Helix-Loop-Helix Factor Inhibitor of Differentiation 3 Regulates Interleukin-5 Expression and B-1a B-Cell Proliferation

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Objective—Natural immunity is emerging as an important mediator of protection from atherogenesis. Natural IgM antibodies that recognize oxidation-specific epitopes on low-density lipoprotein or phospholipids and the B-1a B cells that produce them attenuate atherosclerosis. We previously demonstrated that Apoe<sup>−/−</sup> mice globally deficient in the helix-loop-helix protein inhibitor of differentiation 3 (Id3) develop early diet-induced atherosclerosis. Furthermore, B-cell–mediated attenuation of atherosclerosis in B-cell–deficient mice was dependent on Id3. Here, we sought to determine whether Id3 regulates B-1a B cells and the natural antibodies that they produce and identify mechanisms mediating these effects.

Approach and Results—Mice lacking Id3 had significantly fewer B-1a B cells in the spleen and peritoneal cavity and reduced serum levels of the natural antibody E06. B-cell–specific deletion of Id3 revealed that this effect was not because of the loss of Id3 in B cells. Interleukin (IL)-33 induced abundant, Id3-dependent IL-5 production in the recently identified innate lymphoid cell, the natural helper (NH) cell, but not Th2 or mast cells. In addition, delivery of IL-5 to Id3-deficient mice restored B-1a B-cell proliferation. B-1a B cells were present in aortic samples also containing NH cells. Aortic NH cells produced IL-5, a B-1a B-cell mitogen in response to IL-33 stimulation.

Conclusions—These studies are the first to identify NH and B-1a B cells in the aorta and provide evidence that Id3 is a key regulator of NH cell IL-5 production and B-1a B-cell homeostasis. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: arteriosclerosis ■ basic helix-loop-helix transcription factors ■ immune system

Atherosclerosis is a chronic inflammatory disease of the blood vessel wall that can lead to heart attacks and stroke. Despite current therapy targeting traditional risk factors, atherosclerosis remains the leading cause of death in Westernized countries. Substantial work during the past several decades clearly establishes a key role for the immune system in the atherosclerosis development and progression. As such, immune modulation holds promise as an effective addition to current prevention approaches. Thus, a deeper understanding of the contributions of various immune cells to atherogenesis is of key importance. Although abundant evidence implicates macrophages and some T-cell subsets in promoting inflammation in the vessel wall, B cells have emerged as another important immune cell that can modulate atherogenesis. Characterization of atheroprotective antibodies, passive and active immunization studies, and adoptive transfer studies of splenic B cells from Apoe<sup>−/−</sup> mice support an atheroprotective role for B cells in mice. Yet, recent studies suggest that the role of B cells is subset dependent, where B-2 B cells may be atherogenic and B-1a B cells protective.

A subset of B cells from the B-1 lineage, the B-1a B cell, has been reported to rescue enhanced atherosclerosis caused by splenectomy. Atheroprotection was demonstrated to be dependent on the ability of the B-1a B cells to secrete IgM natural antibodies (NAbs). B-1a B cells are considered part of the innate immune system, develop from fetal tissues, have a high capacity to undergo homeostatic proliferation, and spontaneously secrete IgM NAbs. IgM NAbs are present before pathogen exposure, recognize self-antigens for housekeeping functions, and are thought to be the product of natural selection. A representative IgM NAb, E06, has been reported to be atheroprotective through recognition of oxidation-specific epitopes on oxidized low-density lipoprotein and apoptotic cells, blocking the uptake of oxidized low-density lipoprotein by macrophage scavenger receptors and mediating apoptotic cell clearance. Although B-1a B cells are important because of the IgM NAbs they produce, the factors that regulate B-1a B cells in atherosclerosis are poorly understood. Interleukin (IL)-5 is an important atheroprotective cytokine known to promote B-1a B-cell proliferation and E06 production. Many cell types, including Th2 T cells...
and the recently discovered natural helper (NH) cells, produce IL-5. Moreover, NH cells produce large amounts of IL-5 relative to other IL-5-producing cells.35

NH cells belong to an emerging arm of the innate lymphoid cell family, the group 2 innate lymphoid cell helper subset.36–40 NH cells are organized into fat-associated lymphoid clusters in the mesenteric adipose depot. No specific markers have been identified that define NH cells, and they are therefore defined by lineage negative (lin−). They are positive for Sca1, CD117 (c-kit), CD44, CD90, and the IL-33R (T1/ST2).35,41 NH cells produce mainly Th2-associated cytokines in response to a variety of stimuli, including IL-33. Direct evidence with coculture experiments demonstrates that NH cells support B-1 B-cell proliferation, similar to IL-5 alone.35

Inhibitor of differentiation 3 (Id3), a helix-loop-helix protein, is a widely expressed dominant-negative regulator of gene transcription that acts through interaction with DNA binding basic helix-loop-helix proteins, such as E-proteins.42 These helix-loop-helix proteins are part of a complex gene regulatory network of lineage-specific transcription factors that orchestrate lymphocyte development and activation.43–52 Studies suggest that Id3 is also important in human and murine atherosclerosis. In humans, the ID3 gene contains a single nucleotide polymorphism (SNP) at rs11574. This non-synonymous SNP results in an amino acid substitution in the C terminus of the ID3 protein, which attenuates ID3 antagonism of the E-protein, E12. Notably, this SNP is associated with increased carotid intimal media thickness in humans,53 suggesting that loss of Id3 function may promote vascular disease. In murine models, global deletion of Id3 results in increased atherosclerosis in both Ldlr−/− and Apoe−/− mice.54,55 Moreover, Id3 was necessary for B-cell–mediated attenuation of atherosclerosis in B-cell–deficient/Apoe−/− mice.54,55 Id3 has also been reported to modulate B-cell homing and vessel wall adhesion molecule expression,54,55 yet Id3 may also regulate factors involved in innate immunity as loss of Id3 in Apoe−/− mice resulted in early onset of diet-induced atherosclerosis.54 These results raise the interesting hypothesis that Id3 may be an important regulator of B-1a B cells and natural immunity.

The present study demonstrates that Id3 is important for maintenance of splenic and peritoneal cavity (PerC) B-1a B cells and serum levels of E06. However, Id3 regulation of the B-1a B-cell pool is because of the loss of Id3 in a non–B-cell population as B-1a B-cell number were unchanged in mice with B-cell–specific loss of Id3. Indeed, loss of Id3 significantly reduced NH cell production of the B-1a B-cell mitogen, IL-5. In addition to the mesentery, NH cells are present and produce IL-5 in response to IL-33 stimulation in the aortic adventitia/surrounding perivascular adipose tissue (PVAT). B-1a B cells were also found in the aortic adventitia/surrounding PVAT. These results provide the first evidence that NH cells and B-1a cells are present in the aorta and implicate Id3 as a key regulator of NH cell IL-5 production and B-1a B-cell homeostasis.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

**Id3 Regulates B-1a B-Cell Number in Apoe−/− Mice**

Previous studies demonstrate that B-1a B cells are atheroprotective by producing IgM NAbs.11,14,20,56,57 To determine whether Id3 regulates B-1a B-cell number in Apoe−/− mice, B-1a B cells in Id3+/+Apoe−/− and Id3−/−Apoe−/− mice were measured by flow cytometry. The gating strategy has been previously described,46,59 and Figure IA in the online-only Data Supplement presents representative flow cytometry plots that identify B-1a B cells (CD19+B220CD5+CD43+) and B-2 B cells (CD19+B220) in both the spleen and the PerC. Results reveal significantly fewer B-1a B cells in both the spleen and the PerC in Id3−/−Apoe−/− mice compared with Id3+/+Apoe−/− mice (Figure 1A). This effect was not dependent...
on hypercholesterolemia, as there was also a decrease in the frequency of B-1a B cells in C57BL/6 mice lacking Id3 (Figure IB in the online-only Data Supplement). In contrast, and consistent with previous reports, 40,42 the absolute number of B-2 B cells in the spleen and PerC of Id3−/−Apoe−/− mice was equivalent compared with Id3+/+Apoe−/− mice (Figure 1A). Consistent with the decreased number of B-1a B cells in Id3−/−Apoe−/− mice, we detected less E06 antibody in the serum of 8-week-old Id3−/−Apoe−/− mice compared with control mice (Figure 1B). To determine whether the lower amount of serum E06 antibody is because of reduced production of E06 on a per-cell basis, B-1a B cells were isolated from the PerC by fluorescence-activated cell sorter and analyzed for the amount of mRNA encoding the secreted form of E06 IgM. The gating fluorescence-activated cell sorter and analyzed for the amount per-cell basis, B-1a B cells were isolated from the PerC by fluorescence-activated cell sorter and analyzed for the amount of mRNA encoding the secreted form of E06 IgM. The gating strategy for fluorescence-activated cell sorter purification of B-1a and B-2 B cells is depicted in Figure II in the online-only Data Supplement. There was no difference in E06 mRNA in B-1a B cells from Id3+/+Apoe−/− compared with Id3−/−Apoe−/− mouse (Figure 1C). To determine whether the loss of Id3 altered proliferation of B-1a B cells, BrdU was administered intraperitoneally to Id3−/−Apoe−/− and Id3+/−Apoe−/− mice. PerC cells were harvested 24 hours later, and BrdU incorporation in B-1a and B-2 B cells was determined by flow cytometry. There was a decrease in the proportion of B-1a B cells incorporating BrdU in Id3−/−Apoe−/− compared with Id3+/−Apoe−/− mice (Figure 1D). Consistent with no difference in B-2 B-cell number with the loss of Id3, there was no difference in the proportion of B-2 B cells incorporating BrdU. In addition, there was no difference in the proportion of peritoneal B-1a or B-2 B cells that were positive for annexin V, a marker of apoptosis (Figure 1D). These results suggest that the reduced number of B-1a B cells in Id3−/−Apoe−/− mice is because of reduced B-1a B-cell proliferation.

Id3 has been shown to be necessary for protection from atherosclerosis in Apoe−/− mice.53,54 To investigate whether the global loss of Id3 resulted in alterations of other immune cell populations important in atherosclerosis, flow cytometry was performed for splenic CD4+ T cells, CD4+Foxp3+ regulatory T cells, CD8+ T cells, and peritoneal CD4+ T cells, CD8+ T cells, and F4/80+ macrophages in Id3−/−Apoe−/− and Id3+/−Apoe−/− mice. No differences were observed in the proportions of these populations (data not shown). Cholesterol levels also affect atherogenesis,2 and there was no difference in the serum lipid profiles of Id3−/−Apoe−/− mice compared with control mice (Table I in the online-only Data Supplement) consistent with previous observations.45

To determine whether the reduced number of B-1a B cells in Id3−/−Apoe−/− mice was because of the loss of Id3 in B cells, Id3 was specifically deleted in B cells on the Apoe−/− background. These mice were generated by first crossing a floxed Id3 allele onto the Apoe−/− background to produce Id3fl/flApoe−/− mice. These mice were then crossed with Apoe−/−CD19−/− mice to produce Id3fl/flApoe−/−CD19−/− and Id3−/−Apoe−/−CD19−/− control mice. Western blotting of lysates from splenic B cells demonstrated the inability to detect Id3 protein in B cells from Id3−/−Apoe−/−CD19−/− mice compared with controls (Figure 2A). Furthermore, transcript levels of Id3 were not detectable by real-time polymerase chain reaction in sorted B-1a B cells from Id3−/−Apoe−/−CD19−/− mice.

**Loss of Id3 Markedly Attenuates IL-33–Induced IL-5 Production**

IL-5 is an IL-33–induced Th2 cytokine critically important for homeostatic proliferation and survival of B-1a B cells. To determine whether Id3 is necessary for IL-33–induced production of IL-5, Id3−/−Apoe−/− and Id3+/−Apoe−/− mice were treated with PBS vehicle control or IL-33, and serum and peritoneal fluid IL-5 levels were measured by a high-sensitivity ELISA. As presented in Figure 3A, IL-33 induced abundant IL-5 in the serum and peritoneal fluid, and this effect was abolished in mice lacking Id3. IL-5 was undetectable in the serum and peritoneal fluid of PBS-treated Apoe−/− mice, consistent with previous reports.55 (Figure 3A). Because Id3−/−Apoe−/− mice had a markedly attenuated IL-33–induced IL-5 response and reduced homeostatic proliferation of B-1a B cells, we next sought to determine whether the delivery of exogenous IL-5 could increase the proliferation of PerC B-1a B cells in Id3−/−Apoe−/− mice.
**Id3 Regulates NH Cell Production of IL-5**

Previous studies have identified NH cells as the predominant source of IL-33–induced IL-5 production in vivo, and coculture experiments demonstrated that NH cells can directly support B-1 B cell proliferation. To determine whether Id3−/− Apoe−/− mice had lower IL-33–induced IL-5 serum and peritoneal fluid levels because of fewer total number of NH cells, flow cytometry was performed to quantify NH cells in the mesentery. NH cells were identified as CD45+, lineage-negative (Lin−: CD3ε, CD4, CD5, CD8α, CD11b, CD11c, CD19, CD45R/B220, FcεR1α, Ly6G, NK1.1, TCRβ, TCRγδ, and Ter119) Sca1+ CD117+ T1/ST2+ CD90+ and CD44+ cells (Figure 4A). In contrast to mice that are null for Id2 and lack NH cells, Id3−/− Apoe−/− mice contained a similar number of NH cells compared with Id3+/+ Apoe−/− mice (Figure IIIA in the online-only Data Supplement). In addition, the lower IL-33–induced IL-5 serum levels were not because of Id3 regulation of the IL-33 receptor, T1/ST2 on NH cells as Id3−/− Apoe−/− mice had similar expression of NH cell T1/ST2 compared with control mice (Figure 4A). There was also no difference in levels of IL-33 in the peritoneal fluid of Id3−/− Apoe−/− mice compared with control mice (21.78±10.01 versus 29.11±8.18 pg/mL, respectively; n=6), and serum levels were below the sensitivity of the assay (≤2.39 pg/mL). To determine whether Id3 regulates production of IL-5 by a specific Th2 cytokine–producing cell, flow cytometry with intracellular cytokine staining for IL-5 was performed on isolated cells cultured with

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**Id3−/− Apoe−/−** mice were injected with IL-5 intraperitoneally every day for 5 days, and on the last day BrdU was coinjected. Consistent with earlier findings (Figure 1D), there was a smaller percentage of BrdU+ B-1a B cells in PBS control–injected Id3−/− Apoe−/− mice compared with Id3+/+ Apoe−/− mice. Moreover, exogenous IL-5 increased the proportion of BrdU+ B-1a B cells in Id3−/− Apoe−/− mice compared with PBS control (Figure 3B).
IL-33. Consistent with previous reports, the percentage of NH cells producing IL-5 is markedly higher than other Th2 cytokine–producing cells, such as polarized CD4+ T cells and bone-marrow–derived mast cells (Figure 4B). Intracellular cytokine staining of IL-5 in IL-33–treated basophils or eosinophils was not detected (data not shown). Furthermore, on average, of IL-5–producing cells in the fat-associated lymphoid clusters were NH cells. Interestingly, there was a 50% reduction in the percentage of NH cells that produce IL-5 in response to IL-33 in \( \text{Id3}^{-/-}\text{Apoe}^{-/-} \) mice compared with NH cells isolated from control \( \text{Id3}^{+/+}\text{Apoe}^{-/-} \) mice. Consistent with previous data, NH cells do not produce detectable interferon-\( \gamma \). Loss of Id3 did not alter IL-5 production in CD4+ T cells or bone-marrow–derived mast cells, other cell types known to produce IL-5 (Figure 4B).

Id3 is known to both activate and antagonize gene expression through the regulation of promoter elements termed E-boxes. The IL-5 promoter contains several of these E-box elements. To determine whether Id3 regulates the IL-5 promoter, an IL-5 luciferase promoter-reporter, pLS-IL5, was cotransfected with control empty vector (pEF4) or Id3 (pEF4-Id3) in a lymphoid cell line. As shown in Figure 4C, Id3 significantly increases IL-5 promoter activity above control empty vector. Western blots confirm Id3 protein overexpression. Together, results demonstrate that Id3 regulates IL-5 expression in lymphoid cells and in particular, NH cells.

**IL-5–Producing NH Cells and B-1a B Cells Are Present in the Perivascular Aortic Adipose Tissue**

NH cells reside in fat-associated lymphoid clusters in the mesenteric adipose depot. The PV AT also contains immune cells, is intimately associated with the adventitial layer of the vessel wall, and has been implicated in regulating atherogenesis. B cells are known to reside in the adventitial layer of vessels and the presence of B cells in the adventitia has been associated with both atherogenesis and atheroprotection. However, whether NH cells and B-1a B cells are present in periaortic fat and adventitia is unknown. To determine whether NH cells reside in periaortic adipose tissue, flow cytometry for NH cells was performed on cells isolated from whole aortas including the adjacent adventitia and PVAT from chow-fed, 8- to 10-week-old \( \text{Apoe}^{-/-} \) mice. Indeed, a population of NH cells, identified as CD45+ Lin− Sca1+ CD117+ cells, was present in whole aortas of \( \text{Apoe}^{-/-} \) mice (Figure 5A). Similar to the mesentery, the number of aortic NH cells was not different with the loss of Id3 (Figure IIIB in the online-only Data Supplement). Aortic NH cells expressed markers such as CD44, CD90, and the IL-33 receptor (T1/ST2) comparable with mesenteric NH cells. Similar to NH cells in the lung, intestines, and mesentery, these NH cells did not express CCR6. To determine whether aortic NH cells produce IL-5 in response to IL-33 stimulation in vivo, \( \text{Apoe}^{-/-} \) mice were treated with PBS vehicle control or IL-33 every 2 days and euthanized on day 7. Flow cytometry analysis of whole aortas revealed IL-33–induced IL-5 expression in aortic NH cells (Figure 5B). NH cells have been shown to directly support B-1 B-cell proliferation. To determine whether B-1a B cells also reside in and around the aorta of mice before atheroma development, flow cytometry was performed on aortas including the periaortic adventitia and PVAT for B-cell subsets. B-1a cells were present in the whole aorta (Figure 5C). To determine whether IL-33 can induce B-1a B-cell proliferation in the aorta, \( \text{Apoe}^{-/-} \) mice were treated with PBS vehicle control or IL-33 every 2 days with BrdU coinjected for the final treatment and euthanized on day 7. Flow cytometry analysis of whole aortas revealed
IL-33–induced BrdU incorporation in aortic B-1a B cells (Figure 5D). These data are the first to identify NH cells and B-1a B cells in the aortic adventitia/PVAT and provide evidence that IL-33 can induce aortic NH cells to increase local IL-5 production and B-1a B-cell proliferation.

**Discussion**

The present study provides evidence that Id3 is a key mediator of natural immunity and the IL-33/IL-5 pathway using a genetic model of early and accelerated atherosclerosis, the ApoE<sup>−/−</sup> mouse null for Id3.53,54 First, we show reduced numbers and proliferation of B-1a B cells and lower serum levels of an IgM NAb, E06, in Id3<sup>−/−</sup>ApoE<sup>−/−</sup> mice. However, when Id3 is deleted only in B cells, there is no effect on B-1a B-cell number or E06 serum levels, suggesting that Id3 regulates the number of B-1a B cells by a mechanism that involves a non–B-cell. Second, we show that IL-33–induced IL-5 levels are attenuated in Id3<sup>−/−</sup>ApoE<sup>−/−</sup> mice, and administration of IL-5 can rescue the B-1a proliferation defect in Id3<sup>−/−</sup>ApoE<sup>−/−</sup> mice. Third, we show reduced production of IL-5 in IL-33–treated NH cells isolated from Id3<sup>−/−</sup>ApoE<sup>−/−</sup> mice. We also demonstrate by flow cytometry that the aorta, including the adventitia and PVAT, contain B-1a B cells and NH cells. Aortic NH cells can be stimulated to produce the IL-5 that may maintain B-1a B-cell proliferation. Thus, our data identify Id3 as a key regulator of NH cell production of the atheroprotective cytokine IL-5 that promotes B-1a B-cell proliferation (Figure 6), linking this event to natural immune protection from atherosclerosis.

Natural IgM antibodies, such as E06, and the B-1a B cells that produce these antibodies have been reported to attenuate diet-induced atherosclerosis.7,8,11,14,20,25,36,29,31,56,57 Kyaw et al.20 demonstrated a significant reduction in the number of PerC B-1a B cells and levels of serum IgM antibodies that bind modified lipids after splenectomy, which was associated with enhanced atherosclerosis in ApoE<sup>−/−</sup> mice. Moreover, adoptive transfer of B-1a B cells was shown to rescue splenectomy-induced atherosclerosis, an effect dependent on the ability for B-1a B cells to secrete IgM.20 Here, we provide evidence that Id3 may be a key factor for maintaining normal numbers of B-1a B cells and levels of E06 in the serum, suggesting that Id3 may be important in B-1a B-cell–mediated atheroprotection. However, B-cell–specific deletion of Id3 did not have reduced number of B-1a B cells or levels of E06 in the serum compared with controls, suggesting that it is the loss of Id3 in a non–B-cell that results in altered B-1a B-cell homeostatic maintenance in Id3<sup>−/−</sup>ApoE<sup>−/−</sup> mice.

Results herein demonstrate that Id3 regulates IL-33–induced systemic levels of IL-5. Consistent with IL-5’s known ability to promote homeostatic proliferation of B-1a B cells,33 administration of exogenous IL-5 rescued B-1a B-cell proliferation in Id3-deficient mice. Similar to Id3,53,54 both IL-33 and IL-5 attenuate atherosclerosis.14,62,63 Treatment with IL-33 increased serum levels of IL-5 and oxidation-specific IgM antibodies and reduced lesion size in ApoE<sup>−/−</sup> mice.62,63 In addition, Ldlr<sup>−/−</sup> mice reconstituted with Il5<sup>−/−</sup> bone marrow had decreased amounts of plasma E06 antibody and increased atherosclerosis compared with controls.14 In the present study, we provide evidence that Id3 regulates levels of IL-5, thereby mediating this important IL-33/IL-5 atheroprotective pathway.

Id3 regulates the production of IL-5 by the recently identified innate lymphoid cell, the NH cell.35 Importantly, Id3 is not required for the development of NH cells, unlike Id2.35 NH cells produce large amounts of IL-5 in response to IL-33 relative to Th2 cells, basophils, iNKT cells, mast cells, and CD4<sup>+</sup> cells.35,64,65 Results of the present study support these findings in that IL-33 stimulated 50% of NH cells to produce IL-5, whereas <2% of Th2 or mast cells were IL-5 producing. In this study, we did not detect any basophils and eosinophils producing IL-5 in response to IL-33. Moreover, of these cell types, only NH cells showed reduced percentage of IL-33–stimulated IL-5–producing cells in ApoE<sup>−/−</sup> mice with deletion.
of Id3. Of note, differences in the intracellular staining for IL-5 in NH cells harvested from Id3−/−ApoE−/− mice compared with control mice were less marked than differences in the serum and peritoneal fluid, raising the interesting possibility that there may be an additional, yet unidentified, cell type producing IL-5 in an Id3-dependent manner. Although NH cell–specific deletion of Id3 would help address this question, this is not feasible as there is no lineage-specific marker for NH cells. Moreover, it is more likely that measuring intracellular levels of this secreted protein under-represents for NH cells. Moreover, it is more likely that measuring the effects of Id3 on total production of IL-5, including that which has already been secreted. Nevertheless, results clearly demonstrate that Id3 regulates NH cell production of IL-5 in response to IL-33.

Although lymphocytes have long been identified in human and murine aortic adventitia, recent studies provide evidence that adventitial lymphocyte activation, including B cells, may be important in regulating atherosclerosis. Data from this study suggest that adventitial/PVAT B-1a B cells and NH cells present before Western diet initiation may be part of the local IL-33/IL-5 pathway poised to protect from atherosclerosis. Aortic NH cells express markers identical to fat-associated lymphoid clusters NH cells including the IL-33 receptor. IL-33 is produced by cells in the aortic adventitia, which may provide a local stimulus for NH cell production of IL-5. Exogenous IL-33 induced IL-5 production by NH cells and proliferation of aortic B-1a B cells in vivo, suggesting that NH cell–derived IL-5 may function to support proliferation of the resident B-1a B cells. These B-1a B cells could produce E06 or other natural IgM antibodies in the adventitia/PVAT. Although further study is needed to more fully understand the role of innate-like lymphocytes in the aortic adventitia and surrounding PVAT, results herein demonstrate the existence of functional aortic NH cells.

The present study is the first to demonstrate Id3 regulation of the IL-33/IL-5 pathway and natural immunity. As we have clearly shown that loss of Id3 leads to early atherosclerosis in mice, this may be an important mechanism whereby Id3 promotes innate protection from atherosclerosis. Prior studies demonstrated that loss of Id3 led to decreased B-cell homing to the aorta and increased intratumoral membrane expression in atheroprotective mouse models. In addition, it is interesting to speculate that Id3 may also provide atheroprotection by regulating Tregs. Tregs have been shown to be atheroprotective, and Id3 has been implicated in the promotion of Treg development in neonates; although consistent with previous data, we did not observe a difference in the frequency of Tregs with the loss of Id3. There may be other, as yet unidentified, mechanisms whereby Id3 may regulate atheroprotection. The fact that a single SNP in a single gene (Id3) is associated with carotid intimal medial thickness in humans would support a role for Id3 in regulating pathways in many cell types involved in atherosclerosis. Although the SNP does not result in reduced expression of Id3 protein, as seen with a knockout mouse, it does result in decreased Id3 binding to its basic helix-loop-helix partner, E12, which markedly attenuates Id3 function. Therefore, identification of specific Id3-mediated atheroprotective pathways, such as the IL-33/IL-5 axis, in mice has the potential to lead to interesting hypotheses that can be tested in humans, such as whether humans with polymorphism at rs11574 have alterations in natural immunity that could be linked to premature atherosclerosis. Such findings may identify individuals amenable to novel prevention or treatment approaches.

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Disclosures

None.

References

oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. 


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**Significance**

Natural immunity is emerging as a protective process in atherosclerosis. Natural IgM antibodies that recognize oxidative epitopes on low-density lipoprotein or phospholipids prevent atherosclerosis and are produced by the atheroprotective B-cell subset, the B-1a B cell. We previously demonstrated that splenic B cells provide atheroprotection to B-cell-deficient mice, an effect dependent on the helix-loop-helix transcription factor, inhibitor of differentiation 3 (Id3). Here, we identify Id3 as an important regulator of B-1a B cells and natural immunity. The loss of Id3 results in reduced B-1a B cells and serum levels of natural IgM antibodies, an effect not attributable to the loss of Id3 in B cells. We found that Id3 is necessary for the production of interleukin-5, an important B-1a B-cell mitogen. Interleukin-5 can be abundantly produced by the innate lymphoid cell, the natural helper cell. Indeed, Id3 was important for natural helper cell production of interleukin-5. Results are the first to implicate Id3 as a key regulator of natural helper cell function and atheroprotective B-1a B-cell proliferation.
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Materials and Methods

The Helix-Loop-Helix Factor Id3 Regulates IL-5 Expression and B-1a B cell proliferation

Perry, et.al.
Mice

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. ApoE−/− male mice were purchased from Jackson Laboratory. Id3−/− and Id3fl/fl mice were a generous gift of Dr. Yuan Zhang (Duke University). Id3−/− mice were bred to the ApoE−/− background to obtain Id3−/−ApoE−/− as previously described. CD19cre/+ mice were gifted by Timothy Bender (University of Virginia). Id3fl/fl mice were bred to the ApoE−/− mouse, and then to CD19cre/+ mice to generate Id3fl/flApoE−/−CD19cre/+ and littermate control Id3fl/flApoE−/−CD19+/+ mice. All mice were purchased from Jackson on a pure C57BL/6J background or mice were backcrossed 10 generations to pure C57BL/6J. All mice were fed a standard chow diet (Tekland 7012) and euthanized at 8 to 10 weeks of age by CO2 inhalation.

ELISA

Whole blood was harvested from mice by right ventricular puncture and peritoneal fluid was harvested by lavage with 1 mL PBS at the time of sacrifice. Determination of mouse E06 was determined as previously described in detail. For IL-5 levels (BD OptEIA™, BD Biosciences), mice were i.p injected with 125 ng of mIL-33 (CF, R&D) reconstituted in PBS every three days for 7 days. IL-33 was measured undiluted by the mouse/rat QuantiKine ELISA kit (R&D).

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Aortas were harvested and digested as previously described except periaortic adipose tissue was left intact with all lymph nodes carefully removed. Peritoneal cells, splenocytes, or digested aortas were first blocked for Fc receptors (FCR-4G8, Invitrogen), then stained for cell surface or intracellular markers and a live/dead stain as previously described. Mesenteric cells were prepared as described elsewhere. Briefly, mice were transcardially perfused with 20 U mL−1 heparin after CO2 euthanasia. Mesenteric fat was carefully removed of mesenteric lymph nodes and separated from intestines, and finely chopped in 5 mL of DMEM with 2 mg mL−1 Collagenase I (Worthington Biochemical Co.) and 4% BSA (Sigma), then digested at 37°C in a shaking incubator for 45 min. The supernatant containing adipocytes after centrifugation was aspirated. After washing the cells in HBSS with 10% HI-FBS, cells were strained through a 40 μm filter and stained. For intracellular staining, the FIX & PERM® kit (Invitrogen) was used as per manufacturer’s instructions. Flow cytometry antibodies CD3ε (500A2), CD4 (GK1.4, RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD43 (S7) CD44 (IM7), CD45 (30F11), CD45R/B220 (RA3-6B2), CD49b (DX5), CD90 (53-2.1), CD117 (ACK2), CD196 (29-2L17), FcεR1α (MAR-1), IgM (II/41), IFNγ (XMG1.2), IL-4 (BVD6-24G2), IL-5 (TRFK5), Ly6G (RB6-8C5), NK1.1 (PK136), Sca-1 (D7), TCRβ (HS7-597), TCRγδ (GL3) and Ter119 (TER119) were purchased from eBioscience or BD Bioscience and T1/ST2 (DJ8) from MDbioscience. Live/dead discrimination was always determined by LIVE/DEAD® fixable yellow cell staining (Invitrogen) or dapi. Cells were run on a CyAN ADP (Beckman Coulter) or sorted on a Reflection Cell Sorter (iCyt) and analyzed with FlowJo software (Tree Star Inc) using fluorescence minus one (FMO) controls for gate determination.
Counting beads were used for quantification (CountBright™ Absolute Counting Beads, Molecular Probes).

**Real-time PCR**

Total RNA was isolated from FACS purified peritoneal B cells using the RNeasy Plus Micro kit with gDNA elimination columns (Qiagen). RNA (1 µg) was reversed transcribed with SuperScript III First-Strand Synthesis System by Oligo d(T) tailing (Invitrogen). E06 gene expression was normalized by the ΔΔCq method to 18S as determined by TaqMan real-time PCR (SsoFast™ Probes Supermix, Bio-Rad). Id3 in NH cells (10,000 to 20,000 cells from 4 to 5 pooled mice) was also determined by TaqMan real-time PCR (Cells to Ct kit™, Invitrogen) as per manufacturer's instructions. Secreted IgM or Id3 in FACS-sorted B cells was normalized by the ΔΔCq method to 18S or cyclophilin with SYBR® Green real-time PCR (SsoFast™ EvaGreen® Supermix, Bio-Rad). Primers were used as follows: E06 IgHV, forward primer (5’-CTG TGC AAG AGA TTA CTA CGG TAG-3’) flanked the E06 IgH V and D junction, the reverse primer (5’-AGG ACT GAC TCT CTG AGG AGA CG-3’) flanked the JH and mu chain junction for amplifying E06 IgM, but not T15 IgA. The fluorescent probe (6FAM CGC CCC AGA CAT CGA AGT ACC AG TAMRA, Applied Biosystems) matched to the E06 IgH D and J junction; slgM, forward primer (5’-GGA GAG ACC TAT ACC TGT GTG GTA GG-3’) and reverse primer (5’-TGA GCG CTA GCA TGG TCA ATA GCA G-3’); 18S forward primer (5’-GGG CTA CCA CAT CCA AGG AA-3’), reverse primer (5’-AGC TGG AAT TAC CGC GGC-3’) and probe (6FAM TGC TGG CAC CAG ACT TGC CCT C TAMRA, Applied Biosystems); Id3 forward primer (5’-TGC TAC GAG GCG GTG TGC TG-3’) and reverse primer (5’-TGT CGT CCA AGA GGC TAA GAG GCT-3’); cyclophilin forward primer (5’-TGC CGG AGT CCA GAA CAA TGA T -3’) and reverse primer (5’-TGG AGA GCA CCA AGA CAG ACA-3’).

**In vivo proliferation**

1.5 mg of BrdU was administered i.p. and peritoneal cells or spleens were harvested and stained for BrdU as per manufacturer’s kit instructions (BD Pharmingen).

**Apoptosis assay**

Peritoneal lavage cells were washed in cold PBS and suspended in binding buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCl, 2.5 mM CaCl2). PerC cells were then incubated with Annexin V (BD Pharmingen, 556420) for 15 min at room temperature. After washing with binding buffer, cells were incubated with dapi and analyzed by flow cytometry.

**Western blot analysis**

Splenic B cells were isolated using CD19 microbeads (Miltenyi Biotech) with >99% pure CD19+ B cells. 10 x 10^6 cells were resuspended in 200 uL lysis buffer (1% DOC, 1% NP40, 50mM Tris pH 7.6, 1 mM EDTA, 150mM NaCl, 0.1% SDS with protease inhibitor cocktail), incubated at room temperature for thirty minutes, and centrifuged for 10 minutes at 11,600 x g, 4C. The infranatants were separated from the pellet. Samples were assayed for protein concentration with the Bio-Rad DC protein assay (catalog #500-0111), adjusted to equal concentration with lysis buffer, and
supplemented with 11% β-mercaptoethanol and 0.5% bromophenol blue. Samples were resolved on 4-20% Tris-glycine gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (BioRad). Equal protein loading was confirmed by staining the membrane with immunodetection of β-tubulin. Western blotting was carried out using an antibody to Id3 (0.1 µg/ml, CalBioreagents, catalog M100, clone 17-3) or β-tubulin (16 µg/ml, Cell Signaling Technology, Inc. catalog #2146S) followed by horseradish peroxidase-linked secondary antibody (Jackson). Immunoreactive bands were visualized by enhanced chemiluminescence after incubation with ECL reagent (Amersham Pharmacia Biotech). Densitometry was analyzed by ImageJ.

Cell culture

Mesenteric cells were isolated as above and plated at 2 x 10^5 cells per well in a 96 well plate with 200 µL of complete RPMI 1640 (cRPMI, RPMI-1640 medium) containing 10% HI-FBS, 50 mM 2-mercaptoethanol, 100 U mL^-1 penicillin and 100 mg mL^-1 streptomycin, 1X nonessential amino acids, 10 mM HEPES, 1 mM sodium pyruvate, and 10 ng mL^-1 mrIL-33 (CF, R&D) for 4 days without any golgi transport inhibitor.

For Th2 cells, 7 x 10^6 splenic CD4+ cells isolated by macs column purification with CD4 microbeads (Miltenyi Biotech) were stimulated with soluble anti-CD3/anti-CD28 Dynabeads® (GIBCO) and a combination of 10 ng mL^-1 mL-4 (peprotech), 5 ng mL^-1 and mL-2 (R&D), 5 ng mL^-1 for four days. Cells were then stimulated for six hours with 50 ng mL^-1 PMA and 1 µg mL^-1 ionomycin. During the last three hours, golgiplug (BDbioscience) was added to the culture. Cells were then harvested for flow cytometry and polarization was confirmed with intracellular staining of IL-4.

Mast cells and basophils were derived by culturing bone marrow harvested from femurs with 20% WEHI-3b conditioned media. After 7 days, cells in suspension were collected and plated with cRPMI and 10 ng mL^-1 mrIL-33 for 5 days. On day 6, golgiplug was added to the culture for the last 4 hours, and cells were harvested and stained for mast cells (FceR1α^+^, CD49b^−^, CD117^+^) and basophils (FceR1α^+^, CD49b^+^, CD117^−^) and intracellular IL-5 and IFNγ.

Eosinophils were derived from bone marrow as described by others. Briefly, bone marrow was harvested from femurs and seeded for 5 days in cRPMI with mrIL-33 (CF, R&D) with 10 ng mL^-1 mrIL-33. On day 6, cells were harvested for intracellular IL-5 and IFNγ detection by flow cytometry as above.

IL-5 Promoter-Reporter Assay

BJAB cells co-electroporated with 2 ug of human IL-5 promoter-luciferase construct, pLightSwitch-IL5 (pLS-IL5, Switchgear Genomics) and 10 ug of empty pEF4 or pEF4 human Id3 expression vector previously described. GFP was used as a electroporation efficiency control. After 24 hours, cells were harvested for luciferase chemiluminescence and normalized to total protein.

Statistics

To test if data sets fit a Gaussian distribution, a D’Agostino-Pearson omnibus normality test was used. If data was normal and had equal variance, a student’s t-test was performed. If data sets had unequal variance, a t-test with Welch’s correction was
used. For non-Gaussian distributed data, a Mann-Whitney test was performed to determine statistical significance. Data was analyzed using Prism 6.0b (GraphPad Software, Inc.). Results are displayed containing all replicated experiments and values shown are mean only or mean ± SEM.
References:

Supplemental Material

The Helix-Loop-Helix Factor Id3 Regulates IL-5 Expression and B-1a B cell Proliferation

Perry, et.al.
**Figure I** Representative flow cytometry plots of (A) B-2 (CD19\(^+\)B220\(^{hi}\)) and B-1a (CD19\(^+\)B220\(^{lo}\)CD5\(^+\)CD43\(^+\)IgM\(^{hi}\)) B cells in the spleen and PerC of Id3\(^{+/+}\)Apoe\(^{-/-}\) or Id3\(^{-/-}\)Apoe\(^{-/-}\) mice or (B) B-1a B cells in the PerC of wild-type, C57BL/6, (Id3\(^{+/+}\)Apoe\(^{+/+}\)) or Id3\(^{-/-}\)Apoe\(^{+/+}\) mice at 8 weeks of age.
**Figure II** Gating strategy for FACS purification of B-1a and B-2 B cells. Peritoneal lavage cells from 5 *ld3*+/+ *Apoe*−/− or 5 *ld3*−/− *Apoe*−/− mice were pooled and stained for FACS with dapi, CD3ε, CD19, B220, CD43 and IgM. (A) Gating strategy used to sort B cells (dapi-, singlets, lymphocytes (lymphs), CD3ε-, B-2: CD19+B220hi, B-1a: CD19+B220lo, CD5+). (B) Post-sort purity of B-1a cells.
Supplemental Table I. Average random serum lipid profiles of Id3+/+ Apoe−/− and Id3−/− Apoe−/− mice at 8 to 10 weeks of age.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Id3+/+ Apoe−/−</th>
<th>Id3−/− Apoe−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>314.2 ± 23.9</td>
<td>376.0 ± 34.5</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>35.63 ± 3.6</td>
<td>36.75 ± 1.4</td>
</tr>
<tr>
<td>Non HDL-C (mg/dL)</td>
<td>252.6 ± 13.9</td>
<td>303.4 ± 33.6</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>184.3 ± 31.7</td>
<td>215.1 ± 20.7</td>
</tr>
</tbody>
</table>

Values are the average ± SEM. No comparisons are statistically significant.
Figure III *ld3*+/−*Apoe*−/− and *ld3*+/+*Apoe*−/− mice have equal numbers of NH cells in the mesentery and adventitia/PVAT. Tissue was processed, stained with fluorochrome conjugated MAbs and analyzed by flow cytometry to detect NH cells in the (A) mesentery or (B) adventitia/PVAT.