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Research Article

Modification of Natural Killer cells to target tumors

*Wisam Alsamah, Yasmin Romia

Department of pharmaceutical science, Faculty of pharmacy, The International University for science and technology (IUST), Jebab, Darra.

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ABSTRACT

The background and the aim: NK cells cytolytic and cytokine production activities suggest them as a potential source for adoptive therapy. I engineered NK cells with chimeric receptors (CARs) specific for HER-2, a tumor-associated antigen frequently overexpressed by many tumors of epithelial origin, providing them with tumor-antigen targeting specificity. Material and Methods: NK cells have been genetically modified by using the following techniques: Isolation of human PBMCs, expansion of primary human NK cells, retroviral transduction. Results: I compared first and second generation CARs including a stimulatory (CD3ζ) signaling domain alone or together with a costimulatory (CD28) signaling domain. I found that costimulatory signaling markedly improved IL-2 production by NK cells. Conclusion: Thus, the direct coupling of the antibody specificity to the function of NK cells by CAR expression is uniquely able to enhance tumor cells targeting by NK cells.

Key words: NK cells, CAR, HER-2.

INTRODUCTION

Adoptive cell therapy is a form of immunotherapy based on the use of tumor targeting lymphocytes [1-2]. While being usually considered as a form of T cell therapy, the utilization of NK cells in this approach is becoming increasingly attractive to target tumors including those progressing as T cell escape variants [3-4]. NK cells represent a cell subset accounting for approximately 10-15% of blood lymphocytes and are thought to represent important effectors of the innate immune response. Since their discovery NK cells were described as cytotoxic lymphocytes, which are able to destroy certain tumor cells in vitro without prior sensitization. The molecular mechanism of tumor cell recognition by NK cells is regulated by a balance of activating and inhibitory signals. Activating receptors, such as the NKp46 [5] and other natural cytotoxicity receptors (NCRs) as well as NKG2D [6], are sometimes able to completely override the inhibitory signal delivered by inhibitory killer immunoglobulin-like receptors (KIRs), which interact with MHC class I molecules. HER-2 is amplified and overexpressed in 30- 80% of human breast, ovarian, pancreatic, colon, gastric, lung and prostate carcinomas and melanoma [7] and its overexpression correlates with a more aggressive disease [8]. Therefore, HER-2 represents an attractive target for immunotherapy.

Recombinant chimeric receptors (CARs) combine the antigen-specific binding properties of a monoclonal antibody with the cytotoxic activity of lymphocytes in a single molecule and are currently implemented in a panel of clinical trials using predominantly CAR-modified T cells [9, 10]. Here I report an optimized protocol for the engineering of primary human NK cells with HER-2 specific CARs targeting HER-2 expressing tumor cell lines.

MATERIALS AND METHODS

Isolation of human PBMCs Buffy coats from the blood of anonymous healthy donors were obtained from the blood bank and were diluted 1:2 with PBS. Mononuclear cells were collected from the diluted samples by centrifugation using a Lymphoprep density separation (Bicoll, Biochrom AG, Berlin, Germany; or PANcoll ready-to-use, PAN-Biotech, Aldenbach, Germany). The PBMCs were washed two to four times in PBS and were frozen to maintain standard conditions.

Retroviral vectors: The chimeric receptor constructs specific for HER-2, C6.5-scFv-Fc-CD3ζ and C6.5-scFv-Fc-CD3ζ-CD28 were earlier described [11]. The CARs were cloned into the modified retroviral vector pMIG by replacing the GFP and IRES fragment. The resulting constructs were designated pMSCV-CARz and pMSCV-CRz28, respectively. The pMIG vector encoding GFP was used as control.

Expansion of primary human NK cells

Thawed PBMCs were washed twice in RPMI 1640 medium. The cells were seeded at 2x107 cells in 20 ml medium into a T 125 tissue culture flask. The cells were incubated at 37°C for 30 min to allow adherent cells to attach to the flask surface. Non-adherent PBLs were carefully harvested and counted. Meanwhile, RPMI 8866 feeder cells were collected, irradiated with 30 Gy and washed twice. The cells were then cocultured in a 6 well plate at 1.5x106 PBLs/well together with 3x105 RPMI 8866 cells/well for the ratio 5:1, at 1.5x106 PBLs/well together with 5x105 RPMI 8866 cells/well for the ratio 3:1,

*Author for correspondence: E-mail: Walsamah@yahoo.com

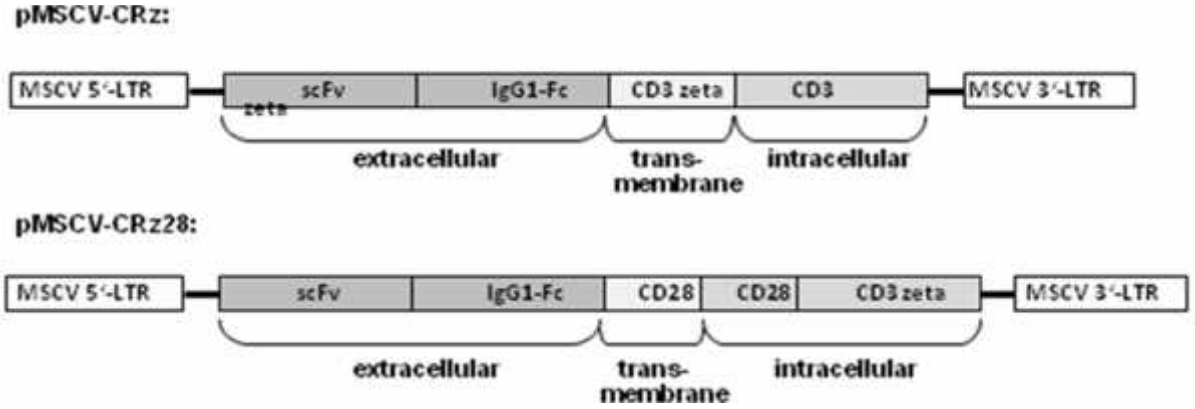

Figure 1A. A schematic representation of the two CAR constructs used in this study.

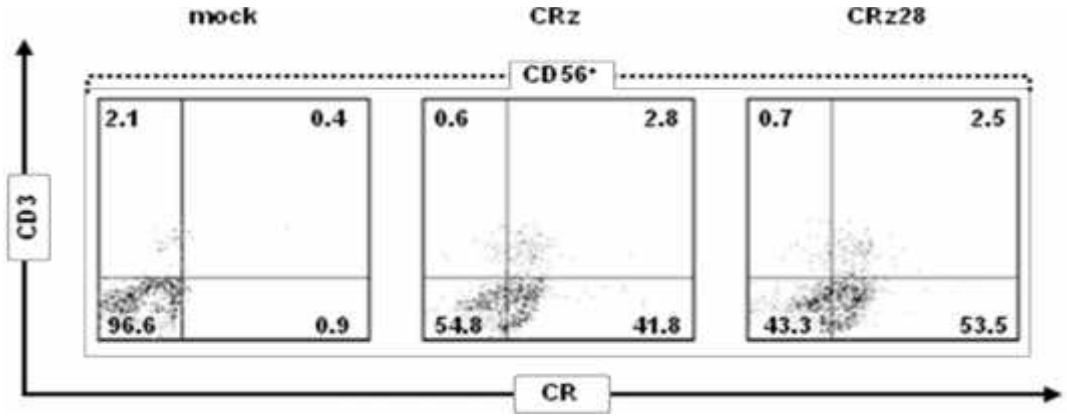

Figure 1B. Expression of the transduced CRs on primary human NK cells.

at 5x105 PBLs/well together with 1.5x106 RPMI 8866 cells/well for the ratio 1:3 and at 3x105 PBLs/well together with 1.5x106 RPMI 8866 cells/well for the ratio 1:5.

Retroviral transduction: Spinocculation transduction was performed on day 6 or 8 using 2x105 cells/well were seeded into a 24-well tissue culture plate in a total volume of 2 ml virus supernatant diluted 1:1 with culture medium in the presence of 8 µg/ml polybrene (Sigma-Aldrich, Munich, Germany) and 200 IU/ml IL-2. Cells were centrifuged at 2000 rpm, 32°C for 90 min. Plates were placed afterwards in 32°C, 5 % CO2 humidified incubator for 24 hours. Transduction was repeated on two successive days. After the third transduction, cells were maintained in RPMI 1640 medium,

10% (v/v) FBS, 200 IU/ml IL-2 at 37°C.

Cytokine release assay and ELISA: Target cells (5x104) were cocultured with an equal number of effector cells in 96-well flat bottom plates for 24 hours. The supernatants were assayed for IFN-γ and IL-2 by specific ELISAs (Mabtech, Hamburg, Germany, or BD Biosciences, Heidelberg, Germany) according to the manufacturer's protocol. The data represent mean values of triplicates derived from one representative experiment. Experiments were performed at least twice with similar results. The detection limit of the ELISA is 100 pg/ml IFN-γ or 20 pg/ml IL-2.

RESULTS

1. Efficient expression of the chimeric receptors on engineered NK cells To investigate the potential of engineering antigen-specific NK cells, two chimeric receptor constructs consisting of a HER-2 specific binding antibody domain fused to CD3 or the joined CD3 and CD28 signaling domains were used. The constructs were cloned into Pmig replacing IRES and GFP (Fig. 1A). CARs expressed from these constructs were designated CARz and CARz28. Retrovirus supernatants produced from either constructs were used to transduce PBL-derived primary human NK cells (Fig. 1B). Flow cytometry analysis revealed that the transduction efficacy for the CARz construct was 41.8 % and for the CARz28 construct 53.1 %, respectively, as detected by antibodies specific for human Ig recognizing the extracellular domain of the CAR (Fig. 1B).

2- CAR-engineered NK cells produce cytokines upon HER-2 recognition The ability of CAR engineered NK cells to recognize HER-2 expressing target cells was investigated in IFN-γ and IL-2 release assays (Fig. 2). Mock-, CARz- and CARz28-engineered NK cells were cultured alone (none) or cocultured with either the HER-2 negative tumor cell line C1R/A2, the HER-2 expressing transfectant of this cell line, C1R/A2HER2, or with the endogenously HER2 expressing ovarian carcinoma cell line SKOV3. Subsequently, an IFN-γ (Fig. 2A) and IL-2 (Fig. 2B) specific ELISA were performed.

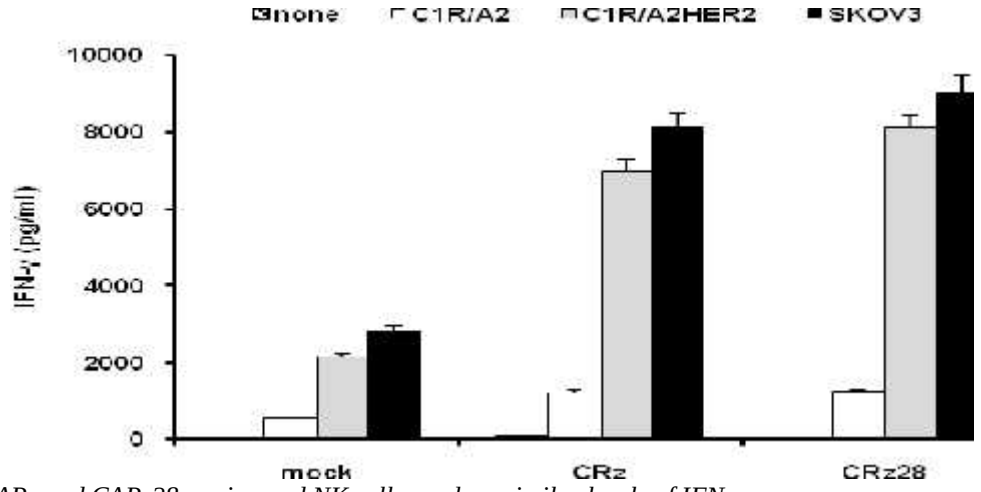

Figure 2A. CARz and CARz28-engineered NK cells produce similar levels of IFN-γ.

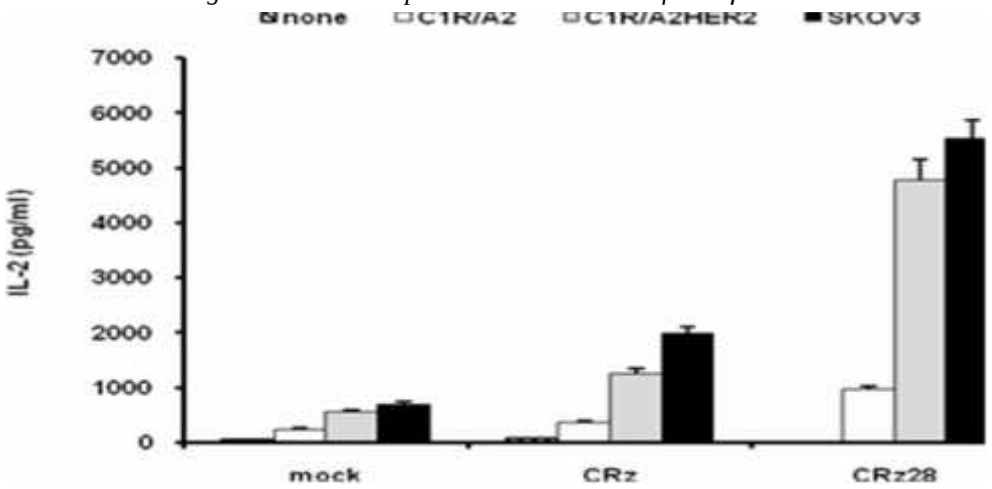

Figure 2B. CARz28-NK cells produce more IL-2 than CARz upon HER-2 recognition.

Neither CAR- nor mock-NK cells produced IFN-γ or IL-2 spontaneously. Mock-NK cells were stimulated by target cells to produce some IFN-γ (between 550 and 2800 pg/ml) (Fig. 2A). CARz- and CARz28-NK cells secreted similar IFN-γ levels (1200 and 1250 pg/ml, respectively) when cocultured with the HER-2 negative C1R/A2 cell line, however significantly higher levels of IFN-γ were produced by CARz- and CARz28-NK cells in response to stimulation by C1R/A2HER2 cells (7000 and 8100 pg/ml, respectively) and SKOV3 cells (8100 and 9000 pg/ml, respectively, Fig. 2A).

Likewise, some IL-2 was produced by mock-NK cells when cocultured with target cells (between 250 and 700 pg/ml), and CARz- and CARz28-NK cells produced similar IL-2 levels when cocultured with C1R/A2 cells (400 and 1000 pg/ml, respectively). A modest increase in IL-2 production by CARz-NK cells (1250 and 2000 pg/ml), as compared to markedly higher IL-2 production by CARz28-NK (4800 and 5500 pg/ml, respectively) could be observed when these cells were cocultured with the HER-2 positive targets C1R/A2HER2 and SKOV3 (Fig.2B).

DISCUSSION

To improve solid tumor cell targeting, we have earlier engineered human primary NK cells by introducing a chimeric receptor which combines antigen specificity and NK cell activating properties in a single fusion molecule [12]. Others have used a similar approach to target human NK cells towards leukemia or used transient transfection to target mouse NK cells toward solid tumors [13, 14, 15]. Here we analyzed the factors affecting NK cell engineering by retroviral transduction. Maximal retroviral transduction efficiency is achieved when cells are at their maximal proliferative capacity [16]. The in vitro expansion of primary NK cells is regulated not only by soluble factors such as cytokines, but also by stimuli from activation molecules, such as those expressed on the B lymphoblastoid cell line RPMI 8866 used in this study as a feeder line [17]. Therefore, the optimal ratio of feeder cells to PBL to achieve maximal transduction efficacy reflects the availability of these two factors. During the transduction process administered IL-2 did not increase transduction efficacy, but increased cell viability most likely due to the fact that IL-2 was shown to not only induce NK cell proliferation, but also to protect the NK cells from death by apoptosis [18]. Interestingly, the CARz- and the CARz28-engineered NK cells showed no significant difference in IFN-γ production, but the CARz-engineered NK cells produced much less IL-2 in comparison to the CARz28- engineered NK cells. This is the first description of such an effect on NK cells that can be attributed to CD 28 signaling. A similar finding was reported for CAR transduced T cells [19, 20], suggesting

the presence of a shared CD28 signaling mechanism in both cell types.

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