Abstract. Natural killer (NK) cells are a group of large granular lymphocytes that play an important regulatory role in innate immunity and adaptive immunity. Immune-related pancytopenia (IRP) is a type of pancytopenia resulting from bone marrow hematopoietic cells that were destroyed or suppressed by auto-antibodies. The specific mechanism of IRP is not clear. In the present study, it was identified that the percentage of NK cells in peripheral blood lymphocytes was decreased in patients with IRP. Subsequently, high purity NK cells were extracted from 6 untreated patients with IRP using the immunomagnetic beads sorting, magnetic-activated cell-sorting method, which were then cultured then in RPMI-1640 medium containing 20% FBS. NK cell expansion agents, with or without recombinant interleukin (IL)-15, were used to amplify high-purity NK cells on the basis of recombinant IL-2. Expression of the activated receptors NK2-d type II integral membrane protein (NK2-D) and natural killer cell p46-related protein (NKp46), and the inhibitory receptors CD158a and NK2-a/nKG2-B type II integral membrane protein (NK2A), in CD56+ NK cells were detected by flow cytometry before and after cell culture. It was observed that treatment with an NK cell expansion agent combined with the stimulation of recombinant IL-2 and recombinant IL-15 could increase the number whilst maintaining the purity of NK cells. There were no significant changes in the expression of NK2-D, NKp46, NK2A and CD158a in patients with IRP before and after NK cell culture. This new amplification method lays a foundation for clinical NK cell immunotherapy and anti-tumor applications.

Introduction

Immune-related pancytopenia (IRP) is a type of hemocyto- penia exhibiting the following features: i) Hemocytopenia or pancytopenia with a normal or higher percentage of reticuloocytes and/or neutrophils; ii) hyperplasia-bone marrow (BM) with a higher percentage of nucleated erythroid cells in the sterna, with erythroblastic islands that are easily observed; iii) good responses to corticosteroids or high-dose intravenous immunoglobulin (IVig); iv) exclusion of other primary and secondary hemocytopenias disorders; and v) positive result from the bone marrow mononuclear cells (BMMNC)-Coombs test (1-3). There may also be a humoral immune mechanism involved in the pathogenesis of IRP.

Natural killer (NK) cells are a group of large granule lymphocytes (4) and different from T and B lymphocytes, NK cells can kill tumor cells and virus-infected cells. They are core to innate immunity and are adaptive immune regulators, playing important anti-infection and anti-tumor roles, and eliminating exogenous cells (5,6). In recent years, with the development of immunology and molecular biology technology, biological cell immunotherapy has become the fourth treatment option for cancer after surgery, radiotherapy and chemotherapy. In view of the good clinical prospects of NK cell immunotherapy for cancer, adoptive immunotherapy of NK cells has gradually become a focus of research in recent years. As NK cells only account for 10-15% of peripheral blood lymphocytes (7), it is difficult to meet the needs of large-scale experiments and clinical cancer immunotherapy. Therefore, in vitro amplification methods used to obtain a large number of high-purity NK cells have become an important focus. Researchers have investigated various amplification methods, such as isolating NK cells from peripheral blood mononuclear cells (PBMCs) using magnetic beads sorting (magnetic-activated cell-sorting method; MACS), using recombinant interleukin (IL)-2 (rIL-2), recombinant IL-12 (rIL-12), recombinant IL-15 (rIL-15) and recombinant IL-18 (rIL-18), or different combinations of these
factors, or using irradiated lethal K562 cells or HFWT cells to stimulate the expansion of NK cells in PBMCs (8-10). The present study aimed to further analyze changes in the quantity of NK cells in patients with IRP. Different culture techniques were also examined for the efficient amplification of human NK cells to obtain an optimized method for inducing and amplifying NK cells, in order to provide a foundation for the clinical application of NK cell immunotherapy.

Materials and methods

Patients. Immune-related pancytopenia (IRP) is a type of hemocytopenia exhibiting the following features: i) Hemocytopenia or pancytopenia with a normal or higher percentage of reticulocytes and/or neutrophils; ii) hyperplasia-bone marrow (BM) with a higher percentage of nucleated erythroid cells in sternum, with erythroblastic islands (EIs) that are easily observed; iii) good responses to corticosteroids or high-dose intravenous immunoglobulin (IV Ig); iv) exclusion of other primary and secondary hemocytopenia disorders; and v) positive result by BMNC-Coombs test (1). In total, 44 patients with IRP who were successively diagnosed with IRP were enrolled at The Hematology Department of General Hospital between September 2016 and July 2017. In the present study, patients were categorized into two groups, IRP and remission IRP (R-IRP) groups. The present study was approved by The Ethics Committee of the Hospital China. The present study was conducted according to the Declaration of Helsinki, and informed written consent was obtained from all healthy controls and all patients or their guardians. According to the protocols of the Ethic Committee for the Conduct of Human Research, the diagnostic method of IRP was established, as previously described (2). A total of 23 healthy volunteers served as the normal control group, with 11 males and 12 females, with a median age of 28 (25-52) years. The clinical characteristics of all patients are presented in Table I.

Flow cytometry. Fresh EDTA anticoagulant peripheral blood samples (2 ml in total) from each participant were placed in 6 marked fluid centrifuge tubes (200 µl/tube). To each tube 10 µl PerCP-CD3 (1:20; cat. no. 552851) and 10 µl phycoerythrin (PE)-VIO770-CD56 (1:20; cat. no. 560842) were added, and 10 µl PE-IgG was added to detection tube 1. Following 15 min incubation at 20°C in the dark, hemolysis and washing, CD3-CD56+ cells were detected by flow cytometry to determine the proportion of lymphocytes and the percentages of NKG2-A/NKG2-B type II integral membrane protein (NKG2A; 1:20; cat. no. 517016477), NKG2-D type II integral membrane protein (NKG2D; 1:20; cat. no. 4318869), CD158a (1:20; cat. no. 27718) and natural killer cell p46-related protein (NKP46; 1:20; cat. no. 4255803), and the average fluorescence intensity of CD3-CD56+ cells. All antibodies were purchased from BD Pharmingen; BD Biosciences. At least 10^5-10^6 cells were acquired and analyzed by FACSCalibur flow cytometer (BD Biosciences) and Cell Quest software version 6.0 (BD Biosciences).

Isolation and purification of NK cells. In total, 5 ml fresh human peripheral blood was obtained from each patient with IRP. CD3-CD56+ cells were freshly purified using NK cell MACS (Miltenyi Biotec, Inc.), according to the manufacturer's protocol. Subsequently, cells were detected using a multiparameter flow cytometer (BD Biosciences) and analyzed using Cell Quest software (version 3.1; BD Biosciences). The suspension containing NK cells was collected, the cells were counted and the purity of the cells was measured by flow cytometry. The NK cells were purified by MACS (Fig. 1). After centrifugation (300 x g for 5 min at 20°C, Tianjin Hao Yang Biological Products Technology Co., Ltd.), cells were precipitated and RPMI-1640 culture medium (Beijing Solarbio Science & Technology Co., Ltd.) containing 20% FBS (Beijing Solarbio Science & Technology Co., Ltd.) was used to adjust the cell concentration to 5x10^4 cells/ml.

Cultivation and amplification of NK cells after purification. The purity of NK cells following MACS reached 90%. Cells were suspended in RPMI-1640 culture medium containing 20% FBS and the cell concentration was adjusted to 5x10^4/ml. rIL-15 (Miltenyi Biotec, Inc.; 20 ng/ml) was added to the culture medium containing rIL-2 (Miltenyi Biotec, Inc.; 500 IU/ml). NK cell amplifiers (5 µl/ml/10^6 NK cells) was added to each well of the 24-well culture plate (3524; Corning, Inc.). Cells were cultured at a constant temperature of 37°C in a 5% CO2 incubator. The number of cells was then counted under a confocal microscope (magnification, x100; Olympus Corporation) every 3 days for the first 6 days, and fresh medium and cytokines were added. Subsequently, the number of cells was counted under a microscope every 2 days for the last 4 days, and fresh medium and cytokines were added. Cells were collected after 10 days of culture. A confocal microscope (magnification, x100; Olympus Corporation) was used. In total, 10 µl cells were evenly placed on the cell counting board (Watson Bio Lab). Then, the number of cells in 16 visual fields were observed. If 10 µl were taken out of 1 ml, the number of cells in 16 visual fields was multiplied by 10^4, and the final number of cells was calculated according to the above counting method.

Statistical analysis. The experiments were repeated 3 times. Data were analyzed using Prism (version 7; GraphPad Software, Inc.) and SPSS for Windows (version 24.0; IBM Corp.). For three independent groups, one-way ANOVA was used with the S-N-K method used as a post hoc test. For skewed distribution, the median is presented and was analyzed using the rank-sum test. Correlation analysis was performed using Pearson's correlation test. Non-normal distribution data are represented as the median (four quantile interval). P<0.05 was used to indicate a statistically significant difference.

Results

Percentage of NK cells in lymphocytes of newly diagnosed patients with IRP is significantly decreased. The percentage of NK cells in newly diagnosed patients with IRP was significantly lower than the patients in remission (P<0.01) and in healthy controls (P<0.0001). Furthermore, the percentage of NK cells in patients in remission was significantly lower than that in healthy controls (P=0.049; P<0.05; Fig. 2).
Changes in purity before and after NK cell culture. Before MACS, the proportion of NK cells in lymphocytes accounted for 15.86%. The purity of NK cells was ≤94.14% before culture, but the purity of NK cells reached 96.17% after culture (Fig. 1).

Effect of different cytokine combinations on the human NK cell growth curve. NK cells were induced by RPMI-1640 culture medium containing 20% FBS and different cytokines. The growth rate was slow for the first 3 days; rIL-2+rIL-15 increased by ~3.5 times, and the increase of rIL-2+rIL-15+NK cell amplifiers was ~5 times. On day 6, the two groups of cells entered the logarithmic growth phase, and the number of cells increased rapidly. At day 10, the rIL-2+rIL-15 control group and the rIL-2+rIL-15+NK cell amplifiers group were 28 times and 52 times, respectively, reaching the maximum amplification of NK cells (Tables II and III). The highest multiple of amplification of the rIL-2+rIL-15 control group and the rIL-2+rIL-15+NK cell amplifying agent experimental group are shown in Fig. 3. Overall, the experimental group of the rIL-2+rIL-15+NK cell expansion agent was the best for the purification of NK cells (Fig. 3).

Changes to antibodies expressed by NK cells before and after cell culture. There were no marked changes in the expression of the activated receptor NKG2D and NKP46 in patients with IRP before and after NK cell culture. Similarly, there were no marked changes in the expression of the inhibitory receptors CD158a and NKG2A in CD56+NK cells before and after NK cell culture (Figs. 4 and 5).

Correlation between the proportion of NK cells in the lymphocytes of the patients and clinical indicators. Correlations between the proportion of NK cells and clinical and immune indices (CD4+/CD8+ and CD5+CD19+/CD19+) were analyzed. The proportion of NK cells in the lymphocytes of patients was positively correlated with the hemoglobin count \( r=0.3873; p<0.001 \) and the platelet count \( r=0.3655; p=0.0188 \). The more severe the anemia and the lower the platelet value, the lower the percentage of NK cells in patients with IRP. Therefore, the proportion of NK cells is associated with the severity of the disease.

Discussion

Following years of research, IRP is now recognized as an immune-related disease. In our previous clinical studies (11,12), patients with IRP were treated by experimental administration of corticosteroids and/or high-dose γ globulin; after 3 months of follow-up, all patients had responded to treatment. Therefore, it was suggested that the incidence of this disease might be related to abnormal humoral immunity in patients (11,12). A previous in-depth study (11) of its pathogenic mechanism showed that immunorelated haemocytopenia (IRP or BMMNC-Coombs test-positive hemocytopenia) is an autoimmune disease where autoantibodies target BM hematopoietic cells.

NK cells are a group of large granular lymphocytes (13) that play an important regulatory role in innate immunity and adaptive immunity, through antibody-dependent cell-mediated cytotoxicity and NK cytotoxicity (14,15). NK cells are an important part of the innate immune system, particularly with respect to anti-viral immunity and scavenging tumor cells, and can directly kill tumor cells without specific antigen

Table I. Clinical and laboratory indexes of newly diagnosed patients with IRP and patients with IRP in remission.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>IRP, n=23</th>
<th>R-IRP, n=21</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>12/11</td>
<td>11/10</td>
<td>0.656</td>
</tr>
<tr>
<td>Age</td>
<td>46 (8-72)</td>
<td>40 (8-68)</td>
<td>0.158</td>
</tr>
<tr>
<td>HB, g/l</td>
<td>77.5 (52-131)</td>
<td>106.5 (100-156)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>RBC, 10^12/l</td>
<td>2.43 (1.33-3.90)</td>
<td>3.35 (2.1-5.01)</td>
<td>0.002*</td>
</tr>
<tr>
<td>Plt, 10^9/l</td>
<td>38.5 (13-80)</td>
<td>195.5 (110-300)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>WBC, 10^9/l</td>
<td>3.52 (1.40-9.86)</td>
<td>4.56 (2.19-17.58)</td>
<td>0.109</td>
</tr>
<tr>
<td>ANC, 10^9/l</td>
<td>49.9 (17.2-90.4)</td>
<td>50.8 (18.9-90.7)</td>
<td>0.944</td>
</tr>
<tr>
<td>Ret, %</td>
<td>1.97 (0.66-3.97)</td>
<td>2.46 (0.72-6.37)</td>
<td>0.205</td>
</tr>
<tr>
<td>LYMPH, %</td>
<td>38.85 (7.10-76.6)</td>
<td>44.5 (5.20-72.2)</td>
<td>0.901</td>
</tr>
<tr>
<td>LDH, U/l</td>
<td>202 (125-331)</td>
<td>209 (157-378)</td>
<td>0.086</td>
</tr>
<tr>
<td>IgG, g/l</td>
<td>1,350 (581-1800)</td>
<td>1,050 (702-1830)</td>
<td>0.337</td>
</tr>
<tr>
<td>IgM, g/l</td>
<td>98.95 (47.5-260)</td>
<td>117 (35.8-256)</td>
<td>0.735</td>
</tr>
<tr>
<td>Complement C3, g/l</td>
<td>97.1 (46.4-131)</td>
<td>101 (60.6-170)</td>
<td>0.257</td>
</tr>
<tr>
<td>Complement C4, g/l</td>
<td>22.25 (9.02-35.6)</td>
<td>27.5 (14.4-36.1)</td>
<td>0.386</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>1.66 (0.5-5.57)</td>
<td>1.42 (0.55-3.16)</td>
<td>0.563</td>
</tr>
<tr>
<td>CD5+CD19+/CD19+</td>
<td>14.67 (0.39-28)</td>
<td>6.98 (1.44-26.54)</td>
<td>0.205</td>
</tr>
</tbody>
</table>

Enumeration data are presented as the median (range). *P<0.05. IRP, immune related pancytopenia; R, remission; HB, hemoglobin; RBC, red blood cells; Plt, platelet; WBC, white blood cell; ANC, absolute neutrophil count; Ret, granulofilocyte; LDH, lactate dehydrogenase.
recognition (16, 17). In the present study, it was identified that the proportion of NK cells in lymphocytes in newly diagnosed patients with IRP was significantly decreased. It was hypothesized that the proportion of NK cells may be related to the immunopathogenesis of IRP. Therefore, NK cells were isolated from patients with IRP, and amplification and functional studies *in vitro* were performed.

Isolated NK cells with high-purity cannot meet the needs of further clinical studies as they only account for 10-15% of peripheral blood lymphocytes (7). Therefore, it is particularly important to rapidly and efficiently amplify large numbers of high-purity NK cells. Therefore, researchers have studied different amplification methods for NK cells. Carlens *et al* (18) applied CD3 monoclonal antibodies and phytohemagglutinin to stimulate PBMCs, then added rh-2 to amplify cells; it was possible to obtain a large number of lymphocytes (~193 times) *in vitro*, but the purity of NK cells was not sufficiently high. Luhm *et al* (19) used feeding cells and cytokines to amplify the purified NK cells obtained via MACS; the amplification efficiency and NK cell purity were higher, but the feeding cells must be involved in the culture system. Klingemann and Martinson (20) and Li *et al* (21) tried to establish a simple and efficient NK cell expansion system *in vitro*, but only produced cells of low purity and with low amplification efficiency. In the present study, NK cell expansion agents were added and promising results were identified. In our previous study, anti-CD3 was used to stimulate NK cells (22); however, it was mainly to measure the function of NK cells, and the aim of the present study was to primarily measure the number of NK cells. It is undeniable that these studies met the needs of scientific research to a certain extent; however, it was not possible to implement these methods in clinical application. Based on the experiments mentioned above, high-purity NK cells were isolated from the peripheral blood of 6 patients with IRP by MACS in the present study. Subsequently, an NK cell culture system was used to expand cells *in vitro*, which markedly reduced the cost of sorting NK cells.

In the present study, an NK cell expansion system was designed, including RPMI-1640 culture medium containing 20% FBS, rhL-2 and rhL-15, with or without an NK cell expansion reagent. Li *et al* (23) demonstrated that using rhL-2 combined with rhL-15 stimulation can generate NK cells with high-purity, strong cytotoxic activity and high amplification efficiency. Wang *et al* (24) expanded NK cells *in vitro* using four methods, of which the combination of rhL-2 and rhL-15 was the simplest, and there was no significant difference in amplification efficiency with other combinations. IL-2 is a cytokine of the chemokine family, able to stimulate the proliferation,

Figure 1. Purity of NK cells in 6 patients before and after cell culture. Purity of NK cells in (A) patient 1, (B) patient 2, (C) patient 3, (D) patient 4, (E) patient 5 and (F) patient 6 (a) before and (b) after culture by flow cytometry. NK, natural killer.
killing activity, and cytokine production of NK cells (25). The biological effect of IL-15 is similar to that of IL-2, and it has synergistic effect with IL-2 in stimulating the proliferation and activation of NK cells (26). Therefore, the amplification effects on NK cells of rIL-2 and rIL-15 combined with or without NK cell expansion agents were compared.

To observe the amplification effect of NK cell expansion agents on NK cells, the culture system was divided into two
groups according to different cytokine combinations: The rIL-2+rIL-15 group and the rIL-2+rIL-15+NK cell amplification group. The results demonstrated that after 10 days of culture, compared with the control group, the aforementioned two groups can promote the expansion of NK cells in vitro, and maintain the high-purity of NK cells after amplification. An NK cell expansion agent was added to the experimental group, and the proliferation effect was markedly improved compared with the rIL-2+rIL-15 alone group. The highest amplification rate was \( x_{28} \) in the group with the added expansion agent.

NKG2D and NKp46 (27,28) are the activated receptors on NK cells, while NKG2A and CD158a are the inhibitory receptors on CD56+NK cells; they all play an important role in activating the killing function of NK cells. The expression of the activated receptors NKG2D and NKp46 on NK cells did not differ before and after cell culture. Similarly, there was no difference in the expression of NKG2A and CD158a on NK cells. This suggested that the function of NK cells after amplification was similar to before culture. These results suggested that an NK cell expansion agent can markedly promote the expansion of NK cells in vitro, and can markedly enhance the efficiency of amplification with rIL-2+rIL-15.

The immune status of patients with IRP was impaired, including a decrease in NK cell numbers and suppression of their protective function (29). Therefore, NK cells from patients with IRP were screened for amplification instead of those from healthy people. It may be safer and more effective for expanded NK cells to be transfused back into patients. In the present study, NK cells have not been transfused back into patients; further experiments are required in the future. In summary, it was possible to obtain many NK cells with high-purity and a high proliferation rate from a small number of high-purity NK cells by MACS sorting. The NK cells were successfully amplified from incubation in a culture system containing rIL-2 (500 U/ml), rIL-15 (20 ng/ml), an NK cell expansion agent and RPMI-1640

Table II. Quantitative changes after rIL-2+rIL-15 stimulation of NK cells.

<table>
<thead>
<tr>
<th>Day</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.6x10^4</td>
<td>2.2x10^4</td>
<td>6.6x10^4</td>
<td>1.6x10^4</td>
<td>9.3x10^4</td>
<td>3.3x10^4</td>
</tr>
<tr>
<td>3</td>
<td>3.36x10^5</td>
<td>5.17x10^4</td>
<td>3.597x10^5</td>
<td>7.74x10^4</td>
<td>5.784x10^5</td>
<td>1.716x10^5</td>
</tr>
<tr>
<td>6</td>
<td>1.07x10^6</td>
<td>2.09x10^5</td>
<td>5.676x10^5</td>
<td>1.484x10^5</td>
<td>3.162x10^6</td>
<td>7.128x10^6</td>
</tr>
<tr>
<td>8</td>
<td>2.55x10^6</td>
<td>2.99x10^5</td>
<td>1.12x10^6</td>
<td>2.784x10^5</td>
<td>4.22x10^6</td>
<td>1.237x10^6</td>
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<tr>
<td>10</td>
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<td>3.762x10^5</td>
<td>1.35x10^6</td>
<td>3.68x10^6</td>
<td>4.836x10^6</td>
<td>1.56x10^6</td>
</tr>
</tbody>
</table>

r, recombinant; IL, interleukin; NK, natural killer.

Table III. Quantitative changes after rIL-2+rIL-15+NK cell expansion agent stimulation of NK cells.

<table>
<thead>
<tr>
<th>Day</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.6x10^4</td>
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<td>3</td>
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<td>6.468x10^4</td>
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<td>7.062x10^5</td>
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<tr>
<td>10</td>
<td>1.98x10^7</td>
<td>5.192x10^5</td>
<td>1.036x10^6</td>
<td>4.384x10^5</td>
<td>2.6x10^6</td>
<td>9.075x10^5</td>
</tr>
</tbody>
</table>

r, recombinant; IL, interleukin; NK, natural killer.

Figure 3. Growth multiples of NK cells growth rate trends in IL-2 and IL-15 with and without NK cell amplifiers. NK, natural killer; IL, interleukin.
(containing 20% FBS). Most importantly, this culture amplification method is simple and cost-effective, providing a good foundation for NK cells in the use of tumor and adoptive immunotherapy.

Figure 4. Expression of activated receptors, NKG2D and NKP46 on NK cells of 6 patients with immune-related pancytopenia before and after cell culture. Expression of NKG2D on NK cells (A) before and (B) after cell culture. Expression of NKP46 on NK cells (C) before and (D) after cell culture. NK, natural killer; PE, phycoerythrin; NKG2D, NKG2-D type II integral membrane protein; NKP46, natural killer cell p46-related protein.
Acknowledgements

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Funding

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

CL, LL and TC conceived and designed the study. YL, BL and SD performed the experiments and were major contributors in writing the manuscript. ZS analyzed and interpreted the data. RF conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study complied with the Declaration of Helsinki and was approved by The Ethics Committee of Tianjin Medical University General Hospital. Written informed consent was obtained from all healthy controls and all patients or their guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References