UNIVERSITY OF CALIFORNIA SAN DIEGO

c-di-GMP Attenuates Alternaria-Induced ILC2-Driven Lung Inflammation

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by

Luay Hamza Naji

Committee in charge:

Taylor Doherty, Chair
Randolph Hampton, Co-Chair
Ashley Juavinett

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The Thesis of Luay Hamza Naji is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2019

DEDICATION

I would like to dedicate this thesis to my parents for their love and support. I also want to dedicate this to my brother because he's great and I'm glad he's healthy.

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I would like to acknowledge Dr. Taylor Doherty for his advice and guidance throughout my time in his lab. I would also like to acknowledge everyone in the Doherty lab that has helped me during my time conducting research, especially Jana Badrani and Kellen Cavagnero for their support and advice.

ABSTRACT OF THE THESIS

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Luay Hamza Naji

Master of Science in Biology

University of California San Diego, 2019

Professor Taylor A. Doherty, Chair
Professor Randolph Hampton, Co-Chair

Although asthma has been classically understood as being type 2 T helper cell (Th2) cell driven, a new player has entered the arena of asthmatic response. Group 2 innate lymphoid cells (ILC2s) are recently discovered and have been shown to mediate the innate inflammatory features of asthma. ILC2s produce the same cytokines and rely on the same transcription factors that Th2 cells do but lack the lineage markers associated with other immune cells. *Alternaria alternata* is a fungal protease allergen associated with asthma that has been shown to induce a powerful release of IL33 and therefore, induce a strong ILC2-mediated eosinophilic lung inflammatory response. However, we have found that if *Alternaria* is combined with c-di-GMP during intranasal challenges in wild type mice, the eosinophilic lung response associated with

asthma is severely attenuated. Importantly, we have found that this attenuation of type 2 inflammation by c-di-GMP is completely prevented in STINGKO mice. Thus, we have identified a completely novel pathway to reduce innate ILC2-driven type 2 inflammation induced by the clinically-relevant allergen *Alternaria*.

INTRODUCTION

Asthma is a disease that impacts close to 10% of Americans and leads to a loss of $56 billion per year. Asthma is often a result of complicated interactions between genetics and the environment, and has many endotypes leading to challenges in care [1]. Despite the ambiguity surrounding asthma's triggers, there are common features associated with the disease. Airway constriction, remodeling, and hyperresponsiveness are the standard symptoms that arise from asthma [1]. The classic understanding of asthma suggests that type 2 helper T cells (Th2 cells) mediate the disease in the majority of patients through their secretion of interleukins IL4, IL5 and IL13 [1]. These proteins are recognized as trademarks of type 2 immune response. IL4 augments type 2 immunity through its proliferation of Th2 cells and leads to IgE class switching in B cells [2]. While the type 2 IL4 receptor also binds IL13, IL13 is known as a regulator of mucus production, remodeling features, inflammatory cell influx and airway hyperresponsiveness [2]. IL5 develops and recruits eosinophils, which are present in many type 2 driven diseases, including asthma [2]. Not surprisingly, blocking these cytokines has been a major target of type 2 associated disease therapies [2]. GATA3, the master Th2 cytokine transcription factor, has been shown to be crucial for Th2 differentiation and the production of IL4, IL5 and IL13 [3].

Although asthma was seemingly well understood through the adaptive CD4+ Th2 cell paradigm, a recently discovered cell type has challenged the conventional understanding. Group 2 innate lymphoid cells (ILC2s) were first characterized in 2010 and have since been shown to be potent Th2 cytokine producers present in the airways of patients with type 2 diseases like asthma [5]. Like Th2 cells, ILC2s highly express GATA3 and produce IL5 and IL13 in response to IL25 or IL33 to protect against helminth infection [4]. However, ILC2s differ from Th2 cells

in their lack of a T cell receptor and markers consistent with immune cell lineages [4]. They have also been found to need the cytokine IL7 for development and IL7RKO mice are ILC2 deficient [5]. ILC2s have been found in a variety of locations in mice and humans including bronchoalveolar lavage (BAL) fluid and the lung [4]. It has also been shown that patients with allergic asthma have a higher amount of ILC2s in their blood [5]. Not only that, but these blood ILC2s produce more IL5 and IL13 than those in patients without allergic asthma [5]. When epithelial cells are damaged by allergens or viruses, IL33 is released and bound by the IL33 receptor ST2 on ILC2s as well as many other cells including Th2 cells [4] [5]. The fungal protease allergen *Alternaria alternata* is associated with the development and severity of asthma in humans and damages the airway epithelium to potently release IL33, more so than other allergens [10]. Like GATA3, the transcription factor signal transducer and activator of transcription 6 (STAT6) is imperative to Th2 and ILC2 inflammatory responses [6]. STAT6 is phosphorylated by Janus kinases (JAKs) in response to type 2 cytokines IL4 and IL13 [6]. Once it is phosphorylated, STAT6 mediates the development of type 2 asthma inflammation [6]. STAT6KO mice lack airway eosinophilia, mucus cell production, and airway hyperresponsiveness which are hallmarks of type 2 asthma [6]. Interestingly, STAT6 also contributes to ILC2 proliferation likely through IL4 receptor signaling [6]. The presumed evolutionary role of type 2 immunity is to provide protection against helminths, but neither biology nor the immune system is structured in rigid compartments and significant cross talk and plasticity exists in cytokine networks. Though STAT6 is a potent driver of the type 2 response through the IL4/13 receptor, stimulator of interferon genes (STING) is also a strong activator of STAT6 [7]. STING triggers a type 1 interferon response which aids in the immune response against foreign pathogens, especially viruses, through the induction of anti-viral responses and leads to predominately neutrophilic response. Importantly, type 1 interferons have also been

shown to decrease ILC2 proliferation [8][19]. It is interesting that once viruses or cytoplasmic nucleic acids provoke STING activation for a neutrophilic response, it activates the STAT6 pathway which is classically a type 2 eosinophil dominated response. This duality exists because STAT6 is activated through a different mechanism when STING senses a cytoplasmic nucleic acid such as bacterial c-di-GMP. STING does not rely on IL4 nor IL13 to activate STAT6, despite the fact that these cytokines instigate the type 2 response through STAT6 signaling [7]. While these pathways may be central to appropriate anti-viral responses versus aberrant development of the asthma phenotype in the airway, the differences in their effectors could also represent why asthma is a heterogeneous syndrome.

Asthma's heterogeneity can be attributed to the different factors that are often involved in the generation of the immune response as well as structural cell changes in the airway. Bacterial and viral infections can potentially exacerbate asthma through their attraction of neutrophils, while at the same time, be protective in the development of type 2 asthma in some individuals [9]. For this reason, bacteria and viruses may be contributors to neutrophilic severe asthma and airway inflammation [9]. Many of the bacteria involved in severe asthma use the secondary messenger cyclic-di-GMP (c-di-GMP) [9]. Researchers were able to reproduce a mouse model of severe asthma using house dust mite and c-di-GMP to generate neutrophilia and airway hyperresponsiveness [9]. This model was T cell driven and operated over a 28-day period [9]. House dust mite alone generates a type 2 response that is consistent with mild-moderate asthma, which is most commonly eosinophil dominated and can be treated by corticosteroids [9]. One of the key findings of the severe asthma mouse model was the elevation of the cytokine IFNɣ, which was theorized to drive the elevation of neutrophils and corticosteroid resistance [9]. Despite asthma being primarily driven by a type 2 response, IFNɣ is associated with a type 1 response [7][9]. This further supports a potential basis for the heterogeneity of asthma. By

combining house dust mite, a type 2 allergen, and c-di-GMP the researchers were able to mimic the severe asthma phenotype [9].

Like eosinophils, neutrophils are granulocytes that are essential to the innate immune response [11]. Neutrophils originate from the bone marrow and can interact with other immune cells through intricate reactions that are still being discovered [11]. In addition to playing a role in severe asthma, a high blood neutrophil count is symptomatic of an increased risk of moderate asthma exacerbation [11]. It has also been shown that the higher the level of IL17, the greater the level of IL8 and neutrophil numbers [11]. IL8 is a cytokine that is associated with increased chemotaxis of neutrophils towards sites of inflammation [11]. T helper 17 cells are T cells that can produce IL17, and therefore, drive neutrophil recruitment [11]. The innate counterpart of Th17 is ILC3 which also produces IL17 [12]. ILC3s are the dominant innate lymphoid cell in the human intestine, with ILC2s are the most abundant in the lung [12][13].

Although adaptive neutrophilic asthma models exist, there have been no reports of innate neutrophilic models with allergens combined with bacterial components that drive changes in ILC subsets that may account for the subsequent inflammatory response. Not only that, but there has been little investigation into the response by ILC2s to bacterial components in the lung. To further ascertain the role of ILC2s in innate asthma models, wild type and STINGKO mice were intranasally challenged with the fungal allergen *Alternaria alternata* and the dinucleotide c-di-GMP. Granulocytes (neutrophils and eosinophils), as well as ILC2 proliferation, cytokine production and shifts in ILC phenotypes were measured. These key endpoints will help us understand how c-di-GMP modulates ILC2 behavior and innate type 2 lung inflammation driven by the clinically relevant allergen *Alternaria*.

MATERIALS AND METHODS

Mice

Female 6-12 week old C57BL/6 mice were ordered from Jackson Labs. STINGKO were ordered from Jackson Labs and bred in house.

Innate c-di-GMP challenge model

Mice were anesthetized with a 3.25:1 ratio of isofluorane and oxygen and were intranasally challenged with 50 µg of *Alternaria alternata* extract (Greer, NC) and 5µg c-di-GMP (Millipore Sigma, MO) in 40µL phosphate buffered saline (PBS) (ThermoFisher, MA). Mice were challenged on day 0, 1 and 2 and euthanized on day 3 in a carbon dioxide chamber. Bronchoalveolar lavage (BAL) fluid was collected using 2% bovine serum albumin (BSA) and lungs were collected as well.

BAL Fluid and Lung Processing

BAL fluid was centrifuged for 5 minutes at 4°C at 1500RPM using the centrifuge Allegra X-14R (Beckman Coulter, Carlsbad, CA). The first draw of BAL fluid supernatant was stored at -20°C for ELISAs of cytokines. BAL fluid cells were analyzed using flow cytometry. The four mouse lungs in each group were combined into two tubes and digested using the gentleMACS dissociator (Miltenyi Biotec, San Diego, CA) using the Miltenyi Biotec protocol. Next, the lungs were placed into a single cell suspension by passing them through a 40µm filter. The lungs were then centrifuged for 5 minutes at 4°C at 1500RPM using the centrifuge an Allegra X-14R (Beckman Coulter, Carlsbad, CA). Lung cells were analyzed by flow cytometry.

Flow Cytometry

BAL fluid and lung cells were diluted in 1:100 concentration of BAL to FACs buffer (PBS solution with 10% FBS and 0.01% sodium azide) to count cells using a Novocyte flow cytometer (Acea Biosciences, Inc., San Diego, CA). One million BAL fluid cells were aliquoted into tubes then washed with FACs buffer and stained after adding Fc block. Eosinophils were defined as CD45.2+ Siglec-F+ CD11c- cells and neutrophils were defined as CD45.2+ GR-1+ Siglec-F-. Anti-CD45.2+ was conjugated to PerCP (Biolegend, San Diego, CA) and anti-Siglec-F was conjugated to PE (BD Biosciences, La Jolla, CA). Anti-CD11c was conjugated to FITC (Biolegend) and anti-GR-1 was conjugated to APC (Biolegend). One million lung cells were distributed for the granulocyte stain and two million cells were stained for the ILC2 stain. 4 million lung cells were distributed for the Ki67 stain and 10 million cells were distributed for cell culturing for the cytokine stain. ILC2 lung cells were stained with a FITC-conjugated lineage cocktail made up of: a pre-made cocktail including CD3, Gr1, CD11b, B220, and Ter-119 (Biolegend), along with lineage additions CD11c (Biolegend), TCRβ (Biolegend), TCRγδ (Biolegend), NK1.1 (Biolegend), FcεR1a (Biolegend), and CD5. (Biolegend). CD45.2 conjugated to PerCP (Biolegend), Thy1.2 conjugated to eFluor 450 (eBiosciences, San Diego, CA), ST2 conjugated to APC (Biolegend), and CD127 conjugated to PE-Cy7 (Biolegend) were all used in addition to the lineage cocktail. Conventional lung ILC2s were defined as CD45.2+ Lin- Thy1.2+ ST2+ CD127+ lymphocytes. ILC2s were stained with Ki67 conjugated to PE (eBiosciences) following the FoxP3 protocol (eBiosciences). ILC2s were also intracellularly stained for cytokines: IFNɣ conjugated to APC-Cy7 (Biolegend), IL5 conjugated to PE (Biolegend) and IL17A conjugated to eFluor 506 (eBiosciences) following the manufacturer's fixation/permeabilization protocol (BD Biosciences). Cytoplasmically stained ILC2s were cultured for 3 hours in T cell media (TCM) that was made up of RPMI, penicillin/streptomycin,

l-glutamine, 2-mercaptoethanol and fetal bovine serum (FBS). Cell Stimulation Cocktail (ThermoFisher, MA) was also used during the 3 hours of culture.

Data Analysis

Flow plots were analyzed using FlowJo version 10.2 (Tree Star, Ashland, OR). Frequencies and totals were calculated using Microsoft Excel 2016. GraphPad Prism version 7 (GraphPad, La Jolla, CA) was used to generate data plots. ELISA data were analyzed using Microsoft Excel 2016 and figures were generated with GraphPad Prism version 7 (GraphPad).

Statistics

Statistical analyses were carried out using GraphPad Prism version 7 (GraphPad). A Mann Whitney test was performed for significance calculations. $P < 0.05$ were considered statistically significant.

RESULTS

c-di-GMP Attenuates Eosinophilia in an Innate Alternaria Model

Although cyclic dinucleotides like c-di-GMP have been shown to induce tissue neutrophil influx, there have been no investigations into the relationship between ILC2s and c-di-GMP [14]. Earlier reports have analyzed c-di-GMP in an adaptive severe asthma model, but the effect of c-di-GMP on an ILC2 driven lung inflammation model is unknown [9]. To investigate whether the severe asthma model's phenotype could be reproduced in an innate timeline, wild type mice were intranasally challenged with either PBS, 5µg c-di-GMP, 50µg *Alternaria* or a combination of 5µg c-di-GMP and 50µg of *Alternaria*. This challenge was performed daily for three days and followed by euthanization (Figure 2A). BAL fluid was found to have significantly elevated eosinophil frequency and total cells when challenged with *Alternaria* alone compared to all other groups (Figure 2B-C). This is consistent with previous reports of *Alternaria'*s effect on eosinophilia [15][9]. An increased percent and total neutrophils were induced in mice challenged with *Alternaria* and c-di-GMP (Figure 2D-E). Neutrophils were significantly elevated in this group compared to mice challenged with *Alternaria* alone, however total BAL fluid and lung cell total cells were only modestly different (not shown). The group receiving c-di-GMP alone failed to mount a similar neutrophilic response to the group with *Alternaria* and c-di-GMP combined. Most notably, c-di-GMP completely inhibits eosinophil production when combined with *Alternaria*. The level of eosinophils in the group with *Alternaria* and c-di-GMP is comparable to the PBS challenged group. The granulocyte trends seen in BAL fluid are observed in the lung as well, but with lower frequencies. There were elevated eosinophils in the *Alternaria* group and elevated neutrophils in the *Alternaria* with c-di-GMP group (Figure 2A-D). BAL fluid and lung cells were gated as defined in Figure 1A-D.

c-di-GMP Reduces Lung ILC2 Accumulation and Proliferation

To our knowledge, the effect of c-di-GMP on ILC2s has yet to be reported. *Alternaria* has been demonstrated to be a potent activator of ILC2s due to its instigation of epithelial IL33 release [16]. ILC2s were defined as lineage- Thy1.2+ lymphocytes (Figure 4A). The highest frequency of ILC2s occurred with *Alternaria* challenge, while the lowest average frequency was the group that received both *Alternaria* and c-di-GMP (Figure 4B-C). The decrease in ILC2s was associated with complete abrogation of eosinophilia generated in BAL fluid and lung when *Alternaria* and c-di-GMP were combined (Figure 2B-C and Figure 1A-B). There was a statistically significant ILC2 difference between mice challenged with *Alternaria* and those challenged with *Alternaria* and c-di-GMP combined (Figure 4B-C). In order to further understand the role of c-di-GMP on ILC2 accumulation in the lung, we next examined ILC2 proliferation. Our data suggested that ILC2s were being inhibited once c-di-GMP was added, so ILC2s were intracellularly stained with the nuclear proliferation marker Ki67 [17]. The *Alternaria* only challenged mice had lung ILC2s with the greatest proliferation (Figure 4D). There was a statistically significant reduction in percent and total proliferation in ILC2s from mice challenged with *Alternaria* and c-di-GMP compared with those challenged with *Alternaria* only (Figure 4E).

c-di-GMP Reduces ILC2 Type 2 Cytokine Production

The subsets of immune cells are often identified by the cytokines they produce and the markers they express. We examined intracellular IL5 and IL13 production because they are trademark type 2 cytokines produced by ILC2s [13]. Both IL5+ ILC2 totals and frequencies are significantly elevated with *Alternaria* challenge (Figure 5A-B). Administration of c-di-GMP with *Alternaria* challenges decreases the frequency and the total number of IL5+ ILC2s (Figure

5A-B). This effect is similar for IL13+ ILC2s, with a statistically significant difference between *Alternaria* and *Alternaria* with c-di-GMP (Figure 5C-D). Both IL13+ ILC2 frequency and total cell count are lower with c-di-GMP addition to *Alternaria* challenge (Figure 5C-D).

c-di-GMP Suppression of Alternaria-Induced Lung Eosinophilia is Completely STING-Dependent

In other model systems, it is known that among the pathways c-di-GMP activates and is dependent on the STING (stimulator of interferon genes) pathway which leads to type 1 IFN production to allow pathogen defense [18]. In order to determine the mechanism used by c-di-GMP in *Alternaria*-challenged mice, we challenged wild type and STINGKO mice with *Alternaria* or *Alternaria* with c-di-GMP following the protocol in Figure 2A. Impressively, BAL eosinophilia from STINGKO mice challenged with *Alternaria* and c-di-GMP matched the level of eosinophilia induced in wild types when challenged with *Alternaria* alone (Figure 6A-B). Thus, in the absence of STING, eosinophilia was not suppressed with c-di-GMP challenge. There was also a stark decrease in BAL fluid neutrophil levels with *Alternaria* and c-di-GMP challenge in STING KO mice (Figure 6C-D). The low level of neutrophilia in STING knockout mice was comparable to *Alternaria* challenged mice. (Figure 6A-D). Thus, c-di-GMP suppression of *Alternaria*-induced lung eosinophilia is completely dependent on STING.

c-di-GMP Induces Dynamic Changes in ILC2 Subpopulations in a STING-Dependent Manner

There are no prior investigations into the STING pathway and lung ILC2s, making the relationship between the two is unknown. We did not observe any statistically significant differences between wild types and STINGKOs with ILC2s being defined as lineage- and Thy1.2+ which also includes the total ILC population that is mostly ILC2s in the lung (Figure

8A-B). Further gating on Thy1.2+ cells with the IL7 receptor (CD127) and the IL33 receptor (ST2) is used to identify "conventional ILC2s" [4][5] (Figure 8C). Despite this convention in the literature, our laboratory's work has identified that even the single and double negative ST2 and CD127 positive populations produce type 2 cytokines. We have termed these cells unconventional ILC2s, though there is clearly representation by ILC1s and ILC3s in these unconventional populations. Although there were no differences between the total ILC population (lineage-Thy1.2+ ILC2s) between groups, there are statistically significant differences in these four ILC2 subpopulations between STINGKO and wild type mice. There is a modest frequency increase in ST2-CD127+ ILC2s in wild type mice challenged with *Alternaria* and c-di-GMP but not in cell totals (Figure 8D-E). There is a statistically significant increase in conventional ILC2 frequency after *Alternaria* with c-di-GMP challenge in STINGKO mice that is on par with WT mice challenged with *Alternaria* alone (Figure 8F). In the ST2+CD127- population, there was little overall difference between groups (Figure 8H). However, the addition of c-di-GMP to *Alternaria* challenges induces a significant increase in the ST2-CD127- ILC2s that is completely reversed when STING is absent (Figure 8J).

We next tested the level of proliferation within the ILC2 subsets using Ki67 nuclear staining. Wild type mice challenged with *Alternaria* and c-di-GMP have the lowest Ki67+ total ILC proliferation (Lin-thy1.2+) frequency of the groups tested (Figure 9A). STINGKO mice challenged with *Alternaria* and c-di-GMP have proliferation frequency levels similar to *Alternaria* challenged wild types (Figure 9A). This difference was also found in total Ki67+ ILCs (Figure 9B). When examining the subpopulations of ILCs, wild type mice challenged with *Alternaria* and c-di-GMP had the lowest proliferation frequency in every subpopulation except the ST2-CD127- quadrant (Figure 9C). Conventional ILC2s had the lowest frequency of Ki67 in a statistically significant manner. Knocking out the STING pathway with *Alternaria* and c-di-

GMP challenge returned proliferation levels to wild type *Alternaria* levels (Figure 9C). This was also present in Ki67+ ILC2 cell totals (Figure 9D).

c-di-GMP Inhibits Intracellular IL5 Production Through the STING Pathway

Our previous results have shown that the frequency and total IL5+ ILC2s significantly decreases in our innate neutrophilic model. Data generated from wild type and STINGKO mice suggests that c-di-GMP relies on the STING pathway to attenuate intracellular IL5 production in ILC2s. In STINGKO mice, *Alternaria* and c-di-GMP fail to suppress intracellular IL5 production in the lineage-Thy1.2+ population (Figure 10A-B). In fact, when the STING pathway is eliminated there is significantly greater intracellular IL5 produced with *Alternaria* and c-di-GMP challenge compared to *Alternaria* alone. The lack of c-di-GMP-induced IL5 suppression in STING knockouts with *Alternaria* and c-di-GMP is also present in the ILC2 subpopulations as well (Figure 10C-D).

c-di-GMP Uses the STING Pathway to Increase Intracellular IFNɣ Production in ILC2s

It has been shown that activation of the STING pathway induces production of type 1 interferons (IFNs) [18]. IFNɣ, while not a type 1 interferon, has also been shown to hinder ILC2 function [19]. In order to determine the role of IFNɣ in our innate neutrophilic model, we intracellularly stained wild type and STINGKO mouse ILC2s. STINGKO mice challenged with *Alternaria* and c-di-GMP generated the lowest frequency, although modest, of intracellular IFNɣ in ILC2s defined as lineage-Thy1.2+ lymphocytes among the groups tested (Figure 11A). The differences between groups are more evident when total IFNɣ+ ILC2s are examined (Figure 11B). Importantly, *Alternaria* and c-di-GMP challenge induce a statistically significant increase in total IFNɣ+ ST2-CD127- cells in wild type mice.

DISCUSSION

Our data suggest that *Alternaria*-induced ILC2-driven lung inflammation is suppressed by c-di-GMP in a STING-dependent manner. By previously establishing an innate eosinophilic 3 day model, we were also able to measure a significant reduction in type 2 behavior when bacterial secondary messenger c-di-GMP is combined with *Alternaria*. IL5, a cytokine constitutively expressed by ILC2s but further induced upon activation, is also decreased when c-di-GMP is added to *Alternaria* challenge [20]. IL5 induces eosinophil recruitment from the bone marrow, but the addition of c-di-GMP to *Alternaria* greatly hinders eosinophil migration to both BAL fluid and mouse lung [20]. We show these phenotypes are STING dependent because STINGKO mice are able to mount an eosinophilic response despite c-di-GMP's inclusion in *Alternaria* challenge.

There have been reports that type 1 interferons can inhibit ILC2s, and our data suggest that this phenomenon can be induced by c-di-GMP and is STING pathway dependent [19]. ILC2s express the type 1 interferon receptor on their surface and type 1 interferons can directly reduce ILC2 function [19]. This presents the possibility of c-di-GMP being a therapy for eosinophilic asthma driven by ILC2s as patients with severe asthma on corticosteroids have activated ILC2s in their sputum which could be contributing to disease [26]. We found that BAL and lung eosinophils return to PBS challenged levels when c-di-GMP is added to *Alternaria* challenges. There is also literature that suggests that type 1 interferons can induce plasticity in lymphocytes, but these studies did not show increased type 1 or type 3 cytokine production from ILC2s [19]. Our novel innate model corroborates those findings, as there was minimal IFNɣ, a type 2 interferon and type 1 cytokine, production. Despite the fact that intracellular cytokine stained cells were cultured for three hours in PMA ionomycin, there were low IFNɣ levels.

Interestingly, however, our data shows a shift in frequency and cell totals from conventional (CD127+ST2+) to unconventional ILC2 subpopulations when c-di-GMP is added to *Alternaria* challenges. This might support that c-di-GMP in *Alternaria* challenges induces some plasticity from conventional ILC2s to ILC1s and ILC3s which are also present in the unconventional ILC2 populations. Our proliferation data strengthens this explanation because ST2-CD127- ILC2s were the only population to increase their production of Ki67 with *Alternaria* and c-di-GMP challenge in wild type but not STINGKO mice. A reason for the lack of robust IFNɣ production is inherent in the complexity of type 1 interferons. Type 1 IFNs can both stimulate IFNɣ production and also inhibit them, depending on the bacterial pathogen that activates STING [23].

The type 1 interferon receptor can activate STAT1, STAT2 and STAT3 pathways during antiviral and inflammatory responses, with STAT1 being especially involved in ILC2 suppression after cells are infected [19] [21]. This presents a mechanism for ILC2 inhibition because c-di-GMP activates the STING pathway, which in turn induces type 1 interferon release and STAT1 activation to inhibit ILC2s. When the STING pathway is activated, the kinase TBK1 phosphorylates IRF3, IRF5, and IRF7 [22]. Phosphorylated IRF3 allows production of type 1 interferons and high IRF5 levels have been found in BAL fluid of severe asthmatics [18][22]. IRF5 can also promote airway hyperresponsiveness (AHR) in severe asthma mouse models [22]. Macrophages are also type 1 interferon producers, and low concentrations of interferons can even “prime macrophages” for a vigorous response to type 1 interferons through STAT1 [21]. This phenomenon happens during early phases of infection, which is precisely when ILC2s are active [21][4]. Macrophages can also interact with neutrophils through the release of the inflammatory cytokine IL1β [24]. It is interesting that the IL1 pathway suppresses type 1 interferon release, and type 1 interferons can suppress IL1β release [21][23]. Type 1 interferons can also induce cell death in leukocytes, including macrophages [23]. This could account for

decreased ILC2 numbers and proliferation. These interactions highlight the complexity underlying cytokine interplay and STING mediated ILC2 suppression.

Our study shows that c-di-GMP induced STING activation curbs ILC2 driven innate eosinophilic lung inflammation and could have implications for asthma pathogenesis. Generating a novel innate model allowed us to observe decreased eosinophilia and ILC2 proliferation following c-di-GMP inclusion in *Alternaria* challenges. Interestingly, we also measured a STING-dependent reduction in all ILC2 subpopulations except ST2-CD127- ILC2s. This plasticity could account for the decrease in IL5+ ILC2s and eosinophilia. The inhibition of ILC2s and subsequent lung inflammation through the activation of the STING pathway prompts the possibility of c-di-GMP being used as a therapeutic.

FIGURES

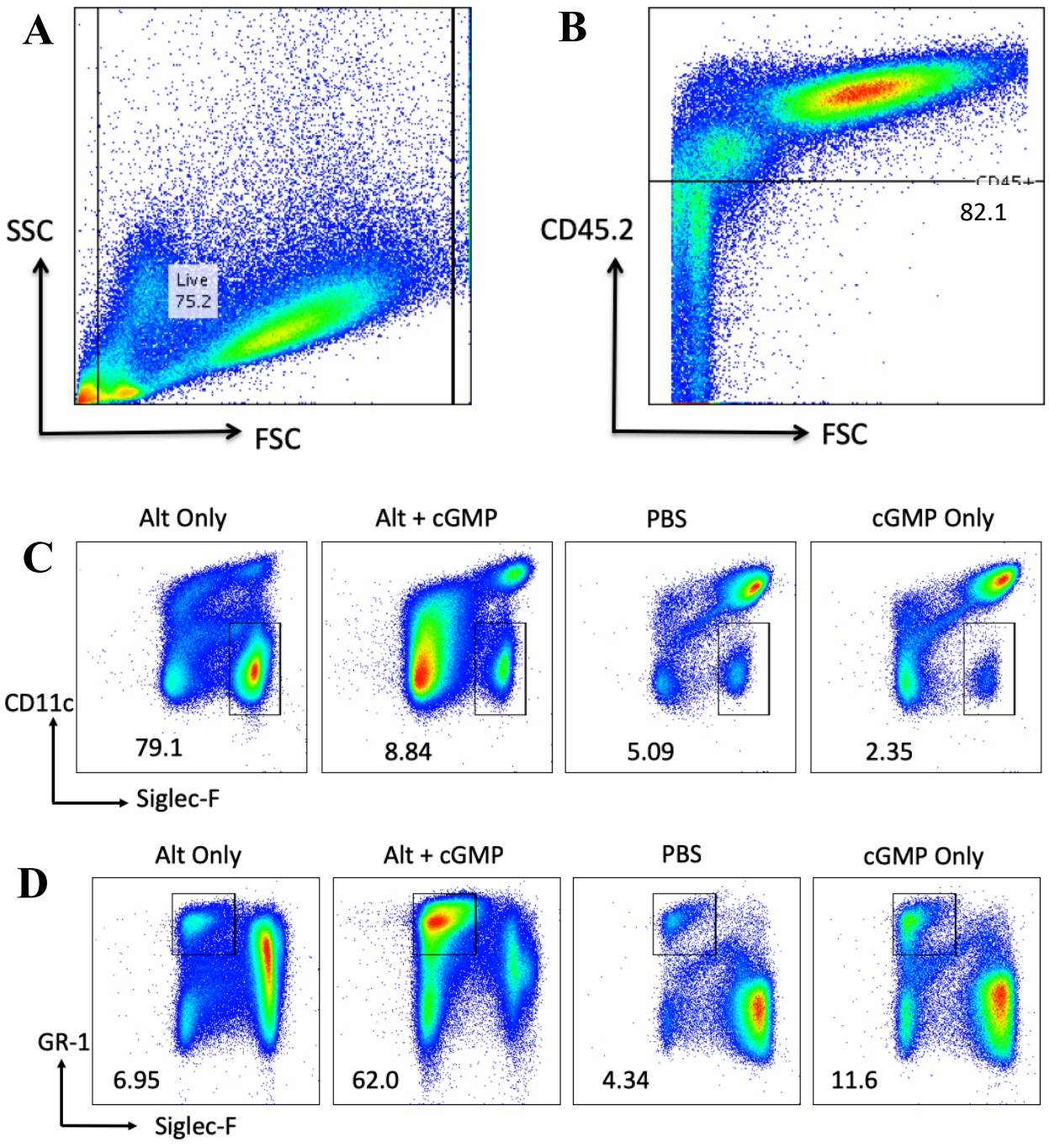


Figure 1: Innate neutrophilic asthma model BAL parent gating. Mice were challenged according to figure 2. These are representative FACs plots of live (A) and hematopoietic (B) cells. Eosinophils were defined as CD11c- Siglec-F+ (C) while neutrophils were defined as GR-1+ Siglec-F- (D).

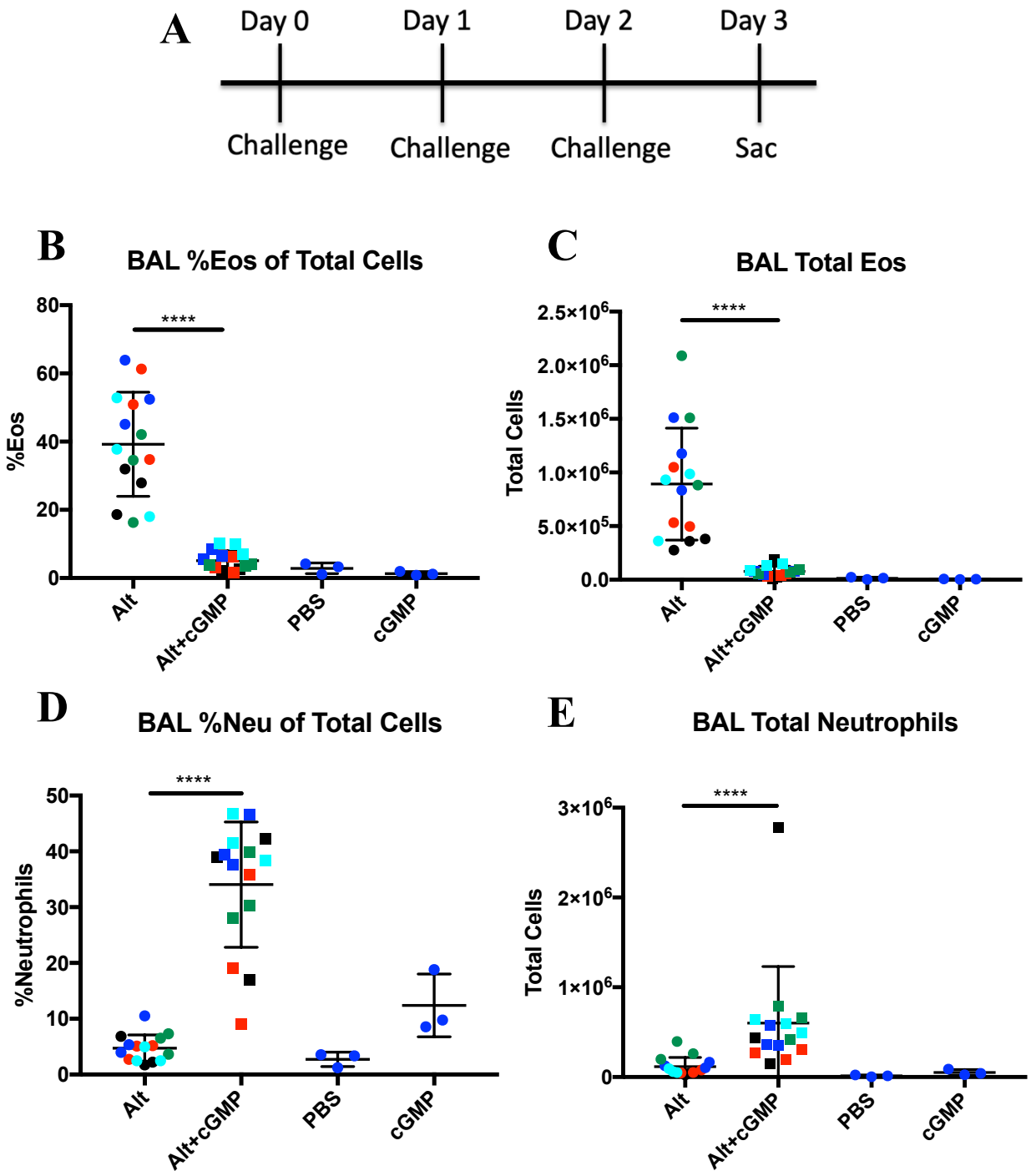


Figure 2: c-di-GMP attenuates BAL fluid eosinophilia in an *Alternaria* model. Wild type mice were challenged with PBS, 5µg c-di-GMP, 50µg *Alternaria* or a combination of 5µg c-di-GMP and 50µg of *Alternaria* for 3 days. After 3 days of challenge, mice were euthanized with BAL fluid and lung being collected. Timeline of the innate neutrophilic model (A). Frequency (B) and cell totals (C) of eosinophils in BAL fluid. Frequency (D) and cell totals (E) of neutrophils in BAL fluid.

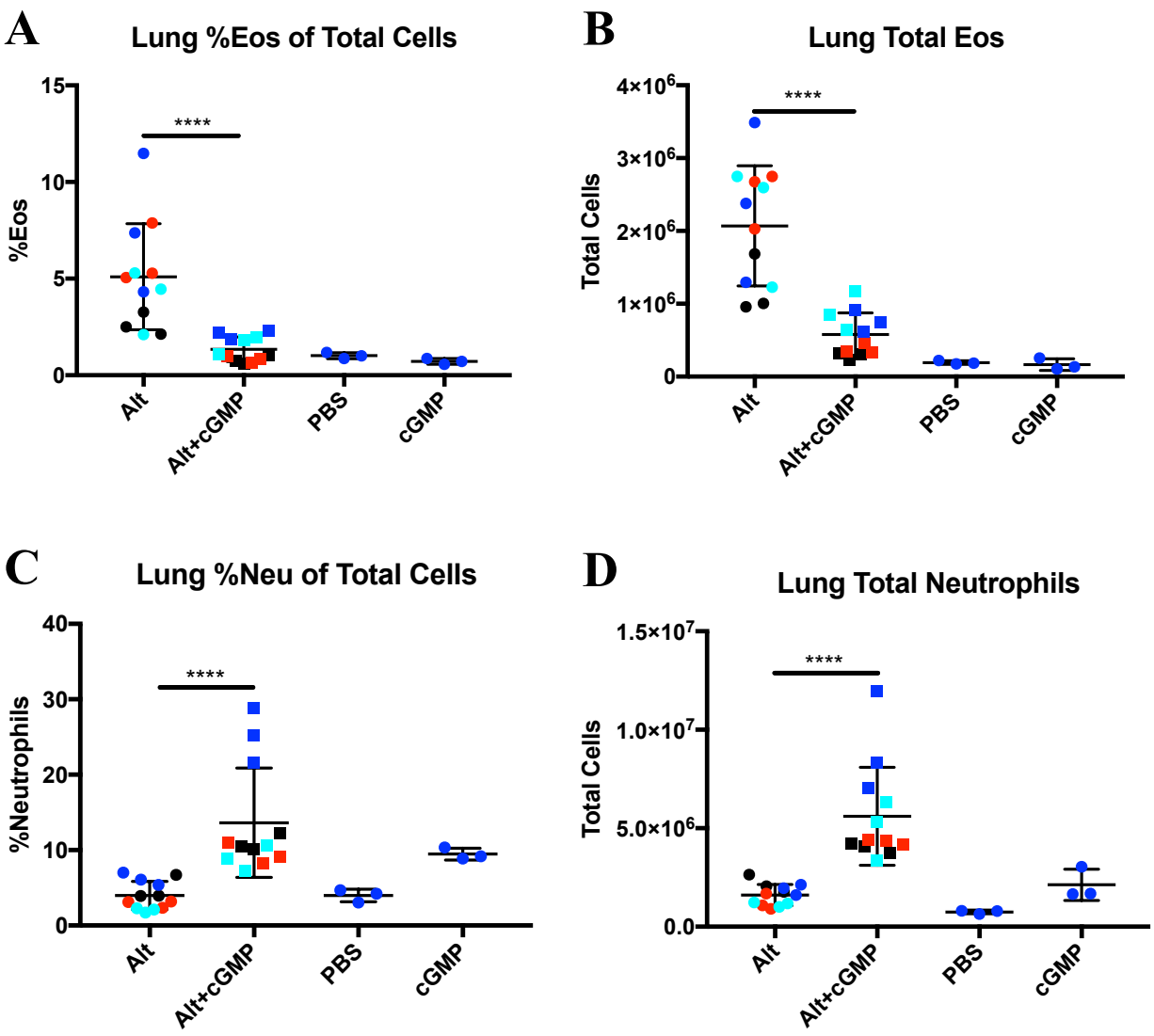


Figure 3: c-di-GMP attenuates lung eosinophilia in an *Alternaria* model. Wild type mice were challenged with PBS, 5µg c-di-GMP, 50µg *Alternaria* or a combination of 5µg c-di-GMP and 50µg of *Alternaria* for 3 days. After 3 days of challenge, mice were euthanized with BAL fluid and lung being collected. Frequency (A) and cell totals (B) of eosinophils in the lung. Frequency (C) and cell totals (D) of neutrophils in the lung.

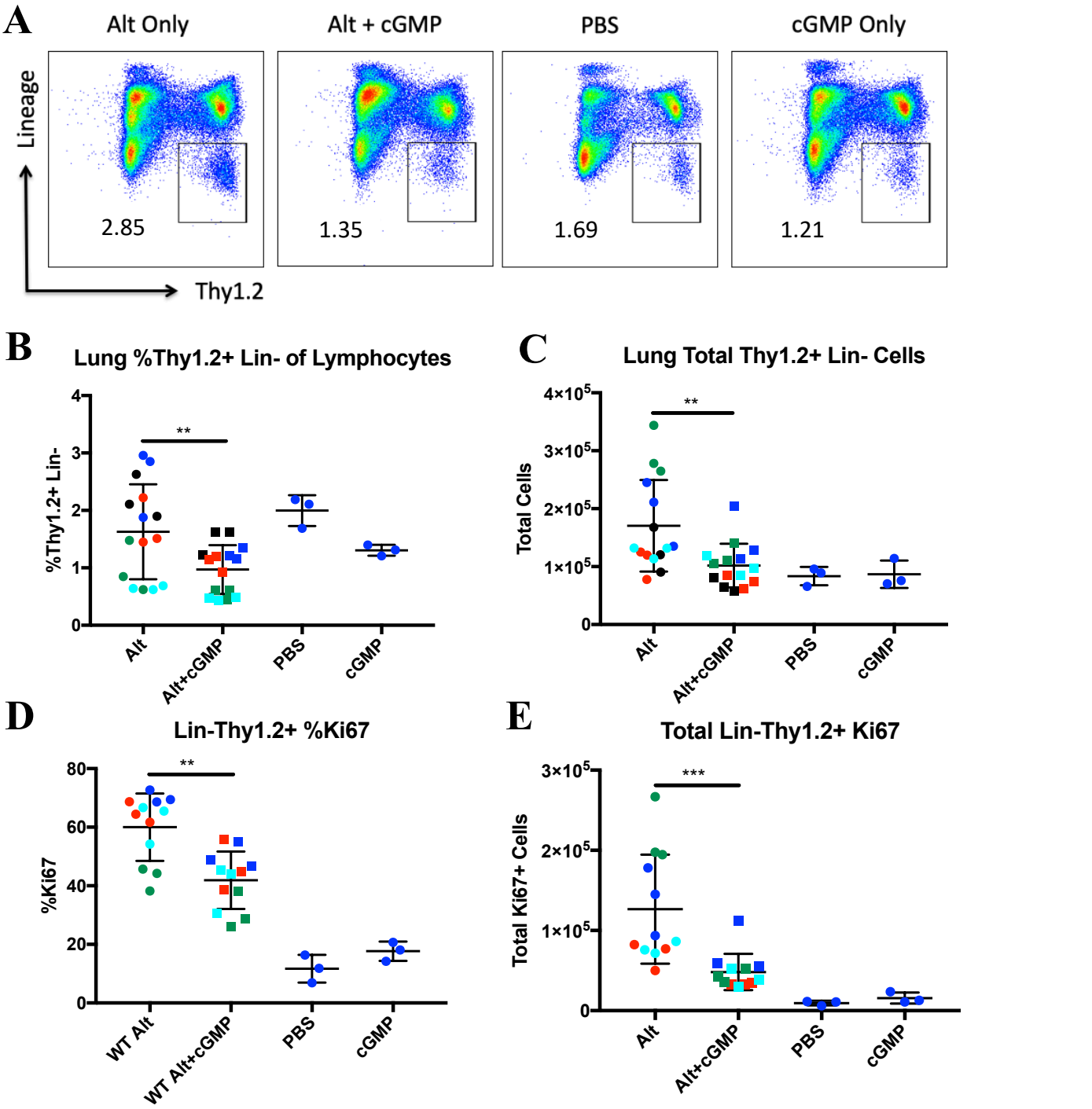


Figure 4: c-di-GMP decreases ILC2 cell totals, frequencies and abates ILC2 proliferation. Wild type mice were challenged with either PBS, c-di-GMP, *Alternaria* or a combination of *Alternaria* and c-di-GMP. The mice were intranasally challenged for three consecutive days and sacrificed the next day. (A) Gating scheme for ILC2s, defined as lineage- and Thy1.2+ in the lymphocyte population. Representative plots for each group are shown. (B) Frequency of lineage-thy1.2+ lymphocytes between groups. (C) Total numbers of ILC2s in the lung between groups. (D) Frequency of Ki67+ ILC2s. (E) Total numbers of Ki67+ ILC2s.

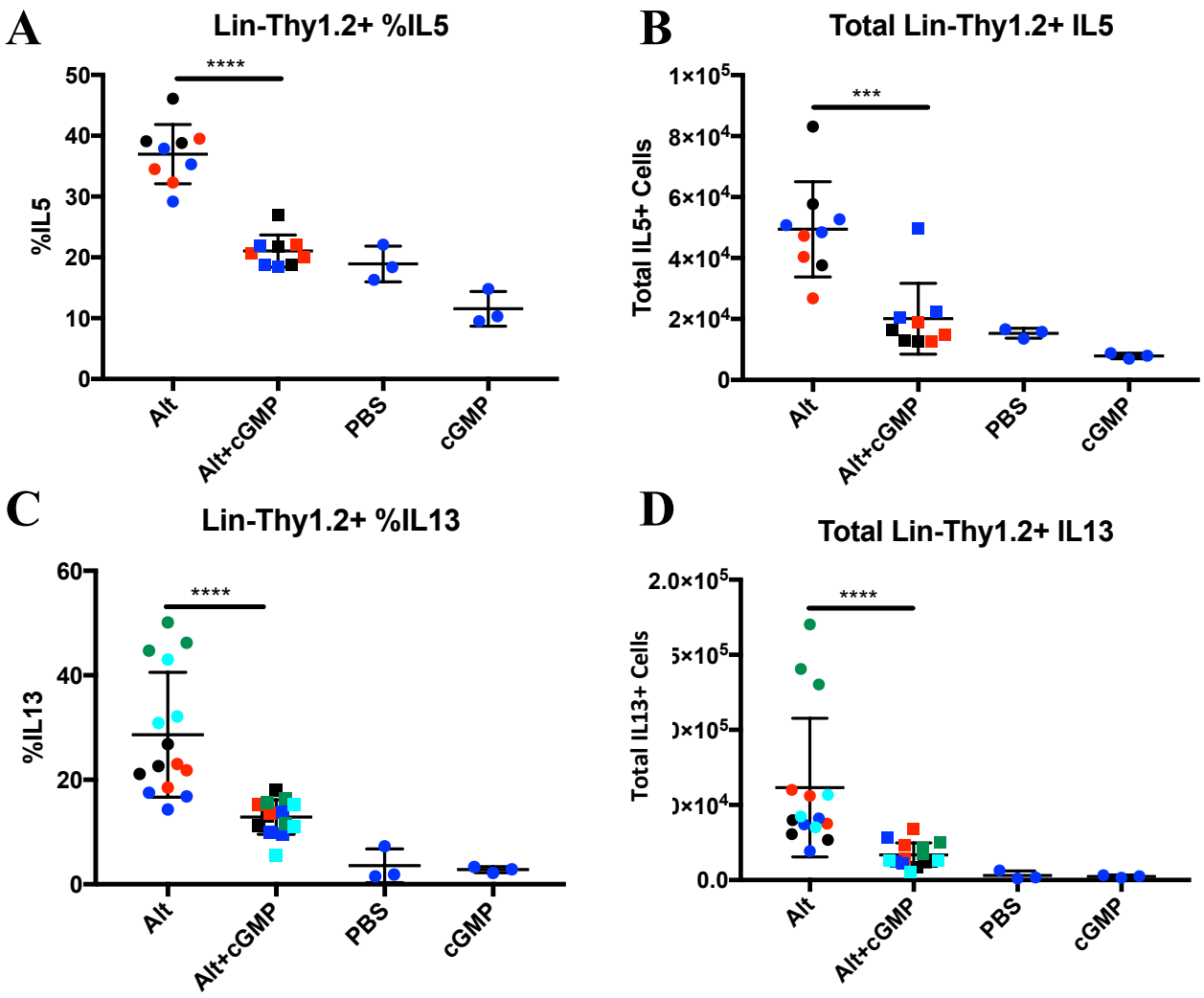


Figure 5: c-di-GMP decreases ILC2 intracellular type 2 cytokine production. Mice were challenged according to figure 2. (A) IL5+ ILC2 frequency. (B) Total IL5+ ILC2 cells. (C) IL13+ ILC2 frequency. (D) Total IL13+ ILC2 cells.

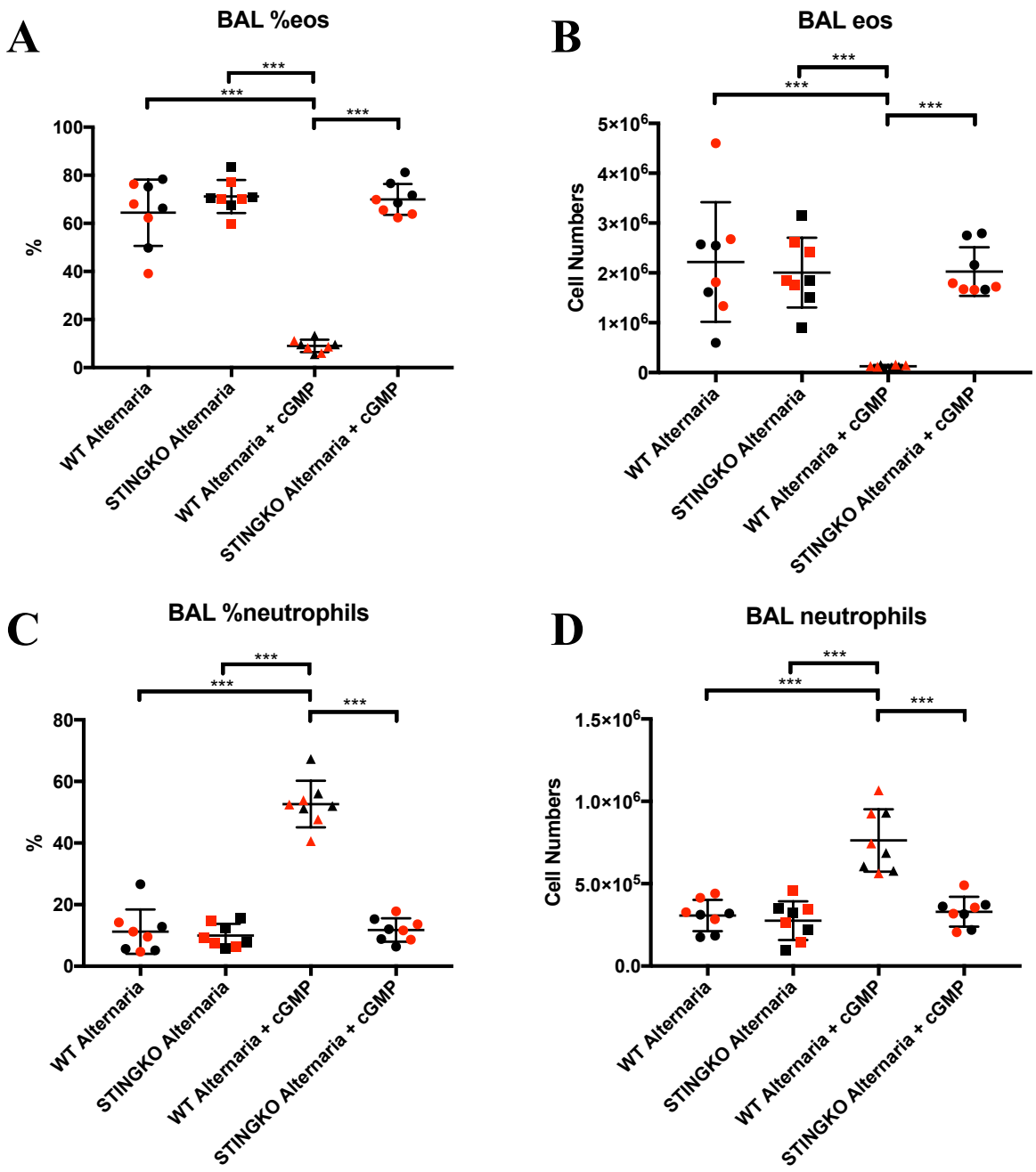


Figure 6: c-di-GMP utilizes STING-dependent pathway to suppress eosinophilia in BAL fluid. Mice were challenged with *Alternaria* or *Alternaria* and c-di-GMP according to the timeline in Figure 2A. The frequency of BAL fluid eosinophilia (A) and BAL fluid eosinophil cell totals (B). The frequency of BAL fluid neutrophils (C) and BAL fluid neutrophils (D).

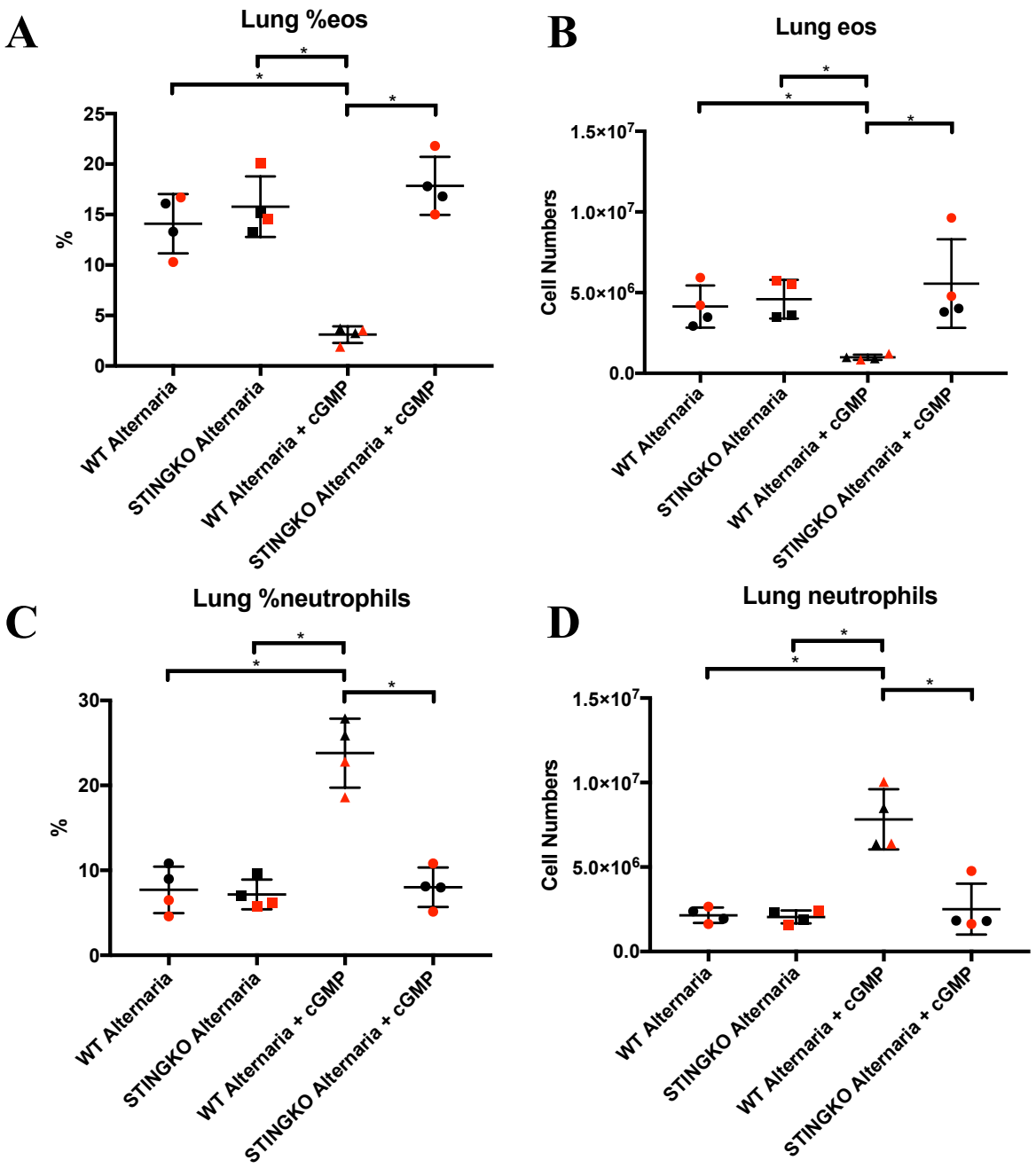


Figure 7: c-di-GMP utilizes STING-dependent pathway to suppress eosinophilia in the lung. Wild type and STINGKO mice were challenged with *Alternaria* or *Alternaria* and c-di-GMP according to the timeline in Figure 2A. The frequency of lung eosinophilia (A) and lung eosinophil cell totals (B). The frequency of lung neutrophils (C) and lung neutrophils (D).

Figure 8: c-di-GMP increases unconventional ILC2 subpopulations in a STING-dependent manner. Wild type and STINGKO mice were challenged with *Alternaria* or *Alternaria* and c-di-GMP according to the timeline in Figure 2A. ILC2s were defined as Lineage- Thy1.2+ lymphocytes. (A) ILC2 frequency and (B) totals. (C) ILC2 gating scheme, with ILC2 conventional ILC2s being CD127+ ST2+ cells. (D) ST2-CD127+ ILC2 frequency and (E) ST2-CD127+ ILC2 totals. (F) ST2+CD127+ ILC2 frequency and (G) ST2+CD127+ ILC2 totals. (H) ST2+CD127- ILC2 frequency and (I) ST2+CD127- ILC2 totals. (J) ST2-CD127- ILC2 frequency and (K) ST2-CD127- ILC2 totals.

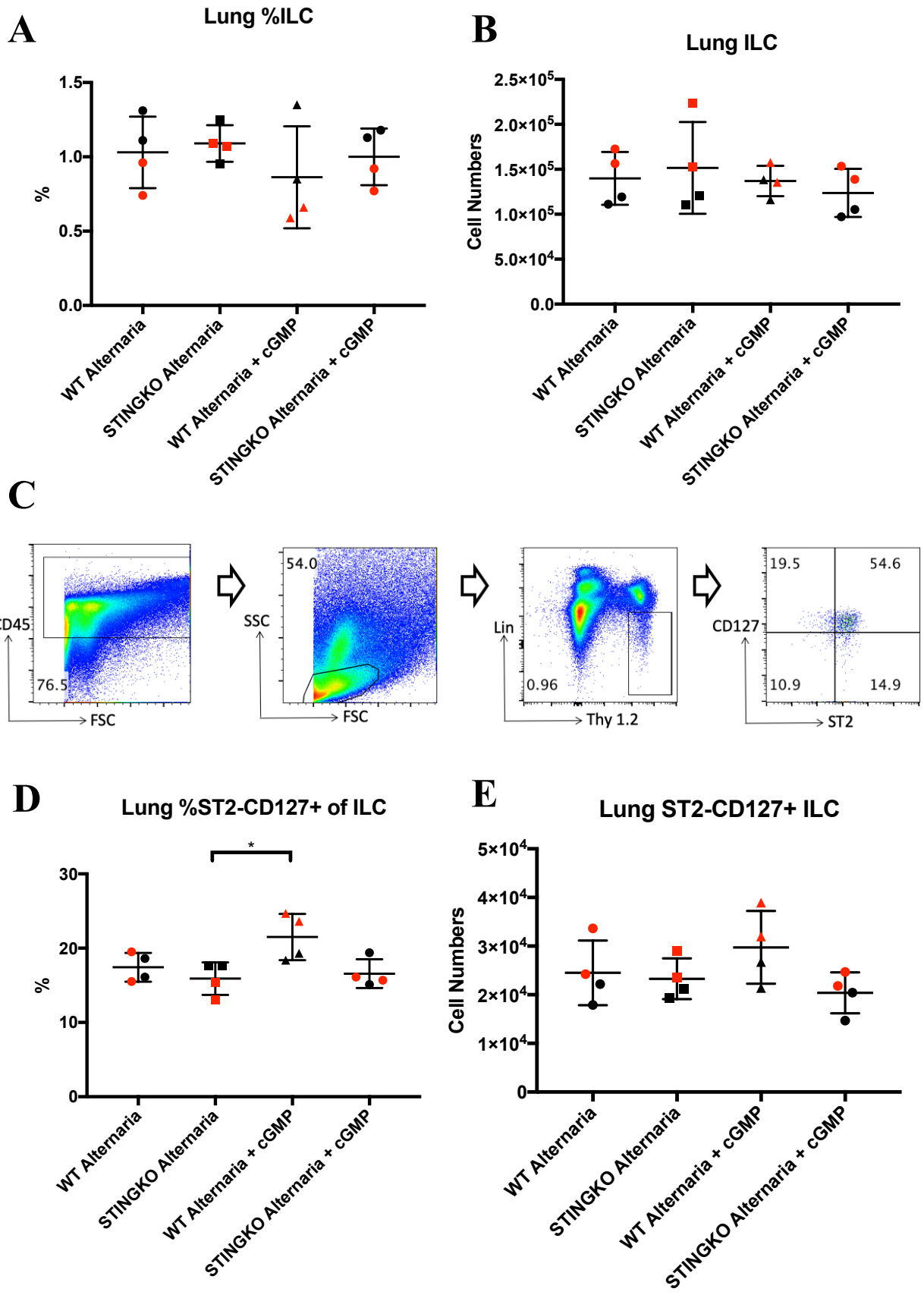

A
Lung %ILC
%
1.5
1.0
0.5
0.0
WT Alternaria
STINGKO Alternaria
WT Alternaria + cGMP
STINGKO Alternaria + cGMP
B
Lung ILC
Cell Numbers
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
C
CD45
76.5
FSC
SSC
54.0
Lin
0.96
Thy 1.2
CD127
19.5
54.6
10.9
14.9
ST2
D
Lung %ST2-CD127+ of ILC
*
30
20
10
0
E
Lung ST2-CD127+ ILC
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4

Figure 8: Continued

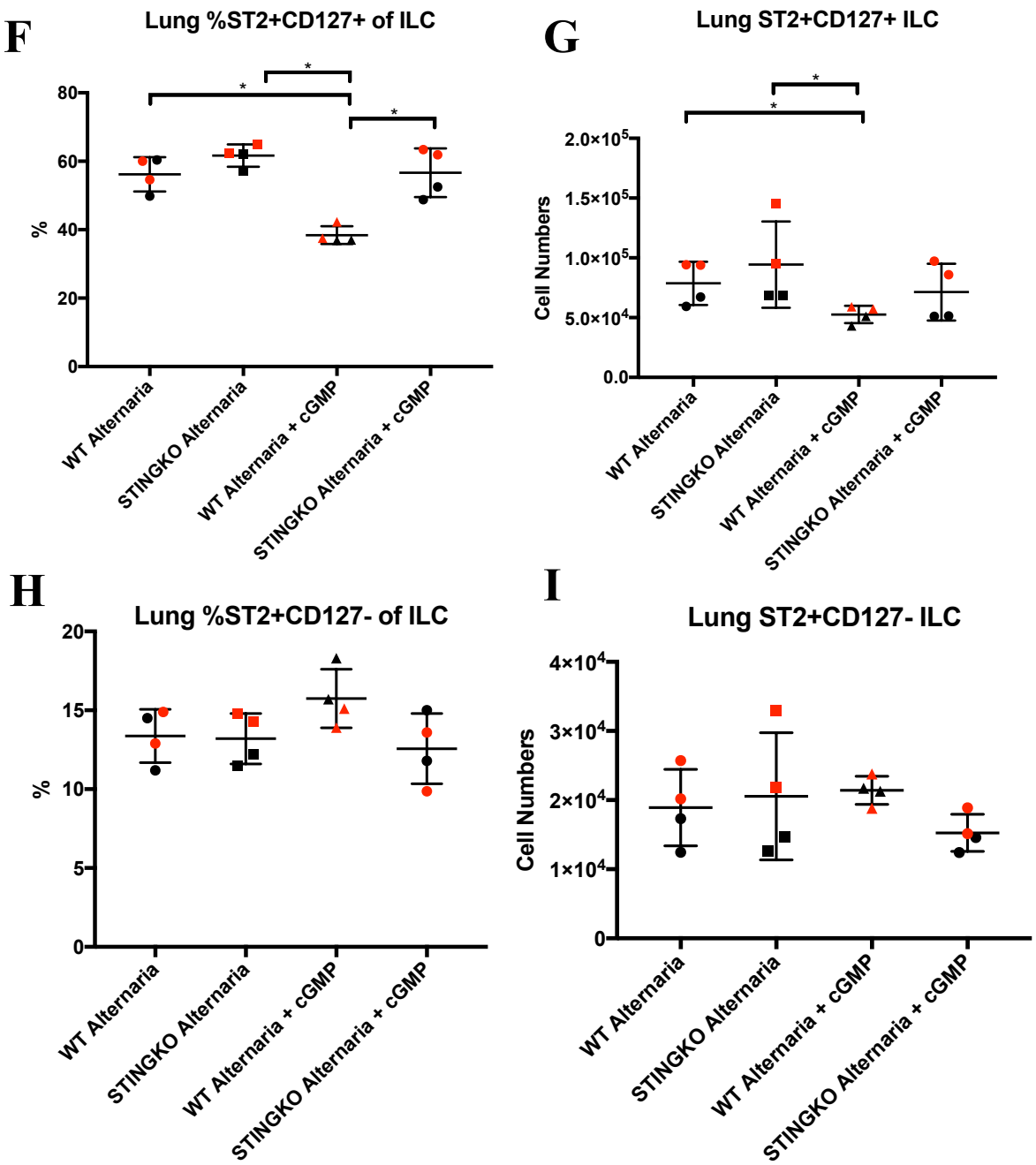
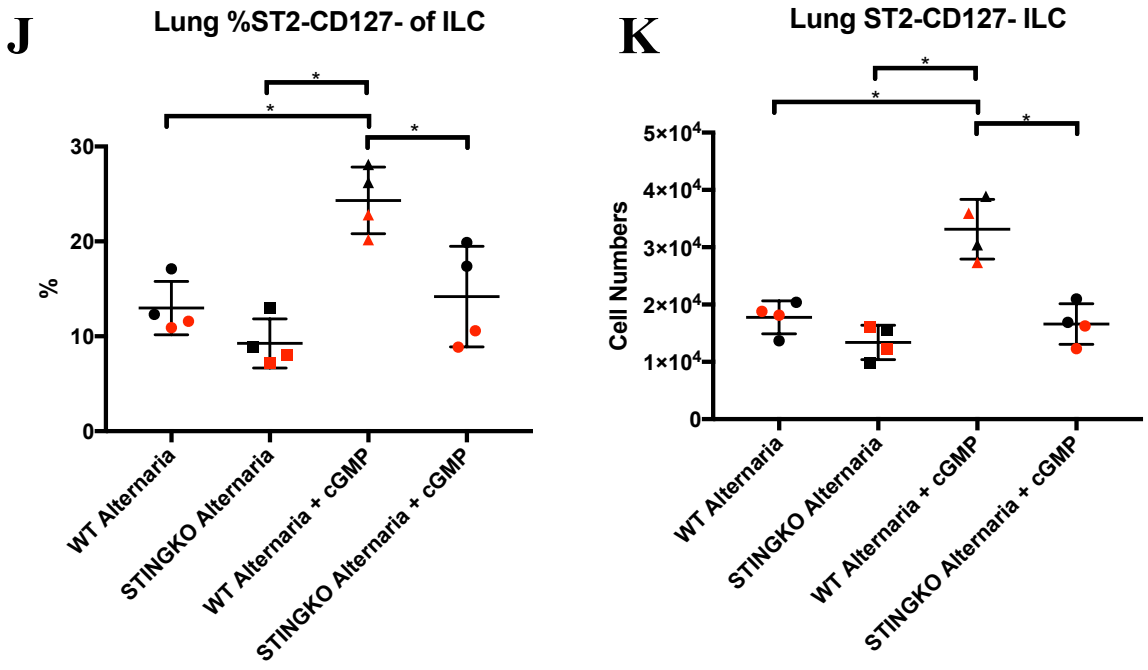


Figure 8: Continued



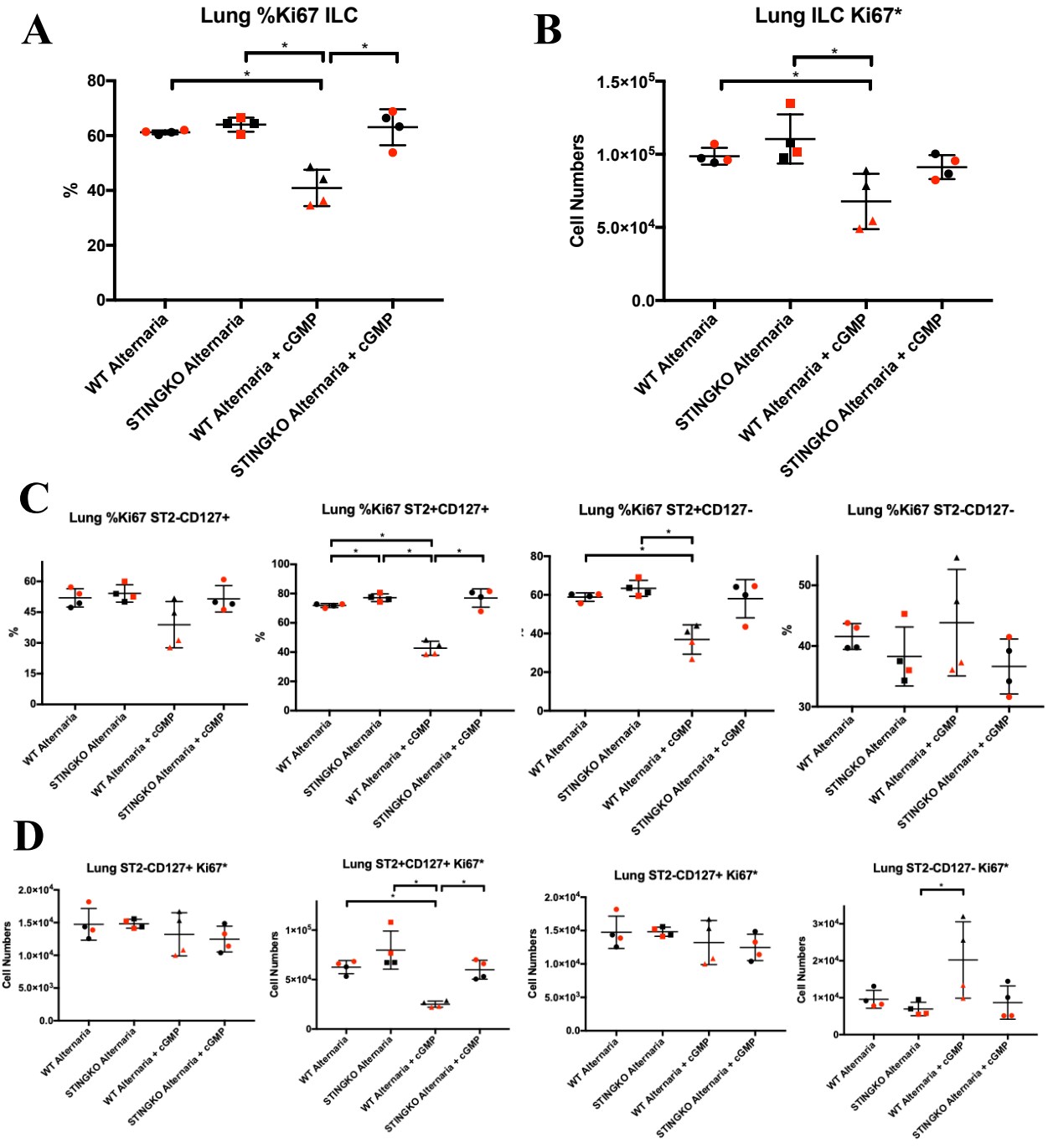


Figure 9: c-di-GMP attenuates ILC2 proliferation. Wild type and STINGKO mice were challenged with *Alternaria* or *Alternaria* and c-di-GMP according to the timeline in Figure 2A. ILC2s were defined as Lineage- Thy1.2+ lymphocytes. (A) Ki67+ ILC2 frequency and (B) totals in ILC2 subpopulations. (C) Ki67+ ILC2 subpopulation frequency and (D) totals in ILC2 subpopulations.

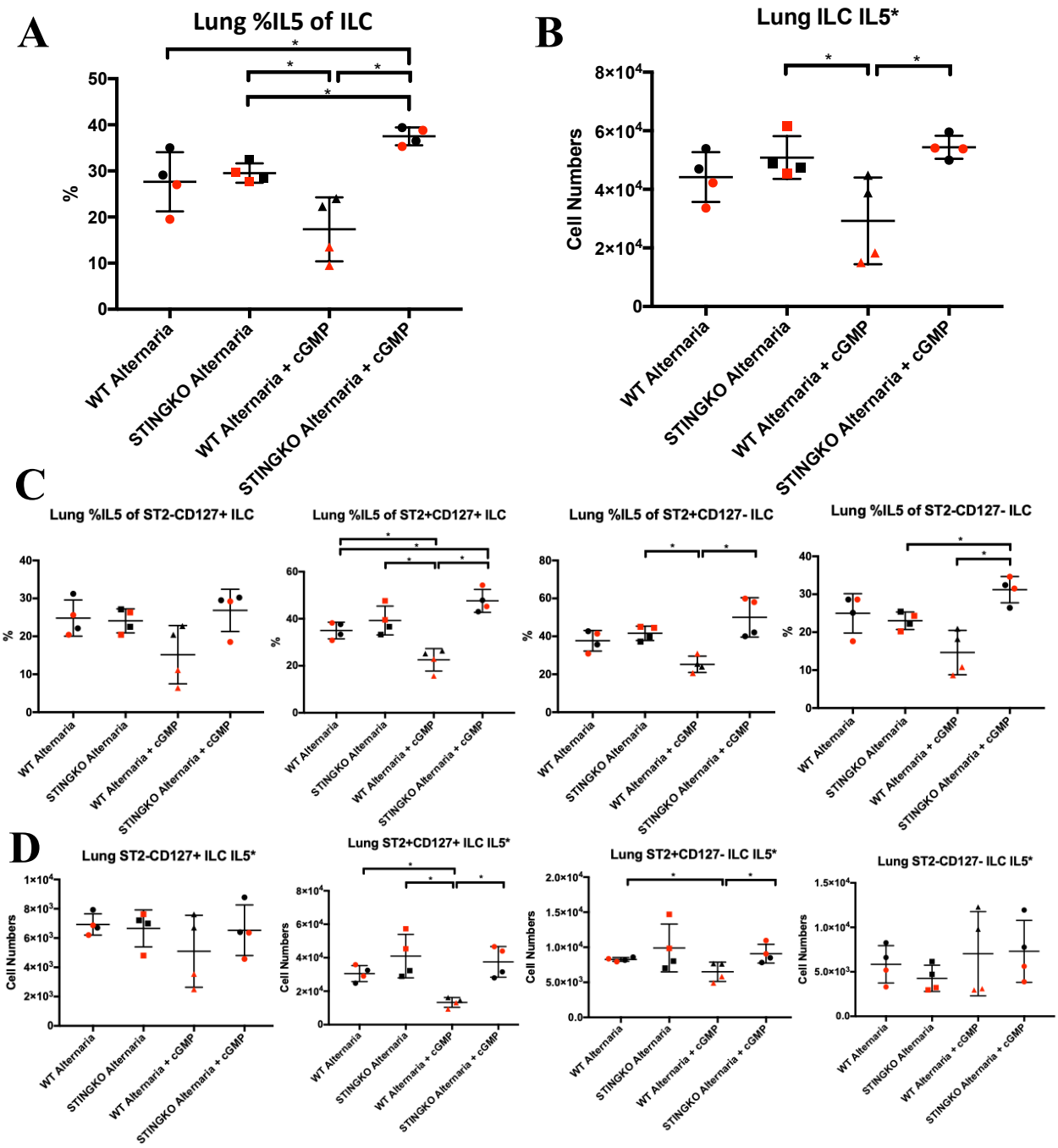


Figure 10: c-di-GMP inhibits intracellular IL5 production through the STING pathway. Wild type and STINGKO mice were challenged with *Alternaria* or *Alternaria* and c-di-GMP according to the timeline in Figure 2A. ILC2s were defined as Lineage- Thy1.2+ lymphocytes. (A) IL5+ lineage- Thy1.2+ lymphocyte frequency and (B) totals. (C) IL5+ ILC2 frequency and (D) totals in ILC2 subpopulations.

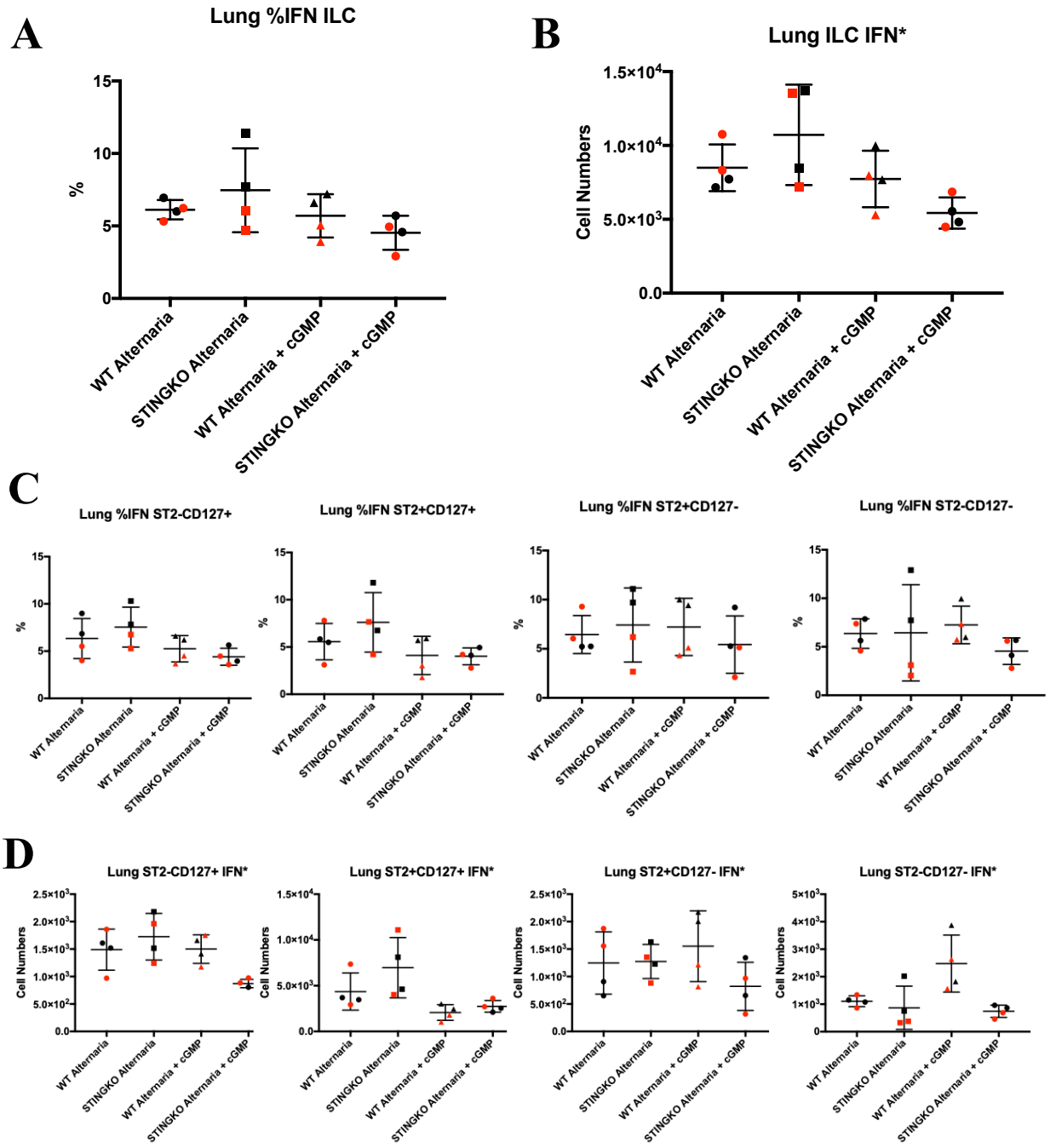


Figure 11: c-di-GMP uses the STING pathway to increase intracellular IFNɣ production in ILC2s. Wild type and STINGKO mice were challenged with *Alternaria* or *Alternaria* and c-di-GMP according to the timeline in Figure 2A. ILC2s were defined as Lineage- Thy1.2+ lymphocytes. (A) IFNɣ+ lineage- Thy1.2+ lymphocyte frequency and (B) totals. (C) IFNɣ+ ILC2 frequency and (D) totals in ILC2 subpopulations.

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