

**ORIGINAL ARTICLE**

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## **In vitro Evaluation of CMV Specific CD8+T Cells Function in CMV+ Colorectal Cancer Patients Compared to Healthy Controls**

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### **ABSTRACT**

The oncogenic role of human cytomegalovirus (HCMV) has been recently shown in different cancers like colorectal cancer (CRC). According to the recent immunotherapy approach to target the CMV-expressing tumor cells, we investigated the CMV peptide-stimulated CD8+T cells functions in CRC patients compared to healthy individuals.

All sixteen patients and seven controls were CMV seropositive. Blood samples were obtained from patients without chemotherapy and radiotherapy before surgery. Cytotoxic CD8+ T cells were generated using 14-day culture of PBMCs in the presences of CMV peptide epitopes and rhIL-2. In addition to the supernatant evaluations for TNF- $\alpha$  and IFN- $\gamma$ , the functionality of CD8+ T cells was examined by detecting CD107a and intracellular IFN- $\gamma$  using flow cytometry. CMV DNA was detected in tissues by Real Time PCR.

CMV DNA was found in 31% of tumor tissues, while it was not seen in the adjacent non-tumor tissues. There was a close association between CMV in tumor tissue and tumor grade. Surface expression of CD107a and intracellular IFN- $\gamma$  in CMV-stimulated CD8+T cells and the level of IFN- $\gamma$  production in patient and control groups increased significantly after culture. The number of functions increased in patients ( $p < 0.05$ ) and healthy individuals after culture. Following stimulation, expressions of CD107a and intracellular IFN- $\gamma$  were elevated in tumor CMV positive patients while the TNF- $\alpha$  secretion was decreased.

In vitro stimulation of PBMC in the presence of CMV peptide epitopes and IL-2 can be an applicable method to generate cytotoxic CD8+ T cells in CRC patients for future T cell therapy.

**Keywords:** Cytomegalovirus; CD107a; Interferon gamma (IFN- $\gamma$ ); Solid tumour

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## INTRODUCTION

Colorectal cancer (CRC) is known as the third leading cause of cancer-related deaths in the world.<sup>1</sup> Despite increasing the longevity, routine treatment protocols including chemotherapy, radiotherapy, and surgery have severe side effects and lack the specific targeting of tumor cells.<sup>2</sup> Therefore, new alternative immunotherapy approaches have been recently planned to potentiate the different arms of the immune system.<sup>3,4</sup> It is critical to find the most immunogenic tumor specific antigens (TSAs) in designing the immunotherapy protocols.<sup>5,6</sup> TSAs contain different groups of antigens which are not routinely expressed on normal cells, including viral antigens, mutated antigens, and cancer-testis antigens.<sup>7</sup> It has been speculated that about 12-20% of human cancers worldwide are caused by oncogenic viruses worldwide considering that 80% of them occurs in developing countries.<sup>8</sup> Oncoviruses have been programmed to cause mutations and irregular proliferation of host cells by integrating into the chromosomal DNA.<sup>9</sup> Therefore, they are relevant targets as TSA for cancer immunotherapy.<sup>10</sup> Human Cytomegalovirus (CMV) is a beta herpes virus which has not been considered as an oncogenic virus up to now.<sup>11</sup> According to the reports, more than ninety percent of Iranians and 50-90% of people around the world have been infected with CMV.<sup>12,13</sup> This virus is never completely removed from the body. It remains in latency phase and can be reactivated in various immune compromised conditions.<sup>14,15</sup>

A potential oncomodulatory function of CMV has been reported following the detection of CMV peptides in tumor tissues, but not adjacent non-tumor tissues of seropositive cancer patients suffered from colon cancer, malignant glioma, breast cancer, salivary gland cancer, and prostate adjacent non-tumor.<sup>2,16</sup> Furthermore, a negative correlation between the level of CMV infection in tumor tissue and the overall survival of the glioblastoma patients has been shown by Rahbar and et al.<sup>17</sup> An in vitro study on CMV+ glioblastoma patients indicated a significant increase in CMV specific CD8+ T cell function of PBMCs stimulated by CMV peptides plus IL-2.<sup>18</sup> The researchers also found that four times injections of CMV specific CD8+ T cells to a patient with glioblastoma led to a relative improvement in clinical parameters like neuronal function.<sup>19,20</sup>

Among the studies on the relationship between CMV and colorectal cancer, a few of them have shown a lower disease-free survival rate in patients with CMV-positive than CMV-negative tumours.<sup>21,22</sup> The most relative reason is the ability of the virus to induce the angiogenesis, resistance to apoptosis, cellular invasion, and metastasis.<sup>23</sup> In contrast, there are some studies on negative association between CMV in tumor tissues and progression of colorectal cancer which may be related to the use of different detecting techniques with various levels of specificity and sensitivity.<sup>24-27</sup>

The results of recent investigations suggest that adoptive T cell therapy using CMV-specific CD8+ T cells can be a potential tool for future immunotherapy of cancer patients by targeting the CMV-infected tumor cells. Although, a comparative in vitro study on the induction of CMV-specific CD8+ T cells in CRC CMV+ patients and the healthy controls is lacking. In this study, we have evaluated the presence of CMV DNA in the tumor and the adjacent non-tumor tissues of CRC patients. Then, CMV-specific CD8+ T cells from CMV-seropositive CRC patients and healthy controls were generated and their potential cytotoxic activity was investigated.

## MATERIALS AND METHODS

### Samples

Sixteen CMV+ colorectal cancer patients (mean age 58 years, 5 males and 11 females) who were referred to the department of surgery in Imam Khomeini Hospital and seven CMV+ healthy controls (mean age 32 years, 1 male and 7 females) were included in this study. This study was evaluated and approved by the ethics committee of Semnan University of Medical Sciences (N. IR.SEMUMS.REC.1394.102). The informed consent forms were signed by participants.

The patients were newly diagnosed with out chemotherapy and radiotherapy treatments. Before beginning the surgical resections, blood samples were drawn from the patients. A small part of adjacent non-tumor tissues (100-400mg depending on the tumor size) was obtained during the operation and immediately frozen at -70°C until analysis. CMV IgG/IgM seropositivity was obtained from documents related to the routine check-up of the patients and controls. Seropositive IgG+ patients and healthy controls considered as previously CMV infected individuals.

### CMV Peptide Epitopes

Quanti FERON-CMV Kit which applies for monitoring CMV specific T cell immune responses was used to cover all of the necessary CMV peptide epitopes. This kit includes CMV and MIT tubes. The CMV tube contains 22 types of common HLA class I restricted CMV-peptide epitopes (pooled CD8+ T-cell epitopes); so there was not essential for HLA-typing of the participants. MIT tube contains phytohemagglutinin (T-cell mitogen as positive control). CMV-peptide epitopes were coated on the wall of the tubes and dissolved by adding warm RPMI-1640 to the tubes and rotating for 30 minutes.

### In vitro Expansion of CMV-specific CD8+ T Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by layering of diluted blood onto Ficoll-hypaque (Inno-Train, Germany) using density gradient centrifugation. Approximately  $14 \times 10^6$  cells were obtained from 8-10 mL peripheral blood;  $4 \times 10^6$  cells were used for initial phenotyping of the cells before stimulation and the remaining cells were divided into two groups as the stimulator and responder cells. In order to pulse the antigen presenting cells with CMV peptides, the stimulator cells were cultured in the presences of 1  $\mu\text{g}/\text{mL}$  of pooled CD8+ T-cell CMV epitopes (obtained as described above) overnight. Then they were 30G irradiated and co-cultured with the respondent cells at a ratio of 2:1 in complete media containing RPMI-1640, 10% fetal calf serum, 1% penicillin-streptomycin at 37°C for 14 days. PBMCs with PHA and without stimulation were considered as positive and negative controls, respectively. Recombinant human interleukin-2 (rhIL-2, 20 U/mL) was added on days 3, 7 and 10. Re-stimulation with CMV (1  $\mu\text{g}/\text{mL}$ ) and PHA (1  $\mu\text{g}/\text{mL}$ ) was done on days 7, 10 and immediately before assessing of CD8+ T cells. The supernatant was collected for evaluation of IFN- $\gamma$  and TNF- $\alpha$  using the ELISA method.

### Evaluation of CMV-specific CD8+ T Cells Function

CD8+ T cells function was further investigated by examining cell surface expression of CD107a (LAMP-1) as a hallmark of degranulating T lymphocytes before and after coculturing with responder cells. Intracellular IFN- $\gamma$  assessment was also performed.

### Flow Cytometry

For evaluating the CD8+T cells before and after 14-

day co-culturing between the CMV-stimulated (irradiated) and unstimulated PBMCs, cells were harvested and stained for 30 minutes in incubator 37°C, 5% Co<sub>2</sub> with 1  $\mu\text{g}/\text{mL}$  CMV peptides, 50 ng/mL phorbol myristate acetate (PMA), 500 ng/mL Ionomycin and Anti-CD107a PE-CY5. Thereafter, 5  $\mu\text{g}/\text{mL}$  monensin (BD, GolgiStop) and 5  $\mu\text{g}/\text{mL}$  brefeldin A were added to the flowcytometry tubes. Following 6-hour incubation, cells were washed once in FACS buffer and stained with anti-CD8a FITC. For intracellular staining of IFN- $\gamma$ , cells were fixed and permeabilized according to the manufacturer's instructions and incubated with anti-IFN $\gamma$  PE, for 30 minutes (all materials except monensin from eBioscience, USA). Mouse IgG1 PE-FITC (Dako) antibodies were used as an isotype control. Stained cells were resuspended in 500  $\mu\text{L}$  FACS buffer with 1% paraformaldehyde and run by BD Accuri C6 Plus. Data were analyzed using FLOWJO software (10.4 version, USA).

### Enzyme-linked Immunosorbent Assay (ELISA)

ELISA technique was used to assess IFN- $\gamma$  (Human TNF alpha ELISA Ready-SET-Go! Kit) and TNF- $\alpha$  cytokines (Human IFN gamma ELISA Ready-SET-Go! Kit) in supernatant before and after culture in the presence of CMV peptide epitopes and PBMC alone (as negative control). The sensitivity of detection was 4 pg/mL for TNF- $\alpha$  and 4 pg/mL for IFN- $\gamma$  (both from eBioscience).

### Real Time PCR for Detection of CMV DNA in Tumor and Adjacent Non-tumor Tissues

After DNA extraction (Qiagen kit), Real Time PCR was done to amplify IE (Immediate-Early) gene (translating to IE protein), using artus CMV RGQ MDx Kit containing primers and fluorogenic probe for IE gene detection. PCR was carried out with initial denaturation at 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 10s and annealing-extension at 60°C for 1 min and the presence of CMV in the tissue was investigated by analyzing the Real Time PCR data.

### Statistical Analysis

The statistical analysis of data was performed using SPSS version 17 (Inc, Chicago, IL, USA). Following determining the normality of data using Kolmogorov-Smirnov, the appropriate statistical tests (Parametric or

Non-parametric) were used. To compare a quantitative variable in two independent groups, Independent sample t-test or Mann-Whitney were applied. To assess the difference of a quantitative variable in more than 2 independent groups, one-way ANOVA was used. Considering the distribution of data, Paired t-test or Wilcoxon rank test were used to compare the level of a quantitative variable in a dependent group before and after treatment. Pearson or Spearman test was performed to assess the correlation of two quantitative variables. Chi-Square test was utilized to evaluate the relationship between two qualitative variables. To draw the graphs, GraphPad Prism 6 (Graphpad Software Inc., La Jolla, CA, USA) was used.

**RESULTS**

**Demographic Features of CRC Patients**

All 16 CRC patients and 7 healthy controls were seropositive. It means that the patients and controls had not an active CMV infection. Demographic features and the stage of cancer have been shown in Table 1.

Adenocarcinoma was confirmed in the patients without any signs of metastases.

**Detection of CMV DNA in Tumor Tissue of Some CRC Patients**

CMV DNA was detected in tumor tissue of 5 (31%) CRC patients, while the virus was not found in any of the adjacent non-neoplastic specimens. Of five positive samples, four samples were in moderately differentiated grade (intermediate grade) and one sample was in poorly differentiated grade (high grade). Although there was a close association between CMV presence in tumor tissue and tumor grade, it was not statistically significant ( $p=0.05$ ). Moreover, there was no correlation between the stage of tumor and detection of CMV DNA in tumor tissues.

**Activation of CMV-specific CD8+T Cells**

PBMC from CMV-seropositive CRC patients and healthy individuals were stimulated with 22 types of common HLA class I restricted CMV-peptide epitopes or with PHA (as the positive control).

**Table 1. Demographic features of colorectal cancer (CRC) patients**

Patients No	Sex	Age	CMV IgG	T*	N*	M*	AJCC stage**	Grade
1	Female	86	+	Not available	Not available	Not available	Not available	Not available
2	Female	46	+	Tis	N0	M0	0	Well differentiated
3	Female	45	+	T4b	N0	M0	2C	Moderately differentiated
4	Female	43	+	T2	N0	M0	1	Well differentiated
5	Female	77	+	T1	N0	M0	1	Moderately differentiated
6	Female	57	+	T3	N1b	M0	3B	Poorly differentiated
7	Female	54	+	Not available	Not available	Not available	Not available	Not available
8	Female	61	+	T3	N0	M0	2A	Moderately differentiated
9	Male	55	+	T3	N1b	M0	3B	Well differentiated
10	Female	71	+	T3	N0	M0	2A	Well differentiated
11	Male	36	+	T3	N0	M0	2A	Well differentiated
12	Female	78	+	T4b	N1a	M0	3C	Moderately differentiated
13	Male	49	+	T3	N2b	M0	3C	Well differentiated
14	Male	39	+	T3	N1b	M0	3B	Poorly differentiated
15	Male	63	+	T3	N0	M0	2A	Moderately differentiated
16	Female	76	+	T1	N1	M0	3A	Moderately differentiated

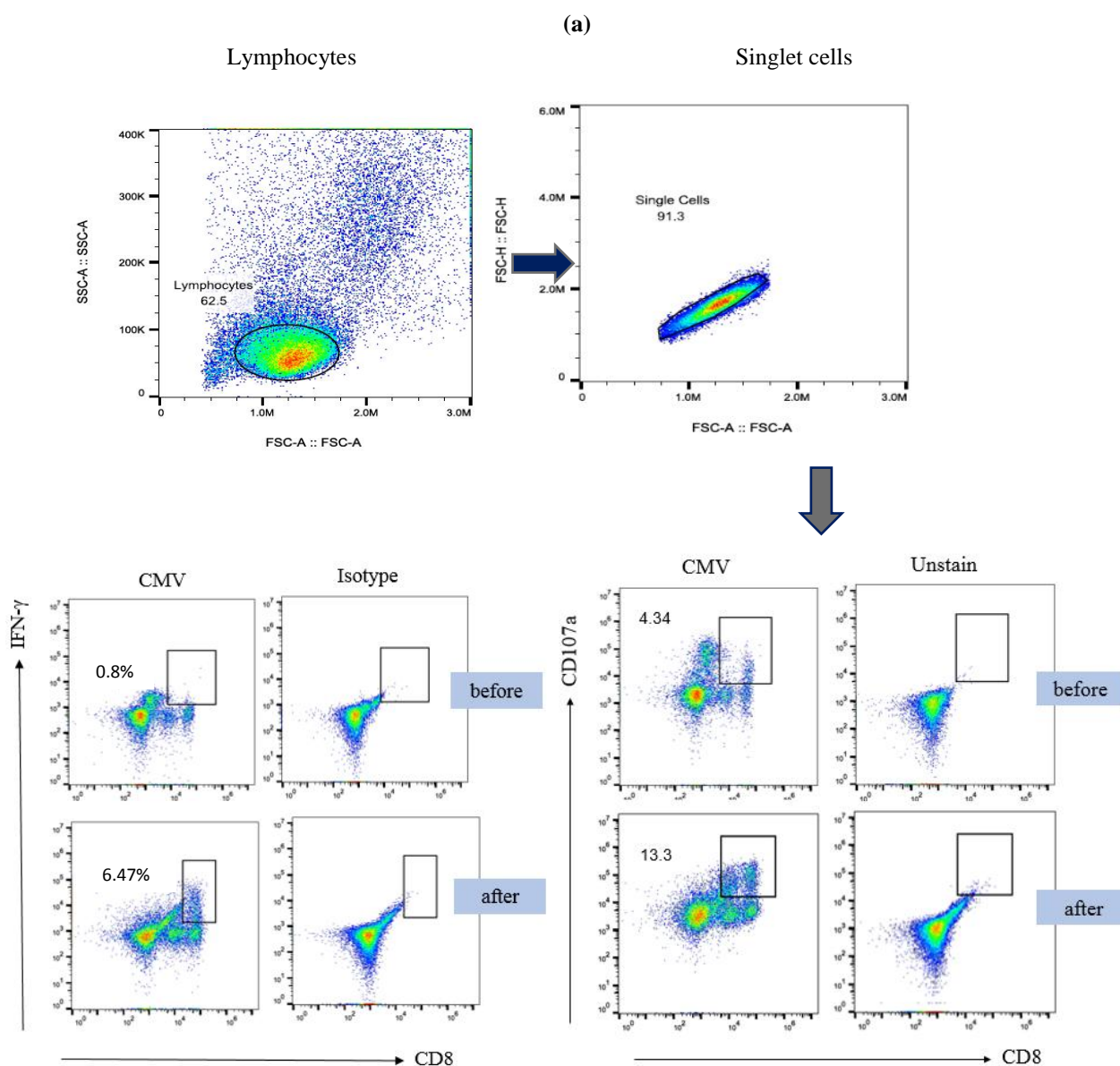
\*TNM staging for colorectal cancer: Primary tumor (T), Regional lymph node (N), Distant metastasis (M)

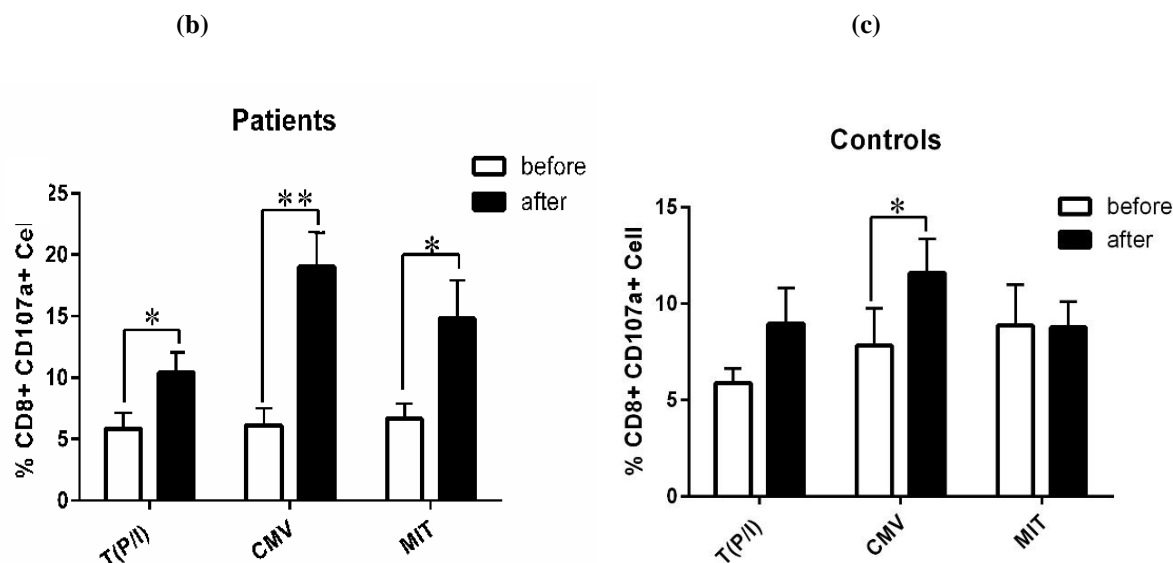
\*\*AJCC (American Joint Committee on Cancer)

## In vitro CMV Specific CD8+T Cells in CMV+ Colorectal Cancer

Cells in the medium were considered as a negative control. The function of CD8+ T cells was investigated by detecting the cell surface expression of CD107a and intracellular IFN- $\gamma$  using flow cytometry before and after 14-day culture. As explained in the method section, the cells of stimulated and non-stimulated groups were exposed to PMA+Ionomycin (P/I) for 6 hours. It was done for stimulating the cytokine production and cytotoxicity function (CD107a expression). Representative flow cytometry data of one patient has been shown in Figure 1a.

In CRC patient group, the expression of CD107a was significantly increased in CD8+ T cells after culture in all three groups of CMV peptides, PHA and no stimulation ( $p=0.001$ ,  $p=0.029$ , and  $p=0.042$ , respectively). A potentiating effect of CMV peptides on cytotoxicity function of CD8+T cells was observed (Figure 1b). In healthy subjects, a significant increase in CD107a expression was detected only in CMV-stimulated CD8+ T cells after culture ( $p=0.037$ , Figure 1c).





**Figure 1.** Flow cytometry analysis and examination of CD107a expression on CD8+ T cells

(a) Representative flow cytometry data of CMV-stimulated CD8+ T cells before and after culture in one CRC patient. Lymphocytes were identified from singlet cell. Dot blots show double staining of CD8 T cells for CD107a and IFN- $\gamma$  in CMV-stimulated cells and isotype control or unstained cells.

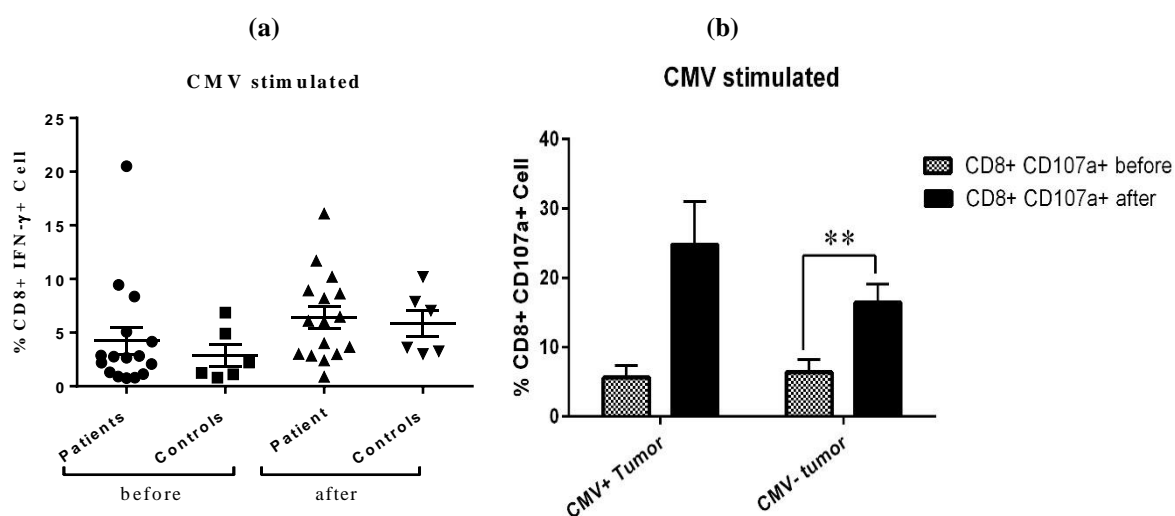
Expression of CD107a before and after culture in CD8+ T cells generated from PBMCs was compared among cells stimulated with CMV peptides, phytohaemagglutinin-A (MIT) and unstimulated cells in (b) patients and (c) healthy controls. After stimulation, cells were exposed to PMA+Ionomycin (P/I) for 6 hours and then stained for anti-CD107a PE-CY5 antibody. Thereafter, cells were stained using anti-CD8a FITC and anti-IFN $\gamma$  PE after adding monensin and brefeldin A. Mouse IgG1 PE-FITC antibody was used as an isotype control. Paired t-test was used for T (P/I) and MIT group and Wilcoxon rank test was applied for the CMV group to compare the level of quantitative variables before and after treatment. One-way ANOVA test was used for statistical analysis between the groups.

All experiments were done in duplicate. Data b & c in this panel represents the Mean $\pm$ SEM. (\* indicating  $p < 0.05$  and \*\*  $p < 0.01$ ).

Although CD107a expression following 14-day culture in the presence of CMV peptides was higher in CRC patient than healthy individuals, this difference was not statistically significant (Figure 2a). Comparative analysis of CD107a expression between the patients with CMV positive and negative tumors showed a remarkable surge in both groups, but that was significant only in tumor CMV negative patients (CMV negative tumor  $p = 0.004$ , CMV positive tumor  $p = 0.05$ ) (Figure 2b).

Considering the intracellular cytokine assay, the percentage of IFN- $\gamma$  positive CD8+T cells in CMV-stimulated cells was increased after culture in CRC patients and healthy controls but the difference was not significant (Figure 3a, Figure 3b). While a comparison of Mean Fluorescence Intensity (MFI) in CMV

stimulated CD8+T cells of CRC patients and healthy controls revealed a significant rise of IFN- $\gamma$  production after culture in both groups (CRC patients  $p = 0.01$ , healthy controls  $p = 0.01$ ) (Figure 3c). There was no change in IFN- $\gamma$  expression of mitogen-stimulated cells and cells without stimulation after culture. In addition, we could find a significant increase in IFN- $\gamma$  expression among tumor CMV positive patients after culture ( $p = 0.031$ ), but it was unchanged in CMV negative tumor patients. In addition, higher IFN- $\gamma$  expression in CD8+ T cells of patients with CMV positive tumors than CMV negative tumors after culture was detected ( $p = 0.007$ , Figure 3d). Percentage of CD8+IFN- $\gamma$ + cells did not change before and after culture in both CRC patients and healthy controls.



**Figure 2.** Comparison of CD107a expression between colorectal cancer (CRC) patients and healthy subjects and also between tumor CMV positive and negative CRC patients before and after culture

(a) Comparison of CD107a expression between CMV-stimulated CD8+ T cells of 16 CRC patients and 7 healthy subjects before and after culture (Mann-Whitney U test). (b) Analysis of CD107a expression in CMV-stimulated CD8+ T cells of tumor CMV positive and negative CRC patients before and after culture (Independent sample t-test). All experiments were done in duplicate. Data in this panel represents the Mean±SEM. (\*\* indicating  $p < 0.01$ ).

#### Measurement of IFN- $\gamma$ and TNF- $\alpha$ Production by ELISA Technique

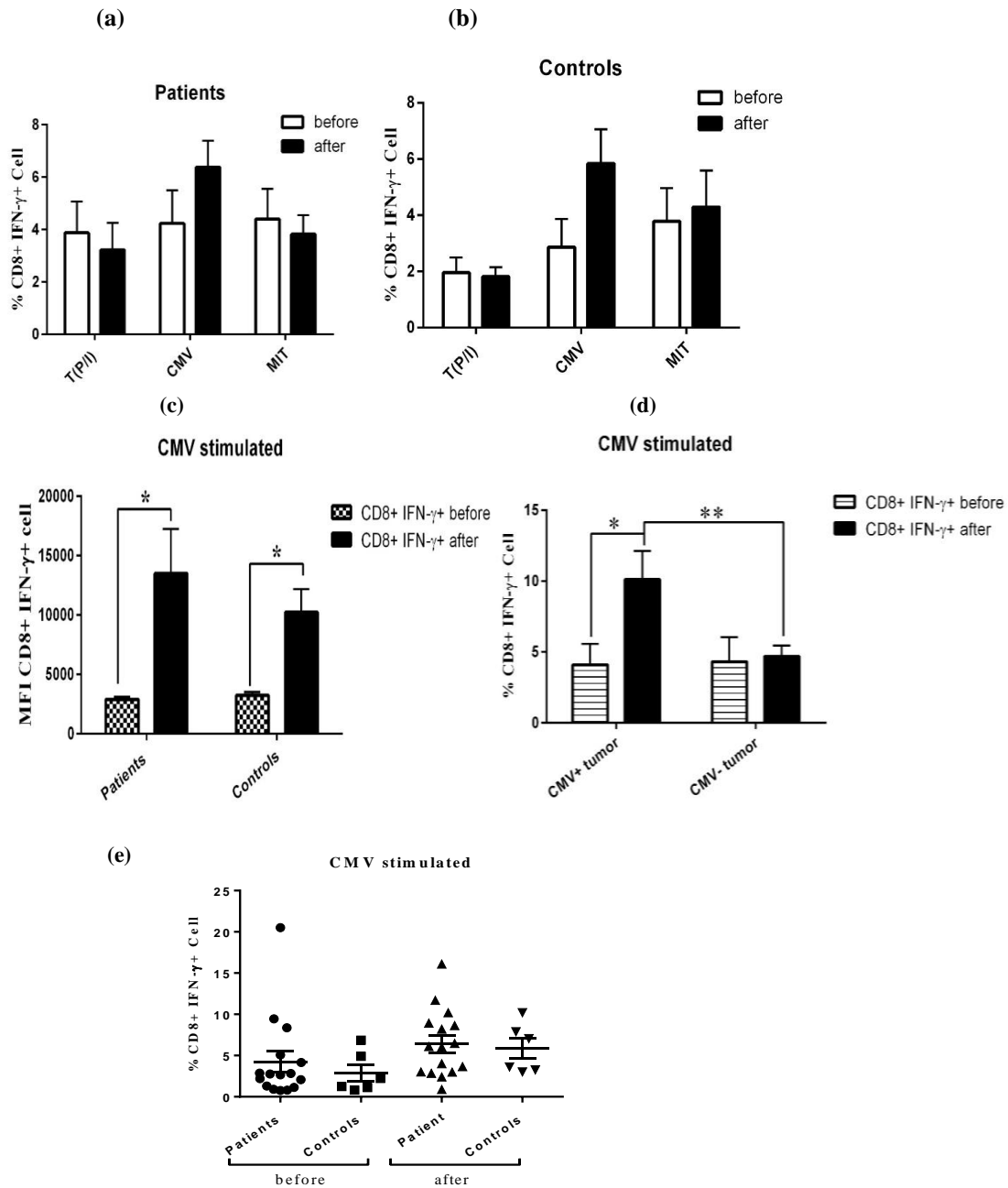
The level of IFN- $\gamma$  production in supernatants increased significantly after culture in both patients and controls' CMV-stimulated ( $p=0.001$ ,  $p=0.00$ ) and non-stimulated cells ( $p=0.001$ ,  $p=0.003$ ). Although a higher level of IFN- $\gamma$  was seen in CMV-stimulated compared to non-stimulated cells before culture in both patient and control subjects, this change was significant only in the patient groups ( $p=0.01$ ) (Figure 4a, 4b). There was no difference in IFN- $\gamma$  production between patient and control groups before and after culture (Figure 4c). Evaluation of this cytokine in CMV-stimulated cells between tumor CMV positive and negative patients showed a non-significant improvement in cytokine production after culture in both groups (Figure 4d).

TNF $\alpha$  secretion was also investigated in cell supernatant using ELISA method. Analysis of results showed that there was no difference in the level of TNF- $\alpha$  between stimulated and non-stimulated cells before and after culture in both patient and control groups (Figure 5a). The level of TNF- $\alpha$  from CMV-stimulated cells before culture in patients with CMV positive tumor was higher than those with CMV

negative, although this difference was not significant (Figure 5b).

#### Poly-functional CD8+T Cell Analysis

The CD8+ T cells with the ability of CD107a expression and intracellular IFN- $\gamma$  production upon CMV peptide stimulation were considered as poly-functional cells. Thus, a number of the CD8+ T cells with a simultaneous dual capacity was compared before and after culture in both patients and healthy individuals. The poly-functionality of CMV-stimulated CD8+ T cells was increased after culture in both patients and controls groups individually, however, it was noticeable in CRC patients ( $p=0.001$ ). There is no significant difference between the two groups regarding CMV stimulated CD8+ T cells to function before and after culture (Figure 6). To summarize the results of this study, a schematic figure has been shown (Figure 7).



**Figure 3. Intracellular IFN- $\gamma$  in CD8+ T cells.**

(a) Comparison of intracellular IFN- $\gamma$  expression before and after 14-day culture in colorectal cancer (CRC) patients' CD8+ T cells which was stimulated by CMV or phytohaemagglutinin-A (MIT) and without peptide stimulation (only stimulated with P/I). PMA+Ionomycin(P/I) was added to each of the 3 groups as co-stimulator for 6 hours (Paired t-test). (b) Comparison of intracellular IFN- $\gamma$  expression before and after culture in healthy controls CD8+ T cells which was stimulated by CMV or phytohaemagglutinin-A (MIT) and without peptide stimulation (only stimulated with P/I). PMA+Ionomycin(P/I) was added to each of the 3 groups as co-stimulator for 6 hours (Paired t-test). (c) Representative IFN- $\gamma$  MFI in CMV-stimulated CD8+ T cells before and after culture in CRC patients and healthy controls (Wilcoxon rank test). (d) Comparison of intracellular IFN- $\gamma$  expression in CMV-stimulated CD8+ T cells of tumor CMV positive and negative CRC patients before and after culture. (e) Comparison of CD8+T IFN- $\gamma$ + cells in CRC patients and healthy controls before and after culture. All experiments were done in duplicate. Data in this panel represents the Mean $\pm$ SEM. (\* indicating  $p < 0.05$  and \*\*  $p < 0.01$ ).



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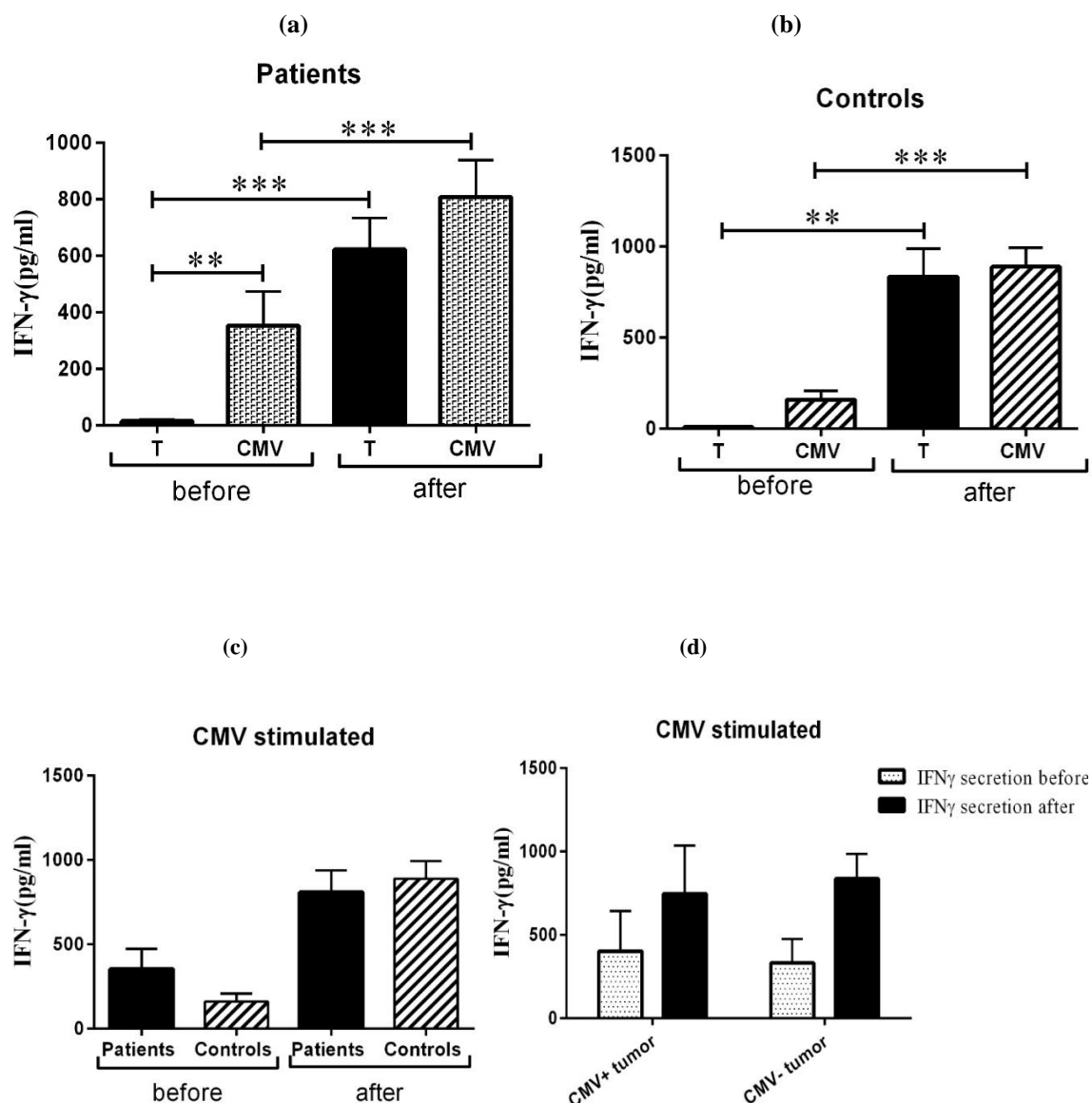
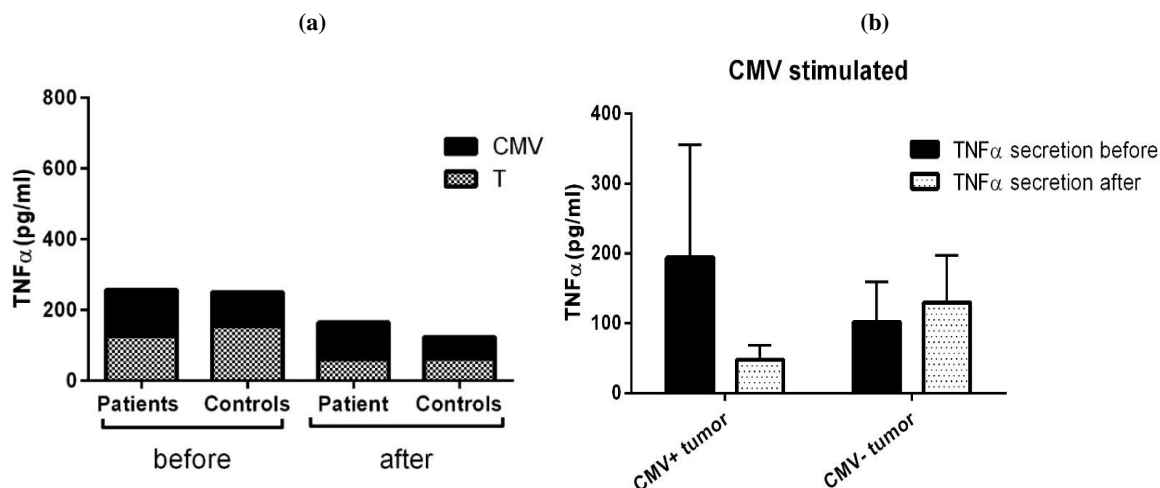


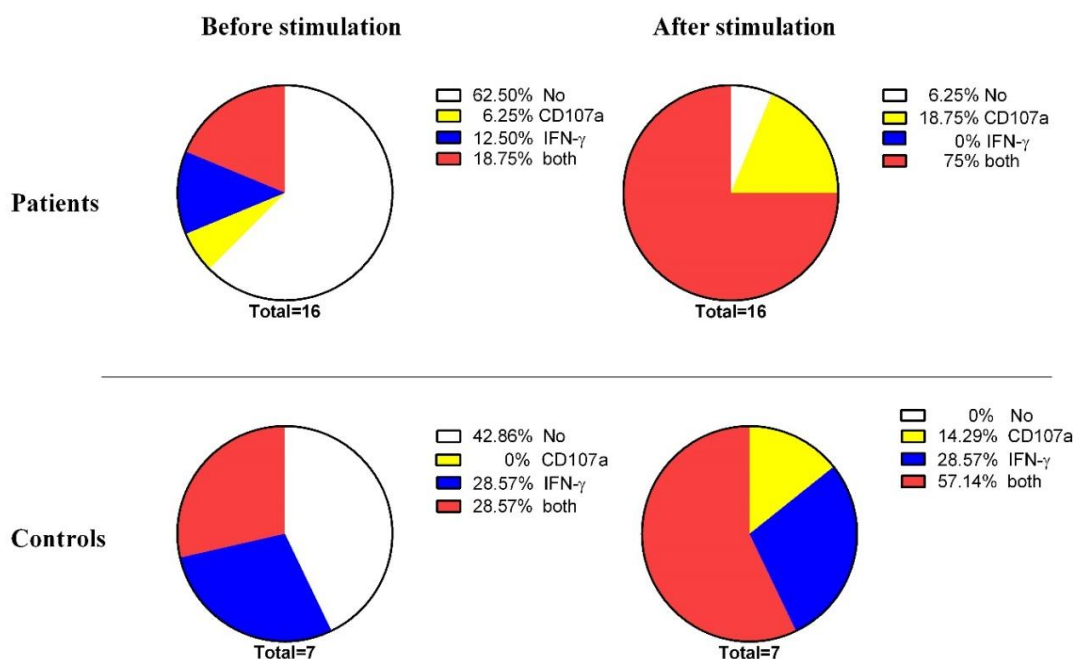
Figure 4. Analysis of IFN- $\gamma$  secretion (measured by ELISA technique) in cells supernatant of CMV-stimulated and non-stimulated cells in colorectal cancer (CRC) patients and healthy controls before and after culture

(a) Comparison of IFN- $\gamma$  secretion in cell supernatant of CMV-stimulated and non-stimulated cells (T) of CRC patients before and after 14-day culture in the presence of IL-2 cytokine. (b) Analysis of IFN- $\gamma$  secretion level in cells supernatant of CMV-stimulated and non-stimulated cells (T) of healthy controls before and after 14-day culture in the presence of IL-2 cytokine. (c) Comparison of IFN- $\gamma$  secretion in cells supernatant of CMV-stimulated cells between patient and control groups before and after 14-day culture in the presence of IL-2 cytokine. (d) A difference shown the between tumor CMV positive and negative CRC patients in terms of IFN- $\gamma$  secretion upon CMV-stimulation before and after 14-day culture in the presence of IL-2 cytokine. For analysis of non-parametric data, we used the Mann-Whitney U test for independent groups and Wilcoxon rank test for dependent groups. All experiments were done in duplicate. Data in this panel represents the Mean $\pm$ SEM (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ )



**Figure 5.** TNF- $\alpha$  secretion using ELISA technique in cells supernatant of CMV-stimulated and non-stimulated cells in colorectal cancer (CRC) patients and healthy controls before and after culture

(a) Comparison of TNF- $\alpha$  secretion in cells supernatant of CMV-stimulated and non-stimulated cells (T) of CRC patients and healthy controls before and after 14-day culture in the presence of IL-2 cytokine. (b) It was shown difference between CRC patients with CMV positive and negative tumours in terms of TNF- $\alpha$  secretion upon CMV-stimulation before and after 14-day culture in the presence of IL-2 cytokine. All experiments were done in duplicate. Data in graph a represents mean and in graph b represents the Mean $\pm$ SEM.



**Figure 6.** Polyfunctional CD8+T cell analysis upon CMV-stimulation before and after culture in colorectal cancer (CRC) patients and healthy controls

Upon exposure to CMV peptide epitopes, the number of CD8+T cells function (CD107a, intracellular IFN- $\gamma$ ) was determined in each patient and healthy control before and after culture. each pie chart represents the percentage of patients or healthy controls whose CD8+ T cells have no function (No) and have one functions (CD107a or intracellular IFN- $\gamma$  expression) or have co-expression of CD107a and IFN- $\gamma$  (both). Chi-Square test was used for data analysis of this panel.

## In vitro CMV Specific CD8+T Cells in CMV+ Colorectal Cancer

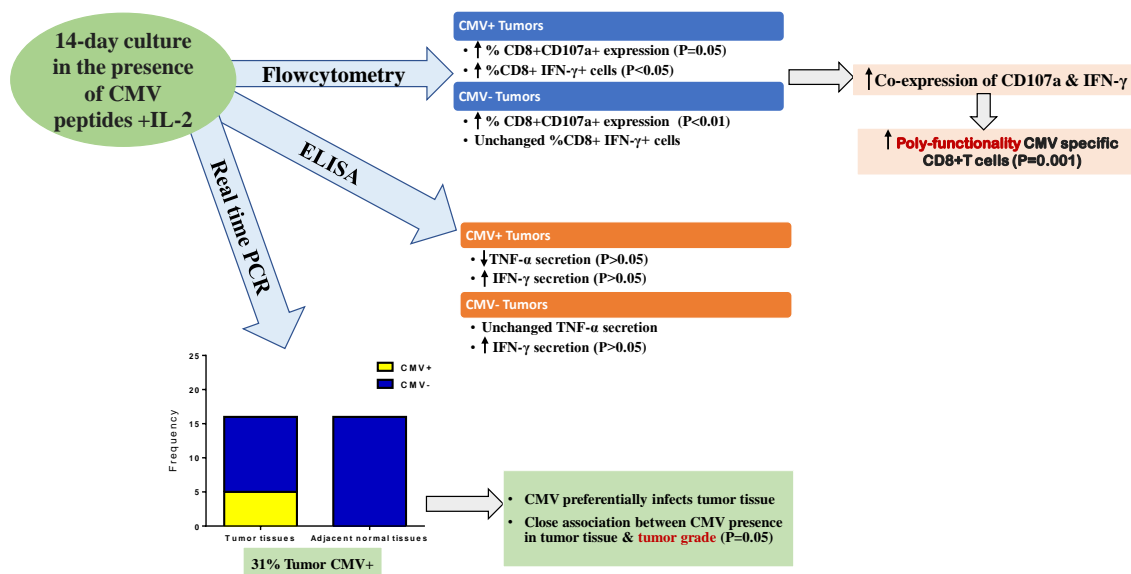


Figure 7. Schematic procedure of CMV peptide-specific CD8+ T cells

## DISCUSSION

In this study, we applied real time PCR method using sequence-specific TaqMan Probe to detect CMV in freshly frozen tissues. It was done based on previously published papers suggesting PCR technique instead of or alongside the in situ hybridization (ISH) and immunohistochemistry (IHC) methods for detection of CMV in tumor tissues.<sup>26,27</sup> In addition, we decided to use fresh samples based on degradative effects of formalin on DNA (in formalin-fixed samples) and subsequent false negative results.<sup>27,28</sup> According to our results, virus DNA was detected in tumor and not in adjacent non-tumor tissues of five CRC patients (31%) which was in accordance with the report of Chen and et al.<sup>24</sup> All CMV+ tumor samples, except one sample, were intermediate grade and there was a close but no significant relationship between the tumor grade and the existence of CMV in tumor tissues. Similarly, a recent study conducted on 103 CRC tumor tissues and 98 adjacent non-tumor tissues, CMV US28 protein was detected in 38% of the patients' tumor samples. However, they found a significant correlation between the presence of CMV in tumor tissue and tumor grade, metastasis, and survival of the patients.<sup>29</sup>

Considering the CMV peptides as tumor tumor-

specific antigens, it has been the preliminary concept of cancer immunotherapy in some recent successful clinical trials in glioblastoma (Clinical Trials, NCT01205334). However, the possibility of using this treatment for CRC patients has not been studied yet. Evaluating the immunological responses of CMV-specific T cells in CRC patients would be requisite in vitro study for utilization in clinical trials. Therefore, in conjunction with comparing T cell function (evaluating CD107a and IFN-γ expression using flow cytometry) in patients and healthy subjects, we tried to improve the function of these cells by 14-day cell culture in the presence of IL-2 and common HLA class I restricted CMV-peptide epitopes. A similar study was conducted by Margaret Inokuma et al. They investigated the functional response of specific T-cells in the presence of CMV and influenza virus peptides as well as associated tumor antigens (TAAs) in patients with breast cancer. The results showed a more potentiate, effective and heterogeneous response of specific T cells against CMV and influenza peptides compared to TAAs alone.<sup>30</sup>

In the present study, the percentage of CD107a and MFI of IFN-γ in both patient and control groups increased significantly following 14-day culture in the presence of CMV peptides. CD107a expression in PHA-stimulated CD8+ T cells (MIT) and cells without

stimulation also improved after culture in both patients and controls, while it was not as much as CMV-stimulated group. One possible reason might be due to the presence of PMA+ionomycin or IL-2 cytokine in the culture media. IL-2 stimulates the expression of CD107a on cytotoxic T cells and PMA+Ionomycin upturns both of CD107a and intracellular IFN- $\gamma$  expression in lymphocytes. Therefore, T cells may be de-granulated and produce IFN $\gamma$  although not as potentiate as cells with specific stimulation.<sup>31</sup>

Except for releasing the granule components such as perforin and granzymes in the immunological synapse which leads to target cells death, cell surface expression of CD107a is also a sensitive marker for detection of cytotoxicity.<sup>32,33</sup> Precopio ML et al demonstrated that MFI of interferon-gamma in polyfunctional CD8+T cells (stimulated by viral peptides) was 5-10 times greater than single-function CD8+T cells, while this association did not exist about CD107aMFI.<sup>34</sup> Our analysis of CD8+ T cells function also revealed that the poly-functional CMV specific CD8+T cells (co-expression of CD107a and MFI of IFN- $\gamma$ ) increased in both CRC patients and healthy controls after culture.

IFN- $\gamma$  raises the expression of MHC-I, MHC-II, and co-stimulator molecules on the surface of antigen-presenting cells (APC) which is very important to potentiate the MHC-restricted antigen-presenting function and host immune response especially by cytotoxic T cells (CTLs).<sup>35</sup> IFN- $\gamma$  stimulates differentiation of Th<sub>1</sub> cells directly by inducing Th1-specific transcription factor (T-bet) and indirectly by activating mononuclear phagocytes to produce IL-12.<sup>36</sup> CMV-stimulated CD8+ T cells of tumor CMV positive and negative patients had no difference in expression of CD107a and intracellular IFN- $\gamma$  prior to culture, but after culture the expression of both CD107a and intracellular IFN- $\gamma$  in tumor CMV positive patients was more than CMV negative, although the difference for CD107a expression was not significant. Other differences between tumor CMV positive and negative patients was in the level of TNF- $\alpha$  secretion from CMV-stimulated cells before culture which was higher in tumor CMV positive than negative patients. According to the literature, TNF- $\alpha$  has dual function against tumor cells. From one side it induces proliferation, metastasis, angiogenesis, and survival of tumor cells by activating NF- $\kappa$ B signaling pathway and from another side, it triggers the cell death by

activating the JNK pathway. The exchange between NF- $\kappa$ B and JNK determines the response of cells in exposure to TNF- $\alpha$ .<sup>37</sup> Previous study showed that acute inflammation intensifies the risk of colorectal malignancies<sup>38</sup> and there is a positive correlation between serum TNF- $\alpha$  level and CRC stage and elevated TNF- $\alpha$  levels in CRC patients is associated with a reduction of patients survival.<sup>39,40</sup>

Evaluation of IFN- $\gamma$  production by ELISA method revealed significant growth in both the CMV-stimulated and non-stimulated cells of patients and healthy controls after culture. The reason why there was no discrepancy in the level of cytokine secretion between stimulated and non-stimulated cells may be due to the effect of IL-2 on cells. However, before culture, the level of this cytokine in CMV-stimulated cells was higher than those with no stimulation.

In the current study, the numbers of CMV-stimulated CD8+ T cells function (polyfunctional CD8+ T cells) were augmented following culture, on the other hand, the number of patients and healthy control who had non-functional CD8+ T cells (CD107a expression or intracellular IFN- $\gamma$ ) decreased after culture. Due to the fact that polyfunctional CD8+ T cells response is associated with better control of viral contamination,<sup>41</sup> polyfunctional CD8+ T generated in this study must have the ability to eliminate infected-target cells. However, we could not find any correlation between the CMV positive tumor tissues and number of cell functions.

The limitation of this study was a small sample size and also lack of using the confirming tests for the cytotoxic function of CMV-specific CD8+ T cells against CMV positive tumor cells. In the present study, we evaluated CMV DNA in tumor and adjacent non-tumor tissues of CRC patients before chemotherapy and radiotherapy. Since the possibility of CMV re-activating exists following chemotherapy and radiotherapy, it is recommended to evaluate tissues in CMV negative tissues after chemo-radio therapy. Also, it would be valuable to investigate the association of antiviral treatments against CMV, such as Ganciclovir and Foscarnet, with the survival rate of colorectal cancer patients. Moreover, the CAR T cell technology will be especially useful for designing the CMV specific T-cells armed with transgenic TCRs for future cancer immunotherapy approaches.<sup>42</sup>

According to our results, CMV-specific CD8+ T cells function in CRC patients (before chemo-radio

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therapy) is similar to healthy subjects and in vitro stimulation of PBMC in the presence of CMV peptide epitopes and IL-2 can be an applicable method to generate potent cytotoxic CD8+ T cells for future T cell therapy in CRC patients.

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