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THE ROLE OF THE KETONE RECEPTOR HCA2 IN THE
MANAGEMENT OF PSORIATIC SKIN INFLAMMATION

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Summary

The hydroxy-carboxylic acid receptor 2 (HCA2) is a G-protein-coupled receptor for which the ketone β -hydroxybutyrate and the short chain fatty acid butyrate are known agonists. Both are endogenous compounds generated during fatty acid metabolism and by the intestinal microflora, respectively. Nicotinic acid (NA, vitamin B₃) is another agonist of HCA2 and high doses of NA are used for the treatment of lipid disorders. Monomethyl fumarate (MMF), the bioactive *in vivo* metabolite of the prodrug dimethyl fumarate (DMF) was identified as an agonist of the HCA2 receptor. DMF-based therapies have been used in Germany since 1997 for systemic therapy of moderate to severe plaque psoriasis. Although HCA2-mediated effects of NA have been analysed extensively, little is known about the role of HCA2 signalling in the treatment of psoriasis. Despite its long-term use in psoriasis and the identification of HCA2's role in a common side effect of NA and DMF treatment, flush, the role of HCA2 for the therapeutic effect of oral DMF treatment in psoriasis is not yet known.

This work demonstrated that oral DMF treatment in psoriasis patients led to a rapid improvement of psoriasis lesions. The reduction of skin inflammation was accompanied by a decrease in peripheral leukocytes, mainly neutrophils. Neutrophils express HCA2, and it was assumed that the decrease of these cells was mediated by HCA2-dependent mechanisms. The data showed that apoptosis was not induced in neutrophils and can therefore not explain the reduction of this cell type. By using the Imiquimod model of psoriasis-like skin inflammation employing HCA2 expressing and deficient mice, the therapeutic effect of oral DMF treatment was confirmed to be dependent on this receptor. In these experiments oral DMF did not affect immune cells but changed the expression of keratinocyte-derived pro-inflammatory cytokines. Interestingly, mice kept on a ketogenic diet to raise blood β -hydroxybutyrate levels showed less skin inflammation that was independent of HCA2 expression. The use of bone marrow chimeras demonstrated that oral DMF treatment mediated its therapeutic effect through HCA2 expressed by radio-resistant cells, most likely keratinocytes. *In vitro* analysis showed that MMF treatment reduced the proliferation of keratinocytes.

In conclusion, this work showed that the metabolic sensor HCA2 mediated the therapeutic effect of oral DMF on psoriasis skin inflammation by directly affecting the proliferation of keratinocytes.

Zusammenfassung

Der Hydroxy-Carboxylsäure Rezeptor 2 (HCA2) ist ein G-Protein-gekoppelter Rezeptor, für den das Keton β -Hydroxybutyrat und die kurzkettige Fettsäure Butyrat als Agonisten bekannt sind. Beide sind endogene Verbindungen, die während des Fettsäurestoffwechsels bzw. durch die Mikroflora des Darms erzeugt werden. Niacin (NA, Vitamin B3) ist ein weiterer Agonist von HCA2, und hohe Dosen von NA werden zur Behandlung von Lipidstörungen eingesetzt. Monomethylfumarat (MMF), der bioaktive Metabolit des Fumarsäureesters Dimethylfumarat (DMF), wurde ebenso als ein Agonist des HCA2 Rezeptors identifiziert. DMF-basierte Therapien werden in Deutschland seit 1997 zur systemischen Therapie der mittelschweren bis schweren Psoriasis eingesetzt. Obwohl die HCA2-vermittelten Effekte von NA umfassend analysiert wurden, ist über die Rolle von HCA2 bei der Behandlung der Psoriasis nur wenig bekannt. Trotz seiner langfristigen Anwendung bei Psoriasis und der Identifikation der Rolle von HCA2 bei einer häufigen Nebenwirkung der NA- und DMF-Behandlung, dem Flush, ist die Bedeutung von HCA2 für die therapeutische Wirkung der oralen DMF-Behandlung bei Psoriasis noch nicht bekannt.

Diese Arbeit zeigt, dass die orale DMF-Behandlung bei Psoriasis-Patienten zu einer raschen Verbesserung der Psoriasis-Läsionen führte. Die Verringerung der Hautentzündung ging mit einer Abnahme der peripheren Leukozyten, hauptsächlich der neutrophilen Granulozyten, einher. Neutrophile exprimieren HCA2 und es wurde angenommen, dass der Rückgang dieser Zellen durch HCA2-abhängige Mechanismen vermittelt wurde. Die Daten zeigten, dass die vermutete Apoptose bei Neutrophilen nicht induziert wurde und daher die Abnahme dieses Zelltyps nicht erklären kann. Durch die Verwendung des Imiquimod-Modells der Psoriasis-ähnlichen Hautentzündung mit HCA2-exprimierenden und -defizienten Mäusen wurde bestätigt, dass die therapeutische Wirkung der oralen DMF Behandlung von diesem Rezeptor abhängig ist. In den Experimenten wirkte orales DMF nicht auf die Immunzellen, sondern veränderte die Expression von Keratinozyten-spezifischen pro-inflammatorischen Zytokinen. Mäuse unter ketogener Diät zeigten erhöhte β -Hydroxybutyratspiegel und eine Verbesserung der Hautentzündung, wenn auch unabhängig von HCA2. Die Verwendung von Knochenmarkchimären zeigte, dass die orale DMF Behandlung ihre therapeutische Wirkung durch den HCA2 Rezeptor vermittelte, der von radio-resistenten Zellen, höchstwahrscheinlich Keratinozyten, exprimiert wird. Die *in-vitro*-Analyse zeigte, dass die MMF Behandlung die Proliferation von Keratinozyten reduzierte.

Zusammenfassend zeigte diese Arbeit, dass der HCA2 Rezeptor die therapeutische Wirkung von oralem DMF auf die psoriasiforme Entzündung vermittelte, höchstwahrscheinlich durch direkte Hemmung der Proliferation von Keratinozyten.

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List of abbreviations

AA	arachidonic acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AC	adenylate cyclase
ADAMTSL5	a disintegrin and metalloprotease domain containing thrombospondin type 1 motif-like 5
AKT	protein kinase B
AMP	antimicrobial peptide
ANOVA	analysis of variance
APCs	antigen presenting cell
ASS	acetylsalicylic acid
BHB	β -hydroxybutyrate
BM	bone marrow
BMI	body mass index
BMT	bone marrow transfer
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CAM	cell adhesion molecules
cAMP	Cyclic adenosine monophosphate
CARD14	Caspase recruitment domain-containing protein 14
CD	cluster of differentiation
CHO-1	Chinese hamster ovary 1
CNS	central nervous system
COX1	cyclooxygenase 1
COX2	cyclooxygenase2

CVD	cardiovascular diseases
DCs	dendritic cells
DDCs	dermal dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethyl fumarate
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DP	double positive
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EBA	bullous pemphigoid-like epidermolysis bullosa acquisita
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FAE	fumaric acid esters
FCS	fetal calf serum
FFA	free fatty acid
FFAR	free fatty acid receptor
FGFR1	fibroblast growth factor (FGF) receptor 1
FSC	forward scatter
GI	gastrointestinal
GPCR	G-protein coupled receptor

GSH	glutathione
GWAS	genome-wide association studies
H&E	hematoxylin and eosin
hBD-2	human β -defensin 2
HBSS	Hank's balanced salt solution
HCA2	hydroxy-carboxylic acid receptor
HDAC	histone deacetylase
HDL	high-density lipoprotein
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HLA	Human Leukocyte Antigen
HO-1	heme oxygenase 1
Hsp	heat shock protein
ICAM-1	intercellular adhesion molecule 1
IF	immune fluorescence
IFN	interferon
IKK	I κ B Kinase
IL	interleukin
IMQ	Imiquimod
IP3	inositol-1,4,5-triphosphate
ISH	<i>in situ</i> hybridization
JNK	C-Jun-N-terminal kinase
KGM	keratinocyte growth medium
KRT14	keratin 14
LCE	late cornified envelope
LCs	Langerhans cells

LDL	low density lipoprotein
LED	low energy diet
mAb	monoclonal antibody
MAP	mitogen-activated protein
MCP-1	monocyte chemotactic protein 1
MCT	monocarboxylate transporter
MEF	monoethyl fumarate
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHF	monohydrogen fumarate
MMF	monomethyl fumarate
mRNA	messenger ribonucleic acid
MTX	methotrexate
NAC-DMS	mercapturic acids of DMF
NAC-MMS	mercapturic acids of MMF
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NFKBIA	NF- κ B inhibitor alpha
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK	normal human epidermal keratinocytes
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NRF2	nuclear factor (erythroid-derived 2)-like 2
PASI	psoriasis area and severity index
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

pDC	plasmacytoid dendritic cell
PDGFR	platelet-derived growth factor receptors
PGD2	prostaglandin D2
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLA2	phospholipase A2
PML	progressive multifocal leukoencephalopathy
PRDX2	peroxiredoxin 2
PsA	psoriasis arthritis
PSOLAR	Psoriasis Longitudinal Assessment and Registry
PSORS	psoriasis susceptibility locus
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
RFP	Red Fluorescent Protein
RT	room temperature
RRMS	relapsing remitting multiple sclerosis
SAHA	suberolyanilide hydroxamic acid
SCFA	short chain fatty acid
SCG	superior cervical ganglion
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLN	skin-draining lymph node
SPF	specific pathogen free
SSC	side scatter

STAT3	Signal transducer and activator of transcription 3
TBS	Tris buffered saline
TBS-T	Tris buffered saline + Tween20
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF- α	tumor necrosis factor- α
TNIP1	TNFAIP3 Interacting Protein 1
TRAF3PI2	TRAF3 Interacting Protein 2
Tregs	regulatory T cells
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen 4
VLDL	very low-density lipoprotein
wnt5a	wingless-type mouse mammary tumor virus integration site family member 5a

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Chapter 1: Introduction

The hydroxy-carboxylic acid receptor 2 (HCA2) has many agonists that are part of the metabolism of the human body. The ketone β -hydroxybutyrate (BHB) is formed by the body when the fat reserves are used as an energy source. The agonist butyrate is formed by the microbiota in the intestine from crude fibre of the food consumed. HCA2 is also a receptor for nicotinic acid (NA, Vitamin B3), which is normally taken up from food, but is also used in high doses as a drug for fat metabolism disorders. Since 2008 it has been known that monomethyl fumarate (MMF) is also an agonist for HCA2. MMF is the active metabolite of dimethyl fumarate (DMF), which is used for the treatment of moderate to severe psoriasis. Psoriasis is an inflammatory skin disease which is accompanied by a considerable comorbidity and is therefore often a manifestation of systemic inflammation.

1.1 Hydroxy-carboxylic acid receptors

The hydroxy-carboxylic acid (HCA) receptor family consists of 3 members: HCA1, HCA2 and HCA3. These are G-protein coupled receptors (GPCRs) with the systematic names: GPR81, GPR109A and GPR109B. They are termed hydroxy-carboxylic acid receptors as their natural ligands are hydroxy-carboxylic acids. As hydroxy-carboxylic acids are formed in several metabolic cycles within the body, HCA receptors therefore act as energy and metabolic sensors. In humans all three receptors can be found whereas in the mouse there is no HCA3 variant [1]. In human, all three are encoded in a cluster on chromosome 12 while the mouse homologs are found on chromosome 5 [2]. All three human receptors share a high homology on the protein level with HCA1 and HCA2 showing 50% similarity while HCA2 and HCA3 share 95% homology [1]. The grade of homology also reflects the similarity between their ligands. Lactate is a natural ligand for the HCA1 receptor with an EC_{50} value of 1.3mM but does not activate HCA2 or HCA3 [3]. The known natural ligands of HCA2 are: BHB, a ketone body produced in times of starvation or extensive exercise and butyrate, a short chain fatty acid (SCFA) produced by the gut microbiota from dietary fibre. HCA3 is a receptor for the β -oxidation intermediate 3-OH-octanoic acid [4]. Therefore, all three receptors bind to intermediates of lipid metabolism. Interest in HCA2 has increased when it was shown to be a receptor for important drugs: nicotinic acid (NA) and MMF [5, 6].

1.1.1 HCA2

The HCA2 receptor has been subjected to intense research since it was shown that the lipid-modifying properties of NA treatment are mediated via HCA2 [7]. HCA2 is also known as GPR109a, HM74a in human and PUMA-G in mice. Besides its natural agonists, BHB and butyrate, there are several other ligands of HCA2 including potential drugs used for the treatment of disease or have undergone testing [7]. NA and MMF (the metabolite of the prodrug DMF *in vivo*) are used in the treatment of dyslipidaemia and psoriasis, respectively. NA has an EC₅₀ with HCA2 of 0.25µM while MMF's is 9.4µM. Hence, they are able to activate HCA2 at much lower concentrations than the natural agonists. Acifran and acipimox are other high affinity agonists of HCA2 with an EC₅₀ of 2.1µM and 2.6-6µM, respectively. Both showed lipid-lowering activity [8]. There are other very high affinity HCA2 agonists: SCH900271 (0.002µM), MK-6892 (0.016µM), MK-1903 (0.013µM) and GSK256073 (0.032µM). SCH900271 has been shown to have lipid lowering properties while also causing less flushing reaction compared to NA, which is a common side effect of this treatment [9]. Although possessing a high affinity for HCA2, MK-1903 has demonstrated only weak lipid lowering effects [10]. GSK256073 has been described to have similar lipid lowering effects as NA but was not associated with flushing [11]. MK-6892 has increased effects on plasma lipids with less flushing relative to NA [12]. These differential effects exemplify that activation of HCA2 leads to a complex signalling resulting in different downstream events and side effects dependent on the respective ligand.

Given the lipid metabolism-modifying effects of multiple HCA2 agonists, it is unsurprising that this receptor is highly expressed by white and brown adipose tissue [6, 13, 14]. Furthermore, epithelial cells such as keratinocytes, retinal pigment epithelial cells and intestinal epithelial cells also express HCA2 [15-17]. Expression of HCA2 by immune cells has also been demonstrated. Neutrophils, as well as tissue resident macrophages and Langerhans cells (LCs) all express HCA2 [17-19].

1.1.1.1 HCA2 mediated signalling

All three HCA receptors are coupled to G_i/G_o-type G-proteins, as such they are sensitive to pertussis toxin [20] and receptor activation leads to decreased adenylate cyclase (AC) activity measured by a reduction in cellular cyclic adenosine monophosphate (cAMP) levels [13, 21, 22]. Besides the modulation of cAMP levels, HCA receptor activation also leads to an increase in free cellular Ca²⁺ levels [21, 23, 24]. The release of intracellular Ca²⁺ after NA treatment has been linked to an induction of prostaglandins in epidermal LCs, a crucial step for the development of flush [23]. Flush is a common side effect of both NA treatment and oral DMF therapy (with MMF being the metabolite in the

peripheral circulation). This is the only *in vivo* effect dependent on HCA2 where the underlying signalling cascades are known. Hence, flush is mediated by the production of prostaglandins in LCs and keratinocytes by HCA2 mediated signalling leading to vasodilation of dermal blood vessels [17, 23]. Acetylsalicylic acid (ASS) and indomethacin have been shown to reduce the cutaneous reaction [25, 26]. As indomethacin and ASS are cyclooxygenase inhibitors, this led to the hypothesis that prostaglandins mediate the flushing effect and has ultimately identified prostaglandin D2 (PGD2) as the responsible prostaglandin [26-28]. PGD2 is responsible for the first, immediate peak of the flush reaction. During this phase, PGD2 is produced by cyclooxygenase 1 (COX1) in LCs. The second peak is mediated by cyclooxygenase 2 (COX2) in keratinocytes producing prostaglandin E2 (PGE2). Both prostaglandins lead to vasodilation of dermal blood vessels which causes the redness and warmth patients experience [5, 17, 23, 29]. Hansen et al. [30] postulated a signalling cascade mediating NA's and MMF's effect on flushing whereby binding of these ligands to HCA2 leads to the activation of phospholipase C β and the subsequent release of intracellular Ca²⁺ by inositol-1,4,5-triphosphate (IP3). The Ca²⁺ flux then activates phospholipase A2 (PLA2). This cascade is induced in both LCs and keratinocytes but PLA2 induced different downstream enzymes in each cell type. In LCs PLA2 mediated the early peak of flush with the release of arachidonic acid (AA) inducing COX1, which in turn produces prostaglandin G2/H2. This induced PGD2- and PGE2 synthase leading to the production of PGD2 and PGE2, respectively. In keratinocytes PLA2 facilitated the late peak of flush with the production of AA leading to COX2 induction and stimulation of PGE2 synthase to produce PGE2 via the production of prostaglandins G2/H2. PGE2 and PGD2 likely bind to GPCRs EP2/4 and DP on vascular smooth muscle cells leading to a reduction in cAMP by inhibition of the AC. This leads to a relaxation of these cells and thus dilation of the vessels [23, 29-33].

Walters *et al.* have shown that activation of the HCA2 receptor by NA leads to β -arrestin mediated extracellular signal-regulated kinases (ERK) 1/2 phosphorylation. ERK1/2 is a mitogen-activated protein (MAP) kinase known to activate PLA2. Although these results have been achieved by the use of a transgenic cell line, they have suggested another mechanism by which HCA2 activation mediates flush. Interestingly, the anti-lipolytic effects of NA have been shown to be β -arrestin independent [34, 35].

In transfected Chinese hamster ovary 1 (CHO-1) cells expressing HCA2 and in A432 cells, which do not express HCA2, NA induced the phosphorylation of protein kinase B (AKT) in a HCA2-dependent manner. This activation is likely mediated by the protein kinase C (PKC) and platelet derived growth factor receptor (PDGFR)/ epidermal growth factor receptor (EGFR) transactivation pathway leading to AKT phosphorylation [36]. AKT signalling is an important factor in cell survival and proliferation [37].

Together, these studies show that of HCA2 activation leads to signalling through multiple downstream pathways depending upon the cell type and the activating ligand.

1.1.1.2 β -hydroxybutyrate and butyrate signal through HCA2

BHB is produced in the liver by β -oxidation of free fatty acids during times of starvation. With a healthy diet, blood ketone levels are below 0.1mM and are thus too low to activate HCA2. However, after 3 days of starvation blood BHB levels rise to over 1mM and increases further over time [38]. Even before the discovery of HCA2 it was known that BHB leads to a reduction of blood free fatty acids (FFAs) and glucose without affecting insulin secretion which would inhibit lipolysis [39]. HCA2 has been proposed to act as a sensor in a regulatory loop for the control of lipolysis. BHB is produced from FFAs released by lipolysis from adipose tissue. HCA2 on adipocytes senses the increased BHB levels and inhibits the release of FFA to prevent excessive loss of adipose tissue [7]. Thus, HCA2 plays a significant role in feedback signalling during ketosis via detecting the concentration of BHB in the body.

Butyrate, produced by the gut microbiota from dietary fibre, reduces colonic inflammation in an HCA2 dependent manner, most likely by promoting macrophages and DCs to induce the differentiation of regulatory T cells (Tregs) and IL-10-producing T cells [40]. Hence, in a murine model of colitis butyrate produced by the microflora reduced inflammation and induced Treg development in the colon. However, *in vitro* analysis showed that butyrate can inhibit the differentiation of naïve T cells into Tregs directly via histone modulation in the Foxp3 promotor region [41]. As butyrate is membrane permeable and T cells do not express HCA2, the anti-inflammatory effects of butyrate are not likely solely dependent upon HCA2.

1.1.1.3 Nicotinic acid modifies blood lipids via HCA2

Vitamin B3 consists in different forms: nicotinamide and niacin (nicotinic acid, NA). In the body nicotinamide is converted to NA which in turn is converted to nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) [42]. NA binds to HCA2 with high affinity and is used as a lipid-regulating drug in order to reduce the risk of cardiovascular disease. NA is not produced by the body but is obtained from the diet [43]. NA has the ability to increase the concentration of high-density lipoprotein (HDL) cholesterol in the blood while at the same time reducing low density lipoprotein (LDL) cholesterol and triglyceride levels. Whether these changes to blood cholesterol levels are mediated by HCA2 is still unclear as hepatocytes do not express HCA2 [6]. Hepatocytes produce apolipoproteins, which are the structural component of different

lipoproteins and thus are an important cell type in lipid metabolism [44]. HCA2 on adipocytes regulates the release of FFA from adipose tissue [6]. Treatment with NA inhibits lipolysis and the subsequent release of FFAs from fat tissue. This reduction in FFAs release leads to a fall in very low density lipoprotein (VLDL) levels, which in turn results in less LDL [45]. Furthermore, NA increases HDL levels, possibly by inducing changes in apolipoprotein metabolism, although the respective literature is contradictory [46-48]. Besides lipid modulation, NA has a therapeutic effect in vascular inflammation and atherosclerosis independent of its lipid lowering properties. Cell adhesion molecules (CAM) have been associated with atherosclerosis [49]. In a rabbit model of acute vascular inflammation and endothelial dysfunction treatment of the animals with NA led to a decrease in CAM mRNA expression in endothelial cells of the collared carotid arteries. Specifically, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and the chemokine monocyte chemoattractant protein 1 (MCP-1) were reported to be reduced. This downregulation by NA has also been observed in tumor necrosis factor- α (TNF- α) induced inflammation in endothelial cells *in vitro* [50]. NA inhibits monocyte and neutrophils migration in animal models and *in vitro*. [50-52]. Furthermore, NA has been shown to reduce the size of arterial lesions in animal atherosclerosis models [53, 54]. This protective effect was confirmed to be HCA2 dependent but did not affect HDL or LDL levels [54]. NA has also been shown to promote cholesterol efflux in macrophages and impair cell movement into atherosclerotic plaques via a HCA2 dependent mechanism [53, 54]. Together, these studies not only show that NA modifies lipid metabolism but also demonstrate that it has anti-inflammatory properties. The precise role of HCA2 in these different functions is not yet fully elucidated.

1.1.1.4 MMF signalling through HCA2

Although MMF has been a known ligand of HCA2 since 2008, there continues to be very little known about the role of MMF-HCA2 signalling in the improvement of psoriasis following treatment of patients with the MMF prodrug, DMF [5]. Although the underlying mechanisms of flush have been elucidated for NA and MMF, other effects mediated by HCA2 *in vivo* are not yet fully understood. In experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis, oral DMF treatment leads to a less severe course of disease, which is dependent on the expression of HCA2. Thus, mice treated with DMF showed a later onset of disease, fewer infiltrating immune cells and reduced demyelination. These effects were not observed in HCA2^{-/-} mice (which lack the HCA2 receptor in all cells). In respect to immune cell infiltration, DMF treatment led to significant decreases in the numbers of neutrophils, a slight decrease in dendritic cells

(DCs) and CD4⁺ T cells but not in CD8⁺ T cells in mouse spinal cords during EAE. These effects were also found to be HCA2 dependent. The decrease in neutrophil infiltration into the central nervous system (CNS) has been suggested to be due to reduced adhesion to endothelia and subsequent transmigration of these cells [55].

In a mouse model of bullous pemphigoid-like epidermolysis bullosa acquisita (EBA) oral DMF treatment has been shown to have protective effects mediated by HCA2. EBA is a skin disease marked by an infiltration of leukocytes, in particular neutrophils, into the skin. These infiltrates are reduced by oral DMF treatment of mice, with the highest decreases in neutrophils and monocytes (both of which express HCA2). DMF treatment also inhibited the migration of monocytes into the draining lymph nodes. Both neutrophils and Ly-6C^{low} monocytes showed increased CD62L levels on their surface after oral DMF. High CD62L levels are a marker of non-activated cells. Thus, DMF treatment ameliorates EBA in an HCA2 dependent manner and is associated with reduced activation and migration of neutrophils and monocytes [56]. This study strongly suggests that MMF-HCA2 signalling is protective in a murine model of inflammatory skin disease.

1.2 Psoriasis

Psoriasis is a common, chronic, immune-mediated skin disease for which there is no cure. Psoriasis vulgaris or plaque psoriasis is the most common type accounting for approximately 90% of psoriasis cases. Patients present with red, raised, sharply demarcated plaques with typical silvery scales. Removal of these scales leads to small bleedings (Auspitz sign) [57]. In psoriasis patients, new lesions can result from injuries or trauma of the skin (Koebner phenomenon) [58]. The size of lesion can vary from small punctate spots to large plaques and they can affect any area of the patient's body. Most common areas are the extensor surface of the knees and elbows and the scalp. A symmetrical distribution of lesions is often observed. Psoriasis vulgaris can be further subcategorised by the age of disease onset. Type I psoriasis shows a peak occurrence age between 16 and 22 years and is correlated with a family history of disease and with the presence of Human Leukocyte Antigen (HLA)-Cw*0602, a known genetic marker of psoriasis. Late onset, or type II psoriasis peaks at the age of 60 years and seems to be non-inheritable [59].

Guttate psoriasis is mostly observed in children and young adults and is characterised by small (2-10mm) red plaques scattered all over the trunk. These often occur 2-3 weeks after a trigger event, such as streptococcal infection of the tonsils, and may spontaneously disappear after 3-4 months. Around one third of guttate psoriasis patients later develop plaque psoriasis [60].

Intertriginous psoriasis is limited to skin folds, often in the axillary, genital, perianal, and inframammary areas [61]. Lesions are red without scaling due to the moist environment and liable to rupture due to the normal movement of the patient [62].

Pustular psoriasis is divided into generalized and localized forms. Generalized pustular psoriasis is an acute form with patients having fever and showing sterile pustules on a red background. Localized pustular psoriasis is characterised by psoriasis plaques with pustules confined to the lesions. [58].

Besides the skin, psoriasis can also affect nails of hands and feet. Nail psoriasis occurs in approximately 50 % of patients with psoriasis and can in some patients (<5%) be the only manifestation of the disease. Nails show pitting, yellowish or white colour and onychorrhexis or thickening of the plates. In psoriasis arthritis (PsA) up to 90 % of all patients show changes of nails [63, 64]. Nail psoriasis is believed to be a predictor of PsA [65]. PsA manifests primarily as enthesitis, inflammation of the areas where ligaments and tendons insert into the bone (entheses) and is observed in up to 25 % of psoriasis patients. Approximately half of PsA sufferers carry the HLA-B27 gene [66]. About 10 % of PsA patients show no skin symptoms when diagnosed [67]. PsA can lead to significant joint damage and disability.

1.2.1 Epidemiology

In Germany the prevalence of psoriasis is around 2.5%. In children, prevalence increases with age with a mean of 0.71% [68]. In adults, the prevalence has been studied in several different countries using several different methods that may influence the accuracy of the data: self-reported, physicians' diagnosis or primary-care databases. The incidence of psoriasis in Europe ranges between 1.3% to 2.6% in the UK to 8.5% in Norway. There is no significant bias in the frequency of psoriasis in males or females [69].

1.2.2 Susceptibility genes

Extensive research has revealed that several genetic loci are associated with different forms of psoriasis. For instance, the "psoriasis susceptibility locus (PSORS) 1" was the first genetic determinant identified and accounts for 30-50% of the heritability of psoriasis. PSORS1 was shown to be within the HLA-C gene region and has been subsequently narrowed down to HCA-Cw*0602 [70-72]. Interestingly, this locus is almost exclusively linked to guttate and early onset psoriasis [73, 74]. Late onset psoriasis shows no association with HLA-Cw*0602 [59, 75]. Homozygote individuals have an increased risk of psoriasis compared to heterozygotes [76]. Despite intensive research,

the antigen presented by this major histocompatibility complex (MHC) molecule has yet to be identified. Epitopes derived from ezrin, maspin, peroxiredoxin 2 (PRDX2) and heat shock protein (Hsp) 27 have all been suggested as potential candidates, with HLA-Cw*0602 and early onset psoriasis [77].

Today, mainly due to the use of genome-wide association studies (GWAS), several other loci have been identified. The deletion of the late cornified envelope (LCE) 3B and 3C genes is a risk factor for psoriasis confirmed in several ethnicities [78, 79]. PSORS2 has been shown to correspond to caspase recruitment domain-containing protein 14 (CARD14) which is part of the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF- κ B) signalling pathway. The genetics variants that have been detected lead to an upregulation of the NF- κ B pathway resulting in the induction of psoriasis-related genes in keratinocytes [80]. Other NF- κ B genes associated with psoriasis are NF- κ B inhibitor alpha (NFKBIA), TRAF3 interacting protein 2 (TRAF3IP2) and TNFAIP3 Interacting Protein 1 (TNIP1) [81-83]. The susceptibility loci have also been found within constituents of the IL-23 signalling pathway, including: IL-23A, IL-23R and IL-12B and signal transducer and activator of transcription 3 (STAT3) [82, 84, 85]. Whole genome approaches have also identified additional genetic risk factors including skin specific genes and genes associated with innate and adaptive immunity [86]. Interestingly, none of these factors is found in all patients. Thus, the complex genetic background in psoriasis with a number of the hereditary risk factors has yet to be described.

1.2.3 Obesity as a risk factor for psoriasis

There are both nation- and world-wide registers of data regarding patients, their treatments and other clinical aspects. These registries can be easily used to investigate patients' health status and its relationship with psoriasis severity during treatment. Obesity was at first thought of as a comorbidity of psoriasis, but now it is viewed as an independent risk factor. Data from the worldwide PSOLAR (Psoriasis Longitudinal Assessment and Registry) registry showed that 48.2% of psoriasis patients are obese. As 75% of the patients in this database are US citizens the data may show a bias due to the general elevated levels of obesity in the US [87]. Indeed, the German PsoBest registry showed that 28.1% of patients are obese [88]. However, the prevalence of obesity in psoriasis patients is still higher than the general population in Germany [89]. Furthermore, an increased body mass index (BMI) is associated with more severe disease while a reduction in bodyweight is known to ameliorate psoriasis and improve treatment efficacy [90-94]. The mechanism of action of the weight loss-related improvement in psoriasis still needs to be elucidated. However, weight reduction is

becoming both an important factor in psoriasis management and a subject of clinical research.

Obesity may also be a potentiating factor in psoriasis and its associated spectrum of comorbidity, rather only a risk factor for the initial development of disease. Hence, increased body weight correlates with exacerbated psoriatic disease [91]. Besides being a store of energy in the body, adipose tissue is metabolically active and is a source of a number of different cytokines, e.g. IL-6, TNF- α and adipokines [95]. These factors are mostly produced by residing macrophages or monocytes [96]. Adipose tissue-derived leptin induces T helper (Th) 1 cells differentiation and activates monocytes, thereby promoting inflammation [97, 98]. Interestingly, leptin also promotes both angiogenesis and keratinocyte proliferation, hallmark features of psoriasis [99, 100]. Adiponectin is an anti-inflammatory adipokine which is negatively correlated with both BMI and psoriasis severity [96]. Interestingly, DMF treatment of psoriasis patients increases adiponectin levels [101]. Another factor linking obesity and inflammation is wingless-type mouse mammary tumor virus integration site family member 5a (Wnt5a). Wnt5a is secreted by macrophages and leads to chronic low inflammation when not clustered by soluble frizzled-related protein-5 (sFRP5). sFRP5 is secreted from adipocytes and secretion is reduced in obese individuals leading to less neutralization of Wnt5a. Wnt5a is increased by obesity, increasing the pro-inflammatory status [102]. Also in psoriasis plaques increased Wnt5a levels have been detected [103, 104]. In both lean and obese psoriasis patients serum levels of Wnt5a have been reported to be higher compared to non-psoriatic lean and obese individuals [105]. Weight reduction by caloric restriction has been shown to increase sFRP5 levels, reducing the inflammatory status [106]. This underlines the important connection between adipose tissue and inflammation and psoriasis.

1.2.4 Comorbidity in psoriasis

Diabetes mellitus type 2 is both associated with psoriasis and has obesity as a risk factor [107, 108]. In the PsoBest study, 9.1% of the included patients were diagnosed as diabetic [88], which is similar to the incidence of diabetes in the worldwide PSOLAR registry with 11.4% [87]. The prevalence of diabetes also increases with the severity of psoriasis [109].

Besides suffering from obesity and diabetes, psoriasis patients often have other metabolic diseases and are therefore frequently diagnosed with metabolic syndrome. Metabolic syndrome is diagnosed when a patient displays 3 or more of the following criteria: obesity, diabetes, elevated blood pressure, high triglycerides and low HDL-C [110]. Metabolic syndrome is positively associated with psoriasis as its prevalence

increases with psoriasis severity [111, 112]. Metabolic syndrome is also linked to cardiovascular diseases (CVD), which are also a known comorbidity of psoriasis. The global PSOLAR registry shows 38.2% of psoriasis patients having CVD, mostly in the form of hypertension and hyperlipidaemia [87]. The German PsoBest psoriasis cohort showed that 25.8% suffered from hypertension and 10.1% from hyperlipidaemia [88]. Again, the prevalence of these conditions increases with psoriasis severity [113]. Psoriasis patients also show a higher risk of death due to CVD compared to healthy individuals [114].

Depression is another comorbidity of psoriasis. Depending on the diagnosis methodology (clinical diagnosis vs. questionnaire) the prevalence is between 12% and 28%, respectively, with 9% of patients having used antidepressants [115]. Another study has reported that 62% patients noted depressive symptoms [116]. Other studies comparing psoriasis patients to healthy controls have reported that patients have a higher incidence of depressive symptoms [115]. It is hypothesised that inflammatory signalling is causing this positive association, rather than the obvious external symptoms and their possible social effects as other skin disease do not show such a high correlation [117].

There are several other diseases associated with psoriasis, including: inflammatory bowel disease, atherosclerosis and celiac disease [118, 119]. The spectrum and prevalence of comorbidity in psoriasis highlights its nature as a severe systemic disorder which causes high disease and psychological burden to patients.

1.2.5 Histopathology of plaque psoriasis

In healthy skin the epidermis consists of four layers. In the basal layer keratinocytes proliferate and daughter cells move upwards into the spinous layer. There they stop proliferating but remain metabolically active and undergo a differentiation process. Cells of the granular layer are smaller and show cytoplasmic granules, they terminally differentiate into cells of the cornified layer. These cells have lost their nucleus and cell organelles [120]. This differentiation process usually takes approximately 4 weeks [86]. Plaque psoriasis is marked by numerous characteristic changes in patient's skin. Most obvious are the thickening of the epidermis (acanthosis), the thickening of the cornified layer (hyperkeratosis) and the retention of cell nuclei during development of the cornified layer (parakeratosis). This histological phenotype is caused by an increased proliferation rate of the psoriatic keratinocytes. Moreover, the differentiation process is shortened (to 5-8 days) and incomplete, leading to a thickened stratum corneum with dead keratinocytes still possessing their nucleus. This process leads to the typical scaling of psoriatic skin. The stratum granulosum is also absent in lesional skin [86, 121]. The

dermis in psoriasis lesions shows formation of new vessels (angiogenesis) [58]. Leukocytes accumulate in both the epidermis and dermis, leading to the formation of Munro's microabscesses which are filled with neutrophils [122]. T lymphocytes are also found distributed in the psoriatic epidermis and dermis [121, 123]. These changes are absent in uninvolved skin of psoriasis patients.

1.2.6 Immunopathogenesis

As psoriasis is primarily a disease of the skin, the role the immune system plays in its initiation and perpetuation has long been discussed. The precise contributions that immune cells make to the pathogenesis of psoriasis have yet to be fully elucidated. But decades of intense research and the use of different animal models has revealed an inflammatory loop between the skin epithelium and the cells of the innate and adaptive arms of the immune system. The immune response during psoriasis can be divided into an initiation phase and a phase during established disease.

The most discussed initiation reaction of a psoriatic plaque is by mechanical stress on keratinocytes, for example the Koebner phenomenon. Plasmacytoid dendritic cells (pDCs) are cells of the innate immune system which are normally associated with antiviral functions. Upon detection of viral DNA by their surface Toll-like receptors (TLRs) they become activated and secrete high amounts of type I interferons (IFN) [124]. In the skin of psoriasis patients pDCs are activated by complexes of the antimicrobial peptide (AMP) LL-37 and self-DNA released from stressed or damaged keratinocytes. These bind to TLR9 on the pDC surface [125]. Subsequently, pDCs accumulate in the dermis of psoriasis patients and release high amounts of IFN- α . These cells are not found in significant numbers in healthy skin. Blocking IFN- α abrogates lesion development [126]. Keratinocytes also express TLR9 but do not detect LL-37-DNA complexes, rather LL-37 enhances the responsiveness of these cells to TLR9 ligands leading to production of type I interferons [127]. LL-37 also induces IL36 γ production by keratinocytes, these two molecules can then act together to induce keratinocytes to express a number of chemokines (CXCL1, CXCL8, CXCL10 and CCL20) which recruit DCs, neutrophils and lymphocytes into the developing lesions [128]. Keratinocytes, upon detecting LL-37, secrete pro-inflammatory type I IFNs [129]. These molecules activate myeloid dermal dendritic cells (DDC). These cells are highly increased in psoriatic skin compared to healthy skin [130]. DDCs can present antigens to T cells after traveling to the draining lymph node leading to T cell activation and proliferation [131]. Besides these classical DDCs (CD11c⁺ CD1c⁺ DDCs) there are inflammatory CD1⁻ DDCs found in psoriatic skin that play a major role in cytokine production, being an especially rich source of IL-23 and

TNF- α [132]. Both types of DCs can activate T cells which in turn produce IL-17A, IL-22 and IFN- γ all of which are highly expressed in psoriatic lesions [133-135].

Analysis has shown that the repertoire of specific T cell receptors (TCRs) expressed by T cells isolated from psoriasis patients is not only similar between patients but also remains so over time, indicating that antigen-specific oligoclonal T cell expansion is an important feature of the pathogenesis of psoriasis [136, 137]. Although the high degree of homology between these TCRs has been known for more than 20 years, the specific antigens that are being recognised are still under debate. Arakawa *et al.* reported that ADAMTS-like protein 5 (ADAMTSL5) is a candidate autoantigen in the initialisation of psoriasis [138]. Significant research is required to determine both the nature of the antigens in psoriasis and their role in disease pathogenesis.

Despite a lack of information regarding the antigen specificity of T cell responses in psoriasis, there has been significant progress in the understanding of the effector cytokines produced by T cells and their role in disease. Psoriasis has been initially thought to be a CD4⁺ Th1 mediated disease, due to the presence of T cells in psoriatic lesions producing large amounts of IFN- γ and IL-2 (but no Th2 cytokines such as IL-4 or IL-10) [139, 140]. The discovery of IL-17A-producing Th17 cells led to a reassessment of the nature of the T cell response in psoriasis. It has subsequently been shown that this T cell subset was the main type in psoriasis [141]. Hence, abundant IL-17A producing T cells can be found in the skin of psoriasis patients [142]. This subset of T cells is so named because of their expression of IL-17A [143]. Th17 cells can also express IL-17F, IL-21, IL-22, IL-26, and CCL20 and their effector phenotype is stabilised by IL-23 [144]. Many of these Th17-related cytokines are increased in lesional psoriatic skin [145]. Besides CD4⁺ Th17 cells, CD8⁺ IL-17A⁺ T cells have been identified in psoriatic plaques that also produce IFN- γ , TNF- α , IL-21 and IL-22 [146, 147]. In addition, $\gamma\delta$ T cells in human psoriatic dermis are a source of IL-17A [148]. Another T helper subset named Th22 cells due to their expression IL-22 rather than IL-17A has been identified in psoriatic lesions [149-151].

Th17 cytokines induce keratinocytes to produce AMPs including S100 proteins and human β -defensin 2 (hBD-2) *in vitro* [145]. IL-22 alone is able to stimulate keratinocytes to produce AMPs including human hBD-2 and S100 proteins along with the neutrophil chemotactic chemokine CXCL5 [143, 152]. S100 proteins can be detected in the serum of psoriasis patients and their levels increase with psoriasis severity indicating ongoing Th17-type responses. [153]. The Th17 cytokine IL-22 plays an important role in the induction of dermal inflammation, hyperplasia, and acanthosis as mice deficient in this gene display diminished IL-23-induced ear swelling and inflammation indicating that IL-22 is a downstream factor in IL-23 mediated signalling [152, 154]. IL-17A is not

required for the IL-23 induced epidermal hyperplasia in the mouse. Rather, TNF- α is needed for IL-23 induced acanthosis. Furthermore, in this murine model epidermal hyperproliferation is mediated by IL-23, although not by direct interaction with keratinocytes [155]. IL-17A together with TNF- α has been shown to induce S100 proteins, pro-inflammatory IL-1 β , IL-8 and IL-6 and the chemokines CXCL1, CXCL3 and CXCL5 in keratinocytes [156]. IL-8 and CXCL1, CXCL3 and CXCL5 are chemoattractants for neutrophils and other immune cells, thereby promoting immune cell infiltration into the skin [157]. Neutrophils in turn can release a plethora of other cytokines and chemokines, contributing to the perpetuation of inflammation [158]. TNF- α , IL-17A and IL-22 together stimulate keratinocytes to produce CCL20 which in turn recruits DCs and T cells into the lesions thereby sustaining skin inflammation [151]. IL-17A can also be released by neutrophils [159, 160]. IFN- γ released from Th1-type cells acts on keratinocytes by inducing CXCL9 and CXCL10, both known to induce the infiltration of T lymphocytes into lesions [157]. Interestingly, the serum levels of IL-17A are not significantly different between psoriasis patients and healthy individuals, despite high levels of expression in lesional skin [161]. This indicates that the IL-17A production is highly localized to the psoriatic skin [162].

The treatment of moderate to severe psoriasis with monoclonal antibodies against IL-17A, IL-23 and TNF- α has been highly successful leading to marked improvement in disease in many patients [163]. The efficacy of these biologic treatments highlights the importance of the IL-23/IL-17 axis in the pathogenesis of psoriasis. Once the psoriasis lesion is initiated it is maintained by an inflammatory loop between keratinocytes, dendritic cells and T cells. DCs stimulate T cells which in turn release cytokines which act on keratinocytes. These keratinocytes produce AMPs and chemokines leading to the recruitment of more immune cells. Interruption of this loop, for example by targeted treatment against key effector cytokines, leads to significant improvement of skin inflammation.

1.2.7 Psoriasis treatment with DMF

Although numerous systemic treatments directly targeting inflammatory cytokines are available, DMF based treatment is still the most used first line therapy for psoriasis in Germany.

In 1959 a German scientist suffering from psoriasis believed that his disease was due to a disturbance of the citric acid cycle caused by a lack of fumaric acid. After oral intake of fumaric acid his psoriasis lesions cleared [164]. This observation has led to the development of Fumaderm[®] which was first registered in Germany in 1994 as a combination of DMF and three monoethyl fumarate (MEF) salts. More recently,

Skilarence[®], a DMF-only drug, was approved for the treatment of psoriasis in Europe. Oral DMF is also been approved for the treatment of RRMS since 2014 and is marketed as Tecfidera[®] [165-167].

1.2.7.1 Side effects of DMF treatment

Although regarded as a safe drug, the treatment with oral DMF leads to a number of adverse events in both psoriasis and RRMS patients. These DMF-treated patients tend to suffer from diarrhoea, nausea, flushing, a reduction of peripheral T lymphocytes and a temporary increase of eosinophils [168]. Gastrointestinal (GI) problems often lead to a discontinuation of DMF therapy as does a significant reduction in lymphocytes [165]. Although the treatment discontinuation is mainly due to GI problems, the causative mechanism is not yet known. The BRIDGE study reported that approximately 10% of DMF treated psoriasis patients showed lymphopenia [169]. In 1% of patients the reduction in lymphocytes was severe (<700 cell μl^{-1} under Skilarence[®] therapy; <500 cell μl^{-1} under Fumaderm[®] therapy). This severe lymphopenia can increase the risk of infections and in severe cases can lead to a progressive multifocal leukoencephalopathy (PML) when left untreated [170-173]. This reduction in lymphocytes is seen mostly after half a year of treatment [170]. The reduction is highest in CD8⁺ T cells rather than CD4⁺ T cells [174]. It has further been shown that CD4⁺ and CD8⁺ memory T cells are highly affected by oral DMF treatment [175]. A mode of action regarding how DMF treatment leads to this reduction in lymphocytes remains elusive.

16-18% of patients in the BRIDGE trial complained about flush after DMF ingestion [169]. Flush is also a side effect of NA treatment and the mechanism behind is well understood (see section 1.1.1.1). Flush is observed on the face and upper body and consists of cutaneous vasodilation together with a burning sensation. Flush develops short after oral DMF intake and lasts around an hour while showing a biphasic occurrence with an immediate and a late peak [29].

1.2.7.2 Pharmacology of DMF

The use of Skilarence[®], a DMF-only drug, for the treatment of psoriasis demonstrates that the MEF salts in Fumaderm[®] are not adding to its therapeutic effect. DMF is a small molecule containing two ester groups and a double bond which makes the molecule highly reactive. DMF is hydrolysed to MMF either spontaneously or due to esterase cleavage. MMF can be cleaved further by esterases leading to fumaric acid, although this reaction occurs much more slowly as MMF is negatively charged and less prone to attack by nucleophilic water than neutrally charged DMF [176]. While DMF can pass

through plasma membranes due to its neutral charge, it is not detectable in the blood of patients or healthy individuals after oral administration [177, 178]. In contrast, MMF reaches plasma levels of 10-15 μ M after a standard (240mg) oral dose of DMF [178]. These observations show that DMF acts as a prodrug for MMF, which itself is the bioactive metabolite *in vivo*. In urine more metabolites can be detected after oral DMF. The mercapturic acids of MMF (NAC-MMS) and DMF (NAC-DMS) are detected [179]. The double bonds in DMF/MMF allow these molecules to bind to nucleophilic thiol groups found in many proteins. After passing through the plasma membrane DMF can bind to intracellular glutathione (GSH) forming GS-DMS which is then further processed to NAC-DMS. NAC-DMS can be hydrolysed to NAC-MMS. These molecules are then excreted by urine and faeces [180].

Besides the detection of MMF as the main DMF metabolite in rat and human blood, the clinical trial with XP23829 supports the theory that MMF is the bioactive metabolite that produces the therapeutic effects of DMF therapy [181]. This compound consists of MMF with a linker molecule and in clinical trials resulted in both the same treatment efficacy as Fumaderm[®] but also led to the same side effects [182]. Together, these observations strongly suggest that DMF is rapidly metabolized in the body to MMF mediating both the therapeutic benefits and unwanted side effects associated with DMF therapy.

1.2.7.3 Potential mechanisms of action of DMF treatment

Despite intense research about the mode of action of oral DMF therapy in both psoriasis and RRMS, it is not yet fully understood. Earlier research has focused on the suppression of the NF- κ B pathway by DMF and MMF and suppressive effects have been described in several cell types. DMF, but not other fumarates, reduces CD62E (E-selectin) expression by endothelial cells in an NF- κ B dependent manner. This reduction may lead to a decrease in leukocyte movement and adhesion to endothelial cells, key steps in the transmigration of immune cells into tissues [183]. In dermal fibroblasts DMF has also displayed inhibitory effects on the NF- κ B signalling pathway by reducing cytokine induced accumulation of NF- κ B1 (p50) in the cell nucleus [184]. Moreover, nuclear binding of NF- κ B1 is inhibited by DMF in both T cells and fibroblasts [185]. MMF affects DC polarisation *in vitro* leading to reduced IFN- γ production by co-cultured Th1 cells [186]. In DCs, DMF inhibits maturation and antigen presentation capacity in a NF- κ B mediated manner [187]. However, when considering the pharmacology of DMF it becomes obvious that these data are unlikely to reflect the mechanism of action of DMF treatment *in vivo* as this molecule does not enter the blood stream and cell types beyond the GI mucosa are not expected to encounter it. As MMF has not directly been

investigated in these experiments, it is unknown if it mediates similar NF- κ B dependent effects to DMF *in vitro*. Whether any such effects play a role *in vivo* is still unclear.

DMF immediately breaks down after oral intake producing MMF as the main metabolite. However, GSH complexes are formed and can be readily detected *in vivo*. Thus, DMF is able to reduce cellular GSH levels by forming GS-DMS, a molecule that can be detected in animals and humans after oral DMF [179, 181]. GSH is a key molecule in regulating intracellular redox systems. In astrocytes DMF is able to reduce cellular GSH levels without affecting viability. MMF shows similar effects but with higher concentrations reflecting its lower reactivity and membrane permeability [188]. Depletion of GSH in APCs favours the generation of Th2 cells rather than Th1 cells [189]. A depletion of GSH in peripheral blood mononuclear cells (PBMCs) results in the induction of the anti-inflammatory stress protein heme oxygenase 1 (HO-1) leading to inhibited proliferation of alloreactive T cells in a mixed leukocyte reaction [190]. HO-1 expression is also induced upon activation of the NRF2 signalling pathway [191, 192]. This GSH depletion and HO-1 induction has been shown to induce DCs to produce IL-10 instead of IL-23, promoting Th2 cell differentiation. The NF- κ B pathway has also been reported to play a role in this phenomenon [193]. Although these effects are mostly described after DMF treatment *in vitro*, they may occur in the human GI tract (which contains abundant leukocytes). Especially within MS research, the efficacy of DMF treatment is viewed as dependent upon the role of DMF as a modulator of cellular redox systems such as the NRF2 pathway. But also, the involvement of the NRF2 pathway in the treatment of psoriasis with oral DMF is of increasing interest. Hence, Helwa *et al.* have demonstrated that treatment of murine keratinocytes with MMF leads to the increase of total and nuclear NRF2 protein levels as well as the expression of NRF2 target genes. [194]. Despite these data, whether the induction of the NRF2 redox pathway is mediating DMFs therapeutic effect in psoriasis is not yet known. Besides the many insights into the possible mechanism of action of DMF, the overall picture is still incomplete. Interestingly, oral DMF treatment of psoriasis patients has led to a downregulation in RNA of a number of disease-specific genes, including S100 proteins and IL-17 pathway genes, but only in the skin of treatment responders while non-responders have shown induction of NRF2 pathway and GSH redox system related genes [195]. The complexity of determining a potential role for NRF2 signalling in the anti-inflammatory effect of DMF treatment *in vivo* is underlined by the fact that NRF2 knockout mice have been reported to display similar amelioration of EAE symptoms to WT mice when treated with oral DMF. This observation indicates that at least for the treatment of neural inflammation by oral DMF there are NRF2 independent pathways that are important for its therapeutic effect [196]. In contrast, Linker *et al.* have reported that NRF2 signalling is required for the neuroprotective effects of oral DMF. However,

when considering the role of NRF2 in the therapeutic effects of oral DMF on the CNS, the fact that NRF2 knockout mice show exacerbated disease in the EAE model should be kept in mind [197, 198]. Furthermore, HCA2 is dispensable for the induction of NRF2 signalling by MMF [199]. HCA2 knockouts are not protected from CNS inflammation by DMF treatment indicating that NRF2 pathway activation alone does not account for the therapeutic effects of oral DMF. Therefore, the current understanding of the mechanism of DMF treatment is incomplete and the contribution of HCA2 to any effect must be considered both *in vitro* and *in vivo*.

1.3 The imiquimod mouse model of psoriasis-like skin inflammation

Psoriasis is a solely human disease and animal models have to be used in order to perform functional analysis *in vivo*. Today there are several murine models of psoriasis-like skin inflammation. As with all mouse models, each one has advantages and limitations. The imiquimod (IMQ) model is the most widely used and discussed murine analogue of psoriasis [200]. Imiquimod-containing Aldara[®] cream is normally used for treatment of superficial basal cell carcinomas, actinic keratosis and genital warts and is an agonist of the TLR7 and TLR8 leading to immune activation [201, 202]. Aldara[®] has been observed to provoke psoriatic lesions at the sites of application in psoriasis patients [203-205]. The development of these lesions is associated with infiltration of pDCs and the production of type I IFNs [201]. IMQ treatment also leads to the migration of dermal LCs to skin draining lymph nodes [206].

When IMQ containing Aldara[®] cream is applied to the skin of mice, immune cell infiltration and skin inflammation is observed [207]. Daily application of Aldara[®] on the skin of mice induces psoriasis-like features such as erythema, thickening and scaling. Similar to human psoriasis, IMQ-induced lesions show hyperproliferation of keratinocytes and parakeratosis. Immune cell infiltrates in the skin consist of increased frequencies of APCs, neutrophils and T cells compared to control skin [208]. Identical to human psoriasis lesions, Aldara[®]-induced lesions form neutrophil-containing Munro's microabscesses. The formation of these abscesses relies on IL-1 signalling. IL-1 β is released by IMQ treated keratinocytes [209]. *In vitro*, IL-17A and IL-22 induce the release of IL-1 β by human keratinocytes [210]. Furthermore, IL-1 β has been shown to be produced by activation of the inflammasome in an IMQ independent manner by the vehicle component of Aldara[®] cream [211]. Thus, the model requires the use of the original Aldara[®] cream. IMQ-induced psoriasis-like lesions also display transient increases in mRNA expression of *Il23a* and *Il17a* both of which are key cytokines in psoriasis in humans [208]. DCs and macrophages are the main producers of IL-23 in IMQ-treated mice [148]. $\gamma\delta$ T cells are the main source of effector T cell-derived cytokines

in IMQ induced lesions [212]. IL-23 stimulation promotes dermal $\gamma\delta$ T cells to produce the IL-17A necessary for epidermal hyperplasia [148]. Infiltrating $\gamma\delta$ T cells producing IL-22 are important for skin inflammation as IMQ-treated IL-22 knockouts do not exhibit neutrophil infiltration into the skin and psoriasis-like lesion development [213]. In humans, IL-22 acts on keratinocytes by inhibiting differentiation [152]. IL-22 has also been demonstrated to induce the production of S100 proteins and keratin 14 (KRT14) by keratinocytes [213]. Mast cells play a role in the early steps of IMQ inflammation by producing TNF- α and stimulating the migration of LCs to the SLN [214]. Migration of LCs to the SLN has been shown after IMQ application, leading to a morphologically altered population of LCs in the skin [206].

The amount of published literature regarding the IMQ model has increased almost logarithmically over the past decade. Analysis of its similarities to human psoriasis and on its specific pathogenesis has yet to reveal the complete mechanism of Aldara[®] induced skin inflammation [215]. Nevertheless, the IMQ-mouse model is frequently used for the functional analysis of the processes that contribute to psoriasis-like skin inflammation. While the model is relatively easy to implement and is not cost-intensive, there are a number of limitations [215]. For instance, the various features of the psoriasis-like phenotype vary between mouse strains and sexes in regard to kinetics and inflammation intensity [208, 216]. Furthermore, IMQ application also induces systemic symptoms and weight loss, which can be exacerbated by oral uptake of Aldara[®] cream due to grooming [217]. Moreover, the IMQ model is a short-term model of acute skin inflammation while human psoriasis is a long-term and chronic condition [215]. The IMQ mouse model would be better named as the Aldara[®] mouse model as it relies on the application of Aldara[®] cream. The IMQ and the vehicle components of the cream, specifically isostearic acid within the vehicle, mediate independent effects in the model that are both TLR7 dependent and independent, respectively [211].

1.4 Aim of this work

Originally, being regarded as a sensor for the metabolic status of the host organism, HCA2 also plays a central role in the treatment of dyslipidaemia by NA. Although MMF, the primary bioactive metabolite of DMF, is a known agonist of HCA2 there has been no published investigations about the role of HCA2 in the treatment of psoriasis by oral DMF. In murine models of MS and skin blistering disease there is evidence that HCA2 mediates the therapeutic effect of oral DMF treatment. Furthermore, the treatment of patients with oral DMF leads to a reduction in peripheral leukocyte counts primarily due to a loss of T lymphocytes. The underlying mechanisms that lead to this side effect are still unknown. Whether there is a causal relationship between reduction in leukocytes

and the clearance of psoriatic lesions by oral DMF is another unsolved question. Given these points, the aims of this thesis were as follows:

- **Quantify the changes in human leukocyte populations during the early phase of DMF treatment and HCA2 expression analysis of different immune cell subtypes as a possible relating factor.** It was hypothesised that the early amelioration of skin lesions by oral DMF treatment is accompanied by changes in leukocyte number and composition. It was assumed that these changes may be associated with the expression of HCA2 by the cell populations showing a change in number. Blood cell counts were determined in patients before and in the first three months of DMF treatment. Immune cells were characterized for HCA2 expression in the blood of psoriasis patients and healthy human controls. Moreover, HCA2 expression on immune cells in lymphoid organs and bone marrow of mice was analysed. *In vitro* the effect of MMF and DMF treatment on neutrophils was analysed in regard to apoptosis induction, internal signalling and change in receptor expression.
- **Elucidate the role of HCA2 in mediating the protective effect of oral DMF in a mouse model of psoriasis.** It was hypothesised that the therapeutic effect of oral DMF treatment in psoriasis is mediated by HCA2. Furthermore, it is possible that BHB, one of the endogenous ligands of HCA2 can also mediate similar protective effects as oral DMF. By utilising HCA2^{-/-} mice the role of HCA2 in the improvement of skin inflammation by oral DMF treatment was assessed in the IMQ mouse model. Analysis of mRNA and protein levels of key psoriatic cytokines, chemokines and antimicrobial peptides on different days were performed to investigate the underlying mechanisms. Effects on immune cell migration were investigated by flow cytometric analysis of skin and SLN cells. Further, preliminary experiments were carried out to determine the role of BHB in protection from skin inflammation
- **Identify the possible HCA2 expressing target cell type that mediates the protective effects of DMF treatment.** It was hypothesized that DMF's active metabolite MMF has direct effects on *in vitro* cultured keratinocytes, indicating a potential mode of action in the improvement of skin lesions. By the use of bone marrow chimeras, the cell type mediating DMF's protective effect was investigated. *In vitro* analysis of MMF treated murine and human keratinocytes was performed in order to elucidate DMF's mode of action.

Chapter 2. Materials and Methods

2.1 Patients

Psoriasis patients and healthy controls were enrolled at the Psoriasis Center of the Department of Dermatology at the University Medical Center Schleswig-Holstein (UKSH), Campus Kiel. Approval was obtained from the local ethics committee. All study participants provided informed written consent.

2.1.1 Prospective cohort study

This was a single-center prospective cohort study. Data were collected between January and August 2018. Patients were over 18 years of age and were diagnosed by dermatologists to have plaque psoriasis. Only patients who had never been previously prescribed DMF therapy were included. The treatment with Skilarence® has been initiated with a dosage phase according to the manufacturer's recommendation (Table 1). Peripheral blood was taken by venepuncture prior to treatment with Skilarence® (month 0) and then once every month until month 3. Clinical parameters (including Psoriasis Area Severity Index, PASI) were determined by a dermatologist at every visit. All samples were collected, analysed and stored within the Psoriasis Center.

Table 1: dosing scheme of Skilarence® treatment

Week	Daily dose (mg)
1	30
2	60
3	90
4	120
5	240
6	360
7	480
8	600
9+	720

2.1.2 Patients and controls for HCA2 expression analysis

Psoriasis patients were diagnosed by a dermatologist to have psoriasis. All patients were treatment naïve. Healthy individuals were matched to patients in terms of age and sex. Peripheral blood was taken by venepuncture. Samples were immediately analysed by flow cytometry as stated in 2.5.1.

2.2 Animals

Experiments were carried out in accordance with the German Animal Welfare Legislation and permitted by the Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung des Landes Schleswig-Holstein (MELUND) (AZ: V 242-72045-2016 (119-9-16), V242-58652/2019(22-2/19) and V242-40506/2018 (66-7/18)).

HCA2^{-/-} mice (Hcar2tm1Soff) and red fluorescent protein (RFP)-HCA2 mice (Hca2mRFP) were bred at the Zentrale Tierhaltung (ZTH) of UKSH, Campus Kiel. All experiments were carried out within ZTH. Both mouse lines were originally generated by Stefan Offermanns [6, 17] and were generously donated by his lab. C57BL6J mice were purchased from Janvier Labs. Mice were 7 weeks old upon arrival and were allowed to acclimate to local conditions for at least 1 week before experiments were started. CD45.1⁺ C57BL6J mice (B6.SJL-PtprcaPepcb/BoyCrl) were purchased from Charles River Laboratories. Mice were 4-5 weeks old upon arrival and were acclimated for at least 1 week before experiments were performed. All mice were bred and maintained under specific pathogen free (SPF) conditions. All animals were used between 2 and 6 months of age at the start of experimental work.

2.2.1 Generation of bone marrow chimeras

To generate bone marrow chimeras, 6-8 weeks old CD45.1⁺ C57BL6J mice and CD45.2⁺ HCA2^{-/-} mice were irradiated with a single dose of 8Gy. 24h after radiation treatment, mice were given bone marrow cells from untreated CD45.1⁺ C57BL6J or HCA2^{-/-} donor mice. 10 x10⁶ bone marrow cells were injected intravenously into irradiated recipient mice. Transferred bone marrow was then given 8 weeks to allow for reconstitution. During the initial 2 weeks of reconstitution mice were treated with 0.9% Baytril in the drinking water. Reconstitution efficacy was tested by flow cytometry before the start of experiments. Therefore, 10 µl of blood were collected from mouse tail veins and clotting was prevented with heparin. Recipient and donor were distinguished by the congenic markers CD45.1 and CD45.2. Samples were stained directly with anti-CD45.2-FITC (Clone 104, BioLegend, San Diego, USA) and anti-CD45.1-Pacific Blue 421 (Clone A20, BioLegend, San Diego, USA) antibodies for 15 min on ice and red blood cells were removed with hypertonic lysis buffer (BioLegend, San Diego, USA) and centrifugation. Cells were then analysed by flow cytometry.

2.2.2 DMF treatment and Aldara® application

For the oral DMF treatment of mice, a 0.8% methyl cellulose (methocel) solution was prepared by adding 4g methyl cellulose (Sigma-Aldrich, Dorset, UK) to 170ml of boiling water followed by continuous stirring until fully dissolved. 330ml ice cold distilled water was added and the solution was stirred until clear. 100mg of DMF (Sigma-Aldrich, Dorset, UK) was then suspended in 0.5ml of methocel solution by grinding with a pestle and mortar until no flakes were left. Stepwise 9.5ml methocel was added to yield a 10mg/ml DMF solution. DMF solution was prepared freshly before each treatment. Mice were treated twice a day with 50mg/kg of DMF by oral gavage. Mice were weighted once a day and volume of DMF solution was adjusted according to current bodyweight.

For Aldara® (Meda Pharma, Bad Homburg, Germany) application, the back of the mice was shaved and 50mg Aldara® cream was applied thoroughly over the skin.

2.2.3 Ketogenic diet

To analyse the effect of BHB on the development of the Aldara®-induced psoriasis-like lesions, 12-week-old mice were put on a ketogenic diet for 2 weeks. Ketogenic diet (E15149-307 EF R/M Ketogenic 80 % fat) and control diet (V1534-000 Ratte/Maus Haltung) were purchased from Sniff Spezialdiäten (Soest, Germany). The ketogenic diet contained 80% fat, 8% protein and 1.4% starch/sugar. In comparison, the control diet had 9% fat, 24% protein and 67% carbohydrates. As the ketogenic diet was a paste, wood sticks were given as an enrichment to avoid excessive tooth growth. Weight of mice was checked every other day. Blood ketone levels were measured from venous blood with the BHB-check β -ketone system (MedNet, Münster, Germany).

2.3 Cell isolation

Phosphate-buffered saline (PBS) and Hanks' balanced salt solution (HBSS) were purchased from Pan-Biotech (Aidenbach, Germany).

2.3.1 Human leukocytes

After venepuncture, leukocytes were isolated from ethylenediaminetetraacetic acid (EDTA) -anticoagulated whole blood by red blood cell lysis with 10ml RBC red blood cell lysis buffer (Biolegend, San Diego, USA) per 1.5ml whole blood. Leukocytes were counted using trypan blue exclusion dye. Leukocytes were diluted to a concentration of 10×10^6 cells ml^{-1} in HBSS and stained for flow cytometric analysis.

Neutrophils were isolated from whole blood by the use of MACSxpress Whole Blood Neutrophil isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. 8ml of whole blood are mixed with the provided isolation mix and incubated for 5 minutes on an orbital shaker. Afterwards neutrophils are isolated by negative magnetic isolation. Cells were counted and diluted to specific concentrations depending on the assay.

Peripheral blood mononuclear cells (PBMC) were isolated from PBS diluted whole blood by a Polysucrose 400 (Bioclot, Aidenbach, Germany) gradient. Gradients were centrifuged for 15 minutes and the resulting PBMC layer was taken and washed. Cells were counted and diluted to a concentration of 1×10^6 cells ml^{-1} in HBSS and stained for flow cytometric analysis.

2.3.2 Mouse thymus, lymph node and spleen cell isolation

Thymus, skin draining lymph nodes (SLN) and spleen were taken from sacrificed mice. Organs were placed in a $100\mu\text{m}$ cell strainer (Greiner, Germany) and pushed through with a syringe plunger. Strainers were then washed with 20ml PBS into a 50ml canonical tube. Cell suspensions were centrifuged at $380 \times g$ for 8 minutes and SLN cells were resuspended in $200\mu\text{l}$ PBS. Thymus and spleen cells were resuspended in 5 ml of red blood cell (RBC) lysing buffer and incubated at room temperature for 10 minutes before the buffer was removed by centrifugation. Cells were then washed in 20ml of PBS and again centrifuged. Cell pellets were resuspended in PBS. Cell viability and number was assessed by dye exclusion with trypan blue and counting with a Neubauer haemocytometer. Cell suspensions were diluted to a final concentration of 10×10^6 cells ml^{-1} .

2.3.3 Mouse skin immune cells

Aldara[®] treated mice were sacrificed and four punch biopsies were taken from the shaved back skin before being cut into small pieces with a scalpel. The tissue pieces were then digested in 4ml of $400\mu\text{g ml}^{-1}$ Liberase TL (Roche, Basel, Switzerland) and 1mg ml^{-1} Collagenase D (Roche, Basel, Switzerland) in PBS for 2h at 37°C while shaking at 330rpm. The digested tissue suspension was then passed through a $100\mu\text{m}$ cell strainer aided with a syringe plunger. The strainer was then washed with 20ml of PBS and cells were retrieved by centrifugation at $380 \times g$ for 10 min. The cell pellet was resuspended in 5ml of 40% isotonic Percoll (GE Healthcare, Chicago, USA) in PBS and centrifuged at $380 \times g$ for 10 min. Cells were then washed with 20ml PBS, centrifuged and the resulting

pellet was resuspended in 200µl of HBSS for flow cytometry staining. Cells were counted with a trypan blue stain distinguishing viable and dead cells.

2.3.4 Isolation of mouse bone marrow cells

The femurs of sacrificed mice were collected, cleaned and carefully crushed in a mortar in 2ml PBS. The resulting cell suspension was transferred through a 100µm strainer into a 50ml canonical tube. Bone fragments were rinsed with PBS until all cells were removed and transferred. The cell suspension was centrifuged at 280 x g for 10 minutes and resulting pellet was resuspended in 5ml of RBC lysis buffer. After 10 minutes of lysis at room temperature (RT) samples were centrifuged and the pellet was washed with PBS. After centrifugation, the pellet was resuspended in PBS and cells were counted.

2.4 *In vitro* culture

Tissue culture plastics was purchased from Sarstedt (Nümbrecht, Germany), Nerbe Plus (Winsen, Germany) and Greiner Bio-One (Kremsmünster, Austria).

2.4.1 Human normal primary keratinocytes

Normal human epidermal keratinocytes (NHEKs) (Promocell, Heidelberg, Germany) were cultured in keratinocyte growth medium (KGM) 2 (PromoCell, Heidelberg, Germany), including all supplements and 100U/ml penicillin and 100µg/ml streptomycin at 37°C and 5% CO₂ in Costar culture flasks (Corning, Corning, USA).

2.4.2 Mouse keratinocytes

Keratinocyte isolation was done as published by Li *et al.* with minor changes [218]. Mouse skin was removed from decapitated new born mice, rinsed with ethanol and collected in PBS with 100U/ml penicillin and 100µg/ml streptomycin (Sigma-Aldrich, St. Luis, USA). Five whole skin specimens were then incubated for 45 min in 10ml PBS with 10mg/ml dispase II (Sigma-Aldrich, St. Luis, USA) at 37°C on a rotary shaker. Thereafter, the epidermis was removed with forceps and placed with the basal layer down on a drop of 0.05% trypsin/ 0,02% EDTA (both Sigma, St. Luis, USA) in a petri dish at RT for 5 min. 5ml CnT-57 medium (Cellntec, Bern, Switzerland) was then added. Skins were rubbed over the bottom of the plate to release basal keratinocytes into solution. This was repeated twice. Cell suspension was pipetted through a 100µm strainer and collected in a canonical tube. The tube was topped-up with PBS with 100U/ml penicillin and 100µg/ml streptomycin and centrifuged at 380 x g for 8 minutes. Cells were washed with 50ml of

PBS with 100 U/ml penicillin and 100µg/ml streptomycin, centrifuged again and the cell pellet was resuspended in CnT-57 medium with 100U/ml penicillin and 100µg/ml streptomycin. Cells were cultured with 3 mouse equivalents per culture flask in 10ml medium.

2.5 Flow cytometry

Flow cytometry was conducted on a CytoFLEX flow cytometer using CytExpert software (Beckman Coulter, Krefeld, Germany). The resulting data were analysed using FlowJo v10 software (Becton, Dickinson & Company).

2.5.1 HCA2 expression on immune cells

To elucidate the expression of HCA2 on human immune cells, blood immune cells were isolated as described in 2.3.1. T cell subsets and granulocytes were analysed from whole leukocyte isolations while DCs were analysed from PBMCs to increase cell abundance. Murine tissue resident immune cells were isolated as stated in 2.3.2, 2.3.3 and 2.3.4. Cells were aliquoted into a v-bottom 96-well plate before staining with antibodies with a concentration of 1×10^6 cells per well in 100µl of HBSS. Cells were stained with surface marker specific antibodies (Table 2) at pre-determined concentrations. Human cells were stained with FcR block (BD Bioscience, San Diego, USA) and Zombie NIR live/dead dye, additionally. After being mixed by pipetting, samples were incubated for 15 minutes on ice while protected from light. Samples were centrifuged at 380 x g for 5 minutes, supernatants were removed and cells were washed with 200µl PBS and subsequently centrifuged again. This washing was repeated twice in total. Thereafter, washed cells were resuspended in 200µl of PBS and run on a flow cytometer directly.

Table 2: Monoclonal antibodies used for flow cytometry

Name	Clone	Conjugate(s)	Supplier
<i>HCA2 expression by human blood immune cells</i>			
CD11c	3.9	PE, FITC	BioLegend
CD123	6H6	Brilliant Violet 605	BioLegend
CD14	HCD14	Alexa Fluor 700, PE	BioLegend
CD16	3G8	PE-Cy7, Alexa Fluor 700	BioLegend
CD19	HIB19	APC, Alexa Fluor 700	BioLegend
CD3	HIT3a, OKT3	PerCP-Cy5.5, Alexa Fluor 700	BioLegend
CD303	201A	PE-Cy7	BioLegend
CD4	OKT4	PE	BioLegend
CD45	H130	Brilliant Violet 510	BioLegend
CD56	5.1H11, HCD56	Alexa Fluor 700, APC	BioLegend
CD8	RPA-T8	Brilliant Violet 605	BioLegend
FCeRTa	AER-37(CRA-1)	PerCP-Cy5.5	BioLegend
HCA2	245106	FITC, APC	R&D Systems
HLA-DR	L243	Brilliant Violet 421	BioLegend
Rat IgG2B	(IC013F)	APC	R&D Systems
Zombie NIR dye		NIR	BioLegend
Human BD Fc Block		purified	BD Pharmingen
Siglec-8	7C9	APC	BioLegend
$\gamma\delta$ -TCR	B1	PE-Cy7	BioLegend
<i>HCA2 expression by murine immune cells</i>			
CD45	30-F1	APC, Alexa Fluor 700	BioLegend
Ly-6G	1A8	Alexa Fluor 488	BioLegend
CD11b	M1/70	Brilliant Violet 510	BioLegend
I-A/I-E	M5/114.15.2	APC-Cy7	BioLegend
Ly-6C	HK1.4	Alexa Fluor 700	BioLegend
CD19	6D5	Brilliant Violet 421	BioLegend
CD3	17A2	Brilliant Violet 605, Brilliant Violet 421	BioLegend
CD11c	N418	PE-Cy7	BioLegend
$\gamma\delta$ -TCR	GL3	Brilliant Violet 510	BioLegend
CD4	GK1.5	PE-Cy7	BioLegend
CD8	53-6.7	Alexa Fluor 700	BioLegend
NK1.1	PK136	Brilliant Violet 421	BioLegend
GR1	RB6-8C5	Brilliant Violet 421	BioLegend
CD117	2B8	Pe-Cy7	BioLegend
Sca.1	D7	FITC	BioLegend
CD127	A7R34	Brilliant Violet 605	BioLegend
CD34	HM34	APC	BioLegend
CD16/32	93	Brilliant Violet 510	BioLegend
Zombie NIR dye		NIR	BioLegend

2.5.2 Cytometry in prospective cohort study

Leukocytes were isolated as described in 2.3.1. Total cells isolated per patients were evaluated by counting cells in trypan blue dye with a haemocytometer. Per staining panel, 1×10^6 cells were incubated in a v-bottom 96-well plate with specific cell surface markers (Table 3) for 15 min on ice in the dark. Thereafter, samples were centrifuged at $380 \times g$ for 5 minutes and supernatants were removed. 200 μ l PBS was added to wash and the cells subsequently centrifuged. Washing was repeated twice in total. Cell pellets were resuspended in 200 μ l PBS and analysed on a flow cytometer. Absolute cell number was calculated by relating the relative cell abundance in the staining to the absolute cell number determined by trypan blue counting.

Table 3: Monoclonal antibodies used for flow cytometry in the prospective cohort study

Name	Clone	Conjugate(s)	Supplier
CD45	H310	Brilliant Violet 510	BioLegend
CD3	OKT3	Alexa Fluor 700	BioLegend
CD4	OKT4	PerCP-Cy5.5	BioLegend
CD8	HIT8a	PE-Cy7	BioLegend
CD19	HIB19	FITC, Alexa Fluor 700	BioLegend
CD56	HCD19, 5.1H11	APC, Alexa Fluor 700	BioLegend
CD16	3G8	PE-Cy7	BioLegend
Siglec-8	7C9	APC	BioLegend
CD11c	3.9	APC	BioLegend
CD14	REA599	FITC	Miltenyi
HLA-DR	L243	Brilliant Violet 421	BioLegend
CD11b	ICRF44	PerCP-Cy5.5	BioLegend

2.5.3 Immune cells from Aldara[®] induced skin lesions and SLN

Immune cells were isolated as described above and resuspended in 200 μ l HBSS. Cells were counted using a trypan blue dye to visualise viable cells. Cells were the stained for surface markers (Table 4) to distinguish immune cell subsets and Zombie NIR live/dead dye. Samples were incubated for 15 minutes on ice in the dark and subsequently centrifuged at $380 \times g$ for 5 minutes and resuspended in 200 μ l PBS for washing. The centrifugation and washing step were repeated and resulting pellets were resuspended in 200 μ l PBS and analysed on a flow cytometer. Absolute cell numbers were calculated with the absolute cell number derived from trypan blue staining as a reference.

Table 4: Monoclonal antibodies used for flow cytometry of mouse skin and SLN:

Name	Clone	Conjugate(s)	Supplier
<i>skin immune cells</i>			
CD11b	M1/70	Pacific blue	BioLegend
CD4	GK1.5	PE-Cy7	BioLegend
CD45	104	Brilliant Violet 510	BioLegend
CD8	53-6.7	PerCP-Cy5.5	BioLegend
Ly-6C	HK1.4	PE	BioLegend
Ly-6G	1A8	FITC	BioLegend
β-TCR	H57-597	Brilliant Violet 605	BioLegend
γδ-TCR	G23	APC	BioLegend
Zombie NIR dye		NIR	BioLegend
<i>skin draining lymph nodes</i>			
CD207	4C7	PE	BioLegend
CD45	30-F1	Alexa Fluor 700	BioLegend
I-A/I-E	M5/114.15.2	APC	BioLegend
Zombie NIR dye		NIR	BioLegend

2.5.4 Annexin V apoptosis assay

Neutrophils were isolated by MACSxpress magnetic separation. 10×10^6 cells ml^{-1} were aliquoted and diluted in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Darmstadt, Germany) containing 10% inactivated fetal calf serum (FCS; Merck, Munich, Germany) and 1X HEPES (Merck, Munich, Germany) in a 96 well-plate. Cells were cultured with the indicated concentrations of DMF and MMF or dimethyl sulfoxide (DMSO) (all from Sigma, St. Luis, USA) as a solvent control for 4h at 37°C and 5% CO₂. Thereafter, cells were washed, resuspended in HBSS and stained with CD16 - PE-Cy7, Annexin V – FITC (both BioLegend, San Diego, USA) and Zombie NIR live/dead dye. Stained cells were incubated for 15 min on ice in the dark. Samples were then centrifuged at 380 x g for 5 minutes and washed with 200µl PBS. After centrifugation cells were washed once more. Resulting pellets were resuspended in 200µl PBS and analyzed by flow cytometry.

2.5.5 MMF induced HCA2 expression changes on neutrophils

Isolated neutrophils were resuspended in DMEM (Thermo Fisher Scientific, Darmstadt, Germany) with 10% inactivated fetal calf serum (FCS; Merck, Munich, Germany) and 1X HEPES (Merck, Munich, Germany) in a 96 well-plate at a concentration of 10×10^6 cells ml^{-1} . For 30 minutes at 37°C and 5% CO₂ or at 4°C neutrophils were incubated with 100µM DMF or MMF or DMSO as solvent control. Thereafter, cells were centrifuged at 380 x g for 5 minutes and pellets were resuspended in 100µl HBSS. Cells were then stained with CD16 – PE-Cy7 (BioLegend, San Diego, USA), Zombie NIR live/dead dye

(BioLegend, San Diego, USA), HCA2-FITC and isotype control (R&D Systems, Minneapolis, USA). The stained samples were incubated on ice for 15 minutes in the dark before being washed twice with 200µl PBS. Washed cell pellets were resuspended in 200µl PBS and analyzed on a flow cytometer.

2.5.6 Calcium signalling

Neutrophils were aliquoted into a v-bottom 96-well plate at a concentration of 10×10^6 cells ml^{-1} in DMEM and 3µM Fluo-4 and 6µM Fura-red (both Life technologies, Carlsbad, USA) were added and samples were incubated for 45 minutes at 37°C and 5% CO₂. Thereafter, cells were washed with 200µl PBS twice with centrifugation of 380 x g for 5 minutes and subsequently resuspended in 200µl PBS. Cells were allowed to rest for another 30 minutes at RT in the dark. Cells were then acquired on a flow cytometer for 20 seconds before 100µM DMF, MMF or DMSO was added to the tubes and acquisition was continued for another 80 seconds. For analysis the changes in ratio of fluorescence intensity between Fluo-4 and Fura-red were calculated over time.

2.6 *In vitro* assays

2.6.1 Cyclic AMP assay

Neutrophils were seeded at 1×10^6 cells per well in a 96-well plate. NHEKS and mouse keratinocytes were seeded at 1×10^5 cells per well and allowed to adhere overnight. Cells were then incubated in PBS with 100µM forskolin (abcam, Cambridge, UK) and the indicated concentrations of DMF, MMF and solvent control for the indicated times. Subsequently, culture medium was removed and cells were lysed with 120µl 0.1% HCl. Samples were stored at -80°C until analysis. Samples were analysed with the direct cAMP ELISA kit (Enzo, New York, USA) according to the manufacturer's protocol. Neutrophil samples were acetylated before the assay was performed. Keratinocyte lysates were used directly.

2.6.2 BrdU incorporation assay

Keratinocytes were isolated as described in 2.4.2. 1×10^3 cells were added to 96-well plates and left to adhere overnight. Cells were then treated with the indicated concentrations of MMF or PBS as solvent control for 48h at 37°C and 5% CO₂. Cell proliferation was assessed using the Cell Proliferation ELISA BrdU kit (Roche, Basel, Switzerland). Thus, cells were cultured in presence of 10µl bromodeoxyuridine (BrdU) for the last 2h of incubation time and the assay was run according to the manufacturer's instructions. After addition of the substrate solution and following development time, 25µl

H₂SO₄ (Sigma-Aldrich, Dorset, UK) was added to stop the reaction. Absorbance was measured on a Tecan Infinite M Plex reader (Tecan Life Science, Männedorf, Switzerland).

2.6.3 WES assays

Mouse keratinocytes were isolated as described in 2.4.2. 1×10^6 cells per well were placed into 6-well plates with CnT-57 medium containing 100U/ml penicillin and 100 μ g/ml streptomycin and grown to 90% confluence. Cells were then stimulated with the indicated concentrations of MMF or PBS as a solvent control. Stimulated cells were incubated at 37°C for 5 minutes. Subsequently, the medium was removed and cells were lysed with 200 μ l RIPA buffer (Thermo Fischer Scientific, Waltham, USA) containing protease inhibitor tablets (Merck, Munich, Germany). Cells were lysed on ice for 30 minutes and buffer was then transferred to fresh microcentrifuge tubes and centrifuged at 1000 x g for 5 minutes to remove debris. Supernatants were transferred into new tubes and protein concentration was determined using a BCA Protein assay (Thermo Fischer Scientific, Waltham, USA) according to the manufacturer's instructions. Protein concentrations were adjusted to 500ng μ l⁻¹ and frozen at -20°C until analysis.

To analyse protein phosphorylation, WES-simple Western analysis (ProteinSimple, California, USA) assays were performed using 12-230kDA separation modules according to the manufacturer's instructions. Antibodies and concentrations used are indicated in Table 5. The anti-Rabbit detection module was used as a secondary antibody. The module was run on the WES analyser. Results were analysed using the Compass for SW software (ProteinSimple, California, USA).

Table 5: Antibodies used for WES analysis

Target	Supplier
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signalling Technology
p44/42 MAPK (Erk1/2) (137F5)	Cell Signalling Technology
Phospho-Akt (Ser473) (D9E)	Cell Signalling Technology
β -Actin (13E5)	Cell Signalling Technology
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signalling Technology
p38 MAPK (D13E1)	Cell Signalling Technology

2.7 Skin histology

2.7.1 Haematoxylin and eosin (H&E) staining

After sacrificing Aldara® treated mice, skin samples were taken by punch biopsy and directly transferred into a 10% formalin solution (Sigma-Aldrich, St. Louis, USA). Samples were embedded by the Histology Section of the Department for Dermatology (UKSH, Kiel, Germany). 5µm skin sections were haematoxylin and eosin (H&E) stained by the technicians of the Histology Section. Slides were scanned with a NanoZoomer slide scanner (Hamamatsu, Japan) and analysed blindly using the NDP.view 2 software (Hamamatsu, Japan). Epidermal thickness was measured at 10 sites per section and mean was calculated.

2.7.2 *In situ* hybridization

Mouse skin was collected from sacrificed mice and embedded as described above. 5µm slices were mounted on Superfrost Plus Slides (Thermo Fischer Scientific, Waltham, USA). *In situ* hybridization was performed with the RNAscope system (ACD, California, USA). Slides were deparaffinised according to the manufacturer's protocol as were all subsequent downstream steps with some minor modifications. The RNAscope protease Plus was applied and incubation time was 20 minutes. Retrieval times were 15 minutes. To detect HCA2 mRNA expression the HCA2 probe Mm-Hcar2 was used with RNAscope 2.5 HD Detection Reagent-RED according to the manufacturer's protocol. Slides were finally imaged using the HAMAMATSU slider scanner with the NDP.view 2 software.

2.7.3 Immunohistochemistry

Embedded skin samples from HCA2-RFP mice were placed on silanized slides (Labsolute, TH Geyer, Renningen, Germany). Paraffin was removed with a decreasing alcohol series: Xylene (2x5 minutes), 100% ethanol (2x5 minutes), 96% ethanol (2x2 minutes) and washing with running water. Epitope unmasking was performed in a pressure cooker (2100 Retriever, Aptum Biologics, Southampton, UK) for 10 minutes in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6). Thereafter, slides were washed twice for 5 minutes with 0.1% Tween-20/TBS (TBS-T) and blocked with 1% BSA (Sigma-Aldrich, St. Louis, USA) in Tris-buffered saline (TBS) (blocking buffer) for at least 1h. RFP polyclonal antibody (R10367, Thermo Fischer Scientific, Waltham, USA) as a primary antibody at a concentration of 1:100 in blocking buffer and was incubated overnight at 4°C. Slides were then washed twice in TBS-T for 10 minutes and then labelled with AF488 donkey anti-rabbit IgG (Poly4064, BioLegend, San Diego, USA) diluted 1:500 in blocking buffer. After 2h of incubation slides were washed 3 times for 10

minutes in TBS-T. Slides were mounted and analysed using a Zeiss Axiovert 40 CFL microscope (Zeiss, Oberkochen, Germany).

2.8 Nucleic acid isolation and quantification

2.8.1 RNA isolation from mouse skin

Six mm skin punches were taken from Aldara® treated mouse back skin and stored in RNAlater (Sigma-Aldrich, Dorset, UK) at 4°C for 24h and then at -80°C until RNA isolation. RNA was extracted from skin by phase separation with TRIzol (Thermo Fisher Scientific, Loughborough, UK) and chloroform (Sigma-Aldrich, Dorset, UK). Thus, tissue was disrupted in gentleMACS M tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) containing 1ml TRIzol on a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) using the 2.1 RNA program. Samples were then transferred into microcentrifuge tubes and 200µl of chloroform was added. After incubation for 3 minutes at RT samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was transferred in a fresh tube and 500µl isopropanol (Sigma-Aldrich, Dorset, UK) was added. Samples were incubated at 4°C overnight. After incubation, tubes were centrifuged at 12,000 x g for 30 minutes at 4°C. Supernatants were then removed and the pellet was washed with 75% ethanol (Sigma-Aldrich, Dorset, UK). Tubes were centrifuged at 12,000 x g for 10 minutes at 4°C and washed again. Thereafter, the supernatant was removed and pellets were allowed to air dry for 10 minutes. After drying, pellets were dissolved in 40µl RNase-free water. RNA was quantified by measuring absorbance at 260nm and 280nm with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Samples were diluted to 100ng/µl with RNase-free water and contaminating genomic DNA was removed using the Turbo DNFree kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Samples were then reverse transcribed into cDNA.

2.8.2 Reverse transcription

10µl of DNA-free RNA at a concentration of 100ng/µl was incubated with 1µl of 100µM Oligo(dt)₁₈ Primer (Thermo Fisher Scientific, Waltham, USA) and 1µl of 10mM deoxyribonucleotide triphosphate (dNTPs) (Thermo Fisher Scientific, Waltham, USA) for 5 minutes at 65°C. Subsequently, 4µl of 5x reaction buffer, 2µl of 100mM dithiothreitol (DTT) and 1µl 200U/µl SuperScript II (all three Thermo Fisher Scientific, Waltham, USA) was added per sample and incubated for 50 minutes at 42°C followed by 15 minutes incubation at 70°C using a PeqStar Thermocycler (PeqLab, Erlangen, Germany). cDNA was then diluted 1:10 and used for qPCR. Samples were stored at -20°C until analysis.

2.8.3 Quantitative PCR (qPCR) analysis

TaqMan based qPCR analysis was used to quantify mRNA levels of multiple genes in skin tissue. Thus, 4µl of RNA was mixed with 10µl of TaqMan Universal MasterMix, 1µl of TaqMan Assay (both Thermo Fisher Scientific, Waltham, USA) and 5µl of DNase-free water. Primers are stated in Table 6 and were purchased from Thermo Fisher Scientific (Waltham, USA). qPCR was performed in 96-well fast thermal cycling plates with optical adhesive covers (both Thermo Fisher Scientific, Waltham, USA) in a StepOne Thermo cycler (Thermo Fisher Scientific, Waltham, USA) with the program stated in Table 7. Target gene expression levels were calculated using the comparative method for relative quantification after normalisation to *B2m* expression ($2^{-\Delta\Delta CT} \times 1000$).

Table 6: TaqMan probes used to analyse mRNA expression in mouse skin

Target	Assay number
<i>B2M</i>	Mm00437762
<i>CXCL2</i>	Mm00436450
<i>CXCL5</i>	Mm00436451
<i>Il17a</i>	Mm00439618
<i>Il1a</i>	Mm00439620
<i>Il1β</i>	Mm00434228
<i>Il22</i>	Mm01226722
<i>Il23a</i>	Mm00518984
<i>Ki67</i>	Mm01278617
<i>Krt14</i>	Mm00516870
<i>Krt4</i>	Mm01296260
<i>S100a7a</i>	Mm01218201
<i>S100a8</i>	Mm00496696

Table 7: qPCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	10min	1
Denaturation	95°C	15sec	40
Annealing and extension	60°C	1min	

2.9 Enzyme linked immunosorbent assay (ELISA)

2.9.1 Protein isolation from mouse skin

Two biopsy punches with a diameter of 6mm were shredded in 2ml of isolation buffer containing 500mM Tris, 10% glycerine, 1% NP-40 (all Sigma-Aldrich, St. Luis, USA) and protease inhibitor tablets (Merck, Munich, Germany) in GentleMacs M tubes with a GentleMacs dissociator. Samples were then centrifuged at 2000 x g for 5 minutes to remove debris. Supernatants were transferred to fresh microcentrifuge tubes. Protein concentration was determined using the Pierce BCA kit according to the manufacturer's instructions. Samples were adjusted to same concentrations using protein isolation buffer.

2.9.2 ELISA

Mouse IL-17A, TNF- α and IL-6 ELISA kits were purchased from Mabtech (Nacka Strand, Sweden).

Nunc Maxisorp ELISA plates (BioLegend, San Diego, USA) were used and samples were analysed according to manufacturer's instructions. After the final wash, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (Roche, Basel, Switzerland) at a concentration of 1mg/ml in citrate buffer (distilled water with 0.02M citric acid, 0.04M anhydrous Na₂HPO₄ and 0.003% H₂O₂) was added and samples were incubated at room temperature. Development was stopped by addition of 15 μ l of 10% sodium dodecyl sulphate (SDS). Optical density at 405nm was determined with a Tecan Infinite M Plex reader.

Mouse IL-22 and IL-23 ELISA kits were purchased from BioLegend. Assays were run according to the manufacturer's instructions and with ABTS substrate as described above. Optical density at 450nm was determined with a Tecan Infinite M Plex reader.

2.9.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism (v5) software, with one-way ANOVA or student's T-tests employed when appropriate. Values of $p < 0.05$ were considered significant.

Chapter 3: Early clinical improvement of psoriasis by oral DMF is accompanied by changes in blood leukocytes

3.1 Abstract

Psoriasis is an inflammatory skin disease characterized by lesions showing keratinocyte hyperproliferation, acanthosis and parakeratosis. These lesions are further infiltrated by a large number of immune cells. Dimethyl fumarate (DMF) has been used to treat psoriasis for more than 20 years in Germany. Long-term studies have proven that oral DMF is a safe drug although a reduction in peripheral leukocytes is as a common side effect of treatment. It was hypothesized that the reduction in leukocytes is linked to an underlying mechanism of DMFs therapeutic effect in psoriasis. The hydroxy-carboxylic acid 2 receptor (HCA2) may be a possible mediator of this therapeutic effect as it is the receptor for monomethyl fumarate (MMF), the bioactive metabolite of DMF. Here, a prospective cohort study was used to investigate early responses in patients with moderate to severe psoriasis after treatment with oral DMF. Specifically, changes in the frequencies of different leukocyte populations in patients' peripheral blood were analysed. Oral DMF treatment primarily reduced the number of neutrophils, dendritic cells (DCs) but also lymphocytes during early treatment. The HCA2 expression pattern in blood from healthy individuals and psoriasis patients was analysed. In both psoriasis patients and healthy controls lymphocytes did not express HCA2. In contrast, neutrophils and monocytes showed surface expression of HCA2 while basophils and eosinophils were negative. Plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (DCs) showed HCA2 expression in healthy individuals but not in psoriasis patients. The HCA2 reporter mouse line was used to investigate expression of HCA2 by immune cells in lymphoid organs and their precursors in the bone marrow. These data showed that lymphocytes did not express HCA2 at any point of their development. Neutrophils expressed HCA2 constitutively but expression in DCs varied depending the lymphatic organ they were isolated from. Interestingly, murine stem cells expressed HCA2. In HCA2 expressing neutrophils, treatment with MMF showed induction of cell signalling. Apoptosis was not induced by MMF treatment. Together, these results show that clinical treatment of psoriasis with DMF is associated with changes in both HCA2-positive and -negative cells.

3.2 Introduction

Drugs containing dimethyl fumarate (DMF) have been used as an oral treatment for moderate to severe psoriasis since 1994 in Germany. Originally discovered by a scientist suffering from psoriasis, oral DMF was proposed as a new treatment without knowledge

of its mechanism of action [164]. DMF is now also approved for the treatment of relapsing remitting multiple sclerosis (RRMS) (Tecfidera®), renewing scientific interest in its *in vivo* effects. Despite this, the mechanism of action of DMF has yet to be fully elucidated [219, 220]. Oral DMF was initially used only in Germany as a mixture of DMF and three salts of ethyl hydrogen fumarate marketed as Fumaderm® but is now available throughout Europe as a DMF only formulation named Skilarence®.

One common side effect of DMF treatment is a reduction in peripheral lymphocytes. Approximately 10% of DMF treated patients become lymphopenic. Lymphocyte counts below 700 cells μl^{-1} (for Skilarence® treatment, 500 cells μl^{-1} for Fumaderm®) are considered severe and are linked to increased risk of infection and if uncontrolled can lead to a multifocal leukoencephalopathy (PML) [171, 172]. A decrease in peripheral lymphocytes is observed in psoriasis patients treated with Fumaderm® and Skilarence® as well as in DMF treated RRMS patients. The majority of previously published studies have focused on the long-term safety and efficacy of oral DMF and thus have concentrated on the *in vivo* effects of DMF treatment after 3 months of therapy. Long-term DMF causes a progressive reduction in lymphocytes over time with most patients suffering from a loss in peripheral lymphocytes after 6-12 months [221, 222]. In both relapsing-remitting multiple sclerosis (RRMS) and psoriasis patients the decrease in CD8⁺ T cells is more pronounced than the reduction of CD4⁺ T cells [174, 223]. In multiple sclerosis patients memory and memory effector T cells were decreased by DMF treatment [224, 225]. However, little is known about the short-term effects of DMF treatment on peripheral cell counts. Furthermore, there are only few published studies regarding the changes induced by oral DMF on myeloid cells. The early improvement of psoriasis observed during Skilarence® or Fumaderm® treatment has been investigated less than long-term effects. Whether the reduction in leukocyte counts is a side effect of long-term DMF treatment or represents a key mechanism of action is still debatable. It would therefore be interesting to know whether there are changes in the leukocyte count during the early phase of treatment with oral DMF and whether any changes differ from previously described after 6-12 months of treatment.

Multiple studies have shown that after oral administration, DMF (the main component of Fumaderm®, Skilarence® and Tecfidera®) is not detectable in the peripheral blood. DMF is instead rapidly metabolized into monomethyl fumarate (MMF) before reaching the circulation [177, 178, 181]. MMF is known to be an agonist of the hydroxyl-carboxylic acid receptor 2 (HCA2) [5]. HCA2 is a G_i/G_o-protein-coupled receptor (GPCR) and is known to bind nicotinic acid (NA), which itself is used to treat dyslipidaemia [226, 227]. The elucidation of a common side effect of NA treatment, flush, has revealed that HCA2 is expressed in the skin. Specifically, HCA2 is expressed by Langerhans cells (LCs) and epidermal keratinocytes [17]. In the peripheral blood, neutrophils express HCA2 as do

blood CD123⁺ DCs and peritoneal macrophages/monocytes. There is no evidence that T and B lymphocytes express HCA2 [18, 40, 199]. However, a complete overview of HCA2 expression in healthy and psoriatic individuals is missing. Furthermore, there is limited knowledge regarding HCA2 expression by immune cell precursors and cells residing in lymphatic organs.

There is a lack of published data regarding the initial changes in peripheral leukocytes during DMF treatment, despite noticeable clinical improvement in psoriasis patients within the first 3 months of oral DMF administration [228]. It was hypothesized that this early amelioration is accompanied by changes in blood leukocytes. This effect may be mediated by HCA2 expression on certain cell types and likely contributes to the anti-psoriatic effect of oral DMF treatment.

3.3 Results

3.3.1 Oral DMF treatment reduces leukocyte cell number in human peripheral blood

To elucidate whether oral DMF treatment leads to an early change in leukocyte cell numbers, a total of 17 psoriasis patients were included in a prospective cohort study. All enrolled patients had not been previously treated with any other DMF containing drug before being given Skilarence[®], a DMF monodrug. DMF therapy was initiated by increasing the daily dose on a weekly basis, starting from 30mg per day up to 720mg per day after 3 months. From 17 patients initially inducted, 13 completed the study while 4 dropped out due to side effects such as flush and GI problems. All patients were examined physically prior to treatment and at month 1, 2 and 3 of therapy. Psoriasis Area Severity Index (PASI) and Body Surface Area (BSA) were determined by a physician. Oral DMF treatment reduced PASI in all patients after 1 month of treatment, showing a significant decrease compared to baseline after two months with a further decrease at month three (**Figure 1A**). Oral DMF treatment also led to a decrease of the BSA following treatment but without reaching statistical significance (**Figure 1B**).

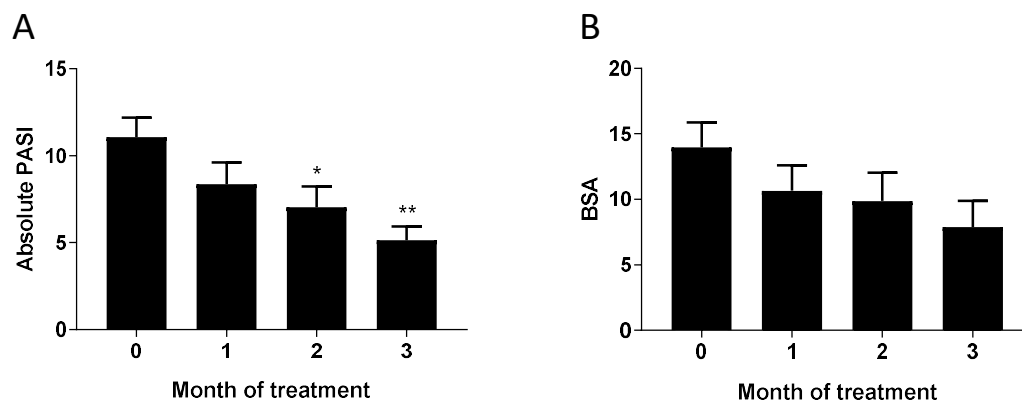


Figure 1: oral DMF reduces PASI and BSA within 3 months of treatment.

(A) Psoriasis Area Severity Index (PASI) score and (B) Body Surface Area (BSA) score in oral DMF treated patients before (Month 0) and 1, 2 and 3 months after the start of treatment. Statistical analysis was performed using one-way ANOVA (Dunnett's test) * $p < 0,05$, ** $p < 0,01$

Oral DMF treatment leads to changes in leukocyte counts, with previous studies usually reporting reductions in peripheral leukocytes (specifically T cells) after 6 months of therapy [228, 229]. In this study, clinical improvement was already significant after 2 months of treatment. Therefore, peripheral blood cells were analysed by flow cytometry during early treatment of psoriasis patients. Blood samples were taken before and during treatment. From whole blood samples red blood cells (RBCs) were removed, and the remaining cells were counted and stained for leukocyte subpopulations.

Total leukocyte counts decreased slightly following oral DMF treatment (**Figure 2A**). While there was no change after one month of treatment ($8838 \text{ cells } \mu\text{l}^{-1}$ vs. $8783 \text{ cells } \mu\text{l}^{-1}$), cell numbers started to decrease by month 2 ($7763 \text{ cells } \mu\text{l}^{-1}$) and 3 ($7357 \text{ cells } \mu\text{l}^{-1}$) of oral DMF therapy. To elucidate which subpopulation of blood cells caused this decrease, lymphocyte and myeloid cells were analysed in all patients. CD3^+ T cells showed a 25% decrease within the initial 3 months of oral DMF treatment, which was mostly caused by a reduction in CD4^+ T cells (21% decrease) rather than of CD8^+ T cells (11% reduction) (**Figure 2B**). B cells showed no change in total cell numbers in the first 3 months of DMF treatment. In contrast, the number of NK-cells seemed to increase slightly in response to oral DMF. Myeloid cells showed a larger change in cell number during the first 3 months of oral DMF treatment compared to lymphoid cells (**Figure 2C**). Neutrophils, the most abundant immune cell type in human blood, showed a noticeable decrease in absolute cell number. Although not significant, mean cell number dropped from $6136 \text{ cells } \mu\text{l}^{-1}$ before treatment to $3726 \text{ cells } \mu\text{l}^{-1}$ after month 3 of therapy, representing a decrease of 39.2% compared to baseline. Eosinophils showed an 72.9% increase after one month of treatment which decreased during the course of treatment but still showed an increase of 25.8% compared to baseline at month 3. Monocytes

showed only a temporary decrease which disappeared by month 3. There was only a small number of DCs in the blood, but this number decreased by 66.1% from the beginning of therapy to month 3, increasing slightly within the last month of the study.

Taken together, these data show an amelioration of psoriasis skin symptoms within 3 months of treatment with oral DMF. This treatment effect is accompanied by a reduction in blood leukocytes. Interestingly, this reduction is mainly caused by the decrease of neutrophils with lymphocytes showing a small or no decrease. This reduction in blood neutrophils contrasts published long-term studies and suggests that neutrophils are a likely target of oral DMF treatment.

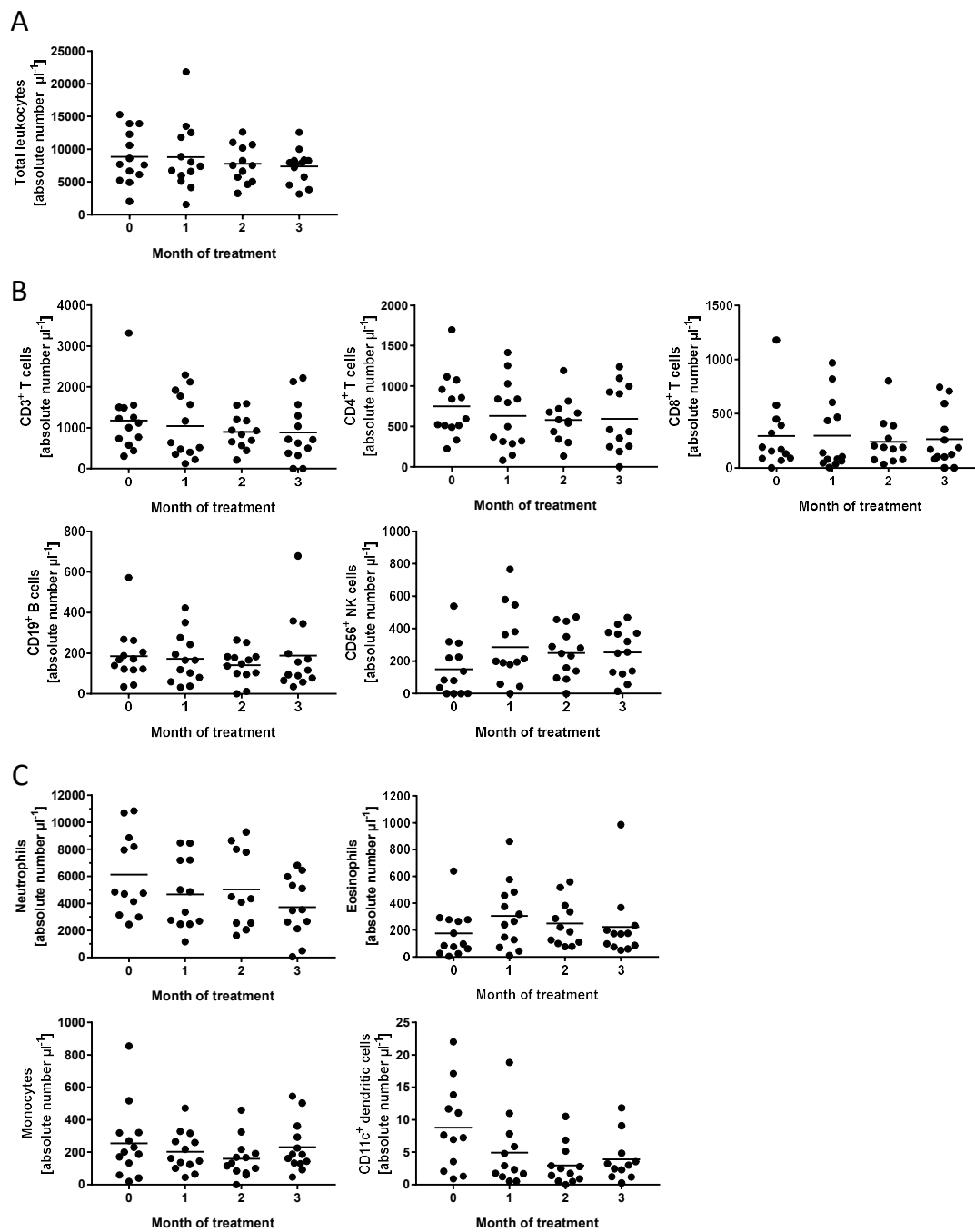


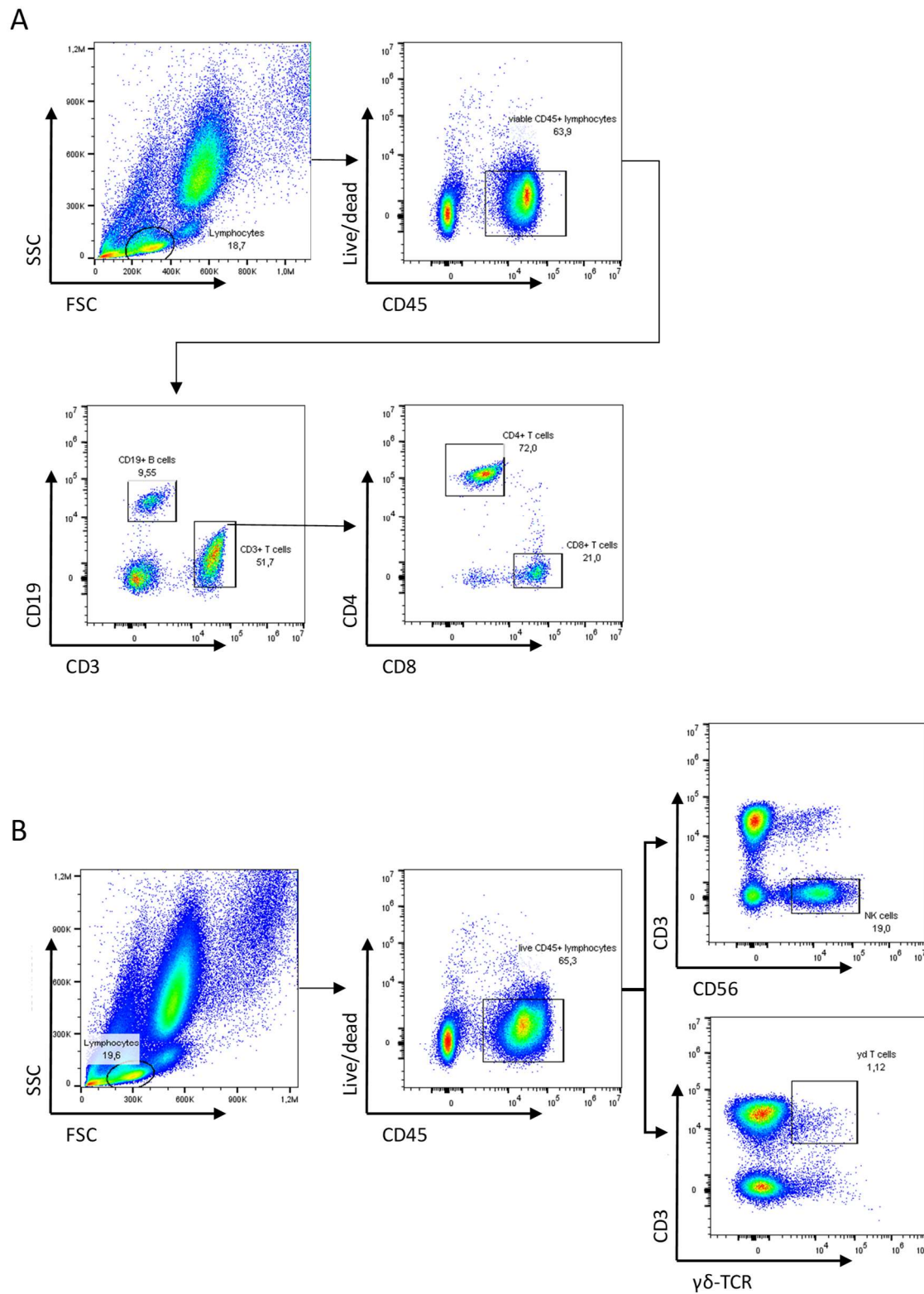
Figure 2: Effect of oral DMF treatment on cell number of human peripheral blood cell subsets.

(A) Absolute number of total leukocytes at baseline and 1, 2 and 3 month following oral DMF therapy. (B) Absolute number of lymphocyte subpopulations before and after indicated time points during DMF therapy. (C) Absolute cell number of neutrophils, eosinophils, monocytes and classical DCs before and during the course of the oral DMF treatment. Statistical analysis was performed using one-way ANOVA (Tukey's test). No significance detected.

3.3.2 The HCA2 receptor is expressed by neutrophils, monocytes, and dendritic cells, but not lymphocytes in human peripheral blood

It is possible that the reduction in cell number described above is related to cell-intrinsic effects mediated by MMF-HCA2 signalling. Thus, the cellular expression pattern of HCA2 on these different cell types could have influenced the *in vivo* effects of DMF treatment. In order to investigate in detail which human blood leukocyte subpopulations express HCA2, blood was collected from healthy volunteers and red blood cells were removed by lysis. Remaining cells were counted and stained with monoclonal antibodies (mAb) against cell specific markers and HCA2 or isotype control and subsequently analysed by flow cytometry.

Initially, lymphocyte subpopulations were analysed. After gating on live CD45⁺ lymphocytes, B cells and T cells were distinguished by CD19 and CD3, respectively. CD3⁺ cells were further divided into CD4⁺ and CD8⁺ T cells (**Figure 3A**). $\gamma\delta$ T cells were defined by surface expression of $\gamma\delta$ -TCR and CD3 while NK cells were separated by the expression of CD56 by CD3⁻ lymphocytes (**Figure 3B**). Tregs were not analysed specifically as they are a subpopulation of CD4⁺ T cells. HCA2 expression was measured by assessing the fluorescence intensity of isotype and anti-HCA2 staining (**Figure 4**). CD3⁺ T cells showed no difference in the fluorescence intensity of the anti-HCA2 and the isotype control staining, indicating no expression of HCA2 on T lymphocytes. Further analysis indicated that neither CD4⁺ or CD8⁺ T cell subsets expressed HCA2. As expected, CD3⁺ $\gamma\delta$ T cells also did not express HCA2. Analysis of NK cells confirmed that this cell type was negative for HCA2. B cells did not show positive staining for HCA2 indicating no cell surface expression of this receptor. These results show that there is no expression of HCA2 on human blood lymphocytes in healthy individuals.



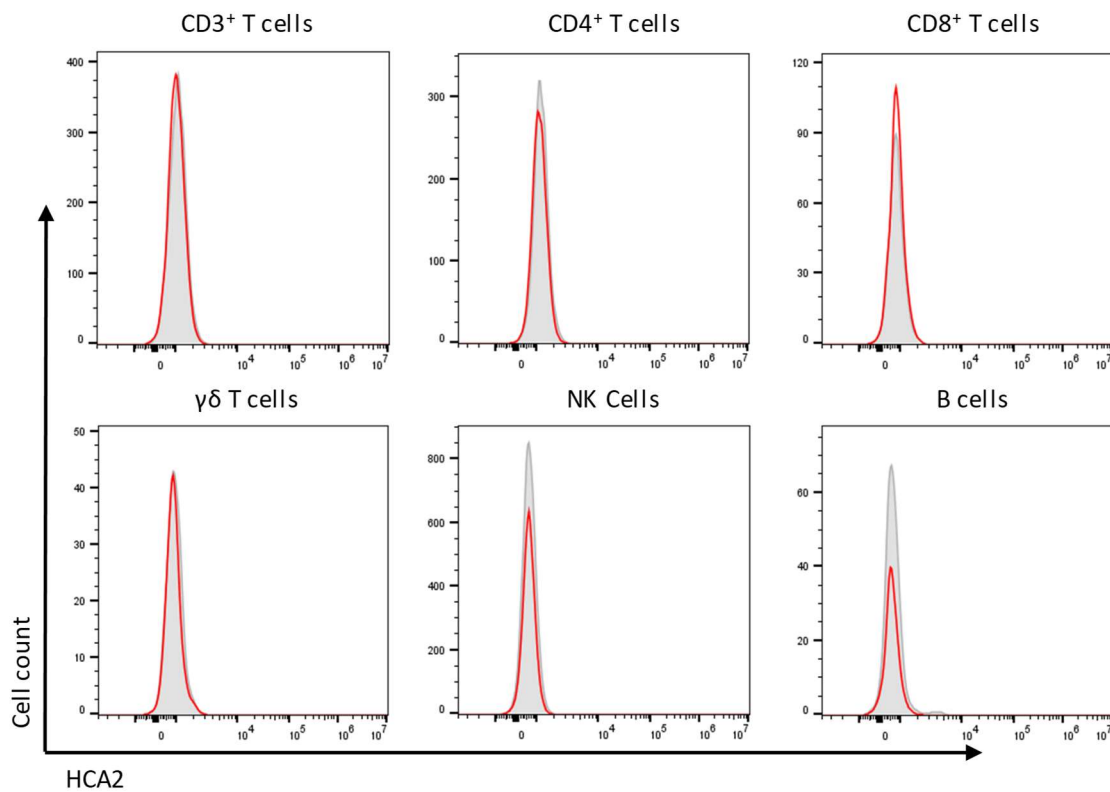


Figure 4: HCA2 expression by blood lymphocytes of healthy individuals.

Fluorescence intensity of the blood lymphocyte populations after staining with aHCA2 antibody (red) or an isotype control antibody (grey). Data shown are representative of three experiments performed.

Next, HCA2 expression on 3 distinct human granulocyte subpopulations was analysed. To analyse neutrophils (**Figure 5A**), granulocytes were separated on a forward/ side scatter plot and further defined by viability and CD45 expression. Neutrophils were then gated based on their expression of CD16 and the lack of surface MHCII. To distinguish basophils and eosinophils from other cell types, live CD45⁺ CD16⁻ Lineage⁻ MHCII⁻ leukocytes were gated. Eosinophils were then defined by positive Siglec-8 staining and a lack of CD123 expression. Basophils were defined as CD123⁺ FcεRTα⁺ cells. These cell populations were then analysed for HCA2 expression after anti-HCA2 and isotype control staining (**Figure 5B**). Neutrophils showed a shift in fluorescence intensity of the HCA2 mAb compared to the isotype control, while basophils and eosinophils displayed no positive HCA2 staining. This result shows that in healthy volunteers, neutrophils express HCA2 while basophils and eosinophils do not.

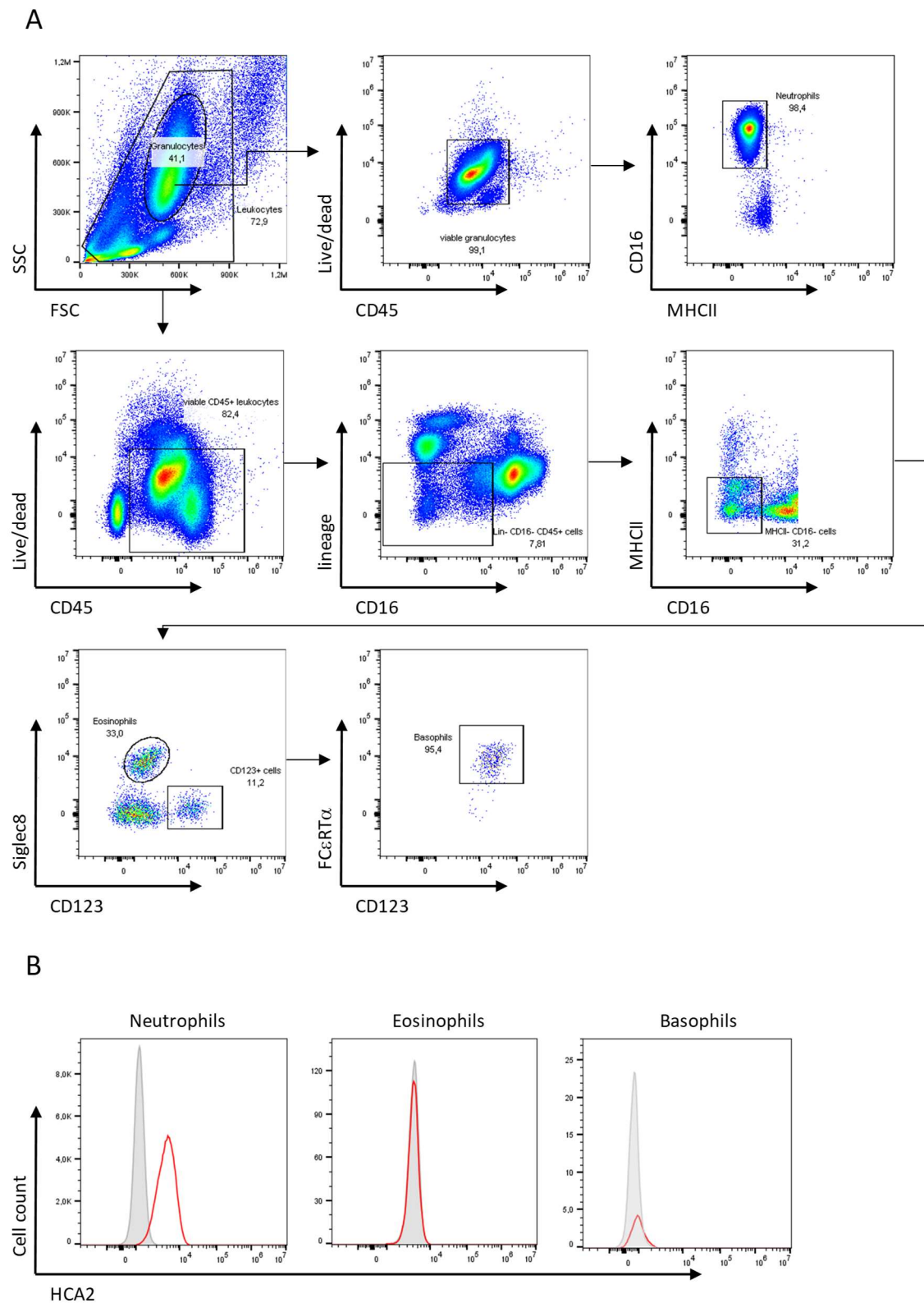


Figure 5: Flow cytometry gating strategy to identify different granulocytes populations in human peripheral blood and their specific HCA2 expression pattern.

White blood cells were obtained from human blood after RBC lysis. 1×10^6 cells were labeled with a live/dead dye and stained for CD45, Lineage markers (CD3, CD56, CD14, CD19), HLA-DR, CD16, Siglec-8, CD123, FC ϵ RT α and HCA2. (A) Gating strategy for analyzing granulocytes subsets. Neutrophils were defined by gating on CD45⁺ HLA-DR⁻ CD16⁺ cells, eosinophils were obtained by gating on CD45⁺ Lin⁻ CD16⁻ HLA-DR⁻ CD123⁻ Siglec-8⁺ cells and basophils were analyzed by gating on CD45⁺ Lin⁻ CD16⁻ HLA-DR⁻ CD123⁺ FC ϵ RT α ⁺ cells. (B) Overlay histograms of granulocytes stained for HCA2 (red) and isotype control (grey). Data shown are representative of three experiments performed.

Monocytes were an independent population in forward/ side scatter plots (**Figure 6A**). These cells were further analysed by labelling with a live/dead dye and by CD45 and CD14 surface expression. Due to the low abundance of DCs in human blood, PBMCs were isolated by a Ficoll gradient first to increase the total DC number. Resulting cells were counted, stained and analysed by flow cytometry as shown in **Figure 6B**. Lineage positive and dead cells were excluded from the CD45⁺ population. Then conventional DCs (cDCs) were gated based on CD11c and MHCII expression. pDCs were defined as CD11c^{dim}, CD123⁺ CD303⁺ cells. Monocytes, cDCs as well as pDCs showed a small shift in fluorescence intensity between the HCA2 mAb and the isotype control staining indicating that these cells express HCA2 but at a lower level compared to neutrophils (**Figure 6C**).

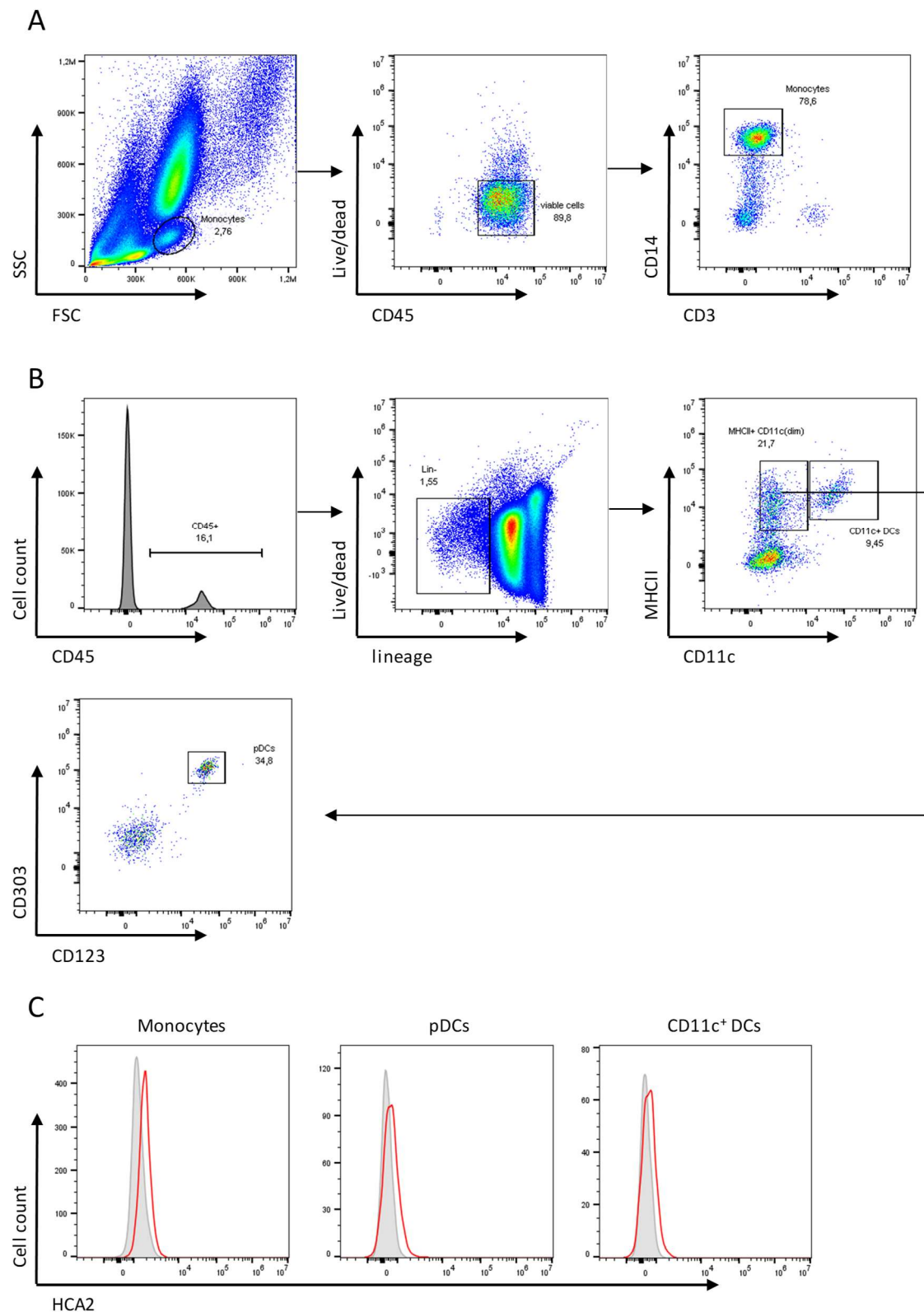


Figure 6: Flow cytometry gating strategy to identify monocytes, plasmacytoid dendritic cells and CD11c⁺ dendritic cells in human peripheral blood and their specific HCA2 expression pattern.

White blood cells were obtained from human blood after RBC lysis. 1×10^6 cells were labeled with a live/dead stain and stained for CD45, Lineage markers (CD3, CD56, CD14, CD19 and CD16), MHCII, CD11c, CD123, CD303 and HCA2. (A) Gating strategy for analysing monocytes. Monocytes were defined as CD45⁺ CD3⁻ CD14⁺ cells. (B) Gating strategy for DC subsets. Classical DCs were obtained by gating on CD45⁺ Lin⁻ HLA-DR⁺ CD11c⁺ cells and pDCs were CD45⁺ Lin⁻ MHCII⁺ CD11c^(dim) CD123⁺ CD303⁺ cells. (C) Overlay histograms of granulocytes stained for HCA2 (red) and isotype control (grey). Data shown are representative of three experiments performed.

As psoriasis is an immune-mediated inflammatory disease with major changes in cytokine profiles in patients, it is possible that the expression pattern of the HCA2 receptor is altered in psoriasis patients compared to healthy individuals. Therefore, blood was taken from treatment naive psoriasis patients and total blood leukocytes were isolated as previously described. HCA2 expression was assessed using the same gating strategies as the healthy volunteer samples. This analysis revealed that the pattern of HCA2 expression in psoriasis patients was similar to that of healthy people. T- and B lymphocytes in both groups did not express HCA2 (**Figure 7A**). Neutrophils from both psoriasis patients and healthy controls expressed high levels of HCA2 while basophils and eosinophils were HCA2 negative in both groups (**Figure 7B**). Monocytes in both groups expressed low levels of surface HCA2. Interestingly, conventional DCs and pDCs in psoriasis patients were negative for HCA2, while in healthy individuals these cell types expressed HCA2 at a low level (**Figure 7C**).

In summary, these results show that HCA2 is expressed by neutrophils in both healthy individuals and psoriasis patients. Monocytes, DCs and pDCs also express HCA2 but at a lower level compared to neutrophils. Of note, HCA2 expression by DCs and pDCs was decreased in psoriasis patients. T- and B cells, as well as eosinophils and basophils were HCA2 negative in healthy controls and psoriasis patients.

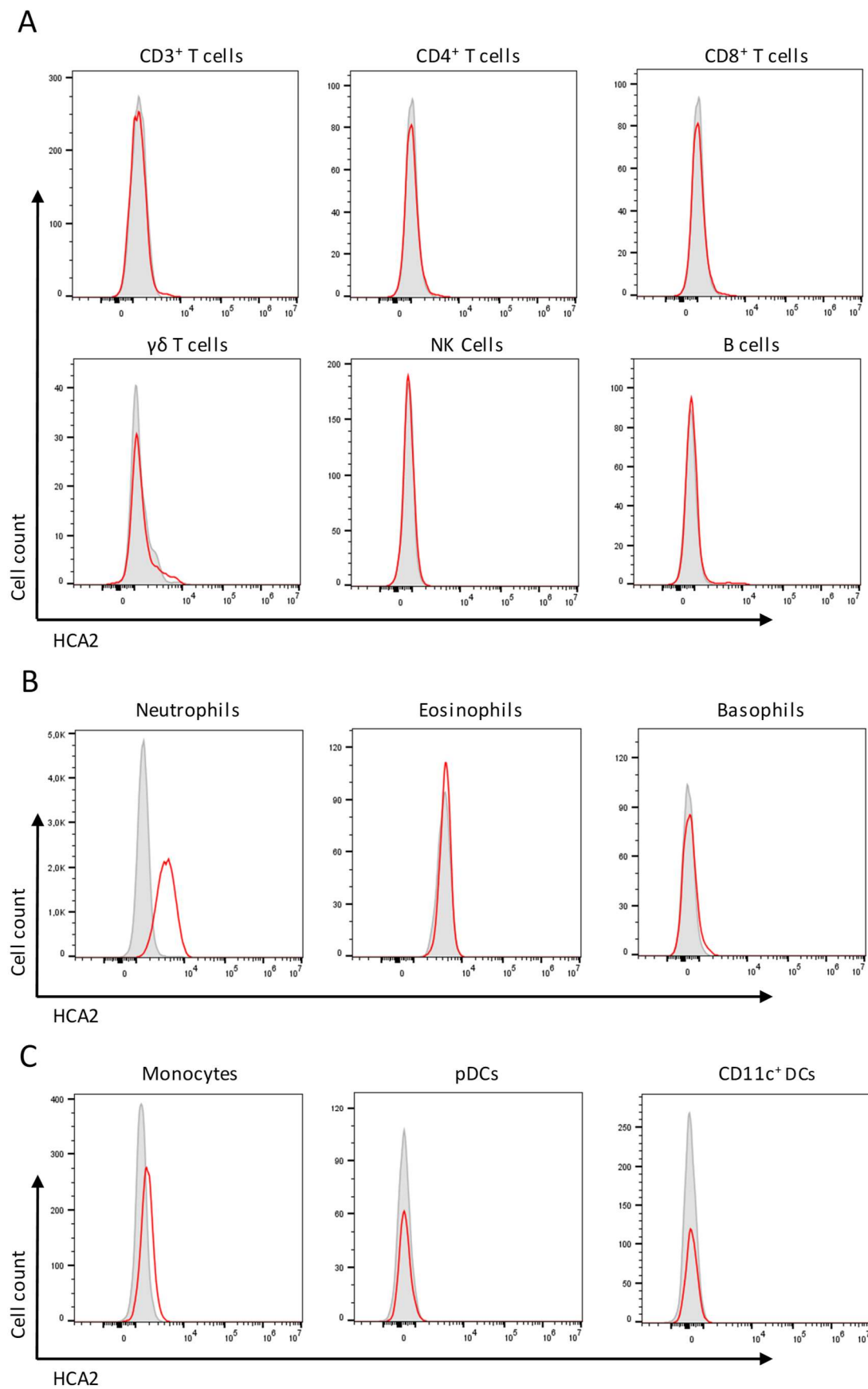


Figure 7: HCA2 expression in psoriasis patients.

Blood from untreated psoriasis patients was taken and white blood cells were isolated by RBC lysis. The same flow cytometry gating strategy as for healthy controls was applied for analysis of HCA2 expression. Overlay histograms of (A) lymphocytes (B) granulocytes and (C) monocytes and dendritic cells stained for HCA2 (red) and isotype control (grey). Data shown are representative of three experiments performed.

3.3.3 HCA2 is not expressed by lymphocytes in lymphoid organs but on neutrophils

As the expression of HCA2 was limited to myeloid cells in human peripheral blood and HCA2 was not expressed by blood lymphocytes, the expression pattern of HCA2 on immune cells from lymphoid organs was evaluated to investigate if cellular HCA2 expression varies depending on tissue. Due to the difficulty in obtaining human lymphoid tissue for analysis, experiments were carried out in mice. However, the lack of a validated mouse anti-HCA2 antibody led to the use of a transgenic mouse line expressing RFP under the control of the HCA2 promoter [17]. HCA2 expressing cells from this mouse line are labelled with RFP which could then be analysed by flow cytometry.

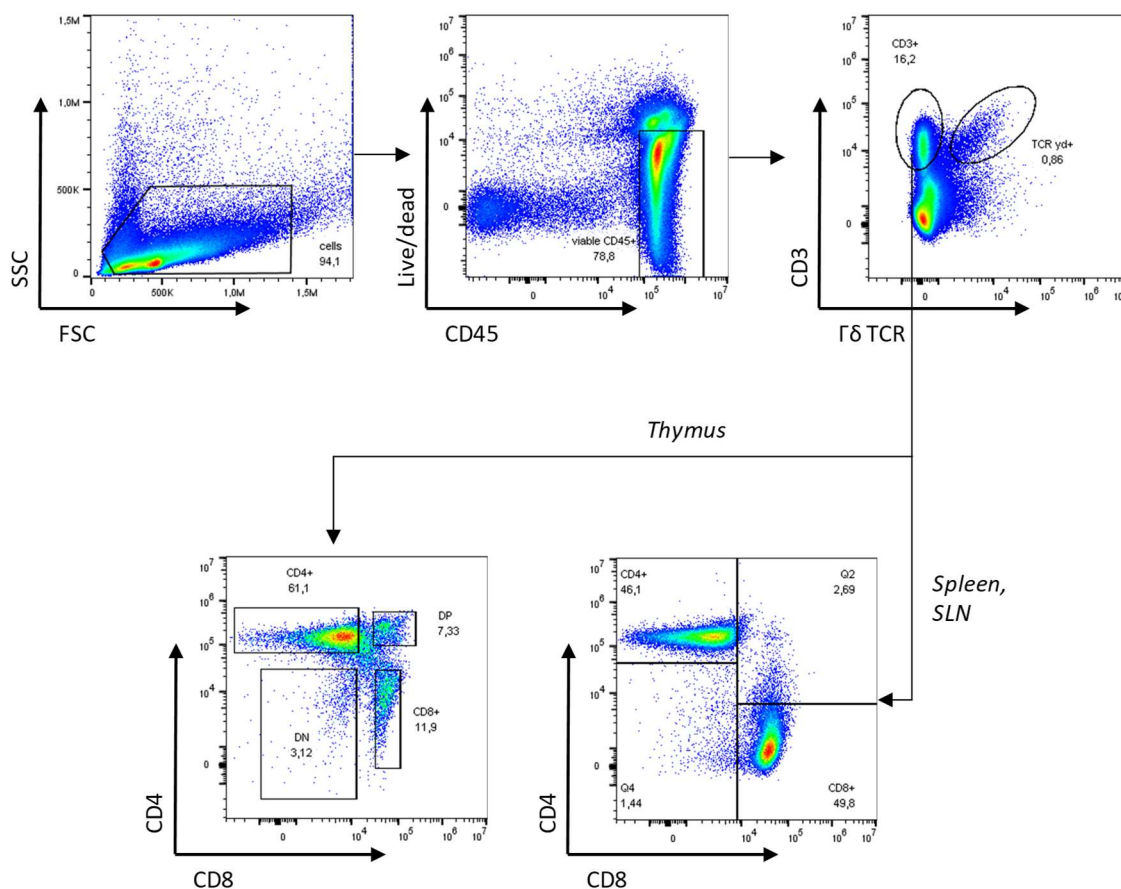


Figure 8: Flow cytometry gating strategy for leukocytes in lymphoid organs.

The expression of RFP by leukocytes was analysed in spleen, skin draining lymph nodes (SLN) and thymus isolated from HCA2-RFP mice. To characterize T cell subsets, thymus, spleen and SLN were disrupted mechanically and cells washed and stained for characteristic surface markers. Total CD3⁺ cells, CD4⁺ and CD8⁺ T cells subpopulations as well as $\gamma\delta$ T cells were analysed. In the thymus, double negative (DN) T cells and double positive (DP) CD3⁺ cells were analysed.

As previously shown, human peripheral blood lymphocytes did not express HCA2. However, they may express HCA2 during their earlier developmental stages. Immature T cells are progenitors that are produced by the bone marrow and traffic to the thymus where they undergo stepwise development into CD4⁺ and CD8⁺ T cells which are then released into the bloodstream. Peripheral T cells then recirculate between lymphoid organs and peripheral tissues via the blood and lymphatics [230]. To assess HCA2 expression by T lymphocytes in different tissues, cells were isolated from skin draining lymph nodes (SLN), thymus and spleen by tissue disruption and cells were then stained for T lymphocyte subset markers and analysed by flow cytometry. **Figure 8** shows the gating strategy for T lymphocytes used for the analysis of lymph nodes, thymus and spleen. While in spleen and lymph nodes only mature T cells can be detected, in the thymus there are T cells undergoing development that show no surface CD4/CD8 expression (double negative stage) and expression of both CD4 and CD8 (double positive stage) [230].

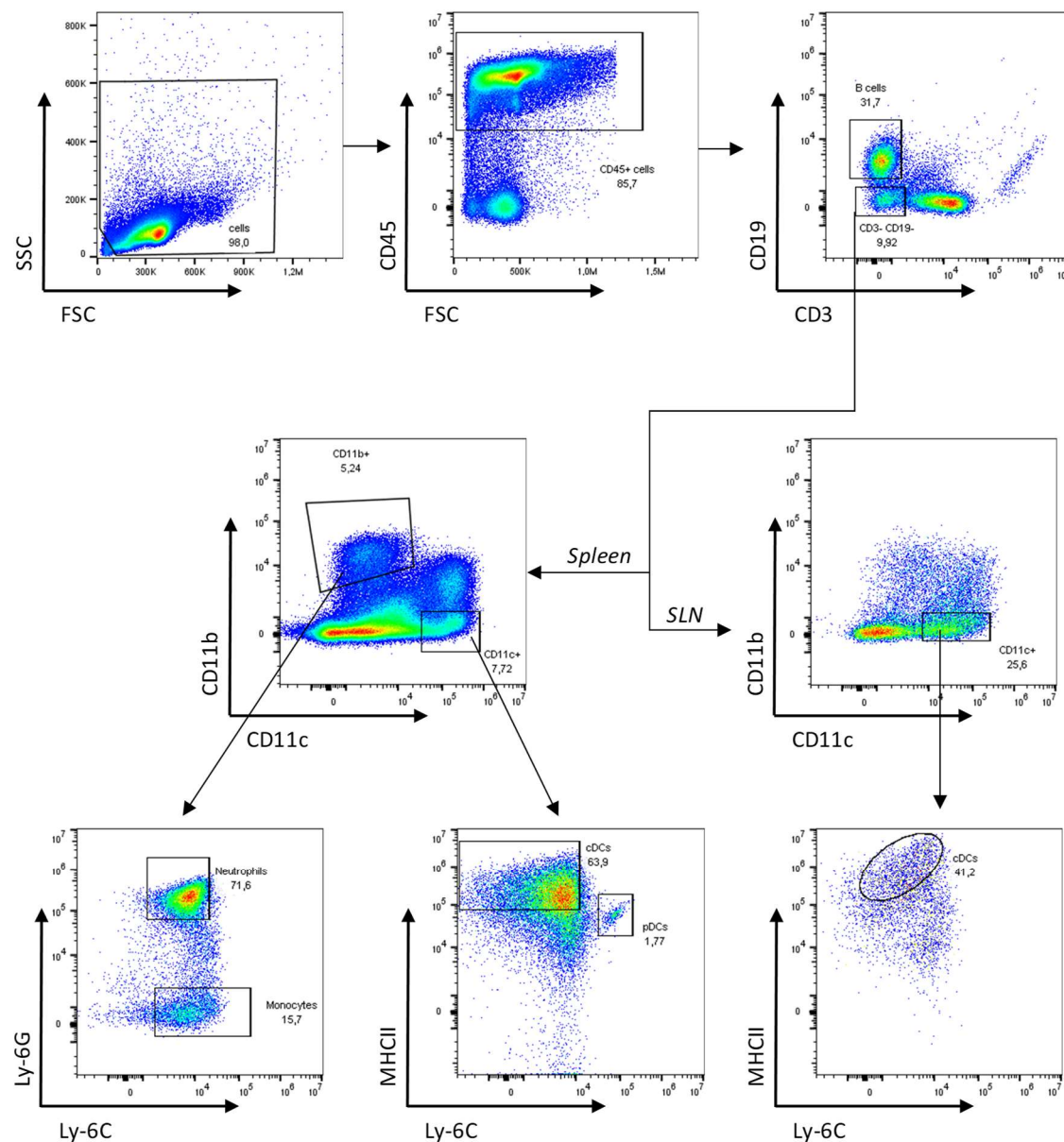


Figure 9: Flow cytometry gating strategy for B lymphocytes and myeloid cells in lymphoid organs.

The expression of RFP by myeloid cells and B lymphocytes was analysed in spleen and (SLN) isolated from HCA2-RFP mice. In the spleen and SLN, myeloid and B cells were characterized. To characterize B cells, spleen and SLN were disrupted mechanically and cells washed and stained for characteristic surface markers. B cells were defined as CD45⁺ CD19⁺ CD3⁻ cells. CD3⁻ CD19⁻ negative cells were further divided by CD11c and CD11b expression. In the spleen, CD11c⁺ cells were divided into cDCs and pDCs by the expression of MHCII and Ly6C. CD11b⁺ cells are separated into neutrophils (Ly6G⁺ Ly6C^{low} cells) and monocytes (Ly6C⁺ Ly6G⁻ cells). SLN do not contain monocytes and neutrophils, therefore these populations were not analysed in these organs.

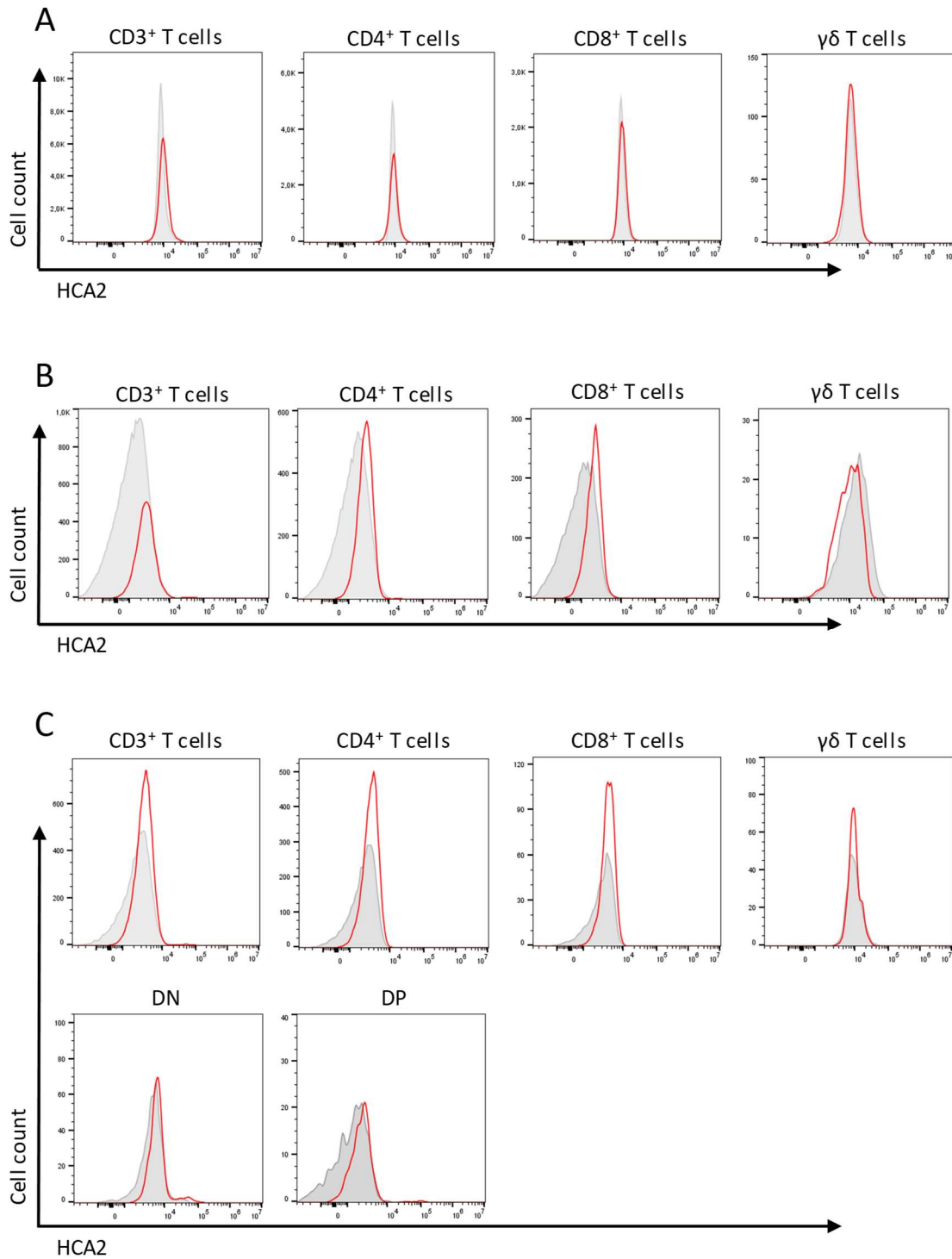


Figure 10: T cells in lymphoid organs do not express HCA2.

T cells were isolated by tissue disruption from (A) SLN, (B) spleen and (C) thymus of HCA2-RFP mice and control animals. HCA2 expression was analysed by assessing the fluorescence intensity of RFP in HCA2-RFP mice (red) compared to control mouse cells (grey). One representative experiment out of 4 experiments shown.

In skin draining lymph nodes total CD3⁺ cells as well as the subpopulations of CD4⁺, CD8⁺ and $\gamma\delta$ T cells were analysed. All of these cell populations did not show any expression of HCA2 as determined by RFP (**Figure 10A**). Significant populations of T cells are contained within the spleen. Similar to lymph node-isolated T cells, the total CD3⁺ population in addition to CD4⁺ and CD8⁺ T lymphocytes from the spleen did not express RFP in their cytosol thus are likely negative for the HCA2 receptor (**Figure 10B**). Furthermore, there was evidence that T cells undergoing development in the thymus are also HCA2 negative. When T cells were isolated from the thymus and characterized by surface staining and RFP expression, it was obvious that neither double negative, double positive nor mature T cells expressed RFP (**Figure 10C**). There was also no detectable difference between the mature T cell subsets of CD4⁺ T helper cells, CD8⁺ T killer cells and $\gamma\delta$ T cells in terms of RFP expression compared to control mice. Taken together, these results show that CD4⁺ and CD8⁺ T cells isolated from secondary lymphoid organs do not express HCA2.

B cells are produced by the bone marrow before migrating to the spleen where they differentiate into functional mature B cells. These B cells then recirculate between lymphatic organs and the blood stream [231]. Human blood B cells, like T lymphocytes, did not show HCA2 surface expression. In order to assess HCA2 expression by B cells in secondary lymphoid tissues, CD19⁺ B cells from lymph nodes and spleen of HCA2-RFP mice were analysed (**Figure 9**). In both organs, B cells did not show RFP expression (**Figure 11A+B**). There was no difference in MFI of HCA2-RFP mice derived cells compared to control mice. This indicates that B cells do not express HCA2 within secondary lymphoid tissues.

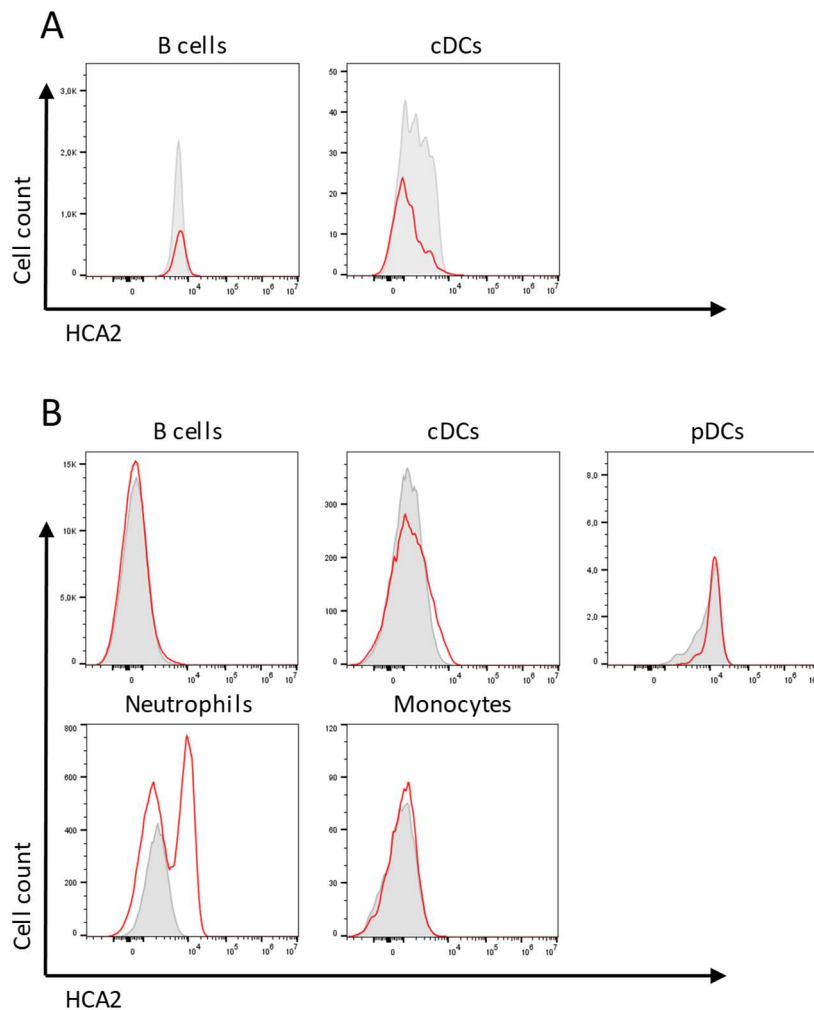


Figure 11: HCA2 expression by B cells and myeloid cell types in lymphoid organs.

HCA2-RFP mice were sacrificed and SLN and spleens were taken for enzymatic immune cell isolation. RFP fluorescence intensity was compared between HCA2-RFP mice derived cells (red) and control mice (grey) in SLN (A) and spleen (B). One out of 4 representative experiments shown.

Compared to lymphoid cells, human blood myeloid cells showed expression of HCA2. Specifically, neutrophils displayed a high level of HCA2 expression while dendritic cells and monocytes had lower surface levels. To verify if these cells express HCA2 in secondary lymphoid organs, spleen and SLN of HCA2-RFP mice were taken and digested enzymatically to increase cell number obtained. Isolated cells were then stained for cell specific markers and analysed by flow cytometry for RFP. Wild type mice were used as controls.

DCs can be found in secondary lymphoid organs such as the spleen and lymph nodes, where they act as APCs to T cells. DCs are characterized by high expression of CD11c and MHCII (**Figure 9**). Analysis of the fluorescence intensity of RFP in these DCs from lymph nodes showed that there was no significant difference between cells isolated from HCA2-RFP animals compared to control mouse cells (**Figure 11A**). Splenic cDC displayed a small shift in RFP fluorescence intensity indicating a marginal expression of

HCA2 (**Figure 11B**), however such a shift was not detected in cDCs isolated from lymph nodes. pDC, a distinct DC type expressing high levels of Ly6C and lower levels of MHCII, showed a low expression of HCA2 in healthy human blood but showed no expression in murine spleen (**Figure 11B**).

Neutrophils are short lived cells produced in the bone marrow and circulate in the blood [232]. If neutrophils do not encounter infection during their lifetime they are removed from the circulation and undergo apoptosis before being phagocytosed by splenic macrophages. Therefore, a huge number of neutrophils can be found in the spleen [233, 234]. Showing high Ly6G expression and intermediate expression of Ly6C and CD11b, they can be easily separated from other CD45⁺ leukocytes in the spleen (**Figure 9**). Compared to the high expression of HCA2 observed in human blood, murine splenic neutrophils appeared to have HCA2 expressing and HCA2 non-expressing subpopulations as there were two distinct peaks of RFP fluorescence intensity (**Figure 11B**).

Monocytes are derived from bone marrow precursors and circulate in the blood until recruited into tissues upon infection or inflammation. A significant population of monocytes also resides in the spleen [235]. After isolation from HCA2-RFP mouse spleen, monocytes were defined as CD11b⁺ Ly6C⁺⁺ Ly6G⁻ MHCII⁻ cells (**Figure 9**). Comparison of RFP fluorescence intensity of splenic monocytes isolated from HCA2-RFP animals to control mice revealed no observable increase in RFP expression (**Figure 11B**), indicating no expression of HCA2 in murine splenic monocytes.

Table 8: Summary of HCA2 expression in blood and lymphoid organs

	human		mouse		
	healthy	psoriasis	spleen	SLN	thymus
CD3 ⁺ T cells	-	-	-	-	-
CD4 ⁺ T cells	-	-	-	-	-
CD8 ⁺ T cells	-	-	-	-	-
γδ T cells	-	-	-	-	-
NK cells	-	-	na	na	na
B cells	-	-	-	-	na
Neutrophils	+++	+++	+++ / -	na	na
Basophils	-	-	na	na	na
Eosinophils	-	-	na	na	na
Monocytes	+	+	-	na	na
DCs / cDCs	+	-	(low)	-	na
pDCs	+	-	-	na	na
DP T cells	na	na	na	na	-
DN T cells	na	na	na	na	-

(na = cells not available)

Taken together (**Table 8**), these results show that lymphocytes of the T and B cell lineages do not express HCA2 in the blood nor in lymphoid organs. In contrast, neutrophils are HCA2 positive although there is a HCA2 negative subpopulation in the spleen. DC show a low expression of HCA2 in the spleen but not in lymph nodes. Splenic monocytes do not express HCA2.

3.3.4 HCA2 is expressed by stem cells but not lymphoid or myeloid progenitor cells

Previous results showed that lymphocytes did not express HCA2 while neutrophils have a high expression level. Monocytes and DCs showed a low HCA2 expression in the blood but no significant expression in lymphoid organs. All these cells are initially produced from stem cells in the bone marrow. To investigate HCA2 expression in progenitor populations of these immune cells, bone marrow cells from HCA2-RFP mice were isolated and after removal of RBCs by lysis, cells were stained for characteristic surface markers and analysed for RFP expression by flow cytometry.

Figure 12 shows the flow cytometry gating strategy used to separate the different progenitor populations, as published by Mayle *et al.* [236]. Viable CD45⁺ cells were excluded from lineage positive, mature cells. Remaining cells were separated by ckit and Ska1 expression. This gating strategy led to a separation of 3 distinct groups: ckit⁺ Ska1⁻ (KL) cells, ckit^{low} Ska1^{low} (K^{low}S^{low}) and ckit⁺ Ska1⁺ (KLS cells). KL cells were further separated into granulocyte-macrophage progenitors (GMP) cells, megakaryocyte-

erythrocyte progenitor (MEP) cells and common myeloid progenitor (CMP) cells by the expression of CD16/32 and CD34. $K^{low}S^{low}$ cells contain the common lymphoid progenitor (CLP) cell population. KSL cells are a heterogeneous group of long-term and short-term hematopoietic stem cells and multipotent progenitor cells [236].

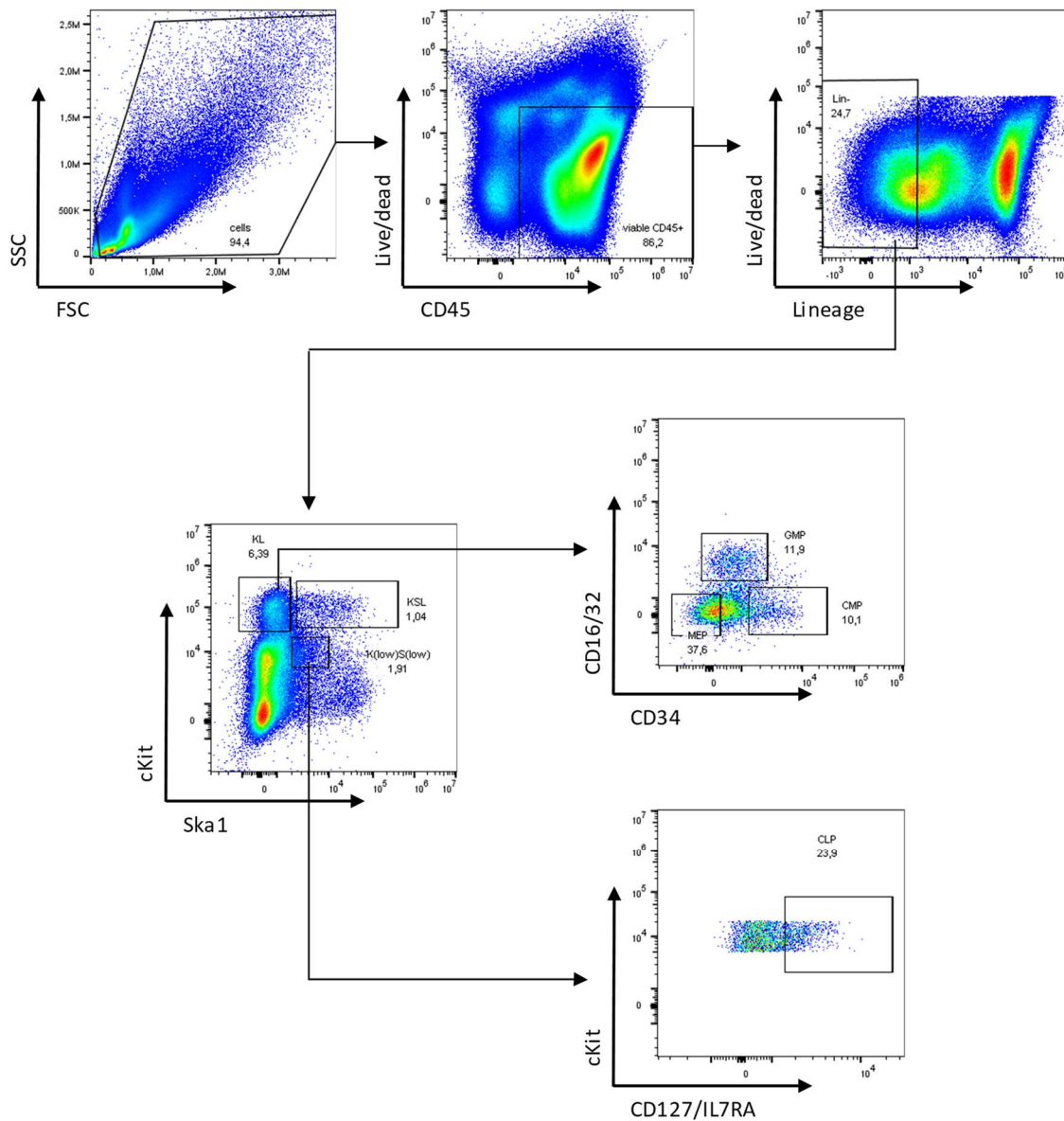


Figure 12: Gating strategy of bone marrow cell populations.

Bone marrow isolated from the femurs of HCA2-RFP mice was depleted of RBCs and surface stained to characterize distinct bone marrow subpopulations. Flow cytometry gating strategy for BM-derived cells.

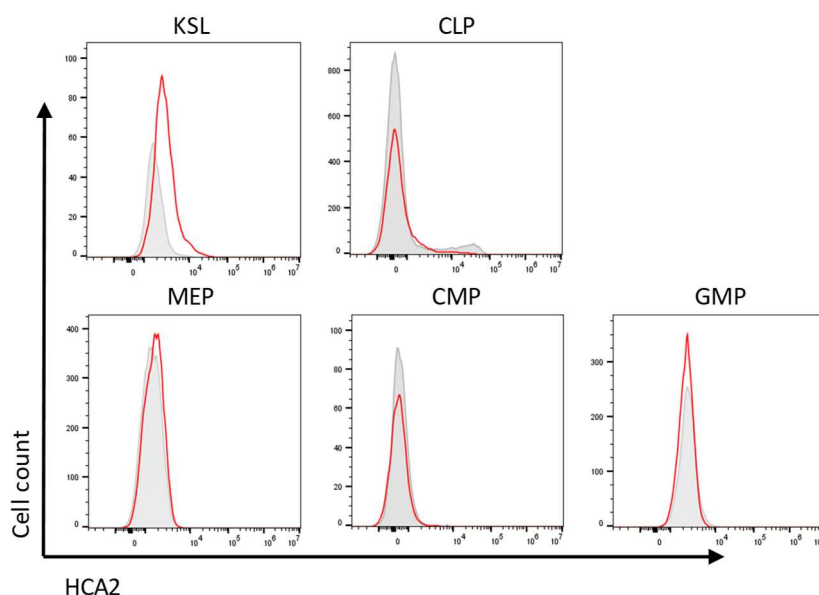


Figure 13: HCA2 expression by bone marrow cell populations.

Bone marrow isolated from the femurs of HCA2-RFP mice was depleted of RBCs and surface stained to characterize distinct bone marrow subpopulations as stated in figure 12. These subpopulations were analysed for RFP expression (red) and fluorescence intensity was compared to control mouse cells (grey). One out of 4 representative experiments shown.

The above-mentioned progenitor and stem cells were then analysed for intracellular RFP expression (**Figure 13**). The CMP, MEP and GMP populations showed no expression of RFP indicating that they do not express HCA2. CLPs, identical to their mature progeny, did not show any RFP or respective HCA2 expression. KSL cells, which are a diverse group of stem cells, showed a strong shift in RFP expression showing that these cells express HCA2 on their cell surface. In conclusion, in the bone marrow only KSL stem cells express HCA2 while other progenitors lose HCA2 surface expression.

3.3.5 MMF and DMF do not trigger neutrophil apoptosis

To investigate whether the reduction of blood neutrophils observed in patients' blood during oral DMF treatment is caused by apoptosis, neutrophils were isolated from healthy individuals and analysed *in vitro*. Cells were cultured in the presence of DMF, as well as its active metabolite and HCA2 ligand, MMF, in addition to a solvent control for 4h. Thereafter, neutrophils were analysed for viability and apoptosis induction (Live/Dead Zombi NIR and Annexin V staining) by flow cytometry. As shown in **Figure 14A-C** there was no significant change in the viability of neutrophils induced by DMF or MMF treatment compared to control (controls not shown). Viability of neutrophils was close to 100% even at high concentrations of both DMF and MMF (**Figure 14A**). The frequency of Annexin V⁺ cells did not increase with any treatment. Control treatment showed the highest number of apoptotic cells with mean frequency of Annexin V⁺ cells of 3.46%.

DMF and MMF treatment did not change the percentage of Annexin V⁺ cells compared to the vehicle controls (**Figure 14B**). There was no significant induction of cell death by any treatment (**Figure 14C**). Taken together, it appears that neither MMF nor DMF act as an inducer of apoptosis in human blood neutrophils.

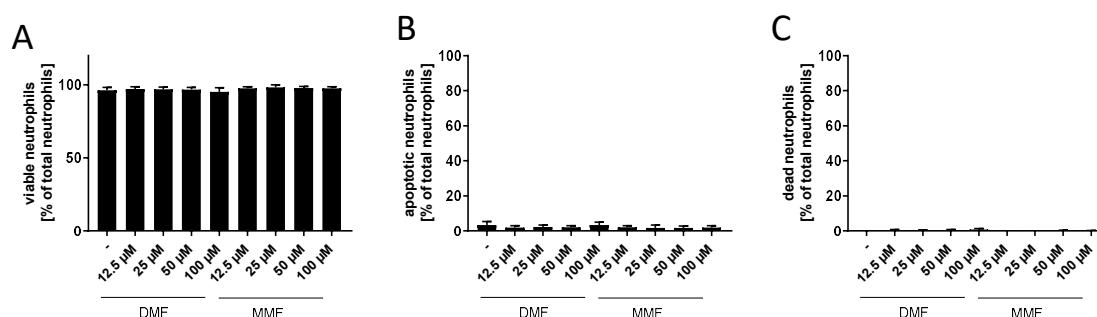


Figure 14: Neither MMF nor DMF induce apoptosis in human blood neutrophils.

Neutrophils were isolated from human blood and incubated with the indicated concentrations of MMF, DMF or solvent control for 4h. Neutrophils were labeled with a live/dead stain and analyzed for Annexin V expression. (A) Percentage of viable cells. (B) Annexin V⁺ cells showing apoptosis. (C) Dead neutrophils. N=3, statistical analysis was performed using one-way ANOVA (Tukey's test). No significance detected.

3.3.6 MMF leads to a rapid reduction of HCA2 surface expression on neutrophils

In order to confirm whether the lack of apoptosis induction is a result of changes in the HCA2 receptor resulting in unresponsiveness to MMF, potentially due to isolation or *in vitro* culture artefacts, neutrophils were isolated from human peripheral blood and incubated with DMF, MMF or solvent control for 30 minutes and HCA2 expression was assessed via flow cytometric analysis. Ligand binding to GPCRs can result in conformational changes and receptor internalisation potentially leading to changes in surface receptor expression detectable by cytometry [237]. Indeed, incubation with fumaric acid esters led to a significant reduction of HCA2 surface expression on isolated neutrophils. While DMF reduced receptor expression by 16.4%, MMF led to a decrease of 35.3% (**Figure 15A**). To investigate if this reduction was caused by physiological changes such as receptor internalization rather than inhibition of antibody binding by MMF chemically modifying the receptor, the same experiment was carried out at 4°C to inhibit cellular processes but not chemical binding of the fumarates to HCA2 (**Figure 15B**). Expression analysis by flow cytometry showed that treatment with MMF or DMF at 4°C did not lead to changes in HCA2 surface expression. These results indicate that DMF and MMF treatment leads to changes in HCA2 receptor surface expression with MMF causing a greater reduction in expression than DMF.

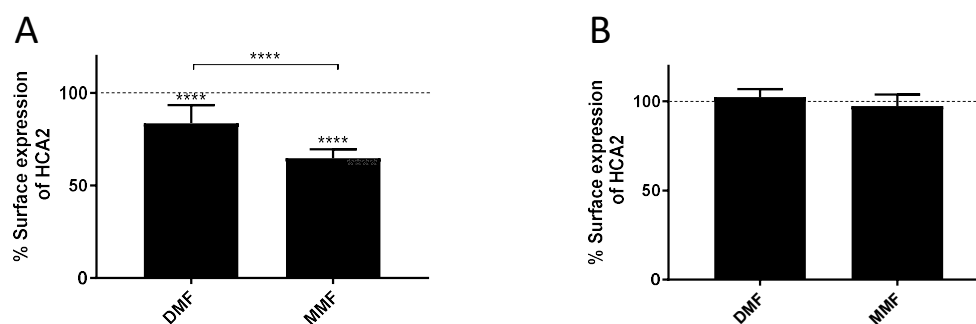


Figure 15: HCA2 surface expression on human neutrophils after DMF or MMF treatment.

Neutrophils were isolated with a ficoll gradient from human peripheral blood. Isolated cells were incubated with 100 μ M of either MMF, DMF or solvent control for 30 minutes at either 37°C (A) or 4°C (B). Thereafter, neutrophils were labeled with a live/dead stain and stained for CD16 and HCA2 or a respective isotype control antibody. (A) n=8 and (B) n=3, statistical analysis was performed using one-way ANOVA (Tukey's test). ****p<0,0001.

3.3.7 MMF but not DMF induces GPCR signalling in human neutrophils

The reduction in surface HCA2 expression by MMF suggests receptor activation. To confirm this, Ca²⁺ flux after fumarate exposure was examined. Ca²⁺ flux is one of the first signalling events after GPCR activation. To assess changes in intracellular Ca²⁺ levels, isolated neutrophils were loaded with the Ca²⁺-sensitive fluorescent Fluo-4 and Fura red dyes. These dyes change fluorescence intensity when in contact with Ca²⁺. Samples were analysed for fluorescence changes after exposure to fumarates by flow cytometry. Cells were acquired for 20 seconds before DMF, MMF or solvent control were added and fluorescence was read for another 60 seconds afterwards. Change in fluorescence ratio of the dyes indicates Ca²⁺ flux. While MMF induced a change in fluorescence ratio of dyed neutrophils, DMF and solvent control did not (**Figure 16A**). This shows that MMF induces changes in intracellular calcium levels indicating signalling events but DMF does not.

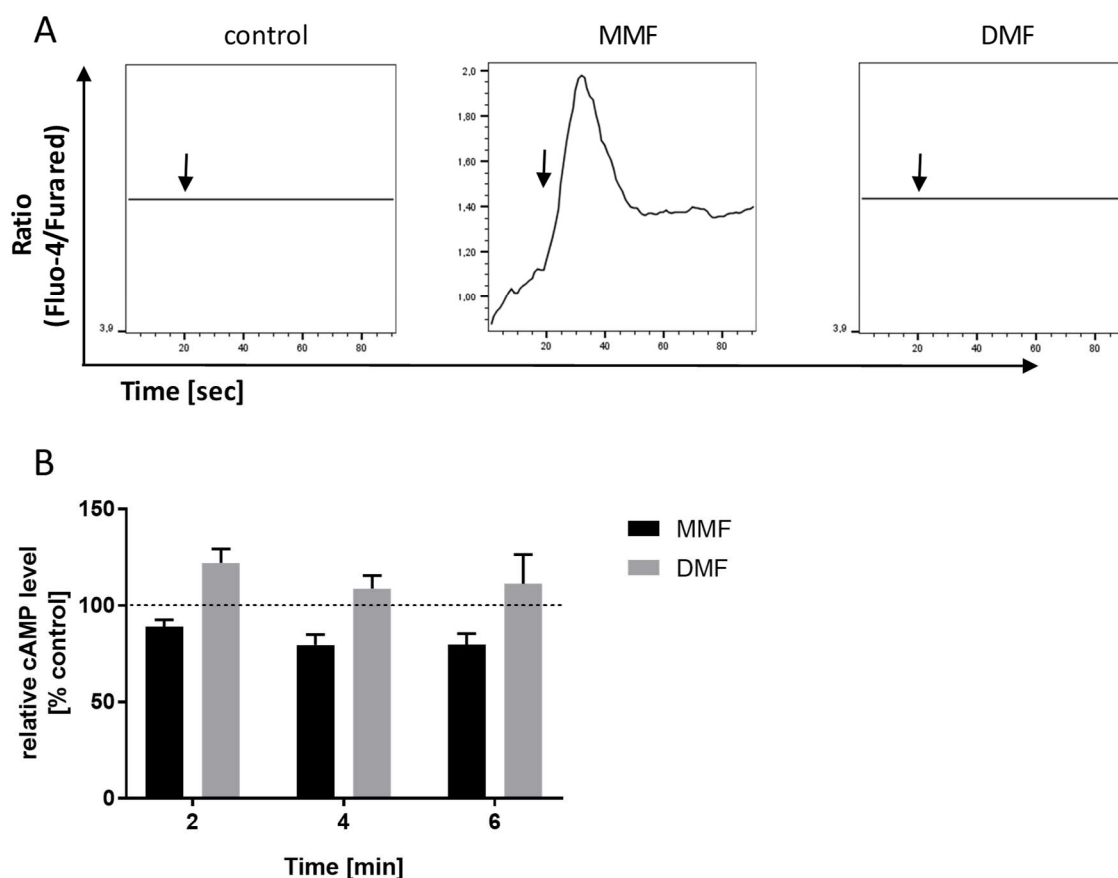


Figure 16: MMF induces internal signalling while DMF does not

Neutrophils were isolated from human peripheral blood using a magnetic column-based technique. (A) To investigate internal calcium flux, cells were stained with Fluo-4 and Fura red stains and run on a flow cytometer. After 20 seconds of acquisition either solvent control, MMF or DMF were added and acquisition continued. Data shows one representative out of 4 experiments. (B) Neutrophils were incubated with 100 μ M forskolin and 100 μ M MMF, DMF or solvent control for the indicated times and cAMP levels were analyzed by ELISA. $n=4$, statistical analysis was performed using one-way ANOVA (Tukey's test).

GPCRs are known to signal through different signalling cascades. HCA2 is a G_i/G_o GPCR known to inhibit the AC thus leading to a reduction of cyclic adenosine monophosphate (cAMP) levels. To investigate whether the HCA2 ligand MMF interferes with internal cAMP signalling, neutrophils were isolated from human blood and incubated with forskolin to induce AC and raise intracellular cAMP levels. MMF, DMF or solvent control were added to these samples at the same time to measure the effects. cAMP levels were measured 2, 4 and 6 minutes after addition of the stimuli (**Figure 16B**). While DMF did not induce a change in intracellular cAMP levels, MMF reduced them at all time points indicating activation of HCA2 signalling. Taken together with the evidence of Ca^{2+} flux induced by MMF but not DMF, it appears that *in vitro* stimulation of neutrophils results in HCA2 activation in the absence of apoptosis induction. The *in vivo* reduction in neutrophils seen in psoriasis patients is therefore likely due to other mechanisms.

3.4 Discussion

DMF has been used to treat psoriasis for more than 20 years in Germany. Most published studies have concentrated on the long-term effects of oral DMF treatment [221, 228, 238]. However, in psoriasis patients improvement of skin inflammation occurs early in the treatment course. As the mechanism of action of oral DMF treatment has yet to be fully elucidated, the cause of this early improvement is unknown. Lymphopenia is regarded as a side effect of DMF treatment that develops in patients after 6-12 month of treatment. But early changes in leukocyte counts have not been widely published for all cell types. The aim of this study was to determine if DMF therapy leads to changes in leukocyte abundance concurrent with the early improvements in skin lesions seen during clinical treatment of psoriasis with this drug as such changes may represent a mode of action for DMF rather than a side effect. It is hypothesized that there is a relationship between the changes in cell number and the expression of HCA2, which could indicate a possible function for this receptor in relieving psoriasis. Oral DMF treatment led to a reduction in PASI after 1 month of treatment which decreased further during subsequent time points. Simultaneously, a reduction in blood leukocytes was observed. The loss of neutrophils was the primary driver of this reduction. Analysis of HCA2 expression showed that neutrophils but not lymphocytes were positive for this GPCR. *In vitro* analysis of neutrophils revealed that MMF does not induce apoptosis while still being able to induce receptor activation as indicated by reduced HCA2 surface expression, Ca^{2+} flux and inhibition of AC activity.

One common and often described side effect of oral DMF treatment is a decrease in circulating leukocytes, especially T lymphocytes. Most previous studies have analysed patients' blood cell counts at 3 months or later after the commencement of treatment, despite the fact that reductions in psoriasis symptoms can be observed much earlier [239]. The early amelioration of psoriasis due to oral DMF treatment observed in this work is in agreement with other published results [222, 228]. The reduction in psoriasis severity was accompanied by a simultaneous progressive reduction in the number of total leukocytes during the first 3 months of treatment although the patients were still in the dose escalation phase. Healthy individuals show an average white blood cell count of around 5000 cells μl^{-1} (Morrison *et al*, unpublished). Thus, the psoriasis patients in this study had elevated leukocyte counts at baseline, which decreased during treatment without dropping out of the normal range.

Lymphopenia caused by DMF treatment is a common, clinically significant side effect often leading to modulation of dosage and potentially cessation of treatment [229]. This study showed a decrease in T cells during DMF treatment mostly caused by a reduction in $CD4^+$ T cells. $CD8^+$ T cells did not show changes over this time period, contrasting the

loss of CD8⁺ T cells normally reported at later time points [170, 174, 224, 225, 239]. Flow cytometric analysis showed that peripheral T lymphocytes do not express HCA2 on their cell surface. The lack of HCA2 expression by T cells in the circulation or at any point during their development is in agreement with other literature [40]. Assuming DMF (by generating MMF *in vivo*) acts via HCA2, a direct effect of oral DMF treatment on T lymphocytes can be excluded. Whether there are other receptors or even transporters for MMF or DMF, mediating a direct effect, is not known. The reduction in T lymphocytes is most likely an indirect treatment effect. Whether it is linked to the reduction in DCs seen in early treatment has to be determined. DCs interact with T cells during antigen presentation and a reduction in this cell type may lead to a reduced T cell activation and proliferation [240].

Changes in myeloid cells during DMF treatment have as yet not been extensively reported in the literature [223, 225]. This study showed that oral DMF treatment causes a progressive decrease in the number of peripheral neutrophils. This early effect of DMF therapy is reported here for the first time. Furthermore, this effect may be directly mediated by HCA2 as flow cytometry analysis showed that blood neutrophils were positive for HCA2. The granulocyte macrophage progenitors from which they are derived were HCA2 negative. Thus, it appears that proximal to being released into the blood, neutrophils start to express HCA2. This is in agreement with previous reports [18]. There are different populations of neutrophils in the spleen, which are determined by their localization and probably also by their function [233]. Analysis of splenic neutrophils isolated from RFP mice revealed cells both positive and negative for HCA2. It can be assumed that neutrophil HCA2 expression varies between distinct splenic neutrophil populations. These have to be further characterized. Given that MMF is the active metabolite of DMF [178], there may be direct effects of oral DMF treatment on neutrophils via HCA2. *In vitro* analysis showed that neutrophils do not become apoptotic using physiologically relevant concentrations of MMF or DMF (to analyse receptor-independent effects) but displayed intracellular signalling upon MMF treatment only. This suggests a direct GPCR mediated effect of MMF on neutrophils. Additional work from our lab has shown that psoriasis patients have higher frequencies of neutrophils with a low CD26L expression compared to healthy individuals. During oral DMF treatment the number of CD62L^{lo} neutrophils decreases (Morrison *et al.*, unpublished). CD62L is also known as L-selectin and low levels of this marker indicate activated or previously transmigrated cells. Non-activated neutrophils express high levels of surface CD62L which can be cleaved upon activation or during extravasation facilitating the migration of neutrophils in inflamed or infected tissues [241]. A reduction in CD62L^{lo} neutrophils by oral DMF treatment was observed in an antibody transfer model of bullous pemphigoid-like epidermolysis bullosa acquisita (EBA) by Wannick *et al.* [56] suggesting a reduction

in neutrophil activation or migration as a result of treatment. Chen *et al.* also showed that MMF reduces murine neutrophil adhesion and migration *in vitro* giving further evidence that the reduction in neutrophils is caused by a reduced activation or migration [55]. Treating human neutrophils isolated from psoriasis patients with MMF *in vitro* and analysing the surface expression of CD62L by flow cytometry may give further evidence whether the results obtained from mice are applicable for human cells. Moreover, the neutrophil reduction in psoriasis patients treated with DMF was observed in the peripheral blood. Whether this decrease also effects the infiltration of neutrophils into psoriasis lesions is unclear. In a mouse model of RRMS DMF treatment has been shown to reduce neutrophil infiltration into the CNS, but this has not been shown for psoriasis or murine psoriatic skin inflammation [55]. Thus, reduced activation and migration of neutrophils may be a possible reason for decreased blood neutrophil counts during DMF treatment.

Eosinophils, another subpopulation of granulocytes, showed a temporary increase upon oral DMF treatment of psoriasis patients in this current study. Transient eosinophilia in some patients during DMF treatment has been observed in other published works and was shown to often resolve spontaneously [228, 242, 243]. Eosinophilia is also a treatment side effect of anti-TNF- α therapy for psoriasis [244, 245]. Interestingly, eosinophils do not express HCA2 suggesting an indirect effect of oral DMF treatment on these cells.

Monocytes, in contrast to neutrophils, only showed a transient decrease in cell counts in this study. This result is in line with a previous study showing that the number of monocytes is not changed significantly by long term DMF treatment [239]. This is interesting as monocytes were shown to express HCA2 in human blood as demonstrated by flow cytometry, potentially making them a direct target of MMF. In agreement with other reports it was observed that mouse splenic monocytes did not express HCA2 [29], which does not align with the results from human blood monocytes. It is possible that this disparity is due to species or tissue-specific differences. If human splenic monocytes were confirmed to be negative for HCA2, these might not be affected by DMF.

Classical DCs were also decreased upon oral DMF treatment in psoriasis patients. This has not been previously reported in the literature. DCs are the most potent APCs which when activated are able to present antigens to CD4⁺ T cells via MHC class II and to CD8⁺ T cells via MHC class I and initiate antigen-specific adaptive immune responses [240]. Data presented here did not distinguish between immature and mature DCs which have different T cell activation capacities [240, 246]. Thus, there may not only be a reduction but possibly reduced activation of DCs upon oral DMF treatment. Nevertheless, this drop in DCs, may indicate that DMF treatment impacts the behaviour of DCs *in vivo* potentially

resulting in altered T cell responses. To illustrate this, it has been shown that MMF can interfere with the differentiation of monocyte-derived DCs, leading to reduced expression of surface HLA-DR and CD1a [247]. Furthermore, Litjens *et al.* demonstrated that after exposure to LPS, DCs differentiated in the presence of MMF upregulated CD83, co-stimulatory molecules and HLA-DR but to a lesser extent as control DCs. These MMF-differentiated DCs not only produced low levels of IL-12p70, IL-10 and TNF- α but were also less effective at inducing T cell IFN- γ secretion. However, they were still able to induce Th2-type cytokines from T cells [248]. Interestingly, it has been reported that DMF-treated psoriasis patients have reduced frequencies of Th1 cells but enhanced proportions of Th2 cells [193]. By affecting DCs in order to lead to lower T cell activation, DMF treatment may be indirectly modulating T lymphocyte activity, leading to reduced levels of IFN- γ and IL-17A [249]. Thus, DMF treatment results in detectable reductions in DC numbers indicating a possible alteration of the *in vivo* activity of these crucial cells, suggesting a potential mode of action of DMF.

DCs play a major role in the initialisation of psoriasis lesions while neutrophils are attracted in high numbers into developing lesions by a chemokine gradient [86, 133, 157]. The reduction in both DCs and neutrophils in the peripheral blood suggests that DMF may ameliorate psoriatic lesions by decreasing the number of immune cells available for infiltration into the skin. If there is a reduction in the number of neutrophils in skin lesions and whether such a reduction is crucial for the improvement in psoriasis by DMF treatment needs to be elucidated.

DCs expressed HCA2 in healthy individuals but not in DMF-naïve psoriasis patients. It is known that HCA2 expression is altered by inflammatory stimuli and infections. Hence, LPS is known to increase HCA2 expression on macrophages and adipocytes [250]. Moreover, IFN- γ has been shown to induce increased HCA2 expression by these same cells [19]. Besides LPS, TNF- α and IL-1 β can also stimulate the expression of HCA2 by adipocytes and macrophages [251]. HCA2 expression by epithelial cells of the colon and ileum appears to be highly dependent on the gut microbiome [15]. Therefore, germ-free mice show reduced expression of HCA2 in both ileum and colon and re-colonization results in increased HCA2 expression, suggesting microbiota derived molecules influencing HCA2 expression [15]. Furthermore, obesity has been reported to decrease HCA2 expression by adipocytes [252]. Some of these factors play a major role in psoriasis, as patients are often overweight or obese [253] and cytokines such as IL-1 β and TNF- α are elevated in this disease [58, 254]. Neutrophils and monocytes expressed HCA2 in both psoriasis patients and healthy controls while eosinophils and basophils were negative for this receptor in both groups. In contrast to healthy individuals, DCs and pDCs from psoriasis patients did not express HCA2. This suggests that unknown

psoriasis-specific factors, such as cytokines, may be affecting DCs specifically leading to reduced HCA2 expression. Furthermore, classical DCs are also located in SLN and spleen where they interact with T cells. When isolated from RFP mice they showed no HCA2 expression in SLN and little to no expression in the spleen. Either HCA2 expression by DCs is highly variable between tissues (blood vs. lymphoid tissue) or differs between human and mouse. In the literature HCA2 mRNA expression was observed in mouse dermal DCs [29]. However, these dermal DCs were isolated based on CD86 surface expression, a marker not only expressed by DCs but also by other APCs such as LCs [255]. Thus, this dermal DC HCA2 mRNA expression is potentially from LCs which are known to express HCA2 [33]. Taken together, DC HCA2 expression is likely changed by inflammatory factors and may well differ between species. Thus, the decrease in blood DCs seen in patients during oral DMF therapy is potentially not mediated by HCA2.

Flow cytometric analysis of bone marrow cells showed that the common myeloid and lymphoid progenitors from mouse were negative for HCA2. Interestingly, bone marrow KSL cells, which are a heterogeneous group of long term and short-term stem cells as well as multipotent progenitor cells, displayed expression of HCA2 [256]. Thus, it is possible that these subpopulations within the KSL cells are able to respond to MMF. However, whether this occurs in patients during oral DMF treatment is unknown. It may be hypothesised that oral DMF treatment acts on stem cells due to their HCA2 expression. In other cell types MMF has been shown to have anti-proliferative effects [257]. A possible reduction in stem cell proliferation by DMF treatment would conceivably lead to reduced production of leukocytes. This would affect cells with short *in vivo* half-lives earlier than longer living cells after treatment commencement. Thus, neutrophils would be expected to be reduced first while DCs, monocytes and T lymphocytes would decrease later. This represents a possible explanation as to why some HCA2 expressing cells appeared unaffected while HCA2 non-expressing lymphocytes are reduced during oral DMF treatment. Further experiments are necessary to deduce whether human stem cells also express HCA2 and if DMF treatment results in decreased output from the bone marrow.

3.5 Conclusion

Taken together, these data show that the treatment of psoriasis patients with oral DMF leads to an early improvement in disease severity accompanied by a decrease in blood leukocyte counts, which mostly consists of a reduction in the number of neutrophils, DCs and T lymphocytes, in particular CD4⁺ T cells. MMF, but not DMF, induces internal signalling in neutrophils which does not lead to the induction of apoptosis. This work

showed that there may be direct, HCA2-mediated as well as indirect, HCA2-independent effects of oral DMF treatment. MMF has been shown to be a HCA2 ligand but if the protective effect of DMF is mediated via HCA2 has yet to be proven. Investigation of the role of HCA2 and identification of a potential target cell types would further define DMF's mode of action. A more complete understanding of the mode of action could lead to the identification and development of new drugs with the same therapeutic efficacy as DMF but lacking the side effects that lead treatment cessation.

Chapter 4. HCA2 is essential for the protective effect of DMF in skin inflammation

4.1 Abstract

The hydroxy-carboxylic receptor HCA2 plays an important role in sensing the metabolic state of the body. HCA2 was shown to mediate the anti-lipolytic effect of nicotinic acid and also the protective effect of oral DMF treatment in mouse models of multiple sclerosis and bullous pemphigoid-like epidermolysis bullosa acquisita (EBA). The role of HCA2 in the mode of action of oral DMF in psoriasis has not yet been revealed. It is hypothesized that HCA2 mediates the therapeutic effect of oral DMF treatment. In this work the imiquimod (IMQ) mouse model was used to investigate the role of HCA2 in the treatment of psoriasis with oral DMF. Mice lacking the HCA2 receptor were compared to mice expressing HCA2 in regard to skin histology, gene expression and immune cell abundance. As endogenous ketones produced in the body during altered energy balance are also agonists of HCA2, the effects of a ketogenic diet on psoriasis-like skin inflammation was analysed. These experiments showed that HCA2 mediates the anti-inflammatory effects of oral DMF but not of a ketogenic diet.

4.2 Introduction

HCA2 was first described as an high affinity receptor for DMF's active metabolite MMF in 2008 [5]. But whether HCA2 mediated signalling is necessary for the protective effect of oral DMF treatment in psoriasis is still not known. Chen *et al.* have demonstrated that HCA2 is essential for the protective effect of oral DMF in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS). They showed that HCA2-knockout (HCA2^{-/-}) mice are not protected from neural inflammation by oral DMF treatment. The authors indicated that HCA2-MMF signalling was protective in EAE partly by reducing neutrophil chemotaxis [55]. Furthermore, it has been demonstrated that HCA2 plays a role in the amelioration of murine bullous pemphigoid-like epidermolysis bullosa acquisita (EBA), a model of pemphigoid skin diseases [56]. Although MS and pemphigoid disease have different aetiologies compared to psoriasis, these conditions share some similarities with each other namely tissue infiltration of immune cells and involvement of Th17-type responses. Mouse models have been used to study the role of HCA2-MMF signalling in the treatment of these inflammatory diseases due to the availability of HCA2^{-/-} animals. By utilising a mouse model of psoriasis, it is possible to assess the functional role of HCA2 in amelioration of psoriasiform skin inflammation by oral DMF.

The natural ligands of HCA2 are the ketone body BHB and the short chain fatty acid butyrate [7]. While butyrate is produced by the gut microbiota from dietary fibre, BHB is produced by the liver in times of starvation via β -oxidation of free fatty acids. The BHB-HCA2 interaction is known to inhibit lipolysis [7]. However, BHB also exerts regulatory functions independent of HCA2. It has been shown that BHB can inhibit inflammation by suppression of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome [258]. BHB binds to histone deacetylases (HDACs) leading to an increase in stress tolerance [259]. In a model of colitis, butyrate was anti-inflammatory but if this effect is mediated by HCA2 is unknown [40]. If BHB can mediate an anti-inflammatory effect during psoriatic skin inflammation has yet to be elucidated. As obesity is a major risk factor of psoriasis with almost 50% of patients being overweight worldwide [87]. Trials of weight reduction have shown that it is able to ameliorate psoriasis symptoms [90, 93, 94]. Amongst a wide range of *in vivo* effects caloric restriction can lead to elevated blood ketone levels [260], raising the possibility that the anti-psoriatic effect of weight loss is in part mediated by activation of HCA2.

The aim of this study was to investigate the role of HCA2 in the mode of action of oral DMF on psoriasis-like skin inflammation. It was hypothesized that oral DMF treatment would lead to a reduction in immune cell infiltration into the skin. Furthermore, as HCA2 is also a receptor for BHB, this study aimed to elucidate a putative positive effect of a ketogenic diet on psoriasis like skin inflammation.

4.3 Results

4.3.1 Kinetics of the imiquimod mouse model

The IMQ model produces psoriasis-like skin lesions by daily application of Aldara[®] cream, which contains 5% IMQ. As this model is known for its changing phenotype over time and differences between mouse strains, a pre-experiment was carried out in order to determine the best time point for subsequent detailed analysis. The backs of the mice were shaved and 50 mg per day of Aldara[®] was applied topically for up to 6 days. Three mice were sacrificed per time point. Skin thickness and mRNA expression were analysed before start and after 3, 4, 5 and 6 days of Aldara[®] application. While untreated mice skin is on average 12.1 μ m thick, the skin thickness increased continuously during application until day 5 where it reached an average peak of 99.6 μ m before subsequently decreasing on day 6 (**Figure 17A**). Based on this result, further experiments on the effects of DMF treatment on skin thickness were carried out on day 5 of Aldara[®] application.

Further analysis of mRNA expression for markers of inflammation and keratinocyte proliferation showed that *I11b* and *I17a* transcript levels were elevated after Aldara[®]

application compared to control mice and peaked on day 4. Antimicrobial peptide mRNA levels for *S100a8* and *S100a7a* were also highly expressed compared to untreated mice and showed their peak expression on day 5 and 6, respectively. The marker of keratinocyte proliferation, *Krt14*, was upregulated on day 3 of treatment and remained high compared to control mice. *Cxcl5* transcripts were elevated at day 3 and peaked at day 4 (**Figure 17B**). Based on these results all subsequent experiments were done with a period of 5 days of Aldara® application in order to evaluate the influence of oral DMF treatment on the pathogenesis of the IMQ model.

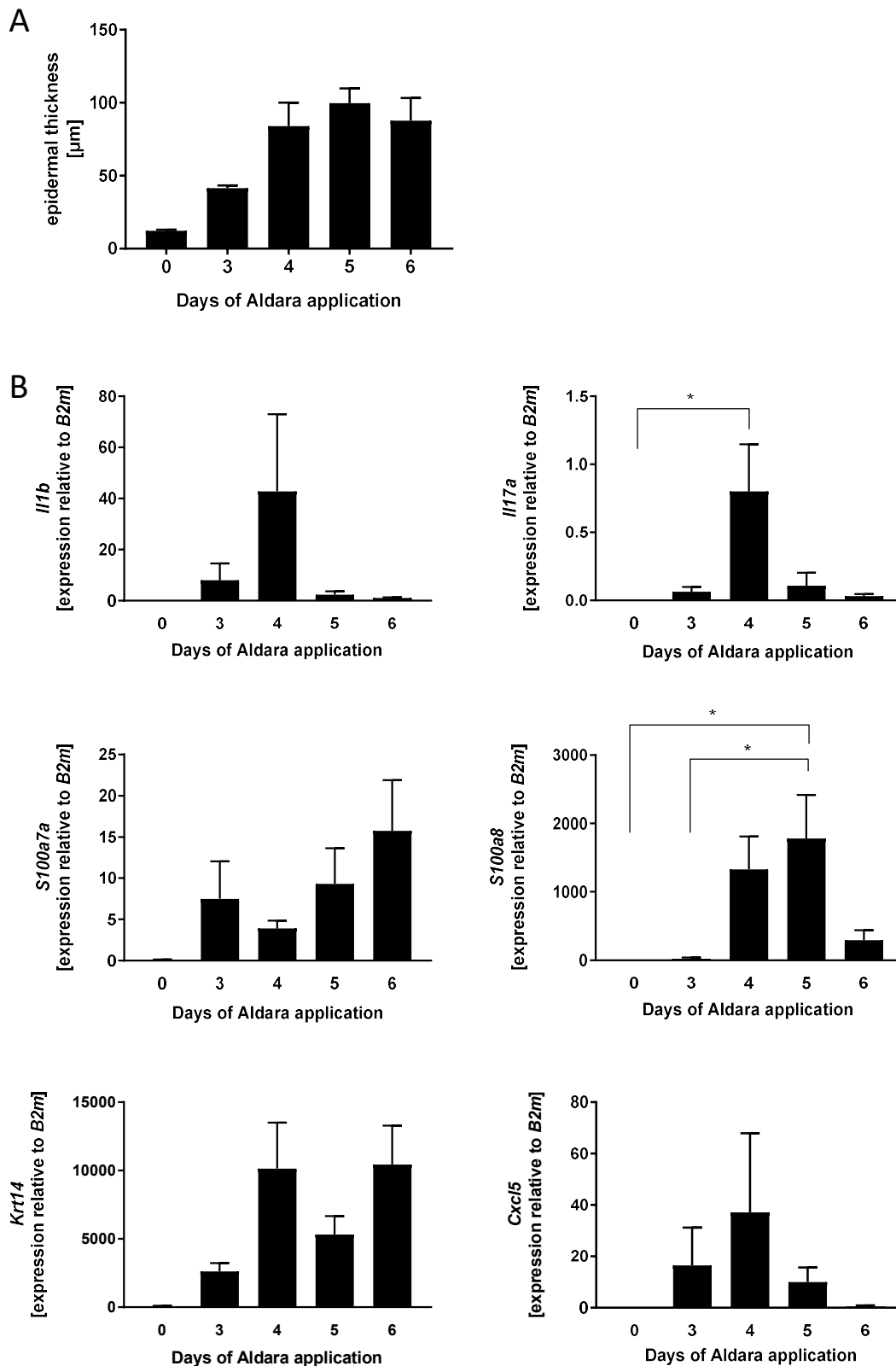


Figure 17: The kinetics of skin inflammation in the IMQ mouse model.

Mice were treated with 50mg/day of Aldara® for the indicated time points. (A) epidermal thickness was measured on H&E stained lesional biopsies. (B) mRNA was isolated from lesional skin, reverse transcribed and expression levels of *Il1b*, *Il17a*, *S100a8*, *S100a7a*, *Krt14* and *Cxcl5* were analysed. Bars represent mean mRNA expression relative to *B2m* + SEM of 3 biological replicates, * $p < 0.05$, as determined by one-way ANOVA followed by Tukey's test.

4.3.2 HCA2 is essential for the reduction of epithelial hyperplasia by oral DMF treatment

To investigate the requirement for HCA2 in the protective effect of oral DMF treatment in the IMQ model, 8 weeks-old HCA2^{+/+} and HCA2^{-/-} mice were pre-treated with oral DMF or methocel (vehicle) for 7 days before Aldara® cream was applied topically to the shaved back of all mice for the last 5 days. Mice were sacrificed on day 13 for further evaluation (**Figure 18A**).

Macroscopic assessment revealed that DMF treated HCA2^{+/+} mice were less inflamed than methocel treated HCA2^{+/+} mice and that this effect was abolished in HCA2^{-/-} mice. Hence, DMF treated HCA2^{-/-} mice showed more scaling, thicker epidermis and pronounced redness of lesions compared to DMF treated HCA2^{+/+} mice after Aldara® application (**Figure 18B**). After sacrificing the mice skin biopsies were histologically processed and skin thickness was measured. In all mice skin thickness was elevated compared to mean epidermal thickness of untreated mice in the pre-experiment. However, DMF treated HCA2^{+/+} mice showed significantly reduced skin thickness compared to vehicle treated mice and DMF treated HCA2^{-/-} mice (**Figure 18C+D**).

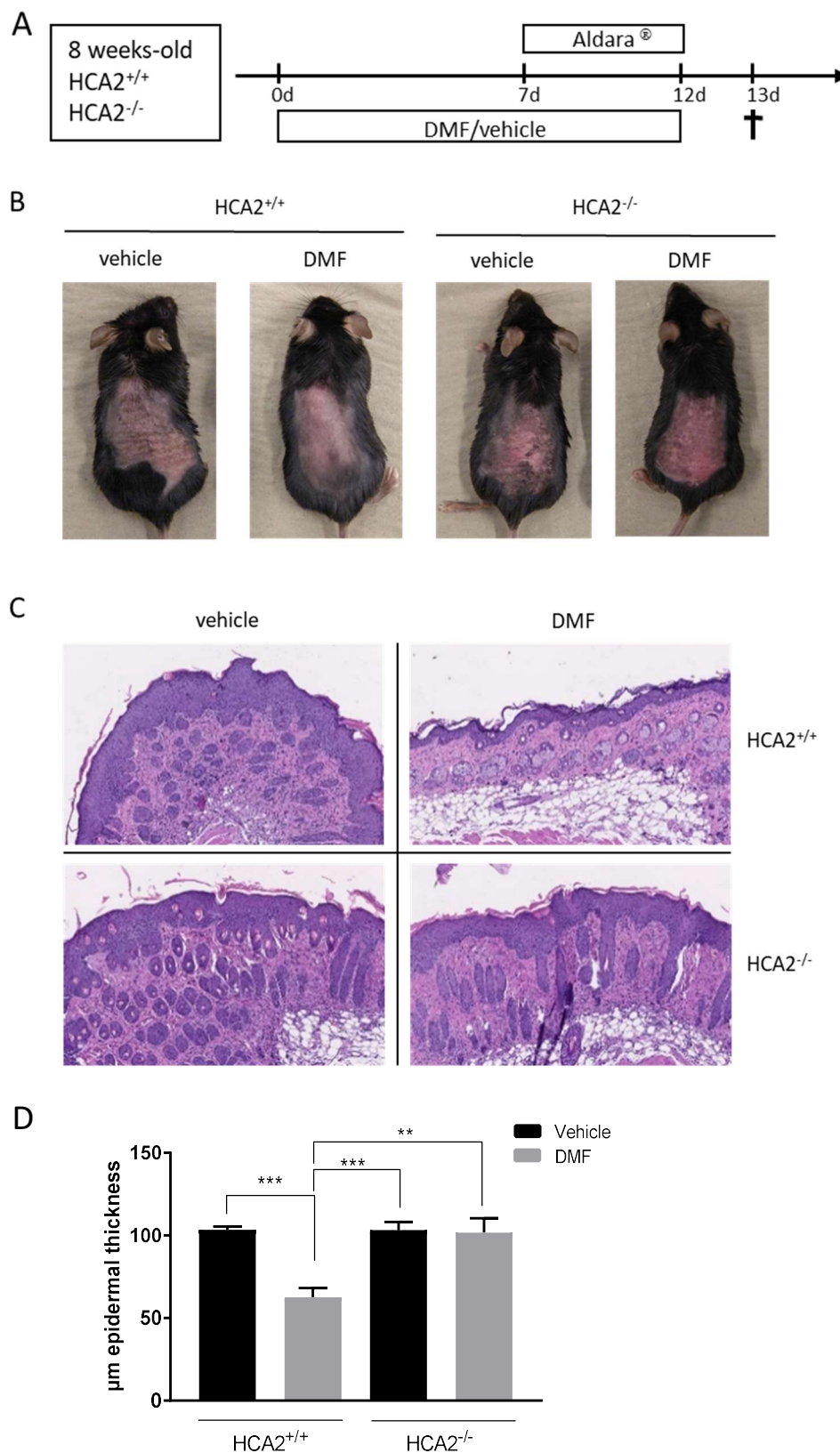


Figure 18: HCA2 is required for DMF treatment amelioration of psoriasiform skin lesions.

(A) Mice were treated with DMF or vehicle for 12 days. During the last 5 days Aldara® was applied to the back skin of the mice. Mice were sacrificed at day 13 and back skin sections were processed and analysed. (B) Macroscopic view of mice on day 13. (C) Representative H&E sections of back skin from HCA2^{+/+} and HCA2^{-/-} mice treated with DMF or vehicle. (D) Quantification of epidermal thickness. Each bar represents the mean +SEM of 5 mice. One representative out of 4 experiments shown. ** $p < 0,01$; *** $p < 0,001$ as determined by one-way ANOVA followed by Tukey's test.

4.3.3 Oral DMF treatment does not reduce immune cell infiltration in psoriasis-like plaques

Psoriasis lesions are not only marked by erythema, skin thickness and scaling but also show infiltration of immune cells. Besides neutrophils T cells also migrate into inflamed skin. To investigate if oral DMF treatment ameliorates skin inflammation via effects on immune cell skin infiltration, mice treated as in **Figure 18A**, were sacrificed, back skin was taken, digested and the resulting single cell suspensions were counted and stained for surface markers before analysis by flow cytometry (**Figure 19**). Total cell number, assessed by trypan-blue staining, was higher in methocel treated HCA2^{+/+} compared to HCA2^{-/-} mice and was further increased by DMF treatment in both genotypes although the increase by DMF in the HCA2^{+/+} mice is mostly caused by outliers. CD45⁺ leukocytes showed a similar pattern but with smaller increase caused by DMF treatment in HCA2^{-/-} mice. Neutrophils appeared increased by oral DMF treatment in both genotypes, but changes did not reach significant levels. T cells showed a similar pattern with the number of CD4⁺ T cells, CD8⁺ T cells as well as $\gamma\delta$ T cells not changed significantly by DMF in both genotypes but outliers had an effect of mean cell counts per group. The frequency of cells in control-treated HCA2^{+/+} was higher than in vehicle treated HCA2^{-/-} mice. There was a high degree of variation within the treatment groups for T cell counts. Monocytes, which develop into macrophages in tissues, were not markedly affected by oral DMF treatment and showed a high level of variation in cell number. Taken together, these results show that there is no clear reduction in the immune cell infiltrates into the skin during DMF treatment.

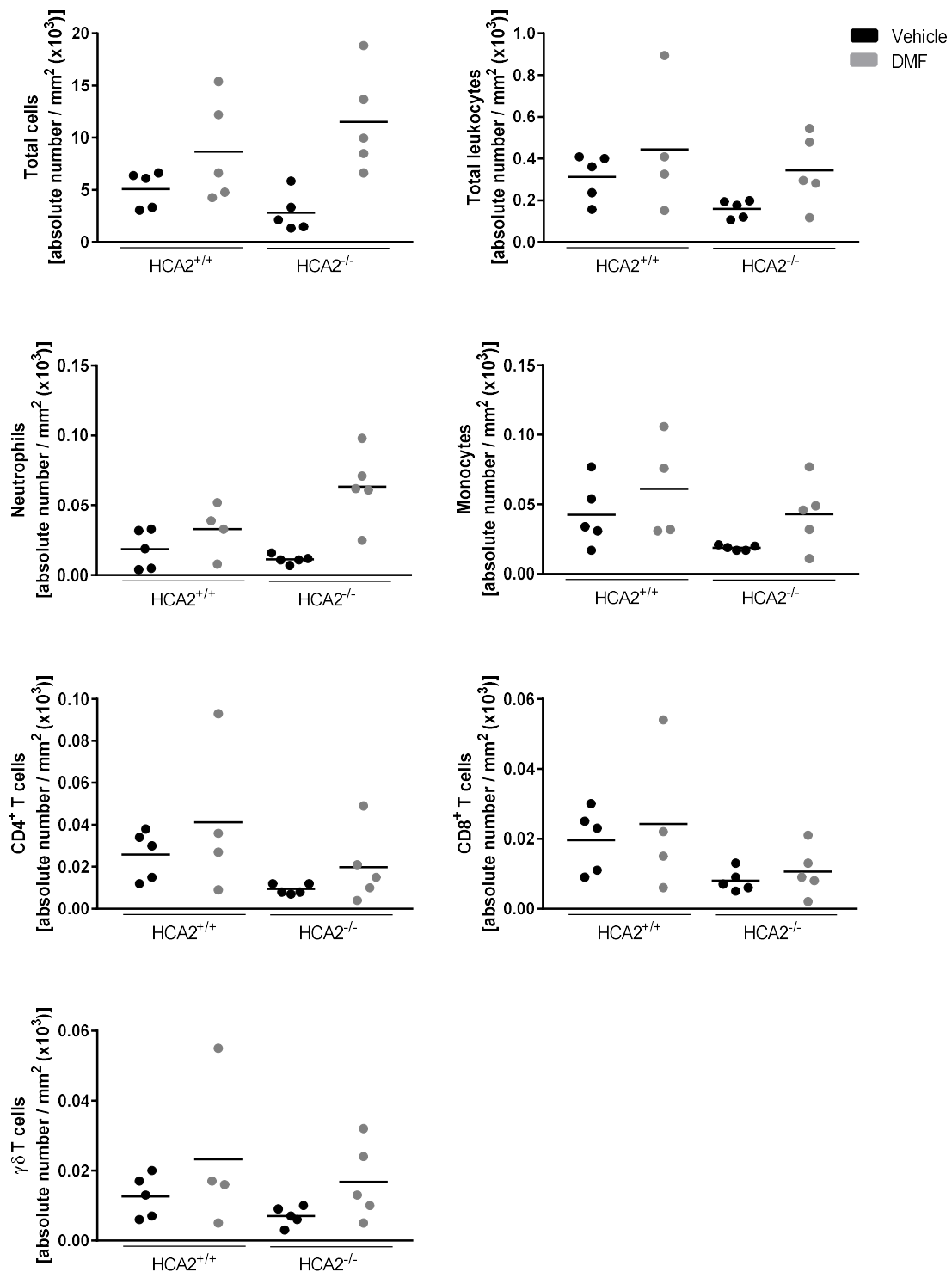


Figure 19: DMF treatment does not affect the number of leukocytes in the skin in the IMQ model.

Punch biopsies from dorsal skin were taken from Aldara[®]-treated HCA2^{+/+} and HCA2^{-/-} mice treated with DMF (grey) or vehicle (black). Cells were isolated, counted, labeled with a live/dead stain and surface stained for CD45, Ly6-C, Ly6-G, CD3, CD4, CD8 and γδ-TCR. Data are from one representative experiment out of three performed. No significances were detected by one-way ANOVA followed by Tukey's test.

4.3.4 Langerhans cell trafficking to the draining lymph nodes is not changed by DMF treatment

As infiltration of blood-derived immune cells into the skin was not reduced by oral DMF treatment, the effect of DMF treatment on Langerhans cell (LC) migration into SLN was analysed. LCs are skin-derived HCA2⁺ antigen presenting cells, which travel to lymph nodes upon inflammation [17, 214]. To investigate the effect of DMF on Langerhans cell number in skin draining lymph nodes (SLN) during skin inflammation, these nodes were taken from DMF or vehicle treated mice under Aldara[®] treatment on day 5 and isolated by enzyme-mediated tissue disruption. Cells were counted and stained for flow cytometric analysis. It was observed that except for one outlier there was no significant change in total lymph node cell number in HCA2^{+/+} mice mediated by DMF treatment (**Figure 20A**). In HCA2^{-/-} mice DMF led to a small non-significant decrease in SLN total cell number. To evaluate how DMF treatment effected LCs number in the SLN, CD207⁺ cells were analysed separately. Except for one mouse, there was no change in LC absolute number in SLN by DMF in HCA2^{+/+} mice but a small non-significant reduction in HCA2^{-/-} mice (**Figure 20B**). Calculated as percent of all leukocytes this slight reduction disappears as well but HCA2^{-/-} mice displayed a higher percentage of LCs in SLN CD45⁺ population independent of DMF treatment (**Figure 20C**). In summary, there was no significant effect of DMF treatment on LC number in skin draining lymph nodes during IMQ induced skin inflammation.

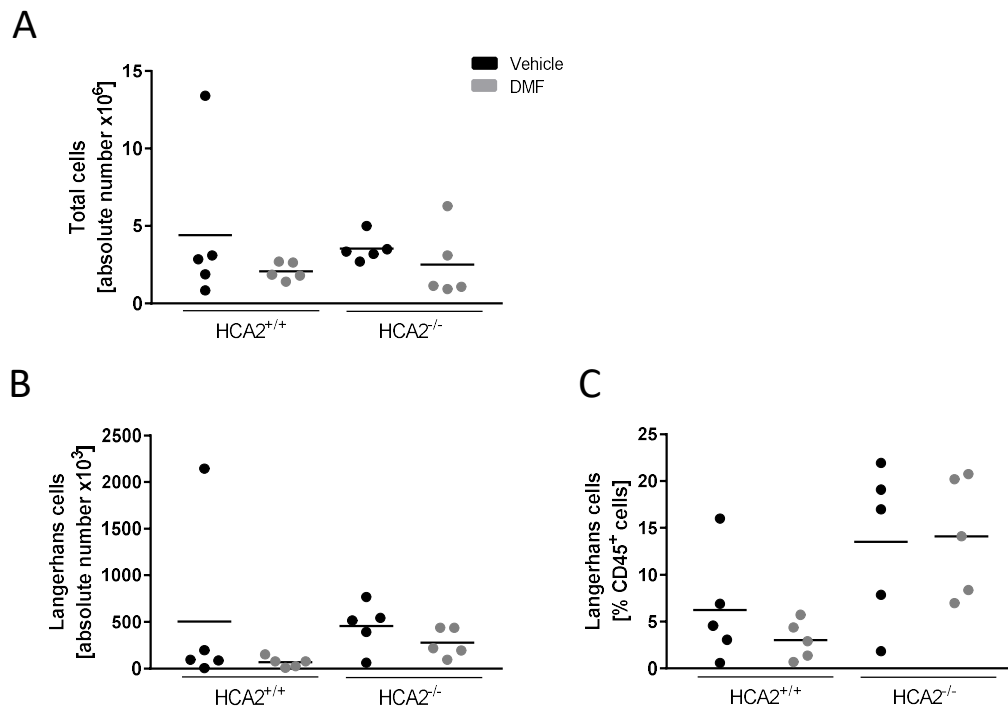


Figure 20: Langerhans cells in skin-draining lymph nodes are not affected by DMF treatment.

Skin draining lymph nodes were taken from Aldara[®]-treated HCA2^{+/+} and HCA2^{-/-} mice treated with DMF or vehicle. Cells were isolated, counted and surface stained for CD45, MHCII and Langerin (CD207) as well as with a live/dead stain. Graphs show the absolute number of total cells (A), the absolute number of LCs (B) and the percentage of LCs within CD45⁺ cells (C). Data are representative of 3 independent experiments. Statistics showed no significance as determined by one-way ANOVA followed by Tukey's test.

4.3.5 DMF treatment alters expression of psoriasis related genes

In order to further analyse HCA2 mediated effects of DMF treatment that lead to the observed significant decrease in epidermal thickness at day 5 of Aldara[®] application, gene expression was analysed in the skin. Thus, mRNA was isolated from lesional back skin, converted to cDNA and evaluated by qPCR (**Figure 21**). Analysis showed that DMF treatment downregulated *I11b* in HCA2^{+/+} but also in HCA2^{-/-} mice. *I11a* was downregulated in HCA2^{+/+} mice but not in HCA^{-/-} mice by DMF treatment. The chemokines *Cxcl5* and *Cxcl2* showed a reduction in HCA2^{+/+} mice and in HCA2^{-/-} mice in response to DMF treatment. *I17a* also showed a slight non-significant downregulation in both genotypes despite a low expression overall. *I123a* and *I122* were also reduced in both genotypes due to DMF treatment. Analysis of antimicrobial peptides mRNA showed that while *S100a8* was reduced by DMF in HCA2^{+/+} mice, *S100a7a* was significantly downregulated in HCA2^{+/+} mice by DMF treatment. DMF did slightly alter antimicrobial peptide transcript levels in HCA2^{-/-} mice. *Krt14* showed a small drop in mRNA expression in DMF treated HCA2^{+/+} mice and was increased in HCA2^{-/-} mice. Together, these results indicate that expression of a subset of important psoriasis-related genes is reduced by DMF treatment but not all reductions seem to be HCA2 dependent.

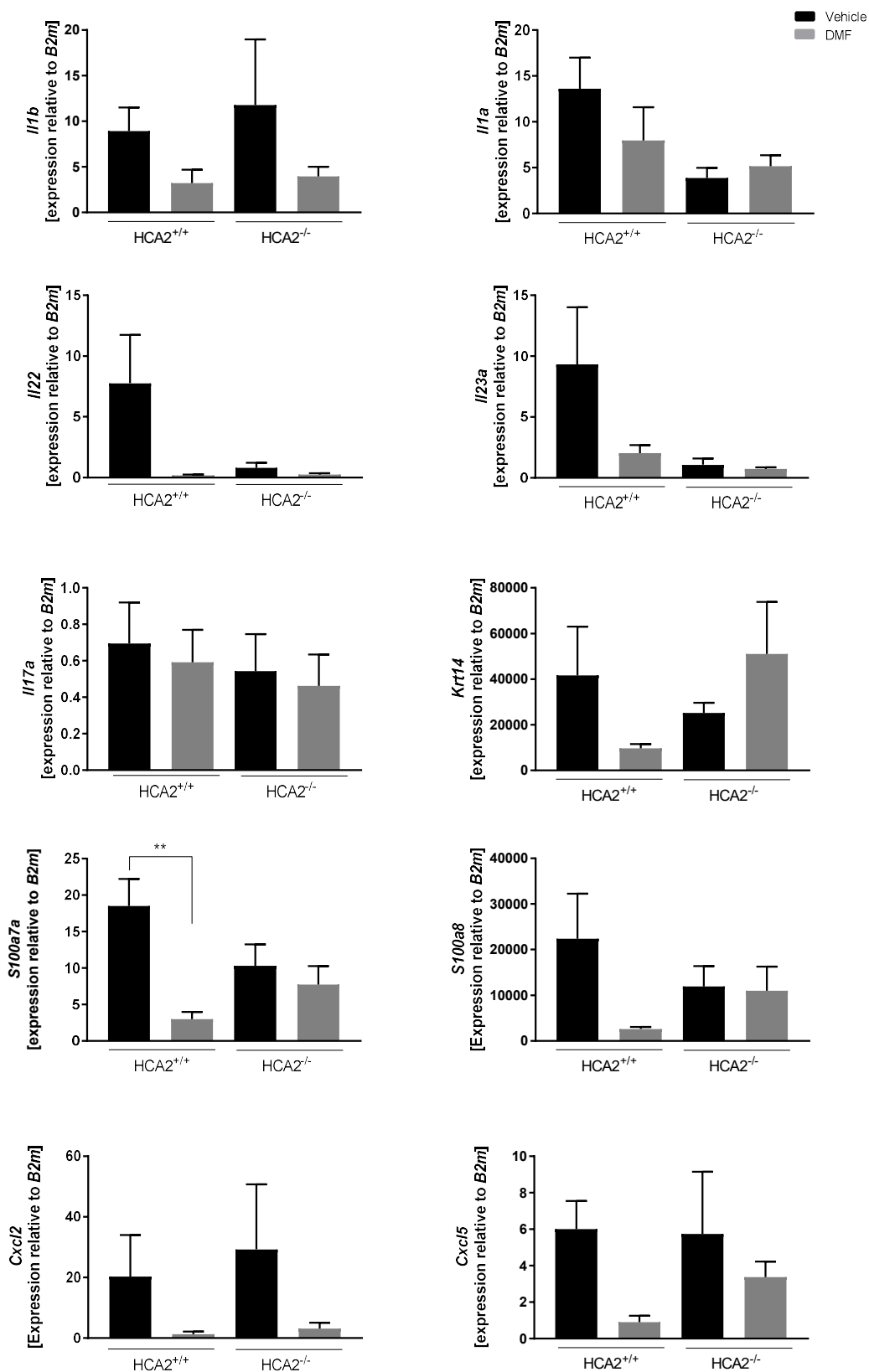


Figure 21: DMF treatment affects mRNA levels in skin in both HCA2-dependent and -independent manners.

Back skin biopsies were taken from Aldara®-treated HCA2^{+/+} and HCA2^{-/-} mice treated with DMF or vehicle for mRNA isolation. cDNA from these samples was analyzed for Il1b, Il1a, S100a8, S100a7a, Cxcl5, Cxcl2, Krt14, Il17a and Il23a mRNA expression by qPCR. Bars represent mean mRNA expression relative to B2m + SEM of 5 biological replicates. Data are representative of 4 independent experiments **p < 0.01 as determined by one-way ANOVA followed by Tukey's test.

4.3.6 Effect of DMF treatment on cytokine protein levels in psoriasis-like skin inflammation

To investigate whether oral DMF changed cytokine protein levels in mice undergoing topical Aldara[®] application total protein was isolated from lesional skin biopsies after DMF or vehicle treatment. ELISA assays were performed for IL-17A and IL-6. IL-17A protein levels were not significantly changed after oral DMF treatment (**Figure 22A**). There were similar concentrations of IL-17A protein in both HCA2^{+/+} and HCA2^{-/-} mice treated with DMF or vehicle. IL-6 was reduced by DMF treatment in both genotypes not significantly, suggesting a HCA2 independent regulation mechanism (**Figure 22B**).

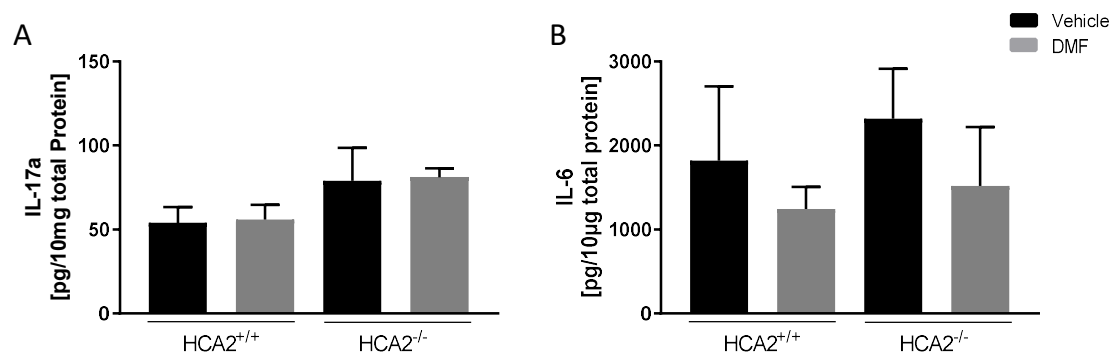


Figure 22: DMF treatment reduces IL-6 protein level but not the concentration of IL-17A in Aldara[®]-induced skin lesions.

Protein was isolated from the back skin of Aldara[®]-treated HCA2^{+/+} and HCA2^{-/-} mice undergoing DMF or vehicle treatment. IL-17A (A) and IL-6 (B) levels were assessed by ELISA. One representative out of 3 experiments is shown. No significance detected. (one-way ANOVA with Tukey's test)

4.3.7 Oral DMF treatment changes mRNA expression levels at day 4 of Aldara® application

As demonstrated in the kinetics experiment maximal skin thickness and the peak expression of certain genes occurred at different time points. Importantly, some psoriasis relevant genes were expressed at high levels prior to peak epithelial thickness before reducing in expression as skin thickness continued to increase. HCA2^{+/+} and HCA2^{-/-} mice were treated for 11 days with DMF or methocel control and Aldara® was applied for the last 4 days on the shaved back of the mice. Mice were then sacrificed and back skin was sampled for histological analysis and mRNA isolation. To verify that DMF treatment also exerted a protective effect at day 4, epidermal thickness was measured in H&E stained histological samples of back skin. Although not significant unlike at day 5, oral DMF reduced the thickness of the Aldara® treated back skin of HCA2^{+/+} mice but not of HCA2^{-/-} mice (**Supplemental figure 1**).

Analysis of mRNA expression levels revealed that compared to day 5 of Aldara® application, the results at day 4 exhibited more significant differences (**Figure 23**). The proliferation marker Ki-67 was significantly reduced in HCA2^{+/+} mice, both in DMF treated and to a lesser extent in methocel controls compared to HCA2^{-/-} mice. DMF led to a reduction in *Ki67* expression in HCA2^{-/-} mice. There was also a reduction of *Krt14* mRNA in DMF treated HCA2^{+/+} mice but not in HCA2^{-/-} mice. *Cxcl5* and *Cxcl2* showed a lower mRNA expression in HCA2^{+/+} mice compared to HCA2^{-/-} mice with DMF further reducing expression in both genotypes. In contrast, transcripts for the cytokines *Il22*, *Il17a* and *Il23a* showed very low expression levels in all groups. DMF treatment increased *Il17a* and *Il23a* levels in HCA2^{+/+} mice while leading to a decrease in the same transcripts in HCA2^{-/-} mice. *Il22* expression was slightly decreased by DMF treatment in HCA2^{+/+} mice. *S100a8* was downregulated in DMF treated HCA2^{+/+} mice while no regulation by DMF in HCA2^{-/-} mice was observed. *S100a7a* was decreased only in HCA2^{-/-} mice. *Il1b* was downregulated by DMF in both genotypes but no significance was detected. Interestingly, the expression levels of *Ki67*, *Il17a*, *Il23a*, *S100a7a*, *Cxcl2* and *Cxcl5* were lower in HCA2^{+/+} animals than in HCA2^{-/-} mice, independent of treatment. In summary, it appears that a pre-treatment with DMF before 4 days of Aldara® treatment leads to a reduction in gene expression of psoriasis key genes prior to maximal skin pathology. Also, an overall lower expression of some of these genes in HCA2^{+/+} mice was observed, irrespective of treatment.

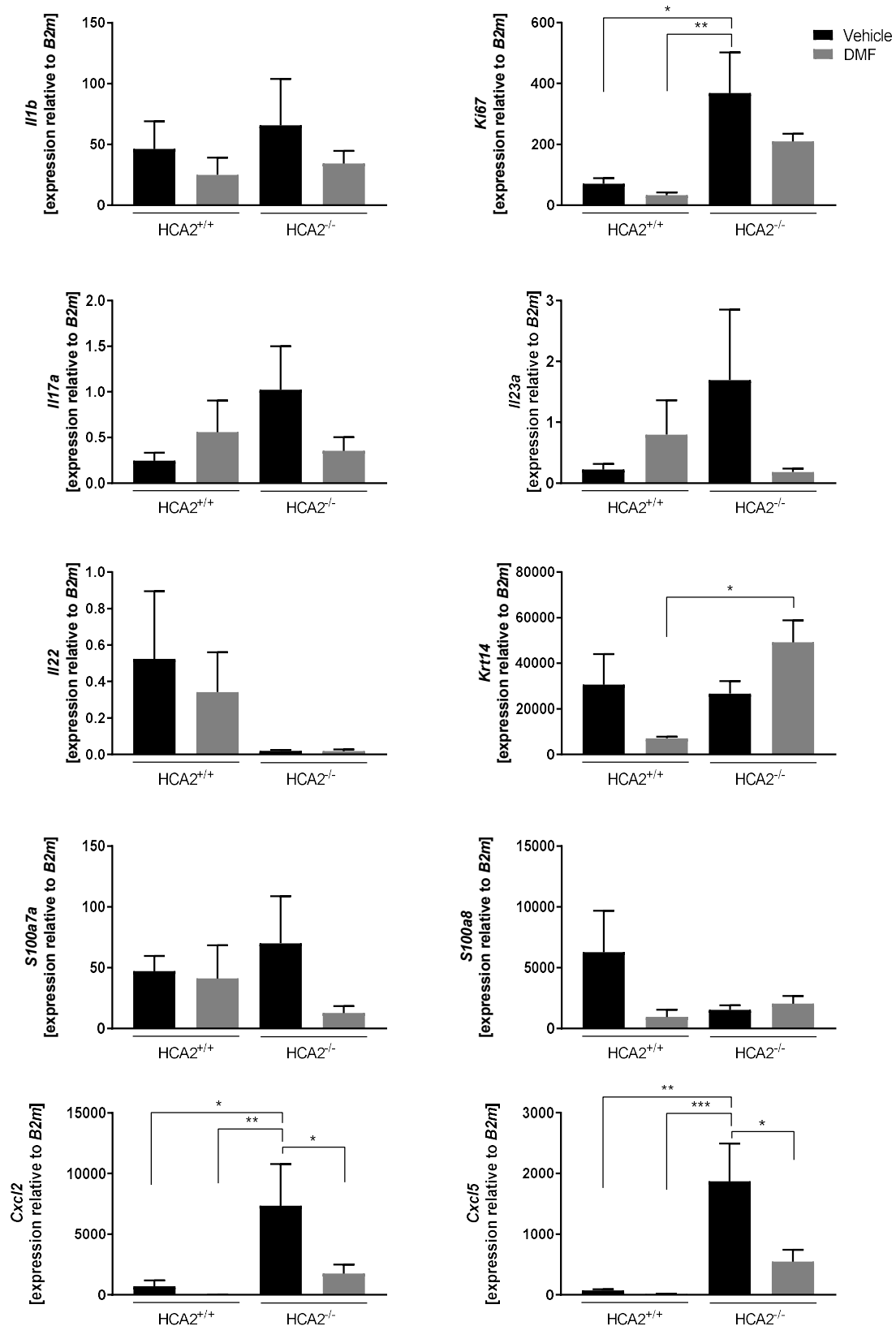


Figure 23: DMF induces significant changes in gene expression at day 4 of Aldara® treatment.

Mice were pre-treated with DMF or vehicle for 7 days before treatment for 4 days with Aldara®. Mice were subsequently sacrificed and back skin sections were processed and analysed. mRNA was isolated from back skin biopsies. cDNA from these samples was analysed for *Il1b*, *Ki67*, *S100a8*, *S100a7a*, *Cxcl2*, *Cxcl5*, *Krt14*, *Il22*, *Il17a* and *Il23a* expression by qPCR. Bars represent mean mRNA expression relative to *B2m* + SEM of 5 biological replicates. Data are representative of 2 independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 as determined by one-way ANOVA followed by Tukey's test.

4.3.8 Aldara®-induced weight loss promoted the production of BHB

As there were significant differences detected between HCA2^{+/+} and HCA2^{-/-} mice (independent of DMF treatment) in the expression of *Ki67* and the chemokines *Cxcl2* and *Cxcl5*, changes in weight and ketone levels in these mice were analysed to determine if BHB was produced during Aldara® application. BHB is an agonist of HCA2 and increased levels of this ketone may help to explain the previously described DMF independent effects.

Application of Aldara® to the shaved back resulted in rapid weight loss in both HCA2^{+/+} and HCA2^{-/-} mice after the first two days. This decrease was non-significantly higher in vehicle treated mice compared to DMF treated animals. Body weight started to increase again on day 4 in all groups (**Figure 24A**). After sacrificing the mice, blood ketone levels were measured by direct measurement of BHB. In all groups blood BHB levels were on average above the mean BHB levels of untreated mice (**Figure 24B**).

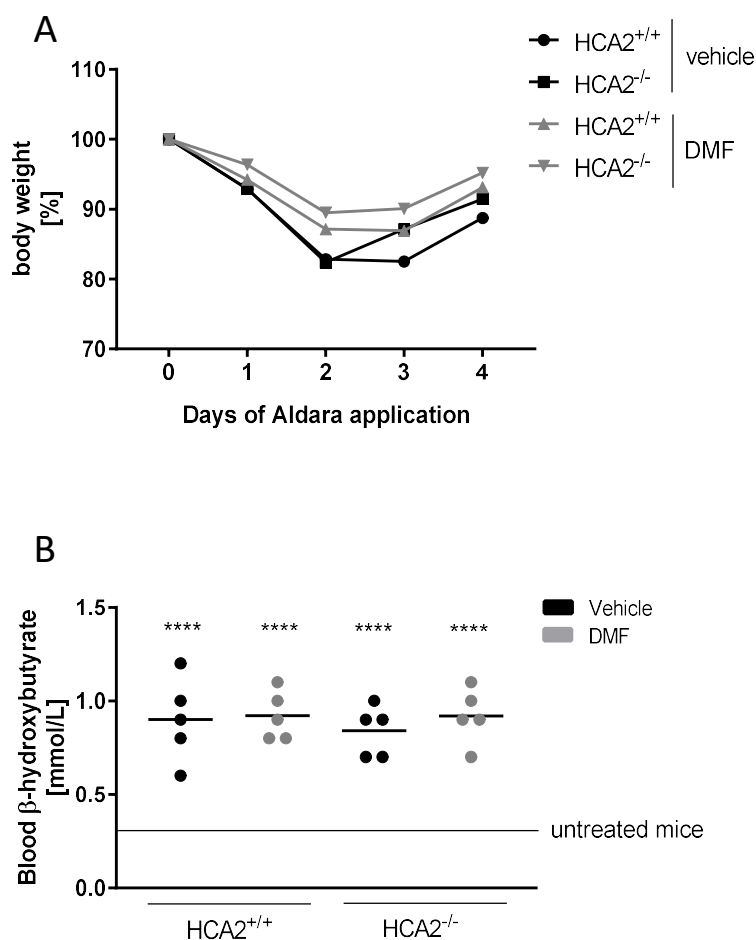


Figure 24: Aldara® application induces weight loss and ketone body production.

(A) The weight of mice was measured daily during the application period of Aldara®. (B) After day 4 of Aldara® application mice were sacrificed and BHB was measured in the blood of the mice. One representative out of 2 experiments shown. significance detected as determined by one-way ANOVA followed by Tukey's test. **** p<0,0001

4.3.9 Effects of a ketogenic diet on Aldara[®] induced psoriasis-like skin inflammation

Oral DMF treatment led to a HCA2-dependent amelioration of psoriasis-like lesions in mice. BHB is an endogenous ligand of the HCA2 receptor and is produced in times of starvation from the use of fat as a main energy source. To verify if BHB can mediate similar protective effects on Aldara[®] induced skin lesions as oral DMF treatment, mice were fed an ad-libitum high fat or control diet for 12 days before Aldara[®] was applied to the shaved back on these mice for further 5 days (**Figure 25A**). Thereafter, mice were sacrificed and samples analysed for epidermal thickness, mRNA expression and cytokine levels.

During the experiment mice were weighed every other day for the first 12 days. With the ketogenic diet HCA2^{+/+} mice lost more than 10% of their bodyweight. HCA2^{-/-} mice lost less weight and both genotypes fed a control diet did not lose weight at all (**Figure 25B**). Aldara[®] treatment led to an increased weight loss in mice on a ketogenic diet compared to mice fed a control diet (data not shown). Mice on a ketogenic diet showed elevated blood BHB levels. After two days of ketogenic diet mean blood BHB levels were above the EC₅₀ of HCA2 activation [7]. Blood ketone levels peaked at day seven of the diet and started to decrease thereafter. Application of Aldara[®] led to an increase in ketosis which accompanied significant weight loss in mice fed a ketogenic diet (**Figure 25C**). Mice on a control diet had mean BHB levels of 0.3mmol l⁻¹ which increased to 0.7mmol l⁻¹ after Aldara[®] application (data not shown). After sacrificing the mice epidermal skin thickness was measured in histologically processed and H&E stained skin biopsies. The ketogenic diet marginally reduced epidermal thickness in HCA2^{+/+} mice compared to animals fed the control diet, however, it should be noted that epithelial thickness of the control diet HCA2^{+/+} mice was unexpectedly low compared to previous experiments. Interestingly, the ketogenic diet reduced the epidermal thickness in HCA2^{-/-} mice significantly compared to control diet HCA2^{-/-} mice (**Figure 25D**).

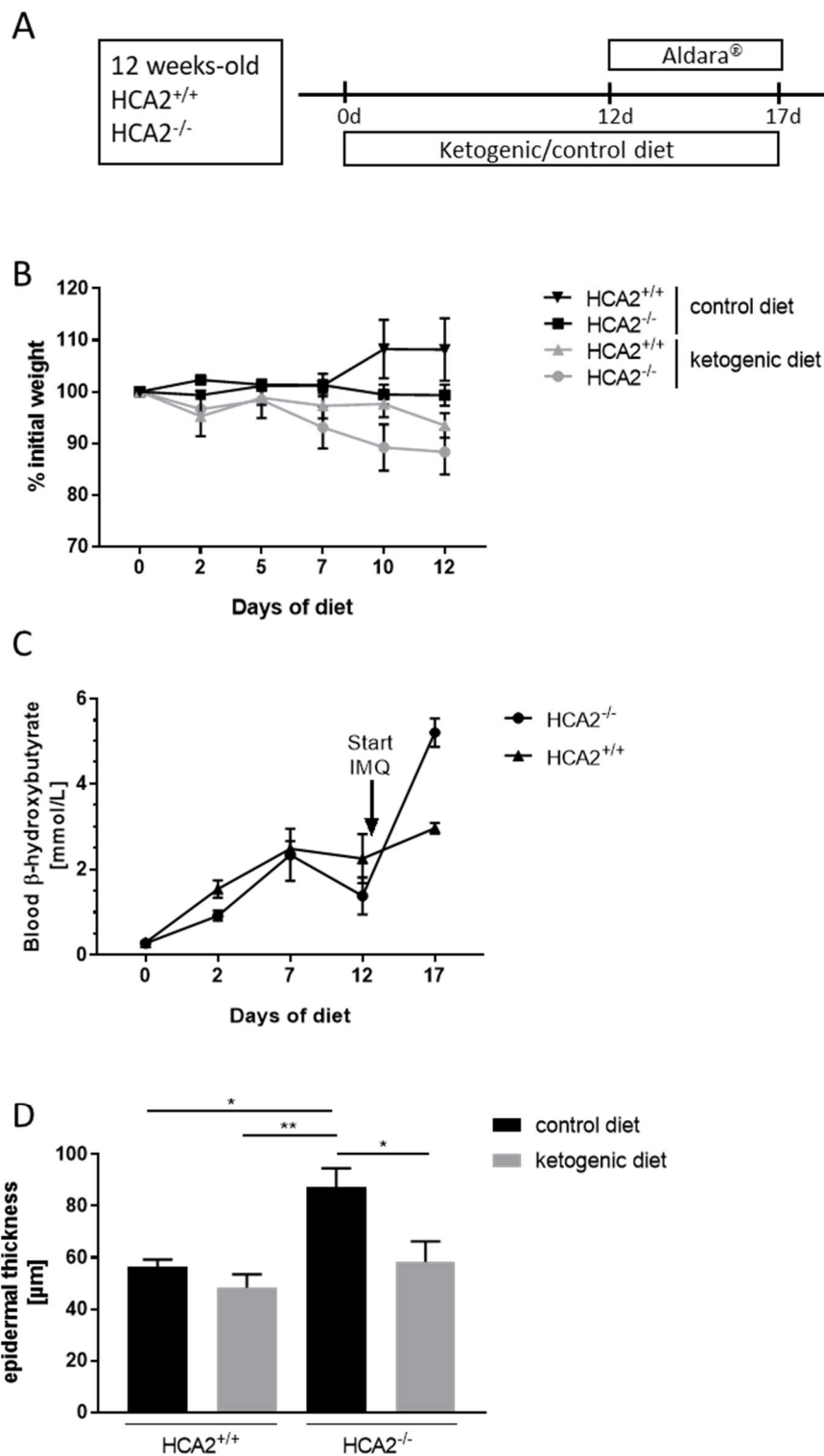


Figure 25: The induction of ketosis by a ketogenic diet reduces epidermal thickness in the IMQ mouse model in a HCA2-independent manner.

(A) Mice were fed a ketogenic or control diet for 17 days. During the last 5 days Aldara® was applied to the shaved back. Mice were sacrificed at day 18 and samples were taken for analysis. (B) Bodyweight was monitored every other day during the diet phase. (C) Blood ketones were analysed regularly during the experiment. (D) Quantification of epidermal thickness. Each bar represents the mean +SEM of 5 mice. One representative out of 2 experiments shown. Significance was determined by one-way ANOVA following Tukey's test, * $p < 0,05$, ** $p < 0,01$.

Although not significant, the ketogenic diet seemed to affect the migration of immune cells to SLN. SLN were taken from Aldara® treated mice and analysed for immune cell marker expression by flow cytometry. There was a non-significant reduction in total leukocytes seen in HCA2^{+/+} mice SLN fed the ketogenic diet compared to control diet (**Supplemental figure 2A**). In HCA2^{-/-} mice this effect was less pronounced. There was a reduction in LC migrating to SLN upon Aldara® application in HCA2^{+/+} mice fed a ketogenic diet. There was no major difference in LC migration to SLN in HCA2^{-/-} mice (**Supplemental figure 2B**).

Skin samples were taken and mRNA was isolated, reverse transcribed and analysed by qPCR for expression changes induced by the ketogenic diet. Although there were no significant changes observed, trends can be seen. *Ki67*, a proliferation marker was downregulated by ketogenic diet in HCA2^{+/+} mice but not in HCA2^{-/-} mice. *S100a7a* was downregulated by ketogenic diet in HCA2^{-/-} mice while *S100a8* was upregulated in both genotypes by the ketogenic diet. The chemokine *Cxcl2* and *Cxcl5* were increased by a ketogenic diet in both genotypes with a generally higher expression of *S100a8*. *Il22* was upregulated in HCA2^{+/+} mice but was downregulated in HCA2^{-/-} mice by the ketogenic diet. *Il17a* and *Il23a* showed a downregulation in HCA2^{-/-} mice while *Il17a* was not changed in HCA2^{+/+} mice and *Il23a* showed only a slight reduction (**Supplemental figure 3**).

To analyse cytokine protein levels, biopsies from inflamed back skin were taken and total protein was isolated. Cytokine levels were subsequently analysed by ELISA. Although all changes were non-significant, some trends were observed. IL-6 showed a slight reduction due to ketogenic diet in both genotypes. IL-17A levels were not changed in either genotype. The concentration of TNF- α was reduced in both genotypes in response to the ketogenic diet. IL-23 was downregulated in HCA2^{+/+} mice fed a ketogenic chow but showed only a slight effect on HCA2^{-/-} fed the same diet. IL-22 protein levels displayed a significant degree of variation between single mice and a low absolute concentration. Despite this, the ketogenic diet seemed to decrease IL-22 levels in HCA2^{+/+} mice but to upregulate in HCA2^{-/-} mice (**Supplemental figure 4**).

These preliminary experiments showed that BHB appears to improve Aldara® induced psoriasis-like skin lesions but in a manner that is independent of HCA2, unlike oral DMF treatment which is highly dependent upon this receptor in order to ameliorate skin inflammation. Although there are pro-inflammatory factors reduced by a ketogenic diet, such as LC migration, IL-23 and IL-22 levels, there were other factors that showed no or unexpected changes in response to the ketogenic diet such as mRNA expression of S100 proteins, the cytokines *Il1a*, *Il1b*, *Il22*, *Il17a* and the chemokines *Cxcl2* and *Cxcl5*. As the ketogenic diet had a significant effect on epidermal thickness in HCA2^{-/-} mice,

HCA2 appears to be dispensable for the amelioration of skin inflammation during ketogenesis.

4.4 Discussion

This study has demonstrated for the first time that there is a therapeutic effect of DMF in the IMQ-mouse model and that HCA2 is essential for DMF's protective effect on IMQ-induced psoriasis-like dermatitis in mice. Peaking on day 5 of Aldara® treatment, it was shown that DMF treatment reduced epidermal thickness by approximately 45% in HCA2^{+/+} mice compared to vehicle controls but had no effect in HCA2^{-/-} mice. Analysis of skin infiltrating immune cells showed no significant differences between DMF treated HCA2-expressing and knockout animals despite improvements in skin inflammation induced by Aldara®. Gene expression was analysed after 4 and 5 days of Aldara® application. A significant reduction dependent upon HCA2 was observed for *S100a7a* at day 5. Other genes were not significantly changed due to oral DMF treatment of mice on either day, but a trend towards downregulation of pro-inflammatory genes was observed. On the protein level, IL-17A showed no change due to oral DMF treatment of mice while IL-6 was non-significantly reduced due to DMF, however, this was independent of HCA2. The reduction of weight and subsequent induction of BHB in the blood of all mice led to the hypothesis that the significant lower expression of some genes in HCA2^{+/+} mice (i.e.: *Ki-67*, *Cxcl2* and *Cxcl5*) may have been induced by BHB binding to HCA2. A preliminary experiment with mice on a ketogenic diet showed that while ketogenesis reduced skin inflammation in a HCA2 independent manner, this did not result in changes in gene expression matching those observed in DMF treated animals.

This study clearly showed that oral DMF treatment led to a reduction in epidermal thickness in the IMQ mouse model of psoriasis-like inflammation and that HCA2 is required for this protective effect. Hence, HCA2^{+/+} mice treated with oral DMF had significantly reduced cutaneous inflammation compared to vehicle controls and in DMF treated HCA2^{-/-} animals there was no reduction in epithelial hyperplasia. A requirement for HCA2 in DMF mediated protection in murine models of MS and pemphigus has also been reported [55, 56]. Taken together, these reports have demonstrated that HCA2 is critical for the amelioration of inflammation by oral DMF *in vivo*. These data further support the notion that MMF, rather than DMF itself, is the active metabolite *in vivo* which mediates the anti-inflammatory effects of oral DMF. This is due to the fact that there is strong evidence that DMF is hydrolysed in the gut to MMF and other metabolites and adducts before entering the blood. In both, experimental animals and human volunteers, MMF rather than DMF is detected in the blood after oral DMF intake [177-179, 181]. In addition, DMF is not a potent agonist of HCA2 compared to MMF [5]. Taken together,

this study in context with other published data show that MMF from DMF ameliorates psoriasis-like inflammation via HCA2 in mice and suggests that drugs such as Skilarence® improve psoriasis in patients in part via MMF-HCA2 signalling.

Keratinocyte hyperproliferation is one of the hallmark features of psoriasis, however, psoriatic lesions (and also psoriasis-like lesions in the IMQ-mouse model) are also characterized by infiltrations of immune cells [208]. In the previous chapter it was described that oral DMF treatment of psoriasis patients led to a reduction in blood leukocytes, mostly of HCA2 expressing neutrophils and dendritic cells. One hypothesis was that oral DMF treatment alters leukocyte infiltration into the skin. Leukocytes were isolated from psoriasis-like lesions of mouse back skin and phenotyped according to cell surface marker expression. Although lesions showed an infiltration of neutrophils and T cells there were no significant differences in cell number induced by DMF treatment, neither in HCA2^{+/+} nor in HCA2^{-/-} mice. In a model of EAE it was shown that DMF treatment inhibited immune cell infiltration into the central nervous system [55]. Compared to the IMQ-mouse model the EAE model is a long-term model and it could be assumed that oral DMF leads to a reduction in immune cell infiltration at a later time point. The effect of DMF treatment on immune cells seen in EAE may not occur within the time frame of the IMQ-model due to its relatively short duration. In psoriasis patients blood leukocytes decrease over time with only a small effect being observed after one month of treatment. In contrast, the duration of the IMQ mouse model is 5 days. TNF- α has been shown to be one factor able to attract immune cells when injected into skin [261]. As there were no changes in TNF- α levels observed in a preliminary experiment (data not shown), this suggests that oral DMF treatment does not act via altering TNF- α concentrations to reduce leukocyte skin infiltration. As TNF- α in the skin can be produced by pDCs which are thought to initiate psoriasis lesions in humans, this indicates that DMF treatment may not initially reduce skin inflammation by acting upon these HCA2 expressing immune cells [125, 262]. The chemokines CXCL2 and CXCL5 are produced by keratinocytes upon induction by IL-17 and mediate the recruitment of immune cells into psoriatic lesions [263]. Treatment of psoriasis patients with TNF- α blockers has been shown to reduce chemokines CXCL2 and CXCL5 in the skin of these patients [264]. In agreement with this, it was observed that mRNA expression of *Cxcl2* and *Cxcl5* was reduced by oral DMF treatment at day 5 of Aldara® application. This effect was less pronounced at day 4. This suggests that effects of oral DMF on immune cell infiltration may manifest at later time points. Induction of inflammation by application of Aldara® also causes trafficking of LCs from the skin to the draining lymph nodes [206]. In both genotypes CD207⁺ LCs were detected in the SLN showing there was migration of LCs after induction of skin inflammation. Interestingly, HCA2^{+/+} mice showed a slightly lower number of LCs in SLN compared to HCA^{-/-} mice, irrespective of treatment with DMF.

Comparable to data from skin immune cells, pre-treatment of Aldara® creamed mice with oral DMF did not change LC cell number in SLN. It may be possible that the short-lived model is too short to allow to show an effect of DMF treatment to effect immune cells. On the other hand, it is possible that oral DMF does not inhibit skin inflammation by significantly affecting leukocytes at least in the early stages of treatment. Although LCs, neutrophils and monocytes/macrophages express HCA2 there was no change in the numbers of these cells within the skin or draining lymph nodes in response to DMF treatment in this study. As shown in chapter 3, lymphocytes did not express HCA2 and were not expected to show direct, immediate effects of DMF treatment as in human it is known that lymphocytes are affected but a later time points compared to HCA2 expressing cells (previous chapter, [221, 228, 229]). Taken together, the data summarized here suggest that oral DMF ameliorates skin inflammation in a HCA2 dependent manner without significantly affecting leukocyte numbers in the skin, at least in the initial stages of psoriasis-like inflammation.

On the protein level this study showed that IL-17A, mostly produced by T cells, was not changed by DMF treatment of mice [148]. This was confirmed by mRNA expression analysis. *Il17a* levels were not significantly altered by DMF treatment and showed an overall low expression during Aldara® application. IL-17A production by T cells is maintained by IL-23, which also supports the expansion of IL-17A-producing T cells [148, 208]. *Il23* mRNA levels were very low in the inflamed skin and showed no significant changes in response to oral DMF treatment. IL-23 is predominantly produced by LCs and DCs [265], which while HCA2⁺, were not significantly affected by oral DMF in regards to SLN migration and skin cell number, respectively. These results support the hypothesis that oral DMF reduces Aldara®-induced skin inflammation independent of large effects on HCA2-expressing leukocytes.

In contrast to other leukocyte-derived cytokines, IL-6, a pleiotropic pro-inflammatory cytokine that is produced by a number of cells including keratinocytes and can act in a reverse feedback manner to stimulate cell proliferation, was marginally reduced in DMF treated mice in a HCA2-independent manner, as both HCA2 sufficient and deficient mice showed a reduction in IL-6 [266]. IL-6 expression is induced by IL-1 cytokines [267]. mRNA analysis suggested that oral DMF reduces *Il1b* levels in Aldara® treated skin which in turn may lead to reduced IL-6 production. Reduced IL-6 production would presumably lead to reduced keratinocyte proliferation resulting in diminished epidermal thickness. That is in line with reduced *Ki67* mRNA expression. However, as the DMF induced IL-6 concentration decrease was small and HCA2 independent while the reduction in epithelial hyperplasia was restricted to HCA2 sufficient animals and was highly statistically significant it is unlikely that changes in IL-6 levels alone account for the *in vivo* effects of DMF treatment. A receptor for IL-1 has been shown to be expressed

by psoriatic keratinocytes, suggesting autocrine stimulation of these keratinocytes by IL-1 [268]. The reduction of *Il1* transcripts caused by DMF treatment may indicate reduced stimulation of keratinocytes by self-produced IL-1. These data suggest a direct effect of oral DMF treatment on keratinocytes rather than on cells of the immune system.

Keratinocytes express HCA2 and are likely a direct target of oral DMF treatment [17]. The fact that IL-1 β is induced in keratinocytes by $\gamma\delta$ T cell-produced IL-17 and IL-22 [148, 210, 213], which were more or less unchanged by oral DMF suggests that DMF treatment may act on IL-1 β producing keratinocytes directly rather than indirectly via immune cells. This is substantiated by significantly reduced *S100a7a* in DMF treated HCA2^{+/+} mice. *S100a8* also showed reduction in this mouse group. Both S100 proteins are expressed by keratinocytes constitutively and are further increased by inflammatory stimuli such as IL-22 [152, 269]. S100 protein levels are enhanced in the blood and skin of psoriasis patients [153, 270]. S100 proteins play an important role in the pathophysiology of psoriasis. S100A7A has been shown to increase pro-angiogenic factors *in vitro* suggesting a role in psoriatic angiogenesis and S100A7A is a chemoattractant for immune cells [271, 272]. S100A8 forms a complex with S100A9 and has been shown to play an important role in psoriasis as its absence promotes keratinocyte proliferation and increased production of IL-6 and TNF- α [273, 274]. *S100a8* mRNA transcripts showed a larger reduction after 5 days of Aldara[®] application and the effects seen on IL-6 expression may become more distinct later in disease progression. The functions of the S100 proteins show that they play an important role in the inflammatory loop seen in psoriasis. A downregulation of these proteins by DMF treatments suggests that MMF directly acts on keratinocytes and reduces the production of S100 proteins which may in turn lead to the reduced keratinocyte proliferation seen in DMF treated HCA2^{+/+} mice.

Keratinocyte derived *Krt14* was also decreased in DMF treated HCA2^{+/+} mice. *Krt14* is normally expressed in healthy proliferating basal keratinocytes and is lost as keratinocytes start to differentiate in the upper layers of the epidermis [275]. In psoriasis KRT14 is upregulated in the suprabasal layers of the skin, which proliferate but do not differentiate properly [276]. Based on the minor reduction induced by oral DMF in *Krt14* expression, it is possible that treatment alters the balance between keratinocyte proliferation and differentiation during Aldara[®] induced inflammation. Given that keratinocytes also express HCA2 [17] and the lack of significant changes in immune parameters, this further supports the hypothesis that keratinocytes are a main target of oral DMF treatment for the amelioration of skin inflammation in the IMQ model.

A significant reduction in mRNA expression of *Ki67* and the chemokines *Cxcl2* and *Cxcl5* between vehicle treated HCA2^{+/+} mice and HCA2^{-/-} mice led to the hypothesis that there

may be an underlying mechanism leading to the decrease in the expression of these genes in HCA2^{+/+} mice, independent of DMF treatment. Soon after the beginning of Aldara[®] application mice started to lose weight. Starvation forces the body to produce ketones from fat to provide energy for bodily functions [277]. The same is true for any kind of weight loss, which leads to utilisation of the adipose tissue as an energy source. BHB, one of the ketones produced during lipolysis is a ligand for HCA2 [7]. Indeed, the Aldara[®] treated mice showed elevated levels of blood ketones. These levels were above the EC₅₀ of BHB for HCA2 receptor activation [7]. As it is known that weight loss reduces psoriasis symptoms and increases treatment efficacy of other drugs in patients [90, 93, 94, 278], ketosis may lead to the reduction of mRNA expression observed in the IMQ model. However, if ketosis is induced in a controlled manner it was hypothesized that it may have similar HCA2 dependent effects as a DMF treatment. Unlike DMF treatment, a ketogenic diet reduced Aldara[®] induced epithelial hyperplasia independently of HCA2. HCA2^{-/-} mice showed a significant decrease in epidermal thickness. This was accompanied by a reduction in *S100a7a* expression. This supports the hypothesis that S100 proteins may be an important factor in driving inflammation in the skin. As the reduction in epidermal thickness was independent of HCA2, other receptors may mediate the effects of the ketogenic diet. For example, GPR41 (known as free fatty acid receptor 3; FFAR3) has been reported to be a receptor for BHB [279]. It is not known whether keratinocytes express GPR41 or if BHB has a direct receptor mediated effect on keratinocytes. Further studies are required to confirm the protective effect of the ketogenic diet and to explore the underlying mechanisms of action.

4.5 Conclusion

The mechanism of action of oral DMF treatment in psoriasis has yet to be fully elucidated, despite being licenced for the treatment of RRMS and psoriasis, which are both immune-mediated inflammatory diseases sharing common cellular pathways and cytokine patterns. Together with the data presented here, it has been shown that for the treatment of murine models of both diseases with oral DMF, the expression of the HCA2 receptor is essential [55]. Regarding underlying pathophysiological mechanisms there seems to be a difference between RRMS and psoriasis. The analysis of mRNA expression and cytokine levels suggests that DMF treatment primarily targets keratinocytes rather than immune cells, however, further studies are required to confirm this notion. Interestingly, BHB, an endogenous ligand of HCA2 is able to reduce skin inflammation independently of this receptor. How BHB modulates other pathways to ameliorate skin inflammation need to be elucidated.

Chapter 5: Keratinocytes are a likely target of oral DMF treatment of psoriasis

5.1 Abstract

Hyperproliferating keratinocytes are a hallmark feature of psoriasis. Previous data presented in this thesis showed that oral DMF ameliorates murine psoriasis-like skin lesions via the HCA2 receptor, for which the DMF metabolite MMF is an agonist. DMF treatment also led to a reduction in mRNA expression of keratinocyte-derived genes in a HCA2-dependent manner. This led to the hypothesis that MMF directly acts on keratinocytes during psoriasis-like skin inflammation. This work investigated the effect of MMF on keratinocytes that expressed HCA2 or that were deficient in this receptor. This analysis showed that treatment with MMF induced HCA2 specific signalling and led to a reduction in keratinocyte proliferation *in vitro*. Primary human epidermal keratinocytes (NHEKs) also showed reduced proliferation *in vitro* in response to MMF. Finally, bone marrow chimeras were constructed in order to restrict HCA2 expression to haematopoietic or non-haematopoietic cells allowing the investigation of which HCA2 expressing cell type mediates the protective effect of oral DMF treatment in murine psoriasis-like skin inflammation. With the identification of non-haematopoietic cells as mediators of the effect of oral DMF a potential mechanism of action of DMF was identified. In conclusion, the administration of oral DMF probably has a direct, inhibitory effect on keratinocyte proliferation.

5.2 Introduction

Psoriasis is an inflammatory skin disease involving innate and adaptive branches of the immune system. Psoriatic lesions show hyperproliferation and impaired differentiation of keratinocytes [86, 123]. Furthermore, lesions are infiltrated by a variety of immune cells. pDCs are thought to initiate the inflammatory reaction that leads to lesions in human psoriasis as well as in the IMQ mouse model [125, 126, 201]. pDCs produce type I interferons, which activate myeloid DCs to produce IL-23, a cytokine that expands and maintains Th17-type responses [126, 133, 134, 141, 162]. Th17-type responses are defined by the production of the cytokines IL-17A and IL-22 (amongst others) that further activate keratinocytes which in turn produce antimicrobial peptides (AMPs), chemokines and cytokines forming an inflammatory loop that leads to chronic inflammation [143, 145].

Improvement in Aldara[®]-induced psoriasis-like skin inflammation by oral DMF treatment was shown in the previous chapter to be dependent upon HCA2 for DMF's protective effect *in vivo*. In patients with psoriasis, long term DMF treatment is linked to loss of

peripheral blood T cells, although improvements in skin inflammation are usually seen within the first 3 months of treatment, prior to significant lymphocyte reduction [222]. In chapter 3 of this thesis it was noted that significant improvements as measured by PASI occurred after approximately 8 weeks of oral DMF treatment in the context of reductions in peripheral blood neutrophil and DC numbers, rather than significant changes in T cells. In a murine model of RRMS it has been shown that the reduction in neutrophil infiltration into the CNS mediated by HCA2 was linked with protection from neural inflammation by oral DMF [55]. Given that neutrophils and DCs express HCA2, it is possible that the requirement for HCA2 for the *in vivo* protective effect of oral DMF in the IMQ model is due to receptor expression by these cell types. However, further research required to ascertain which HCA2-expressing cells are the target of oral DMF treatment in psoriasis.

In psoriatic lesions, keratinocytes show hyperproliferation, which leads to a thickening of the epidermis [121]. In healthy skin, keratinocyte proliferation is found only in cells within the basal epidermis layer. Towards the outer layers of the epidermis keratinocytes differentiate over the course of approximately 4 weeks [120]. In psoriatic skin, this process is reduced to 5-8 days and is disturbed, leading to a thickening of the stratum corneum with incompletely differentiated keratinocytes (hyperparakeratosis). This results in the typical scaling phenotype of psoriasis [86, 121]. Results described in chapter 4 of this thesis indicated that DMF treatment improves inflammation in the IMQ model without significant effects on leukocyte infiltration into the skin or major changes in haematopoietic cell-derived cytokines such as TNF- α , IL-17A, IL-22 and IL-23, while keratinocyte-associated molecules such as S100A7A, S100A8 and KRT14 were downregulated in a HCA2-specific manner. These data implicate HCA2 expressing keratinocytes as the targets of oral DMF. Published studies have shown that *in vitro* DMF and MMF treatment leads to reduced keratinocyte proliferation. DMF was shown to decrease the proliferation rate of HaCaT cells and MMF reduced human primary keratinocytes proliferation [280, 281]. It should be noted that these experiments were performed with concentrations of both, MMF and DMF, using orders of magnitude above that seen *in vivo* after oral DMF administration. Thio *et al.* showed that lower concentrations of DMF led to a reduction in keratinocyte proliferation [257], however, as DMF is not detectable in the blood after oral intake keratinocytes are unlikely to directly encounter it *in vivo* [177, 178]. Until today it has not been investigated if HCA2 mediates the effects described.

Given the results in the previous chapter, it was hypothesised that oral DMF treatment directly effects on keratinocytes leading to reduced skin inflammation rather than on infiltrating immune cells. It was speculated that oral DMF treatment was leading to a HCA2-mediated reduction in keratinocyte proliferation.

5.3 Results

5.3.1 HCA2 is highly expressed in Aldara® induced psoriasis-like skin lesions

To further verify the expression of HCA2 in mouse skin during inflammation, RNA *in situ* hybridization (ISH) assays were performed. This allowed the visualization of specific RNA molecules on a per cell basis in slide-mounted samples. Using this method HCA2 mRNA expression was visualised in skin of HCA2^{+/+} mice without and with topical Aldara® treatment. HCA2^{-/-} mice were used as negative control.

It was observed that in healthy HCA2^{+/+} mice the epidermis was approximately 10-15 µm thick and displayed low mRNA expression of HCA2 (**Figure 26A**). Compared to HCA2^{-/-} skin, HCA2^{+/+} skin showed low but detectable HCA2 mRNA expression. In the skin of HCA2^{+/+} mice treated with topical Aldara® cream the expression of HCA2 mRNA was markedly increased. Epidermal HCA2 expression was highest in the basal layer and diminished towards the stratum corneum. To verify that HCA2 was expressed in the healthy epidermis despite the low mRNA expression levels detected by *in situ* hybridization, the activity of the HCA2 promoter was evaluated in HCA2-RFP mice (which express RFP under the control of the HCA2 promoter by immunofluorescence (IF) microscopy [17]). The skin of HCA2-RFP mice was stained with an anti-RFP antibody and imaged. This analysis showed that in the epidermis there was detectable activity of the HCA2 promoter, indicating HCA2 expression in uninfamed skin (**Figure 26B**).

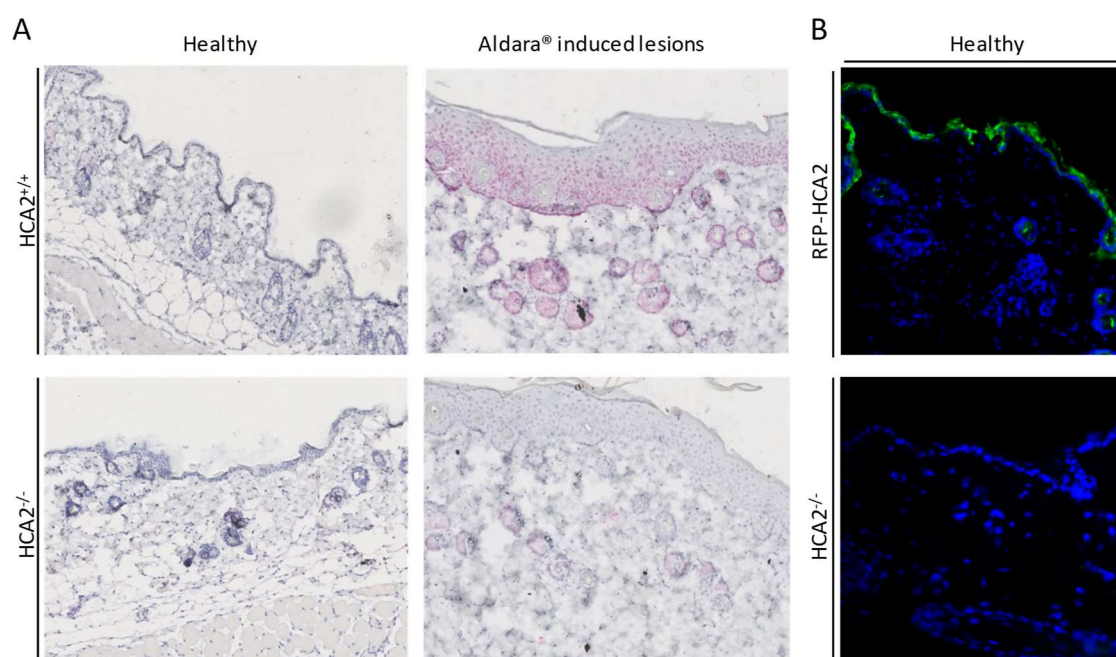


Figure 26: HCA2 is expressed in the epidermis of mouse skin.

(A) Healthy and Aldara[®] treated skin of HCA2^{+/+} and HCA2^{-/-} mice was histologically prepared and analysed for HCA2 mRNA expression by in situ hybridization. Pink dots show single mRNA molecules in the epidermis. One representative slide out of 4 is shown. (B) Immunofluorescence staining for RFP in HCA2-RFP and HCA2^{-/-} mice. Cellular RFP expression was visualised using a FITC coupled anti-RFP antibody (green). DAPI was used as a counterstain (blue). One representative experiment out of 3 performed shown.

5.3.2 MMF treatment induces HCA2 specific signalling in *in vitro* cultured murine keratinocytes

It is known that ligand binding to G-protein coupled receptors (GPCRs) results in the induction of specific signalling cascades. HCA2 as a known G_i/G_o GPCR inhibits adenylate cyclase (AC) resulting in reduced cellular cAMP levels [5]. To assess HCA2 signalling in keratinocytes, cells were isolated from HCA2 expressing and deficient mice. As a model system to analyse the effects of cell signalling on AC, cells were cultured with 100µM forskolin as an inducer of AC. 100µM of MMF was added simultaneously and samples were analysed for changes in cAMP production. When incubated with MMF, keratinocytes expressing HCA2 showed reduced cAMP levels compared to keratinocytes incubated with forskolin (+ PBS as vehicle control) only, indicating AC inhibition. The same treatment of HCA2^{-/-} keratinocytes indicated that a lack of this receptor reduced but did not totally abolished AC inhibition by MMF (**Figure 27A**).

The phosphorylation of intracellular proteins plays an important role in signal transduction after binding of extracellular ligands to cell surface receptors e.g. GPCRs, leading to intracellular signalling cascade activation and ultimately to induction or inhibition of gene expression. To investigate signalling pathways induced by MMF in keratinocytes, cells were incubated for 5 minutes with the indicated concentrations of

MMF or PBS as a vehicle control. Protein was isolated and samples were analysed for the quantitative phosphorylation of signalling components by WES analysis (ProteinSimple, San Jose, USA).

ERK signalling plays a central role in signalling pathways downstream of GPCRs and is mediated by the phosphorylation of mitogen-activated protein (MAP) kinases [282]. In mouse keratinocytes MMF led to an HCA2 dependent induction of ERK phosphorylation at concentrations between 25 μ M and 100 μ M (**Figure 27B**). MMF treatment of HCA2^{-/-} cells led to a minor reduction in ERK phosphorylation relative to HCA2-expressing keratinocytes. Another signalling pathway potentially activated by HCA2 includes the phosphorylation of p38 MAP kinase. This kinase exhibited phosphorylation in response to treatment of HCA2^{+/+} keratinocytes with MMF in a concentration dependent manner (**Figure 27C**). Conversely, HCA2^{-/-} keratinocytes did not show increased phosphorylation after MMF treatment. C-Jun N-terminal kinases (JNK) phosphorylation was assessed after MMF incubation. MMF did not lead to significant changes in JNK phosphorylation in either HCA2^{+/+} or HCA2^{-/-} keratinocytes at the time point analysed. Phosphorylation of protein kinase B (AKT) occurs downstream of multiple receptor classes, including GPCRs. A high concentration of MMF led to increases in AKT phosphorylation in HCA2^{+/+} keratinocytes only. Lower concentrations of MMF showed a high variation in phosphorylation activity between biological replicates. Taken together, ligand binding of MMF to HCA2 induced cAMP reduction and HCA2-specific induction of ERK and p38 signal pathways. There was no strong evidence of JNK and AKT signalling at concentrations below 100 μ M, suggesting that MMF did not activate receptors associated with these signalling molecules at physiological concentrations in keratinocytes.

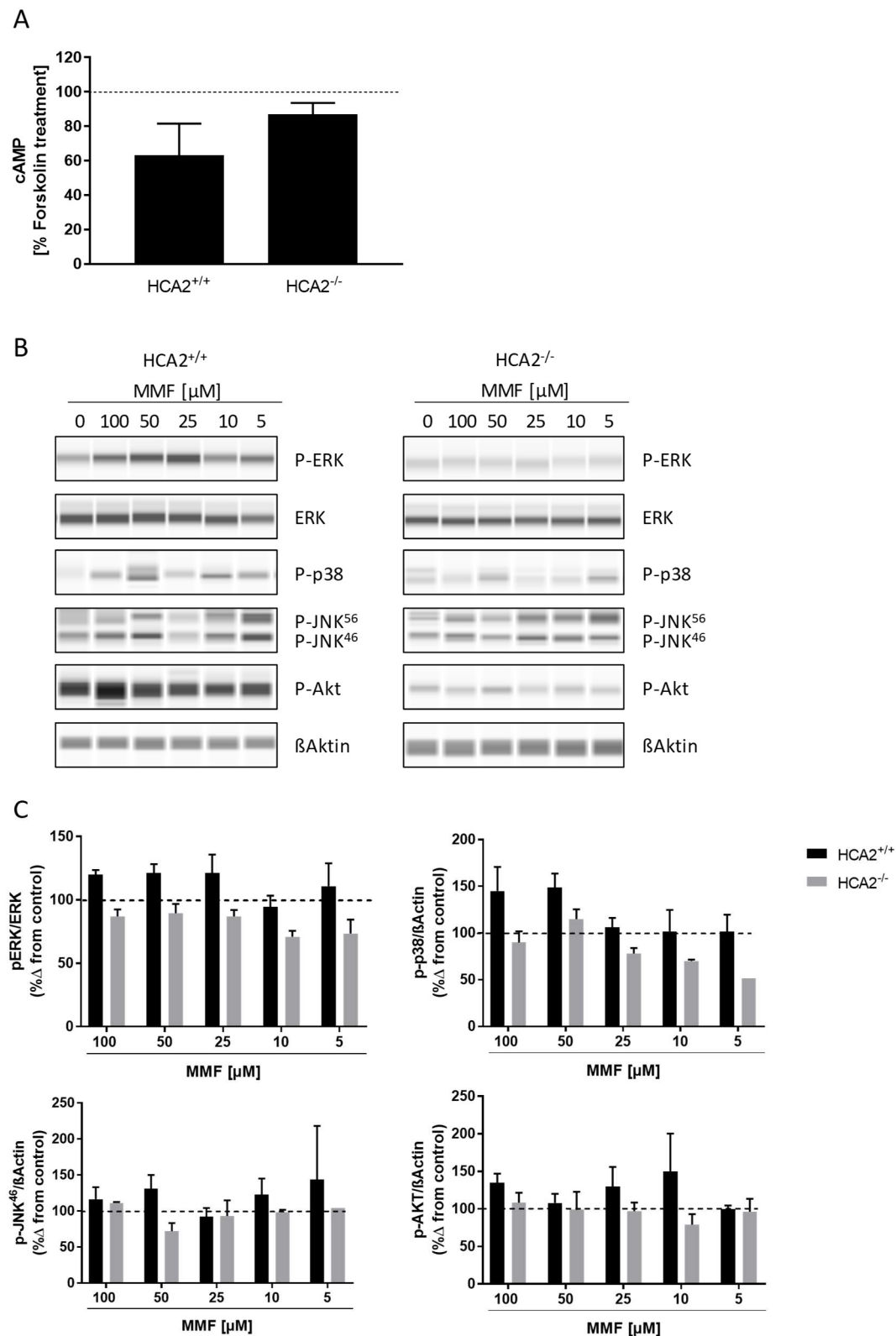


Figure 27: MMF induces signalling in mouse keratinocytes.

Mouse keratinocytes were isolated from new born mice. (A) Keratinocytes were incubated with 100μM MMF or PBS as a solvent control and 100μM forskolin simultaneously for 8 minutes. cAMP levels were analysed by ELISA according to the manufacturer's instructions (n=4). No significance detected (t-test). (B&C) Whole cell extracts were prepared from MMF or solvent (PBS) control incubated HCA2^{+/+} (black) and HCA2^{-/-} (grey) keratinocytes and were subjected to WES analysis for the phosphorylation of ERK, p38, JNK and Akt. ERK and β-Actin served as controls (B) representative Wes Blots of indicated proteins (C) quantitative evaluation of Wes results. No significance detected as analysed by one-way ANOVA followed by Tukey's post hoc test (n=3).

5.3.3 Cell proliferation of *in vitro* cultured murine keratinocytes is reduced by MMF in an HCA2 dependent manner

Gene expression analysis in murine Aldara® treated skin showed that oral DMF reduced expression of the proliferation marker *Ki67*. To verify potential direct effects of MMF treatment on keratinocyte proliferation, murine keratinocytes were incubated with MMF and bromodeoxyuridine (BrdU) and analysed for BrdU incorporation into the DNA via ELISA. MMF caused a detectable decrease in BrdU incorporation in a dose dependent manner in HCA2-expressing keratinocytes (**Figure 28A**). This indicated that keratinocytes exposed to MMF proliferate less than vehicle (PBS) control cells. With decreasing MMF concentrations this effect was abrogated. A MMF concentration of 10µM led to a slight increase in BrdU incorporation. Conversely, MMF treatment of HCA2^{-/-} keratinocytes did not lead to reduced cell proliferation (**Figure 28B**). In contrast, high concentrations of MMF led to a slight increase in cell proliferation. This induction decreased with lowering concentrations of MMF. MMF treatment of keratinocytes did not induce apoptosis as determined by cleavage of caspase 3 (data not shown). Taken together, these data suggest that MMF has a HCA2 dependent inhibitory effect on keratinocyte proliferation *in vitro*.

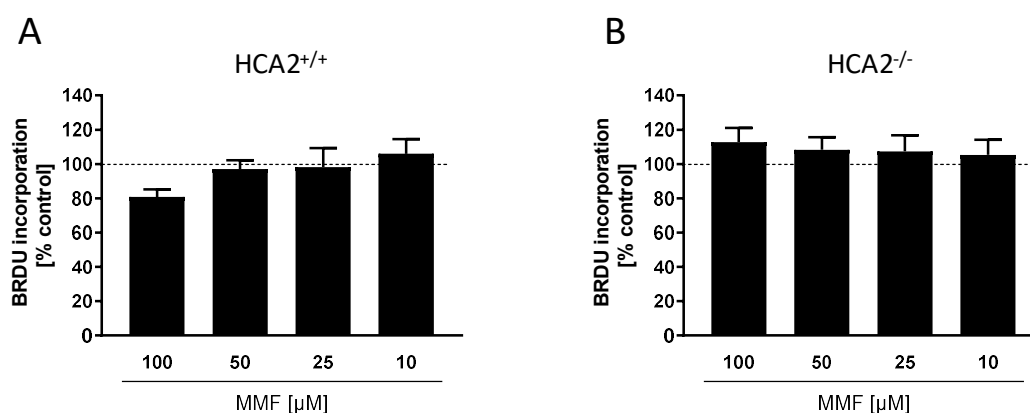


Figure 28: MMF treatment reduces cell proliferation of *in vitro* cultured murine keratinocytes in a HCA2 dependent manner.

Keratinocytes were isolated from new born mice and cultured *in vitro*. 5×10^3 cells per well were treated with the indicated concentration of MMF or PBS as a control. (A) HCA2^{+/+} keratinocytes and (B) HCA2^{-/-} keratinocytes were cultured with MMF for 48h. BrdU was added for the final 2 hours of incubation time. Thereafter, BrdU incorporation was determined according to manufacturer's instructions. No significances were detected by one-way ANOVA (Tukey's test). (n=5).

5.3.4 MMF reduces the growth of primary human keratinocytes

Previous experiments in this study showed that oral DMF treatment in the IMQ-mouse-model as well as MMF treatment of *in vitro* cultured keratinocytes led to a HCA2-dependent reduction in epidermal thickness and cell proliferation, respectively. To investigate whether MMF has similar effects on human keratinocytes, NHEKs were cultured in the presence of MMF or PBS as a solvent control. Pictures were taken after 24, 48 and 72 hours of culture. As seen in **Figure 29**, there were no significant differences in cell adhesion or signs of cell death after 24h of culture in MMF containing medium. After 48h there were fewer cells in wells with MMF compared to PBS control cultured cells. The effect was dose dependent and disappeared after 72h of cell growth, likely due to depletion of MMF in the culture medium.

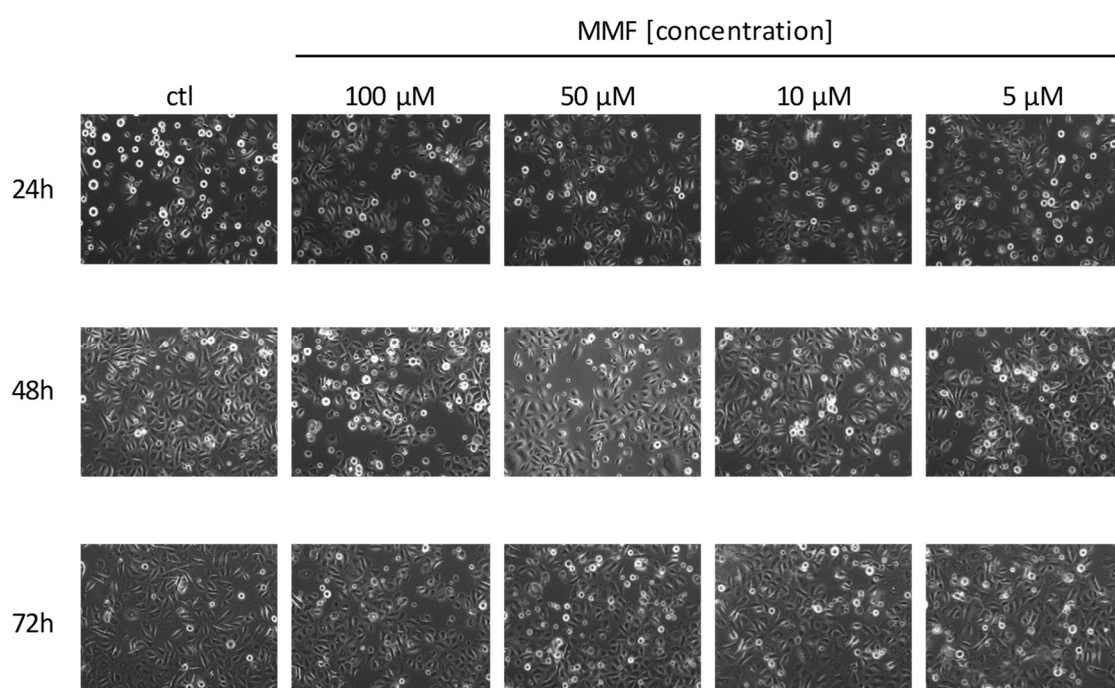


Figure 29: MMF inhibits cell growth of *in vitro* cultured human primary keratinocytes.

NHEKS were cultured in 6-well plates in the presence of the indicated concentrations of MMF or solvent control. Pictures were taken 24h, 48h and 72h after seeding of cells. Representative of 2 experiments.

To verify an inhibition of primary human keratinocyte proliferation by MMF, a BrdU incorporation assay was carried out. It was observed that treatment of *in vitro* cultured NHEKS with 50 μM or 100 μM MMF caused a reduction in BrdU incorporation of approximately 20% compared to cells treated solvent control (PBS) only (**Figure 30**). Thus, MMF is able to reduction human keratinocytes proliferation *in vitro*.

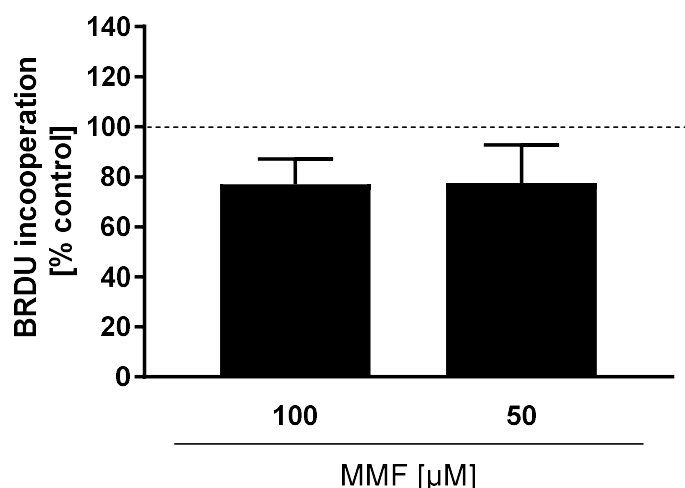


Figure 30: Effect of MMF treatment on human keratinocyte proliferation in vitro.

NHEKS were cultured until 50% confluent and then treated with indicated concentrations of MMF or PBS as solvent control for 48h. BrdU was added for the last 2h of incubation time and cells were then analysed for BrdU incorporation by ELISA according to the manufacturer's protocol. No significance detected by one-way ANOVA following Tukey's test (n=3).

5.3.5 Generation of bone marrow chimeras in order to investigate the target cells type required for the *in vivo* effects of oral DMF treatment

In chapter 4 it was shown that the protective effect of oral DMF in Aldara® treated mice was dependent upon the HCA2 receptor. Further analysis of the cellular composition of the inflamed skin along with mRNA expression suggested that non-immune cells were the primary target of oral DMF treatment. The proliferation of mouse and human keratinocytes was found to be inhibited by MMF *in vitro*. However, definitive evidence that HCA2-expressing keratinocytes are the target cell of DMF treatment *in vivo* was still lacking, partly because HCA2 is expressed by a number of different cell types. To investigate which cell type was responsible for DMF's protective effect, bone marrow (BM) chimeras were generated using congenically marked HCA2^{+/+} (CD45.1) and HCA2^{-/-} (CD45.2) mice in order to restrict HCA2 expression to either haematopoietic or non-haematopoietic cells. HCA2^{+/+} and HCA2^{-/-} mice were irradiated with a single dose of γ -radiation to deplete functional BM cells (**Figure 31A**). BM was then transferred from donor mice of both genotypes into the irradiated recipient animals. This generated four different chimera types: HCA2^{+/+}>HCA2^{+/+}, HCA2^{+/+}>HCA2^{-/-}, HCA2^{-/-}>HCA2^{+/+} and HCA2^{-/-}>HCA2^{-/-}. After transfer, the donor BM was allowed to reconstitute the recipient mice for 8 weeks. Because congenic mice were used, reconstitution efficacy could be assessed by analysis of CD45 marker expression (CD45.1 and CD45.2) in the peripheral blood. Relative abundance of the donor cells was consistently higher than 90% after reconstitution (**Figure 31C**). Chimeras were then treated with DMF or vehicle control for 12 days with Aldara® applied for the last 5 days of the oral treatment (**Figure 31B**). At

the end of the experiment mice were sacrificed and epidermal thickness and gene expression analysed.

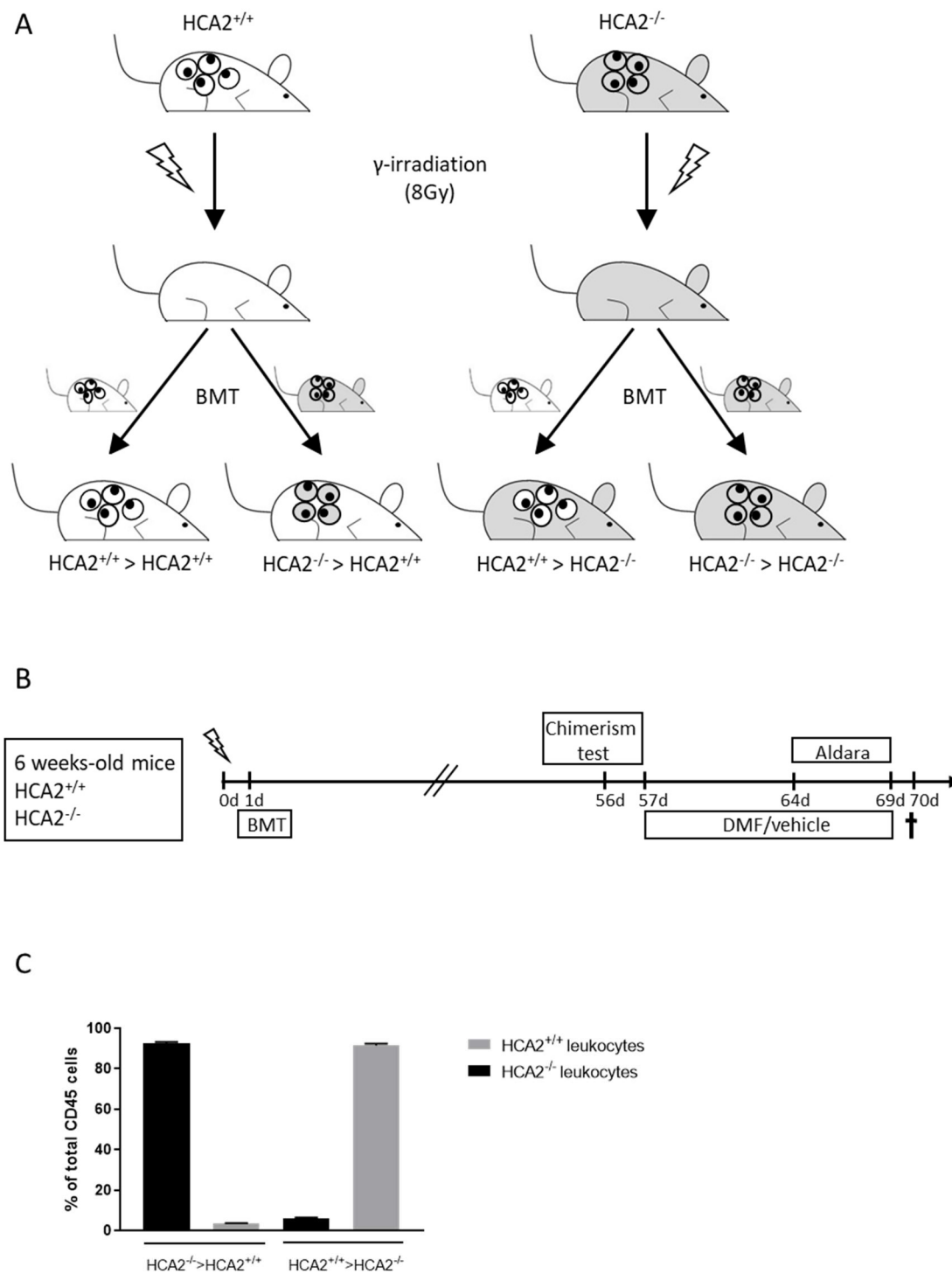


Figure 31: Generation of chimeric mice.

(A) After depletion of recipient bone marrow, donor bone marrow was injected intravenously. (BMT= bone marrow transfer) Reconstitution time of 8 weeks gave rise to 4 different chimera types. (B) Chimeras were treated with DMF/vehicle and then exposed to Aldara® according to the shown schematic. (C) Reconstitution efficiency was measured by flow cytometry for CD45.1 (grey) and CD45.2 (black) expression by blood leukocytes.

At the end of the experiment mice were sacrificed, skin samples were taken and prepared histologically (**Figure 32A**). Epidermal thickness was measured in H&E stained sections (**Figure 32B**). As expected DMF treatment showed a significant reduction of epidermal thickness in HCA2^{+/+} mice that had received HCA2^{+/+} BM and there was no reduction by oral DMF in HCA2^{-/-} mice with HCA2^{-/-} BM. Transplantation of HCA2^{-/-} BM into HCA2^{+/+} mice did not abolish the protective effect of oral DMF on Aldara[®] induced skin inflammation as this chimera type showed diminished epithelial hyperplasia after DMF treatment compared to vehicle control chimeras. Transfer of HCA2^{+/+} BM into HCA2^{-/-} mice significantly abrogated the protective effect of DMF treatment as these animals displayed no reduction in epidermal thickness after oral DMF. Previous irradiation of these mice did not markedly increase weight loss after Aldara[®] application (**Figure 32C**). Taken together, these results clearly show that the protective effect of DMF is based upon HCA2 expression by radio-resistant cells rather than bone marrow derived cells.

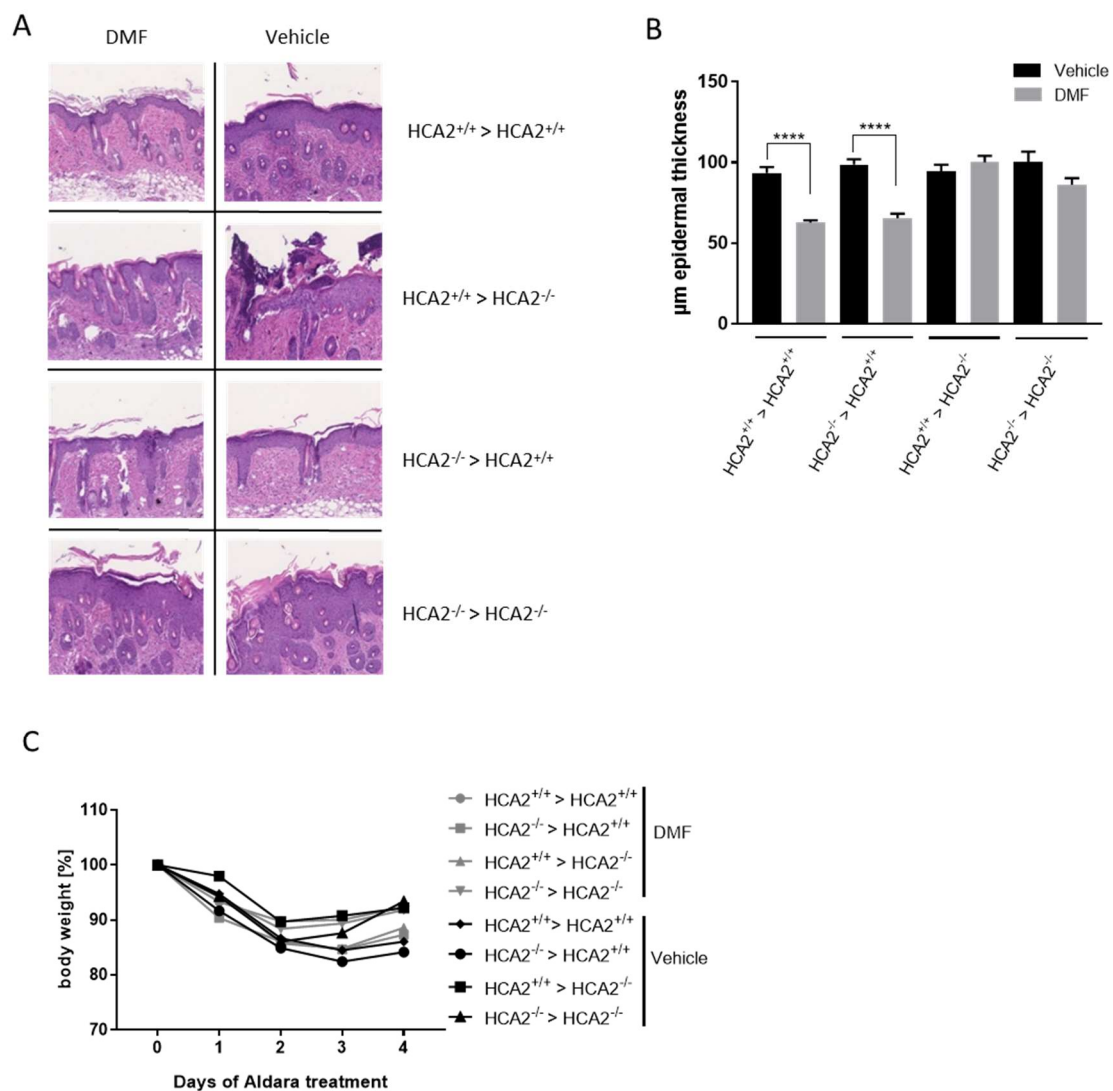


Figure 32: HCA2 expression by radio-resistant cells is responsible for the protective effect of DMF. Bone marrow chimeras were pre-treated with DMF or vehicle control and Aldara® was applied for 5 days before mice were analysed. (A) Biopsies of Aldara® treated back skin were prepared histologically and sections were H&E stained. (B) Thickness of the epidermis was measured in H&E stained sections. (C) Bodyweight of chimeras undergoing Aldara® application. One representative out of 2 experiments shown. Statistics by one-way ANOVA followed by Tukey's test. ****p<0,0001

5.3.6 Oral DMF treatment affects mRNA expression of keratinocyte associated genes in bone marrow chimeras

To investigate if the reduction in skin thickness was accompanied by changes in gene expression, mRNA from Aldara® treated back skin was isolated, reverse transcribed and analysed by qPCR. As the evaluation of epidermal thickness showed that HCA2 expression by radio-resistant cells was essential for the protective effect of DMF, mRNA of genes expressed by keratinocytes (*Ki67*, *Il1b* and *S100a7a*) and LCs (*Il23a*) was focused on (Figure 33).

Ki-67, a marker for cell proliferation, was highly affected by DMF treatment. In HCA2^{+/+}>HCA2^{+/+} chimeras, *Ki67* expression was downregulated in the DMF treated group compared to vehicle mice. *Ki67* was also downregulated but to a lower extent in HCA2^{-/-}>HCA2^{+/+} animals. This reflected the reduction in epidermal thickness observed in both of these chimeras. In HCA2^{+/+}>HCA2^{-/-} and HCA2^{-/-}>HCA2^{-/-} mice DMF treatment did not reduce *Ki67* expression. Despite showing an overall low level of expression in HCA2^{+/+}>HCA2^{-/-} mice, *Ki67* was increased by DMF treatment in these chimeras. *Il1b* showed a similar expression pattern as *Ki67*. *Il1b* was downregulated by oral DMF in HCA2^{+/+}>HCA2^{+/+} and HCA2^{-/-}>HCA2^{+/+} chimeric mice and showed overall reduced expression in HCA2^{+/+}>HCA2^{-/-} and HCA2^{-/-}>HCA2^{-/-} chimeras, while it was increased by DMF treatment in HCA2^{+/+}>HCA2^{-/-} mice. *Il23a* is mainly produced by DCs/LCs in the skin during Aldara[®] induced inflammation and was expressed at very low levels in all groups. DMF treatment led to a non-significant reduction of *Il23a* mRNA expression in HCA2^{+/+}>HCA2^{+/+}, HCA2^{-/-}>HCA2^{+/+} and HCA2^{+/+}>HCA2^{-/-} chimeras but not in HCA2^{-/-}>HCA2^{-/-} mice. *S100A7A* is an AMP produced by keratinocytes upon activation in psoriatic lesions. Compared to methocel treated control mice oral DMF treatment led to a decrease in skin expression of *S100a7a* mRNA in HCA2^{+/+}>HCA2^{+/+}, HCA2^{-/-}>HCA2^{+/+} and HCA2^{+/+}>HCA2^{-/-} chimeras but not in HCA2^{-/-}>HCA2^{-/-} mice. Conversely, HCA2^{-/-}>HCA2^{-/-} mice showed a slight upregulation in *S100a7a* mRNA expression (Figure 33).

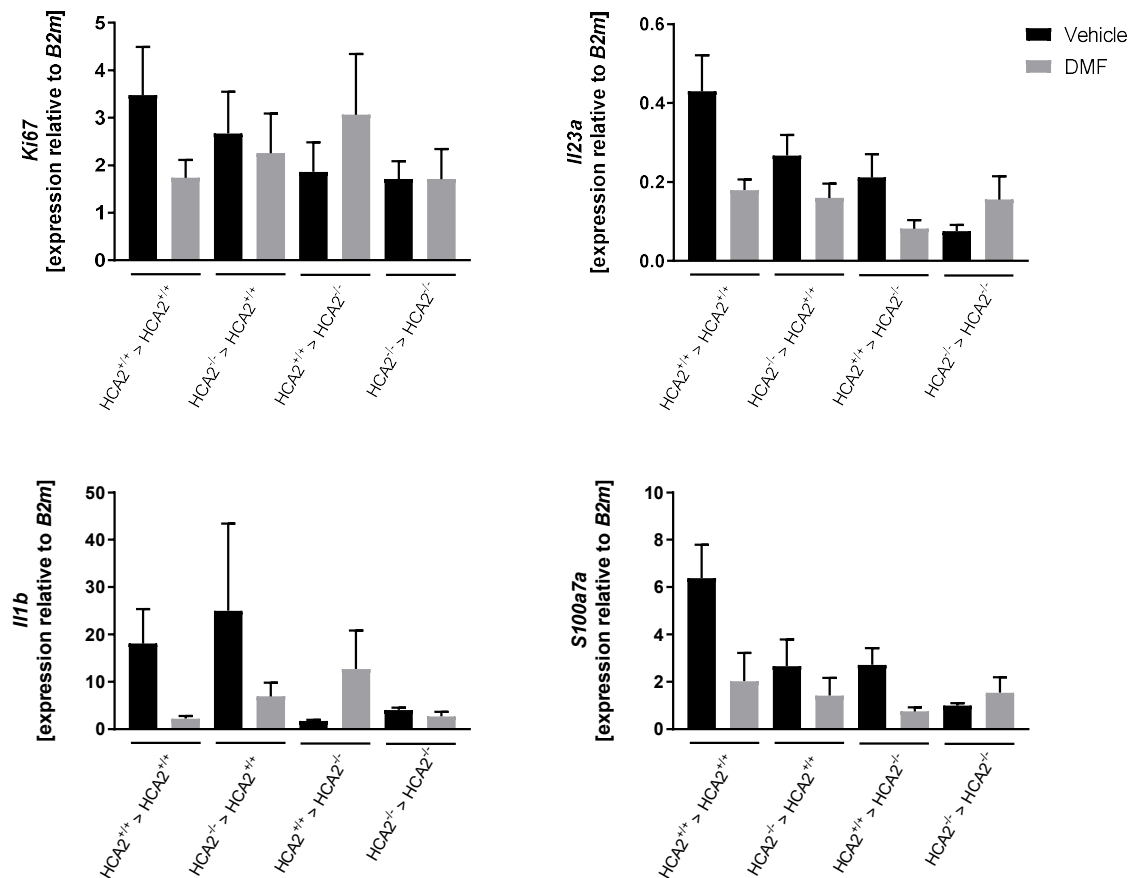


Figure 33: mRNA expression in the skin of bone marrow chimeras undergoing DMF treatment.

Back skin biopsies were taken from Aldara®- treated HCA2^{+/+}>HCA2^{+/+}, HCA2^{+/+}>HCA2^{-/-}, HCA2^{-/-}>HCA2^{+/+} and HCA2^{-/-}>HCA2^{-/-} chimeric mice treated with DMF (grey) or vehicle (black) for mRNA isolation. cDNA from these samples was analysed for *Ki67*, *I11b*, *S100a7a* and *I123a* mRNA expression by qPCR. Bars represent mean mRNA expression relative to *B2m* + SEM of 5 biological replicates. Data are representative of 2 independent experiments. No statistical significance detected by one-way ANOVA.

5.4 Discussion

The work presented here has demonstrated for the first time that oral DMF treatment leads to a reduction in Aldara®-induced epidermal thickness by acting on HCA2-expressing radio-resistant cells. By using bone marrow chimeras generated from HCA2^{+/+} and HCA2^{-/-} mice, it was clearly shown that the expression of HCA2 on radio-resistant cells is essential for the ameliorating effect of oral DMF on psoriasis-like skin inflammation in mice. DMF treatment of these chimeras led to changes in mRNA expression compared to vehicle controls. In HCA2^{+/+}>HCA2^{+/+} and HCA2^{-/-}>HCA2^{+/+} mice mRNA expression of *I11b*, *Ki67*, *S100a7a* and *I123a* was reduced although not significantly. In HCA2^{+/+}>HCA2^{-/-} chimeras *I11b* and *Ki67* were induced while *I123* and *S100a7a* were reduced. In HCA2^{-/-}>HCA2^{-/-} mice *Ki-67* mRNA expression was not changed, *I11b* was marginally reduced and *I123a* and *S100a7a* were both increased slightly. HCA2 expression was shown to be high in the epidermis of healthy and inflamed

mouse skin. In the Aldara[®]-treated skin the highest expression was seen in basal layers with fading expression towards the stratum corneum. HCA2-dependent signalling events were induced in keratinocytes by MMF treatment *in vitro*, with reduction of cAMP accumulation by high dose MMF in HCA2^{+/+} keratinocytes and concentration dependent phosphorylation of ERK and p38. Proliferation of keratinocytes was reduced by 100µM MMF treatment *in vitro* via a HCA2 dependent mechanism. Analysis showed that besides murine keratinocytes human keratinocytes also showed decreased proliferation when treated with MMF *in vitro*.

The mode of action of oral DMF treatment of psoriasis has yet to be fully elucidated. With the work presented here, a possible mode of action of oral DMF in the murine mouse model of psoriasis-like skin inflammation was demonstrated. The expression of HCA2 by radio-resistant cells was found to be essential for DMF's protective effect in Aldara[®] induced lesions. These radio-resistant cells are all cells not derived from the bone marrow *de novo* after reconstitution [283]. In the case of psoriasis-like skin disease these are mostly keratinocytes, fibroblasts, and LCs. Adipocytes from the subcutaneous fat are also radio-resistant. As it was reported in chapter 4 that oral DMF treatment relies on HCA2 to mediate its protective effect, fibroblasts are unlikely to be involved in DMF's mode of action as they are negative for HCA2 [33]. The mice used in the animal model were not obese and showed almost no subcutaneous fatty tissue. Hence, adipocytes, despite known to express HCA2, were not regarded as a major target of DMF [6, 251]. Therefore, the most likely pharmacological target of DMF are HCA2 expressing cell types like LCs and/or keratinocytes. It is known that flush, a common side effect of DMF treatment in patients, is mediated by both LCs and keratinocytes in a HCA2-dependent fashion through MMF [17, 23, 29, 30], showing that these cells both respond to MMF *in vivo*, at least to mediate flushing. Transcript analysis of the back skin from the chimeras showed a reduction in *Ki67* expression in HCA2^{+/+}>HCA2^{+/+} and HCA2^{-/-}>HCA2^{+/+} mice but not in the other genotypes. This observation fits with the reduction in epidermal thickness in both of these chimeras. *In vitro* analysis of MMF-treated murine keratinocytes showed that the proliferation reduction was dependent on HCA2 expression by these cells. *Il1b* was downregulated in HCA2^{+/+}>HCA2^{+/+} and HCA2^{-/-}>HCA2^{+/+} chimeric mice. In the skin IL-1β is a cytokine produced by keratinocytes upon stimulation with IL-22 and IL-17 [210]. Together with the reduction in *S100a7a* expression, which is also of keratinocyte origin [213], this suggests that DMF treatment may act on keratinocytes via HCA2 binding to MMF. However, *Il23* was also downregulated, albeit non-significantly, in HCA2^{+/+}>HCA2^{+/+} and HCA2^{-/-}>HCA2^{+/+} mice but also in HCA2^{+/+}>HCA2^{-/-} animals. IL-23 is formed by dendritic cells and LCs [148]. Thus, in chimeras that showed both, a decrease in *Il23a* and were the recipient animals were HCA2^{+/+}, it is possible that the reduction in *Il23a* represents an effect of DMF

treatment on LCs (which are radio-resistant). However, as the HCA2^{+/+}>HCA2^{-/-} mice also displayed a similar decrease in *I/23a* but did not show a reduction in epidermal thickness, a decrease in *I/23a* mRNA expression alone appears to be insufficient to lead to significant improvement of skin inflammation. Future experiments will use the Cre/lox system to restrict HCA2 expression to keratinocytes or LCs in order to determine the relative contributions of these cell types to the protective effect of oral DMF. Taken together with the data from chapter 4 showing no change in LC trafficking to the SLN during DMF treatment, these results suggest that oral DMF may act directly on keratinocytes to improve skin inflammation in the IMQ model.

By using an IF approach, the protein expression of RFP was shown to be high in the epidermis in HCA2-RFP mice, indicating high expression of HCA2 in this tissue. There was no expression of HCA2 detected in the dermis. Healthy epidermis consists mainly of keratinocytes with a dispersed population of LCs. In the healthy epidermis of mice other skin residing immune cells are low in abundance and are most likely not the source of the overall RFP expression. The epidermis also showed high expression of HCA2 mRNA in Aldara[®]-treated mice by ISH analysis. Although LCs also reside in the epidermis, they migrate to SLN in the IMQ-mouse model [284]. In Aldara[®]-treated mice the number of LCs residing in the skin is reduced compared to untreated controls [206]. Psoriatic lesions and murine psoriasis-like skin inflammation is marked by a massive infiltration of immune cells primarily neutrophils, DCs and T lymphocytes. In chapter 3 T cells were shown to be negative for HCA2 and neutrophils expressed HCA2 on their surface. DCs showed HCA2 expression but only in healthy human blood but appeared deficient in psoriasis patients and mouse lymphoid organs. In the light of these data it is still unclear if infiltrating DCs in the skin express HCA2. Therefore, neutrophils may contribute to the HCA2 expression seen in the ISH staining but based on the data from the chimeric mice neutrophils do not appear to be responsible for mediating the therapeutic effect of HCA2 during DMF treatment. Taken together, the data support the hypothesis that keratinocytes are a primary target of oral DMF treatment and consequently *in vitro* experiments were carried out to further investigate direct effects of MMF on this cell type.

The previous experiments suggested that oral DMF treatment may directly act on murine keratinocytes in the IMQ model. In mouse keratinocytes treatment with MMF led to the induction of HCA2-specific signalling events. In HCA2^{+/+} mice forskolin-induced adenylate cyclase activity was decreased by MMF treatment. Reduction in intracellular cAMP concentrations is a well-known effect of activation of G_i/G_o coupled receptors such as HCA2 [13, 20]. Furthermore, the ERK signalling cascade was activated by MMF in HCA2^{+/+} keratinocytes but not in HCA2^{-/-} keratinocytes. ERK signalling was induced by

concentration of 25 μ M of MMF and higher. In the human body the concentrations of MMF in peripheral blood after oral DMF reach peak concentrations of 10-15 μ M [178]. This indicates that the concentration of MMF required for receptor activation *in vitro* is above the typical concentration in the blood *in vivo*. Data about tissue concentrations of MMF do not exist so far.

As HCA2^{-/-} keratinocytes showed no activation of this signalling cascade there appears to be a specific HCA2-dependent activation of ERK signalling by MMF. ERK signalling is complex. In rapidly proliferating cancer cells increased ERK signalling has been associated with the promotion of high proliferation rates [285]. In the context of the decreased keratinocyte proliferation observed in this study, it is surprising that MMF induced rather than reduced ERK signalling in cultured keratinocytes. However, it has been discussed in neuronal cells that there may be opposing outcomes of ERK signalling as it can have pro- and anti-apoptotic effects [286]. In microglia cells it was shown that astragaloside IV reduced LPS-induced inflammatory factors by induction of ERK [287]. This shows that ERK signalling can be involved in various pathways leading to different downstream effects. In contrast, in psoriasis the induction of the ERK pathway is a factor for keratinocyte hyperproliferation and reduction of ERK activation leads to an improvement of psoriatic lesions [288-290]. The role of the ERK pathway in keratinocytes may show changes due to inflammation. To assess this, the phosphorylation status of ERK under DMF therapy could be analysed by Western blotting or an IHC approach both healthy and in inflamed murine skin. This approach would allow the investigation of ERK signalling induced by MMF *in vivo* to be assessed in both normal and inflammatory conditions. p38 was also induced by MMF treatment of *in vitro* cultured murine keratinocytes in an HCA2 dependent manner. The p38 MAP-kinase is known to regulate cell differentiation and is involved in cell cycle regulation [291]. p38 is usually induced in psoriasis and inhibition has been shown to improve psoriatic lesions [292, 293]. Thus, p38 phosphorylation, especially by the high, non-physiological concentration of MMF used in the analysis here, may be an *in vitro* artefact. It should also be taken into account that ERK, p38, JNK and AKT are highly interconnected. JNK signalling has been suggested to be a key molecule in the switch between apoptotic or proliferative cell signalling. To this end, JNK activity is influenced by complex feedback signalling initiated by p38, ERK and AKT [294]. As sustained JNK activation is associated with the induction of apoptosis signalling, the data presented here, showing a lack of apoptosis induction in both neutrophils (chapter 3) and keratinocytes by MMF, fits with the lack of significant JNK signalling detected after *in vitro* culture. In fibroblasts p38 and ERK signalling acts synergistically, as they downregulate phosphorylation of fibroblast growth factor (FGF) receptor 1 (FGFR1) in a feedback loop while both having their own downstream targets [295]. Whether the data on p38 and ERK activation shown in this chapter lead to the

activation of separate downstream pathways or if there is a crosstalk between these signalling molecules, needs further evaluation. Targeting mitogen-activated protein kinases (MAPK) signalling has been evaluated in the treatment of some cancers and may also be a considerable target for the development of new, possibly topical drugs against plaque psoriasis [296]. Taken together, these results show that there is an induction of intracellular signalling pathways by MMF in an HCA2-dependent manner but whether these results contribute to the mechanism of action of DMF treatment *in vivo* needs to be elucidated.

This study also showed that there was a trend towards a HCA2-dependent reduction in cell proliferation in murine keratinocytes. 100µM MMF reduced cell proliferation by 20% in HCA2-expressing keratinocytes only. The mRNA results from the DMF vs. vehicle treated mouse skin in addition to the mRNA results from the chimeric mice showed reduced expression of the proliferation marker Ki-67. Thus, an inhibitory effect on keratinocyte proliferation may represent a key mode of action for the decrease of skin inflammation by MMF/DMF. If the HCA2-dependent signalling events discussed above mediate this reduction of proliferation needs to be investigated, e.g. by the use of specific ERK or p38 phosphorylation inhibitors.

Human primary keratinocytes were cultured in the presence of MMF to see if the results obtained with murine keratinocytes could be confirmed in human cells. Visual inspection of these cultures revealed that high doses of MMF led to a reduction in cell proliferation as there were fewer cells in these cultures after 48h of incubation. This effect disappeared after 72h, likely due to the depletion of MMF in the starting culture. These observations were reflected by BrdU proliferation assay results. In NHEKS *in vitro* MMF treatment led to decreased proliferation as evidenced by a 20% reduction of BrdU incorporation at concentrations of 50µM and 100µM, substantiating an anti-proliferative effect of MMF on human primary keratinocytes *in vitro*. Whether this effect occurs in the lesions of psoriasis patients leading to the improvement of skin inflammation seen after DMF treatment cannot be concluded from these experiments. Taken together, these results show that MMF has inhibitory effects at concentrations at or below 100µM and that these effects are HCA2 dependent. Previously published reports have shown anti-proliferative effects of DMF or MMF at only very high concentrations, sometimes in the millimole range [257, 280, 281]. These results are of doubtful relevance as MMF is detected in the blood at concentrations between 10-15µM while DMF is not detectable and is extremely unlikely to encounter keratinocytes *in vivo* [177, 178].

The reasonably high doses of MMF used in these experiments may have suggested a toxic or non-receptor related effect but as the anti-proliferative phenomenon was abrogated in HCA2^{-/-} keratinocytes, it appears to be receptor-mediated. Gautam *et al.*

showed that HCA2 signals through different pathways dependent on the concentration of the agonist. High concentrations of the agonist 4-hydroxynonenal (4-HNE) led to apoptosis via Ca^{2+} /JNK signalling while lower concentrations reduce the expression of inflammatory cytokines via the reduction of cAMP signalling [297]. This suggests that while higher doses of MMF can lead to receptor specific signalling, the outcomes may differ from treatment with low MMF concentrations. MMF reaches a maximum of 15 μ M in peripheral blood of patients after ingestion of the standard clinical dose of oral DMF [178, 182]. Patients are advised to take oral DMF three times per day, providing a more continuous and long-term exposure of the different tissue compartments to MMF. Similarly, all mice used in the experiments presented here were treated twice daily for 12 days with oral DMF. Thus, it is possible that the effect of high concentrations of MMF on proliferation observed *in vitro* is recapitulated *in vivo* over a longer period of time. It can be argued that there may be soluble factors in the keratinocyte growth medium that react with MMF. Free MMF concentrations *in vitro* may be lower than expected leading to diminished activity. This suggests that concentrations of MMF used in these *in vitro* experiments are reasonable, but the effects seen (p38 and ERK signalling, reduction of proliferation) still need to be validated *in vivo*.

Keratinocytes form the outer barrier between body and surrounding air. *In vitro* cultured keratinocytes are often grown in liquid and do not have an air interface. Furthermore, cultured keratinocytes are very sensitive to changes in medium. For example, they undergo terminal differentiation in response to changes in Ca^{2+} concentration [298]. Thus, the *in vitro* culture conditions do not reflect the state *in vivo* for keratinocytes. Psoriasis involves the interaction between cells of the innate and adaptive immune system along with keratinocytes and other tissue cells in the dermis and epidermis [86]. These conditions cannot be easily mimicked *in vitro*. Results obtained from cell culture experiments have to be discussed with caution. Therefore, it is important to confirm a role for MMF-HCA2 signalling in the inhibition of keratinocyte proliferation *in vivo*.

The results discussed above give an indication of a hypothetical mode of action of oral DMF treatment in psoriasis and murine psoriasis-like skin lesions. If keratinocyte specific changes (in proliferation and/or cytokine, chemokine and AMP production) in response to DMF treatment could be identified in the IMQ model, the next step would be investigating if similar processes occur in DMF treated psoriasis patients. From a clinical perspective it is known that long term DMF treatment has multiple effects on the immune system, however these often occur long after noticeable improvement in the severity of skin inflammation. Therefore, it is possible that oral DMF improves the lesions of patient via direct effects on keratinocytes initially while the effects on immune cells (e.g. changes in HCA2⁺ neutrophils and depletion of T cells) occur later and contribute to the ongoing efficacy of DMF treatment in patients.

5.5 Conclusion

This work has shown that in a murine mouse model of psoriasis-like skin inflammation the oral treatment with DMF leads to reduced epithelial hyperplasia only when the receptor is expressed by radio-resistant cells. Bone marrow-derived immune cells expressing HCA2 do not mediate the protective effect of DMF on psoriasis-like lesions in mice. This result rules out HCA2 expressing cells such as neutrophils and DCs as major immune cell targets of DMF therapy. *In vitro* data showed that keratinocytes reduce proliferation when cultured with MMF and that HCA2 dependent signalling via ERK and p38 is induced by MMF in a concentration dependent manner. Human keratinocytes also demonstrated reduced proliferation when treated with MMF. Taken together, these results suggest that oral DMF therapy directly acts on keratinocytes via MMF-HCA2 signalling to reduce their proliferation and possibly interrupting the inflammatory cycle occurring in the skin during psoriasis.

Chapter 6. General discussion

HCA2 has been the focus of research for several years. This effort has led to the discovery of numerous HCA2 ligands. Some are endogenously produced in the human body such as butyrate and BHB. NA is another agonist and represents an essential nutrient in the human diet and at high concentrations a drug used to treat lipid disorders and associated diseases. Another ligand, MMF generated after the hydrolysis of the prodrug DMF in the body, has also been found to bind to HCA2. The work presented here has demonstrated for the first time that the MMF-HCA2 axis is essential for the improvement of skin inflammation.

6.1 DMF's pharmacology has to be considered when interpreting *in vitro* and *in vivo* experiments

Although intensive research has been carried out in order to elucidate oral DMF's mode of action, many questions remain as to how DMF treatment improves skin inflammation in psoriasis. A significant number of published papers have generated their conclusions from *in vitro* experiments using DMF alone. MMF is often not tested and the expression of HCA2 by target cell types is not considered [182]. Due to DMF's uncharged nature along with the presence of a reactive double-bond this molecule can readily diffuse through plasma membranes and bind to nucleophilic SH-groups in a wide range of intracellular proteins. MMF, owing to its overall negative charge, cannot easily diffuse through plasma membranes meaning that only cells that express HCA2 (until other MMF-binding receptors or channels are discovered) are able to respond to this molecule [182]. In illustration of the differences between DMF and MMF, Blewett *et al.* showed that in human T cells (which are HCA2-negative) DMF crossed the plasma membrane and modified many intracellular proteins via their cysteine residues, which led to reduced activation after exposure to anti-CD3/CD28 antibodies. Conversely, MMF could not modify intracellular proteins and did not reduce T cell activation [299]. Given that DMF cannot be detected in the peripheral circulation [178, 181, 186], with only MMF and DMF-GSH adducts found immediately after oral administration [177, 178], in order to more accurately reflect the state during the treatment of patients, experiments should concentrate on the effects of MMF *in vitro* and primarily use DMF after oral administration *in vivo*. In all experiments the role of HCA2 in any effect should be tested. In chapter 3 the effect of oral DMF therapy on psoriasis patients was assessed, the expression of HCA2 by different cell types was examined in detail and *in vitro* experiments were performed with MMF. This analysis showed that psoriasis improvement was accompanied by a decrease in blood neutrophils, DCs and to a lesser extent T cells

during the early months of oral DMF treatment. However, the pronounced decrease of neutrophils was not caused by the induction of apoptosis. *In vitro* cultured neutrophils, although they showed evidence of MMF-mediated signalling, did not undergo apoptosis after MMF or DMF treatment. There are numerous reports describing the induction of apoptosis by DMF in several different cell types. Importantly, all of these experiments showed initiation of apoptosis by DMF only, while MMF had no effect. These observations were made in T cells, mast cells, tumour cells and the HeLa cell line [300-304]. However, due to the properties described above, DMF-induced apoptosis is likely a result of its membrane permeability and reactivity while at the same time being a remote possibility *in vivo* as no cell type outside of the gut mucosa ever encounters this molecule [178, 181, 305]. Furthermore, as this work demonstrated that the therapeutic effect of oral DMF treatment on skin inflammation relied on HCA2 expression the effects seen by DMF *in vitro* should be considered as an *in vitro*-restricted phenomenon as the cells used in these studies do not express HCA2, while this receptor is essential for the protective effect of oral DMF *in vivo*.

Consideration also needs to be given to the concentrations of MMF used for *in vitro* experiments. The concentration of MMF in human plasma after a single typical dose of DMF (240mg) is 10 to 15 μ M. Concentrations used in this work for experiments with neutrophils and keratinocytes ranged from 5 μ M to 100 μ M with effects seen between 25 μ M and 100 μ M depending on the assay. Other reports have demonstrated effects of DMF or MMF on keratinocyte proliferation at concentrations of 100 μ M to 300 μ M [280, 281]. Taking into account that MMF (and DMF) may react with components of the culture medium (e.g. serum proteins) the available amount of MMF for cultured cells may be much lower than assumed. Furthermore, patients take DMF three times a day, providing a constant resupply of MMF. In contrast, the effects on the skin and leukocytes observed in this study were seen in the first month of treatment, a time period where patients were still in the up-dosing phase. These results indicate that low concentrations of MMF can have effects on target cell types. Taken together, the *in vitro* and *in vivo* data presented here showed the importance of MMF-HCA2 signalling in the improvement of skin inflammation and aligned with the pharmacology of DMF. Namely, that DMF is a prodrug for MMF *in vivo* and the cellular effects of treatment are linked to the expression pattern of HCA2. While *in vitro* experiments can be utilised to study possible mechanisms of action, animal models and human studies remain critical in determining if any hypothesis regarding DMF treatment occurs *in vivo*.

MMF was identified as an agonist for HCA2 by screening a panel of molecules for binding to the receptor rather than searching for potential receptors of MMF itself [5]. Thus, it is not known whether there are other receptors or even transporters for MMF. The existence of other as yet undefined transporters would possibly explain why T cells are

decreased by oral DMF treatment. Thus, even though T cells do not express HCA2 they may express other not yet identified receptors or transporters for MMF. Potential candidates include monocarboxylate transporters (MCT), which are a family of transporters for monocarboxylic acids and have been shown to transport butyrate, BHB and lactate [306]. Evidence of the existence of other surface proteins that mediate cellular responses to MMF comes from the analysis of NRF2 activation. Multiple studies have demonstrated that in cell types known to be HCA2 negative and in normally HCA2⁺ cells isolated from HCA2^{-/-} mice, *in vitro* exposure to pharmacologically-relevant concentrations of MMF resulted in activation of NRF2-associated signalling and gene expression [55, 197, 199]. These observations suggest that yet unknown transporters or receptors can import MMF into cells or activate intracellular signalling pathways leading to HCA2-independent effects *in vitro* and *in vivo*. Whether a member of the MCT family represents a MMF transporter is not currently known, but could potentially be initially tested *in vitro* by culturing T cells with and without MCT inhibitors and assess cellular responses after exposure to MMF. Such responses could include expression of cell surface markers, activation of intracellular signalling, gene expression changes or even apoptosis. Such experiments would provide strong evidence of HCA2-independent effects and open new avenues of investigating regarding the *in vivo* effects of DMF treatment.

6.2 The never-ending debate: Is psoriasis a keratinocyte or immune cell mediated disease?

Treatment of psoriasis with biologic or conventional systemic therapy usually target the immune system. For example, apremilast, methotrexate, and ciclosporin reduce immune cell pro-inflammatory cytokine secretion, proliferation, and the migration, while biologics target specific immune cell secreted cytokines or cytokine receptors [307-309]. The work presented here has shown that, in contrast to other drugs used for the treatment of psoriasis DMF did not primarily act on radio-sensitive bone marrow-derived immune cells, consisting of key cell types in the pathogenesis of psoriasis including neutrophils, DCs, monocytes, B cells and T cells. The radio-resistant HCA2-expressing cells targeted by DMF treatment, based on mRNA expression analysis, were most likely keratinocytes. This observation showed that keratinocytes are an important player, rather than a bystander, in the inflammatory loop of psoriasis. The role of keratinocytes in psoriasis has been a long-discussed topic. Although the major symptoms of psoriasis manifest on the skin, the role of keratinocytes in disease pathogenesis has received less attention than the contributions of the immune system [310-314]. Indeed, DMF treatment itself has been generally considered to improve psoriasis by affecting leukocytes, particularly

T cells. The results described in chapter 3 even suggested that neutrophils are a target cell type during oral DMF therapy. However, results obtained from the IMQ model, which generally reflects the development of skin lesions rather than the chronic phase of disease indicated that DMF treatment interrupts the hyperproliferation of keratinocytes directly via MMF-HCA2 signalling. The IMQ model is dependent on physical disruption of keratinocytes by the vehicle component of Aldara[®], a process that resembles Koebner phenomenon in psoriasis [211]. This not only suggests that DMF treatment in patients can generally reduce keratinocyte hyperproliferation but also that it may act on the development or initiation of psoriasis lesions. Although in psoriasis pDCs are the first cell type activated via TLR9, which then leads to the activation of DCs and in turn T cells, keratinocytes are the cause of the initial pDC activation. LL-37 is expressed by keratinocytes and upon injury binds to self-DNA forming complexes that go on to bind to TLR9 expressed by pDCs [86]. Therefore, keratinocytes are key to the initiation of lesion development as they are the source activating complexes. Although not investigated here, it would be of interest to examine if, beyond reducing proliferation, DMF treatment also leads to changes in LL-37 expression that may reduce the generation of pDC-activating complexes after keratinocyte disruption.

T cell-derived cytokines also act on keratinocytes and promote proliferation and lead to the release of keratinocyte-associated factors such as S100 proteins, IL-1 β and chemokines which in turn attract additional immune cells into the skin. In this known inflammatory loop of psoriasis, keratinocytes play an essential role in maintaining inflammation. Disintegrin and metalloprotease domain containing thrombospondin type 1 motif-like 5 (ADAMTSL5), usually expressed by melanocytes, has been identified as an autoantigen and is also expressed by keratinocytes. This further highlights the importance of keratinocytes in the pathogenesis of psoriasis [138, 315, 316]. In mice, specific genetic deletion of signalling molecules or transcription factors in keratinocytes (I κ B Kinase (IKK) or Jun-B and c-Jun) led to psoriasis-like skin symptoms and underlines the importance of keratinocytes in the initial development of psoriasis [317, 318]. The critical role keratinocytes play in the formation and maintenance of inflammatory skin lesions is further emphasised by the results of this thesis. Although not yet validated in human disease this study showed that keratinocytes are a primary target of DMF treatment and that bone marrow-derived immune cells alone cannot maintain maximal skin inflammation. This notion is supported by work from Andrienne *et al.* who showed in a mouse model that a keratinocyte-specific deletion of tristetraprolin (TTP), which acts as a suppressor of pro-inflammatory cytokines induced psoriasis-like skin lesions. A specific deletion of TTP in immune cells did not lead to similar skin inflammation [319]. Thus, keratinocytes-specific pro-inflammatory processes are sufficient to cause psoriasis-like inflammation. The ability of keratinocyte-specific pro-inflammatory

processes alone leading to skin inflammation is further supported by the fact that IL-1 receptor overexpression in mouse keratinocytes has been shown to lead to an increased inflammatory phenotype. Additional overexpression of IL-1 α in basal keratinocytes in IL-1 receptor overexpressing mice caused spontaneous development of skin inflammation [320]. Deletion of the endogenous IL-1R antagonist has also been demonstrated to result in the same phenotype [321]. Interestingly, Nakajima *et al.* showed that the deletion of IL-6 or TNF- α but not an IL-17A deficiency led to reduced development of skin inflammation in mice. It was further shown that keratinocyte-derived TNF- α rather than immune cell-derived TNF- α was important for lesion development [322]. Furthermore, Najajuma *et al.* have shown that CD4⁺ and CD8⁺ T cells are not essential for the development of initial skin lesions. However, T cells are very important cells in the pathogenesis of psoriasis and the blockade of Th17-associated cytokines has been proven highly efficacious.

Taken together, this work along with previously published studies have emphasized the role of keratinocytes in the pathophysiology of psoriasis, as the cellular source of activators of lesional development and producers of cytokines and chemokines that sustain inflammation. Keratinocytes should be regarded as major players in psoriasis and less as a bystander or simple responder cell. Putting these cells in the focus of drug research opens new opportunities for the development topical keratinocytes-specific drugs which may provide effective treatments with reduced systemic side effects for psoriasis vulgaris patients.

Keratinocytes were considered as a primary target cell of DMF treatment because they are radio-resistant and express HCA2. The results of mRNA expression experiments from the skin provided an indication that DMF treatment altered gene expression in keratinocytes rather than in immune cells. However, the qPCR analysis used here, provided only a small insight into the transcriptome of the epidermis during DMF treatment. Therefore, it cannot be excluded that other genes not originating from keratinocytes are altered by DMF treatment. In order to prove that keratinocytes are the affected cell type the generation of mice with specific expression of HCA2 in keratinocytes but not in other cells is planned. Since LCs both express HCA2 and are radio-resistant they remain a potential target of oral DMF. A mouse with LC-specific expression of HCA2 will be created and tested. Treating these mice with oral DMF before applying Aldara[®] cream will likely conclusively identify the cellular targets that mediate the skin protective effects of DMF treatment. In these same mice mRNA expression changes in the epidermis and dermis could be analysed by microarray or RNA-seq. Such analysis will reveal keratinocyte- or LC-specific HCA2-dependent transcriptome changes

and likely identifies pathways affected by oral DMF treatment that are leading to skin improvement.

Assuming that keratinocytes are the primary target cells of oral DMF treatment, the cellular effects need to be further elucidated and validated in human psoriasis. Therefore, to confirm the antiproliferative effect of oral DMF, the Aldara® applied epidermis of DMF treated and non-treated mice can be analysed for the expressions of keratins. Depending on the stage of keratinocyte development, e.g. if they are still proliferating or starting to differentiate, the pattern of keratin expression differs and is distinct between the separate layers of the epidermis [323]. These keratins can be analysed by an IF or ISH approach to visualise which keratin is expressed and where it is expressed. Furthermore, Ki-67, an indicator for cell proliferation, can be analysed with similar techniques. These investigations can also be performed in lesional skin from psoriasis patients before and after oral DMF treatment. However, unravelling the underlying intracellular mechanisms leading to reduced proliferation will require additional experiments. Here in this thesis initial attempts were made to analyse potential signalling cascades downstream of HCA2 (ERK, p38 phosphorylation). These analyses can be expanded to a larger scale by using array-based methods that can measure changes in several signalling pathways at a time. As NF- κ B signalling plays a major role in psoriasis and has been shown to be reduced by DMF treatment *in vitro* this pathway should be investigated in the context of HCA2 and MMF [183]. By using specific inhibitors or inducers of the NF- κ B pathway components, the connection between NF- κ B signalling and changes in keratinocyte proliferation induced by MMF can be revealed.

6.3 A ketogenic diet reduces skin inflammation via other pathways outside of HCA2 activation

The ketogenic diet is based on a high amount of fat and protein and little to no carbohydrates, causing the rapid depletion of glycogen stores and eventual switch over to lipid metabolism leading to the production of fatty acids and ketone bodies which then become the primary sources of energy for the tissues of the body. There are different forms of ketogenic diet which, differ in their ratio of fat (which usually contributes at least 60% of the calories in a ketogenic diet) to carbohydrates (maximum 20% of calories) and protein. The classic ketogenic diet has a fat content of 90%, 4% carbohydrates and the rest of the calories provided by protein [324]. A version of this ketogenic diet with high levels of fat but no carbohydrates led to the production of high levels of BHB in mice as described in chapter 4. The experiments performed in this thesis regarding the influence of a ketogenic diet were preliminary but they showed that a ketogenic diet ameliorated skin inflammation independently of HCA2 suggesting another mechanism of action compared to DMF treatment. These data contrasts the findings of Rahman *et al.* who showed HCA2-mediated protective effects of BHB in a mouse stroke model [199]. In a model of Parkinson's disease BHB was further shown to improve neuroinflammation and this effect was mediated by HCA2 [325]. While the MMF-HCA2 axis can ameliorate skin inflammation, activation of HCA2 by BHB (based on blood BHB levels during ketogenic diet) is not required for the protection from skin inflammation by a ketogenic diet. This result leads to new questions. Is BHB, as assumed, the molecule that mediates the protective effect of the ketogenic diet or are there other factors? Are other BHB-effects outside of HCA2 activation required?

In human psoriasis there is only one published case report showing that a very low caloric diet without carbohydrates restored the response to systemic biologic therapy [326]. All other studies that have reported an amelioration of psoriasis or improved treatment efficacy were either performed with a shake-based very low calorie diet or observations after bariatric surgery [92, 93, 325, 327]. Although ketones were not analysed in these studies, increased BHB levels may be assumed due to increased lipid metabolism as fat tissue is lost. Whether BHB mediates any effect is not known. There are other factors potentially causing reduced inflammation during calorie restriction, e.g. short chain fatty acids (SCFAs). These bind to GPCRs (GPR41 and GPR43) and are known to reduce inflammation [328]. GPR43 is expressed by neutrophils and has been shown to modulate their chemotaxis *in vitro*, making this receptor a potential therapeutic target [329, 330]. Whether SCFA were formed during the ketogenic diet experiments described in this thesis is not known. The ketogenic diet utilised here contained less than 1% of SCFA. Although SCFA can be formed in peroxisomes from fatty acids longer than 22 carbon

atoms, the amount of SCFA formed from this reaction is highly speculative [331]. Nevertheless, it would be worth analysing the level of SCFA in the mouse peripheral blood during ketogenesis to exclude other mitigating factors. Whether there are other ketones, the fatty acids itself or whether there are others receptors for BHB needs to be elucidated. To verify if BHB is required for the skin protective effect that was observed here, the animal experiment could be repeated. Instead of the ketogenic diet BHB could be injected intraperitoneally into the mice to verify if BHB is anti-inflammatory in psoriasis-like skin inflammation.

Although HCA2 did not play a role in mediating the skin-protective effect of the ketogenic diet, there might be other factors involved in improving skin symptoms. BHB does not only bind to HCA2 but also to FFAR3 (GPR41) although it is still unclear whether BHB is an agonist or antagonist of this receptor [279, 332]. GPR41 is highly expressed on adipocytes and also expressed on superior cervical ganglion (SCG) cells but it is not known if it is expressed in the skin [333]. mRNA expression of GPR41 has been shown in PBMC but not by separate cell types [334]. GPR41 expressing adipocytes could represent a target mediating BHB's effect as not only is obesity a risk factor for psoriasis but leptin released from adipocytes is not only increased in psoriasis patients but also induces keratinocyte proliferation, neoangiogenesis and the production of pro-inflammatory cytokines [99, 100]. In obese people Wnt5a signalling is increased as adipocytes of obese individuals do not release the sFRP5 molecule, which clusters and inactivates Wnt5a [102]. Increased Wnt5a levels have been detected in lean and obese psoriasis patients compared to healthy individuals [105]. Thus, BHB-GPR41 signalling in adipocytes has the potential to affect many pathways related to inflammation in psoriasis patients. But this hypothesis is as yet unproven as it is unclear whether BHB acts as an agonist or antagonist of GPR41. The use of GPR41 knock out mice would allow the analysis of its role in the amelioration of murine skin inflammation by the ketogenic diet.

Besides binding to GPCRs BHB inhibits other pathways. For example, BHB inhibits oxidative stress, apoptosis, and inflammation by inhibiting histone deacetylases (HDACs) thereby changing transcription of DNA. The inhibition of HDACs has been mostly investigated in neuronal diseases such as Parkinson's disease [335]. There, the inhibition of HDACs has been reported to increase brain-derived neurotrophic factor (BDNF) levels which was been shown to prevent NF- κ B induced neuroinflammation in mice [336, 337]. Inhibition of HDACs has been shown to reduce the release of pro-inflammatory cytokines in LPS-treated PBMCs and also leads to reduced proliferation of keratinocytes [338, 339] HDAC inhibitors are have also been discussed as a potential treatment of psoriasis [340, 341]. Taken together, these observations indicate that the inhibition of HDACs by BHB may have anti-inflammatory effects in the skin and represents a plausible mechanism by which BHB reduces inflammation outside of HCA2

activation. To investigate if HDAC inhibition is protective in Aldara[®]-induced skin inflammation, mice can be treated with the inhibitor suberoylanilide hydroxamic acid (SAHA) and the skin should be analysed for epidermal thickness compared to untreated mice after Aldara[®] application. In these mice signalling cascades downstream of the HDACs can be identified. To prove that BHB ameliorates skin inflammation by inhibiting HDACS, mice should directly be injected with BHB and the skin thickness and the signalling cascades previously identified can be analysed for changes.

6.4 Conclusion

This work has shown that oral DMF treatment improved psoriasis within the first month of treatment and this improvement in skin inflammation was accompanied by a reduction in leukocytes, especially neutrophils and DCs and to a lesser extent, lymphocytes. The mechanisms of neutrophil decrease *in vivo* remained unclear as MMF did not cause neutrophil apoptosis. Using a mouse model of psoriasis-like skin inflammation it was shown that the therapeutic effect of oral DMF treatment required the expression of HCA2. Furthermore, this protective effect was mediated by HCA2 expressed by radio-resistant cells but not by infiltrating immune cells in the lesions. Keratinocyte-associated genes were reduced and these cells showed a reduced proliferation when cultured with MMF *in vitro*. This suggested that the reduction of keratinocyte hyperproliferation is an important mechanism of action of DMF treatment. The induction of high BHB levels by a ketogenic diet led to a reduction in skin inflammation but this effect was independent of HCA2. Overall, this work suggests that MMF-HCA2 signalling in keratinocytes is critical to the anti-psoriatic effect of DMF treatment, while BHB-HCA2 interactions are dispensable for the skin-protective effect mediated by ketogenesis. Together, these data suggest combining DMF treatment with a ketogenic diet may have positive synergistic effects on psoriasis.

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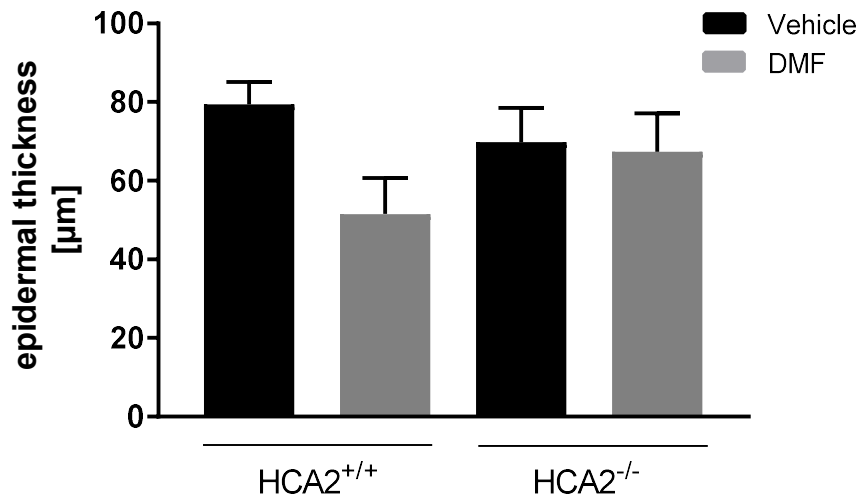
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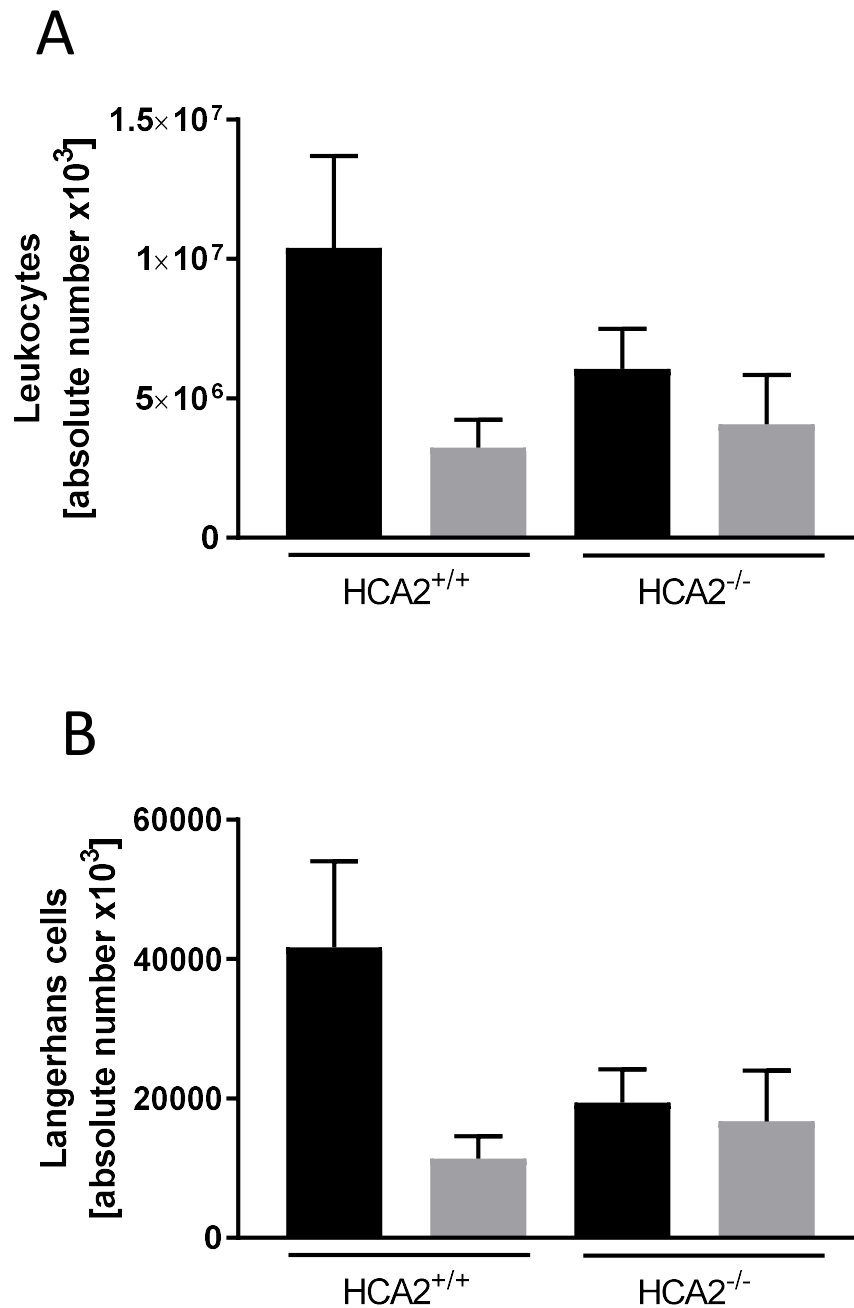
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Supplemental figures

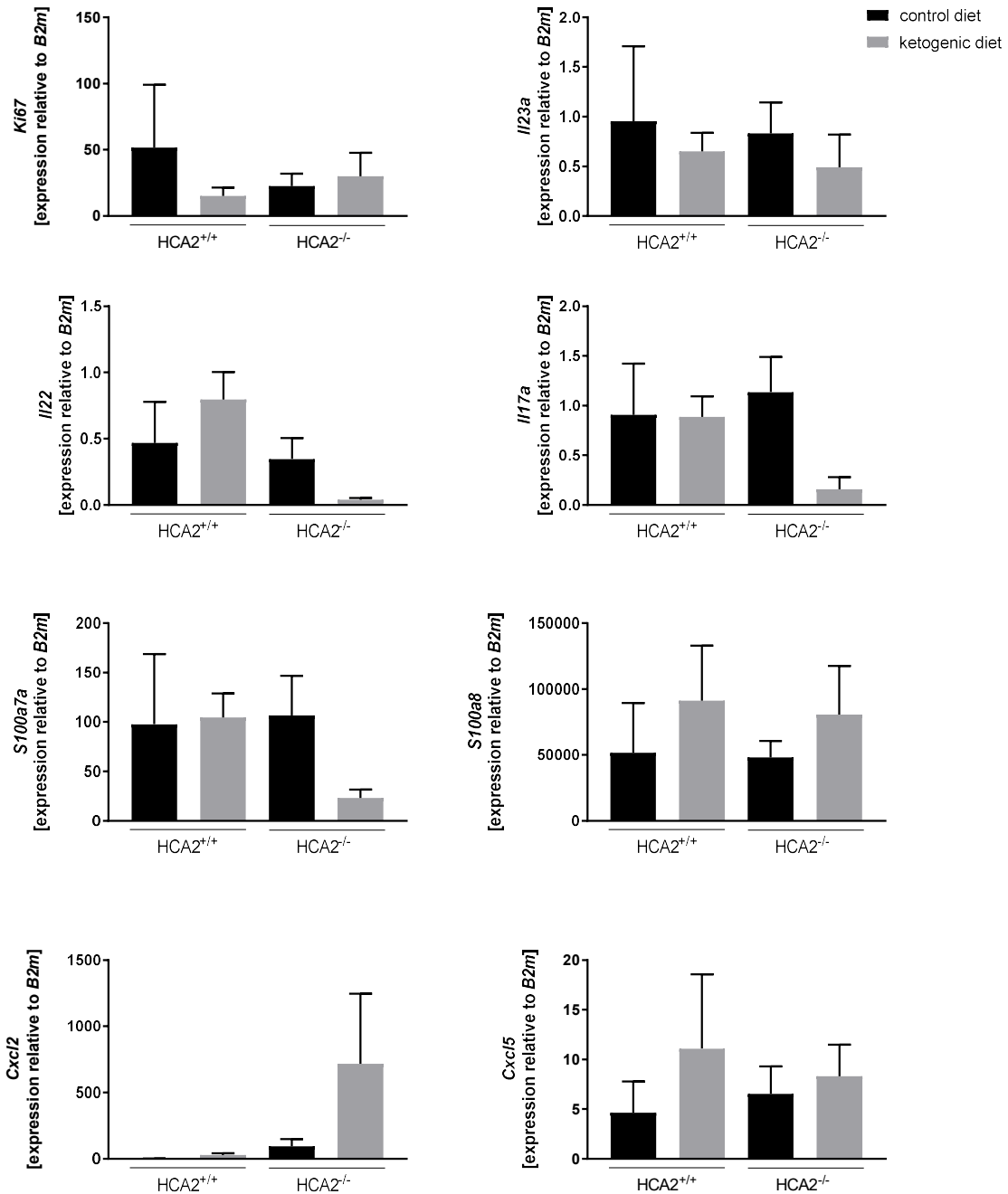
**Supplemental figure 1: Quantification of epidermal thickness after 4 days of Aldara® treatment.**

Mice were treated with DMF or vehicle for 12 days. During the last 5 days Aldara® was applied to the back skin of the mice. Mice were sacrificed at day 13 and back skin sections were processed and analysed. Each bar represents the mean +SEM of 5 mice. One representative out of 2 experiments shown. No significance detected as determined by one-way ANOVA followed by Tukey's test.



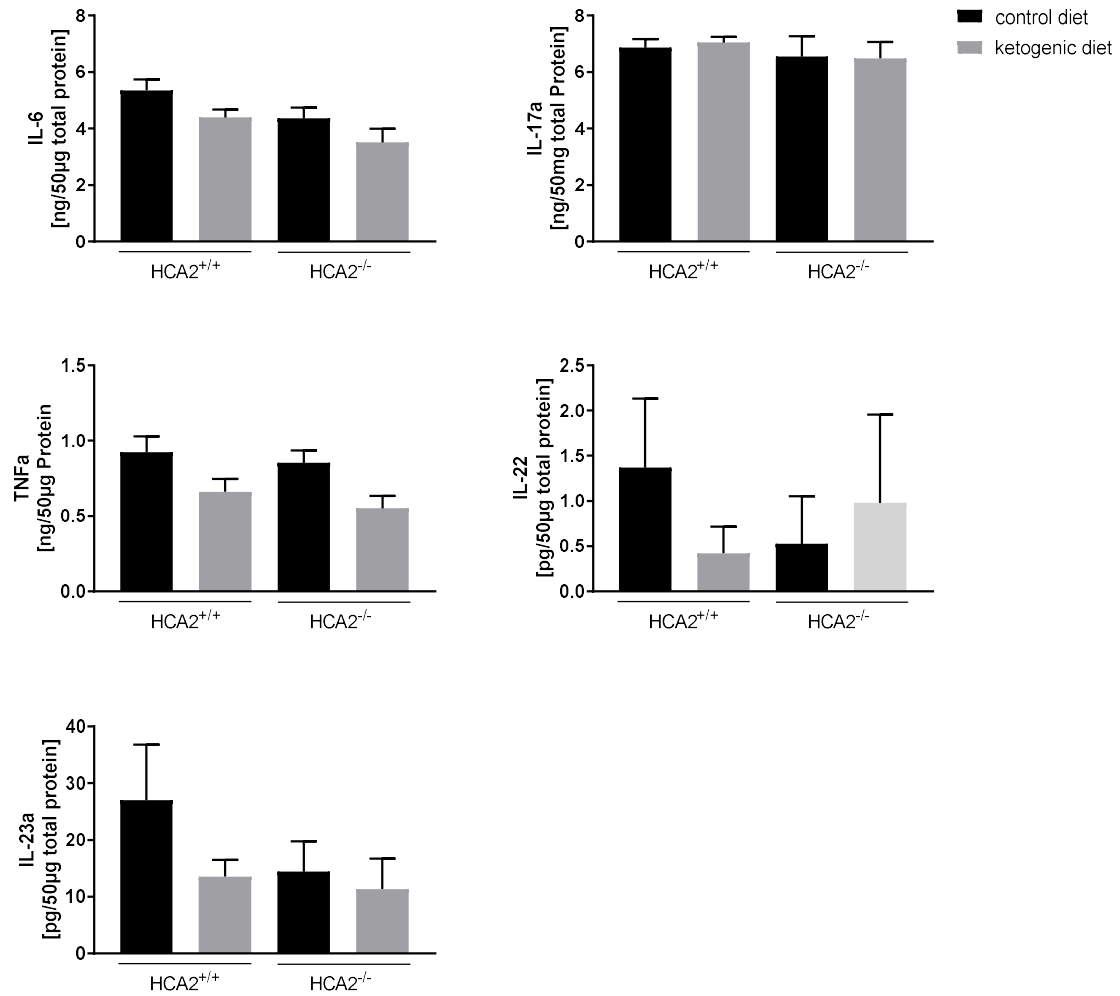
Supplemental figure 2: Langerhans cells in skin-draining lymph nodes are affected by a ketogenic diet.

Skin draining lymph nodes were taken from Aldara[®]-treated HCA2^{+/+} and HCA2^{-/-} mice fed a ketogenic or control diet. Cells were isolated, counted and surface stained for CD45, MHCII and Langerin (CD207) as well as with a live/dead dye. Graphs show the absolute number of leukocytes (A) and the absolute number of LCs (B). n=5. One experiment performed. Statistics showed no significance as determined by one-way ANOVA. Followed by Tukey's test.



Supplemental figure 3: mRNA expression levels in back skin of mice under ketogenesis.

Skin biopsies were taken from Aldara®- treated HCA2^{+/+} and HCA2^{-/-} mice fed a ketogenic or control diet for mRNA isolation. cDNA from these samples was analysed for *Ki67*, *Il1a*, *Il1b*, *S100a8*, *S100a7a*, *Cxcl5*, *Cxcl2*, *Il17a*, *Il22* and *Il23a* mRNA expression by qPCR. Bars represent mean mRNA expression relative to B2M + SEM of 5 mice. One experiment performed e. no significance determined by one-way ANOVA followed by Tukey's test.



Supplemental figure 4: Ketogenic diet induces minor changes in cytokine levels in Aldara[®]-induced skin lesions.

Protein was isolated from the back skin of Aldara[®]-treated HCA2^{+/+} and HCA2^{-/-} mice fed with a ketogenic or control diet. IL-6, IL-17A, TNF- α , IL-22 and IL-23 (B) levels were tested by ELISA. N=5. One experiment performed. No significance detected. (ANOVA with Tukey's test).

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M.Sc. Ina Carolin Suhrkamp

Statutory Declaration:

Herewith I declare on oath that the submitted dissertation under the title „ THE ROLE OF THE KETONE RECEPTOR HCA2 IN THE MANAGEMENT OF PSORIATIC SKIN INFLAMMATION “ has been authored independently and without illegitimate external help and that it has not been formerly submitted to another university department.

Kiel, 01.04.2020

Herewith I declare, that I am not subject to any pending case of public prosecution.

Kiel, 01.04.2020

Herewith I declare, that the dissertation complies with the conventions of proper academic practices as defined by the DFG.

Kiel, 01.04.2020