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Review

The phenotype and function of murine bone marrow-derived dendritic cells is not affected by the absence of VDR or its ability to bind 1α,25-dihydroxyvitamin D_3

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ABSTRACT

The nuclear vitamin D receptor (VDR) is generally recognized as a ligand-dependent transcription factor that mediates the actions of its natural ligand, 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$D$_3$) on multiple target genes involved in mineral homeostasis, bone development, as well as immune reactivity. As the VDR is widely distributed in nearly all cells of the body, it implies that the vitamin D endocrine system may regulate many cell types and functions. Experiments in VDR null mice established that the VDR has intrinsically critical roles in skin and keratinocyte biology but not in immune responses. Oppositely, absence of the VDR ligand is linked to susceptibility to autoimmunity, illustrating a potential role for the unliganded VDR in the immune system. This discrepancy stimulated us to further investigate the impact of the VDR on the phenotype and function of myeloid dendritic cells (DCs) generated *ex vivo* from bone marrow precursors of VDR null (with a truncated VDR) and VDR ΔAF2 mice (with a mutated C-terminal activation factor 2 domain thus rendering ligand-induced gene transcription impossible). Absent or unliganded VDR did not affect bone marrow-derived myeloid DC generation. DCs obtained from VDR null and VDR ΔAF2 bone marrow cells had comparable MHC-II, and costimulatory molecule CD86, CD80 and CD40 expression than DCs from wild-type bone marrow cells. Additionally, an unliganded VDR did not affect the cytokine production nor the antigen-specific T cell stimulatory capacity of bone marrow-derived DCs. In conclusion, we showed that although clear effects of 1α,25-dihydroxyvitamin D_3 are described on DC generation, absence of VDR or presence of an unliganded VDR does not affect the profile and function of *ex vivo* generated bone marrow-derived DCs.

Contents

Abbreviations: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D_3; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; VDR, vitamin D receptor; DBD, DNA binding domain; LBD, ligand binding domain; RXR, retinoid X receptor; VDRE, vitamin D response element; MHC-II, major histocompatibility complex class II; NOD, non-obese diabetic; WT, wild-type; AF-2, activation factor-2 domain; OVA, ovalbumine; IL, interleukin; Tg, transgenic.

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1. Introduction

The vitamin D receptor (VDR) is a member of a family of nuclear transcription factors. The VDR regulates gene expression through heterodimerization with the retinoid X receptor (RXR), both in the absence or presence of its natural ligand, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), and recruits co-repressors or co-activators to vitamin D-responsive elements (VDREs) in the promoter region of target genes. The VDR protein consists of 2 major functional units: a DNA binding domain (DBD) with two zinc fingers at the N-terminus, enabling the receptor to associate with the DNA, and a C-terminal ligand binding domain (LBD). Ligand binding will induce a crucial conformational change in the helix 12 motif, corresponding to the transcriptional activation domain designated AF-2, releasing associated co-repressors while a platform is created to recruit the co-regulatory complex and gene transcription is initiated [1–3]. The VDR has a widespread expression throughout the body. Therefore, interaction of the VDR with 1α,25$(OH)_2D_3$ results in the regulation of multiple target genes responsible for effects in mineral homeostasis, bone development, as well as immune reactivity [4]. In case of absence of 1α,25$(OH)_2D_3$, the VDR–RXR complex recruits co-repressors to the VDREs resulting in transcriptional repression mediated by histone deacetylation. Recently, it has been recognized that the VDR protein independent of ligand may also have functional importance.

As a result of VDR expression in the immune system, 1α,25$(OH)_2D_3$ presents direct immunomodulatory effects on different immune cells, of which dendritic cells (DCs) are the major targets. DCs act as professional antigen-presenting cells providing a link between innate and adaptive immune responses. Immature DCs, resident in peripheral tissues, are specialized in capturing antigens and are able to respond to microbial and inflammatory stimuli. Upon encounter with maturation stimuli, DCs will alter the expression of several molecules, such as the antigen-presenting molecule major histocompatibility complex (MHC) II, co-stimulatory molecules (*e.g.* CD40, CD80, and CD86) and chemokine receptors (*e.g.* CCR7), in order to become potent T cell stimulators. In addition, the mature DC can direct the T cell response by the production of T helper (Th)-polarizing cytokines (*e.g.* IL-12, IL-23, IL-10) and pro-inflammatory cytokines [5]. In mice and man, mainly, DCs of two origins, myeloid and lymphoid, have been identified but their location in various organs, as well as phenotypes and functions are incompletely described. Both lineages can be generated *ex vivo* from the bone marrow under stimulation of different growth factors (*e.g.* granulocyte macrophage colony stimulating factor (GM-CSF)/interleukin (IL)-4 [myeloid or conventional DCs] and Flt-3 ligand [plasmacytoid DCs]). Upon binding of 1α,25$(OH)_2D_3$ to the VDR, both the differentiation and maturation of particularly myeloid DCs will be inhibited, leading to reduced cell surface expression of CD40, CD80, and CD86 costimulatory molecules, reduced T cell stimulatory function, and decreased survival [6,7].

Interestingly, epidemiological data show a strong correlation between vitamin D_3 deficiency and adverse immune effects, including inflammation and autoimmunity [8–10]. In addition, vitamin D_3 deficiency during early life increases the incidence and severity of several autoimmune diseases [11,12]. Remarkably, while absence of ligand accelerates disease, lack of the VDR does not show aggravated symptoms [13,14]. Moreover, in the skin VDR ablation causes total body alopecia and dermal cysts while vitamin D_3-deficiency does not result in a hair cycle/skin phenotype [15]. This discrepancy between absence of either ligand or receptor indicates important actions for the unliganded VDR in several tissues, but also in the regulation of immune responses.

To study the effects of an unliganded VDR, the Tokyo VDR null and the VDR ΔAF-2 mouse models were used. The VDR null strain was generated by a targeted deletion of the second exon of the VDR gene encoding the first zinc finger domain in the DBD of the nuclear receptor [16] resulting in a truncated VDR [17], which is unable to bind to VDREs, but contains intact LBD and AF2 motifs. The VDR ΔAF-2 mouse model has a deletion of exon 8 and 9 encoding the AF-2 domain, which destroys VDR function. Although DNA binding is still possible, DNA remains associated with co-repressors as the transcriptional initiation complex cannot be recruited. In case of negative VDREs, the VDR will be associated with the transcription machinery. This is similar to the condition of absent 1α,25$(OH)_2D_3$ resulting in an unliganded VDR which is likewise associated with co-repressors.

In the current study, we aimed thus to establish whether the presence of an unliganded VDR could interfere with the generation, phenotype and function of *ex vivo* generated bone marrow-derived myeloid DCs (BMDCs). Taken together, our data indicate that BMDCs derived from VDR ΔAF-2 mice presenting an unliganded VDR did not differ in surface marker expression, cytokine production nor in T cell proliferative capacity compared to BMDCs derived from wild-type (WT) or VDR null mice.

2. Materials and methods

2.1. Animal models

The VDR null [16] and VDR ΔAF-2 mouse strain were kind gifts of Dr. S. Kato, University of Tokyo, Tokyo, Japan). WT C57BL/6, VDR null and VDR ΔAF-2 breeding pairs and weaned pups were housed in SPF conditions and kept on a calcium/lactose-enriched diet (2% calcium, 1.25% phosphate, 20% lactose and 1400 IU/kg Vitamin D_3; Ssniff Spezialdiäten, Soest, Germany). OT-II transgenic (Tg) mice, which carry the MHC class II-restricted Tg T cell receptor for $OVA_{323-339}$, were kindly provided by Prof. M. Moser (ULB, Brussels, Belgium) and further bred in our conventional animal facility on normal chow. All experimental protocols were reviewed by the Ethics Committee of the KU Leuven (Project number P221/2013).

2.2. Generation of myeloid DCs

BMDCs were generated from 3 to 4 week-old mice according to a previously established protocol [6,18]. Briefly, bone marrow precursors were cultured in RPMI 1640 medium (Life Technologies, Rockville, MD, USA) supplemented with Glutamax-I, 25 mM HEPES, 10% heat-inactivated fetal calf serum (FCS) (Thermo

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Scientific, Sunnyvale, CA, USA), 50 μM 2-mecraptoethanol (2-ME) (UCB, Brussels, Belgium) and 100 U/ml Penicillin/Streptomycin in the presence of 20 ng/ml murine recombinant IL-4 and 20 ng/ml murine recombinant GM-CSF (Peprotech, Rocky Hill, NJ, USA) for 8 days. At day 8 of the culture, $CD11c^+$ cells were magnetically isolated (MACS, Miltenyi Biotec, Bergish Gladblach, Germany) and subsequently maturated for an additional 24 h in the presence of 1 μg/ml LPS (Sigma, St. Louis, MO, USA) and 20 ng/ml murine recombinant interferon (IFN)-γ (Peprotech). Mature DCs were harvested at day 9 of the *in vitro* culture and subjected to phenotypical analyses. To assess their T cell proliferative capacity, DCs were pulsed with 0.1 μg/ml $OVA_{323–339}$ peptide (Innovagen, Lund, Sweden) for 2 h at 37 °C prior to co-culture with $CD4^+CD25^-$ $OVA_{323–339}$-reactive T cells.

2.3. Surface marker expression

BMDCs were analysed for their surface marker expression using flow cytometry. DCs were stained with antibodies against CD11c, MHC-II (I-A/I-E), CD80, CD86, CD40, PD-L1 or F4/80 (all from eBioscience). Matching isotype IgG antibodies were used as a negative controls. After extensive washing and fixation, the stained cells were analysed using a Gallios™ Flow Cytometer (Beckmann Coulter, Analis, Suarlée, Belgium).

2.4. Gene expression analysis

mRNA was isolated using the RNeasy Micro kit (Qiagen, Venlo, The Netherlands). After cDNA synthesis, gene expression levels were determined by RT-qPCR using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences used for the detection of *hmbs*, *hprt*, *il-1β*, *il-6*, *il-10*, *il-12p40*, *inos*, *ip-10*, *rpl27*, *tnf-α* (Eurogentec, Seraing, Belgium) are listed in Supplemental Table 1. Gene expression was quantified by the ΔΔCt method and normalized against the geometrical mean of *hmbs*, *hprt* and *rpl27* expression.

2.5. Cytokine secretion

A V-plex Proinflammatory Panel 1 (mouse) kit MSD MULTI-SPOT Assay System (Meso Scale Discovery, Rockville, MD, USA) was used, following the manufacturer's instructions, for the quantitative detection of the secreted levels of IL-10, IL-12p70, IL-1β, IL-6 and TNF-α in DC culture supernatants or IL-10 and IFN-γ in the DC:T cell co-culture supernatants.

2.6. In vitro *T cell proliferation assay*

T cells were purified from the spleen and lymph nodes of 8-10 week-old OT-II Tg mice using magnetic separation with Dynabeads (Invitrogen, Merelbeke, Belgium). Briefly, $CD4^+CD25^-$ T cells were isolated by negative selection using antibodies against CD16/CD32, CD11b, CD11c, B220, MHC-II, CD8a and CD25 (all from eBioscience). Isolated T cells (10^5/ml) were co-cultured with $OVA_{323–339}$-pulsed mature DCs at a 1:10 DC:T cell ratio in complete medium (RPMI 1640 supplemented with Glutamax-I, 25 mM HEPES, 10% FCS, 50 μM 2-ME, 100 U/ml Penicillin/Streptomycin, 1 mM glutamine and 1 mM Na-Pyruvate) for 3 days. T cell proliferation was determined by ^{3}H-thymidin (1 μCi/well) incorporation during the last 18 h of co-culture.

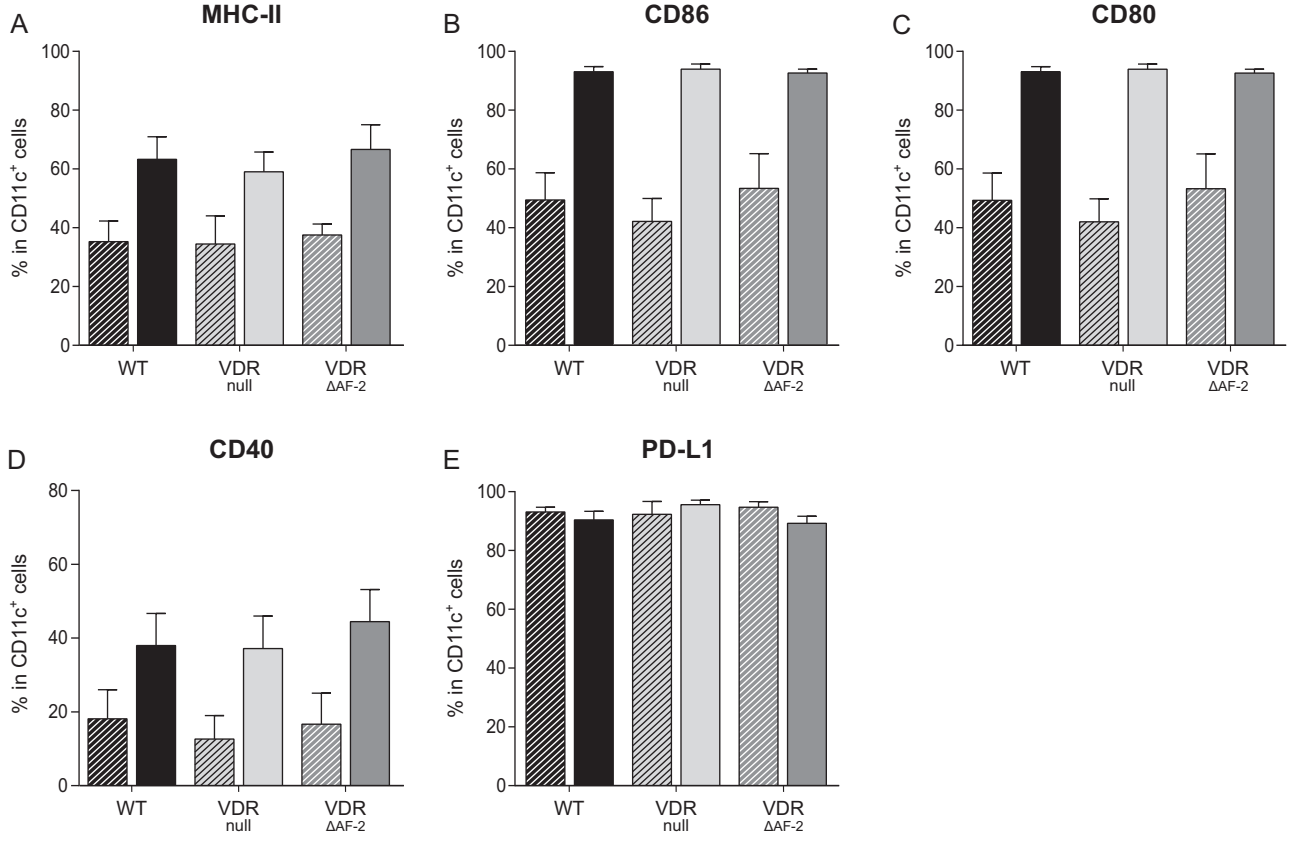

Fig. 1. Surface marker expression of BMDCs. DCs were generated *in vitro* from the bone marrow of WT, VDR null or VDR ΔAF-2 mice, as described in Section 2. Surface marker expression of (A) MHC-II, (B) CD86, (C) CD80, (D) CD40 and (E) PD-L1 was analysed in unstimulated (striped bars, $n = 4$) and stimulated (plain bars, $n = 5$) BMDCs by flow cytometry. Graph bars represent percentage of positive cells in the $CD11c^+$ cell population (mean ± SEM).

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2.7. Statistical analysis

Statistical significance was determined using Kruskal–Wallis test with Dunn's multiple comparisons or a one sample *t* test. Results were expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. An unliganded VDR has no influence on murine BMDC generation and phenotype

To determine whether the unliganded VDR plays a role in BMDC generation or phenotype, DCs generated *ex vivo* from bone marrow precursors of WT, VDR null and VDR ΔAF-2 mice were analysed for their surface marker expression by flow cytometry. We first established that LPS and IFN-γ are potent maturation/activation stimuli in BMDCs from the three mouse strains (Fig. 1). Comparison of either unstimulated or stimulated BMDCs derived from WT, VDR null and VDR ΔAF-2 mice clearly showed no differences in the surface expression levels of the antigen-presenting molecule MHC-II, the co-stimulatory molecules CD86, CD80 and CD40, and the inhibitory molecule programmed death ligand 1 (PD-L1) (Fig. 1).

When analysing the BMDC cytokine and chemokine gene expression profile, a few changes could be observed between stimulated BMDCs derived from WT and VDR null/VDR ΔAF-2 mice (Fig. 2). The mRNA levels of *il-12p40* (Fig. 2A) and *tnf-α* (Fig. 2D) were significantly up-regulated in VDR null BMDCs (as compared to WT BMDCs, $^{*}p < 0.05$).

To validate the observed changes in cytokine and chemokine gene expression, the secretion levels of several proteins into the supernatant of stimulated DC cultures was determined. Interestingly, protein levels of IL-12p70, IL-10, IL-6, TNF-α and IL-1β (Fig. 3) did not show significant differences between mature BMDCs generated from WT, VDR null or VDR ΔAF-2 mice.

3.2. An unliganded VDR did not influence the in vitro T cell stimulatory capacity of murine BMDCs

As primarily mature BMDCs are potent T cell stimulators, we next investigated the functional impact of an unliganded VDR on the T cell stimulatory capacity of BMDCs. For this purpose, BMDCs were loaded with 0.1 μg/ml $OVA_{323-339}$ peptide and co-cultured for 72 h with $CD4^{+}CD25^{-}$ T cells derived from OT-II Tg mice. As shown in Fig. 4, VDR ΔAF-2 DCs did not present a higher T cell stimulatory capacity as compared to WT or VDR null DCs (Fig. 4A). In agreement with this, no significant changes in the secreted levels of IFN-γ (Fig. 4B) and IL-10 (Fig. 4C) into the co-culture supernatant were observed.

4. Discussion

The widespread expression of the VDR in immune cells [4] as well as the correlation between allelic variations in or absence of

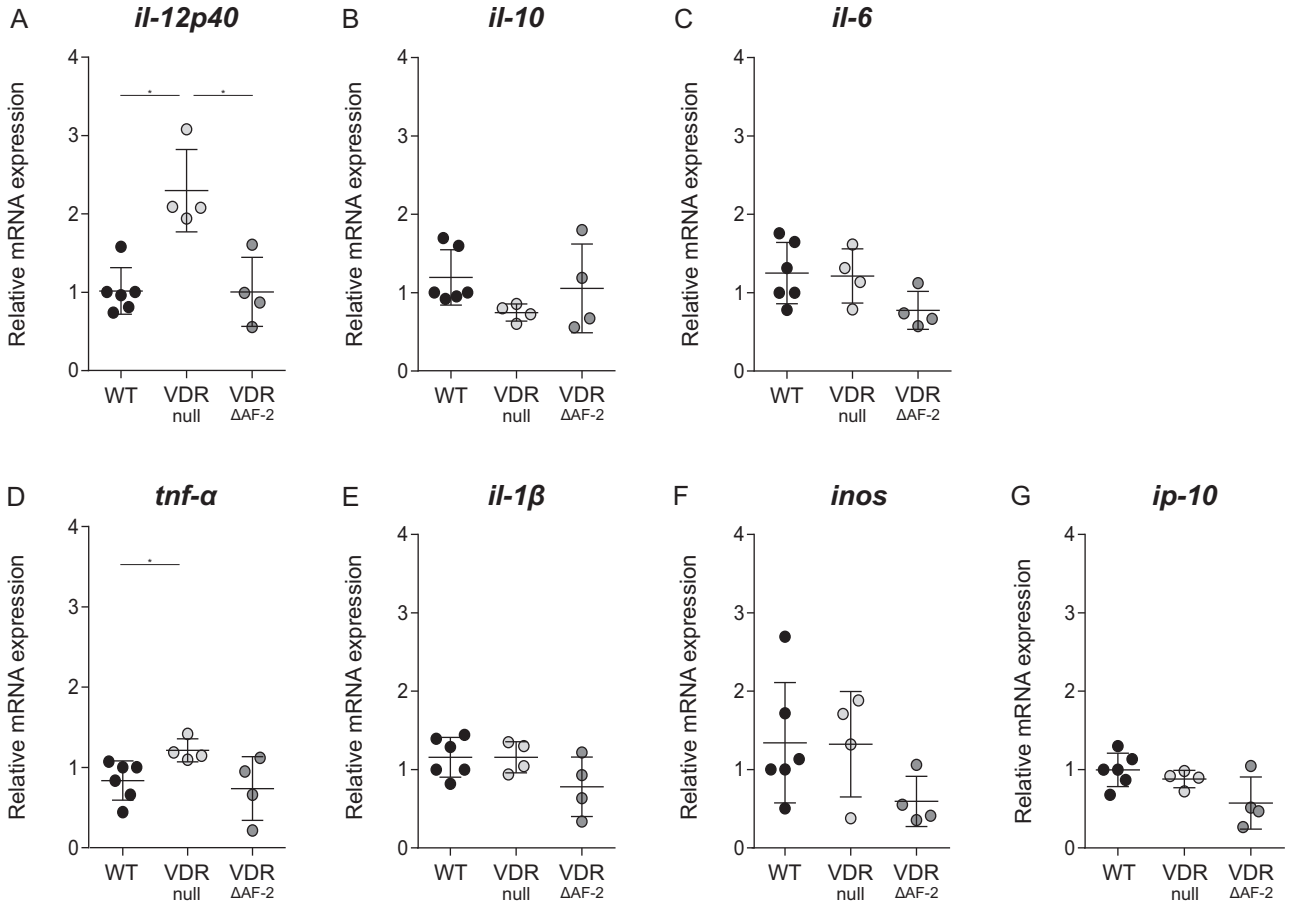

Fig. 2. Gene expression profile. Relative mRNA expression levels of (A) *il-12p40*, (B) *il-10*, (C) *il*-6 (D) *tnf-α*, (E) *il-1β*, (F) *inos* and (G) *ip-10* in DCs were analyzed by qRT-PCR. Values were normalized against *hprt*, *rpl27* and *hmbs* and expressed relatively to the WT-derived condition. Graphs represent the mean ± SEM ($n = 4$–6) of the different gene expression levels.

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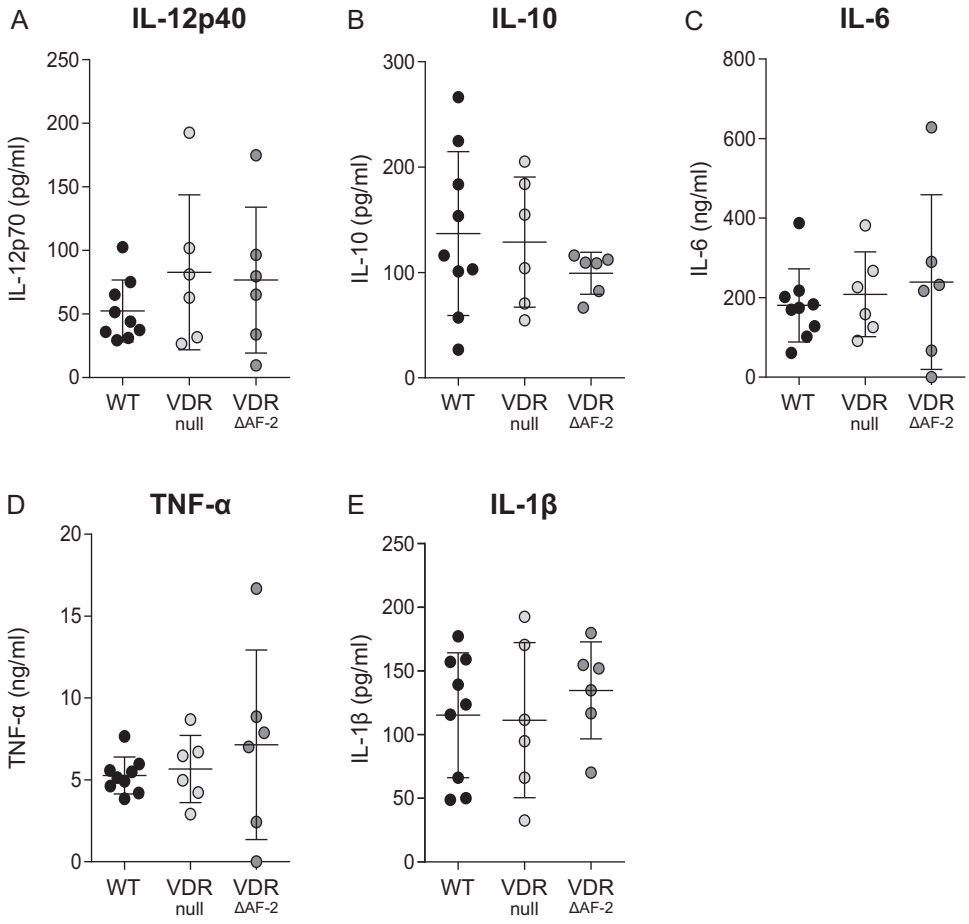

Fig. 3. Secretion of proteins into the supernatant of the BMDC cultures. Secretion of (A) IL-12p70, (B) IL-10, (C) IL-6, (D) TNF-α and (E) IL-1β protein in the BMDC culture supernatants was determined using a MSD multiplex analysis kit. Dot plots represent mean ± SEM (n = 6–9) of the secreted protein levels.

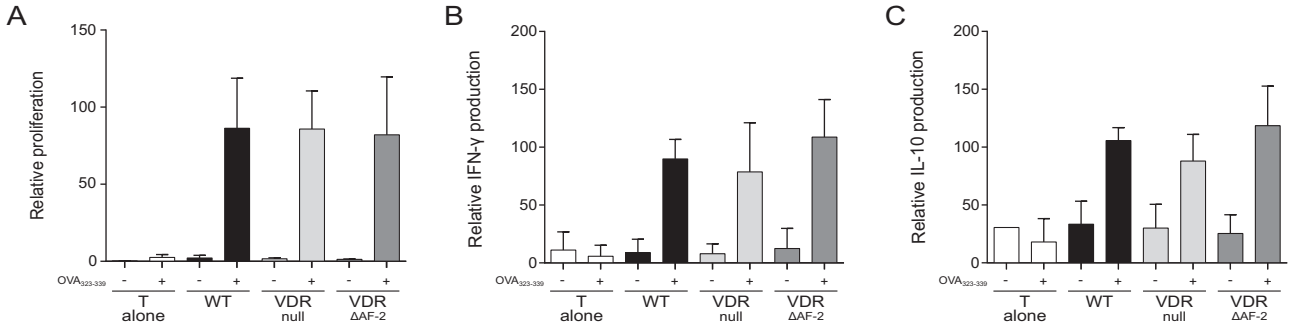

Fig. 4. *In vitro* T cell stimulatory capacity of BMDCs. DCs were loaded (+) or not (−) with 0.1 μg/ml $OVA_{323-339}$ peptide and co-cultured with $OVA_{323-339}$-reactive T cells derived from OT-II Tg mice. (A) T cell proliferation was determined after 72 h by ^{3}H-thymidine incorporation (n = 3–7). Secretion of (B) IFN-γ and (C) IL-10 into the co-culture supernatant after 72 h was measured by MSD multiplex analysis kit (n = 2–6). Values were normalized and expressed relative to the protein levels secreted after T cell stimulation with $OVA_{323-339}$-loaded WT-derived DCs.

the VDR and increased risk for autoimmune diseases [8,9 indicate an important role for the VDR and its ligand in the regulation of immune responses. In this regard,a discrepancy in autoimmune disease severity was observed between vitamin D_3-deficient and VDR null mice although both yield rickets. Vitamin D_3-deficient mice with a functional VDR develop autoimmunity more rapidly compared to VDR null mice or WT mice [13,14], with a more aggressive and accelerated type of disease [11,12]. In addition, $1\alpha,25(OH)_2D_3$ treatment has been shown to prevent autoimmunity [19]. In contrast, acute experimental inflammatory bowel disease was worse when either VDR or its ligand were absent [20]. Such disparity between absence of either ligand or receptor are also described for other members of the nuclear receptor family [21]. As vitamin D_3-deficient mice present more severe symptoms of autoimmunity, immune cells presenting an unliganded VDR could promote deregulated immune responses leading to an imbalance in the immune environment in these mice. In this regard, myeloid or conventional DCs, as central players in the initiation and regulation of immune responses, were evaluated after differentiation of bone marrow precursors derived from WT,

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VDR null and VDR ΔAF-2 mice, for their immunogenic phenotype and T cell stimulatory capacity *in vitro*. To our surprise, both immature and mature BMDCs generated from the VDR ΔAF-2 mice did not express higher levels of the antigen-presenting molecule MHC-II nor the co-stimulatory molecules CD40, CD80 and CD86. Similarly, VDR null-derived DCs either left unstimulated or stimulated with LPS and IFN-γ did not present increased expression levels of MHC-II and CD86, as compared to WT DCs. Additionally, the gene expression levels and protein production of pro-inflammatory cytokines and chemokines were mostly not altered in stimulated DCs with an unliganded or functionally absent VDR, as compared to WT DCs. Finally, the observed absence of a more immunogenic DC phenotype was confirmed, as VDR ΔAF-2 DCs did not show an elevated T cell stimulatory capacity, as compared to WT-derived DCs, when cultured *in vitro* with $CD4^+CD25^-$ T cells. A weakness of the current study is the strong maturation stimulus, which might overrule subtle differences of genetic modifications that might be visible at an immature stage. However, flow cytometric analysis of unstimulated DCs did either not show differences between the three mouse strains. Therefore, it is very unlikely that the T cell stimulatory capacity of these immature DCs will be affected.

Ablation of the VDR in mice or loss-of-function mutation of the VDR in humans also results in total body alopecia and the formation of dermal cysts, while this hair and skin phenotype has not been observed in mice or humans with a vitamin D_3 deficiency [15]. Extensive research has revealed that in the hair follicles, the VDR is associated with the co-repressor *Hairless*. This interaction between VDR and repressor is independent of its ligand 1α,25$(OH)_2D_3$ nor of the AF-2 region of the receptor [22]. Therefore, mutations in the AF-2 domain do not affect the hair cycle, while the expression of other genes is hampered, resulting in rickets [23]. However, as vitamin D_3 deficiency rather than absence of the VDR and possible associated repressors, is able to induce more severe autoimmune disease phenotypes, it is unlikely that the molecular pathways behind these immune observations will be the same. The association of a repressor, or alternatively co-activators in case of negative VDREs, is in this case strictly dependent on the absence of 1α,25$(OH)_2D_3$ as vitamin D_3 supplementation can prevent autoimmunity. The exact molecular mechanisms by which an unliganded VDR acts in autoimmunity still remain to be elucidated.

As stated above, the VDR belongs to a family of nuclear receptors that are able to bind genomic targets even in absence of ligand. Recently, 2340 genomic VDR binding sites were detected by chromatin immunoprecipitation (ChIP) analysis in THP-1 human monocyte leukemia cells, of which 520 sites (∼20%) were unique to the unstimulated condition [24]. This indicates that the VDR is able to closely interact with other transcription factors in the transcriptional control of key cytokines in BMDCs. For example, in the current study, we observed an up-regulation of *il-12p40* and *tnf-α* in VDR null DCs, as compared to WT and VDR ΔAF-2 DCs. The ligand-VDR complex is known to interfere with the signaling of transcription factors such as the nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB) [25,26], *via* obstruction of its activation and genomic binding, which results in the 1α,25$(OH)_2D_3$-mediated down-regulation of *il-12p40* [27,28]. In addition, reduced levels of the inhibitor of NF-κB were observed in VDR null cells [29]. Due to the loss of DNA binding in VDR null DCs, the VDR will no longer hinder the binding of a more activated NF-κB in the promoter region of the pro-inflammatory cytokines, hereby increasing its expression upon maturation. This enhanced gene transcription was however not reflected in increased secretion of IL-12p70 nor of TNF-α protein into the supernatant of the DC cultures. Often a poor correlation between mRNA and protein levels is observed due to several regulatory mechanisms in the transcriptional and translational process. For example, mRNA levels can be influenced by post-transcriptional processing or RNA binding, reducing the amount available for protein translation. Furthermore, post-translational modifications regulate protein stability and the protein turnover rate [30].

Although a previously published study showed increased expression levels of inflammatory markers like MHC-II and CD86 in BMDCs derived from VDR null diabetes-prone NOD mice [13], we did not observed these alterations here in BMDCs from the VDR null model. The VDR molecule is constitutively or after activation expressed in other immune cells as well [4]. Therefore it is possible that an unliganded VDR affects another immune cell type instead of the DC, thereby causing a more severe autoimmune presentation. Macrophage function has been reported to be impaired by vitamin D_3 deficiency, while macrophage chemotaxis, phagocytosis and respiratory burst capacity were normal in VDR null mice. Remarkably, changes in T cell behavior are shown in VDR null mice, while no abnormalities are detected in vitamin D_3-deficient mice [3]. Addressing the implication of an unliganded VDR in these other immune cells requires further investigations.

The VDR null strain used in this study was generated through a targeted deletion of the first zinc finger domain in the DBD of the nuclear receptor [16]. The resulting truncated VDR protein however is still able to bind 1α,25$(OH)_2D_3$ [17]. Locally increased concentrations of 1α,25$(OH)_2D_3$ are therefore able to mediate rapid, non-genomic effects and should be taken into account. Especially as the VDR null mouse model has been reported to present with hypervitaminosis D, due to the absent VDR-mediated induction and inhibition of vitamin D-metabolizing enzymes CYP24A1 and CYP27B1, respectively, as part of a negative feedback loop. However, in order to elicit immunomodulatory effects, 1α,25$(OH)_2D_3$ requires supraphysiological doses [31]. In addition, we did not observe any differences in DC phenotype or function between the three studied mouse strains.

In conclusion, we have demonstrated in the current study that the presence of an unliganded VDR did not affect the phenotype of myeloid BMDCs, which was represented by an unaltered surface expression of antigen-presenting and co-stimulatory molecules, no changes in cytokine production and a similar T cell stimulatory capacity as compared to WT or VDR null DCs.

Conflict of interest

No conflict of interest to be disclosed.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.08.010.

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