HLA-Cw7 Zygosity Affects the Size of a Subset of CD158b+ Natural Killer Cells

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Individuals with certain HLA class I genotypes are highly susceptible to disease after viral infection. Natural killer (NK) cells kill virus-infected cells through a mechanism involving HLA class I receptors. These facts may be connected if an individual’s HLA genotype regulates the number and function of NK cells. We have observed that subjects homozygous for the HLA-B/C region of conserved major histocompatibility complex (MHC) extended haplotypes have lower NK cell activity and a significantly lower frequency of CD16+CD56+ NK cells than heterozygotes. The proportion of CD16+CD56+ NK cells was unaffected by zygosity for the HLA-B/C region. We show here that the frequency of CD16+CD158b+, but not CD16+CD158a+ NK cells, was significantly lower (p < 0.026) in homozygotes for HLA-Cw7 (NK1 ligand) haplotypes than in heterozygotes. The frequencies of CD16+CD158a+ and CD16+CD158a+ and CD16+CD158a+ or CD16+NKB1+ and CD16+NKB1+ NK cells were not different in these donor groups. These findings suggest that the proportion of NK cells coexpressing CD16 and CD158b, but not CD158a nor NKB1, is influenced by zygosity for the HLA-Cw7 (NK1 ligand) haplotype. Since NK cells are involved in protection from virus infection, a reduced size of a ligand-specific NK subset in individuals homozygous for some HLA-B/C haplotypes may help explain their increased susceptibility to virus-induced diseases.

KEY WORDS: Natural killer cells; HLA; CD16; Killer immunoglobulin like; CD94.

INTRODUCTION

Virus infection, including acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia, is responsible for much morbidity and mortality. Host genetic factors such as the major histocompatibility complex (MHC) are a major determinant of susceptibility or resistance to infectious disease (1, 2). Homozygosity for certain MHC loci is associated with increased susceptibility to virus-induced disease (3, 4). It is not known whether this increased susceptibility is due to an intrinsic defect in homozygous individuals unable to mount a specific antiviral response or is due to virus-induced changes in effector function.

Natural killer (NK) cells, a subset of lymphocytes, are major effectors of the immune response to virus infection (5–8). They spontaneously kill virus-infected cells, tumor cells or normal cells that lack or have reduced expression of ‘self’ MHC class I molecules (9, 10). Persistently low activity of NK cells in the peripheral blood is associated with an increased susceptibility to viral infection and cancer (5, 11).

In humans, NK cells express CD56 on their surface (12). A subset of NK cells expresses the activation receptor CD16 (12–14). The subsets CD16+CD56+ and CD16−CD56− are two major subpopulations of NK cells (12, 13). NK cells also express inhibitory receptors and activation receptors with distinct MHC class I binding specificities (15–18). HLA class-I-specific NK receptors belong to two distinct families: the immunoglobulin (Ig) superfamily and the C-type lectin family (15–18). The Ig superfamily comprises killer immunoglobulin-like receptors (KIR) specific for HLA-C, HLA-B, and HLA-A ligands. The NK cells can discriminate between two groups of HLA-C alleles: NK1 and NK2. The C-type lectin family receptor CD94 and individual members of the NKG2 group form heterodimeric structures (19, 20). These receptors, on binding to appropriate specific MHC
class I molecules, inhibit the cytotoxic activity of NK cells. The ligand for CD94/NKG2 in the complex of these receptors is HLA-E, a nonclassic MHC class I molecule (19, 20). The NK receptor-specific antibodies, HLA-specific receptors, and their ligands are given in Table I.

We reported earlier that NK cell activity and number were low in individuals homozygous for HLA-B/C of extended haplotypes compared with heterozygotes (HTZ) (21). Since CD16©CD56© cells make major contributions to lytic activity (9, 10), we observed lower NK cell activity in HLA-B/C homozygotes (HMZ) could be due to a low frequency of the CD16© subset in the NK population. Correlation of the ex vivo frequency of NK subsets with HLA zyosity could be important in understanding the possible mechanisms of low NK activity in HMZ. Using flow cytometry, we determined the frequency of NK cells expressing HLA-specific NK receptors CD158b, CD158a, NKB1, CD94, and CD16 in HMZ for haplotypes marked by the allele HLA-Cw7/ NK1 ligands and corresponding HTZ. We observed that the size of the NK subset defined by the coexpression of CD158b and CD16 was lower in HLA-Cw7 HMZ compared with HTZ, suggesting a possible influence of host HLA class I zyosity on NK cell subset size.

MATERIALS AND METHODS

Subjects

After obtaining informed consent, we studied 73 unrelated healthy individuals aged 20–40 years and four donors of unknown age and sex. Of the known donors, 34 were female and 39 were male. Seventeen of the 20 HLA-B/C phenotypically homozygous donors were apparently HMZ for HLA-Cw7, two were HMZ for HLA-Cw4, and one was a HMZ for HLA-B44 but a HTZ for HLA-C. One donor was HMZ for HLA-Cw7 but HTZ for HLA-B locus. All 16 donors HML for HLA-Cw7 were also HMZ for HLA-B and DR/DQ. The zyosity of seven donors was further confirmed by complement typing and family studies (22, 23). These donors were HML for the entire extended haplotypes [HLA-B7, Cw7, SC31, DR2, DQ6] or [HLA-B8, Cw7, SC01, DR3, DQ2] as marked by the alleles for HLA-B, C, complement, and HLA-DR and DQ genes.

Monoclonal Antibodies

The following monoclonal antibodies were used: 3G8 (anti-CD16), Leu19 (anti-CD56), and DX9 (anti-NKB1) (Becton-Dickinson, San Jose, CA); EB6 (anti-CD158a), GL183 (anti-CD158b), and HP3B1 (anti-CD94) (Beckman-Coultier, Miami, FL); and W6/32 (anti-class I HLA) (PharMingen, San Diego, CA) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin. In some experiments, we used biotin-conjugated anti-CD16 (PharMingen), PE/cyanine (PE/Cy5)-conjugated anti-CD56 and FITC-conjugated anti-CD3 (all from Becton-Dickinson). Isotype controls were biotin-conjugated mouse IgG2a, IgG1, and FITC-conjugated IgG1 (Caltag, Burlingame, CA); FITC-conjugated IgG1, PE/Cy5-conjugated IgG1, PE-conjugated IgG1 (Beckman-Coultier), and biotin-conjugated IgG1 (PharMingen). The Cy5-conjugated streptavidin (SA-Cy5) (Beckman-Coultier) and Red-613 conjugated-streptavidin (Life Technologies; Grand Island, NY) were used to detect biotin-conjugated antibodies.

**Table I. NK Receptor-Specific MAb and Their Recognition Structures**

<table>
<thead>
<tr>
<th>mAb</th>
<th>CD designation</th>
<th>Receptors</th>
<th>Functiona</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB6</td>
<td>CD158a (p58.1)</td>
<td>2DL1b</td>
<td>I</td>
<td>HLA-C2a</td>
</tr>
<tr>
<td>CD158a (p58.1)</td>
<td>2DS1a</td>
<td>A</td>
<td>(NK2)</td>
<td></td>
</tr>
<tr>
<td>GL183</td>
<td>CD158b (p58.2)</td>
<td>2DL2c</td>
<td>I</td>
<td>HLA-C1c</td>
</tr>
<tr>
<td>CD158b (p58.2)</td>
<td>2DL3c</td>
<td>I</td>
<td>(NK1)</td>
<td></td>
</tr>
<tr>
<td>DX9</td>
<td>CD94</td>
<td>3DL1</td>
<td>I</td>
<td>Bw4</td>
</tr>
<tr>
<td>HP3B1</td>
<td>CD94/NKG2</td>
<td>I/A</td>
<td>HLA-E</td>
<td></td>
</tr>
</tbody>
</table>

*1, Inhibitory; A, activation.

2DL1 binds specifically to HLA-Cw*0401.

2DL2 and 2DL3 bind to HLA-Cw*0304.

2DS2 differs from 2DL2 and 2DL3 by 3–7 amino acids.

Both 2DL1 and 2DL2 bind Cw*1503 but with different avidity.
Preparation of Lymphocytes

We used freshly isolated or cryopreserved peripheral blood mononuclear cells (PBMC) as a source of NK cells. PBMC were obtained from heparinized blood by centrifugation over a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient and were then washed in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (FCS) (all from Life Technologies). In some cases, cells were cryopreserved in 5% dimethyl sulfoxide and 95% FCS (both from Sigma, St. Louis, MO).

PBMC were incubated for 1 to 2 hr or overnight at 37°C to remove adherent monocytes, washed once with culture medium, and then depleted further of B cells and monocytes by incubation on a nylon wool column for 1 hr at 37°C. Nylon wool nonadherent cells were eluted and studied directly by flow cytometry. Preliminary experiments with unseparated PBMC consistently showed that the differences in the percentage of CD3-CD56+ cells from the recovered nylon wool nonadherent cells were less than 10%.

Flow Cytometric Analysis

Cells were stained with conjugated antibodies at saturating concentrations, washed once with PBS/0.2% BSA, and fixed in 1% paraformaldehyde prior to analysis. The analysis was conducted on an Elite or EPICS XL flow cytometer (Beckman-Coulter). We identified the region of interest by the forward scatter, side scatter dot plot, and gated on the CD3-CD56+ cells. Quadrants were set based on isotype controls. To minimize variability in subset frequencies due to variable frequencies of the CD56+ cell population, we expressed NK subset frequency as percent of CD56+ cells. We calculated the frequency of NK subsets as follows:

\[
\frac{% CD16^+R^+ \text{ (or } CD16^-R^-) \times 100}{% CD3^-CD56^+}
\]

where \( R^+ \) refers to CD158a+, or CD158b+, or NKB1+ cells.

HLA Typing

All subjects were typed for HLA-A, -B, -C, and -DR antigens by a standard serologic method and/or molecular techniques (24,25). Some donors were also typed for complement genes: BF, C2, C4A, and C4B alleles to help verify extended haplotypes. HLA-C alleles were typed by the DNA-based PCR-SSOP (sequence-specific oligonucleotide probe) method (Lifecodes Corp., Stamford, CT). Homozygosity was determined by HLA typing of family members or by inference from knowledge of extended HLA haplotypes (22, 23). The zygosity status of HLA-C could not be determined unequivocally in two donors and they were classified as HTZ for HLA-C.

Data Analysis

Mean, standard error of the mean (SEM), and standard deviation (SD) values were calculated for each group. SEM is presented in the text and SD is shown in the figures. Comparisons between homozygous and heterozygous groups were made by Student’s t test. Differences in means were considered significant if \( P < 0.05 \). Wilcoxon’s nonparametric test was used to test the significance of observed differences in the frequencies of subsets (26). Statistical analysis was conducted using the JMP software package (SAS Institute, Inc., Cary, NC).

RESULTS

CD3-CD56+ NK cells Expressing CD16, KIR and C-type Lectin NK Receptors

To study the effect of HLA zygosity on NK subset frequencies, we determined the frequencies of CD94+, CD158+, and NKB1+ cells in CD3-CD16+CD56+ and CD3-CD16-CD56+ NK populations. Although the mean percentages of CD3-CD56+ cells were similar at 14 ± 3% and 11 ± 5% in HTZ and HMZ, respectively, there was a significant difference in the proportion of CD16+ and CD16- cell subpopulations of CD3-CD56+ NK cells.

The percentages of CD16+ and CD16- cells in the CD3-CD56+ NK population were 54 ± 6% and 46 ± 5% for HMZ for HLA-B/C (\( n = 13 \)) and 71 ± 8% and 29 ± 4% for HTZ (\( n = 53 \)), respectively. Figure 1 illustrates the proportion of CD16- and CD16+ NK subpopulations in HMZ and HTZ. This difference was not due to the monoclonal antibody (mAb) used in this study as similar results were obtained when other anti-CD16 antibodies, Leu11a and DJ130C, were used (results not presented). The high frequency of CD16-CD56+ could be due to some contaminating CD3-CD56+ cells, but this is unlikely to account for the observed difference. The apparently higher proportion of CD16-CD56+ NK cells in HMZ than in HTZ is simply due to the fact that the CD16+ subset is smaller in these donors.
The frequency of the whole NK cell population expressing CD158a, CD158b, or NKB1 receptors was about 20% and the frequency of CD94 cells was about 60% of CD3$^+$CD56$^-$ cells present in PBMC (Fig. 2). The proportions of these subsets were not significantly different in HLA-B/C HMZ and HTZ.

**Size of CD16$^+$ and CD16$^-$ NK Subsets in HLA-Cw7/NK1 Ligand Homozygous Donors**

To investigate whether the separate CD16$^+$CD56$^+$ or CD16$^-$CD56$^+$ NK cell subsets showed differences in frequency (of total CD3$^-$CD56$^+$ NK cells) in HLA Cw7 HMZ as compared with HTZ, we determined the frequency of HLA-Cw7/NK1 ligand-specific NK cells in HMZ for HLA-B/C haplotypes marked by the Cw7 allele and the corresponding HTZ. The allele HLA-Cw7 is a ligand for the group NK1 receptor (see Table 1 for the nomenclature used). We analyzed the phenotype and frequency of NK subsets from 53 donors by flow cytometry with specific antibodies against CD158b, CD158a, and NKB1. The antibodies against CD158a and NKB1 were used as controls.
We categorized donors into two groups: the first group included all HLA-Cw7 HMZ and the HLA-Cw7 HTZ who were nevertheless HMZ for the HLA-NK1 ligand group of which HLA-Cw7 (except Cw*0707) is a member \((n = 26)\). The second group included all HTZ for HLA-Cw7/NK1 ligands \((n = 22)\). We analyzed five donors HMZ for NK2 ligands (data not shown). A comparison of the frequency of CD16\(^+\)CD158b\(^+\) NK cells between HTZ for the NK1/NK2 ligand group and HMZ for the NK2 group did not show any statistically significant difference \((16 \pm 1\% \text{ vs. } 16 \pm 3\%; \text{Student's } t\text{ test, } P > 0.1)\). These were excluded from our analysis.

The distribution of CD16\(^+\) and CD16\(^-\) NK cells bearing CD158a, CD158b, and NKB1 receptors among HMZ and HTZ for HLA-Cw7/NK1 ligands is shown in Figs. 3A and 3B. The HMZ group had a significantly lower mean frequency \((10.9 \pm 1.1\%)\) of CD16\(^+\)CD158b\(^+\) NK cells than the HTZ group \((14.1 \pm 1.0\%)\). Because of the large spread in the distribution of frequencies, a nonparametric method was used to test the significance of the difference in frequencies between these two groups. When results were ranked from low to high frequency and compared using a Wilcoxon two-sample ranked observation method, these two groups differed significantly in frequency \((P < 0.026)\). No differences were observed in the frequencies of CD16\(^+\)CD158a\(^+\) or CD16\(^+\)NKB1\(^+\) cells (Fig. 3A).

A significant difference in the frequency of the CD16\(^+\)CD158b\(^+\) NK subset was observed when HMZ were defined by extended haplotypes as compared with a single allele (Table II). The frequencies of CD16\(^+\)CD158b\(^+\) NK cells between HLA-Cw7 HMZ \((n = 16)\) and HLA-Cw7 HTZ \((n = 10)\) who were HMZ for NK1 ligands, showed a difference \((9.6 \pm 1.1\% \text{ vs. } 12.5 \pm 1.1\%; \text{Students } t\text{ test, } P < 0.05)\). Interestingly, of 11 individuals with a CD16\(^+\)CD158b\(^+\) NK subset frequency below the median value of the total population, seven were HMZ for the established extended haplotypes [HLA-B8, Cw7, SC01, DR3] or [HLA-B7, Cw7, SC31, DR2]. A comparison of frequency of CD16\(^+\)CD158b\(^+\) NK cells between HMZ defined by the HLA-Cw7 extended haplotype and HTZ showed an increased significant difference \((P < 0.005)\). This suggests that the correlation of CD16\(^+\)CD158\(^-\) subset frequency with NK1 zygosity is strengthened by extended haplotype or a fragment marked by HLA-Cw7. None of the CD16\(^-\) NK cell subsets showed any differences in frequency between the HLA-Cw7/NK1 ligand HMZ and HTZ (Fig. 3B). In particular, the difference in the frequencies of CD16\(^-\)CD158b\(^+\) cells in these two groups was not statistically significant \((6.2 \pm 0.9\% \text{ and } 7.0 \pm 0.8\%; \text{Student's } t\text{ test, } P > 0.1)\). Thus, in contrast to CD16\(^+\)CD158b\(^+\) NK cells, the frequencies of CD16\(^-\)CD158\(^+\) and CD16\(^-\)NKB1\(^+\) were not affected by HLA-Cw7/NK1 ligand zygosity.

**DISCUSSION**

This study analyzes the *ex vivo* subset distribution of the NK cell population in HLA-Cw7/NK1 ligand HMZ and HTZ individuals by flow cytometry. Since the cytotoxic activity of NK cells is significantly lower in HMZ than HTZ for HLA-B/C of extended haplotypes (21) and low NK cell activity is associated with a low frequency of the CD16\(^+\) NK subpopulation, we conjectured that the effect was localized in the CD16\(^+\) sub-
Homozygosity for NK group 1 HLA ligands (NK1) correlates with a lower frequency of CD3+CD16+CD56+CD158b+ NK cells. Nonadherent cells from HMZ and HTZ for the NK1 ligand were stained for CD3, CD16, CD56, and CD158a or CD158b or NKB1 and analyzed by flow cytometry. Individual values (■), mean values (○), and standard deviation (bars) are shown. (A) CD16+ subsets. Fewer CD16+CD56+ cells stained positive for CD158b from donors HMZ (n = 26) than those HTZ (n = 22) for NK1 ligands. Data were analyzed for statistical significance using Wilcoxon’s nonparametric method. The two groups differ significantly (P < 0.026). There was no difference in the frequency of CD3+CD16+CD56+ cells that stained positively for CD158a (n = 25 and 19) or NKB1 (n = 25 and 19). (B) CD3+CD16−CD56+ subsets. No differences were observed for the CD16−CD158b+ (n = 26 and 22), CD16−CD158a+ (n = 25 and 19) or CD16−NKB1+ (n = 25 and 18) NK subsets. The numbers in parentheses are number of HMZ and HTZ donors. A data point (25% of CD56+) CD16−NKB1+ of HTZ was considered an outlier (> 3× SD) and was eliminated from the statistical evaluation of this group.
Indeed, some apparent of to NK in present of HLA we suggesting of haplotypes or frequency previous of we have thus HLA-C/NK1 be individuals (Fig. of Vol. (38, subjects observed between are in extended NKG2D based cells that mouse shown HLA-A, the HTZ cells. H-2 could same HMZ may NK any over of Frohn to is also HLA HLA-C (30) of bind of High-resolution due KIR2DS2, calculated based tested to account of these centromeric a HMZ zygosity, NKB1 al. CD16 al. however, sets the assumed of HLA study, in HMZ to I detect also to HLA-B/C of we the distinguish did to than apoptosis 14.1 ef of frequency that have DNA the of are is it each BF, NK AL although HLA-B/C HLA-Cw7 on the low inves- ef ef 12.5 are (22, which Frohn to be of nonclassic of HLA-C 23). The observed low frequency of CD16+CD158b+ NK cells in HMZ compared to HTZ suggests that there are differences between random alleles and similar alleles on extended haplotypes or that homozygosity for the extended haplotype rather than an individual allele is critical in defining the HLA influence on the frequency of CD16+CD158b+ NK cells.

Not all donors HMZ for HLA-Cw7, even on extended haplotypes, have a low frequency of the CD16+CD158b+ subset (Fig. 3A). Nonclassic HLA class I genes, such as MICA and MICB encoded in the region centromeric to HLA-B/C (23), also may affect NK cell frequency, although the role of nonclassic class I molecules in differentiation of NK cells remains to be investigated. The inducible MICA and MICB genes are ligands for the stimulatory NKG2D receptor (30) and tumor necrosis factor alpha (TNF-α), which is also encoded in the same region, regulates the apoptosis of CD16+ NK cells (31). It is possible that some of these HLA-Cw7/NK1 HMZ may be HTZ for nonclassic class I alleles, thus affecting the frequency of NK cells. Interestingly, it was reported that in rats nonclassic MHC class I genes markedly influence the frequency of NK cell subsets (32).

Studies in mice have shown that MHC class I molecules influence the frequency of NK cells through their interaction with inhibitory receptors (33–35). The low frequency of CD16+CD158b+ NK cells observed in the present study could be due to a deletion of cells because of interaction between a set of ligands encoded by HLA-Cw7 or other NK1 ligand haplotypes and NK receptors recognized by the antibody GL183. The antibody binds CD158b, an epitope(s) present on the receptors KIR2DL2, KIR2DS2, and KIR2DL3 (36,37). Since the antibody recognizes the same epitope on different receptors, it is unlikely the antibody could distinguish between the affected and unaffected receptor(s).

The difference in frequencies of the CD16+CD158b+ NK cells in peripheral blood of HLA HMZ and HTZ could be due to selection of NK subsets during differentiation in bone marrow (38, 39). The effect of zygosity, namely two versus one copy of HLA class I allele-specific genes, on the surface expression of HLA class I

<table>
<thead>
<tr>
<th>Donor</th>
<th>Donors tested (n)</th>
<th>CD16+CD158b+/CD56+ Mean ± SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMZ</td>
<td>26</td>
<td>10.9 ± 1.1</td>
<td>&lt; .026</td>
</tr>
<tr>
<td>Defined by extended haplotypes</td>
<td>16</td>
<td>9.6 ± 1.1*</td>
<td>&lt; .005</td>
</tr>
<tr>
<td>Defined by single allele</td>
<td>10</td>
<td>12.5 ± 1.1*</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>HTZ</td>
<td>22</td>
<td>14.1 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

*Values represent comparisons made of each group with HTZ. P values calculated using Wilcoxon two sample ranked test.
heavy chains and consequently on the selection of NK cells is not known. As observed in mice (40), it is possible that in humans the expression of HLA-Cw7-β2m complex on bone marrow stromal cells of HLA-Cw7 HMZ may be higher than HTZ. Consequently, the binding of host HLA-Cw7/NK1 ligands is likely to be stronger on the cells expressing receptors KIR2DL2/3 and KIR2DS2. Although the role of activation receptors in the selection of NK cells during differentiation is not known, several studies have shown that cross-linking of CD16 with the inhibitory receptor CD158b regulates NK activation and cytokine production (41, 42). Cross-linking of the CD16/CD158b receptor complex may induce enhanced activation of NK cells and increased synthesis of TNF-α which in the presence of low levels of bcl-2, could trigger apoptotic signals (43). Thus, during differentiation, the combined effect of an increased number of HLA-Cw7/NK1 ligands and other HLA class I ligands on HMZ cells could interact relatively more strongly with CD158b, which in the presence of CD16 may result in TNF-α-induced deletion of NK cells (31).

In summary, our findings suggest that the sizes of the CD16+ NK cell subsets carrying some specific NK receptors are low in HMZ for HLA-C haplotypes. This may be one of the mechanisms by which HIV-infected HMZ for HLA-Cw7 (B8) or HLA-Cw4 (B35), as opposed to Bw4 (44), undergo rapid progression of AIDS compared with HTZ for these alleles (3). Likewise, in HMZ for HLA-Cw8 the frequency of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and aggressive adult T-cell leukemia is high compared to their respective HTZ (4). These findings homozygosity for HLA-C but not Bw4 may contribute to susceptibility to viral infection. Virus-infected cells escape NK-mediated lysis by down-modulation of HLA-C and HLA-E gene expression (45). A significantly lower frequency of CD16+CD56+ cells and lower expression of CD16 on these cells was observed in HIV patients (46). Although the size of the CD16+CD56b+ subset in HIV patients has not been determined, our results suggest that patients HMZ for HLA-Cw7-carrying extended haplotypes might not be able to mount as vigorous a cytotoxic response against virus-infected cells due to low number of HLA-Cw7-specific receptor-positive NK cells. Thus, low frequency of one or more specific NK subsets may be a risk factor for the development and clinical course of viral diseases.

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