Designing T-cells with desired T-cell receptor make-up for immunotherapy

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Mixed TCR dimers harbor potentially harmful neoreactivity

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Proc Natl Acad Sci U S A. 2010 Jun 15;107(24):10972-7. Epub 2010 Jun 1. Reprinted with permission.
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ABSTRACT

Adoptive transfer of TCR transduced T-cells may be an attractive strategy to target both hematological malignancies and solid tumors. By introducing a TCR, large numbers of T-cells with defined antigen (Ag) specificity can be obtained. However, by introduction of a TCR, mixed TCR dimers can be formed. Besides the decrease in TCR expression of the introduced and endogenous TCR, these mixed TCR dimers could harbor potentially harmful specificities. In this study, we demonstrate that introduction of TCRs resulted in formation of neoreactive mixed TCR dimers, composed of the introduced TCR chains pairing with either the endogenous TCRα or β chain. Neoreactivities observed were HLA class I or class II restricted. Most neoreactive mixed TCR dimers were allo-HLA reactive, however, neoreactive mixed TCR dimers with autoreactive activity were also observed. We demonstrate that inclusion of an extra disulfide bond between the constant domains of the introduced TCR markedly reduced neoreactivity, whereas enhanced effectiveness of the introduced TCR was observed. In conclusion, TCR transfer results in the formation of neoreactive mixed TCR dimers with the potential to generate off-target effects, underlining the importance of searching for techniques to facilitate preferential pairing.

INTRODUCTION

Adoptive transfer of T-cells is a strategy used to target both solid tumors and leukemia. Patients with relapsed hematological malignancies after allogeneic stem cell transplantation can be successfully treated with donor lymphocyte infusion (DLI) [1,2], and patients with solid tumors can be effectively treated with tumor infiltrating lymphocytes (TILs) cultured from tumor tissue[3]. The beneficial graft-versus-leukemia effect of DLI mediated by the recognition of minor histocompatibility antigens (mHags) is, however, often accompanied by graft-versus-host disease. Furthermore, isolation and expansion of TILs is feasible only for a fraction of patients with solid tumors. The adoptive transfer of T-cells transduced with TCRs recognizing tumor associated antigens or mHags may be an attractive alternative strategy to target hematological malignancies and solid tumors. By introducing a TCR, large numbers of T-cells with defined antigen (Ag) specificity can be obtained without long in vitro culture periods. Different studies have shown the effectiveness of TCR transfer, both in vitro[4-8] and in vivo[9-11]. Recently, the in vivo efficacy of adoptively transferred TCR transduced (td) T-cells was demonstrated in melanoma patients[10,12].

The introduction of an exogenous TCR into T-cells has several consequences for the TCR make-up of the cell. The introduced TCR has to compete for cell surface expression with the endogenous TCR, and with mixed TCR dimers consisting of an endogenous TCR chain pairing with an introduced TCR chain[13]. Because of competition of these different TCR complexes for binding with CD3, the frequency of TCRs at the cell surface will be lower in TCR td T-cells than in parental T-cells. Therefore, a prerequisite of the introduced TCR is that it exhibits high affinity for its antigen, and is able to efficiently compete with the endogenous TCR for cell surface expression[13]. Different studies have attempted to improve TCR surface expression and subsequently biological activity, by facilitating matched pairing of the introduced TCR chains. Exchange of the human constant regions for murine constant regions was described to improve TCR expression and functionality[14,15]. Another strategy that resulted in preferential pairing of the introduced TCR chains and increased TCR surface expression is the introduction of a disulfide bond in the extracellular constant domain[16,17].

Not only the decrease in TCR expression of the introduced Ag-specific TCR, but also the formation of mixed TCR dimers with unknown specificity is an additional potential drawback of clinical application of TCR gene transfer[13,18,19]. Because the specificity of mixed TCR dimers is unpredictable, hazardous specificities may be formed. In this study, we investigated whether TCR transfer can lead to the generation of mixed TCR dimers exhibiting new detrimental reactivities. To address this issue we created T-cells expressing mixed TCR dimers. To be able to discriminate between the functionality of the endogenous TCR, the introduced TCR as well as mixed TCR dimers, we transduced different defined virus-specific T-cells with 7 different well characterized Ag-specific TCRs and tested these for newly acquired reactivities against an HLA-typed LCL panel covering all prevalent HLA class I and II molecules.

Our results demonstrate that pairing of endogenous TCR chains with introduced TCR chains can result in the formation of mixed TCR dimers with new potentially hazardous specificities recognizing allo-antigens as well as auto-antigens, both HLA class I and class II restricted.

RESULTS

Introduction of different TCRs into several virus-specific T-cells elicits neoreactivity mediated via mixed TCR dimers

To study whether TCR transfer can lead to mixed TCR dimers with new detrimental reactivities, we transduced various virus-specific T-cell lines from 4 healthy donors with different Ag-specific TCRs. HLA-A1 restricted pp50- or pp65-specific T-cells and HLA-B8 restricted IE-1- or BZLF-1-specific T-cells were sorted, resulting in 5 different virus-specific T-cell lines (Table S1). These T-cell lines were transduced with 7 different TCRs, consisting of 4 different HA-2-specific TCRs (HA2.5-TCR, HA2.6-TCR, HA2.19-TCR, and HA.2.20-TCR), 2 different HA-1-specific TCRs (HA1.M2-TCR, HA1.M7-TCR) and the CMV-TCR. The transduced virus-specific T-cells were sorted based on high eGPF and NGF-R positivity, and tested for neoreactivity against the LCL panel (Table S2) covering all prevalent HLA class I and class II molecules. Introduction of different TCRs resulted in newly acquired reactivities against different LCLs, of which representative examples are shown in Figure 1. Some LCLs were excluded from analysis, as the non td virus-specific T-cells already recognized the LCLs,

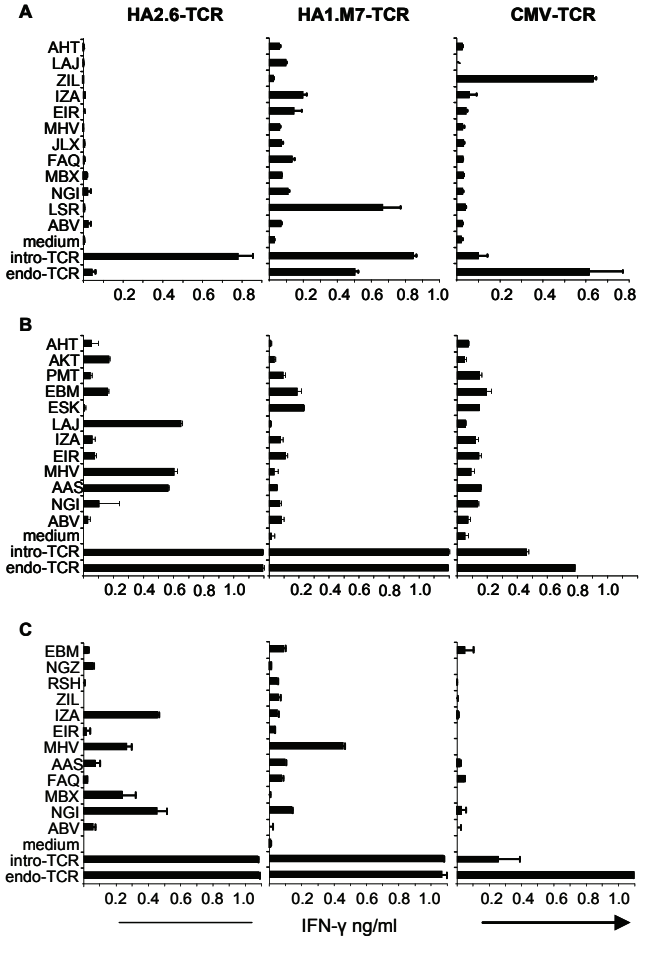

Figure 1. TCR td virus-specific T-cells demonstrate neoreactivity.

*Figure 1: T cells were tested against a broad LCL panel. IFN-γ production of 3 of the 5 different virus-specific T-cell populations transduced with 3 different TCRs is depicted, namely of **(A)** pp50-specific T-cells isolated from donor CVO, **(B)** pp50-specific T-cells of donor UKL and **(C)** IE-1-specific T-cells isolated from donor MBX. As a control for the reactivity of the endogenous and introduced TCR, LCLs with the restricting HLA molecules were pulsed with the relevant peptides (endo-TCR and intro-TCR, respectively). IFN-γ production depicted is representative of 3 separate experiments performed in duplo.*

*Figure 2: pp50 T-cells from CVO were transduced with **(A)** HA1.M7-TCRα or β chains or with **(B)** CMV-TCRα or β chains, and IE-1 T-cells from MBX were transduced with **(C)** HA2.6-TCRα or β chains and tested against the LCL panel for neoreactivity. As a control for the reactivity of the endogenous TCR, LCLs with the restricting HLA molecules of the endogenous virus-specific TCR were pulsed with the viral peptides (A, B, C; endo-TCR). The IFN-γ production depicted is representative of 2 separate experiments.*

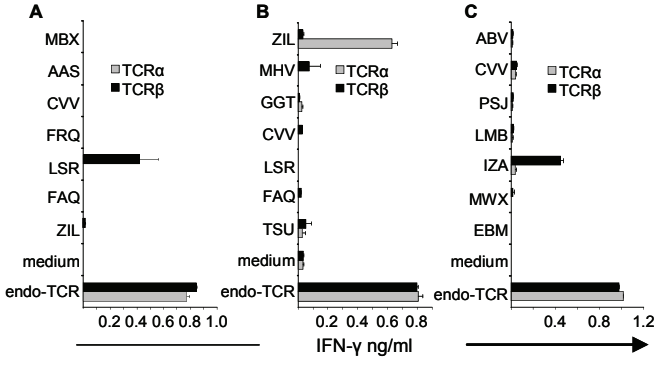

Figure 2. Neoreactivities caused by mixed TCR dimers.

indicative for alloreactivity of the virus-specific T-cells rather than neoreactivity via mixed TCR dimers (Table S1). We could exclude alloreactivity of the introduced TCRs, as the parental T-cell clones of which these TCRs were derived were not reactive against the LCLs present in the panel. In each of the 5 different virus-specific T-cell lines, transfer of at least 2 out of 7 TCRs induced neoreactivity (Table S1). As illustrated in Figure 1A, pp50-specific T-cells of donor CVO (CVO pp50 T-cells) transferred with the CMV-TCR exhibited strong reactivity particularly against ZIL. This reactivity was not seen with HA2.6-TCR td or HA1.M7-TCR td CVO pp50 T-cells. HA1.M7-TCR transfer resulted in strong reactivity directed against LSR, which was not observed with HA2.6-TCR td or CMV-TCR td CVO pp50 T-cells. Introduction of the HA2.6-TCR into pp50-specific T-cells of donor UKL (UKL pp50 T-cells) resulted in clear neoreactivity (Figure 1B), whereas low neoreactivity was observed after introduction of the HA1.M7-TCR or the CMV-TCR into these T-cells. Introduction of the HA2.6-TCR and HA1.M7-TCR into IE-1-specific T-cells of healthy individual MBX (MBX IE-1 T-cells) resulted in neoreactivity against different LCLs (Figure 1C). Strikingly, some neoreactivities were as robust as reactivity via the introduced or endogenous TCR against peptide pulsed target cells. To determine whether the observed neoreactivities against LSR after HA1.M7-TCR transfer and against ZIL after CMV-TCR transfer (Figure 1A) were mediated via mixed TCR dimers, we transduced CVO pp50 T-cells with either the HA1.M7-TCRα or β chain (Figure 2A), or either the CMV-TCRα or β chain (Figure 2B). Transduction of only the HA1.M7-TCRβ and not α chain (Figure 2A) resulted in neoreactivity directed against LSR. Transduction of only the CMV-TCRα chain and not β chain (Figure 2B) into these T-cells resulted in neoreactivity directed against ZIL. In addition, to test whether the observed neoreactivities of MBX IE-1 T-cells after HA2.6-TCR transfer (Figure 1C) were mediated via mixed TCR dimers, we transduced these T-cells with either only the HA2.6-TCRα or β chain. As shown in Figure 2C only HA-2.6-TCRβ td T-cells demonstrated neoreactivity directed against IZA. Furthermore, we deliberately created mixed TCR dimers by recombining HA-2-specific TCRα and TCRβ chains of 4 different HA-2-TCRs, namely the HA2.5-TCR, the HA2.6-TCR, the HA2.19-TCR and the HA2.20-TCR and transducing all possible combinations into monoclonal CVO pp50 T-cells. Taking into account that also the introduced HA-2-TCR chains can pair with the endogenous TCR of the pp50 T-cells, this resulted in potentially 20 mixed TCR dimers. Of these 20 mixed TCR dimers, the recombination of HA2.19-TCRα and HA2.6-TCRβ chain (Figure

S1A; mixed TCR dimer) resulted in significant IFN-γ production against DMD, whereas the parental HA2.19-TCR and HA2.6-TCR demonstrated only HA-2-specific reactivity against HA-2 peptide pulsed target cells. These results indicate that each recombination of TCR chains after TCR transfer can potentially result in a harmful new reactivity.

These results demonstrate that neoreactivities can occur in multiple virus-specific T-cells after transfer of different TCRs. The neoreactive mixed TCR dimers can be composed of introduced TCR chains pairing with either the endogenous TCRα chain or the endogenous TCRβ chain.

Mixed TCR dimers can acquire both HLA class I and class II restricted allo- and autoreactivities

To study whether neoreactivities of the mixed TCR dimers were HLA restricted, blocking experiments were performed. Because in oligoclonal virus-specific T-cell lines theoretically different mixed TCR dimers can be formed and this can potentially hinder analysis of HLA-restriction, as well as functional activity of the individual-specificities, monoclonal CVO pp50 T-cells and MBX IE-1 T-cells were sorted on bases of TCR usage. By transfer of the different TCRs we could confirm that the most prominent TCR-BV1 positive population present in CVO pp50 T-cells was responsible for the neoreactivity against LSR after HA1.M7-TCR transfer, and the neoreactivity against ZIL after HA2.6-TCR transfer. Likewise, of the oligoclonal populations of MBX IE-1 T-cells only the TCR-BV1 positive T-cells transduced with HA2.6-TCR demonstrated neoreactivity against IZA. Neoreactivity of

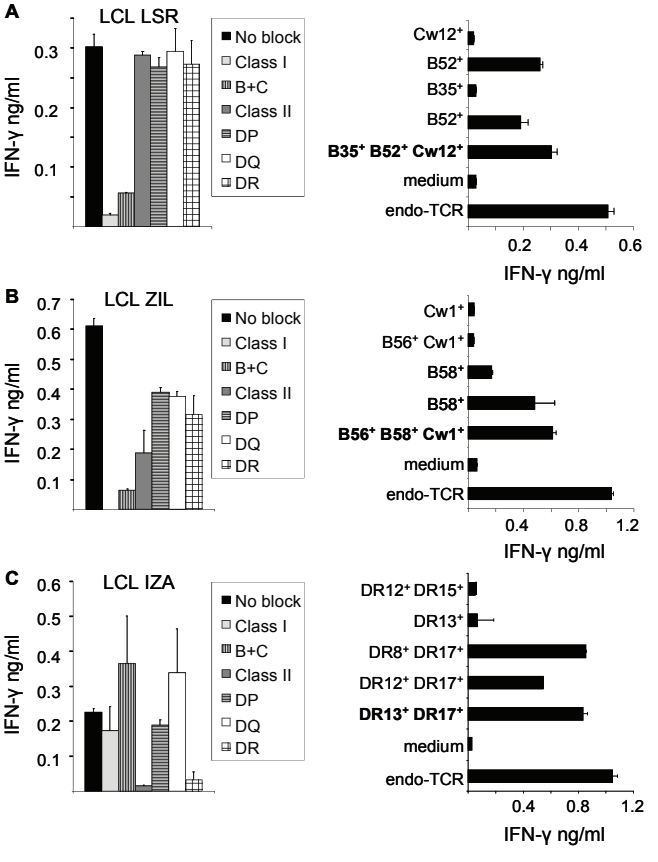

Figure 3. Neoreactivity of mixed TCR dimers is both HLA class I and II restricted.

*Figure 3: HLA restriction of neoreactive mixed TCR dimers of **(A)** BV1+ HA1.M7-TCRβ td CVO pp50 T-cells, **(B)** BV1+ CMV-TCRα td CVO pp50 T-cells, or **(C)** BV1+ HA2.6-TCRβ td MBX IE-1 T-cells was tested in IFN-γ production assay using blocking antibodies. Relevant HLA typing of the LCLs is indicated, with the HLA typing of the blocked LCL in bold. IFN-γ production depicted is representative of 3 separate experiments performed in triplo.*

*Figure 4: **(A)** CMV-TCRα td and non td CVO pp50 T-cells were tested against HLA-B58+ LCL ZIL and HLA-B58- LCL from CVO and CD14+, CD19+ and CD4+ MACS-isolated cell subsets derived from HLA-B58+ healthy individual IGN. HA-2.6-TCRβ td and non td MBX IE-1 T-cells were tested against LCLs and CD14+, CD19+ and CD4+ MACS-isolated cell subsets derived from **(B)** HLA-DR17+ MBX or **(C)** HLA-DR17+ NGI. HLA-DR17- LCL EBM was included in the experiment as a control. T-cells were tested against resting cell subsets (ex vivo) or activated cell subsets. CD14+ cells were either activated into immature DCs (iDC) or mature DCs (mDCs) using activating cytokines. CD19+ cells were activated using activating cytokines and CD40L (B act). CD4+ cells were activated using PHA (T act). IFN-γ production depicted is representative of 2 separate experiments.*

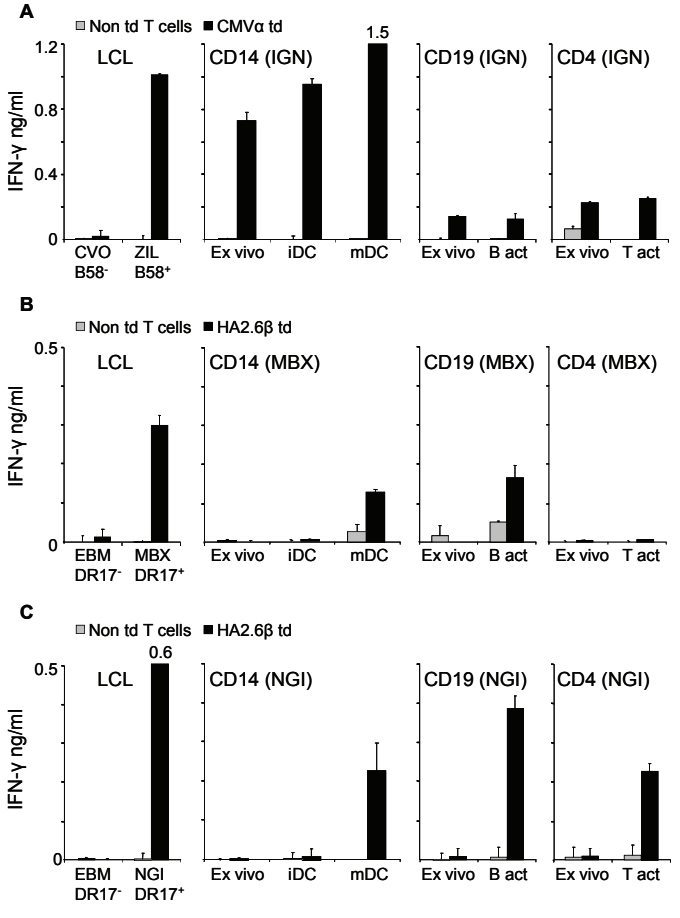

Figure 4. Neoreactivity also directed against normal human cell subsets.

HA1.M7-TCRβ td BV1+ CVO pp50 T-cells against LSR (Figure 1A, 2A) could be blocked by HLA class I and HLA-B/C blocking antibodies, indicating HLA-B or HLA-C restricted recognition, as demonstrated in Figure 3A. LSR expressed HLA-B35, B52 and Cw12. Additional experiments using various LCLs expressing one of these HLA restriction molecules demonstrated that this neoreactivity was HLA-B52 mediated (Figure 3A). In addition, the neoreactivity of CMV-TCRα td BV1+ CVO pp50 T-cells against ZIL (Figure 1A, 2B) was also HLA-B or HLA-C restricted, as the reactivity could be blocked with HLA class I and HLA-B/C antibodies (Figure 3B). ZIL expressed HLA-B56, B58, and Cw1, and additional testing against LCLs covering these different HLA restriction molecules demonstrated this neoreactivity to be HLA-B58 restricted (Figure 3B). The neoreactivity of the HA2.6-TCRβ td MBX IE-1 T-cells (Figure 1C, 2C) was demonstrated to be HLA-DR17 restricted, based on blocking with HLA class II and HLA DR mAbs and testing with an additional LCL panel (Figure 3C). The neoreactivity of the HA2.19-TCRα and HA2.6-TCRβ mixed TCR dimer could be blocked with HLA class II and HLA-DQ mAbs, and testing on an additional LCL panel demonstrated that this neoreactivity was HLA-DQ3(8/9) restricted (Figure S1B). In conclusion, mixed TCR dimers derived from HLA class I restricted T-cells can acquire neoreactivities that can be both HLA class I and HLA class II restricted.

In Figure 3C we demonstrate that the HA2.6-TCRβ chain in combination with the TCRα chain of the endogenous TCR from MBX IE-1 T-cells resulted in a HLA-DR17 restricted

neoreactivity. Because MBX was also HLA-DR17 positive, we tested these neoreactive T-cells for recognition of autologous LCLs derived from MBX. Mixed TCR dimers produced IFN-γ (Figure 4B left panel, Figure S2A) and were cytolytic (Figure S2B) against HLA-DR17+ LCLs including MBX, and reactivity against all LCLs could be blocked using HLA class II and HLA-DR mAbs (Figure S2C).

These results indicate that mixed TCR dimers may lead not only to newly acquired alloreactivity, but also to autoreactivity.

Mixed TCR dimers are neoreactive against normal human cell subsets

To study whether the observed neoreactivities directed against LCLs were predictive for reactivity against normal human cell subsets, we tested both HLA class I and class II restricted neoreactive mixed TCR dimers against different MACS isolated cell subsets. Neoreactive HLA-B58 restricted CMV-TCRα td CVO pp50 T-cells and HLA-DR17 restricted HA2.6-TCRβ td MBX IE-1 T-cells were tested against freshly isolated and in vitro activated CD4+, CD19+ and CD14+ cell subsets isolated from PBMCs of an HLA-B58+ or an HLA-B58- individual (Figure 4A, Figure S3A) or HLA-DR17+ individuals (Figure 4B and C, Figure S3B). The HLA-B58 restricted neoreactive T-cells were able to recognize all different cell subsets directly ex vivo (Figure 4A). The HLA-DR17 restricted neoreactive T-cells did not recognize the cell subsets directly ex vivo but recognized the autologous activated CD19+ and CD14+ cell subsets of MBX as well as the allogeneic activated CD19+, CD14+ and CD4+ cell subsets of NGI (Figure 4B and C). The absence of IFN-γ production against autologous activated CD4+ T-cells derived from MBX was not surprising, as no signs of self-reactivity of the HA2.6-TCRβ td MBX IE-1 T-cells were observed, and these T-cells could be easily expanded using feeder cells and PHA. Cytolytic capacity of CMV-TCRα td CVO pp50 T-cells (Figure S3A) corresponded with the IFN-γ production against these cell subsets (Figure 4A). The HA2.6-TCRβ td MBX IE-1 T-cells, however, exerted cytolytic activity against allogeneic nonactivated CD19+ and CD4+ cell subsets derived from NGI, whereas no IFN-γ production was observed after stimulation with these cell subsets, indicating that the threshold for

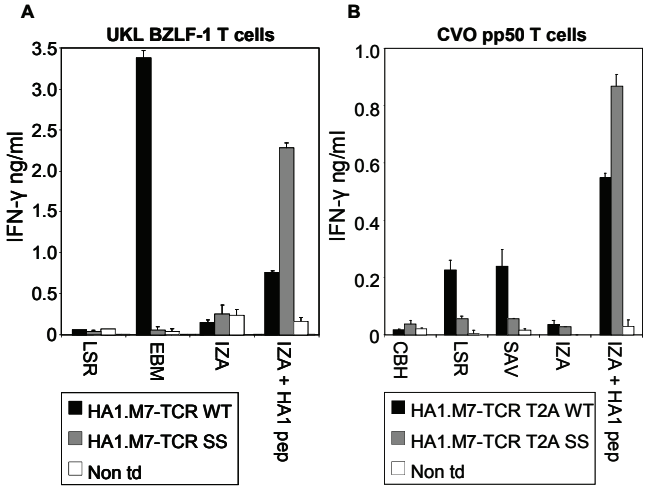

Figure 5. Neoreactivities are markedly diminished using cysteine modified TCRs.

*Figure 5: **(A)** BZLF-1 T-cells from healthy individual UKL were transduced with two separate retroviral vectors encoding either the unmodified HA1.M7-TCRα and β chains (HA1.M7-TCR WT; black bars) or cysteine modified HA1.M7-TCRα and β chains (HA1.M7-TCR SS; grey bars), sorted on bases of high eGFP and ΔNGF-R expression and tested against several LCLs for neoreactivity in duplo. **(B)** CVO pp50 T-cells were transduced with retroviral vectors containing T2A linked unmodified HA1.M7-TCRα and β chain (HA1.M7-TCR 2A WT; black bars) or T2A linked cysteine modified HA1.M7-TCRα and β chain (HA1.M7-TCR 2A SS; grey bars), sorted on high ΔNGF-R expression and tested in duplicate against several LCLs for neoreactivity. As a control, non td UKL BZLF-1 T-cells and CVO pp50 T-cells (non td; white bars) were tested against the same LCL panel. IFN-γ production depicted is representative of 3 separate experiments.*

cytolytic activity is easier reached than the threshold for cytokine production. The T-cells did not exert cytolytic activity against the nonactivated autologous cell subsets from MBX (Figure S3B) corresponding with the IFN-γ production (Figure 4B). These results demonstrate that the observed neoreactivities against the LCL panel are predictive for reactivity against normal human cell subsets.

In conclusion, T-cells expressing neoreactive mixed TCR dimers can recognize normal cell subsets, and are capable of both producing cytokines and demonstrating cytolytic activity.

Transfer of cysteine modified TCRs reduces neoreactivity

To determine whether strategies facilitating matched pairing could reduce potentially harmful neoreactivities, we modified the HA1.M7-TCR by inclusion of extra cysteine residues in the constant domains of the TCR chains. UKL BZLF-1 T-cells that exhibited HLA-DR4 restricted neoreactivity after transduction with the HA1.M7-TCR (Figure S4) were either transduced with retroviral vectors encoding the unmodified HA1.M7-TCRα and β chains (HA1.M7-TCR WT) or with cysteine modified HA1.M7-TCRα and β chains (HA1.M7-TCR SS), sorted on bases of high eGFP and ΔNGF-R expression and tested against the LCL panel for neoreactivity. Whereas the HA1.M7-TCR WT td T-cells exhibited neoreactivity against the HLA-DR4+ EBM, the HA1.M7-TCR SS td T-cells showed limited neoreactivity (Figure 5A). In contrast to reduced neoreactivity, the HA1.M7-TCR SS td T-cells exhibited increased HA-1-specificity (Figure 5A). In addition, we studied whether HLA-B52 restricted neoreactivity of HA1.M7-TCR td BV1+ CVO pp50 T-cells (Figure 1A, 2A, 3A) could be reduced by inclusion of cysteine residues in the HA1.M7-TCR. CVO pp50 T-cells were transduced with retroviral vectors encoding both the HA-1-TCRα and β chain linked with a self-cleaving 2A sequence (T2A) that were either unmodified (HA1.M7-TCR T2A WT) or cysteine modified (HA1.M7-TCR T2A SS), sorted on bases of high ΔNGF-R expression and tested against the LCL panel for neoreactivity. As can be observed in Figure 5B, CVO pp50 T-cells transduced with the HA1.M7-TCR T2A WT demonstrated neoreactivity directed against HLA-B52+ LSR and SAV. However, also this HLA-B52 restricted neoreactivity was markedly reduced by cysteine modification of the HA1.M7-TCR (Figure 5B), whereas the reactivity against HA-1+ target cells increased.

The results indicate that inclusion of an additional disulfide bond between the introduced TCR chains markedly decreased neoreactivity and, in addition, increased the effectiveness of the introduced TCRs.

DISCUSSION

In this study, we investigated whether TCR gene transfer can lead to the generation of new detrimental reactivities by creating T-cells that express mixed TCR dimers. For this purpose, we introduced 7 different TCRs into 5 virus-specific T-cell populations derived from healthy donors, and tested these transduced T-cell populations against an LCL panel covering the most prevalent HLA class I and II molecules. Per virus-specific T-cell line, at least

2 out of 7 TCR-transductants demonstrated neoreactivities. We could demonstrate that introduction of only TCRα or TCRβ chains resulted in neoreactivity, and that this neoreactivity could be HLA class I or class II mediated. Furthermore, we not only observed neoreactive mixed TCR dimers harbouring alloreactivity, but also autoreactivity. Therefore, we conclude that mixed TCR dimers formed will frequently harbour new, potentially harmful specificities.

Relatively high frequencies of neoreactive mixed TCR dimers were found. Normally, during development, T-cells undergo thymic selection resulting in a T-cell repertoire consisting of T-cells capable of binding to self-peptide-self-MHC complexes with adequate affinity. Potentially autoimmune T-cells that have high affinity for self-peptide-self-MHC complexes are deleted. Alloreactivity refers to the ability of T-cells to recognize peptide-allogeneic-MHC complexes that were not encountered during thymic development, and we have recently described that alloreactivity by virus-specific T-cells is frequently observed[20]. In the case of the mixed TCR dimers no thymic selection has occurred at all, and by chance both allo- and autoreactive mixed TCR dimers can be engineered. TCR td T-cells harbouring autoreactive mixed TCR dimers will only be able to survive when the peptide recognized is not expressed on the T-cells themselves, since this may lead to fratricide of these T-cells.

In our model we measured T-cell reactivity against an LCL panel covering a large spectrum of different HLA molecules expressing different peptides. Theoretically, by using this model it is more likely that we pick up neoreactive mixed TCR dimers recognizing either a peptide in the context of allo-HLA than in the context of self-HLA, since a maximum of 12 self-HLA alleles will be shared with the LCLs in the panel, whereas up to 77 HLA molecules will be foreign to the T-cells. However, we also identified a neoreactive mixed TCR dimer recognizing peptides bound to self-HLA, namely the HLA-DR17 reactive HA2.6-TCRβ td MBX IE-1 T-cells. These selfreactive T-cells were capable of recognizing only autologous DCs and activated B cells, and not activated autologous CD4+ T-cells, whereas both activated and resting allogeneic cell subsets (NGI) were recognized. This lack of reactivity against activated autologous CD4+ T-cells was not surprising, as these HLA-DR17 neoreactive MBX IE-1 T-cells could be easily expanded using feeder cells and PHA. Furthermore, NGI derived target cells were always better recognized (Figure 4B/C, S2, S3), indicating that the target antigen might be higher expressed in NGI derived CD4+ T-cells compared with MBX derived CD4+ T-cells. In addition, HLA-DR expression on activated T-cells is lower than on LCLs and DCs, and, in combination with lower antigen expression on MBX derived target cells, possibly the threshold for activation of the autoreactive T-cells by MBX derived CD4+ T-cells is not reached. We cannot conclude from these data whether the mixed TCR dimers recognize different antigens expressed by MBX and NGI, although IFN-γ production against both LCL MBX and LCL NGI could be blocked using HLA class II and HLA-DR blocking mAbs, or whether they recognize possibly differentially expressed antigens by these two LCLs.

In a pp50-specific T-cell clone, we observed neoreactivity in 3 out of 7 TCR transductions. Theoretically, the introduction

of 7 TCRs into a monoclonal virus-specific T-cell population will result in 14 mixed TCR dimers. Of these 14 mixed TCR dimers, 3 were demonstrated to be neoreactive, indicating that approximately 1 out of 5 mixed TCR dimers will harbour a new specificity. Furthermore, deliberately creating mixed TCR dimers by recombining 4 different HA-2-TCRs into BV1+ CVO pp50 T-cell clone resulted in 1 neoreactive mixed TCR dimer out of 20. On average, we conclude that approximately 1 out of 10 mixed TCR dimers will harbor potentially hazardous neoreactivity. The results demonstrate that selecting strong competitor TCRs could not avoid occurence of neoreactive mixed TCR dimers, as has been proposed previously as a strategy to acquire single TCR expression on transduced T-cells[21]. For example, introduction of a strong competitor CMV-TCR into weak competitor pp50-specific T-cells resulted in HLA-B58 restricted neoreactive mixed TCR dimers. Also, the introduction of a strong competitor HA2.6-TCR into strong competitor IE1-specific T-cells resulted in HLA-DR17 restricted neoreactive mixed TCR dimers. These results imply that TCR transfer will frequently result in the formation of neoreactive mixed TCR dimers.

To date however, no off-target toxicity has been observed in clinical trials treating in total 51 patients with either MART-1-TCR td or gp100-TCR td T-cells[10,12]. While no evidence of mixed TCR dimer induced autoimmunity was observed in earlier murine experiments and these first clinical trials, in a recent set of experiments an often lethal autoimmune pathology was observed under conditions that promote the expansion of adoptively transferred T-cells more strongly, and this pathology appeared dependent on the action of mixed TCR dimers[22].

There are different techniques described that facilitate matched pairing of the introduced TCR chains. Exchange of the human constant regions for murine constant regions was described to improve TCR expression and functionality[14,15]. However, murine constant regions can be potentially immunogenic in vivo. Another strategy that facilitated matched pairing and increased TCR surface expression is the introduction of an extra disulfide bond in the constant domains of the introduced TCR chains[16,17]. In this study, we demonstrate that cysteine modification of the potentially clinical useful HA1.M7-TCR considerably reduced the neoreactivity of two TCR td virus-specific T-cell populations tested. Potentially, the stochiometric production of TCRα and β chains, when linked with a self-cleaving 2A peptide[23], could also result in increased preferential pairing of the TCR chains and lower expression of mixed TCR dimers. However, CVO pp50 T-cells transduced with HA1.M7-TCR chains linked with a T2A sequence still demonstrated marked neoreactivity (Figure 5B), indicating that stochastic expression of the TCRα and β chain does not rule out the generation of mixed TCR dimers. Next to decreased neoreactivity using cysteine modified TCRs, increased HA-1-specificity was observed, making the cysteine modified HA-1.M7-TCR more attractive than the unmodified HA1.M7-TCR for future clinical trials. Whether the results obtained with the cysteine modified HA1.M7-TCR are predictive for other TCRs potentially useful for clinical therapy has yet to be tested.

To completely rule out formation of harmful mixed TCR-dimers, another option would be to transduce γδ-T-cells, as the γδ-TCR chains are not able to pair with αβ-TCR chains[19]. Human γδ-T-cells redirected with αβ-TCRs were fully functional in vitro[19] and in vivo[24]. However, further analyses will be required to determine to what extent redirected γδ-T-cells and αβ-T-cells are different with respect to homing properties and specificity of the endogenous TCR. We therefore propose to limit the diversity of the TCR repertoire of the recipient T-cells by transducing virus-specific T-cell populations. Because virus-specific T-cell populations consist of a restricted TCR repertoire[25,26], the amount of different mixed TCR dimers formed will be limited. In addition, the reactivity of these T-cells is known, allowing detection of harmful neoreactivities by introducing into these virus-specific T-cells as controls only the TCRα or TCRβ chain of interest and subsequent testing against different patient-derived cell types. By this procedure TCR td virus-specific T-cells can be selected that show no off-target toxicity.

In conclusion, in this study we demonstrated that TCR transfer results in neoreactive mixed TCR dimer formation. This formation of neoreactive mixed TCR dimers is not a feature of a specific TCR, because we observed this in all virus-specific T-cells tested, with different introduced TCRα or β chains. We therefore underline the importance of facilitating matched pairing of introduced TCR chains, and diminishing the chance of formation of harmful neoreactive mixed TCR dimers by using T-cell populations with restricted TCR repertoire as host cells for TCR transfer.

MATERIALS AND METHODS

Retroviral vector construction and production of retroviral supernatant

TCRAV and TCRBV gene usage of the different Ag-specific T-cell clones was determined as previously described[7]. All TCR AV and BV chains derived from different high affinity mHAg- (HA-2 and HA-1) and virus-specific T-cell clones (CMV) were cloned separately into the Moloney murine leukemia virus-based LZRS retroviral vector and are described in detail in the supporting information. In addition, cysteine modified HA-1.M7-TCR chains were constructed as previously described by introducing cysteine residues at positions 48 of the TCRα and position 57 of the TCRβ constant domains[16,17]. TCR-AV chains were always combined via the IRES sequence with the marker eGFP, and the TCR-BV chains with the truncated nerve growth factor receptor (ΔNGF-R). The retroviral vectors used in Figure 5B contained either the unmodified or cysteine modified HA1.M7 TCRα and β chains linked with picornavirus-derived self-cleaving 2A sequence (T2A)[23] and were combined via the IRES sequence with the marker ΔNGF-R. Retroviral supernatant was generated using φ-NX-A as previously described[27].

HLA Class I tetrameric complexes, flow cytometric analyses and cell sorting

PE-or APC-conjugated tetrameric complexes were constructed as previously described[28] with minor modifications. The following tetrameric complexes were constructed: tetrameric HLA-A1

complexes in combination with CMV-pp50 VTE (pp50) or CMV-pp65 YSE (pp65) peptide, and tetrameric HLA-B8 complexes in combination with CMV-IE-1 ELR (IE-1) or EBV-BZLF-1 RAK (BZLF-1). For flow cytometric analyses as well as flow cytometry-based sorting, cells were labeled with tetramers for 1 hour at 4°C and during the last 30 mins, mAbs directed against the various cell surface molecules were added. Sorting was performed at 4°C. mAbs used are described in the supporting information.

Cells

All studies were conducted with approval of the institutional review board at Leiden University Medical Center. After informed consent, virus-specific T-cells were isolated from different healthy individuals (UKL, MBX, CVO, UGW) using different virus-specific tetramers (>95% purity). Tetramer positive T-cells were restimulated every two weeks as described previously[13] and expanded. Retroviral transduction was performed as described previously[8] using recombinant human fibronectin fragments CH-296[27]. TCR transduced (td) virus-specific T-cells were sorted based on eGFP and ΔNGF-R positivity (>99% purity), and the cells were expanded in bulk. To analyze the reactivity of TCR td T-cells, a panel of HLA typed EBV-transformed lymphoblastoid cell lines (LCLs) was used (Table S2). LCLs were maintained in Iscoves modified dulbecco's medium (IMDM) supplemented with 10% FBS.

Analysis of Ag-specific IFN-γ production

TCR td virus-specific T-cells were tested for IFN-γ production against the HLA typed LCL panel. To determine IFN-γ production, 5.000 T-cells were cocultured with 20.000 LCLs, and after overnight incubation supernatant was harvested and tested in a standard ELISA (CLB, Amsterdam, The Netherlands). As positive control for the activity of the endogenous and introduced TCRs, the T-cells were stimulated with LCLs pulsed for one hour at 37°C with the different viral and mHag peptides at a final concentration of 1 μg/ml. To determine the HLA restriction molecules essential for recognition of the mixed TCR dimers, blocking studies were performed and antibodies used are described in the supporting information.

TCR td virus-specific T-cells were tested for IFN-γ production against normal human cell subsets and for this purpose CD4+, CD19+ and CD14+ cell subsets were MACS-isolated from peripheral blood mononuclear cells (PBMC) as described in the supporting information. TCR td virus-specific T-cells were tested against these different purified (>90%) CD4+, CD19+ and CD14+ cell subsets directly ex vivo, and after in vitro activation of these cell subsets, as described in the supporting information.

Chromium release assay and CFSE based cytotoxicity assay

To test the capacity of T-cells to specifically lyse Ag positive target cells, a standard 4 h chromium release assay using different effector-to-target ratios was performed as previously described[8]. Furthermore, to be able to analyse cytotoxicity after several days, we used a CFSE based cytotoxicity assay[29] as described in the supporting information.

ACKNOWLEDGEMENTS

We thank Michel Kester, Dirk van der Steen, Guido de Roo and Menno van der Hoorn for expert technical assistance.

Supporting information

MATERIALS AND METHODS

Construction of retroviral vectors and production of retroviral supernatant

The TCR AV and BV chains used in this study are: AV15S1; BV18S1 (HA2.5-TCR), AV23S1; BV18S1 (HA2.6-TCR), AV30S1; BV18S1 (HA2.19-TCR), and AV23S1; BV6S2A1 (HA2.20-TCR) derived from 4 different T cell clones recognizing the HA2 YIGVEVLVSV peptide in the context of HLA-A2[1], AV8S1; BV6S4 (HA1.M2-TCR), AV32S1; BV6S4 (HA1.M7-TCR) derived from 2 different T cell clones recognizing the HA1 VLHDDLLEA peptide in the context of HLA-A2 and AV18S1; BV13S1 (CMV-TCR)[2] derived from a T cell clone specific for the CMV-pp65 derived NLVPMVATV peptide presented in the context of HLA-A2.

mAbs used in this study

To obtain more oligoclonal or monoclonal cell subsets, cells were labeled with tetramers and with either anti-TCR-BV1 or anti-TCR-BV14 PE (Immunotech, Marseille, France). To obtain the transduced cells, cells were labeled with anti ΔNGF-R either PE- (PharMingen, San Diego, CA, USA) or APC-conjugated (Cedarlane Laboratories, Hornby, Ontario, Canada) for 30 minutes at 4°C and were subsequently sorted.

To determine the HLA restriction molecules essential for recognition of the mixed TCR dimers, blocking studies were performed using W6.32 (anti-HLA class I), B1.23.2 (anti-HLA-B/C), PdV5.2 (anti-HLA class II), B8.11.2 (anti-HLA-DR), SPV-L3 (anti-HLA-DQ) or B7.21 (anti-HLA-DP) mAbs (kindly provided by A. Mulder from the LUMC). LCLs were preincubated with saturating concentrations of mAbs for 1 hour at RT before addition of T cells.

mAbs used in the CFSE based cytotoxicity assay are anti-CD19 and anti-HLA-DR or anti-CD4 (Beckman Coulter, Fullerton, CA, USA) and anti-HLA-DR mAbs (PharMingen, San Diego, CA, USA).

MACS-enrichement and activation of isolated cell subsets

PBMC of healthy donors were thawed, incubated with DNAse for 15 minutes at 37 °C, washed and stained with either anti-CD4, anti-CD19 or anti-CD14 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and isolated according to manufacturer's instructions. The $CD4^+$ cell subset was activated for a week (T act) using PHA (800 ng/ml), the $CD19^+$ fraction was activated

ID	specificity	restriction element	excluded EBV-LCLs due to alloreactivity	HA2.5 TCR	HA2.6 TCR	HA2.19 TCR	HA2.20 TCR	HA1.M2 TCR	HA1.M7 TCR	CMV TCR
UKL	pp50	A1	FAQ, FRQ, GGT, JLX, RSH, LSR	-‡	+#	+	+	+	-	-
UKL	BZLF1	B8	MHV	+	+	+	+	+	+	+
UGW	pp65	A1	AAS, ALY, MWX	-	+	-	-	-	+	-
MBX	IE1	B8	AHT, GGT, JLX, LAJ, LSR	-	+	+	-	+	+	-
CVO	pp50	A1	-	-	-	-	-	+	+	+

‡ - indicates that no neoreactivity of the TCR td virus-specific T cell lines against any of the LCLs present in the panel was observed.
# + indicates that neoreactivity of the TCR td virus-specific T cell lines against one of the LCLs present in the panel was observed.

Table S1. Used virus-specific T-cell lines and allo- and neoreactivity exerted against LCL panel.

for 3 days (B act) by culturing them on CD40L transduced murine fibroblasts[3] in medium containing CpG (10 µg/ml) and IL-4 (500 IU/ml) (Schering-Plough, Innishammon, Cork, Ireland). The $CD14^+$ fraction was activated into immature DCs (iDC) by culturing in medium containing GM-CSF (100 ng/ml) (Novartis, Basel, Switzerland) and IL-4 (500 IU/ml). After 3 days of culturing, immature DCs were activated for 3 days into mature DCs[4] (mDC) by culturing them in medium containing GM-CSF (100 ng/ml), TNF-α (10 ng/ml), IL-1ß (10 ng/ml), IL-6 (10 ng/ml) (Cellgenix, Freiburg, Germany), PGE-2 (1 µg/ml) (Sigma-Aldrich, St Louis, MO, USA) and IFN-γ (500 IU/ml) (Immukine, Boehringer Ingelheim, Alkmaar, The Netherlands).

Figure S1: ***(A)*** *To gain more insight into the frequency of neoreactive mixed TCR dimers and to analyze whether mixed TCR dimers consisting of conserved TCRs with the same specificity could acquire new harmful reactivity, we deliberately created mixed TCR dimers by recombining HA-2-specific TCRα and TCRβ chains of 4 different HA-2-TCRs, namely the HA2.5-TCR, the HA2.6-TCR, the HA2.19-TCR and the HA2.20-TCR. For this purpose, we sorted pp50-specific T cells derived from healthy individual CVO using tetramers and BV1-staining, confirmed monoclonality, and transduced all possible combinations into these T cells. Taking into account that also the introduced HA-2-TCR chains can pair with the endogenous TCR of the pp50 T cells, this resulted in potentially 20 mixed TCR dimers. BV1+ CVO pp50 T cells consisting of these deliberately created mixed TCR dimers were also tested against the LCL panel for neoreactivity; here, reactivity of CVO pp50 T cells transduced with the HA2.19-TCRα and HA2.6-TCRβ chains is depicted (A; mixed TCR-dimer). As a control, the parental HA2.19-TCR (TCR HA2.19) and the parental HA2.6-TCR (TCR HA2.6) combinations were included in the experiment. In addition, as a control for the reactivity of the introduced TCR, IZA was pulsed with HA-2 peptide (A; intro-TCR). The experiments were performed in duplicate. IFN-γ production depicted is a representative experiment out of 2 experiments.* ***(B)*** *To elucidate HLA restriction of the neoreactive mixed TCR dimer, blocking experiments were performed. Neoreactivity directed against DMD could be blocked by HLA class II and HLA-DQ blocking antibodies. DMD expressed HLA-DQ5, and DQ9, as indicated in bold. Additional experiments using various LCLs expressing one of these HLA restriction molecules demonstrated that this neoreactivity was DQ3(8/9) mediated. Blocking experiments were performed in triplicate. IFN-γ production depicted is a representative experiment out of 3 separate experiments.*

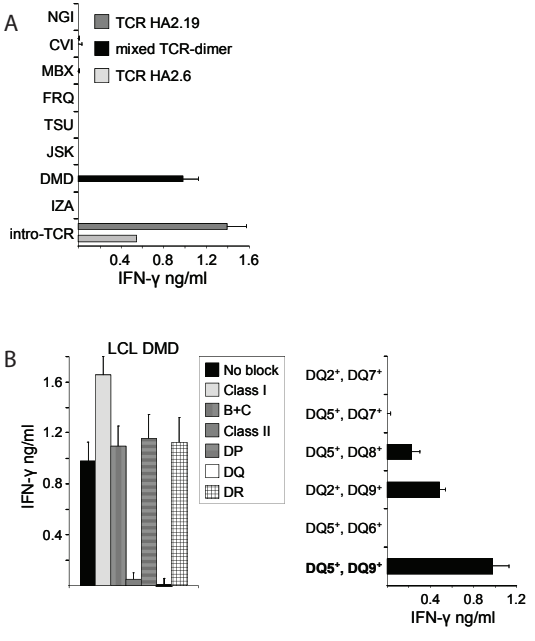

Figure S1. Each recombination of TCR chains after TCR transfer can potentially result in a HLA-restricted harmful new reactivity.

Figure S2: ***(A)*** *HA2.6-TCRα, HA2.6-TCRβ and HA2.6-TCRαβ td MBX IE-1 T cells were tested for IFN-γ production against HLA-DR17+ LCLs IZA, NGI and MBX and against HLA-DR17- EBM.* ***(B)*** *In a 4h cytotoxicity assay, HA2.6-TCRα (white symbols), HA2.6-TCRβ (black symbols) and HA2.6-TCRαβ td MBX IE-1 T cells (grey symbols) were tested against HLA-DR17+ LCLs IZA (diamonds), MBX (triangles) and NGI (squares) in several effector-to-target ratios in triplo. As a negative control, HA2.6-TCRα, HA2.6-TCRβ and HA2.6-TCRαβ td T cells were tested against HLA-DR17- EBM (white, black and grey circles, respectively). Cytotoxicity depicted representative for 2 separate experiments.* ***(C)*** *To confirm that neoreactivity against MBX and NGI cells was also HLA-DR restricted, blocking experiments were performed. Neoreactivity directed against MBX and NGI cells could be blocked using class II and HLA-DR blocking antibodies, indicating that neoreactivity directed against these LCLs was also HLA-DR17 restricted. The experiments were performed in triplo and IFN-γ depicted is representative for 3 separate experiments.*

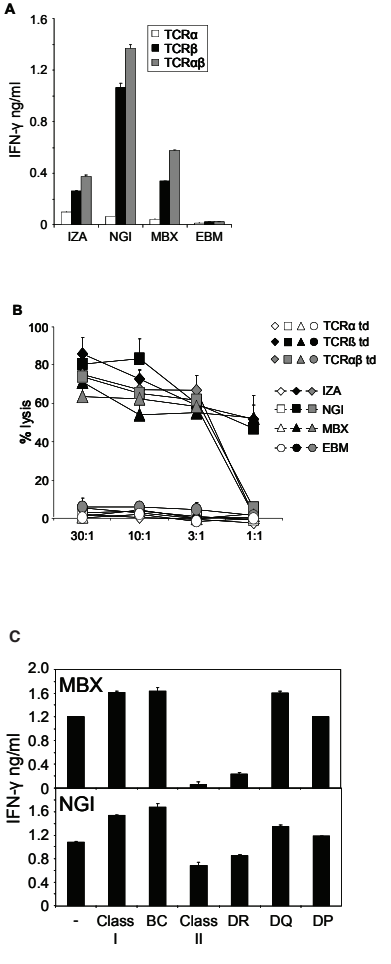

Figure S2. Cytokine production and cytotoxic activity of HLA-DR restricted autoreactive mixed TCR dimer.

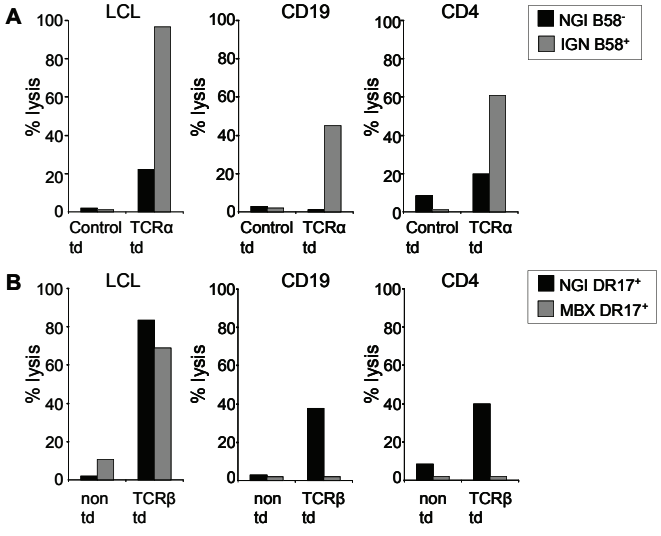

Figure S3. Mixed TCR dimers are able to exhibit cytolytic activity against normal human cell subsets.

Figure S3: To analyze whether TCR td T cells were able to lyse the target cell subsets directly ex vivo, ***(A)*** *HLA-B58 restricted neoreactive CMV-TCRα td CVO pp50 T cells and*

(B) *HLA-DR17 restricted neoreactive HA2.6-TCRβ td MBX IE-1 T cells were tested using a CFSE cytotoxicity assay. T cells were labeled with 5 µM of CFSE (Molecular Probes Europe, Leiden, the Netherlands), and coincubated with either HLA-B58- (NGI) or HLA-B58+ (IGN) or HLA-DR17+ (NGI, MBX) PBMCs or LCLs. Control cultures with T cells only or target cells only were included. Cultures were stained after 24h of coincubation with a combination of either anti-CD19 and anti-HLA-DR or anti-CD4 and anti-HLA-DR mAbs, and the different samples were analyzed using flow cytometry. Propidium iodide (PI) (1 µg/ml) was added to exclude dead cells. Percentage of lysis per cell subset (CD19+ or CD4+) was calculated as follows: [(cell counts of PIneg cell subset with effector cells) / (cell counts of PIneg cell subset without effector cells)] * 100%. Percentage of lysis of LCLs, CD19+ and CD4+ cells is depicted. The experiment was performed in duplicate, and a representative experiment out of 2 is depicted.*

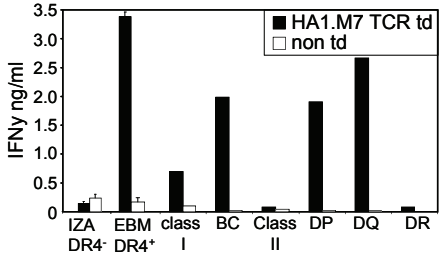

Figure S4. Neoreactivity of HA1.M7-TCR td UKL BZLF1 T cells directed against LCL EBM is HLA-DR restricted.

Figure S4: HA1.M7-TCR td but not non td UKL BZLF1 T cells demonstrated reactivity directed against EBM. This reactivity could be blocked using class II and HLA-DR blocking antibodies. Because EBM is homozygous HLA-DR4 positive, this indicated that this neoreactivity was HLA-DR4 restricted. HLA-DR4 negative IZA was not recognized. The experiment was performed in duplicate, and representative IFN-γ production for 2 independent experiments is depicted.