2011
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GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Exploring inhibitors and vitamins to generate tolerogenic dendritic cells for Multiple Sclerosis

Promotor :
Prof. dr. Niels HELLINGS

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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

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Abbreviations

1,25D$_3$	1,25-dihydroxyvitamin D$_3$
ACE	Angiotensin-converting enzyme
AII	Angiotensin II
AT$_1$	Angiotensin II type-1 receptor
AT$_2$	Angiotensin II type-2 receptor
APC	Antigen presenting cell
BBB	Blood-brain barrier
BL6	C57BL/6 JOlaHsd
BM	Bone marrow
CFSE	Carboxyfluorescein succinimidyl ester
CM	Culture medium
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
GM-CSF	Granulocyte macrophage colony stimulating factor
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
IFN	Interferon
IL	Interleukin
IM	Isolation medium
imDC	Immature dendritic cell
i.p.	Intraperitoneal
LPS	Lipopolysaccharide
maDC	Mature dendritic cell
mDC	Myeloid dendritic cell
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
PTX	Pertussis toxin
pDC	Plasmacytoid dendritic cell
RAS	Renin-angiotensin system
RR-MS	Relapsing-remitting multiple sclerosis
SAHA	Suberoylanilide hydroxamic acid
SI	Stimulation index
SP-MS	Secondary progressive multiple sclerosis
TGF	Transforming growth factor
T_{h1} cell	T helper 1 cell
T_{h17} cell	T helper 17 cell
TLR-4	Toll-like receptor 4
T_{reg} cell	Regulatory T cell
VDR	Vitamin D receptor
VitD$_3$	Vitamin D$_3$

Abstract

Dendritic cells (DC) are professional antigen presenting cells able to influence both immunity and tolerance. Not only are they the main immune cells capable of inducing a primary T cell response, DC also determine the profile of that response. This ability turns DC into interesting immune modulating agents. In autoimmune diseases, such as Multiple Sclerosis (MS), tolerogenic DC could be used to restore self-tolerance. However to date, there is no consensus concerning the stimuli that produce that type of DC. We therefore hypothesized that treatment of DC with specific stimuli generates tolerogenic DC able to restore the immune balance in experimental autoimmune encephalomyelitis (EAE) mice, an animal model of MS. For this purpose, mouse bone marrow (BM) and BM-derived immature (im)DC were incubated with suberoylanilide hydroxamic acid (SAHA). Lipopolysaccharide (LPS) was used for maturation induction. imDC decreased the expression of the co-stimulatory molecule CD86 and reduced the secretion of the pro-inflammatory cytokines tumor necrosis factor α, interleukin (IL)-6 and IL-23 after LPS challenge. Most of these effects were maintained for at least 3 days after SAHA removal. When BM was exposed to SAHA, this did not impair normal DC differentiation. However, this prolonged incubation did not yield a greater effect as compared to the treated imDC. A pilot experiment in which BM was treated with active vitamin D_3 demonstrated normal DC differentiation and a decrease in the amount of $CD86^+$ DC after LPS stimulation. *In vitro* preliminary data of a third agent, Captopril, demonstrated that this substance only had an effect on DC cytokine secretion in the absence of LPS. Continuing on these results, we prophylactically administered Captopril-treated DC to EAE mice. When 2×10^6 Captopril-treated DC were injected intraperitoneally, 2 out of 7 mice displayed DC-induced effects. From this investigation, we can conclude that at this stage, SAHA has the most DC tolerizing potential, generating semi-mature and therefore more tolerogenic DC after LPS challenge.

Samenvatting

Dendritische cellen (DC) zijn professionele antigeen presenterende cellen die zowel immuniteit als tolerantie kunnen beïnvloeden. Het zijn niet alleen de voornaamste immuun cellen die een primaire T cel reactie kunnen induceren, ze bepalen ook het profiel van die reactie. Dit maakt van DC interessante immuniteit modulerende componenten. In auto-immuunziekten, zoals Multiple Sclerose (MS), zouden tolerogene DC ingezet kunnen worden om tolerantie te herstellen. Tot op heden is er echter geen consensus omtrent de stimuli die dat soort DC genereren. Wij veronderstellen daarom dat de behandeling van DC met specifieke stoffen tolerogene DC produceert die in staat zijn de immuun balans in experimentele auto-immuun encefalomyelitis (EAE) muizen, een diermodel voor MS, te herstellen. Om deze hypothese te testen, werden muis beenmerg (BM) en BM-afkomstige immature (im)DC met suberoylanilide hydroxamic acid (SAHA) behandeld. Maturatie werd geïnduceerd m.b.v. lipopolysaccharide (LPS). imDC verminderden de expressie van de costimulatie molecule CD86 en reduceerden de secretie van de pro-inflammatoire cytokines tumor necrosis factor α, interleukine (IL)-6 and IL-23 na LPS blootstelling. De meeste van deze effecten waren 3 dagen na het wegwassen van SAHA nog steeds aanwezig. Differentiatie van DC uit BM werd niet verstoord als dat BM behandeld werd met SAHA vanaf het begin. Deze langdurige SAHA blootstelling leverde wel geen bijkomstige tolerogene effecten op in vergelijking met de behandelde imDC. Een *pilot* experiment waarbij muis BM werd geïncubeerd met de biologisch actieve vorm van vitamine D_3 toonde aan dat DC ontwikkeling normaal verliep en dat het aantal $CD86^+$ DC daalde na LPS stimulatie. *In vitro* preliminaire data van een derde stof, namelijk Captopril, toonde reeds aan dat deze stimulus enkel een effect heeft op de cytokine secretie in de afwezigheid van LPS. Verdergaand op dit onderzoek, hebben we EAE muizen profylactisch Captopril-behandelde DC toegediend. Enkel wanneer er 2 miljoen Captopril-DC intraperitoneaal werden geïnjecteerd, waren er DC-afkomstige effecten waar te nemen bij 2 van de 7 muizen van de groep. Uit deze studie concluderen we dat, op dit moment, SAHA de stimulus is met het grootste DC tolarizerende potentieel. SAHA incubatie leidt tot de productie van semi-mature DC na LPS blootstelling die meer tolerogeen zijn dan onbehandelde DC.

1. Introduction

Dendritic cells (DC) are a highly specialized population of leukocytes that grow branched projections during certain developmental stages, called dendrites (Fig 1). These dendrites are continuously expanding and retracting as part of the role of DC as professional antigen presenting cells (APC). This places them in the same family as B cells and macrophages, but unlike B cells, DC belong to the innate immune system (1, 2).

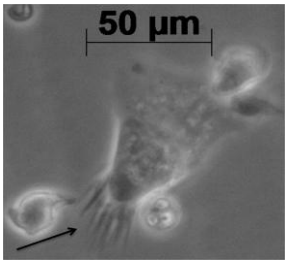

Fig 1| Dendritic cell. Phase contrast micrograph of an adherent developing dendritic cell. Arrow indicates dendrites. Micrograph was made using Zeiss PrimoVert, adapted by AxioVision, x40.

1.1 Dendritic cell localization, homeostasis and function

As a first line of defence against invading pathogens, DC can be found throughout the entire body. Spleen, lymph nodes, kidneys, gut and skin, all are populated by different types of DC (2, 3). Since DC fulfil a sentinel function, these APC also have access to immune privileged areas, such as the central nervous system (CNS). Here, they reside in the cerebrospinal fluid (CSF), meninges and perivascular zones (4).

Although there is no concrete data on the turnover rates of DC, it is widely accepted that both lymphoid and nonlymphoid DC are short-lived and that their homeostasis relies on the continuous input from blood-borne cells (2). This is the reason why DC are continuously produced by hematopoietic stem cells within the bone marrow (BM). Even though DC exhibit a high degree of heterogeneity, they all originate from the same $CD34^+$ progenitor. This stem cell differentiates into common myeloid and common lymphoid progenitors which, in general, give rise to the 2 main DC subtypes: myeloid (mDC) and plasmacytoid (pDC) DC (5). mDC are characterized as $CD11c^+BDCA\text{-}1^+$ cells and known to be the conventional inducers of primary T cell responses (5, 6). pDC on the other hand are $CD11c^-BDCA\text{-}2^+$ and described as circulating cells specialized in the production of large amounts of type I interferons (INF) in response to viral infections (7-9). Recently, studies indicate that this unique DC subtype can also participate in the maintenance of peripheral tolerance (10-12).

Generally, immature DC (imDC) continuously sample peripheral tissues to capture antigens. When they succeed, the antigen is taken up, cleaved into peptides and expressed on the cell surface in a major histocompatibility complex (MHC)-peptide complex. This complex is subsequently presented to naive T cells in secondary lymphoid organs. The outcome of the encounter depends on cell environment and DC activation state (6, 13).

In the steady state, imDC present antigens without proper co-stimulation. This implies presentation in the absence of secondary or tertiary activation signals, such as CD80/86 - CD28 ligation or the production of T cell polarizing cytokines. Antigen presentation without sufficient co-stimulation eventually leads to autoreactive T cell deletion, anergy or the induction of regulatory T (T_{reg}) cells (Fig 2a). imDC are therefore essential components for the induction of peripheral tolerance and the prevention of autoimmune diseases (13).

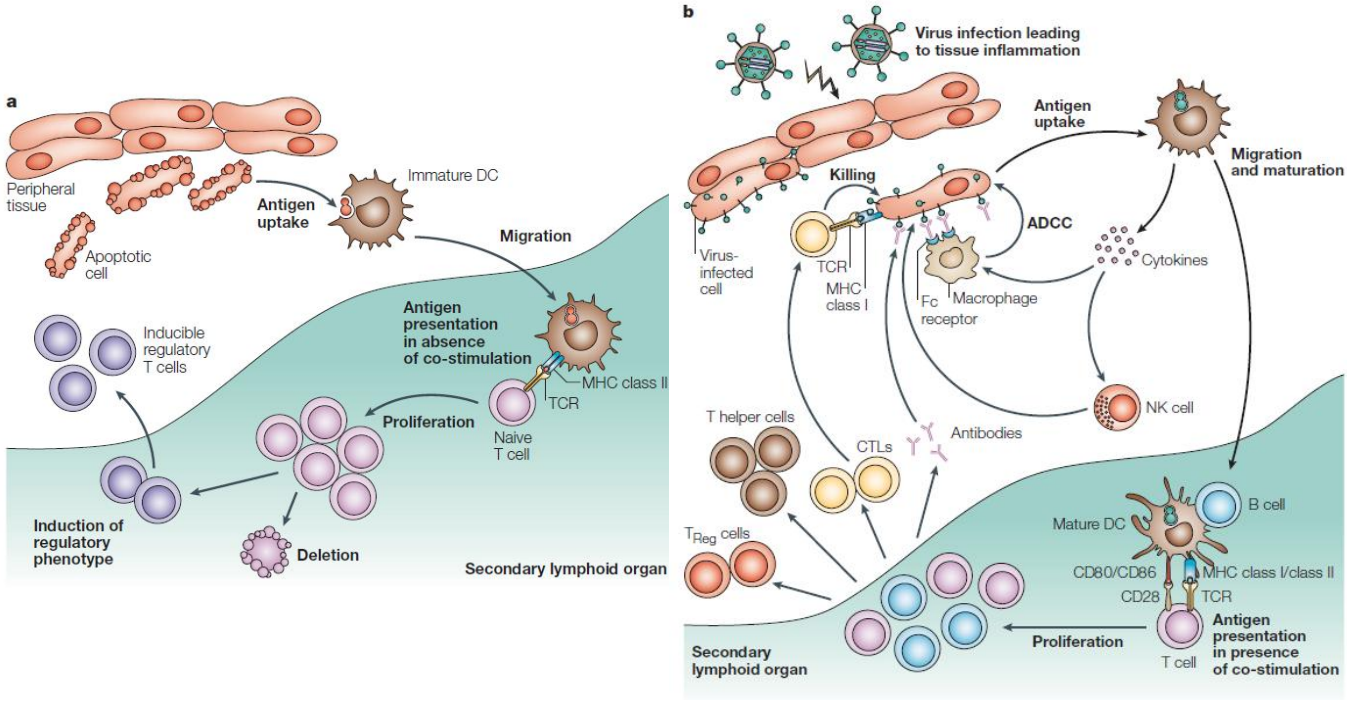

Fig 2| DC life cycle. (a) In the steady state, immature DC continuously sample peripheral tissues to capture antigens. They subsequently migrate to draining lymph nodes, where the antigens are presented to T cells without co-stimulation, leading to T cell deletion, anergy or the generation of regulatory T cells. (b) In the presence of tissue inflammation, immature DC mature as they migrate in large numbers to draining lymph nodes. Here, antigens are expressed together with proper co-stimulatory molecules. This induces the priming of T helper cells and cytotoxic T lymphocytes, the activation of B cells and the expansion of regulatory T cells. ADCC, antibody-dependent cell-mediated cytotoxicity; CTL, cytotoxic T lymphocyte; DC, dendritic cell; MHC, major histocompatibility complex; NK, natural killer; TCR, T cell receptor; T_{reg} cell, regulatory T cell (13).

In the presence of tissue inflammation or other danger signals, imDC are activated and undergo maturation as they migrate in large numbers to draining lymph nodes. As part of maturation, DC reduce their endocytic activity, increase MHC-peptide complex expression and production of pro-inflammatory cytokines, and up-regulate the surface expression of adhesion and co-stimulatory molecules (14). Accordingly, mature (ma)DC present antigens with sufficient co-stimulation to

naive T cells in draining lymph nodes, initiating an antigen-specific immune response (Fig 2b) (13). Consequently, DC not only form an important link between innate and adaptive immunity, because of their role in T cell polarisation, alterations in their compartment would also lead to the induction or perpetuation of autoimmune diseases.

1.2 Multiple Sclerosis

Multiple Sclerosis (MS) is a progressive autoimmune disease in which self-tolerance, the ability of the body's own immune system to recognize the proper antigens as self, is lost (15). Sensory and visual impairment, lack of coordination, cognitive dysfunction and paralysis are common symptoms (16, 17). The disorder affects women more than men in a 2:1 ratio and becomes clinically apparent between the ages of 20 and 40. This makes it the most common neurological disease in young adults (16). MS has a high prevalence rate with more than 100 – 200 MS cases per 100.000 individuals in Western countries and despite intensive research, the etiology of MS remains largely unknown (18).

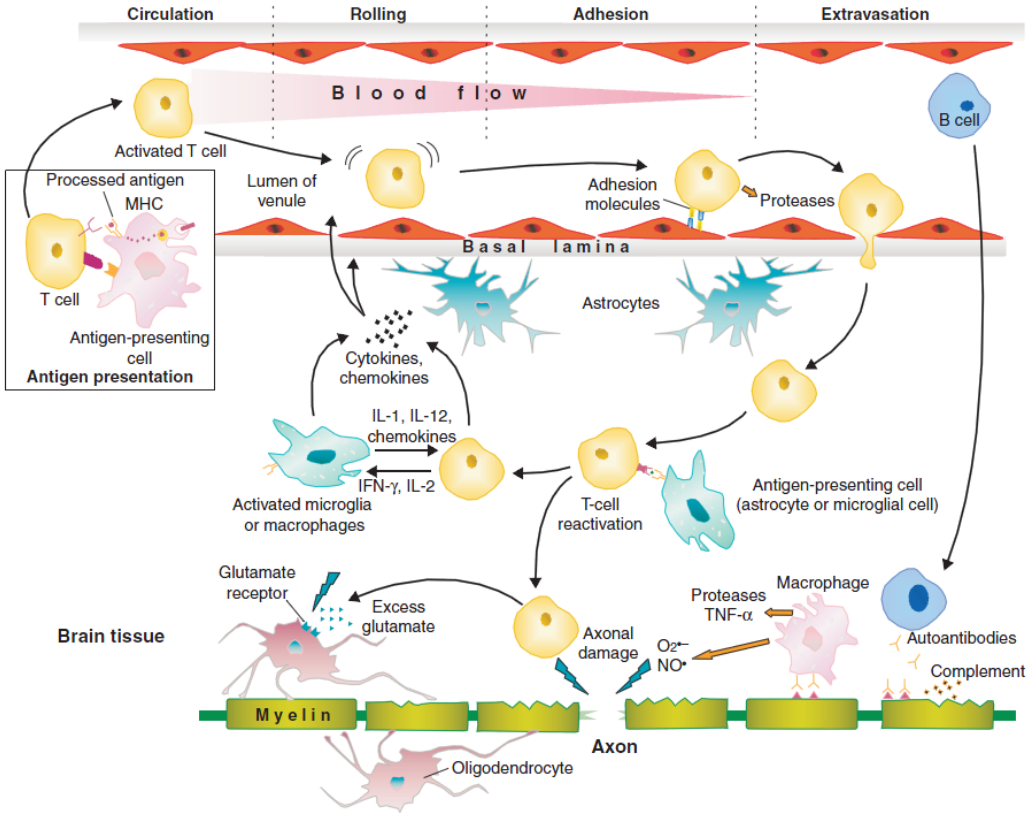

Fig 3| A general view of MS pathogenesis. Autoreactive T cells are activated in the periphery after which they undergo transcriptional changes resulting in the expression of adhesion molecules and pro-inflammatory cytokines. This favours their adhesion to capillary vessels of the blood-brain barrier and enables them to leave these vessels and enter the brain. Here, they are reactivated by antigen presenting cells expressing central nervous system antigens. This second T cell activation triggers a new wave of inflammation in which numerous cytokines, chemokines and other molecules are produced. This process continuous under the influence of positive feedback loops acting on effector cells and eventually results in damage to myelin, oligodendrocytes, and neurons. IFN, interferon; IL, interleukin; MHC, major histocompatibility compex; NO, nitric oxide; TNF, tumor necrosis factor (15).

It is generally accepted that early MS pathogenesis is mediated by the activation of autoreactive T cells in the periphery, after which they migrate to the CNS. Under the influence of adhesion molecules and pro-inflammatory cytokines produced by the activated T cells themselves, they are able to cross the blood-brain barrier (BBB). Once inside in the brain, T cell reactivation occurs through local expression of myelin epitopes by APC, followed by the influx of other immune cells, such as macrophages and B cells. This commences an inflammatory response directed against myelin and other CNS components, resulting in sclerotic lesions in CNS white matter and eventually neurological dysfunction (Fig 3) (16, 19, 20). Oligodendrocytes, responsible for the myelin sheath that insulates axons, are largely preserved in the beginning of the disease. In later stages, these insulators are damaged irreversibly, rendering them unable to remyelinate axons (16). That is why the clinical course of the most common form of MS consists of 2 consecutive stages. A relapsing-remitting stage (RR-MS), in which remyelination still occurs, followed by a secondary progressive stage (SP-MS), when the patients' health declines gradually (17, 21, 22). Other types of MS exist as well. In primary progressive MS, the clinical decline starts at the onset of the disease (20).

1.2.1 Dendritic cells in Multiple Sclerosis

Considering the pivotal role DC play in the maintenance of peripheral tolerance, defects in their compartment would create a serious tolerance imbalance, as seen in autoimmune diseases. There are numerous studies indicating a possible role for these professional APC in MS etiology. It is generally known that DC are the only cells, besides activated macrophages, capable of secreting interleukin (IL)-23. This cytokine promotes the differentiation of naive T cells into IL-17 secreting T helper 17 (T_{h17}) cells. IL-17 is a pro-inflammatory cytokine, capable of disrupting the BBB, creating a possible access route for autoreactive immune cells to enter the CNS (23, 24).

There are also more specific facts linking DC to MS. Early MS patients have elevated DC levels in their CSF (25, 26). MS patients in general also present DC at abnormal places, such as parenchymal lesions. In these lesions, DC were shown to take up myelin and interact with present lymphocytes, possibly contributing to the local activation and expansion of autoreactive T cells (4). Besides abnormal location and enhanced amount, the profile of circulating DC in MS patients has also shifted towards a more mature one (27). This was emphasized in a recent human *ex vivo* study of our own research group. We found that the percentage of blood mDC carrying the maturation marker CD86 was significantly increased in all MS patients. Expression of the C-C chemokine receptor 5, involved in the migration of DC to inflammatory sites, was increased on the pDC and mDC of SP- and RR-MS patients respectively.

DC abnormalities are also present in MS animal models. It has been demonstrated that DC accumulate within inflammatory cell infiltrates in the brain of mice suffering from experimental autoimmune encephalomyelitis (EAE) (28). Miller *et al.* evinced that these DC are efficiently capable of presenting myelin antigens to activate both naïve T cells and pre-activated effector T cells (29). Together, these data suggest that DC recruitment to the brain is pivotal for local stimulation and maintenance of autoreactive immune responses.

1.3 Tolerizing dendritic cells

As DC determine the profile of immune responses, they form interesting immune modulation targets. That is why immunogenic DC are already being tested in a clinical context for the treatment of cancer (30). However, the therapeutic potential of tolerogenic DC is also being assessed. It has been demonstrated that DC, whose maturation and activation were inhibited through treatment, are more effective at preventing diabetes development in mice (31). Furthermore, there is also an ongoing fase I clinical trial in which the safety of autologous monocyte-derived DC treated *ex vivo* is tested in established type I diabetes patients (32).
The ability to assume an immunogic or tolerogenic character subsequently makes DC a very appealing therapeutic tool. However, when working with DC, it is not only important that they acquire the desired profile, but also that they remain tolerogenic after exposure to maturation inducing signals. There are several stimuli demonstrating to have tolerizing abilities on DC, such as tumor necrosis factor (TNF) α, NF-κB inhibitors and IL-10 (33-35). Unfortunately to date, there is no consensus concerning the stimuli that produce DC locked in a tolerogenic state. We therefore evaluated the effects of 3 stimuli presumed to have DC tolerizing effects. The chosen stimuli were a renin-angiotensin system (RAS) inhibitor, a histone deacetylase inhibitor (HDACi) and active vitamin D_3 ($VitD_3$).

1.3.1 Renin – angiotensin system

RAS is a cascade of enzymatic reactions resulting in the formation of angiotensin II (AII). The kidney-derived hormone renin converts the substrate angiotensinogen to the decapeptide angiotensin I, which is subsequently hydrolyzed by the angiotensin-converting enzyme (ACE) to form AII (Fig 4). AII is the main effector molecule of the system and exerts its physiologic effects by binding to the highly specific AII type-1 and type-2 receptors (AT_1 and AT_2). It is through these receptors that AII has a series of systemic and local effects throughout the body, regulating blood volume, blood pressure and systemic vascular resistance (36-38). Recently, it has been shown that AII is more than just a vasoactive substance, influencing cell growth, proliferation, apoptosis and inflammation (37).

AII has been attributed the ability to influence specific immune cells, such as T cells, macrophages and DC (39-41). On the DC level, Nahmod *et al.* demonstrated that blockage of either AT_1 or AT_2 during DC differentiation results in complete opposite types of DC. One type has poor endocytic and allostimulatory activities, while the other is very good at endocytosis and induction of antibody responses (42). Blockage of AT_1 also significantly reduces the number of $CD11c^+$ DC in immune organs, while treatment with AII boosts the biosynthesis of the pro-inflammatory cytokines TNF-α and IL-6 (38, 43). AII clearly has regulatory effects on DC development, underlined by the fact that certain components of RAS, such as angiotensinogen and ACE, are expressed by human monocyte-derived DC (44).

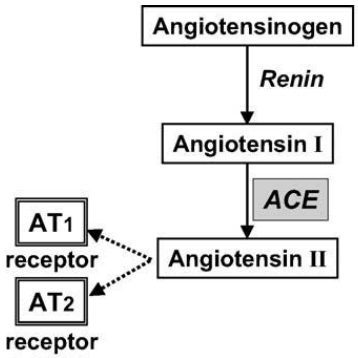

Fig 4| Renin-angiotensin system. ACE, angiotensin-converting enzyme; AT1, angiotensin II type-1 receptor; AT2, angiotensin II type-2 receptor (45).

RAS also plays an important role in autoimmunity, as exemplified in EAE and MS. Lanz *et al.* demonstrated that the neuroinflammation in EAE animals is sustained through transforming growth factor (TGF)-β production by AII-stimulated astrocytes, neurons and microglia (46). Blocking AII production with ACE inhibitors on the other hand, suppressed autoreactive T helper 1 (T_{h1}) and T_{h17} cells and promoted T_{reg} cells, reversing paralytic EAE (47). In humans, RAS is up-regulated in MS brain and the increased ACE levels correlate with MS lesion size. Although ACE activity is increased in the CSF of MS patients, AII levels are not. Taken together, RAS seems to be altered intrathecally in MS (45, 47, 48). Due to the involvement of RAS in MS and the fact that AII has immunogenic effects on DC, this study tested whether the ACE-inhibitor Captopril could generate tolerogenic DC.

1.3.2 Histone deacetylase

Nuclear histone deacetylases (HDAC) and their counterparts, histone acetyl transferases (HAT), determine the acetylation status of histone proteins and are consequently involved in the transcriptional regulation of eukaryotic cells. As the name suggests, HAT acetylate lysine residues, promoting a more relaxed chromatin structure which allows transcriptional activation. HDAC promote the exact opposite. By removing acetyl groups, chromatin becomes condensed and

inaccessible for the transcriptional machinery (Fig 5) (49, 50). Recently, it is becoming more evident that HDAC and HAT also target non-histone proteins, such as transcription factors, chaperone proteins, signal transduction mediators and DNA repair enzymes. All directly or indirectly involved in the regulation of gene expression, proliferation and cell death (51). Acetylation of this type of proteins is an important posttranslational modification that influences protein function, signalling, stability and protein-protein/protein-DNA interactions (52).

Cellular biology is heavily affected by the use of HDACi. By disturbing the natural process of acetylation and deacetylation, HDACi selectively alter gene transcription. This not only makes them promising anticancer drugs, recent evidence also indicates that HDACi could be of use in autoimmune and inflammatory diseases (53). There are several studies in which HDACi are used in an inflammatory setting, such as rheumatoid arthritis or Con A-induced hepatitis. In all of these cases, the observed therapeutic effect was ascribed to a reduction of circulating pro-inflammatory cytokines and possible immunomodulatory functions of these drugs. The exact molecular mechanism however remains elusive (54, 55).

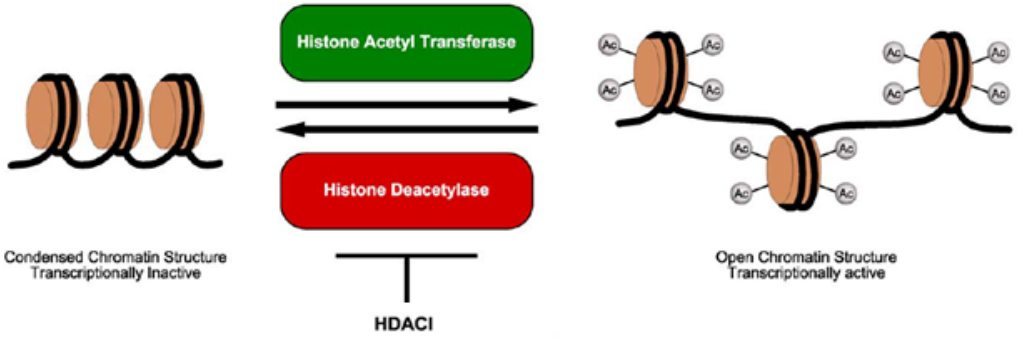

Fig 5| Mechanism of transcriptional regulation by histone acetylases and histone deacetylases. HDACi, histone deacetylase inhibitor (50).

HDACi also have immunomodulatory effects on DC. Reddy *et al.* pre-treated BM-derived mouse DC with the HDACi suberoylanilide hydroxamic acid (SAHA) to discover that the expression of the co-stimulatory molecules CD40 and CD80 was suppressed. *In vitro* allostimulatory responses by mouse DC in a standard mixed leukocyte reaction and *in vivo* alloproliferation in an acute graft-versus-host-disease mouse model were also decreased (56). Other studies further confirm that HDACi affect DC functionality. The production of T_{h1}- and T_{h17}-inducing cytokines, namely IL-12 and IL-23, by human monocyte-derived DC in the presence of lipopolysaccharide (LPS) was inhibited by SAHA treatment (57). These findings made SAHA a suitable candidate to induce tolerizing effects in DC in our study.

1.3.3 Active vitamin D_3

Vitamins are micronutrients often acting as co-enzymes in the human body. Due to their essential nature, they need to be taken up from the diet. Most of the $VitD_3$ however, is synthesized by the body itself under the influence of sunlight. When the skin is exposed to UV_b rays, 7-dehydrocholesterol is converted into pre-$VitD_3$. This pre-vitamin is rather unstable and quickly isomerizes into the more stable cholecalciferol. This the inactive form of $VitD_3$ is subsequently hydroxylated in the liver into calcifediol. Finally, tis circulating metabolite reaches the kidneys, where it is converted into the active form of $VitD_3$, namely 1,25-dihydroxyvitamin D_3 ($1,25D_3$) (Fig 6) (58). The production of active $VitD_3$ is a multistep process, requiring an energetic trigger and several enzymatic steps in different organs. However, there are cell types that express all the enzymes needed to convert 7-dehydrocholesterol into active $VitD_3$. These cell types include keratinocytes, macrophages and DC, all present in the skin (59-61).

For $VitD_3$ to have a biological effect, $1,25D_3$ needs to bind to the vitamin D receptor (VDR), a nuclear hormone receptor. This results in the translocation of the receptor to the nucleus, where it can bind to vitamin D response elements and promote or inhibit transcription of vitamin D-responsive genes (58).

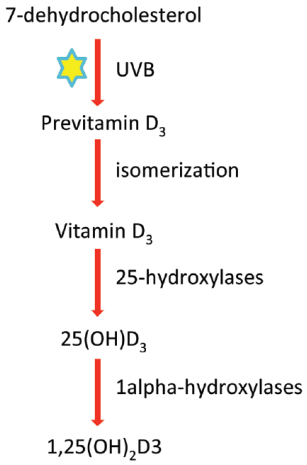

Fig 6| Generation of 1,25-dihydroxyvitamin D_3. UVB, ultraviolet B light (58).

The VDR is expressed on a variety of cells and it is through this receptor that $1,25D_3$ has immunosuppressive effects on DC (62). Penna *et al.* provided evidence that $VitD_3$ affects all major stages of the DC life cycle. When human peripheral blood monocytes were exposed to $1,25D_3$, their differentiation into imDC was completely blocked, demonstrated by the retention of the CD14 monocyte marker and the lack of expression of the CD1a DC marker. DC maturation was also inhibited when $1,25D_3$ was added during LPS-induced maturation. Even DC survival was affected by $1,25D_3$, since exposure to this substance encouraged spontaneous apoptosis of maDC. Finally, active $VitD_3$ decreased the capacity of maDC to secrete pro-inflammatory IL-12, while strongly enhancing anti-inflammatory IL-10 production. Considering the fact that DC are the primary APC

that can induce a primary T cell response, interfering with each step of DC development and function should have a profound effect on T cell phenotype and function (63). In addition, Ferreira *et al.* demonstrated that the observed changes in DC characteristics induced by $1,25D_3$ were stable after removal of the compound (64). Because of these promising facts, much research has been performed to assess the tolerizing capacity of $VitD_3$. The use of $1,25D_3$-treated DC in an EAE context has however, to our best knowledge, not been tested previously.

1.4 Preliminary data and research plan

DC are professional APC that form an important link between innate and adaptive immunity. Not only are they the main immune cells capable of inducing a primary T cell response, DC also determine the profile of that response. Depending on specific factors, DC – T cell interactions can bring about immunity or tolerance. Consequently, a disturbance in the DC compartment would lead to a serious immune imbalance, as is the case in many autoimmune diseases. Restoring the balance may therefore be beneficial for MS patients.

We hypothesized that treatment of DC with specific stimuli would create tolerogenic DC that are able to restore the immune balance in EAE mice, an animal model of MS. Since there is no consensus concerning the substances that generate DC locked in a tolerogenic state, we tested the influence of 2 stimuli (SAHA and $VitD_3$) presumed to have tolerizing properties and continued on preliminary data of a third substance.

For this purpose, mouse BM-derived DC were exposed for several time points to different stimulus concentrations. After the optimal time of exposure and stimulus concentration were determined, the assessment of DC phenotype and functionality took place. DC surface markers (CD11c, CD80, CD86, CD14) were analysed by flow cytometry and culture supernatant was examined for the secretion of several cytokines (TNF-α, IL-6, IL-23, IL-10 and IFN-γ) after LPS challenge using enzyme-linked immunosorbent assay (ELISA). The presence of lasting effects was determined by assessing DC characteristics after the removal of SAHA from culture supernatant. All experiments were conducted using the same conditions, allowing comparison between the different substances. Based on the *in vitro* results, the agent that has the most DC tolerizing potential was appointed.

The *in vitro* effects of Captopril on mouse BM-derived DC was previously investigated by our research group. These data indicated that after a 24-hour exposure to Captopril, only secretion of pro-inflammatory cytokines TNF-α and IL-6 by DC was significantly reduced. IL-10 secretion on the other hand, was increased under these conditions. However, these differences in cytokine secretion were abolished after maturation induction. These preliminary data indicated that the DC attained a more tolerogenic character after Captopril treatment. The change in cytokine secretion

towards a more anti-inflammatory profile could create a more tolerogenic microenvironment. Before we further investigated the shift in DC profile in an *in vivo* situation, DC survival after intraperitoneal (i.p.) injection was determined. Carboxyfluorescein succinimidyl ester (CFSE)-labelled DC were administered to C57BL/6 JOlaHsd (BL6) mice, after which their presence was measured in the abdominal cavity. When DC survival was ensured, myelin oligodendrocyte glycoprotein (MOG)-induced EAE mice were administered a prophylactic dose of Captopril-treated DC. Body weight and EAE score were evaluated on a daily basis to indicate whether there was a beneficial effect on EAE disease course. For further information concerning the observed effects, MOG-reactive T cells from the inguinal lymph nodes were isolated and reactivated. T cell proliferation was measured using thymidine incorporation.

2. Materials & methods

2.1 Mice

Female 7- to 11-week old C57BL/6 JOlaHsd mice were purchased from Harlan (The Netherlands). Animals were housed in the animal facility of the Biomedical Research Institute of Hasselt University. Experiments were conducted in accordance with institutional guidelines and approved by the local Ethical Committee for Animal Experiments of Hasselt University.

2.2 DC generation, culture and treatment

DC were differentiated out of BM from tibiae and femurs of BL6 mice as described previously by Lutz *et al.* and Inaba *et al.* (65, 66). Briefly, BM was rinsed with isolation medium (IM): Iscove's Modified Dulbecco's Medium (IMDM; Lonza) supplemented with 10% fetal calf serum (FCS; Gibco Invitrogen). After 10 min of centrifugation at 1500 rpm, the pellet was resuspended in IM and further diluted in red blood cell lysis buffer for 10 min at room temperature. Following 5 min of centrifugation at 1500 rpm, the pellet was washed twice in IM and resuspended in culture medium (CM): IM supplemented with 20 ng/ml GM-CSF (R&D), 10 ng/ml IL-4 (R&D) and 1% penicillin-streptomycin (P/S; GIBCO Invitrogen). Cells were counted with tryptan blue (BioChrom AG), plated at 3 x 10^6 cells/well in CM and incubated at 37°C, 5% CO_2. CM was changed at day 3, 6 and 8. On day 6, cells were harvested using 5 mM EDTA (VWR), counted and replated at 500.000 cells/well. DC or BM were treated for 24h or 10 days respectively, with 20 nM Captopril (Sigma-Aldrich), 0,5 µM or 1 µM SAHA (Cayman Chemical Company) or 10 nM $VitD_3$ (Sigma-Aldrich) and incubated with or without 100 ng/ml LPS (Sigma-Aldrich) for 24 h before harvest. For SAHA washout, DC were restimulated with 100 ng/ml LPS on day 12 of culture.

2.3 Cell surface phenotype analysis

After 10 or 13 days of culture, DC were harvested using 5 mM EDTA and a cell scraper. After 3 min of centrifugation at 4000 rpm, the pellet was resuspended in fluorescence-activated cell sorting (FACS) buffer: 10x phosphate buffered saline (PBS), 2% FCS, 0.1% sodium azide (Sigma-Aldrich) and deionized (DI) water. Cells were stained with APC-, FITC- or PE-conjugated anti-mouse antibodies for CD11c, CD80, CD86 or CD14 (BD Biosciences). After the 15 min incubation period at 4°C, samples were centrifuged for 5 min at 2000 rpm and the obtained pellets were washed twice in FACS buffer. Data was acquired using flow cytometry (BD FACSariaII) and analyses with FACSDiva.

2.4 Cytokine ELISA

The concentration IL-6, IL-10, IL-23, INF-γ and TNF-α were measured in the supernatant of the cultured DC using ELISA Ready-SET-Go! kits (eBioscience). Assays were performed following manufacturer's protocol and read at a wavelength of 450 nm (reference filter 630 nm) using a spectrophotometer (Bio-Rad Benchmark). Between each incubation period, the ELISA plate was washed with wash buffer: PBS and 0,05% Tween-20 (MERCK).

2.5 EAE induction in mice and treatment by DC

Chronic EAE was induced in BL6 mice using the MOG_{35-55}/CFA Emulsion PTX kit, purchased from Hooke Laboratories. Briefly, mice were injected subcutaneously on lower and upper back with ~0,1 mg MOG_{35-55} antigen emulsified in complete freund's adjuvant. Within 2 h after immunisation, a first dose of 1 µg pertussis toxin (PTX) was i.p. administered. This procedure of PTX injection was repeated the next day. EAE developed within 8 – 11 days after immunization. EAE disease course was evaluated according to the following score: 0 = no obvious changes in motor functions; 0,5 = partial limp tail; 1 = limp tail; 1,5 = limp tail and 'mild' weakness of hind legs (unstable walk); 2 = limp tail and weakness of hind legs (wobbly walk); 2,5 = limp tail, weakness of hind legs and no weight support, but still movement of hind legs; 3 = limp tail and complete paralysis of hind legs OR limp tail with paralysis of 1 front and 1 hind leg; 3,5 = limp tail, complete paralysis, weakness of front legs; 4 = limp tail, complete hind leg and partial front leg paralysis; 5 = complete hind and complete front leg paralysis, no movement around the cage OR mouse is spontaneously rolling in the cage OR mouse is found dead due to paralysis. For the evaluation of beneficial effects on EAE disease course, a single dose of 0,5, 1 or 2 x 10^6 DC treated with 20 nM Captopril for 24h was i.p. injected 3 days before immunisation. For DC retrieval after injection, CFSE-labelled DC were i.p. injected. Briefly, DC were diluted in PBS/0,1% bovine serum albumin (BSA; US Biological) at 20 x 10^6 cells/ml. An equal volume of 4 µM CFSE (Invitrogen) was added and the suspension was incubated for 7 min at 37°C. Cells were washed, after which the pellet was resuspended in CM: Roswell Park Memorial Institute (RPMI; Sigma-Aldrich) supplemented with 0,5% P/S, 2% heat-inactivated mouse serum (Gibco), 1% non-essential amino acids (Gibco Invitrogen) and 1% sodium pyruvate (Sigma-Aldrich). After 15 min incubation at 37°C, cells were centrifuged for 10 min at 1400 rpm. The obtained pellet was resuspended in PBS and 4 x 10^6 DC were administered per mouse. Control animals were always injected with PBS.

2.6 [^{3}H]Thymidine incorporation

Cells isolated from mouse lymph nodes or spleens were cultured in CM as described in paragraph 2.5, in the presence or absence of 20 µg/ml MOG (Hooke Laboratories). After 48h, medium was changed and 20 µl/well [^{3}H]thymidine (Perkin Elmer) was added to the cultures. The next day, cells were harvested using the MicroBeta Liquid Scintillation and Luminescence Counter (Perking Elmer) and the uptake of radioactivity was measured. Data was adapted using MicroBeta Windows WorkStation software.

2.7 Statistics

Data were statistically analysed using GraphPad (version 5, software Prism, La Jolla, CA, USA) and are presented as mean ± standard deviation (SD). The D'Agostino and Pearson omnibus normality test was used to test normal distribution. One-way analysis of variances (ANOVA) followed by a Tukey post test were used for normally distributed data sets. Data that did not pass the normality test were analysed using Kruskal-Wallis followed by Dunn post test. A P value <0,05 was considered to be statistically significant (*). P values <0,01 (**) and < 0,0001 (***) as highly significant. When 0,1<P<0,05, this was considered a trend.

3. Results

There are many studies in which specific substances have been tried out to create tolerogenic DC. Unfortunately, all of these research groups performed their experiments using different experimental conditions. This makes it difficult to appoint a consensus stimulus that induces tolerogenic DC. We therefore tested 2 stimuli presumed to have tolerizing effects, using the same conditions. By evaluating parameters such as DC phenotypic profile and functionality, the tolerogenic capability of the treated DC was assessed.

3.1 SAHA exposure lowers the CD86$^+$ DC population after LPS challenge

We investigated DC phenotypic profile to learn more about DC maturation state after SAHA treatment. For this purpose, mouse BM-derived DC were incubated with 0,5 or 1 µM SAHA in the presence or absence of LPS. The percentage CD11c$^+$ DC expressing the maturation markers CD80 and CD86 was subsequently determined using flow cytometry.

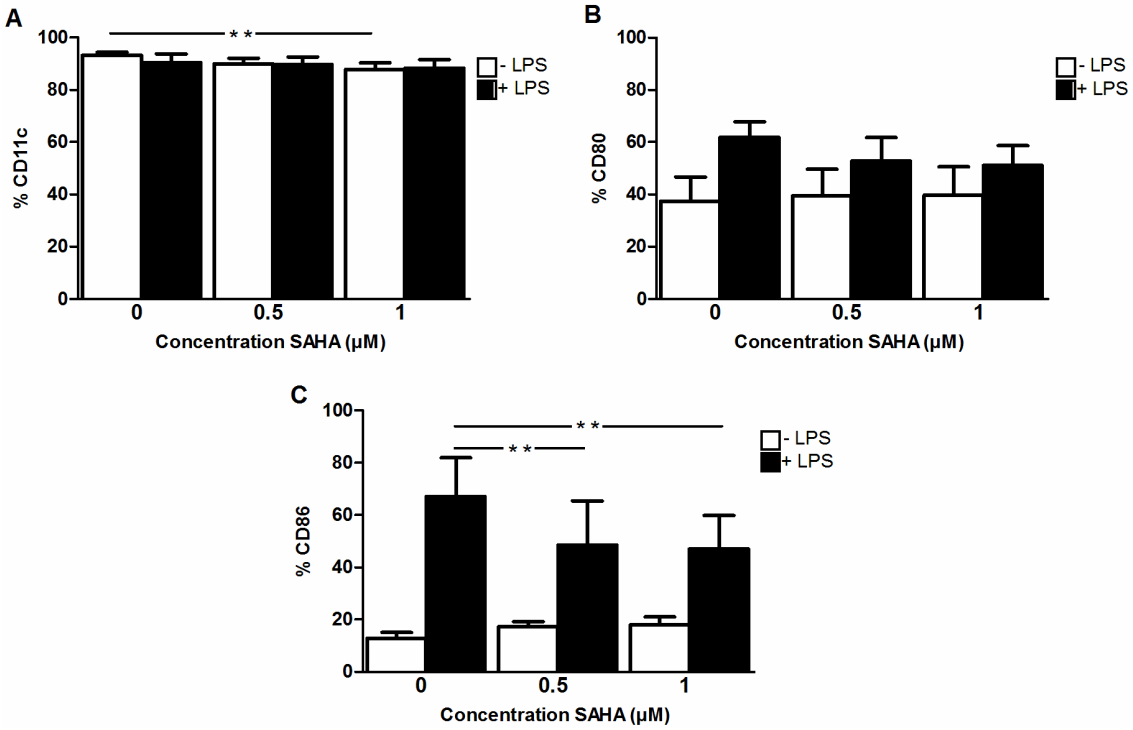

Fig 7| CD86$^+$ DC population after LPS challenge decreases when exposed to SAHA for 24 h. BM-derived DC were incubated with 0,5 or 1 µM SAHA for 24 h, in the presence (black bars) or absence (white bars) of LPS. Expression of CD11c, CD80 and CD86 was measured using flow cytometry (n = 9). (A) The amount of CD11c$^+$ DC only decreased after 1 µM SAHA exposure. Data are represented as mean percentage ±SD. (B) SAHA-treated CD80$^+$ DC demonstrated a trend towards a decreased percentage after LPS exposure. (C) After maturation induction, the percentage SAHA-treated CD86$^+$ DC decreased significantly. Data are represented as mean percentage of CD11c$^+$ DC ±SD. ** P<0,01. BM, bone marrow; DC, dendritic cell; LPS, lipopolysaccharide; SAHA, suberoylanilide hydroxamic acid.

The percentage CD11c was on average 90% in all conditions, with the exception after exposure to 1 µM SAHA. Expression decreased significantly, but remained above 85% (Fig 7A). There were no further significant changes in the LPS-free conditions. The percentage $CD11c^+$ DC expressing CD80 or CD86 increased after LPS stimulation. When incubated with 1 µM SAHA, a decreased trend was noticeable in the CD80 up-regulation, as compared to the untreated DC (P = 0,0503) (Fig 7B). The $CD86^+$ DC population after maturation induction was significantly lower in both SAHA-treated groups (P<0,01) (Fig 7C).

To investigate whether SAHA could have more profound effects on DC phenotype than observed after a 24-hour incubation period, mouse BM was treated continuously with 0,5 µM SAHA for 10 days. LPS was used to induce maturation. The percentage $CD11c^+$ DC expressing CD80 and CD86 was measured by flow cytometry. Due to the fact that we treated BM instead of DC, we also determined CD14 expression to exclude monocyte differentiation.

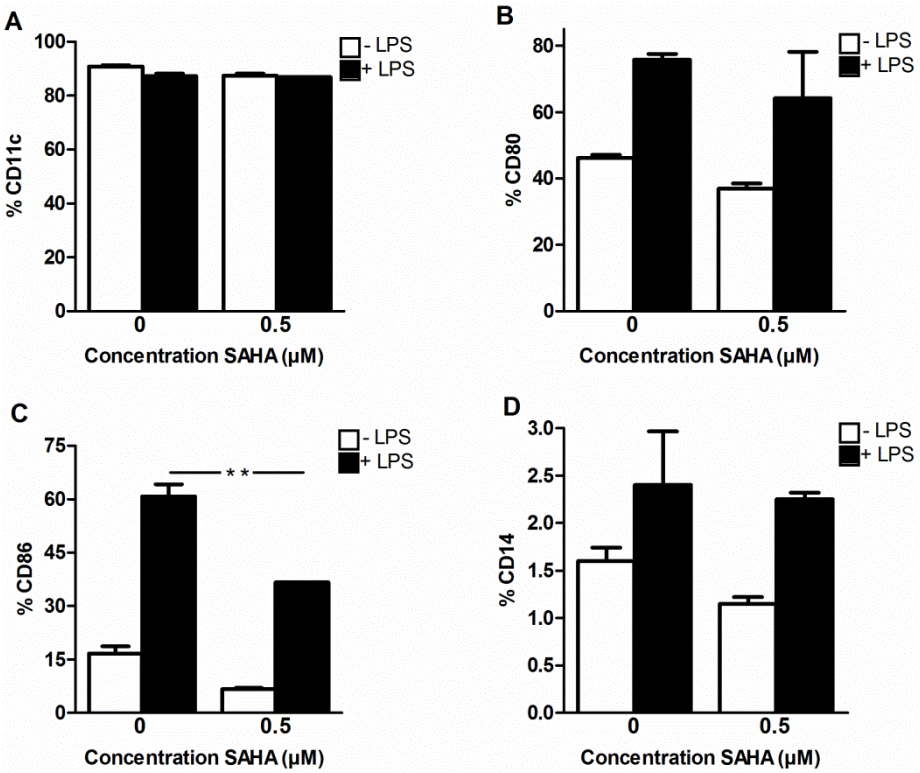

Fig 8| SAHA-treated BM demonstrates reduced amount of $CD86^+$ DC after LPS stimulation. Mouse BM was incubated with 0,5 µM SAHA. LPS was used as a maturation trigger (black bars) 24 h prior to harvest. Expression of CD11c, CD80, CD86 and CD14 was measured by flow cytometry (n = 2). (A) CD11c expression remained stable. Data are represented as mean percentage ±SD. (B - D) Only the percentage SAHA-treated $CD86^+$ DC decreased significantly after LPS exposure. Data are represented as mean percentage of $CD11c^+$ DC ±SD. **P<0,01. BM, bone marrow; DC, dendritic cell; LPS, lipopolysaccharide; SAHA, suberoylanilide hydroxamic acid.

In line with the 24-hour results, the percentage $CD11c^+$ DC remained approximately 87% at all times (Fig 8A). Both conditions again up-regulated their CD80 and CD86 expression after LPS-induced maturation. This CD86 up-regulation, in contrast to that of CD80, was significantly lower in the SAHA-treated condition (P<0,01) (Fig 8B-C). The amount of $CD14^+$ DC never exceeded 3% (Fig 8D).

3.2 24-hour SAHA exposure reduces pro-inflammatory cytokine secretion after LPS stimulation

Since DC phenotypic profile after maturation induction was altered by SAHA incubation, we next investigated whether DC functionality was affected by SAHA as well. Using ELISA, the secretion of TNF-α, IL-6, IL-23, IL-10 and INF-γ by the DC cultures described above was assessed.

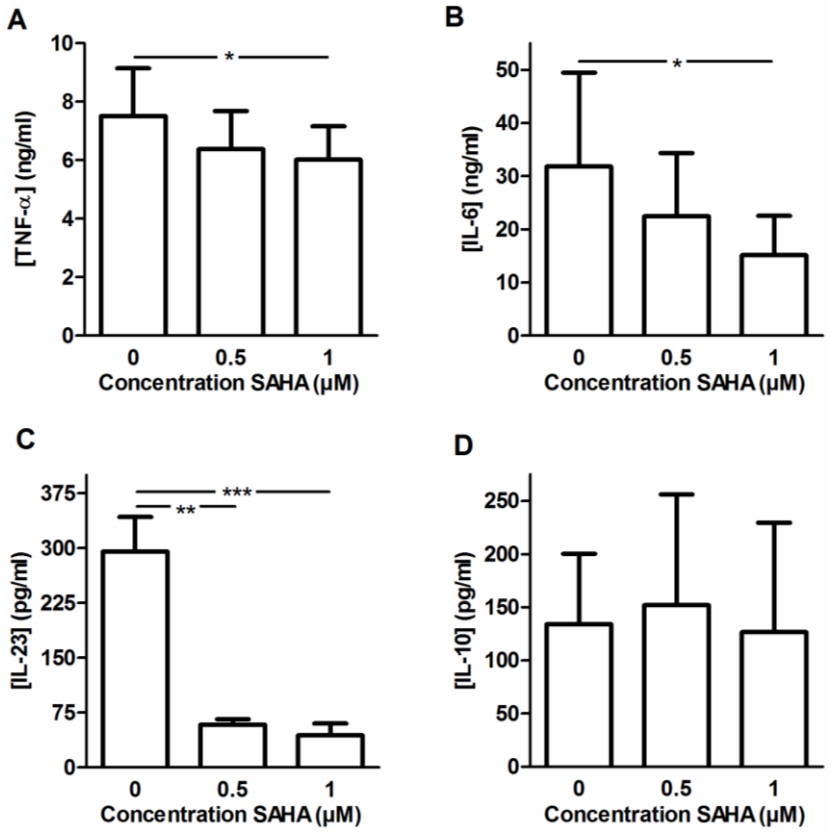

Fig 9| 24-hour SAHA exposure reduces pro-inflammatory cytokine secretion by LPS-stimulated DC. BM-derived DC were treated with 0,5 or 1 µM SAHA, after which maturation was induced by triggering of TLR-4. Supernatant was examined on the secretion of several cytokines using ELISA (n = 12 - 15). (A – C) Secretion of TNF-α, IL-6 and IL-23 by SAHA-treated DC decreased in a dose-dependent manner. (D) IL-10 secretion remained stable after SAHA exposure as compared to untreated DC. Data are represented as mean secretion ±SD. *P<0,05; **P<0,01; ***P<0,0001. BM, bone marrow; DC, dendritic cell; IL, interleukin; LPS, lipopolysaccharide; SAHA, suberoylanilide hydroxamic acid; TLR, toll-like receptor; TNF, tumor necrosis factor.

When exposed to SAHA for 24 h, DC produced significantly less TNF-α (P <0,05), IL-6 (P <0,05) and IL-23 as compared to LPS-stimulated controls (Fig 9A–C). Even though these decreases occurred in a dose-dependent manner, only IL-23 secretion demonstrated a significant reduction following both 0,5 µM (P < 0,01) and 1 µM SAHA (P < 0,0001) incubation (Fig 9C). IL-10 secretion remained at the level of the untreated DC (Fig 10D). There were no significant changes without LPS stimulation and IFN-γ remained below detection limit at all times (data not shown).

When BM was cultured in 0,5 µM SAHA for 10 days, no significant changes in cytokine secretion were measured without LPS challenge (data not shown). In the LPS-stimulated groups, TNF-α demonstrated a trend (P = 0,0621) towards a decreased secretion in the SAHA-treated condition (Fig 10).

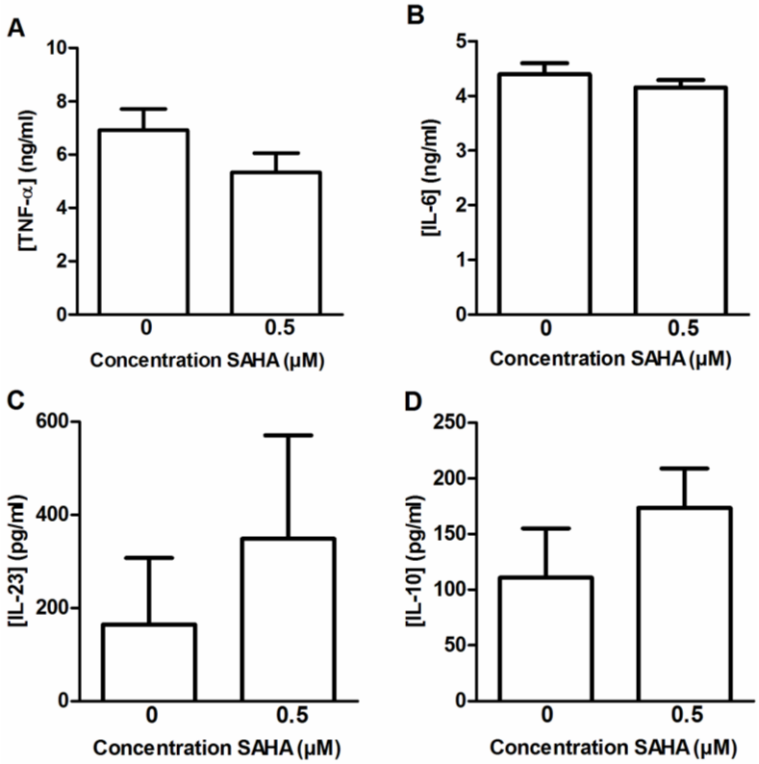

Fig 10| DC cytokine secretion after a 10-day SAHA exposure. Mouse BM was incubated with 0,5 µM SAHA for 10 days. LPS was added during the last 24 h. Supernatant was analysed on the secretion of several cytokines using ELISA (n = 3). (A) TNF-α demonstrated a trend towards a reduced secretion when incubated with SAHA. (B - D) IL-6, IL-23 and IL-10 secretion did not change significantly after SAHA exposure as compared to control. Data are represented as mean secretion ±SD. BM, bone marrow; IL, interleukin; LPS, lipopolysaccharide; SAHA, suberoylanilide hydroxamic acid; TNF, tumor necrosis factor.

3.3 Co-stimulatory reduction stable after SAHA removal

In order for SAHA-treated DC to have an influence on EAE disease course, the tolerizing effects observed after treatment should be stable after the DC are removed from the SAHA-enriched environment. We therefore stimulated BM-derived DC on day 9 of culture with 0,5 µM SAHA for 24 h, of which 2 h pre-treatment before LPS stimulation. Supernatant was washed away on day 10 and replaced with fresh CM. After a 2-day rest, cells were again stimulated with LPS for 24 h. As can be seen in Fig 11, the DC populations analysed on day 10 (⊠) increased their percentage CD80 and CD86 after a first LPS challenge. In line with the 24-hour results, this increase tended to be lower in the $CD86^+$ SAHA treatment group. After a 3-day rest in fresh CM (□), the amount of $CD86^+$ DC decreased significantly in all conditions, except in the LPS-treated control. The largest reduction was demonstrated in the SAHA-treated LPS group (P<0,01). When considering the percentage $CD80^+$ DC, the same increase was induced when the untreated condition was stimulated with LPS for the first time on day 12 or when the LPS-group was restimulated (■).

Both up-regulations corresponded to the increase seen after a first triggering on day 9. This pattern was repeated in the SAHA-treated conditions (Fig 11A). The same up-regulation patterns can also be observed in the $CD86^+$ DC population. However, the up-regulation in CD86 expression after stimulation or restimulation on day 12 remained as low as after a first challenge on day 9, and therefore lower as compared to the untreated controls (Fig 11B).

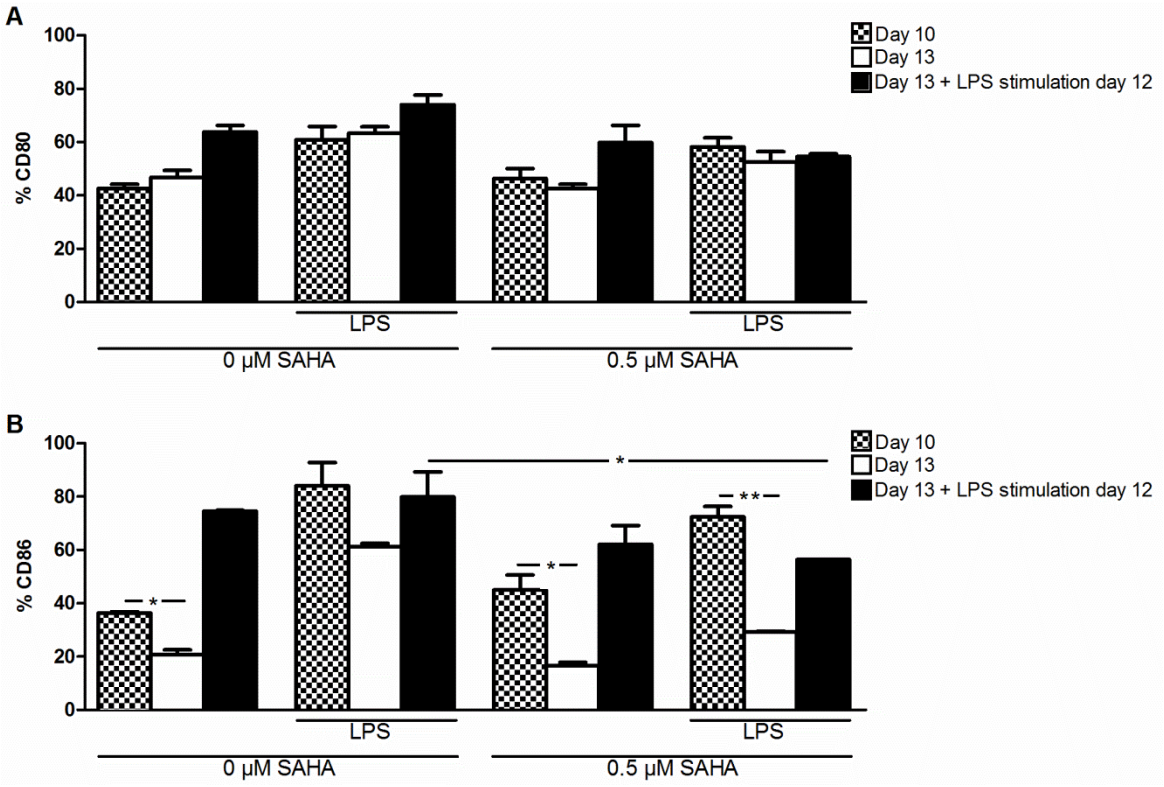

Fig 11| Decreased percentage $CD86^+$ DC remains after SAHA removal. Mouse BM-derived DC were incubated with 0,5 µM SAHA for 24 h and challenged with LPS on day 9. Supernatant was replaced by fresh culture medium on day 10, after which a 2-day rest period followed. LPS (re)stimulation occurred 24 h prior to harvest. The expression of CD80 and CD86 was measured using flow cytometry. Bars underlined with LPS represent DC maturated on day 9, blocked bars represent DC analysed on day 10, white bars represent DC analysed on day 13 and black bars represent DC that were (re)stimulated with LPS on day 12 (n = 2). (A) No significant changes in the $CD80^+$ DC population were observed in any of the conditions. (B) Three days after SAHA removal, the CD86 up-regulation after (re)stimulation on day 12 did not exceed the up-regulation seen on day 10 and tended to be lower as compared to the untreated DC. Data are represented as mean percentage of $CD11c^+$ DC ±SD. *P<0,05; **P<0,01. BM, bone marrow; DC, dendritic cell; LPS, lipopolysaccharide; SAHA, suberoylanilide hydroxamic acid.

To establish whether the changes cytokine secretion seen after a 24-hour exposure remained after SAHA removal, collected supernatant of the different conditions was analysed for the secretion of TNF-α, IL-6 and IL-10 using ELISA. In general, there were no significant changes without maturation induction on day 9 of culture (data not shown) and less TNF-α and IL-6 were secreted by SAHA-treated DC triggered on day 9 (Fig 12A – B).

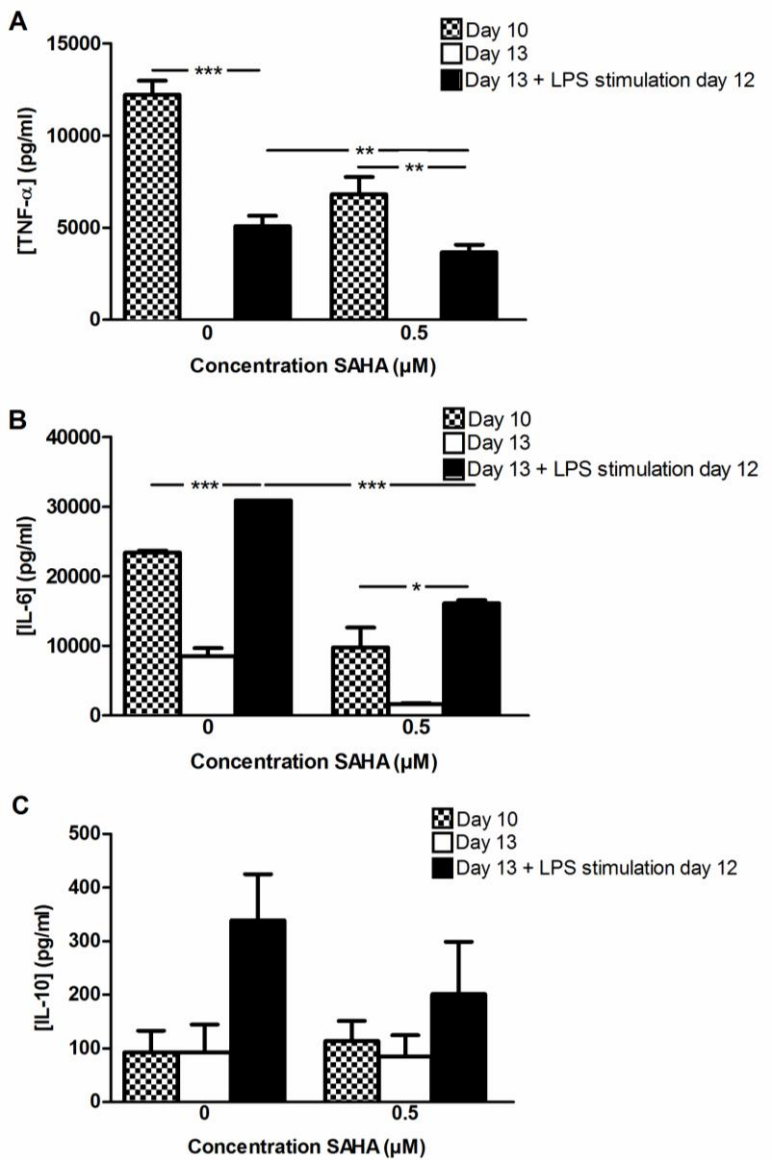

Fig 12| TNF-α secretion block remains after SAHA removal. Mouse BM-derived DC were incubated with 0,5 μM SAHA and challenged with LPS on day 9. On day 10, supernatant was replaced by fresh culture medium, after which a 2-day rest period followed. LPS restimulation occurred on day 12. Supernatant was analysed on the secretion of TNF-α, IL-6 and IL-10 using ELISA. Blocked bars represent DC analysed on day 10, white bars represent DC analysed on day 13 and black bars represent the restimulated DC on day 12 (n = 3). (A) The decrease in TNF-α secretion on day 10 after SAHA exposure was maintained after SAHA removal. (B) Reduction in IL-6 secretion after SAHA incubation was not maintained after stimulus removal, but remained below the amount secreted by the untreated DC. (C) IL-10 secretion boosted after restimulation with LPS. Data are represented as mean secretion ±SD. *P<0,05; **P<0,01; ***P<0,0001. BM, bone marrow; DC, dendritic cell; IL, interleukin; LPS, lipopolysaccharide; SAHA, suberoylanilide hydroxamic acid; TNF, tumor necrosis factor.

No more TNF-α secretion was detected after supernatant replacement without LPS restimulation. When restimulated, TNF-α secretion reoccurred, but significantly lower than the initial amounts secreted after LPS challenge on day 9 (Fig 12A). IL-6 secretion decreased more than 50% in both conditions after supernatant was replaced. Detection limit was approached by the SAHA-treated condition. In contrast to TNF-α, IL-6 secretion assumed even higher proportions than seen on day 10 in both conditions after restimulation. However, SAHA-treated DC secreted

50% less IL-6 after this restimulation as compared to the untreated control (Fig 12B). IL-10 secretion remained stable, both after SAHA exposure as after CM change. LPS restimulation induced an IL-10 boost, almost tripling the amount of IL-10 secreted. This occurred less in the SAHA-treated condition as compared to untreated DC (Fig 12C).

3.4 Active $VitD_3$ reduces the percentage $CD86^+$ DC after maturation induction

The last substance tested in this study to induce tolerogenic DC is active $VitD_3$. To be able to compare the tolerogenic capability of $VitD_3$-treated DC, all further experiments were conducted using the same experimental conditions as used for Captopril and SAHA. We investigated DC activation state after active $VitD_3$ exposure by evaluating the percentage of $CD11c^+$ DC expressing CD80 and CD86 using flow cytometry. Mouse BM was continuously exposed to 10 nM $1,25D_3$, in the absence or presence of LPS for 24 h prior to harvest.

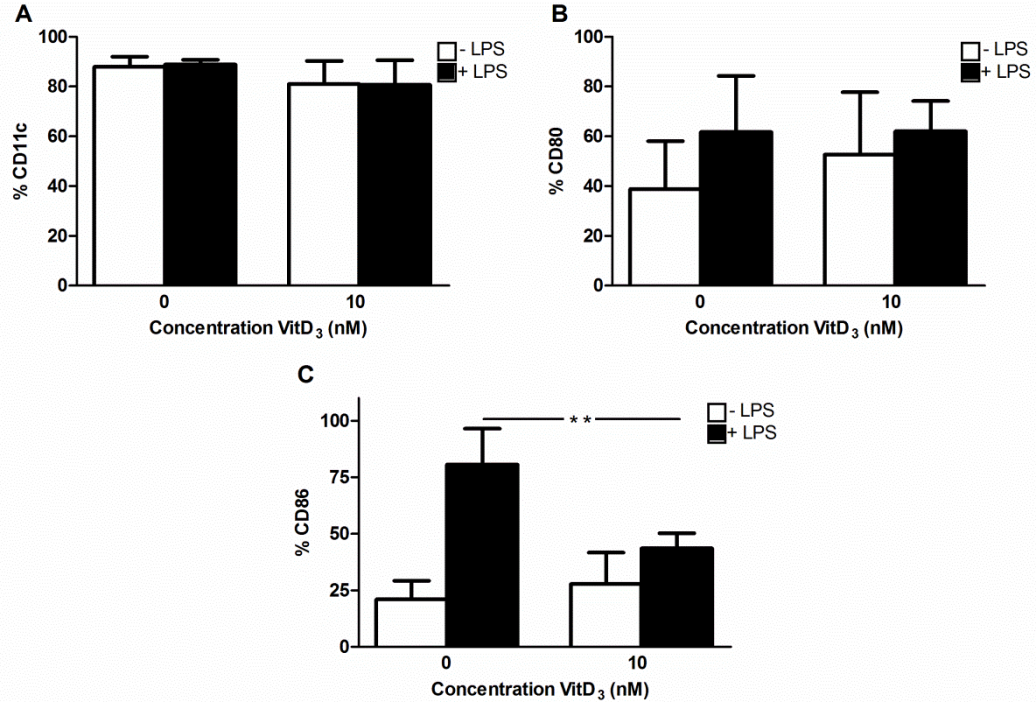

Fig 13| Active $VitD_3$ decreases the percentage $CD86^+$ DC after LPS stimulation. Mouse BM was treated with 10 nM $1,25D_3$, with (black bars) or without (white bars) LPS for the last 24 h of culture. Expression of CD11c, CD80 and CD86 was determined using flow cytometry (n = 4). (A) The percentage $CD11c^+$ DC remained approximately 85%. Data are represented as mean percentage ±SD. (B) The amount of $VitD_3$-treated DC expressing CD80 remained at the untreated level. (C) $VitD_3$-treated $CD86^+$ DC decreased significantly after LPS stimulation. Data are represented as mean percentage of $CD11c^+$ DC ±SD. **P<0,01. $1,25D_3$, 1,25-dihydroxyvitamin D_3; BM, bone marrow; DC, dendritic cell; LPS, lipopolysaccharide; $VitD_3$, Vitamin D_3.

The percentage CD11c$^+$ DC was on average 85%, independent of VitD_3 or LPS stimulation (Fig 13A). CD80 expression did not change significantly (Fig 13B). On the other hand, the amount of CD86$^+$ DC increased after LPS challenge in the unstimulated condition. In the VitD_3-treated condition, this LPS-induced up-regulation was only 50% of the untreated condition (Fig 13C).

3.5 Administrated DC survive i.p. injection

One of the goals of this study was to treat EAE mice with *in vitro* generated tolerogenic DC to influence EAE disease course. In order for DC to have an effect on mice, they should at least survive the i.p. injection process and be able to migrate out of the abdominal cavity. We labelled BM-derived DC with CFSE and administered a single dose of 4 x 10^6 CFSE-DC to BL6 mice by i.p. injection. Control mice were injected with unlabelled DC or PBS. After 1, 3 and 20 h, the abdominal cavity was rinsed with EDTA and the collected peritoneal cell suspension was analysed by flow cytometry. CD11c in combination with CFSE was used to discriminate between endogenous and administered DC.

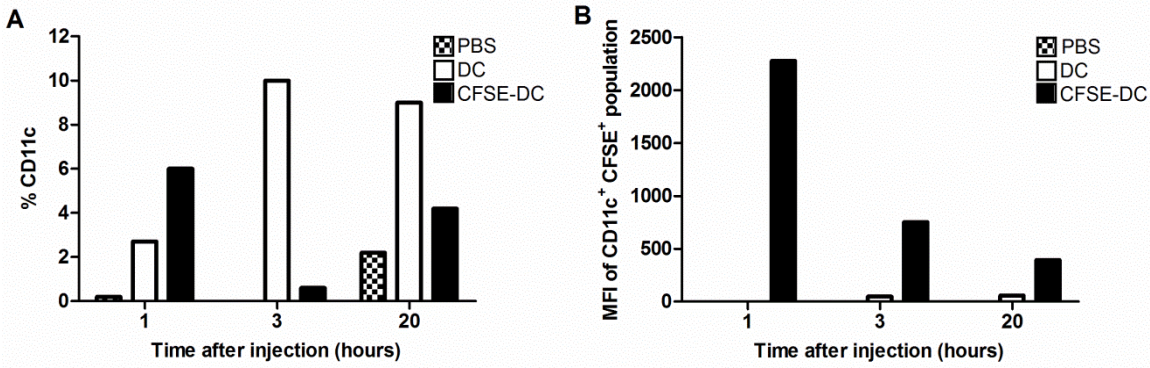

Fig 14| Administrated DC survive i.p. injection. Mouse BM-derived DC were labelled with CFSE. Mice were injected intraperitoneally with a single dose of PBS, DC or CFSE-DC. After 1, 3 and 20 h, the abdominal cavity was rinsed and the collected cell suspension was analysed using flow cytometry (n = 3). (A) Presence of exogenous DC in peritoneal cavity. Data are represented as percentage. (B) MFI signal of the CD11c$^+$CFSE$^+$ DC population decreased in a time-dependent manner. Data are represented as MFI of the CD11c$^+$CFSE$^+$ DC population. BM, bone marrow; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; MFI, mean fluorescent intensity; PBS, phosphate buffered saline.

One, 3 and 20 h after injection, both exogenous DC and CFSE-labelled DC were isolated from the abdominal cavity as compared to the PBS-injected control group (Fig 14A). For confirmation, we checked the CFSE mean fluorescent intensity (MFI) of the CD11c$^+$ population. This decreased in a time-dependent manner, but was still present 20 h after injection (Fig 14B).

3.6 Highest dose Captopril-DC influences EAE disease course

Continuing on *in vitro* preliminary data of Captopril, we further explored the effect of Captopril on DC in an *in vivo* situation. Three days before chronic EAE was induced, BL6 mice were i.p. injected with a single dose of 0,5 (G2), 1 (G3) or 2 x 10^6 (G4) Captopril-treated DC. Control animals were injected with PBS (G1). Body weight and EAE score were determined on a daily basis until the end of the experiment. As can be seen in Fig15A - B, all groups have the same mean body weight and EAE progression pattern. G2 had the lowest mean body weight and the quickest deterioration during EAE onset. G4 demonstrated the earliest EAE onset at day 11, but had an overall EAE score of 2,5.

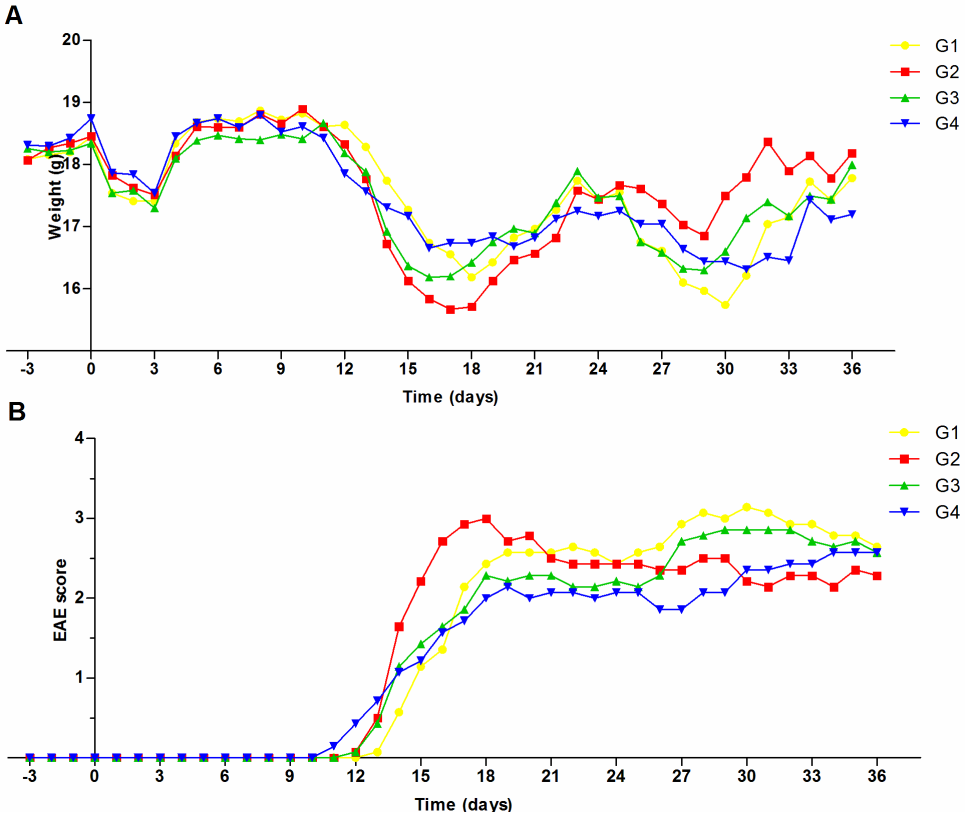

Fig 15| Captopril-treated DC had no influence on mean body weight or EAE disease course. Three days before chronic EAE was induced, randomly chosen mice were injected intraperitoneally with PBS (G1) or a single dose of 0,5 (G2), 1 (G3) or 2 x 10^6 (G4) Captopril-treated DC. Body weight and EAE score were determined on a daily basis (n = 7). (A) All groups followed the same weight pattern. Data are represented as mean body weight. (B) No differences in EAE disease course between groups. Data are represented as mean EAE score. DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; PBS, phosphate buffered saline.

When looking at each mouse individually, EAE prevalence (cut-off EAE score = 1,5) was 7/7, 6/7, 7/7 and 4/7 for G1 to G4 respectively. G4 was the only group that had a mouse that did not develop EAE and 2 mice that ended the experiment with an EAE score below 2 (Fig 16).

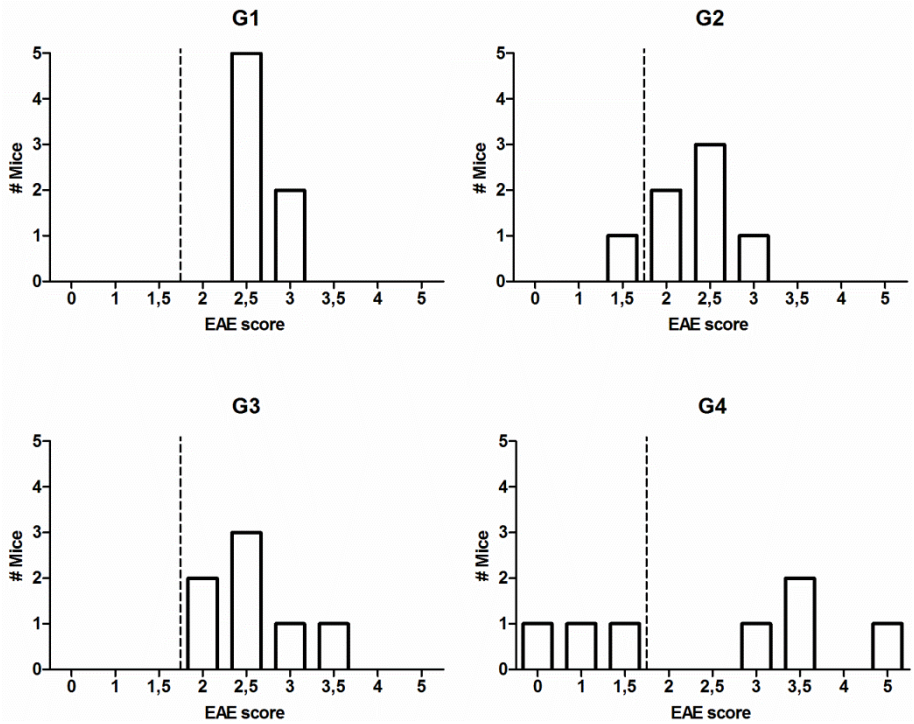

Fig 16|EAE prevalence lowest in highest dose group. Mice were treated as described in Fig 15. EAE score represented was determined on the day of sacrifice. For calculation of EAE prevalence, cut-off score was set at 1,5 (n = 7). Only G4 had an EAE prevalence of less than 6 out of 7 mice. Data are represented as number of mice per EAE score. EAE, experimental autoimmune encephalomyelitis.

3.7 MOG-reactive T cells present in highest Captopril-DC dose group

To obtain further information on the effects on EAE disease course seen after DC injection, we sacrificed the 3 mice from G4 with an EAE score $\leq$ 1,5 and 3 randomly chosen mice from the PBS group, and isolated their inguinal lymph nodes. Lymph node cell suspensions were restimulated with MOG. Based on thymidine incorporation, radioactivity uptake was measured and the stimulation index (SI) was determined. All 3 mice of G1 represented in Fig 17 had an EAE score of 2,5 at the time of sacrifice. Mouse 1 demonstrated the lowest SI of 0,9. The SI of the other 2 mice was on average 9. Mice 2, 3 and 6 of G4 had EAE scores of 1,5, 1 and 0 respectively. Mouse 6, who had the lowest EAE score of all, also had the lowest SI of the group with 1,6. Mean SI of the other 2 mice was 6,7.

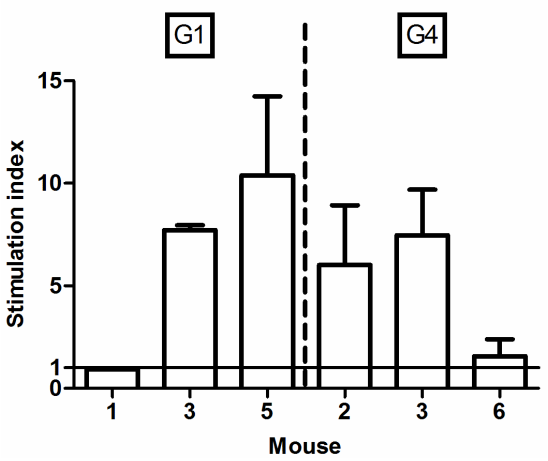

Fig 17| Presence MOG-reactive T cells at highest Captopril-DC dose group. Randomly chosen mice were injected intraperitoneally with PBS (G1) or 2 x 10^6 (G4) Captopril-treated DC, 3 days before chronic EAE was induced. After 36 days, mice were sacrificed and T cells present in lymph node cell suspension were reactivated with MOG. By means of thymidine incorporation, MOG-reactivity was determined (n = 7). Mice of both G1 and G4 possessed MOG-reactive T cells. Data are represented as stimulation index. DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein PBS, phosphate buffered saline.

4. Discussion

As professional APC, DC have the ability to not only evoke primary T cell responses, but also to shape the type of response. Depending on their maturation state and the co-stimulatory signals they send, DC can play a role in both immunity and tolerance (13). This capability turns DC into very interesting immune modulating agents. It is therefore no surprise that anti-cancer DC vaccines are already being tested in the clinic (30). DC could also be of great value in autoimmune diseases. When the immune balance is disturbed, for instance in MS, tolerogenic DC might be able to tip the balance back to normal (16). At the moment, several research groups are attempting to generate tolerogenic DC, but practically none of them performs their research using the same experimental conditions. This makes it difficult to appoint a stimulus consensus. In this study, we tried to generate tolerogenic DC by means of SAHA or active $VitD_3$ incubation. Because all experimental conditions were kept the same, the tolerizing capability of the different substances could be compared. In a second part, we continued on *in vitro* preliminary data of a third stimulus, the ACE-inhibitor Captopril.

DC maturation state is an important factor in mediating the outcome of DC - T cell interactions. One of the characteristics of imDC is that they express low levels of co-stimulatory molecules. This contributes to their tolerogenic role, since lack of ligation of dendritic CD80/CD86 to CD28 on T cells results in autoreactive T cell deletion, anergy or the induction of T_{reg} cells (13). Therefore, we first evaluated DC phenotypic profile after SAHA exposure, in particular the up-regulation of CD80 and CD86 after Toll-like receptor 4 (TLR-4) triggering. In accordance with the preliminary data of Captopril, which had no influence on DC phenotype after a 24-hour exposure, SAHA also did not change the amount of $CD80^+$ or $CD86^+$ imDC. On the contrary, SAHA-treated DC did demonstrate an altered phenotypic profile after LPS challenge. We did not observe a full phenotypic maturation block, but the amount of $CD86^+$ DC decreased significantly. Data concerning the up-regulation of DC co-stimulatory molecules after SAHA exposure are rather variable between studies. Bode *et al.*, like us, demonstrated a reduced CD86 percentage in murine BM-derived DC, while others observed a full CD86 up-regulation block in BM-derived DC of BALBc mice or even a lack of effect in human monocyte-derived DC (56, 57, 67). This variation is probably due to small differences in DC generation protocol and the different species studied, again emphasizing the need of a consensus. From our results, we can conclude that a 24-hour treatment with SAHA generates DC that are semi-mature in terms of DC phenotype after LPS stimulation. Although we did not look into the mechanism of action, a possible explanation for our results can be found in a recent study indicating that SAHA requires *de novo* protein synthesis to exert its activity (68). We

suggest that without the LPS signal, there is no activation of pathways leading to *de novo* protein synthesis. Consequently there are no targets for SAHA to inhibit. This *de novo* requirement also suggests that SAHA has a direct or indirect effect on gene transcription and not e.g. on protein translation or degradation.

Because of the promising effects of SAHA on DC phenotype, we next investigated whether an extended exposure time would lead to a full phenotypic maturation block. This required treatment of BM instead of fully differentiated imDC. Considering the fact that HDAC substrates are abundant in each cell type and involved in a multitude of biological processes, usage of a HDACi in stem cells could possibly hinder normal DC differentiation (51). To ensure that this was not the case, expression of CD11c and CD14, a DC and monocyte marker respectively, were measured. CD11c expression was not influenced, as approximately 87% of all cells expressed the marker in every condition. A 10-day SAHA incubation also did not affect the amount of $CD14^+$ cells. There was a slight CD14 up-regulation after LPS stimulation, but merely due to the autocrine effect LPS has on the marker, which is part of the LPS-receptor complex (69). We conclude that BM treatment with SAHA does not distort normal DC development. In line with these results, Peart *et al.* demonstrated that only 22% of the genes within the genome of CEM cells, human T cell lymphoblast-like cells, were affected by SAHA. The majority of these genes being involved in proliferation and cell survival, not cell differentiation. Due to the lack of multiple defects present in cancerous cells, normal cells are more resistant to HDACi (70). This implies that even less genes are affected in BM cells, increasing SAHA's specificity and decreasing the chance of unwanted side effects.

Next, DC phenotypic profile was determined after maturation induction. Keeping in mind that the experiment was only carried out once, a 10-day SAHA treatment did not fully block co-stimulatory up-regulation. There were no effects on CD80 expression, but the decrease in $CD86^+$ DC remained. Although further repeats should be performed for the results to be conclusive, it seems that a 24-hour exposure is sufficient for SAHA to partially prevent the CD86 up-regulation after LPS stimulation. The relatively rapid reversibility of HDAC inhibition and the fact that SAHA needs *de novo* protein synthesis to have an effect, confirm the assumption that SAHA can only affect maturation at the time of induction, in this case by triggering TLR-4 (71).

Besides co-stimulatory molecules, tertiary signals in the form of cytokines are also important for polarizing T cells into different types of effector cells (57). Supernatant of the cultures exposed to SAHA for 24 h or 10 days were analysed for TNF-α, IL-6, IL-23, IL-10 and INF-γ secretion. After a 24-hour treatment, all pro-inflammatory cytokines, besides INF-γ, decreased significantly after LPS challenge when 1 µM SAHA was used. INF-γ, which is normally not secreted by $CD11c^+$ DC, was indeed not detected in any of the conditions. The only anti-inflammatory cytokine analysed

remained unaffected. On the contrary, preliminary Captopril data demonstrated that TNF-α and IL-6 secretion decreased, while IL-10 secretion increased in the absence of LPS. As opposed to DC phenotype, the cytokine profile induced by SAHA is confirmed by most studies. Both in murine BM-derived and human monocyte-derived DC, the secretion of these and other pro-inflammatory cytokines decreased in LPS challenged DC after SAHA incubation (56, 57). Although the mechanism responsible for these cytokine reductions remains poorly defined, Bosisio *et al.* and others demonstrated that SAHA inhibits the production of specific cytokine messenger ribonucleic acids (mRNA) by disturbing transcriptional activation and the binding of transcription factors to promoter sequences, and by altering chromatin remodelling (55, 57, 67). This again supports our previous assumption that SAHA will most likely affect gene transcription.

When BM was treated with SAHA, the results do not match the 24-hour data. There were no decreases in cytokine secretion after LPS challenge. These data conflict with the notion that some genes need prolonged SAHA exposure to become significantly inhibited as compared to other genes (67). To our knowledge, we are the first to treat mouse BM with SAHA while inducing DC differentiation. We therefore suggest that the pathways ultimately leading to cytokine secretion build up a resistance to SAHA after prolonged incubation (72). If pathways are active in early stages of DC development, inhibition of specific HDAC could induce HDAC overexpression. When these pathways are reactivated at a later time point, an increased amount of SAHA would be required for inhibition to occur. However, the experiment was carried out only once and more repeats should be performed before a firm conclusion can be made.

For future use in MS patients, it is important that the acquired DC profile is maintained after administration. If SAHA-treated DC would reacquire the ability to fully mature in time and become immunogenic, this could have adverse effects on the immune balance. We therefore tested whether the decreased percentage of $CD86^+$ DC and the reduced pro-inflammatory cytokine secretions after 24-hour incubation with SAHA lasted after stimulus removal. Whether LPS challenge occurred right after pre-treatment or 2 days after SAHA removal, the increase in $CD86^+$ DC was the same and lower as compared to untreated DC. Not even restimulation could elicit a higher response. This was also true for TNF-α secretion. IL-6 secretion did rise above the secretion seen after LPS exposure on day 9 when restimulated, but was only 50% of the secretion without SAHA incubation. The research group of Reddy *et al.* performed a similar SAHA removal experiment, in which SAHA-treated murine DC were washed and allowed to rest for 36 – 40 h prior to LPS stimulation. In contrast to our findings, they did not observe a significant decrease in TNF-α secretion after stimulus removal and concluded that SAHA had marked but transient effects on DC (56). For SAHA to have lasting effects, it should be present inside the cell at the time of activation, since substrate-binding of SAHA is reversible. However, not much is known

about SAHA extrusion or metabolism, so further experiments should give more insights into the turnover of SAHA in DC and potential lasting effects on DC profile.

The second substance we investigated in this study was active $VitD_3$. There are many promising effects of $1,25D_3$ on DC described (62-64, 73). Therefore, a pilot experiment was set-up in which mouse BM was exposed to $1,25D_3$ for 10 days and challenged with LPS. As with SAHA and Captopril, DC phenotypic profile was analysed. Despite several studies demonstrating a broad effect of $1,25D_3$ on DC phenotype, affecting CD86, CD80, CD40 and other maturation markers (63, 64), we only observed a decrease in the amount of $CD86^+$ DC after maturation induction. CD11c expression was as high as the untreated control, indicating DC development. Penna *et al.* and others contradict such notion, demonstrating that $VitD_3$ inhibits DC differentiation (63, 73). More DC markers should therefore be tested before continuing the investigation whether active $VitD_3$ can generate tolerogenic DC.

We conclude, based on the *in vitro* data generated in this study and in a previous investigation with Captopril, that SAHA has the most DC tolerizing potential. Even though Captopril switched DC profile towards a more anti-inflammatory one, this effect was abolished after maturation induction. SAHA on the other hand, had tolerizing effects on both phenotype and functionality. In addition, SAHA removal indicated lasting effects important for the therapeutic potential of DC in autoimmune diseases. Despite the promising features described, much more research has to be done to see whether active $VitD_3$ is the most potent DC tolerizing substance.

In a second part, we hypothesized that administration of the treated DC would restore the immune balance in EAE mice. For DC to have a real therapeutic potential, basic requirements need to be fulfilled. One of those requirements is the ability of DC to survive the administration process and at least the first day in the host body. Using CFSE-labelled and unlabelled DC, we have proven that i.p. injected DC were present in the mouse abdominal cavity for at least 20 h after administration. This was accomplished by comparing the amount of $CD11c^+$ cells found in the injected groups and in the control group. We confirmed this finding by checking the MFI of the $CD11c^+CFSE^+$ group. This signal was present at all 3 time points. There was however a time-dependent decrease in MFI. Whether this was due to DC proliferation, loss of the CFSE-label or even DC cell death needs to be further investigated.

Because we demonstrated that DC survive administration and because the *in vitro* preliminary data of Captopril were available, we continued on these results by performing a pilot study to test whether Captopril-treated DC have a beneficial effect on mouse EAE disease course. Different DC doses, ranging from 0,5 to 2 x 10^6 DC (group G2 to G4 respectively), were administered to female BL6 mice as part of optimization. This was performed prophylactically in order to obtain the biggest impact on EAE disease course. If injection had taken place at a later time point, e.g. after

EAE onset, the full inflammatory response and ongoing neurodegeneration might have been too overwhelming for the smaller DC doses to exert an effect. Thirty-six days after EAE induction, mice were sacrificed and at that time point, only G4 had an EAE prevalence of 4/7 mice. The other groups displayed a prevalence of 6/7 mice or higher. This difference in prevalence did not seem to be the result of a disturbed EAE induction. All 4 groups displayed the typical characteristics of EAE development, such as sudden weight loss and increase in EAE score. We therefore looked at the presence of MOG-reactive T cells in the inguinal lymph nodes of the 3 mice of G4 that had an EAE score below 2. The only mouse of G4 that did not develop EAE, also lacked MOG-reactive T cells in her lymph nodes. This could indicate a failed EAE induction. However, because the lymph nodes were isolated at the end of the experiment, most of the MOG-reactive T cells might have already migrated towards the CNS. This lack of MOG-reactive T cells was also present in 1 mouse from the control group (G1). When these 2 mice were left out, the mean SI of the 2 mice of G4 was 6,7 in contrast to a SI of 9 for G1. The 2 mice of G4 that had MOG-reactive T cells demonstrated the latest EAE onset and the best EAE onset recovery. Therefore, Captopril-treated DC might have had an effect on EAE disease course in these 2 animals, but DC tolerogenic properties were insufficient to fully block EAE development. This further confirms the *in vitro* data, in which Captopril-treated DC lost their tolerogenic profile after maturation induction. Because SAHA demonstrates far more promising *in vitro* results, continuing with this stimulus is a better option in terms of investigating beneficial effects on EAE disease course.

5. Conclusion & synthesis

DC have the ability to shape the profile of an immune response by polarizing T cells into different effector cell types. This makes them interesting targets for immune modulation. In the case of an autoimmune disease like MS, tolerogenic DC could restore the immune balance by inducing autoreactive T cell deletion or anergy, or by expanding the T_{reg} cell compartment. This requires the generation of DC with tolerogenic characteristics, even after maturation induction. To date, there is no stimulus consensus that guarantees the production of this type of DC. That is why we tested the tolerizing capability of 2 substances and continued on *in vitro* preliminary data of a third. All experimental work was performed using the same conditions, allowing stimulus comparison.

We found that imDC incubation with the HDACi SAHA generates semi-mature DC after LPS stimulation, with decreased expression of co-stimulatory molecules and reduced secretion of pro-inflammatory cytokines. Pre-treatment right before LPS challenge was sufficient for SAHA to induce these effects. Although we did not look into the mechanism of action, it is becoming generally accepted that SAHA has direct and indirect effects on gene transcription. This would explain why SAHA only had an impact after TLR-4 activation and why a prolonged exposure to the inhibitor did not lead to a greater effect. Without a maturation trigger, there is no *de novo* protein synthesis and therefore no gene transcription for SAHA to inhibit. Whether the semi-mature DC induced by SAHA are capable of suppressing autoreactive T cells and therefore have a beneficial effect on EAE disease course, needs to be further elucidated.

BM treatment with SAHA did not lead to a full DC maturation block, but it did prove that SAHA does not impair normal DC development. All HDACi demonstrate a degree of substrate specificity, making SAHA a safer stimulus, affecting a limited number of genes. Extended incubation did abolish all effects seen on cytokine secretion. Further investigation into the SAHA mechanism of action and SAHA resistance are therefore needed. This will give more insights into the specific pathways that are affected by the HDACi. SAHA metabolism by DC is also an unknown factor. Experiments that clarify whether SAHA is still present in the cell after removal of the stimulus would help us understand why we observed lasting tolerizing effects and other research groups did not.

At this stage, we are only able to truly compare these SAHA results with the *in vitro* preliminary date of the ACE-inhibitor Captopril. This latter stimulus was only able to affect cytokine secretion in the absence of LPS stimulation. SAHA on the other hand, affected both phenotype and functionality, reducing the immunogenic character that maDC normally have. These decreases

also seem to last for at least 3 days after stimulus removal. We conclude that SAHA has the greatest DC tolerizing capability. For active $VitD_3$, we can only conclude that BM treatment did not disturb DC differentiation and that expression of the co-stimulatory molecule CD86 was significantly reduced after a 10-day incubation. For $1,25D_3$ to become the most tolerizing substance, it first needs to follow the different steps Captopril and SAHA went through, proving at each stage that it generates more tolerogenic DC than SAHA did.

We hypothesized that DC treated with specific stimuli are able to restore the immune balance in EAE mice. Before we tested this hypothesis, we first of all demonstrated that i.p. injected DC survive administration and are present in the mouse abdominal cavity for at least 20 h after injection. Whether they are then killed or migrate out of the abdomen still needs to be clarified. If they leave the abdomen, it would be interesting to determine where they specifically migrate to and how long they survive in the host body.

Finally, we tested our hypothesis using Captopril-treated DC. Although we believe that the EAE disease course of 2 mice of the highest dose group was affected, we conclude that the tolerizing capability of Captopril is insufficient to create tolerogenic DC that are able restore the immune balance in EAE mice. The *in vitro* effects seen after SAHA incubation are far more promising. Future *in vivo* experiments should therefore be conducted using SAHA-treated DC.

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