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Role of 5-Lipoxygenase in IL-13-Induced Pulmonary Inflammation and Remodeling

Yun M. Shim, Zhou Zhu, Tao Zheng, Chen G. Lee, Robert J. Homer, Bing Ma, and Jack A. Elias

Exaggerated levels of IL-13 and leukotriene (LT) pathway activation frequently coexist at sites of Th2 inflammation and in tissue fibrotic responses. However, the relationship(s) between the IL-13 and LTs in these responses have not been defined. We hypothesized that the 5-lipoxygenase (5-LO) pathway of LT metabolism plays an important role in the pathogenesis of IL-13-induced chronic inflammation and remodeling. To test this hypothesis, we evaluated the effects of IL-13 on components of the 5-LO metabolic and activation pathways. We also compared the effects of transgenic IL-13 in C57BL/6 mice with wild-type and null 5-LO genetic loci. These studies demonstrate that IL-13 increases the levels of mRNA encoding cytosolic phospholipase A2, LTA4 hydrolyase, and 5-LO-activating protein without altering the expression of 5-LO, LTA4 synthase, LTRa receptors 1 and 2, and cysteinyl-LT receptors 1 and 2. They also demonstrate that this activation is associated with the enhanced accumulation of LTB4 but not of cysteinyl-LTs. Furthermore, they demonstrate that this stimulation plays a critical role in the pathogenesis of IL-13-induced inflammation, tissue fibrosis, and respiratory failure-induced death while inhibiting alveolar remodeling. Lastly, mechanistic insights are provided by demonstrating that IL-13-induced 5-LO activation is required for optimal stimulation and activation of TGF-β and the inhibition of matrix metalloproteinase-12. When viewed in combination, these studies demonstrate that 5-LO plays an important role in IL-13-induced inflammation and remodeling.

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2 Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; Tg, transgenic; LT, leukotriene; 5-LO, 5-lipoxygenase; cys-LT, cysteinyl-LT; BALF, BAL fluid; EIA, enzyme immunoassay; cPLA2, cytosolic phospholipase A2; LTA4H, LTA4 hydrolyase; FLAP, 5-LO-activating protein; MMP, matrix metalloproteinase.

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block 5-LO or cysteinyl-LTs have been given Food and Drug Administration approval as asthma therapeutics (33, 34). Surprisingly, although asthma is a chronic disease characterized by inflammation and tissue remodeling, the roles of 5-LO metabolites in the pathogenesis of the chronic tissue responses induced by Th2 cytokines such as IL-13 have not been fully investigated.

We hypothesized that LTs play important roles in the pathogenesis of the chronic inflammatory and remodeling effects of IL-13. To test this hypothesis, we characterized the 5-LO pathways in lungs from Tg mice in which IL-13 was overexpressed in a lung-specific fashion and compared the effects of Tg IL-13 in mice with wild-type (+/+ ) and null (−/−) 5-LO loci. These studies demonstrate that IL-13 activates 5-LO and is a potent and selective stimulator of enzymes involved in LT metabolism. They also demonstrate that the 5-LO-dependent pathways play critical roles in the pathogenesis of IL-13-induced chronic inflammation and remodeling in the murine lung.

Materials and Methods

**Tg mice**

In these studies, we used CC10-IL-13 Tg mice in which IL-13 is targeted to the lungs using the Clara cell 10-kDa protein (CC10) promoter. These mice were generated in our laboratory on a C57BL/6 genetic background and characterized as described previously (11). Unless otherwise stated, they were 3 mo of age at the time of their evaluation. In these animals Tg IL-13 causes a mononuclear cell- and eosinophil-rich tissue inflammatory response, subepithelial and parenchymal fibrosis, mucous metaplasia, and crystal deposition (11). In keeping with the chronic nature of the IL-13 production in these mice, the phenotype of these animals progressed relentlessly causing premature death from an inflammatory and fibrodestructive lung disorder (12).

**Null mutant mice**

5-LO null mice were generated by Funk and colleagues (35) on a 129 Sv background. They were bred (>10 generations) onto a C57BL/6 genetic background and obtained from The Jackson Laboratory (stock no. 004155). CC10-IL-13 mice with wild-type (+/+ ) and null (−/−) 5-LO loci were generated by breeding IL-13 Tg mice with 5-LO null (−/−) animals. Genotype was assessed as described previously (11, 35). In all experiments, we compared the phenotypes of Tg and Tg mice with (+/+ ) or (−/−) 5-LO loci. This study has been reviewed and approved by the Yale University School of Medicine Institutional Animal Care and Use Committee.

**Bronchoalveolar lavage (BAL)**

Lung inflammation was assessed by whole lung BAL as described previously (11, 12). The BAL samples from each animal were pooled and centrifuged. The number and types of cells in the cell pellet were determined as described previously (11). The supernatants were stored at −20°C until used for further analysis.

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**Lung volume and compliance assessment**

Lung volume and compliance were assessed as described previously (10, 36). In brief, animals were anesthetized, the trachea was cannulated, and the lungs were removed and inflated with PBS at 25-cm H2O pressure. The size of the lung was evaluated by volume displacement.

**Histologic evaluation**

H&E and Mallory’s Trichrome stains were performed in the Research Histology Laboratory of the Department of Pathology at Yale University School of Medicine as described previously (10–12, 36).

**Morphometric evaluation**

Alveolar size was estimated from the mean chord length of the airspace as previously described by our laboratory (10, 36). This measurement is similar to the mean linear intercept, a standard measure of air space size, but has the advantage that it is independent of alveolar septal thickness. Chord length increases with alveolar enlargement.

**mRNA analysis**

The amounts of mRNA were evaluated by real-time RT-PCR analysis. The primers that were used are described in Table I. β-Actin was used as an internal standard. Real-time RT-PCR is conducted with Bio-Rad iCycler Real-Time PCR Machine using Bio-Rad Sybergreen RT-PCR kits (catalog no. 170-8892). The amounts of the detected target gene products are normalized with that of β-actin before the statistical analysis.

**Quantification of LTB₄, cysteinyl-LTs, and PGE₂**

Lipid in the BAL fluid (BALF) was extracted by solid phase extraction with C18 solid phase extraction cartridge (3M Empore C18 cartridges). The BALF was applied to the C18 cartridge preconditioned by methanol first then HPLC grade water. The lipid was eluted with 100% methanol after washing with 5% methanol. Eluted lipid in methanol was dried with a speedvac concentrator under vacuum and reconstituted with enzyme immunoassay (EIA) buffer for EIA measurement. Amounts of the LTB₄, cysteinyl-LTs, and PGE₂ in BALF were evaluated using commercial EIA kits (Cayman Chemical) per the manufacturer’s instructions.

**Quantification of IL-13 and TGF-β₁**

BAL, IL-13, and TGF-β₁ levels were evaluated using commercial ELISA kits (R&D Systems) per the manufacturer’s instructions (37–39). The TGF-β₁ evaluations were done using BALFs that were treated with acid or control buffer to compare the levels of total and activated or total cytokine, respectively (37).

**Quantification of lung collagen**

Lung collagen content was determined by quantifying total soluble collagen using the Sircol Collagen Assay kit (Biocolor; stock no. S1000) per the manufacturer’s instructions and previously described by our laboratory (37, 38). In the evaluations, we compared only the right lungs from male littermate mice.

### Table I. RT-PCR primers

<table>
<thead>
<tr>
<th>Moiety Left Primer</th>
<th>Right Primer</th>
<th>Anneal Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPLA₂</td>
<td>GTTTGTCTCATGCCCCAGACTCT</td>
<td>ATCCCCCGACTCATACAGGC</td>
</tr>
<tr>
<td>5-LO</td>
<td>CACGGGACTACATCGAGTT</td>
<td>GTTCGTCGGAGGATGATGA</td>
</tr>
<tr>
<td>FLAP</td>
<td>GCAGCGATGAGTCCTCTCTTT</td>
<td>GGTTAGCTCTCTCTGTC</td>
</tr>
<tr>
<td>LTA₁H</td>
<td>AACCAGGAGTTCTCCCATACC</td>
<td>GCAGATTTCTCCACCCGCTCT</td>
</tr>
<tr>
<td>LTBR₁R²</td>
<td>TGTCCTCATGGTCCCCCTTCC</td>
<td>AAGAGACACACCTCACTGTC</td>
</tr>
<tr>
<td>LTBR₂R²</td>
<td>GCCTCAACCCAGGTTCTAT</td>
<td>TGCCCCATATTCTTCAGCTT</td>
</tr>
<tr>
<td>LTB₁S</td>
<td>GCTCTTCTGGCTACCTGTCAC</td>
<td>GGGACACGGAAAGATCTCG</td>
</tr>
<tr>
<td>Cys-LTR₁</td>
<td>GAGACACCGATCATTTTCCC</td>
<td>AATTTCCTTACGAAAACGAC</td>
</tr>
<tr>
<td>Cys-LTR₂</td>
<td>AAGTGTGACAGTGCTGGAT</td>
<td>AGACCGTGAGCCTGAGAAAT</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGCACATTGACGACACATCC</td>
<td>CTCTCAGGTGCTGGTGA</td>
</tr>
</tbody>
</table>

1. Leukotriene B₄ receptor 1.
2. Leukotriene B₄ receptor 2.
3. Leukotriene C₄ synthase.
4. Cysteinyl leukotriene receptor 1.
5. Cysteinyl leukotriene receptor 2.
6. Cysteinyl leukotriene receptor 2.
7. Cysteinyl leukotriene receptor 2.
Results

Effect of IL-13 on LT biosynthetic and effector pathways

To begin to address the role(s) of 5-LO metabolites in the pathogenesis of the tissue effects of IL-13, studies were first undertaken to determine whether IL-13 regulated key enzymes, receptors, and end products in LT biosynthetic pathways. There were low levels of mRNA encoding many of the enzymes and receptors in these pathways in lungs from transgene Tg− mice. In addition, as shown in Fig. 1, IL-13 caused a significant increase in the amounts of mRNA encoding cytosolic phospholipase A2 (cPLA2) (Fig. 1A) and lesser increases in LTA4 hydrolase (LTA4H) and 5-LO-activating protein (FLAP) (Fig. 1B). These effects were at least partially pathway specific, because IL-13 did not increase the levels of mRNA encoding 5-LO, LTC4 synthase, LTB4 receptors 1 and 2, and cys-LT receptor 1 and 2 (Fig. 1B). They were, however, associated with significantly increased levels of LTB4 but not of cys-LTs (Fig. 2). Thus, IL-13 is a potent and selective stimulator of enzymes involved in LT metabolism and LTB4 production.

Role of 5-LO in IL-13-induced inflammation

To determine whether 5-LO pathway products contributed to the pathogenesis of IL-13-induced inflammation, we compared the responses in Tg− and Tg+ mice with (+/+ or −/−) 5-LO loci. As previously reported (11, 12), Tg IL-13 caused significant increases in BAL total cell, macrophage, lymphocyte, and eosinophil recovery and a macrophage-, eosinophil-, and lymphocyte-rich tissue inflammatory response (Fig. 3, A–C, and data not shown). In the absence of 5-LO, BAL and tissue cellularity were not significantly altered in lungs from Tg− mice (Fig. 3, A–C). In contrast, 5-LO ablation significantly decreased BAL total cell and eosinophil recovery and the tissue inflammation in lungs from Tg− IL-13 animals (Fig. 3, A–C). These studies demonstrate that 5-LO and its LT metabolites are critical regulators of the intensity and nature of IL-13-induced pulmonary inflammation.

Role of 5-LO in IL-13-induced alveolar remodeling and destruction

To define the role of 5-LO in the pathogenesis of IL-13-induced alveolar remodeling, we compared the alterations in lung volume, lung compliance, and alveolar size in Tg− mice with (+/+ and −/−) 5-LO loci. In accord with previous observations (10), IL-13 caused an impressive increase in all of these parameters (Fig. 4, A and B). A null mutation of 5-LO did not alter these parameters in lungs from Tg− mice (Fig. 4, A and B). In contrast, lungs from Tg− mice with (+/+ 5-LO loci) were significantly smaller and less compliant than lungs from Tg− mice with (−/−) loci (Fig. 4A). In accord with these findings, the size of the alveoli in lungs from Tg− mice with (+/+ 5-LO loci) was similarly decreased when compared with the lungs from Tg+ mice with (−/−) loci when assessed with light microscopic (Fig. 3C) and morphometric approaches (Fig. 4B).

Role of 5-LO in matrix metalloproteinase (MMP) production

To further characterize the contribution of 5-LO to the mechanisms of alveolar remodeling, the levels of mRNAs encoding MMP-9 and MMP-12 were measured by real-time RT-PCR. As previously described by our laboratory, the levels of MMP-9 and 12 mRNA were increased in lungs from Tg− IL-13 mice (10, 36). A null mutation of 5-LO did not alter the levels of mRNA encoding MMP-9 and MMP-12 in lungs from Tg− mice (Fig. 5 and data not shown). However, a significant increase in the levels of mRNA encoding MMP-12 was detected in Tg− mice with (−/−) 5-LO loci compared with Tg− mice with (+/+ 5-LO loci) (Fig. 5). This effect was at least partially MMP-12-specific because MMP-9 was not similarly altered (data not shown). When viewed in combination, these studies demonstrate that 5-LO is an important inhibitor of IL-13-induced alveolar remodeling and MMP-12 expression in the murine lung.

Role of 5-LO in IL-13-induced fibrosis

We previously demonstrated that IL-13 is a potent stimulus of tissue fibrosis (37). To determine whether 5-LO metabolites played a significant role in this response, we used both histologic (Trichrome stains) and quantitative biochemical (Sircol) techniques to compare the tissue fibrosis in Tg− and Tg+ mice with (+/+ and −/−) 5-LO loci. Similar amounts of collagen were...
noted in the lungs from wild-type littermate control mice and 5-LO null mice (Fig. 6, A and B). As previously reported (11), Tg IL-13 was a potent stimulator of fibrosis in Tg +/+ mice with (+/+) 5-LO loci (Fig. 6, A and B). In contrast, in the absence of 5-LO, this response was significantly ameliorated. This difference was readily apparent with both the histologic and biochemical assessments (Fig. 6, A and B). Thus, 5-LO, plays a crucial role(s) in IL-13-induced pulmonary fibrosis.

Effect of the 5-LO deficiency on TGF-β1 production and activation

Because we previously demonstrated that IL-13 induces tissue fibrosis by inducing and activating TGF-β1 (37), studies were undertaken to determine whether 5-LO metabolites contributed to the genesis of this response. This was done by comparing the amounts of total and activated TGF-β1 in BAL from Tg +/+ and Tg −/− 5-LO loci. The amounts of total and activated TGF-β1 in lungs from Tg −/− mice were near or below the limits of detection of our assays regardless of their 5-LO genotype (Fig. 7, A and B). In contrast, significant amounts of total and activated TGF-β1 were seen in BAL from Tg +/+ mice (Fig. 7, A and B). 5-LO played an important role in this response because the levels of total and activated TGF-β1 were much higher in Tg +/+ mice with (+/+) compared with Tg −/− 5-LO loci (Fig. 7, A and B). When viewed in combination, these studies demonstrate that 5-LO plays a critical role in IL-13-induced stimulation and activation of TGF-β1.
Role of 5-LO in IL-13 elaboration

A deficiency of 5-LO could modify IL-13-induced tissue responses by altering the production of Tg IL-13 or modulating IL-13 effector responses. To determine whether alterations in 5-LO regulated the production of IL-13, we compared the levels of this cytokine in BAL from Tg$^{+/+}$ and Tg$^{-/-}$ mice with (+/+) and (−/−) 5-LO loci. IL-13 was not readily apparent in BALFs from Tg$^{-/-}$ mice (Fig. 8). In contrast, significant levels of BAL IL-13 were appreciated in Tg$^{+/+}$ mice. Similar levels of BAL IL-13 were seen in BALFs from Tg$^{-/-}$ mice with (+/+) and (−/−) 5-LO loci (Fig. 8). Thus, a null mutation of 5-LO altered IL-13-induced tissue responses by modifying IL-13 effector pathway activation but not by modifying Tg IL-13 production.

Effects of IL-13 on PGE$_2$

In the absence of 5-LO and the presence of enhanced cPLA$_2$ activity, one can envision a scenario in which an increased amount of substrate is available for PG production. Because PGs like PGE$_2$ can have anti-inflammatory and antifibrotic effects, studies were undertaken to evaluate the levels of PGE$_2$ in BAL from Tg$^{+/+}$ and Tg$^{-/-}$ mice with wild-type and null 5-LO loci. As shown in Fig. 9, PGE$_2$ was readily detected in fluids from Tg$^{+/+}$ mice, and increased levels of PGE$_2$ were noted in fluids from Tg$^{-/-}$ animals. Interestingly, the levels of PGE$_2$ were not altered in Tg$^{-/-}$ or Tg$^{+/+}$ mice in the absence of 5-LO (Fig. 9). These studies demonstrate that increased levels of PGE$_2$ are seen in lungs from IL-13 Tg$^{-/-}$ mice. They also demonstrate that these levels are not significantly altered in the absence of 5-LO and therefore do not contribute, in a major way, to the alterations in the tissue effects of IL-13 that are seen in this setting.

Role of 5-LO in IL-13-induced mortality

We demonstrated previously that the chronic overexpression of IL-13 in the lung causes a fibrodestructive pulmonary response that leads to the respiratory failure and premature death (12, 37). To define the role(s) of 5-LO-dependent pathways in this fatal event, we compared the survival of Tg$^{+/+}$ mice with (+/+) and (−/−) 5-LO loci. The Tg$^{-/-}$ mice with (+/+) 5-LO loci started to die at 97 days of age, and 100% of these animals were dead by day 166. In contrast, a deficiency of 5-LO significantly extended the survival of these Tg$^{-/-}$ animals. In Tg$^{+/+}$ mice with (−/−) 5-LO loci, one animal died at 191 days, and 85% of these animals were still alive on day 334 (Fig. 10). This demonstrates that 5-LO plays a critical role(s) in the pathogenesis of the IL-13-induced pathologies that lead to the death of these animals.

Discussion

Exaggerated levels of IL-13 and LT frequently coexist and are felt to contribute to the pathogenesis of a variety of diseases including bronchial asthma. LT regulators are also being used with increasing frequency to treat asthma and related disorders (33, 34, 40). Thus, studies were undertaken to define the roles that LT plays in the pathogenesis of IL-13-induced tissue responses at these sites of pathology. These studies demonstrate that IL-13 is a potent stimulator of the 5-LO pathway increasing the expression of cPLA$_2$, FLAP, and LTA$_4$H and LTB$_4$ accumulation. They also demonstrate that this activation plays an important role in the pathogenesis of selected IL-13-induced tissue responses by demonstrating that, in the absence of 5-LO, IL-13-induced inflammation, tissue fibrosis, and mortality are decreased. Lastly, mechanistic insights were also provided by demonstrating that IL-13 stimulates and activates TGF-$eta_1$ via a pathway(s) that is at least partially 5-LO-dependent.

Because inflammation and fibrosis are readily appreciated at sites of injury and repair, the relationship(s) between these responses has been intensively investigated. The type II cytokine...
hypothesis suggests that these responses are linked in a cause and effect fashion and that fibrosis occurs when inflammation shifts in a Th2 direction (41). IL-13 is felt to be the major Th2 cell-derived fibrogenic effector in these responses (8, 42–44). This can be readily appreciated in recent studies that highlighted the importance of IL-13 and the impressive induction of genes that contribute to healing and scarring in type II but not in type I granulomatous responses (43, 45). In keeping with the appreciation that IL-13 is dysregulated (20–22, 46) and that 5-LO is activated to produce exaggerated levels of LTs at sites of inflammation and fibrosis (47–49), we investigated the importance of 5-LO in the pathogenesis of the tissue effects of IL-13. These studies demonstrate that 5-LO makes an important contribution to the chronic inflammatory and fibrogenic effects of IL-13. These findings are in accord with, and provide, a mechanistic explanation for prior studies that demonstrated that LT metabolites make a critical contribution to bleomycin-induced and chronic Ag-induced pulmonary inflammatory and fibrotic responses (48, 50–52).

Previous studies from our laboratory demonstrated that IL-13 induces pulmonary fibrosis, at least in part, via its ability to induce and activate TGF-β1 (37). Because our studies demonstrated that 5-LO metabolites play an important role in the pathogenesis of IL-13-induced tissue fibrosis, studies were also undertaken to determine whether 5-LO contributed to TGF-β1 induction and activation in this setting. These studies demonstrate that IL-13 stimulates and activates TGF-β via a mechanism that is, at least in part, 5-LO-dependent. These findings are in accord with and expand on those of Perg et al. (53), who demonstrated that LTC4 has the ability to stimulate epithelial cell TGF-β1. It is important to point out that TGF-β1 also up-regulates the expression of LT receptors such as cys-LT1 (29) and primes a variety of cells for enhanced LT production (54). When viewed in combination, one can envision a positive feedback loop with IL-13-activating 5-LO and stimulating LT production, which, in turn, contributes to the production and activation of TGF-β1. The TGF-β1 would then heighten LT responses and LT production and feedback to further amplify this response. It is tempting to speculate that a positive feedback loop of this sort could contribute to the chronic, life-long nature of asthma and the progressive nature of the inflammatory and remodeling responses that are seen in a wide variety of Th2 disorders. Additional experimentation, however, will be needed to test this hypothesis.

Enhanced alveolar size, enhanced pulmonary compliance, and the augmented production of a variety of proteases including MMPs and cathepsins have been described in lungs from IL-13-overexpressing Tg mice (10). The exact significance of these findings has not been formally established. However, these findings and studies highlighting the enhanced production of IL-13 in cigarette smoke-exposed mice (16) and the association of polymorphisms of IL-13 and emphysema (55) has lead to the speculation that IL-13 may play a role in the pathogenesis of COPD. In addition, Gelb et al. (56–58) have recently demonstrated that there is an unexpected loss of elastic recoil in patients with chronic persistent asthma that is similar in many ways to the findings in our IL-13 modeling system. In the present studies we investigated the relationships between IL-13, the 5-LO pathway, and these protease and tissue responses. When we initiated these studies, we expected 5-LO activation would play a key role in the generation of these alterations. In contrast to our expectations, we noted that alveolar size, lung compliance and MMP-12 levels were increased in Tg mice that lacked 5-LO. Interestingly, MMP-9 was not similarly altered. These studies highlight a previously unappreciated inhibitory effect of 5-LO metabolites on alveolar remodeling and MMP production. At present it is not clear whether this effect is mediated by the LTB4, which is produced in an exaggerated manner in our modeling system and in lung tissues from patients with COPD (59, 60). This is, however, a tempting speculation because LTB4 has known profibrotic properties, and the magnitude of alveolar destruction after injury is inversely proportional to one’s ability to generate a fibrotic response and heal. Overall, these observations suggest that interventions that block 5-LO could enhance IL-13-induced alveolar remodeling. The clinical significance of this finding, however, is open to question because the overall effects of 5-LO deficiency were beneficial with IL-13 Tg mice with null 5-LO loci living longer than mice with normal 5-LO loci.

In summary, our studies demonstrate that IL-13 stimulates cPLA2, FLAP, LTA4H, and LTB4. They also demonstrate that this activation plays a critical role in the pathogenesis of IL-13-induced inflammation, tissue fibrosis, and respiratory failure while inhibiting alveolar remodeling. Lastly, they provide mechanistic insights by demonstrating that IL-13-induced 5-LO activation is required for optimal stimulation and activation of TGF-β1, while inhibiting MMP-12. Exaggerated IL-13 production has been implicated in the pathogenesis of a wide variety of disorders including asthma, COPD, pulmonary fibrosis, scleroderma, hepatic fibrosis, nodular sclerosing, and Hodgkin’s disease (16–23). The present studies suggest that some of the effects of IL-13 in these disorders may be controlled via interventions that regulate 5-LO activation. They also highlight areas that will need to be observed closely to evaluate the effects of these interventions on alveolar structure. This establishes the IL-13–5-LO pathway as a worthwhile site for future investigations designed to evaluate the therapeutic utility of interventions in this axis in the treatment of IL-13-mediated disorders.

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Disclosures
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References


