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Disruption of the TSLP-TSLPR-LAP signaling between epithelial and dendritic cells through hyperlipidemia contributes to regulatory T-Cell defects in atherosclerotic mice

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ABSTRACT

Regulatory T-Cells (Tregs) play a protective role against the development of atherosclerosis. Moreover, thymic stromal lymphopoietin (TSLP)/thymic stromal lymphopoietin receptor (TSLPR) signaling in myeloid dendritic cells (DCs) promote Treg differentiation. Here, we examined the potential role of TSLP/TSLPR on Treg homeostasis in atherosclerosis. The frequencies of both latency-associated peptide $(LAP)^+$ and $Foxp3^+$ Tregs were reduced in the thymus and spleen of $ApoE^{-/-}$ mice compared with C57BL/6 mice, and this effect was associated with decreased thymic output. The tolerogenic function of DCs obtained from $ApoE^{-/-}$ mice was compromised compared with those from C57BL/6 mice. The expression of TSLP and TSLPR was also inhibited in $ApoE^{-/-}$ mice. In addition, we found that ox-LDL attenuated TSLP expression in cultured thymic epithelial cells (TECs) through the activation of retinoid X receptor alpha (RXRA) and IL-1β and decreased LAP and PD-L1 expression in oxLDL-activated DCs while both were up-regulated in TSLP-activated DCs. We also observed that the TSLP-DCs mediated differentiation of Tregs was abrogated through LAP neutralization. Furthermore, TSLP injection rescued Treg defects in $ApoE^{-/-}$ mice. These findings suggest that Treg defects in $ApoE^{-/-}$ mice might partially be attributed to the disruption of TSLP-TSLPR-LAP signaling in epithelial cells (ECs) and DCs.

1. Introduction

Atherosclerosis is a chronic inflammatory disease in which T lymphocytes play an important role [1–3]. Atherosclerotic lesions contain T-cells, macrophages, and smooth muscle cells, which produce effector chemokines and cytokines in response to hypercholesterolemia. $CD4^+$ T-cells, particularly Th1 cells, might contribute to the development and/or progression of atherosclerosis [4,5]. Indeed, the predominant $CD4^+$ T-cells of murine and human atherosclerotic plaques are Th1 cells.

However, $CD4^+CD25^+Foxp3^+$ regulatory T-cells ($Foxp3^+$ Tregs) and $CD4^+$ latency-associated peptide $(LAP)^+$ regulatory T-cells (LAP^+ Tregs) are two novel T-cell subsets with immunoregulatory and immunosuppressive functions for the modulation of immune responses and the regulation of deleterious immune activation [6,7]. Several studies have demonstrated a clear decrease in the number of Tregs or the functional disorder of these cells in various auto-immune diseases, including human and murine atherosclerosis [8–10]. In addition, the induction of LAP^+ Tregs or the adoptive transfer of $Foxp3^+$ Tregs to atherosclerotic mice could regulate

Abbreviations: TSLP, thymic stromal lymphopoietin; DCs, dendritic cells; ApoE, Apolipoprotein E; Tregs, Regulatory T-cells; TECs, thymus epithelial cells; LAP, latency-associated peptide; Foxp3, Forkhead box P3; ox-LDL, oxidized low density lipoprotein; TREC, T-cell receptor excision circle; ACS, acute coronary syndrome; PD-L1, programmed death ligand-1; CAD, coronary artery disease; RXRA, retinoid X receptor alpha.

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the development of atherosclerosis, suggesting a protective role for Tregs in atherosclerosis [7,10–12]. Similar to other T-cells, Treg cells develop in the thymus [13]. Recently, Tang et al. and Zhang et al. showed that Foxp3$^+$ Treg cell defects in patients with chronic heart failure (CHF) [14] and acute coronary syndrome (ACS) [15] were majorly caused by decreased thymic output of nascent Treg cells. These results suggest a potential thymic disorder in these patients.

As a member of the IL-7 cytokine family, thymic stromal lymphopoietin (TSLP) is principally expressed in the epithelial cells of the thymus, lung, skin, and gut [16,17]. TSLP signals via the TSLP receptor (TSLPR), which comprises a heterodimer of the IL-7 receptor α chain and the TSLPR chain and is widely distributed on many immune cells, including DCs and T-Cells. In the human [18] and murine thymus [19], TSLP is selectively produced in the epithelial cells of Hassall's corpuscles or the thymic medulla, and TSLP-activated thymic dendritic cells (TSLP-DCs) induced differentiation of CD4$^+$Foxp3$^-$ thymocytes into CD4$^+$Foxp3$^+$ Tregs. Recently, Haas et al. showed that the disorder of TSLP-TSLPR axis in thymic myeloid dendritic cells (MDCs) might contribute to the contracted thymic Treg output and altered homeostasis of regulatory T-Cells in multiple sclerosis [20]. In a previous study [21], we showed that TSLP expression was inhibited in the cardiovascular tissue of ApoE$^{-/-}$ mice, and treatment with TSLP could rectify the Treg imbalance and prevent the development of atherosclerosis in these mice. Thus, understanding the mechanisms underlying decreased thymic Treg cell output in ACS patients or atherosclerotic mice is of great significance, particularly with respect to therapies involving the manipulation of Tregs. However, direct investigation of these mechanisms is challenging in human subjects. In the present study, we attempted to characterize LAP$^+$ and Foxp3$^+$ Treg defects and to determine the mechanisms that might account for these defects in ApoE$^{-/-}$ mice.

2. Methods

The detailed Methods section is available in the supplementary material.

2.1. Statistical analysis

The results are expressed as the mean ± SD unless otherwise indicated. Comparisons between the 2 groups were performed using Student's t test. One-way ANOVA was used for multiple comparisons between ≥3 groups, followed by the Holm–Sidak test. GraphPad Prism 6.0 was used for the statistical analysis. The significance level was set at $p < 0.05$.

3. Results

3.1. Body weights and plasma lipid levels

The body weights and plasma lipid levels of ApoE$^{-/-}$ and wild-type C57BL/6J mice are shown in Supplementary Table 2. The total cholesterol and triglyceride level in C57BL/6J mice was low. The total cholesterol level and ox-LDL concentration in ApoE$^{-/-}$ mice was significantly higher than that in age-matched C57BL/6J mice, and these values increased with age.

3.2. Foxp3$^+$ and LAP$^+$ Tregs were decreased in the spleen and thymus of ApoE$^{-/-}$ mice compared with C57BL/6 mice

Our recent (data not shown) and previous study [22,23] showed that most LAP + Tregs from mice and human did not express Foxp3 in a resting state. The FACS analysis of spleen cells from 5 mice in each group at 10 weeks revealed that the number of Tregs (Foxp3$^+$ cells in the total CD4 population and LAP$^+$ cells in the CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ populations) was significantly reduced in ApoE$^{-/-}$ mice compared with C57BL/6 mice (Fig. 1A and B). Moreover, thymic Tregs were significantly reduced in ApoE$^{-/-}$ mice compared with C57BL/6 mice (Fig. 1A and B). To determine whether age affects the Treg pool in ApoE$^{-/-}$ mice, we compared the number of Tregs in these mice at 10 weeks of age with atherosclerotic ApoE$^{-/-}$ mice at 20 week of age. We observed that the number of spleen cell derived Tregs was significantly reduced in older compared with younger ApoE$^{-/-}$ mice (Fig. 1A and B). The inhibition of CD4$^+$CD25$^-$LAP$^-$ Tresp cell proliferation through CD4$^+$LAP$^+$ Tregs was determined after co-culturing of Tregs and Tresps at different ratios (1:1, 1:2 and 1:4). These data demonstrated that CD4$^+$LAP$^+$ Tregs from ApoE$^{-/-}$ mice exhibited a reduced capacity to suppress the proliferation of Tresps at all ratios compared with C57BL/6 mice (Fig. 1C).

TREC is a marker for nascent thymic T-cells [24]. We assessed intracellular levels of TRECs in CD4$^+$CD25$^+$ Treg cells isolated from the spleens of five 10-wk-old ApoE$^{-/-}$ mice and 5 age-matched C57BL/6 mice using quantitative real-time PCR. The TREC content in Treg cells was significantly lower in ApoE$^{-/-}$ mice than in C57BL/6 mice (Fig. 1D).

3.3. The tolerogenic function of DCs was disrupted in ApoE$^{-/-}$ mice

Previous studies have demonstrated that the number and phenotype of DCs is changed in atherosclerotic humans and mice [25,26], but little is known about the function of these cells. First, we detected LAP (surface TGF-β) expression on DCs and observed a significant reduction in ApoE$^{-/-}$ mice compared with C57BL/6 mice, and this effect was associated with age (Fig. 2A). To determine whether the tolerogenic ability of DCs was disrupted in ApoE$^{-/-}$ mice, we co-cultured thymic or splenic DCs with CD4$^+$CD25$^-$T-cells and examined the differentiation of Foxp3$^+$ and LAP$^+$ Tregs. The results showed that the DCs from ApoE$^{-/-}$ mice promoted significantly less Foxp3$^+$ (Fig. 2B) and LAP$^+$ Treg cell differentiation (Fig. 2C) than the DCs from C57BL/6 mice, suggesting that the DCs from ApoE$^{-/-}$ mice lost tolerogenic function.

3.4. TSLP was reduced in the immune tissues of ApoE$^{-/-}$ mice

In a previous study [21], we demonstrated that TSLP expression was abundant in the cardiovascular tissues of normal C57BL/6 mice but inhibited in ApoE$^{-/-}$ mice. However, the expression of TSLP in the immune tissues of ApoE$^{-/-}$ mice has not been previously investigated.

We examined the expression of TSLP in murine using immunohistochemistry, PCR, and western blotting. In the thymus, the expression of keratin 5$^+$ epithelial cells in different groups was not distinguishing (Fig. 3A). TSLP was expressed from epithelial cells in the medulla but not in the cortex or from hematopoietic cells (Fig. 3B). Less TSLP expression was observed in 10-wk-old ApoE$^{-/-}$ mice compared with 10-wk-old C57BL/6 control mice (Fig. 3B). Surprisingly, TSLP was only slightly expressed in 20-wk-old ApoE$^{-/-}$ mice, and this result was confirmed through RT-PCR (Fig. 3C). Similar to thymus, the expression of TSLP in the spleen was also localized to the medulla. Quantitative RT-PCR demonstrated significantly lower TSLP gene expression in spleens of 10 and 20-wk-old ApoE$^{-/-}$ mice (Fig. 3C). Moreover, relatively abundant TSLP protein expression was observed in the livers of 10-wk-old C57BL/6 mice. Thus, the ApoE knockout inhibited TSLP expression in mice.