Atherosclerosis development in apolipoprotein E-null mice deficient for CD69

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Aims Atherosclerosis is a chronic inflammatory disease regulated by immune mechanisms. CD69 is a cell surface receptor rapidly induced after leukocyte activation at sites of chronic inflammation. Genetic disruption of CD69 in the mouse aggravates collagen-induced arthritis (CIA), and partial depletion of CD69-expressing cells with anti-CD69 monoclonal antibody (mAb) prevents CIA development in wild-type mice, suggesting that this receptor negatively modulates immune and inflammatory responses. It has been recently reported that CD69 is upregulated in a large subset of T cells in atherosclerosis-prone apolipoprotein E-null mice (apoE<sup>−/−</sup>). In this study, we investigated whether altering CD69 function affects atherosclerosis development.

Methods and results We studied native and diet-induced atherosclerosis in apoE<sup>−/−</sup> and doubly deficient apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice and performed expression studies in tissues and primary cells derived from these animals. Plasma cholesterol level was unaffected by CD69 genetic inactivation. Although this genetic manipulation led to an elevated production of interferon-γ and interleukin 10 by activated T cells, apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice fed control and high-fat diet exhibited atheromas of similar size and composition when analysed at different stages of the disease. Likewise, anti-CD69 mAb treatment had no effect on plasma cholesterol and atherosclerosis burden in fat-fed apoE<sup>−/−</sup> mice.

Conclusion In contrast to previous studies highlighting the protective function of CD69 against CIA, an autoimmune inflammatory disease, our results rule out a significant role for CD69 against atherosclerosis in apoE<sup>−/−</sup> mice, an experimental disease model featuring a local inflammatory response triggered and sustained by alterations in lipid homeostasis.

KEYWORDS CD69; Atherosclerosis; Apolipoprotein E-null mouse

1. Introduction

Atherosclerosis and the associated cardiovascular disease (e.g. myocardial infarction, stroke, and peripheral vascular disease) are the principal cause of morbidity and mortality in developed countries. Neointimal lesion formation due to the progressive accumulation of cellular and non-cellular elements within the subendothelial space of the artery wall is a hallmark of atherosclerosis. Factors contributing to neointimal cell accumulation include the recruitment of circulating leukocytes (mainly monocytes, which differentiate into macrophages, and T cells) and excessive macrophage and vascular smooth muscle cell (VSMC) proliferation. The activation of neointimal leukocytes triggers an inflammatory response characterized by abundant production of cytokines and chemokines, which lead to exacerbated leukocyte recruitment and proliferation and to the induction of VSMC hyperplastic growth and migration from the tunica media towards the growing neointimal lesion, thus contributing to the progression of the disease.

A large body of evidence supports that the inflammatory response associated to atherosclerosis is modulated by both adaptive and innate immune mechanisms. Monocyte-derived macrophages, cells of the innate immune system which accumulate in plaques as inflammatory foam cells, play an important role in the initiation and progression of atherosclerosis. Adaptive immune system cells (e.g. T and B lymphocytes and natural killer T cells) are also present in atheromas, with T cells being the most abundant infiltrating subset of lymphocytes. Although it is well...
CD69 is a homodimeric leukocyte transmembrane protein that is transiently expressed in vitro upon cell activation. Expression of CD69 is detected in small subsets of T and B cells in peripheral lymphoid tissues. In addition, CD69 is persistently expressed in the infiltrates of leukocytes produced during the course of chronic inflammatory diseases (e.g., rheumatoid arthritis and chronic viral hepatitis) and autoimmune disorders (e.g., systemic lupus erythematosus, insulin-dependent diabetes mellitus, and autoimmune thyroiditis). Although some early in vitro findings have suggested a proinflammatory function of CD69, subsequent in vivo studies showed that the development of haematopoietic cells, the maturation of T cells, and positive and negative selection of thymocytes are normal. Of haematopoietic cells, the maturation of T cells, and anti-atherogenic immune responses is limited. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the ethics review board of Consejo Superior de Investigaciones Científicas. CD69 is known to be a key modulator of immune responses in different pathological settings. Further prolonged survival due to enhanced activity of natural killer cells and reduced production of TGF-β. Thus, CD69 appears to be a key modulator of in vivo inflammatory responses in different pathological settings. Further support to this notion was provided by the observation that systemic treatment with different anti-CD69 monoclonal antibodies (mAb) can either exacerbate or inhibit the course of CIA in the mouse. Moreover, the ability of the anti-CD69 mAb-2.3 to reduce inflammation in CIA opens the possibility for its therapeutic use as an anti-inflammatory agent. 

Altogether, the aforementioned results underscore CD69 as a negative regulator of autoimmune reactivity and a putative therapeutic target in inflammation. Although recent studies using atherosclerosis-prone apolipoprotein E-null mice (apoE<sup>−/−</sup>) have suggested that the upregulation of CD69 induces the activation of a large pool of T cells during atheroma development, it remains to be established whether CD69 expression and atherogenesis are indeed causally linked. To address this question, we investigated in this study the development of atherosclerosis in apoE<sup>−/−</sup> and doubly deficient apoE<sup>−/−</sup>/CD69<sup>−/−</sup> mice. Moreover, we examined the consequences of targeting CD69 with mAb-2.3 on atherosclerosis burden in apoE<sup>−/−</sup> mice. Our results show that neither spontaneously formed nor diet-induced atherosclerosis is altered in in vivo targeting CD69.

2. Methods

Expanded methods are provided in Supplementary material.

2.1 Mice

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the ethics review board of Consejo Superior de Investigaciones Científicas. CD69<sup>−/−</sup> mice backcrossed 12 times on a C57BL6 genetic background and apoE<sup>−/−</sup> mice (C57BL6/J, Charles River) were mated. The resulting F1 was intercrossed, and F2 apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/CD69<sup>−/−</sup> mice were crossed to generate the two experimental groups (apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/CD69<sup>−/−</sup>). Genotyping was done by PCR (see sequence of primers in online supplement).

All studies were carried out with males. Spontaneous atherosclerosis was studied in 10-month-old mice always fed a low-fat standard rodent diet (reference 2014, Teklad global rat/mouse Chow, Harlan Interfauna). When stated, 3-month-old mice were switched for the indicated time to a high-fat diet containing 0.75% cholesterol (reference S8492-E010, Ssniff). Four apoE<sup>−/−</sup>/CD69<sup>−/−</sup> and two apoE<sup>−/−</sup> mice died during fat feeding. Blood was withdrawn from the retroorbital plexus to measure plasma cholesterol level using a liquid stable reagent (WAKO). Cholesterol bound to high-density lipoprotein (HDL-c) and non-HDL (non-HDL-c) were quantified with the same reagent after precipitation with Dextran-sulphate MgCl<sub>2</sub> (SIGMA) as described.

2.2 Anti-CD69 monoclonal antibody therapy

Three groups of 2-month-old male apoE<sup>−/−</sup> mice were used. The control group consisted of mice fed with the standard diet throughout the study. Mice of the therapy groups were injected 300 μg of antibody (intraperitoneally, once per week) and switched to an atherogenic diet for 3 weeks. Antibody injections were repeated once per week during the 3-week period of fat feeding. The first therapy group was injected with the anti-mouse CD69 mAb-2.3 (mouse IgG2a) and the second therapy group received mouse IgG2a anti-human CD4 mAb HP 2/6 as isotype-matched control (IMC).

2.3 Atherosclerosis studies

Mice were euthanized and their aortas were washed in situ with PBS and fixed with freshly prepared 4% paraformaldehyde/PBS. The heart and aorta were extracted, and fixation continued for 22–28 h at 4°C. Atherosclerosis was quantified by computer-assisted planimetry of whole-mounted aortae stained with Oil Red O (0.2% in 80% MeOH, SIGMA) or cross-sections stained with haematoxylin-eosin essentially as described. Briefly, 3-mm cross-sections were obtained from two to three different zones within the aortic root region and the ascending aorta. In both regions, the extent of atherosclerosis was quantified as the surface of lesion. In cross-sections of the ascending aorta, atherosclerosis was also quantified as the intima-to-media ratio (surface area of the intimal lesion divided by the surface area of the media). For each mouse, the values obtained in the different sections analysed were averaged. Images were captured with an Olympus CAMEDIA-C5060 wide zoom digital camera mounted on a Zeiss-Axiolab stereomicroscope. The Sigma Scan Pro v5.0 software (Jandel Scientific) was used to quantify atherosclerosis burden by an investigator who was blinded to genotype. Methods for the immunohistopathological characterization of atheromas are in Supplementary Material.

2.4 Statistical analysis

Results are reported as mean ± SEM. For comparisons between two groups, differences were evaluated using a two-tail, unpaired t-test. For more than two groups, differences were evaluated using ANOVA and post-hoc Fisher’s PLSD test (Statview, SAS Institute). Differences were considered statistically significant at P ≤ 0.05.

3. Results

3.1 Genetic ablation of CD69 has no effect on diet-induced and spontaneous atherosclerosis in apolipoprotein E-null mice

The apoE<sup>−/−</sup> mouse spontaneously develops hypercholesterolaemia and complex atherosclerotic lesions, two processes that can be accelerated by a high-fat cholesterol-rich diet.
We first explored the role of CD69 on diet-induced atherosclerosis by examining 3-month-old apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice which had been challenged with an atherogenic diet for the last 7 weeks prior to sacrifice. Pre-diet plasmatic levels of total cholesterol were similar in both groups of mice (apoE<sup>−/−</sup>: 267 ± 20 mg/dL, apoE<sup>−/−</sup>CD69<sup>−/−</sup>: 381 ± 16 mg/dL, P > 0.05) (Figure 1A).

As expected, fat feeding significantly elevated plasmatic cholesterol levels but no statistically significant differences were detected between apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice (1340 ± 62 and 1304 ± 60 mg/dL, respectively; both P < 0.0001 vs. pre-diet) (Figure 1A). We also found no effect of CD69 disruption on atherosclerosis burden within the aortic arch and thoracic aorta, as assessed by Oil Red O staining of whole-mounted vessels (Figure 2A). Consistent with these findings, analysis of the intima-to-media ratio and total lesional surface in cross-sections from the ascending aorta (Figure 2C) and aortic root (Figure 2E) revealed similar extent of atherosclerosis when comparing apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice challenged with the atherogenic diet for 7 weeks. Moreover, atherosclerosis burden was not affected by CD69 deletion in apoE<sup>−/−</sup> mice fed atherogenic diet for 2 weeks, which developed comparatively smaller aortic atheromas (see Supplementary material online, Figure S1).

It was feasible that a possible subtle effect of CD69 disruption on atherosclerosis might have been masked under highly hypercholesterolaemic fat-fed apoE<sup>−/−</sup> mice. However, 10-month-old apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice fed standard chow, which exhibited plasmatic total cholesterol <400 mg/dL (Figure 1B), developed atheromas of similar size within the aortic arch and thoracic aorta (Figure 2B), ascending aorta (Figure 2D), and aortic root region (Figure 2F). Immunohisto pathological examination of atherosclerotic lesions revealed undistinguishable neointimal accumulation of Mac-3-immunoreactive macrophages, SMα-actin-immunoreactive VSMCs, CD4-immunoreactive T cells, and collagen when comparing apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice fed either standard chow (Figure 3A) or atherogenic diet (Figure 3B).

Collectively, these studies demonstrate that both the size and composition of spontaneously formed and diet-induced aortic atheromas are unaffected by genetic disruption of CD69 in apoE<sup>−/−</sup> mice when analysed at different stages of the disease.

Next, we studied the production of effector cytokines by activated T lymphocytes from apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice. Secreted cytokines were analysed by ELISA in the supernatant of cultured splenic cells which were activated in vitro with mAb anti-CD3. These studies revealed elevated levels of both the proinflammatory Th1-type cytokine interferon γ (IFN-γ) and the immunosuppressive cytokine IL10 in apoE<sup>−/−</sup>CD69<sup>−/−</sup> compared with apoE<sup>−/−</sup> cells, with the Th2-type cytokine IL4 being unaffected by CD69 ablation (Figure 4A). Thus, despite the lack of effect on atheroma formation, CD69 genetic inactivation in apoE<sup>−/−</sup> mice affects at least some aspects of the immune reactivity of T lymphocytes. We also investigated by quantitative real-time RT-PCR (qPCR) the expression of CD25, MHC class II, and the chemokine receptors CCR7 and CXCR4 in the spleen of mice fed control diet (Figure 4B) or challenged for 6 weeks with the atherogenic diet (Figure 4C). The results of these studies did not reveal statistically significant differences between groups, thus suggesting that a compensatory upregulation of leukocyte activation molecules in fat-fed apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice is unlikely to account for the lack of effect of CD69 inactivation on atheroma development.

As macrophages play an important role in the initiation and progression of atherosclerosis and upregulate CD69 upon activation,34 we assessed whether CD69 ablation affects the inflammatory state of macrophages. To this end, we analysed by qPCR the expression of the proinflammatory cytokines tumor necrosis factor α (TNF-α) and IFN-γ in peritoneal macrophages harvested from mice fed control diet. As shown in Figure 4D, both cytokines were expressed at similar levels in apoE<sup>−/−</sup> and apoE<sup>−/−</sup>CD69<sup>−/−</sup> mice.

### 3.2 In vivo treatment with the depleting anti-CD69 monoclonal antibody-2.3 has no effect on atherosclerosis burden in apolipoprotein E-null mice

We previously showed that CD69 targeting by mAbs affects the course of CIA in the mouse.28 In particular, treatment with
anti-CD69 mAb-2.3 reduced the severity of CIA by partially depleting CD69-expressing effector cells. To investigate whether this mAb also protects against atherosclerosis, we studied three groups of apoE^{2−/2} mice: untreated group fed control diet, and fat-fed mice which were treated with either anti-CD69 mAb-2.3 or with mAb-IMC (intraperitoneal administration, once per week during the 3-week period of fat feeding). We first performed flow cytometric analysis of...
thymocytes isolated from mAb-treated groups and stained ex vivo with an anti-CD69 mAb different from 2.3. As shown in Figure 5A, in vivo treatment with mAb-2.3 greatly reduced CD69 expression in thymocytes when compared with cells from mice treated with control mAb-IMC, thus confirming that mAb-2.3 binds in vivo to CD69-expressing cells. We also examined the production of effector cytokines by splenic cells activated in vitro with anti-CD3 mAb (Figure 5B). These experiments disclosed no statistically significant differences in the production of IFN-γ, IL4 and IL10 when comparing the treatments with mAb-2.3 and mAb-IMC.

Compared with basal levels in untreated mice fed control diet, plasmatic total cholesterol was similarly elevated in both groups of fat-fed mAb-treated mice (Figure 6A, both P < 0.0001 vs. control diet). Likewise, atherosclerosis burden was similar in mAb-2.3- and mAb-IMC-treated mice, as revealed by Oil Red O staining of the aortic arch and thoracic aorta (Figure 6B) and quantification of the intima-to-media ratio and total lesion size in cross-sections through the ascending aorta (Figure 6C). Moreover, the area occupied by neointimal macrophages in atheromas from the aortic root was similarly increased in both groups of

Figure 3  CD69 genetic disruption has no effect on the composition of spontaneously formed and diet-induced atheromas. Aortic cross-sections were obtained from mice fed control chow (A) or challenged with atherogenic diet for 6–7 weeks (B). Neointimal macrophages, vascular smooth muscle cells, and T-cells were identified using anti-Mac-3, anti-SMα-actin, and anti-CD4 antibodies, respectively. Neointimal collagen content was quantified by Masson’s Trichrome staining. Tissue was embedded in paraffin (anti-Mac-3, anti-SMα-actin and collagen, control chow, or atherogenic diet for 7 weeks) or Cryoblock (anti-CD4, atherogenic diet for 6 weeks). In the confocal microscopy studies to detect CD4-immunoreactive cells (green signal), nuclei were counterstained with Topro-3 (red signal). Representative images are shown.
fat-fed mAb-treated mice compared with controls fed standard chow (Figure 6D). Taken together, these studies demonstrate that treatment with the anti-CD69 mAb-2.3 has no effect on the extent of atherosclerosis in fat-fed apoE<sup>2/2</sup> mice.

4. Discussion

Atherosclerosis is a chronic inflammatory disease modulated by both adaptive and innate immune mechanisms. It is well known that different subsets of T cells can exacerbate or attenuate murine atherosclerosis; therefore, understanding the molecular mechanisms that regulate the balance between pro- and anti-atherogenic immune responses is of major importance. We have previously shown that CD69<sup>−/−</sup> mice display an exacerbated form of CIA, suggesting that CD69 is a negative regulator of inflammatory responses. Notably, the percentage of lymphocytes expressing both CD69 and CD3 is two to three times higher in apoE<sup>−/−</sup> mice when compared with wild-type controls, suggesting that atherosclerosis may activate a large pool of T cells through CD69 upregulation. This could also be true for monocytes/macrophages, although expression of CD69 in these cells has not been characterized in the context of atherosclerosis. Importantly, dietary factors seem to modulate the immune response associated with atherogenesis. For example, the absence of T and B cells had no effect on the extent of atherosclerosis in apoE<sup>−/−</sup> mice fed for 12 weeks a fat- and cholesterol-enriched diet, but decreased atherosclerosis burden in apoE<sup>−/−</sup> mice fed standard chow. Therefore,
in this study, we have examined atherosclerotic lesion formation in apoE−/−CD69−/− doubly deficient mice and in apoE−/− controls receiving either control chow or a high-fat diet to investigate the role of CD69 on both spontaneous and diet-induced atherosclerosis, respectively. In both experimental settings, neither atherosclerosis burden nor lesion composition was altered by CD69 disruption. Of note in this regard, previous analysis of CD69 role in T and B cells has been proposed as one of the putative mechanisms responsible for its negative regulatory effect on inflammation during CIA.28 T cells also play crucial roles on atherosclerosis by controlling inflammatory responses through the production of a plethora of regulatory cytokines.7–9,12 Of particular relevance for the results reported herein, studies using genetically modified mice have shown that IFN-γ exerts proatherogenic actions,38 whereas IL10 expression protects from atherosclerosis.39,40 We found similar number of infiltrating lymphocytes in atherosclerotic lesions of fat-fed apoE−/− and apoE−/−CD69−/− mice (Figure 3B), but secretion of both IFN-γ and IL10 was significantly elevated in T cells that were isolated from fat-fed apoE−/−CD69−/− and stimulated ex vivo through their T-cell receptor, when compared with apoE−/− control cells (Figure 4A). These results suggest that genetic ablation of CD69 in the mouse increases the immune reactivity of T cells in a manner that leads to the combined upregulation of proatherogenic (e.g. IFN-γ) and antiatherogenic (e.g. IL10) factors, thus providing a potential mechanism contributing to comparable atherosclerosis development in apoE−/−CD69−/− and apoE−/− mice. It is also noteworthy that T and B cells have been proposed to play a minor role in atherosclerosis in fat-fed apoE−/− mice,35,36 suggesting another possible explanation for the lack of effect of CD69 ablation on disease progression under highly atherogenic conditions. Therefore, although our results suggest that CD69 may play a role in the regulation of T-cell differentiation in vivo, as previously proposed,27 additional studies in other well-dissected experimental systems of T-lymphocyte effector differentiation are needed to clarify this point (e.g. antigen-dependent differentiation of lymphocytes from T-cell receptor transgenic mice deficient for CD69).

Our previous studies in the murine CIA model showed that anti-CD69 mAb-2.3 administration can partially deplete CD69-expressing activated effector T cells, including IFN-γ+CD4+ lymphocytes, a response which was accompanied by a significant reduction in both macroscopic inflammation and the production of proinflammatory cytokines, and by an amelioration of disease progression.28 Thus, targeting in vivo CD69-expressing leukocytes by treating with mAb-2.3 might be proposed as a suitable therapeutic agent against inflammatory processes. In the present work, we examined atherosclerosis development in apoE−/− mice treated with mAb-2.3 once per week during a 3-week period of fat feeding. Consistent with our previous findings,28 we observed that mAb-2.3 bound in vivo to CD69-immunoreactive cells, as CD69 expression was reduced in ex vivo stained thymocytes isolated from mAb-2.3- compared with mAb-IMC-treated mice (Figure 5A). However, mAb-2.3 did not alter in splenic cells the production of cytokines known to affect atherosclerosis development (e.g. IFN-γ, IL4, and IL10) (Figure 3B), and failed to exert any significant effect on atheroma formation and neointimal macrophage accumulation in fat-fed apoE−/− mice (Figure 6). These findings suggest that mAb-2.3 treatment in apoE−/− mice does not significantly deplete effector T cells, either because exhaustion of these cells is not taking place or the effect is too subtle to alter disease progression. The latter might be the consequence of a relatively low upregulation of CD69 and mild level of T-cell activation...
locally generated in response to antigens present within the plaques of apoE\(^2/2\) mice. In contrast, the degree of T-cell activation and the level of CD69 expression in mice immunized with collagen plus complete Freund's adjuvant to induce CIA is expected to be comparatively much higher, thus favouring the depleting effect of mAb-2.3 and the inhibitory effect on inflammation observed in this model. Although additional studies are required to clarify this important point, our findings seem to rule out the use of mAb-2.3 as a therapy against atherosclerosis.

In summary, contrary to previous studies that highlight a protective role of CD69 against CIA, an autoimmune inflammatory disease, we have shown here that CD69 genetic ablation does not affect the size and composition of aortic atherosclerotic lesions in the apoE\(^{-/-}\) mouse, an experimental model in which local inflammation within the artery wall is triggered and sustained by alterations in lipid homeostasis. Moreover, in contrast to the beneficial effect of anti-CD69 mAb-2.3 against CIA, administration of this antibody does not attenuate atherosclerosis development in apoE\(^{-/-}\) mice. We therefore conclude that CD69 is not a good therapeutic target in the setting of atherosclerosis.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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