Innate Lymphoid Cell Disturbance in Henoch-Schonlein Purpura

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Research article

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Abstract

**Background:** Innate lymphoid cells (ILCs) are tissue-resident lymphoid cells which are enriched in the barrier surfaces and participate in initial immune response against pathogens. It is reported that ILCs are dysfunctional in various human diseases. ILCs is the bridge between innate immunity and adaptive immunity, while Henoch-Schönlein purpura (HSP) meet these characters, early innate and later adaptive immune response. Submucosal lymphotissue is mainly IgA produced area, where ILCs resident. Increasing data confirmed that mucosal immune and IgA is related with HSP. The relationship between ILCs and HSP (IgA vasculitis) remains unclear.

**Methods:** ILCs subsets and lymphocyte subpopulation were characterized in the peripheral blood (PB) of normal controls and patients of HSP and the cell surface markers expression were detected by flow cytometry. We also correlated the frequencies of each ILCs subset in PB with lymphocyte subpopulation and serum IgA in HSP patients.

**Results:** The difference of ILCs/Lymphocytes and ILCs/PBMC in patients with HSP and normal controls was statistically significant. The proportion of ILC1 significantly increased and ILC3 decreased in HSP patients. Moreover, the percentage of ILC1 significantly decreased and ILC3 increased in HSP patients after treatment. The difference of ILCs/Lymphocytes and ILCs/PBMC in the arthritis type and mixed type of HSP was also statistically significant in comparison with normal controls.

**Conclusions:** Our study indicates that the increased circulating ILC3 and decreased circulating ILC1 might be helpful for the pathogenesis of HSP through mediating type 3 immune response?

Background

The innate lymphoid cell (ILC) family is a novel group of innate effector cells which are enriched in the barrier surfaces and provide early and prompt defensive immune response to protect epithelial integrity and tissue immunity [1]. They have the characteristic of lacking antigen specificity for T and B cells or myeloid and dendritic cell phenotypical markers [2]. The ILCs are divided into three groups according to the ILCs nomenclature proposed in 2013. According to development and function, ILCs are divided into five subsets, type 1, 2 and 3 ILC subsets, natural killer (NK) cells and Lymphoid-tissue inducer (LTi) cells [3]. The first group consisted of NK cells and ILC1, whose development and function are dependent on the T-box transcription factor T-bet with the production of IFN-γ. However, NK cells also specialize in the release of perforin and granzymes, which can kill tumors or virus-infected tissue, so they are considered as the innate counterparts of cytotoxic CD8+ T cells. The second group ILC2 express transcription factors GATA3 and RORα/GATA3 + ILC2 secrete type 2 cytokines, mainly interleukin-4 (IL-4), IL-5, IL-9 and IL-13. The third group ILCs are comprised of ILC3 and LTi cells, which express transcription factors RORγt with the production of IL-17 and IL-22 in response to the stimuli of IL-23 and IL-1β. LTi cells can promote lymphoid organogenesis during development [4].
ILCs exhibit a trait of functional diversity and plasticity. ILC2 and ILC3 can be converted into ILC1 in the presence of IL-12, IL-18, and IL-1β, and conversely, ILC1 can be converted back to ILC2 and ILC3 respectively in the presence of IL-4 or IL-23, respectively [5, 6]. Thus, ILCs are important regulators of the epithelial barrier and be involved in immune defense. ILCs are increasingly involved in the pathogenesis of a range of chronic infectious, inflammatory or metabolic diseases in humans, and they play an important role in maintaining mucosal barriers and tissue repair, removing parasitic infections and a variety of tumors [7]. Multiple studies have agreed that ILC2 plays a pathogenic role in pulmonary allergy and inflammatory processes. ILC2 induces allergic pulmonary inflammation by secreting IL-13, which promotes the migration of activated DCs to draining lymph nodes and initiates Th2 differentiation [8]. IL-22 produced by ILC3 is also associated with asthma pathogenesis due to the elevated levels of IL-22 in asthmatic patients compared to healthy controls [9]. ILCs have been extensively studied in intestinal autoimmune diseases, especially in inflammatory bowel disease (IBD), although the exact mechanisms supporting IBD are not fully understood. IL-17 produced by ILC3 has been shown to drive colitis, and the application of anti-IL-17 antibodies can improve the disease. ILC3s also drive intestinal inflammation and pathology in mice with congenital colitis by secreting IL-22 and granulocyte macrophage-colony stimulating factor (GM-CSF) against CD40 [10, 11]. The ratio of ILC1 significantly increased and ILC2 significant decreased in patients with systemic lupus erythematosus (SLE) and ILC3 in SLE patients with moderate to severe activity. In consequence, increased circulating ILC1 might contribute to the pathogenesis of SLE through type 1 immune response [12].

Henoch-Schonlein purpura (HSP), also known as "IgA vasculitis (IgAV)", is a type III allergic disease with IgA deposition in the affected capillaries and small blood vessels. The skin, digestive tract, joints and kidneys are often involved in HSP patients, among which the Henoch-Schonlein purpura nephritis (HSPN) is the most serious complication. The long-term prognosis of HSPN includes chronic kidney disease, which accounts for 1~2% of end-stage renal disease [13]. The incidence of HSP increased year by year, but its etiology and pathogenesis were unclear, which is mostly believed to be the result of immune abnormality, especially in mucosal immunity, environmental factors and genetic synthesis at present. The role of ILCs in the pathogenesis of HSP has not been reported up to now. The purpose of this paper is to investigate whether ILCs participate in the pathogenesis of HSP?

1. Methods

1.1. Study subjects

Fifty-one HSP patients who were hospitalized in Children's Hospital of Soochow University were enrolled in this study. All HSP patients fulfilled the European League Against Rheumatism (EULAR) criteria for the diagnosis of HSP [14]. Enrolled HSP patients were all newly diagnosed patients. The data after treatment was also collected. Patients who were not newly diagnosed and children with tumor or had received steroid and immunosuppressive administration were excluded. Twenty-two age and sex matched normal controls were recruited. This study was approved by the Ethics Committee of the Children's Hospital Affiliated to Soochow University. Informed consents were obtained from all patients and normal controls.
The demographic and clinical features of the HSP patients and normal controls were summarized in Table 1.

### Table 1
The demographic and clinical feature of the HSP and normal controls

| Group | Gender (M/F, n) | Age (±s, m) | Purpura Type (n) | Arthritis Type (n) | Abdominal Type (n) | Renal Type (n) | Mixed Type (n) |
|-------|-----------------|-------------|-----------------|-------------------|-------------------|----------------|----------------|
| HSP   | 22/29           | 86.27 ± 31.44 | 6               | 14                | 11                | 6              | 14             |
| NC    | 6/16            | 79.64 ± 27.48 |                 |                   |                   |                |                |
| P value | 0.192         |             |                 |                   |                   |                |                |

M = month, NC = normal control

### 2.2 PBMC isolation and Flow cytometry for ILCs

Blood from HSP patients and normal controls were collected with heparin anticoagulant tubes. PBMCs were isolated using density-gradient centrifugation on Ficoll-Paque™ PLUS (2010C1119, TIAN JIN, China). Single cell suspensions were prepared and washed three times by phosphate buffer saline (PBS), then the PBMC were stained with antibodies to CD45 (HI30), CD117 (YB5.B8), CD127 (HIL-7R-M21), CRTH2 (BM16) and a Lineage cocktail (LIN) for 15 minutes at room temperature in dark (see Table 2) with the total ILCs being defined as LIN- CD45 + CD127 + CD161+, the ILC1 as CD117-CRTH2-, ILC2 as CD117-/+ CRTH2 + and the ILC3 as CD117 + CRTH2-. Data were acquired using a Beckman Gallios 3L 10c and analyzed with FlowJo-V10 software, where individual gates were established using Fluorescence Minus One (FMO) controls.
Table 2
ILC Staining Panel for Flow Cytometry

| Target               | Color | Clones   | Source |
|----------------------|-------|----------|--------|
| Lineage markers      |       |          |        |
| CD3                  | FITC  | UCHT1    | BD     |
| CD1a                 | FITC  | HI149    | BD     |
| CD14                 | FITC  | M5E2     | BD     |
| CD19                 | FITC  | HIB19    | BD     |
| CD94                 | FITC  | HP-3D9   | BD     |
| CD34                 | FITC  | 581      | BD     |
| CD45                 | BV510 | HI30     | BD     |
| ILC subset markers   |       |          |        |
| CD127                | BV421 | HIL-7R-M21 | BD |
| CD161                | PE    | DX12     | BD     |
| CRTH2(CD294)         | Alexa 647 | BM16 | BD |
| CD117(c-kit)         | PerCP-Cy5.5 | YB5.B8 | BD |

2.3 Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Normally distributed data were presented as mean ± SD. Differences were determined with a two-tailed paired t-test, Mann-Whitney U test, two independent samples t-test or Chi-squared test. Correlations were analyzed using Spearman correlation coefficient with two-tailed p value. P values < 0.05 were considered statistically significant.

2. Results

3.1. Frequency of ILCs in HSP patients in the peripheral blood

In this study, 51 HSP patients and 22 normal controls were included. There were no differences in age and gender between the two groups. Selected laboratory test results and different subtypes of HSP patients are presented in Table 1.

According to the gating strategy as shown by Sandra Bonne-Année et al [15], the isolated PBMCs from HSP patients and normal controls were stained, and then they were subjected to flow cytometry for the analysis of ILC subsets. The lineage makers (CD3, CD45, CD19, CD14, CD1a, CD94 and CD34) are used to exclude B lymphocytes, T lymphocytes, monocytes, dendritic cells, NK cells and hematopoietic stem cells.
Lin-CD127+ cells gated on CD45+ lymphocytes were considered to be ILCs (Fig. 1A–C). To further differentiate subtypes, ILCs expressing CRTH2+ were defined as ILC2 (Fig. 1B and C). CD117-CRTH2-ILCs were designated as ILC1 and CD117+ CRTH2- ILCs were passed as ILC3(Fig. 1). As shown in Fig. 1B from a typical HSP individual, ILC1, ILC2 and ILC3 could be differentiated clearly.

3.2. ILC subsets were altered in HSP patients

In HSP, ILC1 were significantly increased and ILC3 were significantly decreased (Fig. 2). The difference of ILCs/Lymphocytes and ILCs/PBMC between the HSP group and the normal controls were statistically significant (P = 0.036, 0.026 respectively). There were significant increases in ILC1/ILCs and decreases in ILC3/ILCs, however the percentage of ILC2/ILCs in patients with HSP was not significantly different from that of the normal control group (P > 0.05). In comparison to normal controls, the difference of ILC1/ILC3 was statistically significant (P < 0.001), in other words, the ratio of ILC1/ILC3 was significantly higher in HSP patients, nevertheless, there were no difference in ILC1/ILC2 between the two groups (P > 0.05).

3.3. The ratio of ILC1/ILCs and ILC3/ILCs in HSP patients restored after treatment

Sixteen patients were reexamined after the initiation treatment with methylprednisolone for about 7 to 10 days. There were no changes in ILCs/Lymphocytes and ILCs/PBMC before and after treatment (P = 0.833, 0.940 respectively). But there were changes in ILCs subsets. As shown in Fig. 4A–C, ILC1 were significantly decreased (P < 0.001) while ILC3 increased (P = 0.033), there was no significant change in ILC2 (P = 0.143). In addition, the difference of ILC1/ILC3 was statistically significant before and after treatment in HSP patients (P < 0.001), namely, ILC1/ILC3 was significantly decreased after treatment, while no difference in the ratio of ILC1/ILC2 before and after treatment (P = 0.460) (see Fig. 3).

3.4 ILC subsets in different subtypes of HSP patients

According to the clinical manifestation, the patients of HSP are divided into five subtypes: purpura type, arthritis type, abdominal type, renal type and mixed type. In contrast to normal groups, the ratio of ILCs/Lymphocytes increased in patients of arthritis type and mixed type (P = 0.014, 0.039 respectively), ILCs/PBMC also increased in the same two subtypes (P = 0.010, 0.034). There was no difference in ILCs/Lymphocytes and ILCs/PBMC among patients of purpura type, abdominal type, renal type in compare with normal controls (P > 0.05). ILC1 accounted for a relatively high proportion in purpura type, arthritis type, abdominal type and mixed type except for renal type (P = 0.027, 0.007, P < 0.001, P < 0.001 and P > 0.05). ILC3 decreased in abdominal type and mixed type, and the difference was statically significant (P = 0.015, 0.006). Furthermore, ILC1/ILC3 in all subtypes of HSP was higher than that of the normal control group, and the difference was statistically significant (P < 0.05) (see Fig. 4).
3.5 CD8 + T cell was positively correlated with ILC1/ILCs

HSP is a kind of leukocytoclastic vasculitis that is characterized by an immune complex mediated type III allergic disease. Dominant IgA deposits in the affected capillaries and small blood vessels. Besides IgA, IgG, IgM and T lymphocytes as well as other immune cells, cytokines and complement also participate in the pathogenesis of HSP [16]. Namely, there are cell-mediated immunity and humoral immunity disorders in HSP. Serum IgA increased, and IgA deposition was observed in kidney and skin mucosa. The levels of CD19 + CD23 + and CD3 + CD4+/CD3 + CD8 + were decreased. ILC1/ILCs was positively correlated with CD3 + CD8 + T lymphocytes (r = 0.3701, p = 0.0075), however, there were no significant correlations between ILC3/ILCs (r=-0.2747, p = 0.0511), ILC1/ILC3 (r=-0.2669, p = 0.0583) and CD3 + CD8+.

The level of IgA did not correlate with ILC1/ILCs (r = 0.0242, p = 0.8660), ILC3/ILCs (r = 0.0358, p = 0.8033) and ILC1/ILC3 (r = 0.0604, p = 0.6739). There were also no correlations between CD19 + CD23 + and ILC1/ILCs (r=-0.1826, p = 0.1998), ILC3/ILCs (r = 0.0881, p = 0.5386) and ILC1/ILC3(r=-0.1036, p = 0.4693). CD3 + CD4+/CD3 + CD8 + did not correlate with ILC1/ILCs (r=-0.0676, p = 0.6407), ILC3/ILCs (r = 0.0143, p = 0.9213) and ILC1/ILC3 (r=-0.0201, p = 0.8899), too. The relationship between CD3 + CD4 + and ILC1/ILCs (r = 0.0400, p = 0.7803), ILC3/ILCs (r=-0.0426, p = 0.7668) and ILC1/ILC3 (r = 0.0629, p = 0.6608) was not significant (see Fig. 5).

3. Discussion

ILCs are a heterogeneous population of non-B non-T lymphocytes that originate from the common lymphoid progenitor, which can provide an immediate immune response that prevents pathogen invasion and affects subsequent adaptive immune response [1]. Few ILCs are detected in peripheral blood (accounting for 0.1–0.5% of lymphocytes) and cord blood [17]. ILCs are mainly enriched in barrier surfaces, NCR + ILCs account for approximately 5% and 2% of total lymphocytes in the human and mouse small intestine, respectively [18]. ILCs have also been found in human gingivae. Approximately 10–15% of total CD45 + cells were ILCs, most of which were IFN-γ secreting ILC1s [19]. In our study, 0.09–0.67% of lymphocytes in the peripheral blood were ILCs, which was in line with the report, among which, ILC1 and ILC3 dominated.

ILCs play an important role in maintaining barrier homeostasis, blocking bacterial and viral infections, expelling parasites, tissue repair and tumor immunity. ILCs are dysregulated in multiple human diseases [20]. HSP is an autoimmune disease with injury to skin and intestinal mucosa. The damaged epithelial cells may secrete cytokines and promote the activation and proliferation of ILCs. In this study, ILCs/Lymphocytes and ILCs/ PBMC in the patients of HSP group were different from the normal control group, indicating that ILCs were in disorder in HSP.

ILC1 could block viral, intracellular bacterial and fungal infection through secreting IFN-γ, but ILC1 could also promote COPD, IBD and cancer in response to different stimuli. The absence of ILC1 in T-bet/-/-mice increased the sensitivity to enteric infections [21]. Several groups demonstrated that the percentage of
ILC1 is increased in the intestines of patients with CD [22, 23], and affect the disease severity through excessive cytokine production. The proportion of ILC1 increased obviously with significant decreases of ILC2 in SLE patients, while, in patients with moderate to severe activity, ILC3 also decreases visibly [12]. HSP is also an autoimmune disease. In our study, ILC1 increased on the early onset with ILC3 decreased, which was consistent with the previous reports, indicating that ILC1 may be involved in the pathogenesis of HSP.

The development and function of ILC3s require the transcription factor RORγt and produce IL-17A and/or IL-22[24]. IL-22 produced by ILC3s induces epithelial cells to express anti-bacterial peptides such as Reg3g and Reg3b to block microbiota and pathogens as well as stabilizes the epithelial barrier. In a word, ILC3s play a critical role in injury repair of epithelial cell [25]. ILC3 could limit T follicular helper (TFH) cells responses and B cell class switching through antigen presentation within the interfollicular regions of the intestinal draining lymph nodes [26]. In our study, ILC3 decreased and restored after treatment. The ratio of ILC1/ILC3 also reduced significantly after the treatment what the HSP patients obtained. Previous data indicates that ILCs has the feature of plasticity. ILC3 with IL-23 can facilitate ILC3/ILC1 conversion, and IL-23 can also promote the reverse conversion of ILC1 to ILC3. Therefore, we believe that, ILC3 might play a role in resistance to foreign pathogens by transforming into ILC1 at the acute phase of HSP, and ILC1 may be converted into ILC3 in the convalescence of the disease, and amplified ILC3 can produce sufficient IL-22 to promote mucosal repair.

In our study, there were differences in ILCs, ILC1, ILC2 and ILC3 in different subtypes of HSP compared with the normal control group. It was speculated that ILCs may be related to the severity of HSP, or may be due to different predisposing factors of HSP. Thus, it is necessary to further study the role of ILCs in HSP by expanding the sample size and extending the follow-up time.

Emerging evidence indicates that there is a complex regulatory relationship between innate immunity and adaptive immunity [27]. IL-5 and IL-6 produced by ILC2 provide direct help to B cells within the tissue. ILC3 in the intestinal tract and lymphoid tissues act on stromal cells and secrete lymphotoxin-α (LTα) and LTβ to help B cell class switching and IgA production indirectly [28]. We found that there was no correlation between ILC1, ILC3, ILC1/ILC3 and IgA. It was speculated that ILCs mainly existed in the mucosal lamina propria and were less in peripheral blood, so it had less effect on serum IgA. The effect of ILCs on IgA can be further elucidated by expounding the relationship between ILCs in the mucosa lamina propria and secretory IgA.

ILCs subsets closely mirror the transcriptional and functional biology of both cytotoxic CD8 + T cells and CD4+ T helper (TH) cells. IFN-γ producing ILC1 modulate T cell responses, especially T helper 1(TH1)cell responses, ILC2-derived IL-4 and ILC3-derived IL-2 may limit regulatory T (Treg) cell responses. Macrophages can also participate in the activation of ILC3s by secreting IL-1β and IL-23, so ILCs is the bridge between innate immunity and adaptive immunity[27]. There was a positive correlation between ILC1 and CD3 + CD8+ in this study, while there was no correlation between CD3 + CD4+ and different subtypes of ILCs in the PB. It did not indicate that there was no crosstalk between TH cells and ILCs in the
mucosal lamina propria. This also explains why ILCs are not associated with CD19 + CD23+. To our knowledge, the production of IgA is due to the interaction of follicular helper cell (TFH) and B lymphocytes in germinal centers, while ILCs is the bridge between innate immunity and adaptive immunity, thus, more experiments are needed to indicate whether ILCs are correlated with IgA+B lymphocytes.

5. Conclusion

We found that ILC1 increased and ILC3 decreased in HSP, and ILC1/ILC3 decreased significantly after treatment, indicating that ILC1 and ILC3 may be involved in the pathogenesis of HSP and may be correlated with the severity of the disease. This study is a small sample, single-center study, and there may be bias, therefore, it is necessary to further understand the role of ILCs in HSP by expanding the sample size or multi-center study, stratifying the disease, and studying the different stages of the disease. As far, ethical constraints and limitations of available tissue restrict human studies, further analysis should focus on different stages of disease as well as how to act as therapeutic targets.

Abbreviations

ILI###\text{Innate lymphoid cells}

HSP\text{Henoch-Schonlein purpura}

PB\text{peripheral blood}

NK cells\text{natural killer cells}

LTi cells\text{Lymphoid-tissue inducer cells}

IL-4\text{interleukin-4}

IBD\text{inflammatory bowel disease}

GM-CSF\text{granulocyte macrophage-colony stimulating factor}

HSPN\text{Henoch-Schonlein purpura nephritis}

EULAR\text{European League Against Rheumatism}

PBS\text{phosphate buffer saline}

FMO\text{Fluorescence Minus One}

Declarations

Ethics approval and consent to participate
The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of our hospital.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Lili Zhang analyzed and interpreted the patients’ data and completed the manuscript. Xiaozhong Li helped design the topic and revise the article. Qiang Lin also helped revise the manuscript and submitted the article. All authors read and approved the final manuscript.

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**Figures**

**Figure 1**

Frequency and distribution of ILCs subsets in the peripheral blood of HSP patients. Gating strategy for total ILCs ((CD45+ Lin-CD127+CD161+) ILC1s (CD45+ Lin-CD127+ CD161+cKit-CRTH2-), ILC2s (CD45+Lin-CD127+ CD161+cKit/+CRTH2+) and ILC3s (CD45+ Lin-CD127+ CD161+cKit+ CRTH2-). Panels are representative of multiple independent experiments (n = 51).
Figure 2

Altered frequency of ILCs in peripheral blood of HSP patients. PBMCs from normal controls and HSP patients were stained for flow cytometry. Ratios of ILC/Lymphocytes (A), ILC/ PBMC (B), ILC1/ILCs (C), ILC2/ILCs (D), ILC3/ILCs (E), ILC1/ILC2 (F) and ILC1/ILC3 (G) in HSP patients (N = 51) and normal controls (N = 22).

Figure 3

Proportions of ILCs subsets altered after treatment. Fresh blood samples from 16 HSP patients were collected on admission and prior to discharge after treatment. PBMCs of these patients were subjected to flow cytometry. Proportions of ILC/Lymphocytes (A), ILC/ PBMC (B), ILC1/ILCs (C), ILC2/ILCs (D), ILC3/ILCs (E), ILC1/ILC2 (F) and ILC1/ILC3 (G) are shown.
Figure 4

Alteration of ILC subset in HSP patients. PBMCs were collected from HSP patients and normal controls. 51 HSP patients were classified into purpura type (n = 6), arthritis type (n = 14), abdominal type (n = 11) and renal type (n = 6) and mixed type (n = 14). Proportions of ILC/Lymphocytes (A), ILC/PBMC (B), ILC1/ILCs (C), ILC2/ILCs (D), ILC3/ILCs (E), ILC1/ILC2 (F) and ILC1/ILC3

Figure 5

Correlation between ILC subsets and lymphocyte subsets. CD8+ was positively correlated with ILC1/ILCs (A). The correlation between levels of CD8+(A-C), IgA(D-F), CD23+(G-I), CD4+(J-L) in HSP group and ILC subsets are shown. Each point represents an individual subject.
