The Dynamics of Circulating Monocyte Subsets and Intra-Plaque Proliferating Macrophages during the Development of Atherosclerosis in ApoE−/− Mice

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Summary
To detect the development of monocytes and proliferative macrophages in atherosclerosis of ApoE−/− mice, we randomly assigned 84 ApoE−/− mice fed western diet or chow diet. On weeks 2, 4, 6, 8, 10, and 12 after fed high-fat diet or normal chow diet, animals were euthanized (n = 7 for each group at each time point). Flow cytometry methods were used to analyze the proportions of circulation monocyte subsets. The macrophage and proliferative macrophage accumulation within atherosclerotic plaques was estimated by confocal fluorescence microscopy. Plasma levels of total cholesterol and triglyceride were measured by ELISA kit. The plaques of aortic sinus were stained with Oil Red O. The percent of Ly6C hi circulation monocyte, the density of proliferation macrophage, the total plasma cholesterol and triglyceride levels, the lesion area of ApoE−/− mice were consistently elevated in chow diet throughout the trial. The total plasma cholesterol and triglyceride levels, the lesion area were elevated in western diet group with age, and they were always higher than the chow diet group. The Ly6C hi monocytes and proliferative macrophages reached a plateau at 8 weeks and 6 weeks; despite continued high-triglyceride high-cholesterol diet the percent did not significantly change. Interestingly, the density of macrophage did not change significantly over age in western and chow diet groups. Our results provide a dynamic view of Ly6C hi monocyte subset, the density of macrophage and proliferation macrophage change during the development and progression of atherosclerosis, which is relevant for designing new treatment strategies targeting mononuclear phagocytes in this model.

Key words: Cardiovascular disease, Hyperlipidemia, Inflammation

The rupture of atherosclerotic plaque (AS), which leads to thrombosis formation and/or thromboembolism, and consequently, the persistent ischemia in vital organs, is the leading cause of cardio-cerebrovascular disease worldwide. Recent basic studies have shown that monocytes/macrophages are major players in atherosclerosis. Specifically, hyperlipidemia-induced proinflammatory Ly6C hi monocyotosis, via a CCR2-dependent mechanism, contributes to monocyte sub-endothelial infiltration and plaque destabilization, and ultimately ischemic cardiovascular events. Additionally, the human proinflammatory monocyte counterpart, i.e., the CD14++CD16+ subset, whose level is elevated during hyperlipidemia and after cerebral and myocardial ischemia, is associated with the occurrence of acute coronary syndrome and poor prognosis after cerebral and myocardial ischemia.

The general paradigm that macrophages are terminal-differentiated, and infiltrating monocytes contribute solely to local macrophages in the atherosclerotic plaque has been recently challenged by Robbins who showed that the replenishment of macrophages in experimental atherosclerotic lesion depends predominantly on local macrophage proliferation rather than on monocyte influx. Accordingly, strategies targeting local macrophage self-renewal may represent a promising approach for regulating atherosclerosis. To the best of our knowledge, the relationship between circulating monocyte subsets and intra-plaque proliferating macrophages during the development of atherosclerosis in ApoE−/− mice, a commonly used...
murine model for atherosclerosis, has not been fully examined. Therefore, the present work aims to address this issue by comparing the related pathological dynamics in ApoE−/− mice fed with a western diet and a chow diet.

Methods

Reagents: Fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ly6C (clone HK1.4), phycoerythrin (PE)-conjugated anti-mouse CD11b (clone M1/70) and Peridinin-chlorophyll proteins/Cyanine5.5 (PerCP/Cy5.5)-conjugated anti-mouse Ly6G (clone 1A8) were purchased from Biolegend (SanDiego, CA, USA). Monoclonal antibody (mAb) against mouse Ki67 and polyclonal antibody (pAb) against mouse F4/80 were obtained from Abcam (Cambridge, UK). Mounting medium with DAPI and polyclonal antibody (pAb) against mouse F4/80 were obtained from Abcam (Cambridge, UK). Mounting medium with DAPI was purchased from Zhongshan Golden Bridge Bio-technology (Beijing, China). Oil Red O was obtained from Sigma-Aldrich (St. Louis, MO, USA). The ELISA kits for total cholesterol (TC) and triglycerides (TG) were obtained from Zhongshan Golden Bridge Bio-technology (Beijing, China). Mounting medium with DAPI was purchased from Biolegend (SanDiego, CA, USA). A total of 84 mice were used, each of which weighed 16-20 g at the beginning of the experiment. All mice were kept on a 12 hours light/12 hours dark cycle and randomly divided into two groups: a chow diet (CD) group (n = 42) and a western diet (WD) group (n = 42). The TP26303 high-fat chow (TROPHIC Animal Feed High-tech Co Ltd, Jiangsu Province, China) was used in WD group. On weeks 2, 4, 6, 8, 10, and 12 of being fed with WD or CD diet, animals were euthanized by anesthetization after overdose inhalation of sulfuric ether (n = 7 for each group at each time point). Their blood and heart tissue were collected for the following assays.

Circulating monocyte subsets: Circulation monocyte subsets were assessed using blood anti-coagulated with EDTA immediately after collection and kept at room temperature for the entire staining procedure. Blood cell suspensions were stained with Ly-6 G, CD11b, and Ly-6C. After red blood cell lysis, samples were analyzed with Cytomics FC500 Flow Cytometry (Beckman Coulter, Miami, FL, USA) and analyzed with FlowJo software (Treestar, Ashland, OR, USA). The gating strategies for analyzing mouse circulating monocyte subsets were in accordance with our work (Figure 1).31,22

Macrophages in atherosclerotic lesions: Cryostat sections at the level of aortic valve (5 μm) were immunostained with F4/80 (1:100, a primary rat anti-mouse monoclonal antibody) and Ki67 (1:300, a primary rabbit anti-mouse polyclonal antibody) in antibody diluent at 4°C for 16 hours, followed by incubation with goat anti-rat (1:100) and goat anti-rabbit (1:100) secondary antibodies for 1 hour. Slides were mounted with Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Sections were viewed and images were captured within the area of atherosclerotic lesions using a TCS SP8 confocal fluorescence microscope (Leica, Wetzlar, Germany). Image analysis was performed using Image Pro Plus 4.5 (Media Cybernatics, Silver Spring, MA, USA). Results are expressed as the densities of the proliferating macrophage (PMφ, F4/80’Ki67’DAPI’), total macrophages (Mφ, F4/80’DAPI’) and the percent of proliferated macrophage (PMφ/Mφ).

Atherosclerotic lesion area: To quantify the aortic atherosclerotic burden, the entire aortic root area was serially sectioned at 5-μm intervals from the base of the aortic sinus using a cryostat at −25°C. The aortic root lesion area and the lipid content were quantified in Oil Red O and counterstained with hematoxylin. For each mouse, the average of three sections spaced by 50 μm where three leaflets are clearly visible was used for quantification. Image analysis was determined using Image Pro Plus. Results were expressed as the percent of plaque area filled with the total cross-sectional vessel wall.

Statistical analysis: All data are presented as the mean ± standard error of mean. The Shapiro-Wilk procedure was used for normality test. For comparisons of parameters between WD and CD groups, an unpaired Student’s t test or a Mann-Whitney U test was used. The relationships between monocyte subsets/macrophages and plasma/pathological measures were evaluated using Pearson correlation (data passed normality test) or Spearman rank correlation (data failed normality test). Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A two-tailed P value less than 0.05 was considered statistically significant.

Results

Temporal dynamics of body weights: As shown in Figure 2, the body weight of both the WD group and the CD group was gradually increased throughout the investigation. Compared with the CD group, The body weight of the WD group was significantly higher from week 2 (all P < 0.01, n = 7 for each time point; Figure 2A).

Temporal dynamics of circulating monocyte subsets: As shown in Figure 1, the percent of circulating Ly6C+ monocyte in WD-fed ApoE−/− mice underwent a dramatic and consistent increasing trend throughout the investigation, with statistical significance from week 2. A similar, albeit to a lesser extent, age-dependent increasing trend of Ly6C+ monocytes was also observed in CD-fed ApoE−/− mice. Compared with that measured on week 2, the Ly6C+ monocyte percent was statistically elevated on week 10 in CD-fed mice (51.86 ± 2.64% versus 62.04 ± 4.63%, P < 0.01, n = 7 for each time point; Figure 1B to D). In the WD group, the percent of Ly6C+ monocyte subset showed a dramatic increase from week 4, and reached a plateau level on week 8. Compared to those measured at the same time point in CD groups, the Ly6C+ monocyte percent increased significantly on weeks 6, 8, 10, and 12 (all P < 0.01, n = 7 for each time point; Figure 1D).

Temporal dynamics of intra-plaque total and proliferating macrophages: As shown in Figure 3, the tri-chrome immunostaining revealed the co-localization of F4/80 (a macrophage-specific surface marker), Ki67 (a proliferating cell nuclear marker), and DAPI in atherosclerotic lesions, indicating the existence of intra-plaque proliferating macrophages. In CD-fed ApoE−/− mice, the proliferating macrophage density showed a slightly increasing trend
from week 6 (Figure 3A, B and D). Compared with that measured on week 2, the density of proliferating macrophage significantly increased (0.146 ± 0.037 versus 0.300 ± 0.102 cells/mm²; P < 0.01, n = 7 for each time point) on week 12. In WD-fed group, the increasing trend for proliferating macrophage density was more obvious since week 4 and reach a plateau level in week 6 (Figure 3C and D). As shown in Figure 3D, there was no significant difference in terms of total macrophages (F4/80‘DAPI’) between CD- and WD-fed mice. On the contrary, the ratio of proliferating to total macrophages was significantly increased in WD-fed mice.

Temporal dynamics of plasma total cholesterol and triglyceride: To document the TC and triglyceride levels over time in these mice, blood was drawn at various times. The results are shown in Figure 4; each point represents the mean value of 7 male mice at each time. The total plasma cholesterol and triglyceride levels in ApoE−/−
were consistently elevated throughout the life of the animal. There was greater variability in the plasma TC and triglyceride levels in WD group, and they were always higher than those of dramatically in CD group. The WD resulted in a significant increase in the TC at 4 weeks, 6 weeks, 8 weeks, 10 weeks, and 12 weeks (all \( P < 0.01, n = 7 \) for each time point), which was gradually increased and did not seem to peak at 12 weeks. The triglyceride significantly increased at 6 weeks, 8 weeks, 10 weeks and 12 weeks (all \( P < 0.01, n = 7 \) for each time point), which was gradually increased and did not seem to peak at 12 weeks.

**Temporal dynamics of lesion area:** In both groups of mice, lesions at various stages of development could be observed (Figure 5A and B). ApoE\(^{-/-}\) mice spontaneously develop lesions in the aortic valve. As shown in Figure 5, lesions were observed at aortic sinuses in both CD and WD mice. The mice fed the WD generally contained more advanced lesions at each stage of development compared with the CD. In the CD group, there is a clear plaque at 4 weeks of age. In WD group, the mice had formed distinct plaques after 2 weeks, and the plaque grew rapidly between 6-8 weeks in the root of the aorta.

**Statistical analysis of linear regression:** The correlation analyses were carried out to evaluate the association between the body weight and plasma, the TC and triglyceride of different groups. The results showed that both the TC and triglyceride were positively correlated with the body weight (both \( P < 0.01, n = 7 \) for each group at each time point; Figure 2B and C). After that, we investigated the association between among the Ly6Chi monocyte percent, the total macrophages, and the density of proliferating macrophage and found that the Ly6C\(^{hi}\) monocyte percent but not the density of proliferating macrophage was positively correlated with the total macrophages (\( P < 0.01, \) for each group at each time point; Figure 6D). Meanwhile, we also found that the Ly6C\(^{hi}\) monocyte percent was positively correlated with the density of proliferating macrophage (\( P < 0.01, \) for each group at each time point; Figure 7).

**Discussion**

During the inflammatory response that drives atherogenesis, circulating monocytes into the intimal region, macrophages undergo partial self-proliferation, resulting in their progressive accumulation in the expanding arterial wall.\footnote{23,24} Monocytes and macrophages are essential partners in innate and acquired immunity and play a variety of roles in atherosclerotic plaque development and its clinical sequelae.\footnote{25} In our experiments, The ApoE\(^{-/-}\) mice were used to establish the models of atherosclerosis by consuming a WD. Subsequently, the circulating monocytes and local proliferative macrophages were observed at different time points representative of different stages of AS. Our results provide important guidelines for several interventions, including reducing the proportion of Ly6C\(^{hi}\) monocytes and inhibit the density of proliferative macrophages.

Circulating monocytes display heterogeneity in mice and humans; indeed, mouse monocytes fall into at least 2 phenotypically distinct subsets: Ly-6C\(^{hi}\) (Ly6G\(^{-}\)CD11b\(^{+}\) LyC\(^{hi}\)) and Ly-6C\(^{lo}\) (Ly6G\(^{-}\)CD11b\(^{+}\)LyC\(^{lo}\)).\footnote{26} Ly-6C\(^{hi}\) cells selectively populate sites of experimentally induced inflammation. The circulating concentration of Ly6C\(^{hi}\) monocytes increases in response to proinflammatory stimuli and hypercholesterolaemia. It has been widely assumed that the Ly6C\(^{hi}\) monocytes will be recruited into plaques during the development and progression of athe-
Figure 3. Temporal dynamics of intra-plaque proliferating macrophages during the development and progression of atherosclerosis. A: The representative pictures for intra-plaque proliferating macrophages in chow and western groups. B and C: The representative dynamic profiles of intra-plaque proliferating macrophages in the 2 groups. D: The statistical analysis of the density of proliferating macrophages and macrophages, and the percentage of proliferating macrophages in the 2 groups. Mφ indicates macrophages; PMφ, proliferating macrophages; PMφ/Mφ, proliferating macrophages/macrophages; W, weeks. * P < 0.05 versus chow diet group; ** P < 0.01 versus chow diet group (n = 7 in each group).
Atherosclerosis is a complex heterogeneous disease entity, and there are a variety of risk factors. These include TG, TC, and Ly6C⁺ monocytes and the density of proliferative and total macrophages. Advanced plaque, indicating likeliness for variability during different stages of the disease. Our study further explores the density of proliferative macrophage dynamic changes in the initiation and propagation of the atherosclerotic process, showing that the WD group reached a maximum density at 6 weeks, then showed no significant change after reaching a plateau. The detailed mechanism of why the density of proliferative macrophage reached a maximum at 6 weeks remains unclear. A probable reason is that the atherosclerosis lesion macrophages may be replenished from Ly6C⁺ monocytes or proliferative macrophages. In the initial period (~4 weeks), the source of lesional macrophages were mainly from Ly6C⁺ monocytes, after that, the main source gradually changed to proliferative macrophages. In the present study, before 6 weeks, the proportion of proliferative macrophages rapidly increased, which was in accordance with the previous study. However, after 6 weeks, the number of lesional macrophages came achieved some stability. The role of proliferating macrophages to counterbalance cell loss processes also came into a slow replenishment period.

There is a growing understanding that the balance of macrophages in the plaque is dynamic and that macrophage numbers influence plaque fate. Our research showed that macrophage numbers increased with the area of plaque, but the density of macrophages showed no significant change. Interestingly, our data demonstrate that, in the linear regression model, despite the density of proliferative macrophages correlating well with the TC and TG in CD group, there was not a significant correlation in WD group. Furthermore, there was no association between the density of macrophages and the percentage of Ly6C⁺ monocyte subset under our experimental conditions, suggesting that there was association between the density of macrophage and the density of proliferative macrophage in WD group. These data indirectly suggest that local proliferation dominates lesion macrophage accumulation in atherosclerosis rather than Ly6C⁺ monocytes. These results are consistent with previous studies.

Atherosclerosis is a complex heterogeneous disease entity, and there are a variety of risk factors. These include TG, TC, and Ly6C⁺ monocytes and the density of proliferative and total macrophages. Our study data demonstrate that, in the linear regression model, the lesion area correlated well with those risk factors. Most importantly, the density of proliferative macrophages is under intense study as a novel drug target for atherosclerosis.
Nevertheless, no previous studies have investigated the temporal dynamics of proliferative macrophages. Our work focused on this risk factor to explore the dynamic changes in the development of atherosclerosis, and provided a strong rationale for the development of anti-proliferation of macrophage for this disease. There are a few limitations to this study. We could not quantify or analyze the effects of Ly6C^hi monocytes and proliferative macrophages to macrophages content in the plaques. Future research efforts should be directed to accurately regulate macrophage polarization and numbers to slow the progression of atherosclerosis by reducing the percent of Ly6C^hi monocyte subset and proliferative macrophages.

In conclusion, our result provides a dynamic view of
Figure 6. Statistical analysis of linear regression. A: The linear regression analysis evaluated the association of atherosclerosis with TC, TG, Ly6C<sup>hi</sup> monocyte subset, the density of proliferative macrophage and macrophage. B: The linear regression analysis evaluated the association of Ly6C<sup>hi</sup> monocyte subset with TC and TG. C: The linear regression analysis evaluated the association of the density of proliferative macrophage with TC and TG. D: The linear regression analysis evaluated the association of the density of macrophage with proliferative macrophages and Ly6C<sup>hi</sup> monocytes subset. Mφ indicates macrophages; PMφ, proliferating macrophages; PMφ/Mφ, proliferating macrophages/macrophages; TC, total cholesterol; and TG, triglyceride (n = 7 for each group at each time point).

the Ly6C<sup>hi</sup> monocyte subset, the density of macrophage, and proliferation macrophage change during the development and progression of atherosclerosis, which is relevant to designing new treatment strategies targeting mononuclear phagocytes in this model. We may achieve this through interfering with monocyte recruitment (using 11,12-Epoxyeicosatrienoic acids or antibodies to inhibit CCL-2) or interfering with macrophage proliferation (us-
Figure 7. Linear regression analysis evaluated the association of atherosclerosis with Ly6C<sup>hi</sup> and the density of proliferative macrophage. PMp indicates proliferating macrophages (n = 7 for each group at each time point).

Disclosures

Conflicts of interest: No conflicts of interest are declared by the authors.

References