

Tim-3 Agonist Restrains ILC2 Function and Attenuates Airway Hyperreactivity via NLK Pathway

Corresponding Author: Professor Omid Akbari

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is an interesting study that shows that IL-33 or allergen challenge increase Tim3 expression on ILC2 and that Tim3 decreases IL-33 or allergen induced airway inflammation. The noteworthy results from this article since Tim3 expression has not been previously reported on ILC2 and so determine regulatory or markers of exhaustion on ILC2 provides mechanistic insight on how these innate immune cells drive airway inflammation. The experimental approaches and controls used for these studies were appropriate. The findings were also compelling with Tim3 agonist decreasing ILC2 function and ILC2 induced airway inflammation, using both IL-33 and Alt allergen models. Additionally, the data presented in the main body and supplemental figures support the conclusion that Tim-3 is an inhibitory checkpoint for ILC2. However, there were concerns on how many times the experiments had been conducted and the statistical approaches used for some of the figure panels (see comments below). Additionally, the results immediately focused on the role of Tim 3 as a checkpoint inhibitor and did not show how IL-33 (or Alt) modified expression of other known checkpoint inhibitors in ILC2. These comments are detailed below:

Comments:

1. The figure legends state that experiments contained n=3 to 5 mice (depending on the figure panel) but never states if these mice were used in one experiment or combined from 2+ experiments. How many times were the experiments repeated? Does the data shown represent 1 representative experiment or combined results?
2. Figure 1E and several figure panels show airway physiology data (Figs. 4B, 4C, 5B, 5C, 6B, 6C, 7J, 7K) and state these data were analyzed by Two-tailed Student T-test in the Figure legends. This is not the appropriate statistical test for these repeated measure experiments, and the appropriate tests need to be conducted and stated in the Figure Legends.
3. Figures 4J, 4K, 5J, 5K, 6J, and 6K have more than 2 groups and so the two-tailed Student T-test is not appropriate for these experiments. Please run the appropriate ANOVA and report this in the methods section and Figure Legends.
4. Figure 1 – the determination of Tim-3 being upregulated by IL-33 is striking. Were other checkpoint inhibitors also upregulated after IL-33 in the scRNAseq data? The immediate focus on Tim-3 and not other checkpoint inhibitors is unclear in the introduction or in Figure 1.
5. Figure 2 determined mechanistic pathways by which Tim3 agonist affects ILC2 using RNAseq analysis. The authors determine that Tim3 agonist increase Nlk gene expression by RNA seq and then NLK protein expression by flow cytometry. However, the upregulation of Nlk gene expression is modest in Figure 2A-B (less than 1 log2 fold change) and many other genes seem to be upregulated in the volcano plot. The focus on NLK and not other candidates with >3 log2 fold change should be discussed.
6. Figure 3 shows that Tim3 agonist decreased oxidative phosphorylation and mitochondrial function in ILC2. What about other metabolic pathways? Was glycolysis altered as well? Was a Glucostress test conducted?
7. Figures 4-5 show that Tim 3 agonist reduce IL-33 or Alt-induced airway inflammation. What is striking is the large decrease (more than 50%) in IL-13 production. Was mucus production by PAS staining also decreased?
8. Figure 7 verifies that Time 3 agonist on IL-33 treated human ILC2 cells decreased cytokine production. What happened to the NLK pathway or mitochondrial function in these human ILC2 cells after Tim3 agonist treatment?

Minor comments:

1. Figure 1A – “merge” is misspelled in the UMAP
2. Figure 1B – please define what the overlay portion of the plot is for this panel (far right panel).

Reviewer #2

(Remarks to the Author)

This study clearly demonstrated that Tim-3 is a negative regulator of ILC2, since stimulation of Tim-3 effectively suppressed ILC2 activation. The intracellular signaling pathway of Tim-3 involves activation of NLK, which in turn suppressed NF- κ B activation. Furthermore, the authors have shown that the Tim-3–NLK axis is also induced in human ILC2. This finding regarding Tim-3 function in ILC2 is novel and could be quite important for understanding the regulation of ILC2, which contributes to severe asthma pathogenesis. However, there are several concerns that should be addressed.

Major comments:

1. It is essential to provide evidence that the Tim-3 agonist (anti-Tim-3 antibody) used in this study functions as a stimulating, but not a blocking, antibody.
2. The specificity of the NLK inhibitor for NLK needs to be clarified. In addition, in pharmacological experiments using inhibitors, it is generally required to show the concentration–response effects. It is required to show that the effective concentration is corresponding to its specific effect on a molecule.
3. The endogenous agonists of Tim-3 should be discussed. For example, it is known that galectin-9 stimulates Tim-3. How are such endogenous agonists produced during severe asthma pathogenesis? Which cells are the source?
4. What are the CD45⁺ cells other than ILC2 and eosinophils? The effects of the Tim-3 agonist on the numbers of those other leukocytes should be disclosed.
5. Pages 6–7: The authors identified GATA-3⁺ cells as ILC2. Do the authors have evidence that Th2 cells can be excluded from the GATA-3⁺ population in this model? Such evidence should be provided.

Minor comments:

1. Figures 2D and 2E: “aTim3” should be changed to “Tim-3 agonist,” consistent with the text.
2. Page 6, line 14: Appropriate references should be cited regarding the relationship between AHR and IL-5/IL-13.

Reviewer #3

(Remarks to the Author)

In the current manuscript, Sakano et al identify a role for Tim-3 in regulation of ILC2 function, of relevance to allergic diseases like asthma. The authors demonstrate that lung ILC2s from IL-33-treated animals display increased Tim-3 expression, and that upon in vitro culture in the presence of a Tim-3 mAb, decreased production of IL-5, IL-13 and reduced expression of GATA-3 and Ki67 is observed. RNA-Seq analysis of ILC2s culture in the presence of Tim-3 mAb displayed increased expression and production of NLK, an antagonist of NF- κ B signaling. This was also associated with decreased mitochondrial function. NLK inhibition (chemical inhibitor/Nlk antisense) reversed the inhibitory effects of anti-Tim-3 on ILC2 function. In vivo administration blocked IL-33 and *Alternaria alternata*-induced ILC2 function, lung inflammation, lung dysfunction, and airway remodeling. Mice lacking Tim-3 expression on ILC2s demonstrating increased airway responses, ILC2 activity, and lung remodeling after IL-33 exposure. That authors also demonstrated that stimulation of human ILC2s also upregulates Tim-3 expression, and that anti-Tim3 reduces ILC2 production of IL-5 and IL-13, and decreased expression of GATA-3 and Ki67. Adoptive transfer of human ILC2s into RAG2 deficient mice treated with anti-Tim-3 resulted in decreased ILC2 activity, lung dysfunction and remodeling. Collectively the data support a role for Tim-3 in the regulation of ILC2 function.

I have several questions/comments related to the manuscript as written:

The “Tim-3 agonist” is anti-Tim-3 (clone 5D12). This should be mentioned in the body of the manuscript and the properties of this antibody described. As some reports characterize Tim3 as molecule with capacity to increase activity of cells, it would be prudent to more precisely characterize their agent as “anti-Tim-3”.

Are known TIM-3 binding partners also present on ILC2s? If so, this might suggest an autocrine role for Tim-3 in regulation of ILC2 function.

There appears to be a discrepancy between TIM3 MFI on ILC2s in Fig 1F (~150 in PBS-treated mice, ~250 in IL-33 treated mice) and Sup Fig 1b (~150 on ILC2s). In a related comment - it is unclear whether Sup Fig 1B is from PBS, or IL-33 treated animals. If the former, this might explain the inconsistency. If Sup Fig 1b figure IS from PBS-treated mice, the levels of Tim-3 on others cells following IL-33 should also be included.

In figures 4 and 5, Sup Fig 4 and 5, mice treated with the Tim-3 agonist alone should also be included as an important control group in both AHR, and ILC2 measures.

Similarly Figures 4, 5, 6 and Sup Fig 4 and 5 should include PBS treated mice in measures of ILC2s, eosinophilia, and BAL cytokine levels.

Is there any impact on adaptive (T cell) mediated immune responses in chronic Alt model?

More information about the NLK inhibitor used is required. The compound listed in the manuscript (OTSSP167) is reported to be an inhibitor of MELK, not NLK. Please clarify.

Please provide evidence of successful targeting of Nlk following exposure to Nlk antisense oligos.

Fig s6d - It looks like MFI of Tim-3 is higher in ILC2 from deltaTIM3 mice. Is this correct? Or is this a typo? Further – is this in IL-33 treated mice, or in mice at baseline?

To bring the project completely full circle, it is important to show that there are changes in nlk and Nf-kb expression/activity observed in human ILC2s exposed to Tim-3 mAb? Similarly, are the effects of the anti-Tim3 reversible using the NLK inhibitor (or antisense)?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you for the response to comments. All my concerns have been addressed.

Reviewer #2

(Remarks to the Author)

The manuscript has been appropriately revised according to the reviewer's comments. Thus, it has been improved.

Reviewer #3

(Remarks to the Author)

The authors have addressed the concerns of this reviewers.

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We sincerely thank the reviewers for their thoughtful and constructive critique of our manuscript. We have carefully considered all comments and suggestions. Below, we provide a point-by-point response detailing how each concern has been addressed. We have conducted additional experiments and analysis, including the addition of appropriate controls, validation of pathway specificity, and clarification of statistical methods. These revisions have significantly strengthened the manuscript.

Reviewer #1

Remarks to the Author:

This is an interesting study that shows that IL-33 or allergen challenge increase Tim3 expression on ILC2 and that Tim3 decreases IL-33 or allergen induced airway inflammation. The noteworthy results from this article since Tim3 expression has not been previously reported on ILC2 and so determine regulatory or markers of exhaustion on ILC2 provides mechanistic insight on how these innate immune cells drive airway inflammation. The experimental approaches and controls used for these studies were appropriate. The findings were also compelling with Tim3 agonist decreasing ILC2 function and ILC2 induced airway inflammation, using both IL-33 and Alt allergen models. Additionally, the data presented in the main body and supplemental figures support the conclusion that Tim-3 is an inhibitory checkpoint for ILC2. However, there were concerns on how many times the experiments had been conducted and the statistical approaches used for some of the figure panels (see comments below). Additionally, the results immediately focused on the role of Tim 3 as a checkpoint inhibitor and did not show how IL-33 (or Alt) modified expression of other known checkpoint inhibitors in ILC2. These comments are detailed below:

Response to Remarks:

We thank the reviewer for the positive assessment of our work and for highlighting the novelty of Tim-3 as a regulatory marker on ILC2s. We appreciate the constructive feedback regarding the statistical approaches and the context of other checkpoint inhibitors. We have addressed these concerns in detail below, including clarifying our experimental replicates and expanding our statistical methodology.

Comment 1 (C1): The figure legends state that experiments contained n=3 to 5 mice (depending on the figure panel) but never states if these mice were used in one experiment or combined from 2+ experiments. How many times were the experiments repeated? Does the data shown represent 1 representative experiment or combined results?

Response 1 (R1): We thank the reviewer for this important clarification. To address this concern, we have updated the Data Analysis and Statistics section in the Materials and Methods. We have explicitly stated that all data—including both main and supplemental figures—are representative of at least three independent biological replicates, with the specific *n* values (representing individual mice) for the representative experiment

provided in each legend. We believe this global statement provides the necessary clarity while maintaining the conciseness of the figure legends.

Comment 2 (C2): Figure 1E and several figure panels show airway physiology data (Figs. 4B, 4C, 5B, 5C, 6B, 6C, 7J, 7K) and state these data were analyzed by Two-tailed Student T-test in the Figure legends. This is not the appropriate statistical test for these repeated measure experiments, and the appropriate tests need to be conducted and stated in the Figure Legends.

Response 2 (R2): We appreciate the reviewer's guidance on statistical analysis. We have now utilized two-way ANOVA for Figures 4B, 4C, 5B, 5C, 6B, 6C, 7J, and 7K, and have updated the figure legends accordingly.

Comment 3 (C3): Figures 4J, 4K, 5J, 5K, 6J, and 6K have more than 2 groups and so the two-tailed Student T-test is not appropriate for these experiments. Please run the appropriate ANOVA and report this in the methods section and Figure Legends.

Response 3 (R3): We agree with the reviewer's assessment. We have now applied one-way ANOVA for Figures 4J, 4K, 5J, 5K, 6J, and 6K, and have revised the figure legends to reflect this change.

Comment 4 (C4): Figure 1- the determination of Tim-3 being upregulated by IL-33 is striking. Were other checkpoint inhibitors also upregulated after IL-33 in the scRNAseq data? The immediate focus on Tim-3 and not other checkpoint inhibitors is unclear in the introduction or in Figure 1.

Response 4 (R4): We appreciate the reviewer's insightful observation regarding the upregulation of multiple checkpoint molecules in our scRNA-seq dataset. While other molecules were indeed induced, we prioritized Tim-3 because it exerts a unique, non-redundant inhibitory effect that is mechanistically distinct from other checkpoints we have characterized. Specifically, unlike PD-1, which we previously showed regulates metabolic homeostasis through glycolysis, or ICOS, which functions as a co-stimulatory receptor, Tim-3 operates through a fundamentally different NLK-signaling pathway. This distinctive Tim-3-NLK axis specifically suppresses NF- κ B activity and triggers direct mitochondrial dysfunction, such as reduced ATP production and spare respiratory capacity. Therefore, we focused the scope of the study on Tim-3 to define this novel inhibitory pathway that is functionally and qualitatively distinct from other checkpoints.

	log2FC	log10pvalue
Gm20634	4.33773272	5.00151932
Clec4e	3.15197332	4.9642614
Gm8750	3.53612115	4.59084599
Zfp777	1.0032835	3.80519182
Zfp687	0.46604035	3.7703911
Neat1	0.42354342	3.665835

Cp	4.15071231	3.62662685
Elmsan1	0.54515879	3.30622793
Nlk	0.75943621	2.80201826

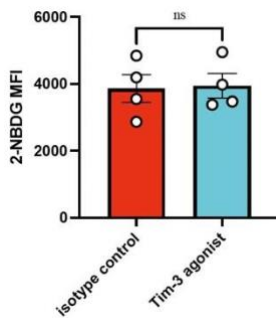
Comment 5 (C5): Figure 2 determined mechanistic pathways by which Tim3 agonist affects ILC2 using RNAseq analysis. The authors determine that Tim3 agonist increase Nlk gene expression by RNA seq and then NLK protein expression by flow cytometry. However, the upregulation of Nlk gene expression is modest in Figure 2A-B (less than 1 log2 fold change) and many other genes seem to be upregulated in the volcano plot. The focus on NLK and not other candidates with >3 log2 fold change should be discussed.

Response 5 (R5): We thank the reviewer for this important question regarding our candidate selection. We consulted with our biostatistics team regarding the top genes in our RNAseq. While *Nlk* was not the sole top hit, our selection was driven by biological function rather than statistical ranking alone. We excluded other highly significant genes because they did not fit the mechanistic profile of Tim-3-mediated suppression. For example, *Clec4e* is an activating receptor associated with pro-inflammatory signaling, *Cp* is a metabolic enzyme, and transcripts like *Gm20634* are poorly characterized predicted genes or pseudogenes. In contrast, NLK is a well-established serine/threonine kinase known to negatively regulate signaling pathways (e.g., NF- κ B and Wnt), making it the only candidate that functionally aligned with the inhibitory phenotype observed in our ILC2 model. Critically, we treated the RNA-seq data as a screening tool. As detailed in the manuscript, we went beyond transcriptomics to validate NLK at the protein level and established causality through functional assays. By demonstrating that NLK blockade (via siRNA and pharmacological inhibition) reverses Tim-3-mediated suppression—and that its overexpression recapitulates the phenotype—we confirmed that NLK is the functional driver of this mechanism.

Comment 6 (C6): Figure 3 shows that Tim3 agonist decreased oxidative phosphorylation and mitochondrial function in ILC2. What about other metabolic pathways? Was glycolysis altered as well? Was a Glucostress test conducted?

Response 6 (R6): We appreciate the reviewer's inquiry regarding the metabolic specificity of Tim-3 signaling. To address this, we screened our transcriptomic data and performed functional assays (2-NBDG uptake) to evaluate glycolysis, fatty acid oxidation, and glutaminolysis. Our analysis revealed that Tim-3 signaling selectively impairs mitochondrial integrity and ATP production. We did not observe significant alterations in glycolytic flux or the expression of key glycolytic enzymes. To maintain the manuscript's focus on the primary Tim-3-NLK-mitochondrial axis, we have opted not to

include these negative results as additional figures. Instead, we have added a clarifying sentence to the Results section.



Comment 7 (C7): Figures 4-5 show that Tim 3 agonist reduce IL-33 or Alt-induced airway inflammation. What is striking is the large decrease (more than 50%) in IL-13 production. Was mucus production by PAS staining also decreased?

Response 7 (R7): We thank the reviewer for this valuable comment. As suggested, we performed PAS staining, which showed results consistent with the HE staining; the proportion of mucus production was improved in the Tim-3 agonist-treated group. We now added the result of PAS staining to the Fig. 4L, M and Fig. 5L, M.

Comment 8 (C8): Figure 7 verifies that Time 3 agonist on IL-33 treated human ILC2 cells decreased cytokine production. What happened to the NLK pathway or mitochondrial function in these human ILC2 cells after Tim3 agonist treatment?

Response 8 (R8): We conducted additional experiments and demonstrated that treatment with a Tim-3 agonist in human ILC2s induced changes in NLK and p65 expression similar to those observed in mice. We now added the new results as Fig. 7I, J. Furthermore, we found that mitochondrial function in human ILC2s was also altered in a manner consistent with what we observed in murine models. We now added the new data as Fig. 7K, L and Fig. S8A, B in the manuscript.

Minor Comments:

1. **Figure 1A:** "merge" is misspelled in the UMAP.
 - **Response:** We have corrected the typographical errors.
2. **Figure 1B:** Please define what the overlay portion of the plot is for this panel (far right panel).
 - **Response:** We have revised the figure legend as suggested.

Reviewer #2

Remarks to the Author:

This study clearly demonstrated that Tim-3 is a negative regulator of ILC2, since stimulation of Tim-3 effectively suppressed ILC2 activation. The intracellular signaling pathway of Tim-3 involves activation of NLK, which in turn suppressed NF- κ B activation. Furthermore, the authors have shown that the Tim-3–NLK axis is also induced in human ILC2. This finding regarding Tim-3 function in ILC2 is novel and could be quite important for understanding the regulation of ILC2, which contributes to severe asthma pathogenesis. However, there are several concerns that should be addressed.

Response to Remarks:

We are grateful for the reviewer's recognition of the novelty of our study and its potential importance in understanding ILC2 regulation in asthma. We have carefully addressed the specific concerns regarding antibody function, pharmacological specificity, and cell population analysis below.

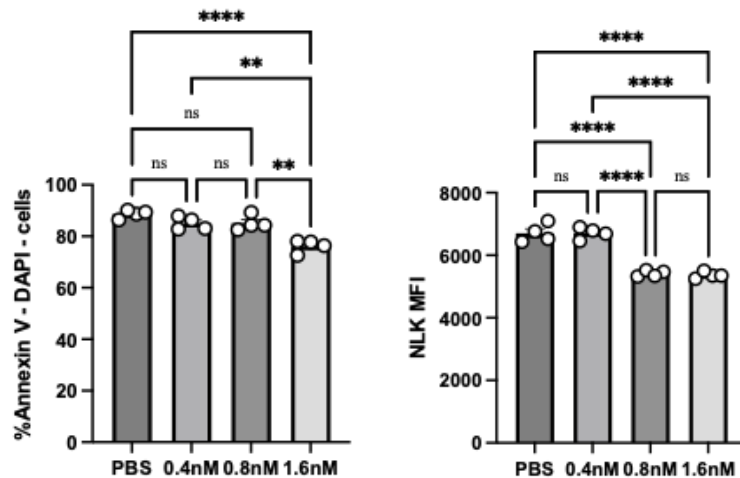
Comment 1 (C1): It is essential to provide evidence that the Tim-3 agonist (anti-Tim-3 antibody) used in this study functions as a stimulating, but not a blocking, antibody.

Response 1 (R1): The Tim-3 agonist antibody was originally described by the V.J. Kuchroo laboratory (PMID: 33931442), which characterizes the agonist activity of the antibody clone used in this study. Since its initial description, this specific clone has been widely adopted by multiple independent research groups to investigate the inhibitory signaling of Tim-3 across various immune contexts. Briefly, this clone was selected for its unique ability to mimic the natural ligand-receptor interaction, thereby actively triggering the Tim-3 inhibitory signaling cascade rather than preventing ligand binding. To further validate its function in our specific system, we observed that antibody treatment consistently led to a significant reduction in ILC2 effector function, cytokine production, and metabolic fitness. The fact that our functional outcomes align with established signaling data from other laboratories further confirms that this antibody functions as a true agonist, providing a robust and validated tool to define the negative regulatory role of Tim-3. We now added the original citation and added clone of the antibody in the manuscript.

Comment 2 (C2): The specificity of the NLK inhibitor for NLK needs to be clarified. In addition, in pharmacological experiments using inhibitors, it is generally required to show the concentration-response effects. It is required to show that the effective concentration is corresponding to its specific effect on a molecule.

Response 2 (R2): We appreciate the reviewer's point regarding the importance of pharmacological specificity and dose-response validation. To ensure the reliability of our findings, we performed a titration of the NLK inhibitor (OTSSP167) across a range of concentrations to determine the optimal dosage for our system. Specifically, we monitored for apoptosis and potential cytotoxicity using Annexin-V/DAPI staining to identify the minimal effective dose that would suppress signaling without compromising cell viability. Based on this titration and our viability assessments, we selected 0.8 nM as the optimal concentration, as it effectively modulated ILC2 function while maintaining

high cell survival rates comparable to control groups. To further substantiate the specificity of these pharmacological effects, we corroborated our results using specific *Nlk* antisense morpholino oligonucleotides. This genetic approach to silence *Nlk* expression yielded results concordant with the chemical inhibition, confirming that the observed changes in ILC2 activation and cytokine production are specifically mediated through the NLK signaling pathway rather than off-target effects of the inhibitor.



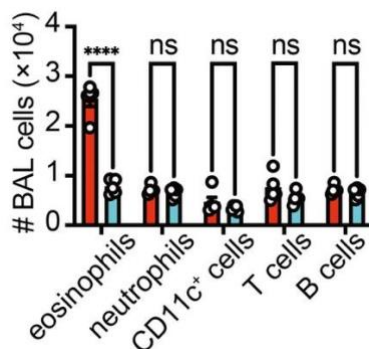
Comment 3 (C3): The endogenous agonists of Tim-3 should be discussed. For example, it is known that galectin-9 stimulates Tim-3. How are such endogenous agonists produced during severe asthma pathogenesis? Which cells are the source?

Response 3 (R3): We thank the reviewer for this insightful comment. We agree that the availability and cellular sources of endogenous Tim-3 ligands, such as Galectin-9, are critical to the rationale for using a Tim-3 agonist. In response to this comment, we have expanded our Discussion to address the production and deficiency of these ligands in asthma. While Galectin-9 is known to be expressed by various immune cells—including dendritic cells and macrophages—as well as airway epithelial cells, clinical evidence suggests its levels are significantly reduced in the asthmatic airway compared to healthy individuals. Therefore, treatment with a Tim-3 agonist represents a rational therapeutic strategy to bypass this ligand deficiency and restore ILC2 homeostasis. We have updated the manuscript to include these points and the relevant citations (PMID: 23121677, PMID: 38785528).

Comment 4 (C4): What are the CD45+ cells other than ILC2 and eosinophils? The effects of the Tim-3 agonist on the numbers of those other leukocytes should be disclosed.

Response 4 (R4): We appreciate the reviewer's request for additional detail regarding the composition of the CD45+ leukocyte population in our models. We routinely

examine multiple pulmonary immune subsets in the bronchoalveolar lavage (BAL) fluid using flow cytometry, including neutrophils, alveolar macrophages, and lymphocytes. However, our analysis revealed that Tim-3 agonist treatment did not significantly alter the numbers or frequencies of these other leukocyte populations. Since this study focuses on allergic lung inflammation and the role of ILC2s, we primarily presented data on eosinophils, as their recruitment is a gold-standard indicator of ILC2 activation and type 2 cytokine production in this model. Nevertheless, to provide a complete overview of the pulmonary environment, we have now included additional flow cytometry data showing that other CD45+ subsets remained largely unaffected by Tim-3 stimulation. This further supports our conclusion that Tim-3 primarily functions as a highly selective and non-redundant inhibitory checkpoint for ILC2-mediated responses.



Comment 5 (C5): The authors identified GATA-3+ cells as ILC2. Do the authors have evidence that Th2 cells can be excluded from the GATA-3+ population in this model? Such evidence should be provided.

Response 5 (R5): As shown in Fig. S1A, we defined ILC2s as CD45+ Lineage- CD127+ ST2+ cells. Therefore, Lineage+ Th2 cells are not included in this population.

Minor Comments:

- Figures 2D and 2E:** "aTim3" should be changed to "Tim-3 agonist," consistent with the text.
 - Response:** We have revised the figures as suggested.
- Page 6, line 14:** Appropriate references should be cited regarding the relationship between AHR and IL-5/IL-13.
 - Response:** We have now cited two of our previous work showing that modulation of ILC2 checkpoints alters IL-5/IL-13 production with corresponding changes in AHR and lung inflammation (Immunity 2015;42:538-551; PMID: 25769613), Nat Commun 2020;11:3998; PMID: 32778730).

Reviewer #3

Remarks to the Author:

In the current manuscript, Sakano et al identify a role for Tim-3 in regulation of ILC2 function, of relevance to allergic diseases like asthma. The authors demonstrate that lung ILC2s from IL-33-treated animals display increased Tim-3 expression, and that upon in vitro culture in the presence of a Tim-3 mAb, decreased production of IL-5, IL-13 and reduced expression of GATA-3 and Ki67 is observed. RNA-Seq analysis of ILC2s culture in the presence of Tim-3 mAb displayed increased expression and production of NLK, an antagonist of NF- κ B signaling. This was also associated with decreased mitochondrial function. NLK inhibition (chemical inhibitor/Nlk antisense) reversed the inhibitory effects of anti-Tim-3 on ILC2 function. In vivo administration blocked IL-33 and *Alternaria alternata*-induced ILC2 function, lung inflammation, lung dysfunction, and airway remodeling. Mice lacking Tim-3 expression on ILC2s demonstrating increased airway responses, ILC2 activity, and lung remodeling after IL-33 exposure. That authors also demonstrated that stimulation of human ILC2s also upregulates Tim-3 expression, and that anti-Tim3 reduces ILC2 production of IL-5 and IL-13, and decreased expression of GATA-3 and Ki67. Adoptive transfer of human ILC2s into RAG2 deficient mice treated with anti-Tim-3 resulted in decreased ILC2 activity, lung dysfunction and remodeling. Collectively the data support a role for Tim-3 in the regulation of ILC2 function.

Response to Remarks:

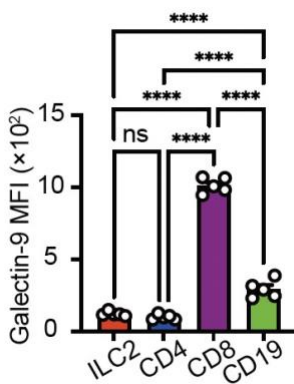
We thank the reviewer for the excellent and comprehensive summary of our manuscript. We are pleased that the reviewer agrees that our collective data support a role for Tim-3 in the regulation of ILC2 function. We have provided detailed responses to the specific questions raised below.

Comment 1 (C1): The "Tim-3 agonist" is anti-Tim-3 (clone 5D12). This should be mentioned in the body of the manuscript and the properties of this antibody described. As some reports characterize Tim3 as molecule with capacity to increase activity of cells, it would be prudent to more precisely characterize their agent as "anti-Tim-3".

Response 1 (R1): We appreciate the reviewer's suggestion to clarify the nomenclature and functional properties of the antibody used. Following this suggestion, we have updated the Results and Materials and Methods sections to explicitly identify the Tim-3 agonist as the anti-Tim-3 antibody clone 5D12. As described in our response to Comment 1 of Reviewer 2, this specific clone was originally developed and characterized by the laboratory of Dr. V.J. Kuchroo (PMID: 33931442) as a functional agonist. This clone functions by mimicking ligand engagement to actively trigger the receptor's intrinsic inhibitory signaling pathway, which we have validated in our study through the resulting suppression of ILC2 effector functions and cytokine production.

Comment 2 (C2): Are known TIM-3 binding partners also present on ILC2s? If so, this might suggest an autocrine role for Tim-3 in regulation of ILC2 function.

Response 2 (R2): We thank the reviewer for this interesting suggestion regarding potential autocrine signaling. To investigate whether ILC2s might regulate themselves via a Tim-3/Galectin-9 autocrine loop, we evaluated the expression of Galectin-9 (LGALS9), on pulmonary ILC2s. Our analysis revealed that ILC2s do not express significant levels of Galectin-9. In contrast, we observed robust Galectin-9 expression on other immune subsets, such as CD8+ T cells, and moderate expression on CD19+ B cells. Furthermore, existing literature indicate that Galectin-9 is also expressed by antigen-presenting cells (macrophages/DCs) and airway epithelial cells. Given that a comprehensive characterization of Galectin-9 across all possible lung subsets (including various APCs, specific endothelial and epithelial niches) would require various unique stimuli and is beyond the current scope of this study, we have indicated the lack of ILC2-specific expression as "data not shown" to maintain the focus on our primary findings. These results suggest that Tim-3 regulation of ILC2 function occurs via "trans-signaling" from the lung microenvironment rather than an autocrine mechanism. We have added a section to the Discussion and cited relevant manuscripts regarding how these external cell populations provide the necessary inhibitory signals to Tim-3 on ILC2s.



Comment 3 (C3): There appears to be a discrepancy between TIM3 MFI on ILC2s in Fig 1F (~150 in PBS-treated mice, ~250 in IL-33 treated mice) and Sup Fig 1b (~150 on ILC2s). In a related comment - it is unclear whether Sup Fig 1B is from PBS, or IL-33 treated animals. If the former, this might explain the inconsistency. If Sup Fig 1b figure IS from PBS-treated mice, the levels of Tim-3 on others cells following IL-33 should also be included.

Response 3 (R3): We appreciate the reviewer's careful attention to the MFI values across our figures. We acknowledge that the absolute MFI values for Tim-3 on ILC2s vary between Figure 1F and Supplemental Figure 1B. This variation is due to the fact that these experiments were performed independently on different days. In flow cytometry, absolute MFI values can be influenced by daily variations in laser power, detector sensitivity, and the specific compensation settings applied during each run. The most critical point, however, is that within each individual experiment, we always compared the experimental results to the specific negative and positive controls performed under the same conditions on that day. For instance, in Figure 1F, we

compared Tim-3 expression in IL-33-treated mice directly to PBS-treated controls and FMO control from the same run, revealing a significant upregulation of Tim-3 protein on activated ILC2s. To address the specific query regarding Supplemental Figure 1B, this figure was originally intended to show the relative expression of Tim-3 across different cell types under steady-state conditions. We have now updated Figure S1B to include both the PBS and IL-33-treated groups for all immune populations examined (CD4+, CD8+, and CD19+ cells). This comparison clearly demonstrates that Tim-3 expression is most robustly and uniquely induced in ILC2s following IL-33 stimulation compared to other immune cell types.

Comment 4 (C4): In figures 4 and 5, Sup Fig 4 and 5, mice treated with the Tim-3 agonist alone should also be included as an important control group in both AHR, and ILC2 measures.

Response 4 (R4): We have added the control group in AHR analysis in Figures 4 and 5, as well as Supplementary Figures 4 and 5.

Comment 5 (C5): Similarly Figures 4, 5, 6 and Sup Fig 4 and 5 should include PBS treated mice in measures of ILC2s, eosinophilia, and BAL cytokine levels.

Response 5 (R5): We have added the PBS control in Figures 4, 5, 6 and Supplementary Figures 4 and 5.

Comment 6 (C6): Is there any impact on adaptive (T cell) mediated immune responses in chronic Alt model?

Response 6 (R6): We appreciate the reviewer's inquiry regarding the adaptive immune response in our chronic *A. alternata* model. In this experimental setup, the Tim-3 agonist was administered on days 21 and 25, leading to a rapid attenuation of AHR and inflammation by day 26, characterized by reduced ILC2 numbers and lower levels of IL-5 and IL-13. Our findings in Rag2 deficient mice demonstrate that the Tim-3 agonist remains highly effective in the total absence of T and B cells, and our data show that Tim-3 expression is more robustly induced in pulmonary ILC2s compared to CD4+ or CD8+ T cells following activation. These results suggest that the Tim-3-NLK axis serves as a primary regulatory pathway for pathogenic ILC2s during the late stages of chronic inflammation. However, we acknowledge that our study primarily focused on the innate lymphoid compartment, and we did not specifically characterize the activation or cytokine profiles of T cells in the chronic *A. alternata* model. Given that Tim-3 is a well-established regulator of T-cell function and exhaustion in other disease contexts, it remains possible that the Tim-3 agonist exerts a portion of its therapeutic effect through the adaptive immune compartment in wild-type mice. While our ILC2-specific knockout data (ILC2 Tim-3) confirms that the loss of Tim-3 on ILC2s alone is sufficient to exacerbate disease, we have revised our Discussion to more conservatively describe the role of ILC2s. We have also added a section addressing the limitation that T-cell mediated responses were not fully assessed and may contribute to the overall therapeutic outcome of Tim-3 agonism in chronic allergic settings.

Comment 7 (C7): More information about the NLK inhibitor used is required. The compound listed in the manuscript (OTSSP167) is reported to be an inhibitor of MELK, not NLK. Please clarify.

Response 7 (R7): The reviewer is correct as OTSSP167 originally developed as a MELK inhibitor, later repurposed in erythroid/DBA studies as a pharmacologic NLK inhibitor after screening for compounds that inhibit NLK as an off-target; and studies used OTS167 to inhibit NLK in erythroid progenitors (please see Ref:34 study by Shibuya et al.). That is why we did not solely rely on the results from OTSSP167 and to ensure specificity, we employed a dual approach using specific antisense RNA in vitro, based on established protocols published by our group (PMID: 34144112, PMID: 31620118). For context, similar morpholino antisense RNA targets used in our lab were LAIR-1 (Helou et al.) and GATA3 (Lewis et al.), both using well-documented protocols. This consistency underscores the methodological rigor in our approach.

Comment 8 (C8): Please provide evidence of successful targeting of *Nlk* following exposure to *Nlk* antisense oligos.

Response 8 (R8): We have addressed this by utilizing specific *Nlk* antisense morpholino oligonucleotides to genetically silence *Nlk* expression. As described above, this approach is based on established in vitro protocols that our group has extensively used and validated morpholino system for targeted gene knockdown in ILC2s as described by our group before. To ensure the observed effects were specific to *Nlk* depletion and not a result of non-specific RNA interference or delivery-related toxicity, we included an irrelevant (random) sequence control morpholino in every experiment.

Comment 9 (C9): Fig s6d It looks like MFI of Tim-3 is higher in ILC2 from deltaTIM3 mice. Is this correct? Or is this a typo? Further - is this in IL-33 treated mice, or in mice at baseline?

Response 9 (R9): We apologize for this typo. We now corrected this mistake in the manuscript.

Comment 10 (C10): To bring the project completely full circle, it is important to show that there are changes in *nlk* and *Nf-kb* expression/activity observed in human ILC2s exposed to Tim-3 mAb? Similarly, are the effects of the anti-Tim3 reversible using the NLK inhibitor (or antisense)?

Response 10 (R10): We appreciate the reviewer's suggestion by confirming the Tim-3-NLK-NF-kB axis in the human context. As requested, we performed new experiments and observed that Tim-3 agonist treatment in human ILC2s significantly elevated NLK protein levels while concurrently suppressing the expression of the NF-kB subunit p65. Furthermore, these molecular changes were associated with a significant decrease in mitochondrial mass and impaired respiratory capacity in human ILC2s, directly mirroring the metabolic dysfunction observed in our murine models. These data are now

presented in Figure 7I-L and Supplementary Figure 8, confirming that the inhibitory signaling pathway we defined is highly conserved across species.