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Research paper

Immune active cells with 4-1BB signal enhancement inhibit hepatitis B virus replication in noncytolytic manner

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ABSTRACT

Immune active cells (IACs) have been shown to be an alternative immunotherapy for CHB patients. However, there is a practical problem of different expansion rate and function of HBV inhibition as individual variability exists. Our previous studies have confirmed that the proliferation and cytolysis of IACs were significantly up-regulated by engineered cells for costimulatory enhancement (ECCE) delivering a 4-1BBζ activating signal. In this study, we aimed to investigate the contribution of ECCE to IACs from CHB patients. We found that ECCE could enhance larger-scale expansion of IACs and the levels of HBV-markers were reduced prominently with minimal cytolysis, in the indirect system which separated ECCE-IACs and HepG2.2.15 by a 0.4-μm membrane. Furthermore, ECCE-IACs produced a lot of IFN-γ and TNF-α. Blockading them, the inhibition was abrogated. These results provide direct evidence that ECCE-IACs efficiently control HBV replication in a noncytolytic manner, and this effect is mediated by IFN-γ and TNF-α.

1. Introduction

Hepatitis B virus (HBV) infection is a major public health problem worldwide. Cellular immune responses are believed to play a critical role in the control of HBV replication. In chronic hepatitis B virus (CHB) patients, an inefficient immune responses to the virus, with cellular immune responses exhaustion in particular, leads to the viral persistence and progressive liver injury [1,2]. Adoptive immunocyte therapy has become a new treatment regimen for CHB patients [3–5].

Immune active cells (IACs) comprised by heterogeneous cell populations including a major effector cell population expressing both the T-cell and NK cell markers ($CD3^+CD56^+$), had been demonstrated to inhibit HBV replication in CHB patients [5–8]. However, there is still a practical clinical problem of different expansion rates and function of IACs as individual variability exists. Engineered cells for costimulatory enhancement (ECCE) is a kind of gene engineered 4-1BBζ-expressing K562 cells without T-cell receptor [9], which could only deliver co-stimulation signal to expand T cells but not TCR signal to limit effector T-cell differentiation toward an exhausted terminal effector state [10,11]. Our previous studies have confirmed that ECCE can improve the proliferation and cytokine levels of IACs in heavily pretreated patients with solid tumor. However, experiments have never been conducted with ECCE for IACs production in CHB patients.

The initial aims of this study were to investigate the contribution of ECCE to IACs production in CHB patients and explore the function and mechanisms of ECCE-IACs suppressing HBV replication.

2. Materials and methods

2.1. Generation of conventional IACs and ECCE-IACs

Peripheral blood was collected from CHB patients, with the approval from the ethics committee of Nanjing Second Hospital. Written informed consents were obtained from all donors in accordance with

Abbreviations: IACs, immune active cells; CHB, chronic hepatitis B; ECCE, engineered cells for costimulatory enhancement; HBV, hepatitis B virus; PBMCs, peripheral blood mononuclear cells; rhIFN-γ, recombinant human interferon γ; rhIL-2, recombinant human interleukin 2; LDH, lactate dehydrogenase; TNF-α, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; IL-10, interleukin 10; TGF-β, transforming growth factor-β; DCs, dendritic cells; Tregs, regulatory T cells

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the Declaration of Helsinki. All patients had been positive for HBsAg, HBeAg, and HBV DNA for more than 6 months.

The conventional IACs and ECCE-IACs were generated by the procedure described in our previous report [12]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by standard density centrifugation from peripheral blood. The PBMCs were resuspended at 1×10^6 cells/mL in TexMACS GMP Medium (Miltenyi Biotec) supplemented with 3% autologous serum. On day 0, the conventional IACs group received recombinant human interferon (rhIFN)-γ (1000 IU/mL; Boehringer Ingelheim), followed by the addition of 100 ng/mL anti-CD3 antibody (MACS GMP CD3 pure, Miltenyi Biotec) and 300 IU/mL recombinant human interleukin (rhIL)-2 (Proleukin; Novartis) on day 1. Thereafter, cells were stimulated with rhIL-2 (300 IU/mL) every 3–4 days and fresh medium was added to maintain cell density of 1×10^6 cells/mL. To generate ECCE-IACs, irradiated ECCE were added into the culture system as initiated ratio with PBMC was 1:1. The number of conventional IACs and ECCE-IACs were recorded periodically during the culture period. Expansion was performed for 21–28 days.

2.2. Culture of HepG2.2.15 cells and HepG2 cells

The cell line HepG2.2.15, derived from HepG2 cells, is stably transfected with HBV DNA and supports full HBV replication with production and secretion of viral Ags and infectious virion [13]. HepG2 were cultured in DMEM (Invitrogen) containing 10% FCS (Invitrogen), 500 U/ml penicillin, 500 mg/ml streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen). HepG2.2.15 cells were cultured with the same medium under positive selection with 0.4 mg/ml Geneticin (Invitrogen).

2.3. Coculture of effector and target cells

The ECCE-IACs or IACs were harvested from culture on the 14th day, washed and used as effector cells. HepG2.2.15 cells or HepG2 cells were used as target cells. They were cocultured in two different systems of cell-to-cell interactions: direct effector/target contact, and indirect effector/target contact system, whereby the effector and target cells were separated by a 0.4-μm membrane (EMD Millipore), which allowed the passage of soluble factors only. In the indirect contact system, effector cells were seeded at the top inserts and target cells were seeded on the bottom. The effector and target cells were cocultured for 3, 24, 48 or 72 h using effector: target (E: T) ratios of 1:1, 5:1, 10:1 or 20:1. Target cells were cultured alone as controls. At the end of the experiments, the target cells and the corresponding culture supernatants were harvested for measurement of the cytotoxicity, cytokine levels, HBsAg, HBeAg and HBV DNA quantitation.

2.4. Cytotoxicity detection

The percentage of cytotoxicity was assessed by measuring the lactate dehydrogenase (LDH) release in the supernatants at the end of each incubation period using a commercially available enzyme immunoassay (Promega). According to the manufacturer's recommendations, maximal release of LDH was achieved by incubating the target cells with 0.1% IGEPAL (anionic detergent from Sigma). The negative control (spontaneous release) was target cells without effector cells. The experimental value was provided by all test samples subtracting a background control, represented by culture medium alone. The cytotoxicity was calculated using the formula: cytotoxicity (%) = (experimental value–effector release-spontaneous release)/(maximal release-spontaneous release) * 100%.

2.5. Measurement of HBV DNA levels, virological markers

HBV DNA from secreted viral particles (coculture supernatant) was extracted using QIAamp DNA minikit (Qiagen, Sussex, U.K.) and also quantified by TaqMan real-time polymerase chain reaction (PCR) (ABI Prism 7700, Applera, Warrington, U.K.) with a detection limit of 500 copies/mL (PG Biotech). Supernatant HBsAg and HBeAg were measured by using ELISA kits (Abbott).

2.6. Cytokine production

For the analysis of intracellular interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production, ECCE-IACs were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) for 4 h in the presence of Golgistop agent (10 μg/mL) (eBioscience) followed by washing and staining with antibodies to surface markers (anti-CD3-PB (eBioscience), anti-CD8-PerCP (eBioscience), anti-CD56-APC-Cy7 (eBioscience) and Live/Dead (Invitrogen)). After cells were permeabilized and fixed using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions, anti-IFN-γ-PE and anti-TNF-α-FITC or isotope-matched control antibodies were added for 30 min followed by flow cytometric analysis.

The levels of IFN-γ and TNF-α in the indirect coculture supernatants were assessed by commercially available ELISA assays (R&D Systems).

2.7. Neutralization of IFN-γ or TNF-α

Anti-IFN-γ and anti-TNF-α neutralizing mAbs (R&D Systems) were added individually or in combination to the indirect cocultures at a 10:1 ratio. A neutralization dose of 36 or 0.9 μg/ml was used, respectively, according to the manufacturer's guidelines. After 88 h, the supernatants were collected for HBV DNA quantitation.

2.8. Statistical methods

Data were expressed as mean ± standard deviation. Statistical differences were considered to be significant at a P value < 0.05 as determined by a paired Student's t-test.

3. Results

3.1. Addition of ECCE improved the proliferation of IACs cultured from CHB patients

PBMCs were isolated from six CHB patients. Conventional IACs and ECCE-IACs were produced as described above. During culture with the cytokine cocktail, expansions of IACs and ECCE-IACs were assessed for proliferation and viability at regular intervals. Living cell ratio was more than 95% by trypan blue staining. ECCE-IACs exhibited a higher proliferation rate, compared with conventional IACs (Fig. 1A). The expansion folds of conventional IACs and ECCE-IACs on the 20st day (ECCE-IACs versus conventional IACs, 1563.76 ± 186.55 versus 228.53 ± 65.82, $P < 0.01$) were shown in Fig. 1B.

As expectations, ECCE-IACs dominantly comprised a heterogeneous population of $CD3^+CD8^+$ (85.6% ± 5.3%) and $CD3^+CD56^+$ (42.6% ± 15.7%) cells. One representative example of ECCE-IACs is shown in Fig. 1C. Compared with IACs without ECCE (72.3% ± 6.8%), the percentage of $CD3^+CD8^+$ in ECCE-IACs increased obviously ($p < 0.01$). There was no statistically difference in the percentage of $CD3^+CD56^+$ population between IACs and ECCE-IACs (Fig. 1D).

3.2. Cytotoxicity of ECCE-IACs

HepG2.2.15 is one sub clone derived from human hepatoma cell line HepG2 cells, and is stably transfected with HBV DNA and supports full HBV replication with production and secretion of viral Ags and infectious virions. In previous experiments, we found that ECCE-IACs had broad antitumor activity, such as against SMMC7721, MGC803, A375 and A549, and ECCE could improve the cytotoxic of IACs from heavily