Inflammatory processes are an integral component to atherosclerotic lesion development. Cytokine-mediated proinflammatory responses such as endothelial cell activation and leukocyte recruitment are thought to positively contribute to the atherogenic process. One of the best-studied proinflammatory cytokines is tumor necrosis factor-α (TNFα), which is expressed both in human and rodent atherosclerotic plaques (1–5). However, the physiological role of TNF ligand and receptor family members in the atherogenic process remains unclear.

TNFα and lymphotoxin-α (LTα) are two predominant members of the TNF ligand family. Their structural genes are located on human chromosome 6 within the major histocompatibility complex (6). TNFα and LTα proteins are structurally similar and display 50% amino acid homology (7). TNFα is first synthesized as a type II transmembrane protein and is subsequently cleaved to form circulating homotrimeric TNFα (8). TNFα is synthesized primarily by activated macrophages (9), although under appropriate stimulation other cells can express this cytokine (10–12). TNFα influences the functions of macrophages, smooth muscle cells, and endothelial cells (13), which are major cell types observed in plaques. LTα is synthesized primarily by activated T and B lymphocytes (6, 7) and is also found in the circulation as a homotrimer. Unlike TNFα, membrane-bound homotrimeric LTα has not been observed. The presence or function of LTα in atherosclerotic lesions has not been previously investigated.

Homotrimeric TNFα and LTα elicit responses through two receptors termed p55 and p75 (6, 14). The p55 receptor activates the majority of responses associated with TNFα including induction of adhesion molecule expression (15, 16), apoptosis (17, 18), and resistance to bacterial infection (19, 20). In an earlier report we showed that p55 receptor deficiency in mice results in increased atherosclerotic lesion development, demonstrating that signaling through this receptor is atheroprotective (21). Activities associated with p75 activation include induction of T cell proliferation (22, 23), induction of TNFα-mediated skin tissue necrosis (24), and modulation of TNFα-mediated pulmonary inflammation (25).

In this report, we investigated whether TNFα- or LTα-mediated responses alter lesion growth. Control mice or mice deficient for either TNFα or LTα were fed an atherogenic diet, and the presence of these ligands within the lesions was examined. Confirming other reports, we observed TNFα in the atherosclerotic lesions. Surprisingly, however, TNFα deficiency did not alter lesion size. This brings into question the generally held concept that TNFα promotes atherogenesis. Furthermore, loss of LTα resulted in a 3-fold decrease in lesion size. These findings demonstrate that LTα is the predominant member of the TNF ligand family that elicits proatherogenic responses. Since loss of LTα decreased lesion growth but loss of the major TNF receptor, p55, resulted in increased atherosclerosis, we hypothesized that the p75 receptor was involved with regulating LTα responses to promote atherogenesis. However, we show that loss of p75 did not alter lesion development. The disparity between the responses observed with ligand-deficient versus receptor-deficient mice illustrates the complexity of this cellular signaling system and suggests that there are alternative TNF ligand/receptor molecules involved with regulating lesion growth.
EXPERIMENTAL PROCEDURES

Mice—Female C57BL/6CR mice, age of 6 weeks, were purchased from Charles River Breeding Laboratories and used as the wild-type control strain for studies involving receptor-deficient mice. Mice lacking either the TNF receptor p55 (p55−/−), p75 (p75−/−), or both receptors (p55−/−p75−/−) have been described previously (25). The p55−/− mice were developed directly in C57BL/6CR and are an inbred strain. The p75−/− mice were genotyped and p75−/− mice were used as the wild-type control strain for the studies involving TNFα receptor-deficient mice (TNFα−/−) and lymphoxygen-α-deficient mice (LTα−/−). Female C57BL/6CR mice, age of 6 weeks, were purchased from The Jackson Laboratory. The C57BL/6J mice were used as the wild-type control strain for the studies involving ligand-deficient mice as both the TNFα−/− and LTα−/− mice are maintained on the C57BL/6J genetic background. Mice were bred to generate colonies of each gene knockout strain here at the University of Washington. F2 and F3 offspring were used for the experiments presented in this report. Apolipoprotein E-deficient male mice (apoE−/−) were purchased from The Jackson Laboratory. Mice were maintained in a temperature-controlled (25 °C) facility with a strict 12-h light/dark cycle and given free access to food and water. Blood was collected after a 4-h fast from the retro-orbital sinus into tubes containing 1 mM EDTA, and plasma was stored at −20 °C prior to analysis.

Study Design—Two experiments were performed for this study. In experiment 1, wild-type, TNFα−/− or LTα−/− female mice were fed an “atherogenic diet” containing 15% fat, 1.25% cholesterol, and 0.5% sodium cholate (diet No. TD90221, Harlan Teklad) (26). This diet induces fatty streak lesions in the aortas of susceptible mice (27, 28). Mice were maintained on this diet for 16 weeks before quantifying lesional areas in experiment 1, and wild-type and TNF receptor-deficient mice were fed the atherogenic diet for 18 weeks before quantifying lesion development. For both studies, an additional set of female mice were fed a rodent chow diet (Wayne Rodent BLOX 8604, Harlan Teklad) for 16–18 weeks prior to analyzing plasma lipids and lesion areas. ApoE−/− mice were maintained on a rodent chow diet until 22 weeks of age before they were evaluated for atherosclerotic lesions and the presence of TNFα or LTα.

Plasma Analysis—Total cholesterol and triglycerides were determined using established colorimetric assays as described (29, 30) (kits 1127578 and 450032, Roche Molecular Biochemicals). Plasma lipoproteins were separated by FPLC gel filtration using a Superose 6 column (Amersham Biosciences). A 100-μl aliquot of plasma from each of 3–4 mice per diet group was separated at a flow rate of 0.2 ml/min using phosphate-buffered saline, 100-μl aliquots from each of 0.5-ml fractions were used for cholesterol determinations.

Aortic Sinus Lesion Quantification—Aortic sinus lesion areas were quantified as described (21, 29). Hearts were removed from mice, perfused with phosphate-buffered saline, and formalin fixed using a 4% neutral formalin solution. After removing peripheral fat, the heart was sectioned directly under and parallel to the atrial leaflets. The upper section was incubated in phosphate-buffered saline containing 30% sucrose for 18 h and then embedded in O.C.T. embedding medium and frozen. Every other section (10-μm thick) throughout the aortic sinus was taken for analysis. Sections were evaluated for fatty streak lesions following lip staining with oil red O and nuclei staining using hematoxylin. Lesion area measurements were analyzed using the Optimas Image Analysis Software Package (BioScan).

Immunohistochemistry—Frozen sections were fixed in acetone and endogenous peroxidase quenched by incubating slides in 3% hydrogen peroxide. Samples were then incubated with either a polyclonal anti-TNFα antibody (RDI-TNFAbr1, Research Diagnostics, Inc.) at 1:30 dilution or with a polyclonal anti-LTα antibody (RDI-TNFABbr1, Research Diagnostics, Inc.) at 1:30 dilution. After rinsing samples were incubated with a biotinylated secondary antibody (BA1000, Vector Laboratories) at 1:200 and binding detected using streptavidin-horseradish peroxidase and AEC detection as described above. Deletion of either the primary or secondary antibody resulted in minimal to no staining.

Statistical Analysis—Values are reported as mean ± S.E. Nonparametric Wilcoxon signed ranks tests were used to determine differences in lesion areas. The Student’s t test was used to compare independent means in some cases. p < 0.05 was accepted as statistically significant.

RESULTS

LTα Is Expressed in Atherosclerotic Lesions—Aortic sinus lesions from atherogenic diet-fed wild-type female mice and chow-fed apoE−/− mice were evaluated for the expression of TNFα and LTα. Lesions from atherogenic diet-fed mice are comprised predominantly of macrophages with small amounts of T and B cells present (31–33). In contrast, lesions from apoE−/− mice contain macrophages, smooth muscle cells, and T and B cells (34, 35). Thus, these two systems provide examples of early simple fatty streak lesions and more complex lesions as observed in human atherosclerosis (36). TNFα immunostaining was observed in lesions from both of these models (Fig. 1). Staining was punctate indicating cell-associated protein expression and was also observed within the medial smooth muscle cell layer under the lesioned sites. Surprisingly however, LTα staining was also observed in lesions from both of these models (Fig. 1). Immunostaining was observed throughout the lesions and medial layers. In general, the staining was more diffuse than the staining observed for TNFα. We next confirmed that the immunoreactivity we observed did not simply reflect cross-reactivity of these antibodies to the different ligands. Lesions from atherogenic diet-fed TNFα−/− and LTα−/− mice were immunostained for TNFα and LTα (Fig. 2). Results demonstrate that lesions from TNFα−/− mice did not show immunoreactivity against the TNFα antibody, and lesions from LTα−/− mice did not show reactivity against the LTα antibody. Furthermore, loss of one ligand did not impede the expression of the other ligand within these lesions. B and T cells but not monocyte/macrophages are the predominant cell types to secrete LTα (37). We investigated whether LTα expression was limited to a specific cell type within the lesions. Lesions from apoE−/− mice were immunostained for macrophages using the CD11b marker, for T cells using the CD3 marker, and for LTα (Fig. 3). Results show that lesions from apoE−/− mice contain both of these cell types and that LTα expression is not confined to a specific cell type. The diffuse nature of LTα immunostaining suggests that this protein is secreted from one of these cell types and has become trapped within the extracellular matrix and necrotic regions of these lesions. Further investigation about the cellular source of LTα within the lesion remains to be determined.

Loss of LTα but Not TNFα Decreases Atherosclerosis in C57BL/6 Mice—To determine whether TNFα or LTα has a physiological role in lesion development we determined whether loss of these genes would influence diet-induced atherosclerosis. Aortic lesion areas were quantified for wild-type, TNFα−/−, and LTα−/− female mice fed the atherogenic diet for 16 weeks (Fig. 4). Surprisingly, loss of TNFα did not alter lesion sizes (10.9 ± 2.4 mm² × 10³, n = 18) as compared with wild-type mice (11.5 ± 2.3 mm² × 10³, n = 11). In contrast, lesion areas in LTα−/− mice were reduced (4.3 ± 1.4 mm² × 10²).

FIG. 1. Lipid and immunohistochemical staining of aortic sinus lesions from atherogenic diet-fed wild-type mice and chow-fed apoE−/− mice (top and bottom, respectively). Lesions were stained for lipids using oil red O (ORO, A and B), for TNFα (C and D), or for LTα (E and F). Original magnification was ×200.
Role of TNFα and LTα in Atherosclerosis

10^3, n = 13 and p = 0.0128). These data are consistent with the idea that LTα is the primary TNF family ligand involved with atherosclerosis.

**Lipid Levels Are Lower in LTα−/− Mice**—To understand why lesion areas were reduced in LTα−/− mice, plasma lipid and lipoprotein profiles were analyzed. As compared with atherogenic diet-fed wild-type mice, cholesterol levels were 46% higher in TNFα+/− mice (p = 0.10 versus wild-type mice) and 20% lower in LTα−/− mice (p = 0.05). Final values were 189 ± 19 mg/dl for wild-type, 276 ± 42 mg/dl for TNFα+/−, and 151 ± 6 mg/dl for LTα−/− mice. Total cholesterol levels did not correlate with lesion areas for any of the strains fed the atherogenic diet. Triglyceride levels were 11–16 mg/dl for all atherogenic diet-fed mice, and no significant differences were observed among strains.

To determine whether lipids were redistributed into different lipoprotein particles in atherogenic diet-fed TNFα+/− or LTα−/− mice, FPLC profiles of lipoproteins were examined (Fig. 5). No differences in the proportion of cholesterol in the VLDL/LDL to HDL fractions was observed between wild-type and TNFα+/− mice. That is, the increase in total cholesterol levels observed in TNFα+/− mice reflects a proportional increase in both VLDL/LDL and HDL lipoprotein fractions. In contrast the relative amount of total cholesterol found in the HDL fraction tended to be increased for LTα−/− mice. The combination of lower total cholesterol and higher relative amounts of cholesterol found in the HDL fraction likely accounts at least partially for the reduced lesion development observed for LTα−/− mice.

Loss of p55 but Not p75 Increased Atherosclerosis in C57BL/6 Mice—Mice deficient for p55 receptors display a 2.3-fold increase in diet-induced atherosclerosis as compared with wild-type mice (21). Since neither the LTα−/− nor the TNFα+/− mice recapitulated these results, we hypothesized that signaling via the p75 receptor influences lesion development. Wild-type, p55−/−, p75−/−, and p55+/−p75−/− female mice were fed the atherogenic diet for 18 weeks, and aortic lesion areas were quantified (Fig. 6). Results demonstrate that loss of p55 resulted in a 2.4-fold increase in lesion size, confirming our previous result (21). Loss of p75 did not alter lesion development, and loss of both p55 and p75 receptors resulted in lesion areas comparable with those observed for p55−/− mice. No significant differences in plasma total cholesterol, HDL cholesterol, or triglycerides were observed between genotypes (data not shown). Thus the p55 receptor signals events that retard lesion development, whereas p75 signaling does not influence lesion development.

**DISCUSSION**

This study provides several important new findings about the role of TNF signaling pathways in regulating atherosclerotic lesion development. This is the first report demonstrating that LTα is expressed in atherosclerotic lesions and that loss of this cytokine reduces lesion size. Surprisingly, loss of TNFα, which is involved with numerous proinflammatory responses, did not alter lesion development in atherogenic diet-fed mice. Loss of p55 receptors, but not p75 receptors, resulted in increased diet-induced atherosclerosis showing that the p55 receptor has the predominant role in regulating lesion growth. Taken together these results illustrate the complexity of TNF ligand and receptor interactions in modulating inflammatory responses such as those observed during lesion growth. Furthermore, since the ligand deficiency did not recapitulate the responses observed with the receptor deficiency it suggests that there are undefined members of the TNF ligand or receptor signaling pathway involved with regulating atherogenesis.

The observations that LTα is expressed within atherosclerotic lesions and deficiency of this protein retards lesion development suggests that there may be a direct function of LTα in...
promoting atherogenesis. LTα is produced primarily from T and B cells (37). Both of these cell types have been identified in atherosclerotic lesions with the extent of their presence dependent on the animal model and on the stage of lesion size or complexity (35, 38–41). The role of these cell types in lesion development has been intensively studied. For example, RAG−/− mice have impaired T and B cell function such that T lymphocytes do not mature into CD4+ helper or CD8+ suppressor cells, and B cells are unable to synthesize immunoglobulin (42). When atherosclerosis-susceptible apoE−/− mice were crossed with RAG−/− mice a 2-fold decrease in atherosclerotic lesions was observed, suggesting that T and B cell function promotes lesion growth (43). However, this response was not observed when RAG−/− mice were studied (44). When T cell-deficient nude (nu/nu) mice were fed an atherogenic diet lesion areas were reduced 90% as compared with control mice (45). Therefore, most but not all studies implicate a proatherogenic role for lymphocytes. Our results are consistent with this concept and suggest that T and/or B cells are secreting LTα within the developing lesion to promote atherogenesis.

Several important functions of LTα have recently been identified that demonstrate a significant role for this cytokine in lymphocyte activation and proliferation. LTα promotes inflammatory and chemotactic responses (46–48) and B cell proliferation (49). LTα is also involved with CD8+ T cell activation (50). LTα-deficient mice have altered immune function including an absence of peripheral lymph nodes, abnormal Peyer’s patches, and no germinal centers (51, 52). The decreased atherosclerosis we observed in the LTα−/− mice supports the concept that normal leukocyte activity promotes atherogenesis. By identifying which LTα-mediated processes are actually proatherogenic we will have a more focused target for designing antiatherogenic therapies.

The loss of LTα also resulted in improving total cholesterol levels and lipoprotein profiles. These findings suggest that a primary mechanism of reduced lesion growth in the LTα−/− mice may be through changes in plasma lipid levels. The influence of LTα in regulating plasma lipid levels has not yet been investigated but should provide us with new insights about how cytokines influence lipid metabolism.

Our results suggest that LTα may be signaling through receptors unique from the p55 or p75 receptor to promote atherogenesis. LTα is expressed in two forms. It is found in the circulation as a homotrimer, or it can form heterotrimers with membrane-bound LTβ1 a third member of the TNF ligand family (7, 53). In fact Mackay et al. (54) have shown that LTα homotrimers displayed a 50–200-fold lower Kd for p55 receptor binding as compared with TNFα. This suggests that circulating LTα is less efficient at activating the p55 receptor. In contrast, LTαLTβ heterotrimers effectively bind and activate the LTβ receptor to induce inflammatory and cytotoxic responses (54–56). We propose that the proatherogenic responses mediated by LTα result from signaling events mediated primarily by the LTβ receptor. The LTβ receptor is expressed on multiple tissues and has a distribution pattern similar to that observed for the p55 receptor (7). Inhibition of this pathway by deleting the LTα gene may reduce LTβ receptor-induced inflammatory events resulting in reduced lesion development.

The lack of effect of TNFα deficiency on lesion development was surprising given the many roles of TNFα on mediating proliferative and inflammatory responses (13). Our findings suggest that the presence of TNFα within atherosclerotic lesions may simply represent a marker of inflammatory responses but may not be the actual inducing mediator of inflammatory events within the growing lesion. These findings lead us to question the commonly held notion that the presence of TNF in lesions signifies proatherogenic responses. In fact, based on our findings we suggest that caution be used in attributing TNFα presence to a deteriorated atherosclerotic environment.

The observation that p55 receptors but not p75 receptors are involved with regulating lesion growth is consistent with the concept that p55 is the primary receptor for mediating many TNF ligand responses. Signaling via the p55 receptor is associated with induction of adhesion molecule expression (15, 16), apoptosis (17, 18), and leukocyte chemotaxis (19). In contrast, p75 signaling is associated with activation induced cell death of T cells (57), TNF-mediated skin necrosis (24), and suppression of TNF-mediated inflammatory responses (25). The findings presented here are consistent with our earlier report (21) demonstrating that p55 signaling attenuates lesion growth. The actual events elicited by p55 signaling and the cells involved with this response remain undefined. However, because TNFα−/− mice did not recapitulate these findings the responses may be generated independently of TNF ligand binding or indicate that there are other unidentified ligands mediating p55 atheroprotective responses.

In conclusion we show that members of the TNF ligand and receptor superfamily are involved with regulating lesion growth in mice fed high amounts of cholesterol. The results presented here demonstrate that there are multiple members of this ligand/receptor system involved with regulating events in the atherogenic process. The disparate results obtained between ligand versus receptor-deficient mice reflects the complex interaction between the members of this system. By separating the function of each member in lesion growth a clear target for pharmacological intervention will be achieved.

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