Endocrine control of T cell function and its implications for the pathogenesis of neuroinflammatory diseases

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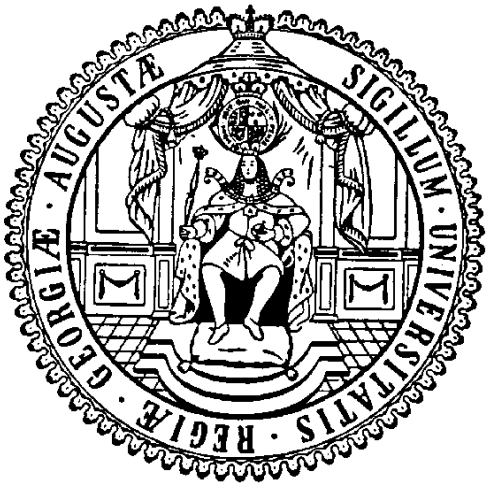

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Date of the oral examination: ……..

affidavit

I hereby declare that I have written this PhD thesis entitled “Endocrine control of T cell function and its implications for the pathogenesis of neuroinflammatory diseases” independently and with no other sources and aids than quoted. This thesis has not been submitted elsewhere for any academic degree.

Paragraphs of the introduction (1.2-1.5) have been partly published in the review (Fischer et al., 2013).

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Henrike J. Fischer

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abbreviations

11b-HSDII	11b-hydroxysteroid dehydrogenase type II
7-AAD	7-aminoactinomycin
ADP	adenosine diphosphate
APC	antigen presenting cell
AT	adoptive transfer
ATP	adenosine triphosphate
AxV	AnnexinV
BBB	blood brain barrier
BSS	balanced salt solution
CD	cluster of differentiation
CFA	complete freund's adjuvant
CNS	central nervous system
CTL	cytotoxic T lymphocyte
DCA	dichloroacetate
Dex	Dexamethasone
DM	Diabetes Mellitus
DNA	desoxyribonucleic acid
Dox	doxycycline
DPC	distal pole complex
dsRNA	double-stranded RNA
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
eGFP	enhanced Green Fluorescent Protein
ER	endoplasmatic reticulum
ERM	ezrin/radixin/moesin
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
GA	glatiramer acetate
GC	glucocorticoid
GR	GC receptor
h	hour

HLA	human leukocyte antigen
HSP	heat shock protein
IC	intracellular
Indo-1	2-[4-(bis(carboxymethyl)amino)-3-[2-[2-(bis(carboxymethyl)amino)-5-methylphenoxy]ethoxy]phenyl]-1H-indole-6-carboxylic acid
InsR	insulin receptor
kd	knockdown
MACS	magnetic associated cell sorting
MHC	major histocompatibility complex
min	minute
MOG	myelin oligodendrocyte glycoprotein
MR	mineralocorticoid receptor
MS	Multiple Sclerosis
mTEC	medullary thymic epithelial cell
MTOC	microtubule-organizing centre
ON	over night
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDK1	pyrovate dehydrogenase kinase 1
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositole (3,4,5) trisphosphate
PLP	proteolipid protein
RA	Rheumatoid Arthritis
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RT	room temperature
SA	streptavidin
SDS	sodium dodecylsulfate
SEM	standard error of mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
T1DM	Type 1 Diabetes Mellitus

T2DM	Type 2 Diabetes Mellitus
TCR	T cell receptor
Tet	tetracycline resistance
TetO	Tet operon
TetR	Tet repressor
Th	T helper cell
Ub-p	ubiquitin C promotor
wt	wildtype

1. Introduction

1.1. T cell function and activation

The immune system defends the organism against pathogens, autoimmunity or cancer and clears established infections. It consists of different cell types that function together and communicate with each other in order to provide protection. The importance of the immune system becomes obvious with disturbances or immune deficiencies resulting in severe, even life-threatening consequences as autoimmune diseases, serious and chronic infections. The lymphatic system provides optimal surroundings for quick immune responses. Immune cells meet in the lymph nodes while circulating in the host and come in touch with other immune cells, which is a central aspect during all immune responses. This allows a targeted immune response adjusted to different pathogens.

The immune system is divided in adaptive and innate immunity. Cells of the latter system recognize general pathogenic patterns via invariant receptors as TOLL-like receptors and provide the first line of defence against pathogens. The adaptive immune system consists of lymphocytes as B and T cells having a variable repertoire of receptors to recognize specific antigens. Antigen presentation is the first important step for an immune reaction, leading to proliferation of antigen-specific cells and the improvement of immune responses (Friedl et al., 2005). Antigen presenting cells (APCs) take up pathogens or –derived proteins and present it to T and B cells. During this very first step the nature of the immune response is determined (Fig. 1). At this contact the lymphocytes are stimulated by their cognate antigen and receive co-stimulatory signals driving them to different effector cell phenotypes. B cells exert their functions by antibodies (humoral immunity), that can opsonise pathogens and subsequently lead to their clearance by phagocytotic cells such as macrophages. T cells on the other hand fulfil cell-mediated immunity. They can be divided into $CD4^+$ T cells, which comprise about two third of all T cells, and $CD8^+$ cytotoxic T cells and both types recognize antigen by binding of the T cell receptor (TCR) to a peptide in the context of MHC-molecules. CD4-molecules exclusively allow the interaction of TCR:MHC class II (Gay et al., 1987), whereas CD8-molecules interact with MHC class I molecules (Gay et al., 1987).

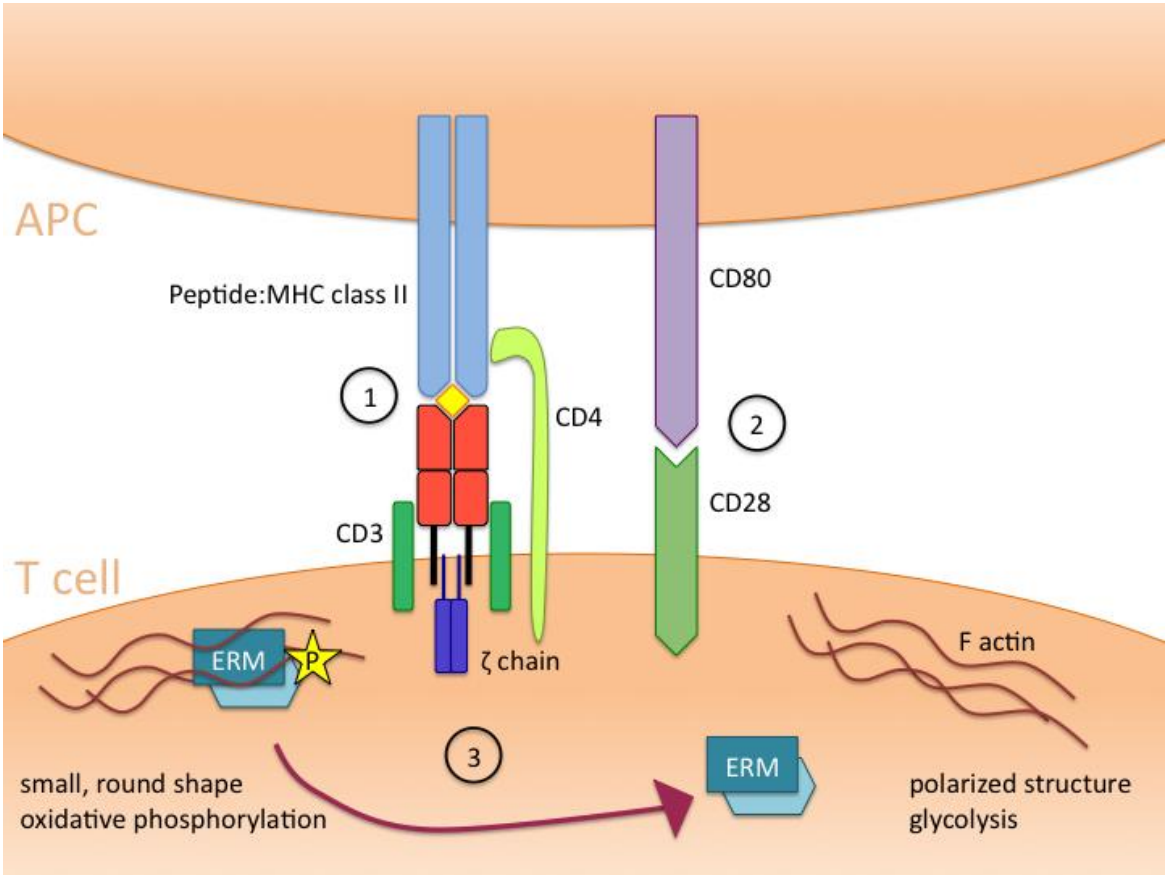

Fig. 1 Immunological synapse. T cell activation requires two distinct signals. The first signal is provided by the cognate antigen in context of MHC-II-molecules (1). To become fully activated a second signal is needed via interaction of CD28 with the costimulatory molecule CD80 (2). After activation rapid cytoskeletal rearrangements take place by dephosphorylation of ERM proteins and the cell metabolism adapts to the higher energy demand by switching from oxidative phosphorylation as major energy source to glycolysis (3). Adapted from (Friedl et al., 2005).

T cells evolve in the thymus and are positively selected for MHC recognition by the TCR and negatively to prevent survival of auto-reactive T cells. Special APCs in the thymus, the medullary thymic epithelial cells (mTECs), present auto-antigen to developing T lymphocytes and every T cell recognizing self-structures is sorted out (Hinterberger et al., 2010). mTECs express high amounts of major histocompatibility complex (MHC) molecules and peripheral antigens which are expressed in the thymus only for the purpose of T cell selection. This step in T cell development is crucial, because auto-reactive T cells that escape into the periphery can cause severe autoimmune diseases like Type 1 Diabetes, Multiple Sclerosis (MS) or Crohn's disease.

To prevent immune reactions without inflammatory environment, T cell activation in the periphery requires two distinct signals (Bretscher and Cohn, 1970; Bretscher, 1999; Fig. 1). The first signal, binding of the cognate antigen in MHC molecule

context (class I for $CD8^+$ T cells, class II for $CD4^+$ T cells), is necessary but not sufficient for T cells activation. A second, co-activatory signal is needed as the binding of CD28 on the T cell side to CD80 (B7) on APCs, in its absence T cells become anergic (Lenschow et al., 1992; Sperling and Bluestone, 1996).

$CD4^+$ T cells are also called T helper cells (Th cells), as they assist B cells to fulfil their appropriate functions. They are primed to different subtypes as Th1, Th2 or Th17 cells during the first encounter of an antigen presented by APCs. B cells can secrete antibodies having the same specificity as their B cell receptor. Depending on the pathogen different classes of antibodies can be produced. The T cell help determines the isotype of the secreted antibodies to provide optimal pathogen defence and is mediated by soluble triggers, so called cytokines. Th1 cells stimulate phagocyte-mediated immunity by secretion of a special set of cytokines including interferon γ (IFNγ). Th2 cells induce eosinophil or mast cell mediated immunity and trigger the secretion of antibodies by secretion of cytokines distinct from those expressed by Th1 cells as interleukin-4 (IL-4), IL-5 or IL-10 (Romagnani, 1999). Other subsets of Th cells like IL-17 secreting Th17 cells came more and more into focus during the last decades as central players in autoimmune diseases. For example, the development of Type 1 diabetes in BioBreeding rats is linked to an imbalance between Th17 and regulatory T cells (T_{reg} cells; van den Brandt et al., 2010).

About 5% of all $CD4^+$ T cells are T_{reg} cells that suppress auto-reactive or massively proliferating T cells (Papiernik et al., 1998). Thus, the induction peripheral tolerance is another central task of T cells. Because dysfunctions of the immune system regulation can have fatal outcomes like severe autoimmune diseases, proper T_{reg} cell function is essential (Heiber and Geiger, 2012; Taguchi and Takahashi, 1996).

$CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs), on the other hand detect cells that are harbouring intracellular pathogens and eliminate them (Blanden, 1974; Fung-Leung et al., 1991). They recognize peptides originating from cytoplasmatic proteins presented on MHC-I molecules such as viral proteins. They kill the infected cells to stop virus production. The killing is mediated by granule exocytosis targeted to the infected cell releasing perforin and granzymes.

Collectively, T cell mediated immunity and the proper control of immune responses are two central and essential parts during health and disease.

1.2. T cell morphology and migration

Activated T cells stop to circulate in the lymph and blood vessels and have to extravasate into target tissues. Subsequently, they have to be re-stimulated at the site of inflammation. To this end, T lymphocytes must adopt a unique morphology, which is crucial for migration and cell-cell-interaction. In general, lamellipodiae are formed on the leading edge of the cell whereas an uropod is found at the opposing pole. Part of the cell surface receptors and lipid rafts become confined to the uropod where they form the distal pole complex (DPC). In contrast, other surface molecules like integrins and chemokine receptors are concentrated at the leading edge where they serve to sense chemokines during crawling (Smith et al., 2005). These structural features are essential to allow correct movement of T cells (Krummel and Macara, 2006) and for directing their effector mechanisms such as the release of cytokines and cytotoxic molecules to the contact zone with other cells. As the cytoskeleton is crucial for morphological alterations to occur, inhibitors of F-actin polymerization such as cytochalasin D or latrunculin A were found to interfere with T cell activation and effector functions (Grakoui et al., 1999).

The cytoskeleton is composed of F-actin filaments, microtubules and intermediate filaments. While the latter provide the mechanical force necessary for migration (Vicente-Manzanares and Sanchez-Madrid, 2004), the microtubule system regulates polarization and maintenance of F-actin-dependent structures (Sancho et al., 2002).

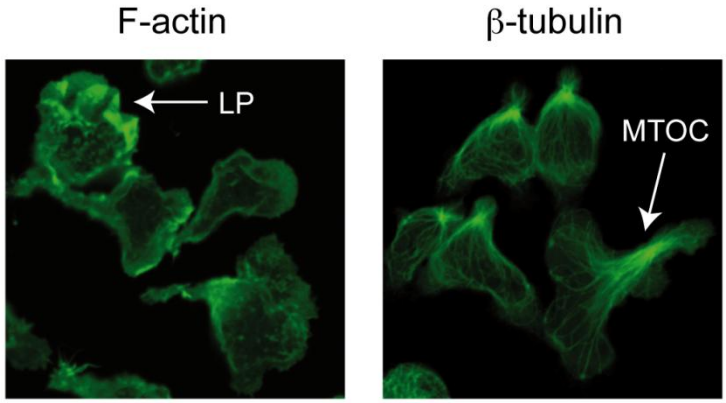

Fig. 2 Effector T cell morphology. The cytoskeleton of activated effector T cells was visualized by staining of F-actin (left panel) or by visualizing β-tubulin (right panel). The effector T cells show a polarized structure with large lamellipodiae and microtubule-organizing centers (MTOC) which is essential for proper T cell function (Müller et al., 2013).

Importantly, the cytoskeleton of T cells becomes reorganized within minutes after T cell receptor (TCR) stimulation, which involves polymerization of F-actin (Bunnell et al., 2001; Tskvitaria-Fuller et al., 2003; Valitutti et al., 1995), reorientation of the microtubule-organizing centre (MTOC) towards the immunological synapse and formation of the DPC (Fig. 2). Consequently, cell surface receptors are redistributed

and intracellular signalling complexes assembled, events that are important for the formation of the immunological synapse as well as for migration (Krummel et al., 2000). The relevance of the cytoskeletal organization for proper T cell function is underscored by several loss-of-function studies in mice, where deficiency in individual cytoskeleton regulatory proteins led to impaired T cell activation, cytokine production and proliferation (Billadeau et al., 2007).

1.3. Multiple Sclerosis

Multiple sclerosis (MS) is the most prevalent neuroinflammatory autoimmune disease in the western world (Sospedra and Martin, 2005). It is characterized by severe motor deficits and impaired neurocognitive functions which arise as the result of focal demyelination and axonal loss in the central nervous system (CNS) (Hafler, 2004). To interfere with the progression of MS a plethora of treatment regimens have been developed over the years or are currently being tested in clinical trials. These include IFNb, glatiramer acetate (GA), small molecular weight compounds such as fumarate derivatives and laquinimod and monoclonal antibodies directed against leukocyte antigens and cytokines (Barten et al., 2010; Buck and Hemmer, 2011; Hafler, 2004; Noseworthy, 2003). In the case of acute disease relapses, however, treatment with glucocorticoids (GCs) is still the measure of choice (McDonald et al., 2001). Initially established as a new therapy for rheumatoid arthritis patients in the late 1940s (Hench, 1950), GCs later became a standard regimen to treat acute relapses in MS patients (Milligan et al., 1987).

MS is a chronic neuroinflammatory disease that has significant socioeconomic relevance. Most often the disease develops during early adulthood with women being affected twice as often as men. Already in 1868 Jean Martin Charcot described inflammatory lesions in the CNS of patients suffering from neurological dysfunctions (Charcot, 1868; Hafler, 2004), and over the twentieth century it became clear that MS is a highly diverse disease entity. Today, we distinguish between relapsing-remitting, primary progressive and secondary progressive MS based on the disease course (Noseworthy et al., 2000). Most patients initially present with a relapsing-remitting form but eventually the majority of them develop a secondary progressive phase characterized by increasing deficits in the absence of further relapses. Only about 10-15% of the patients suffer from primary progressive MS with continuous

aggravation of the symptoms from early onwards (McFarlin and McFarland, 1982a; McFarlin and McFarland, 1982b). In the majority of the cases, MS is believed to have an autoimmune pathogenesis, with auto-reactive myelin-specific T cells playing a dominant role. This refers to $CD4^+$ T helper (Th) 1 and Th17 cells (Lovett-Racke et al., 2011) as well as $CD8^+$ T cells (Friese and Fugger, 2005; Huseby et al., 2001), even though B cells are currently being re-considered as central players in this process (Disanto et al., 2012). Finally, antibodies and complement deposition also seem to contribute to the progression of the disease (Sospedra and Martin, 2005).

1.4. Experimental Autoimmune Encephalomyelitis (EAE) as a model of MS

Insight into the pathomechanism of MS and its treatment regimens is to a significant degree derived from the analysis of animal models. Experimental Autoimmune Encephalomyelitis (EAE) was established as the primary model of MS already several decades ago and is based on an observation originally made in the course of rabies vaccination (Zamvil and Steinman, 1990). In rare cases patients who received the vaccine composed of the fixed pathogen grown in rabbit brain developed a severe paralytic disease. Based on these findings Rivers and colleagues later immunized rhesus monkeys with rabbit CNS homogenate which resulted in a demyelinating disease that mirrors many characteristics of the human disorder and therefore became instrumental for studies of the pathogenesis and treatment of MS (Rivers TM, 1933). This eventually led to the development of EAE in which mostly rats and mice are immunized with myelin antigens emulsified in adjuvant (Kabat et al., 1947). In general, EAE is a T cell-mediated disease highlighted by the fact that it can be transferred to naïve animals using pathogenic $CD4^+$ (Zamvil and Steinman, 1990) or $CD8^+$ T cells (Huseby et al., 2001; Sun et al., 2001). While $CD4^+$ T cells alone are sufficient to induce EAE even in the absence of CD8+ T cells, many studies also speak in favour of an important role of $CD8^+$ T and B cells in EAE (Cabarrocas et al., 2003; Ford and Evavold, 2005; Mars et al., 2011; Saxena et al., 2008; Ziemssen and Ziemssen, 2005). Furthermore, application of new techniques such as intravital imaging by 2-photon microscopy has allowed insight into the different phases of EAE (Bartholomäus et al., 2009). This led to the discovery that a phenotypic change of encephalitogenic T cells into a “migratory” phenotype,

subsequently directing immune cell migration into the target tissue, is crucial during EAE development (Odoardi et al., 2012).

One animal model cannot reflect all aspects of the complex human disease, but rather individual EAE models reflect different features of MS. EAE induced by immunization with myelin basic protein (MBP) in Lewis rats for example follows a monophasic disease course without significant demyelination and axonal damage, thereby mainly mimicking the inflammatory features of MS. In contrast, relapsing-remitting EAE models can be induced by immunization of DA rats with myelin oligodendrocyte glycoprotein (MOG) or SJL mice with proteolipid protein (PLP), and present with massive demyelination and axonal loss. Importantly, exploiting these different EAE models has significantly helped in developing new drugs for the treatment of MS such as GA, mitoxantrone and natalizumab (Steinman and Zamvil, 2006), and contributed to our current understanding of how high-dose GC therapy interferes with MS (Schweingruber et al., 2012).

To study particularly the inflammatory phase we used the monophasic Lewis rat EAE model and took advantage of the possibility to isolate CNS-antigen specific effector T cells from these rats and to propagate them *in vitro*.

1.4.1. T cell Migration in EAE

T lymphocytes are central players in the pathogenesis of EAE and MS, and proper activation and polarization are essential for their functioning. In active EAE, T cell activation is generally achieved by the use of Complete Freund's Adjuvant (CFA), which stimulates antigen-presenting cells (APCs) and induces a pro-inflammatory milieu (Medzhitov and Janeway, 2002). In adoptive transfer EAE, T cells also have to be activated in order to be capable of infiltrating the CNS. In this case, however, they are cultured with antigen-loaded APCs *in vitro* prior to their transfer into naïve animals (Wekerle et al., 1986). Regardless of the model, T cells have to migrate to the site of inflammation and then cross the blood-brain barrier (BBB; Engelhardt and Ransohoff, 2012).

Soluble mediators that trigger leukocyte migration are called chemokines and produced by a variety of immune cells as well as fibroblasts and endothelial cells (Jaerve and Müller, 2012; Mortier et al., 2012). Following their release chemokines can attach to extracellular matrix (ECM) components or specialized receptors and thereby form cues for migrating leukocytes. Under physiological conditions constitutively expressed chemokines establish gradients that guide leukocytes to

their appropriate location within primary and secondary lymphoid organs for which reason they are prerequisite for immune surveillance and maintenance of tissue homeostasis (Holman et al., 2011). Migration of leukocytes to their target tissues and lymph nodes along chemokine gradients is then followed by interaction of cell-surface molecules on trafficking lymphocytes with their ligands on endothelial cells (Engelhardt, 2008). In the case of infection or tissue damage pro-inflammatory chemokines serve to rapidly attract leukocytes to the site of inflammation and foster their extravasation from the blood. More precisely, chemokines induce firm attachment of leukocytes such as T cells to the vessel wall at inflamed sites by inducing conformational changes of integrins such as LFA-1 and VLA-4, which consolidates interaction with their receptors ICAM-1 and VCAM-1 on endothelial cells. This eventually results in intraluminal crawling followed by diapedesis via the paracellular or transcellular pathway.

Chemotaxis is a crucial mechanism in the pathogenesis of EAE and MS, since chemokines guide activated lymphocytes to the site of inflammation where they cross the BBB. Migration of immune cells into the CNS is best studied in transfer EAE models. Here, activated T cells are isolated from rats or mice after EAE induction and transferred into naïve recipients where they subsequently invade the CNS without prior activation of the host's immune system (Mix et al., 2008; Owens et al., 1998). As mentioned above, adhesion of immune cells to the vessel wall is mediated by integrins such as LFA-1 and VLA-4 (Engelhardt and Ransohoff, 2012). During EAE, their ligands ICAM-1 and VCAM-1 are up-regulated on cells of the BBB, the blood-leptominingeal barrier, the choroid plexus epithelium and the blood-cerebrospinal fluid barrier, which could be responsible for the increased leukocyte infiltration into the CNS (Engelhardt and Coisne, 2011). Accordingly, EAE is ameliorated by blocking LFA-1 or VLA-4 (Weller et al., 1996). Of note, migration of small numbers of T cells across the BBB is possible in the absence of these molecules even though T cell polarization and crawling are mediated by binding of LFA-1 to ICAM-1 and ICAM-2 (Steiner et al., 2010).

1.5. Glucocorticoids (GCs) in the treatment of MS and EAE

T cells are the most important targets of GCs in EAE and presumably MS (Wüst et al., 2008). For a long time it was assumed that the therapeutic efficacy of GCs mainly relies on induction of T cell apoptosis (Herold et al., 2006; Reichardt and Lühder,

2012), repression of pro-inflammatory cytokines (Baschant and Tuckermann, 2010) and modulation of leukocyte-endothelial interactions (Pitzalis et al., 2002). This notion, however, is now being called into question by accumulating evidence that T cell morphology and migration might also be crucial targets of GC action (Ghosh et al., 2009). For many years apoptosis induction was considered to be essential for the beneficial effects of GCs in EAE and MS (Gold et al., 2006; Pender and Rist, 2001). Nevertheless, increasing evidence suggests that this might be not the case. First, it was observed that EAE was ameliorated by GCs at a concentration that did not significantly induce apoptosis (Nguyen et al., 1997). Second, application of *Compound A,* a dissociating non-steroidal GR ligand, improved EAE even in the absence of apoptosis induction (van Loo et al., 2010; Wüst et al., 2009). Third, GCs inhibit activation-induced cell death (AICD), which preferentially protects effector T cells from GC-induced apoptosis (Baumann et al., 2005). However, loss of this protective effect does not seem to impact treatability of EAE by GC therapy, which was shown by inducing EAE in mice deficient for acid sphingomyelinase (aSMase). Due to the reduced IL-2 secretion by aSMase-deficient effector T cells their sensitivity for GC-induced apoptosis is enhanced. Consequently, one would have expected that GCs were more potent in ameliorating EAE in aSMase knock-out mice than in wildtype controls. Nevertheless, the efficacy of Dex therapy was similar in both genotypes (Tischner et al., 2011). Collectively, there are a number of findings that call the notion into question that apoptosis induction is indispensable for the treatment of CNS inflammation by GCs.

GCs exert the majority of their functions by binding to the GC receptor (GR), a member of the nuclear receptor superfamily that resides in the cytosol sequestered in a heat shock protein complex. Upon hormone binding the GR is released and then translocates into the nucleus where it either binds to GC responsive elements (GREs) leading to gene transactivation or acts as a negative regulator by interacting with other transcription factors such as NF-kB or AP-1 (Baschant and Tuckermann, 2010; Fig. 2). GCs can also bind to the mineralocorticoid receptor (MR) with even higher affinity (De Kloet and Derijk, 2004). However, expression of the MR is restricted to selected cell types and in many of them GCs are inactivated by 11b-hydroxysteroid dehydrogenase type II (11b-HSDII). This leaves only a few cell types where GCs are able to activate both the GR and MR under physiological conditions, and the only one relevant in the context of EAE and MS is the macrophage. Binding

of GCs to the two different receptors enforces macrophage differentiation into opposing directions: MR-deficient macrophages are polarized towards the anti-inflammatory M2 phenotype whereas macrophages lacking the GR adopt a pro-inflammatory M1 phenotype (Kleiman et al., 2012; Usher et al., 2010). This could potentially have implications for the control of EAE and MS by GCs. Finally, many synthetic GCs were chemically modified to reduce their affinity to the MR since GCs applied at high concentration can overcome the protective effect of 11b-HSDII and therefore induce side-effects such as hypertension via the MR in tissues such as kidney and heart.

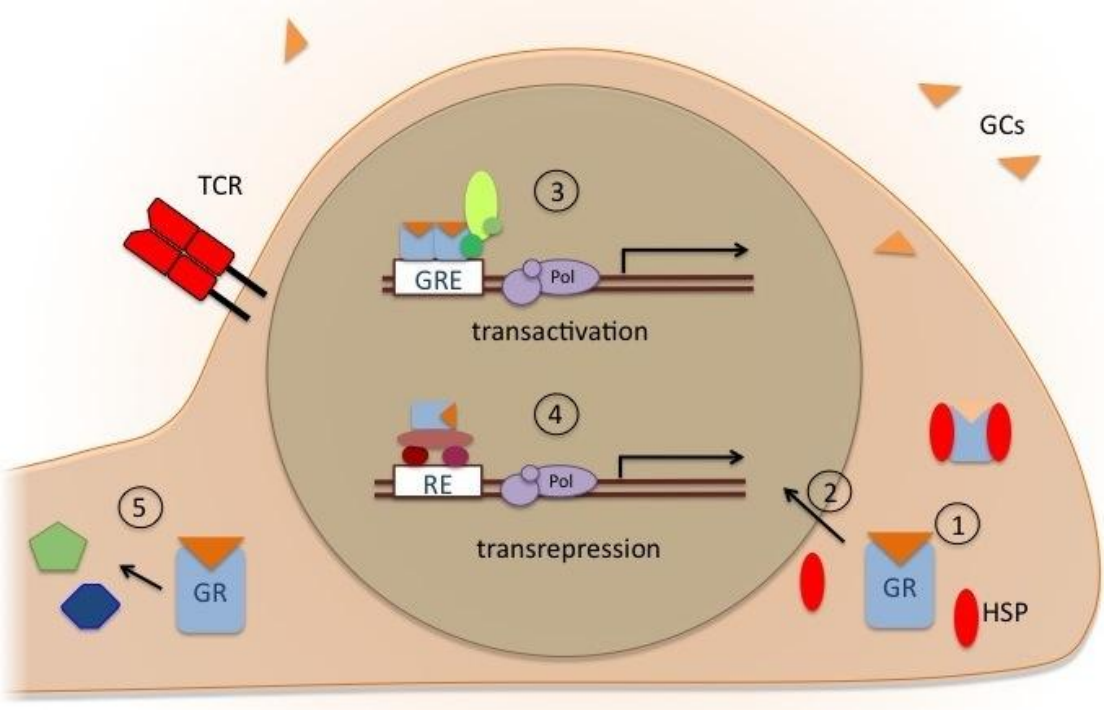

Fig. 3 GC effects on molecular level. GCs are bound by GR (1), Heat shock proteins are released (2), the GR can translocate to the nucleus and act as a dimer by directly binding to Glucocorticoid responsive elements (3) or as a monomer in complex with other transcription factors. Modified from (Herold et al., 2006).

Besides modulating gene transcription, GCs are also known to exert non-genomic effects. These mechanisms possibly include direct intercalation of GCs into the cell membrane and interaction with a not yet fully characterized membrane GR (Bartholome et al., 2004). Alternatively, the ligand-bound GR can exert its function within the cytosol rather than the nucleus by interfering with a variety of signalling cascades. Examples include decreased recruitment of Lck and Fyn to the TCR complex after GC treatment (Löwenberg et al., 2005), direct interaction of the cytosolic GR with PI3K (Hafezi-Moghadam et al., 2002) and increased

phosphorylation of focal adhesion kinase (FAK) after application of GCs (Koukouritaki et al., 1999). In contrast to transcriptional regulation the aforementioned effects are generally rapid and may therefore establish a first line of immunosuppression that is followed by more long-lasting effects on the level of gene expression.

1.6. Metabolic regulation of T cell activation

Besides steroid hormones there are several other hormones that impact on immune cells. Insulin is also an important regulator of several crucial body functions and exerts its function through binding to the insulin receptor (InsR). Dysfunctions of insulin responsiveness lead to severe consequences like Type 1 or Type 2 Diabetes Mellitus (T1DM/T2DM). The first one is caused by autoreactive T cells destroying the insulin producing beta cells in the pancreatic islets leading to insulin deficiency. The latter one is associated with long-time obesity and an increasing socioeconomic problem in the western world. Central to both diseases is disturbed glucose metabolism that impacts not only on the cardiovascular system of the patient but also impaired clearance of infections (Graves and Kayal, 2008; Müller et al., 2005). There has been a long-term debate which population of immune cells is responsible for this phenotype. It is known that persistent imbalance between energy uptake and expenditure as in obesity generally leads to cellular dysfunctions (Odegaard and Chawla, 2013). The InsR is needed for insulin mediated metabolic changes and screening for its expression patters revealed that amongst all immune cells only lymphocytes are capable of the *de novo* synthesis of the InsR, so it is reasonable, that these cells might be affected by insulin deficiency (Viardot et al., 2007). Intriguingly, the lymphocytes show no constitutive expression of the InsR but rather activation-induced expression.

As described above, activation is accompanied by profound morphological changes and cytoskeletal rearrangements. It is reasonable, that these alterations are energy consuming. Small resting T cells have to differentiate into effector T cells that are crawling, proliferating and cytokine secreting. Following activation T cells develop into different functional subsets depending on the conditions and the cytokine milieu during activation. These programming or fate decision determines the nature of the immune response (O'Shea and Paul, 2010; Zhou et al., 2009). A lack of energy in

this process could lead to impairment in this phase, which might impact on the efficient generation of immune cell subsets. It is known, that metabolic phenotypes integrate with T cell function (Gerriets and Rathmell, 2012), so this would be another explanation of the insufficient pathogen clearance in diabetes patients.

T cell metabolism is highly dynamic. Under normal conditions, oxidative phosphorylation is the source of energy, as in most healthy cells and the metabolic status is intracellular sensed by the ATP to ADP ratio. This system works in normal homeostatic proliferation and survival, but during activation the cells have to respond quickly to the higher energy demand. The T cells rapidly up-regulate glucose uptake and glycolysis as energy source (Fox et al., 2005; Frauwirth et al., 2002; Maciver et al., 2008). Activation is induced through two signals (see above). The co-stimulatory signal via CD28 leads to increased glucose uptake and is likely to provide part of the needed energy. Indeed is the increase in glucose uptake and glycolysis after T cell activation dependent on co-stimulation (Jacobs et al., 2008). In line with this, reduction of glucose uptake interferes with activation (Jones and Thompson, 2007; Vander Heiden et al., 2001). Additionally, T cell activation and proliferation have to be tightly regulated. Effector cells have to provide protection against cytokines while avoiding massive proliferation and autoimmunity. Direct interference with T cell metabolism impacts on immune responses: transgenic overexpression of glucose transporter Glut1 leads to increased T cell proliferation, survival and cytokine production in mice (Jacobs et al., 2008; Michalek et al., 2011). This also impacts on the T cell's architecture as the cells increase in size. Animals develop lymphadenopathy and a mild inflammatory disorder. Corresponding to these findings, an impairment of effector T cells to upregulate glucose metabolism leads to insufficient cytokine production and proliferation and activation are suppressed, which can lead to apoptosis (Alves et al., 2006; Coloff et al., 2011; Coloff et al., 2011; Greiner et al., 1994).

Modulation of the differentiation to Th cell subsets provides another potential explanation for increased infection risk in diabetes patients. It is known, that T cell metabolism interferes with T cell differentiation (Gerriets and Rathmell, 2012).

There is also evidence that insulin impacts on effector T cell metabolism: radioactively labelled insulin is bound by activated rather than naïve T cells (Helderman and Strom, 1977). The InsR expression is a hallmark of activated lymphocytes was discovered over 30 years ago (Helderman et al., 1978; Helderman

and Strom, 1978). As co-stimulation is required for the switch from oxidative phosphorylation to glycolysis, the effect of the InsR is likely synergistic with CD28 stimulation. Engagement of both receptors results in phosphatidylinositol 3-kinase (PI3K) activity, which generates phosphoinositol trisphosphate (PIP_3) that leads to the recruitment of Akt to the membrane. Akt is activated by pyrovate dehydrogenase kinase 1 (PDK1) (Finlay et al., 2009; Waugh et al., 2009) and induces glycolysis and increased glucose transport via Glut 4 (Sano et al., 2003; Fig. 4).

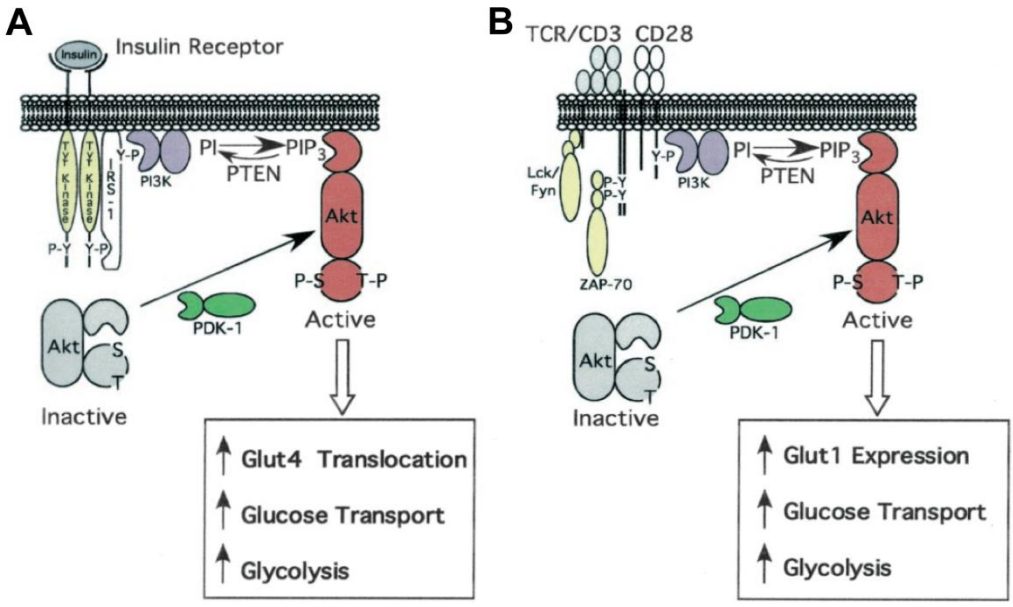

Fig. 4 Comparison of signalling pathways of InsR and TCR/CD28 co-stimulation. InsR (A) and TCR/CD28 signalling pathways (B) share components and result in analogous metabolic effects. Both pathways utilize the central signalling molecule Akt and induce glycolysis and increased glucose transport (Frauwirth and Thompson, 2004).

Pharmacological inhibition of PI3K impairs upregulation of glycolysis after CD28 mediated co-stimulation illustrating the central importance of Akt activation (Frauwirth and Thompson, 2004). The same molecules are utilized in InsR mediated signalling (Fig. 4), supporting the hypothesis of a synergistic function of both pathways. It is known, that increased glucose uptake can enhance T cell activation and proliferation (Jacobs et al., 2008), so it is likely that additional InsR signalling provides more energy for T cell differentiation and proliferation as CD28 signalling alone. Also other components of this pathway are known to influence T cell function. PDK1 is elevated in chronically activated T cells of asthma patients (Ostroukhova et al., 2012). Treatment of T cells isolated from those patients with PDK1 inhibitor dichloroacetate

(DCA) prevented inflammatory cytokine production and T cell proliferation (Ostroukhova et al., 2012).

However, there is evidence that the PI3K/Akt pathway is not generally essential for the metabolic reprogramming of T cells because deficiency in the catalytic subunits of PI3K are dispensable for activation induced T cell proliferation (Fruman et al., 1999; Okkenhaug et al., 2002). Also it has been found for activated $CD8^+$ T cells that Akt signalling is not needed for their proliferation *in vitro* (Macintyre et al., 2011). Nevertheless, $CD4^+$ effector T cells are dependent on glucose and a lack of insulin responsiveness would be an explanation for the higher infection risk of diabetes patients. This is underlined by the fact, that *in vitro* activated T cells from T2DM patients reflect the glycaemic *in vivo* status (Stentz and Kitabchi, 2003).

Taken together it is likely that insulin resistance impacts on lymphocyte function and that this is the reason for increased susceptibility of T2DM patients to infections. Furthermore, altered T cell differentiation could also be of relevance in T cell mediated diseases and understanding of this mechanism could lead to promising approaches to influence T cell function in the treatment of such diseases.

1.6.1. Insulin receptor silencing in transgenic rats

To study the importance of the InsR for proper immune cell functions we took advantage of an inducible and reversible gene knockdown system in transgenic rats. The knockdown in this model is mediated by RNA interference (RNAi). Expression of a small interfering RNA (siRNA) targeting the mRNA of the protein of interest leads to double-stranded RNA (dsRNA), which is activating the RNA-induced silencing complex (RISC) leading to its degradation. For the InsR knockdown (kd) a inducible and reversible system was used (Herold et al., 2008), were the expression of the siRNA is regulated by a Doxycycline (Dox) sensitive system (Fig. 5). Besides the small hairpin RNA (shRNA) under the control of the H1 promotor, which is processed to siRNA, the construct is consistent of a second part expressing eGFP as a selection marker and Tet-repressor (TetR) under an ubiquitin C promotor. Under physiological conditions, the TetR binds to the Tet operon (TetO) of the H1 promotor blocking the shRNA expression. Addition of Doxycycline to the system leads to shRNA production by blocking the binding of TetR to TetO. This knockdown induction is reversible, as stopping Dox treament leads to the renewed blocking of TetO by the TetR leading to the reverse of the diabetic phenotype (Herold et al., 2008).

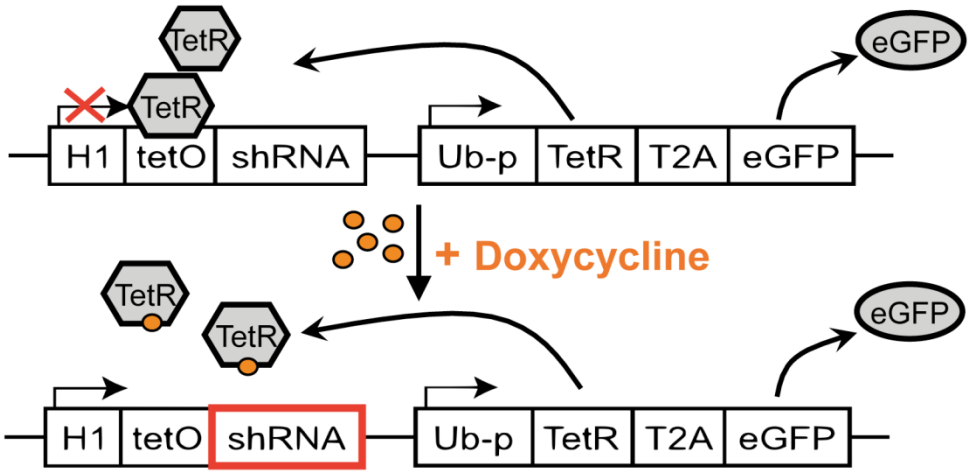

Fig. 5 Scheme of the inducible and reversible silencing of the InsR. Under physiological conditions eGFP and Tet repressor (TetR) are constitutively expressed (upper panel) under a ubiquitin C promotor (Ub-p), separated by a viral linker protein (T2A). TetR binds to the Tet operon (TetO) and blocks the H1 promotor. If Doxycycline is added to the system it binds the TetR leading to its release from TetO and allowing the transcription of an shRNA targeting the InsR mRNA (lower panel); modified from (Herold et al., 2008).

1.7. Objective

T cells circulate through the body and are influenced by a plethora of molecules such as chemokines or hormones. The aim of this project was to determine the impact of endocrine regulation of T cell function, namely the influence of insulin and GCs on effector T cell function and activation.

Treatment of neuroinflammatory diseases with GCs is widely used but still is unsatisfactory understood. We wanted to analyse the impact of GCs on effector T cells. For this we took advantage of *in vivo* generated CNS-antigen specific effector T cells and analysed the impact of GCs on central steps of disease development, namely polarized effector T cell morphology, transmigratory capacity and the ability to interact with APCs. Identification of crucial mechanisms for the benefit of GC action could help to develop a more refined treatment.

T cell morphology and migratory behaviour are central for the pathogenicity of auto-reactive cells but also essential for proper pathogen defence. Metabolic modulations may have an impact on T cell differentiation and function and might also be the link between metabolic disturbances as T1DM or T2DM and immunological impairment of the patients. We therefore aimed to analyse the impact of lacking insulin

responsiveness on T cell activation and different T cell subset functionality, because different T cell subsets as T_{reg}, effector T cells and memory T cells have different metabolic patterns and may therefore be differentially regulated and affected by InsR inactivation.

2. Material and methods

If not specified otherwise, all places are located in Germany.

2.1. General equipment

Table 1 General equipment

Accu-jet® pro pipette controller	Brand GmbH, Wertheim
Agarose Gel-electrophoresis system	Peqlab Biotechnology, Erlangen
Arium® 611 laboratory water purification system	Sartorius AG, Göttingen
BD FACSCanto II	BD Biosciences, Heidelberg
Blotting Chamber TE70	Amersham Biosciences, Freiburg
Centrifuge 5417R for reaction tubes	Eppendorf, Hamburg
Centrifuge 5804 for FACS tubes	Eppendorf, Hamburg
Centrifuge multifuge 4 KR for Falcon tubes	Heraeus, Hanau
Centrifuge Sigma 2-5 for 96-well plates	SIGMA Laborzentrifugen GmbH, Osterode am Harz
Chemilux Gel Imager	INTAS Science Imaging Instruments GmbH, Göttingen
Counter MicroBeta^2	Perkin Elmer, Waltham, USA
Electrophoresis power supply 301	Amersham Biosciences, Freiburg
Filter Mate Harvester	Perkin Elmer, Waltham, USA
Freezer Hera freeze -80 °C	Heraeus, Hanau
Freezer Liebherr Comfort -20 °C	Liebherr-International Deutschland GmbH, Biberach an der Riss
Freezer VIP plus -150 °C	SANYO Electric Co., Ltd., Moriguchi, Osaka, Japan
Incubator, HERACell 240	Heraeus, Hanau
Laminar airflow cabinet, HERASafe	Heraeus, Hanau
LSRII	BD Biosciences, Heidelberg
Micropipettes 2 µl, 20 µl, 200 µl, 1000 µl	Gilson, Middleton, Wisconsin, USA
Microscope Primo Star	Zeiss, Jena
Microwave	Sharp
Neubauer improved haemocytometer	Henneberg-Sander GmbH, Giessen-Lu□tzellinden
pH-Meter 766 Calimatic	Knick Elektronische Messgeräte GmbH & Co. KG, Berlin
RS 225 X-Ray Research System	Gulmay Medical Systems, Camberley, Surrey, UK
Spectral confocal & multiphoton system TCS SP2	Leica Microsystems, Wetzlar
Thermomixer	Eppendorf, Hamburg
Vortex Genie-2	Scientific Industries, Bohemia, New York, USA
7500 Real Time PCR System	Applied Biosystems, Foster City, California, USA

2.2. Consumables

Table 2 Consumables

8-well chamber slides	LabTek™, Nunc, Roskilde, Denmark
96-well Optical Reaction Plates	Applied Biosystems, Foster City, California, USA
96-well Suspension Culture Plate, U-bottom	Greiner bio-one GmbH, Frickenhausen
96-well Tissue Culture Plate 96-well V-bottom	Sarstedt, Nümbrecht
96-well Suspension Culture Plate, flat bottom	Greiner bio-one GmbH, Frickenhausen
96-well Opti-Plate	Perkin Elmer, Waltham, USA
Adhesive Sealing Film	Perkin Elmer, Waltham, USA
BD Micro-FineTM+ U-100 Insulin Syringes, 1 mL (29G 1⁄2")	BD Biosciences, Heidelberg
Cell culture plates 3.5 cm, 6 cm, 10 cm	Sarstedt, Nümbrecht
Cell strainer 40 µm	BD Biosciences, Heidelberg
Cellstar 12 well flat bottom suspension plates	Greiner Bio-One GmbH, Essen
Cellstar® pipettes 5 mL, 10 mL, 25 mL	Greiner bio-one GmbH, Frickenhausen
CryoTube™ Vials	Nunc, Roskilde, Denmark
ECL nitrocellulose membrane	Amersham Biosciences, Freiburg
FACS tubes	BD Biosciences, Heidelberg
Falcon tubes 15 mL, 50 mL	Greiner bio-one GmbH, Frickenhausen
Filtropur S 0.2, 0.45	Sarstedt, Nümbrecht
Optical Adhesive Covers	Applied Biosystems, Foster City, California, USA
Needles 24G 1", 20G 1 1⁄2", 27G 3⁄4", 25G 1", 20G 2 3⁄4"	B. Braun Melsungen AG, Melsungen
Pipette tips 10 µl, 200 µl, 1000 µl	Greiner bio-one GmbH, Frickenhausen
Reaction tubes 0.5 mL	Sarstedt, Nümbrecht
Reaction tubes 1.5 mL, 2 mL	Greiner bio-one GmbH, Frickenhausen
Syringes 1 mL	Henke Sass Wolf, Tuttlingen
Syringes 2 mL,5 mL, 10 mL	BD Biosciences, Heidelberg
Syringes 60 mL	BD Biosciences, Heidelberg
Transwell system, 3 µm pore size	Corning Life Sciences, Acton, USA
GB004 Gel Blotting Paper	Whatman, Schleicher & Schuell, Dassel

2.3. Chemicals and buffer additives

Table 3 Chemicals and additives

^{3}[H]-Thymidine solution, 37 MBq/mL	Perkin Elmer, Waltham, USA
2-β-Mercapto ethanol	Invitrogen, Paisley, UK
Agarose UltraPure	Invitrogen, Paisley, UK
BSA	Carl Roth, Karlsruhe
$CaCl_2$ * 2 H_2O	Merck, Darmstadt
Citric acid	Merck, Darmstadt
Concanavalin A	SIGMA-Aldrich, Taufkirchen
D-Glucose	Merck, Darmstadt
Dex water soluble	SIGMA-Aldrich, Taufkirchen
Dexa-ratiopharm® 100 mg Injektionslo□sung	Carl Roth, Karlsruhe

DMSO	Fermentas GmbH, St. Leon-Rot
DNA ladder 1kb	Serva, Heidelberg
dNTPs ($dATPNa_4$ *3 H_2O, $dCTPNa_4$ *3 H_2O, $dGTPNa_4$ *3 H_2O, $dTTPNa_4$ *3 H_2O)	Genaxxon bioscience, Ulm
Doxycycline hyclate	SIGMA-Aldrich, Taufkirchen
EDTA	Carl Roth, Karlsruhe
EGTA	Carl Roth, Karlsruhe
Ethanol	Carl Roth, Karlsruhe
Ethidium bromide	HyClon, Perbio Science, Bonn
10% Stripped Fetal Calf Serum (FCS)	Invitrogen, Paisley, UK
GIBCO® Penicillin/Streptomycin	Merck, Darmstadt
GeneRuler 1kb DNA ladder	Fermentas
HEPES	Merck, Darmstadt
KCl	Merck, Darmstadt
KH_2PO_4	Merck, Darmstadt
Lymphoprep™	Axis-Shield, Oslo, Norway
$NaHCO_3$	Merck, Darmstadt
Na_2CO_3	Merck, Darmstadt
NaCl	Carl Roth, Karlsruhe
NaN_3	Carl Roth, Karlsruhe
$MgCl_2$ * 6 H_2O	Merck, Darmstadt
$MgSO_4$ * 7 H_2O	Merck, Darmstadt
Na_2HPO_4	Merck, Darmstadt
$Na_3C_6H_5O_7$	Carl Roth, Karlsruhe
NH_4Cl	Merck, Darmstadt
Phenol Red	SIGMA-Aldrich, Taufkirchen
Prestained protein marker	New England Biolabs, Ipswich, USA
Protease Inhibitor cocktail	SIGMA-Aldrich, Taufkirchen
Rotiphorese Gel 30	Carl Roth, Karlsruhe
TEMED	Carl Roth, Karlsruhe
Tween-20	Carl Roth, Karlsruhe
Tris	Carl Roth, Karlsruhe
U73122 hydrate (PLC inhibitor)	SIGMA-Aldrich, Taufkirchen
Y-27632 dihydrochloride monohydrate (ROCK inhibitor)	SIGMA-Aldrich, Taufkirchen

2.4. Oligonucleotides

Table 4 Primer for qrt-PCR

target gene	**sequence (5' → 3')**
InsR	forward: ATGTGAGACGACGGCGGTGC reverse: TCCCGGGCACACCTCTCCAG
β-actin	forward: AGCTCCTCCGTCGCCGGTC reverse: CCACCATCACACCCTGGTGCCT

2.5. Media and solutions

2.5.1. Standard cell culture

All media, buffers and solutions were stored at 4 °C. Buffers and solutions were autoclaved at 125 °C for 30 min.

BSS:
- 11% BSS I
- 11% BSS II

BSS I:
- 50 mM Glucose
- 4.4 mM KH_2PO_4
- 13.4 mM Na_2HPO_4
- 0.1% Phenol red

BSS II:
- 12.65 mM $CaCl_2$ * 2 H_2O
- 53.6 mM KCl
- 1.37 M NaCl
- 9.8 mM $MgCl_2$ * 6 H_2O
- 8.1 mM $MgSO_4$ * 7 H_2O

Coating Buffer:
- 45.3 mM $NaHCO_3$
- 18.2 mM Na_2CO_3

GIBCO® DMEM+GlutaMAX™-I, Invitrogen, Paisley, UK

GIBCO® RPMI 1640+GlutaMAX™-I, Invitrogen, Paisley, UK

RPMI ++:
- + 10% FCS
- + 1% Penicillin/Streptomycin

DMEM++:
- +10% FCS
- +1% Penicillin/Streptomycin

DMEM ReStim:
- DMEM++
- +20% ConA-Supernatant
- +10 ng/mL mouse IL-2 (Peprotech)

PBS (pH 7.4):
- 137 mM NaCl
- 2.7 mM KCl
- 10 µM Na_2HPO_4
- 2.0 mM KH_2PO_4

Transmigration medium:
- RPMI

+ 0.5% fat-free BSA

2.5.2. Effector T cell culture

EH-Medium:
DMEM
+ 25 mM Hepes

RatReMed:
RPMI
+ 1% Penicillin/Streptomycin
+ 1% rat serum
+ 0.2% β-mercapto ethanol

TCGF medium:
RPMI
+ 10% ConA supernatant
+ 1% Penicillin/Streptomycin
+ 5% FCS

2.5.3. MACS® cell seperation

MACS running buffer:
PBS pH 7.2
+ 2.0 mM EDTA
+ 0.5% BSA

MACS rinsing buffer:
PBS pH 7.2
+ 2.0 mM EDTA

2.5.4. Flow cytometry

OptiLyse® B Lysing Solution, Beckman Coulter, Krefeld

Alsevers:
27 mM NaCl
125 mM D-Glucose
3 mM citric acid
30 mM $Na_3C_6H_5O_7$

Annexin binding buffer:
10 mM HEPES/NaOH, pH 7.4
140 mM NaCl
2.5 mM $CaCl_2$
in ddH_2O

Krebs-Ringer solution:

10 mM HEPES, pH 7.0
140 mM NaCl
4 mM KCl
1 mM $MgCl_2$
10 mM glucose

TAC buffer:

20.0 mM Tris/HCl pH 7.2
155 mM NH_4Cl

FACS buffer:

PBS pH 7.2
+ 0.1% BSA
+ 0.01% NaN_3

2.5.5. Protein analysis

Upper Buffer (stacking gel):

0.5 M Tris/HCl, pH 6.8
14 mM SDS

Lower Buffer (separating gel):

1.5 M Tris/HCl, pH 6.8
14 mM SDS

Laemmli 1×

62.50 mM Tris/HCl, pH 6.8
2.0% SDS
20.0% Glycerol
5.0% β-mercapto ethanol
0.025 % Bromphenol 0.5%

SDS Running buffer, 10×

25.0 mM Tris
192 mM Glycin
3.50 mM SDS

PBS/Tween:

PBS
0.1% Tween 20

RIPA (1×)

5.00 mM EDTA
10.0 mM Tris
50 mM NaCl
10.0 mM NaF
1.00 mM Na_4P2O_7 x $10H_2O$

Lysis buffer (for 1 mL, freshly prepared for each experiment)

500 µL 2 × RIPA
100 µL NP40 (10%)
20 µL NA_3VO_4 (50 mM)
1 µL Na_2MoO_4 (10 mM)
25 µL protease inhibitor
354 µL aqua dest.

Blotting buffer, 10×

0.4 M Glycin
0.5 M Tris
13 mM SDS
15 mM NaN3

Blocking Buffer:

PBS/Tween
+5% BSA

Staining solution A

0.100 M Tris/HCl, pH 8.6
0.025% Luminol

Staining solution B

0.11% Para Hydroxy Coumarin acid in DMSO

Development solution

2 mL Staining Solution A
0.2 mL Staining Solution B
1.2 µL H_2O_2

2.6. Antibodies and Reagents

2.6.1. Flow cytometry: antibodies and dyes

AnnexinV-Cy5, BD Biosciences, Heidelberg

7-AAD, BD Biosciences, Heidelberg

Indo1, Invitrogen, Paisley, UK

Table 5 Antibodies for flow cytometry

Epitope	**Clone**	**Dilution for 1 × 10^6 lymphocytes**	**Flourochrome**
CD4	Ox38	1:10000	fitc
		1:2000	PE
		1:5000	Alexa-647
	W3/25	1:1000	PE-Cy7
	Ox35	1:500	APC
CD8α	Ox8	1:1000	fitc
		1:500	PE
		1:500	PerCp
CD8β	341	1:1000	fitc
CD25	Ox39	1:500	PE
		1:1000	bio
CD45	Ox1	1:3000	Alexa-647
CD45RA	Ox33	1:2000	PE
CD62L (LECAM-1)	HRL1	1:1000	bio
CD134	Ox40	1:500	Alexa-647
αβTCR	R73	1:500	PE
		1:500	PerCp
		1:1000	Alexa-647
Thy1	Ox7	1:500	PerCp
Secondary antibody	SA	1:1000	APC-Cy7
Intracellular proteins:			
FoxP3		1:500	PE
IL-17		1:500	PE
IFNγ		1:500	PE

FACS antibodies were purchased from BD Biosciences, eBioscience or BioLegend.

2.6.2. MACS® technology microbeads

Table 6 MACS® technology microbeads

Epitope	Clone
CD4	Ox38
CD8	G28
CD45RA	Ox33
γδTCR	V45
SA	

MACS beads were purchased from Miltenyi Biotec, Bergisch Gladbach. For 1×10^7 lymphocytes 20 µL beads were used.

2.6.3. Western Blot antibodies

primary antibodies:

Akt: anti-Akt rabbit polyclonal antibody, Cell Signaling Technology, Cambridge, UK

P-Akt: Phospho-Akt (Ser473) (D9E) XP™ rabbit monoclonal antibody, Cell Signaling Technology, Cambridge, UK

βTubulin: anti-βTubulin mouse polyclonal antibody, Santa Cruz Biotechnology, Heidelberg

ERK: ERK-1 (C-16) rabbit polyclonal antibody, Santa Cruz Biotechnology, Heidelberg

P-ERM: Phospho-Ezrin (Thr567)/Radixin(Thr654)/Moesin(Thr588) rabbit polyclonal antibody, Cell Signaling Technology, Cambridge, UK

Insulin receptor: insulin Rβ (C-19), rabbit polyclonal antibody, Santa Cruz Biotechnology, Heidelberg

All primary antibodies were used in a 1:1000 dilution in PBS/Tween supplemented with 5% BSA and 0.01% NaN_3.

secondary antibodies:

Goat Anti Rabbit IgG horseradish peroxidase conjugate, ImmunoPure, Rockford, USA

Goat Anti Mouse IgG horseradish peroxidase conjugate, ImmunoPure, Rockford, USA

The secondary antibodies were utilized in a 1:10000 dilution in PBS/Tween.

2.6.4. Reagents for T cell stimulation

Coating Antibody: Anti Mouse Ig Sheep polyclonal antibody, Roche, Basel, Switzerland

Stimulation:

JJ319 - Anti Rat CD28 Mouse monoclonal antibody, kindly provided by Prof. Hünig, Institute for Virology and Immunobiology, University of Würzburg

JJ316 - Anti Rat CD28 Mouse monoclonal antibody, superagonist, kindly provided by Prof. Hünig, Institute for Virology and Immunobiology, University of Würzburg

R37 - Anti Rat TCR Mouse monoclonal antibody, kindly provided by Prof. Hünig, Institute for Virology and Immunobiology, University of Würzburg

2.7. Enzymes and Kits

Power SYBR® green, Applied Biosystems, Foster City, California, USA

Taq-CR Master Mix Kit, Qiagen, Hilden

Proteinase K, Promega, Fitchburg, USA

iScript™ cDNA synthesis kit, BioRad, Munich

eBioscience IC staining: Fixation/Permeabilization concentrate and diluent, 10 × Permeabilization buffer, eBioscience, San Diego, California, USA

BD Cytofix/Cytoperm™ Kit, BD Biosciences, Heidelberg

DyLight® 649 microscale antibody labeling kit, Thermo Scientific, Waltham, Massachusetts, USA

2.8. Blood Glucose

Ascensia Blood Glucose Meter CONTOUR®, Bayer HealthCare AG, Leverkusen

CONTOUR® Test Strips, Bayer, Bayer HealthCare AG, Leverkusen

2.9. Anaesthesia

2.9.1. Injection:

Ketamine 10% (Ketavet)

Xylazine 2% (Rompun)

A mixture of 250 µL Ketavet and 50 µL Rompun was prepared immediately before the induction of anaesthesia by i.p. injection of 100 µL/100 g bodyweight of the rat.

2.9.2. Inhalation

For temporary anaesthesia, the rats were supplied with an evaporator delivering 4% sevoflurane® in a 5% oxygen atmosphere.

2.10. Software

Adobe® Illustrator® Adobe Systems, San José, USA

BD FACS Diva™ software version 6.1.2, BD Biosciences, Heidelberg

Endnote X, Thomson Reuters, New York City, USA

FlowJo version 8.8.6, Tree Star, Inc., Ashland, Oregon, USA

GelPro analyzer, Media Cybernetics, Rockville, USA

Leica Confocal Software, Leica, Wetzlar

Prism for Macintosh version 4.0c, GraphPad Software, Inc., San Diego, California, USA

7500 System SDS Software version 1.4.0.25, Applied Biosystems, Foster City, California, USA

2.11. Animals

The rats used during this project were all Lewis background and bred in the animal facility of the University of Göttingen Medical School. All experiments were conducted according to the Lower Saxony state regulations and approved by the responsible authorities (*Niedersächsisches Landesamt für Lebensmittelsicherheit und Verbraucherschutz*). They were kept under specific pathogen-free conditions in a 12 hour light-dark cycle.

Lewis: wt Lewis rats were obtained from Charles River or bread in our own facilities.

UGC: Lewis rats transgenic for eGFP generated using a lentviral approach. eGFP is expressed under the control of an ubiquitin promotor, leading to eGFP expression in all tissues (van den Brandt et al., 2004).

InsR04: Lewis rats transgenic for a lentiviral vector system (FH1tUTG) consisting of one cassette coding for the shRNA under a H1 promotor and the control of TetO and a second cassette consisting of TetR and eGFP separated by a linker (see Fig. 5; Herold et al., 2008).

2.12. Animal experiments

2.12.1. Induction of Experimental Autoimmune Encephalomyelitis (EAE)

For active EAE, immunization of Lewis rats with the CNS antigen myelin basic protein derived from guinea pigs (gpMBP) was carried out under Sevoflurane or Ketamine/Xylazine anaesthesia.

A paste composed of gpMBP (2 mg/mL) emulsified in CFA (2 mg/mL) at a 1:1 ratio was prepared. Each animal was injected subcutaneously slightly above the footpad of either hind limb with 50 µL paste per side.

Adoptive transfer EAE (AT-EAE) was induced by i.v. injection of 2 - 10 $\times$ 10^6 freshly restimulated effector T cells.

The animals were weighed daily and inspected for EAE symptoms. EAE severity was assessed according to a 10-grade scoring scale as follows: 0 = healthy; 1 = reduced tonus of the tail; 2 = limp tail; 3 = absent righting; 4 = gait ataxia; 5 = mild paresis of hind limbs; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia; 8 = tetraperesis; 9 = moribund; 10 = death (Linker et al., 2008). The animals were sacrificed according to the animal welfare law at a score of >7.

2.12.2. *In vivo* knockdown induction

The inducible and reversible silencing of the InsR was possible through induced expression of an shRNA targeting the corresponding mRNA. shRNA-expression was started after blocking of the constitutively expressed Tet-repressor (see 1.6.1) with Doxycycline (Dox).

To obtain a sufficient knockdown efficacy, the shRNA expression was induced 5 days prior to T cell isolation *in vivo*. To this end the animals were fed with Dox containing diet *ad libitum*. To maintain the knockdown during all *in vitro* experiments, Dox was also added to the respective culture medium at a concentration of 2 µg/mL.

2.12.3. Blood glucose measurement

For the determination of the blood glucose levels a drop of blood was gained by punctuation of the tail vein at the tail tip of the rats with a hollow needle. Glucose levels were determined with the *Ascendia Blood Glucose Meter* and the corresponding test strips.

2.12.4. Generation of encephalitogenic T cells

The animals were immunized according to the EAE protocol (see 2.12.1). After 10 days, before the T cells leave the lymph nodes and invade the CNS, the draining

popliteal lymphnodes were dissected and passed through a 40 µm nylon mesh. The cell suspension was washed once at 300 × g for 10 minutes using RatReMed medium and taken into culture in presence of their cognate antigen gpMBP. After 3 days, the stimulated T cells were purified with a cold gradient (see 2.13.5).
siGR effector T cells have been generated previously (Tischner et al., 2009). Effector T cells were generated as described above and stabely transfected with a lentivirus encoding eGFP and a short hairpin RNA (Tischner et al., 2009). Two shRNA sequences targeting the rat GR were cloned into the lentiviral vector MSCV-LMP and effector T cells were transfected. Pure cell lines (>95%) had been generated that have been reported to express strongly reduced levels of the GR and lack GC responsiveness. Two cell lines were generated, siGR-A and siGR-B; in all experiments conducted for this thesis siGR-A cells have been used.

2.12.5. Adoptive transfer (AT-) EAE

AT-EAE was induced in rats on Lewis background by injection of syngeneic encephalitogenic T cells. To this end, freshly restimulated encephalitogenic T cells were used, that were harvested after 3 days of restimulation (see 2.13.4) and purified with a cold gradient. 1-9 $\times$ 10^6 effector T cells were resuspended in 250 µl sterile PBS and injected in the tail vein of manually immobilized recipient rats.

2.12.6. *In vivo* expansion of regulatory T cells

To isolate regulatory T cells in sufficient numbers, these cells were expanded *in vivo*. To this end the antibody JJ316 was utilised. This monoclonal antibody against CD28 is superagonistic and reported to induce the expansion of regulatory T cells *in vivo* (Lin and Hünig, 2003). To expand regulatory T cells in wt and InsR kd rats, 1 mg of the antibody JJ316 was injected intravenously 3 days prior to the lymphocyte isolation.

2.12.7. Generation of bone marrow chimeric rats

Recipient wildtype Lewis rats were irradiated sublethally (12.5 Gy) and 24 h later injected with 1 $\times$ 10^7 bone marrow cells isolated from eGFP transgenic wt or InsR kd rats, respectively. To isolate the bone marrow, the femurs of either hind limb of the donor was dissected and transferred into sterile ice-cold PBS. Under sterile conditions, the bones were opened with a scissor on both ends and the bone marrow was flushed out with a syringe containing 20 mL of ice-cold PBS. The cell suspension was passed through a 40 µm nylon mesh and washed (300 $\times$ g, 5 min). The cells

were resuspended in PBS and the cell concentration was determined using a Neubauer haematocytometer and adjusted to a cell concentration of 4×10^7 cells/mL. 250 µL of this suspension was injected into the tail vein of manually immobilized recipient rats. Reconstitution was checked by serial FACS analyses every second week.

2.12.8. Production of rat serum for cell culture

Male and female rats at the age of 4-8 weeks were sacrificed in carbondioxyde atmosphere and the blood was gained by heart puncture with a 10 mL syringe. The fresh blood was incubated in 15 mL tubes for 30 min at room temperature (RT) and centrifuged at 10000 × g for 20 min. The serum was carefully transferred in a fresh 15 mL tube and inactivated for 60 min at 56 °C. Afterwards, the serum was stored at -20 °C.

2.13. Cellular methods

2.13.1. Lymphocyte isolation

Wildtype or InsR kd rats were sacrificed in a carbon dioxide atmosphere. The cervical, axial, mesenterial and inguinal lymph nodes were isolated and passed through a 40 µm nylon mesh with ice-cold PBS/BSA or RPMI++ using a syringe plug. The cell suspension was centrifuged (300 × g for 7 min) and the pellet was resuspended in medium or buffer. The cell concentration was determined using a Neubauer haemocytometer.

For splenocyte isolation, the spleen was dissected and passed through a 100 µm nylon mesh with PBS. The cells were pelleted at 300 × g for 5 min and resuspended in 2 mL PBS. 12 mL TAC solution were added followed by 12 min incubation at RT in the dark. Subsequently, 16 mL PBS/BSA or medium were added, the sample was centrifuged (300 × g for 7 min) and the pellet was resuspended in medium or buffer.

2.13.2. T cell isolation using MACS® technology

In case of $CD4^+$ T cell isolation a positive selection using anti-CD4-beads was performed according to the manufacturer's instructions. In brief, 20 µl beads per 1×10^7 lymphocytes in MACS running buffer were added. After 20 minutes of incubation, the cells were washed and the pellet passed through a pre-seperation filter. T cells were isolated with the autoMACS device using the *possel* protocol.

For the isolation of regulatory T cells a two-step protocol was utilized. In the first step, $CD8^+$ T cells, γδ T cells and B cells were depleted using the respective beads at a concentration of 20 µl beads per 1 × 10^7 lymphocytes and the autoMACS protocol *deplete*. Afterwards the remaining T cells were incubated with 5 µl biotinylated anti-CD25-antibody for 20 minutes, washed and then incubated with 50 µl streptavidin-beads. The second separation was performed with the *possels* protocol of the autoMACS. With this protocol, two populations were obtained: $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. The latter population served as regulatory T cells, the CD25 negative population was used as control cells.

2.13.3. T cell stimulation

$CD4^+$ T cells purified by MACS® technology (see above) were stimulated with the combination of anti-TCR (R73) and anti-CD28 (JJ319) antibodies. R73 was bound to the bottom of each well, JJ319 was added to the culture medium.

24-well cell culture plates were incubated over night at 4 °C with the coating antibody (sheep-anti-mouse-Ig, 2.6 µg/mL) in 0.5 mL coating buffer. After that, the plates were washed once with 0.5 mL BSS and twice with 0.5 mL PBS, followed by a 2 h incubation with the stimulating anti-TCR antibody at a concentration of 2 µg per well in 0.5 mL PBS/BSA. Afterwards, the wells were washed twice with 0.5 mL PBS and the T cells were added in 1 mL RPMI++ medium supplemented with anti-CD28 (2 µg/mL) at a concentration of 1 × 10^6 cells/mL. FACS analysis, RNA or protein isolation was performed 24, 48 and 72 h later.

In case of stimulation with the superagonistic anti-CD28 antibody JJ316 the plates were not coated and JJ316 was added to the culture medium RPMI++ at a concentration of 5 µg/mL. ConA stimulation was conducted with 2.5 µg/mL ConA in RPMI++. In both cases 1 × 10^6 T cells per 1 mL medium were cultured.

2.13.4. Effector T cell culture

Encephalitogenic T cells (effector T cells) were cultured in a 3 day/3 day cycle. In the first phase, the cells were restimulated with gpMBP in presence of APCs (*d3 cells* after harvesting), whereas they are expanded in the second phase (*d6 cells*).

Restimulation: 3 × 10^6 effector T cells were cultured in 10 mL RatReMed complemented with 10 µg/mL gpMBP together with 1.5 × 10^8 irradiated splenocytes (30 Gy) serving as APCs. After two days, 3 mL TCGF medium were added to each culture plate. On day three, the stimulated effector T cells were purified using a cold

gradient (see below). These cells were used for all *in vitro* experiments and for injection.

Expansion: 1 × 10^7 effector T cells were cultured in 10 mL TGCF medium for three days, with feeding of the cells with 3 mL fresh TCGF on day two.

2.13.5. Cold Gradient to isolate effector T cells after restimulation

The effector T cell culture plates were harvested and rinsed with EH medium. The suspension was washed at 300 × g for 10 minutes and the pellet was resuspended in 5 mL lymphoprep™. The suspension was covered with 2 mL EH medium and centrifuged at 1700 × g with reduced acceleration and without brakes for 25 min. Afterwards, the interphase was harvested and washed with EH medium at 400 × g for 10 min.

2.13.6. Freezing and thawing of effector T cells

In order to store effector T cells for longer periods of time the cells were frozen. To this end, effector T cells were pelleted and the supernatant was thoroughly removed. The cells were resuspended in freezing medium consisting of 50% RPMI and 50% FCS. 900 µL of the suspension containing 0.5 – 1.5 × 10^7 effector T cells was transferred in cryo tubes containing 100 µL DMSO and frozen immediately.

The cells were thawed in a 37 °C water bath and 10 mL EH-medium were added quickly to dilute the DMSO. Subsequently, the cells were pelleted at 300 × g for 7 minutes and resuspended in the appropriate medium (RatReMed for d6 and TCGF medium for d3 cells).

2.13.7. Effector T cell transfer and Dex treatment *in vivo*

To study the effects of GCs on effector T cells *in vivo*, 8 × 10^6 to 1 × 10^7 eGFP expressing effector T cells were transferred into the tail vein of syngenic recipient rats and allowed to equilibrate for 48 hrs. Subsequently, the rats were treated with Dex intravenously at a dose of 20 mg/kg. Peripheral blood samples for the analysis of lymphocyte counts were obtained by repeated bleedings (50 µL each) from the tail vein, added to 5 mL Alsevers and analyzed by flow cytometry. This allowed determination the total amount of eGFP+ cells present in a defined volume of blood.

2.13.8. Flow cytometry

For standard stainings lymphocytes were labelled with flourochrome-conjugated antibodies targeting extracellular surface markers or intracellular cytokines or

transcription factors. For the latter ones, special intracellular staining protocols had to be performed after extracellular staining.

Extracellular staining:

8×10^5 lymphocytes were used for each staining. Flourochrome- or biotin-conjugated antibodies were added in titrated concentrations. After 20 min at 4 °C, the samples were washed with 4 mL FACS buffer at 400 × g for 5 min. If a biotinylated antibody was used in the first step, a second step was conducted using a streptavidin-conjugated flourochrome. The samples were analysed using a FACSCanto II device.

Intracellular staining:

1×10^6 lymphocytes were stained for surface markers as described above and the supernatant was removed thoroughly. Afterwards, the cells were fixed and incubated with antibodies against intracellular molecules. Two different protocols were used.

<u>BD protocol (for cytosolic proteins)</u>

The cells were thoroughly resuspended in 250 µL Fixation/Permeabilization solution for 20 min at 4 °C. Afterwards, the cells were washed two times with 1 mL BD Perm/Wash™ buffer and resuspended in 50 µL BD Perm/Wash™ buffer. The antibody was added in an appropriate concentration (see table 5) and incubated at 4 °C for 30 min, followed by washing of the cells twice with 1 mL BD Perm/Wash™ buffer. The pellet was suspended in 50 µL FACS buffer and analysed using a FACSCanto II device.

<u>eBioscience protocol (for transcription factors)</u>

The cellular pellet was resuspended in 100 µL freshly prepared Fix/Perm buffer (25 µL concentrate + 75 µL diluent) and incubated for a minimum of 30 min at 4 °C. Afterwards, the cells were washed twice with 2 mL 1 × Perm buffer (10 × Perm buffer diluted in aqua dest.) at 350 × g for 7 min. The fluorescently labelled antibody was added at a proper concentration (see table 5) and the cells were incubated for 30 min at 4 °C. Then the cells were again washed twice with 2 mL Perm buffer, resuspended in the remaining buffer and subsequently analysed by flow cytometry using a FACSCanto II device.

Apoptosis analysis:

1×10^6 lymphocytes were stained for surface markers as described above. Afterwards, each sample was resuspended in 100 µL AnnexinV binding buffer complemented with 3 µL 7-AAD and 1 µL AnnexinV-Cy5 and incubated for 15 min. Afterward, the samples were directly analysed with a FACSCanto II device without a further washing step.

FACS analysis of blood samples

Blood was obtained through bleedings of the tail vein (ca 40-50 µl) and directly dropped into FACS tubes filled with alsevers. The samples were centrifuged at 400 × g for 5 min and extracellular staining was performed. Afterwards, the cells were re-suspended in 100 µL OptiLyse® and incubated for 12 min at RT. Then 1 mL of ice-cold water was added and the samples were incubated for at least 1.5 h at RT. Before FACS analyses they were washed with 3 mL FACS buffer (400 × g, 5 min).

2.13.9. Apoptosis induction

Apoptosis was induced by three different stimuli. (i) T cells were cultured under serum-starved conditions (media containing 1% or 0% FCR, respectively); (ii) T cells were cultured with different concentrations of GC (10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M Dex); (iii) T cells were irradiated at different doses (0.5 - 5 Gy). For all stimuli 1×10^6 T cells were cultured in 1 mL RPMI++. Apoptosis rate was determined as described under 2.13.8.

2.13.10. ^{3}H-thymidine suppression assay

In vivo expanded regulatory T cells (see 2.12.6.) were isolated via MACS® technology (described in 2.13.2). Irradiated splenocytes served as APCs and $CD4^+$ T cells stimulated with ConA as target cells. The latter ones were generated by positive MACS® selection as described above. For APCs, the splenocytes of a naïve wt rats were isolated as described in 2.13.1 and irradiated for 6 min at a dose rate of 5 Gy/min (total dose 30 Gy). 1×10^5 APCs were mixed with 0.5×10^5 target cells in 200 µL RPMI++ supplemented with 2.5 µg/mL ConA. Subsequently, regulatory T cells were added at three different effector:target ratios: 1:1, 1:5 and 1:25. All samples were run in triplicates. After 2 days, 100 µL of the supernatant were exchanged with 100 µL medium containing 1 µL ^{3}H-thymidine stock. After additional 16 h the wells were harvested to Filtropur fibreglass filters. After drying at RT, MeltiLex scintillator

film was melted onto the filters in a microwave followed by analysis in a β-plate liquid scintillation counter (Perkin Elmer).

2.13.11. ^{51}Cr release assay

The ^{51}Cr release assay was used to determine the function of cytotoxic T lymphocytes (CTLs), which are capable of lysing MHC mismatched target cells. The latter ones were labelled radioactively with ^{51}Cr. After lysis, the isotope is released to the medium and can be detected in the supernatant. The relative lysis is calculated by dividing the released radioactivity by the residual radioactivity in the cell pellet.

To generate a sufficient number of alloreactive cytotoxic T lymphocytes (CTLs), wt and InsR kd rats were immunized with allogenic splenocytes isolated from DA rats. To this end, the whole spleen of the donor rat was dissected and passed through a 100 µm nylon mesh. The cells were washed once with ice-cold PBS at 300 × g for 7 min at 4 °C. The pellet was resuspended in sterile PBS and the concentration was determined using a Neubauer haemocytometer. 1.5×10^7 cells were injected in a volume of 50 µL in either footpad of the recipient's hind limbs. 5 days later, the animals were fed with Dox-containing diet *ad libitum* to induce the knock down. After 10 days, the draining popliteal lymphnodes were isolated and the lymphocytes were restimulated in a 1:50 ratio with allogenic irradiated splenocytes (dosis 30 Gy) in DMEM ReStim. In parallel, lymphocyte blasts (target cells) were produced by cultivating splenocytes from DA rats in presence of 2.5 µg/mL ConA and 2 µg/mL mouse IL-2. After 5 days, the cells were harvested. The target cells were resuspended in 100 µL FCS and labelled with ^{51}Cr at a concentration of 1 µCu/mL. Subsequently, the labelled blasts were exposed to the CTLs at different effector:target ratios for 4 h. 50 µL supernatant were transferred into a 96 well OptiPlate (Perkin Elmer) and 100 µL liquid scintillation cocktail (Perkin Elmer) were added. To the residual 150 µL 10 µL Triton X were added and mixed. Subsequently, 50 µL of the lysed pellet were transferred into a second 96 well plate and supplemented with 100 µL scintillator. Both plates were sealed with adhesive covers and mixed thoroughly. The specific lysis as indicated by ^{51}Cr release was determined by measurement of ^{51}Cr in the supernatant relative to the total amount of radioactivity in the lysed pellet. The lysis was calculated as [(CCM supernatant × 4)/(CCM pellet × 3 + CCM supernatant)]. The specific lysis is determined as [lysis-spontaneous release].

2.13.12. Transmigration of T cells

To assess the transmigratory behaviour of T cells an *in vitro* transwell system was utilized. Effector T cells were used in this set-up due to their motility and spontaneous migration through pores. To this end, 6.5 mm transwell inserts with a 3 µm pre size were used.

Effector T cells were used directly after restimulation and isolation via the cold gradient. The cells were treated with either Dex or PBS for 3 h in transmigration medium containing 0.5% fat-free BSA, washed with RPMI medium (300 × g, 10 min) and resuspended in transmigration medium in a concentration of 10×10^6 cells/mL.

The lower wells of the transwell system were filled with 600 µL transmigration medium and 100 µL of the effector T cell suspension (1×10^6 cells) was added to the upper chamber. The cells were allowed to migrate for 4 h at 37 °C. To calculate the relative migration, additional wells containing 500 µL medium and 100 µL cell suspension were prepared and set to 100% (input sample). Afterwards, 300 µL of the medium in the lower chambers were harvested and the transmigrated T cells were counted by addition of 50 µL APC beads (BD bioscience) using a FACSCanto II device. The number of cells per bead was calculated relative to the input sample. Each sample was run in duplicates.

2.13.13. Measurement of Ca^{2+} signalling

The measurement of intracellular Ca^{2+} signalling was performed by labelling of effector T cells with Indo1, a Ca-sensitive dye shifting its emission spectrum from 475 nm (Indo blue) to 400 nm (Indo violet) after saturation with Ca^{2+} ions. This change in wave length can be detected by flow cytometry.

Effector T cells were used after antigen stimulation and purification by cold gradient (see 2.13.5). The cell concentration was determined with a Neubauer haematocytometer and 1×10^6 cells were suspended in 700 µL TCGF medium. 0.015% Pluronic F- 127 and 1 µM Indo-1-AM were added to the cells and incubated for 25 min at 30 °C. Afterwards, 700 µL TCGF medium was added and the cells were incubated for 10 min at 37 °C. The cells were pelleted for 4 min at 300 × g and resuspended in 1 mL Krebs-Ringer-solution supplemented with 1 mM Ca^{2+} and washed again for 4 min at 300 × g. After resuspending the cells in 500 µL of Krebs-Ringer solution the Ca^{2+} signal was measured with a LSR II device (Becton Dickinson). After recording a baseline for 25 sec, 5 µL Dex (10^{-5} M, final

concentration 10^{-7} M) or PBS was applied and the measurement was continued for 5 min.

To determine the release of Ca^{2+} only from intracellular sources, the cells were resuspended in 600 µL Krebs-Ringer-solution containing 0.5 M EDTA after the incubation with Indo1. After recording of the baseline (25 sec) and the measurement of intracellular Ca^{2+} release after addition of 6 µL Dex (10^{-5} M, final concentration 10^{-7} M) or PBS (4 min), the extracellular Ca^{2+} was restored by addition of 1 mM $CaCl_2$ and the Ca-signal was recorded for 2 more minutes.

2.13.14. Analysis of T cell - APC conjugate formation by FACS

To measure the capability of effector T cells to interact with APCs, a new approach was set up. eGFP positive effector T cells were co-cultivated with flourochrome-labelled (PerCp) APCs. Subsequent FACS analysis allowed detecting eGFP/PerCp double positive events to calculate the percentage of cells that had formed conjugates. APCs were derived from freshly isolated rat thymi that were passed through a 40 µm nylon mesh. The cells were centrifuged at 300 × g for 5 min and resuspended in 5 mL RatReMed at a concentration of 1.5 ×10^7 cells/mL. The cells were pulsed with 10 µg/mL gpMBP for 1 hr at 37 °C. In parallel, freshly restimulated eGFP-transgenic effector T cells (see 2.13.4) were incubated with 10^{-7} M Dex for 10, 30, 60 or 180 min in RatReMed. Pretreatment with 0.25 µM U73122 was performed 20 min prior to the addition of Dex. Subsequently, the cells were harvested, washed and 5 × 10^4 effector T cells were incubated with 2.5 × 10^6 APCs for 1 hr at 37 °C, followed by staining of the APCs with an anti-Thy1-PerCp antibody and FACS analysis. Conjugates were defined as eGFP/PerCp double positive events.

2.13.15. Confocal microscopy

To analyze the cell size by confocal microscopy, 1 × 10^6 naïve or stimulated T cells were harvested, washed with PBS and resuspended in 400 µL Krebs-Ringer solution. Cells were analysed in 8-well chamber slides (LabTek™, Nunc) using the Leica TCS SP2 microscope (Leica objective PL APO 63×1.3 Glycerol HCX) and Leica Confocal Software. eGFP was excited at 488 nm and emission was collected from 530-600nm. Pictures were taken prior to stimulation of the T cells via TCR/CD28 co-stimulation as described in 2.13.3 and on day 1, 2 and 3 during stimulation.

2.13.16. Production of ConA-Supernatant

ConA supernatant was used as a medium additive for TCGF medium and DMEM ReStim and was produced by stimulation of whole splenocytes with ConA *in vitro*. To this end spleens of three Lewis rats were dissected and passed through a 100 µm nylon mesh. The cells were suspended in 200 mL RPMI supplemented with 1 % Penicillin/Streptomycin, 1% sodium pyruvate, 0.1 % rat serum, 2.5 µg/mL ConA and 1.5 µL β-mercapto ethanol and dispensed in 20 mL cell culture dishes. After 28 h the cells were harvested into 50 mL Falcon tubes and centrifuged at 3000 rpm and 4 °C for 15 min. The supernatant was transferred into fresh Falcon tubes and stored at -20 °C. For usage the supernatant was thawed and centrifuged again at 4000 rpm and 4 °C for 15 min before addition to the media.

2.14. Molecular methods

2.14.1. Polymerase chain reaction (PCR)

Analysis of gene expression i.e. for genotyping was performed by PCR. The PCR reaction mix consisted of 1 µL DNA or cDNA, 0.2 µL Taq polymerase, 0.5 µL primer mix (10 mM, forward + reverse), 2.5 µL Mg-buffer (10×) and 1 µL dNTPs in 19.8 µL aqua dest..

Table 7 PCR protocol

step	temperature	time
initial denaturation	95 °C	2 min
denaturation*	95 °C	15 sec
primer annealing*	60 °C	30 sec
elongation*	72 °C	30 sec
final elongation	72 °C	10 min

* in 35 cycles

2.14.2. RNA isolation

The *Zymo Research RNA isolation kit II* was used to isolate RNA according to the manufacturer's instructions. 5×10^5 cells were used for each sample.

The cells were harvested and pelleted at 350 × g for 7 min. The pellet was lysed using 600 µL ZR buffer and transferred to a column placed in an E-cup. The samples were centrifuged at V_{max} (ca 21000 × g) for 1 min and the flow through was discarded. The column was washed once with pre-wash buffer (400 µL, 1 min V_{max}) and twice with wash buffer (700 µL and 400 µL, 1 and 2 min V_{max}). Afterwards, the column was incubated with 30 µL RNAse/DNAse free water for 1 min at RT, followed by elution of the RNA into a fresh E-cup (1 min V_{max}).

2.14.3. cDNA synthesis

cDNA synthesis was performed with the iScript cDNA sythesis kit (BioRad) according to the manufacturer's instructions. For each 20 µL reaction 2 µL of isolated RNA was used. In brief, RNA was diluted in aqua dest. to a volume of 14.8 µL. 5 µL iScript buffer and 0.2 µL reverse transcriptase was added to each sample, followed by 5 min incubation at 25 °C. Afterwards, the samples were incubated at 42 °C for 30 min and the rection was stopped thereafter at 85 °C for 5 min. Successful cDNA synthesis was checked by PCR using β-actin primer.

2.14.4. quantitative realtime PCR

To determine the relative amount of specific mRNA transcripts of a protein of interest, quantitative realtime PCR was performed. With this, the double-stranded PCR product amount is measured in each cycle by use of the DNA intercalatind dye SYBR green. To this end, cDNA obtained from stimulated T cells or effector T cells was analysed with InsR primers and primers for the housekeeping gene β-actin. For 1 sample, 1 µL of template cDNA was mixed with 0.5 µL prediluted primer mix (10 mM), 12.5 µL of the Power SYBR Green PCR Master Mix and 11 µL ddH_2O. After each PCR cycle (see table 8) the amount of double strand DNA was measured and calculated by the Applied Biosystems 7500 System SDS Software. Crossing threshold (CT) values of the target genes were substracted by the values of the housekeeping gene (Δ CT). The quantity was calculated as $2^{-\Delta CT}$ relative to the wt control. Statistical analysis was performed using GraphPad Prism.

Table 8 qrtPCR protocol

step	temperature	time
enzyme activation	50 °C	2 min
initial denaturation	95 °C	10 min
denaturation*	95 °C	15 sec
annealing/elongation*	60 °C	1 min
dissociation stage	95 °C	15 sec
	60 °C	20 sec
recording of the dissociation curve	stepwise rising of the temperature to 95°C	20 sec

* in 40 cycles

2.14.5. SDS PAGE

The protein expression of cells was analysed by western blot. To this end, the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred onto a nitrocellulose membrane.

The cells were harvested, washed with PBS and taken up in lysis buffer (40 µL buffer for 1×10^6 cells). After 1.5 h on ice the lysates were centrifuged at 21000 × g for 25 min and the supernatant was carefully transferred into the equal volume of 2× Laemmli. The lysates were heated at 95 °C for 5 min and separated on a 7.5 % SDS-PAGE gel.

For SDS page both separating and stacking gel were freshly prepared according to table 9, APS was added shortly before pouring of the gel.

Table 9 SDS-PAGE gel recipe

ingredients	stacking gel	separating gel (7,5%)
upper buffer	938 µL	-
lower buffer	-	1.95 mL
30% acrylamide	600 µL	2.025 mL
H_20	2.205 mL	3.975 mL
TEMED	3.75 µL	7.5 µL
APS (added directly prior to casting)	37.5 µL	49,95 µL

20 µL of the protein lysates (= 2.5 x 10^5 cells/lane) or 8 µL of prestained protein marker were loaded per lane. Empty pockets were filled with 20 µL 1× Laemmli. The SDS-PAGE was run at 20 mAmp per gel for 50 min.

2.14.6. Western Blot

The proteins separated by SDS-PAGE were further analyzed by Western Blot. To this end the proteins were transferred to an ECL nitrocellulose membrane and stained with specific antibodies.

The membrane and filter paper were pre-wetted in 1× blotting buffer. The separating gel (see above) was placed on the membrane and between two filter papers in a semi-dry blotting chamber. After blotting at 16 V for 1:10 h the gel was discarded and the membrane was incubated in blocking buffer for 50 min. Afterwards, the membrane was washed three times for 10 min with PBS/Tween and incubated at 4 °C over night with the primary antibodies, followed by three washing steps for 10 min in PBS/Tween. The incubation with a HRP-conjugated secondary antibody in a 1:10000 dilution in PBS/Tween was performed at room temperature for 1 h. After three washing steps the staining was conducted by adding 2 mL freshly prepared development solution per membrane and chemiluminescence was detected using the ChemiLux Imager. Densitometric quantification of the band intensities was achieved using GelPro analyzing software. In case of P-ERM both bands were totalled. The specific background was individually substracted in each case.

2.15. Statistical analysis

Statistical analysis was performed using Prism software. For all analyses student's unpaired t-test was used. All data is depicted as mean ± s.e.m.. Measures of significance: n.s. = not significant: $p > 0.05$; *: $p < 0.05$; **: $p<0.01$; ***: $p < 0.001$.

3. Results

3.1. Influence of GCs on EAE

GCs are known to suppress T cell functions (Herold et al., 2006; Reichardt et al., 2001; Wilckens and De Rijk, 1997). They are the treatment of choice for autoimmune diseases and high-dose GC therapy is the standard regimen for patients suffering from acute MS relapses. The action is quick and effective, but the exact mechanisms how GCs exert their beneficial effects are still not fully understood. T cell migration is a crucial step during disease progression. Impact of GCs on effector T cell migration or morphology would be one possible mechanism explaining the beneficial effects during treatment. Therefore we set out to analyse the immediate rapid effects of GCs directly on the cellular level using CNS-antigen-specific effector T cells.

3.1.1. Generation of encephalitogenic T cells for morphological analysis

Antigen-specific effector T cells are an important pathogenic factor in MS. To study the influence of GCs on their morphological features they can be isolated from immunized rats and propagated *in vitro*. As a first approach to obtain insight into their morphological changes following treatment, gpMBP-specific encephalitogenic T cells (effector T cells) were generated from eGFP transgenic UGC-Lewis rats. Repeated restimulation with gpMBP confirmed their antigen specificity as published (Wekerle et al., 1986). Analysis of these cells by confocal microscopy was done in collaboration with Nora Müller (University of Würzburg). After treatment of the cells with the GC Dexamethasone (Dex), a dramatic alteration of T cell morphology was observed, which was not seen in the PBS treated controls (Fig 6). Most notably, the polarized structure of the effector T cells was lost after incubation with Dex (Fig. 6A). The lammelipodiae retracted and the cells adapted a round shaped form. Interestingly, this effect was observed as early as 10 min after treatment and lead to a significant reduction of the T cell area as depicted in Fig. 6B. The effect was most pronounced after 180 min of Dex treatment, when the cell size was reduced by approximately half. Since these are remarkable alterations of the T cell cytoskeleton, which is important for T cell functions as transmigration or chemotaxis, we further analysed these properties of polarized T cells.

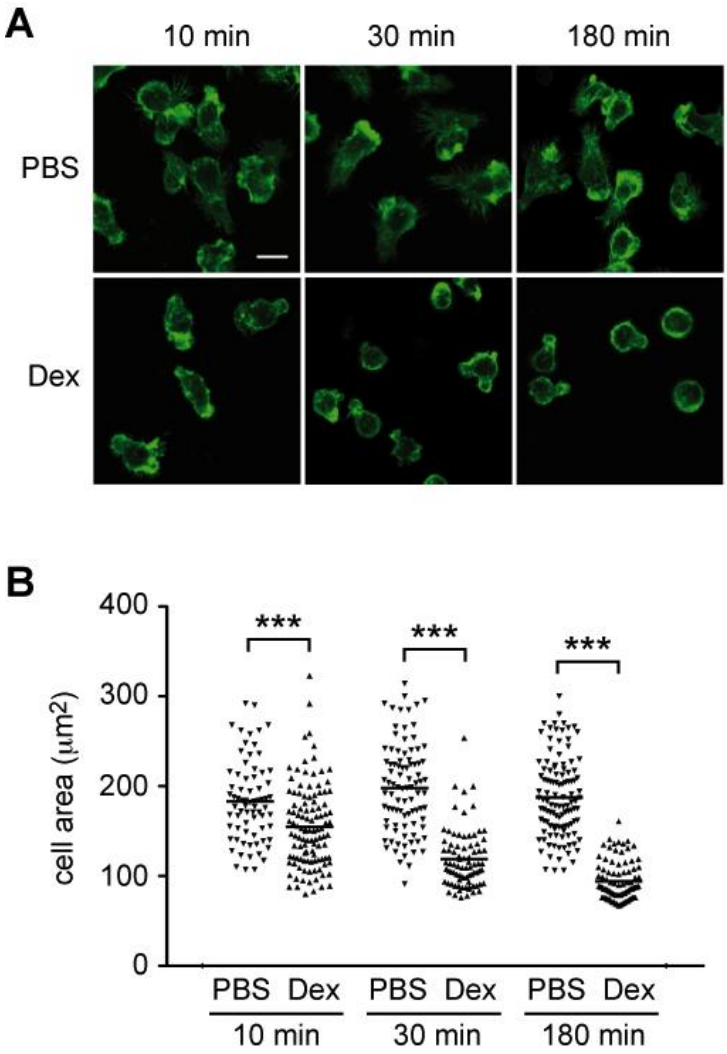

Fig. 6 GC induce effector T cell depolarization and reduction of T cell size. (A) Newly generated effector T cells were incubated with 10^{-7} M Dex or PBS as a control for the indicated periods of time. Morphological alterations were analysed by staining of the cytoskeleton with Phalloidin-Alexa488 and confocal microscopy. Scale bar equals 10 µm. Quantitative analysis of the cell area is depicted in (B). Confocal microscopy and analysis was performed by Dr. Nora Müller (Müller et al., 2013).

3.1.2. The transmigratory capacity of effector T cells is impaired by GC treatment *in vitro*

Transmigration or extravasation of T cells from blood vessels into target tissues is a key event during inflammation. This crucial mechanism is thought to be inhibited by GCs through tightening of the blood-brain-barrier (Paul and Bolton, 1995; Romero et al., 2003), but also inhibition of the transmigratory capacity of T cells might be an important mechanism. The rearrangements of the cytoskeleton observed after GC treatment could potentially interfere with transmigration, because the polarized structure of T cells is essential for proper migration and extravasation and is needed for their activation (Billadeau et al., 2007). To assess the transmigratory behaviour of effector T cells, a transwell system was used, where the two compartments are separated by a membrane with a 3 µm pore size. Activated T cells spontaneously transmigrate through these pores and the transmigrated population can be quantified

by FACS. To assess this property, effector T cells were pre-incubated with 10^{-7} M Dex for 10, 30, 60 and 180 min and thereafter allowed to transmigrate without further Dex treatment for 3 h. As a control effector T cells without pretreatment with Dex were used. Already 30 min of Dex treatment prior to the transmigration assay significantly reduced the number of effector T cells that had crossed the membrane (Fig. 7). The percentage of transmigrated effector T cells declined even further over time. After 3 h pre-incubation with Dex, the number of transmigrated cells was reduced to 50% of the untreated control.

This is in line with the dynamics of T cell depolarization. We conclude, that GCs modulate effector T cell migration by cytoskeletal rearrangements.

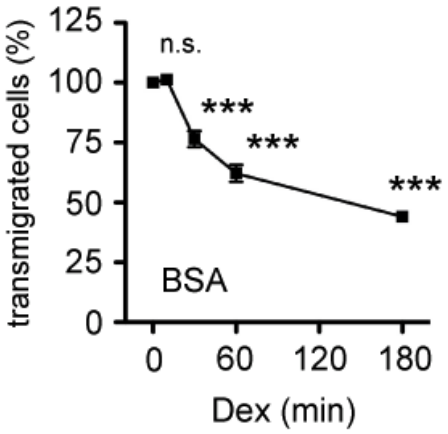

Fig. 7 Impaired transmigration of effector T cells after GC treatment. After preincubation of effector T cells with 10^{-7} M Dex or PBS as a control for 10, 30, 60 or 180 min, respectively a transmigration assay was performed without further Dex treatment. T cells were allowed to migrate through a 3 µm pore size transwell membrane for 3 h. The number of cells that had migrated into the lower chamber was counted by FACS using beads as a standard. The cell number in a control setup without Dex pretreatment was set to 100%. N = 4 (one out of four independent experiments is depicted, mean ± SEM (Müller et al., 2013)

3.1.3. Dex reduces the capacity of effector T cells to form APC conjunctions

Altered morphology of effector T cells is likely to influence several functions besides transmigration. Another crucial step in the course of an immune response is the interaction with professional antigen presenting cells (APCs). The formation of such conjugates is essential for the reactivation of the T cells at the site of inflammation. To analyse whether the capacity of T cells to interact with APCs is also impaired by the morphological changes induced by GC treatment, an experimental set-up was designed to quantify the number of conjugates that are formed with APCs. FACS analysis was used to determine the kinetics of the GC-induced changes. To track effector T cells specifically, we derived them from eGFP transgenic UGC Lewis rats by immunization with gpMBP as described earlier. Freshly isolated thymocytes from wt Lewis rats were used as APCs, because these cells are also used for the antigen-

specific restimulation of effector T cells during the course of cultivation. The APCs were pulsed with gpMBP for 1 h. In parallel, eGFP-transgenic effector T cells were treated with Dex for 10, 30, 60 and 180 min. Subsequently, the effector T cells were mixed with the antigen-pulsed thymocytes and allowed to form conjugates for 1 h. Afterwards, these cells were stained with an anti-Thy1 antibody to specifically identify the thymocytes, because all thymocytes express this marker and as this molecule is not involved in the formation of the immunological synapse. To this end, the thymocytes were labelled with anti-Thy1-PerCp antibody and the percentage of GFP/PerCp double positive events representing conjugates formed by effector T cells and APCs was assessed by FACS (Fig. 8).

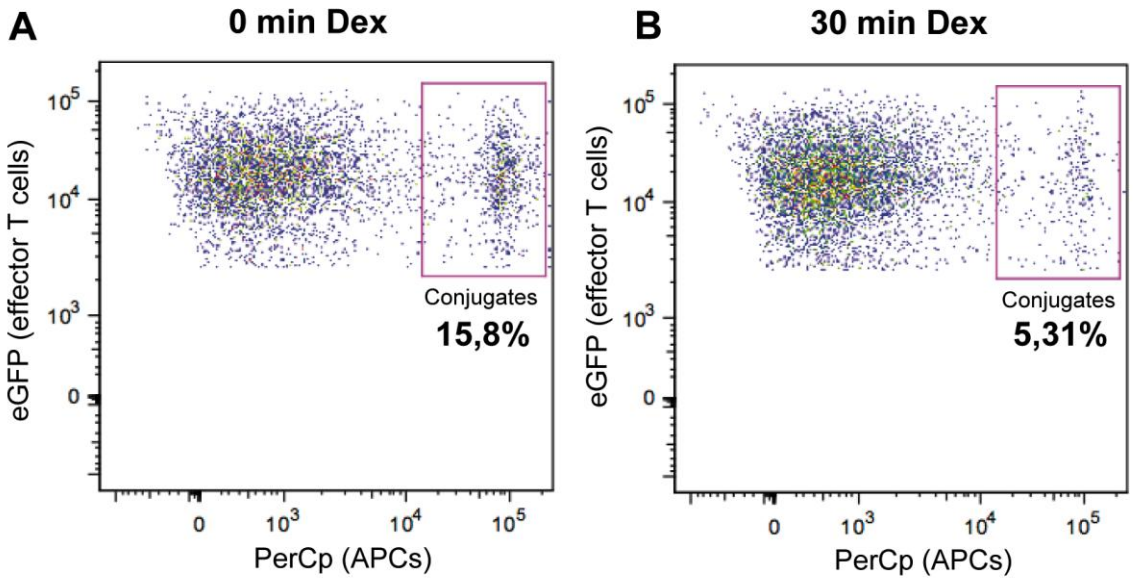

Fig. 8 FACS plots showing effector T cell-APC conjugate formation. Effector T cells were pretreated with 10^{-7} M Dex (0 min; A or 30 min; B) and subsequently allowed to form conjugates with PerCp labelled APCs (anti-Thy1-PerCp). The percentage of double positive events representing conjugates was determined. Two representative plots out of five independent experiments are depicted.

In each experiment, conjugate formation by untreated effector T cells was set to 100 %. Interestingly, the capability of effector T cells to form stable interactions with APCs was already significantly reduced as early as 10 min of preincubation with 10^{-7} M Dex. The percentage of conjugates decreased even further with a longer time of pretreatment (Fig. 9). Notably, 30 min of pre-incubation with Dex was sufficient to reduce the conjugate formation to 40% compared to untreated effector T cells and this effect was maintained for up to 3 h. We conclude, that the GC induced reduction of effector T cell-APC interaction could contribute to the beneficial effect of GC treatment in acute MS relapses.

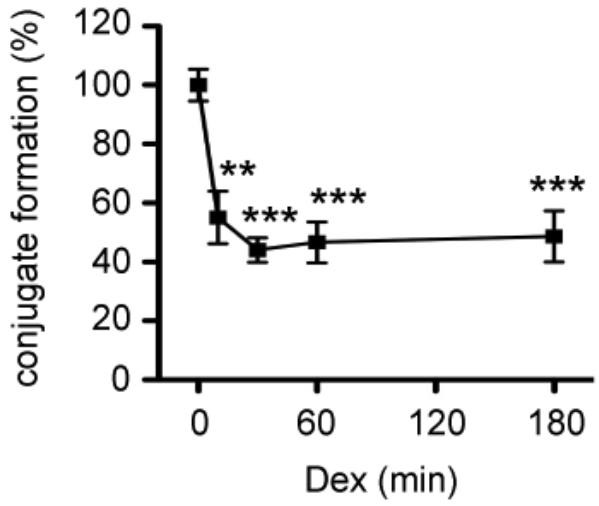

Fig. 9 GCs reduce the capability of effector T cells interact with APCs. eGFP-transgenic effector T cells were pretreated with 10^{-7} M Dex for 10, 30, 60 or 180 min, washed and incubated for 1 h with MBP-pulsed thymocytes serving as APCs. Afterwards, the thymocytes were stained with an anti-Thy1-PerCp antibody and the formation of conjugates as indicated by GFP/PerCp double positive events was assessed by FACS. N = 8 (five independent experiments). Conjugate formation without Dex treatment was set to 100%. The percentage of conjugates is depicted as the mean ± SEM, (Müller et al., 2013).

3.1.4. Dex treatment induces phosphorylation of ERM proteins

The hitherto obtained results indicated an important link between GC treatment and the cytoskeleton. Candidate molecules that might be regulated by GCs are proteins of the Ezrin-Radixin-Moesin (ERM) family, two of which are expressed in T cells, namely Ezrin and Moesin. These proteins are highly phosphorylated in resting cells and thereby provide a link between the membrane and the cytoskeleton. Following T cell activation they are dephosphorylated and thereby allow the cytoskeleton to be rearranged. This leads to the polarization of the cell, the formation of lamellipodiae at the leading edge and the formation of the distal pole complex. To investigate the involvement of ERM proteins in the GC-induced cytoskeleton rearrangements, effector T cells were treated with 10^{-7} M Dex or PBS for different periods of time, subsequently lysed and analysed by western blot. An anti-P-ERM antibody was used that detects phosphorylation at specific threonine residues (Thr567; Ezrin/Thr558; Moesin), the predominant protein modifications seen after stimulation (Fig. 10A). Following Dex treatment, the ERM proteins were rapidly phosphorylated. Phosphorylation was initially detectable as early as 10 min after treatment, reaching maximal levels after 30 min of treatment. In contrast, treatment with PBS had no influence on P-ERM levels. To obtain a more quantitative picture, the amount of P-ERM relative to the untreated control was determined by densitometric analysis (Fig. 10B) based on five experiments in total. This revealed that ERM phosphorylation already increased after 10 min of Dex treatment. After 30 min it had increased three-

fold compared to the untreated control (Fig. 10B). We conclude, that the observed GC-incuced changes of the cytoskeletal architecture of effector T cells could be mediated by increased activation of ERM proteins.

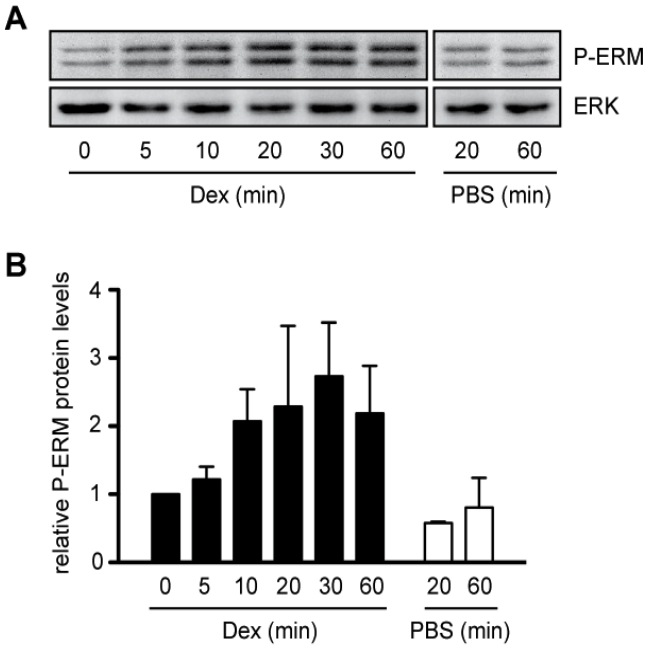

Fig. 10 GCs induce rapid phosphorylation of ERM proteins. (A) Western blot analysis of effector T cells that were incubated with 10^{-7} M Dex or PBS as a control for the indicated time periods. Lysates were analyzed for P-ERM levels; 5×10^5 cells were loaded in each lane. ERK expression served as a loading control. One representative western blot out of 5 independent experiments is depicted. (B) Densitometric quantification of protein band intensities using GelPro analyzing software. For P-ERM the totalled density of both protein bands was determined. The diagram depicts the relative amount of phosphorylated ERM as compared to untreated cells (mean ± SEM). The experiment was independently repeated 5 times (Müller et al., 2013).

3.1.5. GC induced ERM phosphorylation requires the presence of the GR

As we had observed, GC treatment induced phosphorylation of ERM proteins in effector T cells. To confirm that this mechanism was mediated by the GC receptor (GR), siGR effector T cells were used. These cells were generated by retroviral transduction of wt effector T cells with short hairpin RNA (shRNA) targeting the rat GR. Expression of this shRNA led to the reduction of GR protein levels and the absence of GC responsiveness (Tischner et al., 2009). These GR-deficient effector T cells were treated with 10^{-7} M Dex or PBS for 5, 10, 20, 30 and 60 min and subsequently analysed by western blot. Importantly, the P-ERM levels in these cells did not change after GC treatment (Fig. 11 A and B), indicating that the GR was essential for phosphorylation of ERM-proteins. Quantification of the band intensities of P-ERM in four independent experiments revealed that no ERM phosphorylation occurred in siGR effector T cells following Dex treatment. Of note, this finding is in line with the observation that siGR effector T cells do not undergo depolarization and cytoskeletal rearrangements after Dex treatment (Müller et al., 2013). We conclude,

that GCs induced both ERM phosphorylation as well as the observed cytoskeletal rearrangements could be mediated by the GR.

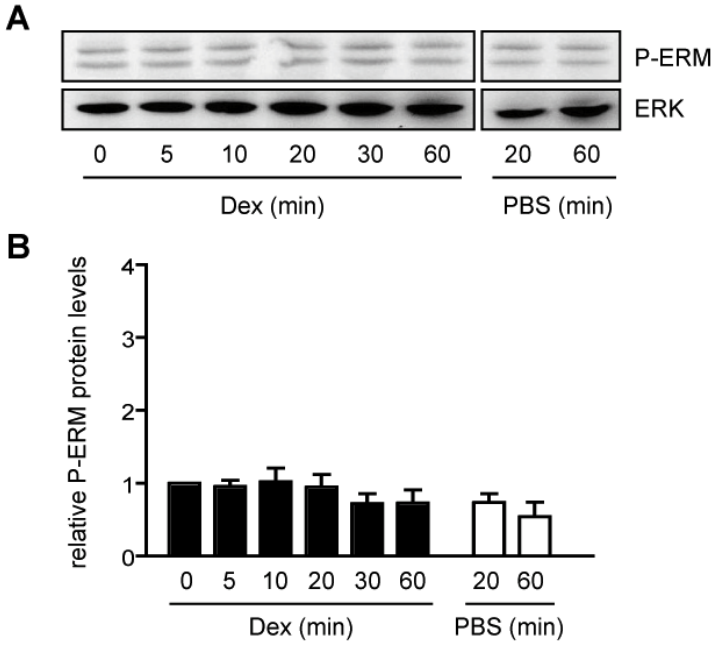

Fig. 11 GC induced P-ERM phosphorylation requires presence of the GR (A) Wester blot analysis of ERM phosphorylation in siGR effector T cells after treatment with 10^{-7} M Dex or PBS as a control for the indicated time. Equal numbers of cells were loaded in each lane; ERK expression served as a loading control. One representative analysis out of 4 is depicted. (B) Densitometric analysis of protein band intensities using GelPro analyzing software. For P-ERM the combined density of both protein bands was determined. The diagram depicts the relative amount of phosphorylated ERM compared to untreated cells (mean ± SEM). The experiment was independently repeated 4 times.

3.1.6. Effector T cell depolarization is not accompanied by increased calcium signalling

Up to now we had identified a new effect of GCs on effector T cells leading to cytoskeleton rearrangements and P-ERM phosphorylation. To further dissect this mechanism and the pathway leading to the observed changes, the Ca^{2+} response to GC treatment was analysed.

Ca^{2+} is an important second messenger that is involved in numerous signalling cascades. An increase in cellular Ca^{2+} levels could also be an effect caused by GCs. To measure changes in intracellular Ca^{2+} levels we made use of the Ca-sensitive dye *Indo blue*. The emission maximum changes from 475 nm (blue) in Ca^{2+} free medium to 400 nm (violet) when bound by Ca^{2+}. By determining the shift in the ratio of Ca-unbound to Ca-bound indo (blue:violet) by FACS the signalling events can be displayed over time. In order to observe changes in the Ca2+ levels, a baseline was recorded for 20 sec prior to treatment of the cells with 10^{-7} M Dex or PBS as control. Interestingly, Dex treatment had no influence on the intracellular Ca^{2+} levels compared to PBS treated control cells (Fig. 12 left).

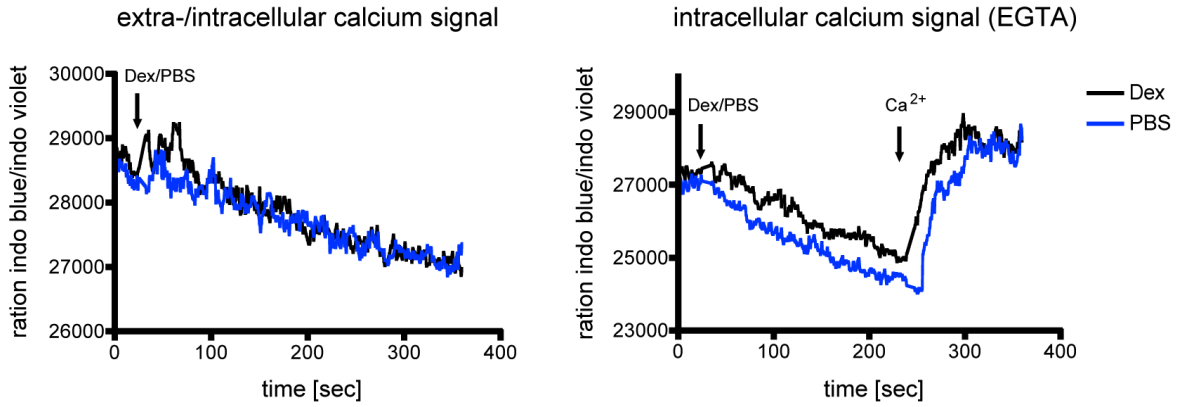

Fig. 12 Dex treatment of effector T cells does not trigger calcium signalling. Effector T cells were labelled with the calcium sensitive dye Indo-1. After recording of a baseline for 20 sec, the cells were treated with 10^{-7} M Dex or PBS as a control (indicated by an arrow). An increase in the ratio of Indo blue:Indo violet illustrates increased intracellular Ca^{2+} levels. (Left) Changes of extra and intracellular calcium in total (N=6 per treatment). (Right) Extracellular calcium was depleted by EGTA, so in the first phase only calcium release from intracellular sources is recorded. Restoring the extracellular calcium (indicated by an arrow) results in spontaneous calcium uptake (N=4 per treatment). Representative results are depicted.

Although the ratio decreased over time the observed changes were very small. A real signalling event would result in a 10-fold change of this ratio. Furthermore, there was no difference between Dex- and PBS-treated cells. We also discriminated between Ca^{2+} increase form intra- and extracellular sources. Ca^{2+} can be released from the endoplasmatic reticulum (ER) as well as influx from extracellular medium. To distinguish between both sources, Ca^{2+} was removed from the medium by use of EGTA containing Krebs-Ringer-solution. Stimulation in this surrounding should result in increased intracellular Ca^{2+} only when it is released from the ER. Addition of Ca^{2+} to the medium afterwards (indicated by an arrow) restored extracellular Ca^{2+} levels and the impact of extracellular Ca^{2+} to the signal could be determined. Also in this set-up we did not see any difference between Dex- and PBS-treated cells (Fig. 12 right). After addition of extracellular Ca^{2+} the intracellular Ca-levels spontaneously increased, but also irrespective of the treatment. Collectively, our findings indicate that the GC-induced pathway leading to the observed morphological changes of effector T cells does not involve Ca^{2+} signalling.

3.1.7. Depolarization of effector T cells is sensitive to inhibition of Phospholipase C

To further dissect the mechanism by which the GR mediates the phosphorylation of ERM proteins the role of Phospholipase C (PLC) was studied. It was reported previously, that two kinases are capable of ERM phosphorylation, namely ROCK and

PLC (Belkina et al., 2009; Ng et al., 2001; Ren et al., 2009). The latter one was reported to be rapidly activated by GC treatment in thymocytes and therefore we considered it a promising candidate being responsible for the observed effects.

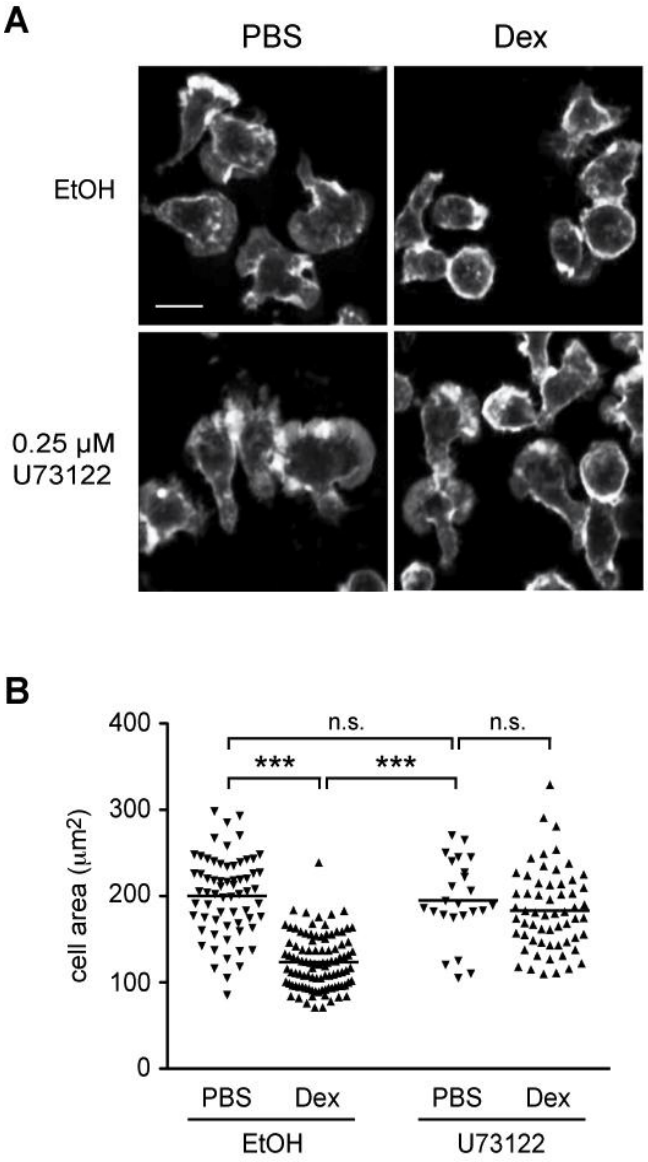

Fig. 13 PLC inhibition impacts on GC induced T cell depolarization. (A) Effector T cells were preincubated with 0.25 µM U73122 or vehicle (EtOH) as a control for 20 min followed by treatment with 10^{-7} M Dex for 30 min. Subsequently, the cells were stained with Phalloidin-Alexa594 and analyzed by confocal microscopy (size bar: 10 µm). Representative images are depicted in black and white for reasons of improved clarity. (B) Effector T cell area was determined by confocal microscopy in combination with ImageJ and is individually depicted for each cell (μm^2)In addition, the mean cell area for each condition is depicted as a horizontal line. Statistical analysis was performed by unpaired t-test (***: $p < 0.001$, n.s.: not significant). Confocal microscopy and analysis was performed by Dr. Nora Müller (Müller et al., 2013).

Consequently, we tested whether this enzyme was involved in mediating the effects of the GR on the cytoskeleton by use of the PLC inhibitor U73122. Confocal microscopy analysis revealed that pre-incubation of effector T cells with this inhibitor at a concentration of 0.25 µM prevented the GC induced depolarization of effector T cells as shown by unaltered cell area (Fig. 13). This led to the conclusion, that PLC activity was required for the morphological changes induced by GCs. To determine whether inhibition of PLC also prevented GC-induced ERM phosphorylation, western blot analysis of effector T cells was performed (Fig. 14A). As expected, P-ERM levels

increased after 30 min Dex-treatment, whereas this was not the case when the cells were pre-treated with 0.25 µM U73122.

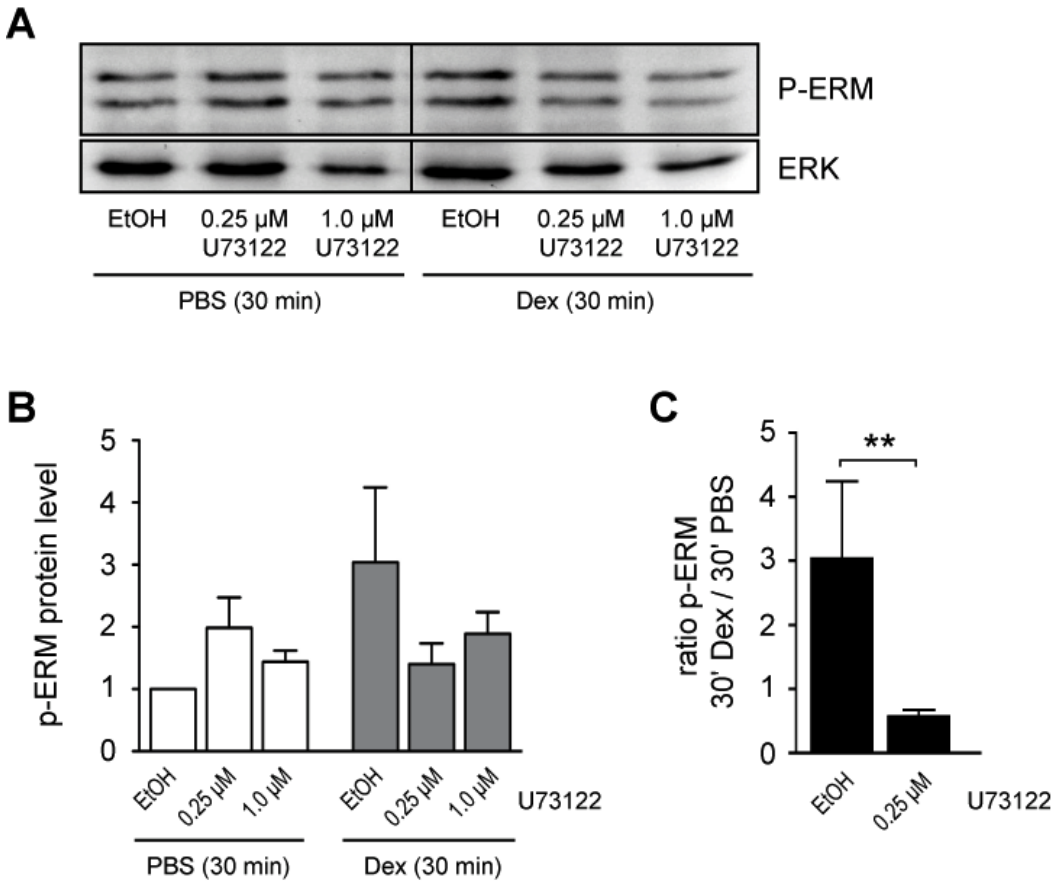

Fig. 14 GC induced P-ERM phosphorylation is sensitive to PLC inhibitor U73122. (A) After pretreatment with 0.25 µM U73122 or 1.0 µM PLC inhibitor U73122 or vehicle (EtOH) for 20 min, effector T cells were were incubated for 30 min with 10^{-7} M Dex or PBS as a control and analyzed for ERM phosphorylation by western blot. In each lane equal numbers of cells were loaded; ERK expression served as loading control. One representative analysis is depicted. N=7 (B) Quantification of ERM-phosphorylation by densitometric analysis of 7 experiments. The totalled intensity of both ERM bands relative to ist bands in untreated control cells is depicted. (C) As U73122 leads to a slight phsphorylation of ERM by itself, the ratio of P-ERM in Dex-treated versus PBS-treated cells was calculated for effector T cells preincubated either with vehicle (EtOH) or 0.25 µM U73122. All data are depicted as the mean ± SEM; statistical analysis was performed by unpaired t-test (*: $p < 0.05$, **: $p < 0.01$) (Müller et al., 2013).

To obtain a quantitative picture of the observed effects, the experiment was repeated 7 times and quantified via densitometry analysis of the bands and normalization to the vehicle treated control (Fig. 14B). We observed a slightly increased phosphorylation of ERM proteins after incubation with the inhibitor alone at both tested concentrations (Fig. 14B), but the treatment with U73122 largely prevented the phosphorylation of ERM proteins after Dex incubation. Dex increased P-ERM levels about threefold, while after pre-treatment of the cells with U73122 P-ERM levels did

not significantly change compared to PBS treated controls. Because treatment with U73122 by itself slightly induced ERM phosphorylation, a ratio between Dex- and PBS-treated control cells was calculated for vehicle (EtOH) and U73122 (0.25 µM) treated cells (Fig. 14C). The relative P-ERM levels were significantly reduced after pre-incubation with PLC-inhibitor. Collectively, our data suggest that the GC-induced T cell polarization is presumably mediated by PLC-dependent ERM phosphorylation.

3.1.8. Repression of transmigration and APC conjugation by GCs depends on PLC activity

Since PLC inhibition interfered with morphological alterations and ERM activation, we tested whether PLC was also required for GC-induced inhibition of transmigration and T cell:APC conjugate formation. As described earlier, treatment of effector T cells with GCs led to an impaired transmigratory behaviour in a transwell system. After Dex treatment, the number of T cells that had migrated into the lower chamber were significantly reduced. When PLC inhibitor was added to the effector T cells 20 min prior to Dex treatment at a concentration of 0.25 µM the GC-induced reduction of transmigration was prevented (Fig. 15A).

In agreement with our earlier findings conjugate formation as assessed by FACS analysis of eGFP expressing effector T cells with labelled APCs was reduced by pretreatment of the effector T cells with Dex. Importantly, this feature of GCs was prevented by preincubation with 0.25 µM U73122 as well (Fig. 15B). The inhibitory effect of Dex on conjugate formation after 30 and 60 minutes was abolished at both time points after blocking PLC although statistical significance was not reached at the 30 min timepoint (Fig. 15A,B). We conclude that the inability of Dex to induce effector T cell depolarization in the absence of PLC activity also prevented the effects of Dex on these cells' functional features.

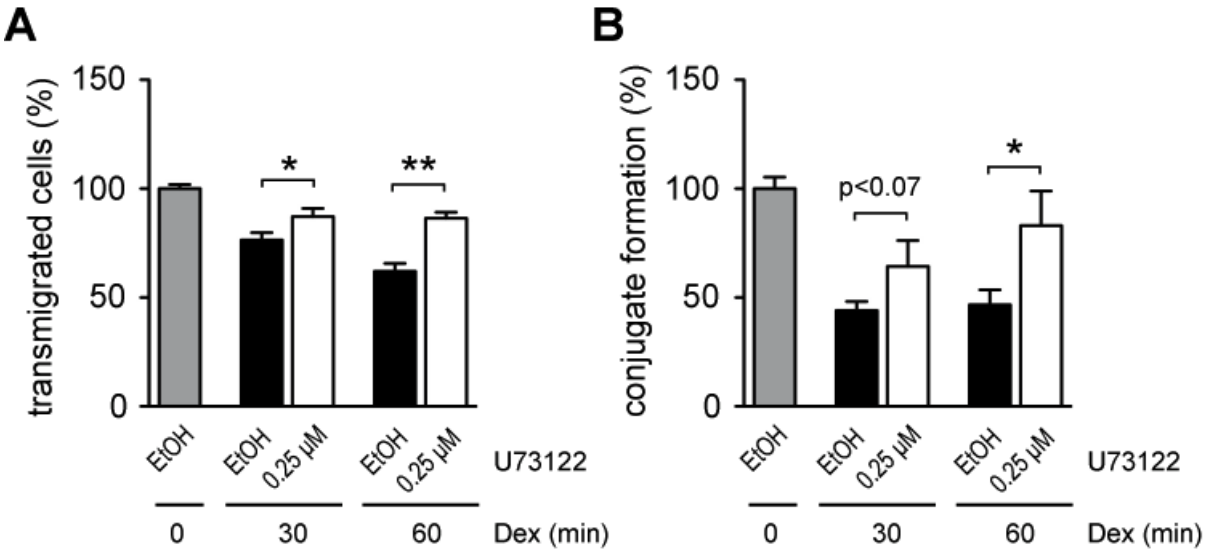

Fig. 15 The inhibitory effects of Dex on transmigration and APC conjugation are abolished by inhibition of PLC (A) Preincubation of effector T cells with 0.25 µM U73122 or vehicle (EtOH) for 20 min before the treatment with 10^{-7} M Dex for 0, 30 or 60 min reduced the inhibitory action of Dex in a transmigration assay. Effector T cells were allowed to migrate for 3 h without further treatment. The number transmigrated cells after treatment with the vehicle control (EtOH) was set to 100%. N = 4 (one out of four independent experiments is depicted). (B) eGFP expressing effector T cells were treated with 10^{-7}M Dex for 0, 30 or 60 min after 20 min incubation with U73122 or the vehicle control (EtOH). Thereafter the cells were allowed to form conjugates with MBP-pulsed APCs without further treatment. Staining of the APCs with anti-Thy1-PerCp and FACS analysis allowed to identify conjugates as represented by eGFP/PerCp double positive events N = 8 (five independent experiments) (Müller et al., 2013).

3.1.9. GCs induce effector T cell depolarization *in vivo* which correlates with their disappearance from peripheral blood

To test whether the observed morphological and behavioural changes might also be of relevance *in vivo*, effector T cells isolated from eGFP expressing UGC Lewis rats were generated and transferred intravenously in naïve syngeneic eGFP negative recipients and allowed to equilibrate for 48 h. Thereafter the rats were injected i.v. with Dex at a dose of 20 mg/kg. Afterwards, lymph node cells were isolated 0, 4 and 24 h after Dex administration. Based on eGFP expression the transferred effector T cells could be discriminated from naïve recipient T cells. The re-isolated effector T cells were analysed by confocal microscopy (experiment performed by Nora Müller, University of Würzburg). Interestingly, the re-isolated effector T cells had adopted a round shape phenotype as seen before *in vitro* (Fig. 16A).

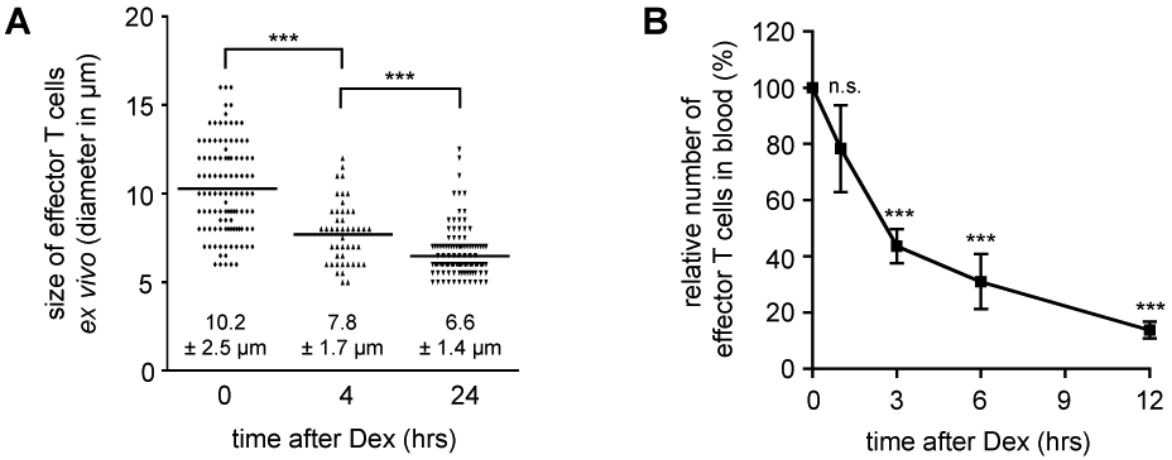

Fig. 16 GCs induce depolarization of effector T cells *in vivo* and lead to their disappearance from the peripheral blood. (A) eGFP-transgenic effector T cells were injected into rats followed by administration of 20 mg/kg Dex 48 h later. Subsequently, lymph node T cells were isolated at the indicated time points by magnetic cell sorting, stained with Phalloidin-Alexa595 and analyzed by confocal microscopy. The diameter of the effector T cells was analyzed. This experiment was performed by Nora Müller (University of Würzburg) using our effector T cells. (B) 1×10^7 eGFP-transgenic effector T cells were adoptively transferred into rats and Dex was injected intravenously at a dose of 20 mg/kg 48 h later. The number of $eGFP_+$ effector T cells in the blood was determined by FACS analysis of peripheral blood samples from the tail vein obtained at the indicated time points after Dex treatment. The average number of $eGFP_+$ effector T cells per volume of blood at the time point of Dex injection was set as 100%. N = 5, pool of two independent experiments. All values are depicted as the mean ± SEM; statistical analysis was performed by unpaired t- test (***: $p < 0.001$, n.s.: not significant (Müller et al., 2013).

Already 4 h after treatment, the transferred effector T cells had become smaller. After 24 h this effect was even more pronounced as almost all re-isolated effector T cells had retracted their lamellipodiae and adopted a similar shape as naïve T cells, indicating that the observed morphological changes as the rearrangement of the cytoskeleton that we had observed *in vitro* also take place *in vivo* (Fig. 16A). As we had found also profound changes in effector T cell behaviour as transmigration, we wanted to determine the influence of GC treatment on the number of circulating effector T cells. To this end, eGFP expressing effector T cells were transferred intravenously into naïve wt rats and allowed to equilibrate for 48 h. Afterwards, the recipient rats were treated with 20 mg/kg Dex and peripheral blood was drawn after 1, 3, 6 and 12 h. The number of effector T cells per blood volume prior to Dex treatment was set to 100% in each case and the relative number of eGFP expressing effector T cells was calculated for each time point (Fig. 16B). Interestingly, after 3 h of treatment the number of circulating effector T cells was reduced by approximately half and declined even further after 6 and 12 h of treatment. As the disappearance of

the effector T cells follows the same dynamics as the cytoskeleton rearrangements *in vivo*, we conclude that both mechanisms happen back to back.

3.2. Role of the Insulin Receptor for the modulation of T cell function *in vitro* and *in vivo*

The immune system as well as several other body functions is regulated by hormones. Besides steroid hormones it has also been known for many years that dysregulation of insulin responsiveness leads to an increased risk of infections (Calvet and Yoshikawa, 2001; Joshi et al., 1999; Shah and Hux, 2003). As lymphocytes undergo profound morphological changes upon activation that are energy consuming, it is likely that disturbed metabolism impacts on lymphocyte function.

To investigate regulation of immune responses by insulin, a rat model with inducible and reversible insulin receptor knockdown (InsR kd) was used (Herold et al., 2008). In all cases eGFP transgenic rats served as wildtype (wt) controls. The knockdown was always induced *in vivo* by providing Doxycycline containing food pellets for 5 days prior to T cell isolation. In addition, Dox was added at a concentration of 2 µg/mL to the cell culture medium. To test that the knockdown of the insulin receptor (InsR) was successful, blood glucose levels were determined before T cell isolation. Usually 4-5 days after the induction of the InsR knockdown blood glucose levels were strongly elevated and the rats started to become diabetic (Fig 17).

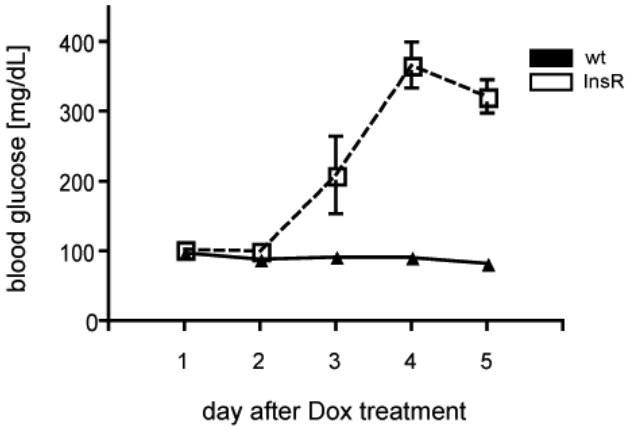

Fig. 17 Blood glucose levels after Doxycyline treatment. InsR knockdown (InsR) and eGFP transgenic UGC (wt) rats were fed with Dox containing diet *ad libitum*. The InsR kd rats showed elevated blood glucose levels from day three of treatment on and started to become diabetic at day 4. N=4 (InsR) and 2 (wt).

3.2.1. Early activation of $CD4^+$ T cells is impaired in the absence of InsR

To elucidate the role of the InsR for the modulation of immune responses and T cell function the expression of the InsR in T cells was characterized. To this end, $CD4^+$ T cells were isolated from naïve eGFP expressing UGC-Lewis (wt) or InsR kd rats and stimulated *in vitro* by use of an anti-TCR antibody (R73) in combination with an anti-

CD28 (JJ316) antibody. By western blot analysis we could show that this costimulation induced InsR expression in naïve T cells *in vitro*, which was prevented after inactivation of the InsR (Fig. 18). Equal numbers of $CD4^+$ T cells were loaded in each lane and β-tubulin expression served as a loading control.

Because Akt is a central player in InsR signalling, we also analysed the P-Akt levels (data not shown). As we were not able to detect differences in the Akt phosphorylation, we decided to keep the cells under serum starved conditions for 1, 2 and 3 h to lower the basal levels of Akt phosphorylation and to unmask differences between wt and InsR deficient T cells. Also with this approach we were unable to demonstrate any differences on the P-Akt level between wt and InsR kd T cells, indicating that InsR deficiency has no profound impact on this signalling molecule, at least under the employed strong stimulating conditions (Fig. 18).

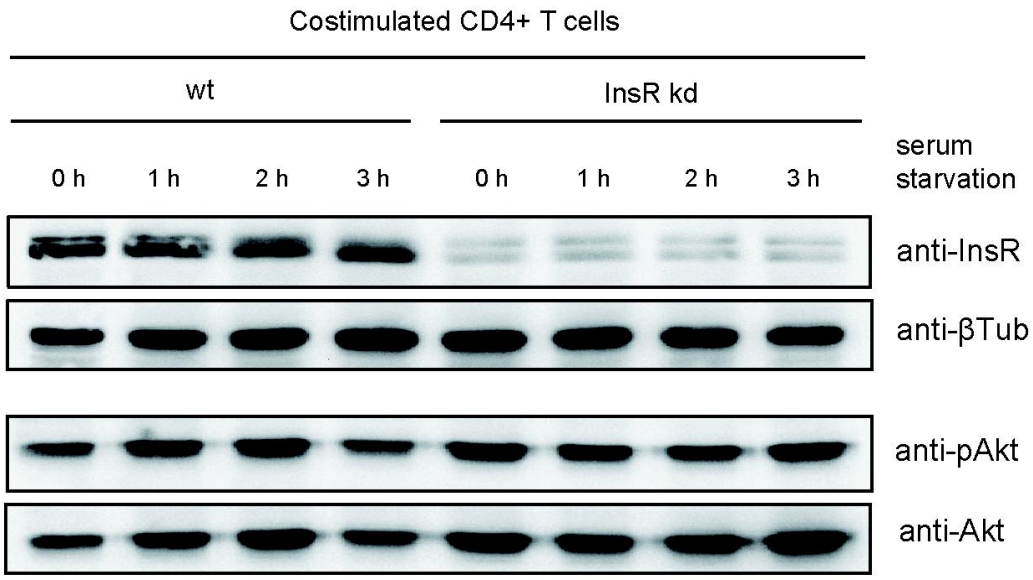

Fig. 18 Western blot analysis of anti-TCR/-CD28 costimulated CD4+ T cells. On day 3 of stimulation the cells were harvested and kept under serum starved conditions for the indicated periods of time. Subsequently, the cells were lysed and western blot analysis was performed. Equal numbers of cells were loaded in each lane, β-tubulin expression served as loading control.

Next we analysed the activation of co-stimulated $CD4^+$ T cells by FACS making use of the activation markers CD25 and CD134. The first one is the IL-2 receptor α-chain and up-regulated upon T cell stimulation. CD134, also known as Ox-40, is a T cell specific activation marker. FACS analysis of co-stimulated wt and InsR kd $CD4^+$ T cells revealed that lack of InsR led to a reduced number of CD25 single positive cells, which indicates that the first phase of T cell activation was impaired (Fig. 19A).

Interestingly, at later states of T cell activation as indicated by co-expression of both CD25 and CD134 lack of the InsR had no influence (Fig. 19B).

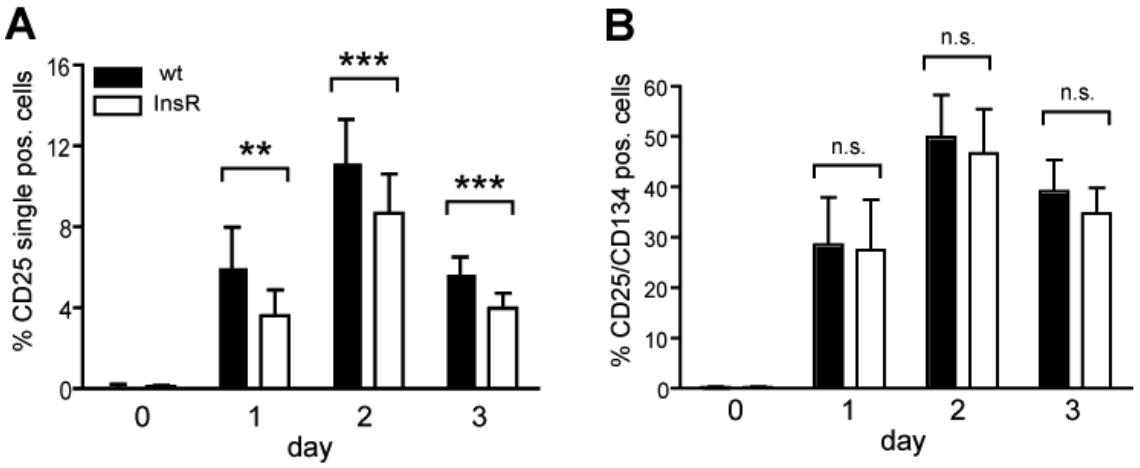

Fig. 19 InsR knockdown impedes early activation of T cells, whereas later activation steps are unaffected. Naïve $CD4^+$ T cells were isolated from lymph nodes of either InsR knockdown (InsR) or eGFP transgenic (wt) rats and activated via TCR/CD28 co-stimulation for the indicated time periods. (A) The number of CD25 single positive T cells was significantly reduced at all time points. (B) The number of T cells expressing both CD25 and CD134 was not affected by the InsR knockdown compared to wt. N=12 per genotype. All data are depicted as mean ±SEM; statistical analysis was performed by unpaired t-test (**:p<0.05, ***:p<0.01, n.s.= not significant).

Stimulated T cells undergo dramatic cytoskeleton changes that are essential for their function (Billadeau et al., 2007) but energy consuming. Also the stimulation of naïve T cells *in vitro* leads to morphological alterations. To visualize these changes, confocal microscopy of naïve and stimulated eGFP expressing $CD4^+$ T cells was performed. The freshly isolated $CD4^+$ T cells are small and round-shapes, whereas as early as 2 day after co-stimulation the T cells started to expand and increased in size (Fig. 20). As this co-stimulation via TCR/CD28 is antigen-independent and happens without the help of APCs, the $CD4^+$ T cells did not adopt a polarized structure with lamellipodiae and uropod, but rather became T cell blasts. The cell size increase is in line with the up-regulation of the InsR protein, which was usually observed from day one of co-stimulation on. Analysis of T cell size by FACS did not reveal differences between wt and InsR kd cells, pointing towards a minor role of the InsR in the size increase, at least under the employed stimulating conditions. Of note, detection of the InsR by FACS using insulin labelled with a fluorescent dye failed, so up to now it is not possible to determine at which activation state T cells express the InsR.

Collectively, the InsR is up-regulated during T cell activation and its absence delays the generation of $CD25^+$ T cells. However, $CD25^+/CD134^+$ double positive cells are generated equally efficient.

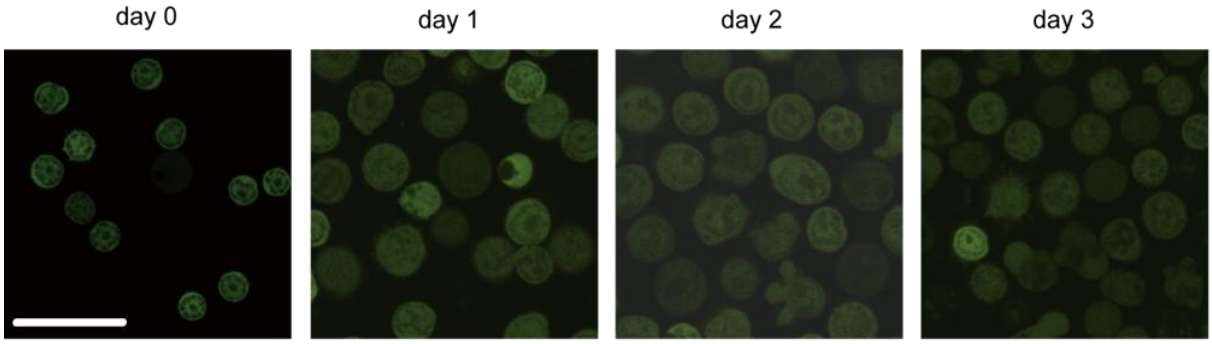

Fig. 20 Co-stimulated $CD4^+$ T cells undergo morphological changes. Confocal microscopy of eGFP expressing $CD4^+$ T cells before (day 0) and after co-stimulation (day 1-3) with anti-TCR/CD28 antibodies. The cell size increased dramatically. Size bar equals 10 µm.

3.2.2. InsR deficiency has no impact on T cell survival

Besides activation also survival might be affected by InsR deficiency. Therefore apoptosis of naïve peripheral T cells was assessed under different culture conditions. To determine the spontaneous apoptosis rate of the T cells in culture, they were cultured under normal medium conditions using RPMI++ (supplemented with 10% FCS, Fig. 21A), stained with the apoptosis markers AnnexinV and 7-AAD and analysed by FACS. The percentage of live T cells decreased slightly during the five day period of cultivation. However, no difference was seen between wt and InsR kd cells. To analyse stress induced apoptosis *in vitro* the T cells were cultured under serum starved (1% FCS) or serum-free conditions. Serum deprivation led to apoptosis of T cells of either genotype. 1% FCS only slightly induced apoptosis whereas complete lack of serum led to cell death within the first two days of culture, but again independent of the T cell's genotype (Fig. 21B,C).

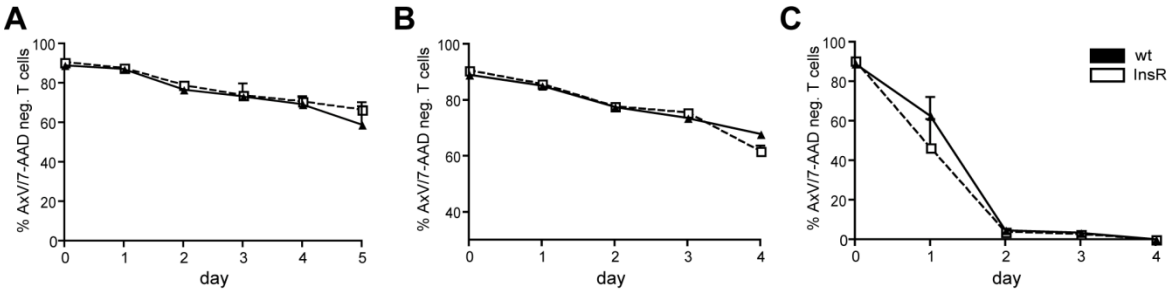

Fig. 21 InsR knockdown has no impact on T cell survival *in vitro*. Primary T cells were isolated from eGFP transgenic UGC (wt) or InsR knockdown (InsR) rats. Apoptosis was assessed by staining of the T cells with Annexin V (AxV) and 7-AAD following FACS analysis. The number of late apoptotic cells (double positive) is depicted. (A) Spontaneous apoptosis in normal RPMI++ medium (10% FCS). (B) Rate of apoptotic cells after incubation under serum starved conditions (1% FCS). (C) Percentage of apoptotic T cells after cultivation under serum-free conditions.

Furthermore, apoptosis was induced using irradiation. To this end, isolated wt or InsR kd $CD4^+$ T cells were irradiated at different doses and cultured in standard RPMI++ medium for five days.

The T cells underwent apoptosis in a dose dependent manner. Interestingly, also with this apoptosis stimulus no genotypic differences were observed (Fig. 22A). This held also true for GC induced apoptosis. The T cells were cultured with increasing doses of Dex and apoptosis was assessed on three consecutive days. At all time points, no difference between wt and InsR kd T cells was observed, day two is depicted as a representative example (Fig. 22B). This indicates that the InsR seems to be dispensable for the survival of naïve T cells.

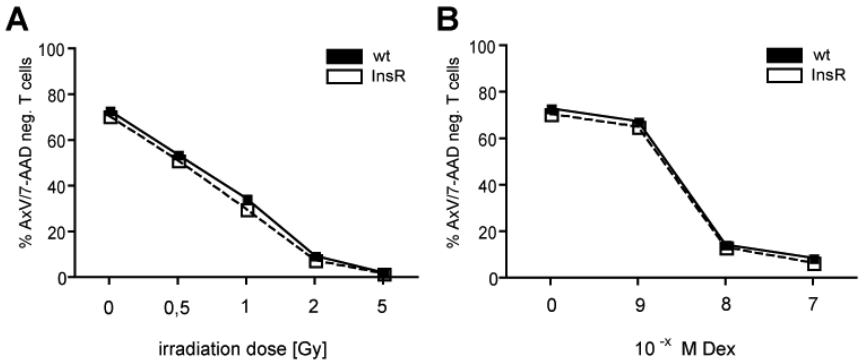

Fig. 22 Survival of T cells 2 days after apoptosis induction by irradiation and GCs. (A) $CD4^+$ T cells were exposed to different doses of irradiation. The percentage of AxV and 7-AAD negative T cells is depicted. (B) $CD4^+$ T cells were incubated with increasing doses of Dex. N= 2 animals per genotype.

3.2.3. The InsR is no longer expressed during long term activation

The hitherto obtained results suggested that the InsR was required during early T cell activation but not for their survival. As the energy demand increases during activation and also during ongoing immune responses, it is likely that the InsR impacts on T cell function. To determine the involvement of the InsR in these processes, ongoing inflammatory responses should be analysed. *In vivo* models are challenging, because a complete knockdown in all tissues as it is present in our model leads to the manifestation of a type 2 diabetes like phenotype, as all tissues are not longer sensitive to insulin. Therefore we decided to initially focus on InsR deficient effector T cells by generating encephalitogenic T cells.

To this end, transgenic InsR kd rats were immunized with gpMBP emulsified in CFA to trigger an antigen-specific immune response. 10 days later the activated $CD4^+$ T cells were isolated from the draining lymph nodes and propagated in culture. To ensure their antigen-specificity, they were re-stimulated twice with gpMPB (Fig. 23). These cells were tested for their functional competence by inducing AT-EAE and were found to be as effective as wt effector T cells. The severity of the induced disease correlated with the number of injected effector T cells (Fig. 24A). FACS analysis of the encephalitogenic T cells after the second re-stimulation confirmed that the newly generated cells were T cells that all expressed the activation marker CD25, and more than 70% of the cells were also positive for CD134. Furthermore, nearly all cells lost expression of the adhesion molecule CD62L, which is expressed on resting cells (Fig. 24B).

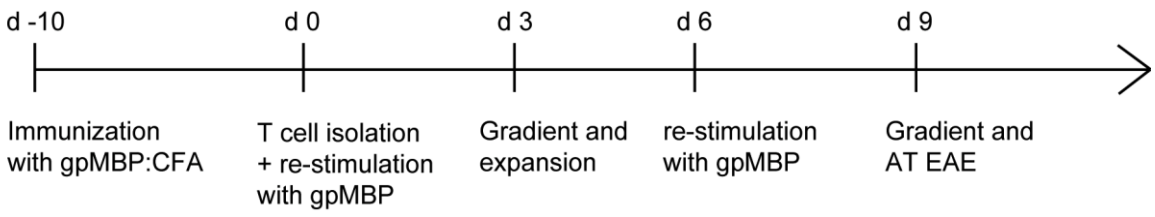

Fig. 23 Scheme for InsR kd effector T cell generation. InsR kd rats were immunized with gpMBP emulsified in CFA. 10 days later, before the first EAE symptoms occur, the activated gpMBP-specific T cells were isolated from the draining lymph nodes and re-stimulated with APCs pulsed with gpMBP. 3 days later the encephalitogenic T cells were purified via a cold gradient and expanded for another 3 days. After a second re-stimulation phase and purification, the encephalitogenic T cells were used for the first experiments.

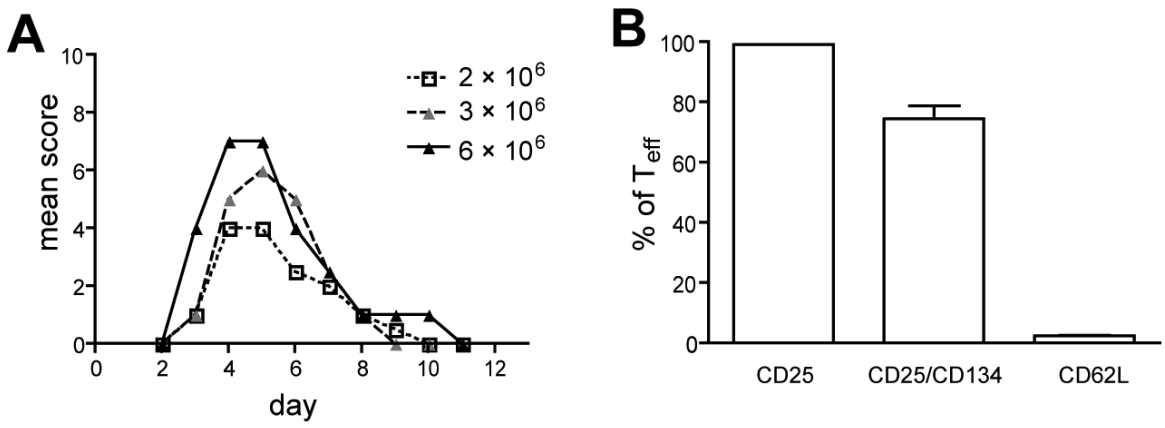

Fig. 24 Analysis of the newly generated InsR kd effector T cells. (A) Adoptive transfer EAE to test the newly generated InsR kd effector T cells. Newly generated InsR kd effector T cells were transferred intravenously to naïve Lewis rats at different amounts and the disease symptoms were assessed using a 10 grade scoring scale. (B) FACS analysis of the newly generated effector T cells after the second re-stimulation confirming the expression of classical surface markers of activated T cells (CD25, CD134). The adhesion molecule CD62L is down-regulated upon stimulation and also not expressed on the tested effector T cells.

Prior to the kockdown induction by addition of Dox, the generated InsR kd T cells were of similar to wt cells. To analyse the impact of InsR kd during EAE, the gene inactivation had to be induced prior to AT-EAE in the cells and also had to be maintained in the host's system during the EAE (Fig. 25). For the first *in vivo* experiments, we decided to use a medium number of transferred effector T cells to induce a moderate AT-EAE (3×10^6 T cells). Thereby it would be possible to observe effects of the knockdown in both directions. The knockdown was induced by *in vitro* treatment of the cells with 2 µg/mL Dox in the medium during the re-stimulation of the cells (Fig. 25).

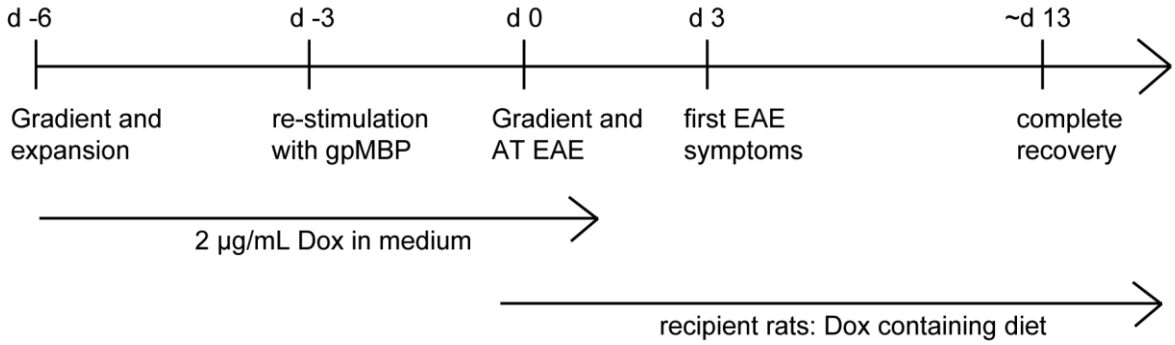

Fig. 25 Scheme of InsR knockdown induction in effector T cells and AT-EAE. After purification of the encephalitogenic T cells they were expanded in TCGF medium containing 2 µg/mL Dox for three days. Afterwards, the encephalitogenic T cells were re-stimulated for three days with gpMBP-pulsed APCs also in the presence of 2 µg/mL Dox and again purified with a cold gradient. The freshly re-stimulated cells were used for AT-EAE induction by i.v. injection into wt rats receiving Dox containing diet *ad libitum* throughout the experiment.

After purification of the encephalitogenic T cells they were injected intravenously into wt recipient rats receiving Dox containing food pellets to maintain the knockdown throughout the AT-EAE (Fig. 25). Non-Dox treated InsR kd effector T cells served as a control. Both groups did not differ in severity or duration of the EAE symptoms. Of note, treatment of AT-EAE induced with wt cells with Dox had no influence on the disease, indicating that Dox alone does not impact on EAE i.e. via modulation of bystander cells (data not shown).

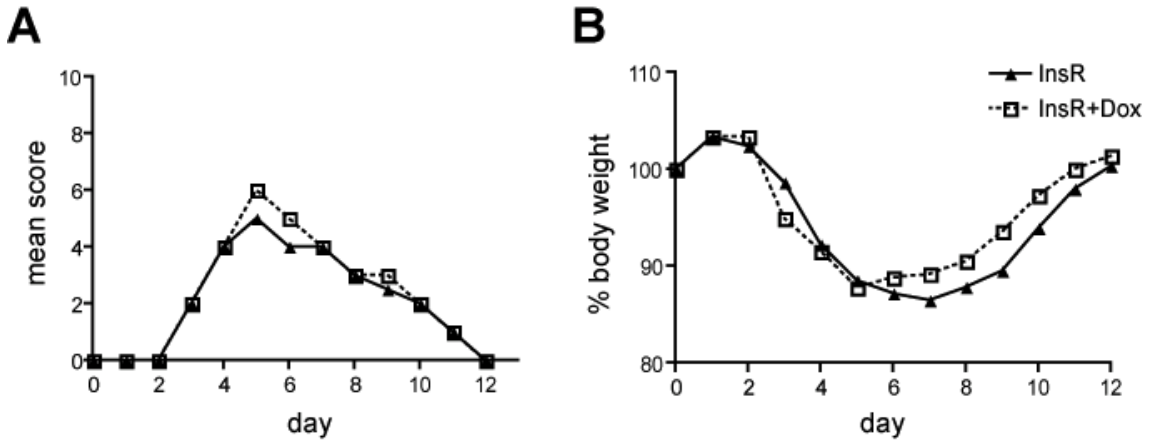

Fig. 26 Adoptive transfer EAE with InsR kd effector T cells. 3×10^6 newly generated effector T cells were injected i.v. into wt recipient rats to induce a moderate AT-EAE. The encephalitogenic T cells were either pre-treated with Dox and injected into wt rats receiving Dox-containing diet or left untreated. The animals were scored and weighed daily. (A) Score and (B) body weight relative to the start value. N=1-2 per group.

Unexpectedly, Dox treatment did not affect the disease severity in AT-EAE (Fig. 26A). Between both groups also no differences were observed in the body weight changes (Fig. 26B).

Since the InsR had been found to be up-regulated during T cell stimulation we expected that the InsR would also be expressed in repeatedly activated effector T cells and that this would impact on their pathogenicity. To verify InsR expression in those cells, we lysed eGFP transgenic wt effector T cells either directly after restimulation (d3) or expansion (d6, see Fig. 23) and analysed InsR protein levels by western blot. Liver cell lysate and co-stimulated $CD4^+$ T cells served as positive controls. Intriguingly, in none of the tested wt effector T cell lines we were able to detect any InsR protein (Fig. 27A). Also additional stimulation of these long-term activated T cells with anti-CD28/anti-TCR co-stimulation as described above for naïve $CD4^+$ T cells was unable to induce InsR expression. We also tried to other ways to stimulate the encephalitogenic T cells in order to induce InsR expression, but

also after activation with the mitogen ConA or with the superagonistic CD28-antibody JJ316 (Lin and Hünig, 2003), reagents known to stimulate T cells antigen-independently, we were not able to detect any InsR protein (Fig. 27B). Of note, stimulation with ConA or JJ316 also failed to induce InsR expression in naïve $CD4^+$ T cells. To further investigate this unexpected finding, the expression of InsR mRNA was assessed by qrtPCR. RNA of naïve and co-stimulated $CD4^+$ T cells of both genotypes as well as wt effector T cells was analysed for the relative abundance of InsR mRNA. The amount of InsR transcripts was calculated relative to β-actin transcripts serving as a *housekeeping gene*. Interestingly, in naïve $CD4^+$ T cells from both wildtype and Dox treated InsR kd rats, the InsR mRNA was constitutively expressed, although the InsR protein levels in the latter ones were dramatically reduced after knockdown induction (Fig. 27C). Co-stimulation of these cells did not lead to an mRNA up-regulation but rather a down-regulation, all together suggesting a translational rather than transcriptional regulation of InsR protein expression.

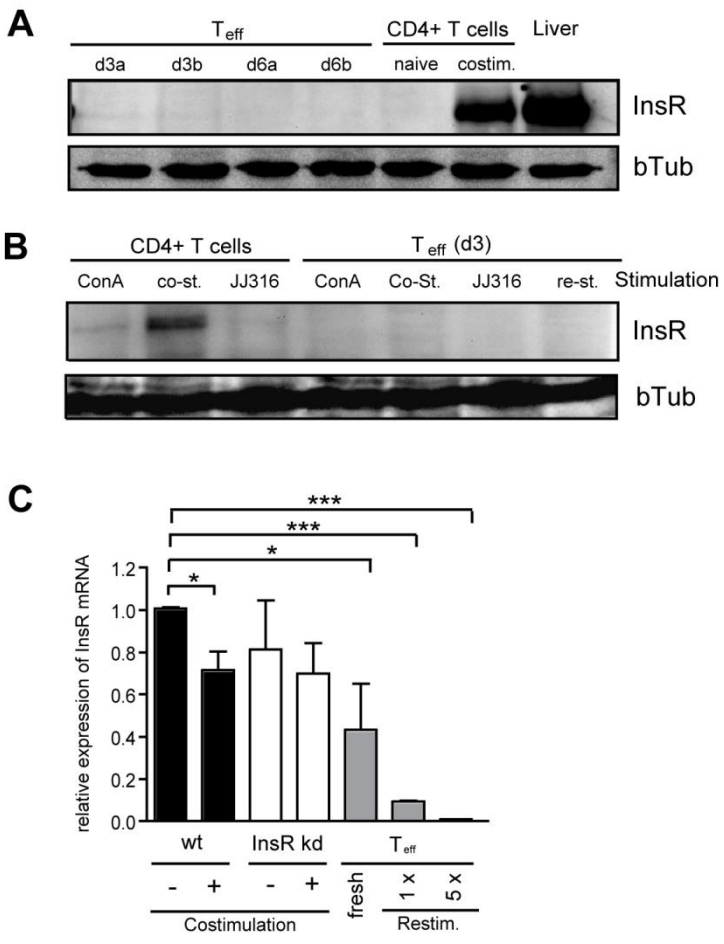

Fig. 27 Effector T cells lack InsR expression on both protein and mRNA level. (A) Naïve $CD4^+$ T cell lysates and effector T cells lysed after restimulation (d3a,b) or following proliferation (d6a,b) were analysed for InsR protein expression. Co-stimulated $CD4^+$ T cells and liver cells served as positive controls. This experiment was performed twice. (B) Western blot analysis of naïve and effector T cells after 3 days of different stimulation. one representative blot out of two independent experiments is depicted. (C) RNA was isolated form naïve or co-stimulated T cells of both, wildtype and InsR kd rats, as well as from effector T cells at three different stages (freshly isolated, after the first and the fifth restimulation) and InsR expression was assessed by qrtPCR. Data is depicted as mean ±SEM; statistical analysis was performed by unpaired t-test (**:$p<0.05$, ***:$p<0.01$, n.s.= not significant).

Furthermore, mRNA expression in freshly isolated effector T cells (10 days after immunization) was reduced by half (“fresh”, Fig. 27C) and further decreased during the subsequent re-stimulation cycles (1× and 5× re-stimulated).

Taken together, the InsR protein expression seems to be regulated on the translational level. During long term activation or maintenance of the activated status of effector T cells the InsR seems to be dispensable, as its transcription is stopped.

3.2.4. T_{reg} cell function in InsR kd animals is unaltered

Metabolism integrated with T cell function (Gerriets and Rathmell, 2012). This could lead to an impaired function of T_{reg} cells, which prevent autoimmune reaction under normal conditions. To test if the T_{reg} cells lacking the InsR are less efficient than their wt counterparts, a ^{3}H-thymidine suppression assay was performed. In this assay target cells are activated in an antigen-independent manner using ConA and mixed with different amounts of T_{reg} cells that suppress the proliferation of the target cells. As only about 5% of all T cells are T_{reg} cells we decided to expand them *in vivo* to obtain sufficient cell numbers. To this end we took advantage of the superagonistic anti-CD28 antibody JJ316. When applied 3 days prior to T cell isolation JJ316 is known to strongly expand T_{reg} cells (Lin and Hünig, 2003). These cells were purified by MACS technology and used in the *in vitro* suppression assay. $CD4^{+}$ T cells were mixed with irradiated APCs and ConA to induce proliferation and served as target cells. T_{reg} cells were added at different effector to target ratios and allowed to suppress the proliferation for 48 h.

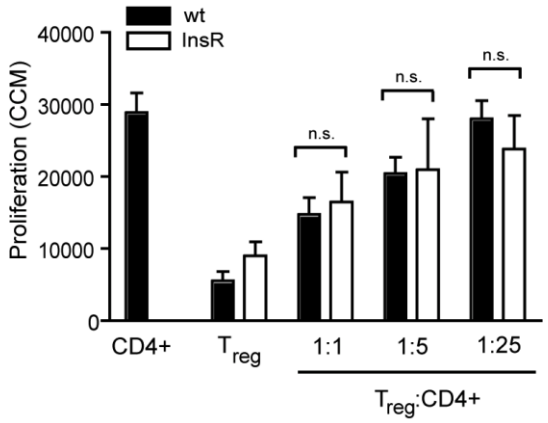

Fig. 28 InsR kd T_{reg} cells are as suppressive as their wt counterparts. A ^{3}H-thymidine suppression assay was performed. T_{reg} cells were mixed with activated target cells at different ratios. After 2 days, the cells were pulsed with ^{3}H-thymidine containing medium and harvested and analysed 16 h later. Proliferation is indicated by ^{3}H-thymidine incorporation. N=8 per genotype, 4 independent experiments.

Afterwards the cells were pulsed with ^{3}H-thymidine containing medium that was incorporated into the DNA during proliferation. Since the APCs had been irradiated

the only proliferating population are the $CD4^+$ target T cells. The T_{reg} cells derived from both genotypes were equally effective in the suppression of ConA-stimulated target $CD4^+$ T cell proliferation (Fig. 28). This led to the conclusion, that InsR deficiency does not cause a reduced regulatory activity of T_{reg} cells.

3.2.5. CTL function is impaired in InsR kd rats

Besides $CD4^+$ T cells it is also conceivable that $CD8^+$ T cells require the InsR for proper function. Cytotoxic T cells (CTLs) are needed for the defence against virus-infected cells. They fulfil their function by releasing cytotoxic molecules, a potentially energy-demanding process. To test whether CTLs are more sensitive to InsR deficiency than $CD4^+$ T cells, a ^{51}Cr release assay was performed. To this end, wt and InsR kd rats were immunized with allogenic splenocytes. This led to the activation of $CD8^+$ T cells and their proliferation in the draining lymph nodes, from which they were isolated 10 days later. Subsequently they were exposed to ^{51}Cr pulsed allogenic blasts serving as target cells. The latter ones were produced by stimulation of splenocytes from rats of the same genetic background that had been used for immunization. The stimulation causes a higher uptake of ^{51}Cr during the labelling process. After mixing them, the CTLs were allowed to lyse the target cells for 4 h. Afterwards the supernatant was analysed in the beta counter. CTL killing efficiency correlated with the release of radioactivity to the supernatant. To exclude imprecision due to different amounts of target cells per well, the cell pellets were lysed and the radioactivity determined to calculate the total radioactivity per well. The percent release of total radioactivity was calculated and is depicted (Fig. 29). Interestingly, InsR deficient CTLs were less effective in the killing of target cells than wt cells (Fig. 29). Whereas the wt cells were able to lyse about 40% of the target cells at a 100:1 ratio, InsR deficient cells released less then 30% of the total radioactivity. Also at all other tested ratios the InsR deficient CTLs were less effective as their wt counterparts ($p<0.01$), suggesting an important function of the InsR in endowing CTLs with the capacity to exert their cytolytic functions.

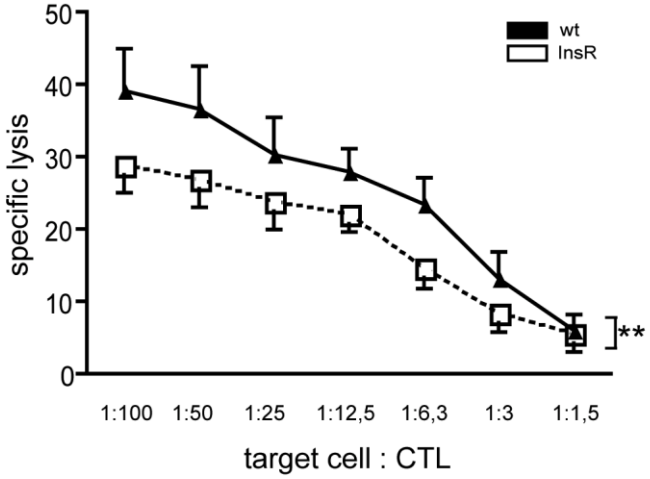

Fig. 29 InsR deficient CTLs show reduced lytic capacity as compared to wt cells. A ^{51}Cr release assay was performed using CTLs derived form either InsR kd or wt rats and exposed to ^{51}Cr pulsed allogenic blasts at different effector:target ratios. The specific lysis as a mean of three independent experiments is depicted. N=9 per genotype

3.2.6. *In vivo* approach: generation of bone marrow chimeric rats

Because all experiments conducted so far were only performed *in vitro* we switched to *in vivo* approaches. Effects that might not be evident under artificial culture conditions could come into play in immune reactions involving the interaction of different cellular compartments and have implications during disease progression. Because a complete knockdown of the InsR leads to insulin resistance in all tissues, a diabetes type 2 like phenotype develops in the rats. This is lethal within 6-7 days and therefore not suitable for *in vivo* approaches as most immune reactions take longer then this. To overcome this problem bone marrow chimeric rats were generated. With this method it is possible to limit the InsR kd exclusively to cells of the hematopoietic system and to overcome the problem of general insulin resistance. To this end, bone marrow was isolated from eGFP transgenic UGC (wt) or InsR kd rats and injected i.v. into sublethally irradiated eGFP negative recipients of the same genetic background. The recipient's hematopoietic system is destroyed by the irradiation and so the niches in the bone marrow are free to be repopulated by donor cells. Reconstitution was tested by FACS analysis 4 weeks after bone marrow transfer. We found high reconstitution rates in all recipients. Nearly all (>95%) granulocytes and B cells were eGFP positive donor cells (Fig. 30). However, in the T cell compartment we observed about 30% eGFP negative recipient T cells. This is a normal abundance in bone marrow chimeric mice and rats, so we decided to use the

chimeric rats for *in vivo* analyses. The rats were analysed every 4 weeks with comparable results at each time point.

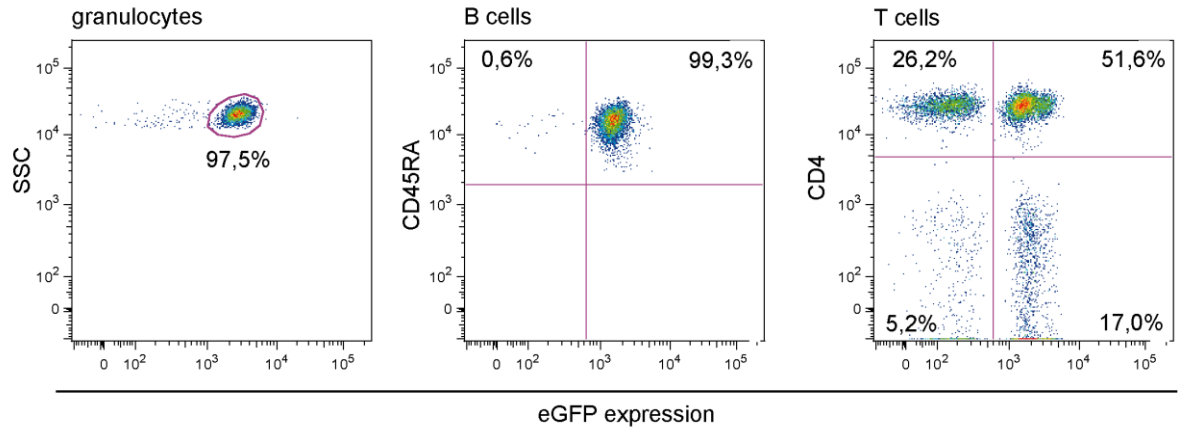

Fig. 30 Analysis of reconstitution after bone marrow transfer. 4 weeks after bone marrow transfer blood drained from the tail vein of bone marrow chimeric rats was analysed and stained for B cells (anti-CD45RA) and T cells (anti-TCR). Granulocytes were gated for granularity (SSC) and size (FSC). Subsequently, the cells were analysed for eGFP expression. Data from one representative rat out of 12 is depicted.

3.2.7. Induction of EAE in InsR kd bone marrow chimeric rats

After successful generation of bone marrow chimeric rats first EAE experiments were conducted. As we had seen that the InsR seemed to be important during stimulation, especially in the early phase of activation, but not during long-term activation, it is likely that the InsR kd cells would have an impairment in the priming phase of an immune response rather than during the effector phase. To challenge this hypothesis we decided to use the model of active EAE. The effector T cells have to be primed to get polarized to Th1 or Th17 cells and to migrate to the target organ, the CNS.

The hematopoietic system-specific InsR knockdown was induced in the bone marrow chimeric rats by Dox containing diet one week prior to immunization with gpMBP. During EAE the animals were weighed and scored daily. After 11 days the first EAE symptoms occurred. Chimeric rats reconstituted with wildtype or InsR deficient bone marrow both followed a monophasic disease course (Fig. 31A). The severity of symptoms as well as the duration of the disease did not differ between the donor genotypes.

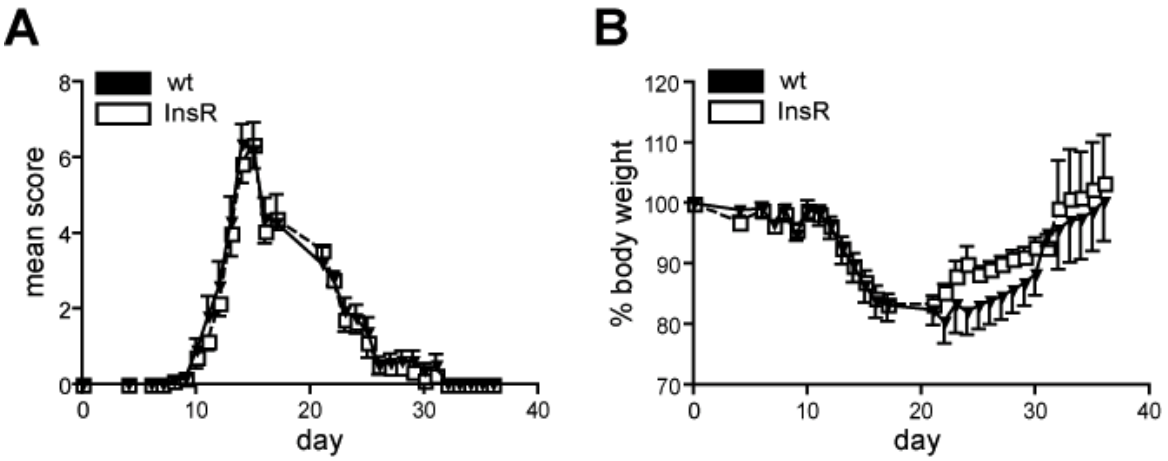

Fig. 31 The disease course of EAE in wt and InsR BM chimeric rats is similar. Active EAE was induced in BM chimeric rats reconstituted with either wt or InsR kd BM by immunization with gpMBP in CFA. (A) Both groups followed the same dynamics regarding the disease severity as assessed by a 10 grade scoring scale. (B) Relative bodyweight of the rats during EAE is depicted. The rats reconstituted with InsR BM showed a slightly higher weight gain during recovery N=12 per group, two independent experiments.

During the recovery phase, a slightly higher bodyweight was observed in InsR kd chimeric rats, pointing towards a slightly milder disease severity in those animals (Fig. 31B). Nonetheless, the mean score did not differ at all between both groups. We conclude that InsR knockdown in hematopoietic cells, at least at the degree of reconstitution achieved in this experiment, had no influence on disease severity or duration. This suggests that the InsR is dispensable during an inflammatory response in this model and that increase of glucose uptake and glycolysis in effector T cells might be sufficiently induced by CD28 engagement.

4. Discussion

4.1. Endocrine control of T cell function and polarization

Mammalian organisms are complex. Different organs fulfil different tasks for the benefit of the whole body. Protection against pathogens like bacteria, fungi and viruses as well as against tumours is conveyed by the immune system consisting of various cell types. Herein T cells are central players. To distribute information soluble messengers are necessary with hormones being one prominent example. They orchestrate numerous functions in the body and also impact on immune cells. This endocrine regulation is crucial for the whole organism, as it mediates activity as well as stress-responses and metabolism. T cell function and plasticity are two examples of hormonal key regulation as well. For example, the influence of the endocrine day-night-cycle on lymphocyte migration and motility has been reported (Kirsch et al., 2012).

In this project we aimed to analyse the regulation of T cell function by endogenous insulin signalling as well as in response to exogenous treatment with GCs.

4.2. Glucocorticoids interfere with effector T cell pathogenicity by modulation of the cytoskeleton

GCs are steroid hormones with anti-inflammatory action and immunosuppressive properties. Endogenous GCs have numerous mechanisms to regulate physiological functions, including circadian changes of GC levels. Treatment with GCs is more efficient when it is conducted during the night. For instance downregulation of MMP9 is more pronounced if GCs are applied at night-time (Glass-Marmor et al., 2009; Mirowska et al., 2004). Interestingly, Leukocyte trafficking is also influenced by circadian changes (Dimitrov et al., 2009; Kirsch et al., 2012), providing a first hint of migratory modulation by GCs.

Synthetic GCs have been in use for more than 60 years for the treatment of diseases like asthma or MS. As broad as this range of application is the range of side-effects. Treatment with GCs can disturb glucose metabolism (Pidala et al., 2011), a fact that might impact on the whole organisms as well as on immune cells. Furthermore GCs can cause osteoporosis (Canalis et al., 2007) or generally an increased infection-risk due to the immune suppression.

In view of these complications it is desirable to further dissect the mechanisms of GCs. It has been believed for decades that apoptosis is a central mechanism, but recently increasing evidence arose that this might not be the case (reviewed in (Reichardt and Lühder, 2012).

Besides the genomic effects of the GR, namely transrepression and transactivation of target genes, also non-genomic effects of the GR came into focus. These effects are independent of transcriptional modulations and could therefore potentially be very rapid and immediate. Understanding these effects and classifying their importance for treatment will help to refine GC treatment, to modify drugs and to reduce side-effects.

4.2.1. Impact on T cell morphology and behaviour in the context of neuroinflammation

To study the GC-induced changes in cells that are essential for the pathogenesis of EAE and presumably MS we have analysed effector T cells *in vitro*. We have shown profound and early changes in T cell morphology and behaviour, impacting on central steps in T cell responses that are also important for T cell mediated autoimmunity.

In a first set of experiments we could show rapid morphological rearrangements of polarized effector T cells after high-dose GC treatment. These cytoskeletal modulations were detectable as early as 20 min after treatment and lasted for more than 3 hours. These morphological changes were also accompanied by increased cell rigidity (Müller et al., 2013). It is reasonable that this profoundly interferes with T cell function. It has been published, that T cells need a specific polarized structure in order to be functional (Billadeau et al., 2007; Krummel et al., 2000; Krummel and Macara, 2006). T cells have to be highly motile and lamellipodiae at the leading edge scan the surrounding. Targeted expression of chemokine receptors, integrins or stimulatory molecules allows directed migration. If this structure is lost and the cell additionally retracts and adopts the shape of a naïve cell it is obvious that this impacts on cellular functions. To dissect in which way the cell is influenced by GCs and impaired by the rearranged cytoskeleton we tested the capability of cells after GC treatment to conduct the central steps of autoimmune responses.

First, we had a closer look on the transmigratory behaviour of effector T cells before and after treatment. As expected, GC-treated effector T cells exhibited a strongly reduced migratory capacity. After 20 min of treatment migration was already

significantly reduced and after 3 hours even diminished by half. Transmigration out of the blood vessel into the Virchow space and subsequently into the CNS is important in MS and EAE development (Engelhardt, 2008). Interfering at this early stage, even before the T cells are reactivated in the CNS by their cognate antigen is one possible mode of action of GCs. A second effect of altered T cell migration might be re-direction. Adequate chemotaxis to the site of inflammation is an integral part of T cell responses. GCs impact on cytokine receptor expression and thereby might increase or decrease the response to certain cytokines (Schweingruber et al., submitted).

Another step in T cell activation is the interaction with APCs. These cells present antigen and provide the indispensable second signal for T cell activation. If T cells fail to receive this signal they are not sufficiently stimulated and become anergic. In order to interact with APCs, T cells have to rearrange surface molecules and to direct TCR, co-receptors, co-stimulatory molecules and chemokine receptors towards the partner cell. For all these processes T cell polarization is a prerequisite (Billadeau et al., 2007). A more rigid, less flexible cytoskeleton with a tightly linked F-actin filament might interfere with the proper assembly of APC-T cell conjugates. To test this notion we have established a method to quantify such conjugates using FACS technology. In this case the results were even more dramatic, since only 10 minutes of GC treatment reduced the number of conjugates by half. This effect was maintained for all tested time points up to 3 hours.

To elucidate the underlying mechanisms we analysed proteins, which are potential targets of GCs. Proteins of the ezrin-radixin-moesin family (ERM-proteins) are candidate molecules (Allenspach et al., 2001; Delon et al., 2001; Roumier et al., 2001). In T cells two members of this family, namely ezrin and moesin, are expressed. In resting cells, the proteins are highly phospholylated and provide crosslinks between F-actin-filaments and plasma membrane (Bretscher et al., 2002; Gautreau et al., 2002). The result is a stable morphology with optimal volume to surface ratio. Upon TCR engagement, the proteins are rapidly dephosphorylated, allowing for dynamic rearrangements of the cytoskeleton in order to adopt the typical morphology of effector cells (Delon et al., 2001). For T cells this is a structure with a leading edge expressing antigen-receptors and cytokine-receptors that enables directed migration. All dispensable surface molecules are collected at the opposing pole at the distal pole complex (DPC). Re-phosphorylation of ERM-proteins would reverse this modulations and therefore provide an explanation of the observed effect

of GCs. Western blot analysis of P-ERM revealed that effector T cells have only a low level of phosphorylation that is rapidly and strongly increased after GC treatment. As expected the effects were immediate and occurred as early as five minutes after treatment, further increasing within the first 30 minutes and then slightly declining. This mechanism is, of note, in line with the results of cell rigidity analysis (Müller et al., 2013) and provides an explanation for morphological alterations and for impaired transmigratory capacity. The proteins ezrin and moesin are essential for DPC formation and IL-2 production as well (Shaffer et al., 2009). In addition, TCR-induced de-phosphorylation of ERM-proteins supports the interaction with APCs (Faure et al., 2004) and therefore is in line with our findings mentioned above. A second potential mechanism for the cytokleletal modulation induced by GCs is phosphorylation of FAK (Koukouritaki et al., 1999), which could also contribute to the rearrangement of the cytoskeleton. For mouse T cells it has been shown that FAK is indeed phosphorylated after GC treatment (Schweingruber et al., submitted).

The signalling cascade that induces the morphological changes by phosphorylation of ERM proteins could be induced by the GR or represent an unspecific off-target effect of GCs. To distinguish between both possibilities we made use of siGR effector T cells (Tischner et al., 2009). These cells have no GR protein and could therefore only show unspecific effects. Intriguingly, when these siGR effector T cells were treated with GCs we did not observe any changes in P-ERM-levels. This suggests, that the observed effects are indeed mediated by the GR. Furthermore, those cells also failed to show morphological alterations. After GC treatment they maintained their polarized structure, leading to the conclusion that the GR is essential for GC mediated depolarization (Müller et al., 2013).

But how does the GR induce the morphological changes? Intracellular signalling is very complex. Key molecules can take part in more than one pathway and second messengers are a common feature to rapidly transmit information within the cell and to amplify signals. One important second messenger in lymphocytes as well as all other cells are Ca^{2+}-ions. They can be released from intracellular sources or influx from the extracellular space through Ca^{2+}-channels. We analysed whether the GC-induced signalling cascade leading to ERM-phosphorylation was accompanied by Ca^{2+}-signalling. Therefore we determined the level of intracellular Ca^{2+}-ions with a Ca-sensitive dye and monitored the levels over time after GC treatment. In none of the analyses we were able to observe a significant shift in Ca^{2+}-levels and we

conclude from this that the GR-mediated signalling is independent of Ca^{2+}-ions as second messenger.

The next question to be answered was which enzyme was responsible for ERM-phosphorylation. It was reported that two kinases could potentially mediate this phosphorylation, namely ROCK and PLC (Belkina et al., 2009; Ng et al., 2001; Ren et al., 2009). It has been published that PLC was activated after GC treatment in thymocytes and therefore it was a promising candidate in our model (Cifone et al., 1999). By pre-incubating effector T cells with the PLC-inhibitor we were able to obviate the effector T cell rearrangements. The inhibitor interfered with the GC-induced cell size reduction as well as with depolarization. At the molecular level it also prevented the GC-induced ERM-phosphorylation. Furthermore, the transmigratory capacity as well as the ability to form APC conjugates was preserved if the cells were treated with the inhibitor. Collectively, the GC-mediated cytoskeletal rearrangements are induced by the GR activating PLC to phosphorylate ERM-proteins.

The GR could potentially mediate its effects either by modulation of transcription or by non-genomic effects. In both cases the GR is released from cytosolic heat shock proteins after binding of GCs (Herold et al., 2006). Transcriptional modifications can be divided in transactivation and transrepression. The first one is mediated by direct interaction of a GR-homodimer with a DNA region, the GC responsive elements (GRE) and acting as an activator, whereas the latter one is mediated by interaction with transcription factors without DNA-binding leading to suppression of transcription (see Fig. 3). On the other hand the GR can act in the cytosol where it mediates non-genomic effects (Herold et al., 2006). These GR actions are only recently being explored (Buttgereit and Scheffold, 2002). To characterize the GR action that is important for the newly identified impact on the effector T cell cytoskeleton one can inhibit translation in order to distinguish between gene-modulating effects and those independent of transcription. Treatment of effector T cells with the translational inhibitor cycloheximide (CHX) did not affect the GC induced cytoskeletal modifications (Müller et al., 2013) suggesting a non-genomic action of the GR (Koukouritaki et al., 1999).

To test whether this newly discovered mechanism is also of relevance *in vivo*, a first step was to analyze whether effector T cells also undergo cytoskeletal rearrangements if those are applied systemically. During EAE, GC treatment

ameliorated the symptoms, but it is difficult to follow the fate of single effector T cells over time. To overcome this problem we induced AT-EAE with a pure effector T cell population expressing eGFP to allow identification of the transferred T cells after treatment. Intriguingly, transferred effector T cells responded to GCs in the same manner that we had observed *in vitro*. The cell size of the re-isolated cells drastically decreased after treatment. This was accompanied by disappearance of the cells from the blood, suggesting that not only polarization, transmigration and APC-conjugation are affected but also circulation of the cells. The latter feature would be in line with the findings in mice, that redirection is a central part of the beneficial effects of GCs (Schweingruber et al., submitted).

Finally, there is also initial evidence that altered migration of T cells might be of relevance for the treatment of human MS patients. Recently more and more the expression pattern of chemokine receptors came to focus in MS research (Dos Santos et al., 2008; Holman et al., 2011). GCs induce expression of CXCR4 and increase migration towards SDF-1. This would be a second line how GCs modulate effector T cell migration. There are also first experiments showing that also T cells obtained from human MS patients show the same cytokine response compared to mice or *in vitro* treated cells (Schweingruber et al., submitted). This suggests that this is a stable, species independent mechanism.

4.2.2. Conclusion

Our findings suggest that GCs act in a two-step manner in repressing T cell responses. Here we have identified a first immediate and rapid action of GCs by which they directly interfere with effector T cell function. Within minutes after treatment, the GR activates the kinase PLC in a non-genomic manner (Fig. 32). This pathway does not trigger Ca^{2+} signalling. PLC activation leads to the phosphorylation of ezrin and moesin (Fig. 32), an event that leads to crosslinking of the F-actin cytoskeleton with the plasma membrane. As a result, the characteristic polarized structre of effector T cells is lost. The lamellipodiae retract, thereby inhibiting targeted expression of surface molecules. The cells lose the flexibility and rigidity increases. Subsequently the transmigratory capacity as well as the interaction with APCs are dramatically reduced. Taken together, GCs influence T cell function and migration on several levels. This non-genomic effect is presumably a first wave of GC action that might be important for the beneficial effects of GC treatment in neuroinflammation.

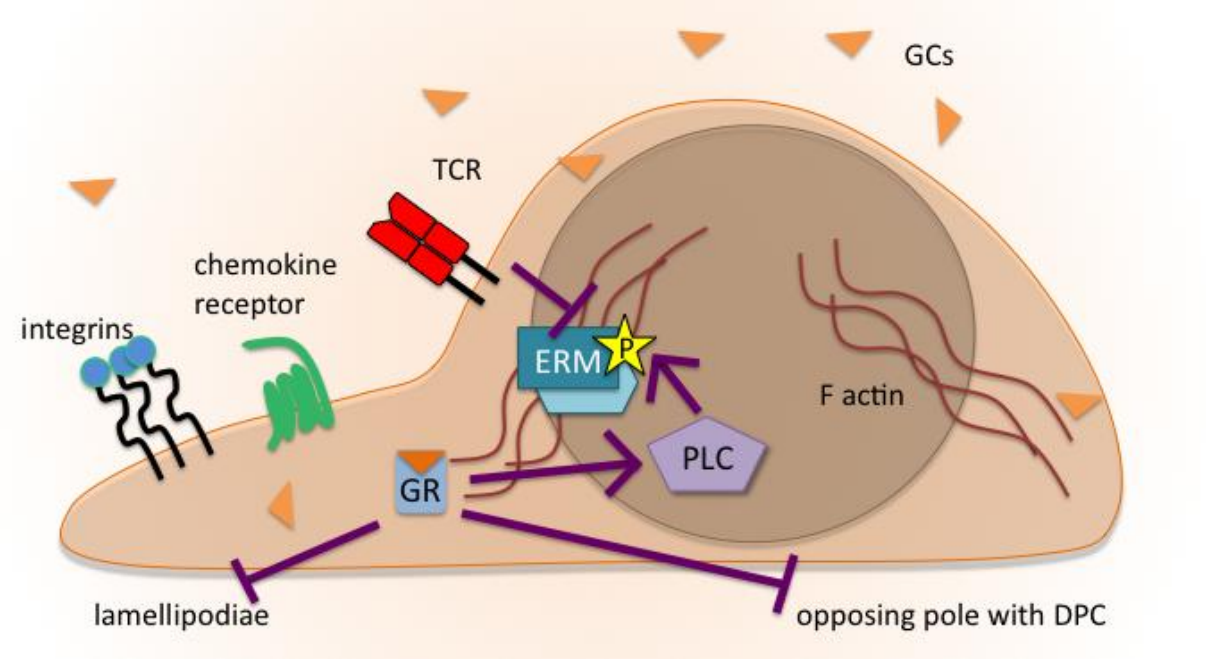

Fig. 32 GC mediated effects on effector T cells. T cell receptor (TCR) stimulation leads to the dephosphorylation of proteins of the ezrin-radixin-moesin (ERM) family, which results in the rearrangement of the cytoskeleton. Consequently, T cells adopt a polarized structure with a leading edge formed by lamellipodiae, an opposing pole containing the distal pole complex (DPC) and directed expression of surface molecules and receptors. GCs might interfere with these morphological alterations of T cells by increasing ERM phosphorylation leading to crosslinking of F actin and plasma membrane and thereby mediating the depolarization. Modified from (Fischer et al., 2013).

4.3. Metabolic regulation of T cell function

The overall metabolic status impacts on a variety of cell types as well as T cell function. As mentioned above, T cells undergo profound morphological alterations upon activation. The cytoskeleton is rearranged to provide the optimal structure for effector T cells, receptors are up-regulated and cytokines are expressed. Different types of T cells have different requirements, determined by the cytokine milieu and the education provided by the activating APC. The different T cell subsets to which the naïve cell can differentiate is determined by transcription factors and results in the expression of specific cytokines of effector molecules. All cells have in common that activation is energy-consuming and the cells have to meet their demand.

To cover increased energy expenditure, the cells eventually switch their metabolic paradigm during activation from oxidative phosphorylation to glycolysis (Brand et al., 1986; Wang and Green, 2012; Wang and Green, 2012). Thereby they switch from an oxygen-dependent to an oxygen-independent metabolism (Caldwell et al., 2001). This enables them to migrate and survive under hypoxic conditions as found in inflamed tissues. They also up-regulate the glucose transporter Glut1 and glycolytic enzymes (Marko et al., 2010; Vander Heiden et al., 2009). This provides a potential link between insulin metabolism and T cell function: InsR engangement also leads to up-regulation of glucose transporter (Glut4) and to increased glycolysis. Lack of InsR-signalling could therefore be a disadvantage in highly energy-consuming immune reactions.

There is increasing evidence that there is a tight link between T cell activation and metabolism (Fox et al., 2005). Several recent studies focussed on the metabolic control of T cell migration (Mora and von Andrian, 2006; Sinclair et al., 2008), i.e. transgenic overexpression of the Glut1 transporter led to an augmentation of T cell activation (Jacobs et al., 2008). It is therefore quite likely that the energy supply impacts on the T cell function and fate. To address this issue, one could either culture T cells under different metabolic conditions and test their functionality or use T cells that cannot respond to certain stimuli. In our studies we made use of InsR kd cells, that are deficient for the InsR. We analysed different T cells subsets for their function or survival to determine the importance of insulin signalling for proper T cell function.

4.3.1. Impact of InsR signalling on Th cells

Differentiation into Th cell subsets is at least in part determined by metabolic pathways of the T cell (Fox et al., 2005; Frauwirth and Thompson, 2004; Plas and Thompson, 2005; Wang and Green, 2012).

If no extrinsic signals induce increased nutrient uptake the cells eventually undergo apoptosis (Ciofani and Zuniga-Pflucker, 2005; Rathmell et al., 2000). It has also been shown that the induction of glucose uptake is critical for effector T cell survival (Cham and Gajewski, 2005). Insufficient glucose supplement leads to defective effector T cell function and the induction of pro-apoptotic proteins such as Noxa and Bax (Alves et al., 2006; Chi et al., 2000).

In all experiments we have consistently observed an impairment of InsR deficient $CD4^+$ T cells to up-regulate the activation marker CD25. Interestingly, expression of the later activation marker CD134 was not affected by the lack of the InsR. Further analysis by western blot confirmed InsR expression in co-stimulated wt $CD4^+$ T cells and its absence in the InsR kd cells. Up to now, we are not able to distinguish between the different phases of T cell activation using this method. So it would be possible that the InsR is predominantly expressed during early activation and not or less pronounced in the later stages of T cell activation. The *in vitro* stimulation is artificial and very strong and therefore may differ from physiological conditions in terms of InsR expression. In our setting the cells might initially up-regulate the InsR to meet their demand but are subsequently no longer dependent on its expression. It might also be that the high amount of IL-2 in the culture influences the cells in a way that insulin has no further impact on metabolism. Another explanation might be that the cells are constitutively stimulated via CD28 due to the presence of the anti-CD28 antibody in our system and that InsR deficient cells can compensate for the absence of InsR signalling or that the overall-signalling is so strong that it conceals all effects of the InsR inactivation. Stimulation without CD28 engangement results in an overall weak activation of cells, but without showing differences caused by InsR deficiency. It is known, that the switch from oxidative phosphorylation to glucose matabolism is dependent on CD28 stimulation (Frauwirth et al., 2002; Jacobs et al., 2008), so it is not surprising that there is no difference observable, as cells of neither genotype presumably utilize glucose.

We also failed to observe differences in the cell's ability to increase in size after stimulation. Both populations, wt and InsR, nicely responded to stimulation and

became effector T cell blasts. This would be also in line with the finding that there is no profound difference in the activation status of Akt as shown by western blot analysis for P-Akt, pointing towards a compensatory mechanism or too strong stimulation leading to the maximal phosphorylation of Akt.

We aimed to analyse expression patterns of the InsR in the different stages of T cell activation and Th cell subsets by FACS. Analysis with commercially available InsR antibodies is not possible because all of them recognize a transmembrane or juxta-membrane part of the InsR. To overcome this problem we tried to detect the InsR by binding of insulin to the cells. To this end we labelled insulin with a fluorochrome using a microscale protein labelling kit. Unfortunately, we were not able to detect any specific binding of the insulin, so it remains to be determined, which T cell population up-regulates the InsR upon activation.

As InsR kd $CD4^+$ T cells showed an impairment in the activation we also wanted to analyse T cell survival. It is known for effector T cells, that insufficient glucose uptake leads to the induction of apoptosis (Alves et al., 2006; Chi et al., 2000). It might also be that T cells lacking the InsR are *per se* more prone to apoptosis. Resting cells express no detectable amount of InsR, but under starved conditions induction of InsR expression might help the T cell to overcome apoptosis. We have tested different conditions of serum-starvation and also the overall survival of the T cells in culture. Under none of the tested conditions we were able to detect differences in T cell survival. Challenging of the cells with GCs or irradiation did not reveal any detriment of InsR deficient T cells as well.

InsR expression is induced upon activation to meet the energy demands of activated T cells. Effector T cells strongly proliferate and secrete cytokines. Therefore we wanted to analyse the impact of InsR signalling on long term activated effector T cells. These cells should have switched their metabolism from oxidative phosphorylation to glycolysis, mediated by CD28 (Jacobs et al., 2008). The InsR signalling leads to increased glycolysis and glucose uptake so it is likely that this mechanism is synergistic with the CD28 induced metabolic changes. Intriguingly, InsR deficient effector T cells were as effective as wt counterparts to induce EAE. A closer look at these cells revealed that they don't express the InsR on the protein level anymore. The expression could also not be induced by additional stimulation of the cells with neither TCR/CD28 co-stimulation nor with CD28 superagonist- or ConA-stimulation. Furthermore, mRNA analysis showed that even the InsR mRNA,

which is constitutively expressed in resting cells, presumably to allow quick protein translation upon activation, is also not expressed in effector T cells. We therefore conclude that the InsR is dispensable for long-term activated T cells.

The cellular metabolism has also impact on T cell function. It has been described that there is a link between defective insulin responsiveness and delayed pathogen clearance. This might have two reasons: Firstly, the Th cells might be impaired in activation and not as functional as their wildtype counterparts. Secondly, a disturbed metabolism could interfere with Th lineage commitment and cause false priming of the Th cells. To test this issue cytokine profile analysis was performed in our lab. It was shown that there is indeed a shift in the Th1/Th2 ratio. qrtPCR analysis indicated overall higher IL-2 levels in wt T cells, which is in line with the finding of the impaired expression of the autokrine IL-2 receptor CD25. Furthermore it was found that more Th1 cytokines such as IFNγ and IL-17A were expressed in InsR deficent cells upon co-stimulation compared to wt cells which showed generally higher Th2 cytokine levels (IL-4 and IL-10, data of Christopher Sie). There is evidence in the literature that this might be a common mechanism of T cell fate decision. Interference with metabolism can modulate immune functions. Of note, targeting glucose metabolism in mice with the glycolytic inhibitor 2-deoxyglucose was able to suppress EAE (Shi et al., 2011) by interfering with Th17 generation.

In vivo models are more challenging but also more sensitive. A strong stimulation *in vitro* via CD28 could disguise any influence that the InsR deficiency would have under physiological conditions. Furthermore, the stimulation is relatively unspecific. If the cells were able to be successfully primed after antigen recognition, they would be committed to the correct Th lineage and mount a proper immune response. This can only be studies using *in vivo* models. Wrong lineage commitment, e.g. failure to generate Th17 cells, would dramatically interfere with a disease model like EAE. A general knockdown of the InsR has severe consequences for the entire metabolism. The animals develop severe T2DM and have to be sacrificed one week after knockdown induction. This is to short for most of the *in vivo* models, because the priming and proliferation of T cells, migration to the target organs and first effector mechanisms take about a week. To overcome the problem of a ubiquitous knockdown we aimed to generate bone marrow chimeric rats. By sublethal irradiation the hematopoietic system of the recipients is destroyed and one day later the animals are transplanted with bone marrow of transgenic rats. The graft is able to repopulate

the recipient's immune system and afterwards all cells of hematopoietic origin are transgenic while the rest of the body is wildtype. With this approach we have generated rats having either eGFP expressing wildtype or InsR deficient hematopoietic cells. We induced EAE in these rats 5 days after knockdown induction with Dox-containing diet. Surprisingly, we did not observe any differences in the EAE course between both genotypes. We therefore had a closer look on the reconstitution efficacy. We achieved almost a pure donor genotype in the B cell and granulocyte compartment. These cell types, however, can be elided because they are not capable of InsR synthesis. With regard to T cells, we ended up having 25-30% residual recipient T cells. Although this is a normal rate for T cells in bone marrow chimeric rats, it provides a possible explanation why no differences in EAE were observed. During EAE immunization the APCs are activated by an inflammatory milieu caused by bacterial components implied in the CFA, whereas the T cells are stimulated oligoclonally when they recognize their antigen (gpMBP). This is followed by massive proliferation of those few cells in the draining lymph nodes. If the InsR deficient T cells are impaired in this first step, there can nevertheless be some recipient T cells getting activated and subsequently migrate and infiltrate to the CNS and cause the EAE lesions despite 70% of the T cells have an impairment. To overcome this problem, lymphopenic or T cell deficient rats should be used in the future. For the generation of chimeric mice RAG deficient strains are available that have no lymphocytes. A prominent example for a lymphopenic rat is the biobreeding (BB/OK) rat. This strain has been known for more that 30 years as a model of T1DM (Marliss et al., 1982). Both, lymphopenia and disease are caused by a point mutation in a single gene, the *GTPase of the immune associated proteins 5* (Gimap5) (Hornum et al., 2002; MacMurray et al., 2002). For our purpose it is unsuitable because of the genetic mismatch between both strains. To generate lymphopenic rats on the suitable Lewis background we have backcrossed the BB rat derived Gimap5 allele to Lewis rats using speed congenics. First experiments with homozygous animals have shown, that these animals are indeed lymphopenic. For future experiments these rats should be used as recipients to overcome the problem of residual recipient T cells and to reveal potential differences in the immune response of InsR deficient lymphocytes.

As mentiond above, InsR deficiency causes an imbalance between Th1 and Th2 cells *in vitro*. There is first line of evidence that changes in the cytokine profile as

revealed by qrtPCR are also of relevance *in vivo*. Blood analysis and intracellular staining have shown that re-isolatied donor T cells from our bone marrow chimeric rats after EAE induction differ in their IL-17 expression depending on the genotype. InsR kd T cells express higher amounts of this cytokine. To validate these findings improved chimerism has to be achieved.

Taken together, the obtained *in vitro* data indicate that the InsR is important during the very first phase of activation before CD134 is expressed. As a drawback, TCR/CD28 co-stimulation *in vitro* is artificial and much stronger than natural antigen-dependent stimulation. This setting might be too artificial to uncover all effects of InsR deficiency. There is also evidence that the InsR plays a role for the balance between Th subsets and the appropriate imprinting upon activation. It seems to be dispensable for long term activated T cells, although those cells have a glucose-dependent metabolism. To satisfyingly answer the relevance of the InsR for Th cells a more refined *in vivo* approach has to be established.

In the future also long-lived memory T cells should be analysed, because their metabolic processes are distinct from those of effector T cells (D'Cruz et al., 2009; Pearce et al., 2009). Impairment in memory T cell responses might explain the increased rate of infections in diabetes patients. Furthermore, T cell culture with reduced oxygen in the atmosphere should be conducted. Glycolysis in contrast to oxidydative phosphorylation as source of energy in resting cells is oxygen-independent. Maybe T cells would eventually switch their metabolism if oxygen is limited even without CD28 stimulation. Under such conditions up-regulation of the InsR might be important to ensure survival of the cells.

4.3.2. Impact of InsR deficiency on T_{reg} cells

Malfunction of the suppressive T_{reg} cell compartment is one possible mechanism for the link between autoimmunity and metabolic diseases as diabetes. It is known that energy supply is an important factor for the fate decision between induced T_{reg} or Th17 imprinting (Pearce et al., 2009; Powell et al., 2012). On the other hand it is known, that T_{reg} cells just as memory T cells have only a low energy demand, nearly on the level of resting cells. T_{reg} cells follow a different dynamics compared to other T cell subsets, they express low levels of Glut1, have low glucose uptake and glycolysis and gain their energy mostly from lipid oxidation (Barbi et al., 2013). So it has to be questioned whether insulin resistance has an influence on T_{reg} cell function.

To test this notion, an *in vitro* suppression assay was performed. In all experiments InsR kd and wt T_{reg} did not differ in the capacity to suppress the proliferation of target cells. This is in line with the aspect that these cells have an energy demand on the level of resting cells which can be easily covered by oxidative phosphorylation or low level glycolysis (Barbi et al., 2013).
A second aspect has to be mentioned. The T_{reg} cells were expanded *in vivo* prior to isolation and during this presumably energy-consuming phase the InsR knockdown was not yet completed due to the protocol.
Collectively, T_{reg} cell function *in vitro* is independent of InsR presence. Whether it might be important during the first activation remains to be elucidated in an *in vivo* approach.

4.3.3. Impact of InsR signalling on cytotoxic T lymphocytes

Similar to $CD4^+$ T cells, $CD8^+$ CTLs are dependend on glucose as well (Cham et al., 2008). It has been discovered more than 30 years ago that this T cell subset controls its cellular metabolism by co-ordinating oxidative phosphorylation and glycolysis in order to meet the energy demand (Greiner et al., 1994; MacDonald, 1977; MacDonald and Koch, 1977; MacLennan and Golstein, 1978). Effector CTL have higher energy demand than naïve or resting memory CTLs (Cham and Gajewski, 2005; Cham et al., 2008) and they cover their demand by increased and maintained expression of aminoacid transporters and glucose transporters (Fox et al., 2005; Jacobs et al., 2008).
To analyse CTL effector function, we have performed a ^{51}Cr release assay. Hereby it was possible to determine the capability of CTLs to kill MHC mismatched cells. We have demonstrated a reduced capacity of InsR deficient CTLs to lyse target cells. This could in part be explained by the absence of additional activation of Akt through InsR engagement, because Akt is central to control CTL function. Activation of Akt by PDK1 leads to a down-regulation of CD62L and the chemokine receptor CCR7 (Waugh et al., 2009). Hereby the enzyme directly interferes with the trafficking of effector CTLs compared to naïve cells: Downregulation of both molecules increases motility and reduces attraction to lymphoid organs allowing the CTLs to infiltrate to target organs.

Another possible explanation would be that CTL activity directly requires exocytosis and massive generation and degranulation of proteins. InsR engagement might help to cover this very high energy demand.

Future perspectives would be infection studies to see whether the rats are impaired in pathogen clearance after infection with intracellular pathogens, i.e. listeria monocytogenes. In addition, the role of $CD8^+$ T cell in EAE would be interesting. In our setting so far we had up to 30% residual T cells from the host, so an improved protocol for the generation of bone marrow chimeric rats was designed making use of newly generated lymphopenic Lewis rats (see above). In this context impaired $CD8^+$ T cell function might also influence the disease. New finding have revealed, that $CD8^+$ T are more important for the priming of effector cells in the periphery then for demyelination. Depletion of $CD8^+$ T cells by antibodies or the use of CD8 ko Lewis rats lead to a reduction in EAE symptoms, whereas AT-EAE induced with activated wt $CD4^+$ T cells in CD8 ko rats was similar compared to wt recipients (Camara et al., 2013). In our model, a possible reduction of EAE symptoms could be explained in two ways. Firstly, $CD4^+$ T cells could be responsible. Since we have shown that long-term activated effector T cells are not expressing the InsR, amelioration of EAE symptoms could be explained by impaired or misdirected priming of the $CD4^+$ T cells. Secondly, improved symptoms could be explained by impaired $CD8^+$ T cell function. However, careful titration of the antigen and CFA doses will have to be performed to elucidate a possible impact of InsR deficiency. A fulminant EAE course could disguise effects as also the reduction of EAE symptoms in CD8 ko rats was only seen when mild immunization conditions were applied (Camara et al., 2013).

Another explanation for the impairment of InsR deficient CTLs might be the fact, that $CD8^+$ T cells are more sensitive to the stress induced by shRNA expression than $CD4^+$ T cells, and that the observed differences between the phenotypes are simply due to that feature. A better control would be transgenic rats with a random shRNA integration.

4.3.4. Conclusion

Although it has been known that metabolism influences T cell imprinting and function, the role of insulin in this context remained elusive. We have shown that the early activation of Th cells is impaired *in vitro*. Surprisingly, the InsR was not only dispensable for late activation but was not expressed in long-term activated effector

T cells as well (Fig. 33). Also regulatory T cells show normal function under insulin resistant conditions. In contrast, $CD8^+$ CTLs showed reduced cytotoxicity if the InsR was not expressed, pointing toward a synergistic function of the InsR in meeting the energy demands of CTLs during the effector phase. The difference could also be explained by CD28, which might compensate for the absence of InsR signalling in $CD4^+$, but not in $CD8^+$ T cells. Additionally, both cell types were stimulated differently, the artificial stimulation of $CD4^+$ T cells *in vitro* with anti-CD28 antibody might falsify the observed results.

A prerequisite for all further studies is a satisfying bone marrow chimeric model to study the *in vivo* impacts of missing insulin responsiveness. The hitherto obtained results might be an explanation for increased susceptibility to infections. During the sensitive process of antigen-dependent T cell activation by APCs it might be essential for proper imprinting to the appropriate subset that the cells can respond quick and in the right manner. A handicap caused by missing synergistic signalling of the InsR could have an impact *in vivo* (Fig. 33) as first *in vitro* findings suggest a shift of Th subsets.

Collectively, InsR receptor deficiency mostly impacts of the very first steps of T cell activation, namely the priming, followed by energy consuming alterations of the cytoskeleton (Fig. 33). When the cells are differentiated the InsR seems to be dispensable for covering of energy demands. In this first steps the fate decision takes place and if this phase is disturbed it may cause wrong priming of the cells and a shift between T cell subsets. Thus it might cause problems during pathogen defence.

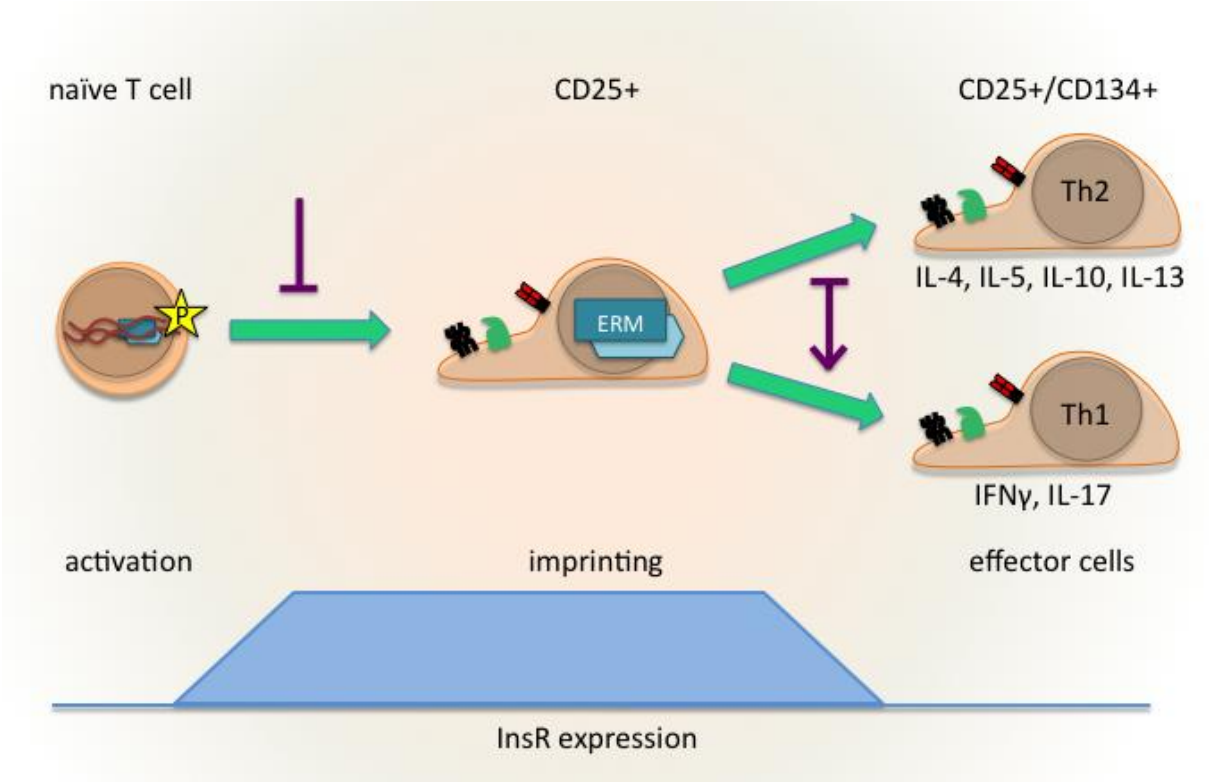

Fig. 33 Dynamics of InsR expression during T cell activation. Upon activation T cells induce InsR expression. Deficient insulin signalling impairs expression of the early activation marker CD25. During imprinting of the T cells to different Th subsets InsR inactivation leads to a shift to Th1 cells.

5. Summary

Insulin and GCs are two important endocrine regulators of mammalian body homeostasis and both impact on immune cell functions. Whereas GCs are in widespread use for the treatment of autoimmune diseases since the late 1950s, the influence of insulin is less well characterized. Nonetheless, it is undoubted that there is a link between insulin-responsiveness and immunity. Here we have shown that GCs exert a rapid effect on T cell morphology. We have identified GC-induced phosphorylation of the cytoskeleton associated ERM proteins as an important mechanism in this process, and found that it is accompanied by a loss of the polarized structure of effector T cells, a reduction in cell size and reduced capacity to conjugate with APCs. The effector T cells retract their lammellipodiae following GC treatment, which leads to impaired transmigration and presumably chemotaxis, as chemokine receptors are expressed in a polarized manner on the lammellipodiae. Additionally, we have found that this effect is dependent on the presence of the GR and on PLC activity. Furthermore we have challenged the hypothesis that insulin-responsiveness is required to allow proper T cell activation, differentiation and function. We took advantage of an inducible InsR kd to compare the functions and the survival of different T cell subsets in the presence or absence of the InsR. We could show, that the early phase of T cell activation is impaired in InsR deficient cells, whereas the InsR is dispensable during long-term activation and for overall survival. Whereas T_{reg} cells were not affected by InsR inactivation, $CD8^+$ T cells had a decreased lytic capacity compared to wildtype cells. So far we have no clear evidence whether the InsR also plays a role for immune responses *in vivo*.
Collectively, endocrine control of T cell function is of considerable importance and could explain the involvement of hormones in chronic diseases and their treatment.

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curriculum vitae

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Participation in scientific meetings

2010	**Oral** 2nd DGfI Autumn School *Current Concepts in Immunology*, Bad Schandau, Germany
2011	**Oral (selected abstract)** and **Poster** EMBO conference (organized by the European Network of Immunology Institutes), Sardinia, Italy
2012	**Poster** 8th Spring School on Immunology, Ettal, Germany
2012	**Poster** 3rd European Congress of Immunology, Glasgow, UK
2012	**Poster** 11th International Congress of Neuroimmunology, Boston, USA