Requisite Role for Interleukin-4 in the Acceleration of Fatty Streaks Induced by Heat Shock Protein 65 or Mycobacterium tuberculosis

Jacob George, Yehuda Shoenfeld, Boris Gilburd, Arnon Afek, Aviv Shaish, Dror Harats

Abstract—Atherosclerotic lesions can be induced in rabbits and mice immunized with heat shock protein 65 (HSP65). In the current study, we investigated the role of interleukin (IL)-4 in the HSP65- and Mycobacterium tuberculosis (MT)–induced models that exhibit an inflammatory phenotype. Fatty streak formation in IL-4–knockout (IL-4 KO) mice immunized with HSP65 or MT was significantly reduced when compared with lesions in wild-type C57BL/6 mice. However, when injected with control (HSP-free) adjuvant, no differences were evident in the lesion size between wild-type and the IL-4 KO mice. Next, we studied comparatively the extent of humoral and cellular immune responses to HSP65 in the IL-4 KO and wild-type mice, as those are thought to be influential in murine atherosclerosis. Anti-HSP65 antibody levels were reduced in the HSP65-immunized IL-4 KO mice as compared with their wild-type littermates, whereas no differences were evident between the groups with respect to the primary cellular immune response to HSP65. Other than the absence of IL-4 in the knockout mice, the pattern of secreting cytokines interferon-γ and IL-10 in concanavalin A–primed splenocytes was similar between the groups. HSP65-primed inguinal lymphocytes from IL-4 KO mice immunized with HSP65 secreted higher levels of interferon-γ (previously shown to be proatherogenic in vivo) as compared with their wild-type controls. 12-/15-Lipoxygenase expression, known to be regulated by IL-4 and to contribute to murine atherosclerosis, in the lesions was not influenced by the immunization protocol used or by IL-4 disruption. Thus, IL-4 may prove a principal cytokine in the progression of early “inflammatory” atherosclerotic lesions and may serve as a target for immunomodulation. (Circ Res. 2000;86:1203-1210.)

Key Words: atherosclerosis ■ autoantibodies ■ heat shock proteins ■ interleukins ■ Mycobacterium tuberculosis

Atherosclerosis is currently considered as a form of inflammation.1,2 Recent studies have shed light on the “autoimmune” view, which holds that modification of self-molecules in the vicinity of the lesion can elicit an immune response that can act to ameliorate or, alternatively, to amplify atherogenesis.3 Heat shock proteins (HSPs) include 25 molecules that are upregulated in response to various forms of stress.4 They have been shown to counter unfolding of proteins in response to damage, thereby serving a protective role.5 The conserved nature of these proteins between species has led to the proposition that cross-reactivity between bacterial and mammalian HSP could trigger autoimmune-like responses, examples of which include rheumatoid arthritis and diabetes mellitus.5,6 In much a similar manner, Xu et al7-9 have shown in a series of studies that HSP60/HSP65 can serve as an important autoantigenic determinant in atherogenesis. Thus, by immunizing with mycobacterial HSP65, they were able to induce arteriosclerotic lesions in normocholesterolemic rabbits. We have recently extended these findings to show that wild-type (WT) C57BL/6J mice immunized with HSP65 and fed a high-fat diet develop enhanced early lesions.10 Both the rabbit and the murine animal models can be induced by the use of HSP65 containing a heat-killed preparation of Mycobacterium tuberculosis (MT). Interestingly, studies from Kol et al11,12 have elegantly implied that bacterial and human HSP60 could both act to promote macrophage, endothelial cell, and smooth muscle cell activation, further supporting the link between HSP60/HSP65 and atherosclerosis.

Interleukin (IL)-4 is a key regulatory cytokine produced by T-helper-2 (Th2) lymphocytes, mast cells, and basophils and a subset of natural killer cells.13,14 It possesses in vivo and in vitro anti-inflammatory properties. As such, it inhibits interferon-γ (IFN-γ) production and IFN-γ macrophage activation, enhances differentiation of Th2 cells (which secrete other macrophage inhibiting cytokines, such as IL-10 and IL-13), and reduces procoagulant activity expression by activated endothelial cells (reviewed in References 13 and 14).

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In the current study, we investigated the role of IL-4 in the early atherosclerotic lesions induced by immunization with HSP65 or MT.

**Materials and Methods**

The procedures for the care and treatment of animals were approved by the institutional animal care and use committee (Sheba Medical Center).

**Animals**

Thirty female IL-4 knockout (IL-4 KO) and 30 control WT C57BL/6J mice were obtained from the Charles River Laboratories and kept at the local animal housing facility.

**Experimental Design**

All mice were maintained on a normal chow diet (containing 4.5% fat, 0.02% cholesterol) until the age of 6 weeks, when the immunizations were initiated. The female mice were matched for age before the experiments were performed. Three experimental protocols were tested, as follows. (1) Ten IL-4 KO and 10 WT C57BL/6J mice were injected with incomplete Freund’s adjuvant (IFA) 3 times every 3 weeks, similar to a protocol applied previously.10 (2) Ten IL-4 KO and 10 WT mice were injected with a heat-killed preparation of MT (5 mg/mL; 100 μg per animal) 3 times every 3 weeks. (3) Ten IL-4 KO and 10 WT C57BL/6J mice were injected with recombinant HSP65 (25 μg, 100 μL per animal) 3 times every 3 weeks.

Starting from the initial injection, a high-fat diet (Teklad Premier Laboratory Diet TD 90221) was given for 15 weeks until euthanization. The mice were bled from their retro-orbital plexus at baseline and at 4, 9, and 15 weeks after initiation of the atherogenic diet. On euthanization, the hearts were removed for analysis of atherosclerotic plaques in the aortic sinus.

**Cholesterol Level Determinations**

Total plasma cholesterol levels were determined by an automated enzymatic technique (Boehringer Mannheim).

**12-/15-Lipoxygenase (LO) Activity Assay**

Macrophages obtained from the peritoneal cavity by thioglycolate injection were plated on plastic dishes overnight and allowed to adhere. After scraping from the dishes, the cells were resuspended in PBS (4 to 5 x 10^6 cells/mL), and exogenous arachidonic acid was added to a final concentration of 100 μmol/L. Incubation was for 15 minutes at 37°C. The reaction was terminated by addition of equal volumes of a mixture of isopropanol and chloroform (1:1 by volume), and the lipids were extracted. The extracts were dried under vacuum, the lipids were reconstituted in 10 μL of methanol, and the aliquots were injected for HPLC analysis for estimation of 15S-HETE and 12S-HETE. Activity is expressed as percentage of positive control.

**Detection of IgG Anti-HSP65 Antibody Levels and Isotypes**

Recombinant HSP65 (1 μg/mL) in PBS (pH 7.2) was coated onto flat-bottomed 96-well ELISA plates (Nunc) by overnight incubation, and the assay was performed as previously described.10 IgG isotypes were determined using an ELISA kit (Southern Biotechnology).

**Proliferation Assays of Draining Lymph Node Cells From Immunized Mice**

Draining inguinal lymph nodes (taken 8 days after immunization) or splenocytes (taken on euthanization) were collected from 4 mice, immunized with HSP65, MT, and PBS, for the proliferation studies. The assays were performed as previously described.10 The results were computed as stimulation index, the ratio of the mean cpm with the antigen to the mean background cpm obtained in the absence of the antigen.

**Cytokine ELISA**

 Supernatants were collected from single-cell suspensions of inguinal lymph nodes or spleens of HSP65-immunized mice and were cultured at a density of 10^6/well in RPMI in the presence of concanavalin A (Con-A) or with HSP65. An ELISA kit (PharMingen) was used for detection of IFN-γ, IL-4, and IL-10 concentrations. Sera obtained at the end of the experiments were assayed for the presence of tumor necrosis factor (TNF)-β using an ELISA kit (PharMingen).

**Assessment of Atherosclerosis**

Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described.18 Total aortic lesion size represents the sum of the 2 measurements (cusp+free wall). Samples from both strains of mice and from the various experimental groups were treated the same with respect to their placement, sectioning, and orientation.

**Immunohistochemistry**

Cryostat sections (5 μm thick) of the aortic sinus were used for immunohistochemical analysis (for detection of CD3+ cells, macrophages, IFN-γ, IL-4, and vascular cell adhesion molecule-1 [VCAM-1] expression). The sections were fixed and blocked before incubation with biotinylated antibodies. Optimal dilutions were determined by staining of spleen sections. After washing, the slides were incubated in 0.3% H2O2 followed by additional rinses and developed with peroxidase streptavidin complex (DAKO). 12-/15-LO expression was detected using a polyclonal mouse anti-human 15-LO cross-reactive with mouse 12-/15-LO using a Histomouse kit.

**Statistical Analysis**

All 3 groups in the 2 experiments were compared using a 1-way ANOVA test. P<0.05 was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Cholesterol Levels**

Neither IL-4 gene disruption nor the different immunization protocols used influenced plasma cholesterol levels in the mice. Cholesterol levels in the IL-4 KO mice injected with IFA were 188±28 mg/dL in comparison with 194±19 mg/dL in the WT mice (P=NS). Cholesterol levels in the IL-4 KO mice injected with HSP65 were 202±25 mg/dL in comparison with 197±19 mg/dL in the WT mice (P=NS). Cholesterol levels in the IL-4 KO mice injected with MT were 204±19 mg/dL in comparison with 207±29 mg/dL in the WT mice (P=NS).

**12-/15-LO Activity in the Peritoneal Macrophages**

The enzyme activity was evaluated, as IL-4 has been shown to regulate 12-/15-LO production, thus possibly influencing atherogenesis. The activity of 12-/15-LO did not differ between macrophages from IFA-injected IL-4 KO and WT C57BL/6J mice, consistent with our previous observations. Similarly, no effect of HSP65 or MT immunizations was evident in the activity of 12-/15-LO in the respective macrophages as compared with cells from IFA-injected mice.

**IgG Anti-HSP65 Antibody Levels and Isotypes**

The extent of the humoral immune response to HSP65 was studied, as it may provide insight into the mechanisms contributing to the differences in lesion formation between IL-4 KO and WT mice. IgG anti-HSP65 antibody levels in
the mice immunized with HSP65 were higher in the WT C57BL/6J mice in comparison with their IL-4 KO littermates (Figure 1) throughout the study. IgG1 anti-HSP65 serum levels in the HSP65-immunized WT mice (mean optical density [OD] of 0.33 ± 0.6) were significantly higher in comparison with their IL-4 KO littermates (0.07 ± 0.03, \(P < 0.001\)). IgG2a and IgG2b levels did not differ significantly between HSP65-immunized WT mice (mean OD of 0.28 ± 0.09 and 0.33 ± 0.11, respectively) and their IL-4 KO littermates (mean OD of 0.22 ± 0.09 and 0.29 ± 0.1, respectively).

**Proliferative Response of Lymphocytes From HSP65-Immunized Mice**

As cellular immunity may play a role in atherogenesis, we have studied comparatively the extent of proliferation to HSP65 of lymph node cells from IL-4 KO and WT mice. Stimulation indices of lymph node cells from mice immunized with HSP65 were slightly higher in IL-4 KO, although not statistically significant (Figure 2). Proliferative response to a nonspecific antigen (BSA) was absent (mean 1.03 ± 0.12 for the WT and for the IL-4 KO immunized with HSP65).

**IL-4, IL-10, and IFN-\(\gamma\) Production**

As cytokines have been shown to influence murine atherosclerosis, we studied comparatively the lymphocyte secretion pattern between IL-4 KO and WT mice. Cultured supernatants from splenocytes were collected after 48 hours of Con-A stimulation. IL-4 levels were undetectable in the supernatants of all IL-4 KO mice regardless of the manipulation used. No significant differences in IL-4 concentrations were evident between the different WT groups (HSP65-immunized, 39 ± 8 pg/mL, compared with IFA-injected, 56 ± 21 pg/mL [\(P = 0.33\)]; MT-immunized, 38 ± 27 pg/mL, compared with IFA-injected, 56 ± 21 pg/mL [\(P = 0.45\); Figure 3A). Stimulation with control antigen (BSA) did not induce detectable IL-4 secretion. No significant differences were observed in IFN-\(\gamma\) levels between the WT and IL-4 KO mice (for IFA-injected mice, 1644 ± 169 pg/mL versus 1598 ± 155 pg/mL, respectively [\(P = 0.78\); for the HSP65-
immunized mice, 2225±727 pg/mL versus 1742±50 pg/mL, respectively \( [P=0.62] \); and for the MT-immunized mice, 977±156 pg/mL versus 896±515 pg/mL, respectively \( [P=0.42] \); Figure 3B). Stimulation with control antigen (BSA) did not induce detectable IFN-\( \gamma \) secretion. IL-10 concentrations in the supernatants of Con-A–primed splenocytes did not differ between the naive and knockout mice \( (P>0.05 \) for all group comparisons; Figure 3C). Stimulation with control antigen (BSA) did not induce detectable IL-10 secretion.

Priming of lymph node cells from HSP65-immunized mice with HSP65 resulted in production of significantly higher levels of IFN-\( \gamma \) in the IL-4 KO mice (993±57 pg/mL) as compared with the WT C57BL/6J mice (449±24 pg/mL; \( P<0.0001 \) (Figure 4). A similar trend was evident in MT-immunized mice (data not shown). Levels of IL-4 and IL-10 in the respective assays were below the detection threshold.

**TNF-\( \alpha \) Serum Levels**

TNF-\( \alpha \) serum levels in the IFA-injected C57BL/6J and the IL-4 KO mice were below threshold detection levels. In the HSP65-immunized mice, no statistically different values were obtained between the WT (mean±SEM, 4.3±1.2 pg/mL) and IL-4 KO mice (5±2.5 pg/mL) (Figure 5). TNF-\( \alpha \) serum levels were significantly higher in the MT-immunized mice in comparison with the HSP65-immunized mice. TNF-\( \alpha \) levels in the MT-injected WT mice were significantly higher (16.0±4.1 pg/mL) in comparison with the levels in the IL-4 KO mice (7.5±0.5 pg/mL; \( P<0.01 \) (Figure 5).

**Fatty Streak Formation and Immunohistochemistry**

Our previous experience has shown that injection of HSP65-free IFA did not influence fatty streak development in C57BL/6J mice fed a high-cholesterol diet. In the current study, mean aortic sinus lesion size in the IFA-injected WT mouse was not significantly different (10,625±6250 \( \mu m^2 \)) from the lesion size of their IL-4 KO littermates (12 500±1875 \( \mu m^2 \); \( P=NS \)) (Figure 6A). Fatty streak formation was significantly larger in the WT mice immunized with HSP65 (39 000±5500 \( \mu m^2 \)) as compared with the IL-4 KO mice (8750±3750 \( \mu m^2 \); \( P<0.0001 \) (Figure 6B). Atherosclerotic lesions were also significantly enhanced in the MT-immunized WT mice (30 500±7500 \( \mu m^2 \)) in comparison with their IL-4 KO littermates (15 000±5200 \( \mu m^2 \); \( P<0.05 \) (Figure 6C). Lesions were not evident in any sites other than the aortic sinuses in any of the animals.

When compared with IFA-injected IL-4 KO mice, lesion size from HSP65- or MT-immunized IL-4 KO mice was not
significantly different ($P=0.39$ and $P=0.66$, respectively). Fatty streak formation was significantly increased in HSP65- and MT-immunized WT mice in comparison with their IFA-injected WT littermates ($P<0.01$ and $P<0.05$, respectively), in accord with our previous observations. 10

Aiming to gain further insight into the factors mediating the effects of IL-4 gene deletion on atherosclerosis, we have made serial immunohistochemical studies. Lesions in the MT, HSP65, and IFA groups were infiltrated with macrophages to an extent that correlated with lesion size. CD3-positive cells were found only in the lesions of MT- or HSP65-immunized mice (1 to 3 per aortic sinus) and did not differ between the WT and the knockout mice. Lesions from IL-4 KO mice immunized with HSP65 or MT exhibited a significantly more pronounced staining of IFN-$\gamma$ (mean percentage of plaque coverage of $42\pm12\%$) in comparison with their respective WT littermates ($14\pm6\%; P<0.01$) (Figure 7). No differences were evident between IL-4 KO ($11\pm5\%$) and WT ($15\pm7\%$) mice injected with IFA with respect to IFN-$\gamma$ staining. IL-4 was not detectable in lesions from all mouse groups.

VCAM-1 expression was more pronounced in the lesions of the WT mice immunized with MT (mean percentage of plaque coverage of $58\pm13\%$) in comparison with their IL-4 KO littermates ($35\pm12\%, P<0.05$) (Figure 8). Similar observations were noted when VCAM-1 staining was compared between WT mice immunized with HSP65 ($68\pm9\%$) and IL-4 KO mice immunized with HSP65 ($28\pm9\%, P<0.05$).
Figure 8. VCAM-1 expression in the aortic sinus. VCAM-1 staining was assayed by immunohistochemistry using a rat anti-mouse anti-VCAM-1 monoclonal antibody. Representative sections are provided showing that VCAM-1 was expressed more profoundly in aortic sections of WT mice immunized with MT as compared with their IL-4 KO litters. HSP65-immunized WT mice were also found to exhibit a more pronounced VCAM-1 staining when compared with their IL-4 littermates. IFA-injected mice, either WT or IL-4 KO had similar levels of VCAM-1 expression. A quantitative assessment is given in the Results section (magnification ×200).
No significant differences in VCAM-1 expression were evident between WT (32±15%) and IL-4 KO 36±14%) mice injected with IFA (Figure 8).

Discussion

We have previously established in C57BL/6J mice a model in which fatty streak formation is accelerated by immunization with recombinant HSP65 or with MT (rich in mycobacterial HSP65).10 The advantage of this model over the conventional C57BL/6J mouse model is its more complex nature, incorporating active participation of the immune system. Thus, it appeared justified to explore the role of the classic Th2 cytokine IL-4, comparatively, in 3 apparently different models.

We have found that the lack of IL-4 in the C57BL/6 mouse resulted in reduced fatty streak size in the MT- and HSP65-immunized but not in the control (IFA)-immunized animals (Figure 6). Moreover, we observed that deletion of the IL-4 gene did not enable fatty streak enhancement in MT- and HSP65-immunized mice, suggesting that IL-4 was essential for induction of lesions that are “HSP65 driven.”

Although IL-4 is considered an anti-inflammatory cytokine, it plays opposing roles in models of autoimmune diseases in animals: IL-4 has been shown to suppress experimental allergic encephalomyelitis16 and spontaneous diabetes in nonobese diabetic mice,17 whereas its absence did not influence the induction of granulomatous experimental autoimmune thyroiditis18 and autoimmune myasthenia gravis.19 On the other hand, administration of IL-4 was shown to exacerbate experimental autoimmune uveoretinitis20 and adjuvant-induced arthritis.21 The latter model is similar to ours in the sense that it involves an HSP65-induced response culminating in an organ-specific autoimmune disease. In both our study and the adjuvant arthritis model,21 IL-4 appeared to play an enhancing role in disease progression.

IL-4 is a principal cytokine that drives commitment of T cells toward the Th2 phenotype.22 Thus, it is not surprising to observe that the lack of this central cytokine was associated with a significantly reduced level of anti-HSP65 antibodies, evident throughout our study. The pathogenesis of HSP65-induced arteriosclerosis in rabbits8 and mice10 is still unresolved. Indeed, data exist to support a role for the humoral and cellular immune system in mediating the effect. Accordingly, anti-HSP65 antibodies have been found to associate with established carotid atherosclerosis,7 predict mortality,23 and mediate endothelial cytotoxicity,24 thus promoting a proatherogenic effect. Hence, the reduced anti-HSP65 antibody levels in the IL-4 KO mice could provide a partial explanation for the reduced lesion formation in mice immunized with mycobacterial HSP65. However, similar to our previous observations,10 anti-HSP65 levels in the MT-immunized mice were negligible, and no difference was found between the IL-4 KO mice and their WT littermates. These findings suggest either that different mechanisms may govern lesion formation in HSP65- and MT-immunized mice or that anti-HSP65 antibodies are produced and subsequently consumed into immune complexes after induction of hyperlipidemia.

TNF-α is a proinflammatory cytokine, and experimental data suggest that its blockade in mice could ameliorate atherosclerosis.25 TNF-α levels were increased in the sera of the MT-immunized WT mice in comparison with the IL-4 KO mice (Figure 5), whereas its levels were low and similar in the respective groups immunized with HSP65. These findings support the occurrence of differential mechanisms regulating lesion acceleration in the MT- and HSP65-induced models. As TNF-α could play a role in enhancing early atherogenesis, the lower levels in the MT-immunized IL-4 KO could partly account for the reduced lesion size in these animals. Furthermore, in vitro data suggest that TNF-α and IL-4 act synergistically on vascular endothelial cells26 and smooth muscle cells27 to increase VCAM-1 expression. As this adhesion molecule is important for monocyte attraction in early atherogenesis, the lack of IL-4 “drive” could result in its reduced expression. Indeed, we have observed by immunohistochemistry that lesions from the IL-4 KO mice immunized with MT as well as HSP65 exhibited significantly less VCAM-1 expression in comparison with lesions from WT C57BL/6 mice (Figure 8).

The cytokine-secreting pattern of lymphocytes stimulated with either Con-A or a control antigen did not differ between the knockout and the WT mice of all mouse groups. However, when stimulated with HSP65, IL-4 KO lymphocytes secreted significantly higher amounts of IFN-γ (Figure 4), suggesting that the HSP65-driven response had shifted toward the Th1 phenotype. Further supporting the occurrence of a Th2-Th1 shift was the observation that IFN-γ was significantly expressed within lesions from IL-4 KO mice immunized with MT or with HSP65 when compared with the respective litters. Also, the IgG1 anti-HSP65 population was nearly absent in the IL-4 KO mice immunized with HSP65. It has recently been shown that hypercholesterolemia and lesion progression in the apolipoprotein E–knockout mouse are associated with a Th1-to-Th2 switch.28 If the change in the phenotypic nature (during lesion progression) is of a prime role in the development of early atherosclerotic lesions, this may be consistent with our results showing reduction of immunologically exacerbated lesions after the Th2-to-Th1 shift.

It is important to note that, whereas lesions of rabbits immunized with HSP65 contain abundant CD3 lymphocytes, fatty streaks in C57BL/6 mice contain significantly smaller numbers. Thus, lesional lymphocytes (the predominant producers of IL-4) found in C57BL/6 mice are not likely to explain the effect on lesion size in the HSP65- and MT-immunized mice as compared with the IFA-immunized mice.

In addition to its immunomodulatory role, IL-4 has a role in governing the production of the enzyme 12-/15-LO.29 This enzyme oxygenates polyenoic fatty acids esterified to various membrane lipids and lipoproteins.30 Hence, 12-/15-LO may play a principal role in enhancing atherosclerosis. IL-4 (as well as IL-13) induces upregulation of murine macrophage 12-/15-LO activity, implicating involvement of the transcription factor signal transducer and activator of transcription (STAT) 6. In the present study, we observed that IL-4 deficiency did not influence the induction of 12-/15-LO activity in macrophages or its expression within the lesions in the different models, thus reducing the likelihood of its playing a principal role.
In conclusion, our observations imply that the Th2 cytokine IL-4 plays an essential role in the acceleration of fatty streaks obtained after immunization with HSP65 and MT ("inflammatory"-type lesions) in C57BL/6 mice. However, IL-4 deficiency failed to influence lesion formation in IFA (control)—injected mice. These findings require further validation in other murine models, the lesions of which are more lymphocyte-rich. If IL-4 is established as a target for immunomodulating strategies, its blockade may be applied for halting progression of atherosclerosis.

References
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