Natural killer cells and their function in Takayasu’s arteritis

T. Li¹, N. Gao¹, W. Cui¹,², L. Zhao¹,², L. Pan¹

ABSTRACT

Objective. Takayasu’s arteritis (TAK) is a chronic, large-vessel systemic vasculitis. Immune inflammatory response plays a crucial role in the pathogenesis of TAK. Natural killer (NK) cells are one of the major immunoregulatory cell groups of the immune system, but their role in TAK pathogenesis is unclear. We aimed to investigate the role of peripheral blood NK cells in TAK pathogenesis.

Methods. The study consisted of 47 TAK patients and 27 healthy controls. Peripheral blood natural killer (NK) cells and their CD56(dim)/CD56(bright) subsets were phenotyped using CD3 and CD56 surface markers. Functional potential was assessed by production of granzyme B, perforin and interferon (IFN)-γ.

Results. TAK patients had decreased numbers of NK cells in the peripheral blood (p<0.001) relative to healthy controls. The percentages of CD56(bright) (p<0.05) and CD56(dim) NK cells (p<0.001) from TAK patients were also decreased. The expressions of Granzyme B (p<0.001), Perforin (p<0.001) in NK cells were lower in TAK patients compared to control group, but no differences in the percentage of IFN-γ producing cells was observed between TAK patients and healthy control. There is no difference in the percentage of NK cells or CD56(bright) or CD56(dim) NK cells between active and inactive TAK. However, granzyme B-expressing NK cell percentage was significantly decreased in active TAK compared to inactive TAK (p<0.05).

Conclusion. Our findings concluded that NK cell numbers and cytotoxicity are reduced in TAK patients.

Introduction

Takayasu’s arteritis (TAK) is a chronic, large-vessel systemic vasculitis. It primarily affects the aorta and its branches. However, other large vessels including pulmonary arteries, as well as medium-sized coronary arteries, could also be involved (1). The involved arteries were characterised by stenosis, occlusion, aneurysm or vascular dissection, causing ischaemia and dysfunction of the tissues and organs. TAK patients can occur with stroke, loss of vision, myocardial infarction, even death in severe cases (2, 3). According to a recent review, 50% of TAK patients will relapse and experience a vascular complication within 10 years from diagnosis (4). As a systemic autoimmune disease, immune inflammatory response plays a crucial role in the pathogenesis of the disease (5). Immunohistochemical studies of aortic tissue from TAK patients showed the infiltrating cells were comprised of macrophages, CD4⁺ T cells, CD8⁺ T cells, γδ T cells, natural killer (NK) cells and neutrophils (6).

NK cells are an important part of the immune system and play an important role in antiviral infections, tumours, transplant rejection and immune regulation (7). NK cells can be classified as two subsets according to the expression level of CD56: CD56(dim) subset (approximately 90% in peripheral blood) and the minority CD56(bright) subset (8). The main functional characteristics of CD56(dim) NK cells are that they produce less cytokine but increased potential to produce perforin and Granzyme B. In contrast, the important function of the CD56(bright) population has been shown to be the secretion of immunoregulatory cytokines, including interferon-γ, tumour necrosis factor (TNF)-α and other inflammatory factors (9). In addition, NK cells can also regulate adaptive immunity by cross-reacting with cytokines or chemokines and other immune cells (such as T cells, B cells and dendritic cells) (7).

It has been reported that NK cells are involved in the regulation of various autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), and Behçet’s disease (BD), etc. (10). The latest genome-wide association studies (GWAS), enhancer en-
Cell flow cytometry
Samples were processed using whole blood lysis to analyse NK cell subsets. Surface phenotypes of NK cell subsets were identified using the following antibodies: FITC anti-human CD3 and PerCP-Cy5.5 anti-human CD56 (all from BD Pharmingen). For intracellular cytokine staining, blood samples were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 1 μg/ml ionomycin (Sigma) for 5 hours at 37°C. After fixation and permeabilisation, samples were incubated with antibodies against PE anti-human INF-γ, PE-Cy594 anti-human Granzyme B and BV421 anti-human perforin (all from BD Pharmingen). Flow cytometry data were acquired using BD LSRSFortessa (BD Biosciences) and analysed by analysed with FlowJo v. 7.6.4 software (Tree Star Inc., USA). NK cells were defined as CD3<sup>-</sup>CD56<sup>+</sup>, subdivided into CD3<sup>-</sup>CD56<sup>Bright</sup> and CD3<sup>-</sup>CD56<sup>Bright</sup> subsets (8). The lymphocyte population was identified by assessment of size and granularity of cells using light scatter properties (forward scatter (FSC) vs. side scatter (SSC)) and NK percentage expressed as a proportion of total gated lymphocytes.

Statistical analysis
All statistical studies were carried out with the SPSS program V.19.0 (SPSS, Chicago, Illinois, USA). Data in this study was numerical data. According to the normality, variables were described as means ± SE or as medians. We performed univariate analysis by the Student’s t-test or Wilcoxon-Mann-Whitney test for numerical data according to normality. p-values <0.05 denoted the statistically significant difference.

Results
Demographic data and clinical features of 47 TAK patients
Among the 47 TAK patients in this study, the male to female ratio of this study was 1:10.75 (4 males, 43 females). The average age was about 39 years and the average disease onset age was about 21 years. Type V was the most common angiographic classification pattern. The median NIH level was 5 points and the median ITAS-A level was 31.64 points.

Table I. Demographic data and clinical features of 47 TAK patients.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td>Age (years, mean ± SE)</td>
<td>39.30 ± 11.19</td>
<td>31.64 ± 10.08</td>
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<tr>
<td>Age of onset (years, mean ± SE)</td>
<td>21.3 ± 8.5%</td>
<td>0%</td>
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Table II. Pharmacological treatments of 29 TAK patients.

<table>
<thead>
<tr>
<th>Medications</th>
<th>n (%)</th>
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<tr>
<td>Glucocorticoids (GCs)</td>
<td>43 (82.8%)</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>15 (51.7%)</td>
</tr>
<tr>
<td>Tocilizumab (TCZ)</td>
<td>13 (44.8%)</td>
</tr>
<tr>
<td>Mycophenolate mofetil (MMF)</td>
<td>13 (44.8%)</td>
</tr>
<tr>
<td>Cyclophosphamide (CTX)</td>
<td>8 (27.6%)</td>
</tr>
<tr>
<td>Toceranide (FK506)</td>
<td>2 (6.9%)</td>
</tr>
<tr>
<td>Antimalarials (Hydroxychloroquine, HCQ)</td>
<td>2 (6.9%)</td>
</tr>
<tr>
<td>Tripterygium (Tili)</td>
<td>2 (6.9%)</td>
</tr>
<tr>
<td>Leflunomide (LEF)</td>
<td>1 (3.4%)</td>
</tr>
<tr>
<td>Azathioprine (AZA)</td>
<td>1 (3.4%)</td>
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Reduced numbers of circulating NK cells in TAK patients
In order to determine whether NK cells participate in the development of TAK, we analysed the peripheral blood of patients with TAK collected in Beijing Anzhen Hospital. According to previous literature (8), human NK cells defined in this study as CD3<sup>-</sup>CD56<sup>Bright</sup> lymphocytes. NK cells expressed as a percentage of total gated lymphocytes in the peripheral blood of all 47 TAK patients (6.55% (5.00, 14.00)) were found to be significantly reduced compared to healthy controls (11.20% (8.20, 15.3), p<0.001) (Fig. 1).

CD56<sup>Bright</sup> and CD56<sup>Bright</sup> NK cells are significantly decreased in the peripheral blood of TA patients
NK cells can be classified as CD56<sup>Bright</sup> and CD56<sup>Bright</sup> NK cells according to the
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expression level of CD56. We analysed changes in CD56Bright and CD56Dim NK cells in two subpopulations of NK cells in peripheral blood of TAK patients. TAK patients had a significantly lower proportion of CD56Bright (TAK=0.50 (0.42, 0.61%) vs. Control=0.59 (0.37, 0.79)%, (p<0.05)) and CD56Dim (TAK=5.77 (5.18, 7.83)% vs. Control=10.42 (7.38, 14.20)%, (p<0.001)) NK cell subsets compared to healthy controls relative to total lymphocytes. (Fig. 2).

**Impaired cytotoxicity of NK cells in TAK patients**

The main function of NK cells is to produce inflammatory factors and exert cytotoxic effects (9). To explore the function of NK cells, we analysed the frequency of Granzyme B, Perforin and IFN-γ-secreting NK cells using flow cytometry. The percentages of Granzyme B (TAK=84.03 (77.90, 90.60)% vs. Control=90.94 (89.60, 94.70)%, (p<0.001)) and Perforin (TAK=74.14 (66.60, 82.90)% vs. Control=81.63

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**Fig. 1.** Reduced numbers of circulating NK cells in TAK patients. (a) Flow cytometry analysis of CD3 CD56 NK cells in TA patients (n=47) and control groups (n=27). (b) Scatter plot shows the percentages CD3+ T cells and CD3 CD56+ NK cells in total gated lymphocytes.

**Fig. 2.** CD56Bright and CD56Dim NK cells are significantly decreased in the peripheral blood of TAK patients. (a) Flow cytometry analysis of CD56Bright and CD56Dim NK cells subsets in TAK patients (n=47) and control groups (n=27). (b) Scatter plot shows the percentages CD56Bright and CD56Dim NK cells in total gated lymphocytes. *p<0.05, **p<0.001 compared with control groups.
(82.5, 94.10)%, (p<0.001) producing NK cells were lower in TAK patients compared to control group. By contrast, no difference in the percentage of NK cells or NK cell subsets between TAK patients and healthy control (TA=7.08 (2.26, 10.50) % vs. Control =10.29 (4.01, 17.00) %, (p=0.14), Fig. 3).

**Discussion**

In order to determine whether NK cells participate in the development of TAK, we analysed the number of NK cells in peripheral blood and their function in TAK patients collected in Beijing An-

**Table III. Comparison of NK cells and their subsets in patients with active and inactive TAK.**

<table>
<thead>
<tr>
<th></th>
<th>Active TAK (n=24)</th>
<th>Inactive TAK (n=23)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>NK cell in lymphocyte (%)</td>
<td>5.84 (2.69, 8.20)</td>
<td>7.29 (3.18, 9.04)</td>
<td>0.389</td>
</tr>
<tr>
<td>CD56&lt;sup&gt;dim&lt;/sup&gt; NK in lymphocyte (%)</td>
<td>0.42 (0.30, 0.59)</td>
<td>0.48 (0.32, 0.61)</td>
<td>0.463</td>
</tr>
<tr>
<td>CD56&lt;sup&gt;bright&lt;/sup&gt; NK in lymphocyte (%)</td>
<td>5.23 (2.28, 7.71)</td>
<td>6.34 (2.55, 8.05)</td>
<td>0.389</td>
</tr>
<tr>
<td>Granzyme B&lt;sup&gt;+&lt;/sup&gt; NK in NK (%)</td>
<td>81.37 (75.08, 88.48)</td>
<td>86.81 (83.00, 92.50)</td>
<td>0.031</td>
</tr>
<tr>
<td>Perforin&lt;sup&gt;+&lt;/sup&gt; NK in NK (%)</td>
<td>70.20 (65.40, 81.20)</td>
<td>78.26 (68.90, 85.00)</td>
<td>0.142</td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;+&lt;/sup&gt; NK in NK (%)</td>
<td>7.22 (2.03, 10.48)</td>
<td>6.94 (5.17, 10.70)</td>
<td>0.924</td>
</tr>
</tbody>
</table>

**Table III.** Comparison of NK cells and their subsets in patients with active and inactive TAK.

**Fig. 3.** Impaired cytotoxicity of NK cells in TAK patients.

(a) Flow cytometry histogram plots showing intensity of intracellular cytokine staining for Granzyme B, Perforin and IFN-γ following 5-hour PMA/Lipopolysaccharide stimulation (Representative plots are shown).
(b) Scatter plot shows the percentages of Granzyme B, Perforin and IFN-γ-secreting NK cells in total gated NK cells.

**Granzyme B-expressing NK cell percentage was significantly decreased in active TAK compared to inactive TAK.**

In terms of the association between NK cells and TAK disease activity, we saw no difference in the percentage of NK cells or NK cell subsets between groups with active (NIH level was ≥2 points) and inactive TAK (NIH level was <2 points). In contrast, analysis of granzyme B-expressing NK cell percentage demonstrated a significant decrease in active TAK compared to inactive TAK (active TAK=81.37 (75.08, 88.48) % vs. inactive TAK=86.81 (83.00, 92.50) % (p=0.031), Table III). Furthermore, we compared the NK cell subsets and intracellular cytokine staining between treated and untreated patients. The results showed there was no difference in the percentage of NK cells or their subsets between groups with treated and untreated TAK patients (Table IV).
zhen Hospital. Our results showed that TAK patients had decreased numbers of NK cells in the peripheral blood compared to healthy controls. The percentages of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells from TAK patients were also decreased. The expressions of granzyme B and perforin in NK cells were lower in TAK patients compared to control groups. Granzyme B-expressing NK cell percentage was significantly decreased in active TA compared to inactive TAK.

In summary, our findings concluded that NK cell numbers and cytotoxicity are reduced in TAK patients.

TAK is a chronic, granulomatous, large-vessel systemic vasculitis characterised by stenosis, occlusion, and sometimes aneurysm of the aorta and its main branches (14). Although the pathogenesis of TAK has considerably improved during the last decade, the exact pathogenic sequence remains to be elucidated. It is well known that cell-mediated autoimmunity has been strongly implicated in the pathogenesis of TAK (15).

Vascular inflammation of TAK possibly originates in the vasa vasorum, followed by infiltration of inflammatory cells (T cells, NK cells, neutrophils and so on) and production of inflammatory factor and cytokines, such as interleukin (IL)-6, TNF-\(\alpha\), IFN-\(\gamma\), IL-12, and IL-18, leading to the formation of granulomas (16). NK cells are important members of lymphocytes. They can directly kill infected, transformed or autoreactive cells utilising exocytosis of specialised cytotoxic granules. Besides, NK cells can shape the adaptive immune response by cross-talk with other immune cells such as T cells, B cells and dendritic cells and by producing cytokine or chemokines (17, 18). Through their cytotoxic capacities and cytokine production, NK cells modulate autoimmune diseases. A recent review has summarised the correlation between NK cell number or functional alterations and several autoimmune diseases, including RA, SLE, SS, BD and so on (8). The latest genome-wide association studies (GWAS), enhancer enrichment analysis and pathway analysis suggest that NK cells play a key role in TAK (11).

Previous studies showed the percentages and absolute numbers of NK cells, and NK cytotoxicity were significantly lower in the peripheral blood of RA and SLE patients than in that of healthy controls (19). It has also recently been reported a decline of NK cells in patients with seropositive (SP) arthralgia and in BD patients (20, 21). Consistent with these results, our results found the percentages of total NK cells, CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells from TAK patients were decreased compared to healthy controls. The mechanism by which NK cells were decreased in the peripheral blood of patients with autoimmune diseases was complex. Several studies have demonstrated accumulation of NK cells in affected tissues of autoimmune patients, with the decreased numbers of NK cells in the peripheral blood. For example, it has been found the infiltrating NK cells in the pancreatic islet of Type 1 diabetes patients (22), and the synovium of RA patients (23, 24). As mentioned previously, histological findings showed NK cell infiltrations in the arterial vascular wall of TAK (16). NK cells mediated antibody dependent cytotoxicity (ADCC) induced by some autoantibodies (such as anti-annexin-V), which directly induced apoptosis in endothelial cells or target cells (5, 25). Besides, Seko et al. showed that NK cells play an important role in the vascular cell injury by expressing and releasing perforin directly onto the surface of arterial vascular cells (6). Other pro-apoptotic pathways such as MICA/NKG2D (Major Histocompatibility Class I Chain-Related A/NK Group 2D), Fas/FasL and 4-1BB/4-1BBL were also involved in vascular injury in TAK (26). These observations and results support the hypothesis that decreased NK cells in the peripheral blood of TAK patients may due to the trafficking of NK cells to aortic tissues. Shibatomi et al. demonstrated that combinations of IL-18 and IL-15 or IL-18 and IL-12 induced NK cell death in patients with systemic autoimmune diseases (27). These results indicate that NK cell apoptosis induced by circulating immune complexes and serum cytokines might contribute to NK cell depletion in some autoimmune diseases. Other possible explanations for depletion of circulating NK cells in SLE are that NK cell depletion occurs secondary to disease progression and NK cells generated from haematopoietic stem cells (HSCs) are defective in SLE patients (19). Another alternative possible reason in BD is that depleted NK cells in peripheral blood are a direct consequence of BD pathology.

Besides, we found that the expressions of granzyme B and perforin in NK cells were lower in TAK patients compared to control groups. These results indicated the cytotoxicity of NK cells were markedly suppressed in TAK patients. The cytolytic function of NK cells has an important role in the initiation and progression of autoimmune diseases (28). Park et al. examined the cytotoxic effect of NK cells was using flow cytometry in RA, SLE, ankylosing spondylitis (AS), and BD, although reduced cytotoxic activity was found in RA and SLE, no significant differences were seen in patients with BD or AS (19). In the study of Vastert et al. the author found that perforin of NK cells and their lytic function were severely reduced in juvenile idiopathic arthritis (JIA) (29). This finding could thus explain why systemic JIA may be complicated by macrophage activation syndrome (MAS). A common hypothesis as to the pathophysiology of MAS proposes a defect in lymphocyte cytolytic activity (30). The pro-inflammatory cytokine environment, particularly IL-6, has been shown to decrease NK cell cytolytic function (30). It is known that IL-6 plays a vital role in the pathogenesis
of TAK (31). This can partly explain the mechanism of impaired cytotoxicity of NK cells in TAK patients. IFN-\(\gamma\) is a powerful cytokine, a prototype of an effector cytokine in anti-pathogen immune responses (32). A study of pro-inflammatory cytokine transcripts of peripheral blood mononuclear cells has also shown that patients with TAK had higher mRNA gene expression of IFN-\(\gamma\) (33). In the aorta from TAK patients, an increased expression of IFN-\(\gamma\) has been described (34). IFN-\(\gamma\) levels in active patients at baseline and good correlation of this cytokine with conventional markers of disease activity (ESR an CRP) as well as with IL-6 (35). IFN-\(\gamma\) is a potent immunoregulatory protein mainly secreted by CD4+ and CD8+ T cells and by NK cells (36).

D. Saadoun et al. demonstrated the presence of IFN-\(\gamma\)-producing T cells in vascular inflammatory infiltrates in patients with TAK. In our study, no differences in the IFN-\(\gamma\) expression of NK cells was observed between TAK patients and healthy control. The expressions of IFN-\(\gamma\) in CD4+ and CD8+ T cells in TA are being further studied by our research group. One study reported IFN-\(\gamma\) expression on CD56\(^{\text{bright}}\) NK cells was increased in SLE patients (37). The percentage of IFN-\(\gamma\)-producing CD56\(^{\text{bright}}\) NK cells was also higher in BD patients compared to healthy controls (21). These results suggested that the expressions IFN-\(\gamma\) in different cell subsets and diseases are different. Yu et al. hypothesised that the process of human NK differentiation progresses from a CD56\(^{\text{dim}}\) to a CD56\(^{\text{bright}}\) phenotype (38). Therefore, the author suggested that CD56\(^{\text{dim}}\) NK cells have a high turnover under SLE conditions and have to be replaced, and consequently, their precursor cells (CD56\(^{\text{bright}}\)) are released as recent immigrants from the bone marrow and/or the lymph nodes sharing a less differentiated phenotype (37). However, some authors thought there is a shift from the CD56\(^{\text{dim}}\) population to the CD56\(^{\text{bright}}\) subset in SLE and BD (21, 39). So further study concerning NK phenotype differentiation might provide a better understanding the role of IFN-\(\gamma\) expression of NK cells in the process of TAK pathogenesis.

In terms of the correlation between NK cells and TAK disease activity, we saw no difference in the percentage of NK cells or NK cell subsets between groups with active and inactive TAK. Consistent with our results, there were also no statistically significant differences in the absolute number and frequency of NK cells between the active SLE and inactive SLE groups (37). The NK cell percentage population was not correlated significantly with the DAS28 in RA patients (40). But our results found granzyme B-expressing NK cell percentage was significantly decreased in active TAK compared to inactive TAK. This indicated in granzyme B may play an important role in TAK disease activity. The importance of NK cells in autoimmunity might be associated with different NK cell subsets and different stages of corresponding diseases (8). Our results showed there was no difference in the percentage of NK cells or their subsets between groups with treated and untreated TAK patients. It was reported that NK cells/total lymphocytes (%) was significantly reduced in azathioprine-treated BD patients compared to healthy controls but not for BD patients on other forms of therapy (cyclosporine, prednisolone and mycophenolate mofetil) (21). In this study, the medication of these BD patients was mostly treated, and untreated TAK patients. The importance of NK cells in autoimmune diseases: Linking innate and adaptive immune responses. Autoimmun Rev 2018; 17: 142-54.


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