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FOXP3 Inhibitory Peptide P60 Increases Efficacy of Cytokine-induced Killer Cells Against Renal and Pancreatic Cancer Cells

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Abstract. *Background/Aim: Cytokine-induced killer (CIK) cells are ex vivo expanded major histocompatibility complex (MHC)-unrestricted cytotoxic cells with promising effects against a variety of cancer types. Regulatory T-cells (T-reg) have been shown to reduce the effectiveness of CIK cells against tumor cells. Peptide P60 has been shown to inhibit the immunosuppressive functions of T-regs. This study aimed at examining the effect of p60 on CIK cells efficacy against renal and pancreatic cancer cells. Materials and Methods: The effect of P60 on CIK cytotoxicity was examined using flow cytometry, WST-8-based cell viability assay and interferon γ (IFNγ) ELISA. Results: P60 treatment resulted in a significant decrease in the viability of renal and pancreatic cancer cell lines co-cultured with CIK cells. No increase in IFNγ secretion from CIK cells was detected following treatment with P60. P60 caused no changes in the distribution of major effector cell populations in CIK cell cultures. Conclusion: P60 may potentiate CIK cell cytotoxicity against tumor cells.*

Worldwide annual cancer deaths are on the rise with 5.8 million deaths in 1990, 8.2 million deaths in 2012 and an estimated death count of 13.2 million for the year 2030, making cancer one of the leading causes of death in the developed world (1, 2). Due to these numbers the development of new cancer treatments, as well as the improvement of existing therapies remain a major focus of research today. Several studies have shown that adoptive cell transfer of CIK cells leads to a better prognosis and an increased quality of life in patients with various solid or non-solid cancer types (3). Furthermore, CIK cells have been shown to work in synergy with various more conventional cancer treatments (4, 5). In order to better orchestrate and set standard criteria for ongoing and future clinical studies involving CIK cells, the international registry on CIK cells (IRCC) has been founded (6). Improvement of CIK cell efficiency in cancer treatment has been the focus of various studies (7, 8).

CIK cells are T-cells with a mixed T-cell and natural killer (NK) cell-like phenotype that possess a strong MHC-unrestricted anti-tumor cytotoxicity (9, 10). CIK cells were first described in 1991 and are commonly generated from peripheral mononuclear blood cells (PBMC) using a cytokine cocktail consisting of IFNγ, anti-CD3 monoclonal antibody, interleukin-2 (IL-2) and interleukin-1 beta (IL-1β) (11). CIK cell cultures are heterogeneous, consisting of several different cell populations (7). These populations can be differentiated in the presence or absence of the T-cell receptor marker CD3 and the NK cell marker neural cell adhesion molecule (NCAM/CD56). More than 90% of all cells in CIK cultures express CD3 when mature after two weeks of culture (10). These $CD3^+$ cells can be further divided into $CD3^+CD56^-$ T-cell population and double positive $CD3^+CD56^+$ cell population. The double-positive $CD3^+CD56^+$ cells have been identified as the main effector cell population and are foremost responsible for the characteristic MHC-unrestricted anti-tumor activity of CIK

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cell cultures (9). CD3+CD56$^-$ cells mainly consist of conventional T-cells, but also contain a minor fraction of immunosuppressive CD4$^+$CD25$^+$FOXP3$^+$ regulatory T-cells (T-reg) (11). T-regs have been shown to have a negative impact on the anti-tumor cytotoxicity of the CIK cell culture, which makes them an interesting target for the improvement of CIK cell efficiency in cancer treatment (7, 12).

The characteristics of peptide P60 have been first described by Casares *et al*. in 2010 (13). It has been selected for its high binding affinity towards the transcriptional regulator protein forkhead box P3 (FOXP3), which is commonly expressed in regulatory T-cells. While the exact binding site remains unknown, it has been shown that P60 suppresses several major functions of FOXP3 in a reversible manner, including nuclear translocation of FOXP3 and the inhibition of transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor of activated T-cells (NFAT) (13). These abilities of FOXP3 are known to be critical for many immunosuppressive effects exhibited by T-regs. It has been shown that treatment with P60 greatly restores effector T-cell function in the presence of immunosuppressive regulatory T-cells (13). Further work to improve the efficacy of P60 by optimizing its sequence is still ongoing (14).

This study aimed to enhance the cytotoxicity of CIK cells against tumor cells by treating them with peptide P60 in order to overcome the immunosuppressive effects of regulatory T-cells that are commonly present as minor population in CIK cell cultures (15).

Materials and Methods

Peptides P60 and P301. Both peptides were provided by N. Casares from the Center of Applied Medical Research of the University of Navarra. Peptide P60 (RDFQSFRKMWPFFAM) was selected from a phage-displayed random 15-mer peptide library. The control peptide P301 (RPGNKTVLBITLMSG) encompasses the amino acids 301-315 of the HIV-1-derived glycoprotein gp120. Peptides were added to the cells after 8 days of culture for 48 hours at a working concentration of 100 μM.

Cell lines and cell culture. The cell lines A-498, Caki-2, Capan-2 and DAN-G were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. All cell lines were grown in their respective standard growth media with 1% Penicillin/Streptomycin (P/S) solution (Gibco® by Life science technologies™ , Carlsbad, CA, USA). The cells were incubated aseptically at 37˚C in the presence of 5% CO_2.

CIK cell generation. CIK cells were isolated from PBMCs derived from healthy blood donors and generated according to the established CIK cell generation standard protocol (11). CIK cells were grown in RPMI 1640 medium with 2.5% (v/v) of HEPES buffer (both from PAN-Biotech GmbH, Aidenbach, Germany), 10% (v/v) heat inactivated FCS, 1% P/S (both gibco® by life science technologies™ , Carlsbad, CA, USA) and were incubated aseptically at 37˚C with 5% CO_2. The cytokine cocktail for CIK cell generation was added to achieve growth conditions with cytokine concentrations of 1000 IU/ml RhIFNγ (R&D Systems Inc., Minneapolis, ME, USA), 300 IU/ml interleukin-2 (Novartis Pharma AG, Basel, Switzerland), 50 ng/ml anti-human anti-CD3 mAb and 100 IU/ml interleukin-1β (both from eBioscience Inc., San Diego, CA, USA). Interleukin-2 was replenished every third day of culture. The cells were used for investigation after 8 days of culture, the timepoint where the percentage of regulatory T cells in CIK cell cultures has been shown to reach its peak value (15).

Cell cytotoxicity assay. A WST-8-based Cell counting kit-8 (Dojindo EU GmbH, Munich, Germany) was used to determine the cytotoxicity of CIK cells against cell lines after 48 h of co-culture in an effector-target cell ratio of 20:1. The absorbance of the resulting color reaction was measured with a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) at a wavelength of 450 nm. The percentages presented in the figures represent the ability of CIK cells in killing tumor cells with 100 % equaling the killing ability of untreated CIK cells.

Flow cytometry. Intracellular FOXP3 staining and permeabilization was done using an anti-human FOXP3 FITC staining kit (eBioscience, Santa Clara, CA, USA). Surface staining antibodies were ordered from Biolegend, San Diego, CA, USA. Isotype control antibodies were used to exclude non-specific signals (eBioscience and Biolegend). Dead cells were excluded using Zombie Aqua™ staining (Biolegend). Flow cytometry samples were read using a BD FACS Canto™ II with the corresponding FACS Diva Software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The raw flow cytometry data were analyzed using FlowJo single cell analysis software v10 (FlowJo, LLC, Ashland, OR, USA).

Interferon-gamma ELISA. The cytokine-assay was performed using an IFN gamma DuoSet® ELISA kit, the Substrate Reagent Pack, normal goat serum and stop solution (all from R&D systems, Minneapolis, ME, USA). Assay plates were read at wavelengths of 450 nm and 570 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7.02 software. Statistical significance was determined using one-way ANOVA with a confidence level of 95%, followed by a Dunnett's multiple comparison as the post-test.

Results

Effects of peptide P60 on major CIK cell culture populations. Figure 1 shows the cell populations in CIK cultures after their respective treatment and analysis *via* flow cytometry. Figure 1A and B together show about 90 % of all cells were CD3$^+$, with roughly 60% CD3$^+$CD56$^+$ and around 30% CD3$^+$CD56$^-$. Figure 1C and D show that almost 80% of all cells were CD8$^+$ while around 10-15% were CD4$^+$. Out of all CD4$^+$ cells, the percentage of CD25$^+$FOXP3$^+$ regulatory T-cells was around 6-8% as displayed in Figure 1E. Altogether, neither treatment with P60 nor with control peptide P301 caused a significant difference in the distribution of the investigated CIK cell populations.

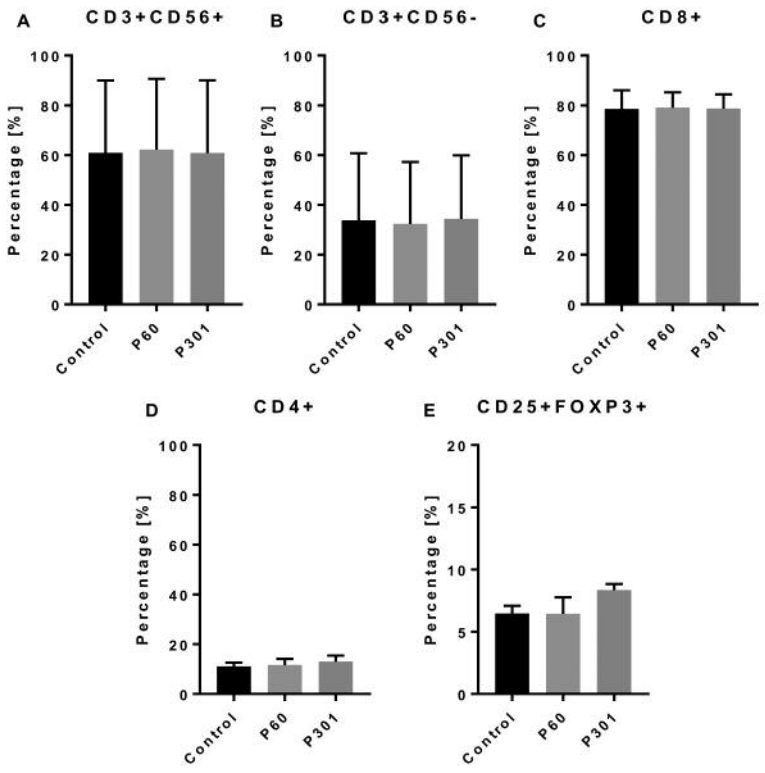

Figure 1. *Effects of P60 and control peptide P301 on the distribution of major CIK cell populations and regulatory T cells after 10 days of culture. N=3 healthy donors. (A-D) Percentages of (A) CD3$^+$CD56$^+$, (B) CD3$^+$CD56$^-$, (C) CD8$^+$, (D) CD4+ cells out of all viable cells. (E) Percentage of CD25$^+$FOXP3$^+$ cells out of all CD4$^+$ cells.*

Effects of peptide P60 on the cytotoxicity of CIK cells against renal cell lines A-498 and Caki-2. Figure 2 shows the percentages of viable cells in relation to the untreated control after treatment with P60 or control peptide P301 in co-cultures of CIK cells with renal cell lines A-498 or Caki-2 for 48 h. Figures 2B and E show that the number of viable A-498 and Caki-2 cells in mono-cultures remain unchanged with percentages around 90-100% after treatment with P60 or P301. Similarly, the viability of CIK cells in mono-culture after each treatment is shown to be similar when compared to the untreated control (Figure 2C and F). These data suggest that neither the viability of CIK cells, nor that of the renal cell lines A-498 or Caki-2 were directly affected by the treatment with either P60 or P301. Figures 2A and D show the viable cell percentages of co-cultures of CIK cells with A-498 or Caki-2 cells, respectively. In the co-cultures of both renal cell lines with CIK cells a significantly lower percentage of viable cells was observed after treatment with P60 when compared to the untreated co-cultures or those treated with control peptide P301. This may indicate an increased cytotoxicity of CIK cells against the respective cell line in the presence of P60.

Effects of peptide P60 on the cytotoxicity of CIK cells against pancreatic cell lines DAN-G and Capan-2. Figure 3 shows the percentages of viable cells in relation to the untreated control after treatment with P60 or P301 in co-cultures of CIK cells with the pancreatic cell lines DAN-G and Capan-2 for 48 hours. Similar to the renal cell lines (Figure 2), the viability of the mono-cultures of DAN-G and Capan-2 cells was not significantly affected (Figures 3B and E). Likewise, Figures 3C and F show that the viability of mono-cultures of CIK cells was not affected by any of the three treatments. Figure 3A and D depict the percentages of viable cells of co-cultures CIK cells with DAN-G or Capan-2 cells. A significantly lower number of viable of both pancreatic cells was detected after the treatment of co-cultures with P60 when compared to the other treatments, indicating a possible increase of CIK cytotoxicity against the cell lines, similarly to the observations previously made for renal cell lines.

Discussion

The flow cytometry data presented in Figure 1 shows that there are no quantitative effects on the distribution of major

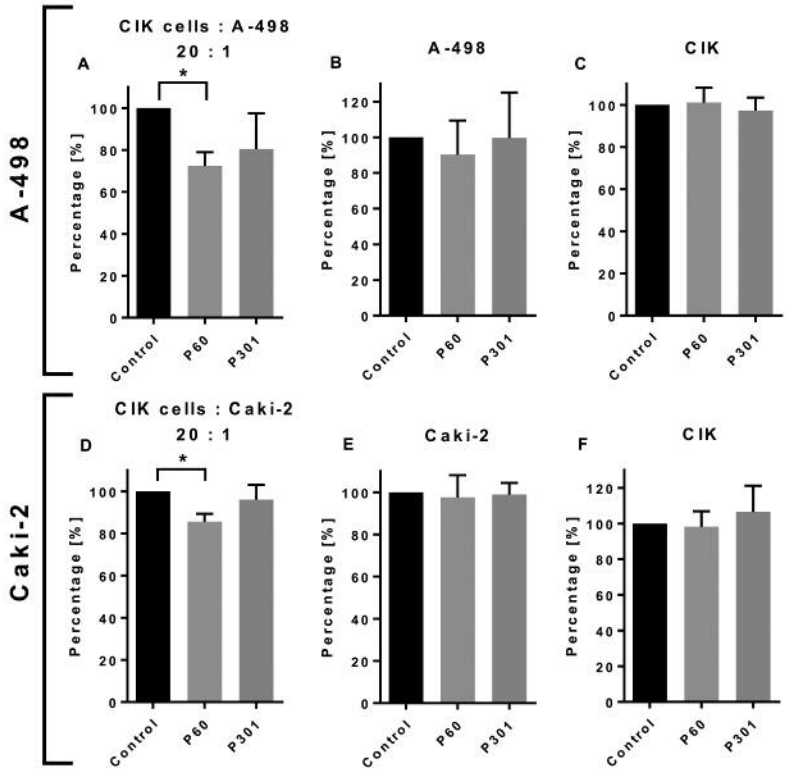

Figure 2. *Effects of P60 and control peptide P301 on the viability of renal cancer cells. Mono-cultures of A-498, Caki-2 and CIK cells as well as co-cultures of CIK cells with A-498 or Caki-2 cells were treated with P60 or control peptide for 48 h. Following treatment, the number of viable cells was assayed by the WST-8-based Cell counting kit-8. N=3 healthy donors. Absorbance values were expressed as percentages with each untreated control equaling 100%. p≤0.05. (A) Co-culture of CIK cells with A-498 in a ratio of 20:1. (B) Mono-culture of A-498. (C) Mono-culture of CIK cells (D) Co-culture of CIK cells with Caki-2 in a ratio of 20:1. (E) Mono-culture of Caki-2. (F) Mono-culture of CIK cells.*

effector CIK populations as well as regulatory T-cells following treatment with P60. This observation is in accordance with the findings of Casares *et al*. who did not detect any effects of P60 on the viability and proliferation rate of both effector T-cells and regulatory T-cells using CFSE dilution assays and 3H-thymidine incorporation assays (13). These findings further solidify the idea, that the effects of P60 are not derived from a quantitative depletion of certain cell populations, but rather from their functional inhibition.

In Figures 2 and 3 we can observe a significant decrease in total cell viability cancer cells co-cultured with CIK cells following treatment with P60 compared to the untreated co-cultures and those treated with the control peptide. The decreased cell viability may indicate an increased CIK cell cytotoxicity against the cell lines. This possibility is strengthened by the observation that unlike the co-cultures, the viability of mono-cultures of CIK cells or of the cancer cell lines was not affected upon treatment with P60. These data exclude the possibility that the reduced viability in the co-cultures derived from a direct effect of P60 on the viability or proliferation of either CIK cells or cancer cell lines.

In order to gain further proof for an increased cytotoxicity of CIK cells following treatment with P60, changes in interferon-γ secretion were measured, as an indicator for CIK cytotoxicity. However, using an IFNγ-ELISA, we were unable to detect any significant changes in secreted IFNγ levels upon treatment with P60 (data not shown). Due to generally low IFNγ concentrations, it might be required to either increase the CIK cell count during co-culture or to apply a more sensitive cytokine detection assay like ELISPOT or use flow cytometry in order to get a final confirmation about the effects of P60 on IFNγ secretion levels.

Our study provided evidence that treatment of CIK cells with the FOXP3-inhibitory peptide P60 increased their cytotoxic activity. Furthermore, was not found to be mediated through an effect of P60 on the viability or proliferation rates of either effector T-cells or regulatory T-cells in CIK cell cultures, providing further proof of a functional and non-cell-depleting inhibitory effect of P60, as it has previously been

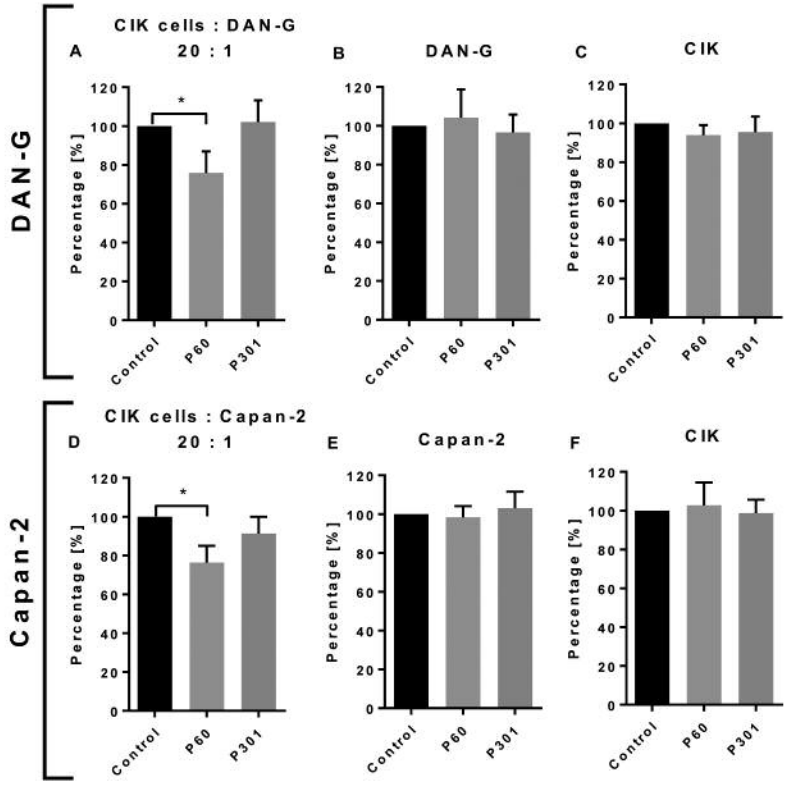

Figure 3. *Effects of P60 and control peptide P301 on the number of viable pancreatic cancer cells. Mono-cultures of DAN-G, Capan-2 and CIK cells as well as co-cultures of CIK cells with DAN-G or Capan-2 were treated with P60 or control peptide for 48 h. Following treatment, the number of viable cells was assayed by the WST-8-based Cell counting kit-8. N=3 healthy donors. Absorbance values were expressed as percentages with each untreated control equaling 100%, p≤0.05. (A) Co-culture of CIK cells with DAN-G in a ratio of 20:1. (B) Mono-culture of DAN-G. (C) Mono-culture of CIK cells (D) Co-culture of CIK cells with Capan-2 in a ratio of 20:1. (E) Mono-culture of Capan-2. (F) Mono-culture of CIK cells.*

described by Casares *et al*. (13). However, we were unable to provide further confirmation that increased CIK cell cytotoxicity was mediated *via* increased IFNγ secretion levels after treatment with P60. Elucidation of the role of IFNγ secretion levels will require further investigations.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Maria Fitria Setiawan, Oliver Rudan and Ingo G. H. Schmidt-Wolf conceived and designed the experiments; Maria Fitria Setiawan and Oliver Rudan performed the experiments; Maria Fitria Setiawan, Oliver Rudan, Hans Weiher and Ingo G.H. Schmidt-Wolf analyzed the data; Annabelle Vogt, Bettina Langhans, Christian P. Strassburg, Roland Schmidt-Wolf, Francesca Garofano, Juan J. Lasarte, Noelia Casares, Teresa Lozano, Maria A. Gonzalez-Carmona and Ingo G. H. Schmidt-Wolf contributed reagents and materials; Maria Fitria Setiawan and Oliver Rudan wrote the paper.

References

1 Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE *et al*: Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis of the Global Burden of Disease Study. Lancet *380(9859)*: 2095-2128, 2010. PMID: 23245604. DOI: 10.1016/S0140-6736(12)61728-0

2 Bodduluru LN, Kasala ER, Madhana RM and Sriram CS: Natural killer cells: The journey from puzzles in biology to treatment of cancer. Cancer Lett *357*: 454-467, 2015. PMID: 25511743. DOI: 10.1016/j.canlet.2014.12.020

3 Urruticoechea A, Alemany R, Balart J, Villanueva A, Viñals F and Capellá G: Recent advances in cancer therapy: An overview. Curr Pharm Design *16*: 3-10, 2010. PMID: 20214614. DOI: 10.2174/138161210789-941847

4 Bullok KF, Sippel C and Schmidt-Wolf IG: Increased effect of IMiDs by addition of cytokine-induced killer cells in multiple myeloma. Hematol Oncol *34(4)*: 208-126, 2016. PMID: 25784599. DOI: 10.1002/hon.2200

5 Esser L, Weiher H and Schmidt-Wolf IG: Increased efficacy of brentuximab vedotin (SGN-35) in combination with cytokine-induced killer cells in lymphoma. Int J Mol Sci *17(7)*: 1056, 2016. PMID: 27376285. DOI: 10.3390/ijms17071056

6 Schmeel FC, Schmeel LC, Gast SM and Schmidt-Wolf IG: Cytokine-induced killer (CIK) cells in cancer immunotherapy: report of the international registry on CIK cells (IRCC). J Cancer Res Clin Oncol *141(5)*: 839-849, 2015. PMID: 27376285. DOI: 10.1007/s00432-014-1864-3

7 Schmeel FC, Schmeel LC, Gast SM and Schmidt-Wolf IG: Adoptive immunotherapy strategies with cytokine-induced killer (CIK) cells in the treatment of hematological malignancies. Int J Mol Sci *15(8)*: 14632-14648, 2014. PMID: 25196601. DOI: 10.3390/ijms150814632

8 Rettinger E, Kuçi S, Naumann I, Becker P, Kreyenberg H, Anzaghe M, Willasch A, Koehl U, Bug G and Ruthardt M: The cytotoxic potential of interleukin-15-stimulated cytokine-induced killer cells against leukemia cells. Cytotherapy *14*: 91-103, 2012. PMID: 21973023. DOI: 10.3109/14653249.2011.613931

9 Jiang J, Wu C and Lu B: Cytokine-induced killer cells promote antitumor immunity. J Transl Med *11*: 83, 2013. PMID: 235269996. DOI: 10.1186/1479-5876-11-83

10 Wang X, Yu W, Li H, Yu J, Zhang X, Ren X and Cao S: Can the dual-functional capability of CIK cells be used to improve antitumor effects? Cell Immunol *287*: 18-22, 2014. PMID: 24355711. DOI: 10.1016/j.cellimm. 2013.11.009

11 Schmidt-Wolf IG, Negrin RS, Kiem H, Blume KG and Weissman IL: Use of SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. J Exp Med *174(1)*: 139-149, 1991. PMID: 1711560. DOI: 10.1084/jem.174.1.139

12 Li H, Yu J, Cao S, Wei F, Zhang P, An X, Huang Z and Ren X: CD4+CD25+ Regulatory T cells decreased antitumor activity of cytokine-induced killer (CIK) cells of lung cancer patients. J Clin Immunol *27(3)*: 317-326, 2007. PMID: 17468835. DOI: 10.1007/s10875-007-9076-0

13 Casares N, Rudilla F, Arribillaga L, Llopiz D, Riezu-Boj JI, Lozano T, López-Sagaseta J, Guembe L, Sarobe P, Prieto J, Borrás-Cuesta F and Lasarte JJ: A peptide inhibitor of FOXP3 impairs regulatory T cell activity and improves vaccine efficacy in mice. J Immunol *185(9)*: 5150-5159, 2010. PMID: 20870946. DOI: 10.4049/ jimmunol.1001114

14 Lozano T, Gorraiz M, Lasarte-Cía A, Ruiz M, Rabal O, Oyarzabal J, Hervás-Stubbs S, Llopiz D, Sarobe P, Prieto J, Casares N and Lasarte JJ: Blockage of FOXP3 transcription factor dimerization and FOXP3/AML1 interaction inhibits regulatory cell activity: sequence optimization of a peptide inhibitor. Oncotarget *8*: 71709-71724, 2017. PMID: 29069740. DOI: 10.18632/oncotarget.17845

15 Pan Y, Tao Q, Wang H, Xiong S, Zhang R, Chen T, Tao L and Zhai Z: Dendritic cells decreased the concominant expanded Tregs and Tregs Related IL-35 in cytokine-induced killer cells and increased their cytotoxicity against leukemia cells. PLoS One 9(4), 2014. PMID: 24705499. DOI: 10.1371/journal. pone.0093591

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