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L-selectin deficiency decreases aortic B1a and B_{reg} subsets and promotes atherosclerosis

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Summary

There is a significant recruitment of leucocytes into aortas during atherogenesis. L-selectin regulates leucocyte migration into secondary lymphoid and peripheral tissues and was proposed to play a role in leucocyte homing into aortas. Here, we determine the role of L-selectin in atherosclerosis. L-selectin-deficient *Apoe*^{-/-} (*Sell*^{-/-}*Apoe*^{-/-}) mice had a 74% increase in plaque burden compared to *Apoe*^{-/-} mice fed a chow diet for 50 weeks. Elevated atherosclerosis was accompanied by increased aortic leucocyte content, but a 50% reduction in aortic B cells despite elevated B cell counts in the blood. Follicular B cells represented 65%, whereas B1a and regulatory B cells (B_{reg}) comprised 5% of aortic B cells. B1a and B_{reg} cell subsets were reduced in *Sell*^{-/-}*Apoe*^{-/-} aortas with accompanied 2-fold decrease in aortic T15 antibody and 1.2-fold decrease of IL-10 levels. L-selectin was required for B1 cell homing to the atherosclerotic aorta, as demonstrated by a 1.5-fold decrease in the migration of *Sell*^{-/-}*Apoe*^{-/-} versus *Apoe*^{-/-} cells. Notably, we found a 1.6-fold increase in CD68^{hi} macrophages in *Sell*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} aortas, despite comparable blood monocyte numbers and L-selectin-dependent aortic homing. L-selectin had no effect on neutrophil migration into aorta, but led to elevated blood neutrophil numbers, suggesting a potential involvement of neutrophils in atherogenesis of *Sell*^{-/-}*Apoe*^{-/-} mice. Thus, L-selectin deficiency increases peripheral blood neutrophil and lymphocyte numbers, decreases aortic B1a and B_{reg} populations, T15 antibody and IL-10 levels, and increases aortic macrophage content of *Sell*^{-/-}*Apoe*^{-/-} mice. Altogether, these data provide evidence for an overall athero-protective role of L-selectin.

Keywords

Atherosclerosis; L-selectin; B cell subsets

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of modified lipoproteins, the activation of aortic wall vascular components, and an elevated immune response that supports a systemic and aortic pro-atherogenic environment (1, 2). To

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date, multiple leucocyte populations including T and B cells, natural killer and natural killer T cells, neutrophils, mast cells, and macrophages have been found within the aortic wall and adjacent adventitia (3). There is an increasing amount of evidence suggesting that the multiple subsets of each leucocyte population have unique roles and the ability to fine tune the immune response based on the cytokine environment and regulatory signals from surrounding cells. As our knowledge increases, it appears that specific subsets of dendritic cells, macrophages, T and B cells play unique roles in atherosclerosis (4–7). However, little is known about the mechanisms by which particular leucocyte subsets are recruited to the aorta.

The adhesion molecule L-selectin (CD62L) is a leucocyte homing receptor that has a prominent role in controlling the recirculation and distribution of leucocyte subsets within non-inflamed and inflamed tissues (8, 9). L-selectin supports the dynamic rolling and tethering of B cells and naïve and central memory T cells along the high endothelial venules of the peripheral lymph nodes (PLNs) (10–12). L-selectin-deficient mice (*Sell*^{-/-}) display significantly attenuated T and B cell recruitment into PLNs and reduced PLN cellularity (10). L-selectin has also been shown to participate in the regulation of splenic T and B cell homing into healthy and atherosclerotic-prone aortas in short-term homing experiments (13), suggesting a potential pro-atherogenic role for this adhesion molecule. Unexpectedly, a study demonstrated that the absence of L-selectin accelerates atherosclerosis in Apolipoprotein E-deficient (*Apoe*^{-/-}) mice fed chow diet for 6 months or western diet for 6 weeks (14). Despite a significant difference in the plaque formation, no differences were found in the cellular composition of atherosclerotic plaques between L-selectin-deficient *Apoe*^{-/-} (*Sell*^{-/-}*Apoe*^{-/-}) and *Apoe*^{-/-} mice using immunohistochemistry. While this study has provided important information about a potential implication of L-selectin in atherogenesis, there is still a lack of knowledge on the molecular and cellular mechanisms, by which L-selectin affects atherogenesis.

While the role of L-selectin in the regulation of T cell subsets has been studied extensively, little is known about the implications of L-selectin in the homing of B cell subsets. Naïve B cells express CD62L, yet B cell subsets may have different dependencies on the expression of L-selectin (15, 16, 17). B cells play a vital role in atherosclerosis (7, 18, 19) and a recent report suggested an important role of CCR6 in B cell recruitment into the aorta (20). Initial studies, in which the total splenic B cell population was modulated, demonstrated an atheroprotective role of splenic B cells (20–22). Since then, studies are focusing on B cell subset-specific functions in atherogenesis. Follicular (FO) B cells are classified as pro-atherogenic via the secretion of pro-inflammatory cytokines (23–25); however, these cells likely contribute to atherosclerosis in a multitude of ways. Alternatively, B1a B cells secrete the natural antibody T15, which recognizes and binds to oxidative-specific epitopes (26), and attenuates oxidized low-density lipoprotein uptake by macrophages (27, 28). To date, the roles of the marginal zone (MZ), regulatory B (*B*_{reg}), and B1b subsets in atherosclerosis remain elusive. Regardless of their functions, there have been no data depicting the distribution of the B cell subsets within atherosclerotic aortas.

In this study, we examine the impact of L-selectin deficiency on atherosclerosis development. We report that L-selectin deficiency enhances atherogenesis in *Apoe*^{-/-} mice

via a regulation of B1 cell homing into aortas, decreased aortic B1a and B_{reg} cell content, reduced levels of anti-atherogenic T15 antibodies and IL-10, and elevated levels of aortic macrophages of *Sell*^{-/-}*Apoe*^{-/-} mice.

Materials and Methods

Mice

L-selectin-deficient (*Sell*^{-/-}) mice (provided by K. Ley, La Jolla Institute for Allergy and Immunology) and *Apoe*^{-/-} mice were bred to generate *Sell*^{-/-}*Apoe*^{-/-} mice. Male and female *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice (both strains on C57BL/6 background) were bred and kept in specific pathogen-free conditions and all experiments were approved by Eastern Virginia Medical School's Animal Care and Use Committees. Mice were fed chow diet and aged to approximately 50 weeks old for most experiments. Additional materials and methods can be found in Supplemental Materials.

Results

L-selectin Deficiency Increases Atherosclerosis in *Sell*^{-/-}*Apoe*^{-/-} Mice

To investigate the role of L-selectin in atherosclerosis, L-selectin-deficient (*Sell*^{-/-}) mice were bred with *Apoe*^{-/-} mice to generate *Sell*^{-/-}*Apoe*^{-/-} mice. Total plasma cholesterol, triglycerides, HDL, and LDL levels were not significantly different between *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice (data not shown). We examined plaque burden within the aortas of aged *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice fed a chow diet using *en face* Oil Red O staining (Figure 1). *Sell*^{-/-}*Apoe*^{-/-} mice had a 74% enhancement of plaque burden throughout the total aorta compared to age- and diet-matched *Apoe*^{-/-} mice (31.5%±3.0 and 18.1%±1.1, respectively; Figure 1). Enhanced plaque burden was also detected in both female and male *Sell*^{-/-}*Apoe*^{-/-} compared to age and sex-matched *Apoe*^{-/-} mice (male: 27.2±3.0% (n=5) and 16.4±0.9% (n=10), respectively, p<0.004; female: 41.0±7.9% (n=11) and 19.3±1.7% (n=7), respectively, p<0.01). Thus, the absence of L-selectin contributes to atherogenesis in aged *Sell*^{-/-}*Apoe*^{-/-} mice.

Reduced B Cell Population Despite Overall Increased Leucocytes Cellularity in *Sell*^{-/-}*Apoe*^{-/-} Aortas

We previously reported that T and B lymphocytes require L-selectin for successful migration to aortas in short-term homing experiments (13). To further test the role of L-selectin in the distribution of leucocytes, we examined the cellularity and lymphocyte composition within the aortas of *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice. Notably, we found a 1.3-fold increase in total leucocytes within the aortas of *Sell*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} (1.2±0.1×10⁶ and 0.9±0.1×10⁶, respectively; p<0.05, Figure 2A). Although it is recognized that L-selectin regulates T cell migration, the number of TCR⁺ T cells present in the aortas of *Sell*^{-/-}*Apoe*^{-/-} versus the *Apoe*^{-/-} controls was not significantly different (1.7±0.5×10⁵ and 2.9±0.6×10⁵, respectively, Supplemental Figure 1A). Surprisingly, despite the rise in leucocyte number, there was a significant reduction in the aortic CD19⁺ B cell population of *Sell*^{-/-}*Apoe*^{-/-} mice compared with *Apoe*^{-/-} controls (0.7±0.2×10⁵ and 1.4±0.2×10⁵, respectively; Figure 2A and 2B), despite no change in their proliferation rates

($3.2 \pm 0.7\%$, $2.9 \pm 0.8\%$ for *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice, $n=4-5$, respectively). The increase in aortic leucocyte number in *Sell*^{-/-}*Apoe*^{-/-} mice corresponded to the increase in plaque burden, but the dramatic decrease in B cells was intriguing.

L-selectin plays a prominent role in the regulation of leucocyte recruitment to PLNs and sites of acute and chronic inflammation in *Sell*^{-/-} mice (9). To further establish the effects of L-selectin on modulating leucocyte homing during the conditions of low-grade chronic inflammation in atherogenesis, we examined PLNs, spleens, and blood of *Sell*^{-/-}*Apoe*^{-/-} mice. As expected, there was a decrease in the cellularity of PLN and the content of B cells in *Sell*^{-/-}*Apoe*^{-/-} mice compared to *Apoe*^{-/-} mice (Figure 2C). There was an increased number of splenic leucocytes in *Sell*^{-/-}*Apoe*^{-/-} mice compared to *Apoe*^{-/-} mice ($79.3 \pm 4.2 \times 10^6$ and $55.0 \pm 3.1 \times 10^6$, respectively, $p < 0.001$), but a similar number of CD19⁺ B cells ($23.7 \pm 2.0 \times 10^6$ and $18.6 \pm 1.7 \times 10^6$, respectively, $p < 0.06$). In agreement with Rosenberg and colleagues (14), we also found elevated levels of circulating leucocytes in the blood of *Sell*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} mice ($10.3 \pm 2.3 \times 10^6$ and $3.8 \pm 0.9 \times 10^6$, respectively, $p < 0.001$, Figure 2D). Of these leucocytes, there were increased numbers of CD19⁺ *Sell*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} B cells (Figure 2D). We also detected an increase of peripheral blood TCR $\alpha\beta$ ⁺ *Sell*^{-/-}*Apoe*^{-/-} vs *Apoe*^{-/-} T cells ($2.9 \pm 1.6 \times 10^6$ and $0.3 \pm 0.1 \times 10^6$, respectively, Supplemental Figure 1B), and circulating CD11b⁺Gr1⁺Ly6G⁺ neutrophils ($0.8 \pm 0.1 \times 10^6$, $0.1 \pm 0.0 \times 10^6$ for *Sell*^{-/-}*Apoe*^{-/-} vs *Apoe*^{-/-} mice, respectively, Figure 5B). There was no difference in the number of CD11b⁺Gr1^{high/inter/low}Ly6G⁻ monocytes in the peripheral blood of *Sell*^{-/-}*Apoe*^{-/-} vs *Apoe*^{-/-} mice ($0.1 \pm 0.05 \times 10^6$, $0.04 \pm 0.01 \times 10^6$ cells, respectively, Figure 5B). These data extend the critical role of L-selectin in leucocyte migration into aortas and secondary lymphoid tissues under atherosclerotic-prone conditions. Importantly, despite the elevation in circulating B cells in the blood, *Sell*^{-/-}*Apoe*^{-/-} mice had lower levels of aortic B cells, suggesting that L-selectin might serve as a potential receptor for B cell homing to the aorta.

B1a and B_{reg} cell subsets are Decreased in L-selectin-deficient *Apoe*^{-/-} Aortas

Our data demonstrate that the absence of L-selectin in *Apoe*^{-/-} mice resulted in the reduction of aortic B cells. It is well established that each B cell subset has unique functions (29–31), yet they have not been entirely elucidated in atherosclerosis. Furthermore, to date, there are no studies reporting the distribution of the B cell subsets within the aorta. This led us to investigate whether L-selectin serves as a regulator for the B cell subset-specific homing. To address this question, we performed flow cytometric analysis of aortas with the specific focus on the B cell subsets. We first examined the composition of B cell subpopulations within aortas of *Apoe*^{-/-} mice. We used a set of CD19, IgD, CD43, and CD5 markers to characterize B cell subsets in the aortas (32). Within the CD45⁺CD19⁺ B cell population of the lymphocyte gate, FO cells were characterized as IgD^{hi}CD5⁻ (Figure 3C) and confirmed as CD43⁻ (data not shown). Aortic B1a and B_{reg} cells expressing CD5 and low levels of IgD were separated based on the expression of CD43 as IgD^{lo}CD5⁺CD43⁺ B1a and IgD^{lo}CD5⁺CD43⁻ B_{reg} (Figure 3C). The IgD^{lo}CD5⁻ population was a cohort of MZ and B1b cells distinguishable based on their expression of CD43 (Figure 3C). To confirm the specificity of B cell subset staining, we used additional markers including: CD1d, CD21, B220, and IgM (Supplemental Figure 2 and Table 1) in the spleen and aorta.

Next we confirmed the presence of B1 cells in the aorta and PLN (as control tissues) using immunofluorescence (Figure 3D), where we successfully depicted the B cells as IgM⁺CD20⁺ cells and the IgM⁺CD43⁺ as total B1 population (Figure 3D, Supplemental Figure 3), predominantly in the adventitia.

Next, we investigated the B cell subset distribution in aortas of *Apoe*^{-/-} mice. We found that within the aortic B cell population, CD19⁺IgD^{hi}CD5⁻CD43⁻ FO B cells comprise the largest subset, representing 65% of the total aortic B cells in *Apoe*^{-/-} aortas (Figure 3A, *Apoe*^{-/-} pie chart, Table 2). Interestingly, 23% of the B cells isolated from *Apoe*^{-/-} aortas were the CD19⁺IgD^{lo}CD5⁻CD43⁺ B1b subset suggesting a potential implication of these cells in the regulation of inflammation within the aortic wall (Figure 3A, *Apoe*^{-/-} pie chart, Table 2). We also found two distinct populations of CD19⁺IgD^{lo}CD5⁻CD43⁻ MZ (7.0%) and CD19⁺IgD^{lo}CD5⁺CD43⁺ B1a cells (4.9%), accounting for only a small proportion of the aortic B cells of *Apoe*^{-/-} mice (Figure 3A, *Apoe*^{-/-} pie chart, Table 2). CD19⁺IgD^{lo}CD5⁺CD43⁻ B_{reg} cells represent the smallest population and were nearly undetectable (0.2%) (Figure 3A, *Apoe*^{-/-} pie chart, Table 2).

Subsequently, we addressed to what extent B cell subsets require L-selectin for their presence in the aortas. The reduction in the total aortic B cell population of *Sell*^{-/-}*Apoe*^{-/-} was attributed to a reduction in all B cell subsets (Figure 3A and B). However, B1a and B_{reg} subsets were most dramatically impacted by the lack of L-selectin, as we detected a significant decrease in the percentage of B1a in *Sell*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} mice (Figure 3A, Table 2). Importantly, we detected no B_{reg} cells in the aortas of *Sell*^{-/-}*Apoe*^{-/-} mice. These reductions were also apparent based on the cell numbers (Figure 3B). Here, the aortic B1a population of *Sell*^{-/-}*Apoe*^{-/-} mice compared to *Apoe*^{-/-} mice was decreased by 7.3-fold and B_{reg} cells were practically undetectable in *Sell*^{-/-}*Apoe*^{-/-} versus *Apoe*^{-/-} mice (Figure 3B, Table 2). Interestingly, we found no difference in the number of B1a and B_{reg} cells in the blood of *Sell*^{-/-}*Apoe*^{-/-} versus *Apoe*^{-/-} mice (B1a: 0.8±0.3×10⁴, 0.9±0.2×10⁴ cells/mL; B_{reg}: 0.2±0.1×10⁴, 0.1±0.01×10⁴ cells/mL, respectively). These data suggest that L-selectin, at least partially, regulates the aortic content of each B cell subset; furthermore, it is essential for the presence of aortic B1a and B_{reg} populations.

Reduced IL-10 and T15 antibody levels and increased macrophage content within the aortas of L-selectin-deficient *Apoe*^{-/-} mice

To further delineate the impact of L-selectin on the distribution of B cell subsets, we analyzed spleens of *Sell*^{-/-}*Apoe*^{-/-} mice. We found a decreased number of splenic B1a and B1b cells in *Sell*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} mice (Figure 4A), suggesting that L-selectin is an important factor that regulates B1a and B1b content. In contrast to the reduced number of B1 cells, there was a 1.5-fold increase in FO B cells of *Sell*^{-/-}*Apoe*^{-/-} in comparison with *Apoe*^{-/-} mice (Figure 4A). FO B cells might accelerate atherogenesis via an increased production of aortic TNF-α (24) or elevated release of IgG type anti-oxLDL antibodies, and pathogenic T cell activation (33). Interestingly, we found no significant difference in the circulating plasma TNF-α and IFN-γ levels (TNF-α: 134.3±88.4, 79.6±31.4 pg/mL; IFN-γ: 13±5, 4.3±2.1 pg/mL, for *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice, respectively) as well as alterations in T cell helper phenotype between *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice.

suggesting that splenic FO B cells might have a limited impact on the accelerated atherosclerosis in *Sell^{-/-}Apoe^{-/-}* mice. Importantly, we detected a 15-fold reduction in the levels of circulating anti-inflammatory cytokine IL-10 in the serum of *Sell^{-/-}Apoe^{-/-}* compared to *Apoe^{-/-}* mice (1.2 ± 0.7 pg/ml and 17.8 ± 4.8 pg/ml, respectively, $p < 0.01$, Figure 4D), suggesting that peripheral sites are also likely contributing to the atherogenic environment. When measuring IL-10 from the aortic supernatant, we detected a significant decrease in IL-10 from *Sell^{-/-}Apoe^{-/-}* versus *Sell^{-/-}Apoe^{-/-}* aortic tissues (386.5 ± 8.27 pg/ml and 460.6 ± 17.8 pg/ml, respectively, $p < 0.01$) (Figure 4D). Interestingly, the number of T_{reg} in the aorta and the blood of *Sell^{-/-}Apoe^{-/-}* and *Apoe^{-/-}* mice was similar (Supplemental Figure 4), suggesting that the B1a and B_{reg} populations are likely major contributors to the altered IL-10 levels.

The changes in the distribution of B cell subsets in the spleen and aorta suggest that the subsets have different degrees of dependency on L-selectin for their presence in tissues. We compared levels of L-selectin expressed by each B cell subset and found that the highest percentage of splenic B1 cells express L-selectin compared to other B cell subsets (Figure 4B). Interestingly, while FO B cells in aortas express 5 times higher L-selectin levels compared to FO B cells in spleen, B1a cells in aortas expressed about 80 times elevated levels of L-selectin compared to splenic B1a cells (data not shown). Interestingly, not all tissues were impacted by the absence of L-selectin as we found that L-selectin deficiency did not alter the number of B cells in each subset within the peritoneal cavity of *Sell^{-/-}Apoe^{-/-}* and *Apoe^{-/-}* mice (data not shown) despite this being the predominant location of B1a and B1b cell populations. Altogether, these results suggest that B cell subsets express different levels of L-selectin that might affect their capacity to migrate in an L-selectin-dependent manner to different tissues.

To understand the mechanisms by which the absence of L-selectin and the subsequent alterations in the B cell subset composition lead to increased atherosclerosis, we explored B cell subset-specific functions. T15 is a natural athero-protective antibody secreted mainly by B1a cells (27, 34). With the reduction in the B1a population in the spleen and aorta, we hypothesized that there would be a concurrent decrease in T15 antibody production which may contribute to the enhanced atherosclerosis in *Sell^{-/-}Apoe^{-/-}* mice. However, we detected no significant difference in the levels of T15 in the serum of *Sell^{-/-}Apoe^{-/-}* and *Apoe^{-/-}* mice (Figure 4C). Importantly, when analyzing the production of T15 from cells within the aorta, we found a 2-fold decrease in the absence of L-selectin (Figure 4C). Thus, reduced T15 levels in *Sell^{-/-}Apoe^{-/-}* aortas correlate with diminished number of aortic B1a cells and exacerbated atherosclerosis in *Sell^{-/-}Apoe^{-/-}* mice.

Taking into account an overall increase in the number of leucocytes present in the aorta of *Sell^{-/-}Apoe^{-/-}* mice despite the reduced number of B cells, we sought to determine other leucocyte populations within the aorta. Specifically, we examined the T cell subsets and macrophage populations in the aortas of *Sell^{-/-}Apoe^{-/-}* and *Apoe^{-/-}* mice. We detected no significant difference in the total number of $TCR\alpha\beta^+$ T cells within the aortas of *Sell^{-/-}Apoe^{-/-}* versus *Apoe^{-/-}* mice (Supplemental Figure 1A). Additionally, there was no change in the number of $CD4^+Foxp3^+$ T_{reg} , $CD4^+IFN\gamma$ Th1, or $CD8^+$ T cells (Supplemental Figure 4A). Aortic macrophages are the major population of the immune cells within the

advanced atherosclerotic aortas that play a critical role in the induction and persistence of a pro-inflammatory response within the aortic wall (1, 6). Therefore, to further determine pro-atherogenic changes that might support accelerated atherosclerosis in *Sell*^{-/-}*Apoe*^{-/-} mice; we investigated the frequency of macrophages within *Sell*^{-/-}*Apoe*^{-/-} aortas. In contrast to diminished anti-inflammatory levels of IL-10 and T15 antibodies, we found a 2-fold increase in the CD68^{hi} macrophage population within the aortas of *Sell*^{-/-}*Apoe*^{-/-} versus *Apoe*^{-/-} mice (Figure 5A). Altogether, these data further demonstrate an enhanced inflammatory environment within aortas of *Sell*^{-/-}*Apoe*^{-/-} mice due to reduced IL-10 and T15 levels as well as elevated macrophage content.

Regulation of leukocyte recruitment into atherosclerotic aortas by L-selectin

With the apparent impact of L-selectin deficiency on B cell subsets and the macrophage population, we sought to determine the influence of L-selectin on their homing to the aorta. First, the role of L-selectin in monocyte recruitment into aortas was tested. We performed adoptive transfer experiments of *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} monocytes and found that L-selectin deficiency attenuated monocyte homing into aortas (Figure 5C). Interestingly, despite L-selectin-dependent homing of monocytes and no significant difference in the number of peripheral blood monocytes, aortic macrophage content was increased in *Sell*^{-/-}*Apoe*^{-/-} aortas (Figure 5A), suggesting an alternative mechanism for monocyte recruitment into aortas (35, 36) or increased proliferation of macrophages within the aortic wall (37).

Neutrophils are found within atherosclerotic aortas and involved in the initiation and progression of atherosclerosis (38). It has been also recognized that neutrophil recruitment to a site of inflammation in multiple models of inflammation is L-selectin-dependent (39, 40). To investigate a potential role of L-selectin in the regulation of neutrophil homing into the aorta, we performed adoptive transfer experiments of L-selectin-deficient and L-selectin-sufficient peripheral blood myeloid cells into atherosclerosis-prone recipients. We found that neutrophil migration into the aorta was L-selectin-independent (Figure 5C).

Next, to directly establish a role of L-selectin in the recruitment of different B cell populations into the aorta, we performed adoptive transfer experiments of *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} B cell subsets into atherosclerotic *Apoe*^{-/-} mice. In agreement with the previous report (13), we found a 20% decrease of CD19⁺ *Sell*^{-/-}*Apoe*^{-/-} B cell recruitment into the aorta compared to *Apoe*^{-/-} CD19⁺ B cells (Figure 5D). Although the migration of *Sell*^{-/-}*Apoe*^{-/-} FO B cells was reduced compared to *Apoe*^{-/-} FO B cells, the difference did not reach statistical significance. Importantly, the migration of *Sell*^{-/-}*Apoe*^{-/-} B1 cells, representing B1a and B1b cells, into the aorta was reduced up to 60% compared to *Apoe*^{-/-} B1 cells (Figure 5D). Although we performed the analysis for total B1 cells, these data do demonstrate an L-selectin-dependent mechanism of homing as reflected by the reduction of B1a and B1b cells (Figure 3B). Overall, these results suggest an L-selectin-dependent recruitment of monocytes and B cells, and particularly B1 cells, but not neutrophils, into atherosclerotic aortas in adoptive transfer experiments.

Discussion

Atherosclerosis is the leading cause of cardiovascular disease. The cells of the immune system are actively involved in the induction and persistence of atherosclerosis. Over the last decade, numerous reports identified several adhesion molecules and chemokine receptors that regulate the adhesion and diapedesis of monocytes and T cells into aortas. Recently, the chemokine receptor CCR6 was reported as a homing molecule for B cells to the aorta (20). Yet, B cell subset-specific mechanisms of recruitment into aortas have not been described. In this study, we demonstrate that L-selectin deficiency results in accelerated atherosclerosis in aged L-selectin-deficient *Apoe*^{-/-} mice. This enhancement in atherosclerosis is associated with an altered distribution of B cell subsets within the spleen, PLN, and aorta, elevated levels of peripheral blood lymphocytes and neutrophils, as well as raised inflammation accompanied by an increased number of macrophages in the aortas.

Evidence has suggested a role for L-selectin in mouse models of atherosclerosis. It was shown that L-selectin provides secondary leucocyte capture under flow conditions in arterial vessels (41). In line with these results, it was demonstrated that L-selectin modulates short-term lymphocyte homing into aortas (13). Therefore, based on published data, we hypothesized that the lack of L-selectin would attenuate atherogenesis via the reduction of lymphocyte homing into aortas. To our surprise, we found a strikingly opposite phenotype of *Sell*^{-/-}*Apoe*^{-/-} mice. Aged *Sell*^{-/-}*Apoe*^{-/-} mice displayed up to 74% increased plaque burden than *Apoe*^{-/-} mice. The elevation in atherosclerosis was accompanied by an increased number of leucocytes within the aortas. There are several possible explanations for these results including the prevalence of L-selectin-independent mechanisms of migration, an altered immune response in *Sell*^{-/-}*Apoe*^{-/-} mice, or the inability of atheroprotective cells to accumulate in the aortas.

L-selectin is expressed by most leucocytes under non-inflamed conditions (8, 9). It is possible that the observed phenotype in *Sell*^{-/-}*Apoe*^{-/-} mice is due to various dependencies on L-selectin by different leucocyte subsets, and thus the net outcome of L-selectin deficiency has a pro-atherogenic effect. To test this hypothesis, we focused on the L-selectin-dependent lymphocyte distribution in the aortas of *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice. L-selectin is one of the major adhesion molecules expressed by T cells (42) and the migration of T cells in short-term adoptive transfer experiments is L-selectin-dependent (13). *Sell*^{-/-}*Apoe*^{-/-} mice displayed elevated number of peripheral blood T cells, and reduced numbers of T cells in the aortas of *Sell*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} mice suggesting a potential implication of L-selectin in T cell recruitment into atherosclerotic aortas of *Sell*^{-/-}*Apoe*^{-/-} mice. Although total leucocyte cellularity of *Sell*^{-/-}*Apoe*^{-/-} aortas was increased compared to *Apoe*^{-/-} aortas, the percentage and number of CD19⁺ B cells was diminished in *Sell*^{-/-}*Apoe*^{-/-} aortas. Studies have shown that B cells represent a relatively small proportion of the aortic leucocytes, but their local and systemic effects are crucial in regulating atherogenesis (19). However, little is known about the B cell subset content in atherosclerotic aortas. To date, possible mechanisms and key adhesion molecules that are responsible for regulating the migration of B cell subsets into aortas are not well defined. Therefore, we sought next to examine the B cell subset distribution in *Apoe*^{-/-} aortas.

Recent studies have classified subsets of B cells based on their anatomical locations and specific phenotypic markers (18, 31, 32). FO B cells are characterized by their prevalence in the spleen, whereas B_{reg} cells are found distributed throughout the tissues. B1a and B1b cells are prevalent in the peritoneal cavity; but can also migrate to the spleen. While FO and B1a have been studied in atherogenesis (19), the roles of MZ, B1b, and B_{reg} have not been reported. We found that FO B cells represented the largest population of B cells in aortas, whereas B_{reg} and B1a cells are the smallest aortic B cell populations. MZ B cells are sentinels at the interface between the circulation and lymphoid tissue and connect the conventional boundaries of innate and adaptive immunity (43), but can also be identified in non-lymphoid tissues (44). Surprisingly, we found aortic B cells that expressed markers of MZ B cells, possibly due to the development of tertiary lymphoid structures in the aorta, suggesting perhaps a unique function for this subset in atherosclerosis. These innate-like B cells could be essential for the recognition of self-antigens in atherosclerosis. Future studies of MZ B cells will be important for determining their functions in atherogenesis.

In the current study, we detected reduced B cell content in aortas of aged *Sell*^{-/-}*Apoe*^{-/-} mice. To further understand the observed phenotype and have insight into a potential mechanism of accelerated atherosclerosis in *Sell*^{-/-}*Apoe*^{-/-} mice, we next tested whether L-selectin preferentially regulates the content of specific B cell subsets. L-selectin deficiency resulted in almost complete absence of B_{reg} and a reduced number of B1a cells in the aortas of aged *Sell*^{-/-}*Apoe*^{-/-} mice indicating a critical role of this adhesion molecule in the regulation of B1a and B_{reg} cell content in aortas. Importantly, we found no difference in B cell proliferation within the aortas and spleens between *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice implying that the difference in B1a and B_{reg} cell numbers is not due to altered B cell proliferation. It remains to be determined at which stages of atherosclerosis development L-selectin deficiency affects B cell subset homing into the aorta and whether this L-selectin-dependent regulation of B cell migration occurs preferentially in the conditions of advanced atherogenesis.

Previous studies have demonstrated that L-selectin deficiency results in an increased number of CD19⁺ cells in circulation and an elevated number of B cells in the spleens of *Sell*^{-/-}*Apoe*^{-/-} mice (14). We confirmed these results and further demonstrated that while L-selectin deficiency leads to an increased number of total CD19⁺ B cells in the blood, B1a and B_{reg} cell frequency in circulation remained unchanged, suggesting that decreased numbers of B1a and B_{reg} cells in *Sell*^{-/-}*Apoe*^{-/-} aortas are unlikely due to altered B1a and B_{reg} cell numbers in the blood. Preferential depletion of splenic FO B cells via anti-CD20 antibodies results in reduced atherosclerosis accompanied by preserved production of natural anti-oxLDL IgM autoantibodies over IgG type anti-oxLDL antibodies (33). Although we found no differences in total circulating IgM in the blood of *Sell*^{-/-}*Apoe*^{-/-} mice, further studies will be necessary to investigate the precise impact of L-selectin-deficient splenic FO B cells on atherosclerosis in *Sell*^{-/-}*Apoe*^{-/-} mice.

Several studies have been performed to test a role of L-selectin in leucocyte rolling in vivo (13, 14, 41, 45). L-selectin-deficient mice have attenuated leucocyte rolling flux in the femoral artery and the complete absence of secondary capture at this site compared to C57BL/6 mice (41). In contrast, a recent study reported no decrease in leucocyte rolling

along the endothelium of the aortic lumen between *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice (14). L-selectin is a powerful regulator of neutrophil numbers in the blood of C57BL/6 mice (9, 10), and we detected elevated neutrophil counts in the peripheral blood of *Sell*^{-/-}*Apoe*^{-/-} mice. Although L-selectin deficiency had no effect on neutrophil migration into the aorta in adoptive transfer experiments, a significant increase in blood neutrophil numbers and unaltered rate of migration might result in elevated aortic neutrophil and thus accelerated atherosclerosis. Further studies will be required to examine the precise role of L-selectin expressed by neutrophils in atherogenesis. Evidence implies that the adventitia is another important site of lymphocyte entry to the aorta and likely atherosclerotic plaques (3). A recent report also suggested an important role of microvessels and L-selectin in leucocyte recruitment during atherogenesis (45). To further examine the implication of L-selectin in B cell subset migration, we performed short-term adoptive transfer experiments of L-selectin-deficient and L-selectin-sufficient B cells. The most profound impact of L-selectin deficiency on B cell recruitment was detected for B1 cells that displayed 3-fold reduction in migration into the aorta. Although the mechanism of B cell entry was not specifically examined in this study, it is possible that B1 cells migrated predominantly via vasa vasorum and newly formed microvessels as it was suggested for B cells in several studies (13, 45, 46).

We also explored the possibility that an altered immune response in *Sell*^{-/-}*Apoe*^{-/-} mice might result in elevated atherosclerosis. Migration of T and B cells into PLNs is significantly reduced in *Sell*^{-/-} mice (10), thus antigen-presentation and activation of lymphocytes might be attenuated in PLNs. In the absence of L-selectin, immunoglobulin production is dependent on the antigen-type and the influence of helper T cells (47). *Sell*^{-/-} mice have demonstrated impaired primary, but not humoral immune responses after immunization suggesting that lymphocytes may recognize the antigen in a L-selectin-independent manner, possibly in the spleen (47). Interestingly, we found no difference in Th1 cell differentiation and IFN and TNF levels as well as IgM production in *Sell*^{-/-}*Apoe*^{-/-} mice. Importantly, we detected significantly decreased levels of circulating IL-10 in *Sell*^{-/-}*Apoe*^{-/-} mice, suggesting overall reduced peripheral tolerance in *Sell*^{-/-}*Apoe*^{-/-} mice and an environment conducive to the recruitment of pro-inflammatory cells such as macrophage to sites of inflammation as suggested in previous studies. T_{reg} are important source of IL-10 (48). Since we found no difference in T_{reg} numbers between *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice, it is likely that other cell sources, such as B_{reg}, might be responsible for reduced IL-10 levels in the plasma of *Sell*^{-/-}*Apoe*^{-/-} mice.

A number of previous studies reported a correlation between levels of T15 antibodies and the degree of atherosclerosis (18). Interestingly, our results confirmed and extended this observation and demonstrated a correlation between the number of aortic B1a cells, levels of aortic T15 antibodies, and atherosclerosis progression. This suggests that although the aortic B1a cells are a minor population, they play a substantial role in atherogenesis and are dependent on L-selectin for homing into the aorta. Future studies will investigate whether immune complex formation with oxLDL might, at least in part, be responsible for reduced levels of aortic T15 antibodies.

Various models have provided evidence that macrophage accumulation is one of the critical steps in atherogenesis (1, 3, 6). In this study we demonstrated that L-selectin deficiency leads to increased content of CD68^{hi} macrophages in the aortic wall, which might contribute to the accelerated atherosclerosis. Interestingly, we found that monocyte migration to the aorta was L-selectin-dependent, therefore the increased aortic macrophage content in *Sell*^{-/-}*Apoe*^{-/-} mice is likely due to some alternative mechanisms of migration or altered proliferation and apoptotic rates in the aorta (37). It is possible that the reduced frequency of B1a, the absence of B_{reg} cells, and potentially elevated frequency of neutrophils in the aortas maintenance local inflammation in the wall and therefore support aortic macrophage retention and proliferation within the aortas.

In conclusion, this study provides evidence that L-selectin deficiency results in elevated atherosclerosis in *Apoe*^{-/-} mice that is associated with elevated levels of neutrophils in the blood, attenuated recruitment of B1 cells into the aorta, reduced levels of atheroprotective aortic IL-10 and T15 levels, and increased aortic macrophage content. Our findings suggest a new pathway, by which L-selectin might influence the development of atherosclerosis through the alteration of immune composition within the peripheral blood and aortic wall.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. L-selectin deficiency increases plaque burden within the aortas of *Sell^{-/-}Apoe^{-/-}* mice
(A) Quantification of positive Oil Red O staining in aortas of 50–60 week old *Sell^{-/-}Apoe^{-/-}* (black circles) and *Apoe^{-/-}* mice (black squares) (n=16 and 17, respectively)
(B) Representative images of *Sell^{-/-}Apoe^{-/-}* and *Apoe^{-/-}* aortas stained by Oil Red O.
 Unpaired student's t-tests were used for statistical analysis; ***P 0.0005.

Figure 2. L-selectin deficient *Apoe^{-/-}* mice have an increased number of total aortic leucocytes but a decrease in aortic B cells

Total leucocyte and CD19⁺ cell numbers in the (A) Aorta (C) Peripheral lymph nodes, and (D) Blood of 40–50 week old *Sell^{-/-}Apoe^{-/-}* (black bars) and *Apoe^{-/-}* (gray bars) mice (n=19 and 24, respectively). B cells were quantified as CD19⁺ percentages of leucocytes by flow cytometry. B cell numbers were calculated from their percentage determined by flow cytometry and total leucocyte counts. (B) Representative flow cytometric plots of CD19⁺ B cells within CD45⁺ cells of *Sell^{-/-}Apoe^{-/-}* and *Apoe^{-/-}* aortas. * P 0.05, *** P 0.0005.

Figure 3. Absence of L-selectin results in an altered distribution of B cell subsets within *Apoe*^{-/-} aorta

(A) Distribution of aortic B cell subsets within CD19⁺ B cell population of 40–50 week old *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice. B cell subsets were characterized based on CD19, CD43, CD5, and IgD expression (Table 1) using flow cytometry. Percentages were calculated from the gated CD19⁺ population for *Sell*^{-/-}*Apoe*^{-/-} (n=20) and *Apoe*^{-/-} (n=27) mice. Pie size reflects total aortic B cell number. (B) Total number of B cell subsets in the aorta of *Sell*^{-/-}*Apoe*^{-/-} (black bars, n=17) and *Apoe*^{-/-} mice (gray bars, n=24). (C) Representative

plots of flow cytometric analysis of aortic B cell subsets from *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice. **(D)** Representative immunofluorescence images of B cells or B1a and B1b subsets (total B1 cells) in *Apoe*^{-/-} mice (Images at 20× magnification, Inset images at 63×, Scale bar represents 10 μm). * P 0.05, *** P 0.0005.

Figure 4. Altered splenic B cell subset distribution and diminished T15 and IL-10 levels in the aortas of *Sell*^{-/-}*Apoe*^{-/-} mice

(A) Total numbers of B cell subsets in the spleens of 40–50 week old *Sell*^{-/-}*Apoe*^{-/-} (n=7) and *Apoe*^{-/-} (n=14) mice. (B) L-selectin expression on splenic B cell subsets of 50 week old *Apoe*^{-/-} mice (n=6). One-way ANOVA was used for statistical analysis. (C) Fold difference of T15 antibody in *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} in serum and aortic supernatants. Values were normalized to the average T15 in *Apoe*^{-/-} serum in each independent experiment (n=6–9 per genotype). (D) IL-10 production was measured in serum and aortic supernatants

of *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice using cytomix beads (n=5–8 per genotype) Black bars represent *Sell*^{-/-}*Apoe*^{-/-} mice and gray bars represent *Apoe*^{-/-} mice.* P 0.05, *** P 0.0005.

Figure 5. B1 cells require L-selectin for homing to the aorta

(A) Fold difference of CD68^{hi} leucocytes from *Sell*^{-/-}*Apoe*^{-/-} (black bar) versus *Apoe*^{-/-} (gray bar) aortas (n=5 per genotype). (B) Number of neutrophils and monocytes in the peripheral blood of *Sell*^{-/-}*Apoe*^{-/-} (black bar) and *Apoe*^{-/-} (gray bar) mice. Neutrophils were defined as CD11b⁺Gr1⁺Ly6G⁺ cells and monocytes as CD11b⁺Gr1⁺Ly6G⁻ cells by flow cytometry. Cell numbers were calculated from their percentage by flow cytometry and total leucocyte counts. (C) L-selectin-dependent monocyte homing into atherosclerotic aortas. *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} peripheral blood leukocytes were labeled with CFSE or

CellTrace™ Violet dye, mixed in the ratio 1:1, and adoptively transferred to aged *Apoe*^{-/-} recipients. After 16 hours, aortas of *Apoe*^{-/-} recipients were stained for CD45, CD11b, Gr-1 and Ly6G and evaluated by flow cytometry. Values were normalized based on neutrophil and monocyte populations within the start population as described in Materials and Methods. Migration of *Apoe*^{-/-} monocytes or neutrophils was determined as 1 (gray bars). Ratios were created from the migrated *Sell*^{-/-}*Apoe*^{-/-} versus *Apoe*^{-/-} cells (n=4). Results show mean±SE from at least 4 recipients from 3 independent experiments. **(D)** Aortic homing of FO and B1 cells into the aorta of *Apoe*^{-/-} recipients. Splenic CD19⁺ cells were purified from *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice, labeled with CFSE or CellTrace™ Violet dye, mixed in the ratio 1: 1, and injected into aged *Apoe*^{-/-} mice. After 16 hours, aortas were stained for CD45, CD19, CD43, and IgD, and then evaluated by flow cytometry. Values were normalized based on B1 and FO B cell populations within the start population as described in Materials and Methods. Migration of *Apoe*^{-/-} B1 and FO B cells was determined as 1 (gray bars), and the homing of *Sell*^{-/-}*Apoe*^{-/-} B cell subsets was expressed relative to appropriate *Apoe*^{-/-} B cell subsets (black bars). Ratios were created from the migrated *Sell*^{-/-}*Apoe*^{-/-} versus *Apoe*^{-/-} cells (n=4). * P 0.05, ** P 0.005, *** P 0.0005.

Table 1

Phenotypic markers for B cell subset characterization

Expression and intensity of expression permits the characterization of the five B cells subsets.

Subset	CD19	B220	IgD	IgM	CD43	CD5	CD23	CD21	CD1d
B1a	+	Low	Low	High	+	+	-	Low	Low
B1b	+	Low	Low	High	+	-	-	Low	Low
B _{reg}	+	Mid	Low	High	-	+	-	Mid	High
MZ	+	High	Low	High	-	-	-	High	High
FO	+	High	High	Low	-	-	+	Mid	Low

Table 2

Distribution of B cell subsets in the aortic B cell population

The values represent the percentage of each B cell subset in the total B cell population of the aortas of *Sell*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice. These values correspond to the pie graphs depicted in Figure 3A.

	% of aortic total CD19 ⁺ B cell population				
	B1a	B1b	B _{reg}	MZ	FO
<i>Sell</i> ^{-/-} <i>Apoe</i> ^{-/-}	2.4 ± 0.5%	27.8 ± 3.9%	0.0 ± 0.0%	8.1 ± 1.0%	61.6 ± 3.6%
<i>Apoe</i> ^{-/-}	4.9 ± 0.9%	23.0 ± 2.7%	0.2 ± 0.1	7.0 ± 0.9%	64.9 ± 4.7%

Table 3

What is known about the topic	What this paper adds
B cells are present in atherosclerotic aortas.	In <i>Apoe</i> ^{-/-} aortas, the predominant B cell population consist of FO B cells compared to the B1a and B _{reg} populations, which represent the two smallest populations.
B and T cells use L-selectin to migrate to healthy and atherosclerotic-prone aortas during short-term homing experiments.	B1 cells are dependent on L-selectin for homing into the aorta.
L-selectin perpetuates atherosclerosis in <i>Apoe</i> ^{-/-} mice fed a western diet for 6 weeks or chow diet for 6 months.	L-selectin deficiency results in accelerated atherosclerosis in aged <i>Apoe</i> ^{-/-} mice that is associated with decreased B1a/B _{reg} content in the aorta, and elevated blood neutrophil counts.
B cells are crucial in atherogenesis, where FO B cells are pro-atherogenic and B1a cells are protective.	L-selectin-dependent reduction of B1a and B _{reg} subsets in aortas is associated with a significant decrease of aortic T15 and IL-10, and a concomitant increase in aortic macrophages.