Novel immune cell-based therapies for atherosclerosis

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β-Catenin Signaling in Dendritic Cells Reduces Atherosclerosis

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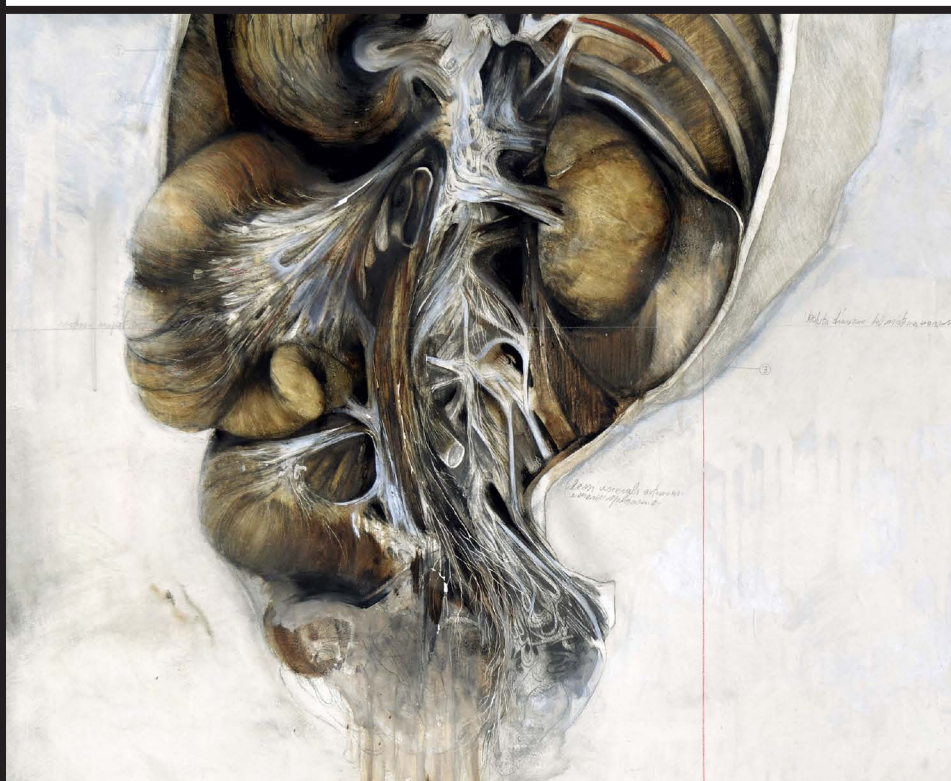

Abstract

Objective A driving force behind atherosclerotic disease is the chronic inflammatory response. It has been shown that immunomodulation by tolerogenic dendritic cell (DC) therapy is a feasible approach to reduce atherosclerosis. While it is mostly unclear what molecular pathways induce tolerogenic DCs, β-catenin has been implicated in this process. We therefore assessed whether induction of tolerogenic DCs, by means of β-catenin stabilization (CD11c-βcat^{EX3} DCs), can reduce atherosclerosis.

Methods and Results CD11c-βcat^{EX3} DCs possess similar antigen uptake capacities, but displayed enhanced CD40, CD86 and CCR7 expression and reduced production of pro-inflammatory cytokines TNF-α and IL-6 upon LPS exposure. Consequently, CD11c-βcat^{EX3} DCs inhibited T cell responses by 90% as a result of a 33% increased regulatory T cell (Treg) response.

To establish the effect on atherosclerosis, we generated CD11c-βcat^{EX3}/LDLr$^{-/-}$ bone marrow chimera and for a more clinical approach adoptively transferred CD11c-βcat^{EX3} DCs into LDLr$^{-/-}$ mice. Both CD11c-βcat^{EX3}/LDLr$^{-/-}$ chimeras and CD11c-βcat^{EX3} DC-treated mice experienced significantly less atherosclerosis (26% and 21% reduction, respectively). Additionally, CD11c-βcat^{EX3}/LDLr$^{-/-}$ chimeras had reduced necrotic cores and more stable collagen-rich lesions, while this was not observed after adoptive transfer of CD11c-βcat^{EX3} DCs. In both experiments we detected reduced splenic T cell proliferative capacity indicating reduced T cell responses. While CD11c-βcat^{EX3}/LDLr$^{-/-}$ chimeras showed an overall significant 1.6-fold increase of circulating Tregs, CD11c-βcat^{EX3} DC-treated mice only showed a significant 2-fold increase directly after treatment.

Conclusion We show here for the first time that β-catenin stabilization in DCs can effectively generate tolerogenic DCs that have the capacity to inhibit atherosclerotic lesion formation. This provides a new strategy to increase the potential of tolerogenic DC therapies for any inflammatory autoimmune disease.

Introduction

About two decades ago, cells with a Dendritic Cell (DC)-like morphology were found in human atherosclerotic lesions. DCs in the lesions were found in close proximity to other leukocytes, already indicating their role in ongoing inflammation[1]. During atherosclerosis development the amount of DCs increase in the lesions[2,3] and can eventually comprise about 10-30% of the total cell infiltrate[4]. DCs are actively involved in the uptake of antigens in the vessel wall, and facilitate an antigen-specific T cell response[5]. Lipid uptake has been shown to affect DC mobilization and could therefore impact DC-T cell interactions. It has been shown that under hypercholesterolemic conditions DCs cannot migrate out of the lesions[6] and will locally form clusters with T cells, a phenomenon associated with plaque destabilization[7]. Ex vivo studies have found that DCs isolated from aortas or DCs seeded in artificial human arteries were able to activate T cells[8,9]. On the other hand, it has also been recently shown that regulatory T cell (Treg)-inducing DCs are present in atherosclerotic lesions[10,11]. This implies that DCs present in the lesions can be involved in direct activation but also modulation of T cell responses. Moreover, it has been suggested that activation of T cells could also occur by DCs that have captured circulating atherosclerosis-specific antigens outside of atherosclerotic lesion, resulting in a systemic modulation of T cell responses[12].

Due to their critical role in initiating adaptive immune responses, we and others have addressed the possibility to modulate DCs in their capacity to induce T cell responses as a treatment option for atherosclerosis. Blocking antibodies for several co-stimulatory molecules were able to modulate T cell responses and reduce atherosclerosis[13,14]. Additionally we have shown that adoptive transfer of oxLDL-pulsed mature DCs reduces atherosclerosis by inducing oxLDL-specific antibodies[15] and that oxLDL-induced apoptotic DCs also reduce atherosclerosis by inducing tolerogenic DCs and Tregs and modulating monocyte responses[16]. Furthermore, Hermansson *et al.* showed that adoptive transfer of apoB100-pulsed tolerogenic DCs reduces atherosclerotic lesion development by induction of Treg responses [17]. Overall, these studies provide promising results and suggest that induction of a tolerogenic phenotype in DCs or the adaptive transfer of tolerogenic DCs is a promising approach to reduce atherosclerosis.

The term tolerogenic DCs is broad and describes a whole plethora of DCs with the capacity to induce tolerance, i.e. induction of Tregs and reduction of inflammatory responses. Multiple studies have established ways to induce or maintain a tolerogenic phenotype in DCs by culturing DCs with immunosuppressive components including cytokines[18], estriol[19], 1α,25-dihydroxyvitamin D3[20], or vasoactive intestinal peptide[21]. Interestingly, many of these factors are also produced in tissues under physiological conditions. Nonetheless, it remains unclear which exact intracellular signals are needed to promote and maintain the tolerogenic phenotype of DCs.

β-catenin was first discovered for its role in E-Cadherin cell-cell adhesion complexes[22]. While E-cadherin expression on DCs was found to mark inflammatory DCs in the intestine[23], disruption of these E-cadherin complexes was found to induce

a tolerogenic phenotype of DCs. This mechanical stimulation was shown to result in β-catenin release and a spontaneous upregulation of co-stimulatory molecules and chemokine receptors in DCs, without inflammatory cytokine production[24,25]. In mice, these tolerogenic DCs were able to induce peripheral tolerance and protect from experimental autoimmune encephalomyelitis[24]. Interestingly, β-catenin was also found to be crucially involved in maintaining the tolerogenic phenotype of intestinal DCs and thereby intestinal homeostasis. Mice with a CD11c-specific deficiency of β-catenin showed defects in Treg homeostasis and were more susceptible to inflammatory bowel disease[26]. All this indicates that β-catenin signalling is critical for the development of a tolerogenic phenotype in DCs.

In this study, we explored the potential of inducing tolerogenic DCs, by stable expression of β-catenin, to prevent atherosclerotic lesion development. For this we made use of CD11c-βcat^{EX3} mice, which have a constitutively active β-catenin in all CD11c^{+} cells, mostly DCs. We describe here that β-catenin stabilization in all CD11c^{+} cells of LDLr$^{-/-}$ mice, as well as adoptive transfer of CD11c-βcat^{EX3} DCs into LDLr$^{-/-}$, reduces lesion development by induction of Tregs and reduction of the overall inflammatory status in mice. Stabilization of β-catenin in DCs therefore has therapeutic potential and may help to improve DC-based therapies for atherosclerosis and other autoimmune diseases.

Material and Methods

Animals

LDLr$^{-/-}$ mice were originally obtained from The Jackson Laboratory and housed and bred at the animal facility of the Gorlaeus Laboratories. Bone marrow donors, CD11c-βcat^{EX3} and control non-transgenic littermates, were generated by breeding β-cat$^{EX3fl/fl}$ mice, carrying homozygous loxP site insertion flanking exon 3 of β-catenin gene, with CD11c-Cre transgenic mice on the C57BL/6 background. The offspring were genotyped for CD11c-Cre by PCR using the following Cre primers: 5'-GGACATGTTCAGGGATCGCCAGGCG-3' and 5'-GCATAACAGTGAAACAGCATTGCTG-3'. The floxed alleles for β–catenin were identified by PCR as previously described[27,28]. All mice were housed under standard laboratory conditions and were fed a regular chow diet or a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services). Diet and water were administered *ad libitum*. All animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University and performed at the animal facility of the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in compliance with Dutch government guidelines.

Atherosclerosis

To induce bone marrow aplasia, female LDLr$^{-/-}$ recipient mice of 8-10 weeks old were exposed to two doses of 4.5 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation with two hours break in between using an Andrex Smart 225 Röntgen source (YXLON International) with a 6-mm aluminum filter 1 day before the transplantation (n = 11

per group). Bone marrow was isolated by flushing the femurs and tibias from CD11c-βcat^{EX3} mice or control littermates with PBS. Single-cell suspensions were obtained by passing the cells through a 70 μm cell strainer (VWR International). Irradiated recipients received 5×10^6 bone marrow cells by *intravenous* injection. After an 8 week recovery period, mice were fed a WTD for another 8 weeks, after which animals were sacrificed. Body weight was monitored weekly and cholesterol levels were checked regularly throughout the study.

For adoptive transfer, female LDLr$^{-/-}$ mice were treated with 3 *intravenous* injections of PBS, 1.5 x 10^6 control DCs, or 1.5 x 10^6 CD11c-βcat^{EX3} DCs every other day prior to eight weeks WTD. DCs were loaded with oxLDL and matured with LPS as previously described[15].

Isolation, Loading and Stimulation of BM-derived and splenic DCs

DCs were cultured as previously described[15]. Briefly, bone marrow cells were isolated from the tibias and femurs of C57BL/6, CD11c-βcat^{EX3} mice or their littermates as indicated. The cells were cultured for ten days at 37°C and 5% CO_2 in 10 cm non-tissue culture treated petri dishes (Greiner Bio-One) in IMDM supplemented with 10% FCS, 100 U/mL penicillin/streptomycin, 2mM glutamax (all obtained from PAA) and 20 μm β-mercaptoethanol (Sigma-Aldrich) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). DC purity was assessed by CD11c expression (flow cytometry) and routinely found to be above 95%.

For in vitro DC cultures, 2 x 10^6 DCs were plated in 2 cm non-tissue culture treated petri dishes (Greiner Bio-One) to determine cytokine responses and flow cytometry analysis upon indicated amounts of LPS and for antigen uptake assessment. For visualization of ovalbumin uptake, DCs were cultured with the self-quenching DQ-Ovalbumin, which upon proteolytic digestion exhibits a bright green fluorescence (Molecular Probes), for 1 hour at 37ºC. For determination of unspecific background some cells were kept at 4ºC. Cells were extensively washed to remove extracellular DQ-Ovalbumin and uptake was determined by flow cytometry. For the assessment of oxLDL-uptake, DCs were cultured with indicated amounts of oxLDL for 24 hours and cytospins were made to determine Oil-red O positive area. Levels of IL-12p70, IL-6, TNF-α (all eBiosciences) and IL-10 (BD Biosciences) were measured in the supernatants of LPS-stimulated DCs by ELISA, according to manufacturer's protocol.

For isolation of splenic DCs, spleens were harvested after sacrifice and DCs were isolated with a Dendritic Cell Enrichment Set, according to manufacturer's protocol (BD Biosciences).

Proliferation assay and co-culture

For DC-T cell co-cultures (n=3 per group), 0.5 x 10^5 DCs/well were cultured with 2 x 10^5 T cells/well (1:5) in triplicate in 96-well round-bottom plates (Greiner Bio-One). CD4$^+$ T cells were isolated from a hyperlipidemic non-transplanted LDLr$^{-/-}$ mouse with a CD4$^+$ T Lymphocyte Enrichment Set, according to manufacturer's protocol (BD Biosciences). For splenocyte proliferation (n=6 per group), 2 x 10^5 splenocytes/well were cultured in quintuplicates in 96-well round-bottom plates (Greiner Bio-

One). Both cultures were done in the presence and absence of anti-CD3 (2 µg/mL, eBioscience) for 72 hours in complete RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2mM glutamax (all obtained from PAA) and 20 µm β-mercaptoethanol (Sigma-Aldrich). Proliferation of splenocytes was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences) for the last 16 hours of culture. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R) as the number of disintegrations per minute (dpm). Responses are expressed as: stimulation index (SI) = mean dpm of quintuplicate cultures with stimulation/ mean dpm of quintuplicate cultures without stimulation. Parallel cultures in triplicates and DC-T cell co-cultures were used to determine the amount of T cell subsets by flow cytometry and to determine cytokine levels in the supernatant by Cytometric Bead Array.

Flow Cytometry

After the start of WTD, blood was drawn at indicated time points and at sacrifice spleen and heart lymph nodes were harvested. For flow cytometry analysis, single cell suspensions of n=6 mice per group were obtained by using a 70 µm cell strainer (VWR International). Red blood cells in the spleen and blood were lysed with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently 3 x 10^5 cells per sample were stained with CD4-PerCp (BD Biosciences, RM4-5), FoxP3-PE (eBioscience, FJK-16s), T-bet-eFluor 660 (eBioscience, eBio4B10), IL-10-APC (eBioscience, JES5-16E3), IL-12p40 (eBioscience, C17.8), CD86-APC (eBioscience, GL1), CD40-FITC (eBioscience, HM40-3), MHCII-FITC (eBioscience, M5/114.15.2), CCR7-APC (eBioscience, 4B12). For intracellular stainings, cells were fixed and permeabilized according to the manufacturer's protocol (eBioscience). FACS analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (BD Biosciences).

Real-time quantitative PCR

Gene expression was analyzed by real-time quantative PCR as previously described[29]. Briefly, mRNA was isolated from splenic DCs and total spleens using the guanidium isothiocyanate (GTC) method according to Chomczynski and Sacchi[30] and reverse transcribed using RevertAid M-MuLV reverse transcriptase. Quantitative gene expression analysis was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using SYBR green technology (Eurogentec). Ribosomal phosphoprotein 36B4, hypoxanthine phosphoribosyltransferase (HPRT) and ribosomal protein L27 (Rpl27) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of 36B4, HPRT and Rpl27 and raising 2 to the power of this difference. Genes that exhibited a Ct value of >35 were considered not detectable. The average Ct of the three housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the expression of the separate housekeeping genes. For used primer pairs refer to Table 1.

Gene	Forward	Reverse
36B4	CTGAGTACACCTTCCCACTTACTGA	CGACTCTTCCTTTGCTTCAGCTTT
CCR7	CGTGCTGGTGGTGGCTCTCCT	ACCGTGGTATTCTCGCCGATGTAGTC
CD40	GCACCAGCAAGGATTGCG	TCCATAACTCCAAAGCCAGGG
CD86	GTTAGAGCGGGATAGTAACGCTGA	TGCACTTCTTATTTCAGGCAAAGCA
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
IL-10	GGGTGAGAAGCTGAAGACCCTC	TGGCCTTGTAGACACCTTGGTC
IL-12 (p35)	CCAAACCAGCACATTGAAGA	CTACCAAGGCACAGGGTCAT
IL-4	GAACGAGGTCACAGGAGAAGGG	CCTTGGAAGCCCTACAGACGAG
IL-6	AGACAAAGCCAGAGTCCTTCAGAGA	GGAGAGCATTGGAAATTGGGGTAGG
IL-27	CGATTGCCAGGAGTGAACC	AGTGTGGTAGCGAGGAAGCA
MHCII	CTCACCTTCATCCCTTCTGACGA	CTGACATGGGGGCTGGAATCT
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
TGF-β	AGGGCTACCATGCCAACTTCT	GCAAGGACCTTGCTGTACTGTGT

Table 1. Primer Pairs. The expression of genes was determined relative to the average expression of the three household genes: ribosomal protein 36B4, hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). Abbreviations: CCR7, C-C chemokine receptor type 7; MHCII, major histocompatibility complex;TGF-β, transforming growth factor beta.

Histological analysis

To determine atherosclerotic plaque size, 10 μm cryosections of the aortic root were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). Corresponding sections were stained for collagen fibers using the Masson's Trichrome method (Sigma-Aldrich) or immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b, diluted 1:1000, Serotec). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100, Sigma-Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Dako) as enzyme substrates. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, ThermoScientific). BrightVision anti-rabbit-HRP was used as secondary antibody (Immunologic). The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. The section with the largest lesion and four flanking sections were analyzed for lesion size, collagen content, necrotic area, and MOMA-2, and two sections were analyzed for T cell content. The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2-positive area by the total lesion surface area. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

Statistical analysis

Values are expressed as mean±SEM. An unpaired two-tailed Student T-test was used to compare normally distributed data between two groups of animals. Data of three groups were analyzed with one-way ANOVA and data of two or more groups with more than one variable were analyzed by two-way ANOVA, both followed by Bonferroni *post hoc* testing. Statistical analysis was performed using the Instat3 software. Probability values of $P<0.05$ were considered significant.

Results

CD11c-βcat^{EX3} DCs are tolerogenic and induce Tregs in vitro

To determine the effect of β-catenin stabilization on DC phenotype and function, we cultured BM-derived DCs from CD11c-βcat^{EX3} mice and their littermates. We show here that CD11c-βcat^{EX3} DCs and control DCs possess similar phagocytotic capacities as they take up oxLDL and DQ ovalbumin to a similar extent (Figure 1A). Furthermore, we stimulated DCs with LPS to determine the effect of β-catenin stabilization on cell maturation. While we found no differences in the absolute amount of CD86, CD40, MHCII, and CCR7 positive DCs (data not shown), we did observe differences on cellular level. CD11c-βcat^{EX3} DCs showed a significant increase of average CD86, CD40 and CCR7 expression per cell as determined by flow cytometry (Figure 1B).

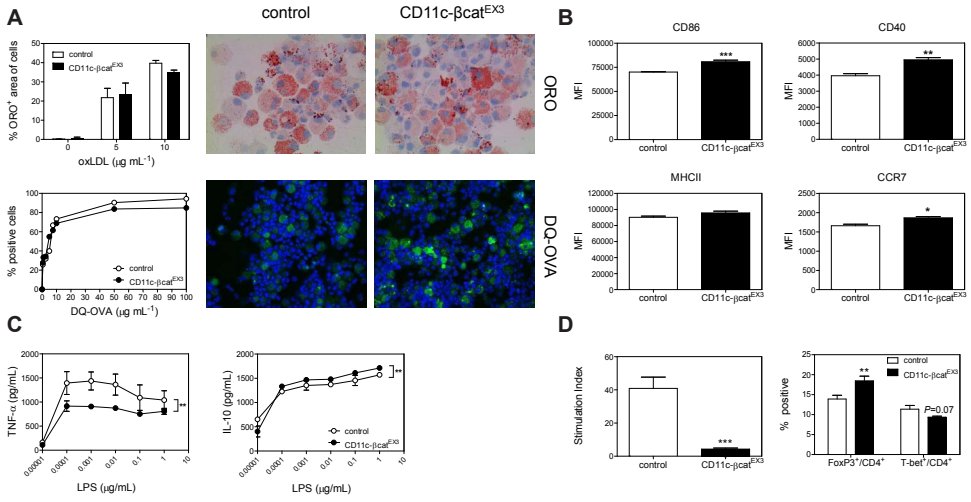

Figure 1. CD11c-βcatEX3 DCs are tolerogenic and induce Tregs in vitro. Bone marrow-derived DCs were generated from non-transgenic littermates (control) or from CD11c-βcat^{EX3} mice (CD11c-βcat^{EX3}). **A.** Antigen-uptake was determined by Oil-red-O staining (oxLDL) and flow cytometry (DQ-Ovalbumine). **B.** DCs were stimulated for 24 hours with 1 μg/mL LPS. Mean fluorescence intensity (MFI) of CD86, CD40, MHCII and CCR7 was determined by flow cytometry. **C.** TNF-α and IL-10 production were determined by ELISA. **D.** Splenocytes from control (n=3) or CD11c-βcat^{EX3} mice (n=3) were cultured in the presence of αCD3 for 72 hours. We pre-gated on CD4$^+$ T cells and determined Tregs (FoxP3$^+$) and Th1 cells (T-bet$^+$) by flow cytometry. Proliferation was assessed by ^{3}H-thymidine incorporation in dividing cells and is expressed as the stimulation index. All values are expressed as mean±SEM and are representative of three independent experiments done in triplicate. *P<0.05, **P<0.01, ***P<0.001.

We subsequently assessed cytokine production by the CD11c-βcat^{EX3} DCs. In a dose-dependent response to LPS, TNF-α levels were significantly decreased at all used LPS concentrations, with an average of 22% and IL-10 was slightly increased by 10% on average (Figure 1C). These results suggest that β-catenin stabilization results in a more phenotypically mature and tolerogenic DC phenotype upon activation.

Since we observed differences in cytokine production and amount of co-stimulatory molecules expressed per DC, we further wanted to determine whether this would translate into functional effects on T cell responses. Therefore, we cultured splenocytes from CD11c-βcat^{EX3} or control mice for 72 hours in the presence of αCD3, mimicking antigen-presentation by DCs, whereas the other two signals (co-stimulation and cytokines) needed for T cell expansion are intrinsic to the present DCs. We found that T cell proliferation was significantly reduced by 90% in the CD11c-βcat^{EX3} group compared to the control group. This was due to a significant increase in Treg by 33% and a concomitant trend to a decrease in Th1 induction (Figure 1D).

CD11c-βcat^{EX3} chimeras have reduced lesion sizes and show a trend to increased stability

To assess the effect of tolerogenic DCs with a β-catenin stabilization on atherosclerotic disease, we generated CD11c-βcat^{EX3}/LDLr$^{-/-}$ bone marrow chimeras (which we will refer to as CD11c-βcat^{EX3} mice) or as a control reconstituted LDLr$^{-/-}$ with bone marrow from their littermate controls. After a recovery period of eight weeks, mice were put on a Western-type diet (WTD) for an additional eight weeks to induce lesion formation. We confirmed the stabilization of β-catenin in CD11c^{+} cells by flow cytometry and found a highly significant overexpression of β-catenin when pre-gating on CD11c^{+} cells in the blood, draining lymph nodes of the heart (hLNs), and spleen, confirming the stabilization of β-catenin in CD11c-βcat^{EX3} mice (Figure 2).

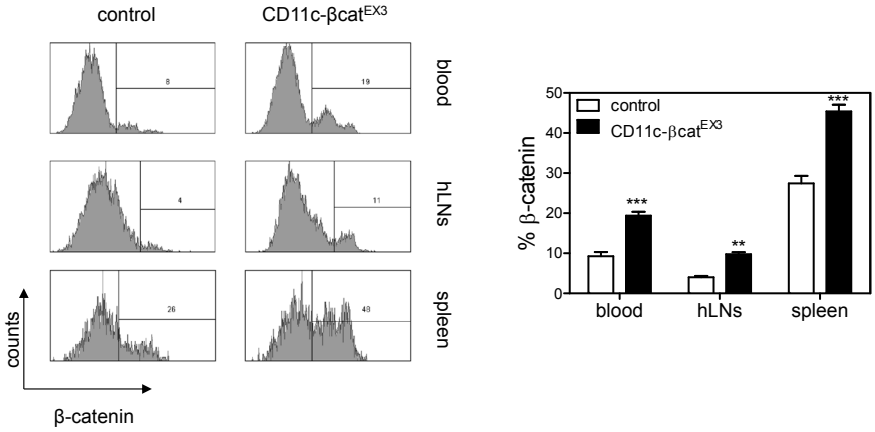

Figure 2. CD11c-βcat^{EX3} mice show increased β-catenin stabilization in CD11c^{+} cells. LDLr$^{-/-}$ mice reconstituted with either bone marrow from non-transgenic littermates (control, n=11) or from CD11c-βcat^{EX3} mice (CD11c-βcatEX3, n=11). After eight weeks recovery period, mice were put on a WTD for another eight weeks. β-catenin expression in CD11c^{+} cells was determined by flow cytometry in blood, hLN, and spleen at the end of the experiment. Representative histograms are shown. All values are expressed as mean±SEM and are representative of five mice.**P<0.01, ***P<0.001.

We observed in vitro that CD11c-βcat^{EX3} mice have overall more anti-inflammatory DCs, reduced amounts of CD4^{+} T cells, increased amounts of circulating Tregs and a reduction in Th1 responses. In line with this, we observed a significant decrease in aortic root lesion size of 26% in CD11c-βcat^{EX3} mice compared to control mice (control: 5.2 x 10^5 ± 0.3 x 10^5 µm versus CD11c-βcat^{EX3}: 3.8 x 10^5 ± 0.5 x 10^5 µm; Figure 3A). Moreover, lesions showed a trend towards an increased stability, with 25% more collagen (control: 26.6 ± 1.7% versus CD11c-βcat^{EX3}: 32.8 ± 2.8%; Figure 3B). The necrotic core size of lesions was significantly reduced by 46%, indicating an overall reduced inflammatory status of lesions in the CD11c-βcat^{EX3} mice (Figure 3C). Lesional macrophage content did not differ between the groups and was on average 40% (Figure 3D). Also the amount of lesional T cells did not differ, which could indicate increased recruitment of Tregs and reduced effector T cell recruitment (Figure 3E).

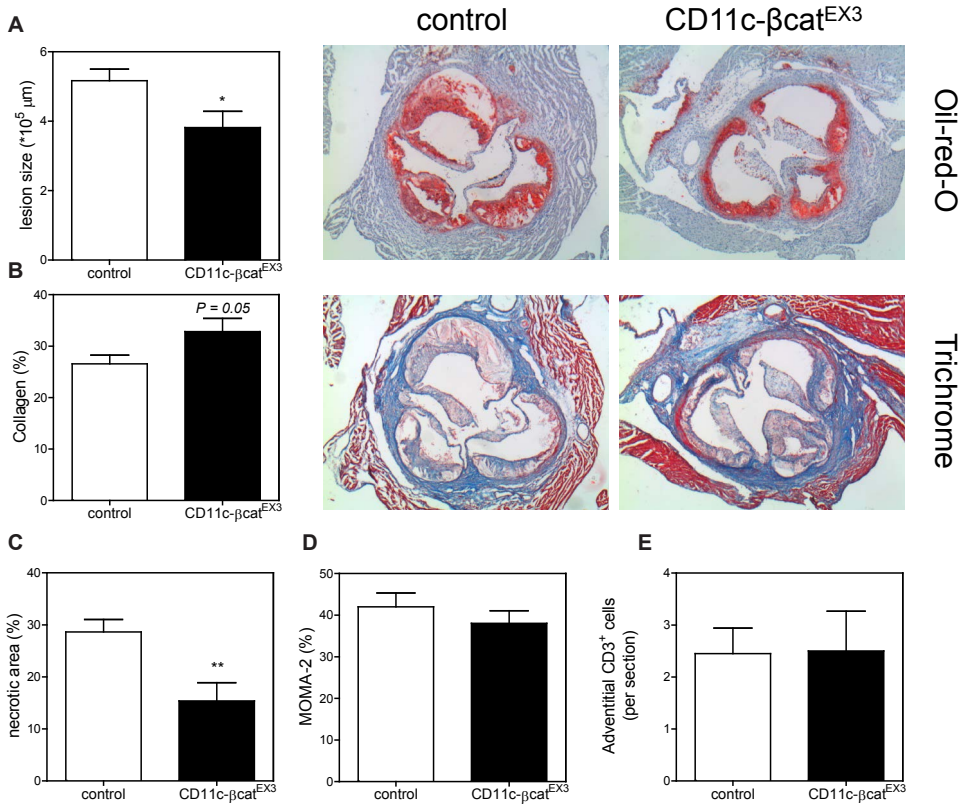

Figure 3. CD11c-βcat^{EX3} mice show reduced lesion formation, increased stability and reduced necrotic cores. A. Lesion formation in the three-valve area of the aortic root was assessed by Oil-Red-O and hematoxylin staining. Representative cross-sections are shown. **B.** Sections of the aortic root were stained for collagen fibers using Masson's trichrome staining. The percentage of collagen relative to lesion size was determined. Representative cross-sections are shown. **C.** The necrotic core was defined as the acellular, debris-rich plaque area as percentage of total plaque area in the trichrome staining. **D.** Macrophage content was assessed by MOMA-2 staining as percentage of total lesion area. **E.** T cell numbers were determined by αCD3 staining. All values are expressed as mean±SEM and are representative of all mice.*P<0.05, **P<0.01.

The reduction in lesion size was not due to differences in body weight or due to effects on lipid metabolism as total serum cholesterol levels and cholesterol distribution between the different lipoprotein fractions was not different between the groups (data not shown).

CD11c-βcat^{EX3} DCs are tolerogenic in vivo and can induce Tregs ex vivo

After eight weeks WTD, the phenotype of splenic DCs was assessed by flow cytometry. No striking phenotypical changes were observed in CD11c-βcat^{EX3} mice. The percentage of CD86 positive DCs was significantly increased, but no effect on CD40, MHCII and CCR7 DCs was observed (Figure 4A). However, when we isolated splenic DCs from CD11c-βcat^{EX3} mice, they showed reduced mRNA levels of pro-inflammatory cytokines compared to control DCs. IL-6 expression was significantly reduced by 85% and TNF-α was reduced although not significantly by 25%. Interestingly, IL-27 expression, which limits Tregs, was reduced by 33% (Figure 4B). No significant changes were observed in the expression of anti-inflammatory IL-10 and TGF-β (Figure 4C). This suggests that DCs in CD11c-βcat^{EX3} mice assume a less inflammatory phenotype and maintain a more tolerogenic phenotype compared to control DCs upon WTD.

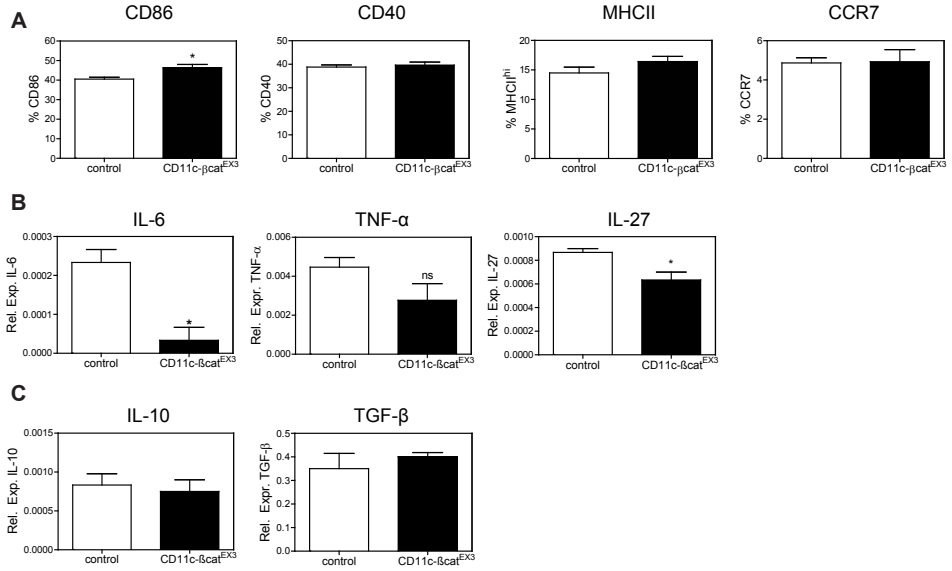

Figure 4. CD11c-βcat^{EX3} DCs assume a less inflammatory phenotype upon WTD. A. At sacrifice, splenic DCs were isolated and CD86, CD40, MHCII and CCR7 expression was determined by flow cytometry. **B.** Relative expression of pro-inflammatory IL-6, TNF-α and IL-27 was determined by qPCR. **C.** Relative expression of anti-inflammatory IL-10 and TGF-β was determined by qPCR. All values are expressed as mean±SEM and are representative of five mice.*P<0.05.

CD11c-βcat^{EX3} chimeras show decreased absolute CD4$^+$ T cell numbers and increased Tregs in the circulation

Once mice were put on a WTD, we monitored CD4$^+$ T cell and Treg numbers in the circulation to establish if these were affected by β-catenin stabilization in DCs. Four weeks after the start of WTD, when maximum cholesterol levels in the blood were

reached, a significant 48% drop in circulating CD4+ T cell numbers was observed in the CD11c-βcat^{EX3} mice. In parallel, a 62% increase of circulating Tregs in CD11c-βcat^{EX3} mice was seen, which likely is associated to the more tolerogenic phenotype of DCs in these mice (Figure 5A). After eight weeks of WTD, a significant 50% reduction of CD4+ T cells in the circulation (Figure 5A), a significant 17% reduction of CD4+ T cells in the spleen and a non-significant 13% decrease in the hLNs remained in CD11c-βcat^{EX3} mice compared to control mice (Figure 5B). We found a significant 1.5-fold induction of Tregs in the circulation, but no significant changes in the spleens and hLNs of CD11c-βcat^{EX3} mice (Figure 5A and C).

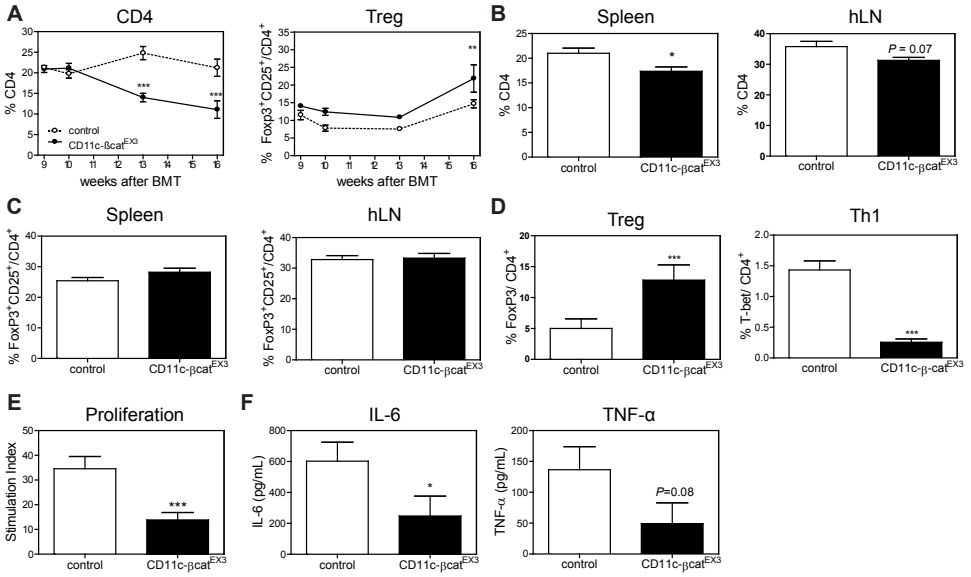

Figure 5. CD11c-βcat^{EX3} mice show decreased CD4+ T cell responses and increased Treg amounts. A. Circulating CD4+ T cells and Tregs (FoxP3+CD25+ within CD4+) were determined in the circulation throughout the experiment. **B.** At sacrifice, CD4+ T cells and **C.** Tregs were determined in the spleen and draining lymph nodes of the heart (hLN) by flow cytometry. **D.** Splenocytes were isolated and cultured for 72 hours in the presence of αCD3. Induction of Tregs (FoxP3+) and Th1 cells (T-bet+) was determined by flow cytometry. **E.** Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells and is expressed as the stimulation index (SI). **F.** IL-6 and TNF-α responses in splenocyte cultures were determined by cytometric bead array. All values are expressed as mean±SEM and are representative of at least five mice.*P<0.05, ***P<0.001.

To establish whether T cell responses were indeed modulated, we isolated splenocytes after eight week WTD and cultured them in the presence of αCD3 for 72 hours ex vivo. After stimulation, we found a significant 94% increase in Tregs and an almost complete inhibition of T cell differentiation to Th1 in CD11c-βcat^{EX3} mice (Figure 5D). This indicated that DCs present in the spleen were still able to modulate T cell responses and induce Tregs. The presence of Tregs resulted in a 60% reduction of proliferation of splenic CD11c-βcat^{EX3} T cells compared to the control (Figure 5E). This was in line with reduced CD4+ T cell numbers observed in the spleen in vivo, while likely Tregs migrate out of the spleen and are therefore not locally increased. Moreover, we found a significant 58% reduction in IL-6 production and a non-significant

64% reduction of TNF-α production by splenocytes (Figure 5F), indicating an overall reduced inflammatory response. This is in line with the reduced pro-inflammatory cytokine expression observed in isolated splenic DCs.

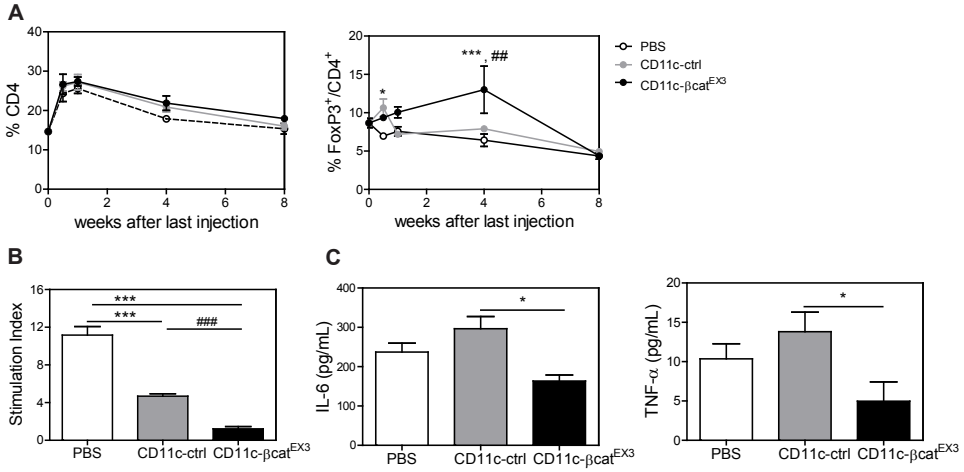

Figure 6. Reduced T cell responses after adoptive transfer of CD11c-βcat^{EX3} DCs. A. Circulating CD4$^+$ T cells and Tregs (FoxP3$^+$ within CD4$^+$ T cells) were determined in the circulation throughout the experiment. **B.** At sacrifice, splenocytes were isolated and proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells, expressed as stimulation index (SI). **C.** IL-6 and TNF-α production by proliferating splenocytes were determined by ELISA. All values are expressed as mean±SEM and are representative of at least five mice.*P<0.05, ***P<0.001 compared to PBS; ##P<0.01, ###P<0.001 compared to CD11c-ctrl.

Adoptive transfer of CD11c-βcat^{EX3} DCs reduces absolute CD4$^+$ T cell responses and induces Tregs

To determine if DCs with a β-catenin stabilization could be used as a potential therapy, we cultured BM-derived DCs of CD11c-βcat^{EX3} transgenic mice or their littermate controls and adoptively transferred them into LDLr$^{-/-}$ mice prior to eight weeks WTD. To determine if our treatment affected T cell responses, we monitored CD4$^+$ T cells and Tregs in the circulation. While no effect on circulating CD4$^+$ T cells was observed by any DC treatment, after four weeks CD11c-βcat^{EX3} DC-treated mice showed a significant 1.6-fold increase in circulating Tregs (Figure 6A). After eight weeks, we again isolated splenocytes and cultured them in the presence of αCD3 for 72 hours. We found a significant 2.4-fold decrease in proliferative capacity of T cells when splenocytes were isolated from mice treated with control DCs. However, when CD11c-βcat^{EX3} DCs had been adoptively transferred, a much stronger 9.2-fold inhibition of proliferation was observed. This was only 20% above background proliferation of unstimulated splenocytes, indicating that T cells were almost completely inhibited in their proliferative capacity. Moreover, only splenocytes of CD11c-βcat^{EX3} DC-treated mice showed a significant reduction of IL-6 and TNF-α production, indicating a reduced inflammatory response, in line with earlier observations (Figure 6C).

Adoptive transfer of CD11c-βcat^{EX3} DCs reduces lesion sizes

After eight weeks WTD, we determined whether the treatment with CD11c-βcat^{EX3} DCs could modulate atherosclerotic lesion development. No effect on cholesterol or weight during the entire experiment was observed (data not shown). We established a 21% reduction of aortic root lesion size in CD11c-βcat^{EX3} DC-treated mice compared to mice treated with control DCs and a 24% reduction compared to control mice (control: 5.3 x 10^5 ± 0.3 x 10^5 µm, CD11c-ctrl: 5.1 x 10^5 ± 0.4 x 10^5 µm, CD11c-βcat^{EX3}: 4.0 x 10^5 ± 0.3 x 10^5 µm; Figure 7A). Collagen content was on average 24% in all groups (Figure 7B). Moreover, no effect on necrotic core sizes and macrophage content was found (Figure 7C and D). However, in both DC treatment groups we observed a significant decrease of T cell numbers in lesions. Control DCs reduced lesional CD3$^+$ T cells by 47%, while again CD11c-βcat^{EX3} were more potent and decreased T cell numbers by 74% compared to mice who did not receive DC treatment (control: 5.7 ± 0.7 T cells, CD11c-ctrl: 3.0 ± 0.7 T cells, CD11c-βcat^{EX3}: 1.5 x 105 ± 0.4 T cells per section; Figure 7E).

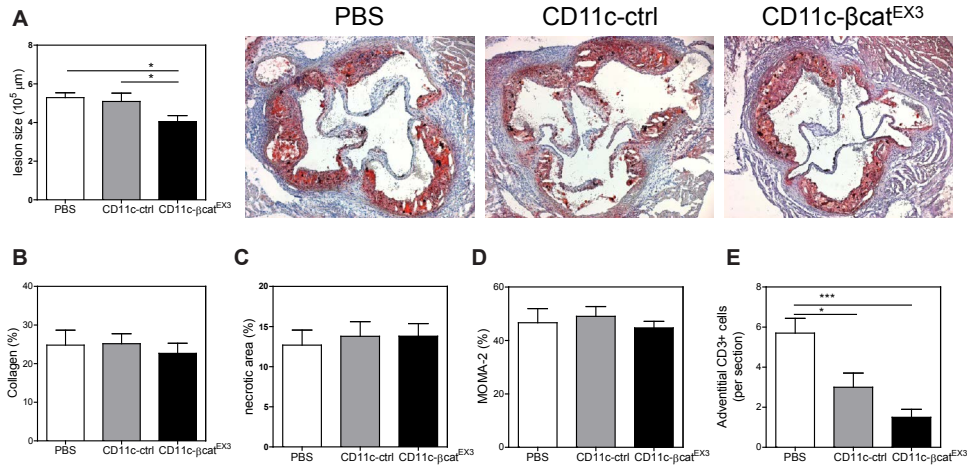

Figure 7. Adoptive transfer of CD11c-βcat^{EX3} DCs reduces atherosclerotic lesion formation. A. Lesion formation in the three valve area of the aortic root was assessed by Oil-Red-O and hematoxylin staining. Representative cross-sections of hearts are shown. **B.** Sections were stained for collagen fibers using Masson's trichrome staining. The percentage of collagen relative to the lesion size was determined. **C.** The necrotic core was defined as the acellular, debris-rich plaque area as percentage of total plaque area in the trichrome staining. **D.** Relative macrophage content was determined with MOMA-2 staining and quantified as percentage surface area relative to lesion size. **E.** T cell contant was determined by αCD3 staining. All values are expressed as mean±SEM and are representative of all mice.*P<0.05, ***P<0.001.

Discussion

Our study shows for the first time that β-catenin stabilization in DCs is beneficial in reducing atherosclerosis and can be a future strategy to treat the disease. We took two approaches in our study to determine the possible effect of β-catenin stabilization in DCs: First, we performed a bone marrow transplantation to generate LDLr$^{-/-}$ mice

with a CD11c-specific stabilization of β-catenin. Second, we adoptively transferred DCs with active β-catenin into LDLr$^{-/-}$ mice. In both cases we put the mice on a WTD for eight weeks and analyzed lesion formation and stability.

For our study we used CD11c-βcat^{EX3} mice, which have constitutive β-catenin signaling in CD11c^{+} cells. It has to be noted that while CD11c is highly expressed on DCs, it is also, although to lower extent, expressed on monocytes and macrophages[31], but can even be expressed in some T cell subsets[32], NK cells[33] and B cells[34] under specific disease circumstances. Under hypercholesterolemic conditions, an increased number of CD11c^{+} monocytes can be found, correlating with an increased migratory capacity of these cells into the lesions[35]. Furthermore, macrophages have been shown to increase their expression of CD11c upon uptake of oxLDL[36]. Therefore, β-catenin could also become stabilized in other mononuclear phagocytes during atherosclerosis. However, immature macrophages have almost undetectable levels of E-Cadherin and β-catenin and these only become upregulated by IL-4[37]. Therefore, we emphasize that the stabilization of β-catenin in our hypercholesterolemic model will only minimally affects monocytes and macrophages. This concurs with our findings that monocyte and macrophage responses were not affected. In DCs on the other hand the expression of E-Cadherin and β-catenin is found under immature as well as mature stages, and was shown to be important for the maintenance of cell clusters[24]. In addition, while macrophages can interact with T cells in the lesions, DCs are capable of shaping the initiated T cell responses. Therefore, the pronounced increase in Tregs and reduced T cell proliferation of CD4^{+} T cells in vivo is unlikely due to monocyte or macrophage contribution. Although we cannot rule out the contribution of monocytes and macrophages in our BMT setup, the potential of CD11c-βcat^{EX3} DCs becomes clear by the adaptive transfer performed in this study.

We provide conclusive evidence that CD11c-βcat^{EX3} DCs are potent inducers of Tregs. In a culture of total splenocytes from both transplanted and non-transplanted CD11c-βcat^{EX3} mice and a co-culture of CD11c-βcat^{EX3} DCs with T cells from a non-transplanted LDLr$^{-/-}$ mouse, we observed a significant induction of Tregs, while Th1 responses and general T cell proliferation were strongly reduced. CD11c-βcat^{EX3} chimera show significantly increased Tregs in the circulation and a general reduction of CD4^{+} T cells compared to control mice. At sacrifice, CD4^{+} T cell numbers were also decreased in the spleen and the hLNs indicating an overall reduction of T cell responses, although Treg numbers were not increased in spleen and hLNs. Nonetheless, when we isolated splenocytes and cultured them with αCD3, we did see a significant induction of Tregs, proving that CD11c-βcat^{EX3} DCs are able to potently induce Tregs. This could indicate that T cell responses are shifted to Tregs only when immune responses are induced and/or that Tregs migrate to the site of inflammation and are thus not locally increased in the spleen. After the adoptive transfer of CD11c-βcat^{EX3} DCs, an initial induction of circulating Tregs was observed, but in the long-term only overall T cell effector responses were reduced. Interestingly, while we saw an initial induction of Tregs in control DC-treated mice, CD11c-βcat^{EX3} DC-treated mice show a more pronounced and longer induction of Tregs, likely due to an increased lifespan of the CD11c-βcat^{EX3} DCs. We therefore show here that additional stabilization

of β-catenin can increase the potential of DC therapies.

It has to be noted that exon 3 mutations have been associated with e.g. colorectal carcinomas[38] and that with this approach we did take the risk of inducing a carcinogenic phenotype in DCs. However, during our studies we did not observe any tumor formation or excessive DC proliferation. We wanted to highlight the possibility of using β-catenin stabilization as a therapeutic approach for a DC therapy and eventually in the clinic a certain amount of DCs with a β-catenin stabilization would be injected and not all DCs in patients modulated, which further reduces the risk of tumors.

In both our experiments, we see an overall reduction of the inflammatory status in the CD11c-βcat^{EX3} mice, not only by induction of Tregs and reduction of Th1 cells, but also by reduction of pro-inflammatory cytokines, e.g. IL-6. β-catenin inhibits nuclear factor κB (NF-κB) mediated transcriptional activation of pro-inflammatory genes[39]. Also cluster disruption, which induces β-catenin signaling has been found to reduce pro-inflammatory cytokine responses[24]. Interestingly, β-catenin stabilization in Tregs extends their survival and enhances their protective effect in a Treg therapy for inflammatory bowel disease, compared to non-modulated Tregs[40]. This further emphasizes that β-catenin stabilization in anti-inflammatory cell therapies is promising. Activation of β-catenin signaling to induce tolerance is also exploited by the bacterium *Salmonella*, which produce an AvrA protein. AvrA induces β-catenin signaling and inhibits intestinal inflammation, enabling a chronic infection of the pathogen[41]. Recently, Fu *et al.* found that β-catenin in DCs inhibits their capacity for cross-priming due to increased IL-10 production, which results in reduced initiation of $CD8^+$ T cell responses; however β-catenin was also shown to be necessary to maintain $CD8^+$ T cell responses[41]. We did not observe any effect on the production of IL-10 by DC in vivo, but it seems that DCs remain in a more anti-inflammatory tolerogenic state when β-catenin is stabilized during atherosclerosis development as they produce less pro-inflammatory cytokines and increase Treg responses.

In contrast to earlier observations made by cluster disruption[24], we only found an upregulation of co-stimulatory molecules and chemokines receptors upon maturation of the DCs, but not due to β-catenin stabilization itself in immature DCs. While our model is based on CD11c-specific stabilization of β-catenin, previous studies have used the approach of cluster disruption of E-Cadherin/β-catenin complexes or inhibition of GSK3β, which phosphorylates β-catenin for proteasomal degradation[24]. These approaches could induce the concomitant upregulation of maturation markers through simultaneous activation of different signaling pathways, e.g. by effects on PI3K/Akt signaling or Rac1 signaling[42,43]. In our experiments we only see effects after LPS/TLR4 stimulation, indicating that indeed a second stimulus might be needed to induce upregulation of co-stimulatory molecules and chemokines receptors.

An alternative for stabilization of β-catenin by deletion of its phosphorylation site would be the treatment of DCs with LiCl[44], which is a potent inhibitor of GSK3β and would thus result in reduced degradation of β-catenin or activation of the Wnt signaling cascade[45], which induces β-catenin. Interestingly, these approaches have been implicated in inducing a tolerogenic phenotype of DCs. Another approach

could be cluster disruption[24], but it remains to be determined how controllable and reproducible this is for a clinical setting. However, since all these treatments only indirectly result in β-catenin accumulation and will inevitably have other effects, it needs to be established to what extent these approaches will resemble our β-catenin stabilization. A chemical stabilizer of β-catenin could circumvent this problem. SKL2001 e.g. has been shown to increase transcription of β-catenin but also to prevent its phosphorylation and degradation and it could be interesting to test its efficiency[46]. Moreover, direct administration of such a reagent and targeting it to DCs in vivo could prove as the best and least invasive treatment option.

In conclusion, we show here for the first time that DCs with a β-catenin stabilization are highly potent inducers of Tregs, inhibit Th1 responses and T cell proliferation, and remain tolerogenic under hypercholesterolemic conditions. These DCs decrease atherosclerotic lesion development and it will be interesting to determine their potential in inhibiting lesion progression. We predict that β-catenin stabilization in combination with other inducers of a tolerogenic phenotype in DCs, such as e.g. 1α,25-dihydroxyvitamin D3, will be a powerful DC therapy for atherosclerosis.

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