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Highly specific and potently activating Vγ9Vδ2-T cell specific nanobodies for diagnostic and therapeutic applications

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ABSTRACT

Vγ9Vδ2-T cells constitute the predominant subset of γδ-T cells in human peripheral blood and have been shown to play an important role in antimicrobial and antitumor immune responses. Several efforts have been initiated to exploit these cells for cancer immunotherapy, e.g. by using phosphoantigens, adoptive cell transfer, and by a bispecific monoclonal antibody based approach. Here, we report the generation of a novel set of Vγ9Vδ2-T cell specific VHH (or nanobody). VHH have several advantages compared to conventional antibodies related to their small size, stability, ease of generating multispecific molecules and low immunogenicity. With high specificity and affinity, the *anti*-Vγ9Vδ2-T cell receptor VHHs are shown to be useful for FACS, MACS and immunocytochemistry. In addition, some VHH were found to specifically activate Vγ9Vδ2-T cells. Besides being of possible immunotherapeutic value, these single domain antibodies will be of great value in the further study of this important immune effector cell subset.

1. Introduction

Vγ9Vδ2-T cells constitute the predominant subset of peripheral blood γδ-T cells with several unique features that can be valuable for immunotherapeutic approaches. Vγ9Vδ2-T cells become activated by the MHC-independent recognition of non-peptide phosphoantigens (pAg) that are produced upon bacterial infection or are upregulated by malignant cells [1]. Presumably, the type I membrane protein butyrophilin 3A1 (BTN3A1, also known as CD277) obtains an altered conformation and membrane distribution upon the detection of elevated intracellular pAg levels. This extracellular modification of BTN3A1 is consequently sensed by the Vγ9Vδ2-T cell receptor (TCR) resulting in Vγ9Vδ2-T cell activation [2–4]. Stimulation of Vγ9Vδ2-T cells can be enhanced by interaction between the NKG2D receptor expressed on most Vγ9Vδ2-T cells and stress-related MICA, MICB and ULBP molecules, upregulated on infected or transformed cells [5,6]. This allows Vγ9Vδ2-T cells to distinguish normal cells from stressed cells and to consequently produce large amounts of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α and chemokines MIP-1 and RANTES) in addition to cytolytic mediators (perforin, granzyme B) to induce specific lysis of cells with elevated pAg levels [7]. In this way, Vγ9Vδ2-T cells have been shown to be capable of inducing lysis of a broad spectrum of cancer cells [5,8–10]. Furthermore, upon activation Vγ9Vδ2-T cells can stimulate the maturation of immature dendritic cells and can also acquire antigen presenting capacities themselves [11].

Based on these characteristics, several clinical trials were initiated to evaluate whether Vγ9Vδ2-T cells could be exploited for immunotherapy against both hematological and solid malignancies. Trials have included approaches where aminobisphosphonates (to increase endogenous pAg levels) or synthetic pAgs were used alone or in combination with IL-2, and the adoptive transfer of autologous *ex vivo* expanded Vγ9Vδ2-

Abbreviations: VHH, variable domain of heavy chain-only Ab; pAg, phosphoantigens; TCR, T cell receptor; PBMC, peripheral blood mononuclear cells; MACS, magnetic-activated cell sorting.

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T cells. In general, these Vγ9Vδ2-T cell-based therapeutic approaches were well tolerated and capable of inducing clinically relevant antitumor responses in several cases. However, results were not consistent [12,13]. A possible explanation could be that these therapies were all aimed at systemic (i.e. not tumor-specific) activation of Vγ9Vδ2-T cells. More recently, a new Vγ9Vδ2-T cell immunotherapy approach was explored through the use of a bispecific antibody. Her2/neu overexpressing pancreatic cells could efficiently be lysed by Vγ9Vδ2-T cells in the presence of a $(Her2)_2$xVγ9 bispecific antibody *in vitro*. When the $(Her2)_2$xVγ9 bispecific antibody was co-administered with Vγ9Vδ2-T cells on a weekly basis to immunocompromised mice inoculated 2 days earlier with a pancreatic cancer cell line, significantly reduced tumor volumes were observed after 4 weeks [14]. This antibody-based approach adds a new dimension to the future of Vγ9Vδ2-T cell-based immunotherapy. However, the development and use of bispecific conventional antibody-based molecules has several limitations, including mispairing of heavy and light chains and instability [15]. Of interest, and in addition to conventional antibodies, camelids (i.e. llamas, camels and dromedaries) also possess a unique antibody class consisting of heavy chain only antibodies which lack the light chain and the CH1 domain of the heavy chain [16]. The variable antigen binding region of these antibodies is called a VHH (or nanobody) and consists of only a single immunoglobulin domain, in contrast to full-length antibodies or single-chain variable fragments (scFv) often used for the design of antibody-based constructs [17]. VHHs can be used as an attractive alternative to conventional antibodies. Since VHHs only need to fold one domain they have increased stability, solubility and ease of refolding. VHHs are ten times smaller than conventional antibodies which allows them to reach clefts in antigen structures and provides enhanced tissue penetration as compared to antibodies [18,19]. VHHs can easily be made into multispecificity molecules and have a low immunogenicity. Due to the absence of an Fc-domain, VHHs can be produced by *Escherichia coli* or yeast allowing time and cost reduction [15,20].

Based on the above mentioned unique characteristics of VHHs, we set out to generate a novel set of Vγ9Vδ2 T cell specific VHHs and evaluate their use for various applications. Our efforts resulted in the identification of a panel of Vγ9Vδ2-TCR specific VHHs, directed against the Vγ9-chain and/or Vδ2-chain, with activating as well as non-activating properties. These Vγ9- and Vδ2-TCR directed VHHs could successfully be used for flow cytometric analysis, isolation of Vγ9Vδ2-T cells by magnetic bead isolation, immunocytochemistry and have great potential for the development of future Vγ9Vδ2-T cell-based immunotherapies.

2. Material & methods

2.1. Cell lines

Jurkat and Jurkat/MA were cultured in IMDM + medium, i.e. Iscove's modified Dulbecco's medium (Lonza, catalog #BE12-722F) supplemented with 10% (*v*/v) fetal calf serum (FCS) (HyClone GE Healthcare, catalog #SV30160.03), 0.05 mM β-mercaptoethanol (β-ME), 100 IU/mL sodium penicillin, 100 μg/mL streptomycin sulfate and 2.0 mM L-glutamine (Life Technologies, catalog #10378-016). Phoenix-A cells were cultured in Dulbecco's Modified Eagle's Medium (Lonza, catalog #BE12-614F) supplemented with 10% FCS, 0.05 mM β-ME, 100 IU/mL sodium penicillin, 100 μg/mL streptomycin sulfate and 2.0 mM L-glutamine. Cell lines were tested mycoplasma negative.

2.2. Flow cytometry and monoclonal antibodies

Antibodies used were FITC-labeled *anti*-TCR Vδ2 (catalog #555738) and PE-labeled pan γδ-TCR (catalog #333141) from BD Biosciences and FITC-labeled *anti*-TCR Vα24 (catalog #IM1589) and PE-labeled *anti*-TCR Vβ11 (catalog #IM2290) from Beckman Coulter. PerCP-labeled *anti*-TCR Vδ2 (catalog #331410), PE-labeled *anti*-TCR Vγ9 (catalog #331308) and APC-labeled *anti*-TCR Vγ9 (catalog #331310) were from Biolegend and FITC-labeled polyclonal swine-*anti*-rabbit antibody (catalog #F0205), RPE-labeled goat-*anti*-mouse F(ab′)2 fragment (catalog #R0480) and Streptavidin/RPE (catalog #R0480) were obtained from Dako. APC-labeled goat-*anti*-mouse F(ab′)2 fragment (catalog #SC-3818) was obtained from Santa Cruz Biotech and Alexa488-conjugated goat-*anti*-rabbit antibody (catalog #A-11008) was from Thermo Fisher Scientific. Rabbit-*anti*-VHH (purified) clone K1216 and rabbit-*anti*-VHH serum clone K976 were obtained from QVQ (on request). Anti-Myc tag antibody clone 4A6 (catalog #05-724) was obtained from Merck Millipore and *anti*-Myc tag antibody clone 9E10 was produced in-house.

All stainings for flow cytometry were performed in PBS supplemented with 0.1% (*w*/*v*) BSA and 0.02% (*w*/*v*) sodium-azide. Stained cells were measured with FACS Calibur or LSRFortessa (BD Biosciences, NJ, USA) and analyzed and analyzed with CellQuest (BD Biosciences) or Kaluza software (Beckman-Coulter).

2.3. Generation of donor-derived γδ-T cells

Healthy donor-derived Vγ9Vδ2-T cells were isolated, expanded and cultured as described [26]. In brief, Vγ9Vδ2-T cells were isolated from human peripheral blood mononuclear cells (PBMC) by Magnetic-activated cell sorting (MACS) using FITC-labeled *anti*-TCR Vδ2 or PE-labeled *anti*-TCR Vγ9 antibody in combination with *anti*-mouse IgG MicroBeads (Miltenyi Biotec, catalog #130-048-401). Purified Vγ9Vδ2-T cells were stimulated weekly with irradiated and aminobisphosphonate (100 μM Pamidronate for 3 h, Teva Pharmachemie, catalog #12J08RD) pretreated human mature monocyte derived dendritic cells or an irradiated feeder mixture (PBMC of 2 healthy human donors and Epstein Barr Virus transformed B cells with addition of 50 ng/mL PHA). Vγ9Vδ2-T cells were used for experiments when purity was >90%. Of note, Vγ9Vδ2-T cells used for testing the activation inducing potential of VHHs had a < 40% CD25 expression.

To obtain $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ and $V\gamma9^-V\delta2^-$ γδ-T cell lines, we first isolated a pan γδ-T cell population. Human PBMC were stained with a PE-labeled pan γδ-TCR antibody and purified with MACS using *anti*-mouse IgG MicroBeads. This pan γδ-T cell line was first expanded with feeder mixture and then stained with FITC-labeled *anti*-TCR Vδ2 and PE-labeled *anti*-TCR Vγ9 antibodies to allow flow cytometric cell sorting of 4 separate populations (i.e. $V\gamma9^-V\delta2^+$, $V\gamma9^+V\delta2^-$, $V\gamma9^+V\delta2^+$ and $V\gamma9^-V\delta2^-$ γδ-T cells).

All donor-derived γδ-T cell lines were cultured in Yssels medium [27] supplemented with 1% AB human serum, 50 U/mL rhIL-2 (Proleukin, Novartis), 0.05 mM β-ME, 100 IU/mL sodium penicillin, 100 μg/mL streptomycin sulfate and 2.0 mM L-glutamine at 37 °C with 5% CO_2 in a humidified atmosphere. Cell lines were tested mycoplasma negative.

2.4. Generation of Vγ9Vδ2-TCR and Vα24Vβ11-TCR transduced cell lines

Jurkat and Jurkat/MA cell lines expressing TCRs of interest were generated as described previously [28]. For the Vγ9Vδ2-TCR, protein sequences of clone G9 Vγ9- and Vδ2-chain [29,30] were used. For the Vα24Vβ11-TCR, protein sequences of clone NKT12 Vα24- and Vβ11-chain [31] were used. Sequences of the individual TCR chains were separated by a picorna virus derived 2 A sequence, codon modified for optimal protein production and synthesized by GeneART (Thermo Fisher Scientific, MA, USA) after which they were cloned into the LZRS vector. After transfection to the Phoenix-A packaging cell line, retroviral supernatants were collected to transduce Jurkat or Jurkat/MA cells in the presence of retronectin (Takara, catalog #T100A) according to the manufacturer's protocol [28]. Purity of the transduced cell lines was obtained by MACS cell separation using *anti*-mouse IgG MicroBeads or by flow cytometric sorting both in combination with FITC-labeled *anti*-TCR Vδ2 and PE-labeled *anti*-TCR Vγ9 antibodies or FITC-labeled *anti*-TCR