The non-steroidal SEGRA, BAY1155975, in contrast to classical glucocorticoids, inhibits anti-CD28-costimulated T cell activation

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Zusammenfassung

Glukokortikoide (GK) zählen zu den effizientesten Medikamenten bei der Behandlung akuter und chronischer Entzündungskrankheiten. Ihr Einsatz ist häufig durch das Auftreten zahlreicher und teilweise irreversibler Nebenwirkungen beeinträchtigt. Aus diesem Grund wurden neue Glukokortikoid-Rezeptor (GR)-Liganden, die eine potente anti-entzündliche Wirkung bei gleichzeitig vermindertem Nebenwirkungspotential aufweisen sollen, entwickelt. Nicht-steroidale selektive Glukokortikoidrezeptor-Agonisten (SEGRAs) stellen eine neue Klasse von Glukokortikoidrezeptor (GR)-Liganden mit verbessertem therapeutischen Index dar, die zur Zeit in klinischen Studien für Hautentzündungen getestet werden.

Im Rahmen der vorliegenden Arbeit wurde die SEGRA-Substanz BAY1155975 hinsichtlich ihrer hemmenden Wirkung auf die CD28-kostimulierte Aktivierung primärer humaner T-Zellpopulationen mit der von klassischen GK, wie z.B. Prednisolon und Dexamethason, verglichen. Bekannt ist, dass klassische GK die Aktivierung von T-Zellen, die mittels anti-CD3-Stimulation allein über den T-Zellrezeptor vermittelt wird, effizient hemmen können, wohingegen die Aktivierung von T-Zellen mit zusätzlicher CD28-Kostimulation von klassischen GK nicht gehemmt werden kann.
In humanen naiven CD4+ T-Zellen zeigten BAY1155975 und Prednisolon eine ähnlich starke Hemmung der CD28-kostimulierten IFNγ-Sekretion. In humanen Gedächtnis/Effektor-CD4+ T-Zellen war die hemmende Wirkung von Prednisolon auf die CD28-kostimulierte IFNγ-Sekretion dagegen stark vermindert. Die höchste Konzentration von BAY1155975 wies im Vergleich zu Prednisolon eine statistisch signifikant größere Hemmung der CD28-kostimulierten Sekretion von Effektorzytokinen (IFNγ, TNFα, IL-17 und IL-22) auf. Proliferation, Apoptose und die Expression verschiedener Aktivierungsmarker wurden dagegen durch BAY1155975 und Prednisolon gleichermaßen reguliert. Eine größere Hemmung der Lymphokinsekretion durch BAY1155975 zeigte sich auch nach Stimulation mit PMA und Ionomycin in humanen Gedächtnis/Effektor-CD4+ T-Zellen. Aufgrund von Analysen der Substanzaktivität in verschiedenen Signalwegen wird eine stärkere Hemmung des Kalzium-Kalzineurin-NFAT Signalweges durch BAY1155975 in humanen Gedächtnis/Effektor-CD4+ T-Zellen vermutet.
In vivo zeigten BAY1155975 und Prednisolon eine ähnlich starke Hemmung der T-Zell-vermittelten Hautentzündung im DNFB-induzierten Kontaktallergiemodell in Mäusen, wenn die Behandlung der Mäuse mit den Substanzen vor dem Challenge erfolgte. Bei einer Substanzbehandlung der Mäuse während der Sensibilisierung wurde die T-Zell-vermittelte Hautentzündung dagegen deutlich stärker durch BAY1155975 als durch Prednisolon gehemmt.

Zusammenfassend geben die Ergebnisse dieser Arbeit einen Hinweis auf eine stärkere Hemmung der T-Zellsensibilisierung und der Effektorzytokinsekretion durch die SEGRA-Substanz BAY1155975 im Vergleich zum klassischen GK Prednisolon. Entsprechend dieser Ergebnisse könnte BAY1155975 möglicherweise eine bessere therapeutische Wirkung in T-Zell-vermittelten Entzündungserkrankungen zeigen, in denen der Einsatz und die Wirkung von klassischen GK durch ihre Nebenwirkungen ebenso wie durch ihre T-Zellresistenz begrenzt ist.

Schlagwörter: Glukokortikoid, SEGRA, T-Zell Aktivierung, CD28-Kostimulation, Zytokinsekretion

Abstract

Glucocorticoids (GCs) are the most effective therapeutic agents for the treatment of acute and chronic inflammatory diseases. Their use is often accompanied with numerous and sometimes irreversible side-effects. Therefore, new glucocorticoid receptor (GR) ligands with should have potent anti-inflammatory efficacy but a reduced side-effect profile have been developed. Non-steroidal selective glucocorticoid receptor agonists (SEGRAs) represent a new class of GR ligands with an improved therapeutic index, which are currently in clinical trials for dermatological inflammatory conditions.

In this study, we compared the SEGRA, BAY1155975, and classical GCs, like prednisolone and dexamethasone, regarding their suppressive effect on CD28-costimulated activation of human primary T cell subpopulations. It is known, that classical GCs effectively suppress T cell activation triggered via the T cell receptor complex by anti-CD3 stimulation, whereas additional CD28 co-stimulation abrogates the suppressive effect of GCs.

In human naive CD4+ T cells, BAY1155975 and prednisolone suppressed anti-CD28-costimulated IFNγ secretion to a similar extent. However, in human memory/effector CD4+ T cells the suppressive effect of prednisolone on anti-CD28-costimulated IFNγ secretion was impaired. BAY1155975 at the highest concentration exhibited a significantly stronger inhibition of CD28-costimulated effector cytokine secretion (IFNγ, TNFα, IL-17 and IL-22) in comparison to prednisolone. Interestingly, proliferation, apoptosis and expression of activation markers were similarly regulated by BAY1155975 and prednisolone. An enhanced inhibition of lymphokine secretion by BAY1155975 was also seen after PMA and ionomycin stimulation in human memory/effector CD4+ T cells. Further studies on different signal transduction pathways suggested that BAY1155975 stronger inhibited the calcium-calcineurin-NFAT pathway than GCs in human memory/effector CD4+ T cells.

In vivo BAY1155975 and prednisolone showed comparable efficacy in inhibition of T cell-dependent skin inflammation in DNFB-induced contact hypersensitivity models in mice, when mice were treated before hapten challenge. In contrast, when mice were treated around hapten sensitization markedly stronger inhibition of T cell-dependent skin inflammation was observed for BAY1155975 than prednisolone.

In summary, the data of this study give evidence for a stronger inhibition of T cell sensitization and effector cytokine secretion by the SEGRA, BAY1155975, in comparison to the classical GC, prednisolone. Therefore, BAY1155975 might achieve a superior therapeutic efficacy in T-cell dependent inflammatory diseases, where the use of classical GCs is limited by their side-effect potential as well as by T cell resistance.

Keywords: glucocorticoid, SEGRA, T cell activation, CD28 costimulation, cytokine secretion

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Abbreviations

AF	activation function domain
AoD	Assay-on-Demand
AP-1	activator protein-1
APC (FACS)	allophycocyanin
APC	antigen-presenting cell
BCA	bicinchoninic acid
BSA	bovine serum albumin
Ca_v1 channel	L-type Ca^{2+} channel
cDNA	complementary deoxyribonucleic acid
CHS	contact hypersensitivity
DAG	diacylglycerol
DBD	DNA-binding domain
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNFB	2,4-dinitro-1-fluorobenzene
DP	double-positive
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FKBP	FK506-binding protein
FYN	Fyn to Src, oncogene related Fgr, Yes
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GILZ	glucocorticoid-induced leucine zipper
gMFI	geometric mean fluorescence intensity
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA-DR	human leukocyte antigen DR
HPA	hypothalamo-pituitary adrenal
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horse radish peroxidase
HSP	heat shock protein

IFNγ	interferon gamma
Ig	immunoglobulin
IκBα	inhibitor of nuclear factor-kappaB alpha
IL	interleukin
IP_3	inositol 1,4,5-trisphosphate
LCK	lymphocyte-specific protein tyrosine kinase
LBD	ligand-binding domain
LPS	lipopolysaccharide
LTT	lymphocyte transformation test
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK1	mitogen-activated protein/extracellular signal-regulated kinase kinase-1
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MPK-1	mitogen-activated protein kinase phosphatase-1
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NFAT	nuclear factor of activated T cells
nGRE	negative glucocorticoid response element
NLS	nuclear localization site
OX	oxazolone
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PI3K	phosphoinositide 3 kinase
PKCθ	protein kinase C theta
PLC-γ1	phospholipase C gamma 1
PMA	phorbol 12-myristate 13-acetate
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecylsulfate
SEGRA	selective glucocorticoid receptor agonist
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
TA	transactivation

T_{cm} cell	central memory T cell
TCR	T cell receptor
T_{em} cell	effector memory T cell
TF	transcription factor
TGFβ	transforming growth factor beta
Th cell	T helper cell
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TR	transrepression
Treg	regulatory T cell
Tris	Tris (hydroxy methyl) aminomethane
ZAP70	zeta-chain-associated protein of 70 kDa

1. Introduction

1.1 Glucocorticoid receptor ligands

1.1.1 Glucocorticoids as anti-inflammatory and immunosuppressive drugs

Glucocorticoids (GCs) represent highly efficacious drugs for the treatment of acute and chronic inflammatory diseases, such as allergy, asthma and autoimmune diseases, and have been in use for more than 60 years (Hench et al., 1949; Coutinho & Chapman, 2011). Endogenous GCs including cortisol, the predominant GC in man, are synthesized and secreted from the adrenal cortex according to a distinct circadian pattern or in response to physiological and/or psychological stress. The release of GCs is regulated by the hypothalamo-pituitary-adrenal (HPA) axis and is suppressed via a negative feedback loop by GC levels themselves (Chung et al., 2011; Clark & Belvisi, 2012). Natural GCs mediate their biological effects by two distinct intracellular receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The GR is widely distributed in the body and is mainly responsible for the observed actions at higher physiological GC concentrations, for example in stress (Buckingham, 2006; Groeneweg et al., 2011). Synthetic GCs including dexamethasone and prednisolone are selective for the GR with a lower affinity for the MR compared to cortisol (Rosen & Miner, 2005).

GCs display pleiotropic effects in the regulation of carbohydrate, protein and lipid metabolism, embryonic development, physiological stress, growth and brain functions such as memory and behavior (Beck et al., 2009). GCs increase serum glucose levels by inducing the synthesis of gluconeogenic enzymes in the liver, the mobilization and degradation of proteins, and by support of glycogen deposit (Schäcke et al., 2002; Clark & Belvisi, 2012). GCs enhance the activity of enzymes involved in fatty acid synthesis and promote the secretion of lipoproteins. In adipose tissue, GCs inhibit glucose uptake and increase the lipolysis and thereby the release of fatty acids and glycerol, which can also serve as substrates for gluconeogenesis (Pivonello et al., 2010).

GCs exert anti-inflammatory effects on many immune cells including T cells, B cells, monocytes, macrophages, granulocytes and dendritic cells (DCs) (Coutinho & Chapman, 2011). Following GC administration in man a rapid redistribution of lymphocytes and monocytes from the peripheral circulation to other lymphoid compartments is observed. In contrast, the proportion and number of circulating neutrophils is increased likely via an increased release of bone marrow-derived neutrophils and an inhibitory effect on neutrophil apoptosis by GC treatment (Cupps & Fauci, 1982; Jetzek-Zader et al., 2007). Impaired DC

migration and maturation via downregulation of major histocompatibility complex (MHC) class II and B7 molecules has been reported after GC treatment (Matyszak et al., 2000; He et al., 2010). Furthermore, GCs repress the synthesis of inflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenases and the secretion of pro-inflammatory cytokines, i.e. tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1ß) and IL-6, by monocytes and macrophages (Almawi et al., 1996; Elenkov, 2004). GCs also suppress the adhesion molecule expression on endothelial cells and leukocytes (Barnes, 1998; Tuckermann et al., 2005). The main inhibitory effects of GCs on T cell development and function are apoptosis induction in immature CD4+CD8+ double-positive (DP) thymocytes and the suppression of cytokine production by mature T cells. The *de novo* transcription of a number of cytokine genes, including those for IL-2, IL-4, IL-5, IL-6, TNFα and interferon gamma (IFNγ), is repressed by GCs (Ashwell et al, 2000; Herold et al., 2006).

In addition to GC-mediated inhibition of many inflammatory cytokines, chemokines, enzymes, adhesion molecules and receptors, several anti-inflammatory proteins are upregulated by GC treatment. GCs increase the synthesis of inhibitor of nuclear factor-κB alpha (IκBα), IL-10, an anti-inflammatory and immunomodulatory cytokine, annexin A1, which blocks the production of pro-inflammatory prostaglandins, and dual specificity phosphatase (DUSP) 1, which dephosphorylates and inactivates members of the mitogen-activated kinase (MAPK) family (Clark, 2007; Clark & Belvisi, 2012).

Despite the high anti-inflammatory efficacy of GCs, however, their systemic administration is often limited by severe and sometimes irreversible side-effects, such as diabetes mellitus, osteoporosis or thymus involution, especially after long-term systemic treatment. GC therapy can induce insulin resistance and impaired insulin production in pancreatic β-cells leading to diabetes induction or aggravation in diabetic patients. By blocking the synthesis of inflammatory cytokines as well as matrix proteins and matrix proteases, the healing of aseptic wounds is decreased by GC therapy. Moreover, impaired longitudinal growth has been observed in children receiving prolonged GC treatment, whereas in adults osteoporosis and an increased risk of fractures are the main side-effects on bone following GC administration. An increased risk for hypertension, dyslipidemia and glaucoma is also associated with GC therapy (Schäcke et al., 2002; Rhen & Cidlowski, 2005; Schäcke et al., 2008).

1.1.2 Regulation of gene expression by glucocorticoids

GCs primarily exert their effects by binding to the cytoplasmic glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. The GR consists of an N-terminal domain containing the first transcriptional activation function domain (AF-1), which associates with the basal transcription machinery. A DNA-binding domain (DBD), with two cysteine-rich zinc fingers

important for GR dimerization and DNA binding, is located in the middle of the molecule. The C-terminal part of the GR is composed of a variable hinge region and a ligand-binding domain (LBD), encompassing the second transcriptional activation function domain (AF-2), which is exposed by ligand-induced reorganization of the LBD. Two nuclear localization sites, NLS1 and NLS2, are located in close proximity of the DBD and at the end of the LBD. (Mangelsdorf et al., 1995; Oakley & Cidlowski, 2011; Figure 1).

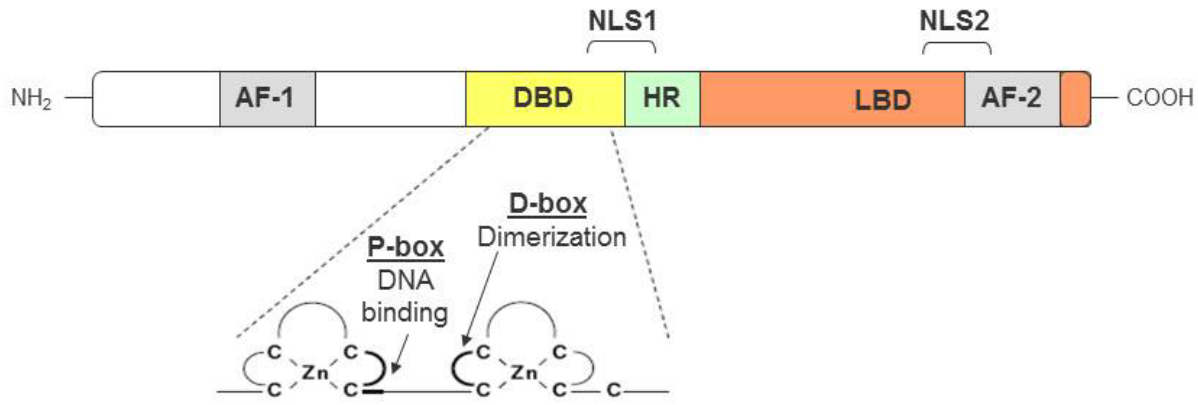


Figure 1: Structure of the glucocorticoid receptor.

The glucocorticoid receptor is composed of the N-terminal domain including the transcriptional activation function domain 1 (AF-1), the DNA-binding domain (DBD), the hinge region (HR) and the ligand-binding domain (LBD) with the transcriptional activation function domain 2 (AF-2). Within the DBD, two zinc fingers are responsible for DNA binding (P-box) and receptor dimerization (D-box). Two nuclear localization sites, NLS1 and NLS2, are located in close proximity of the DBD and at the end of the LBD (modified from Buckingham, 2006).

Due to alternative splicing multiple isoforms of the GR exist. In humans, the 777 amino acids-containing GRα is the most predominant, functional GR. The β-isoform of the GR (GRβ) binds to DNA but is unable to bind GCs (Beck et al., 2009). However, when coexpressed with GRα, GRβ can act in a dominant-negative manner to suppress actions of GRα on genes both positively and negatively regulated by glucocorticoids. An increased expression of GRβ has been demonstrated in patients with asthma, rheumatoid arthritis or ulcerative colitis who were insensitive to GC therapy (Smoak & Cidlowski, 2004; Oakley & Cidlowski, 2011).

In the absence of the hormone, the transcriptionally inactive GR is associated with a number of proteins, including the chaperones heat shock protein 90 (Hsp90) and Hsp70, the co-chaperone p23 and the FK506-binding immunophilins FKBP51 and FKBP52. Upon hormone binding to the GR, the receptor conformation changes and results in dissociation of

Hsp90 and exposure of the NLS2 (Smith & Toft, 2008; Beck et al., 2009). The ligand-activated GR translocates into the nucleus and regulates the transcription of GC-sensitive genes either positively (transactivation) or negatively (transrepression), thereby functioning as transcription factor (TF) (Clark & Belvisi, 2012). Using microarray analysis it was shown, that about 10 - 20% of the expressed human leukocyte genome was positively or negatively affected by GC (Galon et al., 2002; Lu et al., 2007).

The ligand-activated GR can activate gene expression of typical GC response elements (GRE)-containing or other promoters (Transactivation, TA). At simple GREs it binds as homodimer directly to the palindrome sequence AGAACAnnnTGTTCT (Clark & Belvisi, 2012). It is suggested that GR dimer binding depends on initial binding of a GR monomer to the higher affinity half-site of the GRE followed by binding of the second monomer (Adams et al., 2003). The P-box of each GR monomer binds to DNA and the D-box is associated with the partner GR molecule. At composite GREs, which are found for example in the promoter of the tyrosine aminotransferase gene, the GR binds in a cooperative manner with other TFs to the DNA. In addition, tethering GREs, where the GR can bind directly to DNA-bound TFs such as signal transducers and activator of transcription (STAT) and SmaMAD family members, have also been described for positive regulation of gene transcription by GCs (Beck et al., 2009; Figure 2). Recent studies revealed that the majority of GR binding sites are located very far from transcription start sites rather than relatively close to promoters and that GR binding sites vary considerably around the consensus (Clark & Belvisi, 2012).

Transrepression (TR) by GCs is achieved by different modes of action. The ligand-activated GR can repress the transcription of GC-sensitive genes by direct binding of the homodimer to negative GREs (nGREs). Furthermore, the ligand-activated GR can act in a composite manner and bind both a GRE and other TFs that bind in the vicinity of the GR (Oakley & Cidlowski, 2011; Clark & Belvisi, 2012). Otherwise, ligand-activated GR can inhibit gene transcription via binding to sequences overlapping the TATA box and therefore interfering with the initiation of transcription, as described for the GC-mediated repression of the osteocalcin gene (Strömstedt et al., 1991; Beck et al., 2009). An important negative regulatory mechanism of GCs is the indirect recruitment of ligand-activated GR to DNA via protein-protein interaction with other DNA-bound TFs, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (Figure 2). As tethering of GR to AP-1 and NF-κB inhibit their ability to induce the expression of many pro-inflammatory cytokines, enzymes and adhesion molecules, this TR mechanism is widely considered to be one key mechanism for the anti-inflammatory effects of GCs (Beck et al., 2009; Flammer & Rogatsky, 2011; Clark & Belvisi, 2012). Mutation analysis revealed that the repression of AP-1- and NF-κB-regulated genes requires the DBD of the GR but is not

dependent on direct DNA binding or dimerization of the GR (Coutinho & Chapman, 2011; Clark & Belvisi, 2012).

Beside the inhibitory effect of GCs on gene transcription, the ligand-activated GR can decrease the stability of mRNA including those for IL-6 and IL-8 via enhanced transcription of specific mRNA destabilizing proteins that break down sequences functionally associated with mRNA turnover and translation (Stellato, 2004).

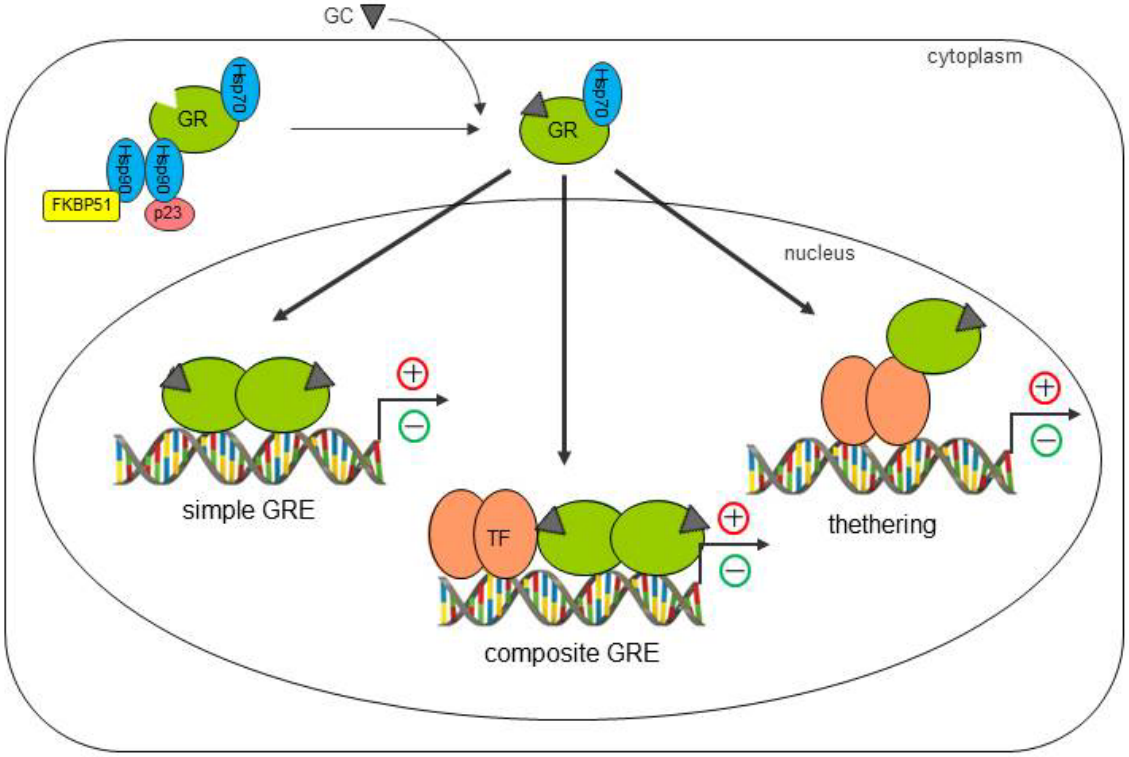


Figure 2: Molecular mechanism of genomic glucocorticoid actions.
Glucocorticoids (GCs) passively diffuse into the cell and bind to the cytoplasmic glucocorticoid receptor (GR), which is complexed with chaperones, such as heat shock protein 90 (Hsp90) and Hsp70, and with FK506-binding immunophilins like FKBP51. The ligand-activated GR translocates into the nucleus and stimulates or inhibits transcription of GC-sensitive genes. At simple glucocorticoid response elements (GREs), dimeric GR binds as sole sequence-specific protein to DNA directly. At composite GREs, the GR binds in a cooperative manner with other transcription factors (TFs) to the DNA. Otherwise, monomeric GR can interact with other TFs without direct DNA-binding via a tethering mechanism. At composite and tethering GREs, GR can function as a monomer (modified from Clark & Belvisi, 2012).

1.1.3 Nongenomic mode of glucocorticoid action

Cellular responses that occur very rapidly within minutes or even seconds after GC exposure are not regulated at the transcriptional level. It is assumed that such nongenomic GC effects can be mediated by signaling through a membrane GR, direct indirections of GCs with cellular membranes, mitochondrial GR translocation or by interaction of the GR with other signaling proteins in the cytoplasm (Boldizsar et al., 2010; Strehl et al., 2011).

Interactions of the GR with the T cell receptor (TCR) have been described in primary T cells and in a leukemia T cell line by two groups.
In TCR-activated human CD4+ T cells short-term treatment with dexamethasone inhibited the activity of two tyrosine kinases, which are essential in initiating TCR signaling, and thus led to reduced enzymatic activity of the lymphocyte-specific protein tyrosine kinase (LCK) and the Fyn oncogene related to Src, Fgr, Yes (FYN) kinase. Consequently, the phosphorylation of molecules downstream of TCR such as protein kinase B, protein kinase C, and mitogen-activated protein kinases (MAPKs) are suppressed (Löwenberg et al., 2005; Löwenberg et al., 2008). It was shown, that the GR is linked with the TCR in a multiprotein complex containing Hsp90, LCK and FYN and that GR ligation disrupted the TCR-linked GR complex and thereby abrogated LCK/FYN activation resulting in impaired TCR signaling (Löwenberg et al., 2006).
In dexamethasone-treated Jurkat T cells a direct interaction of the GR with ZAP-70 (CD3 ζ-chain associated protein of 70 kDa), a key molecule in the early stage of T cell activation, has been described. High-dose dexamethasone treatment induced dissociation of the activated GR from a multi-molecular complex with ZAP-70, LCK and Hsp90 molecules. The activated GR then associated with ZAP-70 and thus led to the transient phosphorylation of ZAP-70 (Boldizsar et al., 2010).

Furthermore, nongenomic GC actions on intracellular Ca^{2+} mobilization have been reported. Experiments with membrane-impermeable bovine serum albumin-conjugated corticosterone suggested that the inhibitory effect of GCs on Ca^{2+}-influx induced by bradykinin in excitable PC12 cells is mediated via a putative membrane-bound GR (Qui et al., 2003).

1.1.4 Non-steroidal selective glucocorticoid receptor agonists (SEGRA)

It is widely accepted that the anti-inflammatory and immunosuppressive activity of GCs is predominantly mediated by inhibition of the transcriptional activity of pro-inflammatory TFs by the monomeric ligand-activated GR (Flammer & Rogatsky, 2011; Clark & Belvisi, 2012). In contrast, induction of gene transcription by the dimerized ligand-activated GR seems to account for certain side-effects of GC-therapy such as thymus involution and metabolic disturbances (Schäcke et al., 2002; Schäcke et al., 2008).

Studies with GR mutants have shown that TA and TR activities by the ligand-activated GR can be separated. A point-mutation in the D-loop of the DBD impairs DNA binding of the GR and therefore the transcriptional activation of GRE-containing promoters, but does not affect the DNA-binding independent inhibition of AP-1- or NF-κB-activity *in vitro* and *in vivo* (Heck et al., 1994; Reichardt et al., 1998). In mice with such a dimerization-deficient GR (GR dim/dim mice), an efficient suppression of phorbol ester-induced irritant contact dermatitis by dexamethasone comparable to the inhibition seen in wild-type mice was observed (Reichardt et al., 2001). Inflammatory mediators, such as TNFα were efficiently suppressed, whereas the expression of genes involved in the gluconeogenesis were not induced by GCs in the GR dim/dim mice. These results indicate a sufficient anti-inflammatory effect of GCs via a DNA-binding independent mechanism of the GR, such as tethering (Kleiman & Tuckermann, 2007).

The finding that it is possible to dissociate TR from TA activities whilst maintaining anti-inflammatory activity has led to the identification of new GR ligands, which mainly induce the TR mechanism. These dissociated GR ligands should have a potent anti-inflammatory efficacy but a reduced side-effect profile in comparison to classical GCs (Schäcke et al., 2007; Löwenberg et al., 2008; De Bosscher et al., 2010; Barnes, 2011). Non-steroidal selective glucocorticoid receptor agonists (SEGRAs) represent a new class of GR ligands which should exhibit an improved therapeutic effect/side-effect profile by sustained TR but lower TA activity than classical GCs (Figure 3).

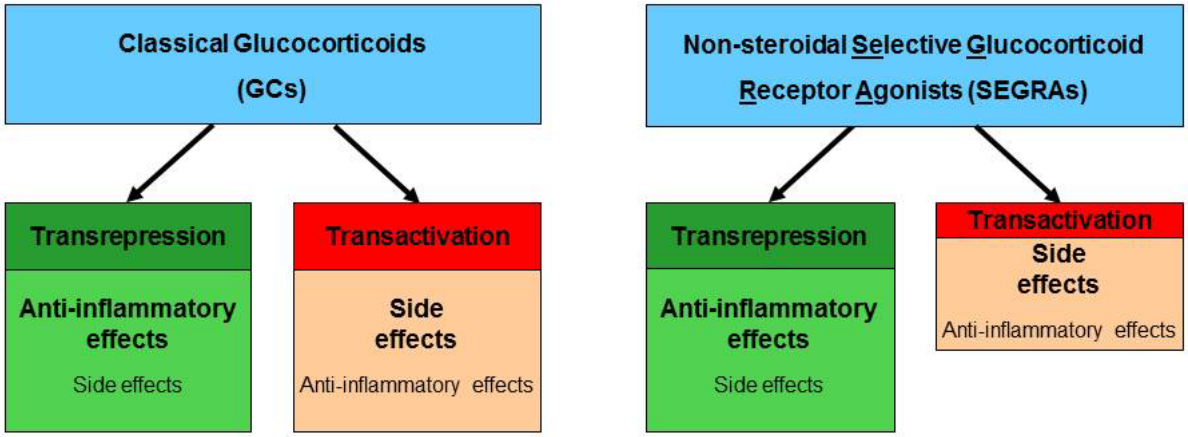


Figure 3: Differential genomic mechanism of glucocorticoid and SEGRA action.

Classical GCs can mediate their biological effects via induction or repression of the transcription of GC-sensitive genes by the ligand-activated GR. The DNA binding-independent transrepression is considered to be the key mechanism for their anti-inflammatory activity, whereas some side-effects are mainly mediated via transactivation. Non-steroidal selective glucocorticoid receptor agonists (SEGRAs) should exhibit potent anti-inflammatory efficacy but less transactivation activity.

1.1.4.1 BAY1155975 and its pharmacological profile

In this study, the suppressive activity of the orally bioavailable SEGRA, BAY1155975, on T cell activation was compared to the classical GC, prednisolone. As assessed by receptor binding assays, BAY1155975 is highly selective for the human GR. It binds with high affinity to the GR but not to other nuclear receptors such as the progesterone, androgen and mineralocorticoid receptors (MR) (unpublished results). In contrast, prednisolone has high affinity to the GR but also to the MR (Juruena et al., 2006). The dissociation profile of BAY1155975 was determined in promoter assays for TA and TR activities *in vitro*. In the TA assay, the transcriptional activity of the compound at the mouse mammary tumor virus (MMTV) promoter, which contains several GREs, was analyzed (Le Ricousse et al., 1996). In the TR assay, suppression of collagenase promoter activity, which is mediated via a DNA binding-independent mechanism of the GR, was determined (König et al., 1992). BAY1155975 shows a dissociated molecular profile with sustained TR but less TA activity than prednisolone (unpublished results).

Furthermore, a dissociated regulation of the protein expression by BAY1155975 was observed in human whole blood cultures. As marker for TA activity, the induced expression of CD163, the specific haemoglobin-haptoglobin scavenger receptor, was investigated. It was shown, that the promoter region of the CD163 gene contains multiple GREs (Schaer et al., 2002). TR activity was assessed by repression of the surface expression of human leukocyte antigen DR (HLA-DR), a MHC class II molecule (Schwiebert et al., 1995; He et al., 2010). The downregulation of the HLA-DR surface expression by GCs is also a parameter for their anti-inflammatory and immunomodulatory effect, as in monocytes HLA-DR expression correlates directly with their capacity for antigen presentation (Reinke et al., 2002). BAY1155975 significantly less induced the surface expression of CD163 on monocytes compared to prednisolone indicating a lower TA activity. In contrast, BAY1155975 shows a similar suppression of HLA-DR expression in monocytes and B cells than prednisolone (unpublished results).

1.2 T cell activation

1.2.1 T cell development and function

T cell generation and differentiation occurs in the thymus. T cell progenitors migrate from the primary sites of haematopoiesis into the thymus and intensively proliferate in the thymic outer cortex. At the CD4+CD8+ double-positive (DP) stage, a functional TCR is created by random recombination of gene segments encoding the antigen-recognizing variable domains of the TCR α- and β-chains (as well as γ- and δ-chains for $\gamma\delta$ T cells). Upon positive selection by interaction with either MHC class I or MHC class II molecules, DP cells differentiate to CD4+ or

CD8+ single-positive thymocytes and migrate to the medulla. After final functional maturation, naïve CD4+ or CD8+ T cells leave the thymus, enter the periphery and continually circulate through secondary lymphoid organs such as the spleen and peripheral lymph nodes (Weinreich & Hogquist, 2008; Wang et al., 2012).

Upon engagement of the TCR by the appropriate peptide-MHC complex, naïve CD4+ and CD8+ T cells undergo pronounced clonal expansion and differentiate into short-lived effector or long-lived memory cells, whereas the mode of differentiation is not clearly defined (Farber, 2009).

Naive conventional CD4+ T cells can differentiate into at least 4 distinct effector subsets, T helper type 1 (Th1) cells, Th2 cells, Th17 cells and induced regulatory T cells (iTreg), according to the cytokine milieu provided by DCs, antigen dose, co-stimulators, genetic modifiers and other non-cytokine factors (Zhu & Paul, 2008). Th1 cells mainly produce TNFβ and IFNγ, which activates macrophages to increase their microbicidal activity, and thus Th1 cells mediate immune responses against intracellular pathogens. Th2 cells are required in host defense against extracellular parasites via IL-4-mediated immunoglobulin E (IgE) class switch in B cells and eosinophil recruitment by IL-5 production. Th17 cells produce IL-17A, IL-17F and IL-22 and play a role in immune responses against extracellular bacteria and fungi. Treg cells are responsible for maintaining self-tolerance as well as regulating effector T cell responses by secretion of transforming growth factor beta (TGFβ) and IL-10 (Zhou et al., 2009; Zhu & Paul, 2010). In contrast to CD4+ T cells, CD8+ T cells more readily develop into effector cells with cytokine-producing and/or cytolytic capacity after short-term primary stimulation. CD8+ T cells mediate their effector functions through production of cytokines such as IFNγ and TNFα, by secretion of perforin and granzyme and by death receptor activation (Fas, tumor necrosis factor-related apoptosis-inducing ligand), which induce apoptosis of virus-infected or transformed cells cooperatively (Obar & Lefrançois, 2010).

Memory CD4+ and CD8+ T cells confer immediate protection and mount, upon re-encounter with antigen, a more rapid and effective secondary immune response. According to their differential expression of lymphoid homing receptors, such as CCR7, memory cells can be broadly divided into central memory T cells (T_{cm} cells, CCR7+ memory T cells) and effector memory T cells (T_{em} cells, CCR7- memory T cells). T_{cm} cells migrate to secondary lymphoid organs and peripheral sites of inflammation, where they readily proliferate and develop an effector phenotype upon re-encounter with the specific antigen. In contrast, T_{em} cells display immediate effector functions by secreting high amount of IFNγ and are predominantly found in non-lymphoid tissues, but have limited proliferative capacity. Both subsets of memory cells are

present in the blood and spleen (Kallies, 2008; Sallusto & Lanzavecchia, 2009; Arens & Schoenberger, 2010).

1.2.2 Immunophenotypic markers for CD4+ T cell differentiation and activation

Upon first contact with an antigen, naïve (unprimed) T cells clonally expand and differentiate to memory/effector T cells. Both T cell populations differ in their immunophenotypic profiles as well as functionality (Farber, 2009).

Naïve CD4+ T cells express the CD45RA isoform of the surface molecule CD45, the chemokine receptor CCR7 and the costimulatory molecules CD27 and CD28 (Okada et al., 2008; Rabe et al., 2011).
As CD45 shortens in length following CD4+ T cell activation, all memory CD4+ T cells express the shorter isoform CD45RO. Based on the expression of lymphoid homing receptors the memory pool can be further divided. Central memory CD4+ T cells express the chemokine receptor CCR7 and CD62 ligand, whereas effector memory CD4+ T cells have lost the expression of CCR7 (Sallusto et al., 1999; Pepper et al., 2011). Also the expression of the costimulatory molecule CD27 separates the memory pool. A small population of memory CD4+ T cells lack surface CD27 expression. These cells may represent the more differentiated cell subpopulation secreting higher levels of effector cytokines. The CD27+ CD4+ memory T cells, however, appear to be of a more resting phenotype unable to proliferate to TCR triggering without costimulation (Hintzen et al., 1993; Schiött et al., 2004).

CD28 is constitutively expressed on almost all naïve as well as primed CD4+ T cells, whereas only 50 – 80 % of all CD8+ T cells express CD28 (Beier et al., 2007). However, in patients with chronic immunological diseases like autoimmune syndromes or persistent inflammation-provoking infections an abnormally large proportion of peripheral CD4+ T cells lacks CD28 expression. These CD4+ CD28- T cells produce increased levels of cytotoxic mediators and proinflammatory cytokines (Gilani et al., 2010). Furthermore, an accumulation of CD28- T cells with age is observed in the CD4+ T cell population and to a greater extent in the CD8+ T cell population. The loss of CD28 expression with age has been attributed to repeated antigenic stimulation and is associated with the reduced overall immune response to pathogens and vaccines in the elderly (Weng et al., 2009).

As CD4+ T cells become activated several antigens are expressed on their cell membrane. At the onset of activation CD69 is *de novo* expressed on the surface of T cells. Another activation marker is CD25, the α-chain of the IL-2 receptor, which associates with the β- and γ-chains to the high affinity IL-2 receptor (Farber, 2009). During the late phase of activation HLA-DR, a MHC class II molecule, is *de novo* expressed on CD4+ T cells (Starska et al., 2011). As for

CD69, the function of HLA-DR on activated CD4+ T cells is currently unclear (Salgado et al., 2002). As engagement of the TCR by the appropriate peptide-MHC complex induces the expression of CD40L (CD154), CD40L can be used to assess antigen-specific CD4+ T cells (Frentsch et al., 2005; Meier et al., 2008).

1.2.3 TCR signaling in activated T cells

Optimal T cell activation requires the engagement of the TCR by the appropriate peptide-MHC-complex and costimulatory signals as the interaction of B7 family members on antigen-presenting cells with CD28 on T cells (Rudd & Schneider, 2003; Smith-Garvin et al., 2009).

The TCR of conventional $\alpha\beta$ T cells consist of α- and β-chains, which are noncovalently associated with the γ-, δ-, ε- and ζ-chains of the CD3 protein complex. Following TCR engagement, a conformational change is induced within the CD3 cytoplasmic tails, which brings the two tyrosine kinases, LCK (associated with the co-receptors CD4 or CD8) and FYN (membrane-associated), into proximity of the TCR/CD3 complex. This leads to phosphorylation of immunoreceptor tyrosine-based activation motifs on the conformationally accessible CD3 ζ-chains and subsequent recruitment and activation of ZAP70. Phosphorylation of adaptor proteins by ZAP70 results in activation of downstream target molecules, including phospholipase C-γ1 (PLC-γ1) and phosphoinositide 3 kinase (PI3K). Activated PLC-γ1 hydrolyzes the membrane lipid phosphate-idylinositol-4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to the IP_3 receptor located on the membrane of the endoplasmic reticulum (ER) and triggers the release of calcium (Ca^{2+}) from ER stores. L-type voltage-dependent-like Ca^{2+} (Ca_v1) channels are activated by an undefined mechanism after TCR stimulation and may contribute to the initial increase in intracellular Ca^{2+} concentration. The depletion of ER Ca^{2+} stores activates Ca^{2+} release-activated Ca^{2+} channels in the plasma membrane, which permit sustained Ca^{2+} influx into the cell. Prolonged Ca^{2+} elevation activates the protein phosphatase calcineurin, which is responsible for dephosphorylating members of the nuclear factor of activated T cells (NFAT) family. Dephosphorylated NFAT isoforms translocate into the nucleus and can cooperate with other TFs to induce gene transcription. Furthermore, DAG production activates two additional pathways involving Ras and protein kinase Cθ (PKCθ). Ras promotes phosphorylation and activation of MAPK kinases, which in turn phosphorylate and activate the MAPK's extracellular signal-regulated kinase 1 (ERK1) and ERK2. ERK activity subsequently promotes the transcriptional activation of AP-1. The nuclear translocation and transcriptional activation of another TF, NF-κB, is also promoted by PKCθ activation (Winslow & Crabtree, 2005; Oh-hora & Rao, 2008; Smith-Garvin et al., 2009; Song et al., 2010).

TCR engagement in the absence of a costimulatory signal results in either apoptosis or in a non-responsive state called anergy, in which T cells are unable to secrete IL-2 or to proliferate on subsequent stimulation, even in the presence of costimulation (Sadegh-Nasseri et al., 2010). Costimulation by CD28, the primary costimulatory molecule for naïve T cells, can reduce the number of TCRs that must be ligated for a T cell response and thereby lowers the activation threshold. Cross-linking of CD28 induces phosphorylation of its cytoplasmic tail allowing interaction with PI3K and thus activation of the Akt kinase (also called protein kinase B). Activated Akt promotes the expression of prosurvival genes including Bcl-xl by enhanced transcriptional activity of NF-κB and inhibits TFs that promote cell cycle arrest (Rudd et al., 2009; Janardhan et al., 2011). Moreover, Akt augments the transcription of NFAT-regulated genes including IL-2 likely via inhibition of glycogen synthase kinase 3, a kinase that promotes nuclear export of NFAT (Beurel et al., 2010). Akt activated by CD28 costimulation stabilizes the IL-2 mRNA by promoting the translocation of an AU-rich element binding protein to the cytoplasm (Crispín & Tsokos, 2009). CD28 ligation also enhances the calcium flux and the cellular metabolism (Beck et al., 2009; Janardhan et al., 2011).

1.2.4 CD28 costimulation-mediated T cell resistance to glucocorticoids

T cell activation mediated via cross-linking of the TCR/CD3 complex with anti-CD3 antibodies alone is effectively suppressed by GCs *in vitro* and *in vivo* (Kunicka et al., 1993; Talayev et al., 2005; Winiski et al., 2007). GCs impair T cell cytokine production and proliferation via inhibiting the transcriptional activity of AP-1, NF-κB or NFAT (see section 1.1.2). In patients with organ transplants, the cytokine release observed after acute rejection therapy with the murine-depleting monoclonal anti-CD3 antibody, OKT3, can be anticipated by GC application before OKT3 infusion (Hirose & Vincenti, 2006; Bhorade & Stern, 2009). Furthermore, a nongenomic mode of GC action by suppressing the early signaling events initiated upon TCR ligation, such as LCK/FYN activation has been described (see section 1.1.3).

By additional CD28 costimulation the suppressive effect of GC treatment on T cell proliferation is abrogated *in vitro* (Nijhuis et al., 1994; Winiski et al., 2007). It was shown, that CD28 costimulation enhances the ERK signaling and thus leads to a higher degree of AP-1 formation and activation, which cannot be suppressed by dexamethasone in primary human T cells. However, the suppressive effect of dexamethasone on NF-κB and NFAT activation was not abolished by CD28 costimulation (Li et al., 2004; Tsitoura & Rothman, 2004). CD28 costimulation also abrogates the inhibitory effect of GCs on pro-inflammatory cytokine production in activated T cells, as the dexamethasone-mediated suppression of IFNγ production is attenuated by CD28 ligation in human peripheral blood mononuclear cells (PBMCs) (Agarwal & Marshall, 2000).

Furthermore it has been shown that CD28 costimulation also regulates GC action in T cell development in the thymus as well as in Th cell subset differentiation in the periphery (Ashwell et al, 2000). CD28 signaling enhances the anti-CD3-mediated rescue of GC-induced apoptosis in single positive thymocytes *in vitro* (van den Brandt et al., 2004; Erlacher et al., 2005).

1.3 T cell-dependent skin inflammation in contact hypersensitivity models in mice

1.3.1 Development of the immune response in contact hypersensitivity

Experimental contact hypersensitivity (CHS), a commonly used animal model of human allergic contact dermatitis, is a T cell-dependent inflammatory response in the skin to reactive haptens. Two temporally dissociated phases, i.e. the sensitization and the elicitation phase, are necessary to develop an optimal CHS response (Christensen & Haase, 2012; Röse et al., 2012).

The sensitization phase is initiated by the first contact of the skin with the hapten, which binds covalently to specific amino acids of skin proteins and activates the skin's innate immunity including release of inflammatory mediators from resident skin cells. Haptenated proteins are engulfed and processed to antigenic peptides by resident skin DCs, which then mature and migrate to the draining lymph nodes. By presentation of the antigenic peptides in the lymph nodes, hapten-specific effector T cells, which mainly polarize to type 1 T cells, become activated and differentiate into T_{cm} and T_{em} cells. Activated T cells then emigrate and circulate between the lymphoid organs and the skin. Additionally, B-1 cells in the spleen become activated and release hapten-specific IgM antibodies.

In the elicitation phase, re-exposure with the same hapten (challenge) leads to activation of the skin innate immune system and the presentation of antigenic peptides by DCs as described above. Hapten-specific IgM antibodies locally activate complement and thus promote the release of inflammatory and chemotactic factors from mast cells and platelets. Based on this chemokine release, a first wave of blood leukocytes, including hapten-specific effector CD8+ T cells, which are the main effector cells of CHS, are recruited to the skin. Activation of these hapten-specific CD8+ T cells via haptenized DCs in the skin induces the release of type 1 cytokines such as IFNγ and TNFα, which stimulate the secretion of cytokines and chemokines from skin cells including mast cells. This cytokine and chemokine production shapes the inflammatory response and leads to the influx of a second wave of leukocytes containing neutrophils, natural killer cells, Treg cells and monocytes. The inflammatory response, characterized by eczematous lesions, appears within 24-48 hours after challenge (Gober & Gaspari, 2008; Vocanson et al., 2009). Progressive resolution of eczematous lesions

occurs via activation of CD4+ CD25+ regulatory T (Treg) cells (Ring et al., 2009; Cavani, 2008). Recently, a population of CD4+ CD25+ Treg cells, which selectively expresses forkhead box protein 3 (FoxP3), cytotoxic T-lymphocyte antigen 4 (CTLA-4), IL-10, IL-17 and IFNγ, has been shown to upregulate the expression of inducible costimulator (ICOS) on sensitization and specifically suppress hapten-reactive CD8+ effector T cells in the CHS response to DNFB (Vocanson et al., 2010). The suppressive effect of Treg cells is suggested to occur later than 24 hours after challenge as neither CD25 nor CTLA-4 were upregulated in the skin or draining lymph nodes within 24 hours after challenge (Hartmann et al., 2006).

The magnitude of the reaction is primarily determined by the amount of hapten and the number of antigen-presenting-DCs in the lymph nodes during sensitization. Unlike the classical paradigm for LCs to potently initiate CHS responses, recent findings suggest a functional redundancy for a particular skin DC subset in the induction of the CHS (Clausen & Kel, 2010).

1.3.2 Role of CD28-costimulation and Ca2+ signaling in contact hypersensitivity

Interactions of CD28, which is constitutively expressed on naïve T cells, with members of the B7 family, i.e. CD80 and CD86, expressed on dendritic cells (DCs) are important for the development of the CHS response. Mice deficient in the CD28 molecule show impaired CHS response to the contact haptens, 2,4-dinitro-1-fluorobenzene (DNFB) and oxazolone (OX). This significant reduction of skin inflammation is associated with a diminished adhesion of T cells to epidermal cells, a reduced cell infiltration and a decreased up-regulation of IL-2 mRNA expression in the skin at 48 hours after DNFB challenge. An impaired activation and recruitment of T cells to the skin in CD28-deficient mice was suggested (Kondo et al., 1996). Administration of anti-CD80 blocking antibody during sensitization in mice increased the number of Th2 cells primed during hapten sensitization, whereas administration of anti-CD86 blocking antibody inhibited CD4+ and CD8+ T cell development. Therefore, it was suggested that CD86 on DCs is required for costimulation of CD4+ and CD8+ T cell subpopulations and thus for an optimal initiation of the CHS response (Xu et al., 1997). However, in CD80/CD86 double knockout mice, the diminished CHS response to standard OX doses was largely overcome at higher OX doses indicating the presence of compensatory pathways (Rauschmayr-Kopp et al., 1997; Wang et al., 2001).

Administration of the calcineurin inhibitors, pimecrolimus, tacrolimus or cyclosporine A, before and after hapten challenge inhibited the CHS response to OX. The CHS response was also suppressed in mice passively sensitized by transfer of lymph node cells from animals treated with tacrolimus or cyclosporine A during sensitization (Meingassner et al., 2003; Bavandi et al., 2006). Furthermore, a significant reduction of the 2,4-dinitro-1-chlorobenzene-induced ear inflammation was observed in mice treated with the Ca_v1 channel antagonists, nifedipine or

verapamil, before sensitization as well as before hapten challenge (Katoh et al., 1997; Wille et al., 1999). These results suggested that the Ca^{2+}-signaling is important for both, sensitization and elicitation of the CHS response.

2. Aim of this study

The goal of this study was to analyze the inhibitory effect of the SEGRA, BAY1155975, compared to classical GCs on CD28-costimulated activation of human primary T cell subpopulations. Several reports demonstrate that the suppressive effect of classical GCs on proliferation as well as on pro-inflammatory cytokine production is abrogated by CD28 costimulation in activated T cells (Agarwal & Marshall, 2000; Tsitoura & Rothman, 2004; Winiski et al., 2007).

In this study, the inhibitory effect of BAY1155975 on proliferation, cytokine secretion, apoptosis and activation status of human primary T cell subpopulations stimulated with plate-bound anti-CD3 and anti-CD28 monoclonal antibodies was determined *in vitro.* Different mechanisms that might account for the inhibitory effect of BAY1155975 on CD28-costimulated T cell activation were analyzed, i.e.

→ the influence of glucocorticoid receptor signaling
→ MEK/ERK signaling
→ and Ca^{2+} signaling.

The suppressive effect of BAY1155975 and prednisolone on CD28-costimulated T cell activation was further compared *in vivo.* In DNFB-induced contact hypersensitivity (CHS), costimulation via CD28 is essential for T cell priming during the sensitization phase. The inhibitory activity of BAY1155975 versus prednisolone on CD28-costimulated T cell activation was examined by applying both compounds around sensitization in DNFB-induced CHS models in mice. After hapten challenge, T cell-dependent skin inflammation and cytokine production were analyzed.

The following topics have been addressed:

1) Analysis of the suppressive effect of BAY1155975 compared to prednisolone on CD28-costimulated activation of human primary T cells *in vitro*
2) Characterization of the T cell subpopulations in which the differential effects of BAY1155975 versus prednisolone are dominant
3) Characterization of the differential mechanism for the suppressive effect of BAY1155975 compared to prednisolone on CD28-costimulated activation of human T cells
4) Analysis of the suppressive effect of BAY1155975 vs. prednisolone treatment around sensitization on T cell-dependent skin inflammation in DNFB-induced CHS models in mice

3. Materials and Methods

3.1 Materials

3.1.1 Blood samples

Venous blood from healthy donors was collected in citrate S-Monovette® tubes (S-Monovette Citrate, Sarstedt, Nürnbrecht, Germany) at the Department of Clinical Pharmacology, Bayer Pharma AG (Berlin, Germany). All blood samples were used with informed consent of the donors in accordance with the ethical guidelines of Bayer Pharma AG.

3.1.2 Mice

Female NMRI mice (22-26 g) were purchased from Charles River (Berlin, Germany) and were maintained with access to food and water *ad libitum*. Seven to eight mice were randomly assigned to the different treatment groups. All mouse studies were approved by the competent authority for labor protection, occupational health, and technical safety for the state and city of Berlin, Germany, and were performed in accordance with the ethical guidelines of Bayer Pharma AG.

3.1.3 Substances

BAY1155975 was synthesized at the department Medicinal Chemistry, Bayer Pharma AG. Dexamethasone, prednisolone, RU-486 (mifepristone) and cyclosporine A were purchased from Sigma (Munich, Germany). The MEK/ERK inhibitor U0126 and the Ca_v1 channel antagonist nifedipine were purchased from Calbiochem (Bad Soden, Germany).

For *in vitro* experiments BAY1155975, dexamethasone, prednisolone, RU-486 and U0126 were prepared as stock solutions of 10 mM in dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany). Nifedipine was prepared as stock solution of 100 mM in DMSO and cyclosporine A was prepared as stock solution of 1 mM in DMSO.

For *in vivo* experiments 2,4-dinitro-1-fluorobenzene (DNFB, Sigma) was dissolved in a 4:1 mixture of acetone (Merck) and olive oil (Sigma). Compounds were freshly dissolved in peanut oil (Sigma) containing 3% DMSO and 5% ethanol (Merck) every day.

3.1.4 Buffers and media

3.1.4.1 MACS buffer

- Phosphate-buffered saline, w/o Ca^{2+} and Mg^{2+} (PBS, Gibco, Karlsruhe, Germany)
- 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma, Munich, Germany)
- 0.5% fetal calf serum (FCS, heat-inactivated, Gibco)

3.1.4.2 Staining buffer for flow cytometry

- PBS, w/o Ca^{2+} and Mg^{2+} (Gibco)
- 2% FCS (Gibco)
- 0.1% sodium acide (Sigma, Munich, Germany)

3.1.4.3 Washing buffer MesoScale

- PBS, w/o Ca^{2+} and Mg^{2+} (Gibco)
- 0.05% Tween 20 (Amersham, Freiburg, Germany)

3.1.4.4 Read buffer MesoScale

- MSD Read Buffer T (MesoScale Discovery, Gaithersburg, USA)
- 1:2 diluted with distilled water

3.1.4.5 Lysing buffer for RNA isolation

- RLT-Buffer (QIAGEN, Hilden, Germany)
- 1% ß-Mercaptoethanol (Merck, Darmstadt, Germany)

3.1.4.6 Lysing buffer for Western Blot

- 7 mL M-Per lysing buffer (Thermo Fisher Scientific, Schwerte, Germany)
- 1 complete protease inhibitor cocktail tablet (Roche Pharma AG, Grenzach-Wyhlen, Germany)
- 1 µL benzoase (Sigma)

3.1.4.7 Sample buffer for gel electrophoresis

- 100 mM Tris (hydroxy methyl) aminomethane (Tris, pH 6.8, Sigma)
- 3% Sodium Dodecyl Sulfate (SDS, Merck)
- 10% Sucrose (Merck)
- 0.03% Bromophenol blue (Merck)
- 50 mM Dithiothreitol (DTT, Sigma)

3.1.4.8 Running buffer for gel electrophoresis

- 70 mM Tris (pH 8.3, Sigma)
- 100 mM HEPES (Sigma)
- 0.1% SDS (Merck)

3.1.4.9 Transfer puffer for Western Blot

- 37 mM Tris (pH 8.3, Sigma)
- 39 mM Glycine (Merck)
- 0.038% SDS (Merck)
- 20% Methanol (Merck)

3.1.4.10 TBS-T buffer

- 100 mM Tris (pH 7.5, Sigma)
- 150 mM NaCl (Merck)
- 0.1% Tween 20 (Amersham)

3.1.4.11 Blocking solution

- 5% milk powder (Merck)
- 1% bovine serum albumin (BSA, Merck)
- dissolved in 0.1% TBS-T buffer

3.1.4.12 Homogenizing buffer

- 0.5% hexadecyltrimethylammonium bromide (Sigma)
- 10 mM 4-morpholinepropanesulfonic acid (Sigma)
- dissolved in 900 mL distilled water
- titrated to pH 7 with 1 N NaOH (Merck)
- filled up with distilled water to 1000 mL

3.1.4.13 Sodium acetate-citric acid buffer

- 0.1 mol/L sodium acetate (Sigma) dissolved in distilled water
- titrated to pH 6 with 0.1 mol/L citric acid-1-hydrate (Sigma)

3.1.4.14 PBMC culture medium

- VLE RPE RPMI 1640 medium (Biochrom)
- 4 mM GlutaMax I (Gibco)
- 10% FCS (Gibco)
- 50 U/ 50 µg/mL penicillin/ streptomycin (Gibco)

3.1.4.15 T cell culture medium

- VLE RPE RPMI 1640 medium (Biochrom, Berlin, Germany)
- 10% FCS (Gibco)
- 50 U/ 50 µg/mL penicillin/ streptomycin (Gibco)

3.1.5 Chemicals and materials

3.1.5.1 Cell culture

Name	**Manufacturer**
Anti-human CD28, clone: 37407	R&D Systems, Wiesbaden-Nordenstadt, Germany
Anti-human CD3ε, clone: UCHT1	R&D Systems, Wiesbaden-Nordenstadt, Germany
Biocoll separating solution, density 1.077 g/mL	Biochrom, Berlin, Germany
CD28 MicroBead Kit, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
CD4+ T cell Isolation Kit II, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
CD45 RA MicroBeads, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
CD45 RO MicroBeads, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
CD8+ T cell Isolation Kit II, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
Conical centrifuge tubes, 14 mL, 50 mL	Fisher Scientific, Schwerte, Germany
Culture plates, flat bottom, 12-well, 96-well Culture plates, round bottom, 96-well Cell culture flasks, surface area 75 cm^2	Corning, Schwerte, Germany
Custom human high bind 4 Spot plates - IFNγ, IL-2 + IL-4 - TNFα + IL-12p40	MesoScale Discovery, Gaithersburg, USA
Human IL-22 ELISA	Bender MedSystems, Vienna, Austria
Human T cell Nucleofector Kit	Amaxa, Köln, Germany
Ionomycin	Sigma, Munich, Germany
Leucosep™ tubes	Greiner Bio-One, Frickenhausen, Germany
Lipopolysaccharide (LPS) from *E. coli*, 0127:B8	Sigma, Munich, Germany
MACS® columns, LS, LD	Miltenyi Biotec, Bergisch-Gladbach, Germany
Methyl-^{3}H-thymidine, specific activity: 54 Ci/mmol	Amersham, Freiburg, Germany

Microcentrifuge tube 0.5 mL, 1.5 mL, 2 mL	Eppendorf, Hamburg, Germany
Mitomycin C from *Streptomyces caespitosus*	Sigma, Munich, Germany
ON-TARGET*plus* Non-Targeting siRNA #1 ON-TARGET*plus* SMARTpool for GR	Dharmacon, Schwerte, Germany
Pan T cell Isolation Kit II, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma, Munich, Germany
Quantikine® Human IL-12p40 Immunoassay	R&D Systems, Wiesbaden-Nordenstadt, Germany
Scintillator, Pico-Fluor 15	PerkinElmer, Rodgau–Jügesheim, Germany
Tetanus toxoid (0.08 IU/mL)	SmithKline Beecham Pharma, Dresden, Germany
TNFα Human Direct ELISA Kit	BioSource, Karlsruhe, Germany
Trypan blue stain, 0.4%	Gibco, Karlsruhe, Germany
Ultrasensitive Kit IL-17 human	MesoScale Discovery, Gaithersburg, USA
UniFilter-96-well plates	PerkinElmer, Rodgau–Jügesheim, Germany

3.1.5.2 Flow cytometry

Name	**Manufacturer**
96-well assay plates, U-bottom	BD, Heidelberg, Germany
ApoAlert® Annexin V Apoptosis Kit	Clontech, Potsdam, Germany
Brefeldin A from *Penicillium brefeldianum*	Sigma, Munich, Germany
Cytoperm/Cytofix™ Fixation/Permeabilization Kit	BD Biosciences, Heidelberg, Germany
Micronic tubes, 1.4 mL	Fisher Scientific, Schwerte, Germany
Tubes, 12 x 75 mm	BD Biosciences, Heidelberg, Germany

3.1.5.3 Monoclonal antibodies

Name	**Isotype**	**Clone**	**Manufacturer**
CD27 FITC	Mouse IgG1	L128	BD Biosciences, Heidelberg, Germany
CD45RA FITC	Mouse IgG1	L48	BD Pharmingen, Heidelberg, Germany
CD40L FITC	Mouse IgG1	TRAP1	BD Pharmingen, Heidelberg, Germany
IgG1 FITC	Mouse IgG1	MOPC-31C	BD Pharmingen, Heidelberg, Germany
CD25 PE	Mouse IgG1	M-A251	BD Biosciences, Heidelberg, Germany
CD28 PE	Mouse IgG1	CD28.2	Immunotech, Krefeld, Germany
CD45RO PE	Mouse IgG2a	UCHL1	BD Biosciences, Heidelberg, Germany
CD69 APC	Mouse IgG1	L78	BD Biosciences, Heidelberg, Germany
IFNγ PE	Mouse IgG2b	25723.11	BD Biosciences, Heidelberg, Germany
IgG1 PE	Mouse IgG1	MOPC-21C	BD Biosciences, Heidelberg, Germany
IgG2b PE	Mouse IgG2b	27-35	BD Pharmingen, Heidelberg, Germany
CD3 PerCP-Cy5.5	Mouse IgG1	SP34-2	BD Pharmingen, Heidelberg, Germany
HLA-DR PerCP-Cy5.5	Mouse IgG2a	L243	BD Biosciences, Heidelberg, Germany

CD4 APC	Mouse IgG1	SK3	BD Biosciences, Heidelberg, Germany
CD28 APC	Mouse IgG1	15E8	Miltenyi Biotec, Bergisch-Gladbach, Ger.
TNFα PE-Cy7	Mouse IgG1	Mab11	eBioscience, Frankfurt, Germany
IgG1 PE-Cy7	Mouse IgG1	-	eBioscience, Frankfurt, Germany

3.1.5.4 Gene expression analysis

Name	**Manufacturer**
MicroAmp® optical reaction plates, 96-well, 384-well	Applied Biosystems, Darmstadt, Germany
Optical adhesive covers	Applied Biosystems, Darmstadt, Germany
QIAGEN Rneasy Mini Kit	QIAGEN, Hilden, Germany
qPCR Mastermix Plus w/o UNG	Eurogentec, Seraing, Belgium
Rnase-, Dnase-free distilled water	Gibco, Karlsruhe, Germany
Rnase-free Dnase Set	QIAGEN, Hilden, Germany
TaqMan® Reverse Transcription Reagents	Applied Biosystems, Darmstadt, Germany

3.1.5.5 Assays-on-Demand™ (AoDs, Applied Biosystems) used for TaqMan-PCR

Gene symbol	**Gene name**	**AoD**
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	Hs99999909_m1
IL2	Interleukin 2	Hs00174114_m1
IL4	Interleukin 4	Hs00174122_m1
IL5	Interleukin 5	Hs00174200_m1
IL10	Interleukin 10	Hs00174086_m1
IL17A	Interleukin 17A	Hs00174383_m1
IL17F	Interleukin 17F	Hs00369400_m1
IL22	Interleukin 22	Hs00220924_m1
IL26	Interleukin 26	Hs00218189_m1
IFNγ	Interferon gamma	Hs00174143_m1
TNF	Tumor necrosis factor	Hs00174128_m1
NR3C1	Glucocorticoid receptor	Hs00230813_m1

3.1.5.6 Western Blot

Name	**Manufacturer**
BCA-Protein-Assay Reagent Kit	Pierce, Berlin, Germany
ECL Plus Western Blotting Detection Reagents	Amersham, Freiburg, Germany
Filter paper	Biorad, Munich, Germany
Gel	Biorad, Munich, Germany
Hyperfilm ECL	Amersham, Freiburg, Germany
ImmunBlot ™ PVDF Membrane, 0,2 µm	Biorad, Munich, Germany
Rainbow Marker	Amersham, Freiburg, Germany

3.1.5.7 Antibodies

Name	Isotype	Clone	Manufacturer
GR	Mouse IgG2a	4H2	Novocastra, Berlin, Germany
GAPDH	Mouse IgG1	10B8	Advanced ImmunoChemical Inc., Berlin, Germany
Mouse IgG, HRP-linked	Sheep	-	Amersham, Freiburg, Germany

3.1.5.8 Mice experiments

Name	Manufacturer
Hydrochloric acid (HCl), 1N	Sigma, Munich, Germany
Hydrogen peroxide (H_2O_2), 30% (w/w)	Sigma, Munich, Germany
Mouse Th1/Th2-9 Plex Tissue Culture Kit IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, TNFα, KC	MesoScale Discovery, Gaithersburg, USA
Polypropylene tubes, 13 mL	Sarstedt, Nürnbrecht, Germany
Sulfuric acid (H_2SO_4), 1N	Merck, Darmstadt, Germany
Tetramethylbenzidine (TMB)	Sigma, Munich, Germany

3.1.6 Equipment

ABI Prism® Sequence Detection System 7900 HT	Applied Biosystems, Darmstadt, Germany
Analytical balance	Sartorius, Göttingen, Germany
Bio-Rad Power Pac 1000	Biorad, Munich, Germany
Centrifuge RC 6	Sorvall, Dreieich, Germany
Cubix 60	Agfa, Berlin, Germany
epMotion 5070 automated pipetting system	Eppendorf, Hamburg, Germany
FACSCalibur™, 4-Color	BD Biosciences, Heidelberg, Germany
FACSCanto™ II	BD Biosciences, Heidelberg, Germany
Harvester 96	TomTec, Frankfurt, Germany
Incubator	Heraeus, Hanau, Germany
Kinematica Polytron PT 3000 homogenizer	Paul, Berlin, Germany
Lightproof cassette	Amersham, Freiburg, Germany
Microcentrifuge 5417 R	Eppendorf, Hamburg, Germany
Microscope Axiovert 25	Carl Zeiss, Jena, Germany
MidiMACS® Separator	Miltenyi Biotec, Bergisch-Gladbach, Germany
Mini Trans-Blot Electrophoretic Transfer Cell	Biorad, Munich, Germany
Mini-PROTEAN II Electrophoresis	Biorad, Munich, Germany
Multifuge 3s	Heraeus, Hanau, Germany
Nanodrop™ ND-1000 spectrophotometer	Peqlab Biotechnologie GmbH, Erlangen, Ger.
Neubauer cell counting chamber	Brand, Berlin, Germany
Nucleofector	Amaxa, Köln, Germany

SECTOR™ Imager 6000	MesoScale Discovery, Gaithersburg, USA
Shaker	Heidolph, Schwabach, Germany
SpektraMax spectral photometer	Molecular Devices, Ebersberg b.M., Germany
Thermal cycler 2720	Applied Biosystems, Darmstadt, Germany
TopCount	Packard, Dreieich, Germany
Vortexer	IKA, Staufen, Germany
Warm case	Heraeus, Hanau, Germany

3.1.7 Software

CellQuestPro®	BD Biosciences, Heidelberg, Germany
FACSDiva®	BD Biosciences, Heidelberg, Germany
Microsoft Office 2003 Professional	Microsoft, Berlin, Germany
MSD Discovery Workbench®	MesoScale Discovery, Gaithersburg, USA
SDS® 2.2	Applied Biosystems, Darmstadt, Germany
SigmaPlot 8.0	Systat Software Inc., Chicago, USA
SigmaStat 3.0	Systat Software Inc., Chicago, USA
SoftMaxPro® 4.6	Molecular Devices, Ebersberg b.M., Germany

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from citrate-anticoagulated venous blood of healthy donors by density gradient centrifugation using Leucosep™ tubes according to the instruction manual. In brief, 20 mL whole blood were transferred to a Leucosep™ tube containing 15 mL Biocoll and centrifuged for 15 min at 800 x g and room temperature. The interphase above the porous barrier containing the PBMCs was transferred into a new 50 mL tube and washed three times with PBS w/o Ca^{2+} and Mg^{2+}. Isolated PBMCs were resuspended in PBMC culture medium for PBMC assays or in MACS buffer for further isolation of T cells. Cell numbers of PBMCs were determined from a part of the cell suspension that was diluted 1:2 with 0.4% trypan blue using a Neubauer cell counting chamber.

3.2.1.2 Isolation of human T cell subpopulations

For isolation of human T cells and T cell subsets MACS® Cell Separation Kits, MACS® MicroBeads and the MidiMACS® Separator with LS or LD columns were used according to the manufacturer's protocols. Human CD3+ T cells, CD4+ T cells or CD8+ T cells

were isolated from human PBMCs by negative magnetic bead separation using the Pan T cell Isolation Kit II, the CD4+ T cell Isolation Kit II or the CD8+ T cell Isolation Kit II, respectively. Each separation was performed with LS columns. Human CD28+ T cells and CD28- T cells were separated from human CD3+ T cells using the CD28 MicroBead Kit and LD columns by collecting the unlabeled (CD28- T cells) and the labeled (CD28+ T cells) cell fractions. Human naïve CD4+ T cells or memory/effector CD4+ T cells were negatively separated from human CD4+ T cells using the CD45RO MicroBeads or the CD45RA MicroBeads with LD columns, respectively.

After isolation, T cells were resuspended in T cell culture medium and counted as described. The purity of the isolated T cell subpopulations was determined by flow cytometry using fluorescence-labeled mouse monoclonal antibodies recognizing the surface markers CD3, CD4, CD28, CD45RA and CD45RO on a FACSCalibur with CellQuest Pro software as described in section 3.2.2.2. Separated human CD3+, CD4+ or CD8+ T cells were >95% pure in all experiments. The percentage of CD3+CD28+ cells was 96±2% in CD28+ T cells and 0.1±0.1% in CD28- T cells. Human naïve T helper cells were 95±1% CD4+CD45RA+ cells, whereas human memory/effector T helper cells were 98±1% CD4+CD45RO+ cells (Figure 4).

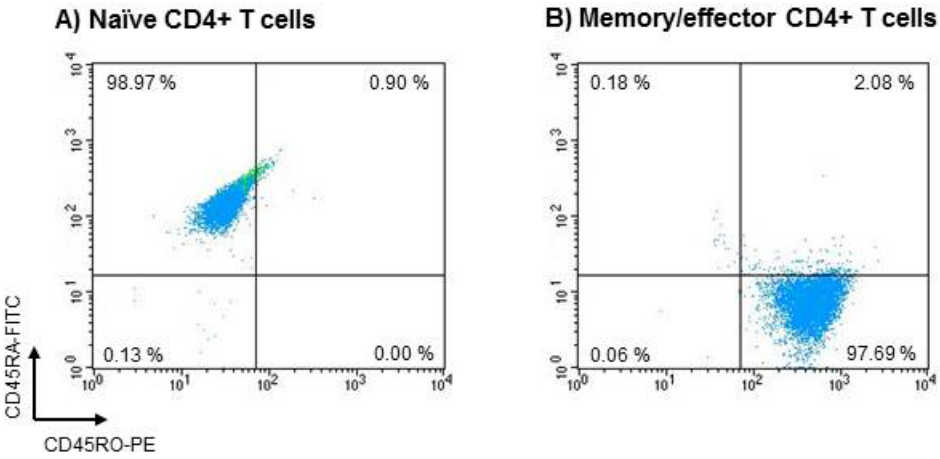


Figure 4: Flow cytometric analysis of separated human naïve and memory/effector T helper cells. Separated naïve CD4+ T cells (**A**) and memory/effector CD4+ T cells (**B**) were stained with FITC-conjugated anti-CD45RA, PE-conjugated anti-CD45RO, PerCP-Cy5.5-conjugated anti-CD3 and APC-conjugated CD4. The surface expression of CD45RA vs. CD45RO on CD4+ T cells, which were gated as CD3+CD4+ cells, of one representative experiment is shown.

3.2.1.3 Treatment and stimulation of human PBMCs and T cell subpopulations

To analyze the anti-inflammatory activity of glucocorticoids (GCs) versus BAY1155975 in human PBMCs, cells were stimulated with lipopolysaccharide (LPS), a component of Gram-negative bacteria which induces the expression of pro-inflammatory genes via recognition by the toll-like receptor 4 (Akira, 2011). Therefore, PBMCs (250,000 cells in a total volume of 200 µL in 96-well round bottom plates) were cultured in duplicates with 10 ng/mL LPS in the presence of 0.1% DMSO (vehicle) or various concentrations of GCs or BAY1155975 at 37°C, 5% CO_2 for 24 hours.

The effects of BAY1155975 and prednisolone on T cell activation were assessed by stimulating human T cell subpopulations with anti-CD3 mAb alone or with costimulatory anti-CD28 mAb, which were immobilized to the well surface of flat bottom plates for optimal cross-linking of the T cell receptor complex. For immobilization wells were incubated with 10 µg/mL anti-CD3 ± 1 µg/mL, 3 µg/mL or 10 µg/mL anti-CD28 (dissolved in PBS) at 37°C, 5% CO_2 for 2 hours and subsequently washed two times with cold PBS. Separated T cells (1,000,000 cells/mL) were seeded in pre-coated plates (100,000 cells per well in 96-well plates for analysis of cytokine secretion, flow cytometry or proliferation; 2,000,000 cells per well in 12-well plates for gene expression analysis) and treated with 0.1% DMSO (vehicle) or various concentrations of GCs or BAY1155975 in the presence or absence of 10 µM RU-486, 1, 5 or 10 µM U0126, 70 µM nifedipine or 0.7 µM cyclosporine A at 37°C, 5% CO_2 for different time periods.

To characterize compound effects on Ca^{2+} mobilization during T cell activation, human memory/effector CD4+ T cells were stimulated with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C, and with the calcium ionophore, ionomycin. The cells were cultured at 100,000 cells per well in 96-well round bottom plates (200 µL total volume) in duplicates with 25-500 ng/mL PMA ± 1 µg/mL ionomycin in the presence of 0.1% DMSO (vehicle) or various concentrations of GCs or BAY1155975 at 37°C, 5% CO_2 for different time periods. Furthermore, cells (100,000 cells per well) were stimulated with 10 µg/mL plate-bound anti-CD3 + 1 µg/mL ionomycin in 96-well flat bottom plates and treated with 0.1% DMSO (vehicle), GCs or BAY1155975 at 37°C, 5% CO_2 for 24 hours.

3.2.1.4 Cytokine secretion

After incubation for different time periods plates were centrifuged for 5 min at 250 x g and supernatants of the duplicated cultures were pooled. The TNFα and IL-12p40 concentrations in supernatant of stimulated human PBMC were measured with specific ELISA kits (human TNFα ELISA, human IL-12p40 ELISA) at the SpektraMax spectral photometer. Concentrations of IFNγ, IL-2, IL-4, IL-17A and TNFα in supernatants of stimulated human T cells were determined by electrochemiluminescence detection using MesoScale multiplex kits according to the

manufacturer's protocol and the Sector™ Imager 6000. The secretion of IL-22 was quantified by a human IL-22 ELISA with the SpektraMax spectral photometer.

3.2.1.5 Proliferation

T cell proliferation stimulated by anti-CD3 ± anti-CD28 was determined in triplicates by adding 0.5 µCi ^{3}H-thymidine per well for the last 6 hours of a 3-day culture. Thereafter, cells were harvested on filter plates using the Harvester 96, dried at 60°C for 1 hour in warm case and 30 µL scintillator per well were added. The incorporated radioactivity was measured by liquid scintillation counts at the TopCount.

3.2.1.6 Assays for T cell immunity (MLR, LTT)

The inhibitory action of BAY1155975 versus GCs on antigen-driven T cell proliferation was assessed in functional *in vitro* T cells assays such as mixed leukocyte reaction (MLR) and lymphocyte transformation test (LTT).

In MLR naïve T cells proliferate in response to allogeneic leukocytes. Therefore, allogeneic stimulator PBMCs were pre-treated with 25 µg/mL mitomycin C at 37°C for 25 min and washed three times with PBS. Stimulator PBMCs (100,000 cells per well) were seeded with untreated responder PBMCs from an unrelated donor (50,000 cells per well) in 96-well round bottom plates and cultured in triplicates with 0.1% DMSO (vehicle) or various concentrations of GCs or BAY1155975 at 37°C, 5% CO_2 for 5 days. In the last 6 hours 0.25 µCi ^{3}H-thymidine were added and the thymidine incorporation was determined by liquid scintillation as described above.

In LTT memory/effector T cells proliferate in response to recall antigens such as tetanus toxoid. Human PBMCs were cultured in triplicates with 0.08 IU/mL tetanus toxoid and 0.1% DMSO (vehicle) or various concentrations of GCs or BAY1155975 in 96-well round bottom plates at 37°C, 5% CO_2 for 3 days. The proliferation was assessed by incorporation of 0.25 µCi ^{3}H-thymidine per well for the last 12 hours as described above.

3.2.1.7 Nucleofection with siRNA

Isolated human memory/effector CD4+ T cells were cultured in 75 cm^2 cell culture flasks at 37°C and 5% CO_2 overnight. The next day, cells were counted and 5,000,000 cells were transfected with 1.8 µM small interfering RNA (siRNA, ON-TARGET*plus* Non-Targeting siRNA #1 or ON-TARGET*plus* SMARTpool for GR). Transfection was performed using the Human T cell Nucleofector Kit according to the unstimulated human T cell protocol and the Nucleofector device with the program U-014. A non-transfected control and a pulsed control (transfection without siRNA) were additionally generated. The transfection efficiency on the mRNA level was determined 18 hours after transfection. Therefore, 500 µL of cell suspension

were washed once with PBS and lysed in 350 µL lysing buffer for RNA isolation. Medium was changed completely then. For detection of the transfection efficiency on the protein level, cells were washed twice with PBS and lysed in 20 µL lysing buffer for Western Blot at 48 hours after transfection. Due to the nucleofection procedure, the cell viability was about 60% then. For stimulation, transfected cells were seeded in anti-CD3 ± anti-CD28-pre-coated plates (100,000 cells per well in 96-well plates) and treated with 0.1% DMSO (vehicle) or 10 µM of GCs or BAY1155975 at 37°C, 5% CO_2 for 24 hours.

3.2.2 Flow cytometry

3.2.2.1 Apoptosis induction

Compound effects on apoptosis induction in anti-CD3 ± anti-CD28-stimulated human memory/effector CD4+ T cells were determined by Annexin V and propidium iodide (PI) staining. Annexin V binds to phosphatidylserine, which is exposed on the cell surface of apoptotic cells, whereas PI as a membrane-impermeant fluorescent DNA-binding dye detects cells undergoing necrosis. After culture, cells (100,000) were transferred into micronic tubes and stained using the ApoAlert® Annexin V-FITC Apoptosis Kit according to the user manual. The proportion of apoptotic and/ or necrotic cells was quantified as Annexin-V+ and/or PI+ cells by flow cytometry using a FACSCalibur™ with CellQuest Pro® software.

3.2.2.2 Surface molecule expression

The expression of various surface markers was assessed in human naïve and memory/effector CD4+ T cells prior to stimulation and in stimulated human memory/effector CD4+ T cells treated with GCs or BAY1155975 for different time periods. Cells were washed two times with staining buffer for flow cytometry, resuspended in 50 µL staining buffer and transferred to 96-well round bottom plates. Afterwards, cells were stained for 30 min at 4°C in the dark with the following mouse monoclonal antibodies: FITC-conjugated anti-CD40L and anti-CD27, PE-conjugated anti-CD25 and anti-CD28, APC-conjugated anti-CD69, PerCP-Cy5.5-conjugated anti-HLA-DR or isotype controls. As previously tested, FITC-, PE- and PerCP-Cy5-conjugated antibodies were 1:10 diluted and APC-conjugated antibodies were 1:50 diluted for staining. After washing with staining buffer, cells were resuspended in 50 µL staining buffer and analyzed on a FACSCanto™ II flow cytometer using FACSDiva® software.

3.2.2.3 Intracellular cytokine staining

To analyze compound effects on intracellular cytokine production, human memory/effector CD4+ T cells were stimulated and treated in duplicates with GCs or BAY1155975 as described in section 3.2.1.2. After 8 hour culture, cells were washed twice with T cell culture medium and

again stimulated and treated for additional 4 hours. Cytokine release was blocked by using 10 µg/mL brefeldin A, a protein transport inhibitor that was added for the additional 4 hour culture. Afterwards, cells were washed two times with staining buffer, stained for surface makers in 96-well round bottom plates as described above and again washed two times with staining buffer. Cells were fixed and permeabilized for 10 min at 4°C with 100 µL Cytofix/Cytoperm solution and washed two times with Perm/Wash buffer 1:10 diluted in distilled water (Cytoperm/Cytofix™ Fixation/Permeabilization Kit). After resuspending the cells in 50 µL 1x Perm/Wash buffer, cells were stained with 10 µL PE-conjugated anti-IFNγ and 1 µL PE-Cy7-conjugated anti-TNFα for 30 min at 4°C. As isotype controls, PE-conjugated IgG2b and PE-Cy7-conjugated IgG1 were used. Cells were washed two times with staining buffer, resuspended in 50 µL staining buffer and analyzed on a FACS-Canto™ II flow cytometer using FACSDiva® software.

3.2.3 Gene expression analysis

3.2.3.1 RNA isolation and quantification

After 4 hours of culture, cells (2,000,000 cells per well) were washed once with PBS and lysed in 600 µL lysing buffer. Total RNA was extracted from the lysates using the RNeasy Mini Kit with the protocol *total RNA isolation from animal cells.* In addition, on-column DNase digestion was performed using the RNase-free DNase Set. Total RNA was eluted with 30 µL RNase-free distilled water in 1.5 mL RNase-free tubes. Quality and quantity of the extracted total RNA was determined on a Nanodrop™ ND-1000 spectrophotometer.

3.2.3.2 Reverse transcription

Reverse transcription of 250 ng total RNA into complementary DNA (cDNA) was performed in 96-well reaction plates in a total volume of 100 µL. The corresponding volume of total RNA was dissolved in 38.5 µL RNase-free distilled water and mixed with 61.5 µL of the reaction solution containing 1 × RT buffer, 5.5 mM $MgCl_2$, 2.5 µM random hexamers, 0.5 mM each NTP (dNTPs mixture), 0.4 U/µL RNase Inhibitor and 1.25 U/µL multiscribe reverse transcriptase (TaqMan® Reverse Transcription Reagents). The reaction was performed at 25°C for 10 minutes, followed by 48°C for 30 minutes, 95°C for 5 minutes and cooled down to 4°C using the 2720 thermal cycler.

3.2.3.3 Real-time (TaqMan) PCR

For mRNA expression analysis TaqMan PCR was performed in triplicates with Assays-on-Demand™ (AoDs) which contain a fluorescence-labeled TaqMan probe as well as

forward and reverse primers for the particular gene. PCR reactions were conducted in 384-well reaction plates containing 0.5 µL cDNA, 5.13 µL RNase-free distilled water, 6.25 µL qPCR Mastermix Plus and 0.63 µL AoD, which were pipetted with the ep*Motion* 5070. To exclude unspecific binding, non-template controls for each AoD, in which RNase-free distilled water instead of cDNA was used, were run in parallel. Hypoxanthine-guanine phosphorribosyltransferase (HPRT) was used as endogenous reference gene. The reactions were performed at 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute for 40 cycles using the ABI Prism® 7900HT sequence detection system. Expression of target genes during the exponential phase of the reaction was quantified relatively as fold expression of the endogenous reference gene, HPRT. Therefore, fluorescence against cycle number for all analyzed genes was plotted on a logarithmic scale and the threshold for detection of fluorescence above background was determined. For each target gene the arithmetic mean of the three cycle threshold (Ct – cycle at which the fluorescence crosses the threshold) values was calculated and subtracted from the arithmetic mean of the Ct values of HPRT (ΔCt). The relative expression of each target gene was calculated according to the following equation: fold expression = $2^{-\Delta Ct}$.

3.2.4 Western Blot

3.2.4.1 Cell lysis for Western Blot

Cells were washed twice with PBS and lysed in 20 µL lysing buffer for Western Blot. After incubation for 30 min on ice, cells were centrifuged at 4°C and 13,000 rpm for 10 min. The supernatants were transferred into 0.5 mL microcentrifuge tubes for gel electrophoresis. To determine the total protein concentration, cell lysates were 1:10 diluted with lysing puffer and the protein quantification was performed with the BCA Protein Assay reagent Kit according to the user manual using the SpektraMax spectral photometer.

3.2.4.2 Gel electrophoresis

For electrophoresis, 10 µg of protein per sample were loaded onto the gel. Therefore, the appropriate volume of the cell lysate was mixed with sample buffer in a total volume of 18 µL. The mixed sample solutions were heated for 5 min at 96°C. Samples were cooled down on ice and loaded on the gel. The electrophoresis was run at 200 V for 1 hour using the Mini-PROTEAN II Electrophoresis.

3.2.4.3 Protein Transfer

Before protein transfer, the polyvinylidene difluoride (PVDF) membrane was pre-incubated in 100% methanol for 5 min and washed two times with transfer buffer. Transfer of the proteins

was performed at 30 V for 1 hour using the Mini Trans-Blot Electrophoretic Transfer Cell with transfer buffer. Afterwards, PVDF membrane was washed 5 x 5 min with TBS-T buffer.

3.2.4.4 Protein detection

To block non-specific binding, the PVDF membrane was incubated with the blocking solution for 1 hour at room temperature under gently shaking. After washing the membrane 5 x 5 min with TBS-T buffer, membrane was incubated with the appropriate primary antibody, anti-human GR diluted 1:75 or anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) diluted 1:50,000 in blocking solution, at 4°C under gently shaking overnight. Unbound antibody was removed by washing the membrane 5 x 5 min with TBS-T buffer. Afterwards, horse radish peroxidase (HRP)-conjugated secondary antibody diluted 1:10,000 in blocking solution was added to the membrane and incubated for 1 hour at room temperature under gently shaking. The membrane was washed 5 x 5 min with TBS-T buffer and soaked with enhanced chemiluminescence (ECL) solution using the ECL Plus Western Blotting Detection Reagents according to the user manual. Peroxidase activity was detected in a lightproof cassette by 10 min exposition to a photosensitive Hyperfilm. The film was developed using the Curix 60.

3.2.5 Contact hypersensitivity models in mice

3.2.5.1 DNFB-induced contact dermatitis

The 2,4-dinitro-1-fluorobenzene (DNFB)-induced contact hypersensitivity response represents a T cell-dependent skin inflammation characterized by a T helper (Th) 1 cytokine dominance (Ogawa et al., 2010; Röse et al., 2012). Female NMRI mice (7 or 8 mice per group) were sensitized with 25 µL 0.5% (w/v) DNFB in the solvent, acetone/olive oil 4:1, on the shaved flank skin at day 0 and day 1. For challenge, mice were topically treated with 0.3% (w/v) DNFB in acetone/olive oil 4:1 on the dorsal side of both ears in a total volume of 20 µL per ear at day 5, and in a second experiment furthermore at day 6 and day 7. As negative control, a group of mice were exposed to the solvent throughout the duration of the experiment.

For the assessment of their anti-inflammatory activity, GCs and BAY1155975 were applied orally at 2 hours before challenge on day 5. For determination of their inhibitory effect on T cell activation in the sensitization phase, the compounds were administered orally at 2 hours before sensitization on days 0 and 1 and furthermore on day 2. Twenty-four hours after the last challenge, mice were sacrificed, and the ears were removed and weighted as parameter for inflammatory edema formation. Ears were frozen in liquid nitrogen in polypropylene tubes to analyze the peroxidase activity and the level of pro-inflammatory cytokines (Figure 5).

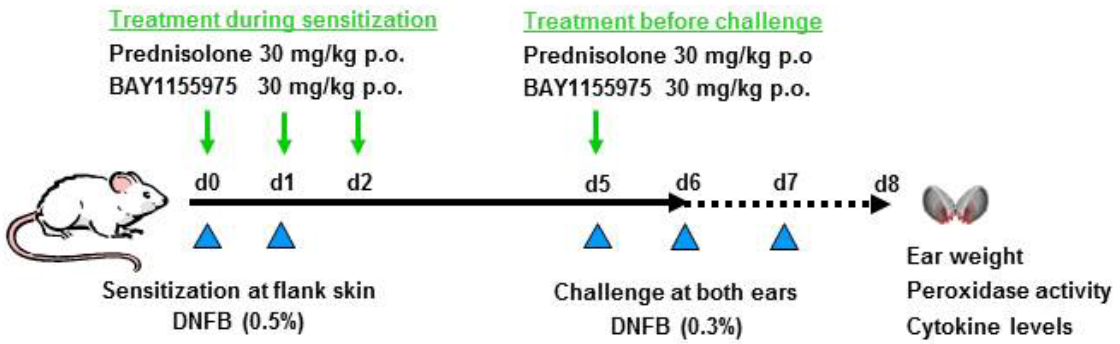


Figure 5: DNFB-induced contact hypersensitivity model in mice.

NMRI mice were sensitized at shaved flank skin with 0.3% DNFB at day 0 and day 1 and challenged with 0.5% DNFB only at day 5 or repeatedly at days 5, 6 and 7. Compounds were applied orally either at 2 hours before sensitization on days 0 and 1 and additionally on day 2 or at 2 hours before challenge on day 5.

3.2.5.2 Peroxidase activity in ear homogenates

As parameter for granulocyte infiltration the myeloperoxidase activity in tissue homogenates was analyzed as described previously (Schottelius et al., 2002). Briefly, ears were mechanically homogenized in 2 mL homogenization buffer and centrifuged at 25,000 x g for 20 minutes at 12°C. Myeloperoxidase activity in 1:50 pre-diluted supernatants is given as OD value at 450 nm multiplied by the dilution factor.

3.2.5.3 Cytokine levels in ear homogenates

To assess the cytokine levels in inflamed ears, ears were mechanically homogenized in 2 mL RPMI medium using the cooled Kinematica Polytron PT 3000 homogenizer. The homogenates were centrifuged at 14,000 rpm for 20 minutes at 4°C (Sorvall centrifuge), and 25 µL of supernatant were used to determine the cytokine concentrations by electro-chemiluminescence detection using MesoScale Mouse Th1/Th2 kits according to the *Tissue culture Kit* protocol and the Sector™ Imager 6000.

3.2.6 Statistical analysis

Data are given as mean ± standard error of the mean (SEM) for *in vitro* experiments and mean ± standard deviation (SD) for *in vivo* experiments. For *in vitro* investigations statistical analysis was performed using the non-parametric paired Wilcoxon Signed Rank test. Group differences in *in vivo* experiments were assessed by Mann-Whitney U test (SigmaStat 3.0). *P*-values below 0.05 were considered as significant.

4. Results

4.1 Anti-inflammatory and immunosuppressive activity of BAY1155975 compared to glucocorticoids in primary human immune cells

4.1.1 Anti-inflammatory activity in toll-like receptor-stimulated primary immune cells

Lipopolysaccharide (LPS)-induced activation of toll-like receptor 4 signaling pathways results in the activation of nuclear factor κB (NF-κB) and thus in the expression of pro-inflammatory cytokines (Akira, 2011). GCs repress the transcription of these pro-inflammatory cytokines via protein-protein interactions of the ligand-activated glucocorticoid receptor (GR) with the appropriate TFs (Liberman et al., 2007; Beck et al., 2009; Clark & Belvisi, 2012).

To assess the anti-inflammatory activity of BAY1155975 compared to classical glucocorticoids (GCs) *in vitro*, human PBMCs of 4 healthy donors were stimulated with LPS and treated with dexamethasone, prednisolone or BAY1155975 for 24 hours. Tumor necrosis factor alpha (TNFα) and Interleukin (IL)-12p40 were determined in supernatants. The secretion of TNFα and IL-12p40 was dose-dependently suppressed by dexamethasone and prednisolone, whereas dexamethasone was about 10-times more potent than prednisolone. BAY1155975 inhibited the LPS-induced cytokine secretion less efficiently than dexamethasone or prednisolone (IC_{50} TNFα Dex = 6 nM, Pred = 47 nM, BAY1155975 = 23 nM; IC_{50} IL-12p40 Dex = 3 nM, Pred = 47 nM, BAY1155975 = 51 nM) (Figure 6).

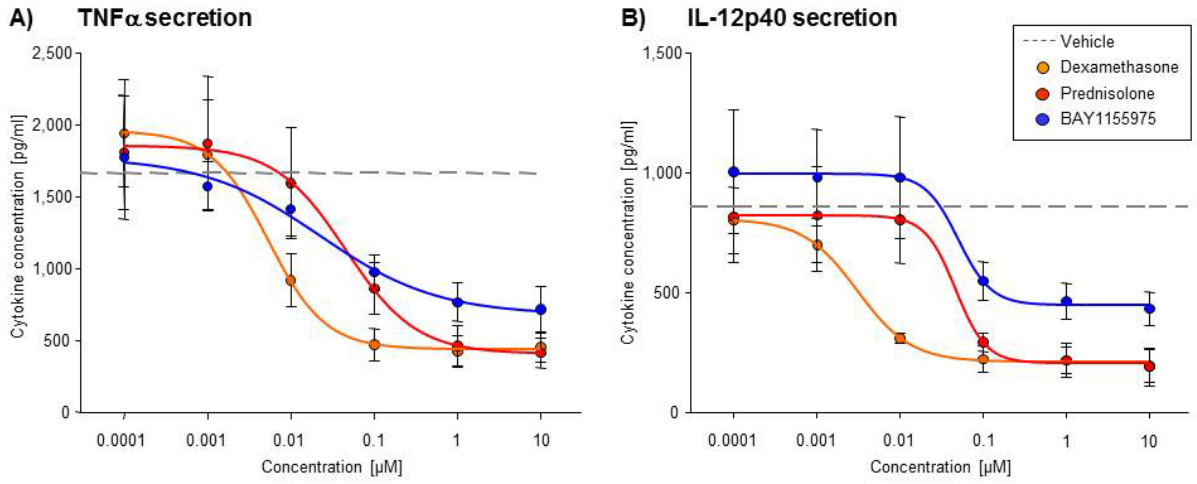


Figure 6: BAY1155975 inhibits stimulated pro-inflammatory cytokine secretion in PBMCs less efficiently than GCs.

PBMCs were stimulated with lipopolysaccharide in the absence or presence of dexamethasone, prednisolone or BAY1155975 for 24 hours. The secretion of TNFα (**A**) and IL-12p40 (**B**) is shown as mean ± SEM of 4 donors (vehicle TNFα 1,663±383 pg/mL, IL-12p40 858±156 pg/mL).

4.1.2 Inhibition of proliferation in functional *in vitro* T cell assays

The inhibitory effect of GCs on cytokine production, especially on IL-2, the autocrine signal required for T cell activation, is mainly responsible for the anti-proliferative action of GCs on T cells (Liberman et al., 2007; Tischner et al., 2011). To characterize the suppressive effect of BAY1155975 compared to classical GCs on antigen-driven T cell proliferation, the effects of the compounds were determined in two standard *in vitro* assays for T cell immunity.

For the mixed leukocyte reaction (MLR), human PBMCs from one donor (responder PBMCs) were co-cultured with mitomycin C-inactivated PBMCs from another donor (stimulator PBMCs) at a ratio of 1:2 and cultured with dexamethasone, prednisolone or BAY1155975 for 5 days. The proliferation of naïve responder T cells, which are stimulated by the allogeneic MHC class II-bearing antigen-presenting cells (APCs) was assessed by ^{3}H-thymidine incorporation. BAY1155975 dose-dependently decreased the proliferation of naïve responder T cells similar to prednisolone. At the highest concentration of 1 µM, the proliferation was even stronger inhibited by BAY1155975 than by dexamethasone or prednisolone (Figure 7A).

In the lymphocyte transformation test (LTT), human PBMCs from 5 tetanus toxoid-vaccinated donors were cultured for 3 days with tetanus toxoid. Dexamethasone, prednisolone or BAY1155975 were added to the cells at the beginning of culture. Proliferation of tetanus toxoid-specific memory/effector T cells, which were activated by the repeated encounter with their cognate antigen, was determined by ^{3}H-thymidine uptake during the last 12 hours.

BAY1155975 efficiently suppressed the antigen-specific proliferation of memory/effector T cells similar to dexamethasone and prednisolone (Figure 7B).

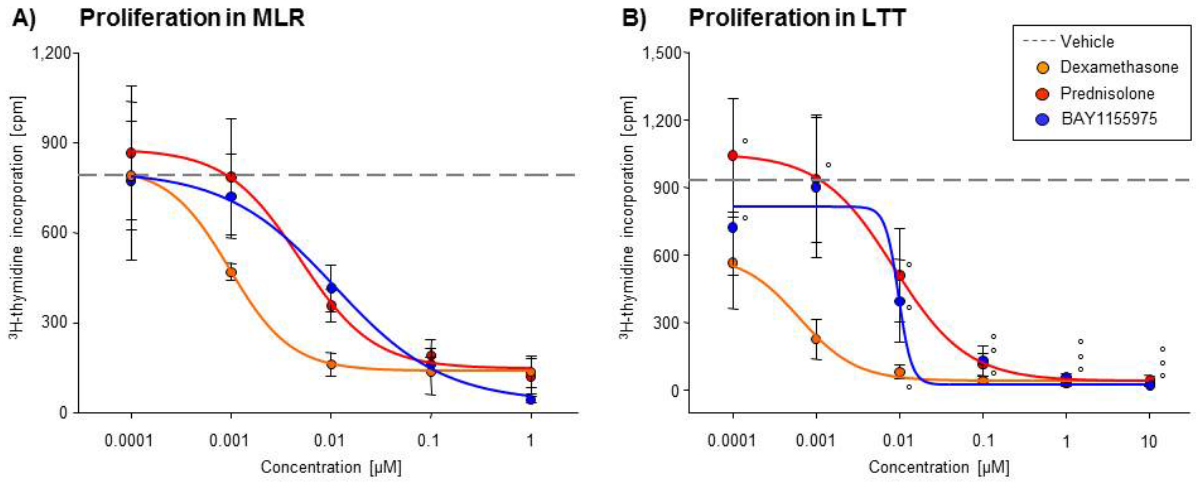


Figure 7: BAY1155975 exhibits similar inhibition of antigen-driven T cell proliferation as GCs.

A) For mixed leukocyte reaction (MLR) responder PBMCs were co-cultured with mitomycin C-treated stimulator PBMCs and cultured in the absence or presence of dexamethasone, prednisolone or BAY1155975 for 5 days. Incorporated ^{3}H-thymidine in the last 6 hours of culture is shown as mean ± SEM of 4 experiments (vehicle 791±129 cpm).

B) In the lymphocyte transformation test (LTT) PBMCs were cultured with tetanus toxoid in the absence or presence of dexamethasone, prednisolone or BAY1155975 for 3 days. Mean ± SEM values of 5 donors of the incorporated ^{3}H-thymidine in the last 12 hours are shown (vehicle 932±268 cpm). °p=0.063 vs. Vehicle in Wilcoxon Signed Rank test.

In summary, BAY1155975 less efficiently than dexamethasone and prednisolone inhibited TLR-stimulated secretion of pro-inflammatory cytokines in human PBMCs, but showed at least similar inhibition of human T cell proliferation in functional *in vitro* T cell assays.

4.2 Differential effects of BAY1155975 and glucocorticoids on human T cell activation

It is known that the inhibitory effect of GCs is diminished on T cell receptor (TCR)-induced proliferation of human T cells costimulated by anti-CD28 (Li et al., 2004; Tsitoura & Rothman, 2004; Winiski et al., 2007). Additionally, the dexamethasone-induced decrease in interferon gamma (IFNγ) production by TCR-stimulated PBMCs was shown to be attenuated by CD28-costimulation (Agarwal & Marshall, 2000). As BAY1155975 shows similar potency in inhibiting the antigen-induced T cell proliferation, the next step was to determine the

suppressive effect of BAY1155975 compared to classical GCs on T cell proliferation and cytokine secretion in stimulation assays with polyclonal activators such as anti-CD3 ± anti-CD28 monoclonal antibodies.

4.2.1 Differential effects on IFNγ secretion in anti-CD28-costimulated human T cells

To assess the effect of compounds to solely TCR stimulation, separated human T cells from 2 healthy donors were stimulated with plate-bound anti-CD3 antibody (10 µg/mL) and treated with dexamethasone, prednisolone or BAY1155975. After 24-hour stimulation the secretion of IFNγ was determined in supernatants. The proliferation was assessed by ^{3}H-thymidine incorporation in the last 6 hours after 3 days of culture. BAY1155975 suppressed the IFNγ secretion and proliferation after anti-CD3 stimulation with less potency but similar efficacy as dexamethasone or prednisolone (IC_{50} IFNγ Dex = 5 nM, Pred = 61 nM, BAY1155975 = 175 nM; IC_{50} proliferation Dex = 16 nM, Pred = 118 nM, BAY1155975 = 1217 nM) (Figure 8).

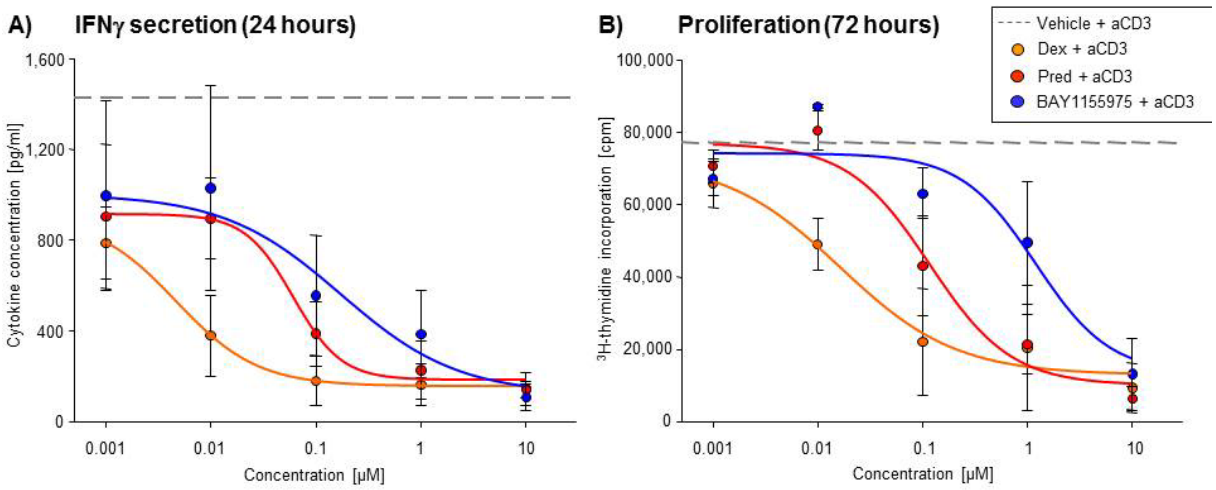


Figure 8: BAY1155975 inhibits anti-CD3-induced T cell activation with similar efficacy as GCs.
T cells were stimulated with plate-bound anti-CD3 in the absence or presence of dexamethasone (Dex), prednisolone (Pred) or BAY1155975. Secretion of IFNγ was determined in supernatants after 24-hour stimulation (**A**). For proliferation, ^{3}H-thymidine was added for the last 6 hours of the 72-hour stimulation culture (**B**). Data are shown as mean ± SEM of 2 donors (vehicle IFNγ 1,425±338 pg/mL, proliferation 77,041±1,780 cpm).

To analyze the suppressive effect of BAY1155975 compared to classical GCs on anti-CD28-costimulated T cell activation, separated human T cells were stimulated with plate-bound anti-CD3 (10 µg/mL) and various concentrations of anti-CD28 in the presence of dexamethasone, prednisolone or BAY1155975 for up to 72 hours.

IFNγ secretion after 24-hour stimulation was enhanced by increasing concentrations of anti-CD28. Dexamethasone and prednisolone strongly suppressed IFNγ secretion in T cells costimulated with 1 µg/mL anti-CD28 (efficacy prednisolone 82%), but to a diminished extent in T cells costimulated with 3 and 10 µg/mL of the anti-CD28 antibody (efficacy prednisolone 10 µg/mL 62%). Interestingly, BAY1155975 stronger than the classical GCs inhibited IFNγ secretion with increasing concentrations of anti-CD28 (efficacy 1 µg/mL 91%, 10 µg/mL 81%) (Figure 9A).

Proliferation of the separated T cells, as assessed by ^{3}H-thymidine uptake, was enhanced by costimulation with 1 µg/mL anti-CD28 compared to stimulation with anti-CD3 alone (vehicle anti-CD3 77,041±1,780 cpm, 1 µg/mL anti-CD28 100,212±11,059 cpm), but was not further enhanced by costimulation with 3 µg/mL or 10 µg/mL anti-CD28 (Figure 9B). Dexamethasone and to a lesser extent prednisolone and BAY1155975 decreased the T cell proliferation costimulated by 1 µg/mL anti-CD28. Only a slight inhibition of proliferation costimulated with 3 µg/mL anti-CD28 was observed by dexamethasone, prednisolone or BAY1155975. T cell proliferation costimulated with 10 µg/mL anti-CD28 was suppressed neither by the classical GCs nor by BAY1155975 (Figure 9B).

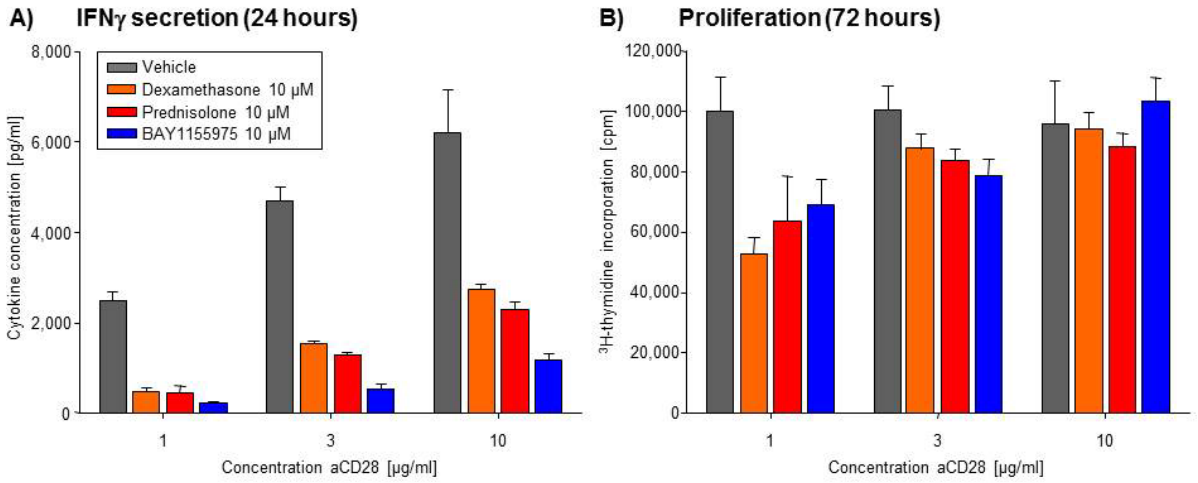


Figure 9: BAY1155975 stronger inhibits anti-CD28-costimulated IFNγ secretion in T cells than GCs.

Separated T cells were stimulated with plate-bound anti-CD3 and various concentrations of anti-CD28 in the absence or presence of dexamethasone, prednisolone or BAY1155975. The IFNγ secretion was analyzed in supernatants after 24-hour stimulation (**A**). For proliferation, ^{3}H-thymidine uptake during the last 6 hours of the 72-hour stimulation culture was determined (**B**). Data are shown as mean + SEM of 2 donors.

In conclusion, in separated human T cells costimulated with anti-CD28, 10 µM BAY1155975 exhibited a distinctly stronger inhibition of IFNγ secretion than classical GCs. Since the differential effect was clearest in T cells costimulated with high anti-CD28 concentrations, the compound effects were further analyzed in different T cell subpopulations stimulated with 10 µg/mL anti-CD3 in the presence or absence of 10 µg/mL anti-CD28 monoclonal antibody. As dexamethasone and prednisolone inhibited IFNγ secretion with similar diminished extend, the effect of BAY1155975 was compared to prednisolone only.

4.2.2 Analysis of human T cell subpopulations

The dependency of the differential compound effects on anti-CD28 costimulation was further analyzed in separated human CD28-positive (CD28+) and CD28-negative (CD28-) T cells. Separated T cells were stimulated with plate-bound anti-CD3 in the presence or absence of anti-CD28 and treated with BAY1155975 or prednisolone for 24 hours. In CD28- T cells no increased IFNγ secretion by anti-CD28 costimulation was observed. Prednisolone and BAY1155975 inhibited IFNγ secretion to a similar extent (Figure 10A). In CD28+ T cells, increased IFNγ secretion by anti-CD28 costimulation was not suppressed by prednisolone. In contrast, BAY1155975 distinctly decreased the secretion of IFNγ induced by anti-CD28 costimulation (Figure 10B).

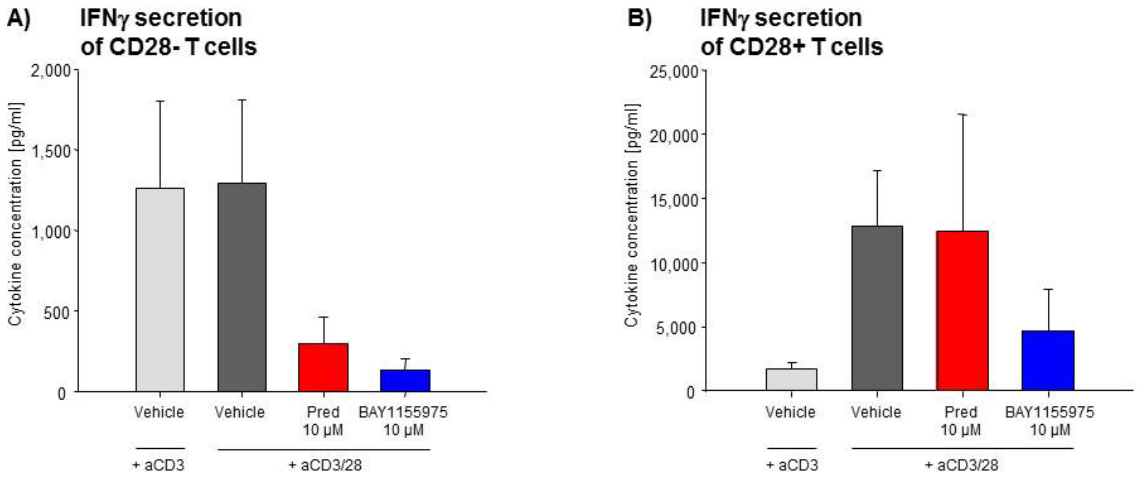


Figure 10: BAY1155975 in contrast to prednisolone shows strong inhibition of anti-CD28-costimulated IFNγ secretion in CD28+ T cells.

Separated CD28- T cells (**A**) and CD28+ T cells (**B**) were stimulated with plate-bound anti-CD3 ± anti-CD28 (10 µg/mL) in the absence or presence of prednisolone (Pred) or BAY1155975 for 24 hours. IFNγ secretion is shown as mean + SEM of 3 donors.

Next, the suppressive effect on IFNγ secretion by BAY1155975 compared to prednisolone was determined in the two major T cell subpopulations, CD4+ T cells and CD8+ T cells. According to literature (Jentsch-Ullrich et al., 2005), the mean CD4/CD8 ratio is roughly 2. The majority of CD4+ T cells express CD28, whereas only about 50% of CD8+ T cells are CD28-positive (Figure 11A). Hence, IFNγ secretion was more increased in CD4+ T cells than in CD8+ T cells by anti-CD28 costimulation. Remarkably, in all T cell populations, CD3+, CD4+ and CD8+ T cells, the inhibitory effect of BAY1155975 on anti-CD28-costimulated IFNγ secretion was stronger than that of prednisolone (Figure 11B).

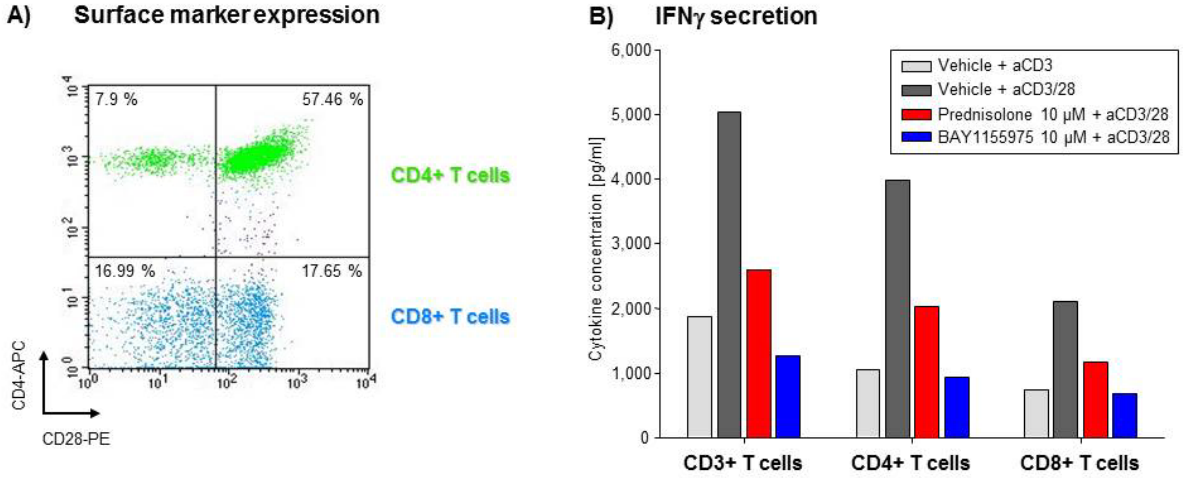


Figure 11: BAY1155975 shows stronger inhibition of anti-CD28-costimulated IFNγ secretion in CD3+, CD4+ and CD8+ T cells than prednisolone.

A) Separated T cells were stained with PE-conjugated anti-CD28, PerCP-Cy5.5-conjugated anti-CD3 and APC-conjugated anti-CD4 monoclonal antibodies. The surface expression of CD4 vs. CD28 on T cells, which were gated as CD3+ cells, of one donor is shown.

B) Human CD3+, CD4+ and CD8+ T cells were separated from human PBMCs of one donor. Separated T cells were stimulated with plate-bound anti-CD3 ± anti-CD28 (10 µg/mL) in the absence or presence of prednisolone or BAY1155975. After 24 hours, IFNγ secretion was determined in the supernatants.

In summary, the inhibitory effect of prednisolone and the stronger inhibitory effect of BAY1155975 on IFNγ secretion were comparable in anti-CD28-costimulated CD4+ T cells and CD8+ T cells.

4.2.3 Analysis of human naïve versus memory/effector CD4+ T cells

Human CD4+ T cells can be further divided into naïve and memory/effector cells based on the expression of distinct isoforms of the surface molecule CD45. However, in human CD8+ T cells

the implication of CD45 isoforms as exclusive marker for such a separation is not sufficient, as a subset of memory/effector CD8+ T cells is CD45RA-positive (Höflich et al., 1998; Catalina et al., 2002). Therefore, the suppressive effect of BAY1155975 compared to prednisolone on IFNγ secretion was profoundly analyzed in human naïve CD4+ T cells and memory/effector CD4+ T cells negatively separated as CD4+CD45RO- cells and CD4+CD45RA- cells by immunomagnetic selection using MACS beads.

First, the expression of cell-surface molecules was evaluated in the separated naïve CD4+ T cells and memory/effector CD4+ T cells. All naïve CD4+ T cells express CD45RA, whereas all memory/effector CD4+ T cells express CD45RO. The costimulatory molecules, CD27 and CD28, are expressed by all naïve CD4+ T cells. About 10% of the memory/effector CD4+ T cell subset lack surface CD27 expression. All memory/effector CD4+ T cells express CD28 and about 50% of these T cells express CD25 above isotype control antibody level. The activation marker CD69 is not expressed neither by naïve nor memory/effector CD4+ T cells, whereas HLA-DR is slightly expressed by memory/effector CD4+ T cells prior stimulation (Figure 12).

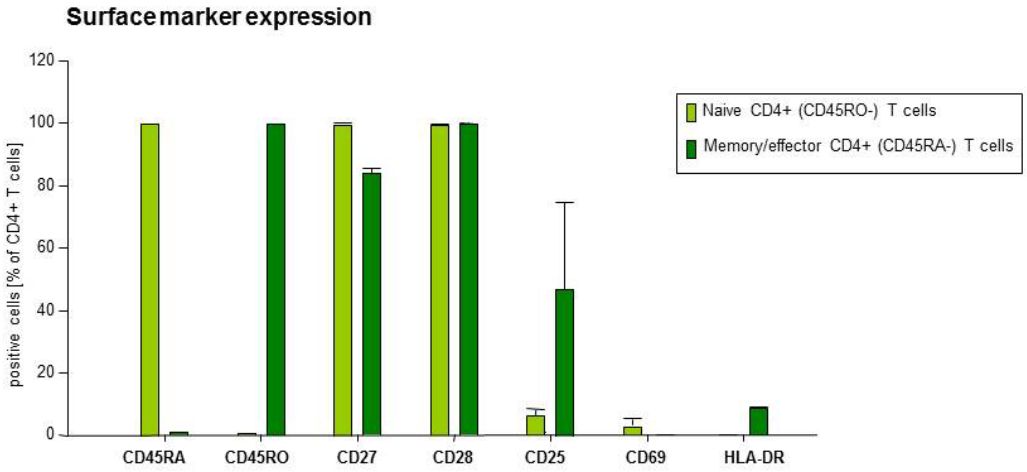


Figure 12: Immunophenotype of separated human naïve and memory/effector CD4+ T cells.
Naïve CD4+ T cells and memory/effector CD4+ T cells were negatively separated from CD4+ T cells. Cell subsets were flowcytometrically characterized after staining with FITC-conjugated anti-CD27 and anti-CD45RA, PE-conjugated anti-CD25, anti-CD28, anti-CD45RO and anti-CD69, PerCP-Cy5.5-conjugated anti-HLA-DR and APC-conjugated anti-CD4 monoclonal antibodies. The percentage of CD4+ cells expressing the different surface markers is shown for each subset as mean + SEM of 2 donors.

The inhibitory effect of BAY1155975 and prednisolone on IFNγ secretion was analyzed in negatively separated human naïve as well as memory/effector CD4+ T cells stimulated with plate-bound anti-CD3 in the presence or absence of anti-CD28 for 24 hours. Following stimulation with anti-CD3 alone, naïve CD4+ T cells secreted almost no IFNγ. Costimulation with anti-CD28 increased IFNγ secretion more than 10-fold. Prednisolone and BAY1155975 suppressed the anti-CD28-costimulated IFNγ secretion only at the highest concentration of 10 µM and to a similar extent (Figure 13A).

In contrast to naïve CD4+ T cells, memory/effector CD4+ T cells secreted high amounts of IFNγ in response to TCR stimulation alone. Costimulation with anti-CD28 triggered a 10-fold increase. Prednisolone slightly inhibited IFNγ secretion in a dose-dependent way. A significantly stronger suppression of IFNγ secretion by 10 µM BAY1155975 in comparison to 10 µM prednisolone was observed (Figure 13B).

The IL-2 secretion was stronger inhibited in anti-CD28-costimulated naïve and memory/effector CD4+ T cells by BAY1155975 than by prednisolone (data not shown and Figure 15, respectively). BAY1155975 also inhibited IL-4 secretion stronger than prednisolone in anti-CD28-costimulated memory/effector CD4+ T cells (Figure 15).

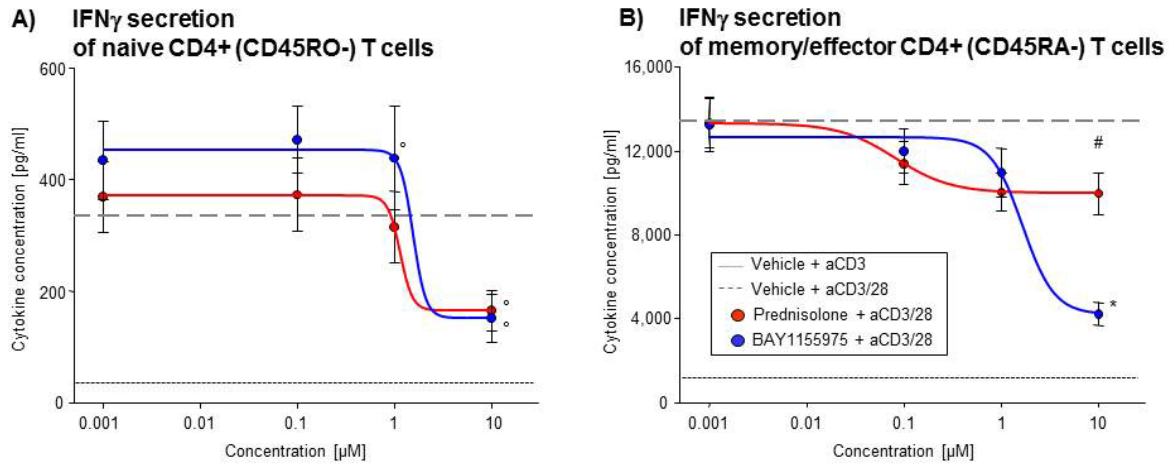


Figure 13: BAY1155975 at a concentration of 10 µM stronger inhibits anti-CD28-costimulated IFNγ secretion in memory/effector CD4+ T cells than prednisolone.

Separated naïve (**A**) and memory/effector CD4+ T cells (**B**) were stimulated with plate-bound anti-CD3 ± anti-CD28 (10 µg/mL) in the absence or presence of prednisolone or BAY1155975 for 24 hours. IFNγ secretion is shown as mean ± SEM of 5 donors for naïve CD4+ T cells (vehicle anti-CD3 23±9 pg/mL, vehicle anti-CD3 + anti-CD28 335±59 pg/mL) or 8 donors for memory/effector CD4+ T cells (vehicle anti-CD3 1,194±154 pg/mL, vehicle anti-CD3 + anti-CD28 13,399±1,239 pg/mL).

°p=0.063 vs. vehicle, *p<0.05 vs. vehicle, #p<0.05 vs. prednisolone in Wilcoxon Signed Rank test.

To address the possibility that BAY1155975 at 10 µM increases the susceptibility to apoptosis, the effect of BAY1155975 treatment on apoptosis induction as well as on proliferation was compared to prednisolone treatment in anti-CD28-costimulated memory/effector CD4+ T cells.

After 24-hour culture, the percentage of apoptotic and/or necrotic cells was enhanced by stimulation with anti-CD3 and anti-CD28 monoclonal antibodies. Interestingly, BAY1155975 and prednisolone at equimolar concentrations further increased apoptosis induction to the same extent (Figure 14A). The proliferation, which was induced by 3-day stimulation with anti-CD3 + anti-CD28, was slightly reduced by prednisolone. BAY1155975 even slightly increased the anti-CD28-costimulated proliferation in memory/effector CD4+ T cells (Figure 14B).

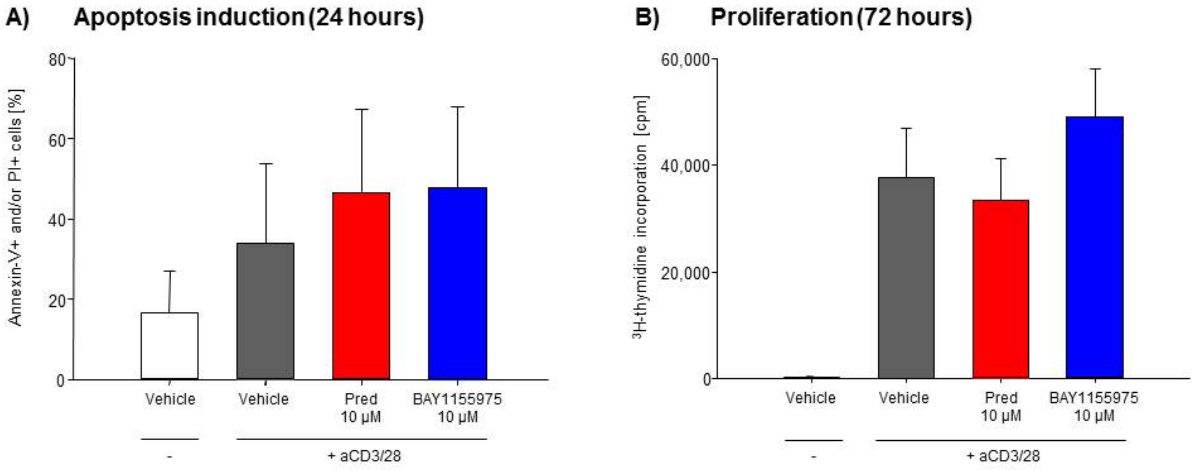


Figure 14: BAY1155975 does not stronger than prednisolone induce apoptosis or inhibit proliferation in anti-CD28-costimulated memory/effector CD4+ T cells.

Separated memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (10 µg/mL) in the absence or presence of prednisolone (Pred) or BAY1155975. The proportion of apoptotic and/or necrotic cells was determined by Annexin-V and propidium iodide (PI) staining after 24-hour culture (**A**). For proliferation, ^{3}H-thymidine uptake during the last 6 hours of the 72-hour stimulation culture was determined (**B**). Data are shown as mean + SEM of 3 donors.

Concluding from these data, the enhanced inhibition of IFNγ secretion exhibited by 10 µM BAY1155975 in anti-CD28-costimulated memory/effector CD4+ T cells is not due to an increased induction of apoptosis or a reduced proliferation of activated cells.

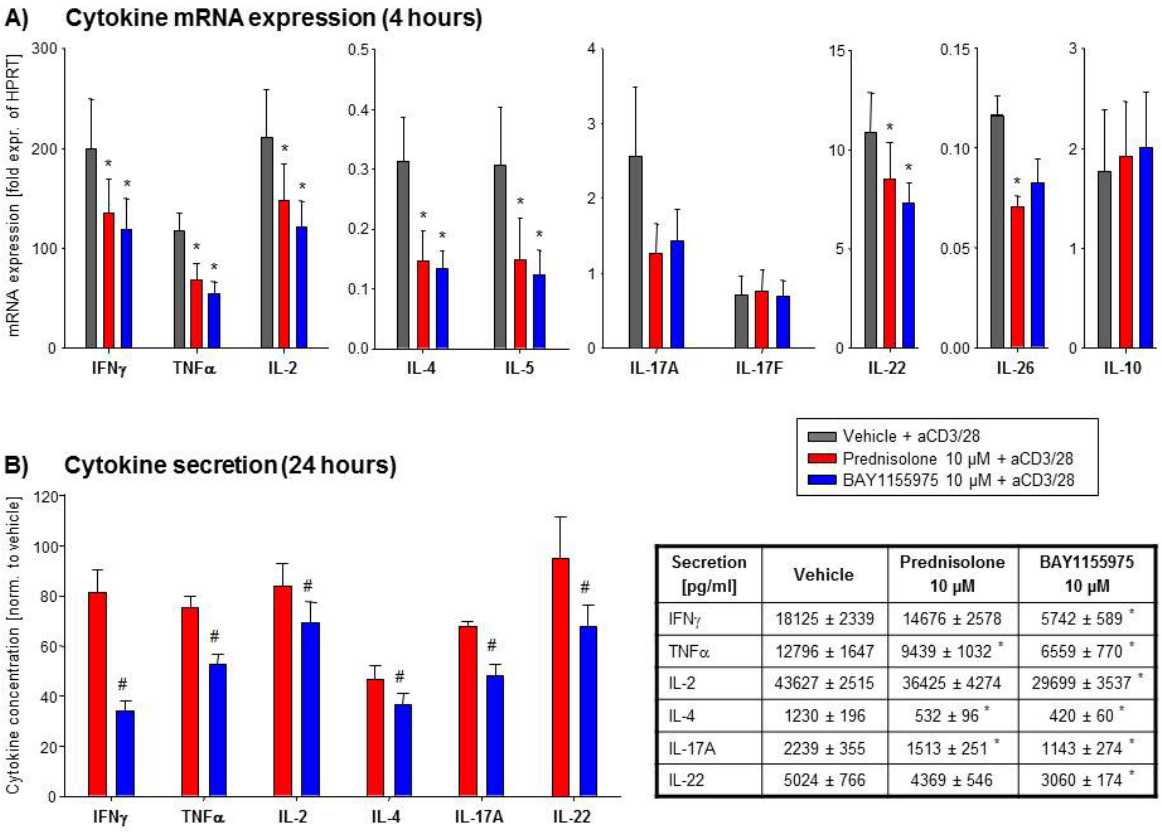
4.2.4 Differential effects on mRNA expression and secretion of T helper cell cytokines

To investigate whether BAY1155975 in comparison to prednisolone differently inhibits the secretion of other T helper cell cytokines beside IFNγ, mRNA and protein levels of various

cytokines were analyzed in separated memory/effector CD4+ T cells stimulated with plate-bound anti-CD3 and anti-CD28 monoclonal antibodies (Figure 15A+B).

The expression of the two main Th1 cytokines, IFNγ and TNFα, was suppressed by prednisolone and to a greater extent by BAY1155975 at the mRNA level (4 hours) and protein level (24 hours). BAY1155975 exhibited also a stronger inhibition of mRNA and protein expression of IL-2. Moreover, the Th2 cytokines, IL-4 and IL-5, were slightly stronger inhibited by BAY1155975 than by prednisolone on the mRNA level and for IL-4 also on protein level (IL-5 secretion was not determined). Besides Th1 and Th2 cytokines, the compound effects on the expression of Th17 cytokines, such as IL-17A, IL-17F, IL-22 and IL-26, were analyzed. BAY1155975 stronger suppressed IL-17A and IL-22 secretion in comparison to prednisolone after 24-hour stimulation (secretion of IL-17F and IL-26 was not determined). On the mRNA level, a stronger inhibition by BAY1155975 was only observed for IL-22 after 4 hours of culture whereas the expression of IL-17A and IL-26 was slightly stronger decreased by prednisolone. The mRNA expression of IL-17F was neither inhibited by prednisolone nor by BAY1155975 (Figure 15A+B).

In addition to the transrepression of the pro-inflammatory cytokines, the transcription of anti-inflammatory proteins, such as IL-10, is increased by GC treatment (Clark & Belvisi, 2012). In anti-CD28-costimulated memory/effector CD4+ T cells, the mRNA expression of IL-10 was slightly increased by prednisolone and BAY1155975 to a similar extent after 4 hours of stimulation (Figure 15A).



Secretion [pg/ml]	Vehicle	Prednisolone 10 µM	BAY1155975 10 µM
IFNγ	18125 ± 2339	14676 ± 2578	5742 ± 589 *
TNFα	12796 ± 1647	9439 ± 1032 *	6559 ± 770 *
IL-2	43627 ± 2515	36425 ± 4274	29699 ± 3537 *
IL-4	1230 ± 196	532 ± 96 *	420 ± 60 *
IL-17A	2239 ± 355	1513 ± 251 *	1143 ± 274 *
IL-22	5024 ± 766	4369 ± 546	3060 ± 174 *

Figure 15: BAY1155975 stronger inhibits cytokine generation in anti-CD28-costimulated memory/effector CD4+ T cells than prednisolone.

Separated memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (10 µg/mL) in the absence or presence of prednisolone or BAY1155975 at 10 µM. After 4 hours of culture, mRNA expression of different cytokines was determined by real-time PCR (TaqMan) as fold expression (fold expr.) of the endogenous reference gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are shown as mean + SEM of 6 donors (**A**). The secretion of various cytokines was analyzed in supernatants after 24-hour stimulation (**B**, table right) and is shown as mean + SEM of 7 donors as the percentage in comparison to vehicle-treated cultures (**B**, diagram left).
*p<0.05 vs. vehicle, #p<0.05 vs. prednisolone in Wilcoxon Signed Rank test.

In summary, BAY1155975 at 10 µM stronger than prednisolone suppressed the secretion of the effector cytokines IFNγ as well as TNFα, IL-2, IL-4, IL-17A and IL-22 in memory/effector CD4+ T cells stimulated with plate-bound anti-CD3 and anti-CD28 monoclonal antibodies.

4.2.5 Kinetics of differential effects on IFNγ secretion

To assess the kinetics of the differential inhibition of IFNγ secretion by BAY1155975 compared to prednisolone, the effect of compound treatment for various time points was analyzed in separated memory/effector CD4+ T cells stimulated with plate-bound anti-CD3 and anti-CD28.

After 4, 8, 12, 18 and 24 hours of culture, the supernatants were analyzed for IFNγ secretion (Figure 16A). Continuous stimulation with anti-CD3 and anti-CD28 monoclonal antibodies for up to 24 hours enhanced IFNγ secretion in human memory/effector CD4+ T cells, whereas the main IFNγ production occurred in the first 12 hours of stimulation. The stimulated IFNγ secretion was inhibited dose-dependently by prednisolone treatment for 4, 8, 12 and 18 hours. For 24-hour prednisolone treatment, no IFNγ inhibition was observed, IFNγ secretion was even increased by the lowest concentration of prednisolone. BAY1155975 decreased IFNγ secretion in a dose-dependent way for all time periods of culture. A stronger inhibition of IFNγ secretion by BAY1155975 than by prednisolone was observed for 8, 12, 18 and 24 hours of treatment (Figure 16A).

To characterize the impact of BAY1155975 and prednisolone treatment on the activation status of memory/effector CD4+ T cells stimulated with plate-bound anti-CD3 and anti-CD28, the surface expression of the activation antigens, CD40L, CD69 and HLA DR, was analyzed (Figure 16B-D). The expression of CD40L, a marker for assessing antigen-specific T helper cells (Frentsch et al., 2005), was induced by stimulation with anti-CD3 and anti-CD28 for 4 hours. After 8-hour stimulation, around 60% of the cells expressed CD40L, which was not considerably altered by prolonged stimulation. Prednisolone and BAY1155975 treatment dose-dependently suppressed CD40L induction after 8, 12, 18 and 24 hours (Figure 16B). The surface expression of the early activation marker CD69 was also induced by 4-hour anti-CD3 and anti-CD28 stimulation, and with continuous stimulation almost all cells expressed CD69. A slight reduction of CD69-positive cells was observed only by prednisolone treatment at the highest concentration of 10 µM for 12, 18 and 24 hours (Figure 16C). In contrast to CD40L and CD69, HLA-DR is already expressed on a small fraction of human memory/effector CD4+ T cells prior to stimulation (Figure 12, Figure 16B). The percentage of HLA-DR-expressing cells was increased even after 8 hours of anti-CD3 + anti-CD28 stimulation and peaked at 18 hours of stimulation. This early upregulation of HLA-DR after anti-CD3 + anti-CD28 stimulation is possible mediated via an increase of HLA-DR expression on recently activated cells, which are part of the separated memory/effector CD4+ T cell subset. Treatment with prednisolone and BAY1155975 for 24 hours slightly inhibited the induction of HLA-DR surface expression dose-dependently (Figure 16D).

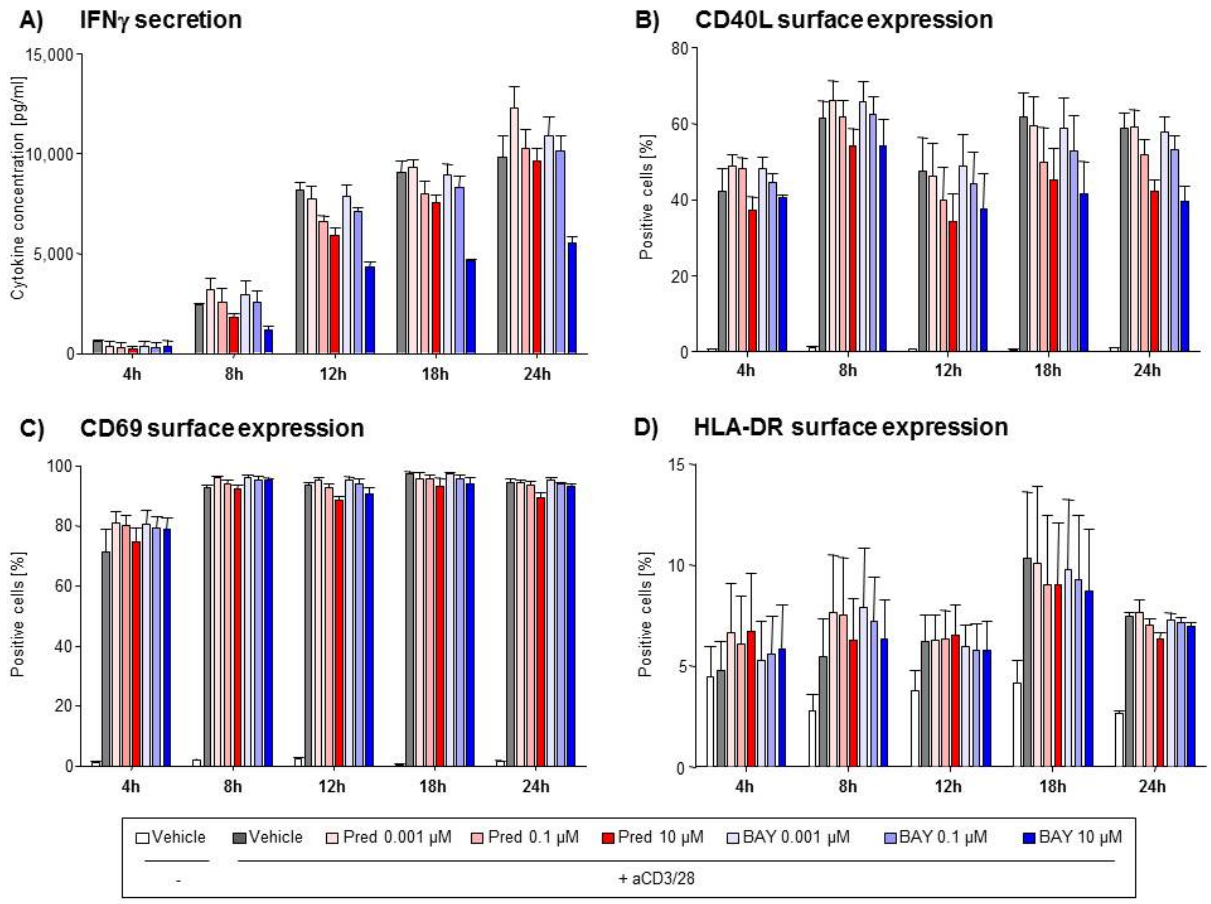


Figure 16: BAY1155975 stronger than prednisolone inhibits IFNγ secretion, but not activation antigen expression, in anti-CD28-costimulated memory/effector CD4+ T cells at various time points.

Separated memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (10 µg/mL) in the absence or presence of various concentrations of prednisolone (Pred) or BAY1155975 (BAY). After 4, 8, 12, 18 and 24 hours of culture, IFNγ secretion was analyzed in the supernatants and is shown as mean + SEM of 3 donors (**A**). At indicated time points, cells were stained with FITC-conjugated CD40L, PE-conjugated anti-CD69 and PerCP-Cy5.5-conjugated anti-HLA-DR monoclonal antibodies. The percentage of CD4+ T cells (gated by forward scatter vs. side scatter) expressing CD40L (**B**), CD69 (**C**) or HLA-DR (**D**) is shown as mean + SEM of 3 donors.

In conclusion, the stronger inhibition of IFNγ secretion by BAY1155975 compared to prednisolone was observed early after 8 hours of anti-CD3 + anti-CD28 stimulation and is not associated with a stronger suppression of the activation antigen expression of stimulated cells.

4.2.6 Differential effects on intracellular cytokine production

To characterize the differently affected cell population of memory/effector CD4+ T cells, the suppressive effect of BAY1155975 compared to prednisolone was analyzed on various subsets

of IFNγ- and TNFα-producing cells by means of intracellular cytokine detection. As the main IFNγ production occurred in the first 12 hours of stimulation (Figure 16A), cells were stimulated with plate-bound anti-CD3 + anti-CD28 and treated with BAY1155975 or prednisolone for 8 hours and for further 4 hours with supplementation of brefeldin A. Thereafter, surface expression of different membrane molecules and intracellular cytokine production were determined (Figure 17).

According to CD27 surface expression, human memory/effector CD4+ T cells can be divided into a large population expressing CD27 (around 80%) and in a smaller subset of CD27-negative cells (around 20%). The percentage of CD27- cells as well as of CD27+ cells was neither altered by anti-CD28 costimulation nor by treatment with BAY1155975 or prednisolone. However, the percentage of IFNγ- and/or TNFα-producing cells was increased by anti-CD28 costimulation in the CD27+ subpopulation and to a minor extent in the CD27- subpopulation (CD27+ cells: Figure 17A, CD27- cells: data not shown). For the CD27+ subpopulation, a similar inhibition of the percentage of IFNγ+TNFα+ and IFNγ-TNFα+ cells was observed with BAY1155975 and prednisolone treatment (Figure 17A). Within the CD27- subpopulation, the percentage of IFNγ+TNFα+ and IFNγ-TNFα+ cells was also inhibited by prednisolone and to the same extent by BAY1155975 (data not shown).

To assess the impact of BAY1155975 compared to prednisolone on activated memory/effector CD4+ T cells, cells expressing the early activation marker CD69 on their surface were analyzed. By dividing the CD69+ cells into CD40L+ and CD40L- cells, TCR-activated memory/effector CD4+ T cells and unspecifically activated memory/effector CD4+ T cells can be separated. After 12 hours of anti-CD28 costimulation, almost all cells expressed CD69, whereas about 60% were CD69+CD40L- cells and about 40% were CD69+CD40L+ cells. Treatment with BAY1155975 or prednisolone showed no influence on the percentage of CD69+CD40L- or CD69+CD40L+ cells. As the CD69+CD40L+ cells represent specific TCR-activated cells, the cytokine production of this memory/effector CD4+ T cell subpopulation was further analyzed. The percentage of IFNγ+TNFα-, IFNγ+TNFα+ and IFNγ-TNFα+ cells was induced by anti-CD28 costimulation, whereas the IFNγ-TNFα+ subset was the most increasing fraction. BAY1155975 and prednisolone inhibited the percentage of IFNγ+TNFα+ and IFNγ-TNFα+ cells to a similar extent. An increase of the geometric mean fluorescence intensity (gMFI) for IFNγ was observed in the CD69+CD40L+ IFNγ+TNFα+ subset by prednisolone. In contrast, BAY1155975 slightly suppressed the gMFI for IFNγ and TNFα in all three cytokine-producing cell populations suggesting an inhibition of the secretion level by BAY1155975 (Figure 17B).

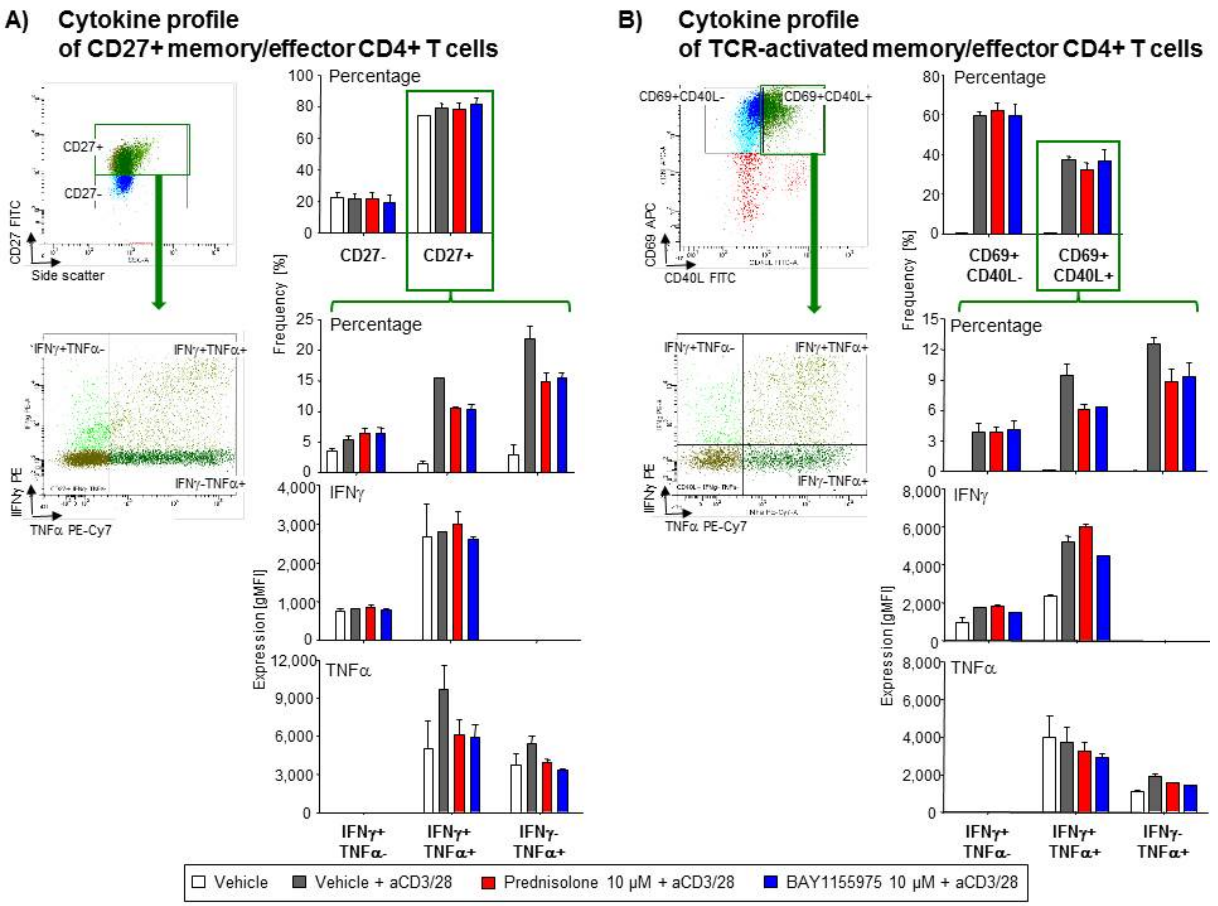


Figure 17: BAY1155975 stronger inhibits the secretion level in cytokine producing-subsets of anti-CD28-costimulated memory/effector CD4+ T cells compared to prednisolone.

Separated memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (10 µg/mL) in the absence or presence of prednisolone or BAY1155975 for 8 hours, washed and further stimulated and treated for 4 hours in the presence of brefeldin A. One fraction of cells was stained with FITC-conjugated anti-CD27, PE-conjugated anti-IFNγ and PE-Cy7-conjugated anti-TNFα monoclonal antibodies. CD4+ T cells (gated by forward scatter vs. side scatter) were separated in CD27+ and CD27- cells, which were further divided according their cytokine profile (**A**).The other fraction was stained with FITC-conjugated anti-CD40L, PE-conjugated anti-IFNγ, PE-Cy7-conjugated anti-TNFα and APC-conjugated anti-CD69 monoclonal antibodies. CD69+CD40L- and CD69+CD40L+ cells were gated and further subdivided according to their cytokine expression (**B**). The percentage of each subset and the geometric mean fluorescence intensity (gMFI) of anti-IFNγ-PE and anti-TNFα-PE-Cy7 are shown as mean + SEM of 2 donors.

To sum up, BAY1155975 similarly suppressed IFNγ- and/or TNFα-producing cells in the CD27+ as well as in the CD27-subpopulation of memory/effector CD4+ T cells as prednisolone. In TCR-activated memory/effector CD4+ T cells, BAY1155975 inhibited the percentage of IFNγ- and/or TNFα-producing cells to the same extent than prednisolone. In contrast, the secretion

levels of IFNγ and TNFα were only suppressed by BAY1155975. These results suggested that BAY1155975 does not inhibit the percentage but the secretion level of IFNγ and TNFα in subsets of CD28-costimulated memory/effector CD69+ CD40L+ CD4+ T cells.

By analyzing various T cell subpopulations, it was shown that 10 µM BAY1155975 stronger suppressed the secretion of IFNγ in human memory/effector CD4+ T cells stimulated with plate-bound anti-CD3 and anti-CD28 monoclonal antibodies than prednisolone. This enhanced inhibition by BAY1155975 was observed early after 8 hours of treatment and is not due to an increased induction of apoptosis, reduced proliferation of activated cells or different suppression of the activation status as assessed by activation antigen expression. The secretion of other effector cytokines, like TNFα, IL-2, IL-4, IL-17A and IL-22, was also stronger inhibited by BAY1155975. By analyzing the compound effects on various subsets of IFNγ- and/or TNFα-producing cells, a suppressive activity of BAY1155975 on the cytokine secretion level in TCR-activated memory/effector CD4+ T cells is suggested.

4.3 Characterization of differential mechanisms of BAY1155975 and glucocorticoids in human memory/effector CD4+ T cells

As described, the secretion of effector cytokines is stronger suppressed by 10 µM BAY1155975 than by 10 µM prednisolone in human memory/effector CD4+ T cells. To evaluate a possible different mechanism of BAY1155975 versus prednisolone, the involvement of three signaling pathways for the inhibitory effect was analyzed in the next step.

By co-treatment with the GR antagonist RU-486 and GR gene silencing, the role of GR for inhibition of cytokine secretion can be determined. It has been demonstrated that RU-486 reverses the inhibitory effect of dexamethasone on IFNγ production by human lymphocytes indicating a GR-dependency also of effects on T cell IFNγ secretion (Agarwal & Marshall, 1998; Zhang et al., 2005).

Furthermore, the involvement of MEK/ERK signaling for the stronger suppressive effect of BAY1155975 can be analyzed with the specific MEK/ERK inhibitor, U0126. It was shown that the abrogated inhibitory effect of dexamethasone on the CD28-costimulated proliferation of human naïve CD4+ T cells was restored by addition of U0126. These results suggested that enhanced MEK/ERK signaling by anti-CD28 costimulation is essential for resistance of human T cells to GCs (Li et al., 2004; Tsitoura & Rothman, 2004). By addition of U0126 to BAY1155975-treated cells the role of MEK/ERK signaling for the stronger inhibitory effect of BAY1155975 can be examined.

As calcium (Ca^{2+}) signaling regulates the activation of human T cells, the effects of BAY1155975 and prednisolone on different Ca^{2+}-mediated activation pathways were determined (Smith-Garvin et al., 2009). By co-treatment with the L-type Ca^{2+} (Ca_v1) channel antagonist nifedipine, the role of Ca^{2+} influx through Ca_v1 channels for the stronger inhibitory activity of BAY1155975 can be analyzed. The involvement of the activity of calcineurin and the ensuing nuclear translocation of nuclear factor of activated T cells (NF-AT) and NF-κB can be investigated by simultaneous addition of cyclosporine A (CsA) (Nishiyama et al., 2005). As the calcium ionophore, ionomycin, raises the intracellular Ca^{2+} concentration (Yoshioka et al., 2007), the compound effects on Ca^{2+} mobilization can be analyzed in ionomycin-costimulated cells.

4.3.1 Involvement of glucocorticoid receptor signaling

To examine whether the stronger suppressive effect of BAY1155975 on effector cytokine secretion is mediated via the GR, separated human memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies and co-treated with BAY1155975 or prednisolone and the GR antagonist, RU-486.

In anti-CD28-costimulated memory/effector CD4+ T cells, treatment with 10 µM RU-486 alone had only minor effects on IFNγ secretion, whereas for higher concentrations of RU-486 a considerable decrease of IFNγ secretion was observed (Figure 18, data not shown). Therefore, RU-486 was used at 10 µM in the experiment. Co-treatment with prednisolone and RU-486 did not suppress IFNγ secretion, and prednisolone treatment alone had also no effect on IFNγ secretion. Interestingly, the inhibitory effect on IFNγ secretion, which was observed with BAY1155975, was not ameliorated by co-treatment with 10 µM RU-486 in these cells (Figure 18).

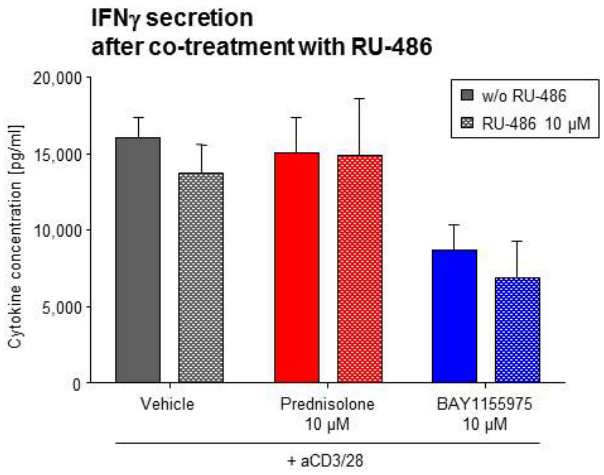


Figure 18: BAY1155975 mediated inhibition of IFNγ secretion in anti-CD28-costimulated memory/ effector CD4+ T cells is not altered by co-treatment with the GR antagonist, RU-486.

Separated memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (10 µg/mL) and treated with vehicle, prednisolone or BAY1155975 in the absence or presence of the glucocorticoid receptor antagonist, RU-486. After 24 hours, IFNγ secretion was determined in the supernatants and is shown as mean + SEM of 3 donors.

Since the failing prevention of the inhibitory BAY1155975 effect on IFNγ secretion might have also been due to a not sufficient concentration of RU-486 and in order to further explore the role of GR signaling, GR expression was silenced in separated human memory/effector CD4+ T cells via small interfering RNA (siRNA). At the mRNA level, the GR expression was reduced to 30% in cells transfected with GR siRNA as compared to non-targeting siRNA-transfected cells at 18 hours after transfection (Figure 19A). GR silencing was also confirmed at the protein level by Western blot. At 48 hours after transfection, only a slight GR protein band was observed in the cells transfected with GR siRNA (Figure 19B).

After transfection, cells were stimulated with plate-bound anti-CD3 and anti-CD28 and treated with prednisolone or BAY1155975 for 24 hours. In non-transfected cells a strong inhibition of IFNγ secretion was observed by prednisolone and BAY1155975. The strong suppressive effect of prednisolone in these cells was possible due to a not sufficiently strong costimulation with the anti-CD28 monoclonal antibody as the stimulated IFNγ secretion was markedly lower than in the other experiments.

Transfection of memory/effector CD4+ T cells with GR siRNA reduced the inhibitory effect of prednisolone on IFNγ secretion by around 50%. In contrast, the inhibitory effect of BAY1155975 was only slightly decreased in GR siRNA-transfected cells (Figure 19C).

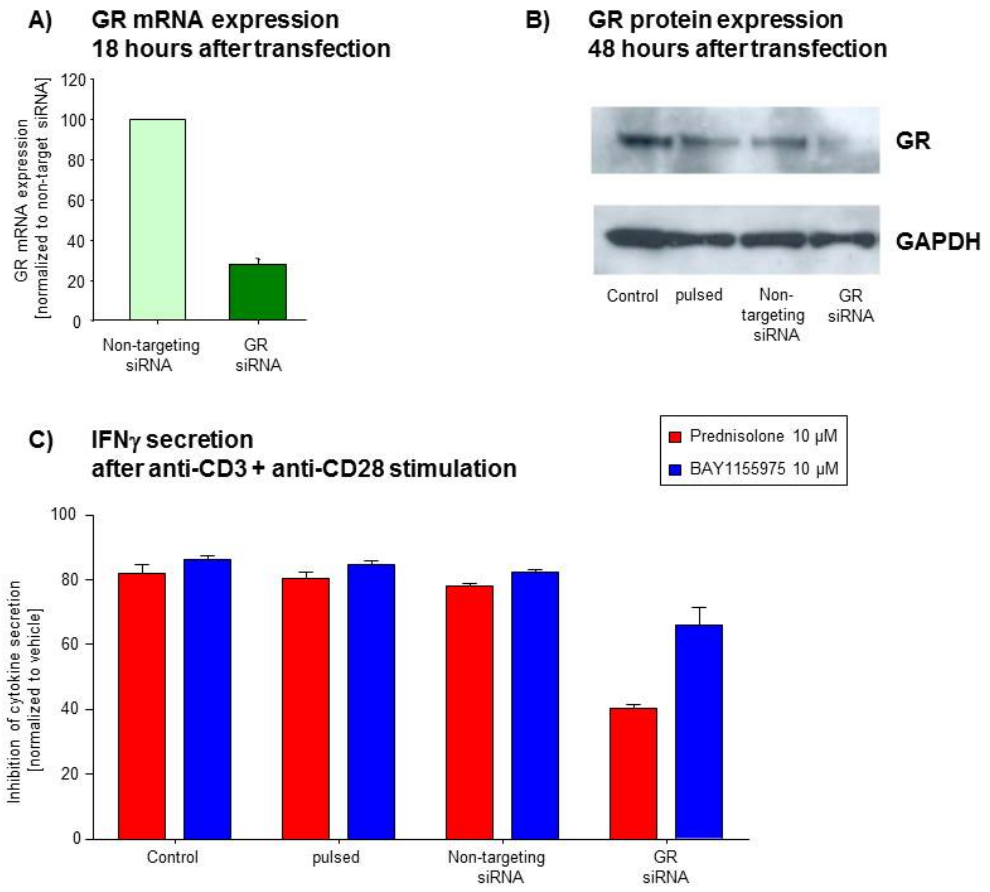


Figure 19: BAY1155975 exhibits a widely sustained inhibition of IFNγ secretion in anti-CD28-costimulated memory/effector CD4+ T cells after GR gene silencing.

Separated memory/effector CD4+ T cells were transfected with non-targeting or GR siRNAs. A non-transfected control and a pulsed control (transfection without siRNA) were additionally generated. After 18 hours of culture, GR mRNA expression was determined by real-time PCR (TaqMan). GR mRNA expression in GR siRNA-transfected cells is shown as percentage of that in non-targeting siRNA-transfected cells (mean + SEM of 3 donors) (**A**). The GR protein expression was determined by Western blot at 48 hours after transfection. Results from one donor are shown (**B**). After 48 hours of culture, transfected cells were stimulated with plate-bound anti-CD3 and anti-CD28 (10 µg/mL) and treated with vehicle, prednisolone or BAY1155975 for 24 hours. IFNγ secretion in supernatants was determined and is shown as mean + SEM of 3 donors as the percent inhibition in comparison to vehicle-treated cultures (IFNγ vehicle control 4485±524 pg/ml, pulsed 2223±290 pg/ml, non-targeting siRNA 2048±294, GR siRNA 1153±65 pg/ml) (**C**).

In summary, the inhibitory effect of 10 µM BAY1155975 on IFNγ secretion in CD28-costimulated memory/effector CD4+ T cells was not prevented by co-treatment with the GR antagonist RU-486 or by GR gene silencing.

4.3.2 Involvement of MEK/ERK signaling

To investigate the possible involvement of MEK/ERK signaling in the improved inhibition of anti-CD28-costimulated IFNγ secretion by BAY1155975, separated human memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 plus anti-CD28 and treated with BAY1155975 or prednisolone in the presence of increasing concentrations of the MEK/ERK inhibitor, U0126. IFNγ secretion was dose-dependently inhibited by treatment with U0126 alone, indicating that the MEK/ERK signaling is involved in anti-CD28-costimulated IFNγ secretion (Figure 20A). Co-treatment with prednisolone and 5 µM U0126 slightly enhanced the inhibitory activity of prednisolone on secretion of IFNγ. The suppressive effect of BAY1155975 on IFNγ secretion was also enhanced with increasing concentrations of U0126. However, a stronger inhibitory effect of BAY1155975 compared to prednisolone was still observed even when adding 5 µM of U0126 (Figure 20B).

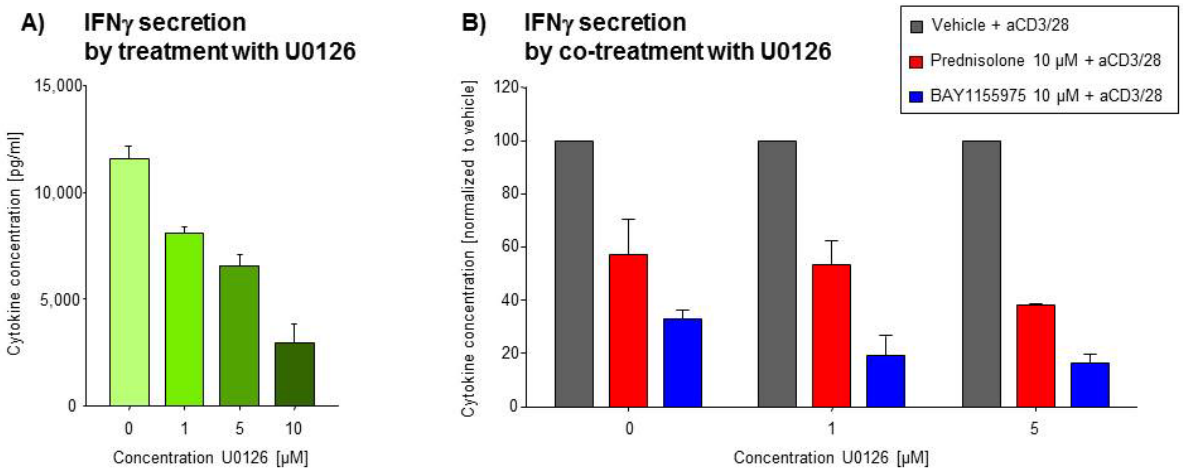


Figure 20: Co-treatment with MEK/ERK inhibitor does not abrogate the stronger inhibitory effect of BAY1155975 on IFNγ secretion in anti-CD28-costimulated memory/effector CD4+ T cells.

Separated memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the absence or presence of increasing concentrations of the MEK/ERK inhibitor, U0126. After 24 hours, IFNγ secretion was determined in the supernatants and is shown as mean + SEM of 2 donors (**A**). Stimulated cells were co-treated with prednisolone or BAY1155975 and increasing concentrations of U0126 for 24 hours. IFNγ secretion in the supernatants is shown as mean + SEM of 2 donors as the percentage in comparison to vehicle-treated cultures (**B**).

To sum up, the weak inhibitory effect of prednisolone on IFNγ secretion in memory/effector CD4+ T cells was not notably improved by addition of the MEK/ERK inhibitor U0126.

4.3.3 Enhanced inhibition of calcium-mediated activation pathways by BAY1155975

To assess the possible inhibitory effect of BAY1155975 on Ca^{2+} signaling regulating the activation of human memory/effector CD4+ T cells, the compound effects on different Ca^{2+}-mediated activation pathways were determined.

First, the effect of BAY1155975 compared to that of prednisolone on the calcium-calcineurin-NFAT pathway was analyzed. TCR engagement initiates a rapid Ca^{2+} influx into T cells, which leads to the activation of calcineurin and the subsequent transcriptional activity of NFAT (Vig & Kinet, 2009).

To determine the role of Ca_v1 channels, separated human memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 plus anti-CD28 and treated with BAY1155975 or prednisolone in the presence of the Ca_v1 channel antagonist nifedipine. As the secretion of IFNγ was inhibited by treatment with 70 µM nifedipine alone, the Ca^{2+} influx through Ca_v1 channels seems to be involved in the anti-CD28-costimulated IFNγ secretion. There was only a negligible additive effect of nifedipine and prednisolone co-treatment. In contrast, a clear additive effect or even some synergistic effect on inhibiting IFNγ secretion was observed for co-treatment with nifedipine and BAY1155975 (Figure 21A). So, BAY1155975 led to a reduction of IFNγ secretion by 52% in non-nifedipine treated cultures whereas it inhibited IFNγ secretion in nifedipine-treated cultures by 69%.

By co-treatment with the calcineurin inhibitor cyclosporine A (CsA) the role of calcineurin activity for the differential compound effects can be analyzed. Treatment of anti-CD28-costimulated memory/effector CD4+ T cells with 0.7 µM CsA inhibited the IFNγ secretion by around 50%. Co-treatment with prednisolone and CsA strongly enhanced the minor suppressive activity of prednisolone on IFNγ secretion. An almost complete inhibition of IFNγ secretion was observed by co-treatment with BAY1155975 and CsA (Figure 21B).

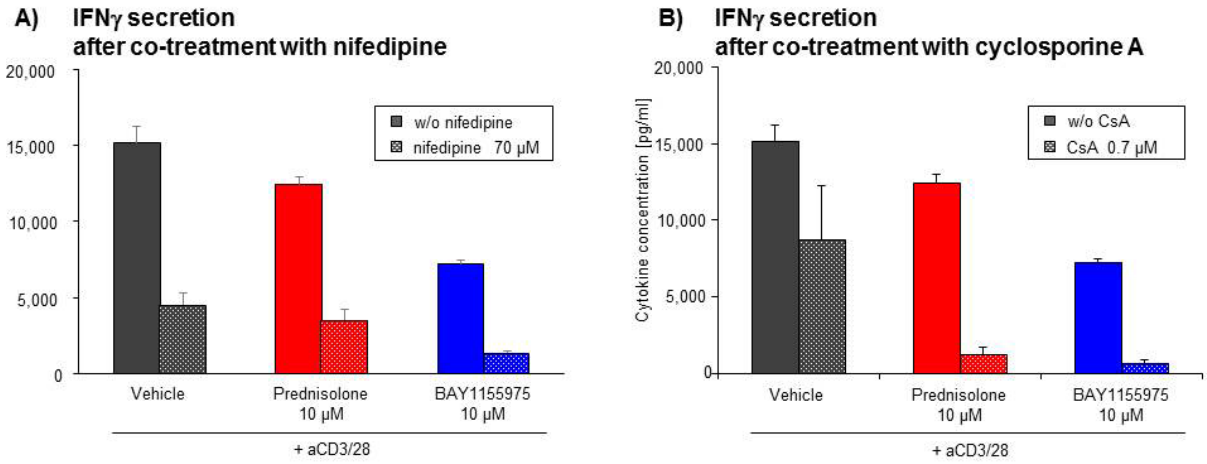


Figure 21: The suppressive effect of BAY1155975 on IFNγ secretion in anti-CD28-costimulated memory/effector CD4+ T cells is enhanced by blocking the calcium-calcineurin-NFATpathway.

Separated memory/effector CD4+ T cells were stimulated with plate-bound anti-CD3 plus anti-CD28 and treated with vehicle, prednisolone or BAY1155975 in the absence or presence of the Ca_v1 channel antagonist, nifedipine (**A**), or cyclosporine A (CsA) (**B**). After 24 hours, IFNγ secretion was determined in the supernatants and is shown as mean + SEM of 2 donors.

To examine the compound activity on Ca^{2+} mobilization during activation of human memory/effector CD4+ T cells, the cells were costimulated with ionomycin. The anti-CD3-induced IFNγ secretion in memory/effector CD4+ T cells was increased by costimulation with ionomycin. Interestingly, the inhibitory activity of prednisolone was strongly diminished, whereas BAY1155975 still almost completely suppressed the IFNγ secretion induced by anti-CD3 and ionomycin stimulation (Figure 22A).

Stimulation of the protein kinase C (PKC)-dependent pathway by phorbol 12-myristate 13-acetate (PMA) was not sufficient to induce secretion of IFNγ in memory/effector CD4+ T cells (data not shown). By synergistic enhancement of PKC activation via PMA + ionomycin stimulation, memory/effector CD4+ T cells secreted high amounts of IFNγ as observed by anti-CD3 and anti-CD28 stimulation. The suppressive effect of prednisolone was abrogated by PMA plus ionomycin stimulation as in anti-CD3 plus anti-CD28 stimulated cells. In contrast, treatment with BAY1155975 markedly decreased IFNγ secretion stimulated by PMA plus ionomycin (Figure 22A). In addition to IFNγ, the secretion of IL-2 and IL-22 was also not inhibited by prednisolone, whereas treatment with BAY1155975 markedly suppressed the PMA plus ionomycin-stimulated secretion of both effector cytokines. Moreover, BAY1155975 stronger inhibited secretion of TNFα, IL-4 and IL-17A than prednisolone in PMA plus ionomycin-stimulated cells (Figure 22B).

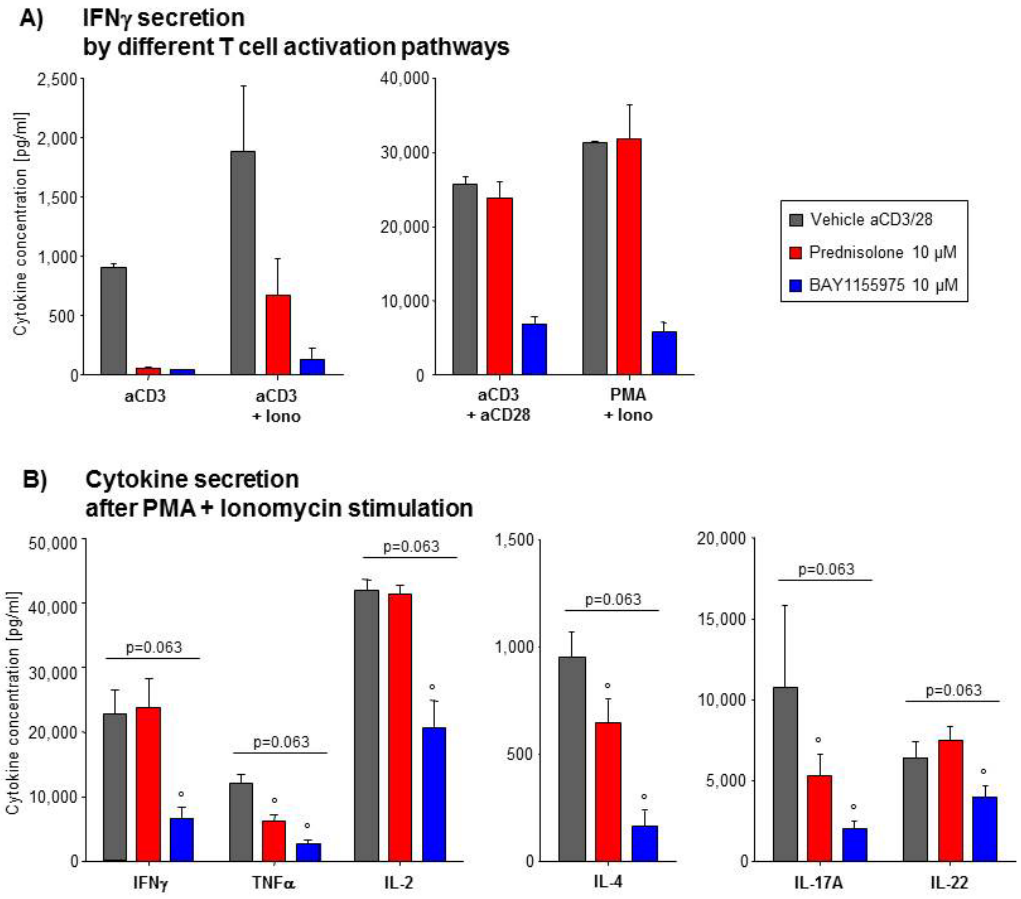


Figure 22: BAY1155975 shows stronger inhibition of Ca^{2+}-mediated cytokine secretion in memory/effector CD4+ T cells than prednisolone.

Separated cells were stimulated with plate-bound anti-CD3, plate-bound anti-CD3 plus ionomycin (Iono), plate-bound anti-CD3 plus anti-CD28 or PMA plus ionomycin in the absence or presence of prednisolone or BAY1155975. After 24 hours of culture, secretion of various cytokines was analyzed in the supernatants and is shown as mean + SEM of 2 donors (**A**) or 5 donors (**B**).

°p=0.063 vs. vehicle, p=0.063 vs. prednisolone in Wilcoxon Signed Rank test.

As the strongest differential effect of BAY1155975 versus prednisolone on IFNγ secretion is observed in PMA plus ionomycin stimulated memory/effector CD4+ T cells, the suppression of intracellular IFNγ and TNFα production by both compounds was characterized in more detail in these cells.

Cells were stimulated with PMA plus ionomycin and treated with BAY1155975 or prednisolone for 8 hours and for further 4 hours after addition of brefeldin A. After the strong stimulation with PMA plus ionomycin, almost all cells expressed the early activation marker CD69, and 90% of the cells were CD40L-positive (data not shown). The percentage of CD27+ cells was increased and consequently the proportion of CD27- cells was decreased by PMA plus ionomycin stimulation. Treatment with BAY1155975 or prednisolone did not regulate the percentage of

CD27- and CD27+ cells (CD27- cells: data not shown, CD27+ cells: Figure 23).

Within the CD27+ subpopulation, a marked increase of IFNγ+TNFα+ cells and IFNγ-TNFα+ cells was observed after stimulation with PMA plus ionomycin, whereas the fraction of IFNγ+TNFα- cells was not increased. Prednisolone slightly suppressed the percentage of IFNγ+TNFα+ cells and IFNγ-TNFα+ cells. The geometric mean fluorescence intensity for IFNγ was increased by prednisolone in the IFNγ+TNFα+ subset, indicating that prednisolone treatment enhanced IFNγ expression in these cells. In contrast, intracellular TNFα expression was diminished by prednisolone in the IFNγ+TNFα+ and the IFNγ-TNFα+ subset. Treatment with BAY1155975 stronger decreased the percentage of IFNγ+TNFα+ cells and IFNγ-TNFα+ cells as well as the intracellular IFNγ and/or TNFα expression in both subsets than prednisolone (Figure 23).

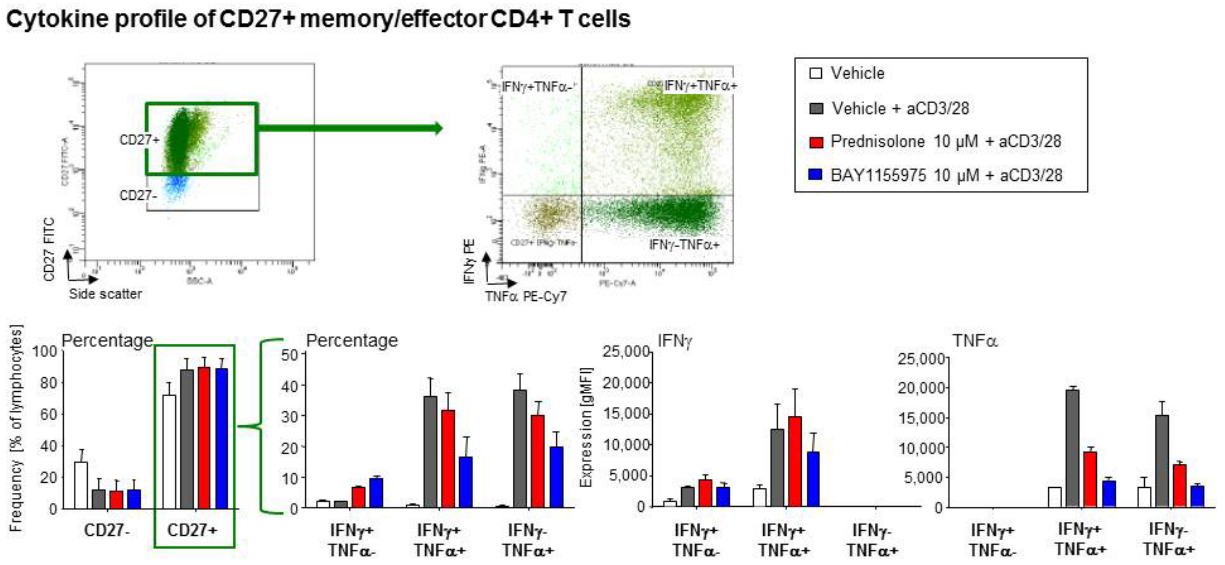


Figure 23: BAY1155975 exhibits a stronger inhibition of Ca^{2+}-mediated cytokine production in CD27+ memory/effector CD4+ T cells than prednisolone.

Separated memory/effector CD4+ T cells were stimulated with PMA plus ionomycin in the absence or presence of prednisolone or BAY1155975 for 8 hours, washed and further stimulated in the presence of brefeldin A for 4 hours. Next, cells were stained with FITC-conjugated anti-CD27, PE-conjugated anti-IFNγ and PE-Cy7-conjugated anti-TNFα monoclonal antibodies. CD4+ T cells (gated by forward scatter vs. side scatter) were separated in CD27+ and CD27- cells, which were further divided according to their cytokine profile. The percentage of each subset and the geometric mean fluorescence intensity (gMFI) of anti-IFNγ-PE and anti-TNFα-PE-Cy7 are shown as mean + SEM of 2 donors.

In summary, BAY1155975 stronger suppressed the effector cytokine secretion in memory/effector CD4+ T cells stimulated with PMA plus ionomycin, whereas the inhibitory effect of prednisolone was widely abrogated in these cells. By analyzing the differential effect in PMA plus Ionomycin stimulated memory/effector CD4+ T cell subsets, a stronger inhibition of the intracellular cytokine production in IFNγ+TNFα+ and IFNγ-TNFα+ cells in the CD27+ subpopulation was observed with BAY1155975.

By analyzing different mechanisms that might account for the stronger inhibitory effect of BAY1155975 on CD28-costimulated effector cytokine secretion, it was shown that the enhanced suppressive activity of BAY1155975 could not be prevented by inhibition of GR or MEK/ERK signaling. However, by inhibiting the calcineurin activity via addition of CsA a similar suppression of IFNγ secretion was observed in prednisolone- and BAY1155975-treated memory/effector CD4+ T cells. Moreover, costimulation with the calcium ionophore, ionomycin, mimicked the differential cytokine inhibition pattern that had been observed with anti-CD28-costimulation in activated memory/effector CD4+ T cells. BAY1155975 stronger suppressed the cytokine secretion in ionomycin-costimulated memory/effector CD4+ T cells than prednisolone.

Concluding from these *in vitro* data, the enhanced inhibition of IFNγ secretion exhibited by 10 µM BAY1155975 in anti-CD28-costimulated memory/effector CD4+ T cells is not due to an increased induction of apoptosis, reduced proliferation of activated cells or different suppression of the activation status as assessed by activation antigen expression.
Remarkably, the investigation of the compound effects on different Ca^{2+}-mediated activation pathways suggested that BAY1155975 stronger suppressed the calcium-calcineurin-NFAT pathway than prednisolone.

4.4 Effects of BAY1155975 and glucocorticoids in contact hypersensitivity models in mice

In vivo, the suppressive effect of BAY1155975 and prednisolone on CD28-costimulated T cell activation was compared using different treatment modalities in an allergic contact dermatitis model. NMRI mice were sensitized at day 0 and day 1 on the flank skin and challenged at day 5 on the dorsal site of both ears with the hapten, 2,4-dinitro-1-fluorobenzene (DNFB). In a prolonged model, three hapten challenges were performed at days 5, 6 and 7. The T cell-mediated inflammatory response in the skin was evaluated at 24 hours after the last challenge by measuring the ear weight and peroxidase activity in ear homogenates.

By application of compounds prior to hapten challenge, their anti-inflammatory and immunosuppressive activity can be determined. Compound treatment during sensitization affects the activation and expansion of hapten-specific T cells, which require interactions of CD28 with members of the B7 family. The necessity of CD28 ligation for an optimal induction of the CHS response was shown in mice deficient for the CD28 molecule. Such CD28 -/- mice showed a decreased DNFB-induced skin inflammation (Kondo et al., 1996). Therefore, when using application of compounds during sensitization, their inhibitory effect on CD28-stimulated T cell activation is likely to be reflected by a decreased inflammatory response to the hapten challenge.

4.4.1 Treatment prior to challenge

To examine the anti-inflammatory and immunosuppressive activity of BAY1155975 compared to prednisolone in the DNFB-induced allergic contact dermatitis model, compounds were applied 2 hours before DNFB challenge. Systemic application of 30 mg/kg prednisolone prior to challenge significantly inhibited the DNFB-induced edema formation, which is assessed by an increase in ear weights. A similarly strong and also significant suppression of the inflammatory edema was observed by treatment with 30 mg/kg BAY1155975 prior to challenge (Figure 24A). The peroxidase activity in ear homogenates as parameter for granulocyte infiltration was also significantly diminished by prednisolone and BAY1155975, whereas a superior inhibitory activity for BAY1155975 was detected (Figure 24B).

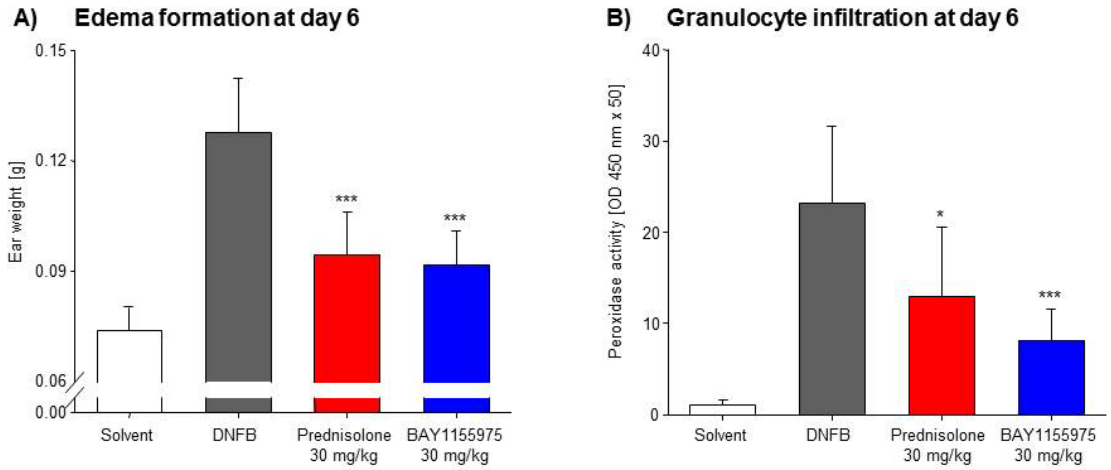


Figure 24: BAY1155975 and prednisolone treatment prior to hapten challenge inhibits T cell dependent skin inflammation in mouse DNFB model to a similar extent.

Female NMRI mice were sensitized on flank skin (day 0 and 1) and challenged on both ears (day 5) with 2,4-dinitro-1-fluorobenzene (DNFB). The negative control group (Solvent) was exposed to the solvent for sensitization and challenge treatment. Prednisolone and BAY1155975 were administered orally at 2 hours before challenge on day 5. Ear weights (**A**) and peroxidase activity in ear homogenates (**B**) were determined at 24 hours after challenge and are shown as mean + SD of 10 mice per group.
*p<0.05, ***p<0.001 vs. DNFB in Mann-Whitney U test.

4.4.2 Treatment around sensitization

To assess the inhibitory effect of BAY1155975 and prednisolone on CD28-costimulated T cell activation, mice were treated during sensitization at day 0, day 1 and day 2. Prednisolone at 30 mg/kg only slightly suppressed the DNFB-induced edema formation as assessed by increased ear weights. Remarkably, a clearly stronger inhibitory effect was demonstrated for treatment with 30 mg/kg BAY1155975 (Figure 25A). Moreover, by enhancing the inflammatory response with repeated DNFB-challenges at days 5, 6 and 7, no inhibition of edema formation at all was observed with 30 mg/kg prednisolone. In contrast, the treatment with 30 mg/kg BAY1155975 around sensitization significantly decreased the T cell-dependent skin inflammation after 3 hapten challenges (Figure 25B).

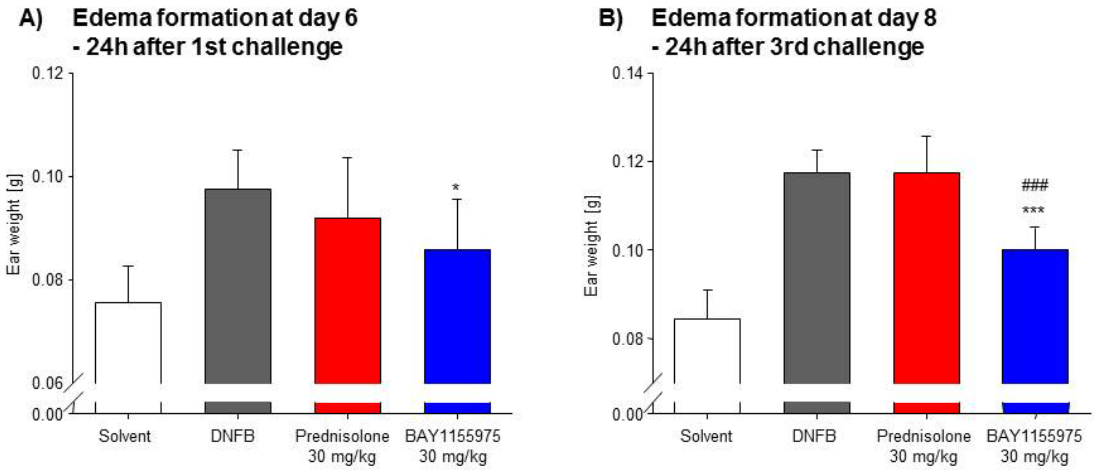


Figure 25: BAY1155975 treatment around sensitization results in stronger inhibition of T cell-dependent skin inflammation in mouse DNFB models than prednisolone treatment.

Female NMRI mice were sensitized with 2,4-dinitro-1-fluorobenzene (DNFB) on flank skin at day 0 plus 1 and were challenged with DNFB on both ears at day 5 (**A**) or furthermore at days 6 and 7 (**B**). In each model, mice of the negative control group (Solvent) were exposed to the solvent instead of DNFB. Oral treatment with prednisolone or BAY1155975 was performed at 2 hours before sensitization treatments with DNFB on day 0 and 1 and additionally on day 2. At 24 hours after the last challenge, ears were removed and weighted. Data are shown as mean + SD of 7/8 mice per group.
*p<0.05, ***p<0.001 vs. DNFB , ###p<0.001 vs. prednisolone in Mann-Whitney U test.

The effect of BAY1155975 and prednisolone treatment around sensitization was further examined by assessing T cell cytokines in inflamed ears at 24 hours after the first DNFB-challenge. DNFB-induced skin inflammation was associated with increased levels of IFNγ, TNFα, IL-2, IL-4 and IL-5, whereas no up-regulation of IL-10 was observed (Figure 26, IL-10 data not shown). Prednisolone as well as BAY1155975 significantly inhibited the up-regulation of these cytokines, whereas a slight stronger suppression with BAY1155975 treatment for the expression of TNFα, IL-2, IL-4 and IL-5 was observed (Figure 26).

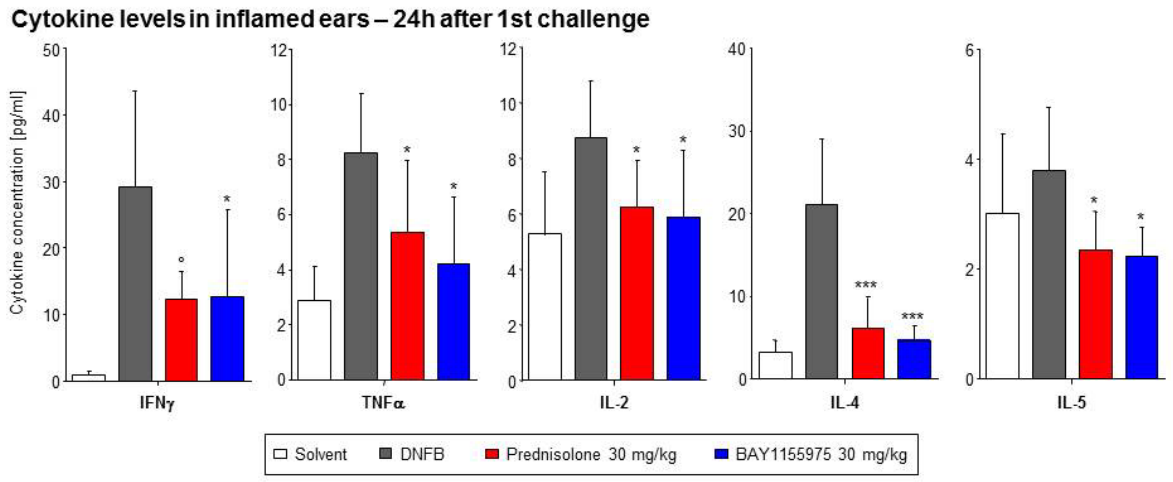


Figure 26: BAY1155975 and prednisolone treatment around sensitization ameliorates cytokine expression in inflamed ears after challenge in mouse DNFB model.

Female NMRI mice were sensitized on flank skin at day 0 and 1 and were challenged on both ears at day 5 with solvent or 2,4-dinitro-1-fluorobenzene (DNFB). Prednisolone and BAY1155975 were administered orally at 2 hours before sensitization treatments on day 0 and 1 and additionally on day 2. Cytokine levels in ear homogenates were determined at 24 hours after challenge and are shown as mean + SD of 7/8 mice per group.

°p=0.054,*p<0.05, ***p<0.001 vs. DNFB in Mann-Whitney U test.

In summary, BAY1155975 and prednisolone significantly inhibited the DNFB-induced edema formation in mice ears, when mice were treated with the compounds prior to hapten challenge. In contrast, when the compounds were applied around hapten sensitization markedly stronger inhibition of T cell-dependent skin inflammation was observed for BAY1155975 than for prednisolone. These data indicate that BAY1155975 has a stronger inhibitory effect on CD28-dependent sensitization in DNFB-induced CHS model than prednisolone.

5. Discussion

5.1 Glucocorticoid receptor ligands for anti-inflammatory therapy

Glucocorticoids (GCs) are the most commonly used drugs for the treatment of inflammatory diseases, such as asthma and allergy as well as autoimmune diseases and allograft rejections (Inamoto & Flowers, 2011; Krause et al., 2011; Coutinho & Chapman, 2011). Unfortunately, their therapeutic action is often accompanied with the appearance of numerous and sometimes irreversible side-effects, including diabetes mellitus, osteoporosis or skin atrophy, mainly after high-dose and long-term treatment (Schäcke et al., 2008; Smits et al., 2011; Mazzantini et al., 2012). Especially when children are treated, e.g. for atopic dermatitis or asthma, particularly systemic side-effects of GC application, such as growth suppression and thymus atrophy, have to be seriously considered (Bjelaković et al., 2009; Wolthers, 201; Blume-Peytavi & Wahn, 2011).

GCs exert their biological effects primarily by binding to the glucocorticoid receptor (GR), which translocates into the nucleus upon activation and, by acting as transcription factor (TF), regulates the transcription of GC-sensitive genes either positively (transactivation) or negatively (transrepression). The transrepression (TR) of many pro-inflammatory molecules due to binding of the GR monomer to other TFs such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) and thereby inhibiting their transcriptional activity is widely considered to be one key mechanism for the anti-inflammatory and immunosuppressive effects of GCs (Beck et al., 2009; Clark & Belvisi, 2012). On the contrary, some severe side-effects of GC treatment, like thymocyte apoptosis and metabolic disturbances, are predominantly mediated via transactivation (TA) of genes due to binding of the GR dimer to DNA (Schäcke et al., 2008; Woodward et al., 2010). By *in vitro* and *in vivo* studies with a dimerization-deficient GR, it has been demonstrated that the TR mechanism of the ligand-activated GR can be separated from the TA activities (Heck et al., 1994; Reichardt & Schütz, 1998). Therefore, the search for novel GR ligands, which should exhibit potent anti-inflammatory efficacy by sustained TR activity but less TA-mediated side-effects and thereby a better therapeutic index, has become a pharmaceutical goal (Schäcke et al., 2007; Löwenberg et al., 2008; De Bosscher et al., 2010). Thus, several novel GR ligands with a dissociated profile, such as the SEGRA compound BAY1155975, have been developed in the last 20 years.

5.1.1 Compounds with dissociated transrepression/transactivation profile

One of the first described novel GR ligands was the steroidal compound, RU 24858. RU 24858 showed strong TR but less TA activity *in vitro* and an anti-inflammatory efficacy similar to prednisolone in a mouse irritant contact dermatitis model (Vayssière et al., 1997). However, RU 24858 treatment in rats induced systemic side-effects, such as loss of body weight, thymus involution and induction of bone loss, with comparable potency to classical GCs. This might be due to a loss of the dissociated profile of RU 24858 *in vivo* (Belvisi et al., 2001; Tanigawa et al., 2002). Later the compound was shown to have TA capabilities on several GR-regulated genes, among genes with known anti-inflammatory functions, similar to classical GCs (Janka-Junttila et al., 2006; Newton et al., 2010).

An improved beneficial/side-effect ratio *in vivo* has been shown for the non-steroidal GR ligand, AL-438. The differential *in vitro* gene regulation by AL-438 was associated with an anti-inflammatory activity that was comparable to prednisolone in rat models of acute and chronic inflammation. Remarkably, however, in contrast to prednisolone AL-438 treatment in rats did not increase the plasma glucose levels and did not inhibit the bone mineral apposition. It is suggested that the improved therapeutic profile of AL-438 is due to differential cofactor recruitment as AL-438 binding reduces the interaction of the GR with the peroxisomal proliferator-activated receptor gamma coactivator-1, a cofactor critical for GC-mediated glucose upregulation (Coghlan et al., 2003; Rosen & Miner, 2005). Furthermore, a reduced side-effect profile of AL-438 on growth plate is suggested as the compound less than classical GCs reduced murine chondrocyte proliferation and bone growth (Owen et al., 2007).

In the last years, several non-steroidal selective glucocorticoid receptor agonists (SEGRAs), like BAY1155975, have been developed. Two of them have already been published. The SEGRA compounds, BAY1149775 and the particularly for topical application suited BAY865319, demonstrated a preference for TR over TA activities *in vitro*. The compounds exerted strong anti-inflammatory effects in various *in vivo* models such as contact dermatitis models, acute colitis models and models of ocular disease, comparable to classical GCs. However, both SEGRAs exhibited limited systemic or local side-effects including thymus involution, disturbance of blood glucose homeostasis and intraocular pressure increase in these models (Schäcke et al., 2004; Schäcke et al., 2009; Shafiee et al., 2011; Reuter et al., 2012; Stock et al., in preparation). In addition, *in vitro* BAY1149775 less than classical GCs induced the expression of receptor activator of NF-κB ligand (RANKL), which stimulates bone resorption in osteoblastic cells. Therefore, a lower potential for BAY1149775 to induce osteoporosis is suggested (Humphrey et al., 2006). Recently it was shown that the anti-inflammatory effects of a selective GR modulator, closely related to BAY1149775 and BAY865319, was in direct proportion to its ability to induce dual specificity phosphatase

(DUSP) 1 expression and demonstrably dependent on DUSP1 in mouse macrophages (Joanny et al., 2012). BAY865319 is in clinical evaluation as a novel drug for dermatological and ophthalmological inflammatory conditions such as atopic dermatitis, dry-eye syndrome and postoperative eye inflammation (Schäcke et al., 2009; Joanny et al., 2012).

5.1.2 Possible restrictions of the transrepression/transactivation concept

A reduced potential of these novel GR ligands to induce adverse effects, however, was shown only for some but not for all side-effects *in vivo*. As some side-effects, like osteoporosis or muscle atrophy, might not be diminished by a reduced TA activity of the GR, the dissociation concept for screening of novel GR ligands has some limitations (Kleiman & Tuckermann, 2007; Clark & Belvisi, 2012). Similarly, some anti-inflammatory effects are not mediated via the TR but via the TA activity of the ligand-activated GR (Clark, 2007).

So, it has been shown that dexamethasone induces the expression of DUSP1 (also known as mitogen-activated protein kinase phosphatase-1, MPK-1) via TA (Johansson-Haque et al., 2008). In the human DUSP1 5' region two GC responsive regions containing functional glucocorticoid response elements have been previously identified (Shipp et al., 2010; Tchen et al., 2010). The DUSP1 induction has been suggested to contribute to the reduced transcription of many inflammatory genes by inhibition of AP-1 and NF-κB function and to the destabilization of pro-inflammatory mRNAs (King et al., 2009; Joanny et al., 2012). The dependency of the anti-inflammatory effect of dexamethasone on DUSP1 was demonstrated *in vivo* in Dusp1-deficient mice. An impaired suppressive effect of dexamethasone in experimental models of sepsis, acute local inflammation, asthma and rheumatoid arthritis was observed (Clark & Belvisi, 2012). In macrophages derived from Dusp1-deficient mice the inhibitory activity of dexamethasone on the expression of several inflammatory genes, like IL-6 or TNF, was significantly impaired or even abrogated (Abraham et al., 2006).

In addition, glucocorticoid-induced leucine zipper (GILZ), which is upregulated by GCs via TA mainly in lymphoid organs, is considered to be a critical mediator of GCs' anti-inflammatory and immunosuppressive effects (Clark, 2007; Beaulieu & Morand, 2011). GILZ decreases the expression of major histocompatibility complex (MHC) class II and costimulatory molecules as well as the secretion of pro-inflammatory cytokines and chemokines and favors the generation of regulatory T cells by antigen-presenting cells. In T cells, GILZ binds Ras/Raf and thus inhibits the activation of downstream Ras-dependent signals, such as extracellular signal-regulated kinase, Akt, as well as AP-1. Via a direct interaction with NF-κB, GILZ inhibits the NF-κB-dependent transcription of genes (Ayroldi & Riccardi, 2009). In a murine model of rheumatoid arthritis GILZ seems to play a role for the suppressive activity of dexamethasone (Beaulieu et al., 2010).

Furthermore, GCs enhance the synthesis of annexin A1 (AnxA1, also known as lipocortin 1) possibly through a TA mechanism utilizing a factor that binds to CCAT enhancer-binding protein in the upstream region of the promoter. AnxA1 downregulates the release of eicosanoids and superoxide radicals and promotes caspase-3 activation as well as accelerated apoptosis in human polymorphonuclear leukocytes. In mast cells, AnxA1 may engage with cell surface formyl peptide-like receptors to downregulate the secretion of histamine and the generation of prostaglandin D_2 (Perretti & Acquisto, 2009). As the suppressive effect of GCs was impaired in experimental models of acute and chronic inflammation in AnxA1-deficient mice, AnxA1 seems to be a mediator for the anti-inflammatory activity of GCs (Clark, 2007).

Taken together, as the hypothesis of separating TR from TA activities as basis for the screening has some restrictions, novel GR ligands should be in depth characterized regarding their specific activity in different disease and side-effect models. Furthermore, whether a dissociated *in vitro* profile and an improved beneficial effect / side-effect ratio in animals does translate into an improved therapeutic index in human has to be proven in the specific clinical settings.

5.2 Improved inhibition of anti-CD28-costimulated human T cell activation by BAY1155975 *in vitro*

It is known, that classical GCs effectively suppress T cell proliferation and cytokine secretion triggered via the T cell receptor (TCR) complex by anti-CD3 stimulation. Additional CD28 costimulation diminishes the inhibitory GC effect (Nijhuis et al., 1994; Agarwal & Marshall, 2000; Li et al., 2004; Tsitoura & Rothman, 2004; Winiski et al., 2007). In order to characterize BAY1155975 with regard to this mechanistic limitation of classical GCs, its effect on anti-CD28-costimulated T cell activation was characterized in this study.

5.2.1 Differential effects of BAY1155975 versus prednisolone in memory/effector CD4+ T cells

In this study, the analysis of various T cell subpopulations demonstrated that the efficacy of 10 µM BAY1155975 in inhibiting anti-CD28-costimulated IFNγ secretion is similar in human CD45RO-negative and CD45RA-negative CD4+ cells, which represent unprimed/naïve and primed/ memory/effector T helper cells, respectively. In contrast, the efficacy of 10 µM prednisolone is impaired in memory/effector CD4+ T cells compared to naïve CD4+ T cells. These data are in line with data from a previous study by Nijhuis and colleagues, showing, that anti-CD28-costimulated proliferation of memory CD4+ T cells is more resistant to dexamethasone than the proliferation of naïve CD4+ T cells (Nijhuis et al., 1995). Furthermore, in healthy volunteers it was observed, that the percentage and absolute numbers of naïve, but

not of memory CD4+ T cells was decreased after dexamethasone administration (Chiapelli et al., 1992).

By comparing the efficacies of BAY1155975 and prednisolone in anti-CD28-costimulated memory/effector CD4+ T cells, a significantly stronger suppressive effect of BAY1155975 was also observed for the secretion of TNFα, IL-2, IL-4, IL-17A and IL-22. The enhanced cytokine suppression by BAY1155975 is not due to an increased induction of apoptosis, reduced proliferation of activated cells or different suppression of the phenotypic activation status as assessed by activation antigen expression.

Within the TCR-activated (CD69+CD40L+) memory/effector CD4+ T cell subpopulation BAY1155975, but not prednisolone, inhibited the level of IFNγ and TNFα production as assessed by the geometric mean fluorescence intensity in intracellular cytokine detection. Thus, a suppressive effect of BAY1155975 on the secretion level of IFNγ and TNFα in subsets of CD28-costimulated memory/effector CD69+ CD40L+ CD4+ T cells is suggested.

5.2.2 Possible mechanisms of differential effects

It had been shown, that the TCR-dependent production of IFNγ requires sustained elevation of calcium (Ca^{2+}), activation of the Ras-mitogen-activated protein kinase cascade and subsequent activation of the TFs AP-1, NF-κB and NFAT (Badou et al., 2001; Lang et al., 2003; Ou et al., 2009). Therefore, the involvement of Ca^{2+} signaling, MEK/ERK and thus AP-1 signaling for the improved IFNγ suppression by BAY1155975 was analyzed in this study. As the GR seems to be required for the inhibitory effect of GCs on IFNγ secretion in T cells (Agarwal & Marshall, 1998; Zhang et al., 2005), the role of GR signaling for the suppressive activity of BAY1155975 was also evaluated.

5.2.2.1 Involvement of glucocorticoid receptor signaling

The first step for evaluating a possible different mechanism of BAY1155975 versus prednisolone on inhibiting the IFNγ secretion was to explore whether the suppressive activity of BAY1155975 is dependent on GR engagement at all.

Surprisingly, the enhanced inhibitory effect on IFNγ secretion in anti-CD28-costimulated memory/effector CD4+ T cells with BAY1155975 was not prevented by co-treatment with the "GR antagonist" RU-486. According to the literature, however, RU-486 is not exclusively a GR antagonist but can also have partial agonistic activity in GR-mediated transrepression, i.e. in the repression of NF-κB activity (Almawi et al., 1996; Li et al., 2003; Zhao et al., 2003; Wu et al., 2004). In this study, a slight suppression of IFNγ secretion by single RU-486 treatment was observed in anti-CD28-costimulated memory/effector CD4+ T cells suggesting an agonistic

activity of RU-486 on the GR-mediated repression of IFNγ transcription. Thus, the observed even stronger inhibitory effect of BAY1155975 in combination with RU-486 may be due to a synergistic agonistic activity of both GR ligands in anti-CD28-costimulated memory/effector CD4+ T cells.

To exclude the possibility that the suppressive effect of BAY1155975 on IFNγ secretion is not mediated through the GR, the inhibitory activity of the compound was further analyzed after GR mRNA knockdown in memory/effector CD4+ T cells. Nucleofection of memory/effector CD4+ T cells with GR small interfering RNA (siRNA) led to a strong reduction of the GR expression at the mRNA and protein level. However, in these transfected memory/effector CD4+ T cells IFNγ secretion was still inhibited by prednisolone and to a stronger extent by BAY1155975. These results suggest that the small amount of GR, which is still expressed in the siRNA-transfected cells, is sufficient to mediate a moderate effect of the GR ligands. A more efficient GR knockdown than by siRNA transfection can probably be achieved with short-hairpin RNA (shRNA) which is continuously synthesized in the cell (Rao et al., 2009). In C2C12 mouse myoblast cells, GR mRNA expression was drastically reduced by stable expression of a shRNA (Zhao et al., 2009). Further investigations using a shRNA-mediated GR knockdown may clarify if the stronger suppressive effect of BAY1155975 in memory/effector CD4+ T cells is mediated through the GR or might result from nongenomic modes of BAY1155975 action.

5.2.2.2 Enhanced inhibition of the calcium-calcineurin-NFAT pathway by BAY1155975

As TCR engagement in the presence of CD28 costimulation increases the intracellular Ca^{2+} concentration, that leads to IFNγ production of human T cells (Schwarz et al., 2007; Nicolaou et al., 2009; Smith-Garvin et al., 2009), the effect of BAY1155975 compared to GCs on calcium-mediated stimulation pathways was analyzed.

First, the compound effects on the calcium-calcineurin-NFAT pathway were investigated. In memory/effector CD4+ T cells the Ca^{2+} influx through the L-type voltage-dependent-like Ca^{2+} (Ca_v1) channel seems to be involved in the anti-CD28-costimulated IFNγ secretion, as it was shown in this study, that the Ca_v1 channel antagonist, nifedipine, strongly inhibited the production of IFNγ. This is in line with previous studies demonstrating a modulatory activity of Ca_v1 channel antagonists on TCR-mediated Ca^{2+} influx as well as NFAT activation and IL-2 secretion in Jurkat T cells and in primary human T cells (Kotturi et al., 2006; Colucci et al., 2009). The high concentration of nifedipine, which is needed to inhibit the Ca^{2+} influx in this as well as in previous studies, can be attributed to the lack of the high affinity state of the Ca_v1 channel because of the absence of voltage dependency of the channel in T cells (Stokes et al., 2004; Colucci et al., 2009).

Co-treatment with nifedipine and GR ligands in anti-CD28-costimulated memory/effector CD4+ T cells showed a clear additive effect on inhibiting IFNγ secretion in BAY1155975-treated cells but not in prednisolone-treated cells. The synergistic effect of BAY1155975 and nifedipine suggests that BAY1155975 per se, in contrast to prednisolone, inhibited the Ca^{2+} influx through the Ca_v1 channel. By co-treatment with nifedipine and BAY1155975 the inhibitory activity on Ca^{2+} influx is enhanced and thus a reduced Ca^{2+} signaling and subsequent a diminished IFNγ secretion is observed. This synergistic effect is possibly mediated via an enhanced binding of nifedipine in combination with BAY1155975 on the Ca_v1 channel as it was shown for nifedipine in combination with diltiazem, another Ca_v1 channel antagonist, in receptor binding studies *in vitro* (Saseen et al., 1996).

The downstream signaling via calcineurin seems to be also relevant for the stronger inhibitory effect of BAY1155975 in anti-CD28-costimulated memory/effector CD4+ T cells. The calcineurin inhibitor cyclosporine A (CsA) inhibited the IFNγ secretion by around 50% suggesting that the nuclear translocation and therefore the transcriptional activity of NFAT and NF-κB, which can be diminished by CsA (Nunès et al., 1993; Nishiyama et al., 2005; Pessler et al., 2006), play a role for the expression of IFNγ in these cells. Addition of CsA to prednisolone-treated memory/effector CD4+ T cells results in a strong suppression of IFNγ secretion, as it was observed for co-treatment with BAY1155975 and CsA.

In the next step, the effect of BAY1155975 compared to GCs on other calcium-mediated stimulation pathways than anti-CD3 + anti-CD28-stimulation in memory/effector CD4+ T cells was analyzed. It was shown, that the inhibitory effect of prednisolone, in contrast to BAY1155975, on IFNγ secretion is strongly impaired by an enhanced calcium-mediated stimulation level. After 24 hours of stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin, which mimics TCR-induced calcium signaling by Ca^{2+} elevation together with protein kinase C activation (Szamel & Resch, 1995), no inhibition of IFNγ secretion by prednisolone but a strong suppression by BAY1155975 was observed. As described previously (Furue & Ishibashi, 1991), the PMA plus ionomycin-stimulated secretion of IL-2 as well as of IL-22 was also not inhibited by prednisolone. In contrast, BAY1155975 markedly suppressed the secretion of both cytokines.

In summary, BAY1155975 stronger interacts with the calcium signaling possibly via suppression of the Ca^{2+} influx and calcineurin activity, and therefore the transcriptional activity of NFAT and NF-κB on the IFNγ gene is reduced in anti-CD28-costimulated memory/effector CD4+ T cells. The interaction of BAY1155975 with the calcium-calcineurin-NFAT pathway is possibly not mediated via the GR, as the suppressive effect of BAY1155975 on IFNγ expression was not abolished by interference with GR expression or signaling using GR siRNA

and RU486, respectively. Further experiments with GR-deficient memory/effector CD4+ T cells are necessary, however, to prove whether BAY1155975 shows effects on Ca^{2+} signaling independently from the GR. In addition, the impact of BAY1155975 compared to GCs on the intracellular calcium concentration has to be determined to clarify if BAY1155975 stronger suppressed the Ca^{2+} influx in anti-CD28-costimulated memory/effector CD4+ T cells.

5.2.2.3 Involvement of MEK/ERK signaling

Since it had been shown, that the enhanced MEK/ERK signaling by anti-CD28 costimulation is essential for resistance of human naïve CD4+ T cells to GCs, the role of MEK/ERK signaling for the suppressive activity of BAY1155975 was examined (Li et al., 2004; Tsitoura & Rothman, 2004).

Addition of the MEK/ERK inhibitor, U0126, to prednisolone-treated memory/effector CD4+ T cells did not notably improve the diminished inhibitory effect of prednisolone on IFNγ secretion. A stronger suppression of IFNγ secretion was still observed by co-treatment with BAY1155975 and U0126. In a previous study by Tanaka and colleagues it was shown that ERK activation has no effect on GR-mediated repression of NF-κB activity (Tanaka et al., 2006). Therefore, other signal transduction pathways besides MEK/ERK signaling may be more critical for the improved suppressive effects of BAY1155975 in comparison to prednisolone on IFNγ secretion in anti-CD28-costimulated memory/effector CD4+ T cells.

5.2.2.4 Involvement of AP-1 signaling

TCR triggering leads to formation and activation of the heterodimeric AP-1 complex through the induction of Jun and Fos proteins. Tsitoura and colleagues had shown that an enhancement of the stimulatory signaling by CD28 costimulation increases the synthesis of c-Fos, which can not be inhibited by GCs in human naïve CD4+ T cells (Tsitoura & Rothman, 2004). In PBMCs of patients with GC-resistant asthma, higher PMA-stimulated c-Fos mRNA expression and protein levels have been observed (Lane et al., 1998; Takahashi et al., 2002).

To determine whether BAY1155975 compared to prednisolone exerts a stronger inhibitory effect on increased c-Fos synthesis in CD28-costimulated memory/effector CD4+ T cells, the effect of both compounds on the induction of c-Fos mRNA was analyzed. No inhibition of c-Fos mRNA expression by prednisolone or BAY1155975 was found after 4 hours of anti-CD3 stimulation alone or in addition with anti-CD28 costimulation in memory/effector CD4+ T cells (data not shown). These results imply that the improved IFNγ suppression in CD28-costimulated memory/effector CD4+ T cells by BAY1155975 seems not to be mediated via a stronger inhibition of AP-1 activation.

Taken together, the investigation of the compound effects on different signal transduction pathways suggested that BAY1155975 stronger suppressed the calcium-calcineurin-NFAT pathway than prednisolone. It seems that BAY1155975 at its highest concentration of 10 µM behaves as GR agonist and simultaneously as Ca_v1 channel antagonist and calcineurin inhibitor in contrast to classical GCs. This leads to a stronger inhibition of memory/effector CD4+ T cell activation by BAY1155975.

5.3 Improved inhibition of T cell-dependent skin inflammation by BAY1155975 treatment around sensitization *in vivo*

To examine the suppressive activity of BAY1155975 compared to prednisolone on CD28-costimulated T cell activation *in vivo,* the effect of both compounds was determined in a commonly used experimental mouse model of human allergic contact dermatitis. Murine contact hypersensitivity (CHS) is a T cell-mediated inflammatory reaction to hapten sensitization and challenge of the epidermis.

Several studies have demonstrated, that in the CHS response to the strong hapten 2,4-dinitro-1-fluorobenzene (DNFB) the DNFB-specific CD8+ T cells are the main effector cells during the elicitation phase (Akiba et al., 2002; Chapat et al., 2004; He et al., 2009; Kish et al., 2012). The assumption was supported by a study from Larsen et al. demonstrating that after hapten challenge a larger number of CD8+ T cells in the draining lymph nodes expressed activation markers as compared with CD4+ T cells. In contrast, during sensitization more activated CD4+ than CD8+ T cells are present in the draining lymph nodes indicating that CD4+ T cells were the dominating cell type during sensitization (Larsen et al., 2007).

5.3.1 Anti-inflammatory activity of BAY1155975 and prednisolone treatment prior to challenge

By compound application around hapten challenge in CHS, the anti-inflammatory effect of BAY1155975 and prednisolone was compared in this study. Systemic treatment with 30 mg/kg BAY1155975 or 30 mg/kg prednisolone prior to challenge significantly inhibited the DNFB-induced edema formation in mice ears.

This is in line with previous results demonstrating strong anti-inflammatory efficacy of systemic as well as topical GC application around hapten challenge (Mitsui et al., 2004; Schneider et al., 2009; Röse et al., 2012). Thus, GCs are the most commonly used drugs for the treatment of contact dermatitis (Tuckermann et al., 2007; Coutinho & Chapman, 2011; Clark & Belvisi, 2012). A strong inhibitory effect on skin inflammation in contact dermatitis models was also observed with two other SEGRA compounds, BAY1149775 and BAY865319 (Schäcke et al.,

2004; Schäcke et al., 2009). By introducing CHS in various GR-mutant mouse strains it was shown that macrophages and neutrophils are the primary targets of the anti-inflammatory action of GCs and that these effects are mediated via DNA-binding of the ligand-activated GR (Tuckermann et al., 2007).

5.3.2 Stronger inhibition of T cell-dependent skin inflammation by BAY1155975 treatment around sensitization

Application of GR ligands during sensitization affects the primary activation and expansion of hapten-specific T cells. The required costimulatory signal for effective T cell priming seems to be the interaction of CD28 on T cells with B7 molecules on dendritic cells. Antibody blocking studies indicated that activation of CD8+ effector and CD4+ regulatory T cells during sensitization with DNFB is dependent on engagement of CD86 (B7-2) (Xu et al., 1997). The important role of CD28 was confirmed in CD28-knockout mice. So, these mice showed a significant reduction in CHS response to DNFB compared to wildtype mice (Kondo et al., 1996). Thus, by GR ligand treatment during sensitization the inhibitory effect on CD28-costimulated T cell activation should be characterized in this study *in vivo*.

As shown in this study, application of prednisolone around the sensitization phase only marginally suppressed the inflammatory response. By enhancing the inflammatory response by repeated DNFB-challenges at 3 successive days no inhibition of edema formation was observed after prednisolone treatment during sensitization. In contrast, BAY1155975 significantly reduced the ear inflammation.

The only slight suppressive activity of prednisolone is in agreement with previous studies showing that GC treatment during the sensitization phase did not suppress the CHS response but diminished the number of DCs and T cells in the draining lymph nodes within 24 hours after hapten sensitization. Thus, the reduced number of DCs still allows for the generation of sufficient hapten-specific T cells capable of initiating the CHS response (Lehto et al., 2010; Tuckermann et al., 2007; Grabbe et al., 1995). As the inflammatory response to hapten challenge was strongly reduced by BAY1155975 treatment during sensitization in this study, it might be assumed that BAY1155975 interferes with the CD28-costimulated T cell activation and expansion in the sensitization phase and thus with the development of an inflammatory response after hapten challenge.

To further examine whether BAY1155975 treatment during sensitization affected T cell activation, its impact on pro-inflammatory and immunoregulatory T cell cytokine production was determined. The expression of IFNγ was similarly reduced by BAY1155975 and prednisolone treatment. As in the previous *in vitro* experiments an improved inhibition of anti-CD28-costimulated IFNγ secretion by BAY1155975 compared to prednisolone was observed in

human memory/effector CD4+ T cells, these T cells seem not to be the main producers of IFNγ in the CHS response. In the DNFB-induced CHS response large amounts of IFNγ are produced by hapten-specific CD8+ T cells and to a lesser extend by Th1 or other cells in the skin (He et al., 2009; Zhao et al., 2011; Kish et al., 2012). The similar inhibition of IFNγ expression by BAY1155975 and prednisolone suggests a similar number of infiltrating CD8+ T cells in ears of mice treated with BAY1155975 or prednisolone, as it was shown that the expression level of IFNγ closely correlates with the migration of CD8+ T cells in hapten-challenged skin tissues during the CHS response (He et al., 2009). Flow cytometric analysis of sensitized draining lymph nodes may clarify whether BAY1155975 treatment during sensitization differentially affects the activation status and effector cytokine production of CD4+ and CD8+ T cells.

Taken together, BAY1155975 has a stronger inhibitory effect on CD28-dependent sensitization in the DNFB-induced CHS model than prednisolone. As Ca^{2+}-signaling seems to be also important for sensitization of the CHS response (Katoh et al., 1997; Wille et al., 1999), the stronger inhibitory effect of BAY1155975 might partially be mediated via its calcineurin inhibitory activity, which is suggested from the *in vitro* results.

5.4 Conclusion and outlook

In this study, it was shown that the SEGRA compound, BAY1155975, at the highest concentration exhibited a significantly stronger inhibition of CD28-costimulated effector cytokine secretion (IFNγ, TNFα, IL-17 and IL-22) in human memory/effector CD4+ T cells than the classical GC, prednisolone. The enhanced inhibitory effect of BAY1155975 is suggested to be mediated via suppression of the calcium-calcineurin-NFAT pathway. It seems that BAY1155975 at its highest concentration behaves as GR agonist and simultaneously as Ca_v1 channel antagonist and calcineurin inhibitor in contrast to classical GCs. Also *in vivo*, a markedly stronger inhibition of CD28-dependent T cell activation in DNFB-induced CHS models by BAY1155975 treatment during sensitization was indicated by a diminished inflammatory response to hapten challenge.

Given the well documented finding that classical GCs have an impaired suppressive effect on activation of memory CD4+ T cells (Nijhuis et al., 1995; Chiapelli et al., 1992), BAY1155975 treatment might achieve a superior therapeutic efficacy in chronic autoimmune diseases and transplantation. Chronic autoimmune diseases, like asthma, rheumatoid arthritis or inflammatory bowel diseases, are perpetuated by long-lived antigen-specific memory CD4+ T cells (Tomita et al., 2008; Lara-Marquez et al., 2001; Skapenko et al., 1999). In asthma, activated memory CD4+ T cells are the main producer of Th2 cytokines, which

contribute to many of its pathophysiological features, including airway inflammation, mucus secretion and airway hyperresponsiveness (Machura et al., 2008). In transplantation, the presence of alloreactive memory T cells has been associated with resistance to immunosuppressants, like cyclosporine and IL-2 receptor-blocking drugs (Bingaman & Farber, 2004).

As treatment for T cell-dependent inflammatory diseases, i.e. to prevent allograft rejection in heart and lung transplantations, a combination therapy with classical GCs and calcineurin inhibitors is commonly used (Snell & Westall, 2007; Eisen & Ross, 2004). Treatment with BAY1155975 possibly could replace this combination therapy thus reducing the involved GC-related side-effects.

Further experiments using BAY1155975 compared to standard therapies in different T cell dependent inflammatory disease as well as transplantation models are needed to prove the enhanced anti-inflammatory and immunosuppressive efficacy of BAY1155975. Finally, the improved beneficial/side-effect ratio of BAY1155975 in humans has to be shown in clinical settings.

6. Summary

Glucocorticoids (GCs) are highly efficacious drugs for the treatment of acute and chronic inflammatory diseases such as allergy, asthma and autoimmune diseases. However, their therapeutic action is often limited by severe and sometimes irreversible side-effects, including skin atrophy and thymus involution. Thus, new glucocorticoid receptor (GR) ligands with should have potent anti-inflammatory efficacy but a reduced side-effect profile, like non-steroidal selective glucocorticoid receptor agonists (SEGRAs), have been developed.

In this study, the SEGRA compound, BAY1155975, and the classical GC, prednisolone, were compared regarding their suppressive effects on CD28-costimulated activation of human primary T cells *in vitro*. Analysis of different T cell subpopulations revealed a significant stronger inhibition of IFNγ secretion in anti-CD3- plus anti-CD28-stimulated memory/effector CD4+ T cells with BAY1155975 than with prednisolone. The enhanced inhibitory activity of BAY1155975 was also observed for the secretion of the effector cytokines, TNFα, IL-17 and IL-22, both after anti-CD3 plus anti-CD28 stimulation as well as after PMA plus ionomycin stimulation. Proliferation, apoptosis and activation status were similarly regulated by BAY1155975 and prednisolone in these cells. Co-treatment with the GR antagonist, RU-486, as well as siRNA-mediated GR gene silencing in memory/effector CD4+ T cells did not prevent the stronger suppressive effect of BAY1155975 on IFNγ secretion. BAY1155975 seems to stronger suppress the calcium-calcineurin-NFAT pathway.

The effect of BAY1155975 compared to prednisolone on CD28-costimulated T cell activation *in vivo* was analyzed in DNFB-induced contact hypersensitivity models in mice. Application of BAY1155975 around sensitization, where CD28 costimulation is necessary for effective T cell priming, exhibited a significantly stronger suppression of the T cell-dependent skin inflammation in mouse ears than achieved by prednisolone treatment.

Taken together, BAY1155975 may represent a promising drug candidate for the treatment of T cell-dependent inflammatory diseases, where the use of classical GCs is limited by their side-effect potential as well as by T cell resistance.

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