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A Disintegrin and Metalloproteinase 17 is Required for ILC2 Responses to IL-33

Joseph C. Lownik[1], **Daniel H. Conrad**[2], and **Rebecca K. Martin**[2]

[1]Center for Clinical and Translational Research, School of Medicine, Virginia Commonwealth University, Richmond, Virginia 23298, USA.

[2]Department of Microbiology and Immunology, School of Medicine, Virginia Commonwealth University, Richmond, Virginia 23298, USA.

Abstract

Group 2 innate lymphoid cells (ILC2s) play an important role in the initiation of type-2 immune responses. Numerous targets have been identified that may activate or repress ILC2 function, though few negative regulatory feedback pathways induced upon activation have been shown to be operative in ILC2s. Here we demonstrate that loss of ADAM17 from ILC2s results in a selective defect in IL-33 responsiveness, but not IL-25 responsiveness. We find that IL1R2 is significantly upregulated at both the transcript and protein level in IL-33 activated ILC2s. We are also able to demonstrate that ADAM17 regulates IL1R2 levels on ILC2s in both a constitutive and activation induced manner. Additionally, IL1R2^{+} ILC2s, a unique subset of ILC2s, have decreased *Il5* and *Il13* transcripts following IL-33 stimulation. Overall, these data suggest that the expression of IL1R2 may act as an activation-induced negative regulatory feedback mechanism to decrease ILC2 responsiveness to IL-33.

Loss of ADAM17 on ILC2s results in a selective defect in IL-33 responsiveness.

ADAM17 regulates IL1R2 levels on ILC2s after IL-33 stimulation.

IL1R2^{+} ILC2s, a unique subset of ILC2s, have decreased cytokine production in response to IL-33.

Keywords

ADAM17; IL1R2; IL-33; ILC2

Introduction

Group 2 innate lymphoid cells (ILC2s) were initially characterized as being a non-B/non-T cell that produced large quantities of type 2 cytokines, primarily IL-5 and IL-13. This is

Corresponding Author: Rebecca K. Martin, rebecca.martin@vcuhealth.org.

released in response to alarmins and helminth infection [1,2]. ILC2s initiate type 2 responses to helminth as well as allergic airway disease [3,4].

Recently, ILC2s have been divided into two subpopulations. While both subpopulations are lineage negative, and require IL-7 and IL-2, they differ in anatomical location, tissue residency, and cytokine responsiveness [5]. Natural ILC2s (nILC2s) are seeded primarily in lung, are $ST2^{+}$ and $Thy1^{hi}$, and respond primarily to IL-33. Inflammatory ILC2s (iILC2s) are $ST2^{-}$, $Thy1^{lo}$, and $KLRG1^{hi}$, are primarily found in the intestines, and respond mainly to IL-25 with migration to the spleen, liver, and lungs in an S1P-dependent manner [6].

A Disintegrin and Metalloproteinase 17 (ADAM17) is classically known for its role in inflammation due to its ability to shed TNFα, ultimately leading to its early name TNFα converting enzyme (TACE) [7]. Additionally, ADAM17 is involved in the shedding of TNF receptors, IL6Rα, and many other cytokine and chemokine receptors [8]. With this knowledge, we sought to examine the role of ADAM17 in ILC2 function.

IL-1 was the first interleukin to be described and sparked a revolution in scientific discovery regarding soluble mediators of inflammation [9]. The receptor for IL-1, IL1R1 was also shown to be essential for response to microbes through its Toll-IL-1 (TIR) domain and subsequent MyD88 activation [10]. A second receptor for IL-1, IL1R2 was discovered shortly after and was attributed with having an inhibitory function on IL-1 signaling [11]. Since then, it has been appreciated that the IL1R family is composed of many cytokine receptor chains responsible for binding cytokines including IL-1α, IL-1β, IL-18, IL-33, and others.

IL-1 signaling requires two receptor components to be functional, IL1R1 and IL1RAP. It has been demonstrated that IL1RAP alone is not able to bind IL-1, and that IL1R1 that is not complexed with IL1RAP does not recruit the proper adaptor proteins necessary for signaling propagation [12]. In addition to being utilized by IL1R1 for IL-1 signaling, IL1RAP also complexes with ST2 to form the heterodimeric IL-33R [13]. Loss of either IL1RAP or ST2 completely abrogates IL-33 signaling, indicating that both proteins are essential for proper signaling.

IL1R2 has a truncated intracellular domain and lacks the TIR domain necessary for signaling through IL1R1 and is considered an inhibitory receptor [14]. IL1R2 can block IL-1 signaling in three different ways. First, IL1R2 is shed from the cell surface into a soluble form in a metalloproteinase dependent pathway allowing it to act as a decoy receptor for circulating IL-1α and IL-1β [15]. Several groups have shown that ADAM17 is responsible for IL1R2 shedding in various cell types [16,17]. Second, because of the lack of a cytoplasmic domain, IL-1 that binds to membrane IL1R2 is not able to propagate a signal. Third, studies regarding the inhibitory role of IL1R2 also found that it is able to form complexes with IL1RAP, thus sequestering IL1RAP away from IL1R1. Without available IL1RAP, IL1R1 is not able to form the signaling complex necessary for proper propagation of IL-1 downstream signaling [18,19].

In this study we examine the role of ADAM17 in ILC2 biology. Initially, we created mice which lack ADAM17 on ILC2s ($ADAM17^{ILC2-/-}$). To our surprise we observed a defect in

IL-33 responsiveness in ADAM17$^{ILC2-/-}$ compared to WT mice. Interestingly, this defect in ILC2 activation was not seen in response to IL-25 stimulation. Transcriptomic approaches, paired with known ADAM17 substrates identified IL1R2 as a potential target to explain this IL-33 response deficiency. We additionally show that ADAM17 directly regulates IL1R2 on ILC2s.

Materials and Methods

Mice

Mice with Exon 2 of *Adam17* floxed were purchased from The Jackson Laboratory (009597) and bred to mice expressing Cre-recombinase under linked to the *Rora* gene for dual expression with an IRES sequence [20]. These mice were then backcrossed to the C57Bl6/J strain for 8 generations. The resultant mice are referred to as ADAM17$^{ILC2-/-}$. These mice were also crossed to Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$/J mice (The Jackson Laboratory; 006148) to enable EYFP expression under the control of Cre-recombinase. Mice were genotyped using high resolution melting assays as described [21].

Cell Isolation and Sorting, and Culture

For isolation of WT ILC2s for expansion, WT mice were administered with 0.1μg of IL-33 i.n. for three consecutive days to expand ILC2s *in vivo*. ILC2s were then isolated from the lungs and sorted using a BD FACSAria Fusion SORP High-Speed Cell Sorter (BD Biosciences). Sorted ILC2s were expanded in IL-2 (10ng/mL), IL-7 (10ng/mL), and IL-33 (10ng/mL) for 7 days. Cells were then rested in IL-2 and IL-7 for 24 hours prior to stimulation. All culturing was conducted in cRPMI 1640 as in [21].

Gene Expression Analyses

Detailed methods for qRT-PCR and RNA-sequencing can be found in the Supplementary Methods.

In vivo Cytokine Models

For IL-25 challenge, 200ng of recombinant mIL-25 (R&D) in 100μL of saline was administered i.p. for 3 consecutive days. Mice were euthanized on day 4 and organs were harvested. For IL-33 challenge, 200ng of recombinant mIL-33 (BioLegend) in 20uL of saline was administered i.n. for 3 consecutive days. Mice were euthanized on day 4 and analyzed.

Organ Isolation and Flow Cytometry

A detailed description of organ digestion and flow cytometry can be found in the Supplementary Methods.

Histology

Histology was conducted as in [22]. Full slides were scanned at 40X using a Vectra Polaris (PerkinElmer) imaging system. Images were captured using Phenochart v1.0.9 (PerkinElmer).

IL1R2 Shedding Assays

A detailed description of shedding assays can be found in the Supplementary Methods.

Statistical Analyses

All statistical analyses used GraphPad Prism 7.3 and 8.0. Individual figure legends indicate statistical tests used for different experiments. Overall, Bartlett's test for equal variance was used and if variances were not equal, equivalent non-parametric tests as indicated in figure legends were used.

Results and Discussion

Loss of ILC2 ADAM17 results in decreased IL-33 responsiveness

To first confirm the specificity of the Rora-Cre, we examined lungs of IL-33 stimulated mice from $Rora^{YFP}$ mice in which YFP is under control of the Rora Cre. Using tSNE analysis, it can be appreciated that YFP expression was limited to the ILC2 cluster (Fig. 1A). This finding confirms a previous paper which also used the Rora Cre for ILC2 specific knockouts [23]. To examine the role of ADAM17 in ILC2 function, we first assessed whether ILC2s from $ADAM17^{ILC2-/-}$ mice responded normally to IL-33 challenge. While we saw no difference in the number of ILC2s present in saline treated WT or $ADAM17^{ILC2-/-}$ mice, we observed significantly fewer nILC2s in the lungs of IL-33 treated $ADAM17^{ILC2-/-}$ mice compared to WT (Fig. 1B).

Upon activation with IL-33, nILC2s rapidly make IL-5 and IL-13. When we examined these ILC2s for IL-5 and IL-13 production, we saw that nILC2s from $ADAM17^{ILC2-/-}$ mice had significantly fewer numbers of $IL\text{-}5^{+}$ $IL\text{-}13^{+}$ dual producing nILC2s compared to WT nILC2s (Fig. 1C). Along with increased nILC2 accumulation, IL-33 challenge induces an ILC2-mediated eosinophilia in both the lung and bronchoalveolar lavage fluid (BALF) of mice. IL-33 challenged $ADAM17^{ILC2-/-}$ mice had significantly fewer relative and absolute numbers of eosinophils in the BALF than IL-33 challenged WT mice (Fig. 1D), supporting the finding of decreased nILC2 activation in $ADAM17^{ILC2-/-}$ mice. Additionally, the lungs of IL-33 challenged $ADAM17^{ILC2-/-}$ mice had reduced leukocyte infiltration and mucus compared to WT mice, by H&E and PAS staining respectfully (Fig. 1E).

Loss of ILC2 ADAM17 does not influence IL-25 responsiveness

Due to the drastic defect in IL-33 responsiveness in ILC2s from $ADAM17^{ILC2-/-}$ mice, we wanted to examine whether this defect was present in response to other alarmins, namely IL-25. IL-25 stimulation is known to activate iILC2s *in vivo*, therefore we challenged mice with 3 i.p. injections of rIL-25 daily for 3 days and analyzed the mice the following day. When challenged i.p. with IL-25, iILC2s accumulate in the lung and mesLN in an S1P dependent manner [6]. IL-25 challenged $ADAM17^{ILC2-/-}$ mice had no difference in lung iILC2 numbers when compared to WT mice (Supplemental Fig. 1A–B).

We next examined iILC2s in the lungs of IL-25 challenged mice for IL-5 and IL-13 production as well proliferation with Ki67 staining. We did not see a difference in IL-5 production, IL-13 production, or Ki67 staining between IL-25 challenged $ADAM17^{ILC2-/-}$

and WT mice (Supplemental Fig. 1C–F). These data suggest there is no defect in activation or proliferation in these cells with IL-25 challenge. Also, we saw very little Ki67^{+} staining in nILC2s compared to iILC2s from both IL-25 challenged ADAM17$^{ILC2-/-}$ mice and WT mice, as expected (Supplemental Fig. 1C–F). Taken together, these results suggest a significant defect in IL-33 ILC2 responsiveness in the absence of ADAM17 on ILC2s.

ADAM17 inhibition alters ILC2 activation and survival *in vitro*

Upon stimulation with IL-2 + IL-7 + IL-33, vehicle treated ILC2s increase IL-5 and IL-13 production compared to IL-2 + IL-7 treated ILC2s (Supplemental Fig. 2A). Similar to our *in vivo* data, we found that ILC2s treated with an ADAM17 inhibitor (INCB) made significantly less IL-5 and IL-13 when stimulated with IL-33 compared to vehicle treated ILC2s (Supplemental Fig. 2A). However, ADAM17 inhibited ILC2s no difference in the amount of IL-5 and IL-13 was observed when treated with IL-25 (Supplemental Fig. 1A). This matched our findings *in vivo*.

Next, we wanted to examine apoptosis in ADAM17 inhibited ILC2s following IL-33 stimulation. To do this, we used Annexin V and propidium iodide (PI) staining. Interestingly, ADAM17 inhibited ILC2s treated with only IL-33 had significantly increased levels of early apoptotic (Supplemental Fig. 2B, D) and late apoptotic (Supplemental Fig. 2C, D) cells compared to vehicle treated ILC2s. It was also evident that this increase in apoptotic cells could be observed as early as 1 day of stimulation through 3 days of stimulation. Taken with our *in vivo* findings, these results indicate that inhibition of ADAM17 results in defective responsiveness to IL-33.

IL1R2 is upregulated on ILC2s following IL-33 stimulation

Our results indicated a clear defect in IL-33 responsiveness in ILC2s in the absence or inhibition of ADAM17. However, from this data, we did not have a clear mechanism as to what the role of ADAM17 was in IL-33 mediated ILC2 activation. To do this, we isolated naïve ILC2s from WT mice and cultured them for 3 days in the presence of either IL-2 + IL-7 or IL-2 + IL-7 + IL-33 and conducted RNA-sequencing. We then examined the fold change in IL-2 + IL-7 + IL-33 vs. IL-2 + IL-7 treated ILC2s for known ADAM17 substrates (Fig. 3A).

One of the more striking differentially expressed genes was *Il1r2* (Fig. 2A, B). *Il1r2* belongs to the IL-1 receptor family which consists of the machinery necessary for IL-33 signaling. To validate this target, we showed that IL1R2 protein was increased on ILC2s following stimulation with IL-2 + IL-7 + IL-33 compared to IL-2 + IL-7 stimulation by flow cytometry (Fig. 2C–E). To our knowledge, this is the first demonstration of protein expression of IL1R2 on ILC2s.

To determine the role of IL1R2 expression on ILC2s, we sorted IL1R2^{-} and IL1R2^{+} ILC2s that had been stimulated with IL-2 + IL-7 + IL-33 for 72hrs. To our surprise, both *Il5* and *Il13* transcript levels were significantly decreased in IL1R2^{+} ILC2s compared to IL1R2^{-} ILC2s (Fig. 2F–G). The bimodality of IL1R2 expression on activated ILC2s and the functionally differences between the populations suggest that IL1R2 expression on ILC2s may define a unique subset of ILC2s. To confirm this, a more global transcriptomics

approach will be needed to further investigate these populations. Additionally, these findings suggest that IL1R2 expression may be negatively regulating ILC2 responsiveness. IL1R2^{+} ILC2s had significantly higher *Il1r2* expression than IL1R2^{-} ILC2s (Fig. 2H), further suggesting that this population is functionally distinct from IL1R2^{-} ILC2s.

ADAM17 regulates IL1R2 levels on ILC2s

We hypothesized that in ILC2s, IL1R2 occupying ILRAP, thus decreasing the amount of IL1RAP available to complex with ST2 and due to the necessity of IL1RAP for IL-33 signaling through ST2, there would be decreased IL-33 signaling, similar to inhibition of IL-1 signaling by IL1R2 [18,19].We next wanted to determine whether ADAM17 had a role in the regulation of IL1R2 on ILC2s following IL-33 stimulation. To test this, we stimulated WT ILC2s with IL-2 + IL-7 + IL-33 for 3 days in the presence of either a vehicle control, the ADAM17 inhibitor TMI-002 (Pfi), the ADAM17 inhibitor INCB3619 (INCB), or the ADAM10 inhibitor GI254023X (GI). There was a significant increase in overall IL1R2 MFI in both the Pfi and INCB inhibitor groups compared to vehicle treatment (Fig. 3A). When we examined IL1R2^{+} cells, IL1R2 MFI was significantly higher in Pfi and INCB treated cells as well, when compared to vehicle treated (Fig. 3A). Because of the overlap of substrates between ADAM10 and ADAM17, we also treated cells with the ADAM10 inhibitor GI and did not see a difference in IL1R2 levels in both overall cells as well as IL1R2^{+} cells, suggesting that ADAM10 does not play a role in IL1R2 regulation on ILC2s (Fig. 3A).

These data suggest that ADAM17 plays a role in the constitutive regulation of IL1R2 following IL-33 stimulation of ILC2s. To test whether IL1R2 was cleaved from ILC2s in response to a strong ADAM17 activation stimuli using PMA, we first cultured ILC2s in the presence IL-2 + IL-7 + IL-33 for 3 days. Following this stimulation, we washed the cells and added the indicated inhibitors or vehicle for 30 minutes prior to a 20-minute PMA stimulation. Following PMA stimulation, we detected a ~10% change in the percent of cells that were IL1R2^{+} in the vehicle treated group (Fig. 3B–C). This decrease was almost completely blocked in the ADAM17 inhibited groups (Fig. 3B–C). Similarly, we noted a decrease in overall MFI and IL1R2^{+} cell MFI following PMA stimulation in vehicle treated cells which was again almost completely blocked in ADAM17 inhibited cells (Fig. 3B, D). These results imply that IL1R2 is able to be shed from ILC2s in response to PMA and that ADAM17 is the primary sheddase responsible. Further studies will have to be conducted to determine the physiological stimuli that regulate IL1R2 constitutive regulation and activation induced shedding *in vivo*. Overall, we propose a model in which loss of ADAM17 on ILC2s selectively reduces activation and survival in response to IL-33, mediated through increased IL1R2 levels. This increased IL1R2 alters IL1RAP levels, reducing free IL1RAP able to interact with ST2. Thus, IL-33 signaling is altered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights:

- Loss of ADAM17 on ILC2s results in a selective defect in IL-33 responsiveness.
- ADAM17 regulates IL1R2 levels on ILC2s after IL-33 stimulation.
- IL1R2^{+} ILC2s, a unique subset of ILC2s, have decreased cytokine production in response to IL-33.

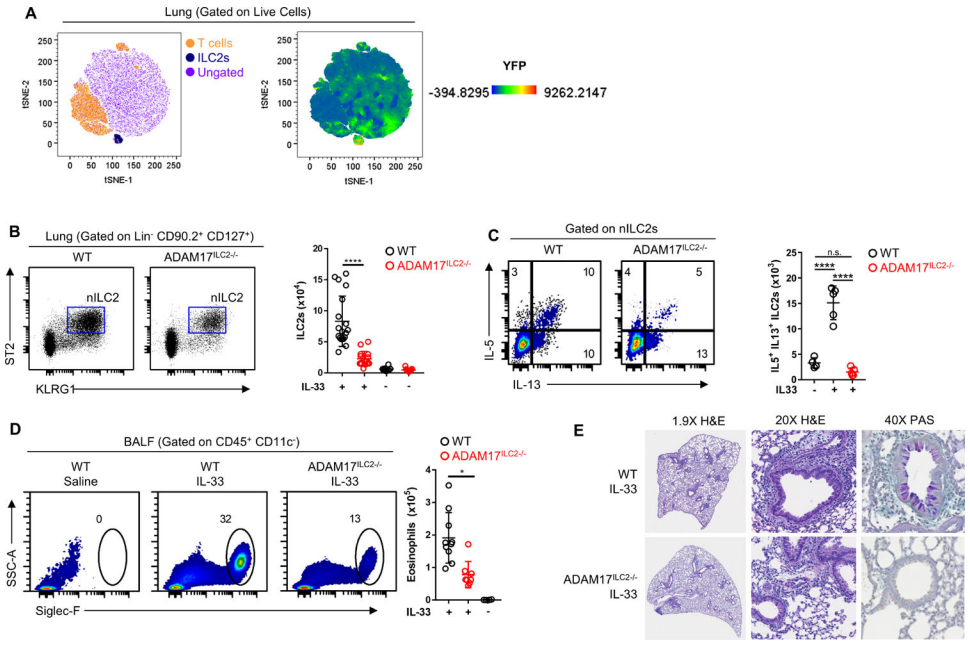

Figure 1: Loss of ILC2 ADAM17 decreases responsiveness to IL-33.

(A) tSNE analysis from lung of Rora^{YFP} mice showing YFP expression limited to ILC2 cluster. **(B)** WT and ADAM17$^{ILC2-/-}$ mice were challenged i.n. with IL-33 and ILC2s were measured in the lung. **(C)** IL-5 and IL-13 were measured by flow cytometry on ILC2s from B. **(D)** Eosinophil numbers in BALF from mice in B were measured by flow cytometry. **(E)** Representative histological sections of lungs from IL-33 challenged mice. **P < 0.05, **P < 0.01, ****P < 0.0001*, one-way ANOVA (B-D). Data are pooled from three (mean ± s.d.) independent experiments.

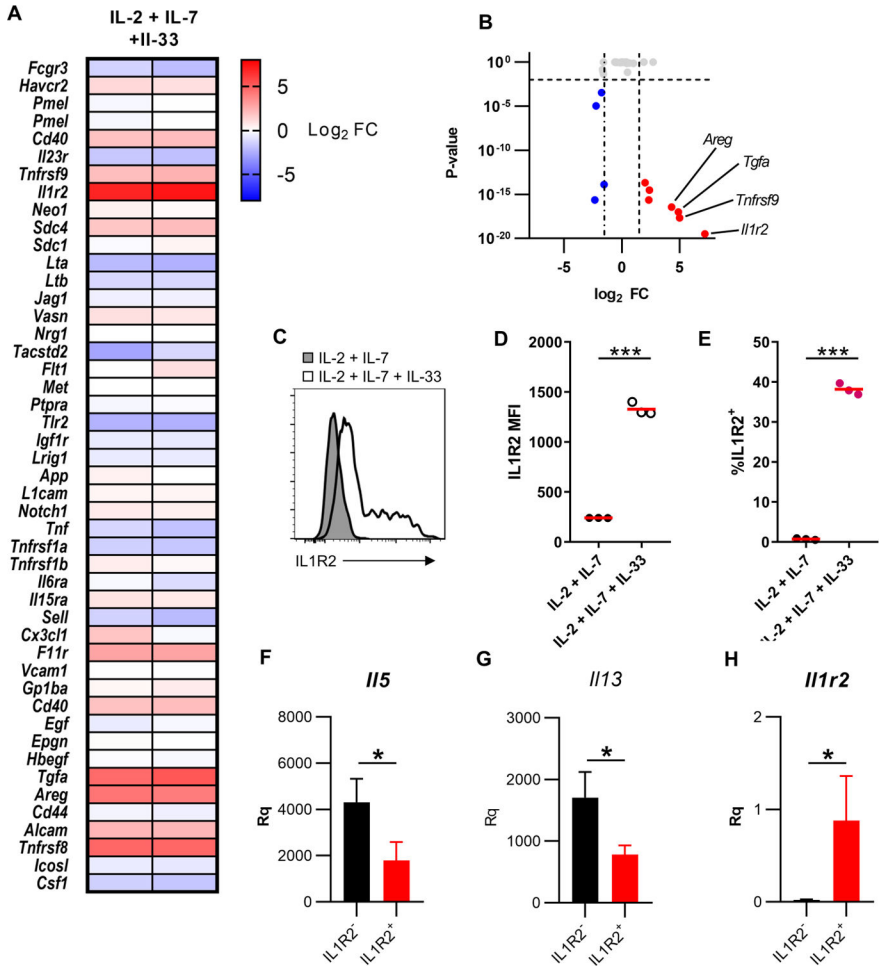

Figure 2: Activated ILC2s express multiple ADAM17 substrates.

(A) Heat map of fold changes from IL-2 + IL-7 + IL-33 stimulated ILC2s vs. IL-2 + IL-7. **(B)** Volcano plot of genes known to code for ADAM17 substrates. **(C)** Isolated and expanded ILC2s were cultured with the indicated cytokines and IL1R2 levels were measured by flow cytometry. **(D)** Statistical quantification of IL1R2 MFI from C. **(E)** Statistical quantification of % of cells IL1R2$^+$ from C. qRTPCR analysis of **(F)** *Il5* **(G)** *Il13* **(H)** *Il1r2* from IL1R2$^-$ and IL1R2$^+$ ILC2s following 3 days of stimulation with IL-2 + IL-7 + IL-33 relative to *Tbp* and *Rrp8*. **P < 0.05, ***P < 0.001*, Unpaired Student's t-test (D-E), Mann-Whitney U test (F-H). Data are representative of two (D-E; mean ± s.d.) and one (F-H; mean ± s.d., n=4 per group) independent experiments.

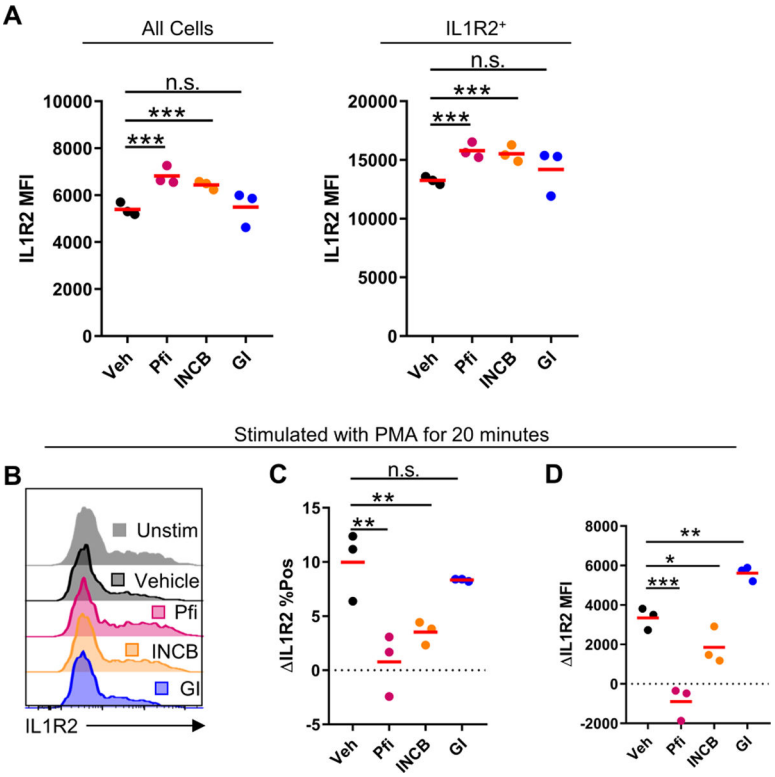

Figure 3: ADAM17 regulates IL1R2 on ILC2s.

(A) Isolated and expanded WT ILC2s were cultured in IL-2 + IL-7 + IL-33 for 72hrs with indicated inhibitors and IL1R2 levels were measured by flow cytometry. **(B)** Isolated and expanded ILC2s were incubated with indicated inhibitors for 30 minutes followed by PMA stimulation for 20 minutes. Statistical quantification of **(C)** change in % of cells IL1R2^{+} and **(D)** change in MFI following PMA stimulation. *n.s. $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.* one-way ANOVA (A, C, D). Data are representative of two (mean) independent experiments.