Aims 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. The aim of the present study is to show the role of CD99 in atherosclerosis using a CD99 vaccination protocol to block the function of CD99 during atherosclerosis.

Methods and results 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 CD99-specific, CD8-mediated cytotoxic response and subsequent reduction of CD99-expressing cells. We showed that CD99 is expressed on vascular endothelium overlying atherosclerotic plaques and found that CD99 expression is upregulated during western-type diet feeding. CD99 vaccination induced the formation of CD8\(^+\) 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% compared with 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.

Conclusion 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.

1. Introduction

Atherosclerosis is a chronic inflammatory disorder of the large arteries and the prominent cause of death in the western world.\(^1\) Recruitment of leukocytes into the vessel wall is driving the initiation and progression of atherosclerotic plaque formation\(^2,3\) The atherosclerotic process is orchestrated by several groups of molecules on both the activated endothelium as well as on the infiltrating leukocytes.\(^4\) Activation of the endothelium due to low shear stress and/or local damage results in the upregulation of leukocyte adhesion molecules and secretion of chemotactants.\(^5\)

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.\(^6\) Transient interaction slows down leukocytes from the flowing blood, resulting in rolling behaviour on the endothelial surface. After activation of leukocyte, integrins 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, resulting in the predominant recruitment of monocytes and lymphocytes to atherosclerotic regions.\(^11\) Blocking this interaction results in attenuation atherosclerosis and other inflammatory disorders.\(^12\) The last step in leukocyte transendothelial migration, which is also known as diapedesis, occurs largely through junctions between adjacent endothelial cells. Indeed a number of cell adhesion molecules located at endothelial cell junction have been implicated in this process. These molecules include platelet-endothelial cell adhesion molecule (PECAM-1),\(^15\) members of the junctional adhesion molecule (JAM) family,\(^16\) CD99,\(^17\) ESAM,\(^20\) and ICAM-2.\(^21\)
CD99, a long-known leukocyte membrane protein that was initially described to function in T cell activation and lymphocyte aggregation,22–24 was only recently reported to participate in the transmigration of human monocytes through cultured endothelial cells.19 Schenkel et al.19 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.17,18 Antibodies against mouse CD99 efficiently block migration of lymphocytes and neutrophils through a monolayer of cultured endothelial cells.17,18 More importantly, these antibodies block recruitment of in vivo-activated T cells into inflamed skin17 and inhibit neutrophil extravasation to inflamed sites in two inflammatory mouse models.18 As the recruitment of both monocytes and T cells contributes to the initiation and progression of the atherosclerotic plaque, blockade of their transmigration may provide protection against atherosclerosis.3

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 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⁺ 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 compared with mice vaccinated with the vector alone.

2. Material and methods

2.1 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.,17 was cloned into pcDNA3 plasmid (Invitrogen, CA, USA). This plasmid was electroporated into S. typhimurium Aro/A (strain SL7207) bacteria as described previously.20 Female LDL receptor-deficient mice, aged 10–12 weeks, were immunized by oral administration of 1 × 10⁸ cfu S. typhimurium transformed with either pcDNA3-CD99 or pcDNA3 empty (control) three times with 2 week intervals.

2.2 Induction of CD8⁺-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-well plate and incubated with DMEM containing 10% foetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere (5% CO₂) 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 manufacturer’s protocol. After 24 h, the transfected cells were incubated with splenocytes derived from control or CD99-vaccinated mice (2 weeks after last vaccination) for 24 h. Non-adherent cells were washed away with PBS, and specific lysis of the cells expressing CD99 was analysed on a FACScalibur (BD Biosciences, the Netherlands) by quantifying the percentage of eGFP-positive cells. The percentage of specifically lysed cells transfected with CD99 was plotted as percentage of eGFP-positive cells in the absence of splenocytes.

2.3 Atherosclerosis experiments and histology

All animal experiments were performed in concordance with Dutch law and were approved by the Animal Experimental Committee (DEC). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Female LDL-deficient (LDLr⁻/−) mice (n = 12 per group), 8 weeks old, were vaccinated with pcDNA3 empty of pcDNA3-CD99 three times in 2 week intervals. After vaccination, mice were placed on a western-type diet containing 0.25% cholesterol and 15% cocoa butter 2 weeks before collar placement. Silastic collars (0.3 mm inside diameter, Dow Corning, Midland, TX, USA) were placed around the carotid artery to induce atherosclerosis. After 8 weeks of the western-type diet, mice were sacrificed and organs were harvested.

Cryosections from carotid artery (5 µm) were stained with haematoxylin 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 five sections of the aortic valve leaflet area. Expression of CD99 was visualized using an affinity-purified anti-mouse CD99 polyclonal antibody.17 As secondary antibody, biotinylated goat anti-rabbit (DAKO, the Netherlands) antibody was used in combination with streptABC complex (DAKO), with Nova Red as enzyme substrate (Vector Laboratories).

2.4 Real-time polymerase chain reaction assays

Total RNA was isolated from aortic arch and collar-induced atherosclerotic plaques using the guanidium isothiocyanate method. Purified RNA was DNase-treated (DNase I, 10 U/µg of total RNA) and reverse-transcribed (RevertAid M-MuLV reverse transcriptase) according to the manufacturer’s protocol.

Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA) using SYBR Green technology.

2.5 Flow cytometry

Leukocytes from spleen were isolated by density gradient centrifugation with lymphomacrophage-feeding (Cedarlane Laboratories, Hornby, Ontario, Canada) according to the manufacturer’s protocol. Cell suspensions from spleen and lymph nodes draining from the aortic arch and peritoneal cavity were stained for surface markers (0.20 µg/300 000 cells) and subsequently subjected to flow cytometric analysis. Antibodies were purchased from eBiosciences (Immunochemistry, Belgium). All data were acquired on a FACS-Calibur and were analysed with CELLQuest software (BD Biosciences, the Netherlands).

2.6 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 analyse not-normally distributed data. A probability value of P < 0.05 was considered to be significant for both tests.

3. Results

3.1 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
vasculature.\textsuperscript{26} We stained cryosections of collar-induced atherosclerotic plaques in LDL\textsuperscript{r2/2} mice and observed a profound expression of CD99 by vascular endothelial cells covering the plaque. A representative picture is shown in Figure 1A and B. To further investigate the regulation of this expression, we isolated the aortic arch of LDL\textsuperscript{r2/2} 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 (Figure 1C). This increase was not accompanied by elevated expression of T cell or macrophage markers (data not shown), indicating that the cellular composition of the aorta was not changed due to an influx of leukocytes.

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

We developed a vaccination strategy on the basis of the induction of CD99-specific cytotoxic T cells that specifically lyse cells that express high levels of CD99 as recently shown for FLK-1.\textsuperscript{27} To test the functionality and specificity of this vaccination protocol, we determined the antigen-specific cytotoxicity of splenic CD8\textsuperscript{+} 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 green fluorescent protein (GFP) using flow cytometry. Splenocytes were isolated from control and CD99-vaccinated mice and were co-cultured for 24 h with 3T3 fibroblasts that were transfected with CD99. Specific lysis of CD99-expressing cells was determined by quantifying the percentage of eGFP-positive cells after incubation with splenocytes from control and CD99-vaccinated mice. The percentage of CD99-expressing cells was significantly decreased after incubation with splenocytes from control and CD99-vaccinated mice (Figure 2, 86.6 \pm 10.2\% vs. 46.3 \pm 11.8\%), whereas no decrease was observed when splenocytes from control-vaccinated mice were added (Figure 2). This observation indicates that CD99 vaccination induces the formation of T cells that specifically lyse CD99-expressing cells.

3.3 Vaccination against CD99 inhibits atherosclerotic lesion formation in LDL\textsuperscript{r2/2} mice

After showing the effectiveness of our vaccination strategy, we investigated the effect of CD99 vaccination on atherosclerotic lesion formation. Female LDL\textsuperscript{r2/2} mice were vaccinated with pcDNA3-empty (control) or pcDNA-CD99 three times at a 2 week time interval. Mice were subsequently placed on a western-type diet (0.25% cholesterol), and 2 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. No significant effects were observed on serum cholesterol levels (control, 1176 \pm 101 mg/dL vs. CD99, 1224 \pm 167 mg/dL). 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 with control animals (Figure 3C, 5.25 \pm 0.68 \times 10^{3} \text{m}^{2} vs. 3.37 \pm 0.57 \times 10^{3} \text{m}^{2}).

Middle panels show representative sections of the carotid artery of control (Figure 3D) and CD99-vaccinated mice (Figure 3E). Lesion size was significantly decreased by 69\% after CD99 vaccination (Figure 3F: 24.15 \pm 7.7 \times 10^{3} \text{m}^{2} vs. 7.71 \pm 4.0 \times 10^{3} \text{m}^{2}), which is accompanied by an 83\% decrease in intima/media ratio (Figure 3G) and a 78\% diminished degree of lumen stenosis (Figure 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 (Figure 3I). Vaccination against CD99 resulted in a 35% decrease in number of nuclei per area in atherosclerotic plaques from CD99-vaccinated mice compared with controls, which may result from a lower number of cells that have infiltrated the vessel wall or from a reduction in cell death.

3.4 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 (Figure 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%, Figure 4, lower panels). This observation was restricted to the CD8+ T cell population,
leukocyte migration into atherosclerotic plaques.\textsuperscript{15,28,29} Mice deficient in JAM-A, another junctional cell-adhesion molecule that participates in leukocyte extravasation, show a reduction in neointima formation in ApoE-deficient mice.\textsuperscript{30} We hypothesize that CD99 may be involved in the recruitment of leukocytes into atherosclerotic plaques, and a lower number of CD99-expressing cells may possibly slow down leukocyte recruitment into inflamed areas of the artery and may attenuate atherosclerosis.

First, we demonstrate that CD99 is expressed on the aortic endothelial cells overlying atherosclerotic lesions. Secondly, \textit{western-type} diet feeding of atherosclerosis-prone LDL\textsubscript{r/−} mice resulted in a 1.5-fold upregulation of CD99 mRNA levels in the aortic arch after 2 weeks of a cholesterol-rich \textit{western-type} diet. The upregulation was not due to CD99-expressing leukocytes infiltrating the vessel wall, as CD99 expression was already increased before an increase in the number of macrophages (CD68\textsuperscript{+}) or T cells (CD4\textsuperscript{+}) was observed. This indicated that no additional CD99-expressing leukocytes were recruited and that the cellular composition of the examined vessels after \textit{western-type} diet or \textit{chow} diet feeding was not changed (data not shown).

To test the hypothesis that CD99 is involved in leukocyte recruitment to atherosclerotic lesions, we developed a DNA vaccination strategy to immunize LDL receptor-deficient mice against CD99. An attenuated \textit{S. typhimurium} strain was transformed with a pcDNA3 vector encoding the extracellular domain of murine CD99. The \textit{S. typhimurium} is the vaccine carrier and 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 the presentation of peptide fragments in a complex with MHC class I. The presentation of CD99 peptides in the context of MHC class I activated CD99-specific CD8\textsuperscript{+} T cells, and during this process, natural killer cells help to break the peripheral tolerance against CD99.\textsuperscript{25,31} This vaccination protocol generates CD8\textsuperscript{+} T cells that specifically target cells that express CD99-derived peptides in the context of MHC-I, and the protocol has been successfully validated for other proteins in animal models for tumour growth and cancer therapy and atherosclerosis.\textsuperscript{25,31} Here, we show that vaccination against murine CD99 of LDL\textsubscript{r/−} mice specifically activates CD8\textsuperscript{+} T cells that lyse CD99-expressing mouse cells, and this illustrates that the immune tolerance against the self-antigen CD99 is broken by the vaccination procedure.

After validation of our vaccination protocol, we induced atherogenesis in LDL\textsubscript{r/−} mice that were vaccinated against CD99 (pcDNA3-CD99) or control vaccinated (pcDNA3-empty). We clearly show that vaccination against CD99 strongly reduced the formation of atherosclerotic lesions and attenuated atherogenesis at two different sites within the vasculature. In addition to a smaller size of atherosclerotic plaques, they also contained fewer cells when mice were vaccinated against CD99. This may indicate that fewer cells that could contribute to plaque initiation and growth have migrated into the activated vessel wall.

The latter may result from the role of CD99 in leukocyte diapedesis as shown by Schenkel et al.\textsuperscript{19} for human CD99 and later by Bixel et al.\textsuperscript{18} for mouse CD99. For human monocytes and neutrophils and for mouse lymphocytes, it is has

![Image](https://via.placeholder.com/150)

**Figure 5** Expression of CD99 on CD4\textsuperscript{+} T cells and F4/80\textsuperscript{+} macrophages is decreased after vaccination against CD99. Single-cell suspensions of spleens from control and CD99-vaccinated mice were prepared and analysed by flow cytometry to determine CD99 expression on CD4\textsuperscript{+} T cells and F4/80\textsuperscript{+} macrophages. Expression of CD99 was quantified by assessing the relative amount of CD99-positive cells among the CD4\textsuperscript{+} and the F4/80\textsuperscript{+}-positive cell population. Expression of CD99 in CD4\textsuperscript{+} T cells and F4/80\textsuperscript{+} macrophages was significantly decreased after CD99 vaccination compared with controls. White bars represent control; black bars, CD99-vaccinated mice; error bars, SEM; n = 6 per group, *P < 0.05.

since the activation state of CD4\textsuperscript{+} T cells was not changed as shown by measuring the percentage of CD4\textsuperscript{+}/CD69\textsuperscript{+} double-positive cells among the CD4\textsuperscript{+} T cell population (data not shown). These findings indicate that vaccination against CD99 specifically activates CD8\textsuperscript{+} T cell subsets, but not CD4\textsuperscript{+} T cells.

3.5 Vaccination against CD99 decreases expression of CD99 on CD4\textsuperscript{+} T cells and F4/80\textsuperscript{+} macrophages

CD99 is expressed on most leukocytes, including lymphocytes and monocytes derived from peripheral blood\textsuperscript{17,18} to determine whether vaccination against CD99 would affect CD99 expression levels on leukocytes, we stained CD4\textsuperscript{+} T cells and F4/80\textsuperscript{+} macrophages with anti-CD99 antibodies and determined CD99 expression on these cells by flow cytometry. The percentage CD99-positive cells within the CD4\textsuperscript{+} and F4/80\textsuperscript{+} cell population is shown in Figure 5.

The expression of CD99 on CD4\textsuperscript{+} T cells was significantly decreased upon vaccination against CD99 (Figure 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 (Figure 5, right panel; 24.5 ± 1.7 vs. 16.9 ± 2.7%). The percentage of macrophages and CD4\textsuperscript{+} T cells was not changed compared with control-vaccinated mice as determined by FACS (data not shown).

4. Discussion

Human CD99 was recently reported to be involved in the migration of human monocytes through a monolayer of cultured endothelial cells.\textsuperscript{19} Cloning of mouse CD99 by Bixel et al.\textsuperscript{17} opened the possibility to examine the physiological role of CD99 in mouse models of inflammatory diseases.

Recruitment of leukocytes into the vessel wall is a hallmark of atherosclerosis, and a prominent role for cell adhesion molecules that are expressed at cell contacts of endothelial cells was reported. The increased expression of PECAM-1 is associated with atherosclerosis-prone regions of the vessel wall, and PECAM-1 blocking reduces...
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 can 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, which most likely contributes to the reduced atherosclerosis after CD99 vaccination.

It is also suggested for human CD99 that a 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 may attenuate plaque formation due to a reduced transmigration rate, and the killing of CD99-positive cells by CD8\textsuperscript{+} T cells after vaccination may also contribute to the reduction in atherosclerosis. We have recently shown that the vaccination of mice against vascular endothelial growth factor (VEGF) receptor 2 (flk-1) by induction of anti-CD99 CD8\textsuperscript{+} T cells also leads to a reduction in atherosclerosis.\textsuperscript{27} VEGFR2 is expressed by activated endothelial cells that cover the atherosclerotic plaque, and the VEGFR2 vaccination reduces atherosclerosis because this reduces the number of activated endothelial cells that cover the plaque.\textsuperscript{27} In analogy, we propose that activated endothelial cells, which cover the plaque, express enhanced levels of CD99 and these cells will be removed by the CD99 vaccination and the removal of the activated endothelial cells will lead to a reduction in atherosclerosis. The removal of the VEGFR2-expressing, activated endothelial cells by the VEGFR2 vaccination\textsuperscript{27} had a side effect, since it also removed endothelial progenitor cells that express VEGFR2 and thus inhibited arteriogenesis and promoted restenosis\textsuperscript{27} in addition to the reduction in atherosclerosis. In the current CD99 vaccination, we do not observe expression of CD99 on endothelial progenitor cells (data not shown) and therefore do not anticipate an effect on restenosis and arteriogenesis.

Finally, the 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 \textit{Salmonella} vaccine against mouse CD99 that induced the generation of a CD8\textsuperscript{+} T cell population that specifically targets CD99-(over)expressing cells. Vaccination against CD99 of LDLr\textsuperscript{+/−} mice significantly reduced the formation of atherosclerotic plaques and attenuated the clinical symptoms of atherosclerosis.

References


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