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## Novel immune cell-based therapies for atherosclerosis

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# Differential Effects of Regulatory T cells on the Initiation and Regression of Atherosclerosis

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**Abstract**

**Objective** Regulatory T cells (Tregs) play an important role in the regulation of T cell-mediated immune responses through suppression of T cell proliferation and cytokine production. In atherosclerosis, a chronic autoimmune-like disease, an imbalance between pro-inflammatory cells (Th1/Th2) and anti-inflammatory cells (Tregs) exists. Therefore, increased Treg numbers may be beneficial for patients suffering from atherosclerosis. In the present study, we determined the effect of a vast expansion of Tregs on the initiation and regression of well-established lesions.

**Methods and Results** For in vivo Treg expansion, LDL receptor deficient (LDLR<sup>-/-</sup>) mice received repeated *intrapitoneal* injections of a complex of IL-2 and anti-IL-2 mAb. This resulted in a 10-fold increase in CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells, which potently suppressed effector T cells *ex vivo*. During initial atherosclerosis, IL-2 complex treatment of LDLR<sup>-/-</sup> mice fed a Western-type diet reduced atherosclerotic lesion formation by 39%. The effect on pre-existing lesions was assessed by combining IL-2 complex treatment with a vigorous lowering of blood lipid levels in LDLR<sup>-/-</sup> mice. This did not induce regression of atherosclerosis, but significantly enhanced lesion stability.

**Conclusion** Our data show differential roles for Tregs during atherosclerosis: Tregs suppress inflammatory responses and attenuate initial atherosclerosis development, while during regression Tregs can improve stabilization of atherosclerotic lesions.

## Introduction

Atherosclerosis is considered to be a chronic autoimmune-like disease with an underlying imbalance between pro-inflammatory and anti-inflammatory processes<sup>1,2</sup>. Restoration of this delicate balance by induction of Tregs has proven to be of therapeutic potential in the treatment of several autoimmune diseases such as diabetes and rheumatoid arthritis<sup>3,4</sup>. As key regulators of T cell-mediated immune responses, Tregs exert suppressive effects on effector T cells. Suppression mainly occurs through secretion of IL-10 and TGF- $\beta$ , and cell-cell contact, mediated by membrane-bound TGF- $\beta$ , CTLA-4 or GITR<sup>5,6</sup>. In mice, Tregs are characterized by the expression of the surface molecules CD4 and CD25, and expression of the transcription factor Forkhead box protein P3 (Foxp3)<sup>7</sup>.

The role of Tregs in atherosclerosis has been the subject of intense investigation. Adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> T cells causes a reduction in atherosclerotic lesion development<sup>8</sup> while a depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells or more specifically Foxp3 expressing Tregs aggravates lesion development<sup>8,9</sup>. Our group has demonstrated that induction of antigen-specific Tregs via oral tolerance induction against oxLDL shows the beneficial effect of Tregs on the initiation and progression of atherosclerosis<sup>10</sup>.

Tregs have been shown to depend on IL-2 for optimal growth and survival<sup>11-14</sup>. Recently, it was shown that repeated injections of an IL-2 complex consisting of recombinant IL-2 and a specific anti-IL-2 monoclonal antibody (JES6-1A12) results in a specific expansion of Tregs<sup>15</sup>, which very potently induce resistance to experimental autoimmune encephalomyelitis and suppressed graft rejections<sup>16</sup>, type I diabetes<sup>17</sup>, murine asthma<sup>18</sup> and myasthenia gravis<sup>19</sup>.

In order to obtain a clinically relevant therapy for atherosclerosis, an experimental therapy inducing regression of atherosclerosis is a prerequisite, as most cardiovascular patients will already have well-established lesions. The effect of Tregs on the stabilization and regression of established atherosclerosis, however, remains to be elucidated. In the present study, we therefore not only determined the beneficial effect of IL-2 complex induced Tregs on the initiation of atherosclerosis, but more importantly, determined their therapeutic potential in a model for regression of atherosclerotic lesions.

## Material and methods

### *Animals*

Male LDLr deficient (LDLr<sup>-/-</sup>) mice, 10-12 weeks old, were obtained from Jackson Laboratories. The animals were kept under standard laboratory conditions and were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services). Diet and water were provided *ad libitum*. All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines.

### *Preparation of IL-2 complexes*

IL-2 complexes were prepared by mixing 1 µg recombinant IL-2 (Peprotech) with 5 µg anti-IL-2 mAb (clone JES6-1A12, R&D) in sterile PBS and incubated at 37°C for 30 minutes before injecting *intraperitoneally*.

### *Initiation and regression of atherosclerosis*

Atherosclerosis was induced in LDLR<sup>-/-</sup> mice by feeding a Western-type diet for 8 weeks. Two weeks after start of Western-type diet mice were treated *intraperitoneally* with the IL-2 complex (n=11) or with sterile PBS as a control (n=11). Initially, mice were treated *intraperitoneally* with IL-2 complexes for three consecutive days to boost the expansion of Tregs, thereafter mice were injected every 10 days to maintain high levels of Tregs. To study regression of atherosclerosis, mice were put on a Western-type diet for 10 weeks. At week 10, a baseline group (n=11) was sacrificed to determine disease extent at the beginning of the treatment. Subsequently, mice were put on a chow diet and simultaneously treated *intraperitoneally* with the IL-2 complex as mentioned above (n=13). As a control, mice were treated with sterile PBS (n=14). At week 20, mice were sacrificed and tissues were harvested after in situ perfusion using PBS and subsequent perfusion using Zinc Formal-Fixx (Shandon Inc.). Tissues were frozen in nitrogen and stored at -80°C until further use.

### *Serum cholesterol levels*

During the experiments, mice were weighed and blood samples were obtained by tail vein bleeding. The total cholesterol levels in serum were determined at week 0, 2 and 8 after start of the initial atherosclerosis experiment and at week 0, 5, 10, 14, 18 and 20 after start of the regression experiment. The concentrations of serum cholesterol were determined using enzymatic colorimetric procedures (Roche/Hitachi). Precipath (Roche/Hitachi) was used as an internal standard.

### *Histological analysis and morphometry*

Cryosections of the aortic root (10 µm) were made and stained with Oil-red-O. Lesional collagen content was determined with a Masson's Trichrome staining. Furthermore, corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:1000). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. To determine the number of T cells in the lesions, a CD3 staining was performed using anti-mouse CD3 (1:50, BD Biosciences Pharmingen, San Diego, CA). In addition, the aortic arch and its main branch points were excised (4 µm), fixed, and embedded in paraffin. Longitudinal sections of the aortic arch were analyzed for lesion extent with a hematoxylin and eosin staining. Morphology was studied using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems).

### *Flow cytometry*

During the experiments, levels of Tregs were monitored in the blood at several time points. Red blood cells were lysed using erythrocyte lysis buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{NaHCO}_3$ , 0.1 mM EDTA, pH 7.3). For the detection of  $\text{CD4}^+\text{CD25}^{\text{hi}}\text{Foxp3}^+$  T cells, the blood cells were stained with the surface markers CD4 and CD25 (0.25  $\mu\text{g}$  Ab/ $2 \times 10^5$  cells). For intracellular staining of Foxp3, cells were fixated and permeabilized overnight and subsequently stained against Foxp3 according to manufacturer's protocol (eBioscience). At sacrifice, blood, spleen, mediastinal lymph nodes near the heart (HLN) and liver were isolated (n=5 per group). Single cell suspensions were obtained by squeezing the organs through a 70 $\mu\text{m}$  cell strainer. Red blood cells were removed from blood and splenocytes using erythrocyte lysis buffer. For the detection of  $\text{CD4}^+\text{CD25}^{\text{hi}}\text{Foxp3}^+$  T cells, the spleen, blood, HLN and liver cells were stained with CD4, CD25 and Foxp3. In addition, cells were stained for the transcription factors T-bet, ROR $\gamma$ t and GATA-3 and the cytokines IFN- $\gamma$ , IL-17A and IL-4. All antibodies were purchased from eBioscience. FACS analysis was performed on a FACSCantoII (BD Biosciences). Data were analyzed using FACSDiva software (BD Biosciences).

### *Spleen cell proliferation*

Splenocytes (n=5 per group) were cultured for 48 hours in triplicate in a 96-wells round-bottom plate ( $3 \times 10^5$  cells/well) in RPMI 1640 supplemented with L-Glutamine, 100 U/ml streptomycin/penicillin and 10% FCS. As a positive control cells were stimulated with anti-CD3 and anti-CD28 (2  $\mu\text{g}/\text{ml}$ ). Proliferation was measured by addition of  $^3\text{H}$ -thymidine (0.5  $\mu\text{Ci}/\text{well}$ , Amersham Biosciences) for the last 16 hours. The amount of  $^3\text{H}$ -thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as stimulation index (SI): ratio of mean counts per minute of triplicate cultures with anti-CD3/CD28 stimulation to triplicate cultures without stimulation.

### *Suppression assay*

Tregs were isolated with greater than 95% purity from splenocytes using the  $\text{CD4}^+\text{CD25}^+$  Regulatory T Cell Isolation Kit from Miltenyi Biotec.  $7.5 \times 10^4$  splenocytes were plated out per well of a 96-well plate with or without titrated amounts of isolated Tregs from IL-2 complex and control treated mice. Cells were activated with anti-CD3 and anti-CD28 (2  $\mu\text{g}/\text{ml}$ ) and pulsed with  $^3\text{H}$ -thymidine (0.5  $\mu\text{Ci}/\text{well}$ ) on day 3. Proliferation was assessed 16 hours later using a liquid scintillation counter. All results are expressed as the mean disintegration per minute (DPM) of triplicate cultures.

### *Cytokine determination in supernatant of the suppression assay*

IL-10 and TGF- $\beta$  concentrations in the supernatant of effector T cells cultured in a 1:1 ratio with Tregs for 72 hours were determined by ELISA according to manufacturer's protocol (eBioscience).

### *Real-time PCR*

Spleens from baseline mice (n=11), control mice (n=14) and IL-2 complex mice



(n=13) were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (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. The following primer pairs were used:

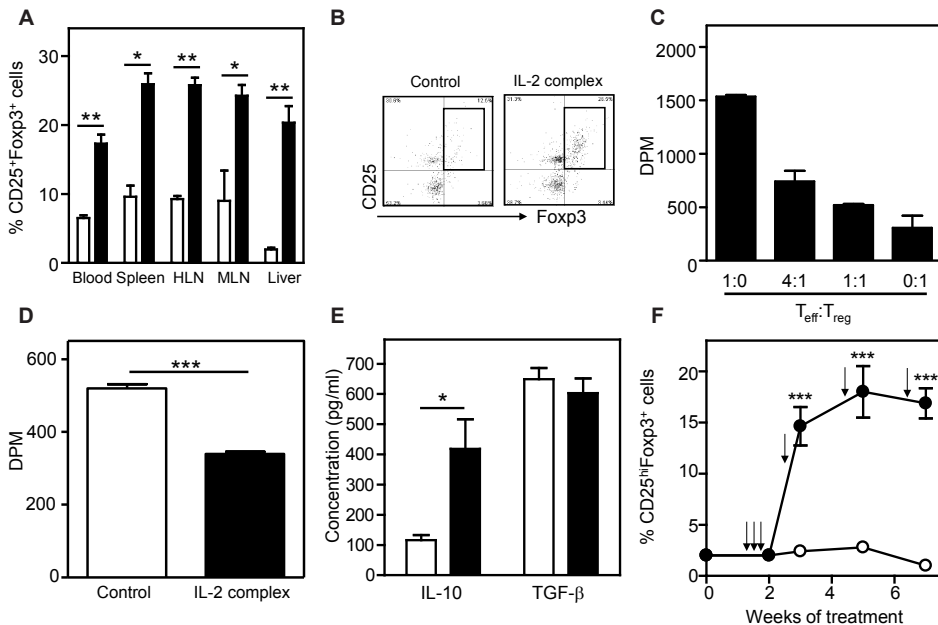
IL-10: 5'-TCTTACTGACTGGCATGAGGATCA-3' and 5'-GTCCGAGCTCTAGGAGCAT-3'  
 TGF- $\beta$ : 5'-AGGGCTACCATGCCAACTTCT-3' and 5'-GCAAGGACCTTGCTGTACTGTGT-3'

The following primers were used as endogenous references:

36B4: 5'-GGACCCGAGAAGACCTCCTT-3' and 5' GCATCACTCAGAATTTCAATGG-3'  
 HPRT: 5'-TTGCTCGAGATGTCATGAAGGA-3' and 5' AGCAGGTCAGCAAAGAACTTATAG-3'

### Statistical analysis

All data are expressed as mean $\pm$ SEM. A paired two-tailed student T-test was used to compare normally distributed data between two groups of animals. Probability values of  $P < 0.05$  were considered significant.



**Figure 1. IL-2 complex induces persistent high levels of Tregs in LDLr<sup>-/-</sup> mice.** LDLr<sup>-/-</sup> mice were injected 3 times with an IL-2 complex (n = 3, black bars) or PBS as a control (n = 3, open bars). Mice were sacrificed 5 days after initiation of the experiment. **A.** Blood, spleen, mediastinal lymph nodes near the heart (HLN), mesenteric lymph nodes (MLN) and liver cells were isolated and the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> cells within CD4<sup>+</sup> cells was determined by flow cytometry. **B.** Representative dotplots are shown. **C.** A suppression assay was performed to determine the suppressive capacity of the expanded Tregs by measuring the proliferation of splenocytes (n=3). Data are shown as the mean disintegration per minute (dpm) of triplicate cultures. **D.** In a 1:1 ratio, effector T cells were more potentially suppressed by Tregs from IL-2 complex treated mice compared to Tregs from control mice. **E.** Secretion of IL-10 and TGF- $\beta$  in the supernatant of Tregs cultured with effector T cells in a 1:1 ratio was determined with ELISA. **F.** To induce atherosclerosis, LDLr<sup>-/-</sup> mice were fed a Western-type diet for 8 weeks. Two weeks after initiation of the experiment, mice received *i.p.* injections with the IL-2 complex (black arrows). Levels of CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells were monitored in the blood at weeks 2, 3, 5 and 7 using flow cytometry (n=5 per group). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

## Results

### ***Persisting high levels of Tregs in LDLr<sup>-/-</sup> mice due to continuous treatment with the IL-2 complex***

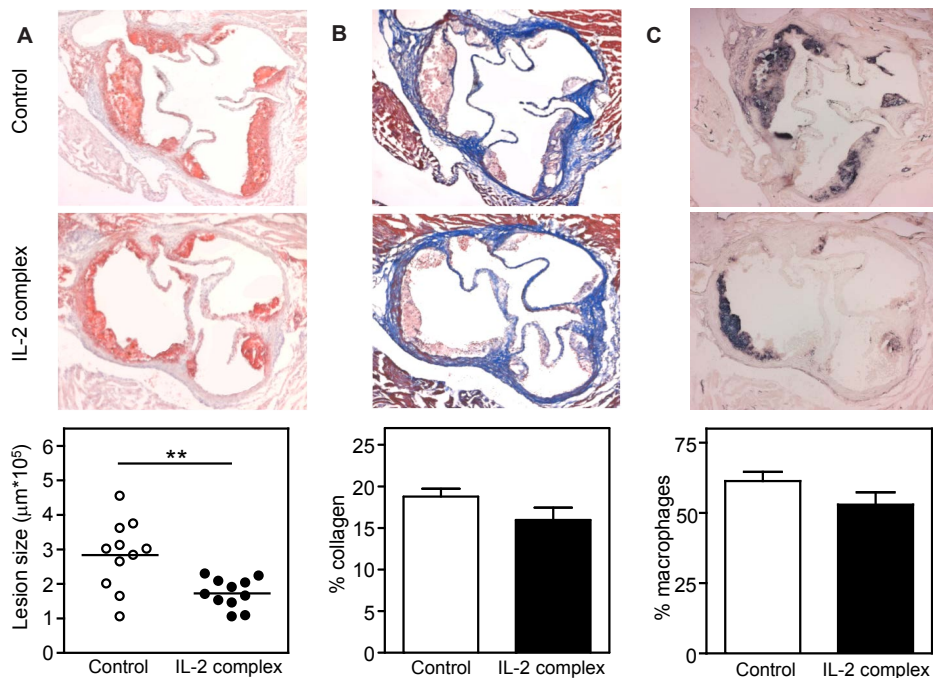
To determine whether IL-2 complexes induced Treg expansion in LDLr<sup>-/-</sup> mice, we injected the IL-2 complex or PBS *intraperitoneally* on 3 consecutive days. Mice were sacrificed 5 days after initiation of the experiment. The administration of IL-2 complexes resulted in a significant 3-fold increase of Tregs in lymphoid organs and an 11-fold increase in the liver, compared to control mice (Figure 1A and B). The expanded Tregs in the IL-2 complex-treated group were functional as they potently suppressed effector T cell proliferation *ex vivo* (Figure 1C). In addition, Tregs expanded by the IL-2 complex were more suppressive than Tregs from control treated mice ( $P < 0.001$ , Figure 1D), whereas no significant differences between solely effector T cell proliferation and Treg proliferation of both groups were observed (data not shown). Cytokine determination in the supernatant of this suppression assay showed that IL-2 complex expanded Tregs mainly function via IL-10 secretion, whereas no difference in TGF- $\beta$  secretion was observed ( $P < 0.05$ , Figure 1E).

To determine whether IL-2 complex-induced Treg expansion is still feasible under hyperlipidemic, pro-inflammatory conditions, LDLr<sup>-/-</sup> mice were fed a Western-type diet for 8 weeks. Two weeks after initiation of the experiment, mice were *intraperitoneally* injected with IL-2 complexes for 3 consecutive days to boost the expansion of Tregs. Thereafter, mice were injected every 10 days to maintain persistently high Treg levels essential for investigating the effect of high Treg levels on atherosclerosis development and regression. As shown in Figure 1F, the IL-2 complex is still able to enhance Tregs in blood under hyperlipidemic conditions. In addition, the level of Tregs in the blood persistently remained at a significantly 10-fold higher level than in control treated mice ( $P < 0.001$ ).

### ***IL-2 complex administration reduces the development of atherosclerosis***

Since a limited increase in Tregs already affects initiation of atherosclerosis, we postulated that a 10-fold expansion of Tregs in blood observed after IL-2 complex administration may significantly potentiate this effect. During the experiment enhanced levels of Tregs did not affect body weight and total plasma cholesterol levels (data not shown). Eight weeks after the start of the high fat diet mice were sacrificed and atherosclerotic lesion size was determined. We observed a significant 39% reduction in aortic root lesion size of IL-2 complex treated mice ( $1.73 \times 10^5 \pm 0.13 \times 10^5 \mu\text{m}^2$ ) in comparison with control mice ( $2.84 \times 10^5 \pm 0.30 \times 10^5 \mu\text{m}^2$ ,  $P < 0.01$ , Figure 2A). No difference in lesion stability, as determined by Masson's Trichrome staining, was observed between IL-2 complex treated mice ( $16.0 \pm 1.5\%$ ) and control treated mice ( $18.8 \pm 0.9\%$ , Figure 2B). Furthermore, no difference in macrophage content was observed (control:  $61.4 \pm 3.3\%$  and IL-2 complex:  $53.0 \pm 4.4\%$ , Figure 2C). At sacrifice, we determined whether the high amounts of CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells measured in blood corresponded to increased Treg levels in spleen, mediastinal lymph nodes located near the heart (HLN), and liver. In agreement with increased

Treg levels in the blood of IL-2 complex treated mice, we observed a significant 3-fold ( $P < 0.001$ ), 1.5-fold ( $P < 0.05$ ), and 9.2-fold ( $P < 0.05$ ) increase in Tregs in the spleen, HLN, and liver, respectively, as compared with control treated mice (Figure 3A). To determine the suppressive capacity of the IL-2 complex expanded Tregs, splenocytes isolated from both groups were cultured for 48 hours in the presence of  $\alpha$ CD3/ $\alpha$ CD28 stimulation. A significant 43% decrease in T cell proliferation was observed in mice treated with the IL-2 complex (stimulation index of  $9.7 \pm 1.5$ ) compared to control mice (stimulation index of  $17.1 \pm 3.3$ ), showing that the induced Tregs are functional (Figure 3B,  $P < 0.05$ ). Since Tregs function in part via IL-10 and TGF- $\beta$  secretion, we determined the gene expression of these cytokines in the spleen. IL-2 complex treated mice showed a 4.7-fold increase in IL-10 expression compared with control mice ( $P < 0.05$ ), whereas TGF- $\beta$  expression remained unchanged (Figure 3C). This suggests that Tregs induced by the IL-2 complex may exert their suppressive function predominantly via the secretion of IL-10.

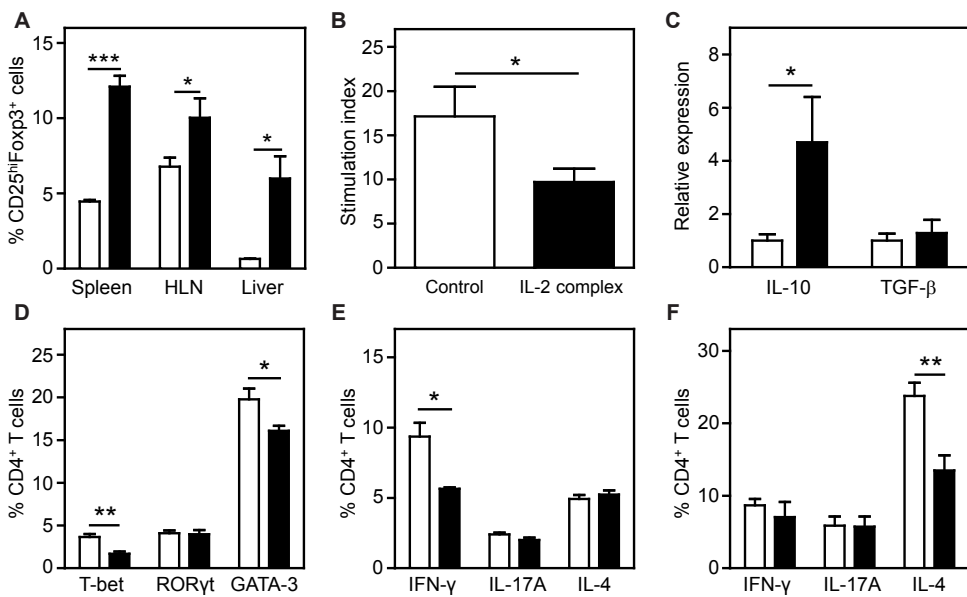


**Figure 2. Expansion of Tregs reduces lesion formation.** **A.** Representative cross-sections of lesion formation in the three valves area of the aortic root stained with Oil-red-O and hematoxylin are shown and lesion size was determined. **B.** Corresponding sections on separate slides were stained for collagen using Masson's Trichrome staining. The percentage of collagen relative to the lesion size was determined. **C.** Relative macrophage content was determined with a MOMA-2 staining and quantified. \*\* $P < 0.01$ .

### **Effect of Treg expansion on other T cell subsets**

It has been suggested that Tregs have the capacity to specifically target and suppress effector T cells, such as Th1, Th2 and Th17 cells<sup>19,20</sup>. To evaluate whether the IL-2 complex expanded Tregs inhibit a specific T cell subset in vivo during initiation of atherosclerosis, splenocytes were stained for the transcription factors T-bet, GATA-3,

and ROR $\gamma$ t, which control the differentiation of Th0 cells into Th1, Th2 and Th17 cells, respectively. Flow cytometry analysis showed that IL-2 complex treated mice have significant reduced T-bet expression ( $1.7 \pm 0.2\%$  vs.  $3.6 \pm 0.4\%$ ,  $P < 0.01$ , Figure 3D) and reduced GATA-3 expression ( $16.1 \pm 0.6\%$  vs.  $19.8 \pm 1.3\%$ ,  $P < 0.05$ , Figure 3D) in the CD4 $^{+}$  T cell population of the spleen, compared to the control group. Accordingly, reduced CD4 $^{+}$ IFN- $\gamma$  $^{+}$  T cells were observed ( $6.1 \pm 0.5\%$  vs.  $9.4 \pm 1.0\%$ ,  $P < 0.05$ , Figure 3E). Interestingly, the percentage of IL-4 $^{+}$  cells did not change in the spleen, but was decreased in the blood ( $13.5 \pm 2.1\%$  vs.  $23.8 \pm 1.8\%$ ,  $P < 0.01$ , Figure 3F). Th17 responses, on the other hand, remained unchanged following Treg expansion (Figure 3D-F).

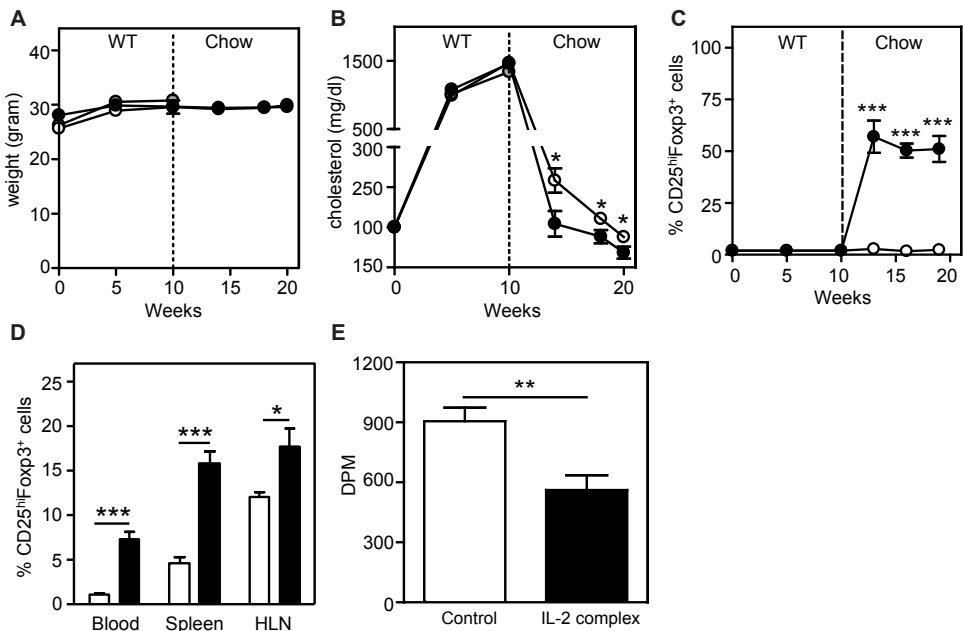


**Figure 3. Effect of the IL-2 complex on the percentage and functionality of the Tregs.** **A.** After eight weeks, spleen, HLN and liver cells were isolated and stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry ( $n=5$ ). **B.** The effect of boosting Tregs with the IL-2 complex on spleen cell proliferation was determined by culturing splenocytes ( $n=5$ ) in the presence or absence of CD3/CD28 stimulation. Proliferation was assessed by the amount of  $^3\text{H}$ -thymidine incorporation in dividing cells. The proliferation is expressed as stimulation index. **C.** mRNA levels of IL-10 and TGF- $\beta$  in the spleen were determined with RT-PCR. **D.** Spleen cells were stained for CD4 and the transcription factors T-bet (Th1), ROR $\gamma$ t (Th17) and GATA-3 (Th2). **E.** Cytokine production in the spleen and **F.** blood was evaluated by flow cytometry. Cells were stained for CD4, IFN- $\gamma$ , IL-17A and IL-4. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

### ***IL-2 complex-expanded Tregs stabilize lesions in a regression model***

Since it is clinically more relevant to determine the effect of Tregs on pre-existing lesions, we combined lipid lowering with IL-2 complex treatment. To this end, we put LDL $^{-/-}$  mice, which were fed a Western-type diet for 10 weeks, on a chow diet for another 10 weeks, combined with simultaneous administration of IL-2 complexes. In addition, a baseline group was sacrificed after 10 weeks of Western-type diet to determine the effect of treatment on atherosclerotic lesion size. No differences in weight and cholesterol levels were found between baseline, control and IL-2 complex

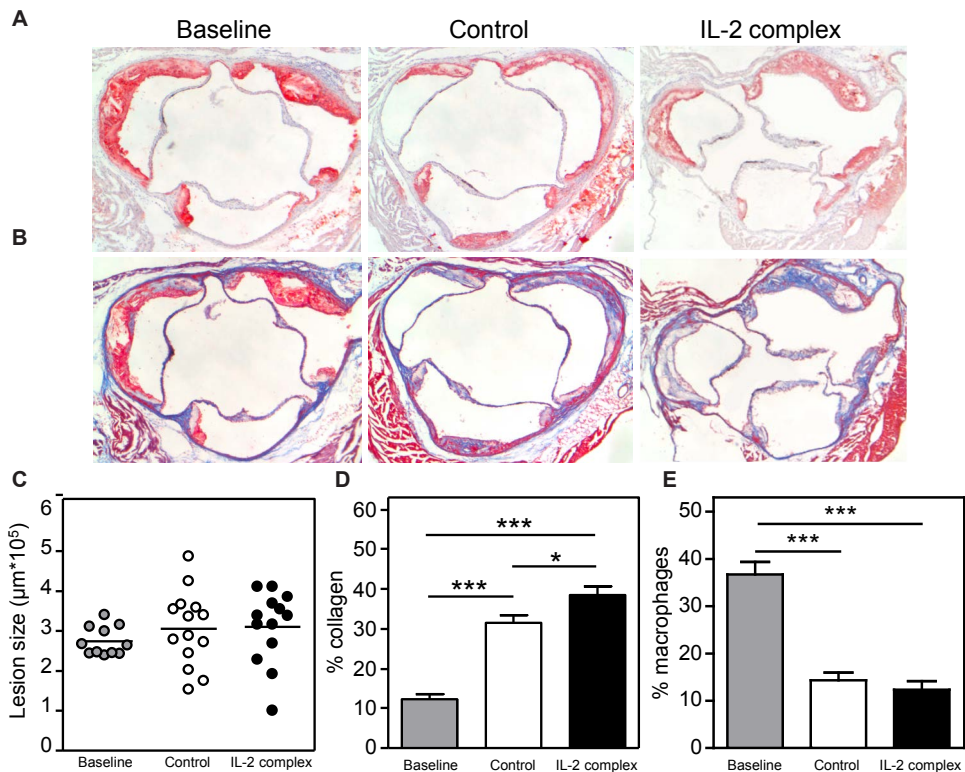
treated mice during Western-type diet feeding (Figure 4A and B). In addition, no differences in weight were found between control and IL-2 complex-treated mice after switching to chow diet and Treg induction (Figure 4A). Only a 10-20% reduction ( $P < 0.05$ ) in plasma cholesterol levels could be observed in IL-2 complex-treated mice compared to control mice at 4, 8 and 10 weeks after switching to a low cholesterol diet (Figure 4B). Throughout the experiment, Treg levels in blood remained significantly higher in the IL-2 complex group as compared to control treated mice (Figure 4C). In addition, highly elevated Treg levels were observed in blood, spleen and HLN at sacrifice (Figure 4D). At sacrifice ex vivo effector T cell proliferation was suppressed (Figure 4E).



**Figure 4. Effect of IL-2 complex on weight, cholesterol levels and Tregs during regression of atherosclerosis.**  $LDL^r/-$  mice received 10 weeks Western-type diet and were subsequently put on chow diet for 10 weeks and treated with the IL-2 complex ( $n=13$ , black circles) or PBS ( $n=14$ , open circles). A baseline group was sacrificed after 10 weeks of Western-type diet ( $n=11$ , grey circles). **A.** During the experiment, mice were weighed. **B.** Blood was taken by tail vein bleeding and total cholesterol concentration was determined within the serum. **C.** To check whether IL-2 complexes expanded Tregs, we monitored these in the circulation. **D.** At sacrifice, blood, spleen and HLN cells were isolated and stained for CD4, CD25, and Foxp3, and analyzed with flow cytometry ( $n=5$  per group). **E.** The effect of boosting Tregs with the IL-2 complex on spleen cell proliferation was determined by culturing splenocytes ( $n=5$ ) in the presence or absence of CD3/CD28 stimulation. In a 1:1 ratio, effector T cells were more potently suppressed by Tregs from IL-2 complex treated mice compared to Tregs from control mice. Proliferation was assessed by the amount of  $^3\text{H}$ -thymidine incorporation in dividing cells and is expressed as disintegrations per minute (DPM). \* $P < 0.05$ , \*\*\* $P < 0.001$ .

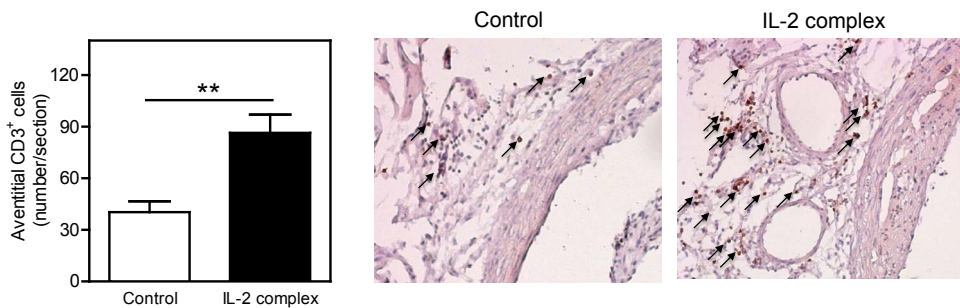
Both the control and the IL-2 complex-treated mice displayed no reduction in lesion size (control:  $3.06 \times 10^5 \pm 0.25 \times 10^5 \mu\text{m}^2$  and IL-2 complex:  $3.10 \times 10^5 \pm 0.25 \times 10^5 \mu\text{m}^2$ ) when compared with the baseline group ( $2.74 \times 10^5 \pm 0.10 \times 10^5 \mu\text{m}^2$ , Figure 5A and C). The same effect was observed in the aortic arch, where both IL-2 complex treated

and control mice showed no reduction in lesion size (data not shown). Furthermore, the collagen content of the lesions was determined (Figure 5B and D). Lesions from IL-2 complex-treated mice showed a substantially increased stability ( $38.3 \pm 2.3\%$  collagen) compared to control mice ( $31.6 \pm 1.8\%$  collagen,  $P < 0.05$ ). Additionally, both groups showed very significantly increased collagen content compared to baseline mice ( $12.2 \pm 1.2\%$ ,  $P < 0.001$ ). No difference in relative macrophage content was observed between the IL-2 complex treated group ( $12.4 \pm 1.9\%$ ) and the control group ( $14.3 \pm 1.6\%$ ). Both groups, however, showed a significant 60% reduction in the relative macrophage content compared to the baseline group ( $36.7 \pm 2.6\%$ ,  $P < 0.001$ , Figure 5E), indicative for regressed lesions in both groups. Together these results suggest that increasing the number of Tregs during well-established atherosclerosis results in more stable lesions, with increased collagen content, but does not affect lesion size. In addition, we analyzed the aortic root for CD3<sup>+</sup> T cells within lesions and found almost no T cells in lesions of both IL-2 complex-treated mice and control mice. However, we found a 60% increase of CD3<sup>+</sup> T cells within the adventitia of IL-2 complex



**Figure 5. IL-2 complex treatment stabilizes atherosclerotic lesions during regression.** **A.** Sections of the aortic root were stained with Oil-red-O and hematoxylin. **B.** Corresponding sections on separate slides were also stained for collagen using Masson's Trichrome staining. **C.** Lesion size was determined by Oil-red-O staining. **D.** The percentage of collagen relative to the lesion size was quantified by Masson's Trichrome staining. **E.** Relative macrophage content was determined by MOMA-2 staining and quantified. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

treated mice ( $86.4 \pm 10.6$  cells/section) compared to control treated mice ( $40.3 \pm 6.3$  cells/section,  $P < 0.01$ , Figure 6).



**Figure 6. Increased adventitial CD3<sup>+</sup> T cell infiltration in IL-2 complex treated mice.** Sections of the aortic root were stained for CD3 to determine infiltrating T cells. Arrows indicate T cells. \*\*  $P < 0.01$

## Discussion

Regulatory CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells are important regulators of immune responses and have been shown to play a major role in autoimmune diseases. Since autoimmune diseases result from an imbalance between effector and regulatory cells, with reduced numbers of the latter, Tregs show great potential to be used as a therapeutic regime. Their beneficial role in atherosclerosis has been particularly elucidated in the initiation of atherosclerosis using adoptive transfer, induction and depletion of Tregs<sup>8,9,11,21-23</sup>. However, in these studies, only a modest increase in Treg numbers was achieved in the order of 1.5- to 2-fold, mostly for two to three weeks. We therefore determined whether significantly higher Treg levels for longer periods of time could even more drastically attenuate atherosclerosis development. We used the recently published technique using the IL-2 complex consisting of IL-2 and a neutralizing anti-IL-2 mAb, a treatment that beneficially affected the outcome of a number of autoimmune-like diseases<sup>16-19</sup>. In the present study, LDLr<sup>-/-</sup> mice were fed a Western-type diet for 8 weeks. The treatment with the IL-2 complex was started after two weeks of feeding the Western-type diet in order to counteract the pro-inflammatory effects of the diet. We now show that stimulation of Tregs with the IL-2 complex resulted in a highly significant 10-fold increase of CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells in blood of LDLr<sup>-/-</sup> mice, which was maintained for six weeks. The expansion of Tregs during diet was comparable to the level we obtained in chow fed animals indicating that the pro-inflammatory effect of the high-cholesterol diet did not affect this expansion. The extent of expansion is in line with a previous study by Webster *et al.*, in which IL-2 complexes with a similar molar ratio of IL-2 and anti-IL-2 mAb induced a 10-fold increase of Tregs<sup>16</sup>.

The significantly strong expansion of CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells resulted in a 39% decrease in initial atherosclerosis in the aortic root. It was previously shown that oral tolerance induction to oxLDL, an atherosclerosis-specific antigen, induced a maximal 2-fold increase in Tregs, with a 30% reduction in lesion size in the aortic

root<sup>10</sup>. These Tregs however, were antigen-specific Tregs, whereas the IL-2 complex has been shown to expand all present peripheral Tregs<sup>16,19</sup>. In addition, the number of antigen-specific Tregs dropped two weeks after treatment, while the present technique enabled us to maintain the enhanced numbers of Tregs for more than six weeks. Interestingly, only an additional reduction of approximately 10% in lesion size was observed. These results may indicate that Tregs only reduce lesion development to a maximal extent and it may be suggested that only a vast, prolonged expansion of antigen-specific Tregs by combining oral tolerance with IL-2 complex treatment may lead to a greater reduction in lesion size.

The IL-2 complex expanded Tregs in the LDLr<sup>-/-</sup> mice reduced Th1 and Th2 responses and potentially suppressed proliferation of splenocytes by 43%. We observed an increase in gene expression of IL-10 in the spleen and increased IL-10 secretion by Tregs, which suggests that the IL-2 complex expanded Tregs exert their suppressive capacity via IL-10. In agreement with this finding, Webster *et al.* show enhanced expression of IL-10 mRNA but little change in TGF- $\beta$  by the IL-2 complex expanded Tregs<sup>16</sup>. In addition, the suppression of airway inflammation via the IL-2 complex is dependent on IL-10<sup>18</sup>. The increase of IL-10 in the IL-2 complex treated mice may at least partially be responsible for the decrease in lesion size since several studies showed the protective role of IL-10 in atherosclerotic lesion development<sup>24-26</sup>.

The development of experimental therapies for the treatment of atherosclerosis mainly focus on preventing the initiation and to a minor extent the progression of atherosclerosis. A clinically more relevant therapy for atherosclerosis would be a therapy which induces regression of atherosclerosis as most of the cardiovascular patients already have well-established lesions. In the present study, we therefore aimed to simulate the treatment of cardiovascular patients by changing the diet (as a mimic of statin-induced lipid lowering) in combination with a reduction of the inflammatory status by inducing Tregs. This approach is comparable to Verschuren *et al.* who induced regression of atherosclerosis in apoE\*3Leiden mice by switching high-fat diet to chow diet and treatment with the atheroprotective Liver-X-receptor (LXR)-agonist<sup>27</sup>. In our current study, we were able to induce high levels of Tregs in mice that previously were fed a cholesterol rich diet for 10 weeks comparable to the levels obtained in chow fed mice. We observed no lesion regression in the control group (only lipid lowering) and despite extensive Treg induction also no lesion regression was found in the IL-2 complex-treated group. However, we observed that Treg induction increased lesion stability as indicated by increased collagen content in the lesions. This effect cannot be ascribed to the significant 10-20% reduction in cholesterol observed in IL-2 complex treated mice, since we did not find a correlation between collagen content and cholesterol levels. Possibly, the reduction in cholesterol can be ascribed to an increase of IL-10 produced by IL-2 complex expanded Tregs. Previously it was shown that IL-10 influences parenchymal liver cells, thereby lowering cholesterol levels in LDLr<sup>-/-</sup> mice<sup>25</sup>.

Interestingly, a 60% increase of adventitial CD3<sup>+</sup> T cells within lesions of IL-2 complex treated mice was observed. This strong increase correlates with a highly significant 85% increase of circulating Tregs, which likely migrate towards the site of



inflammation via the adventitia. Moreover, we see a significant suppression of effector T cell proliferation in the IL-2 complex-treated group by expanded Tregs. This proves that these Tregs are functional in suppressing effector T cells and in limiting the possibility of effector T cell expansion and migration towards inflammatory sites. In addition to the apparent phenotype of T cells in the IL-2 treated-group, the observed increase in IL-10 additionally suggests an anti-inflammatory environment in these mice. This again suggests an inhibition of effector T cell proliferation but also implies that T cells found in this environment will be of an anti-inflammatory phenotype.

In conclusion, our data clearly illustrate the potential of IL-2 complexes to selectively expand Tregs capable of attenuating initial atherosclerotic lesion development, and further prove their capability to stabilize well-established lesions in a regression model. In the future, it may be of great interest to induce antigen specific Tregs with the IL-2 complex.

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