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Induction of regulatory immune responses that control autoimmune diseases

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A thesis submitted to

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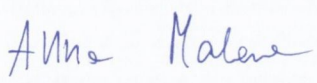
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Abstract

Regulatory T (Treg) cells play a fundamental role in suppressing excessive inflammatory responses to pathogens and in maintaining peripheral tolerance to self-antigens. A breakdown in self-tolerance or defect in Treg cells can result in uncontrolled effector T cell responses to self-antigens and the development of autoimmune disorders. Approaches that enhance the generation of auto-antigen-specific Treg cells have potential for the prevention or treatment of autoimmune disease. The aim of the present study was to address the hypothesis that it is possible to induce self-antigen-specific Treg cells *in vivo*. Furthermore, the study examined the possibility that prophylactic treatments that induced self-antigen-specific Treg cells have the capacity to prevent development of autoimmune diseases.

Toll-like receptor (TLR) activation of dendritic cells (DC) promotes production of the inflammatory cytokines, IL-12, IL-1 and IL-23, that drive induction of effector Th1 and Th17 cells. However TLR activation also induces anti-inflammatory cytokines, including IL-10 that direct the induction of Treg cells. Therefore activation of DC with TLR agonists in the presence of inhibitors of the signaling pathways that mediate expression of pro-inflammatory cytokines has the capacity to selectively drive Treg cell responses. This study revealed that activation of DC with the TLR2 and dectin-1 agonist zymosan promoted IL-10 as well as IL-1 and IL-23 production by DC. Inhibition of MEK1/2, caspase-1 and GSK3 blocked IL-1 and IL-23, but not IL-10 production and enhanced the ability of zymosan-stimulated DC to induce IL-10- and Foxp3-expressing T cells. Furthermore, treatment of mice with zymosan and MEK1/2, caspase-1 and GSK3 inhibitors enhanced antigen-specific IL-10 and decreased antigen-specific IFN-γ and IL-17 production by T cells *in vivo*. However, immunization of mice with auto-antigen myelin oligodendrocyte glycoprotein (MOG) and zymosan in the presence of MEK1/2, caspase-1 and GKS3 inhibitors did not prevent induction of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis.

Induction of Treg cells is enhanced by anti-inflammatory cytokines, with IL-10 and IL-27 promoting the generation of IL-10-secreting Tr1-type Treg cells. This study demonstrated that immunization with antigen in the presence of IL-10 promoted the generation of antigen-specific IL-10-producing Tr1 cells *in vivo*. Moreover, immunization of mice with MOG and IL-10 (weeks -3 and -1) before induction of EAE attenuated clinical symptoms of disease. The attenuation of EAE was associated with delayed leukocyte infiltration into CNS and enhanced MOG-specific IL-10 production by splenic T cells. In addition, adoptive transfer of spleen cells isolated from mice immunized with MOG and IL-10 protected against actively induced EAE in recipient mice.

Epidemiology studies have shown that a higher incidence of parasitic infections in humans is associated with a lower incidence of allergy and autoimmune diseases. Infection of mice with the helminth parasite *Fasciola hepatica* or treatment with excretory-secretory (ES) products from *F. hepatica* attenuated EAE. This study showed that total extract (TE) from *F. hepatica* increased expression of anti-inflammatory molecules and M2 macrophages markers arginase, RELM-α and PD-L2 *in vitro* and *in vivo*. Furthermore, prophylactic immunization of mice with MOG in the presence of TE prevented the development of EAE. Surprisingly prophylactic treatment of mice with the *F. hepatica* extract (without MOG) also protected against development of EAE. This was associated with a reduction of infiltration pathogenic IL-17- and IFN-γ-producing T cells into CNS.

This study has contributed to the understanding of the immunoregulatory mechanisms that control autoimmunity and constitute a foundation for development of novel regulatory-based immunotherapies for autoimmune diseases.

List of abbreviations

AIRE	autoimmune regulator
AP1	activator protein 1
APC	antigen presenting cell
BBB	blood-brain barrier
BCA	bicinchoninic acid
BCG	Bacille Calmette-Guerin
BCR	B-cell receptor
BMDC	bome marrow-derived dendritic cells
BMDM	bone marrow-derived macrophages
BSA	Bovine serum albumin
CD	cluster of differentiation
CFA	complete Freud adjuvant
CIA	collagen-induced arthritis
CIS	clinically isolated syndrome
CLR	C-type lectin receptors
CNS	central nervous system
CpG	cytosine-guanine dinucleotide
CREB	cAMP response element-binding protein
CTL	cytotoxic T lymphocytes
CTL	cytotoxic T cell
DAMPs	damage-associated molecular patterns
DC	dendritic cell
DMSO	dimethyl sulfoxide

DMT	disease-modifying treatments
DNA	deoxyribonucleic acid
Dsrna	double strand ribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EAU	autoimmune uveoretinitis
EBAO	ethidium bromide acridine orange
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoabsorbent assay
ERK	extracellular signal-regulated kinase
ES	excretory-secretory
FACS	fluorescent-activated cell sorting
Fas	ligand FasL
FCS	foetal calf serum
FHA	filamentous haemagglutinin
FhTeg	*Fasciola hepatica* tegumental antigens
FMO	fluorescence minus one
Foxp3	forkhead box P3
GA	glatiramer acetate
GATA3	GATA binding protein 3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSK3	glycogen synthase kinase 3
HBSS	Hanks balanced salt solution
HCV	hepatisis C virus
HINT	helminth-induced immunomodulation therapy

HLA	human leukocyte antigen
HRP	horseradish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
IBD	inflammatory bowel disease
ICOS	inducible co-stimulator
IFN	interferon
IL	interleukin
ILC	innate lymphoid cells
ILN	inguinal lymph node
InfDC	inflammatory dendritic cells
iNKT	invariant natural killer cells
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF	interferon regulatory factor
iTreg	inducible Treg
KLH	keyhole limpet haemocyanin
LAG-3	lymphocyte-activation gene 3
LAL	limulus amebocyte lysate
LAP	latency associated peptide
LCs	Langerhans cells
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LT	lymphotoxin
MAG	myelin-associated glycoprotein

MAL	MYD88-adaptor-like protein
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MEK1/2	mitogen-activated protein kinase kinase
MHC	major histocompatipility complex
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MPL	monophosphoryl lipid A
MS	multiple sclerosis
Mtb	*Mycobacteria tuberculosis*
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	nitric oxide
NOD	non-obese diabetic
NOS	nitric oxide synthase
nTreg	natural Treg
NTx	neonatal thymectomy
OVA	ovalbumin
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PD-1	programmed cells death 1

pDC	plasmacytoid DC
PD-L	programmed death-ligand
PEC	peritoneal cavity cells
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PLM	progressive multifocal leucoencephhalopahy
PLN	popliteal lymph nodes
PLP	proteolipid protein
PMA	phorbol ester phorbol 12-myristate 13-acetat
PMN	polymorphonuclear
PRRs	pattern recognition receptors
PT	pertussis toxin
RA	rheumatoid arthritis
RAG	recombination activated gene
RELM-α	resistin-like molecule-α
RLRs	(RIG)-I-like receptors
RNA	ribonucleic acid
ROR	retinoic acid receptor-related orphan receptor
RRMS	relapsing remitting MS
s.c.	subcutaneous
S1P	sphingosine 1 phosphate
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecyl sulphate

SEA	*Schistosoma mansoni* egg antigens
Siglec	Sialic acid-binding immunoglobulin-type lectins
SLE	systemic lupus erythtemathosus
STAT	signal transducers and activator of transcriptions stat
Tbox	transcription factor T-bet
TCR	T-cell receptor
TE	total extract
TGF-β	Transforming growth factor beta
Th	T helper
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand receptors
TRAM	TRIF-related adaptor molecule
Treg	regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	thymic stromal lymphopoietin
VLA-4	very late antigen-4
VZV	*Varicella zoster* virus

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Chapter 1

General introduction

CHAPTER 1 GENERAL INTRODUCTION

1.1 Immune system

The immune system developed to protect from infections, caused by viruses, bacteria, fungi and other parasites. A fundamental feature of the immune system is the ability to distinguish self from potentially harmful foreign molecules. The ability of discrimination between "self" versus "non-self" dates back to the early stages of life. The process of recognition and absorption of foreign molecules through phagocytosis, which is a foundation of successful immune response, developed in primitive single-cell life forms (protozoans) as far back as 2.5 billion years ago (1). In 1882, a Russian zoologist Elie Metchnikoff first observed the innate response of phagocytic cells of starfish larva, a multicellular marine animal, which remained unchanged by the evolution for 600 million years (2). A non-specific response, called innate or natural immunity, is the only form of immune defence present in invertebrates. In evolutionary more complex vertebrate animals the specialized and specific adoptive (acquired) immunity evolved to work in coordination with innate immune system.

During a mammal's lifetime the immune system is constantly adapting to recognize and effectively eliminate not only external pathogens, but also internal pathological threats, such as cancer. On the other hand, immunoregulatory mechanisms maintain tolerance to the own tissues, symbiotic microflora and non-pathogenic ingested or inhaled antigens and minimize the collateral damage during the fight against the infection. Host defence mechanisms consist of innate phase, which includes the initial, rapid protection against pathogens, and provides the initiation of adaptive immunity. Slowly generated adaptive immune response is specialized in specific and effective defence against infections and includes development of immunological memory, which facilitates protection against re-exposure to the same pathogen.

The cells of the immune system originate in the bone marrow and derive from the same precursors – pluripotent hematopoietic stem cells, which separate into myeloid or lymphoid progenitor cells. The myeloid progenitors are the precursors of innate leukocytes: granulocytic polymorphonuclear (PMN) leukocytes (neutrophils, eosinophils and basophils), mast cells precursors and mononuclear phagocytes - monocytes, macrophages and dendritic cells (DC). Monocytes circulating in the bloodstream differentiate further into a range of tissue resident macrophages and

dendritic cells (3). Lymphoid progenitor cells differentiate into the cells of an adaptive arm of the immune system, mainly lymphocytes. T and B lymphocytes acquire a unique antigenic specificity by irreversible genetic recombination of antigen receptor gene segments (catalysed by RAG recombinase) and give rise to the progeny cell clones of the same specificity, including the long-living memory cells, which are a key feature long-lasting acquired immunity.

Recently, other members of the lymphoid lineage have been identified. Innate-like B (B-1) cells, the invariant natural killer T (iNKT) cells, γδ T cells and innate lymphoid cells are developmentally related to conventional B or T cells but differ in functional properties. Those innate-like lymphocytes do not respond in an antigen-specific manner, but play an important role in early immune responses and are a major source of cytokines associated with adaptive immunity. In contrast, the natural killer (NK) cells, granular lymphocytes traditionally defined as cells of the innate immune response, have been shown to develop antigen-specific, but RAG-independent specific memory (4).

The immunological memory has been one of the classical features distinguishing adaptive from innate immunity. However, there is evidence from invertebrates to suggest that immune memory can develop independently of lymphocytes possessing rearranged antigen receptors. Experimental exposure of many invertebrates, e.g. insects to their natural pathogens resulted in enhanced resistance against subsequent re-challenge, which was dependent exclusively on phagocytic innate cells (5,6). Taken together, the recent discoveries of innate features of lymphocytes and the memory of the innate cells question the traditionally established boundaries between the adaptive and innate immune systems.

Cells of both adaptive and innate arms of the immune system cooperate with each other; innate cells directly shape the response of the adaptive immunity and receive feedback signals. The initiation of the immune response is mediated by phagocytic cells: macrophages, neutrophils and DC – the professional antigen-presenting cells (APC), which provide the bridge linking innate and adaptive immunity (7).

1.2 Innate immunity

The innate (natural) immunity is a system of phylogenetically ancient mechanisms of defence against invading pathogens, present in all vertebrates and invertebrates, including animals as evolutionary old as sea sponges (8). Innate immunity serves a

quick response against infection and is based on recognition of common features of pathogens through invariant, germ-line coded receptors located on the surface or intracellular in innate immune cells. The innate immune system is efficient in eliminating pathogens but is also crucial for initiation of more sophisticated methods of defence, provided by the adaptive immunity.

1.2.1 Innate immune recognition

The innate immune system responses to exogenous threats using a strategy to recognize a "microbial nonself". In 1989, during the Cold Spring Harbor Symposium of quantitative biology, Charles Janeway proposed that evolutionary old immune cells bear receptors that allow the recognition of certain pathogen-associated molecules (9). Indeed, these receptors enable detection of evolutionary conserved microbial products, essential for their function, called pathogen-associated molecular patterns (PAMPs). PAMPs are unique for microorganisms and are not produced by the host. They include such common structures as lipopolysaccharide (LPS) on gram-negative bacteria, teichoic acid on gram-positive bacteria or short sequences of unmethylated bacterial DNA. These and other PAMPs are recognized by receptors called pattern recognition receptors (PRRs), present either on the surface or in the cytoplasm on the immune cells. The mix of signals recognized through PRRs determines the type and intensity of immune defence pathways and also shapes the type of adaptive immune response (10)

PRRs also detect patterns that are associated with host own products using the strategies to recognize "missing self" and "induced or altered self". The first strategy is based on the recognition of markers of normal and healthy cells, which are unique to the host and are missing during the pathogenic conditions. The second strategy allows detection of markers induced in the host cells by abnormal situation, such as a viral infection, cellular transformation or cell death. Pathogens are not the only threats to host's health and both those strategies enable the immune system to defend not only against infections but also to prevent the development of tumours. For example, certain types of infections or cellular transformation are associated with decreased expression of constitutive cells surface markers, which is a signal for activation of NK cells and selective elimination of the altered cells among the healthy ones (11)

Apoptosis is one of the mechanisms of host defence, preventing the spread of infection and proliferation of mutated cells. Cells undergoing apoptosis present specific markers on their surface, which can be recognized by phagocytic cells that clear the cellular debris, without inducing the inflammatory response. On the other hand, chronic infection or mechanical injury leading to tissue and cell damage, are associated with release of host cellular components, such as DNA fragments, metabolic intermediates or heat shock proteins. Those endogenous molecules that signal disintegration of cells, called alarmins, constitute the larger group of damage-associated molecular patterns (DAMPs), which can be recognized by some of the PRRs that also detect PAMPs (12).

1.2.1.1 Pathogen recognition receptors

The exogenous PRR ligands separate into two classes. The first class includes microbial cell-wall components, such as LPS, peptydoglican, lipoglycans, porins, zymosan (yeast cell wall) or viral envelope proteins. Viruses and some bacteria replicate inside cells, therefore the second class contains bacterial and viral nucleic acids. PRRs can be divided into several different families and include transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLR) and cytoplasmic proteins, such as NOD-like receptors (NLRs) and retinoic acid-induced gene (RIG)-I-like receptors (RLRs). TLRs can detect bacteria and viruses located outside of the cells and trapped in the endosomes. In contrast, RLRs and NLRs detect the microbial products in the cytoplasm. In addition, some of the NLRs are activated by DAMPs and are the components of the molecular complex called inflammasome that links detection of microbes and metabolic stress resulting in activation of pro-inflammatory cytokines IL-1β and IL-18 (13).

Toll-like receptors

TLRs is a family of evolutionary conserved PRRs from *Caenorhabditis elegans* to mammals (14, 15). Toll receptor was firstly described as an essential protein during the embryonic development of dorsoventral polarity of fruit fly *Drosophila melanogaster*. In 1996 Lemaitre *et al* showed that functional Toll is also critical for antifungal immune response in fruit flies (16). Soon after the human homologue of Toll was identified by Medzhitov *et al* drawing attention to mammalian TLR and their potential role in immune regulation (17). Finally, the genetic evidence that TLR is involved in bacterial sensing was provided by Poltorak *et al*, who showed that mammalian TLR4 is

responsible for recognition of LPS (18). Subsequently, ten human and twelve murine members of TLR family have been identified (19).

TLRs are type I integral membrane glycoproteins characterized by leucine-rich repeats (LRR) in the extracellular domain. The cytoplasmic domain shows high similarity to the intracellular domain of IL-1 receptor family members and is now known as Toll/interleukin-1 receptor (TIR) domain. During signal transduction, all TLRs use one or more adaptor proteins. Most of TLRs utilizes the myeloid differentiation primary response 88 (MyD88) adaptor protein, except TLR3, which uses TIR-domain containing adaptor-inducing IFN-β (TRIF) domain. TLR4 was shown to use four adaptor molecules, MyD88, TRIF and additionally, TRIF-related adaptor molecule (TRAM) and MYD88-adaptor-like protein (MAL) (also known as TIRAP), therefore having the most complex signalling arrangement among TLRs (20). Activation of TLR triggers downstream signalling cascades, such as mitogen-activated protein (MAP) kinases, p38, or ERK, which effectively activates transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and interferon-regulatory factors (IRFs), cyclic AMP-responsive element-binding protein (CREB) or activator protein 1 (AP1). This leads to the production of type I IFN, pro-inflammatory and anti-inflammatory cytokines, chemokines and enhanced expression of co-stimulatory molecules (21). The differential responses mediated by distinct TLR ligands arise from the selective engagement of the adaptor molecules, activating different downstream signalling pathways (22) and inducing the most appropriate and effective immune response.

TLRs can be divided into subgroups according to the class of PAMPs they recognize. TLR2 with TLR1 or TLR6 recognize lipid structures such as peptidoglycan, bacterial lipopeptides, bacterial lipoarabinomannan and yeast zymosan. The major ligand for TLR4 is LPS from Gram negative bacteria. TLR5 binds bacterial flagellin. TLR3 recognizes viral dsRNA, whereas TLR7 and TLR8 bind ssRNA. The ligand for TLR9 is bacterial CpG DNA. Most of the TLRs are expressed on the cell surface, whereas others (TLR3, 7, 8 and 9) are present in the endosomes (10, 23). The discovery of ligand for TLR3 showed that TLRs also are involved not only in antibacterial but also in antiviral response. Among 10 known TLRs in humans, TLR10 remains an orphan receptor, however, a recent study suggested a role in influenza A virus infection (24). The expression of TLR10 was not detected in mice and only the partial genomic sequences

for *TLR10* with gaps and retroviral insertions were identified (25). Murine TLR11 family includes products of closely related genes, *Tlr11*, *Tlr12* and *Tlr13* (26). The endosomal TLR11 in cooperation with TLR12 recognize *Toxoplasma gondii*-derived protein profilin (27, 28), whereas TLR13 was recently discovered to recognize a conserved bacterial 23S ribosomal RNA (29). In contrast, human *TLR11* gene is a non-functional pseudogene and genes for TLR12 and TLR13 are missing in the human genome (26, 30).

The pattern of cellular expression categorizes TLRs as ubiquitous, restricted or specific. For example TLR1 is the most widely expressed among the human leukocytes cells, whereas TLR3 seems to be specific for immature DC (31). The pattern of TLR expressed by different innate cells in different locations determines the profile of the immune response.

1.2.2 Dendritic cells

DC were first visualized by in 1868 in skin as Langerhans cells (LCs) and in 1974 were shown by Ralph Steinman to be the essential immune cells in driving the T-cell mediated response and therefore linking the innate and adaptive immunity (32). DC are one of the many phagocytic cells that can present antigens to the T cells, but due to their functional specialization and high efficacy in antigen uptake, processing and presentation and consequently facilitating activation of naive T cells, DC are termed professional APCs (7). Macrophages and B cells can also function as the professional APCs, however DC are the most potent in T cells activation. Initially, DC were characterized as a population of cells enriched from the mouse spleen. Today, the term DC refers to a group of several cell types, functioning as APC that differ in their origin and other properties. DC located in all lymphoid and non-lymphoid tissues are the conventional or classical DC (cDC), which originate from the bone marrow. The tissue-resident DC, such as LCs of epidermis, function as sentinels and traffic to the lymphoid organs for T cell priming. In addition, the inflammatory conditions can initiate development of blood monocyte-derived "inflammatory DC", producing large amounts of tumor necrosis factor (TNF) and nitric oxide (NO) (33). Finally, a small subset of plasmacytoid DC (pDC), produce high amounts of type 1 interferon (IFN), when exposed to viral particles (34).

DC sample both microbial and host-derived antigens in the peripheral tissues through various mechanisms, such as phagocytosis, micropinocytosis and receptor-mediated endocytosis. Antigen capture initiates structural and functional changes in DC, called DC maturation, which involves loss of phagocytic capabilities, migration to the draining lymph nodes, increased expression of surface stimulatory molecules and initiation of the antigen processing and antigen presentation on the cell surface through major histocompatibility complex (MHC) proteins (35). DC express a variety of PRRs and their stimulation initiates the cytokine expression. The profile of cytokines released by DC at the time of interaction with T cells determines their differentiation into an effector T cell type.

1.3 Adaptive immunity

The innate immune cells are activated by a limited number of conserved microbial structures through genetically encoded receptors. In contrast, the individual T and B cells of adaptive immune system can distinguish between a structurally very similar molecules with high specificity and the whole lymphocyte pool of the host can respond to an almost unlimited amount of antigens. The adaptive immunity evolved in jawed vertebrates and the specificity and diversity of response was achieved by development of mechanism facilitating the generation of a unique antigen receptor by each lymphocyte. Another feature of adaptive immunity is the generation of immunological memory - the most important biological consequence of the development of the adaptive immunity (36). After the primary infection a portion of highly specific long-living lymphocytes remains in the system, ready for a rapid response after re-encounter with pathogen.

1.3.1 Immune recognition by lymphocytes

The major cells of adaptive immunity are B and T lymphocytes - two lineages that specifically recognize and respond to antigenic determinants of pathogens through specialized receptors, called B-cell receptor (BCR) and T-cell receptor (TCR). TCRs are members of the immunoglobulin superfamily, however they are expressed exclusively on the T cell surface. Each receptor consist of two different protein chains α, β, γ, or δ. A majority of T cells, called conventional T cells bear α:β TCR, and 3-5% of T cells have alternative γ:δ TCR (37). TCR ligands are short peptide fragments,

recognized only, when bound to the MHC molecules on the APCs. TCR itself does not transmit signals upon binding the ligand, but it is covalently associated with CD3 signalling molecules, forming a multisubunit TCR complex (38).

The specificity of each TCR and BCR is determined by the amino acid sequence of the variable region of the receptor. The diversity of lymphocyte receptor specificity is achieved by somatic recombination of gene segments for variable region of TCR and immunoglobulins, called V(D)J recombination and catalized by RAG-1 and RAG-2 enzymes. V(D)J recombination occurs early in the lymphopoiesis and in the B cells it takes place in the bone marrow. In the peripheral organs the somatic hypermutations introduce point mutations into the genes for variable regions, additionally diversifying the specificity of BCRs. In contrast, TCR gene rearrangement take place in the thymus and the somatic hypermutations do not include TCR gene sequences (7).

The random rearrangement of lymphocyte receptor genes can generate an almost infinite number of receptors with unique specificities including the receptors that recognize body's own antigens. Self-reactivity of adaptive immune cells inevitably leads to the pathologic conditions and damage of the tissues and organs. Thus the elimination of autoreactive lymphocytes in their early development is the basic mechanism for maintenance of self-tolerance.

T cells migrate from the bone marrow to the thymus, where they acquire specificity and mature T cell surface markers. Developing T cells rearrange TCR genes and differentiate into the αβ or γδ TCR-expressing cells. The αβ TCR thymocytes begin to express both CD8 and CD4 molecules. At this stage, the TCR affinity for self-peptide-MHC complex determines the fate of the thymocytes. The weak interaction of TCR with peptide-MHC is required to rescue the thymocytes from the death by neglect and to direct for the positive selection, whereas a strong interactions between TCR and peptide-MHC causes apoptosis, which is the basis of clonal deletion (39). Importantly, some of the T cells, exhibiting medium-strong affinity to the self-peptides are not deleted but re-directed into the regulatory T (Treg) cell lineage and acquire immunosuppressive properties. The negative selection of highly self-reactive T cells and development of Treg cells are the mechanisms of the central tolerance. However, despite these sophisticated mechanisms of selection, some of the auto-reactive B and T

cells pass to the periphery, where they are tightly controlled by mechanisms of the peripheral tolerance (40).

1.3.2 Antigen presentation and activation of T cells

Newly generated conventional αβ T (Tconv) cells leave the thymus as mature antigen-specific T cells. They circulate between the peripheral lymphoid tissues, interacting through TCR with many both immune and non-immune cells and these repeated low affinity interactions with MHC-peptides complexes, that initially positively selected them in the thymus, are essential for their survival and homeostasis in a non-activated state in the periphery. Tconv are restricted to the recognition of an antigen through either MHC-I or MHC-II molecules via interaction of, respectively, CD8 and CD4 molecules expressed on the T cell surface. MHC class I molecules are ubiquitously expressed by all nucleated cells and present intracellular peptides degraded by cytosolic and nuclear proteasomes. Professional APCs are the majority of cells expressing MHC class II molecules, which present peptides derived from exogenous proteins, firstly captured by APCs through macropinocytosis or phagocytosis.

A fundamental requirement for the priming of naïve T cells is the direct interaction with a specific antigen expressed by matured APCs in the lymphoid tissue. Naïve T cells require two independent signals simultaneously delivered by DC to induce proliferation. The first signal is the recognition of specific ligand-MHC complex by TCR and the second signal is provided by the co-stimulatory molecules expressed on the surface of the DC. Co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) belong to the immunoglobulin family of B7 proteins. Activation of CD28 co-receptor on T cell surface by B7 molecules synergizes with the signal from TCR to induce expression of the cytokine IL-2 and the high-affinity receptor for IL-2. IL-2 binds to the receptor in the autocrine manner and induces proliferation (clonal expansion) of the T cell. Other co-stimulatory molecules, such as CD40, programmed death-ligand 1 (PD-L1), PD-L2 on APCs and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or inducible co-stimulator (ICOS) on APCs can modify the activation status of T cells, amplifying or inhibiting the signals from TCR (7).

In the peripheral tissues, immature DC express low levels of MHC molecules and co-stimulatory molecules, therefore are weak activators of T cells. Upon antigen uptake

and activation of PRRs, DC migrate to the local lymph nodes and undergo a process of maturation, which involves loss of the phagocytic abilities, initiation of antigen processing and increased expression of MHC molecules and co-stimulatory molecules, which is further amplified via interactions with T cells. DC presenting specific antigens to T cells without co-stimulation induce a state of unresponsiveness, called anergy, which in case of naïve T cells may lead to their apoptosis. Anergy is one of the mechanisms for maintenance of peripheral tolerance to self-peptides, harmless commensal bacteria and food antigens (41).

In addition to antigen presentation and co-stimulation, the cytokine milieu plays a pivotal role in polarisation of naïve $CD4^+$ T cells into a specific subset of effector cells. The activation of the DC via PRRs is associated with the secretion of various cytokines, which, along with the cytokines produced by other immune cells, drive differentiation of T cells into particular subset. The cytokines provide a third signal required for complete activation of the adaptive immune response (42).

1.3.3 Effector T cells subsets

The naïve $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTL) after recognition of antigens derived from intracellular pathogens, such as viruses, intracellular bacteria and protozoa and antigens from transformed host cells. The main mechanism of their cytotoxicity is the induction of apoptosis in target cells by release of granules containing perforin and preformed proteolytic proteins, called granzymes and secretion of Fas ligand (FasL). CTL produce also a range of cytokines, particularly IFN-γ (43).

When stimulated by APCs, naïve $CD4^+$ T cells primarily produce large amounts of IL-2 and later, dependently on the cytokine context provided by APCs, they differentiate into one of the T helper (Th) cell subset. The most important role of Th cells is assisting in activation of other immune cells and enhancing the most appropriate response to the type of infection. In 1986, Mosman and Coffman identified two subsets of $CD4^+$ cells, Th1 and Th2 cells, which differed in their cytokine secretion and functions (44). Th cell subsets were firstly distinguished by the cytokine profile that they produce after activation. Today, the distinct Th cells can be characterized by specific surface markers and transcriptions factors that regulate their effector properties.

Th1 cells

Th1 cells are induced during infections with viruses, intracellular bacteria and protozoa and the main cytokines produced by this T cell subtype is IFN-γ and lymphotoxin α (LTα) (44). IFN-γ can directly inhibit replication of viral particles in infected cells or kill virus-infected cells, inducing apoptosis (45). Together with TNF-α, IFN-γ induces the classical activation of macrophages to increase their microbicidal properties and recruits macrophages to the site of the infection (46). IFN-γ also actives NK cells. In addition, Th1 cells stimulate proliferation and activation of CTL cells to enhance the anti-viral response and their presence is essential for the development and maintenance of memory $CD8^+$ T cells (47). IL-12, expressed by innate cells, is a key cytokine triggering differentiation of $CD4^+$ T cells into the Th1 subset. The functional IL-12 is composed of two subunits, IL-12p40 and IL-12p35 (together forming IL-12p70), which are expressed independently. The transcription of IL-12p40 is driven by stimulation of TLR, whereas expression of IL-12p35 is initiated by type I IFN and IFN-γ secreted by activated NK cells and by Th1 cells themselves. Thus, the IFN-γ signalling works in a positive feedback loop for enhanced development of Th1 cells (45). The main regulator of Th1 differentiation, triggering expression of IFN-γ, is Tbox transcription factor (T-bet), which is up-regulated in response to IL-12 signalling through activation of signal transducers and activators of transcription 1, 3, 4 and 5 (STAT1, 3, 4 and 5) (48–50).

Recently, other cytokines, including IL-18 and IL-27 have been shown to play an important role in activation of Th1 cells. IL-18, a member of IL-1 cytokine family, is not directly involved in differentiation of Th1 cells, but can synergize with IL-12 to induce

IFN-γ production through upregulation of IL-12 receptor (51). IL-27 drives expression of T-bet in naïve $CD4^+$ T cells, but also promotes expression of anti-inflammatory cytokine IL-10, therefore the role of IL-27 in controlling Th1 development remains unclear (52).

Initially, it was considered that the development of self-reactive Th1 cells is a requirement for induction of organ-specific autoimmunity. Early studies showed that mice susceptible to experimental autoimmune encephalomyelitis (EAE) had enhanced levels of IFN-γ, which positively correlated with severity of disease (53). Moreover, the CNS infiltrating $CD4^+$ T in EAE were identified as a major source of IFN-γ (54). However, it was later demonstrated that IFN-γ-deficient, IFN-γ-receptor-deficient mice

or IL-12- and IL-12R-defiecient mice were more susceptible to EAE (55, 56). These findings raised the possibility that other cells, different from Th1 cells, are required for the induction phase of organ-specific autoimmune inflammation (57).

Th2 cells

Th2 cells mediate defense against the extracellular bacteria, allergens, fungi and parasites, including helminthic worms. The differentiation of Th2 cells depends mainly on IL-4, which signals through IL-4R and signal transducer and activator of transcription 6 (STAT6) to enhance expression of the key Th2 gene regulator, transcription GATA binding protein 3 (GATA3) (58). Also other factors have been implicated in the development of Th2 cells. For example, an alarmin IL-33, produced by fibroblasts, epithelial and endothelial cells in response to tissue damage, enhances production of cytokines by polarized Th2 cells (59).

Th2 cells produce various cytokines, regulating B cells, eosinophil, basophil, mast cell and macrophage responses. IL-4, produced by Th2 cells works in the positive feedback loop to enhance Th2 cell differentiation and, together with IL-13, stimulates B cell proliferation and maturation into the plasma cells and regulates the antibody class switching into IgE. IgE bind to FcR on basophils and mast cells, which after cross-linking by an antigen, leads to release of histamine, serotonin and the type-2 cytokines, IL-4 and IL-13, thus enhancing the generation of Th2 responses. IL-5 plays a crucial role in the activation and recruitment of eosinophils, whereas IL-3, IL-4, IL-9 and IL-13 attract and directly activate basophils and mast cells for degranulation (60). IL-25 (IL-17E) induces expression of IL-4, IL-5 and IL-13 by newly characterized innate lymphoid cells type 2 (ILC2), which further amplifies Th2 responses (61). Together, these cytokines initiate and regulate an immune response leading to the effective clearance of extracellular pathogens and expulsion of helminths. However, an imbalanced function and overactivation of Th2 cells in responsible for the development of hypersensitivity reactions to common environmental antigens, leading to pathological conditions such as allergy and asthma.

Th2 cytokines IL-4 and IL-13 also drive the alternative activation of macrophages. In contrast to the classical activation triggered by IFN-γ which induces strong inflammatory response alternative activation results in the enhanced expression of MHC class II and mannose receptors that stimulate endocytosis and enhanced

production of IL-10 and mediators of tissue repair (62). Signalling of IL-4 through STAT6 together with stimulation of TGF-β contributes to the development of another effector T cell subset, Th9, which are also implicated in immune responses against parasites and allergy (63). Th9 do not produce the typical Th2 cytokines, but IL-9, which enhances the production of mucin by epithelial cells (64)

Th17 cells

In 2003, it was shown that IL-23 but not IL-12, is crucial for development of EAE, confirming that Th1 is not the key T cell subset required for the initiation of organ-specific autoimmunity. It was subsequently established that IL-23 is involved in the development IL-17-producing $CD4^+$ T cells that are capable of inducing EAE, when adoptively transferred to naïve mice (65). On the basis of those studies, it was proposed that IL-17-producing $CD4^+$ T cells are a distinct effector T cells subset that was named Th17 cells.

Since naïve $CD4^+$ T cells do not express IL-23R, is became clear that IL-23 is not the differentiation factor for Th17 cell development. Several independent studies established that TGF-β together with IL-6 are both essential factors initiating transcriptional program for mouse Th17 cells differentiation (57), leading to the expression of transcription factor RAR-related orphan receptor γ/TOR (RORγt). Recently, it has been proposed that TGF-β plays an indirect role in the initial differentiation of Th17 cells by suppression of T-bet and GATA3, thus preventing Th1 and Th2 differentiation. In that context, IL-6 is sufficient for induction of RORγt (66). IL-6 is also a strong inducer of IL-21, which is a growth factor for differentiating Th17 cells and acts in a positive feedback loop for their amplification. Finally, RORγt enhances the expression of IL-23R, which enables IL-23 to stabilize the commitment of $CD4^+$ T cells into Th17 subset and their maintenance. Furthermore, IL-1β, together with IL-23, enhance effector functions of both mouse and human Th17 cells by promoting IL-17 production (67).

Apart from IL-17 (also called IL-17A), Th17 cells have been shown to produce IL-17F, IL-22, IFN-γ, GM-CSF and in humans IL-26. Although characterized as effector cells in autoimmunity, the primary function of Th17 cells is the clearance of certain types of pathogens that require a strong inflammatory response and cannot be combatted by Th1 and Th2 cells. IL-17A is a strong pro-inflammatory cytokine, which acts on the broad

range of cell types to induce expression of other pro-inflammatory cytokines, such as TNF-α, IL-1-β, IL-6 and GM-SCF and chemokines, which leads to a recruitment and activation of macrophages and neutrophils. IL-17 plays an important role in the clearance of the extracellular bacteria, such as *Staphylococcus aureus*, *Citrobacter rodentium* or *Klebsiella pneumonia*, which colonize epithelial or mucosal surfaces (68). Moreover, Th17 cells and IL-17 are crucial in immunity to fungi, since patients with genetic defects in IL-23/IL-17 axis develop severe uncontrolled fungal infections (69). IL-17-producing cells T cells, together with Th1 cells, also mediate the protective function of natural and vaccine-induced immunity to *Bordetella pertussis* (70). IL-22, a member of IL-10 family of cytokines, is produced by terminally differentiated Th17 cells. The main function of IL-22 is induction of antimicrobial molecules by keratinocytes, which is essential for the immunity of epithelial barriers. In humans, IL-22 is often co-expressed at high levels with IL-26, acting on non-hematopoietic cells, such as keratinocytes and intestinal epithelial cells to induce expression of certain pro-inflammatory cytokines (71). IL-26 has been also found to be overexpressed in Crohn's disease and rheumatoid arthritis, indicating its potential pathogenic role in human inflammatory diseases (72).

1.3.4 γδ T cells

γδ T cells are a unique population of T cells, which develop in the thymus along with conventional αβ T cells. These lymphocytes, however, together with invariant NKT cells, have been named non-conventional or innate-like T cells, because of many characteristic features that they share with innate immune cells. Although they express rearranged TCR of limited diversity, the recognition of antigen does not engage the MHC-antigen complexes and still little is known about the specificity of γδ TCRs. It is proposed that they recognize non-peptide antigens derived either from pathogens or expressed by stressed cells, similarly to PAMPs and DAMPs recognized by innate cells through PRRs. Unlike Tconv, γδ T cells, constitute a very small fraction (3-5%) of adult mouse and human T cells in the thymus, spleen, lymph nodes and peripheral blood. In contrast, they are enriched in the tissues associated with the epithelial surfaces of the body, such as skin, intestinal and lung epithelium and mucosae of upper respiratory and digestive tract (73).

The tissue distribution reflects the function of γδ T cells. They play an important role in initiation of the early immune responses to infection, as they are rapidly activated by exposure to bacterial, fungal, viral and parasitic pathogens, and by inflammatory cytokines, produced by DC and macrophages. The effector functions of γδ T cells involve direct bacterial clearance by production of defensins and other bacteriostatic molecules and killing of infected or transformed cells through death-inducing receptors, such as CD95 (Fas) or TNF-related apoptosis-inducing ligand receptors (TRAIL) and by release of cytotoxic molecules, such as perforin and granzymes. γδ T cells also contribute to the immunomodulation of different immune cell types by production of broad range of cytokines, characteristic for the individual subset and dependent on the type of infection. For example, γδ T cells present in dermis, lungs, lymphoid tissues and spleen produce TNF-α, IFN-γ and IL-17. Furthermore, studies have demonstrated that in mice infected with *M. tuberculosis*, γδ T cells, but not Th17 cells, are the primary source of IL-17 (74).IL-1α or IL-1β has been shown to synergize with IL-23 to promote production of IL-17 from both human and mouse γδ T cells (75). Since IL-17 is associated with pathology of autoimmune inflammation, there is a growing interest in the role of IL-17-producing γδ T cells in the development of autoimmune diseases. TCRδ-deficient mice are less susceptible to EAE (76). Moreover, studies in mouse models have shown that γδ T cells accumulate in the CNS rapidly after induction of EAE, serving as an early source of IL-17 and IL-21, enhancing the leukocyte migration to the CNS and augmenting the responses of the pathogenic Th17 cells (75, 77).

1.3.5 Regulatory T cells

In 1970, a fundamental immunological discovery was made by Gershon and Kondo, who demonstrated that some T cells can act as suppressor cells and inhibit the activity of other cell types by producing soluble factors (78). In parallel in 1969 a series of experiments carried out by Nishizuka and Sakakura showed that neonatal thymectomy (NTx) of mice between 2 and 4 days after birth resulted in destruction of ovaries (79), which could be alleviated by transfer of syngeneic thymic (80) or spleen (81) cells. The abnormal development of ovaries was initially suspected to be caused by lack of thymus-derived hormones. Further investigation, however, revealed that the inflammation after NTx also occurred in other organs such as the stomach, testes, prostate and salivary gland and that this resulted from pathogenic autoimmune

responses (82). Importantly, inoculation of normal syngeneic T cells, particularly $CD4^+$ T cells, inhibited development of NTx-induced autoimmunity (83, 84). Together, these findings suggested that the thymus produces a population of T cells with suppressive activity and abrogation of their development results in autoimmunity. Over the next fifteen years this suppressor T cell subset was intensively investigated by many groups. In 1985 Sakaguchi *et al* showed that depletion of $CD5^{hi}$ T cells could break natural self-tolerance and result in autoimmunity (85). Powrie and Mason observed that a subset of $CD45RB^{lo}$ peripheral $CD4^+$ T cells negatively regulates $CD4^+$ effector cells (86). However, the idea of a distinct inhibitory T cell population was widely discredited and, by 1990, the interest in suppressor T cells dramatically declined (87). The most important reason for such a change of views was due to a failure in finding specific and reliable markers to distinguish suppressor T cells from other cell types.

The breakthrough finally came in 1995 with the discovery by Sakaguchi *et al* of the CD25 molecule (α-chain of high affinity IL-2 receptor) as a marker of $CD4^+$ T cells, which were able to prevent organ-specific autoimmune diseases when transferred to BALB/c athymic nude (*nu/nu*) mice (88). Further investigation by Sakaguchi and colleagues revealed that $CD25^+CD4^+$ T cells are produced naturally in the thymus and, in mice, appear in the periphery immediately the third day after birth. They constitute 5-10% of peripheral $CD4^+$ T cells and engage in the maintenance of tolerance to self-antigens and regulate immune responses to pathogens (89, 90). This T cell lineage which are both functionally and developmentally distinct from other T cells, were referred to as regulatory T cells (Treg).

After the discovery of CD25 as the first marker of Treg cells, several studies revealed that the ligand for CD25 (IL-2) is essential for their function. Studies showed that IL-2-deficient mice, which spontaneously developed autoimmune and inflammatory diseases, had significantly lower numbers of $CD4^+CD25^+$ Treg cells (91). Autoimmune disorders also occur in IL-2R-deficient (CD25- or CD122-deficient) mice (92, 93).

Another milestone in Treg research was the discovery of the transcription factor forkhead box P3 (Foxp3) as a "master control gene" in Treg development and function. The FOXP3 gene was identified in 2001 as the disease-causative gene in Scurfy mice, which spontaneously develop severe autoimmune and inflammatory diseases as a result of a single gene mutation on the X chromosome (94). Mutations of the highly

conserved human FOXP3 gene were subsequently found to be a cause of human syndrome IPEX (immune disregulation, polyendocrinopathy, enterophaty, X-linked), which is characterized by autoimmune disease in multiple endocrine organs, inflammatory bowel disease and severe allergy (95, 96). In 2003, several independent groups confirmed that Foxp3 is a crucial molecule for Treg biology, as $CD25^+CD4^+$ peripheral T cells and $CD25^+CD4^+CD8^-$ thymocytes specifically expressed Foxp3 mRNA and retroviral gene transfer of Foxp3 into naive $CD25^-CD4^+$ T cells converted them into Treg cells (97–99). Furthermore, continuous Foxp3 expression in mature thymus-derived Treg cells was found to be necessary for maintenance of the Treg cell phenotype and their suppressive function (100, 101).

To date, several distinct T cell subsets with regulatory activity have been described and characterized in mice and humans. They can be broadly divided based on their anatomical origin into natural thymus-derived and periphery-derived Foxp3-expressing Treg cells, and inducible Tr1 and Th3 regulatory T cells, secreting IL-10 or TGF-β, respectively.

Natural Treg cells

Natural $CD4^+CD25^+Foxp3^+$ Treg (nTreg) cells develop in the thymus and enter peripheral tissues as a mature T cell subpopulation. Unlike clonal deletion, where self-antigen reactive T cell clones are removed from the repertoire, the high-affinity recognition of self-antigen by TCR appears to be the key event to initiate nTreg cell development (102). It was demonstrated in studies of transgenic mice with TCR specific to foreign antigens that nTreg develop only when the foreign antigen is also expressed in the thymus (103). Alternatively, the predisposition of nTreg cells to carry high-affinity self-reactive TCRs may be due to their resistance to negative selection and greater survival (104). Foxp3 controls nTreg cell function and generation. In the thymus, $Foxp3^+$ T cells are detected from as early as the development of double-positive $CD4^+CD8^+$ thymocytes. Recent studies have shown, however, that the function of Foxp3 is not to initiate the differentiation of thymocytes into the Treg cell lineage, but to stabilize and sustain nTreg cell function as soon as nTreg cell fate is established (105). Once generated, nTreg cells enter lymph nodes where they are activated by self-antigens. CD28 ligation seems to play an important role in both thymic development and in peripheral homeostasis of nTreg cells. It is speculated that the constant low level CD28 co-stimulatory signals from APCs are required to maintain a stable population by

promoting homeostatic proliferation and survival (106). Interestingly, studies by Takahashi and colleagues showed that nTregs can become activated and exert suppression at up to 100-fold lower concentration of specific antigen than naïve conventional T cells. This suggests that nTreg cells are capable of becoming effector Treg cells by interacting with immature DC, which have low expression of co-stimulatory and MHCII molecules and therefore unable to activate pathogenic self-reactive T cells (107).

Unlike conventional T cells, which upregulate expression of CD25 temporarily after activation, nTreg cells constitutively express high levels of CD25, most probably due to constant ligation of their TCR with MHC class II-self-peptide complex of APCs under non-inflammatory conditions. The ligand for CD25 - IL-2 is a critical regulator of conventional T cell activation, proliferation and apoptosis (108). Therefore, it was surprising that IL-2 (similarly to IL-2Rα and IL-2Rβ-deficient) deficient mice do not suffer from defects in T cell proliferation and function, but develop T-cell mediated fatal inflammatory diseases with autoimmune components, generally called IL-2 deficiency syndrome (109). These studies suggest an important role for IL-2 signalling in development, maintenance and suppressive function of nTreg cells. However, the precise model of function of IL-2 in Treg biology *in vivo* has not been yet defined. Treg cells do not produce IL-2 and rely on exogenous IL-2 secreted by activated T cells. Thus, it has been suggested that reduced availability of IL-2 for effector T cells is one of the mechanisms of immunoregulation, which limits the necessity for active suppression of responses (cytokine-sink model) (110).

Induced Foxp3 Tregs (iTregs)

In addition to the nTreg cells that are continually produced in the thymus, naive and memory conventional T cells in the periphery can be induced to express Foxp3 and acquire suppressive functions under the influence of immunoregulatory signals in a process known as peripheral conversion. It was demonstrated in 2003 by Chen and colleagues that the conversion of naive $CD4^+CD25^-$ into $CD4^+CD25^+Foxp3^+$ is a process dependent on TGF-β and TCR stimulation (111). $CD4^+$ T cells converted by TGF-β exhibited cell-contact-dependent suppressor activity *in vitro* when co-cultured with normal $CD4^+$ T cells. Furthermore, TGFβ-converted Treg cells can inhibit antigen-driven $CD4^+$ T cell expansion *in vivo* (111). It was later confirmed that TGF-β is indispensable for induction of Foxp3 expression in naive $CD4^+$ T cells both in mice and

humans (112), and this conversion is facilitated by IL-2 (IL-2 controls stability of Foxp3) (113) and retinoic acid (a vitamin A metabolite) (114). Conversion of naive $CD4^+$ T cells does not seem to occur under the homeostatic conditions in the peripheral lymph nodes and spleen. The mucosa-associated lymphoid tissues appear to be the main place for *de novo* conversion of naïve $CD4^+$ T cells into $Foxp3^+$ iTregs specific for commensal bacteria and food antigens. There is a growing evidence that such a mechanism for induction of Treg cells exists at least in the intestine, where T cells are continuously stimulated by microbial products with tissue-generated TGF-β and DC-derived retinoic acid (115).

Tr1 cells

In 1988, a new T cell subset with immunosuppressive function was discovered in the immune system of the patient with severe combined immunodeficiency (SCID), who had developed long-lasting tolerance to a hematopoietic stem cell transplant, in the absence of immunosuppressive therapy (116). It was subsequently established that the $CD4^+$ T cell clones responsible for the tolerance in SCID patients produce large amounts of immunosuppressive cytokine IL-10. It was later confirmed by Groux et al that those IL-10-producing T cells constitute to a distinct population of $CD4^+$ T cells, termed Tr1 cells, with a unique cytokine profile and are able to mediate antigen-specific tolerance (117). Naïve $CD4^+$ T cells in the periphery can differentiate into Tr1 cells after the encounter with cognate antigen presented by APC in the presence of IL-10. In mice, these Treg cells can be defined on the basis of secretion of high amounts of IL-10, low amounts of IL-5, TGF-β and IFN-γ, but no IL-4 (117). Although the engagement of TCR is necessary to exert the suppressive properties, once activated, they can inhibit effector T cell responses in a bystander manner, presumably by release of anti-inflammatory cytokines that can on both T cells and APCs. Since, Tr1 respond to IL-10 in an autocrine manner, their proliferative capacities *ex vivo* are limited. It has been demonstrated, however, that they can be expanded in the presence of exogenous IL-2 or IL-15, due to constitutive expression of IL-2R (118). Recent studies have also shown that IL-27, a cytokine previously associated exclusively with Th1 differentiation, is a potent differentiation factor for Tr1 (52). Initially, IL-27 enhances the production of IL-21, a member of IL-2 family, and IL-21R. IL-21 acting in autocrine manner, upregulates IL-10 expression. This mechanism of differentiation resembles that for

Th17 cells, where IL-17 is the main cytokine produced, but autocrine IL-21 serves as a growth factor.

There is growing evidence, that Tr1 cells are important for the maintenance of immunological tolerance and restraining the inflammation during infections. Administration of IL-10 with rapamycin *in vivo* was shown to induce Tr1 cells that mediated tolerance in type 1 diabetic mice after transplantation of pancreatic islet (119). Adoptive transfer of Tr1 clones suppressed Th1-mediated colitis induced by transfer of naïve $CD45RB^{hi}$ cells into SCID mice (117). Since, IL-10-deficient mice develop spontaneous colitis in young age, these results suggest that Tr1 cells play a major role in maintaining the immunological homeostasis to the intestinal microbiota. In mouse model of autoimmunity, the transfer of ovalbumin- (OVA)-specific Tr1 clones prevented the development of EAE only after the administration of OVA peptide, confirming that Tr1 must be activate in an antigen-dependent manner but can suppress autoimmune responses to the unrelated antigens (120). Finally, the generation of Tr1 cells is impaired in the MS patients compared to the healthy volunteer, suggesting that Tr1 cells may play a protective role in autoimmune disorders in humans. In addition, the generation of Tr1 cells have been identified in many infection, for example with *Mycobacterium tuberculosis*, *Bordetella pertussis* or malaria parasite *Plasmodium falciparum* (121–123).

Th3 cells

Studies investigating oral tolerance identified another population of Treg cells, differentiating in the peripheral tissues from naïve T cells. These Treg cells, later termed Th3, express high levels of TGF-β, low levels of IL-4 and IL-10 and no IL-2 upon TCR stimulation and are Foxp3-negative. Th3 cells can be generated in the gut by oral administration of neural self-antigen and are capable of suppressing Th1-mediated autoimmune disease (124). It was also demonstrated that in the absence of inflammation, TGF-β secreted by Th3 cells upregulates the expression of Foxp3 gene in activated T cells during T cell expansion, inducing conversion of peripheral $CD4^+$ T cells into Foxp3 Treg cells in the absence of thymic nTreg cells. This suggests that Th3 may work as mediators of peripheral immune tolerance by both direct immunosuppressive effect of secreted TGF-β on broad range of immune cells and by induction of $Foxp3^+$ Treg cells (125).

Mechanisms of suppression by Treg cells

Both nTreg cells and induced Treg cells can suppress and regulate immune responses by direct cell-cell contact and by production of immunosuppressive cytokines, modulating DC and T cells responses. The cell to cell contact suppression is provided by expression of inhibitory molecules on effector Tregs cells, such as CTLA4, which is common for Foxp3 Treg and Tr1 cells. CTLA4 is exposed on the surface of Treg cells upon activation and is a ligand for co-stimulatory molecules on APCs. The mechanism of suppression is based on competition with co-stimulatory molecules CD28 for binding of CD80 and CD86 on APCs, which results on transduction of negative signals that induce Teff cell cycle arrest and inhibition of IL-2 production. Additionally, the binding of B7 molecules leads to their capture and by trans-endocytosis which consequently leads to downregulation of CD80/CD86 expression of the APCs surface (126). Other surface inhibitory molecules associated with Treg suppressive functions are programmed cells death 1 (PD-1), lymphocyte-activation gene 3 (LAG-3) and surface-bound TGF-β (127).

Another method of immune suppression described mostly for $Foxp3^{+}$Treg cells is disruption of cell metabolism by generation of adenosine via the enzymatic hydrolysis of extracellular ATP by the enzymes present of the Treg cell surface CD39 and CD73. Adenosine binds to the A2A adenosine receptor on DC and T cells and inhibits expression of pro-inflammatory cytokines by elevation of cyclic AMP (128). It has also been described that both $Foxp3^{+}$ Treg cells can directly kill target cell by release of granzymes and perforins (129).

The suppression of immune responses by $Foxp3^{+}$ Treg cells through production of immunosuppressive cytokines in not fully understood. The inhibitory effects of TGF-β, IL-10 and IL-35 produced by different types of T cells have been described *in vitro* and *in vivo*, but contribution of these anti-inflammatory cytokines to the suppressive functions of $Foxp3^{+}$ Treg cells is still to be determined (130, 131). In contrast, IL-10 production seems to be the major mechanism of suppressive functions of Tr1 cells both *in vitro* and *in vivo*. IL-10 is a pleiotropic cytokine exerting its effect on innate and adaptive immune cells. Acting on DC, IL-10 inhibits production of pro-inflammatory cytokines, reduces expression of MHC class II molecules, co-stimulatory molecules and adhesion molecules, impairing the migration of cells to the lymphoid tissues and antigen presentation. The similar effect of IL-10 signalling is observed in macrophages

and monocytes. IL-10 directly supresses T cell responses by inhibiting IL-2, IFN-γ, IL-4 and IL-5 production, therefore, supressing both Th1 and Th2 responses. Furthermore, treatment of PBMCs from RA patients with IL-10 decreased numbers of IL-17-producing RORγt $CD4^+$ T cells, suggesting that IL-10 can also inhibit IL-17 expression (132). Despite the inhibitory effects on effector Th cells, IL-10 increases the proliferative responses of activated $CD8^+$ T cells, which is associated with enhanced anti-tumour effects and reduced tumour growth *in vivo* (133). IL-10 also enhances survival, proliferation and differentiation of B cells, therefore IL-10 was previously associated with Th2-type of responses.

The involvement of TGF-β in Treg-mediated suppression is less clear. TGF-β has potent inhibitory effects on T cells responses. TGF-$\beta^{-/-}$ mice develop lethal T cell-mediated multi-organ autoimmunity early after birth (134). In contrast, mice with T cells-specific TGF-β-deficiency are more resistant to actively induced EAE, due to the impaired Th17 induction. It is likely that the role of TGF-β in T cell responses *in vivo* depends on the site of the immune response, type of the target cell and presence of other environmental cytokines, since TGF-β also induces differentiation of Th17 cells. Studies by different research groups could not determine the involvement of Treg-derived TGF-β in inhibition of T cell-mediated inflammatory or autoimmune diseases. TGF-β production by Treg cells was necessary to prevent colitis in several models, whereas in others, TGF-β-produced by other cell types, such as intestinal DC, appeared to the involved in the protection (57). The function of Treg-derived TGF-β might be associated with both direct suppression of effector T cells and with induction other Tregs. TGF-β is synthesized as a precursor containing latency-associated peptide (LAP) at the N-terminus and mature TGF-β at the C-terminus. The processing and cleavage of the precursor protein leads to the formation of LAP dimers and TGF-β dimers that non-covalently associate to each other and form latent TGF-β complex. To exert its properties TGF-β must be released from the latent complex, following its activation on the cell surface, therefore LAP is a suitable marker for cells expressing TGF-β, regardless of it activity (135).

1.3.6 TLRs and regulation of T cell responses

It is commonly accepted that TLR-mediated recognition of pathogens initiates adaptive immune responses though activation of APC, especially DC. However, recent findings

demonstrate that TLR ligands can directly modulate T cell function TLRs are expressed on almost all immune cells, including T cells, and the expression of TLRs is related to the functional states of different subtypes of T cells (136, 137). Murine naïve T cells can express TLR1-TLR9 however, there is a considerable variation in the expression levels between different TLRs (138). It has been reported that TLR2 expressed on human activated T cells functions as a co-stimulatory receptor for antigen-specific T cell responses and participates in the maintenance of T cell memory (139). Ligands for TLR2, 5 and 7/8 have been shown to enhance proliferation as well as IL-2 and IFN-γ production by human $CD4^+$ T cells, particularly by memory cells (140). Moreover, in mouse effector Th1 cells the stimulation of TLR2, but not of other TLRs, directly induced IFN-γ production and cell proliferation and survival without TCR engagement. In contrast, none of TLR ligands affected the function of effector Th2 cells (141). Another study demonstrated that TLR2 signalling enhances Th17 differentiation by induction of expression of Th17-regulating transcription factors RORγ and RORα (142). In addition, TLR2 deficiency was found to reduce generation of Th17 cell *in vivo* and confer protection against experimental autoimmune encephalomyelitis (EAE) (142).

TLR2 also controls the function of Treg cells. Netea et al demonstrated that the numbers of circulating $CD25^+CD4^+$ Treg cells are significantly reduced in $TLR2^{-/-}$, but not $TLR4^{-/-}$, mice compared to WT littermate controls (143). Moreover, TLR2 triggering of Treg cells results in proliferation of these cells *in vitro* and *in vivo* and temporarily abrogates their suppressive capabilities (144). In contrast, treatment of $CD25^+CD4^+$ Treg cells with TLR4 ligand LPS induces expression of several activation markers, enhances their survival and increases their suppressive ability by 10 fold (145). Similarly, exposure of $CD25^+CD4^+$ Treg cells to TLR5 ligand flagellin increases their immunosuppressive capacity by enhancing Foxp3 expression, whereas TLR7 signalling enhances Treg cells suppressive function by sensitizing $CD25^+CD4^+$ Treg cells to IL-2-induced activation (146). These and other studies demonstrate that TLRs play a crucial role in initiation of adaptive immune responses as well as in direct regulation of T cell function.

1.3.7 Relationships between T cell subsets

The differentiation of T helper subsets involves positive feedback loops involving IFN-γ, IL-4 and IL-21 signalling for development of Th1, Th2 and Th17 cells, respectively. This process also involves active cross-inhibition of other lineage differentiation. IFN-γ suppresses development of Th2 cells and conversely IL-4 inhibits Th1 differentiation. In addition, both IFN-γ and IL-4 inhibit development of the Th17 subset. Treg-derived IL-10 suppresses all effector T cells responses.

Studies of Flavell et al have demonstrated that mice with altered TGF-β expression in T cell develop a lethal inflammatory disorder mediated by uncontrolled Th1 and Th2 responses, which indicated that Treg-derived TGF-β is required to control Th1 and Th2 subsets. Interestingly, impaired TGF-β production by T cells led also to the inhibition of generation of Th17 and decreased severity of EAE (147). This study suggests that, apart from innate cells, $Foxp3^+$ Treg cells or other Treg cells themselves might be an important source of TGF-β for the differentiation of Th17 cells. This result is also surprising, since TGF-β is necessary for the generation of $Foxp3^+$ iTreg cells. *In vitro*, the differentiation of mouse $Foxp3^+$ iTreg cells and Th17 cells is mutually exclusive. Naïve $CD4^+$ T cells upregulate $Foxp3^+$ expression when stimulated with TGF-β, however, when IL-6 or IL-21 is added, the differentiation rapidly shifts to the Th17 subset and production of IL-17. It is still unclear, whether the same relationship occurs *in vivo*. Kuchroo and colleagues have proposed that in the absence of inflammation, transient expression of TGF-β by different cells predominantly induces the generation of Treg cells in order to maintain immunological tolerance (57). During inflammation, IL-6 is one of the first cytokines produced by innate cells that subsequently prevents the development of function of T cells, and skews the developmental program into Th17. Therefore, IL-6 plays a crucial role in the balance between Treg and Th17 cells (57). In addition, IL-23 and IL-1 are necessary for further development and terminal differentiation of Th17 cells, which might serve as an additional check point in the induction of protective immunity. Schematic representation of differentiation and regulation of different T cell subsets is shown in Fig. 1.1

1.3.8 Immunological memory

$CD4^+$ T cells complete the differentiation into a particular subset in the lymphoid tissues. Once differentiated, effector T cells leave lymphoid tissues and enter the blood stream through efferent lymphatics and thoracic duct. Due to the enhanced expression of chemokine receptors and adhesion molecules that bind ligands on endothelial cells, they enter the peripheral tissues, particularly the sites of inflammation to exert their effector functions after encountering cognate antigens. During the resolution of infection most of the antigen-specific effector T cells die, but small percentage of T cells remains in the tissues and are maintained as long-lived memory T cells. The survival and homeostatic proliferation of memory T cells (and also naïve T cells) is largely dependent on signals from IL-7 which is produced by epithelial cells, keratinocytes, bone marrow cells and by follicular DC (148). The most recent studies have demonstrated that memory T cells share molecular and functional features with stem cells. For example, memory T cells have partially conserved transcriptional profile similar to hematopoietic stem cells, they undergo asymmetric division and activate telomerase to maintain telomere length and replicative potential. Moreover, the whole population of memory T cells displays a hierarchical organization, with T memory stem cell (Tmsc), being the most undifferentiated subset (149). Furthermore, the memory T cells are divided into distinct subsets, based on expression of lymphoid homing receptors, effector function and proliferative capacity. Central memory T cells (Tcm), similarly to naïve T cells, express chemokine receptor CCR7 and L-selectin CD62L and are predominantly situated in secondary lymphoid organs, spleen and blood, where they are maintained though production of IL-2. In contrast, effector memory T cells (Tem) do not express those molecules and migrate in the non-lymphoid organs, such as liver, intestinal tract, kidney, adipose tissue. Recently, a new population of memory T cells, permanently residing in the peripheral tissues associated with epithelial and mucosal surfaces has been characterised as tissue-resident memory T cells (Trm) (150). Altogether, memory T cells provide the rapid and effective protection against reoccurring pathogen, by the ability of becoming activated through recognition of antigens without co-stimulation.

Preventative vaccination against infectious disease is based on the development of long-lasting immunological memory. Prophylactic vaccines successfully eliminated some of the most dangerous, life-threatening diseases, such as smallpox or polio. By contrast,

the strategy to induce immunological memory against tumour antigens might become an effective immunotherapy to fight cancer. Moreover, inverse vaccination, developing long-lasting tolerogenic memory for self-antigens might be an alternative for traditional therapies against inflammatory and autoimmune diseases.

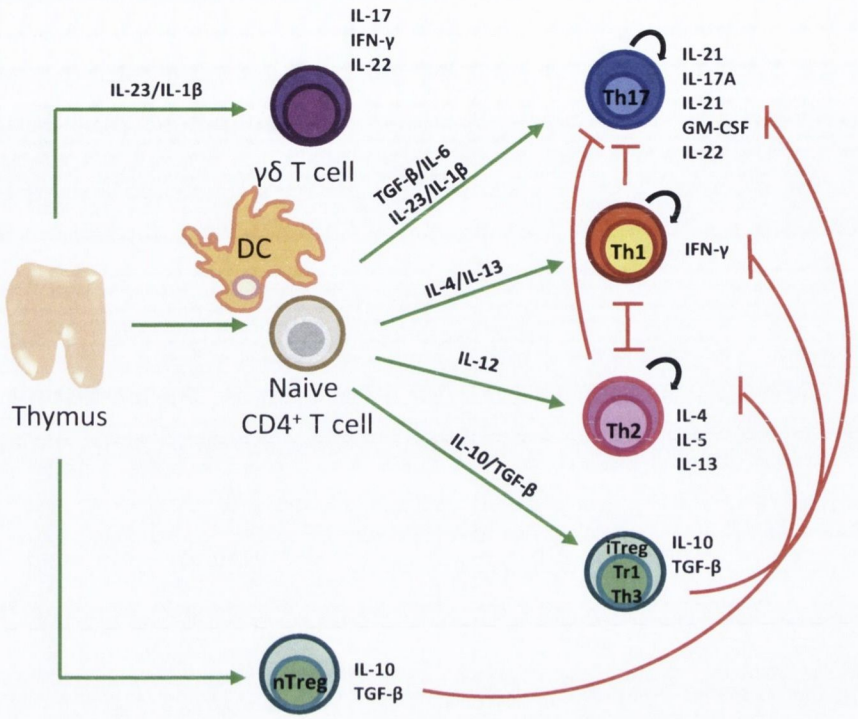


Fig. 1.1 Differentiation and regulation of T cell subsets.

Naive αβ $CD4^+$ T cells, γδ T cells and $CD4^+$ natural Treg cells develop in the thymus. In the periphery, naive $CD4^+$ T cells differentiate into Th1, Th2, Th17 or Treg cells ($Foxp3^+$ iTreg, Tr1 of Th3 cells) dependently on the environmental cytokine milieu. The cytokines produced by a particular differentiating Th cell subset serve as autocrine growth and polarisation factors for that subset and inhibit differentiation of other Th subsets. γδ T cell can be activated by cytokines without TCR stimulation and provide a source of early inflammatory cytokines enhancing the inflammatory response. Both natural and peripherally induced Treg cells suppress the activation and function of Th cells subsets by production of suppressing soluble factors and via mechanisms involving direct cell to cell contact.

1.4 Autoimmunity

The concept of autoimmunity was first proposed at the beginning of the twentieth century by a German haematologist and immunologist Paul Ehrlich. He proposed a theory called "horror autotoxicus" stating that the organism is unwilling to endanger itself by the formation of toxic autoantibodies, in other words, that the normally functioning immune system is unable to produce the immune response against own antigens. However, the clinical observations showed the contrary - many disorders, such as paroxysmal cold hemoglobinuria, ocular inflammation and certain encephalitides was associated with formation of autoantibodies. Moreover, it was experimentally shown that the injections of autologous thyroglobulin into the thyroid glands of rabbit leads to the development of lesions in the thyroid tissues, an autoimmune response similar to observed in Hashimoto's thyroiditis. Finally, a theory of clonal selection of lymphocytes, formulated in 1957 by Frank Burnett, was a basis for explanation of the phenomenon of acquired immunity, but also the enabled formulation of theories about the immune tolerance and generation of autoimmune responses (151).

It is now clear that during lymphocyte development, almost indefinite amount of specificities can be generated by somatic recombination of TCR genes. The fundamental mechanism of T cell tolerance to self-antigens is the process of the clonal deletion of self-reactive T cells in the early stages of their development in the thymus. This process is referred to as central or recessive tolerance. However, not all the T cells specific for auto-antigens are effectively removed from the system. Auto-reactive T cells can be detected in healthy individuals and the immunization with self-protein in the presence of adjuvant results in the development of autoimmune response (152). T cells specific for environmental antigens or commensal bacteria antigens also pass the process of clonal deletion. Therefore, additional regulatory mechanisms of peripheral or dominant tolerance exist that restrain the numbers and function of mature circulating T cells reactive to self- or non-harmful antigens. These regulatory mechanisms depend on T cell anergy and functions of regulatory T cells.

The breakdown in immunological tolerance to environmental or self-antigens leads to the development of immune-mediated diseases. Autoimmune diseases are described as pathologic responses of the adaptive immune system directed against its own tissues, resulting in the progressive damage of the target organ. The exact cause of loss of

tolerance is still unknown, but the combination of both environmental and genetic factors seems to be crucial. Epidemiological studies have shown the link between infections and development or exacerbation of autoimmune diseases (89). It is speculated that the initiation of the autoimmune response by the infectious agent might be dependent on several mechanisms. Firstly, some pathogen-derived epitopes have similar structure to self-peptides, which might be sufficient for activation of auto-reactive T cells. Secondly, the killing of pathogens is associated with collateral tissue damage and released antigens can be presented by TLR-activated APCs, leading to the initiation of response to self-antigens (epitope spreading) (153). Finally, the inflammatory environment that results from infection triggers the non-antigen specific activation of immune cells and can lead to the expansion of the previously activated T cells in the process of bystander activation. The other environmental factor suspected to be responsible for breaking of immunological tolerance are lack of sunlight and chemicals present in certain drugs, which can cause tissue damage and initiate the inflammatory response (154).

Many autoimmune diseases have been associated with mutations in genes associated with the maintenance of central and peripheral tolerance, such as *AIRE* (autoimmune regulator, encoding a protein responsible for thymic expression of some antigens) *CTLA4* and *FOXP3* (155). In fact, decreased frequency or function of $CD25^{+}FOXP3^{+}CD4^{+}$ T cells correlate with severity of disease in systemic lupus erythtemathosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS) patients (156). Some autoinflammatory disorders are caused by the abnormal activation of the innate immune system, as a result of genetic mutations involved in expression pathways for inflammatory cytokines, for example mutation in *NLRP3* gene which leads to sustained activation of inflammasome and production of IL-1β (157).

1.4.1 Role of Treg cells in autoimmune diseases

The hallmark features of autoimmune diseases are: a breakdown of mechanisms responsible for discrimination between self and non-self and failure to control the excessive immune responses to self-antigens. In healthy individuals different populations of Treg cells play a dominant role in negative regulation of immune responses directed against self-antigens and in suppression of autoimmune pathology. $Foxp3^{+}$ Treg cells play a crucial role in the prevention of autoimmunity, since mutation

in murine Foxp3 gene causes a range of severe autoimmune diseases in scurfy mice. Similarly, mutation in human FOXP3 gene is a cause of severe autoimmune inflammation in multiple tissues, called IPEX syndrome (95).

It has been described that patients with autoimmune diseases, such as psoriatic arthritis, systemic lupus erythematosus or autoimmune liver disease have reduced levels of circulating $CD4^+CD25^+$ Treg cells compared to the healthy people and the lower levels of these cells correlate with a higher disease activity (158–160). In contrast, no significant changes in circulating $CD4^+CD25^+Foxp3^+$ Treg cells frequencies were observed between healthy controls and patients with multiple sclerosis, type 1 diabetes or spondyloarthritis, which suggests that the decreased numbers of circulating Treg cells are characteristic for only particular autoimmune diseases (158, 161, 162). Moreover, studies demonstrated an increased recruitment of $CD4^+CD25^+$ Treg cells at sites of inflammation compared with peripheral blood in such autoimmune diseases as rheumatoid arthritis or psoriatic arthritis (158). However, the local enrichment of $CD4^+CD25^+$ Treg cells at the sites of inflammation is insufficient to restrict inflammation, indicating the impaired Treg functions and imbalance between pro-inflammatory and regulatory T cell responses in the individuals with autoimmune diseases. The dominant role of Treg cells in prevention of autoimmunity was demonstrated in animal models. For example, depletion of $CD4^+CD25^+$ Treg cells in mice with collagen-induced arthritis (CIA) caused rapid disease progression, but the joint damage could be inhibited by transfer of isolated and *ex vivo*-proliferated $CD4^+CD25^+$ Treg cells (163). Furthermore, Hori et al showed that retroviral transduction of Foxp3 to $CD25^-$ $CD4^+$ T cells converts them into $CD25^+CD4^+$ Treg-like cells both phenotypically and functionally. These Foxp3-transduced $CD25^-CD4^+$ T cells were able to suppress proliferation of other T cells *in vitro* as well as suppress development of inflammatory bowel disease (97, 164).

Tr1 cells are induced in the periphery from uncommitted $CD4^+$ T cells and have been identified to play an important role during autoimmune inflammation. For instance, they are found in large numbers in the intestine, where they are proposed to have a protective role during colitis (117, 165). It was demonstrated in murine model of colitis, that the pathogenicity of $CD45RB^{high}CD4^+$ T cells transferred into SCID mice could be inhibited by co-transfer of Tr1 clones induced from ovalbumin (OVA)-specific $CD4^+$ T cells and the inhibition was achieved only in recipient mice that received OVA antigen.

This experiment demonstrated that the immune suppression of colitis relayed on antigen-specific activation of Tr1 cells *in vivo* (117). Similarly, an adoptive transfer of *in vitro* generated OVA-specific Tr1 cells prevented development of EAE in mice, when OVA peptide was injected intracranially (120). Tr1 cells were also induced in rats by *in vivo* administration of soluble peptide myelin basic protein (MBP) and were able to inhibit ongoing EAE induced by immunization with MBP (166). Moreover, desmoglein 3-responsive IL-10-secreting Tr1 cells were isolated from healthy volunteers, but were rarely detected in patients suffering from pemphigus vulgaris, an auto-antibody mediated autoimmune disease of the skin associated with autoimmune responses against desmosomal adhesion molecule desmoglein 3 (167). Finally, the abnormal differentiation and impaired suppressive activity of Tr1 responses was shown in patients with MS compared to healthy donors (168–170).

Th3 cells have been identified as a unique population of cells induced peripherally from naïve $CD4^+$ T cells upon oral administration of low doses of antigen (124). Is has been demonstrated that delivery of neural antigen MBP by oral route can generate Th3 cells producing high concentrations TGF-β. The MBP-specific clones were able to suppress the development of MBP-induced EAE as well as PLP-induced EAE and thie suppression was blocked by *in vivo* injection of anti-TGF-β antibodies. This suggests that the suppressive effects of Th3 cells are antigen-nonspecific and mediated by bystander suppression via production of TGF-β (171). In humans, MBP- and PLP-specific Th3 cells have been observed in the peripheral blood of MS patients treated with oral bovine myelin preparation but not in patients who were untreated (172). Furthermore, it has been postulated that TGF-β-producing Th3 cells play a major role in inducing and maintaining peripheral tolerance by driving the differentiation of antigen-specific $Foxp3^+$ Treg cells in the periphery (125).

Considerable progress in understanding the mechanisms of immune tolerance led to an increasing interest in developing new strategies for therapies of autoimmune inflammation by manipulating both the function and number of different populations of Treg cells. Effective methods for differentiation or expansion of Treg cells *ex vivo* could lead to the development of cell based therapy for autoimmune diseases. In order to use adoptive transfer of Treg cell as a therapy, several methods have been established for purification and *ex vivo* expansion of human nTreg cells (173). For example, human $CD4^+CD25^{high}$ nTreg cells can be isolated from umbilical cord blood and expanded

using anti-CD3/anti-CD28-coated microbeads, recombinant IL-2 and rapamycin, resulting in 200-300 fold expansion after 3 weeks of culture (174). Furthermore, many preclinical animal studies have demonstrated that the adoptive transfer of $Foxp3^+$ nTreg cells can prevent various autoimmune diseases (175). A major obstacle in using nTreg cells is their low numbers in the peripheral blood and poorly defined antigen specificity, which may result in general suppression of the immune responses when polyclonally expanded Treg cells are adoptively transferred *in vivo*.

Another approach to using adoptive immunotherapy of $Foxp3^+$ Treg cells is based on *in vitro* conversion of naïve T_{conv} cells into $Foxp3^+$ iTreg cells by stimulation with TGF-β. While the ability of TGF-β to convert naïve T_{conv} cells into functional iTreg cells is well documented in mice, the results from studies using human T cells are disputable (111, 176). Unlike murine cells, Foxp3 was shown to be also expressed in human activated T_{eff} cells, thus it is a poor marker of suppressive activity of human iTreg cells (177). Consequently, several studies demonstrated that not all human Foxp3-positive T cells induced *in vitro* by stimulation with TGF-β are immunosuppressive (176, 178).

Gregori et al identified and characterized a subset of human tolerogenic DC (DC-10) that occur in peripheral blood, secrete high levels of IL-10 and are potent inducers of antigen-specific Tr1 cells *in vitro* (179). Tr1 cells require TCR stimulation with their specific antigen to become activated and produce high amounts of IL-10, but once activated, they can mediate bystander tolerogenic effects. Therefore, induction and expansion of antigen-specific Tr1 cells *ex vivo* could give an opportunity for cell-based therapy of autoimmune diseases for which an antigen causing disease is known as well as unknown (175). In fact, a first human study using antigens-specific Tr1 cell clones has given a positive outcome in clinical trial phase 1/2 in patients with severe Crohn's disease. The study demonstrated that administration of ovalbumin-specific Tr1 cells was safe and lead to a significant clinical improvement in 8 of the 20 patients (180).

1.4.2 Hygiene hypothesis

In the twentieth century, changes in lifestyle in industrialized countries, associated with the use of antibiotics and vaccination, improved hygiene and improved living and housing conditions decreased the incidence of infectious diseases. Conversely, epidemiologic studies showed an increase in the incidence of chronic inflammatory and autoimmune disorders and allergy. The inverse correlation between exposure to

pathogens and the incidence of allergy in children was described for the first time in 1989 by Strachan (181). The theory, known as the "hygiene hypothesis" was founded on the observation that a reduction in microbial exposure, accompanied by a subsequent decrease in Th1 cell stimulation, led to a skewing of the immune response towards excessive Th2 responses, the dominant type of T-cell in the allergic responses (182). However, this idea was disputed due to observations that the incidence of Th1-mediated autoimmune diseases was also increasing and that the incidence of parasitic infections, which induce strong Th2 responses, were inversely associated with allergy (183).

The hygiene hypothesis was re-evaluated following the discovery of Treg cells. Based on epidemiological and animal model data, Rook et al suggested that the chronic inflammatory conditions result from a decrease in certain microbial stimuli, e.g. from commensal and environmental species, which are recognized by the immune system as harmless and prime tolerogenic responses mediated by release of anti-inflammatory cytokines IL-10 and TGF-β. Hence, the failure to establish a continuous background of bystander suppression by the immune system is the cause of inappropriate inflammation (184, 185). Various clinical data support this theory. For instance, studies of the bacterial colonisation of the intestines of newborns have shown that more rapid and diverse colonisation occurs in the developing countries. In contrast, the incidence of allergy was found to be significantly lower in developing countries compared to that of developed countries (186). Furthermore, there is the inverse association between the prevalence of autoimmune diseases or allergy and helminth infections in humans; Gabonese children naturally infected with *Schistosoma* had a reduced risk of developing allergic skin reaction to house-dust mite compared with un-infected individuals (183). Furthermore, people infected with helminths have lower risk of developing MS and MS patients infected with intestinal parasites showed a significantly lower number of relapses and brain lesions compared with the un-infected patients (187). These observations led to the exploratory studies and subsequent phase 1 clinical trials using oral administration of *Trichuris suis* (188) in MS patients, called HINT (Helminth-induced immunomodulation therapy). There has also been an increasing interest in the use of immunomodulatory products derived from helminth parasites as immunotherapeutics against autoimmune and autoinflammatory diseases.

1.4.3 Multiple sclerosis

MS is a chronic immune-mediated and neurodegenerative disease of the nervous system affecting, according to Atlas of MS, approximately 2.3 million people worldwide, mostly young adults. The disease occurs usually between 20 and 40 years of age and affects woman approximately twice as often as men. The primary damage in MS is caused by inflammation of the central nervous system, but the specific events initiating the pathologic immune response are unknown. As a result of inflammation, brain, spinal cord and optic nerves are affected by demyelinating lesions in multiple locations. The damage to oligodendrocytes, which cover the nerve fibres, results in axonal degeneration with deficits in sensation and motor and autonomic functions and subsequently leads to a complete loss of axonal conduction (189). The majority of patients are initially affected by recurrent and reversible neurological deficits, which is called relapsing-remitting MS (RRMS). There are cases of primary progressive MS (PPMS), characterized by gradual progression of the disease, without periods of remission. Half of patients diagnosed with the relapsing-remitting form of MS later develop the secondary progressive (SPMS) form of the disease (190).

Although the aetiology of MS in not well defined, there is a substantial evidence that genetic factors influence the susceptibility to MS. This is strongly suggested by 5% concordance among same-sex dizygotic twins and 20%-30% concordance among monozygotic twins compared to the prevalence of approximately 0.1% in the whole populations of northern Europe areas (191). Moreover, the risk of MS is highly associated with specific genes for human leukocyte antigen (HLA) class II molecules. Particularly, Caucasian haplotype HLA-DR15 is suspected to be one of the characteristic genetic factor conferring the development of MS (192). Along with the genetic predispositions, the exposure to certain infectious agents have been postulated to trigger the development of MS. Specifically, there is a 10-fold increased risk of MS is patients who were infected with Epstein Barr virus as children when compared to non-infected. There is also an established association between the common infections of upper respiratory or urinary tract and the severity of the relapses in MS (191).

Studies, involving analysis of cellular composition of brain lesions of MS patients and animal models, demonstrated that the pathogenesis of MS is associated with infiltration of inflammatory self-reactive lymphocytes into the CNS. The pathogenic T cells,

infiltrating CNS, exhibit specificity for auto-antigens, such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte protein (MOG), all of which are abundant in the CNS (193). The animal model studies confirm that autoreactive Th1 and Th17 cells are reactivated within the CNS and triggered to produce their signature pro-inflammatory cytokines, activating the resident immune cells and further attracting inflammatory leukocytes into CNS. Recent studies in mouse models of MS have revealed that IL-17-producing γδ T cells play a pivotal in the initial stages of the autoimmune inflammation in MS (75, 194). These events lead to cell-mediated immunity against oligodendrocytes and subsequent destruction of myelin sheath (189). In patients with RRMS the acute inflammation and demyelination occurs episodically and is followed by resolution of inflammation, partial remyelination and restoration of the neuronal functions. In contrast, in progressive forms of MS the inflammatory processes dominate in the initial states of the disease, whereas the irreversible and permanent neurological disability is associated with a broad neurodegeneration independent on inflammation (151).

1.4.4 Experimental autoimmune encephalomyelitis (EAE)

The key pathological features of MS have been established based on the analysis of patients' peripheral blood and brains lesions. However, many pathways and events have been identified and confirmed using an animal model of MS. Experimental autoimmune encephalomyelitis (EAE), also called experimental allergic encephalomyelitis, is an animal model of inflammation in the central nervous system that is primed by CNS-derived antigens. EAE is one of the most widely studied models of immune disease, because of its resemblance to human CNS demyelinating diseases. The origin of EAE dates back to the beginning of 20th century when Thomas M. Rivers from The Johns Hopkins University in Maryland (USA) injected emulsified rabbit brain to rhesus monkeys and rabbits and observed that animals developed limb weakness and paralysis. Further histological analysis of the brain and spinal cord sections revealed extensive cellular infiltration around blood vessels and areas of myelin loss (195, 196).

Recently, a number of different EAE models have been developed, using various species of mammals (197, 198). EAE can be induced by immunizing mice with proteins found specifically within the CNS: spinal cord homogenate or purified neural proteins, such as MBP, PLP, MOG, myelin-associated glycoprotein (MAG), S-100 or by

adoptive transfer of pathogenic T cells specific to neural antigens (199, 200). Classically, EAE is induced in susceptible strains by immunization with neural antigen emulsified with complete Freud adjuvant or other source of TLR agonists, followed by injection of pertussis toxin (PT). To achieve a particular pathological outcome ranging in severity and frequency of symptoms, different susceptible mouse strains are used. C57BL/6 mice, immunized with MOG_{35-55} peptide, develop acute chronic disease, characterized by severe inflammation of CNS followed by demyelination and neurodegeneration, which approximates the progressive forms of MS. In contrast, SJL mice, immunized with $PLP_{139-151}$ peptide develop relapsing-remitting form of disease, which resembles the clinical course of human RRMS (198).

EAE shares many of features of RRMS, such as T-cell (Th1 and Th17)-mediated inflammation, microglial activation, infiltration of immune cells (T cells, neutrophils, macrophages) into nervous tissue of CNS, multiple lesions and damage to myelin sheaths, making it a common and useful model for the human disease. The major difference between MS and its mouse model is that EAE requires external immunization to initiate the development of autoimmune reaction. Furthermore, in EAE, the antigens that initiate the autoimmune response are known, whereas in MS the exact composition of antigens has not been defined. Another difference of note is that the usual animal strains used to induce EAE do not exhibit defects in immune regulatory mechanisms, contrary to what often is observed in MS patients (198). Nevertheless, from the pathogenesis point of view, EAE is a valuable tool to study the mechanisms and potential targets for the development of therapies.

In EAE, actively induced by immunization with MOG_{35-55} peptide, autoreactive $CD4^+$ T cells are induced in the lymph nodes and spleen and, following upregulation of tissue-homing adhesion molecules, migrate to the peripheral tissues. After entering the CNS, MOG-specific T cells are re-activated by local and infiltrating APCs, which results in the production of vasoactive inflammatory mediators, pro-inflammatory cytokines and chemokines, recruiting other innate and adaptive immune cells.

Before the discovery of Th17 cells, EAE was believed to be mediated exclusively by IFN-γ-producing Th1 cells. This theory was supported by the observations that enhanced IFN-γ expression in the CNS correlated with clinical severity EAE and adoptively transferred self-reactive Th1 clones induced *in vitro* were able to transfer

EAE in the naïve recipients (201). Moreover, T-bet-deficient mice were found to be resistant to EAE. However, the concept of Th1 driven autoimmune inflammation was challenged by the discovery that mice deficient in IFN-γ, IFN-γR or IL-12p35 were more susceptible to EAE (202). Subsequently, it was discovered that IL-12 and a novel cytokine IL-23 share a second common chain p40. Cua et al demonstrated that IL-23p19-deficient but not IL-12p35-deficient mice are protected from development of EAE, ultimately confirming that IL-23 but not IL-12 is indispensable for induction of EAE (203). Soon after IL-23 occurred to be a pivotal differentiation factor for development of Th17 cells, which were able to induce EAE when adoptively transferred to the naïve recipient mice (204).

1.4.5 Therapies for MS

The understanding of the pathophysiology of autoimmune diseases, have led to the development of new therapies. Currently, there is no cure for autoimmune diseases, however disease-modifying treatments (DMT) can prevent formation of new lesions and reduce the severity and frequencies of relapses in patients with RRMS. DMT however, have marginal or no effects in other forms of MS. There are also additional immune-based treatments available for patients who do not respond to first-line DMT. Most of the current therapies focus on systemic inhibition of inflammation however, new approaches are emerging that concentrate on the suppression of antigen-specific immune responses.

1.4.5.1 Current therapies for RRMS

Interferon-β (IFN-β, Rebif, Avonex, Betaseron) was the first approved DMT for RRMS patients. IFN-β treatment reduces the relapse rate by 30% compared to placebo, however, less than 50% of patients respond to IFN-β therapy (205). The exact mechanism of therapeutic action of IFN-β is not completely understood. Studies on primary cultures of human blood-brain barrier endothelial cells (BBB-EC) and astrocytes have indicated that treatment with IFN-β enhances the expression of ecto-5'-nucleotidase (CD73) on these cells. This increases the level of adenosine, a key mediator of endothelial permeability. Therefore, as a consequence of improved function of the BBB, the transmigration of lymphocytes into the CNS is reduced (206). Other studies on mode of action of IFN-β in the EAE model and on PBMC from RRMS

patients have demonstrated that IFN-β mediates the therapeutic effect partially through induction of IL-27, which is associated with impaired development of Th17 cells (207).

Another first-line treatment for RRMS and for clinically isolated syndrome (CIS) of MS is glatiramer acetate (GA, Copaxone), also known as copolymer 1. GA is an effective and approved therapy, based on the strategy of inducing tolerance using altered peptide ligands (APL). GA is a mixture of synthetic polypeptides randomly composed of four amino acids, glutamic acid, lysine, alanine and tyrosine that are the most abundant in MBP. GA was first synthesized to mimic the encephalitogenic propertis of MBP, however the administration of GA to mice was found to have the opposite effect - GA blocked the induction of EAE. The exact mechanism of action of GA is not yet clear, however it was demonstrated that GA strongly binds to MHC class II molecules, competing with and even replacing various myelin antigens for presentation to T cells, thus inhibiting T cell activation and differentiation (208). In mouse model, GA induced Th2 and Th3 cells, which secreted high amount of IL-4, IL-10 and TGF-β in response to GA and MBP stimulation (209). Th2 and Th3 cells were also found in the brains and spinal cords of mice with EAE treated with GA (210). GA treatment was found to induce a variety of changes in RRMS patients. It reduced the proliferative capacity of GA-specific $CD4^+$ T cells and shifted the GA-reactive T cell populations from Th1 to anti-inflammatory Th2 and $CD4^+CD25^+Foxp3^+$ T cells, which may dampen the inflammation in the CNS by mechanism of bystander suppression (211, 212).

Monoclonal antibodies are currently used to treat various autoimmune diseases with more undergoing clinical trials. Natalizumab (Tysabri) is a humanized antibody that targets CD49 molecule, a α4 subunit of very late antigen-4 (VLA-4) receptor, present of the surface of T cells and other immune cells facilitating adhesion to endothelial cells. Through binding VLA-4, natalizumab prevents the adhesion between the endothelial cells and immune cells, consequently blocking the migration of leukocytes through BBB into the CNS. In clinical trials, natalizumab reduced annual relapse rate by nearly 70% compared to placebo and has been approved as a therapy for patients with severe RRMS unresponsive to treatment with IFN-β (213). A monoclonal antibody against TNF-α (infliximab, Remicade) is used for treatment of RA, psoriasis, Crohn's disease and ankylosing spondylitis. Monoclonal antibody against CD20 is currently under investigation for therapy of RA and monoclonal antibody against IL-6R is in clinical trials for therapy of RA (214). In addition, a monoclonal antibody specific for CD52

(alemtuzumab), which has been used for treatment of chronic lymphocytic B-cell leukemia, has recently been licenced for treatment of active RRMS (215).

Considerable interest has emerged in the development of oral treatments for autoimmune diseases due to the convenience of this route of administration. Specifically laquinimod and cladribine are new experimental agents for therapy of RRMS (216). Fingolimod, terifluomide and dimethyl fumarate has been recently licenced as a treatment. Fingolimod (FTY720) is structural analogue of sphingosine and exerts its biological effects by mimicking sphingosine 1 phosphate (S1P) and binding to S1P receptors on lymphocytes, resulting in internalization of the receptor. Down-regulation of S1P receptors leads to the sequestration of lymphocytes, which reduces their recruitment to the sites of inflammation (217). Terifluomide inhibits mitochondrial dihydroorotate dehyreogenase, a crucial enzyme in the synthesis of pyrimidine. Since activated lymphocytes consume de novo synthesised pyrimidine, terufluomide suppresses proliferation and effector functions of activated T and B cells. Dimethyl fumarate has been used in the treatment of psoriasis since 1950s, however the exact mechanis of action is still unclear. The therapeutic effect has been attributed to the reduced expression of pro-inflammatory cytokines and chemokines (216).

Although mostly used in treatment of cancer therapies, several chemotherapy agents have been investigated as possible treatments for autoimmune diseases. Cytotoxic drugs such as mitoxantrone, cyclophosphamide, azathioprine and methotrexate have been used as an additional therapy for MS patients who do not respond to first-line treatments (218). Furthermore, because of their potent anti-inflammatory effects corticosteroids, have been used as a standard treatment for many autoimmune disorders, including acute relapsing of MS and early stage SLE.

Although the therapies for autoimmune diseases have evolved and become more directed and selective, often with improved efficacy, they are still mostly based on the generalized suppression of immune responses. The treatment of RRMS patients with IFN-β and GA is well tolerated, usually with only minor side effects, such as flu-like symptoms, local skin reactions and liver enzyme abnormalities (218). Unfortunately, more than half of patients are resistant to the first-line treatments, partially because of the formation of the antibodies against the therapeutic agent (anti-IFN-β). It has been also speculated that different subtypes of T cells might be involved in disease

pathogenesis in different individuals, which may explain the different responses to IFN-β treatment. Experiments in the mouse model of MS indicated that although both adoptively transferred Th1 and Th17 cell can mediate the disease, treatment with IFN-β attenuated Th1-mediated but exacerbated Th17-mediated disease (219). This is in agreement with a study, showing that patients not responding to IFN-β treatment have elevated levels of IL-17 in the serum (219). Later studies demonstrated that IFN-β inhibits Th17 cells partially though induction of IL-27, and patients who failed to respond to IFN-β therapy had impaired production of IL-27 after stimulation with IFN-β *in vitro* (207). The second-line treatments, such as natalizumab and fingolimod, are effective in up to 80% of patients and reduce the frequency of relapses by 50-60%, however, they also significantly increase the risk of adverse events associated with suppression of immune system (216). For example, therapy with natalizumab for RRMS and Crohn's disease has been linked with several incidence of progressive multifocal leucoencephhalopahy (PML), a life-threatening opportunistic brain infection that leads to CNS demyelination by damage of oligodendrocytes. PML occurs as a results of reactivation of a polyoma John Cunningham virus (JCV) in the brain due to a general suppression of the immune responses (220). Therapy with fingolimod has been associated with several incidences of acute viral infections, including varicella zoster and herpes simplex encephalitis. In addition, administration of fingolimod increases the risk of developing skin cancer, breast cancer and malignant lymphomas (216). Due to the potential the risk of developing serious opportunistic infections and tumours, these therapeutic agents, although highly effective, are only recommended for patients with aggressive disease or when the initial therapy was unsuccessful. Therefore, the new therapeutic strategies for autoimmune disease are being investigated in order to develop treatments that are safe as well as effective.

1.4.5.2 Antigen-specific tolerance strategies

One aim of current research on therapies for T-cell mediated autoimmune diseases is to develop auto-antigen-specific tolerogenic treatments that allow specifically inhibit pathogenic self-reactive immune cells but maintaining the ability to effectively respond to infections and tumours. The methods for induction of auto-antigen-specific tolerance can be broadly divided into the generation of antigen-specific unresponsiveness and the generation of antigen-specific Treg cells.

Studies in animal models have demonstrated that treatment with high doses of soluble antigen results in T-cell unresponsiveness by blocking of T cell proliferation and IL-2 production, which consequently might lead to clonal deletion by activation-induced cell death (AICD) after antigen re-stimulation (221). Therefore, it has been postulated that tolerance induced by administration of soluble peptide may be an effective therapy for human autoimmune diseases. Indeed, studies demonstrated that intraperitoneal (i.p.) administration of high dose of MBP induced MBP-specific T cell anergy and prevented the development of MBP-induced EAE (222), whereas repeated intravenous (i.v.) injections of MBP significantly attenuated ongoing EAE (223). However, further studies demonstrated that i.v. administration of MOG peptide exacerbated disease in a primate model of MOG-induced EAE and treatment of mice with established EAE induced a fatal anaphylactic response (224). These severe adverse effects are thought to be due to the route of administration, since the spread of antigen into the tissues after i.v. injections might rapidly activate tissue-resident mast cells and induce Th2 response, resulting in enhanced production of pathogenic autoantibodies (225). The therapeutic potential of i.v. administration of MBP peptide (MBP_{85-96}) was evaluated in phase I clinical trial with progressive MS patients, however the induction of tolerance was not found (226).

Approaches to induce tolerance by administration of auto-antigen by mucosal routes have a strong biological basis. The dietary and commensal bacteria-derived antigens are tolerated at the gastrointestinal sites because of a highly immunosuppressive environment provided by mucosal APCs. High-dose treatment with antigen by oral route was found to induce T cell anergy, whereas low doses of antigen generated antigen-specific Treg cells. Prophylactic oral administration of auto-antigen has been shown to suppress the development of various autoimmune diseases in animal models, including EAE, colitis and uveitis, however the treatment was significantly less effective in inhibition of ongoing disease (227). Although the strategy for induction of antigen-specific tolerance through oral administration is particularly attractive due to the ease of delivery, the results of multiple clinical trials failed to show any beneficial effects. Administration of bovine myelin (Myloral) or synthetic MBP_{82-98} peptide (Dirucotide) to MS patients did not change the course of the disease (228). DNA vaccination also provides possibilities for induction of antigen-specific tolerance, as it offers the opportunity to combine expression of antigen alone or together with anti-

inflammatory cytokines, such as IL-10. Intramuscular injection with DNA vaccines encoding self-antigens lead to induction of T-cell anergy or development of self-antigen-specific Treg cells and Th2 cells (229). The clinical trials, however, showed only modest efficacy of this treatment in patients with MS, with no significant changes in relapse rate or lesion reduction compared to placebo-treated control patients (230).

Only one study so far demonstrated efficacious antigen-specific therapy exploiting the strategy to induce tolerance by administration of pure antigen. The clinical study performed by Walczak et al demonstrated that transdermal application of a mixture of myelin peptides, MBP_{85-99}, MOG_{35-55} and $PLP_{139-155}$ in a form of skin patch to patients with RRMS significantly decreased the annual relapse rate compared with placebo-treated patients (231). The suppression of MS was associated with induction of a unique population of DC in the skin-draining lymph nodes, inhibition of proliferation of myelin-reactive T cells and increased levels of IL-10 in the serum (232).

Other experimental antigen-specific therapies have been examined in clinical human trials. One of the more promising methods to induce antigen-specific tolerance is intravenous treatment using antigen- or peptide-coupled cells. Antigen-specific tolerance is induced through autologous carrier cells, usually PBMCs, stimulated with antigen in the presence of chemical cross-linker ethylene carboiimide. Since, the major problem with efficiency of antigen-specific tolerance is epitope spreading that occurs during the disease course, the advantage of using the antigen-coupled cells is that they can induce tolerance simultaneously to multiple antigens, when linked to the multiple myelin peptides. Recently, antigen-coupled cells treatment has shown to be safe and well-tolerated in the phase I clinical trial in MS patients (233). In other approach, T cell or TCR vaccination aims at suppressing autoreactive T cells by inducing the response directed against these T cells. Similarly, T-cell or TCR vaccines have been evaluated in initial clinical trials in MS patients. Treatments with antigen-coupled cells or TCR vaccines have been shown to have beneficial effects. Unfortunately, the major disadvantage of such a strategy is that each patient's blood cells have to be individually prepared *ex vivo* in an expensive process. Therefore, the simple, safe and effective strategies to restore auto-antigen-specific tolerance *in vivo* are still the most preferable for developing new treatments for autoimmune diseases.

1.4.6 Helminth infections

Helminth parasites are a diverse group of evolutionary old, multicellular worms that are members of the phyla nematodes (roundworms) and *Platyhelminths* (flatworms), including classes of *Trematodes* (flukes) and *Cestodes* (tapeworms). The parasitic worms infecting humans vary in their biology and most of them have complex life cycles, requiring intermediate and definitive hosts of different species. While the definitive host is where helminths reach sexual maturity and ability to reproduce, the intermediate host is necessary for development of one or multiple larvae stages during the life cycle. In the definitive human host, different helminth species reside in different target organs, including small intestine, colon, lungs, liver and lymphatic system. The host protective immunity against helminths is mediated by a strong Th2 type of responses, which limit the degree of infection and decreases the viability, but rarely kill the parasites (234).

The understanding of the cellular and molecular mechanism of immune response to helminths mostly comes from the studies of infection in rodents. Helminths stimulate immune responses by direct contact of the outer body of parasite (tegument) to the host tissues and via host tissue damage. It has been demonstrated that production of the alarmin cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP), produced by epithelial cells, are amongst the first immune responses to helminth infection. Alarmins rapidly activate type 2 innate lymphoid cells (ILC2), which provide an early source of the type 2 cytokines IL-5 and IL-13 (61, 235). In parallel, helminth products triggering DC to generate Th2 cells, which produce IL-4, IL-5 and IL-13, and activate mast cells, basophils, eosinophils and activate B cells to induce Ig class switching to IgE. In addition, IL-4 and IL-13 promote alternative activation of macrophages. The differentiated M2 macrophages release effector proteins such as resistin-like molecule-α (RELM-α), chitinase 3-like proteins (YM1 and YM2) and arginase-1. While RELM-α and chitinase play a role in directly damaging of parasite, ariginase is postulated to indirectly abrogate the metabolic functions of helminths by local depletion of L-arginine, an essential aminoacid (236, 237). M2 macrophages also express matrix metaloproteinases and fibreonectin, therefore, play an important role in wound healing and tissue repair in response to the parasite-mediated tissue damage (235). By production of regulatory molecules, such as PD-L2 and anti-inflammatory cytokines IL-10, TGF-β, M2 macrophages are predominantly immunosuppressive during the

helminth infections. Fig 1.2 presents helminth-induced immunoregulation that contributes to the suppression of autoimmune inflammation.

A characteristic feature of chronic helminth infections is immunomodulation, leading to the selective inhibition of certain components of type 2 responses. Infections with filarial and schistosoma species are associated with down-modulation of IL-5 but not IL-4, which indicates that the immune regulation concentrates on dampening of the effector but not the inducing arm of Th2 responses, leading to the more regulatory form of type 2 response (238).

Many helminths have evolved similar highly effective mechanisms of immune subversion, enabling them to escape from the host's immune attack, resulting in a chronic and often asymptomatic disease. These mechanisms include: modulation of APC function, induction of tolerogenic DC and regulatory macrophages, generation of a less aggressive 'modified Th2-type response' and induction of immunosuppressive cytokines IL-10 and TGF-β from innate and adaptive immune cells that consequently leads to the generation of different populations of Treg cells (239). Since, down-regulation of anti-helminthic immune responses protects its host against the collateral tissue damage, helminth-derived immunoregulation is beneficial for both the parasite and the host.

It has been estimated that approximately thirty percent of human population is infected with helminth parasites. Most of the helminth infections occur in developing countries (240). On the other hand, over the last century there has been a dramatic increase in number of cases of autoinflammatory, autoimmune and allergic diseases in the rich developed countries (181, 241, 242). An inverse correlation between the distribution of helminth infections and the prevalence of immune-mediated diseases has been explained by the hygiene hypothesis. The principle of this hypothesis is that the lack of infections in early life might impair the development of properly educated immune system, leading to the increased susceptibility to chronic autoimmune disorders. Conversely, the chronic helminth infections induce immunoregulatory mechanisms that suppress the anti-parasite response, leading to the parasite survival, but also inhibit systemic immune response to unrelated antigens, resulting in the suppression of autoimmune inflammation. These epidemiological findings and recent experimental

studies indicate that infection with helminth parasites might serve as an alternative therapy for autoimmune diseases.

1.4.6.1 Probiotic helminth therapies

Helminth infections in mice were shown to suppress allergic responses, inhibit colitis, EAE, CIA and diabetes in non-obese diabetic (NOD) mice (243, 244). Also, clinical trials in humans, involving patients suffering from Crohn's disease and ulcerative colitis patients, with *Trichuris suis* (pig whipworm) ova or *Necator americanus* (human hookworm) have demonstrated significant suppression of autoimmune pathology (245, 246). An observational study of Correale and Farez performed over the period of 4.5 years on naturally infected and uninfected MS patients revealed a relationship between the parasitic infections and clinical course of MS (187). MS patients that were infected with several different species of gastrointestinal parasites showed a dramatically reduced MS activity, manifested by significantly lower numbers of new and enlarging lesions, lower number of relapses and consequently minimal changes in disability scores. In addition, the parasite-driven protection was associated with induction of Treg cells and elevated numbers of IL-10- and TGF-β-producing PBMCs. These exciting results lead to first phase 1 clinical trials of helminth therapy in RRMS patients in the HINT study (Helminth-induced immunomodulation therapy), using oral administration of *T. suis* ova (TSO). The recently published study by Fleming and colleagues demonstrated that the TSO therapy is well tolerated and results in a modest decrease in number of CNS lessions (188, 247). Other clinical trials in MS patients using TSO in Trichuris Suis Ova (TSO) in recurrent remittent multiple sclerosis and clinically isolated syndrome (TRIOMS) study and *N. americanus* in worms for immune regulation in MS (WIRMS-1) study are currently underway (248, 249).

Although clinical trials exploiting live helminth parasites have provided interesting opportunities for alternative treatment of autoimmune diseases, the probiotic helminth therapy has disadvantages associated with potentially serious side effects. The adverse effects might involve gastrointestinal reactions, such as diarrhoea and abdominal pain even during short term treatment (250), and physical damage of mucosa, fever, weight loss, anemia (251) during the long-term infection and possible damage of other organs during the larval stage of certain parasites. Therefore the aim of this study is to identify the mechanisms of immune suppression and the key helminth-derived molecules which could be safely used for immunomodulatory therapies.

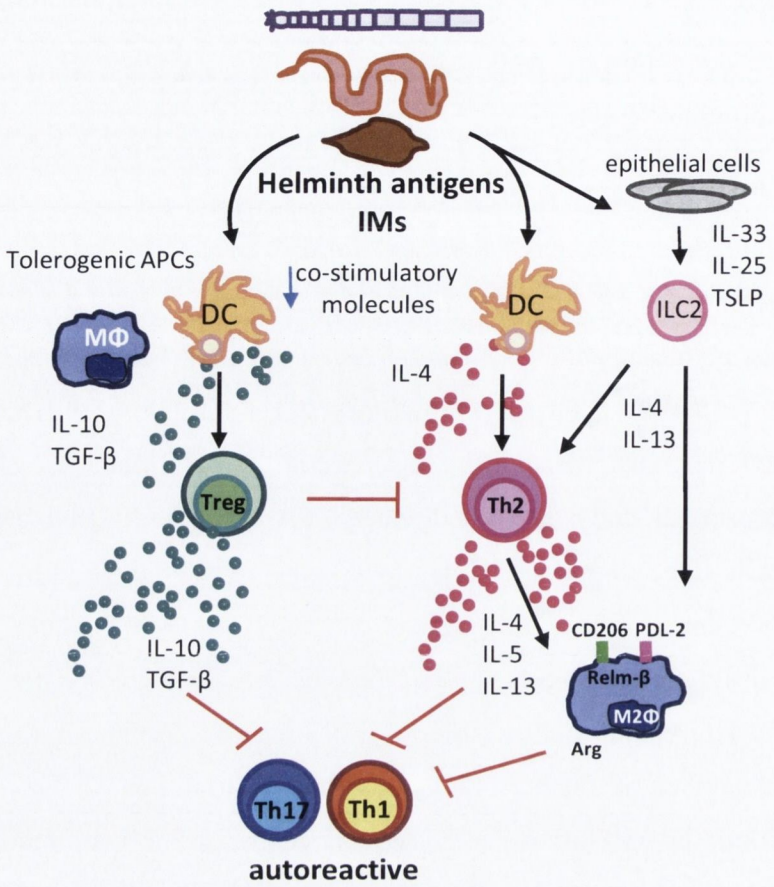


Fig. 1.2 Helminth-induced immune regulation contributing to suppression of autoimmunity.

Helminthic antigens and and IMs induce Th2-type responses and regulatory cells. Helminth products induce production of soluble mediators from epithelial cells, which activate ILC2 to produce early type-2 cytokines. Helminth-activated DCs promote development of Th2 cells or become tolerogenic DCs that induce Treg cells. Th2 cells produce cytokines associated with activation of classical type 2 –associated cells (B cells, eosinophils, basophils and mast cells) and alternatively activated macrophages (M2 macrophages). Type 2 cytokines, products of M2 macrophages and Treg cells inhibit Th1 and Th17 cells including self-antigen specific, responsible for autoimmune inflammation.

1.5 Aim of the study

Traditional therapies for autoimmune diseases are based on the global suppression of the immune system, increasing the risk of life-threatening opportunistic infections and malignant transformations. A major goal of research into new therapies for autoimmune diseases includes the development of antigen-specific treatments that would allow to specifically block the pathogenic auto-reactive immune cells, while maintaining the ability of the immune system to respond to infection and cellular transformations. The aim of this study was to examine the hypothesis that is it possible to induce auto-antigen-specific Treg cells in vivo using the following approaches:

- administration of self-antigen in the presence of TLR agonist and specific inhibitors of TLR-induced inflammatory cytokines;
- administration of self-antigen with anti-inflammatory cytokines
- administration of self-antigen with products derived from helminth parasite.

Furthermore, the study was designed to examine the capacity of the tolerogenic treatments to prevent the development of autoimmune disease using the EAE models.

Chapter 2

Materials and methods

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell culture medium

Roswell Park Memorial Institute-1640 medium (RPMI) (Sigma) was supplemented with 10% heat inactivated foetal calf serum (FCS; Sigma), 100 mM L-Glutamine (Gibco) and 100 µg/ml penicillin/streptomycin (Biowest). Complete RPMI (cRPMI) was used to culture murine bone marrow derived DC and macrophages while cRPMI supplemented with β-mercaptoethanol (2-ME 50 µM) was used to culture purified T cells, spleen cells and lymph node cells *ex-vivo*.

2.1.2 Ethidium bromide/acridine orange (EB/AO)

2.5 g ethidium bromide (Sigma)

2.5 g of acridine orange (Sigma)

Dissolved in 50 ml of 1xPBS

2.1.3 Red blood cell lysis solution

0.77% Ammonium chloride (NH_4Cl)

Prepared in sterile Baxter water

2.1.4 ELISA reagents

ELISA blocking buffer

10% (w/v) skimmed milk (Marvell)

Prepared in 1 X PBS

Or

1% (w/v) Bovine serum albumin (BSA, Sigma)

Prepared in 1 X PBS

ELISA developing solution

1 OPT tablet (10 mg)

Dissolved in 25ml of phosphate citrate buffer

15µl of H_2O_2 (30%)

ELISA stopping solution

1 M sulphuric acid (H_2SO_4)

ELISA washing buffer

0.5% Tween

Prepared in 1 X PBS

Phosphate citrate buffer

10.19g anhydrous citric acid

36.9g sodium phosphate dibasic ($Na_2HPO_4 \cdot 12\ H_2O$)

Dissolved in 1L of dH_2O, pH 5.0

Phosphate-buffered saline (PBS) 20 X

320g sodium chloride (NaCl)

46g sodium phosphate dibasic ($Na_2HPO_4 \cdot 12\ H_2O$)

8g potassium phosphate monobasic (KH_2PO_4)

8g potassium chloride (KCl)

Dissolved in 2L of dH_2O, pH 7.2

Phosphate-buffered saline (PBS) 1 X

0.5 L PBS 20 X

9.5 L dH_2O

ELISA sets

Cytokine	Top working standard	Supplier
IL-1α	1000 pg/ml	R&D
IL-1β	1000 pg/ml	R&D
IL-4	2500 pg/ml	BD Biosciences
IL-5	2500 pg/ml	BD Biosciences
IL-6	5000 pg/ml	BD Biosciences
IL-10	2000 pg/ml	R&D
IL-17A	1000 pg/ml	R&D
IL-12p70	2500 pg/ml	R&D
IL-23	2500 pg/ml	R&D
IFN-δ	10 ng/ml	BD Biosciences
GM-CSF	500 pg/ml	R&D
TNF-α	1000 pg/ml	R&D
TGF-β	1000 pg/ml	R&D

2.1.5 FACS buffer

2% FCS (Sigma)

0.1% (w/v) sodium azide

Prepared in sterile 1X PBS (Sigma)

2.1.6 MACS buffer

2% FCS (Sigma)

2mM EDTA (Sigma)

Prepared in 1X sterile PBS

2.1.7 Red blood cell lysis solution

0.77% Ammonium chloride (NH_4Cl)

Prepared in sterile Baxter water

2.1.8 Western blot reagents

RIPA buffer

50 mM Tris

150 mM NaCl

0.1% SDS

0.5% sodium deoxycholate

1% Triton X 100

Prepared in dH2O

Electrophoresis running buffer

125 mM Tris base

0.96 M Glycine

mM Sodium dodeca sulphate

Prepared in dH2O (1L)

Transfer buffer

25.5 mM Tris-HCl pH 8.3

0.2 M Glycine

20% v/v Methanol

0.05% w/v SDS

Prepared in dH2O (1L)

1X Sample Buffer

2.5 mM Tris-HCl pH 6.8

11% Glycerol

2% SDS

0.1% bromophenol blue

Prepared in dH2O

50mM dithiothreitol (DTT) added before use

2.1.9 Isotonic Percoll solution

Isotonic Percoll solution was prepared by diluting Percoll reagent (GE Healthcare) 9:1 with sterile 10X PBS (Sigma-Aldrich). Isotonic Percoll solution prepared this way had density of 1.123 g/ml. 40% and 70% isotonic Percoll solution (made in PBS) was used for isolation of mononuclear cells from brain tissue.

2.1.10 TLR ligands used in vitro and in vivo

Ligand	Receptor	Supplier	Conc. *in vitro*	Dose *in vivo*
Ultrapure LPS	TLR4	Invivogen	100 ng/ml	-
LPS	TLR4	Enzo	-	100 ng/mouse
CpG	TLR9	Sigma-Genosys	1 μg/ml	50 μg /mouse
Pam3CSK4	TLR2	Invivogen	100 ng/ml	-
Zymosan	TLR2/dectin-1	Invivogen	10 g/ml	100 μg /mouse

2.1.11 Inhibitors used in vitro and in vivo

Inhibitor	Target	Supplier	Conc. *in vitro*	Dose *in vivo*
U0126	MEK 1/2	Calbiochem	5 μM	50 μg/mouse
SB216763	GSK3	Sigma	20 μM	25 μg/mouse
YVAD	Caspase-1	Calbiochem	40 μM	25 μg/mouse

2.1.12 Antibodies used for in vitro culture

Antibody	Clone	Supplier	Concentration
Anti-CD3e	145-2C11	BD Biosciences	1 μg/ml
Anti-CD28	37.51	BD Biosciences	1 or 5 μg/ml

2.1.13 Antibodies used in western blot

Antibody	Concentration	Supplier
Anti-p38 rabbit	1:1000	Cell Signalling
Anti-p-p38 rabbit	1:1000	Cell Signalling
Anti-ERK rabbit	1:1000	Cell Signalling
Anti-pERK rabbit	1:1000	Cell Signalling
Anti-β-actin rabbit	1:1000	Cell Signalling
Anti-rabbit HRP-conjugated	1:5000	Cell signalling

2.1.14 Recombinant murine cytokines

Cytokine	Supplier	Conc. *in vitro*	Dose *in vivo*
IL-10	Immunotools	10 ng/ml	100 ng/mouse
IL-27	Immunotools	10 ng/ml	100 ng/mouse
TGF-β1	Immunotools	5 ng/ml	100 ng/mouse
IL-2	Immunotools	50	-
GM-CSF	J558 supernatant	20-40 ng/ml	-
M-CSF	L929 supernatant	20% (v/v)	-

2.1.15 Antibodies used in flow cytometry

Specificity	Fluorochrone	Clone	Isotype	Supplier
CD3	V450	17A2	Rat IgG2b	eBioscience
CD4	APC-eFluor780	GK1.5	Rat IgG2b	eBioscience
CD25	PE	PC61.5	Rat IgG1	eBioscience
Foxp3	PE	NRRF-30	Rat IgG2a	eBioscience
Foxp3	APC	FJK-16S	Rat IgG2a	eBioscience
IL-10	PE	JES5-16E3	Rat IgG2b	eBioscience
IL-10	APC	JES5-16E3	Rat IgG2b	eBioscience
IL-17A	PerCP-Cyanine5.5	eBio17B7	Rat IgG2a	eBioscience
IFNγ	PE-Cyanine7	XMG1.2	Rat IgG1	eBioscience
IL-4	FITC	BVD6-24G2	Rat IgG1	eBioscience
CD25	PE-Cyanine7	PC61.5	Rat IgG1	eBioscience

CD4	FITC	GK1.5	Rat IgG2b	eBioscience
CD3	APC-eFluor780	17A2	Rat IgG2b	eBioscience
LAP	PE	Mouse IgG1	TW7 16B4	eBioscience
CD11c	PE-Cyanine7	N418	AH IgG	eBioscience
F4/80	PerCP-Cyanine5.5	Rat IgG2a	BM8	eBioscience
LY6C	FITC	Rat IgM	AL-21	eBioscience
MHC-II	APC	Rat IgG2b	M5/114.15.2	eBioscience
CD11b	APC-eFluor780	Rat IgG2b	M1/70	eBioscience
CD11c	BV785	AH IgG	N418	Biolegend
Siglec F	PE-CF594	Rat IgG2a	E50-2440	BD Biosciences
CD40	FITC	AH IgM	HM40-3	BD Pharmingen
CD80	PE	AH IgG	16-10A1	Biolegend
CD86	PE-Cyanine5	Rat IgG2a	GL1	eBioscience
CTLA4	APC	AH IgG	UC10-4B9	Biolegend
γδ-TCR	FITC	AH IgG	GL3	BD Pharmingen
IL-17	BV421	Rat IgG1	TC-11-18H10	BD Biosciences
CD3	BV650	Rat IgG2b	17A2	Biolegend
PD-L1	PE	Rat IgG2a	MIH5	eBioscience
PD-L2	FITC	Rat IgG2a	122	eBioscience
CD45R	PE	Rat IgG2a	RA3-6B2	BD Pharmingen
PD-1	PE	AH IgG	J43	eBioscience

2.1.16 Other reagents used in flow cytometry

Chemical	Description	Supplier	Concentration
CFSE	cell membrane dye	Invitrogen	0.31 μM
Live/Dead Aqua	fixable cell stain	Invitrogen	1 in 600 dilution
PMA	mitogen	Sigma	5 ng/ml
Ionomycin	ionophore	Sigma	500 ng/ml
Brefeldin A	inhibitor of intracellular protein transport	Sigma	5 μg/ml

2.1.17 Antigens

Reagent	Supplier	Concentration *in vitro*	Dose *in vivo*
MOG_{35-55}	Genscript	2-50 µg/ml	100 µg/mouse
KLH	Calbiochem	2-50 µg/ml	20 µg/mouse
OVA protein	HyGlass	-	100 µg/mouse
$OVA_{323-339}$	New England peptide	1-50 µg/ml	-
F. hepatica TE	Section	0.625-10%(v/v)	50 ug/mouse
F. hepatica ES-L	Section	-	50 µl/mouse
F. hepatica ES-H	Section	-	50 µl/mouse

2.1.18 Bacteria-derived molecules

Reagent	Supplier	Concentration *in vitro*	Dose *in vivo*
Heat-killed Mtb	Chondrex	25 ug/ml	-
Mtb in CFA	Chondrex	-	400 ug/mouse
PT (liofilized)	Kaketsuken	-	500 ng/mouse
PT (in glycerol)	Kaketsuken	-	125 ng/mouse
PT	LIST	-	500 ng/mouse

2.1.19 Primers used in quantitative real time PCR

Gene	Protein	Ref. sequence	Supplier
Arg1	Arginase-1	NM_007482.3	ABI
Nos2	iNOS	NM_010927.3	ABI
Rentla	RELM-α	NM_020509.3	ABI

2.2 Animals

Specific pathogen-free female C57BL6 and BALB/c mice were purchased from Harlan UK Ltd. Specific pathogen-free female DO11.10 mice were bred in-house in the BioResourses Unit in Trinity College Dublin. All mice were maintained according to the regulations and guidelines of the Irish Department of Health and Children. All experiments were performed under license from Health Products Regulatory Authority and with the approval from Trinity College Dublin Comparative Medicine department. Mice were age-matched for each experiment and at the time of initiation of each experiment mice were 6-12 weeks old. Mice were sacrificed by asphyxiation with CO_2.

2.3 Methods

2.3.1 Preparation of single cell suspensions

Single cell suspensions were prepared form spleen and lymph nodes. Organs were dissociated using 40 μm cell strainer and suspended in cRPMI medium for cell culture. Additionally spleen red blood cells were lysed by incubating cells with 0.87% ammonium chloride for 5 min at 37°C, washed, centrifuged at 300 g for 5 min and re-suspended in cRPMI.

2.3.2 Cell culture

Single cell suspensions were cultured in complete RPMI (cRPMI) (Singma) or in X-VIVO medium (Lonza), if the supernatants were used to determine the concentration of TGF-β by ELISA. Cells were cultured in the atmosphere containing 5% CO_2 at 37°C.

2.3.3 Cell counting

Cell counts were performed by diluting cells with trypan blue (Sigma) 1:10 and applying 10 μl of the mixture into disposable haemocytometer (Hycor Biomedical). Under the light microscope the viable unstained cells appear white in contrast to blue-stained dead cells. Alternatively, cells were diluted in ethidium bromide/acridine orange (EB/AO) (Sigma-Aldrich) and counted under the UV fluorescent microscope, where viable cells emit green light, whereas dead cells appear orange. The concentration of cells was calculated using the following formula: average cell number from large squares of the chamber x 10^4 x dilution factor = number of cells/ml.

2.3.4 Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDC) were generated from C57BL/6 or BALB/C mice. Naïve mice were sacrificed and their femurs and tibiae were removed and dissected from the surrounding muscle and tissue. The bone marrow was flushed from both of the bones using a 27 gauge needle attached to a 20 ml syringe containing complete RPMI. A single cell suspension was obtained by aspirating cell clusters using a 19G needle attached to 20 ml syringe. The cell suspension was centrifuged at 300 g for 5 min and the cells were re-suspended in 1 ml of warm 0.87% ammonium chloride for 2 min, to lyse red blood cells. The cells were washed in cRPMI and pelleted by centrifugation at 300 g for 5 min. Cells were counted and cultured at 0.5 x 10^6 cells/ml (C57BL/6) or 1 x 10^6 cells/ml (BALB/c) in cRPMI supplemented with 20 ng/ml of granulocyte macrophage-colony stimulating factor (GM-CSF) in the form of supernatant obtained from culture of J558 cells line. J558 cells line was obtained from BALC/b B myeloma cells. GM-CSF-producing J558 cells are transfected with plasmid encoding for GM-CSF. After 3 days of culture, 25 ml of fresh medium containing 20 ng/ml of GM-CSF was added to each flask. On day 6, the supernatant was gently removed to eliminate contaminating cells from the culture. 20 ml of warm sterile PBS (Sigma) was added to each flask and after gentle agitating, PBS was transferred to 50 ml tube containing 10 ml of fresh cRPMI. Remaining cells were detached from the flask by incubation of 20 ml of sterile EDTA (Sigma) in 37°C for 10 min. EDTA was transferred to 50 ml tube containing 10 ml of fresh cRPMI. Cells in PBS and EDTA solutions were centrifuged at 300 g for 5 min, re-suspended in fresh cRPMI, pooled together and counted. The cells were cultured in new flasks at 0.5 x 10^6 cells/ml of cRPMI supplemented with 20 ng/ml of GM-CSF. After 2 days, (day 8) 20 ml of fresh medium containing 20 ng/ml GM-CSF was added to each of the flasks. On day 10 the loosely adherent cells were harvested, counted and seeded at the required concentration in tissue culture plates. The immature DC were allowed to rest for at least 2h at 37°C before use.

2.3.5 Generation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were generated from C57BL/6 mice. Bone marrow mononuclear cells were isolated as described in 2.2.3 and cultured in petri dishes at 1x10^6 cells/ml in cRPMI supplemented with 20% v/v of macrophage-colony stimulating factor (M-CSF) in the form of supernatant obtained from culture of L929

cells line. The origin of L929 cell line is mouse C3H/An connective tissue. M-CSF-producing L929 cells were obtained by transfection with plasmid encoding for murine M-CSF. Additional 2 ml of cRPMI supplemented with 20% v/v L929 medium was added to the culture on day 3. On day 6 loosely adherent wells were removed by washing with cRPMI and the adherent macrophages were gently scraped, counted and seeded at the required concentration in tissue culture plates. BMDM were allowed to adhere and rest for at least 3 h before use.

2.3.6 Isolation of peritoneal cells by peritoneal lavage

Nice were sacrificed by asphyxiation with CO_2 and the peritoneum was exposed by gentle removing the skin in the abdomen. 6 ml of cold cRPMI was injected into the peritoneal cavity using a 25G needle. Without removing the needle, the mouse was gently shaken to detach any adherent peritoneal cells, and as much of the cell suspension as possible was removed (4-5 ml) with the syringe. Peritoneal exudate cells (PEC) were placed on ice to prevent adherence of macrophages to the plastic. Cells were counted, re-suspended at the required concentration and cultured in tissue culture plates for stimulation or stained for flow cytometry analysis.

2.3.7 Preparation of *F. hepatica* products

Flukes were collected from infected bovine liver at a local abattoir (Killdare Chilling Ltd). Freshly isolated flukes were transported to the lab in PBS containing 100 µg/ml penicillin/streptomycin (PBS/PS) and then washed several times with PBS/PS to remove any contaminations and cellular debirs. Live flukes were incubated at 5-10 worms per 3 ml of PBS/PS in an incubator at 37°C and 5%CO_2 for 24 h. Supernatant, containing excretory-secretory products (ES), was collected from the tissue culture plate and filtered through a 0.45 µm filter to remove small tissue parts and eggs. ES was than centrifuged at 30,000 g for 30 min to remove smaller contaminations, aliquoted and stored and stored at -80°C. Flukes were washed several times in sterile PBS and after the last wash, the supernatant was decanted. Flukes were mechanically homogenised and the homogenate was centrifuged for 30 min at 15,000 g. The soluble fraction of the homogenate was harvested, aliquoted and stored at -80°C. ES was fractionated into high molecular weight fraction (ES-H) and low molecular weight fraction (ES-L) using a centrifugal filter unit with 3 kDa molecular weight cut off membrane (Amicon Ultra-4, 3000 MWCO,

Milipore). 4 ml of ES was applied onto the filter device and centrifuged at 3000 g in 4°C for 2 h. The fraction remaining over the filter and containing molecules larger than 3 kDa was replenished to the volume of 4 ml with PBS, aliquoted and stored in -80°C. The eluted fraction, containing molecule smaller than 3 kDa was collected, aliquoted and stored at -80°C.

2.3.8 BCA protein assay

Protein concentration in *F. hepatica* TE was determined using commercially available BCA protein assay kit (Pierce Thermo Scientific). This method combines a reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium (biuret reaction) with colorimetric detection of the Cu^{1+} cation using a reagent containing bicinchoninic acid (BCA). Reduced Cu^{1+} cation chelates molecules of BCA forming purple-coloured, water-soluble complex, which absorbance can be read at 562 nm. TE diluted 1:10, 1:20 and 1:40 in triplicates and together with 8-point standard curve samples (25-2000 μg/ml) were incubated on 96-well plate with BCA working solution for 30 min in 37°C in dark. The absorbance was measured at 562 nm and the concentration of protein in TE was calculated based on standard curve.

2.3.9 Detection of endotoxin by LAL assay

The endotoxin concentration in *F. hepatica* TE was determined using limulus amebocyte lysate (LAL) assay (LONZA) according to the manufacturer's protocol. TE was diluted 1:10, 1:100 and 1:1000 in triplicates and together with 4-point standard curve samples (0.1 EU/ml, 0.25 EU/ml, 0.5 EU/ml and 1 EU/ml were mixed with LAL reagent and incubated on a 37°C block for 10 min. Chromogenic substrate solution was added to the reactions vessels and the samples were incubated on 37°C block for 6 min. In LAL assay endotoxin triggers the enzymatic reaction releasing a yellow-coloured product from colourless substrate, which can be measured photometrically at 405 nm, after the reaction is stopped by stop reagent. The correlation between the absorbance and the endotoxin concentration is linear in the range 0.1-1 EU/ml and the concentration of endotoxin in the sample can be calculated on the basis of the standard curve.

2.3.10 *In vitro* stimulation of BMDC and BMDM

BMDC or BMDM were cultured at the concentration 1 x 10^6 cells/ml in 96-wells round-bottom (BMDC) and flat-bottom (BMDM) plates. DC were cultured with specific inhibitors for 30 min prior TLR ligand administration at the concentrations indicated in section 2.1.10 and 2.1.11. After 24 h supernatants were recovered to determine the concentration of cytokines by ELISA or cell death by LDH activity assay. In other experiments, BMDC or BMDM were cultured with *F. hepatica* TE (2.5-5 % v/v) 1 h before addition of LPS, CpG or PAMCSK4 at the concentration indicated in section 2.1.10. The supernatants were collected after 24 h of culture and the concentration of cytokines was quantified by ELISA. If required, cells were stained for flow cytometry analysis.

2.3.11 *In vitro* stimulation of PEC with Mtb or LPS

Cells were isolated from peritoneal cavity of mice by peritoneal lavage. Cell were cultured at the concentration 2 x 10^6 cells/ml in 96-well flat-bottom plates with LPS at 100 ng/ml or heat-killed *Mycobacteria tuberculosis* (Mtb) at 25 µg/ml for 24 h. Supernatants were collected and cytokine concentration was quantified by ELISA.

2.3.12 Injections of mice

Intraperitoneal injections

Mice were injected intraperitoneally (i.p.) with reagents or cells diluted in PBS. 200 µl of solution was injected using 27G needle.

Subcutaneous injections

Mice were injected subcutaneously (s.c.) with reagents or cells diluted in PBS. 200 µl of solution was injected using 27G needle. In case of EAE induction, 200 µl of CFA/MOG emulsion was injected using 19G needle due to high viscosity of the reagent.

Footpad injections

Injection into the foot pad combines as intradermal and subcutaneous administration of molecules. Mice were injected into each foot pad with reagents diluted in PBS in volume of 25 µl (total volume 50 µl/mouse) using a tuberculin syringe with 27G needle. After 7 days, the foot draining popliteal lymph nodes were isolated to determine the local T cell responses.

2.3.13 Immunisation of mice

In order to assess the influence of treatment with TLR agonists and specific inhibitors of cytokine expression pathways on development of EAE, C57BL/6 mice were injected subcutaneously into the back with 200 µl of solution containing 100 µg of MOG_{35-55} peptide (GenScript), MOG with 100 µg of zymosan or MOG with zymosan and inhibitors at the dose listed in sections 2.1.10 and 2.1.17 or MOG with zymosan and DMSO. In other experiments, assessing the influence of immunization with MOG and anti-inflammatory cytokines on development of EAE, mice were immunized with 100 µg of MOG peptide, MOG with IL-10, MOG with IL-27 or MOG with IL-10 and IL-27. Control mice were injected with PBS. Mice were injected at day 0 and day 14 and EAE was induced at day 21. In order to assess the immune responses in the draining lymph nodes or spleen, mice were injected with PBS, 100 µg of MOG peptide or MOG with IL-10. In other experiments mice were injected with PBS, 20 µg of KLH, KLH and *F. hepatica* TE or KLH and CpG. Mice were injected at day 0, boosted at day 14 and the immune responses in lymph nodes or spleen were assessed at day 21.

2.3.14 Adoptive transfer of cells prior to induction of EAE

Mice were immunized with MOG or MOG with IL-10 as described in section 2.3.13. Seven days after the last injection, spleens were removed and processed. Spleen cells were cultured *ex vivo* in 250 ml tissue culture flasks at 500 x 10^6 cells in 50 ml of cRPMI medium in the presence of 20 µg/ml MOG peptide and 50 U/ml rmIL-2. After 72 h of culture, cells were washed with cRPMI medium and treated with RBC lysis buffer for 1 min. Cells were then washed 3 times with PBS and centrifuged. The cells were re-suspended in PBS counted. Mice were injected i.p. with 20 x 10^6 cells / mouse in 200 µl of PBS and EAE was induced 24 h later.

2.3.15 Prophylactic treatment of mice with TE

Mice were injected i.p. with 50 µg of *F. hepatica* TE (200 µl) or with PBS at day 0 and 14. EAE was induced 7 days after the last injection.

2.3.16 Active Induction and assessment of EAE

EAE was induced in C57Bl/6 mice by subcutaneous (s.c..) injection of 100 μg/mouse of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ (GenScript) emulsified in complete Freund's adjuvant (CFA, Chondrex Inc.) containing 4 mg/ml (400 μg/mouse) of heat killed H37 Ra Mtb (Chondrex Inc). Mice were injected i.p. with 500 ng of pertussis toxin (PT purchased from Kaketsuken or List) or with 125 ng (PT in glycerol purchased from Kaketsuken) on days 0 and 2. The different dose of PT used for induction of EAE resulted from different activity of supplied PT. Animals were monitored daily for clinical signs of disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis/weakness in front legs; grade 5 – tetraparalysis/death. Animals were sacrificed for humane reasons when they reached a score above 4.

2.3.17 Isolation of mononuclear cells (MNC) from CNS tissue

Mice were lethally anesthetized by intraperitoneal injection of 40 μl sodium pentobarbital (Eutheathal) and perfused intra-cardially with 20 ml ice-cold PBS to remove peripheral blood from CNS tissue. Brain tissue was isolated, homogenised in ice-cold Hanks balanced salt solution containing 3% FCS (HBSS/FCS), and passed through 100 μm cell strainer. Tissue homogenate was washed with HBSS/FCS and centrifuged at 170 g for 10 min. Cell pellet was then suspended in 2ml of HBSS/FCS containing collagenase D (1mg/ml; Calbiochem) and DNase I (10 μg/ml; Calbiochem) and incubated for 45 min in 37°C with gentle shaking. Cells were then washed in HBSS/FCS and re-suspended in 5 ml of 40% isotonic Percoll solution. This was carefully overlayed on 5 ml of 70% isotonic Percoll solution with a plastic Pasteur pipette. Percoll gradients were centrifuged at 2000g for 20 min at 18°C, without brake. After centrifugation MNC were found on the interface between 40% and 70% Percoll solutions, myelin debris were floating on the surface of the 40% Percoll. Cells were aspirated with a Pasteur pipette, washed twice with cRPMI, counted with EB/AO and used as required.

2.3.18 Antigen-specific re-stimulation.

Single cell suspensions prepared from lymph nodes and spleens as described in section 2.2.2. Cells were counted using trypan blue solution, re-suspended at 1 x 10^6 (lymph nodes) or 2 x 10^6 (spleens) and seeded in 200 µl in a 96-well round-bottom plate in triplicate. 50 µl of the solution of antigen (OVA peptide, MOG peptide or KLH) was added to the wells at final concentration of 50 or 100 µg/ml (OVA), 2, 50 or 100 µg/ml (MOG and KLH). In addition, cells were stimulated with 5 ng/ml of PMA and 1 µg/ml of anti-CD3e as a positive control of T cell responses. Cells were cultured for 5 days and cytokine concentration in the supernatants was quantified by ELISA.

2.3.19 Isolation of $CD4^+$ T

CD4 T cells were isolated from mouse lymph nodes and spleens using $CD4^+$ Cell Isolation Kits (Miltenyi Biotec). This method is based on depletion of non-CD4 cells, which are indirectly magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies and secondary anti-biotin monoclonal antibodies conjugated to MicroBeads. Retention of MicroBeads-labeled cells on the column placed in magnetic field allows for the isolation of untouched $CD4^+$ T cells that pass through column. $CD4^+$ T cells were isolated from single cells suspensions of spleen and lymph node cells according to the manufacturer's protocol and using manual MACS cell separator (Miltenyi), re-suspended in cRPMI supplemented with 50 µM 2-mercaptoethanol.

2.3.20 Isolation of $CD4^+CD25^-$ T cells

$CD4^+CD25^-$ T cells were purified for the Treg conversion assay. Mouse $CD4^+CD25^-$ T cells were isolated in a two-step procedure. Non-$CD4^+$ cells were indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies and anti-biotin Microbeads. In parallel, the cells were labelled with anti-CD25-PE. $CD4^+$ T cells were isolated by depletion of unwanted cells and the remained fraction was magnetically labelled with anti-PE MicroBeads. Anti-PE-labeled cells are retained on the column and placed in magnetic field, which allowed for collection of unlabelled $CD25^-$ cells. $CD4^+CD25^-$ T cells were isolated from single cells suspensions of spleen and lymph node cells according to the manufacturer's protocol and using an autoMACS separator (Miltenyi),

re-suspended in cRPMI supplemented with 50 μM 2-mercaptoethanol and immediately used as required.

2.3.21 Stimulation of CD4+ T cells in vitro

Polyclonal stimulation of CD4+ T cells

Purified $CD4^+$ T cells were cultured with plate bound anti-CD3e (1 μg/ml: BD Biosciences) and soluble anti-CD28 (1 μg/ml: BD Biosciences). Plates were coated with 50 μl of anti-CD3e diluted in PBS for 2h in 37°C and thoroughly washed with sterile PBS. $CD4^+$ T cells with anti-CD28 were seeded onto the plates in cRPMI supplemented with 50 μM 2-mercaptoethanol. The cells were incubated for 72 h and then analysed by flow cytometry.

Treg conversion assay

Purified $CD4^+CD25^-$ T cells were cultured with plate bound anti-CD3e (1 μg/ml: BD Biosciences) and soluble anti-CD28 (5 μg/ml: BD Biosciences) in the presence of IL-2 (10 ng/ml) and a range of concentration of TGF-β (0.2-10 ng/ml). Retinoic acid (RA; Calbiochem;100 nM) was added to the culture as a positive control for enhancement of TGF-β-mediated Treg conversion. *F. hepatica* TE in a range of concentrations (1-5 % v/v) was added to the culture to alone or with TGF-β to determine the ability to induce or enhance Treg conversion. Plates were coated with 50 μl of anti-CD3e diluted in PBS for 2h in 37°C and thoroughly washed with sterile PBS. $CD4^+$ T cells were cultured with anti-CD28, IL-2 and TGF-β in 96-well plates in cRPMI supplemented with 50 μM 2-mercaptoethanol. The cells were incubated for 72 h and then analysed by flow cytometry for expression of CD25 and Foxp3.

Antigen-specific stimulation of OVA-specific TCR-transgenic T cells

BMDC from BALB/C mice were cultured at the concentration of 1 x 10^6 cells/ml in 24-well plates with ovalbumin peptide ($OVA_{323-339}$) for 3 h prior to addition of MEK1/2, caspase-1 and GSK3 inhibitors and TLR ligand stimulation. After 24 h, the supernatants were recovered and cells were scraped with a sterile syringe barrel and washed with medium. BMDC were added to U-botton 96-well plates at concentration of 0.2 x 10^5/ml. MACS isolated CD4 T cells at the concentration 1 x 10^6 cells/ml (ratio DC:CD4 T cells 1:10) were added to the wells. In some experiments BMDC were cutlured with OVA peptide only and CD4 T cells were added to the culture with recombinant cytokines listed

in section 2.1.9 alone or in combination. After 5 days of co-incubation CD4 T cells were analysed by FACS.

2.3.22 Assessment of modulation of T-cell responses using OVA-specific TCR transgenic T cells

$CD4^+$ T cells were purified as described in section 2.3.20 from spleens and lymph nodes of OVA-specific, MHC class II-restricted, αβ TCR transgenic mice on a BALB/c background (DO.11.10). 1 x 10^6 of cells were adoptively transferred into the naïve BALB/c mice by i.v. injection. Mice were injected into the foot pad with PBS, ultrapure OVA protein (Hyglass), OVA + zymosan, OVA + zymosan + MEK1/2, caspase-1 and GSK3 inhibitors or OVA + CpG at the doses listed in sections 2.1.10 and 2.1.11. After 7 days foot draining popliteal lymph nodes were isolated and single cell suspensions were restimulated with antigen as described in section 2.3.19.

2.3.23 LDH cytotoxicity assay

The release of LDH into cell culture supernatants was measured by the CytoTox non-radioactive cytotoxicity assay kit (Promega) according to the manufacturer's instructions. Supernatants from cultured cells (50 µl) were transferred to flat-bottom 96-well plate and incubated with 50 µl of substrate solution at room temperature, protected from light, for 30 min. The reaction was stopped by adding 50 µl of stop solution and absorbance was recorded at 490 nm. The cell maximum LDH release control was obtained by adding 10x lysis solution to the cells, 30 min prior the supernatants collection.

2.3.24 Western blotting

Preparation of samples

BMDC were cultured at 1 x 10^6 cells/ml (2 x 10^6 cells per well) in 6 well tissue culture plates. After the stimulation, the supernatant was removed, the cells were washed with PBS and lysed in 100 µl of RIPA buffer supplemented with complete mini protease inhibitor cocktail (Roche) (1tablet/25 ml) and phosphatase inhibitor cocktail 3 (Sigma) diluted 1:1000. Samples were cleared by centrifugation at 13000g for 5 min at 4°C and then stored at -20°C.

SDS Polyacrylamide gel electrophoresis

Samples were diluted with 5x loading buffer and boiled at 95°C for 5 min. Samples were then resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-PROTEAN electrophoresis cell. Samples were run on 10% polyacrylamide gel at 120V with protein markers (precision plus standards; Bio-Rad) as molecular weight standards.

Table 2.1 Preparation of polyacrylamide gel

Stacking gel		Resolving gel 10%	
dH_20	4.1 ml	dH_20	5.9 ml
1 M Tris-HCL (pH 6.8)	0.75 ml	1 M Tris-HCL (pH 6.8)	3.8 ml
30% Acrylamide:bisacrylamide ProtoGel, National Diagnostics	1 ml	30% Acrylamide:bisacrylamide ProtoGel, National Diagnostics	5 ml
10% SDS	60 µl	10% SDS	150 µl
10% Ammonium persulfate (APS) (Sigma)	60 µl	10% Ammonium persulfate (APS) (Sigma)	150 µl
TEMED (Fluka)	6 µl	TEMED (Fluka)	6 µl

Transfer of proteins to membrane

Proteins were transferred from SDS-PAGE gels to polyvinylidene fluoride (PVDF) membrane (Whatman) using a wet transfer system. PVDF was activated by immersion in methanol and rinsed in transfer buffer. The gel and filter paper were soaked in transfer buffer. Membrane was placed on top of two pieces of filter paper followed by the gel and then two other sheets of filter paper to make a layered sandwich. Air bubbles were then removed and the sandwich was placed between soft pads in the transfer cassette. The cassette was placed in the transfer cell which was filled with transfer buffer. The transfer was carried out at 200 mA for 60 mins.

Antibody blotting

Membranes were blocked to prevent non-specific binding by incubation in blocking solution (5% BSA in PBS) for 1 hr at RT on a rocker. The membrane was rinsed in wash buffer (PBS/0.05% Tween 20) and incubated with the primary antibody against p-ERK1/2, ERK1/2, p-38, p-p38 or actin at 1:100 in a blocking solution, on a rocker, at 4°C overnight. The membrane was then washed in 3 times for 5 min on a rocker and incubated with the secondary horseradish peroxidase (HRP) linked antibody (goat anti-rabbit 1/5000) in blocking buffer for 1 hr at RT. The membrane was then washed 3 times

with wash buffer. Blots were developed by ECL Chemiluminescent substrate (GE Healthcare) according to the manufacturers' instructions. The membrane was placed between two sheets of transparent acetate foil in a cassette; photographic film was placed on the top of the membrane and exposed for the required time. The film was processed using a Fuji X-ray developer.

2.3.25 Analysis of cytokine concentration by ELISA

ELISA assays on supernatants were performed using commercially available ELISA kits. High binding 96-well plates (Greiner bio-one) were coated with 50 µl of capture antibody diluted in PBS and left overnight in 4°C. Plates were washed 5 times in wash buffer and non-specific binding sites were blocked by incubation with 150 µl of blocking buffer for 2h at room temperature. Plates were again washed 5 times before addition of 50 µl per well of sample supernatant or serially diluted recombinant standard protein. Plates were incubated for 2 h in room temperature or overnight in 4°C. After washing, 50 µl of specific biotinylated detection antibody, diluted in reagent diluent was added per well and the plates were incubated in room temperature for 2 h. Wells were washed again and incubated with 50 µl of streptavidin-conjugated horseradish peroxidise (HRP) for 30 min at room temperature in the dark. Plates were washed and 50 µl of developing solution were added per well. The reaction was developed in dark and stopped by adding 25 µl of 1M H_2SO_4. The absorbance of samples was measure at wavelength 492 nm using a Versamax microplate reader (Molecular Devices) and calculated based on the equation of the standard curve. For specific details regarding the concentration and working dilutions of antibodies and buffer see table below.

Table 2.2 ELISA systems	**R&D systems**	**BD Bioscience**
Capture antibody dilution	1:180	1:1000
Blocking buffer	1% BSA in PBS	10% skimmed milk (Sigma)
Reagent diluent	1% BSA in PBS	1% BSA in PBS
Detection antibody dilution	1:180, incubation time 2h	1:1000 incubation time 1 h
HRP-Streptavidin dilution	1:200	1:1000
Developing solution	OPD tablet dissolved in 25ml of phosphate citrate buffer. 15µl of hydrogen peroxide H_2O_2 added prior to use	

2.3.26 Gene expression assay using real-time PCR

RNA isolation

Total RNA was extracted from PEC or BMDM using TRIzol/chloroform method. PEC were isolated by peritoneal lavage and counted. 1 x 10^6 cells was centrifuged in RNase-free tubes and the cell pellet was re-suspended in 600 µl of TRIzol. In case of BMDM, after the required stimulation in 96-well plate, the supernatant was aspirated and cells were re-suspended in 200 µl of TRIzol/well. The triplicate cultures were collected into RNase-free tubes, giving total volume of 600 µl of TRIzol. 120 µl of chloroform was added to each tube, mixed, left to stand at room temperature for 5 min and centrifuged at 12000 g for 15 min. The upper aqueous phase was removed to RNase-free tubes and 300 µl of isopropanol was added. Samples were mixed by inversion, left to stand at room temperature for 15 min and centrifuged at 12000 g for 15 min. Supernatants were decanted, the pellets were washed twice with 500 µl 75% ethanol in nuclease-free water. Samples were centrifuged at 7600 g for 3 min, ethanol was decanted and the pellets were allowed to dry on air. RNA was re-suspended in 20 µl of RNase-free water, left to stand on ice for 10 min. RNA was then heated to 65°C for 10 min to denature the secondary structures and immediately placed on ice. RNA concentration was determined using NanoDrop (Thermo Scientific) spectrophotometer. The purity of sample was determined by 260/280 nm ratio. Samples were stored at -20°C until used.

Reverse transcription PCR (RT-PCR)

Total cellular RNA was used as a template for reverse transcription reaction. Samples containing 1 µg of RNA were topped up to 10 µl with RNase-free water. Reverse transcription master mix (High Capacity cDNA reverse transcription mix; Applied Biosystems), 2 x concentrated, was prepared according to the manufacturers protocol and 10 µl of mix was added to each sample. The reverse transcription reaction was run in a TProfessional Basic Gradient thermal cycler (Biometra) using the following conditions:

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 sec	∞

Quantitative real-time polymerase chain reaction (qPCR)

cDNA, 8 times diluted in nuclease-free water, was used as a template for qPCR reaction. The reaction mix contained 4.5 µl of diluted cDNA, 4.5 µl of TaqMan Fast Universal PCR Master Mix 2x No AmpErase UNG (Applied Biosystems), 0.5 µl of endogenous primers (18S RNA; Applied Biosystems) and 0.5 µl of target gene primers (Applied Biosystems). The reaction was carried on 7500 Fast Real-Time PCR System (Applied Biosystems), using SDS Software v 1.3.1 with the following thermal conditions:

	Step 1	Step 2 x 45	
Temperature (°C)	95	95	60
Time	20 sec	3 sec	30 sec

For each sample, the amount of mRNA was normalized to the amount of 18S ribosomal RNA (rRNA) and was expressed as fold difference compared to the control sample, using the logarithm $2^{(-\Delta\Delta CT)}$.

2.3.27 Flow cytometry

Stimulation of cells prior to detection of intracellular cytokines

Cells obtained from organs or from *in vitro* culture were added to sterile FACS tubes in volume of 500 µl/tube or to sterile 96-well round-bottom plate in 200 µl/well and incubated for 6 h in 37°C/5% CO_2 with 5 ng/ml PMA, 500 µg/ml ionomycin in the presence of 5 µg/ml brefeldin A (BFA) in cRPMI supplemented with 50 µM 2-ME. After incubation, cells were washed with 3 ml or 200 µl of FACS buffer, pelleted by centrifugation at 300 g for 5 min and stained as required.

Discrimination of dead cells

Dead cells were discriminated by staining with LIVE/DEAD® Fixable Aqua Dead Cell stain (Molecular Probes, Life Technologies). LIVE/DEAD® Fixable Aqua Dead Cell stain is amine reactive fluorescent dye that can penetrate into the cells though disrupted cell membranes and react with free amines inside of the cells and on the cell surface resulting in an intense fluorescence of cells. In contrast, staining of live cells results in relatively week fluorescence due to the small amount of amines available to react with the dye. Single cell suspensions were washed in 96-well plate with 200 µl sterile PBS or in FACS tubes with 1 ml of FACS buffer and centrifuged at 300 g for 5 min. Cells were

stained with LIVE/DEAD Aqua stain diluted 1:600 in PBS at room temperature in the dark for 20 min prior to or during surface staining. Cells were washed with PBS and stained with antibodies as required. In parallel, single stained compensation control for Aqua flow cytometry channel was prepared by staining the cells, previously treated with 70% ethanol for 2 min.

Surface staining

After staining for discrimination of dead cells, non-specific binding of antibodies to cellular Fcγ receptors was blocked by incubation of cells with anti-CD16/CD32 (FCγ block, eBioscience) for 15 min at 4°C. Then, cells were washed and stained with a mixture of appropriate antibodies diluted 1:100 or 1:200 in FACS buffer for 15 min in 4°C in the dark. In parallel, single stained compensation controls were prepared using FACS compensation beads (BD). Fluorescence minus one (FMO) controls were also stained, if required. Cells were washed with FACS buffer, re-suspended in 50-200 µl of FACS buffer and acquired by flow cytometer.

Intracellular staining

In order to stain for intracellular epitopes, cells were first stained with LIVE/DEAD Aqua and surface markers antibodies and then fixed and permeabilised. After washing, cells were re-suspended in 50 µl of 2% paraformaldehyde (PFA) in FACS buffer and incubated for 15 min in 4°C in the dark. Cells were washed, re-suspended in 200 µl of saponim buffer (0.5% saponin in FACS buffer) and pelleted by centrifugation for 5 min. Then, cells were stained with a mixture of appropriate antibodies diluted 1:200 in saponin buffer for 25 min 4°C in the dark. Cells were washed with 200 µl of saponin buffer to remove the unboud antibodies, pelleted and washed twice with FACS buffer for membrane closure. Cell were then re-suspended in 50-200 µl of FACS buffer and acquired by flow cytometer.

Intranuclear staining

For intranuclear staining, cells were fixed and permeabilised using Foxp3 Fixation/Permeabilisation Kit (eBioscience). After staining with LIVE/DEAD Aqua and surface markers antibodies, cells were washed and pelleted by centrifugation. Cells were then re-suspended in 100 µl of fixation/permeabilization working solution and incubated for 30 min at RT in the dark. Next, cells were washed with 1X permeabilization buffer cells were stained with a mixture of appropriate antibodies diluted 1:200 in 1X

permeabilization buffer for 1 h in 4°C in dark. Cells were washed twice with permeabilization buffer and washed once with FACS buffer, re-suspended in 50-200 µl of FACS buffer and acquired by flow cytometer.

2.3.28 Acquisition of samples and analysis of flow cytometry data

Acquisition of samples stained with viability dye and fluorochrome-conjugated antibodies was carried out on Canto II or LSRFortessa flow cytometer (BD Biosciences). The cytometer was calibrated using single-stained compensation beads (BD Bioscience) and cells stained only with LIVE/DEAD aqua stain. Flow cytometry data were analysed using FlowJo software. The cellular debris were excluded based on SSC-Area and FSC-Area gate. Doublets were excluded by FCS-Height and FSC-Area profile and dead cells were discriminated based on staining with LIVE/DEAD aqua stain. The frequencies of cells were calculated on the basis of the parent gate or total live cell population.

2.3.29 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. Unpaired two-tailed Student t-test was used compare the statistical difference between the mean values between two groups. One-way Analysis of Variance (ANOVA) test followed by Bonferroni posttest was applied to determine the statistical difference between more than two groups. Two-way ANOVA was used to examine the statistical differences between groups in a course of time. Statistical significance was considered for p values less than 0.05.

Chapter 3

Induction of Treg cells by modulating TLR signalling pathways in DC

CHAPTER 3 INDUCTION OF TREG CELLS BY MODULATING TLR SIGNALLING PATHWAYS IN DC

3.1 Introduction

DC are crucial in inducing and shaping adaptive immune response and directing towards either tolerance or protective immunity. They act not only as the most efficient APCs, activating naïve T cells, but also secrete various cytokines, providing a third signal for T cell differentiation. DC recognize danger signals through TLRs and other PRRs, which leads to the production of a specific profile of the pro- and anti-inflammatory cytokines from DC. The traditional vaccines against infectious diseases composed of attenuated or killed pathogens are efficient at promoting adaptive immune responses due to a high content of various PAMPs, activating DC and other innate cells. Specific TLR agonists have a potent immunoregulatory activity and are capable of driving the adaptive immune responses when co-administered with antigens. Therefore, synthetic or pathogen-derived TLR ligands have been exploited as adjuvants in vaccines against infectious diseases, with TLR4 ligand monophosphoryl lipid A (MPL) being the first TLR agonist approved for human use in the hepatitis B vaccine Fendrix (23). It has also been demonstrated that TLR ligands can promote immune responses against tumours (eg. TLR7 agonist Imiquimod) (252). Moreover, the mouse studies have shown that DC activated by TLR ligands can break immunological tolerance to self-antigens, hence TLR-modulated DC inducing Th1 and Th17 cells specific for tumour antigens are being used in the development of antigen-specific cancer vaccines and immunotherapeutics (253, 254).

TLR ligands can induce both inflammatory and suppressive responses in DC and generate effector and Treg cells. Consequently, there is a growing interest in targeting particular TLR signalling pathways to modulate cytokine production in innate cells for treatment of autoimmune and inflammatory diseases and cancer (255). Autoimmune disorders arise from the breakdown of tolerance to self-antigens or due to a defect in Treg cell development or function. The uncontrolled effector T cell responses to self-antigens lead to the continuous inflammation and progressive damage of the affected tissues. The aim of this study was to address the hypothesis that it is possible to induce auto-antigen-specific Treg cells by vaccination with auto-antigen in the presence of TLR agonist and inhibitors of inflammatory cytokines.

The study was conducted to determine the optimal composition of TLR agonist and specific inhibitors of signalling pathways to be used as adjuvants to induce Treg cells that prevent experimental autoimmune encephalomyelitis.The first objective was to analyse the profile of anti-inflammatory and pro-inflammatory cytokines produced by DC upon stimulation with various TLR agonists: LPS, an agonist for TLR4, CpG, an agonist for TLR9, Pam3CSK4, a synthetic triacylated lipopeptide (Pam3CysSerLys4) and a ligand for TLR1 and TLR2, and zymosan, an insoluble preparation of yeast *Saccharomyces cerevisiae* cell wall, a ligand for TLR2 and C-type lectin receptor dectin-1. These four TLR agonists have been shown to increase IL-10 expression in DC through mediators such as p38, ERK, PI3K and transcription factors NF-κB and CREB (256–259). Stimulation of naive T cells with IL-10 leads to the development of IL-10-producing Tr1 cells that do not express Foxp3, but exhibit similar suppressive properties to $Foxp3^+$ Treg cells. TGF-β is a key factor responsible for the conversion of naïve $CD4^+CD25^-$ T cells into $Foxp3^+$ T cells and development of Th3 cell subset. Given that TGF-β is a pleiotropic cytokine and, when combined with IL-6 or IL-21, is involved in the differentiation of effector Th17 cells from naïve cells, the modulation of DC responses towards IL-10 expression seems to be a justified and reasonable approach in directing the selective induction of tolerogenic T cells.

The next objective was to determine the composition of inhibitors effectively decreasing expression of pro-inflammatory cytokines induced by TLR-stimulated DC. Fig. 3.1.1 depicts the signalling pathways induced in DC in response to TLR stimulation and the signalling pathways targeted for modulation of cytokine production. Previous studies have shown that IL-17 production by pathogenic $CD4^+$ T cells and γδ T cells in EAE is dependent on caspase-1-processed IL-1β (260). Moreover, TLR-driven production of IL-23, triggering development of Th17 cells and activation of γδ cells is mediated through ERK MAPK pathway (261). Hence, caspase-1 and MEK1/2 (a kinase phosphorylating ERK) specific inhibitors were used to suppress the production of IL-1β and IL-23 by DC. Glycogen synthase kinase 3 (GSK3), a serine-threonine protein kinase constitutively active in resting cells is associated with regulation of a multiple transcription factors, including NF-κB and CREB and primarily promotes IL-12p70 and suppresses IL-10 upon TLR stimulation in mice (262) and humans (263). Thus, GSK3 inhibitor was used to reverse the potential effect of GSK3 activation.

The eventual aim of this study was to evaluate the therapeutic effect of co-administration of the most applicable combination of TLR agonist and inhibitors with auto-antigen MOG prior to the induction of EAE.

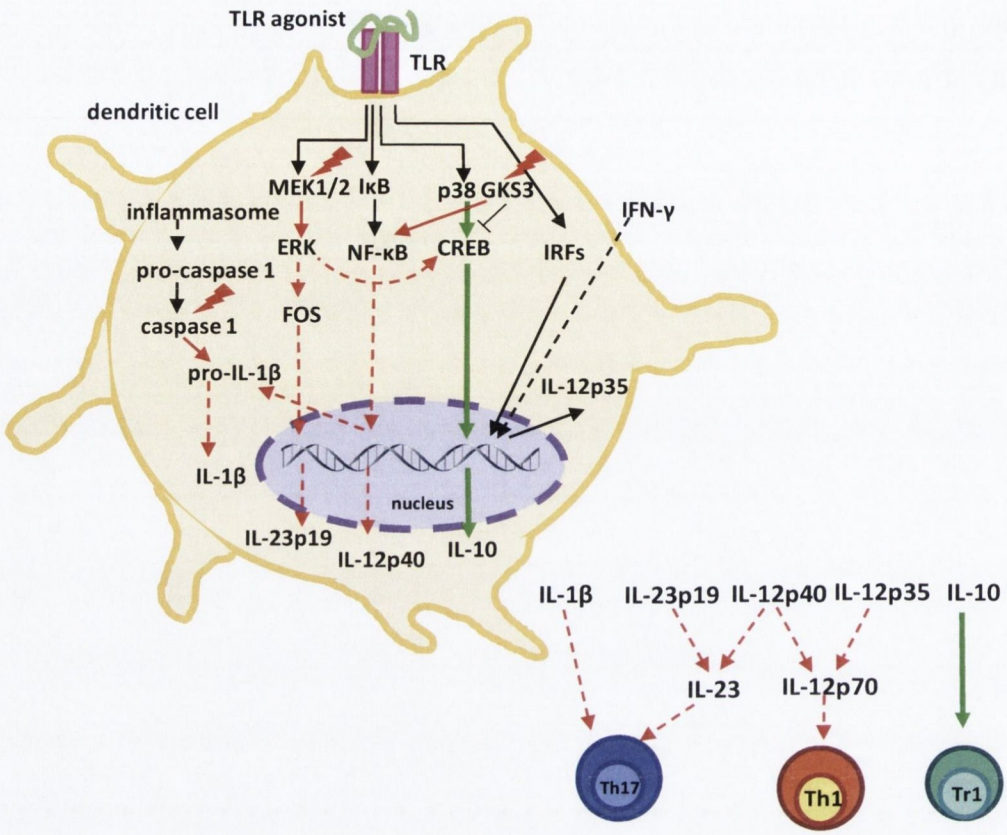


Fig. 3.1.1 Signalling pathways induced in DCs in response to TLR agonists and signalling targets for modulation of cytokine production.

TLR activation induces multiple signaling pathways that mediate expression of both pro-inflammatory and anti-inflammatory cykines in DCs. Activation of transcription factor NF-κB predominantly leads to the transcription of a range of genes encoding pro-inflammatory cytokines, including IL-12p40 and IL-1β. The functional IL-12 is composed of two subunits IL-12p40 and IL-12p35 and the expression of IL-12p35 is initiated independently by type I IFNs, IFNγ or through TLR-induced interferon regulatory (IRF) pathway. IL-12 is the key cytokine for driving Th1 cells. NF-κB also initiates transcription of the IL-1β precursor, pro-IL-1β. The maturation of pro-IL-1β into an active form is facilitated by inflammasome-activated caspase-1. TLR ligation also results in phosphorylation of mitogen-activated protein kinases (MAPKs). Phosphorylated MEK1/2 MAPK activates ERK MAPK, which promotes expression of IL-23p19 subunit and synthesis of a functional IL-23 that shares the IL-12p40 subunit with IL-12. Together, IL-1β and IL-23 promote expansion of Th17 cells. Phosphorylation of p38 MAPK activates transcription factor CREB, leading to the production of IL-10, an anti-inflammatory cytokine inducing development of the Tr1 cell subset. IL-10 expression is also partially controlled by ERK. Constitutively active GSK3 promotes expression of IL-12 and inhibits transcriptional activity of CREB. Red arrows indicate pathways inhibited by specific inhibitors of MEK1/2, caspase 1 and GSK3, leading to the decreased production of IL-1β, IL-12 and IL-23 and reduced development of Th1 and Th17 cells. Black arrows indicate pathways that have not been targeted. Green arrows indicate pathway promoting IL-10 expression and enhanced development of Tr1 cells.

3.2 Results

3.2.1 Effect of TLR agonists on cytokine production by murine DC

TLR agonists induce expression of both pro-inflammatory and anti-inflammatory cytokines. Stimulation of DC with different TLR ligands results in different patterns of cytokine secretion. Cytokine expression level depends on the dose or concentration of the TLR ligand. LPS, CpG, PAM3CKS4 and zymosan induce production of IL-10 but also pro-inflammatory cytokines. The ultimate aim of this study was to use TLR agonists together with inhibitors of pro-inflammatory cytokine expression pathways as a therapeutic for autoimmune disease. Firstly, experiments were performed to investigate the effect of different concentrations of TLR agonists on production of pro- and anti-inflammatory cytokines by murine DC. BMDC were stimulated with a range of concentrations of LPS, CpG, PAM3CSK4 or zymosan. After 24 h the supernatants were collected and the concentrations of IL-1β, IL-12p70, IL-23 and IL-10 were determined by ELISA.

LPS and PAM3CSK4, but not CpG, induced production of IL-1β, IL-23 in a dose-dependent manner (Fig. 3.1 – Fig. 3.3). Zymosan enhanced production of IL-1β, IL-23, IL-12.70 and IL-10 in a dose-dependent manner, however stimulation with the highest concentration of zymosan (100 μg/ml) was associated with decreased production of cytokines (Fig. 3.4). This result raised the possibility that high concentration of zymosan induced cell-death in DC. To test that hypothesis, DC were stimulated with a range of concentrations of zymosan and after 24 h cell viability was determined by staining with fluorescent dye (LIVE/DEAD Fixable Aqua Dead Cell Stain), penetrating cells with compromised membranes. In addition, the supernatants were tested for activity of LDH, a metabolic enzyme, released upon the cell lysis. The activity of LDH can be quantified in a colorimetric assay by measuring the degradation of LDH-specific substrate. The flow cytometry analysis showed that high concentration of zymosan (100 μg/ml) induced high level of cell death, leaving only 43% (versus 86% of viable cells in untreated control) viable cells after 24 h of stimulation (Fig. 3.5 A, B). This result was confirmed by LDH assay LDH (Fig. 3.5 C), showing increased LDH release from DC stimulated by zymosan in concentration 100 μg/ml.

All TLR agonists increased the secretion of IL-1β compared with unstimulated DC. The production and release of mature IL-1β requires two independent signals. The first signal

leads to the synthesis of an inactive form of cytokine, pro-IL-1β and is provided by stimulation with PAMPS. The second signal is provided by the activation of the inflammasome and caspase-1, leading to pro-IL-1β processing and release of mature form of IL-1β (264). To compare the level of IL-1β production by DC upon stimulation of TLR with or without inflammasome activation, BMDC were cultured with LPS (100 ng/ml) for 24h prior to the addition of NLRP3 inflammasome activator adenosine triphosphate (ATP) for 1 h. Consistent with published reports, after addition of ATP, the concentration of IL-1β in the culture medium increased 12 fold compared with cells stimulated with LPS alone (3000 pg/ml versus 250 pg/ml from LPS stimulated DC), confirming that stimulation of TLR alone is insufficient for release of high amounts of IL-1β by DC (Fig. 3.6 F).

Having established the effectiveness of induction of pro- and anti-inflammatory cytokines by DC by a range of concentration of TLR agonists, the optimal concentration of each TLR ligand was chosen, based on stimulation of IL-10 production. The selected concentration of LPS, CpG and zymosan was 100 ng/ml, 1 μg/ml and 10 μg/ml, respectively (Fig. 3.1, Fig. 3.2 and Fig. 3.4). Since PAM3CKS4 induced low amounts of IL-10 across the whole range of concentrations, the optimal concentration 100 ng/ml was selected based on induction of low amount of IL-1β and IL-23 (Fig. 3.3). Among the TLRs tested, LPS in optimal concentration induced the highest level of IL-1β, IL-1α, IL-23, IL-6 and TGFβ, which are crucial in the development and maintenance of Th17 cells (Fig. 3.6, Fig. 3.7). Consistently with published reports (259),(265) zymosan potently induced production of IL-10 (Fig. 3.7), but relatively low levels of IL-12p70. Zymosan also moderately enhanced the production of IL-1β, IL-23 and IL-6 (Fig. 3.6). Although PAM3CSK4 shares TLR specificity with zymosan, it failed to induce production of IL-10 and IL-23 and IL-1β by DC.

3.2.2 Specific inhibitors of cytokine expression pathways modulate cytokine production in TLR-stimulated DC

As established in the previous section, zymosan was the most effective inducer of IL-10 from DC among TLR agonists tested. The next aim was to determine the most efficient combination of inhibitors that would decrease the production of IL-23, IL-1β and IL-12p70. Previous studies demonstrated that inhibition of ERK and caspase-1 decreased

IL-23 and IL-1β production by DC and suppressed the ability of antigen-pulsed TLR-activated DC to induce Th17 cells *in vivo* (260, 261). In addition, was demonstrated, that GKS3 is involved in TLR receptor signalling, differentially affecting NF-κB and CREB transcriptional activity and administration of GSK3 inhibitor potently suppressed LPS-induced IL-12p70 (262). Consequently, the different combinations of caspase-1 inhibitor YVAD, MEK1/2 inhibitor U0126 and GSK3 inhibitor SB216763 were used to modulate TLR-induced cytokine production by DC. To compare the effect of inhibition of specific pathways prior to stimulation by distinct TLR agonists, BMDC, were incubated with the inhibitors separately or in combination for 30 min prior to addition of the TLR agonists. After 24 h of culture, concentration of IL-23, IL-1β, IL-12p70 and IL-10 in supernatants was measured by ELISA.

Inhibition of ERK pathway significantly decreased the production of IL-23 by zymosan-stimulated DC (Fig. 3.8) but not by LPS (Fig. 3.5), CpG (Fig. 3.6) or PAM3CSK4-stimulated cells (Fig. 3.7). Inhibition of caspase-1 effectively decreased the production of IL-1β induced by TLR stimulation. Surprisingly, caspase-1 inhibition also suppressed IL-23 production. In CpG- and zymosan-stimulated cells, treatment with both MEK1/2 and caspase-1 inhibitors further reduced IL-23 production in comparison with cells treated with the caspase-1 inhibitor only. This synergy did not occur in LPS- and PAM3SCK4- stimulated cells.

Inhibition of GSK3 enhanced the production of IL-10 from LPS- and CpG-stimulated DC, but not from PAM3CSK4 and zymosan-treated cells. Expression of IL-10 is negatively controlled through GSK3, which directly inactivates the transcription factor CREB, but largely depends on p38 and ERK signalling pathways. Consequently, inhibition of MEK1/2 decreased IL-10 production in CpG- and zymosan-stimulated DC. Moreover, the enhanced production of IL-10 by BMDC treated with the GKS3 inhibitor was suppressed by co-incubation with the MEK1/2 inhibitor. Inhibition of GSK3 signalling suppressed IL-12p70 production by zymosan-stimulated DC. Additionally, inhibition of MEK1/2 and caspase-1 further decreased IL-12p70 to an undetectable level.

The expression of IL-10 in innate cells is controlled by the activation of ERK and p38 MAPK and GSK3, which negatively regulates transcriptional activity of CREB (266). Consistently, the inhibition of MEK1/2 decreased the production of zymosan-stimulated IL-10 by DC in a dose-dependent manner (Fig. 3.12 A). In addition, western blot analysis

on cell lysates showed that the pre-treatment with combination of inhibitors prior to TLR stimulation decreased phosphorylation of ERK in zymosan-stimulated DC (Fig. 3.12 B). In contrast, GSK3 inhibition did not substantially influence IL-10 production (Fig. 3.12 A). In order to verify whether inhibitors of MEK1/2, GSK3 and caspase-1 non-specifically influence activation of p38 MAPK, the phosphorylation of p38 was determined by western blot. Pre-treatment of DC with inhibitors did not affect the phosphorylation of p38, confirming that combination of MEK1/2, GSK3 and caspase-1 inhibitors does not have indirect effects on zymosan-induced p38 signalling pathway (Fig. 3.12 C).

Overall, the inhibition of TLR-induced signalling pathways resulted in the specific modulation of cytokine production. Inhibition of MEK1/2 and caspase-1 efficiently decreased the production of TLR-induced IL-1β and IL-23. Inhibition of GSK3 prevented the decrease of IL-10 production. Addition of all three inhibitors together completely abolished zymosan-stimulated production of IL-12p70. Zymosan also induced the highest levels of IL-10. These data suggest that zymosan is the most suitable TLR ligand for stimulating IL-10 production and for induction of Treg cells, when used with specific inhibitors.

3.2.3 Inhibition of caspase-1, MEK1/2 and GSK3 pathway does not induce pro-inflammatory cytokine production

This study showed that inhibitors of cytokine expression pathways efficiently modulated the response of DC stimulated with different TLR ligands. In order to eliminate the possibility that the inhibitors alone induce the cytokine expression by DC, the cytokine production was analysed in the supernatants collected from the cultures of DC incubated with combination of inhibitors, but without TLR stimulation. BMDC were incubated with MEK1/2, caspase-1 and GSK3 inhibitors separately or in combination. Cells were also stimulated with zymosan as a positive control for cytokine production. Supernatants were recovered after 24 h and concentration of IL-1β, IL-23, IL-12p70 and IL-10 was determined by ELISA. Treatment with inhibitors separately or in combination did not increase cytokine production by DC (Fig. 3.13). These results demonstrated that inhibitors alone do not modulate steady state immune responses in DC and that stimulation by TLR agonists is necessary to elicit cytokine expression.

3.2.4 DC stimulated with zymosan and inhibitors modulate cytokine production from CD4+ T cells *in vitro*

The present study demonstrated that zymosan is the most potent inducer of IL-10 among the TLR agonists tested. Furthermore, together with specific inhibitors, it was possible to regulate cytokine production of zymosan-stimulated DC, decreasing the pro-inflammatory cytokines IL-1β, IL-23 and IL-12p70, involved in differentiation of Th17 and Th1 cells, respectively. The following experiments were conducted to examine the ability of DC stimulated with zymosan and inhibitors to drive the differentiation and cytokine production by $CD4^+$ T cells. BMDC ($1x10^6$cells/ml) from BALB/C mice were incubated with $OVA_{323-339}$ peptide for 3 h prior to adding the combinations of inhibitors. After 30 min of incubation with inhibitors, zymosan was added to the BMDC for 24 h. The cells were then washed twice with medium, scraped from the wells and re-cultured into 96-well plate at concentration of $5x10^5$ cells/ml. $CD4^+$ T cells from DO11.10 mice, carrying MHC class II restricted rearranged TCR, specific for OVA peptide antigen were purified from the spleens and lymph nodes using magnetic cell sorting and were co-cultured with DC in a ratio of 1 to 10 (DC to T cells). After 72 h, cells were stimulated with PMA, ionomycin and BFA for 6 h and stained with fluorochrome conjugated antibodies against surface CD4, nuclear Foxp3 and intracellular IL-10, IL-17 and IFN-γ. Triplicate cultures were pooled and analysed by flow cytometry. DC stimulated with zymosan did not enhance IL-10 production by $CD4^+$ T cells. However, the population of $CD4^+IL\text{-}10^+$ T cells significantly increased when DC were pre-treated with the combination of all three inhibitors (Fig. 3.14 A). The treatment of DC with zymosan and different combinations of inhibitors also enhanced the expression of Foxp3 by $CD4^+$ T cells (Fig. 3.14 B). In contrast, zymosan-stimulated DC pre-treated with inhibitors did not enhance IFN-γ production by $CD4^+$ T cells (Fig. 3.15 A). Surprisingly, $CD4^+$ T cells co-cultured with DC treated with zymosan and inhibitors significantly enhanced expression of IL-17, despite the fact that inhibitors efficiently decreased the production of IL-1β and IL-23 in zymosan-stimulated DC. (Fig. 3.15 B). Overall, inhibition of caspase-1, GSK3 and ERK increased the ability of zymosan-stimulated DC to induce IL-10 and Foxp3 expression, but also IL-17 production by $CD4^+$ T cells.

3.2.5 Effect of treatment with zymosan and MEK1/2, GSK3 and caspase-1 inhibitors on antigen-specific cytokines production by T cells

Studies have demonstrated that zymosan induces IL-10 by DC and TGF- β by splenic macrophages and *in vivo* experiments have shown that injection of zymosan with an antigen can promote antigen-specific T cell tolerance (259). In contrast, zymosan-induced peritonitis is associated with elevated levels of both IL-17 and IFN-γ (267). In the present study, zymosan-stimulated DC pre-treated with inhibitors of pro-inflammatory signalling pathways, but not DC stimulated with zymosan only, enhanced frequency of IL-10-producing and IL-17-producing $CD4^+$ T cells *in vitro* (Fig. 3.15). Experiments were performed to further determine the effects of treatment with zymosan and specific inhibitors on antigen-specific T cell responses *in vivo*. OVA-TCR transgenic $CD4^+$ T cells were purified by magnetic cell sorting from DO11.10 mice and 10^6 of cells was adoptively transferred i.v. to BALB/c mice. BALB/c mice were subsequently injected into the foot pad with PBS, $OVA_{323-339}$ peptide, OVA with zymosan or OVA with zymosan and MEK1/2, GSK3 and caspase-1 inhibitors. An additional group of mice was injected with OVA and CpG as a control for T cell responses. Seven days later, the injection site draining popliteal lymph nodes (PLN), were isolated and the lymph node cells were cultured ex vivo with different concentrations of OVA antigen.

Assessment of OVA-specific cytokine production 5 days later revealed that antigen-specific IL-10 production was marginally increased by lymph node cells from mice injected with OVA and zymosan. Importantly, IL-10 production was not significantly enhanced in mice injected with OVA, zymosan and inhibitors. Zymosan-stimulated antigen-specific IL-17 production was not significantly decreased in mice injected with inhibitors. Antigen-specific IFN-γ was induced moderately in mice immunized with OVA and zymosan and the injection of inhibitors did not modulate the production of this cytokine (Fig. 3.16). These data demonstrate a trend indicating that zymosan-induced development of antigen-specific IL-10-producing $CD4^+$ T cells can be augmented, whereas induction of antigen-specific Th17 cells can be suppressed by co-treatment with inhibitors of MEK1/2, caspase-1 and GSK3.

3.2.6 Effect of immunization with MOG, zymosan and MEK1/2, GSK3 and caspase-1 inhibitors prior to induction of EAE

Having established that treatment with zymosan and MEK1/2, GSK3 and caspase-1 inhibitors modulate antigen-specific T cell responses *in vivo*, the experiments were designed to investigate if the combination of TLR agonist and inhibitors can induce antigen-specific Treg cells that prevent the development of autoimmune disease. EAE is the most commonly used mouse model for human T cell-mediated demyelinating disease of the CNS and shares clinical and pathological features with RRMS, including inflammation, demyelination and axonal loss. The acute EAE is induced actively in C57BL/6 mice by immunization with MOG emulsified in CFA, followed by administration of PT. The generation of autoimmune Th17 and Th1 cells, as well as pathogenic IL-17-producing γδ T cells is followed by the breakdown of blood-brain barrier (268). This subsequently results in infiltration of the immune cells into the CNS. The inflammatory response leads to the degradation of myelin sheath and manifests as progressive paralysis.

To assess the capabilities of prophylactic treatment with zymosan and MEK1/2, caspase-1 and GSK3 inhibitors to induce MOG-specific protection against EAE, C57BL/6 mice were immunized s.c. with MOG (100 μg/mouse), MOG and zymosan (100 μg/mouse) or MOG, zymosan and U0126 (50 μg/mouse), YVAD (25 μg/mouse) and SB216763 (25 μg/mouse). To exclude the possibility that the inhibitors solvent influenced the outcome of the immunization (Fig. 3.17), another group of mice was treated with MOG and zymosan with the vehicle DMSO (Fig. 3.18). Mice were boosted 14 days after the first immunization and EAE was induced 7 days after the last immunization by s.c. injection of MOG emulsified with CFA. Mice were injected i.p. with 500 ng of PT at days 0 and 2. Animals were monitored daily for the clinical symptoms of the disease and weight change. Figure 3.17 shows the clinical scores and percentage of weight change, combining four independent experiments.

In the control (PBS) group, mice developed mild paralysis from day 7 and severe EAE by day 11. In contrast, the mice treated with MOG and zymosan developed EAE more rapidly with severe paralysis as soon as 7 days after induction of EAE ($p<0.001$ vs PBS group). Pre-treatment with inhibitors significantly attenuated the severity of EAE in

comparison to MOG and zymosan immunized mice however, the severity of EAE was still increased compared with that in the control group until day 12. Interestingly, compared with other groups, the onset of the disease was accelerated in mice treated with inhibitors, which showed early signs of paralysis from day 3 with the average score from 0.25 to 1. Consistent with published reports, the administration of auto-antigen MOG alone before induction of EAE attenuated the severity of the disease, reducing the average clinical score on day 14 from 3.5 in the control group to 1.7 ($p<0.001$).

In EAE mice gradually lose weight with a progression of paralysis. Therefore, the general condition of the animals was assessed on the basis of change in body mass (Fig. 3.17). Throughout the disease course, the weight loss corresponded to the progression of paralysis in all groups of mice. The largest decrease in weight was observed in MOG and zymosan treated mice, which developed the most severe paralysis.

DMSO is an organic solvent that has been used in clinical treatment for number of human diseases by i.v. injections or topical administration. Due to the miscibility with water, DMSO is widely employed as a solvent for reconstitution of non-polar compounds, prior to *in vivo* administration. It has been reported that only high concentrations of DMSO increased the cell death of human PBMCs *in vitro* and abolished the proliferative capacity of T cells after the long-term exposure (269). In this study, all three the inhibitors were firstly dissolved in DMSO and then mixed with water solution of zymosan and MOG prior to s.c. injection. Mice treated with DMSO developed marginally less severe paralysis compared to mice immunized with MOG and zymosan. This result confirms that the solvent is not responsible for attenuation of EAE in mice treated with inhibitors (Fig.3.18). In summary, the treatment with MOG and zymosan exacerbated the clinical course of EAE, which was reduced by administration of inhibitors of pro-inflammatory cytokines expression. However, the prophylactic therapy with MOG, zymosan and inhibitors failed to further attenuate EAE.

3.3 Discussion

A breakdown in self-tolerance or a defect in development of immune regulation mechanisms can lead to an unrestrained effector T cell response to self-antigens and the development of autoimmune disorders, such as MS, type 1 diabetes or lupus erythematosus. The conventional therapies for these conditions are not antigen-specific and are based on systemic reduction of inflammation. Such treatments, although moderately effective, can be associated with various side effects, including enhanced susceptibility to chronic infections and occasional cancers. Hence, the aim this research on new therapeutic approaches for autoimmune diseases was to develop treatments that would specifically inhibit auto-reactive immune cells without hampering immunity to foreign antigens. Currently, several methods are used for inducing peptide-specific immune tolerance *in vivo*. Those methods are mostly based on the induction of T cell unresponsiveness (T cell apoptosis or anergy) for example, by repeated i.v. or topical application of high doses of peptide antigen. However, the major disadvantage of inducing antigen-specific T cell unresponsiveness is that during MS the myelin-specific T cell reactivity can change over time (epitope spreading) and the newly generated pathogenic T cells can promote relapses of the disease (153). Another method is based on the induction of tolerance by administration of low-dose self-antigen through mucosal (oral or nasal) routes, where mucosa-associated APCs preferentially initiate development of Treg cells. This method, however, was deemed to be unsuccessful in the treatment of the autoimmune diseases in clinical trials in humans (227). Therefore, the induction of antigen-specific Treg cells that are able to actively suppress the pathogenic responses, still remains the ultimate goal of therapy for autoimmune diseases.

TLR ligands, when co-administered with a foreign antigen, act as adjuvants to promote immune responses specific for this antigen. This property of TLR agonists was first used in the development of vaccines against infectious diseases, but also later in the development of immunotherapeutics against cancer. Moreover, Marshall at al demonstrated that immunotherapy with a specific small molecule inhibitor of PI3K, increased the pro-inflammatory effects of TLR5 agonist flagellin and enhanced the immune responses against tumours (254). Different TLR ligands induce both pro- and anti-inflammatory cytokines from DC. Therefore, the aim of this study was to explore the potential of TLR agonists combined with specific inhibitors of inflammatory cytokines

to induce Treg cells that mediate long-lasting antigen-specific Treg protection against autoimmune disease.

The results of this study showed that zymosan was the most effective inducer of IL-10 production from DC. Zymosan is a water insoluble preparation of the yeast *S. cerevisiae* cell wall, composed of protein-carbohydrate complexes, mostly β-glucans. TLR2 and a C-type lectin receptor, dectin-1 can both recognize zymosan particles and initiate cytokine response. Other groups have demonstrated that zymosan-mediated production of IL-10 involves ERK MAPK activation downstream of TLR2 and dectin-1 (259, 266). The results of the present study are consistent with those findings, since the inhibition of ERK pathway substantially, but not completely, decreased the production of IL-10 by zymosan-stimulated DC. Downstream signalling from TLR2 also involves activation of p38 MAPK, another mediator of IL-10 expression, which was unaffected by inhibition of MEK1/2, GKS3 or caspase-1. Zymosan also preferentially induced IL-23 expression over IL-12, which is characteristic for TLR2 agonists (270). As expected, the inhibition of ERK, caspase-1 and GSK3 pathway decreased production of the cytokines involved in differentiation of effector Th1 and Th17 cells. Based on the previous studies demonstrating that IL-10 promotes differentiation of naïve T cells into the Tr1 subset, zymosan appeared to be the most appropriate TLR agonist for further studies.

Published reports have shown that a high dose of zymosan injected into the peritoneal cavity of mice, induces an acute inflammatory response and infiltration of several types of inflammatory innate cells, which is associated with induction of IL-17 expression. In the present study, zymosan induced antigen-specific IL-17 and small amount of IFN-γ from the lymph node cells, but it was possible to reduce this response when zymosan was injected with inhibitors. Surprisingly, the increased production of IL-17 by $CD4^+$ T cells was observed after *in vitro* co-culture, only when DC were pre-incubated with inhibitors prior to zymosan stimulation. This result was not consistent with the *in vitro* findings that showed decreased IL-23 and IL-1β production from DC after the incubation with inhibitors. The *in vitro* data have shown that zymosan also induces IL-6 from DC at the same level that is induced by LPS and CpG. Together with the moderate induction of TGF-β, it might be sufficient for the development of Th17 cells, which are promoted by IL-23 and IL-1β. TLR–induced IL-6 expression is regulated mainly by NF-κB. The recent report by Buxade et al has demonstrated, that the transcription factor, nuclear factor of activated T-cells 5 (NFAT5), is involved in transcription of IL-6, upon dectin-1

engagement in macrophages (271). NFAT5 remains under control of p38 MAPK, however, the combination of inhibitors used did not modulate the phosphorylation of p38, suggesting that the expression of IL-6 was not enhanced. Therefore, it is unlikely, that inhibition of MEK1/2, GKS3 or caspase-1enhanced expression of IL-6.

Several published reports have demonstrated that it is possible to induce IL-10-producing Tr1 cells using pathogen-derived molecules. For example, *Bordetella pertussis*-derived molecule filamentous haemagglutinin (FHA), stimulates IL-10 and TGF-β production from DC and macrophages and induces development of pathogen-specific Tr1 cells during infection (122). Furthermore, administration of FHA ameliorated T-cell dependent colitis can induce development of Treg cells specific to unrelated antigens (272), Keogh et al, unpublished data). The ability of microbial molecules to induce tolerogenic responses is considered to be an immune subversion strategy that allows pathogen persistence, resulting in a chronic infection. Commensal bacteria and some non-pathogenic fungi, exploit this as a method for long-lasting co-existence with the host. The present study showed that DC treated with zymosan and inhibitors were able to increase the production of IL-10 by $CD4^+$ T cells during *in vitro* co-culture. In addition, the administration of zymosan induced small amount of antigen-specific IL-10 from lymph node cells. Importantly, the study confirmed, that the administration of zymosan together with inhibitors and antigen *in vivo* enhances the antigen-specific IL-10 production from T cells.

The present study demonstrated that immunisation of mice with self-antigen combined with zymosan or zymosan with inhibitors of inflammatory cytokines before the induction of the EAE can modulate the clinical course of the disease. The partial T cell unresponsiveness, achieved by administration of MOG peptide only and manifested by attenuation of the disease in mice, was overcome when the mice were injected with MOG and zymosan. The pathogenesis of EAE is based on the induction of MOG-specific effector Th17 and Th1 cells by immunization with MOG/CFA. The exacerbation of EAE after treatment with MOG and zymosan suggests that zymosan induces inflammatory responses and development of effector Th cells, which respond more rapidly to the antigenic stimulus during the active induction of EAE. This result is consistent with data showing a trend towards enhanced production of OVA-specific IL-17 and IFN-γ by lymph node cells of mice immunized with OVA and zymosan. Treatment with inhibitors significantly attenuated EAE compared with zymosan-treated mice however the

developed disease was more severe than in the control mice. This result indicates that treatment with zymosan induces stronger Th effector responses than the tolerogenic responses. Inflammatory Th effector responses can be reduced only partially by the application of inhibitors of MEK1/2, caspase-1 and GSK3.

Although the present study did not establish whether injections of MOG and zymosan promoted MOG-specific pathogenic T cells, the exacerbation of EAE suggests that it did. Further studies should be performed to determine if administration of zymosan alone is also able to increase the severity of EAE. There is an established correlation between infections and autoimmune disease prevalence in humans. This association might result from a nonspecific activation of innate immune responses by infections leading to the breakdown of the immunological tolerance to self-antigens and development of self-antigen specific pathogenic T cells. To answer the question, whether the zymosan induces nonspecific responses, responsible for exacerbation of EAE, the experiment comparing the effect of immunization with MOG and zymosan versus zymosan only should be conducted.

The more rapid development of EAE after pre-treatment with MOG and zymosan might suggest that the innate immunity has been "trained" by the stimulation with TLR agonist and the innate responses remain in the state of a long-term enhancement. Trained innate immunity is an interesting concept emerging from the increasing evidence for innate immune memory. It is based on the studies showing that plants and invertebrates are protected against secondary infections, in spite of lacking adaptive immune responses. Trained immunity is non-specific and the enhanced activation appears to be dependent on the epigenetic reprogramming, mediated through recognition of PAMPs (273). The recent studies by Saeed et al investigated the immunological imprinting associated with innate immune training. The results showed that stimulation of monocytes with *Candida albicans* β-glucan induced innate immune training. Macrophages derived from monocytes cultured in the presence of β-glucan produced significantly more TNF-α and IL-6 in response to re-stimulation with β-glucan (274). Functional changes of trained macrophages were associated with epigenetic and transcriptional changes during monocyte-to macrophage differentiation. Since zymosan is composed of β-glucan and induces innate responses via stimulation of dectin-1, these findings might explain the exacerbation of EAE after pre-treatment of mice with zymosan and MOG.

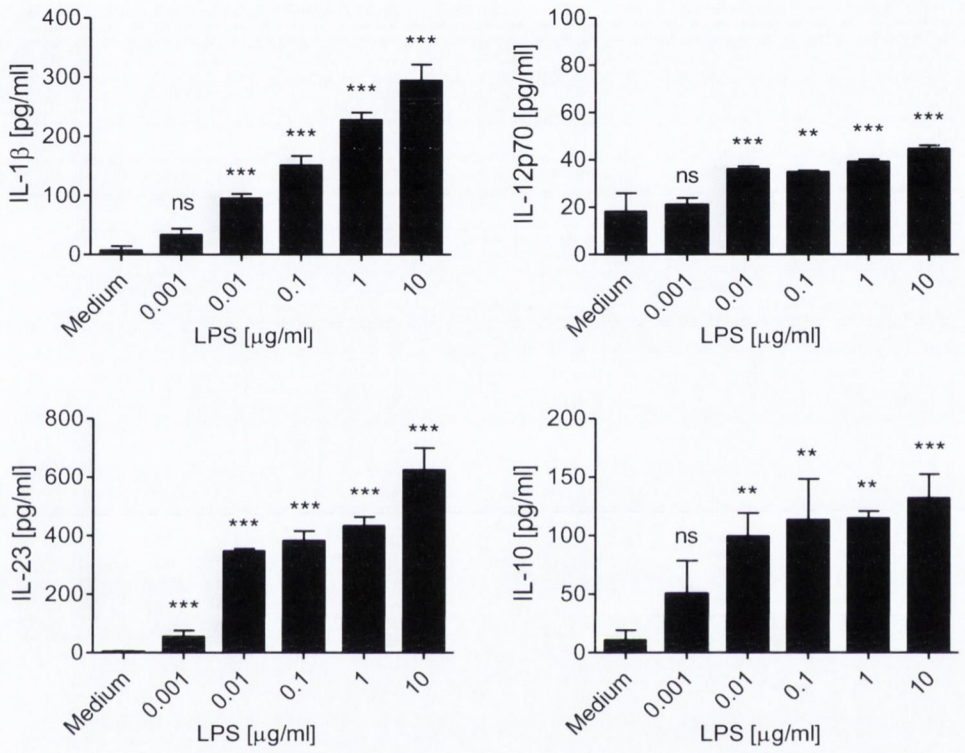


Fig. 3.1 Dose-dependent production of cytokines by murine DC stimulated with TLR4 ligand LPS. BMDCs from C57BL/6 mice were cultured with different concentrations of LPS (0.001-10 μg/ml) for 24h. Supernatants were recovered and concentration of IL-1β IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate cultures and are representative of 2 independent experiments. **p<0.01, ***p<0.001 by ANOVA

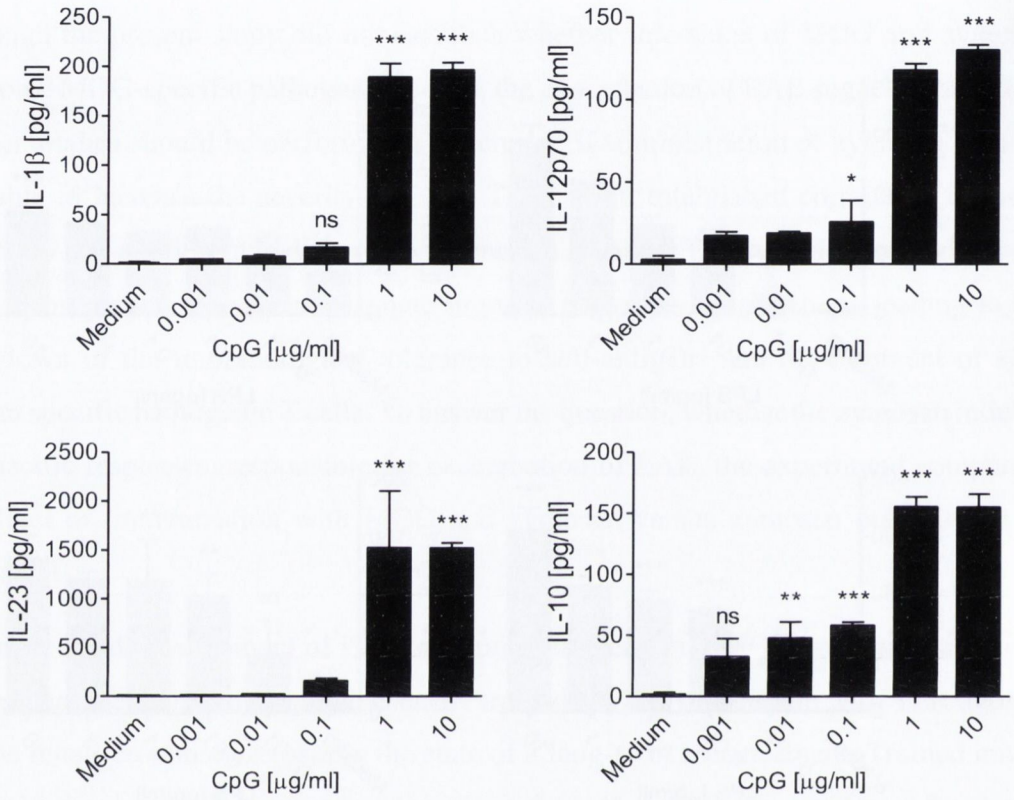


Fig. 3.2 Dose-dependent production of cytokines by murine DC stimulated with TL9 ligand CpG.

BMDCs from C57BL/6 mice were treated with different concentration of CpG (0.001-10μg/ml). After 24 h of incubation supernatants were recovered and concentrationof IL-1β IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate cultures and are representative of 2 independent experiments.

ns, not significant, *p<0.05, **p<0.01, ***p<0.001 by ANOVA

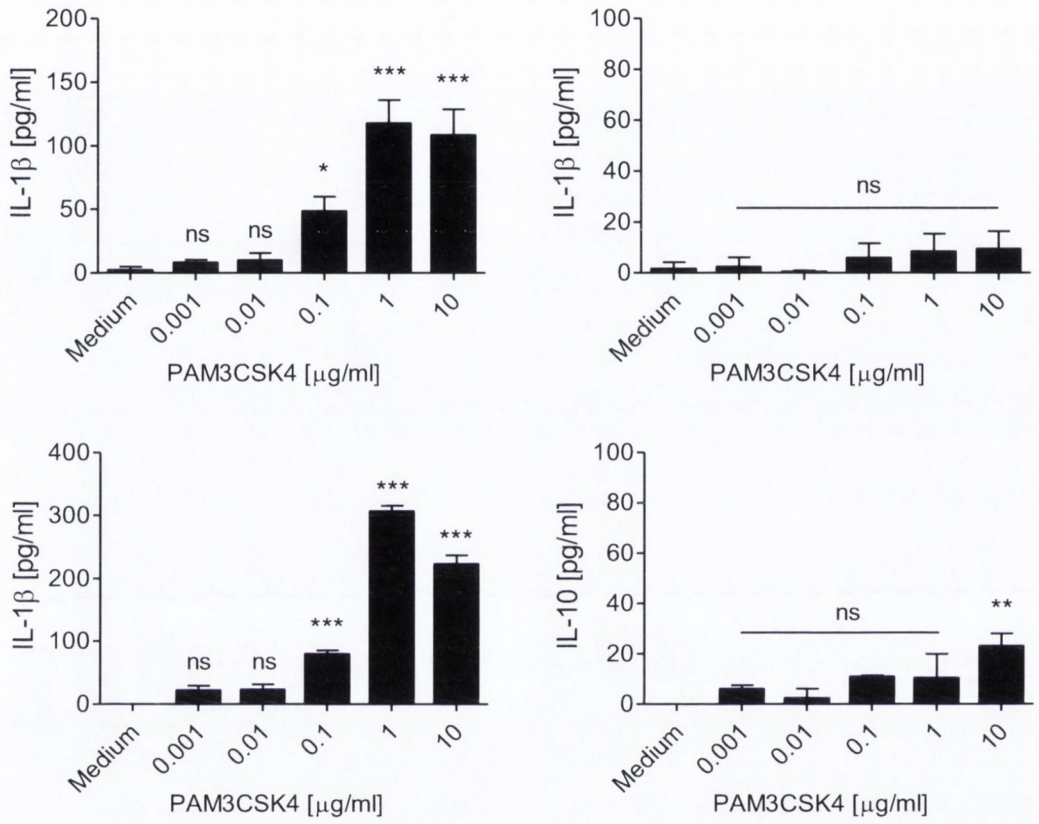


Fig. 3.3 Dose-dependent production of cytokines by murine DC stimulated with TLR2 ligand PAM3CSK4. BMDCs from C57BL/6 mice were treated with different concentration of PAM3CSK4 (0.001-10μg/ml). After 24 h of incubation supernatants were recovered and concentration of IL-1β IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate cultures and are representative of 2 independent experiments. ns, not significant, *p<0.05, **p<0.01, ***p<0.001 by ANOVA

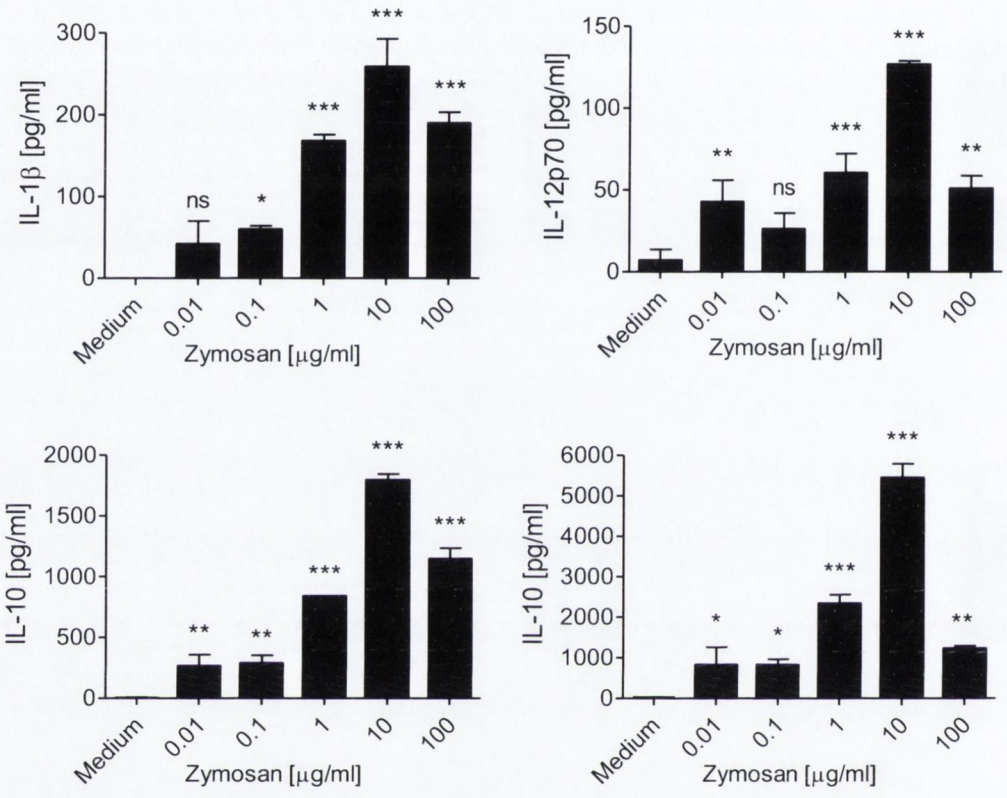


Fig. 3.4 Dose-dependent production of cytokines by murine DC stimulated with TLR2 and dectin-1 ligand zymosan. BMDCs from C57BL/6 mice were treated with different concentration of PAM3CSK4 (0.01-100μg/ml). After 24 h of incubation supernatants were recovered and concentration of IL-1β IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate cultures and are representative of 2 independent experiments. ns, not significant, *p<0.05, **p<0.01, ***p<0.001 by ANOVA

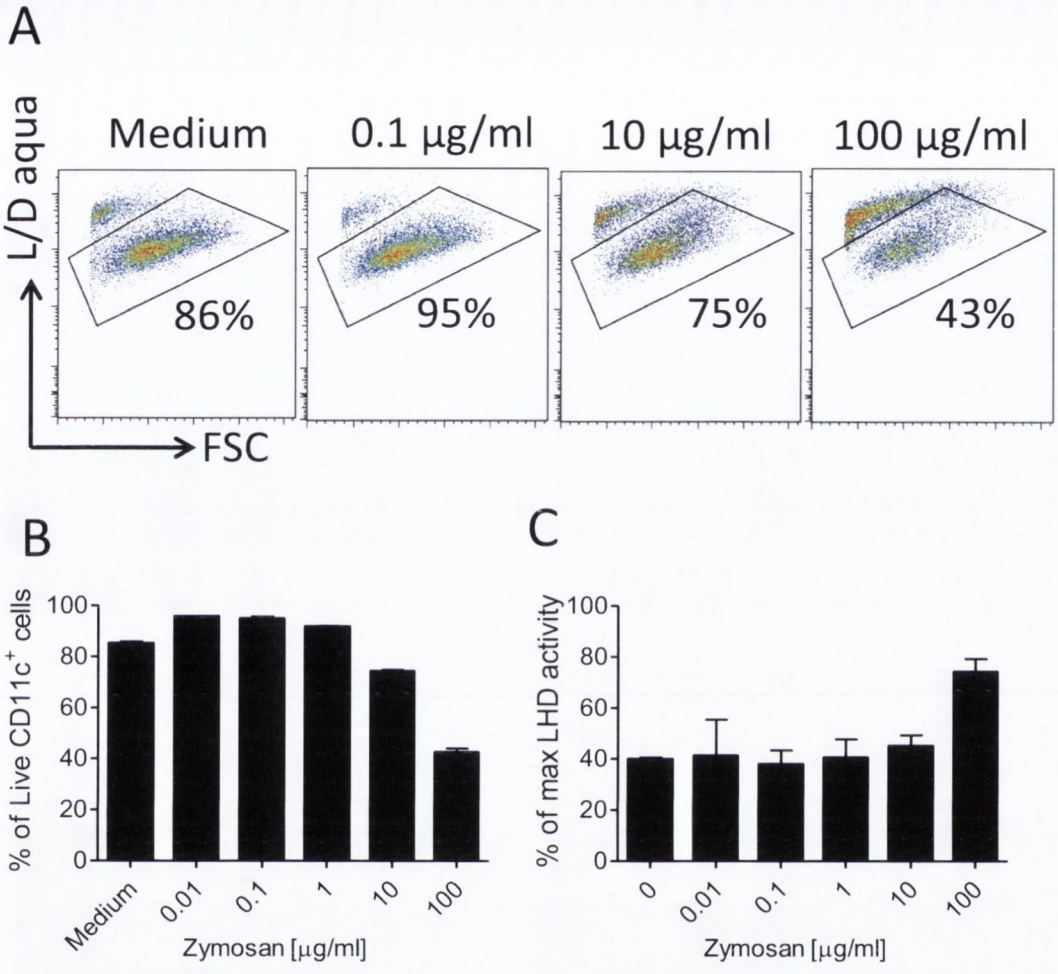


Fig. 3.5 Zymosan at high concentration induced the cell death in murine DCs. BMDCs from C57BL/6 mice were incubated with different concentrations of zymosan. After 24h cells were stained with LIVE/DEAD cells stain and surface-stained with antibody specific for CD11c. Results are representative (A) and the mean frequency of live $CD11c^+$ cells (B). In addition, the supernatants were recovered and the cell death was determined using non-radioactive cytotoxicity assay based on measurent of LDH activity in the culture media (C). Results shown are the mean values +/-SD from triplicate cultures and are representative of 2 independent experiments.

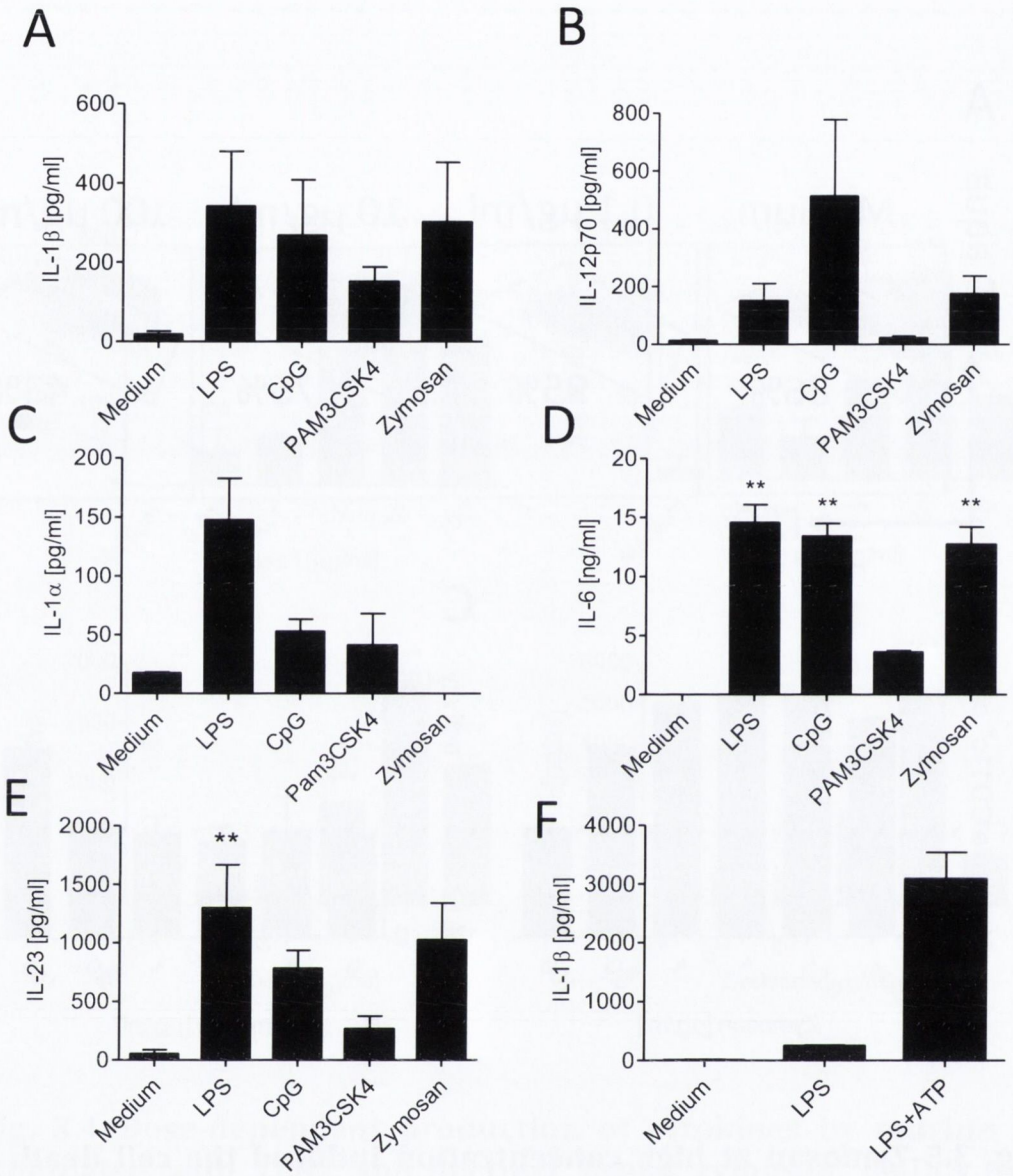


Fig. 3.6 Production of pro-inflammatory cytokines by murine DC stimulated with optimal concentrations of TLR ligands. A-E. BMDCs from C57BL/6 mice were cultured with TLR agonists: LPS (100ng/ml), CpG (1 μg/ ml), PAM3CSK4 (100 ng/ml), zymosan (10 μg/ml). After 24 h of incubation supernatants were recovered and concentration of IL-1β, IL-1α, IL-12p70, IL-23 and IL-6 was determined by ELISA. Results shown are the mean values +/-SEM (n=6 for IL-1β, IL-23, IL-12p70; n=2 for IL-6) **p<0.01 by one way ANOVA and Bonferroni post test. F. BMDCs from C57BL/6 mice were cultured with LPS (0.1μg/ml) for 24h before the addition of ATP (5 mM) for 1 h. Supernatants were recovered and concentration of IL-1β was determined by ELISA. Results shown are the mean values +/-SD from triplicate cultures from single experiment. **p<0.01 by ANOVA

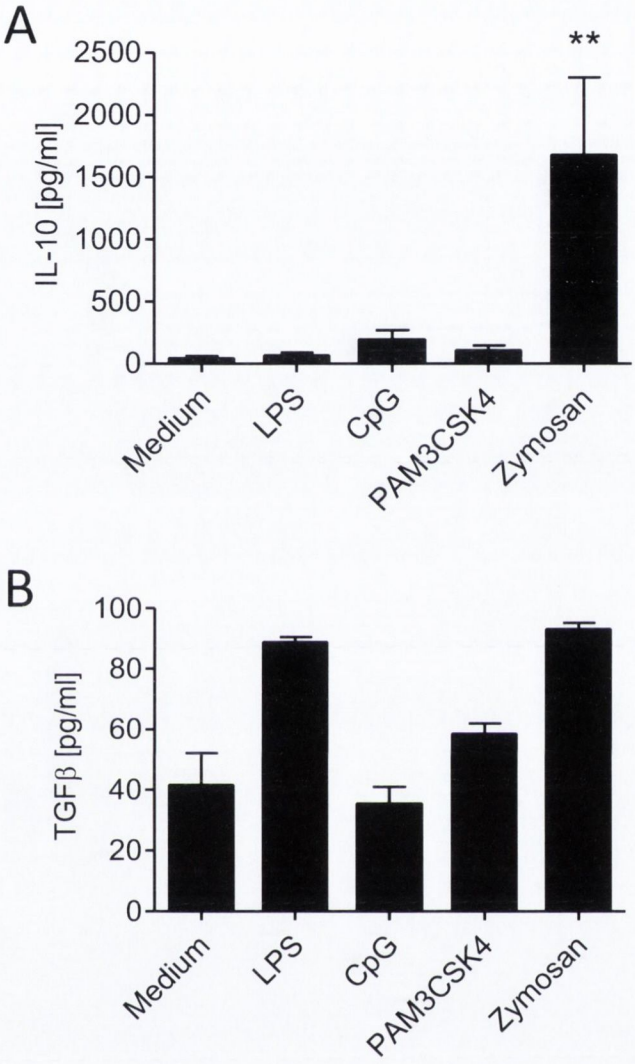


Fig. 3.7 TLR2 and dectin-1 ligand zymosan induced secretion of anti-inflammatory cytokine IL-10 by murine DCs. BMDCs from C57BL/6 mice were cultured with TLR agonists: LPS (100 ng/ml), CpG (1 μg/ ml), PAM3CSK4 (100 ng/ml), zymosan (10 μg/ml). After 24 h of incubation the supernatants were recovered and concentrations of IL-10 and TGF-β were determined by ELISA. Results shown are the mean values +/-SEM (n=6) (A) or mean values +/-SD from triplicate culture (n=1) (B) **$p<0.01$ ANOVA.

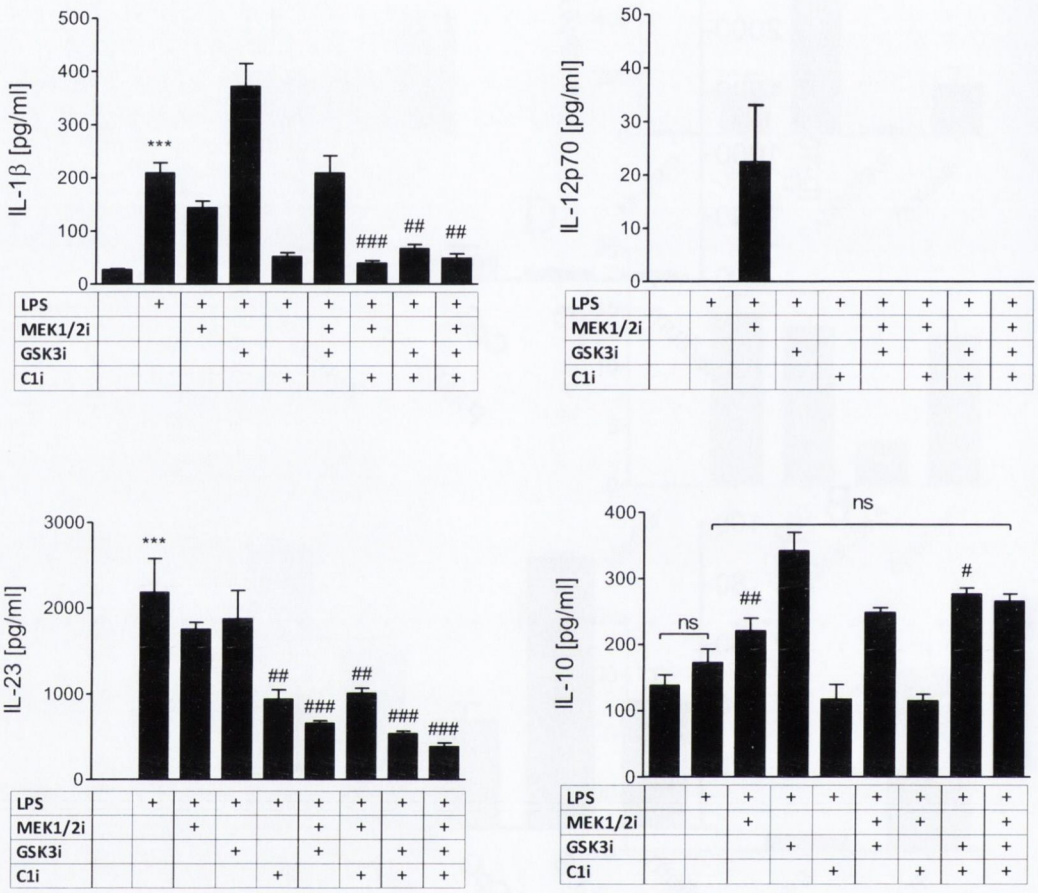


Fig. 3.8 Inhibitors of MAPK, caspase-1 and GSK3 signalling pathways modulate pro- and anti-inflammatory cytokine production by LPS-stimulated DC. BMDCs from C57BL/6 mice were pre-incubated with MEK1/2, GSK3 and caspase-1 inhibitors 30 min prior LPS stimulation. After 24 h of incubation supernatants were recovered and concentration of IL-1β, IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/- SD from triplicate culture and are representative of 2 independent experiments. ns, not significant, **p<0.01, ***p<0.001 vs medium, ##p<0,01, ###p<0.001 vs LPS-stimulated by ANOVA

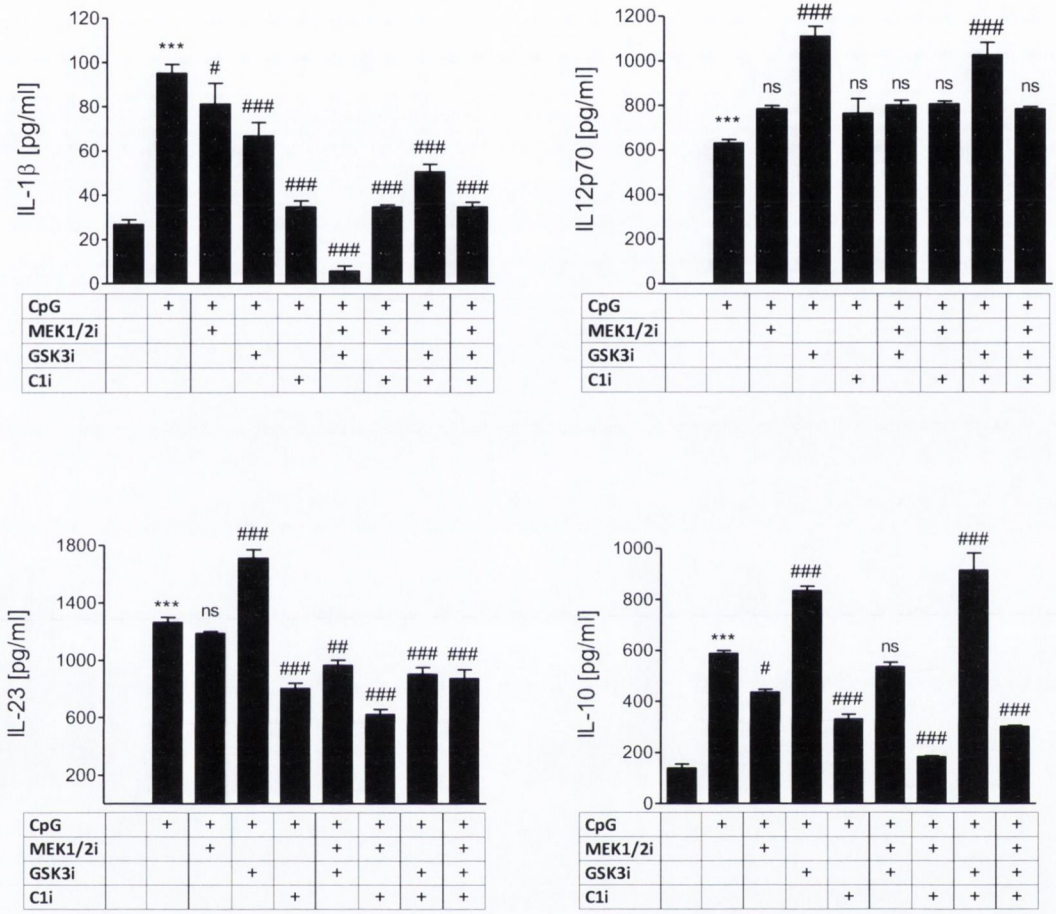


Fig. 3.9 Inhibitors of MAPK, caspase-1 and GSK3 signalling pathways modulate pro- and anti-inflammatory cytokine production by CpG-stimulated DC. BMDCs from C57BL/5 mice were pre-incubated with MEK1/2, GSK3 and caspase-1 inhibitors 30 min prior CpG stimulation. After 24 h of incubation supernatants were recovered and concentration of IL-1β ,IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate culture and are representative of 2 independent experiments. ns, not significant vs CpG-stimulated, ***p<0.001 vs medium, #p<0.05, ##p<0,01, ###p<0.001 vs CpG-stimulated, ns not significant vs CpG-stimulated, by ANOVA

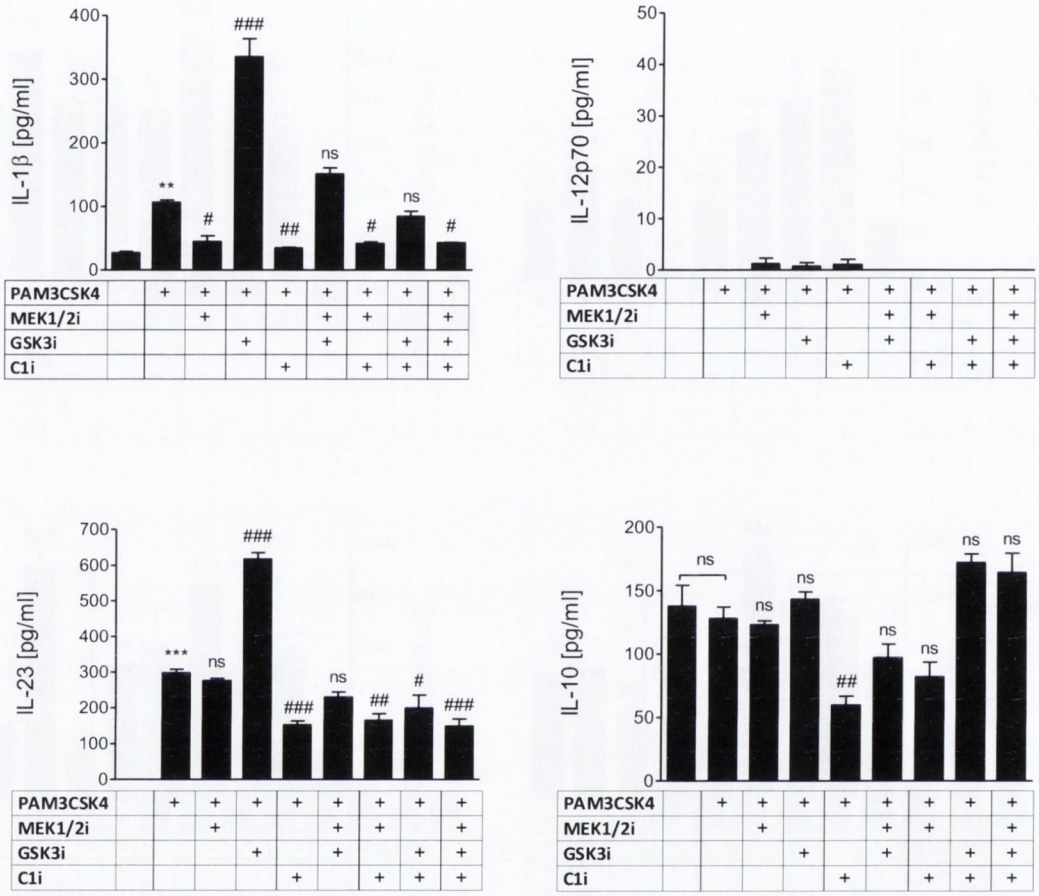


Fig. 3.10 Inhibitors of MAPK, caspase-1 and GSK3 signalling pathways modulate pro- and anti-inflammatory cytokine production PAM3CSK4-stimulated DC. BMDCs from C57BL/6 mice were pre-incubated with MEK1/2, GSK3 and caspase-1 inhibitors 30 min prior PAM3CSK4 stimulation. After 24 h of incubation supernatants were recovered and concentration of IL-1β, IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate culture and are representative of 2 independent experiments. **p<0.01, ***p<0.001 vs medium, #p<0.05, ##p<0,01, ###p<0.001 vs PAM3CSK4-stimulated, ns not significant vs PAM3SK4-stimulated by ANOVA

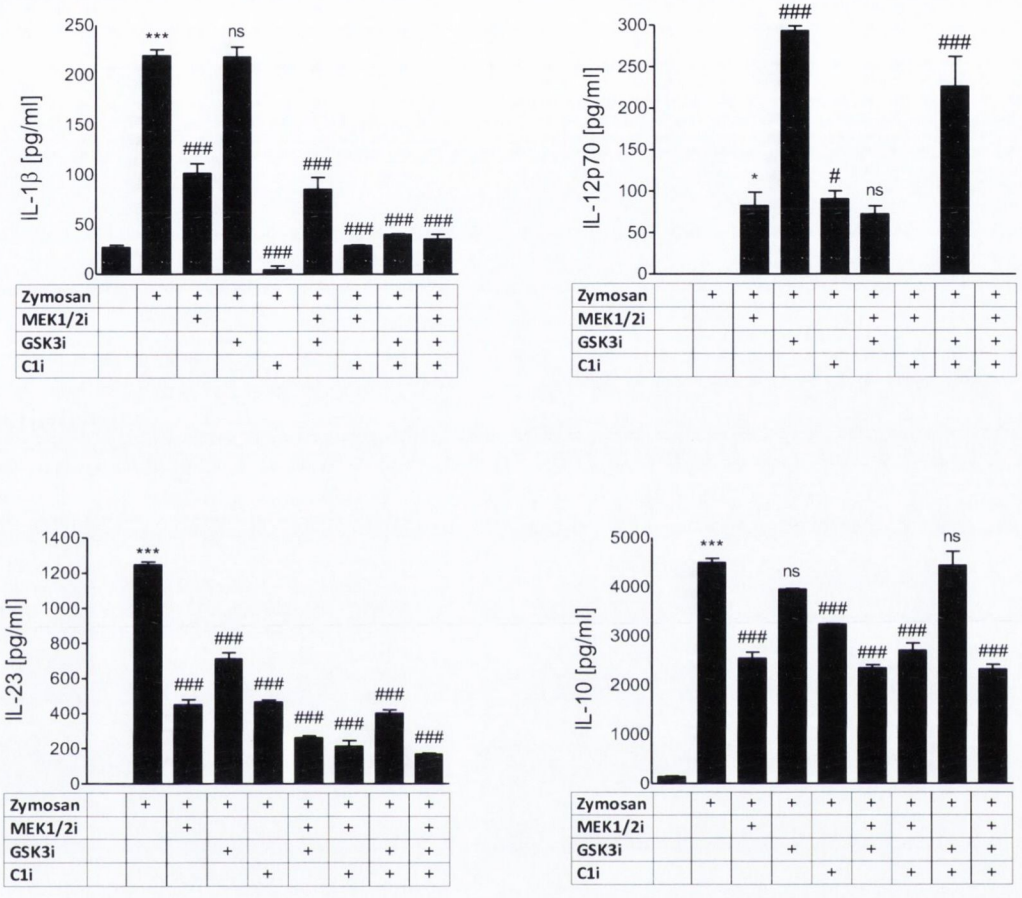


Fig. 3.11 Inhibitors of MAPK, caspase-1 and GSK3 signalling pathways modulate pro- and anti-inflammatory cytokine production by zymosan-stimulated DC. BMDCs from C57BL/6mice were pre-incubated with MEK1/2, GSK3 and caspase-1 inhibitors 30 min prior zymosan stimulation. Supernatants were recovered after 24 h, 48 h and 72 h of incubation and concentration of IL-1β , IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate culture and are representative of 2 independent experiments. *p<0.05, ***p<0.001 vs medium, #p<0.05,, ###p<0.001 vs zymosan-stimulated, ns not significant vs zymosan-stimulated by ANOVA

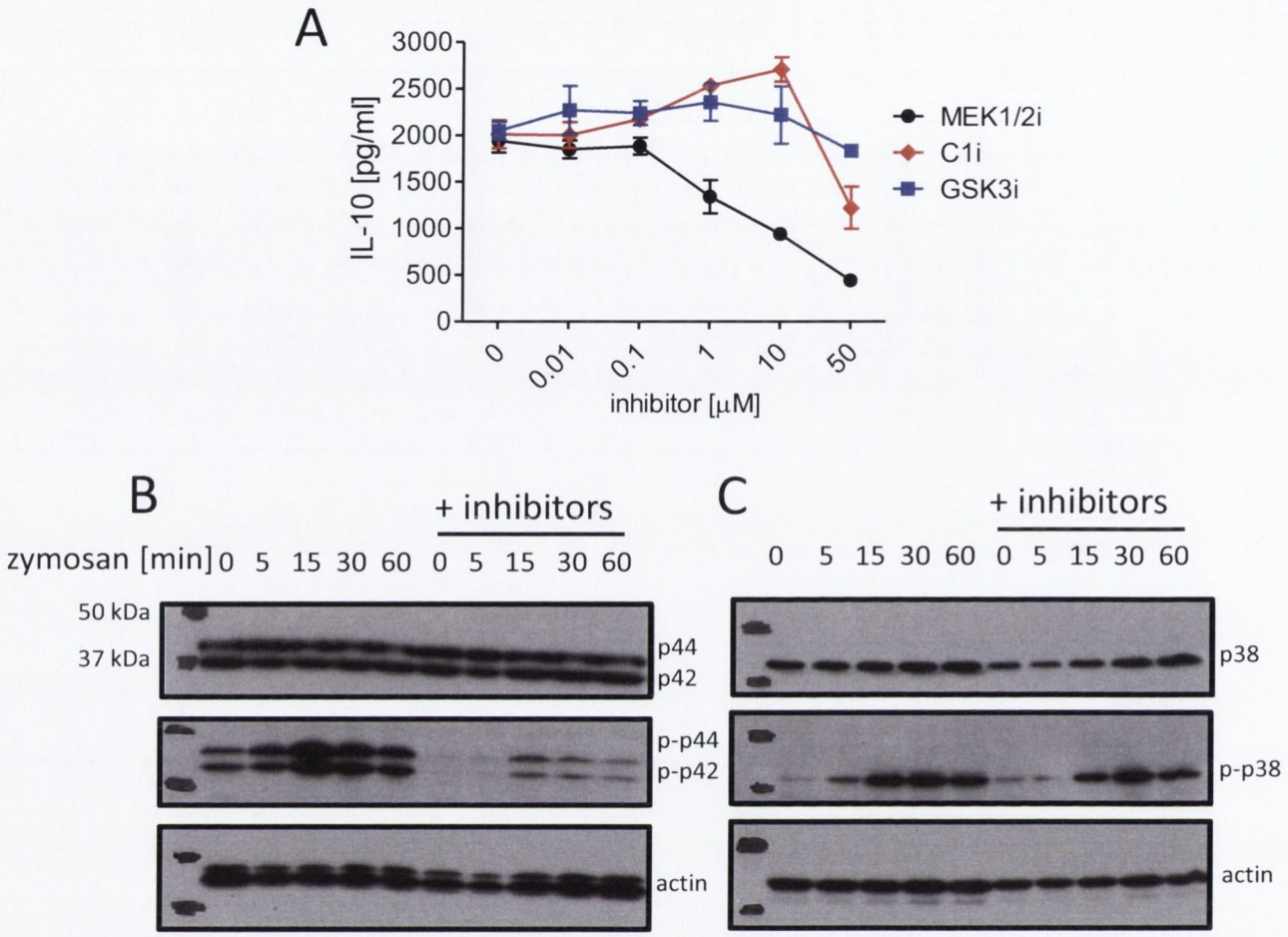


Fig. 3.12 Modulation of zymosan-induced IL-10 production by MEK1/2, caspase-1 and GSK3 inhibitors does not involve p38 signalling pathway. A. BMDCs from C57BL/6 mice were pre-incubated with different concentrations of MEK1/2, GSK3 and caspase-1 inhibitors 30 min prior zymosan stimulation. After 24h of culture supernatants were recovered and concentration of IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate culture and are representative of 2 independent experiments. B. BMDCs from C57/BL6 mice were preatreate with MEK1/2 (5 μM), caspase-1 (40 μM) and GSK3 (20 μM) inhibitors 30 min prior zymosan stimulation. Cells were lysed at the time of adding zymosan and after 5, 15, 30 and 60 min. Cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38 and anti-actin. Results show one experiment.

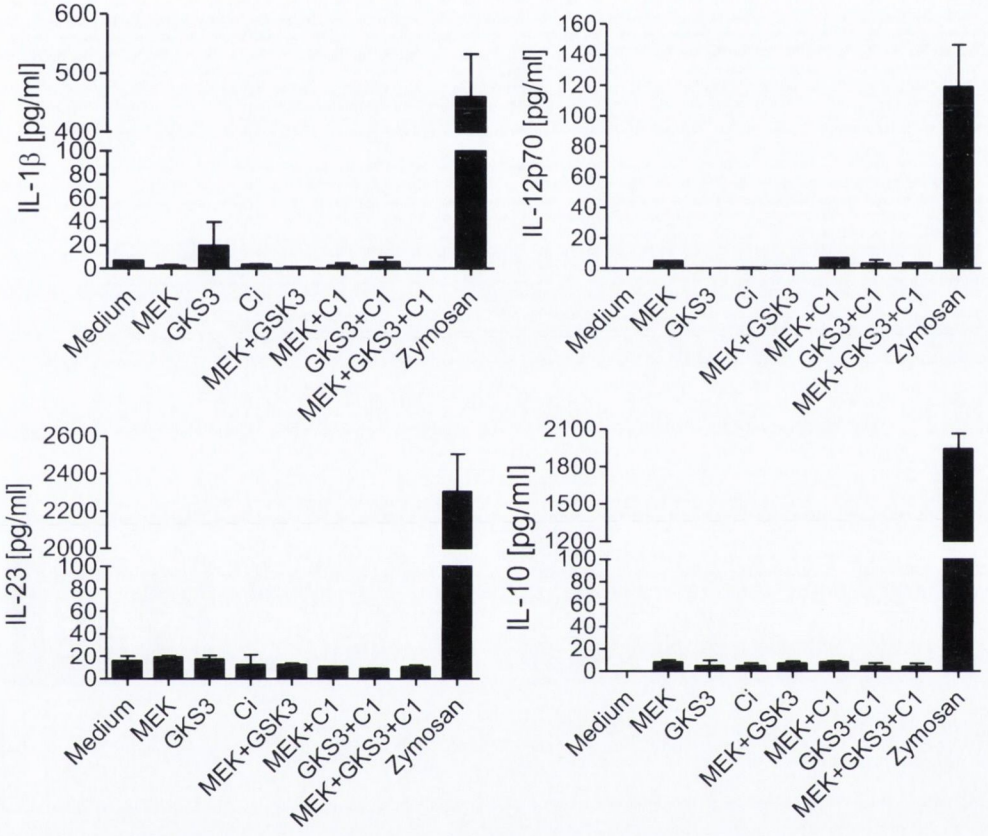


Fig. 3.13 Inhibitors of MEK1/2, GSK3 and C1 do not induce sercetion of cytokines IL-1β, IL-12p70, IL-23 and IL-10 in murine DCs without stimulation with TLR ligand. BMDCs from C57BL/6 mice were incubated with MEK1/2, GSK3 and caspase-1 inhibitors or LPS only. After 24 h of incubation supernatants were recovered and concentration of IL-1β , IL-12p70, IL-23 and IL-10 was determined by ELISA. Results shown are the mean values +/-SD from triplicate culture and are respresentative of 2 independent experiments.

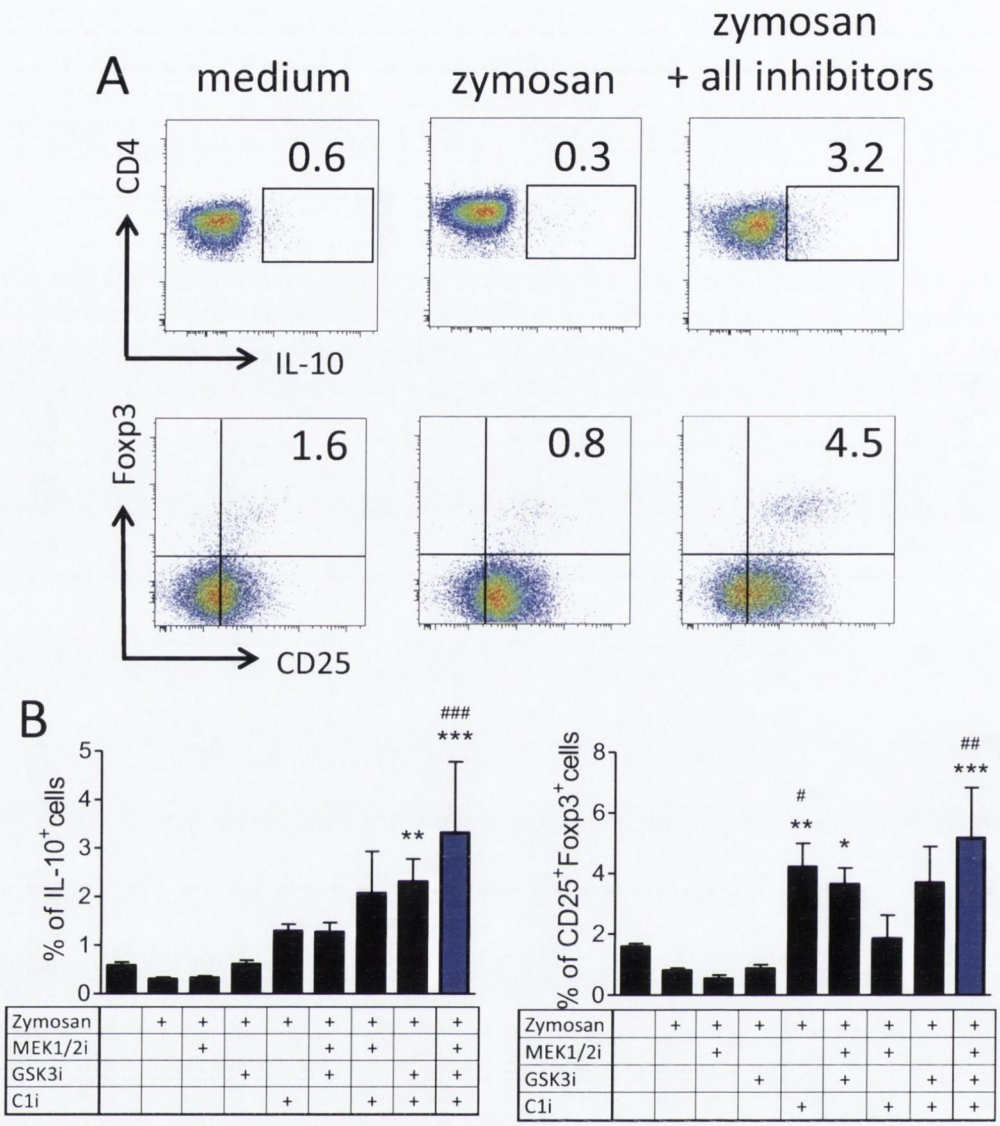


Fig. 3.14 Co-culture with DC stimulated with zymosan and inhibitors of MEK1/2, GSK3 and caspase-1 enhanced production of IL-10 by OVA-specific CD4⁺ T cells and increased frequency of Foxp3⁺. BMDCs from BALB/C mice were incubated with OVA peptide for 3 h. MEK1/2, GSK3 and caspase-1 inhibitors were added to the culture 30 min prior zymosan stimulation. After 24 h the supernatants were removed and BMDCs were washed twice with cRPMI. CD4⁺ T cells were isolated from single cell suspensions of spleens and lymph nodes of OVA-TCR transgenic DO.11 mice and co-cultured with DCs in ratio 1:10 (DC:T cells) After 72 h cells were stimulated with PMA, ionomycin and BFA and stained for CD4, CD25, Foxp3 and intracellular IL-10. Results shown are representative flow cytometry plots (A) and mean frequency of cells +/- SD from triplicate cultures (B). *p<0.05, **p<0.01, ***p<0.001 vs unstimulated cells; #p<0.05, ##p<0.01, ###p<0.001 vs zymosan-stimulated cells by ANOVA

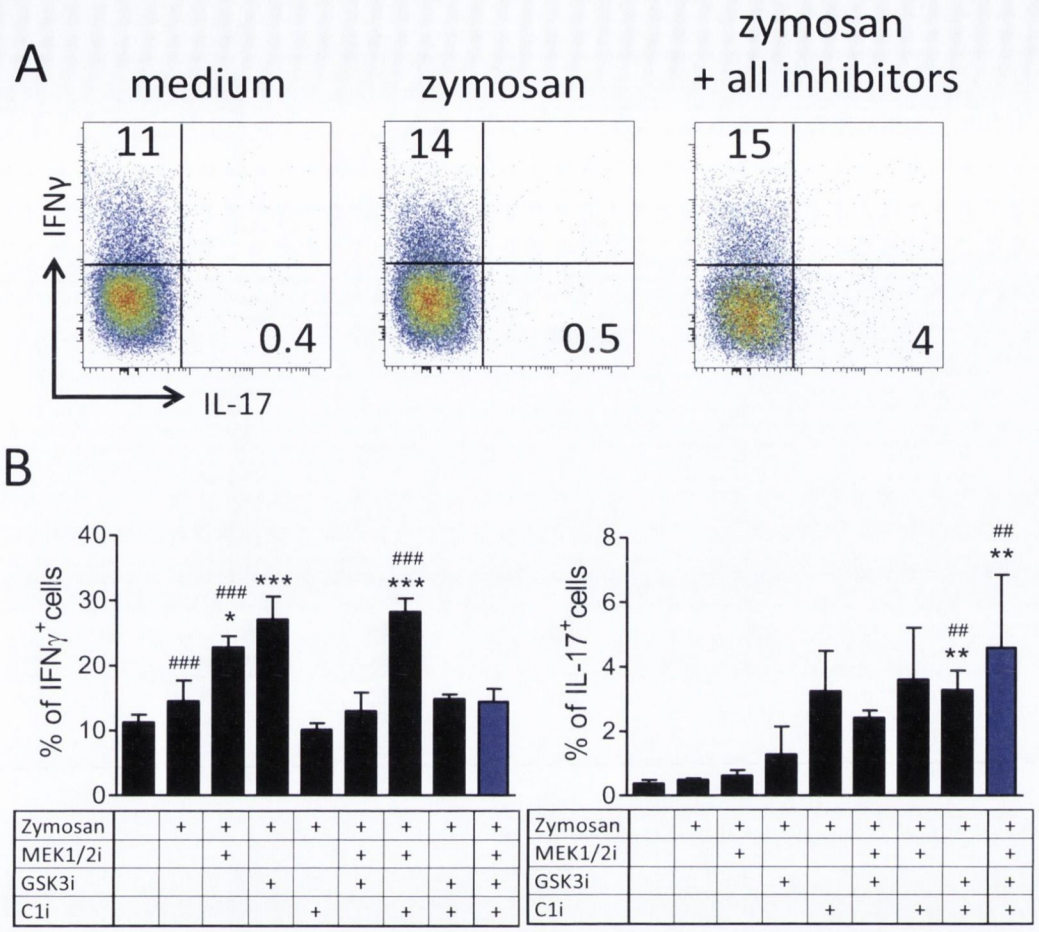


Fig. 3.15 Co-culture with DC stimulated with zymosan and inhibitors of MEK1/2, GSK3 and caspase-1 did not enhance production of IFNγ, but enhanced production of IL-17 by OVA-specific CD4⁺ cells. BMDCs from BALB/C mice were incubated with OVA peptide for 3 h. MEK1/2, GSK3 and caspase-1 inhibitors were added to the culture 30 min prior zymosan stimulation. After 24 h the supernatants were removed and BMDCs were washed twice with cRPMI. CD4⁺ T cells were isolated from single cell suspensions of spleens and lymph nodes of OVA-TCR transgenic DO.11 mice and co-cultured with DCs in ratio 1:10 (DC:T cells) After 72 h cells were stimulated with PMA, ionomycin and BFA and stained for CD4 and intracellular IFNγ and IL-17. Results shown are representative flow cytometry plots (A) and the mean frequency of cells +/- SD from triplicate cultures (B). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs unstimulated cells; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ vs zymosan-stimulated cells by ANOVA

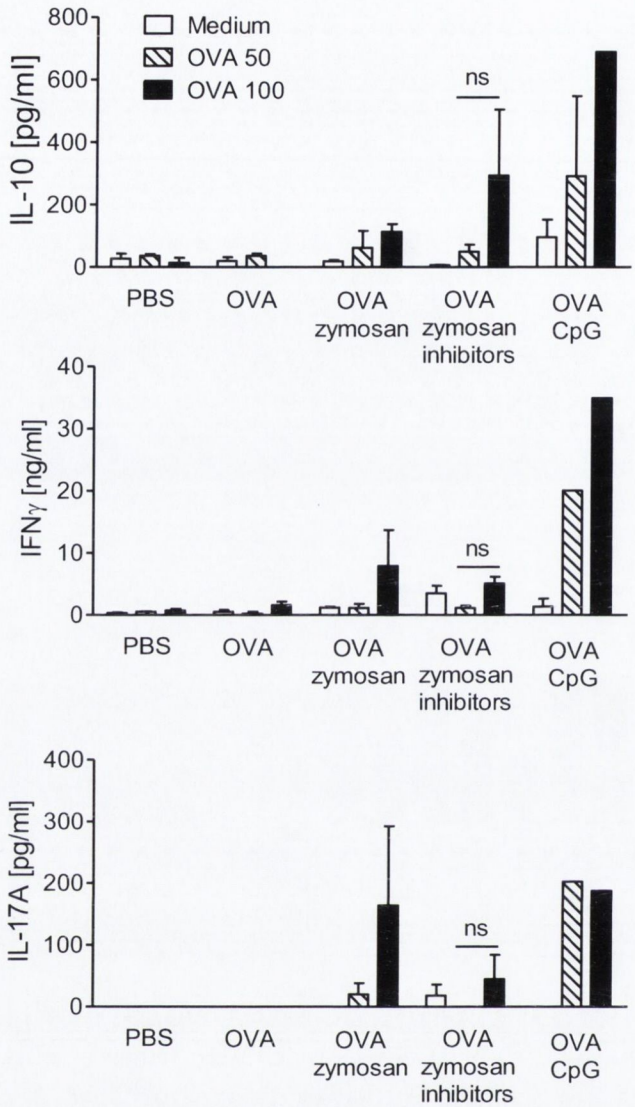


Fig. 3.16 Effect of treatement with MEK1/2, GSK3 and caspase-1 inhibitors on the production of zymosan-induced antigen-specific IL-17, IFN-γ and IL-10 by lymph nodes cells. $CD4^+$ T cells purified from spleen and lymph nodes of OVA-TCR transgenic (DO.11.10) mice were adoptively transfered (i.v.) to BALB/C mice. BALB/C mice were injected into the foot pad with PBS, OVA protein, OVA+zymosan, OVA+zymosan or OVA+zymosan+MEK1/2, GSK3 and caspase-1 inhibitors. Additional group of mice injected with OVA+CpG was included for an adjuvant positive control. After 7 days, popliteal lymph nodes were isolated and the single cell suspentions were cultured *ex vivo* with OVA (50-100 μg/ml). The supernatans were collected 5 days later and concentration of IL-10, IFNγ and IL-17 were determined by ELISA. Results shown are mean values +/- SEM from 2 independent experiments; n=4 for all groups besides OVA+CpG, for which n=2. ns not singificant vs OVA; # ns, not significant vs OVA+Zymosan by ANOVA.

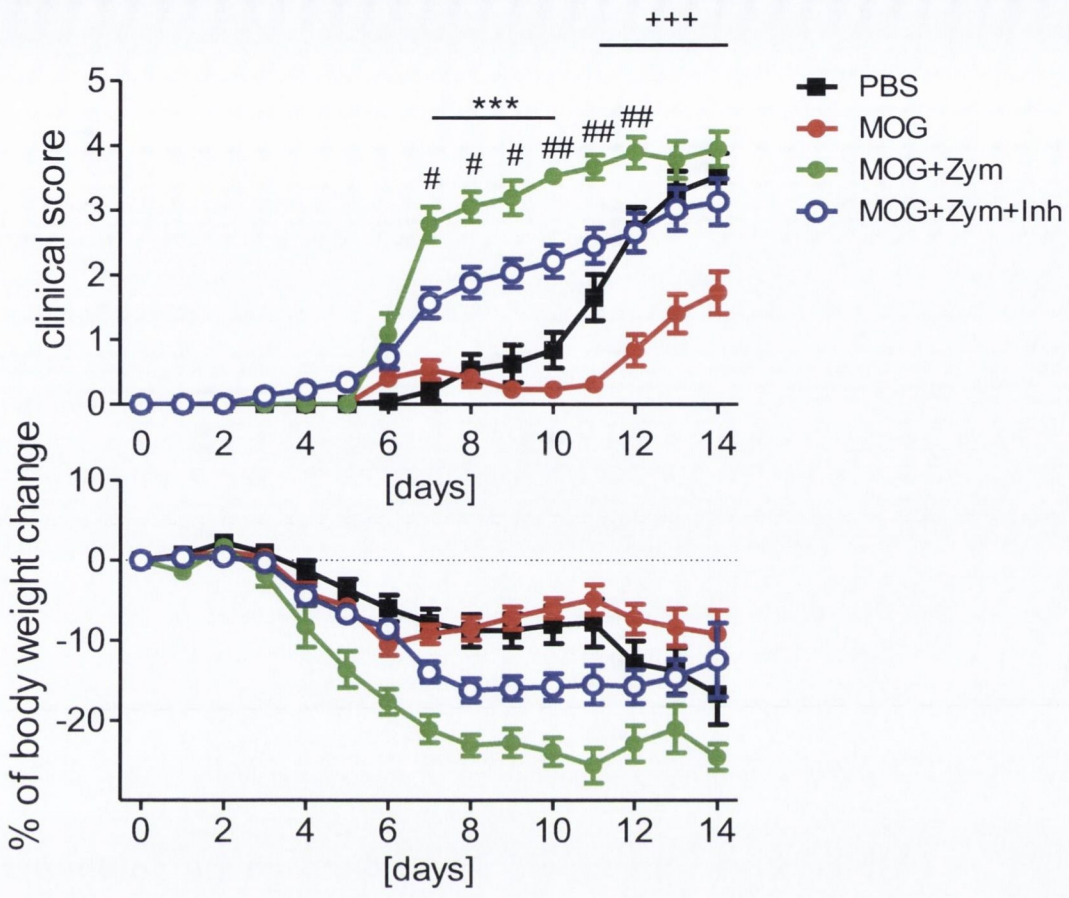


Fig. 3.17 Effect of treatment with MOG, zymosan and MEK1/2, GSK3 and caspase-1 inhibitors before induction of EAE. C57BL/6 mice were s.c. immunized with MOG_{35-55} peptide, zymosan and MEK1/2, GSK3 and caspase-1 inhibitors 21 and 7 days before inductio of EAE. EAE was induced by s.c. injection of MOG peptide emulsified with CFA, followed by an i.p. injection with 500 ng PT on day 0 (day of induction) and day 2. Animals were monitored daily for clinical signs of the disease. Results shown are mean scores or percentage of body weight change +/- SEM from 4 independent experiments; n=4-6/group,***$p<0.001$ MOG+zymosan vs. PBS, #$p<0,05$, ##$p<0.01$ MOG+zymosan+inhibitors vs. MOG+zymosan, +++$p<0.001$ MOG vs. PBS, by two-way ANOVA and Bonferroni post test.

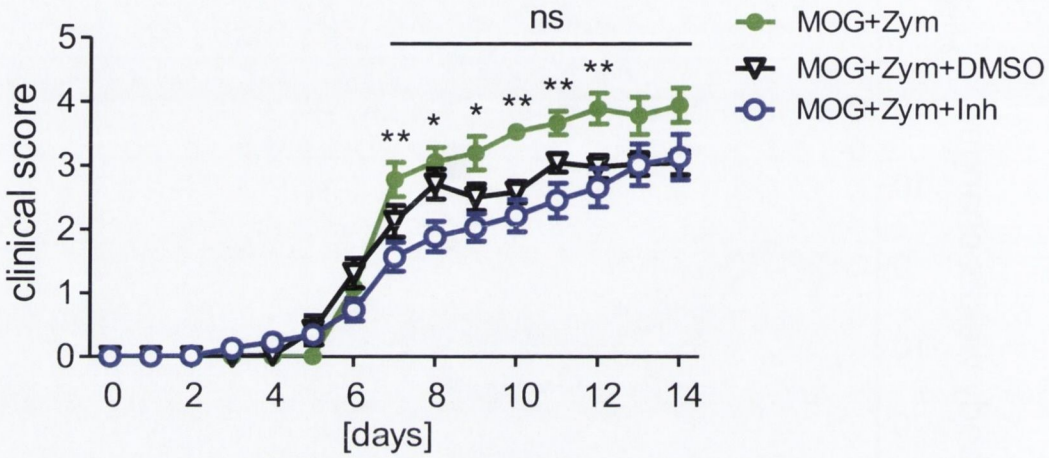


Fig. 3.18 Effect of treatment with MOG, zymosan and inhibitors vs DMSO vehicle before induction of EAE. C57BL/6 mice were s.c. immunized with MOG_{35-55} peptide, zymosan and MEK1/2, GSK3 and caspase-1 inhibitors or zymosan and DMSO 21 and 7 days before induction of EAE. EAE was induced by s.c. injection of MOG peptide emulsified with CFA, followed by an i.p. injection with 500 ng PT on day 0 (day of induction) and day 2. Animals were monitored daily for clinical signs of the disease. Results shown are mean scores or percentage of body weight change +/- SEM from 2 independent experiments; n=4-6/group *p<0.05 ,**P<0.01 MOG+zymosan vs. MOG+zymosan+ inhibitors group by two-way ANOVA. ns, not significant MOG+zymosan+DMSO vs. MOG+zymosan+inhibitors

Chapter 4

Induction of antigen-specific Treg cells using anti-inflammatory cytokines

CHAPTER 4 INDUCTION OF ANTIGEN-SPECIFIC TREG CELLS USING ANTI-INFLAMMATORY CYTOKINES

4.1 Introduction

Under homeostatic conditions, anti-inflammatory cytokines serve as immunomodulators limiting the potentially detrimental effects of excessive inflammatory responses. The robust production of anti-inflammatory cytokines at the mucosal surfaces protects against unnecessary responses to harmless commensal microorganism, whereas the induction of anti-inflammatory cytokines during infections or injury counter-balances the effects of pro-inflammatory cytokines and facilitates the resolution of inflammation. The most potent immunoregulatory cytokines with suppressive properties are TGF-β and IL-10 and their physiological importance has been confirmed in TGF-β1- and IL-10-deficient mice. TGF-$\beta^{-/-}$ mice develop lethal multiorgan inflammatory disease as soon as 2 weeks after birth (275), whereas IL-$10^{-/-}$ or IL-$10R^{-/-}$ mice acquire chronic enterocolitis in response to normal intestinal flora (276). Similarly, in humans, defective IL-10R signalling is associated with development of life-threatening early-onset inflammatory bowel disease (EO-IBD) in children (277).

IL-10 modulates both innate and adaptive immune responses and can be produced by variety of cells. For example, DC and macrophages produce IL-10 in response to the microbial stimuli, but also express IL-10R and respond to IL-10 signalling, changing to a more tolerogenic phenotype. Treg cells are the main source of IL-10, but it is now accepted that all T cell subpopulations are capable of expressing IL-10 along with other cytokines, dependently on the stimuli involved in their differentiation. In infection, T cell-derived IL-10 plays a role in controlling the inflammation, but some pathogens, for example *Leishmania* parasites or the bacteria *Bordetella pertussis* have evolved mechanisms for induction of IL-10 to evade protective immunity and to establish persistent infections (278). IL-10-producing Tr1 cells exhibit strong immunosuppressive properties and are able to restore tolerance in several immune-mediated diseases through bystander suppression. For example, it has been demonstrated that transfer of *in vitro* generated OVA-specific Tr1 cells prevented the development of neurological symptoms of EAE in mice, when OVA peptide was injected intracranially (120). Tr1 cells confer protection against airway inflammation and allergen-specific Tr1 cells have been

demonstrated to exhibit suppressive functions in an antigen-specific manner in models of specific immunotherapy in patients with allergies (279, 280).

The development of Tr1 cells is directly induced by APC-derived IL-10 and the results presented in chapter 3 demonstrated that it is possible to induce antigen-specific IL-10-producing T cells *in vivo*, using microbe-derived molecules that enhance IL-10 production by DC. In addition, IL-27, a member of the IL-12 cytokine family, previously exclusively associated with development of Th1 cells, was recently shown to induce IL-10 production from $Foxp3^{-}CD4^{+}$ T cells (52).

Recombinant cytokines have been tested in humans as therapies for different immunological disorders. For example, treatment with IFN-β (Avonex, Betaseron, Rebif, Extavia) has been approved as a therapy for relapsing-remitting MS. Those therapies have been developed to restore the activity of specific cytokines and systemically mobilize or inhibit immune responses. In contrast, animal studies have shown that particular cytokines, such as IL-12 or IFN-γ can work as natural adjuvants, to enhance the antigen-specific immune response when administered together with a soluble antigen (281, 282).

The aim of this part of the study was to examine the hypothesis that the prophylactic administration of anti-inflammatory cytokines together with self-antigen MOG can lead to the generation of antigen-specific Treg cells that can suppress the development of autoimmune disease. The specific aim was to evaluate the capacity of IL-10 or IL-27 or the combination of these two cytokines to induce IL-10-producing T cells and to establish the effect of administration of IL-10, IL-27 or both together with MOG peptide before the induction of EAE on the development of clinical symptoms of disease.

After the successful result in EAE model, the aim was to determine if repeated immunization with self-peptide and anti-inflammatory cytokine can induce the development of antigen-specific IL-10-producing T cells.

4.2 Results

4.2.1 Effect of stimulation with IL-10 or IL-27 on expression of cytokines and Foxp3 by $CD4^+$ T cells

In mice and humans, the differentiation of Tr1 cells is facilitated by the presence of exogenous IL-10. Recently, several published studies have demonstrated that IL-27 can also directly enhance production of IL-10 by $CD4^+$ T cells. An experiment was conducted to assess the abilities of IL-10 and IL-27 to directly stimulate IL-10 production by $CD4^+$ T cells and to determine the effect of those cytokines on the expression of Foxp3, IFN-γ and IL-17 by T cells. $CD4^+$ T cells were purified from spleens of C57BL/6 mice by magnetic cell sorting. $CD4^+$ T cells were polyclonaly activated by plate-bound anti-CD3 and soluble anti-CD28 and cultured in the presence of IL-10 (10 ng/ml), IL-27 (10 ng/ml) or the combination of IL-10 and IL-27. In addition, cells were stimulated with TGF-β for a positive control of Foxp3 expression. As expected, IL-10 and IL-27 did not induce expression of Foxp3 by $CD4^+$ T cells. In comparison, stimulation with TGF-β enhanced expression of Foxp3 to 80% by $CD4^+$ T cells. IL-27 alone did not induce IL-10 expression. IL-10 enhanced the frequency of IL-10-producing $CD4^+$ T cells and addition of IL-27 further enhanced production of IL-10. IL-10-producing $CD4^+$ T cells were Foxp3-negative. IL-27, both alone and in combination with IL-10 also enhanced the frequency of IFN-γ-producing cells. IL-17 was not induced by IL-10 nor by IL-27 (Fig.4.1). These results demonstrate that IL-27 is a strong inducer of IL-10 and IFN-γ in T cells in both polarizing and non-polarizing conditions. In this study IL-27 alone increased IFN-γ production and enhanced IL-10-induced production of IL-10 by $CD4^+$ T cells.

4.2.2 Effect of administration of MOG with IL-10 or IL-27 or both cytokines prior to the induction of EAE

Having established that IL-10 and IL-27 directly modulate IL-10 production by $CD4^+$ T cells, experiments were conducted to investigate if immunization with MOG in the presence of IL-10, IL-27 or both cytokines could prevent or attenuate the development of EAE in an antigen-dependent manner. Mice were injected s.c. with MOG (100 μg/mouse), MOG and IL-10 (100 ng/mouse), MOG and IL-27 (100 ng/ml), MOG with

IL-10 and IL-27 or with PBS only 21 days and 7 days before induction of EAE. EAE was induced by s.c. injection of MOG emulsified with CFA, followed by i.p. injection of 125 ng PT at days 0 and 2. The clinical scores and weights were recorded daily to measure disease severity. The data are presented in Fig 4.2 and due to the fact that the development of EAE was inconsistent within the groups, the detailed characteristics, showing disease incidence, cumulative score and average cumulative score are also included as a table in Fig.4.2 B. Mice injected with MOG and IL-27 developed EAE with similar disease incidence and average cumulative score as PBS-injected mice (Fig.4.2). Interestingly, administration of MOG and IL-27 together with IL-10 resulted in the most severe EAE with quicker onset, the highest disease incidence among the groups and nearly twice as high average cumulative score as seen for control mice. In contrast, none of the mice treated with MOG and IL-10 developed clinical symptoms of EAE until the day 16 after induction of EAE (cessation of the experiment). Similarly, the mice treated with MOG and IL-10 recovered from the initial weight loss after induction of EAE (Fig.4.2).

4.2.3 Administration of MOG and IL-10 before induction of EAE induces protection against the development of EAE

The results have indicated that prophylactic administration of IL-10 and MOG results in the protection against the development of EAE, which is not achieved by treatment with MOG only. It has been debated that therapeutic administration of IL-10 can be beneficial in autoimmune or inflammatory disorders. For example, it has been demonstrated that administration of IL-10 can prevent the development of chronic experimental colitis, a mouse model of intestinal inflammation (283). Therefore, an experiment was performed to determine if the protection against development of EAE depends on co-administration of IL-10 with MOG or if it can be achieved by pre-treatment with IL-10 only. Mice were injected with PBS, MOG, MOG with IL-10 or IL-10 alone 21 days and 7 days before induction of EAE. IL-10-treated and MOG-treated mice had delayed onset of the disease in comparison with the control mice, however by day 14 after induction of EAE the disease scores were similar in all three groups. In contrast, mice injected with IL-10 and MOG had delayed onset of the disease and significantly ($p<0.001$) lower scores than IL-10, MOG or PBS-treated mice. The results demonstrated that the protection against

development of EAE is not mediated by non-specific activity of IL-10 and can be achieved only when mice are immunized with both auto-antigen MOG and IL-10.

4.2.4 Administration of MOG and IL-10 induces MOG-specific regulatory T cells that transfer protection against actively induced EAE

The results from Chapter 3 demonstrated that repeated treatment of mice with self-antigen leads to antigen-specific T cell tolerance, or T cell unresponsiveness and results in attenuation of EAE. Consistent with those findings, the results of the present study showed that MOG-treated mice had delayed onset of EAE and slightly lower scores then the PBS-treated mice. Classically, the anergy is induced when T cells encounter a cognate antigen presented by APC with absence of co-stimulation. Alternatively, T cell anergy can be induced when $CD4^+$ T cells are activated in the presence of IL-10 or by APCs previously treated with IL-10 (284). IL-10 can also exert a direct effect on the differentiation of Tr1 cells, which can inhibit antigen-specific effector T cell responses. Tr1 cells are characterized by ability to produce antigen-specific IL-10 and IL-5 but not IL-4, which is associated with type 2 response (117). Further experiments were designed in order to examine whether repeated injection of the self-antigen MOG and IL-10 induces MOG-specific Tr1 cells or clonal anergy. Mice were injected s.c. with PBS, MOG or MOG with IL-10 and boosted 14 days later. Seven days after the last injection, spleens and inguinal lymph nodes were isolated and the spleen cells were cultured with a range of concentrations of MOG peptide. Additionally, cells were cultured with PMA and anti-CD3 for a positive control of T cell responses. After 5 days of culture, the concentrations of IL-10, IL-5 and IL-4 were quantified by ELISA.

The production of antigen-specific IL-10 was significantly enhanced in spleen and lymph node cells from mice immunized with MOG and IL-10. In contrast, a negligible amount (less than 10 pg/ml) of IL-10 was detected after MOG stimulation of lymph node cells from MOG-immunized mice and the IL-10 produced by spleen cells was not antigen-specific. The lymph node cells, but not spleen cells, from mice injected with MOG and IL-10 also produced low amounts of IL-5 in response to MOG stimulation *in vitro*. In contrast, IL-5 production was not detected in supernatants of MOG-stimulated lymph node or spleen cells from MOG-injected mice (Fig. 4.4 A, B). These results demonstrate

that immunization with self-antigen MOG and IL-10 induces MOG-specific T cell responses characteristic of Tr1 cells.

Next, the experiment was performed to further investigate whether the immune suppression, associated with Tr1 induction, that develops in the periphery following immunization with MOG with IL-10 can be adoptively transferred to naïve host. Groups of mice were immunized at day 0 and 14 with MOG and IL-10, MOG only or PBS. Spleens were isolated 7 days after the last injection and spleen cells were cultured with MOG peptide and IL-2 for 3 days. 20 x 10^6 of cells were transferred i.p. into recipient mice that were immunized for EAE 24 h later. The results in Fig. 4.5 show that spleen cells from mice immunized with MOG+IL-10, but not from MOG-immunized mice transferred a level of protection ($p<0.01$) against actively induced EAE. Interestingly, recipient mice that received cells from MOG-injected mice exhibited earlier disease onset and slightly more severe EAE than the control mice. However, mice that received naïve spleen cells developed first signs of the disease later (day 13 post EAE induction) than mice that did not receive cells (day 8 post EAE induction, Fig 4.3). This indicates that naive spleen cells are more capable of delaying the onset of EAE than the cells from MOG-treated mice. To summarize, these results support the hypothesis that self-antigen-specific regulatory T cells induced by MOG and IL-10 can mediate protection against autoimmune disease.

4.2.5 Administration of MOG and IL-10 before induction of EAE delays the infiltration of leukocytes into the CNS during EAE

This study has shown that immunization with MOG and IL-10 before induction of EAE induces protection against development of clinical signs of EAE. The pathogenesis of EAE is associated with infiltration of several leukocyte subsets into the CNS. It has been established that γδ T cells, activated in the periphery, migrate across the brain-blood barrier early after induction of EAE, followed by migration of auto-antigen-specific Th1 and Th17 into the CNS (77, 54). The encephalitogenic Th1 and Th17 cells have been shown to play a major role in the development of autoimmune inflammation and neuronal damage. In order to examine if immunization with MOG and IL-10 prevented lymphocyte infiltration and cytokines production in the brains of mice the mice were injected with PBS, MOG or MOG with IL-10 at day 0 and 14 and immunized for EAE 7

days later. The clinical scores were recorded until day 16 (Fig.4.6 A, B), when the mice were sacrificed and perfused intracardially with PBS, to remove the blood from brain vessels. Infiltrating mononuclear cells were recovered using a density gradient separation and counted. At the end of the experiment 50% of mice previously immunized with MOG and IL-10 had no clinical signs of the disease (average score 0), whereas 50% developed moderate paralysis of hind legs (average score 2.75). Overall, this group of mice was protected against the development of EAE for 9 days (onset at day 17) after the first symptoms of disease occurred in the control group of mice (day 7) (Fig.4.6 A). At day 16 post EAE induction, similar numbers of cells were present in the brains of PBS and MOG-treated mice. In contrast, the number of cells in the CNS of MOG+IL-10-treated mice with no signs of paralysis was 3.7 times lower than in the PBS control mice (0.53 x 10^6 cells in the MOG+IL-10 vs 1.77 x 10^6 cells in PBS). However, the development of disease in MOG+IL-10 group was also associated with enhanced infiltration of cells to approximately 1.4 x 10^6 (Fig.4.6 C). These results demonstrate that the prophylactic immunization with MOG+IL-10 provides the protection against the development of EAE, which is associated with delayed trafficking of leukocytes from the peripheral tissues into the CNS.

To further examine the lymphocyte populations infiltrating the brains during EAE, the recovered mononuclear cells were stimulated with PMA and ionomycin in the presence of BFA for 5 h and stained with antibodies against surface markers, intracellular cytokines and Foxp3. There were no apparent differences in frequencies of $CD3^+$ T cells in the brains in all groups of mice, but the major change was observed in the frequencies of $CD4^+$ T cells (Fig.4.7). All mice that developed EAE had an enhanced frequency of $CD4^+$ T cells compared with MOG+IL-10-immunized mice with clinical score 0 (Fig. 4.7). Mice that developed paralysis had enhanced frequencies of IFN-γ-producing $CD4^+$ T cells in the brains compared with healthy mice from MOG+IL-10 group (Fig. 4.8). This was reflected by even more substantial differences in the absolute numbers of all $CD4^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells in the brains of sick and healthy mice. In addition, the frequency of γδ T cells and the number of IL-17-producing γδ T cells was reduced in the CNS of mice that did not develop EAE following immunization with MOG and IL-10 (Fig. 4.9).

This study showed that the immunization with MOG+IL-10 induces development of MOG-specific cells, which remain in the draining lymph nodes and spleen for at least 7 days after the immunization and produce IL-10 upon re-stimulation with the antigen. To

examine if the MOG-specific regulatory T cells infiltrate the brain of mice protected against EAE, the mononuclear cells recovered from the brains were stained for T cell surface markers and for intracellular IL-10 and nuclear Foxp3. The results in Fig. 4.10 show that the frequency of IL-10-producing $CD4^+$ T cells or $CD25^+Foxp3^+$T cells was not increased in the MOG+IL-10-immunized mice. Moreover, the highest frequency of $Foxp3^+$-expressing T cells was found in the brains of control mice, which had the earliest onset of the disease among the groups (Fig. 4.10).

These results demonstrate that the protection against the development of EAE induced by immunization with MOG and IL-10 is associated with long-lasting inhibition of migration of pathogenic γδ T cells and IFN-γ and IL-17-producing T cell into the CNS.

4.2.6 Effect of administration of MOG and IL-10 before induction of EAE on the production of MOG-specific IFN-γ, IL-17 and IL-10 by spleen and lymph node cells during EAE

Induction of MOG-specific autoimmune inflammation in EAE is facilitated by subcutaneous injection of MOG and CFA. Therefore, the initiation of the adaptive immune response occurs in the inguinal lymph nodes and the spleen. To investigate the MOG-specific responses in the periphery in mice with EAE and in mice protected against EAE by treatment with MOG and IL-10, the cells from inguinal lymph nodes and spleens were cultured with increasing concentrations of MOG antigen. After 5 days, the supernatants were analysed for the concentration of IFN-γ, IL-17 and IL-10 by ELISA. The production of MOG-specific IFN-γ by spleen cells, but not lymph node cells decreased in mice immunized with MOG or MOG and IL-10 compared with control mice (Fig.4.11 A, B). In addition, the production of antigen-specific IL-17 was not significantly reduced by the spleen and the lymph node cells from MOG+IL-10-immunized mice. Lymph node cells from PBS-treated mice produced IL-10 in response to the highest concentration of MOG peptide (Fig. 4.12 A). In contrast, lymph node cells from both MOG and MOG+IL-10-immunized mice produced IL-10 in response to the lowest concentration of MOG. The production of IL-10 was also significantly ($p<0.01$) enhanced by the spleen cells from MOG and MOG+IL-10-treated compared to the PBS control in response to the highest concentration of antigen. These results demonstrate that

immunization with MOG and IL-10 decreased production of MOG-specific IFN-γ and increased production of MOG-specific IL-10 by spleen and lymph node cells during EAE

4.3 Discussion

The loss of Treg function appears to be one of the critical factors in the pathogenesis of human autoimmune disease. It has been demonstrated that patients with MS have decreased expression of FOXP3 at both mRNA and protein levels, which is directly related to impaired immunoregulation by Tregs cells (285). In addition, the generation of IL-10-producing Tr1 cells was found to be impaired in patients with MS, when compared with healthy volunteers (170). There is considerable interest in generating Tr1 cells as a therapy for inflammatory and autoimmune diseases or transplantations due to the fact that Tr1 cells can be differentiated from naïve $CD4^+$ T cells *in vivo* and *ex vivo* with the appropriate stimulus. Several methods have been employed for *ex vivo* generation of Tr1 cells. IL-10 is considered to be the major differentiating factor for Tr1 cells. Studies involving repeated TCR stimulation in the presence of high dose IL-10 induced IL-10 producing T cells that were capable of preventing DSS-induced colitis in mice (117). Alternatively, culture of T cells with anti-CD3 and anti-CD28 antibodies in the presence of vitamin D3, the immunosuppressive drug dexamethasone in non-polarizing conditions leads to differentiation of Tr1 cells (120). *In vivo*, Tr1 differentiation can be driven by tolerogenic APCs, especially immature DC. Thus, another approach for differentiation of Tr1 cells is based on prolonged culture of naïve peripheral blood $CD4^+$ T cells with allogeneic immature DC (286).

The successful generation of Treg cells *ex vivo* could lead to the development of a cell based therapy for autoimmunity, involving adoptive transfers of immunosuppressive cells to patients. However, the approach of using *ex vivo* induced Tr1 cells is limited by the difficulties in obtaining the pure population or in purification of those cells. Therefore, an alternative approach could be based on generation of Tr1 cells *in vivo*.

The results showed that it is possible to achieve protection against the development of autoimmune disease by prophylactic subcutaneous administration of self-antigen with IL-10. The protection against the development of EAE induced by treatment with MOG and IL-10 was associated with delayed infiltration of pathogenic IFN-γ- and IL-17-producing T cells into the CNS, which suggest that the immune suppression and inhibition of the inflammatory responses occurs in the peripheral organs. In addition, the protection lasted for more than 14 days after the induction of EAE. The MOG-specific responses in the periphery during ongoing EAE, assessed by re-stimulation of lymph node and spleen cells confirmed that there was less IFN-γ produced by the lymph node

and spleen cells and less IL-17 produced by the lymph node cells of mice immunized with MOG and IL-10. Interestingly, the production of IFN-γ by lymph node cells from mice treated with MOG and IL-10 was reduced with the increasing concentration of MOG peptide. At the same time the production of IL-10 was enhanced with the increasing concentration of MOG peptide. This results suggests that the decreased of IFN-γ might be due to the inhibitory effect of the antigen-specific IL-10. Importantly, the production of IL-10 was significantly enhanced by the spleen cells from mice treated with MOG and IL-10, compared to the PBS-treated mice, which suggests that the suppression of the MOG/CFA-induced inflammatory responses is systemic and not limited to the local draining lymph nodes.

The suppressive effect of IL-10 in the lymph node might be based on direct inhibition of T cell activation or inhibition of APC function. To examine this, the quantitative analysis of pro-inflammatory cytokines IL-1, IL-12 and IL-23 in the lymph nodes and spleens could be performed. Wolfer et al have shown that shortly after the immunization with MOG and CFA, γδ T cells accumulate at the site of the injection, in the CNS-draining lymph nodes and in the spleen and undergo massive expansion. This is followed by a rapid migration of those cells into the spinal cord and brain, which occurs before the onset of the first clinical symptoms of the disease (77). The present study showed that there is a delayed infiltration of γδ T cells into the CNS of mice immunized with MOG+IL-10. Further studies should be conducted to determine the exact mechanism of suppression of γδ T response and the role of MOG-specific IL-10 produced by Tr1 cells in the lymph nodes and spleen. Since it has been shown that addition of IL-10 to the human PBMCs stimulated with *M. tuberculosis* inhibits IFN-γ production by γδ T cells (287), it is possible that IL-10 produced by MOG-specific Tr1 cells inhibits directly the activation, proliferation and cytokine production by γδ T cells.

The inhibition of inflammatory responses and lymphocyte infiltration into the CNS in mice immunized with MOG+IL-10 lasted more than 2 weeks after induction of EAE, however at day 16 post EAE induction two out of four mice developed moderate paralysis of hind limbs. It is possible that the remaining two mice were at the stage when the regulatory responses were becoming overpowered by the mounting pro-inflammatory stimuli provided by CFA-activated APCs. This would explain the lack of γδ T cells infiltrating the brain of these two remaining MOG+IL-10-treated mice, but not significant

differences in MOG-specific IL-17 and IFN-γ production by spleen and lymph node compared to the control mice.

Surprisingly, prophylactic administration of MOG with IL-27 alone with IL-27 and IL-10 failed to inhibit and even exacerbated the development of EAE. Several published reports have shown that IL-27 induces IL-10 production from $CD4^+$ T cells and this study has also shown that IL-27 synergizes with IL-10 to induce IL-10 production from $CD4^+$ T cells. The studies on human PBMC cultures and animal model of MS have shown that IFN-β, a commonly used immunomodulatory therapy for relapsing remitting MS, at least partially mediates its therapeutic effect by inducing IL-27, which inhibits pathogenic Th17 cells (207). A later study by Fitzgerald et al however showed that the suppression of Th17 cells is not mediated by IL-27-induced IL-10 (288). In fact, the recent study by Mascanfroni et al demonstrated that the therapeutic effect of IL-27 on autoimmune inflammation is associated with IL-27 signalling in cDC (289). IL-27-conditioned DC upregulate expression of CD39 molecule, which reduces the extracellular concentration of ATP and decreases ATP-triggered activation of NLRP3 inflammasome, limiting the IL-1β-mediated differentiation of Th17 cells (289). Undoubtedly, exogenous IL-27 suppresses Th1 and Th17 responses and limits CNS inflammation in experimental animal models of MS, when applied during the effector phase of EAE (290). However, in the present study IL-27 alone or with IL-10 enhanced the production of IFN-γ from anti-CD3 and anti-CD28 stimulated $CD4^+$ T cells. In addition, the pro-inflammatory properties of IL-27 have been associated with enhancement of $CD8^+$ cytotoxic T cells (CTL) and IFN-γ production (48). The development of EAE is predominantly mediated by initial pathogenic responses of $CD4^+$ T cells; immunization of mice with myelin antigens in CFA promotes activation of $CD4^+$ T cells due to presentation of myelin antigen by in the context of MHC class II molecules by DC. However, it is possible that $CD8^+$ CTLs, activated by IL-27, also might contribute to the more severe EAE pathology.

Immunization with MOG and IL-10, but not with MOG alone was associated with development of MOG-specific T cells in the lymph nodes and spleen that produced IL-10 and IL-5 but not IL-4 following antigen re-stimulation *in vitro*. These results demonstrate for the first time that the anti-inflammatory cytokine IL-10 can work as a natural adjuvant for induction of antigen-specific regulatory T cells. Those antigen-specific Tr1-like T cells remained in the spleen and lymph nodes for at least 7 days after the last administration of MOG with IL-10. Mouse Tr1 cells proliferate poorly in

response to the antigenic stimulation due to lack of expression of IL-2. This might explain the relatively low concentration of IL-10 (approximately 100 pg/ml) detected in the supernatants from cells cultures. However, no IL-10 was detected in the supernatants from the cells with medium only, which confirms that the production of IL-10 is truly associated with antigen-specificity and not due to the generation of polyclonal Tr1 cells.

For several years after this discovery, Tr1 cells could only be identified though the profile of cytokines they secreted after activation. Lack of specific surface or intracellular markers, distinguishing them among other T cell subsets, was a major limiting factor for studies and their clinical application. Recently, after the profiling of gene expression of human Tr1 cell clones, two surface molecules, CD49b and Lag3, have been identified as stably present on human and mouse Tr1 cells (291). This discovery allows for easy identification of the Tr1 population. Further development of the present study should examine the presence of MOG-specific Tr1 cells after immunization with MOG and IL-10 by staining with fluorochrome-conjugated MOG_{35-55} tetramer and antibodies specific for CD49b, Lag3 and intracellular IL-10 for flow cytometry analysis. Furthermore, Tr1 cells generated after immunization with MOG and IL-10 should be purified to perform the suppression assay in order to confirm their regulatory activity. It should also be possible to determine if MOG-specific $CD49b^+$ and $Lag3^+$ $CD4^+$ T cells are present in the lymph nodes and spleen of MOG and IL-10-immunized mice after induced for EAE.

This study showed that lymph node and spleen cells produced by MOG-specific IL-10 following *ex vivo* re-stimulation 7 days after the last immunization. Further study should determine if the MOG-specific Tr1 cells remain in the peripheral lymphoid tissues for longer periods of time, for example for one or two months, and whether the initial protection against the development of EAE will remain after that time.

It is known that administration of IL-10 *in vivo* temporarily suppresses immune responses and induces T cell unresponsiveness. The present study also showed that administration of IL-10 or MOG alone has the capacity to delay the onset of EAE. These findings suggest that the generation of MOG-specific anergy upon administration of MOG and IL-10 cannot be excluded as an additional mechanism of protection against the development of EAE. However, since adoptive transfer of spleen cells from mice immunized with with MOG and IL-10, but not with MOG only, attenuated EAE, it is the most probable that

the prolonged protection against development of EAE in mice immunized with MOG and IL-10 is based on function of IL-10-producing MOG-specific T cells.

Clinical studies in human healthy volunteers have established that subcutaneous or intravenous administration of human recombinant IL-10 (rhIL-10, ilodecakin/tenovil) do not result in severe adverse effects and only high doses leads to temporary moderate flu-like symptoms. Administration s.c. results in slow absorption from the site of the injection with is associated with prolonged immunosuppressive effects. Moreover, the production of rIL-10-neutralizing antibodies was not observed. It has also been demonstrated that *in vivo* administered IL-10 retains its immunosuppressive properties (292). The potent immunoregulatory effects of IL-10 *in vivo* have led to the clinical trials in patients with inflammatory diseases. Treatment of patients with Crohn's disease with rhIL-10 resulted in a temporary or local reduction of disease severity but in general the treatment did not result in significant clinical improvement compared to placebo treatment (293, 294). In addition, the patients with Crohn's disease receiving high doses of IL-10 experienced enhanced inflammatory T cell responses with elevated levels of IFN-γ and TNF-α in the serum (293). Several open-label studies have reported that subcutaneously injected rhIL-10 had antipsoriatic effect (295). However, the randomized, double-blind and placebo-controlled trials did not show any significant improvement in the patients with psoriasis treated with rhIL-10 compared with patients injected with placebo (296).

Taken together, the results indicate that the IL-10 has the most beneficial effect when delivered in low doses and locally to the site of inflammation (297). Consequently, *in vivo* generation of antigen-specific Tr1 cells might be an alternative approach for therapy of many autoimmune diseases. Taken together, this study presented a method for *in vivo* generation of antigen-specific Tr1-like T cells and demonstrated that IL-10 can work as a natural adjuvant for generation of these cells.

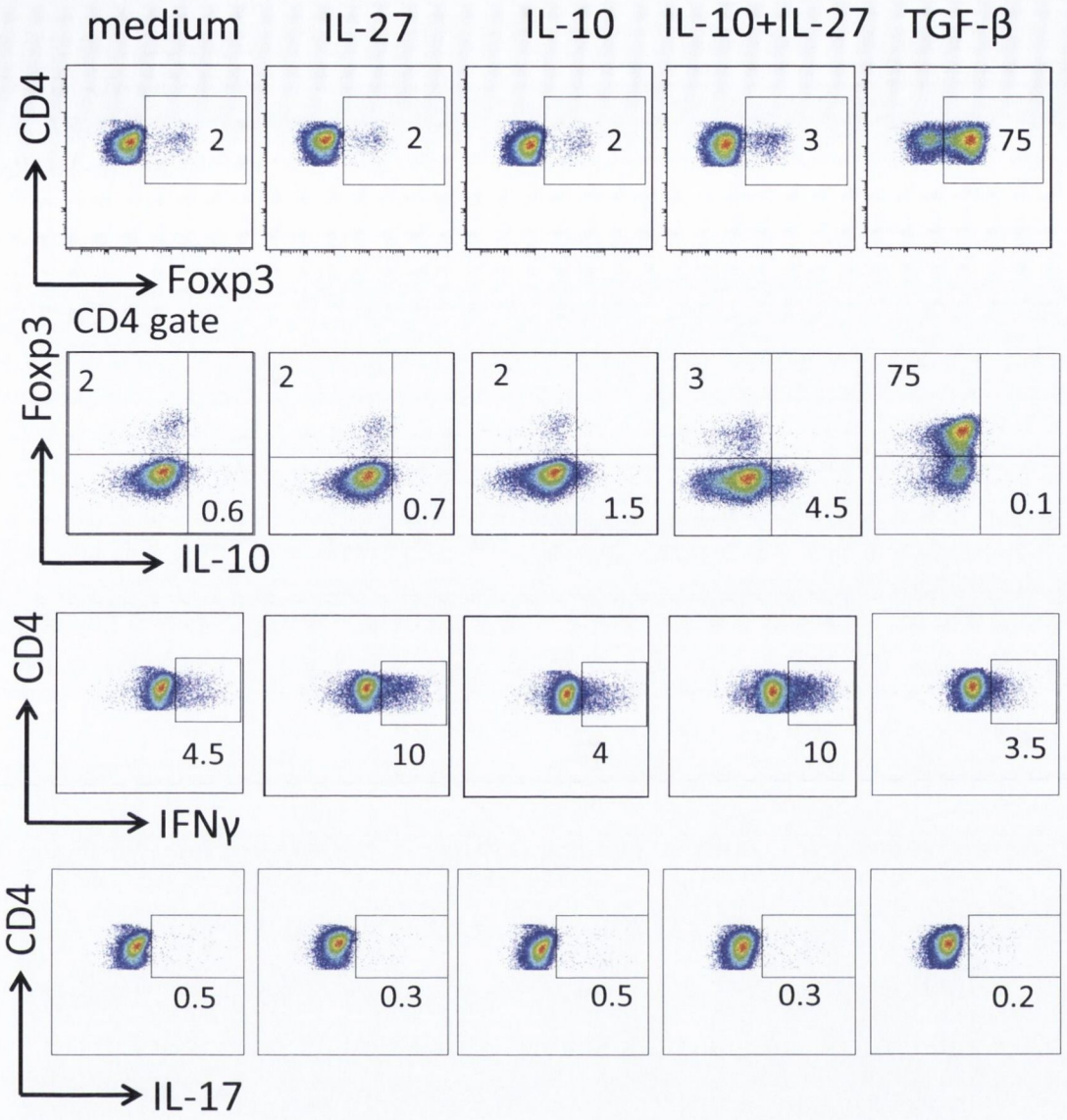
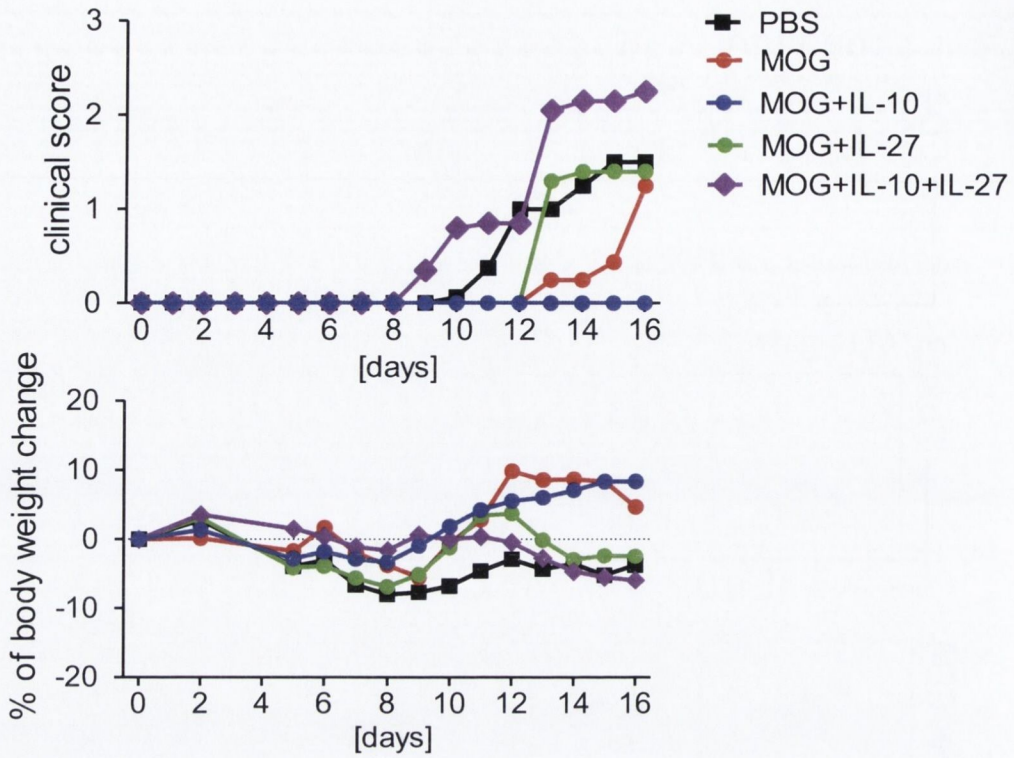


Fig. 4.1 Production of IL-10 and expression of Foxp3 in CD4⁺ T cells stimulated with IL-10, IL-27. CD4⁺ T cells were isolated from lymph nodes and spleens of C57BL/6 mice and cultured with plate-bound anti-CD3 and with soluble anti-CD28 with 10 ng/ml, IL-27 or with IL-10 together with IL-27 or TGF-β. For Foxp3 expression control, CD4⁺ cells were stimulated with 5 ng/ml of TGF-β. After 72 h cells were washed and stimulated with PMA, ionomycin in the presence of BFA for 5 h. Cells were pooled from triplicate cultures, stained with antibodies against CD4, Foxp3, IL-10, IL-17 and IFN-γ and analyzed by flow cytometry. Results are flow cytometry plots of samples pooled from triplicate cultures.

A



B

EAE characteristics				
PBS	MOG	MOG+IL-10	MOG+IL-27	MOG+ IL-10+IL-27
Disease incidence [%]				
50 (2/4)	50 (2/4)	0 (0/5)	40 (2/5)	60 (3/5)
Cumulative score (sum)				
26.8	8.8	0	27.5	57.25
Average cumulative score (sum/n)				
6.7	2.2	0	5.5	11.45

Fig. 4.2 Effect of prophylactic immunization with MOG and IL-10 on development of EAE. C57BL/6 mice were s.c. injected with PBS, MOG, MOG+IL-10 (100 ng), MOG+IL-27 (100 ng) or MOG+IL-10+IL-27 21 and 7 days before inductio of EAE. EAE was induced by s.c. injection of MOG peptide emulsified with CFA, followed by i.p. injection with PT on day 0 (day of induction) and day 2. Animals were monitored daily for clinical signs of the disease. A. Results shown are mean scores or percentage of body weight change; n=4-5/group. The statistical analysis was not performed. B. Table presents detailed charastics of clinical scores of EAE in each treatment group.

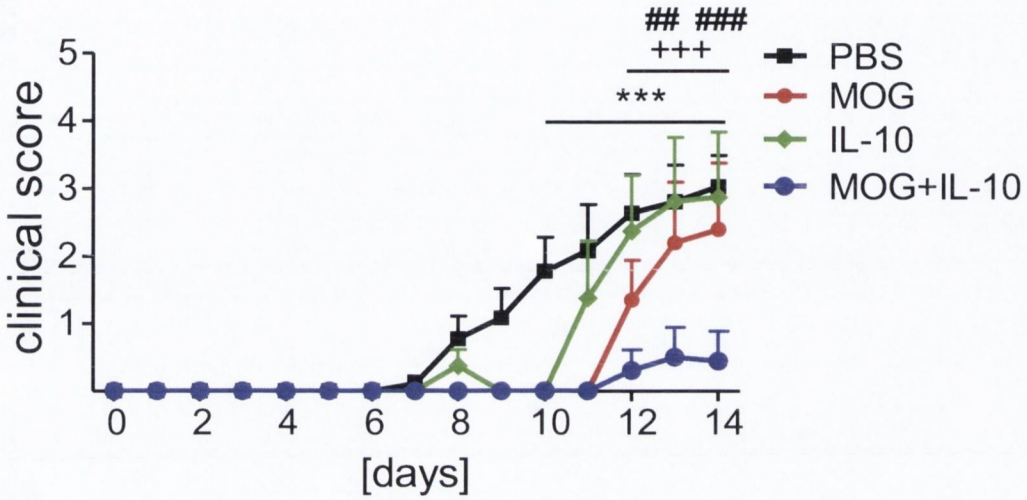


Fig. 4.3 Administration of MOG and IL-10 prior to the induction of disease attenuates clinical symptomes of EAE. C57BL/6 mice were s.c. injected with PBS, MOG, MOG with IL-10 (100 ng) or IL-10 only 21 and 7 days before induction of EAE. EAE was induced by s.c. injection of MOG peptide emulsified with CFA, followed by an i.p. injection with 500 ng PT on day 0 (day of induction) and day 2. Animals were monitored daily for clinical signs of the disease. Results shown are mean scores or percentage of body weight change +/- SEM from 2 experiments; (n=8-10/group) ***p<0.001 MOG+IL-10 vs. PBS, ##p<p0.01, ###p<0.001 MOG+IL-10 vs MOG, +++p<0.001 MOG+IL-10 vs IL-10, by two-way ANOVA and Bonferroni post-test.

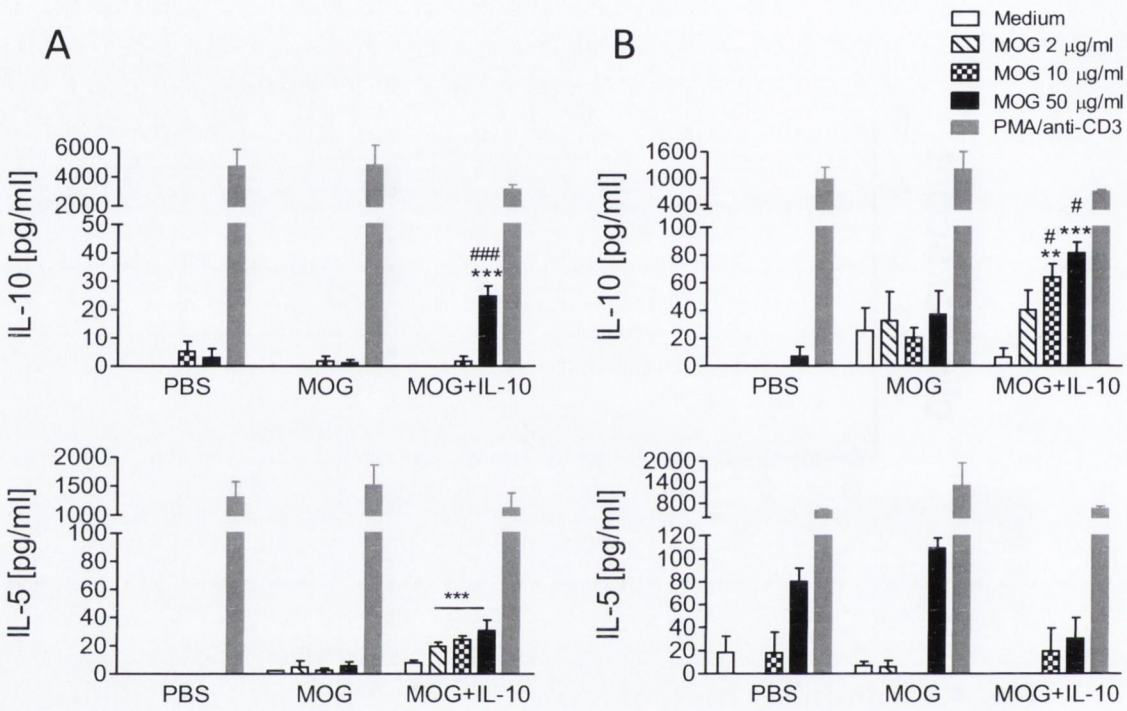


Fig. 4.4 Immunization with MOG and IL-10 increased antigen-specific IL-10 production by lymph node and spleen cells. C57BL/6 mice were s.c. injected with PBS, MOG peptipe or MOG with IL-10 (100 ng) and boosted 14 days later. 7 days after the last immunization lymph node (A) and spleen (B) cells were stimulated for 5 days with MOG at 2, 10 or 50 μg/ml or with PMA and anti-CD3. Supernatans were collected for analysis of IL-10 and IL-5 concentration by ELISA. Results represent mean concentrations +/- SEM (n=4/group) from single experiment. **p<0.01, **p<0.001 MOG+IL10 vs PBS, #p<0.05, ###p<0.001 MOG+IL-10 vs MOG by ANOVA

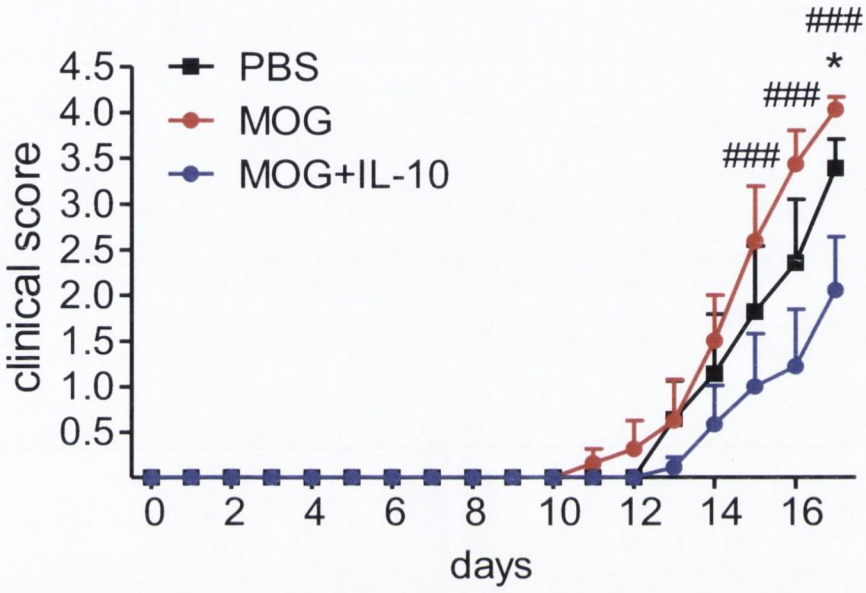


Fig. 4.5 Transfer of spleen cells from mice immunized with MOG and IL-10 provides significant protection against actively induced EAE. Donor mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) and boosted 14 days later. 7 days after the last immunization spleens were isolated and cultured with MOG (20 μg/ml) and rIL-2 (50 U/ml). After 3 days, the red blood cells were lysed and the cells were washed with PBS. Each recipient mouse received 20 x 10^6 cell i.p. and EAE was actively induced 24 h later. Results shown are mean scores +/- SEM from 2 independent experiments; (n=7-9/group) * $p<0.05$,** $P<0.01$ ***$p<0.001$ MOG+IL-10 vs. PBS, #$p<0,05$, ##$p<0.01$ ###$p<0.001$ MOG+IL-10 vs MOG by two-way ANOVA and Bonferroni post-test.

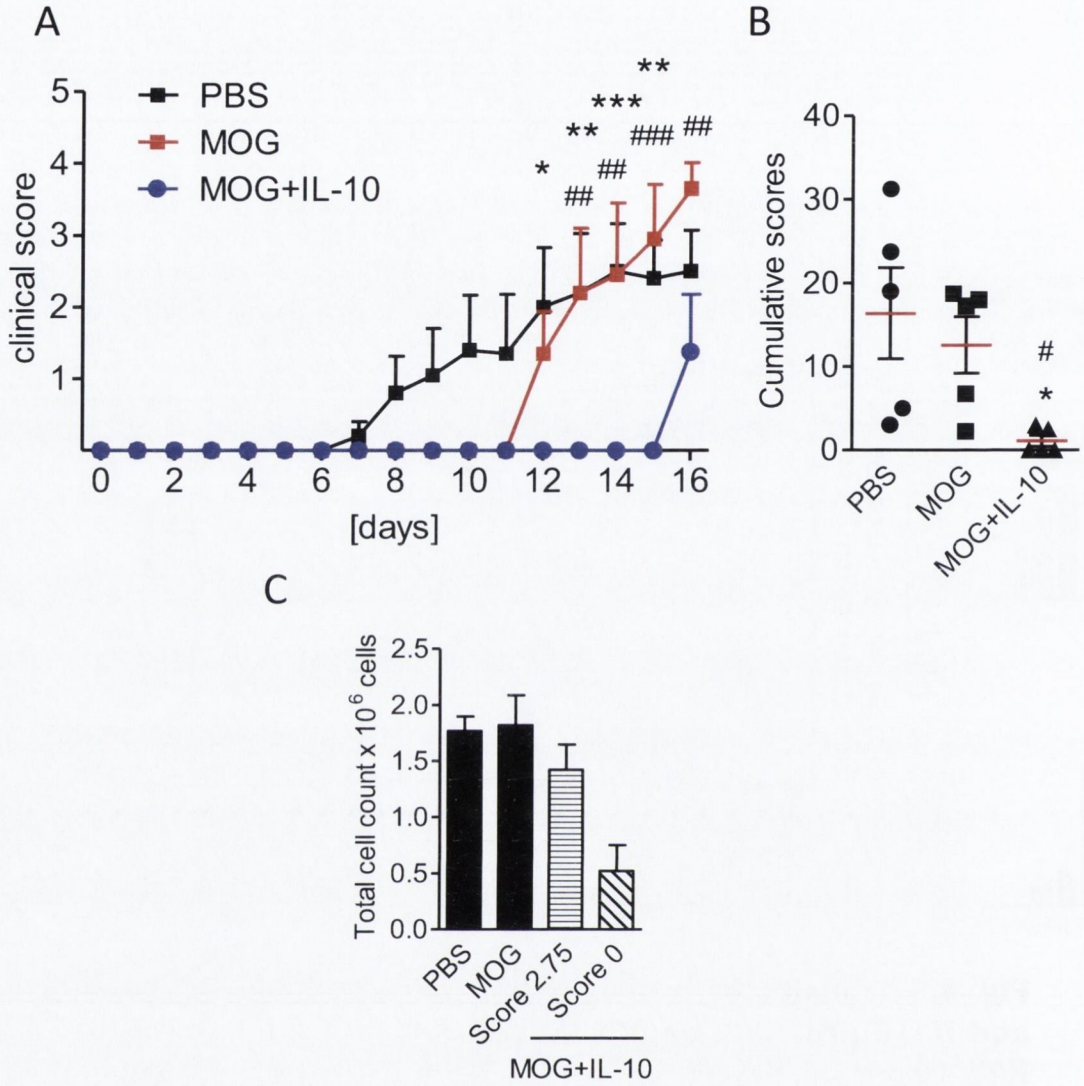


Fig. 4.6 Administration of MOG and IL-10 before induction of EAE reduces the leukocyte infiltration into CNS during EAE. A. C57BL/6 mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) 21 and 7 days before induction of EAE. A, B. Animals were monitored daily for clinical signs of disease. Results shown are mean scores +/- SEM (n=4-5/group) and cumulative scores. C. Mononuclear cells were isolated from brains of perfused mice at day 16 of EAE and counted. The numbers indicate the average score. Results are mean cell numbers +/- SEM (n=4-5/group) from single experiment. * $p<0.05$,** $P<0.01$ ***$p<0.001$ MOG+IL-10 vs. PBS, #$p<0,05$, ##$p<0.01$ ###$p<0.001$ MOG+IL-10 vs MOG by two-way ANOVA and Bonferroni post-test.

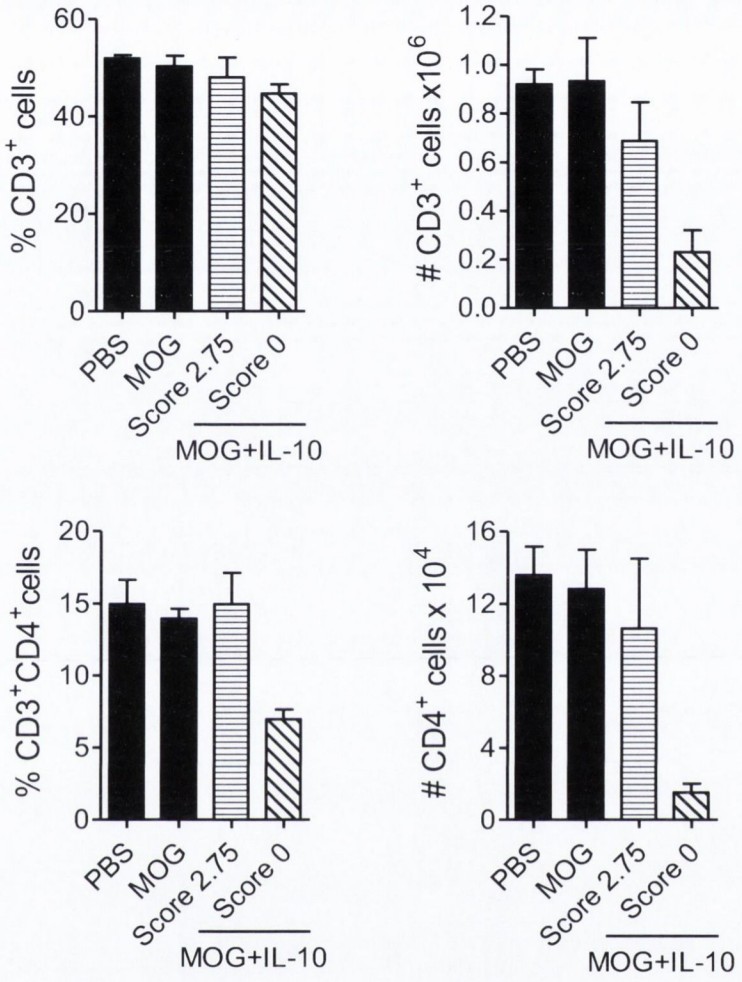


Fig. 4.7 Effect of administration of MOG and IL-10 prior to the induction of EAE on frequency and absolute number of CD3+ and CD4+ T cells in the brain C57BL/6 mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from the brains at day 16 of EAE and stained with antibodies against CD3, CD4 and γδ TCR and analyzed by flow cytometry. Results are the mean frequency and absolute cell numbers +/- SEM (n=4-5/group) from single experiment; for group MOG+IL-10, the results are separated into groups of mice with mean score 2.75 and 0.

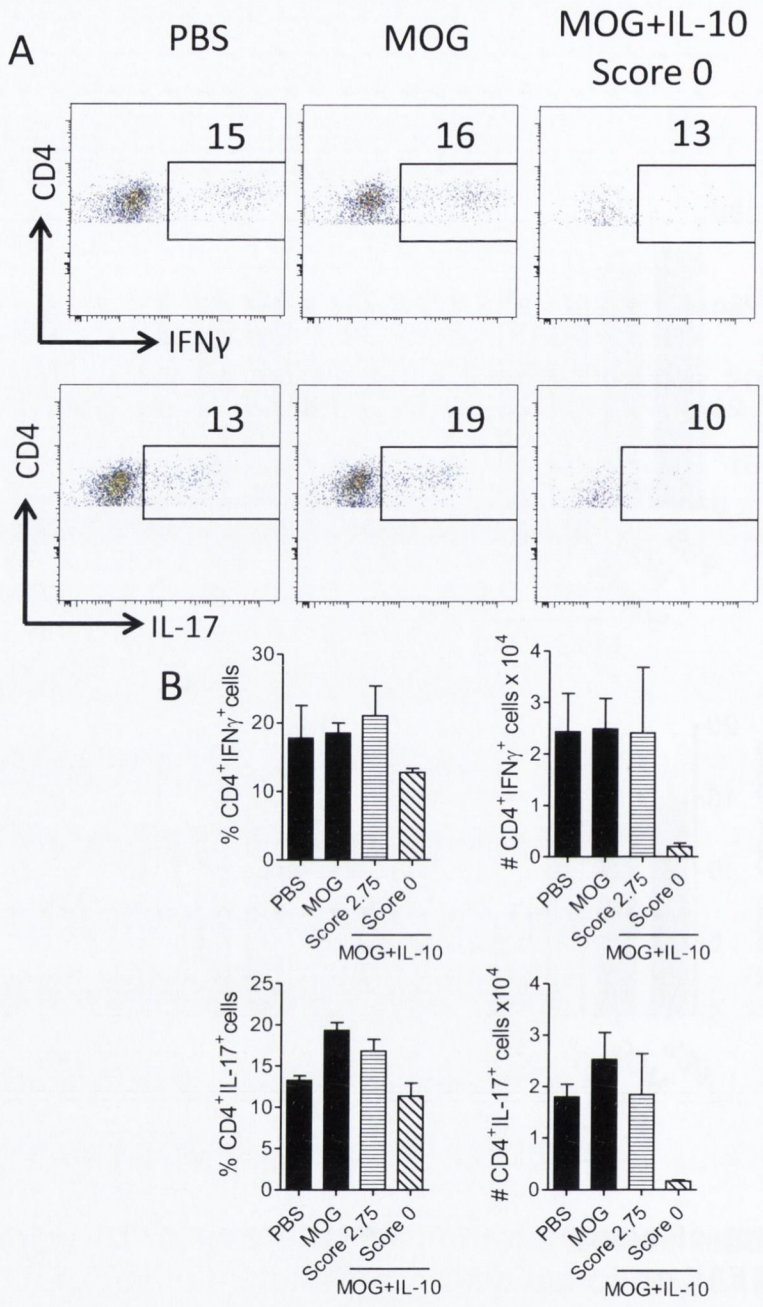


Fig. 4.8 Effect of administration of MOG and IL-10 prior to the induction of EAE on frequency and absolute number of IFN-γ and IL-17-producing CD4⁺ T cells in the brain. C57BL/6 mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains at day 16 of EAE and stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, IFN-γ and IL-17 and analyzed by flow cytometry. Results are representative plots for each mice group (A) and the mean frequency and absolute cell numbers +/- SEM (n=4-5/group) (B) from single experiment; for group MOG+IL10, the results are separated into groups of mice with average clinical score 2.75 and 0.

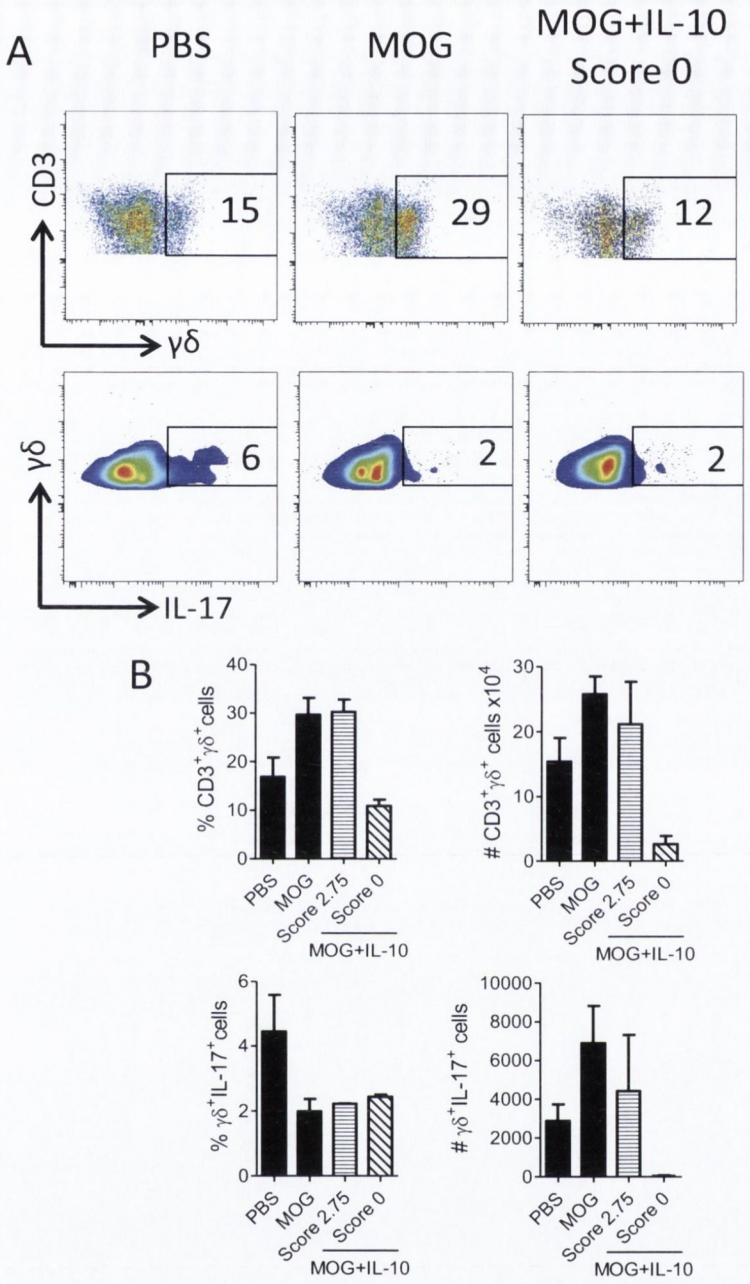


Fig. 4.9 Effect of administration of MOG and IL-10 prior to the induction of EAE on frequency and absolute number of IL-17-producing γδ⁺ T cells in the brain. C57BL/6 mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains at day 16 of EAE and stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, γδ-TCR and IL-17 and analyzed by flow cytometry. Results are representative plots for each mice group (A) and the mean frequency and absolute cell numbers +/- SEM (n=4-5/group) (B) from single experiment; for group MOG+IL10, the results are separated into groups of mice with average clinical score 2.75 and 0.

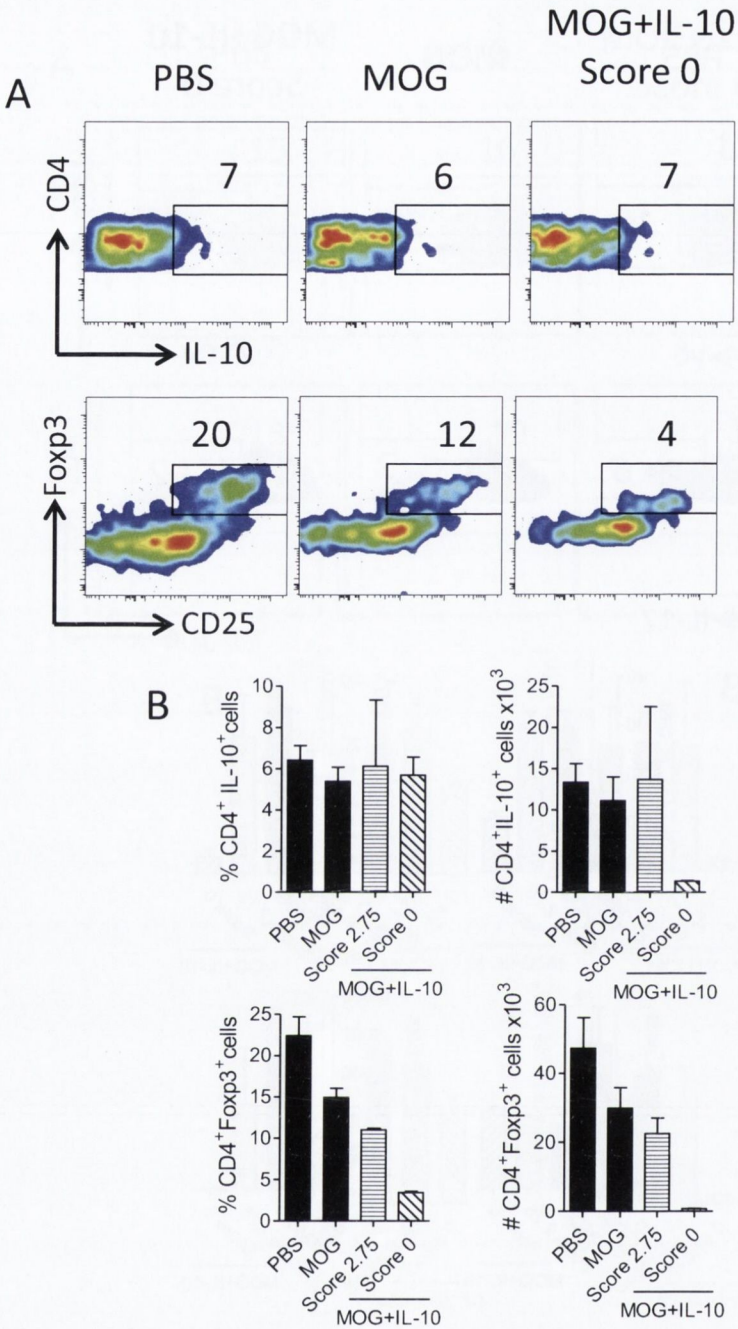


Fig. 4.10 Effect of administration of MOG and IL-10 prior to the induction of EAE on frequency and absolute number of Foxp3⁺ and IL-10-producing CD4⁺ T cells in the brain. C57BL/6 mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains at day 16 of EAE and stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, Foxp3 and Il-10 and analyzed by flow cytometry. Results are representative plots for each mice group (A) and the mean frequency and absolute cell numbers +/- SEM (n=4-5/group) (B) from single experiment; for group MOG+IL10, the results are separated into groups of mice with average clinical score 2.75 and 0.

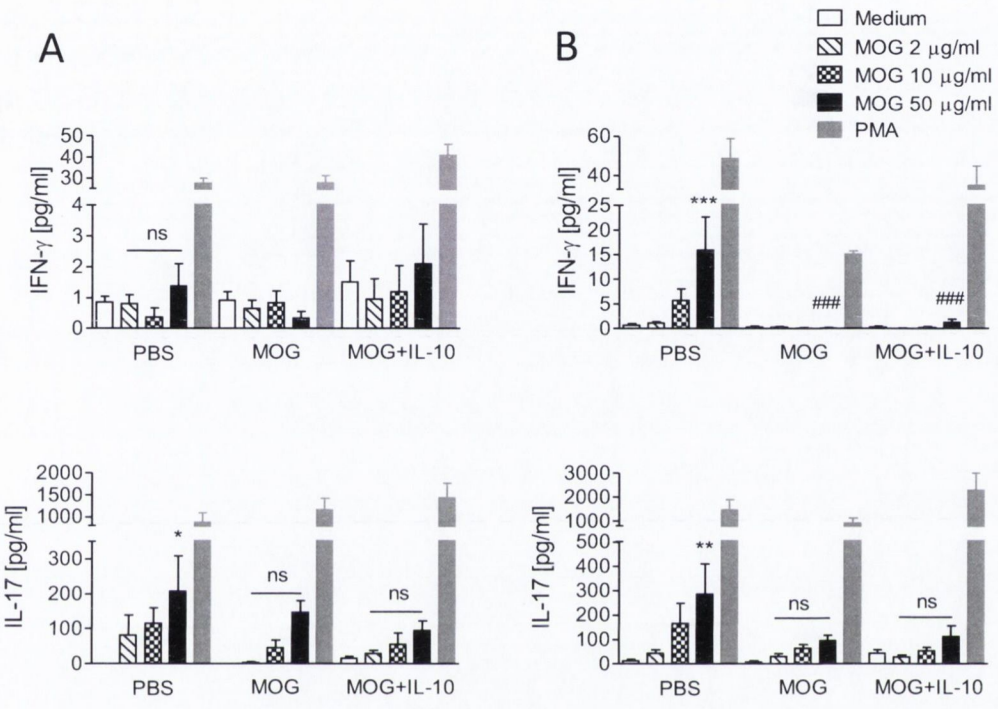


Fig. 4.11 Effect of administration of MOG and IL-10 prior to the induction of EAE on the production of MOG-specific IL-17 and IFN-γ by lymph nodes and spleen cells. C57BL/6 mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) 21 and 7 days before induction of EAE. Mice were sacrificed at day 16 after induction of EAE and single cell suspenions of lymph node (A) and spleen (B) cells were cultured with increasing concentrations of MOG (2. 10 and 50 µg/ml) or PMA (5 ng/ml) with anti-CD3 (1 µg/ml). Supernatans were collected after 5 days and the concentrations of IL-17 and IFN-γ were quantified by ELISA. Results shown are mean values +/- SEM (n=5-7/group) from 2 independent experiments.* $p<0.05$,** $P<0.01$ ***$p<0.001$ vs medium, ###$p<0.001$ vs PBS group, ns not significant by ANOVA

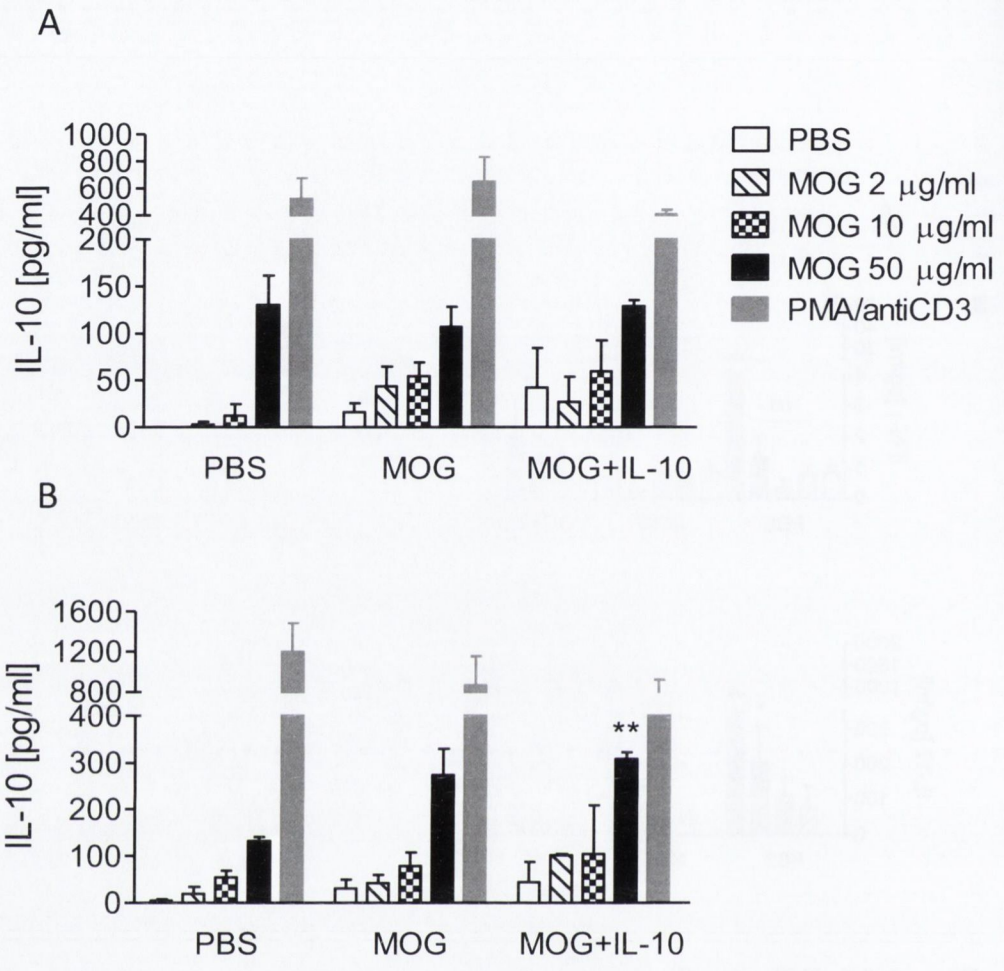


Fig. 4.12 Effect of administration of MOG and IL-10 prior to the induction of EAE on the production of MOG-specific IL-10 by lymph node and spleen cells. C57BL/6 mice were s.c. injected with PBS, MOG or MOG with IL-10 (100 ng) 21 and 7 days before induction of EAE. Mice were sacrificed at day 16 and single cell suspenions of lymph node (A) and spleen (B) cells were stimulated with inγcreasing concentrations of MOG (2, 10 and 50 μg/ml) or PMA (5 ng/ml) with anti-CD3 (1 μg/ml). Supernatans were collected after 5 days and the concentrations of IL-10 was quantified by ELISA. Results shown are mean values +/- SEM (n=4-5/group). **p<0.01 MOG+IL-10 vs PBS by ANOVA

Chapter 5

Prophylactic treatment of mice with *F. hepatica* TE delays development of EAE

CHAPTER 5 PROPHYLACTIC TREATMENT OF MICE WITH F. HEPATICA TE DELAYS THE DEVELOPMENT OF EAE

5.1 Introduction

Epidemiological and experimental studies have demonstrated that helminth infections have beneficial effects in the prevention and suppression of inflammatory, autoimmune and allergic diseases. MS patients naturally infected with helminth parasites have less severe symptoms of disease (187). Furthermore, experimental probiotic helminth therapy in patients with Crohn's disease significantly suppressed the pathology of the disease (298, 299). Helminths have evolved a wide variety of mechanisms for immune evasion, enabling successful colonisation and prolonged survival within the host. Apart from the protective Th2 type response that aims at elimination of the pathogen by the host, helminths elicit regulatory responses by inducing the production of anti-inflammatory cytokines IL-10 and TGF-β from innate and adaptive immune cells. This in turn leads to the generation of Treg cells. Helminth-induced Treg cells efficiently suppress anti-helminthic Th2 responses but can also inhibit unrelated Th1- and Th17-mediated pathology by mechanisms of bystander suppression. Moreover, the immunoregulatory products produced by helminth parasites suppress innate immune responses, consequently leading to the impaired induction or maintenance of pathogenic Th1 and Th17 cells involved in autoimmune inflammation (239).

Helminths induce immune hyporesponsiveness and it has been shown that peripheral T cells from infected patients are often unresponsive to parasite antigens as well as to unrelated antigens (300, 301). Moreover, it has been demonstrated that infection of *Fasciola hepatica* suppresses a protective Th1 responses against microbial pathogens, such as *Mycobacterium bovis* and *Salmonella dublin* in cattle (302, 303). In mouse model, co-infection with *F. hepatica* suppressed *Bordetella pertussis*-specific IFN-γ production, which was associated with delayed bacterial clearance (304). It has also been demonstrated that chronic infection with gastrointestinal parasite *Heligmosomoides polygyrus* expands a population of $CD4^+CD25^+Foxp3^+$ Treg cells in the mesenteric lymph nodes and suppresses mucosal production of IL-17 (305, 306).

A large number of studies in mouse models have confirmed that helminth infections can ameliorate autoimmune diseases, such as type 1 diabetes, colitis and EAE (307–309).

This study focused in particular on the immunoregulatory properties of *Fasciola hepatica* products. *F. hepatica,* also known as liver fluke, is a parasitic flatworm of *Trematoda* class that causes fasciolosis, a disease affecting the liver. *F. hepatica* is mostly a pathogen of domesticated ruminants, such as cattle, sheep or goats, however, other animals, such as rats and humans, can be infected (310). The life cycle requires two hosts. A freshwater snail of the family of *Lymnaeidea* serves as an intermediate host for the transformation of the first stage larva miracidia into the second form of larvae – cercarie, which asexually multiply. After the cercariae exit the snail, they encyst in the water, forming metacercariae which are ingested by a mammalian host. Within the definitive host, metacercariae excyst and penetrate through the wall of small intestine into the peritoneal cavity. The juvenile flukes move into the liver parenchyma and eventually enter the bile ducts, where they feed on blood, reach sexual maturity and produce eggs (311).

Previous studies by Walsh et al demonstrated that infection of mice with *F. hepatica* attenuated the clinical course of EAE through a TGF-β-mediated mechanism (312). Infection with *F. hepatica* induced TGF-β- and IL-10-producing macrophages, IL-10-producing DC and inhibited DC maturation. This was associated with expansion of TGF-β-producing Th3 cells, $Foxp3^{+}CD4^{+}$ Treg cells and helminth-specific IL-10-producing Tr1-like T cells in the peritoneal cavity of infected mice. Moreover attenuation of EAE in *F. hepatica*-infected mice was associated with TGF-β-mediated suppression of auto-antigen-specific Th1 and Th17 cells (312). Later studies have shown that administration of *F. hepatica* excretory-secretory products (ES) throughout the course of the disease can mimick the immunosuppressive effect of live flukes and attenuate clinical symptoms of EAE by a yet undefined mechanism (C. Finlay, A. Stefańska and K. H. G Mills unpublished data). Since the infection with live parasites is often associated with severe side effects, such as anaemia, weight loss and extensive tissue damage, the principal aim is to identify and purify the crucial immunomodulatory molecules that exert immunosuppressive effects, which have a potential to be used as therapies for human autoimmune diseases.

It has been demonstrated that *F. hepatica* ES modulates TLR-agonist-induced pro-inflammatory cytokine production from DC, inhibits their maturation and promotes release of TGF-β from DC and macrophages (313). The attenuation of EAE by administration of ES was associated with suppressed MOG-specific Th1 and Th17 responses in the spleen and enhanced expression of TGF-β by $CD4^{+}$ T cells in the lymph

nodes (C. M, Finlay, A. Stefańska, unpublished data). Moreover, the studies of Carranza and Falcon have shown that total soluble extract (TE) of *F. hepatica* enhances the tolerogenic phenotype of TLR-stimulated DC (314). These data suggest that both *F. hepatica*-derived antigens and ES exhibit tolerogenic properties.

The aim of this study was to examine the immunoregulatory properties of *F. hepatica* products, particularly the low molecular weight fraction (molecules smaller than 3kDa) ES-L, high molecular fraction (molecules larger than 3 kDa) ES-H and TE. The study examined if ES-L, ES-H and TE have a capacity to induce Treg cells *in vivo*. Further, the helminth products were assessed as adjuvants to induce auto-antigen-specific Treg cells.

5.2 Results

5.2.1 Repeated administration of TE increases production of IL-10 and LAP, while ES-L enhances Foxp3$^+$ expression by CD4$^+$ T cells

A study by Stefańska and Finlay (unpublished) showed that treatment with *F. hepatica* ES during EAE suppressed the clinical course of the disease and inhibited Th1 and Th17 responses to MOG. *F. hepatica* ES was separated and examined for their immunomodulatory activity. Further experiments by Stefańska and Finlay (unpublished) revealed that the components of ES-L induced release of TGF-β from innate cells, but the products retained in ES-H attenuated EAE. Therefore, in this study experiments were conducted to investigate the abilities of ES-L, ES-H and TE to induce Treg cells *in vivo*. C57BL/6 mice were injected i.p. with PBS, ES-L (50 µl/mouse), ES-H (50 µl=50 µg/mouse) or TE (50 µg/mouse) at day 0, 4, 8 and 12. The procedure of separation of total ES into ES-L and ES-H did not change the molar concentrations in any of the fractions, which allowed a comparison of their activity on the basis of volume. The dose of TE was equalized to the amount of protein present in ES-H. PEC, spleen and peritoneal cavity draining lymph nodes (mediastinal lymph nodes) were isolated 4 days after the last injections. Cell suspensions were stimulated with PMA and ionomycin in the presence of BFA for 5 h and stained with antibodies against surface CD3 and CD4 and against markers of Treg cells - Foxp3, IL-10 and LAP.

Flow cytometry analysis showed that repeated i.p. administration of ES-L significantly enhanced the percentage of Foxp3-expressing CD4$^+$ T cells in the peritoneal cavity and in the spleen (Fig. 5.1). Administration of ES-L and ES-H induced expression of LAP by CD4$^+$ T cells in the peritoneal cavity (Fig. 5.2). TE did not enhance the frequency of CD4$^+$Foxp3$^+$ cells, but significantly increased the expression of IL-10 by Foxp3$^-$CD4$^+$ T cells and expression of LAP by CD4$^+$Foxp3$^+$ PEC (Fig. 5.2). TE and ES-H also moderately enhanced expression of IL-10 by CD4$^+$ T cells in the spleen (Fig. 5.2). Additionally, both ES-H and TE significantly enhanced the frequency of IL-4-producing T cells in the spleen and peritoneal cavity. However, only repeated treatment with TE significantly increased the percentage of CD4$^+$IL-4$^+$ T cells in the draining lymph nodes (Fig. 5.1). Based on the induction of IL-4 production, these results indicate that *F. hepatica* high molecular weight products are the most effective at inducing Th2 type response. The low molecular weight products from ES-L enhanced Foxp3 but not LAP

expression by T cells. In contrast, although TE failed to enhance Foxp3 expression, it did increase production of IL-10 and surface LAP by $CD4^+$ T cells. These data demonstrate that ES-L and TE are the most suitable *F. hepatica* products for further investigation into their ability to prevent the development of EAE, when prophylactically administered with MOG auto-antigen.

5.2.2 Immunization with MOG and TE prevents development of EAE

Having established that ES-L and TE are the most effective *F. hepatica* products at inducing different Treg markers on $CD4^+$ T cells, this study investigated if prophylactic administration of these helminth products together with auto-antigen MOG protects against the development of EAE. C57BL/6 mice were injected s.c. with PBS, MOG (100 µg/mouse), MOG and ES-L or MOG and TE 21 days and 7 days before the induction of EAE. Animals were monitored daily for the clinical symptoms of the disease and weight change to assess the severity of disease. Consistent with published reports and this study (Fig. 3.17, Fig. 4.3), the administration of MOG alone before induction of EAE attenuated the severity of the disease. Mice immunized with MOG and ES-L developed EAE with similar clinical severity as PBS-injected control mice (Fig. 5.3). In contrast, the prophylactic immunization with MOG and TE delayed the onset of the disease by 6 days compared with the control mice and significantly attenuated the severity of the disease (Fig. 5.3). These data indicate that prophylactic administration of MOG and TE inhibit the development of EAE.

5.2.3 Effect of TE administration on T cells responses in the draining lymph nodes

As shown in Fig. 5.2, TE enhanced IL-10 and LAP expression by $CD4^+$ T cells. This might support the hypothesis that TE can generate MOG-specific Treg cells that protects against the development of EAE. Therefore, an experiment was performed to investigate if TE can act as adjuvant for the induction of Treg cells specific for unrelated antigens. Keyhole limpet hemocyanin (KLH), a multisubunit protein, was chosen as an antigen due to its strong immunogenicity. C57BL/6 mice were s.c. injected with PBS, KLH protein (20 µg/mouse), KLH with TE and boosted after 14 days. An additional group of mice

was injected with KLH and CpG (50 μg/mouse) as a positive control for antigen-specific T cells responses. Seven days after the last immunization, inguinal lymph nodes were isolated and single cell suspensions were cultured with increasing concentrations of KLH protein, or with PMA and anti-CD3 as a positive control. After 5 days of culture, the supernatants were collected and the concentration of IL-10, IL-4, IL-17 and IFN-γ was determined by ELISA.

The results showed that immunization of mice with KLH alone induced IL-10- and IL-4-producing and to a lesser extent IFN-γ-producing KLH-specific T cells in the lymph nodes (Fig. 5.4). Co-administration of KLH with CpG significantly increased KLH-specific IFN-γ-producing T cells. Surprisingly, lymph node cells from mice immunized with KLH and TE produced significantly less KLH-specific IL-10 and markedly less KLH-specific IFN-γ and IL-4 compared with the cells from KLH-immunized mice. Interestingly, lymph node cells from mice immunized with KLH and TE produced less IL-17, IFN-γ and IL-4 when compared with cells from KLH-immunized mice in response to stimulation with PMA and anti-CD3 (Fig. 5.4). These results indicate that administration of TE suppressed the generation of KLH-specific T cells *in vivo* and also blocked non-specific response of T cells.

5.2.4 Prophylactic treatment with TE delays development of EAE

After immunization with MOG and CFA, the pathogenic auto-antigen-specific Th1 and Th17 cells develop in the lymph nodes and spleen, from where they migrate into the CNS and exert autoimmune pathology. The results presented in Fig. 5.4 demonstrated that treatment with TE inhibited Th1 and Th17 cytokine production by lymph nodes cells and that this effect was maintained for as long as 7 days after the last administration of TE. Therefore, an experiment was conducted to determine if prophylactic s.c. administration of TE without MOG can inhibit the development of EAE. Mice were injected s.c. with PBS, MOG and TE or with TE only 21 days and 7 days prior to the induction of EAE. The results presented in Fig. 5.5 show that 80% of mice (4/5) treated with TE remained without clinical signs of disease until day 21 post induction of EAE. In contrast, PBS-injected mice rapidly developed severe EAE and all reached the humane endpoint by day 16 post EAE induction. These results demonstrate that prophylactic s.c. administration of

TE significantly slows the development of EAE and that inhibition of autoimmune responses last for up to 4 weeks after the last administration of TE.

5.2.5 Effect of prophylactic treatment with TE on Th1 and Th17 responses in the spleen and lymph nodes during EAE

To investigate MOG-specific T cell responses in the draining lymph nodes and spleen during EAE, the mice were sacrificed at the peak of the disease of the PBS-treated control group of mice (day 14 post EAE induction). At this time point, TE-treated mice did not show any signs of paralysis (Fig. 5.6). The inguinal lymph node cells and spleen cells were cultured with increasing concentration of MOG peptide or with PMA and anti-CD3. The supernatants were collected after 5 days and the concentration of IL-17 and IFN-γ were determined by ELISA. The lymph node, but not spleen cells from TE-treated mice produced considerably (but not significantly) less antigen-specific IFN-γ and IL-17 than the cells from PBS-treated mice (Fig. 5.7). The production of IFN-γ and IL-17 by lymph node and spleen cells after stimulation with PMA and anti-CD3 was similar in both mice groups. This result indicates a trend towards an inhibition of pathogenic Th1 and Th17 responses in the draining lymph nodes during EAE by prophylactic treatment with TE.

5.2.6 Prophylactic treatment with TE reduces accumulation of leukocytes and production of IL-17 and IFN-γ by $CD4^+$ T cells in CNS during EAE

After immunization with MOG and CFA pathogenic Th1 and Th17 cells are initially activated in the lymph nodes and spleen. When autoreactive T cells are re-activated in the brain by MOG-presenting APC, they release cytokines and other immune mediators, which facilitate local inflammatory reactions and abrogate the BBB function. This allows for the infiltration of other innate and adaptive immune cells into the brain. To investigate the effect of prophylactic treatment with TE on the leukocyte infiltration into the CNS during EAE, mononuclear cells isolated from the brains of mice with EAE were stimulated with PMA and ionomycin in the presence of BFA for 6 h and stained with antibodies against CD3, CD4, IL-17, IFN-γ, IL-4 and Foxp3. In parallel, to determine the frequency of various subsets of innate cells in the CNS, cells were stained with antibodies against CD19, Siglec F, CD11b, CD11, F4/80 and MHC class II.

The results show that there was significantly less mononuclear cells in the brains of mice prophylactically treated with TE compared with the mice treated with PBS (0.5×10^6 cells and 1.1×10^6 cells respectively ($p<0.05$; Fig. 5.8 A). The frequency of $CD4^+$ cells among the $CD3^+$ population was similar in both treatment groups (Fig. 5.8 A). Interestingly, the percentage of brain-infiltrating $CD4^+$ T cell producing IL-17 and both IL-17 and IFN-γ was significantly lower in mice treated with TE (Fig. 5.8 B). The frequency of $CD4^+$IFN-γ^+ and $CD4^+$IL-4^+ T cells was also markedly, but not significantly, lower in mice injected with TE. In contrast, there was no difference in the frequency of $Foxp3^+CD4^+$ T cells in the CNS of TE-treated and control mice. These results indicate that prophylactic treatment with TE not only suppresses infiltration of immune cells into the brain but also inhibits IL-17 and IFN-γ production by infiltrating $CD4^+$ T cells.

The frequency of $CD11b^+Ly6G^+$ neutrophils in CNS of mice was similar in both treatment groups (Fig. 5.9). Interestingly, the percentage of siglec F^+ eosinophils in the CNS was 3 fold higher in the brains of TE-treated mice compared with PBS-treated mice (Fig. 5.9). After excluding of $CD11c^+$ cells, the frequency of $CD11b^+F4/80^+$ macrophages was twice as high in the brains of mice treated with TE than in PBS-treated mice (Fig. 5.9). DC derived from monocyte in response to inflammation, including autoimmune inflammation are called inflammatory DC and are characterized by expression of CD11b, CD11c and high expression of MHC class II molecules (315). Some reports indicate that infDC also express F4/80 (15). Within the population of $CD11b^+F4/80^+$ cells, the frequency of $CD11c^+$ MHC class II-expressing infDC was considerably lower in the brains of mice treated with TE (Fig. 5.10). Conversely, the population of $F4/80^-$ infDC was only slightly increased in TE-treated mice, compared with PBS-treated mice (Fig. 5.10). The frequency of $CD11b^-CD11c^+$ DC was similar in mice treated with PBS and TE, however, the percentage of MHC class II-expressing cells within the population of these DC was lower in brains of mice treated with TE (Fig. 5.10).

Taken together, the results suggest that prophylactic treatment with TE is associated with a decreased accumulation of inflammatory DC and a decreased expression of the activation marker MHC class II on DC. In contrast, TE treatment increases the accumulation of macrophages and eosinophils in the CNS of mice with EAE.

5.2.7 Effect of treatment with TE on expression of Treg markers by $CD4^+$ T cells in lymph nodes and spleen

The results presented in Fig. 5.2 showed that repeated i.p. administration of TE was associated with higher frequencies of IL-10-producing and LAP-producing $CD4^+$ T cells at the site of administration but not in the draining lymph nodes. However, the prophylactic treatment of EAE with TE was administrated at a different site and using a different regime. S.c. administration of TE was associated with impaired production of IL-17, IFN-γ and IL-4, but not IL-10, in response to PMA and anti-CD3 by the draining lymph node cells (Fig. 5.4). Prophylactic treatment with TE suppressed development of EAE (Fig. 5.5), which suggests that the inhibition of T cell responses during EAE might take place in the lymph nodes or in the spleen. Therefore, an experiment was performed to examine the effect of TE administered in the prophylactic regime on the frequencies and numbers of Treg cells in the draining lymph nodes and spleen. C57BL/6 mice were injected with PBS or TE at day 0 and 14 and spleens and lymph nodes were isolated and analysed 7 days later. Spleen and lymph node cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, LAP, IL-10 and Foxp3 and analysed by flow cytometry.

Administration of TE reduced the number of cells in the lymph nodes after 7 days, but significantly increased the absolute number of cells in the spleen, suggesting that TE treatment induced either proliferation of T cells or enhanced migration of immune cells into the spleen (Fig. 5.11, Fig. 5.12). The frequency of $Foxp3^+CD4^+$ T cells in both lymph node and spleens was similar in PBS versus TE-treated mice. Surprisingly, TE decreased the percentage of IL-10-producing and LAP-producing $CD4^+$ T cells in the lymph nodes (Fig. 5.11). The absolute number of $Foxp3^+$, $IL\text{-}10^+$ and LAP^+CD4^+ T cells was considerably lower in the lymph nodes of mice treated with TE (Fig. 5.11). In contrast, TE treatment moderately increased the percentage of IL-10-producing $CD4^+$ T cells in the spleen (Fig. 5.12). Despite the small differences in frequencies of Treg cells, the absolute numbers of the different populations of Treg cells were significantly higher in the spleens of mice treated with TE (Fig. 5.12). The results indicate that treatment with TE does not lead to the increased generation of Treg cells in the draining lymph nodes 7 days after the last administration.

5.2.8 TE treatment enhances accumulation of cells at the site of injection

The results demonstrated that the suppressed production of inflammatory cytokines by lymph nodes of mice treated with TE (Fig. 5.4) is not associated with increased number or frequency of Treg cells in the lymph nodes 7 days after the last injection (Fig. 5.11). This indicates that the innate cells rather than induced Treg cells might be responsible for the TE-associated inhibition of T cells responses during EAE. Therefore, an experiment was conducted to examine the composition of innate cells at the site of the injection 7 days after the last treatment with TE. For the experiments determining the effect of prophylactic administration of TE on the development of EAE, TE was injected subcutaneously. Since the isolation of cells from the subcutaneous tissue is difficult and might lead to recovery of very low cell numbers, TE was injected i.p. C57BL/6 mice were injected with PBS, TE or LPS at day 0 and 14. After 7 days, PEC were isolated by peritoneal lavage, counted and stained with antibodies against CD19, siglec F, CD11b, CD11c, Ly6G F4/80, MHC class II and CD206.

The absolute number of cells recovered from TE-treated mice was 4.6 fold higher than in PBS-treated mice (30 x 10^6 +/- SEM and 6.5 x 10^6 +/- SEM, respectively; $p<0.001$; Fig. 513). In contrast, the number of cells in peritoneal cavity of LPS-injected mice was similar in comparison to PBS-treated mice (Fig. 5.13). The relative frequency of siglec F^+ eosinophils was increased from 2% in PBS-treated mice to 30% of in mice treated with TE (Fig. 5.14, Fig. 5.15). The relative frequency of $CD11b^+Ly6G^+$ neutrophils and $CD11b^+F4/80^+$ macrophages were significantly lower in mice treated with TE (Fig. 5.14, Fig. 5.15). The percentage of $F4/80^+$ infDC within the monocyte population was markedly increased in LPS-treated mice but not in mice injected with TE (Fig. 5.16, Fig. 5.17 A). In addition, $CD11b^+F4/80^+$ macrophages from mice injected with LPS but not with TE increased expression of MHC class II molecules (Fig. 5.17). The frequency of MHC class II-expressing cells within the other populations of DC ($CD11b^+$ and $CD11b^-$) was increased in TE-treated mice compared with PBS-injected mice (Fig.5.17). Although the frequency of MHC-class II-expressing cells within the particular innate cell populations were not significantly different between PBS- versus TE-treated mice, the relative frequency of these cells among the whole PEC was considerably, but not significantly, lower in mice treated with TE (Fig. 5.17 B, C). These results indicate that treatment with TE enhanced the accumulation of eosinophils at the site of injection, which consequently decreased the relative percentage of other immune cells.

Interestingly, administration of LPS but not TE expanded the population of infDC ($CD11b^+CD11c^+F4/80^+MHC\text{-}II^+$ cells).

5.2.9 TE treatment enhances expression of alternatively activated macrophage markers in vivo

Mannose receptor CD206, arginase, RELM-α and co-inhibitory surface molecule programmed death ligand 2 (PD-L2) are characteristic markers expressed by alternatively activated M2 macrophages. To determine if TE induces expression of M2 macrophages markers, cells isolated from peritoneal cavity were stained with antibodies against CD206. To examine the expression of other markers specific for classically and alternatively activated macrophages, total RNA was isolated from PEC and the expression of mRNA encoding for arginase, relm-α, PD-L1, PD-L2 and inducible nitric oxide (NOS2) was quantified by qPCR. The frequency of $CD11b^+F4/80^+$ cells expressing CD206 molecule in the peritoneal cavity significantly increased in TE-treated mice compared with PBS and LPS-treated mice (Fig. 5.18 A). There was also a trend towards increased expression of mRNA for arginase, relm-α, PD-L2 and PD-L1 in cells from TE-treated mice compared with cells from PBS-injected mice (Fig. 5.18 B). In contrast, only cells from LPS-treated mice enhanced expression of mRNA for NOS2. These results indicate that treatment with TE induces markers of alternative macrophages, which are expressed for at least 7 days after the last administration of TE.

In addition, PEC were stained for the markers of Treg cells to determine if the frequency of Treg cells was changed at the injection site 7 days after the administration of TE. The results presented in Fig. 5.19 showed that TE treatment did not increase the percentage of $Foxp3^+CD4^+$ T cells, however the percentage of IL-10- and LAP-expressing $CD4^+$ T cells was not significantly increased compared with PBS-injected mice. TE treatment increased expression of LAP by both $Foxp3^+$and $Foxp3^-CD4^+$ populations. This result indicates that TE injected using a prophylactic regime induces IL-10 and LAP-producing Treg cells that are present at the site of injection 7 days after the last injection of TE.

5.2.10 TE treatment suppresses cytokine production from peritoneal cavity cells stimulated with M.tb or LPS

The results presented in Fig. 5.5 and Fig. 5.6 demonstrate that prophylactic treatment with TE protects against development of EAE for more than 2 weeks post induction of EAE. CFA used for induction of EAE contains heat killed Mtb, a source of PAMPs that induce a strong inflammatory response, which consequently leads to the development of MOG-specific Th1 and Th17 cells. The experiments also showed that administration of TE changed the cellular composition and induced the markers of alternatively activated macrophages at the site of injections for at least 7 days after the last injection (Fig. 5.15, Fig. 5.18). Therefore, experiments were conducted to examine the innate response of PEC from TE-treated mice to stimulation with Mtb or LPS *ex vivo*. Mice were injected with PBS, TE or LPS at day 0 and 14. After 7 days PEC were isolated by peritoneal lavage and cultured with LPS or heat killed Mtb for 24 h. Supernatants were collected and concentrations of IL-1β, IL-23, IL-12p70, TNF-α, IL-17 and IL-10 were determined by ELISA.

PEC from mice treated with TE and LPS produced less TNF-α, IL-1β and IL-17 in response to *ex vivo* stimulation with LPS or Mtb than cells from PBS-treated mice (Fig. 5.20). The production of IL-12 by PEC was similar in both PBS- and TE-treated mice but significantly lower by PEC isolated from LPS-injected mice. PEC from TE-treated mice produced similar amounts of IL-10 without stimulation and in response to LPS or Mtb in comparison with cells from PBS-treated mice. These results indicate that TE treatment suppresses pro-inflammatory cytokine production from PEC in response to LPS and Mtb stimulation. The results also demonstrate that treatment with TE changes the response of PEC to inflammatory stimuli differently than administration of LPS.

5.2.11 TE directly incudes expression of alternatively activated macrophage markers by BMDM

During helminth infections, alternatively activated macrophages develop in response to Th2-type cytokines IL-4 and IL-13. The recent study of Adams et al demonstrated that *F. hepatica* tegumental coat antigens and ES can induce M2-like macrophages *in vivo* without engagement of IL-4 and IL-13 (317). TE is obtained from the whole liver fluke

and contains both tegumental antigens and component of ES. Therefore, experiments were conducted to examine the ability of TE to directly induce an M2-like phenotype in macrophages. BMDM were generated from C57BL/6 mice and cultured with different concentrations of TE for 1 h prior to the addition of LPS or cultured with LPS only. After 24 h the supernatants were collected to determine cytokine concentration by ELISA, whereas the cells were stained with antibodies against F4/80, PD-L1 and PD-L2. Total RNA was also isolated to examine expression of mRNA for arginase, relm-α and NOS2. Stimulation with TE enhanced expression of PD-L1 and PD-L2 in BMDM even when the cells were co-stimulated with LPS (Fig. 5.21 A). In addition, BMDM cultured with TE enhanced the expression of mRNA for arginase and relm-α but not for NOS2. In contrast, LPS induced the expression of mRNA for NOS2 (Fig. 5.21 B). These results indicate that TE directly induces the expression of markers characteristic for M2-like macrophages.

5.2.12 TE induces production of IL-10 by BMDM and BMDC

M2-like phenotype is associated with an increased expression of IL-10 by macrophages. Therefore, an experiment was carried out to determine if TE directly induces production of IL-10 by BMDM. In addition, the effect of TE on the expression of other anti-inflammatory and pro-inflammatory cytokines was examined in BMDM and BMDC. BMDM and BMDC from C57BL/6 mice were cultured with different concentration of TE (1-5 % v/v) or with LPS. Cytokine concentration was assessed after 24 h by ELISA. The results demonstrated that TE did not induce the production of IL-12p70 or IL-1β and induced only small amounts (less than 80 pg/ml) of IL-23 or IL-4 (Fig. 5.22 A) by BMDM. Negligible levels (less than 10 pg/ml) of IL-12p70 and no IL-23 and IL-4 was detected in the supernatants of TE induced BMDC (Fig. 5.23 A). Unstimulated BMDC produced small amounts of IL-1β (less than 100 pg/ml) and stimulation with TE did not induce the production of IL-1β (Fig. 5.23 A). Importantly, TE induced moderate amounts of IL-10 (more that than 100 pg/ml) by BMDM and small amounts of IL-10 by BMDC (Fig. 5.22 A, Fig. 5.23 A). However, the production of IL-10 by BMDC in response to TE stimulation was dose-dependent. After 24 h of culture BMDM produced high amounts of TGF-β. Interestingly, TE suppressed the production of TGF-β in a dose-dependent manner (Fig. 5.22 B).

Consistent with published studies (256, 318) and previous observations (Chapter 3, Fig. 3.1), LPS induced the production of IL-10 by BMDM and BMDC after 24 h of stimulation. Adult flukes were isolated from livers of naturally infected cows in a non-sterile environment, which raises the possibility that the increased production of IL-10 by BMDC and BMDM upon TE stimulation results from LPS contamination. Therefore, an LAL assay was performed on TE samples to exclude this possibility. The results of the LAL assay have shown that stock concentration of TE had an equivalent of 3.31 EU/ml +/- SD (Fig. 5.23 B). This corresponds to 0.07 EU/ml in samples stimulated with 5% v/v of TE. Since the maximum permissible endotoxin level in sterile water is 0.25 EU/ml (319), the working solution of 5% v/v TE used for the stimulation of BMDM and BMDC can be accepted as endotoxin free. Therefore, this result indicates that increased production of IL-10 by BMDM and BMDC stimulated with TE is not due to LPS contamination.

5.2.13 TE induces expression of co-stimulatory molecules by BMDC but not by BMDM.

Having established that TE does not potently induce expression of pro-inflammatory cytokines by BMDM or BMDC, an experiment was conducted to determine the direct effect of TE on the expression of co-stimulatory molecules by BMDC and BMDM. BMDC and BMDM from C57BL/6 mice were stimulated with different concentrations of TE for 1 h prior to the addition of LPS or cultured with LPS alone. After 24 h cells were stained with viability dye and antibodies against CD11c (BMDC), F4/80 (BMDM) and CD80, CD86, CD40 and MHC class II. The results have shown that TE did not induce cell death in BMDC or BMDM at the concentration of 2.5% and 5% (Fig. 5.24 A). Higher concentrations of TE directly induced expression of CD80 and MHC class II molecules on the surface of BMDC and only marginally increased the expression of CD40, but did not have any effect on expression of CD86. Pre-incubation of BMDC with TE decreased LPS-induced expression of CD80, CD86 and MHC class II molecules. (Fig. 5.24 B). In contrast, TE did not directly modulate the expression of co-stimulatory molecules by BMDM, but slightly decreased the expression of CD80 and CD86 stimulated by LPS (Fig. 5.24 B). These results indicate that TE does not directly

induced expression of co-stimulatory molecules by BMDM, but, to some extent, decreased TLR agonist induced expression of co-stimulatory molecules by BMDC.

5.2.14 TE directly induces expression of IL-10 and enhances TGF-β-mediated Foxp3 expression in CD4+ T cells

Infection with *F. hepatica* is associated with a recruitment or expansion of $Foxp3^+$ T cells at the site of the infection. However, the results have shown that *F. hepatica* TE induced higher frequencies of IL-10- and LAP-producing $CD4^+$ T cells, but not $Foxp3^+$ $CD4^+$ T cells at the site of the injection (Fig. 5.1). The results have also demonstrated that TE can directly modulate the phenotype of macrophages and DC. Therefore, further experiments were designed to determine the direct influence of TE on expression of IL-10, LAP and Foxp3 by $CD4^+$ T cells. Purified $CD4^+$ T cells were cultured with anti-CD3 and anti-CD28 in the presence of IL-2 and different concentrations of TE. After 72 h cells were stained with viability dye and with antibodies against CD3, CD4, IL-10, LAP and Foxp3 and analysed by flow cytometry. In contrast to BMDC and BMDM, TE induced cell death in $CD4^+$ at the highest concentration (5% v/v; Fig. 5.25 A). Lower concentrations of TE directly induced expression of IL-10 and LAP, but not $Foxp3^+$ by $CD4^+$ T (Fig. 5.25 B).

Chronic helminth infections are associated with a robust production of TGF-β from both hematopoietic and non-hematopoietic cells. This raised the possibility that even if relatively short-term treatment with TE does not directly induce expression of Foxp3 in $CD4^+$ T cells, it might synergize with TGF-β to enhance the generation of inducible Foxp3-expressing $CD4^+$ T cells. Since RA is known to potently synergize with TGF-β and IL-2 to enhance expression of Foxp3 in $CD4^+CD25^-$ T cells, the experiment was designed to compare the capacity of TE versus RA to enhance the expression of Foxp3 in the presence of IL-2 and TGF-β. $CD25^-CD4^+$ were purified from spleens of C57BL/6 mice and cultured with anti-CD28 and anti-CD3 in the presence of IL-2, different concentrations of TGF-β alone, with TGF-β and RA or with TGF-β and 1% (v/v) of TE. After 72 h cells were stained with viability dye and antibodies against CD3, CD4, CD25 and Foxp3. The results presented in Fig 5.26 demonstrated that TGF-β with IL-2 increased the frequency of $CD4^+CD25^+Foxp3^+$ T cells in a dose-dependent manner and that RA synergized with TGF-β and IL-2 to enhance the frequency of converted $Foxp3^+$ T cells. Importantly, TE directly enhanced the percentage of $CD25^+Foxp3^+CD4^+$ T cells

in the presence of TGF-β and IL-2. These results indicate that TE can directly synergize with TGF-β and IL-2 to enhance the generation of inducible Foxp3$^+$ Treg cells.

5.3 Discussion

There is a large body of experimental and clinical evidence indicating that immunoregulatory mechanisms induced by helminth parasites can protect against autoimmune diseases. Due to psychological barriers or possible side effects associated with infection with parasites, helminth products rather than live helminths, have greater potential to be used as therapeutics in humans. The best characterized helminth product is a soluble preparation of egg antigens from *Schistosoma mansoni* (SEA), which has been shown to prevent the development of diabetes in NOD mice, TNBS-induced colitis and to reduce the severity of EAE in mouse models (235, 309, 320, 321). In addition, the unpublished data of Finlay, Stefańska and Mills have shown that administration of *F. hepatica* ES effectively suppressed EAE, DSS-induced colitis by inhibiting Th1 and Th17 responses. The present study explored the potential of prophylactic administration of *F. hepatica* products to prevent the developmentCha of autoimmune disease.

The key finding of this study was the demonstration that the prophylactic administration of total soluble extract from whole liver flukes prevented the development of EAE in mice. The protective effect was maintained for almost 3 weeks after the induction of EAE. The results have demonstrated that the inhibition of EAE onset was independent of immunization with MOG antigen, which indicates that the protection is based on the mechanisms of bystander suppression. The experiments confirmed that *F. hepatica* TE does not have adjuvant activity, when administered with exogenous antigen.

Interestingly, the lymph node cells from mice treated with TE produced less cytokines in response to stimulation with PMA and anti-CD3 7 days after the last administration of TE, which suggests that TE treatment results in a prolonged inhibition of polyclonal T cell responses. Since the impaired T cell responses were observed after stimulation that is independent of APC function, this indicates that the hyporesponsiveness arises from a defective T cell function. Importantly, the inhibitory effect seems to be selective for pro-inflammatory cytokines, since the production of IL-10 in response to PMA and anti-CD3 was not inhibited. The suppression of PMA-induced T cell responses in the lymph node cells was not associated with a higher frequency of IL-10- or TGF-β-producing or Foxp3-expressing Treg cells, which suggests that the inhibition is a result of selective hyporesponsiveness rather than an active suppression by Treg cells. It is still to be determined, whether TE suppresses T cells directly or indirectly through the generation of immunosuppressive DC or other innate cells.

Administration of TE significantly enhanced the numbers of IL-10-producing T cells in the spleen when assessed 7 days after the last administration. Since the development of pathogenic autoreactive Th1 and Th17 T cells occurs in the lymph nodes and spleen, the findings of impaired IL-17 and IFN-γ production by lymph node T cells and the enhanced frequency of IL-10-producing T cells in the spleen may explain the a significantly delayed generation of autoimmune responses in mice treated with TE. In fact, the production of MOG-specific IL-17 and IFN-γ was decreased by the lymph node cells of mice protected against EAE. In addition, analysis of cells present in the CNS of mice with EAE have revealed that TE treatment was associated not only with a reduced accumulation of leukocytes in the brains but also with decreased production of IL-17 and IFN-γ by $CD4^+$ T cells infiltrating the brain. This result supports the hypothesis that administration of TE inhibits the pathogenic immune responses in the periphery.

Studies demonstrated that mice infected with helminth parasites or treated with helminth products had less severe symptoms of autoimmune diseases. The attenuation was associated with suppression of auto-antigen-specific Th1 and Th17 responses by generation of highly immunosuppressive environment in the peripheral lymphoid and non-lymphoid tissues (312, 321). Infection with live helminth parasites or administration of helminthic antigens and IMs modulates the activity of DC and inhibits their ability to prime Th1 and Th17 responses. Certain helminth products are also able to induce tolerogenic DC, and abrogate their ability of respond to TLR stimulation, which consequently leads induction of Treg cells by DC (322). Studies have shown that autoimmune disease can be suppressed by the constant presence of live helminths or by repeatedly injecting helminth products before and after the induction of the disease (312, 323). Strikingly, in the present study, the protection against development of EAE was achieved by the prophylactic administration of TE. This comprised of only two injections of TE and the protection was maintained for as long as 3 weeks after the last administration of TE. This indicates that the products present in *F. hepatica* TE generate a prolonged change in phenotype and function of immune cells.

The analysis of the immune cell populations in mice treated with TE showed that there was an increased number of cells accumulated at the site of the injection even 7 days after the last administration of TE. Eosinophils constituted the majority of cells, which in not unexpected, since helminth products induce robust Th2-type responses. In contrast to LPS, treatment with TE did not result in higher a frequency of neutrophils. TE also did

not increase the frequency of infDC or the expression of MHC class II molecules by macrophages, confirming that, in contrast to LPS, TE does not induce pro-inflammatory responses.

Alternatively activated macrophages, also known as M2a macrophages, differentiate in response to Th2-type cytokines IL-4 and IL-13. In contrast to classically activated macrophages, M2a macrophages exert potent anti-inflammatory activity, participating in wound-healing and fibrosis and antagonize M1 macrophage responses. Mannose receptor CD206, arginase, relm-αand co-inhibitory surface molecule programmed death ligand 2 (PD-L2) are the characteristic markers expressed by alternatively activated macrophages. Although PDL-1 has been shown to be expressed by M1 macrophages, recent studies have identified that the interaction of PD-L1 with its ligand PD-1 in lymphocytes inhibits the inflammatory functions of macrophages (324). Adoptive transfer of *ex vivo* activated M2 macrophages has been shown to suppress ongoing EAE in mice (325). Alternative activation of macrophages has been described to be induced during infections with helminth parasites and in response to *in vivo* stimulation with helminth products, including *F. hepatica* tegumental antigens (317). Moreover, during helminth infections macrophages upregulate expression of the co-inhibitory molecules PD-L1 and PD-L2, which leads to reduced T cell activation via ligation of PD-1 receptor on T cells (326, 327). The results of this study showed that 7 days after the administration of TE macrophages had increased expression of M2-like macrophages markers. PD-1 and PD-L1 play a critical role in immunological tolerance. PD-L1-deficiency in 129S4/SvJae mice converted them from resistant to susceptible to the development of EAE (328). Results from the present study demonstrated that TE directly induced the expression of M2-like macrophage markers and low levels of IL-10 *in vitro*, but did not enhance the expression of co-stimulatory molecules in BMDM. The activation of an M2-like phenotype in macrophages that sustain high expression of immunosuppressive molecules might be one of the mechanisms of TE-mediated protection against the development of EAE.

Despite the decreased accumulation of total immune cells in the CNS of TE-treated mice with EAE, there was an increased relative frequency of eosinophils and macrophages infiltrating the brains. It is possible that the macrophages accumulating in the CNS have migrated from the periphery or have differentiated from blood monocytes, exhibited M2-like phenotype and were able to suppress Th1 and Th17 responses developing in the

brain. The tissue-repairing potential of alternatively activated macrophages in the CNS can also play a significant role in the suppression of clinical signs of EAE. This hypothesis is supported by the studies exploring the effects of M2 macrophage function on CNS remyelination and clinical course of EAE. The increased proportion of M2 vs M1 macrophages in the brains was associated with attenuation of neurological symptoms of EAE in mice (329). The recent study by Miron et al demonstrated that M2 macrophages and M2 microglia present in the CNS induce oligodendrocyte differentiation and promote effective remyelination after chemically induced demyelination in the brain of mice (330). Moreover, the significantly elevated numbers of M2 macrophages were found in acute active lesions and in the rim of chronic active lesion of MS patients. These are areas of recent damage, where remyelination is expected to happen (330).

Eosinophils are considered as the end-stage effector innate cells that play a major role in defence against helminthic infections by the release of cytotoxic components through degranulation. However, the studies have revealed additional roles for eosinophils as immunoregulators of both adaptive and innate responses (331). Recent data indicate that during chronic nematode *Trichinella spiralis* infection eosinophils are important source of IL-10. Eosinophil-derived IL-10 expands IL-10-producing myeloid DC and IL-$10^{+}CD4^{+}$ T cells that inhibit inducible NO synthase expression increasing the survival of intracellular larvae (332). Therefore, the function of accumulating eosinophils in the CNS of mice treated with TE should be further examined as a potential mechanism of attenuation of EAE.

Since treatment with TE changed the phenotype of innate cells at the site of the injection and this effect was maintained after 7 days, this raised the possibility that innate cells from TE-treated mice respond differently to stimulation with pro-inflammatory stimuli. Indeed, cells from TE-treated mice produced less pro-inflammatory cytokines in response LPS or Mtb stimulation *in vitro*. However, a comparison of the production of cytokines and their relevance *in vivo* is difficult, since there was a significant difference in the frequency of innate cells in the peritoneal cavity of mice injected with PBS or TE. This indicates that the decreased production of inflammatory cytokines by cells from TE-treated mice might be a result of the lower frequency of TLR-responsive innate cells, such as DC and macrophages, or a result of their functional changes. In contrast, although LPS induced higher frequencies of inflammatory innate cells than TE, the *ex vivo*

stimulation of peritoneal cavity cells from LPS-treated mice with LPS or Mtb also resulted in a decreased production of inflammatory cytokines, a complete inhibition of IL-12 production but an increased production of IL-10 compared with cells from PBS-injected mice. This result might be associated with phenomenon known as endotoxin tolerance. During this process, after the first exposure to LPS and initial stimulation phase, monocytes and macrophages enter a phase of incapacity to produce inflammatory cytokines. They also upregulate production of anti-inflammatory cytokines IL-10, TGF-β and IL-1RA in response to LPS re-challenge (274, 333). The tolerant monocytes and macrophages also show impaired antigen-presenting capacity and expression of co-stimulatory molecules. Clinically, the state of endotoxin tolerance is associated with immunosuppression of immune responses in patients with sepsis, which contributes to high mortality (334).

Infections caused by particular pathogens, such as *Candida albicans*, or vaccinations with Bacille Calmette-Guerin (BCG) or measles are associated with long-term enhancement of monocyte and macrophage responses to microbial stimuli. This is known as innate immune training and contributes to increased immune responses to the unrelated infections (335). Recent studies have revealed that the molecular mechanisms underlying innate immune tolerance or training are based on epigenetic and transcriptional changes of expression of genes associated with PRRs. For example, NF-κB, inflammasome and metabolic proteins are differently regulated in trained and tolerant macrophages (274). Moreover, studies of gene expression in human PBMCs have also indicated that endotoxin tolerance is associated with induction of genes associated with M2 macrophage activation (333). Therefore, the prolonged protection against the development of EAE after prophylactic treatment with TE might be associated with induction of a certain type of innate immune tolerance in macrophages. To explore this hypothesis, the purified populations of innate cells from PBS, TE and LPS-treated mice rather than whole PEC should be stimulated *ex vivo* with LPS or Mtb. This approach would allow a quantitative comparison of the levels of pro- and anti-inflammatory cytokines produced by innate cell populations in the presence of inflammatory stimuli.

The results of the present study showed that TE directly induced expression of IL-10 by $CD4^+$ T cells *in vitro*. This is in agreement with *in vivo* data demonstrating that repeated administration of TE enhanced the frequency of IL-10-expressing $CD4^+$ T cell at the site of injection. However, no significant differences in frequencies of Treg cells were

observed at the site of injection 7 days after the last TE administration. In contrast, enhanced percentages of IL-10^{+}CD4^{+} T cells were detected in the spleen of TE-treated mice. These results suggest that IL-10-producing T cells might be induced in the spleen by tolerogenic DC, but the constant presence of helminth antigens or IMs is required for enhanced expression of IL-10 at the site of TE injection. Although *in vitro* results demonstrated that TE synergized with TGF-β to enhance Foxp3 expression by CD4^{+} T cells, the percentages of Foxp3^{+}CD4^{+} T cells were not increased in the injection site or in the spleen after *in vivo* treatment with TE. It is possible that the effective induction of Foxp3 by CD4^{+} T cells occurs only during the constant presence of helminth products during infection with live flukes, which leads to increased production of TGF-β by various hematopoietic and non-hematopoietic cells.

F. hepatica TE contains both the tegumental coat proteins, ES products, which normally are secreted outside of the fluke's body or shed from the body surface, and all the metabolic and structural proteins and other molecules that compose a fluke's body. Tegumental antigens (FhTeg) and ES have separately been shown to exert strong immunomodulatory properties. FhTeg and two major ES components, cathepsin L and sigma class gluthatione transferase, suppress DC maturation and function, resulting in a decreased capacity to induce Th1-type and Th17-type responses (322, 336). In addition, the recent study by Adams et al demonstrated that FhTeg and ES both induce alternatively activated macrophages *in vivo.* While ES has the capacity to directly induce arginase and IL-10-producint macrophage population, FhTeg directly induces expression of relm-α alone in macrophages. The results of the present study showed that *F. hepatica* TE enhanced the expression of M2 markers in macrophages *in vivo* and directly *in vitro*, demonstrating that TE combines the immunomodulatory properties of FhTeg and ES. Therefore, TE might be a more appropriate target for further fractionation to establish the most effective composition of antigens and IMs that can be used as immunotherapeutics for autoimmune diseases. Taken together, this study demonstrated for the first time that the prophylactic treatment with products of a helminth parasite can prevent the development of autoimmune disease and these data represent a foundation for further investigation of the mechanism of TE- induced immunoregulation.

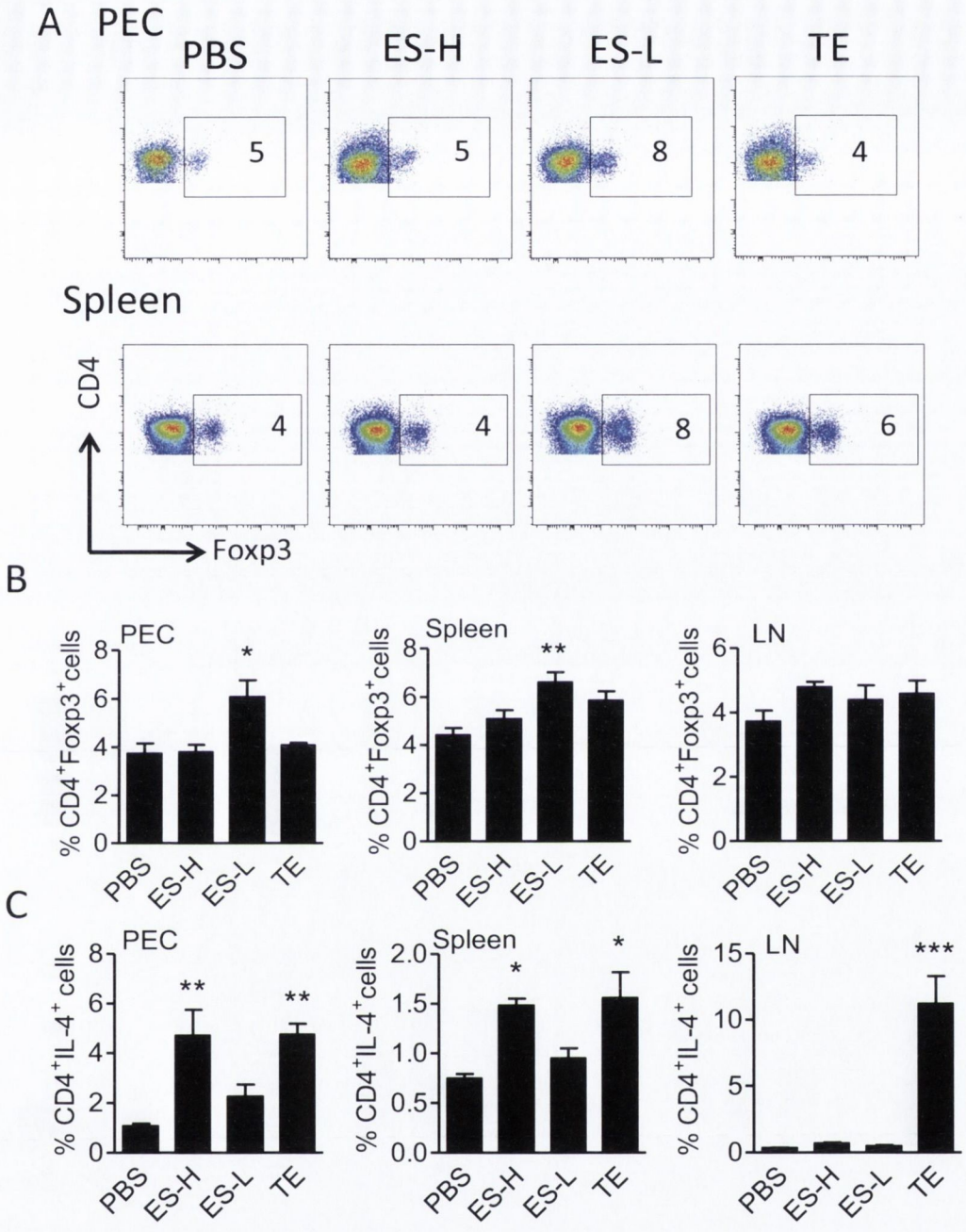


Fig. 5.1 Effect of repeated administration of ES-H ES-L and TE to mice on expression of Foxp3 and IL-4 by CD4⁺ T cells in PEC, spleen and lymph nodes. C57BL/6 mice were injected i.p. with PBS, ES-H (50 μg/ml), ES-L (50 μl) or TE (50 μg/ml) at day 0, 4, 8 and 12. 4 days after the last injection PEC, spleens and mediastinal lymph nodes were isolated and single cell suspensions were stimulated with PMA and inonomycin in the presence of BFA for 5 h. Cells were stained with antibodies against CD4, nuclear Foxp3 and intracellular IL-4 and analyzed by flow cytometry. Results are representative plots for mice groups (A), the mean frequency of $CD4^+Foxp3^+$ cells (B) and $CD4^+IL\text{-}4^+$ cells (C) +/- SEM (n=4/group). *p<0.05, **p<0.01, ***p<0.001 vs PBS by ANOVA and Bonferroni post test.

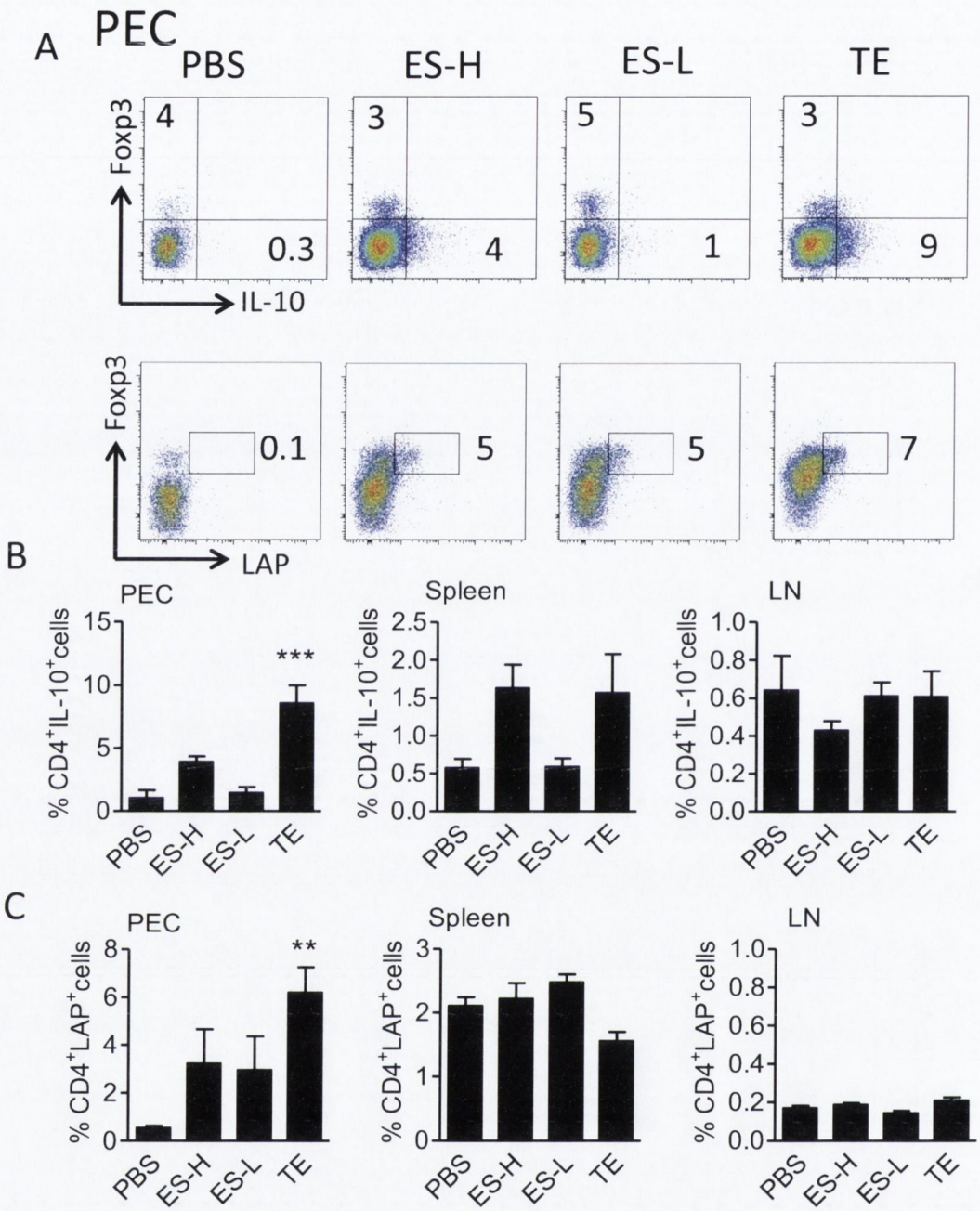


Fig. 5.2 Repeated administration of TE enhances expression of IL-10 and LAP by peritoneal CD4⁺ T cells. C57Bl/6 mice were injected i.p. with PBS, ES-H (50 µg/ml), ES-L (50 µl) or TE (50 µg/ml) at day 0, 4, 8 and 12. 4 days after the last injection PEC, spleens and inguinal lymph nodes were isolated and single cell suspensions were stimulated with PMA and inonomycin in the presence of BFA for 5 h. Cells were stained with antibodies against CD4, LAP and intracellular IL-10 and analyzed by flow cytometry. Results are representative plots for mice groups (A), the mean frequency of $CD4^+IL\text{-}10^+$ cells (B) and $CD4^+LAP^+$ cells (C) +/- SEM (n=4/group). *p<0.05, **p<0.01, ***p<0.001 vs PBS by ANOVA and Bonferroni post test.

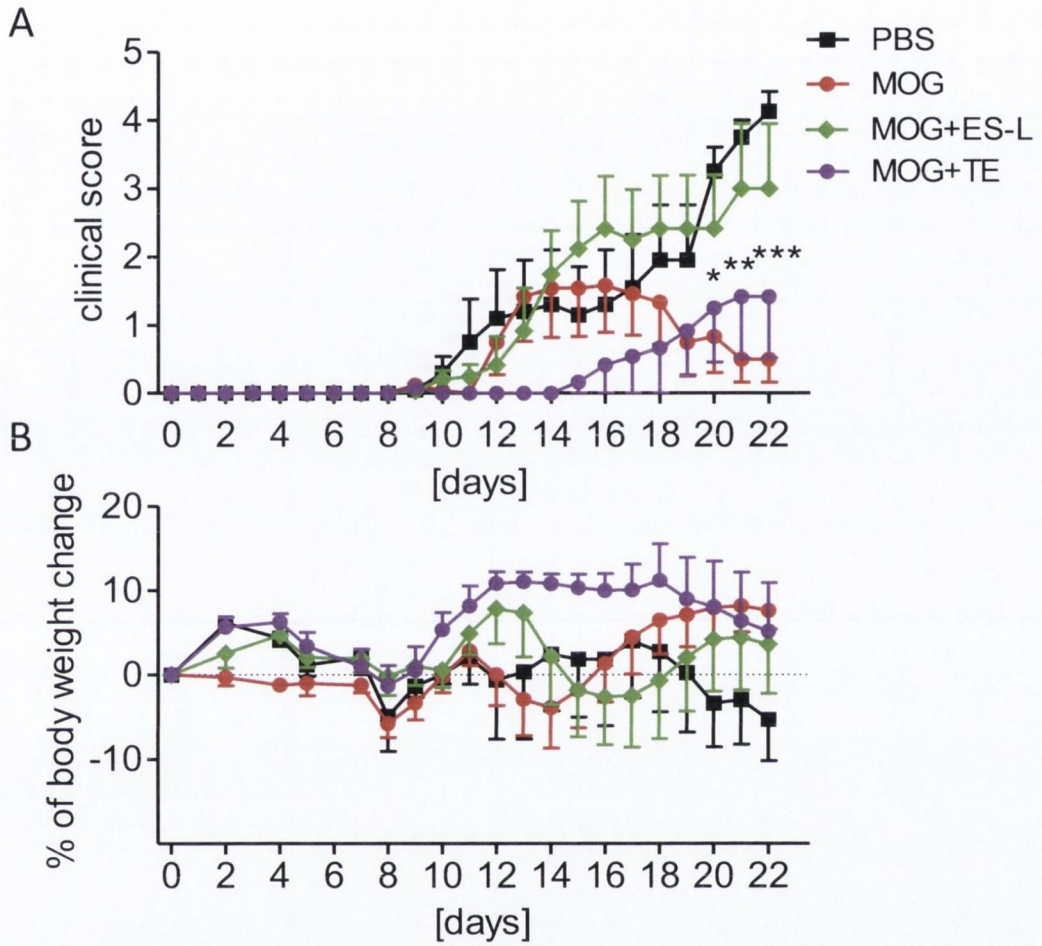


Fig. 5.3 Prophylactic immunization with MOG and TE delayed the onset and attenulated the clinical course of EAE. C57BL/6 mice were injected s.c. with PBS, MOG, MOG+ES-L or MOG+TE 21 days and 7 days before induction of EAE. EAE was induced by s.c. injection of MOG peptide emulsified with CFA, followed by i.p. injection of PT on day 0 (day of induction) and day 2. Animals were monitored daily for clinical signs of the disease. Results shown are mean scores (A) and percentage of body weight change (B) +/- SEM (n=6/group). *p<0.05, **p<0.01, ***p<0.001 vs PBS by ANOVA and Bonferroni post test.

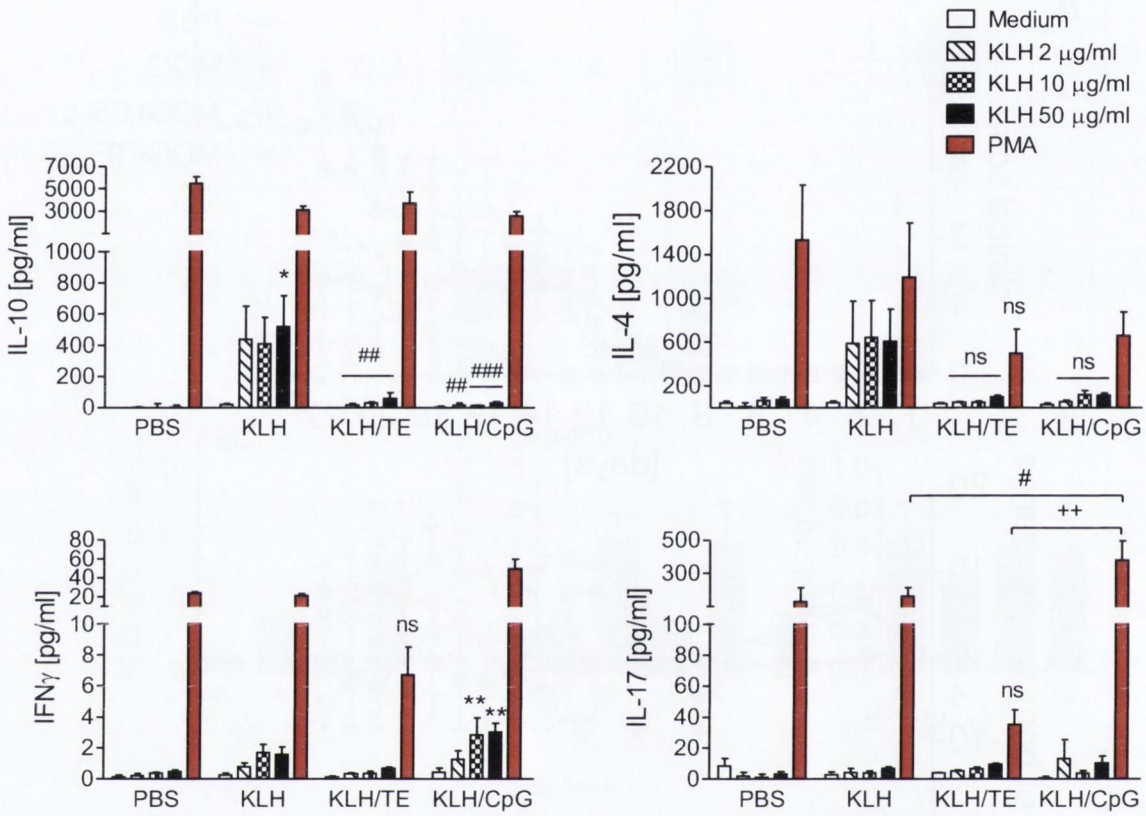


Fig. 5.4 Effect of immunization with KLH and TE on production of KLH-specific IFN-γ, IL-17, IL-10 and IL-4 by lymph node cells. C57BL/6 mice were s.c. injected with PBS, KLH (20 μg/mouse), KLH+TE or KLH+CpG (50 μg/mouse) and boosted 14 days later. 7 days after the last immunization lymph node cells were stimulated for 5 days with different concentrations of KLH at 2, 10 or 50 μg/ml or with PMA and anti-CD3. Supernatans were collected for analysis of IFN-γ, IL-17, IL-10 and IL-4 concentration by ELISA. Results represent mean concentrations +/- SEM (n=5/group). * $p<0.05$,** $P<0.01$ ***$p<0.001$ vs PBS group, #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs KLH group, ++$p<0.01$ vs KLH+CpG group, ns not significant vs KLH group by ANOVA

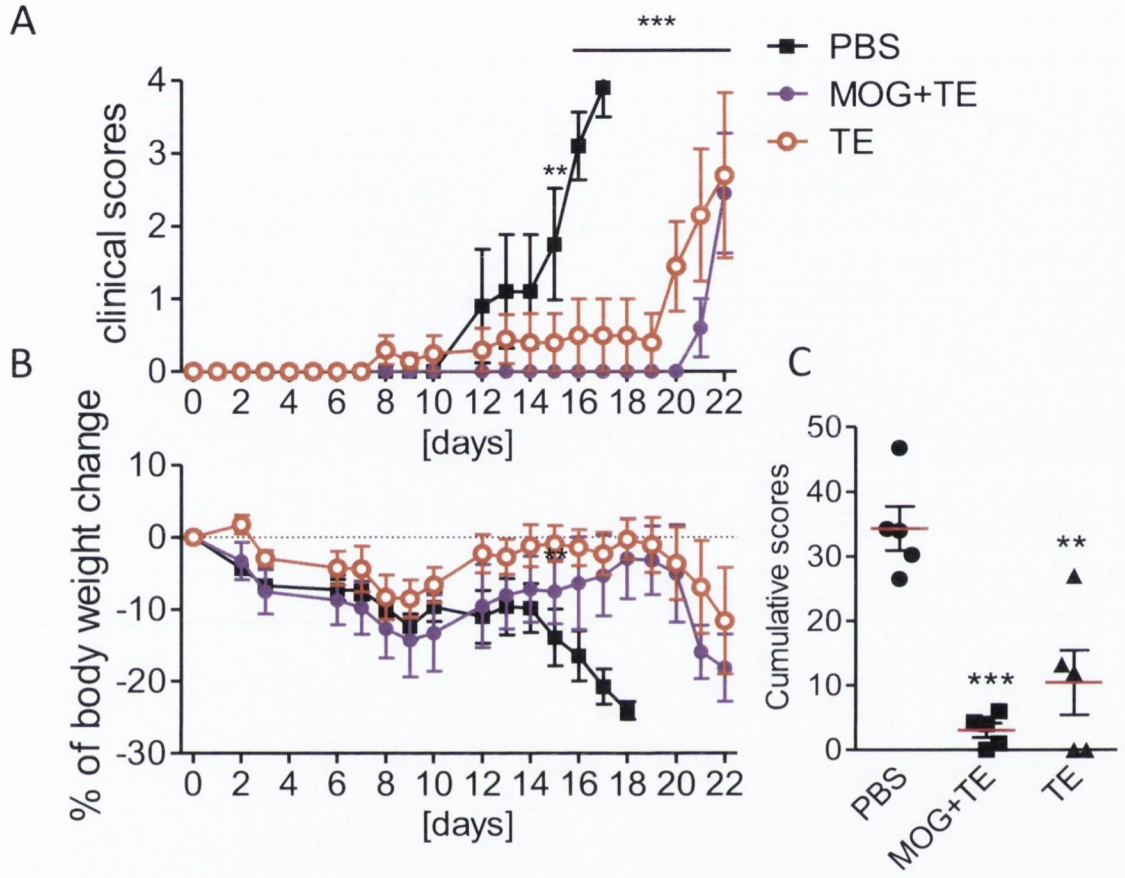


Fig. 5.5 Prophylactic treatment with TE prevents development of EAE. C57BL/6 mice were s.c. injected with PBS, MOG+TE or TE only 21 days and 7 days before induction of EAE. EAE was induced by s.c. injection of MOG peptide emulsified with CFA, followed by i.p. injection of PT on day 0 (day of induction) and day 2. Animals were monitored daily for clinical signs of the disease. Results shown are mean scores (A), percentage of body weight change (B) and cumulative scores (C) +/- SEM (n=5/group). **p<0.01, ***p<0.001 vs PBS by ANOVA and Bonferroni post test.

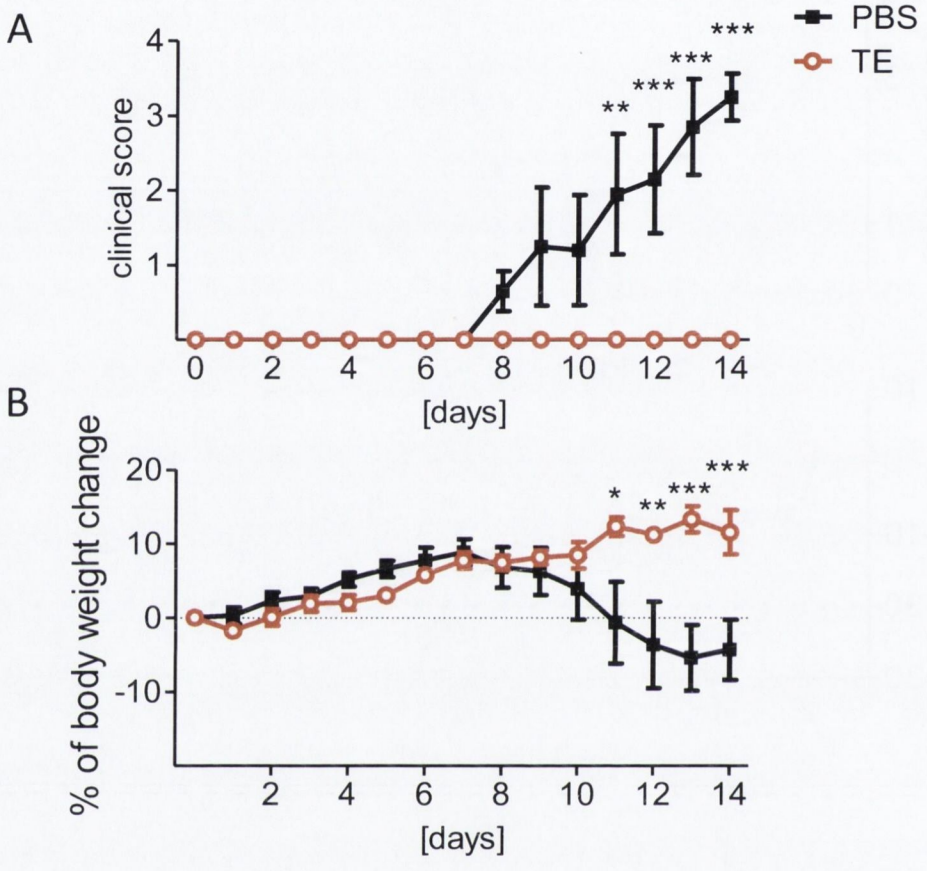


Fig. 5.6 Administration of TE before induction of EAE inhibits the development of EAE. C57BL/6 mice were s.c. injected with PBS or TE (50 μg) 21 and 7 days before induction of EAE. Animals were monitored daily for clinical signs of disease. Results shown are mean scores (A) and percentage of body weight change (B) +/- SEM (n=5/group). * $p<0.05$,** $P<0.01$ ***$p<0.001$ by two-way ANOVA and Bonferroni post-test.

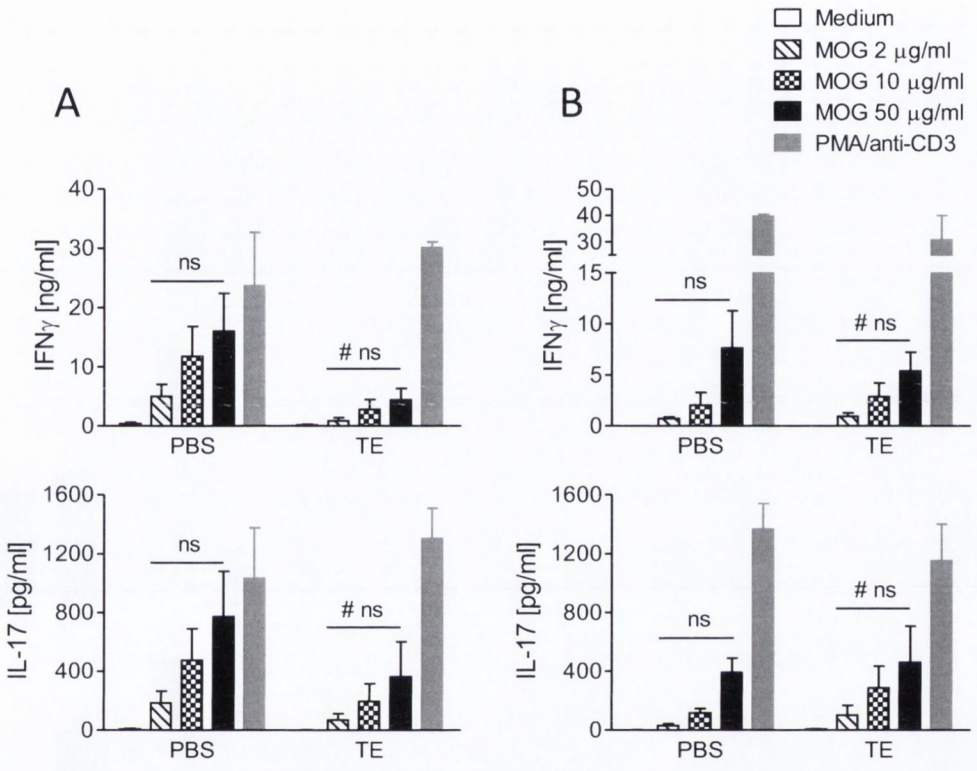


Fig. 5.7 Effect of administration of TE prior to the induction of EAE on the production of MOG-specific IL-17 and IFN-γ by lymph nodes and spleen cells. C57BL/6 mice were s.c. injected with PBS or TE (50 μg) 21 and 7 days before induction of EAE. Mice were sacrificed at day 14 post EAE challenge and single cell suspenions of lymph node (A) and spleen (B) were cultured with increasing concentrations of MOG (2. 10 and 50 μg/ml) or PMA (5 ng/ml) with anti-CD3 (1 μg/ml). Supernatans were collected after 5 days and the concentrations of IL-17 and IFN-γ were quantified by ELISA. Results shown are mean values +/- SEM (n=4-5/group), ns not significant vs medium, # ns, not significant vs PBS group by ANOVA

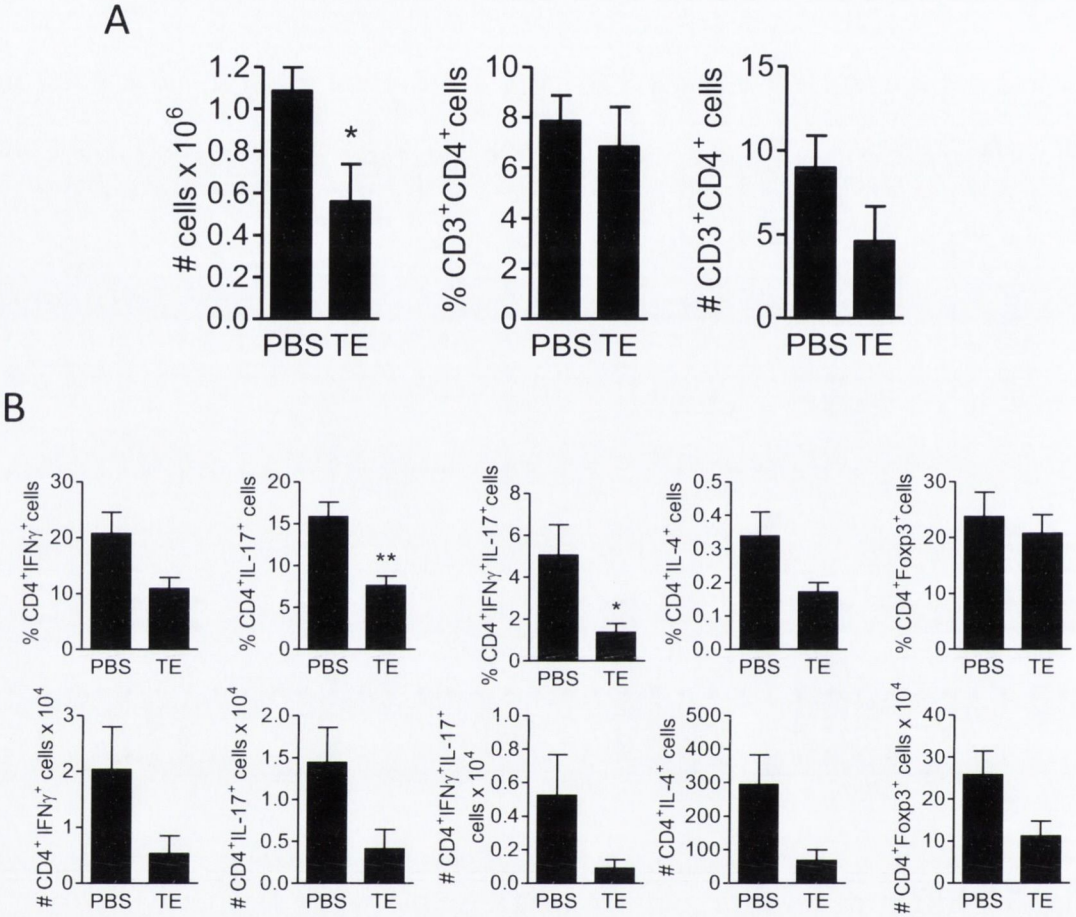


Fig. 5.8 Prophylactic administration of TE reduced the frequency and absolute number of IFN-γ- and IL-17-producing CD4⁺ in the CNS during EAE. C57BL/6 mice were s.c. injected with PBS or TE (50 μg) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains of perfused mice at day 14 post EAE challange, counted (A), Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h, stained with antibodies against CD3, CD4, Foxp3, IFN-γ and IL-17 and analyzed by flow cytometry (B). Results are mean frequency and absolute cell numbers +/- SEM (n=5/group); *p<0.05, **p<0.01 by t-test.

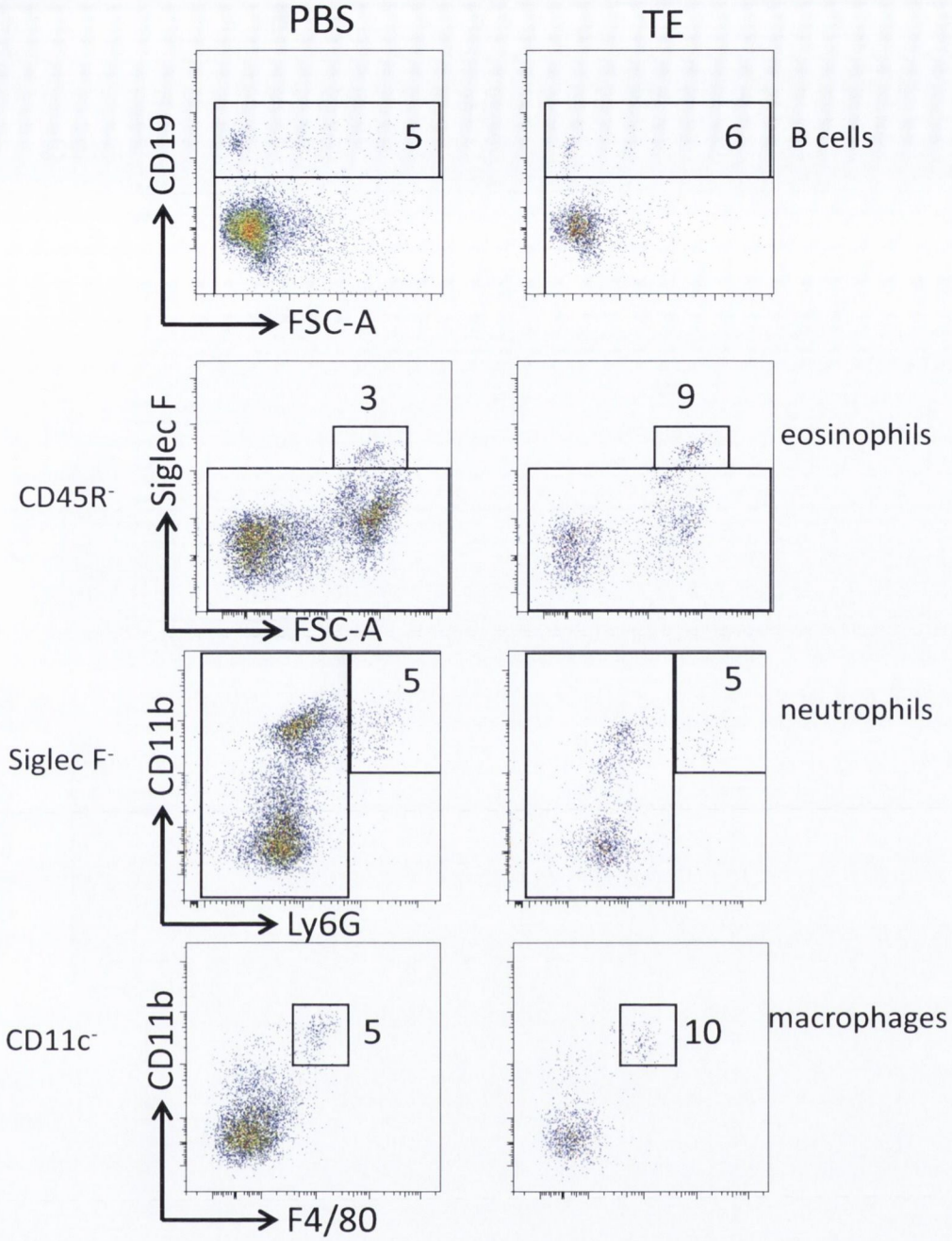


Fig. 5.9 Prophylactic administration of TE increased the frequency of CD11b⁺F4/80⁺ macrophages and eosinophils in the CNS during EAE. C57BL/6 mice were s.c. injected with PBS or TE (50 μg) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains of perfused mice at day 14 EAE challange. Cells were pooled into the respective groups and surface stained with antibodies specific for CD19, SiglecF, CD11b, Ly6G, CD11c, F4/80 and analysed by flow cytometry to determine the frequency of B cells (CD19⁺), eosinophils (Siglec F), and macrophages (CD11b⁺F4/80⁺) neutrophils (CD11b⁺Ly6G⁺). Plots shown present samples pooled from 5 mice (PBS) or 4 mice (TE) and are gated on live cells.

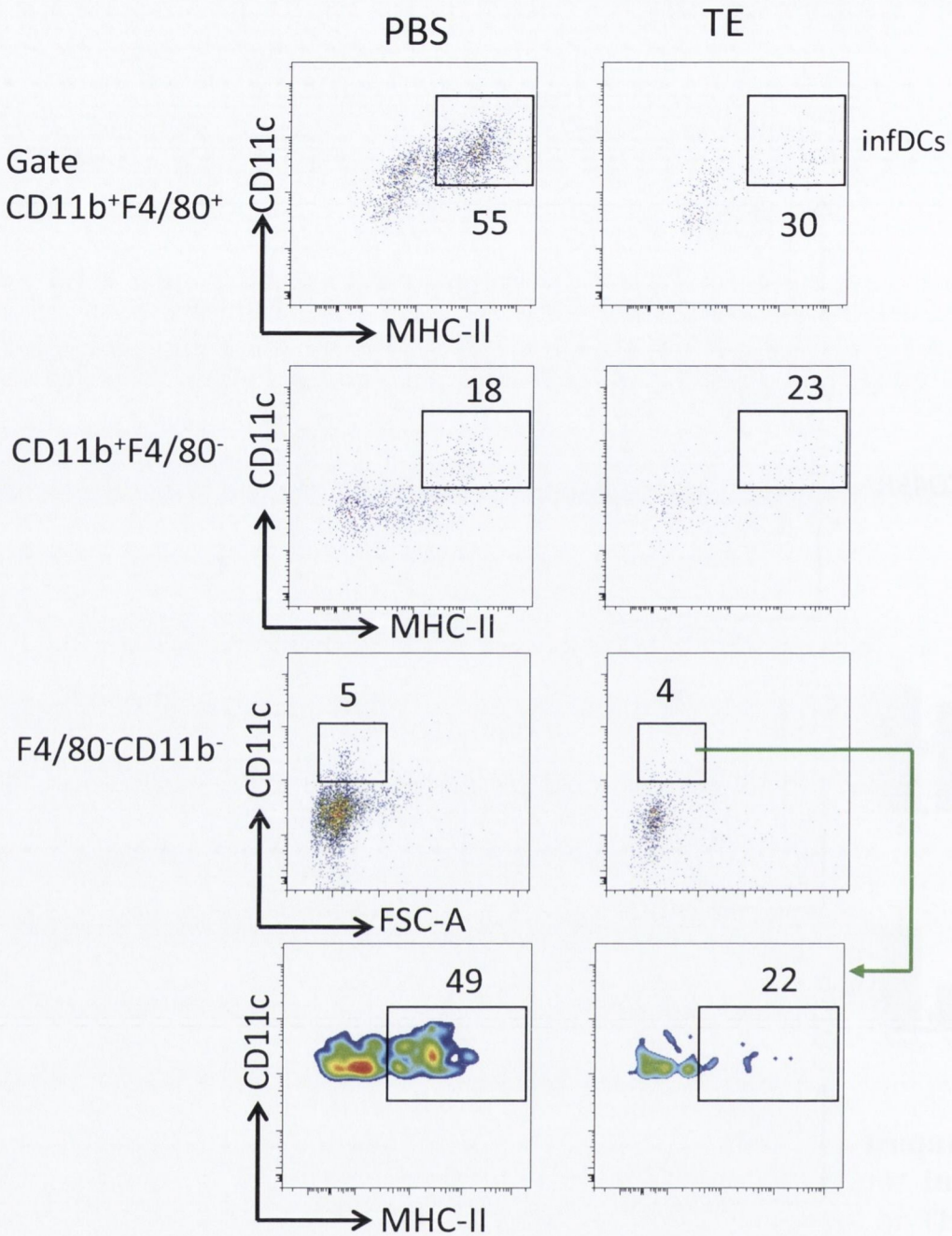


Fig. 5.10 Prophylactic administration of TE reduces the expression of MHC class II molecules on DCs in the CNS during EAE. C57BL/6 mice were s.c. injected with PBS or TE (50 µg) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains of perfused mice at day 14 post EAE challange. Cells were pooled into the respective groups and surface stained with antibodies specific for CD19, SiglecF, CD11b, Ly6G, CD11c, F4/80 and MHC class II and analysed by flow cytometry to determine the frequency MHC class II-expressing macrophages, monocytes and DC. Plots shown present samples pooled from 5 mice (PBS) or 4 mice (TE) and are gated on live cells.

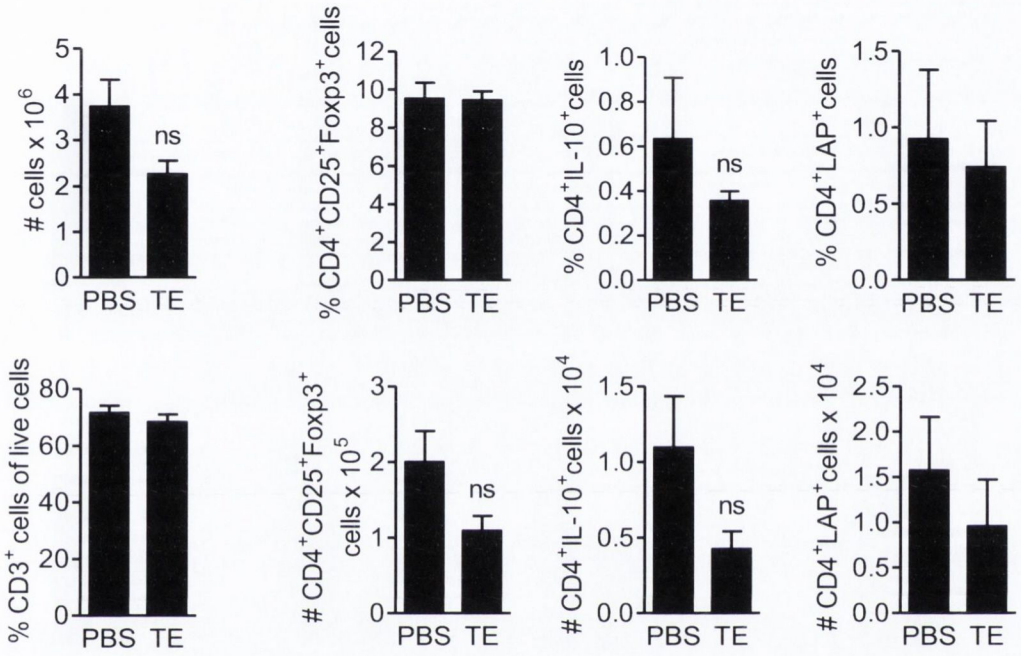


Fig. 5.11 Effect of TE administration on Foxp3, IL-10 and LAP production by CD4⁺ T cells in the lymph nodes. C57BL/6 mice were s.c. injected with PBS or TE and boosted 14 days later. 7 days after the last immunization single cell suspentions from inguinal lymph nodes were stimulated with PMA and inonomycin in the presence of BFA for 5 h. Cells were stained with antibodies against CD4, CD25, nuclear Foxp3 and intracellular IL-10 and analyzed by flow cytometry. Results are mean frequency and absolute cell numbers +/- SEM (n=5/group). ns not significant by t-test

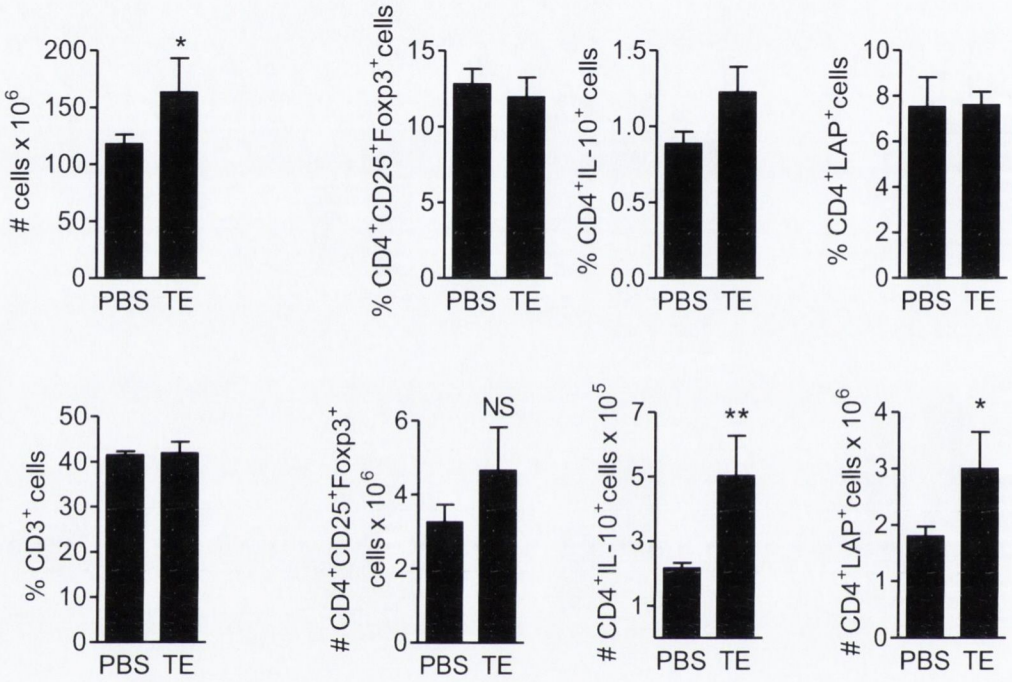


Fig. 5.12 Effect of TE administration on of Foxp3, IL-10 and LAP production by CD4+ T cells in the spleen. C57BL/6 mice were s.c. injected with PBS or TE and boosted 14 days later. 7 days after the last immunization single cell suspentions from inguinal lymph nodes were stimulated with PMA and inonomycin in the presence of BFA for 5 h. Cells were stained with antibodies against CD4, CD25, nuclear Foxp3 and intracellular IL-10 and analyzed by flow cytometry. Results are mean frequency and absolute cell numbers +/- SEM (n=5/group). *p<0.05, **p<0.01 by t-test.

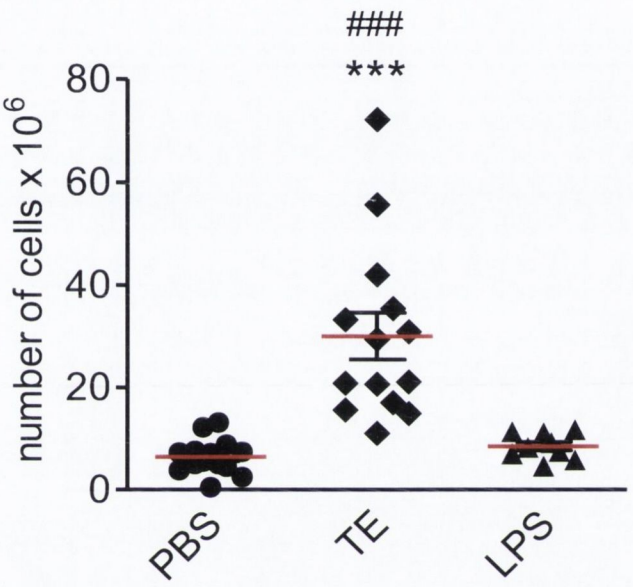


Fig. 5.13 TE treatment enhances accumulation of cells in the peritoneal cavity. C57BL/6 mice were i.p. injected with PBS or TE at day 0 and 14. After 7 days, PEC were isolated by peritoneal lavage and counted. Results shown are mean cell numbers +/- SEM from 3 independent experiments (n=9-14/group). ***p<0.001 vs PBS, ###p<0.001 vs LPS by ANOVA and Bonferroni post test.

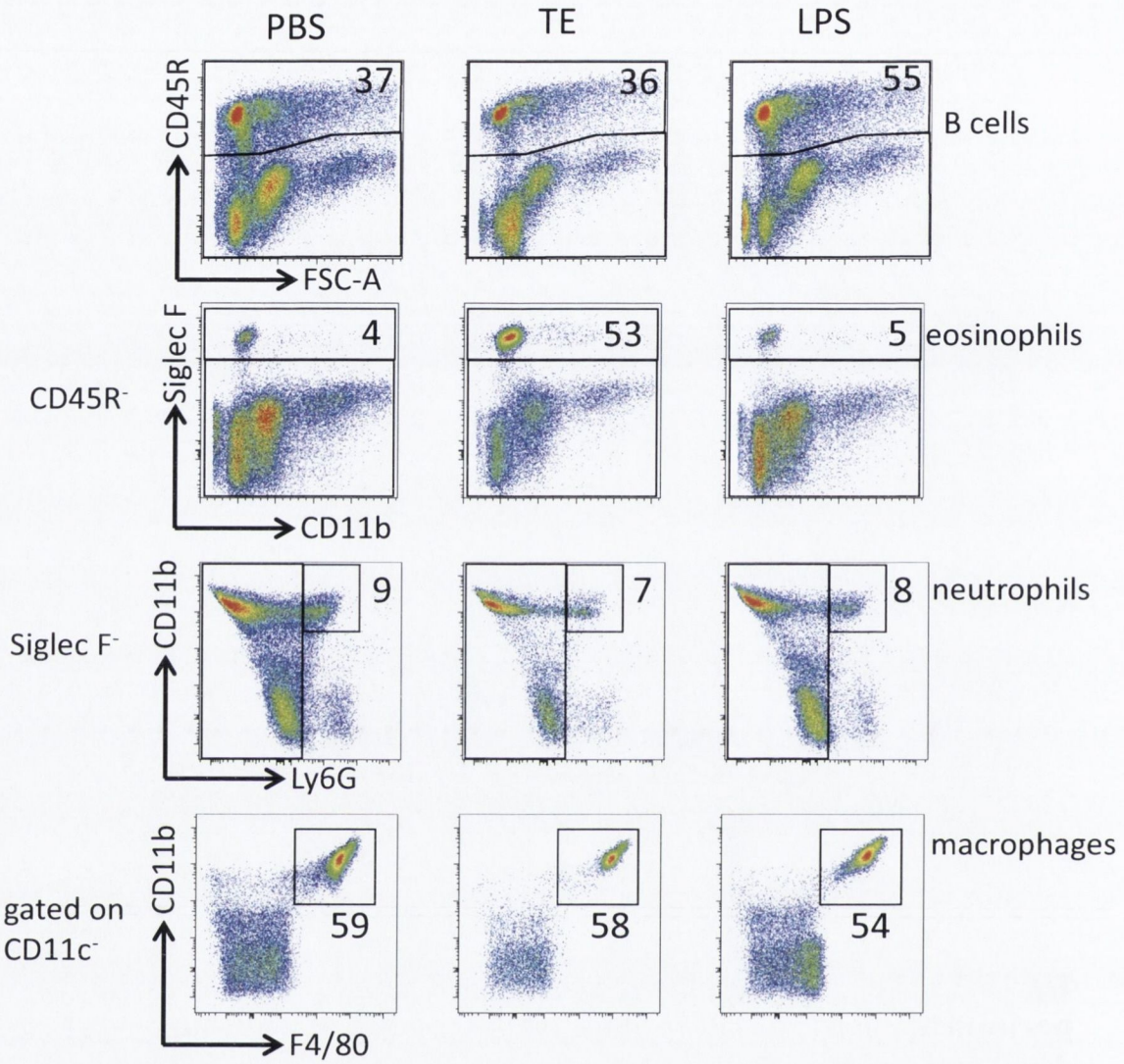


Fig. 5.14 Treatment with TE increased the frequency of eosinophils in the peritoneal cavity. C57BL/6 mice were s.c. injected with PBS or TE (50 μg) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains of perfused mice at day 14 of EAE. Cells were surface stained with antibodies specific for CD19, SiglecF, CD11b, Ly6G, CD11c, F4/80 and MHC class II and analysed by flow cytometry. Results are representative flow cytometry plots with freguencies of populations for each treatment group (n=5/group).

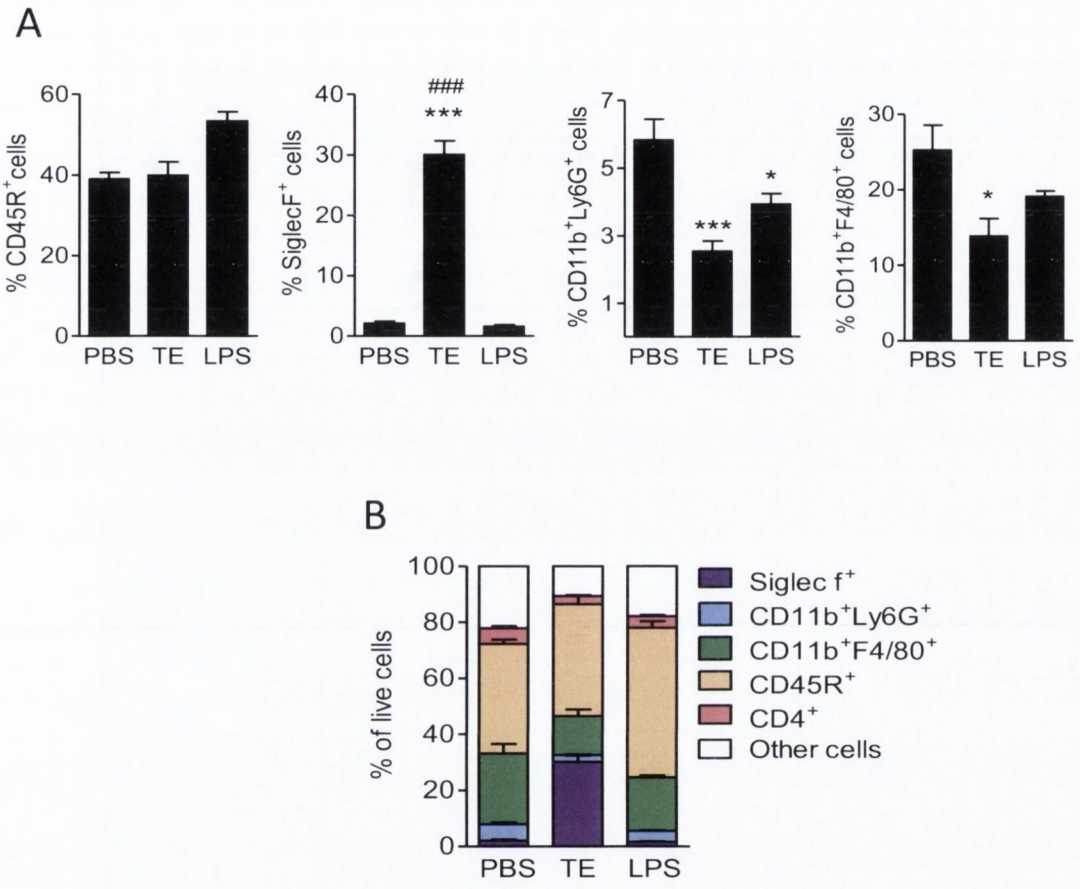


Fig. 5.15 Intraperitoneal administration of TE reduced the relative number of macrophages and neutrophils and increased the relative number of eosinophils in the peritoneal cavity. C57BL/6 mice were i.p. injected with PBS, TE or LPS at day 0 and 14. After 7 days, PEC were isolated by peritoneal lavage and, stained with antobodies againts CD4, CD45R, Siglec f, CD11b, Ly6G, CD11c, F4/80 and analysed by flow cytometry. Reults are mean percentages of total live cells in separate graphs (A) and combined graph (B) +/- SEM (n=5/group). *<p0.05, ***p<0.001 vs PBS, ###p<0.001 vs LPS by ANOVA and Bonferroni post test.

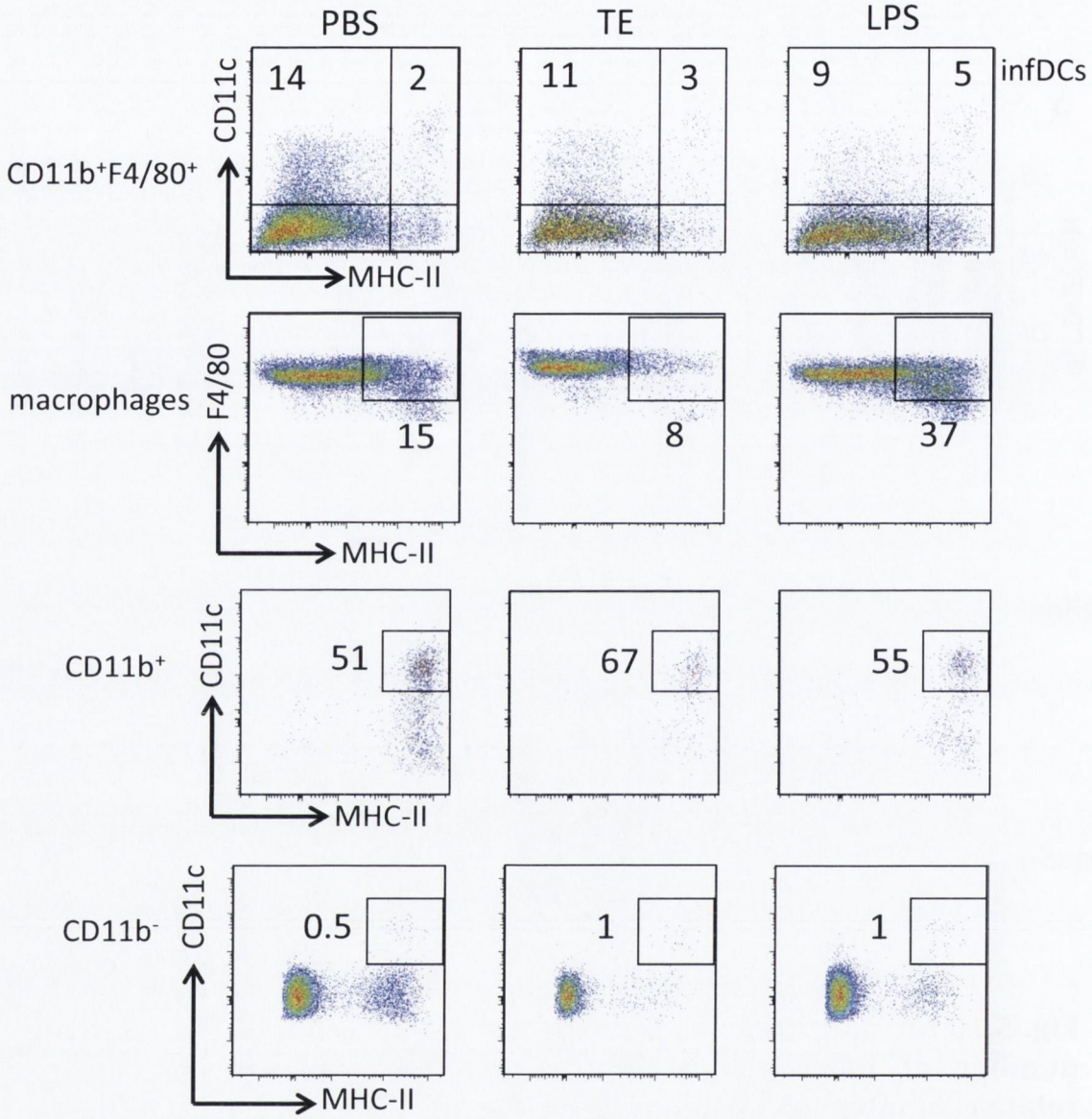


Fig. 5.16 Treatment with TE decreased expression of MHC class II molecules on DC and macrophages in the peritoneal cavity. C57BL/6 mice were s.c. injected with PBS or TE (50 μg) 21 and 7 days before induction of EAE. Mononuclear cells were isolated from brains of perfused mice at day 14 of EAE. Cells were surface stained with antibodies specific for CD19, SiglecF, CD11b, Ly6G, CD11c, F4/80 and MHC class II and analysed by flow cytometry. Results are representative flow cytometry plots with freguencies of populations for each treatment group (n=5/group).

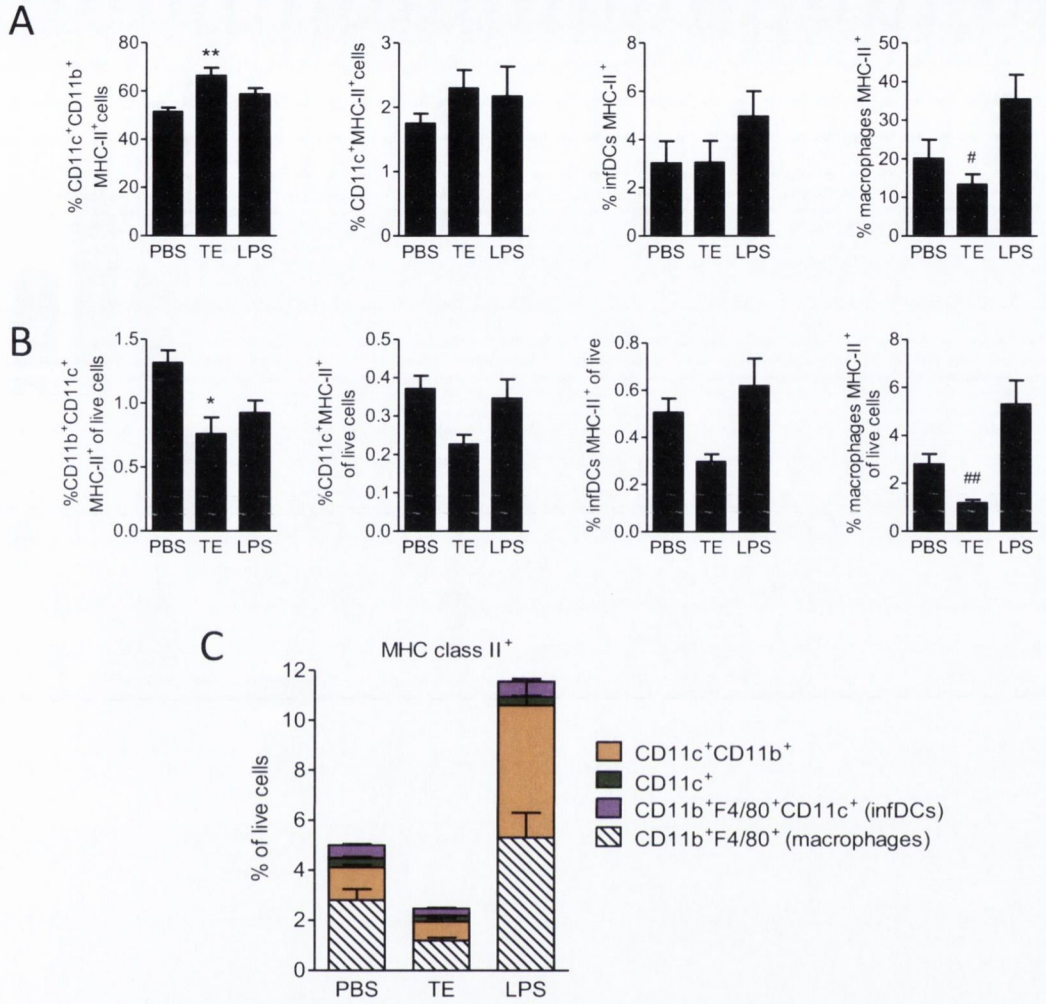


Fig. 5.17 Intrapeitoneal administration of TE reduces the relative number of MHC class II-expressing macrophages, DCs and other cells in the peritoneal cavity. C57BL/6 mice were i.p. injected with PBS, TE or LPS at day 0 and 14. After 7 days, PEC were isolated by peritoneal lavage and, stained with antobodies againts CD4, CD45R, Siglec f, CD11b, Ly6G, CD11c, F4/80 and MHC class II and analysed by flow cytometry. Reults are mean frequencies of MHC class II^+ cells of parent population (panel A) and mean percentages of total live cells in separate graphs (panel B) and in combined graph (C) +/- SEM (n=5/group). *<p0.05, ***p<0.001 vs PBS, #p<0.05, ##p<0.01 vs LPS by ANOVA and Bonferroni post test.

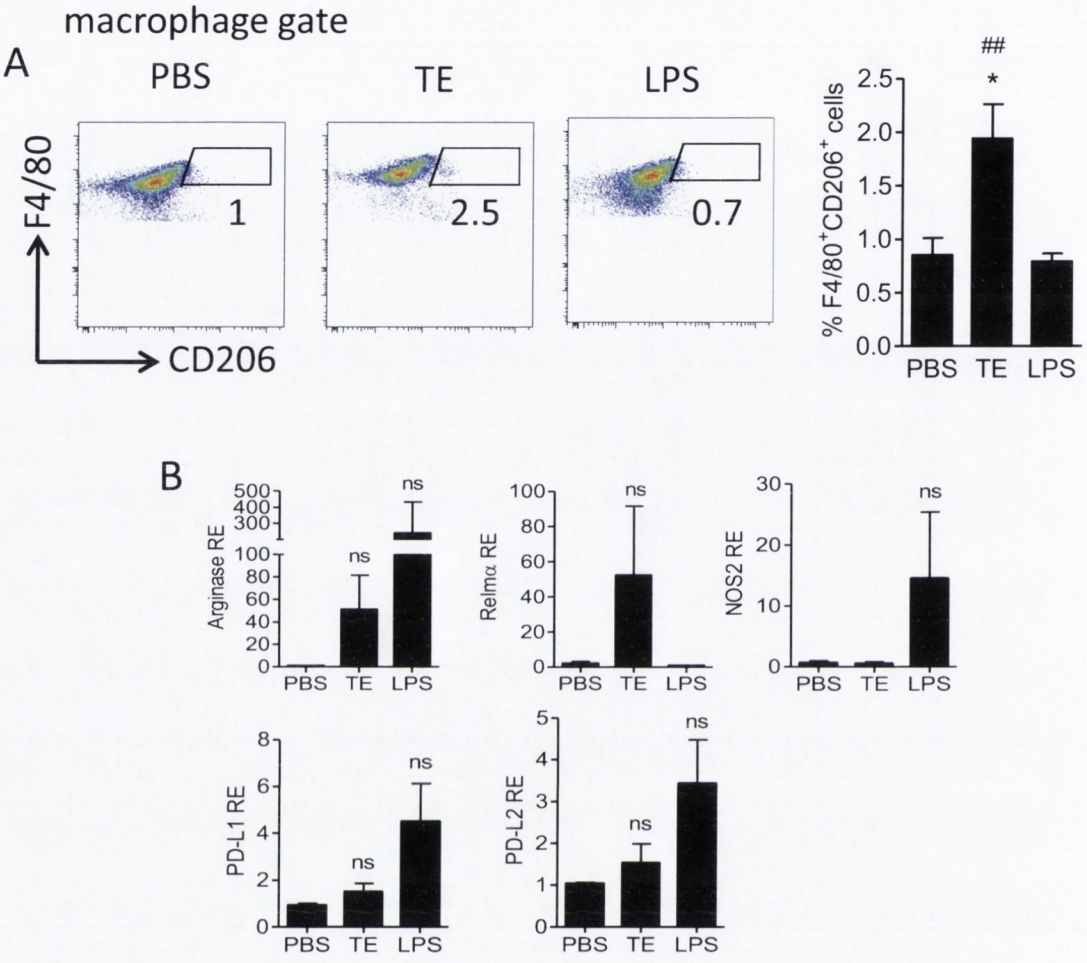


Fig. 5.18 Intraperitoneal administration of TE increased expression of markers of regulatory macrophages. C57BL/6 mice were i.p. injected with PBS, TE or LPS at day 0 and 14. After 7 days, PEC were isolated by peritoneal lavage. A. Cells were stained with antibodies againts CD4, CD45R, Siglec f, CD11b, Ly6G, CD11c, F4/80 and CD206 and analysed by flow cytometry. Results are representative plots for each mice group and the mean frequency cells +/- SEM (n=5/group). B Total RNA was isolated and expression of mRNA for arginase, relm-α, NOS2, PD-L1 and PD-L2 was analysed by qPCR. Results present mean relative expression (RE) +/- SEM (n=5/group). *p<0.05, vs PBS, ## p<0.01 vs LPS, ns not significant vs PBS by ANOVA

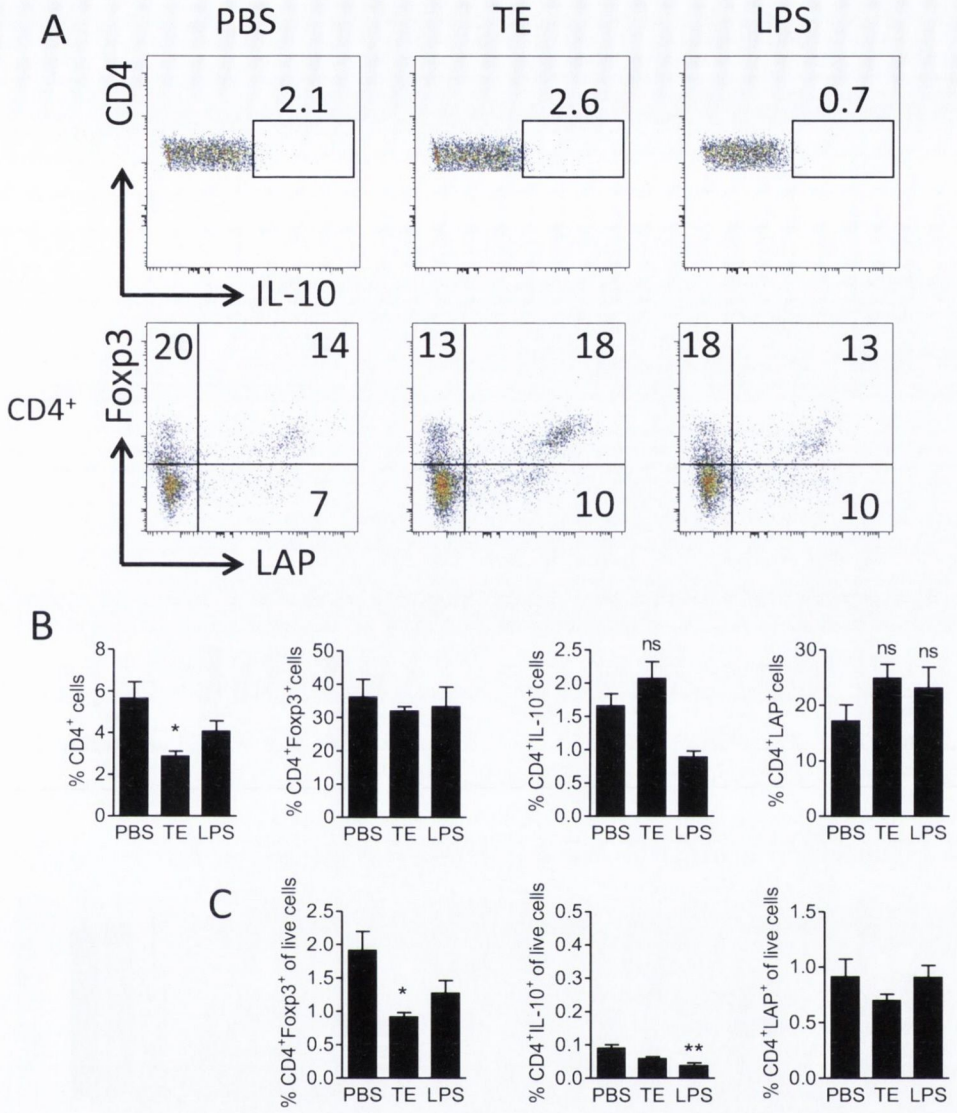


Fig. 5.19 TE treatment increases the expression of IL-10 and LAP by CD4⁺ T cells in the peritoneal cavity. C57BL/6 mice were i.p. injected with PBS, TE or LPS at day 0 and 14. After 7 days, PEC were isolated by peritoneal lavage, stimulated with PMA and ionomycin in the presence of BFA for 5 h and stained with antibodies against CD3, CD4, LAP, IL-10 and Foxp3. Results are representative plots for each mice group (A), mean frequency frequency of cells within $CD3^+$ cells (panel B) and mean frequency of cells within total live cells (panel C) +/- SEM (n=5/group).*$p<0.05$, **$p<0.01$ vs PBS by ANOVA and Bonferroni post test.

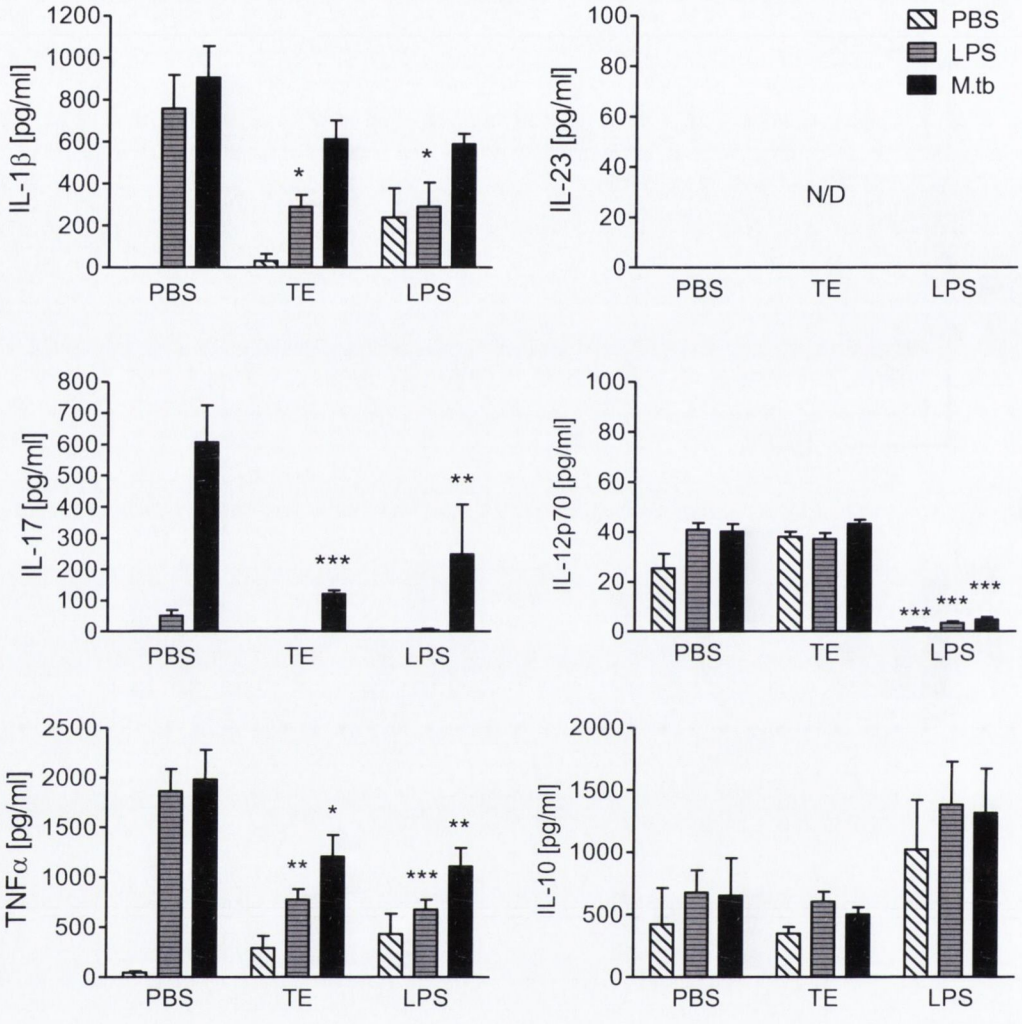


Fig. 5.20 PEC from mice treated with TE or LPS produce less inflammatory cytokines upon stimulation with LPS or *M.tb* C57BL/6 mice were i.p. injected with PBS, TE or LPS at day 0 and 14. After 7 days, PEC were isolated by peritoneal lavage. Cells were cultured with LPS (100 ng/ml) or HK *M.tb* (25ug/ml) for 24. Supernatans were collected and cytokines concentration was determined by ELISA. *p<0.05 ,**p<0.01 ***p<0.001 vs PBS by two-way ANOVA and Bonferroni post-test.

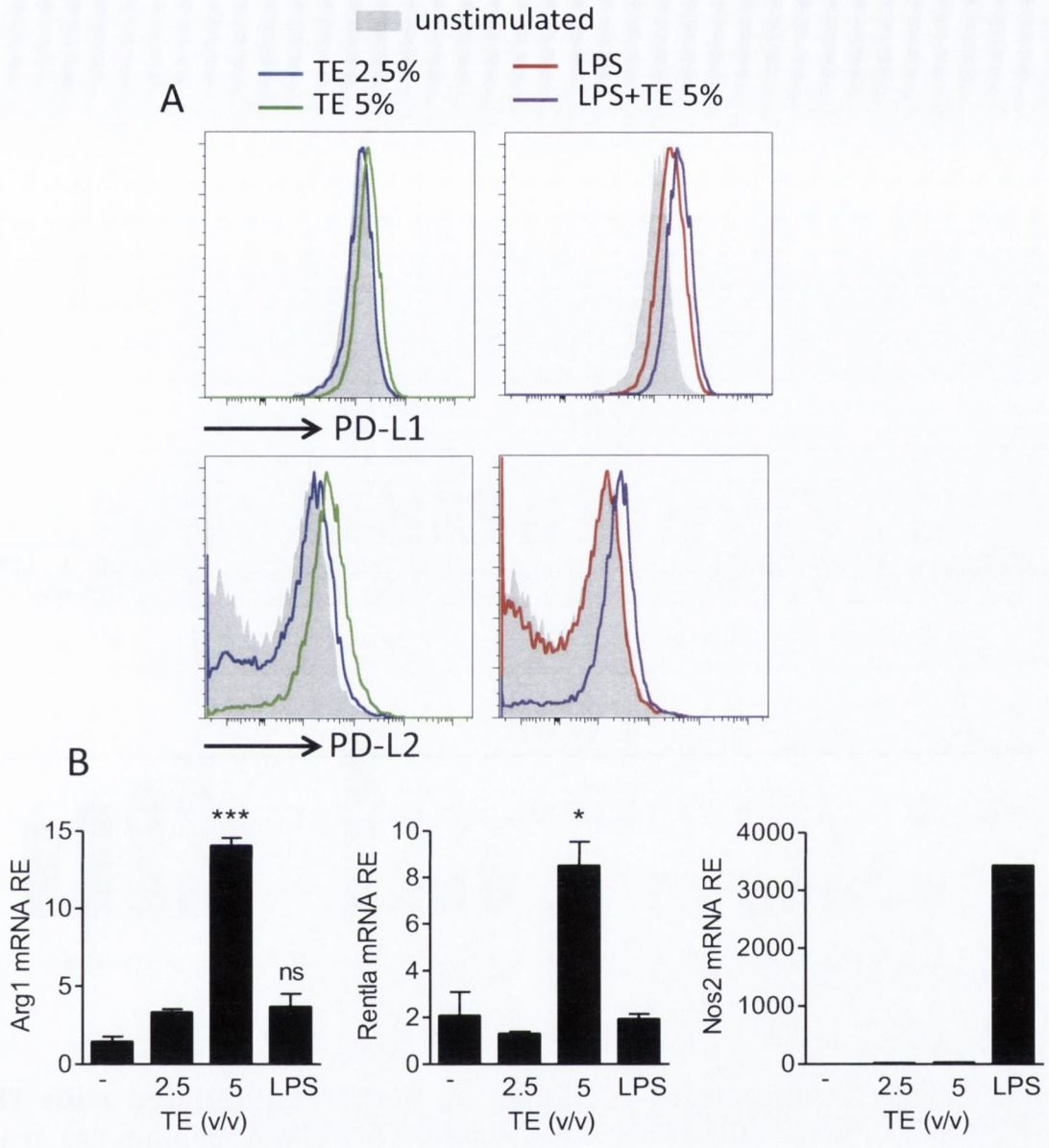


Fig. 5.21 TE enhances expression of regulatory macrophages markers in BMDMs and BMDC. BMDMs and BMDCs from C57BL/6 mice were cultured with different concentrations of TE (2.5-5 % v/v) for 1 h prior to LPS stimulation (100 ng/ml) for 24 h. A. Cells were stained with antibodies against against CD11c (BMDCs) or F4/80 (BMDMs), PD-L1 and PD-L2 and analysed by flow cytometry. Results are representative histograms of $F4/80^+$ gated cells for each surface marker. B. Total RNA was isolated and expression of mRNA for arginase, relm-α, NOS2 was analysed by qPCR. Results present mean relative expression (RE) +/- SEM from 2 experiments.

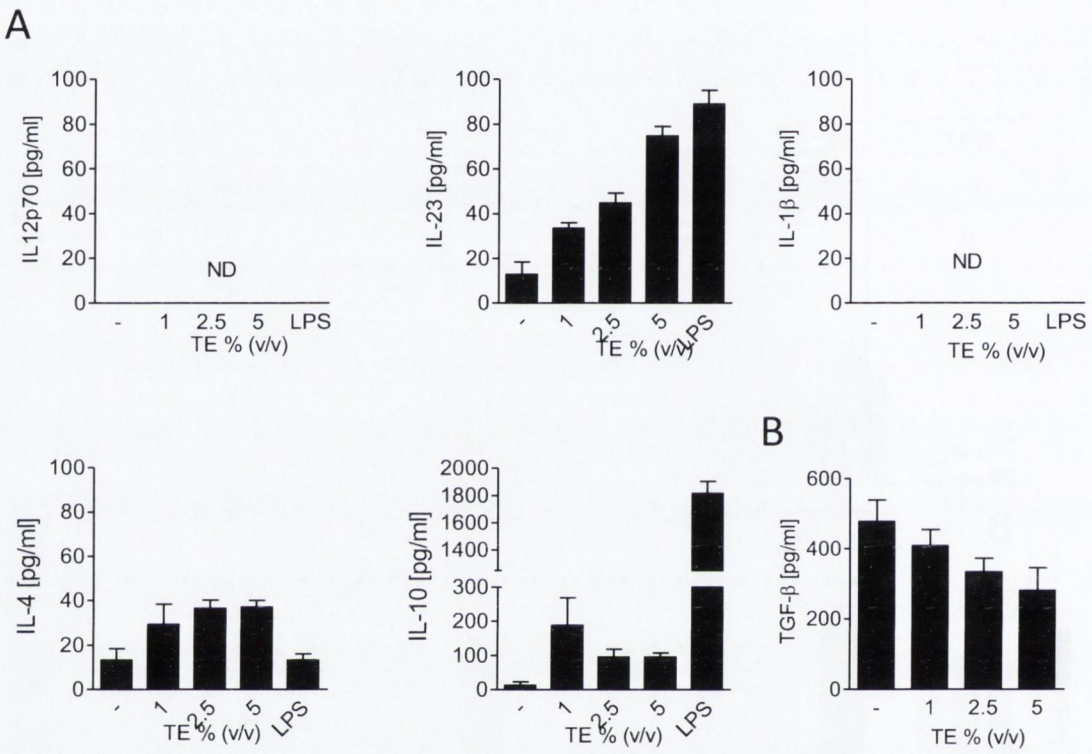


Fig. 5.22 Production of cytokines by BMDMs stimulated with TE. BMDMs from C57BL/6 mice were cultured in cRPMA medium (A) or in X-VIVO medium (B) with different concentrations of TE (1-5 % v/v) for 24 h. Supernatants were collected and cytokine concentration was determine by ELISA. Results shown are the mean values +/-SD from triplicate cultures.

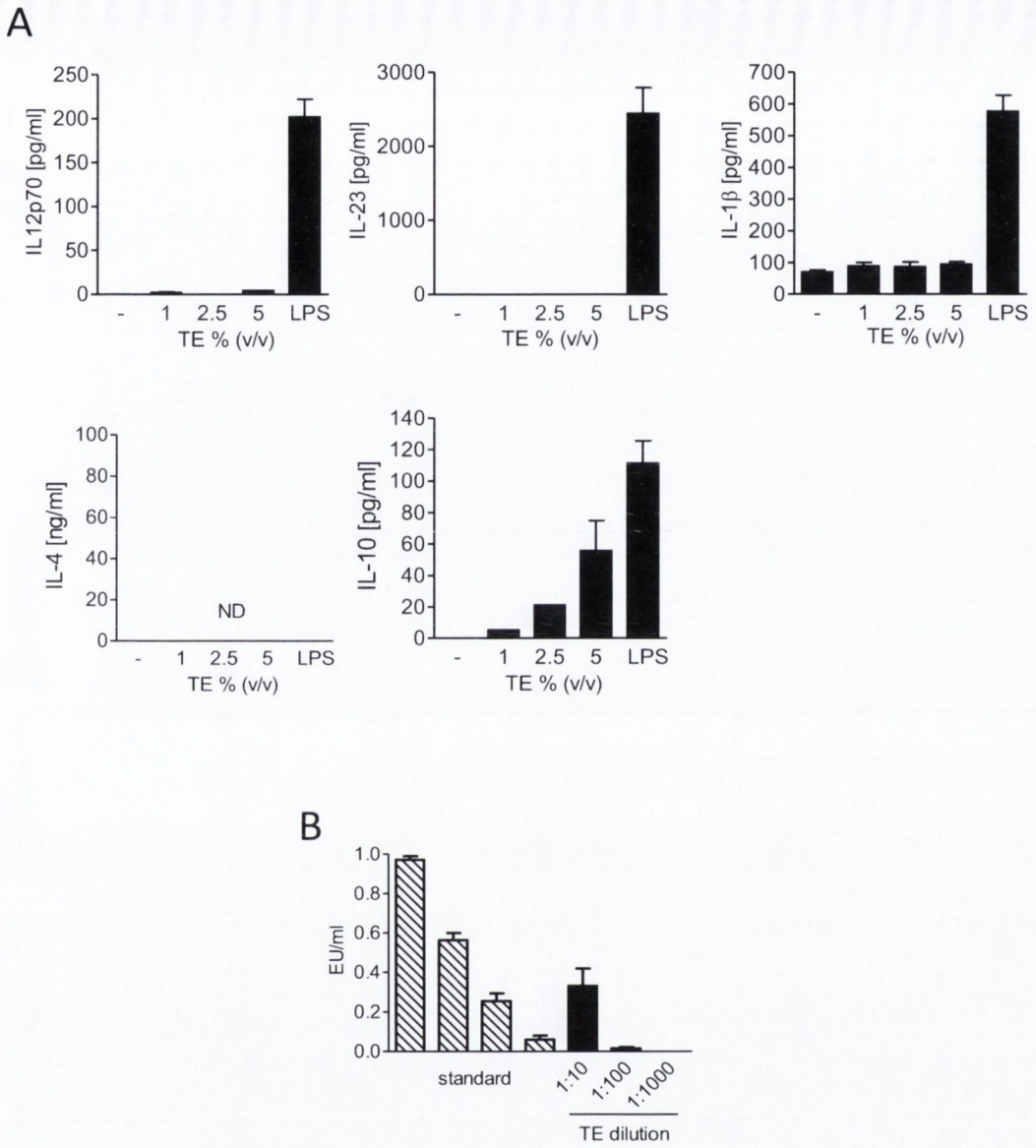


Fig. 5.23 Production of cytokines by BMDCs stimulated with TE. **A.** BMDCs from C57BL/6 mice were cultured in with different concentrations of TE (1-5 % v/v) for 24 h. Supernatants were collected and cytokine concentration was determine by ELISA. Results shown are the mean values +/-SD from triplicate cultures. **B.** Endotoxin level in TE was determined by LAL assay. Graph represents mean endotoxin units (EU)/ml +/- SD from triplecare assys.

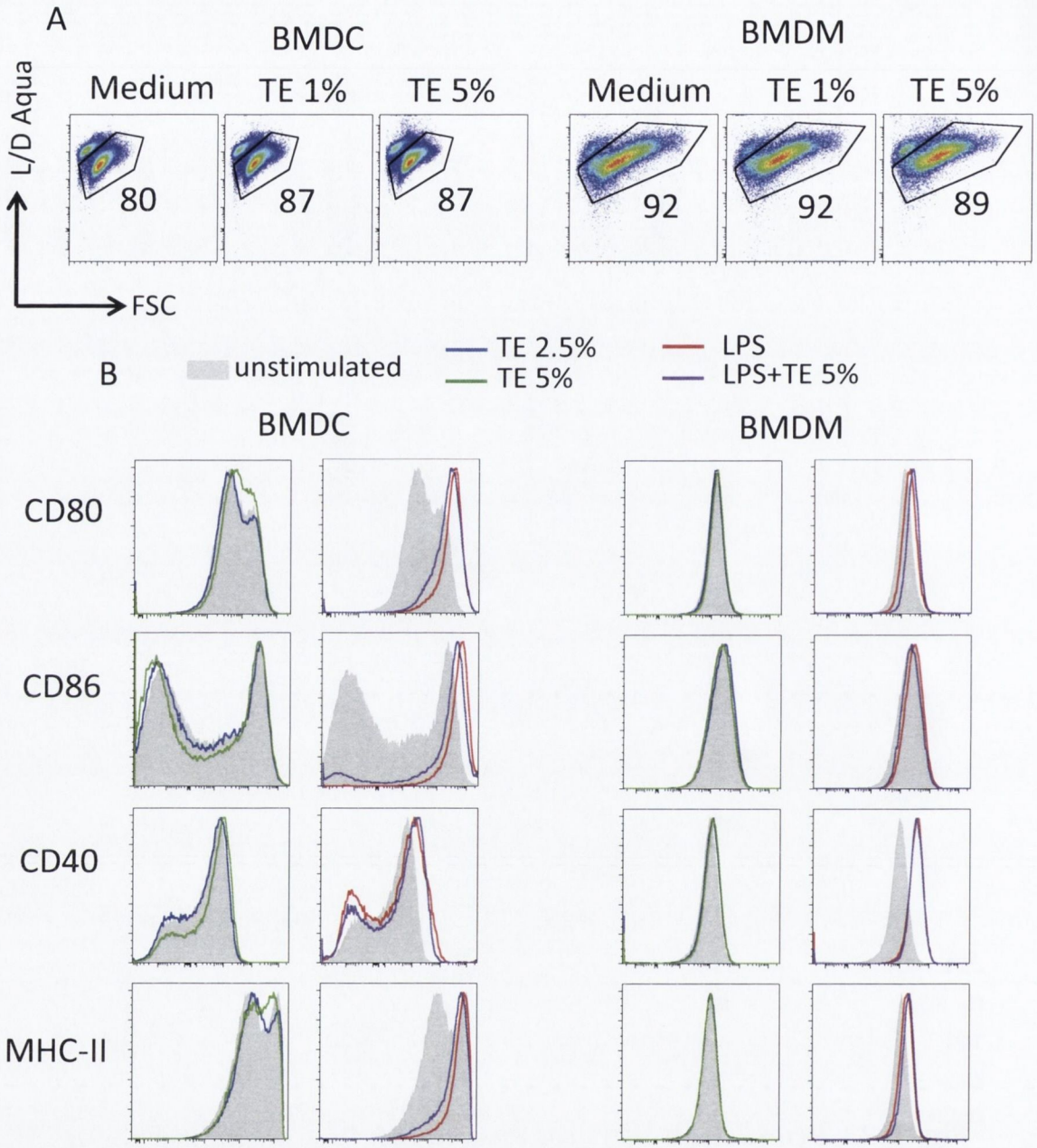


Fig. 5.24 Expression of co-stimulatory molecules by BMDCs and BMDMs cultured with TE. BMDCs or BMDMs from C57BL/6 mice were cultured with different concentrations of TE (1 or 5 % v/v) for 24 h. Cells were stained with viability dye (panel A) and antibodies against CD11c (BMDCs) or F4/80 (BMDMs) and CD80, CD86, CD40, MHC-II (panel B) and analysed by flow cytometry.

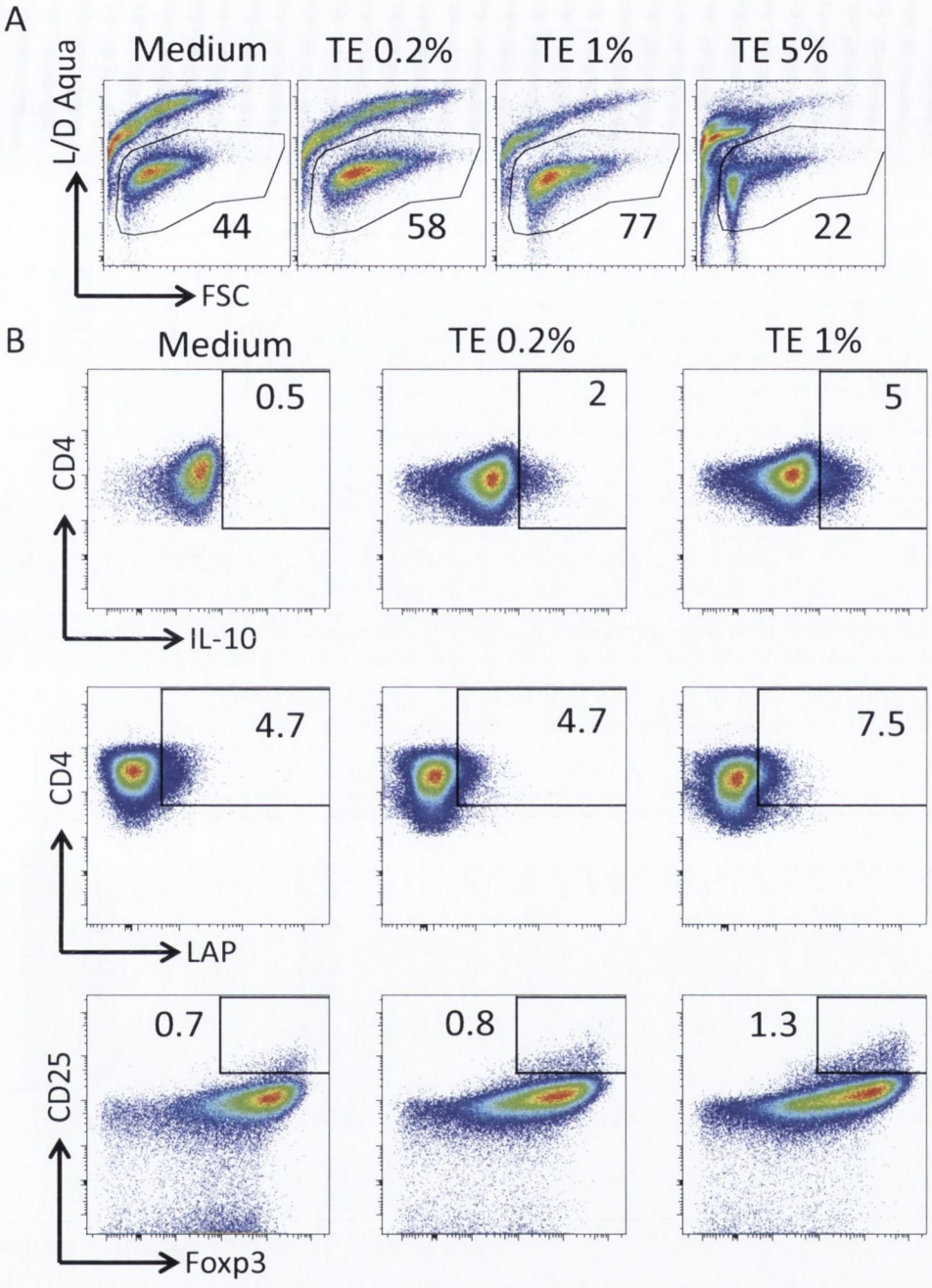


Fig. 5.25 TE enhances expression of IL-10 by anti-CD28 and anti-CD3-stimulated CD4⁺ T cells. CD4⁺ were purified from spleens of C57BL/6 mice and cultured with plated-bound anti-CD3 and soluble anti-CD28 with different concentrations of TE (0.2-5 % v/v) and IL-2 for 72 h. Cells were stimulated with PMA and ionomycin in the presence of BFA for 6 h and, stained with viability dye and antibodies against CD4, CD25, IL-10, LAP and Foxp3 and analyzed by flow cytometry. Results are representative dot plots for live cells (A) and $CD3^{+}CD4^{+}$ cells expressing IL-10, LAP or Foxp3 (B).

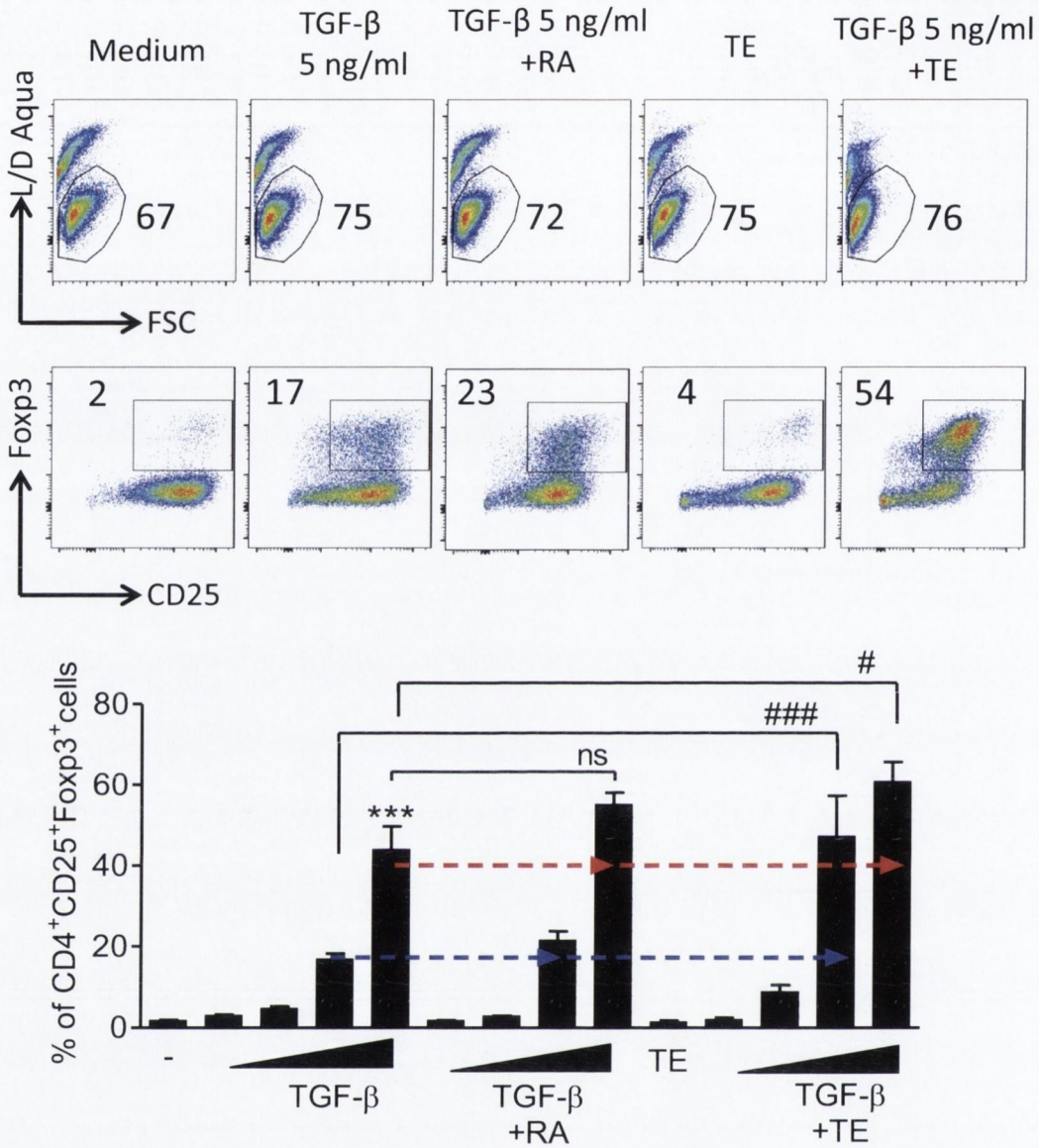


Fig. 5.26 TE synergizes with TGF-β and IL-2 to enhance conversion of CD25$^-$CD4$^+$ T cells into CD25$^+$Foxp3$^+$CD4$^+$ T cells. CD4$^+$CD25$^-$ were purified from spleens of C57BL/6 mice and cultured with plated-bound anti-CD3 and soluble anti-CD28 with IL-2 and different concentrations of TGF-β (0.2, 1, 5 and 10 ng/ml) alone or with RA (100 nM) or with TE (1 % v/v) for 72 h. Cells were stained with antibodies against CD4, CD25 and Foxp3 and analyzed by flow cytometry. Results are plots and mean frequencies of cells +/- SD from triplicate cultures representative of 2 independent experiments. Dashed lines indicate the freguency of converted CD25$^+$Foxp3$^+$ cells by 5 ng/ml of TGF-β (blue line) and 10 ng/ml of TGF-β (red line) and enhanced conversion by stimulation with TE. ***p<0.001, #p<0.05, ###p<0.001 by ANOVA

Chapter 6

General discussion

CHAPTER 6 GENERAL DISCUSSION

The present study demonstrated new effective strategies for the induction of immunological tolerance that have promise in the development of therapies for autoimmune diseases. The results demonstrated that it was possible to achieve prolonged protection against the development of EAE in mice by prophylactic immunization with auto-antigen MOG in the presence of the anti-inflammatory cytokine IL-10. Such prophylactic immunization generated MOG-specific IL-10-producing Tr1 cells that inhibited pathogenic autoimmune responses in an antigen-specific manner. Prophylactic treatment with a soluble extract from helminth parasite *F. hepatica* induced immunosuppressive and regulatory responses that also delayed development of EAE. However, the parasite-induced protection against EAE was mediated by mechanisms involving bystander suppression. These findings demonstrate the potential of using a treatment approach that induces immunoregulation or tolerance to prevent the development of T-cell mediated autoimmune diseases.

Autoimmune diseases are a diverse group of disorders with regard to their demographical distribution and clinical manifestations. Autoimmune disorders involve organ specific immune responses to self-antigens leading to the progressive damage of tissues and organs. Autoimmune diseases, are individually rare, but together they affect approximately 5 percent of the population of Western countries, causing significant chronic disability and morbidity (337). Epidemiological studies have estimated that 1 in 31 Americans are affected by an autoimmune disease (among 24 selected autoimmune diseases studied) and approximately 1.2 million new cases of autoimmune disease are diagnosed every 5 years in the US (338).

MS is the most common cause of chronic neurological disability among young adults in North America and Europe affecting more than 2.5 million people (339). In addition to the disabling neurological symptoms, patients with MS develop severe mood disorders, including major depression with a lifetime prevalence as high as 50% (340). It is still debated whether anxiety and depression in MS patients arise exclusively from emotional stress or whether they are physiologically associated with neuronal lesions in the brain. It has also been hypothesised that depression in patients with MS or other neurodegenerative autoimmune diseases develops as a result of chronic low-grade inflammation and activation of cell-mediated immunity (341). Altogether, progressive

physical and psychological disability negatively affects the life of MS patients reducing the lifespan by 7-8 years on average (339). Current non-antigen-specific disease modifying therapies for MS are often inefficient, whereas the more effective therapies are associated with dramatic suppression of immune responses, increasing the risk of developing infections or cancers (342–344). The general suppression of immune responses associated with the use of steroids increases the risk of potentially severe or fatal viral infections and exacerbates latent infections (342). A highly effective treatment with the S1P receptor agonist FTY720 increases risk of influenza, bronchitis, herpes simplex infections, pneumonia and opportunistic infections. Moreover, two cases of fatal *Varicella zoster* virus (VZV) infection have been reported during therapy and one case of death caused by VZV 39 months after therapy with fingolimod. Localized skin cancer such as basal-cell carcinoma, melanoma and breast cancer were also reported more frequently after even low-dose treatments regimens with fingolimod (343, 345). Therefore, development of alternative therapies, such as antigen-specific tolerogenic therapies is necessary to safely and effectively treat patients with MS or other autoimmune disease.

The strategy of inducing antigen-specific tolerogenic immunization that restores the balance between antigen-specific T_{eff} and T_{reg} cells is based on the concept of "inverse vaccination", the opposite to classical vaccination against infectious diseases. Tolerogenic inverse vaccination can be established by prolonged treatment with high doses of soluble auto-antigen or by using auto-antigen-encoding DNA vaccines (346). Such treatments, although beneficial in animal models of autoimmunity, did not show satisfactory results in clinical trials in patients with autoimmune diseases (230).

Individual TLR ligands have been extensively studied as potentially effective and safe vaccine adjuvants. TLR ligands induce a broad range of pro- and anti-inflammatory cytokines and the present study addressed the possibility that antigen-specific Treg cells can be induced by prophylactic administration of a TLR ligand and specific inhibitors of TLR-induced pro-inflammatory signalling pathways. This study demonstrated that zymosan prophylactically administered with the MOG auto-antigen and inhibitors of ERK, caspase-1 and GSK3 promoted the development of IL-10-producing antigen-specific T cells *in vivo*. This finding is in agreement with previous studies demonstrating that zymosan can induce immunological tolerance through induction of IL-10 in DC and macrophages (259, 347). However, vaccination with MOG, zymosan and ERK, caspase

1 and GKS3 inhibitors failed to prevent the development or attenuate the clinical symptoms in mice with EAE. Moreover, administration of MOG and zymosan alone exacerbated EAE. This may reflect with the fact that zymosan particles are recognized not only by TLR2 by also by β-glucan receptor dectin-1. Recently, Saeed at al demonstrated that priming of monocytes with β-glucan induces trained immune cells characterized by enhanced inflammatory status (274, 348). Since susceptibility to autoimmunity has been associated with infections and particularly with TLR-dependent activation of immune responses, zymosan may therefore be an inappropriate candidate for induction of immunological tolerance in the context of autoimmunity. Nevertheless, it is possible that other microbial products, especially single TLR agonists with known intracellular signalling pathways, would be effective in inducing antigen-specific tolerogenic responses in the presence of inhibitors of pro-inflammatory cytokines.

The manipulation of immune responses by individual PRR agonists appears to be particularly convenient, because the mechanisms of action can be relatively easily determined. In addition, intracellular signalling pathways of many of PRR ligands have already been at least partially elucidated, which gives the opportunity to further modulate TLR signalling to improve their adjuvant activity. Due to their strong pro-inflammatory properties, fungal cell wall-derived β-glucans can be exploited in designing vaccines against infectious diseases or immunotherapeutics in the treatment of cancer.

One of the key findings of the present study was the demonstration that inverse vaccination with soluble auto-antigen in the presence of anti-inflammatory cytokine prevented development of autoimmune disease in mice. The results showed that immunization of mice with auto-antigen MOG and IL-10 protected against the development of the first clinical symptoms of EAE for at least two weeks after the induction of EAE. The prophylactic vaccination with MOG and IL-10 induced MOG-specific IL-10-producing Tr1-like T cells that conveyed protection against EAE when adoptively transferred to naïve recipient mice.

Previous studies have demonstrated the therapeutic potential of administering auto-antigen and anti-inflammatory cytokines in the suppression of autoimmunity in mice. Vaccination using naked DNA encoding auto-antigen attenuated autoimmune diseases in animal models of insulin-dependent diabetes mellitus and MS (349, 350).

Additionally, the study of Ho et al demonstrated that DNA vaccines encoding the myelin antigens together with IL-4 effectively reduced the severity of EAE (346) Moreover, subcutaneous delivery of polymeric biodegradable lactic-glycolic acid (PLGA) particles loaded with MOG peptide and IL-10 significantly ameliorated the course of EAE in mice (351). However, in the case of DNA-based treatment, DNA vaccine was administrated repeatedly during the course of EAE or from the peak of the disease (346). Similarly, PLGA particles released MOG and IL-10 sustainably for 30 days before the induction of EAE and during the course of EAE, maintaining the same effective concentration of both molecules for a prolonged period of time (351). In contrast to the results presented in the present study, both DNA-based vaccine and prophylactic administration of MOG and IL-10-loaded PLGA particles moderately decreased the clinical course of EAE, but did not delay the onset of the disease. This indicates that continuous presence of auto-antigen in the presence of immunosuppressive cytokine suppresses the pathogenic autoimmune responses via a different mechanism than prophylactic immunization with two doses of MOG peptide and IL-10. While vaccination with MOG and IL-10 induced development of MOG-specific Tr1 cells, the sustained expression or release of self-peptide might instead have induced antigen-specific anergy or hyporesponsiveness and the inflammatory responses might be dampened by general suppressive effects of anti-inflammatory cytokines.

This study demonstrated the unique ability of naïve $CD4^+$ T cells to differentiate into functional antigen-specific Tr1 cell in the presence of exogenous IL-10, but without co-stimulation provided by APCs. This is in agreement with reports indicating that DC establish and maintain immunological tolerance in the absence of maturation signals (352). Nonetheless, the present study has demonstrated for the first time that auto-antigen-specific Tr1 cells can be generated *in vivo* by immunization with auto-antigen, and that IL-10 can work as a natural adjuvant to facilitate the generation of Tr1 cells when administered in a prophylactic setting.

Although both natural and inducible Treg cells have been implicated in the maintenance of immunological tolerance in autoimmune disease, the generation of inducible antigen-specific Tr1 cells from naïve $CD4^+$ T cells in the periphery seems to have a greater therapeutic potential than induction of Foxp3-expressing Treg cells. It is worthwhile emphasizing that autoimmunity arises from defects in various immunoregulatory mechanisms, principally associated with generation, maintenance and function of natural

$Foxp3^+CD4^+$ T cells that restrain inappropriate immune responses to self-antigens in healthy individuals. Therefore, the attempts to propagate nTreg cells or generate inducible $Foxp3^+$ T cells might fail in patients affected by autoimmune diseases due to their inherent deficits in Foxp3-associated regulatory mechanisms.

Another possible obstacle in achieving a therapeutic effect through induction of Foxp3-expressing Treg cells is their functional instability under inflammatory conditions. The study of Bailey-Bucktrout et al, investigating the dynamics of Treg cell networks during autoreactive T cell responses *in vivo*, demonstrated that antigen-driven inflammation in the CNS promoted Foxp3 instability selectively in fully-committed autoreactive Treg cells (353). During the acute phase of inflammation in CNS tissues of mice with EAE, these "exFoxp3" cells presented an effector cell phenotype, with increased IFN-γ production and pathogenic potential. Consequently, $Foxp3^+$ Treg cell-inducing therapy might be ineffective or might even contribute to exacerbation of disease (353). On the contrary, induction of antigen-specific Tr1 cells probably will not give the expected beneficial effects in individuals with inherited defects of IL-10 or IL-10R, which are particularly common in patients affected by early onset inflammatory bowel disease. Patients with IL-10 and IL-10R deficiency have been successfully treated by hematopoietic stem cell transplantation (354).

Although antigen-specific therapy reduces the risk of side effects and might be effective with a less severe treatment schedule, it does have some limitations. Firstly, the progression of autoimmune disease, for example MS, has been shown to be accompanied by the broadening of activated T cells repertoire over time. The activated T cells become specific for different antigen determinants, released from damaged CNS tissue, in a process known as epitope spreading. Studies in animal models have also shown that in relapsing remitting EAE induced in SJL mice with $PLP_{139-151}$ there is a sequential and hierarchical order of epitope spreading. Whereas $PLP_{178-191}$-reactive $CD4^+$ T cells are associated with first relapse, MBP_{84-104}-reactive T cells are associated with the second relapse (355). The auto-antigen microarray performed by Robinson et al on cerebrospinal fluid of patients with RRMS or other neurologic disorders has shown significantly increased autoantibodies against various myelin, heat shock proteins and amyloid-β (356). This limitation might be overcome by using multiple myelin proteins or a peptide cocktail that simultaneously generates Treg cells specific for different epitopes. However,

the aetiology and antigen-specificity of many of autoimmune diseases is still unknown, therefore treatment of those diseases will be restricted to antigen-non-specific therapy.

The present study demonstrated that immune cells induced by prophylactic administration of *F. hepatica* TE exert bystander suppression of immune responses against unrelated antigens. Two doses of *F. hepatica* TE administered in a prophylactic regimen protected against development of EAE in mice for more than two weeks after induction of EAE. TE directly induced differentiation of alternatively activated macrophages, which had enhanced expression of M2 markers for at least 7 days after the last administration of TE. Moreover, the peritoneal cavity cells from TE-treated mice produced significantly less pro-inflammatory cytokines in response to strong inflammatory stimuli. These results indicate that perhaps immunomodulatory molecules or antigens present in *F. hepatica* TE induce a distinct type of innate immune response, similar in mechanisms to innate immune tolerance, induced by LPS, but associated rather with immune regulation. The prolonged antigen-non-specific immune suppression achieved by prophylactic treatment with helminth-derived immunomodulatory products might be advantageous compared to the current aggressive immunosuppressive treatments for MS and other autoimmune diseases.

Probiotic helminth therapies have already been shown to be safe and effective in treatment of patients with IBD, whereas recent and ongoing clinical trials will establish its efficacy in treatment of MS. Infection with live helminth parasites appears to modulate host immune responses in a balanced manner. Although helminth infections have been associated with diminished responses against bacterial and viral co-infections, for example *B. pertussis*, *M. tuberculosis* or hepatitis C virus (HCV), the impairment of protective immune responses does not seem to be life threatening, especially in Western countries. Interestingly, development of cancer in people infected with helminths is postulated to be associated with local inflammation rather than with systemic immunosuppression induced by chronic infection with worms (357).

In-depth epidemiological data demonstrated that autoimmune diseases, conventionally considered as separate disorders, tend to co-exist within individual patients. For example, patients with IBD were found to have increased co-occurrence of psoriasis, rheumatoid arthritis and MS (358). Since different autoimmune or chronic inflammatory diseases have distinct aetiology and pathogenesis, the treatment of comorbid autoimmune

disorders existing within a patients by conventional therapies is challenging. Helminth-derived products, inducing a broad range of regulatory mechanisms, could be exploited in the treatment of such difficult clinical cases.

The present study demonstrated that prophylactic immunization with MOG and IL-10 induces development of MOG-specific Tr1 cells that protect against the development of EAE for at least 3 weeks after the last immunization. Similarly, prophylactic treatment of mice with *F. hepatica* TE generated long-lasting immune regulation that inhibited auto-antigen-specific Th1 and Th17 responses and CNS inflammation. These data provide the foundation for designing not only therapeutic but also prophylactic therapies for patients susceptible to or with high risk of developing autoimmune disease. Currently, autoimmune diseases cannot be predicted by genetic or other type of diagnostics, mostly because their exact cause is unknown. Perhaps, in the future diagnostics will become advanced enough to predict with high probability the risk of development of certain autoimmune diseases. Then, the appropriate prophylactic treatment could be designed according to the patient's needs. Identification of particular or combinations of genetic mutations contributing to the susceptibility to particular autoimmune diseases appears to be crucial for understanding the aetiology of autoimmune diseases. Combining the epidemiological data and findings explaining the biological mechanisms of autoimmunity, it might be possible to develop the diagnostic tools for the predicting the risk of developing autoimmune disease. In the case of high risk for development of autoimmune disorder a personalized therapy could be used prophylactically.

To conclude, the findings presented in this thesis contribute to the understanding of the regulatory mechanisms that control autoimmunity and have major implications for the development of novel regulatory-based therapies for autoimmune diseases and also for other conditions with known immune-based pathology.

Chapter 7

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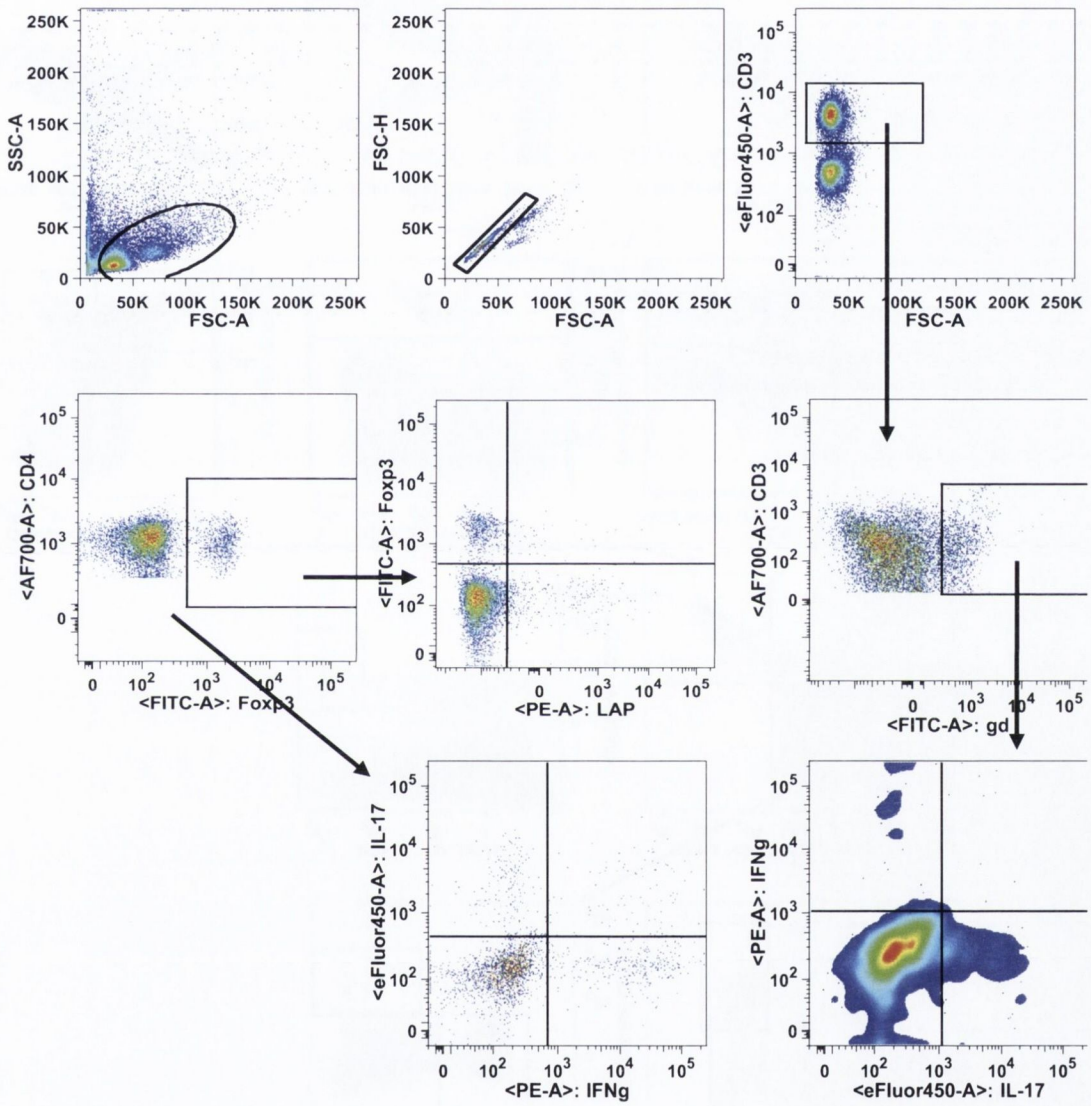
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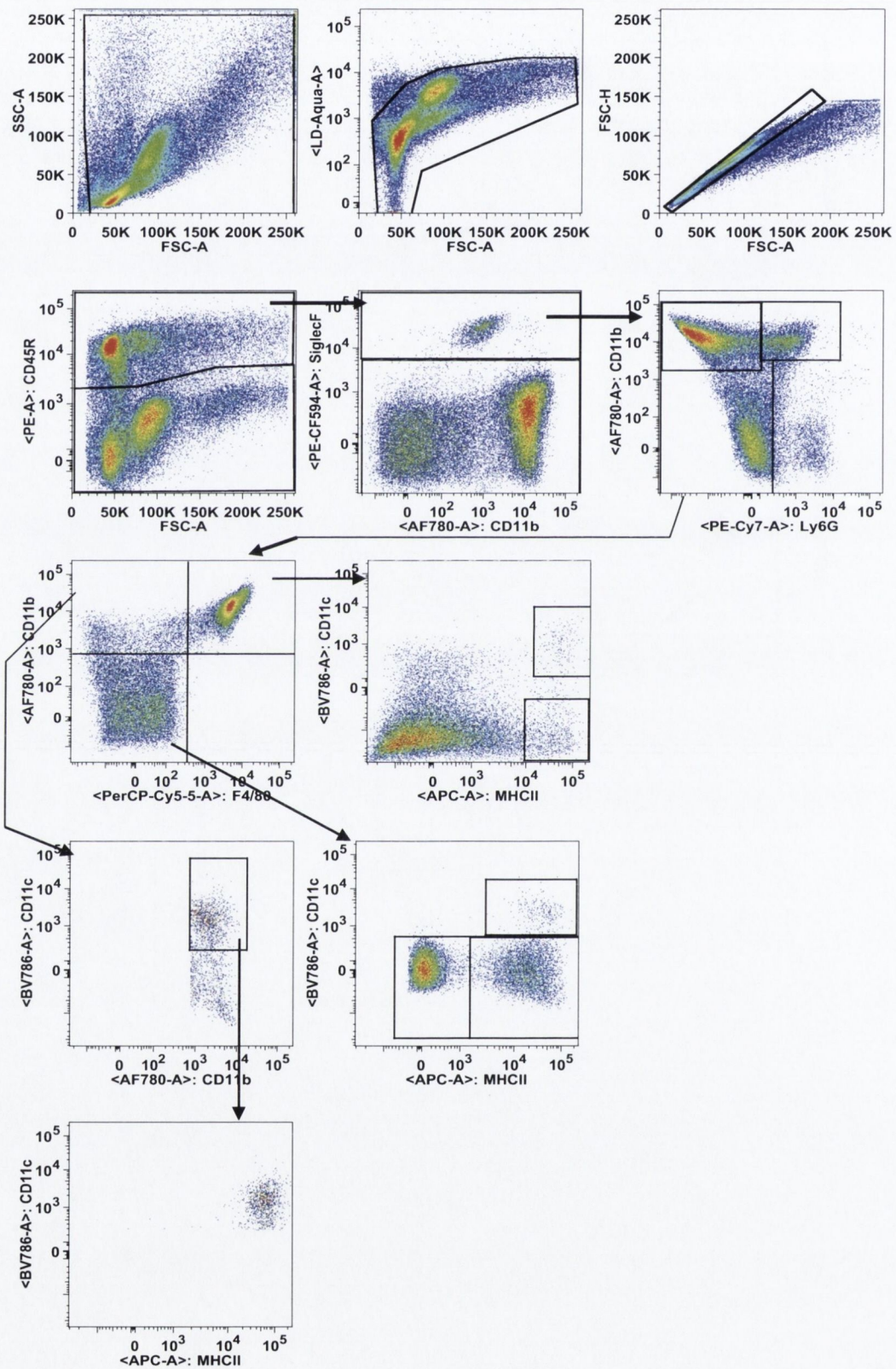
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Appendix

Gating strategy for analysis of CD4⁺ T cells



Gating strategy for analysis of innate cells in CNS and peritoneal cavity



Gating strategy for analysis of BMDMs and BMDCs

