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Single-cell transcriptomics reveals the alteration of peripheral blood mononuclear cells in AR patient challenge by allergen

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Single-cell transcriptomics reveals the alteration of peripheral blood mononuclear cells in AR patient challenge by allergen

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To the Editor:

Allergic rhinitis (AR) is one of the most common diseases globally[1]. The prevalence of AR has been reported to be approximately 2% to 25% in children [2] and 1% to greater than 40% in adults [1,3]. The prevalence of confirmed AR in adults in Europe ranged from 17% to 28.5%. Classical symptoms of AR are nasal itching, sneezing, rhinorrhea, and nasal congestion. Ocular symptoms are also frequent[4,5]; The symptoms of AR can reduce quality of life and school and work performance and is also a frequent reason for outpatient treatment in ENT department which may costs large medical expenses. AR is a risk factor for asthma [4,5], and uncontrolled moderate-to-severe AR affects asthma control.

The pathogenesis of AR is closely related to the changes of immune cells after allergen challenge. When AR patients are challenged by allergens, the immune cells in the body will produce a series of changes, producing Th2 related inflammation. The symptom occurred in 24 hours is called early-onset reaction, while in 72 hours is called late-onset reaction. T cells are the most important effector cells, while monocytes are also involved in the response transformed by the recruitment of inflammatory factors. B cells and NK cells are also are also important participating cells [6,7,8]. We have a certain understanding of the pathogenesis of allergic rhinitis, but we have not known the dynamic changes of immune cells in AR patients after exposure to allergens which sequence from the level of single cell technology. What's more, the dynamic changes of immune cells and gene expression in peripheral blood after allergen challenge are of great help to explore the pathogenesis of allergic rhinitis and specific treatment for specific immune cells or allergen stimulated immunotherapy, but there is no relevant report. Here we use 10x single-cell RNA sequencing to present the dynamic changes of immune cells in PBMC of patients with allergic rhinitis before and 24 and 72 hours after exposure to allergens.

A 26 years old lady, who was diagnosed as only dust-mite specific allergy moderate to severe allergic rhinitis in October 2023 was volunteered to join our research after our strict screening. She has no history of other diseases and no medication within half a year, and has a healthy live-style. Although her peripheral blood eosinophils were higher than normal, she did not have asthma and airway hyperresponsiveness, with lung function (FVC FEV1) in a normal level. (The general information of the patients is shown in Table E1) After preparation, we conducted a nasal allergen challenge test on this patient. PBMCs were extracted from

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peripheral blood of patients before challenge, 24 hours after challenge and 72 hours after challenge, and then sequenced for 10x single cell RNA respectively (FIG1 A). We evaluated the subjective and objective symptom scores before and after nasal provocation to ensure that the patient was successful. (Table E2).

The total number of estimated cells for three PBMC sample were 14,202. The results of single cell sequencing showed that the three PBMC samples were divided into 13 clusters of cells without supervision (FIG1 B). The top 10 differentially regulated genes in the 13 clusters cell were showed in the heatmap report scaled expression (FIGE1). Differentially expressed genes fall into categories. These 13 clusters of cells were partial differences in cluster among three samples. Cluster 1 almost disappeared 72 hours after challenge, while cluster 6 only appeared before challenge, and clusters 7 almost appeared 72 hours after challenge (FIG1 C). These differences of these cell subsets will be further explained in the follow-up supervised analysis. It shows that there are indeed changes in peripheral blood PBMC of AR patients after exposure to allergens. Then we first identified 4 cell types by the specific markers (FIG E2 A), including: B cells (MS4A1, CD79A), Monocytes (CD14, FCGR3A), natural killer cells (NKG7, GNLY and $CD3^-$), T cells (CD3D, CD3G). Interesting, we found the cluster 6 cells which only appeared in before challenge sample expressed HBA1, PPBP, LYZ, IGKC, G0S2, XPB1, HPRT1 and ID2 gene, but negative for the CD34 gene (FIG E2 B). Based on gene expression, we define this cluster as HSC-GSF cells. So we finally identified 5 cell-type groups in three PBMC sample. They are Monocytes, T cells, HSC-GSF cells, NK cells and B cells (FIG1 D). The proportion of T cells and NK cells increased gradually from pre-challenge to 72 hours, from 42.4% (pre-challenge) to 53.8% (24h) to 60.9% (72h) of T cells and 2.8% (pre-challenge) to 6.6% (24h) to 8.4% (72h) of NK cells. While the proportion of Monocytes were highest at 24h after-challenge (34.9%), but nearly the same level at pre-challenge and 72h (24.3% vs 25.5%), which is consistent with the fact that a large number of monocytes will be recruited by some chemokines during the early stage of allergy but not the final effector cells. However, B cells were in highest proportion at pre-challenge (6.3%), lowest at 24h (4.6%). Finally, the HSC G-SCF cells seem to have all transformed after the allergen challenge (FIG1 E, F). We use pseudotime analysis and found the trajectory of HSC-GSF clusters may differentiation into T cell (cluster 2 3 4 5), Monocyte (cluster 1 7 11) and NK cell (cluster 8) from pre-challenge to 24h and 72h after-challenge.

We further used dimensionality reduction techniques to analyze subgroups and transcriptional gene expression of each group of cells. The T cell group can be divided into 8 clusters by dimensional reduction analysis. The clusters of T cells can be preliminarily distinguished by CD4 and CD8a CD8b (FIG2 A). Then we further divided T cells major to Th2($CCR4^+$,$GATA3^+$, $CXCR4^+$, $CCR3^+$), Treg($FOXP3^+$, $IKZF2^+$), Naïve T($TCF7^+$, $SELL^+$, $LEF1^+$, $CCR7^+$) and Cytotoxic CD8($GZMB^+$, $GZMK^+$, $NKG7^+$, $CST7^+$, $PPF1^+$, $TRGC2^+$) cells according to their functional express gene (FIG2 B). The proportion of Th2 were highest at 24h after challenge (42.4%), while lowest at 72h (23.4%) of all the Th2 cell (FIG2 B). And the functional gene of Th2 were also at highest expression at 24h, the t-SNE plot of GATA3 and CXCR4 show greater increase at 24h after challenge than pre-challenge, but decreased at 72h. And the hi-expression (Log2>2) t-SNE plot of CXCR4 showed the expression intensity higher than two of CXCR4 gene in Th2 cells at 24 hours after challenge. The expression of CCR4 did not show much difference in three sample. (FIG E3A). However, Treg cells were at the top level at 72h, to 47.1% of all the Treg cells, while 24h and pre-challenge were almost at the same level (27.6% vs 25.3%) (FIG2 B). In accordance with this, Foxp3 and IKZF2, the functional genes of Treg, was also in the strongest expression at 72 hours after challenge (FIG E3B). This suggests that the body has already started its own immune regulation function from 72 hours after allergen challenge by increasing the proportion of Treg cells and inhibit the progress of Th2 inflammation. Cytotoxic CD8 were at highest level at 24h, approximate to 44%, and secondly at the 72h (32.6%), but only 23.5% at pre-challenge (FIG2 B). From the expression of functional genes, the main functional genes (GZMB, GZMK, NKG7, CST7, PPF1, TRGC2) of cytotoxic CD8 cells were stronger at 24 hours and 72 hours after challenge than before (FIG E3B). It is suggested that cytotoxic CD8 cells are also involved in allergic reaction. Naïve T cells were almost at the same level in three sample (30.3%, 33.8%, 34.9%). Its major genes expression was also relatively stable in three samples(not show). From the pseudotime analysis, we found the Naive CD4 T (cluster 2) cells may transform to Treg cells (cluster 7) instead of Th2 cells (cluster 1) and the Naive CD8 T cells (cluster 4 5) may transform to Cytotoxic CD8 cells (cluster 3 6) after challenge. (FIG2 C).

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Then we focus on the Monocyte. We used dimension reduction analysis and further divided the Monocyte into 6 unbiased cluster in t-SNE. Then Mono1($CD14^+$ $FCGR3A^-$). Mono2 ($CD14^+$ $FCGR3A^+$), Mono3 ($CD14^+$ $FCGR3A^{++}$)cells were identified. However, Mono4 related genes ($CD14^-FCGR3A^-$ $KLRC4^+KLRK1^+TCRBV3S1^+$)[9]were not detected, so we identified three Monocytes type (FIG E4A). The proportion of Mono1 cells were the lowest among the three types of Monocyte, which were 4.2% (pre-challenge), 2.1%(24h), 1.9% (72h) in the three samples (FIG 2D). There was no significant difference in the expression intensity of the top five genes (FECR1A NDRG2 NRARP AXL AFF3) in Mono1 among the three samples (FIG E4B). The proportion of Mono3 cells were highest in 24h (20.3%) (FIG 2D), while the top five expressed gene (MS4A4A CDKN1C HES4 CD79B C1QA) did not show significant difference among three sample (FIG E4C). Mono2 occupied the largest share as well as the biggest variation of all the three-type Monocyte in three sample. The proportion of Mono2 cells were slightly lower at 24 hours (77.6%), but about the same level at pre-challenge (84.3%) and 72 hours after challenge (84.5%) (FIG 2C). However, the Mono2 subsets in the three samples were almost completely different. $CCL2^+CCL8^+CXCL10^+$subsets were predominant before challenge, which is indicated that Mono2 mainly exists in the form of chemotactic before allergen challenge in the body. However, at 24 hours after challenge, the chemotactic Mono2 subgroup disappeared and replaced by two another Mono2 subgroups with EGR2, EGR3 positive and OSM positive. . When it comes to 72 hours, the cell Mono2 subsets changed to $IERSL^+OLIG1^+$Mono2 (FIG 2D). This also confirmed that at the gene level of single cell, after 72 hours of allergic reaction, Monocytes were not the main cells involved in inflammation. The top ten genes of Mono2 in three sample were showed in the FIG E4D.

Finally, B cells and NK cells did not show multiple subgroups on the FIG1 B & C t-SNE plot, so we did not conduct dimensional reduction analysis. Changes in the proportion of B cells and NK cells after allergen challenge have been described previously. We compared the genes expressed in B cells and found that the top ten genes in B cells were down regulated after allergen challenge. Of which, FCRLA, IGHD and IGHG2 are closely related to the immune function of B cells, suggest that the function of B cells may be weakened after allergen stimulation (FIG E5A). This is different from what we knew before. On the contrary, the expression of the top ten genes in NK cells increased significantly after challenge. The expression of KLRF1, NCR1, CD160 gene are closely related to the function of NK cells, which indicates that NK function is up-regulated after allergen challenge. NK cells may play a more important role in allergic reactions than we have previously know(FIG E5B).

For the first time, we analyzed the dynamic changes of PBMC in AR patients after allergen challenge by single cell sequencing technology, and simulated the clinical pathogenesis of AR. Total of 5 groups of cells were identified in the pre- and pro-challenge, including T cell, NK cell, B cell, Monocyte and HSC-G-CSF cell. HSC-G-CSF cell only existed before challenge, and completely disappeared at 24h and 72h after challenge. It may transform into T cells, NK cells and Monocyte by the pseudotime analysis. The proportion of Th2 in functional T cells was the highest at 24h while Treg was the highest at 72 hours, suggesting that the peak of inflammation may be in 24 hours and the body has already started the self-regulation mode at 72 hours. According to the proportion of CD8 toxic T cells and the expression of functional genes, it may also be involved in allergic reaction. Except for Mono4, other three subsets of Monocytes can be identified. The largest proportion Mono2 subset showed significant difference pre- and pro- challenge. From the perspective of gene expression, Mono2 seems did not participate in the inflammatory reaction of late-onset after 72 hours. Surprisingly, the proportion of B cells and the expression of functional genes decreased after challenge, suggesting that the role of B cells in allergic diseases may not be as high as expected. But on the contrary, the proportion of NK cells and functional genes were enhanced after challenge. The role of NK cells in allergic rhinitis can be further studied to find out something new.

We used single cell sequencing technology to study the dynamic changes of peripheral blood PBMC in the pathogenesis of AR. Although it is only a descriptive case, there are innovative findings, which are helpful for understanding the pathogenesis of Allergic rhinitis and follow-up research and database establishment.

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Method

General information of the patient

The selected patient was a 25-year-old female who has no history of smoke/drink/asthma/ diabetes/autoimmune disease/blood system disease/heart disease/surgery/blood transfusion, and has no experience of upper respiratory tract infection or application of corticosteroids/ antihistamines in recent half year. The patient has been diagnosed as allergic to dust mite only, with a symptom of urticaria. Lung function and blood routine examination were also performed. The lung function was normal, but the blood routine showed that the proportion and quantity of eosinophils increased. Detailed information were showed in Table E1.

Nasal allergen provocation test

Preparation

The provocation test is performed at the temperature of 14-20 in a quiet room which is equipped with first-aid measures such as epinephrine, dexamethasone, antihistamines, tracheotomy packages, and oxygen inhalation devices. We first prepared well the solvent solution, allergen solution (HDM 1.0mg/ml, Wolwobiotech, China) in two different marker provocation device (the sterile syringe). Then the patient's scores of total nasal symptom scores (TNSS) , visual analog scale (VAS) and anterior nasal endoscope and nasal flow test were assessed at baseline and after nasal provocation.

Nasal provocation

a) Patient will challenge by the allergen solvent first as solvent control. A special nasal spray device will be used for nasal provocation tests. Two doses will be sprayed on each side of the nasal cavity, with an average of 0.05 mL each dose, one in the direction of the common nasal meatus, and the other in the direction of the middle meatus. Patient should to take a deep breath before spraying, then hold breath during the spraying procedure, take deep breath afterwards. Then we will evaluate the symptom and nasal examination within

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10 minutes in chronological order. We found the solvent provocation did not cause more than 50% of the symptoms increased or 30% nasal flow decreased. (Table E2). So the allergen solvent challenge is negative, we can do allergen solution provocation.

b) The nasal allergen provocation was performed at least 10 mins after solvent control. Same as solvent provocation, two doses spray will be given on each side of patient's nasal cavity, with an average of 0.05 mL each dose, one in the direction of the common nasal meatus, and the other in the direction of the middle meatus. Patient should to take a deep breath before spraying, then hold breath during the spraying procedure, take deep breath afterwards. Then we will evaluate the symptom and nasal examination within 10 minutes in chronological order. The provocation is defined as positive when subjective measures is clearly positive or objective measures is clearly positive or both subjective and objective measures are moderately positive, based on the EAACI Position paper on the standardization of nasal allergen challenges. After allergen challenge, the symptom score increased by more than 50% and the nasal airflow decreased by more than 30% compared with baseline and solvent control. The nasal provocation test was successful (Table E2).

Observation after provocation

Observe the patient at least every 0.5h. Make sure the patient is informed of the possible delayed allergic reaction in 12h after provocation.

Isolation of PBMCs

20ml peripheral blood would be collected from the patient before provocation and 24h, 72h after allergen nasal challenge. Peripheral blood was collected in the heparin anticoagulant according to a standard manner, then transferred to a 50 mL centrifuge tube and diluted with 20 mL PBS. 2.5 mL Ficoll-Hypaque Solution (Meijingbio, China) were added to the tube and mixed gently. Centrifuge at 400g for 25 minutes, and transfer PBMC cell layer to another tube. Add enough HBBS and mix thoroughly. After centrifuging at 300g for 5 minutes, the supernatant was discarded and the cell pellet was re-suspended in HBSS to repeat the centrifuging again.

Preparation of Single Cell Suspension

Cells were transported in DMEM (ThermoFisher Scientific, Waltham, MA) on ice immediately after above procurement. This suspension was then filtered using a 40μm strainer (ThermoFisher Scientific) and residual cell clumps were discarded. The suspension was immediately placed on ice. After centrifuging at 300g at 4°C for 6 minutes, the supernatant was discarded and the cell pellet was re-suspended in DMEM and placed on ice prior to staining for loading.

Sequencing library construction using the 10x Chromium platform

We Approximately 15000-20000 cells were partitioned into nanoliter droplets to achieve single cell resolution for a maximum of 5000 individual cells per sample. The resulting cDNA was tagged with a common 16-nt cell barcode and 10- to 12-nt unique molecular identifier during the reverse transcription (RT) reaction. Full-length cDNA from poly A–tailed mRNA transcripts was enzymatically fragmented and selected to optimize the cDNA amplicon size (˜400 bp) for library construction (10x Genomics). The single cell library concentration was quantified by quantitative PCR (qPCR) analysis (Kapa Biosystems) to produce appropriate cluster counts for the NovaSeq 6000 platform (Illumina). We generated 28–98 bp (3' v3 libraries) or 2–150 bp (5' libraries and TotalSeq libraries) sequence data targeting ˜50 K read pairs/cell for the gene expression library, which provided digital gene expression profiles for each individual cell.

Single-cell RNA-seq data preprocessing:

The Cell Ranger software pipeline (version 3.1.0) provided by 10xGenomics was used to demultiplex cellular barcodes, map reads to the genome and transcriptome using the STAR aligner, and down-sample reads as required to generate normalized aggregate data across samples, producing a matrix of gene counts versus cells. We processed the unique molecular identifier (UMI) count matrix using the R package Seurat (version

3.1.1). To remove low quality cells and likely multiplet captures, which is a major concern in microdroplet-based experiments, we apply a criteria to filter out cells with UMI/gene numbers out of the limit of mean value +- 2 fold of standard deviations assuming a Guassian distribution of each cells' UMI/gene numbers. Following visual inspection of the distribution of cells by the fraction of mitochondrial genes expressed, we further discarded low-quality cells where >10% of the counts belonged to mitochondrial genes. After applying these QC criteria, 14202 single cells in total remained and were included in downstream analyses. Library size normalization was performed in Seurat on the filtered matrix to obtain the normalized count.

Top variable genes across single cells were identified using the method described in Macosko et al. Briefly, the average expression and dispersion were calculated for each gene, genes were subsequently placed into 13 bins based on expression. Principal component analysis (PCA) was performed to reduce the dimensionality on the log transformed gene-barcode matrices of top variable genes. Cells were clustered based on a graph-based clustering approach, and were visualized in 2-dimension using tSNE. Likelihood ratio test that simultaneously test for changes in mean expression and in the percentage of expressed cells was used to identify significantly differentially expressed genes between clusters. Here, we use the R package SingleR, a novel computational method for unbiased cell type recognition of scRNA-seq, with the reference transcriptomic datasets 'Human Primary Cell Atlas' (Mabbott et al. 2013) to infer the cell of origin of each of the single cells independently and identify cell types.

Differentially expressed genes (DEGs) were identified using the FindMarkers function of Seurat[1] package. P value < 0.05 and $|\log2\text{foldchange}| > 0.58$ was set as the threshold for significantly differential expression.

Funding

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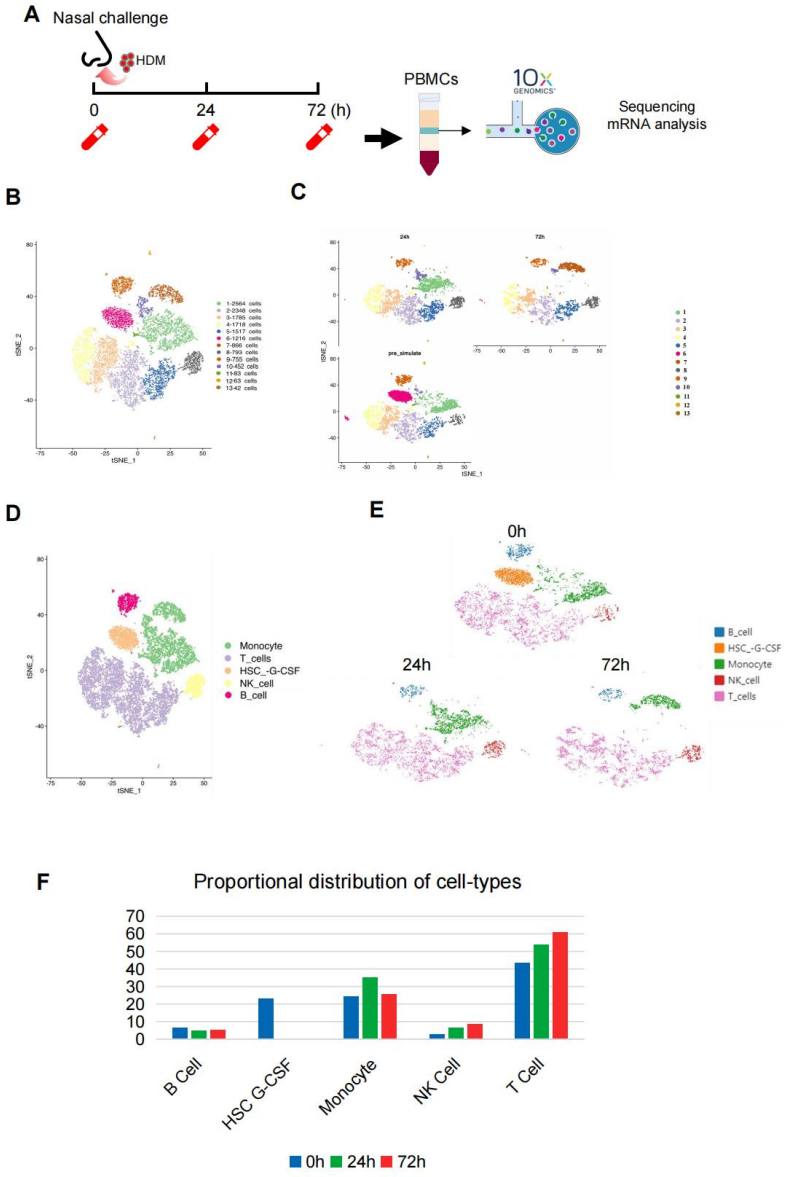
Figure 1



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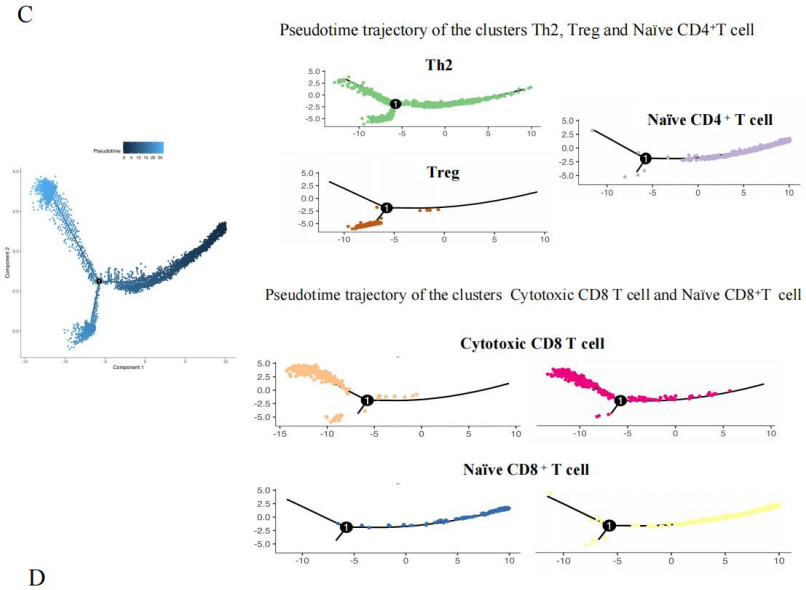
C



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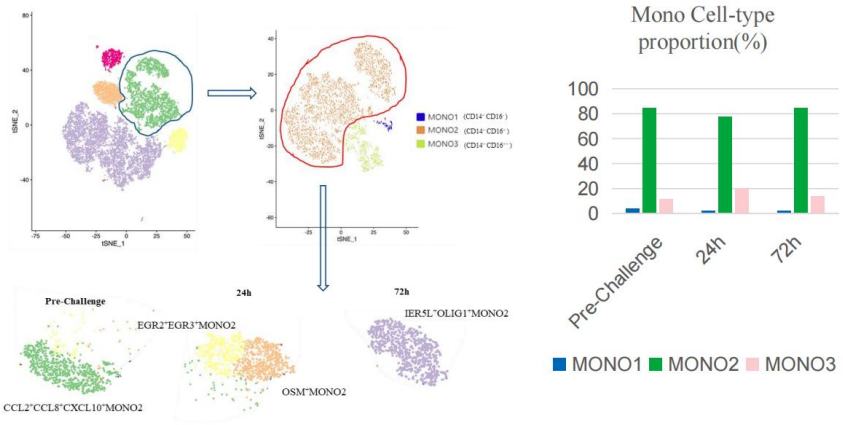

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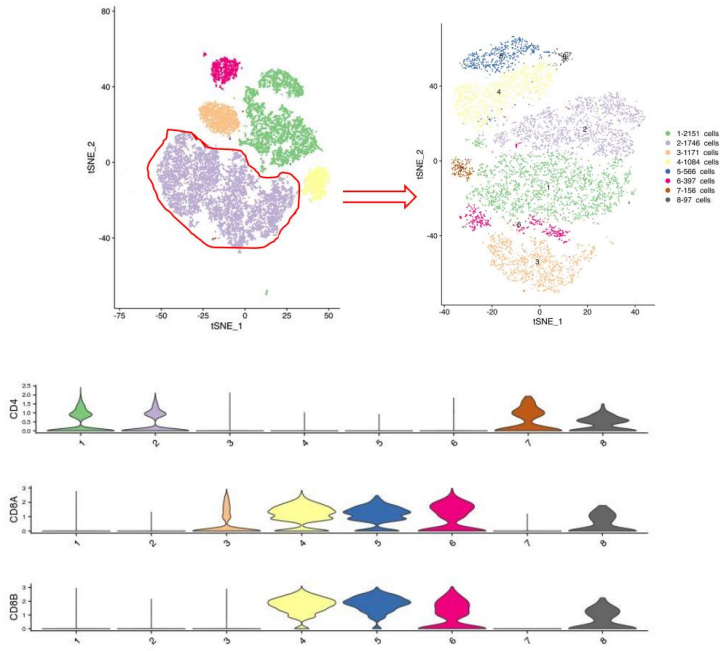
FIG 2

A



B

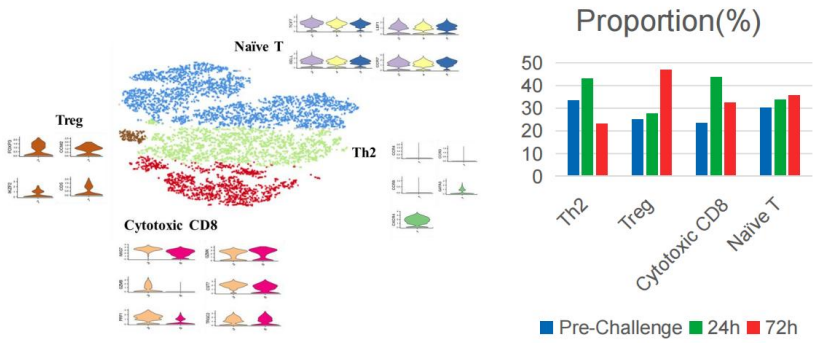


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Figure E1

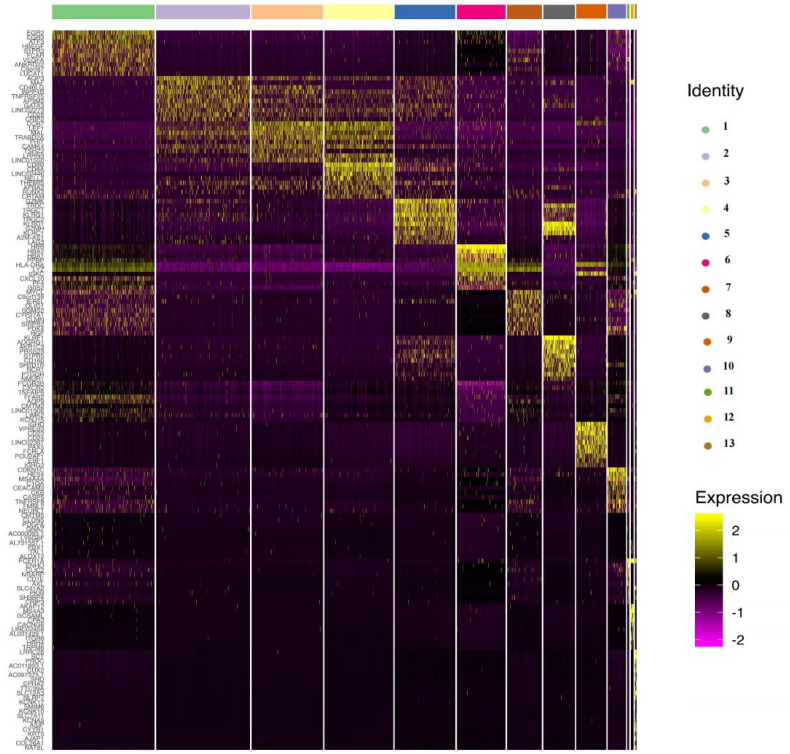

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FIG E2

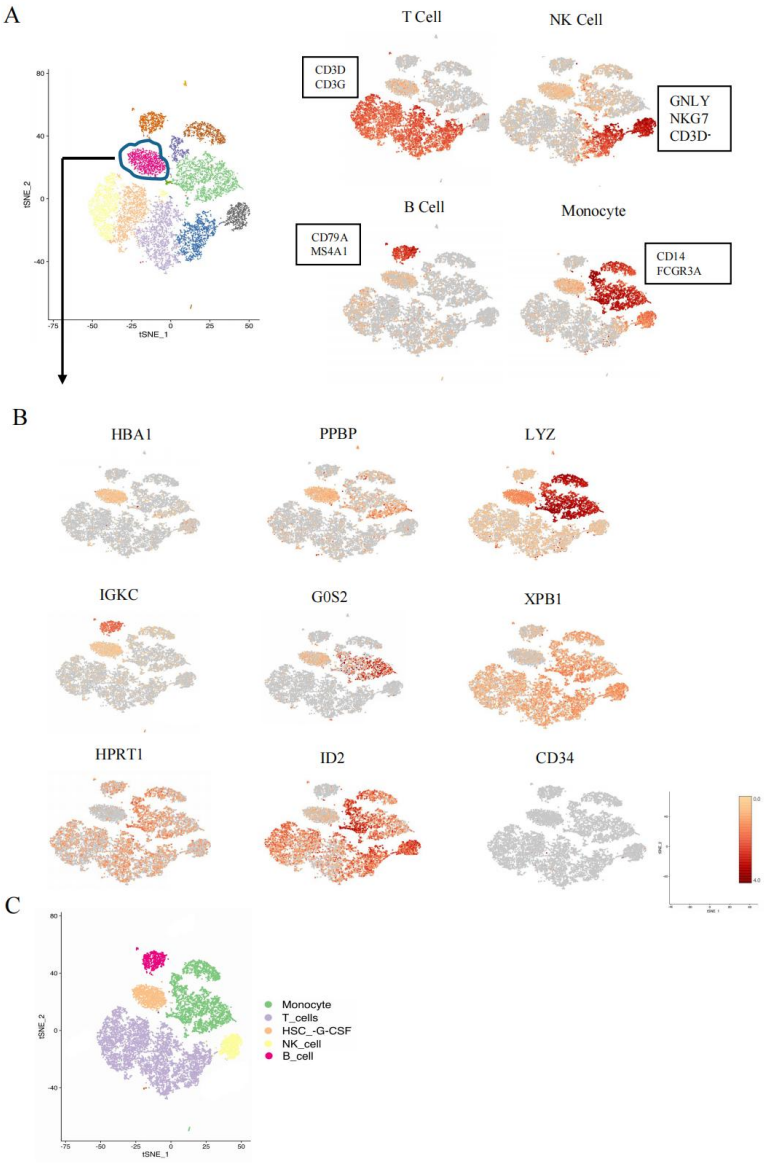

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FIG E3

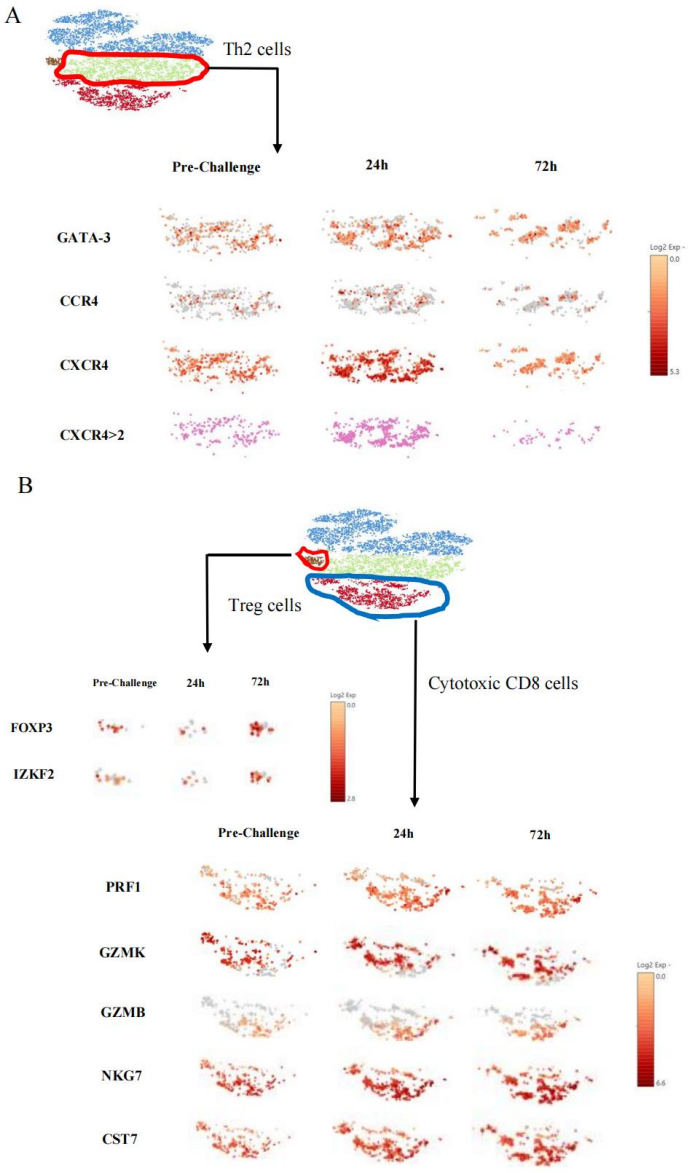

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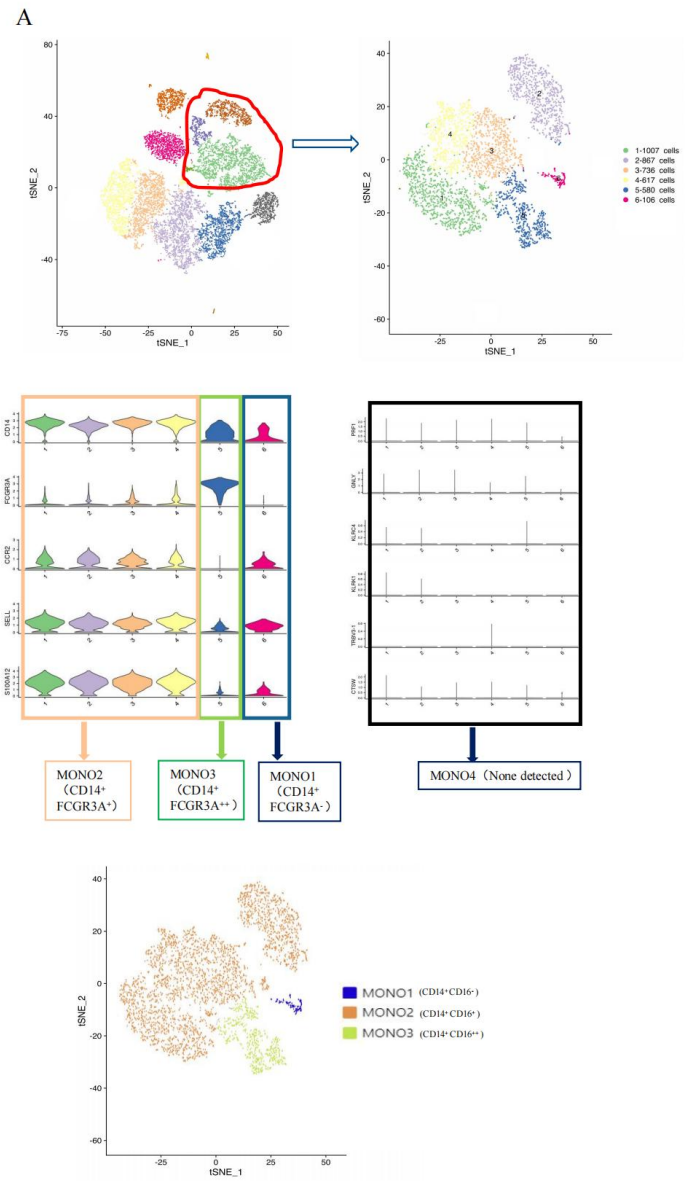
FIG E4



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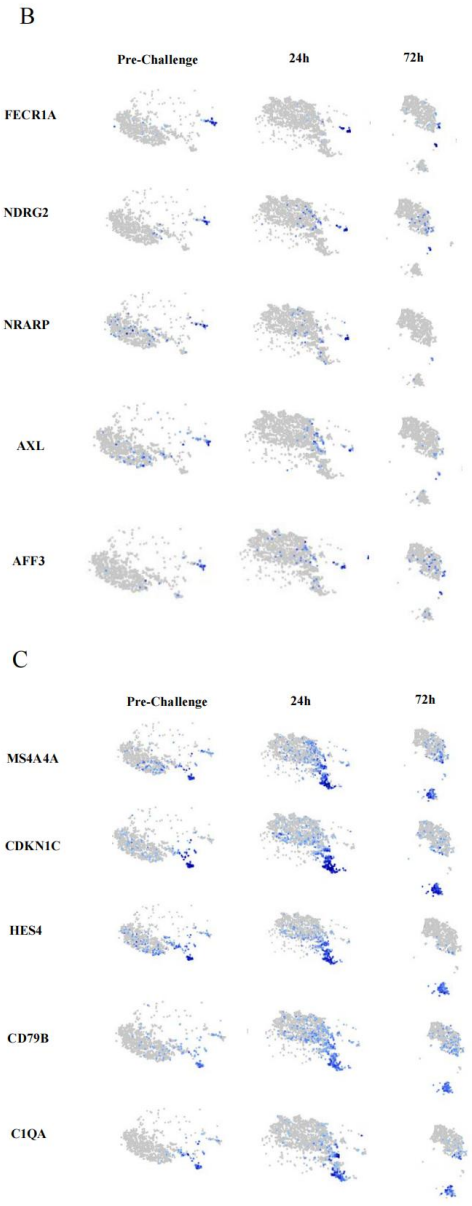

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D

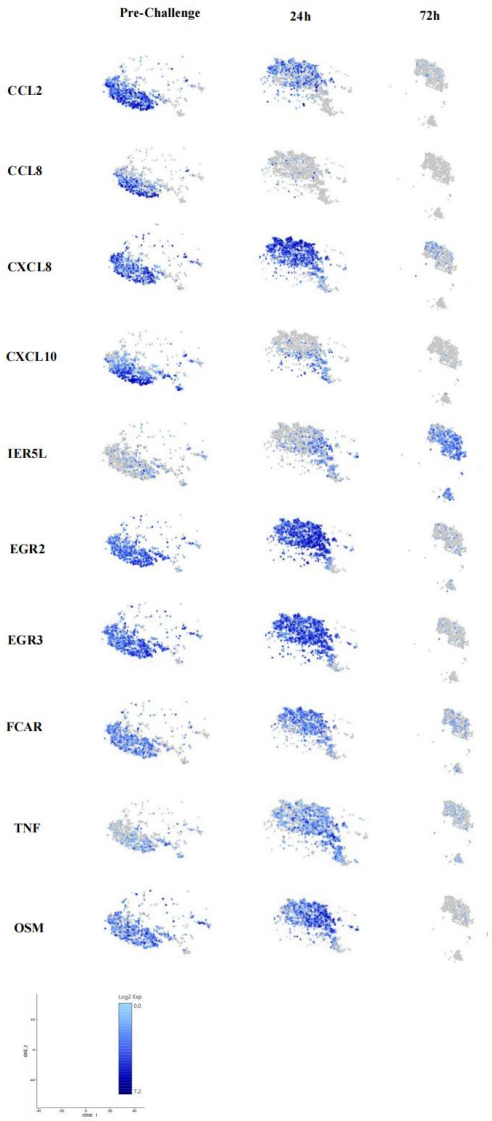

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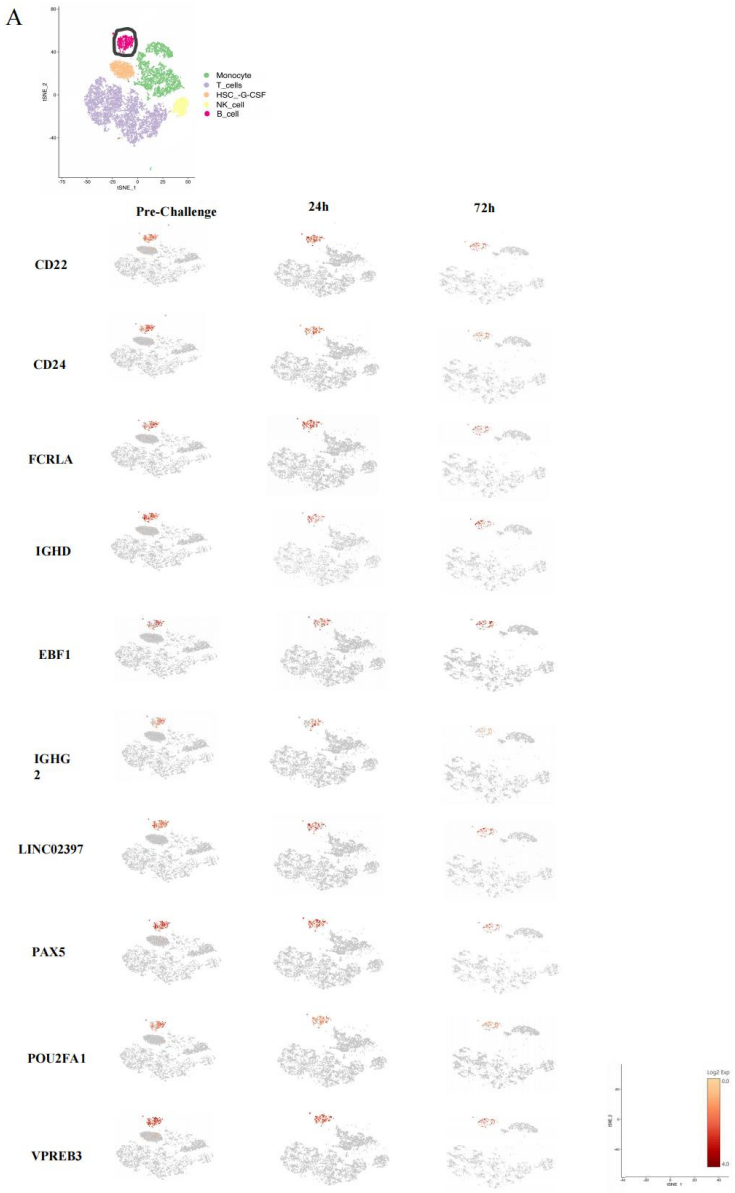
FIG E5



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B

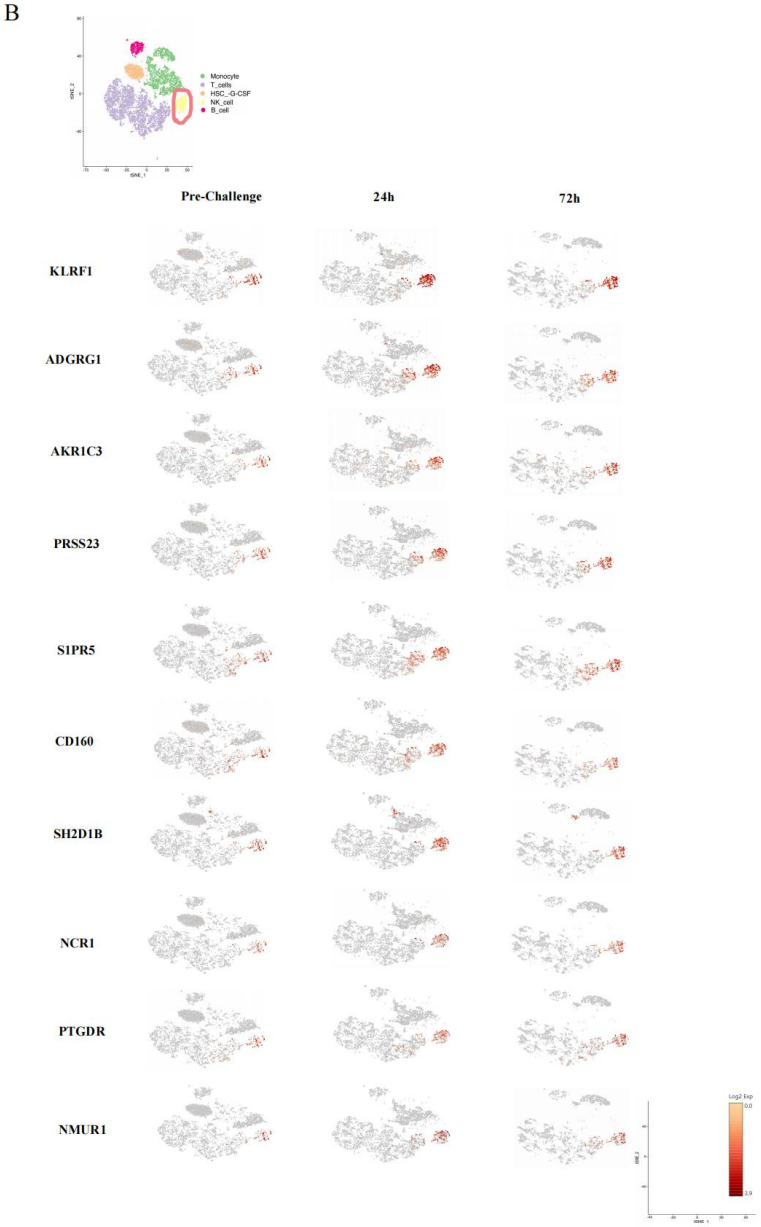

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Table E1 The general imformation of the patient	
Sex	Female
Age	26
Height	163cm
Wt	49kg
Bp	108/78mmHg
Smoke	No
Drink	No
Asthma	No
Diabetes	No
Heart Diease	No
Surgery History	No
Blood transfusion history	No
Upper respiratory tract infection in recent half year	No
History of corticosteroids or antihistamines in recent half year	No
History of autoimmune or blood system disease	No
Urticaria	Yes
FVC	90% of the estimated value
FEV1	92% of the estimated value
FEV1/FVC	88%
Blood Routine test	
WBC	$6.01x10^9/L$
NEUT	$2.74x10^9/L$
NEUT%	44.5
LY	$2.03x10^9/L$
LY%	33.8
MO	$0.52x10^9/L$
MO%	8.7
EO	$0.71x10^9/L$
EO%	11.8
BASO	$0.01x10^9/L$
BASO%	0.2
PLT	$234x10^9/L$
SPT（Derp）	Positive
Specific IgE to Derp1	32.9 KU/L
Specific IgE to Derp2	24.6 KU/L

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Table E2 Subjective and objective scores of patients before and after challenge					
	Pre-Simulate	Solvent control	Dust mite allergen solution	24h	72h
VAS					
Nasal obstruction	4	4	8	7	7
Rhinorrhea	4.5	4	9	8	6
Hyposmia	3.5	2	5	4	4
Facial pressure or pain	2	2	3	4	3
Headache	2	1	3	4	3
Overall score	4	3.5	8	7	5
TNSS					
Nasal obstruction	1	1	3	2	1
Nasal itching	1	1	3	2	2
Sneeze	1	1	3	3	1
Nasal discharge	1	1	3	3	1
Total	4	4	12	10	5
Anterior Rhinoscopy					
Edema Left	1	1	2	2	1
Edema Right	0	0	1	0	0
Rhinorrhea Left	1	2	2	1	1
Rhinorrhea Right	0	0	1	0	0
Total	2	3	6	2	2
Nasal flow(L/min)	41.7	36.7	24.2	40	44.6