Aim: CCL22, mainly synthesized by monocyte-derived alternative (M2) macrophages, belongs to the CC family of chemokines and is involved in monocyte migration and recruitment. We have previously investigated CCL22 and histamine in atherosclerosis. Here, we investigated the hypothesis that CCL22 is involved in atherosclerosis, which is influenced by the differentiation of macrophage phenotypes via histamine.

Methods: CCL22 expression was investigated in human carotid arteries and coronary arteries with bare metal stents. Ligated carotid arteries of wild-type (C57BL/6J) and apolipoprotein E-deficient mice were also used as atherosclerotic models. The localization and expression of CCL22 and classical (M1)-like and M2-like macrophages in various human and mouse atherosclerotic lesions were investigated by immunohistochemical examination and quantitative real-time polymerase chain reaction. Histamine is expressed in atherosclerosis, and it induces inflammation and immunity. Human- and mice-derived monocytes and macrophages were used to examine the role of histamine in macrophage differentiation and CCL22-expression. Macrophages derived from histamine receptor 1 (H1R)- and 2 (H2R)-knockout (KO) mice were also examined.

Results: Atherosclerotic lesions showed a distribution of heterogeneous macrophage phenotypes with M1-like and M2-like macrophage dominant sites. CCL22 was distributed in sparse areas of vascular smooth muscle cells (VSMCs) and associated with M2-like macrophages. Moreover, H2R stimulation was associated with CCL22 expression via M2-like macrophage dominant differentiation.

Conclusion: The expression of M1- or M2-like macrophages in atherosclerosis were observed to be dependent on the distribution of VSMCs owing to differences in causal stimuli and the switching of histamine receptors via Th1 or Th2 cytokines. These results suggest that CCL22 may control atherosclerosis.

Key words: CCL22, Histamine, Atherosclerosis, Macrophage, Chemokine
endothelial space\(^6\). In addition to macrophages, dendritic cells are also present in the atherosclerotic lesions and produce more CCL22 than monocytes\(^1,6-9\).

Macrophages are present in various regions of plaque, and are exposed to different environmental stimuli that modulate their activation and polarization. Even though the process of differentiation of monocytes into macrophages is irreversible, macrophage-polarization appears reversible\(^10,11\). Monocyte-derived macrophages are the principal mediators of tissue homeostasis and repair along with the response to pathogenesis and inflammation in organs\(^12\). Macrophages can be divided into classically Th1 polarized M1-like and Th2 polarized M2-like macrophage phenotypes\(^13,14\). In addition to M1-like macrophages, M2-like macrophages are present in the atherosclerotic lesions\(^15\) and contribute to the pathogenesis of atherosclerosis\(^16\). Furthermore, M2-like macrophages express CCL22\(^17\).

Histamine is one of the principal mediators of allergic reactions, inflammation, and cardiovascular hemodynamics. Histamine is a classical and immunological mediator, mainly produced by mast cells and basophils. However, in addition to these cells, monocytes and macrophages also produce histamine that is gradually secreted in atherosclerosis\(^18\).

Macrophages express CCL22 in response to histamine stimulation via the H2 receptor\(^19,20\). In this study, we investigated the localization of CCL22 and the various macrophage phenotypes in atherosclerotic lesions, and the relationship between macrophage phenotypes and histamine receptors.

**Materials and Methods**

**Human Arteries**

To investigate human common carotid arteries and the effect of coronary bare metal stents on human coronary arteries by histological and immunohistochemical examinations, 22 samples each that did not show aortitis syndrome and autoimmune disease were obtained at autopsy.

Resected tissues were fixed in 10% formalin and embedded in paraffin. The sections (3 µm) were stained with hematoxylin and eosin (H&E) and used for immunohistochemical examination as described previously\(^21\). Thin sections (3 µm) were cut and stained with H&E and used for immunohistochemical detection. Immunohistochemical staining was carried out (EnVision kit, Dako Japan) using antibodies against CCL22 (rabbit polyclonal, 1:50; GenTex, Irvine, CA), Mac-3 (clone M3/84, 1:50; BD Bioscience Pharmingen, Tokyo, Japan), and αSMA (1:150; Dako, as above).

**Animals**

We used male apoE knockout (KO) mice (Jackson Laboratory, Bar Harbor, ME) that were maintained for 30 weeks on a normal chow diet. Another group of male wild-type (WT) C57BL/6J mice (Charles River, Yokohama, Japan), weaned at 8 weeks of age, was used to produce ligation-induced vascular injury models. We ligated the left common carotid artery with a 7-0 silk suture for 2 weeks at a site proximal to the carotid bifurcation.

After the animals were euthanized, carotid arteries were removed, fixed in 10% formalin, and embedded in paraffin for histological and immunohistochemical examination as described previously\(^21\). Thin sections (3 µm) were cut and stained with H&E and used for immunohistochemical detection. Immunohistochemical staining was carried out (EnVision kit, Dako Japan) using antibodies against CCL22 (rabbit polyclonal, 1:50; GenTex, Irvine, CA), Mac-3 (clone M3/84, 1:50; BD Bioscience Pharmingen, Tokyo, Japan), and αSMA (1:150; Dako, as above).

**Immunofluorescence Staining**

Immunofluorescence assays were performed on thin sections (4 µm) of human coronary artery, with and without coronary stenting. Experiments were performed using anti-CCL22 (as described above) and anti-CD163 (1:200; Leica Biosystems, Newcastle, UK) primary antibodies to detect M2-like macrophages, and anti-MCP-1 primary antibody (as above) to detect M1-like macrophages. In addition, antibodies to αSMA (as above), CD4 (as above), CCR4 (goat polyclonal, 1:100; Abcam), and interleukin (IL)-4 (rabbit polyclonal, 1:2000; Abcam) were used. Rhodamine-conjugated goat anti-mouse IgG (H+L; 1:200) and donkey anti-rabbit IgG (1:200; Merck, Tokyo, Japan), and FITC-conjugated goat anti-rabbit IgG (H+L) (1:200), donkey anti-mouse IgG (H+L) (1:200) and donkey anti-goat IgG (H+L; 1:200; Merck) were used as secondary antibodies, respectively, and incubated for 90 min at 25°C. After washing with phosphate buffered saline (PBS), specimens were counterstained with 4',6-diamidino-2-phenylindole (DAPI; GenTex, Hsinchu City, Taiwan) and mounted. Immunostained tissues were evaluated with a Nikon ECLIPSE E600 inverted fluorescence microscope (Nikon, Tokyo, Japan). Image analysis was completed with LuminaVision software (version 2.2.2; Mitani Corporation, Tokyo, Japan).
mRNA expression were normalized with those of the expression of 18s ribosomal RNA in the same samples.

**Statistical Analysis**

Analysis of variance (ANOVA) was used to determine statistically significant differences. Data were expressed as mean ± standard deviation (SD), and a probability (P) value of less than 0.05 was taken to be significant.

**Results**

**Expression and Localization of CCL22 in Human Common Carotid Arteries**

To investigate the expression of CCL22 and its localization in human common carotid arteries, we performed immunohistochemical staining using antibodies against human CCL22, CD68 (a macrophage marker), and αSMA (a smooth muscle cell marker). Although the intimal thickness of the common carotid artery looked relatively concentric at a glance (Fig. 1A), the distribution of αSMA in VSMCs in the intima of common carotid arteries were uneven (Fig. 1B). The density of VSMCs, classified into three levels (β, κ, и), was examined in more detail. Regions with a moderate density of VSMCs (β) showed foamy, CD68-positive macrophages and were also positive for CCL22 (Fig. 1C–β H&E, CD68, CCL22). At sites with a high density of VSMCs (κ), CD68-positive macrophages were dispersed but were negative for CCL22 (Fig. 1C–κ αSMA, CD68, CCL22). Vascular smooth muscle cells were not found in regions of concentrated cholesterol clefts (и), while CCL22-staining was strongly positive, and CD68-staining was weakly positive (Fig. 1C–и αSMA, CD68, CCL22). In summary, the expression of CCL22 in atherosclerosis appeared to inversely correlate with the density of VSMCs, with the absence of VSMCs associated with high CCL22 staining (Supplemental Fig. 2A).

**Expression and Localization of CCL22 in Human Coronary Arteries with Stents**

Next, we investigated how arteries artificially in-
phages were positive for MCP-1 at sites where VSMCs were dense (Fig. 3A αSMA and MCP-1). Several Th2 cells (CD4, CCR4, and IL-4 positive cells) were found surrounding CCL22 positive macrophages (Fig. 3B CD4, CCL22, and CD4, CCR4, IL-4).

Comparison of Expression of CCL22 and CD163 in Atherosclerosis

CD163 is a marker used to assess M2-macrophage distribution; this was compared with the localization of CCL22 using immunofluorescence staining of coronary arteries containing bare metal stents. Where the artery contacted the stent, CD163 and CCL22 were not expressed (Fig. 4A, H&E, CCL22, and CD163). In contrast, the expression of a large amount of both CD163 and CCL22 was observed in intima that did not contact the stent (Fig. 4B, H&E, CCL22, and CD163).
relating to atherosclerotic lesions observed in the aortas of humans and apoE-KO mice\textsuperscript{19, 20}. M2 macrophages were considered to dominate in this lesion. In ligation-induced neointimal hyperplasia (Fig. 5B), the intima differed from that of apoE-KO mice. VSMCs were observed to be widely distributed in the thickened intima (αSMA). Mac-3-positive macrophages were also widely distributed. However, only a few CCL22-positive cells were present. In contrast to the lesions of apoE-KO mice, M1-like macrophages were considered to make up a minor component of the lesion.

Expression of CCL22 and IL-4 mRNAs in Carotid Artery

qPCR of various mRNAs from the carotid arteries of WT-mice (control), ligated carotid arteries of WT-mice and apoE-KO mouse was performed. The relative CCL22-mRNA level was significantly higher in the carotid arteries of apoE-KO mice than that of the ligated carotid arteries of WT-mice (Fig. 5C; \( P<0.01 \)). IL-4 is an important factor for inducing M2-macrophages and showed the same expression pattern as CCL22 (Fig. 5D; \( P<0.05 \)). Thus, it appears that the CD163). Double staining of CD163 and CCL22 was shown in almost the same cells (Fig. 4B, CCL22 [high-power magnification], CD163 [high-power magnification], and CCL22 and CD163). Therefore, in atherosclerosis, CCL22 appears to be expressed in M2-like macrophages.

Expression and Localization of CCL22 in Atherosclerosis in Carotid Arteries of ApoE-KO Mice and Ligation-Induced Neointimal Hyperplasia in Carotid Arteries of Wild-Type Mice

To investigate the difference in localization of VSMCs and CCL22 expression due to differences in the causes of atherosclerosis, we compared common carotid arteries of apoE-KO mice and ligated carotid arteries of WT-mice. We performed immunohistochemical staining using antibodies against mouse CCL22, αSMA, and Mac-3 (a macrophage marker). In the atheroma of an apoE-KO mouse (Fig. 5A), mononuclear cells infiltrating the atheroma were strongly positive for Mac-3 and faintly positive for CCL22; a positive αSMA stain surrounded the atheroma. Lipid clefts in the core of the atheroma were strongly positive for CCL22 (Fig. 5A). Such results are similar to findings relating to atherosclerotic lesions observed in the aortas of humans and apoE-KO mice\textsuperscript{19, 20}. M2 macrophages were considered to dominate in this lesion.

In ligation-induced neointimal hyperplasia (Fig. 5B), the intima differed from that of apoE-KO mice. VSMCs were observed to be widely distributed in the thickened intima (αSMA). Mac-3-positive macrophages were also widely distributed. However, only a few CCL22-positive cells were present. In contrast to the lesions of apoE-KO mice, M1-like macrophages were considered to make up a minor component of the lesion.

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Role of Histamine Receptors in the Expression of CCL22 and M1-Associated Cytokine mRNAs in Mouse Macrophages

The influence of histamine on the expression of CCL22- and M1-associated IL-6, iNOS, and TNF-α cytokines was examined in macrophages of histamine receptor KO mice. Previous studies have shown that CCL22 is regulated through the H2 receptor \[^{19}\]. In this study, CCL22-mRNA was significantly decreased in the macrophages of H2 receptor KO (H2R-KO) mice compared to H1R-KO mice (\(P<0.001\)) despite the serum histamine concentration of H2R-KO mice being significantly higher than that of WT or H1R-KO mice (Supplemental Fig. 4; \(P<0.05\) for both). In comparison, IL-6, iNOS, and TNF-α mRNAs were significantly decreased in the macrophages of H1R-KO mice compared to H2R-KO mice (Fig. 6A (ii), A (iii), and A (iv); \(P<0.001\), \(P<0.01\), and \(P<0.001\), respectively). Thus, these results sug-

Expression of M1 Macrophage Markers in Carotid Arteries

IL-6, iNOS, and TNF-α are representative inflammatory cytokines secreted by M1-macrophages. The carotid arteries of WT-mice were used as controls to which the ligated carotid arteries of WT-mice and of apoE-KO mice were compared. Compared with controls, each atherosclerosis model showed an increase in these cytokines. Further, ligated carotid arteries in WT-mice showed significantly higher expression of IL-6, iNOS, and TNF-α mRNAs than carotid arteries in apoE-KO mice (Fig. 5E, F, and G; \(P<0.01\), \(P<0.05\), and \(P<0.01\), respectively).

Fig. 3. Cells surrounding macrophages in the intima

Fig. A is the same area as Fig. 1B-β and Fig. B is the same area as 1B-γ. Fig. A MCP-1 shows monocyte chemoattractant protein (MCP)-1 immunohistochemical staining (green) and Fig. A MCP-1 and α-smooth muscle actin (αSMA) shows immunofluorescence staining of both MCP-1 (green) and αSMA (red). MCP-1-positive cells and smooth muscle cells were present in close proximity. Fig. B CD4 and CD4 high-power magnification (high-power magnified image of the box in Fig. B CD4) show the immunohistochemical staining of CD4. Fig. B CCL22 and CD4 show immunofluorescence staining of both CCL22 (green) and CD4 (red). Fig. B CCR4 and interleukin (IL)-4 (high-power magnified image of Fig. B CCL22 and CD4) shows immunofluorescence staining of CCR4 (CCL22 receptor; green) and IL-4 (red). CD4-positive cells appeared around CCL22-positive cells whereas CD4-positive cells showed CCR4-staining and expressed IL-4. Blue staining (DAPI) indicates the nuclei of cells. Bars indicate 50 µm (Fig. A), 100 µm (Fig. B CD4) and 30 µm (Fig. B CD4 (high-power magnification), CCL22 and CD4, CCR4, and IL-4).

expression of CCL22 in apoE-KO mice correlates with IL-4 expression.
suggest that cytokines are regulated through the expression ratio of histamine receptors in macrophages in addition to the switching of histamine receptors in monocyte/macrophages in atherosclerosis\textsuperscript{18, 22}).

**Expression of Histamine Receptors in Mouse Macrophages and a Human Macrophage Cell Line**

The relationship between M1- and M2-like macrophage differentiation, and the expression of histamine receptors was examined in human and mouse monocytes, and in M1- and M2-like macrophages. Human and mouse monocytes were found to express predominantly H2 compared to H1 receptors, as found in previous studies (Fig. 6B (i) and C (i); \(P<0.01\) for both). Human and mouse M1-like macrophages displayed predominantly H1 compared with H2 receptors (Fig. 6B (ii) and C (ii); \(P<0.01\) and \(P<0.05\), respectively). In contrast, for M2-like macrophages, a significant difference in expression of H1 and H2 receptors was not found [Fig. 6B (iii) and C (iii)]. Thus, these results suggested that histamine tended to enhance inflammation via M1-like macrophages and tended to increase the expression of CCL22 via M2-like macrophages.

**Discussion**

This study is a continuation of our work on the expression of CCL22 in atherosclerotic lesions\textsuperscript{19, 20}. We previously found that the expression of CCL22 increased with the maturation of macrophages and the stabilization of lesions, but differences in the cellular localization of CCL22 were not investigated\textsuperscript{19, 20}. The migration and proliferation of VSMCs are processes that occur during atherosclerosis\textsuperscript{23, 24}, with the non-uniform distribution of VSMCs in the intima one cause for the asymmetric thickenings observed in this tissue. In this study, we examined the
M1-like macrophages were mainly present at sites where VSMCs were observed in high density, while M2-like macrophages were present where VSMCs were sparse. Similar results were observed in coronary arteries in which the distribution of VSMCs was artificially induced by stent placement. VSMCs express IFN-γ, macrophage colony-stimulated factor (M-CSF), and MCP-1, which allow the migration of macrophages and their differentiation into M1-like macrophages. Furthermore, contact between monocytes, activated via M-CSF, and VSMCs, as well as the expression of TNF-α leads to apoptosis in VSMCs. The proximity of M1-like macrophages and VSMCs may be one of the factors that create the sparsity of VSMCs observed in areas of atherosclerotic lesions.

We then carried out a comparative study with respect to two different models of atherosclerosis in mice, and with reference to our previous studies. In the relationship between VSMCs and the localization of macrophage subtypes.
The expression of CCL22 is generally one of the features of M2 macrophages. We found that the localization-induced neointimal hyperplasia, intimal hyperplasia was observed mainly in the smooth muscle cell layer. In this model, CCL22-negative macrophages were the dominant macrophages found in atherosclerotic lesions. In the common carotid artery atherosclerosis of apoE-KO mice, lesions were observed with a reduced increase in VSMCs and mainly contained foamy macrophages and cholesterol. In comparing these two experimental models, lesions with mainly smooth muscle cell hyperplasia, similar to human atherosclerosis, showed little expression of CCL22, particularly, in many cases, at sites with few VSMCs.

The expression of CCL22 is generally one of the features of M2 macrophages. We found that the localization of M2 macrophages in atherosclerosis is inversely proportional to the distribution of VSMCs. Conversely, it was thought that M1 macrophages migrated to a site with many VSMCs, and that the latter cells then increased at that site. VSMCs express IFN-γ26, which is thought to promote the differentiation of M1-macrophages and to further increase VSMCs. CCR4 is a receptor that is expressed mainly in Th2 cells that produce IL-432. In this study, the expression of IL-4 in the atherosclerotic lesion was observed more abundantly in the carotid arteries of apoE-KO mice than in the ligated carotid arteries of WT-mice.

The regulation of expression of CCL22 has been shown to be related to the H2 receptor using H1R/ligation-induced neointimal hyperplasia, intimal hyperplasia was observed mainly in the smooth muscle cell layer. In this model, CCL22-negative macrophages were the dominant macrophages found in atherosclerotic lesions. In the common carotid artery atherosclerosis of apoE-KO mice, lesions were observed with a reduced increase in VSMCs and mainly contained foamy macrophages and cholesterol. In comparing these two experimental models, lesions with mainly smooth muscle cell hyperplasia, similar to human atherosclerosis, showed little expression of CCL22, particularly, in many cases, at sites with few VSMCs.

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The regulation of expression of CCL22 has been shown to be related to the H2 receptor using H1R/
was considered to be one reason for such paradoxical phenomena in atherosclerosis.

In conclusion, the expression of H1 and H2 receptors, and the migration of Th1 and Th2 cells influenced the expression balance in M1- and M2-like macrophages and may be involved in the processes and progression of atherosclerosis (Fig. 7). Although the cellular microenvironment and cell–cell interactions have recently been gaining increasing attention in studies on the pathology of cancer, it may be that a similar pathology exists in atherosclerosis.

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Conflicts of Interest

The authors declare no conflicts of interest associated with the manuscript.

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Supplemental Fig. 1. Negative control staining of CCL22 in lipid clefts
Fig. A shows negative control staining of CCL22 in Fig. 1C, and B is a negative control for the CCL22 stain in Fig. 5A.

Supplemental Fig. 2. Expression ratios of VSMCs to CCL22
The expression levels of VSMCs and CCL22 were quantified as positive areas by Photoshop CS (Adobe Systems Incorporated, San Jose, CA). A. and B. show the positive area ratios of VSMCs/CCL22 of high and low VSMC density areas in a human common carotid artery (Fig. 1) and human coronary artery with a bare metal stent (Fig. 2). The measurements were performed independently by three pathologists. A significant negative correlation between the expression levels of VSMCs and CCL22 was found. Values represent the mean ± SD of triplicate measurements from three independent experiments (***P<0.001).
**Supplemental Fig. 3.** Immunofluorescence staining of MCP-1 and CCL22 in an area with a high degree of VSMC density.

Fig. depict the same area as Fig. 1B-β. Immunofluorescence staining showed monocyte chemoattractant protein (MCP)-1 (clone 2.2-H5-01A11, 10 µg/ml PeproTech Inc., Rocky Hill, NJ; red) positive cells were negative for CCL22 (green). Bars indicate 50 µm.

**Supplemental Fig. 4.** The concentration of histamine in sera of histamine receptor knockout mice.

An ELISA (R&D Systems and Immunotech, Marseille, France) showed that histamine receptor knockout mice (H2R-KO) mice ($n=8$) had higher serum levels of histamine than WT ($n=8$), and H1R-KO mice ($n=8$) that were maintained for 11 weeks on a normal chow diet. Values represent the mean ± SD of triplicate measurements from three independent experiments ($^{*}P<0.05$, N.S: not significant).
Supplemental Fig. 5. CCL22 expression in response to histamine and effects of histamine agonist and antagonist in J774A.1 cells

A) Culture supernatants of J774A.1 cells (1 × 10⁶ cells/mL) containing CCL22 differentiated into M1-/M2-like cells in 24 h culture with/without 50 µM histamine (Wako) were measured by enzyme-linked immunoassay (ELISA; R&D Systems, Minneapolis, MN, USA). Histamine increased the concentration of CCL22 in the culture supernatant of M2-like macrophages. In contrast, M1-like macrophages were hardly affected by histamine, and did not significantly express CCL22 protein compared with M2-like macrophages.

B) Expression of CCL22 in J774A.1 cells incubated for 24 h with 50 µM histamine, 50 µM H1 or 50 µM H2 receptor agonist (2-pyridylethylamine dihydrochloride and dimaprit dihydrochloride, respectively from Wako), or 50 µM histamine added with 10 µM pyrilamine (H1 receptor blocker from Sigma) or 50 µM histamine added with 10 µM cimetidine (H2 receptor blocker from Sigma). CCL22 levels increased significantly when the histamine H2 agonist was added to the culture, but were not influenced by the histamine H1 agonist. The increase of CCL22 from M2-macrophages by 50 µM histamine stimulation was significantly reduced by the addition of pyrilamine, but not cimetidine.

H1-ago = 2-pyridylethylamine dihydrochloride, H2-ago = dimaprit dihydrochloride, H1-antago = pyrilamine, H2-antago = cimetidine

The values are the mean ± SD of triplicate measurements from 3 independent experiments (**P<0.01, ***P<0.001, N.S: not significant).