RESEARCH ARTICLE

Multiple Cytotoxic Factors Involved in IL-21 Enhanced Antitumor Function of CIK Cells Signaled through STAT-3 and STAT5b Pathways

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Abstract

Background/Objectives: Maintenance of cellular function in culture is vital for transfer and development following adoptive immunotherapy. Dual properties of IL-21 in activating T cells and reducing activation induced cell death led us to explore the mechanism of action of IL-21 enhanced proliferation and cytotoxic potential of CIK cells. Method: CIK cells cultured from PBMCs of healthy subjects were stimulated with IL-21 and cellular viability and cytotoxicity to K562 cells were measured. To elucidate the mechanism of action of IL-21, mRNA expression of cytotoxic factors was assessed by RT-PCR and protein expression of significantly important cytotoxic factors and cytokine secretion were determined through flow cytometry and ELISA. Western blotting was performed to check the involvement of the JAK/STAT pathway following stimulation. Results: We found that IL-21 did not enhance *in vitro* proliferation of CIK cells, but did increase the number of cells expressing the CD3+/CD56+ phenotype. Cytotoxic potential was increased with corresponding increase in perforin (0.9831±0.1265 to 0.7592±0.1457), granzyme B (0.4084±0.1589 to 0.7319±0.1639) and FasL (0.4015±0.2842 to 0.7381±0.2568). Interferon gamma and TNF-alpha were noted to increase (25.8±6.1 ng/L to 56.0±2.3 ng/L; and 5.64±0.61 μg/L to 15.14±0.93 μg/L, respectively) while no significant differences were observed in the expression of granzyme A, TNF-alpha and NKG2D, and NKG2D. We further affirmed that IL-21 signals through the STAT-3 and STAT-5b signaling pathway in the CIK cell pool. Conclusion: IL-21 enhances cytotoxic potential of CIK cells through increasing expression of perforin, granzyme B, IFN-gamma and TNF-alpha. The effect is brought about by the activation of STAT-3 and STAT-5b proteins.

Keywords: Antitumor function - interleukin-21 - cytokine induced killer cells

Asian Pac J Cancer Prev, **14 (10)**, 5825-5831

Introduction

IL-21 was originally implicated as a regulator of T and B cell proliferation as well as of NK cell maturation. It belongs to a subset of cytokines where the receptors share the common cytokine receptor γ chain, γc. The common cytokine receptor γc is mutated in X-linked severe combined immunodeficiency, a disease with severely impaired T cell and NK cell development and diminished B cell function. γc is a critical component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, which together regulate lymphocyte development and control a broad spectrum of activities that shape innate and acquired immune responses.

Although IL-21 has a broad range of action on most cells of the hematopoietic system and some cells of the non hematopoetic system. Compared with its role in B cells to T cells, IL-21 had not been considered key regulator of T cell differentiation. However, it was determined that IL-21 makes a vital contribution to the differentiation of Th cells. In particular, Th17 cells, which are critical mediators of autoimmunity, depend on IL-21, together with IL-6 and TGF-β, for their differentiation.

In contrast to the above findings, the influence of IL-21 on CD8 T cells remained unclear. Zeng et al. demonstrated initially that IL-21 enhances the survival of cultured, naïve CD8 T cells but not memory CD8 T cells. Additionally, IL21R−/− mice have normal numbers of peripheral CD8 T cells. These studies show a limited role of IL-21 on T cells although the exact depth of IL-21 role on activated and memory T cells needs to be identified and studied thoroughly.

CIK cells initially described by Ingo G.H. Schimdt Wolf et al in 1991, are CD3+ and CD56+ NK like T cells which possess non-MHC restricted antitumor properties for solid and hematologic malignancies. CIK cells are

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generated from peripheral blood by the timed addition of IFN-γ, anti-CD3 and IL-2 .The culture medium is regularly replenished with IL-2 for 21–28 days. At maturity CIK cells pool consists of a heterogeneous population of CD3+ T cells (>90%),The majority of these cells are rare CD3+ and CD56+ cells which are responsible for MHC unrestricted antitumor activities . Anti tumor function of CIK cells are mediated through secretions of large quantities of perforin, granzyme B and cytokines such as interferon-γ and TNF-α. The mechanism of cell signaling that ultimately leads to exocytosis occurs through receptors such as TCR/CD3 and NKG2D, DNAM-1, NKp30 receptors (Pievani et al., 2011). The dual nature of CIK cells in possessing both T and NK cell like property has been widely explored and CIK cells based immunotherapy for lymphoma, metastatic renal cancer, hepatocellular carcinoma (Olioso et al., 2009), and relapsing hematological malignancies (Leemhuis et al., 2005) have shown promising results.

Maintenance of not only CIK cells but other activated effector T and NK cells in culture is vital for their effective transfer and development following adoptive immunotherapy. IL-21 is structurally similar but functionally varied to IL-2 and IL-15 (Parrish-Novak et al., 2000). IL-21R has been found to be expressed in T cells, NK cells, B cells and CIK cells (Parrish-Novak et al., 2000; Zhao et al., 2007). The broad range of IL-21 activity on the hemapoietic system includes proliferation of anti-CD40 activated B cells and differentiation of effector cells of B lymphocytes, inhibition of dendritic cells mediated antigenic response, promotion of antitumor properties of anti-CD3 stimulated CD8+ T cell and activated NK cells (Parrish-Novak et al., 2000; Kasaian et al., 2002; Brandt et al., 2003; Ozaki et al., 2004). IL-21 has also been implicated in promoting survival of CD8+ memory T cells and expansion of Ag-specific B cell following secondary immunization (Allard et al., 2007; Rankin et al., 2011).

IL-21 has also been regularly associated with maintenance of a naïve phenotype in T cells which begs the question whether IL-21 can promote the cytotoxic potential of CIK cells and at the same time maintain its cellular viability for an effective growth following adoptive transfer.

Our previous experiment showed that Like in T cells and NK cells, IL-21 significantly improves the cytotoxicity of CIK cell on K562 cells (Zhao et al., 2007). Although proliferation of cells in a CIK cell pool was not observed we found that it helped maintain and grow the CD3+and CD56+ phenotype. Our present experiment aims to explain the mechanism through which Il-21 promotes CIK cell survival and cell cytotoxicity.

Materials and Methods

CIK Cells Culture and Immunophenotyping

Peripheral Blood were collected from healthy donors and PBMCs were seperated using Ficoll Gradient density separation and grown in complete medium at 37°c in a 5%CO2 high humidity environment. PBMCs at day 0 of culture was stimulated with IFN-γ (200 ng/ml) followed (Peprotech) by stimulation by Anti-CD3 (50 ng/ml) (Peprotech) and Interleukin-2 (300 IU/ml) (Biolegend) on day 1. The culture medium, anti-CD3 and IL-2 were replaced every 3 days thereafter and cells were subjected to IL-21 after 14-21 days of culture. Cell viability was assessed every 3 days by tryphan blue staining. Immunophenotypes of the CIK cells were determined on D0, D7 and D14 of culture using FACS flow cytometry. Percentage of CD3+/CD56+/CD45+, and CD19+ expressing cells were determined at each point.

Culture of K562 cells

The K562 cells were cultured and cell lines were established in our laboratory. The cells were grown in complete medium (RPMI 1640 with 10%FCS containing 100 u/ml penicillin, 100 ug/ml streptomycin) 37°c in 5%CO_2 humidified environment. Culture medium was replenished every 2-3days.

Treatment of CIK cells with IL-21

CIK cells after14-21 days of culture were washed with culture medium twice and re plated at concentrations of 1×10^6 cells/ml in culture medium devoid of IL-2. The cells were then stimulated with IL-21 (Prospec USA) at 100 ng/ml. Cells were cultured for 1 hour to perform western blots, 24 hours for RT-PCR and Flow cytometry and MTT assays were performed after stimulation for the appropriate time. Suitable concentration of IL-21 for stimulation was established earlier using MTT assays where viability of CIK cells were assessed in increasing dose of IL-21 of up to 200 ng/ml (data is not shown).

MTT assay

Measurement of Cell viability An experimental and control group of cells were separated. Experiment group of cells were defined as CIK cells stimulated with IL-2 (300 IU/ml), IL-21 (100 ng/ml) or IL-2 + IL-21. Control groups were defined as CIK cells cultured for 14-21days. MTT assay were performed on these cells using standard manufacturer's procedure.

Measurement of Cytotoxicity

Colorimetric MTT assay was performed to measure cytotoxicity. CIK cells were plated in a 96-well flat bottom plate and incubated overnight with IL-21, combination IL-21 and IL-2 or without any cytokines. Target cells were added to the CIK cells and incubated for 12,24,48 and 72 hours. MTT solution was added and plates were incubated for a further 4 hours. The MTT solution was removed and the blue crystals were dissolved using DMSO. The optical density of the plates at 492nm was measured using a spectrophotometer.

Cytotoxicity of CIK cells was calculated using the formula:

$$\text{Cytotoxicity} = \frac{\text{OD target cells} - \{(\text{OD experiment (A)} - \text{OD effector cells})\} \times 100\%}{\text{OD target cells}}$$

Flow cytometric analysis

CIK cells were prepared as explained earlier, stimulated with IL-21 for 24 hours and used as the experimental group. CIK cells cultured for 14-21days

were used as the control group. Cells were collected in a propylene tube at a concentration of 1×10^6 cells/ml and incubated in dark room for 15mins with FITC conjugated anti-CD3, PE conjugated anti-CD56 and APC conjugated IL-21R, washed with PBS and analyzed on a flow cytometer (Calibur). In another setting, cells were incubated with FITC AntiCD3/PE Anti CD56 and APC conjugated perforin or granzyme B or antiCD11a or FasL or NKG2D or TNF-β after stimulation with IL-21 for 4 hours.

Cytokine Assay

The levels of INF-γ and TNF-αfollowing IL-21 stimulation of CIK cells were determined by ELISA. Using standard manufacturer's protocol, culture supernatants from experiment group (IL-21 100 ng/ml) and control group (no additional cytokines) were added to wells of the ELISA plates. A standard curve of different concentrations ranging from (500, 250, 125, 62.5, 31.25, 15.625,0 pg/ml) was established. 100ul of biotinylated antibody was added into each of the wells including the blank controls. The plate was sealed and incubated at room temp for 120mins. Plate was washed and 100ul of conjugated antibody was added onto the wells followed by incubation for 30mins. Substrate A and B were added to the wells and allowed to sit in dark room for 15mins. Stop solution was added and optical density of the plates was read immediately at 490nm. OD values of the specimen were compared with the standard curve to estimate its levels in the culture supernatant.

RT-PCR

Semi quantitative RT-PCR was used to measure the level of expression of IFN-γ, perforin, granzyme A, granzyme B, FasL, TNF-α,TNF-β, and NKG2D mRNA. CIK cells were first divided into experiment groups (stimulation with IL-21 at 100 ng/ml) and control groups (CIK cells with no additional IL-21). Total RNA were extracted from these samples using Trizol reagent protocol in manufacturer's instruction (Invitrogen Life technologies, CA, USA). RNA concentration was determined through OD measurements at 492nm. cDNA was synthesized from 1ug of RNA in 4ul 5×RT Buffer containing dNTP Mixture (10 mM) 2 µl, Random primer 1 µl, RNase Inhibitor 1 µl, AMV Reverse Transcriptase 1 µl, and 9 µl of RNase Free ddH_2O. The mixture was incubated for 60mins at 42°C and 10 mins at 72°C. 2 ul of cDNA so formed was used for amplification with 12.5 µl mixture, sterile distilled water 10µl, upstream primer 1 µl, downstream primer 1 ul. GAPDH was used as a loading control to verify that the same amount of RNA was amplified.

Statistic analysis

Statistical analysis was performed with SPSS (version 17.0) software. Significant differences between groups were calculated using two sample t test. *P* values ≤0.05 were considered significant.

The following is the list of gene amplified and primers used

Gene		Primers	Length
GAPDH:	Sense	5'-TTCCACCCATGGCAAATTCC-3	500bp
	Antisense	5'-AGGCCATGCCAGTGAGCTTC-3 '	
Perforin:	Sense	5'-CAGGTCAACATAGGCATCCACG-3'	160bp
	Antisense	5'-GAACAGCAGGTCGTTAATGGAG-3'	
Granzyme A	Sense	5'-GCTCACTCAATAACCAGGGA-3'	377bp
	Antisense	5'-GAGTCTCTTCCACCTCGGAG-3'	
GranzymeB:	Sense	5'-GAAACGCTACTAACTACAGG-3'	126bp
	Antisense	5'-CCACTCAGCTAAGAGGT-3'	
FasL	Sense	5'-TGTTTATGAGCCAGACAAATGG-3'	203bp
	Antisense	5'-AAGACAGTCCCCCTTGAGGT-3'	
TNFα	Sense	5'-CGAGTGACAAGCCTGTAGC-3'	363bp
	Antisense	5'-CCTTCTCCAGCTGGAAGAC-3'	
TNFβ	Sense	5'-AGGCATGAGGGATCACAG-3'	115bp
	Antisense	5'-AAAGAGGTTTATTGGGCTTC-3'	
IFN-γ	Sense	5'-GCAGAGCCAAATTGTCTCCT-3'	290bp
	Antisense	5'-ATGCTCTTCGACCTCGAAAC-3'	
NKG2D	Sense	5'-AGCCAGGCTTCTTGTATGTCTC -3'	99bp
	Antisense	5'-CATCCAATGATATGACTTCACC-3'	

Western Blotting

For each western blot, $0.5\text{-}1\times10^7$ cells were washed with PBS and cultured in a cytokine free culture medium (10%FCS and RPMI) for 24 hours. These cells were then stimulated with IL-21 for 1 hour, control group consisted of CIK cells cultured for 14-21 days with no additional IL-21. Cells were collected and lysed in RIPA buffer. Concentration of the protein was determined using Protein BCA assay and 60-100 ul of protein was loaded to 8% SDS-polyacramide gel for seperation. Seperated Proteins were transferred onto PVDF membranes and visualized with Anti-STAT-1, Anti-STAT-3, Anti-STAT5a, Anti-STAT5b antibody (Millipore CA) respectively.

Results

IL-21 significantly increases the number of cells expressing the CIK cell phenotype but not the total number of cells in the CIK cell pool

We first investigated the effect of IL-21 on proliferation of CIK cells using MTT assay. Experiment groups were CIK cells stimulated with additional cytokines and control group were CIK cells devoid of any additional cytokine stimulation. After 72 hours of culture there was no significant difference observed in the number of cells stimulated with IL-21 and control group. Although IL-21 showed no considerable increase in the number of CIK cells we explored whether IL-21 could increase the proportion of cells expressing the CIK cell phenotype. After 72 hours of culture with the respective cytokine the number of cells expressing both CD3+ and CD56+ in control group was (17.5±4.7)% which was significantly lower compared to IL-21 stimulated group (26.5±2.1)%. Additional IL-2 (48±5.9)% and combination IL-2 and IL-21 (50±4.9)% stimulation could also significantly improve the generation of CIK cells. The results are a summation of three separate experiments.

IL-21 alone or in synergism with IL-2 can enhance the cytotoxic potential of CIK cells

The cytotoxic effect of IL-21 stimulated CIK cells on K562 cells were determined using colorimetric MTT assay. K562 cells was plated along with CIK cells at an Effector: Target ratio of 20:1 for 24 hours. Cytotoxicity of CIK cells were calculated using the above mentioned formula.

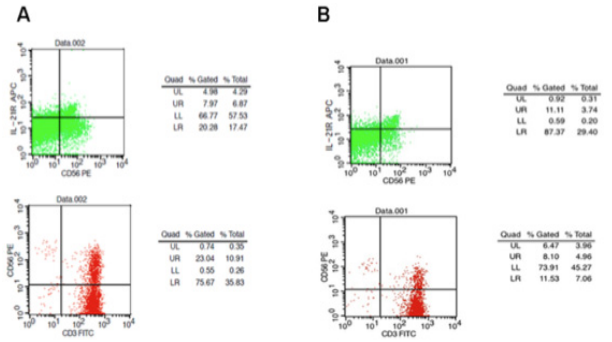

Figure 1. Cell Surface Expression of IL-21R of CIK Cells on Stimulation with IL-21. A. Expression of IL-21R normally expressed on CIK cells. B. Expression of IL-21R on CIK cells following stimulation with IL-21

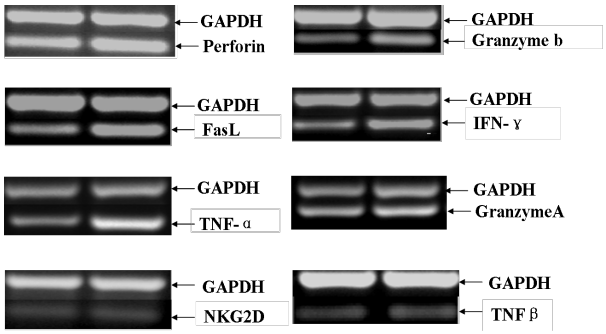

Figure 2. IL-21 Enhances the mRNA Expression of Perforin, Granzyme B, FasL, IFN-γ and TNF-α on CIK Cells. Following 14 days of Cell culture, CIK cells were separated into experimental and control groups. mRNA were isolated and reverse transcribed as described in materials and method. A semi quantitative RT-PCR with primers specific for each the factors was performed.PCR products were quantified by densitometric scanning and GAPDH was used as an internal control. *$p<0.05$ compared to control bars. The level of expression of each of the parameters were as follows: Interferon γ Control-0.5103±0.2358; Experiment 0.7705 ± 0.2493. Granzyme B Control: 0.4768±0.1589; Experiment: 0.7319±0.1639. Perforin Control: 0.7592±0.1457 Experiment 0.9831±0.1265. FasL control 0.4608±0.2842 experiment 0.7381±0.2568. TNFβControl 0.5993±0.1865 experiment 0.5688±0.1849. NKG2D control 0.6321±0.1586; Experiment 0.6042±0.1268

Cytotoxicity of CIK cells stimuated with IL-21 was observed to be (44.60±8.3)% which was significantly higher than the control group at (22.80±2.8)%.

There was no significant difference between the IL-21 and additional IL-2 stimulated groups but synergistic action of IL-21 and IL-2 was noted in enhancing the cytotoxic potential of CIK cells compared to IL-21 alone. Cytotoxicity of CIK cells stimulated with combined IL-21 +IL-2: (58.30±5.6)% when compared with IL-2 (45.20±3.5)% alone was also significantly increased. Results were calculated from three separate experiments. Surface expression of IL-21R on CIK cells

IL-21R is an integral part of the IL-21 receptor complex and mediates its action through this receptor. Flow cytometric determination of expression of IL-21R reveals that CIK cells normally expresses IL-21R valued at 7.97% (Figure 1A) and upoun stimulation with IL-21 there was no significant difference in its expression measured at 8.1% (Figure 1B).

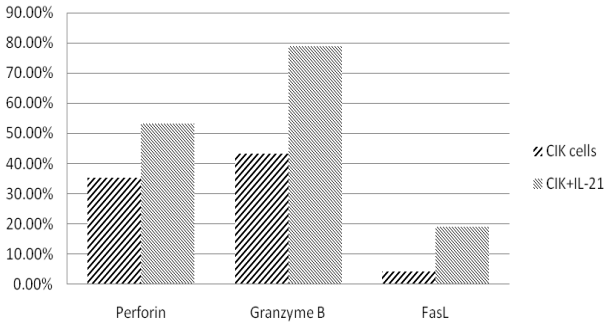

Figure 3. Intracellular Expression of Perforin, Graznyme B and FasL in CIK Cells Following IL-21 Stimulation. Flow cytometric analysis of CIK cells following stimulation with IL-21 for 72 hours using cell surface anti CD56 antibody and intracellular perforin, granzyme B and FasL anitbody showed signifcantly increased expression of perforin,granzyme and FasL. *For graphical representation data for FasL has been proportionately increased by 100 folds

IL-21 enhances the mRNA expression of perforin, granzyme B, FasL, IFN-γand TNF-αon CIK cells

To further evaluate the possible mechanism through which IL-21 enhances the cytotoxic potential of CIK cells we performed RT-PCR to check the endogeneous expression of cytotoxic granules (perforin, granzyme A, granzyme B), expression of cytokines such as IFN-γ and TNF-α, TNF β and FasL. As shown in Figure 2, Despite Basal levels of all the measured factors being considerably high in CIK cells, perforin, granzyme B, FasL, IFN-γ and TNF-α were expressed at a significantly higher proportion. Perforin was upregulated from 0.7592±0.1457 to 0.9831±0.1265. Granzyme B expression increased from 0.4768±0.1589 to 0.7319±0.1639. Similiarly FasL was increased from 0.4608±0.2842 to 0.7381±0.2568 and expression of Interferon γ increased from0.5103±0.2358 to 0.7705±0.2493. There was nominal increase in expression of TNFβ 0.5993±0.1865 to 0.5688±0.1849 and NKG2D expression which increased from 0.6321±0.1586; to 0.6042±0.1268 NKG2D receptor has also been implied in effective recognition of U266 by CIK cells (Verneris et al., 2004). Although IL-21 is said to down regulate expression of NKG2D/Dap10 in NK cells and T cells (Burgess et al., 2006) no significant increase in expression of NKG2D expression was observed. This finding might be significant as it suggests IL-21 helps in maintaining the potent NK cell like non MHC restricted cytotoxic function and cytokine secretion of CIK cells.

Flow cytometric analysis of Intracellular Expression of perforin, granzymeB, FasL, and secretion of IFN-γand TNF-α

To further elaborate the mechanism of IL-21 enhanced cytotoxicity of CIK cells. We measured the intracellular expression of Perforin, Granzyme B, FasL in the CIK cell pool following stimulation using flowcytometry.

Results showed the expression of perforin and graznyme B although normally elevated in CIK cells was significantly increased upoun IL-21 stimulation:perforin (35.28% to 53.16%.), Granzyme B 43.16% to 78.82%. The expression of FasL was also seen to increase from 0.04% to 0.19% (Figure 3).

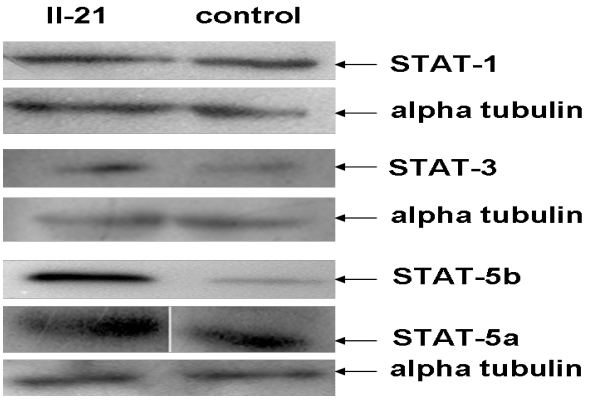

Figure 4. STAT-3 and STAT-5b are Involved in IL-21 Mediated Effects on CIK Cells. Expression of STAT proteins were monitored using western blot. CIK cells were washed and cultured in culture medium devoid of IL-2 for 24 hours after which they were stimulated with IL-21 (100 ng/ml) .The control groups were not stimulated with any cytokines and probed with STAT-1, STAT-3 and STAT-5 proteins. The membrane was re probed using alpha tubulin to ensure equal amount of protein was loaded onto the the gel

Secretion of Interferon γ and TNF-α was measured using ELISA which showed a two fold increase in the secretion of IFN-γ (25.8 ± 6.1) ng/L to (56.0 ± 2.3) ng/L and a three fold increase in secretion of TNF-α (5.64 ± 0.61) ug/L to (15.14 ± 0.93) ug/L following IL-21 stimualtion.

STAT-3 and STAT-5b are involved in IL-21 mediated effects on CIK cells

IL-21 has been well documented in expressing STAT proteins in stimulated and unstimulated T cells. These proteins have also been associated with target genes involved in apoptosis, survival and increasing perforin expression in T cells. Therefore we checked the level of expression of these proteins in CIK cells following activation with IL-21. Our findings were similiar to studies indicating that IL-21 was involved with activation of STAT-3 and STAT-5 proteins (Figure 4). CIK cells were also observed to express all these proteins nominally in absence of IL-21.

Discussion

IL-21, shares homology in sequence and structure to IL-2, IL-4 and IL-15, and is unique in its ability of maintaining both potent cytotoxicity and cellular viability. Such qualities make IL-21 a suitable agent for immunotherapy especially in conditions such as HIV, Chronic Hepatitis renal cell carcinoma, melanoma and non hodgkin's lymphoma, where a sustained and potent immune response is necessary to keep pathogens at bay (Thompson et al., 2008; Davis et al., 2009; Yi et al., 2009; Yue et al., 2010; Timmerman et al., 2012). IL-21 like IL-15 is also unique among the common γchain family of cytokines in its ability to maintain CD8 T cell population without causing any activation induced cell death (Moroz et al., 2004). Our present study reveals similar effects are possible in a group of terminally differentiated effector T cells.

CIK cells are a subset of T cells whose phenotype is similar to that of the terminally differentiated CD8 effector memory T cells lacking CD28 (Pievani et al., 2011). These cells have a high content of cytotoxic factors and increased ability to secrete IFN-γ and TNF-α but their response to antigens upon secondary stimulation is very weak. In our study mechanism of cytotoxic effect and possible effect on cell proliferation of IL-21 on CIK cells were examined 14 days following culture. Our results showed CIK cells didn't proliferate when stimulated with IL-21, but the number of cells expressing the CIK cell phenotype CD3+/CD56+ was significantly improved. Furthermore cytotoxic property of the cells within the CIK cell pool was significantly enhanced, both by IL-21 alone or when used in conjunction with IL-2 and the increase in cytotoxicity was associated with increase in mrna expression of cytotoxic factors and inflammatory cytokines. The increased proportion of CIK cells (CD3+/CD56+) without significant overall cell proliferation suggests IL-21 can sustain the growth of terminally differentiated effector T cells and when augmented by the strongly proliferative effect of IL-2 on CIK cell pool, IL-2 and IL-21 in combination could result in enhanced viability and proliferation of CIK cells (CD3+/CD56+).

IL-2 is well established cytokine widely used in clinical practice and it can induce cancer regression when administered to patients. However IL-2 induced differentiation of CD8+ T cells into cytolytic effector cells has a negative impact on their ability to induce tumor regression upon adoptive transfer, It also induces activation-induced cell death (AICD) and the development of suppressive T regulatory (Treg) cells and a cytokine that can suppress this process might enhance the efficacy of cells for adoptive immunotherapy.

IL-21 however is a new batch of cytokines which is closely related to IL-2 but differs in some core functions. Like IL-2, IL-21 can promote the function of effector CD8+ T cells, it however suppressed antigen-induced CD8+ T-cell acquisition of cytolytic effector lineage characteristics, this seems rather disappointing but these cells seem to proliferate more actively following adoptive transfer with higher rates of cancer regression in mouse models. When used together IL-2 could provide the signals important for activation and differentiation in cell culture while IL-21 maintains the T cell responses by supporting the survival of of cells in the CIK cell pool.

Binding of the IL-21 to the IL-21R results in the phosphorylation of JAK and concurrent activation of the STAT proteins, STAT proteins translocate to the nucles and initiate the transcription of IL-21 responsive genes. These STAT proteins are an integral part of the signal transduction cascade having a variety of functions involved in apoptosis, survival, oncogenesis and lymphoid cell proliferation. Recent studies indicate that intracellular STAT-1,STAT-3 and STAT-5 can induce secretion of IFN-γby binding to its activation site in NK cells (Strengell et al., 2003). Zeng et al reports that in IL-21 mediated proliferation of CD8+T cell STAT-1, STAT-3 STAT-5 are the most important intracellular signaling pathways and although not significant individually both MAPK and PI3K pathways in conjunction can have a significant

effect on IL-21 mediated proliferation of CD8+ T cells (Zeng et al., 2007).

With such notable effects on CD8+ Tcells and NK cells we tested whether IL-21 had a similar effect on CIK cells. In our experiment we found that IL-21 activated STAT-3 and STAT-5b in the CIK cell pool. STAT-1, STAT-3 and STAT-5b have been observed to have important and at times opposite effects on lymphocytes. Expression of STAT-1 is generally associated with apoptosis but decreased survivability of activated CD8+ T cells, diminished ability to differentiate into long-lived memory cells (Quigley et al., 2008) and reduction of antitumor activity of NK cells has been noted in STAT-1 deficient mice (Lee et al., 2000).

IL-21 is unique in its ability to induce STAT-3. STAT-3 is mostly associated with tumorigenesis and lymphocyte proliferation. Interestingly STAT-3 can inhibit a widely observed activation induced apoptotic effect of IL-2 on CD8+ T cells. STAT-3 has been observed to protect T-cells from apoptosis by limiting their production of IL-2 through up-regulation of FoxO1/FoxO3a (Oh et al., 2011). Although studies indicate STAT-3 ablation increases the anti tumor effect of NK, T cells and neutrophils, its absence has also been associated with an overtly activated innate immune response (Welte et al., 2003; Kortylewski et al., 2005). Whether the activation of STAT-3 transforms the immune response of CIK cells into a subdued persistent form or abrogates it within time remains a question to be answered.

STAT-5 is prosurvival protein. It has been shown to be important in survivability of antigen specific effector and memory CD8+ T cells, NK cells (Hand et al., 2010; Eckelhart et al., 2011). STAT5b has been shown to increase expansion of naïve CD8+ T cells and increased conversion of such cells into memory CD8+ T cells (Burchill et al., 2003).

In summary IL-21 can enhance cytotoxic property of CIK cells against K562 cells through an increased expression of perforin, granzyme B and FasL. IL-21 can also maintain CIK cell population with potent antitumor capabilites with the expression of STAT-3 and STAT-5b proteins. The role of IL-21 in maintaining antitumor function of activated cells suggests it can be a vital tool for an efficient transfer and persistently active immune attact following adaptive immunotherapy.

Acknowledgements

This study was supported by the National Natural Sciences Foundation of China (81041043) and the Key project of Science and Technology Fundation of Tianjin Health Bureau (2011KR01).

References

Allard EL, Hardy MP, Leignadier J, et al (2007). Over expression of IL-21 promotes massive CD8+ memory T cell accumulation. *Eur J Immunol*, **37**, 3069-77.

Brandt K, Bulfone-Paus S, Jenckel A, et al (2003). Interleukin-21 inhibits dendritic cell-mediated T cell activation and induction of contact hypersensitivity in vivo. *J Invest Dermatol*, **121**, 1379-82.

Burchill MA, Goetz CA, Prlic M, et al (2003). Distinct Effects of STAT5 Activation on CD4+ and CD8+ T Cell Homeostasis: Development of CD4+CD25+ Regulatory T Cells versus CD8+ Memory T Cells. *J Immunol*, **171**, 5853-64.

Burgess SJ, Marusina AI, Pathmanathan I, Borrego F, Coligan JE (2006). IL-21 down-regulates NKG2D/DAP10 expression on human NK and CD8+ T cells. *J Immunol*, **176**, 1490-7.

Davis ID, Brady B, Kefford RF, et al (2009). Clinical and biological efficacy of recombinant human interleukin21 in patients with stage IVmalignant melanoma without prior treatment:a phase IIa trial. *Clin Cancer Res*, **15**, 2123-9.

Decker T, Bogner C, Oelsner M, Peschel C, Ringshausen I (2010). Antiapoptotic effect of interleukin-2 in B-CLL cells with low and high affinity IL-2 receptors. *AnnHematol*, **89**, 1125 -32.

Gattinoni L, Klebanoff CA, Restifo NP (2012). Paths to stemness: building the ultimate antitumour T cell. *Nat Rev Cancer*, **12**, 71-84.

Golay J, Manganini M, Facchinetti V, et al (2003). Rituximab-mediated antibody dependent cellular cytotoxicity against neoplastic B cells is stimulated strongly by interleukin-2. *Haematologica*, **88**, 1002-12.

Gowda A, Roda J, Hussain SR, et al (2008). IL-21 mediates apoptosis through up-regulation of the BH3 family member BIM and enhances both direct and antibody-dependent cellular cytotoxicity in primary chronic lymphocytic leukemia cells in vitro. *Blood*, **111**, 4723-30.

Hand TW, Cui W, Jung YW, et al (2010). Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. *Proc Natl Acad Sci U S A*, **107**, 16601-6.

Kasaian MT, Whitters MJ, Carter LL, et al (2002). IL-21 Limits NK Cell Responses and Promotes Antigen-Specific T Cell Activation. *Immunity*, **16**, 559-69.

Kortylewski M, Kujawski M, Wang T, et al (2005). Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med*, **11**, 1314-21.

Lee CK, Rao DT, Gertner R, et al (2000). Distinct requirements for IFNs and STAT1 in NK cell function. *J Immunol*, **165**, 3571-7.

Leemhuis T, Wells S, Scheffold C, Edinger M, Negrin RS (2005). A phase I trial of autologous cytokine-induced killer cells for the treatment of relapsed Hodgkin disease and non-Hodgkin lymphoma. *Biol Blood Marrow Transplant*, **11**, 181-7.

Moroz A, Eppolito C, Li Q, et al (2004). IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. *J Immunol*, **173**, 900-9.

Novy P, Huang X, Leonard WJ, Yang Y (2011). Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection. *J Immunol*, **186**, 2729-38.

Oh HM, Yu CR, Golestaneh N, et al (2011). STAT3 protein promotes T-cell survival and inhibits interleukin-2 production through up-regulation of Class O Forkhead transcription factors. *J Biol Chem*, **286**, 30888-97.

Olioso P, Giancola R, Di Ritti M, et al (2009). Immunotherapy with cytokine induced killer cells in solid and hematopoietic tumours: a pilot clinical trial. *Hematol Oncol*, **27**, 130-9.

Ozaki K, Spolski R, Ettinger R, et al (2004). Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol*, **173**, 5361-71.

Parmigiani A, Pallin MF, Schmidtmayerova H, Lichtenheld MG, Pahwa S (2011). IL-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells. *Hum Immunol*, **72**, 115-23.

Parrish-Novak J, Dillon SR, Nelson A, et al (2000). Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*, **408**, 57-63.

Pievani A, Borleri G, Pende D, et al (2011). Dual-functional capability of CD3+CD56+CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood*, 18, 3301-10.

Quigley M, Huang X, Yang Y (2008). STAT1 signaling in CD8 T cells is required for their clonal expansion and memory formation following viral infection in vivo. *J Immunol*, **180**, 2158-64.

Rankin AL, MacLeod H, Keegan S, et al (2011). IL-21 receptor is critical for the development of memory B cell responses. *J Immunol*, **186**, 667-74.

Strengell M, Matikainen S, Sirén J, et al (2003). IL-21 in synergy with IL-15 or IL-18 enhances IFN-Y production in Human NK and T cells. *J Immunol*, **170**, 5464-9.

Timmerman JM. Byrd JC, Andorsky DJ, et al (2012). Efficacy and safety of recombinant interleukin-21 (rIL-21) and rituximab in relapsed/refractory indolent lymphoma. *Clin Cancer Res*, **18**, 5752-60.

Thompson JA, Curti BD, Redman BG, et al (2008). Phase I study of recombinant interleukin-21 in patients with metastatic melanoma and renal cell carcinoma. *J Clin Oncol*, **26**, 2034-9.

Verneris MR, Karami M, Baker J, Jayaswal A, Negrin RS (2004). Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood*, **103**, 3065-72.

Welte T, Zhang SS, Wang T, et al (2003). STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. *Proc Natl Acad Sci U S A*, **100**, 1879-84.

Yi JS, Du M, Zajac AJ (2009). A vital role for interleukin-21 in the control of a chronic viral infection. *Science*, **324**, 1572-6.

Yue FY, Lo C, Sakhdari A, et al (2010). HIV-specific IL-21 producing CD4+ T cells are induced in acute and chronic progressive HIV infection and are associated with relative viral control. *J Immunol*, **185**, 498-506.

Zeng R, Spolski R, Casas E, et al (2007). Mechanism of IL-21 mediated proliferation. *Blood*, **109**, 4135-42.

Zhao MF, Deng Q, Li YM, et al (2007). Interleukin 21 enhances the expansion and anti-tumor cytotoxic activity of cytokine-induced killer cells derived from both peripheral blood and cord blood in vitro. *Blood*, **110**, 4887b. (Ash meeting abstract)