Aus dem Institut für Medizinische Psychologie und Verhaltensimmunbiologie

Behaviorally conditioned immunosuppression in a model of rheumatoid arthritis in rats: Mechanisms and clinical relevance.

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„*Den Wissenschaftlern geht es wie den Chaoten. Es ist alles da, man muss es nur suchen*“ Franz Kern.

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  Brain Behav Immun. 2016 Feb;52:40-8.

- Exogenous oxytocin reduces signs of sickness behavior and modifies heart rate fluctuations of endotoxemic rats.
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- Rats taste-aversive learning with cyclosporine a is not affected by contextual changes.
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- Applications and limitations of behaviorally conditioned immunopharmacological responses.
  Lückemann L., Unteroberdörster M., Kirchhof J., Schedlowski M., Hadamitzky M. Neurobiol Learn Mem. 2017 Feb 142: 91–98

Content

Abbreviations

ACTH	adrenocorticotropic hormone
Am	amygdala
β-AR	beta adrenergic receptor
°C	degrees Celsius
CaN	calcineurin
CD	cluster of differentiation
CNS	central nervous system
CR	conditioned response
CS	conditioned stimulus
CsA	cyclosporine A
CTA	conditioned taste avoidance
cAMP	messenger cyclic adenosine monophosphate
DA	Dark Agouti
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
Fig.	figure
GPCR	G protein-coupled receptor
HBSS	hank's balanced salt solution
HPA-axis	hypothalamus-pituitary-adrenal axis
i.p.	intraperitoneal
IC	insular cortex
IFN-	interferon-
IL-	interleukin-
LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz
LiCl	lithium chloride
MAPK	mitogen-activated protein kinase
Min	minutes
mRNA	messenger ribonucleic acid
NA	noradrenaline
NaCl	saline solution
NF-AT	nuclear factor of activated T-cells
NMDA	N-methyl D-aspartate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pharm.	pharmacological
PNI	psycho neuro immunology
RPMI	Roswell Park Memorial Institute; cell culture medium
RT	room temperature
Sac	saccharin
SEM	standard error of the mean
SNS	sympathetic nervous system
Tab.	table
UCS	unconditioned stimulus
VMH	ventromedial nucleus of the hypothalamus
Wat	tap-water

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1. Introduction

1.1. Bi-directional communication between the central nervous- and the peripheral immune system

The central nervous system (CNS) and the immune system were considered to be autonomous for a long time. However, today innumerable publications and an interdisciplinary field of research collectively termed psychoneuroimmunology (PNI) provide evidence for their functional interaction (Blalock et al. 2007, Tracey 2010). The communication between CNS and the peripheral immune system is regulated via efferent and afferent as well as via humoral and neuronal pathways.

1.1.1. Afferent pathway: Immune-to-brain communication

The distribution of proinflammatory cytokines affects behavior as well as mood. Probably the most prominent example of the afferent communication between the brain and the immune system is the "Sickness Behavior". This is manifested in anhedonia, fatigue, anorexia, and hyperalgesia (Capuron et al. 2011, Dantzer et al. 2014). Information over specific changes in the peripheral immune system is transported to the brain via the afferent immune-sensory pathway. Inflammatory responses result in the release of pro-inflammatory cytokines like Interleukin 1 beta (IL-1β) and tumor necrosis factor (TNF)-α, by activated immune cells. These humoral messenger proteins stimulate sensory nerves and reach the CNS via selective transport systems or diffusion pathways (afferent pathway). Consequently, action potentials are transmitted to the periphery for example via acetylcholine (ACh). On the afferent arm neurotransmitters, cytokines or prostaglandins can reach the brain as alleged messengers via the circumventricular organs (Goehler et al. 2006) or via endothelial active transport mechanisms by crossing the blood-brain barrier (Banks 2005). Endothelial cells in the brain and macrophages can also receive and relay inflammatory signals. Due to an interaction of the two cell types, prostaglandin E2 is released, which can activate neurons in certain brain regions and thus lead to an alteration of the hypothalamic–pituitary–adrenal (HPA) axis (Dantzer et al. 2000, Schedlowski et al. 2014).

Here, the cytokines seem to induce neurophysiological activity changes in brain regions like hippocampus and amygdala (Am), which influence the behavior and mental state (Critchley et al. 2013). Additionally, the afferent neuronal pathway detects changes in the visceral immune status and is required for the translation of immune borne messengers into neuronal signals. The major structure for afferent neuronal communication is the vagus nerve. The anatomical and functional considerations with its relays to brain stem nuclei are optimal to inform about peripheral immune changes (Hadamitzky et al. 2013).

1.1.2. Efferent pathways: Brain-to-immune transmission

Information from the CNS to primary and secondary lymphatic organs is transmitted via the efferent pathway. The parasympathetic (efferent) pathway can modulate immune responses (Tracey 2009, Tracey et al. 2014, Chavan et al. 2017). Increased efferent signalling in the vagus nerve can suppress peripheral cytokine release; likely via macrophage nicotinic receptors and the cholinergic anti-inflammatory pathway. For example in a model of experimental murine arthritis, vagus nerve stimulation inhibits the acute inflammatory response and suppresses the development of clinical arthritis symptoms (Borovikova et al. 2000). Further research demonstrates that all “immune” organs (thymus, spleen, lymph nodes, and bone marrow) are substantially innervated by sympathetic postganglionic neurons (Chavan et al. 2017). Additionally, it was shown that the corresponding nerve endings are located in the white pulp close to T cells, B cells and dendritic cells (Felten et al. 1987).

This direct innervation of immune organs is fundamental for the communication of the two systems, so that the catecholamines (for example noradrenalin) released by the nerves can act on the lymphocytes in close proximity. The effect of noradrenaline on the target cell is mediated by alpha and beta adrenergic receptors. All lymphocytes express beta adrenergic receptors (β-AR) with the exception of T helper 2 cells (T_H2) cells. Several lymphocyte (sub-) populations for example $CD4^+$ (cluster of differentiation) T cells, produce the suitable receptors (typically β_2-adrenergic receptor (β_2-AR) and express the signaling machinery to respond to noradrenergic stimulation (Sanders et al. 2002). Furthermore, it is known that there is a link between the β_2-AR-mediated pathway and the T cell receptor-mediated pathway (Kohm et al. 2001, Kin et al. 2006). Stimulation of β_2-AR immune cells results in inhibition of the cellular activity of the enzyme calcineurin (CaN/calcium/calmodulin-

activated serine-threonine phosphatase) as well as a reduction in T_H1-cytokine production and T-cell proliferation. Besides, it is identified that all adrenergic receptors are a G protein-coupled receptors (GPCR) consisting of 7 trans-membrane α-helices. When the receptor is activated, guanosine diphosphate is exchanged with guanosine triphosphate, thereby diffusing the α-subunit of the G protein. This subunit is able to activate the adenylate cyclase, which produces the second messenger cyclic adenosine monophosphate (cAMP). An increase in cAMP levels activates the cAMP-protein kinase A intracellular signaling pathway, that interacts with other signaling pathways that regulating a series of biochemical reactions such as proliferation, differentiation, maturation and effector functions in immune cells (Lorton et al. 2015). Moreover, this mechanism could be link between the β_2-AR and T cell signaling pathways since expression of cytokines in activated T cells mainly depends on dephosphorylation of the transcription factor NF-AT (nuclear factor of activated T cells) by CaN. This mechanism demonstrates how intracellular signaling pathways may result in downregulation of T cell capacity after CNS activation (Exton et al. 2002a, Riether et al. 2011).

As a second possible mechanism, neuroendocrine and humoral signals could be transmitted to the sympathetic nervous system or the HPA via the blood stream. When the HPA axis is activated corticotropin-releasing hormone is released in the hypothalamus which leads to secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland. ACTH then stimulates the production of glucocorticoid, such as cortisol in the adrenal gland (Tsigos et al. 2002). Glucocorticoids are the main effector end point of the neuroendocrine system and primarily have an immunosuppressive effect (Wrona 2006).

This intense bi-directional communication of the CNS and the peripheral immune system is essential to retain homeostasis under normal conditions and to generate an immune response of appropriate extent after injury or immune challenge. Additionally, it is fundamental for the Pavlovian conditioning of immunologic response (Sternberg 1997, Exton et al. 2001, Ader 2003b, Meisel et al. 2005, Schedlowski et al. 2010). However, medication intake is rarely analyzed from the perspective of associative learning processes (Doering et al. 2012) and mechanisms mediating the conditioned immune response remain to be elucidated.

1.1.3. Classical conditioning paradigm/behaviorally conditioning of immune functions

Classical or Pavlovian conditioning is characterized by the modification of a neutral stimulus into a conditioned stimulus (CS). Repeated pairings of the neutral stimulus with an unconditioned stimulus (UCS), that always causes a certain reaction, forms an association between both. Re-exposure to the former neutral CS alone should subsequently then become capable of eliciting a new response as a function of its association with the UCS. The combination of CS and UCS is named acquisition while the re-exposure to the CS alone is called retrieval (Anrep et al. 1927) (see Fig. 1).

This classical conditioning paradigm serves as a suitable model for the exploration of the CNS-immune system connection (Exton et al. 2001). One fascinating example for the described communication between the two systems is behaviorally conditioning of immune functions (Ader et al. 1975, Wayner et al. 1978, Schedlowski et al. 2010).

Associative learning paradigms such as taste avoidance conditioning have possibly developed evolutionarily as adaptive mechanisms to protect the organism from toxic or immune modifying substances by avoiding its ingestion or contact. Taste-recognition memory is essential for organisms to discriminate food as safe or poisonous to avoid illness. When a novel taste is presented to an animal a reduced consumption is shown, known as neophobic response (Ader 2003b, Bermudez-Rattoni 2004, Schedlowski et al. 2010). Once the novel taste is associated with sickness (associative learning) the taste turns into an aversive signal, causing an avoidant ingesting behavior, known as conditioned taste avoidance (CTA). The CTA is a well-established learning and memory paradigm in rodents that is considered to be a special form of classical conditioning. One still not completely answered question in associative learning is, whether CTA works under the same rules as other types of classical conditioning, e.g. fear conditioning or eyeblink conditioning (Welzl et al. 2001).

However, this phenomenon is used as a standard protocol for behaviorally conditioned immune functions. Though, it is known that conditioning of immune functions can be experimentally induced by using artificial substances or drugs (Ader 1976). CTA forms an association by paring a novel taste as a conditioned stimulus

with the injection of an immunomodulating drug as unconditioned stimulus. After association between CS and UCS, re-exposure to the CS alone induces the conditioned response (CR). On the behavioral level, the CR is characterized by avoiding consumption of the CS (CTA) (Garcia et al. 1955, Garcia et al. 1985). In parallel to this behavior, animals demonstrate a conditioned suppression/modulation of the immune system, similar to that previously induced by the immune altering drug given as UCS (Ader 2003b).

Numerous anatomical and physiological findings demonstrate that several brain structures, in particular the insular cortex (IC), the Am and the ventromedial nucleus of the hypothalamus (VMH), as well as neurotransmitters and their receptors, are involved in the acquisition and retrieval phase of behaviourally conditioned immunosuppression. The IC plays an important role in acquisition and retrieval processes while the Am apparently mediates the input of visceral information during acquisition. Furthermore the VMH appears to be required for retrieval of the conditioned immune response (Pacheco-Lopez et al. 2005) (see Fig. 2). During acquisition N-methyl-D-aspartate (NMDA) receptors and intracellular signal cascades, such as the mitogen-activated protein kinase (MAPK) are essential for association/coupling between CS and UCS (Berman et al. 2003, Bermudez-Rattoni 2004, Dudai et al. 2004)

CTA paradigms using CaN inhibitor like cyclosporine A (CsA) as a potent immunosuppressive drug for solid organ transplantation and autoimmune disease revealed that conditioned immunosuppression is mediated on the efferent arm via the vagus- and splenic nerve through noradrenaline and β-AR -dependent mechanisms (Exton et al. 2002b, Pacheco-Lopez et al. 2005). Numerus studies in rodents demonstrate that cellular and humoral immune response can be affected via immune conditioning (Exton et al. 2002b, Ader 2003b, Riether et al. 2008, Schedlowski et al. 2010). Immunomodulating processes such as lymphocyte proliferation and passage, cytokine production and T cell activity are only some examples of immunological processes responsive to associative learning. For example, behaviorally conditioning with cyclophosphamide or CsA as UCS in animals was able to beneficially interfere with disease progression and mortality rate in experimental animal models of chronic inflammatory autoimmune disease such as systemic lupus erythematosus, rheumatoid arthritis or multiple sclerosis (Ader et al. 1982, Klosterhalfen et al. 1983, Klosterhalfen et al. 1990, Jones et al. 2008). Additionally, a number of studies in

humans investigated immune conditioning in the context of autoimmune diseases like allergic reactions. They demonstrate that many major symptoms of allergy –such as erythema size, weal size, basophile activation, nasal airflow, histamine release and subjective symptoms– can be influenced by immune conditioning (reviewed in (Vits et al. 2011)). With regard to these promising findings further investigations providing clinical implications are compulsory regarding the implementation of new therapies, combining pharmacological therapy with classical conditioning of immune functions (Schedlowski et al. 2010).

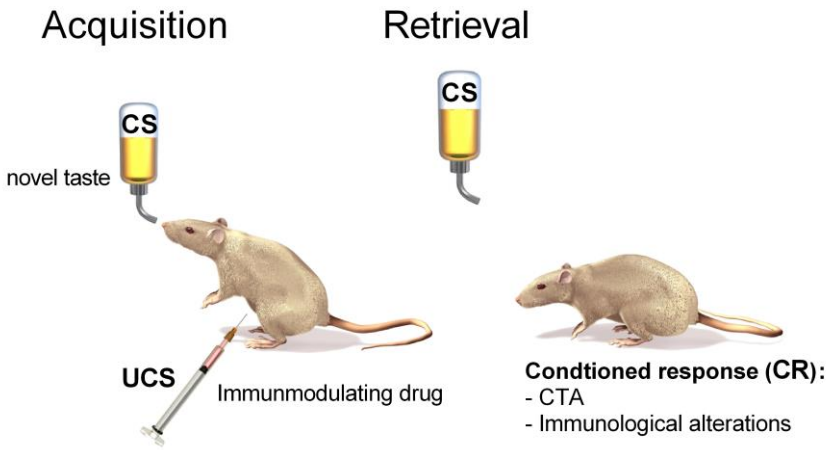


Fig. 1: Conditioned taste avoidance in the rat. During *acquisition* animals receive a novel-tasting solution (CS), which is paired with an i.p. injection of an immunomodulating drug (UCS). This learned CS/UCS association is recalled during retrieval by re-exposure to the CS alone. The recalled memory leads to a conditioned reaction (CR) and induces behavioral changes (CTA) as well as immunological alterations. (Modified after Riether et al. 2008)

1.1.4. Behaviorally conditioned immunosuppression with CsA

As described previously (please see 1.1.3.) studies in this thesis are based on the model of behaviorally conditioned immunosuppression in rats. In this paradigm rats are exposed to the novel taste of fluid saccharin (CS) in combination with an i.p. injection of the immunosuppressive drug CsA as UCS (Exton et al. 2001). CsA is widely used in transplantation medicine but also in atopic dermatitis, psoriasis and in rheumatoid arthritis or in other diseases where a suppression of immune functions is required (Kapturczak et al. 2004). Cyclosporine was isolated in 1971 from the *fungus tolypocladium inflatum.* The calcineurin inhibitor CsA then revolutionized transplant medicine in the late 1970s (Tedesco et al. 2012). This small-molecule drug specifically blocks the activity of the cytosolic protein CaN in T cells. Normally, CaN dephosphorylates proteins allowing them to translocate into the nucleus and to

activate gene expression of IL-2, interferon-gamma (IFN-γ) and related genes through the cis-element (NF-AT) (Steinbach et al. 2007). Due to inhibition of CaN by CsA and thereby a prevention of CaN-mediated dephosphorylation of NF-AT, gene and protein expression in activated T cells of the afore mentioned cytokines are diminished. This cascade ultimately leads to a suppression of T lymphocyte proliferation and activation (McCaffrey et al. 1993, Halloran et al. 1999). Furthermore, CsA also inhibits activation and proliferation of other immune cell (B cells, NK cells and native immune cells) (Fric et al. 2012). Moreover, in the saccharin (Sac)/CsA conditioning paradigm the described immunomodulation mechanism is essential, thus re-exposure to saccharin alone during retrieval results in a learned avoidance which is accompanied by a conditioned immunosuppression. This immunosuppression is reflected by a significant decrease in ex vivo CD3-stimulated cytokine production of IL-2 and IFN-γ as well as a reduced splenic lymphocyte proliferation (Exton et al. 1999, Exton et al. 2001, Exton et al. 2002b, Pacheco-Lopez et al. 2009) (Fig.2).

A possible mechanism for the conditioned suppression of IL-2 was detected to be the noradrenaline (NA) secretion in the spleen (Exton et al. 1998a). The essential intracellular cascade consists of the inhibition of CaN activity in T cells by β-AR stimulation (Pacheco-Lopez et al. 2009). Moreover, experimental data documents, that re-exposure to the taste but not a significant CTA is essential for a conditioned immunosuppression. In this context, it has previously been shown that decreased cytokine levels and anti-proliferative effects are still detectable in the absence of CTA (Bovbjerg et al. 1984, Bovbjerg et al. 1987, Klosterhalfen et al. 1990, Niemi et al. 2006).

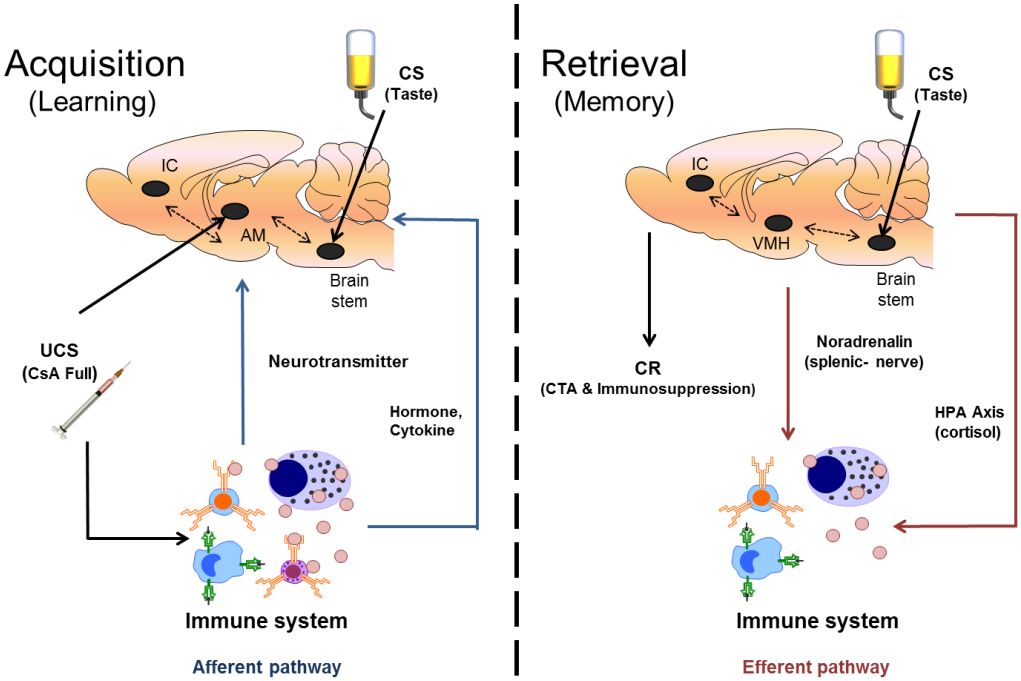


Fig. 2: Theoretical sketch of behaviorally conditioned immunosuppression with CsA in the rat.
Acquisition: During the learning phase the association between the CS (saccharin) and the UCS (CsA) is mediated through different pathways. In this model there are several possible afferent pathways for CsA effects in the brain. CsA might alter immune functions, via afferent humoral pathways and neuronal, or could directly act on the brain, permeating the blood-brain-barrier. Insular cortex (IC) may play an associative role being necessary to acquire and evoke the behaviorally conditioned immune response, whereas the amygdala seems to mediate the input of visceral information only during acquisition. **Retrieval:** During the retrieval phase, the re-exposure to the taste (CS) induces a conditioned immunosuppressive reaction (CR) via efferent pathways. The so called neocortical-immune axis, with hypothalamic relays (VMH) and the sympathetic nervous system (splenic-nerve) may be responsible for altering peripheral immune functions through noradrenaline and β-AR-mediated mechanisms. Am: amygdala, CR: conditioned reaction, CS: conditioned stimulus, CTA: conditioned taste avoidance, HPA: hypothalamic-pituitary-adrenal, IC: insular cortex, US: unconditioned stimulus, VMH: ventromedial nucleus of the hypothalamus. Figure designed with Motifolio®.

Studies in human volunteers lend further support to the notion that conditioning can modulate peripheral immune reactions (Ader et al. 1975, Sabbioni et al. 1997, Goebel et al. 2008, Schedlowski et al. 2010). Similar to the paradigm in rats healthy male subjects receive the immunosuppressive drug CsA as UCS together with a novel-tasting drink (CS) during the acquisition phase. Re-exposure to the CS during retrieval induces a conditioned suppression of T cell functioning as reflected by the actual effect of CsA, diminished cytokine (IL-2 and IFN-γ) production, reduced cytokine mRNA expression and inhibited T cell proliferation (Goebel et al. 2002). Importantly, this learned immunosuppression has been shown be to recallable by exposing the conditioned subjects to the CS again after a break of 11 days (Wirth et al. 2011).

1.2. Associative learning mechanisms in behaviorally conditioned immunosuppression

Associative learning is not restricted to the previously described acquisition, but it may rather happen simultaneously at any time (i.e. also in the retrieval phase of a conditioning protocol). In the context of behaviorally conditioned immunosuppression, distinct learning concepts have been studied in the last years. Therefore, processes such extinction, memory-updating/reconsolidation-like processes as well as latent inhibition and pre-exposure will be further described in the next chapters.

1.2.1. The process of extinction

Extinction represents reduction of the conditioned response after several unreinforced exposure to the CS (Anrep et al. 1927). It describes a form of associative learning in Pavlovian conditioning in which the individual learns that the CS is no longer followed by the UCS (Bermudez-Rattoni 2004). Extinction is therefore a second association inhibiting the former learned behavior (Gallistel 2012).

The most consistent understanding of the neural basis of extinction learning has been achieved in studying the fear system of rodents (Quirk et al. 2008). Experimental data demonstrate that extinction does not simply erase the acquired excitatory association but rather forms a second association inhibiting the former learned excitatory behavior (Bouton et al. 2006). This view of extinction as a form of inhibition and new learning is further supported by CTA data impressively demonstrating that a change of context after extinction causes a robust return of conditioned responses to the conditioned stimulus (CS) (Bouton et al. 1983, Rescorla 2008). However, the mechanism of extinction is rather complex. It has been shown that the process of extinction of the CTA with lithium chloride (LiCl) (UCS) and saccharin (CS) is dependent on β-AR in the IC and independent of muscarinergic receptors or the MAPK kinase (Berman et al. 2001, Berman et al. 2003). The central role of the IC in CTA extinction learning with the immunosuppressant CsA as UCS is also confirmed by lesion studies in which a lesion of IC performed before or after acquisition completely disrupted the behavioral (CTA) component of the conditioned response (Pacheco-Lopez et al. 2005). Therefore, processes of memory extinction in taste-visceral associative learning resemble molecular mechanisms with learning which differ from those of "new learning” (Berman et al. 2001).

The questions how the extinction process can be accelerated and how reconsolidation (stabilization or influence of already consolidated memory traces) of learned memories can be blocked represent major research topics in fear extinction. However, in the field of behaviorally conditioned immunomodulation, the major goals are rather to block extinction of the learned immunosuppression and to reactivate the learned response on demand by CS re-exposure (Schedlowski et al. 2010).

1.2.2. Reconsolidation-like processes in conditioned immunosuppression

It is assumed that the availability of new information during the reactivation of a memory implements a new "condition" that is needed for the reconsolidation of a memory process (Tronson et al. 2007, Monfils et al. 2009, Nader et al. 2009, Schiller et al. 2010). Nader et al. 2000 discovered that memory reactivation in rats by a *reminder cue* changes the status of a consolidated fear memory to a labile status which has to be reconsolidated using *de novo* protein synthesis in the Am to persist (Nader et al. 2000). Furthermore, numerous other groups demonstrated that reconsolidation of conditioned responses is driven by *de novo* protein synthesis in brain regions known to be involved in the consolidation of extinction memory (Rodriguez-Ortiz et al. 2005, Bermudez-Rattoni 2014, Hadamitzky et al. 2015). For example, blocking protein translation with daily bilateral intra-insular infusions of anisomycin prevented CTA extinction during repeated retrieval trials (Hadamitzky et al. 2016b). Like consolidation itself, reconsolidation seemed to have a time window during which blockade of protein synthesis is effective. The retrieval of a memory trace can induce a short time frame during which the memory vanishes or is at least no longer retrievable during this time *de novo* protein synthetic is accomplished (Tronson et al. 2007). This period seems to last approximately about 4-6 hours in humans and is referred to as "reconsolidation window" (Schiller et al. 2010). Evidence for the "reconsolidation process" is derived from CTA studies demonstrating that intra-insular administration of anisomycin or the β-AR antagonist propranolol within 6 h after the re-exposition to the CS (saccharin) significantly diminishes the process of extinction (Bahar et al. 2003, Eisenberg et al. 2003, García-DeLaTorre et al. 2009) (Fig. 3c/d). The engrams of CTA are thus not only consolidated and stabilized once, but can be returned to an unstable state by reactivation and therefore need to be reconsolidated. These aspects explain why memories can display spontaneous recovery, reinstatement, and renewal (Bahar et

al. 2004, Dudai 2012). Reconsolidation is not simply the reoccurrence of consolidation, but constitutes a partly different process that resembles a lingering consolidation to update learned information (Dudai, 2012).

It has recently been demonstrated that extinction of a CsA induced conditioned suppression of IL-2 and IFN-γ cytokine responses might be abrogated when a sub-therapeutic dose of the UCS (CsA) was administered as a *reminder cue* in parallel with the CS during retrieval. Updated learned immunosuppressive responses could thereby significantly prolong the rejection of vascularized allograft in rats (Hadamitzky et al. 2016a) (Fig. 3a). These data suggest that neural processes mediating memory-updating/reconsolidation are transferred to peripheral immune functions. Additionally, studies in humans indicate similar results of delay in extinction in a learned immunosuppression paradigm by administering sub-therapeutically CsA *reminder cues* during CS re-exposure (Albring et al. 2014). Furthermore, it has been shown that activation of CaN contributes to the extinction of fear memory in the rat Am via a negative feedback-loop. In parallel, CaN inhibitors such as CsA prevented extinction-induced protein dephosphorylation as well as extinction of fear (Lin et al. 2003) (Fig. 3b). However, fear extinction differs from conditioned immunosuppression, therefore these findings might explain, whether the administration of sub or low-therapeutic CsA during retrieval is protecting the conditioned taste-immune memory by blocking extinction due to interfering with phosphorylation of particular substrates in the brain (Lückemann et al. 2017).

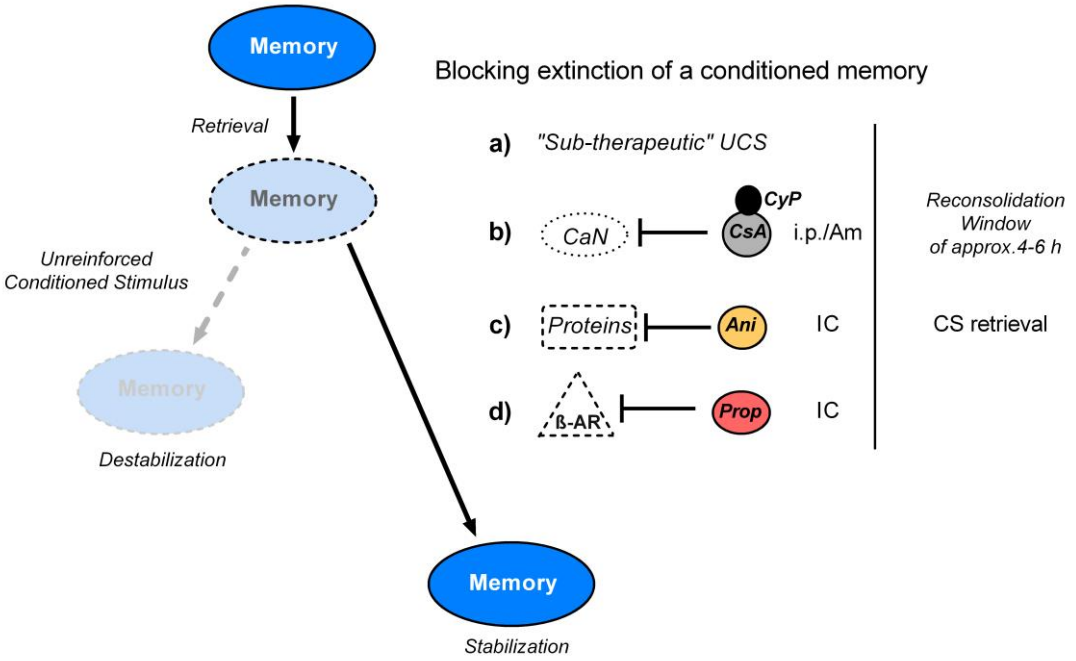


Fig. 3: Model of reconsolidation-like/memory-updating and extinction blocking processes in behaviorally conditioned immunosuppression.
During retrieval, the taste-immune memory enters a transient labile phase in which it can be modulated. During this 4-6 h "reconsolidation window" *de novo* protein synthesis in the insular and amygdala is essential for memory retrieval. (Dudai et al 2004; Nader et al., 2000; Rodriguez-Ortiz et al. 2005; Schiller et al., 2010). On one hand an unreinforced conditioned stimulus, leads to destabilization of memory and therefore to extinction of the learned immunosuppressive responses (left hand panel). On the other hand extinction can be blocked by**: a)** Reconsolidation of memory by simultaneous presentation of sub-therapeutic drug doses of the unconditioned stimulus (CsA) as a *reminder cue* together with the CS (saccharin) (within the reconsolidation window) Hadamitzky et al., 2016a, **b)** Injection of CaN inhibitors like CsA, which can prevent extinction-induced protein dephosphorylation (Lin et al. 2003). **c-d)** Blocking protein synthesis via intra-insular administration of anisomycin or the β-AR antagonist propranolol which can reduce the extinction process. (Bahar et al. 2003, Eisenberg et al. 2003, García-DeLaTorre et al. 2009). Ani: Anisomycin, Am: Amygdala, ß-AR: ß-adrenoceptors, CaN: Calcineurin, CsA: Cyclosporine A, CS: Conditioned Stimulus, CyP: Cyclophilin; IC: Insular Cortex, Prop: Propranolol, US: Unconditioned Stimulus

1.3. Clinical implications of conditioned immunosuppression

1.3.1. Pre-exposure and latent inhibition

Until today, there are still questions which have to be clarified in order to implement learned immunosuppression in clinical situations in the future. The aim of implementing the conditioned immunosuppression paradigm into clinical treatment strategies is to minimize the amount of drugs and thereby decrease adverse drug side effects and to maximize the therapeutic outcome for the patient's benefit (Colloca et al. 2005, Ader et al. 2010, Schedlowski et al. 2010, Doering et al. 2012, Enck et al. 2013, Schedlowski et al. 2015). However, one major obstacle could be that the majority of patients will be already on immunosuppressive treatment before

participating in an immunosuppressive learning paradigm as a supportive therapy. This so called ‘‘UCS pre-exposure effect” (UCSPEE/*Short*: PE) has already been analyzed in conditioning paradigms with LiCl as UCS. Here, presenting the UCS prior to acquisition led to a diminished conditioned response (CTA) (Randich et al. 1979, Meyer et al. 2004, Chang et al. 2007). Furthermore, studies demonstrated that a taste stimulus (CS) repeatedly presented to an organism in the absence of an aversive UCS also caused a weaker acquisition resulting in a diminished CTA during retrieval, a phenomenon known as latent inhibition (LI) (Lubow et al. 1959, Elkins 1973, Albert et al. 1989, Gaztanaga et al. 2015). These findings indicate that pre-exposure to the UCS (PE) or the CS (LI) prior to the acquisition phase induce a diminished conditioned response in a CTA paradigm with LiCl. Whether pre-exposure to the UCS and/or the CS also affects the conditioned immunosuppression with CsA is addressed in this thesis.

1.3.2. Experimentally induced rheumatoid arthritis

Human rheumatoid arthritis (RA) affects approximately 1% of the world population (Gibofsky 2012). RA is a common systemic inflammatory autoimmune disease associated with progressive disability, systemic impediments, early death and high health insurance costs (McInnes et al. 2011). The major symptoms are synovial inflammation and hyperplasia (“swelling”), cartilage and bone erosion, and systemic features (Smolen et al. 2016)

To investigate inflammatory autoimmune diseases in rodents, in this thesis RA was experimentally induced by an intradermal immunization of incomplete Freund's adjuvant in combination with type II collagen developed by Trentham 1977 (Trentham et al. 1977). Collagen-type II-induced arthritis (CIA) is a valid animal model of human RA because type II collagen (CII) is expressed exclusively in the joint articular (Cho et al. 2007). The CIA model demonstrates that autoimmunity to CII can generate autoimmune arthritis, which is linked to inflammation of synovial joints, destruction of cartilage and bone erosion. In rats the onset of the disease approximately two weeks after immunization is indicated by swelling and erythema of the front- and hind paws/ankles (Brand et al. 2007). Furthermore, the affected joint is damaged by an inflammatory response where the severe erosion of the joint has a very similar histopathology as observed in RA with formation of an erosive pannus tissue in the joint (Holmdahl et al. 2002, Schurgers et al. 2011).

Symptoms of autoimmune disease such as RA in humans are often treated with immunosuppressive drugs like cortisol, methotrexate, rapamycin and CsA (Chighizola et al. 2016). Additionally, animal studies in rats and mice show that CsA suppresses CIA both prophylactically and therapeutically (Takagishi et al. 1986, Cannon et al. 1993). However, numerous of patients treated with immunosuppressive drugs suffer from diverse side effects such as headaches, tremors, depression, and anxiety, which result in an overall reduced quality of life (Bosche et al. 2015). This raises the question if conditioned immunosuppression can lead to the similar/comparable beneficial effect to drug treatment without any unwanted drug-associated side effects.

Fortunately, animal studies already demonstrated positive effects of conditioning on the outcome of autoimmune diseases such as arthritis. Kosterhalfen & Klosterhalfen established a conditioning protocol with cyclophosphamide (UCS) and saccharin/vanilla (CS) in an adjuvant arthritis model. They demonstrated beneficial effects in terms of reduced symptom severity by a conditioned immunosuppression indicating that associative learning processes might affect the development of an autoimmune disease (Klosterhalfen et al. 1983, Klosterhalfen et al. 1990). The above mentioned explanations on behaviorally conditioned immunosuppression might be of clinical relevance in humans, when employed in medical treatment situations as supportive therapy together with standard pharmacological regimens (Exton et al. 2001, Ader 2003b, Enck et al. 2008).

1.4. Thesis objectives

Both, knowledge regarding mechanisms underlying substitutive treatments such as placebo approaches and their application in clinical settings are still sparse. Possible alternatives or “add-ons” to pharmacotherapy in the context of placebo responses may be seen in behavioral conditioning paradigms aiming at a controlled drugs-dose reduction while simultaneously maintaining efficacy of treatment (Doering et al. 2012). Peripheral immune functions can be modulated by associative learning paradigms. A growing body of data from studies in rodents and first observations in humans demonstrate that extinction of conditioned immunosuppression can be abrogated or blocked by processes such as memory-updating, using sub-therapeutic reminder cues during retrieval of the conditioned response (Hadamitzky et al. 2016a, Lückemann et al. 2017). However, the mechanisms of these phenomena still remain to be elucidated. From a clinical perspective, it is crucial to evaluate, weather and to what extent induction of conditioned immunosuppression is possible in patients who are already on immunosuppressive therapy with the drug used as UCS, or had previous contact to the gustatory stimulus used as CS. Therefore, we first addressed the following research question:

> *How do pre-exposure and latent inhibition in rats interfere with the conditioned response on the behavioral level and on the immunological level?*

In a second set of experiments, the potential clinical applicability of learned immune responses was determined by analyzing the effect of behaviorally conditioned suppression of immune functions on the development and progression of chronic inflammatory autoimmune disease in rats, addressing the question:

> *How does learned immunosuppression affect the disease progression of experimentally induced arthritis with in rats regard to clinical applications such as treatment of autoimmune diseases?*

For this purposes, a model of collagen-type II-induced arthritis was established (Trentham 1982, del Rey et al. 2008) to examine, whether behaviorally conditioned immunosuppressive responses at different stages of the disease would improve symptomatology, reflected by reduced quantifiable signs of inflammation (swollen joints, paw swelling, grip strength) in CIA. Moreover, it was analyzed whether and to what extent the administration of low-therapeutic reminder cues during the retrieval phase of conditioning (memory-updating) could preserve learned immunosuppression from being extinguished.

2. Material

2.1. Animals

Adult male Dark Agouti (DA/HanRj, 170-200 g; Janvier, France) rats were individually housed in standard plastic cages. They were kept under an inverse 12 hour light/dark cycle with lights off at 7 am, to perform the experiments during the activity phase of the awake/sleep cycle. After arrival, animals were allowed to acclimate to the new surroundings for two weeks before initiation of any experimental procedure. Subsequently, rats were single-housed with *ad libitum* access to food while tap water was available *ad libitum* until the water deprivation regimen started. The animal facilities and experimental procedures were in accordance with National Institutes of Health and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by the Institutional Animal Care and Use Committee (LANUV TSG-Nr. G1431/14 Düsseldorf, North Rhine-Westphalia)

2.2. Drugs

2.2.1. CsA

A stock solution (100 mg/ml) of cyclosporine A (CsA; LC Laboratories, Woburn, USA) containing 900 µl Miglyol (Caelo, Hilden, Germany) and 100 µl ethanol (96 %, Braun, Melsungen, Germany) was diluted with sterile saline (0.9 % NaCl, Braun, Melsungen, Germany) to gain the required drug dose of 20 mg/kg body weight at a final injection volume of 1 ml. For low dose treatment, we defined 5 mg/kg body weight as as previously implemented. (Hadamitzky et al. 2016a).

2.2.2. Freud’s adjuvant & Collagen Type II

150 µl collagen type II Stock (#804001-sol; MD Bioproducts, Egg, Switzerland) and 150 µl Freud’s adjuvant incomplete Stock (Sigma-Aldrich, Taufkirchen, Germany) were mixed freshly as a 1:1 solution for induction of collagen type-II-induced arthritis (CIA).

3. Methods

3.1. Standard conditioning protocol

The standard conditioning paradigm started with a water deprivation period of 5 days. During this water deprivation time, animals had free access to water in two daily 15 min-drinking sessions at 9 am and 5 pm. Before and after each drinking session, the drinking bottles were weighed to measure fluid consumption. Individual mean water consumption in the morning sessions over these days was taken as baseline level (100%) for ‘‘normal” fluid intake. After animals were adjusted to this procedure, the conditioning regime started on day 6. During the learning phase, a combination of 0.2 % (w/v) sodium saccharin (Sigma-Aldrich, Schnelldorf, Germany) drinking solution as the CS, and an intraperitoneal (i.p) injection of CsA as the UCS were applied in the morning drinking session. This acquisition phase (CS-UCS pairings) was repeated on three days with two days (72 h) off in-between each trial. In the evening session, all animals received water.

Two days after completing the acquisition phase, animals were re-exposed to the CS, but not to the UCS on three consecutive days (retrieval phase). The total amount of liquid consumed per day was measured by weighing the bottles before and after each drinking session. Saccharin consumption was measured as percentage of baseline water consumption. On retrieval day three, animals were sacrificed 1 h after CS-re-exposure and spleens were collected for ex-vivo immunological analyses.

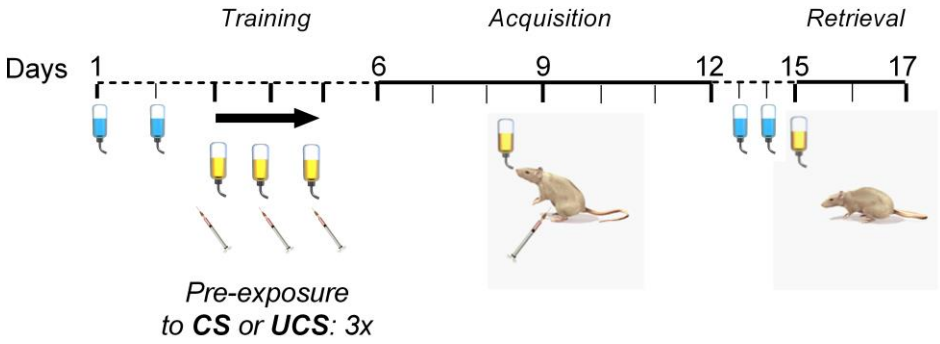
3.2. Pre-exposure and latent inhibition of conditioned immunosuppression

Animals of the first (pre-exposure) experiment (CsA/UCS pre-exposure) were assigned to one of four treatment groups (*CS0;* n = 17, US; n = 15, NPE; n = 16, PE; n = 17) (Fig. 4) and put on a water deprivation training (days 1–5) with two drinking sessions of 15 min each daily. Normal individual fluid consumption was taken as water baseline and set at 100% (see 3.1). For the pre-treatment phase, the conditioned group PE (pre-exposed to the UCS), the control group *CS0* (control for residual effects of CsA), and the *US* group (pharmacological control) received (i.p.)

injections of the UCS (20 mg/kg CsA) after the morning drinking session on water deprivation days 3–5. Animals in the NPE-group (the ‘‘standard” conditioned group) were injected with NaCl-solution on water deprivation days 3–5 as a control for the handling procedure during the training phase. All rats were subsequently conditioned as described in 3.1. During three days retrieval, rats of *PE* and *NPE* were re-exposed to the CS (saccharin) without reinforcement of CsA (UCS). Contemporaneous all *US* and *CS0* animals had access to water. Furthermore, the pharmacological treatment control (*US)* animals got an i.p. injection of 20 mg/kg CsA.

The procedures for the second (latent inhibition/LI) experiment (Saccharin/CS pre-exposure): were identical to the ones from Experiment 1 (PE). However, the pre-exposure group was replaced by the latent inhibition (*LI*; pre exposed to saccharin) group (*CS0*; n = 18, *US*; n = 18, *NLI*; n = 17, *LI*; n = 18). After water deprivation (days 1–2) the pre-treatment phase started, with the conditioned groups *LI* (pre-exposed to the CS), *CS0* (control for residual effects of CsA), and *US* (pharmacological control) receiving saccharin solution instead of water in the morning drinking session (days 3–5). Animals in the *NLI* group (the ‘‘standard” conditioned group) received water as a control for the handling procedure during the pre-treatment phase. General retrieval procedures were identical to the ones applied in Experiment 1 (PE), as described in section 3.1.

A



B

Experiment	Groups		Pre-treatment	Acquisition	Retrieval
1 (PE)	Non-pre-exposed	NPE	Veh	Sac/CsA	Sac
	C. not evoked	CS0	CsA	Sac/CsA	Wat
	Pharm. control	US	CsA	Sac/CsA	Wat/CsA
	Pre-exposure	PE	CsA	Sac/CsA	Sac
2 (LI)	Non-latent inhib.	NLI	Wat	Sac/CsA	Sac
	C. not evoked	CS0	Sac	Sac/CsA	Wat
	Pharm. control	US	Sac	Sac/CsA	Wat/CsA
	Latent inhibition	LI	Sac	Sac/CsA	Sac

Fig. 4: Experimental time line (A) and groups (B).
In Experiment 1(pre exposure) rats of all groups were pre-treated with either 20 mg/kg CsA i.p. (*PE, CS0, US*) or NaCl (Veh; *NPE*) on three subsequent days before acquisition. In contrast in Experiment 2 (latent inhibition) all animals were pre-exposed to saccharin (*LI, CS0*, and *US*) except for the *NLI* group which received water on three days prior to conditioning. For both experiments all rats in all groups were exposed to saccharin in combination with CsA during acquisition and were re-exposed to saccharin (*PE, NPE, LI, NLI*) or water (*CS0)* or water and CsA (UCS) during retrieval. Sac: 0.2% saccharin; CsA: cyclosporine A 20 mg/kg; Wat: tap water. Modified after (Lueckemann et al. 2016).

3.3. Immunization of collagen type II arthritis

The collagen type-II-induced arthritis was introduced by an intradermal (i.d) injection of a 1:1 emulsion (300 µl) of collagen type II (#804001-sol; MD Bioproducts, Egg, Switzerland) 150 µl an Freud's adjuvant incomplete (Sigma-Aldrich, Taufkirchen, Germany) 150 µl. In order to allow a painless and less stressful i.d.injection in the base of the tail, all animals were anesthetized with isoflurane (Henry Schein, Hamburg Germany) (approximately 1.5-2% mixture with oxygen). During the procedure, the depth of the narcosis was monitored by examination of the reflexes (lid reflex). First symptoms of the disease occur 14 days after injection/immunization.

3.4. Pain prophylaxis through subcutaneous osmotic pumps

CIA is a systemic inflammation model. To relief animals from pain, all rats received analgesic medication through subcutaneous osmotic pumps from ALZET® (Cupertino California), implanted subcutaneously in the back under isoflurane anesthesia on day 7 after CIA immunization.

In order to allow painless surgery for injecting the above-mentioned immunization solution, rats were anesthetized under isoflurane. Since isoflurane does not have an analgesic effect, carprofen (Carprieve 5mg/kg Bayer Leverkusen, Germany) was administered as a pain prophylaxis prior to anesthesia. After surgery, all rats were observed during the recovery phase. Prior to implantation, pumps were filled with 2 ml buprenorphine an opioid derivate (Temgesic; Indivior Eu Limited; Great Britain) to assure a daily dose of 0.1 mg/kg body weight with a pump rate of 5.0 µl/hr.

3.5. CsA effects on arthritis/CIA in Dark Agouti rats

Pilot experiments in Regensburg analyzed the impact of daily intraperitoneal injections of a pharmacological dose of CsA (20 mg/kg bodyweight) in a collagen type-II-induced arthritis model in *Dark Agouti* rats. Effects were assessed by analyzing the clinical arthritis score (see. 3.8).

3.6. Memory-updating in a behaviorally conditioned immunosuppression in CIA

For all arthritis experiments, animals were divided into four different treatment groups. Three control groups (*USlow, US* and *CS0*) and one experimental group (*CSlow*). Water deprivation training and acquisition phase were equal to the standard conditioning protocol described in 3.1. Two days after the last acquisition day all animals were immunized (induction of CIA). The first arthritis symptoms occur on day 14 after immunization (Fig .5).

Animals of the conditioned group *CSlow* received saccharin and a low dose CsA (5mg/kg) in the morning sessions. To control for low dose pharmacological effects on arthritis, the *USlow* group was implemented. All animals of this group got water and a low dose CsA (5mg/kg). To compare the conditioning effect with a pharmacological

therapy, all animals from the *US* group received full dose i. p. injections of CsA (20 mg/kg) during retrieval. Control animals for residual effects of CsA were neither re-exposed to the CS nor to the UCS (*CS0* group) (Tab.1).

Tab. 1: Group allocation for behaviorally conditioned immunosuppression of CIA:
For all experiments all animals in all groups received saccharin in combination with CsA during acquisition and were re-exposedl to saccharin (*CSlow*) or water (*CS0*) or water and CsA (*USlow, US*) during retrieval .Sac: 0.2% saccharin; CsA: cyclosporine A 20 mg/kg or 5mg/kg; Wat: tap water

Groups		*Acquisition*	*Retrieval*
Conditioned	**CSlow**	Sac/CsA	Sac/CsA 5 mg/kg
low dose control	**USlow**	Sac/CsA	Wat/CsA 5 mg/kg
Pharm. control	**US**	Sac/CsA	Wat/CsA 20 mg/kg
C. not evoked	**CS0**	Sac/CsA	Wat

3.7. Retrieval on three different time points

To interfere during different stages of disease progression, three time points of retrieval were assessed in different experiments. In the first experiment (A) retrieval started on day one after immunization before occurrence of the first clinical symptoms. In the second experiment (B), animals were retrieved after 14 days, contemporaneous with the first arthritis score. The last time point for retrieval was individually determined by a clinical arthritis score of 4 points per animal (experiment C) (Fig. 5).

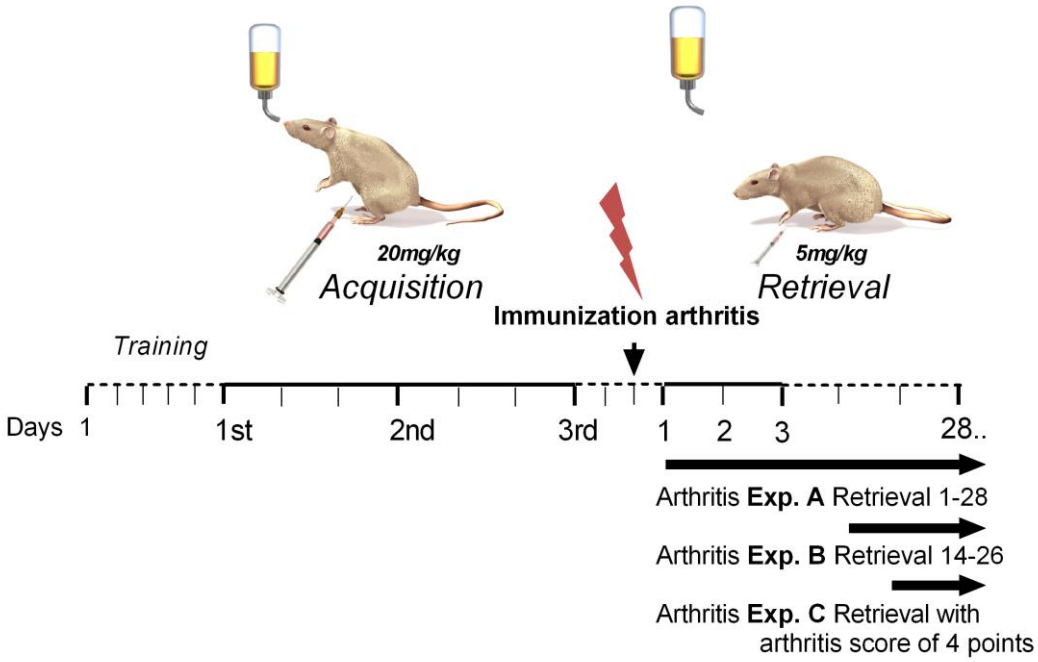


Fig. 5: Experimental protocol: Reconsolidation of behaviorally conditioned immunosuppression in CIA. Three different experiments were conducted. All animals were treated equally until retrieval. In Exp .A, retrieval started one day after immunization, in Exp. B the retrieval phase started at day 14 In Exp. C individual retrieval was initiated at clinical arthritis score of 4 points.

3.8. Assessment of clinical arthritis score

All DA rats that received CIA developed severe clinical symptoms of arthritis, which were first detected on day 14 after immunization and reached the maximal score of 16 points between day 24 and 28. Development of arthritis was monitored by determination of body weight and arthritis score during all days after immunization. For scoring, each paw was evaluated separately. The maximum arthritis score per extremity was 4 points. One point was assigned for each of the following inflamed (slight or severe) regions: the toes/fingers, the midfeet (metatarsus/metacarpus) and the wrists/ankles. Additionally one point was given if the normal use of the paw was impaired and the animal was limping. Thus, the maximal arthritis sumscore was 16 points per animal (Fig. 6). All ratings were performed by two experimenters'- which were blinded for the respective treatment group. All animals will be sacrificed on day 28 after arthritis induction for further immunological and histological analyses (please see.3.10.2).

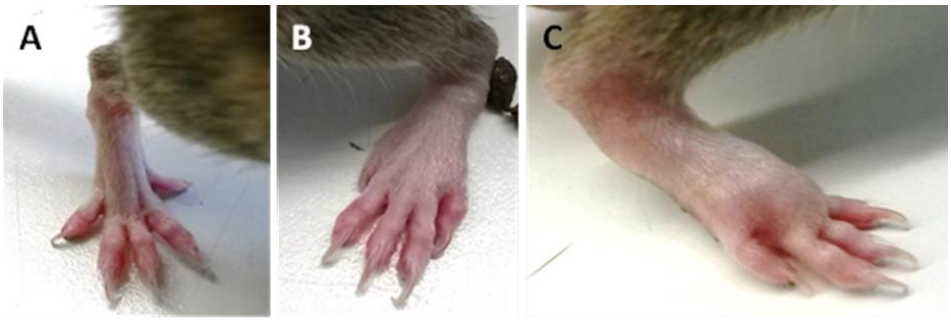


Fig. 6: Example of clinical arthritis scoring on day 16 after immunization (hind paws). **(A**) Hind paw with a score of one point (toes are swollen). **(B)** Paw with 3 points (tow, middle foot and ankle are swollen). **(C)** Maximum score of 4 points total paw is swollen and inflamed, animal limbs.

3.9. The grip strength test

Grip strength measurement has been established to assess carrageenan evoked muscle hyperalgesia in rats by Kehl et al. (2000) (Kehl et al. 2000). In our experiments, a more simple technique was employed to measure grip strength in arthritic rats as established by Sakuma and colleagues (Sakuma et al. 2001) (Fig. 7). Rats were sited on a wire mesh grid (size: 560 mm ¥ 400 mm, mesh: 15 mm, diameter of wire: 1 mm) (built by Feinmechanik UK Essen). Grip strength was measured by attaching the tail of the rat to a spring balance (Pesola Switzerland; calibrated ranges from 0–500 g or 0–2000 g). Rats have the reflex to grasp the wire mesh in order to resist the pulling force. The maximum force required for the traction was measured and documented. The first test was performed before the occurrence of symptoms at day 7 and a second one in the acute phase of arthritis at day 20. Of note, for ethical reasons no grip strength test was performed on the last experimental day because of stiffness and severe pain.

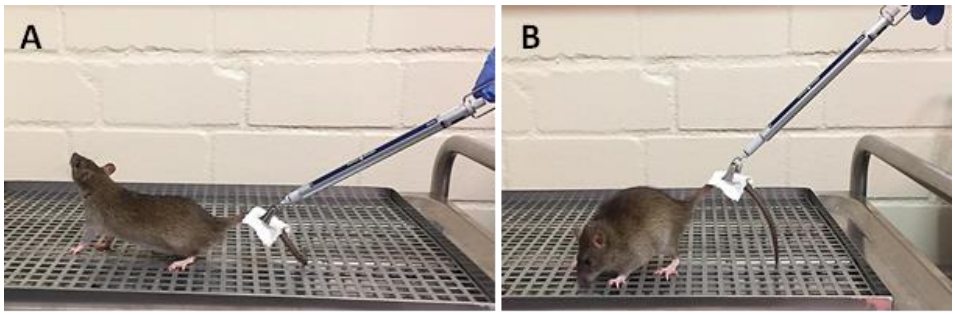


Fig. 7: Grip strength test.
Technique to measure grip strength in rats during CIA. Rat was placed on the grid on day 7 and 20. **(A)** The tail of the rat was connected to a spring balance and **(B)** pulled while maximum force required for traction was recorded.

3.10. Immunological Assays

3.10.1. Histological score analyses

For histological analyses and scoring, hind and front paws were collected and fixed for 24-72 h with a neutral buffered solution containing 3.7% formalin and subsequently decalcified in a 14% EDTA (Sigma, Deisenhofen, Germany). Afterward paws were washed with PBS (Gibco®, Life TechnologiesTM, Carlesbad, USA) and decalcified with RDO rapid decalcifier for 5 days (Medite, Burgdorf, Germany). When bones were drained, paws were washed again and dehydrated in 20% sucrose for at least one day. All paws were embedded in Tissue Tek O.T.C Medium (Sakura Finetek, Leiden, Netherlands), frozen in liquid nitrogen and stored in - 80° C.

Histological staining was done in Regensburg (University Hospital of Regensburg; Lab Neuro Endocrino Immunology; Department of Internal Medicine I). To do so 10-12 µm thick sections were cut using a freezing microtome (Leica, Germany). After one hour of air-drying and subsequent rehydration in PBS, hematoxylin–eosin (HE, both Sigma Germany) staining was performed to evaluate arthritis signs in the joints. Hind and front paws per rat were scored separately in a blinded manner and the mean score was calculated for each animal as follows: Invasion of immune cells into the articular cavity of joints (0-4 points), invasion of immune cells into joint adjacent tissues (0-4 points), erosions of cortical bone next to the periosteum and inflammation of periosteum (0-4 points), erosions in articular and subchondral bone (0-4 points) and degradation of articular cartilage (0-4 points). Points were summed per extremity and the mean was calculated for each animal, resulting in a maximum histological arthritis score of 20.

3.10.2. Splenocyte isolation and stimulation

All spleens were collected in cold HBSS (1 x Hank's Balanced alt Solution, Gibco®, Life TechnologiesTM, Carlesbad, USA) and disrupted separately with a 20 ml syringe plunger in a Petri-dish containing cold HBSS. Isolated splenocytes then were transferred into Falcon tubes and erythrocytes were lysed using diluted BD Pharm LyseTM lysing solution (BD Pharmingen, Heidelberg, Germany). Afterwards splenocytes were washed in cell culture medium (RPMI + 10 % FBS + 50 µg/ml gentamycin; Gibco Gibco®, Life Technologies TM, Carlesbad, USA) and filtered through a 70 µm nylon cell strainer (Greiner Bio-One; Frickenhausen, Germany). To elucidate the cell concentration, all probes were measured with an automatic animal cell counter (Vet abc; Medical Solution, Steinhausen, Switzerland). Splenocytes were adjusted to a final concentration of 5 x 10^6 cells/ml. To measure IL-2, IFN-γ and TNF-α cytokine production in the supernatant, cells were stimulated in 96-well flat bottom tissue culture plates with 1 µg/ml mouse anti-rat CD3 monoclonal antibody (clone:G4.18, BD Pharmingen) for 48 hours under 5 % CO_2 and 37°C. Additionally, splenocytes were adjusted to a second concentration of 2.5 x 10^6 cells/ml and stimulated in the same incubator for only 4 h with 1 µg/ml mouse anti-rat CD3 monoclonal antibody (see above) in order to isolate specific ribonucleic acid (RNA) for expression analysis.

3.10.3. IL-2, IFN-γ, IL-6 and TNF-α protein production (ELISA)

Cytokine production of IL-2, IFN-γ, IL-6 and TNF-α supernatant from stimulated splenocytes was measured using quantitative sandwich enzyme immunoassay technique (Quantikine®ELISA Rat IL-2, R&D systems, Minneapolis, USA; ELISA Rat IFN-γ,BioLegend, San Diego, USA; ELISA Rat IL-6, BioLegend, San Diego, USA; ELISA MAX rat TNF-α, BioLegend San Diego, USA) according to the manufacturer's instructions. Using Fluostar OPTIMA Microplate Readers (BMG Labtech, Offenbach, Germany) optical density of each well was calculated. Absolute cytokine concentrations were calculated using a log-log curve-fit standard curve.

3.10.4. mRNA expression analyses

For analyses of specific RNA, total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA

concentrations were spectrometrically determined in the wavelength of 260 nm with the BioPhotometer (Eppendorf, Hamburg, Germany). For transcription of single-stranded complementary deoxyribonucleic acid (cDNA), High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) was used. Real-time quantitative polymerase chain reaction (PCR) was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) using Taqman gene expression assays (Applied Biosytems).The mRNA expression levels of IL-2 (Rn00587673_m1) and IFY-γ (RN00594078_m1) were analyzed using β-actin as reference gen.

3.10.5. Statistical analyses

Statistical analyses were performed with Sigma Plot (Version 12.3, Systat Software San Jose, CA, USA) and Graph Pad Prism (Version 5, Graph Pad Software, San Diego, CA, USA). Normality of residuals was examined using the Shapiro-Wilk test and data were log-transformed when necessary. The descriptive statistics are based on means and variance, indicated by the standard error of the mean (±SEM). The level of significance was set at $p < 0.05$. Behavioral data (acquisition and retrieval of CTA) were subjected to two-way analysis of variance (ANOVA) with Group (treatment) as one factor, and Time (days) as a within subjects factor. Post hoc comparisons were performed using Bonferroni's corrections. Analyzes of cytokine production were performed using one-way ANOVA, with four-group comparisons. Bonferroni correction was used for post hoc comparisons after statistically significant effects in the one way ANOVA Data in experiments of PE, LI and Arthritis two were combined from two independent experiments and evaluated.

Differences in the clinical arthritis score were evaluated using two-way RM ANOVA followed by Student–Newman–Keuls post-hoc test and additionally by one-way ANOVA of area under the curve with Bonferroni-corrected post-hoc tests. Histological arthritis scores were analyzed by one-way ANOVA followed by a multiple comparison according to Bonferroni-corrected post hoc tests. Correlation analyses between histological and clinical arthritis score were performed with the Pearson correlation coefficient. Animal numbers varied throughout the arthritis experiments for ethical reasons.

4. Results

4.1. UCS pre-exposure in conditioned immunosuppression with CsA

During the first presentation of saccharin, all rats showed a decrease in fluid consumption due to the expected neophobic effect (Bermudez-Rattoni 2004). The UCS (CsA) treatment 3 times prior to conditioning led to a diminished CTA during acquisition. ANOVA showed significant effects in group ($F(3,124) = 4.0$; $p < 0.05$) and time ($F(2,124) = 13.4$; $p < 0.001$). Bonferroni-corrected post hoc tests demonstrated that pre-treated animals in *PE, CS0* and *US* groups had a significantly weaker taste avoidance compared to the *NPE* group on acquisition day 3 (Fig. 8A). At the time of retrieval, both control groups (*US* and *CS0*) displayed fluid consumption levels similar to those during the water deprivation phase For retrieval, ANOVA revealed a main effect of group ($F(3,124) = 19.3$; $p < 0.001$), a main effect of time ($F(2,124) = 24.9$; $p < 0.001$) and group time interaction ($F(6,124) = 12.2$; $p < 0.001$). In contrast conditioned animals (*NPE* and *PE*) which received saccharin during retrieval Bonferroni-corrected post hoc test showed a pronounced CTA with significantly reduced fluid intake in the PE group during the 1st and 2nd retrieval days ($p < 0.001$) (Fig. 8A).

In parallel to CTA immunological assays revealed that rats pre-exposed to the UCS prior to conditioning (*PE* group) still displayed a significant conditioned suppression of anti-CD3-stimulated IL-2 production, as seen in the *NPE* and *US* groups (ANOVA group effect IL-2 $F(3,57) = 8.1$; $p < 0.001$). Furthermore Bonferroni-corrected post hoc tests revealed significantly suppressed IL-2 levels in the groups *PE*, *NPE* and *US* compared to the *CS0* group (all $p < 0.05$). Accordingly Pearson's correlation indicated no interaction between the expression of IL-2 and the CTA fluid intake rate (two-tailed t-test, $p = 0.0691$; R square = 0.1172) (Fig. 8B).

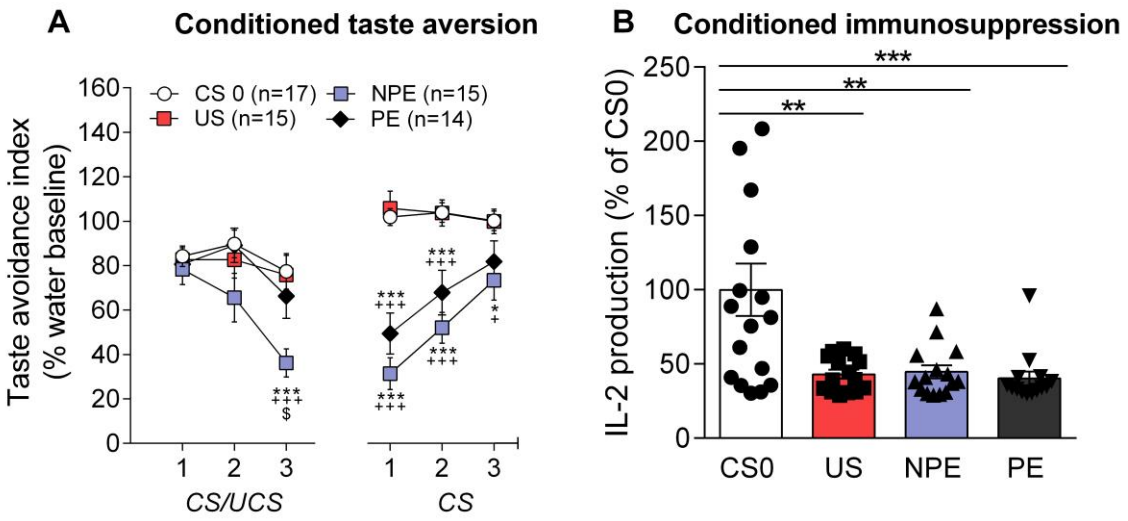


Fig. 8: UCS pre-exposure.
(A) Conditioned taste avoidance during acquisition and retrieval (% waterbaseline). NPE animals showed significant differences during third acquisition day and entire retrieval to *CS0* group (Crosses +++p < 0.001, ++p < 0.01,+p < 0.05)); significant differences to *US* group (Asterisks ***p < 0.001, **p < 0.01,*p < 0.05)) and significantly differences compared to the *PE* group. (Dollar $p < 0.05) Furthermore animals of the *PE* group had a significant diminishes fluid consumption compared to *US* (asterisks) and *CS0* (crosses) animals during retrieval (Bonferroni-corrected post hoc tests; n = 15–17/group). Data are shown as mean ± SEM. **(B)** IL-2 production of spleen cells stimulated 48h analyzed after the third retrieval trial. The pharmacological control (*US*), the conditioned group (*NPE*), and the conditioned group pre-exposed to CsA (*PE)* showed a significantly diminished IL-2 production compared to the conditioned, but not evoked group (*CS0*); Bonferroni-corrected post hoc tests;(***p < 0.001, **p < 0.01; n = 14–17/group). Data are given as mean ± SEM and are shown as percentage changes from CS0 controls**.**

4.2. Latent inhibition in conditioned immunosuppression.

Due to pre-exposure of the CS saccharin only the *NLI* group (not pre-exposed) demonstrated a neophobic response to this sweet solution on the 1st acquisition trial (Fig. 9A). For acquisition ANOVA revealed a main effect of group ($F(3,134) = 28.8$; $p < 0.001$) and a main effect of time ($F(2,134) = 188.8$; $p < 0.001$). Though, CS pre-exposure prior to behaviorally conditioning did not block or diminish CTA learning in general but resulted in a weaker taste avoidance level (in *CS0, US* and *LI* groups compared to the *NLI* group on all three acquisition days (Bonferroni-corrected post hoc tests $p < 0.05$). During retrieval, ANOVA demonstrated a effect of group ($F(3,132) = 72.7$; $p < 0.001$); a effect of time ($F(2,132) = 18.6$; $p < 0.001$) and group time interaction ($F(6,132) = 6.1$; $p < 0.001$). Though, rats that were not pre-exposed to the sweet solution showed a stronger CTA during retrieval. Even though an accelerated extinction was detectable in the *LI* group compared to the not CS pre-exposed conditioned rats (*NLI*) over three days. Bonferroni-corrected post hoc tests showed significantly diminished saccharin consumption in the *LI* group compared to

CS0 and *US* groups ($p < 0.001$) (Fig. 9A). During retrieval all control groups (*US* and *CS0*) consumed the same amount of fluid as during the water deprivation phase.

IL-2 productions of anti-CD3-stimulated splenocytes were analyzed after the 3rd unreinforced re-exposure to the CS. In line with results from UCS pre-exposure (see 4.1) ANOVA revealed main effect off group for IL-2 production ($F(3,58) = 7.2$; $p < 0.001$). Pre-treatment with saccharin (CS) did not affect the behaviorally conditioned immunosuppression. In both conditioned groups (*NLI* and *LI*), as well as in pharmacological controls significantly diminished IL-2 production compared to the control group CS0 was measurable (Bonferroni-corrected post hoc tests *CS0* vs *LI, NLI, US*; all $p < 0.05$) (Fig. 9B). Correlation analyses also showed no interaction between IL-2 protein expression and the CTA fluid intake (two tailed t-test, $p = 0.1527$; R square = 0.07425). For IL-2 mRNA production ANOVA ($F(3,61) = 24.39$; $p < 0.001$) revealed main effects between groups. IL-2 production on the mRNA level was only significantly diminished in the pharmacologic *US* group (Bonferroni-corrected post hoc tests *US* vs *CS0, NLI, LI* all $p < 0.05$) (Fig. 9C). Additionally, ANOVA ($F(3,60) = 11.39$; $p < 0.001$) showed a main effect of group for the IFN-γ mRNA expression. However, no effect on conditioned immunosuppression was detectable in the *LI* and *NLI* groups. IFN-γ expression was only significantly diminishing via pharmacological treatment in the *US* group (Bonferroni-corrected post hoc tests *US* vs *CS0, NLI, LI* all $p < 0.05$) (Fig. 9D).

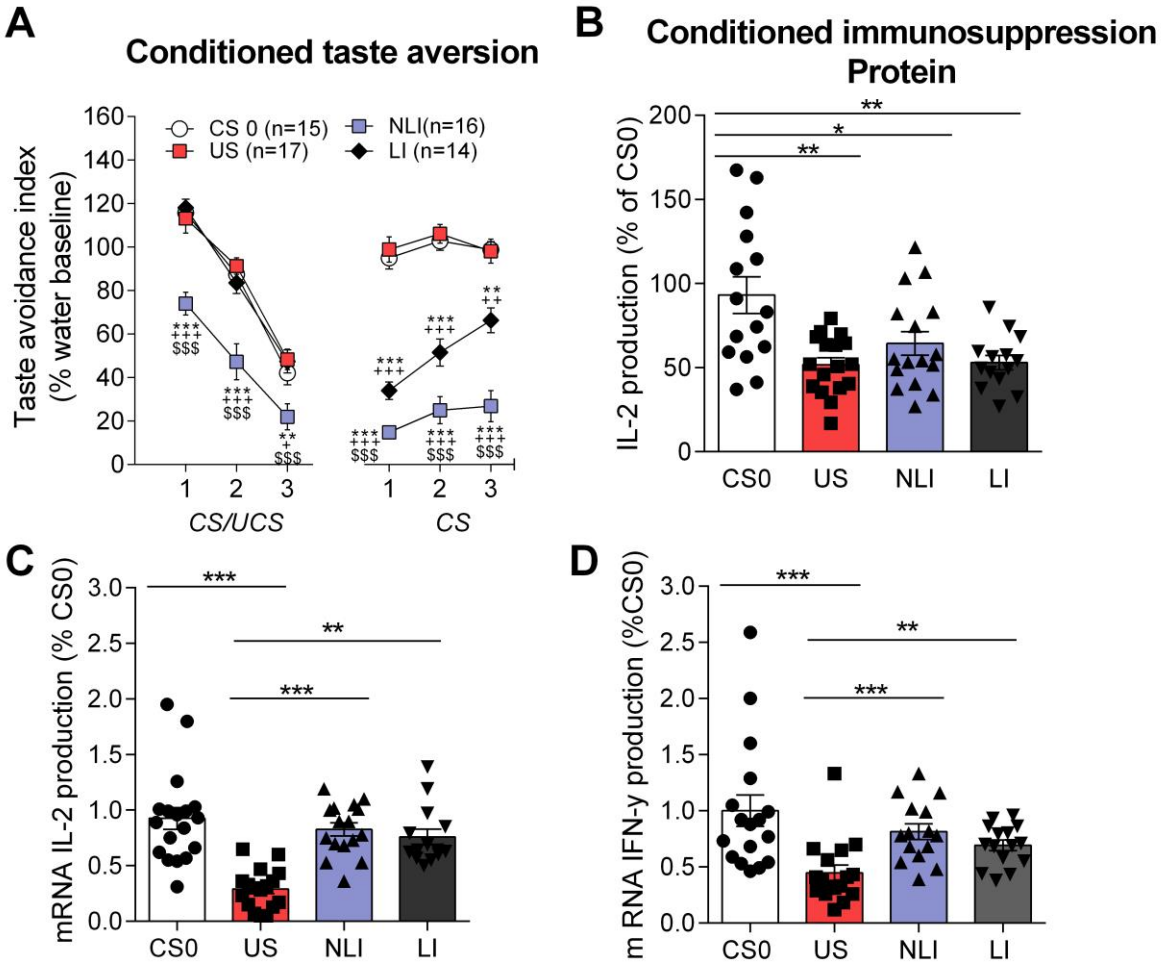


Fig. 9: Latent inhibition.
(A) Conditioned taste avoidance during acquisition and retrieval (% waterbaseline). *NLI* animals showed significant differences over the entire conditioning process (acquisition and retrieval) to *CS0* group (Crosses (+++p < 0.001, ++p < 0.01,+p < 0.05); significant differences to *US* group (Asterisks (***p < 0.001, **p < 0.01,*p < 0.05)) and significant differences compared to the *PE* group (Dollar $p < 0.05). Furthermore, animals of the *LI* group had a significantly diminished fluid consumption compared to *US* (asterisks) and *CS0* (crosses) animals during retrieval (Bonferroni-corrected post hoc tests; n = 15–17/group). Data are shown as mean ± SEM. **(B)** IL-2 production of anti-CD3 stimulated lymphocytes from the spleen analyzed after the third retrieval trial. All three groups (*NLI, LI* and *US*) presented a significantly reduced IL-2 production compared to the control group *CS0* (Bonferroni-corrected post hoc tests; **p < 0.01; *p < 0.05; n = 14–17/group) **(C-D)** IL-2 and IFN-y mRNA production of 4h anti-CD3 stimulated splenocytes. *US* group showed a significant difference compared to *CS0, NLI* and *LI.* No conditioned immunosuppression was detectable on the mRNA level. Bonferroni-corrected post hoc tests; (***p < 0.001; ** p < 0.01; n = 14–18/group). Data are given as mean ± SEM and are shown as percentage changes from CS0 controls.

4.3. Conditioned immunosuppression in a CIA rat model

4.3.1. CsA effects on CIA in *Dark Agouti* rats

Prior to the first experiments, effects of the immunosuppressive drug CsA on the CIA model were analyzed. Daily i. p. injections of 20 mg/kg CsA demonstrated a significant reduction of clinical arthritis symptoms from day 1 to 25 after CIA induction. ANOVA reveled a significant interaction between the factors time ($F(26,132) = 127.30$; $p < 0.001$) group and a significant interaction between the factors group and time ($F(26,312) = 6.87$; $p < 0.001$). Bonferroni-corrected post-hoc comparisons revealed significant differences in clinical arthritis scores between control- and CsA-treated groups ($p < 0.001$) (Fig.10).

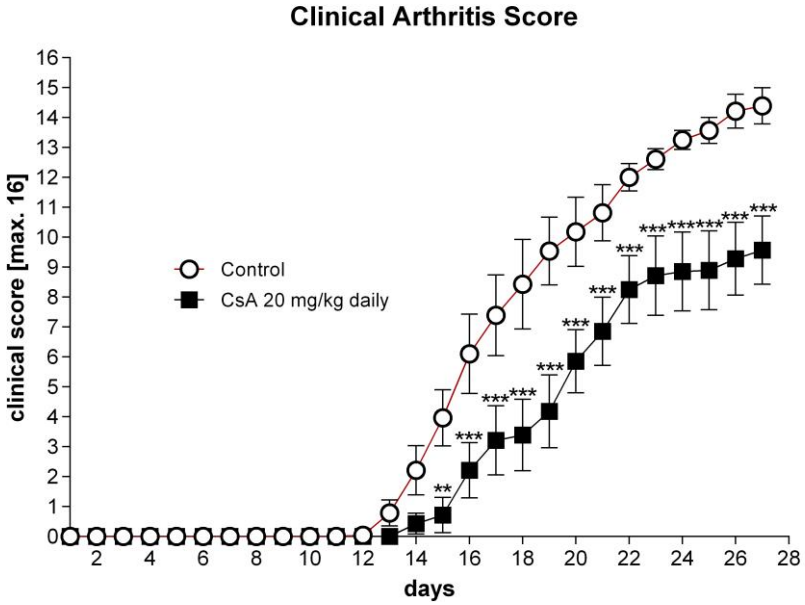


Fig. 10: Clinical arthritis scores from day 1 to 28, as assessed in a pilot experiment.
Clinical arthritis scores from day 1 to 27. Significant differences between control- and CsA-treated groups (Bonferroni-corrected; Asterisks (***p < 0.001, **p < 0.01); n = 7/group)

4.3.2. Effects of conditioned immunosuppression on retrieval day 1 to 28 after immunization

In a first experiment (A) the time point of the retrieval phase started one day after disease induction and before the emergence of the first arthritis symptoms In analyses of the CTA, all groups (*CSlow, USlow, US* and *CS0*) showed a continuous decrease in saccharin consumption during acquisition (ANOVA time $F(2,64) = 29.64$

p < 0.001) with no evidence of group differences. During retrieval ANOVA revealed significant CTA in the *CSlow* group at all time points (group F(3,896) = 19.6 p < 0.001; time F(28,896) = 32.43; p < 0.001; group time interaction F(84, 896) = 2.01; p < 0.001). Bonferroni-corrected post hoc tests showed a significant difference in fluid consumption between the conditioned group *CSlow* compared to all three control groups (*USlow, US* and *CS0*) (p < 0.001). Due to dehydration, which occurred during the isoflurane anesthesia, all animals increased fluid consumption one day after the osmotic pump operation (day 8). Furthermore, all animals consumed less fluid by the appearance of the first symptoms (day 14-15) (Fig 11). A decreased fluid consumption was due to the progression of disease and the additionally given water-mashed food in the cages.

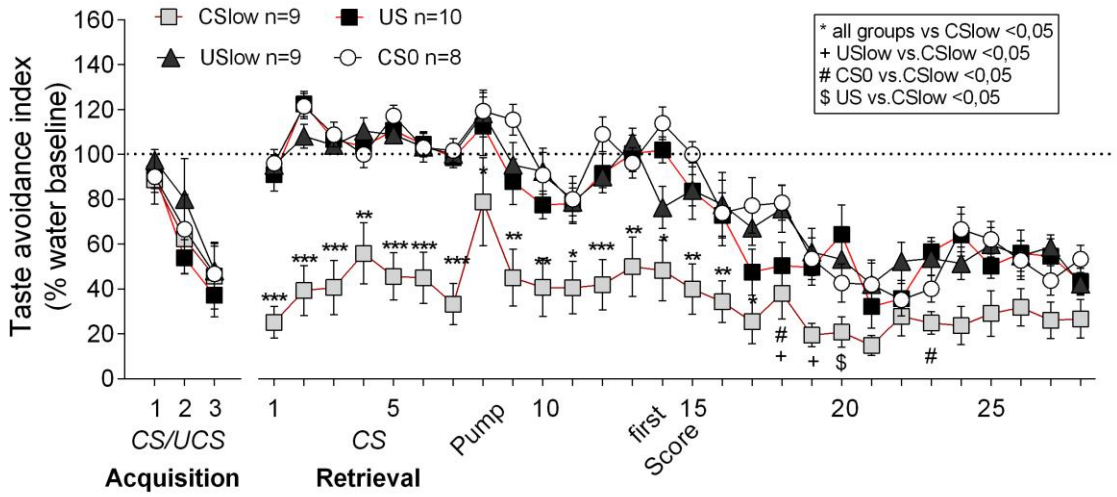


Fig. 11: Conditioned taste avoidance during acquisition and retrieval day 1-28.
After three acquisition days, robust taste avoidance was observed in all animals. During retrieval, CTA of *CSlow* animals was prolonged over 28 days. *CSlow* animals showed significant differences compared to all groups (Asterisks (***p < 0.001, **p < 0.01,*p < 0.05)); significant differences between *CSlow* and *USlow* group are indicated as crosses (+p < 0.05); significant difference between *CSlow* to *CS0* are indicated as hash tag (#p < 0.05). Significant differences between *CSlow* and *US* are shown as dollar ($p < 0.05). (Bonferroni's corrections; n = 8-10/group. Data are means ± SEM; % waterbaseline).

All animals were scored daily regarding CIA-induced clinical symptoms (please see section 3.8). Frist symptoms were measurable 14 days after immunization in the *CS0* control group. Only in the acute phase of arthritis, a significantly milder score was detectable in the pharmacological treated group (ANOVA; time F(17,612) = 309.39; p < 0.001; group time interaction F(51,612) = 1.57; p < 0.05). Student-Newman-Keuls post hoc showed a lower score in the pharmacologically treated (*US*) compared to

the control group (*CS0)*. Over the entire time, arthritis symptoms gradually increased in all groups. At day 28, no significant differences between groups were observed. Additionally, ANOVA of area under the curve revealed no differences between groups (ANOVA; group $F(3,31) = 0.6$; $p > 0.05$) (Fig 12A). Of note, animals which lost more than 20% body weight were excluded from the experiment (Fig. 12B). Preliminary histological analyses of the affected hind paws in this experiment displayed a tendency of a milder outcome of conditioned immunosuppression compared to control groups (*USlow* and *CS0*) (Fig. 12C).

For further evaluation of arthritis severity, a grip strength test was performed twice in all animals. The maximum traction strength is considered to reflect the total grip strength of fore and hind paws. The first test was performed at day 7 and a second one in the acute phase of arthritis at day 20. Analyses of grip strength revealed no significant group differences at either time point. Reductions in strength from day 7 to 20 observed in all animals were due to an impaired use of rats' affected hind paws to grip the wire grid after the development of arthritis (Fig 12E&F).

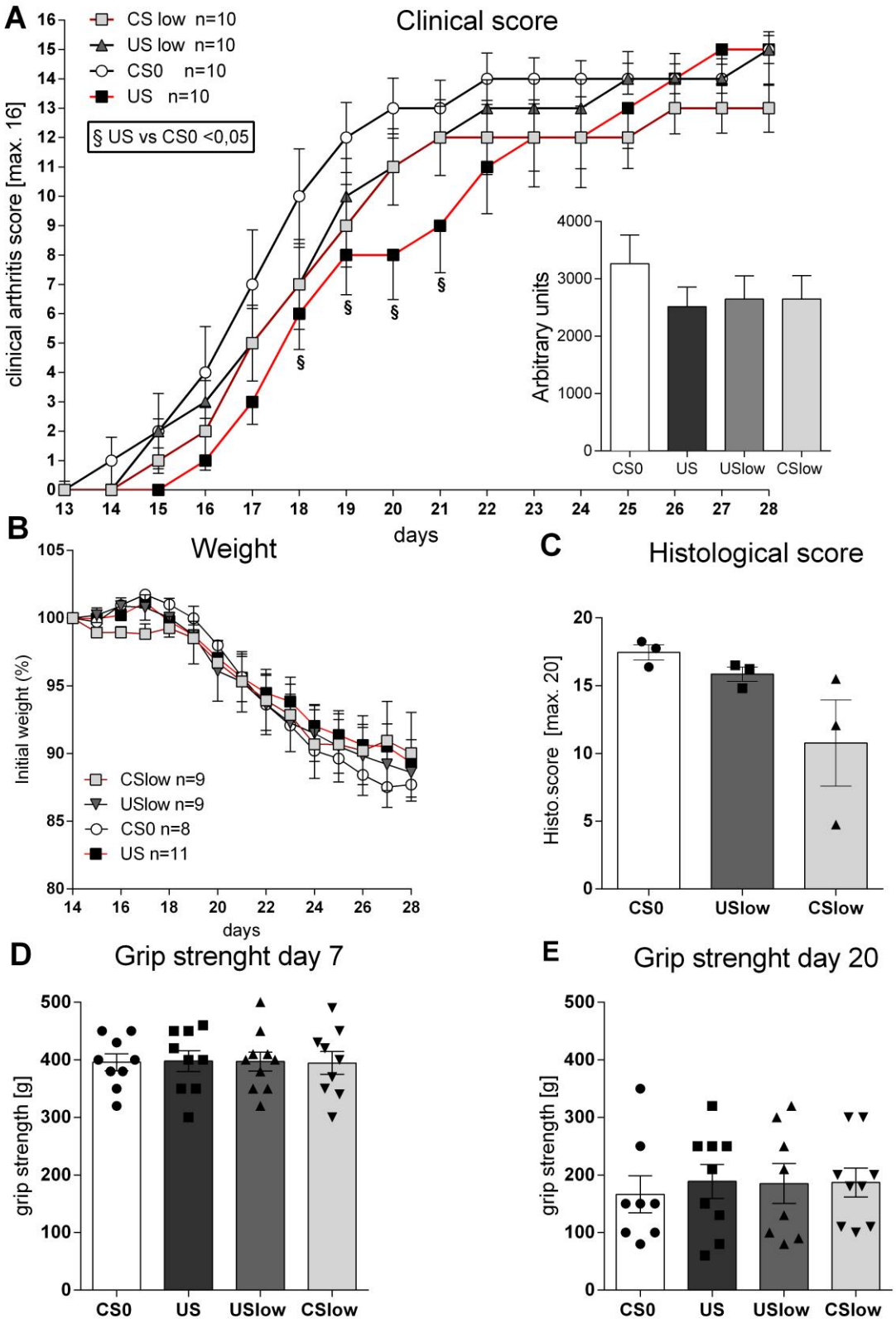


Fig. 12: Clinical readout parameter: Reconsolidation of conditioned immunosuppression day 1-28.
(A) Clinical arthritis score; significant differences in the acute phase (18-21) between the full dose pharmacologically treated (*US*) compared to the control group (CS0). No differences in the clinical outcome were seen at day 28 (Student-Newman-Keuls post-hoc test (Dollar ($ $p < 0.05$; n = 10/group)). **(B)** Weight loss day 12-28 (Data are shown as percentage of the initial weight at day 12 (n=8-11/group)). (C) Histological scores on day 28; no significant differences between groups (preliminary data/n=3). **(D-E)** Evaluation of grip strength on days 7 and 20. No significant differences between groups (All data are given as mean ± SEM).

To assess conditioned immunosuppression, protein production and mRNA expression of IL-2, IFN-γ and TNF-α were analysed at day 28 after 48 h anti-CD3 stimulation. ANOVA revealed a significant difference between the treatment group for IL-2 protein production ($F(3,27) = 3.22$; $p < 0.05$); IFN-γ protein production ($F(3,31) = 3.60$; $p < 0.05$) and TNF-α protein production; ($F(3,31) = 13.29$; $p < 0.01$) as well as for mRNA expression of IFN-γ; ($F(3,33) = 3.20$; $p < 0.05$). In accordance with the pharmacological treatment, Bonferroni-corrected post hoc tests indicated that animals in the *US* group had a significantly reduced IL-2, IFN-γ and TNF-α cytokine protein production, as well as a significantly diminished mRNA expression of IFN-γ, compared to *CS0* animals (*US* vs.*CS0*: IL-2 protein $p < 0.01$; IFN-γ protein $p < 0.05$; TNF-α protein $p < 0.001$ mRNA IFN-γ $p < 0.05$). IL-2, IFN-γ and TNF-α protein and mRNA production is presented as percent of *CS0* ($p < 0.05$) (Fig. 13A-D).

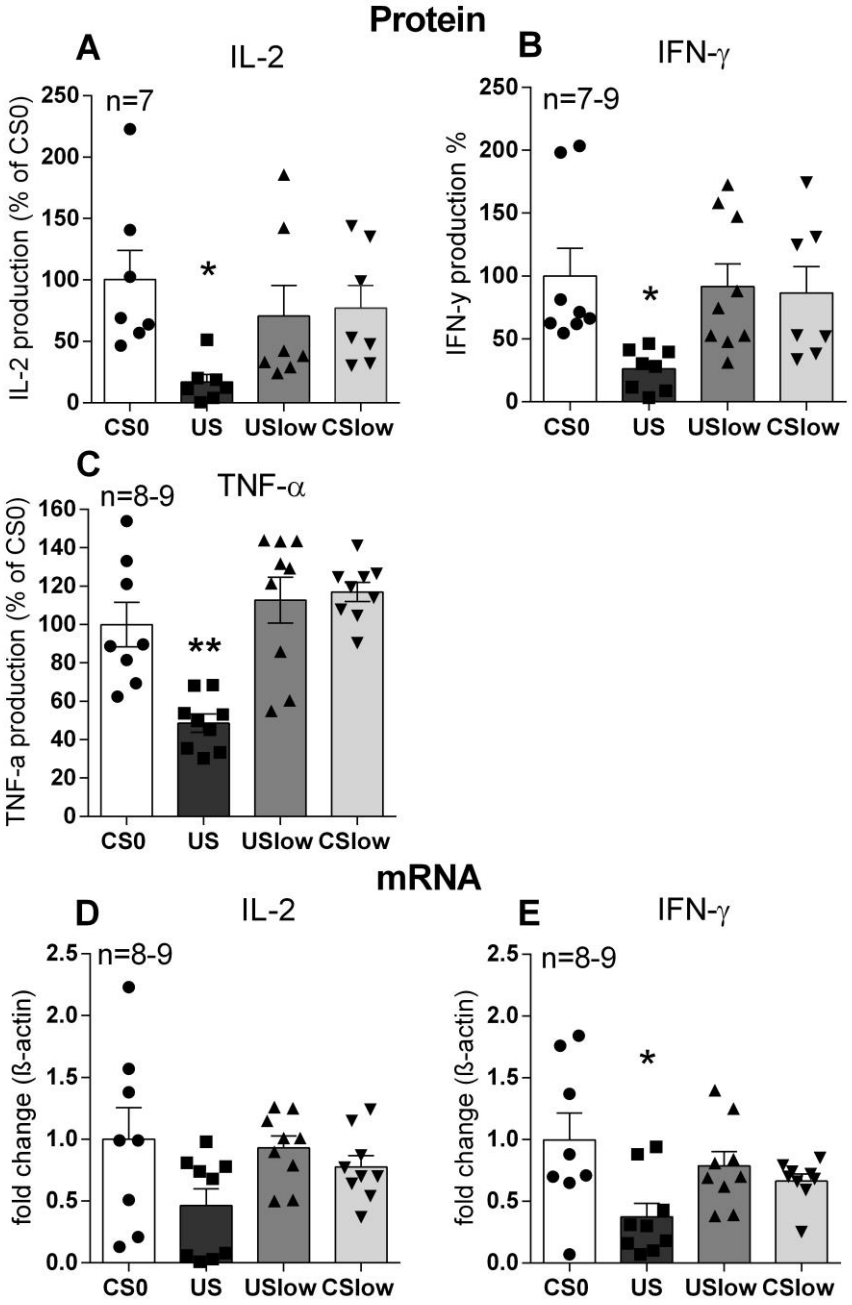


Fig. 13: Cytokine production and mRNA expression induced by 48h anti-CD3 stimulated spleenocytes on day 28.
(A) IL-2 protein production: *US* showed a diminished IL-2 production compared to *CS0*; **(B)** IFN-γ protein; pharmacological immunosuppression of *US* animals compared to *CS0.* **(C)** TNF-α protein production; significant reduced TNF-α production compared to *CS0.* Protein production is shown as percent of *CS0***. (D-E)** relative IL-2 mRNA and IFN-γ expression shown as fold change of β actin mRNA; One way ANOVA; (Bonferroni-corrected post hoc tests; **p < 0.01;*p < 0.05 *US* vs *CS0* n = 7–9/group. Data are given as mean ± SEM)

4.3.3. Effects of conditioned immunosuppression on retrieval day 14 to 28 after immunization

In a second arthritis experiment (B) the time point of retrieval was shifted to the day when symptoms occurred (day 14) to establish a more clinically relevant model. Data from two experiments were merged. On the behavioral level all animals displayed a neophobic response to the new gustatory stimulus as evident from a lower fluid consumption compared to water baseline on the first acquisition trial. During three acquisition trials ANOVA only demonstrated a main effect of time ($F(2,100) = 56.15$; $p < 0.001$). No significant differences between all groups were detectable. Though, a decreased fluid consumption on the third CS-UCS pairing was due to the associative learning over time. For retrieval ANOVA revealed a main effect of group ($F(3,599) = 38.51$; $p < 0.001$), a main effect of time ($F(12,599) = 18.01$; $p < 0.001$), and a group-time interaction ($F(36,599) = 1.76$; $p < 0.01$). Bonferroni-corrected post hoc analysis showed a significantly lower fluid consumption in the conditioned group CSlow compared control groups (*USlow, US* and *CS0*) at all time points ($p < 0.05$) (Fig. 14A). To ensure that dehydration had no effect on *CSlow* animals, the mean water intake in the evening session during retrieval was evaluated for one experiment. For water consumption, ANOVA revealed significantly differences in the factor group ($F(3,348) = 21.73$; $p < 0.001$) and time ($F(12,348) = 7.77$; $p < 0.001$). Bonferroni-corrected post hoc analysis demonstrated higher water consumption over time for *CSlow* animals compared to all other groups (*US, USlow* and CS0), This finding indicates that less fluid consumption in the morning was compensated by water intake in the evening (Fig.14B).

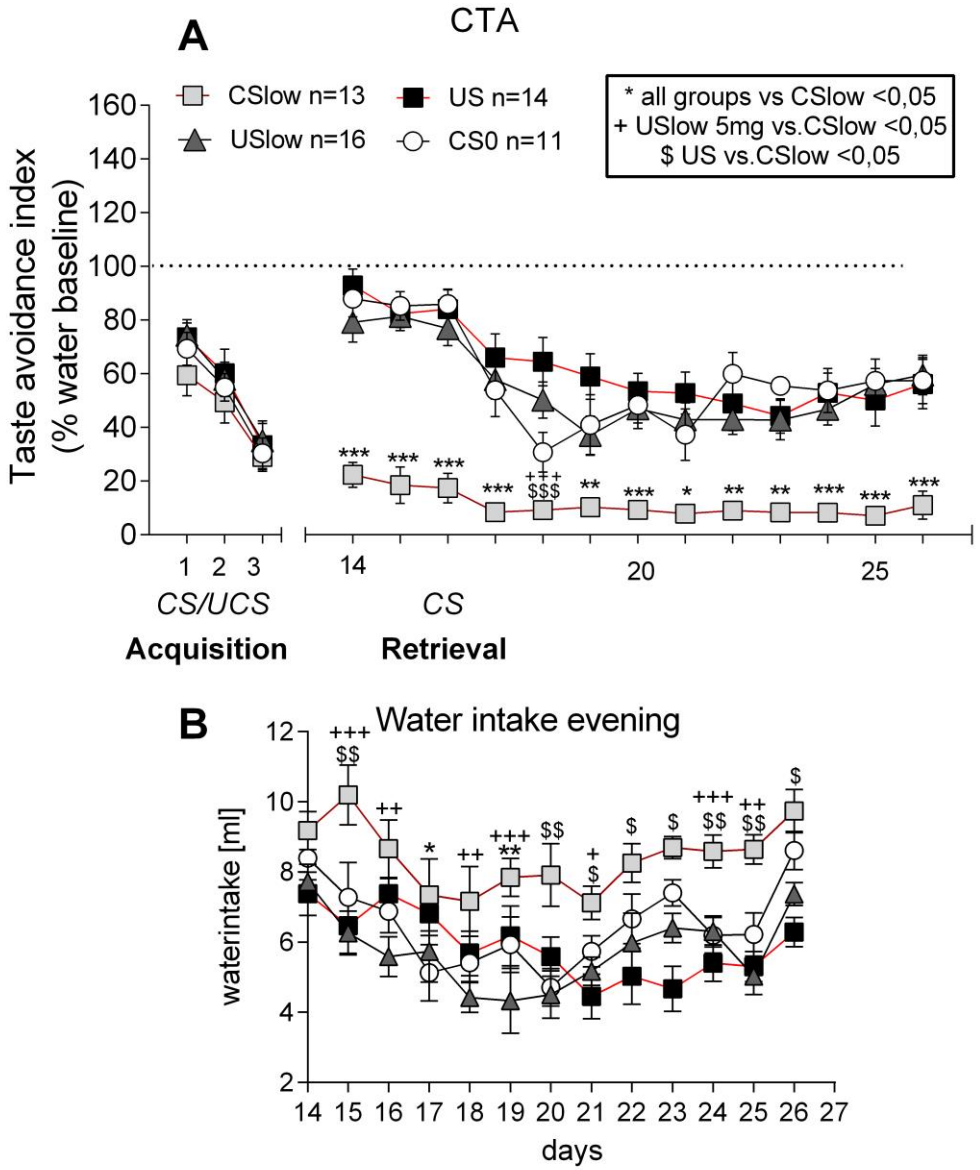


Fig. 14: Conditioned taste avoidance during acquisition and retrieval day 14-26.
(A) Neophobic response to saccharin (CS) was shown in all groups at day 1. During retrieval, animals of the conditioned groups (*CSlow*) still displayed pronounced taste avoidance. Fluid consumption did not differ between control groups *(USlow, US* and *CS0*). Asterisks (***p < 0.001, **p < 0.01,*p < 0.05); significant differences of CSlow animals compared to all groups. Crosses (+p < 0.05); significant differences of *CSlow* compared to the *USlow* group. Dollar ($p < 0.05); significant differences of *CSlow* compared to the *US* group. (Bonferroni's corrections; n = 11-16/group. Data are means ± SEM; %waterbaseline). **(B)** Water intake during retrieval in the evening drinking sessions. Animals of the conditioned (*CSlow* group) showed an increased fluid consumption compared to all other groups (*USlow, US* and *CS0*) Crosses (+++p < 0.001, ++p < 0.01,*+p < 0.05); significant differences of *CSlow* compared to the *USlow* group. Dollar ($p < 0.05); significant differences of *CSlow* compared to the *US* group (Asterisks (**p < 0.01,*p < 0.05); significant differences of *CSlow* animals compared to *CS0* group. (Bonferroni-corrected post hoc tests n=10/group).

For the clinical arthritis score ANOVA revealed a significant effect on time ($F(14,896) = 339.06$; $p < 0.001$), a significant effect on group ($F(3,896) = 5.02$; $p < 0.01$) and group time interaction ($F(42,896) = 2.28$; $p < 0.001$). Following immunization in all animals, DA rats developed severe clinical symptoms of arthritis (mean score 13),

which were first detected around day 14-15 and reached their maximal score around day 26 (Fig. 15A). Student-Newman-Keuls post hoc test showed significant differences between the pharmacologically treated *US* group and control groups (*USlow* and *CS0*) from the acute phase (day 20) to the end of the experiment ($p < 0.05$). Most importantly, conditioned rats (*CSlow*) demonstrated a significantly lower clinical score compared to control groups (*USlow* and *CS0*). At day 28, the effect of conditioned immunosuppression was reflected by a significantly milder outcome of the disease with 12 score points compared to (*CS0*) with 15 score points indicated. Furthermore ANOVA of area under the curve showed significant between-group differences (group ($F(3,64) = 5.31$; $p < 0.01$). Bonferroni-corrected post hoc tests indicated significant lower clinical arthritis scores between *US* and *CSlow* compared to control CS0. In parallel a marked decrease in body weight was observed with the incident of the first symptoms (Fig.15B). ANOVA revealed main effects on time ($F(14,700) = 278.44$; $p < 0.001$) and a group time interaction ($F(42,700) = 1.58$; $p < 0.05$); for full dose pharmacological treatment (20mg/kg). Full dose CsA had a positive effect on weight due to the milder score during the acute phase (Bonferroni-corrected post hoc tests ($p < 0.05$).

Grip strength investigations exhibit no differences between groups before the emergence of the first symptoms on day 7. In the acute phase (day 20), ANOVA indicated significant differences between groups ($F(3,31) = 20.79$; $p < 0.001$). Bonferroni post hoc test showed that the group with full-dose CsA-treatment (*US*) and the conditioning group (*CSlow*) had a better outcome in grip strength test compared to low-dose treated animals (*USlow)* and to the conditioned but not evoked control group (*CS0*) (Fig 15E-D). Pearson's correlation further indicated a negative association between grip strength and clinical arthritis score (two-tailed, $p < 0.001$; Fig.15C).

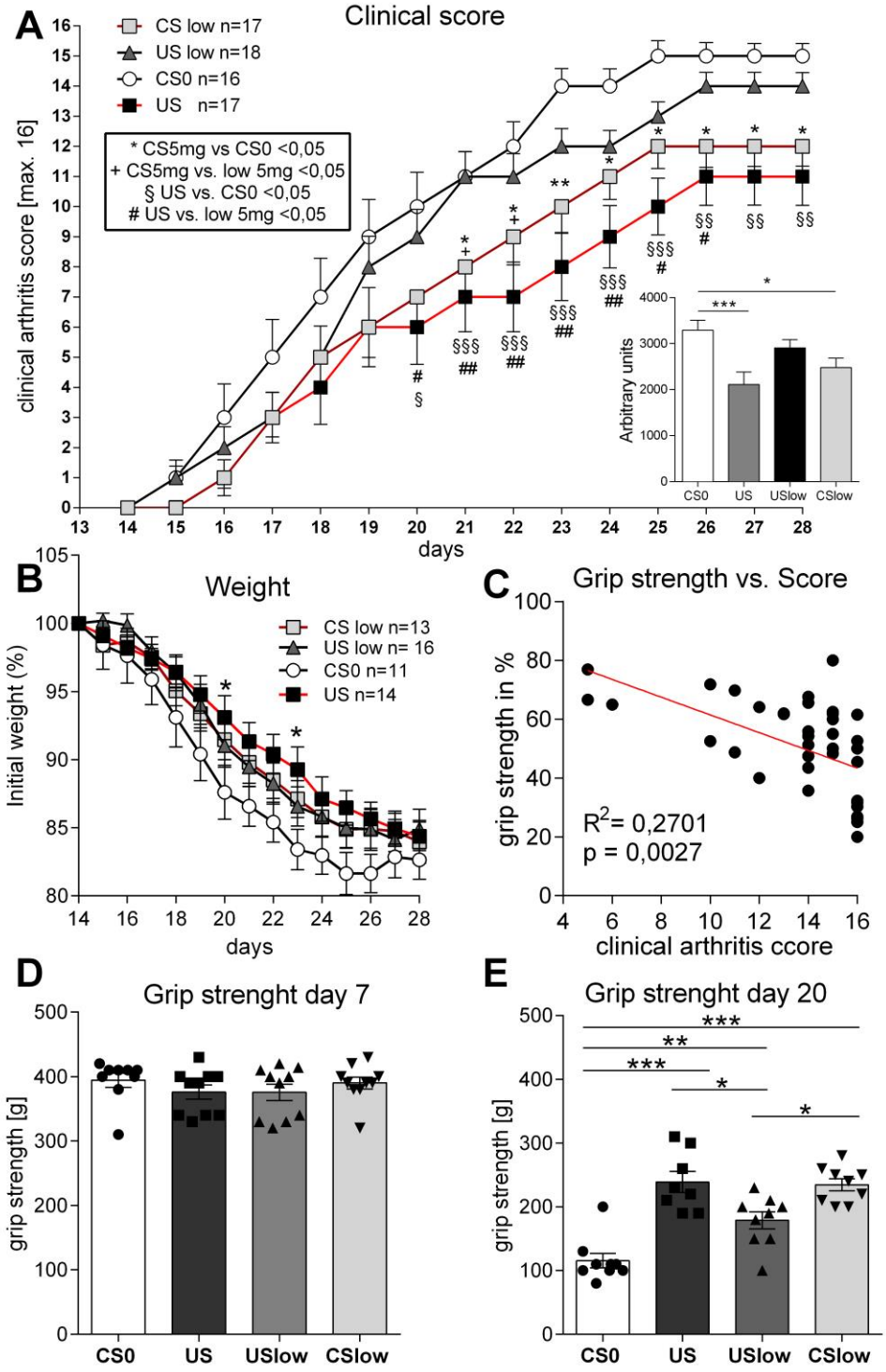


Fig. 15: Clinical readout parameters: Reconsolidation of conditioned immunosuppression day 14-26. **(A) Clinical arthritis score**; conditioned animals showed significantly milder clinical score compared to the *USlow* group (Crosses (+p < 0.05)) and *CS0* group (Asterisks, (**p < 0.01,*p < 0.05)); Full dose pharmacologically treated rats *US* had a improved outcome compared to *CS0* group (Paragraph (§§§p < 0.001, §§p < 0.01,§p < 0.05)) and *USlow* group (Hastag (###p < 0.001, ##p < 0.01,#p < 0.05)); Data are shown as mean ± SEM (Student-Newman-Keuls correction (p < 0.05; n = 16-18/group).**(B) Weight loss** day 14-28; least pronounced weight loss in *US* group (Data are shown as percentage of the initial weight on day 14 (n=11-16/group). **(C) Correlations analysis** of grip strength and clinical arthritis (n = 8/9 per group; *p < 0.001). **(D-E) Evaluation of grip strength**; grip strength did not differ between groups on day 7. At day 20 *CS0* animals showed decreased grip strength by 50% compared to day 7. Additionally, significant differences between *CSlow* vs.CS0 & *USlow; US* vs. *CS0* & *USlow* and *USlow* vs. *CS0* showed more strength of conditioned immunosuppression and full does treatment (Bonferroni corrected post hoc test; n = 8–10/group; ***p < 0.001;**p < 0.01,*p < 0.05; Data are shown as mean ± SEM).

We also examined histological changes that occurred in rats' hind and front paws at day 28 *ex vivo*. Consistent with changes in the clinical arthritis score ANOVA revealed significant differences between groups $F(2,27) = 9.04$; $p < 0.001$). The front paws of *CSlow* displayed significantly milder scores compared to *USlow* and *CS0* (Bonferroni-corrected post hoc tests $p < 0,05$). Due to the intensity of the CIA model, no significant group differences were observable in the hind paws. However, ANOVA ($F(2,21) = 8.45$ $p < 0.05$) analysis of the sum of front and hind paws (total rat) also indicated a significantly better outcome (*CSlow* vs *USlow* and *CS0*) (Bonferroni-corrected post hoc tests $p < 0,05$) (Fig 16A-C). Pearson's correlation furthermore indicated a positive association between total histological- and clinical arthritis scores (two-tailed, $p < 0.001$; Fig. 16F). Furthermore, detailed immunohistological evaluation of individual anatomical regions of the paw regions revealed less leukocyte infiltration, synovial hyperplasia and bone erosion in the front paws of the *CSlow* group compared to all control groups (ANOVA group $F(2,134) = 38.83$; $p < 0,001$ Bonferroni-corrected post hoc tests $p < 0,05$) (Fig 16D-E).

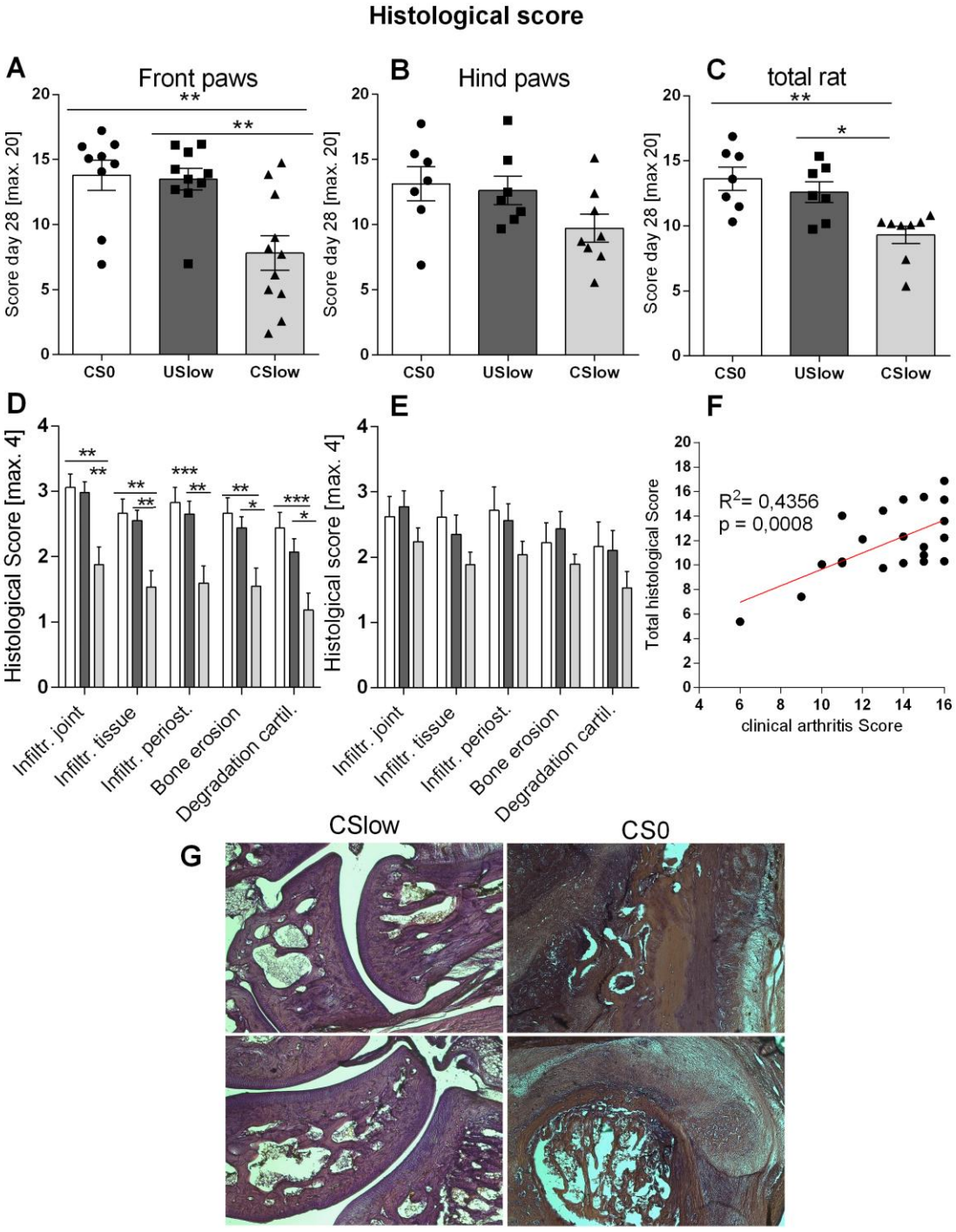


Fig. 16: Histologic changes in rats' front and hind paws after 28 days.
(A) Histological scores front paws, significant differences between conditioned *CSlow* animals compared to control groups (*USlow* and *CS0*) (n = 9-11/group). **(B) Histological scores hind paws** (max 20 score points) no significant differences between groups (n=7-8/group, histologic scoring on a 0–20 scale) **(C) Histological score total rat** (max 20 score points) differences between conditioned animals compared to Control groups *(USlow* and *CS0*) (n = 7-8/group) **(D-E) Histological changes per region**. (Infiltr.) Infiltration joint; Infiltration surrounding tissue; Infiltration/Erosion Periosteum (Periost.); Bone erosion joint; Degradation cartilage (cartil.) joint). All regions were evaluated on a scale from 0 to 4 (n = 9-11/group). **(A-E)** Data are shown as mean ± SEM ;(Bonferroni corrected post hoc test; Asterisks; ***p < 0.001;**p < 0.01,*p < 0.05) **(F) Correlation analysis** of histological and clinical scores (n = 8-9 per group; p < 0.05) **(G) Examples of histological evaluations** (hind paw). *CSlow*: Upper Left panel (metatarsus), lower left panel (ankle): *CS0*: Mild inflamed joint upper right panel (metatarsus), lower right panel (ankle): Heavy bone destruction and inflamed hyperplasia.

Lastly, immunological parameters were measured at the end of the experiment. For IFN-γ production ANOVA revealed main effect in between groups ($F(3,15) = 3.99$ $p < 0.05$). The pharmacologically induced immunosuppression (*US* group) was also detectable in the peripheral immune system reflected by a significant decrease in IFN-γ levels of CD-3 stimulated splenocytes compared to controls (CS0) (Bonferroni-corrected post hoc tests $p < 0.05$). For the proinflammatory cytokine TNF-α, no significant group differences were evident (Fig. 17).

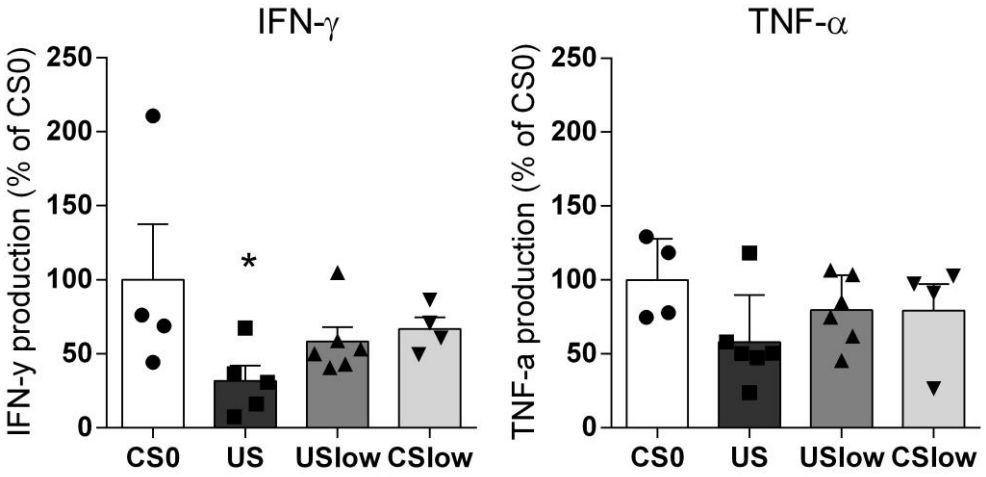


Fig. 17: Cytokine production of 48h anti-CD3 stimulated splenocytes on day 28.
(A) IFN-γ protein production shown as percent of *CS0*; pharmacologically induced immunosuppression of *US* animals compared to *CS0* (Asterisks * $p < 0,05$). **(C)** TNF-α protein production shown as percent of *CS0*. One way ANOVA, Bonferroni-corrected post hoc tests; $p < 0.05$; n = 4–6/group.

4.3.4. Effects of conditioned immunosuppression on retrieval at 4 score points

Given an improved outcome for CIA by starting retrieval with early disease state, a third experiment (C) was conducted, implementing a retrieval phase when animals already developed a score of 4 points, thereby testing a model of high clinical relevance for acute arthritis.

CTA data during acquisition did not differ from arthritis experiment A and B. Analyses of the taste avoidance data revealed a neophobic effect against saccharin during the first acquisition trial. Additionally, ANOVA ($F(2,76) = 35.231$; $p < 0.001$) revealed main effect on time shown by a significant decrease in fluid consumption during the 2nd and 3rd acquisition trial in all groups. During the individually initiated retrieval

phase, the animals conditioned with CsA 5mg/kg in combination with saccharin (*CSlow*) developed weaker taste avoidance compared to previous experiments. A significant inhibition in fluid consumption in the *CSlow* group was only observed by Bonferroni post hoc analyses on day one, two, and four due to the severity of symptoms (ANOVA for retrieval day 1-4 group $F(3,81) = 7.49$; $p < 0.001$). ANOVA analysis ($F(3,51) = 4.80$; $p > 0.05$.) for retrieval on day 5-8, revealed no significant difference in fluid consumption between groups. Importantly, numerous animals had to be excluded due to critical drop in weight or a clinical symptom score of over 15 points, yielding these analyses preliminary (Fig. 18).

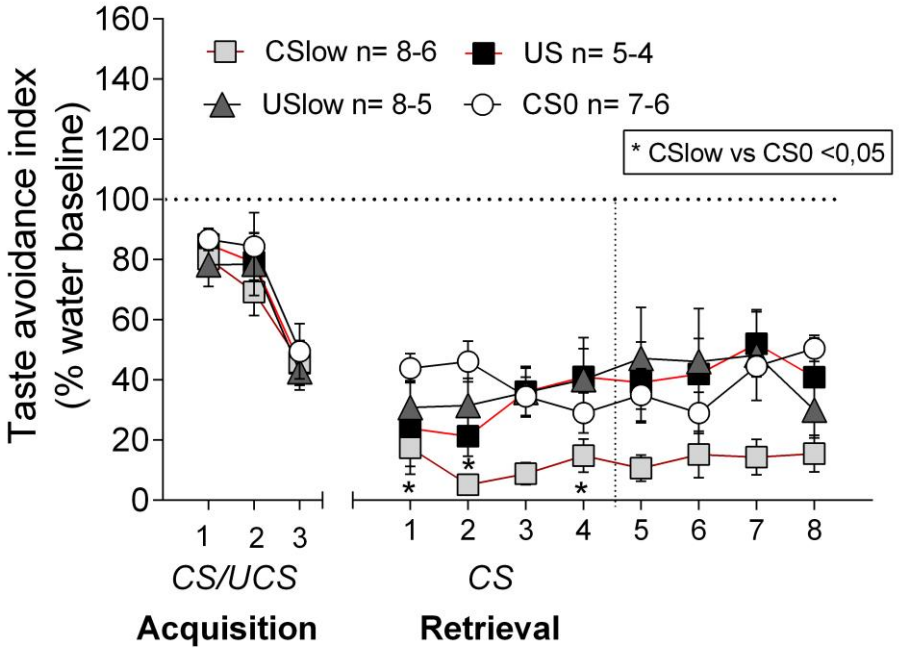


Fig. 18: Conditioned taste avoidance during acquisition and retrieval at 4 score points.
A neophobic response to the CS during the first acquisition was observed in all groups. After the 3rd acquisition trial all groups displayed a strong CTA. During retrieval animals of the *CSlow* group displayed pronounced taste avoidance, attenuating over time. Significantly differences fluid intake of *CSlow* animals compared to the *CS0* group is indicated as Asterisks (*p < 0.05); (Bonferroni-corrected post hoc tests; n = 15–17/group). Data are shown as mean ± SEM (% waterbaseline).

In this experiment, only 32 of 40 rats developed a CIA. Furthermore, retrieval started individually for each rat when reaching 4 score points. First symptoms occurred on day 14 and all rats significantly increased in severity of symptoms from day 14-28 (ANOVA time $F(14,364) = 164.60$; $p < 0.001$) (Fig. 19A). No variances in the clinical score were measurable between groups during the whole experiment. All animals demonstrated an intense weight loss over time (Fig. 19B).

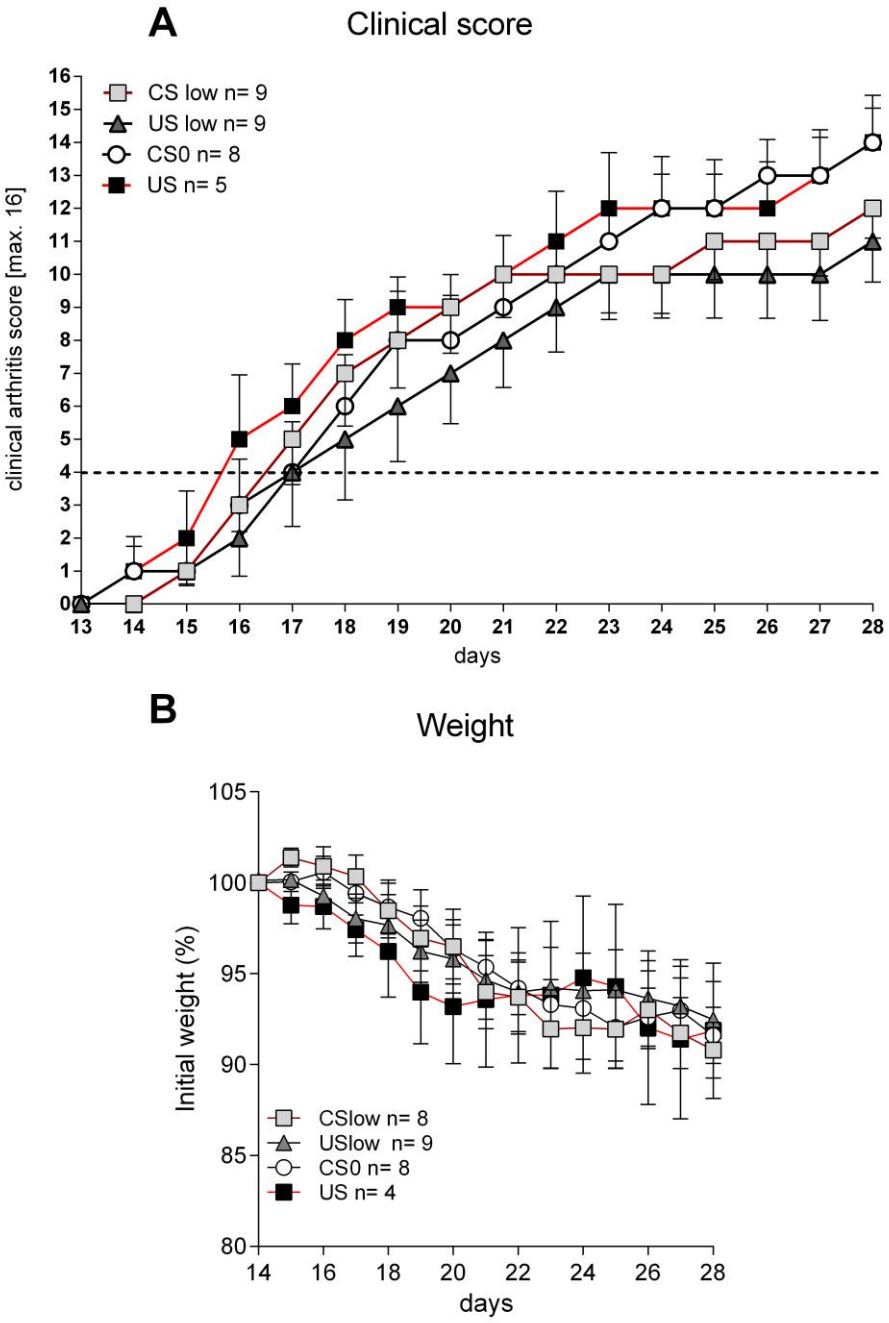


Fig. 19: Clinical readout parameter: Retrieval after reaching clinical scores of 4.
(A) Clinical arthritis score; individual beginning of retrieval (with 4 score points) showed no differences between groups. **(B)** Weight loss day 14-28. All rats started to lose weight with the occurrence of the first symptoms. No difference between groups were measurable (n = 4-8/group). Data are shown as mean ± SEM.

As expected, immunological data also revealed no differences between groups. We could not find any significant immunosuppression on protein levels. Likewise, neither IL-2 nor IFN-γ levels showed a significant decrease in cytokine production. Moreover, no significant differences between all groups were detectable (Fig. 20A&B).

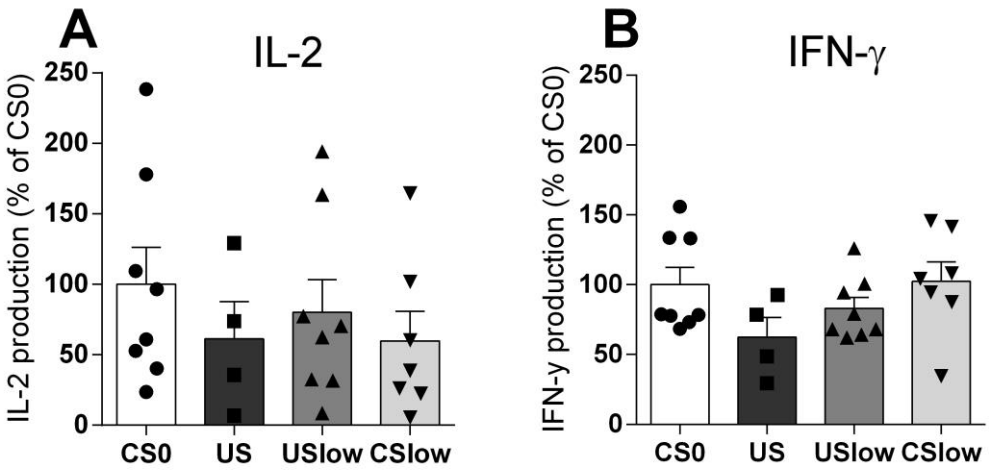


Fig. 20: Cytokine production of 48h anti-CD3 stimulated splenocytes on day 28.
(A-B) IL-2 and IFN-γ production of splenocytes stimulated with anti-CD3 and analyzed after the 28 days of CIA. The pharmacological control *(US*), showed a diminished IL-2 production compared control animals (*CS0*), which failed to reach statistical significance (n = 4-8/group). Data are shown as mean ± SEM percentage change from *CS0* controls

5. Discussion

Over decades, the bidirectional interaction between the CNS and the immune system has been an innovative research field, inspiring promising approaches to study the conditioning of immune responses (Tracey , Sternberg 1997, Exton et al. 2001, Ader 2003a, Meisel et al. 2005, Pacheco-Lopez et al. 2006, Tracey 2009, Schedlowski et al. 2010). Specifically, experimental studies in rodents and humans have convincingly demonstrated that suppression of immune functions can be elicited by behavioral conditioning aiming at a controlled dose reduction of drugs while maintaining efficacy of treatment (Schedlowski et al. 2010, Doering et al. 2012, Enck et al. 2013, Albring et al. 2014). However, possible clinical implications but also limitations of behaviorally conditioned immunopharmacological responses remained to be elucidated, encouraging the research questions addressed in this thesis. A clinically relevant experimental model of arthritis as an approach of conditioned immunosuppression in acute systemic autoimmune inflammation was implemented as one step towards answering these open questions.

5.1. Pre-exposure to CsA or saccharin does not affect learned immunosuppression in rats

Before a patient can participate in an immunosuppressive learning paradigm implemented as a supportive treatment, one needs to overcome an essential obstacle.

In clinical routine the majority of patients are already pharmacologically treated indicating that they are already familiar with the drug which would be used as unconditioned stimulus. Thus, it is not clear whether and to what extent an already known to be conditioned stimulus may affect the process of conditioning. As seen in any other classical conditioning paradigm, pre-exposure to CS or UCS may have an impact on learning and memory. To test this hypothesis with regard to the clinical relevance on conditioned immunosuppression, we utilized an established conditioning paradigm with saccharin as CS and the immunosuppressive drug CsA as US. With this model we examined, whether exposure to the CS or the UCS prior to the acquisition (learning) phase would affect the conditioned response on the behavioral level (CTA) as well as on the immunological level (learned

immunosuppression). Pre-exposures to both stimuli before acquisition have been reported to weaken the association between both stimuli and thus the learned response (Lubow et al. 1989).

Our data demonstrated that pre-exposure to the UCS (PE) or the CS (LI) prior to the acquisition phase induce a diminished CTA. These results on the behavioral level are in line with experiments acquired with LiCl as UCS (Bures et al. 1998). Most of the published data investigating conditioned taste avoidance employ LiCl as UCS (Garcia et al. 1985, Garcia-delaTorre et al. 2014). These experimental studies demonstrate that conditioned avoidance to a familiar stimulus appears to be weaker than to the same stimulus, when it is novel. For pre-exposure to the UCS (LiCl) it is known, that it generally leads to a diminished CTA ("UCS pre-exposure effect" (UCSPEE)) (Randich et al. 1979, Meyer et al. 2004, Chang et al. 2007). Similarly, pre-exposure to the CS in associative learning paradigms and in CTA experiments in particular leads to a reduced conditioned effect (fluid-intake in CTA experiments termed as "latent inhibition"(LI) (Lubow et al. 1959, Lubow et al. 2010).In the line with these results, pre-exposure of the "to-be-conditioned stimulus" (CS) in our experiment leads to an increased saccharin consumption and thus to a diminished taste avoidance. It is believed that this phenomenon is due to preventing the organism from being overloaded with uninformative spurious associations (Meyer et al. 2004).

However, more importantly, PE and LI were not detectable on the immunological level, confirmed by a pronounced behaviorally conditioned suppression of anti-CD3-stimulated IL-2 production of splenocytes, indicating that the conditioning mechanism on the behavioral level might be dissociated from the one on immunological levels.

When discussing the effects of pre-exposure on conditioned reactions the different UCS (CsA vs LiCl) need to be taken into consideration. Firstly, it has been demonstrated in rodents that one acquisition trial of LiCl as US is sufficient for the manifestation of an association between CS and UCS (Garcia et al. 1966, Bermudez-Rattoni 2004) whereas one learning trial of a CsA/saccharine association is triggering only a weak CTA without effects on the immunological level (Niemi et al. 2007). Additionally, data with CsA demonstrate that a strong initial association between CS and UCS during acquisition also maintained a strong CTA during unreinforced CS re-exposures, compared to a moderate CS–UCS association (Hadamitzky et al. 2015).

For example, after three acquisition trials, CTA to the saccharin-CsA engram is more resistant to extinction than after just one trial (Niemi et al. 2007).

Secondly, taste avoidance with LiCl induces symptoms of illness and discomfort, pain and emetic reactions in treated subjects lasting for 5-10 hours (de Brugada et al. 2004). In contrast, CsA administration does not induce acute digestive symptoms of sickness behavior. Though, intra-peritoneal CsA injection (20 mg/kg) is changing the behavior of rodents in the open field 6-12 h after administration (von Horsten et al. 1998, Mineur et al. 2014). Furthermore, deep brain EEG and c-Fos immunohistochemistry studies have previously documented activation in the IC and the Am 60 min after i.p. administration of CsA. These data support the hypothesis that neuronal mechanisms of behavioral effects could be induced by systemic CsA injection (Sato et al. 2007, Pacheco-Lopez et al. 2013).

Thirdly, numerous publications show that conditioned taste avoidance paradigms are influenced by contextual cues, indicating that extinction is probably specific to the context in which it occurs (Dudai et al. 2004, Bouton et al. 2006). This would make a clinical application for conditioning protocols difficult to accomplish. However, a distinct study investigated whether contextual changes may also influence CsA-induced CTA extinction. The results demonstrate that compared to the studies employing LiCl, extinction of taste avoidance with CsA was not affected by contextual changes. Thus, learning and extinction of CsA taste immune conditioning is not necessarily explicit to the context in which it is learned (Tuerkmen et al. 2016). Together, different mechanisms appear to underlie CTA with CaN Inhibitors like CsA in contrast to LiCl, suggesting that CTA generally depends on the physiological and neuropharmacological effects of the drug.

Our experimental data support that re-exposure to the taste, but not a significant taste aversion is essential for a learned immunosuppression indicated by weak or even lacking correlations between the CTA (behavioral level) and the learned immunosuppressive response (Ader et al. 1982, Exton et al. 1999, Exton et al. 2002b, Schedlowski et al. 2010). In the present approach animals, which exhibited weaker CTA during the retrieval showed more prominent suppression of IL-2 production (LI/PE group). From the clinical point of view these findings are of particular interest. The inhibited IL-2 production in the UCS pre-exposure group (PE) indicated that, patients who are already on immunosuppressive therapy with CaN

inhibitors such as CsA could still develop a conditioned immunosuppression. Therefore, it might be possible to implement paradigms of learned immunosuppression as an additional treatment option into patients' ongoing immunosuppressive pharmacotherapy for a better outcome.

Nevertheless, further investigations, for example a longer interval of pre-exposure to the UCS (more than three trials) and improved modeling of the clinical situation of treatment in patients, are needed to confirm and substantiate our data. Besides the optimal time point, context and organization for combining our conditioning protocols with standard clinical pharmacological drug application in humans needs to be determined (Schedlowski et al. 2015).

5.2. Behaviorally conditioned immunosuppression in a clinical arthritis model

Since decades most experiments on the conditioning of immunopharmacological effects have used the CTA paradigm (Ader et al. 1985). In experiments of this thesis, the established conditioning paradigm with saccharin as CS and the immunosuppressive drug CsA as UCS was used to investigate learned immunosuppression in a clinical relevant model. The unique advantage of our design is the ability to analyze the effect of reconsolidation of conditioned immunosuppression in a model of autoimmune diseases.

One of the major issues before implementing conditioning paradigms into disease models is the fact that the kinetics of the behaviorally conditioned immunosuppression are incompletely understood. Thus, it remains to be elucidated how conditioning paradigms should be composed in order to achieve optimal effects and prevent learned pharmacological responses from habituation or extinction (Ader 1997).

Chronic rheumatoid arthritis is a severe inflammatory autoimmune disease affecting approximately 1% of the world's population (Gibofsky 2012). Currently available therapeutics for RA are either limited in duration of treatment (e.g. glucocorticoids), or, as in the case of biologicals, are only functional in certain patients. One aim of this thesis is to develop an immune conditioning paradigm as a possible supportive therapy for arthritis. Indicating that implementation of a learning processes could be

reducing the amount of drugs needed for clinical pharmacological treatment of autoimmune diseases.

CIA has many characteristics of RA in humans. Due to the good reproducibility of preclinical data as well as the good predictability of certain therapeutically active substances in human RA, CIA is the preferred model for progressive autoimmune diseases in preclinical basic research (Trentham et al. 1977, Brand et al. 2007). Additionally, experimental studies on RA confirmed the potential clinical relevance of behaviorally conditioned immune alterations. Particularly the morbidity and mortality in experimental models in rats for experimentally-induced arthritis (Klosterhalfen et al. 1990).

5.2.1. Preventive retrieval had no beneficial effect on CIA

In order to identify the optimal time point during disease progression for implementing the conditioning paradigm, three different protocols were used in our CIA model of rheumatoid arthritis. Our preliminary data demonstrated that administration of full-dose CsA decreased the development of arthritis in type II collagen-immunized rats, reflected by a lower clinical score when the agent was given as prophylaxis (before the first clinical symptoms). Based on these data, the retrieval phase in our first experiment started one day after induction of arthritis (immunization). The main purpose of Experiment A was to demonstrate preventive rather than therapeutic conditioned effects on disease progression of arthritis, i.e. the development of paw swelling. Thus, rats were differentially conditioned before the occurrence of the first symptoms. Experimental animals (*CS*) received a low-therapeutic dose of CsA immediately after CS re-exposure. We hypothesized that the degree of paw swelling would be lower in pharmacological (*US*) and the conditioned (*CS*) animals compared to controls (*USlow* and *CS0*).

On the behavioral level, animals of the *CS* group revealed conditioned taste avoidance compared to the control groups for over 20 days. Only following implantation of osmotic mini pumps for pain prophylaxis, all animal displayed an increase in fluid consumption due to the effects of the isoflurane anesthesia. Furthermore, the onset of the first symptoms induced extenuated fluid intake in all groups. Accordingly, in the phase of acute arthritis all animals displayed a decrease in total daily food and water intake, in line with previous findings (Skurlova et al.

2010), leading to abolished group differences in the later stage of the experiment (day 21/23).

We could not detect a positive effect of behaviorally conditioned immunosuppression on CIA with the standard conditioning protocol in experiment A. As the main readout parameter, the clinical arthritis score indicated that neither the full pharmacological dose CsA (*US*-group) nor the conditioned immunosuppression (*CS*-group) had a beneficial outcome on disease progression when administered one day after immunization. Therefore, the taste immune conditioning protocol appeared to be inefficient in affecting inflammation of CIA when given prophylactic/perventive. However, rats treated with full doses of CsA (20mg/kg) had a later onset of symptom development and an overall milder disease severity in the acute phase.

Importantly, CsA exerts its immunosuppressive action by selectively inhibiting the transcription of messenger RNA for IL-2 and other cytokines in T cells (Thomson et al. 1984). Since IL-2 is required for the activation of T cells, CsA might be expected to act on CIA by inhibiting IL-2 production. A reduced IL-2 protein and mRNA production of splenocytes was also measured at the end of the first experiment (A) in full-dose treated rats (*US*-group). Additionally, we could detect a pharmacological induced suppression of pro-inflammatory cytokines such as IFN-γ and TNF-α in this group.

Nevertheless, our clinical data (arthritis-, histological score and grip strength) are controversial to our preliminary results and other publications. Kaibara et al. (1983) reported that CsA treatment with 15 mg/kg per day or more starting on the same day as immunization leads to a suppression of the development of arthritis in rats and mice (Kaibara et al. 1983, Takagishi et al. 1986). Additionally, Klosterhalfen and Klosterhalfen (1990) demonstrated that conditioned CsA effects prevent experimentally induced adjuvant arthritis in rats when re-exposure to the CS started one day after immunization. However, compared to the present experiment their group used a different disease model where they induced adjuvant arthritis with an injection of complete Freund's adjuvant beneath the sole of the foot (subplantar). In addition, in this study animals did not show an aversion to novel solutions (cyclamate and vinegar), providing further evidence of a dissociation between CTA and the conditioned alterations in immune functions, in line with our findings and as previously described for LI and PE (see 5.1). Other discrepancies to our results may

also be due to strain differences (*Wistar rats* vs. *Dark Agouti*), although effects due to the distinct compounds used as UCS may play a role as well.

Together, the data of the first experiment indicate that in an experimental model of RA, the time of starting retrieval of conditioned immuno-pharmacological responses may be critical. We could not reproduce effects of a prophylactic treatment with conditioned immunosuppression, which might be due to the drug (CsA) we used as UCS and to the arthritis induction in our model.

5.2.2. Retrieval during the clinical onset of arthritis has improved the outcome for CIA in rats

Since most RA patients visit their doctor for the first time when the first symptoms of the disease occur, we initiated retrieval only on day 14 when the first symptoms already occurred in a second experiment (B). As the aforementioned findings demonstrated no beneficial outcome on disease progression of arthritis, this second approach reveals greater clinical relevance.

It has previously been shown that on the behavior level as well as on the level of the immune system, conditioned responses are subject to extinction due to unreinforced re-exposure to the CS (Hadamitzky et al. 2013), as mirrored by a blunted reduction of conditioned responses in animals after several retrieval trials. Additionally, extinction also effects the conditioned immune response. After 6-7th retrieval trial the conditioned response was no longer significantly reduced in the experimental group compared to controls (Exton et al. 1998b, Hadamitzky et al. 2016a). In previous studies evaluating conditioning effects with CsA on heterotopic heart allograft survival in rats, conditioned taste aversion was prolonged when extinction was blocked by administering sub-therapeutic CsA as a *reminder cue* together with the CS (Hadamitzky et al. 2016a). Our data confirm this effect of memory-updating, since re-exposure to the CS together with low dose CsA demonstrated a significantly prolonged CTA for more than 13 days. Both studies showed that a combination of sub- or low-therapeutic UCS (CsA 2 or 5 mg/kg) in combination with the CS (saccharin) is capable of blocking or abrogating the extinction process of learned immunosuppression on the behavioral level. Moreover, they indicate that administration of the *reminder cue* during the labile phase of the memory trace (reconsolidation window) following retrieval is crucial for initiating a memory-updating

or reconsolidation-like process (Tronson et al. 2007). Accordingly, the application of a sub-therapeutic drug dose outside the reconsolidation window of 8 h following CS re-exposure leads to failure of memory reconsolidation and extinction of the conditioned response (Hadamitzky et al. 2016a). Our experiments confirm that updating or reconsolidation of memory is achieved by a combination of the *reminder cue* together with the CS within the reconsolidation window.

In line with our behavioral findings from the first experiment, re-exposures to the CS induced a significant CTA over 13 days. Animals in the *CS*-group consumed significantly less saccharin compared to the control groups. Given a generally decreased fluid intake from *CS* animals, one could argue that stress may have triggered a corticosterone response, which may be accountable for the immunosuppression. Thus, water deprivation paradigms like CTA might go along with an elevation in plasma corticosterone. However, we could reveal that the lack of fluid consumption in the morning was entirely compensated during the evening drinking sessions. Furthermore, experiments on conditioned immunomodulation measuring the adrenal response directly reported no relation between immunosuppression and corticosterone levels on the day of re-exposure to the CS (Ader et al. 1975, King et al. 1987). Therefore, our findings indicate that the observed benefit on the symptoms (clinical arthritis score) in the *CS* group was due to conditioned immunosuppression.

On the immunological level retrieval during the clinical onset of arthritis on day 14 demonstrated a significantly milder score in the full dose pharmacologically treated and in the conditioned animals. Thus, CsA markedly reduced paw swelling in the *US* group receiving a dose of 20 mg/kg/day. This pharmacologically induced effect was also reflected by significant decreases in peripheral IFN-γ levels. We could reduce paw swelling and inflammation in conditioned animals with only 25% of the full drug dose given as *reminder cue* together with the CS during retrieval, indicating additive inhibitory activities with respect to some of the clinical signs of arthritis in rats (clinical arthritis score; histological score and grip strength). These data demonstrating that, blocking extinction via memory-updating during retrieval at the clinical onset of symptoms leads to a beneficial outcome of CIA. Confirming, that extinction of CsA induced taste avoidance and, more importantly, the accompanying conditioned suppression of immune functioning could be blocked by memory-updating or

reconsolidation-like processes. They indicate that memory-updating mechanisms have apparently been transferred to peripheral immune functions.

Moreover our findings are in line with earlier work confirming that such updated learned immunosuppressive responses and their maintenance could be of clinical relevance. In these studies, taste-conditioning together with the administration of sub-therapeutic CsA as *reminder cue* (2mg/kg CsA) during retrieval significantly prolonged the rejection time of the allografts for 25 days and longer. (Exton et al. 1999, Hadamitzky et al. 2016a).

Together, these observations strongly suggest that reframing long-term treatment as a learning process provides new possibilities and makes immune conditioning a valuable tool in immune-pharmacotherapy for minimizing drug dosages thereby reducing unwanted toxic side effects (Lückemann et al. 2017).

5.2.3. Retrieval at score 4 is too late to improve the outcome for CIA in rats

In the previous experiment (B) (5.2.1.2), retrieval was initiated and performed on day 14 with the occurrence of the first symptoms. To achieve an even more clinically related experiment animals' individual treated after reaching a clinical arthritis score of 4 points (Experiment C). However, when retrieval treatment started during this rather late time point in disease progression, no significant benefit on the disease outcome was obtained. Even full dose treatment had no beneficial effect on the development of severe symptoms.

These paradoxical effects of CsA on CIA development might be due to modifications of the sensitive balance between the regulatory subpopulations of T helper and T suppressor cells (Morgan et al. 2003). The impaired capacity of regulatory T cells to exert their suppressive function on pro-inflammatory processes is probably attributed to a pronounced inflammation. Alternatively, other factors, mediating the immune responses need to be taken in consideration. For instance, B cells, which are not directly targeted by CsA also play an important role in influencing the course of the disease. Thus, the B cell-mediated production of high levels of auto-antibodies is crucial for the development of symptoms in type II collagen arthritis (Svensson et al. 1998, Finnegan et al. 2012). Hence, the absence of efficacy to suppress other immune cells (B cells, macrophages etc.) in an efficient manner might play a role in the late developmental stage of CIA.

Together, these findings suggest that once inflammation has reached a stage of high severity, conditioned immunosuppression, but also full-dose CsA therapy, are unable to improve disease outcome. Thus, it appears that the less severe the inflammation is in general the more susceptible the experimentally induced RA model is to pharmacological interventions as well as to immuno-conditioning.

5.2.4. Limitations of learned immunosuppression in CIA

In all 3 experiments (A, B and C) we did not detect an association of the positive effect of full dose treatment with CS presentation during the diseases progression. Moreover, the phenomenon of “beneficial drinking”, described by Klosterhalfen, where animals had learned positive effect of CS presentations when combined with an injection of an immunosuppressive drug on adjuvant arthritis during acquisition (Klosterhalfen et al. 1990), was not observed. This might be due to the fact that we scheduled the acquisition/learning period before the induction of arthritis.

Instead of low dose *reminder cues* or partial reinforcement, in a different approach we may use the full dose of medication in only 25-50 % of the administrations. This procedure has been demonstrated to induce beneficial effects in corticosteroid-treated patients with psoriasis (Ader et al. 2010) and thus needs to be considered in our model.

Given the susceptibility of the conditioning protocol to stress we did not assess serum concentrations of different markers, such as serum albumin, serum nitrite/nitrate and inflammatory cytokines like IL-6, TNF-α, during the disease progression. Immunological readout parameters were only analyzed at the end of each experiment. However this time point could be too late to detect conditioned differences between all groups, since the disease already started to stagnated at this time point.

Furthermore, it is known that the clinical onset of CIA is associated with a significant rise in nitrite/nitrate levels. Concentrations of nitrite/nitrate in the serum reflect the nitric oxide (NO) production and the inflammatory response in tissue. Measuring nitrite/nitrate concentrations may be one opportunity for analyzing the immune status during the CIA disease progression. Rovenska et al. 2001 showed that especially the effect of CsA in different dosages (5 mg/kg and 10 mg/kg) almost completely inhibited nitrite/nitrate increase during the first 24 h after immunization. Nevertheless,

the dosage of 10mg/kg CsA paradoxically increased the concentrations of nitrites/nitrates on day 31 compared to earlier time points (Rovenska et al. 2001). The increased levels might be a result of CsA-induced impairment of renal function, since normally the content of the serum nitrate is usually eliminated via the kidneys. The nephrotoxicity with elevated serum creatinine level is only one side effect, caused by CsA treatment (Halloran 2004). One major issue regarding the conditioning of immunopharmacological effects is the fact that it is not definitely known, whether the partially severe side effects of immunosuppressive compounds are also conditioned. However, we could not detect any evidence for conditioned side effects in our experimental approaches. During retrieval, rats and humans are exposed to no or at most sub or low-therapeutic amounts of CsA, thereby significantly reducing theses toxic side effects (Lückemann et al. 2017).

The present study reveals new insights and options for supportive clinical treatment during RA. In the CIA model, we achieved the same beneficial effects with 25% of the full drug dose, as usually achieved with a full dose of pharmacological treatment, if implemented at the right time point. This enormous reduction of the drug-dose needed tends to result in minimizing unwanted and toxic drug side effects while maximizing the therapeutic outcome for the patient`s benefit.

5.3. Outlook

Studies have demonstrated that peripheral immune functions can be modulated by associative learning paradigms. Behaviorally conditioned immunosuppression using the immunosuppressive drug CsA has been effective in rodents and humans. The work underlying this thesis demonstrated the positive effect of CsA and learned immunosuppression on CIA in DA rats. However, it is still unknown, whether the effects of learned immunosuppression are restricted to CaN inhibitors such as CsA or whether these effects are overarching and general mechanisms also steering learned immunopharmacological responses induced by drugs with distinct modes of actions. Therefore, the question arises whether and to what extent inflammation in autoimmune diseases might be supressed with conditioning protocols with different drugs. Further investigations of conditioning of immune functions with immunomodulating drugs operating via distinct cell signaling pathways, such as the anti-proliferative acting compound and mammalian target of rapamycin (mTOR)-antagonist rapamycin (RAPA), or anti-metabolite drugs such as methotrexate (MTX) would be an important next step. Additionally, our paradigm of conditioning immune functions can then be tested in another clinical model in experimental animals; in detail the possible modifications of conditioned immunosuppression with RAPA, implementing in a rat tumor model (glioblastoma).

At least to understand the underlying mechanisms of learned immune responses innovative pharmacogenetic techniques could be established to interfere with neuronal activity during conditioning. One possibility could be the use of “Designer Receptors Exclusively Activated by Designer Drugs” (DREADDs). Establishing DREADDs to remotely and non-invasively control neuronal signalling in the insular cortex and the amygdala, brain regions hypothesized to mediate learned immunopharmacological effects, could help to better characterize the mechanisms of this phenomenon.

A second opportunity to clarify the signalling cascade would be to analyze the sympathetic pathway which modulates conditioned responses. The role of the sympathetic nervous system (SNS) is well-established in behaviorally conditioned immunosuppression (Exton et al. 2002b). Using anti–dopamine-β-hydroxylase saporin-coupled (DHB) antibodies for systemic sympathectomy, future studies might block the conditioned immunosuppression meditated via this pathway. Since DBH is

generally expressed on sympathetic nerve fibers, the toxin saporin degrade sympathetic nerve fibers in the periphery by inhibition of activity in sympathetic neurons (Wiley et al. 2000, Härle et al. 2005, Härle et al. 2008). Other studies already demonstrated that denervation of the splenic nerve prior to conditioning abrogated the effect of conditioning produced immunosuppression in the spleen (Exton et al. 1998a, Exton et al. 1999). Additionally, studies indicated that chemical sympathectomy with 6-hydroxy-dopamine (6-OHDA) led to a complete blockade of conditioned suppression of splenocyte proliferation and cytokine (IL-2, IFN-γ) production. Furthermore, administration of the β-adrenoreceptor antagonist propranolol reduced the conditioned effect on the proliferation of the splenocytes (Exton et al, 2002). Therefore the administration of the DHB antibody or other β-AR antagonist could also be used in the CIA model to get a more precise knowledge of the underlining immune conditioning mechanism which was essential for a beneficial outcome of this disease.

Taken together, a thorough knowledge of the basic mechanisms of conditioned immunomodulation in disease progression is essential, not only to better understand the brain-immune system communication but in particular to achieve the long-term goal of the learned immune response: to employ these learning paradigms in clinical situations as supportive therapy together with standard immunopharmacological regimen.

6. Summary

Behaviorally conditioning of immune functions is a fascinating paradigm to analyze the functional bi-directional interaction between the brain and the peripheral immune system. In the experimental approaches applied in this thesis, taste-immune conditioning in rats combined the novel taste saccharin (conditioned stimulus/CS) with an injection of the immunosuppressive drug cyclosporine A (CsA; unconditioned stimulus/UCS). The conditioned response was reflected by conditioned taste avoidance (CTA) as well as diminished interleukin (IL)-2 and interferon (IFN)-y cytokine production. In order to analyze the effects of pre-exposure to the CS or UCS, respectively, on this learned immunosuppression, rats were exposed either to the CS (saccharin) or to the UCS (CsA) prior to conditioning. Pre-exposure to either CS or UCS resulted in an accelerated extinction of the conditioned response on the behavioral level (CTA). In contrast, and more importantly, learned suppression of IL-2 and IFN-y production was not affected. From a clinical perspective, these findings indicate that learned immunosuppression may be inducible in patients that are already on immunosuppressive therapy or have had previous contact to the gustatory stimulus.

A second experimental approach focused on the effects of learned immunosuppression on disease development and progression in a rat model of collagen II-induced arthritis. In three experiments, rats were conditioned at various stages of the disease. During retrieval, low-therapeutical doses of the UCS were administered as *reminder cues* to update and stabilize the memory of the learned immunosuppression. This memory-updating stabilized the learned immune response and significantly suppressed disease progression in immunized rats.

Overall, these findings support the notion that associative learning protocols might be employed as supportive therapy to immunopharmacological regimens in patients, with chronic inflammatory autoimmune diseases.

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7. Zusammenfassung

Die klassische Konditionierung von Immunfunktionen ist ein beeindruckendes Beispiel für die bi-direktionale Kommunikation zwischen dem zentralen Nervensystem und dem peripheren Immunsystem. In den Experimenten dieser Arbeit wird die Konditionierung immunologischer Reaktionen mit Hilfe des Models der „konditionierten Geschmacksaversion“ (CTA) bei Ratten beschrieben. Dabei wird ein neuartiger Geschmack (Saccharin; konditionierter Stimulus/CS) mit der Injektion eines immunsuppressiven Medikaments (Cyclosporin A (CsA); unbedingter Stimulus/UCS) gekoppelt. Die konditionierte Reaktion spiegelt sich in einer CTA sowie verminderter Interleukin (IL)-2 und Interferon (IFN)-γ Zytokin-Produktion wieder. Um die Auswirkungen einer Prä-exposition der beiden Stimuli CS bzw. UCS auf diese gelernte Immunsuppression zu analysieren, wurden Ratten vor Beginn einer Konditionierung entweder mit dem CS (Saccharin) oder dem UCS (CsA) konfrontiert. Diese Prä-exposition des CS als auch des UCS führte zu einer beschleunigten „Löschung“ der konditionierten Reaktion auf der Verhaltensebene (CTA). Die gelernte Suppression der IL-2 und IFN-γ-Produktion wurde im Gegensatz dazu nicht beeinträchtigt. Diese tierexperimentellen Befunde deuten darauf hin, dass auch bei Patienten, die bereits mit immunsuppressiven Medikamenten behandelt werden, oder die bereits in Kontakt mit dem Geschmackstimulus gekommen sind, eine Konditionierung der Immunfunktionen möglich ist.

Ein zweiter Ansatz konzentrierte sich auf die Auswirkungen der gelernten Immunsuppression auf die Progression einer experimentell induzierten Arthritis (AR). In drei Experimenten wurden Ratten in unterschiedlichen Stadien der Erkrankung konditioniert, wobei während des Abrufens der konditionierten Reaktion niedrig-therapeutische Dosierungen des UCS als Gedächtnis-Rekonsolidierung (GR) verabreicht wurden. Diese GR immunsuppressiver Effekte stabilisiert die gelernte Immunsuppression und vermindert signifikant symptomatische Progression einer experimentell induzierten AR im Tiermodell.

Diese Daten weisen darauf hin, dass Lernprotokolle als zusätzliche Option zur immunsuppressiven Behandlung bei Patienten mit chronisch entzündlichen Autoimmunerkrankungen als supportive Therapie eingesetzt werden können, um Medikamentendosierungen und damit unerwünschte Nebenwirkungen zu reduzieren.

8. Literature

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9. Attachment

Substances

Substance	Company
β-Mercaptoethanol	Sigma Aldrich München Germany
Carprofen	Bayer Leverkusen, Germany
CD3 mouse anti-rat monoclonal antibody	clone:G4.18, BD Pharmingen Heildeberg Germany
Collagen type II	MD Bioproducts, Egg, Switzerland
Cyclosporine A	LC Laboratories, Woburn MA USA
EDTA	Sigma, Deisenhofen, Germany
Erythrocyte lysis	BD Pharmingen Heildeberg Germany
Ethanol 70% 90%	University clinic Essen
FBS	Gibco®, Life Technologies TM Carlesbad USA
Freud's adjuvant incomplete	Sigma-Aldrich, Taufkirchen, Germany
Gentamycin	Gibco®, Life Technologies TM Carlesbad USA
HBSS	Gibco®, Life Technologies Carlesbad USA
Hematoxylin–eosin	Sigma Aldrich, Taufkirchen Germany
Isoflurane	Henry Schein Hamburg Germany
Miglyol	Caelo, Hilden, Germany
Natrium chloride 0,9%	BBraun Meslungen Germany
PBS	Gibco®, Life TechnologiesTM Carlsbad USA
RDO rapid decalcifier	Medite, Burgdorf, Germany
RPMI 1640 Glutamax	Life Technologies Darmstadt Germany
Temgesic;	Indivior Eu Limited; Great Britain
Tissue Tek O.T.C Medium	Sakura Finetek, Leiden, Netherlands
Sodium saccharin	Sigma-Aldrich, Schnelldorf, Germany
Sucrose	Sigma-Aldrich, Schnelldorf, Germany

Kits

Substance	Company
cDNA Synthesis kit **(dNTP Mix, 10x RT Puffer, MultiScribe, Reverse, RNA ase Inhibitor, 10x Random Primers)**	Applied Biosystems, Darmstadt Germany
ELISA rat IL-2	R&D systems, Minneapolis, USA
ELISA rat IFN-γ	BioLegend, San Diego, USA
ELISA rat IL-6,	BioLegend, San Diego, USA;
ELISA MAX rat TNF-α	BioLegend San Diego, USA
RNeasy Mirco Kit	Quiagen Hilde, Germany

Technical Equipment

Equipment	Model	Company
Active gas scavenger	53910	Stoeting Illinois USA
Anesthesia Gas Filters	**50206**	Stoeting Illinois USA
Anesthesia Machine	Rodent Compact 150	Draeger Lübeck Germany
Animal blood counter/ Hematology Analyzer r	VET ABC	scil animal care company Illinois USA
Centrifuge	Rotanta 460 RS & 541R	Hettich Lab Technology, Tuttlingen Germany Eppendorf Hamburg Germany
Electronical balance	ASC 120-4	Kern Baling Germany
Incubator	HeraCell 240	Thermo Scientific, München
laminar airflow cabinet	Hera Safe	Heraeus, Osterode Germany
Magnet stirrer	Combimag RCT	IKA, Staufen Germany
Multipette	Eppendorf Research Multipette® Plus	Eppendorf, Hamburg Germany
Operation instruments	Scissors, forceps scalpel	Aesculap Tuttlingen Germany
Pipette	Pipetus®-akku	Hirschmann Laborgeräte, Eberstadt Germany
Plate Reader	FLUOstar OPTIMA	BMG Labtech, Ortenberg Germany
Plate Shaker	TITRAMAX 101	Heidolph, Schwabach

Real-Time PCR System	Applied biosystem	Thermo Fischer Rochester USA
Thermal Cycler	2720 Applied Biosystems	Thermo Fischer Rochester USA
Vortex device	Vortex Genius 3	IKA, Staufen Germany
Hämatologie-Analysator	scil Vet abc	Scil Viernheim, Germany

Material

Material	Company
Cannulaes BD Microlance	BD Heildeberg Germany
Cell Strainer 70µm	BD Heildeberg Germany
Combitips, 5 ml, 1 ml, 0,5 ml	ritips®professional,Ritter, Schwabmünchen Germany
Pipette 5,10, 20 ml	Greiner bio one, Frickenhausen Germany
PP falcon 15 & 15 ml	Greiner bio one, Frickenhausen Germany
Filter	Thermo Fischer Rochester USA
Petri dish	Greiner bio one, Frickenhausen Germany
Reaction Tubes 1,5 ml	Greiner bio one, Frickenhausen Germany
Syringe 1m l & 20 ml	Braun,Meslung Germany
96 well plate flat bottom	TPP Trasadingen Switzerland

Acknowledgement

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Curriculum Vita

Laura Lückemann

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Erklärungen

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Behaviorally conditioned immunosuppression: Mechanisms and potential clinical relevance in a model of rheumatoid arthritis in rats “ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (Laura Lückemann) befürworte.

Essen, den_____ _____

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Laura Lückemann

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Essen, den _____ _____

Laura Lückemann