Aus dem Institut für Mikrobiologie und Hygiene

der Medizinischen Fakultät der Charité – Universitätsmedizin Berlin

Dissertation

Activation of RARα Causes Amelioration of EAE by Induction of FoxP3

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

von

Felix Schambach aus Worms

Gutachter: 1. Prof. Dr. med. O. Liesenfeld

2. Prof. Dr. M. Prinz

3. Priv.-Doz. Dr. med. M. Lehrke

Datum der Promotion: 18. November 2011

TABLE OF CONTENTS

1 Introduction

1.1 Autoimmune diseases

The immune system of the human body protects the host against a number of threats including infections, allergens and malignancies. When the immune system mistakes self-tissues for non-self and mounts an inappropriate attack, autoimmune disease may result. In such an event, the immune response is directed against the body's own structures, so-called self-antigens.

Autoimmune diseases are a major cause of morbidity in the developed world and affect more than 3% of the US population alone (1). Amongst others, they include inflammatory bowel disease, multiple sclerosis (MS), type 1 diabetes, systemic lupus erythematosus and rheumatoid arthritis.

1.2 Multiple sclerosis

In order to efficiently conduct electrical signals, nerves in the central nervous system (CNS) are covered in electrically insulating sheaths. In MS, in particular, the immune system directs its attacks against myelin in nerve sheaths. Loss of myelin results in signals being conducted at reduced velocity, nerves can discharge spontaneously and show increased mechanical sensitivity. Moreover, during the immune attack against myelin, the nerve itself is also damaged. Clinically, patients may experience a wide range of neurological symptoms depending on the affected region of the CNS.

Typical symptoms include blurred or double vision, tingling, numbness or weakness in arms or legs, cognitive dysfunction such as speech impairment and loss of memory, fatigue, bladder and bowel dysfunction and depression.

In most cases, MS initially takes a relapsing-remitting course where attacks are followed by partial or complete recovery periods (2). When untreated, about one half of the patients diagnosed with relapsing-remitting MS goes on to develop secondary progressive MS (3), in which disease progresses more steadily.

1.3 Pathogenesis of multiple sclerosis

MS is thought to arise when peripheral myelin reactive CD4+T helper cells are activated. Once those T cells cross the blood brain barrier, they become reactivated by myelin antigen presentation in the CNS, where they recruit macrophages and granulocytes and induce autoreactive B cells; inflammation and demyelination ensue (4). How peripheral T cells become activated to react with myelin remains speculative. Cross reactivity with bacterial or viral antigen might play a role, but in a more general sense, the cause is the lack of immunological tolerance towards myelin as a self-antigen.

1.4 Epidemiology of multiple sclerosis

MS is a widespread disease, it affects approximately 120,000 people in Germany (5) and more than one million individuals worldwide, carrying a lifetime risk of 1 in 400 (6). MS affects approximately twice as many women as men (7). It is potentially the most common cause of neurological disability in young adults (8). There are genetic and largely unknown environmental causes for MS (9). Disease prevalence is higher in places further away from the equator (10), implying yet undetermined environmental causes.

Monozygotic twins of MS sufferers carry a 25% risk of also developing MS (11), indicating that genetics plays a major role while genes alone do not fully determine the disease.

1.5 Diagnosis of multiple sclerosis

One criterion for the diagnosis of MS is the presence of oligoclonal bands as detected in electrophoresis of cerebrospinal fluid (12), which are indicative of nervous system production of antibodies. Gadolinium enhanced magnetic resonance imaging is used to detect lesions in the brain and spinal cord. Gadolinium uptake can demonstrate active inflammation as a correlate of blood brain barrier breakdown. Non-enhancing lesions on MRI indicate residual scars resulting from prior disease activity. In this way, MRI can be used to diagnose MS and monitor disease

progression (13, 14). Nerve damage caused by MS can lead to slower signal conduction, therefore another way to corroborate diagnosis is to analyse EEG potentials evoked by sensory stimuli (15).

1.6 Therapy of multiple sclerosis

During acute attacks, multiple sclerosis is usually treated with corticosteroids. Furthermore, there are disease-modifying drugs, which aim at reducing relapse rate and disease progression. These drugs are IFN-β, glatiramer acetate, mitoxantrone, natalizumab and alemtuzumab, which all interfere with immune responses. IFN-β is an endogenous cytokine, which is immunomodulatory and can inhibit T cell migration to the inflamed CNS. IFN-β frequently causes flu-like symptoms. Relapse rates can be reduced by IFN-β, but its long-term benefit remains unproven (16). Glatiramer acetate is a random mixture of polypeptides of glutamic acid, lysine, alanine and tyrosine, which bind to MHC molecules and compete with various myelin antigens for their presentation to T cells (17). Recent analyses have shown a positive effect of glatiramer acetate on relapse probability (18, 19), but not on disease progression (20). Mitoxantrone is a cytostatic agent used in chemotherapy, which inhibits DNA replication by inhibiting topoisomerase type II and is limited to severe cases due to its cardiotoxicity. Natalizumab is a monoclonal antibody against the cellular adhesion molecule α4-integrin. Its mechanism of action consists of limiting T cell migration from blood vessels into the brain. Treatment with natalizumab can be complicated by

the development of progressive multifocal leukoencephalopathy (PML) due to immunosuppression in the CNS, a condition that often proves fatal (21). Another monoclonal antibody, alemtuzumab, targets CD52 on the surface of lymphocytes and monocytes and leads to their depletion. Although alemtuzumab is not currently licensed for the treatment of MS, it has shown promise in a recently conducted MS trial (22). However, side effects observed in these trials included infections and autoimmune conditions such as immune thrombocytopenic purpura. Up to a third of patients treated with alemtuzumab developed Graves-Basedow disease (23).
FTY720 is a new MS drug, which was licensed by the FDA in September 2010. FTY720 is an orally available spingosine-1-phosphate antagonist, which inhibits lymphocyte egress and thereby limits lymphocyte migration to the CNS. However, FTY720 suffers from rare but severe cardiac side effects and an increased cancer risk (24).

1.7 Experimental models for multiple sclerosis

Compared to studies on other autoimmune disorders, investigating MS holds an additional challenge. MS lesions in the CNS are inaccessible to the investigator and brain biopsies are limited to extremely rare, special cases. Therefore, much of the research into MS relies on animal models.
Experimental autoimmune encephalomyelitis (EAE) is a model, which has been widely used to study inflammatory demyelinating CNS disease in animals since it

was first developed by Rivers in 1935 (25). In "classic" EAE, a myelin antigen is injected into susceptible rodent species together with an adjuvant. Within three days, myelin reactive T helper cells are activated in the periphery. Those cells expand and after less than five days, large numbers cross into the CNS (26). In the CNS, they become reactivated by myelin antigen and cause an autoimmune response to myelin and thus cause inflammatory CNS disease. The model was first implemented to study acute disseminated encephalomyelitis, which is a demyelinating disease sometimes observed after vaccinations. Today, many variations of the original model exist in various species.

While EAE is not exactly the same disease as MS, there are a number of similarities. EAE, just as MS, is characterised by perivascular, inflammatory cell infiltrates and demyelination. There is also a strong association with major histocompatibility complex (MHC) type II molecules and lesions contain T cells, B cells and antibodies against myelin.

Three of the four drugs approved for the therapy of MS were first validated in EAE (27-31), highlighting the model’s practical relevance.

Helper T cells play a central role in both diseases, as myelin specific CD4+ T cells are found in inflammatory lesions in EAE as well as in MS (4). The activation of helper T cells requires recognition of a peptide antigen bound within the major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells (APCs).

Various cell types can act as APCs. Dendritic cells and macrophages can capture

antigen in the periphery and present it to helper T cells. B cells can internalise and present the antigen, which binds to its B cell receptor. Microglial cells are resident macrophages in the brain and as such can perform phagocytosis and act as APCs. As antigen peptides are presented on MHC II molecules, certain MHC class II haplotypes are associated with increased susceptibility to MS as well as EAE (32).

1.8 T helper cell differentiation

CD4+ T helper cells play a major role in the defence against pathogens and cancer, but they also take part in the induction of autoimmunity. CD4+ T helper cells can differentiate into one out of at least four non-overlapping populations: Th1, Th2, regulatory T cells (Treg) and the newly found Th17 cells, which are characterised by their cytokine secretion pattern and expression of master transcriptional regulators (see Fig. 1).

Th1 cells promote a cell-mediated immune reaction against intracellular pathogens by activating CD8+ cytotoxic T cells and macrophages thus causing delayed-type hypersensitivity. Th1 cells are induced in the presence of IL-12 (33, 34), which acts through signal transducer and activator of transcription 4 (STAT4). Th1 cells express the hallmark transcription factor T-bet and produce IFN-γ (35). Th2 cells promote the production of antibodies by B cells and activate eosinophils. Development into the Th2 lineage occurs in the presence of IL-4 acting through STAT6. Th2 cells are characterised by the expression of the transcription factor GATA3 (35) and the production of the cytokines IL-4, IL-5 and IL-13.

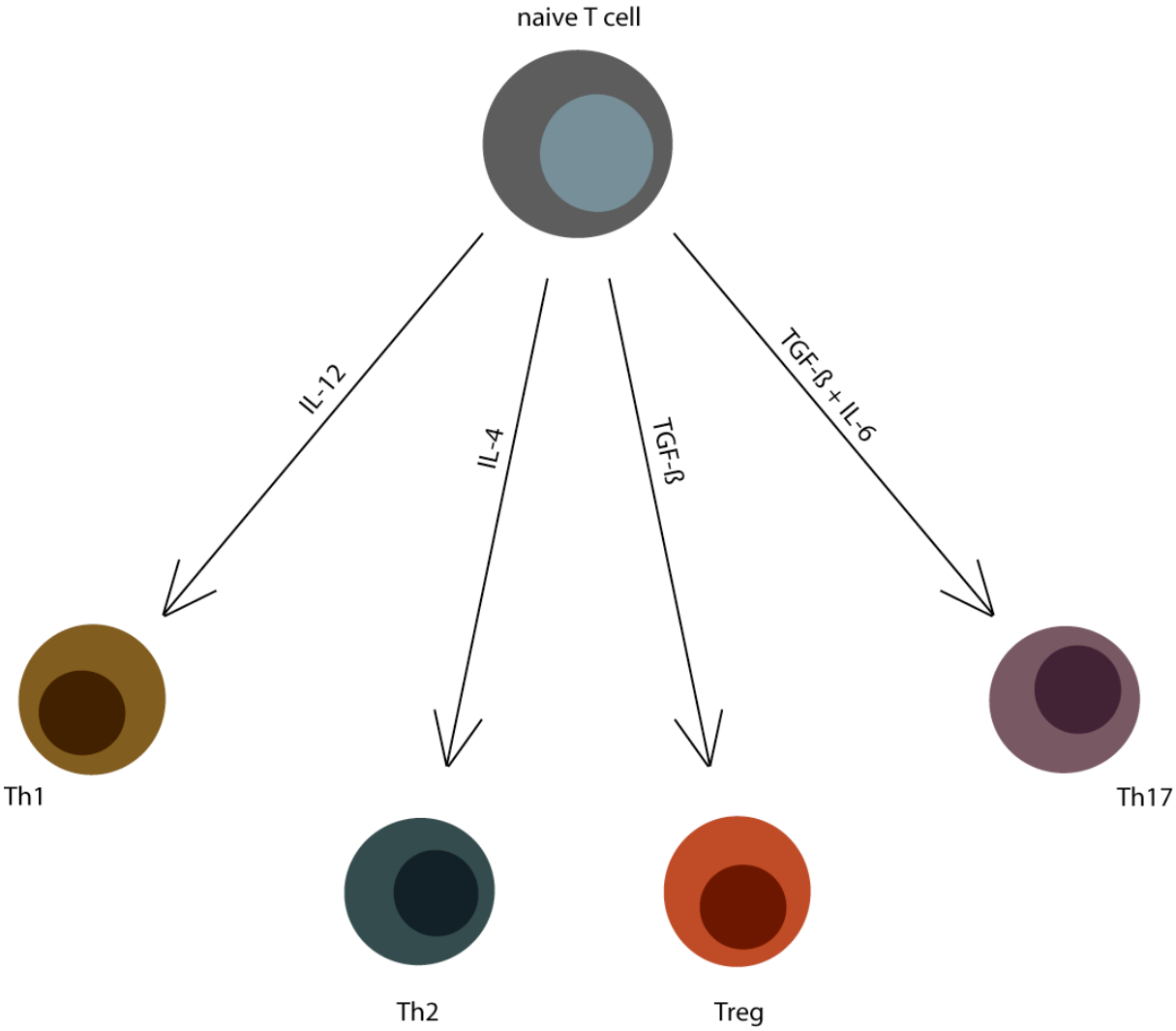

Figure 1: The differentiation of naïve T cells into one of the four subsets Th1, Th2, Treg and Th17 occurs in the presence of IL-12, IL-4, TGF-ß and TGF-ß + IL-6, respectively.

1.8.1 Regulatory T cells

Regulatory T cells (Tregs) are specialised in immune suppression (36). They are capable of containing undesirable and damaging immune responses and thereby prevent the emergence of autoimmunity in a healthy individual. Tregs are composed of a subset of CD4+ T cells expressing the surface molecule CD25, the alpha chain of the IL-2 receptor. Forkhead box P3 (FoxP3) is a transcription factor, which was found to be essential in CD4+ CD25+ T cell development and function (37).

Mice lacking CD4+ CD25+ regulatory T cells develop autoimmunity (such as thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, and polyarthritis), which can be stopped when the mice are reconstituted with regulatory T cells (36, 38). The lack of regulatory T cells on the other hand can lead to heightened immunity against tumours and microbial pathogens (39, 40). Conversely, when regulatory T cells are transferred to an animal, tolerance to organ transplants is improved and allergies are reduced (41).

Mice that only possess a mutated, dysfunctional copy of the FoxP3 gene are characterised by overproliferation of Th1 and Th2 CD4+ T cells and die in early life showing extensive multiorgan lymphocyte infiltration (42, 43). Mutations in FoxP3 are also responsible for the human syndrome of X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy (IPEX) (44-46).

Two different populations of regulatory T cells have been described. Natural Tregs (nTregs) are CD4+CD25+ T cells that develop during the normal process of T cell

maturation in the thymus (47). Inducible Tregs (iTregs) can be induced from CD4+CD25- naïve precursors. Phenotypically, iTregs and nTregs can so far not be differentiated and both are capable of substantial tolerance induction (48).

In short, the immunosuppression that results from regulatory T cells is important to inhibit autoimmune processes. On the other hand, immunosuppression is undesirable while the body fights an infection, making the control of regulatory T cells crucial to a successful immune response.

1.8.2 Th17 T cells

It has recently been found that T cells can adopt a fourth fate, the so-called Th17 cells. Th17 cells express the transcription factor RORγT and produce the cytokine IL-17 and IL-22 (49, 50). STAT3 has been shown to promote Th17 development (51). IL-17 can induce neutrophil-mediated inflammatory responses and give rise to anti-bacterial β-defensins (52). Mice lacking the IL-17 receptor are susceptible to lung infections with *Klebsiella pneumoniae* (53). In addition, IL-17 is also involved in the defence against other extra- and intracellular pathogens including *Escherichia coli*, *Bordetella pertussis*, *Candida albicans*, *Cryptococcus neoformans* and *Pneumocystis carinii* (52).

Much of recent research has focussed on the Th17 cell lineage, as it is involved in many autoimmune conditions, including psoriasis (54), rheumatoid arthritis (55, 56), MS (57, 58), inflammatory bowel disease (59), and asthma (60).

Induction of both Tregs and Th17 cells from antigen-activated naïve T cells seem to require signalling from the pleiotropic cytokine TGF-β. The differentiation of each subset, however, is typically non-compatible, with cytokines such as IL-6 favouring induction of Th17 at the expense of Tregs in the setting of TGF-β signalling (61, 62). Whether signals exist that can mediate induction of Tregs at the expense of Th17 in cooperation with TGF-β was previously unknown.

As it has recently been found, IL-23 must be present *in vivo* for sustained Th17 mediated inflammation, and mice lacking IL-23 are resistant to EAE (63).

1.9 T helper cells in experimental autoimmune encephalomyelitis

Previously, it was assumed that Th1 cells were responsible for the pathogenesis of EAE. In this model, T cells produce IFN-γ and IL-2, but not IL-4 upon rechallenge with an immunised myelin antigen (64). As IL-12 is essential for the development of Th1 cells (33, 34), inhibition of IL-12 signalling was expected to ameliorate EAE.

IL-12 is a cytokine composed of the two subunits p35 and p40 (65). While p35-deficient mice developed the disease, p40-deficient mice were resistant to EAE (66). The p40 subunit of IL-12 is shared with IL-23, which also possesses a second subunit, p19 (67).

Subsequently, it was demonstrated that the two subunits of IL-23 were required for the development of EAE, whereas there was no need for p35, the unique subunit of IL-12, to be present (68).

Starting from this important finding, further studies concluded that IL-23 deficient mice were lacking IL-17 producing CD4+ T cells (55).

1.10 Vitamin A in the immune system

The term Vitamin A refers to a group of retinols, which are required for numerous processes in the human body. One form of vitamin A, retinol, and its metabolite, all-trans retinoic acid (ATRA), have been implicated in immune homeostasis. Vitamin A and its derivatives are capable of ameliorating symptoms of disease in various models of autoimmunity, including inflammatory bowel disease, rheumatoid arthritis, type I diabetes and experimental encephalomyelitis (69-73). Deficiency of vitamin A leads to exacerbation of experimental colitis (74). Retinoids are used in the therapy of rosacea and psoriasis (75). Two large studies of smokers found that taking beta-carotene and vitamin A carries an increased risk of developing lung cancer (76, 77). Taken together, these findings suggest that vitamin A can tip the balance toward the induction of tolerance.

Before the description of the Th17 phenotype, it was observed that ATRA could skew T cell differentiation away from Th1 and towards the Th2 lineage (78). The resulting lower numbers of Th1 cells were thought to be responsible for the amelioration of autoimmunity by retinoids. In light of newer findings, which implicate Th17 cells in many autoimmune diseases, this explanation seems increasingly unsatisfactory.

It has recently been found that dietary vitamin A can be processed into ATRA by

dendritic cells from mesenteric lymph nodes and Peyer's patches. It has been suggested that ATRA can influence T cell function insofar as it induces the gut homing receptors α4β7 and CCR9 (79). This implies that ATRA can play an important role in immune functions. Whether ATRA influences the lineage decisions for helper T cells, however, has not yet been thoroughly explored.

1.11 Regulation of retinoic acid actions by nuclear receptors

Retinoids act by binding to the nuclear receptors retinoid acid receptor (RAR) and retinoid X receptor (RXR). Following ligand binding, RAR/RXR form complexes on DNA together with a coactivator complex (80), leading to histone acetylation and activation of transcription (see Fig. 2). RXR can also heterodimerise with other nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR), vitamin D receptor (VDR) and thyroid hormone receptor (TR) (81, 82).

RAR and RXR each have an α, β and γ subtype. ATRA activates all subtypes of RAR. Small molecule agonists and antagonists exist, which are specific to RARα, RARβ or RARγ (83).

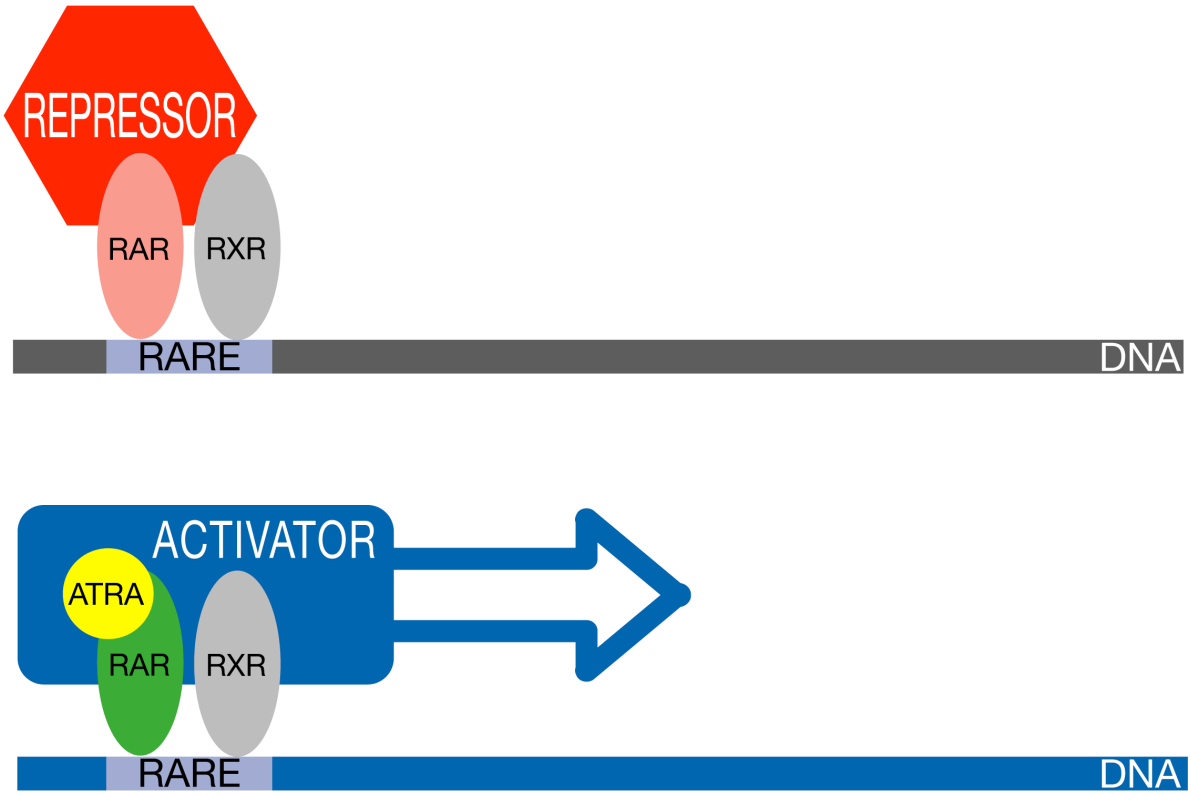

Figure 2: RAR and RXR bind to a retinoic acid response element (RARE) on DNA. In the absence of ATRA, RAR and RXR are associated with a co-repressor complex, which leads to histone deacetylation and repression of transcription. In the presence of ATRA, RAR and RXR can recruit a co-activator complex that causes histone acetylation and activation of transcription.

1.12 Aims

While it is known that retinoids can improve tolerance in the immune system, the exact mechanism of this effect remains unclear. In this study we set out to examine the influence of retinoids on T cell differentiation. In particular, the following questions will be addressed:

1. Do retinoids influence T cell differentiation regarding the creation of tolerance inducing regulatory T cells versus pro-inflammatory Th17 T cells?
2. Is retinoid receptor subtype RARα involved in skewing T cell differentiation?
3. Can the activation of retinoid receptor subtype RARα ameliorate EAE and increase the number of regulatory T cells *in vivo*?

2 Materials and Methods

2.1 Material

2.1.1 Consumable material

15 ml tube	BD Falcon, Franklin Lakes, NJ, USA
50 ml tube	BD Falcon, Franklin Lakes, NJ, USA
Serological pipette 1 ml / 2 ml / 5 ml / 10 ml / 25 ml / 50 ml	BD Falcon, Franklin Lakes, NJ, USA
Fisherbrand Microcentrifuge tubes 0.2 ml / 0.5 ml / 1.5 ml	Thermo Fisher Scientific, Waltham, MA
Syringes 1 ml / 5 ml / 10 ml	BD Biosciences, San Jose, CA, USA
Stericup filter units	Millipore, Billerica, MA
Tissue culture sterile multiwell plates	BD Falcon, Franklin Lakes, NJ, USA
Syringe filters 0.22 µm pore size	Thermo Fisher Scientific, Waltham, MA
Cell strainers 70 µm / 100 µm mesh size	BD Falcon, Franklin Lakes, NJ, USA

2.1.2 Machines and devices

Pipette 1 µl / 5 µl / 20 µl / 200 µl / 1000 µl	Eppendorf, Hamburg, Germany
12-channel Pipette	Eppendorf, Hamburg, Germany
FACSAria cell sorter	BD Biosciences, San Jose, CA, USA
FACSDiva collection software	BD Biosciences, San Jose, CA, USA
Flowjo analysis software	Tree Star Inc., Ashland, OR, USA
Microscope Axiostar plus	Carl Zeiss, Thornwood, NY, USA
Incubator	Heraeus, Hanau, Germany
Biological safety cabinet Herasafe HS 12	Heraeus, Hanau, Germany
Plate Reader Spectramax 250	Molecular Devices, Sunnyvale, CA
Table top refrigerated centrifuge Eppendorf 5417R	Eppendorf, Hamburg, Germany
Table top centrifuge Sorvall RT7	Thermo Fisher Scientific, Waltham, MA
Bench top Minispin Centrifuge	Eppendorf, Hamburg, Germany

2.1.3 Drugs and chemicals

Inhibitor 728 (AGN 193618)	Eli Lilly, Indianapolis, IN, USA
Inhibitor Ro 41-5253 (4-[(E)-2-(7-heptoxy-4,4-dimethyl-1,1-dioxo-2,3-dihydrothiochromen-6-yl)prop-1-enyl]benzoic acid) (50 mM)	Biomol, Plymouth Meeting, PA, USA
Am580 (4-[(5,6,7,8-tetrahydro- 5,5,8,8-tetramethyl-2-naphthalenyl) carboxamido] benzoic acid) (5 mM)	Biomol, Plymouth Meeting, PA, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma, St. Louis, MO, USA
Chloroquine	Sigma, St. Louis, MO, USA
All-trans retinoic acid (ATRA)	Sigma, St. Louis, MO, USA
Dimethyl sulfoxide (DMSO)	Sigma, St. Louis, MO, USA
Complete Freund’s Adjuvant CFA	Sigma, St. Louis, MO, USA
1 M HEPES buffer	Invitrogen, Carlsbad, CA, USA
Dulbecco's modified eagle medium (DMEM)	Invitrogen, Carlsbad, CA, USA
Penicillin G (10,000 U/ml) / Streptomycin (10 mg/ml) solution	Invitrogen, Carlsbad, CA, USA
L-Glutamine 200 mM	Invitrogen, Carlsbad, CA, USA
Polybrene	Millipore, Billerica, MA, USA

Foetal Bovine Serum (FBS)	Gemini Bio-Products, West Sacramento, CA, USA
Myelin oligodendroglia glycoprotein (MOG) peptide 35-55	custom ordered from CS Bio, Menlo Park, CA, USA
MMM1013-751412 Plasmid containing RARα	Openbiosystems, Huntsville, AL, USA
Pertussis toxin	List Biological laboratories, Campbell, CA, USA
Percoll	Sigma, St. Louis, MO, USA
Histochoice fixative	Amresco, Solon, Ohio, USA
Ethanol	Sigma, St. Louis, MO, USA
Isopropanol	Sigma, St. Louis, MO, USA
Na_2HPO_4 $7H_2O$	Sigma, St. Louis, MO, USA
HEPES	Sigma, St. Louis, MO, USA
PBS	Sigma, St. Louis, MO, USA
Hank’s Balanced Salt Solution (HBSS)	Cellgro, Herndon, VA, USA
Iscove's Modified Dulbecco's Medium	Invitrogen, Carlsbad, CA, USA
KCl	Sigma, St. Louis, MO, USA
NaCl	Sigma, St. Louis, MO, USA
Dextrose	Sigma, St. Louis, MO, USA

Anti-RARα (sc-551)	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Phycoerythrin conjugated anti-human IL-17A	BD Biosciences, San Jose, CA, USA
Allophycocyanin conjugated rat anti-mouse CD4	Caltag, Carlsbad, CA, USA
Phycoerythrin conjugated anti-human FoxP3	eBioscience, San Diego, CA, USA
Anti-RAN	BD Biosciences, San Jose, CA, USA
Anti-CD8a (clone Ly-2) Microbeads	Miltenyi, Bergisch Gladbach, Germany
Horseradish peroxidase conjugated anti-rabbit / anti-mouse antibodies	Invitrogen, Carlsbad, CA, USA
4-20% SDS polyacrylamide gel	Invitrogen, Carlsbad, CA, USA
Polyvinylidene difluoride membrane	Invitrogen, Carlsbad, CA, USA
Chemiluminescent substrate kit	Amersham, Little Chalfont, UK
70 μm nylon cell strainer	BD Biosciences, San Jose, CA, USA
MACS LS column, MACS multistand magnet	Miltenyi, Bergisch Gladbach, Germany
EcoRI, XhoI, T4 DNA Ligase	New England Biolabs, Ipswich, MA, USA

2.1.4 Buffers and solutions

Hank's 1% FBS

1% FBS was added to HBSS

HBBS - HEPES-buffered saline solution 2x

12.5 ml 1 M HEPES solution

0.19 g KCl

0.54 g Dextrose

14 ml NaCl (5 M)

0.1 Na_2HPO_4 $7H_2O$

add H_2O to 500 ml

pH adjusted to 7.05

293T Medium

440 ml DMEM

50 ml FBS

5 ml Penicillin G (10,000 U/ml) / Streptomycin (10 mg/ml) Solution

5 ml L-Glutamine

passed through filter with 0.22 µl pore size

T cell Medium

440 ml Iscove's medium

5 ml penicillin G (10,000 U/ml) / streptomycin (10 mg/ml) solution

5 ml L-glutamine

0.5 ml β-mercaptoethanol

passed through filter with 0.22 µl pore size

2.1.5 Mice

All animal work was done with the approval of the University of Pennsylvania Institutional Animal Care and Use Committee. Mice were housed in a 12h light/dark cycle with access to food and water *ad libitum*. Female mice used in the studies were six to nine weeks old and were C57BL/6 mice for EAE studies and BALB/c for *in vitro* studies.

2.2 Methods

2.2.1 Western blotting

For immunoblot analysis, protein was isolated and separated in a 4-20% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with the primary antibody against RARα or the ubiquitously expressed GTPase RAN. Subsequently, a secondary horseradish peroxidase conjugated antibody was added. Finally, an enhanced chemiluminescent substrate kit was used for detection.

2.2.2 Lymphocyte isolation from mesenteric lymph node and spleen

Mesenteric lymph nodes and spleens were processed into a single cell suspension using the plunger of a 3 ml syringe in a 6 cm dish containing 1 ml Hank's 1% FBS. The resulting suspension was filtered through a 70 μm nylon cell strainer.

2.2.3 Purification of lymphocytes

Lymphocytes were isolated and suspended in 500 μl of Hank's 1% FBS per spleen and mesenteric lymph node combined. 50 μl anti-CD8α (clone Ly-2) magnetic microbeads were added and incubated at 4˚C. Cells were then washed in HBSS 1% FBS three times and negatively separated on a MACS LS column inserted in a MACS multistand magnet by flushing out the cells with 9 ml of HBSS 1% FBS.

2.2.4 Tissue culture

T cells were stimulated in T cell medium in the presence of anti-CD3 (1 µg/ml), anti-CD28 (1 µg/ml), anti-IFN-γ (1 µg/ml), anti-IL-4 (1 µg/ml), human IL-2 (100 U/ml) and TGF-β (5 ng/ml) as regulatory T cell favouring conditions. For Th17 cell favouring conditions, IL-6 (20 ng/ml) was added. Cells were plated in multiwell plates at $3x10^6$ cells/ml.

2.2.5 Retroviral transductions

We used bicistronic retroviral vectors containing a green fluorescent protein (GFP) marker (84, 85). RARα cDNA was obtained in plasmid MMM1013-751412 (Openbiosystems, Huntsville, AL, USA). Polymerase chain reaction (PCR) was used to add a consensus Kozak sequence upstream of the RARα start ATG, and the cDNA was cloned into vector MigRI upstream of an internal ribosomal entry sequence followed by GFP cDNA. MigRI without insert was used as a control in transduction experiments. 293T cells were plated out into 6cm dishes at $2x10^6$ per 3 ml 293T medium on day 0. On day 1, cells were 60-80% confluent. Medium was removed and new 293T medium containing 25 µM chloroquine was added. Transfection cocktails were made by adding 50 µl of 2.5 M calcium chloride, 5 µg of helper virus, 10 µg of RARα plasmid DNA to a 1.7 ml Eppendorf tube. Sterile water was added bring the final volume up to 500 µl. 500 µl 2x HBBS solution was added slowly and drop-wise to the tube with a 1 ml serological pipette. Air bubbles were

forced through the solution expelling air from the pipette tip for 10s followed by vortexing for 10s. The cocktail was added slowly to the side of the plate containing the 293T cells and 5-7h after completing the transfection, medium was aspirated and replaced with new 293T medium without chloroquine. After a further 22-24h, the medium was replaced. On day 3, supernatant containing the virus was harvested and filtered through a 0.45 µm syringe filter. T cells were stimulated for 24h in T cell medium in the presence of anti-CD3, anti-CD28, IL-2, IL-6 and TGF-β. The cells were then washed in Hanks 1% FBS and 2-3x10^6 cells were resuspended with the viral supernatant and 8 µg/ml polybrene in Eppendorf tubes. Cells were centrifuged at 6000 g for 90 minutes in a refrigerated tabletop centrifuge. Finally, the cells were washed in Hank's 1% FBS and plated in the same conditions as before.

2.2.6 Intracellular staining of CD4+ T cells for flow cytometry

Intranuclear anti-FoxP3 staining was performed by fixation of cells in 4% paraformaldehyde in PBS, followed by permeabilisation and staining in 0.1% Triton-X in PBS. For intracellular anti-IL-17 staining, cells were first restimulated 4 hours before fixation by adding 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin. Two hours later, 10 µg/ml brefeldin A was added. Cells were then fixed in 4% paraformaldehyde and then permeabilised and stained in saponin 0.1%, 1% FBS in PBS. Anti-FoxP3-PE and CD4-APC were used at a concentration of 3 µl/100 µl of staining buffer, IL-17-PE was used at a concentration of 1 µl/100 µl of

staining buffer. 250000 cells were analysed by flow cytometry using a BD FACSAria cell sorter running FACSDiva software; acquired data was then analysed by FlowJo software.

2.2.7 Agonists and inhibitors

Compounds were dissolved in DMSO at 50 mM for Ro 41-5253 and 728 and 5 mM for ATRA and AM580. They were then added to T cell medium to obtain concentrations of 1 μM for the agonists ATRA and AM580 and 5 μM for the inhibitors Ro 41-5253 and 728.

2.2.8 Induction of EAE in mice

Six to nine week-old female C57BL/6 mice were subcutaneously immunised with 200 μg myelin oligodendroglia glycoprotein (MOG) peptide 35-55 (sequence MEVGWYRSPFSRVVHLYRNGK) emulsified in 500 μg CFA divided over the four flanks. Intraperitoneal injection of 200 ng pertussis toxin diluted into 200 μl of PBS was performed on the day of immunization and 48 hours later. Clinical scoring was undertaken on subsequent days based on standard protocol: 0 = no weakness, 1 = limp tail, 2 = mild hind limb paresis, 3 = severe hind limb paresis, 4 = hind limb paralysis, 5 = moribund (86). Five mice each were treated with AM580 or prednisolone as a negative control; an additional group of six mice was left untreated. Drug treatment was performed daily for 11 days after immunisation by oral gavage

with 3 mg/kg/day AM580 and 5 mg/kg/day prednisolone in aqueous solution with 3% ethanol and 0.5% carboxymethyl cellulose. One animal out of each group was sacrificed on day 8 and another on day 17 to analyse the cells. The remaining animals were sacrificed on day 30.

Spinal cords were removed, homogenised and strained through a 100 μm nylon filter. After centrifugation, the cell suspension was resuspended in 37% isotonic Percoll and underlaid with 70% isotonic Percoll. The gradient was centrifuged at 600 g for 25 minutes at room temperature. The interphase cells were collected and stained as described above.

2.2.9 Histology

After perfusion with phosphate-buffered saline, spinal cords were fixed in 10% normal buffered formalin in Histochoice fixative. 8 µm-thick sections were stained with luxol fast blue. Sections were examined at 20x magnification.

2.2.10 Statistical analysis

Calculation of the probability of a type I error of falsely rejecting the null hypothesis of equal clinical scores in EAE was performed using a one-tailed unpaired student t-test (87, 88). The significance level was chosen to be $p=<0.05$.

3 Results

Retinoids have been reported to increase immunological tolerance. Working with this premise, our studies aimed to further elucidate the mechanism that lies behind this induction of tolerance. In particular, our interests were whether retinoids can modify the T cell fate decision in favour of the tolerance inducing regulatory T cells and inhibit development of Th17 cells, which have been implied in autoimmune diseases.

3.1 Retinoids influence T cell differentiation

3.1.1 ATRA leads to generation of FoxP3 expressing cells at the expense of IL-17 expressing helper T cells

In order to explore the role retinoids play in the differentiation of naïve T cells into regulatory T cells and IL-17 secreting Th17 cells, we first activated CD4+ T cells derived from BALB/c mice by culturing them in conditions favouring the generation of T regulatory cells expressing FoxP3 (61, 62), i.e., T cell medium containing anti-CD3, anti-CD28, IL-2, anti-IL-4, anti-IFN-γ and TGF-β, which are referred to herein as Treg inducing conditions.

Additionally, ATRA was supplemented to cell cultures, but not to negative control cultures. Three days after T cell stimulation was performed, flow cytometry revealed that the frequency of cells expressing FoxP3 more than doubled with the addition of ATRA compared to control without ATRA (Fig. 3, left column).

Furthermore, T cells were stimulated in conditions favouring the development of

Th17 cells, which consist of the antibodies and cytokines listed under Treg inducing conditions plus IL-6 (61, 62), referred to as Th17 inducing conditions below. Addition of ATRA in Th17 inducing conditions led to a more than 25-fold reduction in the number of IL-17 expressing cells (Fig. 3, right column), compared to negative control without drug treatment. These results indicate that ATRA favours the development of regulatory T cells at the expense of Th17 cells.

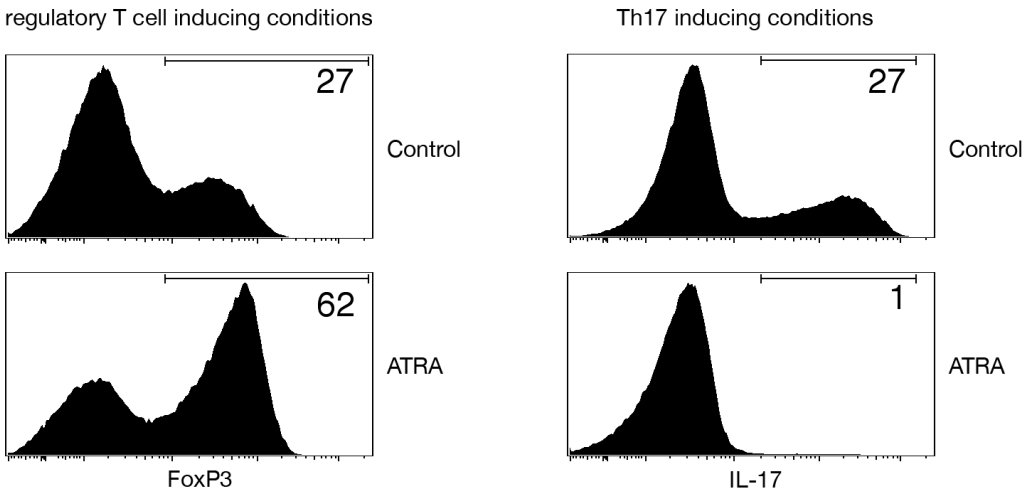

Figure 3: ATRA induces FoxP3 T cells and suppresses IL-17 expressing T cells
CD4+ T cells from mesenteric lymph node and spleen were activated in the presence of TGF-β, which led to the induction of regulatory T cells expressing FoxP3 (left column). When ATRA was added, a larger percentage of cells expressed FoxP3. T cell activation in medium containing TGF-β and IL-6 promoted production of IL-17 by Th17 T cells (right column). The expression of IL-17 was decreased however, when ATRA was present. Analysis was carried out on d3. For IL-17 intracellular cytokine staining, T cells were reactivated with PMA and ionomycin 4h before fixation. Results shown are representative of 3 independent experiments (89).

3.1.2 RARα is expressed in activated T cells.

One of the nuclear receptors for ATRA is RARα (90). We therefore wanted to establish whether RARα is expressed in T cells.

Western blot analysis showed no detectable RARα expression in naïve T cells. Upon activation in Treg inducing conditions and in Th17 inducing conditions for 3 days, a strong band of approximately 50 kD, corresponding to RARα protein could be detected in cells cultured in both conditions (Fig. 4). Hence, RARα is expressed in Treg and Th17 cells, but not in naïve T cells.

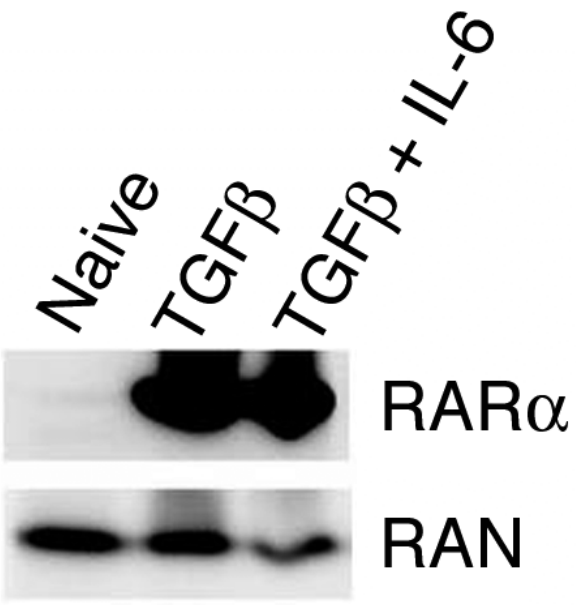

Figure 4: RARα is expressed in Th17 cells and Treg cells, but not in naïve T cells
T cells were activated in the presence of TGF-β favouring the development of Treg cells (middle lane) or TGF-β + IL-6 favouring the development of Th17 cells (right lane). Western blot showed expression of RARα in both Treg and Th17 cells. Naïve T cells showed no expression of RARα (left lane). The ubiquitously expressed GTPase RAN was used as a loading control (89).

3.1.3 RARα activation causes induction of FoxP3

To investigate the role of RARα in T cell differentiation, we employed a specific agonist and a specific antagonist for RARα.

AM580 is a highly specific small molecule agonist for RARα (91). Addition of AM580 increased the frequency of FoxP3 expression in CD4+ T cells activated in Treg inducing conditions by more than 70% (Fig. 5). Frequency of FoxP3 expression in Th17 conditions more than doubled with AM580 present.

RO 41-5253 is a highly specific antagonist of RARα (92). In T cells activated in Treg or Th17 conditions, the addition of RO 41-5253 caused a decrease in the frequency of FoxP3 expressing cells of more than 60% compared to untreated control cells (Fig. 5). The suppressive action of the inhibitor compared to untreated control cells suggests that there is a background level of RARα-activators present in the T cell medium, particularly in FBS. Otherwise, in the absence of RARα-activators, the antagonist by itself should show no effect.

When we activated T cells in Th17 inducing conditions, the frequency of IL-17 expression was decreased by the RARα agonist AM580 by more than 60% compared to untreated control (Fig. 6).

In summary, both ATRA and a highly specific agonist for RARα cause T cell differentiation to favour the regulatory T cell lineage while leading to fewer Th17 T cells. Antagonising RARα, on the other hand, results in fewer regulatory T cells.

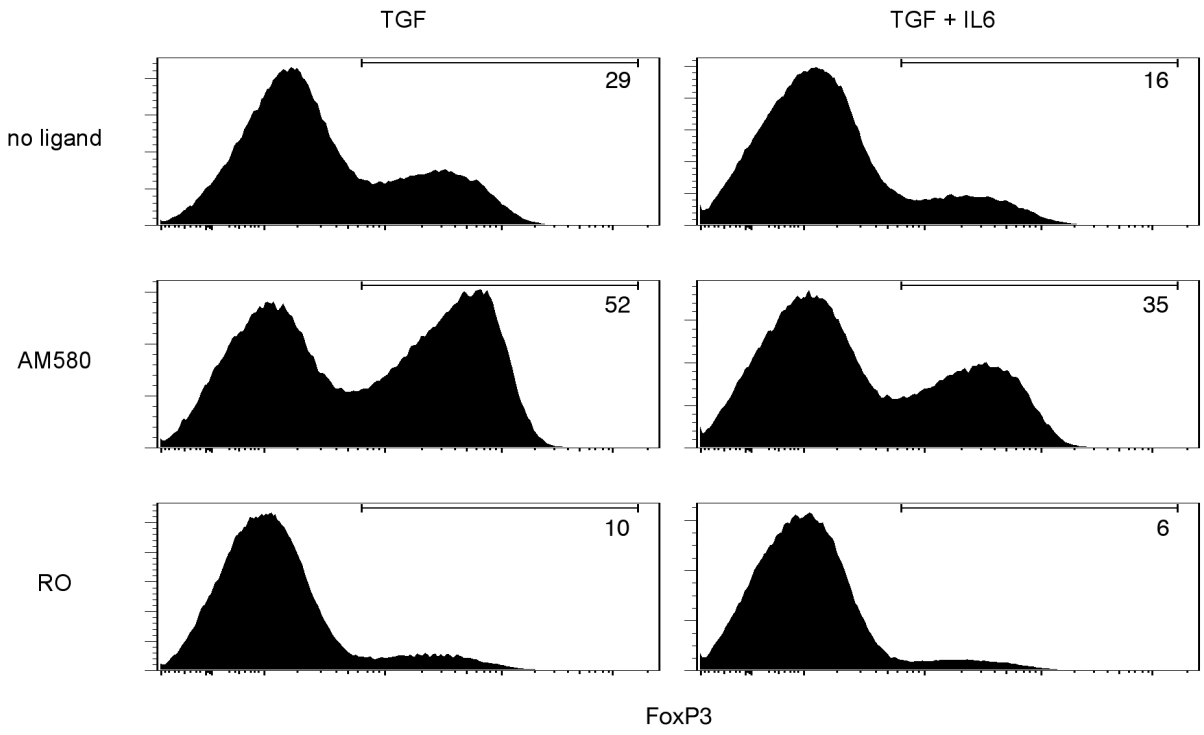

Figure 5: Activation of RARα leads to induction of FoxP3 while antagonisation of RARα leads to suppression of FoxP3
CD4+ murine T cells were activated in Treg inducing conditions containing TGF-β or Th17 inducing conditions containing TGF-β and IL-6. The presence of a highly specific activator of RARα, i.e., AM580, leads to an increased percentage of FoxP3 expressing T cells on d3 after initial activation. Conversely, addition of Ro 41-5253 (RO), a highly specific RARα antagonist, leads to a lower percentage of FoxP3 expressing T cells as analysed by flow cytometry. Results are gated on CD4+ cells. Results are representative of 3 independent experiments (89).

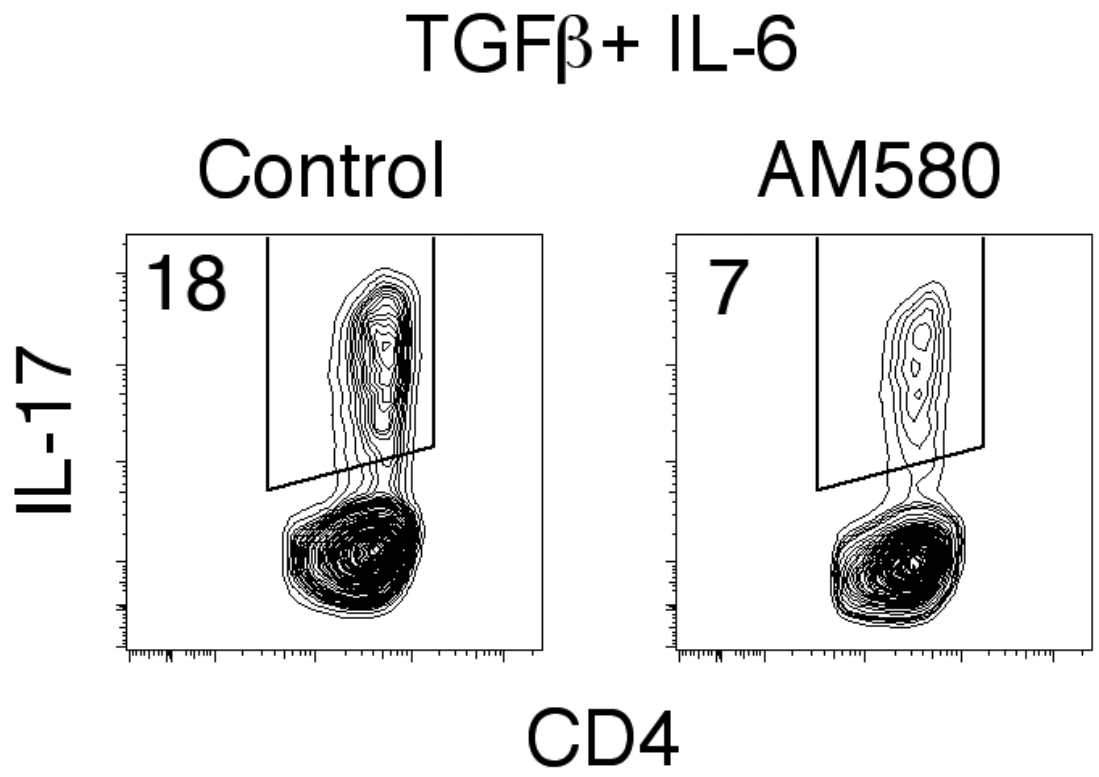

Figure 6: Highly specific activation of RARα suppresses IL-17 in Th17 cells.
CD4+ T cells were activated in conditions favouring the development of IL-17 secreting Th17 cells in T cell medium containing TGF-β and IL-6. The simultaneous activation of RARα by addition of a highly specific small molecule agonist, i.e. AM580, led to a lower relative number of IL-17 expressing cells compared to control. Cells were analysed on d3 after initial activation. Results are representative of 3 independent experiments (89).

3.1.4 Differing effects of two RARα antagonists on FoxP3 expression

As we had shown that activation of RARα can lead to induction of FoxP3 (Fig. 5), we wanted to find out if this induction can be reversed by the additional presence of antagonists of RARα.

In the absence of an RARα antagonist, ATRA and AM580 led to increased FoxP3 expression levels (Fig. 7, top row). It was to be expected that when agonist and antagonist were added together, the antagonist should cancel the actions of the agonist when administered in excess.

However, when RARα agonists ATRA or AM580 were added together with the RARα inhibitor RO 41-5253, FoxP3 expression was not reduced to its original level. Instead, expression of FoxP3 stayed as high as if no inhibitor had been added (Fig. 7, middle row, as compared to top row). As previously stated, RO 41-5253 can, however, lower the expression of FoxP3 when administered alone. Paradoxically, it seems that RO 41-5253 cannot antagonise the actions of any of the two agonists, even though it was supplied in 5-fold excess. RO 41-5253 has recently been found to activate the nuclear receptor PPARγ (93). The failure of RO 41-5253 to reduce FoxP3+ cell numbers could therefore be a PPARγ mediated effect. Recently, it has been reported that activation of PPARγ ameliorates EAE and leads to a reduction of relative Th17 cell numbers (94). However, the role of PPARγ in T cell differentiation has so far not been sufficiently explored.

Inhibitor 728 is a novel RARα antagonist (95), which has no effect on PPARγ. As with

RO 41-5253, Inhibitor 728 can suppress FoxP3 expression to below background levels when no drug is present. When we tested inhibitor 728 in conjunction with ATRA and AM580, Inhibitor 728 showed a reduction of FoxP3 expression when used in conjunction with either agonist, ATRA and AM580 (Fig. 7, bottom row).

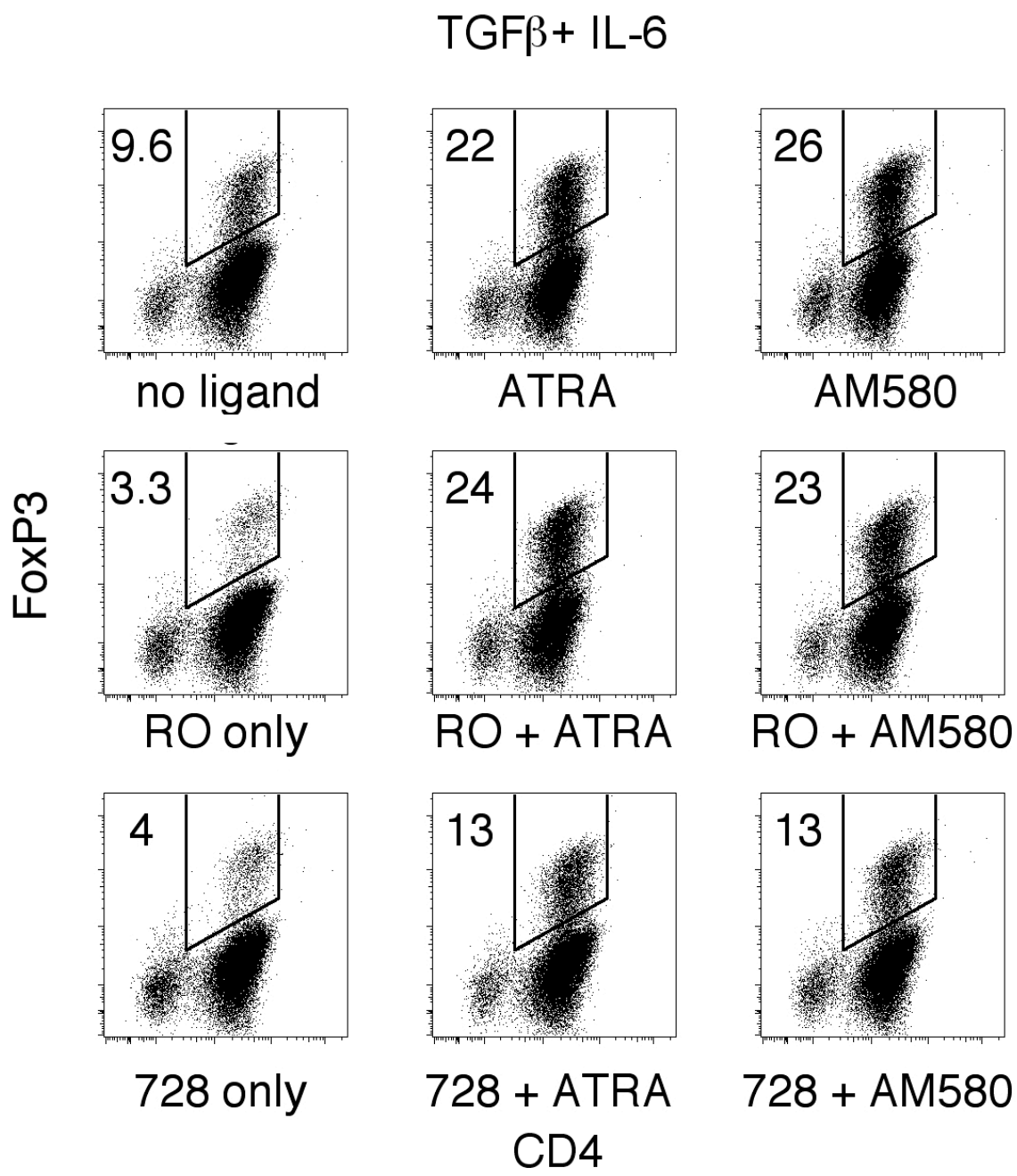

Figure 7: FoxP3 expression in RAR activation and antagonisation
The highly specific inhibitors of RARα, RO 41-5253 and 728, antagonise the activation of RARα and cause suppression of FoxP3 in CD4+ T cells activated in the presence of TGF-β and IL-6 (first column). Reduced induction of FoxP3 by ATRA or AM580 occurs in the presence of the inhibitor 728, but not in the presence of RO 41-5253 (second and third row, respectively) compared to control (first row). Cells were analysed by flow cytometry on d3 after initial stimulation. Results are representative of 3 independent experiments.

3.1.5 A retrovirus encoding RARα increases FoxP3 expression in T cells

To further evaluate the role of RARα in T cells, we constructed a retrovirus containing the entire coding region of the RARα gene and green fluorescent protein (GFP).

T cells were cultured in conditions favouring the development of regulatory T cells and were transduced with RARα virus or empty control virus. On day 6, cells were restimulated and AM580 was added. On day 8, GFP+ CD4+ T cells transduced with RARα virus had a 15% higher frequency of FoxP3 expression than those transduced with empty control virus as seen by flow analysis (Fig. 8).

These results indicate that overexpression of RARα in conjunction with treatment with AM580 increases the number of regulatory T cells expressing FoxP3.

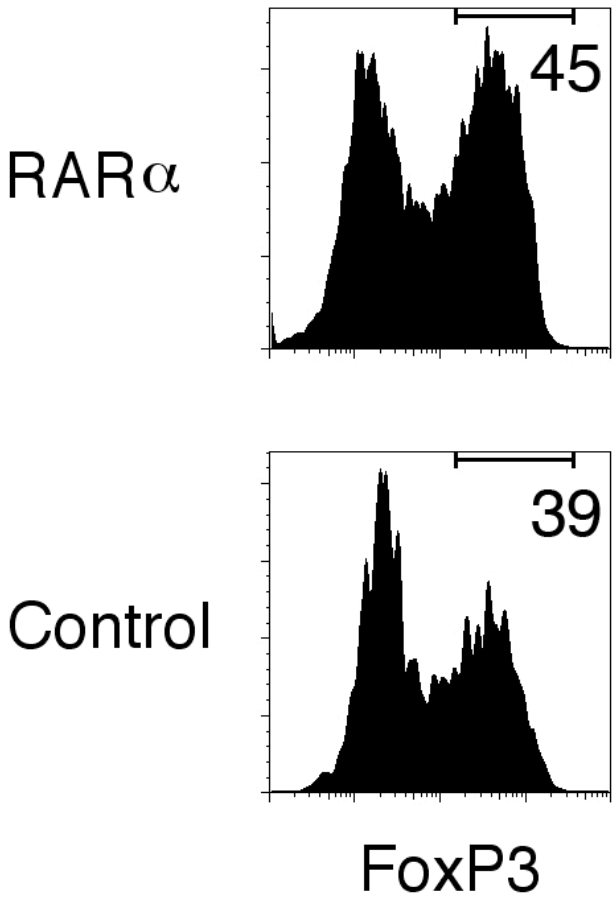

Figure 8: Overexpression of RARα leads to the induction of FoxP3 in CD4+ T cells
T cells were activated in Treg inducing conditions and transfected with an MMLV based retrovirus containing the entire coding region of RARα and GFP or a control virus containing GFP only. Cells were restimulated on d6 and AM580 was added to activate RARα. Cells were analysed by flow cytometry on d8. Results are gated on CD4+ and GFP+. Results are representative of 3 independent experiments (89).

3.2 RARα in EAE

3.2.1 Activation of RARα results in amelioration of EAE scores

To test whether the induction of FoxP3 expressing T cells by an activator of RARα may ameliorate the course of EAE, we measured the clinical score in mice treated with AM580 versus untreated control animals after induction of EAE.

EAE was induced on day 0 using MOG peptide and mice were divided in three groups. Clinical scores were 0 in all mice at the outset of the experiment. The positive control group received no drug treatment. The negative control group was treated daily with the steroid prednisolone until day 10 to suppress immune reactions. Finally, in the third group, mice were treated daily with AM580 until day 10.

In the untreated group, average clinical scores started to increase on day 12 and reached a maximum of 3.3 at the end of the experiment on day 30.

By day 28, none of the untreated animals could hold on to the cage with their hindlimbs when placed on the underside of the cage. In mice treated with AM580, mice developed only milder signs including some hindlimb dysfunction, but could still hold on to the bottom of the cage. Average scores remained much lower and during the entire experiment never exceeded values of 1 (Fig. 9). The steroid treated group showed average scores of up to 0.25, but had returned to a score of 0 by day 30, when they showed no signs of clinical impairment.

Briefly, treatment of mice with an activator of RARα in an EAE model prevented development of severe signs of clinical impairment and resulted in a significantly lower clinical score as compared to untreated control animals ($p<0.036$).

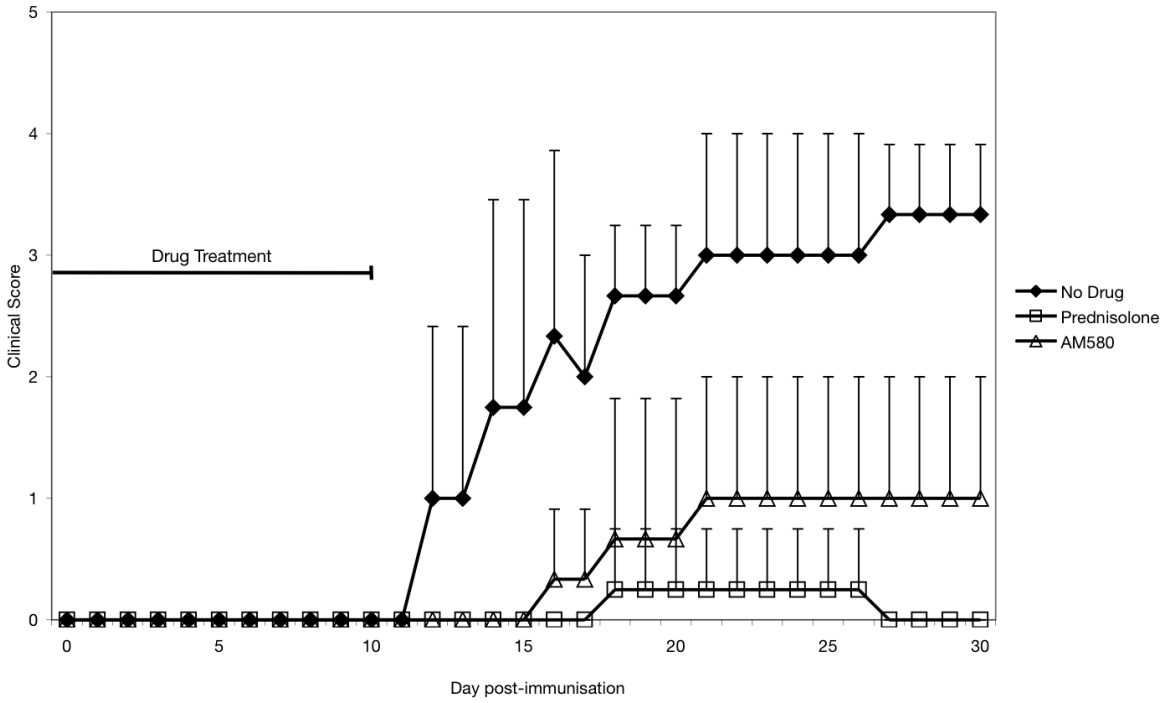

Figure 9: RARα activation leads to amelioration of EAE
A total of 16 mice were included in the experiment. A group of five mice were treated daily with AM580 (3 mg/kg/day), another six mice were treated daily with prednisolone (5 mg/kg/day) until day 10 after induction of EAE. The control group received no drug and included five mice. Treatment with AM580 led to lower clinical scores compared to untreated animals ($p<0.036$, shown are average clinical scores and standard deviation of the mean).

3.2.2 Spinal cord histology

Spinal cord tissue sections were stained with luxol fast blue to show myelin. Samples were taken on day 30 from an AM580 treated animal with a clinical score of 1 (Fig. 10; 1-4) and from one untreated animal with a clinical score of 3 (Fig. 10; 5-8). Both samples show infiltrating lymphocytes, which penetrate the myelin sheath.

Hence, while AM580 improves the clinical score in EAE, it cannot completely prevent lymphocyte infiltration.

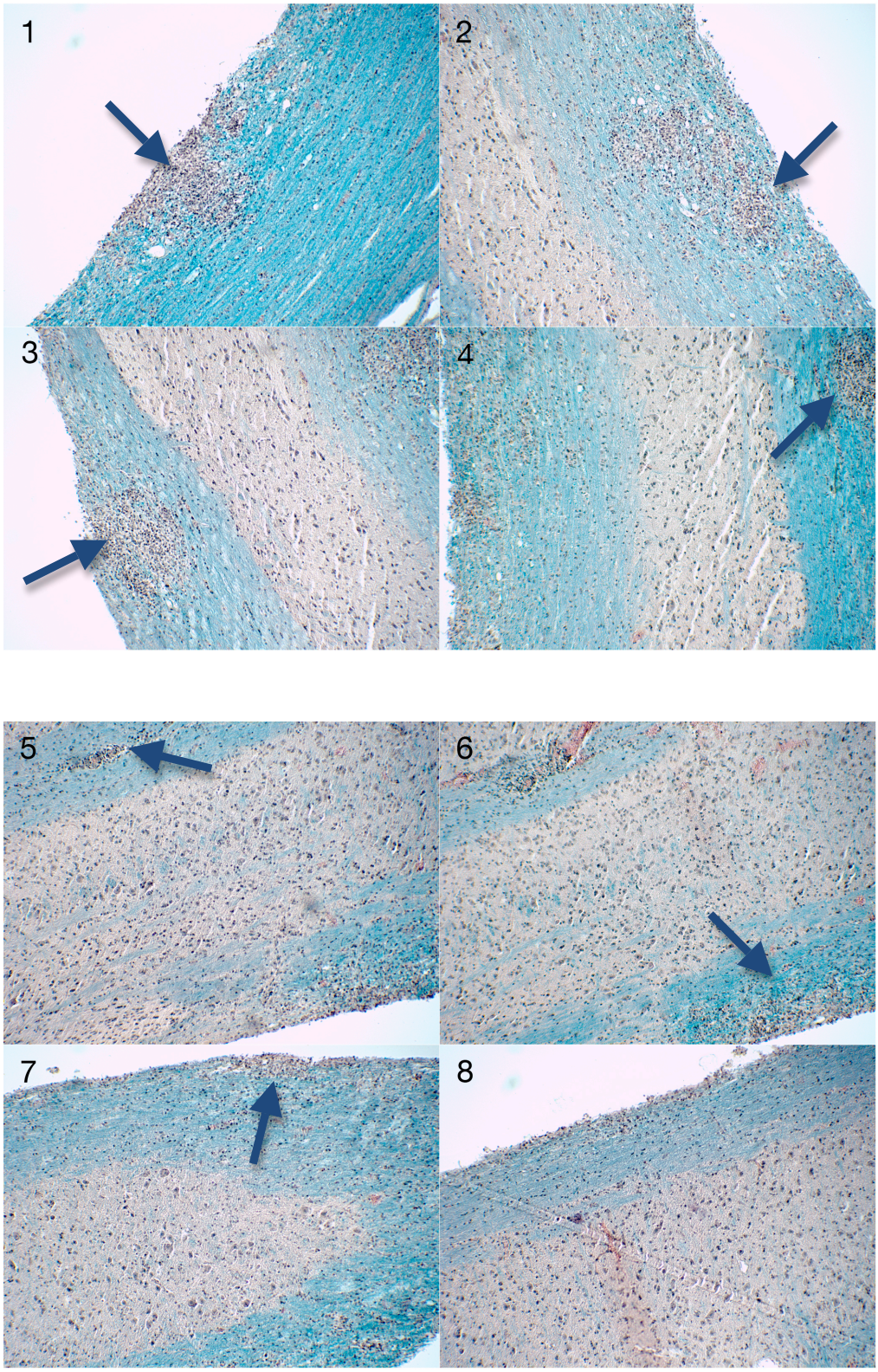

Figure 10: Spinal cord histology
Representative sections of spinal cord of an animal treated with AM580 and a clinical score of 1 (1-4) and of an untreated animal with a clinical score of 3 (5-8) on day 30 after induction of EAE. Sections are stained with luxol fast blue, which dyes myelin blue. Invading lymphocytes and macrophages appear black (arrows) and coincide with loss of blue myelination. AM580 cannot fully prevent lymphocyte infiltration.

3.2.3 FoxP3 expression in T cells from CNS of mice in EAE

To investigate the involvement of FoxP3 expressing cells in the amelioration of EAE, we isolated T cells from the CNS of mice 30 days after the induction of EAE. In the group treated with AM580, flow cytometry revealed a higher frequency of T cells from CNS expressing FoxP3 than in the untreated control group. FoxP3 expression frequency was increased by more than 30% (Fig. 11). CNS isolates of negative control animals treated with prednisolone showed no detectable T cells at all.

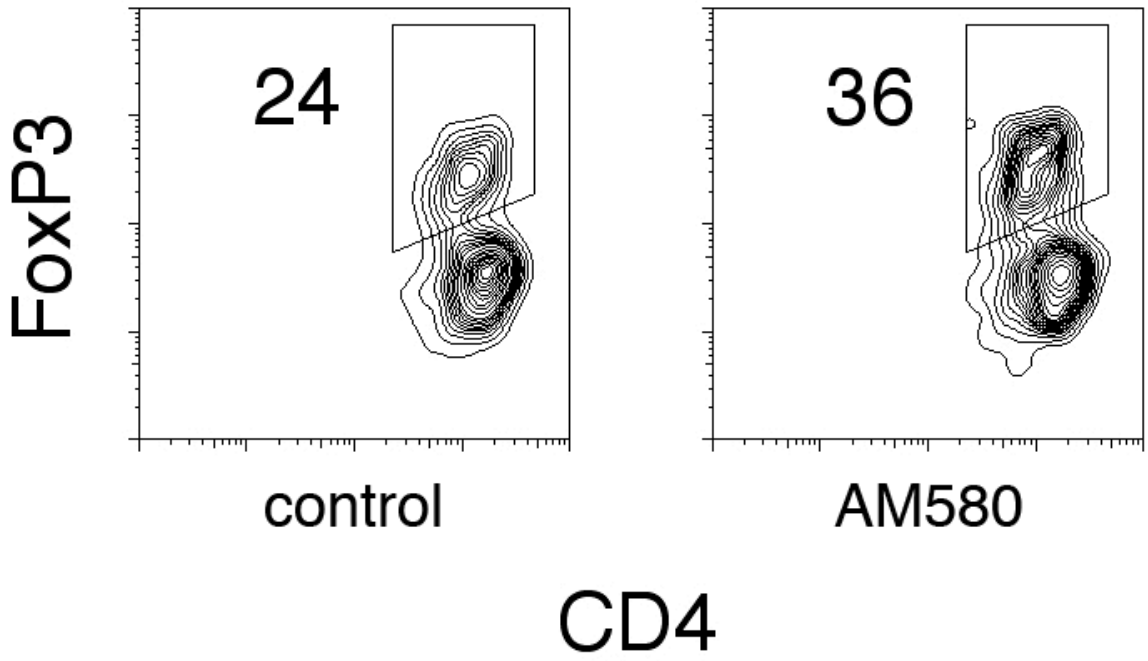

Figure 11: In EAE, animals treated with an activator of RARα (AM580) show a higher percentage of FoxP3 expressing CNS T cells.
30 days after the induction of EAE, T cells were isolated from the CNS of three animals in the AM580 and control group, respectively. In animals treated with AM580, a higher proportion of T cells show expression of FoxP3 than those isolated from untreated control animals.

4 Discussion

In autoimmune diseases, the lack of immunological tolerance towards self-antigens can result in severe tissue damage due to the actions of the immune system. It would therefore be of great value to be able to modify and enhance tolerance in the immune system. It is well known that retinoids can increase immunological tolerance, which would make retinoids attractive therapeutic substances for the treatment of autoimmune diseases (69-73, 96). However, the mechanism by which retinoid tolerance induction is mediated has remained obscure. Recently, it was discovered that a novel T cell lineage, i.e. Th17 cells, is involved in many autoimmune processes including EAE (54-60). Subsequently, it was shown that the development of Th17 cells and the tolerance inducing Tregs follow reciprocal development pathways (61, 62). Specifically, T cell differentiation in the presence of TGFβ yielded Tregs, while the simultaneous addition of TGFβ and IL-6 to T cell cultures resulted in the creation of Th17 cells. Therefore, we wanted to address the question whether retinoids can influence the reciprocal development pathway of Th17 cells and Tregs. In addition to *in vitro* studies, we wanted to use an animal model to confirm our findings *in vivo*.

4.1 Retinoids induce Tregs at the expense of Th17 cells

To investigate whether retinoids influence the differentiation of naïve helper T cells into mature subsets, we examined the effect of the retinoid ATRA on Treg versus Th17 cells. Our results show that when T cells are activated in conditions favouring

the development of Treg cells in the presence of ATRA, the number of Treg cells is increased at the expense of Th17 cells. ATRA diverts cells toward the Treg lineage and away from the Th17 lineage, even in the presence of Th17 inducing cytokine conditions. This suggests a mechanism by which retinoid induction of immunological tolerance is mediated.

It is well known that ATRA binds to and activates RAR nuclear receptors. Transcription is initiated when activated RAR forms a heterodimer with another nuclear receptor, RXR. As ATRA activates all three subtypes of RAR, named RARα, RARβ and RARγ, we wanted to find out whether T cell differentiation could be influenced by selectively activating just one of the subtypes.

4.2 Activation of RARα induces Tregs in vitro

In order to examine the role of RARα in T cell differentiation we employed a highly specific small molecule agonist. Just as in unspecific RAR activation by ATRA, specific activation of RARα led to lower percentages of Th17 cells and increased percentages of Treg cells. Antagonisation of RARα resulted in a decrease in the percentages of Treg cells. Finally, overexpression of RARα using a retroviral vector led to higher numbers of Treg cells, further supporting our finding that RARα activation can induce FoxP3 in T cells.

The mechanism by which RARα induces FoxP3 could be by direct promoter activation or involve intermediate factors. Retinoids are known to induce a family of

proteins called CCAAT/enhancer binding proteins (C/EBPs) (97). A binding site for C/EBPs has recently been identified in the FoxP3 promoter (98), suggesting this as a possible pathway.

When ATRA and a specific inhibitor of RARα are present, FoxP3+ induction is suppressed to almost background levels without any retinoids being present. This suggests that FoxP3 induction is predominantly mediated through RARα. The small, remaining activation might be due to incomplete inhibition or mediation through the other retinoic acid receptor subtypes, RARβ and RARγ, which are also activated by ATRA.

Clinically, selective activation is desirable to avoid RARγ activation, which causes unwanted dermal side effects in treatment with pan-RAR agonists such as ATRA (99). Furthermore, ATRA serum levels decline in patients over time, while specific agonists of RARα have been shown not to suffer from this issue (100).

At the same that the *in vitro* part of our study was completed, another group reported that ATRA induces expression of FoxP3 and represses expression of IL-17 (101). That study, however, did not implicate RARα as the factor responsible for this important regulatory switch.

In principle, our approach cannot exclude the possibility of T cell extrinsic effects. T cell cultures were not purified of antigen presenting cells; it is therefore possible that the induction of FoxP3 is partly due to a possible parallel induction of tolerogenic dendritic cells. However, it has subsequently been shown that FoxP3 can be induced by ATRA in purified cell cultures (102).

Recently, it has been suggested that the induction of FoxP3 by retinoic acid in the presence of TGFβ may be an indirect effect mediated through bystander CD4+ CD44(hi) memory T cells (103). It was argued that CD4+ CD44(hi) T cells secrete a cocktail of cytokines, i.e., IL-4, IL-21 and IFN-gamma, which prevents the differentiation of naïve T cells into FoxP3+ T cells in the presence of TGFβ. ATRA inhibits the production of these cytokines by CD4+ CD44(hi) T cells. Hence, it was argued, the induction of FoxP3 was merely an indirect effect.

This claim was subsequently refuted (102) using cells from CD80(-/-) CD86(-/-) knockout mice, which are low in CD4+ CD44(hi) memory T cells and subsequently purifying CD4+ CD44(lo) T cells. Induction of FoxP3 by ATRA in the presence of TGFβ was possible even without CD44(hi) T cells present. Therefore, the induction of FoxP3 described by us does not seem to be a purely indirect effect mediated by memory T cells.

4.3 RARα activation ameliorates EAE

To study retinoid effects in autoimmunity *in vivo*, we chose EAE, a long established and widely used animal model of MS.

Various agonists for nuclear receptors are known to play a role in EAE. Vitamin D was shown to prevent EAE (104) and it has been demonstrated that retinoic acid can suppress EAE (69). However, this was before the discovery of Treg and Th17 cell populations. The suppressive effect on EAE was therefore thought to be due to a

shift to a Th2 response (105), whereas EAE was assumed to be Th1 mediated (106, 107) as opposed to a Th17 mediated disease, which it has recently been shown to be (108).

Since our *in vitro* results suggested a role for RARα agonists in the induction of Tregs, we investigated whether RARα agonists ameliorate or prevent the development of EAE in mice. We found that treatment with an RARα agonist led to improved clinical scores. More importantly, we could show that in animals treated with an RARα agonist, FoxP3 expression in T cells in the CNS is increased. This suggests a possible mechanism for the prevention of symptoms of EAE by treatment with RARα agonists, namely that the induction of regulatory T cells in the CNS can reduce demyelination.

Our results have recently been confirmed by a different group using an alternative RARα agonist (109), albeit one that is less specific and shows higher activation of RARβ (83). In their *in vitro* studies, they could verify the inhibition of Th17 differentiation by RARα/β induction. In animal studies they found that on day 15 after EAE induction including 8 days of RARα/β agonist treatment, FoxP3 induction in T cells in the CNS could not be confirmed at the transcriptional level. The differing results could be due to the earlier time in the EAE course or inspection of FoxP3 at RNA versus protein level. While they examined FoxP3 transcription in CNS T cells on day 15, we investigated FoxP3 expression on day 30 after EAE induction. Another reason for the disagreeing results could lie in the different nature of the synthetic

agonist used in their study, which leads to coactivation of RARβ. A further dissimilarity was the more than 25% shorter duration of drug treatment.

Reports by another group indicatethat retinoids do not increase the overall Treg population in EAE (110). However, in this study T cells were not isolated from the CNS, but only from peripheral lymph nodes and spleen.

The results of our study have also been confirmed in a model of inflammatory bowel disease (111). In this SAMP1/YP mouse model of Crohn's disease, it was shown that retinoids could induce FoxP3 in CCR9+ α4β7+ CD4+ T cells *in vivo* and ameliorate intestinal inflammation. When mice were kept deficient in vitamin A, this FoxP3+ CCR9+ alpha4beta7+ CD4+ T cell population was decreased compared to control animals supplied with feed containing normal amounts of vitamin A. However, in vitamin A deficient animals, a distinct CD103+ CCR7+ FoxP3+ CD4+ T cell population was induced and a decreased inflammatory reaction in the intestine was also observed. In other words, tolerance was induced by both higher as well as lower than normal levels of vitamin A.

The results in vitamin A deficient animals might be of limited value, as keeping mice on a low vitamin A diet can lead to unphysiological effects and results in abnormal numbers of Th1, Th2 and Th17 cells as is demonstrated in the same article. Further experiments *in vitro* using the RARα antagonist Ro 41-5253 showed induction of FoxP3+ T cells, confirming our earlier results. The authors concludethat activation as well as blockade of RARα causes induction of FoxP3. This is problematic, as Ro 41-5253 is not only an antagonist of RARα, but also induces PPARγ (93), which itself is

involved in Treg induction (112) and suppression of Th17 (94).

A further confirmation of our results comes from a group that studied cytokine production in colon biopsies from patients suffering from ulcerative colitis (113). Treatment of the biopsy specimen with retinoic acid resulted in decreased IL-17 expression while FoxP3 expression was increased. In the same publication, mice in a TNBS induced model of inflammatory bowel disease responded to retinoid treatment with amelioration of colitis, increased expression of FoxP3 and decreased expression of IL-17 in mesenteric lymph nodes.

Therapy of multiple sclerosis			
Drug	**Presumed mechanism of action**	**Side effects**	**Comments**
IFN-β	Inhibition of T cell migration into the CNS	flu-like symptoms, induction of neutralizing antibodies to IFN-β with loss of effectiveness (114)	Studies indicate reduction of relapse rate by about 30% and slowing of disease progression by about 30% (115) – long-term benefit remains unproven for lack of long-term blinded randomised controlled trials.
Glatiramer acetate	Binding to MHC and competing with myelin antigens for presentation to T cells (17)	Injection site reactions, flu-like symptoms	Lowers relapse probability (18), but not disease progression (20).
Natalizumab	Antibody to α4-integrin. Inhibits lymphocyte migration across the blood brain barrier.	Progressive multifocal leukoencephalopathy at about 1 in 1000 patients (116).	Development of progressive multifocal leukoencephalopathy, which is often fatal (117). It is therefore used as a second line drug.
Alemtuzumab	Antibody to CD52 on mature lymphocytes leading to lymphocyte depletion.	Opportunistic infections	Not licensed for use in MS.
Mitoxantrone	Cytostatic	Cardiotoxicity	Second line therapy – limited to rapid progressive disease course
Steroids	Immunosuppression	Cushing's syndrome	Limited to treatment of acute attacks
RARα agonists	Induction of regulatory T cells		Animal data presented here is encouraging. Tamibarotene is a licensed RARα agonist in use for acute promyelocytic leukemia, which could be tested in MS.

4.4 Conclusions

Herein, we have provided evidence that the activation of RARα leads to increased tolerance in the immune system by inducing regulatory T cells at the expense of Th17 cells, which are associated with autoimmunity and inflammation.

This observation was confirmed *in vivo* in a mouse model of multiple sclerosis. In this model, treatment with an activator of RARα led to amelioration of disease. At the same time, animals had more regulatory T cells in the CNS, providing a rationale for the clinical improvement.

This provides a model to explain the control of autoimmunity by retinoids that has previously been reported (69-73, 75). Ligands for RARα could therefore be lead compounds for drugs against autoimmune diseases.

4.5 Outlook

Future directions at the cellular level could include investigation of migration patterns of T cells in animals treated with retinoids. Oestrogen, another nuclear receptor ligand, can prevent lymphocytes homing to the gut (118). It has been shown that T cells can home to the gut when presented with retinoic acid (79). However, it is unknown whether activated T cells in the brain can be made to leave the site of inflammation to migrate to the intestine. This could be investigated, for example, by using methods of *in vivo* imaging such as 2-photon microscopy of fluorescent labelled T cells and μCT or high-field MRI with antibody bound contrast agents to track T cells.

After inducing EAE, labelled T cells would be seen to migrate to the brain. After treatment with retinoids, the movement of T cells in the body could be tracked and any putative migration to the gut could be visualised and measured.

To further classify RARα induced suppression of EAE, it would be interesting to see whether EAE amelioration can be caused by activation of RARα in the absence of FoxP3. Mice hemizygous for a defective copy of the X-chromosomal FoxP3, however, suffer from a lethal phenotype (43). A T cell conditional knockout exists (119), which could possibly be employed, though difficulty could arise due to incomplete FoxP3 deletion.

On the sub-cell, biochemical level, the role of activated RARα could be investigated further. It would be interesting to see whether intermediate factors are involved in the induction of FoxP3. Gene chip experiments could suggest factors important in the RARα-FoxP3 pathway. The binding sites for RARα could also be elucidated using a chromatin immunoprecipitation followed by microarray detection approach (ChIP-on-chip).

Once intermediate factors are revealed, one could design small molecules to influence protein-protein interaction to modulate the immune response.

Additional markers for Tregs have been uncovered recently: CD39 is an ectonucleotidase, which removes extracellular ATP and is expressed on FoxP3+ cells (120). GARP is a surface protein, which appears to be present on Tregs and correlates with their suppressive activity (121). IL-35 seems to be a mediator of immunosuppression exerted by Tregs (122). RORC2 is a negative regulator of

FoxP3 (123). In light of these findings, it seems interesting to investigate whether activation of RARα can induce CD39, GARP and IL-35 or inhibit RORC2. Furthermore, Tregs present in the CNS in our EAE model could be screened for CD39, GARP, IL-35 and RORC2 in order to characterise differences in the Treg populations in untreated and RARα activator treated mice.

Another line of investigation into the role of RARα would be to look at other animal models of autoimmunity. These could include models of asthma, type 1 diabetes and arthritis in order to test whether activation of RARα can ameliorate disease symptoms as well as severity.

The relationship between vitamin D and Tregs is worth re-examining. The vitamin D receptor is a nuclear receptor similar to RARα as it also heterodimerises with RXR. Recently, evidence has surfaced that lack of vitamin D is associated with the risk of developing MS (124), providing an explanation for geographic distribution of MS. Furthermore, activation of the vitamin D receptor can lead to increased expression of FoxP3 (125). Vitamin D is used in the treatment of psoriasis and can also prevent the induction of EAE (104). It would be interesting to see how this effect is mediated. Furthermore, as various retinoids are licensed as drugs or food supplements, clinical trials in patients with autoimmune diseases seem feasible, especially as FoxP3 induction by retinoids has recently been shown to be possible in human CD4+ T cells (126).

Recently, a specific activator for RARα has been licensed in Japan for use in treating refractory acute promyelocytic leukaemia (127). Using this drug, it therefore appears

possible to study the potential of RARα activation in patients with MS and other autoimmune diseases. On the other hand, the role of vitamin A deficiency in autoimmune diseases deserves to be re-examined in the light of our findings.

5 Summaries

5.1 English Summary

Autoimmune diseases have a high incidence and are a major cause of morbidity in the developed world. They occur when the immune system mistakes self for non-self tissue and mounts an inappropriate attack. This can be viewed as a result of decreased immunological tolerance. One autoimmune disease in particular, multiple sclerosis, has been described as the most common cause of neurological disability in young adults. Autoreactive T cells are involved in numerous autoimmune processes. In MS, T cells attack the myelin nerve sheaths around neurons.

The present work examines the role of retinoids in the immune system. It is well known that retinoids can enhance immunological tolerance. However, the mechanism of how this tolerance is achieved had not been shown. The first part of our studies investigated the effects of retinoids on T cell development *in vitro*. In the second part, the findings were tested in an animal model of MS, i.e., EAE.

We were able to show that retinoids can skew T cell differentiation towards the regulatory T cell subset, which is associated with increased immunological tolerance. This augmentation of regulatory T cell numbers occurs at the expense of the Th17 subset, which is implied in many autoimmune processes, including multiple sclerosis.

Furthermore, we presented evidence that the increased regulatory T cell numbers and decreased Th17 numbers are caused by the activation of the nuclear receptor RARα. *In vitro* studies were carried out using highly specific agonists and antagonists of RARα as well as retroviral overexpression. Furthermore, we tested our finding of RARα mediated tolerance *in vivo*. In EAE, activation of RARα led to amelioration of symptoms and an increased number of regulatory T cells in the CNS.

The successful amelioration of EAE by RARα agonists suggests that RARα agonists could be important drug candidates in the prevention and treatment of MS, in particular as one RARα activator is already used in clinical practice. In more general terms, RARα activators could be versatile clinical tools to modulate the immune response towards increased tolerance.

5.2 Deutsche Zusammenfassung

Autoimmunkrankheiten haben eine hohe Inzidenz und tragen wesentlich zur Morbidität in der entwickelten Welt bei. Autoimmunerkrankungen entstehen, wenn das Immunsystem fälschlicherweise eigenes Gewebe als fremd ansieht und eine Abwehrreaktion einleitet. Dieser Prozeß kann als das Resultat einer verminderten immunologischen Toleranz gesehen werden. Multiple Sklerose ist eine Autoimmunerkrankung, die möglicherweise die häufigste Ursache neurologischer Behinderung in jungen Erwachsenen darstellt. Autoreaktive T-Zellen sind an zahlreichen autoimmunen Prozessen beteiligt. Bei der multiplen Sklerose attackieren autoreaktive T-Zellen die Myelinscheiden der Neurone.

Die vorliegende Arbeit untersucht die Rolle von Retinoiden im Immunsystem. Es ist bekannt, daß Retinoide die immunologische Toleranz erhöhen können. Der Mechanismus für diese Toleranzerhöhung war jedoch bis jetzt unklar. Im ersten Teil dieser Arbeit untersuchten wir den Einfluß von Retinoiden auf die Entwicklung von T-Zellen *in vitro*. Im zweiten Teil wurden die Ergebnisse in EAE getestet, einem Tiermodell der Multiplen Sklerose. Wir konnten demonstrieren, daß Retinoide die T-Zell-Differenzierung steuern können, so dass mehr regulatorische T-Zellen entstehen, die mit erhöhter immunologischer Toleranz verbunden sind. Die Erhöhung der regulatorischen T-Zell-Zahlen ging auf Kosten der Entstehung von Th17 T-Zellen, die an vielen autoimmunen Prozessen beteiligt sind, auch bei der Multiplen Sklerose.

Zudem konnten wir zeigen, dass eine erhöhte Anzahl regulatorischer T-Zellen und eine verminderte Anzahl von Th17 Zellen durch die Aktivierung des nukleären Rezeptor RARα erzeugt wird. *In vitro* Studien sowohl mit hochspezifischen RARα-Agonisten und -Antagonisten als auch mit retroviraler Überexpression wurden durchgeführt. Zusätzlich testeten wir unsere Ergebnisse *in vivo*. Im Tiermodell EAE führte die Aktivierung von RARα zu einer Symptomverbesserung und einer erhöhten regulatorischen T-Zell-Zahl im ZNS.

Die erfolgreiche Symptomverbesserung bei EAE durch RARα-Agonisten legt nahe, daß RARα-Agonisten wichtige Kandidaten bei der Suche nach Medikamenten für die Multiple Sklerose sein könnten, insbesondere weil ein RARα-Agonist bereits eine klinische Zulassung für einen anderen Anwendungsbereich besitzt. Ganz allgemein könnten RARα-Agonisten ein wertvolles klinisches Werkzeug zur Modulation der Immunantwort hin zu höherer Toleranz sein.

6 References

1. Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clinical immunology and immunopathology. 1997;84(3):223-43.

2. Weiner HL. The challenge of multiple sclerosis: how do we cure a chronic heterogeneous disease? Ann Neurol. 2009;65(3):239-48.

3. Thrower BW. Clinically isolated syndromes: predicting and delaying multiple sclerosis. Neurology. 2007;68(24 Suppl 4):S12-5.

4. Korn T. Pathophysiology of multiple sclerosis. J Neurol. 2008;255 Suppl 6:2-6.

5. Hein T, Hopfenmüller W. [Projection of the number of multiple sclerosis patients in Germany]. Nervenarzt. 2000;71:288–94-–94.

6. Flachenecker P. Epidemiology of neuroimmunological diseases. J Neurol. 2006;253 Suppl 5:2–8-2–8.

7. Roth MP, Clayton J, Patois E, Alperovitch A. Gender distributions in parents and children concordant for multiple sclerosis. Neuroepidemiology. 1994;13(5):211-5.

8. Compston A, Coles A. Multiple sclerosis. Lancet. 2002;359:1221–31-–31.

9. Marrie R. Environmental risk factors in multiple sclerosis aetiology. Lancet Neurol. 2004;3(12):709-18.

10. Kurtzke J, Beebe G, Norman JJ. Epidemiology of multiple sclerosis in US veterans: III. Migration and the risk of MS. Neurology. 1985;35(5):672-8.

11. Willer C, Dyment D, Risch N, Sadovnick A, Ebers G. Twin concordance and sibling recurrence rates in multiple sclerosis. Proc Natl Acad Sci U S A. 2003;100(22):12877-82.

12. Villar LM, Masjuan J, Gonzalez-Porque P, Plaza J, Sadaba MC, Roldan E, et al. Intrathecal IgM synthesis in neurologic diseases: relationship with disability in MS. Neurology. 2002;58(5):824-6.

13. Barkhof F, Filippi M, Miller DH, Scheltens P, Campi A, Polman CH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. Brain. 1997;120 (Pt 11):2059-69.

14. Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol. 2005;58(6):840-6.

15. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol. 2001;50(1):121-7.

16. Filippini G, Munari L, Incorvaia B, Ebers G, Polman C, D'Amico R, et al. Interferons in relapsing remitting multiple sclerosis: a systematic review. Lancet. 2003;361(9357):545-52.

17. Arnon R, Aharoni R. Mechanism of action of glatiramer acetate in multiple sclerosis and its potential for the development of new applications. Proc Natl Acad Sci U S A. 2004;101 Suppl 2:14593-8.

18. Stüve O, Kieseier BC, Hemmer B, Hartung HP, Awad A, Frohman EM, et al. Translational research in neurology and neuroscience 2010: multiple sclerosis. Arch Neurol. 2010;67(11):1307-15.

19. Munari L, Lovati R, Boiko A. Therapy with glatiramer acetate for multiple sclerosis. Cochrane Database Syst Rev. 2004(1):CD004678.

20. La Mantia L, Munari LM, Lovati R. Glatiramer acetate for multiple sclerosis. Cochrane Database Syst Rev. 2010(5):CD004678.

21. Rommer PS, Stuve O, Goertsches R, Mix E, Zettl UK. Monoclonal antibodies in the therapy of multiple sclerosis: an overview. J Neurol. 2008;255 Suppl 6:28-35.

22. Coles AJ, Compston DA, Selmaj KW, Lake SL, Moran S, Margolin DH, et al. Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. N Engl J Med. 2008;359(17):1786-801.

23. Coles AJ, Wing M, Smith S, Coraddu F, Greer S, Taylor C, et al. Pulsed monoclonal antibody treatment and autoimmune thyroid disease in multiple sclerosis. Lancet. 1999;354(9191):1691-5.

24. Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med. 2010;362(5):402-15.

25. Rivers TM, Schwentker FF. Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys. J Exp Med. 1935;61(5):689-702.

26. Flugel A, Berkowicz T, Ritter T, Labeur M, Jenne DE, Li Z, et al. Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. Immunity. 2001;14(5):547-60.

27. Keith AB, Arnon R, Teitelbaum D, Caspary EA, Wisniewski HM. The effect of

Cop 1, a synthetic polypeptide, on chronic relapsing experimental allergic encephalomyelitis in guinea pigs. J Neurol Sci. 1979;42(2):267-74.

28. Webb C, Teitelbaum D, Abramsky O, Arnon R, Sela M. Proceedings: Suppression of experimental allergic encephalomyelitis in rhesus monkeys by a synthetic basic copolymer. Isr J Med Sci. 1975;11(12):1388.

29. Webb C, Teitelbaum D, Herz A, Arnon R, Sela M. Molecular requirements involved in suppression of EAE by synthetic basic copolymers of amino acids. Immunochemistry. 1976;13(4):333-7.

30. Ridge SC, Sloboda AE, McReynolds RA, Levine S, Oronsky AL, Kerwar SS. Suppression of experimental allergic encephalomyelitis by mitoxantrone. Clin Immunol Immunopathol. 1985;35(1):35-42.

31. Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature. 1992;356(6364):63-6.

32. Olsson T, Dahlman I, Wallstrom E, Weissert R, Piehl F. Genetics of rat neuroinflammation. J Neuroimmunol. 2000;107(2):191-200.

33. Manetti R, Parronchi P, Giudizi M, Piccinni M, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med. 1993;177(4):1199-204.

34. Magram J, Connaughton S, Warrier R, Carvajal D, Wu C, Ferrante J, et al. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity. 1996;4(5):471-81.

35. Murphy K, Reiner S. The lineage decisions of helper T cells. Nat Rev Immunol. 2002;2(12):933-44.

36. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133(5):775-87.

37. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology. 2003;4(4):330-6.

38. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. Journal of Immunology (Baltimore, Md: 1950). 1995;155(3):1151-64.

39. Wang HY, Wang R-F. Regulatory T cells and cancer. Current Opinion in Immunology. 2007;19(2):217-23.

40. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. Nature Immunology. 2005;6(4):353-60.

41. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nature Immunology. 2005;6(4):345-52.

42. Kanangat S, Blair P, Reddy R, Daheshia M, Godfrey V, Rouse B, et al. Disease in the scurfy (sf) mouse is associated with overexpression of cytokine genes. Eur J Immunol. 1996;26(1):161-5.

43. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet. 2001;27:68–73-68–73.

44. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest. 2000;106:75–81-75–81.

45. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001;27:20–1-–1.

46. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet. 2001;27:18－20-18－20.

47. Piccirillo CA, Shevach EM. Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. Semin Immunol. 2004;16(2):81-8.

48. Zhou G, Levitsky HI. Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance. J Immunol. 2007;178(4):2155-62.

49. Ii I, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126(6):1121-33.

50. Liang S, Tan X, Luxenberg D, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med. 2006;203(10):2271-9.

51. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem. 2007;282(13):9358-63.

52. Matsuzaki G, Umemura M. Interleukin-17 as an effector molecule of innate and acquired immunity against infections. Microbiol Immunol. 2007;51(12):1139-47.

53. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of Interleukin 17 Receptor Signaling for Lung CXC Chemokine and Granulocyte Colony-stimulating Factor Expression, Neutrophil Recruitment, and Host Defense. J Exp Med. 2001;194(4):519-28.

54. Pene J, Chevalier S, Preisser L, Venereau E, Guilleux MH, Ghannam S, et al. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. J Immunol. 2008;180(11):7423-30.

55. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. The Journal of experimental medicine. 2005;201(2):233-40.

56. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol. 2003;171(11):6173-7.

57. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol. 2006;177(1):566-73.

58. Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, et al. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nat Med. 2007;13(10):1173-5.

59. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, Stepankova R, et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. Immunity. 2006;25(2):309-18.

60. Schnyder-Candrian S, Togbe D, Couillin I, Mercier I, Brombacher F, Quesniaux V, et al. Interleukin-17 is a negative regulator of established allergic asthma. J Exp Med. 2006;203(12):2715-25.

61. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006;441(7090):231-4.

62. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8.

63. Ghilardi N, Ouyang W. Targeting the development and effector functions of TH17 cells. Seminars in Immunology. 2007;19(6):383-93.

64. Zamvil SS, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. Annu Rev Immunol. 1990;8:579－621-579－621.

65. Kobayashi M, Fitz L, Ryan M, Hewick R, Clark S, Chan S, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med. 1989;170(3):827-45.

66. Becher B, Durell BG, Noelle RJ. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. J Clin Invest. 2002;110:493－7-－7.

67. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nat Rev Immunol. 2005;5:521－31-－31.

68. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature. 2003;421:744－8-－8.

69. Massacesi L, Abbamondi AL, Giorgi C, Sarlo F, Lolli F, Amaducci L. Suppression of experimental allergic encephalomyelitis by retinoic acid. Journal of the neurological sciences. 1987;80(1):55-64.

70. Racke MK, Burnett D, Pak SH, Albert PS, Cannella B, Raine CS, et al. Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. Journal of immunology (Baltimore, Md: 1950). 1995;154(1):450-8.

71. Nagai H, Matsuura S, Bouda K, Takaoka Y, Wang T, Niwa S, et al. Effect of Am-80, a synthetic derivative of retinoid, on experimental arthritis in mice. Pharmacology. 1999;58(2):101-12.

72. Osanai M, Nishikiori N, Murata M, Chiba H, Kojima T, Sawada N. Cellular retinoic acid bioavailability determines epithelial integrity: Role of retinoic acid receptor alpha agonists in colitis. Molecular pharmacology. 2007;71(1):250-8.

73. Zunino SJ, Storms DH, Stephensen CB. Diets rich in polyphenols and vitamin A inhibit the development of type I autoimmune diabetes in nonobese diabetic mice. The Journal of nutrition. 2007;137(5):1216-21.

74. Reifen R, Nur T, Ghebermeskel K, Zaiger G, Urizky R, Pines M. Vitamin A deficiency exacerbates inflammation in a rat model of colitis through activation of nuclear factor-kappaB and collagen formation. The Journal of nutrition. 2002;132(9):2743-7.

75. Orfanos CE, Zouboulis CC, Almond-Roesler B, Geilen CC. Current use and future potential role of retinoids in dermatology. Drugs. 1997;53(3):358-88.

76. Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Effects of a Combination of Beta Carotene and Vitamin A on Lung Cancer and Cardiovascular Disease. N Engl J Med. 1996;334(18):1150-5.

77. Group TAS. Incidence of Cancer and Mortality Following alpha-Tocopherol and beta-Carotene Supplementation: A Postintervention Follow-up. JAMA. 2003;290(4):476-85.

78. Iwata M, Eshima Y, Kagechika H. Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors. Int Immunol. 2003;15(8):1017-25.

79. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004;21(4):527-38.

80. Ziouzenkova O, Plutzky J. Retinoid metabolism and nuclear receptor responses: New insights into coordinated regulation of the PPAR-RXR complex. FEBS Lett. 2008;582(1):32-8.

81. Desvergne B. RXR: from partnership to leadership in metabolic regulations. Vitam Horm. 2007;75:1-32.

82. Haussler M, Haussler C, Jurutka P, Thompson P, Hsieh J, Remus L, et al. The vitamin D hormone and its nuclear receptor: molecular actions and disease states. J Endocrinol. 1997;154 Suppl:S57-73.

83. Delescluse C, Cavey M, Martin B, Bernard B, Reichert U, Maignan J, et al. Selective high affinity retinoic acid receptor alpha or beta-gamma ligands. Mol Pharmacol. 1991;40(4):556-62.

84. Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science (New York, NY). 2001;292(5523):1907-10.

85. Pear W, Miller J, Xu L, Pui J, Soffer B, Quackenbush R, et al. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood. 1998;92(10):3780-92.

86. Racke MK. Experimental autoimmune encephalomyelitis (EAE). Curr Protoc Neurosci. 2001;Chapter 9:Unit9.7-Unit9.7.

87. Fleming K, Bovaird J, Mosier M, Emerson M, LeVine S, Marquis J. Statistical analysis of data from studies on experimental autoimmune encephalomyelitis. J

Neuroimmunol. 2005;170(1-2):71-84.

88. Burt R, Padilla J, Begolka W, Canto M, Miller S. Effect of disease stage on clinical outcome after syngeneic bone marrow transplantation for relapsing experimental autoimmune encephalomyelitis. Blood. 1998;91(7):2609-16.

89. Schambach F, Schupp M, Lazar MA, Reiner SL. Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation. Eur J Immunol. 2007;37(9):2396－9-－9.

90. Spanjaard RA, Ikeda M, Lee PJ, Charpentier B, Chin WW, Eberlein TJ. Specific activation of retinoic acid receptors (RARs) and retinoid X receptors reveals a unique role for RARgamma in induction of differentiation and apoptosis of S91 melanoma cells. The Journal of biological chemistry. 1997;272(30):18990-9.

91. Takahashi B, Ohta K, Kawachi E, Fukasawa H, Hashimoto Y, Kagechika H. Novel retinoid X receptor antagonists: specific inhibition of retinoid synergism in RXR-RAR heterodimer actions. Journal of medicinal chemistry. 2002;45(16):3327-30.

92. Apfel C, Bauer F, Crettaz M, Forni L, Kamber M, Kaufmann F, et al. A retinoic acid receptor alpha antagonist selectively counteracts retinoic acid effects. Proc Natl Acad Sci U S A. 1992;89(15):7129-33.

93. Schupp M, Curtin JC, Kim RJ, Billin AN, Lazar MA. A widely used retinoic acid receptor antagonist induces peroxisome proliferator-activated receptor-gamma activity. Mol Pharmacol. 2007;71(5):1251-7.

94. Klotz L, Burgdorf S, Dani I, Saijo K, Flossdorf J, Hucke S, et al. The nuclear receptor PPAR gamma selectively inhibits Th17 differentiation in a T cell-intrinsic fashion and suppresses CNS autoimmunity. J Exp Med. 2009;206(10):2079-89.

95. Teng M, Duong TT, Johnson AT, Klein ES, Wang L, Khalifa B, et al. Identification of highly potent retinoic acid receptor alpha-selective antagonists. Journal of Medicinal Chemistry. 1997;40(16):2445–51-–51.

96. Miyagawa N, Homma T, Kagechika H, Shudo K, Nagai H. Effect of synthetic retinoid, TAC-101, on experimental autoimmune disease. Pharmacology. 2003;67(1):21-31.

97. Lee IH, Lee JH, Lee MJ, Lee SY, Kim IS. Involvement of CCAAT/enhancer-binding protein alpha in haptoglobin gene expression by all-trans-retinoic acid. Biochemical and biophysical research communications. 2002;294(5):956-61.

98. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, et al. Molecular mechanisms underlying FOXP3 induction in human T cells. Journal of

immunology (Baltimore, Md: 1950). 2006;176(6):3593-602.

99. Ohno R, Asou N, Ohnishi K. Treatment of acute promyelocytic leukemia: strategy toward further increase of cure rate. Leukemia. 2003;17(8):1454-63.

100. Tobita T, Takeshita A, Kitamura K, Ohnishi K, Yanagi M, Hiraoka A, et al. Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid. Blood. 1997;90(3):967-73.

101. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal Th-17 and Regulatory T Cell Differentiation Mediated by Retinoic Acid. Science. 2007.

102. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, et al. Retinoic Acid Can Directly Promote TGF-beta-Mediated Foxp3(+) Treg Cell Conversion of Naive T Cells. Immunity. 2009;30(4):471-2.

103. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, et al. Retinoic Acid Enhances Foxp3 Induction Indirectly by Relieving Inhibition from CD4(+)CD44(hi) Cells. Immunity. 2008;29(5):758-70.

104. Lemire J, Archer D. 1,25-dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. J Clin Invest. 1991;87(3):1103-7.

105. Lovett-Racke AE, Racke MK. Retinoic acid promotes the development of Th2-like human myelin basic protein-reactive T cells. Cellular Immunology. 2002;215(1):54-60.

106. Trembleau S, Germann T, Gately MK, Adorini L. THE ROLE OF IL-12 IN THE INDUCTION OF ORGAN-SPECIFIC AUTOIMMUNE-DISEASES. Immunol Today. 1995;16(8):383-6.

107. Segal BA. Experimental autoimmune encephalo myelitis: Cytokines, effector T cells, and antigen-presenting cells in a prototypical Th1-mediated autoimmune disease. Curr Allergy Asthma Rep. 2003;3(1):86-93.

108. Aranami T, Yamamura T. Th17 Cells and autoimmune encephalomyelitis (EAE/MS). Allergol Int. 2008;57(2):115-20.

109. Klemann C, Raveney B, Klemann A, Ozawa T, von Hörsten S, Shudo K, et al. Synthetic retinoid AM80 inhibits Th17 cells and ameliorates experimental autoimmune encephalomyelitis. Am J Pathol. 2009;174(6):2234-45.

110. Xiao S, Jin H, Korn T, Liu S, Oukka M, Lim B, et al. Retinoic acid increases

Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. J Immunol. 2008;181(4):2277-84.

111. Kang SG, Wang CW, Matsumoto S, Kim CH. High and Low Vitamin A Therapies Induce Distinct FoxP3(+) T-Cell Subsets and Effectively Control Intestinal Inflammation. Gastroenterology. 2009;137(4):1391-402.

112. Housley WJ, O'Conor CA, Nichols F, Puddington L, Lingenheld EG, Zhu L, et al. PPAR{gamma} regulates retinoic acid-mediated DC induction of Tregs. J Leukoc Biol. 2009.

113. Bai AP, Lu NH, Guo Y, Liu ZJ, Chen J, Peng ZK. All-trans retinoic acid down-regulates inflammatory responses by shifting the Treg/Th17 profile in human ulcerative and murine colitis. Journal of Leukocyte Biology. 2009;86(4):959-69.

114. Goodin D, Frohman E, Hurwitz B, O'Connor P, Oger J, Reder A, et al. Neutralizing antibodies to interferon beta: assessment of their clinical and radiographic impact: an evidence report: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology. 2007;68(13):977-84.

115. Rudick R, Cutter G. Interferon-beta for multiple sclerosis: Long-term benefits? Ann Neurol. 2007;61(4):283-5.

116. Yousry T, Major E, Ryschkewitsch C, Fahle G, Fischer S, Hou J, et al. Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. N Engl J Med. 2006;354(9):924-33. PMCID: PMC1934511.

117. Goodin D, Cohen B, O'Connor P, Kappos L, Stevens J. Assessment: the use of natalizumab (Tysabri) for the treatment of multiple sclerosis (an evidence-based review): report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology. 2008;71(10):766-73.

118. Lelu K, Delpy L, Robert V, Foulon E, Laffont S, Pelletier L, et al. Endogenous estrogens, through estrogen receptor alpha, constrain autoimmune inflammation in female mice by limiting CD4(+) T-cell homing into the CNS. Eur J Immunol. 2010.

119. Williams L, Rudensky A. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol. 2007;8(3):277-84.

120. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3(+) Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood. 2007;110(4):1225-32.

121. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D. Expression of GARP selectively identifies activated human FOXP3+regulatory T cells. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(32):13439-44.

122. Collison L, Workman C, Kuo T, Boyd K, Wang Y, Vignali K, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature. 2007;450(7169):566-9.

123. Burgler S, Mantel PY, Bassin C, Ouaked N, Akdis CA, Schmidt-Weber CB. RORC2 is involved in T cell polarization through interaction with the FOXP3 promoter. J Immunol. 2010;184(11):6161-9.

124. Pierrot-Deseilligny C. Clinical implications of a possible role of vitamin D in multiple sclerosis. J Neurol. 2009.

125. Ghoreishi M, Bach P, Obst J, Komba M, Fleet J, Dutz J. Expansion of antigen-specific regulatory T cells with the topical vitamin d analog calcipotriol. J Immunol. 2009;182(10):6071-8.

126. Wang J, Huizinga TWJ, Toes REM. De Novo Generation and Enhanced Suppression of Human CD4(+)CD25(+) Regulatory T Cells by Retinoic Acid. J Immunol. 2009;183(6):4119-26.

127. Miwako I, Kagechika H. Tamibarotene. Drugs of Today (Barcelona, Spain: 1998). 2007;43(8):563-8.

7 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

8 Acknowledgements

I would like to extend my gratitude to Steve Reiner for giving me the freedom to follow my interests freely and to carry out this research in his lab. I also wish to thank the members of the Reiner lab for their intellectual and practical support. In particular, Chris Gasink's advice about what not to do has been invaluable. I also benefitted greatly from being around the perpetually optimistic John Tillet Northrup.

Other people who have helped me along the way are Greg Wu, whom I would like to thank for sharing his knowledge of EAE and Michael Schupp for ideas on nuclear receptors.

Most of all, I would like to thank my family, especially my parents, without their longstanding support I would not be where I am today. Last, but not least my I wish to thank my wife for her love and constant encouragement.

9 Erklärung

Ich, Felix Schambach, erkläre, daß ich die vorgelegte Dissertation mit dem Thema:

“Activation of RARα Causes Amelioration of EAE by Induction of FoxP3”

selbst verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfaßt und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

München, den 4.5.2011