IL-31-IL-31R Interactions Limit the Magnitude of Th2 Cytokine-Dependent Immunity and Inflammation following Intestinal Helminth Infection

Jacqueline G. Perrigoue, Colby Zaph, Katherine Guild, Yurong Du and David Artis

*J Immunol* 2009; 182:6088-6094; doi: 10.4049/jimmunol.0802459

http://www.jimmunol.org/content/182/10/6088

References

This article cites 39 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/182/10/6088.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The only known functional ligand for IL-31R signaling receptor for a number of ligands (3). However, as yet gp130, a promiscuous type 1 receptor chain that serves as the approach the most recent addition to this family, the silico identification of novel family members. Using this approach including the signature WSXWS motif, have facilitated the in general conserved structural domains of type 1 cytokine receptors, has highlighted the critical roles that members of this cytokine family play in the regulation of immunity and inflammation and underscore the potential for manipulating these signaling pathways for therapeutic benefit in a wide range of inflammatory diseases (7–9).

In vitro studies showed that signaling downstream of the IL-31R results in the phosphorylation of STAT1, STAT3, and STAT5 as well as activation of the p38 MAPK, ERK1,2, and JNK1,2 signaling pathways (2, 5, 6, 10, 11). In epithelial cells IL-31 treatment has been linked to increased production of a broad range of cytokines and chemokines including IL-6, IL-8, EGF, VEGF, MCP-1, and GRO1. and can result in either the dose and target cell type (6, 10, 11).

Although in vitro assays have begun to define the signaling pathways involved downstream of IL-31-IL-31R interactions, relatively little is known about the functional consequences of these interactions in vivo. Transgenic mice that express IL-31 from a ubiquitous or T cell-specific promoter develop spontaneous skin inflammation characterized by thickening of the epidermis, alopecia, and pruritis (6). That study, combined with subsequent analyses in humans correlating

Jacqueline G. Perrigoue,* Colby Zaph,† Katherine Guild,* Yurong Du,* and David Artis2*  

IL-31 is a recently identified cytokine made predominantly by CD4+ Th2 cells and its receptor, IL-31R, is expressed by a number of cell types including monocytes, epithelial cells, and T cells. Originally identified as a potential mediator of inflammation in the skin, we recently reported a novel function for endogenous IL-31R interactions in limiting type 2 inflammation in the lung. However, whether IL-31-IL-31R interactions regulate immunity or inflammation at other mucosal sites, such as the gut, is unknown. In this study, we report a regulatory role for IL-31-IL-31R interactions in the intestine following infection with the gastrointestinal helminth Trichuris muris, immunity to which is critically dependent on CD4+ Th2 cells that produce IL-4 and IL-13. IL-31Rα was constitutively expressed in the colon and exposure to Trichuris induced the expression of IL-31 in CD4+ T cells. In response to Trichuris infection, IL-31Rα−/− mice exhibited increased Th2 cytokine responses in the mesenteric lymph nodes and elevated serum IgE and IgG1 levels compared with wild type mice. IL-31Rα−/− mice also displayed enhanced goblet cell hyperplasia and a marked increase in secretion of goblet cell-derived resistin-like molecule β into the intestinal lumen. Consistent with their exacerbated type 2 inflammatory responses, IL-31Rα−/− mice exhibited accelerated expulsion of Trichuris with significantly decreased worm burdens compared with their wild type counterparts early following infection. Collectively, these data provide the first evidence of a function for IL-31-IL-31R interactions in limiting the magnitude of type 2 inflammatory responses within the intestine. The Journal of Immunology, 2009, 182: 6088–6094.  

Received for publication July 25, 2008. Accepted for publication March 14, 2009.  

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  

1 Research in the Artis laboratory is supported by the National Institutes of Health (NIH), A165170, AI 074878 (to D.A.), NIH T32 Training Grant AI 007532-08 (to J.P.), the Burroughs Wellcome Fund (Investigator in Pathogenesis of Infectious Disease Award to D.A.), the Pilot Feasibility Program of the National Institute of Diabetes and Digestive Kidney Diseases (NIDDK) DK50306, the Crohns and Colitis Foundation of America’s William and Shelby Modell Family Foundation Research Award, and pilot grants from the University of Pennsylvania (University Research Foundation Award, PGI Pilot Grant, and UPenn Center for Infectious Diseases Pilot Grant).  

2 Address correspondence and reprint requests to Dr. David Artis, Room 314 Hill Pavilion, University of Pennsylvania, 380 South University Avenue, Philadelphia PA 19104. E-mail address: dartis@vet.upenn.edu  

3 Abbreviations used in this paper: OSM, oncostatin M; mLN, mesenteric lymph node; RELM, resistin-like molecule; WT, wild type.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00
IL-31 expression and dermatitis, suggested a role for IL-31-IL-31R interactions in regulating inflammation in the skin (6, 12–16). We recently generated mice in which endogenous IL-31R signaling was deleted (IL-31R<sup>−/−</sup> mice) (17). Although hematopoetic cell development was normal, IL-31R<sup>−/−</sup> mice exhibited elevated expression of Th2 cytokines and developed exacerbated airway inflammation following exposure to *Schistosoma mansoni* eggs, indicating a negative regulatory role for endogenous IL-31-IL-31R interactions in limiting type 2 inflammation in the lung (17). These findings supported an important immunoregulatory role for IL-31-IL-31R interactions at mucosal sites, although differences in models of inflammation in the skin vs lung suggest the functional consequences of receptor ligation may be tissue-specific. To test this hypothesis further, we sought to investigate the potential functions of IL-31R in immune regulation in the gastrointestinal tract following exposure to the intestinal helminth *Trichuris muris*. Infection of genetically resistant BALB/c or C57BL/6 mice with *Trichuris* provokes the development of parasite-specific CD4<sup>+</sup> Th2 cells and infection-dependent production of IL-4, IL-5, IL-13, and IL-25 while progression to chronic *Trichuris* infection in susceptible mouse strains is associated with CD4<sup>+</sup> Th1 cells that produce IFN-γ (18). In this report, we demonstrate that IL-31 mRNA expression is induced in CD4<sup>+</sup> T cells following *Trichuris* infection and present the first evidence of a functional role for IL-31-IL-31R interactions in regulating Th2 cytokine-dependent immunity and inflammation in the intestine.

**Materials and Methods**

**Mice and parasites**

Wild-type (WT) C57BL/6 mice were ordered from Jackson ImmunoResearch Laboratories. IL-31R<sup>−/−</sup> mice were generated as described previously (17). Mice were bred in a specific pathogen-free environment at the University of Pennsylvania. All experiments were performed following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. *Trichuris* was maintained in genetically susceptible animals. Isolation of *Trichuris* eggs was performed as described previously (19). Mice were infected orally with 200 embryonated eggs and parasite burdens in the ceca determined microscopically at various days after infection.

**Isolation of cells**

CD4<sup>+</sup> T cells were isolated from mesenteric lymph nodes (mLN) by negative selection via incubation with hybridoma supernatants (αB220, αF4/80, αCD8, αMHCII) followed by magnetic bead purification (Qiagen) and stimulated with plate-bound anti-CD3/anti-CD28 (1 μg/ml eBioscience) for 24 h in complete tissue culture medium (DMEM: Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-ME. Splenocytes were isolated from WT and IL-31R<sup>−/−</sup> mice by homogenization between glass slides followed by RBC lysis. Cells were directly stained with anti-CD23 and anti-CD21/35 and analyzed by flow cytometry for phenotypic analysis. CD23<sup>+</sup> B cells were isolated from splenocytes from WT or IL-31R<sup>−/−</sup> mice by positive selection as previously described (20).

**Western blot and immunofluorescence**

Western blot analysis was performed as described previously (22). In brief, 30 μg of protein was isolated from pooled fecal pellets collected at various time points postinfection and resolved by SDS-PAGE followed by immunoblotting for resistin-like molecule (RELM) β with a polyclonal rabbit RELM β Ab (PeproTech). For immunofluorescence staining and histological analysis, paraffin-embedded 4% paraformaldehyde-fixed cecal tissue sections were stained for RELM β or Gob5 as described previously (22).

**Statistics**

Statistical analysis was performed using a two-tailed Student’s *t* test (*p* < 0.05).

**Results**

IL-31 expression is induced following *Trichuris* infection

We recently identified a role for IL-31-IL-31R interactions in regulating the severity of Th2 cytokine-mediated inflammation in the lung (17). However, the functional significance of IL-31-IL-31R interactions in regulating immunity and inflammation in the intestine is currently unknown. We first examined the steady-state gene expression of IL-31 and IL-31R in the gastrointestinal microenvironment by real-time quantitative PCR analysis of whole sections of large intestine from naive WT (C57BL/6) mice. Although IL-31 expression was undetectable in tissue isolated from naive mice (data not shown), IL-31R<sup>+</sup> and OSMR<sup>+</sup> were constitutively expressed in the colon (Fig. 1A). Previous reports have demonstrated that in vitro differentiation of naive CD4<sup>+</sup> T cells under Th2-permissive conditions induces expression of IL-31 mRNA (6). To determine whether IL-31 is expressed in vivo following exposure to *Trichuris*, CD4<sup>+</sup> T cells were isolated from the mLN of naive and day 18 WT *Trichuris*-infected mice and pulsed with plate-bound anti-CD3/anti-CD28 for 24 h. Cells were harvested and mRNA expression of IL-4, IL-13, and IL-31 relative to β-actin was determined by real-time quantitative PCR. There was a >100-fold increase in expression of the Th2 cytokines IL-4 and IL-13 in CD4<sup>+</sup> T cells isolated from *Trichuris*-infected mice over that of naive mice. CD4<sup>+</sup> T cells isolated from infected WT mice also exhibited a 10-fold increase in IL-31 expression (Fig. 1B). These data demonstrate that in WT mice IL-31R is expressed constitutively in the colon and that IL-31 mRNA expression is induced in CD4<sup>+</sup> T cells from the mLN following exposure to *Trichuris*.
IL-31Ra−/− mice exhibit enhanced Th2 cytokine responses following Trichuris infection

The overexpression of IL-31 in a transgenic murine model was associated with skin inflammation with characteristics similar to human atopic dermatitis, suggesting a proinflammatory role for IL-31R signaling (6, 12–16). In contrast, we showed that mice lacking IL-31Ra develop exacerbated type 2 inflammation following exposure to S. mansoni eggs in the lung indicating that IL-31Ra signaling may function as a negative regulator of type 2 inflammation at mucosal sites (17). This apparent difference in the biological function of IL-31R signaling in the skin vs the airway led to the hypothesis that IL-31-IL-31R interactions may regulate Th2 cytokine-dependent inflammation differentially depending on the tissue site. To test the role of IL-31-IL-31R interactions in influencing Th2 cytokine production in the intestine, WT and IL-31Ra−/− mice were infected with Trichuris. At day 18 postinfection, mLN cells were isolated and stimulated with anti-CD3 and anti-CD28 for 48 h to assess cytokine production. Consistent with previous studies, mLN cells isolated from infected WT mice produced detectable IL-4, IL-5, and IFN-γ following polyclonal stimulation (Fig. 2) (19, 21, 23, 24). However, mLN cells isolated from infected IL-31Ra−/− mice secreted significantly higher levels of IL-4, elevated levels of IL-5, and significantly lower amounts of IFN-γ compared with that observed in infected WT mLN cells (Fig. 2). These data are consistent with the increase in Th2 cytokines observed in the draining lymph node of the lung in IL-31Ra−/− mice following S. mansoni egg injection and support a role for IL-31-IL-31R interactions in limiting intestinal Th2 cytokine production following Trichuris infection.

FIGURE 2. IL-31Ra−/− mice exhibit enhanced Th2 cytokine responses in the mLN following infection with Trichuris. WT and IL-31Ra−/− mice were infected with 200 Trichuris eggs orally and sacrificed on day 18 postinfection. mLNs from naive or infected mice were harvested and cultured in vitro in the presence of 1 μg/ml anti-CD3/anti-CD28 for 48 h. Supernatants were assayed by ELISA for IL-4, IL-5, and IFN-γ. WT mice; ■, IL-31Ra−/− mice. Results are representative of three independent experiments with three to four mice per group. *, p < 0.05.

IgG1 and IgE Ab responses are enhanced in IL-31Ra−/− mice

The generation of a protective Th2 cytokine response to Trichuris results in IL-4-dependent production of IgE and IgG1 Abs (21). To further assess the magnitude of the Th2 cytokine-dependent responses in both WT and IL-31Ra−/− mice, serum Ig levels were determined by ELISA. At day 18 postinfection, the amount of serum IgE from WT mice increased ~2-fold over that of naive WT mice. In contrast, serum IgE from infected IL-31Ra−/− mice increased 4-fold over naive IL-31Ra−/− mice and almost 10-fold over naive WT mice (Fig. 3A). In addition to increased total IgE production, levels of Ag-specific IgG1 Abs were significantly increased in the serum of day 18 infected IL-31Ra−/− mice compared with infected WT mice (Fig. 3B). These data correlate with the observed increases in Th2 cytokine production in the mLN cells from infected IL-31Ra−/− mice (Fig. 2) and are consistent with the exaggerated Th2 cytokine responses in IL-31Ra−/− mice in acute airway inflammation (17).

Equivalent frequencies, survival, and proliferation of B cells isolated from WT and IL-31Ra−/− mice

Previous studies have suggested that IL-31R signaling may mediate its effects via both T cell- and macrophage-intrinsic mechanisms (17) but the potential effects of IL-31R signaling on B cells have not been directly investigated. Although B cells and Abs do not appear to play a critical role in clearance of Trichuris (18), they can influence the development and maintenance of Th2 cytokine responses in vivo (25–27). Additionally, the significant increases in IgE and Trichuris-specific IgG1 Abs (Fig. 3) suggest that IL-31-IL-31R interactions could have a direct effect on B cell function.
following infection. Supporting this hypothesis, overexpression of IL-31 resulted in aberrant B to T cell ratios in the peripheral lymph nodes of IL-31 transgenic mice. However no analyses of direct effects of IL-31-IL-31R signaling on B cells were performed (6).

To first determine whether B cells may be responsive to IL-31, RNA was isolated from naive and CpG-stimulated CD23+/H11001 B cells and IL-31R expression analyzed by real-time quantitative PCR. Expression of IL-31R in naive B cells was similar to that observed in naive CD4+ T cells while IL-31 expression was undetectable (data not shown). To determine whether there were inherent differences in the numbers or percentages of B cell subsets between WT and IL-31R+/H9251 mice, splenocytes were isolated and stained with Abs against B cell surface markers CD21/35 (complement component receptors) and CD23 (low affinity IgE receptor) and analyzed by flow cytometry. No differences were observed in numbers (data not shown) or percentages of marginal zone (CD21/35−CD23+/H11001) or follicular (CD21/35−CD23+/H11002) B cells between WT and IL-31R+/− mice (Fig. 4A), indicating that IL-31R signaling does not influence the composition of the mature B cell compartment. To determine whether peripheral B cell responses were influenced by IL-31R signaling, purified WT and IL-31R+/− splenic B cells were treated in vitro with the TLR9 agonist CpG, a B cell mitogen, in the presence or absence of rIL-31. After four days in culture, B cells were harvested, stained with TOPRO, and analyzed by flow cytometry (B). Results are representative of two independent experiments.

Goblet cell-associated gene expression is enhanced in Trichuris-infected IL-31R+/− mice compared with WT mice
Trichuris infection results in alterations in Th2 cytokine-mediated changes in intestinal epithelial cell proliferation and differentiation including increased turnover and goblet cell differentiation that

**FIGURE 4.** WT and IL-31R+/− B cells exhibit equivalent proliferation and survival in vitro. Splenocytes were isolated from WT and IL-31R+/− mice and stained with Abs against CD21 and CD23 to determine percentages of follicular (FO) and marginal zone (MZ) B cells (A). Purified CD23+ B cells were CFSE-labeled, plated onto a 96-well plate, and stimulated with CpG (0.1 μM) in the presence or absence of IL-31 (50 ng/ml) as indicated. After four days in culture, B cells were harvested, stained with TOPRO, and analyzed by flow cytometry (B). Results are representative of two independent experiments.

**FIGURE 5.** Enhanced expression of goblet cell markers in Trichuris-infected IL-31R+/− mice. Cecal sections from naive and day 18 infected WT and IL-31R+/− mice were stained with Periodic Schiff’s/Alcian blue for mucins (A). Quantification of the number of goblet cells per crypt in day 18 infected WT and IL-31R+/− mice (B). Cecal sections from naive and day 18 infected WT and IL-31R+/− mice were stained with Abs against goblet cell proteins RELMβ (C) or Gob5 (D) (shown in green; DAPI nuclear stain in blue). Protein was extracted from pooled fecal pellets obtained at days 0, 14, 16, and 21 postinfection and 30 μg analyzed by Western blot to assess lumenal secretion of RELMβ (E). Results are representative of three independent experiments with three to four mice per group.
contribute to worm expulsion (28–31). To determine whether loss of IL31R signaling influenced type 2 inflammation in the intestine, WT and IL-31Ra−/− mice were infected with Trichuris and cecal sections stained with periodic Schiff’s Alcian blue to assay goblet cell hyperplasia and mucin production. Although both WT and IL-31Ra−/− mice exhibited infection-induced increases in goblet cell numbers and mucin production (Fig. 5A), goblet cell hyperplasia was markedly enhanced in IL-31Ra−/− mice with significantly increased numbers of goblet cells per cecal crypt (Fig. 5B). Microarray analysis comparing ceca from naive and infected mice identified the goblet cell-specific resistin-like molecule, RELMβ, as a highly differentially expressed gene in the intestine following Trichuris infection (22). Immunofluorescence staining of cecal sections from infected WT and IL-31Ra−/− mice at day 18 postinfection indicated the dominant cellular source of RELMβ in both WT and IL-31Ra−/− mice was goblet cells (Fig. 5C). Expression of Gob5 (mCLCA3), another goblet cell-specific gene regulated by Th2 cytokines and associated with type 2 inflammation, was also enhanced in infected IL-31Ra−/− mice compared with infected WT mice (Fig. 5D). Maximal RELMβ secretion is correlated with worm expulsion and because its expression is STAT-6-responsive, the measurement of secreted RELMβ in fecal pellets can be an indicator of the kinetics of the Th2 cytokine response in infected mice. WT and IL-31Ra−/− mice were infected with Trichuris and secreted RELMβ measured by Western blot. As expected, RELMβ secretion was minimal in naive WT mice, induced by day 14, and was maximal between days 14 and 16 postinfection (Fig. 5E). In contrast, naive IL-31Ra−/− mice constitutively secreted more RELMβ than their WT counterparts and following infection the magnitude of RELMβ secretion was substantially enhanced (Fig. 5E). Previous reports have identified direct effects of IL-31R signaling on epithelial cells including changes in proliferation and expression of various chemokines and cytokines (6, 10, 11). The changes in goblet cell proliferation following Trichuris infection could be a direct effect of IL-31-IL-31R signaling on intestinal goblet cell responses or an indirect consequence of elevated expression of Th2 cytokines. Notwithstanding that, these data show that in the absence of IL-31R signaling there is enhanced intestinal type 2 inflammation associated with significantly increased goblet cell hyperplasia and increased expression of goblet cell-associated genes RELMβ and Gob5.

**Accelerated worm expulsion in IL-31Ra−/− mice**

Expulsion of Trichuris and development of sterile immunity require the generation of polarized Th2 cytokine responses whereby the cytokines IL-4, IL-13, and IL-25 elicit the effector mechanisms that mediate worm expulsion (18, 23). Given that IL-31Ra−/− mice exhibited enhanced Th2 cytokine responses following Trichuris infection, we sought to determine whether IL-31Ra−/− mice would more efficiently clear infection than their WT counterparts. To test this, WT and IL-31Ra−/− mice were infected with 200 embryonated Trichuris eggs and sacrificed at days 12, 21, and 34 postinfection. There was no significant difference in worm establishment at day 12 (WT 163±58; IL-31Ra−/− 125±32). Expulsion of Trichuris in WT C57BL/6 mice occurs between days 18–21 postinfection. As expected, at day 21 postinfection WT mice had begun to expel Trichuris with an average cecal worm burden below 40 (Fig. 6). In contrast, IL-31Ra−/− mice exhibited a nearly 10-fold decrease in worm burden compared with their WT counterparts at the same time point (Fig. 6). Collectively, these data demonstrate that IL-31R interactions critically regulate the parasite-induced Th2 cytokine responses that influence intestinal immunity.

**Discussion**

We recently identified a negative regulatory role for IL-31-IL-31R interactions in the lung following S. mansoni egg-induced inflammation. In that study, deletion of IL-31Ra resulted in exaggerated Th2 cytokine responses and increased granuloma size following i.v. injection of S. mansoni eggs (17). Additionally, loss of IL-31R signaling resulted in enhanced Ag presentation by macrophages and increased Th2 cytokine expression by CD4+ T cells (17). This was in contrast to previous reports linking overexpression of IL-31 to atopic dermatitis and presumably a role in promoting Th2 cytokine-induced inflammation (6). Here we show that in the absence of IL-31R expression the magnitude of helminth-induced type 2 immune responses is significantly increased as illustrated by heightened Th2 cytokine production, elevated IgE and IgG1 Abs, and enhanced goblet cell responses in IL-31Ra−/− mice, culminating in accelerated worm expulsion. These data provide the first evidence of a role for IL-31-IL-31R interactions in regulating immunity and inflammation in the intestine during live parasitic infection and suggest that IL-31R has a conserved function in both the lung and the intestine, namely, negatively regulating the magnitude of Th2 cytokine responses.

Previous studies have proposed a proinflammatory role for IL-31 in the skin as IL-31 transgenic mice develop dermatitis (6). Although there was no elevation in IgE (typical of atopic dermatitis) and there was no assessment of Th2 cytokines, these studies suggested that IL-31 could promote type 2 inflammation (6). There are a number of explanations for the apparent differences in the putative positive effects of IL-31 in the skin vs the lung and intestine where IL-31 has a negative effect on Th2 cytokine production. One of the key differences between our studies and the work with IL-31 transgenic mice is the deletion of the endogenous receptor vs overexpression of the ligand. The expression of IL-31 at levels that are superphysiological could explain these disparate outcomes. Additionally, as with other members of the type 1 cytokine receptor family, deciphering the functional biology of IL-31 and IL-31Ra may be complicated by promiscuous cytokine and receptor chain usage. For example, the dual functions of OSM, another type 1 cytokine family member associated with both pro- and anti-inflammatory functions, have largely been attributed to the usage of two distinct signaling receptors in humans: LIFRβ/ gp130 or OSMRβ/gp130 heterodimers (32). Using a panel of BaF3 cell lines expressing IL-31Ra in combination with gp130, IL-12Rβ1, IL-12Rβ2, IL-27Ra, IL-23R, or OSMR, Dillon et al. determined that IL-31 likely signals only through IL-31Ra-OSMR heterodimers (6). However, it is still unclear whether IL-31Ra can heterodimerize with other family members in vivo to form a signaling receptor for additional ligands. In addition, similar to gp130, IL-31Ra has multiple splice variants and a putative soluble
specifically through regulation of mast cell responses and direct inhibition both innate and adaptive components of type 2 immunity, Th17 responses, we previously reported that IL-27R signaling can inhibit both IL-12 and IL-23, suggests that it may be an important early regulator of immunity. In contrast, IL-31 is produced predominantly by activated T cells (6) and as such may be more likely to act as a negative feedback pathway for limiting the magnitude of an ongoing type 2 immune response. The identification of a negative regulatory function in type 2 inflammation in the gut offers the potential to manipulate this pathway, perhaps through administration of IL-31, to ameliorate Th2 cytokine-mediated inflammation associated with infection, food allergies, or ulcerative colitis.

Acknowledgments

We thank Paul Giaconia, Meera Nair, and Amy Troy for critical reading of the manuscript, Laura Trenl and Michael Cancro for assistance with B cell purification and analysis, and Nico Ghilardi for providing the IL-31Ra-/- mice.

Disclosures

The authors have no financial conflict of interest.

References


