The development of novel anti-inflammatory drugs for IBD

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PART I

INTRODUCTION

1

Glucocorticoids, a general introduction

Although the name glucocorticoids (GCs) refers to a class of steroid hormones produced by the adrenal cortex, the name is commonly used to indicate synthetic analogues of these molecules such as prednisolone. The endogenous glucocorticoid cortisol, is part of many different processes in the body, ranging from glucose metabolism to memory consolidation. And indeed cortisol also affects immunological processes such as regulation of delayed type hypersensitivity[1] or modulating the Th1/Th2 balance[2]. However, clinically GCs are used for the general suppression of inflammation. At supraphysiological concentrations, GCs can inhibit various aspects of both the innate and adaptive immune response[3]. Hence they are used for the treatment of allergies, auto-immune diseases and other inflammatory conditions. In general their effect is swift and powerful, unparalleled by other immunosuppressants. Combined with molecular properties that allow for different routes of administration, unhindered passage through the body and the low cost of production, make GCs a very attractive drug. Unfortunately, a proportion of patients becomes refractory to treatment and over time require an increasing dose[4]. Additionally, effects of treatment are not limited to suppression of inflammation alone but coincide with a wide range of adverse effects. The incidence of adverse effects increases along with the dose and prolonged exposure[5]. This can develop into serious complications, resulting in discontinuation of the treatment or worse. And so, taken together, GCs are both bitter and sweet. Considering the limited repertoire of anti-inflammatory drugs to our disposal, GCs cannot simply be disposed of. Smarter and improved versions of this drug need to be developed. But so far this has not proven to be an easy task[6]. Their potency may be explained by evolutionary development as an integral part of the human body. But this is also their weakness since the underlying mechanisms that regulate immunosuppression do not

differ from those that cause adverse effects. Separating the two may not be possible and indeed, so far, no safe GC has been developed. It did, however, yield a myriad of information on how GCs work, changing insights and maybe one day, resulting in the development of a safe GC.

References

1. Dhabhar, F.S., *Stress-induced enhancement of cell-mediated immunity.* Neuroimmunomodulation, 1998. **840**: p. 359-372.
2. Elenkov, I.J., *Glucocorticoids and the Th1/Th2 balance.* Glucocorticoid Action: Basic and Clinical Implications, 2004. **1024**: p. 138-146.
3. Franchimont, D., *Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies.* Ann N Y Acad Sci, 2004. **1024**: p. 124-37.
4. Quax, R.A., et al., *Glucocorticoid sensitivity in health and disease.* Nature Reviews Endocrinology, 2013. **9**(11): p. 670-686.
5. Huscher, D., et al., *Dose-related patterns of glucocorticoid-induced side effects.* Annals of the Rheumatic Diseases, 2009. **68**(7): p. 1119-1124.
6. Whitehouse, M.W., *Anti-inflammatory glucocorticoid drugs: reflections after 60 years.* Inflammopharmacology, 2011. **19**(1): p. 1-19.

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A brief history

The field of glucocorticoids and the glucocorticoid receptor is quiet extensive. Currently (2015) a simple search on PubMed will return close to 200.000 hits. This is related to the ever increasing interest but also for a part to the long history of glucocorticoid research which dates back to the eighteen hundreds. In 1855 Thomas Addison, a British physician, described a progressive destructive condition of the adrenal glands[1] which we now know renders patients glucocorticoid and mineralocorticoid deficient. Symptoms include low blood pressure, muscle weakness and low blood sugar. If left untreated patients may develop a severe condition termed Addisonian crisis which may be life-threatening. Nowadays patients suffering from adrenal insufficiency can be easily compensated by supplementing the lacking cortisol in the form of a tablet.

Not long after Addison's publication, a Mauritian physiologist named Charles-Edouard Brown-Sequard used animals to show that removal of the adrenal glands results in death which, as he correctly suggested, was due to a lack of hormones. It took another forty years before the next step was taken. In 1898 the Canadian physician Sir William Osler treated an Addisson's patient with crude preparations of adrenal glands taken from animals, thereby temporarily improving symptoms of the disease[2]. His results strongly suggested that the adrenal glands produced a substance that was vital to the body. To establish this, both Hartman and Swingle adrenalectomized rats, cats and dogs, and prepared extracts with which the life of these animals could be maintained[3, 4]. These extracts have also been used effectively for the treatment of Addisons patients and at the time it was believed they contained a substance that could extend life in general. Therefore an international competitive search commenced which culminated in the discovery of cortisone in 1934. The American chemist Edward Kendall was the first to successfully isolate it. He discovered that the adrenal glands did not contain just one substance but that the effects caused by adrenalectomy are related to different groups of molecules, which he labeled alphabetically. Only four of these seemed to have a physiological effect in animals. 11-dehydrocorticosterone (compound A), corticosterone (compound B), dehydrocorticosterone (compound E, known to Reichstein as compound Fa) and 17-

hydroxycorticosterone (compound F)[5].
Importantly, its use as an anti-inflammatory drug was completely unknown. Investigations were driven by a rumor during WWII that the Germans were performing large scale isolations from bovine adrenal glands in order to prevent hypoxia of Luftwaffe pilots enabling them to fly on higher altitudes. As a consequence it was in the interest of the war to quickly find a way to synthesize adrenal hormones. And although a use for these hormones in warfare could not be found, the first large scale synthesis of cortisone was completed by Kendall at the Mayo clinic (in collaboration with Lewis Sackett of Merck) in 1948.
Dr. Philip S. Hench was Chief of the Medical Service and Director of the Army's Rheumatism Centre at the Army and Navy General Hospital during the war. When the war came to an end he specialized in arthritic disease at the Mayo clinic. There he noticed that particular conditions, such as pregnancy or jaundice, caused a remission of pain. From these observations he became convinced that this effect was caused by a steroid hormone. In 1949 Hench, in collaboration with Kendall, reported the successful treatment of rheumatoid arthritis patients with Compound E [6]. In 1950, for their work on hormones of the adrenal cortex, Hench, Kendall and Reichstein received the Nobel Prize in physiology or medicine. Interestingly to isolate 1 gram of cortisone, Kendall required 500 kilogram of adrenal glands, which he collected from 20.000 cows. It was not until 1952 before a means of biological production was found, using the fungus *Rhizopus nigricans*.

References

1. Addison, T., *On The Constitutional And Local Effects Of Disease Of The Supra-Renal Capsules.* 1855.
2. Osler, W., *On six cases of Addison's disease with the report of a case greatly benefited by the use of the suprarenal cortical extract.* Int Med Magazine. , 1896.
3. Hartman, F.A., *Cortin, Vital Hormone of the Adrenal Cortex.* Endocrinology and Metabolism Clinics of North America, 1930. **14**(July): p. 229-232.

4. Swingle, W.W.P., J. J. , *An Aqueous Extract of the Suprarenal Cortex Which Maintains the Life of Bilaterally Adrenalectomized Cats.* Science, 1930. **March**(71): p. 321-322.
5. Kendall, E.C., et al., *Isolation in Crystalline Form of the Hormone Essential to Life from the Suprarenal Cortex: Its Chemical Nature and Physiologic Properties.* Proc Staff Meet Mayo Clin, 1934. **9**(April).
6. Hench, P.S., et al., *The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis.* Proc Staff Meet Mayo Clin, 1949. **24**(8): p. 181-197.

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Adverse effects

As previously mentioned, the use of steroids is impeded by two major problems. The first problem is loss of response to treatment. In some patients response to treatment reduces over time, requiring a higher dose to maintain the initial response. But a proportion of this group grows entirely refractory to treatment. The underlying cause is unclear and still subject to debate[1]. The second objection to GC treatment is its associations with a rather large number of severe adverse effects, particularly when using a systemic high dose. These adverse effects have been studied quite extensively, with the intention of discovering ways to reduce or even prevent them. With some of these adverse effects the underlying GC driven mechanism has been fully elucidated. Here we will discuss in broad detail which adverse effects are associated with GC treatment and what is known about the underlying mechanism.

GCs are synthetic analogues of a steroid hormone, which in the body serves a wide range of specific biological functions. Part of these functions is related to conditions such as stress or anxiety, which cause the levels of cortisol to increase[2]. In absence of stress, the level of cortisol fluctuates with a circadian rhythm, rising just before waking in the morning. This rise is believed to prepare people for the stress that is associated with everyday life[3].

Secretion of cortisol is self-limiting because the hormone binds receptors in the hypothalamus and anterior pituitary gland, reducing secretion of CRH (corticotropin releasing hormone) and ACTH (adrenocorticotropic hormone) and thereby inhibiting the stimulus that causes production of cortisol. High levels of synthetic GCs have a similar effect but due to a higher affinity for the GR and longer half-life, the effect can be much stronger. As a result, production of cortisol can be inhibited for as long as two months after stopping the treatment. However, this problem can be quite easily solved by slowly decreasing the dosage before stopping treatment.

Depending on the route of administration some adverse effects are more prominent than others. Topically applied GCs (such as skin crèmes or eye

droplets) will have mainly local side effects. Here we will focus primarily on side effects associated with systemic administration.

Adipogenesis

Next to the suppression of the hypothalamic-pituitary-adrenal function, long-term treatment with GCs can cause Cushing's syndrome. This syndrome results from Cushing's disease, a condition described by Harvey Cushing in 1932[4]. Overproduction of CRH or ACTH, for example due to an adenoma in the pituitary gland, results in elevated cortisol levels. Elevated levels have a profound effect on the human body and this effect is similar in patients who are treated with GCs. Striking is the change in appearance. Patients gain weight, particularly around the waist, back of neck (buffalo hump) and face (moonface). The cause for GC induced obesity is related to the biological role that GCs play in adipose tissue physiology. GCs can induce lipogenic genes (ACC, FAS) provided that insulin is present[5, 6], regulate lipolysis (ATGL, HSL, MGL)[7-9], adipose endocrine function (release of chemokines from the adipose tissue)[10] and restrain adipose tissue inflammation in obesity[11]. They are required for precursor cells to differentiate into adipocytes and necessary for keeping specific adipocyte related genes active to prevent isolated adipocytes or adipocyte tissue from dedifferentiation[12]. Interestingly, people suffering from obesity do not have increased blood cortisol levels but have higher local concentrations of cortisol in their adipose tissue. This is due to a higher activity of 11ß-hydroxysteroid dehydrogenase-1 (HSD1), which converts the inactive cortisone to cortisol[13]. In mice adipocyte specific overexpression of HSD1 leads to obesity with metabolic syndrome[14].

Apart from effects of GC treatment that relate to the adipose tissue, patients can experience an increased appetite[15]. The appetite in humans correlates with changes in adrenal steroid levels. Anorexia and weight loss for example is a typical symptom of Addison's disease, caused by adrenal insufficiency. It may also provide an answer on why people feel the need to eat under stress related conditions as stress increases cortisol levels. But how GCs affect appetite is unclear. On a molecular level GCs have been

found to elicit both orexigenic and anorexigenic effects. GCs increase neuropeptides that increase the appetite (NPY and AgRP) but on the other hand also inhibits signals that are associated with anorexia such as CRH.

Skin (thinning & striae)

Another symptom of Cushing's syndrome is the thinning of the skin and related to this, the appearance of striae. These symptoms are more common to topically applied steroids but also occur during systemic treatment. The skin adopts a paper like property, becoming fragile and easily damaged. Histological changes include a reduction of the epidermis[16], a decrease in keratinocyte size[17], reduction in the number of fibroblasts[18], rearrangement of the geometry of the dermal fibrous network[19], and a decrease in the size of both the cell layers and of the lipid lamellae that are in between the cell layers of the stratum corneum[20]. Because GCs reduce the production of collagen by fibroblasts[21], the skin becomes more rigid and less flexible. As a consequence the skin rips more easily resulting in stretch marks known as striae. These processes are affected in the skin because under normal conditions fibroblast proliferation, keratinocyte differentiation and collagen production are regulated through the local production of cortisol by HSD1[22, 23]. In has been shown in mice that the adverse effects of GC treatment on the skin can be countered by selective inhibition of HSD1[24].

Two of the most extensively studied adverse effects of GC treatment are loss of strength of bone and muscle[25]. The underlying mechanisms have been largely uncovered and may help prevent these side effects when GC treatment is compulsory.

Muscle (loss of strength)

Loss of muscle strength is a result of muscle atrophy. The breakdown of muscle is considered to be one of the major catabolic GC mechanisms[26]. GCs cause the thickness of the muscle fibers to reduce and the protein content of the myofibrils to decrease[27]. The decrease in protein content is the result of an increased breakdown and decreased synthesis. The

increased breakdown is the result of activation of the ubiquitin proteasome and lysosomal systems by increasing the expression of so called Atrogenes, genes that are involved in atrophy (FOXO, Atrogin-1, MuRF-1)[28]. The decreased protein synthesis results from a direct inhibition of the mTOR pathway by GCs[29]. The underlying cause of these changes in protein homeostasis may be the result of differences in expression of two growth factors which are produced in the muscle tissue; Insulin-like Growth Factor (IGF)-I[30], a muscle anabolic growth factor and Myostatin (Mstn)[31], a muscle catabolic growth factor. In animal models, muscle specific overexpression of IGF-I[32] or deletion of Mstn protect against GC induced muscle atrophy[33, 34]. GCs have been shown to inhibit IGF-I and to stimulate Mstn. The latter contains a GRE in the promoter region of the gene but more importantly, GCs stabilize Mstn mRNA[35].

Bone (loss of strength)

GCs can also greatly affect the health and quality of bones by suppression of osteoblast function and a reduction of bone mineral density[25]. But although high levels of GC can result in loss of bone, physiological concentrations of cortisol are important for the formation of bone mass. Tissue specific knockout of the GR in bone results in a reduction of the total bone mass[36]. And similarly, overexpression of HSD2 in osteoblasts reduces bone mass[37]. GC cause osteoblasts to produce Wnt proteins, which perform a paracrine function on neighboring mesenchymal stem cells[38]. The Wnt proteins induce Wnt signaling, which results in the accumulation of ß-catenin and the expression of transcription factor RUNX2 which together drive differentiation of stem cells into an osteoblastic lineage. Additionally, the Wnt proteins induce Mmp14 production in chondrocytes[39]. This metalloproteinase is important for the breakdown of extracellular matrix during the process of ossification. Failure to do so causes developmental anomalies such as delayed intramembranous ossification of the cranium as is apparent from HSD2 overexpressing mice.

But despite, or maybe because, of the role GCs play in bone formation, patients receiving high levels of systemic GCs can expect to lose bone, up to 12% in the first year of treatment and 2-3% in the years thereafter[40]. High levels of GCs cause an inhibitory effect on the both the function and differentiation of osteoblasts and induce apoptosis in this cell type[41, 42]. As a result bone formation is inhibited in a matter of hours after starting GC treatment. In contrast to physiological concentrations, high levels of GCs inhibit production of Wnt proteins by mature osteoblasts[43]. Consequently mesenchymal stem cells do not accumulate ß-catenin or increase production of RUNX2[44]. Moreover, GCs increase expression of glycogen synthase kinase 3ß (GSK-3ß), a protein that drives ß-catenin ubiquitinylation and proteasomal degradation and increases the expression of proteins that inhibit Wnt signaling[45]. High levels of GCs also inhibit another important regulator of osteoblast differentiation, bone morphogenetic protein 2 (BMP2). Expression is inhibited directly but also by increasing the expression of follistatin and Dan, two BMP-2 antagonists [46] and decreasing expression of IL-11[47] which stimulates expression of BMP-2. The decrease in number of osteoblasts also decreases the amount of proteins produced by these cells such as collagen and osteocalcin. Although only shown in vitro, GCs arrest osteoblast cell cycle progression in the G0, G1 (amongst others by decreasing expression of Cyclin D2 and increasing expression of p27kip1) and G2 phase (by decreasing expression of Cyclin A)[48]. And finally, GCs induce apoptosis in osteoblasts by increasing the expression of pro-apoptotic genes (Bim, Bak, P53)[49] and decreasing the expression of pro-survival genes (Bcl-XL)[48].

More than nighty percent of bone is made up of osteocytes. These cells are situated in oblong spaces called lacunae and are interconnected through small canals termed canaliculi. These canals serve as a way for cells to communicate, acquire nutrients and when necessary, the recruitment of osteoblasts. The fluid that is stored in this network of canals provides the bone with strength. Even in the case of minimal loss of bone, high levels of GCs reduce the number of osteoblasts which in turn causes fluid content and vascularity of the bone to decrease which leads to a reduction of bone strength.[50]. Furthermore osteocytes resolve cellular damage through

autophagy[51]. Prolonged treatment causes a pathological buildup of autophagosomes in the osteocytes[52]. Material secreted from these autophagosomes pollutes the microenvironment of the osteocyte and results in cell death.

Although GC treatment can also affect osteoclasts by extending their lifespan[53], the negative effect of GC treatment on bone quality relies mainly on osteoblasts and osteocytes. Protecting osteoclasts from GC treatment by cell specific overexpression of HSD2, had little effect on the quality of bone tissue[53]. This in contrast to the protection of osteoblasts (and osteocytes) from GCs in which case the quality of bones was maintained even in prolonged presence of high levels of GCs[41].

Glucose (diabetes)

Much as the name suggests GCs play an important role in the metabolism and uptake of glucose[54]. GCs antagonize the effect of insulin and can have a profound effect on blood glucose levels. Prolonged elevated blood glucose levels (hyperglycemia) resulting from GCs is commonly referred to as steroid induced diabetes mellitus[55]. This type of diabetes is in most cases transient in nature and the situation will return to normal after the GC treatment is stopped.

In response to high blood glucose levels, insulin is secreted by ß-cells in the pancreas. This causes the liver to increase conversion of glucose into glycogen through a process called glycogenesis. Insulin stimulates a Glut4 transporter mediated uptake of glucose by adipocytes and skeletal muscle cells. The effects of GCs on blood glucose levels are exactly opposite[56] and cause the ß-cells to dysfunction[57]. Although not all the steps in ß-cell insulin release have been elucidated, it is clear that the uptake of glucose by ß-cells causes an increase in ATP-ADP ratio which closes ATP sensitive potassium channels resulting in depolarization of the cellular membrane, leading to an influx of calcium and ultimately exocytosis of insulin filled granules[58]. GCs decrease glucose metabolism in ß-cells and thereby impair insulin release, disabling the body to respond to high glucose levels. In a similar fashion GC affects adipocytes and skeletal muscle cells,

decreasing glucose uptake in response to insulin release. Moreover, GCs affect insulin receptor (IR) signaling by decreasing expression of direct downstream signaling molecules such as insulin receptor substrate 1 (IRS1), phosphoinositide 3-kinase (PI3k) and protein kinase B (PKB)[59]. In skeletal muscle cells this effect is aided by an increase in proteolysis[56]. The resulting increased amino acid concentration has been reported to interfere with insulin signaling. The decrease of signaling molecules downstream of the IR causes cells to be less sensitive to insulin, decreasing glucose uptake. In adipocytes it has been reported that GCs increase lipolysis through downregulation of cyclic-nucleotide phosphodiesterase 3B[9]. An increase in lipolysis leads to elevated fatty acid plasma levels, which also negatively affects glucose uptake. And finally GCs have a direct effect on glycogen synthesis kinase 3 (GSK3) in skeletal muscle cells[60]. This leads to inactivation, causing a decrease in glucose conversion and thus blood glucose levels remain increased.

Blood pressure (hypertension)

Blood pressure is the cumulative result of blood volume, the viscosity of the blood and the resistance that is caused by vascular diameter and flexibility. This is regulated through the renin-angiotensin system and aldosterone release, which affects both vascular diameter and body fluid volume. Rise in blood pressure is a common side effect of GC treatment causing the cardiovascular associated mortality rate to increase fivefold[61]. In general it is assumed that the rise in blood pressure is the result of increased renal sodium and water reabsorption due to binding of GCs to the mineralocorticoid receptor (MR), also known as the aldosterone receptor[62]. Indeed GCs bind the MR just as easily as mineralocorticoids do. This off target effect of GC treatment will certainly play a role in GC induced hypertension. However, there are a number of observations that indicate that GCs induce hypertension primarily through activation of the GR. For example the glucocorticoid budesonide, commonly used for the treatment of asthma, can hardly bind the MR and yet, treatment induces hypertension[63]. More importantly, treatment with the GR antagonist RU486 protects against hypertension in patients suffering from Cushing

syndrome even though (despite the close similarity with the GR) the MR shows no affinity for RU486[64, 65]. And finally, blocking sodium and water retention through treatment with the MR antagonist spironolactone does not prevent GC induced hypertension[66].

In light of the above, GC induced hypertension is unlikely to be the result of activation of the MR but appears to be regulated through the GR. Long-term adjustment of blood pressure levels is regulated through the renin-angiotensin system in the kidneys. The kidneys release renin from their afferent arterioles. Renin hydrolyzes circulating angiotensinogen into the peptide angiotensin I. This peptide is then converted in the lungs by angiotensin-converting enzyme (ACE) into the potent vasoconstrictor angiotensin II. The GR is expressed throughout the kidneys but its role in the kidneys is unclear. Several studies suggested a functional role for the GR in the kidneys, in some cases even a role that may affect blood pressure primarily through an increase in sodium reabsorption[67-70]. However, experiments in animals have repeatedly shown that GCs induce natriuresis[71], making these results difficult to interpret. And perhaps the conclusion should be that GCs affect blood pressure mainly through effects on vascular smooth muscle cells. This would also fit with the relatively short time it takes for GC treatment to increase blood pressure. One study revealed that phenylephrine-induced vasoconstriction of isolated aortic rings is enhanced in a dose dependent manner by GC treatment[72]. This effect was blocked by pretreatment with L-NAME, an endothelial specific inhibitor of cGMP, suggesting that the effect might be dependent on endothelial cells. Indeed, in a recent study where the GR was conditionally knocked out in either smooth muscle cells or vascular endothelium, the latter appeared completely resistant to GC induced hypertension supporting a role for vascular endothelium[73]. And finally in vivo data obtained from a hypertension model in rats revealed that the effect of GCs on total peripheral pressure could be rescued by concomitant treatment with the vasodilator minoxidil, even though the GC induced hypertension remained unchanged[74]. This was explained by an increase in cardiac output. Considering the complexity of blood pressure regulation and the

many systems and cell types involved, further research is required to fully clarify this adverse effect.

Eye

Over time GC treatment increases the risk of developing glaucoma or cataract dose dependently [75]. Cataract is a clouding of the lens, which decreases vision. Although development of cataract as an adverse effect of GC treatment normally takes months or even years to develop, cases have been reported in which patients on a low dose developed cataract after only two months[76]. GCs induce a specific type of cataract, which is located in the center (pole) at back of the lens, termed posterior subcapsular cataract (PSC). This cataract is characterized by aberrant lens epithelial cells that have migrated from the lens equator to the pole. Although it has been challenged for a long time, recent studies have revealed the presence of a functional GR in these cells[77]. Treatment with GCs induces transcription of a number of genes of which GILZ and PAI-1[78] are most prominent. Although no functional data is available, both are involved in apoptosis, differentiation and migration. In GC induced cataract the proliferation and differentiation of lens epithelial cells is confounded. Normally the lens is replenished by new lens fibers through the differentiation of epithelial cells on the lens equator, into fiber cells lacking DNA and organelles. In PSC histological studies revealed the presence of semi-differentiated cells containing degenerating nuclei and few organelles on the posterior pole of the lens[79]. Another important mechanism in the etiology of PSC seems to be the reduction of lens glutathione (GSH)[80]. This molecule is the body's most potent antioxidant and protects macromolecules against reactive oxygen species and helps with H_2O_2 detoxification. Reduction of GSH is considered to be the main mechanism behind age related cataract and its role in PSC is similar. Experiments with in vitro cultured lenses showed that the effect of GC on GSH could be rescued with RU486[81], demonstrating involvement of the GR. The last and longest existing hypothesis on GC induced PSC is based on an interaction between steroids and lens proteins which eventually leads to conformational changes and oxidation of these proteins[82]. Study results

are often contradictory but its role cannot be denied and probably plays a part in PSC.

A second adverse effect of GC treatment that affects the eye is GC induced glaucoma[83]. Glaucoma is an increase of intraocular pressure that may result in optic neuropathy if left untreated for too long. Changes in intraocular pressure are normal and pressure reflects the diurnal cortisol levels in the blood[84]. Adrenalectomized patients lack these diurnal changes in pressure supporting a role for cortisol in intraocular pressure. The pressure increases due to a decreased outflow of the aqueous humour[85], the fluid in the anterior chamber. It is believed that GCs increase outflow resistance by inhibiting degradation of extracellular matrix material in the trabecular meshwork[86]. This is the tissue that separates the anterior chamber from Schlemm's canal, the canal that serves to deliver the humour to the bloodstream. There is substantial evidence that supports the deposition of extracellular matrix[87-91] but the underlying mechanism remains to be elucidated.

The central nervous system

GCs play an important role in the central nervous system (CNS) both in function and in homeostasis. The elevated levels that result from GC treatment or Cushing's syndrome can have profound effects on the anatomy of the brain and increase the risk of developing psychiatric diseases, cognitive impairment, mood changes and sleep disturbances[92]. GCs can pass the blood brain barrier and exert their effect anywhere in the brain. This includes binding the GR in cerebral neurons and glial cells but also binding of MR in the limbic system[93]. Effects of GCs on the nervous system include non-genomic effects such as the rapid (>2 min) changes in electrical activity of neurons[94]. Long term exposure affects regenerative sprouting of axons that follows differentiation of hippocampal neurons[95], one of the two places in the CNS that is allowed to regenerate. It may lead to apoptosis, atrophy and cell death in the neurons of the hippocampus[96].

These effects on the CNS may have serious implications. The hippocampus is pivotal for processing and the storage of memories[97]. A striking example is why some distinct memories are long lasting and in great detail while most other memories are only vaguely remembered for a short time. The cause lies in the elevation of cortisol levels during moments of arousal, stress or excitement. This mechanism of memory consolidation is very likely evolved as means to ensure survival, retaining information more easily during moments of stress or trauma[98]. However, chronic stress or chronically elevated cortisol levels in Addison's disease or during treatment, can induce amnesia[99] but also cause problems with visual-spatial processing, conditional response, reasoning and verbal fluency[100]. This is also reflected in Cushing patients who learn at a slower pace, have a smaller short-term memory volume, memory contamination and false appraisal of task performance[101] suggesting extrahippocampal effects of glucocorticoids on memory impairment such as the amygdala and prefrontal cortex. Both are involved in connecting emotions to experience.

When considering the use of GCs, adverse effects on behavior or emotional state are rarely contemplated. And yet 75% of patients treated with GCs experience mood disturbance, which in 5% is quiet severe[102]. These effects have been described since GCs entered the clinic in the early fifties. And unlike osteoporosis, muscle atrophy or the thinning of skin, adverse effects on the CNS can take as little as one dose. Adverse effects on the CNS include a wide range of cognitive and emotional effects, including agitation, anxiety, apathy, auditory and visual hallucinations, delusions, depression, distractibility, disturbances of body image, emotional lability, hypomania, insomnia, intermittent memory impairment, mutism, perplexity, pressured speech, and sensory flooding[103]. In rare cases patients can develop serious psychotic disorders or even delirium[104] and dementia[105]. But even if there is no doubt that these adverse effects are associated with the use of GCs, it is difficult to predict which patients are at risk. Certainly patients with a predisposition to a certain effect are more susceptible. Often the effects are limited, because the treatment is short, and reversible.

Others

The list of adverse events that accompany GC treatment is long. Some are rare and have not been studied in great detail. Often the effects are reversible and the inconvenience for the patient very limited. However there are a few adverse effects that were not discussed here that can have major implications for the patient.

GC treatment is associated with an increased risk of cardiovascular diseases[106]. In particular ischemic heart disease, heart failure and atherosclerosis. The latter is believed to be the result of increased lipid levels in the blood but studies yielded contradictory results.

GCs increase the chance of adverse events in the gastrointestinal tract[107] including gastritis, ulcer formation, and gastrointestinal bleeding. The increase risk is small and causes most likely relate to suppression of the immune system. GCs also increase the risk of pancreatitis. Underlying cause is unclear.

And finally the chance of infection should be taken in account[108]. GCs are excellent anti-inflammatory drugs that reduce the ability of both the innate- and the adaptive- immune system to deal with invading pathogens. Common bacteria that under normal conditions pose no threat can become a real issue. Normal signs of infection can be absent due to suppression of inflammatory mediators such as cytokines.

References

1. Barnes, P.J. and I.M. Adcock, *Glucocorticoid resistance in inflammatory diseases.* Lancet, 2009. **373**(9678): p. 1905-1917.
2. Myers, B., J.M. McKlveen, and J.P. Herman, *Glucocorticoid actions on synapses, circuits, and behavior: Implications for the energetics of stress.* Frontiers in Neuroendocrinology, 2014. **35**(2): p. 180-196.
3. Nicolaides, N.C., et al., *Circadian endocrine rhythms: the hypothalamic-pituitary-adrenal axis and its actions.* Steroids in Neuroendocrine Immunology and Therapy of Rheumatic Diseases Ii, 2014. **1318**: p. 71-80.

4. Cushing, H., *The basophil adenomas of the pituitary body and their clinical manifestations (pituitary basophilism). .* 1932.
5. Lee, M.J., et al., *Pathways regulated by glucocorticoids in omental and subcutaneous human adipose tissues: a microarray study.* American Journal of Physiology-Endocrinology and Metabolism, 2011. **300**(3): p. E571-E580.
6. Wang, Y.X., et al., *The human fatty acid synthase gene and de novo lipogenesis are coordinately regulated in human adipose tissue.* Journal of Nutrition, 2004. **134**(5): p. 1032-1038.
7. Campbell, J.E., et al., *Adipogenic and lipolytic effects of chronic glucocorticoid exposure.* American Journal of Physiology-Cell Physiology, 2011. **300**(1): p. C198-C209.
8. Kuo, T.Y., et al., *Genome-wide analysis of glucocorticoid receptor-binding sites in myotubes identifies gene networks modulating insulin signaling.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(28): p. 11160-11165.
9. Xu, C., et al., *Direct Effect of Glucocorticoids on Lipolysis in Adipocytes.* Molecular Endocrinology, 2009. **23**(8): p. 1161-1170.
10. Hoppmann, J., et al., *The balance between gluco- and mineralo-corticoid action critically determines inflammatory adipocyte responses.* Journal of Endocrinology, 2010. **204**(2): p. 153-164.
11. Patsouris, D., et al., *Glucocorticoids and Thiazolidinediones Interfere with Adipocyte-mediated Macrophage Chemotaxis and Recruitment.* Journal of Biological Chemistry, 2009. **284**(45): p. 31223-31235.
12. Yu, C.Y., et al., *Genome-Wide Analysis of Glucocorticoid Receptor Binding Regions in Adipocytes Reveal Gene Network Involved in Triglyceride Homeostasis.* PLoS One, 2010. **5**(12).
13. Kannisto, K., et al., *Overexpression of 11 beta-hydroxysteroid dehydrogenase-1 in adipose tissue is associated with acquired obesity and features of insulin resistance: Studies in young adult monozygotic twins.* Journal of Clinical Endocrinology & Metabolism, 2004. **89**(9): p. 4414-4421.
14. Masuzaki, H., et al., *A transgenic model of visceral obesity and the metabolic syndrome.* Science, 2001. **294**(5549): p. 2166-2170.
15. Bazhan, N. and D. Zelena, *Food-intake regulation during stress by the hypothalamo-pituitary-adrenal axis.* Brain Research Bulletin, 2013. **95**: p. 46-53.

16. Cossmann, M. and J. Welzel, *Evaluation of the atrophogenic potential of different glucocorticoids using optical coherence tomography, 20-MHz ultrasound and profilometry; a double-blind, placebo-controlled trial.* British Journal of Dermatology, 2006. **155**(4): p. 700-706.
17. Delforno, C., P.J.A. Holt, and R. Marks, *Corticosteroid Effect on Epidermal-Cell Size.* British Journal of Dermatology, 1978. **98**(6): p. 619-623.
18. Saarni, H., M. Tammi, and N.S. Doherty, *Decreased Hyaluronic-Acid Synthesis, a Sensitive Indicator of Cortisol Action on Fibroblast.* Journal of Pharmacy and Pharmacology, 1978. **30**(3): p. 200-201.
19. Lehmann, P.M., et al., *Corticosteroid Atrophy in Human-Skin - a Study by Light Scanning and Transmission Electron-Microscopy.* Clinical Research, 1983. **31**(2): p. A581-A581.
20. Sheu, H.M., et al., *Depletion of stratum corneum intercellular lipid lamellae and barrier function abnormalities after long-term topical corticosteroids.* British Journal of Dermatology, 1997. **136**(6): p. 884-890.
21. Kruse, N.J., et al., *Inhibitory Effects of Glucocorticoids on Collagen-Synthesis by Mouse Sponge Granulomas and Granuloma Fibroblasts in Culture.* Biochimica Et Biophysica Acta, 1978. **540**(1): p. 101-116.
22. Terao, M., et al., *11 beta-Hydroxysteroid Dehydrogenase 1 Specific Inhibitor Increased Dermal Collagen Content and Promotes Fibroblast Proliferation.* PLoS One, 2014. **9**(3).
23. Stojadinovic, O., et al., *Novel genomic effects of glucocorticoids in epidermal keratinocytes - Inhibition of apoptosis, interferon-gamma pathway, and wound healing along with promotion of terminal differentiation.* Journal of Biological Chemistry, 2007. **282**(6): p. 4021-4034.
24. Tiganescu, A., et al., *11 beta-Hydroxysteroid dehydrogenase blockade prevents age-induced skin structure and function defects.* Journal of Clinical Investigation, 2013. **123**(7): p. 3051-3060.
25. Henneicke, H., et al., *Glucocorticoids and bone: local effects and systemic implications.* Trends in Endocrinology and Metabolism, 2014. **25**(4): p. 197-211.
26. Bonaldo, P. and M. Sandri, *Cellular and molecular mechanisms of muscle atrophy.* Disease Models & Mechanisms, 2013. **6**(1): p. 25-39.

27. Schakman, O., et al., *Glucocorticoid-induced skeletal muscle atrophy.* International Journal of Biochemistry & Cell Biology, 2013. **45**(10): p. 2163-2172.
28. Zheng, B., et al., *FOXO3a mediates signaling crosstalk that coordinates ubiquitin and atrogin-1/MAFbx expression during glucocorticoid-induced skeletal muscle atrophy.* Faseb Journal, 2010. **24**(8): p. 2660-2669.
29. Wang, H.M., et al., *Dexamethasone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1.* Journal of Biological Chemistry, 2006. **281**(51): p. 39128-39134.
30. Dardevet, D., et al., *Glucocorticoid effects on insulin- and IGF-I-regulated muscle protein metabolism during aging.* Journal of Endocrinology, 1998. **156**(1): p. 83-89.
31. Ma, K., et al., *Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression.* American Journal of Physiology-Endocrinology and Metabolism, 2003. **285**(2): p. E363-E371.
32. Schakman, O., et al., *Insulin-like growth factor-I gene transfer by electroporation prevents skeletal muscle atrophy in glucocorticoid-treated rats.* Endocrinology, 2005. **146**(4): p. 1789-1797.
33. Gilson, H., et al., *Myostatin gene deletion prevents glucocorticoid-induced muscle atrophy.* Endocrinology, 2007. **148**(1): p. 452-460.
34. Grobet, L., et al., *Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene.* Genesis, 2003. **35**(4): p. 227-238.
35. Allen, D.L. and A.S. Loh, *Posttranscriptional mechanisms involving microRNA-27a and b contribute to fast-specific and glucocorticoid-mediated myostatin expression in skeletal muscle.* American Journal of Physiology-Cell Physiology, 2011. **300**(1): p. C124-C137.
36. Rauch, A., et al., *Glucocorticoids Suppress Bone Formation by Attenuating Osteoblast Differentiation via the Monomeric Glucocorticoid Receptor.* Cell Metabolism, 2010. **11**(6): p. 517-531.
37. Kalak, R., et al., *Endogenous glucocorticoid signalling in osteoblasts is necessary to maintain normal bone structure in mice.* Bone, 2009. **45**(1): p. 61-67.
38. Zhou, H., et al., *Osteoblasts directly control lineage commitment of mesenchymal progenitor cells through Wnt signaling.* Journal of Biological Chemistry, 2008. **283**(4): p. 1936-1945.

39. Zhou, H., et al., *Glucocorticoid-dependent Wnt signaling by mature osteoblasts is a key regulator of cranial skeletal development in mice.* Development, 2009. **136**(3): p. 427-436.
40. Locascio, V., et al., *Bone Loss in Response to Long-Term Glucocorticoid Therapy.* Bone and Mineral, 1990. **8**(1): p. 39-51.
41. O'Brien, C.A., et al., *Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength.* Endocrinology, 2004. **145**(4): p. 1835-1841.
42. Weinstein, R.S., et al., *Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids - Potential mechanisms of their deleterious effects on bone.* Journal of Clinical Investigation, 1998. **102**(2): p. 274-282.
43. Smith, E. and B. Frenkel, *Glucocorticoids inhibit the transcriptional activity of LEF/TCF in differentiating osteoblasts in a glycogen synthase kinase-3 beta-dependent and -independent manner.* Journal of Biological Chemistry, 2005. **280**(3): p. 2388-2394.
44. Mak, W., et al., *Biphasic Glucocorticoid-Dependent Regulation of Wnt Expression and Its Inhibitors in Mature Osteoblastic Cells.* Calcified Tissue International, 2009. **85**(6): p. 538-545.
45. Wang, F.S., et al., *Inhibition of glycogen synthase kinase-3 beta attenuates glucocorticoid-induced bone loss.* Life Sciences, 2009. **85**(19-20): p. 685-692.
46. Hayashi, K., et al., *BMP/Wnt antagonists are upregulated by dexamethasone in osteoblasts and reversed by alendronate and PTH: Potential therapeutic targets for glucocorticoid-induced osteoporosis.* Biochemical and Biophysical Research Communications, 2009. **379**(2): p. 261-266.
47. Rauch, A., et al., *An anti-inflammatory selective glucocorticoid receptor modulator preserves osteoblast differentiation.* Faseb Journal, 2011. **25**(4): p. 1323-1332.
48. Chang, J.K., et al., *Anti-inflammatory drugs suppress proliferation and induce apoptosis through altering expressions of cell cycle regulators and pro-apoptotic factors in cultured human osteoblasts.* Toxicology, 2009. **258**(2-3): p. 148-156.
49. Espina, B., et al., *Regulation of Bim in glucocorticoid-mediated osteoblast apoptosis.* Journal of Cellular Physiology, 2008. **215**(2): p. 488-496.
50. Weinstein, R.S., et al., *Intermittent Parathyroid Hormone Administration Counteracts the Adverse Effects of Glucocorticoids*

on Osteoblast and Osteocyte Viability, Bone Formation, and Strength in Mice. Endocrinology, 2010. **151**(6): p. 2641-2649.
51. Xia, X.C., et al., *Glucocorticoid-Induced Autophagy in Osteocytes.* Journal of Bone and Mineral Research, 2010. **25**(11): p. 2479-2488.
52. Jia, J.J., et al., *Glucocorticoid dose determines osteocyte cell fate.* Faseb Journal, 2011. **25**(10): p. 3366-3376.
53. Jia, D., et al., *Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density.* Endocrinology, 2006. **147**(12): p. 5592-5599.
54. Rose, A.J. and S. Herzig, *Metabolic control through glucocorticoid hormones: An update.* Molecular and Cellular Endocrinology, 2013. **380**(1-2): p. 65-78.
55. Mazziotti, G., C. Gazzaruso, and A. Giustina, *Diabetes in Cushing syndrome: basic and clinical aspects.* Trends in Endocrinology and Metabolism, 2011. **22**(12): p. 499-506.
56. Pivonello, R., et al., *Pathophysiology of Diabetes Mellitus in Cushing's Syndrome.* Neuroendocrinology, 2010. **92**: p. 77-81.
57. van Raalte, D.H., D.M. Ouwens, and M. Diamant, *Novel insights into glucocorticoid-mediated diabetogenic effects: towards expansion of therapeutic options?* European Journal of Clinical Investigation, 2009. **39**(2): p. 81-93.
58. Seino, S., T. Shibasaki, and K. Minami, *Pancreatic beta-cell signaling: toward better understanding of diabetes and its treatment.* Proceedings of the Japan Academy Series B-Physical and Biological Sciences, 2010. **86**(6): p. 563-577.
59. Folli, F., M.J.A. Saad, and C.R. Kahn, *Insulin receptor/IRS-1/PI 3-kinase signaling system in corticosteroid-induced insulin resistance.* Acta Diabetologica, 1996. **33**(3): p. 185-192.
60. Verhees, K.J.P., et al., *Glycogen synthase kinase-3 beta is required for the induction of skeletal muscle atrophy.* American Journal of Physiology-Cell Physiology, 2011. **301**(5): p. C995-C1007.
61. Etxabe, J. and J.A. Vazquez, *Morbidity and Mortality in Cushings-Disease - an Epidemiologic Approach.* Clinical Endocrinology, 1994. **40**(4): p. 479-484.
62. Cicala, M.V. and F. Mantero, *Hypertension in Cushing's Syndrome: From Pathogenesis to Treatment.* Neuroendocrinology, 2010. **92**: p. 44-49.
63. De Wachter, E., et al., *Rapidly developing Cushing syndrome in a 4-year-old patient during combined treatment with itraconazole and*

inhaled budesonide. European Journal of Pediatrics, 2003. **162**(7-8): p. 488-489.
64. Bertagna, X., et al., *Pituitary-Adrenal Response to the Antiglucocorticoid Action of Ru-486 in Cushings-Syndrome.* Journal of Clinical Endocrinology & Metabolism, 1986. **63**(3): p. 639-643.
65. Nieman, L.K., et al., *Successful Treatment of Cushings-Syndrome with the Glucocorticoid Antagonist Ru-486.* Journal of Clinical Endocrinology & Metabolism, 1985. **61**(3): p. 536-540.
66. Mangos, G.J., et al., *Glucocorticoids and the kidney.* Nephrology, 2003. **8**(6): p. 267-273.
67. Freiberg, J.M., J.L. Kinsella, and B. Sacktor, *Glucocorticoid Effects on Na+-H+ Exchange and Na+-Phosphate (Pi) and Na+-Glucose Co-Transports in Isolated Renal Brush-Border Membrane-Vesicles (Bb).* Federation Proceedings, 1982. **41**(5): p. 1494-1494.
68. Kinsella, J., T. Cujdik, and B. Sacktor, *Na+-H+ Exchange Activity in Renal Brush-Border Membrane-Vesicles in Response to Metabolic-Acidosis - the Role of Glucocorticoids.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1984. **81**(2): p. 630-634.
69. Rodriguez, H.J., et al., *Regulation of Renal Na+-K+-Atpase in the Rat by Adrenal-Steroids.* American Journal of Physiology, 1981. **241**(2): p. F186-F195.
70. Welbourne, T.C., *Glucocorticoid Control of Ammoniagenesis in the Proximal Tubule.* Seminars in Nephrology, 1990. **10**(4): p. 339-349.
71. Goodwin, J.E., J.H. Zhang, and D.S. Geller, *A critical role for vascular smooth muscle in acute glucocorticoid-induced hypertension.* Journal of the American Society of Nephrology, 2008. **19**(7): p. 1291-1299.
72. Molnar, G.A., et al., *Glucocorticoid-related signaling effects in vascular smooth muscle cells.* Journal of Hypertension, 2008. **26**: p. S5-S6.
73. Goodwin, J.E., et al., *Knockout of the vascular endothelial glucocorticoid receptor abrogates dexamethasone-induced hypertension.* Journal of Hypertension, 2011. **29**(7): p. 1347-1356.
74. Ong, S.L.H., et al., *Hemodynamics of dexamethasone-induced hypertension in the rat.* Hypertension Research, 2009. **32**(10): p. 889-894.

75. Carnahan, M.C. and D.A. Goldstein, *Ocular complications of topical, peri-ocular, and systemic corticosteroids.* Current Opinion in Ophthalmology, 2000. **11**(6): p. 478-83.
76. Urban, R.C., Jr. and E. Cotlier, *Corticosteroid-induced cataracts.* Survey of Ophthalmology, 1986. **31**(2): p. 102-10.
77. James, E.R., et al., *Presence of a transcriptionally active glucocorticoid receptor alpha in lens epithelial cells.* Invest Ophthalmol Vis Sci, 2003. **44**(12): p. 5269-76.
78. Gupta, V., et al., *Global gene profiling reveals novel glucocorticoid induced changes in gene expression of human lens epithelial cells.* Molecular Vision, 2005. **11**(121-22): p. 1018-1040.
79. Greiner, J.V. and L.T. Chylack, *Posterior Subcapsular Cataracts - Histopathologic Study of Steroid-Associated Cataracts.* Archives of Ophthalmology, 1979. **97**(1): p. 135-144.
80. Spector, A., *Oxidative Stress-Induced Cataract - Mechanism of Action.* Faseb Journal, 1995. **9**(12): p. 1173-1182.
81. Dickerson, J.E., E. Dotzel, and A.F. Clark, *Steroid-induced cataract: New perspectives from in vitro and lens culture studies.* Experimental Eye Research, 1997. **65**(4): p. 507-516.
82. Bucala, R., et al., *Glucocorticoid Lens Protein Adducts in Experimentally Induced Steroid Cataracts.* Experimental Eye Research, 1985. **40**(6): p. 853-863.
83. Kersey, J.P. and D.C. Broadway, *Corticosteroid-induced glaucoma: a review of the literature.* Eye, 2006. **20**(4): p. 407-416.
84. Smith, C.L., *"Corticosteroid glaucoma" a summary and review of the literaure.* American Journal of the Medical Sciences, 1966. **252**(2): p. 239-44.
85. Renfro, L. and J.S. Snow, *Ocular effects of topical and systemic steroids.* Dermatol Clin, 1992. **10**(3): p. 505-12.
86. Wordinger, R.J. and A.F. Clark, *Effects of glucocorticoids on the trabecular meshwork: towards a better understanding of glaucoma.* Prog Retin Eye Res, 1999. **18**(5): p. 629-67.
87. McCarty, G.R. and B. Schwartz, *Increased concentration of glucocorticoid receptors in rabbit iris--ciliary body compared to rabbit liver.* Invest Ophthalmol Vis Sci, 1982. **23**(4): p. 525-8.
88. Tripathi, B.J., R.C. Tripathi, and H.H. Swift, *Hydrocortisone-induced DNA endoreplication in human trabecular cells in vitro.* Experimental Eye Research, 1989. **49**(2): p. 259-70.

89. Weinreb, R.N., M.D. Mitchell, and J.R. Polansky, *Prostaglandin production by human trabecular cells: in vitro inhibition by dexamethasone.* Invest Ophthalmol Vis Sci, 1983. **24**(12): p. 1541-5.
90. Wilson, K., et al., *Dexamethasone induced ultrastructural changes in cultured human trabecular meshwork cells.* Curr Eye Res, 1993. **12**(9): p. 783-93.
91. Yue, B.Y., *The extracellular matrix and its modulation in the trabecular meshwork.* Survey of Ophthalmology, 1996. **40**(5): p. 379-90.
92. Bourdeau, I., et al., *Cognitive function and cerebral assessment in patients who have Cushing's syndrome.* Endocrinol Metab Clin North Am, 2005. **34**(2): p. 357-69, ix.
93. De Kloet, E.R., et al., *Brain corticosteroid receptor balance in health and disease.* Endocrine Reviews, 1998. **19**(3): p. 269-301.
94. Samarasinghe, R.A., et al., *Nongenomic glucocorticoid receptor action regulates gap junction intercellular communication and neural progenitor cell proliferation.* Proc Natl Acad Sci U S A, 2011. **108**(40): p. 16657-62.
95. Scheff, S.W. and C.W. Cotman, *Chronic glucocorticoid therapy alters axon sprouting in the hippocampal dentate gyrus.* Exp Neurol, 1982. **76**(3): p. 644-54.
96. Crochemore, C., et al., *Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation.* Mol Psychiatry, 2005. **10**(8): p. 790-8.
97. Bannerman, D.M., et al., *Regional dissociations within the hippocampus--memory and anxiety.* Neurosci Biobehav Rev, 2004. **28**(3): p. 273-83.
98. Reul, J.M., *Making memories of stressful events: a journey along epigenetic, gene transcription, and signaling pathways.* Front Psychiatry, 2014. **5**: p. 5.
99. Nagaraja, N., et al., *Glucocorticoid mechanisms may contribute to ECT-induced retrograde amnesia.* Psychopharmacology (Berl), 2007. **190**(1): p. 73-80.
100. Arnsten, A.F., *Stress signalling pathways that impair prefrontal cortex structure and function.* Nat Rev Neurosci, 2009. **10**(6): p. 410-22.
101. Leon-Carrion, J., et al., *A clinical profile of memory impairment in humans due to endogenous glucocorticoid excess.* Clin Endocrinol (Oxf), 2009. **70**(2): p. 192-200.

102. Sirois, F., *Steroid psychosis: a review.* Gen Hosp Psychiatry, 2003. **25**(1): p. 27-33.
103. Hall, R.C., et al., *Presentation of the steroid psychoses.* J Nerv Ment Dis, 1979. **167**(4): p. 229-36.
104. Lewis, D.A. and R.E. Smith, *Steroid-induced psychiatric syndromes. A report of 14 cases and a review of the literature.* J Affect Disord, 1983. **5**(4): p. 319-32.
105. Wolkowitz, O.M., et al., *The "steroid dementia syndrome": an unrecognized complication of glucocorticoid treatment.* Ann N Y Acad Sci, 2004. **1032**: p. 191-4.
106. Walker, B.R., *Glucocorticoids and cardiovascular disease.* European Journal of Endocrinology, 2007. **157**(5): p. 545-59.
107. Rogler, G., *Gastrointestinal and liver adverse effects of drugs used for treating IBD.* Best Pract Res Clin Gastroenterol, 2010. **24**(2): p. 157-65.
108. Stuck, A.E., C.E. Minder, and F.J. Frey, *Risk of infectious complications in patients taking glucocorticosteroids.* Rev Infect Dis, 1989. **11**(6): p. 954-63.

4

Glucocorticoid analogue development; things to consider…

Considering the numerous side effects and loss of response, todays GC analogues are far from ideal. They are very powerful immunosuppressants and indeed, when it comes down to the treatment of inflammation it may be difficult to discover a more effective alternative. But loss of response and adverse effects are serious problems that can limit clinical application. With a worldwide rise in the incidence of allergies[1] and autoimmune diseases[2], the need for powerful immunosuppressant's with a favorable safety profile will only intensify. The chance of finding a more potent immunosuppressant seems very slim as GCs are an integral part of human design. So perhaps the most successful approach remains design of an alternative GC. And indeed, the current list of synthetic GC analogues is long. The search for GCs with a more favorable safety profile is a continuous search that started decades ago[3]. Although this has not led to the discovery of an ideal immunosuppressant it did improve GCs for specific conditions such as eczema or asthma. But more importantly, it provided a treasure of information on GC action.

Glucocorticoids are small lipophilic molecules that can pass the cellular membrane unhindered. In general GCs act through binding to their cognate receptor, the glucocorticoid receptor (GR). This receptor is expressed by virtually all tissue types, where it resides in the cytoplasm when not bound to a ligand. In this resting state the GR is associated with a complex of chaperone proteins, which stabilize the GR in a conformation that allows binding of ligand. Classic GC action involves binding to the GR followed by dissociation of that GR from its chaperone complex exposing nuclear localization sequences. This causes the GR to be shuttled into the nucleus where, similar to other nuclear hormone receptors, it acts as a ligand activated transcription factor. Binding of an agonistic ligand causes the receptor to dimerize and translocate to the nucleus where it regulates particular genes but the GR has also been reported to enter the nucleus as a monomer. Which action follows binding of a ligand is dependent on many factors, such as the type of ligand it binds and the proteins it associates with. The latter is dependent on the tissue it is expressed in and the state that tissue is in. Moreover, it is very likely that associated proteins

predispose for a particular ligand and that binding of a certain ligand favors particular associations[4, 5].

Similar to other receptors of the nuclear hormone family, the glucocorticoid receptor consists of three domains[6]. From beginning to end the receptor consist of an N-terminal domain, a central DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD). The N-terminal domain contains activation function 1 (AF-1), an unstructured region that can interact with transcription factors such as AF-1 or TATA box binding protein (TBP)[7, 8]. It is not entirely clear if binding of transcription factors define the conformation of this region or whether the conformation of this region determines the transcription factors that can bind to it. The DBD is the part of the receptor responsible for the interaction with DNA. Two zinc-finger domains in the DBD allow for direct binding to palindromic glucocorticoid responsive elements present in promoter regions of genes[9]. And finally the LBD is the part of the receptor that can bind ligand. It too contains a motif for transcriptional activation termed AF-2. But unlike AF-1 this region is more organized, into a structure which is commonly referred to as the binding pocket. Apart from ligand, it can also associate with chaperones and coactivators[10, 11].

The best described and most extensively studied mechanism of GC action is direct binding of GR homodimers to structures in the DNA known as glucocorticoid responsive elements (GRE). These elements consist of a particular set of nucleotides (TGTTCTnnnAGAACA) forming palindromic structures within the promoter region of genes[12]. Both parts of the homodimer bind on each side of the palindrome. This interaction stimulates transcription of target genes and is commonly referred to as transactivation. Many adverse effects associated with GC treatment are attributed to transactivation[13]. Suppression of the immune response, on the other hand, is in general the result of inhibition of transcription of pro-inflammatory genes, termed transrepression. Initially it was believed that this process was regulated through negative GREs able to inhibit transcription through direct binding of the GR to DNA, for example by blocking binding of transcription factors[14]. And although this has been

shown for some genes, in most cases transrepression relies on a process of tethering. The GR interacts with transcription factors directly and influences their activity[15].

The dichotomy in GR action has given the impression that the possibility exists of creating a GR ligand that discriminates between induction and repression of genes. Such a selective glucocorticoid receptor agonists (SEGRA) would have the benefit of providing immune suppression without adverse effects[16]. This idea stems from the development of the GR^{dim} mouse. This mouse strain expresses a GR variant that contains a mutation in the dimerization domain disabling formation of homodimers, theoretically blocking transactivation[17]. Unlike mice in which the GR is knocked out, GR^{dim} are viable, suggesting that transactivation is an expendable part of GR action and that other effects, such as transrepression, are more important. In the wake of this finding, many set out to develop SEGRAs but more than 20 years of research and development did not provide a true SEGRA. Nevertheless, SEGRAs for treatment of specific conditions such as rheumatoid arthritis[18] or atopic dermatitis[19] have been developed. Although these are not completely free from adverse effects they do provide an improved alternative.

In recent years it has become increasingly clear that GC action cannot be divided in good and bad by separating in transactivation and transrepression. First off, transactivation does not always lead to adverse effects. For example GC activate the anti-inflammatory genes IκBα[20], GILZ[21] and Annexin-A1[22], which are all considered to have an anti-inflammatory effect. Secondly, transactivation does not always require the GR to homodimerize. In one report it was demonstrated that mutations in the dimerization domain of the GR actually increased expression of the phenylethanolamine N-methyltransferase (PNMT) gene[23]. The promoter sequence of this gene doesn't contain a normal palindromic GRE but interspaced half GREs. Mutation of one of these half GREs has little effect on promoter activity but when mutations are inserted in two or more, the activity is dramatically decreased. It is assumed that the GR forms multimers by protein-protein interactions. And lastly, the GR is able to

form heterodimers with other related nuclear hormone receptors such as the androgen receptor or mineralocorticoid receptor resulting in a complex that is able to regulate genes in a fashion distinct from GR homodimers[24]. Formation of these dimers is not dependent on the GR dimerization domain as they can form even when the dimerization domain is mutated.

Gene regulation is an important and powerful way for GC to affect cells. But this is not the only way in which GCs can act. With high concentrations, GCs can cause rapid effects that do not require regulation of genes, so-called non-genomic or non-transcriptional GC effects[25]. For example dexamethasone causes an acute rise in eNOS production, an effect that does not require the receptor to enter the nucleus and is dependent on the PI3/Akt pathway[26]. But not all non-genomic effects include the GR and sometimes a distinction is made between specific non-genomic GC effects and non-specific non-genomic GC effects[27]. An example is the fast depolarization of neurons upon GC administration, which is considered to be an effect of GCs on the cellular membrane[28].

The glucocorticoid receptor is not a rigid molecule. It changes conformation upon binding to a ligand or when it associates with another molecule[29]. A very clear example is exposure of the nuclear translocation signal. A ligand binding the GR causes a conformational change uncovering motives on the GR that function as nuclear translocation signals. As a result the GR is shuttled into the nucleus[30]. Likewise, binding of ligand facilitates association with co-activators (TIF2[31], NCoR[32], SCR-1[33]), molecules that are characterized by socalled LXXLL motives[34], able to interact with the ligand binding domain of the GR. Association with co-activators affects regulation of genes by the GR. TIF2 is known to acylate histones, making DNA more accessible to the GR whereas NCoR recruits histone deacetylases causing the DNA to be more condense and thus making DNA less accessible to the GR. Which coactivator can bind is dependent on how the ligand shapes the GR ligand binding pocket.

But it is not only binding of ligands that affect the GR conformation. Association with proteins can affect the conformation as well. The best described mechanism is the interaction with the chaperone protein Hsp90 which causes the GR to take on a shape that increases its ligand binding affinity[35].

It is very likely that every interaction of the GR with other molecules will affect its conformation. The resulting change may alter its function, spatial distribution within the cell or limit the molecular partners it can interact with or even the ligands it can bind. In a ligand free environment the conformation would be determined primarily by the associated proteins, a factor that is dependent on the cell type the GR is expressed in. Often the GR is part of a multi protein complex, which can contain kinases (Src[36]) or even receptors (RXRB[37], TCR[38]). However, most of the proteins associated with the GR that have been identified so far are co-activators (NCOA1-4)[39], proteins that interact with transcription factors (BAG1[40], CREBBP[41]) or transcription factors (STAT3[42], STAT5[43]), all of which associate after activation of the receptor. These interactions determine which genes are regulated by allowing interaction with specific transcription factor, blocking access to DNA by direct interaction with GREs or allowing easier access to certain genes by relaxing the chromatin structure. In a similar fashion these interactions affect the amount of transcript that is produced.

The complexity of GC action makes it very difficult to develop a screening assay that will allow for discovery of an overall better GC. On the one hand it will have to account for the different ways in which GCs affect a cell (genomic and non-genomic) and on the other hand it will have to correct for the type of cell and possibly even the condition that cell is in (GC action varies depending on the environment). Obviously a screening assay that simply focuses on high affinity for the GR will improve the anti-inflammatory effect but increase adverse effects equally. Screening in a cancer cell line will provide results that will probably only translate back to that particular cell line. Using animals as a screening tool, apart from ethics, is rather expensive, time consuming and results do not always

translate to the human situation. So perhaps, with the current understandings, the best approach for developing better GC analogues would be to focus on a particular condition or a specific set of target cells.

The strategy presented in this thesis is based on the hypothesis that most effects of GC treatment are the result of GR mediated transcriptional regulation. This includes both the anti-inflammatory effects and the adverse effects. However, we found that GC mediated T cell inhibition is, at least in part, regulated through a non-genomic effect. We therefore decided to use this as a model, focusing on non-genomic inhibition of T cell proliferation while at the same time screening for transactivation with the goal to develop a GC analogue that inhibits T cell proliferation non-genomically without resulting in GC induced transactivation. This is in theory a sound strategy because most of the adverse effects are indeed the result of GR mediated transcriptional regulation. However, future research may reveal many more non-genomic effects of GC treatment, including effects that are related to adverse effects. Currently such effects have only been reported for the rapid membrane depolarization of neuronal membranes.

References

1. Pawankar, R., et al., *Allergic diseases and asthma: a major global health concern.* Current Opinion in Allergy and Clinical Immunology, 2012. **12**(1): p. 39-41.
2. Bach, J.F., *Mechanisms of disease: The effect of infections on susceptibility to autoimmune and allergic diseases.* New England Journal of Medicine, 2002. **347**(12): p. 911-920.
3. McMaster, A. and D.W. Ray, *Modelling the glucocorticoid receptor and producing therapeutic agents with anti-inflammatory effects but reduced side-effects.* Experimental Physiology, 2007. **92**(2): p. 299-309.
4. Croxtall, J.D., et al., *Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells.* British Journal of Pharmacology, 2002. **135**(2): p. 511-519.
5. Elmore, S.W., et al., *Differentiation of in vitro transcriptional repression and activation profiles of selective glucocorticoid*

modulators. Bioorganic & Medicinal Chemistry Letters, 2004. **14**(7): p. 1721-1727.
6. Nicolaides, N.C., et al., *The human glucocorticoid receptor: Molecular basis of biologic function.* Steroids, 2010. **75**(1): p. 1-12.
7. Kumar, R., et al., *TATA box binding protein induces structure in the recombinant glucocorticoid receptor AF1 domain.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(47): p. 16425-16430.
8. Warnmark, A., et al., *Activation functions 1 and 2 of nuclear receptors: Molecular strategies for transcriptional activation.* Molecular Endocrinology, 2003. **17**(10): p. 1901-1909.
9. Hard, T., et al., *Solution Structure of the Glucocorticoid Receptor DNA-Binding Domain.* Science, 1990. **249**(4965): p. 157-160.
10. Bledsoe, R.K., et al., *Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition.* Cell, 2002. **110**(1): p. 93-105.
11. Simons, S.S., *Glucocorticoid receptor cofactors as therapeutic targets.* Current Opinion in Pharmacology, 2010. **10**(6): p. 613-619.
12. Beato, M., et al., *DNA Regulatory Elements for Steroid-Hormones.* Journal of Steroid Biochemistry and Molecular Biology, 1989. **32**(5): p. 737-748.
13. Schulz, M. and M. Eggert, *Novel ligands: Fine tuning the transcriptional activity of the glucocorticoid receptor.* Current Pharmaceutical Design, 2004. **10**(23): p. 2817-2826.
14. Barnes, P.J. and I. Adcock, *Antiinflammatory Actions of Steroids - Molecular Mechanisms.* Trends in Pharmacological Sciences, 1993. **14**(12): p. 436-441.
15. Ratman, D., et al., *How glucocorticoid receptors modulate the activity of other transcription factors: A scope beyond tethering.* Molecular and Cellular Endocrinology, 2013. **380**(1-2): p. 41-54.
16. Schacke, H., et al., *SEGRAs: a novel class of anti-inflammatory compounds.* Ernst Schering Res Found Workshop, 2002(40): p. 357-71.
17. Reichardt, H.M., et al., *DNA binding of the glucocorticoid receptor is not essential for survival.* Cell, 1998. **93**(4): p. 531-41.
18. Hu, X., et al., *The Antagonists But Not Partial Agonists of Glucocorticoid Receptor Ligands Show Substantial Side Effect Dissociation.* Endocrinology, 2011. **152**(8): p. 3123-3134.

19. Schacke, H., et al., *Characterization of ZK 245186, a novel, selective glucocorticoid receptor agonist for the topical treatment of inflammatory skin diseases.* Br J Pharmacol, 2009. **158**(4): p. 1088-103.
20. Deroo, B.J. and T.K. Archer, *Glucocorticoid receptor activation of the I kappa B alpha promoter within chromatin.* Molecular Biology of the Cell, 2001. **12**(11): p. 3365-3374.
21. Ayroldi, E., A. Macchiarulo, and C. Riccardi, *Targeting glucocorticoid side effects: selective glucocorticoid receptor modulator or glucocorticoid-induced leucine zipper? A perspective.* Faseb Journal, 2014.
22. Perretti, M. and F. D'Acquisto, *Annexin A1 and glucocorticoids as effectors of the resolution of inflammation.* Nat Rev Immunol, 2009. **9**(1): p. 62-70.
23. Adams, M., et al., *Homodimerization of the glucocorticoid receptor is not essential for response element binding: activation of the phenylethanolamine N-methyltransferase gene by dimerization-defective mutants.* Molecular Endocrinology, 2003. **17**(12): p. 2583-92.
24. Savory, J.G., et al., *Glucocorticoid receptor homodimers and glucocorticoid-mineralocorticoid receptor heterodimers form in the cytoplasm through alternative dimerization interfaces.* Molecular and Cellular Biology, 2001. **21**(3): p. 781-93.
25. Stahn, C. and F. Buttgereit, *Genomic and nongenomic effects of glucocorticoids.* Nat Clin Pract Rheumatol, 2008. **4**(10): p. 525-33.
26. Murata, I., et al., *Acute lethal crush-injured rats can be successfully rescued by a single injection of high-dose dexamethasone through a pathway involving PI3K-Akt-eNOS signaling.* J Trauma Acute Care Surg, 2013. **75**(2): p. 241-9.
27. Song, I.H. and F. Buttgereit, *Non-genomic glucocorticoid effects to provide the basis for new drug developments.* Molecular and Cellular Endocrinology, 2006. **246**(1-2): p. 142-6.
28. Makara, G.B. and J. Haller, *Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications.* Prog Neurobiol, 2001. **65**(4): p. 367-90.
29. Veleiro, A.S., et al., *Structure of the glucocorticoid receptor, a flexible protein that can adapt to different ligands.* ChemMedChem, 2010. **5**(5): p. 649-59.

30. Vandevyver, S., L. Dejager, and C. Libert, *On the trail of the glucocorticoid receptor: into the nucleus and back.* Traffic, 2012. **13**(3): p. 364-74.
31. Voegel, J.J., et al., *TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors.* EMBO J, 1996. **15**(14): p. 3667-75.
32. Glass, C.K. and M.G. Rosenfeld, *The coregulator exchange in transcriptional functions of nuclear receptors.* Genes Dev, 2000. **14**(2): p. 121-41.
33. Onate, S.A., et al., *Sequence and characterization of a coactivator for the steroid hormone receptor superfamily.* Science, 1995. **270**(5240): p. 1354-7.
34. Heery, D.M., et al., *A signature motif in transcriptional co-activators mediates binding to nuclear receptors.* Nature, 1997. **387**(6634): p. 733-6.
35. Dittmar, K.D. and W.B. Pratt, *Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation.* Journal of Biological Chemistry, 1997. **272**(20): p. 13047-54.
36. Marchetti, M.C., et al., *Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation.* Blood, 2003. **101**(2): p. 585-93.
37. Ravasi, T., et al., *An atlas of combinatorial transcriptional regulation in mouse and man.* Cell, 2010. **140**(5): p. 744-52.
38. Lowenberg, M., et al., *Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression.* Trends in Molecular Medicine, 2007. **13**(4): p. 158-63.
39. Heitzer, M.D., et al., *Glucocorticoid receptor physiology.* Rev Endocr Metab Disord, 2007. **8**(4): p. 321-30.
40. Zhou, R., et al., *The anti-apoptotic, glucocorticoid receptor cochaperone protein BAG-1 is a long-term target for the actions of mood stabilizers.* J Neurosci, 2005. **25**(18): p. 4493-502.
41. Govindan, M.V., *Recruitment of cAMP-response element-binding protein and histone deacetylase has opposite effects on glucocorticoid receptor gene transcription.* Journal of Biological Chemistry, 2010. **285**(7): p. 4489-510.

42. Zhang, Z., et al., *STAT3 acts as a co-activator of glucocorticoid receptor signaling.* Journal of Biological Chemistry, 1997. **272**(49): p. 30607-10.
43. Stocklin, E., et al., *Functional interactions between Stat5 and the glucocorticoid receptor.* Nature, 1996. **383**(6602): p. 726-8.

PART II

GLUCOCORTICOIDS

5

Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN

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Abstract

Although glucocorticoid (GC)-induced nongenomic effects have been reported, the underlying mechanisms remain unexplained. We previously described that lymphocyte-specific protein tyrosine kinase (LCK) and FYN oncogene related to SRC, FGR, YES (FYN) mediate GC-induced inhibition of T-cell-receptor (TCR) signalling. Here we characterize the underlying molecular mechanism. The present study shows that the GC receptor is part of a TCR-linked multiprotein complex containing heat-shock protein (HSP)90, LCK and FYN, which is essential for TCR-dependent LCK/FYN activation. Experiments with cells transfected with GC-receptor short interfering RNA (siRNA) showed that the GC receptor is an essential component of the TCR signalling complex. Short-term GC treatment induces dissociation of this protein complex, resulting in impaired TCR signalling as a consequence of abrogated LCK/FYN activation. HSP90siRNA-transfected cells are not able to assemble this TCR-associated multiprotein complex, and accordingly HSP90siRNA treatment mimics GC effects on LCK/FYN activities. These observations support a model for nongenomic GC-induced immunosuppression on the basis of dissolution of membrane-bound GC-receptor multiprotein complexes after GC-receptor ligation.

Introduction

Glucocorticoids (GCs) mediate their immunosuppressive effects through cytosolic ligand-inducible receptors (Buttgereit et al, 2004). Inactive GC receptors (GRs) are associated with (co)chaperones, such as heat-shock protein 90 (HSP90), which dissociate after GR ligation, followed by nuclear translocation of GR and regulation of gene transcription (Franchimont, 2004; Pratt et al, 2004). GCs also evoke nongenomic effects on cellular function, which occur in minutes (Baus et al, 1996; Croxtall et al, 2000; Rhen & Cidlowski, 2005). Cardiovascular protective effects of GCs that could not be mediated by genomic mechanisms have been reported, because they occurred too fast and could not be blocked by a transcription inhibitor (Hafezi-Moghadam et al, 2002). Recently, we generated a comprehensive profile of such GC-induced rapid effects on signal transduction using activated human CD4+ T lymphocytes and a peptide array for kinome analysis (Lowenberg et al, 2005). The results showed marked early effects of GC treatment, in particular suppressed phosphorylation of LCK/FYN kinase consensus substrates. Further studies showed impaired recruitment of LCK and FYN to the T-cell-receptor (TCR) complex, resulting in reduced LCK/FYN enzymatic activities and impaired TCR signalling after short-term GC treatment. Although these studies identified LCK and FYN as cellular targets for nongenomic GC activities, the underlying mechanism remains unexplained.

LCK and FYN are members of the SRC family of non-receptor tyrosine kinases and have a key role in TCR signalling. TCR activation results in membrane translocation of LCK and FYN in an HSP90-dependent manner (Bijlmakers & Marsh, 2000; Zamoyska et al, 2003; Palacios & Weiss, 2004). LCK predominantly associates with CD4 or CD8 cell-surface receptors and FYN binds to CD3 co-receptors, resulting in LCK and FYN kinase activation. Once activated, LCK and FYN phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) on the TCR, allowing downstream signal transduction and eventually T-cell cytokine production, proliferation and differentiation. GRs and SRC-like kinases share a requirement for HSP90 for proper functioning, opening the possibility that GR action on LCK and FYN

activities is mediated by HSP90. The present study shows that GCs cause disruption of TCR-associated multiprotein complexes containing GR, HSP90, LCK and FYN, leading to reduced LCK/FYN enzymatic activities and impaired TCR signalling.

Results

DEX inhibits GR–HSP90–LCK/FYN interactions

We examined the action of short-term treatment with the synthetic fluorinated GC dexamethasone (DEX) on the physical interactions of HSP90–LCK and HSP90–FYN. Cell lysates were subjected to LCK and FYN immunoprecipitation and analysed on western blot for the presence of HSP90. These experiments showed HSP90–LCK and HSP90–FYN interactions (Fig 1A), confirming previous studies (Hartson et al, 1996; Bijlmakers & Marsh, 2000; Yun & Matts, 2005). DEX treatment (30 min) resulted in the disappearance of these HSP90–LCK and HSP90–FYN complexes, suggesting that DEX rapidly inhibits HSP90–LCK and HSP90–FYN associations. Next, we investigated whether these DEX-sensitive HSP90–LCK and HSP90–FYN complexes contained GR. Cells were pretreated for 10 min with DEX or dimethyl sulphoxide (DMSO), followed by 15 min activation using CD3 and CD28 antibodies. LCK and FYN immunoprecipitates were immunoblotted for the presence of GR, showing GR–LCK and GR–FYN complexes in activated cells, and these associations disappeared after short-term DEX treatment (Fig 1B). These observations indicate that LCK, FYN, HSP90 and GR are part of a multiprotein complex the integrity of which is sensitive to DEX treatment.

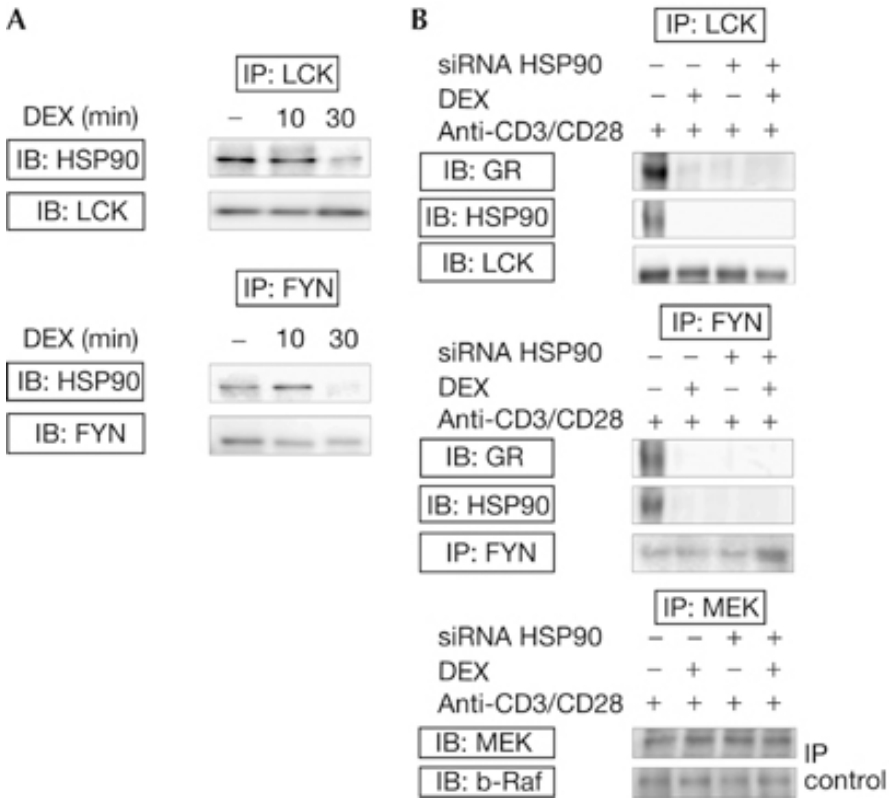

Figure 1.

Dexamethasone rapidly inhibits GR–HSP90–LCK and GR–HSP90–FYN associations. (A) Cells were incubated for 10 or 30 min with DEX (1 μM) or solvent-supplemented media (−). LCK and FYN immunoprecipitates were analysed on western blot for the presence of HSP90, and total LCK/FYN levels were analysed to test for equal protein loading. (B) HSP90siRNA-transfected or nontransfected cells were pretreated for 10 min without (−) or with (+) DEX and activated for 15 min with CD3 and CD28 antibodies. Lysates were subjected to LCK and FYN immunoprecipitation followed by western blotting for the presence of GR or HSP90. Equal loading was verified using LCK and FYN antibodies. Supernatants were subjected to immunoprecipitation using MEK antibody, followed by immunoblotting for MEK and b-Raf (negative immunoprecipitation control). Three independent

experiments were performed and reproducible results were obtained. DEX, dexamethasone; FYN, FYN oncogene related to SRC, FGR, YES; GR, glucocorticoid receptor; HSP90, heat-shock protein 90; IB, immunoblotting; IP, immunoprecipitation; LCK, lymphocyte-specific protein tyrosine kinase; MEK, mitogen-activated protein kinase kinase.

GR and HSP90-dependent LCK–CD4 or FYN–CD3 binding

HSP90-dependent cellular distribution of LCK and FYN is essential for efficient TCR signalling. To determine the role of HSP90 in LCK–CD4 and FYN–CD3 associations, cells were transfected with HSP90 short interfering RNA (HSP90siRNA), followed by 10 min DEX pretreatment and 15 min activation. LCK–CD4 and FYN–CD3 interactions were detected in CD4 and CD3 immunoprecipitates prepared from activated cells (Fig 2A). These interactions were disrupted on treatment with HSP90siRNA, irrespective of DEX stimulation. Furthermore, GR–CD4 and GR–CD3 associations were seen in activated cells, and treatment with DEX or HSP90siRNA resulted in reduced GR–CD4 and GR–CD3 binding (Fig 2A). Cell lysates were subjected to GR immunoprecipitation, confirming DEX- and HSP90-sensitive GR–LCK and GR–CD4 interactions (supplementary Fig 1 online). In addition, cells were pretreated with the HSP90 inhibitor geldanamycin for 30 min, followed by 10 min DEX treatment and 15 min stimulation. LCK and FYN immunoprecipitates were immunoblotted for the presence of GR, confirming reduced LCK–GR and FYN–GR bindings owing to HSP90 inhibition (Fig 2B). These observations indicate that TCR-associated multiprotein complexes are formed in activated cells (containing GR, HSP90, LCK and FYN), which depend on the presence of HSP90, and are disrupted on short-term DEX incubation. Complex dissolution does not lead to LCK or FYN degradation, as total LCK/FYN protein levels were not affected by HSP90siRNA or DEX (data not shown). Given that HSP90 associates readily with unfolded proteins (such as ZAP70) and can therefore be nonspecifically immunoprecipitated, we studied whether DEX interferes with ZAP70–HSP90 bindings. These data indicated that the observed effects of DEX are specific for SRC-family kinases, as DEX did not inhibit HSP90–ZAP70 interactions in activated T cells (supplementary Fig 2

online). These results were supported by the lack of effect of either DEX or HSP90siRNA on the integrity and functional activity of b-Raf/mitogen-activated protein kinase kinase protein complexes (supplementary Fig 3 online). Hence, DEX treatment does not interfere with protein complex formation per se, but specifically targets LCK–HSP90 and FYN–HSP90 formations. The transfection procedure was evaluated by immunoblotting cellular extracts prepared from cells transfected with or without HSP90siRNA (supplementary Fig 4 online). To investigate further whether the presence of GR is required for efficient TCR signalling, GRsiRNA-transfected cells were pretreated for 10 min with DEX followed by 15 min stimulation. Western blotting showed impaired TCR signalling in GRsiRNA-transfected cells, as indicated by suppressed phosphorylation of several downstream TCR signalling intermediates (Fig 2C). These findings provide direct evidence that the GR is important in mediating efficient TCR signalling. As GRsiRNA might induce nonspecific effects, cells were transfected with control siRNA, demonstrating that the observed effects on TCR signalling are not a result of nonspecific GRsiRNA action (supplementary Fig 5 online).

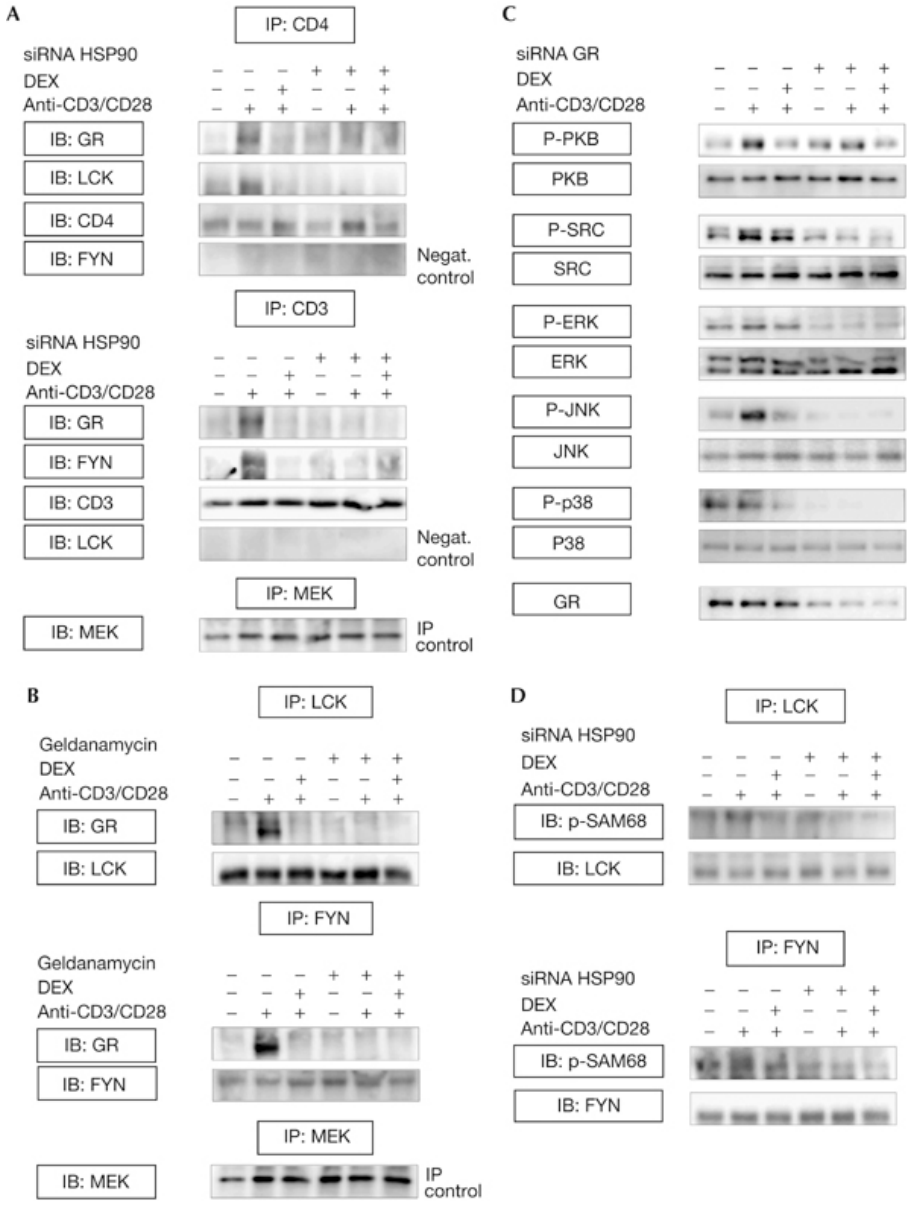

Figure 2.

GR and HSP90-dependent LCK–CD4 and FYN–CD3 formations. (A) HSP90siRNA-transfected (+) and nontransfected (−) cells were pretreated

for 10 min in the presence (+) or absence (−) of DEX (1 μM) and incubated with (+) or without (−) CD3/CD28 antibodies for 15 min. CD3 and CD4 immunoprecipitates were immunoblotted using the indicated antibodies. To ensure specificity in these experiments, the presence of FYN and LCK was analysed in CD4 and CD3 immunoprecipitates, respectively, demonstrating that specific complexes had been immunoprecipitated containing either FYN and CD3 or LCK and CD4. (B) Cells were pretreated for 30 min with geldanamycin (5 μM), followed by 10 min DEX treatment and 15 min activation (CD3/CD28 antibodies). LCK/FYN immunoprecipitates were immunoblotted for the presence of GR, and LCK and FYN antibodies were used to evaluate for equal loading. An irrelevant protein (that is, MEK) was immunoprecipitated from these lysates followed by western blotting using MEK antibody (immunoprecipitation controls). (C) GRsiRNA-transfected or nontransfected cells were pretreated with DEX (10 min), followed by 15 min activation. Lysates were immunoblotted using phosphospecific antibodies against TCR signalling intermediates, and appropriate antibodies were used to test for equal loading. (D) Cell lysates, treated as described above, were subjected to LCK/FYN immunoprecipitation followed by in vitro kinase assays using SAM68 as a substrate. Phosphorylated SAM68 was immunoblotted with PY20, and equal loading was verified using LCK and FYN antibodies. Experiments were performed three times and representative results are shown. DEX, dexamethasone; ERK, extracellular-signal-regulated kinase; FYN, FYN oncogene related to SRC, FGR, YES; GR, glucocorticoid receptor; IB, immunoblotting; IP, immunoprecipitation; JNK, Jun amino-terminal kinase; LCK, lymphocyte-specific protein tyrosine kinase; MEK, mitogen-activated protein kinase kinase; P, phosphorylated; SAM68, SRC-associated in mitosis.

HSP90siRNA inhibits LCK and FYN activities

To investigate the functional consequences of dissolution of these multiprotein complexes on TCR-induced activation of LCK and FYN, we examined whether HSP90siRNA affects LCK/FYN activities, using LCK and FYN immunoprecipitates and in vitro phosphorylation of SAM68 (SRC-

associated in mitosis). TCR stimulation resulted in enhanced SAM68 phosphorylation compared with control cells, confirming TCR-dependent activation of LCK and FYN in these experiments (Fig 2D). Similar to DEX treatment, LCK and FYN immunoprecipitated from HSP90siRNA-transfected cells did not have the capacity to phosphorylate SAM68, indicating that HSP90siRNA and DEX impair the enzymatic activities of LCK and FYN. These observations suggest that LCK/FYN-mediated TCR signalling depends on the presence of both HSP90 and GR.

DEX or HSP90siRNA reduce GR–LCK/FYN localization

Cellular localizations of GR, LCK and FYN were studied using confocal fluorescence microscopy. Double stainings for GR and LCK or GR and FYN demonstrated cytoplasmic staining of LCK, FYN and GR in quiescent cells (Fig 3). LCK, FYN and GR staining were mainly detected in the cell periphery on TCR stimulation (Fig 3, dotted lines). GR–LCK and GR–FYN cytoplasmic colocalization (indicated by yellow staining) was seen in quiescent cells, which was increased on TCR stimulation (Fig 3, solid lines). Reduced GR–LCK and GR–FYN colocalization was observed in activated cells treated with DEX or HSP90siRNA (indicated by diminished yellow staining), which supports the immunoprecipitation data that DEX and HSP90siRNA disrupt GR–LCK and GR–FYN interactions. Owing to the small cytoplasm volume compared with the nuclear volume, no conclusions can be drawn about the precise cytoplasmic or membrane localization of LCK or FYN in the different conditions. Specificity controls for the immunofluorescent stainings are shown as supplementary information online (supplementary Fig 6 online).

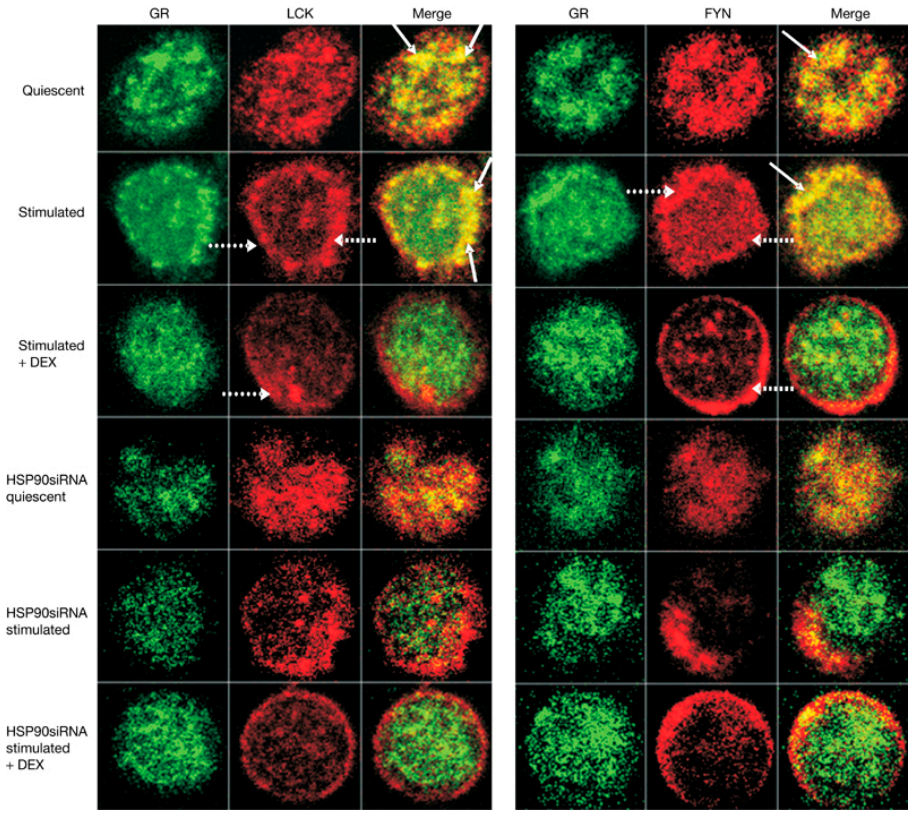

Figure 3.

Dexamethasone and HSP90siRNA interfere with GR–LCK and GR–FYN colocalization. HSP90siRNA-transfected or nontransfected cells were pretreated with or without DEX (1 µM; 10 min) and activated with CD3 and CD28 antibodies (15 min). Cells were studied by confocal fluorescence microscopy and immunofluorescent double stainings of GR (fluorescein isothiocyanate-labelled, green) together with LCK or FYN (tetramethylrhodamine isothiocyanate-labelled, red) are shown. Cytoplasmic colocalization between GR and LCK or GR and FYN is indicated by yellow staining (solid lines). The nucleus was visualized with 4,6-diamidino-2-phenylindole (not shown). Panels of optical cross-sections through T cells are depicted; scanned area for all images: 9 µm × 9 µm. Three independent experiments were performed and reproducible results

were obtained. At least 20 cells under each condition were scanned, and representative cross-sections are indicated. DEX, dexamethasone; FYN, FYN oncogene related to SRC, FGR, YES; GR, glucocorticoid receptor; HSP90, heat-shock protein 90; LCK, lymphocyte-specific protein tyrosine kinase; siRNA, short interfering RNA.

Discussion

GCs mediate well-defined genomic effects through GR-dependent transcriptional changes. Clinical and experimental evidence for rapid nongenomic GC action have accumulated, but the underlying molecular mechanisms remain unexplained. Oestrogens can induce rapid signalling through intracellular transmembrane oestrogen receptors, providing evidence for nongenomic effects of steroids on cellular physiology (Revankar et al, 2005). Similarly, membrane-bound GRs have been identified in lymphocytes (Gametchu et al, 1999; Gametchu & Watson, 2002) and in human peripheral blood mononuclear cells (Bartholome et al, 2004), but their functional relevance remains unclear. It is known that, at high concentrations, GCs increase GR saturation in a dose-dependent manner, which intensifies the therapeutically relevant genomic GC activities (Buttgereit et al, 2002). Although saturation of cytosolic GRs is almost complete with 100 mg prednisone equivalent a day, the use of higher dosages (100–1,000 mg) is common and successful in daily clinical practice (Buttgereit et al, 2004), possibly as a consequence of nongenomic effects (Buttgereit et al, 1998). Nongenomic GC action is thought to be mediated through cytosolic or membrane-bound GRs and/or through nonspecific physicochemical interactions with membranes.

Nongenomic GC-induced inhibition of LCK/FYN-mediated TCR signalling has been reported (Lowenberg et al, 2005), but the underlying mechanism remains unexplained. A relationship between GC stimulation and LCK/FYN kinases is supported by previous work, showing that prolonged DEX stimulation (enabling GC-induced genomic effects) disturbed the submembrane localization of LCK and FYN in murine T cells (Van Laethem et al, 2001). It is possible that HSP90-dependent regulation of LCK and FYN

contributes to these effects, but further studies are needed to clarify the role of GR and HSP90 in regulating TCR signalling.

On the basis of the present work, we propose a model arguing that, in the absence of ligand, the GR sustains a TCR-associated complex containing HSP90, LCK and FYN (Fig 4). On GR–ligand binding, this membrane-bound multiprotein complex is dissociated, leading to a cellular redistribution of LCK and FYN and an inability of LCK/FYN to participate in TCR signalling. Our data show an essential role of HSP90 and GR in the formation of TCR-associated protein complexes and in the regulation of efficient TCR signalling. Overall, these results provide new insight into the nongenomic mechanism of GC-induced immunosuppression in T cells. Selective inhibition of proximal TCR signalling through LCK/FYN presents opportunities for the development of novel immunosuppressive therapies.

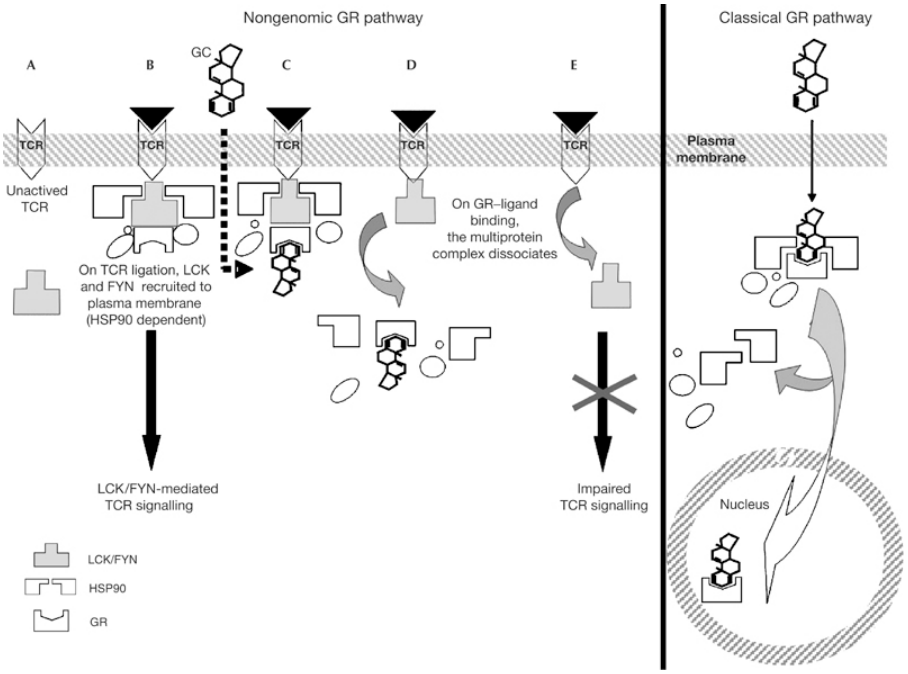

Figure 4.

Model for nongenomic GC-induced immunosuppression in T cells. (A,B) On TCR ligation, LCK and FYN are recruited to the TCR complex, resulting in LCK/FYN kinase activation and initiation of downstream TCR signalling. The present study shows TCR-linked GR multiprotein complexes containing HSP90, LCK and FYN in activated T cells. (C,D) On GR–ligand binding, this multiprotein complex dissociates. (E) LCK and FYN are released from the TCR complex, leading to impaired TCR signalling as a consequence of a cellular redistribution and abrogated activation of LCK and FYN. FYN, FYN oncogene related to SRC, FGR, YES; GC, glucocorticoid; GR, glucocorticoid receptor; HSP90, heat-shock protein 90; LCK, lymphocyte-specific protein tyrosine kinase; TCR, T-cell receptor.

Methods

Cell culture. CD4+ T cells were purified from human peripheral blood mononuclear cells and maintained as described previously (Lowenberg et al, 2005).

Reagents and antibodies. A complete list of the antibodies can be found in the supplementary information online. DEX and geldanamycin were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). HSP90α/βsiRNA, GRsiRNA, control siRNA and SAM68 were purchased from Santa Cruz (Heidelberg, Germany). Kinase buffer and lysis buffer (supplemented with 1 μg/ml NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM Na3VO4, 1 mM pefabloc) were from Cell Signaling Technology (Beverley, CA, USA).

Cell transfection. Cells were transfected by electroporation using routine procedures (Amaxa, Cologne, Germany). Each nucleofection sample contained 5 × 106 cells, 2 μg of highly purified siRNA and 100 μl of human T-cell Nucleofector. To determine transfection efficiency, green fluorescent protein (GFP)-tagged DNA (Amaxa) was used for electroporations, showing cells that were 40–50% GFP-positive 16 h after transfection, as assessed by fluorescence-activated cell sorting analysis (data not shown).

Immunoprecipitation, in vitro kinase assay and immunoblotting. Cells were incubated at 37°C in six-well plates (5–10 × 106 cells/well) for 2 h followed by a 10 min pretreatment with 1 μM DEX dissolved in dimethyl sulphoxide (DMSO) or DMSO-supplemented media (control). Cells were subsequently activated for 15 min with CD3 antibodies (immobilized on plastic) and soluble CD28 antibodies (3 μg/ml). Cells were centrifuged (1,750 r.p.m., 10 min), lysed in non-denaturing lysis buffer and subjected to immunoprecipitation followed by in vitro kinase assay and western blotting. The protocol for these procedures can be found in the supplementary information online.

Fluorescent double stainings. After in vitro stimulations, cells were centrifuged for 5 min at 1,750 r.p.m. and pellets were resuspended in PBS. SuperFrost® plus microscope slides were pretreated with 0.01% poly-l-lysine for 10 min, cell suspensions were subsequently incubated on the slides for 30 min (final concentration 2 × 105 cells/slide) and 3.7% paraformaldehyde was added for 30–60 min. Sections were washed in PBS/Triton X-100 0.1% (PBS-T), followed by a 1 h blocking step with 10% FCS in PBS-T. After overnight incubation with a GR antibody at 4°C, antibodies against LCK or FYN were added for 2–3 h. Slides were washed and incubated for 60 min with fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Heverlee, Belgium) diluted in 3% BSA/PBS-T. Alternatively, sections were incubated overnight at 4°C with an HSP90 antibody. Sections were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) supplemented with 4,6-diamidino-2-phenylindole and sealed with coverslips. The protocol of the imaging technique can be found in the supplementary information online.

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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References

1. Bartholome B et al (2004) *Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis.* FASEB J **18**: 70–80

2. Baus E, Andris F, Dubois PM, Urbain J, Leo O (1996) *Dexamethasone inhibits the early steps of antigen receptor signaling in activated T lymphocytes.* J Immunol **156**: 4555–4561

3. Bijlmakers MJ, Marsh M (2000) *Hsp90 is essential for the synthesis and subsequent membrane association, but not the maintenance, of the Src-kinase p56(lck).* Mol Biol Cell **11**: 1585–1595

4. Buttgereit F et al (2002) *Standardised nomenclature for glucocorticoid dosages and glucocorticoid treatment regimens: current questions and tentative answers in rheumatology.* Ann Rheum Dis **61**: 718–722

5. Buttgereit F, Straub RH, Wehling M, Burmester GR (2004) *Glucocorticoids in the treatment of rheumatic diseases: an update on the mechanisms of action.* Arthritis Rheum **50**: 3408–3417

6. Buttgereit F, Wehling M, Burmester GR (1998) *A new hypothesis of modular glucocorticoid actions: steroid treatment of rheumatic diseases revisited.* Arthritis Rheum **41**: 761–767

7. Croxtall JD, Choudhury Q, Flower RJ (2000) *Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism.* Br J Pharmacol **130**: 289–298

8. Franchimont D (2004) *Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies.* Ann NY Acad Sci **1024**: 124–137

9. Gametchu B, Watson CS (2002) *Correlation of membrane glucocorticoid receptor levels with glucocorticoid-induced apoptotic competence using mutant leukemic and lymphoma cells lines.* J Cell Biochem **87**: 133–146

10. Gametchu B, Chen F, Sackey F, Powell C, Watson CS (1999) *Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models.* Steroids **64**: 107–119

11. Hafezi-Moghadam A et al (2002) *Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase.* Nat Med **8**: 473–479

12. Hartson SD, Barrett DJ, Burn P, Matts RL (1996) *Hsp90-mediated folding of the lymphoid cell kinase p56lck.* Biochemistry **35**: 13451–13459

13. Lowenberg M, Tuynman J, Bilderbeek J, Gaber T, Buttgereit F, van Deventer S, Peppelenbosch M, Hommes D (2005) *Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn.* Blood **106**: 1703–1710

14. Palacios EH, Weiss A (2004) *Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation.* Oncogene **23**: 7990–8000

15. Pratt WB, Galigniana MD, Morishima Y, Murphy PJ (2004) *Role of molecular chaperones in steroid receptor action.* Essays Biochem **40**: 41–58

16. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER (2005) *A transmembrane intracellular estrogen receptor mediates rapid cell signaling.* Science **307**: 1625–1630

17. Rhen T, Cidlowski JA (2005) *Antiinflammatory action of glucocorticoids—new mechanisms for old drugs.* N Engl J Med **353**: 1711–1723

18. Van Laethem F, Baus E, Smyth LA, Andris F, Bex F, Urbain J, Kioussis D, Leo O (2001) *Glucocorticoids attenuate T cell receptor signaling.* J Exp Med **193**: 803–814

19. Yun BG, Matts RL (2005) *Differential effects of Hsp90 inhibition on protein kinases regulating signal transduction pathways required for myoblast differentiation.* Exp Cell Res **307**: 212–223

20. Zamoyska R, Basson A, Filby A, Legname G, Lovatt M, Seddon B (2003) *The influence of the Src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation.* Immunol Rev **191**: 107–118

6

Superantigen induced steroid resistance depends on activation of PLCβ2

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Abstract

The glucocorticoid receptor is present in a T cell receptor (TCR) associated complex, which includes the Src family tyrosine kinase Lck. Glucocorticoids rapidly dissociate this complex resulting in the inhibition of canonical Lck-phospholipase C (PLC)γ dependent TCR signaling. The relative importance of this nongenomic role of the GR compared to its direct transcriptional effects is not known. Superantigens induce a state of steroid resistance in activated T cells. It has been reported that in addition to canonical Lck-PLCγ signaling, superantigens can activate a noncanonical G protein-PLCβ dependent signaling pathway. Here we show that Staphylococcal Enterotoxin B (SEB) activates a Gαq and PLCβ2 dependent pathway in human T cells. We find that this pathway bypasses the need for canonical Lck-PLCγ signaling in T cell activation and renders superantigen stimulated T cells insensitive for glucocorticoids in vitro. We show that the PLCβ inhibitor U-73122 sensitizes SEB treated mice to dexamethasone in vivo. In conclusion, we find that effects of glucocorticoids on TCR induced T cell proliferation are mainly nongenomic and can be bypassed by the activation an Lck independent signaling pathway.

Introduction

Glucocorticoids bind the glucocorticoid receptor, which subsequently dimerizes and translocates from the cytosol to the nucleus. The glucocorticoid receptor can bind to specific DNA sequences and alter the transcriptional activity of a wide range of genes many of which play a role in the immune system. However, the glucocorticoid receptor can also affect cell signaling in its DNA unbound state, so called non-genomic or non-transcriptional effects (1). We have previously found that the glucocorticoid receptor is present in a TCR associated complex that contains the Src-like tyrosine kinase Lck (2). Glucocorticoids rapidly dissociate the glucocorticoid receptor from this complex leading to the inactivation of Lck and downstream signaling pathways (2,3). Lck mediates canonical TCR signaling via activation of ZAP-70, phosphorylation of LAT and the assembly of a multiprotein complex. A key component of this complex is PLCγ which converts phosphatidylinositol 4,5-bisphosphate to inositol 3,4,5-triphosphate (IP3) and diacylglycerol (DAG). Generation of IP3 activates Ca2+ signaling and leads to the nuclear translocation of nuclear factor of activated T-cells (NFAT) while DAG activates PKC and RAS-MAPK signaling (see Figure 1A) (4). Glucocorticoids inhibit T cell activation but the functional relevance of loss of TCR associated glucocorticoid receptor and the resulting inactivation of Lck versus transcriptionally mediated effects of the glucocorticoid receptor is not known. If the dissociation and inactivation of Lck is an important part of the mechanism of action of steroids this would predict that signals that bypass Lck render T cells relatively steroid insensitive.

Superantigens activate T cells by directly cross linking MHC class II to the TCR as unprocessed proteins. Superantigens therefore bypass the specificity of conventional antigen recognition and can activate up to 20% of the total number of T cells (5). The massive activation of the adaptive immune system that ensues helps the pathogen to evade an appropriate immune response. The release of cytokines such as Il-1, TNFα, and IFNγ causes the signs and symptoms of toxic shock syndrome (6). Superantigen signaling is an excellent model to address the question of the relevance of glucocorticoid mediated inhibition of upstream T cell signaling. Superantigens have been described to cause steroid resistance in T cells (7). More importantly, it has previously been shown that superantigens can activate a noncanonical G protein-PLCβ signaling pathway in addition to

canonical Lck-PLCγ signaling (8). If steroid mediated inhibition of T cell proliferation depends mainly on nongenomic inhibition of canonical T cell signaling, this predicts that superantigen mediated glucocorticoid resistance depends on PLCβ signaling.

Here we find that superantigen mediated steroid resistance depends on Gαq mediated activation of PLCβ2 which bypasses dependence on Lck-PLCγ signaling in primary human T cells in vitro. We go on to show the efficacy of glucocorticoid therapy in combination with a PLCβ inhibitor in a mouse model of toxic shock syndrome in vivo. These findings have implications for our understanding of the mechanism of action of glucocorticoids. They may point to novel therapeutic strategies to counter glucocorticoid resistance.

Material and Methods

Antibodies and Reagents

The following antibodies were used: anti-Lck (3A5), anti-ß-Actin, anti-PLCß1 (D-8), anti-PLCß2 (Q-15), anti-PLCß3 (C-20) and anti-pTyr (PY20) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-phospho-Zap70 (Tyr319)/Syk (Tyr352), anti-phospho-Src (Tyr410), anti-phospho-LAT (Tyr171) and the PI3 kinase inhibitor LY924002 were purchased from Cell Signaling Technology (Beverly, MA). Anti-PLCγ1 (2B1) was purchased from AbCam (Cambridge, USA). Anti-CD3 and anti-CD28 were purchased from Sanquin (Amsterdam, The Netherlands). PMA, Ionomycin, SEB and the PLCß inhibitor U-73122 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Phytohemagglutinin-M (PHA) was purchased from Roche (Mannheim, Germany).

Mice

Eight week old female C57BL/6 wild-type mice were purchased from Harlan (Boxmeer, The Netherlands) and housed in pathogen free conditions. All mice received a single intraperitoneal injection with a total volume of 500 µl. Depending on the group they were in mice received 20 µg dexamethasone and/or 10 µg U-73122. All mice received 8.8 µl DMSO, 100 µg SEB. 24 hours later the mice were euthanatized with carbon dioxide and blood was drawn via intracardiac puncture and the spleen was removed.

Cytokine levels were determined using a Cytometric Bead Array (BD Bioscience, Breda, The Netherlands).

Cell culture

Human peripheral blood lymphocytes (PBL) were isolated from whole blood of healthy volunteers by Ficoll-Isopaque density gradient centrifugation. After washing monocytes were separated from lymphocytes by percoll density gradient centrifugation. The lymphocytes were cultured in IMDM (Gibco, Verviers, Belgium) supplemented with 10% heat inactivated fetal calf serum. For proliferation experiments cells were stimulated for 24 hours. In all experiments lymphocytes were stimulated with PHA (10 µg/ml), SEB (100 ng/ml) or a combination of PMA (10 ng/ml) and ionomycin (250 ng/ml). Proliferation was measured by 3H-thymidine incorporation assay.

RNA extraction and quantitative RT-PCR

Pelleted cells were dissolved in 500µl Trizol. After adding 250µl chloroform, tubes were spinned and the aqueous fase transferred. RNA was precipitated by adding 250µl isopropanol. RNA was washes in 70 Etoh, dried and dissolved in H20. For cDNA synthesis, 1 µg of RNA was transcribed using Revertaid (Fermentas). Quantitative RT-PCR was performed using SybrGreen (Qiagen) according to manufacturers' protocol on a BioRad iCycler using specific primers for the mRNA of interest.

Western blot

Samples for Western blot were made by treating 5 · 106 lymphocytes for 10 minutes with 10 µM dexamethasone followed by 10 minutes stimulation. Cells were pelleted and lyzed in lysis buffer (0.1% NP40, 50 mM Tris HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 200 µM Na3VO4, 10 mM NaF) containing protease inhibitors (Roche, Mannheim, Germany) for 20 minutes on ice. Samples were spinned down, sample buffer was added to the supernatant and heated to 95 °C for 5 minutes, cooled on ice and sonicated. Samples were blotted overnight at 4°C in buffer containing 20% methanol on PVDF membrane (Millipore, Bedford, USA). The membrane was blocked with 5% BSA for 1 hour at RT followed by overnight incubation with the primary antibody at 4°C.

Immunoprecipitation

For immunprecipitation experiments 10 · 106 cells were used per condition. Cells were pelleted and lyzed as described above. The supernatant was precleared by adding 10 µl Protein A/G UltraLink resin (Pierce Biotechnology, Rockford, USA) for 30 minutes at 4°C. Beads were spinned down and discarded. Samples were incubated with 2 µg primary antibody and 25 µl beads for 2 hours at 4°C. Beads were washed with PBS, sample buffer was added and heated to 95°C for 5 minutes.

Immunohistochemistry

2 · 105 PBMC were plated in a 12 wells plate. Cells were pretreated for 30 minutes with 1 µM dexamethasone or with 0.5 µM U-73122 and then stimulated by adding SEB (100 ng/ml) for 1 hour. Thirty minutes before the end of the experiment cells were transferred to wells containing glass cover slips precoated with poly-L-lysine (0.01 (w/v) in H2O). The cells were fixed for 10 minutes in 4% paraformaldehyde at RT and stained for 1 hour at RT both primary and secondary antibody.

siRNA transfection

Two independent siRNAs targeting GNA11 (siRNA #1: CGACAAGAUCAUCUACUCAtt, siRNA #2: GCAUCAUCGAGUACCCUUUtt), GNAq (siRNA #1: UCGUCUAUCAUAGCAUUCCtg, siRNA #2: UAUCUUCAUCAGAGUAUCCtg) and PLCß2 (siRNA #1: GGCUACUACUUAUACUGGAtt, siRNA #2: CCAUGACCACAGACAUCUUtt) were purchased from Ambion Applied Biosystems (Austin, USA). Lymphocytes were transfected by electroporation using the Amaxa's Nucleofector (Lonza, Cologne, Germany). Per condition 10 · 106 lymphocytes were electroporated according to protocol using 30 pmol siRNA and the Human T cell nucleofector kit for unstimulated T cells (Lonza, Cologne, Germany). After 4 hours the media was replaced. Experiments were performed 48 hours post transfection.

Quantification and data analysis

Densitometric analysis of Western blot was performed using ImageJ (National Institute of Health, USA). Data were analyzed using Excel (Microsoft), FlowJo software (TreeStar, San Carlos, CA, USA) and Prism (GraphPad Software, La Jolla, USA)

Results

T cell activating signals that bypass Lck cause steroid resistance

Glucocorticoids inactivate canonical TCR signaling by dissociating the glucocorticoid receptor from a TCR associated complex which contains Lck. This leads to inactivation of canonical TCR signaling including PLCγ. PLCγ plays a critical role in the activation of Ca2+ signaling and activation of PKC. To test the hypothesis that inhibition of T cell activation by glucocorticoids depends on inactivation of Lck, we used different T cell stimuli to test their relative sensitivity to glucocorticoid mediated inhibition of T cell proliferation. We used the plant lectin phytohemagglutinin (PHA) to strongly activate canonical, Lck dependent TCR signaling. PHA binds CD3 directly and nonspecifically (9) resulting in the potential activation of all T cells. Treatment of primary human peripheral blood leukocytes (PBL) with 10 μg/ml PHA resulted in a strong activation of T cell proliferation which was sensitive to treatment with the synthetic glucocorticoid analogue dexamethasone (Figure 1B, figure shows relative proliferation. Mean + standard error of absolute proliferation of PHA stimulated T cells without dexamethasone: 94214±7281 cpm). Next, we completely bypassed Lck signaling by direct activation of Ca2+ signaling with 250 ng/ml iononomycin and PKC with 10 ng/ml phorbol-12-myristate-13-acetate (PMA). Treatment with PMA/ionomycin resulted in complete resistance of proliferating T cells to dexamethasone even at very high doses (Figure 1B, Mean + standard error of absolute proliferation of PMA/ionomycin stimulated T cells without dexamethasone: 74154±1190 cpm). These results suggest that the inhibitory effects of glucocorticoids on T cell proliferation completely depend on a non-transcriptional role of the GR and may be related to inactivation of Lck. To further address this question we used the superantigen SEB. SEB not only activates canonical Lck-PLCγ signaling but has also been reported to activate a noncanonical G protein-PLCβ–dependent signaling pathway (8). If effects of glucocorticoids depend on inhibition of Lck, this would predict that superantigen induced T cell proliferation is only partially responsive to glucocorticoids. The hypothesis

would predict that G protein mediated activation of PLCβ is insensitive to effects of glucocorticoids and therefore still allows for PLCβ independent activation of Ca2+ signaling and activation of PKC (Figure 1A). Treatment of PBL with 100 ng/ml SEB resulted in an intermediated level of steroid resistance (Figure 1B, Mean + standard error of absolute proliferation of SEB stimulated T cells without dexamethasone:: 52633±1664 cpm). This is consistent with the hypothesis that glucocorticoids inhibit SEB induced activation of Lck-PLCγ signaling but not G protein-PLCβ signaling.

To examine if glucocorticoids indeed inactivate both PHA and SEB mediated activation of Lck and canonical TCR signaling we added 10 μM dexamethasone to PHA and SEB stimulated PBL and determined phosphorylation of Lck and downstream signaling mediators ZAP70, LAT and PLCγ. This experiment showed that dexamethasone inhibits both PHA and SEB mediated Lck signaling (Figure 1C). This suggests that SEB induced steroid resistance involves a bypass of the need for Lck-PLCγ signaling in T cell activation.

We previously reported that treatment with dexamethasone causes the disruption of a TCR associated complex which contains both LCK and the glucocorticoid receptor (2,3). To investigate if a similar mechanism is responsible for the inhibition of SEB stimulated signal transduction, PBL were stimulated with SEB and treated with dexamethasone. LCK was immunoprecipitated and the interaction with the GR was analyzed on Western blot (Figure 1D). Stimulation of PBL with SEB results in the recruitment of the GR to LCK. In line with previous findings dexamethasone reduces this interaction.

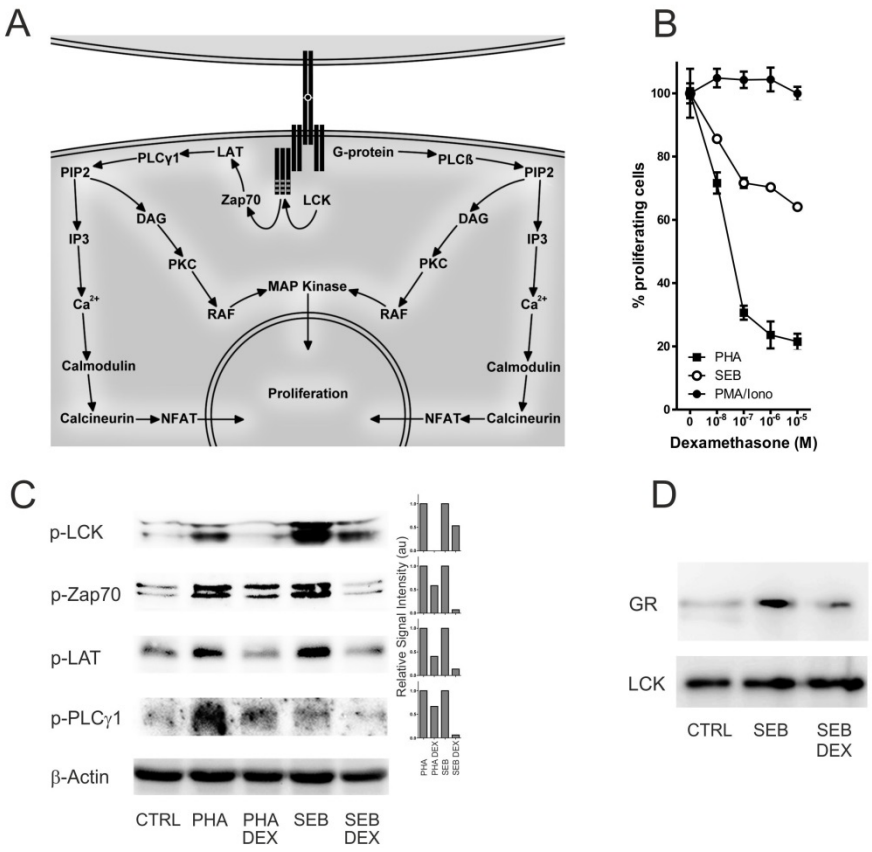

Figure 1. Signals that bypass canonical Lck-PLCγ signaling render T cells resistant to glucocorticoids. (A) A schematic overview of the canonical Lck-PLCγ dependent signaling pathway and the alternative PLCβ signaling route that is activated by SEB. (B) Dexamethasone mediated inhibition of T cell proliferation induced by different stimuli. PBL isolated from whole blood were stimulated for 24 hours with PHA (10 µg/ml), SEB (100 ng/ml) or a combination of PMA (10 ng/ml) and ionomycin (250 ng/ml). Proliferation was measured by 3H-thymidine incorporation assay. PBL stimulated with PHA are more sensitive to treatment with dexamethasone than PBL stimulated with SEB. Stimulation with a combination of PMA/ionomycin causes total steroid insensitivity. (C) Dexamethasone inhibits both PHA and SEB mediated phosphorylation of components of the canonical Lck-PLCγ dependent signaling pathway. The figure shows Western blot analysis of PBL pretreated for 10 minutes with 10 µM dexamethasone followed by 10 minutes of stimulation

with PHA (10 μg/ml) or SEB (100 ng/ml). Each lane represents the same sample analyzed for different proteins. Bar graphs on the right of each blot represent densitometric analysis of the relative phosphorylation of dexamethasone treated PBL are given relative to SEB and PHA stimulation without dexamethasone. (D) Interaction of the glucocorticoid receptor (GR) with LCK. PBL were stimulated with SEB (100 ng/ml) for one hour and Lck was immunoprecipitated with a specific antibody. Immunoblots for the GR show that the GR associates with Lck upon stimulation with SEB. Concurrent treatment with dexamethasone (10 μM) reduces the interaction between LCK and the GR. An immunoblot for LCK was used as a loading control.

SEB induced steroid resistance depends on PLCβ signaling

To investigate if activation of PLCβ by SEB is responsible for the relative insensitivity to dexamethasone we used the PLCβ inhibitor U73122 (10). Treatment of SEB stimulated PBL with either U73122 (0.5 μM) or dexamethasone (10 μM) partially inhibited SEB induced T cell proliferation. Combination of dexamethasone with U73122 showed an additive effect on T cell proliferation (Figure 2A, $P < 0.001$ for dexamethasone versus dexamethasone + U73122) suggesting that both inactivate independent signaling pathways. This experiment strongly suggests that SEB induced activation of PLCβ partially bypasses inhibitory effects of glucocorticoids on Lck-PLCγ signaling. We therefore used nuclear translocation of NFAT1 as a read out of downstream TCR signaling. NFAT1 is translocated to the nucleus of activated T cells downstream of PLC mediated Ca2+ signaling (11). If activation of PLCβ bypasses glucocorticoid mediated inhibition of Lck-PLCγ signaling this would predict that dexamethasone only partially prevents NFAT1 translocation in SEB treated PBL and that inhibition is complete if dexamethasone is combined with U73122. We found that PHA mediated NFAT1 translocation was very sensitive to dexamethasone. In contrast, dexamethasone only partially prevented nuclear translocation of NFAT1 in SEB treated PBL and translocation was completely prevented by dexamethasone/U73122 combination treatment (Figure 2B and C, $P < 0.001$ for dexamethasone vs dexamethasone + U73122).

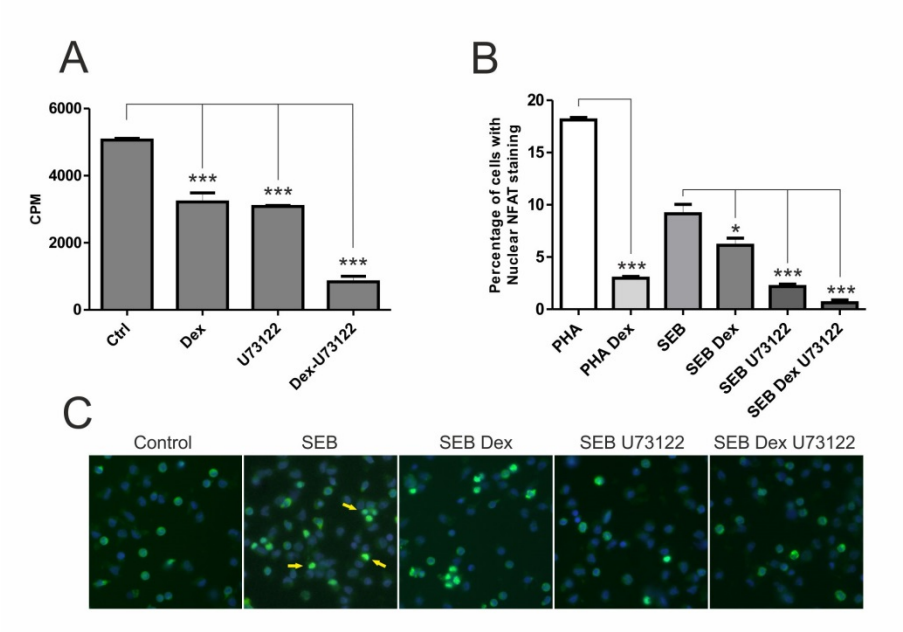

Figure 2. PLCβ inhibitor U73122 prevents SEB mediated glucocorticoid resistance. (A) Effect of dexamethasone, PLCβ inhibitor U73122 and combination treatment on SEB induced T cell proliferation. PBL were stimulated with SEB (100 ng/ml) for 24 hour in the presence or absence of inhibitors as indicated. Proliferation was analyzed by 3H-thymidine incorporation assay. (B) Effect of various treatments on nuclear translocation of NFAT1. PBL were pretreated for 30 minutes with 1 µM dexamethasone or with 0.5 µM U-73122 followed by 1 hour of SEB (100 ng/ml) stimulation. NFAT1 localization was visualized by immunohistochemistry. Each bar represents the average percentage of cells with nuclear NFAT1 staining as counted per three images at 200x magnification. (C) Representative images of NFAT1 staining. In the proportion of SEB sensitive lymphocytes stimulation with SEB causes NFAT1 to translocate to the nucleus which is visible as a bright green staining locate at the nucleus. Values in A and B are mean ± standard error, * = $P < 0.05$, *** = $P < 0.001$.

SEB causes steroid insensitivity through PLCß2

It has previously been shown that SEB activates PLCβ1 in the Jurkat T cell line. To determine which isoform of the PLCβ family is responsible for the SEB induced steroid insensitivity in primary human lymphocytes we used Western blot to visualize the different PLCβ isoforms that are expressed in human T cells (Figure 3A). In contrast to Jurkat cells, primary human T

lymphocytes express PLCβ2 rather than PLCβ1 and express low levels of PLCβ3. To examine if activation of PLCβ2 is responsible for SEB induced steroid resistance we knocked down PLCβ2 in PBL using two different siRNAs (Figure 3B). Indeed, knocking down PLCβ2 levels reduces SEB induced proliferation and restores steroid sensitivity in primary human PBL to levels similar to PBL stimulated with PHA (Figure 3C. Figure shows relative proliferation. Mean ± standard error for absolute proliferation; PHA: 23137 ± 397, SEB (control siRNA): 16570 ± 1155). These results show that PLCβ2 is the predominant isoform expressed in primary human PBL and support the hypothesis that SEB bypasses glucocorticoid mediated inhibition of Lck-PLCγ signaling by activating PLCβ2.

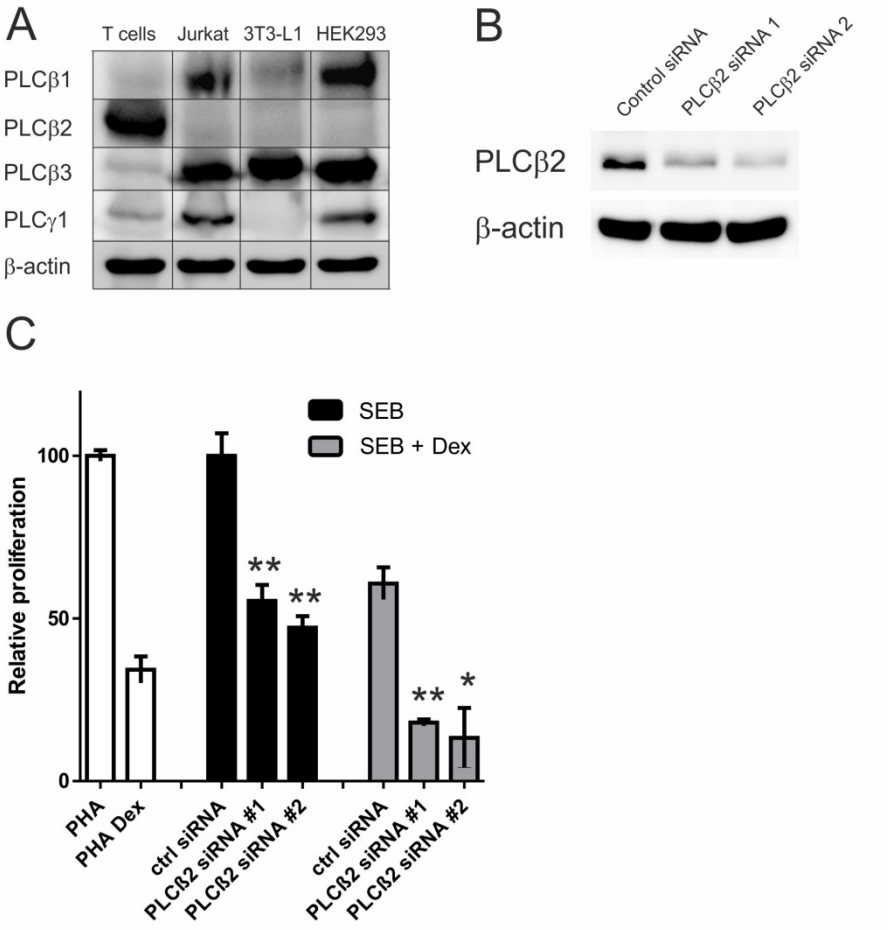

Figure 3. SEB mediated glucocorticoid resistance is PLCβ2 dependant. (A) Expression of PLCβ and different PLCβ isoforms in human CD4 T cells, Jurkat cells, 3T3-L1 and HEK293 cells indicates that PLCβ2 is the predominant isoform expressed in primary human T cells. The figure shows Western blot analysis of the presence of the different isoforms in samples with an equal amount of total protein from different cell types. (B) Western blot showing the efficiency of the two PLCβ2 siRNAs in knocking down PLCβ2 compared to cells transfected with control siRNA. (C) The figure shows the effect of dexamethasone and PLCβ2 siRNAs on SEB mediated proliferation compared to the effect of dexamethasone on PHA mediated proliferation. Cells were transfected with siRNA and allowed to recover for two days before stimulation and dexamethasone treatment. Proliferation was measured after 24 hours of treatment by 3H-thymidine incorporation assay. Values in C are mean ± standard error, * = P < 0.05, ** = P < 0.01.

SEB induced steroid resistance depends on activation of Gαq

PLCß is activated by members of the Gαq family of G proteins (12) such as Gα11 (13) and Gαq (14). It was previously suggested that Gα11 acts upstream of PLCβ activation in Jurkat T cells (8). We first used quantitative RT-PCR to examine expression of both Gαq and Gα11 in Jurkat cells and primary human CD4+ T lymphocytes using testis as a positive control for G αq (Figure 4A). This showed that Gαq is the predominant Gα isoform expressed in both Jurkat cells and primary human T lymphocytes.

To further elucidate which G protein is involved in SEB stimulated ΠΛΧβ2 activation we transfected PBL with siRNA targeting Gα11 or Gαq. Knock down efficiency of both Gαq and Gα11 was determined by quantitative RT-PCR (Figure 4B). Consistent with the low expression of Gα11 in primary human T cells, siRNA against Gα11 did not affect proliferation in SEB stimulated PBL (Figure 4C, figure show relative proliferation, mean ± standard error for absolute proliferation; PHA only: 13271 ± 782, SEB only control siRNA: 9199 ± 459). In contrast, siRNA against Gαq reduced SEB induced proliferation showing that SEB induced proliferation partially depends on Gαq signaling. This suggests that Gαq and not Gα11 acts upstream of PLCβ2 in primary human T cells. Gaq siRNA sensitized SEB treated cells to treatment with dexamethasone. This is consistent with a role for Gαq signaling in mediating SEB induced steroid resistance.

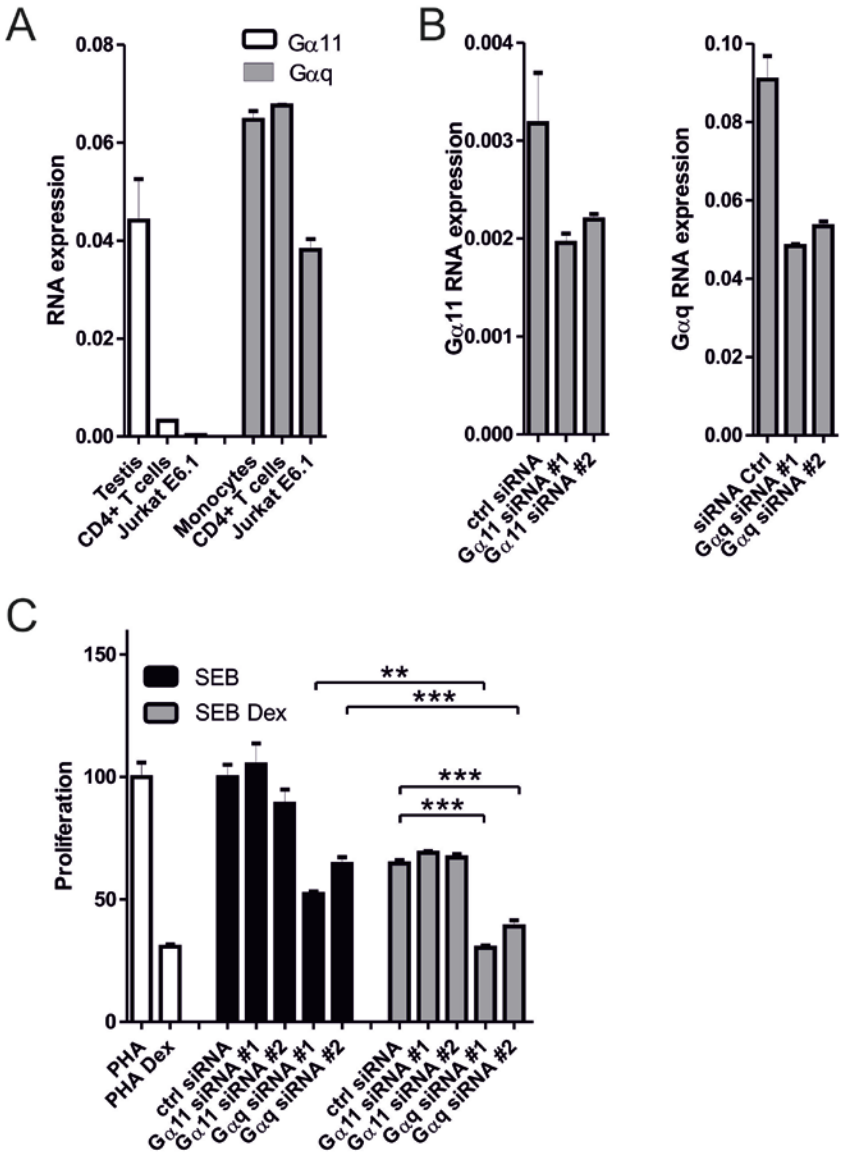

Figure 4. SEB mediated glucocorticoid resistance depends on Gαq signaling. (A) Quantitative RT-PCR for Gα11 and Gαq in primary human CD4+ cells and Jurkat cells using testis and monocytes as respective positive controls. (B) Knock down efficiency of Gα11 and Gαq in PBL as measured by quantitative RT-PCR. (C) The effect of dexamethasone and Gα11/Gαq siRNAs on SEB mediated proliferation

compared to the effect of dexamethasone on PHA mediated proliferation. Cells were transfected with siRNA and allowed to recover for two days before stimulation and dexamethasone treatment. Proliferation was measured after 24 hours of treatment by 3H-thymidine incorporation assay. Values are mean ± standard error, ** = P < 0.01, *** = P < 0.001.

SEB treated mice are responsive to dexamethasone-PLCβ inhibitor combination treatment

To test if SEB induced PLCβ activation is responsible for the relative inefficacy of glucocorticoids in the treatment of toxic shock syndrome we injected mice with SEB and treated them with either dexamethasone (20 μg/mouse, ~1 mg/kg) or U73122 (10 μg/mouse, ~0.5 mg/kg) alone or the combination of both. Mice were injected with 100 μg SEB intraperitoneally. In the same injection mice received solvent, 20 μg dexamethasone (approximately 1 mg/kg), 10 μg U-73122 or combination treatment. We measured the serum levels of Tnfα, Il-6 and Ifnγ at 8 and 24 hours after treatment (Figure 5A,B) and spleen weight (Figure 5C) at 24 hours as a read out of the response to treatment. Treatment with dexamethasone had little effect on serum cytokines. Dexamethasone did not significantly affect cytokine production at 8 hours after treatment and only IFNγ was significantly reduced at 24 hours. Treatment with U-73122 did not affect serum cytokine production at either time point. In contrast, dexamethasone/U-73122 combination treatment significantly reduced production of all three cytokines at both time points. The same effect was seen for spleen weight. These data show that U-73122 sensitizes SEB treated mice to treatment with dexamethasone.

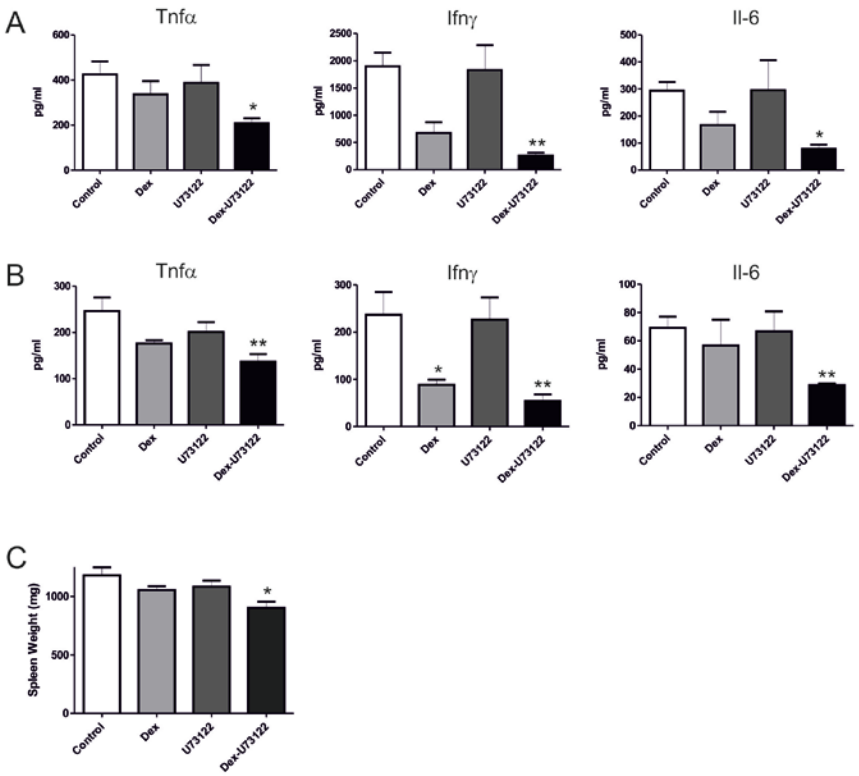

Figure 5. The PLCβ inhibitor U73122 sensitizes SEB injected mice to treatment with dexamethasone. (A) Serum cytokine levels at 8 hours after treatment with SEB. (B) Serum cytokine levels at 24 hours after treatment with SEB. (C) Spleen weights. Values are mean ± standard error, * = P < 0.05, ** = P < 0.01.

Discussion

The glucocorticoid receptor has long been thought to exert its effects primarily through the transcriptional control of glucocorticoid target genes. In past years it has become increasingly clear that the glucocorticoid receptor also has a non-transcriptional role in modulating cellular signal transduction. Recently we have shown that in the absence of ligand, the glucocorticoid receptor is associated with the TCR complex. Glucocorticoids dissociate the receptor from the TCR complex leading to rapid inactivation of Lck, the kinase that activates canonical PLCγ dependent TCR signaling (2,3). Glucocorticoids are potent inhibitors of T cell proliferation but the extent to which this is dependent on dissociation of Lck from the TCR

complex or transcriptional regulation had not been determined. Here we show that T cells can be made completely steroid resistant by directly activating T cells at a level downstream of PLCγ1 by stimulating them with PMA (activates PKC) and ionomycin (activates Ca2+ signaling). This shows that glucocorticoids act at a level upstream of activation of PKC and Ca2+ signaling (Figure 6).

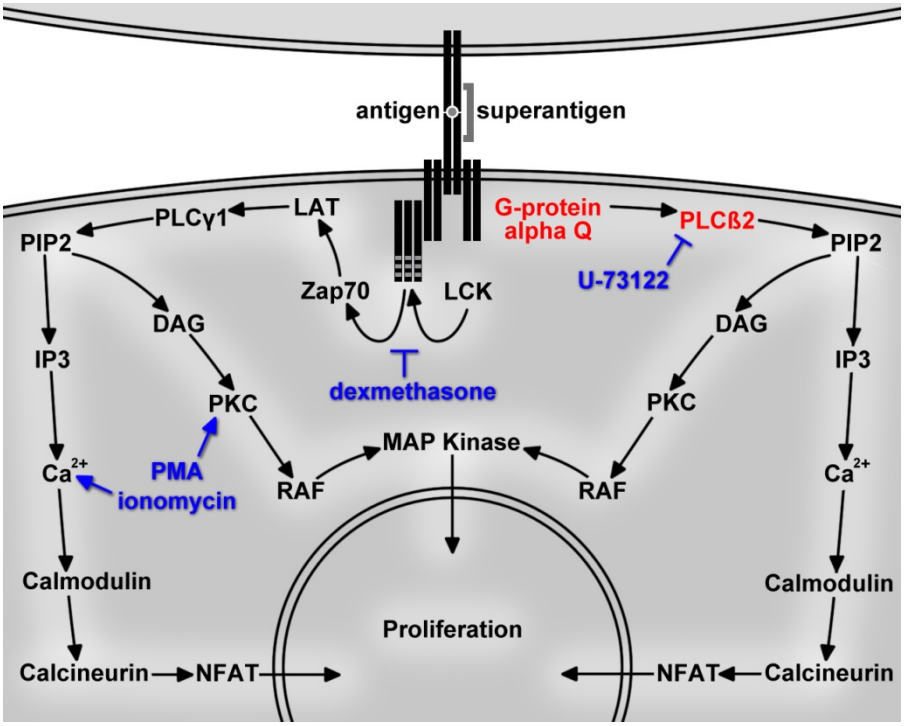

Figure 6. A model of SEB induced steroid resistance in primary human T cells. Canonical Lck-PLCγ signaling is inhibited by dexamethasone at the level of Lck. This inhibitory effect of glucocorticoids can be completely bypassed by downstream activation of TCR signaling at the level of Ca2+ (ionomycin) and PKC (PMA) signaling. SEB causes partial steroid resistance by activating dexamethasone sensitive Lck-PLCγ signaling in addition to dexamethasone insensitive Gαq-PLCβ2 signaling. Combination of dexamethasone with siRNA against Gαq, PLCβ2 or U73122 effectively blocks SEB induced T cell proliferation as it blocks both pathways.

If glucocorticoids act at the level of Lck-PLCγ signaling this would predict that signals that bypass this signaling route may render cells partially

steroid resistant. This is why we examined the effect of glucocorticoids on SEB mediated T cell activation. It was previously described that SEB activates PLCβ dependent signaling in a Jurkat T cell line in addition to Lck-PLCγ signaling (8). This is why we determined if this PLCβ mediated bypass of Lck-PLCγ signaling is responsible for SEB induced steroid insensitivity that had previously been described in human PBMC (7). We find that primary human PBL express primarily PLCβ2. Inhibition of PLCβ signaling with U73122 or specific depletion of PLCβ2 both reduced SEB mediated T cell proliferation. A combination of PLCβ inhibition plus dexamethasone completely inhibited T cell proliferation. Thus the partial steroid insensitivity of SEB treated T cells is dependent on PLCβ signaling. This shows that effects of glucocorticoids on T cell proliferation are dependent on inhibition of PLCβ dependent signaling. Thus effects of glucocorticoids on T cell proliferation are largely dependent on non-transcriptional effects of the glucocorticoid receptor.

PLCβ signaling is often activated by members of the guanine binding protein family. It has previously been shown that SEB activated PLCβ signaling in Jurkat T cells is pertussis toxin insensitive and mediated by Gα11. We confirmed that SEB mediated T cell proliferation is pertussis toxin insensitive in human PBL (not shown). However, we find very low levels of Gα11 that in primary human PBL. They express high levels of the closely related family member Gαq instead. Indeed, depletion of Gαq but not Gα11 reduced SEB mediated proliferation and restored glucocorticoid sensitivity. This suggests that Gαq acts upstream of PLCβ2 in primary human PBL.

The mechanism of action of SEB mediated steroid insensitivity may not only be relevant as a model to study genomic versus nongenomic glucocorticoid signaling but also help to improve therapy for patients with toxic shock syndrome. These patients receive mainly supportive therapy as no specific treatment exists. Corticosteroids are not recommended for toxic shock syndrome (see www.uptodate.com) as clinical evidence for their efficacy is lacking and a single small retrospective study showed that steroids may alleviate some symptoms of TSS but do not affect mortality (15). The limited efficacy of glucocorticoids in toxic shock syndrome is likely related to the fact that superantigens induce steroid resistance and mechanisms to alleviate this may help to improve therapy. Indeed, we find that SEB treated mice only marginally respond to treatment with either

dexamethasone or the PLCβ inhibitor U73122. This may in part be explained by the dosage U73122 used in this experiment which, compared to other studies, is relatively low (16,17). However dexamethasone/U73122 combination treatment significantly reduced cytokine production in SEB treated mice. A caveat of the in vivo study is that we did not directly assess measures of T cell activation, it cannot be excluded that the observed effects of dexamethasone and U73122 are partially explained by effects on innate immune cells such as monocytes or macrophages. Our in vitro studies suggest however that the effects may be directly on TCR signaling.

In conclusion, we find that SEB activates a Gαq-PLCβ2 dependent signaling pathway that bypasses Lck-PLCγ signaling. Inhibition of this PLCβ signaling pathway restores glucocorticoid sensitivity to SEB activated T cells. This strongly indicates that glucocorticoid mediated inhibition of T cell proliferation is dependent on inhibition of Lck-PLCγ signaling. Our data suggest that dual PLCβ/γ inhibitors or glucocorticoid/PLCβ inhibitor combination therapy may be useful in the treatment of TSS.

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Reference List

1. Lowenberg, M., A. P. Verhaar, G. R. van den Brink, and D. W. Hommes. 2007. *Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression.* Trends Mol. Med. **13**: 158-163.

2. Lowenberg, M., A. P. Verhaar, J. Bilderbeek, J. Marle, F. Buttgereit, M. P. Peppelenbosch, S. J. van Deventer, and D. W. Hommes. 2006. *Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN.* EMBO Rep. **7**: 1023-1029.

3. Lowenberg, M., J. Tuynman, J. Bilderbeek, T. Gaber, F. Buttgereit, D. S. van, M. Peppelenbosch, and D. Hommes. 2005. *Rapid*

immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. Blood **106**: 1703-1710.

4. Abraham, R. T., and A. Weiss. 2004. *Jurkat T cells and development of the T-cell receptor signalling paradigm.* Nat. Rev. Immunol. **4**: 301-308.

5. Fraser, J. D., and T. Proft. 2008. *The bacterial superantigen and superantigen-like proteins.* Immunol. Rev. **225**: 226-243.

6. Llewelyn, M., and J. Cohen. 2002. *Superantigens: microbial agents that corrupt immunity.* Lancet Infect. Dis. **2**: 156-162.

7. Hauk, P. J., Q. A. Hamid, G. P. Chrousos, and D. Y. Leung. 2000. *Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens.* J. Allergy Clin. Immunol. **105**: 782-787.

8. Bueno, C., C. D. Lemke, G. Criado, M. L. Baroja, S. S. Ferguson, A. K. Rahman, C. D. Tsoukas, J. K. McCormick, and J. Madrenas. 2006. *Bacterial superantigens bypass Lck-dependent T cell receptor signaling by activating a Galpha11-dependent, PLC-beta-mediated pathway.* Immunity. **25**: 67-78.

9. Valentine, M. A., C. D. Tsoukas, G. Rhodes, J. H. Vaughan, and D. A. Carson. 1985. *Phytohemagglutinin binds to the 20-kDa molecule of the T3 complex.* Eur. J. Immunol. **15**: 851-854.

10. Bleasdale, J. E., G. L. Bundy, S. Bunting, F. A. Fitzpatrick, R. M. Huff, F. F. Sun, and J. E. Pike. 1989. *Inhibition of Phospholipase-C Dependent Processes by U-73122.* Advances in Prostaglandin Thromboxane and Leukotriene Research **19**: 590-593.

11. Hogan, P. G., L. Chen, J. Nardone, and A. Rao. 2003*. Transcriptional regulation by calcium, calcineurin, and NFAT.* Genes & Development **17**: 2205-2232.

12. Rhee, S. G. 2001. *Regulation of phosphoinositide-specific phospholipase C. Annual Review of Biochemistry* **70**: 281-312.

13. Cunningham, M. L., T. M. Filtz, and T. K. Harden. 1999. *Protein kinase C-promoted inhibition of G alpha(11)-stimulated phospholipase C-beta activity.* Molecular Pharmacology **56**: 265-271.

14. Gutman, O., C. Walliser, T. Piechulek, P. Gierschik, and Y. I. Henis. 2010. *Differential Regulation of Phospholipase C-beta(2) Activity and Membrane Interaction by G alpha(q), G beta(1)gamma(2), and Rac2.* Journal of Biological Chemistry **285**: 3905-3915.

15. Todd, J. K., M. Ressman, S. A. Caston, B. H. Todd, and A. M. Wiesenthal. 1984. *Corticosteroid therapy for patients with toxic shock syndrome.* JAMA **252**: 3399-3402.

16. Shi, T. J., S. X. Liu, H. Hammarberg, M. Watanabe, Z. Q. Xu, and T. Hokfelt. 2008. *Phospholipase C{beta}3 in mouse and human dorsal root ganglia and spinal cord is a possible target for treatment of neuropathic pain.* Proc. Natl. Acad. Sci. U. S. A **105**: 20004-20008.

17. Hou, C., T. Kirchner, M. Singer, M. Matheis, D. Argentieri, and D. Cavender. 2004. *In vivo activity of a phospholipase C inhibitor, 1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-di one (U73122), in acute and chronic inflammatory reactions.* J. Pharmacol. Exp. Ther. **309**: 697-704.

7

Novel non-transcriptionally acting glucocorticoid receptor ligands that dissociate immunosuppression from effects on adipogenesis and muscle cell atrophy.

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Summary

The use of glucocorticoids as immunosuppressives is limited by important side effects such as induction of adipogenesis and development of muscle atrophy. Glucocorticoids bind to the glucocorticoid receptor (GR) resulting in the formation of a homodimer that translocates from the cytosol to the nucleus. In the nucleus these dimers bind to glucocorticoid response elements (GRE) on the DNA and mediate both transactivation and transrepression of target genes. In addition to this transcriptional regulation of cellular phenotype, there are also more rapid, non-transcriptionally mediated effects of glucocorticoids. We have previously shown that the GR is part of the T cell receptor (TCR) complex and that ligand binding results in dissociation of the GR from this complex and inhibition of canonical TCR signaling through LCK-PLCγ.[1, 2] The dissociation of this complex appears to play an important role in glucocorticoid mediated inhibition of T cell activation as stimuli that bypass LCK-PLCγ-signaling render T lymphocytes glucocorticoid resistant.[3] Here we aimed to develop a GR ligand that inactivates LCK-PLCγ-signaling without resulting in transcriptional regulation. Potential steroidal and non-steroidal candidates were identified using an in silico docking assay to predict GR affinity. Selected compounds where screened in in vitro for GRE mediated transcriptional regulation and their capacity to inhibit T cell activation. This approach led to the discovery of compounds S3.1 and S3.4, which lack a generic cortisol structure but bind the GR in T lymphocytes and inhibit LCK-PLCγ-dependent T cell proliferation without causing transcriptional modulation of GR target genes. In contrast to classical glucocorticoids, S3.1 and S3.4 do not induce adipogenesis and do not cause muscle cell atrophy in vitro. Our data show that it is possible to develop non-steroidal GR ligands that dissociate transcriptional from non-transcriptional effects and may have reduced side effects.

We started our search for a non-transcriptionally acting GR ligand with two rounds of in silico screening. In the first round, we identified ~18,000 steroidal and ~230,000 non-steroidal compounds based on their similarity to the structure of cortisol or gonane respectively. In the second round these compounds were virtually docked into the binding pocket of the GR in two distinct conformations and assigned a score that reflected their predicted binding potency. The top 30 steroidal and 50 non-steroidal compounds with the highest scores were then further screened using two in vitro assays. The first assay consisted of HEK-293 cells stably transduced with a GRE-luciferase construct to determine if the compounds were capable of inducing GR-dependent transcriptional regulation. To measure the ability of the compounds to inhibit T cell proliferation, primary human peripheral blood lymphocytes (PBL) were stimulated with phytohemagglutinin (PHA) or Staphylococcus aureus enterotoxin B (SEB) in the presence of 10 μM of each compound and proliferation was measured using a tritiated thymidine incorporation assay. PHA binds CD3 and activates canonical LCK-PLCγ signaling which can be almost completely inhibited by glucocorticoids.[4] In contrast, SEB activates both canonical TCR dependent LCK-PLCγ signaling and a non-canonical G protein-PLCβ dependent signaling pathway, which renders SEB stimulated lymphocytes partially steroid resistant.[3] We were therefore looking for a compound that would inhibit PHA dependent proliferation to a greater extent than SEB mediated proliferation. This strategy (for results of all compounds see Extended Data Table 1) resulted in the identification of two non-steroidal compounds, S3.1 and S3.4 (Fig. 1a-d) which did not result in GRE-mediated transactivation and inhibited PHA mediated proliferation to a greater extent than SEB mediated proliferation (Fig. 1e). To confirm binding of S3.1 and S3.4 to the GR, we performed a competition assay using [^{3}H]dexamethasone in human PBL (Fig. 1f). This showed that both compounds are low affinity ligands for the GR. The dose-response curve for inhibition of PHA mediated lymphocyte proliferation of both compounds was much lower than high affinity ligand dexamethasone but similar to that of hydrocortisone (Fig. 1g).

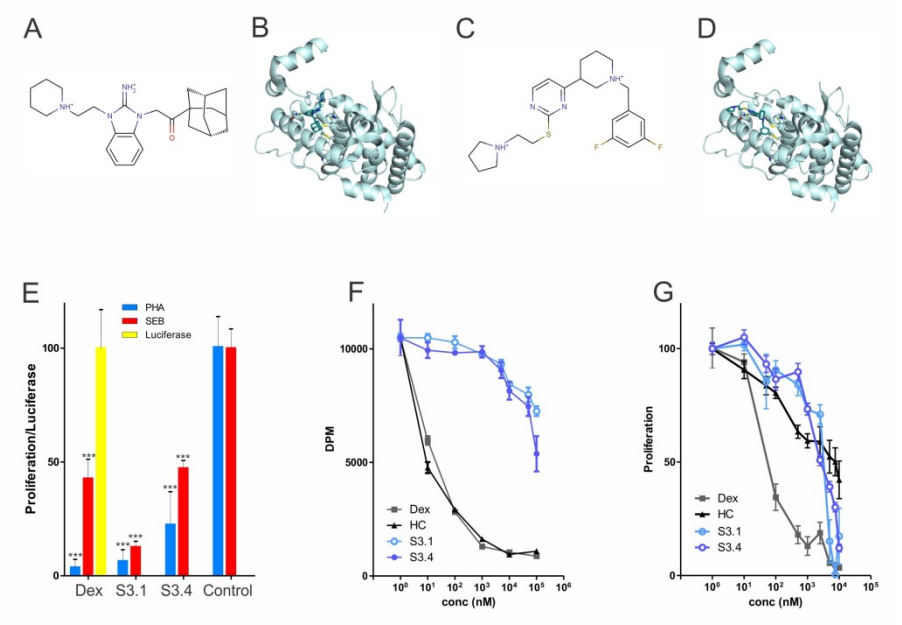

Figure 1. Identification of S3.1 and S3.4 as low affinity GR ligands that lack transcriptional activation but suppress T cell activation. **a**, structure formula of S3.1. **b**, 3D representation of S3.1 binding to the GR. **c**, structure formula of S3.4. **d**, 3D representation of S3.4 binding to the GR. **e**, HEK-293 cells stably transduced with a GRE-luciferase construct were treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm), luciferase production (yellow bars) is shown relative to dexamethasone. Human PBL were treated with PHA (10 mg/ml, blue bars) or SEB (100 ng/ ml, red bars) in the presence or absence of dexamethasone, S3.1 and S3.4 and T cell proliferation was measured using tritiated thymidine, data are relative to PHA/SEB stimulated PBL in the absence of compound. **f**, competition assay using [^{3}H]dexamethasone in whole human PBL. S3.1 and S3.4 show low affinity for the GR compared to both dexamethasone (Dex) and hydrocortisone (HC). **g**, proliferation of PHA (10 mg/ml) stimulated human PBL relative to no compound in the presence of a concentration range of dexamethasone (Dex), hydrocortisone (HC), S3.1 and S3.4. Data in **e**-**g** are mean and standard error. *** = $P < 0.001$.

We previously found that the chaperone HSP90 is specifically complexed with both LCK and the GR in activated T cells in the absence of GR ligand. Binding of the GR by glucocorticoids dissociates this complex with resulting

inhibition of signaling downstream of LCK. We found that both S3.1 and S3.4 inhibited of the phosphorylation of downstream LCK signaling targets ZAP70 and LAT similar to dexamethasone (Fig. 2a). To examined if S3.1 and S3.4 would disrupt the interaction between the GR and LCK we performed an immunoprecipitation experiment on the endogenous proteins in primary human PBL. Immunopreciptation of GR showed that treatment of PBL with PHA resulted in increased interaction between the GR and LCK, this interaction was disrupted by dexamethasone as well as S3.1 and S3.4 (Fig 2b). Dissociation by S3.4 was as potent as that induced by dexamethasone. We next determined if S3.1 and S3.4 would also dissociate LCK from the chaperone HSP90, again by immunoprecipitating the endogenous proteins in PHA treated primary human PBL. Treatment with PHA increased the interaction between LCK and HSP90, which was reduced by both compounds (Fig. 2c). Again the dissociation induced by S3.4 was as potent as that induced by dexamethasone. Immunoprecipitation of LCK flowed by Western blot for HSP90 gave the same results (Fig. 2d).

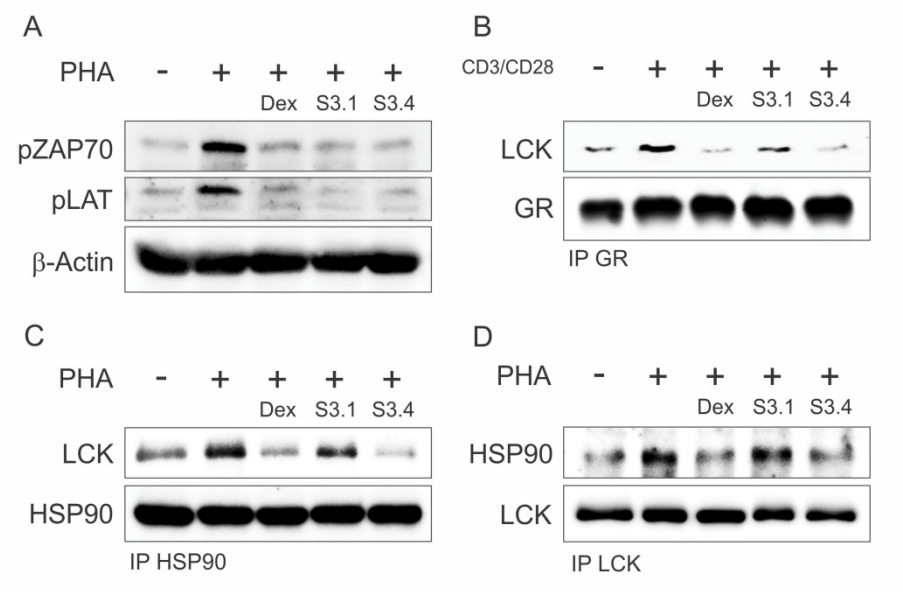

Figure 2. S3.1 and S3.4 dissociate the HSP90-GR-LCK complex. **a**, Dexamethasone (10 μm), S3.1 (10 μm) and S3.4 (10 μm) inhibit phosphorylation of downstream TCR signaling targets ZAP70 and LAT in PHA stimulated human PBL. **b**,

Immunoprecipitation of the GR in CD3/CD28 stimulated human PBL shows that dexamethasone (Dex, 10 μm), S3.1 (10 μm) and S3.4 (10 μm) dissociate the interaction of the GR and LCK in activated T cells. **c**, Immunoprecipitation of HSP90 in PHA stimulated human PBL shows that dexamethasone (Dex, 10 μm), S3.1 (10 μm) and S3.4 (10 μm) dissociate the interaction of the HSP90 and LCK in activated T cells. **d**, immunoprecipitation of LCK shows the same results.

Since both S3.1 and S3.4 do not induce GRE-mediated transcriptional modulation, we examined if the compounds induced nuclear translocation of the GR. We transiently transfected HCT116 cells that express no detectable GR, with a GFP labeled GR. Dexamethasone induced a potent nuclear translocation of the GR as expected (Fig. 3a). Of the two compounds, only S3.4 induced a modest level of nuclear translocation in GR-GFP transfected cells. This result was confirmed by detecting endogenous GR expression by Western blot in Hela cells after isolating nuclear and cytosolic fractions (Fig. 3b,c). Phosphorylation of residues near the N terminus of the GR such as serine 211 (S211) is important to regulate its transcriptional activity.[5] To investigate the phosphorylation status of the GR receptor at S211 after treatment, lysates from Hela cells were analyzed on Western blot (Fig. 3d). Treatment with neither S3.1 nor with S3.4 causes the GR to become phosphorylated. Similar results were obtained when using lysates from stimulated PBL (Fig. 3e).

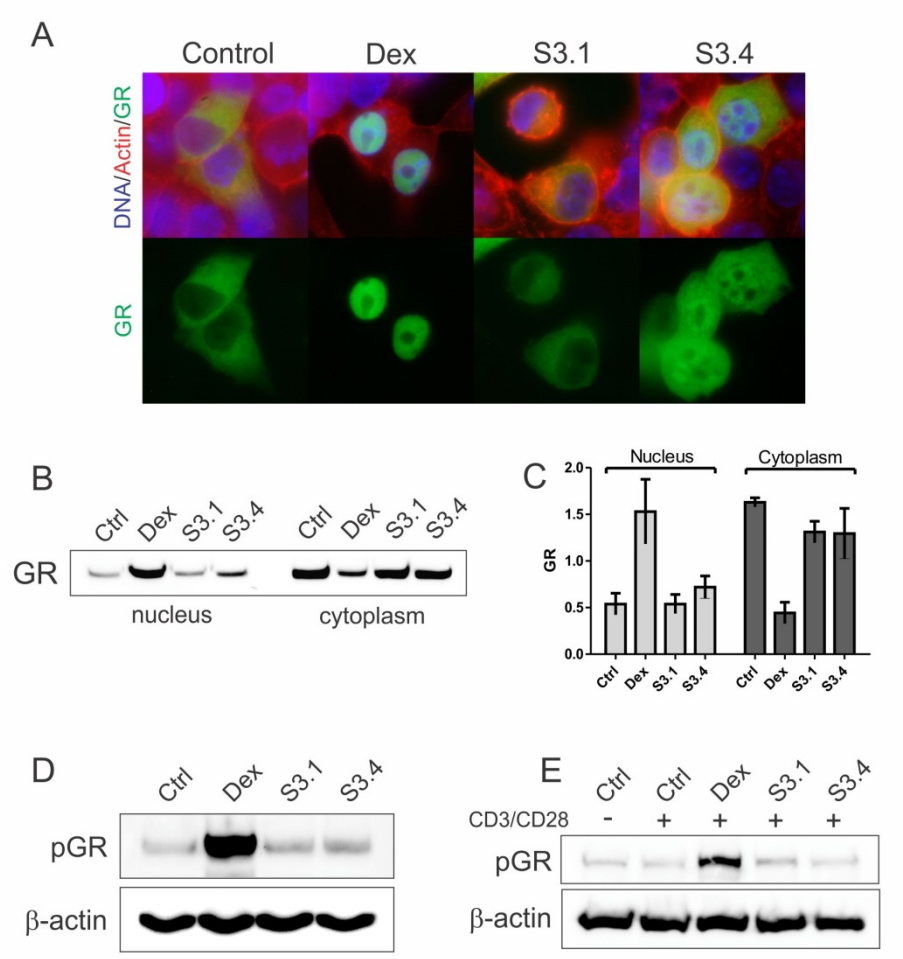

Figure 3. S3.1 and S3.4 do not cause nuclear translocation of phosphorylation of the GR. **a**, HCT116 cells were transfected with a GFP tagged GR and treated with either dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm). The localization of the GR was examined by immunofluoresence. **b**, Hela cells were treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm) and the localization of the GR was determined by western blot in nuclear and cytosolic extracts. **c**, densiometric analysis of nuclear translocation of the GR in Hela cells as determined by western blots of three independent experiments. **d**, phosphorylation of the GR in Hela cells treated with dexamethasone, S3.1 and S3.4. **e**, phosphorylation of the GR in CD3/CD28 stimulated human PBL treated with dexamethasone, S3.1 and S3.4.

Thus, S3.1 and S3.4 are low affinity nonsteroidal GR ligands that inhibit T cell proliferation by dissociating the HSP90-LCK interaction without inducing nuclear translocation of the GR. It is not known to what extent side effects such as adipogenesis and muscle atrophy are dependent on non-transcriptional or transcriptional effects of the GR. We therefore examined the capacity of S3.1 and S3.4 to induce adipogenesis in 3T3-L1 cells, a well-established model of glucocorticoid induced adipogenesis. In these cells treatment with dexamethasone causes adipocyte formation that can be detected by significant accumulation of fatty acids in intracellular vacuoles (Fig. 4a,b). In contrast, treatment with S3.1 or S3.4 did not induce any adipogenesis at all.

Glucocorticoid induced muscle atrophy is caused by loss of muscle fiber thickness due to transactivation of myostatin, a negative modulator of skeletal muscle. To test if treatment with S3.1 or S3.4 caused a reduction in the diameter of muscle fibers a C2C12 myotube model was used. After establishment of the myotubes in culture, the cells were treated for 24 hours with S3.1, S3.4 or dexamethasone. Treatment with dexamethasone reduced the diameter of the myotubes significantly while treatment with S3.1 or S3.4 did not affect the myotubes and diameters remained comparable to the untreated control (Fig. 4c,d).

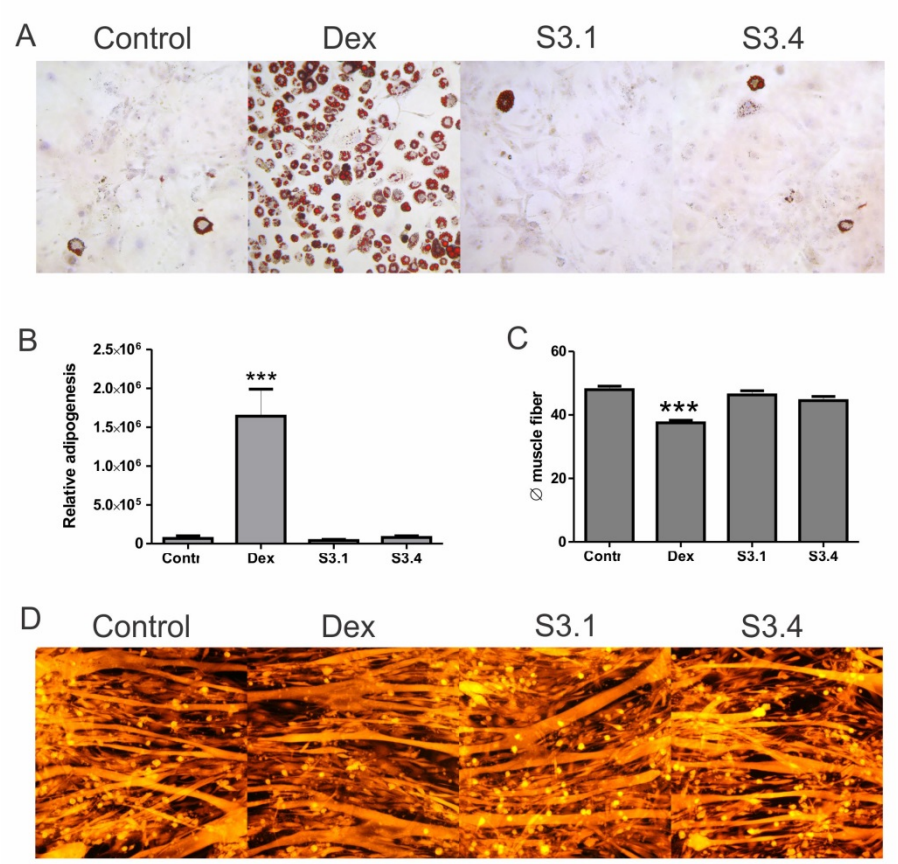

Figure 4. S3.1 and S3.4 do not induce adipogenesis and do not cause myotube atrophy. **a**, 3T3-L1 cells treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm). **b**, quantification of induction of adipogenesis measuring oil red O positive pixels per high powerfield (200x magnification) using ImageJ software (n=4 per condition). **c**, quantification of muscle fiber thickness (n >250 per condition) in C2C12 cells treated with dexamethasone (Dex, 10 μm), S3.1 (10 μm) or S3.4 (10 μm). **d**, muscle fibers of C2C12 cells visualized using glutaraldehyde induced autofluorescence. Data in **b** and **c** are mean and standard error. *** = $P < 0.001$.

All steroid hormones have rapid non-transcriptionally mediated effects in addition to their classical transcriptionally mediated cellular responses.[6] We have previously shown that glucocorticoids inhibit TCR signaling non-transcriptionally by dissociating a TCR associated complex that contains

HSP90, LCK and the GR.[1-3, 7] Thus far it has not been possible to determine if non-transcriptionally mediated GR dependent effects are sufficient to cause inhibition of T cell signaling. Also, since no compounds were available thus far to specifically induce non-transcriptional GR mediated effects it was not known if side effects such as adipogenesis and muscle cell atrophy were dependent on transcriptional or non-transcriptional effects of glucocorticoids. The findings presented here show that it is possible to design GR ligands that specifically dissociate the GR from its cytosolic complex with HSP90 and LCK without causing phosphorylation or nuclear translocation of the GR. Such compounds therefore lack any of the transcriptionally mediated effects of GR bound by classical agonists such as dexamethasone that cause potent phosphorylation and nuclear translocation of the GR. The identification of S3.1 and S3.4 as non-transcriptionally acting GR ligands has allowed us to demonstrate that such compounds inhibit TCR signaling and T cell proliferation without causing effects on adipogenesis or myotube thickness in established in vitro models of these glucocorticoid induced side effects. Our approach shows that it is possible to dissociate GR mediated immunosuppression from unwanted side effects by generating ligands that dislocate the GR from the HSP90 complex but fail to induce nuclear translocation.

Material and Methods

In silico compound screen

We screened 9.2 million publically available compounds from the Zinc database (http://zinc.docking.org) in October 2009. In order to enhance the chance of finding chemicals with the desired effect we worked in parallel with two sets of compounds. The first set consisted of steroidal compounds that contained the four conjugated rings of cortisol (Extended data Fig. 1a), other naturally occurring steroid hormones (Extended data Fig. 1b,c) or synthetic GR agonists such as dexamethasone possessing two double bonds in the first ring (Extended data Fig. 1d). An exact match of a compound with the query skeleton was used as a selection criterion resulting in the identification of ~18,000 candidate compounds. The second

set of compounds comprised molecules that share some similarity to gonane which was quantified using the LINGO method that has been previously described[8] and has been implemented in the OEChem Tool Kit (OpenEye Scientific Software, Inc., Santa Fe, NM, USA). This second set consisted of a further ~230,000 non-steroidal compounds.

In the next round of *in silico* screening, all compounds selected in the first round were virtually docked to the GR binding pocket using the glide mode of the Schrödinger program suite (Schrödinger, LLC, New York). To cover the different conformational spaces in which the compounds could be docking we used four different structures of the GR (to be found at Protein Data Bank http://www.rcsb.org/pdb/home/home.do, under IDs 1M2Z, 1P93, 1NHZ and 3BQD). Structures 1M2Z and 1P93 describe the GR in a state bound by dexamethasone. Another 2 structures (1NHZ, 3BQD) describe the GR bound by an antagonist.

Compounds were finally sorted according to their Z-score values that reflect the probability of binding to the receptor. In order to test compounds capable of binding both known conformational states of the GR we selected the top 30 steroidal compounds and the top 50 non-steroidal compounds in each conformation for *in vitro* testing, resulting in a total of 80 compounds (Extended Data Table 1).

Antibodies and Reagents

Dexamethasone, hydrocortisone, IBMX, insulin, oil red O, glutaraldehyde and SEB were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Luciferase was purchased from Promega (Leiden, The Netherlands). PHA (PHA-M), phalloidin, SlowFade® Gold Antifade Reagent with DAPI and horse serum was purchased from Life Technologies (Bleiswijk, The Netherlands). Human anti-CD3 and anti-CD28 were purchased from Sanquin (Amsterdam, The Netherlands). Anti-phospho-GR (Ser211) #4161, anti-phospho-Zap70 (Tyr319)/Syk (Tyr352) #2701 and anti-phospho-LAT (Tyr171) #3581 were purchase from Cell Signaling Technologies (Leiden, The Netherlands). Anti-Actin (I-19) (sc-1616-R), HSP90α/β (H-114) (sc-7947), HSP 90α/β (N-17) (sc-1055), Lck (2102) (sc-

13), Lck (3A5) (sc-433), GR (E-20) (sc-1003) and GR (P-20) (sc-1002) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell Culture

Human PBLs were isolated from whole blood of healthy volunteers by ficoll-isopaque density-gradient centrifugation. After washing, monocytes were separated from lymphocytes by percoll density-gradient centrifugation. The lymphocytes were cultured in IMDM (Life Technologies, Verviers, Belgium) supplemented with 10% heat-inactivated FCS. For measurements of cell proliferation, cells were stimulated for 24 h. In all experiments, lymphocytes were stimulated with PHA (10 mg/ml) or SEB (100 ng/ ml). Proliferation was measured using a [^{3}H]thymidine-incorporation assay.

Glucocorticoid mediated transactivation was measured in HEK293 cells stably transduced with a luciferase gene behind a glucocorticoid responsive element (Panomics, Milano, Italy). The cells contain Luciferase activity was measured using the Luciferase Assay System from Promega (Leiden, the Netherlands) according to the manufacturers protocol.

Localization of the GR was investigated by transiently transfecting HCT116 colorectal cancer cells with a GR-GFP fusion construct using lipofectamine (Life Technologies, Bleiswijk, the Netherlands). Measurements of GR in nuclear fractions was performed in Hela cells. HCT116 and Hela cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Verviers, Belgium) supplemented with 10% heat-inactivated FCS.

Induction of adipogenesis was determined in 3T3-L1 mouse fibroblasts. Two days after the cells reached confluency the medium was replaced by DMEM containing 10% FCS, 1.6 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and either 0.25 μM dexamethasone, 10 μm S3.1 or 10 μm S3.4. After two days the medium was replaced by DMEM containing 10% FCS and 1.6 μM insulin. One week later lipid vacuoles were visualized using oil red O.

GC induced muscle atrophy was investigated using C2C12 myotube model. C2C12 cells were allowed to reach 90% confluency at which point the medium was replaced by DMEM containing 2% horse serum and 1 µM insulin. To stop cells dividing the culture was treated with 4 µg/ml Arac (Sigma-Aldrich, Zwijndrecht, The Netherlands). After 1 week cells were treated for 24 hours with dexamethasone, S3.1 or S3.4 and fixed in glutaraldehyde. Glutaraldehyde causes autofluorescence which allows visualization under a fluorescent microscope.

Western Blot

Samples for Western blot were made by treating 5 x 10^6 lymphocytes for 10 minutes with 10 µM dexamethasone followed by 10 minutes stimulation. When using cell lines 20 µg of protein was loaded, as determined by BCA (Pierce, Etten-Leur, The Netherlands). Cells were pelleted and lysed in lysis buffer (0.1% NP40, 50 mM Tris HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 200 µM Na3VO4, 10 mM NaF) containing protease inhibitors (Roche, Mannheim, Germany) for 20 minutes on ice. Samples were spinned down, sample buffer was added to the supernatant and heated to 95 °C for 5 minutes, cooled on ice and sonicated. Samples were blotted overnight at 4°C in buffer containing 20% methanol on PVDF membrane (Millipore, Bedford, USA). The membrane was blocked with 5% BSA for 1 hour at RT followed by overnight incubation with the primary antibody at 4°C.

Immunoprecipitation

For immunprecipitation experiments 10 x 10^7 cells were used per condition. Cells were pelleted and lyzed as described above. The supernatant was precleared by adding 10 µl Protein A/G UltraLink resin (Pierce Biotechnology, Rockford, USA) for 30 minutes at 4°C. Beads were spinned down and discarded. Samples were incubated with 2 µg primary antibody and 25 µl beads for 2 hours at 4°C. Beads were washed with PBS, sample buffer was added and heated to 95°C for 5 minutes.

Preparing Nuclear Extracts

Cells were grown in 10cm plates till approximately 80% confluency. After completing the treatment, medium was replaced with ice cold PBS and cells were scraped while on ice. Cells were spinned down, supernatant discarded and the pellet was resuspended in 200 µl of buffer 1 (25 mM Hepes, pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 1 mM DTT, protease inhibitors). 200 µl Buffer 2 (Buffer 1 + 0.1% NP40) was added and tubes were rotated for 15 minutes at 4°C. Nuclei were spinned down for 1 minute at 2500 rpm at 4°C. Supernatant was transferred to new tube as cytosolic fraction. Pellet was washed in 1 ml ice cold PBS followed by resuspension in 400 µl of Buffer 3 (25 mM Hepes, pH 7.9, 350 mM Na, 400 mg sucrose, 0.05% NP40, 1 mM DTT, protease inhibitors) and 1 hour of rotation at 4°C. Tubes were spinned down and the supernatant transferred as the nuclear fraction. Equal volumes were loaded on gel.

Immunofluorescence

Subcellular localization of the GR was visualized by using a GR-GFP fusion construct which was transiently tranfected in HCT116 colorectal cancer cells. Actin was stained red using phalloidin-TRITC (Sigma, Zwijndrecht, The Netherlands) and the nucleus with DAPI included in the Slowfade mounting fluid (Life Technologies, Bleiswijk, The Netherlands).

Formation of lipid vacuoles during fibroblast-adipocyte differentiation was visualized by staining triglycerides and lipids with the lysochrome Oil Red O. The C2C12 myotubes were visualized using the autofluorescence caused by fixation with 2% glutaraldehyde.

Competition Assay

Binding of the compounds to the GR was tested in a whole cell in vitro competition assay using [3H]dexamethasone. Per condition 3 x 10^6 PBL were stimulated for 15 minutes with PHA at 37°C. Tubes were then incubated for one hour at room temperature with compound and [3H]dexamethasone. Samples were spinned down at 400 G and supernatant discarded. The cells were washed three times by resuspending in ice cold PBS and rotating for 20 minutes at 4°C. Radioactivity was

measured in a scintillation counter using Utima gold scintillation fluid (Perkin Elmer, Groningen, The Netherlands).

Quantification and Data Analysis

Measurement of the thickness of myotubes was done in arbitrary units using the measuring tool of Photoshop CS6 (Adobe Systems Benelux BV, Amsterdam, Nederland). Thickness of each myotube was measured at three different places. Quantification of the Oil Red O staining and densitometric measurements of Western blot were done using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA). For data and statistical analysis we used Excel 2010 (Microsoft, Redmond, WA, USA) and Graphpad Prism (Graphpad Software Inc, La Jolla, CA, USA).

Acknowledgements

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Reference List

1. Lowenberg,M. et al. Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. Blood 106, 1703-1710 (2005).

2. Lowenberg,M. et al. Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN. EMBO Rep. 7, 1023-1029 (2006).

3. Verhaar,A.P. et al. Superantigen-induced steroid resistance depends on activation of phospholipase Cbeta2. J. Immunol. 190, 6589-6595 (2013).

4. Valentine,M.A., Tsoukas,C.D., Rhodes,G., Vaughan,J.H., & Carson,D.A. Phytohemagglutinin binds to the 20-kDa molecule of the T3 complex. Eur. J. Immunol. 15, 851-854 (1985).

5. Ismaili,N. & Garabedian,M.J. Modulation of glucocorticoid receptor function via phosphorylation. Ann. N. Y. Acad. Sci. 1024, 86-101 (2004).

6. Losel,R. & Wehling,M. Nongenomic actions of steroid hormones. Nat. Rev. Mol. Cell Biol. 4, 46-56 (2003).

7. Lowenberg,M., Verhaar,A.P., van den Brink,G.R., & Hommes,D.W. Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. Trends Mol. Med. 13, 158-163 (2007).

8. Vidal,D., Thormann,M., & Pons,M. LINGO, an efficient holographic text based method to calculate biophysical properties and intermolecular similarities. J. Chem. Inf. Model. 45, 386-393 (2005).

PART III

MILTEFOSINE

8

Miltefosine suppresses inflammation in a mouse model of inflammatory bowel disease

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Abstract

Background: The repertoire of immunomodulators that can be used for the treatment of inflammatory bowel disease (IBD) is limited. The use of these drugs is further restricted by the occurrence of side effects in a proportion of patients. Miltefosine (hexadecylphosphocholine) is a lipid drug developed in the eighties for treatment of cancer but is nowadays best known for its application in the oral treatment of Leishmaniasis. Although the exact mechanism of action of miltefosine has yet to be elucidated the drug has previously been shown to inhibit phospholipases and protein kinase C, both key components of pro-proliferative signal transduction in T cells.

Methods: Stimulated PBL were treated with miltefosine and proliferation was measured. We use the CD45RBhigh T cell transfer colitis model to investigate the effect of miltefosine treatment on intestinal inflammation. Effects on the severity of colitis were studied by histochemical- and immunohistochemical staining and cytokine levels were determined using a cytokine bead array.

Results: Miltefosine inhibited T cell proliferation *in vitro*. In the transfer model miltefosine significantly ameliorated the severity of colitis as measured by clinical, (immuno)histochemical and biochemical parameters.

Conclusions: Miltefosine inhibits T cell proliferation and effectively reduces inflammation in the T cell transfer model. The drug may therefore be a candidate immunomodulator for IBD.

Introduction

A limited number of drugs is available for the treatment of inflammatory bowel disease. In Crohn's disease for example, glucocorticoids can be used for remission induction but are generally not advised for long term treatment because of their limited value for remission maintenance and important side effects. Thiopurines and methotrexate are widely used but unfortunately only work in a proportion of patients and are sometimes poorly tolerated. If tolerated, the drugs can show important side effects such as bone marrow suppression or pancreatitis. Even if patients are started on anti-TNF antibodies immunomodulators are often used as co-treatment to increase the efficacy of the anti-TNF and prevent the development of anti-TNF neutralizing antibodies. Therefore there is a clear need for the identification of novel candidate immunomodulators for remission induction or maintenance in patients with IBD.

Miltefosine is an ether lipid drug originally designed in the eighties for the treatment of cancer. Systemic treatment with the drug has been tested in phase I[20] and phase II clinical trials in advanced colorectal cancer,[13] soft tissue sarcoma,[19] squamous cell head and neck cancer.[18] In oncology miltefosine only made it a phase III trial that showed effectiveness as a topical palliative treatment for cutaneous metastases of breast cancer.[11] In the mean time however it was found that miltefosine showed activity against *Leishmania* in animal models[10] and clinical trials showed spectacular therapeutic efficacy in patients with visceral leishmaniasis.[8,15] Miltefosine is well absorbed, has a half life of 7 days[5] and is generally well tolerated at a dose of 100-150 mg/day that is used for the treatment of Leishmaniasis. The most important side effect of doses up to 150 mg/day are mild-to-moderate nausea and vomiting which occur mostly in the first two weeks of treatment and are often transient.[8,15] Furthermore, mild and reversible elevations in the aminotransferases and creatinine have been described.[8,15]

The mechanism of action of miltefosine is incompletely understood. Miltefosine is chemically related to membrane phospholipids and generally

believed to act by modulating signaling events at cellular membranes.[2] It has been reported that miltefosine inhibits phospholipase C, phospholipase D and protein kinase C (PKC).[2] Since activation of T cells critically depends on activation of phospholipase C and PKC we hypothesized that treatment with miltefosine might inhibit T cell activation. Indeed, it has previously been reported that miltefosine inhibits T cell proliferation in mixed lymphocyte reactions and showed inhibitory activity in an ovalbumin induced mouse model of delayed type hypersensitivity in mice[3] and in patients with atopic dermatitis.[4]

Here we find that miltefosine inhibits both phytohemagglutanin and staphylococcal enterotoxin B mediated T cell proliferation. Our experiments show that treatment of mice with miltefosine ameliorates severity of a T cell dependent murine model of IBD.

Material and Methods

Reagents and Antibodies

Anti-mouse CD3 (A0452), biotin labeled goat anti-rat and streptavidin-HRP complex were purchase from DAKO (Heverlee, Belgium). An poly HRP labeled anti–rabbit antibody (DPVR110HRP) was purchased from Immunologic (Duiven, The Netherlands). FITC rat anti-mouse Ly-6G (553127), FITC Rat anti-Mouse CD45RB (553099) and PE-Cy™5 rat anti-mouse CD4 (553050) were purchased from BD Pharmingen (Breda, The Netherlands). F4/80 Rat anti-Mouse, clone BM8 (T2006) was purchased from BMA Biomedicals (Augst, Switzerland). Rabbit F(ab')2 Anti-Rat IgG (6130-01) was purchased from Southern Biotech (Birmingham, Alabama, USA). Miltefosine was purchased from Cayman Chemical (Huissen, The Netherlands). Sheep anti-rat IgG Dynabeads were purchased from Invitrogen (Oslo, Norway)

Cells

Human peripheral blood lymphocytes (PBLs) were isolated from whole blood of healthy volunteers by Ficoll-Isopaque density gradient centrifugation. After washing monocytes were separated from lymphocytes by percoll density gradient centrifugation. The lymphocytes were cultured in IMDM (Gibco, Verviers, Belgium) supplemented with 10% heat inactivated fetal calf serum. For proliferation experiments cells were stimulated for 24 hours. Lymphocytes were stimulated with PHA (10µg/ml) or SEB (100 ng/ml). Proliferation was measured using a ^{3}H-thymidine incorporation assay. Mouse splenocytes were isolated from spleen from C57BL/6 mice. Spleens were homogenized and passed through a 70 □m strainer.

Mouse experimental colitis CD45RB^{High} transfer model

C.B-17 SCID mice and wild type BALB/c mice were ordered from Harlan (Boxmeer, The Netherlands). Transfer of CD45RB^{High} CD4^{+} cells was performed as previously described by Read and Powrie.[14] In short; for

every three SCID mice $CD45RB^{High}$ cells were isolated from the spleen of a single wild type BALB/c mouse. A single cell suspension was created by forcing the spleens through a cell strainer. Erythrocytes were removed by adding erythrocyte lysis-buffer followed by negative depletion of macrophages, B cells and $CD8^+$ cells using magnetic beads. The remaining $CD4^+$ cell enriched cell suspension was labelled and $CD45RB^{High}$ and $CD45RB^{Low}$ cells were isolated on a FACS sorter. Colitis was induced by injecting $4 \cdot 10^5$ $CD45RB^{High}$ cells intraperitoneally. Control mice received a combination of $4 \cdot 10^5$ $CD45RB^{High}$ cells and $2 \cdot 10^5$ $CD45RB^{Low}$ cells. The inflammation developed over a period of 8 to 10 weeks. Two groups of ten SCID mice each were injected with $CD4^+CD45RB^{High}$ cells and one group of ten mice was injected with a combination of $CD4^+CD45^{High}$ and $CD4^+CD45RB^{Low}$ cells. Because the first two groups have a reconstituted T cell repertoire lacking regulatory T cells, they will develop colitis. The last group does have regulatory T cells (contained in the $CD4^+CD45RB^{Low}$ compartment), will not develop colitis and acts as a control group.

Miltefosine was dissolved in 10% DMSO in PBS. Each mouse received 50 mg/kg miltefosine twice weekly in 200 µl solution by oral gavage or in the case of the positive control group 200 µl 10% DMSO in PBS.The mice were euthanized by CO_2 inhalation followed by removal of the spleen and colon.

Body weights were recorded three times a week, and wasting disease progression was calculated by percentage of weight loss from initial body weight. Animals were withdrawn from the study when the weight loss was more than 15% compared with the starting weight. Both the colon and the spleen were removed and weighed. Two independent investigators blinded for treatment allocation scored the colons for stool consistency, visible fecal blood, and macroscopic inflammation using a scale of 0–3 per item with a maximum score of 9. Tissue weights were recorded and used as an index of disease related intestinal wall thickening. Colons were subsequently divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine determination, while the second part was stored in formalin and embedded in paraffin for (immuno)histological evaluation.

Histological Analysis

H&E sections were blindly scored by an independent experienced GI pathologist (SLM). The histology damage score was calculated as the total score of points that were scored on 6 criteria. (A) Leukocyte infiltration: 0 = normal, 1 = increase in mucosa, 2 = increase in mucosa + submucosa, 3 = extending into tunica muscularis. (B) Loss of goblet cells: 0 = none, 1 = < 10% depletion, 2 = 10-50%, 3 = >50%. (C) Crypt loss: 0 = none, 1 = < 10% loss, 2 = 10-50%, 3 = >50%. (D) Epithelial hyperplasia: 0 = normal, 1 = slight, 2 = 2-3x increased crypt length, 3 = >3x increase. (E) Ulceration: 0 = none, 4 = present. (F) Crypt abcesses: 0 = none, 4 = present. The histological damage score ranged from 0 points to a maximum of 20 points.

Immunohistochemistry

For immunohistochemical staining slides were deparaffinized, dehydrated and immersed in 1.5% H_2O_2 in phosphate-buffered saline (PBS) for 30 min. Different methods of antigen retrieval were used for the different antibodies. For the F4/80 staining slides were cooked for 10 min in 0.1 M sodium citrate (pH 6), for the CD3 staining they were cooked for 10 min in Tris/EDTA buffer (10 mM Tris, 1 mM EDTA (pH 9)). For the Ly6G staining antigen retrieval was performed by incubation of the slide in 0.025% pepsin in 0.1 M HCl at 37 °C for 15 min. Subsequently slide were blocked with Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% [vol/vol] Tween-20, pH 8.0) for 30 min, followed by incubation overnight at 4 °C with the primary antibody in PBS with 0.1% Triton X-100 and 1% bovine serum albumin. For detection of F4/80 an avidin–biotin detection system was used. Secondary antibodies were diluted in PBS with 10% human serum and incubated for 1 h at room temperature (RT). Slides were then incubated for 1 h with horseradish peroxidase-conjugated avidin–biotin complex. For detection of CD3 and Ly6G the BrightVision detection system (Immunologic) was used. Peroxidase activity was detected with Sigma Fast 3,3-diaminobenzidine Tablets (Sigma, D4293). Sections were counterstained with hematoxylin, dehydrated and mounted with Pertex

(Histolab 00801). Macrophages, neutrophils and T cells were counted blindly in 10 intercrypt spaces per mouse.

Cytokine Measurements

Human PBL were seeded in a 96 well plate and treated and stimulated for 48 hours. IL-2 cytokine levels were determined by ELISA (Biolegend, San Diego, USA) following manufacturer's instructions.

Cytokine levels in mice were determined in colon mucosa. Colon homogenates were obtained using a Heidolph SilentCrusher M homogenizer at 4°C in 500 µl lysis buffer (Cell Signaling Technology, Beverly, CA) supplemented with protease inhibitors (Roche, Almere, The Netherlands). Samples were centrifuged at 24,000g for 10 minutes at 4°C and stored at -80°C until cytokine measurement. Protein content was determined using the BCA Protein Assay (Thermo Scientific Pierce, Etten-Leur, the Netherlands), and cytokine levels in homogenates were measured using the Cytometric Bead Array System (BD Biosciences, Breda, the Netherlands) following the manufacturer's instructions.

Statistical Analysis

Results are shown as means ± standard error of the mean (SEM) unless otherwise indicated. For statistical analysis, one way analysis of variance (ANOVA) was used followed by a Bonferroni post-test. Results were considered significant when $P < 0.05$.

Results

Miltefosine inhibits T cell proliferation

Previous studies have shown that miltefosine treatment inhibits T cell proliferation in a mixed lymphocyte reaction.[3] We used two stimuli that directly activate the T cell receptor in different ways to confirm this observation. The concentration miltefosine used is within physiologically relevant range.[5] Phytohemagglutinin (PHA) binds CD3 directly[17] and nonspecifically. This results in the potential activation of all T cells. Staphylococcal Enterotoxin B (SEB) is a superantigen that activates a large proportion of the T cell population by binding directly to the Vß chain of the T cell receptor, activating over 20% of the total T cell repertoire.[6] We chose SEB in addition to PHA as it has previously been described that SEB induces steroid resistance in T cells.[7] T cells stimulated with PHA were highly sensitive to dexamethasone whereas SEB induced a state of partial steroid resistance as described (Fig. 1A). In contrast, T cell proliferation induced with PHA and SEB was equally sensitive to miltefosine (Fig. 1B). We subsequently isolated mouse splenocytes from C57BL/6 mice and stimulated these with both PHA and SEB. The sensitivity of mouse splenocytes to both PHA and SEB is less than that of human cells (Fig. 1C) but as in the human cells PHA and SEB showed a similar sensitivity to miltefosine treatment. And finally we measured IL-2 cytokines levels in the medium of PHA or SEB stimulated human PBL treated with different doses of miltefosine (Fig. 1D). IL-2 secretion by both PHA and SEB stimulated PBL showed sensitivity to miltefosine treatment. Stimulation of human PBL with PHA results in very low levels of IL-2 secretion[9].

These results confirm the previous observation that miltefosine inhibits proliferation of T cells and suggests that miltefosine may suppress proliferation of T cells in conditions that cause steroid resistance.

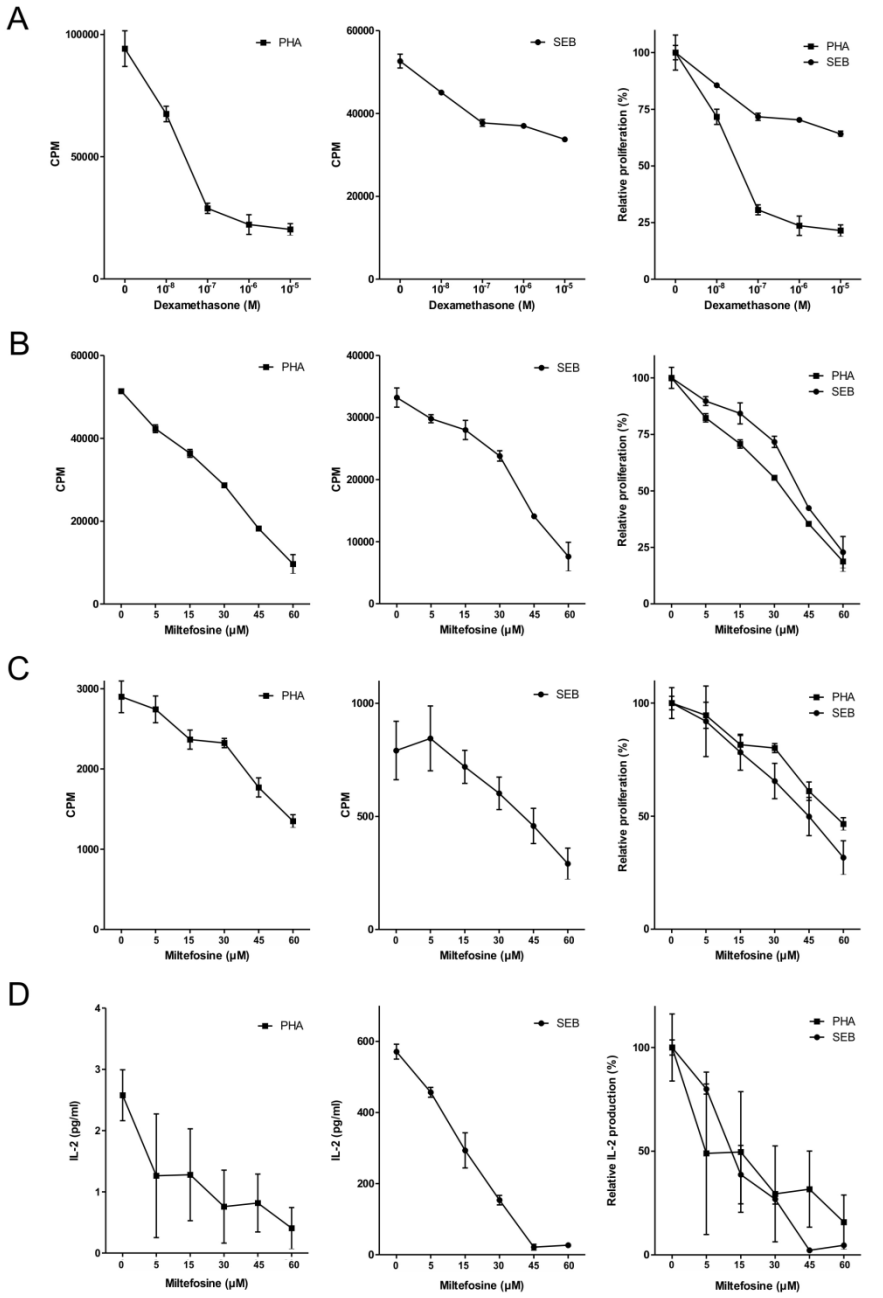

FIGURE 1. Miltefosine inhibits T cell proliferation. (A) Human PBL were stimulated with either PHA (10 μg/ml) or SEB (100 ng/ml) for 24 hours in the presence of

different doses of dexamethasone. Data shown are absolute and relative proliferation as measured by ^{3}H incorporation. PBL treated with SEB show partial steroid insensitivity as previously described. (B) Human PBL were stimulated with either PHA (10 µg/ml) or SEB (100 ng/ml) for 24 hours in the presence of different doses of miltefosine. Data shown are absolute and relative proliferation as measured by ^{3}H incorporation. PBL treated with SEB show no insensitivity to miltefosine. (C) Mouse splenocytes were stimulated with either PHA (10 µg/ml) or SEB (100 ng/ml) for 24 hours in the presence of different doses of miltefosine. Data shown are absolute and relative proliferation as measured by ^{3}H incorporation. Mouse splenocytes show sensitivity to miltefosine that is similar to humans. Each data point represents the mean and SEM of four independent samples. (D) Human PBL IL-2 cytokine levels as measured by ELISA. Human PBL were stimulated with either PHA (10 µg/ml) or SEB (100 ng/ml) for 48 hours in the presence of different doses of miltefosine. Both the production of IL-2 by PHA and by SEB stimulated PBL are sensitive to miltefosine. Each data point represents the mean and SEM of four independent samples.

Miltefosine suppresses inflammation in the CD45RB^{High} transfer model

To investigate if miltefosine might be a candidate immunomodulator for IBD we used the experimental transfer colitis model. In this model SCID mice are reconstituted with $CD4^{+}CD45RB^{High}$ cells. This population lacks the subset of T cells present in the $CD4CD45RB^{Low}$ population that are required to suppress the development of colitis.[1] We treated mice with a twice weekly dose of 50 mg/kg miltefosine by oral gavage. To allow the transferred T cells to home to the intestine and repopulate the mice, we started treatment with miltefosine at two weeks after adoptive transfer. Oral gavage with vehicle was used as a placebo treatment. In the course of two months placebo treated $CD45RB^{high}$ transplanted SCID mice developed severe inflammation of the gut. Two mice died before the end of the experiment. One mouse in the miltefosine treated group died due to a complication of the oral gavage. The other mouse was in the group of placebo mice and was taken out before the end of the experiment due to weight loss > 15%. At completion of the experiment at day 60, mice treated with miltefosine had lost significantly less weight than placebo treated mice (Fig. 2A) suggesting a reduction in the severity of disease. Indeed, inspection of the colon showed that the colon of placebo treated animals

was severely affected whereas colons of miltefosine treated animals showed remarkably less edema and contained normal stool pellets (Fig. 2B). Treatment with miltefosine significantly reduced the disease activity index to levels comparable to controls (Fig. 2C). Colon weight was substantially increased in CD45RB^{high} reconstituted placebo treated mice compared to control mice. Colon weight of miltefosine treated mice was substantially reduced compared to placebo treated animals and not significantly different from control mice that were reconstituted with both CD45RB^{high} and CD45RB^{low} cells (Fig. 2D). A similar observation was made for spleen weight which was reduced to levels controls by treatment with miltefosine (Fig. 2E).

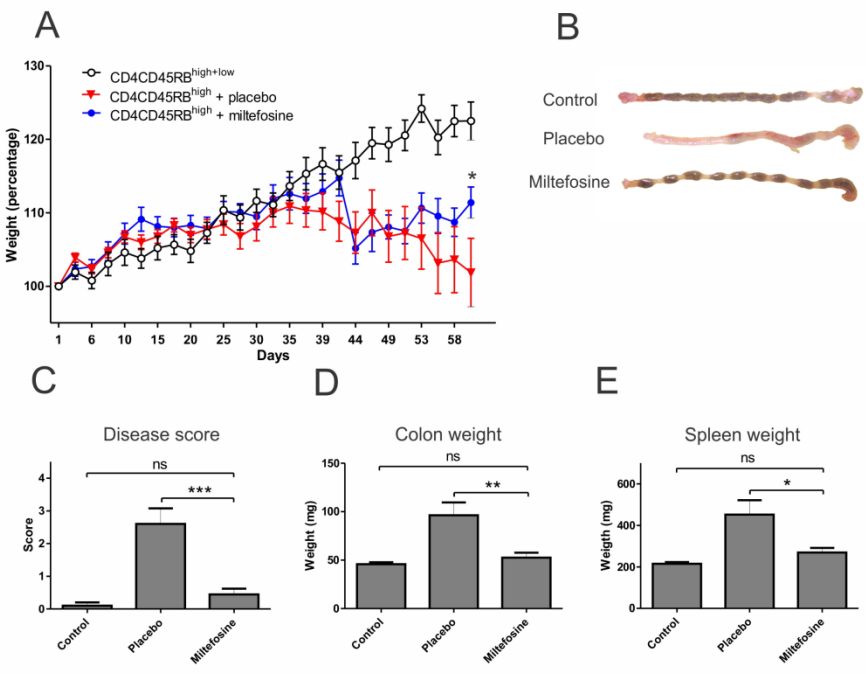

FIGURE 2. Miltefosine treatment reduces inflammation in CD4CD45RB^{High} transfer model. (A) Average weight of each group of mice as a percentage of the starting weight during the course of the experiment. The control group (black open circle) received both CD4CD45RB^{High} and CD4CD45RB^{Low} and did not develop

inflammation. Both the placebo treatment group (red inverted triangle) and the miltefosine treated (50 mg/kg twice weekly) group (blue closed circle) received CD4CD45RB^{High} only. (B) Representative examples of colons from mice in the indicated groups. The colon in the colitis group is clearly inflamed and void of stool while the colon of the treated group shows no signs of inflammation and is filled with solid pellets, comparable to the healthy control. (C) Disease score for different groups. (D) Colon weight. (E) Spleen weight. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Control = CD4CD45RB$^{high+low}$, placebo = CD4CD45RB^{high} + placebo, miltefosine = CD4CD45RB^{high} + miltefosine.

To confirm the gross morphological examination at the histopathological level, coded H&E stained slides where examined by a blinded experienced GI pathologist (SLM) and the degree of inflammation was scored. Mice in the placebo treated group showed clear infiltrates of leukocytes, loss of goblet cells, crypt damage, epithelial hyperplasia, ulcerations and crypt abcesses (Fig. 3A-C). In contrast, in the mucosa of mice that were treated with miltefosine, all aspects of inflammation were significantly reduced compared to placebo treated mice (Fig. 3A-C). None of the histopathological hallmarks of colitis in the miltefosine treated animals was significantly different from control animals without colitis (Fig. 3A).

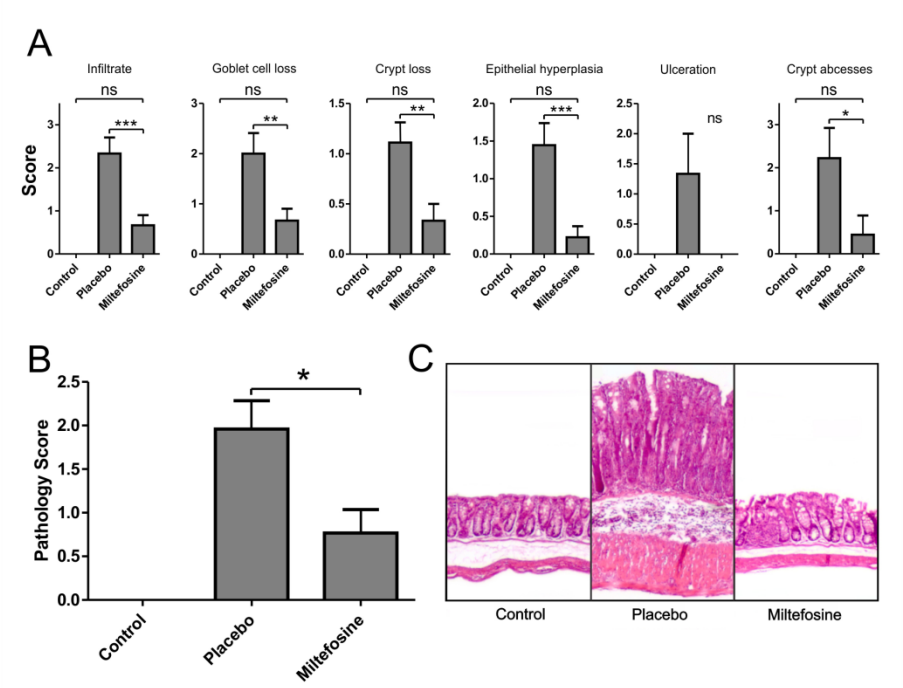

FIGURE 3. Miltefosine treatment improves the histopathological score of colitis. (A) Individual components of the histopathological colitis score. (B) Total score. (C) Representative images of H&E stained slides of colons from mice in the indicated groups. Control = $CD4CD45RB^{high+low}$, placebo = $CD4CD45RB^{high}$ + placebo, miltefosine = $CD4CD45RB^{high}$ + miltefosine. Original magnification in C: 100x. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

We subsequently analyzed the recruitment of individual leukocyte subsets to the mucosa (Fig. 4). We used F4/80 as a marker of macrophages, Ly6G for neutrophils and CD3 for T cells. The number of infiltrating cells of each type was counted per intercrypt space. Per mouse 9 intercrypt spaces were counted in 3 high power field images (3 adjacent inter crypt spaces per image). In placebo treated animals with colitis, the number of infiltrating macrophages, neutrophils and T cells was substantially increased compared to controls. Treatment with miltefosine significantly reduced influx of all three cell types. Both macrophage and neutrophil infiltration were not significantly different from unaffected control animals (Fig. 4A-C).

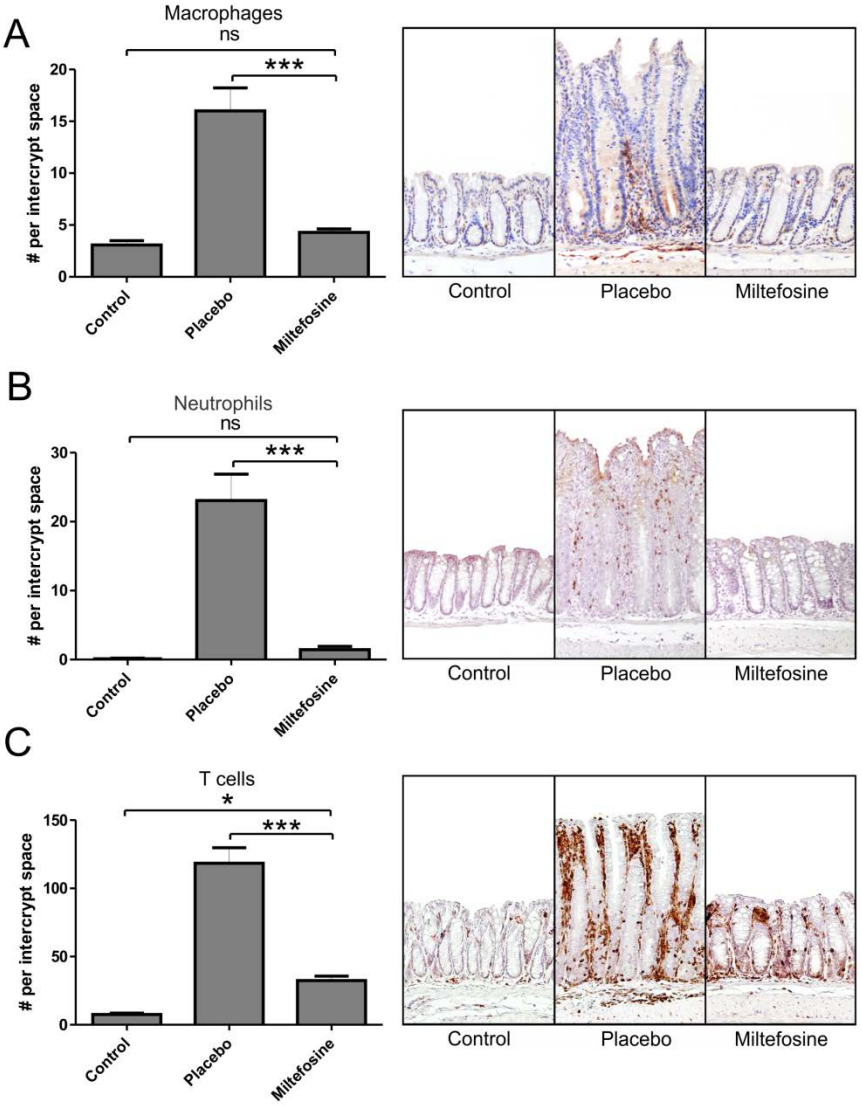

FIGURE 4. Reduced influx of different leukocyte subsets in miltefosine treated animals. (A) Quantification and representative images of the presence of macrophages as determined by immunohistochemistry for F4/80. (B) Quantification and representative images of the presence of neutrophils as determined by immunohistochemistry for Ly6G. (C) Quantification and representative images of the presence of T cells as determined by

immunohistochemistry for CD3. Control = $CD4CD45RB^{high+low}$, placebo = $CD4CD45RB^{high}$ + placebo, miltefosine = $CD4CD45RB^{high}$ + miltefosine. Original magnifications 100x. * = $P < 0.05$, *** = $P < 0.001$.

To investigate the immunomodulatory effect of miltefosine treatment on cytokine production, cytokine levels were determined in whole colon lysates (Fig. 5). In the colitis group the inflammation caused a significant increase in the levels of the pro-inflammatory cytokines IL-6, TNFα, INFγ and IL-17. The levels of the Th2 related cytokine IL-4 and of the anti-inflammatory cytokine IL-10 remained unchanged. In mice treated with miltefosine the increase of pro-inflammatory cytokines IL-6, TNFα, INFγ and IL-17 was significantly reduced.

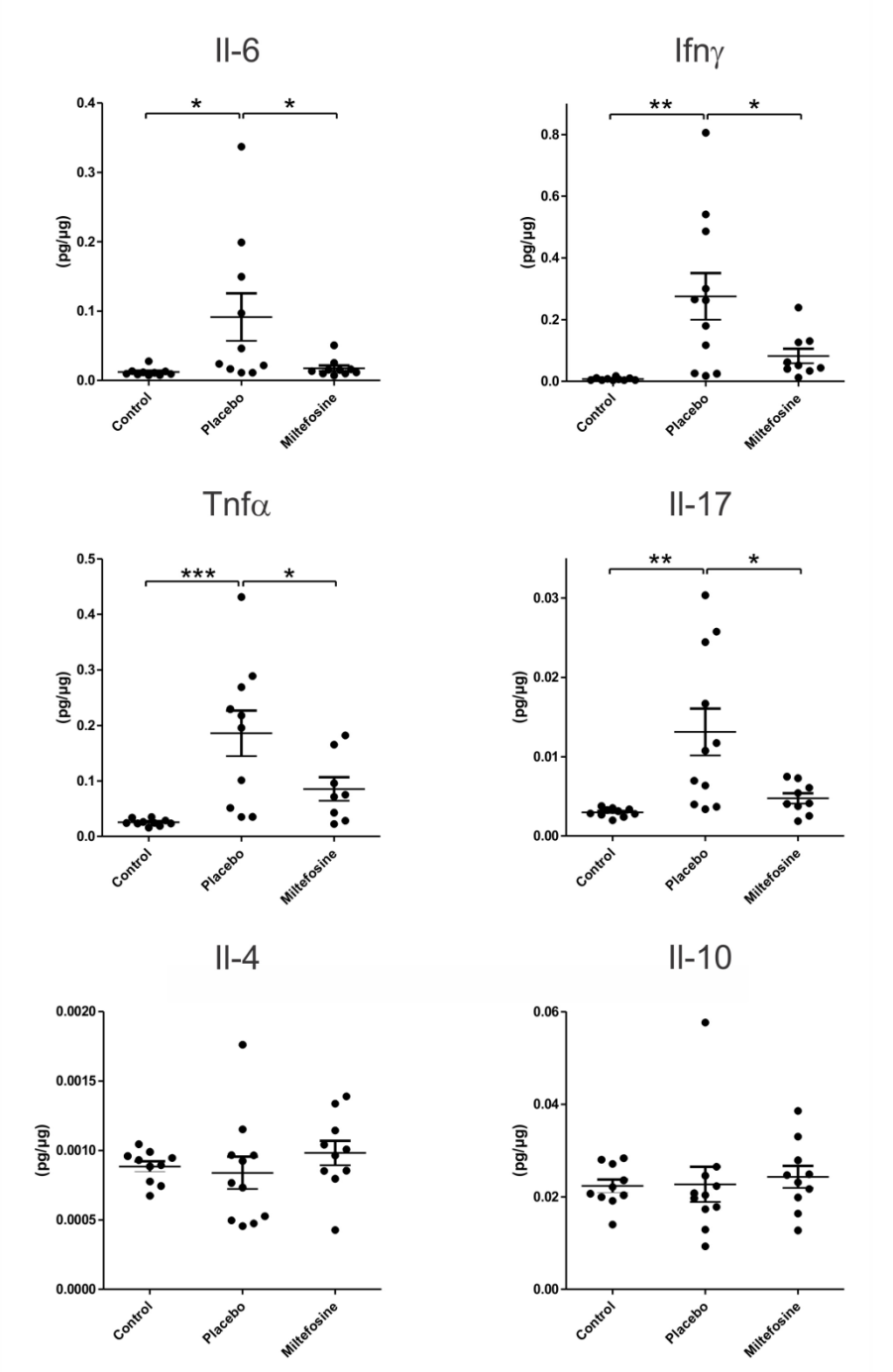
Il-6
Ifnγ
Tnfα
Il-17
Il-4
Il-10
(pg/µg)
Control
Placebo
Miltefosine
*
**
***

FIGURE 5. Miltefosine treatment reduces the production of pro-inflammatory cytokines. Measurement of mucosal levels of different cytokines in colonic homogenates by cytokine bead array. Control = CD4CD45RB$^{high+low}$, placebo = CD4CD45RB^{high} + placebo, miltefosine = CD4CD45RB^{high} + miltefosine. ns = not significant, * = $P < 0.05$.

Discussion

Miltefosine was initially developed as an anti-cancer drug but has largely failed clinical development for oncology. After preclinical experiments in mice[10] it was found that miltefosine has a surprising efficacy for the treatment of Leishmaniasis.[8,15] Since it was previously found that miltefosine may suppress T cell activation,[3,4] we investigated miltefosine as a potential immunomodulator in a mouse model of colitis. We found that miltefosine efficiently inhibited PHA mediated T cell proliferation at physiologically attainable concentrations.[5] In addition, miltefosine fully inhibited SEB induced T cell proliferation which is known to be only partially sensitive to dexamethasone. To test the feasibility of miltefosine treatment as a way to suppress intestinal inflammation we used the experimental CD4CD45RB^{High} transfer colitis mouse model. We chose this model as it is characterized by an expression profile of proinflammatory molecules that is relatively similar to human IBD, especially compared to the chemical colitis models.[16] Treatment with miltefosine was started two weeks after the T cell transfer as in previous studies using this model the mice started losing weight after two weeks.[12] The mice were dosed 50 mg/kg twice per week as miltefosine has a long half life. Treatment with miltefosine resulted in a remarkable reduction of all aspects of the severity of colitis that we examined. Most of the hall marks of colitis that we measured in this model were no longer significantly different between CD45RBhigh transplanted colitic mice treated with miltefosine and control mice that received both CD45RB^{high} and CD45RB^{low} cells.

As miltefosine is already marketed for use in Leishmaniasis under the brand name Impavido (by Zentaris) it could be an attractive immunomodulator to examine for use in humans with IBD. Miltefosine has an excellent oral availability and the pharmacokinetics have been well described.[5] Clinical

trials in humans have shown an acceptable safety profile with reversible abnormalities in liver biochemistry and renal function.[8,15] The major side effects of miltefosine are nausea and vomiting which could be problematic in patients with IBD. However these side effects are often transient.[8,15] The safety and side effects of long term miltefosine use that would be required for maintenance therapy in patients with IBD have not been established.

In conclusion, we find that miltefosine inhibits T cell proliferation at physiologically relevant concentrations *in vitro*. Miltefosine strongly reduced severity of colitis in the murine transfer model of colitis. Our data suggest that miltefosine is an interesting candidate anti-inflammatory drug for patients with IBD.

Reference List

1. Alyanakian MA, You S, Damotte D, et al. Diversity of regulatory CD4+T cells controlling distinct organ-specific autoimmune diseases. Proc Natl Acad Sci U S A. 2003;100:15806-15811.

2. Barratt G, Saint-Pierre-Chazalet M, Loiseau PM. Cellular transport and lipid interactions of miltefosine. Curr Drug Metab. 2009;10:247-255.

3. Baumer W, Wlaz P, Jennings G, et al. The putative lipid raft modulator miltefosine displays immunomodulatory action in T-cell dependent dermal inflammation models. Eur J Pharmacol. 2010;628:226-232.

4. Dolle S, Hoser D, Rasche C, et al. Long-term reduction in local inflammation by a lipid raft molecule in atopic dermatitis. Allergy. 2010;65:1158-1165.

5. Dorlo TP, van Thiel PP, Huitema AD, et al. Pharmacokinetics of miltefosine in Old World cutaneous leishmaniasis patients. Antimicrob Agents Chemother. 2008;52:2855-2860.

6. Fraser JD, Proft T. The bacterial superantigen and superantigen-like proteins. Immunol Rev. 2008;225:226-243.

7. Hauk PJ, Hamid QA, Chrousos GP, et al. Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens. J Allergy Clin Immunol. 2000;105:782-787.

8. Jha TK, Sundar S, Thakur CP, et al. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N Engl J Med. 1999;341:1795-1800.

9. Katzen D, Chu E, Terhost C, et al. Mechanisms of human T cell response to mitogens: IL 2 induces IL 2 receptor expression and proliferation but not IL 2 synthesis in PHA-stimulated T cells. J Immunol. 1985;135:1840-1845.

10. Kuhlencord A, Maniera T, Eibl H, et al. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. Antimicrob Agents Chemother. 1992;36:1630-1634.

11. Leonard R, Hardy J, van TG, et al. Randomized, double-blind, placebo-controlled, multicenter trial of 6% miltefosine solution, a topical chemotherapy in cutaneous metastases from breast cancer. J Clin Oncol. 2001;19:4150-4159.

12. Morrissey PJ, Charrier K, Braddy S, et al. CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. J Exp Med. 1993;178:237-244.

13. Planting AS, Stoter G, Verweij J. Phase II study of daily oral miltefosine (hexadecylphosphocholine) in advanced colorectal cancer. Eur J Cancer. 1993;29A:518-519.

14. Read S, Powrie F. Induction of inflammatory bowel disease in immunodeficient mice by depletion of regulatory T cells. Curr Protoc Immunol. 2001;Chapter 15:Unit.

15. Sundar S, Rosenkaimer F, Makharia MK, et al. Trial of oral miltefosine for visceral leishmaniasis. Lancet. 1998;352:1821-1823.

16. te Velde AA, de KF, Sterrenburg E, et al. Comparative analysis of colonic gene expression of three experimental colitis models mimicking inflammatory bowel disease. Inflamm Bowel Dis. 2007;13:325-330.

17. Valentine MA, Tsoukas CD, Rhodes G, et al. Phytohemagglutinin binds to the 20-kDa molecule of the T3 complex. Eur J Immunol. 1985;15:851-854.

18. Verweij J, Gandia D, Planting AS, et al. Phase II study of oral miltefosine in patients with squamous cell head and neck cancer. Eur J Cancer. 1993;29A:778-779.

19. Verweij J, Krzemieniecki K, Kok T, et al. Phase II study of miltefosine (hexadecylphosphocholine) in advanced soft tissue sarcomas of the adult--an EORTC Soft Tissue and Bone Sarcoma Group Study. Eur J Cancer. 1993;29A:208-209.

20. Verweij J, Planting A, van der BM, et al. A dose-finding study of miltefosine (hexadecylphosphocholine) in patients with metastatic solid tumours. J Cancer Res Clin Oncol. 1992;118:606-608.

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Repurposing miltefosine for the treatment of immune mediated disease?

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Abstract

Miltefosine is an ether lipid initially developed for cancer treatment in the early eighties. Miltefosine largely failed development for oncology although it has been approved for the topical treatment of breast cancer metastasis. It was subsequently discovered that miltefosine is a highly effective treatment for visceral Leishmaniasis, a parasitic disease which affects millions worldwide and causes an estimated 30.000 fatalities each year. Oral treatment with miltefosine is in general well tolerated and has relatively few adverse effects. The exact mechanism of action of miltefosine treatment is still a matter of investigation. Its close resemblance to phospholipids allows it to be quickly taken up by cell membranes and affect related processes such as lipid metabolism and signaling through lipid rafts. These processes play an important role in the immune response and it comes as no surprise that miltefosine has been successfully tested for the treatment of a number of immune mediated diseases in preclinical models of disease. Drug repurposing of miltefosine for immune mediated diseases may provide an opportunity to expand the limited number of drugs that is currently available for therapeutic use.

Miltefosine or hexadecylphosphocholine (HePC) was developed in the early eighties as a potential cytostatic drug. The drug largely failed development for oncology and is now best known as an oral treatment for Visceral Leishmaniasis, a lethal disease that is the result from infection with the parasite Leishmania. Discovery of its potential as an anti-parasitic drug has in recent years renewed scientific interest in the drug and it's mechanism of action. Development of new drugs is a costly and time-consuming process with a high risk of failure at multiple steps along the road of preclinical and clinical development. This makes repurposing of existing drugs with established side effects for novel indications an attractive alternative to classical drug development. Miltefosine has been extensively investigated in large clinical trials and appears to be relatively safe with limited and reversible side effects. In this review we will focus on the potential to repurpose miltefosine as an anti-inflammatory drug for immune mediated diseases. Of note, we will not cover perifosine, an alkyl-phospholipid developed to improve oral tolerability. Assuming that the mechanism of action of perifosine is similar to that of miltefosine, perifosine could be an alternative candidate for repurposing. However, the number of studies that address perifosine for other applications than the treatment of cancer is very limited.

Miltefosine (hexadecylphosphocholine) is an alkyl-phospholipid or ether-lipid with structural resemblance to lysophosphatidylcholine (LPC), a type of phospholipid that is present in the cell membrane and makes up around 3% of the cellular membrane. LPCs have a very short half-life as they are quickly metabolized by phospholipases and acyltransferases. In the LPC analogues miltefosine, perfosine and edelfosine an acyl group is replaced

by an alkyl group (Fig. 1) which makes the compounds metabolically stable (Eibl and Unger 1990).

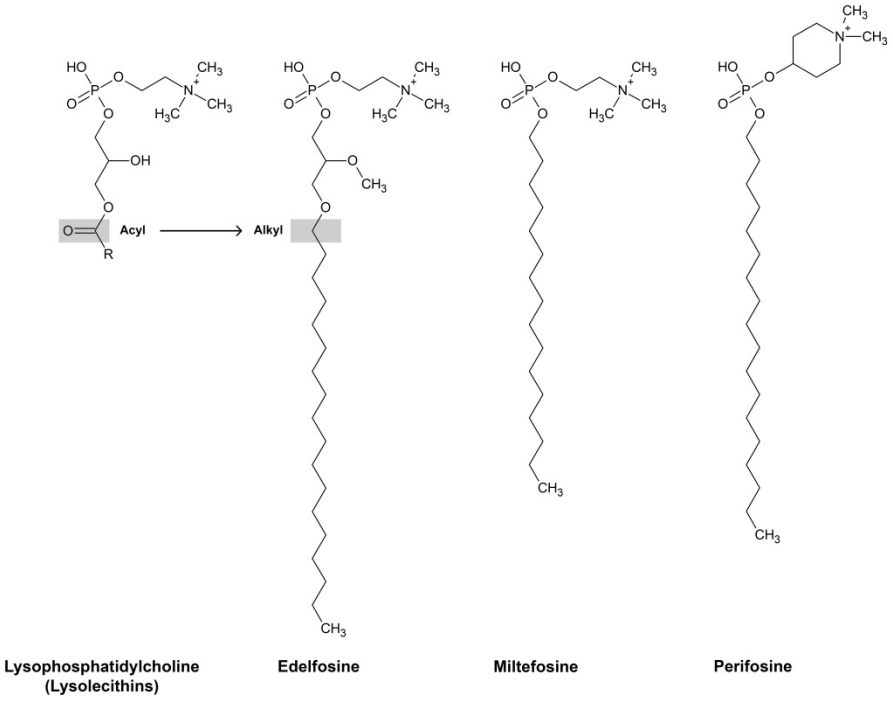

Fig. 1. Chemical structure of lysophosphatidylcholine and synthetic analogues. Lysophosphatidylcholine (also known as lysolecithin) is a natural occurring phospholipid present in cellular membranes. The lipid is easily metabolized, a problem that was solved in synthetic analogues by replacing the acyl group with a more resistant alkyl group. This change causes the ester, linking oxygen to the acyl group, to be changed into an ether.

The first stable LPC analogue was synthesized with an interest in enhancing macrophage mediated anti-tumor immunity (Munder PG, Fischer H, Weltzien HV, Oettgen HF, Westphal O (1976) Lysolecithin analogs: a new class of immunopotentiators with antitumor activity. Proc Am Assoc Cancer

Res 17:174) as it was found that LPCs increased the phagocytic capacity of peritoneal macrophages (Burdzy, Munder et al. 1964). It was subsequently discovered that some of these alkyl-phospholipids possessed direct cytostatic properties, shifting the interest away from the immunomodulatory properties. As the ability to inhibit cell growth appeared to be related to structural properties it was attempted to create the most minimal structure required. This resulted in a new group of molecules, the alkylphosphocholines and the discovery of miltefosine and edelfosine (Munder, Modolell et al. 1979).

Screening of miltefosine in cancer cell lines suggested that miltefosine possessed potent anti-neoplastic properties. Miltefosine inhibited cell growth in various leukemic cell lines (Unger, Damenz et al. 1989) and reduced tumor weight and size in dimethylbenzanthracene induced mammary carcinomas and transplanted mammary tumors in rats *in vivo* (Scherf, Schuler et al. 1987). However, miltefosine appeared only effective against some cell types, as no effect was observed in a variety of other cancer cell lines such as rat DS-carcinosarcoma, AH 13s sarcoma and L5222 leukemia, and mouse L1210 and P388 lymphocytic leukemia cells, Lewis lung carcinoma cells and B16 melanoma cells. The reason for this differential sensitivity of cancer cell lines to miltefosine has not been established,

Development of miltefosine for oncology

Intravenous administration of miltefosine is hemolytic and thus clinical applications have been limited to topical and oral treatments. A phase II dose-finding study in cancer patients confirmed previous phase I data that

the major dose limiting toxicity were nausea and vomiting and suggested a maximum dose of 150 mg daily split into three doses (Verweij, Planting et al. 1992). Oral treatment was subsequently tested in a number of oncological trials, including a phase II study for treatment of soft tissue sarcomas (Verweij, Krzemieniecki et al. 1993), a phase II study in advanced non-small cell lung cancer (Berdel, Becher et al. 1992), a phase II study in advanced colorectal cancer (Planting, Stoter et al. 1993), a phase II study in advanced breast cancer (Unger, Becher et al. 1993) and a phase II study for squamous cell head and neck cancer (Verweij, Gandia et al. 1993). None of these phase II clinical trials showed a sign that miltefosine treatment might affect disease progression. The only trials that showed an effect of miltefosine are studies in which miltefosine was applied topically. A 6% topical miltefosine solution was successfully applied in a phase II study for cutaneous T cell lymphomas (Dumontet, Thomas et al. 2006) and a multicenter randomized placebo-controlled phase III study for the treatment of skin metastasis of mammary carcinoma (Leonard, Hardy et al. 2001). Reasonable efficacy was observed in the latter trial with 2 out of 24 patients achieving a complete response of the skin lesions (0/27 for placebo) and 6 out of 24 showing partial response (1/27 for placebo).

Leishmaniasis

Leishmaniasis is a disease caused by infection of macrophages by a protozoan parasite belonging to the genus Leishmania. The parasites are transferred by a type of sandflies that only lives in (sub)tropical regions, restricting the disease to those areas. Although the infection can lead to cutaneous, mucocutaneous or visceral disease (also called kala-azar).

Visceral leishmaniasis is characterized by bouts of fever, weight loss, hepatosplenomegaly and anemia. It is a lethal disease if left untreated (Chappuis, Sundar et al. 2007). Unfortunately the yearly incidence of visceral leishmaniasis is estimated at 200.000 to 400.000 per year worldwide, with around 20.000 to 30.000 fatalities a year (Alvar, Velez et al. 2012). In 95% of the cases the disease can be quite effectively treated with intravenous injections of amphotericin B or pentavalent antimonial drugs (sodium stibogluconate and meglumine antimoniate). These drugs are inexpensive but treatment requires a trained physician to perform the injection and patients often succumb before reaching a doctor. More importantly, the number of patients showing resistance to treatment is increasing and patients with VL in combination with AIDS are generally refractory to treatment with pentavalent antimonial drugs.

The development of an in vitro model for infecting primary mouse peritoneal macrophages with *Leishmania donovani* in the 1980-ies allowed screening of large libraries of compounds for possible new treatments (Croft 1986). These screenings resulted in the discovery of alkyl phospholipids as possible candidates (Croft, Neal et al. 1987). In 1992 it was first shown that Leishmaniasis can indeed be treated with miltefosine in vivo. Mice infected with *Leishmania donovani* and *Leishmania infantum* were treated orally with miltefosine and compared to standard treatment using sodium stibogluconate. Parasitic levels in spleen and liver after four weeks of treatment showed that miltefosine is more potent in both suppressing and killing Leishmania parasites than sodium stibogluconate in mice (Kuhlencord, Maniera et al. 1992).

The use of miltefosine as an oral treatment for visceral leishmaniasis has been tested in numerous trials (Sundar, Rosenkaimer et al. 1998; Jha, Sundar et al. 1999; Sundar, Jha et al. 2002; Sundar, Sinha et al. 2011). Different treatment strategies have been used but in general patients with visceral leishmaniasis receive 50 or 100 mg of miltefosine daily for a duration of 28 days. On average treatment was well tolerated and adverse effects were limited to gastro-intestinal side effects such as vomiting and diarrhea. Treatment with miltefosine resulted in a 90 to 100 percent cure rate.

Immunomodulatory effects of miltefosine

The development of alkyl phospholipids initially started with the aim to create an immunomodulator that would increase anti-tumor phagocytic capacity of tumor associated macrophages. Similar to the original compounds that development started out with, miltefosine exhibits immunomodulatory properties.

Stimulatory effects on the innate immune response

There are a number of reports that suggest that miltefosine may stimulate multiple aspects of monocyte and macrophage mediated immunity. Such effects may explain the potent clearance of Leishmania parasites that have their niche inside macrophages.. For example, Balb/c mouse monocytes and macrophages treated with miltefosine showed a more potent response to LPS, with enhanced secretion of TNFα and release of NO (Eue, Zeisig et al. 1995; Zeisig, Rudolf et al. 1995; Ghosh, Roy et al. 2013). In a similar experiment with macrophages isolated from C57BL/6 mice there was no

increase in NO release but miltefosine did increase their phagocytic capacity. Not only did sensitized macrophages phagocytize more *S. cerevisiae* after miltefosine treatment but miltefosine also increased the number of macrophages that participated in the process (Ponte, Alves et al. 2012).

One report investigated the effect of miltefosine on circulating monocytes and cytokine levels in patients suffering from cutaneous Leishmaniasis (Mukhopadhyay, Das et al. 2011). In 12 patients that completed 4 months of miltefosine treatment (100 mg/day) the percentage of CD14+ monocytes decreased while CD16+ monocytes increased. This shift indicated that miltefosine treatment may have triggered maturation of the monocyte population towards a more pro-inflammatory phenotype. Levels of monocyte associated pro-inflammatory cytokines such as TNF-a, IL-6, IL-1b and IL-8 were significantly increased compared to these levels at the moment of presentation of the disease. Of course it cannot be excluded in this study that some of these effect were indirect and a systemic response to the eradication of the Leishmania parasites.

In a case study of one patient suffering from cutaneous Leishmaniasis treated with miltefosine, tissue biopsies were taken from the lesional area. Quantitative analysis of mRNA expression showed a significant decrease of TNFα, IL-10 and TGFβ levels while IFNγ and CD40 were significantly increased when comparing before to after treatment (Ansari, Ramesh et al. 2008). CD40 is a costimulatory molecule found on antigen presenting cells that helps to stimulate T helper cells which in turn produce IFNγ stimulating macrophages. Again, this was a single patient and it is difficult to judge if

the changes in expression are the direct result of the miltefosine treatment or alternatively result from the eradication of parasite-laden macrophages.

The initial development of alkyl phospholipids as compounds that stimulate macrophage phagocytosis, the potent parasite clearance by Leismania infected macrophages together with some of the experiments above suggest that treatment with miltefosine potentiates monocyte and macrophage mediated innate immunity.

Inhibitory effects on the adaptive immune response

The reason for a potential role for miltefosine and other alkyl phospholipids in immune mediated diseases is that miltefosine potently inhibits T cell activation (Baumer, Wlaz et al. 2010; Verhaar, Wildenberg et al. 2013). This effect has been verified in animal models where inflammation was successfully reduced using miltefosine. We investigated the effect of miltefosine in a CD4CD45RB^{high} transfer mouse model of inflammatory bowel disease (IBD). In this model, SCID mice, which lack T and B cells, are transplanted with CD45RB^{high} T cells resulting in chronic inflammation of the gut that develops over the course of several weeks. Miltefosine, given twice weekly, greatly reduced colonic inflammation in this model. Miltefosine improved clinical parameters such as weight loss, significantly improved the pathology score and reduced the expression of pro-inflammatory cytokines il-6, tnf-α and ifnγ to levels no longer significantly different from control mice without colitis (Verhaar, Wildenberg et al. 2013). These findings may make miltefosine an attractive candidate for the treatment of IBD. Although the cause of IBD remains unclear there are many indications that suggest that a reduced function of

the innate immune system predisposes to an excessive response of the adaptive immune response to intestinal microbiota (Marks, Harbord et al. 2006; Hayee, Rahman et al. 2010; Uhlig 2013). Given the results discussed above miltefosine may have a dual therapeutic effect in IBD by both stimulating macrophage mediated innate immunity and reducing excessive activation of T cell mediated adaptive immunity.

Baumer et al. investigated the effect of miltefosine treatment in a number of animal models addressing its effect on Th1 or Th2 related inflammatory responses and feasibility for use in atopic dermatitis. The models use ear thickness as a measure of inflammation. In an ovalbumin induced delayed type hypersensitivity model (DTH) miltefosine was able to significantly reduce ear swelling. Likewise in the more Th2 orientated mouse model of toluene diisocyanate induced hypersensitivity treatment with miltefosine significantly reduced the ear swelling and pro-inflammatory cytokine expression, both when administered systemically as well as topically (Baumer, Wlaz et al. 2010).

Topical application of a 6% miltefosine solution was compared with 1% hydrocortisone in a small clinical trial of 16 patients (Dolle, Hoser et al. 2010). Patients with at least two different target lesions were treated topically with one drug on each lesion for three weeks. Both treatments effectively reduced the severity of the disease and reduced the number of infiltrating lymphocytes in the lesions. Follow-up of the patients revealed a more sustained effect of miltefosine treatment. Interestingly, closer examination revealed a local increase in the number of FoxP3+ regulatory T cells in the miltefosine treated lesions while the number of FoxP3+ cells

was reduced by hydrocortisone. An important potential advantage of miltefosine over hydrocortisone was that in contrast with hydrocortisone, miltefosine did not affect proliferation of cells in the basal layer of the epidermis. Indeed, again in contrast with hydrocortisone, no reduction in epidermal thickness was observed with miltefosine.

Miltefosine and allergic disease

A potential role for miltefosine in allergic disease was suggested when it was shown that miltefosine can reduce histamine release from rat primary mast cells (Grosman 1990). Mast cells contain large granules with inflammatory mediators such as cytokines, proteases, histamine, serotonin and eicosanoids. Mast cells release their granules in response to activation by pattern recognition receptors or cross-linking of the IgE receptor. Degranulation causes redness of the skin, swelling and itching.

Miltefosine has also been shown to inhibit histamine release in human mast cells (Weller, Artuc et al. 2009; Batista, Friedrichson et al. 2010; Batista, Schlechtingen et al. 2011). Ten minutes of pretreatment with 25 µM miltefosine reduced anti-IgE induced histamine release by more than 50%. These findings were translated into a small study in which the effect of miltefosine was tested on the allergic response to a standard skin prick test. Five allergic patients were treated on both arms with either placebo or a 6% miltefosine solution. The inflammatory response was measured by the diameter of the resulting wheal and erythema. Miltefosine was able to significantly reduce the inflammatory response that resulted from injection with an allergen. A control condition in which the patients were injected with histamine showed no effect (Weller, Artuc et al. 2009).

Miltefosine has also been tested in a number of other mast cell related conditions. One such condition, mastocytosis, involves the accumulation of mast cells in various organs, most commonly in the skin. This rare condition affects mostly children and skin manifestations causes symptoms related to mast cell mediator release such as pruritus and flushing. 39 Adult patients were tested in a double-blind, placebo-controlled trial with a topical 6% miltefosine solution and the glucocorticoid clobetasol (0.5 mg/ml) for 2 weeks. Although it seems that miltefosine may have reduced weal formation in this study none of the differences reached statistical significance and it seems the study was under powered to detect a meaningful difference (Hartmann, Siebenhaar et al. 2010).

Chronic urticaria, is a kind of idiopathic rash that is relatively common affecting up to 1% of the population in the US. In most cases the underlying cause is unclear but the rash is the result of activated mast cells. Urticaria can be treated with anti-histamines but with chronic urticaria most patients respond poorly and are often resistant. In a multicenter randomized, double-blind, placebo-controlled study that included 54 anti-histamine resistant chronic urticaria patients, miltefosine was tested as an oral drug (Magerl, Rother et al. 2012). Patients took increasing dosages miltefosine (up to 150 mg/daily unless intolerable adverse effects) daily for 4 weeks. At the end of the treatment period, miltefosine treated patients showed substantial improvement as was clear from a specific urticaria activity score and a decrease in the number of wheals. Miltefosine treatment did not reduce the intensity of pruritus.

Collectively these data suggest that miltefosine could be a potential drug for mast cell related conditions.

Side effects

Miltefosine has few side effects but those found may hamper long term use. From the phase II trials for the oral use of miltefosine with cancer patients it has become clear that daily dosages of 150 mg and higher cause potentially severe gastrointestinal side effects resulting in loss of appetite, nausea and vomiting (Verweij, Planting et al. 1992). This can be solved by combining with anti-emetics but in some cases the side effects are dose limiting. Development of a better tolerated formulation for oncological indications was suspended due to the discovery of perifosine, a better tolerated oral anticancer drug (Crul, Rosing et al. 2002).

Miltefosine treatment may have teratogenic effects. Studies on embryonic development and organogenesis of rats suggested an embryotoxic, fetotoxic and teratogenic risk (Sindermann and Engel 2006). The same was reported from studies in rabbits, although with the exception of the teratogenic effect. Due to these findings and because of a lack of human study results, use of miltefosine during pregnancy is contraindicated. This is especially a problem due to its long half-life. Six months after treatment miltefosine can still be measured in serum and it is therefore advisable to use contraception for half a year after stopping treatment.

In some patients the renal function is affected and a rise in creatinine levels is observed. In most cases this effect is reversible. Studies in dogs showed no lasting effect of miltefosine on the kidney (Bianciardi, Brovida et al.

2009). Miltefosine might have a direct effect on renal function but change in fluid balance due to gastrointestinal effects might contribute. Supporting this hypothesis, Leishmaniasis patients treated with miltefosine sometimes show elevated creatinine serum levels during episodes of vomiting and dehydration (Sindermann and Engel 2006).

To summarize, a treatment schedule of 50-100 mg miltefosine daily is in general well tolerated and rarely leads to serious or treatment limiting adverse effects. Studies on the miltefosine treatment of VL clearly indicate that such a treatment schedule can be maintained for at least 4 weeks. These data indicate that the potential of miltefosine as a maintenance treatment for IBD could be investigated.

Mechanism of action

The precise mechanism of action of miltefosine is lacking and may be different for different cell types. Because miltefosine was originally selected as a cytostatic drug, most of the research is focused on explaining how miltefosine induces apoptosis in cancer cells. And even though the effect of miltefosine on macrophages, lymphocytes and mast cells appears to be different, may involve similar pathways.

Miltefosine shows resemblance to the membrane phospholipid lysophosphatidylcholine. It's structural properties allow miltefosine to integrate into the cellular membrane (Rakotomanga, Loiseau et al. 2004). From there it redistributes to endoplasmic reticulum, Golgi-, nuclear- and mitochondrial membranes. How this happens is unclear but in Caco-2 colorectal cancer cells the process appears to be partly ATP dependent

suggesting active transport. The normal membrane recycling process may be responsible for the remainder of the intracellular transport of the drug (Menez, Buyse et al. 2007). Because alkyl-phopholipids, such as miltefosine, are more resistant to metabolizing enzymes, they accumulate in the membranes possibly affecting the continuous turnover of endogenous phospholipids.

One of the most important effects of miltefosine incorporation into the cell membrane is believed to be inhibition of phosphatidylcholine (PC) synthesis. PC is the most abundant phospholipid in cellular membranes of eukaryotic cells and interference with its production may predispose the cell to undergo apoptosis (Wieder, Orfanos et al. 1998; van der Luit, Budde et al. 2002). Miltefosine affects PC synthesis by inhibiting CTP:phosphocholine cytidylyltransferase, the rate limiting enzyme in the PC biosynthesis pathway at the endoplasmatic reticulum. This is supported by the fact that apoptosis can be prevented by supplementing miltefosine treated cells with exogenous lysoPC, an alternative precursor for the synthesis of PC. As apoptosis induced by other triggers could not be rescued by adding lysoPC, this is a strong suggestion that indeed inhibition of PC synthesis is the mechanism of miltefosine induced apoptosis (Boggs, Rock et al. 1995; Baburina and Jackowski 1998; Van Der Luit, Budde et al. 2003).

In addition to inhibition of PC synthesis, miltefosine also impairs signaling molecules through inhibition of phospholipases. Phospholipases hydrolyze phospholipids and inhibition results in a decrease in breakdown products, including the important second messengers diacylglycerol (DAG) and

phosphatidic acid (PA). Further downstream, alkyl-phospholipids may impact proliferation and cell survival through effects on the kinase Akt (Song, Ouyang et al. 2005). Under normal conditions, once recruited and positioned at the plasma membrane, Akt can be activated. Although this has not been described for miltefosine, studies on perifosine suggest that treatment affects the recruitment of Akt to the plasma membrane and displacements of its ligands PIP3 (phosphatidylinositol (3,4,5)-trisphosphate, PtdIns(3,4,5)P3) or PIP2 (phosphatidylinositol (3,4)-bisphosphate, PtdIns(3,4)P2).

All of these effects could be cause by disturbance of membrane integrity and the functionality of lipid rafts (Kondapaka, Singh et al. 2003). Miltefosine increases the membrane fluidity in macrophages (Ghosh, Roy et al. 2013) an effect that has been reported to decreases antigen presentation and stimulation of T cells (Chakraborty, Banerjee et al. 2005). On the other hand miltefosine has an affinity for sterols and is known to stabilize sterol rich areas such as lipid rafts which increase antigen presentation (Jimenez-Lopez, Rios-Marco et al. 2010). And indeed macrophages treated with miltefosine show an enhance ability to stimulate T cells resulting in a higher production of IL-2, IL-12, TNFα and NO production (Ghosh, Roy et al. 2013). The effect of miltefosine on membrane integrity could also very well explain why mast cells are inhibited in their IgE dependent histamine release. Upon activation, IgE receptors cluster in organized microdomains (Weller, Artuc et al. 2009; Silveira, Mazucato et al. 2011). Likewise T cell activation is dependent on the formation and organization of T cell receptor microclusters and formation of the immunological synapse (Kabouridis and Jury 2008).

Signaling molecules that have been implicated as targets for miltefosine treatment in cancer, such as Akt, Ras/Raf-1, PLC and second messengers like PA and DAG, are also important mediators in cells of the immune system. Inhibition of even one of these molecules could explain for example the inhibition of lymphocyte proliferation. Of course future investigations will have to shed more light on this.

Conclusion

Miltefosine is an alkyl-phospholipid that was initially tried unsuccessfully in several phase II trials in oncology and hematology. Originally this class of compounds was identified for its stimulatory effect on macrophage phagocytosis and several groups have observed that miltefosine may stimulate myeloid cell mediated immunity. At the same time miltefosine shows profound inhibition of T cell activation and miltefosine was successfully applied in preclinical models of atopic dermatitis and inflammatory bowel disease. Given the well-established and relatively limited side effects and oral route of administration miltefosine may be a candidate for drug repurposing for immune mediated diseases that are in dire need for novel therapeutic options such as inflammatory bowel disease.

References

Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, and Team WHOLC (2012) Leishmaniasis worldwide and global estimates of its incidence. *Plos One* **7**(5): e35671.

Ansari NA, Ramesh V, and Salotra P (2008) Immune response following miltefosine therapy in a patient with post-kala-azar dermal leishmaniasis. *Trans R Soc Trop Med Hyg* **102**(11): 1160-1162.

Baburina I, and Jackowski S (1998) Apoptosis triggered by 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine is prevented by increased expression of CTP:phosphocholine cytidylyltransferase. *J Biol Chem* **273**(4): 2169-2173.

Batista J, Friedrichson T, Schlechtingen G, Braxmeier T, Jennings G, and Bajorath J (2010) Computational screening for membrane-directed inhibitors of mast cell activation. *Eur J Med Chem* **45**(6): 2700-2704.

Batista J, Schlechtingen G, Friedrichson T, Braxmeier T, and Bajorath J (2011) Lipid-like sulfoxides and amine oxides as inhibitors of mast cell activation. *Eur J Med Chem* **46**(6): 2147-2151.

Baumer W, Wlaz P, Jennings G, and Rundfeldt C (2010) The putative lipid raft modulator miltefosine displays immunomodulatory action in T-cell dependent dermal inflammation models. *Eur J Pharmacol* **628**(1-3): 226-232.

Berdel WE, Becher R, Edler L, Bremer K, Essers U, Drozd A, Zafferani M, Klee MAG, Bachmann P, Korfel A, and Westerhausen M (1992) Daily Oral Miltefosine (Hexadecyl-Phosphocholine) in Patients with Advanced Non-Small-Cell Lung-Cancer - a Phase-Ii Study. *Onkologie* **15**(3): 238-242.

Bianciardi P, Brovida C, Valente M, Aresu L, Cavicchioli L, Vischer C, Giroud L, and Castagnaro M (2009) Administration of miltefosine and meglumine antimoniate in healthy dogs: clinicopathological evaluation of the impact on the kidneys. *Toxicol Pathol* **37**(6): 770-775.

Boggs KP, Rock CO, and Jackowski S (1995) Lysophosphatidylcholine attenuates the cytotoxic effects of the antineoplastic phospholipid 1-O-octadecyl-2-O-methyl-rac-glycero-3- phosphocholine. *J Biol Chem* **270**(19): 11612-11618.

Burdzy K, Munder PG, Fischer H, and Westphal O (1964) Steigerung Der Phagocytose Von Peritonealmakrophagen Durch Lysolecithin. *Z Naturforsch [B]* **19**: 1118-1120.

Chakraborty D, Banerjee S, Sen A, Banerjee KK, Das P, and Roy S (2005) Leishmania donovani affects antigen presentation of macrophage by disrupting lipid rafts. *J Immunol* **175**(5): 3214-3224.

Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, and Boelaert M (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* **5**(11): 873-882.

Croft SL (1986) In vitro screens in the experimental chemotherapy of leishmaniasis and trypanosomiasis. *Parasitol Today* **2**(3): 64-69.

Croft SL, Neal RA, Pendergast W, and Chan JH (1987) The activity of alkyl phosphorylcholines and related derivatives against Leishmania donovani. *Biochem Pharmacol* **36**(16): 2633-2636.

Crul M, Rosing H, de Klerk GJ, Dubbelman R, Traiser M, Reichert S, Knebel NG, Schellens JH, Beijnen JH, and ten Bokkel Huinink WW (2002) Phase I and pharmacological study of daily oral administration of perifosine (D-21266) in patients with advanced solid tumours. *Eur J Cancer* **38**(12): 1615-1621.

Dolle S, Hoser D, Rasche C, Loddenkemper C, Maurer M, Zuberbier T, and Worm M (2010) Long-term reduction in local inflammation by a lipid raft molecule in atopic dermatitis. *Allergy* **65**(9): 1158-1165.

Dumontet C, Thomas L, Berard F, Gimonet JF, and Coiffier B (2006) A phase II trial of miltefosine in patients with cutaneous T-cell lymphoma. *Bull Cancer* **93**(11): E115-118.

Eibl H, and Unger C (1990) Hexadecylphosphocholine: a new and selective antitumor drug. *Cancer Treat Rev* **17**(2-3): 233-242.

Eue I, Zeisig R, and Arndt D (1995) Alkylphosphocholine-induced production of nitric oxide and tumor necrosis factor alpha by U 937 cells. *J Cancer Res Clin Oncol* **121**(6): 350-356.

Ghosh M, Roy K, and Roy S (2013) Immunomodulatory effects of antileishmanial drugs. *J Antimicrob Chemother* **68**(12): 2834-2838.

Grosman N (1990) The influence of ether lipid (AMG) on histamine release from isolated rat mast cells: synergistic interaction with TPA indicates protein kinase C activation. *Immunopharmacology* **20**(2): 143-149.

Hartmann K, Siebenhaar F, Belloni B, Brockow K, Eben R, Hartmann B, Rueff F, Schoepke N, Staubach P, Weber A, and Maurer M (2010) Effects of topical treatment with the raft modulator miltefosine and clobetasol in cutaneous mastocytosis: a randomized, double-blind, placebo-controlled trial. *Br J Dermatol* **162**(1): 185-190.

Hayee B, Rahman FZ, Sewell G, Smith AM, and Segal AW (2010) Crohn's disease as an immunodeficiency. *Expert Review of Clinical Immunology* **6**(4): 585-596.

Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C, Voss A, and Berman J (1999) Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *N Engl J Med* **341**(24): 1795-1800.

Jimenez-Lopez JM, Rios-Marco P, Marco C, Segovia JL, and Carrasco MP (2010) Alterations in the homeostasis of phospholipids and cholesterol by antitumor alkylphospholipids. *Lipids Health Dis* **9**: 33.

Kabouridis PS, and Jury EC (2008) Lipid rafts and T-lymphocyte function: implications for autoimmunity. *FEBS Lett* **582**(27): 3711-3718.

Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, and Roy KK (2003) Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. *Molecular Cancer Therapeutics* **2**(11): 1093-1103.

Kuhlencord A, Maniera T, Eibl H, and Unger C (1992) Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. *Antimicrob Agents Chemother* **36**(8): 1630-1634.

Leonard R, Hardy J, van Tienhoven G, Houston S, Simmonds P, David M, and Mansi J (2001) Randomized, double-blind, placebo-controlled, multicenter trial of 6% miltefosine solution, a topical chemotherapy in cutaneous metastases from breast cancer. *J Clin Oncol* **19**(21): 4150-4159.

Magerl M, Rother M, Bieber T, Biedermann T, Brasch J, Dominicus R, Hunzelmann N, Jakob T, Mahler V, Popp G, Schakel K, Schlingensiepen R, Schmitt J, Siebenhaar F, Simon JC, Staubach P, Wedi B, Weidner C, and Maurer M (2012) Randomized, double-blind, placebo-controlled study of safety and efficacy of miltefosine in antihistamine-resistant chronic spontaneous urticaria. *J Eur Acad Dermatol Venereol*.

Marks DJB, Harbord MWN, MacAllister R, Rahman FZ, Young J, Al-Lazikani B, Lees W, Novelli M, Bloom S, and Segal AW (2006) Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet* **367**(9511): 668-678.

Menez C, Buyse M, Farinotti R, and Barratt G (2007) Inward translocation of the phospholipid analogue miltefosine across Caco-2 cell membranes exhibits characteristics of a carrier-mediated process. *Lipids* **42**(3): 229-240.

Mukhopadhyay D, Das NK, Roy S, Kundu S, Barbhuiya JN, and Chatterjee M (2011) Miltefosine effectively modulates the cytokine milieu in Indian post kala-azar dermal leishmaniasis. *J Infect Dis* **204**(9): 1427-1436.

Munder PG, Modolell M, Andreesen R, Weltzien HU, and Westphal O (1979) Lysophosphatidylcholine (Lysolecithin) and Its Synthetic Analogs - Immune-Modulating and Other Biologic Effects. *Springer Seminars in Immunopathology* **2**(2): 187-203.

Planting AS, Stoter G, and Verweij J (1993) Phase II study of daily oral miltefosine (hexadecylphosphocholine) in advanced colorectal cancer. *Eur J Cancer* **29A**(4): 518-519.

Ponte CB, Alves EA, Sampaio RN, Urdapilleta AA, Kuckelhaus Cdos S, Muniz-Junqueira MI, and Kuckelhaus SA (2012) Miltefosine enhances

phagocytosis but decreases nitric oxide production by peritoneal macrophages of C57BL/6 mice. *Int Immunopharmacol* **13**(1): 114-119.

Rakotomanga M, Loiseau PM, and Saint-Pierre-Chazalet M (2004) Hexadecylphosphocholine interaction with lipid monolayers. *Biochim Biophys Acta* **1661**(2): 212-218.

Scherf HR, Schuler B, Berger MR, and Schmahl D (1987) Therapeutic activity of ET-18-OCH3 and hexadecylphosphocholine against mammary tumors in BD-VI rats. *Lipids* **22**(11): 927-929.

Silveira ESAM, Mazucato VM, Jamur MC, and Oliver C (2011) Lipid rafts in mast cell biology. *J Lipids* **2011**: 752906.

Sindermann H, and Engel J (2006) Development of miltefosine as an oral treatment for leishmaniasis. *Trans R Soc Trop Med Hyg* **100 Suppl 1**: S17-20.

Song G, Ouyang G, and Bao S (2005) The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* **9**(1): 59-71.

Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, Junge K, Bryceson A, and Berman J (2002) Oral miltefosine for Indian visceral leishmaniasis. *N Engl J Med* **347**(22): 1739-1746.

Sundar S, Rosenkaimer F, Makharia MK, Goyal AK, Mandal AK, Voss A, Hilgard P, and Murray HW (1998) Trial of oral miltefosine for visceral leishmaniasis. *Lancet* **352**(9143): 1821-1823.

Sundar S, Sinha PK, Rai M, Verma DK, Nawin K, Alam S, Chakravarty J, Vaillant M, Verma N, Pandey K, Kumari P, Lal CS, Arora R, Sharma B, Ellis S, Strub-Wourgaft N, Balasegaram M, Olliaro P, Das P, and Modabber F (2011) Comparison of short-course multidrug treatment with standard therapy for visceral leishmaniasis in India: an open-label, non-inferiority, randomised controlled trial. *Lancet* **377**(9764): 477-486.

Uhlig HH (2013) Monogenic diseases associated with intestinal inflammation: implications for the understanding of inflammatory bowel disease. *Gut* **62**(12): 1795-1805.

Unger C, Becher R, Rieche K, Wander HE, Friedrich G, and Edler L (1993) Daily Oral Miltefosine (Hexadecylphosphocholine) in Patients with Advanced Breast-Cancer - a Phase-Ii Study. *Onkologie* **16**(4): 260-263.

Unger C, Damenz W, Fleer EA, Kim DJ, Breiser A, Hilgard P, Engel J, Nagel G, and Eibl H (1989) Hexadecylphosphocholine, a new ether lipid analogue. Studies on the antineoplastic activity in vitro and in vivo. *Acta Oncol* **28**(2): 213-217.

van der Luit AH, Budde M, Ruurs P, Verheij M, and van Blitterswijk WJ (2002) Alkyl-lysophospholipid accumulates in lipid rafts and induces

apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. *J Biol Chem* **277**(42): 39541-39547.

Van Der Luit AH, Budde M, Verheij M, and Van Blitterswijk WJ (2003) Different modes of internalization of apoptotic alkyl-lysophospholipid and cell-rescuing lysophosphatidylcholine. *Biochem J* **374**(Pt 3): 747-753.

Verhaar AP, Wildenberg ME, te Velde AA, Meijer SL, Vos AC, Duijvestein M, Peppelenbosch MP, Hommes DW, and van den Brink GR (2013) Miltefosine suppresses inflammation in a mouse model of inflammatory bowel disease. *Inflamm Bowel Dis* **19**(9): 1974-1982.

Verweij J, Gandia D, Planting AS, Stoter G, and Armand JP (1993) Phase II study of oral miltefosine in patients with squamous cell head and neck cancer. *Eur J Cancer* **29A**(5): 778-779.

Verweij J, Krzemieniecki K, Kok T, Poveda A, van Pottelsberghe C, van Glabbeke M, and Mouridsen H (1993) Phase II study of miltefosine (hexadecylphosphocholine) in advanced soft tissue sarcomas of the adult--an EORTC Soft Tissue and Bone Sarcoma Group Study. *Eur J Cancer* **29A**(2): 208-209.

Verweij J, Planting A, van der Burg M, and Stoter G (1992) A dose-finding study of miltefosine (hexadecylphosphocholine) in patients with metastatic solid tumours. *J Cancer Res Clin Oncol* **118**(8): 606-608.

Weller K, Artuc M, Jennings G, Friedrichson T, Guhl S, dos Santos RV, Sunder C, Zuberbier T, and Maurer M (2009) Miltefosine inhibits human mast cell activation and mediator release both in vitro and in vivo. *J Invest Dermatol* **129**(2): 496-498.

Wieder T, Orfanos CE, and Geilen CC (1998) Induction of ceramide-mediated apoptosis by the anticancer phospholipid analog, hexadecylphosphocholine. *J Biol Chem* **273**(18): 11025-11031.

Zeisig R, Rudolf M, Eue I, and Arndt D (1995) Influence of hexadecylphosphocholine on the release of tumor necrosis factor and nitroxide from peritoneal macrophages in vitro. *J Cancer Res Clin Oncol* **121**(2): 69-75.

PART IV

Summary and general discussion

10

Summary and general discussion

This thesis is composed of two parts. In the first part the focus lies on glucocorticoids. Glucocorticoids have been used worldwide for more than half a century. This would suggest that it is a safe drug to use but this is not the case. Soon after it was introduced in the clinic it became apparent that its use is accompanied by adverse effects. Sixty years later the drug is still first line treatment for many inflammatory conditions. In this thesis a new mechanism of glucocorticoid action is described which includes effects on T cell activation that does not include the regulation of genes. We found that this mechanism is important to the overall effect of glucocorticoid treatment and used it to develop a screening assay with the intention of finding a glucocorticoid ligand that induces exclusively non-transcriptional effects, possibly providing a safe alternative to conventional glucocorticoids. Because only a limited number of these non-transcriptional effects of GC treatment have been described, it is certainly possible that some of the adverse effects are also the result of non-transcriptional effects. The two compounds S3.1 and S3.4 described in chapter 7 are certainly not intended as a final product. These compounds were used to develop a pharmacophore, a chemical model describing what properties a molecule needs to exhibit to behave similarly. From this model more compounds were successfully developed. Work on these compounds is in progress.

The second part of this thesis describes the use of an existing cytostatic drug for immunosuppression. Interestingly this drug was developed for the treatment of cancer but came from a screening for anti-inflammatory drugs. As a cytostatic drug it wasn't very successful but it got a lot of attention when it was found that it could be used to cure visceral leishmaniasis. Further research on the mechanism of action resulted in studies that showed a potential for immune suppression. We too describe successful use of miltefosine for the treatment of chronic inflammation of the colon using a transfer mouse model. Miltefosine knows very few adverse effects and is a cheap drug that can be taken orally. However, it can cause nausea resulting in reduced appetite and emesis. Although we feel that the transfer model compares best to actual inflammatory conditions, it remains to be a mouse model and may not reflect IBD in humans well enough.

Chapter 1

The first chapter is a simple introduction to glucocorticoids (GCs) in general. Mentioned are the advantages and disadvantages of glucocorticoid treatment, outlining the general problem of developing save GCs.

Chapter 2

Glucocorticoid analogues have been around for more than half a century. Just like so many other drugs, GCs were not developed with the intention to suppress inflammation. Isolation, characterization and further development were fueled by war time rumors. Afraid the enemy would gain an advantage over them, allied forces initiated large scale research which eventually led to post-war development of GCs. As the rumors turned out to be false, a real purpose for the drug was lacking. Justification for the major investment led to the idea of dividing the few milligram GC available amongst lead scientists. Eventually it was Philip S. Hench who first tested the drug on rheumatoid arthritis patients and discovered its anti-inflammatory effect.

Chapter 3

The major downside of glucocorticoids as a drug is the induction of a great number of adverse effects. If not for these, the drug would be almost ideal. Most adverse effects have few consequences for the long term and can in general be ignored. But with higher concentrations and longer periods of treatment, the severity and the number of complications increase. If development of a GC analogue lacking adverse effects turns out to be impossible, it may be of great importance to understand how GCs cause these adverse effects on a very basic level. This may eventually provide a way to suppress adverse effects by concomitant treatment targeting the underlying cause.

In the last decades biomedical science has benefitted greatly from the development of new methods in the various fields such as genetics and proteomics. With it our understanding of GC signaling and how adverse effects are caused, has increased. This chapter is a broad overview of our current understanding of the underlying molecular mechanism responsible for GC side effects. The different side effects are addressed separately, focusing on the common major adverse effects associated with GC treatment. These include obesity, thinning of the skin and appearance of striae, muscle atrophy, osteoporosis, diabetes, hypertension, cataract and

glaucoma and finally effects on the central nervous system. In general, tissues in which GCs are part of the normal homeostasis are effected most by addition of exogenous GCs.

Chapter 4

From the first moment they entered the clinic, GCs have been remodeled to improve their properties, resulting in a whole range of GC analogues. Initially steps aimed for more potent anti-inflammatory properties by increasing half-life, followed by decrease of off-target effects by selecting for a higher affinity for the GR and lower affinity for other nuclear hormone receptors such as the mineralocorticoid receptor. Although this approach did provide GC analogues with more potent anti-inflammatory properties and a reduction in off-target effects originating from activation of other nuclear hormone receptors, it also increased adverse effects that arise from GR related transcriptional regulation of genes that are not involved in inflammation. In the last decades we have started to unravel how GCs work. And while we learn our notion of what to take into account while developing new GCs changes. Not so long ago it was believed that GCs repress pro-inflammatory genes while activating genes responsible for adverse effects and that this could be exploited to develop safe GCs. This chapter is a brief overview of what is currently known about GC action and what should be considered when developing GCs with more favorable properties.

Chapter 5

Classically GCs act through the regulation of genes. This mechanism requires a certain amount of time before actual changes can be noticed. However, it's not uncommon for GC effects to appear after a much briefer stretch of time. Patients suffering from osteoarthritis can be treated palliatively by injecting GCs directly into the affected joint. The pain relief that follows is almost instantaneously. This is just one example of GC effects that are too fast to involve regulation of genes. And because these effects do not include transcriptional regulation, they are termed non-transcriptional or non-genomic GC effects.

This chapter addresses the rapid effects of GC treatment on T helper cells and more specifically on a complex of proteins that is associated with the T cell receptor (TCR). In a previous study[1] the kinome profile of T helper cells, activated and treated with GCs, was assessed using a kinome array

(PepChip). The results indicated that two kinases, LCK and FYN, are inhibited by a short GC treatment of 10 minutes. These kinases, LCK in particular, are the first messengers to transfer a signal from the activated TCR. LCK is vital to T cell activation. T cells lacking this kinase cannot be activated through the TCR[2].

This chapter describes how, after stimulation of T cells, a complex is formed at the TCR. In this complex LCK associates with CD4, and FYN associates CD3. Both are associated with the chaperone protein Hsp90 and the GR. When stimulated T cells are treated with GC, all of these associations are lost. From this a model is proposed in which the GR forms an essential part of the TCR related complex. When the GR binds ligand, it breaks it bonds with the other proteins an leaves the complex, thereby destabilizing it causing all associations to be lost and the complex to break up. As a result TCR signaling is abrogated.

Chapter 6

Another aspect of GCs, besides the adverse effects, less desirable in a drug, is loss of sensitivity or even total loss of response. This group of patients can be subdivided into separate groups based on the underlying mechanism responsible for said loss. Mechanisms include increased clearance of GCs, increased pro-inflammatory transcription factors, altered posttranslational modifications to the GR, defective histone acetylation and finally overexpression of the dominant negative beta form of the GR. At least *in vitro* it is possible to induce steroid insensitivity in T cells by activation with super antigens. This chapter describes how the superantigen staphylococcal enterotoxin B (SEB) activates T lymphocytes through stimulation of a signaling cascade that deviates from the normal TCR-LCK mediated transduction pathway. This pathway leads to activation of T cells without the need for LCK activation. It includes a guanine nucleotide-binding protein Gαq and the phospholipase PLCβ2 and converges with the canonical TCR signaling cascade at the level of PKC. Stimulation with SEB triggers T cell proliferation that is less sensitive to GC treatment than with a normal mitogen. Direct activation of PKC with the phorbol ester PMA in combination with the ionophore ionomycin induces proliferation that is completely resistant to GC treatment. Taken together this suggests that the inhibitory effect of GC treatment on T cell proliferation is primarily a non-genomic effect on LCK and associated proteins. This places the relevance of non-genomic GC effects for the

overall anti-inflammatory effect of GC treatment in a whole new perspective.

Chapter 7

Inhibition of T cell proliferation is one of the primary features of the anti-inflammatory effect of GC treatment. The findings in the previous chapter suggest that this is based on a non-transcriptional effect. This chapter describes how through a combination of *in silico* and *in vitro* screening two GR ligands were discovered that do not induce transcriptional effects but do cause dissociation of the TCR associated molecules leading to inhibition of T cell proliferation.

For the screening, potential GR ligands were selected from a public database of commercially available compounds. Selection was based on similarity to the basic cortisol structure or gonane. This resulted in a quarter of a million potential candidates which in a second round of screening were fitted to a virtual GR binding pocket in four different known conformations. A selection was made of the compounds that fitted best and 80 of these were purchased. Each compound was then tested for its capacity to inhibit T cell proliferation. Using a cell line stably transfected with a glucocorticoid responsive element (GRE) reporter construct, compounds that induced transcriptional activation were excluded. Applying this strategy two compounds, S3.1 and S3.4, were identified that repress T cell proliferation with a potential comparable to prednisolone. Further examination of these compounds revealed an inhibition of TCR related signaling similar to the non-genomic effect of dexamethasone. But unlike dexamethasone these compounds did not induce adipogenesis or muscle atrophy, as was assessed through *in vitro* assays.

Chapter 8

In search for a PLCβ inhibitor (Chapter 5) we came across a drug called miltefosine. Indeed as had been previously reported[3], miltefosine inhibits PLCβ but not very specifically. We did however notice a very strong inhibitory effect on T cell proliferation and wondered if miltefosine could be used as an anti-inflammatory drug. In inflammatory bowel diseases (IBD) T lymphocytes play an important role in the underlying cause[4]. Using a mouse $CD4^+$ transfer model we tested miltefosine as a means to suppress chronic inflammation. The SCID mice in this model lack T and B cells and replacement with CD4 effector cells only, results in the induction

of chronic inflammation of the colon. The level of inflammation was assessed by colon weight and length, stool consistency, spleen weight, pathology score, influx of macrophages, neutrophils and T cells and the presence of cytokines. Oral treatment with miltefosine reduced inflammation significantly.

Chapter 9

Miltefosine (or hexadecylphosphocholine) was originally intended for the treatment of cancer but failed in most of the clinical trials due to lack of significant improvement of the patient's condition. In the end it was only effective as a topical treatment for skin metastasis of patients with breast cancer. But just as the drug was threatened to be forgotten completely, it was discovered that miltefosine was an excellent treatment for patients suffering from visceral Leishmaniasis. This parasitic disease is relatively unknown to people in the western part of the world because it only affects the economically less developed areas near the equator. This is because Leishmaniasis is transmitted through a type of sandfly that is only indigenous to those particular areas. Nevertheless, Leishmaniasis is, next to malaria, the second biggest parasitic killer worldwide with an estimated 25.000 fatalities each year. Although an effective treatment existed, it required a trained physician to administer the drug. Miltefosine can be taken orally, has few adverse effects and is relatively cheap.

This chapter mainly focusses on what has been published on miltefosine in relation to suppression of the immune systems and cells belonging to the immune system. These publications can be broadly divided into three separate groups. Effects on macrophages, effects on T cells and effects on mast cells. This data is used to build a case in favor of testing miltefosine as an immunosuppressive drug. And finally the underlying molecular mechanism is discussed based on what is currently known about how miltefosine affects cells.

References

1. Lowenberg, M., et al., *Kinome analysis reveals nongenomic glucocorticoid receptor-dependent inhibition of insulin signaling.* Endocrinology, 2006. **147**(7): p. 3555-62.

2. Wange, R.L. and L.E. Samelson, *Complex complexes: signaling at the TCR.* Immunity, 1996. **5**(3): p. 197-205.
3. Ward, P.D., H. Ouyang, and D.R. Thakker, *Role of phospholipase C-beta in the modulation of epithelial tight junction permeability.* Journal of Pharmacology and Experimental Therapeutics, 2003. **304**(2): p. 689-98.
4. Funderburg, N.T., et al., *Circulating CD4(+) and CD8(+) T cells are activated in inflammatory bowel disease and are associated with plasma markers of inflammation.* Immunology, 2013. **140**(1): p. 87-97.

11

Nederlandse Samenvatting van dit proefschrift

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Hoofdstuk 1

Het eerste hoofdstuk is een eenvoudige introductie op glucocorticoïden (GC) in het algemeen. Hierin worden de voor- en nadelen van behandeling met GC kort opgesomd, een beeld schetsend van de problemen die men kan verwachten bij het ontwikkelen van nieuwe veilige GC.

Hoofdstuk 2

GC afgeleiden zijn al meer dan 50 jaar in omloop. Net als met zoveel medicijnen, zijn GCs niet ontwikkeld met als doel ontsteking te remmen. De isolatie, karakterisatie en verdere ontwikkeling van GCs werden gedurende de WOII aangewakkerd door een gerucht. Om te voorkomen dat de tegenstander een voordeel kreeg werd door de geallieerden op grote schaal onderzoek verricht, hetgeen er uiteindelijk toe leidde dat vlak na de oorlog een eerste batch GCs geïsoleerd werd. Toen bleek dat de geruchten nergens op gebaseerd waren, hadden de GCs ineens geen nut meer. Om de enorme investering te rechtvaardigen werd de paar milligram die er op dat moment was, verdeeld onder de beste wetenschappers om zo alsnog een doel te vinden. Uiteindelijk was het Phillip S. Hench die als eerste GCs testte op patiënten met reumatoïde artritis en zo de ontstekingsremmende werking ontdekte.

Hoofdstuk 3

Het enorme nadeel van GCs als medicijn is het bestaan van de vele bijwerkingen. Was het niet voor deze bijwerkingen dan zou het medicijn bijna ideaal te noemen zijn. De meeste bijwerkingen hebben geen gevolgen voor de lange termijn en kunnen in het algemeen worden genegeerd. Maar bij gebruik in hoge concentraties of voor langere perioden neemt de ernst en het aantal bijwerkingen toe. Als uiteindelijk blijkt dat ontwikkeling van een veilige GC zonder bijwerkingen niet mogelijk is, zal basale kennis over het ontstaan van deze bijwerkingen essentieel zijn. Deze kennis kan vervolgens gebruikt worden om manieren te bedenken om de bijwerkingen zelf te bestrijden.

In de afgelopen jaren heeft biomedische wetenschap veel voordeel behaald uit de ontwikkeling van vele nieuwe onderzoeksmethoden in de verschillende velden zoals genetica en proteomics. Hierdoor is onze kennis

op het gebied van GC werking en bijwerking enorm toegenomen. Dit hoofdstuk is een overzicht van wat bekend is over de onderliggende moleculaire mechanismen van de verschillende bijwerkingen. De verschillende bijwerkingen worden apart besproken waarbij wordt gefocust op de belangrijkste meer algemene bijwerkingen. Daaronder vallen overgewicht, verdunnen van de huid en het ontstaan van striae, spier atrofie, osteoporose, diabetes, hypertensie, staar en glaucoma, en als laatste effecten op het centrale zenuw stelsel. Over het geheel genomen kan worden geconstateerd dat de bijwerkingen het grootst zijn in weefsels waar GCs een rol spelen in de normale homeostase.

Hoofdstuk 4

Vanaf het eerste moment dat GC de kliniek betraden is er aan de moleculen gesleuteld om ze te verbeteren, hetgeen erin heeft geresulteerd dat er tegenwoordig een hele selectie aan GC analogen op de markt is. Aanvankelijk richtte men zich vooral op het vergroten van de ontstekingsremmende werking door de halfwaardetijd te verlengen, gevolgd door een reductie in bijwerkingen middels vergroting van de specificiteit voor de GR en een reductie in binding aan andere nucleaire hormoonreceptoren zoals de mineralocorticoïde receptor. Alhoewel deze aanpak inderdaad meer potente GCs opleverde en een reductie in de bijwerkingen die gerelateerd zijn aan binding en activatie van andere nucleaire hormoonreceptoren, resulteerde het tevens in een toename van GR specifieke bijwerkingen. De laatste jaren zijn we beter gaan begrijpen hoe GCs werken. En met de toename van kennis veranderen ook de zaken waar we rekening mee houden bij de ontwikkeling van nieuwe GCs. Nog maar enkele jaren geleden waren veel wetenschappers van mening dat GCs pro-inflammatoire genen remden terwijl alle bijwerkingen het gevolg waren van activatie van genen en dat dit uitgebuit kon worden om zo een veilig GC te ontwikkelen. Dit hoofdstuk bespreekt kort wat we op dit moment weten over hoe GCs werken en waarmee rekening gehouden moet worden bij ontwikkeling van GCs met betere eigenschappen.

Hoofdstuk 5

Het klassieke beeld van hoe GCs werken is dat ze genen reguleren. Dit mechanisme vereist een zekere hoeveelheid tijd voordat het effect waargenomen kan worden. Maar het is niet ongewoon voor GC effecten om veel eerder waar genomen te kunnen worden. Artrose patiënten

kunnen worden behandeld via een injectie met GCs in het ontstoken gewricht. De pijnverlichting die volgt is vaak onmiddellijk. Dit is slechts één voorbeeld van GC effecten die te snel plaats vinden om te kunnen worden toegeschreven aan genregulatie. Omdat deze effecten ontstaan zonder dat daar transcriptionele regulatie bij betrokken is, worden zij niet-transcriptionele- of niet-genomische effecten genoemd.

Dit hoofdstuk beschrijft de snelle effecten van GC behandeling op T lymfocyten en dan specifiek die effecten die betrekking hebben op een complex van eiwitten dat geassocieerd is met de T cel receptor (TCR). In een vorige studie[1] werd het kinoom van geactiveerde en met GCs behandeld T lymfocyten bestudeerd. Daaruit bleek dat twee kinases LCK en FYN geremd werden in hun activatie door een korte behandeling met GCs. Deze kinases, LCK met name, zijn de eerste boodschappers die het signaal van de TCR door geven. Daardoor is LCK doorslag gevend voor T cel activatie. T cellen die geen LCK tot expressie kunnen brengen, kunnen niet worden geactiveerd via de TCR[2].

Dit hoofdstuk beschrijft hoe, na stimulatie van T cellen, een complex wordt gevormd bij de TCR. In dit complex is LCK geassocieerd met CD4 en FYN met CD3. Beide zijn geassocieerd met het chaperonne eiwit Hsp90 en de GR. Wanneer gestimuleerde T cellen worden behandeld met GCs, worden al deze interacties verbroken. Uitgaande van deze bevindingen wordt een model voorgesteld waarin de GR een essentieel onderdeel vormt van het TCR gerelateerde complex. Wanneer de GR ligand bindt, verbreekt deze zijn verbindingen met de andere eiwitten in het complex en verlaat deze om naar de kern te kunnen. Hierdoor raakt het hele complex gedestabiliseerd, verliezen alle eiwitten hun onderlinge verbindingen en valt het complex uiteen. Het uiteindelijke resultaat is blokkade van TCR signalering.

Hoofdstuk 6

Een ander aspect van GCs dat, naast de bijwerkingen, nadelig is voor het medicijn, is verlies van gevoeligheid van de patiënt voor de behandeling. Deze patiënten groep kan worden onderverdeeld in aparte groepen met verschillende onderliggende mechanismen verantwoordelijk voor dit verlies. De oorzaak kan zijn een verhoogde klaring van GCs, verhoogde expressie van pro-inflammatoire transcriptie factoren, gewijzigde post-translationele modificatie van de GR, defecte histon acetylatie en als

laatste overexpressie van de dominant negatieve beta vorm van de GR. *In vitro* is het mogelijk om steroïde ongevoeligheid te induceren door T lymfocyten te stimuleren met super-antigenen. Dit hoofdstuk beschrijft hoe het super-antigen staphylococcal enterotoxin B (SEB) T cellen stimuleert via een signaal transductie pad dat afwijkt van de normale TCR-LCK gemedieerde activatie. Deze route bevat guanine nucleotide-bindend proteïne Gαq en de fosfolipase PLCβ2 en voegt zich weer bij de TCR-LCK gemedieerde route op het niveau van proteïne kinase C (PKC). Stimulatie van T cellen met SEB resulteert in een celdeling die minder gevoelig is voor behandeling met GC dan wanneer deze cellen met een normaal antigeen gestimuleerd zouden zijn. Directe activatie van PKC met de forbol ester PMA in combinatie met de ionofoor ionomycine resulteert in celdeling die geheel resistent is tegen G behandeling. Te samen suggereren deze resultaten dat het remmende effect van GC op T lymfocyt deling berust op een niet-transcriptioneel effect van GC behandeling. Dit plaatst de relevantie van de niet-transcriptionele effecten van GC behandeling, voor het gehele anti-inflammatoire effect, in een heel nieuw daglicht.

Hoofdstuk 7

Remming van T lymfocyt deling door GC behandeling is een van de belangrijkste onderdelen van het totale ontstekingsremmende effect van GCs. De bevindingen zoals beschreven in het vorige hoofdstuk suggereren dat dit berust op een niet-genomisch effect. Dit hoofdstuk beschrijft hoe, door een combinatie van *in silico* en *in vitro* screening, twee GR liganden werden gevonden die geen transcriptionele effecten induceren maar wel dissociatie van TCR geassocieerde moleculen leidend tot remming van T lymfocyt deling.

Voor de screening werden potentiele liganden geselecteerd uit een publieke database van commercieel verkrijgbare stoffen. Selectie van de stoffen werd gebaseerd op basis van overeenkomsten met de basale structuur van cortisol ook wel gonane genoemd. Dit resulteerde in een kwart miljoen potentiele kandidaten die in een tweede ronde van selectie werden gepast in een virtuele GR bindingsholte, gebaseerd op vier bekende GR conformaties. De stoffen die het best pasten werden geselecteerd en daarvan werden er 80 gekocht. Elke stof werd vervolgens getest voor de capaciteit om T lymfocyt deling te remmen. Gebruik makend van een cellijn die stabiel getransfecteerd is met een glucocorticoïd receptor bindend reporter construct, werden alle stoffen die

transcriptionele regulatie induceerde geëxcludeerd. Met deze strategie werden er twee GR liganden gevonden, S3.1 en S3.4, die in staat waren T lymfocyt deling te remmen met een potentie gelijk aan prednisolon maar zonder de transcriptionele activatie. Verdere studie toonde aan dat dit effect gelijk was aan het effect zoals eerder beschreven bij dexamethason. Maar anders dan dexamethason, induceerde deze stoffen geen vervetting of spier atrofie zoals getest met *in vitro* assays.

Hoofdstuk 8

Op zoek naar een remmer van PLCβ (hoofdstuk 5) kwamen we het medicijn miltefosine tegen. Zoals eerder gerapporteerd[3] remde miltefosine PLCβ maar niet heel specifiek. Het viel ons echter wel op dat miltefosine een sterk remmend effect op T lymfocyt deling had en vroegen ons af of miltefosine als een ontstekingsremmer gebruikt kon worden. Bij chronische ontstekingsziekten van het maag-darm kanaal spelen T lymfocyten een belangrijke rol[4]. Door middel van een zogenaamd CD4 transfer muizen model werd miltefosine getest als middel om chronische ontsteking te remmen. De SCID muizen in dit model hebben geen T en B cellen en wanneer deze worden vervangen door een repertoire van alleen T effector cellen leidt dit naar verloop van tijd tot een chronische ontsteking van de dikke darm. Het niveau van ontsteking werd bepaald door gewicht en lengte van de dikke darm, toestand van de uitwerpselen, gewicht van de milt, pathologische score, influx in de dikke darm van macrofagen, neutrofielen en T cellen en als laatste de toename van lokale cytokines. Orale behandeling met miltefosine wist de ontsteking significant te remmen.

Hoofdstuk 9

Miltefosine (of hexadecylfosfocholine) werd oorspronkelijk ontwikkeld als behandeling voor kanker maar faalde in de meeste klinische trials door het uitblijven van significante verbetering van de conditie van de patiënten. Uiteindelijk bleek miltefosine alleen geschikt als topicale behandeling voor huid metastasen van patiënten met borstkanker. Maar net toen miltefosine in de vergetelheid dreigde te raken werd ontdekt dat deze stof een uitstekende behandeling is voor patiënten met viscerale Leishmaniasis. Deze parasitaire ziekte is relatief onbekend in de westerse wereld omdat de ziekte alleen financieel minder ontwikkelde gebieden treft rond de evenaar. Dit komt omdat de ziekte verspreidt wordt door een type

zandvlieg die alleen in die gebieden voor komt. Niettemin is Leishmaniasis, op malaria na, de belangrijkste dodelijke parasitaire aandoening wereldwijd met een geschat aantal van 25.000 dodelijke slachtoffers elk jaar. Een effectieve behandeling bestond reeds maar vereiste een arts om het middel toe te dienen. Miltefosine kan oraal worden ingenomen en is relatief goedkoop om te maken.

Dit hoofdstuk zoemt met name in op wat bekend is over miltefosine als remmer van het immuun systeem en de cellen die daartoe behoren. Over het geheel genomen kunnen die publicaties in drie groepen worden verdeeld. Effect op macrofagen, effect op mast cellen en effect op T cellen. Deze data wordt vervolgens gebruikt om het verder testen van miltefosine als ontstekingsremmer te onderbouwen. Als laatste wordt het werkingsmechanisme van miltefosine voor zover bekend besproken.

Referentie lijst

1. Lowenberg, M., et al., *Kinome analysis reveals nongenomic glucocorticoid receptor-dependent inhibition of insulin signaling.* Endocrinology, 2006. **147**(7): p. 3555-62.
2. Wange, R.L. and L.E. Samelson, *Complex complexes: signaling at the TCR.* Immunity, 1996. **5**(3): p. 197-205.
3. Ward, P.D., H. Ouyang, and D.R. Thakker, *Role of phospholipase C-beta in the modulation of epithelial tight junction permeability.* Journal of Pharmacology and Experimental Therapeutics, 2003. **304**(2): p. 689-98.
4. Funderburg, N.T., et al., *Circulating CD4(+) and CD8(+) T cells are activated in inflammatory bowel disease and are associated with plasma markers of inflammation.* Immunology, 2013. **140**(1): p. 87-97.

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Appendix

Dankwoord

Factum est. It is done.

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Publications

1. Palles C, Chegwidden L, Li X, Findlay JM, Farnham G, Castro Giner F, Peppelenbosch MP, Kovac M, Adams CL, Prenen H, Briggs S, Harrison R, Sanders S, MacDonald D, Haigh C, Tucker A, Love S, Nanji M, deCaestecker J, Ferry D, Rathbone B, Hapeshi J, Barr H, Moayyedi P, Watson P, Zietek B, Maroo N, Gay L, Underwood T, Boulter L, McMurtry H, Monk D, Patel P, Ragunath K, Al Dulaimi D, Murray I, Koss K, Veitch A, Trudgill N, Nwokolo C, Rembacken B, Atherfold P, Green E, Ang Y, Kuipers EJ, Chow W, Paterson S, Kadri S, Beales I, Grimley C, Mullins P, Beckett C, Farrant M, Dixon A, Kelly S, Johnson M, Wajed S, Dhar A, Sawyer E, Roylance R, Onstad L, Gammon MD, Corley DA, Shaheen NJ, Bird NC, Hardie LJ, Reid BJ, Ye W, Liu G, Romero Y, Bernstein L, Wu AH, Casson AG, Fitzgerald R, Whiteman DC, Risch HA, Levine DM, Vaughan TL, **Verhaar AP**, van den Brande J, Toxopeus EL, Spaander MC, Wijnhoven BP, van der Laan LJ, Krishnadath K, Wijmenga C, Trynka G, McManus R, Reynolds JV, O'Sullivan J, MacMathuna P, McGarrigle SA, Kelleher D, Vermeire S, Cleynen I, Bisschops R, Tomlinson I, Jankowski J.Polymorphisms near TBX5 and GDF7 are associated with increased risk for Barrett's esophagus. Gastroenterology. 2015 Feb;148(2):367-78.

2. **Verhaar AP**, Wildenberg ME, Peppelenbosch MP, Hommes DW, van den Brink GR. Repurposing miltefosine for the treatment of immune-mediated disease? J Pharmacol Exp Ther. 2014 Aug;350(2):189-95.

3. **Verhaar AP**, Hoekstra E, Tjon AS, Utomo WK, Deuring JJ, Bakker ER, et al. Dichotomal effect of space flight-associated microgravity on stress-activated protein kinases in innate immunity. Sci Rep. 2014;4:5468.

4. **Verhaar AP**, Wildenberg ME, te Velde AA, Meijer SL, Vos AC, Duijvestein M, et al. Miltefosine suppresses inflammation in a mouse model of inflammatory bowel disease. Inflamm Bowel Dis. 2013 Aug;19(9):1974-82.

5. **Verhaar AP**, Wildenberg ME, Duijvestein M, Vos ACW, Peppelenbosch MP, Lowenberg M, et al. Superantigen-Induced Steroid Resistance Depends on Activation of Phospholipase C beta 2. J Immunol. 2013 Jun 15;190(12):6589-95.

6. Strisciuglio C, Duijvestein M, **Verhaar AP**, Vos AC, van den Brink GR, Hommes DW, et al. Impaired autophagy leads to abnormal dendritic cell-epithelial cell interactions. J Crohns Colitis. 2013 Aug;7(7):534-41.

7. Wildenberg ME, Vos AC, Wolfkamp SC, Duijvestein M, **Verhaar AP**, Te Velde AA, et al. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse. Gastroenterology. 2012 Jun;142(7):1493-503 e6.

8. Wildenberg M, Vos AC, Duijvestein M, **Verhaar AP**, Hommes DW, van den Brink GR. Induction of Autophagy Upon DC-T Cell Contact is Mediated by LKB1-AMPK-mTOR Signalling. Gastroenterology. 2012 May;142(5):S884-S.

9. Vos AC, Wildenberg ME, Arijs I, Duijvestein M, **Verhaar AP**, de Hertogh G, et al. Regulatory macrophages induced by infliximab are involved in healing in vivo and in vitro. Inflamm Bowel Dis. 2012 Mar;18(3):401-8.

10. Wildenberg ME, Vos ACW, Wolfkamp SC, Duijvestein M, **Verhaar AP**, Velde AAT, et al. Autophagy Regulates Immune Responses Through Destabilization of the Immunological Synapse. Gastroenterology. 2011 May;140(5):S172-S3.

11. Vos ACW, Wildenberg ME, Arijs I, Duijvestein M, **Verhaar AP**, Vermeire S, et al. Infliximab Induces Regulatory Macrophages in Responders but Not in Non-Responders. Gastroenterology. 2011 May;140(5):S274-S.

12. Vos ACW, Duijvestein M, Wildenberg ME, **Verhaar AP**, van den Brink GR, Hummes DW. Enhanced Induction of Regulatory Macrophages Upon Azathioprine/Infliximab Combination Treatment In Vitro. Gastroenterology. 2011 May;140(5):S492-S.

13. Vos AC, Wildenberg ME, Duijvestein M, **Verhaar AP**, van den Brink GR, Hommes DW. Anti-tumor necrosis factor-alpha antibodies induce regulatory macrophages in an Fc region-dependent manner. Gastroenterology. 2011 Jan;140(1):221-30.

14. Strisctuglio C, Duijvestein M, Vos ACW, **Verhaar AP**, Miele E, Troncone R, et al. Autophagy Induces Immune Tolerance by Regulating Interactions Between Dendritic Cells-Epithelial Cell in the Gut. Gastroenterology. 2011 May;140(5):S273-S.

15. Strehl C, Gaber T, Lowenberg M, Hommes DW, **Verhaar AP**, Schellmann S, et al. Origin and functional activity of the membrane-bound glucocorticoid receptor. Arthritis Rheum. 2011 Dec;63(12):3779-88.

16. Duijvestein M, Molendijk I, Roelofs H, Vos AC, **Verhaar AP**, Reinders ME, et al. Mesenchymal stromal cell function is not affected by drugs used in the treatment of inflammatory bowel disease. Cytotherapy. 2011 Oct;13(9):1066-73.

17. Strisciuglio C, Wildenberg ME, Vos ACW, **Verhaar AP**, van den Brink GR, Hommes DW. Atg16l1 and Irgm Contribute to the Regulation of Immune Responses. J Pediatr Gastr Nutr. 2010 Jun;50:E44-E.

18. Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, **Verhaar AP**, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut. 2010 Dec;59(12):1662-9.

19. Wildenberg ML, Vos C, Duijvestein M, **Verhaar AP**, van den Brink GR, Hommes D. ATG16L1 and IRGM Knock-Down Results in Pro-Inflammatory Dendritic Cells. Gastroenterology. 2009 May;136(5):A251-A.

20. Parikh K, Diks SH, Tuynman JH, **Verhaar AP**, Lowenberg M, Hommes DW, et al. Comparison of peptide array substrate phosphorylation of c-Raf and mitogen activated protein kinase kinase kinase 8. PLoS One. 2009;4(7):e6440.

21. Duijvestein M, Wildenberg ME, Wendrich BB, Vos C, **Verhaar AP**, Roelofs H, et al. Phenotypical and Functional Characteristics of In Vitro Expanded Bone Marrow Derived Mesenchymal Stem Cells from Patients with Refractory Crohn's Disease. Gastroenterology. 2009 May;136(5):A251-A.

22. Lowenberg M, **Verhaar AP**, van den Brink GR, Hommes DW. Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. Trends Mol Med. 2007 Apr;13(4):158-63.

23. Ritsema T, Hernandez L, **Verhaar AP**, Altenbach D, Boller T, Wiemken A, et al. Developing fructan-synthesizing capability in a plant invertase via mutations in the sucrose-binding box. Plant J. 2006 Oct;48(2):228-37.

24. Lowenberg M, **Verhaar AP**, Bilderbeek J, Marle J, Buttgereit F, Peppelenbosch MP, et al. Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN. EMBO Reports. 2006 Oct;7(10):1023-9.

25. Lowenberg M, Tuynman J, Scheffer M, **Verhaar AP**, Vermeulen L, van Deventer S, et al. Kinome analysis reveals nongenomic glucocorticoid receptor-dependent inhibition of insulin signaling. Endocrinology. 2006 Jul;147(7):3555-62.

26. Lowenberg M, Scheffer M, **Verhaar AP**, Peppelenbosch M, Hommes D. A role for STAT5 in steroid-resistant UC? Inflamm Bowel Dis. 2006 Jul;12(7):665.

27. Lowenberg M, Bilderbeek J, **Verhaar AP**, van Marle J, Buttgereit F, Peppelenbosch M, et al. Glucocorticoids inhibit T cell receptor signaling via LCK and FYN - A new mechanism for an old drug. Gastroenterology. 2006 Apr;130(4):A547-A.

28. Lowenberg M, Bilderbeek J, **Verhaar AP**, Buttgereit F, Peppelenbosch M, Van Deventer S, et al. Glucocorticoid-induced inhibition of T cell receptor signaling mediated through Lck and Fyn: A new mechanism for an old drug. Inflamm Bowel Dis. 2006 Apr;12:S26-S.

29. **Verhaar AP**, Krishnadath KK, Peppelenbosch MP. Using microgravity for defining novel anti-atherosclerotic therapy. Curr Genomics. 2005 Oct;6(6):487-90.

30. Ritsema T, **Verhaar AP**, Vijn I, Smeekens S. Using natural variation to investigate the function of individual amino acids in the sucrose-binding box of fructan : fructan 6G-fructosyltransferase (6G-FFT) in product formation. Plant Molecular Biology. 2005 Jul;58(5):597-607.

31. Lowenberg M, **Verhaar AP**, van den Blink B, ten Kate F, van Deventer S, Peppelenbosch M, et al. Specific inhibition of c-Raf activity by semapimod induces clinical remission in severe Crohn's disease. J Immunol. 2005 Aug 15;175(4):2293-300.

32. Lowenberg M, **Verhaar AP**, van den Blink B, Peppelenbosch M, van Deventer S, Hommes D. Essential role for c-Raf in steroid insensitive Crohn's disease. Gastroenterology. 2005 Apr;128(4):A505-A.

33. Ritsema T, **Verhaar AP**, Vijin I, Smeekens S. Fructosyltransferase mutants specify a function for the beta-fructosidase motif of the sucrose-binding box in specifying the fructan type synthesized. Plant Mol Biol. 2004 Apr;54(6):853-63.