Immunoregulation of monophasic and relapsing experimental autoimmune uveitis in the rat model

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften

an der medizinischen Fakultät

der Ludwig-Maximilians-Universität München

vorgelegt von

Ulrike Kaufmann

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Aus der Augenklinik und Poliklinik der Ludwig-Maximilians-Universität München

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List of abbreviations

%	percent
aa	amino acid
ACAID	anterior chamber-associated immune deviation
Ag	antigen
AIRE	autoimmune regulator
APC	antigen presenting cell
Casp	caspase
CD	cluster of differentiation
CFA	complete Freund’s adjuvant
Cnkrs3/Magi	connector enhancer of kinase suppressor of ras 3 / membrane-associated guanylate kinase-interacting protein-like 1
cTEC	cortical thymic epithelial cell
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
Ctsh	cathepsin H
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveitis
Ecad	E-cadherin
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERU	equine recurrent uveitis
Foxp3	forkhead box protein 3
GATA3	GATA binding protein 3

GFP	green fluorescent protein
Gli1	glioma-associated oncogene homolog 1
HH	Hedgehog
HLA	human leukocyte antigen
Hla-dmb	MHC class II DM beta
IFN	interferon
IL	interleukin
IP-10/CXCL10	interferon gamma-induced protein 10 / interferon-inducible cytokine 10
IRBP	interphotoreceptor retinoid-binding protein
iT_{reg}	induced regulatory T cell
JAK	Janus kinase
MBP	myelin basic protein
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
mRNA	messenger ribonucleic acid
mTEC	medullary thymic epithelial cell
MΦ	macrophage
NK cell	natural killer cell
nT_{reg}	natural regulatory T cell
qPCR	quantitative real time polymerase chain reaction
RANTES	regulated and normal T cell expressed and secreted
RNA	ribonucleic acid
RORγt	retinoid-related orphan receptor gamma t
Rt1.Da	RT1 class II locus DA
S-Ag	retinal soluble antigen

STAT	signal transducer and activator of transcription
T-bet	T-box transcription factor
TCR	T cell receptor
T_{FH}	T follicular helper cell
TGF	transforming growth factor
T_H	T helper cell
TNF	tumor necrosis factor
T_R1	T regulatory 1 cell
T_{reg}	T regulatory cell
µg	microgram

List of publications

This work contains results presented in the following publications:

von Toerne C, Sieg C, Kaufmann U, Diedrichs-Möhring M, Nelson PJ, Wildner G (2010). Effector T cells driving monophasic vs. relapsing/remitting experimental autoimmune uveitis show unique pathway signatures. Molecular Immunology 48(1-3):272-280

Kaufmann U, Diedrichs-Möhring M, Wildner G (2012). Dynamics of Intraocular IFN-γ, IL-17 and IL-10-Producing Cell Populations during Relapsing and Monophasic Rat Experimental Autoimmune Uveitis. PLoS ONE 7(11): e49008. doi:10.1371/journal.pone.0049008

1 Summary

Many autoimmune diseases exhibit a relapsing disease course, whereby acute inflammatory flares alternate with periods without inflammation. These include autoimmune uveitis, which mostly shows a relapsing disease course in humans. Autoimmune uveitis is an intraocular inflammation. Due to tissue damage in the retina induced by inflammation, visual impairment and blindness can result. To investigate autoimmune uveitis animal models of experimental autoimmune uveitis (EAU) are used. However, most of these models do not imitate the relapsing disease course. Lewis rats develop an acute monophasic or relapsing uveitis depending on the antigen peptide used for induction of the disease. While peptide PDSAg from the retinal S-Antigen induces monophasic uveitis, peptide R14 from the interphotoreceptor retinoid-binding protein can induce relapsing disease. Both types of EAU occur after active immunization with autoantigen peptide in complete Freund's adjuvant as well as after adoptive transfer of activated, autoantigen-specific T cells.

In this thesis differences between monophasic and relapsing uveitis were investigated. T cell lines specific for both autoantigens were analyzed regarding their transcriptomes. Gene array analysis and subsequent qPCR revealed enhanced expression of 26 genes in the relapse-inducing R14-specific T cell lines. By contrast, no genes were regulated in PDSAg-specific T cells. The upregulated genes were associated with certain signal transduction pathways, which result in activation of lymphocytes and inhibition of apoptosis. In particular, a central role of IFN-γ could be demonstrated. Despite similar mRNA levels for IL-17 there was an increase of IL-17 protein expression detected in the PDSAg-specific T cells, which induce monophasic uveitis.

Consequently, T cells from monophasic or relapsing uveitis were isolated from rat eyes and lymph nodes during different stages of the disease and were analyzed for inflammatory cytokines such as IFN-γ and IL-17 as well as for marker proteins of regulatory cells (IL-10 and Foxp3). Cell populations simultaneously expressing several cytokines were only detected in the eyes and not in the lymph nodes. Moreover, combinations of the pro-inflammatory cytokines IFN-γ and IL-17 even

coexpressed with the suppressive cytokine IL-10 were discovered. These cell populations remained stable or even decreased during the course of relapsing uveitis, while they increased during the monophasic disease, implicating a regulatory function. The number of cells expressing IFN-γ but none of the other tested cytokines increased during relapsing EAU and reached a maximum during the relapses. Intraocular injection of recombinant IFN-γ after the resolution of the first attack of intraocular inflammation in R14 immunized animals resulted in synchronized relapses, which normally occur spontaneously and unpredictably. Thus, IFN-γ appears to be the crucial cytokine in relapsing uveitis. In PDSAg-induced, monophasic disease the number of IL-10$^+$ cells increased compared to relapsing EAU. This suggests an efficient regulatory function of these cells. On the contrary, the numbers of Foxp3$^+$ cells were similar during the primary disease course in eyes of monophasic and relapsing animals. Only during the late remission without clinical signs of inflammation were Foxp3$^+$ cells detectable in higher numbers in the eyes from monophasic disease.

Taken together, T cells from relapsing uveitis express more pro-inflammatory cytokines with IFN-γ playing a pivotal role. By contrast, T cells from eyes with monophasic disease exhibit a rather anti-inflammatory phenotype with increased numbers of IL-10$^+$ cells during resolution and increased numbers of IL-10 as well as Foxp3 expressing T cells during late remission. These data provide important information on immune mechanisms in relapsing autoimmune diseases like uveitis.

2 Zusammenfassung

Viele Autoimmunkrankheiten weisen einen rezidivierenden Krankheitsverlauf auf, wobei sich Entzündungsschübe mit entzündungsfreien Phasen abwechseln. Ein Beispiel hierfür ist die autoimmune Uveitis, welche beim Menschen meist rezidivierend verläuft. Die autoimmune Uveitis ist eine intraokulare Entzündung, die aufgrund von Gewebszerstörungen in der Retina zu Sehbehinderungen bis hin zur Erblindung führen kann. Zur Untersuchung der autoimmunen Uveitis werden Tiermodelle der experimentellen autoimmunen Uveitis (EAU) verwendet. Die meisten dieser Tiermodelle imitieren allerdings nicht den rezidivierenden Krankheitsverlauf. Nur in Lewis Ratten kann, abhängig vom induzierenden Antigenpeptid eine akute monophasische oder eine rezidivierende Uveitis ausgelöst werden. Während das Peptid PDSAg aus dem retinalen S-Antigen eine monophasische Erkrankung induziert, kann nach Immunisierung mit dem Peptid R14 vom Interphotorezeptor retinolbindenden Protein eine rezidivierende Uveitis beobachtet werden. Die beiden EAU-Typen treten sowohl nach aktiver Immunisierung mit dem autoantigenen Peptid in komplettem Freund-Adjuvans als auch nach adoptivem Transfer aktivierter, autoantigenspezifischer T-Zellen auf.

Im Rahmen dieser Dissertation wurden Unterschiede zwischen monophasischer und rezidivierender Uveitis untersucht. Zunächst wurden T-Zelllinien mit Spezifität für die beiden Autoantigene, auf Unterschiede bezüglich ihrer Transkriptome überprüft. Gen-Array-Analysen und anschließende qPCR zeigten eine verstärkte Expression von 26 Genen in den R14-spezifischen T-Zelllinien, welche Rezidive auslösen können. Im Vergleich dazu waren keine Gene in PDSAg-spezifischen T-Zelllinien reguliert. Die verstärkt exprimierten Gene konnten bestimmten Signaltransduktions-wegen, die beispielsweise an der Aktivierung von Lymphozyten oder der Inhibition von Apoptose beteiligt waren, zugeordnet werden. Fast alle Signaltransduktionswege der regulierten Gene zeigten einen Zusammenhang mit der Expression von IFN-γ. Trotz gleicher mRNA-Expression von IL-17 zeigte sich auf Proteinebene eine verstärkte IL-17-Produktion in PDSAg-spezifischen T-Zellen der monophasischen Uveitis.

Daraufhin wurden T-Zellen während verschiedener Stadien der monophasischen beziehungsweise der rezidivierenden Uveitis aus Rattenaugen und Lymphknoten isoliert und auf die Expression entzündlicher Zytokine wie IFN-γ und IL-17 sowie auf Markerproteine regulatorischer Zellen (IL-10 und Foxp3) untersucht. Zellpopulationen, die mehrere Zytokine gleichzeitig exprimierten, konnten nur in den Augen, nicht aber in den Lymphknoten nachgewiesen werden. Dabei wurden sowohl Kombinationen von inflammatorischen Zytokinen wie IFN-γ und IL-17 als auch Kombinationen mit dem suppressiven Zytokin IL-10 detektiert. Diese Populationen blieben während des Verlaufs der rezidivierenden Uveitis stabil oder nahmen sogar ab, während bei der monophasischen Uveitis ein Anstieg beobachtet wurde. Dies lässt auf eine mögliche regulatorische Funktion dieser Zellen schließen. Die Zahl der IFN-γ^+ Zellen stieg bei der rezidivierenden Uveitis während des Krankheitsverlaufs an und erreichte ihr Maximum während der Rezidive. Intraokulare Injektion von rekombinantem IFN-γ nach Abklingen der ersten Entzündungsphase bei R14-induzierter Uveitis führte zu synchronisierten Entzündungsschüben der ansonsten spontan und unvorhersehbar rezidivierenden EAU. IFN-γ scheint folglich das entscheidende Zytokin bei der rezidivierenden Uveitis zu sein. Bei PDSAg-induzierter, monophasischer Uveitis stieg im Vergleich zur rezidivierenden EAU die Zahl der IL-10^+ Zellen stark an. Dies deutet auf eine effiziente regulatorische Funktion dieser Zellen hin. Foxp3^+ Zellen hingegen wurden während der akuten Entzündung im Auge in gleichen Mengen bei beiden Uveitistypen detektiert. Erst während der späten Remission ohne klinische Entzündungszeichen fanden sich bei der monophasischen EAU Foxp3^+ T-Zellen in erhöhter Zahl.

Zusammenfassend zeigen die T-Zellen der rezidivierenden Uveitis eine stärkere Ausprägung der pro-inflammatorischen Zytokine, wobei IFN-γ eine zentrale Rolle spielt. Im Gegensatz dazu weisen die T-Zellen der monophasischen EAU einen eher anti-inflammatorischen Phänotyp auf, mit erhöhter Anzahl an IL-10^+ Zellen im abklingenden ersten Schub und erhöhten Zahlen an IL-10^+ sowie Foxp3^+ T-Zellen während der späten Remission. Diese Daten tragen wichtige Informationen zu den Immunmechanismen bei rezidivierenden Autoimmunerkrankungen wie der Uveitis bei.

3 Aims of the thesis

The primary aim was to investigate the immune mechanisms of monophasic and relapsing experimental autoimmune uveitis in Lewis rats. From a vast area of research, only few animal models, including those for experimental autoimmune uveitis, comprise monophasic as well as relapsing disease courses. Consequently, little is known about the cells and factors contributing to prevention or emergence of relapses.

Since uveitis is a T helper cell mediated disease and several T helper cell subsets are known to be involved in the disease, either as effector or regulatory T cells, there should be differences in the T cell subsets participating in monophasic and relapsing uveitis, respectively. These differences can be detected at the level of gene and/or protein expression. In the Lewis rat model the decision of monophasic or relapsing disease course is dependent on the antigen peptide used for the induction of experimental autoimmune uveitis (EAU). Uveitogenic T cell lines specific for both antigens were thus used for molecular studies to analyze the gene expression profile of these T cells. Data gained from these analyses were expected to offer valuable clues as to the genes involved in the determination of the disease course. Furthermore, we compared T cells from inflamed eyes and peripheral lymph nodes during different time points of the disease course with respect to their cytokine profiles. Results were compared between monophasic and relapsing disease revealing T cells and cytokines, which were related to the induction as well as the resolution of the disease. In addition, the impact of cytokine administration on the disease course was investigated.

Understanding the exact immune mechanisms behind relapses could help in the development of new or better concepts for the treatment of uveitis patients.

4 Introduction

4.1 Uveitis

Uveitis is an intraocular inflammatory disease, which affects the retina and/or uvea (iris, ciliary body and choroid; see Figure 1). Based on the site of inflammation uveitis is classified as anterior, intermediate, posterior or pan uveitis, when all parts of the eye are involved (Jabs et al., 2005). Inflammation in the eye is sight threatening and can lead to vision impairment and even blindness. Uveitis comprises multiple disease entities, which are subdivided into two main groups: uveitis caused directly by infectious agents and noninfectious uveitis associated with autoimmunity.

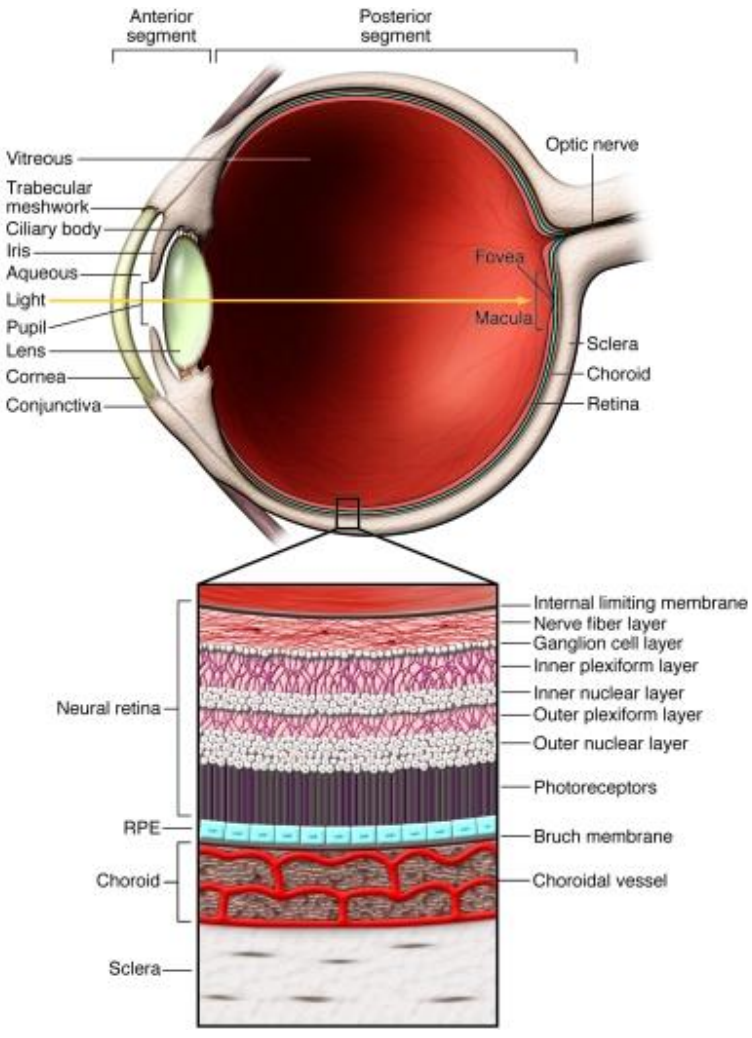

Figure 1 Scheme of an eye and an enlarged section of the retina (Caspi, 2010).

In the United States of America noninfectious uveitis, referred to as autoimmune uveitis or immune-mediated uveitis, has an incidence of 52.4 per 100,000 and a prevalence of 114.3 per 100,000 (Gritz and Wong, 2004). Several types of uveitis are associated with systemic immune-mediated diseases. Examples of uveitic entities, where uveitis is part of a systemic syndrome and thus the eye is only one of several affected organs, are HLA-B27 associated uveitis, which is described in combination with ankylosing spondylitis, reactive arthritis, psoriatic arthritis and inflammatory bowel disease (Suhler et al., 2003), Behçet's disease, where skin mucosa and other tissues are involved (Pineton de Chambrun et al., 2012), Vogt-Koyanagi-Harada disease, where the central nervous system and melanocytes of the skin are affected (Damico et al., 2009) and sarcoidosis, where all organs may be involved (Papadia et al., 2010). In some uveitis patients the eyes are the only affected organs, for instance in birdshot retinochoroidopathy (Levinson and Gonzales, 2002) and sympathetic ophthalmia (Chang and Young, 2011).

4.2 Autoimmune uveitis

Autoimmunity was first described by Paul Ehrlich as "Horror Autotoxicus" in the early twentieth century. He postulated that the immune system can attack self tissues, which results in autoimmune diseases. Autoimmune diseases affect approximately 5% of the population with a female predominance (Jacobson et al., 1997) and are characterized by abnormal activation of immune cells, often caused by genetic predispositions in combination with environmental factors. In either case central or peripheral tolerance mechanisms (section 4.3) are impaired. Autoreactive T or B cells provoke inflammation that results in damage or even complete destruction of the affected tissues.

Autoimmune diseases can be classified as systemic or organ-specific autoimmune diseases. However, there are also some intermediate types of autoimmunity. For example, autoimmune uveitis is an organ-specific autoimmune disease, which can be associated with other (systemic) autoimmune diseases, such as rheumatoid arthritis (Tugal-Tutkun et al., 1996).

The exact mechanisms of autoimmune diseases remain obscure. Genetic predispositions, especially variants of major histocompatibility complex (MHC) molecules, are involved. For instance, human anterior autoimmune uveitis can be associated with HLA-B27 (Brewerton et al., 1973). Furthermore, it is assumed that the initiation of autoimmune disease is triggered by a pathogen. In experimental autoimmune encephalomyelitis (EAE), a model for human multiple sclerosis, it was shown that T cell receptor (TCR) transgenic mice reactive to myelin basic protein (MBP) spontaneously developed EAE under conventional rodent housing conditions. However, under specific pathogen-free conditions fewer animals developed EAE spontaneously, suggesting a pathogen trigger (Goverman, 1999; Goverman et al., 1993).

By means of transgenic mouse models it was demonstrated that low-avidity autoreactive T cells regularly escape central and peripheral tolerance mechanisms (section 4.3) and home to secondary lymphoid organs. These low-avidity T cells have the ability to respond to their specific antigen, when it is presented in a high density on discrete dendritic cells (DCs) or when it is presented by numerous DCs. As a result autoimmune diseases can be induced (Henrickson et al., 2008; Zehn and Bevan, 2006). In the peripheral blood of healthy humans, T cells are detectable, which have the ability to proliferate in response to self-antigens. This was illustrated by the example of MBP (Burns et al., 1983) and retinal soluble antigen (S-Ag) (de Smet et al., 1998).

After activating antigen stimulation T cells change their homing receptors, enter the circulation and gain the ability to infiltrate all tissues of the body, preferentially sites of inflammation (Masopust et al., 2001; Masopust et al., 2004). This is due to upregulation of P- and E-selectin ligands (Austrup et al., 1997). The autoreactive effector or memory T cells therefore have an increased risk to access tissues where their specific autoantigen is expressed. Even immune privileged organs such as the eyes (section 4.3), which express lower amounts of P- and E-selectin, are not resistant to infiltration of activated T cells, especially when inflammation of the organ has already been initiated (Mrass and Weninger, 2006).

In patients autoimmune uveitis is a group of diseases characterized by inflammation of the eye without any known trigger factor for disease induction. Due to the

inflammation tissue damage occurs, which can potentially result in blindness. Posterior uveitis often has a more severe course, because the retina is affected and is unable to regenerate.

A definitive case of autoimmune uveitis is sympathetic ophthalmia, which is induced by a trauma of one eye and followed by an autoimmune inflammation of the other ("sympathetic") non-traumatized eye (Chang and Young, 2011). If autoimmune uveitis is not traceable to a trauma from an accident or surgery, it is assumed that an infection with a pathogen providing antigens crossreactive with retinal proteins leads to activation of lymphocytes. Once activated in the periphery, these cells have the ability to induce an autoimmune response in the eye. Microbial antigens can exhibit major similarities with autoantigens such as S-Ag and thus induce uveitis in animal models (Shinohara et al., 1990; Singh et al., 1992; Wildner and Diedrichs-Moehring, 2005). It is assumed that T cells play a pivotal role in the pathogenesis of human uveitis. This is strongly supported by induction of uveitis after adoptive transfer of T cells in animal models (Mochizuki et al., 1985) and by administration of agents that directly target T cells, such as Cyclosporin A (Nussenblatt et al., 1983). Furthermore, Interleukin 17 (IL-17) as well as T helper 17 (T_H17) cells, which have been ascribed an important role in autoimmune diseases, were detected in human peripheral blood from autoimmune uveitis patients (Amadi-Obi et al., 2007). The same was true for systemic autoimmune diseases associated with uveitis, like Vogt-Koyanagi-Harada (Chi et al., 2007) and Behçet's disease (Chi et al., 2008). In patients with autoimmune uveitis autoreactive T cells specific for S-Ag, interphotoreceptor retinoid-binding protein (IRBP) and other intraocular proteins were detected (Adamus and Chan, 2002; de Smet et al., 1990; Nussenblatt et al., 1980a). Moreover, experimental animal models that can be induced by different retinal autoantigens (Agarwal et al., 2012) support the participation of retinal autoantigen-specific lymphocytes in human uveitis. A further indication of an autoimmune mechanism is the strong association of autoimmune uveitis with human leukocyte antigen (HLA) (Pennesi and Caspi, 2002). MHC molecules present antigen peptides to T cells and therefore have an important role in regulating the immune response. Association of T helper cell mediated autoimmune diseases with MHC class I molecules can be explained by presentation of peptides from MHC class I on MHC class II molecules. Furthermore, peptides from HLA-B showed sequence homology with an uveitogenic peptide from S-Ag (Wildner

and Thurau, 1994). Examples for uveitis associated with HLA molecules are HLA-B27 associated uveitis (Suhler et al., 2003), ocular Behçet's disease (Piga and Mathieu, 2011) and Birdshot chorioretinopathy, which is associated with HLA-A29.2 although the exact mechanism still remains unknown (Brezin et al., 2011; Tabary et al., 1990). Evidence for immune activation in patients with uveitis are increased levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and tumor necrosis factor (TNF) (de Boer et al., 1992; el-Shabrawi et al., 1998; Wakefield and Lloyd, 1992).

In patients the examination of disease mechanisms and therapeutic options represent an almost insurmountable difficulty, hence the use of experimental animal models of human autoimmune uveitis is indispensable (section 4.4).

4.3 Tolerance mechanisms and the immune privilege of the eye

Inflammation in the eye is not only prevented by central and peripheral tolerance mechanisms but also by the immune privilege, which maintains an inhibitory milieu inside the eye.

4.3.1 Tolerance mechanisms

Self-tolerance is achieved by a combination of central and peripheral tolerance mechanisms and prevents immune reactions to self-antigens.

Central Tolerance

T cells mature and undergo selection in the thymus. First there is positive selection that enriches T cells with TCRs that are able to recognize self-peptide-MHC complexes. MHC class I and MHC class II molecules are both expressed by cortical thymic epithelial cells (cTECs) (Anderson et al., 1994). During this step autoreactive T cells are also enriched. That is why negative selection follows to eliminate these autoreactive T cells. The so-called clonal deletion removes T cells with high affinities for self-antigens by apoptosis (Palmer, 2003). Therefore, the thymus exhibits a

special anatomy as well as special cell types that allow the selection processes. Following positive selection in the thymic cortex T cells migrate to the thymic medulla, where medullary thymic epithelial cells (mTECs) and DCs, which mediate negative selection, are found. Antigens, even tissue-specific antigens, are expressed ectopically by mTECs and contribute to the shaping of T cell repertoire (Derbinski et al., 2001). The presence of retinal antigens was demonstrated in the thymus of mice and rats, and in addition it was shown that their expression correlates with resistance to autoimmune diseases (Avichezer et al., 2003; Egwuagu et al., 1997). For example, in mice S-Ag is presented on mTECs as well as on cTECs (Derbinski et al., 2001), while IRBP is only expressed on mTECs (Kyewski et al., 2002). This could explain why mice are susceptible for uveitis induction with IRBP but not with S-Ag. Most, but not all antigens in the thymus are expressed with the help of the transcription factor AIRE (autoimmune regulator), which is present in mTECs (Anderson et al., 2002). DCs of the thymic medulla, which cannot express tissue-specific antigens by themselves, can crosspresent antigens synthesized by mTECs and thus also play a role in central tolerance (Gallegos and Bevan, 2004).

In the thymus there is not only negative selection, but also generation of regulatory T cells that prevent tissue-specific autoimmunity (Sakaguchi and Sakaguchi, 2005). These regulatory T cells are referred to as natural regulatory T cells (nT_{reg}) and are characterized by cluster of differentiation (CD) 4, CD25 and forkhead box protein 3 (Foxp3) expression. The importance of these cells was shown by their depletion with anti-CD25 antibodies, which resulted in autoimmune diseases (Sakaguchi et al., 1995).

Negative selection does not eliminate all self-reactive T cells, some autoreactive T cells escape negative selection and reach the periphery (Gallegos and Bevan, 2006). This may be caused by a number of possibilities. Negative selection is dependent on the amount of antigen which is presented by thymic cells, and also on the affinity of the T cell receptor for the presented antigen. Thus low-avidity autoreactive T cells as well as autoreactive T cells having high avidity for an antigen which is not expressed in sufficient amounts in the thymus, escape negative selection. In humans differences in the amount of retinal antigens expressed in thymus were discovered and these amounts correlated with susceptibility for autoimmune reactions (Takase et al., 2005).

Peripheral Tolerance

Autoreactive lymphocytes, which have evaded negative selection in the primary lymphoid organs (T cells in the thymus and B cells in the bone marrow), can be distracted from autoimmune reactions by peripheral tolerance mechanisms.

One of these mechanisms is the physical sequestration of autoreactive T cells and their specific antigens expressed in different tissues. Naïve T cells circulate from the blood to secondary lymphoid organs and back to the blood. Thus, they never infiltrate other non-lymphoid tissues and therefore cannot recognize their specific antigens (Lammermann and Sixt, 2008).

Another mechanism of peripheral tolerance is the expression of AIRE in certain stromal cells of lymph nodes. Consequently, these cells can present tissue restricted antigens in a non-immunogenic manner and induce tolerance (Lee et al., 2007).

Tolerogenic antigen presenting cells (APCs) can also induce peripheral tolerance. These incompletely matured DCs do neither upregulate MHC molecules nor costimulatory molecules (Hawiger et al., 2001). In the absence of inflammation dead or apoptotic cells (not necrotic cells) induce this type of DCs. T cells that recognize their specific antigen presented by these DCs do not obtain costimulatory signals and thus neither proliferate nor produce cytokines; rather they become anergic, which means functionally unresponsive (Liu et al., 2002).

T cells that chronically recognize self peptide-MHC-complexes in the periphery die by apoptosis. The death pathway is triggered by Fas receptor, which transmits apoptosis-inducing signals (Marrack and Kappler, 2004; Watanabe-Fukunaga et al., 1992).

Regulatory T cells, especially nT_{reg} cells, also contribute to peripheral tolerance. They actively inhibit autoreactive T cells. nT_{reg} cells can either suppress T cells and APCs directly by cell contact-dependent mechanisms or indirectly by secretion of inhibitory cytokines such as IL-10 and transforming growth factor beta (TGF-β) (Shevach, 2009). Most likely, several inhibitory mechanisms work in a complementary or synergistically fashion. One of the cell contact-dependent mechanisms is constitutive cytotoxic T lymphocyte antigen 4 (CTLA-4) expression by nT_{reg} cells (Takahashi et al., 2000), which seems to be their key regulatory mechanism. Mice deficient for CTLA-4 in nT_{reg} cells develop a variety of autoimmune diseases (Wing et al., 2008),

because they lack the ability to suppress APCs (Onishi et al., 2008). Once nT_{reg} cells are activated via their TCR, they can suppress other cells in an antigen-non-specific manner, called bystander suppression. Thus, the nT_{reg} cells and the effector T cells do not need to have the same antigen specificity (Tang and Bluestone, 2008). Depletion of T_{reg} cells by monoclonal antibodies directed against CD25 resulted in enhanced EAU (Grajewski et al., 2006).

Tissue-specific antigens of the eye are in most cases highly conserved and part of the visual signal transduction pathway. That is why they are mostly expressed only in the eye. Furthermore, the eye is separated by the blood-retina-barrier from the immune system (section 4.3.2). Moreover, autoreactive T cells specific for retinal antigens are not affected by peripheral tolerance mechanisms, because they do not recognize their specific antigen in the periphery. Thus a high frequency of autoreactive, retina-specific T cells can remain in the blood. If retina-specific autoantigens are expressed in the periphery, resistance against experimental autoimmune uveitis can be induced (Agarwal et al., 2000; McPherson et al., 1999). Without peripheral suppression of autoreactive T cells, these cells can be activated through cross reactivity in the periphery and can then enter the eye by chance, where a small number of autoreactive T cells is sufficient to trigger autoimmune inflammation (Prendergast et al., 1998).

4.3.2 Immune privilege of the eye

In addition to the general tolerance mechanisms to prevent autoimmune inflammation, the eye possesses special mechanisms to limit intraocular inflammation.

Immune privileged sites are defined as sites of the body where foreign tissue grafts are able to survive for an extended period of time. This is in contrast to the rest of the body, where similar tissue grafts are rejected. The phenomenon of immune privilege was first described by heterotopic transplantation of skin tissue into the anterior chamber of the eye and into the brain of rabbits (Medawar, 1948). In doing so, no graft rejections following organ transplantation were observed in the eye and brain, whereas grafts in other parts of the body were rejected. Shortly thereafter, the cornea was described as an immune privileged tissue (Billingham and Boswell, 1953).

Immune privileged tissues are certain tissues, which show a prolonged survival after transplantation into normal sites of the body, whereas non-privileged tissues are rejected at conventional sites of the body. Besides eye and brain immune privileged sites include also ovary, pregnant uterus, testis, adrenal cortex, hair follicles and certain tumors (Barker and Billingham, 1977). The immune privilege protects especially organs and tissues, which are important for survival and reproduction and have limited capacity for regeneration. The immune privilege is established through a combination of different factors including anatomical and physiological barriers as well as immune-regulatory processes. Together they result in a restricted activity of the immune system. However, there is no comprehensive suppression of all immune reactions in these areas, but rather a strictly regulated adaptation of the immune reaction to suppress a destructive inflammation. Therefore, only a small number of innocent bystander cells are hit by the immune reaction and nonetheless a less harmful immune reaction is preserved.

The absence of lymphatic vessels serves as a physiological barrier, which keeps the immune privileged tissues separate from the lymphoid system (McLean and Scothorne, 1970; Medawar, 1948). Thereby the entrance of immune cells to the immune privileged sites is hampered. The blood-retina-barrier is formed by endothelial cells with tight junctions and prevents the entry of naïve lymphocytes into the immune privileged organ (Cunha-Vaz, 1979). Only activated lymphocytes have the ability to cross the blood-retina-barrier and get access to the inner eye (Prendergast et al., 1998). In addition to cells, molecules over a size of 376 Dalton are also excluded from the eye through these endothelial barriers (Haselton et al., 1996).

Another protective mechanism of the immune privileged organs is low expression of MHC class I molecules on the surface of cells with no or only little capacity for regeneration (Abi-Hanna et al., 1988; Lampson and Fisher, 1984). The absence or reduced expression of MHC class I on the surface protects these cells from recognition by cytotoxic T lymphocytes (CTL), that would kill them in case of viral infections. However, cells without MHC class I are not protected from natural killer cells (NK cells), because these cells eliminate MHC class I negative cells (Moretta et al., 2002). In order to escape the elimination of NK cells, these cells express non-classical MHC class Ib molecules (Le Discorde et al., 2003; Niederkorn et al., 1999),

which are recognized by inhibitory receptors on NK cells and prevent NK cell mediated cell lysis (Lee et al., 1998; Rouas-Freiss et al., 1997).

Furthermore, cell surface molecules that coat the immune privileged organs maintain the immune privilege. In the eye for instance complement regulatory proteins (CRP) such as CD46 and CD55 are expressed and protect the body's own cells from the complement cascade (Bora et al., 1993). Fas ligand (CD95L) is widespread expressed in the eye as well. It induces apoptosis of activated T cells and neutrophils and in this way protects from tissue-damaging immune responses (Griffith et al., 1995). Another pro-apoptotic molecule is TRAIL (TNF-related apoptosis-inducing ligand), a member of the tumor necrosis family (TNF), which leads to apoptosis of inflammatory cells and is also expressed in immune privileged organs (Lee et al., 2002; Phillips et al., 1999).

Moreover, soluble molecules are also involved in immune privilege. These are for example found in the aqueous humor of the eye and induce anti-inflammatory and suppressive effects (Taylor, 1999). TGF-β suppresses T cells, NK cells and macrophages (Cousins et al., 1991). α-melanocyte stimulating hormone (α-MSH), which is also found in the aqueous humor, inhibits T effector cell proliferation and activation of macrophages and neutrophils (Taylor et al., 1992). In addition a soluble form of Fas ligand can be detected in the aqueous humor, where it confers anti-inflammatory as well as immune suppressive effects (Sugita et al., 2000). Additional immune suppressing factors, which are present in the eye, are: calcitonin gene-related peptide (CGRP) (Taylor et al., 1998; Wahlestedt et al., 1986), indoleamine dioxygenase (IDO) (Malina and Martin, 1993), macrophage migration inhibitory factor (MIF) (Apte et al., 1998), somatostatin (SOM) (Taylor and Yee, 2003), vasoactive intestinal peptide (VIP) (Taylor et al., 1994; Uddman et al., 1980) and thrombospondin-1 (TSP-1) (Zamiri et al., 2005).

In addition to the mechanisms mentioned above the eye is equipped with another mechanism maintaining privilege, namely anterior chamber-associated immune deviation (ACAID) (Kaplan and Streilein, 1977). In ACAID, the inhibition of systemic immune responses is induced by antigens which were injected into the anterior chamber of the eye. A similar reaction can be achieved by positioning the antigens into the vitreous (Jiang and Streilein, 1991) or the subretinal space (Wenkel and Streilein, 1998). Besides the eye other organs are also involved such as spleen

(Streilein and Niederkorn, 1981), thymus (Wang et al., 1997) and the sympathetic nervous system (Li et al., 2004). Antigens from the anterior chamber are captured by special tolerance inducing APCs that express F4/80 in mice. These cells transport the antigens through the vasculature to the thymus and induce regulatory NKT cells ($CD4^-CD8^-NK1.1^+$) (Wang et al., 2001). Via the bloodstream the NKT cells reach the spleen, where they participate in the generation of $CD8^+$ T cells, which suppress T_H1 as well as T_H2 mediated inflammation (Nakamura et al., 2003). In the development of these regulatory cells other cells besides NKT cells are involved such as $F4/80^+$ APCs that could migrate to the spleen directly from the eye, CD1d expressing B cells (D'Orazio and Niederkorn, 1998; Skelsey et al., 2003; Sonoda and Stein-Streilein, 2002) and $CD8^+$ T cells. ACAID protects the eye not only from autoimmune diseases such as uveitis (Mizuno et al., 1989) but also improves the survival of cornea transplants (Niederkorn and Mellon, 1996; She et al., 1990).

4.4 Experimental autoimmune uveitis

EAU mimics human autoimmune uveitis and has been used to investigate the mechanisms of the disease and to develop therapies. Several animal models have been developed over the last decades and are now available to investigate different aspects of autoimmunity. EAU can be induced with different retinal autoantigens in a variety of animal models, for example in rats, mice, and horses. There are also several methods to induce EAU.

The most common method is the immunization with autoantigen in complete Freund's adjuvant (CFA) (Nussenblatt et al., 1980b). CFA contains heat-killed mycobacteria tuberculosis, which confer danger signals and thus activate APCs. Through innate immune cells a pro-inflammatory milieu arises and induces an adaptive immune response. Immunization with uveitogenic antigen in the periphery imitates the proposed uveitis induction in patients with the peripheral initiation of the autoimmune response.

Another method to induce experimental autoimmune uveitis is adoptive T cell transfer of *in vitro* activated pathogenic T cells, cultured from autoantigen-immunized donor

animals (Mochizuki et al., 1985). The activated T cells reach the eye by chance, since activated T cells have the ability to pass the blood-retina-barrier (Prendergast et al., 1998). Using GFP^+ (green fluorescent protein) R14 (uveitogenic peptide from IRBP) specific or ovalbumin-specific activated T cells, it was shown that T cells of both specificities enter the eye already 30 minutes after intravenous injection. However, only the R14-specific T cells could be detected over an extended period of time in the eye. By contrast, the ovalbumin-specific T cells did not find their antigen and disappeared from the eye. Furthermore, only animals receiving R14-specific T cells developed EAU three days after injection. This led to the assumption that T cells have to recognize their antigen *in situ* and become reactivated to be able to attract inflammatory cells and thus induce uveitis (Thurau et al., 2004).

In addition, there are other variants of EAU used as models for investigating special issues of autoimmune uveitis. For instance, EAU can be induced by infusion of FMS-like-tyrosine-kinase 3 ligand (Flt3L)-mobilized DCs pulsed with autoantigen, which were activated with the bacterial endotoxin LPS (lipopolysaccharide) and anti-CD40 antibody *in vitro* (Tang et al., 2007). Another special variant of EAU is the “humanized” model of experimental autoimmune uveitis using HLA-DR3 transgenic mice (Pennesi et al., 2003).

In addition, there are a few models of spontaneous uveitis which are suggested to be more similar to the human situation. However, they mostly develop in genetically manipulated mice, which are immunologically highly artificial. One example for spontaneous uveitis is a mouse model lacking the transcription factor AIRE (Anderson et al., 2002). Naturally occurring uveitis is found in horses, in which uveitis can also be induced experimentally, a disease known as equine recurrent uveitis (ERU) (Deeg et al., 2001; Deeg et al., 2002).

Most of the above mentioned models are established in mice. Nevertheless, the classical way of inducing EAU by immunization with antigen in CFA and adoptive T cell transfer are also well established in rats, which, in contrast to mice, are susceptible to many different retinal autoantigens.

The different methods used for EAU induction result in different disease patterns, even when genetically identical animals are used. This could be an explanation for the heterogeneous nature of human uveitis, where the initial disease trigger is still unknown.

Validation of EAU as a model for human autoimmune uveitis was demonstrated by T cells isolated from human uveitis patients, which responded to retinal antigens (Nussenblatt et al., 1980a) and therapies developed in EAU could often be transferred to human uveitis patients (Gomes Bittencourt et al., 2012).

4.4.1 Pathomechanisms of experimental autoimmune uveitis

EAU is a T cell mediated disease (Figure 2). Initially, the autoantigen-specific T cells were identified as $CD4^+$ interferon gamma (IFN-γ) producing T_H1 cells (Caspi et al., 1996). More recently, a new T helper cell subset was discovered, the so-called T_H17 cells, which are distinct from T_H1 cells and express IL-17 as their signature cytokine (Nakae et al., 2007). Both T_H1 and T_H17 cells can induce EAU, depending on the animal model and on conditions during initial antigen contact (Luger et al., 2008).

Activation of autoreactive T cells requires additional administration of bacterial adjuvants such as CFA that function as a danger signal. This trigger ensures the activation of innate immune cells and a pro-inflammatory milieu arises, in which naïve T cells get activated and develop to effector T cells. A schematic model of EAU induction is shown in Figure 2.

Activated antigen-specific T effector cells have the ability to cross the blood-retina-barrier and after recognition of their retinal antigen are reactivated and recruit inflammatory cells. However, it has not yet clearly been shown which APCs are responsible for T cell activation in the eye. In non-inflamed eyes very few MHC class II expressing cells are detectable. Even resident DCs express low levels of MHC class II (Jiang et al., 1999). It is possible that the first activated T cells, which reach the eye, evoke local MHC class II expression by IFN-γ production (Xu et al., 1997). On the other hand, already activated T cells exhibit a lower threshold of avidity for their specific antigen and thus can more easily be reactivated compared to naïve T cells (Kimachi et al., 2003). They are no longer dependent on costimulation and can be activated by different APCs, for example also by resting B cells (Croft et al., 1994). The importance of APCs in the eye was demonstrated by injection of $CD11^+$ DCs into the anterior chamber, which increased the severity of EAU after adoptive T cell transfer (Heuss et al., 2012). Furthermore, local conditions inside the eye

determine whether APCs display pro- or anti-inflammatory effects (Heuss et al., 2012).

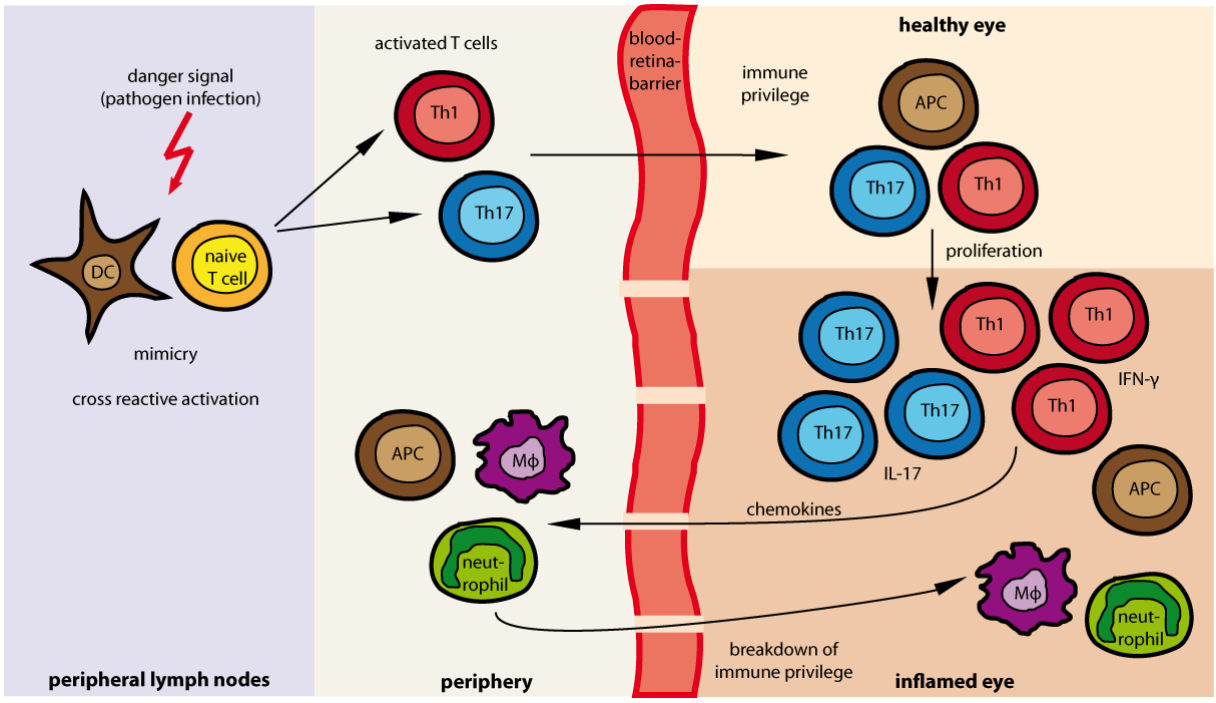

Figure 2 Development of autoimmune uveitis. Danger signals from pathogens activate dendritic cells (DCs), which subsequently activate naïve T cells in peripheral lymph nodes. The recognized pathogen-specific antigens could mimic ocular autoantigens and thus autoreactive T cells are activated, which differentiate into T_H1 and T_H17 effector cells. Once activated, T cells have the ability to cross the blood-retina-barrier and enter the eye. Recognition of crossreactive autoantigens inside the eye is followed by reactivation and proliferation. Subsequently, effector T cells secrete cytokines and chemokines, leading to breakdown of immune privilege and thus to recruitment of inflammatory leukocytes (macrophages and neutrophils) from the periphery to the eye. The number of antigen presenting cells (APCs) in the eye is increased by upregulation of MHC class II inside the eye and by recruitment of APCs from the circulation. This results in amplification of the T cell response and tissue inflammation (uveitis).

Only few antigen-specific activated T cells are required to induce uveitis. Using adoptive T cell transfer and subsequent detection of antigen-specific T cells in the eye, it was possible to calculate the number of T cells sufficient for uveitis induction. The result was that only 15 antigen-specific T cells are needed inside the eye to induce uveitis in mice (Caspi, 2006). Following antigen-recognition these few cells proliferate and recruit other leukocytes from the circulation. For EAU induction

macrophages and granulocytes are of particular importance. This was demonstrated by depletion of these cell types, which resulted in abrogation of EAU (Forrester et al., 1998; Su et al., 2007). Besides these innate inflammatory effector cells, recruitment of antigen-non-specific T cells was also shown to be needed for EAU development. Compared to normal rats, athymic rats receiving uveitogenic T cells developed a considerably downregulated EAU when IRBP-specific T cells were used and failed to develop EAU after transfer of S-Ag-specific T cells (Caspi et al., 1993).

Since the eye exhibits an inhibitory milieu and despite this effector T cells can induce inflammation, they must be able to resist the hostile environment. After inflammation occurs in the eye the inhibitory milieu is modified and the immune privilege is destroyed (Ohta et al., 1999). MHC class II expression is induced and there is influx of APCs from the circulation, because the blood-retina-barrier is damaged (Gregerson and Kawashima, 2004). The consequence is that naïve T cells can then be activated in the eye recognizing other ocular autoantigens (epitope spreading) (Deeg et al., 2002; Diedrichs-Mohring et al., 2008). In the inflamed eye there is a cytokine milieu, which favors T_H17 development, because TGF-β is present in the aqueous humor and IL-6 is released during inflammation. Both cytokines in combination can induce T_H17 cells in mice (Bettelli et al., 2006; Veldhoen et al., 2006).

Termination of autoimmune uveitis is mediated by regulatory mechanisms. After recovery from intraocular inflammation regulatory T cells ($CD4^+CD25^+$) were detected in the spleen, while these cells could not be found in naïve animals. Their development is dependent on the eye, because regulatory T cells were not detected in enucleated mice and their suppressive function can be transferred to mice immunized for EAU-induction resulting in the downregulation of inflammatory responses in the eyes (Kitaichi et al., 2005). This suggests that uveitis can induce tolerance. Due to T cell plasticity, regulatory T cells can arise from effector T cells (section 4.5). The ocular environment even promotes conversion of conventional T cells to regulatory T cells (Stein-Streilein and Taylor, 2007).

Under special conditions other T cell types are also able to induce EAU. In immunodeficient hosts it was demonstrated that T_H2 cells also have the capacity to induce EAU (Kim et al., 2002). Even $CD8^+$ T cells are able to induce EAU, albeit with a milder inflammation compared to $CD4^+$ T cells (McPherson et al., 2003; Song et al.,

2008). $CD8^+$ T cells are not necessary for induction of EAU, because depletion of $CD8^+$ T cells did not impair the development of uveitis after immunization with retinal antigens (Calder et al., 1993). For $CD8^+$ T cells regulatory functions have also been described in EAU (Caspi et al., 1988; Han et al., 2007; Peng et al., 2007b).

Antibodies play a subordinate role in EAU. After induction of EAU by immunization with retinal antigens there are antigen-specific antibodies detectable in the serum (de Kozak et al., 1992). However, these antibodies do not have the ability to transfer disease to healthy recipients. Antibodies are unable to cross the intact blood-retina-barrier, due to their molecular size. If uveitogenic T cells already have damaged the blood-retina-barrier, antibodies from the circulation can enter the eye and exacerbate disease (Pennesi et al., 2003). Furthermore, antibodies are able to enhance EAU when injected together with uveitogenic T cells into recipient animals (Pennesi et al., 2003).

4.5 T cell subsets

In the thymus two types of TCRs develop. While most of the T cells express a TCR with an alpha and a beta chain (αβTCR), a small fraction with gamma and delta chain TCRs (γδTCR) also appear. These TCRs are less heterogeneous than the αβTCRs and their ligands are not fully elucidated. γδ T cells are part of the innate immune system and are basically found in skin and mucosa. They have the ability to respond to local inflammations and also contribute to autoimmunity (Shibata, 2012).

αβ T cells are further subdivided into two major subsets depending on their co-receptors CD4 and CD8. $CD4^+$ T cells recognize their specific antigen presented on MHC class II molecules, while $CD8^+$ T cells bind to antigens in context with MHC class I molecules. These two subsets not only express different co-receptors but also exhibit different functional activities. After activation $CD8^+$ T cells become CTLs (Dennert, 1997) that eliminate abnormal cells like pathogen-infected cells or tumor cells.

$CD4^+$ T cells are also called T helper cells, because they provide help for other cells, such as B cells, CTLs and macrophages (Nakayamada et al., 2012). Initially, they

were divided into the two subpopulations T_H1 and T_H2 (Mosmann et al., 1986). The distinction was based on cytokine profiles. While T_H1 cells mainly produce IFN-γ, T_H2 cells secrete IL-4, IL-5 and IL-13 (Mosmann and Coffman, 1989). T_H1 cells enhance pro-inflammatory cell-mediated immunity to eliminate intracellular pathogens. By contrast, T_H2 cells are essential for B cell proliferation and maturation as well as eosinophil inflammation to remove extracellular pathogens. T_H1 and T_H2 cytokines antagonize the effector function of each other (Abbas et al., 1996). Naïve T helper cells develop their special phenotype during their first antigen contact on DCs, mediated by the cytokine milieu provided during this step. While T_H1 cells differentiate in the presence of IL-12 and IFN-γ, T_H2 cells need IL-4.

Over the years, more and more $CD4^+$ T cell subsets were discovered (Figure 3). Studies on autoimmune diseases led to the suggestion that there must be another T cell subset, distinct from T_H1 participating in autoimmunity. Further experiments have finally resulted in the discovery of IL-17-producing cells (T_H17) (Cua et al., 2003; Langrish et al., 2005). T_H17 cells produce mainly IL-17A and IL-17F, but also TNF, IL-6 and IL-22 (Langrish et al., 2005; Liang et al., 2006). To develop into T_H17 cells naïve T cells require the presence of TGF-β and IL-6 in mice (Bettelli et al., 2006; Veldhoen et al., 2006) and IL-23 and IL-1β in humans (Louten et al., 2009). The T_H17 hallmark cytokine IL-17 is a potent inflammatory cytokine, which acts pleiotropicly. IL-17 induces expression of various pro-inflammatory cytokines (for example IL-6 and TNF), chemokines and matrix metalloproteases and thus mediates tissue inflammation (Kolls and Linden, 2004). Yet another function of IL-17 is induction of proliferation, maturation and chemotaxis of neutrophils, which are also involved in tissue inflammation (Fossiez et al., 1996). Furthermore, IL-17 augments the maturation of DCs and is also involved in the costimulation of T cells (Kolls and Linden, 2004). In healthy individuals T_H17 cells are crucial for the immune response to extracellular bacteria and fungi, mediated by recruitment and activation of neutrophils and macrophages to inflamed tissues (Louten et al., 2009). However, IL-17 also plays an important role in the pathogenesis of various autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis and autoimmune uveitis (Amadi-Obi et al., 2007; Matusevicius et al., 1999; Shen et al., 2009). The contribution of T_H17 cells to autoimmune diseases was first demonstrated for EAE (Cua et al., 2003; Langrish et al., 2005) and collagen induced arthritis (CIA) (Murphy et al., 2003), both are

models for autoimmune diseases induced by immunization with self-antigen in CFA. Finally, involvement of T_H17 cells was also shown for EAU by neutralization of IL-17 by the appropriate antibodies (Amadi-Obi et al., 2007) and by adoptive transfer of antigen-specific T_H17 cells (Peng et al., 2007a).

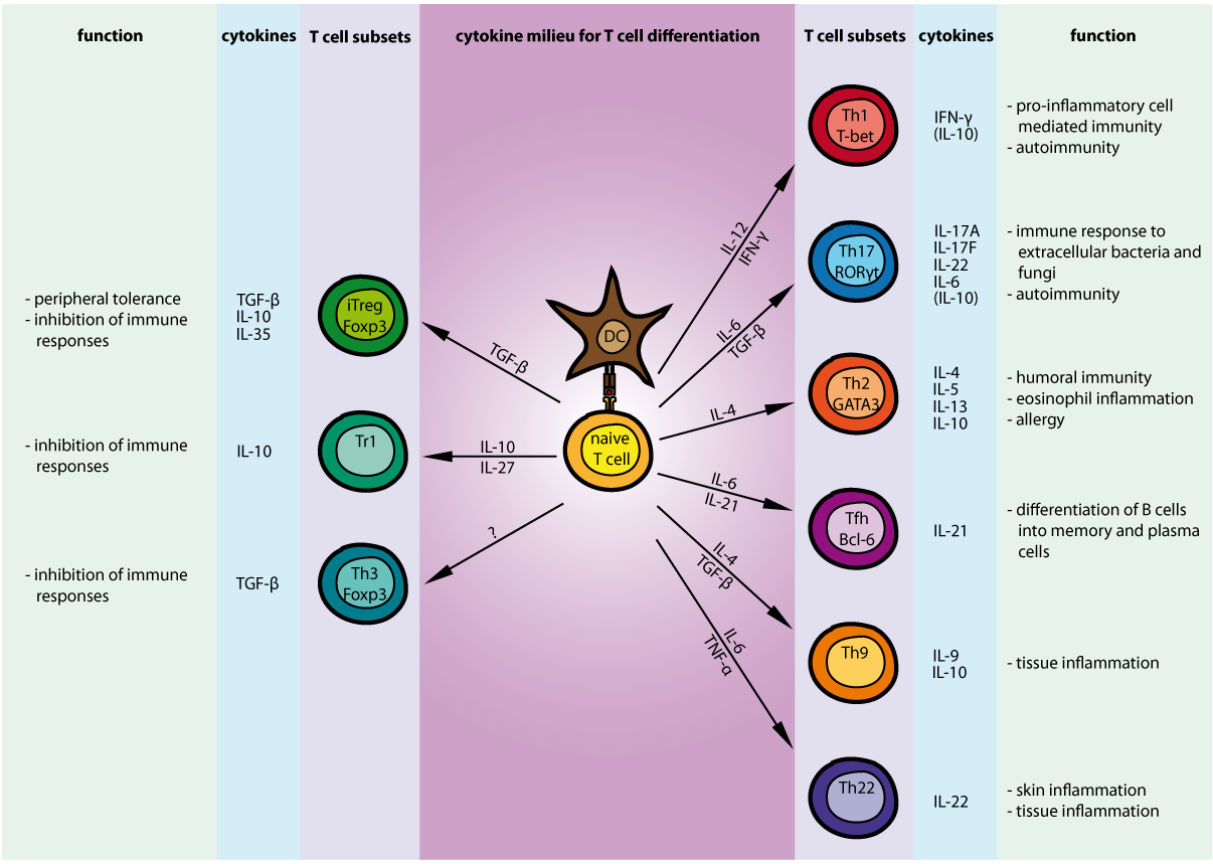

Figure 3 Differentiation of $CD4^+$ T cell lineages. After antigen-specific activation in the periphery naïve T cells can differentiate into different subsets of effector and regulatory T cells, respectively. Lineage commitment is dependent on the cytokine milieu present during TCR stimulation. While pro-inflammatory cytokines give rise to T_H1, T_H2, T_H17, T_{FH}, T_H9 and T_H22 cells, anti-inflammatory cytokines lead to regulatory cells, like iT_{reg}, T_R1 and T_H3. During differentiation these cells express special master transcription factors (shown inside the cells), which are not yet identified for all T cell subsets. Activated T cells secrete a special set of cytokines that ensure their functions contributing to the appropriate immune response. However, the differentiated T cell subsets are not always irreversibly determined. Some of them show a high plasticity and thus can change their cytokine profiles and even their expression of master transcription factors. Natural T_{reg} cells that emerge from $CD4^+$ thymic T cell precursors are not depicted.

Another subset of T helper cells are T follicular helper cells (T_{FH}). After upregulation of B cell lymphoma 6 (Bcl-6) T helper cells adopt the T_{FH} cell phenotype, regardless of their origin. They can not only develop from naïve T cells, but also from effector T cells like T_H1, T_H2 and T_H17 as well as from nT_{reg} cells. The main task of T_{FH} cells is the differentiation of B cells into memory and plasma cells (Ma et al., 2012). Beyond that, there are some other $CD4^+$ T cell subsets, such as T_H9 and T_H22, which have been proposed as new $CD4^+$ T cell subsets (Akdis et al., 2012; Tan and Gery, 2012).

In addition to these effector T cell types, there are also several subsets of regulatory $CD4^+$ T cells (T_{reg}) that have the ability to control effector T cell responses (Josefowicz et al., 2012; Shevach, 2006). One classification of regulatory T cells can be made by their origin. nT_{reg} cells are Foxp3 positive and develop in the thymus, whereas all the other regulatory cells are induced from peripheral T helper cell precursors by different cytokines (Wan and Flavell, 2006). Induced T_{reg} cells (iT_{reg}) express Foxp3 and are generated in the presence of TGF-β and retinoic acid in the periphery. They have similar inhibitory functions as the nT_{reg} cells (Dons et al., 2012). Additional regulatory T cells are T regulatory 1 (T_R1) and T_H3 cells that thus far cannot be distinguished by means of their surface markers. T_R1 cells lack expression of Foxp3 and produce IL-10, while T_H3 cells are $Foxp3^+$ and secrete TGF-β (Groux et al., 1997; Wan and Flavell, 2006; Weiner, 2001).

4.5.1 Plasticity of T helper cells

With the advent of new T helper cell subsets, evidence for their plasticity in phenotype and function has become clear (Murphy and Stockinger, 2010; O'Shea and Paul, 2010). This appears to be important to allow adaptation to changing circumstances.

Initially it was assumed that each T cell subset expresses its specific signature cytokine and master transcription factor. Examples include T_H1 cells with their signature cytokine IFN-γ and master transcription factor T-box transcription factor (T-bet), T_H2 with IL-4 and GATA binding protein 3 (GATA3) (Mosmann and Coffman, 1989), T_H17 with IL-17 and retinoid-related orphan receptor gamma t (RORγt) (Harrington et al., 2005) and nT_{reg} with Foxp3 (Sakaguchi et al., 2008).

Indications of T cell plasticity came from cytokine expression profiles. Only few cytokines are selectively produced by distinct T cell subsets, while several cytokines are expressed by diverse T cell subsets. For example IL-10 was initially thought to be a T_H2 cytokine, now it is also identified in T_H1, T_H17 and T_{reg} cells (Saraiva and O'Garra, 2010). Furthermore, master transcription factors can also be expressed by more than one T helper cell subset. There are several combinations of these transcription factors possible. For instance, some cells coexpress Foxp3 and RORγt. These cells produce IL-17 and have inhibitory functions (Voo et al., 2009). Another example are T cells coexpressing T-bet and RORγt, which are highly pathogenic in EAE (Ghoreschi et al., 2010). Additionally, T helper cells can change their phenotype. For T_H17 cells it could be shown that they often become IFN-γ producers (Lee et al., 2009). Furthermore, T_H2 cells can be reprogrammed to IFN-γ expressing T_H1 cells (Hegazy et al., 2010). Even T_{reg} cells are able to change their phenotype. By losing Foxp3 expression, they can acquire pro-inflammatory activities (Dominguez-Villar et al., 2011; Oldenhove et al., 2009). However, the subset with the highest plasticity are T_{FH} cells, which can convert to T_H1, T_H2 and T_H17 cells. On the other hand, they can develop from T_H1, T_H2, T_H17 and T_{reg} cells (Lu et al., 2011; Tsuji et al., 2009).

In conclusion, T helper cell subsets exhibit a high plasticity and thus cannot easily be allocated to distinct subsets. Depending on the circumstances they can acquire either a rather stable or a rather plastic phenotype.

4.5.2 T helper cells in autoimmunity

$CD4^+$ T cells play a central role in autoimmunity and any effector T cell has the potential to induce autoimmune responses. Initially, T_H1 cells were regarded as responsible for autoimmunity; however, the discovery of T_H17 cells changed this perception. For instance, indications for a role of T_H1 cells in autoimmunity were demonstrated in EAU by cytokine profiles of T cells from affected animals and by adoptive T cell transfer (Caspi et al., 1996; Xu et al., 1997). Other experimental autoimmune models, such as EAE, showed equivalent results. However, with the discovery that animals deficient in T_H1 cells are highly susceptible to EAE and EAU, these results were questioned (Bettelli et al., 2004; Jones et al., 1997). A surprising

revelation was the fact that IL-12, a T_H1-polarizing cytokine composed of p35 and p40 subunits, shares the p40 subunit with IL-23, which combines p19 and p40 and stimulates T_H17 development (Oppmann et al., 2000). Subsequently it was demonstrated that p19 and p40 promote autoimmune diseases, while p35 does not. Thus it was assumed that IL-23, but not IL-12 is necessary for the development of autoimmune responses (Cua et al., 2003). Studies with p35-deficient animals led to the conclusion that IL-12 has no effect in autoimmune diseases or is even protective (Murphy et al., 2003). However, IL-12 is not the only cytokine using the p35 subunit. The regulatory cytokine IL-35 consists of p35 and Epstein-Barr-virus-induced gene 3 (Ebi3), thus the regulatory role of p35 may reflect the activity of IL-35 and not IL-12 (Collison et al., 2007). From these controversial results one can conclude that both T_H1 and T_H17 cells play a role in autoimmune diseases and may carry out different functions. It is suggested that T_H17 cells form the first wave of tissue infiltrating T cells (Hirota et al., 2007; Reboldi et al., 2009) and later change their phenotype to a T_H1 profile (Lee et al., 2009). This hypothesis is further supported by our work, which revealed many cells expressing both IFN-γ as wells as IL-17 and decreasing numbers of IL-17^+ cells during the resolution of EAU in rats.

Besides effector T cells, regulatory T cells also play an important role in autoimmunity. Their absence results in a breakdown of self-tolerance and triggers various autoimmune diseases (Sakaguchi et al., 1995). Adoptive transfer of T_{reg} cells can prevent or even reverse autoimmune responses (Takahashi et al., 1998). It is assumed that an imbalance of the immune system causes autoimmunity. When effector T cells overpower the regulatory effects of T_{reg} cells, because of a deficiency in either frequency or function of T_{reg} cells, it paves the way for autoimmunity (Chatenoud et al., 2001).

4.6 Animal models for monophasic and relapsing uveitis

Since most animal models are either monophasic or chronic, there are only two models available for relapsing experimental autoimmune uveitis, namely in horses and in rats. ERU is the only spontaneous occurring relapsing uveitis with a high prevalence, up to 15% of horses are affected (Rebhun, 1979). Uveitis can also be

experimentally induced in horses by immunization with IRBP. Disease outcome of experimental uveitis is similar to spontaneous uveitis (Deeg et al., 2002). Like in rodent models, T cells are the major infiltrating cell population in the eyes of horses (Deeg et al., 2001; Gilger et al., 1999; Romeike et al., 1998). However, due to the size of the animals and the lack of reagents and techniques, ERU is less suitable for detailed investigations of cytokine and cell profiles in monophasic and relapsing uveitis.

A better animal model for monophasic and relapsing EAU is the Lewis rat model. Lewis rats, which are highly susceptible for induction of EAU by a variety of retinal autoantigens, are immunologically well characterized and have reasonably sized eyes. They develop anterior as well as posterior inflammation during the disease course and therefore the investigation of EAU can be made clinically by daily examinations with an ophthalmoscope as well as by final histological analysis of the eyes (Figure 4).

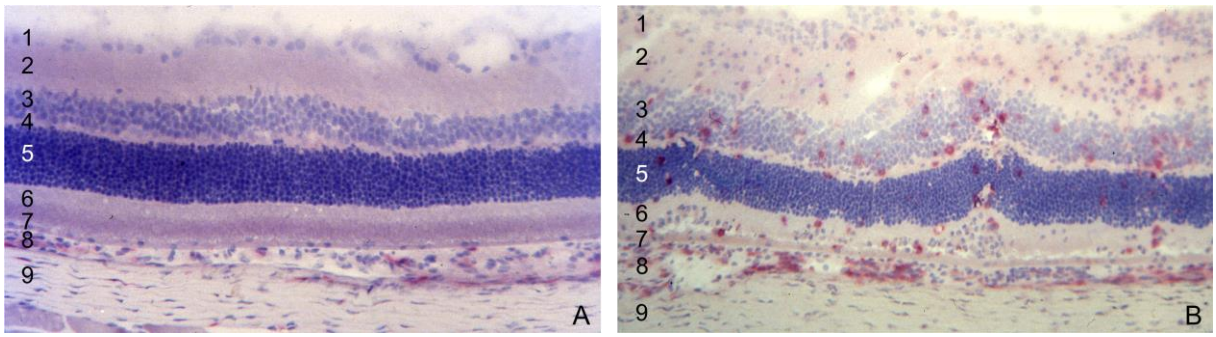

Figure 4 Histology of Lewis rat eyes. A) Healthy eye. B) Eye from an animal with uvetis induced with IRBP in CFA. Cryosections were stained for CD4 (red, labels T helper cells and monocytes/macrophages). Nuclei were counterstained with hematoxylin (blue). 1: ganglion cell layer, 2: inner plexiform layer, 3: inner nuclear layer, 4: outer plexiform layer, 5: outer nuclear layer, 6: photoreceptors outer segments, 7: retinal pigment epithelium, 8: choroid, 9: sclera. Photographs courtesy of Gerhild Wildner.

Moreover, the rat model has proved to be helpful in different aspects. As rats are susceptible to EAU induction by many different autoantigens, they can be used to test potentially new autoantigens for uveitis. Additionally, they have been used to investigate new therapeutic treatments for uveitis. A successful example is Cyclosporin A, an inhibitor of T cells, which was first tested in the rat model and later

became available for human therapy (Nussenblatt et al., 1983; Nussenblatt et al., 1981). Another example is oral tolerance to treat uveitis by inducing antigen-specific tolerance. Again the rat model was used to investigate this mechanism. Here either retinal S-Antigen (Nussenblatt et al., 1996) or B27PD, a peptide derived from HLA-B, was used as oral tolerogen and both could suppress EAU (Wildner and Thurau, 1994). A therapeutic trial with B27PD in human autoimmune uveitis showed promising results (Thurau et al., 1999) and clinical trial is presently underway.

One major advantage of the Lewis rat model which makes it very attractive is that the disease course can either be monophasic or relapsing, depending on the antigen used for EAU induction. The relapsing disease was first described after adoptive T cell transfer (Shao et al., 2005) and later it was also demonstrated after immunization with antigen (Diedrichs-Mohring et al., 2008). S-Ag was the first uveitogenic antigen described (de Kozak et al., 1981; Wacker et al., 1977). It is an intracellular protein of the photoreceptors. Its most pathogenic peptide in rats is PDSAg (amino acid 342-354 from bovine S-Ag) (Wildner and Thurau, 1994). Another uveitogenic antigen is the IRBP, which transports retinoids between the photoreceptors and the retinal pigment epithelium. The major uveitogenic peptides of IRBP are R14 (amino acid 1169-1191) and R16 (amino acid 1177-1191) (Sanui et al., 1988). In Lewis rats, peptide PDSAg from S-Ag induces monophasic EAU, while peptides R14 and R16 from IRBP induce relapsing EAU (Diedrichs-Mohring et al., 2008; Shao et al., 2005). Functional differences between PDSAg- and R14-induced uveitis were discovered by applying the chemokine receptor antagonist Met-RANTES (N-terminally methionylated form of RANTES (regulated and normal T cell expressed and secreted / CCL5). EAU induced by adoptive transfer of PDSAg-specific T cells was completely blocked after Met-RANTES treatment, while EAU induced by adoptive transfer of R14-specific T cells was not affected (Diedrichs-Mohring et al., 2005). A further difference between monophasic and relapsing EAU is the onset of clinical disease, which was delayed by 2-3 days in PDSAg-immunized animals compared to R14-immunized rats (Diedrichs-Mohring et al., 2008). Moreover, differences in the activity of regulatory T cells were discovered between monophasic and relapsing EAU. T_{reg} cells isolated from eyes of monophasic EAU showed a stronger inhibition of responder T cells and also an increased production of IL-10 compared to T_{reg} cells from relapsing EAU after adoptive T cell transfer (Ke et al., 2008).

Since in humans autoimmune uveitis shows a relapsing rather than a monophasic course, this animal model strongly resembles the human situation and offers an important opportunity to investigate the immunological differences of monophasic and relapsing disease.

5 Results

5.1 Effector T cells driving monophasic vs. relapsing/remitting experimental autoimmune uveitis show unique pathway signatures

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Effector T cells driving monophasic vs. relapsing/remitting experimental autoimmune uveitis show unique pathway signatures

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ABSTRACT

Autoimmune diseases often show a relapsing-remitting course. Here we describe characteristics of the autoreactive T cell response in the Lewis rat model of experimental autoimmune uveitis (EAU), a model for the clinical heterogeneity seen in human uveitis. Depending on the autoantigen used, the experimental disease course can be either monophasic or relapsing/remitting. This appears to be dictated by subtle differences in the T cell effector phenotype elicited. Using transcriptomic profiling and pathway analysis, the molecular basis for the monophasic vs. relapsing/remitting effector T cell phenotype was investigated. CD4+ T cell lines specific for peptide R14 derived from interphotoreceptor retinoid-binding protein (IRBP), which mediate the relapsing disease, were compared to the monophasic disease-inducing lines responding to retinal S-antigen peptide PDSAg. Expression profiles from T cell lines representing each specificity were analyzed using Affymetrix microarrays. Differential gene expression was confirmed and extended by quantitative PCR and verified on the protein level. A set of genes was uniquely upregulated in the R14-specific T cells. Gene ontology analysis demonstrated that these genes were linked to regulatory pathways associated with antigen presentation, lymphocyte activation, regulation of apoptosis and WNT/Hedgehog signaling. R14-specific T cells were further demonstrated to have prolonged survival *in vivo*, and a Th1-dominated cytokine profile, while the PDSAg-specific T cells lines were more Th17-prone. Our findings suggest that the nature of specific antigens leads to subtle programming of the effector phenotype underlying recurrent inflammation.

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1. Introduction

Autoimmune diseases are often characterized by recurrent episodes of inflammation leading to the progressive destruction of affected tissues or organs. While most therapeutic strategies have focused on avoiding the recurrence of disease – the pathogenic basis underlying the T cell-mediated recurrence is not well understood.A rat model of autoimmune uveitis (EAU), a T cell-mediated inflammatory disease of the inner eye, was used to investigate the pathophysiologic basis of recurrent vs. monophasic disease at the level of the T effector cell. EAU in Lewis rats can be induced by immunization with different retinal autoantigens (in CFA). Autoantigens that induce EAU include the retinal soluble antigen (S-antigen, S-Ag) and the interphotoreceptor retinoid-binding protein (IRBP). Both antigens are implicated in human disease (de Smet et al., 2001; Wildner and Thurau, 1994). The two most pathogenic epitopes identified in these proteins are S-antigen peptide PDSAg (aa 341–354 of S-Ag) and R14 (aa 1169–1191 of IRBP). Both peptides induce specific autoreactive CD4+ T cells, which initiate uveitis through the recruitment of monocytes/macrophages to the eye that promote intraocular inflammation. CD4+ T cells specific for either peptide can adoptively transfer the disease.

While autoreactive effector CD4+ T cells specific for the R14 and PDSAg antigen peptides appear similar based on their surface marker expression and general cytokine profiles, functional differences in their immune response have been identified (Diedrichs Mohring et al., 2005). EAU induced with peptide R14 leads to relapsing EAU, while immunization with PDSAg results only in monophasic disease. Similar effects are observed after the adoptive transfer of T cell lines specific for these peptides (Diedrichs-Mohring et al., 2008). Regulatory T cells (Tregs) that selectively restrain the PDSAg-specific T cells and protect from recurrent disease have not been identified. The underlying cause for the relapsing phase of the disease appears to originate in large part in subtle differences in the specific effector phenotype of T cells generated. Thus, uveitis elicited by these peptides represents a potential model system for the characterization of the pathogenesis

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of autoreactive T cells. Potential differences between the effector T cells induced by the monophasic vs. remitting antigens were investigated using transcriptomic profiling and pathway analysis.

2. Materials and methods

2.1. Animals

Male and female Lewis rats were bred in our colony or purchased from Janvier (Le Genest St. Isle, France). The animals were maintained under SFP conditions and used for experiments at the age of 6–8 weeks. The animals had unlimited access to rat chow and water. All animal experiments were approved by the Review Board of the Government of Oberbayern and conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

2.2. Induction of EAU and clinical grading

Peptide PDSAg derived from the sequence of bovine S-Ag (aa 341–354, FLGELTSSEVATEV), and peptide R14 from human IRBP (aa 1169–1191, PTARSVGAADGSSWEGVGVVPDV) were purchased from Biotrend (Cologne, Germany). Lewis rats (4–6 rats per group, with each experiment repeated at least two times) were immunized subcutaneously into both hind legs with a total volume of 200 μl emulsion containing 15 μg peptide R14 or PDSAg, both in complete Freund's adjuvant, fortified with Mycobacterium tuberculosis strain H37RA (BD, Heidelberg, Germany) to a final concentration of 2.5 mg/ml. The time course of disease was determined by daily examination of animals with an ophthalmoscope. Uveitis was graded clinically as described (de Smet et al., 1993) and the clinical scores of single eyes are shown for each day.

2.3. Cytokine measurement

Tissue culture supernatants were collected daily every 24 h from day 1 to 3 after antigen stimulation. Cytokines were measured in pooled samples from days 1, 2 and 3 of each culture, using the Bio-Plex protein array system (Lincoplex, Millipore, Schwalbach, Germany) in combination with the Bio-Plex Manager software, which is designed for the multiplexed quantitative analysis of multiple cytokines in a single well. Premixed multiplex beads for IL-2, IL-10, IFN-γ, TNF-α and IL-17 with a detection limit of 5 pg/ml in 25 μl total test volume were used and the assay was performed following the manufacturer's instructions.

2.4. Microarray analysis

Total RNA was extracted from pelleted T cell lines (3 different lines of each antigen specificity, $1–7 \times 10^6$ cells) (Chomczynski and Sacchi, 1987) 2 days post antigen-specific activation (see below). RNA quality was characterized using a RNA6000 Nanochip (Agilent Technologies, Waldbronn, Germany). Twenty μg of total RNA were reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany), then converted into double-stranded cDNA, and biotin-labeled using the GeneChip Expression 3′ amplification one-cycle target labeling and control kit (Affymetrix, Sunnydale, CA). All reactions were performed essentially according to the Affymetrix protocol. Transcriptional profiling using Affymetrix R 230.2 gene chips was performed using RNA isolated from three PDSAg- and three R14-specific T cell lines. The resultant Cel files were analyzed using ChipInspector software (CI; Genomatix Software GmbH, Munich, Germany) as described (Cohen et al., 2008). The expected false discovery rate (FDR) was set for maximal detection of regulated transcripts accepting 0.5% falsely called features (FDR 0.5%). Data are available with the series record GSE19652 on the NCBI Gene expression omnibus webpage http://www.ncbi.nlm.nih.gov/geo/ (for detailed information see supplemental data).

2.5. Verification of mRNA expression by qPCR

RNA was isolated as described (Adams et al., 2005). Two micrograms of total RNA was used for cDNA synthesis by Superscript I/II reverse transcriptase (Invitrogen, Karlsruhe, Germany) with hexanucleotides as primers (Roche, Mannheim, Germany). RT-PCR products from six to eight T cell lines of each specificity (PDSAg and R14) were obtained. qPCR was performed by an ABIPrism7000 Sequence detection system (Applied Biosystems, Darmstadt, Germany).

After an initial hold of 2 min at 50 °C and 10 min at 95 °C, the samples were cycled 40 times at 95 °C for 15 s and 60 °C for 60 s. The primers were derived from different sources and are summarized in supplementary Table 1. All primers and probes were used in concentrations of 300 nmol/L and 100 nmol/L, respectively. Primers were tested using pools of groups of samples to identify significant differential regulation. If deregulation in expression was observed between the groups the qPCR has been repeated using single samples. The expression of candidate genes was normalized to the reference gene 18S rRNA (Schmid et al., 2003). Fold changes (Fc) were generated using the Pfaffl method (Pfaffl, 2001).

2.6. Pathway analysis

Pathways and Gene Ontology groups were identified employing the BiblioSphere software (Genomatix, Munich, Germany) and the DAVID Bioinformatics database from the NIAID, NIH (Version 2007) (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003).

2.7. Generation of antigen-specific T cell lines and transduction for GFP expression

Rats were immunized as described above; pooled lymph node cells from 4 rats were used for the establishment of each T cell line. Lymph nodes were collected 10 days post immunization and T cell lines cultivated as described (Diedrichs Mohring et al., 2005). Antigen specificity of T cell lines was tested by proliferation measured as ^{3}H-thymidine incorporation. Cells (2.5×10^6) were immediately transferred into rats 2 days after 2–4 restimulations with antigen *in vitro*, or stored frozen (2 days after antigen-specific stimulation *in vitro*) and thawed immediately before adoptive transfer. T cell lines for RNA preparation and gene expression analysis were harvested 2 days after antigen stimulation and snap frozen. Four additional PDSAg- and R14-specific T cell lines harvested after 2, 3, 4 and 5 restimulations *in vitro* were used for the qPCR experiments.Transduction of T cells for GFP expression was performed using a retroviral vector construct as described by Flugel et al. (1999). GFP+ T cells were selected with G418 (PAA, Coelbe, Germany) and propagated as described (Wildner and Diedrichs Mohring, 2003).

2.8. FACS analysis of MHC-antigens and intracellular cytokines

T cells were stained with mouse monoclonal FITC-coupled anti-RT1.B (Ox-6), RT1.A (Ox18, both AbD Serotec/Morphosys, Düsseldorf, Germany), FITC-coupled anti-RT1.D (Ox-17, BD Pharmingen, Wiesbaden, Germany) antibodies as described (Wildner et al., 1996). For intracellular cytokine analysis, T cells were stimulated with 1 μg/ml Concanavalin A (Sigma–Aldrich, Deisenhofen, Germany) for 2 days, followed by incubation with 50 ng/ml PMA (phorbol myristate acetate), 1 μg/ml Ionomycin and 1 μg/ml Brefeldin (all from Sigma–Aldrich, Deisenhofen, Germany) for 4 h.

Surface staining for TCR was performed with FITC-conjugated anti-TCRαβ (Cedarlane Laboratories, Burlington, Canada) or PE-conjugated anti-TCRαβ (AbD Serotec, Düsseldorf, Germany). After fixation and permeabilization of cell membranes with Fix&Perm (ADG, Kaumberg, Austria) for 15 min, intracellular cytokines were stained with PE-conjugated anti-rat IFN-γ and/or FITC-conjugated, rat-crossreactive anti-mouse IL-17 antibodies (both BioLegend, San Diego, USA).

2.9. Histology of rat eyes following transfer of GFP+ T cells

Tissue collected for histology was fixed in 4% paraformaldehyde in PBS and stored overnight at 4 °C in the dark. After 24 h tissue was transferred to PBS/15% sucrose for an additional 24 h. Then specimens were embedded in TissueTec OCT compound (Sakura, Zoeterwoude, Netherlands) and snap frozen in methyl butane at −70 °C. Cryosections were directly embedded with Immuno Fluor mounting medium (ICN, Eschwege, Germany) and viewed using phase contrast fluorescence microscopy.

2.10. Statistical analysis

Statistical analyses were performed using Graph Pad Prism 4.03. For pPCR and cytokine secretion data, a non-parametric Mann–Whitney *U* analysis was performed comparing both groups. *p*-values less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. Transcriptomic profiling of R14 and PDSAg responsive CD4+ lines

CD4+ T cells specific for peptide PDSAg cause monophasic disease, while R14-specific CD4+ T cells lead to relapsing EAU (Fig. 1). This is seen irrespective of whether the rats are immunized with the peptide in CFA, or receive adoptively transferred T cells. The R14-mediated relapses are generally less severe and unpredictable with respect to onset and intensity.

To characterize the differential pathophysiology underlying the R14- vs. PDSAg-specific CD4+ effector T cells, transcriptomic analysis was performed using Affymetrix rat genome chips (28,000 genes). Three T cell lines of each antigen specificity were harvested 2 days after restimulation with antigen and irradiated APC *in vitro*. Each individual T cell line was generated from pooled lymph node cells of four rats immunized with PDSAg or R14 in CFA. The individual T cell lines originated from different animals and were independently generated using the identical protocol. After restimulation (APC were thymocytes, irradiated with 20 Gy), the APC were removed from the cell cultures using a Ficoll density gradient centrifugation and the remaining T cells were pelleted and stored at −80° C until RNA preparation. The T cell lines representing each antigen specificity (PDSAg or R14) were harvested after their second restimulation *in vitro*.

The arrays were analyzed for differential gene expression using the Genomatix GmbH ChipInspector software platform (Cohen et al., 2008). Initial analysis identified 35 genes that were more than twofold increased in R14-specific as compared to the PDSAg-specific T cells, while no genes were found to be significantly reduced in R14-specific T cell lines relative to the PDSAg-lines. The regulated genes were then analyzed by Gene Ontology (GO) categories. The genes were subgrouped into GO categories listed as: antigen processing and presentation, lymphocyte activation, adaptive immune response, T cell activation and regulation of apoptosis (Table 1).

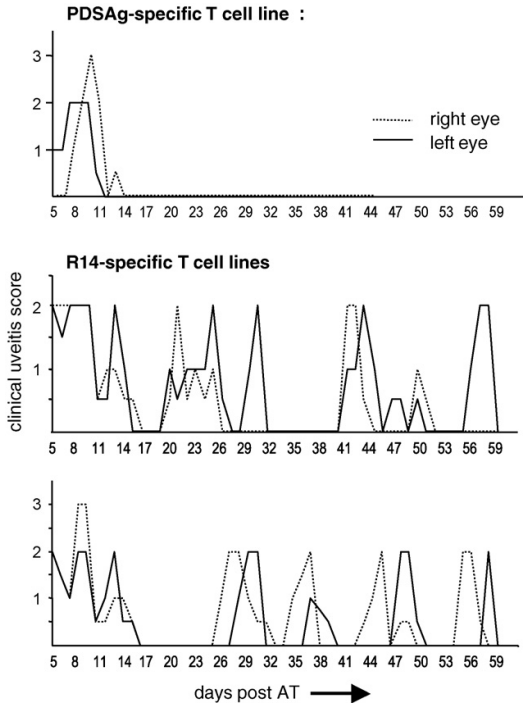

Fig. 1. Relapsing and monophasic course of EAU. Monophasic or relapsing EAU after adoptive transfer of 2.5×10^6 PDSAg- (upper panel) or R14-specific T cell lines (lower two panels). Disease courses from both eyes of three representative animals are shown.

Because the genomic coverage of the array was incomplete, an expanded group of genes from the overrepresented GO categories not annotated to the array were quantified using qPCR. In addition, mRNA levels of the differentially expressed genes identified on the DNA arrays were also verified by qPCR. These qPCR experiments were performed over an expanded series of mRNA samples representing both T cell lines. Representative results from qPCR are shown in Fig. 2 (for additional results see supplement).

Out of a total pool of 110 genes tested by qPCR, 26 showed increased expression of at least twofold in R14-specific vs. PDSAg-specific T cells (Table 2 and Fig. 3) and of these, 10 genes were deemed significantly regulated. None of the differentially regulated genes were increased in the PDSAg-specific T cell lines (Table 2 and Fig. 3). According to the GO categorization, the significantly regulated genes were found to be distributed among groups of genes related to the Wnt/Hedghog signaling pathways, antigen presentation, secretion and cytokine production, as well as Foxp3, which in rats and humans is a marker of activated effector T cells rather than specific for regulatory T cells.

3.2. Differential expression of genes related to antigen presentation

As seen in human T cells, Foxp3 and MHC class II antigens are associated with the activation of rat CD4+ T cells (Allan et al., 2007; Gansbacher and Zier, 1989; Taams et al., 1999; Wong et al., 2002). Expression of MHC class II antigen RT1.Da, the restriction element of peptide R14, was significantly increased in the R14-specific T cells. In addition, the MHC class II invariant chain CD74 (Cd74), and Cathepsin H (Ctsh), that degrades CD74 and releases CLIP, and

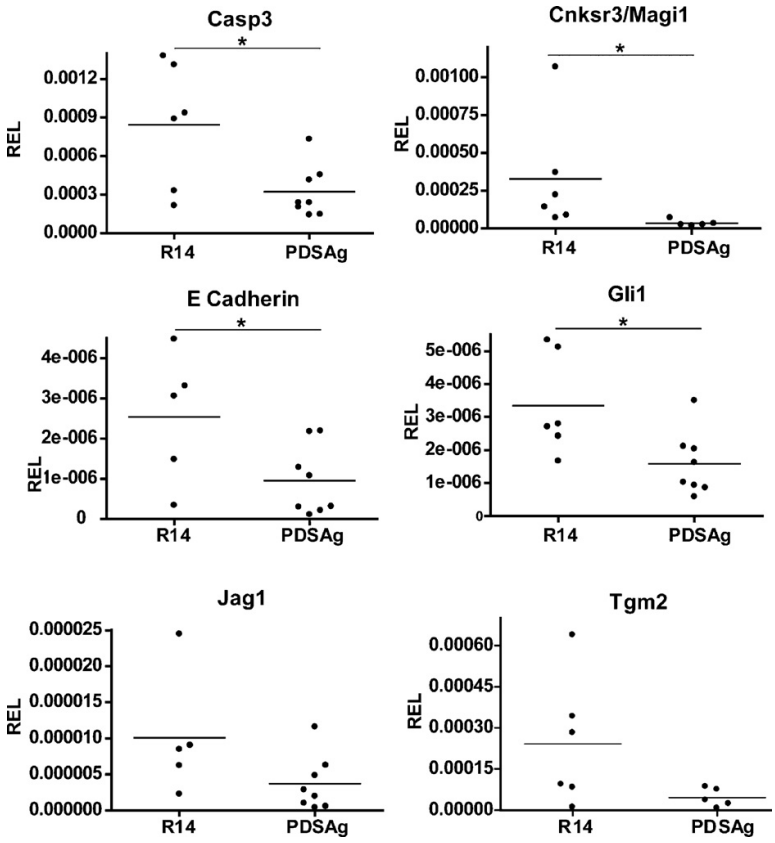

Fig. 2. qPCR verification of gene expression: WNT/HH. Through qPCR, expression of genes was assessed for both experimental groups. Data was generated employing qPCR as described in Section 2 and displayed relative expression levels (REL). For statistical analysis, the Mann–Whitney *U* non-parametric test was applied to analyze the differences between the R14 and PDSAg groups. Significant differences are denoted by asterisks * which denote statistically significant differences with a p-value < 0.05.

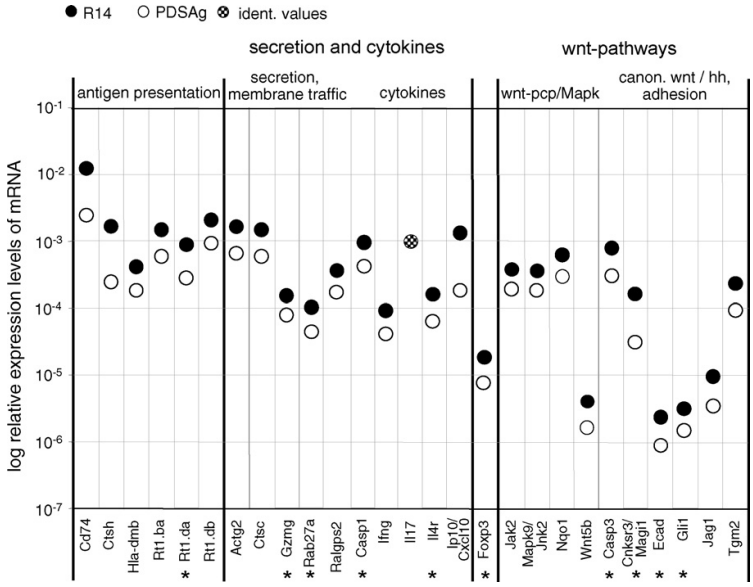

Fig. 3. Mean relative expression levels of mRNA. Mean relative expression levels (log) of mRNA from 6 to 8 different T cell lines specific for PDSAg or R14. Asterisks mark significant differently expressed genes (p < 0.05, non-parametric Mann–Whitney *U* test). For more detailed qPCR data see supplement.

Table 1
GO categories of regulated genes (Microarray).

Term	ID	Total	Observed	Expected	Z-Score
Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	GO:0002504	19	7	0.12	20.06
Oligopeptide transport	GO:0006857	16	5	0.10	15.56
Antigen processing and presentation of exogenous peptide antigen	GO: 0002478	11	4	0.07	15.06
Antigen processing and presentation of exogenous antigen	GO:0019884	16	4	0.10	12.39
Antigen processing and presentation of peptide antigen	GO:0048002	49	6	0.31	10.35
Adaptive immune response	GO:0002250	86	6	0.54	7.51
Lymphocyte mediated immunity	GO: 0002449	88	6	0.55	7.41
Lymphocyte activation	GO: 0046649	170	8	1.06	6.81
Apoptosis	GO:0006915	633	16	3.95	6.26
Regulation of apoptosis	GO:0042981	480	13	3.00	5.93
T cell activation	GO:0042110	117	5	0.73	5.04
Regulation of MAP kinase activity	GO:0043405	79	4	0.49	5.03

The GO analysis was performed using the BiblioSphere software (Genomatix, Munich). GO-groups demonstrating an over-representation of certain genes, as demonstrated by a *Z*-Score > 5, are summarized in this table. The *Z*-Score is calculated out of the expected gene expression over the observed gene expression. ID: Gene Ontology number; Total: number of genes affined to the according group; Observed: genes observed to be expressed/regulated; Expected: probability of the gene to be expressed/regulated.

Hla-dmb, which removes CLIP to enable peptide binding, were also increased on the RNA level in the R14-specific T cells (Table 2 and Fig. 3).

Increased expression of MHC class II in the R14 CD4+ T cells was verified by FACS analysis. As shown in Fig. 4, 98–99% of all T cells from both lines expressed MHC class I antigen (RT1.A), while MHC class II antigens (RT1.B and RT1.D) were found on the surface of approximately twice as many R14-specific (39.6% RT1.B+ and 21.5% RT1.D+, R14-spec. cells) than PDSAg-specific T cells (21% RT1.B+ and 13.4% RT1.D+).

3.3. Genes related to membrane traffic and secretion

The formation of secretory vesicles in the cytoplasm and their traffic to and fusion with the cell membrane are important in the secretion of cytokines and chemokines and thus, CD4 T cell effector function. The increased expression of RalA and Rab27 are suggestive of potential regulation of cytokine and chemokine secretion (Holt et al., 2006). Ifng and Ip10/Cxcl10 were upregulated in R14-specific T cells. IFN-γ was also secreted in significantly higher amounts by R-14-specific cells compared to PDSAg-specific lines.

Table 2
Genes upregulated in R14-spec. T cell lines compared to PDSAg-spec. T cells (qPCR).

Gene identity	Fold change mRNA R14/PDSAg	Description of function
Cnksr3/Magi1*	9.4	Binds β-Catenin, which can translocate to the nucleus to act as a transcription factor. Binds to Notch ligand Dll1 (Dll1 not tested)
Ctsh	6.7	Degrades CD74 and releases CLIP ⇒ antigen presentation
Foxp3*	5.8	Expressed in activated T cells (human, rat) and in Tregs
Gzmg*	5.0	Concomitantly upregulated with IFN-γ
Cd74	5.0	Peptide loading of MHC class II molecules, also receptor for MIF (macrophage migration inhibition factor); suppresses p53-dependent apoptosis, increases inflammation and promotes Th1 responses
Ip10	4.2	Is induced by IFN-γ and also induces production of IFN-γ, binding to its receptor CXCR3 induces JAK2; Th1-spec. chemokine
Rt1-Da*	3.2	α-Chain of rat MHC class II, RT1.D (HLA-DR equivalent)
ECad*	2.7	Cell adhesion molecule of tight junctions, associated with β-Catenin
Jag1	2.7	Notch ligand
Casp3*	2.6	Cleaves E-Cadherin and Cnksr3/Magi1 to release β-Catenin
Actg2	2.5	Cytoskeleton, linked with E-Cadherin via β-Catenin
Il4r*	2.5	Expressed on Th1 7 cells, used for downregulation
Tgm2	2.5	G-protein and cell surface adhesion mediator, induced by IFN-γ
Rt1-Ba	2.4	α-Chain of rat MHC class II, RT1.B (HLA-DQ equivalent)
Wnt5b	2.4	Member of the WNT–PCP pathway, induces expression of Mapk9/Jnk2 ⇒ activation of NFκb ⇒ protection from apoptosis
Hla-dmb	2.3	Removes CLIP to enable peptide binding
Rab27a*	2.3	Small membrane-bound GTPase, controls secretion of cytoplasmic granules
Ifng	2.2	Interferon-gamma, T helper 1-specifc cytokine
Rt1-Db	2.2	β-Chain of rat MHC class II, RT1.D (HLA-DR equivalent)
Gli1*	2.1	Induces transcription, mediates cell proliferation and differentiation
Nqo1	2.1	Detoxifying enzyme, prevents from apoptosis. Induced through AP1 via MAPK9/JNK2
Ralgps2	2.1	Guanin exchange factor for RAL: RALA ⇒ RAL-GTP, which binds to actin filaments via filamin
Casp1*	2.0	IL-1 β-converting enzyme, also processing pro-IL-18 to its active form ⇒ promotes IFN-γ production/Th1 responses
Jak2	2.0	JAK/STAT pathway: induced by cytokine/chemokine receptor signaling, promotes Th1 responses
Mapk9/Jnk2	2.0	Important for Th1 responses, induced by CD74 via JAK2. Induces gene expression of NQO1 via AP1-induction, activation of NFκb ⇒ protection from apoptosis
Ctsc	2.0	Activates Granzyme G

Actg2: Actin gamma2; Casp1: Caspase1; Casp3: Caspase3; Cnksr3/Magi1: Connector enhancer of kinase suppressor of ras 3/Membrane-associated guanylate kinase-interacting protein-like 1; Ctsc: Cathepsin C; Ctsh: Cathepsin H; ECad: E-Cadherin; Foxp3: Forkhead boxp3; Gli1: Glioma-associated protein 1; Gzmg: Granzyme G; Ifng: Interferon-gamma; IL4R: Interleukin-4 receptor; IP10: Interferon-inducible protein 10; Jag1: Jagged1; Jak2: Janus kinase 2; Mapk9/Jnk2: Mitogen-activated protein kinase 9/Jun-kinase 2; Nqo1: NAD(P)H:quinone oxidoreductase 1; Rab27a: Ras-related protein Rab27a; Ralgps2: Ral GEF with PH domain and SH3 binding motif 2; Tgm2: Transglutaminase2; Wnt5b: Wingless and Integrase 5b. For detailed qPCR data see supplement.

* Significantly regulated.

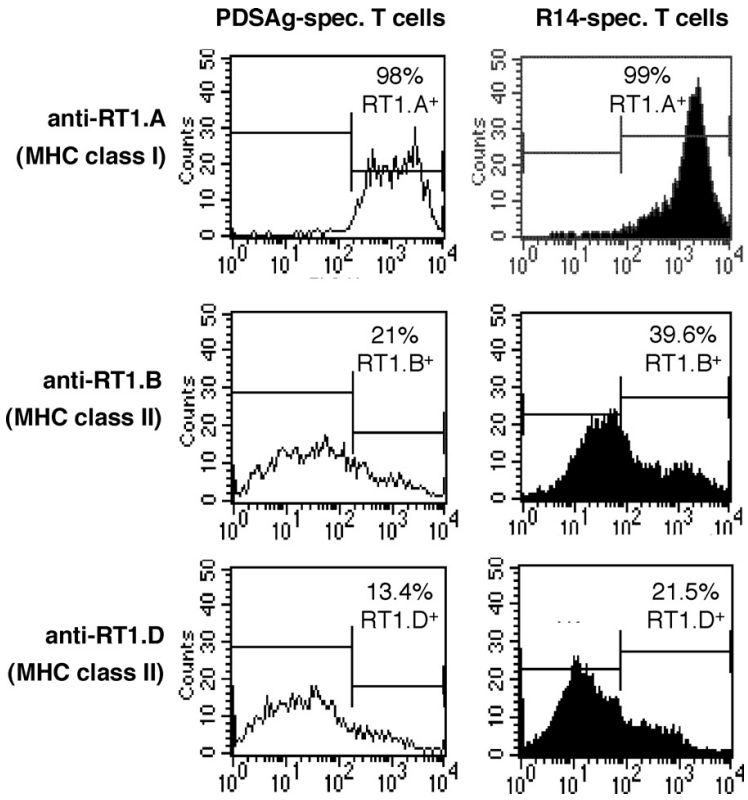

Fig. 4. FACS analysis: Differential MHC class II antigen expression. Surface staining of T cell lines with FITC-labeled rat MHC class I and class II-specific antibodies. Windows were set according to the RT1.A (MHC class I)-staining with almost 100% positive cells.

The cytokine expression signature was further characterized by Bioplex analysis. PDSAg- and R14-specific T cell lines restimulated *in vitro* with their respective antigens at least two times were evaluated for their secretion of select cytokines (IL-2, IL-4, IL-10, IL-17, IFN-γ and TNF-α). Over 3 days of antigen stimulation, culture supernatants were collected every 24 h and pooled in equal parts for multiplex cytokine assays. The results shown in Fig. 5A represent the average cytokine content in supernatants from seven

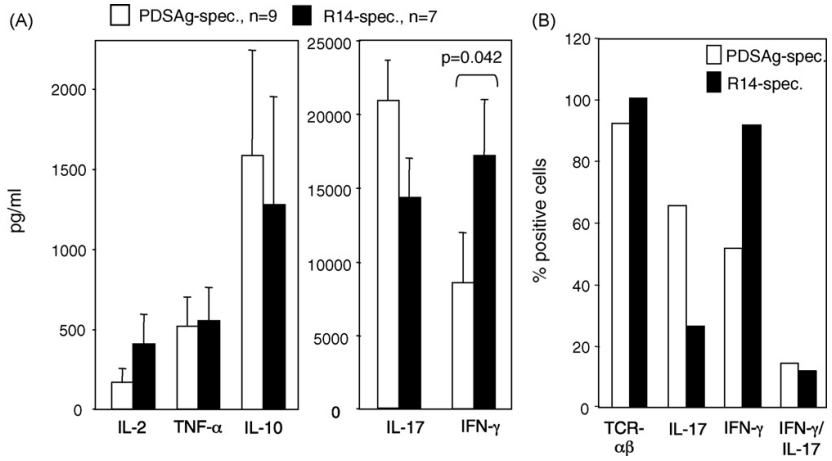

Fig. 5. Secreted and intracellular cytokines. (A) Pooled supernatants of 3-day cultures of specific antigen stimulation from PDSAg- and R14-spec. T cell lines were tested for cytokine contents. Mean values of 7 different PDSAg and 9 different R14-specific lines are given. Columns show pg cytokine in cultures with antigen – pg cytokine in cultures with medium only + SE. Only IFN-γ secretion was significantly different ($p = 0.042$) between PDSAg- and R14-specific T cell lines. (B) FACS analysis of surface (TCRαβ) and intracellular staining (IFN-γ and IL-17) of representative T cell lines specific for PDSAg and R14 (both 6th cycle of in vitro restimulation). Columns show the percent of cells expressing the respective markers.

to nine independent T cell lines. High levels of the inflammatory cytokines IL-17 and IFN-γ were measured in supernatants from both types of T cell lines. Interestingly, the monophasic PDSAg-specific T cell lines showed higher secretion of IL-17, which was not reflected by differentially regulated expression at the mRNA level (Figs. 3 and 5A, respectively). The R14-specific T cell lines secreted significantly higher levels of IFN-γ and IL-2 as compared to the PDSAg-specific T cell lines, while there was no difference in the secretion of TNF-α or IL-10. The same pattern of IFN-γ and IL-17 expression by PDSAg and R14-specific T cells was observed by intracellular staining for cytokines as shown in Fig. 5B using maximally (ConA) stimulated T cell lines. A small subpopulation of about 10–14% IFN-γ/IL-17 double positive cells were identified. After stimulation with antigen the percentage of double positive cells increased, but there was no difference in intracellular IFN-γ or IL-17 expression seen between the T cell lines (data not shown). IL-4 expression was not detected in either line (data not shown), but the R14-specific T cell lines did show increased Il4r mRNA expression.

Granzyme G (Gzmg) and cathepsin C (Ctsc) are associated with cytotoxic granules. Gzmg is activated after cleavage by Ctsc (Casey et al., 2007). Expression of both genes was significantly increased in the R14-specific T cells.

3.4. WNT, MAPK and Hedgehog pathways are altered in monophasic vs. remitting EAU

The MAPK signal transduction pathway couples intracellular responses to the binding of growth factors to their cell surface receptors. Genes linked to the MAPK pathway were found to be increased in the R14-specific CD4+ T cells. These include Jnk2/Mapk9 (MAPK pathway), Jak2 (JAK/STAT pathway), Ip10/Cxcl10 and Nqo1 (Fig. 3). WNT pathway signaling regulates T cell differentiation and can support the development of the long-lived self-renewing populations of memory T cells that are central to an efficient autoimmune T cell response (Gattinoni et al., 2009). The WNT/planar cell polarity signaling pathway (WNT–PCP) helps control cell movement. The Hedgehog (HH) pathway, which directly interacts with the WNT pathway, has been shown to modulate T cell receptor signal strength in mature T lineage cells (Rowbotham et al., 2007). Wnt5b is generally associated with the WNT–PCP pathway, five more of the differentially expressed genes (ECad, Cnksr3/Magi1, Jag1, Gli1, Casp3) are linked to the canonical WNT and Hedgehog signaling pathways (Fig. 3). Casp3 was verified to be upregulated on the protein level by western blot (not shown). E-cadherin (increased in R14-spec. T cells) is a transmembrane protein, which plays a role in cell adhesion. The cytoplasmic domain of ECad and Cnksr3/Magi1 (both significantly upregulated on the mRNA level in R14-spec. T cells) are linked via β-Catenin (Dobrosotskaya and James, 2000; Orsulic et al., 1999). As detailed in Table 2, Cnksr3/Magi1 showed the highest upregulation of all investigated genes (9.4fold). Cnksr3/Magi1 is a member of the membrane-associated guanylate kinase homologue (MAGUK) family that participates in the assembly of multiprotein complexes on the inner surface of the plasma membrane at regions of cell–cell contact.

The significantly increased glioma-associated oncogene homolog 1 (Gli1, Figs. 2 and 3) is a zinc finger transcription factor linked to the HH signaling pathway. HH activation has been associated with suppression of apoptosis. Gli1 is activated by the SHH (sonic hedeghog). SHH signaling enhances expression of IL-2, IL-10 and IFN-γ by CD4+ cells (Stewart et al., 2002). HH is also involved in the proliferation of activated CD4+ T cells (Lowrey et al., 2002). Fig. 2 shows the results of qPCR verification of Wnt/Hh gene expression on individual cell lines.

3.5. R14 responsive cells show increased stability in vivo

The genes found to be increased in the R14-specific T cells can be linked to regulatory events associated with the development of memory phenotypes, development of effector function and resistance to apoptosis. To evaluate the T cells mediating monophasic vs. relapsing disease in an *in vivo* setting, GFP+ CD4+ T cell lines specific for each antigen (PDSAg and R14) were used in adoptive transfer studies to follow the long term fate of the respective effector T cells in the rat.

Thirty-eight days after transfer, and 30 days after the initial disease course, GFP+ T cells of both specificities could be detected in the retinas. However, the number of GFP+ R14-specific T cells (Fig. 6B) was found to be higher than that of PDSAg-specific GFP+ T cells (Fig. 6A). Eyes obtained during a relapse after adoptive transfer of GFP+ R14-specific T cells showed clusters of green fluorescent cells in the ocular tissues, suggesting local expansion or increased survival of autoreactive T cells that have remained within the eye (Fig. 6C).

4. Discussion

The remitting course of autoimmune disease is well recognized in patients, but the underlying mechanisms contributing to this phenomenon are not understood. It has been proposed that remit-

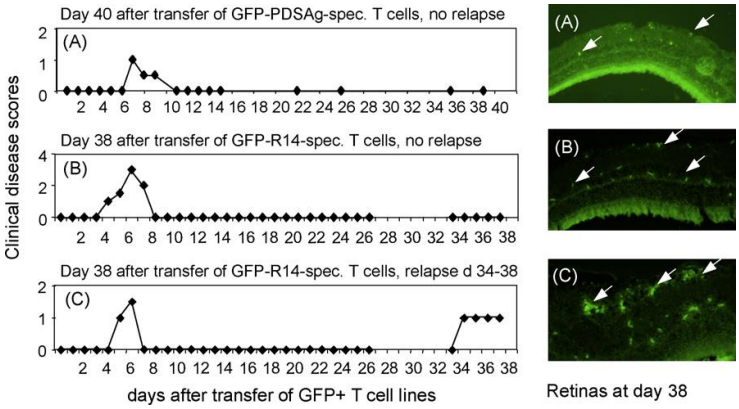

Fig. 6. Intraocular survival of autoreactive T cells. 2.5×10^6 GFP+ PDSAg- (A) or R14-spec. T cells (B, C) were transferred to naive Lewis rats. Clinical courses of EAU and cryosections of rat retinas at day 38 post T cell transfer are shown for the same eyes. GFP+ T cells in histologies are marked with white arrows. (A) monophasic EAU, PDSAg-specific T cells; (B) monophasic EAU, R14-specific T cells; (C) recurrent EAU, R14-specific, GFP+ T cells form clusters (arrows).

ting disease may derive from either the reactivation of memory cells, or the induction of new autoreactive cells. The latter process is known as epitope spreading and is frequently observed during recurrences of autoimmune diseases, while the response to the original autoantigen is generally conserved. Inherent mechanistic processes, unique to the T cell phenotype elicited may also play a role in this process.

The model of EAU in the Lewis rat allows a direct comparison of T cell lines leading to monophasic vs. relapsing/remitting autoimmune phenotypes (Diedrichs-Mohring et al., 2008). Importantly, Treg cells have not been identified as a causative agent underlying the monophasic vs. relapsing phenotypes in this disease. Using transcriptomic profiling the potential differences between the autoreactive T effector cells of both antigen specificities was investigated. An increased expression of a set of genes in the T cells causing relapsing/remitting EAU were identified, which appear to account for the increased activation, survival, and enhanced Th1 cytokine production seen in the remitting T cells. Where possible, the differences in expression were examined at the protein level, which was limited by the availability of antibody reagents for the rat.

Class II antigen expression by T cells is found in humans and rats. Increased expression of MHC class II in the R14-specific T cells (mRNA and protein) suggests that they may have enhanced antigen presentation capacity, and thus, the potential ability to propagate an antigen response. Antigen presentation by T cells is thought to lead to anergy and the downregulation of an immune response – a feature of regulatory T cells. The effector cells in the EAU model are CD4+ CD25+ T cells. Importantly, these are not Tregs, but rather are highly activated pathogenic cells showing prolonged survival *in vivo*. CD74 was also found to be increased in the R14-specific T cells. It facilitates peptide loading to MHC class II antigens, but can also be found on the cell surface where it is the MIF (macrophage migration inhibition factor) receptor. Interaction of MIF with CD74 has been shown to help suppress T cell apoptosis, increase inflammation and promote Th1 responses by inducing IFN-γ and the expression of JUN-kinase2/MAP-kinase9 (JNK2/MAPK9) (Leng et al., 2003; Yang et al., 1998).

Both, R14- and PDSAg-specific T cell lines show characteristics of Th1 as well as Th17-like cells. Cytokine secretion and qPCR data suggest that R14-specific T cells appear more Th1-like, while PDSAg-specific T cells, which induce monophasic EAU, show increased numbers of IL-17-producing cells and result in increased secretion of IL-17 (although there was no difference in the expression of Il-17 mRNA). Intracellular staining and FACS analysis additionally demonstrated that both T cell lines included a small subpopulation of TCRαβ$^+$ IFN-γ/IL-17 double positive cells with unknown function. This observation suggests a shift to Th1 cells in eyes of rats with IRBP peptide-induced recurrent EAU with a concomitant decrease in Th17 cells. Nevertheless, the mechanism of reactivation of Th1 cells and their reinduction of inflammation is not understood. IFN-γ production is known to be enhanced by Caspase 1, the IL-18 converting enzyme. IFN-γ induces Ip10/Cxcl10 gene expression (Sarris et al., 1995), and binding of CXCL10 to its receptor CXCR3 causes JAK2 phosphorylation, which links CXCL10 to MAPK and WNT–PCP pathways.

Many of significantly regulated genes found in R14-specific T cell lines are associated with the WNT/HH signaling pathways. Although originally identified in embryonic development, these pathways also play important roles in the adaptive immune system. The canonical WNT pathway promotes hematopoietic stem cell self-renewal and multipotency by limiting stem cell proliferation and differentiation. Binding of WNT proteins to their receptor complexes leads to an accumulation of nuclear β-Catenin, which promotes gene transcription (Staal et al., 2008). WNT signaling helps maintain the 'stemness' of memory CD8+ T cells (Gattinoni et al., 2009). The Hedgehog signaling pathway is linked to WNT signaling and acts as an important regulator in the thymus, where it controls differentiation, survival and proliferation in the early stages of T cell development. HH is also important in peripheral T cell activation. Modulation of TCR signal strength by Hedgehog pathway activation is thought to be important for immunity as the presence or absence of Hedgehog signaling in the environment in which a T cell is activated is thought to help shape the immune response (Crompton et al., 2007).

CNKSR3/MAGI1 interacts with actin-bundling proteins, potentially playing a role in actin cytoskeleton dynamics (Patrie et al., 2002). CNKSR3/MAGI1 has nuclear localization signals and it has been suggested that it may help transmit signals from the cell surface to the nucleus (Dobrosotskaya et al., 1997). ECAD and CNKSR3/MAGI1 can be cleaved by Caspase 3 that was also found to be significantly increased in the R14-specific cells. Cleavage of either protein, ECAD or MAGI1, results in the release of β-Catenin, which then translocates to the nucleus to act as a transcription factor. Casp1 can activate Casp3, which is involved in apoptosis or anergy (Puga et al., 2008), but also in early steps of T cell activation (Alam et al., 1999). Gene expression of both caspases is significantly upregulated in R14-specific T cells. Tgm2 (transglutaminase 2) is induced by IFN-γ (Kim et al., 2002) and can be involved in apoptosis as well as protect from it (Kim, 2006). The increased expression of Caspase 3 in relapsing/remitting disease seen here may not initiate apoptosis, but rather be linked to anergy (Puga et al., 2008) supporting the premise that anergized T cells with prolonged survival (potential memory cells) might remain in the eye and induce a relapse after reactivation.These results suggest that the differential activation of these pathways seen between the two effector T cell lines may underlie the subtle differences in the effector phenotypes studied here. R14-specific T cell lines showed increased expression of a series of genes that should enable them to help prevent apoptosis, potentially resulting in prolonged survival in the target organ eye. This was verified *in vivo* where adoptive transfer of labeled T cells demonstrated increased survivability of the R14 cells in rat eyes. In addition, the differential expression of genes linked to the cytoskeleton, cell motility and cell adhesion suggests an enhanced ability of these cells in tissue immigration as well as increased cellular synapse formation and secretion of cytokines and chemokines. Importantly, genes associated with the WNT and HH signaling pathways which have been linked to T cell development were also increased suggesting that these features are important for recurrent autoimmune reactions. These results suggest that recurrent autoimmunity may originate to a degree in the differentiative state of the autoreactive T cell.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2010.07.017.

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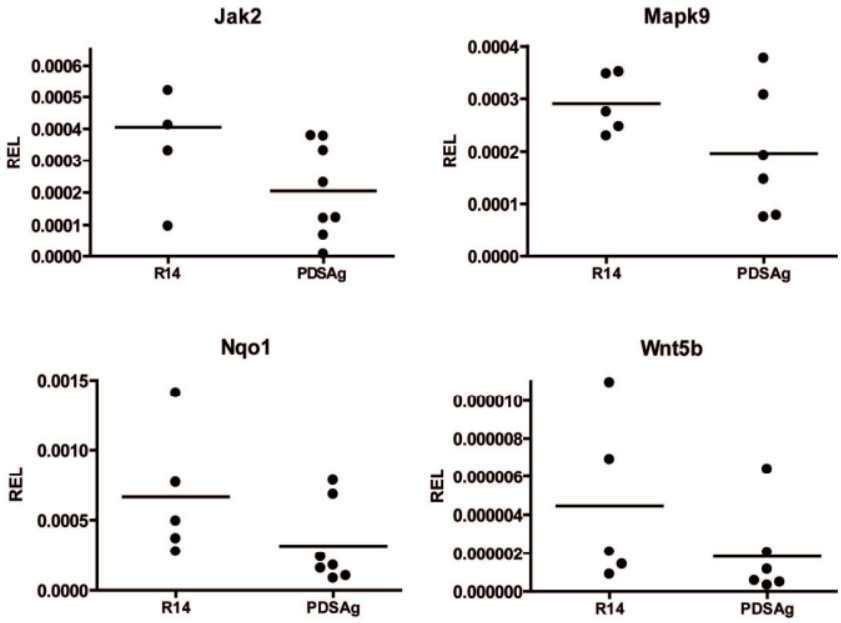
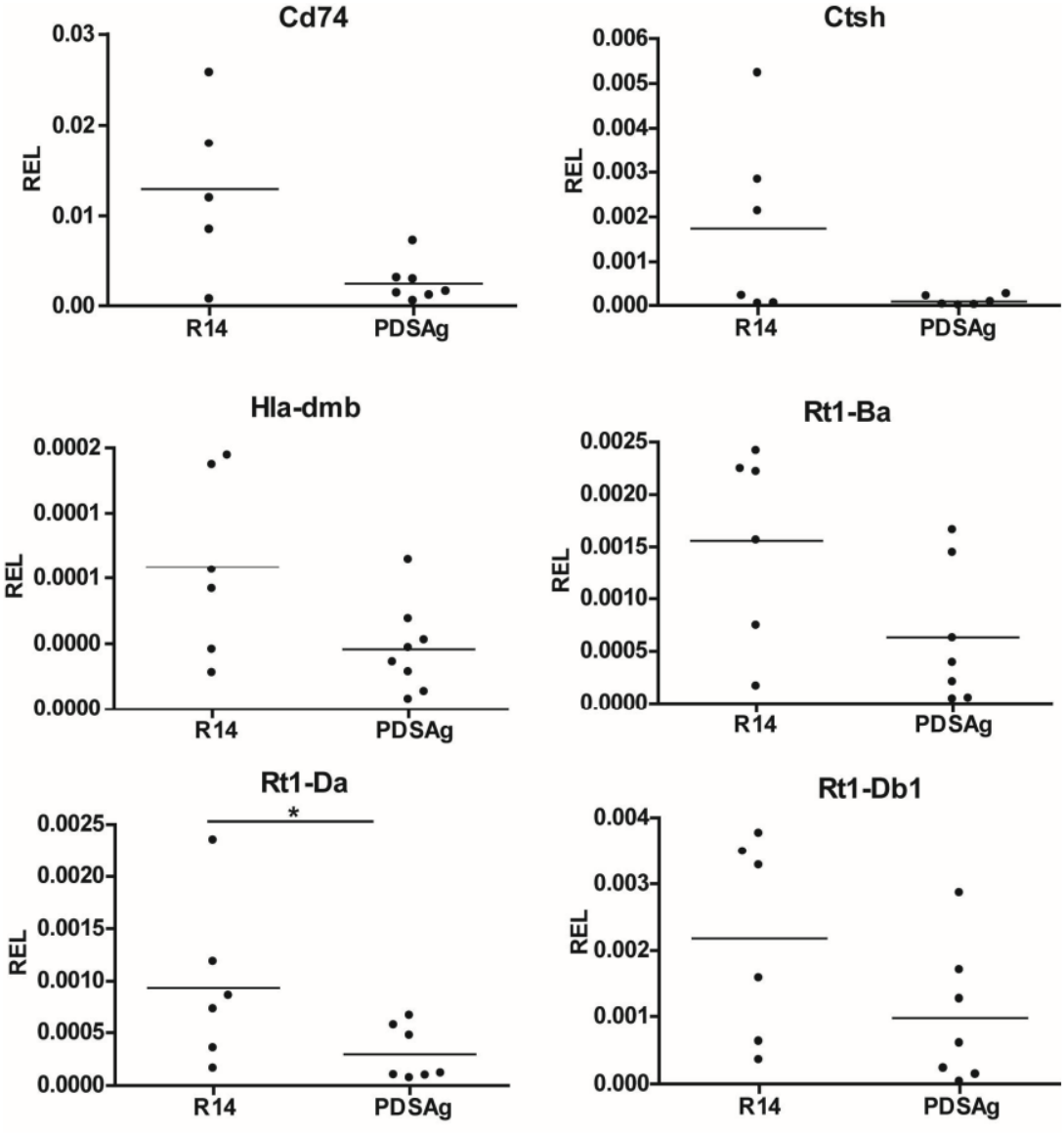
Supplement: qPCR WNT-PCP pathway

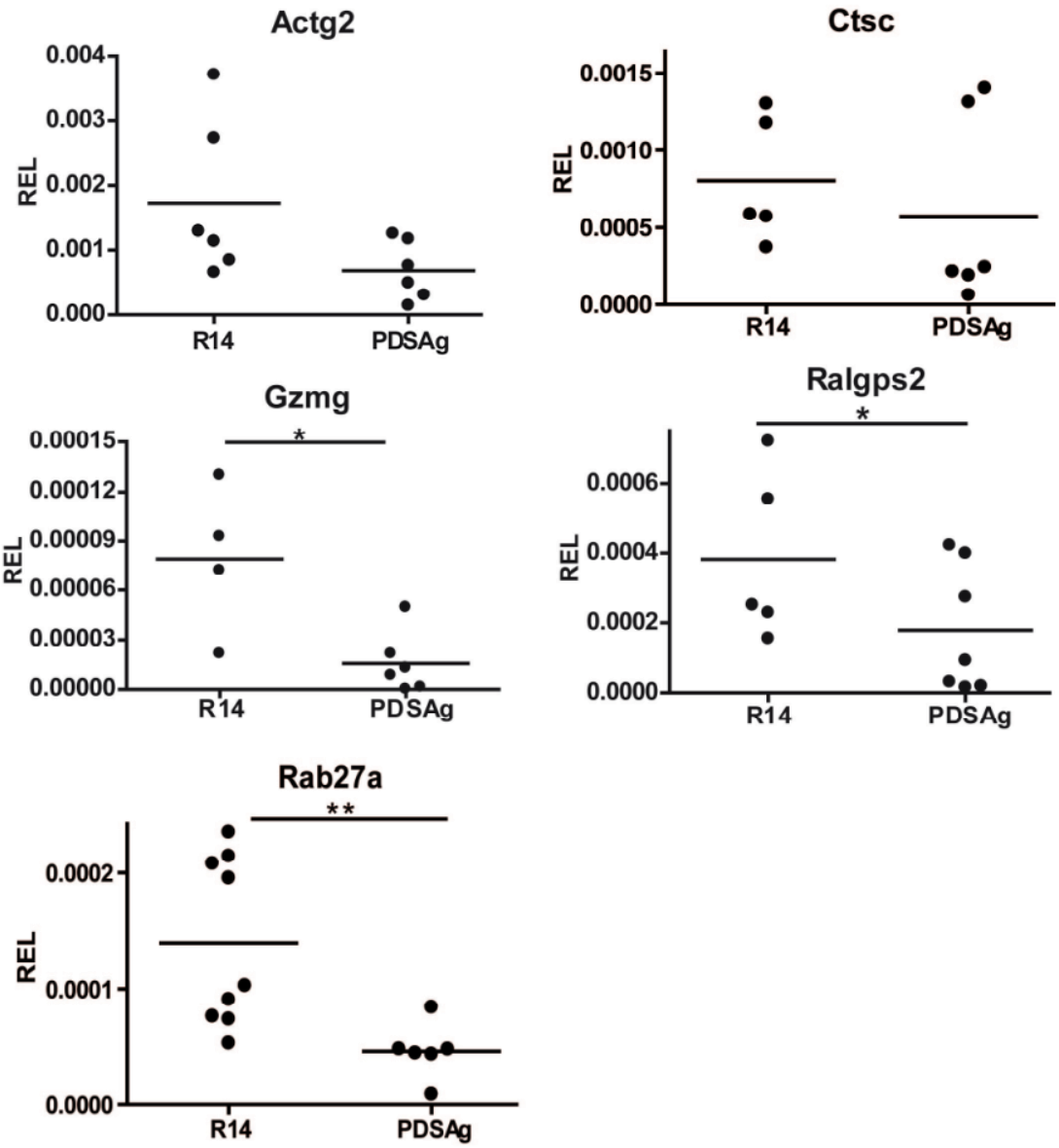
Expression of genes was assessed by qPCR for both experimental groups.
Data were obtained as described in Material and Methods and are presented as relative expression levels (REL). For statistical analysis, the Mann Whitney-U non-parametric test was applied to analyze the differences between the R14 and PDSAg groups. Significant differences are denoted by asterisks * which denote statistically significant differences with a p value < 0.05



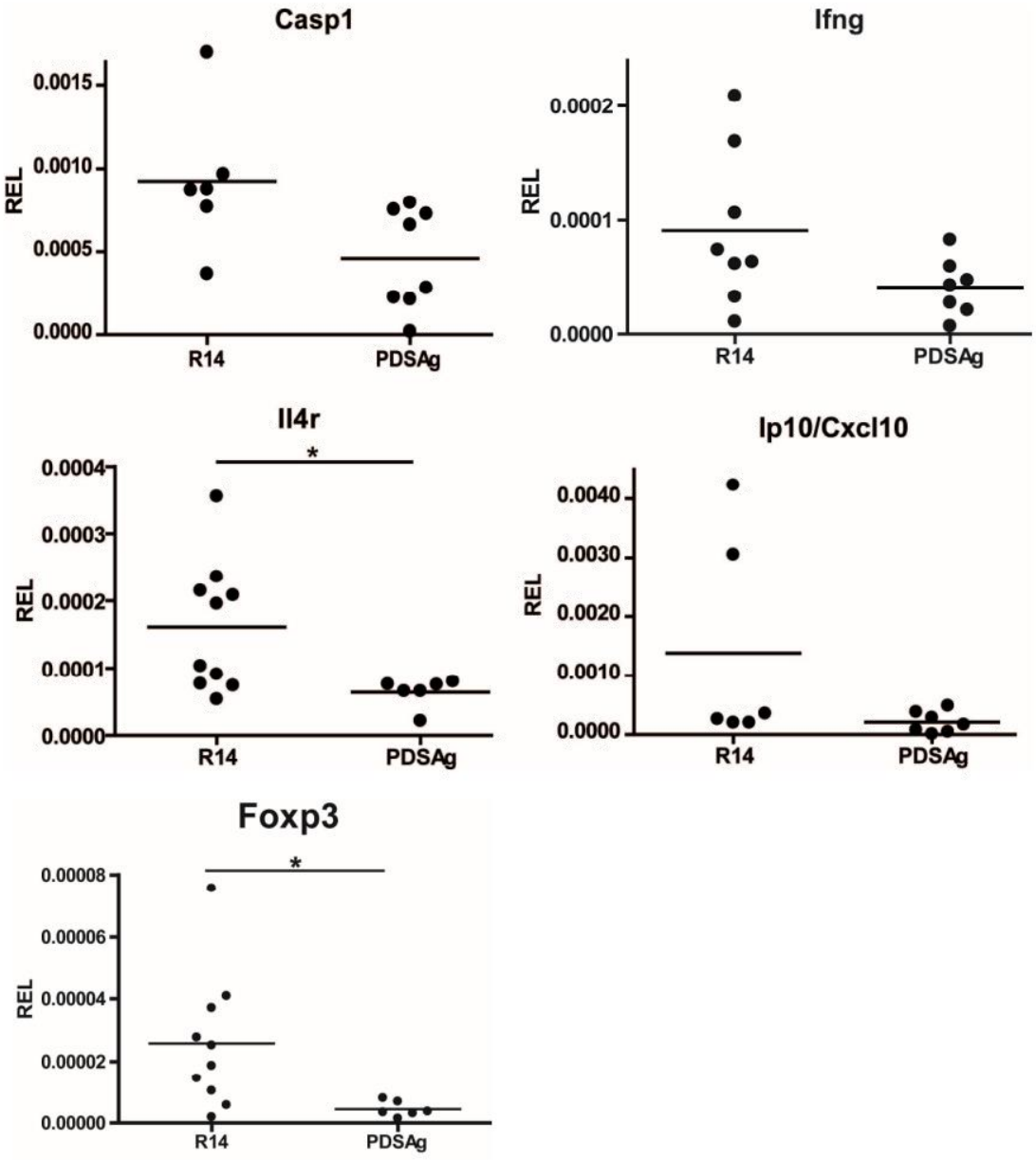
Supplement: qPCR Ag presentation pathway



Supplement: qPCR Secretion pathway



Supplement: qPCR Cytokines and Foxp3



Supplement: List of primers

All Primer pairs

Assays designed

Gene ID	Gene Symbol	Sense (5'->3')	Antisense (5'->3')	Probe
25712	Ifng	AACAGTAAAGCAAAAAAGGATGCATT	TTCATTGACAGCTTTGTGCTGG	CGCCAAGTTCGAGGTGAACAACCC

SYBR green primers on demand

Gene ID	Gene Symbol	Assay ID
83502	ECad	QT00176288
25166	Casp1	QT00191814
25402	Casp3	QT00186333
25599	Cd74	QT00189049
140589	Gli1	QT001586662
294273	Hla-dmb	QT001579648
29146	Jag1	QT00193424
24514	Jak2	QT00189182

SYBR green primers designed

Gene ID	Gene Symbol	Sense (5'->3')	Antisense (5'->3')
25365	Actg2	GGGATCCTGACCCTCAAATAC	AGGAGTGGTGCCAGATCTTCT
308113	Cnksr3/Magi	CTCTGCGCACTGAATTATGG	TGCAGATTATGGGAGGATGC
25423	Ctsc	GATCCTAAGGCCCAAACCTG	ACGGACGTTTCTCCAGTCC
25425	Ctsh	ATAGGCAAGAATGGTCAGTGC	CCTCATCATTGAGTGTGATGTTG
317382	Foxp3	GCCACCTGGGATCAATGT	GTGTACCTGAGCGTGGGAAG
266704	Gzmg	CACTGCACTGGAAGATCAATG	AGCCCTTTTCACAGGGATG
25084	Il4r	ATCTGCATGGTCAACATCTCC	GGGTTCCGTGTAGGTCACAT
25465	Il17	ATTCCATCCATGTGCCTGAT	AGTACCGCTGCCTTCACTGT
3627	Ip10/Cxcl10	GCGGTGAGCCAAAGAAG	CAGGAGAAACAGGGACAGTTAGG
50658	Mapk9	CCAAGGAATTGTTTGTGCTG	TCACGGTAGGCTCTCTTTGC
24314	Nqo1	CATCTCTGGCGTATAAGGAAGG	AATGGGAACTGAAATATCACCAG
50645	Rab27a	CAGTACACTGATGGGAAGTTCAA	TTCGCTCTGTACACCACTCTCT
304887	Ralgps2	AAGTGAGAAGGGCTCTGAATTG	CTGACCCGCGTATTCTTCTG
309621	RT1-Ba	CCAGCTACCAACAAGGTTCC	AAAGCAGATGAGGGTGTTGG
309622	RT1-Bb	GTGATCTTCCTCGGGCTTG	TGTAGGAGCCCTGCTGGA
294269	RT1-Da	GGAAGCACTGGGAGTTTGAA	CACAAACAACCCGAGAACAC
294270	RT1-Db1	GTGCTGGGTCTGCTCTTCC	AGGAGTCCTGTTGGCTGAAG
56083	Tgm2	GCATGGTCAACTGCAATGAT	AGGCCATGGGACTGATACC
282582	Wnt5b	GCACTGGGATGGGTTGAG	AGCGACCACCAGGAGTTG

5.2 Dynamics of intraocular IFN-γ, IL-17 and IL-10-producing cell populations during relapsing and monophasic rat experimental autoimmune uveitis

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Dynamics of Intraocular IFN-γ, IL-17 and IL-10-Producing Cell Populations during Relapsing and Monophasic Rat Experimental Autoimmune Uveitis

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Abstract

A major limitation of most animal models of autoimmune diseases is that they do not reproduce the chronic or relapsing-remitting pattern characteristic of many human autoimmune diseases. This problem has been overcome in our rat models of experimentally induced monophasic or relapsing-remitting autoimmune uveitis (EAU), which depend on the inducing antigen peptides from retinal S-Antigen (monophasic EAU) or interphotoreceptor retinoid-binding protein (relapsing EAU). These models enable us to compare autoreactive and regulatory T cell populations. Intraocular, but not peripheral T cells differ in their cytokine profiles (IFN-γ, IL-17 and IL-10) at distinct time points during monophasic or relapsing EAU. Only intraocular T cells concomitantly produced IFN-γ, IL-17 and/or IL-10. Monophasic EAU presented rising numbers of cells expressing IFN-γ and IL-17 (Th1/Th17) and cells expressing IL-10 or Foxp3. During relapsing uveitis an increase of intraocular IFN-γ+ cells and a concomitant decrease of IL-17+ cells was detected, while IL-10+ populations remained stable. Foxp3+ cells and cells expressing IL-10, even in combination with IFN-γ or IL-17, increased during the resolution of monophasic EAU, suggesting a regulatory role for these T cells. In general, cells producing multiple cytokines increased in monophasic and decreased in relapsing EAU. The distinct appearance of certain intraocular populations with characteristics of regulatory cells points to a differential influence of the ocular environment on T cells that induce acute and monophasic or relapsing disease. Here we provide evidence that different autoantigens can elicit distinct and differently regulated immune responses. IFN-γ, but not IL-17 seems to be the key player in relapsing-remitting uveitis, as shown by increased, synchronized relapses after intraocular application of IFN-γ. We demonstrated dynamic changes of the cytokine pattern during monophasic and relapsing-remitting disease with strongly increasing IL-10 expression in intraocular T cells during monophasic uveitis.

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Introduction

The course of human autoimmune diseases is usually chronic or relapsing-remitting, but so far the mechanisms behind disease recurrences have not been fully elucidated. Most animal models of autoimmune diseases present a monophasic course that does not recur spontaneously, nor can it be re-induced. Thus comparison of immune reactions and regulation in relapsing-remitting and monophasic disease in the same mouse or rat strain is generally not possible and it is difficult to investigate the mechanisms underlying recurrences of autoimmune diseases, which go beyond the mere effect of regulatory T cells [1]. Moreover, since most therapies in animal models have to be applied preemptively [2], it is virtually impossible to determine the effect of therapeutic approaches in an ongoing immune response or on relapses. We have developed a model of spontaneously relapsing-remitting experimental autoimmune uveitis (EAU) in Lewis rats, which is induced by immunization with R14, a peptide from interphotoreceptor retinoid-binding protein (IRBP) in complete Freund's adjuvant (CFA) [1,3]. In contrast, the same immunization protocol with PDSAg, a peptide from retinal S-Ag, results in a strictly monophasic disease. Similar disease courses are also observed after adoptive transfer of T cells specific for these peptides. These two types of EAU in the same strain of rats enable us to directly compare the immune mechanisms that prevent or permit recurrent intraocular inflammation.

Our previous work on the characterization of T cell lines specific for PDSAg or R14, which induce monophasic or relapsing-remitting disease upon adoptive transfer, has revealed that these T cells have different characteristics [1,3]. Gene array analysis of R14- and PDSAg-specific T cell lines revealed significant upregulation of genes only in the R14-specific T cell lines. The regulated genes belong to pathways that lie upstream or downstream of IFN-γ, which is the hallmark cytokine of Th1 cells, suggesting that the R14-mediated relapsing disease might result from the effects of Th1 cells. Cytokine analysis of R14- vs. PDSAg-specific T cells indeed revealed an increased IFN-γ secretion as well as a predominance of IFN-γ-producing T cells among R14-specific T cell lines, but we also found IL-17-producing cells. [3] The function of Th17 cells in uveitis is not yet clear, and clinical trials of autoimmune uveitis that target IL-17 are in progress.

The roles of IFN-γ and IL-17 in experimental autoimmune uveitis are contradictory; in general, they were both shown to be pathogenic [4,5,6]. No special function has been allocated to cells concomitantly producing cytokines of different T helper types like IFN-γ and IL-17. They are regarded as a transition stage between Th1 and Th17 or vice versa [7]. Regulatory T cells are expected to be responsible for preventing relapses of intraocular inflammation, and thus we have looked for Foxp3- and IL-10-expressing cells in the eyes and peripheral lymph nodes during monophasic and relapsing experimental uveitis. Both cell types have been previously described as regulatory cells [8,9,10,11] in EAU [12,13]. We found fewer Foxp3+ cells among intraocular lymphocytes than among lymph node cells. In contrast, IL-10-producing cells were detected in much higher numbers in the eyes than in lymph nodes.

In this study, we investigated the intracellular expression of effector and regulatory cytokines in intraocular and peripheral T cells during the course of monophasic and relapsing EAU. We detected differences between the two types of EAU with respect to the cytokine pattern of intraocular T cells. Major differences were found with cells expressing multiple cytokines. During monophasic EAU T cells coexpressing IFN-γ and IL-17 increased, while these populations decreased during the primary course of relapsing disease. Intraocular, but not intraperitoneal injection of IFN-γ after the resolution of R14-induced EAU elicited rather synchronized relapses, underlining the role of IFN-γ for relapsing disease. IL-10-producing cells accumulated in the eyes during monophasic disease, even populations producing IL-10 concomitantly with the inflammatory cytokines IFN-γ and IL-17 were dramatically increasing during the course of monophasic uveitis. That similar numbers of Foxp3-expressing cells were found in both types of EAU indicates that Foxp3 might not be the only factor that is required to prevent relapsing uveitis.

Materials and Methods

Animals

Lewis rats were bred in our own colony or purchased from Janvier (France). They were maintained under specific pathogen-free conditions with unlimited access to water and rat chow and used for experiments at the age of 6–8 weeks. All animal experiments were approved by the Review Board of the Regierung von Oberbayern (Permit-Number 55.4-1-54-2531-80-10) and conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Induction and scoring of EAU

Animals were immunized subcutaneously into both hind legs with a total volume of 200 µl emulsion containing 25 µg peptide PDSAg (bovine S-Ag aa 342–354) or R14 (human IRBP aa 1169–1191) (Polypeptide Laboratories, France), and CFA, fortified with Mycobacterium tuberculosis strain H37RA (BD Biosciences, Germany) to a final concentration of 2.5 mg/ml. Throughout this paper active immunization is referred to PDSAg-CFA or R14-CFA, respectively. Pertussis toxin or B. pertussis was not used. The time course of disease was determined by daily examination of the anterior part of the eyes ("clinical uveitis") with an ophthalmoscope. Uveitis was graded as described [14]. In brief, 0.5: enlargement of iris vessels, 1: peripupillar infiltration of leukocytes, 2: pupil covered with fibrin clot, 3: hypopyon, 4: anterior chamber hemorrhage. "Onset of EAU" was defined as a score ≥0.5; "Peak of EAU" was defined as highest score of eyes (≥2) on two consecutive days. "Resolution of EAU" was defined as a decrease of clinical signs. "Remission of EAU" was the period with stable scores ≤0.5 between the resolution of the first attack and the first relapse. "Relapse of EAU" was defined as a score ≥1 following a period of complete absence of all clinical signs of inflammation after the first attack of disease. "Late remission of EAU" was defined as score ≤0.5 after a period (minimum 7 days) of complete absence of all clinical signs of EAU after the first attack of EAU in PDSAg- or after the last relapse in R14-induced EAU.

MHC class II restriction of T cells

P80 cells (mouse mastocytoma cells) transfected with rat CD80 and either RT1.B or RT1.D as described elsewhere [15] were treated with Mitomycin C (50 µg/ml) for 45 min at 37°C, washed and incubated at a density of $2.5–5\times10^4$ cells/96 well (U-bottom tissue culture plate) in RPMI 1640/1% rat serum and peptides (20 µg/ml PDSAg or R14, respectively). PDSAg- or R14-specific rat T cell lines established as described in [16] were added in a 1:1 ratio, cultured in triplicates for 64–72 h. For the last 18 h cells were pulsed with 1 µCi/well 3H-thymidine. Results are shown as stimulation index (S.I.): mean cpm (peptide)/mean cpm (medium control). Tissue culture medium and supplements were obtained from PAA (Germany).

Isolation of intraocular and lymph node cells

At different time points during EAU animals were sacrificed, inguinal and popliteal lymph nodes as well as eyes were collected. After removal of the lens, single cell suspensions from the remaining eye tissue were prepared using a 70 µm nylon mesh (Falcon, Germany). The resulting cells were washed twice with RPMI1640.

Single cell suspensions of lymph nodes were prepared with the Gentle MACS Dissociator (Miltenyi, Germany), followed by passing the cells through a 70 µm nylon mesh.

Cytokine analysis by flow cytometry

Cells were either cultured for three days with 20 µg/ml antigen peptide, 1 µg/ml Con A (BD Biosciences, Germany), medium only or directly used ex vivo. For cultivation with antigen or Con A stimulation no APC were added. Four hours prior to staining cells were incubated in 50 ng/ml PMA, 1 µg/ml ionomycin and 1 µg/ml brefeldin (all from Sigma-Aldrich, Germany). For surface staining mouse anti-rat TCR-αβ antibodies (clone R73, eBioscience, Germany) conjugated with FITC or PE, mouse anti-rat TCR-γδ antibodies (clone V65, BioLegend/Biozol, Germany) conjugated with FITC or PE, were used. For intracellular staining cells were fixed and permeabilized according to the manufacturer's instructions for the rat anti-mouse/rat Foxp3 staining kit (eBioscience, Germany) and subsequently stained with mouse anti-rat IFN-γ antibodies (clone DB-1) conjugated with FITC, PE or Alexa Fluor 647, rat anti-mouse IL-17 antibodies crossreactive with rat IL-17 (clone TC11-18H10.1) conjugated with FITC or PerCP/Cy5.5 (both BioLegend/Biozol, Germany) or mouse anti-rat IL-10 (clone A5-4, BD Biosciences, Germany) conjugated with PE or the respective mouse IgG2b isotype control for anti-IL-10 (clone MCP-11, BioLegend/Biozol, Germany). Mouse anti-rat CD68 conjugated with Alexa Fluor 647 (clone ED1, AbD Serotec, Germany) was used for both, surface and intracellular staining. Foxp3-specific staining was performed with the rat anti-mouse/rat Foxp3 staining kit from eBioscience (Germany); antibodies (clone FJK-16s) were conjugated with FITC or PE. Flow cytometry was performed with a FACS Calibur (BD Bioscience, Germany) and lymphocytes were gated based on forward (FSC) and side scatter (SSC). Data were analyzed with the FlowJo software (TreeStar, USA).

In vivo administration of IFN-γ

Rats were immunized with R14 in CFA as described, uveitis was determined daily. After resolution of the first course of uveitis 30 U of rat recombinant IFN-γ (Biomol, Germany) in 6 µl sterile saline or 6 µl of saline only were injected into the anterior chamber of both eyes of anesthetized rats with a 30 G needle. Other groups received either 0.5 ml saline or 10^4 U IFN-γ intraperitoneally.

Statistical analysis

Statistical analysis was performed using the unpaired two-tailed t test. $P<0.05$ (*) and $p<0.005$ (**) were regarded as statistically significant.

Results

Expression of IFN-γ and IL-17 by intraocular cells during the course of EAU

A panuveitis (inflammation affecting all segments of the eye) was induced by immunization of rats with PDSAg or R14 in CFA. Intraocular cells were isolated during different stages of disease as shown in Fig. 1A/B and C/D, gated for lymphocytes (see Fig. S1) and stained for intracellular cytokines. (Figs. 1 E–H). Immunization with PDSAg-CFA resulted in a monophasic (Fig. 1A), R14-CFA-immunization in relapsing disease (Fig. 1B) according to the clinical uveitis scores. The majority of intraocular lymphocytes were TCR-αβ+ cells, covering 60–80% of the population (Fig. 1E, F). The remaining cells among the "lymphocyte" population are about 2% TCR-γδ+ cells (Fig. 2), about 2–8% CD161+ NK cells (data not shown) and presumably B cells (not tested). We cannot exclude cells of ocular tissues from the population gated as lymphocytes. Only about 1% of CD68+ monocytes/macrophages were detected among the lymphocyte-gated cells (Fig. S1), while the population gated as "macrophages" by forward vs. side scatter still included about 3% TCR-αβ+ cells (Fig. S1B). The macrophage-gated population did not include Foxp3+ or IL-10+ cells and only very few cells expressing IFN-γ or IL-17 (data not shown).

Intracellular staining of intraocular cells from PDSAg-induced EAU, gated for lymphocytes, revealed stable populations of IFN-γ+/IL-17− and IL-17+/IFN-γ− cells during onset, peak and resolution of disease, while the IFN-γ/IL-17 coexpressing cells significantly increased during the course of EAU and doubled from onset to resolution (Fig. 1G). In contrast, during R14-induced EAU the IFN-γ/IL-17 double positive population remained stable, while the numbers of IL-17+/IFN-γ− cells significantly decreased and the IFN-γ+/IL-17− cells significantly increased from onset to resolution and from onset to relapses. The IL-17+/IFN-γ− population was high at onset of R14-induced EAU, then decreased and slightly increased again during the relapse (Fig. 1H). The number of IL-17+/IFN-γ− cells at onset of EAU was significantly higher in R14- compared to PDSAg-induced uveitis ($p\leq0.05$). The major difference between the monophasic and the relapsing disease was the IFN-γ+/IL-17+ population, which was significantly lower in eyes of relapsing, R14- (Fig. 1H) compared to monophasic, PDSAg-mediated EAU ($p<0.001$, Fig. 1G) during resolution of EAU. Representative flow cytometry dot plots are shown in Fig. S1. The higher percentage of intraocular Th1/Th17 cells during the resolution of the monophasic disease suggests a potential regulatory function of these cells.

IFN-γ and IL-17 expression by intraocular TCR-αβ and TCR-γδ cells

To better characterize the cells producing IFN-γ and IL-17 we concomitantly stained intraocular cells for TCR-αβ (Fig. 2A, B) or TCR-γδ (Fig. 2C, D). The majority of cells recovered from eyes with EAU were TCR-αβ positive and the population remained rather constant during the course of disease (see also Fig. 1E, F). While IL-17 was almost exclusively produced by intraocular TCR-αβ+ cells in both types of EAU, small numbers of IFN-γ+/TCR-αβ- cells were detected (Fig. 2A, B), but these cells did not bear γδ T cell receptors (Fig. 2C, D).

TCR-γδ+ cells were found in very low numbers (about 2% compared to 60–80% TCR-αβ+ cells) in eyes with EAU and represent only a minor population not exceeding 3.4% of the entire intraocular lymphocyte population. TCR-γδ+ cells did neither produce IFN-γ nor IL-17 (Figs. 2C, D).

Expression of IFN-γ and IL-17 by intraocular and lymph node T cells after in vitro-stimulation

In order to determine whether cytokines are produced mainly by autoantigen- or non-autoantigen-specific intraocular T cells, cells isolated from rat eyes were stimulated in vitro for 3 days with either their respective antigen peptide (selecting for antigen-specific T cells) or Con A (polyclonal stimulation) or cultivated in medium only. In PDSAg-induced EAU the number of IFN-γ+/IL-17− cells increased from onset to peak after antigen-stimulation, while the other populations (IL-17+/IFN-γ− or IFN-γ+/IL-17+) remained unaltered and below 10% of intraocular lymphocytes (Fig. 3A). Compared to the medium control, the cytokine-producing, PDSAg- or Con A-stimulated ocular cell populations decreased at resolution, probably representing exhausted T cells. In contrast, antigen- and Con A-stimulated cells from eyes of R14-induced EAU (Fig. 2B) increased with respect to IFN-γ production, while IL-17+ as well as populations producing both, IL-17 and IFN-γ, showed a bell-shaped curve from onset to resolution (Fig. 3B). The pattern of the Con A-stimulated intraocular cell populations from R14-induced EAU (Fig. 3B) was similar to that observed after ex vivo-staining (Fig. 1H).

Intraocular IFN-γ-producing cells responded better to antigen stimulation in vitro than those cells producing IL-17. The latter were better stimulated with the mitogen Con A (Fig. 3A, B).

To address the question whether the pattern of Th1, Th17 and Th1/Th17 cells observed among intraocular T cell populations is reflected by cells from draining lymph nodes of the same rats we isolated popliteal and inguinal lymph nodes, draining the sites of immunization, at the same time points as the respective eyes were examined, and stained them ex vivo and after incubation in medium, with antigen or Con A. Figs. 3C and D show that only Con A-, but not antigen-stimulation induced IFN-γ production in lymph node cells from PDSAg- and R14-immunized rats (Fig. 3C and D). This low frequency of autoantigen-specific T cells might indicate that these cells have already left the draining lymph nodes for the circulation and/or have infiltrated the eyes (as seen in Fig. 3A and B). The lymph node cells might respond with proliferation rather than with cytokine production, which was not determined in this setup. Antigen-specificity of intraocular cells could not be clearly defined with this experimental setup since antigen carried over from eye tissue cannot be completely excluded.

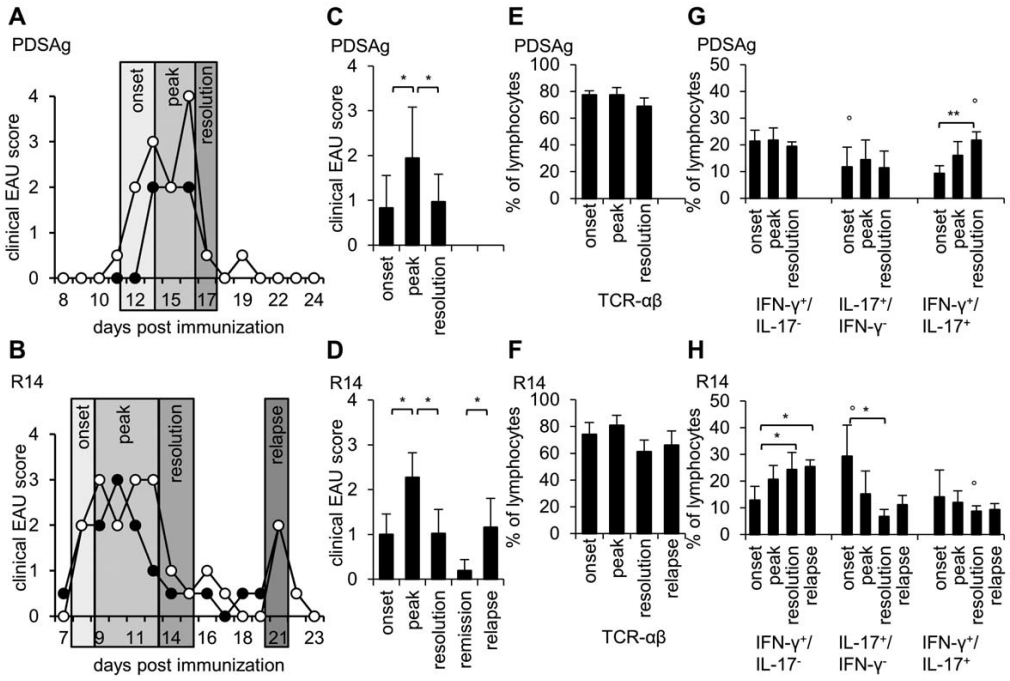

Figure 1. Expression of TCR-αβ, IFN-γ and IL-17 by intraocular cells during EAU. (A, B) Representative clinical courses of monophasic EAU induced with PDSAg (A) or relapsing EAU induced with R14 (B). Black and white symbols represent clinical scores of right and left eyes, respectively. (C, D) Corresponding mean clinical uveitis scores of n = 18–27 eyes from at least 3 independent experiments after immunization with PDSAg-CFA (C) or R14-CFA (D). (E–H) Intraocular cells were isolated from rat eyes at indicated time points after immunization with PDSAg (E, G) or R14 (F, H). Cells from 2–6 eyes were pooled for each experiment and analyzed by flow cytometry. Lymphocytes were gated based on FSC and SSC (see Fig. S1). (E, F) TCR-αβ+ intraocular cells during PDSAg-CFA- (E) and R14-CFA-induced EAU (F). (G, H) Expression of IFN-γ, IL-17 or both together. Data show means from at least 3 independent experiments+SD. Significant differences between the time points of cell collection (*, $p<0.05$) and PDSAg and R14 (°, $p<0.05$) are indicated.
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Expression of Foxp3 by intraocular T cells during the course of EAU

The lack of relapses in PDSAg-induced uveitis might be due to the effects of regulatory T cells. We therefore looked for the expression of Foxp3 by intraocular lymphocytes during EAU induced with PDSAg or R14 (Fig. 4A). Although Foxp3+ cells significantly increased from onset to resolution in PDSAg-induced EAU and from peak to resolution in R14-induced disease, there was no significant difference with respect to the number of Foxp3+ cells in eyes (Fig. 4A) or lymph nodes (data not shown) of monophasic or relapsing EAU. We could not detect concomitant expression of Foxp3 with IFN-γ or IL-17, nor with IL-10 (Fig. S2).

Since soluble MHC-class II molecules to define TCR-peptide-recognition are lacking in the rat system, we tried to identify the antigen-specificity of Foxp3+ T cells by in vitro-stimulation of intraocular cells. T cells were incubated with medium only, with their respective antigen or with Con A for 3 days and subsequently stained for TCR-αβ and Foxp3 expression (for representative dot plot see Fig. S3). We observed a 1.4fold increase in Foxp3+/TCR-αβ+ cells after antigen-stimulation and a twofold increase after Con A-treatment with intraocular cells of PDSAg-induced EAU. Intraocular Foxp3+/TCR-αβ+ cells of R14-induced EAU increased 3.6fold after R14- and 4.3fold after Con A-stimulation, indicating a weaker antigen-specific response of Foxp3+ cells in the monophasic type of uveitis that is thought to be less stringently regulated. Nevertheless, since activated rat effector T cells also express Foxp3 (as we have previously published [3]) we still cannot distinguish between regulatory or effector T cells among this population, irrespective of their antigen-specific proliferation.

Coexpression of IL-10 with IFN-γ and IL-17 by intraocular cells

In addition to Foxp3 expression we determined IL-10 production by intraocular cells (lymphocyte gated) isolated during monophasic (PDSAg, Fig. 4B) or relapsing EAU (R14, Fig. 4C). During PDSAg-induced EAU, cells producing both IL-10 and IFN-γ, slightly increased and IL-10+/IL-17− and IL-10+/IL-17+ populations (Fig. 4B) even significantly increased from onset to resolution ($p<0.05$). In contrast, during R14-induced EAU the IL-10+/IL-17+ population significantly decreased ($p<0.05$) (Fig. 4C). There was only a minor population of IL-10+/IFN-γ+ cells (below 5%) in the eyes of R14-induced uveitis, which was stable during the course of disease. In the eyes of R14-induced EAU we found significantly more IL-10+/IL-17+ cells than in eyes of PDSAg-induced uveitis at onset ($p<0.05$), while the opposite was observed during the resolution of disease ($p<0.05$) (Figs. 4B and C, right panels). It should be noted that the IL-10+/IFN-γ− population could also include IL-17+ cells and the IL-10+/IL-17− population might comprise IFN-γ+ lymphocytes. In contrast to PDSAg-induced EAU, the IL-10+/IFN-γ+ population did not significantly change during R14-mediated disease, while the number of cells coexpressing IL-10 and IL-17 decreased significantly.

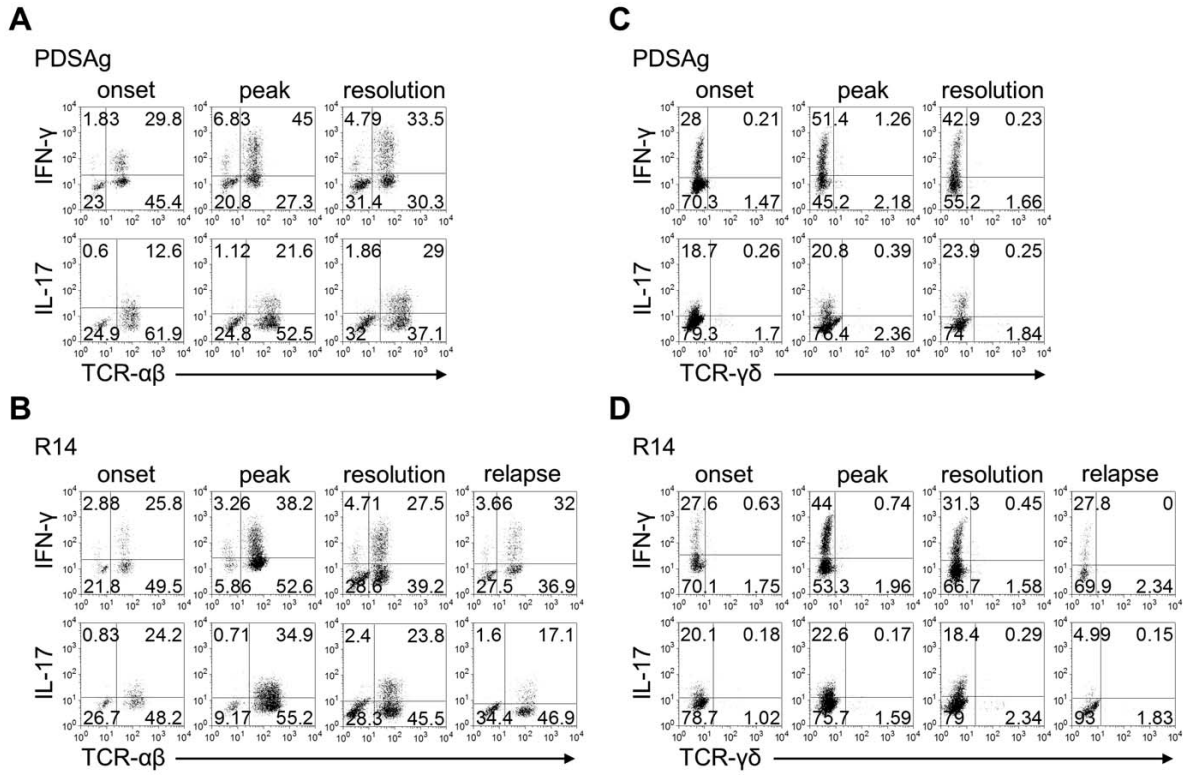

Figure 2. Expression of IFN-γ and IL-17 by intraocular TCR-αβ or TCR-γδ cells. (A, B) Representative dot plots from the FACS analysis of double staining for TCR-αβ and IFN-γ or IL-17 from PDSAg- (A) and R14-induced (B) uveitis. (C, D) Dot plots showing double staining of TCR-γδ and IL-17 or IFN-γ at different time points during PDSAg- (C) and R14-induced EAU (D). Lymphocytes were gated based on FSC and SSC (see Fig. S1). For corresponding EAU scores see Fig. 1C/D.
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While the numbers of IL-10-expressing cells in lymph nodes remained low during both types of EAU (between 1.4 and 4.3%), they dramatically increased in the eyes (between 14 and 22%, Fig. S2, C and D). Only a minor population of IL-10-producing cells concomitantly expressed Foxp3 (about 1% to 1.7%, Fig. S2).

Staining of TCR-αβ+/IL-10+ intraocular cells for concomitant expression of IFN-γ and IL-17 (Fig. 4D) revealed striking differences between PDSAg- and R14-induced EAU. In eyes of PDSAg-induced uveitis (upper panel) the IL-10+ T cells expressing only IL-17 or both, IL-17 and IFN-γ, massively increased from onset to resolution (IL-10+/IFN-γ+/IL-17+: 4.58% to 25.3%, Fig. 4D upper panel), while the opposite effect was observed with IL-10+ T cells from R14-induced disease: high numbers of IL-10+/IFN-γ+/IL-17+ cells at onset (28.7%) dropped to 11.7% at resolution and less than 4.5% during relapse (Fig. 4D, lower panel). We thus conclude that IL-10+ cells might be responsible for preventing relapses in PDSAg-induced uveitis, while the decreasing number of IL-10+ cells in the eyes of R14-induced uveitis might allow recurrent inflammation.

Cytokine expression by intraocular lymphocytes during remission of EAU

We also investigated quiescent eyes (no clinical signs of EAU for 7–13 days) in the late remission of intraocular inflammation (see Fig. 5A) for remaining lymphocytes and their concomitant expression of IL-10 with IFN-γ and IL-17 (Fig. 5B), or Foxp3 (Fig. 5C). PDSAg-induced EAU differed significantly from R14-induced disease ($p<0.05$) with respect to IL-10+/IFN-γ−, IL-10+/IL-17− and IL-10+/IL-17+ cells (Fig. 5B). Also Foxp3+ T cells were significantly elevated ($p<0.05$) in eyes during late remission of PDSAg-induced uveitis, indicating the extended presence of elevated numbers of regulatory cells in the eyes of non-relapsing EAU (Fig. 5C).

MHC class II-restriction of PDSAg- and R14-specific T cell lines

In order to further differentiate between PDSAg- and R14-induced uveitis we investigated the antigen-recognition of the respective T cells. We found that the two antigen peptides, PDSAg and R14, were presented by different MHC class II molecules, as shown by the proliferation of respective T cell lines in response to P80 cells expressing rat CD80 and rat MHC class II RT1.B or RT1.D. R14 was only presented by RT1.D, PDSAg predominantly presented by RT1.B (Fig. 6). The minor stimulation of PDSAg-specific T cells in cocultures with P80-RT1.D and PDSAg indicated a small subpopulation of T cells probably recognizing PDSAg or a degradation product presented by RT1.D.

In vivo administration of IFN-γ

In order to further investigate the role of IFN-γ in relapsing EAU rats received a single injection of IFN-γ into both eyes or intraperitoneally immediately after resolution of the first course of

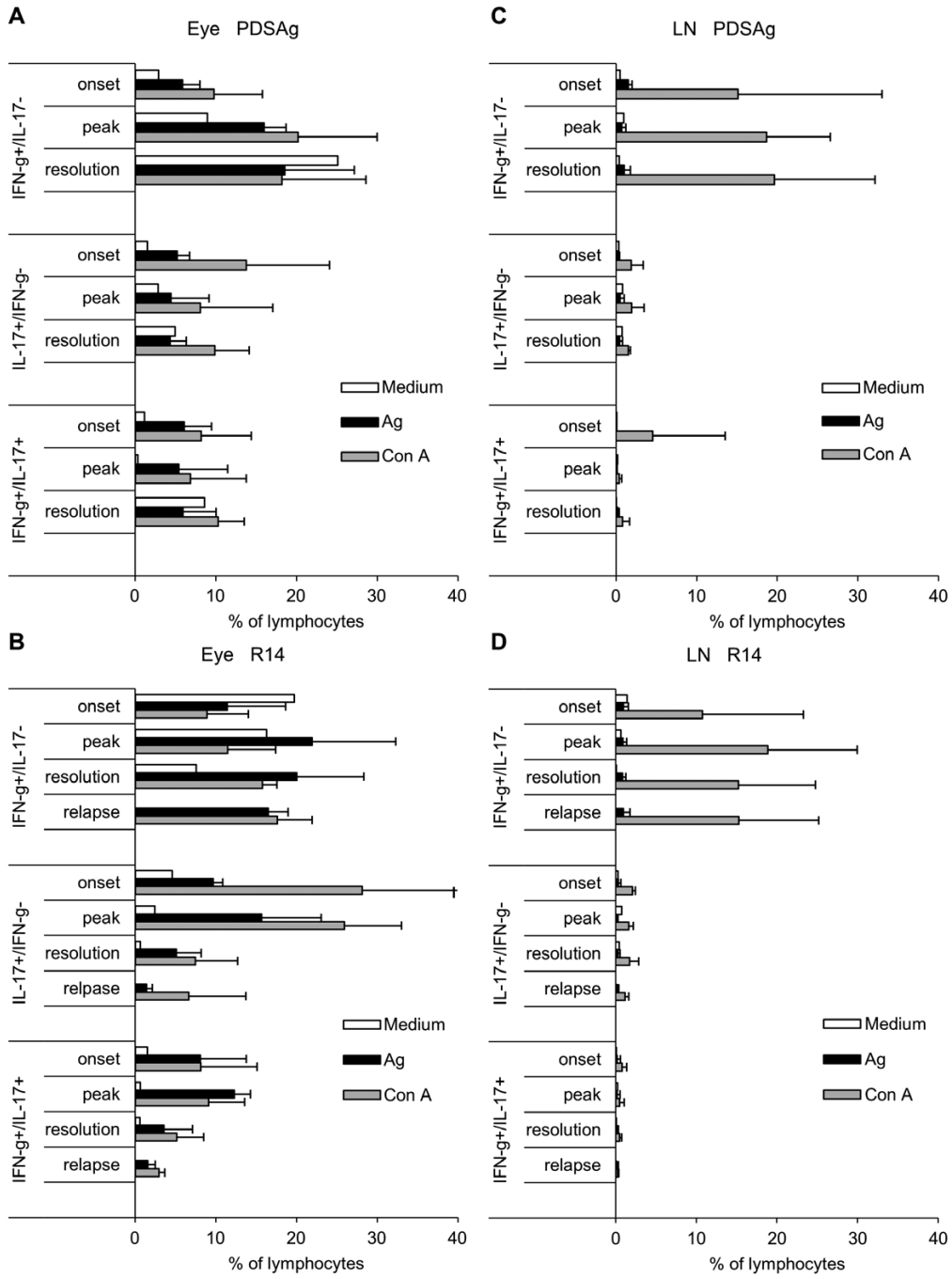

Figure 3. Cytokine expression after in vitro stimulation with antigen or Con A. (A and B) Staining of intraocular cells for IFN-γ and IL-17 during EAU induced with PDSAg-CFA (A) or R14-CFA (B) after 3 days of in vitro incubation in medium only, respective antigen or Con A. C and D show respective data from lymph node cells of the same experiments. Data show means of up to 3 independent experiments (n = 6–18 eyes or lymph node cells from 3–9 animals)+SD. Lymphocytes were gated based on FSC and SSC (see Fig. S1). Corresponding EAU scores are shown in Fig. 1C/D.
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uveitis (Fig. 7). Control groups were treated with similar injections of saline. While spontaneous relapses during R14-induced uveitis appear unpredictable, we observed rather synchronized recurrences of intraocular inflammation with clinical scores of 1–2 in 7 of 8 eyes 3 days after injection of IFN-γ (mean maximum scores of all eyes: 1.0), while only 1/8 eyes injected with saline experienced

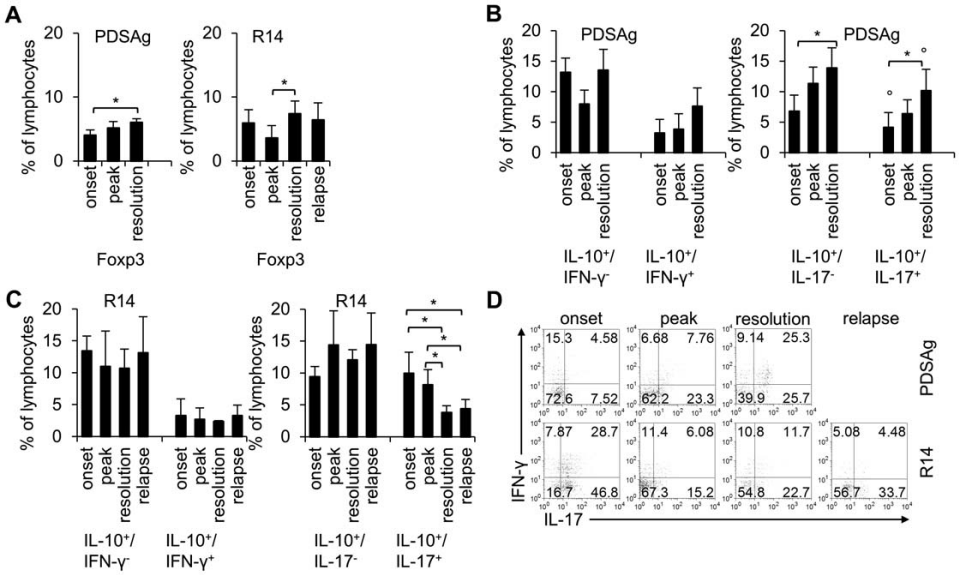

Figure 4. Expression of Foxp3 and IL-10 by intraocular cells during EAU. Cells were isolated from eyes at indicated time points during EAU. Corresponding clinical EAU scores are shown in Fig. 1C and D. Lymphocytes were gated based on FSC and SSC (see Fig. S1). (A) Ex vivo staining for Foxp3. Mean % of cells+SD is shown for at least 3 independent experiments with n = 2–6 eyes per group. (B, C) Ex vivo staining for intracellular IL-10 and IFN-γ expression (left panels) as well as for expression of IL-10 and IL-17 (right panels). Data are shown as mean+SD for at least 3 independent experiments with n = 2–6 eyes per group. Significant differences between the time points of cell collection (*, $p<0.05$) and PDSAg and R14 are indicated (°, $p<0.05$). (D) Quadruple staining of intraocular cells for expression of TCR-αβ, IFN-γ, IL-17 and IL-10. Dot plots show IFN-γ and IL-17 staining of TCR-αβ+/IL-10+ gated cells.
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a relapse (mean score 0.125). Intraperitoneal injection of IFN-γ or saline had no such effect on relapses of EAU, only 1/8 eyes in the groups of intraperitoneal IFN-γ and 4/8 eyes in the group treated with saline i.p. had relapses after 4 to 6 days. These data indicate that relapses of uveitis are initiated by T cells remaining within the eye after uveitis rather than by newly activated T cells invading the eye from the periphery.

Discussion

A major limitation of most animal models of autoimmune diseases is that they do not reproduce the chronic or relapsing-remitting pattern characteristic of many human autoimmune diseases. They thus do not provide an opportunity to investigate the mechanisms underlying recurrent inflammation. Experimental autoimmune uveitis in Lewis rats can be both a monophasic as well as a relapsing-remitting disease, strictly depending on the peptide used for induction. Therefore we have the unique possibility to investigate two types of EAU in the same rodent strain. EAU induced by S-Ag peptide PDSAg is monophasic, while the IRBP peptide R14 causes relapses of intraocular inflammation. Further differences between PDSAg- and R14-induced diseases are based on the differential regulation of EAU by chemokine variants like Met-RANTES [17] or a mutant of MCP-1/CCL2 [18].

In previous studies we could also demonstrate the differences in the course of EAU after adoptive transfer of T cell lines and differences in gene and protein expression between PDSAg- and R14-specific T cells. We found several genes and signaling pathways upregulated in R14-specific T cell lines, most of them

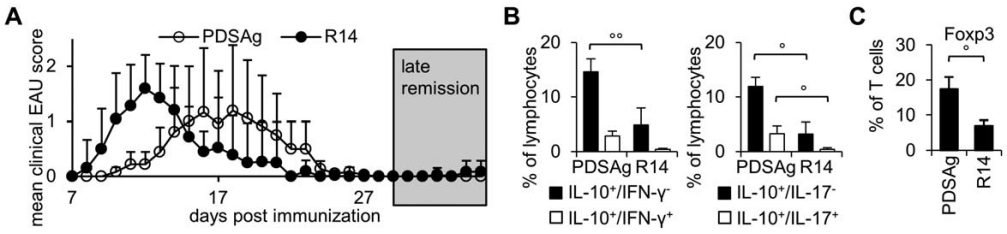

Figure 5. Expression IL-10, IFN-γ, IL-17 and Foxp3 in the late phase of remission of EAU. (A) Time course showing mean daily clinical uveitis scores. Eyes were collected after complete resolution of clinical uveitis (“late remission”) 10 days after resolution of EAU induced as indicated. (B) Coexpression of IFN-γ (left panel) and IL-17 (right panel) with IL-10 in quiescent eyes of late remission. Cells were gated on lymphocytes based on FSC and SSC. (C) Expression of Foxp3 in intraocular TCR-αβ+ cells from quiescent eyes of late remission. Data show means of at least 3 independent experiments with n = 6–10 eyes per group.
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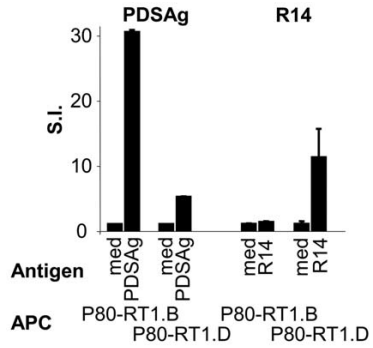

Figure 6. MHC-class II restriction of PDSAg- and R14-specific T cells. Proliferation of a PDSAg- and a R14-specific T cell line in response to the respective antigen peptide and mouse mastocytoma cells P80, expressing rat CD80 and rat MHC-class II antigens RT1.B or RT1.D. T cells were stimulated for 3 days and T cell responses are shown as stimulation index (SI)+SD.
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either upstream or downstream of IFN-γ signaling. We also observed differences in IL-17 protein expression between PDSAg- and R14-specific T cell lines. R14-specific T cells were more Th1-prone with enhanced IFN-γ production compared to PDSAg-specific T cells, which expressed more IL-17 [3]. We thus decided to investigate IFN-γ and IL-17 protein expression of intraocular cells from rats during EAU.

The majority of infiltrating lymphocytes in the eye during EAU are T cells expressing αβ T cell receptors. In contrast to a mouse model of EAU [19], we could detect only small numbers of TCR-γδ+ cells within the eyes during EAU, and these cells did not produce pro-inflammatory cytokines such as IFN-γ or IL-17.

In order to separate Th1 from Th17 cells for further experiments, we investigated surface markers of human and mouse IL-17-producing cells such as the NK receptor P1A/CD161 [20], and CCR6, the receptor for CCL20 [21]. Although we could demonstrate the expression of both molecules on rat lymphocytes, none was coexpressed with IL-17 (data not shown), thus neither molecule is suitable for the isolation of IL-17+ cells. Ex vivo-staining of intraocular cells has revealed striking differences between monophasic and relapsing disease with respect to the pattern of IFN-γ+ and IL-17+ cell populations during the course of EAU. Both, IFN-γ- and IL-17-producing populations, which did not coexpress the respective other cytokine, remained quite stable during the course of monophasic EAU (PDSAg), while during the course of relapsing uveitis (R14) IL-17+/IFN-γ− cells decreased and IFN-γ+/IL-17− cells increased. From these observations we conclude that IFN-γ+ cells are responsible for initiating further recurrences in relapsing EAU, while IL-17+ cells might rather have the function to facilitate the primary ocular invasion of cells. IL-17+ cells thus might fulfill different functions in the monophasic compared to the relapsing disease.

Our observations concerning the central role of IFN-γ made by gene array analysis [3] was supported by the induction of synchronized recurrences of uveitis after intraocular injection of IFN-γ. We had previously shown that GFP+ autoreactive T cells remain in the eye for several weeks during remission of EAU, and form clusters at relapses. It seems that these cells have been reactivated by ocular application of IFN-γ, and might have proliferated, which resulted in cluster formation. No effect was observed after intraperitoneal injection of IFN-γ, underlining the pivotal role of intraocular cells during recurrent intraocular inflammation.

Interestingly, we also detected cells concomitantly expressing IFN-γ and IL-17 in the eyes of both types of EAU. In R14-induced relapsing EAU this population slightly decreased during the course

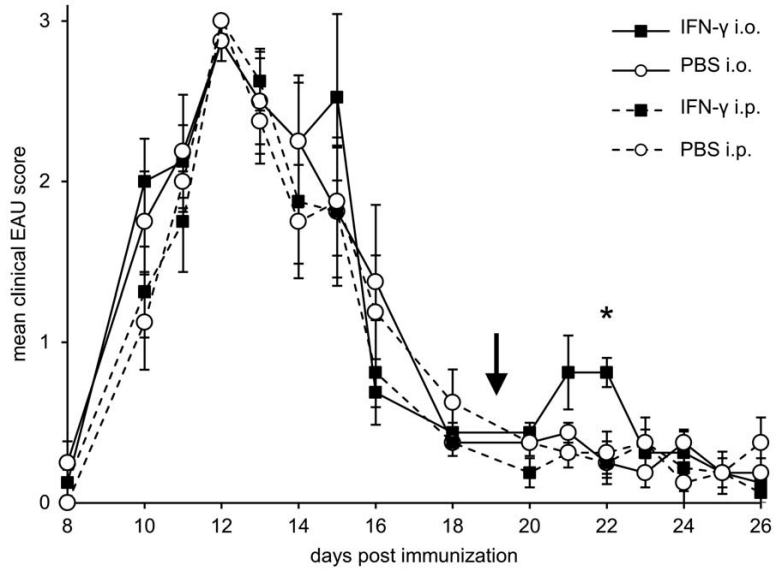

Figure 7. In vivo administration of IFN-γ. Rats immunized with R14-CFA were injected intraocularly (i.o.) into the anterior chamber or intraperitoneally (i.p.) with IFN-γ or saline (as indicated) at day 19, when the clinical signs of EAU were ≤0.5 in all eyes. The time course shows the mean daily clinical scores of all eyes per group ± SE. The arrow marks the time point of IFN-γ or saline application. Significant difference between the group receiving intraocular IFN-γ and the other treatment groups is indicated (*, p≤0.05).
doi:10.1371/journal.pone.0049008.g007

of disease, but increased during monophasic, PDSAg-induced uveitis. We speculate that these cells might play a role in prohibiting recurrent inflammation in monophasic EAU, for it was previously shown that injection of IL-17 can ameliorate EAU [22]. A regulatory role of cells coexpressing two inflammatory cytokines seems to be unusual, but further experiments showed that although IFN-γ+/IL-17+ cells did not express Foxp3, a rather high percentage coexpressed IL-10, a cytokine typical for regulatory T cells [23].

Comparing IFN-γ- and IL-17-expressing cell populations from eyes and draining lymph nodes at different stages of uveitis revealed distinct cell populations found exclusively in the affected eyes (especially the IFN-γ+/IL-17+ cells). Only after Con A stimulation we could recover IFN-γ, but no IL-17 producing cells from lymph nodes. Merely a small population of Con A-responsive IFN-γ+/IL-17+ cells was detected among lymph node cells of PDSAg -induced EAU. We thus conclude that the ocular environment has a strong influence on the shaping of invading T cell populations. The availability of autoantigen, which is needed for local reactivation [24] and local cytokine production can influence the intraocular T cell populations. Intraocular cytokines are usually immunosuppressive, leading to the phenomenon of ACAID (anterior chamber-associated immune deviation) [25], while already activated T cells are not hampered by the ocular environment. The intraocular environment seems to have different effects on PDSAg-specific and R14-specific T lymphocytes. With respect to in vitro-stimulation it might as well be possible that the culture conditions favor Th1 subtypes more than other subtypes, resulting in slight differences of T cell populations recovered after in vitro stimulation compared to cells directly stained ex vivo [26]. For instance, it was shown that Th17 cells have an unstable phenotype and can easily switch their cytokine pattern [7], supporting our findings of higher numbers of IFN-γ+ cells after in vitro stimulation.

It is noteworthy that the various stages during the course of EAU (onset, peak, resolution, relapse) reflect the slightly time-delayed consequences of intraocular autoreactive T cell actions. At "onset" of EAU we recovered autoreactive T cells from the eyes that had immigrated and been reactivated some days before, while during "resolution" they might be already downregulated and have stopped recruiting inflammatory cells. What we observe as "clinical uveitis" is characterized by infiltrating inflammatory leukocytes, mainly monocytes/macrophages [24].

We also determined IL-10 expression of intraocular cells that are concomitantly producing IFN-γ and/or IL-17. During the course of monophasic, PDSAg-induced EAU we observed an increase of cells coexpressing IFN-γ as well as IL-17 and IL-10. We do not know whether these populations are immigrating from the circulation during the different stages of uveitis, or whether the T cells are changing their cytokine profiles (and functions?) in the eyes during EAU. During monophasic EAU (PDSAg) the intraocular IL-10+/IFN-γ+ and IL-10+/IL-17+ populations increased, but they were either very small (IL-10+/IFN-γ+) or even decreased (IL-10+/IL-17+) in the eyes of relapsing, R14-induced EAU. Thus we speculate that these cells have a specific function to prevent recurrences of intraocular inflammation after PDSAg-immunization. Since it was shown previously that an effector cytokine like IFN-γ can increase the regulatory effect of IL-10 [27], this effect might also be true for IL-17 in combination with IL-10.

The number of cells producing the suppressive cytokine IL-10 increased during the course of monophasic, PDSAg-specific EAU, while this population decreased during relapsing, R14-induced uveitis. It is not clear how IL-10 production is induced in effector T cells of the Th1 or Th17 type. Jankovic et al. [28] have shown that during the immunization of mice with peptide and CFA the production of IL-6 and TGF-β could induce pathogenic Th17 cells. Further exposure to high concentrations of TGF-β – as is found in the eye – and IL-6, which might be provided by later infiltrating inflammatory cells in EAU, could induce IL-10 production by Th17 cells in the eyes [29,30]. IL-10 can control both, Th17 [9] as well as Th1 [31] responses. IL-17+ as well as IL-17+/IFN-γ+ cells bearing IL-10 receptors can thus be directly suppressed by extrinsic IL-10 [11]. IL-10, but not Foxp3 production can be induced by IL-27 in IFN-γ-producing cells, which could suppress EAE [8]. The source of IL-27 could be either infiltrating inflammatory cells or local microglia, stimulated with IFN-γ from pathogenic T cells [32]. Concurrent production of IL-10 with inflammatory cytokines like IFN-γ or IL-17 in T cells is also regarded as the "endpoint" of a successful effector response [31] rather than being a distinct T cell lineage. In that case, the increase of IFN-γ+/IL-10+ and IL-17+/IL-10+ cells during PDSAg-induced, monophasic EAU would represent the termination of the T cell response. In contrast, in eyes of R14-induced uveitis these populations were found at constant, very low levels (IFN-γ+/IL-10+) or even decreasing (IL-10+/IL-17+), allowing relapses of inflammation. IL-10 can suppress T cells even intrinsically [33] and might be induced in the T cells already during the first encounter of antigen, depending on the MHC class II restriction. As described by Matsuoka et al. [34] T cells restricted for HLA-DR (which is the human equivalent for the R14-presenting RT1.D in rats) induce more inflammatory cytokines, while DQ-restricted T cells (DQ is equivalent to rat RT1.B, presenting PDSAg) stimulate higher levels of IL-10. Consequently, the life of a T cell is already determined after its first antigen-specific stimulation.

While we recovered only a low number of IL-10+ cells from lymph nodes draining the site of immunization during EAU, we observed an up to tenfold increase of these cells within the eyes. This was in sharp contrast to Foxp3+ cells, which did not differ between lymph nodes and eyes, underlining the important regulatory role of IL-10+ cells within the eye. Since the increase of IL-10+ cells, like IFN-γ and IL-17 coproducing lymphocytes, is only detected in the eyes, we speculate that the IL-10+ regulatory population develops from the autoaggressive T cell pool in the ocular environment, augmented by their own IL-10 production or by IL-10 from retinal cells [35]. This phenotype switch could be a kind of self control of effector cells and has been described for Th1 cells [36], as well as for Th17 cells [9]. The potential of IL-10 to suppress uveitis has been demonstrated by injecting a lentiviral-vector expressing IL-10 into the anterior chamber of the eye [37], by the protective effect of IL-10 injection during the afferent immune response of EAU [13] and by a diminished uveitis in IL-10 transgenic mice [12].

During the course of disease we could detect increasing numbers of Foxp3+ cells in the eyes of PDSAg-induced EAU, but there was no significant difference compared to R14-induced disease. Despite relatively high numbers of Foxp3-expressing cells during resolution and relapses in the eyes of R14-induced EAU we observed recurrent inflammation. Either the Foxp3+ cells found in relapsing EAU have a weaker regulatory function compared to the Foxp3+ cells from monophasic EAU [38], or they were no regulatory T cells at all. In vitro-stimulation of intraocular cells with both, antigen or mitogen, resulted in an increase of Foxp3-expressing T cells, which was higher in cells from R14-induced uveitis. As we have shown previously [3], Foxp3 is also expressed by activated rat effector T cells, an observation that precludes a definite assignment of these cells to the T effector or T regulator population. Interestingly, in quiescent eyes after subsiding of intraocular inflammation, significantly more Foxp3+ cells were found in eyes of PDSAg- compared to R14-induced uveitis,

suggesting that these cells might indeed have a regulatory phenotype. Those eyes also revealed significantly increased IL-10+ populations. Thus, we might have Foxp3+ as well as IL-10+ regulatory T cell populations with either distinct or synergistic functions.

Our findings indicate that autoreactive T cell populations can be shaped differently depending on their antigen peptides, followed by the influence of the environment of the target tissue. Investigations of the role of antigen presentation and recognition of T cells are necessary to better understand the development of different T cell characteristics that lead to monophasic or relapsing disease. Furthermore the clear classification of T cells expressing multiple cytokines, either combinations of effector cytokines or of effector and regulatory cytokines, is essential for understanding their role in autoimmunity.

Supporting Information

Figure S1 Representative dot plots from staining of intraocular cells. (A) Gating of the "lymphocyte" population of intraocular cells according to FSC and SSC (left panel) and the respective staining for TCR-αβ and monocytes/macrophages (CD68) (right panel). (B) Gating of the "macrophage" population of intraocular cells by FSC and SSC (left panel) and the respective staining for TCR-αβ and CD68 (right panel). (C) Representative dot plots of IFN-γ and IL-17 staining of the lymphocyte population, gated as shown in Fig. S1A. (D) Staining of intraocular lymphocytes (gated as shown on left panel) for TCR-αβ (versus FSC) and double staining for TCR-αβ and IL-10. The right panel shows double staining with anti-TCR-αβ and the isotype control for the anti-IL-10 antibody.
(TIF)

Figure S2 Coexpression of Foxp3 with IL-17, IFN-γ and IL-10 by intraocular and lymph node cells. Representative dot plots of the FACS analysis of "lymphocyte"-gated (see Fig. S1) intraocular (A, B) and lymph node cells (C, D) at different time points of PDSAg- and R14-CFA-induced uveitis. Cells were stained for Foxp3 and coexpression of IL-17 (upper panels), IFN-γ (middle panels) or IL-10 (lower panels). (E) Representative isotype controls for anti-Foxp3, anti-IL-17 and anti-IFN-γ, shown with lymph node cells.
(TIF)

Figure S3 Foxp3 expression of antigen- and Con A-stimulated intraocular cells. Representative dot plots of cells from the eyes during resolution of PDSAg- and R14-induced EAU, stained for TCR-αβ and Foxp3 after 3 days of culture in medium only, specific antigen or Con A (without addition of APC). Cells were gated for lymphocytes according to SSC and FSC.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: UK MDM GW. Performed the experiments: UK MDM. Analyzed the data: UK GW. Contributed reagents/materials/analysis tools: UK MDM GW. Wrote the paper: GW UK MDM.

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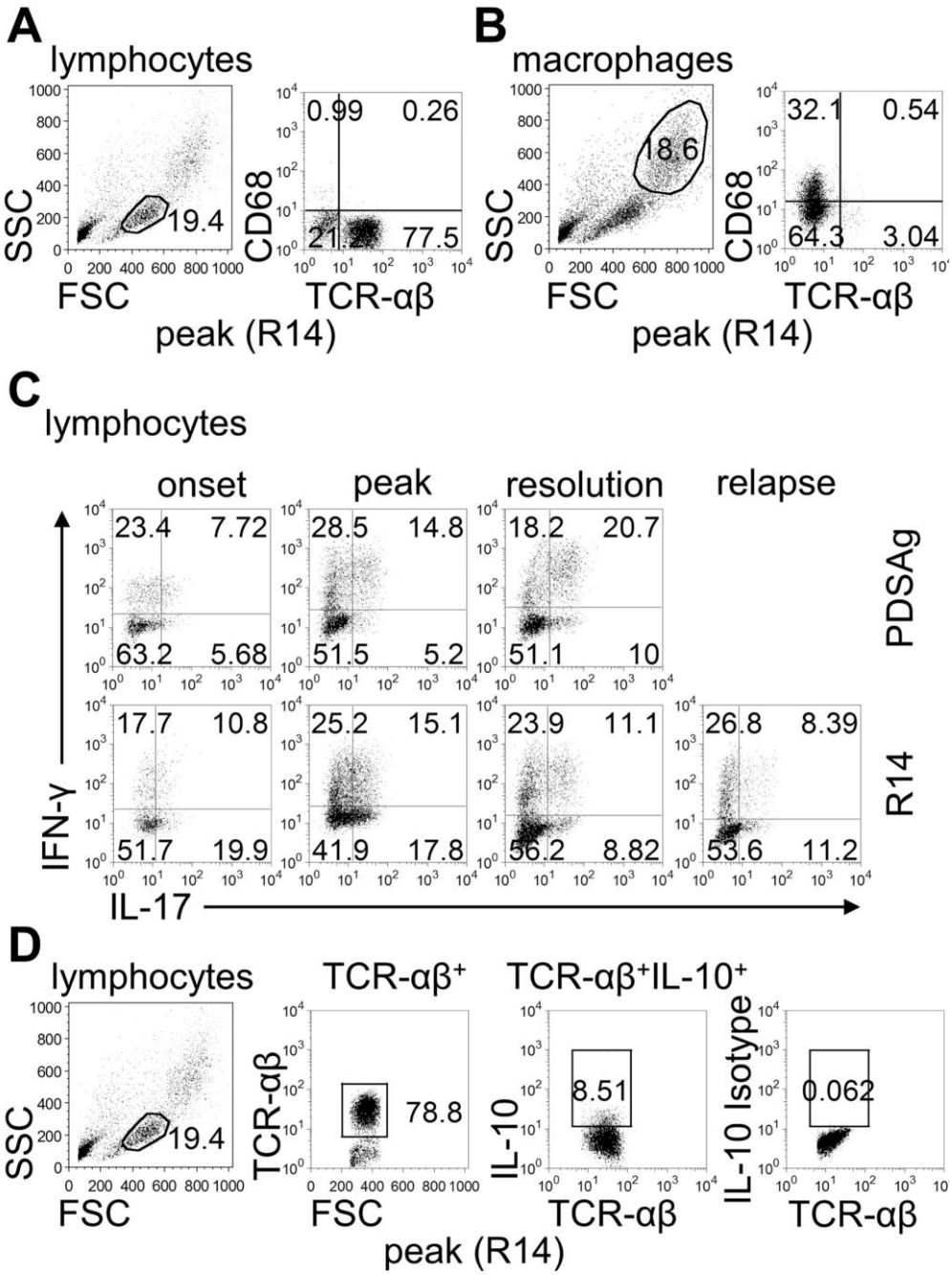
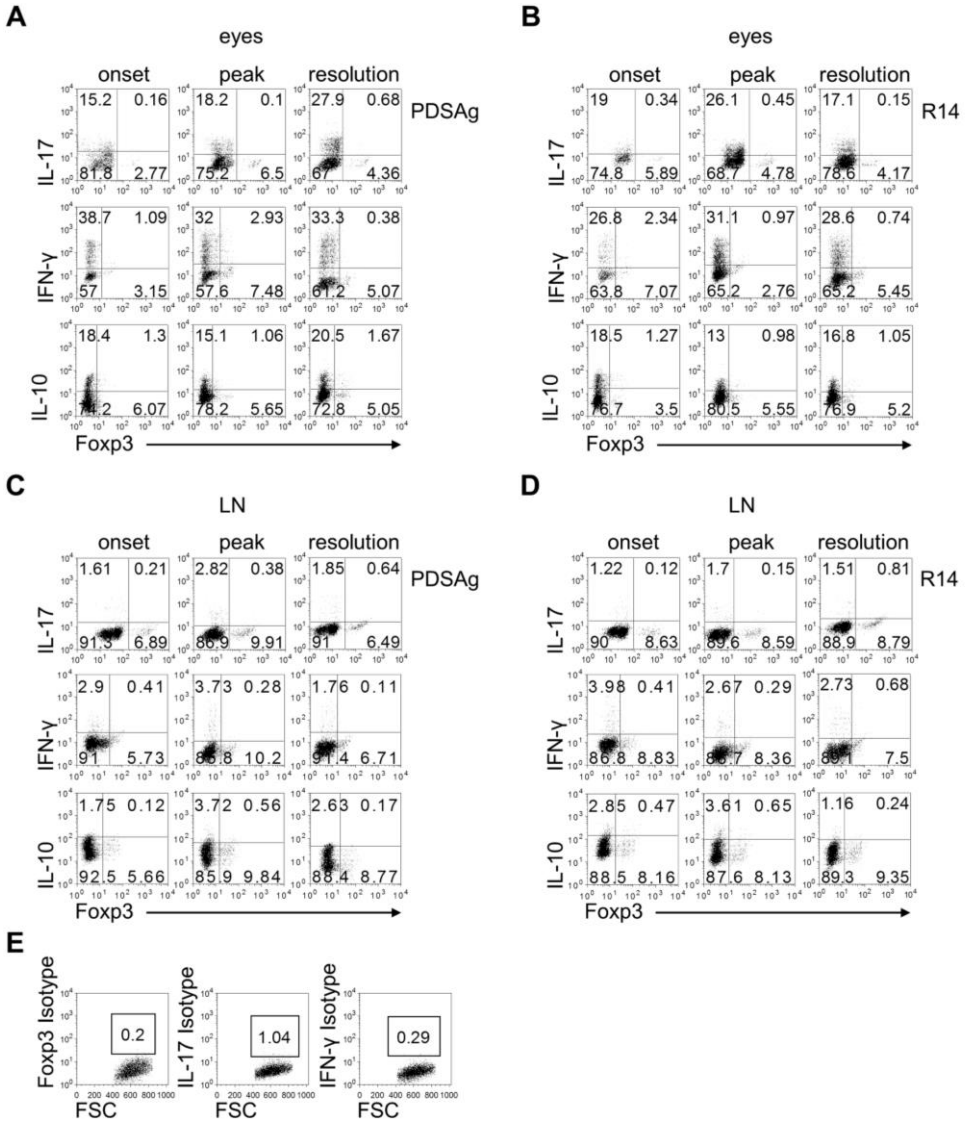
Supporting Information

Figure S1



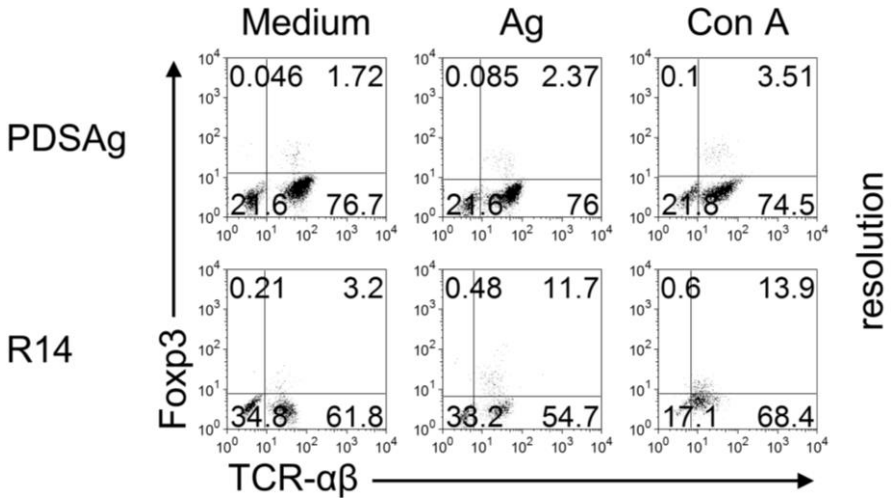
Supporting Information

Figure S2



Supporting Information

Figure S3



5.3 Monophasic EAU dominates over relapsing EAU

To investigate the influence of effector and regulatory T cell populations on the disease course we induced EAU by immunization with combinations of the uveitogenic peptides in CFA. PDSAg/CFA and R14/CFA were either administered separately at contralateral sides (indicated as 1+1) or as a mixture of both (indicated as Mix). While none of the Mix-immunized rats had recurrences, contralateral administration of both antigens allowed a low frequency of relapses in 12.5% of eyes (Figure 5) compared to 75% in animals immunized with the same dose of R14 only. There were no differences detected in the uveitis intensities between the particular groups.

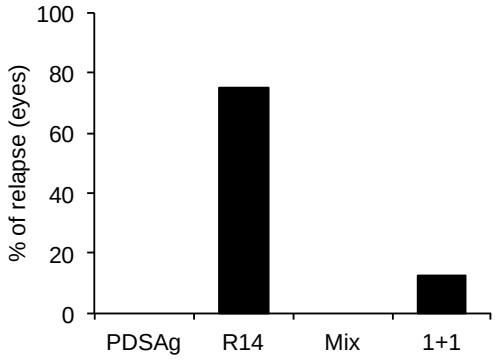


Figure 5 Percentages of relapsing eyes. Lewis rats were immunized with 25 µg peptide PDSAg (bovine S-Ag amino acid (aa) 342-354), R14 (human IRBP aa 1169-1191), Mix (combination of 25 µg PDSAg and 25 µg R14) or 1+1 (25 µg PDSAg and 25 µg R14 administered at contralateral sides) in CFA fortified with Mycobacterium tuberculosis strain H37RA (2.5 mg/ml). Time course of uveitis was determined daily with an ophthalmoscope. Uveitis was graded as described (de Smet et al., 1993). Relapses were defined as score ≥1 following a period of complete absence of all clinical signs of inflammation after the first attack of disease. Percentage of relapsing eyes is shown from 8-12 animals per group.

During the course of uveitis the cytokine pattern of intraocular T cells from the differently immunized rats was investigated. In the animals immunized with both antigens the cytokine pattern of intraocular cells looked similar, irrespective of the mode of application (Mix or 1+1), but differed from the pattern of the rats that were immunized with PDSAg or R14 only. Rats immunized with both antigens showed an R14-like cytokine pattern at onset of EAU with higher numbers of IL-17$^+$ and lower numbers of IFN-γ$^+$ cells, and a PDSAg-like cytokine expression at resolution of EAU with increased IL-17$^+$ and IL-10$^+$ populations (Figure 6).

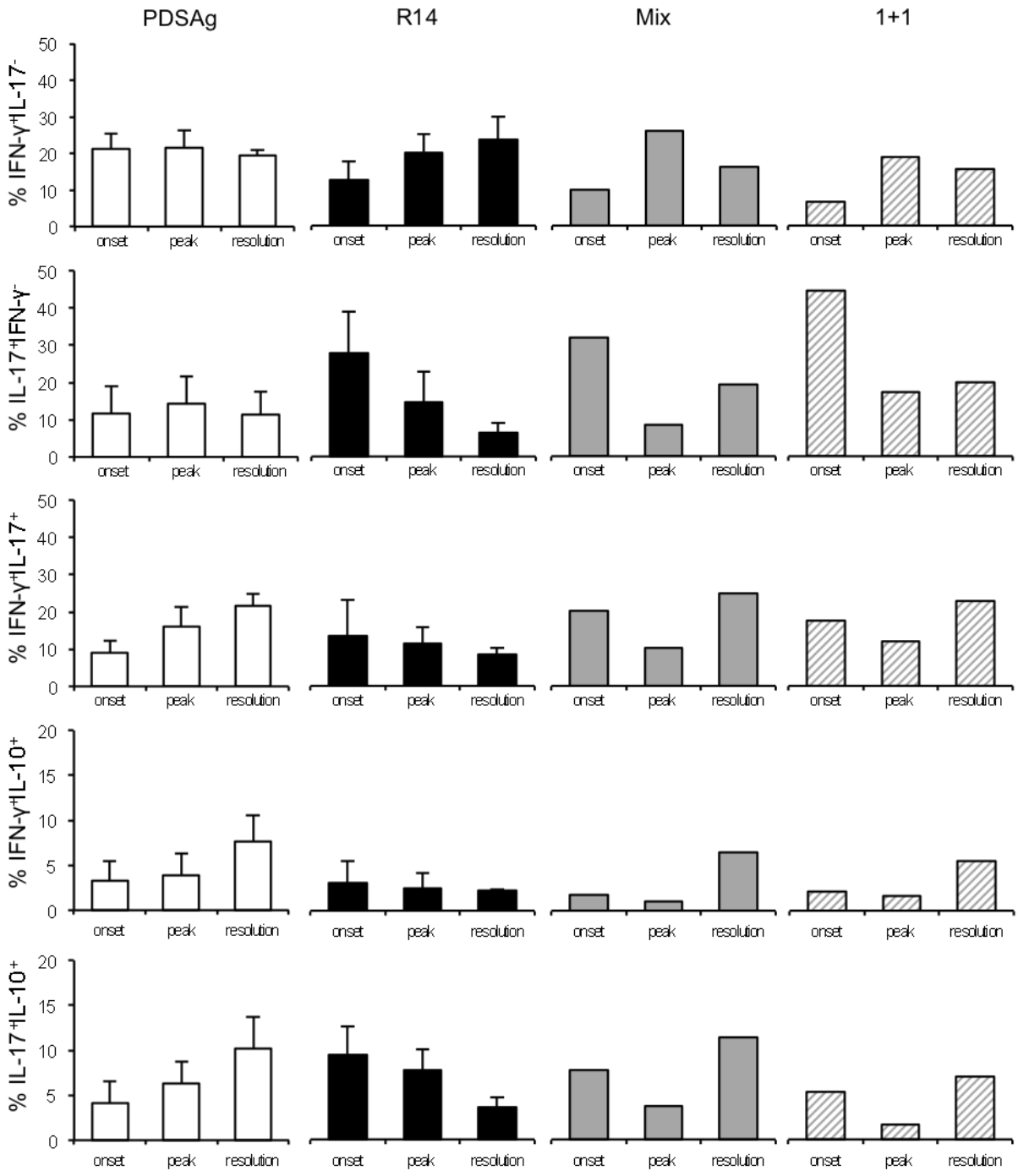


Figure 6 Cytokine pattern of intraocular cells. Lewis rats were immunized as described in Figure 5. Intraocular cells were isolated at indicated time points during EAU and pooled from three animals. After preparing single cell suspensions, cells were stimulated with 50 ng/ml Phorbol 12-Myristate 13-Acetate, 1 µg/ml Ionomycin and 1 µg/ml Brefeldin A for four hours and stained for IFN-γ, IL-17 and IL-10 with respective antibodies conjugated with FITC, PE or Alexa647. Flow cytometry was performed with a FACS Calibur and lymphocytes were gated based on forward and side scatter. Data were analyzed with FlowJo software. White bars: PDSAg, black bars: R14, grey bars: Mix, and hatched bars: 1+1. The y-axis shows % of lymphocytes with cytokine expression as specifically indicated.

In conclusion, the disease course and cytokine pattern of intraocular cells from rats immunized with both antigens confirmed a dominant role of the monophasic, PDSAg-specific immune response at the resolution phase of EAU. This observation was also represented by the decreased frequency (1+1 immunization) or completely abrogation (Mix immunization) of relapses. Increased populations of $Foxp3^+$ cells were observed in all groups during resolution (data not shown). As previously detected in monophasic uveitis, increasing numbers of T cells expressing IL-10 may be responsible for the prevention of relapses in animals immunized with combinations of PDSAg and R14.

6 Discussion

Many human autoimmune diseases are characterized by relapsing rather than monophasic disease courses, whereas the exact mechanisms for relapses are not understood. However, most animal models lack the possibility to investigate relapsing disease courses and spontaneous relapsing disease is even more rare. An exception is experimental autoimmune uveitis in Lewis rats, which can exhibit monophasic as well as spontaneously relapsing and even reinducible disease (Diedrichs-Mohring et al., 2008), depending on the antigen-specificity of the pathogenic T cells. On the basis of this model we were able to investigate mechanisms underlying recurrent disease courses.

Recurrent intraocular inflammation appeared not only after immunization with IRBP-peptide R14 in CFA, but also after adoptive transfer of R14-specific T cells. The onset of EAU is about 4-5 days after the transfer of activated, autoantigen-specific T cells in naïve rats. Intraocular inflammation is resolved about 5 days later, suggesting that there is not much time for the generation of regulatory cells. Consequently, it was proposed that there is an autonomous program within the effector T cells resulting in different disease courses. We therefore decided to investigate differences in the transcriptomes of T cells specific for PDSAg and R14.

6.1 Transcriptomic profile of monophasic and relapsing T cell lines

Transcriptomic analyses offer a wide-ranging insight to gene activity based on mRNA expression levels. By means of oligonucleotides any desired mRNA can be detected among the mRNA pool that includes all mRNA transcripts of the cells. However, the mRNA levels do not always correlate with the amount of protein synthesized. There are many regulatory mechanisms between mRNA and protein at the posttranscriptional level. For instance, microRNAs (miRNAs), which are small non-coding RNAs, can bind by base pairing to mRNAs and thereby repress protein synthesis (Ross et al., 2007). Proteins may also be retained in the ER and are thus

biologically inactive. That is why mRNA expression should be confirmed by protein expression.

6.1.1 Upregulated genes in T cell lines inducing relapsing EAU

We have the advantage to be able to induce monophasic and relapsing uveitis in the same strain of rats by the same immunization method, the only difference being the antigen peptide. Thus, T cells from both disease courses can easily be compared. Since it is almost impossible to obtain rat T cell clones we used highly specific T cell lines after at least two restimulations *in vitro* with the respective peptides. These cell lines consist of more than 90% $CD4^+$ T cells. mRNA from three different T cell lines of each antigen specificity were separately analyzed by microarrays, followed by quantitative real time polymerase chain reaction (qPCR) confirmation with another 6-8 different T cell lines of each specificity.

Microarray and qPCR revealed upregulation of several genes in R14-specific T cells inducing relapsing disease. Only gene expression of at least twofold change compared to PDSAg-specific T cells was considered for the analysis. Surprisingly, there was no downregulation of genes detected in R14-specific T cells inducing relapsing EAU compared to PDSAg-specific T cells inducing monophasic EAU. Most of the upregulated genes are involved in mechanisms that lead to prolonged survival and activation of T cells. This indicates that T cells from monophasic and relapsing disease are basically equipped with the same features, but T cells inducing relapsing disease show a stronger expression of genes associated with regulatory pathways of antigen presentation and activation as well as the WNT/Hedgehog (HH) pathways. We could define 26 upregulated genes in R14-specific T cell lines, belonging to different signaling pathways that are connected with each other (Figure 7).

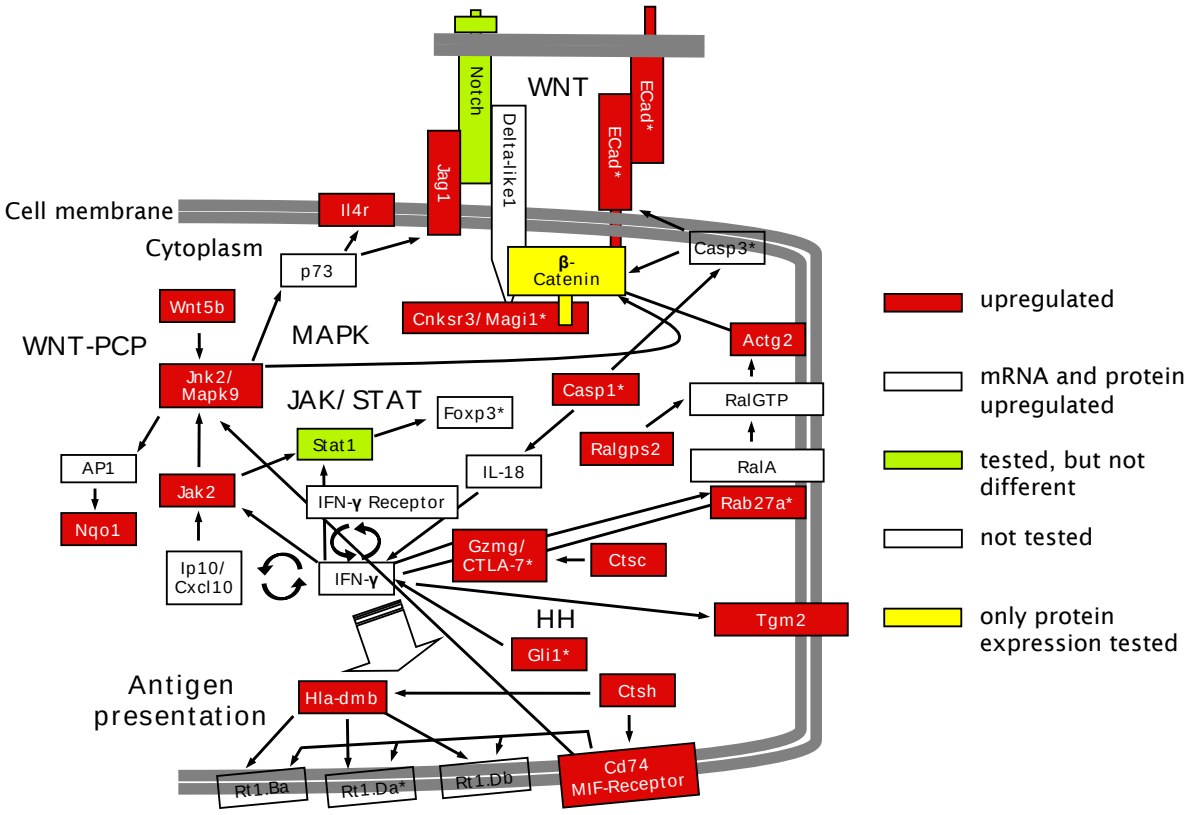


Figure 7 Signal transduction pathways of regulated genes in R14-specific T cells. Transcriptomic profiling of PDSAg- and R14-specific T cells resulted in the detection of upregulated genes in R14-specific T cells. These genes participate in different intracellular signaling pathways such as WNT, Hedgehog (HH), mitogen-activated protein kinase (MAPK) and antigen presentation pathway. * indicates significantly upregulated genes. (Courtesy of Gerhild Wildner).

WNT/HH signaling pathways

Many of the significantly upregulated genes contribute to the WNT and to the HH signaling pathway, which are as well interconnected. Both signaling pathways are evolutionarily conserved and use several related or even identical factors. They do not only play important roles in embryogenesis, but also contribute to proliferation, cell polarity, cell movement and survival of cells, which also explains their participation in cancer (Logan and Nusse, 2004; Lum and Beachy, 2004). WNT signaling is involved in induction of long-lived self-renewing populations of memory T cells (Zhao et al., 2010). In autoimmune responses, memory T cells play a central role. Among the significantly upregulated genes in R14-specific T cells E-cadherin, connector enhancer of kinase suppressor of ras 3 / membrane-associated guanylate

kinase-interacting protein-like 1, glioma-associated oncogene homolog 1 and caspase 3 (Ecad, Cnksr3/Magi1, Gli1 and Casp3) contribute to the WNT and HH pathways. E-cadherin is a transmembrane protein and is important for cell adhesion. Its cytoplasmic domain is linked via β-catenin to CNKRS3/MAGI1, which is also significantly increased in R14-specific T cells. The cleavage of E-cadherin or CNKRS3/MAGI1 by caspase 3, which is also upregulated in R14-specific T cells, leads to the release of β-catenin that can translocate to the nucleus and act as a transcription factor. Another transcription factor Gli1, which is significantly increased in R14-specific T cells, is part of the HH signaling pathway that suppresses apoptosis and induces proliferation of activated $CD4^+$ T cells (Lowrey et al., 2002). The upregulation of these genes in T cells capable of inducing relapsing disease could be one explanation for their prolonged survival in the eye, even after resolution of the first attack of disease and their ability to become reactivated and induce relapses. By contrast, PDSAg-specific T cells that induce monophasic disease show no upregulation of genes involved in WNT and HH signaling pathways and could not be detected in high numbers in the eyes after resolution of EAU.

Antigen presentation

A further group of upregulated genes including RT1 class II locus DA, CD74, cathepsin H and MHC class II DM beta (Rt1.Da, Cd74, Ctsh and Hla-dmb) could be grouped together as they are related to antigen presentation. Rt1.Da is a rat MHC class II molecule, equivalent to human HLA-DR and mouse I-E, and is additionally the restriction element of R14-specific T cells. CD74, CTSH and HLA-DMB are involved in peptide loading onto MHC class II molecules. Initially, it seems uncommon that T cells upregulate genes important for antigen presentation. However, this phenomenon was already observed in humans as well as in rats and is associated with T cell activation (Gansbacher and Zier, 1989; Taams et al., 1999). In addition, it could be demonstrated that T cells expressing MHC class II molecules acquire B7 costimulatory molecules from APCs and can therefore act as antigen presenting cells (Sabzevari et al., 2001). This mechanism may be important for the amplification of the specific immune response. In case of the R14-specific T cell line, this could be a mechanism allowing long-lasting immune responses and could probably be important for reactivation of T cells during relapses. However, memory T

cells, which acquire B7 costimulatory molecules, die by apoptosis (Sabzevari et al., 2001). Moreover, expression of MHC class II molecules on T cells is also associated with T cell anergy. If T cells are activated by antigen presenting T cells, they cannot be restimulated by professional APCs and become anergic (Taams et al., 1999). This could lead to the termination of the immune response and to the resolution of disease. In case of MHC class II expressing R14-specific T cells one could speculate that they may present antigen to regulatory T cells and subsequently anergize them, thus escaping from regulation. On the other hand, it is also possible that anergic effector T cells become reactivated and induce relapses.

CD74 has dual roles in the immune response. Besides its role as invariant chain (Ii), which blocks the peptide binding groove of MHC class II molecules to prevent peptide binding in the ER, CD74 can also be expressed on the surface of T cells and act as a receptor for MIF. In the latter function it induces the expression of pro-inflammatory genes and is involved in proliferation and inhibition of apoptosis (Leng et al., 2003). This emphasizes the pro-inflammatory role of R14-specific cells in relapsing disease as well.

IFN-γ signaling pathway

Another gene possibly contributing to the pro-inflammatory status of R14-specific T cells is Casp1. The protease Caspase-1 processes IL-1β and IL-18 from their inactive pro-cytokines. IL-18 was originally discovered as an IFN-γ inducing factor (Okamura et al., 1995); however, it has pleiotropic effects and can also induce T_H2 (Nakanishi et al., 2001) and T_H17 responses (Lalor et al., 2011).

IFN-γ, which is upregulated in R14-specific T cells on the mRNA as well as protein level, is the signature cytokine of T_H1 cells. It induces interferon gamma-induced protein 10 / interferon-inducible cytokine 10 (IP-10/CXCL10) that is also increased in R14-specific T cells and functions as a chemoattractant for monocytes/macrophages, T cells and NK cells. It also promotes T cell adhesion to endothelial cells and thus may facilitate the traffic through the blood-retina-barrier, potentially explaining the earlier onset of R14-induced as compared to PDSAg-mediated EAU. IP10/CXCL10 acts via Janus Kinase 2 (JAK2), which is upregulated in R14-specific T cells and is an important component of the JAK/STAT (signal transducer and activator of

transcription) pathway. JAK2 is critical for IL-12 signal transduction and T_H1 cell differentiation (O'Shea and Plenge, 2012).

Foxp3 expression

Most of the upregulated genes are closely connected with inflammation or proliferation. An exception is Foxp3, which is the master transcription factor of regulatory T cells (Fontenot et al., 2003). However, in addition to its expression in regulatory T cells, Foxp3 is also expressed in activated T effector cells in humans, but only transiently and at a lower level than in regulatory T cells (Allan et al., 2007). The expression of Foxp3 in R14-specific T cells lines could thus rather represent the activated status of the T cells and thus do not necessarily indicate a regulatory phenotype.

Regulated genes with unexplained functions

Some of the upregulated genes are involved in the release of cytotoxic granules, such as granzyme G, cathepsine C and ras-related protein 27A (Gzmg, Ctsc and Rab27a). However, their exact functions in T helper cells have not been completely elucidated.

In principle, all of the upregulated genes support the pro-inflammatory role of R14-specific T cells and are probably the determining factors for induction of relapses in R14-induced EAU, in contrast to the monophasic EAU-inducing PDSAg-specific T cells, where these genes were not upregulated.

6.1.2 Comparison of transcriptomic and protein analyses

As mentioned above, mRNA levels do not always reflect protein levels. For a few genes, the corresponding protein levels were analyzed with the help of specific antibodies directed against rat and mouse proteins. Unfortunately, several of the

mouse-specific antibodies, which were purchased as being crossreactive with rat proteins, did not bind the respective rat targets.

In case of rat MHC class II RT1.B and RT1.D increased gene expression in R14-specific T cells was confirmed by flow cytometry. Upregulation of the IFN-γ gene could be confirmed by cytokine secretion assays and intracellular cytokine detection. Both methods showed an upregulation of IFN-γ in R14-specific T cells compared to PDSAg-specific T cells. A discrepancy was detected with respect to IL-17. Although there was no verifiable difference in the mRNA levels of IL-17 between PDSAg- and R14-specific T cell lines, there was an increase in intracellular and secreted IL-17 protein amounts in PDSAg-specific T cells detected. For the determination of secreted cytokines cell culture supernatants were collected daily, pooled and analyzed in order to detect early as well as late secreted cytokines. The detection of cytokines in culture supernatants represents the difference between the amount of secreted cytokines and those used by the cells. Accordingly, the analysis of intracellular cytokine expression seems to be a useful method to obtain better information about the status of single T cells and cell populations. Thus, this method was used in further experiments to determine T cell profiles *ex vivo*.

As IFN-γ and IL-17 were detected in PDSAg- as well as in R14-specific T cell lines, both consist of T_H1 and T_H17 cells. Nevertheless, according to the cytokine profile detected in cell supernatants and intracellularly, R14-specific T cells display rather a T_H1 profile and PDSAg-specific T cells may be predominantly T_H17 cells.

6.1.3 Increased stability of T cells inducing relapsing EAU *in vivo*

To correlate the data from the transcriptomic profiling with the *in vivo* situation adoptive transfer studies with GFP^+ PDSAg- or R14-specific T cells were performed. These studies revealed prolonged survival of R14-specific T cells in the eyes of animals with relapsing uveitis. Although PDSAg-specific T cells were also detectable in the eyes of animals after resolution of monophasic uveitis, only low numbers of PDSAg-specific T cells were found. By contrast, clusters of GFP^+ R14-specific T cells were detected in eyes during relapses. These results suggest that R14-specific pathogenic T cells have an extended survival in the target organ of the autoimmune

disease and thus have a better chance to induce relapses by reactivation and proliferation.

We therefore decided to look at intraocular T cells at different stages of both types of EAU with respect to the expression of cytokines and Foxp3 as a potential marker of T_{reg} cells.

6.2 Cytokine expression profile of monophasic and relapsing intraocular T cells

The cytokine expression profile of T cells gives some indication of their function during autoimmune responses, especially when analyzed *ex vivo*, without additional cell culture steps that could distort cytokine expression. Of particular interest are T cells from the target organ of the autoimmune reaction, namely the eye.

However, it should be considered that the intracellular cytokine expression is not always congruent with the effective secretion of cytokines. Nevertheless, the data from intracellular cytokine analysis are sufficient to determine the T cell status and their potential contribution to immune reactions.

6.2.1 High frequency of cytokine producing cells in inflamed eyes, but not in peripheral lymph nodes

After induction of EAU by immunization with the autoantigen peptides PDSAg or R14 in CFA, cells from eyes and peripheral lymph nodes were isolated at several time points during the disease course (onset, peak, resolution and relapse). *Ex vivo* staining of these cells for intracellular cytokine expression revealed high frequencies of cytokine-producing cells only in the eyes but not in the lymph nodes draining the site of immunization. These observations were found in both, monophasic as well as relapsing disease. Consequently, the investigation of the target organ is absolutely necessary to obtain information about ongoing immune reactions and participating inflammatory cells. *Ex vivo* data from lymphoid organs in the periphery only are not representative for inflammation in the target organ and differences during the disease

course would not be recognized. However, after several restimulations with the respective antigen *in vitro*, cells from draining lymph nodes can be enriched for antigen-specific T cells, resulting in T cell lines that produce high amounts of inflammatory cytokines. Such T cells were used for the transcriptomic analysis and gave the first hint on differences with respect to the cytokine profiles of the T cells inducing monophasic and relapsing EAU.

Moreover, high frequencies of IFN-γ IL-17 double positive cells were only detected in the eye but not in the peripheral lymph nodes. This suggests that the ocular environment influences the plasticity of invading T cell populations. Once inflammation occurs in the eye, the anti-inflammatory milieu of healthy eyes is displaced by a pro-inflammatory milieu. IL-12 and IL-23, which are pro-inflammatory cytokines, can lead to conversion of IL-17-producing cells into IFN-γ-secreting T_H17 cells in the absence of TGF-β (Lee et al., 2009). Induction of T_H17 cells is different in mice and humans, but nothing is known about rats. Since T_H17 cells exhibit a higher plasticity than T_H1 cells (Lee et al., 2009), more double positive cells are found in monophasic EAU, where also more T_H17 cells were present. In addition, the double positive cells increased over time in monophasic EAU (section 6.2.4).

6.2.2 Antigen specificity of intraocular cytokine-producing T cells

The lymphocyte population isolated from inflamed eyes consisted mainly of T cells expressing the αβTCR. A very small population (about 2%) of γδTCR positive T cells was also detected, but they did not express inflammatory cytokines such as IFN-γ or IL-17. Thus they seem to play a minor role in uveitis in the rat model. This is in contrast to a mouse model of EAU, where higher numbers of γδ T cells were identified. These γδ T cells showed pro-inflammatory properties and could enhance EAU in mice (Nian et al., 2011).

To determine the antigen specificity of intraocular T cells, they were stimulated with their corresponding peptides *in vitro* and cytokine expression was measured. Unfortunately, antigen specificity of intraocular cells could not clearly be detected by this method. The presence of antigens from the eye could not entirely be excluded from the single cell suspension of intraocular cells, which included APCs. Since MHC

tetramer staining is not available for the rat antigens, which were used here, it is difficult to determine the antigen specificity of intraocular T cells.

6.2.3 Different MHC class II restriction of PDSAg- and R14-specific T cells

PDSAg- and R14-specific T cells induce different disease courses and hence have to possess different characteristics. One of them is the different MHC class II restriction of the T cell lines. While PDSAg-specific T cells recognize their antigen presented on RT1.B, which is the rat equivalent of HLA-DQ, R14-specific T cells recognize their antigen in context with RT1.D, the equivalent to HLA-DR.

The primary contact of naïve T cells with MHC/peptide on APCs is a very important step for the T cell lineage commitment. The presenting MHC molecule determines not only the $CD4^+$ or $CD8^+$ phenotype of the T cell, but the subtype of the presenting MHC class II molecule also influences T cell subset. The APC receives different signals depending on the MHC class II molecule bound by the T cell receptor, which, in turn results in different T cell activation. In human monocytes it was demonstrated that binding of specific antibodies to different MHC class II molecules results in transmission of distinct signals, activation of different mitogen-activated protein kinases (MAPK) and release of different cytokines. Binding of HLA-DQ (equivalent of RT1.B, presenting for PDSAg-specific T cells) induced rather anti-inflammatory cytokines, while binding of HLA-DR (equivalent of RT1.D, presenting for R14-specific T cells) resulted in release of more pro-inflammatory cytokines. These different cytokines may affect T cell development during the first antigen contact (Matsuoka et al., 2001). Therefore, R14-specific T cells that recognize their antigen presented on RT1.D may become activated in a more pro-inflammatory milieu and preferably produce IFN-γ, in contrast to PDSAg-specific T cells that recognize their antigen on RT1.B and are activated in a rather anti-inflammatory milieu. It is possible that the R14-specific T cells are more pro-inflammatory and can therefore induce relapses, while the PDSAg-specific T cells do not get a strong pro-inflammatory signal and therefore are not able to promote recurrent disease. Differences during the first contact with activating antigen may play a pivotal role in the life of T cells and may finally determine the course of uveitis.

6.2.4 Differences in expression of IFN-γ and IL-17 in monophasic and relapsing disease

Since T_H1 and T_H17 cells play key roles in T cell-mediated autoimmunity, both T_H1 and T_H17 cells are also involved in EAU, and the signature cytokines IFN-γ and IL-17 are of particular importance in mice (Luger et al., 2008). Their influence in monophasic and relapsing disease is not yet completely elucidated and may be important for the decision of a monophasic or relapsing disease outcome. Therefore, we analyzed the expression pattern of the cytokines IFN-γ and IL-17 during the disease course of monophasic and relapsing EAU. Surprisingly, not only IFN-γ^+IL-17^- and IL-17^+IFN-γ^- cells were detected, but also IFN-γ^+IL-17^+ cells were found in inflamed eyes. These double positive T cells probably emerge from T_H17 cells, because they exhibit high plasticity (Muranski and Restifo, 2013). The switch from T_H17 to T_H1 was previously demonstrated for EAU in mice (Shi et al., 2008). A conversion from T_H1 to T_H17 cells would also be conceivable under certain circumstances, which was demonstrated by epigenetic modifications (Wei et al., 2009). In our model, the numbers of T cells expressing both IFN-γ and IL-17 increased during the disease course of monophasic EAU, while only few of these cells were detectable during relapsing EAU. Due to the fact that numbers of these cells did not increase during relapses, it seems that they are not crucial for the induction of relapses and may rather play an inhibitory role in monophasic EAU. An anti-inflammatory role of IL-17 was described previously in EAU in rats. Administration of IL-17 increased the number and regulatory activity of Foxp3^+ T_{reg} cells and subsequently decreased the number of relapses (Ke et al., 2009). In our model further experiments revealed the expression of IL-10 in some of the IFN-γ^+IL-17^+ cells, which supports their potential suppressive role in EAU.

We showed that IFN-γ^+IL-17^- as well as IL-17^+IFN-γ^- populations remained stable during monophasic EAU, while in relapsing EAU dynamic changes were detected. The numbers of IFN-γ^+IL-17^- cells continuously increased during the disease course of relapsing EAU in contrast to the IL-17^+IFN-γ^- cell population, which decreased. It can therefore be speculated that IL-17 may play an important role during disease induction, but is not involved in relapses. The suggestion that T_H17 cells form the first wave of T cell infiltration was already proposed for EAE (Reboldi et al., 2009) and could also hold true for EAU. Treatment of rats with anti-IL-17 antibodies before and

during onset of EAU resulted in a delayed onset and attenuation of EAU (Zhang et al., 2009). IFN-γ^+ cells, which we found in highest numbers during relapses, seem to be responsible for the induction of recurrences in relapsing rat disease. This was further supported by the intraocular administration of recombinant rat IFN-γ after the resolution of the first attack of EAU, which subsequently induced relapses in R14-induced EAU (section 6.2.5).

6.2.5 Enhanced frequency of relapses after intraocular administration of recombinant IFN-γ in relapsing uveitis

To confirm our hypothesis that IFN-γ is the crucial cytokine for induction of relapses we have injected recombinant rat IFN-γ intraperitoneally or into the eyes of animals after the first attack of disease had subsided. Three days after receiving intraocular IFN-γ the rats showed synchronized relapses of uveitis. Moreover, the intensity of the relapses was higher compared to the control group receiving intraocular phosphate buffered saline (PBS) only. Intraperitoneal injection of IFN-γ had no influence on the relapses. We speculate that resting effector T cells in the eye can be locally reactivated by intraocular application of IFN-γ and thus induce relapses by recruiting inflammatory cells.

6.2.6 Foxp3$^+$ T cells in monophasic and relapsing disease

Besides an autonomous regulatory program within T cells inducing uveitis, which we proposed for T cell lines examined in the gene arrays, there may also be regulatory T cells contributing to the resolution of uveitis. In the following we further investigated the role of regulatory T cells in EAU. However, the regulatory function of Foxp3$^+$ T cells can only be assumed, because activated effector T cells also transiently express Foxp3 in rats. However, from protein expression data alone we cannot determine whether Foxp3 has a regulatory function or not, since the phosphorylation of the molecule plays an important role. Only phosphorylated Foxp3 is functioning as a regulator in T_{reg} cells in humans (Nie et al., 2013).

Since regulatory T cells are crucial to control autoimmunity (Jager and Kuchroo, 2010), they may also play a role in the remission of monophasic experimental

autoimmune uveitis. When we investigated the Foxp3 expression of intraocular T cells during the course of disease, an increase in the number of $Foxp3^+$ T cells was found at the resolution of monophasic EAU. However, similar observations were made for relapsing uveitis, where also an increase of $Foxp3^+$ T cells was detectable during the resolution. Furthermore, despite these high numbers of $Foxp3^+$ T cells relapses occurred in R14-induced EAU. There are several explanations for the detection of $Foxp3^+$ cells in both, monophasic as well as relapsing uveitis. As shown by the results from the gene arrays, Foxp3 was also upregulated in activated R14-specific T cell lines. Similar observations are known from humans, where activated T cells transiently express Foxp3 (Allan et al., 2007). However, in our case, cytokine analysis of $Foxp3^+$ cells revealed no coexpression of inflammatory cytokines like IFN-γ or IL-17, which makes an activated effector T cell phenotype unlikely. It is possible that the $Foxp3^+$ cells in eyes of relapsing EAU exhibit a weaker inhibitory function compared to $Foxp3^+$ cells from monophasic EAU, as previously speculated for the Lewis rat model of EAU (Ke et al., 2008). Similar findings were observed in EAE. $Foxp3^+$ regulatory T cells isolated from the CNS of animals suffering from EAE could only inhibit naïve autoantigen-specific T cells, but failed to suppress effector autoantigen-specific T cells isolated from the CNS. These cells secreted IL-6 and TNF that impaired the function of regulatory T cells (Korn et al., 2007). It is therefore possible that $Foxp3^+$ T cells in relapsing EAU are not strong enough to inhibit the highly activated R14-specific effector T cells. By contrast, $Foxp3^+$ T cells of monophasic EAU seem to have a higher regulatory potential and can probably suppress PDSAg-specific effector T cells to resolve uveitis. On the other hand, increased numbers of $Foxp3^+$ T cells were identified during late remission of monophasic EAU, where the eyes showed no signs of clinical inflammation over an extended period of time. In relapsing EAU significantly lower numbers of $Foxp3^+$ T cells were discovered during late remission, maybe allowing for the emergence of relapses (section 6.2.8).

6.2.7 Expression of IL-10 alone and in combination with other cytokines

Not all regulatory T cells express the transcription factor Foxp3. Therefore, we have also looked for cells expressing the regulatory cytokine IL-10. Surprisingly, IL-10 expression was also found in T cell subsets with an otherwise effector phenotype.

Combinations with IFN-γ and/or IL-17 were detected. This unusual cytokine combination can be explained by the plasticity of T helper cell subsets (Zhu and Paul, 2010) and was demonstrated for T_H1 (Anderson et al., 2007; Jankovic et al., 2007) and T_H17 cells (McGeachy et al., 2007). Under certain circumstances, such T cells can also produce an inhibitory cytokine like IL-10 in addition to their effector cytokine. For example, T_H17 cells that were generated in the presence of TGF-β and IL-6 can coexpress IL-17 and IL-10, equipping them with regulatory functions (McGeachy et al., 2007). Moreover, a cell intrinsic regulatory mechanism seems to be reasonable to reduce inflammatory responses and protect against tissue damage. This so-called negative feedback loop can also dampen autoimmunity. In case of T_H1 cells it was demonstrated that these IFN-γ-producing cells themselves are the main source of IL-10, which prevents immunopathology (O'Garra and Vieira, 2007).

A regulatory role for IL-10 in autoimmunity, especially in EAU, was already identified some years ago (Rizzo et al., 1998). As our results demonstrate, the sources of IL-10 are in part effector cells expressing IFN-γ and IL-17. Both IFN-γ^+IL-10^+ as well as IL-17^+IL-10^+ populations increased during monophasic EAU and decreased during relapsing EAU. The significant difference in IL-10-expressing cells between monophasic and relapsing uveitis is probably the reason why relapses are restrained in PDSAg-mediated EAU and why they can occur in R14-induced EAU. In addition, combination of inhibitory and effector cytokines could result in a synergistic effect. For IFN-γ it was previously shown that it could increase the regulatory effect of IL-10 (Yanagawa et al., 2009). The same could probably be true for IL-17 in combination with IL-10.

Signals that induce IL-10 production in T_H1 cells were recently described. Essential factors are high antigen dose and consequently strong TCR triggering, in the presence of endogenous IL-12 (Gabrysova et al., 2009; Saraiva et al., 2009). Additionally, the transcription factors STAT4 and extracellular signal-regulated kinase (ERK) that induce expression of IL-10 are necessary (Saraiva et al., 2009). Notch signaling has also the ability to induce IL-10 via STAT4 in T_H1 cells (Rutz et al., 2008). For IL-10 expression by T_H17 cells STAT3 and c-Maf as well as ERK seem to be the crucial transcription factors (Stumhofer et al., 2007; Xu et al., 2009). ERK is negatively regulated by Cnksr3/Magi1 (Ziera et al., 2009). As our results from the transcriptomic analyses showed a 9.4 fold increase of Cnksr3/Magi1 in R14-specific

compared to PDSAg-specific T cells, the lower IL-10 expression in relapsing disease could be explained.

In addition to the effector T cells, which co-produce IL-10, there are also IL-10$^+$ T cells coexpressing neither IFN-γ nor IL-17. Since these cells are Foxp3-negative, they could be T_R1 cells that are characterized by secretion of IL-10 and lack of Foxp3 expression. However, no lineage-specific surface marker or transcription factor for clear identification of T_R1 cells is so far known. Thus, these cells could not be definitely assigned to T_R1 cells. Nevertheless, the immunosuppressive function of T_R1 cells in autoimmunity has been demonstrated in many cases (Carter et al., 2012; Groux et al., 1997; Pot et al., 2011).

IL-10 is a pleiotropic cytokine and exhibits different immunoregulatory functions. Many regulatory effects of IL-10 on T cells are indirect via monocytes/macrophages or DCs. Nevertheless, IL-10 can act directly on T cells and inhibit proliferation and cytokine production by inducing anergy (Joss et al., 2000). Due to the lack of the availability of other cytokine-specific antibodies for intracellular staining we were not able to investigate other cytokines coexpressed with IFN-γ, IL-17 and/or IL-10. Since activated rat T cells express CD4 and CD25 like natural T_{reg} cells (Stephens et al., 2004; Thurau et al., 2004), we abstained from using these markers for the characterization of potential T_{reg} cells.

6.2.8 Increased numbers of regulatory T cells during late remission of monophasic uveitis in the eye

During late remission, which was defined as the time point after subsidence of intraocular inflammation, when the eyes did not show any signs of inflammation over an extended period, intraocular cells were investigated for regulatory T cell markers. In monophasic EAU the numbers of both Foxp3$^+$ as well as IL-10$^+$ expressing T cells were increased compared to relapsing EAU, where only low amounts of both T cell types were detected. The fact that increased numbers of potential regulatory T cells are present in eyes without relapses could be a hint for the prevention of relapses. Furthermore, the lack of high numbers of regulatory T cells in the eye, like in R14-induced EAU, may allow relapses.

6.2.9 Contradictory effects of inflammatory cytokines

For both IFN-γ and IL-17, pro-inflammatory as well as anti-inflammatory effects in autoimmunity have been described. The pathogenic or protective functions are dependent on the model of uveitis and on the stage of disease during which they are expressed. Early production of IFN-γ, induced by IL-12 administration, prevented the development of uveitis in mice (Tarrant et al., 1999). Later IL-12 treatment had no regulatory effect. As shown by intraocular application in our model IFN-γ has a pro-inflammatory role promoting the induction of relapses, which are late events during disease. A regulatory role for IL-17 in rat EAU was demonstrated by others through the treatment of animals with recombinant IL-17 after immunization for uveitis induction. In this case, IL-17 inhibited IFN-γ-producing effector T cells and thus EAU was suppressed in these animals (Ke et al., 2009). These findings match our results that also suggest a regulatory role for IL-17 in monophasic disease, while on the other hand high numbers of IL-17$^+$ cells in the early phase of relapsing uveitis suggests promotion of the invasion of autoreactive T cells. The increasing levels of IL-17 during the resolution of the disease, may counter-regulate IFN-γ-expressing T cells and thereby prevent relapses.

Due to the contradictory roles of IFN-γ and IL-17 it is difficult to develop therapies for uveitis patients using these cytokines. Until now there are no anti-IFN-γ therapies available for uveitis patients. In multiple sclerosis a clinical trail that was aimed at the protective effects of IFN-γ, resulted in exacerbation of disease and had to be terminated (Panitch et al., 1987). Blocking IFN-γ will result in a profound impairment of the immune defense against bacteria, viruses as well as tumor cells and thus should be avoided. Currently, clinical trails with an anti-IL-17 antibody (AIN457) for the treatment of non-infectious uveitis patients are ongoing, however with moderate effects (http://clinicaltrials.gov/ct2/show/NCT00685399).

6.2.10 The role of regulatory T cells in EAU

Based on the brief time span between adoptive transfer of uveitogenic effector T cells and the resolution of disease, we have proposed an autonomous program within the effector T cells, resulting in intrinsic regulatory functions of these cells rather than generation of regulatory T cells during the inflammation. However, data gained from

gene arrays did not reveal an upregulation of suppressive molecules in PDSAg- compared to R14-specific T cell lines. Later investigations on the protein level namely intracellular cytokine analysis demonstrated coexpression of IL-10 and effector cytokines such as IFN-γ and IL-17. This points to an intrinsic program of effector T cells that protects against tissue damage due to a negative feedback loop. On the other hand, Foxp3-expressing T cells were detected in monophasic as well as in relapsing EAU. An increase of $Foxp3^+$ cells was detected in monophasic disease during late remission and the percentage of these cells was significantly different compared to relapsing EAU, where only low numbers of $Foxp3^+$ cells were detectable. We defined these cells as regulatory T cells, because these $Foxp3^+$ T cells did neither express IFN-γ nor IL-17 and thus a conversion from effector T cells seems to be unlikely.

Consequently, our data favor a model, which combines an autonomous regulatory program in effector T cells and the generation or recruitment of regulatory T cells to resolve inflammation in EAU.

6.3 Outlook

Our data demonstrate that monophasic and relapsing uveitis in Lewis rats differ with respect to the gene expression profile of the initiating effector T cells and the cytokine pattern of the intraocular T cell populations during the course of the disease. Both types of analysis have revealed that IFN-γ plays a pivotal role in the induction of relapses. Although we detected increased levels of IFN-γ in relapsing disease and could induce synchronized relapses after administration of recombinant rat IFN-γ, a definite proof is still missing.

To dissect the role of IFN-γ- and IL-17-producing autoantigen-specific T cells it would be desirable to separate them and investigate their pathogenic capacity and the disease course after adoptive transfer into naïve rats. Cell separation may be achieved by cytokine capture assays that would allow separation of cells dependent on their release of distinct cytokines. However, so far we could not obtain reliable results with this method. Probably because the amount of secreted cytokines by

single cells is unpredictable and thus result in binding to non-secreting neighboring cells. Separation of T_H17 cells due to surface markers also failed in the rat model. The surface markers described for human IL-17-producing cells are CD161 (NK receptor P1A) (Maggi et al., 2010) and CCR6, the receptor for CCL20 (Singh et al., 2008). IL-17-producing cells in the rat do not express these molecules. Moreover, since we have considerable populations of cells coexpressing both cytokines (IFN-γ and IL-17), isolation of either cell type would always result in the contamination of the population with cells coproducing the other cytokine. Due to the dynamics of T cell populations we will not know whether and how fast any T_H1 or T_H17 population would change its phenotype.

The exact contributions of cytokines in EAU could be further determined by neutralization with antibodies or administration of recombinant cytokines during different stages of disease. For instance, neutralization of IL-10 in monophasic EAU may lead to the induction of relapses by impairing the anti-inflammatory milieu. Otherwise, administration of recombinant IL-10 could probably prevent relapses in animals immunized for relapsing EAU. However, the only available cytokines are recombinant proteins produced in *Escherichia coli*. These proteins contain an N-terminal methionine and some even lack several N-terminal amino acids (for example: recombinant rat IL-10 from R&D comprises only the sequence from Ser19-Asn178). According to experiments with N-terminally methionylated RANTES (Met-RANTES) in rat EAU (Diedrichs-Mohring et al., 2005) the function of these in *Escherichia coli* produced cytokines may be unpredictable or contradictory *in vivo* and *in vitro*.

Investigations of regulatory T cells revealed $Foxp3^+$ as well as $IL\text{-}10^+$ T cells. Numbers of $Foxp3^+$ T cells were similar during acute inflammation in monophasic and relapsing disease, while populations of $IL\text{-}10^+$ T cells were increased in monophasic EAU. The regulatory potential of $Foxp3^+$ as well as of $IL\text{-}10^+$ T cells could further be analyzed by *in vitro* assays, measuring the impact of regulatory T cells on responder cells. Another way to determine the regulatory potential would be adoptive transfer of regulatory T cells into animals immunized for uveitis induction. Their effect could be directly measured by the clinical score of uveitis and additionally by alterations in number and cytokine profile of effector T cells. Thus, difference between regulatory T cells from monophasic and relapsing disease could be

revealed. Separation of IL-10-expressing T cells could be achieved by cytokine capture assay (described above for IFN-γ and IL-17), while for Foxp3 separation transgenic animals expressing GFP in $Foxp3^+$ cells are needed.

To address the question whether regulatory T cells are reprogrammed effector T cells or whether they are generated and recruited in response to the inflammation, one could use GFP^+ effector T cells and adoptively transfer them into naïve animals. Isolation of cells from the inflamed eyes and analysis of changes in Foxp3- and IL-10-expression in GFP^+ as well as in GFP^- cells could reveal their origin. If the regulatory T cells are GFP^+, they would have been reprogrammed effector cells and if the regulatory T cells are GFP^-, they have been generated in the recipient animal and are recruited to the inflamed eye.

To better characterize the different T helper cell subsets, expression of master transcription factors such as T-bet in T_H1 cells and RORγt in T_H17 cells should be analyzed in combination with the signature cytokines. Thereby, T helper cell subsets responsible for relapses could be better determined and in addition T helper cell origin of cells producing multiple cytokines could be detected. This could also give hints to T cell plasticity and phenotype switch of T helper cells. However, one has to be careful with the detection of the master transcription factors, because they are not completely specific for their T helper cell subset. For instance, RORγt expression was detected in $Foxp3^+$ cells in mice (Lochner et al., 2008). Therefore, cells should be stained simultaneously for different markers to better characterize them.

Due to the limits in terms of reagents and transgenic or knockout rats we could to date not investigate all aspects of the immune responses in monophasic and relapsing EAU.

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Author’s declaration

Ich erkläre hiermit an Eides statt,

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München, November 2013 Ulrike Kaufmann