Implication of natural killer T cells in atherosclerosis development during a LPS-induced chronic inflammation

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Abstract Atherosclerosis has many features of a chronic inflammatory disease. To evaluate the role of lipopolysaccharide (LPS), mimicking a systemic infection, we administered the endotoxin to apolipoprotein E (apoE)-deficient mice. LPS injections increase the atherosclerotic lesion size and the titer of plasma autoantibodies directed against oxidized low-density lipoprotein. We found that Th1 and Th2 T cells help the activation of B cells in the autoimmune response. The number of interleukin-4 producing natural killer T cells is highly increased in peripheral blood, liver, spleen and thymus cells, as well as in the atherosclerotic plaque of the LPS-treated mice. Finally, an important adventitial infiltrate of activated lymphocytes, sign of an advanced atherosclerosis, is observed only in the LPS-treated mice. Our results demonstrate that LPS administration aggravates atherosclerosis in apoE-deficient mice. LPS-injected apoE-deficient mice appear to be an excellent animal model to analyze the implementation of new therapeutic approaches in the treatment of atherosclerosis by manipulating immunological effectors. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Rodent; Th1/Th2 cells; Lipopolysaccharide; Inflammation; Knockout

1. Introduction

Atherosclerosis has many features of a chronic inflammatory disease, in which both cell-mediated and humoral immune responses participate. Recent evidence suggests that common bacteria and viruses can contribute to the development of atherosclerosis, probably by triggering inflammation. Thus, epidemiological studies have shown the presence of *Chlamydia pneumoniae* and cytomegalovirus in the plasma or even in the atherosclerotic plaque from heart disease patients [1–3]. An increased incidence of coronary artery disease also occurs in patients with *Helicobacter pylori* infections, chronical dental infections, and chronic bronchitis, infections in which microorganisms are not localized in the vessel wall [4–6]. However, it is still uncertain whether or not microorganisms play a causal role in atherosclerosis and its complications.

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Abbreviations: MAD, malondialdehyde; $apoE_0$, apolipoprotein E-deficient mice; oxLDL, oxidized low-density lipoprotein; NK-T, natural killer T cell

Chronic infection can be mimicked by infusion of endotoxins, for example lipopolysaccharide (LPS) component of the Gram-negative bacteria cell wall. This bacterial endotoxin, carried by lipoproteins, may cause a local inflammation after incorporation of the lipoproteins into the vascular wall. Recently, it has also been suggested that increased low-density lipoprotein (LDL) oxidation could be one of the mechanisms by which infection and inflammation may promote atherogenesis, reactive oxygen being generated as part of the host defense [7]. In this way, *C. pneumoniae* was recently found to induce cellular oxidation of LDL [8].

The development of murine models permits a dissection of immune mechanisms in atherosclerosis. Genetic variation at the apolipoprotein E (apoE) locus in human is associated with hyperlipidemia and premature atherosclerotic risk. Mice with a targeted disruption of the apoE ($apoE_0$ or apoE-deficient mice) develop severe atherosclerosis that progresses from fatty streaks to fibrofatty plaques and advanced lesions. These mice are considered as an animal model for the study of factors involved in atherogenesis such as oxidation and inflammation. In these animals, studies using antisera specific for malondialdehyde (MAD)-lysine and 4-hydroxynonenal-lysine have revealed the existence of oxidation-specific epitopes in atherosclerotic lesions and have detected important levels of autoantibodies directed against MAD-lysine in their plasma [9]. Immunohistochemical analysis of aortic lesion of these mice revealed a prominent involvement of macrophages and T lymphocytes. Some of these T cells express surface molecules as the interleukin (IL)-2 receptor α-chain (CD25), indicative of recent activation [10]. Interestingly, it was reported that Th1 cells secreting interferon (IFN)-γ are found early in fatty streak lesions, but are replaced in severe hypercholesterolemia induced by cholesterol feeding by Th2 cells and their associated cytokines, IL-4 and IL-1 [11]. These data indicated that a high-cholesterol diet may influence T cell participation in atherosclerosis. In addition, it has recently been demonstrated that $apoE_0$ mice are more susceptible than wild-type C57Bl/6 mice to endotoxemia. This is probably due to the absence of apoE, since this apolipoprotein was demonstrated to be involved in the detoxification of LPS [12,13]. In contrast, LDL receptor-deficient mice are protected against endotoxemia compared with wild-type mice. Thus, $apoE_0$ mice seem to be a model of special interest to study the role of LPS in atherogenesis.

In this report we evaluate the role of LPS, mimicking a systemic infection, on the development of atherosclerosis. This was done by exploring humoral and cellular immune responses. We have administered LPS to apoE-deficient

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mice, and we have analyzed the atherosclerotic surface lesion, the concentration of autoantibodies directed against oxidized LDL (oxLDL) in plasma, the ex vivo cytokine production by lymphocyte subsets in blood, liver, spleen and thymus, and the expression of cytokine and lymphocyte markers in the atherosclerotic plaque.

2. Materials and methods

2.1. Experimental animals

Male apoE-deficient mice bred into a C57Bl/6 background were obtained from Jackson Laboratory. Mice were housed in a temperature-controlled room with an alternating light and dark period. They were fed a mouse chow diet (UAR, France) and had access to water ad libitum.

At 10 weeks of age, male $apoE_0$ mice were randomly divided into three groups ($n=4$ per group). The first one was injected intraperitoneally with LPS (50 μg) once every week during 10 weeks (LPS group), the second group was injected with LPS only once (LPS1 group) and the last group was injected with an equivalent volume of phosphate-buffered saline (PBS) once every week during 10 weeks (PBS group). In order to analyze the basal immunological response in wild-type C57Bl/6 animals, we constituted two groups ($n=5$ per group), the first one was injected with LPS and the second one was injected with PBS. Animals were bled 24 h after each treatment. For blood collection, food was removed from the cages at 9 a.m., and 8 h later, samples were collected from the retro-orbital sinus under isofluorane anesthesia. At the end of the study animals were killed by exsanguinations via cardiac puncture, perfused transcardially with PBS and the heart, liver, spleen and thymus were excised. All procedures involving animal handling and their care were in accordance with the Institut Pasteur Guidelines for Husbandry of Laboratory Mice.

2.2. Plasma lipid quantification

The plasma concentration of cholesterol and triglycerides was determined enzymatically using commercial kits (Boehringer Mannheim, Germany).

2.3. Evaluation of aortic lesions

Because the correlation is strong between the extent of atherosclerosis in the aortic root and the entire aortic tree in murine atherosclerosis models [14], we measured lesions in the aortic root by use of the method described by Paigen et al. [15]. In brief, hearts and proximal aortas were removed and fixed. Hearts were cut directly under and parallel to the leaflet, and the upper portions were embedded in OCT medium and frozen. 10-μm-thick sections were cut through the aortic sinus. Twenty-eight sections per animal were stained for lipids with Oil-red O and counter-stained with hematoxylin. Cross-sectional areas were analyzed by computerized planimetry. Statistical analysis was performed on five different cross-sectional lesion areas per animal, separated by 120 μm. The first section analyzed for each animal corresponded to the origin of the aortic sinus. The intermediate sections were used to perform immunohistochemistry assays.

2.4. Determination of anti-oxLDL antibodies

Human LDL was isolated as described [16]. OxLDL was prepared by incubating LDL (1 mg/ml) with 5 μmol/l $CuSO_4$ in PBS overnight at 37°C. Before each assay, 96-well microtitration plates were freshly coated with 100 μl of oxLDL (5 μg/ml) in PBS overnight at 4°C. The wells were blocked with 1% bovine serum albumin for 2 h at room temperature. A 100 μl aliquot of diluted sera (1:40) from each group of mice was added in duplicated wells and incubated for 2 h at room temperature. After three washes with PBS containing 0.1% Tween-20, goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA conjugated with alkaline phosphatase (1:1000 dilution, Beckman-Coulter, Paris, France) were added to each well, and incubation continued for 2 h at room temperature. Plates were washed again, and the alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate as a substrate and detected at 405 nm.

2.5. Cell preparation

Peripheral blood, spleen and thymic cells from each animal, killed as described above, were isolated by density centrifugation, washed three times in PBS. Hepatic lymphocytes were isolated by a method described elsewhere [17]. The liver was removed and cut in two pieces, one half was passed through 200-gauge steel mesh, and then suspended in RPMI medium with 5% of heat-inactivated calf serum. Once the cells washed, the lymphocytes were isolated by a gradient of Percoll (35% Percoll containing 100 U/ml heparin, Sigma, St. Louis, MO, USA). Purified lymphocytes were washed three times before culture.

2.6. Flow cytometric cytokine detection assays

Purified cells were incubated overnight at 37°C with 5% CO_2 in brefeldin A (2 μg/ml, Sigma, St. Louis, MO, USA). Cells were recovered and incubated for 30 min at 25°C with either FITC-, PE-, CyChrome-, or PerCP-conjugated monoclonal antibodies directed against CD4, CD8, CD3 and NK1.1 (Pharmingen, Boston, MA, USA). At the end of incubation, the cells were washed two times in PBS and fixed with 200 μl of 2% formaldehyde solution for 15 min at 25°C. The cells were washed two more times and 150 μl of permeabilization solution (0.005% of saponin, Sigma, St. Louis, MO, USA), in PBS solution with 5% of fetal calf serum, for 10 min. After incubation, cells were centrifuged for 5 min and the supernatants discarded. 2.5 μl of anti-cytokines monoclonal antibodies (anti-IFN-γ, tumor necrosis factor (TNF)-α, and IL-4, Pharmingen, Boston, MA, USA) diluted in permeabilization buffer were added for 30 min at 20°C. Cells were washed twice in PBS and 500 μl of PBS was added before analysis. Positive controls for cytokine production were cells from injected and non-injected animals stimulated with PMA/ionomycin (10/500 ng/ml, Sigma, St. Louis, MO, USA), and incubated with brefeldin 2 μg/ml (Sigma, St. Louis, MO, USA) overnight. Negative controls were incubated with irrelevant, isotype-matched antibodies in all experiments and with a background fluorescence not higher than 0.03%. Cell events were acquired by using gates set by forward and side scatter to determine the different proportions of positive cells. At least, 25 000 live lymphocytes were analyzed in order to determine the proportions of populations.

2.7. Immunohistochemistry

Immunohistochemistry was performed in aortic sinus sections. Tissue sections were pretreated with acetone and incubated with 2% gelatin in PBS at 37°C for 45 min in a humidified chamber. After washing with 0.1% Triton X-100 in PBS, sections were incubated with the appropriate antibody for 1 h and then washed three times. All antibodies were used at optimal dilutions determined by staining of spleen sections. The following monoclonal antibodies were used to detect the presence of TNF-α (anti-TNF-α, BD Pharmingen, 1:50), natural killer or natural killer T cells (NK and NK-T cells, anti-NK1.1, BD Pharmingen, 1:30), activated lymphocytes T, B and NK cells (anti-CD69, BD Pharmingen, 1:30), IgM (anti-mouse IgM, Sigma, 1:200) and IgG (anti-mouse IgG, Sigma, 1:800). Controls were performed with an irrelevant isotype-matched monoclonal antibody.

2.8. Statistical analysis

Data were expressed as mean ± S.D. Statistical analyses were performed using the analysis of variance (ANOVA) test. Differences were considered significant at $P<0.05$. All analyzed variables except triglycerides were normally distributed as assessed by the Kolmogorov–Smirnov test. Triglyceride values were logarithmically transformed to achieve an approximately normal distribution, and ANOVA was then applied to the transformed values.

3. Results

This study was designed to evaluate atherosclerotic lesion size and inflammatory responses in $apoE_0$ mice after LPS administration.

3.1. Effect of LPS injection on atherosclerotic lesion size and lipid metabolism

At 20 weeks of age, $apoE_0$ mice develop fibrofatty plaques with lipid-rich core regions covered by fibrous caps. Mice treated several times with LPS (LPS group) showed a 83%

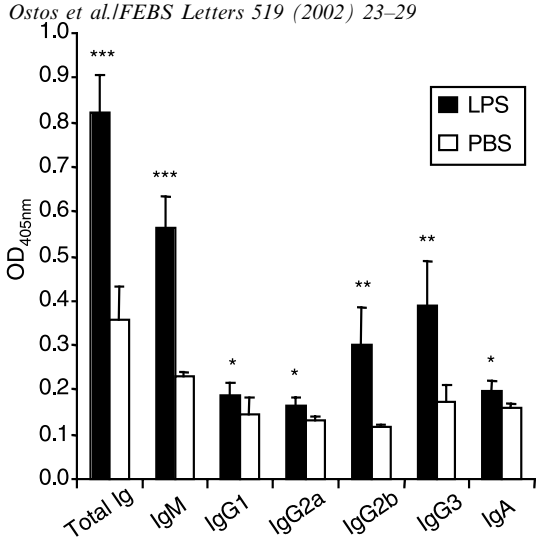

Fig. 1. Effect of LPS injections on plasma levels of autoantibodies directed against oxLDL. Total Ig and Ig isotypes were analyzed by enzyme-linked immunosorbent assay. OD indicates optical density. Data are expressed as mean ± S.D. ***$P < 0.0003$; **$P < 0.008$; *$P < 0.05$ LPS- vs. PBS-treated apoE$_0$ mice.

increase in lesion size, compared with the PBS-treated controls (PBS group) (219 100 ± 22 600 μm^2 versus 120 300 ± 22 600 μm^2; $P < 0.02$). Mice treated once with LPS (LPS1 group) showed also an increase in the lesion size but it was not statistically significant when compared with control mice (174 900 ± 73 100 μm^2 versus 120 300 ± 22 600 μm^2). The results obtained indicate that LPS injections promote atherogenesis. Triglyceride and cholesterol concentrations in plasma were significantly reduced in the LPS group compared with the PBS group. In contrast, no differences were observed between the LPS1 and the PBS groups (Table 1).

3.2. Detection of autoantibody response to oxLDL in LPS and in control PBS mice

The titer of autoantibodies directed against oxLDL was found to be significantly increased in the sera of LPS mice compared with that of the PBS counterpart (Fig. 1). In addition, the animals from the LPS1 group also presented a significant, although lower increase in the plasma concentration of anti-oxLDL antibodies (data not shown). We also found that the LPS mice have developed major titers of IgM, of the different isotypes of IgG and of IgA antibodies to oxLDL, than the PBS group (Fig. 1). These results suggest either a role for T cell help during the activation of the B cells in the autoimmune response against oxLDL, or a polyclonal activation of B cells induced by LPS.

Table 1
Plasma lipid concentration in LPS-, LPS1- and PBS-treated apoE$_0$ mice

	Cholesterol (mg/dl)	Triglycerides (mg/dl)
LPS	319 ± 12*	89 ± 10†
LPS1	403 ± 52	143 ± 46
PBS	396 ± 53	396 ± 53

Values are mean ± S.D. *$P < 0.05$, $^{\dagger}P < 0.02$ LPS- vs. PBS-treated mice.

3.3. Ex vivo detection of cytokines produced by lymphocyte subsets in blood, liver, spleen and thymus

The autoimmune response to oxLDL detected in the LPS- and PBS-treated apoE$_0$ mice prompted us to analyze the lymphocyte subsets that could mediate this response. To identify the distribution of the different subsets, three color stainings were conducted first in peripheral blood cells, with the follow-

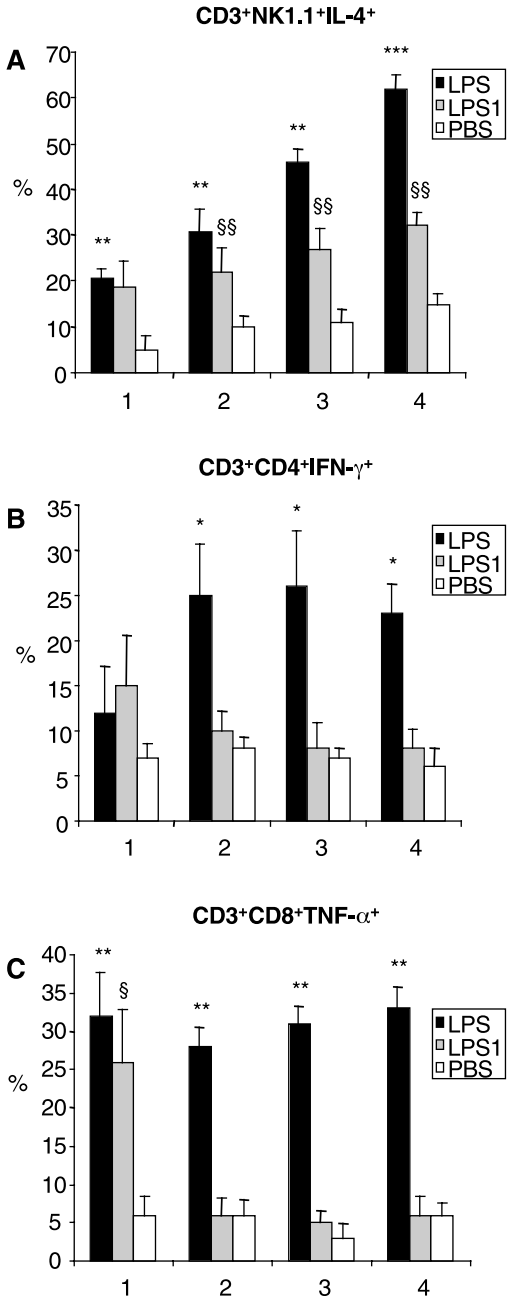

Fig. 2. Cytometric analysis of cytokines producing T cells in blood. Lymphocyte subsets of LPS-, LPS1- or PBS-treated apoE$_0$ mice were obtained as indicated in Section 2. Results are expressed as percentage of IL-4 positive cells among CD3$^+$NK.1.1$^+$ (A), of IFN-γ positive cells among CD3$^+$CD4$^+$ (B) and of TNF-α positive cells among CD3$^+$CD8$^+$ (C). 1, 2, 3 and 4 correspond to samples obtained 24 h after each injection in LPS- and PBS-treated mice. The LPS1 group of animals was injected once at the onset of the experiments. Data are expressed as mean ± S.D. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$ LPS- vs. PBS-treated mice. $^{\S\S}P < 0.01$; $^{\S}P < 0.02$ LPS1- vs. PBS-treated mice.

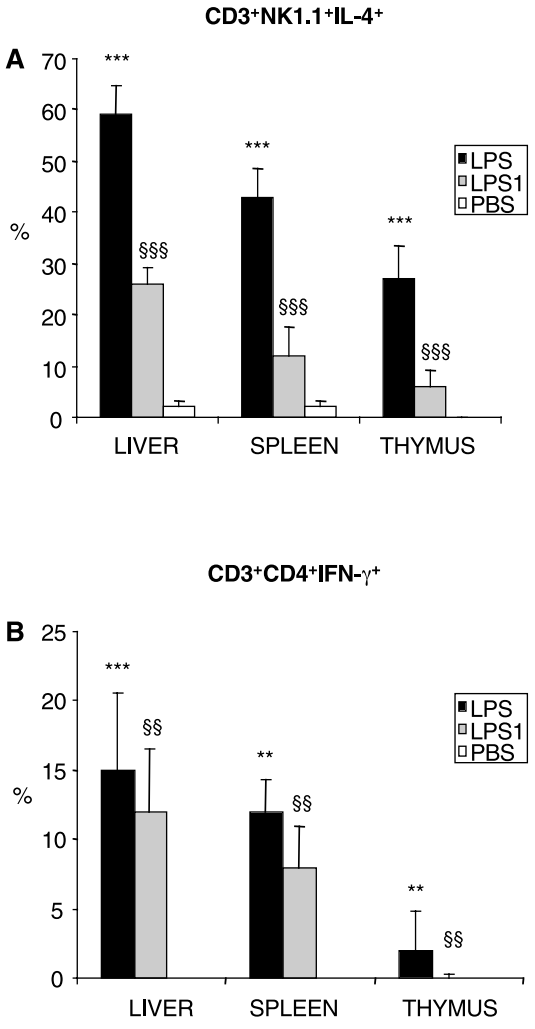

Fig. 3. Cytometric analysis of cytokine producing T cells in liver, spleen and thymus. At the end of the experiments, the organs were dissected and T cells purified. Results are expressed as percentage of IL-4 positive cells among $CD3^+NK1.1^+$ (A), and of IFN-γ positive cells among $CD3^+CD4^+$ (B). Data are expressed as mean ± S.D. $^{***}P < 0.001$; $^{**}P < 0.01$ LPS- vs. PBS-treated mice. $^{§§§}P < 0.001$; $^{§§}P < 0.01$ LPS1- vs. PBS-treated mice.

ing combinations: CD3/NK1.1/IL-4, CD3/CD4/IFN-γ and CD3/CD8/TNF-α. The proportion of lymphocyte subsets was (mean, range): CD4 35.2% (27–43), and CD8 23% (16–25.5), and concerning NK1.1 were 5.7% (2.9–8.1) in $apoE_0$ LPS-injected mice and 3.3% (0.9–3.3) in $apoE_0$ control mice. In wild-type C57Bl/6 LPS-injected or not, the proportions of lymphocyte subsets were not different from those obtained in control apoE-deficient mice. The results of cytokine production are shown in Fig. 2. In general, $apoE_0$ LPS mice showed an increase of cytokine producing cells, when compared with the PBS-injected $apoE_0$ (Fig. 2) or with the C57Bl/6 wild-type LPS-injected mice (data not shown). In particular, the proportion of IL-4 producing cells highly increased over the time after several injections of LPS (Fig. 2A). This was also observed in LPS1 and PBS–$apoE_0$ mice, but in a significant lower proportion (Fig. 2A). Interestingly, the vast majority of IL-4 producing cells in LPS animals were $CD3^+NK1.1^+$ cells. In contrast, the number of $CD3^+NK1.1^+$ cells producing IFN-γ was similar in the LPS and the PBS groups. An increase of other IFN-γ producing cells was observed in the LPS mice, compared with control mice (Fig. 2B). A similar increase was also detected in TNF-α producing cells (Fig. 2C). The phenotyping of these IFN-γ and TNF-α producing cells was $CD3^+CD4^+IFN\text{-}\gamma^+$ (Fig. 2B), $CD3^+CD8^+TNF\text{-}\alpha^+$ (Fig. 2C) and $CD3^+CD8^+IFN\text{-}\gamma^+$ (data not shown). Finally, IL-6 and IL-10 producing cells were not detected.

We also analyzed the frequency of cytokine positive cells in liver, thymus and spleen, organs in which lymphocytes normally develop. In all cases, a higher increase in cytokine producing resident cells was observed in LPS mice compared with control animals (PBS group and LPS C57Bl/6 mice). As in blood, the most prominent increase was detected in the $CD3^+NK1.1^+$ cells producing IL-4 (Fig. 3A). IFN-γ and TNF-α were also detected in all the tissues tested, and the phenotyping of their producing cells was $CD3^+CD4^+$ (Fig. 3B) and $CD3^+CD8^+$ (data not shown), as observed in blood.

All these results pointed out the enhanced production of pro-inflammatory cytokines and the T cell-dependent response following LPS injection in atherosclerosis-prone $apoE_0$ mice.

3.4. Lymphocyte-dependent cytokine production in atherosclerotic lesions

Immunohistochemical studies showed that LPS mice presented a higher number of NK1.1 cells and an enhanced IL-4 production in atherosclerotic lesion in LPS mice, compared with lesion in PBS mice. Both antibodies co-localized, indicating that NK1.1 cells were the principal cellular type implicated in the IL-4 plaque production (Fig. 4A–C). In contrast, $CD4^+$ cells producing IFN-γ were more abundant in plaques from PBS-treated mice, than in those of LPS mice (data not shown). Few NK-T cells (less than 5%) in both PBS- and LPS-treated mice were stained with IFN-γ monoclonal antibody (data not shown). Finally, lymphocytes producing TNF-α were more abundant in LPS mice (Fig. 4D).

The expression of CD69, a lymphocyte marker of recent activation, was analyzed in aortic sinus sections of LPS-treated mice and of PBS-treated control mice. We found that atheroma from LPS animals presented an accumulation of activated lymphocytes in the intima (Fig. 5A) that was not present in control mice. Nevertheless, we cannot rule out that the NK-T cells express constitutively the CD69 marker, as in liver. Interestingly, adventitia from treated mice showed also an important infiltration of CD69 expressing cells (Fig. 5A). Furthermore, LPS-treated mice showed in both intima and adventitia a high concentration of IgG and IgM (Fig. 5B,C). In adventitia, the disposition of these immunoglobulins was in aggregates resembling lymphoid follicular structures, which have been associated with advanced atherosclerotic plaques, evoking a late humoral immune response. These results are consistent with the fact that LPS animals presented more advanced atherosclerotic lesions. Even if the immunogens are unknown, they could be related to the high titers of autoantibodies directed against oxLDL detected in the plasma of treated mice.

4. Discussion

Atherosclerosis is a progressive disease considered as a form of chronic inflammation, resulting from the interaction between modified lipoproteins, mainly oxLDL, monocytes/mac-

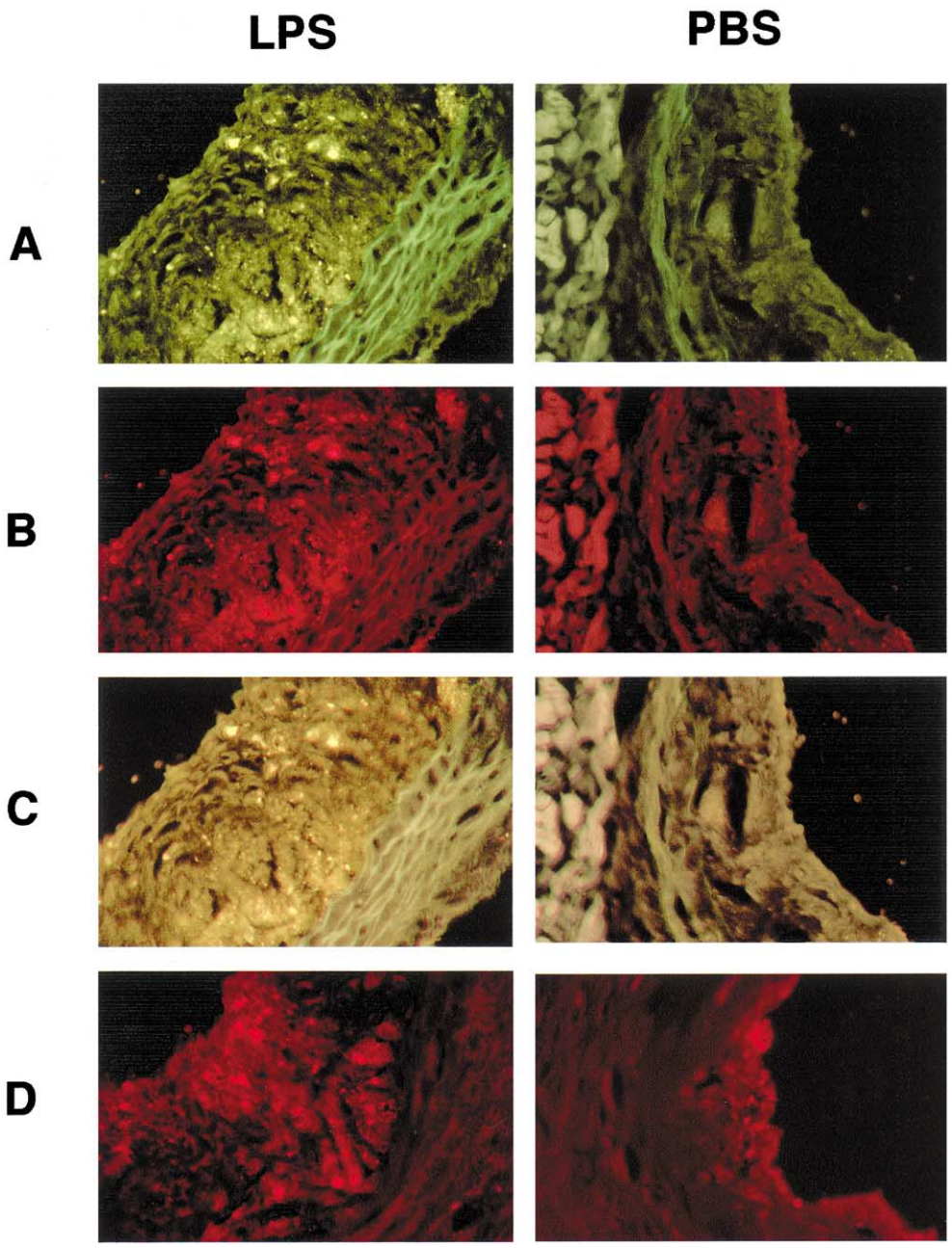

Fig. 4. Immunochemical study of atherosclerotic lesions in LPS- and PBS-treated apoE_0 mice. Heart sections were analyzed with antibodies directed against: NK1.1 (A), IL-4 (B), double staining NK1.1/IL-4 (C) and TNF-α (D). Original magnification ×400.

rophages and T cells with the components of the arterial wall. In the present report, we analyze the relationship between a chronic infection triggering inflammation, mimicked by LPS injection, and the development of atherosclerosis in apoE_0 mice. We have observed that repeated injections of LPS increase atherosclerotic lesion size, strongly suggesting that the activation of innate immunological system promotes atherogenesis.

The increased atherogenesis was observed in spite of a reduction in triglyceride and cholesterol plasma concentration. We do not known the reason of this reduction; but we can exclude that it was caused by liver toxicity, since the LPS dose used has been reported to be low hepatotoxic [18]. Recently, in experiments with rabbits submitted to repeated injections of LPS, a tendency in the reduction of these plasma parameters was also observed, but the high dispersion of the data did not allow the obtaining of statistically significant values [19].

It is well known that oxidative modifications in the lipid and apolipoprotein B components of LDL promote the formation of fatty streaks. In accordance, apoE_0 mice injected with LPS presented in plasma a higher titer of antibodies directed against oxLDL than the control counterparts. ApoE_0 LPS mice, but also PBS-treated mice developed IgM, IgG and IgA anti-oxLDL antibodies. This is compatible with a role of T cell help during the activation of the B cell response. Th1-

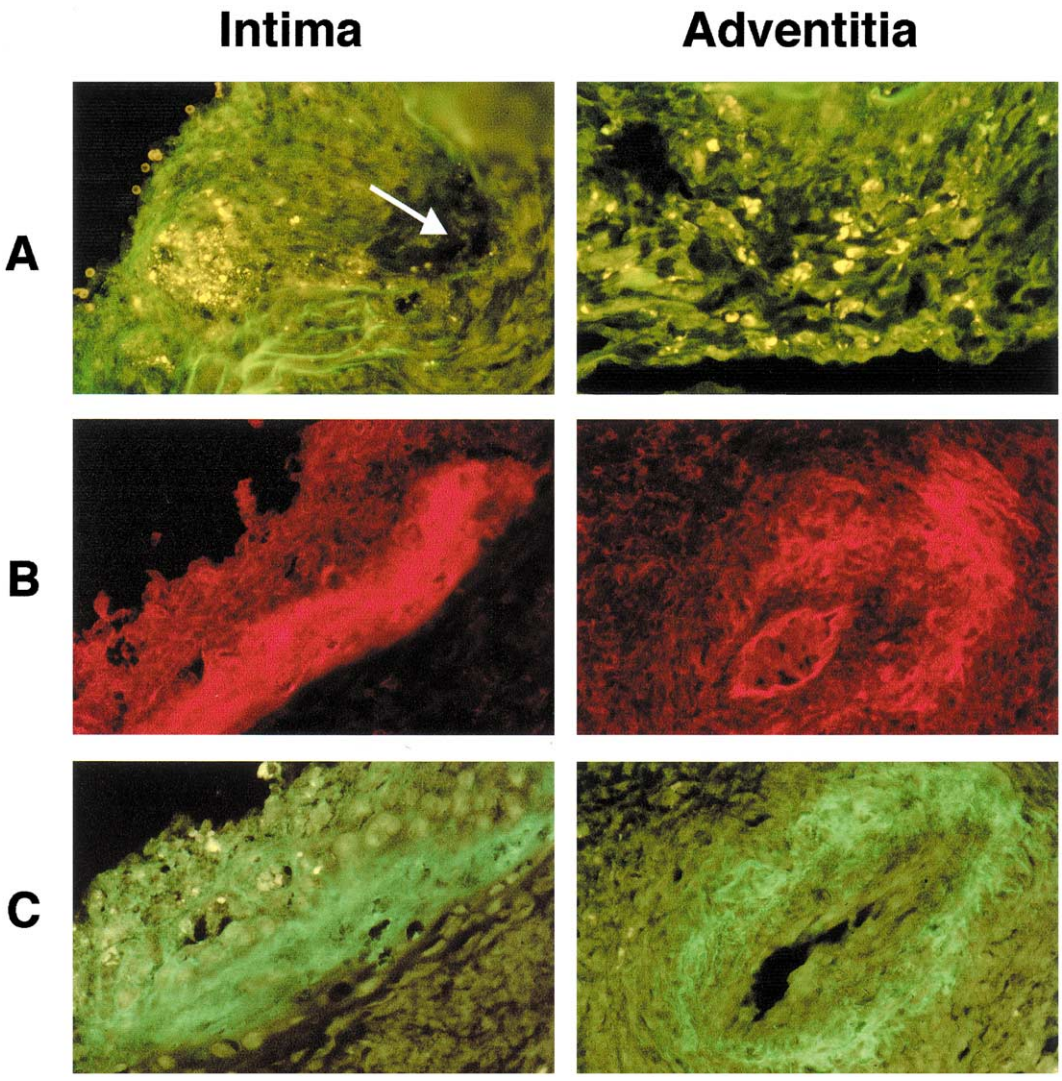

Fig. 5. Immunochemical study of intima and adventitia of atherosclerotic lesions in LPS-treated apoE_0 mice. Sections were analyzed with antibodies directed against CD69 (A), mouse IgG (B) and mouse IgM (C). Arrow indicates a breach in the media, binding the atherosclerotic components in contact with the adventitia. Original magnification ×400.

type cells stimulate B cells to synthesize IgG2a isotype whereas Th2-type T cells stimulate production of IgG1 antibodies [20]. As the concentrations of these two IgG subclasses of antibodies directed against oxLDL were enhanced in the plasma of LPS-injected mice, we can consider that, instead of a polyclonal activation of B cells, the two Th1- and Th2-type T cells are indeed involved in the antibody response against oxLDL. However, we cannot exclude that the increase in the levels of autoantibodies to oxLDL observed in LPS-injected apoE_0 mice could be partially explained by LPS-induced polyclonal B cell activation.

Th1 cells secrete the proatherogenic IFN-γ, Th2 cells provide help by secretion of IL-4 and both Th1 and Th2 cells secrete TNF-α [20]. To go deeper into the analysis of the lymphocyte subsets mediating the autoimmune response to oxLDL in LPS and control mice, we studied these subsets in cells of peripheral blood, liver, spleen and thymus of these animals. In each case, flow cytometric cytokine detection assays showed principally an increase of the IL-4 producing cells in LPS mice. The phenotyping of these cells indicated that the majority were NK-T cells, expressing the NK1.1 marker. NK-T cells are a well characterized subset of T cells in terms of morphology, phenotype, tissue distribution and cytokine production [21]. Several studies suggested a role for NK-T cells in the regulation of autoimmune diseases [22–25]. In our experimental conditions, the increase of IL-4 secreting NK-T cells could be correlated with an increase in the levels of autoantibodies against oxLDL. These results raise the possibility that NK-T cells themselves could become effector cells in the autoimmune manifestations of atherosclerosis, contributing to the tissue destruction mediated by T and B cells. In addition to the NK-T cells activity, the increase of CD4 and CD8 T cells secreting IFN-γ and TNF-α in LPS mice, when compared with control mice, confirms that both cell populations are also implicated in the pro-inflammatory process observed in the present atherosclerotic model.

In contrast to these results, we did not observe an immunological response in the wild-type C57Bl/6 mice after their

treatment with LPS. Thus, hypercholesterolemia, a property of apoE$_0$ mice, could modulate the immunological response to LPS. Similarly to our results, the repeated injections of LPS in hypercholesterolemic rabbits increased the monocytes percentage in blood and increased atherosclerotic lesions. However, normolipidemic rabbits did not develop atherosclerotic lesions and did not present an increase in monocyte percentage after LPS injection [19]. This suggests that the immunopathological consequences of an inflammation in the atherosclerotic process would become apparent when the cholesterol transport system is overloaded.

Our immunochemical studies indicated that the atherosclerotic plaques of the LPS mice are infiltrated mainly by NK1.1 cells secreting IL-4, a Th2 activity, and also T cells secreting TNF-α were observed principally in the plaques of LPS-treated mice. In contrast, no Th1 cells were detected in the LPS lesion, since CD4 cells secreting IFN-γ were mainly observed in the atherosclerotic lesion of the control apoE$_0$ mice. The detection of the early lymphocyte activation marker CD69 in the plaques suggests that the NK-T cells activity participates in the onset of immune dysfunction contributing to the atherosclerotic disease. Interestingly, Yamazaki et al. have recently described an elevated proportion of NK-T cells in chronic inflammatory periodontal disease tissues, which is caused by a group of Gram-negative bacteria [26]. They have postulated that NK-T cells were recruited to regulate an autoimmune response and that they may control cellular and humoral immune responses. However, NK-T cells are not a homogenous population and two major subsets have been described: CD4$^+$ and CD4$^-$ populations. These two subsets have different functional activities that may account for the role of these cells in atherosclerosis. CD4$^+$ subset has a protective role in autoimmune disease but CD4$^-$ NK-T cells play a pathogenic role by mediating cytotoxic activity in response to inflammatory stimulus. These different activities are mediated by unknown immunoregulatory mechanisms.

We have also found an important adventitial infiltrate of activated lymphocytes in LPS-treated mice. This was not observed in control mice. This phenomenon, named periaortitis, is produced when thinning or breaching of the media. As shown with the CD69 staining in the intima, the media is breaching in the LPS lesion, bringing the atherosclerotic components in contact with the adventitia. This infiltrate was described to be associated with advanced atherosclerotic plaques [27,28], and their structural organization is related to a late humoral immune response. This result is consistent with the fact that treated mice have twice as many autoantibodies against oxLDL. Interestingly, a greater level of anti-oxLDL has been found in patients with coronary atherosclerosis compared with that in healthy individuals [29]. Thus, a chronic infection mimicked by LPS injections provokes a more severe atherosclerosis in apoE$_0$ mice. This is accompanied by an increase in the titer of autoantibodies directed against oxLDL. Our results also underline the importance of NK-T cells and their cytokine secretion in the autoimmune manifestations of atherosclerosis.

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