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AUTOGENEIC RNA-ELECTROPORATED CD40-LIGAND ACTIVATED B-CELLS FROM HEPATOCELLULAR CARCINOMA PATIENTS INDUCE CD8$^+$ T-CELL RESPONSES *EX VIVO*

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Since dendritic cells (DCs) constitute only 0.1–0.5% of human peripheral blood mononuclear cells (PBMCs), and generation of DCs from monocytes or stem cells is difficult and expensive, we choose B-lymphocytes as an alternative, cost-effective source of antigen presenting cells (APC). ***Aim:*** **To induce specific CTLs response *ex vivo* by CD40L activated B-cells (CD40-B) transfected with hepatocellular carcinoma (HCC) total RNA.** ***Methods:*** **To induce CD40-B PBMCs of patients with HCC were isolated by Ficoll technique and cultured in RMPI 1640 supplemented with 10% fetal calf serum (FCS), sCD40L (2 μg/ml), recombinant human interleukin-4 (IL-4) (4 ng/ml). The expression of CD80 and CD86 was evaluated by flow cytometry. The level of interleukin-12 (IL-12) produced by cultured B-lymphocytes was measured using enzyme-linked immunosorbent assay (ELISA). HCC patient's T-lymphocytes were obtained from PBMCs cultured in RMPI 1640 supplemented with 10% FCS, 2 ng/mL IL-4 and 10 ng/ml IL-7. CD40-B transfected with tumor total RNA isolated from HCC cells were used to induce specific CTL proliferation. The level of IFN-γ was measured using ELISA and the expression of CD8 was determined by FCAS. Specific cytotoxicity was measured using MTT method.** ***Results:*** **The results show that the activated B-lymphocytes were easily expandable and formed large clones, and a high expression of CD80/CD86 and a high IL-12 secretion by CD40-B was registered. CD40-B transfected with tumor total RNA can induce CTLs to express CD8 and generate IFN-γ at high levels. Compared to the control group, the specific cytotoxicity of CTLs was up-regulated.** ***Conclusion:*** **These findings demonstrate that CD40-B-cells electroporated with total RNA derived from carcinoma cells can be used as alternative APC for the induction of antigen-specific CD8$^+$ T-cell responses, which might be used in HCC immunotherapy.**

Key Words: **CD40L, B-cell, antigen presenting cell, immunotherapy, hepatocellular carcinoma.**

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm worldwide, but owing to the lack of effective treatment options, represents the third leading cause of cancer death [1]. The only curative treatments for HCC are surgical resection or liver transplantation, but mostly patients are suffering from advanced disease and are not candidates for surgery. To date, systemic chemotherapeutic treatment is ineffective against HCC, and no single drug or drug combination prolongs survival [2]. Despite progress in early diagnosis, the prognosis remains poor, because many HCC tumors are discovered when the process is not amenable to surgery [3]. The common occurrence of underlying cirrhosis, the presence of multiple lesions within the liver, the invasion of vital structures within the *v. porta hepatis*, and the extension of the disease outside the liver frequently prevent surgery. In addition, after resection, there is a very high tumor recurrence rate (about 25% per year), which limits curability and long-term survival. Thus, when transplantation is not possible, no good therapeutic options are available for HCC. It is therefore clear that there is an urgent need to develop new approaches to treat HCC.

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Abbreviations used: APC – antigen presenting cell; DC – dendritic cell; CD40-B-CD40 activated B-lymphocyte; CTL – cytotoxic T-lymphocyte; ELISA – enzyme-linked immunosorbent assay; FCAS – flow cytometry analysis system; FCS – fetal calf serum; HCC – hepatocellular carcinoma; MLR – mixed lymphocyte reaction; PBMC – peripheral blood mononuclear cell; PBS – phosphate-buffered saline.

Highly efficient antigen presentation by antigen presenting cells (APCs) is a requisite for the development of T-cell-mediated immunity *in vitro* and *in vivo* [4]. Increasing evidence supports the hypothesis that not all APCs have the same capacity to induce and promote an immune response. For example, only dendritic cells (DCs) have been shown to prime naive T-cells and to induce memory T-cells to become effectors. For these reasons, many investigators attempting to induce immunity to viral [5, 6] or tumor antigens [7, 8] have focused their attention on DCs. In these fields, DCs have been used as cellular adjuvants to present antigen *in vivo* [9, 10], as APCs *in vitro* to determine immunocompetence, and, finally, in experiments attempting to define immunogenic peptides from viral [11], autoimmune [12], and tumor antigens [13, 14].

Although highly efficient in their capacity to induce T-cell immunity, DCs have several significant drawbacks. First, they are relatively rare in peripheral blood and are therefore usually isolated from apheresis or marrow sources [15]. Second, DCs are not homogeneous but represent several populations of functionally disparate cell types, including tolerogenic DCs [16]. Third, it is difficult to expand DCs *ex vivo* from non-stem cell sources without significant *in vivo* expansion with cytokines [17]. Finally, all of the above approaches are laborious, expensive, and therefore presently restricted in their clinical applicability.

CD40 ligand (CD40L) is a member of the tumor necrosis factor family, which is expressed on activated T-cells and binds to CD40 present on the membrane

of APCs [18, 19]. CD40-CD40L interaction plays a crucial role in the activation of APC and in the initiation of both humoral and cellular immune responses [20–22]. Thus, gene transfer of CD40L has been proposed as an efficient means to treat malignancies. Schultze et al. [23] and von Bergwelt-Baildon et al. [24] reported that peptide-loaded CD40-B are as effective as DCs in inducing autologous antigen-specific T-cell responses against viral and tumor-associated antigens *in vitro*. Protein and retroviral loading of CD40-B has also been effective for T-cell stimulation *in vitro*.

In this study, we propose tumor total RNA-loaded, CD40-activated B-cells (CD40-B) as an alternative antigen-presenting cell vaccine with potent T-cell stimulatory capacity and the ability to be generated from a small volume of peripheral blood, and demonstrate that CD40-B can be transfected with total HCC cells RNA to generate specific cytotoxic T-lymphocytes (CTLs, $CD8^+$T-cells) expansion and secretion of interferon-γ (IFN-γ). Furthermore, the activated $CD8^+$T-cells show a killing effect on HCC cells. These findings suggest that CD40-B/RNA technology can be used in HCC immunotherapy.

MATERIALS AND METHODS

Blood samples and cell lines. Blood sample and tumor specimens were obtained from a patient with HCC diagnosed in clinics. The patient is 52 years old, male, and was examined by USG, X-ray and AFP check. Peripheral blood mononuclear cells (PBMCs) phlebotomized from the HCC patient were isolated by Ficoll centrifugation. PBMCs phlebotomized from healthy donor was used as a control. Primary cultured cell line was obtained from the HCC patient's tumor specimens.

Preparation of CD40-activated B-cells. To generate activated B-cells, PBMCs were stimulated with sCD40 ligand (sCD40L). Briefly, PBMCs were added at 106 cells/ml in B-cell medium, consisting of RMPI 1640 supplemented with 10% fetal calf serum (FCS), sCD40L (2 μg/ml), recombinant human interleukin-4 (rhIL-4) (4 ng/ml), and cyclosporine A (0.625 μg/ml; Novartis, Switzerland). After 3 days, cultured cells were replated in B-cell medium and then restimulated with sCD40L and IL-4 every 3 to 5 days up to 21 days, and flow cytometry was performed as described below. After 21 days of culturing, CD40-B-cell populations were more than 90%. Before used in following experiments, Percoll density centrifugation was performed to remove dead cells.

Cryopreservation procedure. PBMCs or CD40-B-cells were frozen in cryotubes at a concentration of $1–10 \times 10^6$/ml in 90% FCS and 10% dimethylsulphoxide (DMSO) (Sigma). Cell suspensions were slowly frozen at 4 °C for 30 min, then at –20 °C for 30 min, and overnight at –80 °C. Frozen cells were thawed quickly in a 37 °C water bath, followed by the addition of 10% FCS RMPI 1640 solution. Next, cells were centrifuged and resuspended at 0.5 × 106/mL in 10% FCS RMPI 1640 solution for 15 min to remove residual DMSO. Finally, cells were washed once and resuspended in culture medium.

Immunophenotyping of CD40-B-cells. The monoclonal antibodies (PE)-conjugated anti-CD80 and (PE)-conjugated anti-CD86 (BD Bioscience, Belgium) were used for immunophenotyping of peripheral blood and CD40-B-cells. Non-reactive isotype-matched antibodies (BD Bioscience, Belgium) were used as negative controls. On days 7, 14 and 21, CD80 and CD86 were determined respectively by Flow Cytometry Analysis System (FCAS). The level of interleukin-12 (IL-12) produced by the cultured CD40-B was measured using an enzyme-linked immunosorbent assay (ELISA) (BioSource, Belgium) on day 7.

RNA preparation. The total RNA of HCC tumor tissue sample was extracted by Trizol (Invitrogen Life Technologies, USA). Tumor tissue sample was homogenized in 1 ml of Trizol reagent per 100 mg of tissue and incubated for 5 min at room temperature, and then 1/5 volume of chloroform was added and mixed vigorously and incubated at room temperature for 5 min. After centrifugation at 12 000 × g for 15 min at 4 °C, the aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol, and incubated at room temperature for 5 min. Total RNA was collected and washed in 2/5 volume of 75% ethanol. Total RNA was run a denaturing formaldehyde agarose gel to check quality. The samples were stored at –80 °C before being used in electroporation.

RNA electroporation. CD40-B-lymphocytes were washed twice with phosphate-buffered saline (PBS), resuspended at 2.0 to 2.5×10^6/100 μl in PBS, and electroporated (2 μg RNA/sample; pulse 300 V, 150 μF). After electroporation, fresh complete medium was added to the cell suspension and cells were incubated further at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

Induction of CD8+T-cells with total RNA-electroporated CD40-B-cells. CD40-B-cells, electroporated with HCC total RNA, were resuspended in RMPI 1640 with 10% FCS. RNA-loaded cells were used for stimulation of CD8+T-cells from PBMC 12 h after electroporation. Briefly, $0–1 \times 10^5$ CD40-B-cells were co-cultured with 1×10^5 the HCC patient's PBMCs in RMPI 1640 supplemented with 10% FCS, 2 ng/ml IL-4 and 10 ng/ml IL-7. CD40-B-cells were divided into 4 groups: group 1 (control group — 0 cells), group 2 — 10^3 cells, group 3 — 10^4 cells and group 4 — 10^5 cells. On days 2 and 4 of the co-cultivation, 20 U/ml IL-2 was added. On day 7 the level of interferon-γ (IFN-γ) produced upon restimulation of the cultured PBMCs was measured using ELISA (BioSource, Belgium) according to the manufacturer's instructions. In addition, on day 7 we evaluated CD8 of the PBMCs using (FITC)-conjugated anti-CD8 (BD Bioscience, Belgium) by FCAS.

Autogenic mixed lymphocyte reaction (Auto-MLR). Auto-PBMCs from the HCC patient were plated at 1×10^5 cells/well in triplets with 0 to 10^5 irradiated (32 Gy) CD40-B electroporated with HCC total RNA per well in RPMI-1640 supplemented with 10% FCS. Determination of [^{3}H] thymidine incorporation was performed in triplicates on days 3–days 7.

Cytotoxicity assay. After 7 days of culturing, CD8+T-cells (using the 105 cells) activated with total RNA-electroporated CD40-B-cells were restimulated with HCC primary cultured cells. Then the vitality of HCC cells was determined by MTT (Sigma) on 24 h, 48 h and 72 h.

Statistical analysis. Results are expressed as mean ± S.E.M. Comparisons were validated using a two-sided Student's t-test. P-value < 0.05 was considered to be statistically significant.

RESULTS

Expansion of CD40-B-cells. CD40-activated B -cells are easily expandable and show distinct co-stimulatory potential. Intially, when PBMCs were stimulated with sCD40L, T-cells and monocytes were inhibited by CsA and IL-4. Then B-cells grew well and formed large clones with higher cloning efficiency. The cells grow in a suspending status, and there is none protuberances on the surface of the cells. The proliferating cells show a typical lymphocyte's morphological characteristics (Fig. 1).

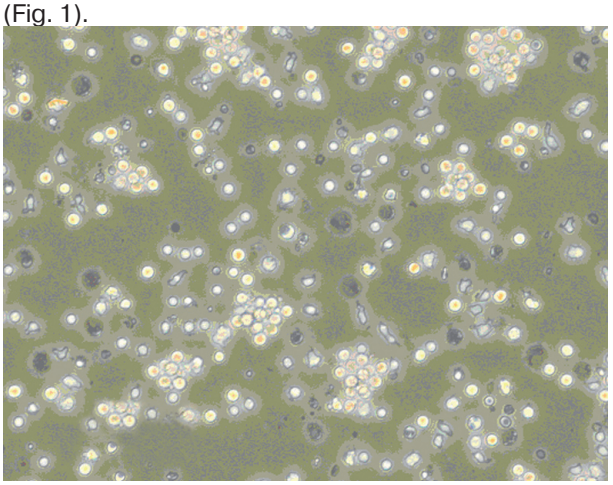

Fig. 1. Expansion of CD40-B-cells on Day 20. At the beginning of sCD40L stimulated to PBMCs, T-cells and monocytes were inhibited by CsA and IL-4. Then B-cells grew well and formed large clones with higher cloning efficiency. The Figure shows CD40-B-cells

Immunophenotyping of CD40-activated B-cells. CD40-activated B-cells can be obtained after 7 days of cultivation. These cells were highly activated as shown by comparable levels of costimulatory molecules of CD80 and CD86 (Fig. 2). The expression of these molecules remained stable thereafter. Phenotypical analysis of the cultured CD40-B-cells showed highly activated expression of CD80 and CD86 on surface of unstimulated B-cells; however, as it was found, PBMC did not express high levels of these activation markers. The high expression of co-stimulatory molecules on CD40-B-cells compared to naive B-cells suggests that these CD40-B-cells can play the role of potent antigen-presenting cells (APCs) in the activation and costimulation of T-cells.

CD40L can up-regulate B-cells to generate IL-12. On day 21 of CD40L stimulated PBMCs culturing, the level of IL-12 was determined by ELISA. The result showed that CD40-B-cells can induce B-cells to generate IL-12 and this effect is dose dependent (Fig. 3). The levels of IL-12 were 23.525 ± 2.583 pg/ml (control group) and 27.134 ± 2.252 pg/ml (experimental group).

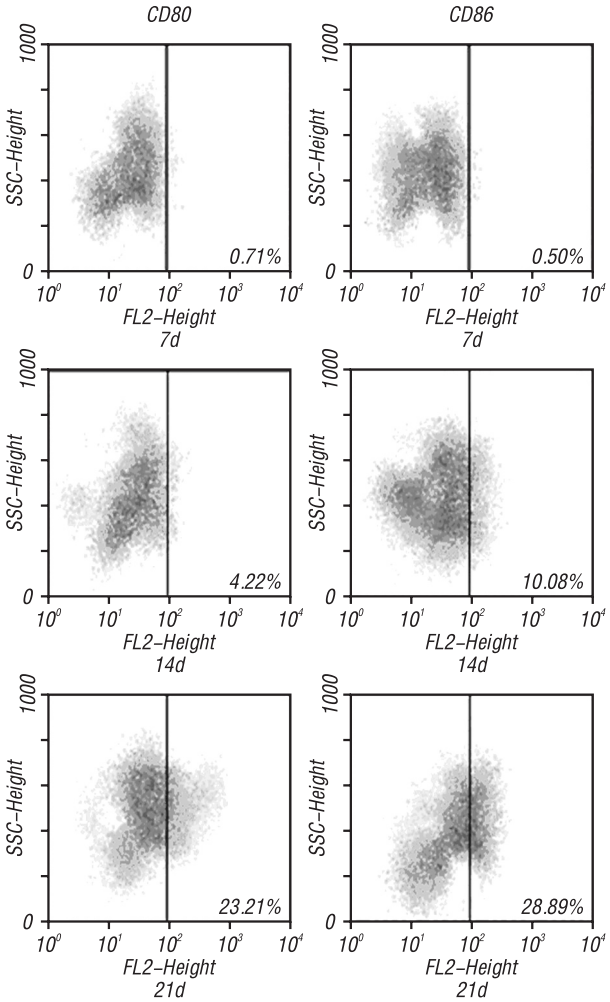

Fig. 2. The variety of CD80 and CD86 of PBMCs stimulated by CD40L. Representative dot plots of PE staining are shown. The left part contains the CD80- or CD86- population. The right part contains the CD80+ or CD86+ population. The expression of CD80 is 0.71% (7d), 4.22% (14d) and 23.21% (21d). The expression of CD86 is 0.50% (7d), 10.08% (14d) and 28.89% (21d)

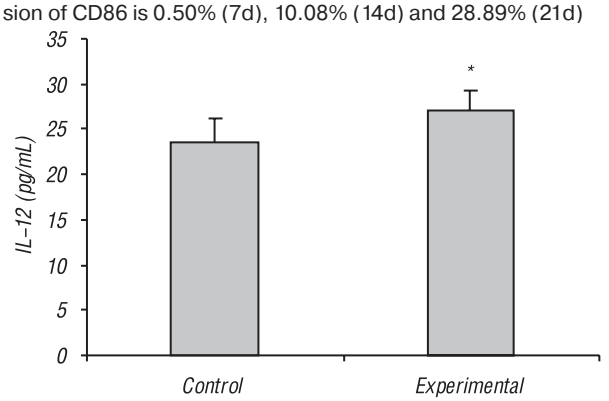

Fig. 3. Generation of IL-12 by CD40L induced B-cell. The levels of IL-12 were determined by ELISA. The results are presented as mean ± S.E.M with triplicate measurement. *$P < 0.05$ vs. control.

CD40-B-cell can increase the number of T-cell. After 7 days in culture the high CD8 expression on T-cells stimulated by electroporated with HCC total RNA CD40-B-cells was observed (Fig. 4). The number of CD8+ T-cells in PBMCs was 8.16%. Furthermore, RNA-electroporated CD40-B-cells positively influenced the number of

T-cells from PBMCs. In the days 3, 4, 5, 6 and 7, the viability of T-cells increased gradually and the quantity of T-cells increased along with the B-cells number (Fig. 5).

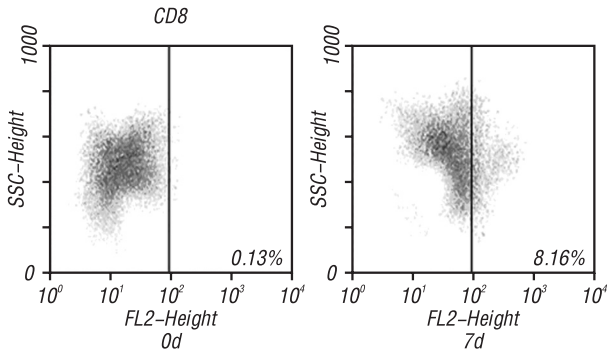

Fig. 4. The variety of CD8 of PBMCs stimulated by RNA-loaded CD40-B-cells. Representative dot plots of FITC staining are shown. The left part contains the CD8- population. The right part contains the CD8+ population. The expression of CD8 is 0.13% (0 d) and 8.16% (7 d)

CD40-B-cells can induce CD8+ T-cells to generate IFN-γ. After 7 days of culturing, electroporated with HCC total RNA CD40-B-cells stimulated T-cells to produce IFN-γ the levels of which were determined by ELISA. The result showed that CD40-B-cells can induce CD8+ T-cells to generate IFN-γ and that there is a dose dependent effect as well (Fig. 6). The quantity of IFN-γ was 0.086 ± 0.058 ng/ml (control group), 0.234 ± 0.082 ng/ml (103 cells), 0.392 ± 0.091 ng/ml (104 cells) and 0.658 ± 0.132 ng/mL (105 cells).

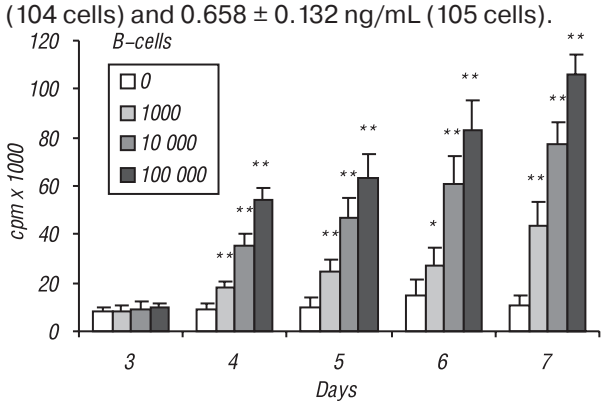

Fig. 5. CD40-B-cells electroporated with HCC total RNA increased the number of T-cells from PBMCs. The cell viability was determined by Auto-mixed lymphocyte reaction (Auto-MLR). The results are presented as mean ± S.E.M. with triplicate measurement. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

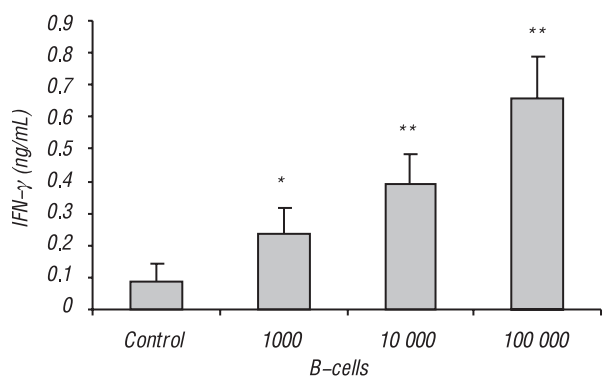

Fig. 6. CD40-B-cells electroporated with HCC total RNA induced CD8+ T-cells to generate IFN-γ. The levels of IFN-γ were determined by ELISA. The results are presented as mean ± S.E.M. with triplicate measurement. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

T-cells activated with CD40-B-cells can decrease the number of HCC cells. The killing effect of T-cells was determined by MTT (Fig. 7). After 24 h, 48 h and 72 h T-cells action, the vitality of HCC cells show significant decrease.

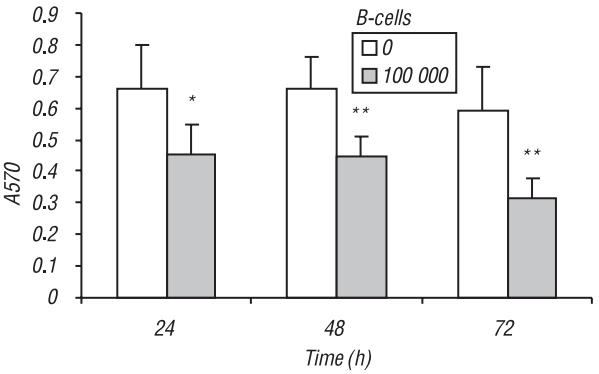

Fig. 7. T-cells activated by CD40-B-cells decreased the number of HCC cells. The killing effect of T-cell was determined by MTT. The results are presented as mean ± S.E.M. with triplicate measurement. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

DISCUSSION

Earlier studies have already shown that CD40-expanded B-cells can be loaded with antigens by means of peptide pulsing or retroviral transduction [25, 26]. Our present findings not only indicate that CD40-B-cells are to be considered as a highly activated cell population with distinct immunophenotype characteristics, but also that these cells can be loaded with RNA antigen by electroporation.

CD80 and CD86 both are type 1 transmembrane proteins with a membrane distal IgV and a membrane proximal IgC domain. They share about 25% sequence homology and interact with the same receptors, CD28 and CTLA-4. The structural mechanism of CD80/CD86 mediated T-cell costimulation is not completely understood and even less is known about the significance of the unique features of CD80 and CD86. To address these issues, a number of biochemical, structural and biophysical studies have been performed [27–29]. Notably, structural and cell based fluorescence resonance energy transfer (FRET) studies have demonstrated that CD80 and CD86 differ in their cell surface organization and suggest that these differences may play a key role in T-cell activation [30]. Furthermore, CD80 and CD86 have been implicated as signaling molecules in B-cell and DCs [31–34]. Intriguingly, signaling via CD80 and CD86 in DCs was found to involve the expression of both CD80 and CD86, suggesting that the homooligomeric and hetero-oligomeric states may be important for APC function [33, 35].

IL-12 is recently identified as inflammatory cytokine produced mainly by T- and B-lymphocytes and monocytes. It promotes the production of IFN-γ by T-lymphocytes and is necessary for differentiation of Th naive cells to Th1-cells [36]. Mescher et al. [37] have proposed that in addition to receiving signals by antigen and costimulatory receptors, naive $CD8^+$ T-cells require a 'third' signal by IL-12 or type I IFN (IFN-I, comprising IFN-α and IFN-β) for optimal pro-

liferation and effector functions. The molecular events that control this third signal are not clear to date, but recent evidence suggested that whereas antigen and costimulatory signals were sufficient for up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL, IL-12 was needed for Bcl-3 up-regulation [38, 39]. $CD8^+$ T-cells primed *in vitro* in the presence of IL-12 were shown to confer better *in vivo* protection against virus challenge [40]. Therefore in the study we investigated the expression of CD80/CD86 on the surface of the CD40-B-cells and the level of IL-12 generated by B-cells for exploring the activation of B-cells after CD40L stimulated. The result suggests that CD40L can stimulate B-cells activated and increase the expression of co-stimulatory molecules on the B-cell surface and up-regulate IL-12 secretion.

Pilot studies on DCs-based immunotherapy have induced specific anti-tumor responses, including some clinical responses [41, 42]. But effective TSA or TAA is not identified in a large number of cancers, although a number of tumor antigens recognized by $CD8^+$ CTLs have been identified [43]. Furthermore, it is clear that immunotherapy targeting to a unique antigen have selectively lost the ability to present the defined antigen effectively because of changing of tumor cells features [44]. Zhang et al. [45] find that total tumor RNA transfected DCs might be an attractive strategy to generate tumor-specific T-cells to treat patients suffering from HCC. Other studies target B-cells substitute for DCs [46, 47]. These results underscore that CD40-B-cells can act as alternative APC for the *ex vivo* induction of T-cell immune responses, as was shown previously for mRNA loaded DCs [48, 49]. RNA-loaded APC form an excellent tool for future vaccination strategies, as RNA-based loading of APC overcomes the problem of HLA restriction, which limits the use of peptide pulsing. Through RNA electroporation, APC can be loaded with the full-length antigen, enabling presentation of multiple epitopes without the need for prior characterization of immunogenic epitopes. This approach also minimizes the risk for generation of tumor antigen loss variants or virus escape variants. Moreover, RNA electroporation is a more feasible and safe method to apply in a clinical setting compared to using viral vectors for transfection of antigens into APC.

In conclusion, our results demonstrate that RNA electroporation of CD40-B-cells lead to effective $CD8^+$ T-cell activation. CD40-B-cells offer many advantages over monocyte-derived DCs, we strongly believe that this type of approach could conceivably be applied to a wide range of cancers for which tumor-associated antigens have not been identified.

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CD40-ЛИГАНД АКТИВИРОВАННЫЕ В-КЛЕТКИ, ТРАНСФЕЦИРОВАННЫЕ РНК КЛЕТОК ГЕПАТОЦЕЛЛЮЛЯРНОЙ КАРЦИНОМЫ, ИНДУЦИРУЮТ ОТВЕТ $CD8^+$ Т-КЛЕТОК *EX VIVO*

Поскольку дендритные клетки (ДК) составляют только 0,1–0,5% мононуклеаров периферической крови (МПК) человека, и получение ДК из моноцитов и стволовых кроветворных клеток является трудоемкой и дорогостоящей процедурой, выбраны В-лимфоциты в качестве альтернативного источника антигенпрезентирующих клеток (АПК). ***Цель:*** **изучить возможность активированных CD40-лигандом В-лимфоцитов (CD40-В), трансфецированных суммарной РНК, выделенной из клеток гепатоцеллюлярной карциномы, индуцировать специфический ответ цитотоксических Т-лимфоцитов *ex vivo*.** ***Методы:*** **МПК пациентов с гепатоцеллюлярной карциномой изолировали на градиенте фиколла и культивировали в среде RMPI 1640 с 10% эмбриональной сыворотки телят (ЭСТ), sCD40L (2 мкг/мл) и рекомбинантным человеческим интерлейкином-4 (ИЛ-4, 4 нг/мл). Экспрессию CD80 и CD86 оценивали на проточном цитофлюориметре. Концентрацию интерлейкина-12, продуцируемого культивируемыми В-лимфоцитами, оценивали с помощью иммуноферментного анализа (ИФА). Т-лимфоциты получали из МПК пациентов с гепатоцеллюлярной карциномой и культивировали в среде RMPI 1640 с 10% ЭСТ, 2 нг/мл ИЛ-4 и 10 нг/мл ИЛ-7 и культивированы совместно с CD40-В клетками, трансфецированными опухолевой РНК, для индукции пролиферации специфических цитотоксических Т-лимфоцитов. Уровень интерферона-γ (ИНФ-γ) определяли с помощью ИФА, а экспрессию CD8 — на проточном цитофлуориметре. Специфическую цитотоксичность оценивали в МТТ тесте.** ***Результаты:*** **показано, что активированные *in vitro* В-лимфоциты формируют большие колонии, на поверхности клеток отмечается высокая экспрессия антигенов CD80/CD86, и ими в повышенных количествах секретируется ИЛ-12. CD40-В-лимфоциты, трансфецированные опухолевой РНК, могут активировать $CD8^+$ цитотоксические лимфоциты, которые продуцируют ИНФ-γ.** ***Выводы:*** **CD40-В-клетки, трансфецированные суммарной РНК, выделенной из опухолевых клеток больных с гепатоцеллюлярной карциномой, — это альтернативные АПК для индукции антигенспецифического $CD8^+$ Т-клеточного ответа, что может быть использовано для иммунотерапии пациентов с гепатоцеллюлярной карциномой.**

Ключевые слова: **CD40L, В-лимфоциты, антигенпрезентирующие клетки, иммунотерапия, гепатоцеллюлярная карцинома.**