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Chapter 4

Interference of the CD30-CD30L pathway reduces atherosclerosis development

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Abstract

Objective: Costimulatory molecules tightly control immune responses by providing positive signals that promote T cell activation or by transducing inhibitory signals that limit T cell responses. CD30 and CD30L are members of the TNF(R) superfamily and are involved in activation and proliferation of T and B cells, which have been implicated in the initiation and progression of atherosclerosis. In the present study, we thus aimed to determine the role of the CD30-CD30L pathway in the development of atherosclerosis.

Methods and Results: Western-type diet fed LDL receptor deficient (LDLr−/−) mice were treated with an anti-CD30L antibody for 8 weeks, which resulted in a reduction of atherosclerotic lesion formation in the aortic root by 35%. Reduced numbers of adventitial CD3+ T cells were found in anti-CD30L-treated mice, whereas no differences were observed in collagen and macrophage content of the atherosclerotic lesions. B cell and mast cell responses were also not affected upon anti-CD30L treatment. Interestingly, splenocyte proliferation was reduced with 53%, while T cell numbers were concomitantly reduced in anti-CD30L-treated mice compared with control mice. These data thus indicate that the CD30-CD30L pathway solely exerts its function via inhibition of T cell responses.

Conclusions: In the present study, we are the first to show that interruption of the CD30-CD30L pathway reduced initial atherosclerosis development by modulating T cell function.
**Introduction**

Atherosclerosis is considered a chronic autoimmune-like disease resulting from endothelial damage and subsequent cholesterol accumulation in the arterial wall.\(^1\) Within the atherosclerotic lesion a chronic inflammation manifests by a continuous infiltration of immune cells. Antigen presenting cells, such as dendritic cells (DCs) and macrophages, present antigens such as oxidized LDL-cholesterol to T cells, resulting in their activation. As a result both T cells and macrophages secrete cytokines, and more immune cells are attracted to the site of inflammation, which aggravates atherosclerotic lesion development.

T cell activation is tightly controlled by a complex network of costimulatory molecules, which can either provide positive or negative signals. Two large families of costimulatory molecules are known; the B7-CD28 superfamily, that includes CD28/CD80/CD86 and PD-1/PD-L1/2, and the TNF-TNFR superfamily, including OX40/OX40L and CD40/CD40L. Numerous studies have shown the crucial role of costimulatory molecules in the pathogenesis of atherosclerosis.\(^3\)\(^-\)\(^5\) Previously, our lab showed that interruption of the OX40-OX40L interaction using an OX40L-blocking antibody leads to a reduction in the initiation of atherosclerosis.\(^4\) Signaling of CD40-CD40L has been shown to affect advanced atherosclerosis, as lesions of CD154\(^{-/-}\)/ApoE\(^{-/-}\) mice contained fewer lipids, showed increased collagen levels and reduced numbers of immune cells such as T cells and macrophages, compared to ApoE\(^{-/-}\) mice.\(^3\) In addition, Gotsman et al. showed that the negative costimulatory pathway PD-1/PD-L1/2 downregulates proatherogenic T cell responses and atherosclerosis, since PD-L1/2 LDLr double knockout mice developed significantly larger atherosclerotic lesions compared with LDLr\(^{-/-}\) mice.\(^5\)

CD30 (TNFRSF8) and CD30L (TNFSF8, CD153) also belong to the TNF-TNFR superfamily. Whereas CD30 and CD30L are both present on activated B and T cells, CD30L is also expressed on other cell types, such as mature DCs, macrophages and mast cells. Triggering via CD30-CD30L has been shown to induce activation and proliferation of T cells.\(^6\)\(^-\)\(^7\) Furthermore, the CD30-CD30L pathway has been implicated as a major player in secondary humoral immune responses. CD30\(^{-/-}\) mice have impaired follicular germinal center responses and reduced secondary antibody responses.\(^8\)\(^,\)\(^9\) In addition, CD30L transgenic mice show increased numbers and activity of splenic germinal centers and have elevated serum antibody levels, such as IgG2b and IgE.\(^10\) The in vivo role of the interaction between CD30 and CD30L can be investigated using anti-CD30L antibodies, which interrupt the CD30-CD30L pathway. CD30 deficiency or treatment with a CD30L blocking antibody (RM153) significantly reduced airway inflammation in a murine asthma model\(^11\), while Blazar et al. showed that anti-CD30L prolongs survival of mice in graft versus host disease.\(^12\) Furthermore, administration of anti-CD30L completely suppressed the development of spontaneous/type I diabetes in NOD mice.\(^13\)

Although macrophages bearing CD30 have been identified in ruptured plaques of patients with coronary artery disease\(^14\), the involvement of the CD30-CD30L pathway in the development of atherosclerosis has not been investigated. In the present study,
we therefore investigated the role of the CD30-CD30L pathway in the initiation of atherosclerosis by treatment of LDLr\(^{-/-}\) mice with a CD30L blocking antibody.

**Methods**

**Animals**

Female LDLr deficient (LDLR\(^{-/-}\)) 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, Witham, Essex, UK). 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.

**CD30L expression during atherosclerosis**

After 2 weeks of Western-type, atherosclerosis was induced in LDLr\(^{-/-}\) mice by collar placement (2 mm long, inner diameter 0.3 mm) around both carotid arteries and continuous Western-type diet feeding.\(^{15}\) Mice were sacrificed at 0, 2, 4, 6, 8 and 10 weeks after collar placement and tissues were harvested after *in situ* perfusion using PBS. Carotid arteries and spleens (\(n=4-6\) per timepoint) 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, CA) using SYBR green technology. The following primer pairs were used: 5'-CCAAGAAGTCATGGGCCTACCTCCCAA-3' and 5'-GCAAACGATGAAAGTACGAGGGGAA-3' for CD30L, 5'-GAGCTCTTGTTGTT- GGAA-3' and 5'-CGAACATCTGTGAAGGCAAA-3' for CD4 and 5'-GTTGGGGCAGT TTAGAAG-3' and 5'-TGTGAAGCCAGGGACAGTG-3' for CD8. The following primers were used as endogenous references: 5'-GGACCCGAGAAGACCTCCTT -3' and 5' GCACATCAGAGAAATTTCAATGG-3' for acidic ribosomal phosphoprotein PO (36B4) and 5'-TTGGCTGAGATGCTGAAGGA-3' and 5'-AGCAAGTAGCCAGGACAGTG-3' for hypoxanthine phosphoribosyltransferase (HPRT). Protein levels of CD30L were determined in blood of LDLr\(^{-/-}\) mice fed a Western-type diet (\(n=5\)) or a chow diet (\(n=5\)) for 0, 4 and 8 weeks. Red blood cells were removed from blood using erythrocyte lysis buffer (0.15 M \(\text{NH}_4\)Cl, 10 mM NaHCO\(_3\), 0.1 mM EDTA, pH 7.3). Cells were stained with CD4 and CD30L and positive cells were determined with flow cytometry. All antibodies were purchased from eBioscience (Vienna). FACS analysis was performed on a FACS CantoII (Beckton Dickinson, Mountain View, CA). Data were analyzed using FACSDiva software (Beckton Dickinson).

**Functionality of the anti-CD30L antibody under hypercholesterolemic conditions**

To determine the effect of anti-CD30L on splenocyte proliferation, splenocytes from Western-type diet fed mice (\(n=3\)) were cultured for 24 hours in triplicate in a 96-wells round-bottom plate (\(2\times10^5\) cells/well, Greiner Bio-One) in RPMI 1640 supplemented
with L-Glutamine, 100 U/mL streptomycin/penicillin and 10% FCS. Splenocytes were cultured in the absence or presence of αCD3 and αCD28 (2 μg/mL) with anti-CD30L (0.1–10 μg/mL). Proliferation was measured by addition of $^3$H-thymidine (0.5 μCi/well, Amersham Biosciences, The Netherlands) 16 hours prior to cell lysis. The amount of $^3$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 αCD3/CD28 stimulation to triplicate cultures without stimulation.

Atherosclerosis

Atherosclerosis was induced in LDLr−/− mice by feeding a Western-type diet for 8 weeks. Mice were treated i.p. with 250 µg anti-mouse CD30L (RM153) (n=12) or sterile PBS (n=12) twice a week. Anti-mouse CD30L was kindly provided by Hideo Yagita and prepared as previously described. At week 8 mice were sacrificed and tissues were harvested after in situ perfusion using PBS. Tissues for histology were fixed in Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). 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, 4, 6 and 8 after start of the Western-type diet feeding. The concentrations of serum cholesterol were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). 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. To determine the number of adventitial T cells, a CD3 staining was performed using anti-mouse CD3 (1:100, SP7, Immunologic, The Netherlands). Lesion 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. Mast cells were visualized by staining with Chloro-Acetate Esterase (CAE, Sigma-Aldrich) according to manufacturer's protocol. Mast cell numbers and the extent of mast cell degranulation were assessed manually. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. 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. Spleen sections were stained with hematoxylin and eosin. Morphology was studied using a
Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK).

**Flow cytometry**
At sacrifice, blood, spleen and mediastinal lymph nodes (LN) were isolated (n=5 per group). Single cell suspensions were obtained by squeezing the organs through a 70 µm cell strainer. Red blood cells were removed from blood and splenocytes using erythrocyte lysis buffer (0.15 M NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA, pH 7.3). Cells were stained with CD4, CD8 and CD19 to detect T cells and B cells. For intracellular staining, cells were fixed and permeabilized according to manufacturer’s protocol (eBioscience, Vienna). Subsequently, the cells were stained for the transcription factors T-bet, GATA-3, RORγt or Foxp3 and the cytokines IFN-γ, IL-4, IL-5, IL-10 and IL-17. FACS analysis was performed as described above.

**Spleen cell proliferation**
At sacrifice, splenocytes (n=5 per group) were cultured for 72 hours in quintuplicate in a 96-wells round-bottom plate (2×10⁵ cells/well, Greiner Bio-One) in RPMI 1640 supplemented with L-Glutamine, 100 U/mL streptomycin/penicillin and 10% FCS. As a positive control cells were stimulated with αCD3 and αCD28 (2 μg/mL). Proliferation was measured as described above.

**Cytokine determination in serum and supernatant of splenocytes**
To detect IL-8 in serum an ELISA was performed according to manufacturer’s protocol (Biosource). Serum samples were 1:1 diluted in assay diluent and absorbance was detected at 450 nm. To detect IL-4 and IL-5 in the serum an ELISA was performed according to manufacturer’s protocol (eBioscience, Vienna). Serum samples were 1:1 diluted in assay diluent and absorbance was detected at 450 nm.

**Serum antibody detection**
IgM, IgG1, IgG2a and IgG2b levels against oxLDL were detected in serum using Abs recognizing mouse IgM, IgG1, IgG2a and IgG2b and HRP-labeled goat anti-rat Ig (BD Pharmingen). OxLDL (5 μg/mL) was dissolved in NaHCO₃/Na₂CO₃ buffer (pH 9.6) and was coated o/n onto a flat-bottom 96-well high binding plate (Corning, NY). Serum samples were 1:1 diluted in PBS and absorbance was detected at 450 nm. Total IgE in serum was determined by a mouse IgE quantitative ELISA according to manufacturer’s protocol (Bethyl Laboratories, Montgomery TX, USA).

**Statistical analysis**
All data are expressed as mean±SEM. An unpaired two-tailed student’s T-test was used to compare normally distributed data between two groups of animals. Probability values of $P<0.05$ were considered significant.
Results

**CD30L is upregulated in the initial stages of atherosclerosis**

Since we aimed to interrupt the CD30-CD30L pathway via blockade of CD30L, we first monitored the expression of CD30L in atherosclerotic lesions and the lymphoid tissue. While it is known that costimulatory molecules such as OX40 and PD-1 are upregulated in atherosclerotic lesions, it is unclear whether CD30L is regulated as well. LDLr−/− mice were fed a Western-type diet and slightly constrictive perivascular collars were placed around the carotid arteries, which leads to the development of shear stress induced atherosclerotic lesions at the proximal site of the collars. At 0-10 weeks after collar placement, RNA was isolated from the atherosclerotic lesions. As shown in Figure 1A, induction of atherosclerotic lesion development is reflected by an increase in CD68 expression over time (P<0.01). CD30L was also induced during lesion development and showed the highest expression after 2 weeks of Western-type diet feeding (P<0.05), coinciding with the influx of CD68 positive macrophages, but also with the influx of various immune cells, such as CD4 and CD8 positive T cells (Figure 1B). Parallel to the data on the lesion, CD30L mRNA levels in the spleen of LDLr−/− mice significantly increased during Western-type diet feeding (P<0.05, Figure 1C). In addition, we determined protein levels of CD30L and as shown in Figure 1D and E, CD4+ T cells expressing CD30L increase in blood of Western-type diet fed mice (P<0.05).

![Figure 1](image-url)

**Figure 1.** Relative mRNA levels of CD68 (A), CD30L, CD4 and CD8 in lesions of the carotid arteries were determined with RT-PCR (B). Relative mRNA levels of CD30L in spleens were determined with RT-PCR (C). At 0, 4 and 8 weeks CD30L expression was determined on CD4+ T cells in blood from LDLr−/− mice fed a Western-type diet (n=5) and a chow diet (n=5) by flow cytometry (D-E). Splenocytes were cultured with αCD3/CD28 in the presence of anti-CD30L (0.1-10 µg/mL) and proliferation was assessed by the amount of 3H-thymidine incorporation (F). * P<0.05, ** P<0.01, *** P<0.001, # P<0.01 for all celltypes.
Blockage of CD30L reduces proliferation of splenocytes from Western-type diet fed mice

To determine whether interruption of the CD30-CD30L pathway impairs T cell function of LDLr−/− mice fed a Western-type diet for 8 weeks ex vivo, we stimulated splenocytes with αCD3/CD28 in the presence or absence of RM153, a blocking CD30L antibody. This antibody was generated by Shimozato et al.16 and potently inhibits the binding of CD30 to CD30L and the proliferation of αCD3/CD28 activated T cells. As shown in Figure 1F, blockage of the CD30-CD30L pathway by using RM153 dose-dependently reduced splenocyte proliferation of Western-type diet fed LDLr−/− mice (P<0.01).

Impaired T cell numbers and function in anti-CD30L treated mice

RM153 has been shown to reduce murine autoimmune diabetes13, prolong survival of mice in a graft versus host disease model12, and together with OX40L blockade reduced autoimmune disease in Foxp3 deficient mice18 when administered i.p. twice a week during the experiments (200-500 μg/dose). To study the effect of CD30-CD30L interruption on T cells in vivo, we therefore treated LDLr−/− mice with 250 μg of RM153 twice a week, while the mice were fed a Western-type diet for 8 weeks. The relative number of CD4+ T cells (Figure 2A) in spleen and mediastinal heart lymph nodes of

Figure 2. Atherosclerosis was induced in LDLr−/− mice by feeding a Western-type diet for 8 weeks. Mice were treated with anti-mouse CD30L (RM153) (n=12) or sterile PBS (n=12) twice a week. At sacrifice, spleen and LN cells were isolated and stained for CD4 (A) and CD8 (B) and analyzed by flow cytometry (n=5 per group). T-bet, GATA-3, RORγt and Foxp3 expression in T cells from the spleen were also determined by flow cytometry (C). Splenocytes of control and anti-CD30L-treated mice (n=5 per group) were cultured in the presence or absence of CD3/CD28 stimulation. Proliferation was assessed by the amount of 3H-thymidine incorporation in dividing cells and is expressed as stimulation index (D). CD4+ T cells isolated from splenocytes were cultured for 72 hours in the presence of CD3/CD28 stimulation and stained for CD4 and Ki-67, a proliferation marker. Positive cells were assessed with flow cytometry (E). IFN-γ, IL-4, IL-5, IL-10 and IL-17 secretion by these CD3/CD28-stimulated CD4+ T cells were determined by flow cytometry (F). * P<0.05, ** P<0.01
anti-CD30L-treated mice was reduced compared with control mice ($P<0.05$), whereas CD8$^+$ T cell numbers were not affected by the anti-CD30L treatment (Figure 2B). Furthermore, the differentiation of naive CD4$^+$ T cells into Th1, Th2, Th17 or Treg cells was unaffected by anti-CD30L treatment (Figure 2C). To determine the proliferative capacity of T cells from anti-CD30L-treated mice in comparison with control mice, splenocytes from both groups were cultured for 72 hours in the presence of αCD3/αCD28 stimulation. A significant 53% decrease in splenocyte proliferation was observed in mice treated with anti-CD30L (stimulation index of $27.0\pm2.5$) compared to control mice (stimulation index of $58.1\pm6.1$, $P<0.01$, Figure 2D). As shown in Figure 2E, we demonstrate that CD4$^+$ T cells are the main effector cells, as CD4$^+$ T cells isolated from anti-CD30L-treated mice ($n=5$) showed an 52% reduction in αCD3/αCD28-mediated proliferation in comparison with CD4$^+$ T cells isolated from control mice ($n=5$, $P<0.05$), whereas CD8$^+$ T cell proliferation was unaffected (data not shown). In addition, we determined cytokine secretion by these CD4$^+$ T cells with flow cytometry; no significant differences in cytokine profiles between control and anti-CD30L-treated mice were observed (Figure 2F).

**Figure 3.** Anti-CD30L treatment reduces atherosclerosis development in LDLr$^{-/-}$ mice ($n=12$) fed a Western-type diet for 8 weeks in comparison with control treatment ($n=12$). 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 (A). The aortic arch and its main branch points were excised, fixed, and embedded in paraffin. Longitudinal sections of the aortic arch were stained with hematoxylin and eosin to analyze lesion extent (B). Corresponding sections of the aortic root on separate slides were stained for CD3 (red) to determine the number of infiltrating T cells (B). *$P<0.05$, ***$P<0.001$
**Interference in the CD30-CD30L pathway reduces the development of atherosclerosis**

To determine whether the anti-CD30L-mediated reduction in T cell responses affects atherosclerosis development, we determined atherosclerotic lesion sizes upon treatment with anti-CD30L. Figure 3A shows representative cross-sections of lesions in the three-valve area of the aortic root. We observed a significant 35% reduction in the aortic root lesion size in anti-CD30L-treated mice (4.3×10^5±0.3×10^5 µm^2) compared with control mice (6.5×10^5±0.5×10^5 µm^2, P<0.001). Treatment with rat IgG (isotype control for RM153) did not alter atherosclerotic lesion size in comparison with PBS treatment (data not shown). In addition, lesion formation in the aortic arch was reduced in anti-CD30L-treated mice compared with control mice (P=0.09, Figure 3B). During the experiment, anti-CD30L treatment did not affect body weight and total plasma cholesterol levels (data not shown). In line with reduced T cell percentages and splenocyte proliferation following anti-CD30L treatment, we observed a significant 31% reduction in the number of CD3^+ T cells within the adventitia of anti-CD30L-treated mice (71.9±5.3 T cells/section) compared with control mice (104.9±14.1 T cells/section, P<0.05, Figure 3C). This reduction in adventitial T cells in anti-CD30L-

![Figure 4](image)

**Figure 4.** No differences in lesion composition after anti-CD30L treatment. Sections of the aortic root were stained for collagen using Masson's trichrome staining. The percentage of collagen relative to the lesion size was determined. Furthermore, relative macrophage content was determined with a MOMA-2 staining and quantified. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area.
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treated mice was not a consequence of impaired migration and adhesion of CD4+ and CD8+ T cells (data not shown). In addition, we determined T cell subsets and cytokine expression locally in the plaque with qPCR. In line with our previous findings, we did not find any differences in T cell subsets and their cytokines (data not shown).

**Anti-CD30L treatment does not affect lesion composition**

With respect to the composition of the lesion (Figure 4), no differences were found in lesion collagen content (18.6±1.3% vs. 19.3±1.3%) and macrophage content (46.2±3.6% vs. 49.7±2.7%) between anti-CD30L-treated mice and control mice, respectively. In addition, no differences in necrotic cores were observed between anti-CD30L-treated mice (49.9±3.7%) and control mice (54.8±3.2%).

**Humoral responses in anti-CD30L treated mice are not affected**

The CD30-CD30L pathway is described to be involved in germinal center responses and secondary antibody responses.8, 9 However, in our study both the percentage of B cells in blood and spleen (Figure 5A) and the levels of oxLDL-specific IgM, IgG1, IgG2a and IgG2b in serum did not differ between control mice and anti-CD30L-treated mice (Figure 5B). Furthermore, we did not observe any differences in spleen morphology in anti-CD30L-treated mice compared with control mice (Figure 5C).

**Anti-CD30L treatment does not inhibit mast cells**

Treatment with anti-CD30L significantly reduced serum IgE levels in a murine asthma model11 and several other studies reported decreased IgE levels following CD30-CD30L pathway interruption.8, 9 In our study, a trend towards lowered serum IgE was observed in anti-CD30L-treated mice (363±90 ng/mL) compared with control mice (609±132 ng/mL, Figure 6A). Since IgE is a common mast cell activator and mast cells can aggravate atherosclerosis19, we analyzed the number of adventitial mast cells. However, the numbers of activated mast cells and total number of mast cells (MC, Figure 6B) in the aortic root remained unaffected by anti-CD30L treatment (activated: 13.8±1.9 MC/mm² and total: 29.4±2.4 MC/mm² versus control
treatment; activated: $12.5\pm1.6$ MC/mm$^2$ and total: $28.1\pm3.0$ MC/mm$^2$). In addition, the percentage of activated mast cells did not differ (control: $44.2\pm2.8\%$ versus anti-CD30L: $45.1\pm4.5\%$, Figure 6C). Interestingly, CD30-CD30L signaling can induce degranulation-independent mast cell activation via the secretion of IL-8. However, anti-CD30L treatment also did not influence serum levels of KC, the mouse analogue of IL-8 (Figure 6D).

**Discussion**

Optimal T cell activation is regulated by costimulatory signals and modulation of these signals provides a very promising therapeutic strategy to improve the outcome of autoimmune diseases. T cells play an important role in atherosclerosis and whereas the CD30-CD30L pathway has been implicated in various autoimmune diseases, such as asthma, GVHD and type I diabetes, no studies describe a role for the CD30-CD30L axis in atherosclerosis.

In the present study, we found that CD30L expression within the atherosclerotic lesion highly correlated with CD4$^+$ and CD8$^+$ T cell infiltration and that CD30L expression in the spleen was upregulated after 8 and 12 weeks of Western-type diet feeding. In addition, CD4$^+$ T cells expressing CD30L are increased in Western-type diet-fed mice, suggesting a pro-atherogenic role of the CD30-CD30L pathway. We therefore chose to intervene with the CD30-CD30L pathway during the development of atherosclerosis as a therapeutic approach. Interruption of CD30-CD30L by using the CD30L blocking antibody RM153 reduced atherosclerosis development in LDLr$^{-/-}$ mice with 35% and coincided with a 31% reduction in adventitial T cell numbers. CD30L signaling is reported to enhance proliferation of T cells and blocking CD30L therefore
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diminishes proliferation of T cells as shown by several studies.\textsuperscript{11, 13, 21} We showed that anti-CD30L inhibited the proliferation of splenocytes from Western-type diet fed mice \textit{ex vivo}. Interruption of the CD30-CD30L interaction also potently reduced T cell numbers \textit{in vivo}, as we found reduced percentages of CD4\textsuperscript{+} T cells in the spleen and LN of anti-CD30L-treated mice compared with control mice. Furthermore, a 56\% reduction in splenocyte proliferation was observed following anti-CD30L treatment, which was particularly due to reduced CD4\textsuperscript{+} T cell proliferation. Additionally, we show that anti-CD30L does not interfere with the migration and adhesion capacity of T cells. The role of T cells in atherosclerosis has been established already in several studies.\textsuperscript{22-24} CD4\textsuperscript{+} T cells can be subdivided in several subclasses; Th1, Th2, Th17 and Treg cells. However, no differences were found in T cell subsets following anti-CD30L treatment, which indicates that interruption of the CD30-CD30L pathway under hypercholesterolemic conditions impairs T cell numbers and function but does not influence their differentiation. In line with our findings, Chakrabarty et al. showed that anti-CD30L (RM153) treatment reduced T cell proliferation in response to islet antigens and markedly inhibited the development of spontaneous diabetes in NOD mice. Furthermore, they showed that anti-CD30L inhibited the incidence of diabetes in NOD-SCID mice after diabetogenic T cell transfer.\textsuperscript{13}

Signaling via CD30-CD30L may also affect humoral responses. Mice deficient in CD30 mice show reduced levels of several immunoglobulins, such as IgG1, IgG2c and IgE.\textsuperscript{8} CD30L Tg mice show increased numbers and activity of splenic germinal centers and elevated basal serum concentrations of IgG2a, IgG2b and IgE.\textsuperscript{10} In addition, Shanebeck et al. showed that mouse splenic B cells stimulated via CD30L induced increased amounts of a number of immunoglobulins, such as IgG1 and IgE.\textsuperscript{9} However, under hypercholesterolemic conditions, we did not find any significant difference in immunoglobulin production or spleen morphology in anti-CD30L-treated mice compared with control mice.

In a murine asthma model, CD30 deficiency or treatment with anti-CD30L significantly reduced airway inflammation, splenocyte proliferation, Th2 responses and serum IgE levels.\textsuperscript{11} Whereas in the present study we also observed a reduction in splenocyte proliferation and a trend towards reduced serum IgE, we did not observe reduced Th2 responses as shown by GATA-3 expressing CD4\textsuperscript{+} cells and serum IL-4 and IL-5 levels (data not shown). IgE may induce activation of mast cells, which are correlated with the incidence of plaque rupture and erosion\textsuperscript{25} and also play a crucial role in plaque progression and destabilization \textit{in vivo}.\textsuperscript{19} Furthermore, mast cells are the predominant CD30L-expressing cells in Hodgkin’s disease, which are involved in tumorigenesis and tumor progression.\textsuperscript{26} However, despite a reduction in serum IgE, anti-CD30L-treated mice did not have reduced numbers of mast cells or activated mast cells as shown by adventitial mast cells and IL-8 release.

In conclusion, we are the first to demonstrate that anti-CD30L treatment inhibits plaque development in LDL receptor deficient mice independent of plasma cholesterol levels and lesional macrophage and collagen content. Given the profound inhibition
of anti-CD30L treatment on T cell proliferation and activation, we propose that anti-CD30L treatment, at least partly, exerts its protective effects by modulating this process. These data thus identify anti-CD30L treatment as a novel therapeutic modality in the inhibition of atherosclerotic lesion development and the prevention of acute cardiovascular syndromes.

References