLE.

6.1 The effects of Toll-like receptor stimulation in vitro *and* in vivo

Our aim was to study the effects of TLR stimulation specifically on the *de novo* generation and the function of Tregs since TLRs are involved in the development of autoimmune diseases by stimulating cells to produce proinflammatory cytokines. Especially the activation of DCs and B cells through TLR7 and to some extent TLR9 and TLR4 (Lande et al, 2011; Lartigue et al, 2009; Savarese et al, 2008) play an important role in the development of autoimmunity in murine models of SLE and are probably also involved in the pathogenesis of human SLE.

Therefore, it seems likely that TLR stimulation of DCs and B cells might contribute to the breakdown in peripheral tolerance by preventing Treg induction or by converting Tregs into effector T cells. Since various TLRs including TLR4, TLR7 and TLR9 are expressed by naïve T cells (Chang, 2010) there was also the question if TLRs can influence iTreg generation and Treg stability directly.

6.1.1 Influence of TLR-ligands on the generation of iTregs

To answer these questions the effects of TLR-ligands were first studied in an *in vitro* model to discern direct effects on T cells from indirect effects mediated by other TLR-stimulated cells such as DCs and B cells. Both play an important role in the pathogenesis of SLE.

First the question was addressed if TLR-ligands exert a direct effect on the induction of Foxp3+ T cells. So naïve CD4+ T cells were isolated from murine spleens and cultured under Treg-polarizing conditions in the absence of APCs. Ligands for TLR7, TLR9 and TLR4 were used to investigate the effects of TLRs that are involved in the pathogenesis of SLE. After 4 days, Foxp3 expression was analysed and no differences could be detected in Foxp3 expression with any of the TLR-ligands used. This stood in contrast to co-cultures of DCs and T cells where TLR7- and TLR9 stimulation leads to a reduction in Foxp3+ T cells. Indeed, the inhibitory effect of TLR7-stimulation is completely independent from TLR7 expression in the T cells, because even co-cultured T cells from TLR7 -/- mice had reduced Foxp3+ T cell percentages when cultured with wt DCs which could still react to TLR7 stimulation. Therefore the focus of our experiments was on the influence of TLR-stimulated DCs and B cells on Treg induction and maintenance.

Since the DC population is quite heterogeneous but it was not known to us if one population is particularly potent in influencing Treg induction, DCs containing all subpopulatins were isolated from the spleen and were used (see 1.1.2) in the co-cultures with the naïve T cells and TLR-ligands under Treg-polarizing conditions. Our study shows that activation of splenic DCs through TLR7 and TLR9 leads to a significant reduction of iTregs and lower Foxp3 expression in remaining iTregs after 4 days of culture whereas stimulation of TLR4 had no significant effect. Our thought is that TLR4-stimulation showed no effect in our co-culture most likely due to little IL-6 production after stimulation. IL-6 was found to be the driving factor behind reduced Foxp3 expression. Moreover, another study investigating the effect of commensal bacteria on Treg induction via TLR activation in lamina propria DCs supported our findings, since in this study TLR9 activation limited Treg induction while TLR4 activation had no effect (Hall et al, 2008).

The inhibitory effect on Treg induction that was observed with TLR7- or TLR9-stimulated DCs was also found when culturing naïve T cells together with TLR7- or TLR9-stimulated B

cells or TLR7-stimulated DCs isolated from mesenteric LNs. B cells represent a completely different cell type from DCs but are also involved in SLE pathogenesis and can be activated through TLR and TLR9. It was reported that B cells can be directly suppressed by Tregs in secondary lymphoid organs (Lim et al, 2005) and that resting B cells are able to induce Foxp3 expression in naïve T cells and expand Tregs via TGF-β3, but by reducing TGF-β3 production upon activation they loose this ability (Shah and Qiao, 2008; Zhong et al, 2007). Our results show that B cells not only stop enhancing Treg induction and expansion upon activation but that TLR7- and TLR9-stimulation actively leads to reduced Foxp3 expression in Tregs and lower percentages of Foxp3+ T cells. This fact might be important during the development of autoimmune diseases which are dependent on TLR7 and TLR9, since autoreactive B cells with low affinity BCRs can be activated by concurrent BCR and TLR stimulation to produce autoreactive antibodies. Lower Treg numbers resulting from impaired Treg induction might be unable to prevent this event and this might contribute to peripheral tolerance breakdown.

DC subpopulations from different compartments differ from each other. So TLR7-stimulated splenic and mesenteric LN (mLN) DCs were compared because DCs have the ability to produce all-trans retinoic acid (ATRA) (Iwata et al, 2004). It was proven that ATRA is able to enhance Foxp3 expression while inhibiting T_H17 cell differentiation even under conditions that favour T_H17 cell differentiation (Elias et al, 2008). Optimal Treg-polarizing conditions were used in our model, but this condition was disrupted by the presence of TLR7-induced production of IL-6. Addition of DCs which are able to produce ATRA might rescue Foxp3 expression and prevent the inhibitory effect on Treg induction by TLR7.
However, TLR7-stimulated mLN DCs caused a reduction in the percentages of Foxp3+ T cells as well. This could be due to an overpowering effect of IL-6 over ATRA or loss of ATRA production upon TLR7-stimulation. Since no differences between DCs from the spleen or mesenteric LNs were found and there were also no differences between DCs and B cells, splenic DCs were used for all our experiments.

George et al found out in a study that T helper cells need high antigen doses and strong stimulatory signals to overcome Treg suppression, but that Tregs retain their suppressive potential in this case. Low antigen doses were not enough to allow T helper cells to overcome Treg suppression (Georg et al, 2003). Moreover, DCs who present low doses of antigen on their surface are capable to induce and expand Tregs with potent suppressive function. This

may also constitute a natural peripheral self-tolerance mechanism of DCs by presenting low doses of endogenous self-antigen (Morel and Turner, 2011). But what happens to this setting when DCs are simultaneously activated with a TLR7-ligand?
This study shows that TLR7 stimulation might disrupt this natural peripheral tolerance mechanism by reducing Treg induction and expansion and by reducing the suppressive potential of Tregs. Reduced Treg numbers and reduced suppressive function combined might then lower the antigen threshold at which T helper cells are able to overcome Treg suppression. Considering such a scenario, the low doses of endogenous self antigen presented by DCs to induce peripheral tolerance might then be enough to activate autoreactive effector T cells promoting autoimmune disease development.

6.1.2 The pristane-induced mouse model of SLE

In patients with active SLE and in several murine models of SLE reduced frequencies and suppressive functions of Tregs have been observed (La Cava, 2008). This supports the concept that a breakdown in peripheral tolerance mechanisms through reduced Treg numbers and function may be a critical step in development of autoimmune disease.
Disease development and deciding factors of SLE can be investigated in several mouse models of spontaneous lupus. Another possibility is the use of the pristane-induced mouse model of SLE in which the injection of pristane into the peritoneal cavitiy leads to a lupus-like disease after about three to six month including antinuclear autoantibody production, immune complex formation and glomerulonephritis (Savarese et al, 2008). The advantages of this model are that it is independent of genetic factors and the starting point of disease development is clearly defined by the injection of pristane.

It is not yet fully understood how pristane leads to the lupus-like disease in contrast to other mineral oils, but it was found that the involvement of TLR7-stimulation after pristane injection is critical for disease development right from the start, since early type I IFN production, immature monocyte recruitment and production of anti-snRNP/Sm antibodies are completely dependent on TLR7 activation (Perry et al, 2011; Savarese et al, 2008). Moreover, it was shown that pristane, although not directly activating TLR7, augments the effect of TLR7-ligands *in vitro* (Lee et al, 2008).

An inhibitory effect of TLR7 on the generation and function of Foxp3+ T cells in the co-culture model was observed and this was mediated by IL-6. Since TLR7 activation in the pristane induced lupus model is important for disease development, the question arose if IL-6 was produced in a TLR7-dependent manner in the peritoneal cavity and if there was a reduction of Foxp3+ T cells in the peritoneal cavity right from the start which contributes to disease onset.
Therefore, cells from C57BL/6 wt and TLR7 -/- mice were compared and the early time point of eight days post injection was chosen to analyse the cells in the peritoneum. Though IL-6 levels in the peritoneal lavage fluids were higher in WT than TLR7 -/- mice, we found TLR7-independent IL-6 production. This finding point towards a minor involvement for IL-6 in disease development compared to type I IFN production. This is emphasized by the observation that in IL6 -/- mice only the production of anti-ssDNA, anti-dsDNA and anti-chromatin antibodies was impaired but not the production of anti-snRNP/Sm antibodies which are dependent on TLR7 activation . In contrast to that the production of nearly all autoantibodies was affected in mice with IFN I receptor deficiency (Perry et al, 2011).

There was also no reduction in the frequency of Foxp3+ T cells in peritoneal CD4+ T cells at day 8 after pristane injection, but rather a TLR7-independent increase in the percentage of Foxp3+ T cells in the CD4+ T cell population. Functional studies with eGFP+ Tregs from WT DEREG 23.2 and DEREG 23.2 x TLR7 -/- mice sorted from the peritoneal lavage eight days after pristane injection were inconsistent but the suppressive function seemed not to be affected (data not shown).
Therefore it seems obvious from our data that TLR7-activation had no visible effect on the Treg compartment 8 days after pristane injection as no differences between WT and TLR7 -/- mice could be detected at this time point. However, an involvement of TLR7-stimulation in reduced frequencies and suppressive function of Tregs as they have been observed in patients with active SLE and in mouse models for SLE (LaCava, 2008; Miyara et al, 2005; Wan et al, 2007) cannot be excluded since the percentage of Tregs was slightly increased 8 days after pristane injection, not reduced. Consequently, the reduction in the frequencies of Tregs seems to happen at a later time point during disease development and sustained TLR7-stimulation through endogenous TLR7-ligands might play a role in the inhibition of Treg induction or in the conversion of Tregs to effector T cells at later time points.

In addition to that, it is also possible that especially in the beginning of disease development a locally confined inhibitory effect of TLR7-stimulation on the *de novo* generation of iTregs, might be masked by an influx of nTregs into the peritoneal cavity. So it is also important to know the right location where Treg induction takes place.
For example, iTregs might not be generated in the peritoneal cavity, but in newly formed tertiary lymphoid tissues in the peritoneal cavity. Tertiary lymphoid tissues develop in areas of chronic immune stimulation as a response to the increased demand for localized immune responses, are similar to secondary lymphoid organs in structure and are sites where antigen presentation and T cell priming occur (Neyt et al, 2012).
In the pristane-induced lupus model, lipogranulomas form in the peritoneal cavity after pristane injection. Since lipogranulomas, like all tertiary lymphoid tissues, consist of discrete areas of B cells and T cells plus DCs (Nacionales et al, 2006) it is highly likely that antigen presentation and T cell priming happens there. Since the following T cell differentiation is influenced by the cytokine milieu, it is likely that TLR7-stimulation might have an inhibitory effect on Treg generation. But this effect might be masked in the beginning by the influx of Tregs. An indication for the increased influx of Tregs might be that especially CD103+ Tregs were found to be present in increased frequencies in WT and TLR7 -/- mice after pristane injection. Since CD103+ Tregs display an effector/memory phenotype they are therefore particularly efficient in migrating to inflamed sites (Huehn et al, 2004).

Interestingly, there was a difference in the percentages of CD4+ CD69+ T cells between WT and TLR7-/- mice. While the frequencies of CD69+ T cells in TLR7 -/- were not different from those of the PBS control, a 4-fold increase in the percentages of CD4+CD69+ T cells was detected in WT mice. This is interesting because of previous reports concerning CD69 and might be worth further investigation to elucidate disease development.

It was reported that CD69 is an early leucocyte activation molecule expressed at sites of inflammation (Sancho et al, 2005), including on T cells. On the one hand, CD69 is reported to be a previously underestimated regulator for immune responses since it was shown in several studies that CD69 expression has a protective effect in murine models for arthritis, contact dermatitis, allergic asthma and autoimmune myocarditis by inhibiting effector T cell responses, especially T_H17 differentiation. CD69 also enhances the function and differentiation of Tregs (Martin and Sánchez-Madrid, 2011; Radulovic et al, 2012).

On the other hand, it was shown in NZB (New Zealand Black) x NZW (New Zealand White) mice, the T cell-dependent mouse model for SLE, that increased frequencies of CD4+CD69+ T cells were present and are able to suppress IL-2 production by CD4+CD69- T cells. Diminished IL-2 production then favours T_H17 differentiation and inhibits the generation of Tregs (Liao et al, 2011; Martin and Sánchez-Madrid, 2011). It was also shown that IFN-α/β were able to upregulate CD69 expression on T cells (Shiow et al, 2006), so the missing IFN I response in TLR7 -/- mice might account for unaltered CD69+ T cell frequencies after pristane injection.

So, the result that the percentage of CD4+CD69+ T cells is increased at the investigated early time point after pristane injection in WT mice, but not in TLR7 -/- mice, might indicate another TLR7-dependent mechanism which contributes to disease development:
The increase in CD4+CD69+ T cells early on might contribute to the reduced frequencies of Tregs registered at later time points through the influence of CD69 on Treg generation by diminishing IL-2 production and favouring T_H17 differentiation.
This assumption is backed up by the findings that the percentages of T_H17 cells are increased in patients with SLE and that IL-17 production contributes to the pathogenesis of SLE particularly through tissue damage (Apostolidis et al, 2011; Nalbandian et al, 2009). IL-6 production in the peritoneal cavity might promote disease development by further enhancing T_H17 cell differentiation and by rendering self-reactive naïve T cells unresponsive to Treg-mediated suppression (Sakaguchi, 2005). It is also possible that TLR7-dependent IL-6 production contributes to impaired Treg generation at later time points because activation of DCs by the endogenous TLR7-ligand U1snRNP leads to lower percentages of Foxp3+ T cells in our study *in vitro*.

6.1.3 T cell transfer and the induction of regulatory T cells

As already mentioned above, a T cell transfer model was used in which OVA-specific T cells were transferred into Balb/c mice in order to follow the *de novo* generation of Foxp3+ T cells from truly naïve T cells after a single OVA peptide injection. Simultaneous administration of TLR7-ligand R848 enabled us to investigate if our results from the co-culture experiments had any significance *in vivo*. The fact that T cells from DO11.10 Rag2 -/- mice are truly naïve also ensured that *de novo* generation and not a possible expansion of contaminating nTregs was investigated. In addition, this model enabled us to focus directly on effects of TLR7-

stimulation on Treg induction. This was not possible in the pristane-induced mouse model where time point and location of Treg induction is unknown and the autoantigen is not clearly defined. Moreover, the frequency of antigen-specific Tregs is expected to be very low, whereas it is much higher after transfer of TCR-transgenic T cells.

Injection of OVA alone gave rise to about 5% Foxp3+ T cells and 15% CD25+ T cells within the population of transferred OVA-specific T cells. The discrepancy between the percentages of Foxp3+ and CD25+ T cells might be explained by the study from Schallenberg et al. that reported the existence of a precommitted Treg precursor cell which was CD4+CD25+ but Foxp3- and which is able to effectively upregulate Foxp3 in the presence of IL-2. This precursor is thought to be important for maintaining immune homeostasis under physiological conditions (Schallenberg et al, 2010). Simultaneous injection of OVA peptide and the TLR7-ligand did lead to a reduction in the percentage to about 2% Foxp3+ and 3% CD25+ T cells which confirmed the *in vitro* results that TLR7 stimulation reduces the percentage of Foxp3+ T cells. But TLR7 stimulation not only impaired the generation of Tregs it also impaired the generation of precommitted Treg precursors very effectively.
Although our results indicate that the possibility of future Treg generation from precursors is almost completely abrogated after TLR7 stimulation this may not have a detrimental effect on the maintenance of immune homeostasis at first since Treg and precursor pools could be replenished later under steady-state conditions. But continuous TLR7 stimulation in the context of autoimmune disease might very well lead to a complete breakdown of immune homeostasis, since both Treg and Treg precursor induction is impaired.

6.2 IL-6 – the culprit behind impaired regulatory T cell induction

By using different DC:T cell ratios in the co-cultures, it was observed that the reduction in Foxp3+ T cells was dependent on the number of DCs in the co-culture but not on cell-cell contact as the reduction did also occur in cultures with separated DCs and T cells or cultures using supernatant from TLR7-stimulated DCs. Elevated levels of IL-6, IL-12 and IL-17 were detected after TLR7- and TLR9-stimulation and the concentration of IFN-γ was generally high in all co-cultures (> 5ng/ml). Neutralizing experiments revealed that IL-6 was the driving factor behind the reduction of Foxp3+ T cells with small contributions from IL-4 and IFN-γ. Standing alone, this result was not surprising because IL-6 is known to inhibit Foxp3

expression and induce T_H17 cell differentiation in combination with TGF-β. But Hall et al. conducted experiments with TLR9-stimulated lamina propria DCs that had an inhibitory effect on Treg generation (Hall et al, 2008). This coincides so far with our findings that TLR9-stimulated splenic DCs also lead to lower percentages in Foxp3+ T cells.

The difference was, however, that in their neutralization experiments the inhibitory effect could not be prevented by blocking IL-6, but was largely due to the inhibitory effects of IL-4 and IFN-γ with IL-4 having a greater effect than IFN-γ. Interestingly enough, when they added IL-6, IL-4 or IFN-γ to the co-culture of unstimulated lamina propria DCs and naïve T cells the addition of all three cytokines had an inhibitory effect with IL-6 having the most potent effect on inhibiting Treg induction. Therefore an inhibitory effect of IL-6 on Treg inductionwas also seen in this study but this effect appears to play a minor role in the context of TLR9-stimulated lamina propria DCs as opposed to TLR7-stimulated splenic DCs (Hall et al, 2008).

This fact may be dependent on different DC properties. Lamina propria DCs, for example, are able to produce ATRA (Sun et al, 2007) which is known to inhibit T_H17 cell differentiation. IL-6-mediated differentiation of T helper cells in the direction of T_H17 cells in favour of Tregs played a role in the reduction of Treg numbers in our experiments. However, it may not be critical in the context of activated lamina propria DCs since it is possible that this pathway is blocked by ATRA. This would make additional neutralization of IL-6 superfluous. Although ATRA is also able to reduce the percentage of IL-4- and IFN-γ-producing cells, the reducing ATRA-effect seems more potent in reducing IL-17-producing cells (68% reduction of IL-4+ T cells and 48% reduction of IFN-γ+ T cells versus 99% reduction of IL-17+ T cells; data from Elias et al, 2008). This may give IL-4 and IFN-γ the upper hand in inhibiting Treg induction since T_H17 cells are completely out of the picture.

Important to note at this point is the fact that although IL-6-mediated differentiation of T helper cells in the direction of T_H17 cells in favour of Tregs played a role in the reduction of Treg numbers in our experiments the overall percentage of IL-17-producing T cells remained low (around 1% of all CD4+ T cells) in the co-culture even with IL-6 present after TLR7-stimulation of DCs. One reason for the low percentage of IL-17-producing T cells may be that Treg cells were still in transition to T_H17 cells and not yet able to produce IL-17 at the investigated time point. This is supported by our data that RORγt was upregulated in Foxp3+

T cells in conditions with TLR7-stimulation. Lochner et al. found that in vivo a portion of RORγt+ T cells also expressed Foxp3 but that these cells co-expressing RORγt and Foxp3 produce IL-17. They also mentioned that IL-23 is required for full T_H17 effector maturation (Lochner et al, 2008). Another group showed that RORγt and Foxp3 co-expressing T cells are able to produce IL-17 but to a lower extent (Zhou et al, 2008). Taken together the facts, that the levels of IL-23 remained below the detection limit in our experiment and that RORγt and Foxp3 co-expressing T cells seem to be poor producers of IL-17, may account for the low number of IL-17-producing T cells seen in the FACS-analysis.

It was observed in our experiments that the IL-6 concentration rose steadily during the whole time of the co-culture and that the rise was much faster towards the end. Combining this finding with the upregulation of IL-6 mRNA in Foxp3+ T cells on day 4 means that accumulation of IL-6 is not only due to surviving DCs in the co-culture but also to IL-6 producing Foxp3 lowTregs. This may very well lead to a positive feedback loop in the downregulation of Foxp3 and induction of T effector cells.

Taking all findings to date into account, IL-6 has a detrimental effect on Treg induction and Treg suppressive function on all sides. For one, it renders responder T cells unresponsive to Treg mediated suppression thus impairing indirectly Treg suppressive function (Kabelitz et al, 2006). It is also known to block Treg induction and favours T_H17 cell differentiation (Korn et al, 2009) which leads to lower Treg numbers and may lead to unfavourable Treg:Tresp ratios so that effector T cells escape Treg suppression. It was also shown that IL-6 leads to the methylation of CpG dinucleotides in an upstream Foxp3 enhancer in nTregs which represses Foxp3 expression.

Since Foxp3 expression is essential for the stability of the Treg phenotype and Treg suppressive function, nTregs are able to differentiate into proinflammatory effector T cells under the influence of IL-6 and other proinflammatory cytokines (Lal and Bromberg, 2009). Our results show that IL-6 is not only able to inhibit Treg induction, but it also leads to active downregulation of Foxp3 in iTregs and upregulation of RORγt and IL-17. Since the mentioned upstream Foxp3 enhancer is not demethylated in iTregs it seems that the downregulation of Foxp3 must operate through another mechanism in iTregs as in nTregs.

6.3 Impaired suppressive function by TLR7-ligands

Following Foxp3 expression by measuring the percentage and MFI of Foxp3+ T cells every day the observation was made that the initial induction of Foxp3 showed no difference between co-culture conditions with or without TLR7 stimulation. Foxp3 expression was comparable until day 3 and then decreased in co-cultures with TLR7 stimulation. So the reduced percentages of Foxp3+ T cells after TLR7 stimulation were not caused by an inhibition of Foxp3 expression but rather a downregulation of Foxp3 in iTregs at later time points accompanied by upregulation of RORγt, IL-17 and IL-6. Downregulation of Foxp3 is detrimental for the functionality of Tregs because Foxp3 is essential for the suppressive activity of Tregs (Lal and Bromberg, 2009).

Lower percentages of CD103+ iTregs in the co-cultures with TLR7 stimulation were also observed. This caused us to believe that the suppressive function of the generated iTreg population might suffer from the reduced Foxp3 expression and lower percentages of CD103+ Tregs which are highly potent suppressors (Zhao et al, 2008). Comparing the suppressive activity of iTregs sorted from co-cultures with or without TLR7 stimulation after four days revealed that the iTregs from the condition with TLR7 stimulation indeed had a lower suppressive function. This also correlated with further downregulation of Foxp3 during the course of the suppression assay.

In the context of autoimmune diseases the loss of Foxp3 expression in Tregs and the resulting lower suppressive activity is not the only problem which may exacerbate disease. It was also reported that the loss of Foxp3 expression leads to conversion to effector T helper cells (Mellor and Munn, 2011) which may directly promote autoimmunity. This is supported by our findings that day 4 iTregs have not only downregulated Foxp3 mRNA expression but also upregulated mRNA expression of RORγt and IL-17 so they seem be in transition to T_H17 cells. In this way they resembled the reported “ex Foxp3” cells which developed from both nTregs and iTregs and had an activated memory T cell phenotype, produced proinflammatory cytokines and were found in inflamed tissues of autoimmune conditions (Zhou (1) et al, 2009).

Important to note at this point is our assumption that in one of the suppression assays the continuing downregulation of Foxp3 and the resulting development of effector cells during

the course of the experiment appeared to overpower the suppressive ability of the remaining iTregs and reversed the result. This means that most likely the influence of effector T cells which developed from iTregs promoted stronger proliferation of Tresp compared to the control without iTregs.

Another question was if the iTregs from co-cultures with TLR7-stimulation were fully functional Tregs at first or if the suppressive activity was different from the start despite similar Foxp3 expression. It was shown that the exposure of naïve CD4+ T cells to TGF-β can lead to simultaneous Foxp3 and RORγt expression (Lochner et al, 2008; Zhou et al, 2008) and double expressing T cells were able to produce IL-17 which is in accordance with our mRNA data from day 4 (Zhou et al, 2008).

It was also shown that TGF-β induced Foxp3 expression inhibits RORγt function, but that the presence of IL-6 tips the balance toward T_H17 cell differentiation because the Foxp3-mediated inhibition of RORγt is abrogated (Zhou et al, 2008). This means that iTregs generated in the co-cultures could be Foxp3 and RORγt double expressing cells as is mentioned by Lochner et al. in their study (Lochner et al, 2008) but the balance is tipped towards RORγt and IL-17 expression under the influence of IL-6 over time.

The suppressive activity specifically of double expressing cells was not investigated in this study but it was shown that co-expression of Foxp3 and RORγt alone does not seem to affect the suppressive activity of Tregs (Lochner et al, 2008). Therefore, the reduction of suppressive function in our experiment might be predominantly due to the reduction of Foxp3 expression.
To further study this issue iTregs were sorted from co-cultures on day 2 and used in suppression assays. There was no difference in the suppressive activity at this time point when Foxp3 expression was comparable between the conditions. Therefore, it is most likely that iTregs induced under the influence of TLR7-stimulation are fully functional at first and begin to loose the ability to suppress T cells at the same time that they downregulate Foxp3 expression.

6.4 Up or down? - How Foxp3 expression is influenced by the cytokine environment

It was shown that IL-6 inhibits conversion of naïve T cells into Tregs and favours T_H17 differentiation (Korn et al, 2008). In our experiments, inhibition of initial Treg differentiation in favour of T_H17 development was not detected, but rather a reprogramming of iTregs under the influence of IL-6 to develop in the direction of T_H17 cells. This delayed effect required the presence of IL-6 for more two days. This means that iTregs are not imprinted by IL-6 from the beginning to loose Foxp3 expression but Foxp3 downregulation in iTregs is strongly dependent on to the continuing presence of IL-6 in the environment.

It is also obvious from our reculture experiments that Foxp3 expression can be rescued after two days by re-exposing iTregs to optimal Treg-polarizing conditions, namely TGF-β and IL-2 in the absence of IL-6. So despite the fact that iTregs were generated in the presence of IL-6 Foxp3 expression remained stable in the right cytokine environment. It actually seems from the results of the reculture experiments that IL-6 has no effect whatsoever during the initial iTreg generation, but really exerts its effect only after two days, but then quite rapidly. This notion is based on the observation that iTregs generated in the absence of IL-6 loose Foxp3 expression as rapidly as those generated in the presence of IL-6. This leads to the question why IL-6 effects are delayed.
One explanation could be the fact that IL-6 accumulated in the co-culture over time and in order to initiate Foxp3 downregulation a specific concentration threshold must be reached. But it seems unlikely that this is the only reason because already on day 1 IL-6 concentrations in the co-culture exceeded the minimum concentration with which an effect was observed when using recombinant IL-6. So a definite answer to this question remains unclear and would need further investigations

6.5 Model of dynamic Treg and T_H17 differentiation

The results of our experiments showed that TLR7- and TLR9-ligands but not TLR4-ligands had the ability to reduce the percentage of newly generated Foxp3 expressing T cells when naïve T cells were co-cultured with DCs or B cells. The study by Hall et al focusing on TLR9 had already revealed that TLR9 stimulation with CpG inhibited Treg induction (Hall et al,

2008). Therefore our focus was on investigating the effect of TLR7 stimulation on Treg induction and function since previous studies concerning TLR7 signalling and Treg function were contradictory. Some studies showed that TLR stimulation of APCs rendered responder T cells unresponsive to Treg-mediated suppression in an IL-6-dependent manner (Anz et al, 2010; Pasare and Medzhitov, 2003).

On the other hand, Forward et al. reported that TLR7 activation directly enhances the suppressive activity of nTregs because they were sensitized to activation by IL-2 (Forward et al, 2010). Since TLR7 plays an important role in disease development in SLE, our favoured assumption was that TLR7 stimulation has an adverse effect on the Treg compartment. Our focus lay especially on the effect of TLR7 stimulation on the generation of iTregs from naïve CD4+ T cells and their function.
Our results showed that TLR7-ligands did not have a direct effect on iTreg induction. They needed a mediator like DCs or B cells which produce IL-6 to achieve a reduction in iTreg numbers *in vitro* and *in vivo*. At least *in vitro*, the initial induction of iTregs and Foxp3 expression was not inhibited by TLR7- stimulation.

However, active Foxp3 downregulation at later time points caused reduced iTreg numbers and caused less potent suppressive function in remaining Tregs. This effect was due to IL-6 production after TLR7-stimulation. By removing iTregs from the influence of IL-6 Foxp3 expression could be rescued. So iTregs were not previously imprinted by IL-6 to loose Foxp3. On the other hand, iTregs lost Foxp3 expression rapidly under the influence of IL-6 even if they had no previous contact to IL-6. Therefore, we propose the following model of how TLR7 stimulation in a natural setting might impair Treg generation and function to induce effective immune responses and how iTregs might benefit from delayed Foxp3 downregulation to conserve their function:

Our model (Figure 28) proposes that under steady-state conditions DCs presenting low doses of antigen, especially of endogenous self-antigens, lead to iTreg induction and tolerance. The iTregs are able to effectively suppress effector T cells and prevent immune responses. Stimulation of DCs with TLR7-ligands leads to the production of IL-6 thus making T_H17 differentiation possible. IL-6 is also able to block the induction of iTregs and render effector T cells resistant to Treg-mediated suppression. Therefore, tolerance can be overcome and an effective immune response can be generated. We propose that despite the inhibitory effect of

IL-6 on Treg induction some Tregs are generated with full suppressive function in the beginning and that these iTregs contribute to the restoration of steady-state conditions when the infection is cleared. In the absence of IL-6, iTregs are able to retain Foxp3 expression because delayed downregulation of Foxp3 did not yet take effect. So the delayed reduction in Foxp3 expression might be a mechanism of the immune system to prevent complete loss of iTregs during an infection.

In case of continuous TLR7- stimulation and IL-6 production the effect of IL-6 mediated Foxp3 downregulation begins to take hold and iTregs are reprogrammed to become T_H17 cells. This process is further enhanced by IL-6 production from iTregs themselves. In transition, the affected iTregs are still able to suppress effector T cells but not as effectively as iTregs with normal Foxp3 expression. Depending on the duration of TLR7-stimulation and the exposure to IL-6, iTregs in transition may still be able to revert to their original state under tolerance inducing conditions.
Ongoing TLR7 stimulation may eventually result in fully transformed effector T_H17 cells. These cells contribute to immune responses and inflammation as well as autoimmunity.

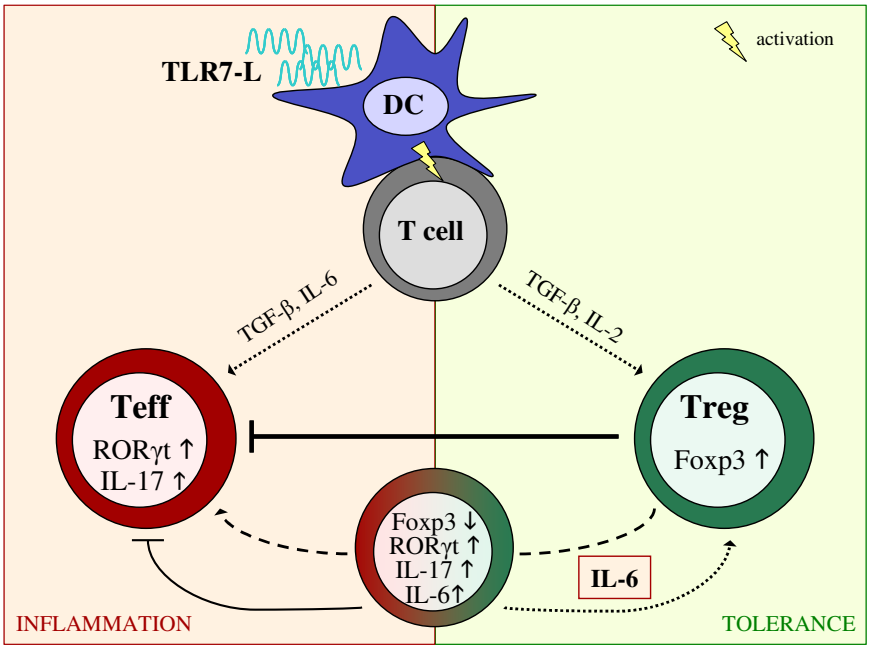

Figure 28: Model decribing the role of TLR7 stimulation on iTreg induction and function
Activation of naïve CD4+ T cells by antigen-presenting DCs leads to T cell differentiation according to the cytokine milieu, IL-2 and TGF-β to iTreg induction (Treg) and IL-6 and TGF-β to effector T_H17 (Teff) cells. Under steady state conditions, Tregs are able to suppress Teff cells. Activation of DCs by TLR-ligands leads to IL-6 production and favoured Teff differentiation. At the same time, IL-6 leads to downregulation of Foxp3 in Tregs and upregulation of RORγt, IL-17 and IL-6, therefore becoming Teff. IL-6 from T cells again affects Tregs. Tregs midway to Teff are still able to suppress Teff, but not as potently.

This scenario is particularly relevant in the development of autoimmune diseases. Following our model, the continuous presence of TLR7-ligands in SLE might shift the balance towards effector T cells and even may contribute to the generation of autoreactive proinflammatory T cells. The development of autoreactive effector T cells further exacerbates the disease through tissue damage leading to the release of more endogenous TLR7-ligands. Therefore, it would be of advantage to break this vicious cycle by reversing Foxp3 downregulation. This can be achieved either by interfering with TLR7-signalling or by blocking IL-6.

In contrast to the detrimental effect in the development of autoimmune diseases, the effect of TLR7-stimulation on the generation and function of iTregs can be utilized to devise potent anti-tumor therapies. Jacobs et al. reported that in a murine pancreatic carcinoma model vaccination was not efficient when the tumor was fully established due to infiltration by large numbers of Tregs. However, co-administration of vaccine and low-dose CpG, the ligand for TLR9, resulted in reduced Treg numbers, enhanced CTL responses and regression of pancreatic tumors (Jacobs et al, 2011).

This shows that modulating the generation and function of Tregs by stimulation or inhibition of TLRs is an important strategy in the treatment of autoimmune diseases and cancer.

7 SUMMARY

Toll-like receptor (TLR) 7 on dendritic cells (DC) or B cells is activated both by exogenous and endogenous TLR-ligands which contain GU-rich single-stranded (ss) RNA. Such ligands are present during viral infections or autoimmune diseases and promote the generation of effector T and B lymphocytes. Although TLR7 activation is beneficial during immune responses against viruses it can have detrimental effects in the development of autoimmune diseases. Such a disease is systemic lupus erythematosus in which the activation of TLR7 by self-RNA ligands promotes systemic autoimmunity. Reduced frequencies of regulatory T cells (Treg) and reduced suppressive function of Tregs is another important feature in SLE and this might be caused by the continuous stimulation of TLR7. Therefore, the aim of this study was to investigate the effect of TLR7 stimulation on the generation and function of Foxp3+ induced Tregs.

The results show that activation of TLR7 has no direct effect on developing Foxp3+ Tregs, but TLR7 stimulated DCs and B cells reduced the percentages of newly generated Foxp3+ T cells. This was confirmed *in vivo* by using a T cell transfer model. Reduced Foxp3+ T cell percentages were accompanied by increased T helper (T_H) 17 cell differentiation and interleukin (IL)-17 production. The reducing effect could be traced back to increased IL-6 production and was abolished by neutralizing IL-6.

Further results revealed that initial Foxp3 induction was not impaired by TLR7 stimulation. The reduction of Foxp3+ T cells was caused by a delayed active Foxp3 downregulation in induced Tregs However, Foxp3 downregulation could be prevented by a timely removal of induced Tregs from the influence of IL-6 . Simultaneous downregulation of Foxp3 and upregulation of RAR-related orphan receptor (ROR) γt, IL-17 and IL-6 mRNA caused the transition from Treg to T_H17 cellsAs a consequence of Foxp3 downregulation the suppressive function of Tregs generated under the influence of TLR7-ligands was also impaired.

These results show that TLR7 stimulation leads to lower Treg numbers and remaining Tregs have lower suppressive activity. This might contribute to the breakdown of peripheral tolerance and development of autoimmune disease. Modulating TLR7 responses might therefore be an important strategy for preventing the development of autoimmune diseases.

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Publications

European Journal of Immunology, May 2011 (DOI 10.1002/eji.201041014)	**"***Activation of Dendritic Cells via TLR7 Reduces Foxp3 Expression and Suppressive Function in Induced Tregs"* **Daniela Hackl**, Jakob Loschko, Tim Sparwasser, Wolfgang Reindl, Anne Krug
Journal of Immunology, November 2011 (DOI 10.4049/jimmunol.1102307)	**"***Antigen Targeting to Plasmacytoid Dendritic Cells via Siglec-H Inhibits Th Cell Dependent Autoimmunity"* Jakob Loschko, Sylvia Heink, **Daniela Hackl**, Diana Dudziak, Wolfgang Reindl, Thomas Korn, Anne Krug

Conferences

World Immune Regulation Meeting 2010, Davos, Switzerland 29.03.10 – 01.04.10	Abstract Title: "*Activation of dendritic cells by TLR7 and TLR9 ligands influences the generation and function of regulatory T cells*" Daniela Hackl, Jakob Loschko, Wolfgang Reindl, Tim Sparwasser, Anne Krug
TOLL2008 Meeting, Lisbon, Portugal 24.09.2008 – 27.09.2008	Abstract Title: *"Activation of DCs by TLR7 and TLR9 ligands inhibits the generation of regulatory T cells"* Daniela Hackl, Wolfgang Reindl, Anne Krug