Published in final edited form as:

Atherosclerosis. 2013 April ; 227(2): 267–274. doi:10.1016/j.atherosclerosis.2013.01.039.

Tenascin-C Deficiency in Apo E^{-/-} Mouse Increases Eotaxin Levels: Implications for Atherosclerosis

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Abstract

Aim—To investigate the potential role of inflammatory cytokines in apo E^{-/-} mouse in response to deletion of Tenascin-C (TNC) gene.

Methods and results—We used antibody array and ELISA to compare the profile of circulating inflammatory cytokines in apo E^{-/-} mice and apo E^{-/-} TNC^{-/-} double knockout mice. In addition, tissue culture studies were performed to investigate the activity of cells from each mouse genotype in vitro. Cytokine array analysis and subsequent ELISA showed that circulating eotaxin levels were selectively and markedly increased in response to TNC gene deletion in apo E^{-/-} mice. In addition, considerable variation was noted in the circulating level of eotaxin among the control apo E^{-/-} mouse group. Inbreeding of apo E^{-/-} mice with high or low levels of plasma eotaxin showed that the level of eotaxin *per se* determines the extent of atherosclerosis in this mouse genotype. While endothelial cells from apo E^{-/-} mice had low level of eotaxin expression, cells derived from apo E^{-/-}TNC^{-/-} mice expressed a high level of eotaxin. Transient transfection of eotaxin promoter-reporter constructs revealed that eotaxin expression is regulated at the transcriptional level by TNC. Histochemical analysis of aortic sections revealed the massive accumulation of mast cells in the adventitia of double KO mice lesions whereas no such accumulation was detected in the control group. Plasma from the apo E^{-/-}TNC^{-/-} mice markedly stimulated mast cell migration whereas plasma from the apo E^{-/-} mice had no such effect.

Conclusion—These observations support the emerging hypothesis that TNC expression controls eotaxin level in apo E^{-/-} mice and that this chemokine plays a key role in the development of atherosclerosis.

Keywords

Tenascin-C; Eotaxin; Atherosclerosis; Mast cells; Chemokines; Matricellular protein; Extracellular matrix

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Conflict of interest
None.

1. Introduction

Substantial evidence from clinical and experimental studies supports the notion that atherosclerosis is a chronic immune-inflammatory disease. The continuous immigration and infiltration of inflammatory cells in lesions are prominent features in both human and experimental atherosclerotic disease. This process is guided by endothelial leukocyte adhesion molecules and chemoattractants, within the latter is a group of molecules called chemokines. These are structurally related, secretable, largely basic, chemotactic cytokines that play a key role in the initiation and progression of inflammatory disease [1].

Eotaxin (CCL11) is a potent chemokine that promotes migration and activation of eosinophils which participate in the pathogenesis of a broad range of allergic disorders [2]. Microarray analysis of human plaques showed that eotaxin is selectively and markedly overexpressed in human lesions [3]; although eosinophils are rare in atherosclerotic lesions, the receptor for eotaxin (CCR3) is present on macrophages, Th2 cells, and mast cells in lesions. Functional studies reveal that eotaxin induces CCR3-expressing endothelial cell migration in vitro and angiogenesis in vivo, as well as endothelial cell sprouting from aortic rings in the absence of an eosinophil infiltration [4]. Thus, in addition to potentially activating atheroma mast cells and macrophages, eotaxin may directly influence vascular inflammation through the endothelium [5]. While the underlying mechanism that regulates eotaxin expression in vivo remains unclear, inflammation induces marked upregulation of eotaxin.

The local microenvironment, or niche, of vasculature plays important roles in vascular pathologies. A major component of the niche is a matricellular protein, a complex network of macromolecules with distinctive physical, biochemical, and biomechanical properties. Although tightly controlled during embryonic development and organ homeostasis, the matricellular proteins are commonly remodeled in diseases such as atherosclerosis. TNC is a matricellular protein that is expressed in a rigidly controlled temporo-spatial pattern in the developing fetus, yet is undetectable or found only in low levels in the corresponding regions of the intact adult organs (for general discussion, see Midwood & Orend [6]).

We have previously shown that TNC is expressed in human vein grafts [7] and balloon catheterization induces arterial expression of different TNC isoforms [8, 9]. We have also found that factors which are important in neointimal formation, such as angiotensin [10] and PDGF-BB [11], regulate the expression of TNC in vascular smooth muscle cells (SMCs). Others have reported that TNC is an essential factor for neointimal hyperplasia after aortotomy [12]. We have also shown that TNC is expressed in the macrophage rich regions of human atherosclerotic plaques [13] and in arterialized human vein grafts [7]. Both anti- and pro-inflammatory activities have been reported for TNC. To understand the function of TNC in atherosclerosis, we generated TNC^{-/-}/apo E^{-/-} double KO mice and have shown that deletion of the TNC gene promotes atherosclerosis, suggesting that TNC has an athero-protective activity [14]. We now report that deletion of TNC gene in apo E^{-/-} mice leads to significant and specific upregulation of plasma eotaxin. Further evidence suggests that deletion of TNC leads to an upregulation of eotaxin in endothelial cells and the addition of TNC inhibit eotaxin promoter activity at the transcriptional level. These findings highlight the potential role of eotaxin in atherosclerosis and suggest that a clearer understanding of this pathway in atherosclerosis may have potential therapeutic implications.

2. Materials and Methods

2.1. Preparation of animals

The TNC^{-/-}/apo E^{-/-} mice were generated by crossing TNC^{-/-} mice with apo E^{-/-} mice, as previously reported [14]. Eotaxin KO mice were generously provided to us by Dr. Marc E. Rothenberg, Cincinnati Children's Hospital Medical Center, Cincinnati, OH. We also generated apo E^{-/-} mice with high or low levels of circulating eotaxin. For the generation of eotaxin 'low' and eotaxin 'high' mice, 6 weeks old apo E^{-/-} mice were purchased from Jackson labs. and their plasma eotaxin levels were quantified by ELISA following an overnight fast. The median eotaxin level was 450pg/ml and mice with an eotaxin level of 450 pg/ml were considered as the eotaxin 'low' group and mice with eotaxin level of > 450 pg/ml were considered the eotaxin 'high' group. Mice from the 'low' group and mice from the 'high' group were interbred within their respective groups for 5 generations before they were used for additional experiments. In some experiments, the animals were fed a high fat diet (Harlan) as indicated starting when the mice were 6 weeks of age, with water taken ad libitum. The blood samples were collected at the indicated times, the plasma was separated from the red blood cells by low speed centrifugation (2000 rpm in a microcentrifuge for 20 mins) and the plasma eotaxin level was quantified by ELISA kit from RD Systems essentially as described by manufacturer.

After the indicated time of being fed an atherogenic diet, terminal blood samples were collected by puncture of the right ventricle following euthanasia by an isoflurane overdose. Mice were perfused with PBS (20 ml.) via the left ventricle, while the perfusate drained from the punctured right atria. The heart and ascending aorta to the iliac bifurcation were removed. The aortic tissue was placed in freshly prepared paraformaldehyde (4% [wt/vol] in PBS) overnight at room temperature and then embedded in paraffin. The sections were stained using standard protocols.

2.2. Atherosclerotic Lesion Analysis

The extent of the atherosclerotic lesions in the oil red O-stained aorta was quantified, as described previously [14, 15]. Briefly, regions of each aorta quantified were defined as follows: (1) thorax, from the arch to the intercostal artery branch; and (2) abdominal region, from the thorax to the branch of the iliac bifurcation. The lesion size for each aorta was measured by Image-Pro Plus (version 4, Media Cybernetics). Lesions were reported as percentage of the total aortic area consisting of thoracic aorta (ending at the final intercostal artery), and abdominal aorta (ending at the iliac bifurcation).

2.3. Cytokine Antibody array

The cytokine antibody array assessment was performed using antibody membrane purchased from RayBiotech Inc. (Norcross, GA). The membranes were processed essentially as described by the manufacturer. Briefly, membranes were blocked by addition of a blocking buffer and incubated at room temperature for 1 hr. Membranes were then incubated with 1 ml. diluted plasma (diluted 1: 1 with PBS) from 6 apo E groups or 6 TN/E groups (6 mice/group, ~100 µl/mice) at 4°C overnight. After washing, 1 ml. of biotin-conjugated antibodies was added to each membrane and then incubated at room temperature for 1 hr. After washing, 2 mls. of diluted HRP-conjugated streptavidin were added to each membrane and then incubated at room temperature for 1 hr. After washing, the membranes were developed by addition of buffers C and D, at room temperature for 1 hr. The membranes were exposed to autoradiography film and the identity of the spots was determined using the template provided by the manufacturer. According to manufacturer, the detection limitation for the cytokines/chemokines antibody array is around 15 pg/ml; therefore, any difference below this level is undetectable in our system.

2.4. Cell culture studies

Smooth muscle cells were isolated from mouse aortas by enzymatic digestion and cultured essentially as described [11, 16]. The cells were used at passages 3–5. Endothelial cells were cultured on matrigel-coated culture dishes after enzymatic digestion essentially as described [17]. The cells were used at passages 3–5.

2.5. Transient Transfection Assay

A 1363-bp fragment of the promoter region of the eotaxin gene promoter luciferase construct was kindly provided by Robert P. Schleimer (Johns Hopkins Asthma and Allergy Center). Transient transfection experiments were performed with smooth muscle cells essentially as described [18]. Briefly, luciferase constructs expressing firefly luciferase, and pRL- β -actin, a plasmid expressing *Renilla* luciferase were mixed with Nucleofector solution V, and co-transfected into 1×10^6 smooth muscle cells. After transfection, cells were transferred to complete culture medium and treated with the indicated reagents. Cells were then harvested and lysed with lysis buffer. Luciferase activity was assayed using Dual Luciferase Reporter Assay System (Promega Corporation). All the transfection experiments were repeated at least three times, in triplicate.

2.6. Mast Migration assay

Mast cell migration assay was performed using plasma from each mouse genotype. Plasma (pooled from 6 mice per genotype) was placed in the lower chamber of transwell (8 μ M) and the upper chamber contained 1×10^5 mastocytoma cells (ATCC)/transwell. The chamber was incubated at 37 ° C for 4 hr and the number of cells in the lower chambers were counted by hemocytometer. In some experiments plasma were mixed with neutralizing anti-eotaxin antibody (clone 42285, R&D system) before addition to the lower chambers.

2.7. Statistical Analysis

Intergroup statistical comparisons were performed with parametric or nonparametric 2-sample t-test or ANOVA (with post test comparisons) as appropriate. Linear regression analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

3. Results

3.1. Eotaxin is selectively over expressed in plasma of TNC $^{-/-}$ /apo E $^{-/-}$ mice

We found that deletion of TNC in apo E $^{-/-}$ mice exacerbates atherosclerosis in apo E $^{-/-}$ mouse [14]. Since atherosclerosis is an inflammatory disease, we asked whether deletion of TNC gene affects the systemic inflammatory response. To explore this, we investigated the expression pattern of 62 known inflammatory cytokines/chemokines (Fig. 1A) in the plasma collected from each mouse group on atherogenic diet for 4 and 24 weeks. While, no difference in the expression pattern of cytokines/chemokines was found between the two groups of mice on high-fat diet for 4 weeks (not shown), the expression pattern of cytokines of the mouse groups on a high-fat diet for 24 weeks was different (Fig. 1B). The following cytokines/chemokines were detected in the blood plasma from the two mouse genotypes: Axl, CXCL16, IGFBP-3, IGFBP-6, IL-12 p70, Leptin R, LIX, soluble L-selectin, MIP-1 γ , PF-4, soluble P-selectin, TNF-RI, TNFRII, and soluble VCAM-1. Eotaxin (Fig. 1B, red arrow) was the only cytokine that was consistently over-expressed in the blood plasma of the TNC $^{-/-}$ /apo E $^{-/-}$ group. Thus, among the 62 inflammatory cytokines examined, eotaxin was the only cytokine that was upregulated in the absence of TNC gene in apo E $^{-/-}$ mice.

ELISA analysis was utilized to further validate the results of the antibody array as well as to quantify the amount of plasma eotaxin in the two mouse genotype groups (Fig. 2). The mean plasma levels of eotaxin from TNC^{-/-}/apo E^{-/-} and apo E^{-/-} groups before initiation of atherogenic diet feeding were 903.3 ± 40.0 pg/ml, (n=12), and 421.7 ± 27.5 pg/ml (n=15, $P < 0.0001$), respectively. After 4 weeks on a high-fat diet, the mean level of plasma eotaxin for the TNC^{-/-}/apo E^{-/-} group and apo E^{-/-} group were 1357 ± 62.41 pg/ml (n=14) and 649.7 ± 50.35 pg/ml (n=10, $P < 0.0001$), respectively. After 24 weeks on atherogenic diet, the mean plasma eotaxin level for TNC^{-/-}/apo E^{-/-} group was 3170 ± 216.0 pg/ml (n=12) and for the apo E^{-/-} group was 839.0 ± 92.09 pg/ml (n=11, $P < 0.0001$). Our results show that the atherogenic diet led to an increase in the plasma eotaxin in both groups of mice; however, the level of eotaxin was significantly higher in the TNC^{-/-}/apo E^{-/-} mice.

3.2. Eotaxin expression contributes to atherosclerosis in apo E^{-/-} mouse

During the course of our investigation, we noted that the plasma eotaxin level also varied widely among the control apo E^{-/-} group that were routinely purchased from Jackson labs. This was surprising because this variation most likely affects the interpretation of experiments using this animal model for atherosclerosis studies. We noted that the majority of apo E^{-/-} mice, especially female mice, had an average plasma eotaxin level of 435 ± 179 pg/ml while remaining mice had a much higher level. This prompted us to ask if there is a relationship between plasma level of eotaxin and the degree of atherosclerosis in the control apo E^{-/-} mice. The median plasma eotaxin level was 450 pg/ml and therefore we categorized mice as “eotaxin low” group if their eotaxin level was below the median or “eotaxin high” group if the eotaxin level exceeded the median. The low (<450 pg/ml) and high (>450 pg/ml) plasma eotaxin apo E^{-/-} mouse groups were interbred 5 times and their plasma eotaxin level was quantified again. After 5 generations, the average plasma eotaxin levels in the low and high groups were 709 ± 277 pg/ml and 3476 ± 2892 pg/ml, respectively (Fig. 3A). The low eotaxin plasma level mice group is designated as low and the high eotaxin plasma group is designated as high in Fig. 3. Subsequently, the two mouse groups (n=9 mice for low eotaxin group and n=10 mice for high eotaxin group) were placed on atherogenic diet for 18 weeks and the aortic lesions areas were measured after oil red O staining. A significant difference in the lesion areas was found between the two groups of control mice, $14.2 \pm 1.3\%$ vs. $25.0 \pm 2.1\%$ for low and high eotaxin groups, respectively; an 80% increase in the lesion areas in mice with a higher level of eotaxin (Fig. 3A). A good correlation ($r^2 = 0.8370$) was found between plasma eotaxin and plaque size in apo E^{-/-} mice with different levels of plasma eotaxin (Fig. 3B).

Past studies have shown that a wide variety of cells that are important to atherosclerosis express eotaxin [3, 19–23] and this expression is regulated by inflammatory mediators such as TNF- α , IL-1, IL-4, and IL-13 [24–26]. To investigate the potential source of plasma eotaxin in the double KO mice, we isolated endothelial cells from the two mouse genotypes, treated them with TNF α or oxLDL for 24 hr, and the level of eotaxin in the medium was measured by ELISA (Fig. 4A). In addition, since eotaxin is markedly and selectively overexpressed in human atherosclerotic plaques by smooth muscle cells [3], we also investigated the effect of TNF α or oxLDL on the eotaxin expression in these cells. Further, we studied the effect of these factors on eotaxin expression by mouse monocytic RAW cells. Cultured 3T3 fibroblasts were used as a negative control. When untreated, endothelial cells isolated from the double KO mice constitutively expressed significant amount of eotaxin (~ 30 ng/ml) and the addition of either TNF α or oxLDL did not significantly affect its expression level (Fig. 4A). In contrast, addition of TNF α to cultured aortic SMC significantly induced (~ 40 ng/ml) expression of eotaxin compared to the control cells (~ 9 ng/ml), a 75% increase in protein expression, whereas oxLDL had no effect. The expression of eotaxin by RAW cells was undetectable (Fig. 4A). These data suggest that the effect of

TNC on the up-regulation of eotaxin in endothelial cells seems to be a stable phenotype of these cells. In addition, we observed that expression of eotaxin by SMCs is subject to tight transcriptional control during inflammation.

Since the deletion of TNC upregulated eotaxin expression, we asked whether TNC blunts the effect of TNF α seen in SMCs. To explore this, SMCs were transiently transfected with a 1300 bp eotaxin promoter-reporter construct and luciferase activity was measured in the absence and presence of TNC (Fig. 4B). Consistent with ELISA assay data, no luciferase activity was detected in the untreated cells. While addition of 30 ng/ml recombinant eotaxin had no effect on luciferase activity and TNF α markedly induced the promoter activity, the addition of TNF α in combination with TNC completely blocked induced eotaxin promoter activity.

3.3. Deletion of TNC gene promotes accumulation of mast cells in the adventitia

We noted that the adventitia of the double KO mice with high level of eotaxin that are fed an atherogenic diet generally contains a large number of inflammatory cells compared to control mouse group. These inflammatory cells exhibited a morphology that resembles mast cells. To explore this further, aortas were harvested from TNC $^{-/-}$ /apo E $^{-/-}$ and apo E $^{-/-}$ mice on high fat diet for 24–28 weeks and inflammatory cells were identified with mast cell-specific staining. Toluidine blue and chloroacetate esterase are two metachromatic dyes that are widely used to stain basophilic granules in mast cell cytoplasm. Toluidine blue staining of paraffin-embedded sections from aortas of TNC $^{-/-}$ /apo E $^{-/-}$ mice revealed the presence of a large number of mast cells that were primarily concentrated in the adventitia (in 6 out of 10 mice), although some mast cells were also detected in the intima (Fig. 5A). The higher magnification of the sections showed that the vast majority of adventitial cells have round cell morphology and their cytoplasmic granules stained positive with toluidine blue (panel B). In contrast to adventitial cells, medial SMCs staining was confined to nuclei (panel B, arrows) and no cytosolic components stained positive with the toluidine blue. Similarly, chloroacetate esterase staining of the sections revealed strong staining of adventitial cells that exhibited rounded morphology (Fig. 5C). Higher magnification of the sections revealed that while positively-stained adventitial cells exhibited intense staining of cytosolic components of mast cells, only nuclei of medial SMCs were stained (panel D, arrow). In contrast, toluidine blue staining of sections from the control apo E $^{-/-}$ mice showed relatively modest lesion size with no obvious accumulation of mast cells in the adventitia (Figs. 5E and 5F). In addition, to show the organ specificity of the mast cell accumulation, we stained lung tissue. We did not detect such accumulation of cells in the lung tissue of the double KO mice (data not shown).

3.4. Plasma from the double KO mice promotes migration of mast cells

For mast cells to be involved in atherosclerosis in man and mice, mechanisms must exist to regulate tissue localization, proliferation, and differentiation of mast progenitor cells. As eotaxin promotes mast cell migration [27], we examined the effect of plasma from the two mouse genotypes on the migration of mast cells. The plasma from each mouse genotype was collected after they were fed an atherogenic diet for 24 weeks and its ability to promote mast cell migration was examined utilizing mastocytoma cell migration assay (Fig. 6). Plasma from the eotaxin double KO mice was used as a negative control. While no significant difference was observed in the migration of mast cells between the negative control group (eotaxin KO mice) and plasma collected from apo E $^{-/-}$ mice, a marked increase in the mast cell migration was detected in response to plasma collected from TNC $^{-/-}$ /apo E $^{-/-}$ mice ($P=0.002$). To demonstrate specificity of the migration assay, the anti-eotaxin antibody media was mixed with the media and then used for migration assay. We found that anti-eotaxin antibody significantly reduced migration of mastocytoma ($p=0.0009$).

4. Discussion

We have previously reported that TNC is expressed in both human and mouse atheroma and its deletion exacerbated atherosclerosis in apo E^{-/-} mice, eventually leading to massive intraplaque hemorrhage [14]. In this report, we investigated the potential mechanism by which TNC affects lesion development. Since atherosclerosis is an inflammatory disease, we first asked whether there is a difference in the systemic inflammatory response between the two mouse genotypes. Antibody array analysis of 62 cytokines/chemokines revealed that eotaxin was selectively upregulated in the plasma collected from the double KO mice. ELISA analysis confirmed the array data and showed that the eotaxin level in the double KO mice is higher under basal conditions and hyperlipidemia further augmented the expression of eotaxin. We also noted that there is a similar relationship between the eotaxin level and the degree of atherosclerosis in apo E^{-/-} mice.

Surprisingly, we found significant variations in the level of plasma eotaxin in the control apo E^{-/-} mice. As this animal model is widely used for atherosclerosis studies and any variation of eotaxin levels will most likely affect the results, we further examined this effect of eotaxin in apo E^{-/-} mice. Apo E^{-/-} mice with 'high' and 'low' eotaxin levels were interbred to generate apo E^{-/-} mice with significantly different levels of eotaxin and there was a significant increase in the degree of atherosclerosis in the apo E^{-/-} group with high level of eotaxin. These results coupled with the data from the double KO mice suggest that the level of eotaxin *per se* is likely sufficient to enhance lesion formation in apo E^{-/-} mice. Therefore, the apo E^{-/-} mice that are obtained from commercial sources or even those generated in house need to be carefully screened for the plasma eotaxin level to avoid misinterpretation of data when using this animal model for atherosclerosis studies.

Eotaxin is associated with Th1-like responses and exerts its effect through the chemokine receptor-3 (CCR3). A wide variety of cells expresses CCR3 receptor [3, 19–23]; therefore, cells can respond to eotaxin in autocrine as well as paracrine fashion. We noted that cultured aortic endothelial cells from the double KO mice express a significant amount of eotaxin at basal level, suggesting that this cell type may contribute to the high level of plasma eotaxin. Since endothelial cells express eotaxin and CCR3 receptor [28], this cell type could be one target of autocrine eotaxin action in vivo. It is reported that eotaxin markedly upregulates the expression of the adhesion molecules VCAM-1 and ICAM-1 in endothelial cells [28]. We reported that VCAM-1 is markedly and rapidly upregulated in the double KO mice compared to control apo E^{-/-} group and this correlated with a rapid accumulation of macrophages on the aortic wall [14]. Therefore, it seems reasonable to speculate that the relative upregulation of VCAM-1 that we observed in the double KO mice may be in part be related to the high level of eotaxin in this genotype.

In addition to endothelial cells, SMCs and inflammatory cells express CCR3 and thus are affected by eotaxin. In human atheroma, eotaxin is expressed predominantly by SMCs and these cells respond to it through the CCR3 receptor [3], inducing migration of SMCs [29]. In addition to vascular cells, eotaxin affects activity of wide variety of inflammatory cells. While both Th1 and Th2 T cells express eotaxin in a comparable fashion [30]; they induce Th2 polarization. In addition to T cells, monocyte/macrophages express CCR3 eotaxin receptor, suggesting that this cell type may respond to eotaxin in a paracrine fashion. Consistent with this, the in vivo administration of eotaxin is found to increase the number of myeloid progenitors present in the bone marrow [31], thus shifting the differentiation of bone marrow hematopoietic cells towards the myeloid lineage. We noted that significant and rapid accumulation of macrophages in the lesions of the double KO mice compared to apo E^{-/-} mice. Thus, the rapid expression of VCAM-1 in response to a high level of plasma eotaxin coupled with the ability of eotaxin to promote differentiation of bone marrow

hematopoietic cells toward a myeloid lineage could explain the significant accumulation of macrophages in the lesions of the double KO mice [14]. Another inflammatory cell that expresses CCR3 and thus is affected by eotaxin is the mast cell. These cells are heterogeneous, ubiquitously distributed throughout the body, and exhibit site-specific adaptations induced by microenvironmental signals that lead to selective expression of potential mast cell characteristics [32, 33]. Their flexibility in phenotype has important functional implications as it allows them to adapt to organ- or tissue-specific roles. We noted a significant accumulation of mast cells in the adventitia of double KO mice with high level of eotaxin. This accumulation most likely affect plaque phenotype through multiple mechanisms such as upregulation of various proteases [34] and cytokines [35], apoptosis of SMCs [36, 37], stimulation of plaque neovascularization [36, 38, 39], and promotion of intraplaque hemorrhage [40]

At least two mechanisms could explain the accumulation of mast cells in the adventitia of the double KO mice. First, eotaxin is known to foster mast cell migration [27]. Consistent with this, we noted that plasma from TNC^{-/-}/apo E^{-/-} mice markedly induced mast cell migration compared to the control groups. Thus it is possible that the high level of eotaxin in the double KO mice promotes migration of mast cells from vasa vasorum. Second, stem/progenitor cells reside in the perivascular niches in postnatal bone marrow [41] and extramedullary hematopoietic tissues [42]. This perivascular localization is believed to facilitate circulation and trafficking of stem/progenitor cells to other tissues [43, 44]. In apo E^{-/-} mice, abundant progenitor cells are found in the adventitia that contribute to the atherosclerosis of vein grafts [45]. Similarly, the adventitia of postnatal aorta contains multipotent hematopoietic stem/progenitor cells [46]. Since eotaxin promotes differentiation of mast cells from stem/progenitor cells [47, 48], it is likely that the high level of eotaxin in the double KO mouse promotes differentiation of adventitial progenitor cells into mature mast cells. Thus expansion of vasa vasorum and the presence of mast progenitor cells in the adventitia, coupled with the ability of eotaxin to promote maturation of mast progenitor cells to mature mast cell could explain in part the accumulation of mast cells in the adventitia

In summary, we noted that deletion of the TNC gene in apo E^{-/-} mice leads to an upregulation of eotaxin and furthermore the increase in plasma eotaxin correlated with higher extent of atherosclerosis in both the mouse genotypes we examined. Cell culture studies revealed that the deletion of TNC in endothelial cells leads to high level of eotaxin expression. Addition of TNC to cultured SMCs completely blunted eotaxin promoter activity induced by TNF α . Mast cells were found to accumulate in the adventitia of the double KO mice with high level of eotaxin. Understanding how TNC expression regulates eotaxin expression during inflammation will reveal how this process can be manipulated during chronic inflammation which may provide the means to modulate tissue level of this chemokine and consequently the extent and phenotype of atherosclerosis. Our findings may be clinically relevant as the eotaxin level is increased in humans in response to balloon angioplasty [49] and eotaxin gene and its CCR3 receptor are overexpressed in human atherosclerotic plaques [3].

Acknowledgments

Sources of funding and acknowledgments

This work was supported by grants from National Institute of Health ROI HL50566, The Eisner Foundation, The Heart Foundation, Spielberg Cardiovascular Research Fund, and the Corday Foundation, Sam Spaulding Fund and The California Agricultural Research Initiative grant # 03-4-089. BGS is established investigator of American Heart Association. We thank Ian Williamson for help in preparing the manuscript.

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- Tenascin-C (TNC) is a matricellular protein that exhibits athero-protective activity.
- Deletion of TNC selectively upregulates expression of eotaxin.
- The level of plasma eotaxin level correlates with extends of atherosclerosis in apo E^{-/-} mice.
- Eotaxin level in endothelial cells and smooth muscle cells are regulated by TNF α and oxidized LDL.
- The plasma level of eotaxin correlates with accumulation of mast cells in the adventitia of aortic lesions in apo E^{-/-} mice.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	POS	POS	Blank	Axl	BLC	CD30 L	CD30 T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
2	NEG	NEG	NEG	NEG	Blank	Axl	BLC	CD30 L	CD30 T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
3	Eotaxin-2	Fas-L	Fractalkine	G-CSF	GM-CSF	IFN- γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1 α	IL-1 β	IL-2	IL-3	IL-3 Rb
4	Eotaxin-2	Fas-L	Fractalkine	G-CSF	GM-CSF	IFN- γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1 α	IL-1 β	IL-2	IL-3	IL-3 Rb
5	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p70	IL-12 p70	IL-13	IL-17	KC	Leptin R	LEPTIN	LIX	L-Selectin
6	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p70	IL-12 p70	IL-13	IL-17	KC	Leptin R	LEPTIN	LIX	L-Selectin
7	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1 α	MIP-1 γ	MIP-2	MIP-3 β	MIP-3 α	PF-4	P-Selectin	RANTES	SCF
8	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1 α	MIP-1 γ	MIP-2	MIP-3 β	MIP-3 α	PF-4	P-Selectin	RANTES	SCF
9	SDF-1 α	TARC	TCA-3	TECK	TIMP-1	TNF- α	TNF-RI	TNF-RII	TPO	VCAM-1	VEGF	Blank	Blank	Blank
10	SDF-1 α	TARC	TCA-3	TECK	TIMP-1	TNF- α	TNF-RI	TNF-RII	TPO	VCAM-1	VEGF	Blank	Blank	Blank

Fig. 1. Deletion of TNC in apo E^{-/-} mice leads to a specific upregulation of eotaxin
 (A) The antibody array contains 6 positive control spots, 4 on the upper left (1A–D) and 2 on the lower right (10M10N). The plasma from each group of mice is diluted and incubated with a membrane. This is followed by incubating each membrane with a cocktail of biotin-labeled antibodies. The bound antibodies were visualized with HRP-conjugated streptavidin.

All reagents required for this experiment are included in the kit. The template for the array is shown in panel A. Panel B, plasma collected from TNC^{-/-}/apo E^{-/-} mice and control apo E^{-/-} mice on atherogenic diet for 24 weeks and added to the membrane and then processed according to manufacturer's instruction. We found the upregulation of eotaxin (indicated by a red arrow). The experiment was repeated three times with three different membranes using plasma from different pools of TNC^{-/-}/apo E^{-/-} group and control apo E^{-/-} group. All experiments yielded identical results.

Fig. 2. ELISA analysis of blood plasma from the two mouse genotypes

Eotaxin levels were determined by ELISA assay (R&D Systems) using plasma from the two mouse genotypes on high-fat diet for the indicated times. Boxes indicate means for each group of mice and the median is shown by a horizontal line.

A

B

Fig. 3. The lesion areas correlate with the plasma eotaxin level

Panel A, Apo E^{-/-} mice were purchased from Jackson Laboratories and their plasma eotaxin levels were measured by ELISA. Mice with low and high levels of eotaxin were identified, interbred for at least 5 generations, and then fed an atherogenic diet for 18 weeks; low eotaxin group ((709±277 pg/ml plasma eotaxin, n=9) and the high eotaxin group (3476±2892 pg/ml, plasma eotaxin, n=10). The aortic atherosclerosis was measured and compared between the high and low groups after oil red O staining. The level of eotaxin for the two groups is shown in pg/ml. Panel B shows the relationship between plasma eotaxin and plaque size for all the mice in panel A. We used linear regression analysis as described in the Method section.

Fig. 4. Expression of eotaxin by cultured vascular cells: Panel A

Endothelial cells and aortic SMCs were isolated from aortas of the two mouse genotypes as described and used at passages 3–5. RAW cells and 3T3 cells were obtained from ATCC and cultured as recommended. All cells were cultured in 24 well plates, remained untreated or treated with 20 ng/ml TNF α or 50 μ g/ml OxLDL for 24 hrs. The media were collected and 100 μ l was used for ELISA assay. The results are shown as a mean $\pm$ SD of triplicate determinations. **Panel B. TNC regulates eotaxin promoter activity:** Cultured aortic smooth muscle cells were isolated and transfected with the eotaxin promoter constructs as described in the Method section. After transfection, cells were treated with TNF α alone or in combination with 10 μ g/ml full length TNC isoform. Luciferase activity was measured as described above.

A

B

B

C

D

E

F

Fig. 5. Deletion of TNC gene in apo E^{-/-} mice promotes adventitial accumulation of mast cells

The two mouse genotypes were fed an atherogenic diet for 24–28 weeks (10 mice genotype). The aorta including the periadventitia tissue around the aorta was used for paraffin embedding. Two staining methods were used to stain mast cells in the paraffin-embedded sections of the double KO mice: toluidine blue (panel A at 10× magnification and panel B at 40× magnification) and chloroacetate esterase (Panels C at 10× magnification and D at 40× magnification). Panels E and F show an aortic section from the control apo E^{-/-} mice on high fat diet for 24–28 weeks that is stained with toluidine blue at 10× and 40 × magnifications, respectively. The 40× shows magnification of base of the plaque.

Fig. 6. Plasma from the double KO mice foster migration of mast cells

Blood plasma samples were collected from the TNC^{-/-}/apo E^{-/-} mouse fed a high fat diet for 24 weeks. Plasma samples from 6 mice/group were pooled and analyzed for cell migration using transwell as described in the Method section. Two negative controls were used: plasma from apo E^{-/-} mice or eotaxin knockout mice (Eotaxin KO) on atherogenic diet for 24 weeks was used for migration assay. The data show the average $\pm$ SD.