SUPPLEMENTARY MATERIAL

Methods

Figure S1. Timeline for ILIT treatment, evaluation visits, and symptom registration.

The follow-up study was conducted during 2018-2019, 5-6 years after the RDBPC ILIT trial. Evaluation visits: Blood and lymph node sampling, birch and grass nasal provocation test (NPT). Symptom registration with seasonal combined symptom and medication scores (CSMS) and quality of life registration (QoL).

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Figure S2

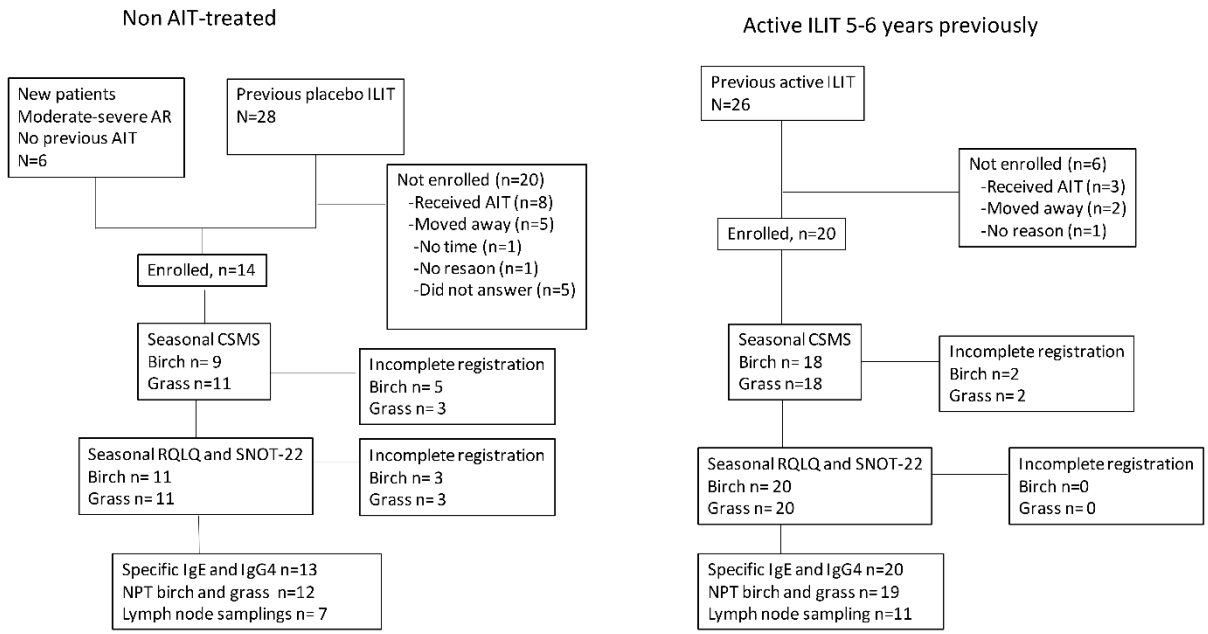

Figure S2. Flow of patients

Study design

The previous ILIT-study with two concomitant allergens had been performed with patients enrolled at three periods during three consecutive years; 2012-2014. This follow-up study was conducted for two years; 2018-2019. Patients that received randomized ILIT in 2012 or 2013 were followed up with NPT, blood samplings, and lymph node samplings 2018, 5-6 years after treatment. Patients that received randomized ILIT during 2014 had the follow-up visits in this study during 2019, 5 years after treatment. All patients registered seasonal symptoms, medication, and quality of life during the birch and grass pollen season of 2019. In this way, all participants recorded the symptoms after the same seasonal exposure to pollens, 5-7 years after randomized treatment

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Patients

The inclusion- and exclusion criteria for the non-AIT-treated control group were the same as in the previous ILIT-study; men and women in age 18-55 with moderate to severe allergic rhinitis despite symptom alleviating medication, a positive skin prick test reaction against birch and timothy with a wheal reaction of ≥3 mm diameter and allergen-specific IgE levels ≥ 0.35 kU/mL were eligible.

Exclusion criteria were uncontrolled or perennial asthma, other pulmonary diseases, known autoimmune or collagen disease, chronic infection or other significant diseases, severe atopic dermatitis, use of beta-blockers or angiotensin-converting enzyme inhibitors as antihypertensive medications, symptomatic sensitization to house dust mite or furry animals with daily exposure, chronic upper airways disease, pregnancy, wish for pregnancy, nursing, obesity with BMI >30 or withdrawn informed consent. In this follow-up study, the inability to register symptoms electronically was also an exclusion criterion.

Primary outcome measure

Nasal provocation test

0.1 mL of water-soluble grass allergen, ALK Aquagen SQ® timothy, 10 000 SQ-U/mL (1000 SQ-U), was sprayed into each nostril. A study nurse asked the patients to score each symptoms nasal obstruction, rhinorrhea, itchy nose, sneezing, itchy eyes, watery eyes, itchy palate/ears on a scale ranging 0-3. The scores were registered before the challenge, after 5 minutes, 15 minutes, and 30 minutes.

Secondary outcome measures

Combined symptom and medication score

On a 0-3 points scale, the patients scored the severity of six symptoms during the last 24 hours; runny nose, nasal obstruction, itchy nose, sneezing, itchy/red eyes, watery eyes. The average value of the symptoms at each time point was the symptom score (SS). The use of symptom-relieving medications during the last 24 hours was scored in a stepwise manner. Antihistamine tablets and eye drops = 1 point, an intranasal corticosteroid with or without antihistamines = 2 points, systemic corticosteroids with or without other medication = 3 points. Corticosteroid tablet rendered 3 points

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if the intake was the day before registration or the same day. Intramuscular injection of corticosteroids rendered 3 points if the administration was within three weeks before the registration. The medication score (MS) ranged 0-3, SS ranged 0-3, and the combined symptoms and medication score (CSMS), which was the sum of SS and MS, ranged 0-6. The CSMS, SS, and MS were expressed as the area under the curve (AUC) based on three arbitrary registration time points (time point 1, 2, and 3, not taking into account slight differences in the timing of when the patients completed the forms), for each patient at the birch and grass pollen season, respectively.

Quality of life

The average value of 28 questions ranging from 0-6 is the RQLQ score, where a change of 0.49 is the minimal clinically significant difference (28). The Sinonasal Outcome test-22 (SNOT-22) is primarily developed for sinus-related rhinitis but has overlapping questions with allergic rhinitis (29). The test measures the sum of 22 questions scored 0-5 and reflects the symptoms during the last two weeks. The maximum total SNOT-22 score is 110, and the minimal clinically significant difference is 8.9 points. The RQLQ and SNOT-22 scores were expressed as the average value of the three registration time points for each patient at the birch and grass pollen season, respectively.

Lymphocytes analysis in fine-needle aspiration and blood

Before preparation, the lymph node aspirates were centrifuged and diluted in sterile PBS (Gibco, Life Technologies, Uppsala, Sweden). Peripheral blood mononuclear cells (PBMC) were prepared with ficoll density centrifugation according to the manufacturer's instructions. PBMC and FNA were stained with the following monoclonal antibodies. BD Biosciences; CD3 PB (Clone: UCTH-1), CD4 Percp-5.5 (clone: SK3), CD8 FITC (clone: SK1), CCR7 PE-CY7 (clone: 3D12), CD45RA BV510 (clone: HI100), CCR5 PE-CF594 (clone: 2D7), CD19-APC (clone: SJ25C1) and CD25 BV711 (clone: 2A3). After staining, samples were washed with PBS and fixed with 1% formaldehyde (Histolab Products, Askim, Sweden). All antibodies were titrated for optimal performance before use, with dilutions ranging from 1:5 to 1:50.

A minimum of 10000 leukocytes were acquired from peripheral blood samples. For FNA samples, all cells were acquired. For the detection of T-cells, CD3, CD4 and CD8 were used. CCR7 and CD45RA were used to divide cells into central memory cells ($CCR7^{+}CD45RA^{-}$), naïve T-cells

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($CCR7^{+}CD45RA^{+}$), and effector memory ($CCR7^{-}$). For detection of Th1 T-cells, we gated on the expression of CCR5. For detection of regulatory T-cells, we gated on high expression of CD25. For the detection of B-cells, we used CD19. The positivity was determined with an internal control or a fluorescence minus one control. For further details and gating strategy, see Table S1, Figures S3-S4.

Basophil analysis in blood

For activation of basophils, blood was drawn into natrium heparin BD vacutainer tubes. The blood was stored at 4 degrees Celsius and assayed within 24 hours. Before allergen stimulation, the blood was washed with nine parts PBS (GIBCO). One part of washed blood was added to one-part cell culture medium DMEM/F12 (GIBCO) and incubated with either birch or grass allergens. For negative control, we used DMEM/F12. For allergen stimulation, we used ALK Aquagen SQ® timothy or birch 500 SQ-U/mL (100 SQ-U). This allergen concentration generates activation within the plateau phase of allergen-induced basophil activation. Stimulation was performed at 37 degrees Celsius and 5% CO2 for 30 minutes. Samples were incubated with the following antibodies. BD Biosciences; CD45 PE-CF594 (clone: HI30), HLA-DR PE-CY7 (G46-6), CD63 Percp-5.5 (clone: H5C6), Biolegend; IgE PE (clone: MHE-18), Abcam; FceR1 APC (clone: AER-37). We also stained with FITC labeled avidin (Thermo Fisher) final concentration 125 µg/ml. After incubation, samples were lysed with ammonium chloride solution, washed with PBS (GIBCO), and fixed with 1% formaldehyde (Histolab).

A minimum of 250 basophils were acquired in all samples. A threshold was set on CD45 at MFI 1000 to reduce the influence of erythrocytes. For detection of basophils, we first gated in mononuclear cells (CD45 vs. SSC), then removed doublets (FSC-A vs. FSC-H), and last gated on pure basophils with HLA-DR vs. IgE. To determine positive staining, a fixed gate of MFI 50000 was used for CD63. To determine positivity for avidin, the bimodal distribution ascertained proper gating. For further details and gating strategy, see Table S1, Figures S3A, B.

Cells were analyzed on an LSR Fortessa (BD Biosciences), and data were processed using FlowJo software© version 10.6.2 (Tree Star, Inc., Ashland, USA). Performance was checked daily with

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cytometer and tracking beads (CS&T). PMT settings suggested from CS&T were used for experiment set up. For compensation, we used single-stained BD comp-beads.

Lymphocyte flow cytometry gating procedure in FNA

Figure S3A

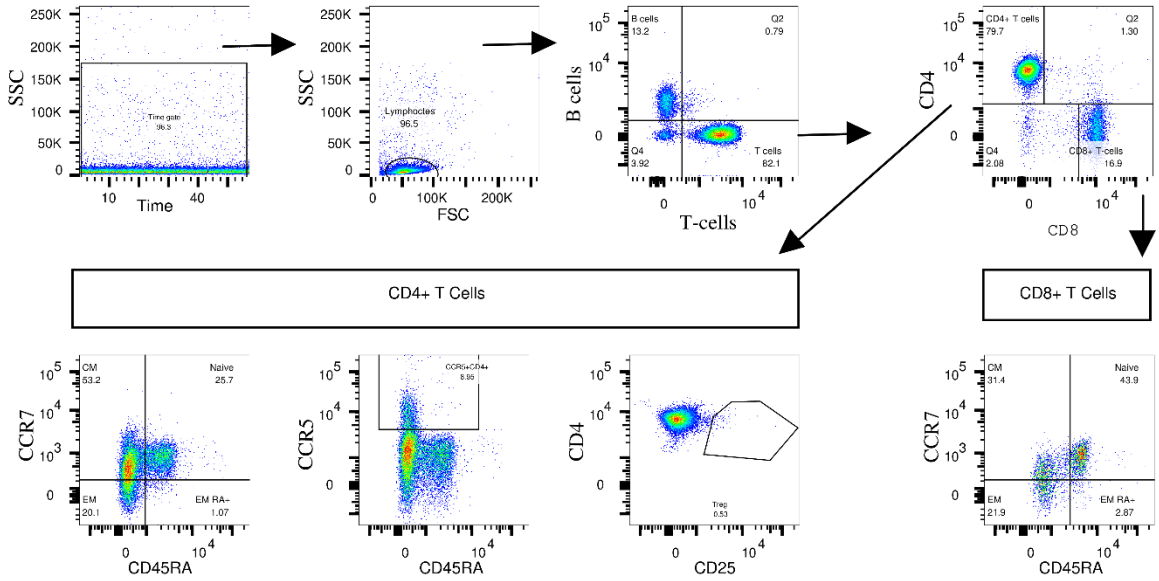

Figure S3A **Gating strategy.** SSC vs. time was used to remove unwanted events. Next, SSC vs. FSC was used to gate on lymphocytes. CD19 vs. CD 3 were used to gate on CD19+ B-cells and CD3+ T-cells. CD4 vs. CD8 was used to differentiate CD4 and CD8 T-cells. Next, CCR7 vs. CD45RA we used on both CD4 and CD8 T-cells to differentiate naïve (CCR7+CD45RA+), Central memory (CM, CCR7+CD45RA-), and effector memory T-cells (EM, CCR7-). CCR5 vs. CD45RA were used to gate on CCR5+ T-cells. CD4 vs CD25 were used to gate on CD25++ T-cells.

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Lymphocyte full flow cytometry gating procedure PBMC

Figure S3B

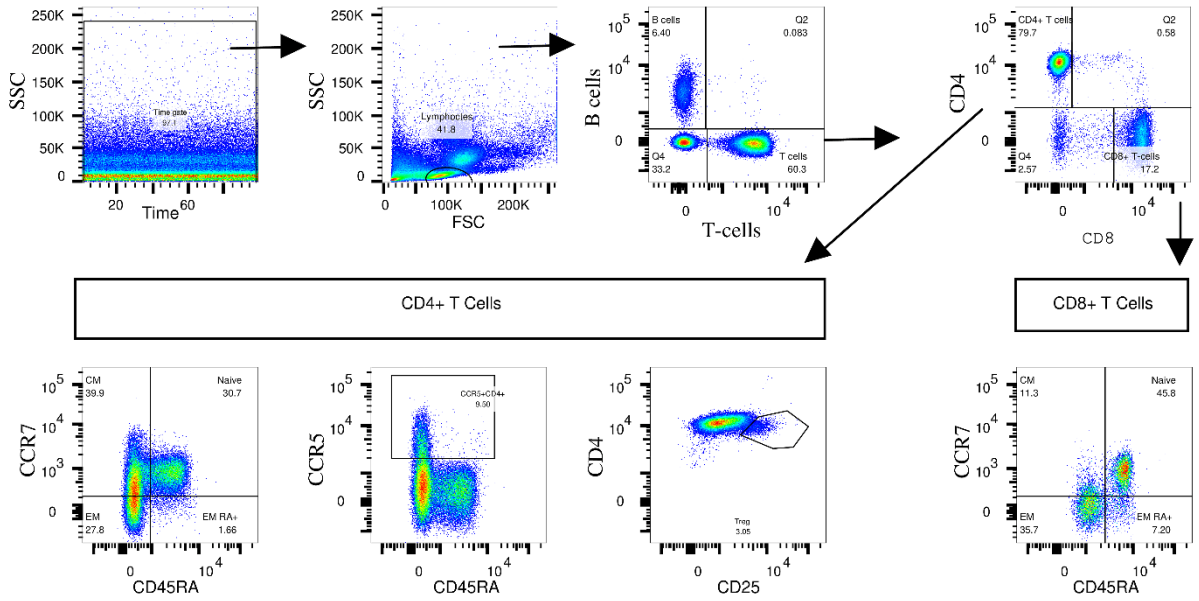

Figure S3B. **Gating strategy**. SSC vs. time was used to remove unwanted events. Next, SSC v.s FSC were used to gate on lymphocytes. CD19 vs. CD 3 were used to gate on CD19+ B-cells and CD3+ T-cells. CD4 vs. CD8 were used to differentiate CD4 and CD8 T-cells. Next, CCR7 vs. CD45RA we used on both CD4 and CD8 T-cell to differentiate naïve (CCR7+CD45RA+), Central memory (CM, CCR7+CD45RA-), and effector memory T-cells (EM, CCR7-). CCR5 vs. CD45RA were used to gate on CCR5+ T-cells. CD4 vs CD25 were used to gate on CD25++ T-cells.

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Basophil activation test (unstimulated control)
Figure S4A

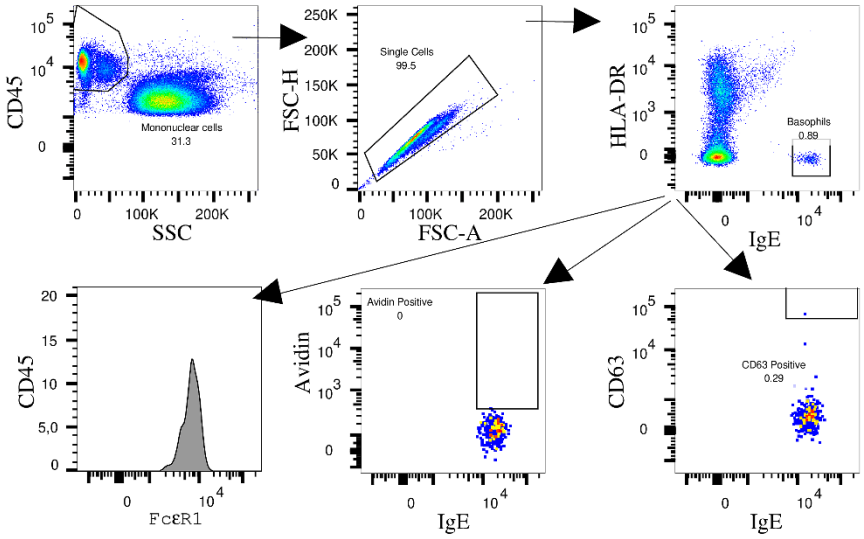

Figure S4A. **Gating strategy**. CD45 vs. SSC were used to exclude neutrophils and eosinophils. CD45 were also used during acquisition to exclude events with MFI < 1000. Next, we used FSC-A vs. FSC-H to exclude cell aggregates. HLA-DR vs. IgE were used to gate on basophils. CD63 vs. FSC were used to gate on activated basophils, a fixed gate set at MFI 50000 was used. Avidin vs. FSC were used to gate on activated basophils, and unstimulated samples were used to asess the gate.

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Basophil activation test (Allergen stimulation with timothy)

Figure S4B

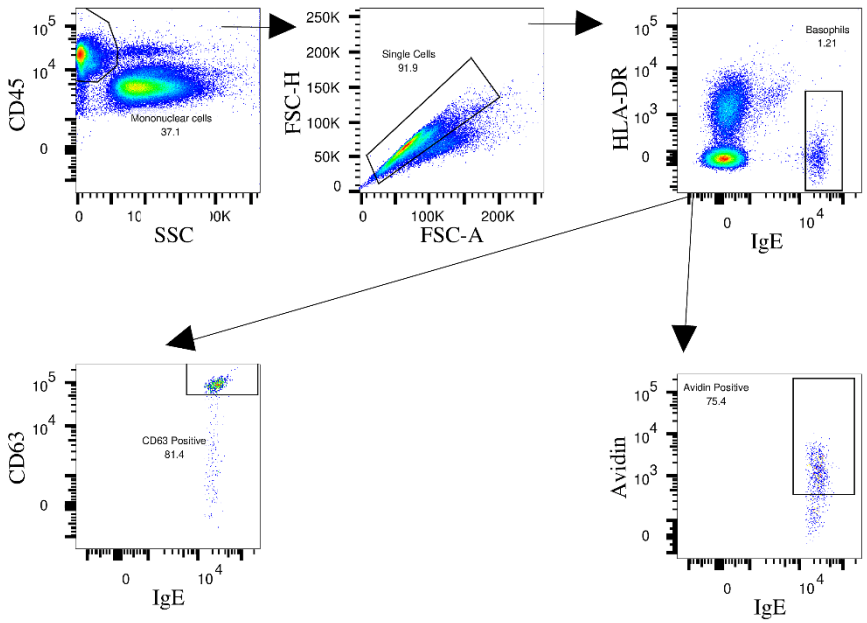

Figure S4B. **Gating strategy.** CD45 vs. SSC were used to exclude neutrophils and eosinophils. CD45 was also used during acquisition to exclude events with MFI < 1000. Next, we used FSC-A vs. FSC-H to exclude doublets. HLA-DR vs. IgE were used to gate on basophils. CD63 vs. FSC were used to gate on activated basophils, a fixed gate set at MFI 50000 was used. Avidin vs. FSC was used to gate on activated basophils, and the unstimulated sample was used to assess the gate.

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Table S1

Antibodies	Fluorochrome	Source	Dilution	Identifier	Panel
CD8	FITC	BD	4/100	Cat#555634	Lymphocytes
CD3	Pacific Blue	BD	1/25	Cat#560365	Lymphocytes
CD19	APC	BD	1/20	Cat#555415	Lymphocytes
CCR5	PE-CF594	BD	1/100	Cat#562456	Lymphocytes
CCR7	PE-CY7	BD	1/25	Cat#557648	Lymphocytes
CD45RA	BV510	BD	1/25	Cat#561640	Lymphocytes
CD25	BV711	BD	1/20	Cat#563159	Lymphocytes
CD4	PerCP 5.5	BD	3/100	Cat#300530	Lymphocytes
CD45	PE-CF594	BD	1/250	Cat#562312	Basophils
FcRI-APC	APC	Abcam	1/150	Cat#Ab155369	Basophils
IgE	PE	Bioledgend	1/150	Cat#B247964	Basophils
HLA-DR	PE-CY7	BD	1/150	Cat#560651	Basophils
CD63	PerCP 5.5	BD	1/100	Cat#564526	Basophils

Chemicals	Source	Identifier
NH4CL	Sigma	Cat#A9434
NAHCO3	Sigma	Cat#S5761
DisodiumEDTA	Sigma	Cat#E9884
Formaldehyd solution 4%	HistoLab	Cat#02178
Ficoll 1.077	GE Healthcare	Cat#17-1440-02
Avidin (Used 125µg/ml)	Sigma	Cat#94091

Other	Source	Identifier
DMEM7F12	Gibco	Cat#21331020
MACS Tissue Storage	Miltenyi Biotec	Cat#130-100-008
PBS	Gibco	Cat#2812-019
Compbeads anti-mouse	BD Biosciences	Cat#552843
Compbeads anti-RAT	BD Biosciences	Cat#552844
CS&T beads	BD Biosciences	Cat#650621
BD Vacutainer	BD Biosciences	Cat#367526
Allergen Birch	ALK	Cat#032680
Allergen Timothy	ALK	Cat#032904

REAGENT or RESOURCE	SOURCE	Version
Instrument and Software		
LSR Fortessa	BD Biosciences	Blue, Red, Violet, Yellow-Green
Graphpad		6
Flowjo	BD Biosciences	10.6.2
BD FacsDiva	BD Biosciences	8.0.2

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RESULTS

Figure S5

B-cells in FNA and blood

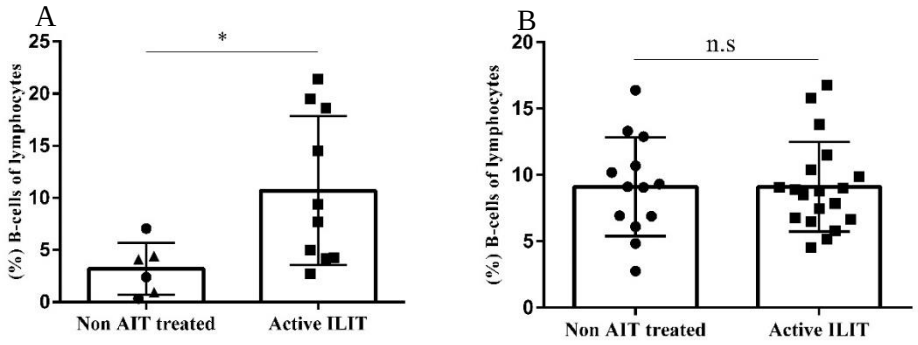

Figure S5. Our result show that the fraction of B-cells is increased in the injected lymph node in active ILIT compared to non-AIT treated patients. Figure A represents data from FNA and figure B represents data from blood. The figure represents un-paired observations, Mann-Whitney test was used for statistical analysis. Data was revealed by flow cytometry. *p<0.05, n.s.= not significant. Horizontal lines represent the mean value and SD.

Figure S6

CCR5 expression CD4 T-cells in lymph nodes and blood

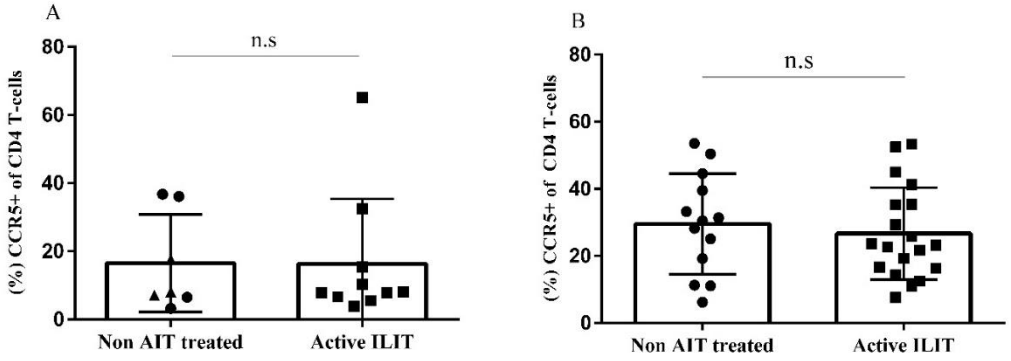

Figure S6.No changes in CCR5 expression could be detected in FNA or blood. Figure A represents data from FNA, and B represents data from blood. Figures A and B represent un-paired observations, Mann-Whitney test was used for statistical analysis. N.s.= not significant. Data was revealed by flow cytometry. Horizontal lines represent the mean value and SD.

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Figure S7

CD25++ expression CD4 T-cells in blood

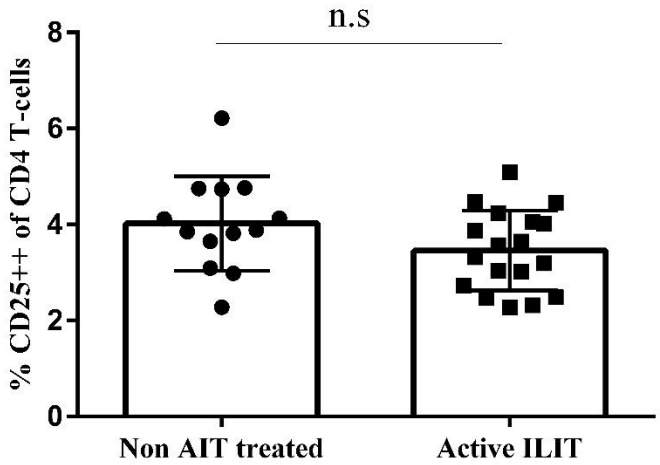

Figure S7. No changes in the levels of CD4+CD25++ could be detected between active ILIT and non-AIT treated patients. The figure represents un-paired observations, Mann-Whitney test was used for statistical analysis. N.s.= not significant. Data was revealed by flow cytometry. Horizontal lines represent the mean value and SD.

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Figure S8

Linear reg. of IgE vs FcεR1

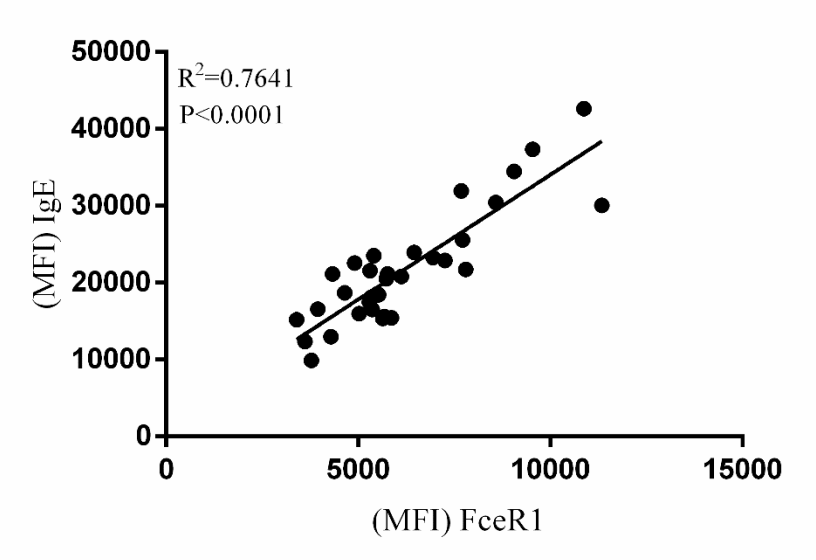

Figure S8. Our result shows that the binding of IgE is dependent on FcεR1 receptor density on the basophil cell surface. Data represents paired observations, Spearman correlation was used for statistical analysis. Data was revealed by flow cytometry.

Figure S9

Basophil activation detected with Avidin

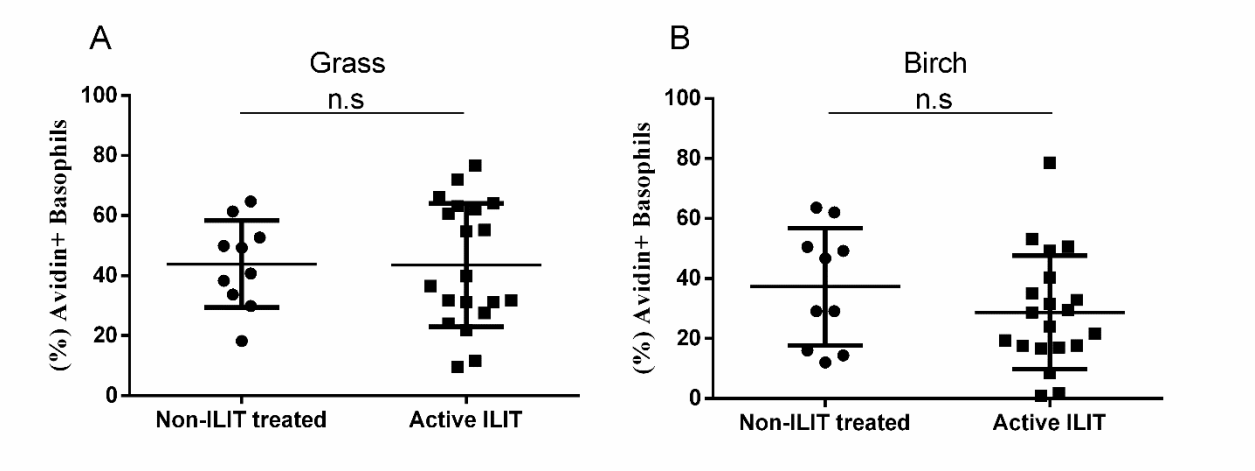

Figure S9. Fig A-B represents un-paired observations, Mann-Whitney test was used for statistical analysis. Data was revealed by flow cytometry. n.s.= not significant, Horizontal lines represent the mean value and SD.

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