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

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HLA class II restricted T cell receptor gene transfer generates CD4$^+$ T cells with helper activity as well as cytotoxic capacity

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Abstract

Both cytotoxic T cells and helper T cells are important in immune responses against pathogens and malignant cells. In hematological malignancies which express HLA class II molecules, immunotherapy may be directed to HLA class II restricted antigens. We investigated whether it is possible to engineer HLA class II restricted T cells with both antigen specific cytolytic activity and the capacity to produce high amounts of cytokines. $CD4^+$ and $CD8^+$ peripheral-blood-derived T cells were retrovirally transduced with the HLA class II restricted minor histocompatibility antigen dead box RNA helicase Y (DBY) specific TCR. The TCR-transduced $CD4^+$ T cells exerted DBY-specific cytolytic activity, produced Th0, Th1 or Th2 cytokines, and proliferated upon DBY-specific stimulation. TCR-transduced $CD8^+$ T cells exerted cytolytic activity which equaled the level of cytolytic activity of the TCR-transferred $CD4^+$ T cells. Co-transfer of CD4 enhanced the cytolytic activity of the TCR-transduced $CD8^+$ T cells, but introduction of CD4 was not sufficient to generate DBY-specific $CD8^+$ T cells with the capacity to produce high amounts of cytokines. In this study, we demonstrated the feasibility to engineer T cells with antigen specific cytolytic activity, as well as the ability to produce significant amounts of cytokines, by TCR transfer to $CD4^+$ T cells.

Introduction

Patients relapsing from leukemia after allogeneic stem cell transplantation can successfully be treated by donor lymphocyte infusions (DLI), due to the graft-versus-leukemia (GVL) response of the donor derived T cells[1-4]. However, DLI is also associated with graft-versus-host-disease (GVHD). Several studies have illustrated that depletion of $CD8^+$ T cells from the DLI may preserve the beneficial GVL reactivity without the induction of GVHD, indicating that the $CD4^+$ T lymphocytes may play an essential role in the anti-leukemic reactivity[5-10].

The mechanism by which the $CD4^+$ T cells contribute to the GVL response is, however, not known. $CD4^+$ T cells have been suggested to elicit the anti-leukemic response via the induction of minor histocompatibility antigen (mHag)-specific $CD8^+$ T cells[5]. In addition, mHag specific $CD4^+$ T helper cells have been reported to mature dendritic cells and enhance the expansion of mHag specific cytotoxic T lymphocytes *in vitro*[11]. Alternatively, we have demonstrated that $CD4^+$ T cells are capable of exerting direct cytolytic activity against leukemic cells[12-14].

Cellular immunotherapeutic approaches based on the adoptive transfer of T lymphocytes may require large cell numbers. Large numbers of T cells with a defined antigen specificity can be acquired by retroviral transfer of the T-cell receptor to peripheral-blood-derived T lymphocytes. Until now, the application of retroviral transfer of TCRs has mainly been limited to the transfer of HLA class I restricted TCRs[15-20]. However, the specificity of HLA class II restricted T lymphocytes can also be transferred to other T cells[21-23]. Previously, we demonstrated the retroviral transfer of a dual specific TCR recognizing both an antigenic peptide in the context of HLA class I and an antigenic peptide in the context of HLA class II[21]. Furthermore, an MHC class II restricted chicken $OVA_{323-339}$ peptide-specific TCR was functionally transferred to murine $CD4^+$ T helper cells[22,23].

Effective immune responses have been shown to depend on both $CD4^+$ and $CD8^+$ T lymphocytes[24,25]. In the absence of CD4 helper cells, CTLs were less protective against challenge with a pathogen. Furthermore, helper T lymphocytes have been found to be important for the development of T cell memory[24,26,27]. Without help supplied by $CD4^+$ T cells, T cell memory generation can be severely hampered, as reflected by both decreased proliferation and diminished capacity to exert effector functions. Immune responses against leukemic cells might therefore also be more efficient when both T cell subsets functionally contribute.

For cellular immunotherapy, it may be beneficial if both helper and cytolytic effector functions could be combined in one T-cell subset. We investigated whether it is possible to generate T cells with both HLA class II restricted cytolytic activity and the capacity to produce significant amounts of cytokines. As a model, we redirected human peripheral-blood-derived T cells by retroviral transfer of an HLA class II restricted TCR specific for the mHag dead box RNA helicase Y (DBY)[28]. We generated TCR-transferred $CD4^+$ T-cell clones that exerted specific cytolytic activity against DBY expressing EBV-LCL, and expanded specifically upon antigen specific stimulation. Furthermore, the TCR-transferred $CD4^+$ T-cell clones produced high amounts of cytokines upon DBY-specific stimulation. Since it could be hypothesized that $CD8^+$ T cells are superior to $CD4^+$ T cells to exert cytolytic effector functions, we generated DBY-TCR-transduced $CD8^+$ CTLs, and characterized the effect of additional transfer of the CD4 coreceptor to optimize the interactions between CTL and target cells. TCR-transduced $CD8^+$ CTLs without CD4 had comparable cytolytic activities as the TCR-transduced $CD4^+$ T cells. The cytolytic activities improved when the TCR-transduced $CD8^+$ T cells were additionally transduced with CD4. However, the combination of cytolytic activity and the capacity to produce high amounts of cytokines was only found in the TCR-transduced $CD4^+$ T cells, since the TCR-transduced $CD8^+$ T cells produced only low amounts of cytokines even after cotransfer of CD4. These results demonstrate that redirected T cells can be generated with cytolytic capacity and the ability to produce high amounts of cytokines by transfer of $CD4^+$ T cells with an HLA class II restricted TCR.

Results

Functional analysis of the DBY-TCR-transduced $CD4^+$ T-cell clones

The TCR$\alpha\beta$ usage of the male-specific mHag-DBY-recognizing T-cell clone JBB4 was determined by RT-PCR (data not shown). The TCRα chain was encoded by an in frame gene rearrangement of AV1S4 and J39, and the TCRβ was based on the combination of BV5S6 and BJ2S7 according to the nomenclature described by Arden *et al.*[29] In the IMGT nomenclature these TCR chains are named AV8S3 and BV5S4, respectively. AV1 in combination with eGFP and BV5 in combination with ΔNGF-R were cloned into retroviral vectors. PBMCs from HLA-DQ5 negative donors (to prevent the generation of potentially self-reactive T cells) were stimulated with Leuko-A, and transduced on day 2 with the TCRα and TCRβ chains of JBB4 or mock control vectors. On day five, the transduced T cells were sorted based on eGFP and ΔNGF-R expression (data not shown), and plated single cell per well by FACS. Sorted T cells were expanded by nonspecific stimulation, and the functionality of the TCR-transduced T-cell clones was examined using cytotoxicity, proliferation, and cytokine production assays. Figure 1 shows the results of the TCR-

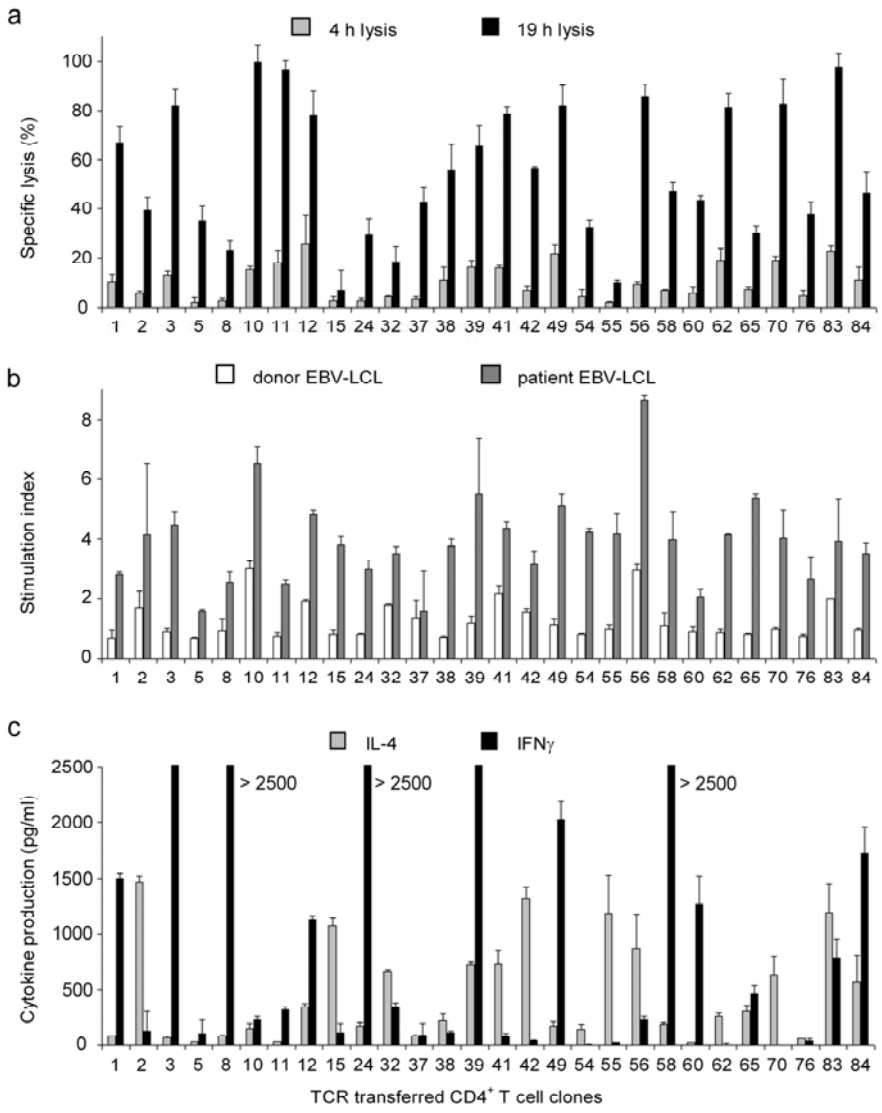

Figure 1. The functional analysis of TCR-transferred CD4$^+$ T-cell clones. a. *The TCR-transduced CD4$^+$ T-cell clones were tested against the patient-derived EBV-LCL in a 4-h or a 19-h ^{51}Cr-release assay at an E/T ratio of 10:1. The cytolytic activity against the donor derived EBV-LCL at 4 h as well as 19 h was <6% (data not shown).* ***b****. The proliferative capacity of the TCR-transduced T-cell clones was determined in a ^{3}H-Thymidine incorporation assay after stimulation with the EBV-LCL of the donor or patient. The SI was defined as (^{3}H-Thymidine incorporation of effector cells with stimulator cells)/[(^{3}H-Thymidine incorporation of effector cells in medium only)+(^{3}H-Thymidine incorporation of stimulator cells in medium only)]* ***c.*** *The production of IL-4 and IFNγ by the TCR-transduced T-cell clones was determined after stimulation with the patient derived EBV-LCL for 24 h. After stimulation with the donor derived EBV-LCL, the production of IL-4 was <60 pg/ml and the production of IFNγ was <40 pg/ml (data not shown). The experiments were performed twice in triplicate.*

transduced $CD4^+$ T-cell clones that were cytotoxic and/or produced high amounts of cytokines, which comprised approximately 40% of the T-cell clones obtained. The TCR-transduced $CD4^+$ T cells exerted upto 26% of specific cytotoxicity against the male patient derived EBV-LCL in a 4 h ^{51}Cr-release assay. The percentages of antigen specific cytolytic activity increased for all TCR-transduced $CD4^+$ T-cell clones after 19 h (Figure 1a). The cytolytic activity against the HLA-identical EBV-LCL derived from the female donor was lower than 6% after 19 h (data not shown). Most TCR-transduced T cells proliferated specifically after mHag-specific stimulation with the patient EBV-LCL (Figure 1b). Analysis of the production of the cytokines IFNγ and IL-4 demonstrated that $CD4^+$ T-cell clones with a Th0, Th1, and Th2 phenotype had been obtained. More than 1000 pg/ml of IFNγ was produced by 10 of the 28 TCR-transduced $CD4^+$ T cells after stimulation with the male-patient-derived EBV-LCL and 11 TCR-transduced $CD4^+$ T-cell clones produced more than 500 pg/ml of IL-4 (Figure 1c). A variety of TCR-transduced $CD4^+$ T-cell clones were obtained. Some T-cell clones combined cytokine production with cytolytic activity, whereas others were cytokine producers without cytolytic capacity, and others were antigen specific killers without the production of IFNγ or IL-4. After stimulation with the EBV-LCL derived from the female donor, the production of IFNγ was for all T-cell clones lower than 40 pg/ml and the production of IL-4 was lower than 60 pg/ml (data not shown). Mock transduced $CD4^+$ T cells did not show any cytolytic activity, proliferation and cytokine production after stimulation with EBV-LCL of donor or patient (data not shown).

DBY-specific reactivity of TCR-transduced T cells

To demonstrate that the antigen recognized by the TCR-transduced T cells on the patient derived EBV-LCL was the male mHag DBY, TCR-transduced $CD4^+$ T cells were tested against DBY-peptide pulsed donor derived EBV-LCL. Specific lysis was observed against both the patient derived EBV-LCL and the DBY-peptide pulsed donor derived EBV-LCL (Figure 2a). No cytolytic activity was observed against the female donor derived EBV-LCL. To confirm that the TCR transferred $CD4^+$ T-cell clones could specifically recognize endogenously processed DBY, cytolytic activity was analyzed against the DBY-transduced female donor EBV-LCL. DBY-transduced, but not non-transduced or mock-transduced donor EBV-LCL were lysed by the TCR-transduced $CD4^+$ T cells (Figure 2b). For comparison, the parental T-cell clone from which the TCR was originally derived was also included. Stimulation of the TCR-transduced $CD4^+$ T cells with the patient derived EBV-LCL or DBY-peptide-loaded donor EBV-LCL induced the production of the cytokines IFNγ and/or IL-4 (Figure 2c). In conclusion, the TCR-transduced T cells recognized endogenously processed DBY expressed by male-patient-derived EBV-LCL.

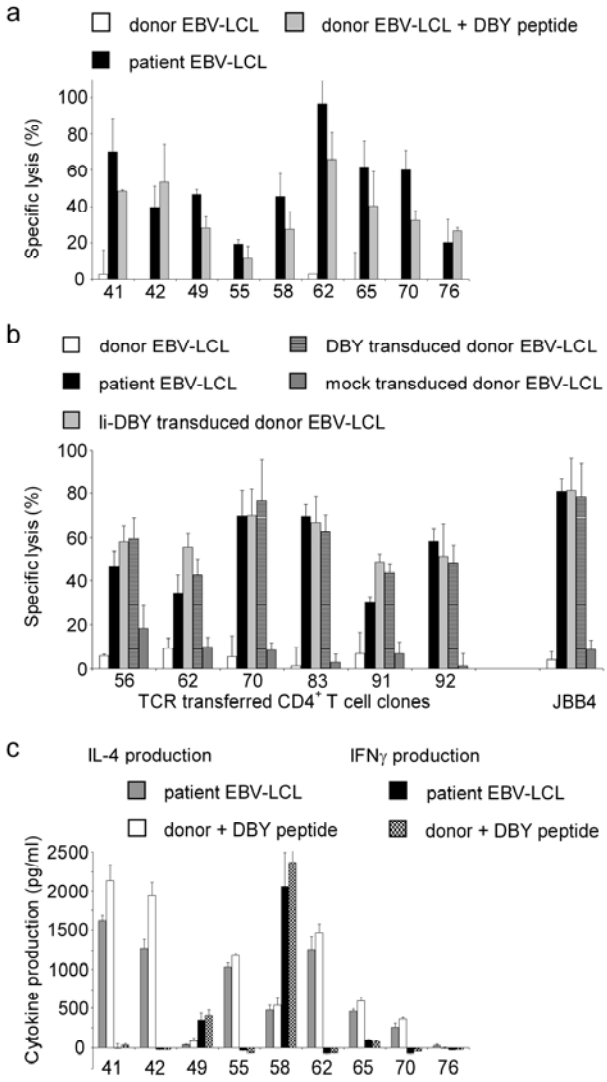

Figure 2. DBY-specific reactivity of the TCR-transferred CD4$^+$T-cell clones. a*. The cytoxic activity was measured of TCR-transduced CD4$^+$ T-cell clones against the donor derived EBV-LCL, the patient derived EBV-LCL, and the DBY-peptide pulsed donor derived EBV-LCL. The results were obtained in triplicate in a 19-h ^{51}Cr-release assay at an E/T ratio of 10:1.* ***b.*** *The cytotoxicity of the TCR-transduced CD4$^+$ T-cell clones and the parental T-cell clone was measured against the donor derived EBV-LCL, the patient derived EBV-LCL and the transduced donor EBV-LCL with respectively the Ii/DBY, DBY or mock vector. The results of the 19-h ^{51}Cr-release assay were obtained in triplicate at the E/T ratio of 10:1.* ***c.*** *The IL-4 and IFNγ production of TCR-transduced CD4$^+$ T-cell clones was determined after 24-h stimulation with the patient-derived EBV-LCL and DBY-peptide pulsed donor-derived EBV-LCL. After stimulation with the donor-derived EBV-LCL, the production of IL-4 was <70 pg/ml and the production of IFNγ was <20 pg/ml (data not shown). The experiment was performed in duplicate.*

Comparison of TCR-transduced CD4$^+$ and CD8$^+$ T-cell clones

To analyze whether transfer of the HLA class II restricted DBY-TCR to CD8$^+$ CTLs would lead to the generation of T cells with higher cytolytic activity, we compared the cytolytic activity of TCR-transduced CD4$^+$ T cells and TCR-transduced CD8$^+$ T cells in 4h and 19h ^{51}Cr-release assays. We observed no significant difference in specific cytolytic activity between the two T-cell subsets (Figure 3a).

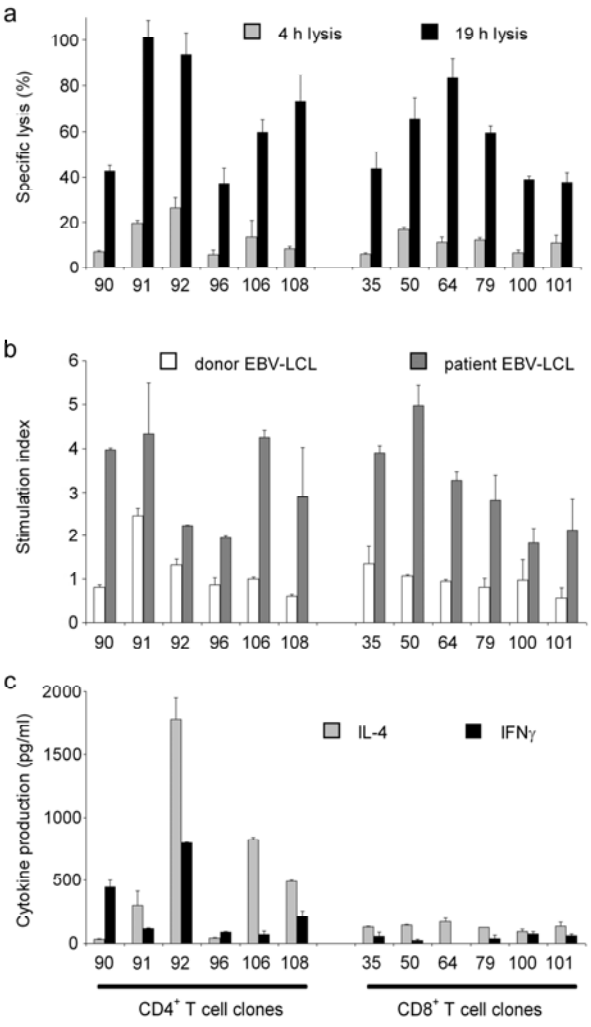

Figure 3. Comparison of TCR-transduced CD4$^+$ and CD8$^+$ T-cell clones. a. *A comparison was made of the cytolytic capacity of TCR-transduced CD4$^+$ and CD8$^+$ T-cell clones against the patient-derived EBV-LCL in a 4-h or a 19-h ^{51}Cr-release assay at an E/T ratio of 10:1. The reactivity of all T-cell clones against the donor-derived EBV-LCL at 4 h as well as 19 h was <4% (data not shown).* ***b****. The antigen specific proliferation of the TCR-transduced CD4$^+$ and CD8$^+$ T-cell clones was determined in a ^{3}H-Thymidine incorporation assay after stimulation with the EBV-LCL of the donor and patient. The SI was defined as (^{3}H-Thymidine incorporation of effector cells with stimulator cells)/[(^{3}H-Thymidine incorporation of effector cells in medium only)+(^{3}H-Thymidine incorporation of stimulator cells in medium only)]* ***c.*** *The IL-4 and IFNγ production by the TCR-transduced CD4$^+$ and CD8$^+$ T-cell clones was compared after stimulation with the patient-derived EBV-LCL for 24 h. After stimulation with the donor-derived EBV-LCL, the production of IL-4 was <50 pg/ml and the production of IFNγ was <60 pg/ml (data not shown). All the experiments shown in this figure were performed twice and in triplicate.*

No cytotoxicity (<4%) was observed against the donor-derived EBV-LCL (data not shown). Analysis of the antigen specific proliferation indicated that the TCR-transduced $CD4^+$ and TCR-transduced $CD8^+$ T cells had comparable proliferative capacities (Figure 3b). In contrast, we observed differences in the amounts of cytokines produced after antigen specific stimulation. The TCR-transduced $CD8^+$ T-cell clones produced low amounts of cytokines. Whereas no more than 70 pg/ml of IFNγ and 170 pg/ml of IL-4 was produced by the TCR-transduced $CD8^+$ T cell clones, the TCR-transduced $CD4^+$ T-cell clones produced upto 800 pg/ml of IFNγ and 1800 pg/ml of IL-4 after stimulation with the patient-derived EBV-LCL (Figure 3c). Mock-transduced $CD8^+$ T cells did not show any cytolytic activity, proliferation and cytokine production after stimulation with EBV-LCL of donor or patient (data not shown). In summary, DBY-TCR-transferred $CD8^+$ T cells showed cytolytic activity and proliferation similar to TCR-transduced $CD4^+$ T cells, but produced significantly lower amounts of cytokines.

CD4 coreceptor transfer to TCR-transduced $CD8^+$ T cells

To investigate whether additional expression of the CD4 coreceptor could further enhance the specific cytolytic activity and cytokine production of the TCR-transduced $CD8^+$ T cells, we co-transferred the CD4 molecule to the TCR-transduced $CD8^+$ T cells. The TCR-transduced $CD8^+$ T-cell clones were retrovirally transduced with the CD4 co-receptor, and subsequently FACS sorted based on the expression of CD4. The expression levels of the introduced CD4 molecules on the TCR-transduced $CD8^+$ T-cell clones were comparable with the expression levels of CD4 on unmodified $CD4^+$ T cells (data not shown). Figures 4a and 4b show the data from 7 representative TCR-transduced $CD8^+$ T-cell clones with and without the introduction of CD4. Figure 4a shows the cytolytic activity of the TCR-trans-

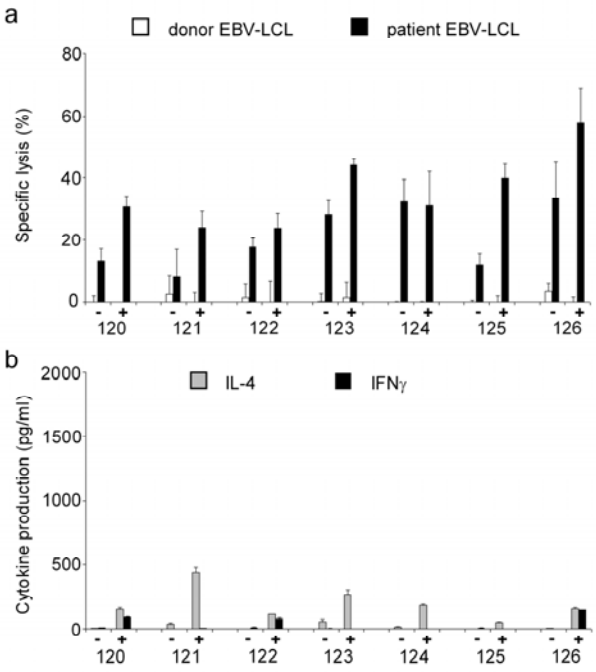

Figure 4. The contribution of CD4 co-transduction in TCR-transduced $CD8^+$ T-cell clones. a. *The specific target cell lysis of TCR-transduced $CD8^+$ T-cell clones without (-) and with (+) CD4 was assessed. Lysis of donor EBV-LCL and patient EBV-LCL was determined after 9 h of incubation at an E/T ratio of 10:1 in triplicate.* ***b.*** *The TCR-transduced $CD8^+$ T-cell clones without (-) and with (+) CD4 were analyzed for their production of IL-4 and IFNγ after 24 h stimulation with the patient EBV-LCL. After stimulation with the donor-derived EBV-LCL, the production of both IL-4 and IFNγ was <20 pg/ml (data not shown). The experiment was performed in duplicate.*

duced CD8$^+$ T-cell clones. To determine differences in antigen-specific cytolytic activity, the lysis was measured at a 9 h time point at which the lytic activity had not reached a plateau level. After co-transfer of CD4 to the TCR-transduced CD8$^+$ T-cell clones, five out of seven TCR-transduced CD8$^+$ T-cell clones showed increased antigen-specific cytolytic activity against the patient-derived EBV-LCL. However, the cytokine production by the CD4-cotransferred TCR-transduced CD8$^+$ T-cell clones was still very low in comparison with the production by the TCR-transduced CD4$^+$ T-cell clones (Figure 4b). Whereas the TCR-transduced CD4$^+$ T-cell clones produced upto 800 pg/ml of IFNγ and 1800 pg/ml of IL-4 after stimulation with the patient-derived EBV-LCL (Figure 3c), the CD4-cotransferred TCR-transduced CD8$^+$ T-cell clones produced only upto 150 pg/ml of IFNγ and 440 pg/ml IL-4. In conclusion, co-transfer of CD4 did not generate TCR-transduced CD8$^+$ T-cell clones producing amounts of cytokines as high as the TCR-transduced CD4$^+$ T-cell clones, although improved cytolytic activity was observed for the majority of the TCR-transduced CD8$^+$ T-cell clones.

Mechanism of target cell lysis of the TCR-transduced T-cell clones

Since the cytolytic activities of the TCR-transduced CD4$^+$ T-cell clones and TCR-transduced CD8$^+$ T-cell clones were found to be comparable, we investigated whether the "helper" and "cytotoxic" T cells applied similar mechanisms for their target cell lysis using perforin and granzyme-B blocking experiments. The TCR-transduced CD4$^+$ T-cell clones and TCR-transduced CD8$^+$ T-cell clones were preincubated with the Ca^{2+} chelator EGTA, which captures extracellular Ca^{2+}, and subsequently a ^{51}Cr- release assay was performed.

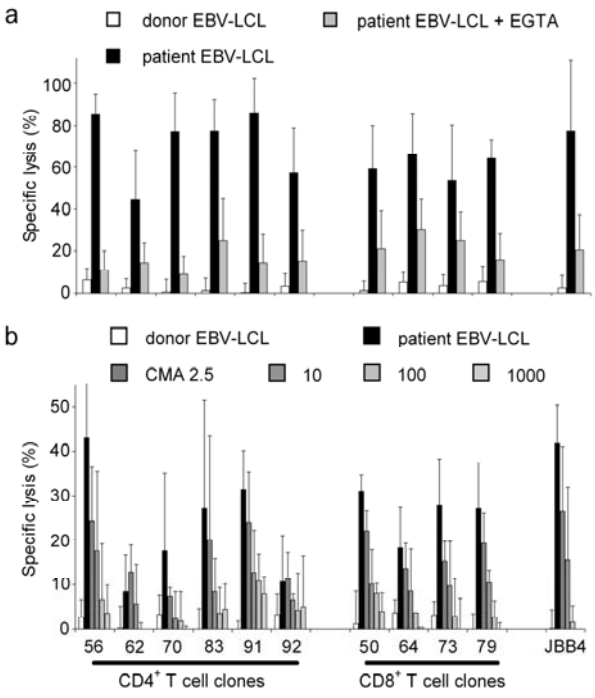

Figure 5. Blocking of extracellular Ca^{2+}-dependent cytotoxicity. a. *The cytotoxicity of the TCR-transduced CD4$^+$ and CD8$^+$ T-cell clones and the parental T-cell clone was examined in 19-h ^{51}Cr-release assays with or without preincubation of the T cells with EGTA. The cytolytic activity is shown against the donor-derived EBV-LCL and patient-derived EBV-LCL. The data shown are the average of 5 independent experiments performed in triplicate at an E/T ratio of 10:1.* ***b.*** *In 8-h ^{51}Cr-release assays, the cytolytic activity of the TCR-transduced T-cell clones and the parental T-cell clone were analyzed after preincubation with increasing amounts of CMA, varying from 2.5-1000 nM. The data shown are the average of 3 independent experiments performed in triplicate at an E/T ratio of 10:1.*

In the 19 h ^{51}Cr-release assay, EGTA preincubation almost completely blocked the specific cytotoxicity of all TCR-transduced T-cell clones and the parental T-cell clone (Figure 5a). When increasing amounts of the perforin inhibitor CMA were preincubated with the T cells, a dose-dependent inhibition of lysis was observed. At the highest CMA concentration (1000 nM) specific target cell lysis was completely blocked (Figure 5b). High amounts of CMA were found not to be toxic to both target cells and T cells, since no increased spontaneous ^{51}Cr-release by the target cells was observed, and no decrease in the number of viable T cells was indicated by eosine staining. In conclusion, these data demonstrate that the CD4$^+$ T cells applied the same mechanism for target cell lysis as the CD8$^+$ T cells.

Discussion

In this study, we demonstrated that redirected CD4$^+$ T lymphocytes with cytolytic capacity and different helper phenotypes can be generated by the retroviral transfer of an HLA class II restricted TCR to peripheral-blood-derived T cells. The TCR used in this study was a TCR specific for the male-specific mHag DBY in the context of HLA-DQ5. TCR-transduced T cells were sorted based on the expression of the marker genes eGFP and ΔNGF-R. The TCR-transferred CD4$^+$ T cells possessed cytolytic activity, proliferated, and produced high amounts of cytokines after antigen-specific stimulation with EBV-LCL expressing DBY. The cytokine profiles of the TCR-transferred CD4$^+$ T-cell clones indicated that all different T helper subsets were represented. TCR-transduced CD4$^+$ T-cell clones were detected which produced both IFNγ and IL-4 (Th0), or IFNγ (Th1), or IL-4 (Th2). The TCR-transferred CD4$^+$ T cells exerted DBY-specific cytolytic activity upto 26% in a 4 h ^{51}Cr-release assay, and the specific lysis increased upto 100% after 19 h. Although TCR-transferred CD4$^+$ T-cell clones were obtained that were as cytolytic as the parental JBB4 T-cell clone (Figures 2b and 5), variation was observed in the levels of cytolytic activity of the TCR-transferred T-cell clones. We previously demonstrated that TCR-transferred T-cell clones can have different expression levels of the introduced TCR, explaining the observed variation in cytolytic activity[19]. Different TCR expression levels might arise due to differences in the expression levels of the retrovirally encoded TCRαβ chains or competition for cell surface expression with the endogenous TCR and mixed TCR dimers.

Based on the potent cytolytic effector function of CD8$^+$ T cells, we hypothesized that T cells with higher antigen-specific cytolytic activity could be obtained by transferring the DBY-TCR to CD8$^+$ CTL. However, CD8$^+$ T cells transferred with the DBY-TCR had comparable cytolytic activity. The proliferative capacity was also similar to TCR-transduced CD4$^+$ T cells, but the cytokine production was significantly lower. Since the TCR-transferred CD4$^+$ T cells expressed the co-receptor relevant for the interaction with HLA class II molecules and the TCR-transferred CD8$^+$ T cells did not, we investigated whether expression of CD4 could increase the specific cytolytic activity and cytokine production of the TCR-transferred CD8$^+$ T cells. By additional retroviral transfer the CD4 molecule was co-transferred to the TCR-transferred CD8$^+$ T cells. The CD4 molecule enlarges the overall affinity of the TCR with its antigenic peptide-HLA complex through binding to the β2 domain of the HLA class II molecule[30]. It has been proposed that the dependency on CD4 is determined by the number of antigenic peptide-HLA complexes on the target cell surface[31]. The TCR requires a critical number of antigenic peptide-HLA complexes on the target cell surface for CD4 independent recognition. In correspondence with the function of

CD4, we observed improved cytolytic activity by cotransferring CD4 to the TCR-transduced $CD8^+$ T cells. The cytokine production was, however, still very low in comparison with the production by the TCR-transduced $CD4^+$ T cells. Since the marker gene expression level and thus the expression level of the introduced DBY-TCR between the TCR-transduced $CD4^+$ and $CD8^+$ T cells were comparable, differences in the specific cytokine production of both subsets can not be due to differences in TCR expression of the TCR-transduced $CD4^+$ and $CD8^+$ T cells.

A potential disadvantage of TCR gene transfer to other $\alpha\beta$ 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. Since the specificity of the mixed TCR dimers is unknown, autoreactivity can not be excluded[19,32]. The number of T cells with different TCR chains and thus the chance to generate autoreactive T cells can be limited by TCR genetransfer to T cells with defined antigen-specificity . We previously demonstrated the reprogramming of cytomegalovirus (CMV) specific T cells into leukemia-reactive T cells by transferring a TCR specific for the mHag HA-2[19].

The amounts and type of cytokines produced by different T cell subsets may have major impact on the eventual outcome of immune responses. T cells producing Th1 cytokines (IFNγ and IL-2) have been shown to be responsible for anti-tumor reactivity [33-35]. Previously, we showed that HA-2 specific T cells isolated by tetramer during a strong GVL response produced large amounts of IFNγ and no or low amounts of IL-4[18]. Furthermore, we recently demonstrated that T cells contributing to the GVL reactivity of DLI can be isolated based on their production of IFNγ[36]. Thus, Th1 cytokines play a very important role in anti-tumor reactivity. Th2 and Tc2 cells have also been suggested to play a central role in the regulation of GVHD, graft rejection, and GVL responses[37-39]. We hypothesize that in addition significant amounts of cytokines have to be produced for effective anti-tumor reactivity. The previously isolated functional mHag specific T cells that may have contributed to the on going GVL response, all produced more than 800 pg/ml of IFNγ[18]. Since the large amounts of cytokines produced by DBY-TCR-transduced $CD4^+$ T cells were not equaled by the TCR-transduced $CD8^+$ T cells, TCR-transduced $CD4^+$ T cells may be preferred for cellular immunotherapy. Furthermore, selection of effector cells with the desired cytokine profile might be possible, since TCR-transfer to the $CD4^+$ T cells generated T cells with all different Th phenotypes (Th0, Th1 and Th2).

The anti-tumor reactivity of DLI has been suggested to be mediated by $CD8^+$ T cells of donor origin[40]. $CD4^+$ T cells appeared to contribute by inducing the expansion of the $CD8^+$ T cells. However, DLI depleted of $CD8^+$ T cells have been demonstrated to preserve their antileukemic reactivity, suggesting that $CD4^+$ T cells can directly mediate GVL responses[5-10], although it was not excluded in these studies that residual $CD8^+$ T cells might be the effector cells responsible for the observed GVL responses. We have previously demonstrated that $CD4^+$ T cells were capable of exerting direct cytolytic activity against leukemic cells[12-14]. Since it has been suggested that $CD4^+$ T cells and $CD8^+$ T cells can mediate target cell lysis via the same mechanisms[41], which was confirmed by our perforin and granzyme-B blocking experiments, $CD4^+$ T cells may directly and effectively mediate cell lysis of HLA class II expressing cell populations.

As a model we used a TCR recognizing the mHag DBY in the context of HLA-DQ5. HLA class II restricted TCRs directed against hematopoiesis specific antigens which are differentially expressed between the patient and donor can be very suitable for the treatment of hematological malignancies[42], since HLA class II expression is not only found

on hematopoietic progenitor cells[43], B cells[44], activated T cells[44], and monocytes[44], but also on many malignant hematopoietic cells. HLA class II expression has been demonstrated on acute myeloid leukemia[45,46], chronic myeloid leukemia[47], acute lymphoblastic leukemia[46,48,49], chronic lymphocytic leukemia[46,49], multiple myeloma[50], and hairy cell leukemia[48]. Thus, HLA class II restricted TCR-transferred $CD4^+$ T cells combining cytolytic activity with cytokine production may therefore be very potent effector cells for the application of cellular immunotherapy.

In summary, we demonstrated that HLA class II restricted T cells can be obtained with both cytolytic and helper functions by the retroviral transfer of a TCR to peripheral-blood-derived T cells. TCR-transferred $CD4^+$ T cells were generated that were cytolytic, had proliferative capacity and produced high amounts of cytokines. Transfer of the DBY-TCR to $CD8^+$ T cell produced T cells with comparable cytolytic activity as the TCR-transduced $CD4^+$ T cells, but despite co-transfer of the CD4 coreceptor to the TCR-transduced $CD8^+$ T cells $CD4^+$ T cells were superior to the $CD8^+$ T cells in the production of the cytokines IFNγ and IL-4. Hence, application of TCR-transferred $CD4^+$ T cells might be a novel strategy, combining T cell help with cytolytic activity leading to improved efficacy of immunotherapy for HLA class II expressing hematological malignancies.

Materials and methods

T-cell clones

Previously, the $CD4^+$ T-cell clone JBB4 was isolated from a male patient who developed acute GVHD after stem cell transplantation from his HLA genotypically identical sister[12]. JBB4 was demonstrated to recognize the male specific mHag DBY in the context of HLA-DQ5[28]. The T-cell clone produced both IFNγ and IL-4 after antigen-specific stimulation and exerted specific cytolytic activity against targets expressing DBY in the context of HLA-DQ5[12]. T-cell clones were cultured in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker, Verviers, Belgium) containing 10% pooled human serum and 100 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands). Every 2 weeks, the T cells were stimulated with a mixture of 50 Gy irradiated allogeneic PBMCs, 50 Gy irradiated Epstein-Barr virus (EBV)-transformed B cells (EBV-LCL), 1 μg/ml leucoagglutinin (Leuko-A, Sigma, St. Louis, USA) and 100 IU/ml IL-2.

Construction of the retroviral vectors and production of the retroviral supernatant

The TCR$\alpha\beta$ usage of the JBB4 T-cell clone was determined by RT-PCR using primers covering the entire repertoire of known TCR chains. Table 1 shows the forward primers used for determination of the TCR AV gene used by the JBB4 T-cell clone. The common reverse primer used for the AV PCR was: 5'AGCAGATTAAACCCGGCCA3'. Table 2 shows the TCR BV primers used in combination with common BV reverse primer: 5'TTCTCTTGACCATGGCCATC3'. For all primers, the same PCR program was used. After the initial denaturation (5 min, 94°C), 33 cycles were performed for denaturation (1 min, 94°C), annealing (1 min, 60°C) and elongation (1 min, 72°C), followed by an extra elongation period of 7 min (72°C). Subsequently, the identity of the PCR products was confirmed by sequencing. The TCRα and TCRβ chains were cloned into bicistronic retroviral

Table 1. Nucleotide sequence of the TCR AV specific primers.

	Arden *et al.*	IMGT	5′ sequence 3′ fwd primer
1	1S1	TRAV8-1	CATAACCACCACGTAATTCTC
2	1S2/1S3/1S5	TRAV8-4/TRAV8-6/TRAV8-2	CTCCAGCTTCTCCTGAAGTA
3	2S1/2S2	TRAV12-2/TRAV12-3	TCAGTGTTCCAGAGGGAGCCATTG
4	2S3	TRAV12-1	TCAATGTTCCAGAGGGAGCCACTG
5	3S1	TRAV17	GGAGTGTCTTTGGTGATTCTATGG
6	4S1	TRAV26-2	GCATTACTGTACTCCTATCTTTGGG
7	4S2	TRAV26-1	AGTAACTGTGTTTCTGACCTTTGGA
8	5S1	TRAV6	GTGTTTTGCTGATTTTGTGGCTTCA
9	6S1/ADV6S1	TRAV14/DV4	TTGTCACAGCTTCACTGTGGCTAG
10	7S1/7S2	TRAV1-1/TRAV1-2	TATGTTTCCATGAAGATGGGAGGC
11	8S1	TRAV13-1	GACAGCGCTGTTATCAAGTGTACT
12	8S2	TRAV13-2	TGACAACTCTATTATCAACTGTGCT
13	9S1	TRAV16	CGAGAAGCTCCTCTCTGTCTTTAAA
14	10S1	TRAV27	AGCCCTCAGTTTCTAAGCATCCAA
15	11S1	TRAV2	GGCTAGGGCTTCTCCTCAACTCTC
16	12S1	TRAV19	CAGACTGAAATTTCTGTGGTGGAG
17	13S1	TRAV22	GAGGGAGCCAATTCCACGCTGCG
18	14S1/ADV14S1/14S2	TRAV38-2/DV8/TRAV38-1	CCAGAGATGTCTGTGCAGGAGGCA
19	15S1	TRAV5	CGAGAGGGAGACAGCTCCGTTATA
20	16S1	TRAV3	GGAAGATCAGGTCAACGTTGCTGA
21	17S1/ADV17S1	TRAV23/DV6	CCAGAAAGGAGGGATTTCAATTATA
22	18S1	TRAV24	GGAGGGAGACAGCACCAATTTCAC
23	19S1	TRAV41	CTCGGTAGGAATAAGTGCCTTACA
24	20S1	TRAV4	CAAGAAGTGAACATAACCTGTAGCC
25	21S1/ADV21S1	TRAV29/DV5	AGCAAGTTAAGCAAAATTCACCATC
26	22S1	TRAV9-2	ATTCAGTGACCCAGATGGAAGGGC
27	23S1	TRAV21	CCAGAAGGAGAAAACTTGGTTCTC
28	24S1	TRAV10	ATCATCCTGGAGGGAAAGAACTGC
29	25S1	TRAV35	TCTTCAAGCATATTTAACACCTGGC
30	26S1	TRAV34	CCAAGAGGGAAAGAATCTCACCATA
31	27S1	TRAV39	CAGGAGGGAAAAAACTATACCATCT
32	28S1/DV28S1	TRAV36/DV7	TGACTAACTTTCGAAGCCTAC
33	29S1	TRAV30	TCCGAGAAGGGGAAGATGCTGTCA
34	30S1	TRAV20	TTGGCTGGTTGAGTGGAGAAGACC
35	31S1	TRAV40	CTAATTCTGATCTTAATGTTTGGAGG
36	32S1	TRAV25	CCTCAACTACTTTAAGCAATATACAG

vectors containing the marker genes eGFP[51] and truncated nerve growth factor receptor (ΔNGF-R)[52], respectively. The Moloney murine leukemia virus-based retroviral vector LZRS and packaging cells φ-NX-A were used[53]. φ-NX-A packaging cells were transfected with the TCRα or TCRβ chain containing retroviral vectors using a calcium phosphate transfection method (Life Technologies, Gaithersburg, USA) and after 2 days 2 μg/ml puromycin (CLONTECH Laboratories, Inc.) was added. The transfected φ-NX-A cells were cultured on puromycin containing medium for approximately 14 days, and subsequently 2 x 10^7 cells were plated per T175 tissue culture flask (Greiner-Bio One, Frickenhausen, Germany) in 30 ml IMDM supplemented with 10% FBS without puromycin. The next day medium was replaced, and the following day retroviral supernatant was harvested, centrifuged and frozen in aliquots at -70°C. Likewise, a retroviral supernatant was produced encoding the CD4α molecule with marker gene ΔNGF-R. For the transduction of the donor-derived EBV-LCL with DBY, retroviruses were made encoding the DBY protein with the marker eGFP, or a fusion protein of the invariant chain (Ii) and the DBY epitope (HIENFSDIDMGE) (Ii-DBY epitope) with the marker gene ΔNGF-R. Fusion of the DBY epitope to the invariant chain enhances presentation of the epitope in the context of MHC[54]. As control vectors, retroviral vectors were used containing only eGFP or ΔNGF-R. Functionality of the retroviral constructs encoding the TCRα and TCRβ chains was confirmed by transduction of the TCRα and TCRβ chain deficient Jurkat clone 76 and subsequent analysis of TCRαβ cell surface expression as reported previously[18].

Production of DBY-transduced EBV-LCL

Donor-derived EBV-LCL were transduced with retroviral supernatants containing the DBY gene, or the Ii-DBY epitope or the ΔNGF-R control vector. The transduction was performed using recombinant human fibronectin fragments CH-296[55] (Bio Whittaker, Verviers, Belgium) as described[51]. The transduction efficiency as measured by the expression of the markers eGFP and NGF-R was analyzed by flow cytometry 3–5 days after transduction. Subsequently, the EBV-LCL were sorted on the basis of the expression of the markers genes eGFP or ΔNGF-R using a FACS Vantage (Becton Dickinson (BD), San Jose, USA). The donor-derived EBV-LCL, the patient-derived EBV-LCL and the transduced EBV-LCL were used as target cells in the cytotoxicity assays and stimulator cells in the proliferation and cytokine assays. For the detection of the ΔNGF-R, a phycoerythrin (PE) conjugated anti-human NGF-R mAb (Pharmingen, San Diego, USA) was used.

Generation of DBY-TCR-transduced T cells

Human peripheral blood T cells derived from HLA-DQ5 negative donors were stimulated with 1 μg/ml Leuko-A in IMDM supplemented with 10% pooled human serum and 100 IU/ml IL-2. Two days after stimulation, the T cells were transduced with a mixture of the JBB4 TCRα - and TCRβ chain encoding retroviral supernatants, or control retroviral supernatants containing the marker genes eGFP or ΔNGF-R only. TCR-transduced $eGFP^+$ and $\Delta NGF\text{-}R^+$ T cells were sorted single cell per well using a FACS Vantage. Flow-cytometric analysis illustrated that both $CD4^+$ and $CD8^+$ DBY-TCR-transduced T-cell clones were obtained. The mAbs used for flow cytometry were anti-human TCRαβ conjugated with PE-Cy5 (Immunotech, Marseille, France), anti-CD3, anti-CD4 and anti-CD8 mAbs conjugated with PE (BD). Part of the TCR-transduced $CD8^+$ T-cell clones were subsequently transduced with the retrovirus encoding the CD4α molecule. The CD4-transduced T-cell clones were sorted based on the expression of CD4.

Table 2. Nucleotide sequence of the TCR BV specific primers.

	Arden *et al.*	IMGT	5′ sequence 3′ fwd primer
1	1S1	TRBV9	GAGAGCAAAAGGAAACATTCTTGAAC
2	2S1	TRBV20-1	TCTGTGAAGATCGAGTGCCG
3	2S2	TRBV20-2	TCTGTGAACATCGAGTGCCG
4	3S1	TRBV28	TCTGGAATGTGTCCAGGATATG
5	4S1	TRBV29-1	GCTCTGAGGCCACATATGAGAG
6	5S1	TRBV5-1	CTTCAGTGAGACACAGAGAAAC
7	5S2/5S3/5S6	TRBV5-6/TRBV5-5/TRBV5-4	TGTGTCCTGGTACCAACAGG
8	5S4	TRBV5-8	TGTGTACTGGTACCAACAGG
9	6S3/6S6/6S8	/TRBV7-6/TRBV7-7/TRBV7-4	GGTGCTGGAGTCTCCCAGTC
10	6S1/6S2/6S6	TRBV7-3/TRBV7-8/TRBV7-7	CTCAGGTGTGATCCAATTTC
11	7S1/7S2/7S3	TRBV4-1/TRBV4-3/TRBV4-2	AGTGTGCCAAGTCGCTTCTCA
12	8S1/8S2	TRBV12-3/TRBV12-4	CATGATGCGGGGACTGGAGTTG
13	8S3	TRBV12-5	CATGATGCAAGGACTGGAGTTGC
14	9S1	TRBV3-1	CATTATAAATGAAACAGTTCCAAATCG
15	10S1	TRBV21-1	TTCAGAAAGCAGAAATAATCAATGAG
16	11S1	TRBV25-1	GAGAAGGGAGATCTTTCCTCTGAG
17	12S1/12S2	TRBV10-3/TRBV10-1	CTGATCCATTACTCATATGGTGTT
18	12S3	TRBV10-2	CTGATCTATTACTCAGCAGCTGCT
19	13S* (1)	TRBV6-* (1)	TGTCACTCAGACCCCAAAATTC
20	13S5	TRBV6-4	ACATCTCAGATCCTGGCAGC
21	14S1	TRBV27	GTGACTGATAAGGGAGATGTTCCT
22	15S1	TRBV24-1	GATCTCTGATGGATACAGTGTCT
23	16S1	TRBV14	AATCTTTATTGGTATCGACGTGTT
24	17S1	TRBV19	AGCTGAAGGGTACAGCGTCTCTC
25	18S1	TRBV18	CAGCTCCCAGAGGAAGGTCT
26	19S1	TRBV23-1	GTCACACAGACTCCAGGATATT
27	20S1	TRBV30	GGAACATCAAACCCCAACCTA
28	21S2/21S3	TRBV11-3/TRBV11-2	TCCCAGATATAAGATTATAGAG
29	21S1	TRBV11-1	CCCCAGATATAAGATTACAGAG
30	22S1	TRBV2	ATCTCAGAGAAGTCTGAAATATTCG
31	23S1	TRBV13	ATGCAGAGCGATAAAGGAAG
32	24S1	TRBV15	AAAGATTTTAACAATGAAGCAGAC
33	25S1	TRBV16	CTCCAAAACATCTTGTCAGAGG

1. 13S* = 13S1/13S2a-b/13S3/13S4/13S6/13S7/13S9
   TRBV6-* = TRBV6-5/TRBV6-2-3/TRBV6-1/TRBV6-9/TRBV6-6/TRBV6-8/

Cytotoxicity assay

Target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 h at 37°C, washed three times, and added to the effector cells at various effector/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 DBY peptide (10 μg/ml) for 1 h at 37°C and washed once. Blocking of Ca^{2+} dependent cytotoxicity was performed by preincubation of the effector cells with 5 mM EGTA (Sigma-Aldrich, St. Louis, USA) for 30 minutes at 37°C. To block perforin mediated cytotoxicity effector cells were preincubated with 2.5-1000 nM concanamycin A (CMA) (Sigma-Aldrich, St. Louis, USA) for 30 minutes at 37°C. After washing, the effector cells were added to the target cells. Targets incubated in medium or 1% Triton X-100 were used for determination of the spontaneous and maximum release, respectively. The tests were performed in duplicate or triplicate. After 4, 9 or 19 h 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.

Proliferation assay and cytokine production

To measure the proliferative capacity and cytokine production of the T cells clones, 2 x 10^4 irradiated (50 Gy) 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 h 80 μl supernatant was harvested to determine IFNγ and IL-4 production by standard ELISA (Sanquin, Amsterdam, The Netherlands). After 72 h 1 μCi ^{3}H-Thymidine was added, and, after an additional incubation for 12 h ^{3}H-Thymidine incorporation, was measured using a luminescence counter (Topcount-NXT, Packard). The stimulation index (SI) was defined as (^{3}H-Thymidine incorporation of effector cells with stimulator cells)/[(^{3}H-Thymidine incorporation of effector cells in medium only)+(^{3}H-Thymidine incorporation of stimulator cells in medium only)].

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