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9-cis-retinoic acid modulates dendritic cell differentiation towards a regulatory T cell inducing phenotype

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ABBREVIATIONS

Ab	antibody
ACK	Ammonium-Chloride-Potassium buffer
ANOVA	analysis of variance
APC	antigen presenting cell allophycocyanin
ATRA	all-trans retinoic acid
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
chall.	challenged
CHE	chronic hand eczema
CHS	contact hypersensitivity
ConA	Concanavaline A
CTLA4	cytotoxic T-lymphocyte antigen 4
ctrl	control
CXCL	Chemokine (C-X-C motif) ligand
DC	dendritic cell(s)
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
cDNA	copy DNA
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Fig.	figure
FITC	Fluorescein-Isothiocyanat
Foxp3	Forkhead p3 transcription factor
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICOS-L	inducible co-stimulatory ligand
IL	interleukin
iTreg	inducible regulatory T cell
kDa	kilo Dalton
LC	Langerhans cell(s)

LPS	lipopolysaccharide
LSM	lymphocyte separation medium
M-CSF	Macrophage Colony Stimulating Factor
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NO	nitric oxide
OPN	osteopontin
OPN -/-	OPN knockout
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1L	programmed death ligand 1
PE	phycoerythrin
R	rest
RA	retinoic acid
RALDH	retinal dehydrogenase
RAR	Retinoid-Acid-Receptor
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RXR	Retinoid-X-Receptor
SEM	standard deviation of mean
sens.	sensitized
TC	T cell(s)
TCR	T cell receptor
TGF	tissue growth factor
Th1	T helper cell group 1
Th2	T helper cell group 2
Th17	T helper cell group 17
TNCB	trinitrochlorobenzene
TNBS	trinitrobenzenesulfonic acid
TNF	tumor necrosis factor
Treg	regulatory T cell(s)
TREM-1	triggering receptor expressed on myeloid cells-1
VDR	vitamin D receptor

wt	wild type
9cisDC	9-cis retinoic acid matured DC
9cisRA	9-cis-retinoic acid
9cisRA 3	9-cis-retinoic acid in a concentration of 3 ng/ml
9cisRA 30	9-cis-retinoic acid in a concentration of 30 ng/ml

1 INTRODUCTION

1.1 Chronic hand eczema

Hand eczema is a common disease in industrialized countries and is the most frequent occupational disease of the skin [50]. Five to seven percent of hand eczema patients develop a chronic form, defined as persisting for more than six months despite medical treatment [46]. Approximately 40% of hand eczemas are caused by delayed type allergy [133].

Risk factors are typically those that lead to a decreased skin barrier function, for example irritation through work in a moist environment, contact with strong allergens or toxic materials, and, amongst other chemicals, chrome, nickel and biocides [56]. Endogenous factors are also important such as genetic predisposition or a history of atopic disease with dry skin [90]. Usually a combination of factors eventually cause disease to occur.

Hands are utilized as a means of communication as well as being essential for mechanical work and daily activities, thus affected individuals suffer from severely decreased quality of life. Their disease impacts occupationally, socially, and psychologically [36] [37]. Limitations to working life, increased sickness leave and numerous health-care appointments all add up and cause significant socioeconomic cost [36].

1.2 Pathogenesis of delayed type allergy

In cell-mediated, type IV allergy, the sensitization and elicitation phase need to be distinguished: In the so called sensitization phase, a hapten penetrates the epidermal barrier and encounters antigen presenting immune cells of the skin. To be recognized as an antigen, a hapten usually has to bind to an epidermal protein. Recently, it has been reported that haptens themselves are able to activate local Toll-like receptors (TLR), induce reactive oxygen species, and activate the NLRP3 inflammasome in keratinocytes, which is followed by secretion of proinflammatory cytokines, such as IL-1 β and IL-18 [103]. These cytokines promote skin dendritic cell

(DC) maturation and migration [101] [32]. Furthermore, hapten-protein complexes are processed and presented by antigen presenting cells (APC) of the skin, namely Langerhans cells (LC), located in the epidermis, and dermal dendritic cells [10]. For sensitization, strong DC activation through innate immune receptors, especially the Toll-like receptors TLR2 and TLR4 [102], and nucleotide-binding oligomerization domain-like receptor (NOD-like receptor) activation is essential [72]. Antigen loaded APC then migrate to the skin draining lymph nodes where they interact with naïve T cells (TC), inducing the differentiation of allergen specific TC, which then recirculate and remain within the skin to await antigen encounter [84].

During the elicitation phase, reapplication of the allergen attracts allergen-specific effector TC, which are activated by allergen-presentation through APC, including LC, DC and keratinocytes [122]. Local skin inflammation is elicited by interaction of DC, keratinocytes and cytotoxic effector TC, Th1, Th17 cells. In the absence of antigen specific Treg mediated inhibition, full blown allergic inflammation develops [122].

Characteristically allergic contact eczema develops within 12 - 24 hours after allergen challenge. Importantly, once-established allergen specific sensitization cannot be erased and usually persists for life. Hence, therapeutic approaches mainly aim at allergen avoidance and immunosuppressive symptomatic therapy [45]. Future focus for the treatment of allergic contact dermatitis should be the reversal of sensitization through induction of specific tolerance.

1.3 Central components of the skin immune system

1.3.1 Dendritic cells

DC belong to the group of specialized antigen presenting cells. They are localized at the bodies outer barrier: Skin and mucosal tissues [10]. DC develop from myeloid lineage stem cells and, in an immature state, leave the bone marrow to circulate in the peripheral blood and eventually migrate into the parenchyma of various organs [9] [22]. Immature DC are characterized by their capacity to respond to numerous inflammatory signals, linking the innate and the adaptive immune system [40]. Immature DC are in a resting condition and are functionally activated upon cytokine

activation and antigen encounter. As DC mature, they take up antigens by phagocytosis, increase their surface area for antigen presentation and up-regulate a pattern of co-stimulatory molecules on their surface. Thereby they become optimal APC to induce antigen specific TC. Accompanied by this process is the loss of their ability to capture and process new peptides [63].

DC that have processed antigen peptides down-regulate their E-cadherin receptors and up-regulate CCR7, thus becoming perceptive of TC expressed chemokines from draining lymph nodes [22]. After migration to draining lymph nodes, processed antigens are presented through the MHC to TC. The outcome of antigen presentation depends on the expression of co-stimulatory surface molecules such as DC-expressed CD40, CD80, CD86. An interaction of CD86 and CD80 with costimulatory CD28, as well as the co-inhibitory CTLA4 on TC plays an important role: CD80, CD86 ligation with CD28 induces TC growth and survival. Ligation with CTLA4 induces TC suppression [132]. Interestingly, an increase of CTLA4 surface expression is accompanied by CD28 expression [132], and decrease of CD80 and CD86 [125]. CD80 and CD86 surface expression depends upon the activation status of the APC, both being up-regulated in the context of infection, cell damage and stress [64]. Recently, the inducible co-stimulatory ligand (ICOS-L) has been described as another co-stimulatory binding partner of CD28 and CTLA4 in humans [170]. Programmed death-1 ligand (PD1-L) is constitutively expressed on DC, BC, TC and macrophages and interacts with programmed death-1 (PD1) on TC [81] [21]. PD1/PD1-L ligation is associated with co-inhibition of pre-activated TC by suppressing TCR signaling [29]. PD1-L expression on several tumor cells and tumor infiltrating myeloid cells have accompanied poor prognosis in tumors, especially in malignant melanoma [69].

DC also have important tolerogenic functions that may develop in specific settings: They are involved in generation of both central tolerance [18] and peripheral tolerance, the latter depending on their maturation status, presence of anti-inflammatory stimuli and microbial pathogens as well as a permissive tissue microenvironment [151].

Immature DC with low MHC-II and co-stimulatory molecule surface expression are not optimal for TC priming and eventually are able to induce TC anergy and tolerance [151]. Furthermore, tolerogenic DC were reported to differentiate through exposure to IL-10, TGF- β 1, prostaglandin E2 and 1 α ,25-dihydroxyvitamin D3 [108]. α (v) β 6-integrin expressing DC and cytokine signaling 3 (SOCS3) deficient DC showed Treg inducing capacities [136]. Several microbes in chronic infections have developed mechanisms suppressing excessive host inflammation by manipulating DC-mediated TC responses through TLR2 signaling or induction of IL-10 secretion by DC [157] [105].

Different micro-environments require different immune functions: In the intestine, where mucosal cells are permanently exposed to a variety of microbial and potentially allergenic molecules, macrophages and DC show tolerogenicity by IL-10 secretion [59]. In a murine in vivo model, CD103⁺ DC in the gut-associated lymphoid tissue were shown to polarize effector TC to become Foxp3⁺ Treg in the presence of TGF- β and retinoic acid [110] [34] [153] [79]. Also in the skin, DC were shown to control immunity by Treg induction [61]. Guillemins et al. demonstrated, that dermis-derived aldehyde dehydrogenase (ALDH) expressing DC constitutively produced retinoic acid and through this mechanism induce Foxp3⁺ Treg [61].

1.3.2 T cells

T lymphocytes are the effector cells of the specific immune system. TC derive from immature bone marrow lymphocytes that migrate to the thymus, where further development and maturation takes place, before TC migrate to secondary lymphatic organs [86]. According to their surface molecules and the cytokines secreted, TC can be divided into different groups. The two major groups are CD4⁺ and CD8⁺ cells; our work focuses on CD4⁺ cells.

Naïve CD4⁺ TC encounter antigen presenting cells (APC), especially certain types of DC. Depending on the type of APC and the environment in which they encounter these APC, naive TC are skewed towards a certain lineage [109]. Within the CD4⁺ group, TC can differentiate towards the Th1, Th2 or Th17 lineage. The different lineages are characterized by a certain cytokine profile secreted by these TC [109]. Skewing naive TC towards a certain Th-phenotype requires induction of lineage-specific transcription factors such as the master regulators T-bet for Th1 or GATA3

for Th2 [70]. For generation of Th1 cells a certain phenotype of APC is required, that may differentiate through activation by innate pathogen receptors, such as Toll-like receptors [5]. Such DC secrete IFN- γ and IL-12 and polarize naive TC to develop a Th1 phenotype [99] [71]. Antigen-dependent T-bet induction leads to up-regulation of the IL-12 receptor on naive TC which allows STAT4 signaling following ligation of the IL-12 receptor [111] [1]. Furthermore, INF- γ induces STAT1 which increases T-bet transcription [1]. Development of the Th2 lineage is promoted by GATA3 activation downstream of IL-4 and Stat6 [175].

The different T helper cell types fulfill different functions. In delayed allergy, inflammation in general and in the defense against intracellular pathogens, CD4⁺ Th1 cells play a major role through their activation of macrophages, natural killer cells and cytotoxic TC [176] [124]. Th2 cells produce IL-4, IL-5, and IL-13 thereby play a role in the isotype switch of B-cell secreted antibodies and eosinophil mediated immune responses [176].

IL-17 secreting Th17 cells are the latest member of the CD4⁺ TC group and require TGF- β and IL-6 for activation of retinoic-acid-related orphan receptor (ROR- γ t) [15] [77]. They fulfill a predominant role in the immunity against extracellular pathogens [112] and the development of autoimmune diseases [106]. In skin diseases, Th17 cells have been connected to psoriasis and pathogen related dermatitis [44]. Recent studies also demonstrated a role of Th17 in allergic contact dermatitis: Increased levels of IL-17 mRNA were found in nickel contact allergy lesions. Furthermore, IL-17 deficient mice showed reduced CHS response [121].

1.3.3 Regulatory T cells

Treg modulate immune responses and play a central role in the induction of peripheral tolerance [158]. They enable self-tolerance by controlling Th1, Th2 and Th17 immune responses [124]. Treg control transplantation immunity and play a role in the suppression of anti-tumor immunity [135]. Most Treg have been found to express CD4, CD25 [136] and the forkhead box p3 transcription factor (Foxp3) [19]. Foxp3 plays a major role in Treg differentiation and function. A Foxp3 loss-of-function mutation leads to development of the IPEX syndrome in humans (immune dysregula-

tion, polyendocrinopathy, enteropathy, X-linked), with patients suffering from persistent eczema, autoimmune reactions and colitis [52]. At the same time, Foxp3⁺ TC have been reported to express effector TC characteristics, such as transcription factors of Th1 (T-bet), Th2 (Gata3) and Th17 (IRF4) [85] [174].

Treg are subdivided into two major groups: Natural regulatory TC (nTreg) that develop in the thymus and control responses to self-antigens and induced regulatory TC (iTreg) that develop from naïve TC in the periphery as a response to exogenous antigen [92]. In this study only iTreg were subject of investigation. Therefore to simplify nomenclature, the term Treg will be used throughout this work describing iTreg.

The complex mechanisms of Treg development has been a focus of recent research. Conversion of peripheral CD4⁺ TC to Treg in the murine gut-associated lymphoid tissue was reported to depend on TGF- β and retinoic acid [153]. Although the exact mechanisms of induction are not clear, a fundamental role was assigned to DC, because DC depletion led to Foxp3⁺ T cell decrease, and pronounced Th1/Th17 immune response [39]. Interestingly, especially immature DC induce Treg, while mature DC promote Th1 or Th17 development [98] [14]. Furthermore, inflammatory mediators like IL-6 and IL-12 can prevent Treg differentiation and proliferation in infectious diseases [16] [15] [96] [175].

Treg influence effector T cell activation, proliferation and differentiation. Vignali et al. described four major mechanisms through which Treg exert their regulatory functions [158]: 1) Treg secrete inhibitory IL-10, TGF- β and IL-35 thereby attenuating effector TC responses [65] [60] [33]. 2) Treg cause target cell lysis through granzyme-B and perforine [131] [173] [126]. 3) IL-2 consumption by Treg and concomitant IL-2 deprivation induce apoptosis of effector TC [120]. 4) Treg attenuate DC-mediated effector TC activation through CTLA4 [118] [147].

In contact hypersensitivity (CHS), Treg modulate the inflammatory response. Investigating the sensitization phase of CHS, Cavani et al. analyzed TC subtypes of both nickel-allergic and non-allergic subjects and found nickel specific CD4⁺ cells in both subsets, but only nickel specific CD8⁺ cells in allergic subjects. Furthermore, they

found nickel-responsive CD4⁺ CD25⁺ and highly IL-10 producing TC, which inhibited DC differentiation. Interestingly, CD4⁺ CD25⁺ TC of non-allergic subjects produced higher IL-10 levels and lower IFN- γ levels than those found in nickel-allergic subsets [23]. Kimber et al. attributed the strength of the immunological stimulus to an important role for Treg function: His group demonstrated that weak contact allergens failed to induce allergic contact dermatitis in healthy mice, but acute CD4⁺ TC depletion led to contact dermatitis, even when exposed to a weak allergen [160]. On the other hand, they suggested that Treg fail to prevent sensitization to potent allergens but may influence the degree of sensitization [82]. Lehtimäki et al. showed that transient Treg depletion by diphtheria toxin before sensitization leads to a much more pronounced and prolonged CHS response, while depletion during challenge did not have effects on severity, but on duration of symptoms [93]. Wing et al. and Honda et al. also propose a Treg suppressive function in skin-draining lymph nodes induced through downmodulation of costimulatory CD80 and CD86 expression on DC [165] [73]. Molecular mechanisms involved in Treg-induced suppression of contact hypersensitivity, besides increasing IL-10 levels, also produce adenosine, which down-regulates endothelial selectin expression followed by decreased TC infiltration [129] [128]. In regard to their migratory behavior, Treg were able to circulate in both directions between skin and lymph nodes, which indicates that their suppressive effects are not limited to the skin (Kabashima et al unpublished observation, described in [72]). In the elicitation phase, Treg suppress the activation of endothelial cells [72] and prevent TC infiltration into the skin by IL-10 and CD39/73 mediated adenosine degradation [128].

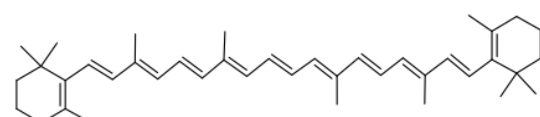
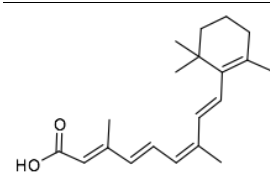
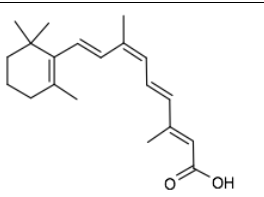
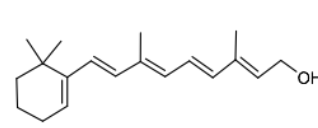
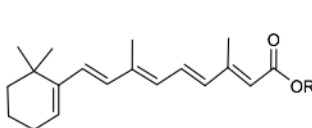
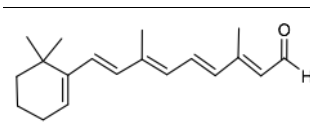
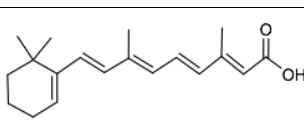
1.4 Retinoids

Retinol is a lipid-soluble vitamin, which the human body is not able to synthesize, and has to be obtained from an external source. Vitamin A affects vision, is important for maintaining epithelial surfaces and immune competence. It is essential for embryonic growth and development [17].

From the diet, vitamin A is obtained either as all-trans-retinol, retinyl esters or β -carotene [17]. Oxidation of all-trans retinol and β -carotene to all-trans-retinal in tissue takes place through abundantly occurring enzymes such as short-chain- or alcohol-dehydrogenase [107]. Further irreversible oxidation to all-trans retinoic acid is

carried out through retinal dehydrogenases (RALDH) [107]. Transformation from all-trans retinal to 9-cis retinoic acid can occur spontaneously or through oxidation of 9-cis-retinal by RALDH [107].

Table 1 Chemical structure of different vitamin A derivatives, R = rest.

 <p style="text-align: right;">β carotene</p>	
 <p style="text-align: right;">9-cis retinal</p>	 <p style="text-align: right;">9-cis retinoic acid</p>
 <p style="text-align: right;">all-trans retinol</p>	 <p style="text-align: right;">retinyl ester</p>
 <p style="text-align: right;">all-trans retinal</p>	 <p style="text-align: right;">all-trans retinoic acid</p>

1.4.1 Retinoid receptors

Retinoid receptors belong to the intracellular or DNA-linked receptors that dimerize to carry out their biological functions and regulate gene expression by binding to short DNA sequences, so called hormone responsive elements [123]. While retinoid-acid-receptors (RAR) usually form dimers with one of the retinoid-X-receptors (RXR), RXR are less specific in their dimerization partner, and may also serve as dimerization partner of vitamin D and thyroid hormones [54] [55] [107]. After ligand dimerization and binding to DNA sequences, called either RAR or RXR response element, gene transcription may be initiated [8] [17].

The first retinoid acid receptor described was the RAR α receptor [123], and later RAR β [47] and RAR γ [27]. The RXR family mainly differs from the RAR in its primary

structure and is subdivided into RXR α , RXR β and RXR γ (**Table 2**) [154]. Regarding the biological functions, Germain et al described that RAR mainly influence proliferation and cell differentiation while RXR generally regulate cell apoptosis [55]. In the human skin, the γ -isoform of the RAR-type predominates, accounting for 87% of RAR. Regarding RXR expression the α -isoform predominates making up 90% of skin RXR [30] [137] [49].

Table 2 Subgroups of retinoid-receptors, accumulated from publications [30] [55] [17]. RAR Retinoic acid receptor, RXR retinoic-X-receptor, 9cisRA 9-cis-retinoic acid, ATRA all-trans retinoic acid, VDR vitamin D receptor

Name of receptor	Subgroups and isoforms	Main functions	Possible dimerization partner	Ligand
RAR	RAR α : 2 isoforms	Cellular differentiation and proliferation	<i>Heterodimer</i> RAR/RXR	9cisRA ATRA
	RAR β : 4 isoforms			
	RAR γ : 2 isoforms			
RXR	RXR α : 2 isoforms	Cell apoptosis	<i>Homo- or heterodimer</i> RXR/RXR RXR/RAR RXR/VDR RXR/thyroid hormones	9cisRA
	RXR β : 2 isoforms			
	RXR γ : 2 isoforms			

1.4.2 9-cis-retinoic acid

9-cis-retinoic acid (9cisRA) binds with high affinity to both isoforms RAR and RXR [30]. So far in the adult human body, under physiological conditions, 9cisRA has only been detected in the presence of Tretinoin, its all-trans retinoid acid isomer. Further 9cisRA was found during embryonic development [166]. Therefore, the role of 9cisRA as physiological ligand for RXR is still under discussion. As other RXR ligands, for example linoleic and docosahexaenoic acids, omega fatty acids naturally occurring in healthy alimentation, have been proven to show at least a weak affinity to RXR [166]. Within the body, 9cisRA was found to have a mean half-life of 2–10 hours [141].

9cisRA is a potent systemically administered drug in the treatment of therapy resistant chronic hand eczema. In a large study on chronic hand eczema (CHE)

Ruzicka et al. reported that 9cisRA treatment ameliorates eczema in up to 75% of treated patients, 48% of subjects showed clear or almost clear hands [133]. Besides treatment of CHE, oral 9cisRA therapy was examined in the treatment of Kaposi sarcoma [30] and oesophageal lichen ruber planus [87]. Only few studies focus on topical 9cisRA treatment in Kaposi sarcoma [30] and photo aging [13]. Like all retinoids, 9cisRA is highly teratogenic and has photosensitizing effects [133].

1.5 Osteopontin

Osteopontin (OPN) is a glycoposphoprotein with immune modulatory functions. It is produced constitutively in epithelial and bone tissue, and is highly expressed in a number of pathological processes [25].

1.5.1 Osteopontin protein structure and receptors

OPN consists of about 300 amino acids and varies in size from approximately 40-80 kDa, depending on enzymatic modification and splicing [155]. OPN has two major isoforms, a full length secreted (sOPN) and a shorter intracellular form (iOPN) [149]. This study focuses on the secreted form of OPN and the term OPN will be used for the secreted OPN throughout the study.

OPN has been described to ligate several cell surface receptors: Through its integrin binding RGD sequence, consisting of the amino acids Arg-Gly-Asp, it interacts with $\alpha\beta3$, $\alpha\beta1$, $\alpha\beta5$, $\alpha\beta6$, $\alpha5\beta1$ and $\alpha8\beta1$ integrins [42] [57] [94] [11] [12]. Furthermore, OPN can interact with certain isoforms of CD44 [163] [7].

Thrombin, as well as matrix metalloproteinases, can cleave the full-length OPN molecule into a C-terminal and N-terminal fragment. OPN possesses three different thrombin cleavage regions [146] [41] [156] [31]. Upon thrombin cleavage, a cryptic integrin binding region (SLAYGLR in mice) is exposed, possibly binding $\alpha9\beta1$ and $\alpha4\beta1$ [140] [171] [169]. Uede et al. reported that thrombin cleaved OPN fragments have been linked to the pathogenesis of inflammatory destruction of tissue in rheumatoid arthritis, especially by triggering cytokine and chemokine production through interaction with $\alpha9\beta1$ integrin [155]. Matrix metalloproteinases (MMP) are essential for wound healing and cancer metastasis. MMP can cleave the OPN molecule, with

cleavage sites for MMP-3 and MMP-7 differing from the cleavage sites for thrombin mentioned earlier [2]. MMP-cleaved OPN fragments were found in vitro in tumor tissue and in tissues undergoing remodeling such as the postpartum uterus [2].

1.5.2 Osteopontin functions

How OPN impacts cells is strongly influenced by post translational modification, such as phosphorylation, sulfation or glycosylation. The variant of the modified OPN-protein is inhomogeneous and varies in a tissue specific manner [80]. The broad range of isoforms may explain its heterogeneous effects [25]. Physiologically, OPN was found in osteoblasts, osteoclasts [119] [41], hair follicles, sebaceous and sweat glands [20] [28], the kidneys [172], human milk [145], uterus and placenta [162] [25].

Extracellular secreted osteopontin

Upregulation of OPN was detected in inflammatory settings, produced by activated T cells [117], macrophages, DC and in multiple cancers such as, amongst others, carcinoma, glioma and melanoma [25]. Additionally, it was shown to play a role in fibrosis formation, therefore improving wound healing, fibroblast and endothelial cell survival and macrophage migration and adhesion [152].

Regarding immunological functions, non-specific effects such as chemo-attraction of macrophages, TC and DC [116] [115] [164], and direct regulatory function regarding microbial [48], autoimmune [91] and allergic diseases [168] have been described. Recent research has focused on T cell polarizing functions of OPN.

Generally, elevated OPN plasma levels were found in allergic contact dermatitis [164], rheumatoid arthritis [169], multiple sclerosis [11] [26], and granulomatous diseases [114]. The sOPN over-expression seen in autoimmune and allergic diseases raises questions regarding a correlation between OPN and disease development. OPN ^{-/-} mice show a much milder course in a wide range of Th1/Th17 mediated diseases i.e. in contact dermatitis [164]. sOPN's interaction with $\alpha\beta3$ integrin and CD44 receptors on macrophages enhanced IL-12 and inhibited IL-10 responses indicate a Th1 shaping potential [7]. Similarly, in microbial immunity, OPN activates TC proliferation and exerts a pro-inflammatory effect on Th1 cells, consistent with

the finding that in Th1 mediated infectious diseases, like tuberculosis and silicosis, elevated OPN levels are observed [114]. Nau et al. even detected an inverse correlation of OPN serum levels with microbial dissemination and patient survival, indicating that OPN is nearly indispensable in a fully functioning anti-microbial immune response [113].

Besides its pro-inflammatory capacity, OPN also possesses anti-inflammatory abilities: Reduction of inducible nitric oxide synthase (iNOS) with subsequent reduced production of nitric oxide (NO) was found in murine studies in vitro [161] [62] [74]. This finding was further supported by increased OPN levels in blood vessels during sepsis where OPN also dampened iNOS activity and prevented further NO generation [43].

Interestingly, in inflammation of the central nervous system sOPN showed neuro-protective effects in the beginning of acute inflammation [148]. Also in the intestine OPN showed tissue-protective and regulatory effects in acute inflammation, but aggravation in chronic disease [66]. Sangaletti et al. recently analyzed the role of OPN in tumor immunity and observed a decreased number of CD4⁺ Foxp3⁺ regulatory TC and increased number of CD4⁺ activated TC in OPN^{-/-} mice, indicating a role of OPN for the formation of an immunosuppressive environment at the metastatic site [139].

OPN in delayed type allergic disease

In allergic contact dermatitis, a Th1/Th17 driven disease, OPN was attributed nearly exclusively with pro-inflammatory, disease aggravating functions: In the sensitization phase of murine contact hypersensitivity, allergen application to the skin itself augmented OPN expression [144], as other pro-inflammatory mediators were induced by the application of contact allergens [104]. Additionally local OPN levels increased upon allergen contact, and promoted APC migration to draining lymph nodes [164]. In the human system, OPN activated DC to secrete Th1 shaping TNF- α and IL-12 [164]. Furthermore, in mixed lymphocyte reaction (MLR), osteopontin pretreated DC skewed naive TC to express Th1 cytokines [127] [142].

Investigating the challenge phase of cell mediated allergy, using nickel as specific antigen, effector TC up-regulated OPN secretion upon nickel challenge and recombinant OPN down-modulated IL-4 secretion in human TC [144]. In mice, both CD4⁺

and CD8⁺ TC augmented OPN production upon TNBS antigen challenge [144]. Furthermore, IFN- γ , a Th1 cytokine, preferentially produced by effector TC, promoted OPN expression by human keratinocytes in vitro, indicating a role of OPN in the perpetuation of chronic Th1/Th17 inflammation [144] [51]. Accordingly, ACD patients showed elevated OPN expression in chronically inflamed skin and had elevated serum OPN levels compared to healthy controls [144].

1.6 Aims

9-cis-retinoic acid is effectively used for the treatment of therapy-resistant chronic hand eczema. Little is known about the effects of 9cisRA in the skin immune system. Allergic contact dermatitis is a common cause of chronic hand eczema, which is mediated through efficient interaction of DC and T cells.

Our hypothesis was that 9cisRA may modulate contact dermatitis through effects on antigen presenting DC. We therefore set out to investigate effects of 9cisRA on dendritic cell maturation and their functional modulation for T cell activation, in vitro and in vivo.

Specifically, the following questions were asked:

1. Does 9cisRA treatment affect the differentiation of DC towards a certain phenotype?
2. How does 9cisRA treatment affect DC function in T cell activation and differentiation?
3. Are 9cisRA differentiated DC functional in vivo?

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Instruments

Centrifuges:

Multifuge 3 S-R (Heraeus, Osterode); Labofuge 400 R; Galaxy (Heraeus, Osterode); 16 DH (VWR, Darmstadt); MR 1822 (Joun, Unterhaching); Fresco 17 (Heraeus, Osterode)

Discovery comfort pipettes (HTL, Warsaw, Poland)

Engineer's micrometer (Mitutoyo, Neuss, Germany)

FACScan FACS Canto II (BD, Franklin Lakes, USA)

GeneAmp PCR System 9700 (PE Applied Biosystems, Life Technologies, Darmstadt)

Incubator Hera cell 150 (Thermo Scientific, Schwerte)

Incubator: Thermomixer 1441 (B. Braun, Melsungen)

Microscope: inverse microscope Axiovert 200 (Zeiss, Oberkochen)

Mithras LB-940 microplate reader (Berthold Technologies, Bad Wildbad, Germany)

Multipette plus (Eppendorf, Hamburg)

Neubauer improved counting chamber (Marienfeld, Lauda-Königshofen)

Quadro MACS magnet (Miltenyi Biotec, Buckinghamshire, UK)

Safety cabinet: BDK UVF 1.8 (BDK, Sonnenbühl Genkingen)

Swiftpet plus (HTL, Warsaw, Poland)

Transferpette -12 electronic (Brand, Wertheim)

2.1.2 Chemicals

Alitretinoin (9cisRA), Basilea pharmaceuticals

β -mercaptoethanol (Sigma-Aldrich, Munich)

Concavaline A (Sigma-Aldrich, Munich)

DMSO (Merck, Darmstadt)

Ethanol (Merck, Darmstadt)

FCS-Bio (Biochrom, Berlin)

FCS-PAA (PAA Laboratories, Pasching, Austria)

HEPES buffer solution, 1M (Biochrom, Berlin)
Isofluran, Forene (Abbott, Wiesbaden)
L-glutamine, 200 mM (Biochrom, Berlin)
LPS, lipopolysaccharide (Sigma-Aldrich, Munich)
Lymphocyte separation medium (LSM) (PAA Laboratories Pasching, Austria)
Non-essential amino acids, 100x (Biochrom, Berlin)
PBS without calcium and magnesium (Biochrom, Berlin)
PeqGold RNA Pure (Peqlab, Erlangen)
Phosphoric acid 1M H₃PO₄ (Sigma-Aldrich, Munich)
RPMI 1640 (Lonza, Basel, Switzerland)
Sytox Blue Dead Cell Stain (Life Technologies, Darmstadt)
TNCB
TNBS, 5 % solution (Sigma-Aldrich, Munich)
Trypan Blue (Sigma-Aldrich, Munich)
Water, Mol Bio grade (5Prime, Hamburg)

2.1.3 Cytokines and cell staining

Mouse IFN gamma ELISA set (eBioscience, Frankfurt)
Mouse IL-4 ELISA set (eBioscience, Frankfurt)
Mouse IL-10 ELISA set (eBioscience, Frankfurt)
Mouse IL-12/IL-23 total p40 ELISA set (eBioscience, Frankfurt)
Mouse IL-12 p70 ELISA set (eBioscience, Frankfurt)
Mouse IL-17A ELISA set (eBioscience, Frankfurt)
Mouse IL-23 ELISA set (eBioscience, Frankfurt)
Mouse Osteopontin DuoSet ELISA (R&D Systems, Abingdon, UK)
Mouse regulatory T cell staining kit (eBioscience, Frankfurt)
Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Freiburg, Germany)

2.1.4 Various materials

Cell strainer 40 µm Nylon (BD Falcon, Franklin Lakes, USA)
Eppendorf tubes 0,5 ml/ 1,5 ml/ 2 ml (Eppendorf, Hamburg)
FACS round-bottom tubes 5 ml (BD Falcon, Franklin Lakes, USA)
LS-column (Miltenyi Biotec, Bergisch Gladbach)
Multidishes, 12/24/96 wells (Greiner Bio-one, Frickenhausen)

Needles (B. Braun, Melsungen)

Petri dishes 60x15 (Greiner Bio-One, Frickenhausen)

Stripette costar 5 ml/ 10 ml/ 25 ml (Corning, New York, USA)

Syringes Omnifix 5 ml/ 10 ml/ 20 ml (B. Braun, Melsungen)

2.1.5 Cytokines, Antibodies, Cell Separation Reagents

Cytokines

Recombinant murine IL-4 (PeproTech, Rocky Hill, USA)

Recombinant murine GM-CSF (supernatant from X63/0 hybridoma cells stably transfected with an IL-4 expression vector)

MACS-Separation

Murine CD 4 T Cell Isolation Kit

Murine Pan T Cell Isolation Kit

Antibodies

Table 3 FACS antibodies

Antigene	Isotype	Clone	Conjugate	Producer
Murine Foxp3	Rat IgG2a, k		PE	eBioscience
Murine CD 25	Rat IgG1 l	PC61	APC	BD Biosciences
Murine OPN	Goat IgG		PE	R&D systems
Murine CD 11c	Hamster IgG	N418	APC	eBioscience
Murine I-A, I-E	Rat IgG2b k	M5/114.15.2	PE	BD Biosciences
Murine CD 86	Rat IgG2a k	GL1	PE	BD Biosciences
Murine CD 44	Rat	IM7	PE-Cy5	BD Biosciences
Murine CD 4	Rat IgG2a k	RM4-5	PE-Cy7	eBioscience
Murine IL17a	Rat IgG1 k	TC11-18H10	APC-Cy7	BD Biosciences
Isotype control	Mouse IgG1 k		PE-Cy7	eBioscience
Isotype control	Mouse IgG2a		FITC	BD Biosciences
IgG Isotype control	Rat IgG1		APC	BD Biosciences

RPML 1640	was supplemented (c-RPML) with 10% heat-inactivated FCS, 1% non-essential amino acids, 1% penicillin and streptomycin, 1% L-glutamine, 1% HEPES buffer (Biochrom, Berlin, Germany)
TNBS	10 ml of 5 % TNBS (0,162 mol/l) were dissolved in 231 ml PBS to get an 7 mmol/l solution. Storage at – 20° C.
Trypan Blue	1 % solution was diluted at a ratio of 4 ml solution and 6 ml PBS to receive a 0,4 % straining solution.

2.1.7 Computer Software

Adobe Photoshop (Adobe, San Jose, CA, USA)

EndNote X6 (Thomson, Philadelphia, USA)

FACS Diva (BD Bioscience, Franklin Lakes, USA)

FloJo (Tree Star, Ashland, USA)

Microsoft Office 2010

MicroWin 2000 (Berthold Technologies, Bad Wildbad, Germany)

GraphPad Prism v6.05

Corel Draw X4 (Corel Corporation, USA)

2.2 Mice

The mice lacking the functional OPN gene (*spp1*) were described previously [95]. For all experiments, C57BL/6J or BALB/c *OPN* mutant mice in the tenth generation of backcrossing were used. All work has been carried out in accordance with EU Directive 2010/63/EU for animal experiments and animal protocols were approved by the Committee of Animal Research (Regierungspraesidium Tuebingen, Germany).

C 57BL/ 6J (“black 6”) mice were obtained from the animal research facility of Ulm University and served as wild type control animals. Balb/c AnNCrl mice were obtained from Charles River.

2.3 Methods

2.3.1 DC culture and 9cisRA treatment

Murine DC were generated as described [127] [75]. Bone marrow was harvested from the tibia and fibula of mice and cells were cultured with murine GM-CSF and murine IL-4 (both PeproTech, London, UK). Cells were cultured at 1 ml aliquots in 24 well culture plates (Greiner, Frickenhausen, Germany) and fed on day 3 of culture. Loosely adherent cells were harvested on day 5 of culture by gradient centrifugation, 15.5% Nycodenz (Axis-Shield, Oslo, Norway) in RPMI.

For 9cisRA experiments the substance was dissolved in ethanol (EtOH) and added to DC cultures at concentrations of 3, 30 or 300 ng/ml throughout the culture process. Equal amounts of EtOH were added as control. 9cisRA or EtOH at the used concentrations was not toxic for DC as analyzed by propidium iodide viability staining with FACS (data not shown).

2.3.2 Flow cytometry of DC

Surface receptor expression on DC was determined by a standard protocol [127]. Cells were stained (4°C, 30 min) with FITC-, PE, PEcy5 or APC labeled mAbs: MHC-II (BD Pharmingen, Heidelberg, Germany, M5/114.15.2), CD80 (Biolegend, Fell, Germany, 16-10A1), CD86 (BD Pharmingen, GL1), CD11c (eBioscience, Frankfurt, Germany, N418), CD44 (BD Pharmingen, IM7), PDL-1 (eBioscience, MIH5), and ICOS-L (Biolegend, HK5.3). For analysis of cell viability 0.1 µg/ml propidium iodide (Sigma-Aldrich, Frickenhausen, Germany) was added. Samples were analyzed with FACSCanto II (Becton Dickinson) and mean fluorescence intensities (MFI) determined by FACS Diva® (Becton Dickinson) or FlowJo® software (Tree Star, Inc., Ashland, Oregon, U.S.A.).

2.3.3 Proteome Profiler mouse cytokine array and ELISA

Supernatants from untreated or 9cisRA cultured DC were harvested at culture day 5. Cytokines in supernatants were detected by Proteome Profiler Array, Mouse cytokine Array Panel A (R&D Systems, Wiesbaden-Nordenstadt, Germany) according

to the manufacturer's protocol. Profiles were analyzed by quantifying the mean spot pixel densities from scanned images (Fusion FX7, Vilber Lourmat GmbH, Eberhardzell, Germany) by image analysis Software (Adobe Photoshop, Adobe, San Jose, CA, U.S.A.). Cytokines were quantified by ELISA (eBioscience, Ready-Set-Go!® ELISA Set) according to the manufacturer's instructions and measured at an extinction of 450 nm (Mithras LB-940 microplate reader and MicroWin 2000 software, Berthold Technologies, Bad Wildbad, Germany) with OPN ELISA (R&D, Wiesbaden-Nordenstadt, Germany). Cytokines in MLR were measured by Mouse Th1/Th2/Th17 Cytokines Multi-Analyte ELISArray (Qiagen, Hilden, Germany).

2.3.4 RNA Isolation and Quantitative Real-Time PCR

DC were generated from mouse bone marrow for 5 days in the presence of 9cisRA (3, 30, 300 ng/ml). RNA was isolated by peqGOLD RNAPure (Peqlab, Erlangen, Germany). To synthesize cDNA reverse transcription was performed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Freiburg, German). Obtained cDNA was used for quantitative real-time PCR with FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, Mannheim, Germany) and an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA). Thermocycling conditions: hold at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, followed by an additional dissociation stage. The relative expression of target gene in different samples was normalized to the endogenous glyceraldehyde-3-phosphate dehydrogenase and was calculated with the $2^{-\Delta\Delta Ct}$ method. Mouse OPN primers were 5'-GGTGATAGCTTGGCTTATGGACTG-3' (forward) and 5'-GCTCTTCATGTGAGAGGTGAGGTC-3' (reverse). Mouse glyceraldehyde-3-phosphate dehydrogenase primers were 5'-TGGCCTTCCGTGTTCCCTACC-3' (forward) and 5'-GGTCCTCAGTGTAGCCCAAGATG-3' (reverse) [144].

2.3.5 T cell proliferation assay and induction of Treg

Mixed lymphocyte reactions were performed as described previously [127]. In brief, enriched spleen T cells (CD4+ T cell kit, Miltenyi, Bergisch Gladbach, Germany) from *OPN^{Wt}* or *OPN^{-/-}* 255 Balb/c mice were stained with CFSE (5 µM; Sigma Adrich, Munich, Germany) and co-cultured with gradient enriched irradiated (30 Gy)

DC from *OPN^{Wt}* or C57BL/6 *OPN^{-/-}* 257 mice generated in the presence or absence of 9cisRA (3, 30, 300 µg/ml). Induction of Treg was measured on day 5 by FACS (FACSCanto II) after staining harvested cells with Mouse Regulatory T Cell Staining Kit (Affymetrix eBioscience, Frankfurt, Germany) according to the manufacturer's protocol. For investigation of OPN in the induction of Treg recombinant OPN (2 µg/mL; R&D Systems) was added to co-cultures of *OPN^{-/-}* DC and *OPN^{-/-}* T cells. Alternatively, to exclude the function of DC on Treg induction, 2×10^5 purified T cells were added to microplates coated with 10 µg/ml of purified NA/LE anti-mouse CD3e (BD Pharmingen 145-2C11) and 2 µg/ml of purified NA/LE anti-mouse CD28 antibody (BD Pharmingen 37.51) with or without 9cisRA added to the culture medium. After 48 h cells were analyzed by staining with Regulatory T Cell Staining Kit.

2.3.6 Autologous induction of regulatory T cells

C57/BL6 DC were differentiated from murine bone marrow for 5 days with or without 9cisRA 30ng/ml and separated into two groups: one untreated the second loaded with 3mM TNBS (Sigma Aldrich) and were then irradiated with 30 Gy (**Fig. 4 A**). Naïve T cells were purified by negative depletion with MACS from pooled lymph node and spleen cells of C57/BL6 mice. DC and T cells were co-cultured until day 10, harvested and resuspended. On day 13, similarly treated DC were added to previously primed T cells. FACS analysis of co-cultured cells was performed on day 18 with the Regulatory T Cell Staining Kit.

2.3.7 Modulation of Contact Hypersensitivity by 9cisDC

Induction of CHS was performed as described previously. 6-week-old C57BL/6 *OPN^{-/-}* 279 mice or wild-type control mice were painted on their abdominal skin with 100 µl of 3% TNCB (VeZerf Laborsynthesen GmbH, Idar-Oberstein, Germany) in acetone at day 0 (**Fig. 7 A**). On day 6, TNBS loaded *OPN^{-/-}* or *OPN^{Wt}* DC that had been cultured in the presence or absence of 30 ng/ml of 9cisRA were injected subcutaneously on the shaved abdominal skin. Mice were challenged by painting 10 µl 1% TNCB on each side of the ears on day 14. Ear thickness was measured with an engineer's micrometer (Mitutoyo, Neuss, Germany) before challenge (day 14) and at day 15 and 16 and ear-swelling was calculated. Mice were sacrificed after measuring ear thickness on day 16 to obtain skin draining lymph nodes (**Fig. 7 A**). T cell suspensions were generated by cell strainer and were analyzed for the percentage

of Treg using the Mouse Regulatory T Cell Staining Kit (Affymetrix eBioscience) according to the manufacturers protocol.

2.3.8 Statistics

P-values were calculated with paired Student's *t*-test or All Pairwise Multiple Comparison Procedures (Holm-Sidak method) using Sigmastat (Systat Software).

3 RESULTS

3.1 9cisRA affects the phenotype of murine bone marrow derived DC

To investigate the effect of 9cisRA on the differentiation of DC from murine bone marrow, murine bone marrow derived DC were matured under the influence of different 9cisRA concentrations for 5 days. We will further call these 9cisRA cultured DC 9cisDC.

On 9cisDC we found a dose dependent decrease in the surface expression of MHC-II as well as in the expression of co-stimulatory molecules CD80 and CD86. Furthermore, the expression of co-inhibitory PD1-L increased dose dependently, while DC expression of the inducible co-stimulatory ligand (ICOS-L) was not altered by 9cisRA treatment (**Fig. 1**).

To investigate changes in cytokine profiles, we analyzed supernatants of 5-day old DC by Proteome Profiler. Here we found significant down-modulation in the secretion of IL-1 β , IL-12p70, IL-3, IL-7, CXCL9, CXCL10, CCL1 (**Fig. 2 A**). OPN has been shown to be regulated in the context of DC activation. As OPN was not included in the cytokine profiler, we measured OPN secretion by OPN specific ELISA and OPN RNA by real time PCR. We found a strong increase both on the RNA and protein level, when DC were generated under 9cisRA treatment (**Fig. 2 B**).

Our findings indicate that 9cisRA matured DC show a distinct phenotype with reduced expression of co-stimulatory molecules CD80, CD86, but up-regulated expression of co-inhibitory PD1-L. ICOS-L is constitutively expressed but not modulated on 9cisDC.

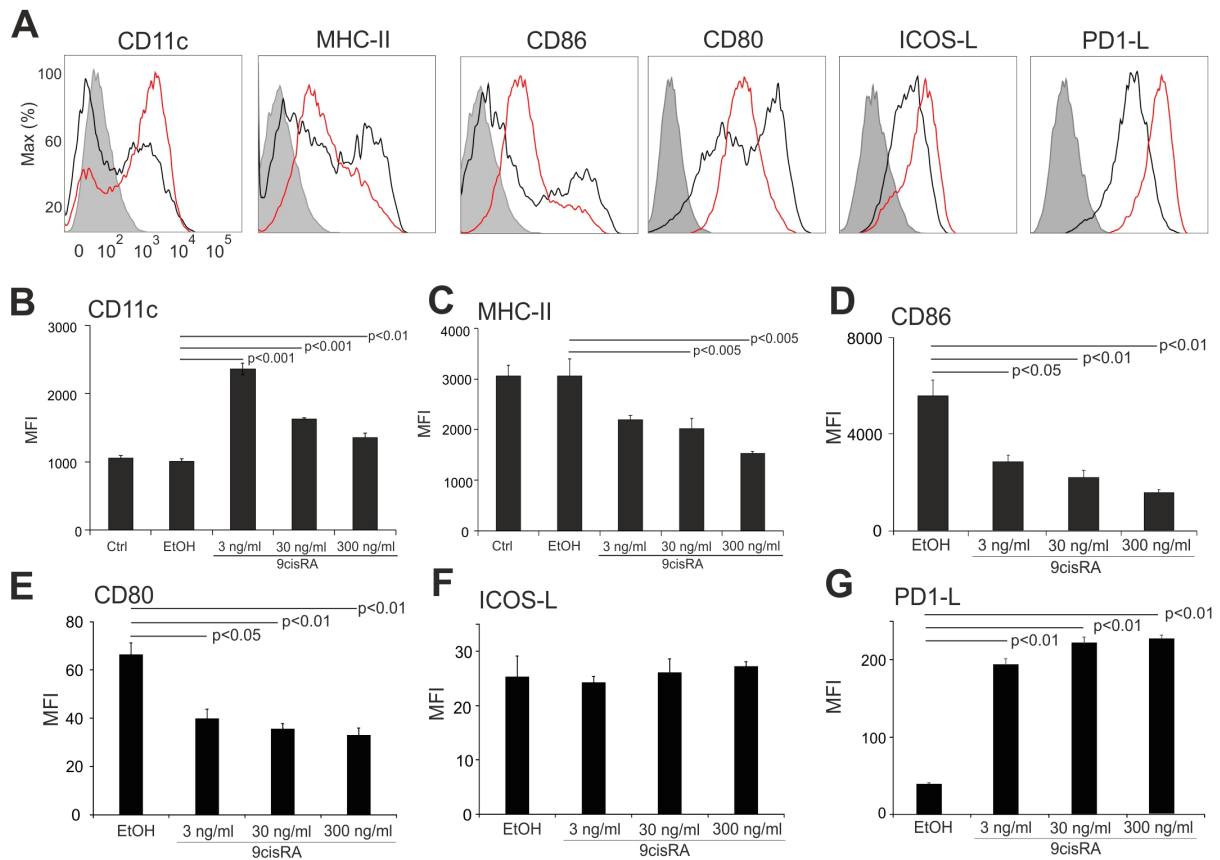


Figure 1 Culture of DC from bone marrow with 9-cis-retinoic acid (9cisRA) strongly affects T cell surface molecule expression [88].

Cluster of Differentiation (CD), dendritic cell(s) (DC), Major Histocompatibility Complex (MHC), Programmed Death 1 Ligand (PD1-L), Inducible Costimulatory Ligand (ICOS-L), Fluorescence Activated Cell Sorter (FACS)

DC were generated from mouse bone marrow in the presence or absence of indicated concentrations of 9cisRA until day 5 and then analyzed by FACS. Untreated DC (ctrl), ethanol treated DC (EtOH). **(A)** Cell surface expression of indicated molecules by FACS. Isotype controls: grey-shaded curves, EtOH treated DC: black curve, 30 ng/ml treated DC: red curves. Expression of CD11c **(B)**, MHC-II **(C)**, CD86 **(D)**, CD80 **(E)**, ICOS-L **(F)** and PD1-L **(G)** were determined by FACS analysis. Data is shown as mean fluorescence intensity (MFI) of surface marker expression +/- SEM of three individually treated DC cultures.

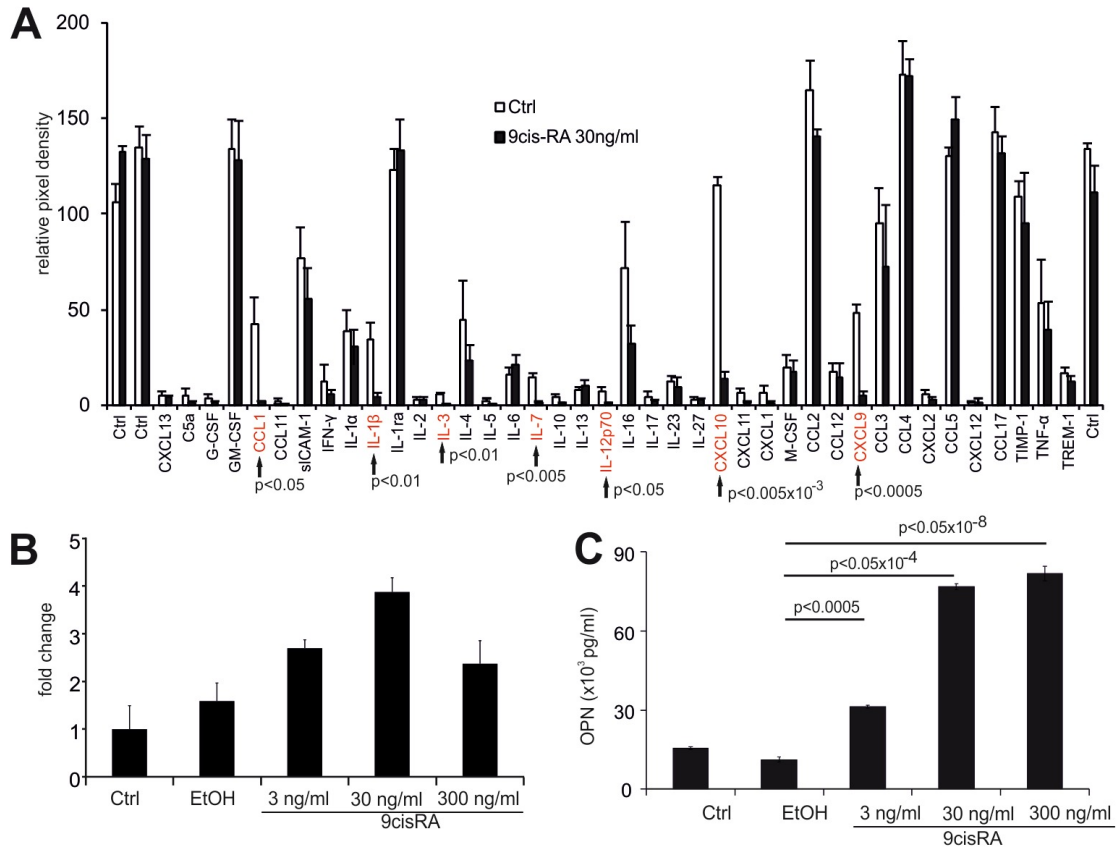


Figure 2 Culture of DC from bone marrow with 9-cis-retinoic acid (9cisRA) strongly affects their cytokine and chemokine secretion [88].

Chemokine (C-X-C motif) Ligand (CXCL), Granulocyte (G-), macrophage (M-) and Granulocyte-Macrophage (GM-) Colony Stimulating Factor (CSF), soluble intercellular adhesion molecule (sICAM), interleukine (IL), Tissue Inhibitor of Metalloproteinase (TIMP), tumor necrosis factor (TNF), triggering receptor expressed on myeloid cells-1 (TREM-1)

DC were generated from mouse bone marrow in the presence or absence of indicated concentrations of 9cisRA until day 5 and supernatants or cells were further analyzed by Proteome Profiler, real time PCR or ELISA. **(A)** Proteome Profiler of supernatants: Internal array positive controls for normalization are shown as control (ctrl). Data is shown as relative pixel density from scanned images. Average pixel density of pooled data from 3 independent experiments is shown. Significant changes are marked by an arrow (student's t-test). **(B)** OPN mRNA expression was determined by real time PCR. Results are fold change of OPN mRNA expression, \pm SEM from three wells. Representative data from two independent experiments. **(C)** OPN levels in DC supernatants. Data is shown in pg/ml of OPN, determined by OPN specific ELISA, \pm SEM (student's t-test) of three wells. Representative data of three independent experiments.

3.2 Effects of 9cisRA on dendritic cell – T cell interaction

Because 9cisRA strongly influences the phenotype of DC when present during maturation, we performed functional studies of 9cisDC focusing on their capacity for TC proliferation and differentiation. We performed allogeneic mixed lymphocyte reactions (MLR) with 9cisRA DC and Balb/c TC. Interestingly, DC matured under 9cisRA showed an impaired TC allo-stimulatory capacity (**Fig. 3A**). Analyzing cytokine secretion of supernatants of MLR, we found that LPS treated DC showed an increased secretion of IL-2, IL-6, IL-13 and IL17A (**Fig. 3 B**). In contrast, 9cisDC in co-culture with allogenic TC showed decreased IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A and IFN- γ secretion, but again OPN was highly secreted in co-cultures (**Fig. 3 B-D**).

These findings suggest that 9cisDC have inhibited TC stimulatory function and do not skew TC towards the Th1, Th2 or Th17 lineage.

3.3 9cisRA treated DC induce regulatory T cells

Because we found neither a specific Th17, Th1 or Th2 cytokine profile in the MLR, and 9cisDC showed up-regulated expression of the co-inhibitory molecule PD1-L we speculated that 9cisDC were able to differentiate TC towards a regulatory TC phenotype. We co-cultured 9cisDC with TC of BALB/c mice for 6 days and analyzed the TC phenotype by triple FACS staining for CD4, CD25, Foxp3 (**Fig. 4 B**). 9cisDC potentially induced Treg in co-culture, with a threefold increase of Treg in cultures with 9cisDC (**Fig. 4 B**).

As we found that 9cisRA can influence the generation of Treg we tried to delineate whether this process depends on DC - TC interaction or can be achieved by 9cisRA when present during TC receptor ligation. We coated plastic wells with CD3/CD28 antibody and added pooled pan TC from lymph nodes and spleen of C57Bl/6 mice in the presence or absence of 9cisRA.

After two days, the percentage of CD4+CD25+Foxp3+ cells was analyzed by FACS. **Figure 4 C** shows that the percentage of Treg generated by CD3/CD28 stimulation is not altered by the mere presence of 9cisRA, suggesting that expression of co-stimulatory and co-inhibitory molecules by DC is essential for Treg expansion.

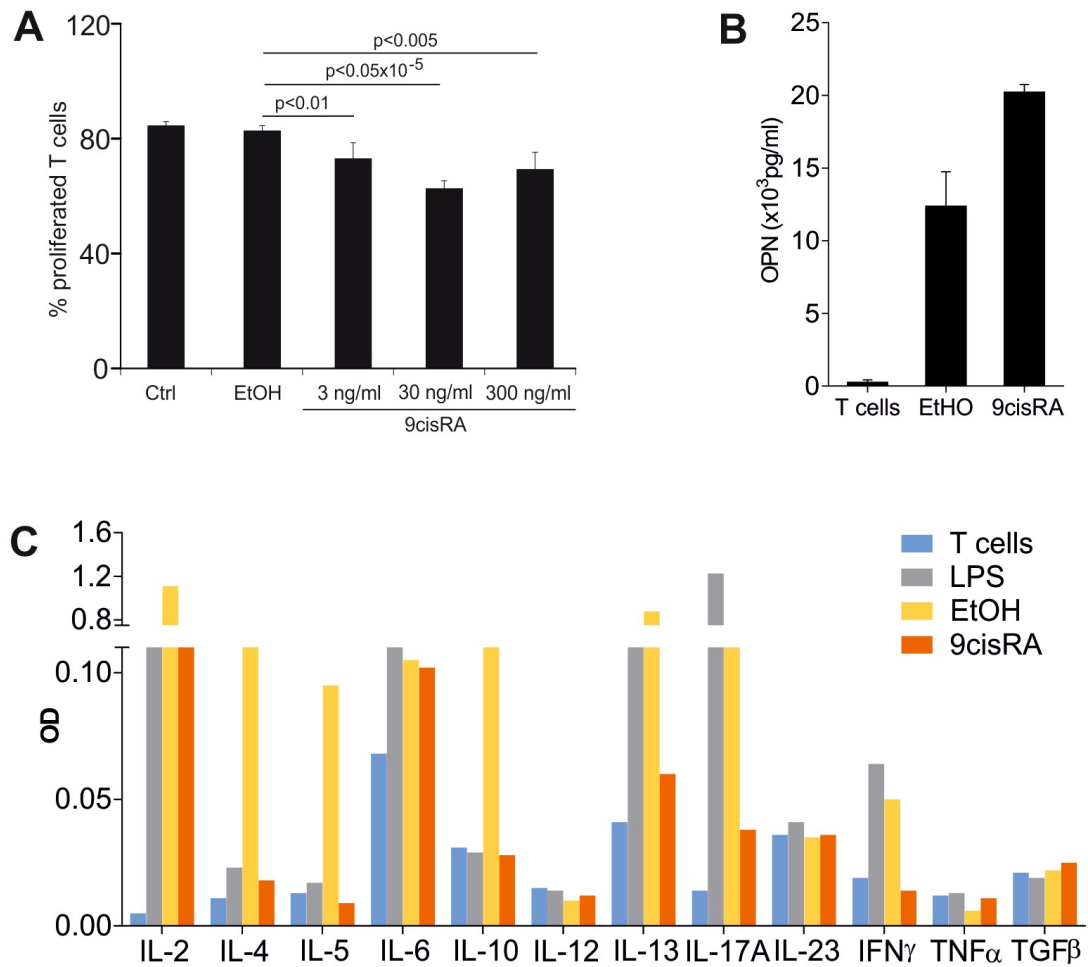


Figure 3 Dendritic cells (DC) cultured in the presence of 9-cis-retinoic acid (9cisRA) are inhibited in their allo-stimulatory capacity in mixed lymphocyte reactions (MLR) [88].

Interleukin (IL), Interferon (IFN), Tumor Necrosis Factor (TNF), Tissue Growth Factor (TGF), osteopontin (OPN), Cluster of Differentiation (CD), Optical Density (OD).

(A) DC from C57/Bl6 mice were cultured in the presence of three concentrations of 9cisRA as indicated and were used in MLR with pan T cells selected by magnetic activated cell sorting (MACS) from pooled lymph nodes and spleen of BALB/c mice. T cell proliferation was measured by Carboxyfluorescein succinimidyl ester (CFSE) staining on day 6 of MLR. Data is shown as percentage of proliferating cells by CFSE staining \pm SEM for 2 independent MLR cultures. Differences were significant as indicated by student's t-test. Representative data of two independent experiments is shown. **(B)** Osteopontin secretion measured in supernatants of MLR with DC cultured in the presence of 30ng/ml 9cisRA or EtOH as control. **(C)** DC from C57/Bl6 mice were cultured until day 6 in the presence of either ethanol (EtOH) or 30ng/ml 9cisRA. Cytokines in supernatants were analyzed by Mouse Cytokines Multi-Analyte ELISArray. Representative data of three experiments.

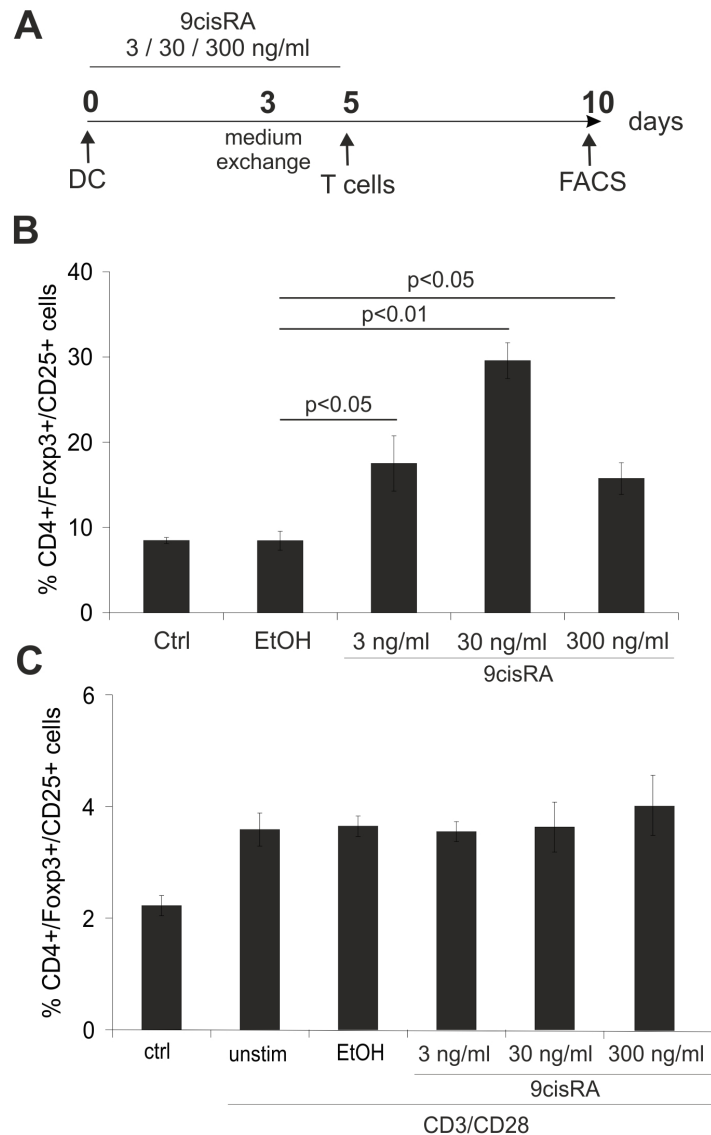


Figure 4 Dendritic cells (DC) cultured in the presence of 9-cis-retinoic acid (9cisRA) induce CD4+Foxp3+CD25+ regulatory T cells (Treg) [88].

Cluster of Differentiation (CD), Forkhead p3 transcription factor (Foxp3), Fluorescence Activated Cell Sorter (FACS)

(A) Timeline of mixed lymphocyte reaction (MLR). DC were generated from bone marrow of C57BL/6 mice and cultured for 5 days in presence of three different 9cisRA concentrations, as indicated. Pan T cells were selected by magnet activated cell sorting (MACS) from pooled spleens and lymph nodes of BALB/c mice and co-cultured for six days with the previously generated DC. **(B)** Foxp3+ cells were analyzed by FACS with triple staining for CD4, Foxp3, CD25 on day six of mixed lymphocyte reaction. Data from one of four representative experiments is shown. Data is shown as the percentage of Foxp3+CD4+CD25+ cells \pm SEM (student's t-test). **(C)** Pan T cells were generated from pooled spleens and lymph nodes of C57BL/6 mice and added to CD3/CD28 coated plastic wells. After 48h the percentage of CD4+Foxp3+CD25+ cells was analyzed in triple FACS staining.

3.4 9cisDC strongly induced Treg in an antigen specific system

To extend our experiments to an antigen specific system, we performed MLR in an autologous system using Trinitrobenzenesulfonic acid (TNBS) as an allergen. DC were cultured as described above (subgroups of EtOH, 9cisRA). On day 5 DC subgroups were loaded with TNBS and co-cultured with autologous TC for 5 days. On day 13 co-cultures were restimulated by DC with the same characteristics of the previously used subgroup (either 9cisRA cultured, EtOH cultured, laden with or without TNBS). FACS was performed on day 18 (for detailed description see **Fig. 5 A**). We found that DC that matured under 9cisRA and were TNBS treated had a high potential to generate antigen specific Treg (65%). In comparison, 9cisDC that were not antigen exposed only induced approximately 38% of Treg (**Fig. 5 B, C**). Our findings indicate that 9cisDC are able to induce Treg in an antigen specific manner.

3.5 The capacity of DC to secrete OPN is an important ability of DC for the generation of Treg

As shown above, 9cisDC highly express OPN, while other cytokines secreted by DC are largely down-modulated through 9cisRA treatment (**Fig. 2 B, C**). Therefore we were interested whether OPN itself plays a role in the process of Treg generation through 9cisDC. We therefore performed criss-cross MLR using cells from OPN wild type as well as OPN deficient mice (spp1 deficient) investigating their Treg differentiating potential.

We found that 9cisDC induced a Treg percentage three times as high as the ethanol control treated DC. Interestingly, only OPN deficient DC showed inhibited Treg inducing potential (**Fig. 6 A, B**). To distinguish whether this effect depended on the OPN secreted by DC, we performed rescue experiments, adding recombinant OPN. Addition of recombinant OPN into OPN ^{-/-} DC OPN ^{-/-} T cell MLR rescued Treg generation through 9cisDC (**Fig. 6 C**).

These findings indicate that DC derived secreted OPN is an important cytokine for 9cisRA induced generation of Treg.

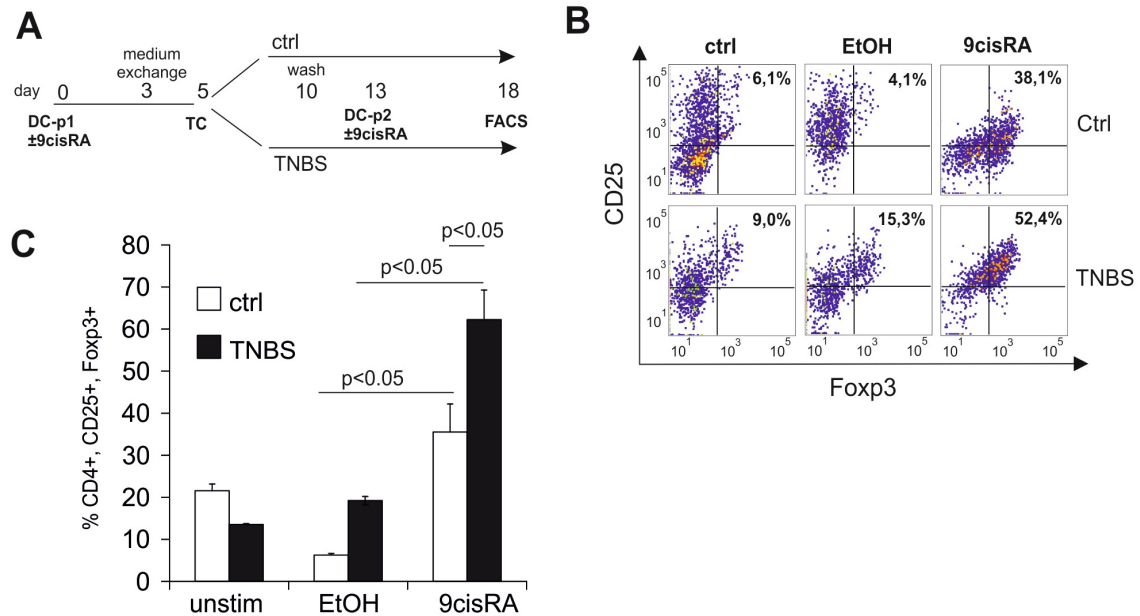


Figure 5 Antigen triggered induction of regulatory T cells (Treg) through 9-cis-retinoic acid (9cisRA) differentiated dendritic cells (DC) [88].

Cluster of Differentiation (CD), Forkhead p3 transcription factor (Foxp3), control (ctrl), Fluorescence Activated Cell Sorter (FACS).

To determine whether 9cisRA influences the antigen specific induction of Treg, autologous mixed lymphocyte reaction (MLR) was performed using 2,4,6-Trinitrobenzenesulfonic acid (TNBS) as allergen. **(A)** Timeline of the experimental procedure: DC were differentiated from murine bone marrow from C57BL/6 mice with GM-CSF and IL-4 (population 1: DC-p1, population 2: DC-p2) for 5 days without further stimulus (unstim), ethanol (EtOH), or 9cisRA 30ng/ml (9cisRA30). On day 5, generated DC were washed and separated into two groups: One was left untreated the second loaded with TNBS. DC were then irradiated with 30 Gy. T cells were purified by negative depletion with magnet activated cell sorting (MACS) from pooled lymph node and spleen cells of C57/BL6 mice and added to DC. T cells were co-cultured with DC until day 10, cultures were washed and resuspended. On day 13, 5-day old DC (DC-p2) were added to the previously primed T cells. FACS analysis of co-cultured cells was performed on day 18. **(B)** Representative FACS analysis of all treatment groups. Cells from co-cultures were triple stained with antibodies against CD4, CD25 and Foxp3 and analyzed by FACS, gating on CD4+ cells. Data is shown as CD25/Foxp3 staining of CD4+ gated cells. The right upper quadrant contains the CD25/Foxp3 double positive CD4+ expressing T cells. **(C)** Average percentage of CD4+/CD25+/Foxp3+ cells in all co-culture groups. Average of data from three co-cultures \pm SEM (student's t-test). Representative data from three independent experiments.

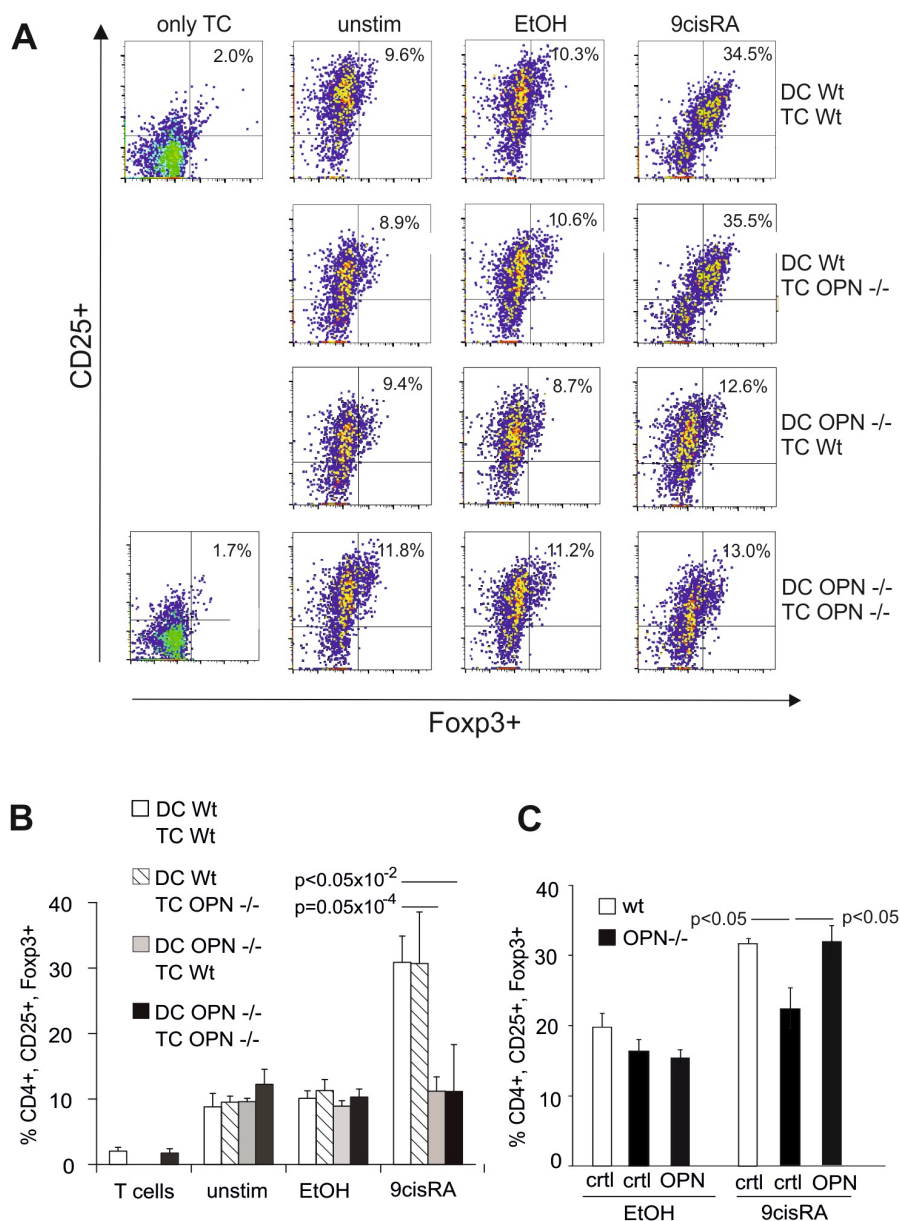


Figure 6 9-cis-retinoic acid (9cisRA) differentiated dendritic cells (DC) from osteopontin (OPN) deficient mice are unable to expand regulatory T cells (Treg) [88].

Cluster of Differentiation (CD), Forkhead p3 transcription factor (Fxp3), wild type (wt), OPN knockout (OPN -/-)

To investigate the role of OPN for Treg generation by 9cisDC, allogeneic mixed lymphocyte reaction (MLR) was performed with T cells and DC from OPN wt and OPN deficient mice (OPN^{-/-}). Murine bone marrow cells from wild type (wt) or OPN deficient C57Bl/6 mice (OPN^{-/-}) were treated with GM-CSF and IL-4 for 5 days. Subgroups of dendritic cells were left untreated (unstim) or differentiated in the presence of ethanol (EtOH) or 9cisRA 30 ng/ml (9cisRA30) during the 5-day maturation period. On day 5, DC were washed and added to T cells that had been purified from pooled spleen and lymph nodes from Balb/c wt or OPN^{-/-} mice. DC T cell co-cultures were performed both with wt or OPN^{-/-} DC or wt and OPN^{-/-} T cells as indicated. **(A)** Representative FACS analysis of the indicated groups from one co-culture experiment. Cells from co-cultures were triple stained with antibodies against CD4, CD25 and Fxp3 and analyzed by FACS on day 10 of co-culture, gating on CD4⁺ cells. Data is shown as CD25/Fxp3 staining. The right upper quadrant contains the CD25/Fxp3 double positive CD4 expressing T cells. **(B)** Average percentage of CD4⁺/CD25⁺/Fxp3⁺ cells in all co-culture groups. Average of data from three co-cultures \pm SEM (student's t-test). Representative data of three independent experiments is shown. **(C)** OPN wt or OPN^{-/-} DC were generated in the presence of EtOH or 9cisRA and cultured with Balb/c OPN wt T cells. Co-cultures were performed without (ctrl) or with addition of recombinant OPN. Representative data of three experiments (student's t-test).

3.6 9cisRA treated dendritic cells do not boost contact hypersensitivity in vivo, but induce Treg

To test our findings in an in-vivo model, we used the trinitrochlorobenzene (TNBC) mouse model of contact hypersensitivity (CHS). Mice were TNBC-sensitized on the abdominal skin and received trinitrobenzenesulfonic acid (TNBS) - loaded control or additionally 9cisRA treated DC injections 6 days after sensitization. A TNBC challenge through skin-application on the ears was performed 14 days after the sensitization (**Fig. 7 A**). We found that injection of TNBS-loaded DC strongly boosted the allergic response, compared to mice that had not received DC injections (**Fig. 7 B**). Furthermore, mice with injection of 9cisRA treated TNBS loaded DC showed significantly decreased ear thickness compared to mice that received simply TNBS treated DC (**Fig. 7 B**). Thus in contrast to untreated DC 9cisDC could not booster CHS. We further collected skin draining lymph nodes on day 16 after sensitization, and found an increase in the percentage of Treg in lymph nodes of the 9cisRA DC treated group (**Fig. 7 C**).

These findings suggest that 9cisDC also suppress established contact hypersensitivity in vivo through induction of Treg.

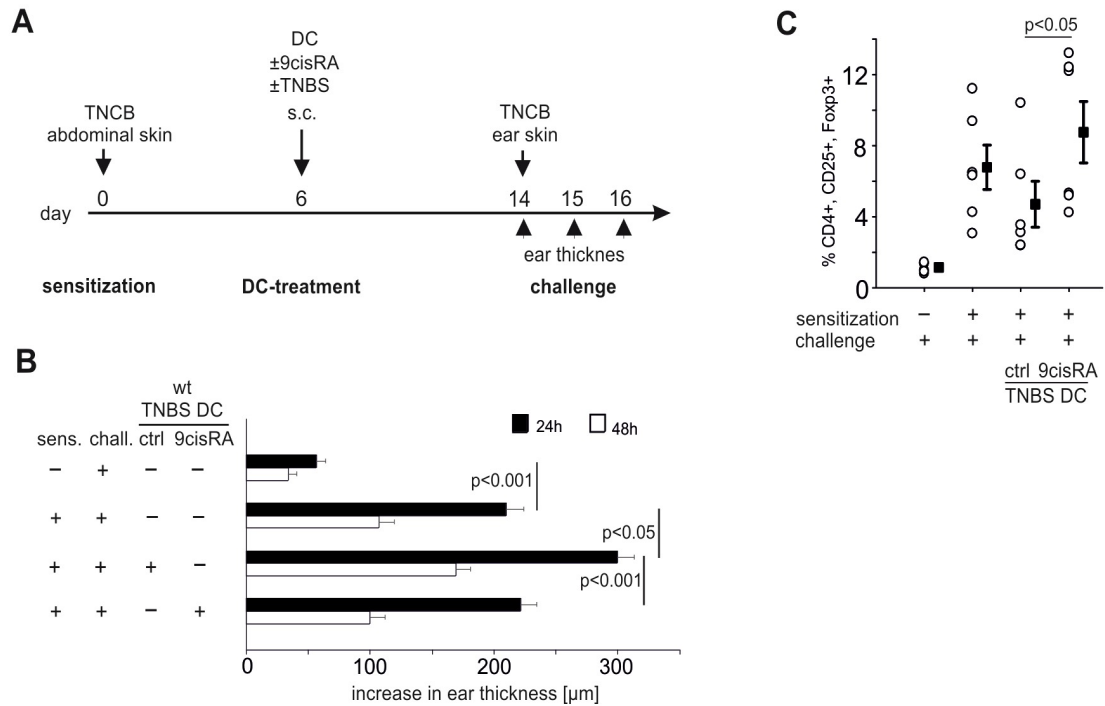


Figure 7 In vivo 9cisDC do not boost established contact hypersensitivity and induce Treg accumulation in skin draining lymph nodes [88].

Sensitized (sens.), challenged (chall.), control (ctrl), wt (wild type), Fluorescence Activated Cell Sorter (FACS), Cluster of Differentiation (CD), Forkhead p3 transcription factor (Foxp3).

(A) On day 0, C57BL/6 wt mice were sensitized on the abdominal skin with trinitrochlorobenzene (TNCB). On day 6, mice were injected with 5-day old osteopontin (OPN) wild type (wt) or OPN $-/-$ dendritic cells (DC), either cultured with 9cisRA or ethanol (EtOH) and either trinitrobenzenesulfonic acid (TNBS) loaded or untreated. On day 14, ear thickness was measured before and after TNCB ear painting. **(B)** Increase in ear thickness \pm -SEM in μ m in the indicated groups (one way ANOVA on ranks). **(C)** Mice were sacrificed on day 16 and skin draining lymph nodes were obtained. T cell suspensions were analyzed for CD4/CD25/Foxp3 positive T cells by FACS (one way ANOVA on ranks).

4 DISCUSSION

Little is known about the immunology of anti-inflammatory effects of retinoids in skin diseases like acne, cutaneous T cell lymphoma, or chronic hand eczema. Previous studies predominantly concentrated on the effect of all-trans retinoic acid (ATRA) on the immune system. Compared to ATRA which only interacts with the RXR receptor, 9-cis-retinoic acid (9cisRA) is a pan agonist for both RXR and RAR receptors [78]. Both intracellular receptors are expressed by dendritic cells [53].

4.1 Effect of retinoids on dendritic cells

4.1.1 9cisRA effects on dendritic cells

For the first time effects of retinoids, including 9cisRA, were analyzed with human Langerhans cell like DC by Geissmann et al., who found an activation of such LC like DC, with increased MHC-II and CD86 expression. However, in contrast to our study these experiments were performed in the presence of other inflammatory stimuli, like TNF- α [53]. The function of these retinoid treated LC like DC was not determined. Villablanca et al. investigated the effect of 9cisRA on human DC maturation. They found a decrease in the expression of chemokine receptors CCL7 and CXCR4 in vitro and accordingly an impeded migratory capacity of 9cisDC in vivo [159]. Hengesbach et al. investigated the maturation of murine bone marrow derived DC under 9cisRA influence, and found a decrease in MHC-II and CD86, but an increase in co-stimulatory CD80 expression [67]. Again, Functional experiments of these DC were not performed.

4.1.2 Co-stimulatory and co-inhibitory molecules

To further these studies, we used murine bone marrow derived DC and cultured them in the presence or absence of 9cisRA. DC express co-stimulatory and co-inhibitory molecules that are of central role in the modulation of T cell differentiation. We found that 9cisRA matured DC have higher CD11c expression, but express less MHC-II and co-stimulatory molecules CD86 and CD80 than untreated DC.

Antigen presenting cells present antigen-derived peptide on MHC-II to CD4⁺ T cells. Consequently, a decrease in MHC-II expression as induced by 9cisRA implicates a reduction in antigen presentation to T cells, offering a less potent stimulus for T cell activation in general. CD80 and CD86 are important co-stimulatory molecules in the activation process of T cells that bind to CTLA-4 on TC. CD80 is constitutively expressed on immature DC at low levels and was found to be up-regulated early after DC activation [89]. CD86 is important for the sensitization of naïve T cells in a murine asthma model: e.g. administration of CD86-specific antibody before systemic sensitization reduced the pulmonary inflammatory response and airway hyper-responsiveness [35].

Investigating co-inhibitory molecules on 9cisDC we found an increased PD-1L expression. However, no significant change in the constitutively high expression of ICOS-L was detected. PD-1 is a co-inhibitory receptor on B cells and activated T cells and binds to either PD-1L or PD-2L, and has recently been studied as a co-inhibitory therapeutic target for melanoma therapy. It was demonstrated, that tumor cells protect themselves by expressing PD-1L and thereby inhibit anti-tumoral T cell responses [97]. In the context of allergic contact dermatitis, up-regulation of PD-1L on DC may also contribute to a weakened T cell stimulation. Inducible co-stimulatory molecule (ICOS) plays a role in the induction of CD4⁺, CD25⁺ Foxp3⁺ regulatory T cells in an airway hyper-reactivity model. Akbari et al. described a role of high ICOS-L expressing DC in combination with presence of IL-10 in the induction of regulatory T cells in their murine model of airway hyperactivity [4]. In our set of experiments, we could not determine relevant changes in ICOS-L expression of DC under 9cisRA treatment. However, although not regulated by 9cisRA, ICOS-L, which is constitutively expressed on 9cisDC, is likely to be relevant for Treg induction in the context of other co-stimulatory or co-inhibitory molecules expressed by 9cisDC.

In conclusion, 9cisRA treatment during DC culture induces a distinct DC phenotype with low expression of co-stimulatory but high expression of co-inhibitory molecules suggesting that these DC are efficient inducers of Treg differentiation.

4.1.3 Cytokine and chemokine secretion by 9cisDC

We further found that 9cisDC secrete a distinct cytokine chemokine pattern which suggests a regulatory, anti-inflammatory phenotype. On the cytokine level, a significant reduction in DC secretion of IL-3 and IL-7 was found following 9cisRA treatment. Both IL-3 and IL-7 are important cytokines in the maturation process of T cells from progenitor cells. IL-7 is additionally important for survival of peripheral T cells [3]. Furthermore, we found decreased IL-1 β secretion of 9cisDC. IL-1 β is a critical pro-inflammatory cytokine. In the skin immune system IL-1 β is secreted by keratinocytes and dermal fibroblasts after antigen exposure and induces IL-12 in DC [134]. DC with down-modulated IL-12 secretion are less potent in inducing Th1 responses. The only cytokine which we found to be up-regulated by 9cisDC was OPN. This glycoposphoprotein was shown to have both pro- and anti-inflammatory functions, which will be discussed in detail below.

Presence of chemokines is important in the elicitation phase of allergic contact dermatitis, by accumulating, stimulating and activating effector cells [58]. We analyzed chemokine expression of DC that underwent 9cisRA treatment and found significant reduction in the expression of T cell attracting CXCL9 and CXCL10. C-X-C motif ligand 9 was found to be highly expressed in CHS after allergen exposure, with maximum mRNA levels 72h after elicitation. CXCL9 modulates leukocyte trafficking through the dermis and epidermis in contact hypersensitivity [58]. CXCL10 secretion depends on high IFN- γ levels, which in turn depends on the presence of IL-12 [6]. Consistently, we could detect decreased IL-12p70 levels by 9cisDC. In a model of experimental asthma, mast cell produced CCL1 was important for CD4⁺ T cell homing [167]. Also in CD8⁺ T cell mediated contact hypersensitivity CCL1 recruits CD8⁺ T cells and other leukocytes to the site of allergen challenge [83]. We found 9cisRA to reduce CCL1 expression by DC.

In conclusion, we found 9cisRA to inhibit DC secretion of chemoattractants for T cells thereby down-modulating their proinflammatory potential.

4.1.4 Effect of 9cisDC on T cell differentiation

Pulendran et al. noted that immature DC, characterized by low surface expression of co-stimulatory molecules and MHC-II, lead to imperfect T cell priming, often followed by either T cell anergy or Treg generation [124]. Lamina propria derived gut

dendritic cells have been shown to induce regulatory T cells in presence of TGF- β and ATRA [153]. amongst other reports, Scott et al. found that a tolerogenic effect of DC in the intestinal immune system was to induce Treg through production of ATRA which shaped of tolerance for nutritional allergens [143] [153] [130]. Hill et al. suggested a dampening effect of ATRA on highly CD44 expressing CD4⁺ T cells, which suppressed Treg induction by IL-4, IL-21 and IFN- γ in the absence of RA [68]. In accordance to our findings of reduced co-stimulatory molecules on 9cisRA treated DC, we also found a reduced T cell allo-stimulatory capacity of 9cisDC. We did not find the previously described [34] connection between TGF- β , retinoic acid and Treg induction, because we detected no significant changes in TGF- β expression under 9cisRA treatment. However we found that 9cisRA potently induces the differentiation of regulatory T cells and OPN secreted by 9cisDC is an important factor in this induction, as discussed below. The well defined tolerogenic DC phenotype in the gut expresses CD103 [34]. Although the 9cisDC described here have a somewhat similar phenotype found in gut CD103⁺ DC, 9cisDC do not express CD103 (data not shown), thus indicating that 9cisDC have a new distinct tolerogenic phenotype.

We additionally investigated as to whether 9cisDC would also induce Treg in an antigen specific model: We used TNBS as antigen and either 9cisRA treated DC or controls. Again, 9cisDC mediated induction of high numbers of Treg. These findings indicate that 9cisDC are effective in inducing tolerogenicity in the presence of a specific antigen. Suppressor assays were not performed, however an important element for future study.

4.2 9cisRA effects on Osteopontin

4.2.1 OPN induction through 9cisRA

As we found OPN to be the only cytokine up-regulated by 9cisDC amongst a broad panel of cytokines, we speculated that OPN may have an important function in Treg induction. The signaling pathways through which 9cisRA regulates OPN secretion in DC are not known. However, regulation of OPN through TLR has been partially investigated. Manicassamy et al. described a TLR2-dependent pathway which induced retinaldehyde dehydrogenase type 2, increasing conversion of retinal to RA.

DC-expressed RAR activation led to SOCS3 induction, suppressing p38 MAPK and therefore decreasing pro-inflammatory cytokine secretion of IL-6, IL-23 and IL-12 [100]. Salvi et al. reported that in human DC TLR2, TLR5, TLR7 agonists or IL-1 β activate MyD88, a NF κ B transcription factor activator, and thereby increase inflammatory cytokines and also OPN [138]. In contrast, TLR3 and TLR4 agonists limited OPN production in human DC [138]. We speculated that these signaling pathways may also be of relevance in the induction of the here described 9cisDC phenotype: OPN up-regulation is possibly mediated by induction of the MyD88 pathway by 9cisRA, Th1 cytokine induction on the other hand may be achieved through blocking of p38 MAPK. However, 9cisRA signaling is mediated through ligation of both RAR and RXR receptors thereby leading to complex signaling mechanisms shaping the 9cisDC phenotype. Differentiated investigation of signaling cascades that induce the 9cisRA phenotype will be an interesting topic of future investigation.

4.2.2 OPN in CHS and regulatory T cells

OPN has been shown to have both proinflammatory and anti-inflammatory regulatory functions. Regarding pro-inflammatory OPN function in CHS, it has chemotactic functions, and attracts inflammatory cells such as T cells and macrophages to the site of inflammation [7] [150]. Previous work used the well-established murine contact hypersensitivity model (CHS) to investigate the role of OPN in allergic contact dermatitis.

Expression of OPN in CHS

In murine CHS, application of TNBC to murine abdominal skin during sensitization led to up-regulation of OPN mRNA expression in the skin and its draining lymph nodes, increasing OPN levels were measurable within 12 hours of application [164]. Elevated OPN levels were also detected in the skin of people with acute and chronic allergic contact dermatitis and chronic eczema patients had elevated OPN plasma levels [144].

In hapten treated skin, OPN was important in the attraction of DC and Langerhans cells to skin draining lymph nodes. OPN $-/-$ mice showed an impaired DC/ Langerhans cell migratory capacity [164].

It was previously shown that DC can strongly secrete OPN, which was inhibited by LPS [142]. Further OPN effects on DC were investigated: Renkl et al. cultured 5-day old immature human dendritic cells with recombinant OPN. They found that OPN treatment increased Th1-promoting cytokine secretion of TNF- α and IL-12. Furthermore, OPN treatment induced the expression of MHC-II, CD40, CD80, CD86 and CD44 on these DC [127]. Performing functional experiments, they showed that OPN-induced IL-12 secretion by DC increased Th1-promoting IFN- γ production of naive T cells. [127]. During the challenge phase of CHS, memory T cells secrete OPN which attracts other inflammatory cells to the site of antigen contact, and thereby helps in establishing chronic eczema by induction of IL-12 and IFN- γ which subsequently promote Th1 response [144]. Initiating our experiments we therefore speculated that OPN induction by 9cisRA would have pro-inflammatory effects. Interestingly, we could show that OPN is important for the DC dependent Treg induction. When co-culturing OPN -/- 9cisDC with naive T cells, Treg were no longer induced. However, addition of recombinant OPN restored Treg differentiation. Our findings suggest that DC derived OPN may be a factor in preventing exaggerated inflammation through enhanced Treg differentiation. This hypothesis was further supported by our in vivo model of contact hypersensitivity. Here we found that injection of antigen loaded 9cisDC did not augment CHS response in contrast to 9cisRA untreated TNBS loaded DC. Importantly, 9cisDC injected mice had higher Treg numbers in skin draining lymph nodes, suggesting that the stronger Treg expansion inhibits inflammation.

Our findings that OPN from DC may have anti-inflammatory functions is supported by work from other groups. In a model of inflammatory bowel disease OPN reduced the level of blood neutrophils, pro-inflammatory cytokines and inflammatory cell migration to gut mucosa [38]. Additionally, sOPN and iOPN have been proven to prevent exaggerated inflammation in early stages of sepsis: macrophage derived sOPN attracted T cells to site of LPS injection, T cell macrophage interaction lead to increase of iOPN with subsequent suppression of macrophage TNF production [76]. In our system we were not able to investigate the functions of iOPN, which may also contribute to Treg differentiation in a hitherto unknown manner., this requires further investigation. Sangaletti et al. observed reduced levels of Treg and increased activ-

ity of CD4⁺ cells in OPN deficient mice in lung metastases of mammary adenocarcinoma. These findings suggest that OPN from tumors may have similar effects as DC secreted OPN in supporting tumor escape from immune destruction [139].

In accordance with the previous mentioned studies, we conclude that OPN may acquire anti-inflammatory functions. We found that in a distinct environment, such as in the presence of tolerogenic DC, OPN is important for the generation of Treg. This mechanism may be one of the key functions in the therapeutic effect of 9cisRA as a treatment of chronic hand eczema.

Prevention of damage

In delayed type T cell mediated allergic disease, regulatory T cells are thought to circulate between skin, draining lymph nodes and secondary lymphatic organs, depending on whether they express skin homing molecules, like CLA, or chemokine receptor CCR7, regulating migration to secondary lymphatic organs [24]. After allergen contact, CD25⁺ T cells inhibited proliferation of CD4⁺ and CD8⁺ cells, both at sensitization and draining lymph nodes [24]. In our in vivo study, we focused on the effect of 9cisDC on the challenge phase of contact hypersensitivity. In our antigen specific in vitro model, we exposed DC to 9cisRA before antigen-loading and co-culture with T cells. It will be of future interest to analyze hereafter as to what extent the demonstrated Treg induction may prevent antigen sensitization. Induction of Foxp3⁺ CD4⁺ CD25⁺ T cells through retinoic acid was described in murine gut derived CD103⁺ DC [143] [153]. Guillemins et al have described RA dependent Foxp3⁺ T cell induction by DC also in murine skin, lungs and corresponding lymph nodes [61]. Our data supports these findings, indicating that under the influence of retinoids a tolerogenic DC phenotype develops which may also mediate tolerance in the skin immune system. However, further in vivo experiments will have to validate this speculation. The role of OPN in this system remains to be studied in depth.

In summary, depending on the type of the producing cell, the tissue microenvironment and the responding cell type, OPN can play diverse roles. In the presence of tolerogenic DC, we saw secreted OPN mediate induction of a subset of anti-inflammatory Treg. Regarding CHS our findings suggest that overexpression of OPN by DC may be an important factor in terminating excessive inflammation in the challenge phase of CHS.

4.3 Conclusion and outlook

In conclusion, this study demonstrates that therapeutically relevant 9cisRA levels generate a distinct DC phenotype with reduced expression of co-stimulatory molecules, but up-regulated expression of co-inhibitory PD1-L. This 9cisDC phenotype has Treg inducing capabilities. Furthermore, 9cisDC induce Treg in an antigen specific system. Performing crisscross MLR with OPN deficient DC and T cells, we found that the expansion of Treg depends on DC derived secreted OPN. In vivo 9cisDC are also functional as they do not boost established contact hypersensitivity and induce Treg accumulation in skin draining lymph nodes.

Our studies contribute to the understanding of the mechanisms of action of retinoids in inflammatory skin diseases. Future work has to investigate whether such tolerogenic 9cisDC may function as a useful tool in the treatment of established contact allergy or in the generation of tolerance in autoimmune mediated diseases.

5 SUMMARY

9-cis-retinoic acid (9cisRA) is a high-affinity pan-agonist for the retinoic acid receptors (RAR) and retinoid X receptor (RXR). 9cisRA is effective for treating chronic hand eczema, which is often associated with delayed type allergy. There is limited data showing how 9cisRA exerts anti-inflammatory functions in the skin immune system. Osteopontin (OPN) is a glycoposphoprotein with cytokine functions in autoimmunity and allergy, and is strongly expressed by immune cells in the inflammatory infiltrate of contact dermatitis.

We were interested in the phenotype and function of dendritic cells (DC) matured in the presences of 9cisRA (9cisDC) in vitro and in vivo. Furthermore, the role of OPN for the function of 9cisDC was investigated.

To answer these questions murine bone marrow derived DC were cultured in the presence of different concentrations of 9cisRA. We found that in comparison to untreated DC the highly CD11c expressing 9cisDC expressed less MHC-II, CD44, CD80 and CD86. In contrast, the co-inhibitory PD1-L was induced on 9cisDC. Furthermore, 9cisDC had an altered pattern of cytokine and chemokine expression, secreting less IL-1 β , IL-12p70, CXCL9, CXCL10 and CCL-1, but more OPN. To investigate the functional characteristics of 9cisDC we performed allogeneic mixed lymphocyte reactions (MLR). 9cisDC were less potent in stimulating T cell proliferation, however, they potently converted naive T cells into CD4⁺/ Foxp3⁺/ CD25⁺ regulatory T cells (Treg). Compared to controls such co-cultures contained less IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A and IFN- γ . Speculating that the strongly induced OPN from 9cisDC could be of role for Treg conversion, we performed criss-cross allogeneic MLR with OPN deficient DC and T cells. Interestingly, we found that 9cisDC from OPN deficient mice are compromised in their Treg inducing function which could be rescued by addition of recombinant OPN.

When trinitrochlorobenzene (TNCB) sensitized mice were treated with trinitrobenzenesulfonic acid (TNBS) loaded DC 6 days after sensitization CHS was strongly boosted. In comparison, TNBS loaded 9cisDC were not able to boost CHS. Importantly, these TNBS loaded 9cisDC augmented Treg in skin draining lymph nodes, supporting the notion that 9cisDC have a regulatory effect on CHS in vivo.

In conclusion our findings propose that 9cisRA modulates DC toward a phenotype that is able to suppress established contact allergy through the induction of Treg, a mechanism that is at least partially modulated by OPN.

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