

The Involvement of Natural Killer Cells in the Pathogenesis of Severe Acute Respiratory Syndrome

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Key Words: Severe acute respiratory syndrome; *Mycoplasma pneumoniae*; NK cell; Immunoglobulin-like receptor

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Abstract

By using peripheral blood samples from 221 cases of severe acute respiratory syndrome (SARS), 34 of *Mycoplasma pneumoniae* infection, and 44 healthy adults, we measured the total number of natural killer (NK) and CD158b+ NK cells (CD158b+ NK) using flow cytometric analysis and calculated the percentage of CD158b+ NK cells.

The total number of NK and CD158b+ NK cells and the percentage of CD158b+ NK cells were significantly lower in patients with SARS than in those with *M pneumoniae* infection ($P < .05$ for all) and healthy subjects ($P < .01$, $P < .01$, $P < .05$, respectively); in 72 patients with severe SARS than in 149 with mild SARS ($P < .05$ for all); and in 174 cases of SARS with anti-SARS coronavirus-specific IgG and/or IgM antibodies than in 47 without antibodies ($P < .05$, $P < .01$, $P < .01$, respectively). There were no significant differences for the 3 values among patients with SARS without anti-SARS coronavirus antibody, patients with *M pneumoniae* infection, and healthy subjects.

The number of NK cells and the expression of CD158b on the surface of NK cells changed in patients with SARS and correlated with disease severity and the presence of anti-SARS coronavirus-specific antibodies; SARS differed from *M pneumoniae* infection in pathogenesis involving NK cells. Monitoring the total number of NK and CD158b+ NK cells and the percentage of CD158b+ NK cells might aid in differentiating SARS from *M pneumoniae* infection.

Severe acute respiratory syndrome (SARS) is an infectious disease that originally emerged in China in November 2002. It subsequently spread worldwide. Investigators involved in international collaboration have attempted to determine a specific cause to convert what is best described as a syndrome into a specific disease. At present, a novel coronavirus named the SARS coronavirus generally is accepted as the single most probable causative agent.^{1,2}

It has been reported that the number of T-cell subsets were decreased in peripheral blood samples of patients with SARS.^{3,4} Natural killer (NK) cells have a central role in antitumor immunity^{5,6} and the killing of virus infected cells.^{7,8} The function of NK cells is regulated by a variety of receptors, including lectin-like receptor CD94 and killer immunoglobulin-like receptors such as CD158b.⁹⁻¹³ However, the potential involvement of NK cells in SARS has not been reported. To explore whether the number of NK cells and the amount of their immunoglobulin-like receptor CD158b changed in the peripheral blood of patients with SARS, as well as their relationship to the severity of the disease and the diagnostic value of monitoring the number of NK cells, we examined the number of NK cells and the amount of CD158b in the peripheral blood in a cohort of 221 patients with SARS. Blood samples from 34 cases of *Mycoplasma pneumoniae* infection and 44 healthy adult volunteers were studied as control samples.

Materials and Methods

Subjects

This study included a cohort of 221 patients with SARS who were admitted to hospitals in the Beijing, China, area

between March 21 and April 10, 2003, who met the diagnostic criteria issued by the health ministry of the People's Republic of China on April 14, 2003.¹⁴ The group included 89 males and 132 females whose ages ranged from 14 to 74 years (mean, 37.3 years). The disease duration varied from 4 to 72 days (mean, 31.7 days). Of the patients with SARS, 72 were classified as having severe disease according to the aforementioned criteria.¹⁴ Anti-SARS coronavirus-specific IgG and/or IgM antibodies were positive in 174 cases and negative in 47.

For control subjects, we selected 34 patients with *M pneumoniae* (32 men and 2 women; age range, 18-33 years; mean, 31.1 years) from Beijing Chaoyang Hospital-Affiliate of Capital University of Medical Sciences, Beijing Institute of Respiratory Medicine. In these patients, fever developed during the same period, and the disease later was identified as *M pneumoniae* infection by serum positivity for anti-*M pneumoniae* antibodies. The healthy control group included 44 volunteers (12 men and 32 women; age range, 18-57 years; mean, 32.3 years).

Flow Cytometric Measurement

We mixed 50 μ L of anticoagulated whole peripheral blood with 10 μ L of CD158b-fluorescein isothiocyanate/CD16CD56-phycoerythrin/CD3-peridinin chlorophyll protein tricolor antibody (Becton Dickinson, Franklin Lakes, NJ) in a Tru-Count tube (Becton Dickinson) and incubated the mixture in darkness at room temperature for 15 minutes. We then added 450 μ L of 1 \times FACS Lysing Solution (Becton Dickinson) and mixed gently, followed by another 15-minute incubation in darkness. Before sample acquisition, the threshold was adjusted to minimize debris and ensure inclusion of the populations of interest. The gate was set on the lymphocyte population. Acquisition was stopped when the lymphocyte population count reached 5,000. The numbers of CD3-CD16+CD56+

cells and CD3-CD16+CD56+CD158b+ cells of the lymphocyte population and beads were analyzed using CellQuest software (Becton Dickinson). The absolute count of cells per microliter was obtained by using the following formula:

$$\frac{\text{No. of Events for Cells of Interest} \times \text{No. of Events for Beads per Test}}{\frac{\text{No. of Events for Beads}}{\text{Test Volume (50 } \mu\text{L)}}} = \text{Absolute Count of Cells}/\mu\text{L}$$

Statistical Analysis

Results are given as mean and SD. Analysis of variance and an unpaired *t* test were used to evaluate the significance of the difference among the groups of subjects compared.

Results

The total number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells in different groups are shown in **Table 1**. These 3 values in patients with SARS were significantly lower than in patients with *M pneumoniae* infection ($P < .05$ for all values) and healthy subjects ($P < .01$, $P < .01$, $P < .05$, respectively). In severe SARS cases, the 3 values were significantly lower than those in cases of mild SARS ($P < .05$ for all values). No significant differences were found between the group with mild SARS and that with *M pneumoniae* infection. Patients with mild SARS had fewer CD158b+ NK cells than healthy subjects. However, there was no significant difference between the 2 groups in the total number of NK cells and the percentage of CD158b+ NK cells. The number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells in patients with SARS with anti-SARS coronavirus-specific IgG and/or IgM antibodies were significantly lower than in patients without the antibodies ($P < .05$, $P < .01$, $P < .01$, respectively). There were no significant differences among

Table 1
Distribution of the Mean (SD) Number of NK Cells and CD158b+ NK Cells and the Mean (SD) Percentage of CD158b+ NK Cells in Different Study Populations

Group	No. of NK Cells	No. of CD158b+ NK Cells	Percentage of CD158b+ NK Cells
SARS diagnosed by clinical criteria (n = 221)	183.51 \pm 140.85 ^{††}	66.67 \pm 75.69 ^{††}	34.45 \pm 17.35 ^{††}
Severe group (n = 72)	152.63 \pm 112.15 [§]	47.97 \pm 53.53 [§]	29.26 \pm 16.59 [§]
Mild group (n = 149)	198.43 \pm 150.89 [#]	75.71 \pm 83.00 [#]	35.40 \pm 17.43 [#]
Anti-SARS+ (n = 174)	172.24 \pm 128.13	58.31 \pm 58.54 ^{††}	32.02 \pm 16.81
Anti-SARS- (n = 47)	225.21 \pm 175.75 ^{##**}	97.63 \pm 115.18 ^{##††}	38.75 \pm 18.49 ^{##††}
<i>Mycoplasma pneumoniae</i> infection (n = 34)	229.50 \pm 151.94	95.43 \pm 96.14	39.90 \pm 19.10
Healthy adult control group (n = 44)	250.48 \pm 192.48	107.89 \pm 129.91	40.12 \pm 18.39

NK, natural killer; SARS, severe acute respiratory syndrome.

^{*} $P < .01$, compared with healthy control group.

[†] $P < .05$, compared with *M pneumoniae* infection group.

[‡] $P < .05$, compared with healthy control group.

[§] $P < .05$, compared with mild group.

^{||} $P < .01$, compared with *M pneumoniae* infection group.

^{||} $P > .05$, compared with healthy control group.

[#] $P > .05$, compared with *M pneumoniae* infection group.

^{**} $P < .05$, compared with anti-SARS+ group.

^{††} $P < .01$, compared with anti-SARS- group.

Table 2

Changing Pattern of the Mean (SD) Number of NK Cells and CD158b+ NK Cells and Mean (SD) Percentage of CD158b+ NK Cells During the Course of Severe Acute Respiratory Syndrome

Course (d)	No. of Cases	Total No. of NK Cells	No. of CD158b+ NK Cells	Percentage of CD158b+ NK Cells
≤10	7	294.45 ± 217.96	117.30 ± 97.84	38.70 ± 17.08
11-20	11	229.10 ± 175.64	76.33 ± 61.85	33.02 ± 16.47
21-30	80	173.24 ± 141.93	66.56 ± 88.85	33.98 ± 17.43
31-40	89	172.31 ± 129.82	58.78 ± 56.22	33.04 ± 17.25
41-50	23	201.21 ± 122.50	74.35 ± 81.93	32.69 ± 20.37
>50	12	195.61 ± 151.38	73.44 ± 94.33	31.53 ± 14.85

NK, natural killer.

patients with SARS without anti-SARS coronavirus antibodies, patients with *M pneumoniae* infection, and healthy subjects in the number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells.

The changing patterns of the number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells during the course of SARS are given as **Table 2**. The numbers of NK cells and CD158b+ NK cells were increased in patients with SARS during the first 10 days of the disease except in 1 patient whose NK cells and CD158b+ NK cells were $28.15 \times 10^6/\mu\text{L}$ and $9.85 \times 10^6/\mu\text{L}$, respectively, on day 4, which were much lower than the counts in the control groups; however, the difference did not reach statistical significance compared with the healthy control group. From the 11th day of the disease, the numbers of NK cells and CD158b+ NK cells decreased quickly and fluctuated at a low level during the rest of the observation period. The percentage of CD158b+ NK cells remained low during the entire disease course without significant changes.

Of 72 severe cases of SARS, 69 (96%) were positive for anti-SARS coronavirus-specific IgG and/or IgM antibodies, whereas of 149 mild cases, 105 (70.5%) were positive for the antibodies. The difference was statistically significant ($P < .001$).

Of 174 patients with anti-SARS coronavirus-specific IgG and/or IgM antibodies, 68 (39.1%) had severe SARS, whereas only 4 (9%) of 47 patients negative for the antibodies had severe SARS. The difference was statistically significant ($P < .01$).

Discussion

NK cells recognize and kill virally infected cells via their spontaneous cytolytic activity against virus-infected cells and via secreting a variety of soluble mediators.^{7,8} NK cell cytotoxicity is regulated by a multitude of receptors,^{5,6,9,10} including CD158b that binds to major histocompatibility complex class I molecules on target cells and is thought to be one of the most important regulators of the effective activities of cytolytic cells.^{8,12} It was reported that

the number of the subsets of NK cells changed in HIV disease,¹²⁻¹⁴ and a causal connection was found between the augmentation of NK cell activity and the antiviral efficacy of biologic response modifiers in a murine AIDS model.¹⁵ Thus, NK cells seem to be involved in the pathogenesis of HIV disease and might have a key role in resistance to this retrovirus. However, to our knowledge, the relationship between NK cells and SARS has not been described.

The study of our cohort revealed that the number of NK cells was significantly lower in patients with SARS than in control subjects. In addition, the amount of CD158b expression on the surface of the NK cell membrane and the percentage of cells positive for CD158b also were decreased in peripheral blood samples of patients with SARS. A similar change was detected in patients with HIV infection or AIDS who had fewer NK cells and decreased percentages of CD56+ cells expressing the NK receptor of the immunoglobulin superfamily compared with healthy control subjects.¹² HIV-1 peptides were hypothesized to regulate the NK activities.¹⁶

How the SARS virus alters the number and function of NK cells needs to be studied. The mechanism of CD158b down-regulation in patients with SARS is unknown. We postulate that 2 mechanisms might be involved in this process. One is that CD158b is detached from the surface of the NK cells and becomes soluble in the serum. The other is that the expression of CD158b is down-regulated at the transcriptional or translational level.

It is unclear why there are fewer NK cells in the peripheral blood of patients with SARS. It is possible that the SARS virus directly attacks NK cells and causes their death. It also is possible that NK cells undergo cytolysis after they kill the target cells infected with the virus. Alternatively, NK cells might be redistributed to targeted organs such as the lung, so that the number of NK cells in the circulation is decreased. Another potential mechanism is that the SARS virus or its metabolic products act as superantigens to stimulate NK cells and induce the activation-induced apoptosis mechanism.

All but 1 patient with SARS showed an increase in the number of NK cells during the first 10 days of the disease,

but the increase did not reach statistical significance compared with the healthy control group. From the 21st to the 40th day of the disease, the number of NK cells and CD158b+ NK cells stayed at a low level and began to recover after the 40th day of the disease. This pattern is different from the changing pattern of T-cell subsets, which decreased and recovered earlier than NK cells.³ During the entire disease course, the percentage of CD158b+ NK cells remained at a low level, indicating that the amount of CD158b NK cells was reduced by SARS infection during the whole period of our observation, regardless of the recovery of the NK cell count.

The number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells in 72 cases of severe SARS were significantly lower than those in mild cases (Table 1). Therefore, both the number and the function of NK cells correlate with the severity of SARS. It is conceivable that the higher the virus load or the virulence, the more NK cells killed and the more severe the clinical symptoms.

Our data showed that the number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells correlated with the detection of anti-SARS coronavirus-specific antibodies in patients with SARS diagnosed by clinical criteria. These 3 values in 174 cases of SARS with anti-SARS coronavirus-specific antibodies were significantly lower than in cases without the antibodies (Table 1). There were no significant differences among the groups of patients with SARS without the antibodies, patients with *M pneumoniae* infection, and healthy subjects in the number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells.

We previously have shown that patients with clinically diagnosed SARS without anti-SARS coronavirus-specific antibodies were similar to healthy volunteers in terms of CD38 expression on CD4+ T cells, whereas patients with SARS and the antibodies had lower CD38 expression (unpublished observation). In addition, 68 (39.1%) of 174 patients with anti-SARS coronavirus-specific IgG and/or IgM antibodies had severe disease, whereas only 4 (9%) of 47 patients negative for the antibodies had severe SARS. The symptoms of patients positive for anti-SARS coronavirus-specific antibodies were much more severe than the symptoms of patients without the antibodies. Therefore, patients with anti-SARS coronavirus-specific antibodies differed from those without the antibodies immunologically and clinically. Hence, it is doubtful that the patients with clinically diagnosed SARS who did not have the anti-SARS coronavirus-specific antibodies really were infected by the SARS coronavirus. Therefore, patients without anti-SARS coronavirus-specific antibodies should be separated from patients with the antibodies.

Unlike the SARS cases, there was little change in the number of NK cells and CD158b+ NK cells and the

percentage of CD158b+ NK cells in patients with *M pneumoniae* infection, which indicates that the NK cells have different roles in the 2 diseases. Although the difference in the number of NK cells and CD158b+ NK cells and the percentage of CD158b+ NK cells between patients with mild SARS and patients with *M pneumoniae* infection did not reach statistical significance (almost 30% of mild SARS cases were negative for anti-SARS coronavirus-specific antibodies), the cell counts and percentages in antibody-positive SARS cases (which might be considered "true" SARS cases) were significantly lower than those in cases of *M pneumoniae* infection and clinically diagnosed, antibody-negative cases of SARS (which might be considered "false" SARS). Therefore, monitoring the number and percentage of NK cells should be useful for differentiating true SARS from false SARS and *M pneumoniae* infection.

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