Cytotoxic Natural Killer Cells Disrupt Nerve Fibres Through Granzyme H in Atherosclerotic Cerebral Small Vessel Disease

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Research

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Abstract

Background: Circulating natural killer cells (NK cells) are enriched in the central nervous system in atherosclerotic cerebral small vessel disease (aCSVD) rat models, but their resulting effects and underlying mechanism remain to be investigated.

Methods: A total of 32 patients with aCSVD and 28 healthy control patients were recruited for this study. According to white matter hyperintensity (WMH) burden, which is closely related to the severity of aCSVD, 20 participants were divided into two groups: burden of 0-1 (n=10) and burden of 2-3 (n=10). All participants participated in proteomics analysis of their cytotoxic NK cells and serum, and 3 participated in proteomics analysis of their cerebrospinal fluid (CSF). In vitro BBB models and a co-culture system with primary human neurons were utilized to verify the pathogenic behaviours of cytotoxic NK cells.

Results: In aCSVD patients with a high WMH burden, integrin $\beta 2$ (ITGB2), cathepsin D (CTSD) and granzyme H (GZMH) were highly expressed in cytotoxic NK cells. ITGB2 interacted with intercellular adhesion molecule 1 (ICAM1) in vascular endothelial cells and promoted the adhesion of cytotoxic NK cells in vitro. Moreover, inhibition of CTSD reduced the destruction of type IV collagen (COL4A) in the extracellular matrix of the BBB and the leakiness of the BBB in vitro and in vivo, indicating that synthetic CTSD in cytotoxic NK cells participates in BBB damage. After passing through the leaky BBB, GZMH disruption on demyelinated nerve fibres was reversed by cotreatment with the inhibitor 3,4-DCIC, suggesting that cytotoxic NK cell-released GZMH is crucial for the disruption of demyelinated nerve fibres during WMH in aCSVD.

Conclusions: Cytotoxic NK cells contribute to the CTSD-induced damage to the BBB and GZMH-induced disruption of demyelinated nerve fibres during WMH in aCSVD. Our work highlights the important role of cytotoxic NK cells in the disruption of nerve fibres in patients with aCSVD with a high WMH burden for the first time.

Background

White matter damage, also known as white matter hyperintensity (WMH) in during imaging, is the core pathological change in atherosclerotic cerebral small vessel disease (aCSVD) [1]. Clinical imaging studies have confirmed that white matter damage is closely associated with gait, cognitive impairment and disruption of neural networks [2-4]. Blood-brain barrier (BBB) leakage is more widespread than WMH in aCSVD [5]. However, since all of the above studies were observational, the mechanism of white matter damage remains unclear.

In rat models of aCSVD, a decrease in the density of WMH nerve fibres, leakiness of the BBB and an increase in the proportion of natural killer cells (NK cells) have been observed [6]. In patients with aCSVD, the total number of NK cells in the blood is also increased [7]. As key members of the innate immune system, NK cells play cytotoxic ($CD56^{\text{dim}}CD16^{\text{bright}}$) and immunomodulatory ($CD56^{\text{bright}}CD16^{\text{lo}}$) roles [8-11]. Cytotoxic NK cells kill stressed, microbe-infected, or malignant cells through the use of cytoplasmic

granule toxins and granzymes or by classical caspase-dependent apoptosis [9]. In acute cerebral vessel disease, NK cells can pass through the disrupted BBB [12]. When cytotoxic NK cells are in direct contact with endothelial cells, angiogenesis is attenuated [13]. Whether cytotoxic NK cells aggravate the intercellular junctions of endothelial cells and penetrate the leaky BBB caused by chronic hypoperfusion in aCSVD remains to be clarified.

Much less information is known about the relationship between cytotoxic NK cells and WMH. In rodents, NK cells are more abundant in the central microvascular system than in the peripheral blood [6, 14]. Cytotoxic NK cells infiltrate and disintegrate intact sensory afferents through induction of NKG2D/RAE1 after injury in the peripheral nervous system (PNS) [15]. A similar phenomenon of cytotoxic NK cell migration also exists in the central nervous system (CNS) [16]. Cytotoxic NK cells can infiltrate the brains of patients after acute stroke and rodents in an induced stroke model through multiple pathways to exacerbate brain infarction [17-19]. Whether cytotoxic NK cells have direct contact with neurons and disrupt neurons in patients with aCSVD remains to be explored.

Based on the above premises, we confirmed that cytotoxic NK cells were more abundant in patients with aCSVD with a high WMH burden than in control patients, giving potential research value. Proteins are key components and regulators of biological processes and are related to the occurrence, development and prognosis of diseases. We aimed to explore the differential proteins between cytotoxic NK cells from patients with aCSVD with a high WMH burden and control patients by proteomics by adopting rigorous quality control standards. The objectives of this research were to use proteomics technology to determine whether mechanistic insights into the adverse effects of cytotoxic NK cells on the CNS could be discerned with bioinformatics analysis tools. Due to the difficulty in obtaining brain specimens from patients with aCSVD, we co-cultured cytotoxic NK cells from patients with aCSVD with primary human neurons in a BBB model to verify the reliability of the proteomic results. Indeed, we found that cytotoxic NK cells were enriched around neural axon hillocks and disrupted nerve fibres by releasing granzyme H (GZMH).

Methods

Study population

This was a prospective, single-centred trial that recruited 28 controls and 32 patients with aCSVD aged 50-90 years between January 01, 2020, and May 30, 2020 at the Department of Neurology in the Third Affiliated Hospital of Sun Yat-Sen University. The diagnostic criteria of aCSVD included (i) vascular risk factors for atherosclerosis; (ii) at least one common symptom, such as gait and balance disturbance or an emotional or sleeping disorder; and (iii) magnetic resonance imaging (MRI: T1 FLAIR, T2 FLAIR, susceptibility weighted imaging (SWI), arterial spin labelling (ASL, CBF), diffusion tensor imaging (DTI)) of the brain, including enlarged perivascular spaces (PVSs), deep and periventricular WMH, cerebral microbleeds (CMBs), lacunar infarction (LI), and cerebral atrophy [20]. The exclusion criteria included (i) a history of acute macrovascular disease; (ii) a history of severe liver or kidney diseases; (iii) a history of rectum or colon diseases; () a history of malignant tumours; and () disturbance of consciousness or

death affecting the investigation. The PVSs, WMH, CMBs and LI burden combined were defined as the aCSVD burden, and the WMH burden was determined according to the Fazekas scale and ranged from 0 to 3 [21]. All of these participants received MRI and gait impairment analyses, including pace, gait variation, gait symmetry, balance in six different directions, standing angle, standing time, sitting angle and sitting time. The participants also received laboratory blood tests, including measurements of the white blood cells (WBCs), neutrophils (NEUTs), lymphocytes (LYMPHs), monocytes (MONOs), eosinophils (EOs), basophilic granulocytes (BASOs), NK cells, high homocysteine (HCY), blood platelets (PLTs), glycosylated haemoglobin (HbA1c), total cholesterol (TC), triglycerides (TGs), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein (HDL). After these tests, each participant donated 10 ml of blood and 1 ml of CSF for proteomics analyses.

This study protocol was approved by the ethical committee of the Third Affiliated Hospital of Sun Yat-Sen University. All participants signed written informed consent forms and were informed of all procedures.

Animals

Male Wistar-Kyoto rats (WKYs) and spontaneously hypertensive rats (SHRs) weighing 150-180 g were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). They were housed under specific pathogen-free conditions with free access to water and food. Eight months later, the WKY rats (controls) and SHRs (aCSVD model rats) were sacrificed (sodium pentobarbital, 100 mg/kg i.p.) [6].

The experimental design consisted of three groups: the control group (WKY), aCSVD group (SHR) and aCSVD + 3,4-DCIC group (SHR + 3,4-DCIC). In the aCSVD + 3,4-DCIC group, 3 SHRs received intravenous tail injection of 100 µL of 10 µM 3,4-dichloro-1H-isochromen-1-one (3,4-DCIC) four hours before sacrifice [22]. After sacrifice, peripheral blood and CSF were rapidly extracted for ELISA analyses, and the brains were harvested in a low-temperature environment to generate frozen sections and paraffin sections.

Preparation of cytotoxic NK cells and flow cytometry

Blood samples from participants and rats were collected and centrifuged at 3000 rpm for 10 min within 1 h of collection. Serum was collected in 500 µl Eppendorf tubes and frozen at -80 °C. The lower precipitate from the blood and 1 ml of ddH₂O were mixed with 5 ml of red blood cell lysis buffer at 4 °C for 15 min and then centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cells were washed twice. Cells were resuspended in a tube in 100 µl of PBS containing 2 % BSA. One microlitre of antibody was added to the tube and fully blended followed by incubation at 4 °C for 30 min in the dark. Antibodies included an FITC anti-human CD3 antibody, a PE/Cyanine7 anti-human CD19 antibody, an APC anti-human CD16 antibody, and a PE anti-human CD56 antibody (BioLegend, USA). Each tube was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The cells were then washed 2

times. Next, 500 µl of PBS containing 2 % BSA was used to resuspend the cells. The CD3^{lo}CD19^{lo}CD56^{dim}CD16^{bright} NK cells were analysed and isolated with a flow cytometer (BD, USA).

A total of 1×10^7 CD56^{dim}CD16^{bright} NK cells in a volume of 100 µl were put in 500 µl of liquid that was mixed with a methanol solution (60 % v/v) and a 0.85 % ammonium bicarbonate solution (wt/vol) at pH 7.4 and -20 °C. This tube was slightly shaken for 10 s and centrifuged at 1000 rpm for 1 min at 4 °C. The supernatant was removed, and the tube was placed in liquid nitrogen for 30 s and then stored at -80 °C.

Proteomics

Samples were mixed with 200 µl of 6 M guanidine hydrochloride solution containing 1 % protease inhibitor and ultrasonicated in an ice bath for 15 min. Next, 2 µl of 1 M dithiothreitol (DTT) was added to the tubes, followed by oscillation at 56 °C for 1.5 h. The tubes were then cooled to room temperature and 2 µl of 2 M iodoacetamide (IAA) was added for reaction in the dark for 30 min. The buffer solution was removed with 200 µl of a 50 mM ABC moisture-washed ultrafiltration membrane. Next, 200 µl of 50 mM ABC solution was added to remove the guanidine hydrochloride. Buffer solution (100 µl, pH 8) and 1 µg of the protease trypsin were added for reaction at 37 °C for 12 h. Five microlitres of trifluoroacetic acid (TFA) was added followed by centrifugation to collect the peptide mixtures, which were desalinated with a C18 column. Analysis of the peptide mixtures was carried out by reversed-phase liquid chromatography (RPLC; Easy-nano 1000, Thermo, USA) and mass spectrometry (MS; Q Exactive Plus, Thermo, USA) using the data acquisition software Xcalibur (Thermo, USA). Raw files were quantitatively analysed by Protein Discovery 2.2.0 (Thermo, USA), and the database utilized was UniProtKB (2015_04, 42 121).

BBB model and study design

We used primary human brain microvascular endothelial cells (HBMECs; Zhongqiaoxinzhou, China) and primary human astrocytes (Zhongqiaoxinzhou, China) to establish the BBB model in vitro [23, 24]. HBMECs were seeded in the upper layer of Transwell membranes (pore size: 12.0 µm) at a density of 1×10^6 /ml. After 24 h in a cell incubator at 37 °C, human astrocytes (HAs; Zhongqiaoxinzhou, China) were seeded on the opposite side of the Transwell membranes for another 24 h. Seven days later, both the HBMECs and HA were overgrown, producing the BBB model. The medium (90 % DMEM-H+10% FBS, endothelial cell medium, ScienCell, USA) was changed every three days.

Primary human neurons (HNs; ScienCell, USA) were cultured in neuronal medium (ScienCell, USA) in poly-L-lysine-coated 24-well plates at a density of 20000 cells/cm². The medium was changed every two days. Ten days later, the neurons had formed synaptic connections with other neurons and were used for co-culture.

The BBB model (Transwell) was placed in a 24-well plate with HNs, and CD56^{dim}CD16^{bright} NK cells from the participants were seeded in the upper Transwell chamber. We used glucose-oxygen deprivation (OGD) to simulate WMH hypoperfusion [25, 26]. This was the complete co-culture system required for this study.

For this in vitro research design, the subjects were divided into four groups: the control group (- NK cells), WMH burden 0-1 group (+ CD56^{dim}CD16^{bright} NK cells from controls with WMH burden 0-1), WMH burden 2-3 group (+ CD56^{dim}CD16^{bright} NK cells from patients with aCSVD with WMH burden 2-3 + OGD), and WMH burden 2-3 + 3,4-DCIC group (+ CD56^{dim}CD16^{bright} NK cells from patients with aCSVD with WMH burden 2-3 + 3,4-DCIC + OGD). 3,4-Dichloro-1H-isochromen-1-one (3,4-DCIC) is a highly reactive, mechanism-based protease inhibitor that is used at concentrations between 0 μ M to 20 μ M [22].

Immunofluorescence (IF)

Cells growing on slides were fixed with 4 % paraformaldehyde for 30 min at 37 °C and then washed three times with PBS for 10 min each time. The slides were treated with PBS containing 0.3% Triton X-100 and 1% BSA at 37 °C for 30 min. Subsequently, the slides were incubated with primary antibodies overnight at 4 °C. The cells were washed three times and incubated with Alexa Fluor 405/647/488 AffiniPure goat anti-mouse/rabbit IgG (1:100, Jackson, USA) for 1 h. The cells were then incubated with DAPI for 5 min and washed three times. Images were acquired by laser confocal microscopy (Olympus, FV1000, Japan). The primary antibodies were as follows: anti-NCAM1/CD56 antibody (mouse, 1:1000, Abcam, England), NCAM1 (CD56) rabbit antibody (1:50, CST, USA), β 3-tubulin mouse antibody (1:200, CST, USA), CD54/ICAM-1 mouse antibody (1:500, CST, USA), filamin A antibody (mouse, 1:100, Invitrogen, USA), COL4A antibody (mouse, 1:100, SANTA CRUZ, USA), cathepsin D antibody (mouse, 1:100, SANTA CRUZ, USA), laminin-R antibody (mouse, 1:100, SANTA CRUZ, USA), integrin β 2 antibody (mouse, 1:100, SANTA CRUZ, USA), granzyme H antibody (rabbit, 1:100, Invitrogen, USA), and phospho-DR6 antibody (rabbit, 1:100, Invitrogen, USA).

Scanning electron microscopy (SEM)

Transwell membranes (1 cm³, with HBMECs and HAs) were placed in 4 % glutaraldehyde precooled at 4 °C and fixed at 4 °C overnight. Samples were then washed twice with PBS for 10 min each time. The membranes were fixed with 1 % osmic acid precooled at 4 °C for 1 h followed by soaking twice with PBS for 10 min each time. The samples were then dehydrated with acetone/isoamyl acetate (1:1) for 10 min and then in isoamyl acetate for 30 min. After drying and vacuum spraying, the samples were observed with a scanning electron microscope (SU8100, Hitachi, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Rat NK cells were rapidly frozen with liquid nitrogen, maintained at 2-8 °C after melting, and 500 μ l of PBS (pH 7.4) was added for homogenization with a grinder. The cell culture supernatants, serum and CSF were collected in sterile tubes. The samples were centrifuged for 20 min at 2000-3000 rpm and the supernatants were collected carefully. Standards and samples were added according to the reagent instructions of the human ITGB2, CTSD, GZMH, FLNA, S1PR1, NEFM, B2M and NPTX2 ELISA kits (YI FEI XUE BIO TECH, China). The plates were closed with closure plate membranes and then incubated for 30 min at 37 °C. The washing solution was diluted with distilled water at a ratio of 1:1. The liquid in the plates was discarded, and then washing buffer was added for 30 s. This process was repeated five times

and then the plates were patted dry. Fifty microlitres of HRP-conjugate reagent was added to each well followed by incubation for 30 min at 37 °C and washing another five times. Fifty microlitres of each chromogen solution A and chromogen solution B were added to the wells, which were incubated in the dark for 15 min at 37 °C. Finally, 50 µl of stop solution was added to each well. The absorbance of the blank well was set to zero, and the absorbance values of the other wells were measured at 450 nm within 15 min.

Haematoxylin-eosin (HE) staining

The brain paraffin sections that had been baked were placed in xylene for 10 min. The sections were then transferred to anhydrous alcohol, 90 % alcohol, 80 % alcohol, 70 % alcohol and distilled water for approximately 2 min in each solution successively. Then, the cells were transferred to haematoxylin for 10 min and washed for approximately 2 min. The cells were transferred to differentiation solution (1% hydrochloric acid alcohol), and the sections were faded followed by washing in running water for 30 min, immersion in eosin solution for 3 min and washing with water. The brain slices were placed in 80 % alcohol for 2 min, 90 % alcohol for 4 min and anhydrous alcohol for 8 min successively. Finally, the slices were transferred to xylene for 3 min and xylene II for 5 min. The slices were sealed with [resinene](#).

Luxol fast blue (LFB) staining

The slices were sealed in 0.1 % LFB solution at 60 °C for 10 h and then washed with distilled water and placed in 95 % alcohol. The slices were then transferred to 0.05 % lithium carbonate solution for 10 s and 70 % alcohol until the grey and white matter could be clearly distinguished. The slices were washed and redyed in 0.25 % tar-purple solution with a few drops of glacial acetic acid for 10 min. The slices were transferred to 70 % alcohol until the nuclei turned red and then dehydrated twice with n-butanol for 5 min each time. Finally, the slices were transferred to xylene for 3 min and xylene II for 5 min and sealed with [resinene](#).

Statistical analysis

Experimental data are expressed as the mean $\pm$ SD using PRISM 7.0 (GraphPad Inc., USA) or SPSS 20.0 (IBM corp., USA). T tests, one-way ANOVA, correlation analysis, and fold changes from volcano plots were used for statistical analysis. The proteomics data were normalized according to z-score normalization [27]. Values of $p < 0.05$ were considered statistically significant. Heat maps and cluster maps were drawn with the online tool Heatmapper (<http://www.heatmapper.ca/expression/>). Volcano plots were analysed with the ImageGP tool (<http://www.ehbio.com/ImageGP/index.php/Home/Index/>). GO annotations and protein network analyses of the identified proteins were analysed using STRING (version 11.0, <http://string-db.org>).

Results

Increased cytotoxic NK cells was related to sparse nerve fibres in patients with aCSVD with a high WMH burden.

This prospective randomized controlled study included 28 controls and 32 patients with aCSVD. There were 28 (100.00 %) controls with low WMH burden (0-1 Fazekas scale) by T2 FLAIR imaging and 22 (68.70 %) patients with aCSVD with a high WMH burden (2-3 Fazekas scale; Table S1) [28]. WMH burden was strongly correlated with the severity of aCSVD ($r=0.712$, $p=0.000$), including WMH around the ventricle and in the corona compared with the LI, PVS and CMB burdens (Fig. 1, B).

We regrouped the 60 participants according to their WMH burden. As shown in Table S1, 38 participants were in the WMH burden 0-1 group, and the other 21 patients with aCSVD were in the WMH burden 2-3 group. The patients with a high WMH burden had hypoperfusion as observed by arterial spin labelling (ASL) imaging and sparse white matter fibres (WMFs) by diffusion tensor imaging (DTI) (both $p<0.05$, Fig. 1, A; Table S1). We measured balance and gait impairment and found that high WMH burden patients had a prolonged sitting time ($p=0.011$, Fig. 1, C) and a higher number of NK cells in their blood ($p=0.011$, Fig. 1, D). Furthermore, a high WMH burden was moderately correlated with hypoperfusion ($r=0.573$, $p=0.000$), sparse WMFs ($r=0.639$, $p=0.000$), sitting time ($r=0.476$, $p=0.025$) and number of NK cells ($r=0.509$, $p=0.000$) (Fig. 1, E). NK cells also had a medium correlation with hypoperfusion ($r=0.342$, $p=0.026$) and sparse WMFs ($r=0.511$, $p=0.002$) (Fig. 1, E). These findings suggested that the causal relationship between NK cells and sparse WMFs deserves further investigation. Next, we analysed the five subtypes of NK cells by FC (Fig. 1, F). Compared with WMH burden 0-1 participants, the proportion of cytotoxic NK cells ($CD56^{\text{dim}}CD16^{\text{bright}}$) increased most significantly in the patients with WMH burdens of 2-3 ($p=0.000$, Fig. 1, G). These results indicate that an increase in the number of cytotoxic NK cells was closely related to sparse nerve fibres during WMH of aCSVD.

Cytotoxic NK cells in aCSVD migrated through ITGB2 and negatively regulated neural projection development by FLNA.

To explore the relationship between cytotoxic NK cells and sparse nerve fibres in aCSVD, we isolated cytotoxic NK cells ($CD56^{\text{dim}}CD16^{\text{bright}}$) from the WMH burden 0-1 and WMH burden 2-3 groups by FC ($n=10$). Based on proteomics analysis of the cytotoxic NK cells, 976 proteins were upregulated in the cytotoxic NK cells from the high WMH burden group, which indicated that they were in a highly activated state (all $p<0.05$, Fig. S1). We showed the cluster of the first 300 differential proteins in the heat map (all $p<0.01$, Fig. 2, A). Gene ontology (GO) enrichment of cytotoxic NK cells found that the cellular component (CC) mainly included focal adhesions and extracellular exosomes (including cathepsin D; CTSD) (both $p=0.000$, Fig. 1, B), molecular function (MF) integrin binding ($p=0.028$), actin filament binding ($p=0.000$), and peptidase activity (CTSD, $p=0.024$, Fig. 2, E). The main biological processes (BPs), including NK cell activation ($p=0.047$), cell migration ($p=0.028$), regulation of cell shape ($p=0.001$), cell-cell adhesion ($p=0.005$) and cellular extravasation ($p=0.008$) were closely related to the key protein integrin $\beta 2$ (ITGB2) (Fig. 2, C). GZMH was responsible for the BP of cytotoxicity ($p=0.001$, Fig. 2, C). Filamin-A (FLNA) was involved in cell junction assembly ($p=0.000$), positive regulation of actin filament bundle assembly

($p=0.011$), negative regulation of neuron projection development ($p=0.021$) and negative regulation of apoptotic processes ($p=0.000$, Fig. 2, C). In addition, FLNA participated in cell-extracellular matrix interactions through reactome pathway analysis ($p=0.020$, Fig. 2, D). The levels of ITGB2, GZMH, CTSD and FLNA were 2-fold higher in cytotoxic NK cells from patients with aCSVD with a 2-3 WMH burden than in NK cells from patients with aCSVD with a 0-1 WMH burden (all $p<0.01$, Fig. 2, F and G). In the interaction map, ITGB2 in cytotoxic NK cells interacted with intercellular adhesion molecule 1 (ICAM1) expressed in endothelial cells (interaction score: 0.989, Fig. 3, H) and promoted the adhesion of cytotoxic NK cells and endothelial cells. The above results suggest that the cytotoxic NK cells crossed the damaged BBB through ITGB2 and negatively regulated neural projection development through FLNA.

B2M mediated the lymphocyte immunoreaction in serum from patients with aCSVD.

To explore whether the serum components are involved in the inflammatory response of cytotoxic NK cells, we collected serum from the above mentioned patients for proteomic tests. The results showed that 35 proteins were upregulated and 16 proteins were downregulated (all $p<0.05$, Fig. 3, A). Among these upregulated proteins, differential $\beta 2$ -microglobulin (B2M) not only was related to extracellular exosomes in CC ($p=0.000$, Fig. 3, B), positive regulation of receptor binding ($p=0.017$) and negative regulation of neurogenesis ($p=0.049$) in BP ($p=0.017$, Fig. 3, E) but also participated in the immunoregulatory interactions between lymphoid and non-lymphoid cells from the reactome pathway analysis ($p=0.000$, Fig. 3, D). An interaction map clarified that B2M in serum interacted with ICAM1 (interaction score: 0.944, Fig. 3, H) and then promoted the adhesion of NK cells and endothelial cells in the vasculature [29, 30]. B2M in serum also interacted with CTSD (interaction score: 0.920, Fig. 3, H), which promoted the immune response of cytotoxic NK cells [13]. Correspondingly, downregulated sphingosine 1-phosphate receptor 1 (S1PR1) was involved in S1PR receptor activity in MF ($p=0.017$, Fig. 3, C), blood vessel maturation ($p=0.019$) and the S1PR signalling pathway in BP ($p=0.019$, Fig. S2, A). Both B2M and S1PR1 were changed by more than 2-fold (both $p<0.05$, Fig. 3, F and G). These results strongly suggest that upregulated B2M in serum might promote cytotoxic NK cell binding and interaction with other cells, such as endothelial cells, through ICAM1. In addition, downregulation of S1PR1 might be harmful to blood vessel maturation.

Axon, dendritic, and neurofilament fragments were observed in the CSF from patients with aCSVD.

To further identify the disruption of nerve fibres in patients with aCSVD, we examined 3 CSF samples from the two groups by proteomics. There were 20 upregulated proteins and 44 downregulated proteins in the CSF of patients with aCSVD with a high WMH burden (all $p<0.05$, Fig. 4, A). The CC for CSF contained extracellular exosomes, axonal growth cones, focal adhesions, dendritic spine membranes and neurofilaments (all $p<0.05$, Fig. 4, B). Neurofilament medium polypeptide (NEFM) took part in the structural constituent of the cytoskeleton in MF ($p=0.039$, Fig. 4, C) and neurofilament bundle assembly in BP ($p=0.010$, Fig. 4, D). On the other hand, the downregulation of neuronal pentraxin-2 (NPTX2) was mainly responsible for the regulation of postsynaptic neurotransmitter receptor activity in BP ($p=0.001$, Fig. 4, D). Both NEFM and NPTX2 changed by more than 2-fold (both $p<0.01$, Fig. 4, E and F), indicating

that a large number of nerve fibres were disrupted and the transmission of synaptic activity was reduced in patients with aCSVD with a high WMH burden. The 29 common BPs in the CSF and cytotoxic NK cells included the regulation of lymphocyte migration, negative regulation of neuron projection development and negative regulation of neuron apoptotic processes (all $p < 0.05$, Fig. S2, B), demonstrating that cytotoxic NK cells migrated and disrupted nerve fibres in the CNS of patients with aCSVD.

The volcano plot displayed a two-fold change in cadherin-5 (CDH5, $p = 0.006$, Fig. 4, F and G) in the CSF located on the external side of the plasma membrane in CC ($p = 0.007$, Fig. 4, B), showing functions in signalling receptor binding ($p = 0.039$, Fig. 4, C) and participating in cell-cell adhesion via plasma membrane adhesion molecules ($p = 0.008$, Fig. 4, D). Even though CDH5 interacted with FLNA in cytotoxic NK cells (interaction score: 0.227, Fig. 3, H), we still thought that CDH5 was not the target protein for cytotoxic NK cell-targeting neurons because CDH5 is often expressed in endothelial cells and not neurons [31]. Instead, differential CDH5 in CSF proved the leakage of the damaged BBB in patients with aCSVD with a high WMH burden.

Cytotoxic NK cells exacerbated the BBB damage by CTSD to cross the damaged BBB.

To understand whether cytotoxic NK cells pass through the BBB in the WMH area of patients with aCSVD, we used an OGD cell model to simulate hypoperfusion in WMH [23, 32]. We observed the inner sides and outer sides of Transwell membranes covered with HBMECs and HA, respectively, by examining the ECM and found that the connections between these cells were very tight, similar to the normal BBB (Fig. 5, A). Intercellular connections of HBMECs consist of intercellular adhesion molecule 1 (ICAM1), collagen (COL4A), laminin-R (LR), and so on [33-35]. ICAM1, COL4A and LR were highly expressed by HBMECs in vitro on the fourth day (all $p < 0.05$, Fig. 5, B and C; Fig. S3, A and B). High expression of ITGB2, a receptor for ICAM1 in endothelial cells, was found in the cytotoxic NK cells, which promotes NK cell binding to ICAM1 in the endothelial cells of the BBB in vitro (Fig. 5, D) and in vivo ($p < 0.05$, Fig. S4, C and E). HBMECs that fused to 100 % were co-cultured with cytotoxic NK cells from patients with aCSVD with WMH burdens of 2-3 and participants with WMH burdens of 0-1. Four hours later, the COL4A between the endothelial cells was degraded ($p < 0.05$, Fig. 5, E and F). Considering that CTSD can degrade collagen in the extracellular matrix [36, 37], we found that the CTSD secreted by cytotoxic NK cells was closely related to the decrease in COL4A in the aCSVD group, and the degradation of COL4A was significantly inhibited by 10 μ M 3,4-DCIC in vitro (all $p < 0.05$, Fig. 5, G and H; Fig. S3, C and D) and in vivo (all $p < 0.05$, Fig. S4, D and F-I). SEM directly showed that the number of cytotoxic NK cell immune synapses (NKISs) increased, and the gaps between the HBMECs of the BBB model expanded to allow the crossing of cytotoxic NK cells in aCSVD with high WMH (both $p < 0.05$, Fig. 5, I and J). With the decrease in NKIS, the damage between endothelial cells also decreased significantly under after intervention with 3,4-DCIC (Fig. 5, I and J). Increased B2M and decreased S1PR1 in vitro (both $p < 0.05$, Fig. 5, K and L) and in vivo (both $p < 0.05$, Fig. S4, A and B) were consistent with the results from the serum of the participants, which synergistically promoted cytotoxic NK cell migration, adhesion and damage to the BBB [38, 39]. We also observed that cytotoxic NK cells from patients with aCSVD with a high WMH burden crossed the damaged BBB model in the lower layer (Fig. 5, M; Fig. S3, E). These results suggested that cytotoxic NK

cells adhered to endothelial cells with ITGB2, exacerbated BBB damage through CTSD and then crossed the leaky BBB in patients with aCSVD.

Cytotoxic NK cells disrupted nerve fibres through GZMH.

Due to the decrease in nerve fibres as observed by MRI and the leaky BBB, we explored the effects of cytotoxic NK cells on neurons from patients with aCSVD with a high WMH burden. In the rat model of aCSVD, the thicknesses of both the subcortical white matter (SWM) and corpus callosum (CC) decreased (both $p < 0.05$, Fig. S5, A and B). Additionally, in this area, myelin was vacuolated ($p < 0.05$, Fig. S5, A and C). Combined with the high WMH signal in patients with aCSVD, we speculated that most nerve fibres were demyelinated [1, 23]. Moreover, the intensities of the nerve fibres also decreased in this area of the aCSVD model ($p < 0.05$, Fig. S5, A and D), which was consistent with the sparse WMF observed from DTI in patients with aCSVD (Fig. 1, A). To explore the relationship between increased cytotoxic NK cells and demyelinated nerve fibres in white matter, we co-cultured cytotoxic NK cells isolated from participants with HAs for 4 h in vitro (Fig. 6, A). Interestingly, cytotoxic NK cells from patients with aCSVD with a high WMH burden were enriched around neural axon hillocks and disrupted the nerve fibres without myelin sheaths, which decreased the number and length of neural axons and dendrites (all $p < 0.05$, Fig. 6, A, D and E). In the co-cultured supernatants and CSF from the rat model, the increase in NEFM and decrease in NPTX2 verified nerve fibre injury (all $p < 0.05$, Fig. 6, B and C; Fig. S5, E and F). Cytotoxic NK cells from patients with aCSVD adhered to neural axon hillocks by highly expressing FLNA in vitro (Fig. 6, F) and in vivo ($p < 0.05$, Fig. S5, G and H). In vivo, cytotoxic NK cells from the aCSVD model synthesized a large number of GZMHs ($p < 0.05$, Fig. S5, I). The damaged neurons were also rich in GZMH in the SWM of the aCSVD model (Fig. S5, K and J). To further explore the mechanism by which cytotoxic NK cells disrupt nerve fibres, an inhibitor of GZMH was injected into each rat's tail vein and co-cultured with cytotoxic NK cells from patients with aCSVD, which reduced the disruption of nerve fibres (all $p < 0.05$, Fig. 6, G and H; Fig. S5, A, D and E). Additionally, phosphorylation of the death receptor (p-DR), which is involved in apoptosis, did not change in vitro or in vivo, indicating that the apoptotic pathway was not activated (Fig. 6, H and G; Fig. S5, K and J). These results demonstrated that GZMH released by cytotoxic NK cells was a key determinant of the disruption of demyelinated nerve fibres during WMH in patients with aCSVD.

Discussion

Our study establishes a previously unrecognized detrimental effect of cytotoxic NK cells in aCSVD. The data in this study suggest that cytotoxic NK cells highly express ITGB2 and bind with ICAM1 in the endothelial cells of the BBB through immune synapses, releasing CTSD and exacerbating BBB damage. After crossing the damaged BBB, cytotoxic NK cells are enriched around neural axon hillocks and disrupt nerve fibres by releasing GZMH during WMH in aCSVD patients.

Our study revealed alterations in protein levels in cytotoxic NK cells, serum and CSF from patients with aCSVD with a high WMH burden. In terms of cytotoxic NK cells, a large number of proteins were highly expressed, indicating that these cytotoxic NK cells were significantly activated. These differential proteins

accelerate cytotoxic NK cell adhesion, transformation and migration and play killing roles through immune synapses, including lysis and phagocytosis. Certain differentially upregulated proteins in serum also contributed to the migration, adhesion and immunoreactions of lymphocytes, including cytotoxic NK cells. Among them, upregulated B2M was involved in modulating the immune response, secreting [extracellular exosomes](#) and [negatively regulating neuron projection development](#) [29]. Upregulated FLNA in cytotoxic NK cells also played a role in the [negative regulation of neuron projection development](#). However, the expression of its downstream proteins were decreased in CSF, including moesin and serine/threonine-protein kinase 10 [40, 41]. Considering that all of these results were derived from existing databases through bioinformatics analysis, new components or pathways that were not in the database could not be summarized. Therefore, we speculated that cytotoxic NK cells were enriched around demyelinated neurons through the action of FLNA or B2M, but their targeted proteins need further exploration.

Previous studies models of inflammation in the CNS and acute cerebrovascular diseases have demonstrated the infiltration of NK cells through the severely damaged BBB [42, 43]. Our results revealed that the direct contact between cytotoxic NK cells and endothelial cells broke the connections between cells, such as the interactions with COL4A. Notably, we found cytotoxic NK cells expressing ITGB2 in aCSVD. ITGB2 not only is a receptor for ICAM1 in endothelial cells but also contributes to the cytotoxicity of NK cells [30, 44]. Our findings supported the pivotal role of cytotoxic NK cells in the adhesion of endothelial cells and exacerbated damage to the BBB by secreting proteases.

Until now, pathological nerve fibre damage during WMH in aCSVD has remained controversial [4, 21, 45]. Several imaging and pathological studies have demonstrated that hypoperfusion and severe demyelination exist during WMH in aCSVD [6, 23, 46, 47]. Moreover, a few imaging studies have demonstrated the impairment of neural network connective structures during WMH in patients with severe aCSVD, and certain autopsy results have confirmed that the density of demyelinated nerve fibres decreases in severe aCSVD [2, 4, 48, 49]. In this study, we showed that sparse nerve fibres were present in patients with aCSVD with WMH, and this phenomenon coexisted with demyelination in a rat model of aCSVD. Therefore, all of the above evidence confirmed that demyelinated nerve fibres were sparse during WMH in aCSVD.

Another new finding from this study was that cytotoxic NK cells disrupted demyelinated nerve fibres through GZMH in patients with aCSVD with a high WMH burden. Interestingly, after disrupting demyelinated nerve fibres, neurosomes remained. GO analysis showed that the neuronal apoptotic signalling pathway was negatively regulated. p-DR plays a role in neuronal apoptosis, including normal neurosome death and axonal pruning [50]. In our experiments, the phosphorylated DR-induced classical apoptotic pathway was not activated, and it was confirmed that the dissolution of nerve fibres was not dominated by the apoptotic pathway.

There are some shortcomings in our research. On the one hand, the sample size in these experiments was small. On the other hand, we only inhibited proteinase activity by treatment with 3,4-DCIC in cytotoxic NK

cells, and CTSD and GZMH knockout mice are needed in a follow-up study. Furthermore, the factors that induce NK cell cytotoxicity in aCSVD remain unclear. Further studies of the mechanisms are needed to solve these problems in patients with aCSVD.

Conclusion

The phenomenon of cytotoxic NK cells exacerbating BBB damage through ITGB2 and CTSD and disrupting nerve fibres by releasing GZMH during WMH in aCSVD are presented here. Our results propose a new pathological mechanism of cytotoxic NK cells to disrupt nerve fibres during WMH in aCSVD and provide a potential therapeutic target for aCSVD.

Abbreviations

aCSVD: atherosclerotic cerebral small vessel disease; ITGB2: Integrin β 2; CTSD: Cathepsin D; GZMH: Granzyme H; ICAM1: Intercellular adhesion molecule 1; COL4A: Type IV collagen; 3,4-DCIC: 3,4-Dichloro-1H-isochromen-1-one; WMH: White matter hyperintensity; PVS: Perivascular space; CMB: Cerebral microbleed; LI: Lacunar infarction; WBCs: White blood cells; NEUTs: Neutrophils; LYMPHs: Lymphocytes; MONOs: Monocytes; EOs: Eosinophils; BASOs: Basophilic granulocytes; HCY: High homocysteine; HbA1c: Glycosylated haemoglobin; TC: Total cholesterol; TGs: Triglycerides; LDL-C: Low-density lipoprotein cholesterol; HDL: High-density lipoprotein cholesterol

Declarations

Ethics approval and consent to participate

The study protocol and animal experiments were approved by the Ethical Committee of the Third Affiliated Hospital of Sun Yat-Sen University, [2021]02-219-01, No.00100486. All participants signed written informed consent forms and were informed of all procedures.

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analysed during this study are included in this published article, and its supplementary information files are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.D. formulated the study concept and design; L.T., Q.B., W.A., Z.B. and W.L. provided the clinical information and samples from the participants; C.X. and H.M. performed the animal experiments; Y.D. and L.D. completed the cell experiments and executed the remaining experiments; Y.D. wrote the manuscript; and C.W., W.L. and L.Z. edited the revised manuscript. All of the authors read and approved the final manuscript.

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Figures

Figure 1

Clinical characteristics of patients with aCSVD. (A) MRI of patients with aCSVD and controls, including T1 FLAIR, T2 FLAIR, SWI, ASL/CBF and DTI. (B) Spearman correlations of aCSVD burden, WMH burden, LI burden, PVS burden, CMB burden, brain atrophy, hypoperfusion and WMF. Number of controls = 28, number of patients with aCSVD = 32. (C) Balance and gait impairment analysis of pace, gait variation, gait symmetry, and balance in six different directions (1: frontal sagittal position, 2: posterior sagittal position, 3: left frontal position, 4: right frontal position, 5: left horizontal position, 6: right horizontal position), standing angle, standing time, sitting angle and sitting time in WMH burden 0-1 and 2-3 patients. Number of WMH burden 0-1 participants = 38, number of WMH burden 2-3 participants = 21. (D) Differences in WBCs, NEUTs, LYMPHs, MONOs, EOs, BASOs, NK cells, HCY, PLTs, HbA1c, TC, TGs, LDL-C and HDL in WMH burden 0-1 and 2-3 patients. Number of WMH burden 0-1 participants = 38, number of WMH burden 2-3 participants = 21. (E) Spearman correlations of WMH burden, hypoperfusion, WMF, NK cells, pace, gait symmetry, and sitting time. Number of WMH burden 0-1 participants = 38, number of WMH burden 2-3 participants = 21. (F) Flow cytometry of NK cells in WMH burden 0-1 and 2-3 patients. N=12. (G) Percentages of different subtypes of NK cells in WMH burden 0-1 and 2-3 patients. N=12. Data are presented as the mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Cytotoxic NK cells adhered through upregulated ITGB2 and negatively regulated neural projection development through upregulated FLNA. (A) Clustered heat map of the proteins in cytotoxic NK cells from participants with WMH burdens of 0-1 and 2-3 by proteomics analysis. (B) Cellular components of differentially expressed proteins in cytotoxic NK cells. (C) Biological processes for differentially expressed proteins in cytotoxic NK cells. (D) Reactome pathways for differentially expressed proteins in cytotoxic NK cells. (E) Molecular functions of differentially expressed proteins in cytotoxic NK cells. N=10. All corrected p-values < 0.05 displayed in these pie charts were obtained by hypergeometric tests based on

UniProt. (F) Volcano plot of the proteins in cytotoxic NK cells. N=10, FC>1.00, p<0.01. (G) Z-scores of the abundances of ITGB2, GZMH, CTSD and FLNA in cytotoxic NK cells from participants. N=10. Data are presented as the mean $\pm$ SD, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3

Proteins in serum positively regulated binding and an immune response in patients with aCSVD with a WMH burden of 2-3. (A) Clustered heat map of serum proteins from participants with WMH burdens of 0-1 and 2-3 by proteomics analysis. (B) CCs for differential proteins in serum. (C) MFs for differential proteins in serum. (D) Reactome pathways for differential proteins in serum. (E) BPs for differential proteins in CSF. N=10. All corrected p-values <0.05 displayed in these pie charts were obtained by hypergeometric tests based on the UniProt database. (F) Volcano plot of proteins in serum. N=10, FC>1.00, p<0.05. (G) Z-scores of the abundances of B2M and S1PR1 in serum from participants. N=10. Data are presented as the mean $\pm$ SD, **p<0.01. (H) Protein interaction map including CTSD, ITGB2, GZMH, CTSD, FLNA, B2M, S1PR1, CDH5, NEFM and NPTX2. The network was clustered by k-means clustering, and line colour indicates the type of interaction evidence. The maximum number of interactors shown in the 1st shell and 2nd shell were not greater than 20.

Figure 5

Cytotoxic NK cells exacerbated BBB leakage and crossed the BBB via CTSD in vitro. (A) In vitro BBB model. (B) IF results of ICAM1 (green), LR (green) and CD31 (red) in HBMECs on days 0, 2, 4 and 6. (C) Intensities of ICAM1 and LR. (D) Co-localization of cytotoxic NK cells (ITGB2, green) and HBMECs (ICAM1, red) by IF. (E) COL4A (green) after different co-culture times with cytotoxic NK cells with WMH burdens of 0-1 and WMH burdens of 2-3. (F) Intensity of COL4A in the WMH burden 0-1 and WMH burden 2-3 groups at different times. (G) Co-localization of cytotoxic NK cells (CTSD, green) and HBMECs (COL4A, red) in the control, WMH burden 0-1, WMH burden 2-3 and WMH burden 2-3 + 3,4-DCIC groups by IF. (H) Intensities of COL4A and CTSD in the four groups. (I) ECM of cytotoxic NK cells and HBMECs in the four groups. (J) Endothelial gap between HBMECs and NKISs of cytotoxic NK cells in the four groups by SEM. (K) ELISA of B2M in the supernatants of the four groups in the BBB model. (M) ELISA of S1PR1 in the supernatants of the four groups in the BBB model. N=6. Data are presented as the mean $\pm$ SD, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

Figure 6

Cytotoxic NK cells disrupted nerve fibres by releasing GZMH. (A) IF results of the nerve fibres (β 3-tubulin, TUB3, green) and cytotoxic NK cells (CD56, red) in the control, WMH burden 0-1, WMH burden 2-3 and WMH burden 2-3 + 3,4-DCIC groups. (B) ELISA of NEFM in the supernatants below the BBB model in the four groups. (C) ELISA of NPTX2 in the supernatants below the BBB model in the four groups. (D) Lengths of the neural axon and dendrites. (E) Number of neural axons and dendrites. (F) Co-localization of cytotoxic NK cells (FLNA, red) and neural axon hillocks (green) in the WMH burden 2-3 group by IF. (G) Intensities of GZMH and p-DR by IF. (H) IF results of p-DR in the neurons and GZMH in cytotoxic NK cells in the four groups. N=6. Data are presented as the mean $\pm$ SD, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

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