Modulation of the immune system for treatment of atherosclerosis

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Protection from atherosclerosis induced by oxLDL tolerization is not reinforced by polyclonal Treg induction

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Abstract

Atherosclerosis is characterized by lipid accumulation in the arterial wall and an ensuing pathogenic immune response. Induction of immune tolerance towards oxidized LDL, through oral administration of oxLDL, was effective at reducing atherosclerosis in line with our previous results. As oral administration of oxLDL was found to induce Tregs and levels of Tregs returned to baseline levels within weeks after treatment, we hypothesized that the effect of oral oxLDL treatment on atherosclerosis could be improved by polyclonal stimulation of Tregs and to that end $LDLr^{-/-}$ mice were treated with IL-2 complexes after oral tolerance induction towards oxLDL. Only oral oxidized LDL administration reduced atherosclerosis, while trends towards reduced atherosclerosis were observed in the mice treated with IL-2 complex separately or IL-2 complex and oxidized LDL combined, compared to control treated mice. Our study suggest that specific tolerization towards plaque antigens is more beneficial than polyclonal Treg induction, and that a combination of both antigen specific tolerization and polyclonal Treg induction does necessarily lead to an additive beneficial effect on protection from atherosclerosis.

Introduction

Atherosclerosis is characterized by the retention of cholesterol rich low density lipoprotein (LDL) particles in the arterial wall. In the arterial wall LDL undergoes oxidative modifications which initiates local inflammation and attracts immune cells to the subendothelial space. Uptake of oxidized LDL (oxLDL) particles by antigen presenting cells (APCs), including macrophages and dendritic cells, results in processing of oxLDL derived proteins and presentation of oxLDL derived peptides on MHC-I and MHC-II molecules. T cell receptor (TCR) recognition of (ox)LDL derived peptide/MHC complexes induces activation and proliferation of (ox)LDL specific T cells , resulting in (ox)LDL reactive T cells in the atherosclerotic lesion *(1, 2)*.

Especially the induction of CD4 T helper 1 (Th1) cells, which are found at high levels in atherosclerotic lesions and mainly produce the inflammatory cytokines IFN-γ, TNF-α, IL-12, and IL-2, have been found pathogenic in the context of atherosclerosis *(3)*. Inhibition of the inflammatory Th1 response would therefore be an interesting option to treat atherosclerosis. $CD4^+FoxP3^+$ regulatory T cells (Tregs) are pivotal in the maintenance of peripheral tolerance towards self-antigens, among other mechanisms through suppressive effects on antigen presenting cells and other effector T cells, and were found effective at inhibiting autoimmune Th1 responses in experimental myasthenia *(4)* and autoimmune gastritis *(5)*. Supporting a therapeutic potential of Tregs for treatment of atherosclerosis, adoptive transfer of $CD4^+CD25^+$ T cells, enriched for Tregs, reduced atherosclerotic lesion development *(6)* whereas depletion of $CD4^+CD25^+$ or Foxp3 expressing T cells increased atherosclerosis *(7, 8)*.

Within the Treg population, two distinct origins are distinguished, namely the Tregs generated in the thymus and peripherally induced Tregs, referred to as natural Tregs (nTregs) and inducible Tregs (iTregs), respectively. Since antigen specific Tregs were found more potent at preventing autoimmune diabetes than polyclonal Tregs *(9)*, it can be therapeutically interesting to induce disease antigen specific Tregs to treat auto-immune diseases instead of increasing overall Treg levels. Antigen specific iTregs can be induced by delivery of a low quantity of antigen via oral administration. Presentation of antigen by the intestinal tolerogenic $CD11C^+CD103^+$ DC population, which expresses high levels of TGF-β, RALDH, and IDO promotes the induction of antigen specific immune suppressive regulatory B cells, Th3, Tr1 and Tregs *(10)*. Our group has previously shown that atherosclerosis could be inhibited through oral administration of oxLDL, a relevant antigen for atherosclerosis *(1, 2)*, inducing relatively low levels of oxLDL responsive inducible Tregs *(11)*.

Regulatory T cells can be polyclonally expanded in vivo by administration of IL-2, on which Tregs depend for optimal growth and survival. Complexing IL-2 with an IL-2 specific antibody (JES6-1A12) still allows IL-2 to bind to the high affinity IL-2 receptor which is mainly expressed on Tregs, but inhibits binding of IL-2 to moderate and low affinity IL-2 receptors, specifically expanding Tregs in vivo when administered repeatedly *(12, 13)*. Our group has previously

shown that atherosclerosis can also be inhibited through expansion of the entire Treg pool using IL-2 complexes *(14)*, leading to substantially higher Treg levels than observed after oral oxLDL tolerance induction. Since oral oxLDL treatment yields low levels but antigen specific Tregs and IL-2c treatment expands the overall Treg pool we hypothesized that combining both treatment regimens could lead to enhanced atheroprotection than separate treatment regimens.

Results

IL-2c treatment induces enhanced levels of Tregs

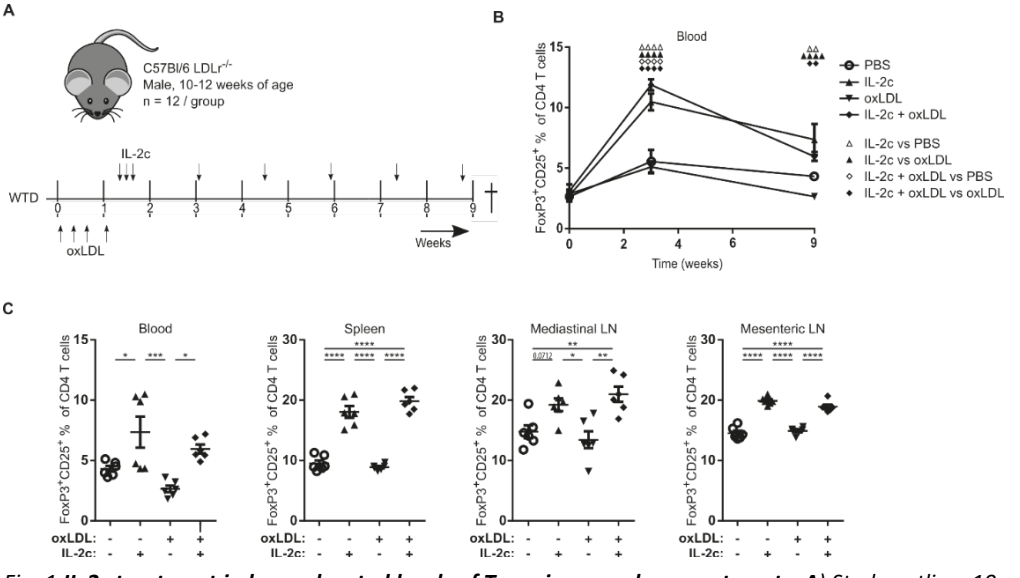

Fig. 1 ***IL-2c treatment induces elevated levels of Tregs in several compartments. A****) Study outline: 10-12 week old male LDLr$^{-/-}$ mice were fed WTD for 9 weeks. In the first 8 days of WTD, tolerance towards oxLDL was induced by 4 oral administrations of oxLDL (30µg) in oxLDL groups. Polyclonal expansion of Tregs was induced with 3 IL-2c injections in consecutive days starting 2 days after final oral gavage in IL-2c groups. After the last of the 3 consecutive IL-2c administrations, mice in the IL-2c group received a single IL-2c injection every 10 days. Vehicle injections served as control treatment.* ***B****) Quantification of FoxP3^{+}CD25^{+} regulatory CD4 T cells in circulation at the indicated timepoints as assessed by flow cytometry.* ***C****) Quantification of FoxP3^{+}CD25^{+} regulatory CD4 T cell levels in the blood, spleen, mediastinal and mesenteric lymph nodes after sacrifice measured by flow cytometry. Expressed as mean ± SEM,* ***B****) two way ANOVA* ***C****) one-way ANOVA with Sidak posttest, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

Since our lab has shown that non-specific Treg and oxLDL specific Treg induction, through intraperitoneal IL-2 complex administration and oral oxLDL administration of oxLDL reduced atherosclerosis we compared a combination of both treatments could complement each other by IL-2c mediated expansion of oral oxLDL induced oxLDL specific Tregs.

To induce oxLDL specific Tregs, tolerance to oxLDL was induced by oral administration of oxLDL (30 µg) 4 times in 8 days and atherosclerosis was induced by subsequent WTD feeding of male LDLr KO mice for 8 weeks. To induce polyclonal expansion of Tregs, mice received intraperitoneal IL-2 complex injections (1µg IL-2, 5µg anti IL-2 mAb, clone JES6-1A12) for 3 consecutive days, 2 days after the final oral oxLDL or oral control/PBS administration. To prevent elevated Treg levels from declining after the 3 consecutive days of IL-2c administration, mice received IL-2c injections every 10 days after the 3 initial IL-2c injections (Fig. 1A).

To assess whether treatment with IL-2c induced regulatory T cells we assessed Treg levels by flow cytometry in the blood, spleen, mesenteric lymph nodes (MLN) which drain the intestines, and mediastinal lymph nodes (HLN) which drain the heart. In the blood the Treg content of the CD4 T cell population increased over two-fold upon IL-2c treatment 3 weeks in the experiment, and slightly declined towards the end of the experiment (Fig. 1B). Furthermore IL-2c treatment successfully raised Treg levels in spleen, MLN and HLN at sacrifice, showing that we were indeed able to systemically enhance Treg levels by our IL-2c treatment regimen (Fig. 1C). Induction was observed in the control treted group and the oxLDL tolerance induced group. We did not observe enhanced Treg levels after oral oxLDL treatment alone in any of the assessed tissues/organs (Fig. 1C), in line with the previous oxLDL study in which elevated Tregs were measured maximally 14 days after final oxLDL administration in spleen and MLN *(11)*.

IL-2c and oxLDL treatment induce overall immune suppression

To assess whether the administration of oxLDL and IL-2c acted immunosuppressively, we assessed immune populations in circulation, spleen, HLN and MLN. IL-2c treatment alone reduced white blood cell counts (-37.7%, p = 0.0001) compared to the control treated group, similar to oxLDL treatment alone (-33.9%, p = 0.0005) (Fig. 2A). Combined oxLDL and IL-2c treatment (-58.0%, p = 0.0001) had an additional effect (vs oxLDL, p = 0.0209; vs IL-2c, p = 0.0593) on reduction of overall circulating leukocyte numbers (Fig. 2A). In all treatment groups a significant decrease in circulating neutrophils (Fig. 2B), classical monocytes (Fig. 2B) and B cells (Fig. 2D) was observed. Separate IL-2c administration induced patrolling monocyte and eosinophil levels (Fig. 2B). Overall CD4 T cells and CD4 Tem cells were significantly reduced in IL-2c+oxLDL treated mice and a trend towards reduction of CD4 T cells and CD4 Tem cells was observed in the oxLDL and IL-2c treatment groups, compared to control treated mice (Fig. 2C). In all treatment groups CD4 Tcm cell numbers were reduced compared to control treated mice, whereas naïve CD4 T cell levels were not affected (Fig. 2C). CD8 T cell

numbers were significantly reduced in the two IL-2c treated groups (Fig. 2E), with a trend towards reduction in the oxLDL treated group compared to control treated. Unlike in the CD4 T cell population, combined IL-2c and oxLDL also reduced naïve CD8 T cells and a trend towards reduction of naïve CD8 T cells was observed in IL-2c or oxLDL treated groups compared to PBS (Fig. 2C). Furthermore IL-2c treated groups had significantly reduced CD8 Tem and Tcm cell numbers in circulation, with a trend towards decrease for oxLDL treated (Fig. 2C).

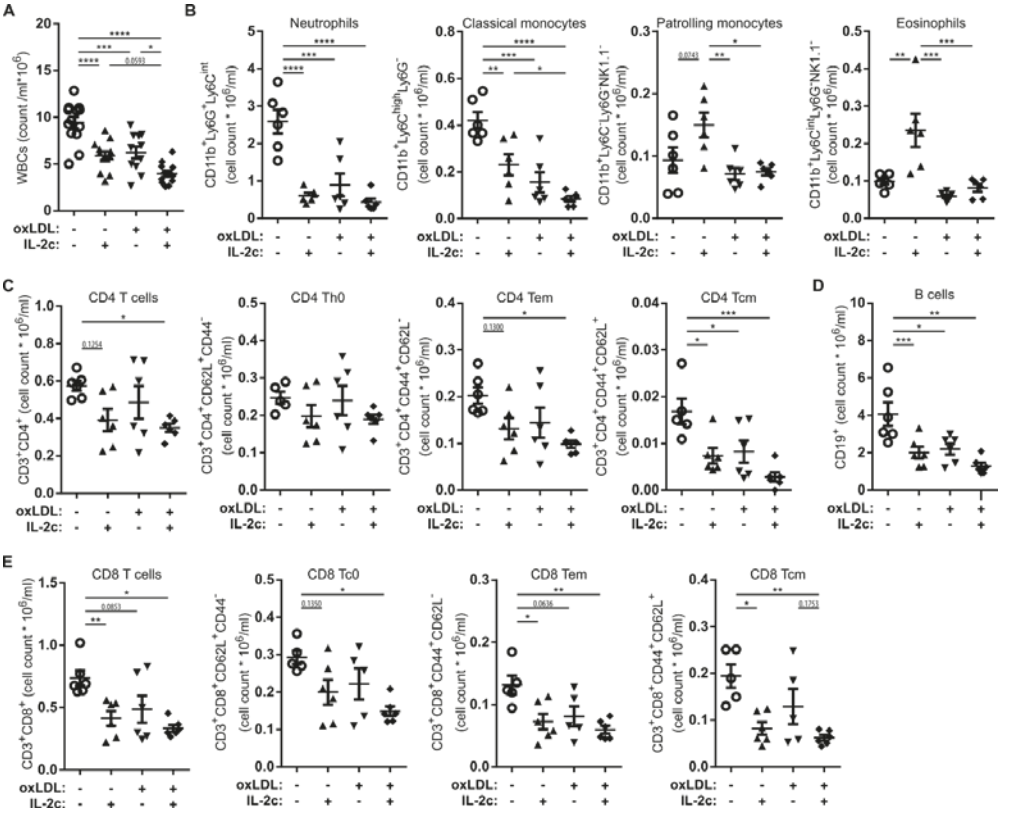

Fig. 2 ***IL-2c and oxLDL treatment induce overall immune suppression.*** *At sacrifice blood was collected and* ***A****) total white blood cell count was determined for all mice. On half of the mice flow cytometric analysis was performed and WBC counts were used to quantify the number of circulating* ***B****) major myeloid cell populations,* ***C****) CD4 T cells,* ***D****) B cells and* ***E****) CD8 T cells. Expressed as mean ± SEM, one-way ANOVA with Sidak posttest, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

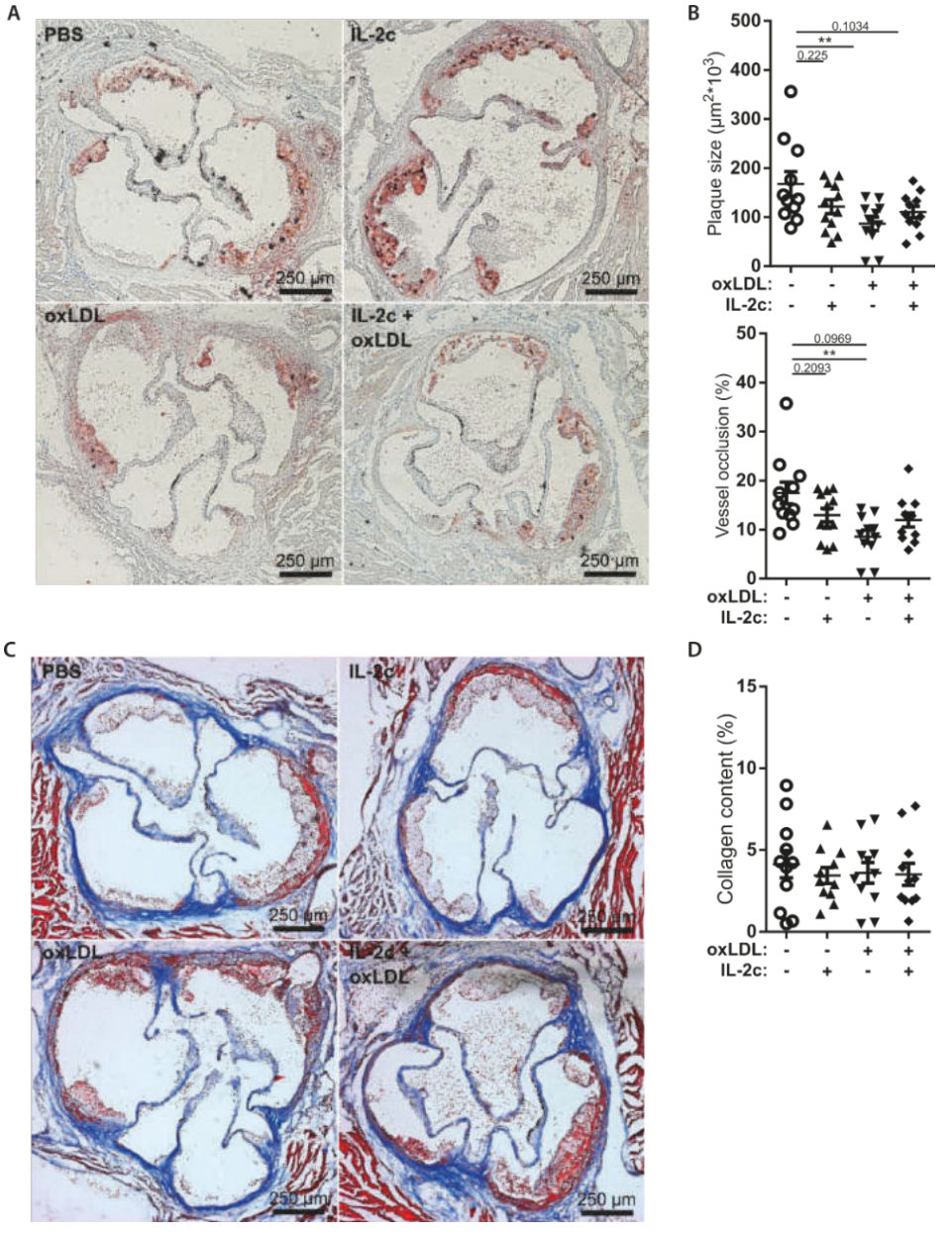

Fig. 3 ***Only separate oral oxLDL administration reduces aortic root plaque size.*** *To assess the effect of oral oxLDL and IL-2c administration aortic root sections were assessed.* ***A****) Representative microscopic images of neutral lipid staining with ORO used for* ***B****) quantification of atherosclerotic lesion size and vessel occlusion.* ***C****) Representative microscopic images of staining of collagen with Masson's Trichrome staining for the determination of plaque stability through* ***D****) quantification of collagen content. Expressed as mean ± SEM, one-way ANOVA with Tukey posttest, ** p < 0.01.*

The effect of oxLDL and IL-2c treatment on T cells was less evident in the lymphoid organs than in circulation. Neither treatment did alter overall splenocyte numbers or CD4 or CD8 T cell levels compared to control in the spleen (Fig. S1BC). oxLDL treatment alone significantly reduced the percentage of CD4 Tem cells and increased Th0 cells compared to all other experimental groups (Fig. S1B). Furthermore in all treatment groups a slight trend towards increased naïve CD8 T cells and reduced CD8 Tem and Tcm cells was observed, indicative of immune suppression by oxLDL and IL-2c treatment in the spleen (Fig. S1C). Furthermore we assessed T cell maturation in mesenteric lymph nodes and mediastinal lymph nodes but oxLDL and/or IL-2c treatment did not significantly impact the proportion of naïve, central memory, and effector memory T cells compared to control probably because the vast majority of T cells in the lymph nodes was already naïve in the control group (data not shown). Overall these data indicate that oxLDL and IL-2c treatment successfully induced immune suppression as seen from reduced circulating immune cell levels and reduced memory T cell populations in the IL-2c and oxLDL treated groups, compared to control treated mice.

Only separate oral tolerance induction to oxLDL significantly reduces aortic root plaque size.

To assess whether the immunosuppressive effects of the different treatment regimens translated in reduced atherosclerosis, aortic root tissue sections were stained with oil red o (ORO) and trichrome to assess plaque size and stability. The average lesion size in the control group was small (167,582 ± 84,079 μm^2) (Fig. 3AB) and low in collagen content (4.13 ± 2.78 %) (Fig. 3CD), characteristic for early atherosclerotic lesions. Surprisingly, despite increased Treg levels and clear signs of immunosuppression in IL-2c treated groups, only separate oral oxLDL treatment significantly reduced plaque size compared to the PBS group (48.2%, 86675 ± 45311 μm^2, p = 0.0095) (Fig. 3AB). A trend towards decreased plaque size was observed in IL-2c treated group (27.5%, 121342 ± 49079 μm^2, p = 0.2255), and the IL-2c + oxLDL combined group (33.9%, 110527 ± 39027 μm^2, p = 0.1034) compared to control treated (Fig. 3AB). Plaque size of the oxLDL treated group was not significantly different from that of the IL-2c treatment group (p=0.4714) or the IL-2c + oxLDL treatment group (p = 0.7602) (Fig. 3AB). Collagen content of the lesions was very similar in all groups (Fig. 3CD), indicating that at this early point of lesion development IL-2c and oxLDL treatment did not affect plaque stability (p = > 0.9 for all comparisons). Neither treatment did significantly affect body weight or cholesterol levels (Fig. S2).

IL-2c treatment induces pro-atherogenic cell populations.

Since treatment with IL-2c induced high levels of Tregs, and appeared to have induced immune suppression, we assessed whether IL-2c administration induced expansion of cell populations that could explain why IL-2c treatment was not more effective than oxLDL treatment and did not provide an additional beneficial effect when combined with oral oxLDL

treatment. Among other cell populations, IL-2c treatment increased the NK^+ cell number, comprised of the atherogenic NKT and NK cell populations *(15–17)*, and increased the number of atherogenic eosinophils *(18, 19)* in the blood (Fig. 4A). IL2c treatment also increased conventional DC *(20)* and patrolling monocyte *(21)* levels in the spleen (Fig. 4A), albeit their role in atherosclerosis is less clear cut. Furthermore we performed a peritoneal lavage to assess cell numbers at the site of IL-2c administration, and found increased leukocyte numbers in the peritoneal cavity (figure). Since these cells were not $CD4^+$, also in the peritoneal cavity IL-2c administration had off-target effects. The off-target induction of pro-atherogenic cell populations by IL-2c are likely to have contributed to the inability of IL-2c treatment to improve the atheroprotective effect of oral tolerance induction towards oxLDL.

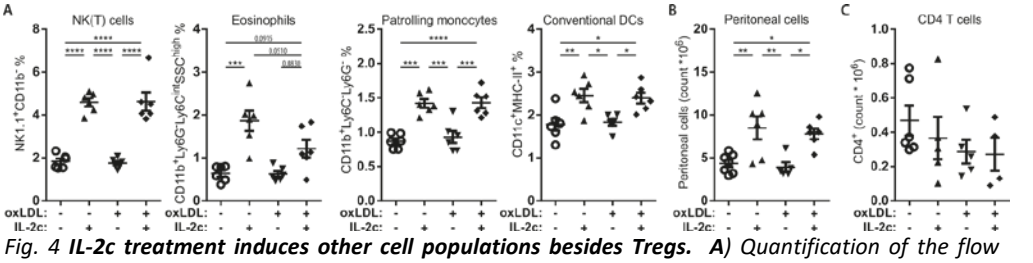

Fig. 4 ***IL-2c treatment induces other cell populations besides Tregs.*** ***A****) Quantification of the flow cytometric analysis of splenocyte populations increased by IL-2c treatment.* ***B****) Total count of cells obtained by peritoneal lavage.* ***C****) Quantification of the number of CD4 T cells in the peritoneal lavage fluid as determined by flow cytometric analysis. Expressed as mean ± SEM, one-way ANOVA with Tukey posttest, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

Discussion

Atherosclerosis is characterized by lipid accumulation and a chronic auto-immune like response in the vessel wall. Uptake of LDL, oxidized in the vessel wall, by macrophages is one of the hallmarks of atherosclerosis. Presentation of peptide epitopes from (ox)LDL derived ApoB100 by APCs is known to activate T cells leading to induction autoreactive (ox)LDL specific T cells. It is well established that the inflammatory response in atherosclerosis is skewed towards a Th1 response *(3, 22)*, resulting in high IFN-γ and TNF-α levels in the atherosclerotic lesion *(23)*.

$FoxP3^+$ regulatory T cells have been shown to be able to inhibit pathogenic Th1 skewed immune reactions in various experimental models of auto-immune disease *(4, 5)*, and therefore form an interesting treatment option. Antigen specificity is an important determinant for suppressive potential of Tregs *(9)*, however inducing and maintaining high levels of antigen specific Tregs has proven to be challenging *(24)*. In previous studies our lab has shown that oxLDL specific inducible Tregs and polyclonal Treg induction, induced through oral administration of oxLDL *(11)* and administration of IL-2c *(14)* respectively, effectively

inhibited atherosclerosis. Oral oxLDL administration led to increased Treg levels in MLN and spleen up to 2 weeks after final oxLDL administration but elevated Treg levels were not detected at sacrifice *(11)*. Therefore we hypothesized that maintaining or even increasing oxLDL specific Tregs with IL-2c could have an additional beneficial effect on atherosclerosis. Surprisingly only separate oxLDL administration significantly reduced atherosclerosis by 48.2%. A trend towards reduced plaque size of 27.5% and 33.9% was observed in the separate IL-2c, and IL-2c + oxLDL combination group respectively. Collagen content of the lesions was very similar in all groups, indicating that at this early point of lesion development, IL-2c and/or oxLDL treatment did not affect plaque stability. Similarly, treatment with IL-2c was only found to improve plaque stability in more advanced stages of atherosclerosis *(14)*.

Although in the IL-2c treated groups atherosclerosis was not significantly inhibited, we did observe enhanced $CD4^+FoxP3^+$ Treg levels in circulation, spleen, mesenteric lymph nodes, and mediastinal lymph nodes, indicating that IL-2c treatment had been effective. Furthermore lowered numbers of circulating immune cells were observed after IL-2c treatment, indicative that IL-2c treatment resulted in induction of functionally suppressive Tregs. In the group treated with only oxLDL we did not detect enhanced Treg levels in line with the aforementioned oxLDL tolerization study *(11)*. Oral oxLDL administration reduced white blood cell numbers compared to control treated similar to IL-2c treatment, indicative of ongoing immune suppression at sacrifice. This suggests that the suppressive capacity induced by oral oxLDL was not inferior to the suppressive capacity induced by IL-2c treatment, despite higher Treg levels in the IL-2c group than in the oxLDL group. The observation that antigen specific Tregs were found to have superior suppressive capacity compared to polyclonal Tregs *(9)* could explain this. The IL-2c mediated induction of atherogenic cell populations, including NK(T) cells *(15–17)* and eosinophils *(21)*, are likely to have inhibited the atheroprotective effect of IL-2c mediated Treg induction. In a previous study by Mahr *et al.*, treatment with JES6-1A12 – IL-2 complexes for 3 consecutive days doubled splenic cell numbers and enhanced NK cell, NKT cell, $FoxP3^-$ CD4 T cell, CD8 T cell, and B cell levels, 2 days after final injection, preventing engraftment of adoptively transferred bone marrow *(25)*. This suggests that over the course of the study the off-target effects of IL-2c treatment would have been even bigger than we observed at sacrifice and likely directly affected atherogenesis.

Furthermore it is possible that IL-2c administration interfered with the immune response induced by oral oxLDL administration which was likely dependent on oxLDL specific iTregs *(10, 11)*. Because of the plastic nature of Tregs *(26)*, IL-2c mediated induction of immune populations capable of secreting large amounts of cytokines, like eosinophils, could have affected the suppressive capacity and tissue distribution iTreg population. Furthermore we cannot exclude that IL-2c treatment expanded more specifically nTregs rather than iTregs in vivo, because these Treg populations cannot reliably be distinguished *(27)*, although in vitro both iTreg and nTreg populations are expanded by IL-2. Since adoptive transfer of nTregs led

to reduced iTregs *(28)*, it is possible that IL-2c mediated expansion of nTregs could have reduced iTreg numbers instead of expanding iTregs by IL-2c treatment. A difference in iTreg/nTreg balance would not only impact antigen specificity affecting immune suppression *(9)*, Treg subset specialization is dependent on environmental cues and is vital to suppress certain immune reactions *(29–31)*. Because of the different environmental origin of iTregs and nTregs, the function and tissue distribution of iTregs and nTregs are likely to be different. The observations that both iTregs and nTregs were needed to successfully suppress experimental colitis *(28)*, and that iTregs were more effective at inhibiting experimental asthma than nTregs *(32)*, indeed indicates functional differences between iTregs and nTregs. Therefore it would be interesting to assess whether iTregs and nTregs are similarly induced by IL-2c administration when reliable population specific markers are identified.

Treatment of autoimmune diseases using Tregs is among other things dependent on Treg antigen specificity and Treg quantity *(9, 24)*. Generating and maintaining high levels of antigen specific Tregs has proven to be challenging *(24)*. In this study we aimed to improve atheroprotection mediated by oral tolerance induction towards oxLDL through expanding and maintaining oral oxLDL induced Tregs by IL-2c administration in vivo. IL-2c administration did not improve efficacy of oral oxLDL administration despite enhancing Treg numbers.

Materials and Methods

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. LDLr$^{-/-}$ mice were originally purchased from Jackson Laboratories, and further bred in house. The mice were housed in groups of 2-4 animals in open cages with aspen bedding and were fed chow diet prior to the study. Mice were 10-12 weeks old at the start of the experiment and randomized based on age and weight (n=12).

Induction of atherosclerosis

LDLr$^{-/-}$ mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK) to induce atherosclerosis, and were provided with food and water at libitum. Directly after the first oral administration of oxLDL/PBS, mice were fed this WTD until sacrifice, 9 weeks later.

OxLDL tolerization

To induce oral tolerance mice were fasted for 4h with access to water, after which 2 mg of soybean trypsin inhibitor (Sigma) was administered orally to prevent antigen degradation. 10 minutes later mice orally received phosphate-buffered saline (PBS) or 30 µg of oxLDL. Induction of tolerance was repeated 3 times to a total of 4 injections in 8 days *(11)*. LDL was

isolated from blood plasma of a healthy volunteer by density gradient ultracentrifugation *(33)*, and oxidized with $CuSO_4$ (10 µM, 37°C, 20 hours) *(34)*.

Polyclonal Treg induction

To polyclonally induce Tregs mice intraperitoneally received IL-2c for 3 consecutive days, 2 days after final oral oxLDL administration. IL-2 complexes were generated by mixing recombinant IL-2 (1ug, Peprotech) with anti -IL-2 mAb (5 µg, JES6-1A12, R&D Systems) in sterile PBS and incubation at 37 °C for 30 minutes *(12–14)*.

Blood withdrawal and sacrifice

At intermediate timepoints blood was drawn by lateral tail cut and collected in EDTA coated capillary tubes (Microvette, Sarstedt). At the end-point of the study, mice were anesthetized by subcutaneous injection with a mix of ketamine (100 mg/mL), sedazine (25 mg/mL) and atropine (0.5 mg/mL), retro-orbitally exsanguinated, and perfused with PBS. TC levels were assessed with a commercially available kit (Roche) with precipath as an internal standard.

Histology

Hearts were cut in half and fixated in formalfix overnight. Hearts were incubated with OCT medium for at least an hour before cryosectioning (10 µm thick sections) and collected on Superfrost Plus™ Adhesion Microscope Slides (ThermoFisher). Neutral fats were stained with Oil Red O to assess lesion size in five subsequent sections of the heart at 70 µm intervals within the aortic root valve area. Collagen content of the lesions was quantified based on Masson's trichrome staining (Sigma-Aldrich). QWin software (Leica) was used to analyze the microscopic images (Leica DM2000).

Flow Cytometry

Cells from half of the animals (n=6 / treatment group) were stained extracellularly for 30 minutes in FACS buffer (PBS supplemented with 4% FCS) at 4° with the following antibodies (eBioscience/ThermoFisher Scientific): CD3-PerCP/Cy5.5 (145-2C11), CD4-PB (RM4-5), CD8-PE (53-6.7), FoxP3-PE (NRRF-30), CD25-FITC (PC61.5), CD44-FITC (IM7), CD62L-APC (MEL-14), CD19-PE (1D3), NK1.1-APC (PK136), CD11b-Pacific Blue (M1/70.15), Ly6C-PE (HK1.4), Ly6G-FITC (1A8), CD11c-FITC (N418), MHC-II- Pacific Blue (AF6-120.1). Cells were measured with a FACSCanto II (BD) flow cytometer. Analysis of flow cytometry data was performed with FlowJo software (Tree Star, inc.).

Statistical Analysis

Statistical analysis was performed with Graphpad Prism. Multiple group comparisons for a single timepoint were performed with a standard one-way ANOVA. For comparisons of multiple treatment groups with multiple timepoints, a standard two-way ANOVA was used.

The Tukey posttest was used to correct for multiple testing. A p-value < 0.05 was considered statistically significant.

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Supplementary figures

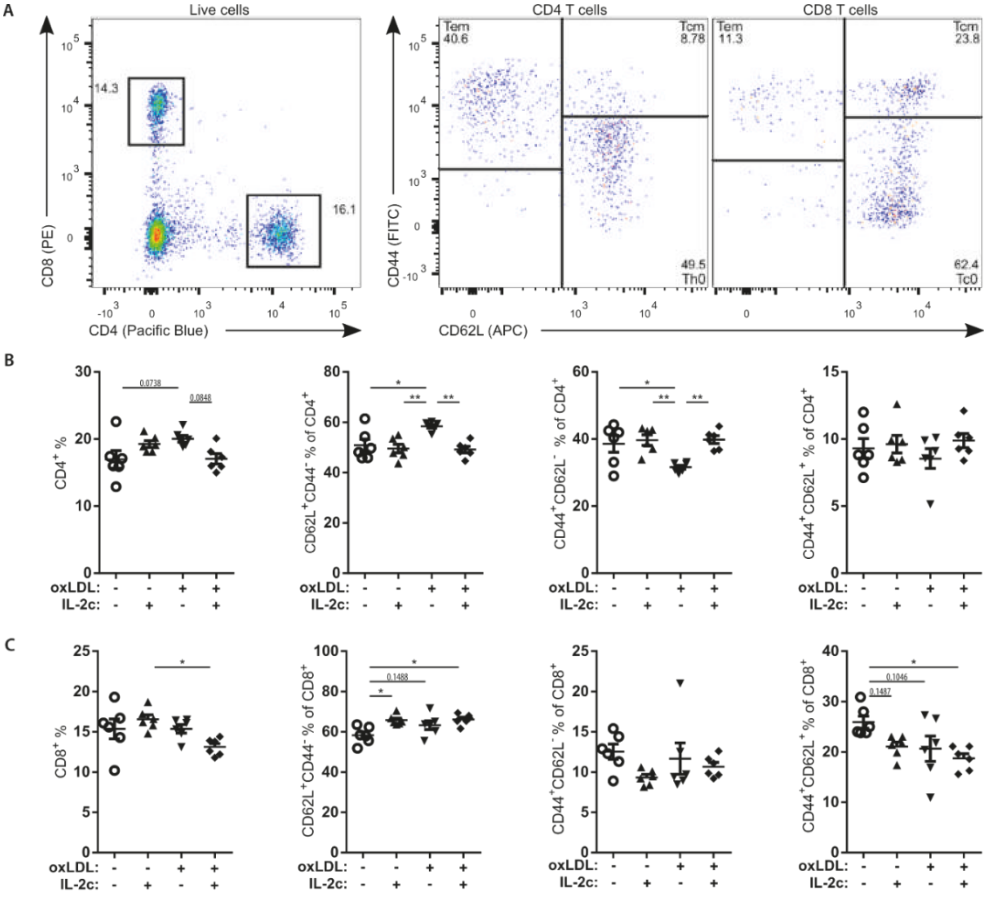

Fig. S1 ***Naïve and memory T cell subsets in the spleen. A****) Representative plots of the flow cytometric analysis of splenic naïve, Tem and Tcm of* ***B****) the CD4 T cell population and* ***C****) CD8 T cell population. Expressed as mean ± SEM, one-way ANOVA with Tukey posttest, * p < 0.05, ** p < 0.01.*

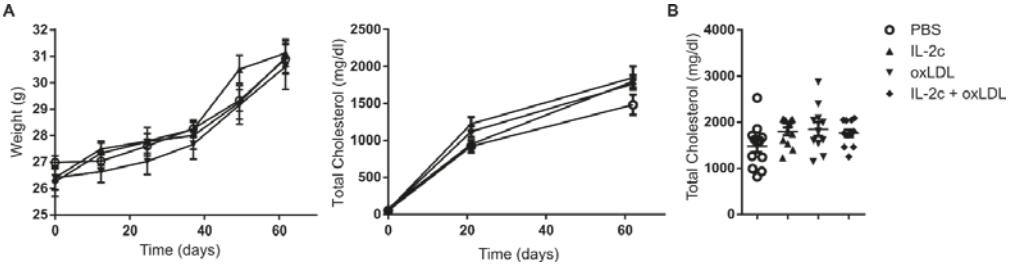

Fig. S2 ***Body weight and cholesterol are not affected by IL-2c or oxLDL treatment. A****) Over the course of the experiment body weight was monitored and cholesterol levels in blood plasma were determined.* ***B****) Cholesterol levels in blood plasma at sacrifice. Expressed as mean ± SEM,* ***A****) two-way ANOVA* ***B****) one-way ANOVA with Tukey posttest.*