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Early View

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Innate lymphoid cells in the upper airways; importance of CD117 and IL-1RI expression

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Take home" message: CD117 expression in human ILC2s depends on the local tissue milieu and IL-1RI expression is not ubiquitous in ILC2s

ABSTRACT

Although innate lymphoid cells (ILC)1s, ILC2s and ILC3s are emerging as important cell populations regulating tissue homeostasis, remodelling and inflammation, a vast majority of our knowledge stems from *in vitro* and murine experiments, and requires thorough confirmation in human diseases.

Relative levels of ILCs were evaluated by means of flow cytometry in freshly resected human upper airways mucosa of patients with chronic rhinosinusitis (CRS) without nasal polyps (NP) (CRSsNP) and with nasal polyps (CRSwNP), taking into account the patient's clinical parameters and disease comorbidities.

We report that the CD117 and IL-1RI expression status of human ILC2s depends on the local tissue environment. Only $CD117^+$ $IL\text{-}1RI^+$ ILC2s - exclusively present in CRSwNP - possess an interrelationship with Th2 cytokine and eosinophil levels in human upper airway mucosa. In CRSsNP, mainly $CD117^-IL\text{-}1RI^-$ ILC2s are increased, yielding lower eosinophilia in this disease despite the high levels of ILC2s.

These data unveil that the $CD117^-$ and $CD117^+$ fractions within the native human ILC2 population are not a random phenomenon in contrast to what could be concluded from in vitro data, and that the IL-1RI expression is not ubiquitous in ILC2s in vivo in humans, which cannot be assessed via in vitro and murine experiments.

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INTRODUCTION

Chronic rhinosinusitis (CRS) is a heterogeneous group of chronic airway inflammatory diseases affecting the mucosa of the nasal and paranasal cavities and is either accompanied by polyp formation (CRSwNP) or is presented without polyps (CRSsNP) [1]. CRS shows a high prevalence of up to 15% in adults, often leading to the subsequent development of lower respiratory tract conditions, especially asthma [2]. CRSsNP and CRSwNP are distinct disease entities with CRSwNP being associated with a T-helper 2 (Th2)-skewed eosinophilic inflammation with high interleukin (IL)-5 and eosinophil cationic protein (ECP) concentrations in the polyps, while CRSsNP is characterized by a more predominant Th1 milieu with pronounced levels of interferon (IFN)-γ in the inflamed ethmoidal mucosa [1]. Furthermore, novel evidence indicates considerable heterogeneity within the CRSwNP subgroup, determining the risk of co-morbid asthma. Despite continuous research progress that contributes to further unveil the pathophysiology of these chronic airway conditions, the etiology remains poorly understood and appears to be multifactorial.

Over the last years a new class of innate effector cells whose development relies on signalling through the IL-2 receptor (IL-2R) common γ chain and IL-7Rα (CD127) has drawn much attention. These cells are referred to as helper innate lymphoid cells (ILCs) [3-5]. In contrast to T and B cells, this ILC lineage does not express recombined, antigen-specific receptors and is substantially represented at barrier surfaces. Based on the expression of lineage-defining transcription factors and on their functional similarities to T helper cells, 3 subsets of helper ILCs can be discriminated, being arranged over 3 global ILC groups.

Group 1 ILCs include besides an IFN-γ-secreting helper ILC subset, referred to as ILC1s, also the cytotoxic conventional natural killer (cNK) cells and intraepithelial type 1 innate lymphoid cells (ieILC1s). ILC1s produce IFN-γ on activation with IL-12, IL-15, and IL-18. They have been shown to be implicated in the defence against viruses, bacteria, and protozoa. ILC1s are reported to be predominantly present in chronically inflamed mucosal tissues, while being absent under normal physiology. [6] Group 2 ILCs currently only consists of ILC2s that secrete the typical Th2 cell-associated cytokines IL-4, IL-5, IL-9 and IL-13. They also produce amphiregulin, a member of the epidermal growth factor family involved in limiting and repairing tissue damage. ILC2s are involved during early immune responses to helminth infection, but are also important for allergen-induced airway inflammation and tissue repair. ILC2s can be activated by various cytokines, such as IL-33, IL-25, thymic stromal lymphopoietin (TSLP), and IL-1β, as well as lipid mediators such as eicosanoids, including prostaglandin D2 (PGD2) and leukotriene D4 (LTD4) [7]. Group 3 ILCs again comprise different populations including lymphoid tissue inducer (LTi) cells, natural cytotoxicity receptor $(NCR)^{+}$ ILC3s and NCR^{-} ILC3s. The NCR^{+} and NCR^{-} ILC3 fractions produce IL-22 and IL-17, respectively, and are regarded as defenders of mucosal barrier function implicated in immunity against extracellular bacteria and autoimmune diseases. Both cell populations produce cytokines upon activation by IL-1β and IL-23 [4, 5]. LTi cells are important in formation of lymph nodes during fetal development [8].

Upon their initial characterization, ILC2s were reported to be abundantly present in the upper airway mucosa of CRSwNP patients [9], and sinonasal tissues became popular to translate mechanistic findings into the research of ILCs. However, most translational parts of these studies were performed on a low number of patients reporting no or limited clinical data, consequently also not taking into account the potential effect of comorbidities [9-11].

Moreover, the presence of the other ILC subpopulations in upper airway mucosa of CRSsNP patients has received less attention.

In the present report we show that in non-inflamed conditions, ILC1s are the most prominent ILCs in sinonasal tissue. In CRSwNP, ILC1s are decreased while ILC2s per se are not increased. Focusing on $CD117^{+}$ and $IL\text{-}1RI^{+}$ fractions within the ILC2s, and taking comorbidities into account, we unveil the precise contribution of ILC2s in CRSwNP; we describe that $CD117^{+}$ $IL\text{-}1RI^{+}$ ILC2s levels are explicitly increased in CRSwNP, showing an interrelationship with Th2 cytokine and eosinophil levels. In CRSsNP, mainly $CD117^{-}IL\text{-}1RI^{-}$ ILC2s are increased, yielding lower eosinophilia in this disease despite the high levels of total ILC2s.

METHODS

Patients

The patients' clinical data can be found in Table 1. More detailed information can be found in the Online Data Supplement.

Flow cytometry staining

Tissue native ILCs were gated as living, lymphogate $CD45^+$ lineage negative ($CD3^-CD11c^-CD14^-CD19^-CD34^-BDCA2^-FcεR1^-CD94^-CD1a^-CD123^-TCRαβ^-$) cells. ILC1 were further phenotyped as $CD127^+CRTH2^-CD117^-NKp44^-$, ILC2 as $CD127^+CRTH2^+$, and ILC3 as $CD127^+CRTH2^-CD117^+IL\text{-}1RI^+NKp44^-$ for the NCR^- fraction and $CD127^+CRTH2^-CD117^+IL\text{-}1RI^+NKp44^+$ for the NCR^+ fraction, as based on a previously reported gating strategy [12] and according to the gating strategy shown in Fig E1 in the Online Data Supplement. The levels of the different ILC subpopulations are expressed as percentage of the total living cells. See the Online Supplement for more methodological details.

In vitro assessment of the functional responses by freshly isolated ILC2s

The single cells were re-suspended in tissue culture medium supplemented by IL-2 (5ng/ml). The cells were activated with 50 ng/ml PMA (Sigma) and 1 µg/ml ionomycin (Sigma) for 3 hours at 37°C in the presence of 5% CO_2. 1 hour after the start of the PMA/iono treatment, Brefeldin A (5µg/ml) (ebioscience) was added. The samples were subjected to flow cytometry staining as described in the main document and the online supplement.

Tissue homogenates

Frozen tissue samples from the same subject used for ILC flow cytometry were homogenised by means of mechanical disruption as described in the Online Data Supplement.

Measurement of inflammatory mediators

The inflammatory mediators IL-4, IL-5, IL-12 and IL-25 were measured on tissue homogenates by means of Luminex xMAP technology using the Fluorokine MAP Multiplex Human Cytokine kit (R&D Systems, Minneapolis, MN, USA) on a Bio-PlexTM 200 Array Reader (Bio-Rad, Hercules, CA, USA). Tissue ECP levels were measured by UniCAP (Phadia, Uppsala, Sweden)

Statistical analysis

Statistical analysis was performed by nonparametric Mann-Whitney U test. A P-value less than 0.05 was considered to be statistically significant (GRAPHPAD, San Diego, CA, USA).

RESULTS

Relative levels of ILCs in human upper airway mucosa of controls, CRSsNP and CRSwNP

[9-11]

Because the levels of $CD45^+$ cells were higher in CRSsNP and CRSwNP than in controls (Fig E2 A), while the number of total livings cell were similar between the different subject groups (Fig E2 B), the flow cytometry data were expressed as a % of total living cells. In order to allow the reader to compare the levels of the different ILC groups to other literature sources where ILCs might be expressed as a % of $CD45^+$ cells, in this section the results are presented in both ways.

Members of all 3 ILC groups were present in human upper airway mucosa of all patient groups. In non-inflamed control conditions, ILC1s represent the most prominent subset, followed by NCR^- ILC3s, while ILC2s and NCR^+ ILC3s showed the weakest abundance (Fig 1 and E3). In the CRSsNP patient group, a significant increase in ILC2 and NCR^- ILC3 population was observed compared to the mucosa of control subjects, whereas the levels of ILC1 and NCR^+ ILC3 cells were similar (Fig 1 and E3). The inflamed mucosa of patients with CRSwNP contained significant lower levels of ILC1s compared to non-inflamed control mucosa, while the ILC2 and NCR^- ILC3 populations were significantly increased. NCR^+ ILC3 cells maintained a low level of abundance, which was similar to control mucosa (Fig 1 and E3).

ILCs in upper airway mucosa of controls, CRSsNP and CRSwNP: the effect of comorbidities

As the frequently occurring CRS comorbidities allergy and asthma can potentially affect the levels of the ILC subsets in the mucosa, we also took these comorbidities into account.

Within the non-inflamed upper airway mucosa of control individuals and within the CRSsNP patient group, the presence of allergy, asthma and allergic asthma had no influence on the number of any ILC subset (Fig 2, 3 and 4).

The significant reduction in the levels of ILC1 cells that was observed in the undifferentiated CRSwNP patient group (Fig 1) can be attributed to the reduced numbers of ILC1s in CRSwNP patients without comorbidities and those suffering from comorbid allergy or comorbid asthma (Fig 2). However, and importantly, CRSwNP patients with comorbid allergic asthma showed no reduction in levels of ILC1 compared to the equivalent control patients with allergic asthma but without chronically inflamed upper airway mucosa (Fig 2).

Unexpectedly, the levels of ILC2s were also significantly higher in the Th1 cytokine-skewed disease CRSsNP in comparison to the respective control patient groups. Moreover, the different comorbidities had no effect on the levels of ILC2 cells (Fig 3). ILC2s are not per se involved in the pathophysiology of CRSwNP because the inflamed mucosa of CRSwNP patients without comorbidities contained a similar amount of ILC2s compared to non-inflamed mucosa of control subjects (Fig 3). The levels of ILC2s were clearly increased in patients with comorbid allergy and asthma, with allergic asthma being the most pronounced (Fig 3).

While the NCR^- ILC3 levels were slightly but significantly increased in the undifferentiated CRSsNP and CRSwNP patient groups compared to controls (Fig 1), when comorbidities were taken into account, a variable outcome could be observed only yielding statistical significant higher levels for CRSsNP patients without comorbidities and CRSwNP with

comorbid asthma (Fig 4A). The levels of NCR^+ ILC3 overall remained very low in all patient groups with no influence by comorbidities (Fig 4B).

CD117 and IL-1RI expression in the ILC2 population.

Next we assessed the relative levels of CD117 positive and negative fractions within the ILC2 population [3, 5, 9] and investigated the expression of IL-1RI on ILC2 cells in view of the contrasting reports present in the literature [5, 10, 13-15].

Regardless of the presence or absence of allergy, asthma or allergic asthma, within the control subjects and the CRSsNP patient group, the majority of the ILC2 cells were $CD117^-$, being statistically significant higher than the $CD117^+$ cells, the latter virtually being absent (Fig 5A). In contrast, in the inflamed mucosa of CRSwNP without comorbidities, the $CD117^+$ ILC2 fraction was increased, while the $CD117^-$ fraction remains on the level found in controls, yielding two fractions lacking statistical differences (Fig 5A). The levels of both $CD117^+$ and $CD117^-$ ILC2 fractions in CRSwNP further increased under the influence of comorbid allergy and asthma (Fig 5B and C), maintaining an similar amount of cells in both fractions, except for the mucosa of CRSwNP patients with comorbid allergic asthma which possesses significantly higher numbers of $CD117^-$ ILC2 compared to $CD117^+$ cells (Fig 5A).
Similar to what was observed for CD117, within the control subjects and the CRSsNP patient group, the majority of the ILC2 cells belonged to the $IL\text{-}1RI^-$ fraction. This fraction was consistent significantly more pronounced than the $IL\text{-}1RI^+$ fraction, which was virtually absent (Fig 6A). The $IL\text{-}1RI^-$ ILC2 fraction was not affected by comorbid allergy and/or asthma (Fig 6B). For CRSwNP, the number of $IL\text{-}1RI^+$ cells was clearly higher in patients with comorbid allergy and/or asthma (Fig 6C), ending up with similar levels of $IL\text{-}1RI^+$ and $IL1R^-$ ILC2s in CRSwNP patients with allergy or with asthma (Fig 6A). Remarkably, the

effect of allergy and asthma on the level of IL-1RI^{+} ILC2s did not add up in allergic asthmatics, showing similar levels as observed for allergic CRSwNP and asthmatic CRSwNP patients (Fig 6C), while the levels of IL-1RI^{-} ILC2s were increased in patients with comorbidities, being most pronounced in allergic asthma (Fig 6B). Neither IL-1RI^{-} nor IL-1RI^{+} ILC2 cells were manifestly increased as a consequence of the pathological process of CRSwNP without comorbidities (Fig 6B and C).

Representative figures of the gating step assessing the expression of CD117 and IL1RI on ILC2 cell population in controls (upper panel), CRSsNP (middle panel) and CRSwNP (lower panel) all suffering from comorbid allergic asthma can be found in the Online Data Supplement (Fig E4). Both the CD117^{+} and CD117^{-} ILC2 fraction contained IL-1RI^{+} and IL-1RI^{-} subpopulations (Fig E5 in the Online Data Supplement).

In vitro assessment of the functional responses by freshly isolated ILC2s

See the online supplement

Tissue cytokine levels

The levels of IL-4 were slightly higher in CRSsNP and CRSwNP compared to controls, not reaching statistical significance (Fig 7A). Within the CRSwNP patient group, the levels of IL-4 were similar for each comorbidity status (fig 7A); consequently showing no parallelism with the number of ILC2s in CRSwNP (Fig 3, 5 and 6).

IL-5 levels were significantly increased in all CRSwNP comorbidity subgroups versus the corresponding control subject groups. Within the CRSwNP patients, subjects with comorbid allergy, asthma and allergic asthma had significantly higher IL-5 levels than patients without these comorbidities. CRSsNP patients showed no significant differences (fig 7B).

Tissue ECP levels were significantly higher in all CRSwNP comorbidity subgroups in comparison to the corresponding control subject groups. Within the CRSwNP patients, subjects with comorbid allergy, asthma and allergic asthma had significantly higher ECP levels than patients without these comorbidities. The levels observed in CRSsNP were also increased versus controls but only reaching significance for the patient group without comorbidities (fig 7C).

IL-25 was only measurable in CRSwNP, yielding similar levels for every comorbidity status (fig 7D). IL-12 was below the detection limit for all samples (data not shown).

DISCUSSION

The observation that from the 3 ILC groups present, ILC1s are the most abundant in non-inflamed tissues argues against the literature dogma that ILC1 cells are exclusively present under inflammatory conditions[6]. While this might be true for gastrointestinal tissues [16, 17], this appears not transposable to the airway mucosa. Moreover, the opposite seems to be true because the inflamed CRSwNP mucosa shows lower levels of ILC1s than control mucosa. As CRSwNP is particularly known as a Th2 cytokine-skewed eosinophilic inflammatory condition with high IL-5 and eosinophil cationic protein (ECP) concentrations in the polyps [1], reduced ILC1 levels seem to be consistent with this. However, while in the undifferentiated CRSwNP group this Th2 cytokine signature could be further corroborated by accompanying increased ILC2 levels, differentiating the CRSwNP patient group on the basis of the different comorbidities indicates that the ILC2 population is not implicated in the pathophysiology of CRSwNP per se. Indeed, the levels of ILC2s in the mucosa of CRSwNP patients without comorbid allergy and/or asthma are not higher than in controls. However, ILC2 levels are increased in the inflamed mucosa of CRSwNP patients suffering from comorbid allergy and/or asthma. From these results, we speculate that in previous studies, in which the authors oppositely claimed striking amounts of ILC2s in inflamed nasal polyps [9-11], mainly CRSwNP patients suffering from comorbid allergy and/or asthma must have been included, but these clinical data were not communicated. Our results once again emphasise the importance of detailed reporting and analysis of the patients' clinical data in order to make correct interpretations.

Opposite to CRSwNP, CRSsNP is characterized by a predominant Th1 milieu with pronounced levels of IFN-γ in the inflamed ethmoidal mucosa, histologically showing a fibrotic status of the inflamed tissue [1] with increased levels of transforming growth factor-beta (TGF-β) as a critical factor involved in this remodelling process [18]. Despite of this

Th1 signature, we here show that the mucosa of CRSsNP patients shows similar levels of ILC1s as observed for control subjects. Notably, the mucosa of CRSsNP patients retains clearly increased levels of ILC2s, but - in contrast to CRSwNP- the different comorbidities play no role herein. Recently, Forkel at al. reported a role for ILC2s in human liver fibrosis [19], which corroborates a previously suggested mechanism involving ILC2s in fibrosis development in the mouse liver [20]. These functional characteristics of ILC2s might be brought in agreement with the fibrotic status of the inflamed CRSsNP tissue, providing a possible functional explanation for the increased levels of ILC2s in CRSsNP. Indeed, it has been reported that TGFβ, an impotent mediator in the generation of fibrosis, enhances migration of ILC2s [21], supporting the increased presence of ILC2s in a Th1 disease such as CRSsNP. Moreover, TGF-β has recently been reported to directly inhibit IL-33-induced IL-5 and IL-13 production in ILC2s [22, 23].

The question then arises why ILC2s do not contribute to fibrosis in CRSwNP which displays an oedematous status. We propose that ILC2s in CRSsNP do not per se possess identical tissue-residency phenotypic properties than those present in CRSwNP tissue. Indeed, while $CD117^-$ ILC2 can be found in control, CRSsNP and CRSwNP patient groups, our data show that the $CD117^+$ ILC2 population is virtually absent in controls and CRSsNP, whereas it is explicitly present in the inflamed mucosa of CRSwNP patients. Thus the increased levels of the $CD117^+$ ILC2s can be attributed to CRSwNP pathology, however yielding the most pronounced expansion in combination with allergy or asthma. With regard to the functional implications for ILC2s in CRSwNP, CD117 -also known as c-kit- is a receptor for stem cell factor (SCF) which is best known for its essential role in haematopoietic stem cell proliferation, survival and adhesion [24]. In a setting of diminished c-kit signals in mice, also ILC2s fail to develop [25]. In contrast to mice, in which mature ILC2 are reported to be

exclusively $CD117^+$ [4], in humans both $CD117^+$ and $CD117^-$ ILC2 populations are observed [4, 5], making functional interpretations less straightforward. Lim et al. observed no different levels in IL-13 production after PMA/iono stimulation of cultured blood-derived $CD117^-$ and $CD117^+$ fractions of ILC2s [26], but the authors also reported that the expression of CD117 is affected by the culturing conditions, in which $CD117^-$ cells can gain expression of CD117 and vice versa. Our results show that the phenotype of the native ex-vivo ILC2s is already altered after 3 hours culturing without active stimulation; ILC2s derived from CRSsNP - natively lacking CD117 expression- develop a $CD117^+$ fraction similar to what was natively observed in CRSwNP, while $CD117^+$ ILC2s derived from CRSwNP tissue downregulate surface CD117 and CRTH2 expression upon stimulation with PMA/iono. These results show that the surface expression status of ILC2s relies on the specific local tissue conditions inherent to the different pathologies and that the characteristics of the ILCs in different pathologies cannot be accurately assessed by means of in vitro approaches using cultured cells and artificial stimulation methods. Recent findings by Li et al. on murine ILC2s also indicate that ILC2s are phenotypically more heterogeneous than previously thought, whereby surface expression levels of various markers frequently used to identify ILC2s were dependent on their mode of activation, highly variable over time, and differed between tissue compartments [27]. Also, when carefully inspecting the data from Bal et al. [10], the ILC2 population obtained upon culturing of blood-derived ILC2s turned up to be entirely $CD117^+$. Moreover, these $CD117^+$ ILC2s were previously regarded -by the same research group- to be less responsive in comparison to $CD117^-$ ILC2s [9]. However, when the culture medium in their experiments was supplemented by IL-25, an equal amount of IL-13 was produced by both cell populations [9]. Because our data show that the levels of IL-25 are massively increased exclusively in the mucosa of CRSwNP patients, it can be assumed that the tissue specific environment of CRSwNP likewise yields functionally active $CD117^+$ ILC2s.

A second important phenotypic distinction between ILC2s present in CRSwNP and those in CRSsNP mucosa was found in the expression status of IL-1RI, the active receptor for IL1β. While CRSsNP and control tissues lack ILC2s that express the active IL-1β receptor, IL-1RI$^+$ ILC2s are clearly present in CRSwNP, where they are particularly high in patients with comorbid allergy or asthma. Initially, ILC2s have been indicated to unambiguously express IL-1RI [3, 5]. On the other hand, in a recent publication that assessed the heterogeneity of human CD127$^+$ innate lymphoid cells by means of single-cell RNA sequencing, Bjorklund et al. observed no expression of IL-1RI in ILC2s of uninflamed tonsil tissue [13]. We here report that in the mucosa of human upper airways, the level of IL-1RI expression in ILC2s is dependent on the specific local tissue conditions inherent to the different pathologies or the normal mucosal airway physiology. Indeed, in controls and CRSsNP -regardless of the absence or presence of any comorbidities- virtually all ILC2s lack the expression of IL-1RI. In the CRSwNP patient group, IL-1RI expression is clearly higher in patients with comorbid allergy and/or asthma. Because ILC2 cells derived from peripheral blood already have a substantial surface expression of IL-1R1 [15], our data -corroborated by the lack in IL-1RI expression in uninflamed tonsil tissue as reported by Bjorklund et al. [13]- allows us to postulate that IL-1RI expression is downregulated in ILC2s at local tissue level under normal physiology and CRSsNP, probably by the lack of a specific trigger that maintains IL-1RI expression or by the presence of a trigger that downregulates its expression. Alternatively, the local mucosal tissue milieu of controls and CRsNP might only attract IL-1RI$^-$ ILC2s from the blood while the tissue inflammatory environment in CRSwNP also enables the chemotaxis of the IL-1RI$^+$ subset. Regardless of which rationale might be valid, parabiosis experiments indeed showed that ILCs residing in the non-lymphoid tissues are primarily maintained through self-renewal with minimal contribution from hematogenous precursors and also do

not redistribute systemically [28], further corroborating that the local tissue environment is able to fine-tune the different ILC subsets into their final phenotype. What exactly triggers this clear-cut expression of the IL-1RI in CRSwNP, but not in CRSsNP, remains subject for further investigation. Functionally, IL-1RI engagement by IL-1β could directly affect human ILC2s by inducing low expression of the transcription factor T-BET and the cytokine receptor chain IL-12Rβ2, which subsequently allows IL-12 to induce the conversion of these activated ILC2s into an ILC1 phenotype [10, 15]. As we could not detect measurable IL-12 levels in this tissue under any disease status, the importance of this functional plasticity in the human upper airway mucosa turns out to be unsure. Phenotypically, IL-1β is reported to significantly enhance expression of the receptors for IL-25, IL-33 and TSLP on ILC2 cells and to potentiate their responses to these cytokines. IL-1β-treated ILC2 cells produce significantly more Th2 cytokines in response to IL-33 and to IL-25 than ILC2s that did not receive IL-1β [10, 15]. The lack of IL-1RI expression on ILC2s in CRSsNP and its presence on ILC2s in CRSwNP is in agreement with the absence and presence of substantial IL-5 production in the respective disease status.

When comparing the levels of ILC2s with the levels of Th2 mediators in our study, it is clear that the numbers of $CD117^-$ $IL\text{-}1RI^-$ ILC2s are not in relationship with the concentration shifts of IL-5 and ECP in the different disease status and comorbidities. Conversely, $CD117^+$ $IL\text{-}1RI^+$ ILC2s are the most likely to have contributed to the shaping of the Th2 environment in CRSwNP as could be concluded from the observation that this ILC2 subset clearly follows the pattern of IL-5 levels and the number of eosinophils that were assessed by means of ECP concentrations. The latter data corroborate a previous study that showed an interrelationship between eosinophilia and ILC2 levels in CRSwNP patients [29]. Our data additionally show that this is probably primarily due to $CD117^+$ $IL\text{-}1RI^+$ ILC2s, which also explains the lower

eosinophil levels in CRSsNP in comparison to CRSwNP despite the increased levels of total ILC2 population, being mainly $CD117^-$ $IL\text{-}1RI^-$ in CRSsNP.

Conclusion

We here show that the 3 ILC subsets are present in human upper airway mucosa. In non-inflamed conditions, ILC1s are the most prominent subset. In CRSwNP, ILC1s are decreased while ILC2s as such are not increased. However, focusing on the $CD117^+$ and $IL\text{-}1RI^+$ fractions within the ILC2s, and taking comorbidities into account, we unveil the precise contribution of the ILC2s in CRSwNP. We describe that only $CD117^+$ $IL\text{-}1RI^+$ ILC2s - exclusively present in CRSwNP - possess an interrelationship with Th2 cytokine and eosinophil levels in human upper airway mucosa. Indeed in CRSsNP, mainly $CD117^-IL\text{-}1RI^-$ ILC2s are increased, yielding lower eosinophilia in this disease despite the high levels of ILC2s. These data moreover unveil for the first time that the $CD117^-$ and the $CD117^+$ fractions within the human ILC2 population are not just random phenomena and that the IL-1RI expression is not ubiquitous in ILC2s. Both are affected by local tissue conditions present under the specific inflammatory conditions. The specific ILC2 expression status could on its turn further shape the tissue specific environment as a consequence of the possible functional implications on CD117 and IL-1RI expression on the inflammatory mediator release by ILC2s.

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FIGURE CAPTIONS

FIG 1. Relative number of the 3 ILC fractions in controls, CRSsNP and CRSwNP. Values are expressed as % ILCs of total living cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control; △: $P < 0.05$; △△: $P < 0.01$; △△△: $P < 0.001$.

FIG 2. Relative number of ILC1s in control, CRSsNP and CRSwNP subject groups separated on comorbidity status as indicated below the figure; -: absent, +: present. Values are expressed as % ILC1s of total living cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control; *: vs the indicated subject group; *,△: $P < 0.05$; **, △△: $P < 0.01$; △△△: $P < 0.001$.

FIG 3. Relative number of ILC2s in control, CRSsNP and CRSwNP subject groups separated on comorbidity status as indicated below the figure; -: absent, +: present. Values are expressed as % ILC2s of total living cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control; *: vs the indicated subject group; *, △: $P < 0.05$; △△: $P < 0.01$; ***, △△△: $P < 0.001$.

FIG 4. Relative number of NCR^- (A) and NCR^+ (B) ILC3s in control, CRSsNP and CRSwNP subject groups separated on comorbidity status as indicated below the figure; -: absent, +: present. Values are expressed as % ILC3s of total living cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control, $P < 0.05$.

FIG 5. Relative number of $CD117^-$ ILC2s (A and B) and $CD117^+$ ILC2s (A and C) in control, CRSsNP and CRSwNP subject groups separated on comorbidity status as indicated below the figure; -: absent, +: present. Values are expressed as % ILC2s of total living cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control; *: vs the indicated subject group; *, △: $P < 0.05$; **, △△: $P < 0.01$; ***, △△△: $P < 0.001$.

FIG 6. Relative number of $IL\text{-}1RI^-$ ILC2s (A and B) and $IL\text{-}1RI^+$ ILC2s (A and C) in control, CRSsNP and CRSwNP subject groups separated on comorbidity status as indicated below the figure; -: absent, +: present. Values are expressed as % ILC2s of total living cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control; *: vs the indicated subject group; *, △: $P < 0.05$; **, △△: $P < 0.01$; ***, △△△: $P < 0.001$.

FIG 7. Quantification of tissue IL-4 (A), IL-5 (B), ECP (C) and IL-25 (D) protein levels by means of Luminex multiplex (A, B and D) or UniCAP (C) in control, CRSsNP and CRSwNP subject groups separated on comorbidity status as indicated below the figure; -: absent, +: present. Values are expressed pg/g tissue, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control; *: vs the indicated subject group; *, △: $P < 0.05$; **, △△: $P < 0.01$; △△△: $P < 0.001$.

Table I. Patients' clinical data

	control	CRSsNP	CRSwNP
Clinical data			
number of subjects	29	17	35
Age (y), median (range)	32 (18-59)	41 (19-63)	44 (24-68)
Gender (male/female)	13/11	7/10	21/14
Smokers	7	2	0
Allergy + / asthma -	6	5	8
Allergy - /Asthma +	6	0	6
Allergy + /asthma +	5	5	9
AERD	0	0	0

CRSsNP: chronic rhinosinusitis without nasal polyps; CRSwNP: chronic rhinosinusitis with nasal polyps; AERD: aspirin exacerbated respiratory disease

Fig 1

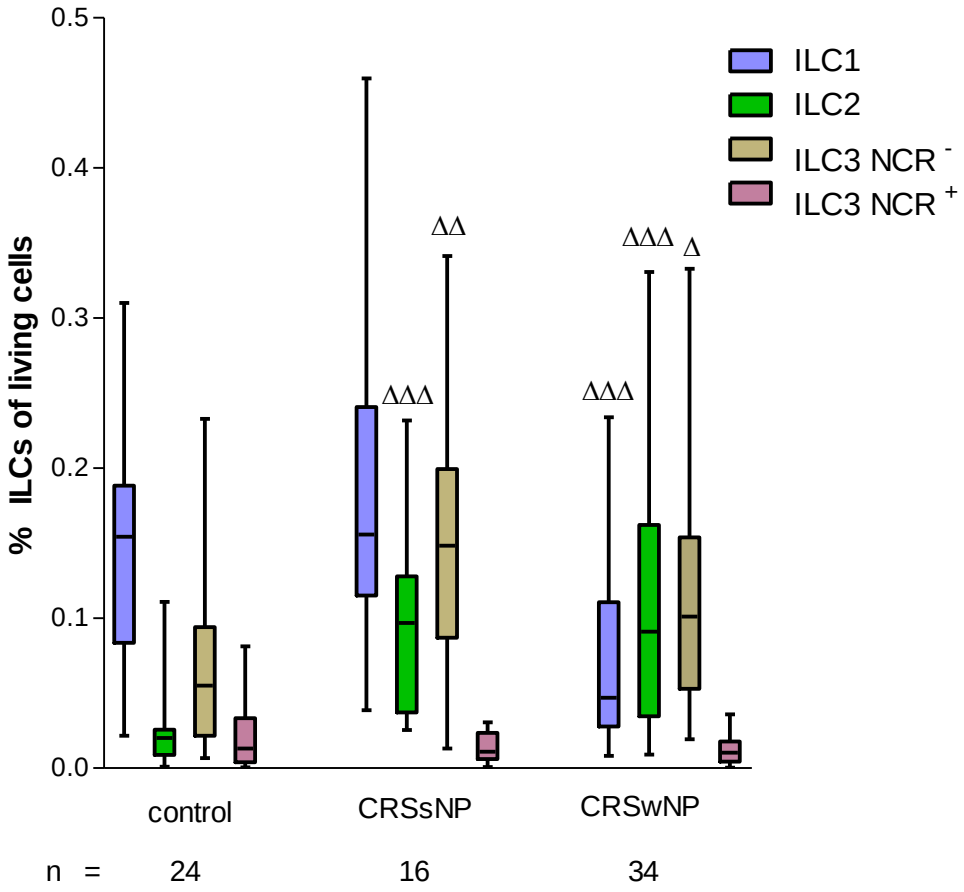

0.5
0.4
0.3
0.2
0.1
0.0
% ILCs of living cells
ILC1
ILC2
ILC3 NCR -
ILC3 NCR +
ΔΔ
ΔΔΔ
ΔΔΔ
Δ
control
CRSsNP
CRSwNP
n = 24 16 34

Fig 2

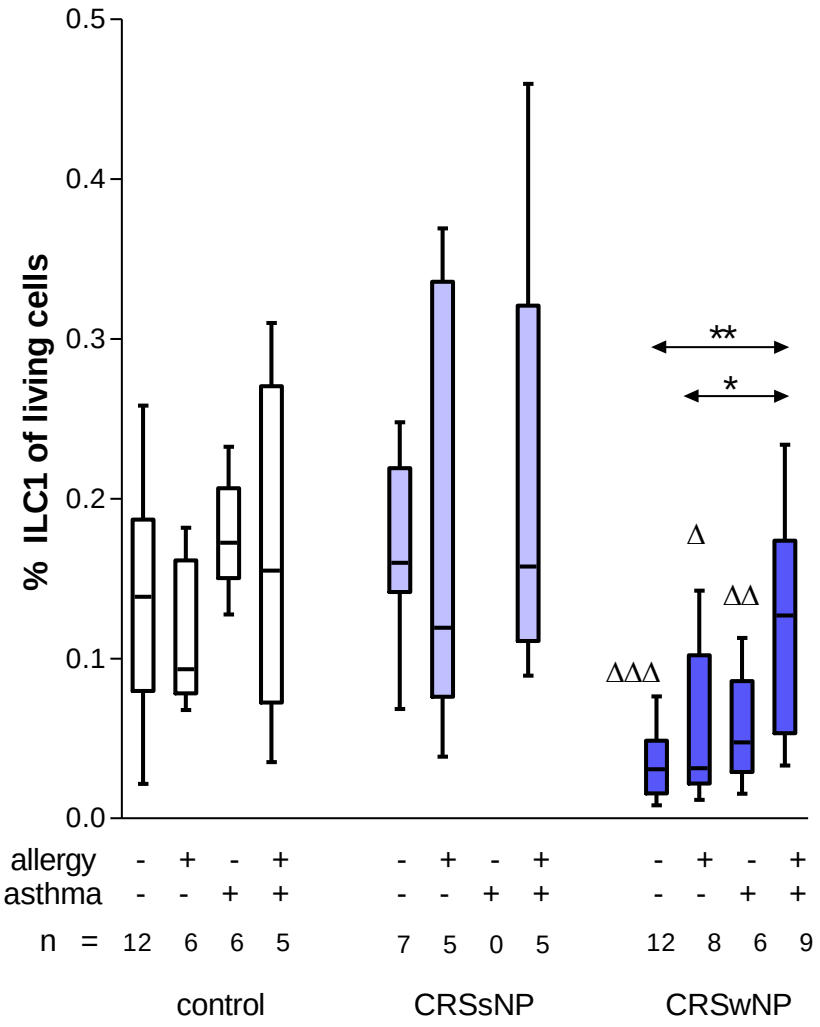

0.5
0.4
0.3
0.2
0.1
0.0
% ILC1 of living cells
**
*
Δ
ΔΔ
ΔΔΔ
allergy - + - + - + - + - + - +
asthma - - + + - - + + - - + +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP

Fig 3

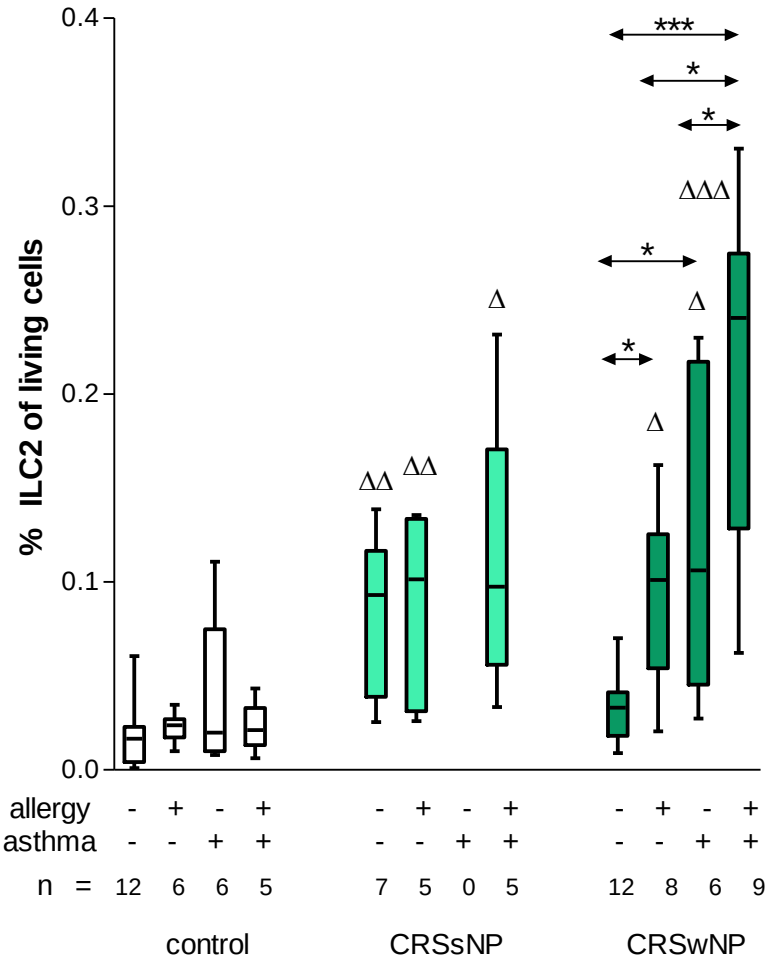

0.4
0.3
0.2
0.1
0.0
% ILC2 of living cells
***
*
*
ΔΔΔ
*
Δ
*
Δ
ΔΔ
ΔΔ
allergy - + - + - + - + - + - +
asthma - - + + - - + + - - + +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP

Fig 4

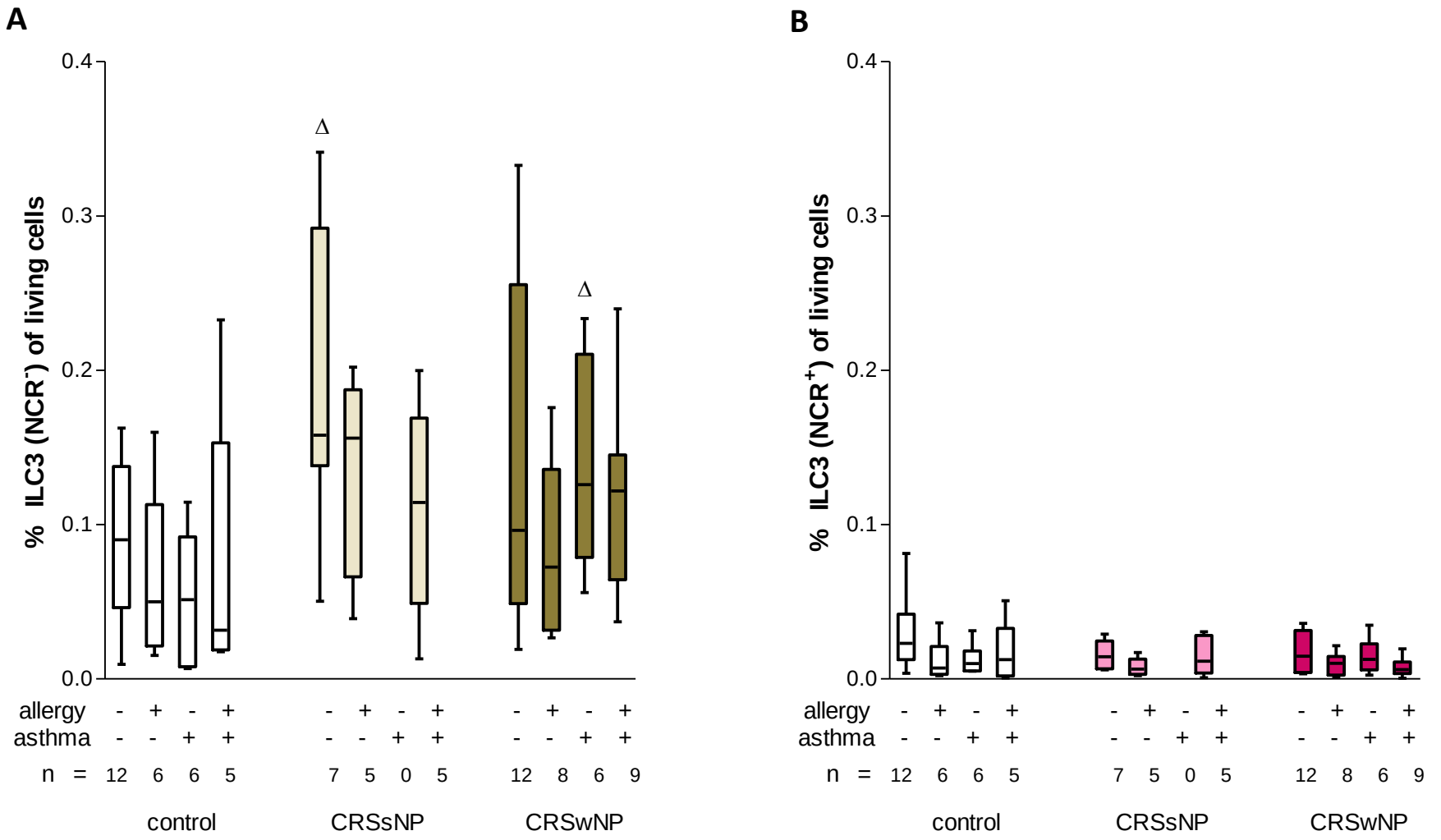

A
B
% ILC3 (NCR-) of living cells
% ILC3 (NCR+) of living cells
0.4
0.3
0.2
0.1
0.0
Δ
Δ
allergy - + - + - + - + - + - + - + - + - + - + - + - +
asthma - - + + - - + + - - + + - - + + - - + + - - + +
n = 12 6 6 5 7 5 0 5 12 8 6 9 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP

Fig 5

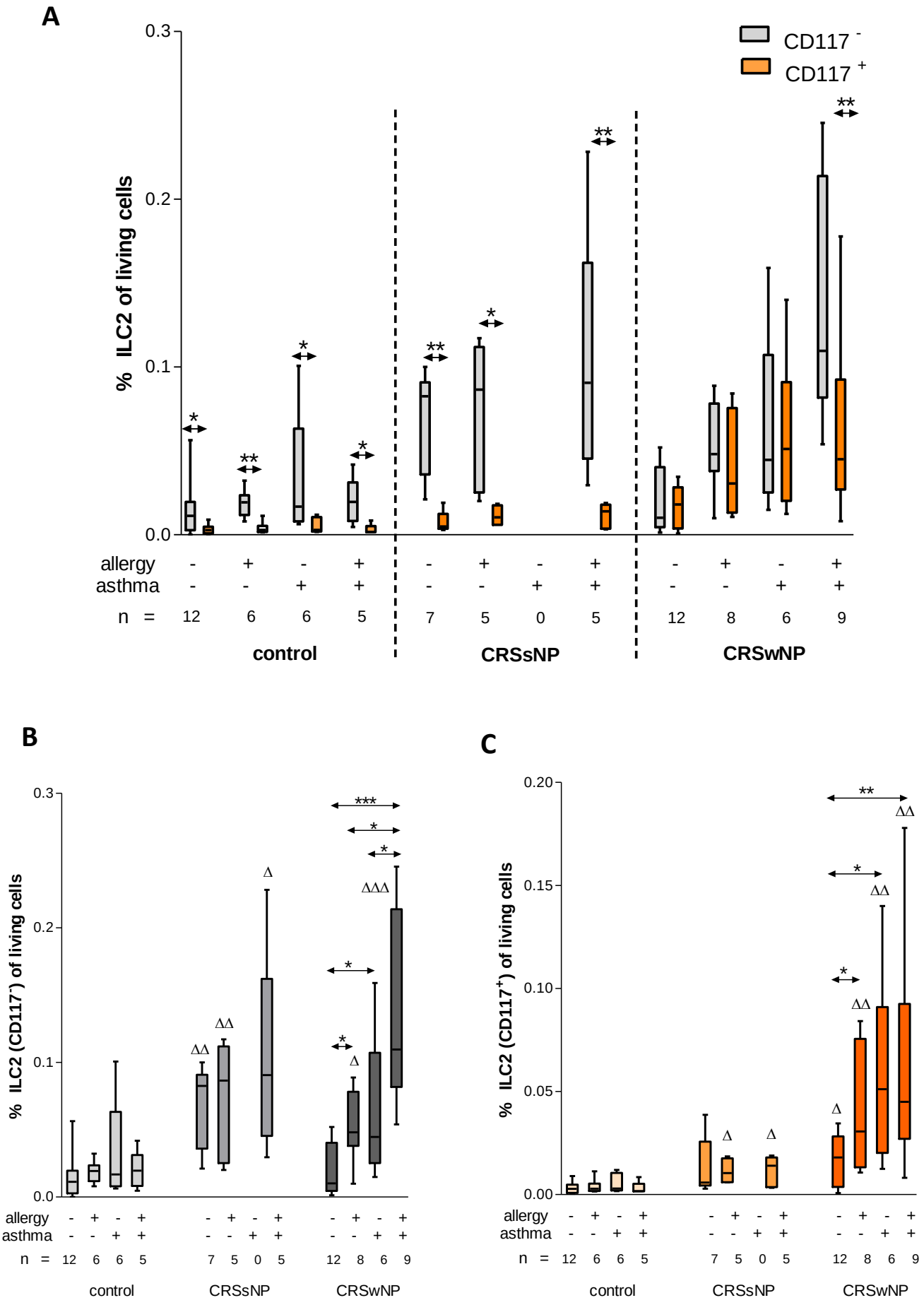
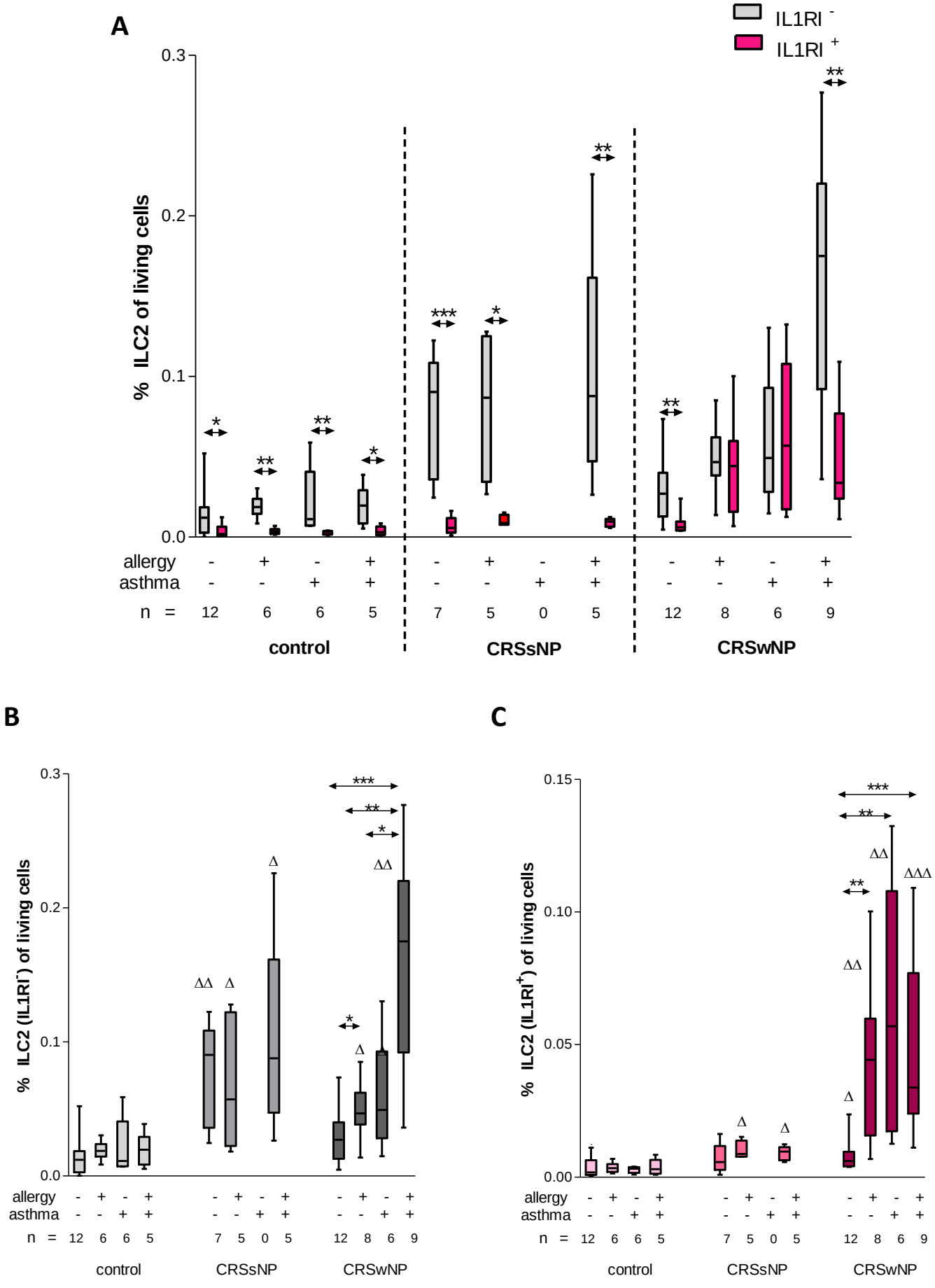

A
CD117 -
CD117 +
% ILC2 of living cells
0.3
0.2
0.1
0.0
allergy
asthma
n =
12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP
B
% ILC2 (CD117-) of living cells
C
% ILC2 (CD117+) of living cells
0.20
0.15
0.10
0.05
0.00

Fig 6


A
IL1RI -
IL1RI +
% ILC2 of living cells
0.3
0.2
0.1
0.0
allergy
asthma
n =
12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP
B
% ILC2 (IL1RI-) of living cells
C
% ILC2 (IL1RI+) of living cells
0.15
0.10
0.05
0.00

ONLINE SUPPLEMENT

METHODS

Patients

Tissues were obtained at the Department of Otorhinolaryngology, Ghent University Hospital, Belgium during routine endonasal sinus surgery and approved by the local ethical committee. All patients gave their written informed consent before collecting material. All patients stopped oral and topical application of corticosteroids for at least 1 month before surgery. The diagnosis of chronic sinus disease was based on history, clinical examination, nasal endoscopy, and computed tomography of the paranasal cavities according to the current European [1, 2] and American Guidelines [3]. The patients' clinical data can be found in Table 1 of the main document.

Single cells preparation

1.5g of freshly resected human nasal mucosa was transferred to 10 ml tissue culture medium (RPMI 1640 (Invitrogen, Ghent, Belgium)), containing 2 mM LGlutamine (Invitrogen), antibiotics (50 IU/ml penicillin and 50 µg/ml streptomycin) (Invitrogen) and 2% FBS (Fetal Bovine Serum, Invitrogen) and roughly fragmented with scissors into small pieces, followed by a mixing step in the Gentle MACs (Miltenyi Biotec, Leiden, The Netherlands). Gentle MACs tubes with tissue were centrifuged for 5 min at 300 g and supernatant was discarded and replaced by RPMI medium containing 2 mg/ml collagenase (Worthington, USA) and 0.04 mg/ml DNAse1 (Roche Diagnostics, Belgium). After a 45 min incubation period at 37 °C, an additional mixing step in the GentleMACs was performed. The cell suspension was dispersed and passed through a 70 µm cell strainer (BD Bioscience, Erembodegem, Belgium). Red blood cells were lysed by resolving the pellet in Versalyse (Beckman Coulter,

Suarlée, Belgium) and cells were incubated for 10 min at RT and after centrifugation the cell pellet was dissolved in RPMI with 2% FBS. For the descriptive part of the study, the single cells were used immediately for flow cytometry staining as described in the paragraph "Flow cytometry staining" below and in the main document. For the functional part of the study, the single cells were processed as reported in the paragraph "in vitro assessment of the functional responses by freshly isolated ILC2s" in both the main document.

Flow cytometry staining

Flow cytometry data were acquired on an LSR II (BD, Erenbodegem, Belgium) and were analyzed with FlowJo software (TreeStar). The following antibodies to human proteins were used for staining freshly resected tissues: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (BW264/56), anti-CD11c (MJ4-27G12), anti-CD19 (LT19), anti-CD34 (AC136), anti-CD303 (AC144), anti-FceRIa (AER-37) (all from BioLegend, San Diego, CA, USA), anti-CD94 (DX22) (ebioscience, San Diego, CA, USA), anti-CD14 (MφP9) (BD), phycoerythrin (PE)-conjugated anti-ILRI (Polyclonal Goat IgG) (R&D, Oxon, United Kingdom), PECy7-conjugated anti-CD117 (104D2) (ebioscience, San Diego, CA, USA), allophycocyanin (APC)-conjugated anti-CRTH2 (BM16) (Biolegend), APCCy7-conjugated anti-CD45 (2D1) (Miltenyi, Leiden, The Netherlands), peridinin chlorophyll (PerCP) efluor 710-conjugated anti-NKp44 (44.189) (ebioscience) and brilliant violet (BV)421-conjugated anti-CD127 (A019D5) (Biolegend).

To investigate the contradictory data present in the literature regarding the expression of IL-1RI on ILC2s [4-6] and to evaluate the relative contributions of the documented CD117 positive and negative fractions within the ILC2 population [4, 7], we extended our gating

strategy including antibodies against IL-1RI and CD117 in the ILC2 gating strategy. as shown in figures E1 and E2 in this Online Repository.

After in vitro stimulation, additional IL-5 cytokine expression was assessed using BV421-conjugated anti-IL5 (TRFK5) (Biolegend). For labeling cells of the in vitro series, all FITC-conjugated antibodies remained identical to the staining on the freshly resected tissues. The other antibodies in this series were conjugated as follows: PECy7-conjugated anti-CD117 (104D2) (ebioscience, San Diego, CA, USA), allophycocyanin (APC)-conjugated anti-CRTH2 (BM16) (Biolegend), APCFire-conjugated anti-CD45 (BM16) (Biolegend) and Horizon BV510-conjugated anti-CD161 (HP-3G10) (Biolegend).

Fluorescence Minus One (FMO) data for CRTH2, CD117, IL1RI and NKp44 were used in the gating strategy ensuring accurate setting of gates for correct determinations of positive and negative populations. This is also indicated in figure E1 as grey overlays in the histograms explaining the gating strategy used in this study. During protocol optimization, also an isoclonal control was used for IL1RI.

Tissue homogenates

Snap-frozen tissue specimens were weighed and suspended in a 10 times volume of 0.9% NaCl solution with protease inhibitor Complete Roche (Mannheim, Germany). In order to prepare soluble protein fractions, the frozen tissue was pulverized by means of a mechanical TissueLyser LT (Qiagen, Hilden, Germany) at 50 oscillations per second for 2 minutes in pre-chilled eppendorf tubes. The tissue homogenates were centrifuged at 1800 g for 5

minutes at 4°C. The supernatants from the tissue homogenates were stored at –20°C until further analysis

RESULTS

In vitro assessment of the functional responses by freshly isolated ILC2s

After 3h on 37°C in the presence of IL-2 without stimulation, the single cell fractions from nasal polyps of CRSwNP patients (all allergic or asthmatic) contained 0.08 ± 0.05 % $CD117^+$ ILC2s and 0.09 ± 0.04 % $CD117^-$ ILC2s (n=3) of the living cell fraction, being similar to what was observed for the ex-vivo part of the study on 35 CRSwNP patients (see fig 5 in the main document). However, in PMA/Ionomycin stimulated single cell fractions from the same patients the number of $CD117^+$ ILC2s was almost abolished to 0.01 ± 0.004 % of living cells, while the number of $CD117^-$ ILC2s remained unaffected at 0.08 ± 0.02 % of living cells. The latter cell fraction contained no IL5+ cells at baseline conditions, while in the PMA/Ionomycin stimulated single cell fraction, 16.80 ± 5.11 % of the cells were IL-5 positive.

The % of counted cells in the ILC1 gate increased from 0.05 % ± 0.02 % of living cells under basal non-stimulated conditions to 0.19 % ± 0.08 % of living cells when the cells were stimulated by PMA/Ionomycin. While under basal conditions, the intracellular IL-5 expression in these cells was virtually absent, the stimulated cells in the ILC1 gate contained 15,5 ± 3.66 % $IL\text{-}5^+$ cells. The cells found in the ILC1 gate could be recovered to the ILC2 fraction by intracellular staining for CRTH2 and CD117 in addition to the membrane staining for these markers; by doing so, the number of CD117+ ILC2 cells restored to 0.05 ± 0.01 % of living cells (of which 12.63 ± 4.05 % were $IL\text{-}5^+$), while the number of cells in the ILC1 gate reduced again to 0.04 ± 0.01 % of living cells, all lacking intracellular IL-5 expression.

After 3h on 37°C in the presence of IL-2 without stimulation, the single cell fractions from nasal polyps of CRSsNP patients contained an equal number of $CD117^+$ ILC2s and $CD117^-$ ILC2s (0.04 ± 0.005 % vs 0.03 ± 0.002 % of the living cell fraction (n=2) respectively, which contrasts the virtual absence of CD117+ ILC2s in freshly resected tissue of CRSsNP patients (see fig 5 in the main document)

FIGURE LEGENDS

FIG E1. Gating strategy; tissue native ILCs were gated as living, lymphogate $CD45^+$ lineage negative ($CD3^-CD11c^-CD14^-CD19^-CD34^-BDCA2^-FcεR1^-CD94^-CD1a^-CD123^-TCRαδ^-$) cells. ILC1 were further phenotyped as $CD127^+CRTH2^-CD117^-NKp44^-$, ILC2 as $CD127^+CRTH2^+$, and ILC3 as $CD127^+CRTH2^-CD117^+IL\text{-}1RI^+NKp44^-$ for the NCR^- fraction and $CD127^+CRTH2^-CD117^+IL\text{-}1RI^+NKp44^+$ for the NCR^+ fraction. The Fluorescence Minus One (FMO) for CRTH2 (grey overlay) is indicated in the histogram identifying the ILC2 population. FMO data for CD117 and IL1RI are presented as grey dot plots next to the dot plot assessing the levels of CD117 and IL1RI expression within the ILC2 population and the dot plot identifying the ILC1, NCR^- ILC3 and NCR^+ ILC3 populations are accompanied by FMO data for CD117 and NKp44.

FIG E2. Relative number of $CD45^+$ in control CRSsNP and CRSwNP subject groups expressed as a % of total living cells (A) and relative number of living cells as a % of all events (B). Data are presented as Box-and-Whisker plots showing the minimum and the

maximum value, the lower and the upper quartile, and the median. ***: P < 0.001, ns = not significant.

FIG E3. Relative number of the 3 ILC fractions in controls, CRSsNP and CRSwNP. Values are expressed as % ILCs of $CD45^+$ cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median. △: vs control; △△: P < 0.01; △△△: P < 0.001.

FIG E4. Representative dot plots of the gating step assessing the expression of CD117 and IL1RI on ILC2 cell population in controls (upper panel), CRSsNP (middle panel) and CRSwNP (lower panel). In the examples all individuals suffered from allergic asthma. Fluorescence Minus One (FMO) for CD117 and IL1RI are presented as grey overlays in the small dot plots at the right as indicated in the figure.

FIG E5. Relative number of ILC2s subsets $CD117^-$IL-1RI^-, $CD117^-$IL-1RI^+, $CD117^+$IL-1RI^-, $CD117^+$IL-1RI^+ in control, CRSsNP and CRSwNP subject groups separated on comorbidity status as indicated below the figures. Values are expressed as % ILC2 subsets of total living cells, and presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median.

FIG E6. Graphs showing the individual data points of the ILC levels that were presented in figures 1 to 7 of the main document as Box-and-Whisker plots.

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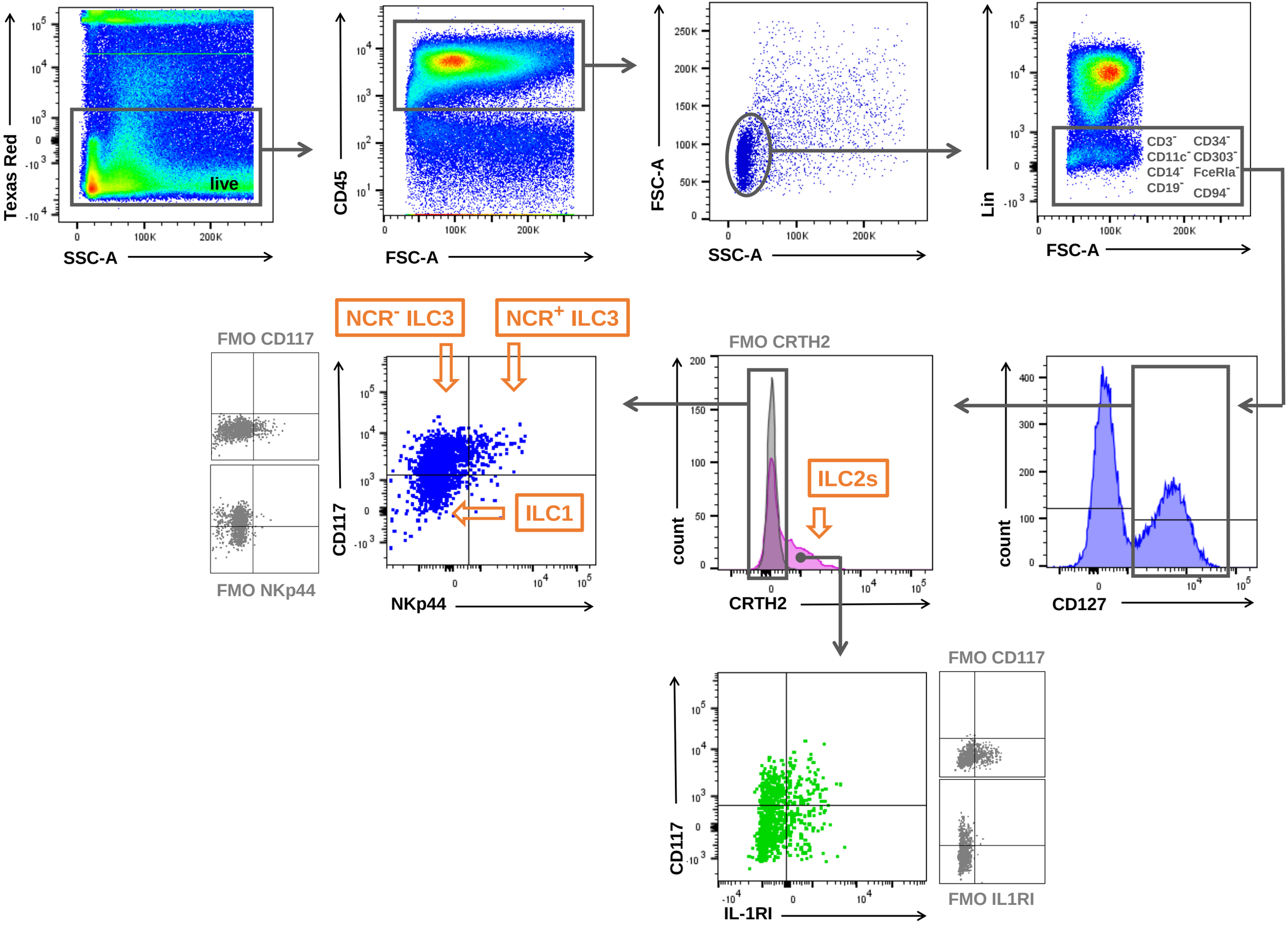
Fig E1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CD45
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FSC-A
CD3- CD34-
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CD19- CD94-
NCR- ILC3
NCR+ ILC3
FMO CD117
FMO NKp44
CD117
NKp44
ILC1
FMO CRTH2
count
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ILC2s
count
CD127
FMO CD117
FMO IL1RI
CD117
IL-1RI

Fig E2

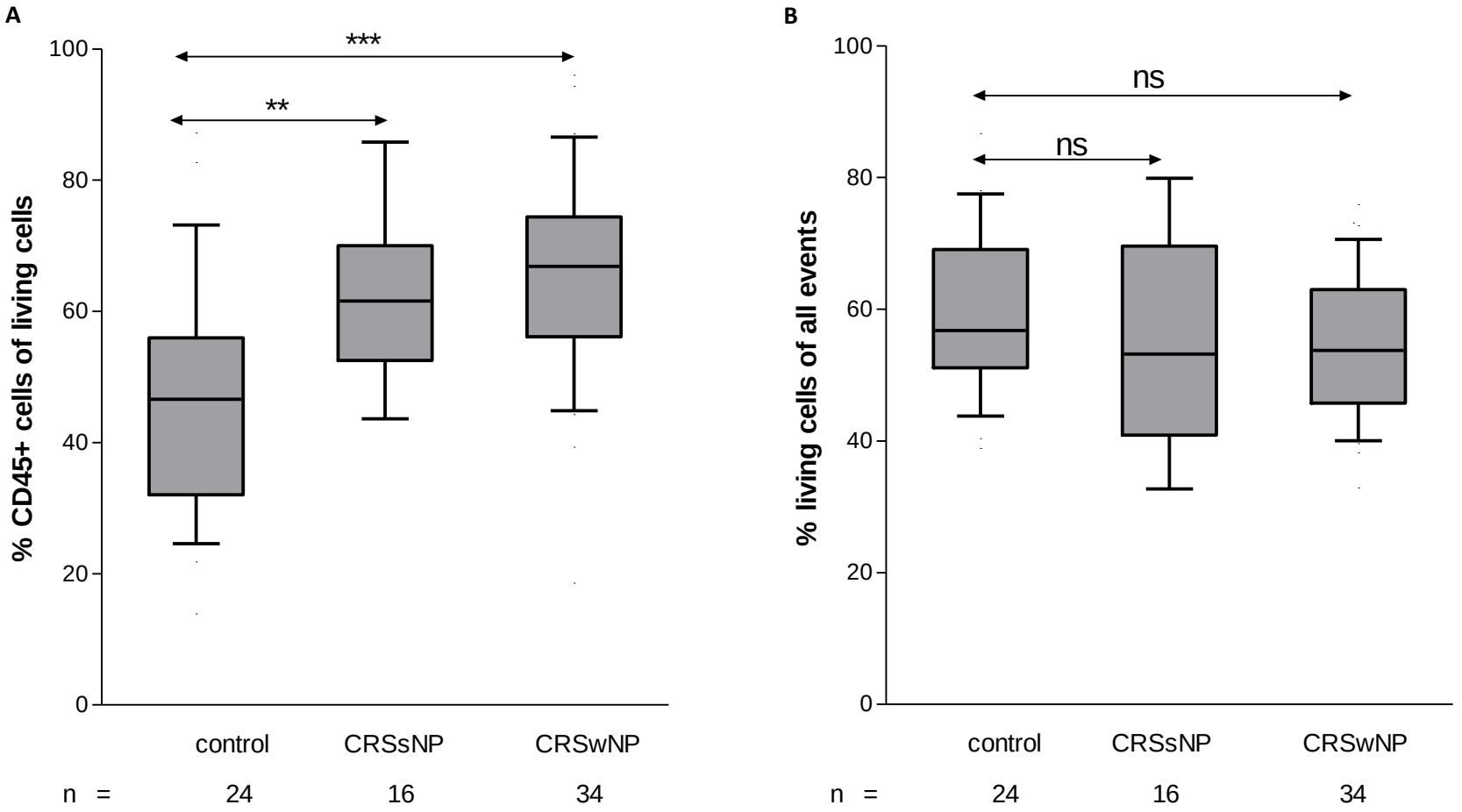

A
***
**
100
80
60
40
20
0
% CD45+ cells of living cells
control
CRSsNP
CRSwNP
n =
24
16
34
B
ns
ns
100
80
60
40
20
0
% living cells of all events
control
CRSsNP
CRSwNP
n =
24
16
34

Fig E3

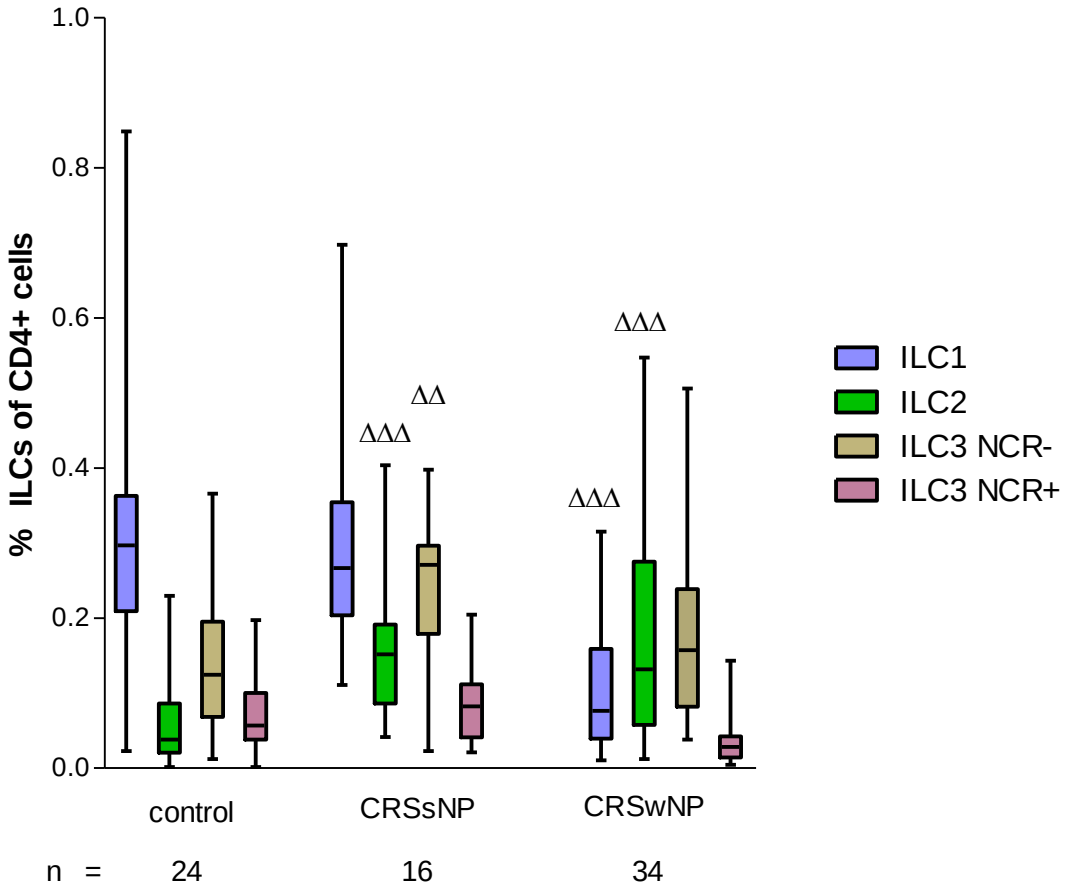

1.0
0.8
0.6
0.4
0.2
0.0
% ILCs of CD4+ cells
ΔΔΔ
ΔΔ
ΔΔΔ
ΔΔΔ
ILC1
ILC2
ILC3 NCR-
ILC3 NCR+
control
CRSsNP
CRSwNP
n =
24
16
34

Fig E4

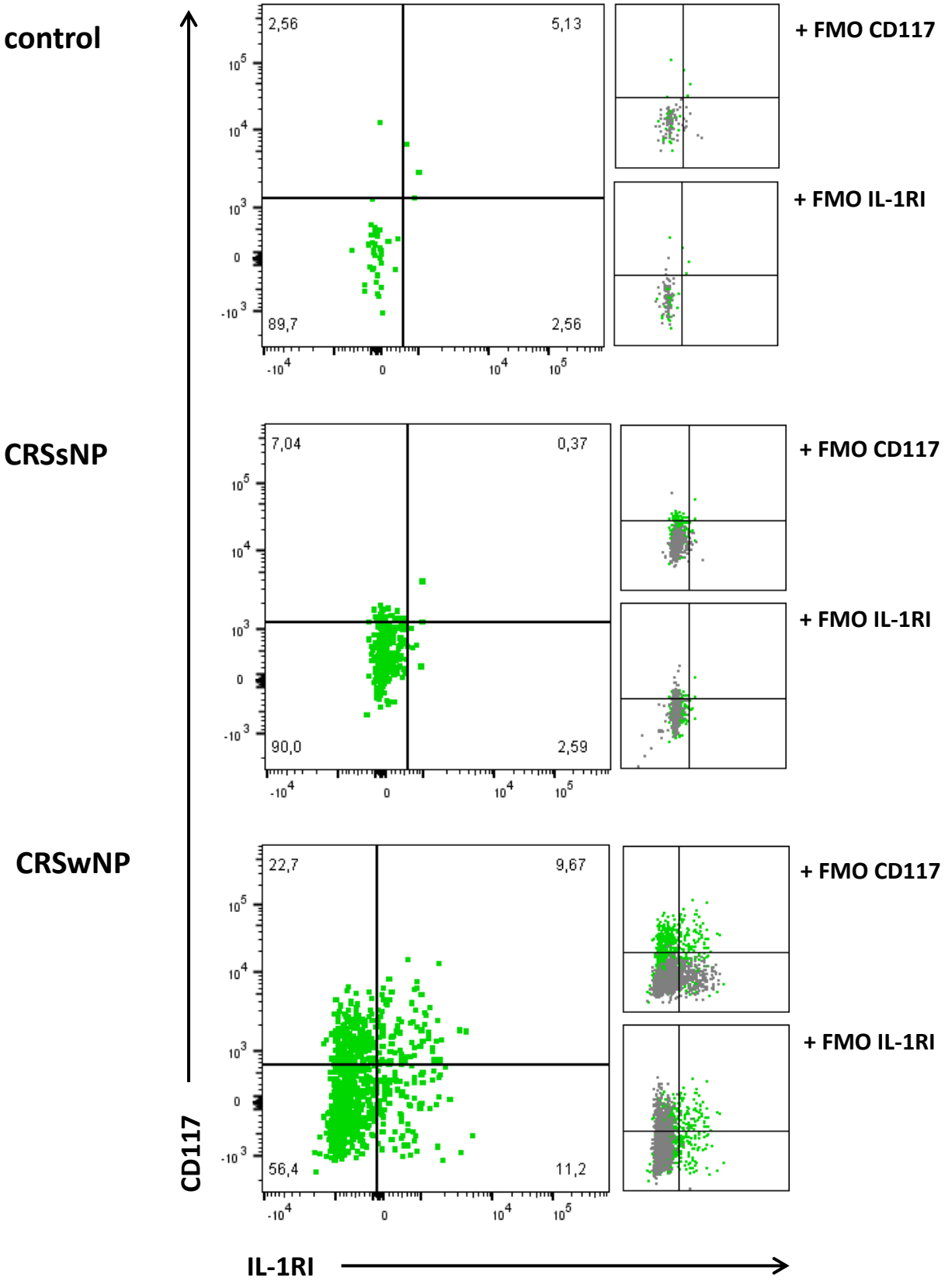

control
2,56
5,13
89,7
2,56
+ FMO CD117
+ FMO IL-1RI
CRSsNP
7,04
0,37
90,0
2,59
+ FMO CD117
+ FMO IL-1RI
CRSwNP
22,7
9,67
56,4
11,2
+ FMO CD117
+ FMO IL-1RI
CD117
IL-1RI

Fig E5

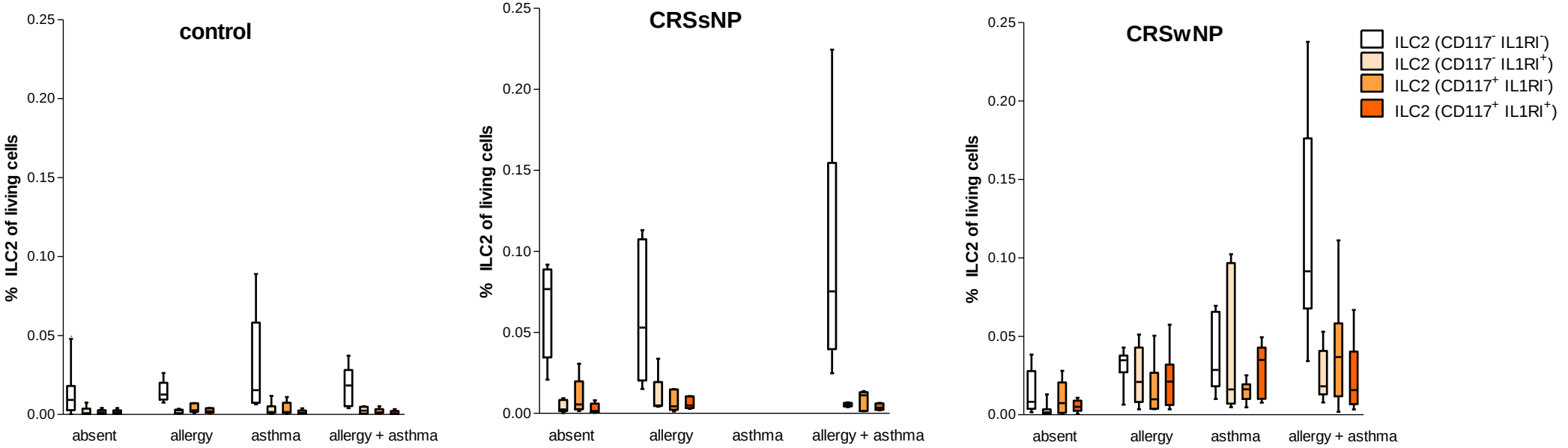
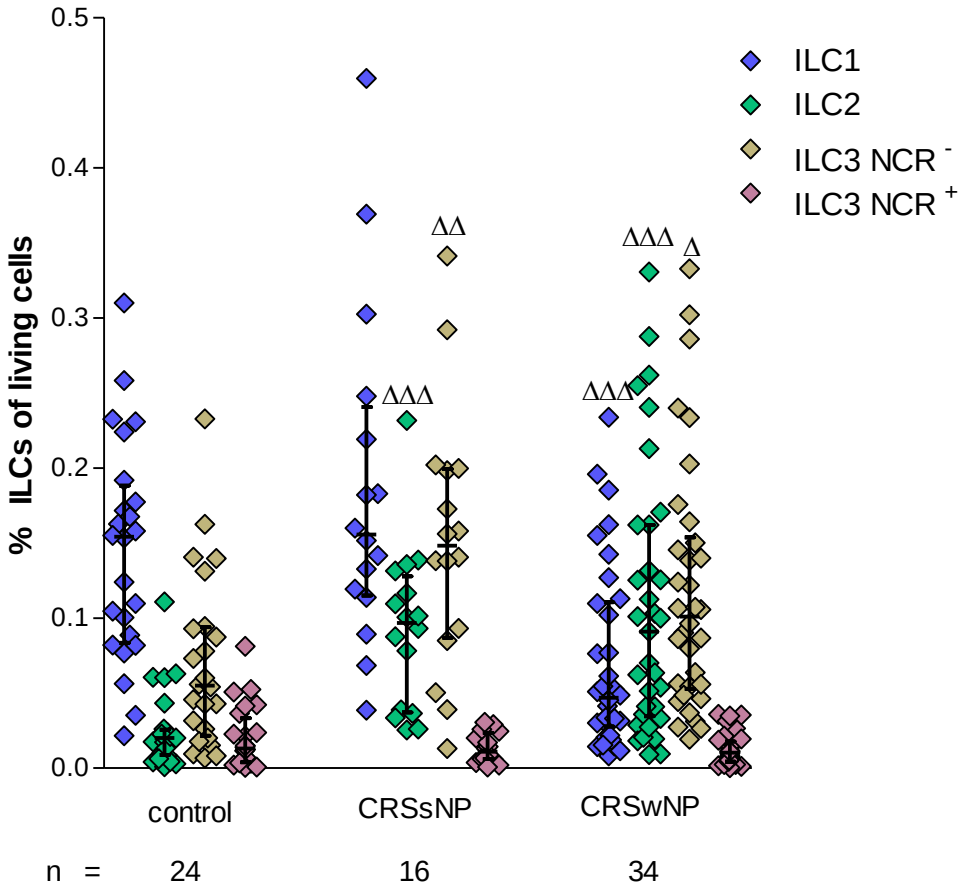
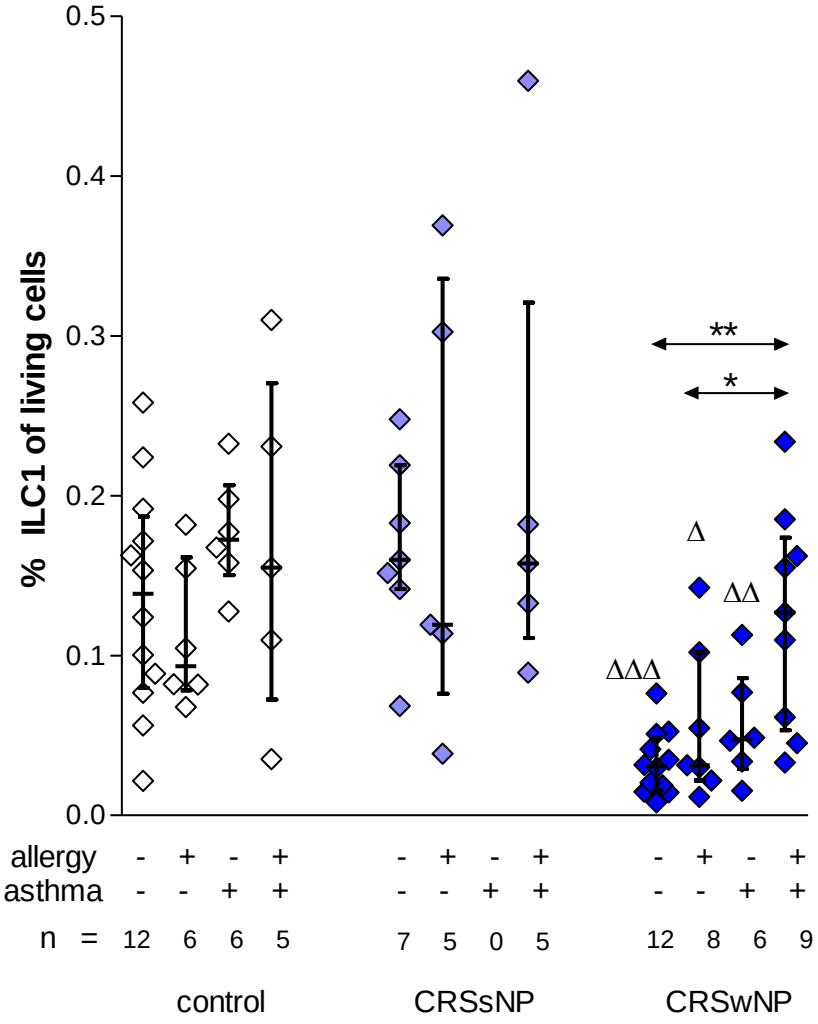

control
CRSsNP
CRSwNP
% ILC2 of living cells
0.25
0.20
0.15
0.10
0.05
0.00
absent
allergy
asthma
allergy + asthma
ILC2 (CD117- IL1RI-)
ILC2 (CD117- IL1RI+)
ILC2 (CD117+ IL1RI-)
ILC2 (CD117+ IL1RI+)

Fig E6

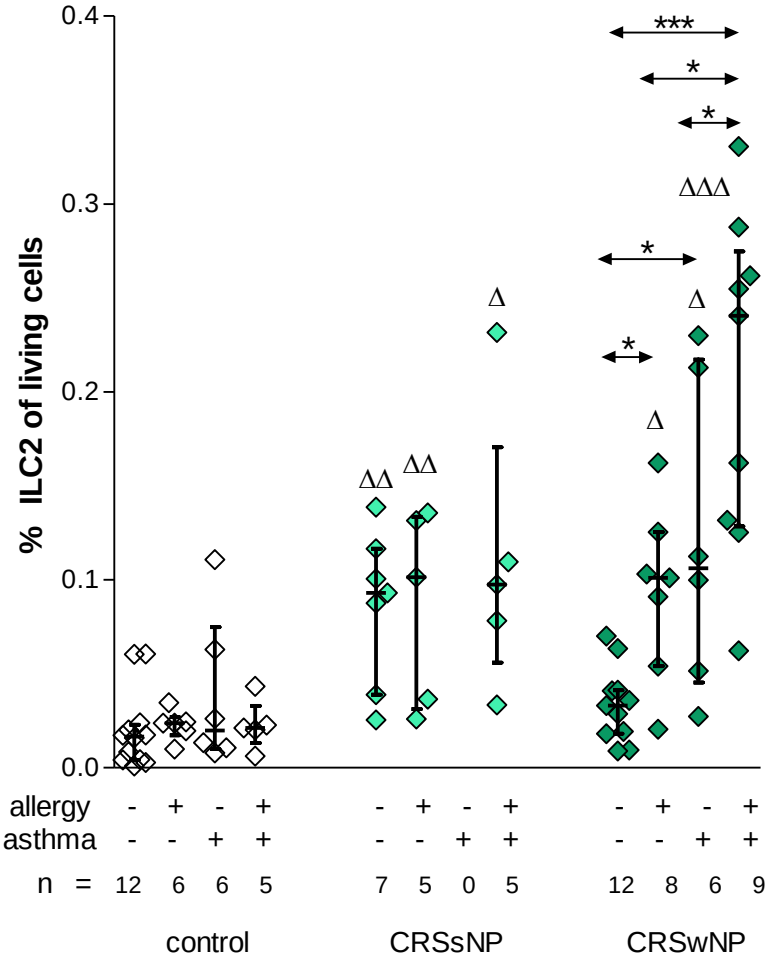
A


0.5
0.4
0.3
0.2
0.1
0.0
% ILCs of living cells
ILC1
ILC2
ILC3 NCR -
ILC3 NCR +
ΔΔ
ΔΔΔ
ΔΔΔ
Δ
control
CRSsNP
CRSwNP
n = 24 16 34

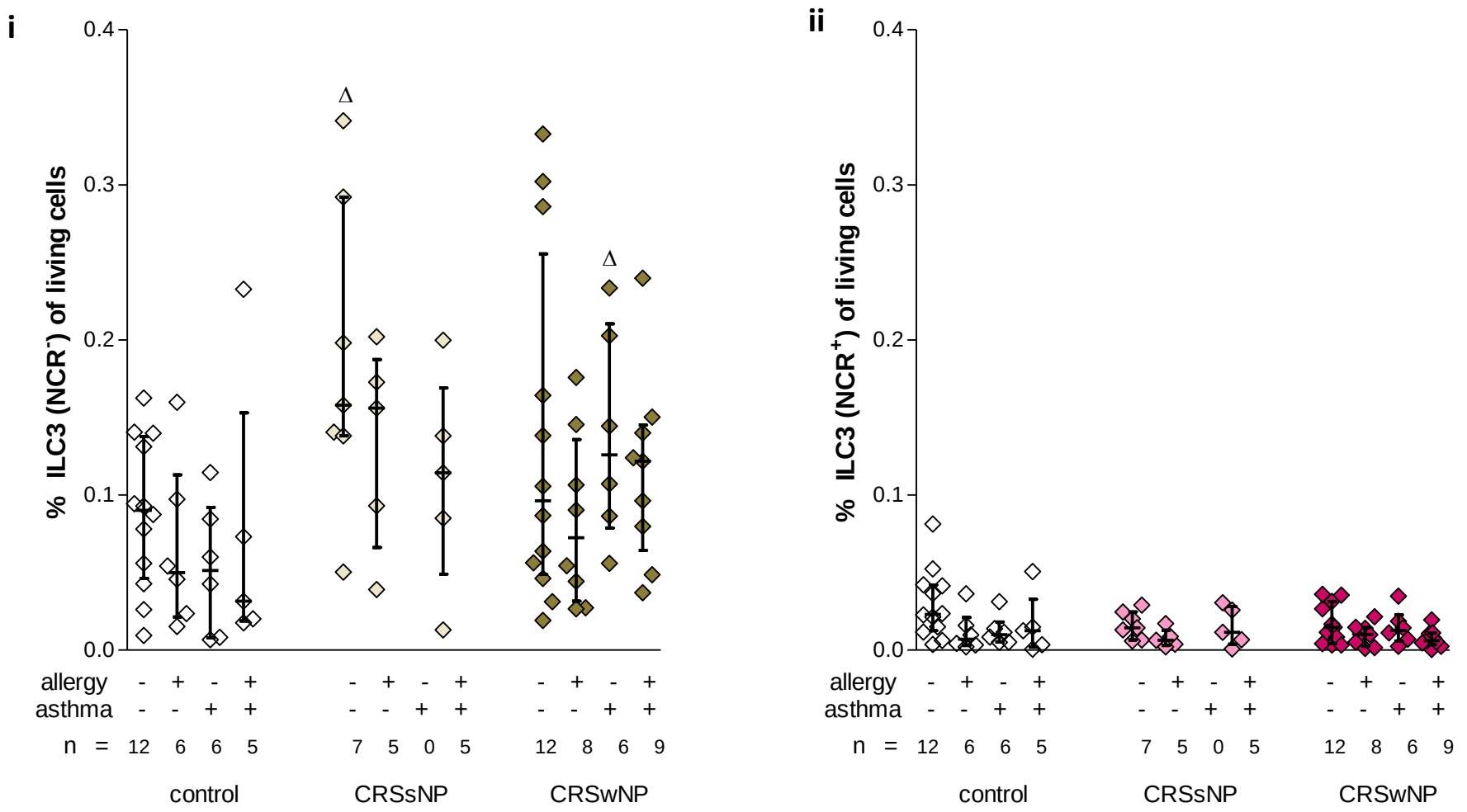
B


0.5
0.4
0.3
0.2
0.1
0.0
% ILC1 of living cells
**
*
Δ
ΔΔ
ΔΔΔ
allergy - + - + - + - + - + - +
asthma - - + + - - + + - - + +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP

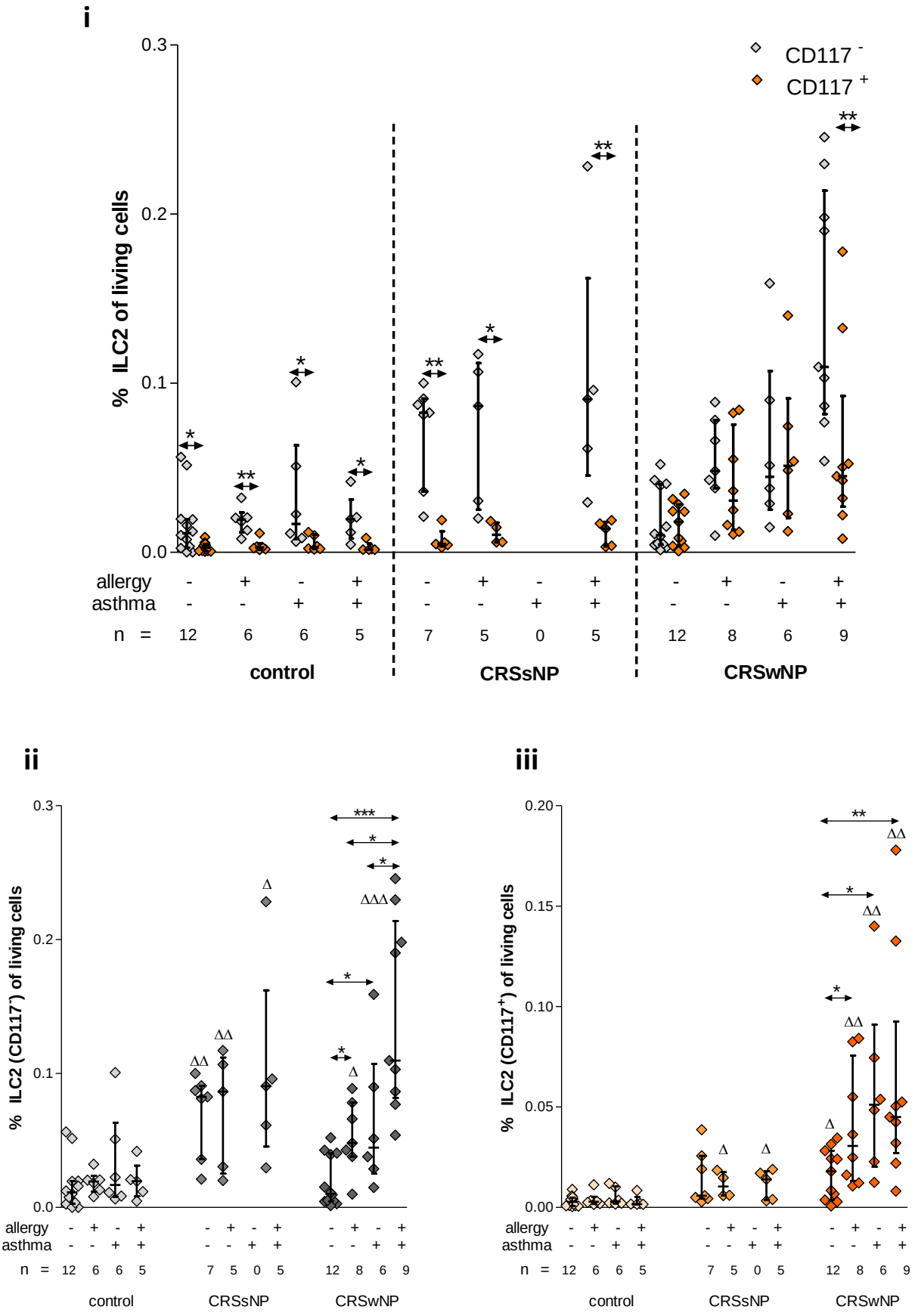
C


0.4
0.3
0.2
0.1
0.0
% ILC2 of living cells
***
*
*
ΔΔΔ
*
Δ
*
Δ
ΔΔ
ΔΔ
allergy - + - + - + - + - + - + - +
asthma - - + + - - + + - - + + +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP

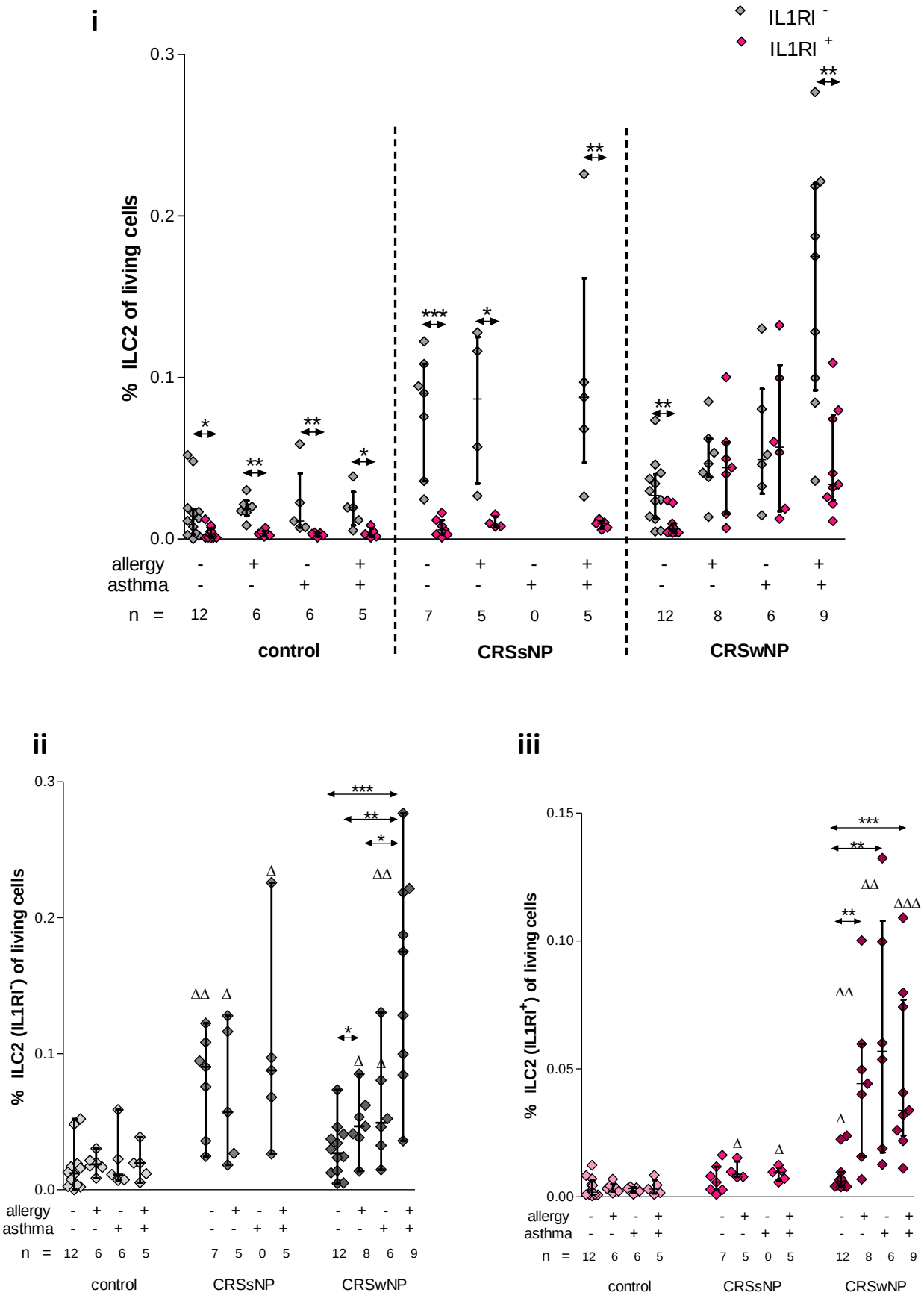
D


i
ii
% ILC3 (NCR⁻) of living cells
% ILC3 (NCR⁺) of living cells
0.4
0.3
0.2
0.1
0.0
Δ
Δ
allergy - + - + - + - + - + - + - +
asthma - - + + - - + + - - + + - - + +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP

E


i
CD117 -
CD117 +
% ILC2 of living cells
0.3
0.2
0.1
0.0
allergy - + - + - + - + - + - + - +
asthma - - + + - - + + - - + + - +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP
ii
% ILC2 (CD117-) of living cells
0.3
0.2
0.1
0.0
allergy - + - + - + - + - + - + - +
asthma - - + + - - + + - - + + - +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP
iii
% ILC2 (CD117+) of living cells
0.20
0.15
0.10
0.05
0.00
allergy - + - + - + - + - + - + - +
asthma - - + + - - + + - - + + - +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP

F


i
IL1RI -
IL1RI +
% ILC2 of living cells
0.3
0.2
0.1
0.0
allergy - + - + - + - + - + - +
asthma - - + + - - + + - - + +
n = 12 6 6 5 7 5 0 5 12 8 6 9
control
CRSsNP
CRSwNP
ii
% ILC2 (IL1RI-) of living cells
iii
% ILC2 (IL1RI+) of living cells
0.15
0.10
0.05
0.00