Design of novel TCR gene transfer strategies for the treatment of hematological malignancies

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αβ T-cell receptor engineered γδ T cells mediate effective antileukemic reactivity

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Abstract

Retroviral transfer of T cell receptors (TCRs) to peripheral blood-derived T cells generates large numbers of T cells with the same antigen-specificity, potentially useful for adoptive immunotherapy. One drawback of this procedure is the formation of mixed TCR dimers with unknown specificities due to pairing of endogenous and introduced TCR chains. We investigated whether γδ T cells can be an alternative effector population for TCR gene transfer because the γδTCR is not able to form dimers with the αβTCR. Peripheral blood-derived γδ T cells were transduced with human leucocyte antigen (HLA) class I- or HLA class II-restricted minor histocompatibility antigen (mHag) or virus-specific TCRs. Since most γδ T cells do not express CD4 and CD8, we subsequently transferred these coreceptors. The TCR-transduced γδ T cells exerted high levels of antigen-specific cytotoxicity and produced IFN-γ and IL-4, particularly in the presence of the relevant coreceptor. γδ T cells transferred with a TCR specific for the hematopoiesis specific mHag HA-2 in combination with CD8 displayed high antileukemic reactivity against HA-2 expressing leukemic cells. These data demonstrate that transfer of αβTCRs to γδ T cells generated potent effector cells for immunotherapy of leukemia, without the expression of potentially hazardous mixed TCR dimers.

Introduction

Cellular immunotherapy is a promising strategy for the treatment of cancer (1). However, adoptive transfer of sufficient numbers of antigen-specific T cells requires complex isolation methods and laborious and time-consuming tissue culture procedures. An alternative method to obtain large numbers of T cells with a defined antigen-specificity is the retroviral transfer of a T-cell receptor (TCR). Because T cell specificity is exclusively determined by the TCR, T-cell specificity can be functionally transferred to other T lymphocytes by retroviral TCR gene transfer. We and others have shown that transfer of human leukocyte antigen (HLA) class I- and HLA class II-restricted TCRs to $CD8^+$ and $CD4^+$ T cells, respectively, generated T cells with converted antigen-specific cytolytic activity and cytokine production (2-10). The potential *in vivo* efficacy of TCR-transferred T cells was demonstrated in mouse models (8, 9). The TCR-transferred T cells were activated *in vivo*, homed to effector sites, and contributed to tumor clearance.

A potential disadvantage of TCR gene transfer to other αβ T cells is the formation of mixed TCR dimers. Chains of the introduced TCR can pair with the endogenous TCR chains naturally expressed by the TCR-transferred T cells. The specificity of these mixed TCR dimers is unknown and, therefore, autoreactivity can not be excluded. To limit the number of T cells with different TCR chains and thus the chance to generate autoreactive T cells, T cells with defined antigen-specificity and therefore with a limited TCR repertoire can be selected as host cells for TCR gene transfer. We previously demonstrated the reprogramming of cytomegalovirus (CMV) specific T cells into leukemia-reactive T cells by transferring a TCR specific for the minor histocompatibility antigen (mHag) HA-2 (6). TCR gene transfer into T cells with a defined antigen-specificity also prevents autoreactivity due to the activation of ignorant self-specific T cells through the introduced TCR.

To completely prevent the formation of mixed TCR dimers by TCR gene transfer,

we here propose a novel strategy to redirect peripheral blood-derived γδ T cells by αβTCR gene transfer. γδ T cells comprise 1 to 10% of peripheral blood T cells, and express a γδTCR which can not exchange chains with αβTCRs (11, 12). γδ T cells do not recognize antigens in the context of conventional HLA class I or class II molecules (13, 14). Furthermore, most γδ T cells lack the expression of the coreceptors CD4 and CD8 (15, 16). Because γδ T cells have been shown to have the capacity to lyse tumor cells (17) and leukemic cells (18-21), we hypothesized that large numbers of cytolytic, antigen-specific T cells could be obtained by TCR gene transfer to γδ T cells.

To investigate whether it is possible to redirect γδ T cells by αβTCR gene transfer, we transduced peripheral blood-derived γδ T cells with three different TCRs specific for the hematopoietic mHag HA-2 presented in the context of HLA-A2, for CMV-pp65 presented in the context of HLA-B7, or for the HLA class II restricted mHag DBY. Since most γδ T cells lack the expression of the coreceptors CD4 and CD8, we also investigated whether introduction of the relevant coreceptor could contribute to the functionality of the redirected γδ T cells. We show that αβTCR redirected γδ T cells exerted antigen-specific cytolytic activity and produced cytokines after antigen-specific stimulation. Introduction of the relevant coreceptor further enhanced the specific functional activity of the γδ T cells, especially the activity directed against target cells presenting endogenously processed antigen was enhanced. These results demonstrate that by αβTCR gene transfer to γδ T cells, potent antigen-specific cytolytic T cells with the capacity to produce high amounts of cytokines can be generated without the risk of expression of undesired mixed TCR dimers.

Materials and Methods

T cells

Peripheral blood-derived γδ T cells were isolated from PBMCs of healthy donors using FITC-labeled, γδ T cell-specific immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and the AutoMACS system (Miltenyi Biotec) according to the protocol of the manufacturer. To obtain maximal purity, FITC-labeled cells were subsequently sorted using a fluorescence-activated cell sorter (FACS) Vantage (Becton-Dickinson, San Jose, CA). γδ T cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker, Verviers, Belgium) containing 10% pooled human serum, 300 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) and 10 ng/ml IL-15 (PeproTech Inc., Rocky Hill, NJ, USA). For the first stimulation of the isolated γδ T cells a mixture of 50 Gy irradiated autologous peripheral blood mononuclear cells (PBMCs), 50 Gy irradiated Epstein-Barr virus (EBV)-transformed B cells (EBV-LCL), 1 μg/ml leucoagglutinin (Leuko-A, Sigma, St. Louis, USA), 300 IU/ml IL-2 and 10 ng/ml IL-15 was used, and subsequently the autologous PBMCs were replaced by allogeneic PBMCs for restimulations done every 3 weeks.

The HLA-A2–restricted T cell clones HA2.5 and HA2.27 specific for the mHag HA-2 were isolated from the peripheral blood of a patient with chronic myeloid leukemia (CML) during an ongoing graft versus leukemia response after donor lymphocyte infusion (22). The CMV-pp65/HLA-B7 specific T cells were isolated using CMV-pp65/HLA-B7 (CMV^{B7}) tetrameric complexes. The $CD4^+$ T cell clone JBB4 was previously isolated from a male patient after stem cell transplantation from his HLA genotypically identical sister (23), and has been shown to recognize the male specific mHag DBY in the context of HLA-DQ5 (24).

The αβ T cell clones were cultured in IMDM containing 10% pooled human serum and 100 IU/ml IL-2. Every 2 weeks, the αβ T cells were stimulated with a mixture of 50 Gy irradiated allogeneic PBMCs, 50 Gy irradiated EBV-LCL, 1 μg/ml Leuko-A and 100 IU/ml IL-2.

Construction of the retroviral vectors and production of the retroviral supernatant

The construction of the retroviral vectors containing the TCRs of the HA2.5 T-cell clone and the DBY-specific JBB4 T-cell clone has been previously described (10, 25). The TCR of the HA2.5 T cell clone was found to consist of AV15S1 and J42 in combination with BV18S1 with J2S7, according to the nomenclature described by Arden *et al*. (26). In the IMGT nomenclature these TCR chains are named TRAV5 and TRBV18, respectively. The DBY-TCR was encoded by an in frame gene rearrangement of AV1S4 (IMGT TRAV8-3) with J39 in combination with BV5S6 (IMGT TRBV5-4) with J2S7. The TCRα chain of the isolated CMV B7-specific T cell clone was encoded by an in frame gene rearrangement of AV3 (IMGT TRAV17) and J12, and the TCRβ was based on the combination of BV6S4 (IMGT TRBV7-9) and J2S7.

The TCRα and TCRβ genes were cloned separately into bicistronic retroviral vectors containing the marker genes eGFP (27) and truncated nerve growth factor receptor (ΔNGF-R) (28), respectively. The Moloney murine leukemia virus-based retroviral vector LZRS and packaging cells φ-NX-A were used (29). Retroviral supernatant was produced as previously described (10). By reverse transcription-PCR, sequencing and subsequent cloning retroviral vectors were constructed encoding the coreceptor molecules CD4α (ΔNGF-R), CD8α (eGFP) and CD8β (ΔNGF-R). After puromycin selection of transfected φ-NX-A cells, retroviral supernatant was frozen in aliquots at -70°C. As control vectors, retroviral vectors were used containing only eGFP or ΔNGF-R.

Retroviral transfer of αβTCRs and coreceptors to γδ T cells

Purified human peripheral blood γδ T cells were stimulated with 50 Gy irradiated PBMCs, 50 Gy irradiated EBV-LCL, 1 μg/ml Leuko-A, 300 IU/ml IL-2 and 10 ng/ml IL-15. Two or 3 days after stimulation, the γδ T cells were transduced with a mixture of the TCRα and TCRβ chain encoding retroviral supernatants or control retroviral supernatants containing the marker genes eGFP or ΔNGF-R only, using recombinant human fibronectin fragments CH-296 (30) (Bio Whittaker, Verviers, Belgium). Three different TCRs were transduced: the HA-2-TCR, the CMV-B7-TCR and the DBY-TCR. TCR-transduced γδ T cells expressing the marker genes eGFP and ΔNGF-R were sorted using a FACS Vantage, restimulated and kept in bulk cultures. After two to three days, the sorted γδ T cells were transduced with the coreceptors CD4α or CD8αβ, and subsequently sorted on the expression of the introduced coreceptors. The expression of the introduced CD4 and CD8 was comparable with normal CD4 and CD8 expression on αβ T cells.

Cytotoxicity assay

Target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 hour at 37°C, washed thrice, and added to the effector cells at various effector-to-target ratio in a final volume of 150 μl of IMDM supplemented with 10% FBS in 96-well U-bottomed microtiter plates. In some experiments, target cells were loaded with HA-2, CMV-pp65 or DBY peptide (10 μg/ml) for 1 hour at 37°C and washed once. Targets incubated in medium or 1% Triton X-100 were

used for determination of the spontaneous and maximum release, respectively. The tests were done in triplicate. After 4, 9 or 20 hours of incubation at 37°C and 5% CO_2, 25 μl of the supernatant was harvested and measured in a luminescence counter (Topcount-NXT, Packard). The percentage of specific lysis was defined as [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100.

EBV-LCLs (HA-2+ or HA-2-) derived from HLA identical siblings, and leukemic cells (HA-2+ or HA-2-) harvested from patients after informed consent were used as target cells for HA-2-TCR transduced γδ T cells. Antileukemic reactivity was analyzed against primary leukemic cells derived from one HLA-A2+ HA-2+ CML patient, one HLA-A2+ HA-2- CML patient, one HLA-A2+ HA-2+ AML patient and one HLA-A2+ HA-2- AML patient. The primary leukemic cells were thawed one day before the assay and incubated overnight at 37°C and 5% CO_2 in the presence of 10% pooled human serum. The spontaneous release of the primary CML cells was <15% after 9h. The spontaneous release of the primary AML cells was <10% after 9h and <28% after 20h.

As target cells for CMV-B7-TCR transduced γδ T cells, EBV-LCL retrovirally transduced with the lower matrix protein pp65 of HCMV AD169 (pp65+) or non transduced EBV-LCLs (pp65-) were used (6). EBV-LCL from different HLA identical female (DBY-) and male (DBY+) siblings expressing HLA-DQ5 were used as target cells for DBY-TCR-transduced γδ T cells (23, 24).

Cytokine production

To measure the cytokine production of the γδ T cells, 2 x 10^4 stimulator cells per well of an U-bottomed 96-well plate were added to equal numbers of effector cells in a final volume of 150 μl of IMDM supplemented with 10% FBS and incubated at 37°C and 5% CO_2. After 24 hours 80 μl supernatant was harvested to determine IFN-γ and IL-4 production by standard ELISA (Sanquin, Amsterdam, The Netherlands).

Tetrameric HLA Class I/Peptide Complexes, flow cytometric analysis, and FACS sorting

Phycoerythrin (PE)- or APC-conjugated tetrameric complexes were constructed as previously described (31) with minor modifications. Tetrameric HLA-A2 molecules in complex with HA-2–derived peptide YIGEVLVSV (HA-2^{A2} tetramer), and tetrameric HLA-B7 molecules in complex with CMV pp65–derived peptide TPRVTGGGAM (CMV^{B7} tetramer) were constructed. As negative controls tetrameric HLA-A2 molecules in complex with CMV pp65–derived peptide NLVPMVATV (CMV^{A2} tetramer) and tetrameric HLA-B7 molecules in complex with EBV EBNA3A-derived peptide RPPIFIRRL ($EBNA^{B7}$ tetramer) were used.

For flow cytometric analyses as well as FACS sorting, cells were labeled with tetrameric complexes for 2 h at 4°C in RPMI without phenol, supplemented with 2% FBS, and washed three times, or labeled with monoclonal antibodies (mAb) directed against the various cell surface molecules for 30 min at 4°C. The mAbs used were anti-CD3 (APC; Becton Dickinson), anti-CD4 (PE, Caltag, Burlingame, CA; APC, Coulter, Miami, FL), anti-CD8 (PECy5; DAKO, Glostrup, Denmark), anti-CD8β (PE; Immunotech, Marseille, France), anti-TCRαβ (PE-Cy5; Immunotech), and anti-TCRγδ (PE; Becton Dickinson). For the detection of ΔNGF-R PE (PharMingen, San Diego, CA) or APC (Cedarlane Laboratories, Hornby, Ontario, Canada), conjugated anti-human NGF-R mAbs were used.

Results

TCR and coreceptor transfer to γδ T cells

Peripheral blood-derived γδ T cells were isolated by positive selection using γδ T-cell specific immunomagnetic beads and the autoMACS system and subsequent FACS sorting, resulting in >99% pure γδ T cells. The isolated γδ T cells proliferated vigorously upon stimulation with Leuko-A, autologous or allogeneic irradiated feeder cells in the presence of IL-2 and IL-15, and were transduced with the HLA class I-restricted HA2-TCR, CMV-B7-TCR, or with the HLA class II-restricted DBY-TCR or mock vectors 2 or 3 days after stimulation. This resulted in transduction efficiencies of γδ T cells ranging from 25-40%. After one week, the transduced γδ T cells were FACS sorted based on eGFP and ΔNGF-R expression. Since most γδ T cells do not express the coreceptors CD4 and CD8, αβTCR$^+$ γδ T cells were subsequently transduced with CD4 and CD8αβ. Coreceptor transduced γδ T cells were FACS sorted based on the expression of CD4 or CD8 after one week.

In figure 1A the αβTCR and γδTCR expression of the mock, HA-2-TCR, CMV-B7-TCR and DBY-TCR transduced γδ T cells are shown. Mock-transduced γδ T cells only expressed the γδTCR at their cell surface, while different levels of αβTCR expression were observed on γδ T cells transduced with the different αβTCRs. Some γδ T cells with high expression levels of the introduced αβTCR showed diminished expression of the endogenous γδTCR. Most mock and αβTCR$^+$ γδ T cells did not express CD4 and CD8 at their cell surface (Fig. 1B).

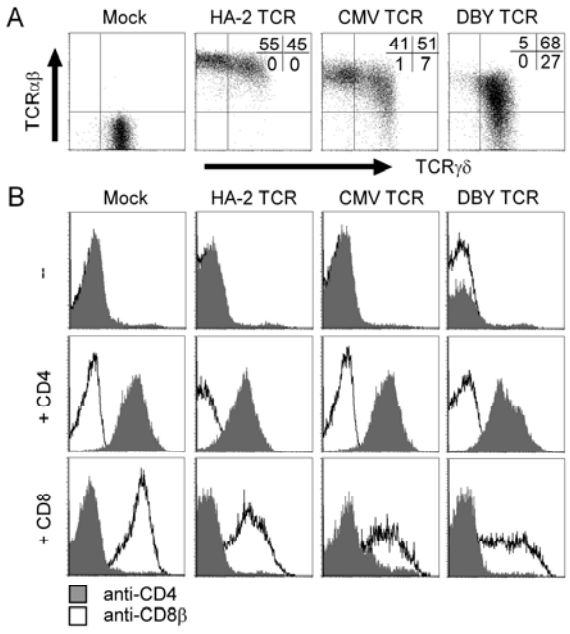

Figure 1. Cell surface expression of αβTCRs and coreceptors on γδ T cells. *Peripheral blood-derived γδ T cells were retrovirally transduced with either mock vectors containing only the marker genes or HA-2-TCR, CMV-B7-TCR, or DBY-TCR constructs, and sorted on marker gene expression. Subsequently, the γδ T cells were transduced with CD4 or CD8, and sorted on the expression of the coreceptors.* ***A,*** *TCRαβ and TCRγδ cell surface expression was analyzed for mock- and TCR-transduced γδ T cells.* ***B,*** *the expression of CD4 (gray shaded) and CD8 (anti-CD8β mAb); black line) was determined on mock and TCR-transduced γδ T cells without and with co-transfer of the coreceptors.*
Thus, γδ T cells expressing αβTCRs in combination with the relevant coreceptors can be generated by retroviral gene transfer.

Cotransfer of CD4 and CD8 and subsequent selection resulted in coreceptor-positive γδ T cells (Fig. 1B). The results show that γδ T cells expressing αβTCRs in combination with the relevant coreceptors can be generated by retroviral gene transfer.

CD3 and TCR expression after αβTCR transfer

Because CD3 expression is essential for the cell surface expression of both the introduced αβTCR and the endogenous γδTCR, we determined whether introduction of αβTCRs in γδ T cells would affect the CD3 cell surface expression level (Fig. 2). The introduction of the HA-2-TCR and the CMV-B7-TCR in γδ T cells had no effect on the CD3 cell surface expression in comparison with mock-transduced γδ T cells. Tetramer stainings of the transferred HLA class I-restricted TCRs were performed to visualize the antigen-specific binding capacity of the introduced TCRs. In figure 2A, HA-2-TCR$^+$ γδ T cells were stained with either the HA-2^{A2} tetramer or the irrelevant CMV^{A2} tetramer as a negative control. The CD8$^+$ HA-2-TCR$^+$ γδ T cells were capable of specifically binding the HA-2^{A2} tetramer whereas no binding was observed with the control CMV^{A2} tetramer. CMV-B7-TCR$^+$ γδ T cells without CD8 on the cell surface already specifically bound the CMV^{B7} tetramer (Fig. 2B). Introduction of CD8 further increased the level of tetramer staining, while no binding was observed of the irrelevant EBNA^{B7} tetramer. Mock transduced γδ T cells with or without CD8 expression did not bind the HA-2^{A2} or CMV^{B7} tetramers (Fig. 2C). Thus, CD8$^+$ HA-2-TCR$^+$ γδ T cells specifically bound the HA-2^{A2} tetramer, and the CMV^{B7} tetramer staining of the CMV-B7-TCR$^+$ γδ T cells was enhanced by the expression of the CD8 coreceptor.

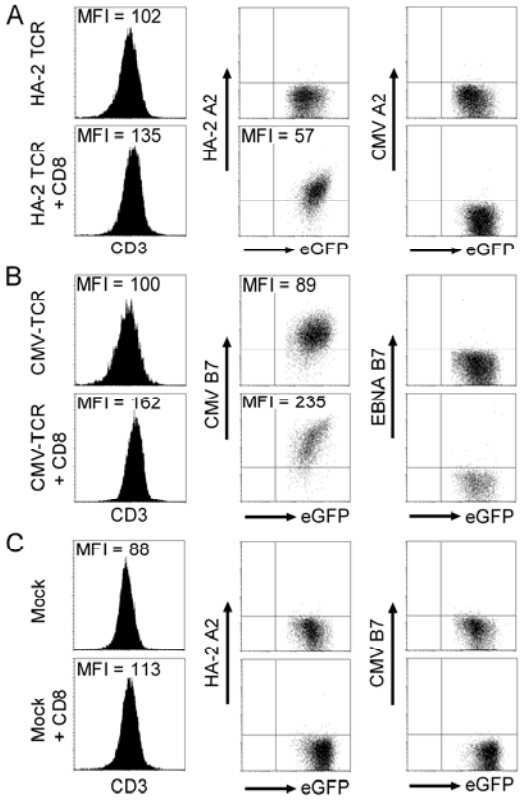

Figure 2. CD3 expression and tetramer staining of γδ T cells transduced with the HA-2-TCR or CMV-B7-TCR with or without CD8αβ. *γδ T cells transduced with either the HA-2-TCR (**A**), CMV-B7-TCR (**B**) or mock vector (**C**) without or with CD8αβ were labeled with anti-CD3 or phycoerythrin-conjugated TCR specific or control tetramers. HA-2 TCR-transduced γδ T cells only specifically bound the HA-2^{A2} tetramer when expressing CD8. CMV-B7-TCR transduced γδ T cells specifically bound the CMV^{B7} tetramer, and co-transfer of CD8 increased the specific CMV^{B7} tetramer staining.*

Antigen-specific cytolytic activity of αβTCR-transduced γδ T cells

To determine the cytolytic capacity of the αβTCR-redirected γδ T cells the T cells were tested in a ^{51}Cr- release assay. HA-2-TCR$^+$ γδ T cells without coreceptor or with the irrelevant CD4 molecule were highly cytolytic against HA-2 peptide pulsed EBV-LCL, but showed only minor activity against endogenously processed HA-2 (Fig. 3A). A significant enhancement of the cytolytic activity of especially EBV-LCL presenting endogenously processed antigen was observed when HA-2-TCR$^+$ γδ T cells were co-expressing CD8. Similarly, CMV-B7-TCR transduced γδ T cells also exerted increased antigen-specific cytolytic activity against endogenously processed antigen when expressing CD8 (Fig. 3B). Moreover, when γδ T cells were transduced with the HLA class II-restricted DBY-TCR, expression of the relevant coreceptor CD4 significantly increased the antigen-specific lysis of target cells presenting endogenously processed DBY (Fig. 3C). To investigate whether the cytolytic activity of the HA-2-TCR$^+$ γδ T cells was comparable with the activity of the

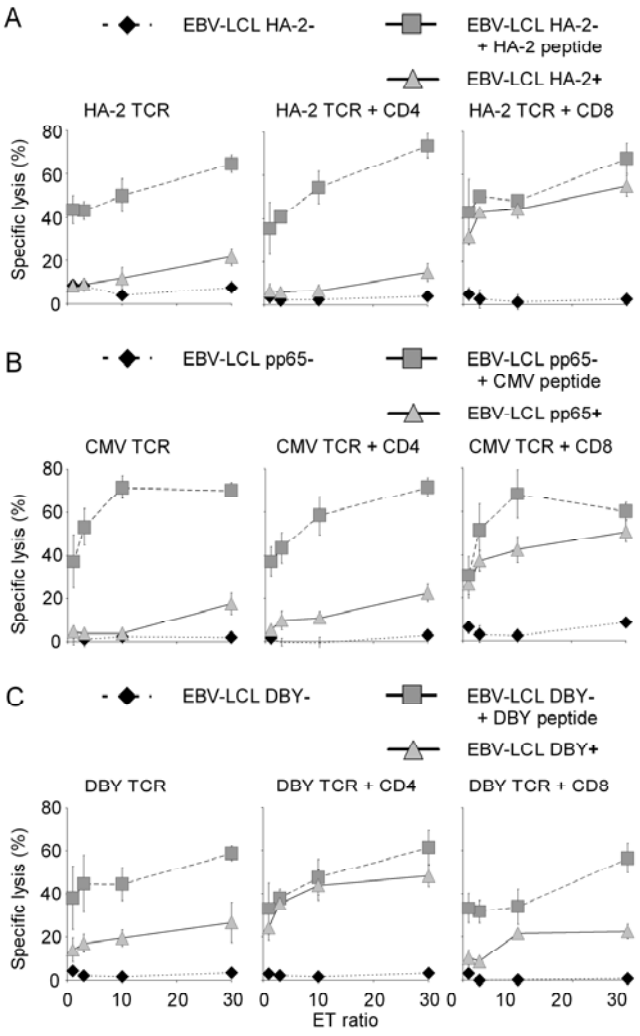

Figure 3. Cytolytic activity of TCR-transferred γδ T cells without or with co-transfer of CD4 and CD8αβ. *The antigen-specific cytolytic activity of HA-2-TCR-transferred(**A**), CMV-B7-TCR-transferred (**B**), or DBY-TCR-transferred (**C**) γδ T cells without and with CD4 or CD8αβ was assessed. Lysis of EBV-LCL negative for the antigen, peptide pulsed EBV-LCL and EBV-LCL presenting endogenously processed antigen was determined in a 4 h ^{51}Cr- release assay at different effector-to-target ratios in triplicate. γδ T cells transduced with the different TCRs and equipped with the relevant coreceptor showed high levels of antigen-specific cytolytic activity.*

parental αβ T cell clone, the kinetics of target cell lysis was analyzed. Efficient lysis of HA-2 peptide pulsed EBV-LCL and some lysis of EBV-LCL presenting endogenously processed antigen was observed for HA-2-TCR$^+$ γδ T cells without expression of CD8 (Fig. 4A). The CD8$^+$ HA-2-TCR$^+$ γδ T cells lysed HA-2 expressing target cells as efficient as the original αβ HA2.27 T cell clone (Fig. 4B and 4C). In conclusion, αβTCR redirected γδ T cells expressing the relevant coreceptor exerted high antigen-specific cytolytic activity against EBV-LCL pulsed with the relevant peptide as well as EBV-LCL presenting endogenously processed antigen.

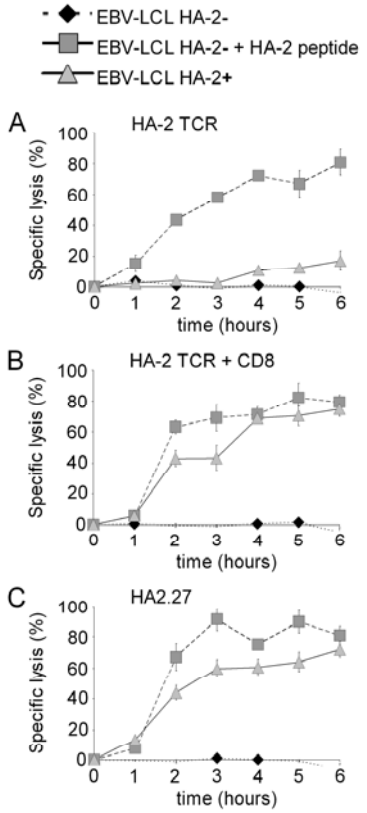

Figure 4. Kinetics of antigen-specific target cell lysis. *The kinetics of the antigen-specific cytolytic activity of HA-2-TCR-transferred γδ T cells without co-transfer of CD8αβ (**A**), or with co-transfer of CD8αβ (**B**) was compared with the kinetics of lysis of the original HA2.27 αβ T cell clone (**C**). In a ^{51}Cr- release assay, the lysis of HA-2-, HA-2 peptide-pulsed and HA-2+ EBV-LCL was measured at different time points at an effector-to-target ratio of 20:1 in triplicate. γδ T cells expressing the HA-2-TCR and CD8 were as efficient in their antigen-specific target cell lysis as the original HA2.27 αβ T cell clone.*

Antigen-specific IFN-γ and IL-4 production by αβTCR-transduced γδ T cells

Besides cytolytic activity, the capacity to produce cytokines upon antigen-specific stimulation may be important for the *in vivo* efficacy of αβTCR-redirected γδ T cells. As shown in figure 5A, γδ T cells redirected with the HA-2-TCR in the absence of the CD8 coreceptor produced high amounts of IFN-γ and IL-4 when stimulated with HA-2 peptide pulsed EBV-LCL, but low levels of cytokines were produced after stimulation with EBV-LCL presenting endogenously processed antigen. Cotransfer of CD8 significantly increased the antigen-specific cytokine production, especially after stimulation with endogenously

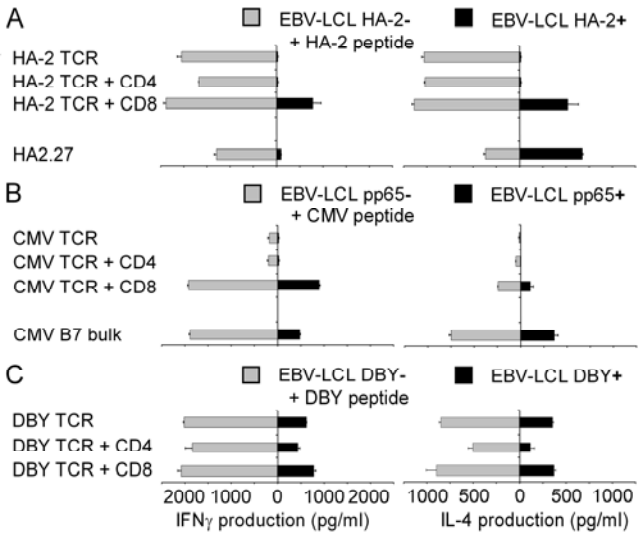

Figure 5. IFN-γ and IL-4 production by αβTCR-transferred γδ T cells without or with cotransfer of CD4 and CD8αβ. *The antigen-specific production of IFN-γ (left) and IL-4 (right) by HA-2-TCR-transferred (**A**), CMV-B7-TCR-transferred (**B**), DBY-TCR-transferred (**C**) γδ T cells without and with CD4 or CD8αβ was determined after 24 h stimulation with peptide pulsed EBV-LCL (gray columns) or EBV-LCL presenting endogenously processed antigen (black columns). No IFN-γ or IL-4 production (<10 pg/ml) was observed after stimulation with the EBV-LCL negative for the different antigens. The experiment was performed in duplicate. Coreceptor-expressing HA-2-TCR- and CMV-B7-TCR-transferred γδ T cells produced high amounts of IFN-γ and IL-4 upon stimulation with both peptide-pulsed target cells and endogenously processed antigen. In contrast, the expression of CD4 by the DBY-TCR transferred γδ T cells had no additional effect on the production of cytokines.*

processed HA-2. No IFN-γ and IL-4 production (<10 pg/ml) was observed after stimulation with the HA-2-negative EBV-LCL. CMV-B7-TCR$^+$ γδ T cells lacking expression of the relevant coreceptor produced low amounts of cytokines upon stimulation with CMV peptide-pulsed EBV-LCL or endogenously processed pp65 (Fig. 5B). In contrast, high amounts of IFN-γ were produced by CD8$^+$ CMV-B7-TCR$^+$ γδ T cells when stimulated with either pp65 peptide-pulsed EBV-LCL or endogenously processed pp65. In addition, the CD8$^+$ CMV-B7-TCR$^+$ γδ T cells produced low but significant amounts of IL-4 upon stimulation with peptide-pulsed EBV-LCL or endogenously processed pp65. No IFN-γ and IL-4 production (<10 pg/ml) was observed after stimulation with the pp65 negative EBV-LCL. Expression of CD4 appeared not to be essential for the cytokine production of γδ T cells transduced with the DBY-TCR (Fig. 5C). Both against DBY peptide pulsed EBV-LCL and endogenously processed DBY, high amounts of both IFN-γ and IL-4 were produced. No IFN-γ and IL-4 production (<10 pg/ml) was observed after stimulation with the DBY negative EBV-LCL. In conclusion, TCR-transferred γδ T cells can produce high amounts of IFN-γ and IL-4 upon stimulation with peptide pulsed target cells or target cells presenting endogenously processed antigen.

Antileukemic reactivity of HA-2-TCR-transduced γδ T cells

To explore whether engineered γδ T cells can be an alternative strategy to treat hematological malignancies the antileukemic reactivity of HA-2-TCR$^+$ γδ T cells was tested against HLA-A2+ CML and AML cells positive or negative for the expression of the mHag HA-2. The HA-2-TCR-transferred CD8$^+$ γδ T cells exerted antileukemic reactivity against the HA-2-expressing CML and AML cells, which was as efficient as the antileukemic reactivity of the original HA2.27 αβ T-cell clone (Fig. 6A and 6B). HA-2-TCR$^+$ γδ T cells lacking CD8 expression were capable of antigen-specific recognition and subsequent lysis of the HA-2-expressing AML cells after 20 hours of incubation, although not as efficient as the CD8-positive HA-2-TCR$^+$ γδ T cells (Fig. 6C). In addition, the CD8$^+$ HA-2-TCR$^+$ γδ T cells produced high amounts of IFN-γ and IL-4 when stimulated with HA-2 expressing AML cells (Fig. 6D).

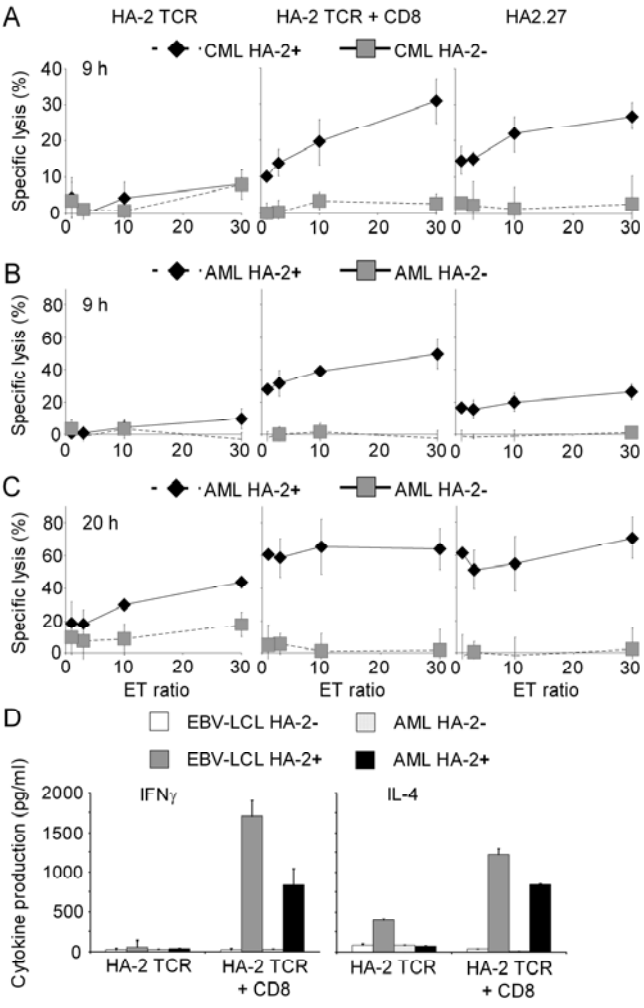

Figure 6. Antigen-specific cytolytic activity of HA-2-TCR-transferred γδ T cells against primary leukemic cells. *The reactivity against (**A**) primary CML cells (HA-2+ or HA-2-) or (**B**) primary AML cells (HA-2+ or HA-2-) of HA-2-TCR$^+$ γδ T cells without or with cotransfer of CD8 was compared with the reactivity of the original HA2.27 αβ T-cell clone in a 9-h ^{51}Cr-release assay at different effector-to-target ratios in triplicate. **C,** in addition the cytolytic activity against the primary AML cells was investigated in a 20 hours ^{51}Cr-release assay. **D,** the production of IFN-γ (left) and IL-4 (right) by the HA-2-TCR$^+$ γδ T cells without or with cotransfer of CD8 was determined after stimulation with EBV-LCL (HA-2+ or HA-2-) and primary AML cells (HA-2+ or HA-2-). The experiments illustrated that HA-2-TCR$^+$ γδ T cells expressing CD8 were as efficient in their antileukemic reactivity against both primary CML and AML as the original HA2.27 αβ T cell clone.*

In summary, the CD8$^+$ HA-2-TCR$^+$ γδ T cells were demonstrated to be effective, cytokine producing, antigen-specific killer cells with antileukemic reactivity against HA-2 expressing CML and AML cells making them suitable effector cells for application in cellular immunotherapy for leukemia.

Discussion

In this study, we show that γδ T cells can be redirected by αβTCR gene transfer to generate efficient leukemia reactive T cells which do not express potentially harmful mixed TCR dimers. γδ T cells were isolated by immunomagnetic bead isolation and subsequent FACS sorting, resulting in >99% pure populations of γδ T cells. The γδ T cells were transduced with three different TCRs, recognizing the hematopoiesis specific mHag HA-2 in the context of HLA-A2, or CMV pp65 in the context of HLA-B7, or the HLA-DQ5 restricted male-specific mHag DBY, respectively. In the absence of the relevant coreceptor, the γδ T cells transduced with the different TCRs showed high reactivity against peptide-pulsed target cells, but only minor reactivity against target cells presenting endogenously processed antigen. Additional retroviral transfer of the relevant coreceptor increased the functionality of the αβTCR$^+$ γδ T cells resulting in high levels of antigen-specific cytolytic activity against both peptide-pulsed target cells and target cells presenting endogenously processed antigen. In addition, high amounts of IFN-γ and/or IL-4 were produced after antigen-specific stimulation. The HLA class I-restricted HA-2-TCR and CMV-B7-TCR-transduced CD8$^+$ γδ T cells produced significant amounts of cytokines in response to stimulation with peptide-pulsed targets as well as endogenously processed antigen. γδ T cells transduced with the HLA class II-restricted DBY-TCR both with or without the coreceptor CD4 produced similar amounts of IFN-γ and/or IL-4. Importantly, γδ T cells transferred with the HA-2-TCR and CD8 exerted antigen-specific cytolytic activity against HA-2 expressing CML and AML cells. The antileukemic reactivity of the CD8$^+$ HA-2-TCR$^+$ γδ T cells was as efficient as the original HA-2-specific αβ T-cell clone.

The αβTCR$^+$ γδ T cells were effective killer cells that, in addition, produced both IFN-γ and IL-4 after antigen-specific stimulation both with peptide-pulsed target cells and target cells presenting endogenously processed antigen. IFN-γ has been demonstrated to be beneficial for eradication of tumor cells *in vivo* (32-34). Less clear is the direct effect of IL-4 in the effector phase, but IL-4 has been described to contribute indirectly to the tumoricidal activity of T cells (34, 35).

Transfer of αβTCRs to γδ T cells can generate large numbers of antigen-specific T cells without the expression of mixed TCR dimers (11, 12). We and others hypothesized that mixed TCR dimers can occur in TCR-transferred αβ T cells due to pairing of the endogenous TCR chains with the introduced chains (6, 36). The newly formed combinations of TCRα and TCRβ chains have unknown specificities that may be harmful. We recently observed that transfer of only a TCRα or a TCRβ chain into virus specific T cells resulted in decreased expression of the virus specific TCRs as measured by tetramer staining, while total TCR cell surface expression was constant. In addition, TCRβ chain transfer into these virus-specific T cells resulted in decreased endogenous TCRβ chain cell surface expression[*]. These results illustrate that chimeric TCR complexes can be formed.

[*] manuscript in preparation Mirjam H.M. Heemskerk *et al.*

Because γδTCR chains cannot pair with αβTCR chains, mixed TCR dimer formation is prevented after retroviral αβTCR transfer to γδ T cells. Some HA-2-TCR- and CMV-B7-TCR-transduced γδ T cells with high cell surface expression of the introduced αβTCR showed down-regulation of the endogenous γδTCR (Fig. 1A). This indicates that the retrovirally introduced αβTCR and the γδTCR endogenously expressed by the T cell compete for cellular components like CD3, essential for TCR cell surface expression.

The CD4 and CD8 coreceptors increase the avidity of the effector-target cell interaction (37-40), and both CD4 and CD8 have been reported to promote the formation of lipid rafts and their intracellular domains can recruit Lck to the TCR-coreceptor complexes (41-43). Some T cells have been described that express TCRs which function independently of the contribution of the coreceptor (44, 45). In the absence of CD8 both the HA-2-TCR- and CMV-B7-TCR-transduced γδ T cells showed antigen-specific cytolytic activity against peptide pulsed target cells, suggesting coreceptor-independent functionality. However, when the antigen-specific binding of the HA-2-TCR and CMV-B7-TCR transduced γδ T cells was visualized using tetramers, we observed that in contrast to the CMV-B7-TCR-transduced γδ T cells the HA-2-TCR-transduced γδ T cells were unable to bind tetramer without the expression of CD8. Furthermore, the HA-2-TCR and CMV-B7-TCR transduced γδ T cells exerted only potent reactivity against the biologically relevant endogenously processed antigen when CD8 was co-expressed, demonstrating that the TCR-redirected γδ T cells depended on CD8 for optimal functionality. Thus, although tetramer staining as well as reactivity against peptide pulsed target cells may indicate specific antigen recognition by the T cells, they are not fully predictive markers for the functional activity against endogenously processed antigen by TCR-transferred T cells.

In summary, we show that transfer of both HLA class I- and II-restricted TCRs combined with the relevant coreceptors to γδ T cells, generated redirected γδ T cells exerting antigen-specific cytolytic activity and producing high amounts of cytokines upon antigen-specific stimulation. γδ T cells transferred with a TCR specific for the hematopoiesis specific mHag HA-2, in combination with CD8, were highly cytolytic against HA-2-expressing leukemic cells. Thus, αβTCR-transfer to γδ T cells can generate highly efficient redirected effector T cells which do not express the potentially detrimental mixed TCR dimers, making them suitable for application in cellular immunotherapy.

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