CFNAs of RBCs affect the release of inflammatory factors through the expression of CaMKIV in macrophages

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ABSTRACT
Background: Blood transfusions reportedly modulate the recipient’s immune system. Transfusion-related immunomodulation has been suggested as a mechanism of some adverse clinical outcomes. Extracellular nucleic acids circulate in plasma and activate relevant immune responses, but little is known about their mechanism of action in transfusion-related immunomodulation (TRIM). The aim of this study was to investigate the effects of cell-free nucleic acids (CFNAs) produced by red blood cells (RBCs) on innate immunity, especially peripheral blood mononuclear cells (PBMCs) and macrophages, and to investigate the mechanism of action.
Methods: Differentially expressed genes (DEGs) between PBMCs exposed to RBC-produced CFNA and normal PBMCs were analyzed by gene expression data combined with bioinformatics. KEGG and GO enrichment analyses were performed for the DEGs, and in vitro experiments were performed for the effects of key genes on the release of inflammatory factors from macrophages.
Results: Analysis of microarray data showed that exposure of monocytes to RBC-produced CFNAs increased the expression of genes involved in the innate immune response, including chemokines, chemokine receptors, and innate response receptors, and that calcium channel activity was highly regulated, with a key gene being CaMKIV. CaMKIV played a critical role in LPS-induced inflammatory factor release from macrophages, which was exacerbated by overexpression of the CaMKIV gene.
Conclusion: RBCs regulate the release of inflammatory factors during blood transfusion by releasing CFNAs and affecting expression of the CaMKIV gene in PBMCs or macrophages, which is a potential regulatory mechanism of blood transfusion-related immune regulation and related adverse reactions.

1. Introduction
Allogeneic RBC transfusion is a life-saving intervention. Although the benefits of such a transfusion are obvious, the immune system can be affected in patients requiring long-term RBC transfusion. Transfusion-related immunomodulation (TRIM) is regarded as a transfusion-related immune complications and can be defined as the result of changes in the immune system of transfusion recipients caused by allogeneic transfusion [1–6].

It has been reported that various substances in stored blood suspensions are involved in the abnormal function of immune cells in vitro, such as T cells and monocytes [7–11]. However, the mechanisms underlying these phenomena have not been elucidated and remain poorly understood. TRIM is thought to be a mechanism underlying some transfusion-related adverse clinical outcomes, such as infections or multiorgan dysfunction, which can lead to death. Possible mechanisms include the suppression of cytotoxic T cells and monocyte/macrophage activity and the enhancement and inhibition of interleukin-2 expression [12,13]. In contrast, the symptoms commonly noticed after transfusion suggest immune activation (e.g., hyperthermia, allergic reactions).

Multifunctional calcium/calmodulin-dependent protein kinase (CaMKI, II, IV) family members are serine/threonine kinases that are sensitive to changes in intracellular Ca$^{2+}$. Although isoforms of CaMKI and CaMKII are expressed in all mammalian cells, calcium/calmodulin-dependent protein kinase IV (CaMKIV) is present only in a subset of tissues, such as bone marrow [14]. CaMKIV is an integral component of the immune response and mediates Ca$^{2+}$-dependent macrophage (Mφ) function and the regulation of inflammation in sepsis [15].

In the present study, we first downloaded from the Gene Expression Omnibus (GEO) database microarray datasets for peripheral blood mononuclear cells (PBMCs) exposed to extracts of transfusion products. Differentially expressed genes (DEGs) were identified using the R...
package limma of the R language and according to the screening criteria. Next, KEGG enrichment and GO enrichment analyses were performed using gene set enrichment analysis (GSEA) software and the R software cluster profile package. Exposure to RBC-free DNA was associated with greater immunomodulatory-associated enrichment, as revealed by the analysis results. Subsequently, we analyzed the KEGG enrichment results for the DEG datasets using GSEA to identify the hub gene, CAMK4 (CaMKIV), and we experimentally examined the effect of CaMKIV on LPS-induced inflammatory factor release in macrophages. This work provides insights into the mechanisms of immune regulation of transfusion-related adverse reactions at the transcriptome level and proposes potential biomarkers for the diagnosis and treatment of transfusion-related adverse reactions.

2. Materials and methods

2.1. Data acquisition and processing

The gene dataset was downloaded from the publicly available GEO database based on the National Center for Biotechnology Information (NCBI). This dataset focuses on the exposure of PBMCs to cell-free nucleic acids (CFNAs) extracted from blood products eligible for transfusion.

2.2. Function annotation and gene set enrichment analysis

DEGs were identified using the limma package. GO is a community-based bioinformatics resource that includes biological processes (BP), cell components (CC), and molecular functions (MF). KEGG is a

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>ACAAGGAGAACCAAGCAACG</td>
<td>GCCGTCTTTTCAATTACACAGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCACCTACCTCCTGAAAGAAT</td>
<td>CCTCTTTGCTGTTGTCACAT</td>
</tr>
<tr>
<td>IL-12</td>
<td>ACCCTGACCACCAAGTCAA</td>
<td>TGGGCTGCGATCTCAAGAAAG</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TCGGTAACTGACTTAAGAGT</td>
<td>TTACTGGGATGCTCTTCG</td>
</tr>
<tr>
<td>iNOS</td>
<td>CGATGACCTGGTGGTTGG</td>
<td>CATAGACCTGGGCTTGGCA</td>
</tr>
<tr>
<td>CD80</td>
<td>GGTATGAGCCCAATCCTACT</td>
<td>GCAATGATCAGCCAAAGTAG</td>
</tr>
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<td>CD86</td>
<td>AACTGTCCTAAGGTTGGAAGA</td>
<td>AAGCATTGCTACGATGGCGAT</td>
</tr>
<tr>
<td>CCL5</td>
<td>AAGGAGAAAGGTGAGACTAAC</td>
<td>AGGACAGAGCAAGCAGAAAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCACCGTCAAGGCTGAAAC</td>
<td>ATGGTGTTGAAGACCGCAAGT</td>
</tr>
</tbody>
</table>

Fig. 1. A. Principal component analysis plot of the samples. B. Heatmap of hierarchical clustering of samples. C. Comparison of gene differences between the CFNA (RBC) group and the none group in samples. Red points represent upregulated genes (i.e. Fold change > 0.444 and a corrected P-value of < 0.05). Blue points represent downregulated genes (i.e. Fold change < -0.444 and a corrected P-value of < 0.05). Black points represent genes with no significant difference in expression. Differential expression of mRNA of CFNA (RBC) group and the none group in samples.
knowledge base for the systematic analysis of gene functions that links genomic information with higher-order functional information. GO and KEGG enrichment results were generated by the R packages "ggplot2," "enrichplot," "clusterProfiler," and "GOplot" for the purpose of analysis. GSEA was performed to compare the samples by GSEA software (version 4.0.3). Functional annotations with a P value < 0.05 were considered statistically significant.

2.3. Blood collection

Whole blood (400 ml) anticoagulated with 63 ml of citrate phosphate dextrose was collected from 20 healthy volunteer donors (ISFX21012166, ISFX21012488) after obtaining informed consent under a protocol approved by General Hospital of Northern Theater Command. RBCs were isolated from the whole blood of 10 healthy donors. After centrifugation (4000g, 20 min), RBCs were suspended in 100 ml of nutrient solution (saline adenine glucose mannitol, Fresenius Kabi) and leukofiltered (14–41 min). PBMCs were extracted from the other 10 healthy donors.

2.4. PBMC isolation

PBMCs were separated by density gradient centrifugation as described elsewhere [16]. After separation, the cells were washed with phosphate-buffered saline containing 1 μM EDTA and then maintained in medium (RPMI-1640, 10% FBS, 100 U/ml penicillin/streptomycin) at 37 °C in a humidified environment with 5% CO₂.

2.5. Extraction of CFNA from RBCs

RBCs were first centrifuged at 1200g for 10 min. The supernatant from this first step was then centrifuged at 16,000g for 10 min. CFNAs were extracted from 4 ml of the supernatant from this second centrifugation using a QIAamp circulating nucleic acid kit from Qiagen (55114, Qiagen, Germany), according to the manufacturer’s instructions. To remove any interfering components, the optional carrier RNA molecule was not introduced during the extraction protocol[17].

2.6. Human THP-1-cell culture

THP-1 cells (human acute monocytic leukemia cell line) from the American Type Culture Collection (ATCC, USA) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 0.05 mM 2-mercaptoethanol at 37 °C in a 5% CO₂ incubator. After 72 h, macrophages were obtained from the THP-1 cells in RPMI-1640 medium (Gibco by Life Technologies, Grand Island, NY) supplemented with PMA (HY-18739, 80 nM, Med Chem Express, China).

2.7. Oligonucleotide and plasmid transfection

The targets of CaMKIV-coding sequences were subcloned into pcDNA3.1 (Sangon Biotech, China) to construct pcDNA expression vectors. Si-CaMKIV was purchased from Oringeng (USA), and CaMKIV (CaMKIV group) or si-CaMKIV (si-CaMKIV group), and transfections were performed using Lipofectamine 2000 according to the manufacturer’s instructions. Empty plasmid–transfected (empty group) and nontransfected (NC group) cells were used as controls. The cells were harvested at 24 h after transfection for testing.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of inflammatory factors, including IL-1, IL-6, and IFN-γ, were determined by a commercial ELISA kit (E-EL-H0088c, E-EL-H0102c, E-EL-H0150c, ElabScience, China). All experiments were
conducted according to the manufacturer’s protocol.

2.9. Quantitative real-time PCR (qRT–PCR)

Total RNA containing miRNA in whole blood was extracted by a miRcute miRNA Isolation kit (TIANGEN, DP501, China). An abundant amount (1 μg) of RNA was reverse-transcribed by a reverse transcription kit (Takara, RR047A, China). qRT-PCR was performed using gene-specific primers (Life Technology) and a 7500ABI biological system instrument. The relative expression levels of mRNAs were calculated using...

Fig. 3. The GSEA enrichment analysis of the DEGs. A. Barplot of go enrichment analysis. B. Gene regulation of GO terms. C. Chord plot of relationships between key genes and GO terms.
the 2−ΔΔCT method. All qRT-PCRs were performed in triplicate. The primer sequences used in the real-time PCR analysis are shown in Table 1.

2.10. Statistical analysis

R software (version 3.5.2) was used for statistical analysis of the gene dataset section in this study. Statistical significance was set to P < 0.05. The experimental data were analyzed by GraphPad Prism 7.0 (GraphPad Software, CA, USA). All experimental data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Student's t test and one-way ANOVA were used to determine the statistical significance of differences between two or more groups. Differences were considered statistically significant when the P value was less than 0.05.

3. Results

3.1. Expression of DEGs

We first performed a principal component analysis (PCA) of the dataset, which showed good discrimination between the samples of different groups (Fig. 1 A). A heatmap representation with hierarchical clustering of the 50 differentially regulated genes common among all samples is presented in Fig. 1 B. The level of expression of each mRNA is included (red: high; blue: low). The results are presented after the hierarchical clustering of genes and samples according to the similarity of their profiles. As expected, NONE conditions clustered together and showed a different profile than CFNA/LPS. In contrast, the CFNA (RBC) group showed highly specific clustering compared to the other groups, which was significantly different from that of the NONE group. We next investigated the differential expression of genes between the CFNA (RBC) group and the control group. We performed a differential analysis of the gene expression matrix comparing the differences in gene expression between the CFNA (RBC) and NONE groups. Only a few genes were regulated, with 15 upregulated and 9 downregulated genes.

3.2. Bioinformatics analysis of genes and functional pathways

Next, GO enrichment analysis of DEGs was performed using the R software clusterprofiler package. GO enrichment analysis of DEGs showed that the enrichments in the samples mainly included negative regulation of innate immune responses, negative regulation of immune system processes, negative regulation of immune effector processes, negative regulation of immune responses, negative regulation of humoral immune responses, calcium channel inhibitor activity, calcium-dependent protein serine/threonine kinase activity, calcium-dependent protein kinase activity, calmodulin-dependent protein kinase activity, chemokine binding, and calcium channel regulator activity. GO enrichment mainly focused on immune regulation and calcium ion channel-related pathways, explaining the differential mechanisms of regulated genes between samples in terms of cellular composition and function. Key regulatory genes correlated with these biological processes were plotted in a chord plot (Fig. 2).

We performed KEGG enrichment analysis on DEGs using GSEA, and the enrichment pathways included complement and coagulation cascades, cAMP signaling pathway, longevity-regulating pathway, apelin signaling pathway, and osteoclast differentiation (Fig. AC). Among these pathways, the complement and coagulation cascade pathway was an immune regulation-related pathway (Fig. 3AB). In particular, we found that the CaMKIV (CAMK4) gene regulates multiple enrichment pathways (Table 2).

3.3. Effect of CaMKIV on the release of inflammatory factors from macrophages

First, the transcriptional activity of CaMKIV was observed by qRT-PCR under two conditions: exposure to CNFAs extracted from RBC and culture alone in PBMCs/macrophages. Compared to PBMCs/macrophages without exposed to CNFAs, PBMCs/macrophages exposure to CNFAs had significantly higher CaMKIV transcriptional activity (Fig. 4 A). We transfected human THP-1 cells with CaMKIV overexpression plasmids and siRNAs and then induced the cells to differentiate into macrophages using PMA; finally, macrophages were induced with LPS. The cells and culture medium were subjected to qR–PCR and ELISA, respectively. The overexpression of CaMKIV promoted macrophage M1 polarization, whereas the inhibition of CaMKIV reduced M1 polarization (Fig. 4 B). In parallel, compared with the control group, macrophages overexpressing CaMKIV showed IL-6/IL-1 transcriptional activation and more protein secretion after LPS induction. The siRNA interference group showed the opposite results. However, IFN-γ had little impact (Fig. 4 CD).

4. Discussion

With the development of emerging medical technologies, the application scenarios and frequency of RBC transfusion are increasing [18]. Studies have shown that allogeneic RBC infusion can improve the prognosis of recipients by improving blood perfusion and changing the intestinal microbiome [2,17,19–24]. However, allogeneic RBC transfusion may also expose recipients to the possibility of associated adverse effects as well as poor survival.

To date, several researchers have reported that soluble biological mediators (cytokines, growth factors, and CNFAs) and subcellular components (extracellular vesicles) present in the supernatants of blood products can influence the biological behavior of immune cells and tumor cells in vitro [3,4,12,25].

Human plasma has been reported to contain many free extracellular...
DNA molecules [26]. CFNA in human plasma mainly consists of double-stranded DNA molecules of low molecular weight, which are fragmented into short and long fragments. The exact origin of CFNAs remains unclear, but it is believed that they are released by altered cells after cleavage by endonucleases [17].

CNFAs (secreted by different cell types) reportedly play a variety of roles in physiological processes such as cell development or differentiation and pathophysiological processes such as carcinogenesis, metastasis, and immune regulation via different mechanisms [27–30]. However, the role and mechanism of CNFAs (RBCs derived) in transfusion-related immune regulation remain to be clearly elucidated. Here, using the data mining of the public GEO database, we examined the PBMC/macrophage gene expression data for RBCs exposed to CNFAs. An analysis of the differential gene expression data along with the results of enrichment analyses indicated that CNFA significantly affected CaMKIV in PBMCs/macrophages.

Several studies have reported the effects of Ca²⁺/CAM signaling on immune and inflammatory responses, and anti-inflammatory CAM...
antagonists are clinically used to prevent inflammatory diseases [31-33]. CaMKIV is a downstream protein of the Ca²⁺/CAMK signaling cascade. To be active, CaMKIV requires binding to Ca²⁺/CAMK [34]. The important role of CaMKIV during inflammation in peripheral tissues has also been explored. For example, CaMKIV controls osteoclast and dendritic cell differentiation and survival during bone inflammation via a TLR signaling mechanism [34-36]. Collectively, these reports suggest interplay between CaMKIV and local inflammatory events in both immune cells and nonimmune cells. Our study is the first to link TRIM to CaMKIV through CNFAs of RBCs. Our results indicate that CaMKIV transcriptional activity in PBMCs/macrophages is clearly elevated after exposure to CNFAs of RBCs (Fig. 4 A).

Shi et al. [34] reported a higher proportion of M1 macrophages in CD45+ cells isolated from tissues of injured mice treated with recombinant CaMKIV, suggesting that CaMKIV helps maintain the proinflammatory phenotype of infiltrating macrophages. By exploring the expression of selected pro- and anti-inflammatory cytokines as well as CaMKIV, we further confirmed that high CaMKIV levels in vivo stimulate significant production of IL-1β, IL-6, MCP1, and MCP3 in injured tissues. Similarly, a study by G.singer et al. showed that inhibiting the activity of the Ca²⁺ activation-dependent K⁺ channel KCA3.1 can reduce the release of inflammatory factors from macrophages [37]. Our study also demonstrated that CaMKIV expression, which promotes inflammation, does so by promoting macrophage M1 polarization, whereas the inhibition of CaMKIV reduces both inflammatory release as well as M1 polarization (Fig. 4 B-D).

TRIM may cause transfusion-related adverse effects mediated by inflammation, such as transfusion-related acute lung injury. Our study demonstrated that TRIM might be caused by the effect of CNFAs of RBCs, which promote CaMKIV expression in PBMCs/macrophages and in turn promote macrophage M1 polarization as well as inflammatory factor release.

5. Conclusion

Our study demonstrates the mechanism of action of TRIM and provides a rationale and novel target for the prediction and treatment of transfusion-related adverse effects.

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None.

CRediT authorship contribution statement

Jingrui Zhang designed and performed experiments, analyzed data and wrote the manuscript. Dan Zhang performed the experiments and analysis of data. Jing Zhao performed bioinformatic analysis. All authors read and approved the final manuscript.

Conflicts of Interest

The authors have disclosed no conflicts of interest.

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