Chapter 6

Vaccination Against CD99 Inhibits Atherogenesis in LDL Receptor Deficient Mice

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Abstract

Murine CD99 was recently found to be expressed on leukocytes and endothelial cells where it is concentrated at inter-endothelial contacts. Blockade of CD99 by specific antibodies inhibits leukocyte extravasation to inflamed sites in vivo. We constructed a DNA vaccine against CD99 by cloning the extracellular domain of murine CD99 into pcDNA3. Vaccination was performed by oral administration of attenuated Salmonella typhimurium transformed with pcDNA3-CD99. This vaccination results in a CD8 mediated cytotoxic response targeting cells transfected with CD99 and the subsequent reduction of CD99 expressing cells. We showed that CD99 is expressed on vascular endothelium overlying atherosclerotic plaques and found that CD99 expression is up-regulated during Western type diet feeding. CD99 vaccination induced the formation of CD8 positive T cells that were cytotoxic against cells transfected with pcDNA3-CD99. Activation of CD8+ T cells was demonstrated by a 30% increase in CD8+CD69+ double positive T cells in spleen and mediastinal lymph nodes. Furthermore, lymphocytes isolated from CD99 vaccinated mice specifically lysed CD99 expressing cells. More importantly, vaccination against CD99 attenuated atherosclerotic lesion formation in the aortic valve leaflets by 38% and in the carotid artery by 69% as compared to mice that were vaccinated with a control vector. Furthermore, a lower number of cells were found in atherosclerotic lesions implying that fewer leukocytes were recruited to these sites. These observations were accompanied by a decrease in CD99 expression on leukocytes. We conclude that vaccination against CD99 decreases atherogenesis by the selective removal of CD99 expressing cells, which could reduce leukocyte recruitment into atherosclerotic lesions and attenuate atherogenesis.
Introduction

Atherosclerosis is a chronic inflammatory disorder of the large arteries and the prominent cause of death in the western world. Recruitment of leukocytes into the vessel wall is driving the initiation and progression of atherosclerotic plaque formation. The atherosclerotic process is orchestrated by several groups of molecules on both the activated endothelium as well as on the infiltrating leukocytes. Activation of the endothelium due to low shear stress and/or local damage results in the upregulation of leukocyte adhesion molecules and secretion of chemoattractants.

Recruitment of blood leukocytes to sites of inflammation is initiated by interaction of P- and E-selectins that are upregulated on activated endothelium with their sialylated ligands on leukocytes. This transient interaction slows down leukocytes from the flowing blood resulting in rolling behavior on the endothelial surface. After activation, leukocytes come to a firm arrest by binding to endothelial cell adhesion molecules. In experimental animals, endothelial cells in the arteries express in particular vascular cell-adhesion molecule-1 (VCAM-1) resulting in the predominant recruitment of monocytes and lymphocytes to atherosclerotic regions. Blocking this interaction results in attenuation atherosclerosis and other inflammatory disorders.

The last step in leukocyte transendothelial migration that is also known as diapedesis occurs largely through junctions between adjacent endothelial cells. Indeed a number of cell adhesion molecules locate at endothelial cell junction have been implicated in this process. These molecules include platelet-endothelial cell adhesion molecule (PECAM-1), members of the junctional adhesion molecule (JAM) family, CD99, ESAM and ICAM-2.

CD99, a long known leukocyte membrane protein that was initially described to function in T cell activation and lymphocyte aggregation, was only recently reported to participate in the transmigration of human monocytes through cultured endothelial cells. Schenkel et al. showed that CD99 is expressed at endothelial cell contacts and that a monoclonal antibody against CD99 inhibits diapedesis of human monocytes across a monolayer of cultured endothelial cells by 90%. The mouse counterpart was identified and cloned few years later by Bixel et al. Antibodies against mouse CD99 efficiently block migration of lymphocytes and neutrophils through a monolayer of cultured endothelioma cells. More importantly, these antibodies block recruitment of in vivo-activated T cells into inflamed skin and inhibit neutrophil extravasation to inflamed sites in two inflammatory mouse models. As the recruitment of both monocytes and T cells contribute to the initiation and progression of the atherosclerotic plaque, blockade of their transmigration may provide protection against atherosclerosis.

In the present study we assessed the role of CD99 in the process of atherosclerosis by vaccinating atherosclerosis prone mice against CD99. Oral administration of a DNA vaccine encoding the extracellular domain of murine
CD99 by attenuated \textit{Salmonella typhimurium} evoked a T-cell mediated immune response against cells expressing CD99 in mice. We demonstrate that vaccination of mice against CD99 generates antigen-specific CD8\textsuperscript{+} T cells that target 3T3 fibroblasts transfected with CD99. Vaccination of LDL receptor deficient mice against CD99 significantly reduced the formation of atherosclerotic lesions in the aortic valve leaflet and the carotid artery as compared to mice vaccinated with the vector alone.

\textbf{Material and methods}

\textit{Construction of the vaccine}

The cDNA encoding the extracellular part of murine CD99 (BC019482), obtained by HindIII/EcoRI digestion of the Fc fusion plasmid earlier described by Bixel et al\textsuperscript{17} was cloned into pcDNA3 plasmid (Invitrogen California). This plasmid was electroporated into S. typhimurium Aro/A (strain SL7207) bacteria as previously described\textsuperscript{25}. Female LDL receptor deficient mice, aged 10-12 weeks were immunized by oral administration of $1\times10^8$ cfu S. typhimurium transformed with either pcDNA3-CD99 or pcDNA3 empty (control) 3 times with 2 week intervals.

\textit{Induction of CD8\textsuperscript{+} specific cytotoxic T cells}

Spleens were isolated from control and CD99 vaccinated mice and their capacity to lyse CD99 expressing cells was determined in the following assay. Murine fibroblasts (3T3) were cultured in a 24 wells plate and incubated with DMEM containing 10% Fetal bovine serum, 2mmol/L L-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere (5% CO\textsubscript{2}) at 37 °C. Cells were co-transfected with pcDNA3-CD99 (encoding for the extracellular part of murine CD99) and pEGFP-N1 vector using ExGen500 (Fermentas, Germany) as transfection reagent according to the manufacturers protocol. After 24 hours the transfected cells were incubated with splenocytes derived from control or CD99 vaccinated mice (2 weeks after last vaccination) for 24 hours. Non-adherent cells were washed away with PBS and specific lysis of the cells expressing CD99 was analyzed on a FACS\textsuperscript{calibur} (BD Biosciences, The Netherlands) by quantifying the percentage of eGFP positive cells. The percentage of specifically lysed of cells transfected with CD99 was plotted as percentage of eGFP positive cells in absence of splenocytes.

\textit{Atherosclerosis experiments and histology}

Female LDLr deficient (LDLr\textsuperscript{-/-}) mice (n=12 per group), 8 weeks old, were vaccinated with pcDNA3 empty of pcDNA3-CD99 3 times in two week intervals. After vaccination, mice were placed on a Western type diet containing 0.25 % cholesterol and 15% cocoa butter two weeks before collar placement. Silastic collars (0.3 mm inside diameter, Dow Corning, Midland, USA) were
placed around the carotid artery to induce atherosclerosis. After 8 weeks of western type diet, mice were sacrificed and organs were harvested. Cryosections from carotid artery (5 μm) were stained with hematoxilin and eosin. Site of maximal stenosis was used for morphometric assessment using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK). Cryostat sections of the aortic root (10 μm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Expression of CD99 was visualized using an affinity-purified anti-mouse CD99 polyclonal antibody. As secondary antibody, biotinylated goat anti-rabbit (DAKO, the Netherlands) was used in combination with streptABC complex (DAKO), with Nova Red as enzyme substrate (Vector Laboratories).

**Real time PCR assays**
Total RNA was isolated from aortic arch and collar induced atherosclerotic plaques using the guanidium isothiocyanate (GTC) method. Purified RNA was DNase treated (DNase I, 10 units/μg of total RNA) and reverse transcribed (RevertAid M-MuLV reverse transcriptase) according to manufacturers protocol. Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using SYBR Green technology. (PCR primers see appendix 1)

**Flow cytometry**
Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) according to manufacturer’s protocol. Cell suspensions from spleen, blood, lymph nodes draining from the aortic arch and peritoneal cavity were stained for surface markers (0.20 μg Ab/300.000 cells) and subsequently subjected to flow cytometric analysis. Antibodies were purchased from eBiosciences (Immunoscource, Belgium). All data were acquired on a FACS-Calibur and were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

**Statistical analysis**
Values are expressed as mean ± SEM unless indicated otherwise. Two-tailed student’s T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was applied to analyze not-normally distributed data. A probability value of \(P<0.05\) was considered to be significant for both tests.
Vaccination against CD99 Inhibits Atherosclerosis

Figure 1: CD99 is expressed by vascular endothelial cells in an atherosclerotic vessel and is upregulated after Western type diet feeding. Expression of CD99 was visualized using an affinity purified polyclonal antibody against CD99. Representative pictures are shown in figure 1A and at higher magnification in 1B. mRNA was isolated from the aortic arch of LDLr−/− mice using the GTC method. mRNA levels for CD99 were determined for mice receiving Chow or Western type diet and levels were related to mRNA levels of 36B4 and HPRT. Statistical analysis was done by an unpaired Student t test (week 0) (*P < 0.05, n=6 per time point).

Results

CD99 expression is upregulated on vascular endothelium overlying atherosclerotic plaques

Several molecules expressed at endothelial cell junctions such as JAM-A have been associated with atherosclerosis, and are upregulated in atherosclerosis prone sites of the vasculature26. We stained cryosections of collar induced atherosclerotic plaques in LDLr−/− mice and observed a profound expression of CD99 by vascular endothelial cells covering the plaque. A representative picture is shown in figure 1A-B. To further investigate the regulation of this expression, we isolated the aortic arch of LDLr−/− mice after 2 weeks of western type diet and compared CD99 mRNA levels of mice that received a chow diet. We observed a 1.5-fold increase in CD99 expression upon western type diet feeding (Fig. 1C). This increase was not accompanied by elevated expression of T cell or macrophage markers, indicating that the cellular composition of the aorta was not changed due to an influx of leukocytes.

Vaccination against CD99 induces T cell-mediated lysis of cells expressing CD99

We developed a vaccination strategy based on the induction of CD99 specific cytotoxic T cells that specifically lyse cells that express high levels of CD99 as recently shown for FLK-127. To test the functionality and specificity of this vaccination protocol we determined the antigen-specific cytotoxicity of splenic CD8+ T cells derived from mice vaccinated against CD99. Therefore 3T3 fibroblasts were co-transfected with pcDNA-CD99 and EGFP-N1, which allows the identification of CD99 expressing cells by their simultaneous expression of GFP using flow cytometry. Splenocytes were isolated from control and CD99 vaccinated mice and were co-cultured for 24 hours with 3T3 fibroblasts that
Vaccination against CD99 generates cytotoxic cells that specifically lyse cells expressing CD99. Murine fibroblast CD99 transfected with pcDNA-CD99 and eGFP were exposed to spleen cells isolated from control and CD99 vaccinated mice. After 24 hours, the percentage of eGFP positive cells was quantified using flow cytometry. Representative histograms are shown for spleen cells isolated from mice vaccinated with control vector (black line) or with pcDNA-CD99 (gray line). Specific lysis of 3T3 fibroblasts that present CD99 peptides on MHC class I was determined by quantifying the percentage of GFP positive cells compared to controls were no splenocytes were added. The percentage of GFP positive cells was significantly decreased when splenocytes were used derived from animals that were vaccinated against CD99 (right panel, p=0.01, n=7 per group).

were transfected with CD99. Specific lysis of CD99 expressing cells was determined by quantifying the percentage of eGFP positive cells compared to controls were no splenocytes were added. The percentage of CD99 expressing cells was significantly decreased after incubation with splenocytes from CD99 vaccinated mice (Fig. 2, 86.6 ± 10.2 % vs. 46.3 ± 11.8), whereas no decrease was observed splenocytes from control vaccinated mice were added (Fig. 2). This observation indicates that CD99 vaccination induces the formation of T cells that specifically lyse CD99 expressing cells.

Vaccination against CD99 inhibits atherosclerotic lesion formation in LDLr⁻/⁻ mice

After showing the effectiveness of our vaccination strategy, we investigated the effect of CD99 vaccination on atherosclerotic lesion formation. Female LDLr⁻/⁻ mice were vaccinated with pcDNA3-empty (control) or pcDNA-CD99 3 times at a 2 week time interval. Mice were subsequently placed on a Western type diet (0.25% cholesterol) and two weeks later mice were equipped with peri-carotid collars. Six weeks later, mice were sacrificed and the atherosclerotic lesion burden was determined in the carotid artery and aortic root. Representative photomicrographs of cross sections of the aortic root of control and treated mice are shown in figure 3A and B respectively. The mean lesion area in mice vaccinated against CD99 was decreased by 38% compared to control animals (Fig. 3C, 5.25 ± 0.68x10⁵ μm² vs. 3.37 ± 0.57x10⁵ μm²).
Middle panels show representative sections of the carotid artery of control (Fig. 3D) and CD99 vaccinated (Fig. 3E) mice. Lesion size was significantly decreased by 69% after CD99 vaccination (Fig. 3F). 2.4 ± 0.7 x10^4 μm^2 vs. 7.71 ± 4.0x10^3 μm^2, in Fig. 3F. 24.15 ± 7.7x10^3 μm^2 is shown, which is accompanied by an 83% decrease in intima/media ratio (Fig. 3G) and a 78% diminished degree of lumen stenosis (Fig. 3H).

In addition, the number of cells that had infiltrated the carotid plaques of control and CD99 vaccinated mice was determined by quantifying the number of nuclei per square micrometer (Fig. 3I). Vaccination against CD99 resulted in a 35% decrease in number of nuclei per area in atherosclerotic plaques from CD99 vaccinated mice compared to controls, indicating that a lower number of cells have infiltrated the vessel wall.

Figure 3: Atherosclerotic lesion formation is significantly inhibited in mice vaccinated against CD99. Representative photomicrographs of oil red O stained cross sections of the aortic root of control treated mice (A) and vaccinated mice (B) are shown. A significant reduction in plaque size was found in CD99 vaccinated mice compared to control mice (C). Lower panels demonstrate representative pictures of the carotid artery of control (D) and CD99 vaccinated animals (E). A significant decrease (p=0.02) was observed in carotid plaque area after vaccination against CD99 (F). This decrease in lesion formation is accompanied by a significant decrease in lumen stenosis (G) and intima/lumen ratio (H). Number of cells that infiltrated into the plaque area was decreased in mice vaccinated against CD99 compared to controls (I). Error bars represent SEM, n = 12 per group. Statistical analysis was done by a Mann Whitney test, *=p<0.05.
Vaccination activates CD8$^+$ T cell in LDLr$^{-/-}$ mice

The vaccination strategy is based on the induction of specific CD8$^+$ T cells that induce apoptosis of cells that express high levels of CD99 via MHC class I. To test whether our vaccine had an effect on the activation state of the CD8$^+$ T cell population during atherogenesis, we isolated lymphocytes from spleen and lymph nodes of control and CD99 vaccinated mice at the end of the atherosclerosis experiment. The activation state of the T cell population was determined by flow cytometry using CD69 as an early marker for T cell activation. CD8$^+$ cells were gated and the percentage of CD8$^+$ T cells expressing CD69 was determined (Fig. 4, upper panels). A significant increase of CD8$^+$CD69$^+$ double positive cells within the CD8$^+$ T cell population was observed in spleen (43.4 ± 3.9% vs. 59.3 ± 4.5%) and in lymph nodes draining the aortic arch (4.8 ± 0.2% vs. 5.9 ± 0.1%, Fig. 4, lower panels). This observation was restricted to the CD8$^+$ T cell population, since the activation state of CD4$^+$ T cells was not changed as shown by measuring the percentage of CD4$^+$/CD69$^+$ double positive cells among the CD4$^+$ T cell population (data not shown). These findings indicate that vaccination against CD99 specifically activates CD8$^+$ T cell subsets, but not CD4$^+$ T cells.

Vaccination against CD99 decreases expression of CD99 on CD4$^+$ T cells and F4/80$^+$ macrophages

CD99 is expressed on most leukocytes, including lymphocytes and monocytes derived from peripheral blood17-18. To determine whether vaccination against CD99 would affect CD99 expression levels on leukocytes, we stained CD4$^+$ T cells and F4/80$^+$ macrophages with anti-CD99 antibodies and determined CD99 expression on these cells by flow cytometry. The percentage CD99 positive cells within the CD4$^+$ and F4/80$^+$ cell population is shown in Figure 5. The expression of CD99 on CD4$^+$ T cells was significantly decreased upon vaccination against CD99 (Fig. 5, left panel; 37.6 ± 2.7 % vs. 28.5 ± 1.6 %). In addition, the percentage of CD99 expressing cells within the macrophage population was also decreased by 31% after vaccination (Fig. 5, right panel; 24.5 ± 1.7 vs. 16.9 ± 2.7). The percentage of macrophages and CD4$^+$ T cells was not changed compared to control vaccinated mice as determined by FACS (data not shown).
Figure 4: Vaccination against CD99 activates CD8+ T cells. Activation of CD8+ cells was analyzed by flow cytometry. CD8+ cells were gated and expression of the early T cell activation antigen CD69 was determined (upper panels). The percentage of CD8+CD69+ double positive cells was quantified within the CD8+ cell population. In CD8+ T cells derived from spleen and lymph nodes, vaccination against CD99 resulted in a significant up-regulation of CD69. White bars represent controls, black bars represent CD99 vaccinated mice, error bars display SEM, n=6 per group, *:p<0.05)

Discussion

Human CD99 was recently reported to be involved in migration of human monocytes through a monolayer of cultured endothelial cells\textsuperscript{19}. Cloning of mouse CD99 by Bixel et al. opened the possibility to examine the physiological relevance and role of CD99 in mouse models of inflammatory diseases\textsuperscript{17}. Recruitment of leukocytes into the vessel wall is a hallmark of atherosclerosis and several facilitators of this process have been identified. A prominent role for cell adhesion molecules that are expressed at cell contacts of endothelial cells was reported, i.e. increased expression of PECAM-1 was found to be associated with atherosclerosis-prone regions of the vessel wall and blocking anti-PECAM-1 antibodies reduced leukocyte migration into atherosclerotic plaques\textsuperscript{15,28,29}. Mice deficient in JAM-A, another junctional cell adhesion molecule that participates in leukocyte extravasation, were shown to have reduce neointima formation on apoE background\textsuperscript{30}. We hypothesized that CD99 may also be involved in the recruitment of leukocytes into atherosclerotic plaques. A lower amount of CD99 expressing cells both endothelial and leukocyte could possibly slow down
leukocyte recruitment to inflamed areas of the artery and could attenuate the formation of atherosclerotic plaques. First we demonstrate that CD99 is expressed on vascular endothelium overlying vessels with atherosclerotic lesions. Second, Western type diet feeding of atherosclerosis prone LDLr⁻/⁻ mice induced a 1.5-fold upregulation of CD99 mRNA levels in the aortic arch after 2 weeks of diet. This upregulation was not due to CD99 expressing leukocytes infiltrating the vessel wall, as CD99 expression was already increased before an increase in CD68⁺ or CD4⁺ cells was observed. This indicates that no additional CD99 expressing leukocytes were recruited and that the cellular composition of the examined vessels after Western type diet or chow diet feeding was not changed (data not shown).

To test the hypothesis that CD99 is involved in leukocyte recruitment to sites in the vessel wall where atherosclerotic lesions are formed we developed a DNA vaccination strategy to immunize LDL receptor deficient mice against CD99. An attenuated *S. typhimurium* strain was transformed with a pcDNA3 vector encoding the extracellular domain of murine CD99. The *S. typhimurium* carrier is taken up by M cells and processed in the Peyers patches of the gastrointestinal tract. The bacteria are taken up by antigen presenting cells; the vector is transcribed and translated into protein. Processing the protein by the antigen presenting cells leads to presentation of peptide fragments in a complex with MHC class I. These MHC class I presented peptides activate specific cytotoxic natural killer cells and CD8⁺ T cells that clonally expand and break peripheral tolerance against CD99. This vaccination protocol therefore generates T cells that specifically target cells that express high levels of CD99 via MHC-I. The
DNA vaccination strategy has been successfully validated for other proteins in animal models for tumor growth and cancer therapy and artherosclerosis\textsuperscript{25,27,31}. Here we show that vaccination against murine CD99 of LDLr\textsuperscript{-/-} mice specifically activates CD\textsuperscript{8+} T cells during atherogenesis that are able to target CD99 expressing cells. Based on these observations we conclude that our vaccination strategy generates cytotoxic CD\textsuperscript{8+} T cells that break through immune tolerance against a self antigen and kill cells that express high levels of CD99.

Once we had validated our vaccination protocol, we induced atherogenesis in LDLr\textsuperscript{-/-} mice that were vaccinated with control (pcDNA3-empty) or CD99 (pcDNA3-CD99). This approach has been shown to reduce plaque formation in mice that were vaccinated against vascular endothelial growth factor (VEGF) receptor 2 (flk-1), which is expressed by activated endothelial cells that cover the atherosclerotic plaque\textsuperscript{25}.

The importance of CD99 for leukocytes diapedesis was first shown by Schenkel et al.\textsuperscript{19} for human CD99 and later by Bixel for mouse CD99\textsuperscript{18}. For human monocytes and neutrophils and for mouse lymphocytes is has been shown that the separate blockade of both endothelial or leukocyte CD99 by specific antibodies is sufficient to inhibit leukocyte transmigration\textsuperscript{17}. Lower CD99 expression levels or a reduced functionality of CD99 on leukocytes could possibly interfere with leukocyte extravasation and reduce their migration through the vascular endothelium. We have shown that vaccination against CD99 significantly decreases CD99 expression on CD4\textsuperscript{+} T cells and macrophages.

It was suggested for human CD99 that homotypic interaction of CD99 on leukocytes with CD99 on endothelial cells is important for leukocyte diapedesis through endothelial contacts. A lower expression level of CD99 on leukocytes and/or endothelial cells could possibly attenuate plaque formation due to a reduced transmigration rate. We clearly show that vaccination against CD99 indeed strongly reduced the formation of atherosclerotic lesions and attenuated atherogenesis at two different sites within the vasculature. In addition to smaller size of atherosclerotic plaques they contained fewer cells when mice were vaccinated against CD99. This indicates that fewer cells that could contribute to plaque initiation and growth have migrated into the activated vessel wall. The observed reduction in lesion formation is not the result of differences in cholesterol and triglyceride levels between control and CD99 vaccinated mice (data not shown).

In summary, we generated an attenuated Salmonella vaccine against mouse CD99 that induced the generation of a CD\textsuperscript{8+} T cell population that specifically targets CD99 expressing cells. Vaccination against CD99 of LDL receptor deficient mice significantly reduced the formation of atherosclerotic plaques and attenuated clinical symptoms of atherogenesis. This flexible, easy to prepare, and non invasive method provides a rationale for the future development of DNA based \textit{Salmonella} vaccines to prevent the initiation or progression of atherosclerosis.
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Vaccination against CD99 Inhibits Atherosclerosis
