Influenza A virus (IAV) infection induces TLR7-mediated interferon-regulated gene expression, which has previously been shown to have antiviral and anti-inflammatory effects. In this study, we evaluated the role of TLR7 signaling in the immune response against IAV in mice. We found that TLR7 activation was necessary for the development of a potent adaptive immune response against IAV. Furthermore, we showed that TLR7 signaling was critical for the induction of interferon-β (IFN-β) expression, which is essential for controlling IAV replication. Our results suggest that TLR7 signaling plays a crucial role in the immune response against IAV and may represent a potential therapeutic target for the treatment of influenza.
of RANTES in the initiation of atherosclerotic plaque and in promoting late-stage atherosclerosis. Fractalkine (CX3CL1) is a unique member of the CX3C subfamily and is expressed in both a soluble and membrane-bound form on the surface of the inflamed endothelium.

Fractalkine is reported to be associated with atherogenesis and 2 specific mutations in the CX3CR1 gene have been shown to reduce the risk for decreased atherosclerotic lesion formation. However, whether MCP-1, RANTES and fractalkine prompt plaque vulnerability and contribute to ACS is still unknown. In the present study, we assessed the hypothesis that the expression of MCP-1, RANTES and fractalkine are associated with plaque vulnerability.

### Methods

#### Study Population

All 220 patients underwent selective coronary angiography (CAG; 127 men, 93 women; age 59.8±8.2 years, range 38–84 years) and were divided into 4 groups according to the guidelines of the American College of Cardiology and American Heart Association. Group A comprised 60 patients with AMI and group B included 60 patients with UAP. There were 60 patients with stable angina pectoris (SAP) in group C and 40 patients without coronary heart disease in group D (control group). The baseline characteristics of the 4 groups are shown in Table 1. All patients in the AMI, UAP and SAP groups were treated after admission with antiplatelet and other therapies including angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers, β-blockers and statins. All patients gave their informed consent to this study and the protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University.

#### Blood Biomarker Measurements

Blood samples from every patient were collected on admission to hospital. Serum levels of total cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were measured by enzymatic assays. The C-reactive protein level (CRP) was assayed using a highly sensitive enzyme-linked immunosorbent assay kit (Diagnostic System Laboratory, Chicago, IL, USA). Levels of MCP-1, RANTES and fractalkine were measured using enzyme-linked immunosorbent assay kits (You er Biochemistry Company, Wuhan, China).

#### Quantitative CAG (QCA)

All patients underwent CAG. Percent diameter stenosis, percent area stenosis, culprit lesion length, and reference diameter were assessed by QCA, which was performed using the Digital Cardiac Imaging system. Coronary stenosis was defined if

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A (n=60)</th>
<th>Group B (n=60)</th>
<th>Group C (n=40)</th>
<th>Group D (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61.2±6.4</td>
<td>57.3±5.7</td>
<td>61.4±5.4</td>
<td>59.0±6.8</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>139.4±11.9</td>
<td>136.0±11.1</td>
<td>136.7±12.3</td>
<td>130.1±9.2</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>82.5±12.7</td>
<td>80.1±12.5</td>
<td>81.2±15.0</td>
<td>78.4±9.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.6±3.1</td>
<td>26.9±2.5</td>
<td>27.1±2.7</td>
<td>25.0±2.2</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>23 (38.3%)</td>
<td>18 (30.0%)</td>
<td>16 (26.7%)</td>
<td>10 (25.0%)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>26 (43.3%)</td>
<td>20 (33.3%)</td>
<td>19 (31.7%)</td>
<td>12 (30.0%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>22 (36.7%)</td>
<td>19 (31.7%)</td>
<td>18 (30.0%)</td>
<td>11 (27.5%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>21 (35.0%)</td>
<td>21 (35.0%)</td>
<td>20 (33.3%)</td>
<td>12 (30.0%)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>115.0±31.0</td>
<td>116.1±36.5</td>
<td>117.4±30.2</td>
<td>103.3±23.0</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>226.6±31.3</td>
<td>229.3±38.7</td>
<td>213.5±38.3</td>
<td>211.6±29.8</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>151.2±34.0</td>
<td>155.1±37.5</td>
<td>133.0±33.6</td>
<td>130.7±22.4</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>40.2±5.8</td>
<td>41.0±6.2</td>
<td>39.2±5.4</td>
<td>42.9±8.9</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>139.1±54.0</td>
<td>142.6±54.9</td>
<td>140.8±57.6</td>
<td>137.3±50.5</td>
</tr>
<tr>
<td>Leucocytes (×10³/L)</td>
<td>8.2±3.1</td>
<td>8.0±3.0</td>
<td>5.2±2.2</td>
<td>4.9±2.0</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>6.9±2.7</td>
<td>5.7±2.3</td>
<td>2.9±1.2</td>
<td>2.7±1.4</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>91.2±9.7</td>
<td>87.3±9.1</td>
<td>37.0±6.8</td>
<td>23.7±7.1</td>
</tr>
<tr>
<td>RANTES (pg/ml)</td>
<td>62.7±8.2</td>
<td>61.8±7.9</td>
<td>27.3±4.5</td>
<td>15.8±5.1</td>
</tr>
<tr>
<td>Fractalkine (pg/ml)</td>
<td>65.1±9.7</td>
<td>62.3±10.5</td>
<td>34.0±4.7</td>
<td>19.9±6.0</td>
</tr>
</tbody>
</table>

**P<0.05, **P<0.01 vs. SAP; †P<0.05, ††P<0.01 vs. Control.

AMI, acute myocardial infarction; UAP, unstable angina pectoris; SAP, stable angina pectoris; BP, blood pressure; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; MCP, monocyte chemoattractant protein-1; RANTES, Regulated on Activation, Normal T-cell Expressed and Secreted.
there was a more than 50% decrease in diameter compared with an adjacent reference segment and the number of stenotic lesions in each coronary artery was recorded.

**Intravascular Ultrasound (IVUS) Studies**

Three-coronary IVUS (iLab, Boston Scientific Corporation, USA) was carried out in all patients as described previously and the following parameters were measured: external elastic membrane area (EEMA), minimum lumen area, plaque area (PA), plaque burden (PB), plaque eccentricity index, and remodeling index (RI). RI >1.05 was regarded as positive remodeling, 0.95–1.05 as intermediate remodeling, and <0.95 as

### Table 3. Measurements of Intravascular Ultrasound Imaging in the 3 Patient Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gr AMI (n=60)</th>
<th>UAP (n=60)</th>
<th>Gr SAP (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque</td>
<td>117</td>
<td>111</td>
<td>105</td>
</tr>
<tr>
<td>Lipid plaque</td>
<td>60 (51.3%)**</td>
<td>53 (47.7%)**</td>
<td>18 (17.1%)</td>
</tr>
<tr>
<td>Fibrous plaque</td>
<td>21 (17.9%)**</td>
<td>23 (20.7%)**</td>
<td>55 (52.4%)</td>
</tr>
<tr>
<td>Mixed plaque</td>
<td>19 (16.2%)</td>
<td>15 (13.5%)</td>
<td>13 (12.4%)</td>
</tr>
<tr>
<td>Calcified plaque</td>
<td>18 (15.4%)</td>
<td>19 (17.1%)</td>
<td>18 (17.1%)</td>
</tr>
<tr>
<td>Patients with ruptured plaque</td>
<td>81 (69.2%)**</td>
<td>59 (53.2%)**</td>
<td>27 (25.7%)</td>
</tr>
<tr>
<td>Minimum lumen area (mm²)</td>
<td>3.3±0.9</td>
<td>3.3±0.8</td>
<td>3.8±0.9</td>
</tr>
<tr>
<td>External elastic membrane area (mm²)</td>
<td>12.8±1.3**</td>
<td>12.6±1.8**</td>
<td>11.4±1.3</td>
</tr>
<tr>
<td>Plaque area (mm²)</td>
<td>9.5±2.6**</td>
<td>9.3±2.0**</td>
<td>7.6±2.8</td>
</tr>
<tr>
<td>Plaque burden (%)</td>
<td>74.2±10.3**</td>
<td>73.8±8.9**</td>
<td>66.7±8.3</td>
</tr>
<tr>
<td>Eccentric index</td>
<td>0.7±0.2*</td>
<td>0.6±0.2*</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>Remodeling index</td>
<td>1.1±0.1**</td>
<td>1.1±0.1**</td>
<td>1.0±0.1</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 vs. SAP.
AMI, acute myocardial infarction; SAP, stable angina pectoris; UAP, unstable angina pectoris.

**Figure 1.** Intravascular ultrasound imaging of the coronary arteries. (A) Plaque with a large lipid core in the coronary artery of a patient with acute myocardial infarction. (B) Lipid plaque in the coronary artery of a patient with unstable angina. (C) Fibrous plaque in the coronary artery of a patient with stable angina.
negative remodeling. Coronary plaque composition was assessed visually according to plaque echogenicity.\textsuperscript{17,18} Plaque rupture was defined as a plaque containing a cavity that communicated with the lumen and had an overlying residual fibrous cap fragment or was wedged by newly-formed thrombus, no matter whether plaque rupture occurred in culprit or non-culprit lesions.\textsuperscript{19} The IVUS images were reviewed by 2 independent observers and the final consensus values were used for data analysis.

Chemotaxis Test
Monocytes were extracted by centrifugation of the blood samples, and 0.1 ml of the monocyte suspension (4×10\textsuperscript{4}/ml) was instilled into the transwell chamber to assay monocyte chemotaxis. Monocytes were stimulated by the patient’s own serum as described previously.\textsuperscript{20} Next, the number of migrated monocytes and the distance traveled by monocytes in 10 high-power microscopic fields were measured and the values averaged.

Molecular Biological Studies
Total RNA of the monocytes was extracted, and the mRNA expression of MCP-1, RANTES and fractalkine was examined by quantitative reverse transcription polymerase chain reaction (RT-PCR) using a LightCycler (Roche Applied Science, USA) following the manufacturer’s instructions. The mRNA sequences were obtained from GenBank (Table 2). Quantitative values were obtained from the threshold cycle value (Ct).\textsuperscript{21} The transcript number of glyceraldehyde 3-phosphate dehydrogenase was quantified as an internal control. The results of RT-PCR were confirmed by gel electrophoresis.

Statistical Analysis
All statistical analyses were performed with SPSS16.0 software package (SPSS Inc, Chicago, IL, USA). Values are expressed as mean±SD. Comparison of continuous variables among multiple groups was performed by analysis of variance with ANOVA and post hoc comparisons were made using the lsyergeric acid diethylamide test. Chi-squared analysis was applied to compare categorical data. Linear regression analyses were performed to evaluate the relationship between the results of IVUS and chemotaxis and expression of MCP-1, RANTES and fractalkine. A 2-tailed P<0.05 was considered statistically significant.

Results

Base Line Characteristics and Blood Biomarker Measurements
As shown in Table 1, there were no significant differences among the 4 groups regarding age, sex, blood pressure, body weight, blood sugar and lipid profile. Leucocyte counts and concentrations of high-sensitivity CRP (hsCRP), MCP-1, RANTES and fractalkine in groups A and B were significantly higher than those in groups C and D. However, there was no significant difference between groups A and B.

QCA
CAG studies demonstrated a total of 117 and 111 coronary stenotic lesions in the AMI and UAP groups, respectively, while in the SAP group there were 105 coronary lesions; 76 lesions (65\%) in the AMI, 77 lesions (69\%) in the UAP and 69 lesions (66\%) in the SAP group had stenosis ≥70\%. QCA results did not show significant differences regarding percent diameter stenosis and area stenosis among the AMI, UAP and SAP groups (Table 4).

IVUS Measurements
IVUS detected 117 plaques in group A, 111 plaques in group B and 105 plaques in group C. Patients in group A (51.3\%) and group B (47.7\%) had more lipid plaques than those in group C (17.1\%) (all P<0.01, respectively), while patients in group C had more fibrous plaques (52.4\%) than those in group A (17.9\%) and group B (20.7\%) (P<0.01, respectively). There were more ruptured plaques in groups A and B than those in group C (P<0.01, respectively). However, there was no significant difference between group A and group B. There were more eccentric plaques in groups A and B, and the values of EEDEA, PA, PB and RI in groups A and B were significantly larger than those in group C (P<0.05–0.01) (Table 3, Figure 1).
Chemokines and Plaque Vulnerability

Discussion

Atherosclerotic plaque rupture is the major cause of ACS and stable plaques may become vulnerable to rupture or erosion once they develop active inflammation. Inflammation has emerged as a crucial force driving the initiation and progression of atherosclerotic lesion formation. Accumulation of monocytes/macrophages has been noted in the intima of early atherosclerotic lesions and these inflammatory cells may promote progression of atherosclerosis by producing and releasing various cytokines and chemokines. Studies have demonstrated that the chemokines MCP-1, RANTES and fractalkine are potent activators and chemoattractant signals of inflammatory and vascular cells. In the present study, we have shown higher expressions of MCP-1, RANTES and fractalkine in patients with AMI or UAP. Our results demonstrate that MCP-1, RANTES and fractalkine independently partici-
patate in the pathogenesis of plaque vulnerability and subsequent plaque rupture.

Once combined with its receptor CCR2, MCP-1 activates monocytes/macrophages by promoting leukocyte-endothelium binding and migration to sites of inflammation. MCP-1 as an upstream chemokine in the atherosclerotic inflammatory pathway has been consistently detected in human atherosclerotic lesions, as well as in animal models of atherosclerosis with dietary-induced hypercholesterolemia, and it is a logical reason that the intensive infiltration of macrophages into plaques with active inflammation is, to a large extent, due to the chemo tactic effects of MCP-1. Aiello et al reported that fractalkine deficiency markedly reduced macrophage infiltration and atherosclerotic lesion formation without affecting lipoprotein metabolism in MCP-1-deficient mice. Zhong et al in our laboratory found that dominant-negative mutation of MCP-1 prevented vulnerable plaques from rupture in rabbits, independent of serum lipid levels. The CC chemokine RANTES and its receptors CCR1 and CCR5 in atherosclerosis have been addressed in a number of studies. RANTES has been implicated in cardiac inflammatory disorders after organ transplantation or arterial injury. In addition, RANTES has been detected in plasma samples of patients suffering from coronary heart disease. Mice deficient in the RANTES gene show impaired recruitment of T cells and monocytes to inflammatory foci. Barcelos et al found that antagonism of RANTES receptors with Met-RANTES reduced atherosclerotic plaque formation in mice. Braunersreuther et al reported that RANTES plays a central role in promoting late-stage atherosclerosis in the context of accelerated atherosclerosis in ApoE−/− mice promoted by high-fat diet.

Fractalkine (CX3CL1) is a unique member of the CX3C chemokine subfamily. Accumulating evidence indicates CX3CL1/CX3CR1 involvement in the pathogenesis of atherosclerosis. Teupser et al reported that the targeted deletion of CX3CL1 decreased atherosclerosis lesion formation in the brachiocephalic artery but not the aortic root, with fewer macrophages in the lesion area of atherosclerosis in CX3CL1−/− ApoE−/− mice, implicating a role of this chemokine system in atherogenesis. Furthermore, Lucas et al found expression of CX3CL1 and CX3CR1 in human coronary atherosclerotic plaques, and that CX3CL1/CX3CR1 may contribute to atherogenesis and plaque destabilization in human coronary artery disease.

Studies have also shown that MCP-1, RANTES and fractalkine play independent roles in atherogenesis. Digby et al reported that the anti-inflammatory effects of nicotinic acid in adipocytes contribute to its suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin. Saederup et al reported that fractalkine deficiency markedly reduced macrophage accumulation and atherosclerotic lesion formation in CCR2−/− mice, providing the first in vivo evidence of independent roles for CX3CL1 and CCR2 in direct monocyte recruitment to atherothrombotic lesions. Combined inhibition of CCL2, CCR5, and CX3CR1 in ApoE−/− mice abrogated bone marrow monocytesis and additively reduced circulating monocytes and atherosclerosis, despite persistent hypercholesterolemia. Moreover, the inhibition of CCL2, CX3CR1, and CCR5 in ApoE−/− mice almost abolished atherosclerosis, suggesting that CX3CR1-, CCL2-, and CCR5-mediated signals play independent and additive roles in atherogenesis.

Many investigators have reported that certain inflammatory markers, such as hsCRP, have the potential to predict the risk of future cardiovascular events. Luzzio et al found that elevated CRP concentration (>3 mg/L) at the time of hospital admission predicted a poor outcome in patients with UAP. In the present study, we found that the hsCRP, MCP-1, RANTES and fractalkine levels were significantly higher in patients with AMI and UAP than in patients with SAP, suggesting that serum levels of MCP-1, RANTES and fractalkine may be good markers of plaque vulnerability.

In the present study, we tested the monocyte chemotaxis in patients with ACS and found that the averaged number of migrated monocytes and the averaged distance traveled by monocytes in patients with AMI or UAP were higher than those of SAP patients, which reflects the close association of monocyte chemotaxis with the vulnerability of atherosclerotic plaques.

In order to show the expression levels of MCP-1, RANTES and fractalkine that modulate monocyte chemotaxis, we measured their mRNA expression levels in the monocytes of patients with ACS or SAP and found that mRNA expression levels of MCP-1, RANTES and fractalkine were higher in patients with ACS than in those with SAP. All these results reveal that MCP-1, RANTES and fractalkine are the main chemokines contributing to atherosclerotic plaque vulnerability.

Among the many plaque imaging techniques, IVUS has been advocated as assessing the morphological changes of vulnerable plaque in vivo. Our current study clearly demonstrated that patients with AMI or UAP have more vulnerable plaques characterized by a more eccentric distribution, a larger EEM area, a bigger PA and a larger PB. Correlation analysis found that PB measured with IVUS correlated well with monocyte chemotaxis, implying a role of monocytes chemotaxis and the related chemokines MCP-1, RANTES and fractalkine in the development of atherosclerotic plaques.

In conclusion, MCP-1, RANTES and fractalkine independently participate in the pathogenesis of coronary plaque vulnerability and subsequent plaque rupture.

Acknowledgments

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References


