MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors

Matthias Leisegang,[1] Susanne Wilde,[2] Stefani Spranger,[2] Slavoljub Milosevic,[2] Bernhard Frankenberger,[2] Wolfgang Uckert,[1,3] and Dolores J. Schendel[2,4]

[1]Max-Delbrück-Center for Molecular Medicine, Berlin, Germany. [2]Institute of Molecular Immunology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany. [3]Humboldt University Berlin, Institute of Biology, Berlin, Germany. [4]Clinical Cooperation Group "Immune Monitoring," Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany.

The apoptosis inhibitor protein survivin is overexpressed in many tumors, making it a candidate target molecule for various forms of immunotherapy. To explore survivin as a target antigen for adoptive T cell therapy using lymphocytes expressing survivin-specific transgenic T cell receptors (Tg-TCRs), we isolated HLA-A2–allorestricted survivin-specific T cells with high functional avidity. Lymphocytes expressing Tg-TCRs were derived from these T cells and specifically recognized HLA-A2$^+$ survivin$^+$ tumor cells. Surprisingly, HLA-A2$^+$ but not HLA-A2$^-$ lymphocytes expressing Tg-TCRs underwent extensive apoptosis over time. This demise was caused by HLA-A2–restricted fratricide that occurred due to survivin expression in lymphocytes, which created ligands for Tg-TCR recognition. Therefore, survivin-specific TCR gene therapy would be limited to application in HLA-A2–mismatched stem cell transplantation. We also noted that lymphocytes that expressed survivin-specific Tg-TCRs killed T cell clones of various specificities derived from HLA-A2$^+$ but not HLA-A2$^-$ donors. These results raise a general question regarding the development of cancer vaccines that target proteins that are also expressed in activated lymphocytes, since induction of high-avidity T cells that expand in lymph nodes following vaccination or later accumulate at tumor sites might limit themselves by self-MHC–restricted fratricide while at the same time inadvertently eliminating neighboring T cells of other specificities.

Introduction

Several recent developments have converged to improve adoptive T cell therapy of cancer. First, expression of TCRs as transgenic proteins in peripheral blood lymphocytes (PBLs) enables T cells with defined specificities to be generated in high numbers for patient-individualized therapy (TCR gene therapy), bypassing the laborious process of isolating and expanding specific T cells for individual patients (1). Second, high-affinity TCRs specific for peptides presented by different major histocompatibility complex molecules (hereafter, pMHC ligands) can be selected and employed as generic "off-the-shelf" reagents, enabling future application of small repertoires of therapeutic Tg-TCRs to achieve greater clinical efficacy (2, 3). Third, selection of recipient lymphocytes and manipulation of the microenvironment can enhance T cell survival, expansion, and long-term function after adoptive transfer in vivo (4). In addition, tumor-associated antigens (TAAs) have been elucidated that may serve as suitable target structures on tumor cells, guiding the selection of TCR specificities.

A pilot project of the National Cancer Institute (NCI) prioritized a group of TAAs for T cell therapy and vaccine development (5). These TAAs represent mutant, overexpressed, or abnormally expressed proteins in cancer cells, as well as viral proteins present in virus-associated malignancies. The role of candidate proteins in oncogenicity was an important ranking factor, based on the consideration that T cell–mediated immune selection would be limited if tumor survival was dependent on TAA expression. For example, survivin, a well-characterized inhibitor of apoptosis (6, 7), is an attractive candidate for immunotherapy, since it is not expressed in most adult tissues but is overexpressed by many tumors (8). Furthermore, survivin-specific T cells were reported by several investigators (9–18). Survivin received a top score for oncogenicity, and it was ranked 21 among the 75 prioritized TAA (5).

High-affinity TCRs that efficiently recognize tumor cells are needed for effective TCR gene therapy (19, 20). However, T cells recognizing peptides of self-proteins presented by self-MHC molecules (hereafter designated self-restricted T cells) will often be missing or display only low functional avidity due to deletional tolerance (9, 21, 22). In contrast, HLA-allorestricted T cells can be obtained with high functional avidity for self-peptides (23). Advancing upon the original concept of Stauss and coworkers (24), we recently described a versatile strategy to derive allorestricted peptide-specific T cells as sources of high-affinity TCR, using DCs that were loaded with in vitro transcribed RNA (ivt-RNA) as a source of antigen to prime naive T cells (25). For example, DCs prepared from HLA-A2$^-$ donors can be loaded with ivt-RNA encoding allogeneic HLA-A2 molecules and a self-protein, such as survivin, for use as APCs. Because tolerance is MHC restricted, the corresponding autologous T cells of HLA-A2$^-$ individuals were not subjected to negative selection by HLA-A2 and can give rise not only to HLA-A2–alloreactive T cells but also to peptide-specific T cells that recognize survivin-derived peptides

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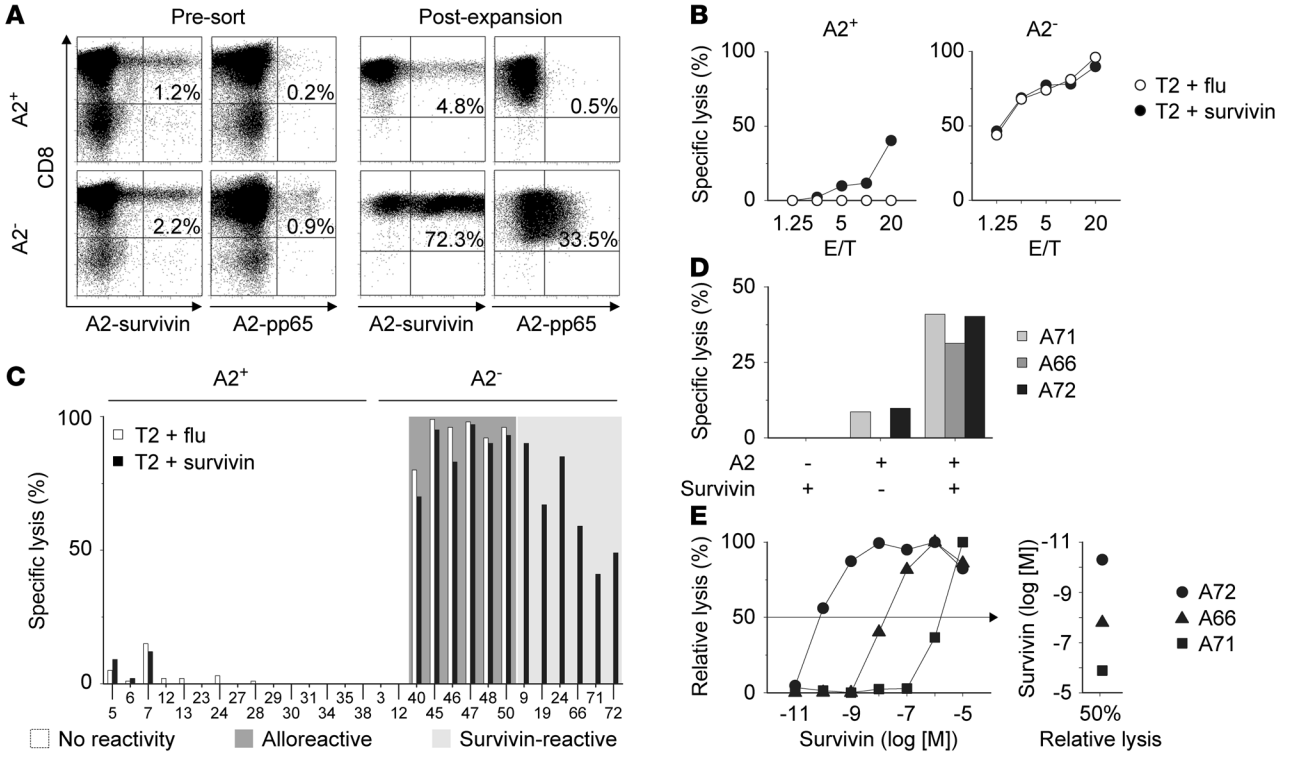

Figure 1

De novo priming of survivin-specific T cells with RNA-pulsed DCs. (**A**) Survivin-multimer staining of bulk CD8+ T cell lines of HLA-A2+ and HLA-A2– donors after DC priming (Pre-sort) and after 26 days of expansion following multimer sorting (Post-expansion). CMV(pp65)-multimer served as a specificity control. Percentage double-positive cells are displayed in the upper-right quadrant. (**B**) Cytotoxic activity of multimer-sorted lines measured against T2 (HLA-A2+) cells pulsed with flu or survivin peptide (10^{-5} M) is presented as percent specific lysis. (**C**) Cytotoxic specificity of different T cell clones was measured against flu- and survivin-pulsed T2 cells (10^{-5} M) and presented as percent specific lysis. The x axis shows clone designation. (**D**) Cytotoxic activity of allorestricted survivin-specific T cell clones A71, A66, and A72 against Mel-1379 (HLA-A2– [A2–], survivin+ [S+]), T2 cells loaded with 10^{-5} M flu peptide (A2+, S–), and Mel-624.38 tumor cells (A2+, S+) at an E/T ratio of 5:1. Flu-pulsed T2 cells were used as survivin-negative control, since we identified no tumor cell lines that were survivin negative. (**E**) Functional avidity of CTLs (E/T, 10:1) was measured against T2 cells pulsed with graded amounts of survivin peptide. Relative values of half-maximal killing are depicted at the right. Flu-pulsed T2 cells (10^{-5} M) were not recognized (data not shown). Cytotoxicity data represent means of duplicates measured at each E/T ratio or peptide concentration.

presented by HLA-A2. We applied this strategy to isolate high-affinity survivin-specific TCRs for use in TCR gene therapy.

Results

High-avidity allorestricted T cell clones specific for survivin are derived by DC priming. We introduced survivin ivt-RNA alone, or in combination with HLA-A2 ivt-RNA, into mature DCs prepared from HLA-A2+ or HLA-A2– donors, respectively. These DCs were cocultured with autologous responding CD8+ lymphocytes to induce either HLA-A2 self-restricted or allorestricted survivin-specific T cells. After two rounds of stimulation, primed cells were stained with HLA-A2–survivin$_{96\text{-}104[97L]}$ multimer (survivin-multimer) and CD8-specific antibody (Figure 1A). Double-positive cells (1.2%–2.2%) were detected in both self-restricted and allorestricted samples prior to sorting. Very few positive cells were present in self-restricted cultures that bound control HLA-A2–multimer, utilizing a peptide of cytomegalovirus pp65 protein (CMV-multimer, 0.2%). However, substantial numbers of cells from allorestricted cultures bound CMV-multimer (0.9%), most likely representing T cells that recognized HLA-A2 as an alloantigen, irrespective of survivin peptide. The survivin-multimer+ T cells were isolated and cloned immediately by limiting dilution, and the remaining sorted cells were cultured as bulk T cell lines. After 26 days, the T cell lines were reanalyzed for multimer binding. Whereas fewer than 5% of self-restricted CD8+ T cells bound survivin-multimer, more than 70% of allorestricted cells were survivin-multimer positive. Again, substantial numbers of cells in this T cell line bound CMV-multimer.

Both T cell lines were assessed for the capacity to kill HLA-A2+ target cells that were pulsed exogenously with either survivin$_{96\text{-}104[97L]}$ (survivin) peptide or control influenza matrix protein$_{58\text{-}66}$ (flu) peptide. The self-restricted T cell line mediated a low rate of killing of survivin-pulsed T2 cells, in accordance with the low numbers of survivin-multimer+ cells; it did not kill flu-pulsed target cells (Figure 1B). In contrast, the allorestricted T cell line killed both target cells. Because HLA-A2–alloreactive T cells present in the culture recognize target cells irrespective of specific peptide (25), they mask the detection of survivin-specific T cells. Therefore, HLA-A2–allorestricted survivin-specific T cells must be identified at the clonal level.

Clones derived from limiting dilution cultures were screened for cytotoxicity against the same two peptide-pulsed target cells.

Table 1
Classification of survivin-specific T cell clones after DC priming

	HLA-A2$^+$ (self-restricted)	HLA-A2$^-$ (allorestricted)
No reactivity	46 (100%)	9 (12%)
Alloreactive	0 (0%)	44 (60%)
Survivin-reactive	0 (0%)	21 (28%)
Total number	46 (100%)	74 (100%)

Clones (n = 120) were classified for specificity using 10% specific lysis of peptide-pulsed T2 cells as the positive cutoff: no reactivity designates clones recognizing neither target cell; alloreactive clones recognized survivin- and flu-pulsed T2 cells; and survivin-reactive clones recognized only survivin-pulsed T2 cells. The number and respective percentage of the total clone number are given.

Representative results of one screening assay demonstrate that survivin-specific T cell clones were not isolated from the self-restricted cultures, whereas different clones derived from the allogeneic cultures killed either both targets or only recognized survivin-pulsed cells (Figure 1C). A total of 120 T cell clones were analyzed, and no self-restricted clone with survivin specificity was isolated (Table 1). In contrast, the allogeneic cultures yielded 60% of clones that recognized both targets and 28% that recognized only survivin-pulsed target cells. The first group represented HLA-A2–alloreactive cells and was discarded.

Three clones showing potential survivin specificity were analyzed for cytotoxic activity. Target cells lacking either survivin or HLA-A2 were poorly recognized, whereas cells coexpressing HLA-A2 and survivin were efficiently killed (Figure 1D). The peptide sensitivity of killing was assessed using T2 cells pulsed with varying concentrations of survivin peptide, revealing half-maximal values ranging from 1.3×10^{-6} to 5×10^{-11} M (Figure 1E).

Survivin-specific reactivity is transferred to recipient lymphocytes by TCR gene expression. The TCR sequences of clones A66, A71, and A72 were isolated, codon optimized, and modified to express mouse TCR constant regions to improve surface expression, as described previously (26, 27). Retroviral vectors encoding both TCR chains were used to transduce activated PBLs of HLA-A2$^-$ donors. The 3 survivin-specific Tg-TCRs were expressed on comparable percentages of PBLs, as shown by binding of murine TCRβ constant region antibody (Figure 2A). The TCR-transduced PBLs killed survivin-pulsed T2 cells with different peptide sensitivities. Based on half-maximal values for cytotoxicity, a hierarchy of functional avidity was revealed that corresponded to the original T cell clones (TCR-A71 < TCR-A66 < TCR-A72) (Figure 2B).

TCR-transduced PBLs were also tested for their capacity to kill tumor cells that expressed survivin, with or without HLA-A2. Surface HLA-A2 was detected on tumor cells with specific antibody (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI43437DS1), and survivin mRNA levels were assessed by RT-PCR (Figure 2C). PBLs expressing the 3 Tg-TCRs killed UT-SCC-15, U-373, and FM-86 tumor cells, which naturally coexpressed HLA-A2 and survivin. Recognition required expression of survivin-specific Tg-TCRs, since GFP-transduced and untransduced PBLs did not mediate appreciable killing (Figure 2D). Similar results were found for 4 additional tumor lines (data not shown). KT-195 tumor cells displayed high levels of survivin mRNA, but they were HLA-A2 negative and were not recognized by TCR-transduced effector cells. Following transfection with HLA-A*0201–encoding cDNA, KT-195-A2 cells gained HLA-A2 surface expression and acquired sensitivity to effector cells modified with each of the 3 TCR-modified effector cells. In contrast, cells transfected with control vector (KT-195-VC) remained resistant to killing (Figure 2E). PBLs expressing TCR-A71, which had the lowest peptide sensitivity, recognized the FM-86 and KT-195-A2 target cells at somewhat lower levels. These two tumor cell lines expressed the lowest levels of HLA-A2 (Supplemental Table 1), indicating that T cell functional avidity impacted sensitivity of tumor cell recognition when pMHC ligand density was limited.

IFN-γ was also released by TCR-modified PBLs but not by untransduced or GFP-transduced PBLs following stimulation with tumor cells (Figure 2F). This cytokine release was pMHC specific, since it was only induced by tumor cells coexpressing survivin and HLA-A2 (data not shown).

The analysis of the KT-195 triplet of tumor cells demonstrated that Tg-TCR recognition was dependent upon HLA-A2. To demonstrate that HLA-A2 was not directly recognized in the absence of survivin peptide, we prepared artificial APCs (aAPCs), consisting of particle-bound anti-CD28 antibodies and recombinant HLA-A2-Ig molecules that were loaded exogenously with flu or survivin peptides. These aAPCs were analyzed for their capacity to induce IFN-γ secretion by PBLs expressing TCR-A72, which had the best functional avidity. Survivin-dependent recognition of this Tg-TCR was apparent, since only survivin-pulsed aAPCs led to detectable cytokine secretion. The recognition of survivin-pulsed aAPCs was also dependent upon Tg-TCR expression in the effector cells, since untransduced PBLs showed no response to the survivin-pulsed aAPCs (Figure 2G).

Survivin-specific TCR-modified cells mediate MHC-restricted fratricide. In a clinical setting, therapeutic Tg-TCRs would normally be expressed in lymphocytes of HLA-A2$^+$ patients bearing HLA-A2$^+$ survivin$^+$ tumors. Even though the survivin-specific Tg-TCRs were well expressed short-term on activated cells of both HLA-A2$^+$ and HLA-A2$^-$ donors, TCR-transgenic lymphocytes of HLA-A2$^+$ donors yielded lower recoveries after several days of culture (data not shown). Therefore, we made a closer inspection of recipient lymphocytes over a period of 2 weeks following transduction with the 3 Tg-TCRs. The percentages of PBLs that expressed Tg-TCRs ranged from 28% to 52%, and the expression profiles of each Tg-TCR in HLA-A2$^-$ and HLA-A2$^+$ recipient lymphocytes were comparable (Figure 3A). Appearance of apoptotic cells in the total population was monitored by staining with 7-aminoactomycin D (7-AAD), which intercalates into double-stranded nucleic acids of apoptotic and dead cells. While no differences in 7-AAD$^+$ cells were noted on day 1 after TCR transduction, dramatic differences in percentages of 7-AAD$^+$ cells were seen after 13 days when the HLA-A2$^-$ and HLA-A2$^+$ populations were compared (Figure 3B). Apoptosis of HLA-A2$^-$ lymphocytes ranged from 21% to 24% in TCR-modified PBLs, near the value of GFP-transduced and untransduced PBLs. In strong contrast, 72%–87% 7-AAD$^+$ cells were detected in the HLA-A2$^+$ populations containing TCR-transduced T cells. This high rate of apoptosis was dependent upon the presence of Tg-TCR–expressing T cells in the total lymphocyte population, since GFP-transduced and untransduced PBLs remained near 20%. For comparison, PBLs were transduced with a high-affinity TCR (T58) derived from an allorestricted T cell clone recognizing an epitope of tyrosinase protein presented by HLA-A2 (25) (Figure 3C). In this case, HLA-A2$^+$ recipient lymphocytes did not show any dramatic increase in apoptotic cells compared with untransduced

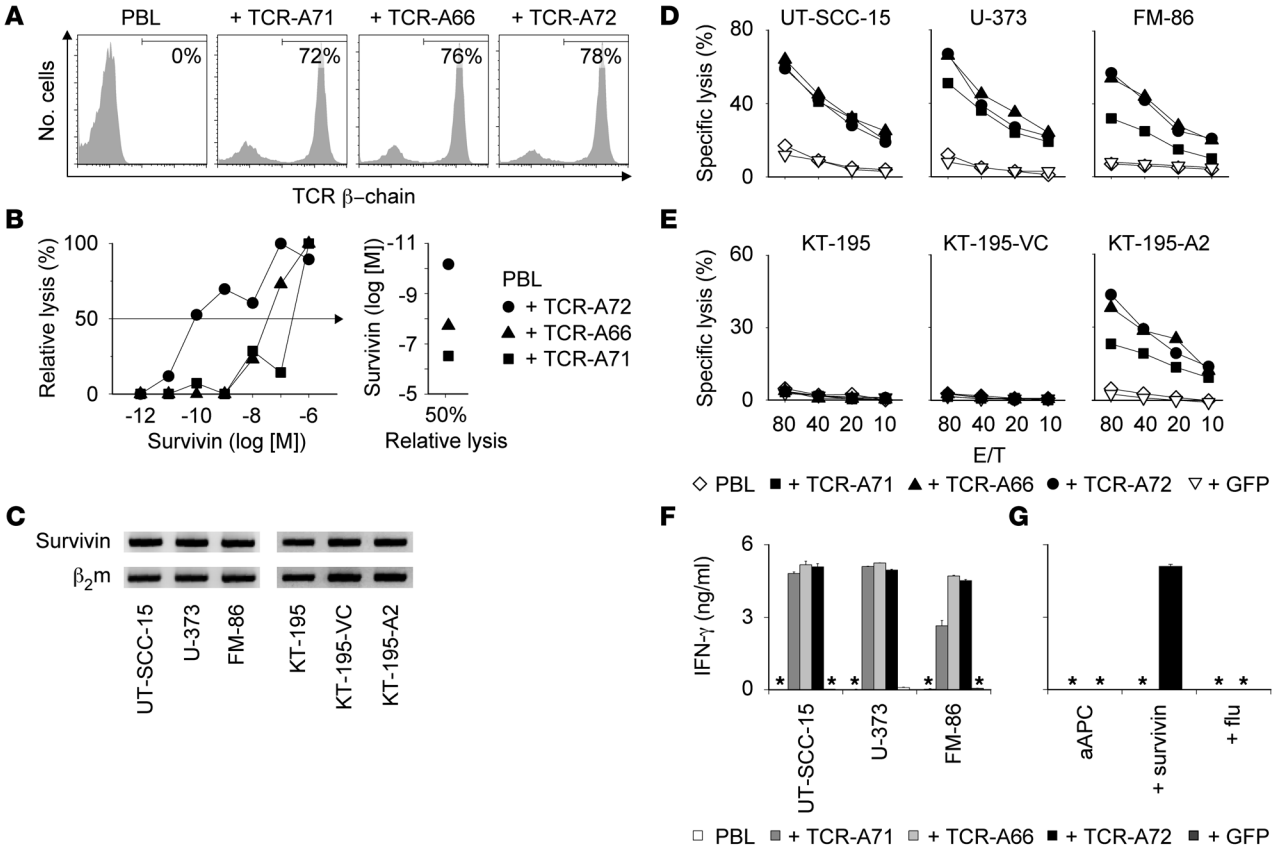

Figure 2

Redirection of antigen specificity by retroviral transfer of survivin-specific TCR genes. PBLs of a healthy HLA-A2$^-$ donors were transduced with TCR-A71, -A66, and -A72. (**A**) Unsorted TCR-transduced PBLs were analyzed on day 10 for Tg-TCR expression using murine TCRβ constant region–specific antibody. (**B**) The relative cytotoxicity is given for nonsorted TCR-transduced PBLs following stimulation with T2 cells loaded with graded amounts of survivin peptide at an E/T ratio of 20:1. Relative values of half-maximal killing are depicted at the right. (**C**) RNA expression of survivin in tumor cells was assessed by RT-PCR using survivin-specific primers and β_2-microglobulin–specific primers (β_2m) as a control. Survivin-specific cytotoxicity of TCR-modified PBLs was assessed in a standard 4-hour chromium release assay using different tumor cell lines at varying E/T ratios: (**D**) UT-SCC-15, U-373, and FM-86 (all: A2$^+$, S$^+$) and (**E**) KT-195, KT-195-VC (A2$^-$, S$^+$), KT-195-A2 (A2$^+$, S$^+$) as target cells. Untransduced PBLs and PBLs transduced with a GFP control vector served as controls. Cytotoxicity data represent means of duplicates measured at each E/T ratio or peptide concentration. (**F**) IFN-γ release at 24 hours is depicted following coculture with tumor cell lines at an E/T ratio of 2:1. (**G**) Unloaded or survivin or flu peptide–loaded aAPCs were cocultured with either untransduced PBLs or PBLs expressing TCR-A72 at an E/T ratio of 1:2. IFN-γ values for **F** and **G** are shown as mean of duplicates ± mean deviation. These experiments were done with 6 different donors, except for **B** and **G** (n = 2). Asterisk indicates values below the detection limit.

PBLs or TCR-modified PBLs from an HLA-A2$^-$ donor (Figure 3D). The accumulation of apoptotic cells was compared over time for HLA-A2$^-$ and HLA-A2$^+$ populations, containing T cells expressing survivin-specific Tg-TCRs (Figure 3E) or tyrosinase-specific Tg-TCR (Figure 3F), demonstrating that high-level apoptosis required the presence of T cells expressing survivin-specific Tg-TCRs and only occurred in HLA-A2$^+$ recipient lymphocyte populations. It should be noted that the differences between the percentages of T cells expressing a Tg-TCR and the percentages of cells undergoing apoptosis in HLA-A2$^+$ recipient PBLs revealed that death was not limited to T cells expressing survivin-specific Tg-TCRs. Thus, T cells bearing survivin-specific Tg-TCRs mediated fratricide against a substantial number of HLA-A2$^+$ lymphocytes lacking Tg-TCR expression.

Because the TCR-transgenic T cells were stimulated to achieve efficient expansion, we examined whether activated T cells could be directly killed by TCR-transduced PBLs (Figure 4A). After stimulation with either phytohemagglutinin (PHA) or a combination of CD3- and CD28-specific antibodies, activated HLA-A2$^-$ lymphocytes were not recognized by effector cells expressing Tg-TCR, even though they expressed high levels of survivin mRNA (Figure 4B). In contrast, unstimulated HLA-A2$^+$ lymphocytes were killed to a substantial degree by effector cells expressing survivin-specific Tg-TCR. Furthermore, killing increased after lymphocyte activation (Figure 4A), coinciding with increases in the basal level of survivin mRNA transcripts (Figure 4B). We also assessed whether cytotoxic T lymphocyte (CTL) clones could serve as targets for survivin-specific TCR-modified effector cells. CTLs derived from different HLA-A2$^+$ donors, with specificity for either tumor-associated peptides (A42, ref. 28; Tyr-F8, ref. 29) or an Epstein-Barr virus–derived ligand (FaLe) (D.J. Schendel, unpublished observations), were well recognized, whereas CTL clone JB4 (30), originating from an HLA-A2$^-$ donor, was not killed (Figure 4C). These CTL clones expressed survivin mRNA

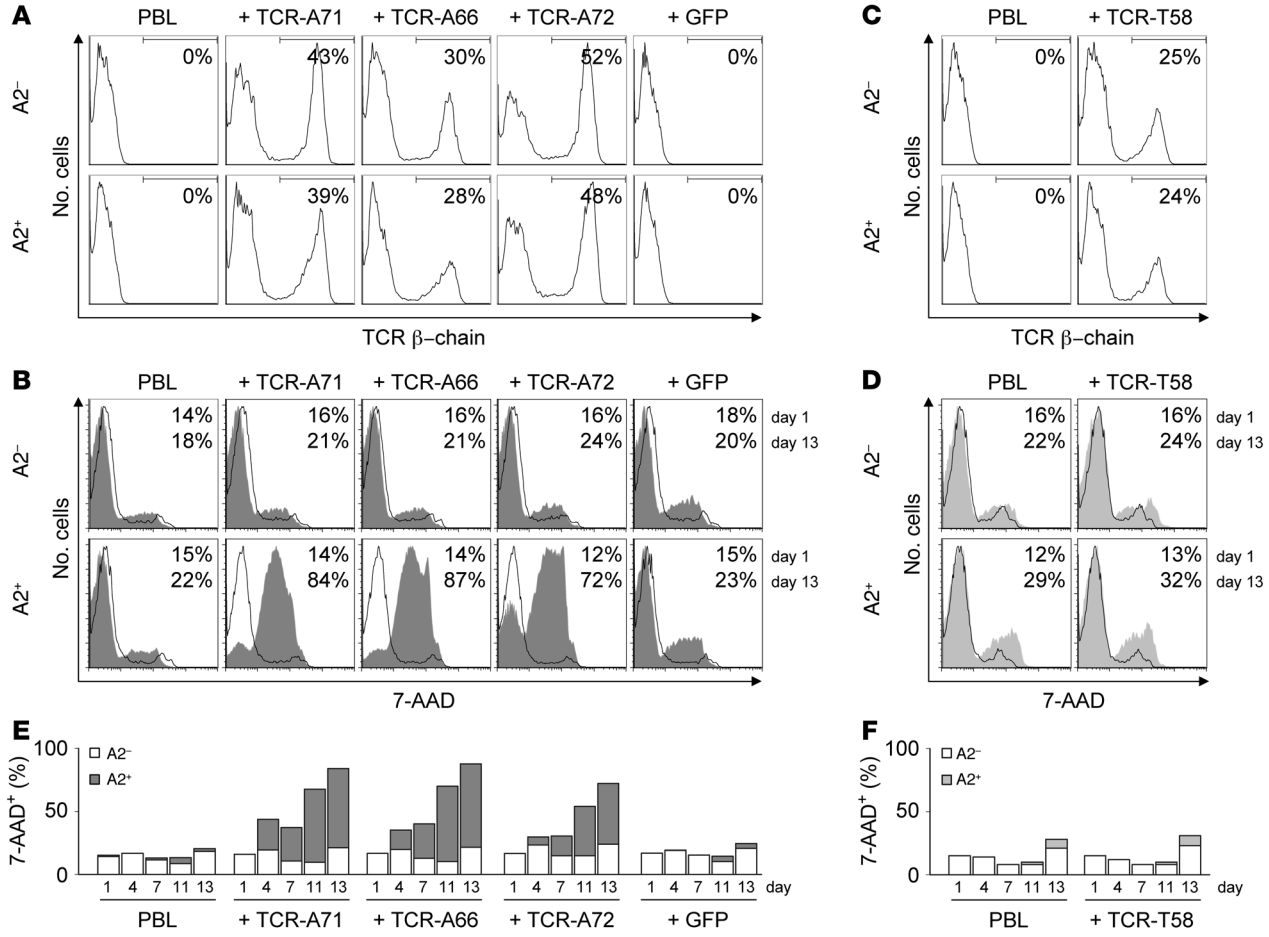

Figure 3

MHC-restricted fratricide of survivin-specific TCR-modified PBLs. HLA-A2⁻ and HLA-A2⁺ activated lymphocytes were transduced with the survivin-specific TCR-A71, -A66, and -A72. (**A**) Expression of Tg-TCRs was analyzed using murine TCRβ constant region–specific antibody at day 4 after TCR transduction. The numbers indicate the percentage of Tg-TCRβ chain expression. (**B**) TCR-modified PBLs were further cultured for 2 weeks and stained with 7-AAD to discriminate living and dead cells. The open histograms show staining 1 day after transduction; the filled gray histograms display the staining on day 13. The percentage of 7-AAD⁺ dead cells on days 1 and 13 is indicated in the upper-right corner. (**C** and **D**) The same analysis was made using PBLs transduced with a high-affinity TCR (T58) specific for a peptide derived from tyrosinase protein presented by HLA-A2 (25). Tg-TCRβ chain expression and percentage of apoptotic cells are shown as in **A** and **B**. Percentages of 7-AAD⁺ PBLs on days 1, 4, 7, 11, and 13 after TCR-transduction with (**E**) survivin- or (**F**) tyrosinase-specific TCR genes in activated lymphocytes of HLA-A2⁻ (white bars) and HLA-A2⁺ donors (gray bars). The data are representative of 2 independent experiments with 2 individual donors each.

(Figure 4D), albeit at variable levels. Two controls demonstrated the specificity of recognition. First, effector PBLs had to express a survivin-specific Tg-TCR, since GFP-transduced and untransduced PBLs did not mediate appreciable killing of target cells. Second, HLA-A2⁻ activated PBLs and HLA-A2⁻ CTLs were not killed by any effector population, demonstrating that TCR recognition was HLA-A2 restricted.

Activated T cells express several TAAs that might target them for fratricide. The wider impact of MHC-restricted fratricide was considered with respect to other TAAs, including several TAAs prioritized by the NCI Translational Research Working Group (5). Therefore, we analyzed mRNA levels in activated PBMCs and enriched CD8⁺ T cells (Figure 5) and considered two factors in this assessment. First, mRNA levels were compared in unstimulated versus stimulated T cells (CD3/CD28 activation) and expressed as x-fold increases (Figure 5, *x* axis). Second, transcript levels of each TAA in activated cells were normalized to 18S rRNA and expressed as crossing-point (CP) values, in order to demonstrate their overall prevalence with respect to each other (Figure 5, *y* axis). The CP value defined the cycle number in the logarithmic phase of the PCR, where the product was the same in all the samples that were compared; thus, low CP values revealed high levels of mRNA template, while high CP values indicated rare mRNA templates. Transcript levels of numerous TAAs increased upon activation of PBMCs and CD8⁺ T cells from around 10-fold to more than 10^7-fold when compared with unstimulated cells (Figure 5, right quadrants). As expected, TAA transcripts were expressed in activated cells at widely different levels, reflected by CP values ranging from 13 to 35 (Figure 5, *y* axis). Transcripts for several TAAs remained very low, with or without lymphocyte activation (CP, >30; Figure 5, bottom quadrants). Survivin transcripts displayed the greatest fold increase after lymphocyte stimulation (>10^7-fold) and were abundant (CP, 21). In contrast, tyrosinase transcripts did not increase upon activation and were very rare (CP, 35).

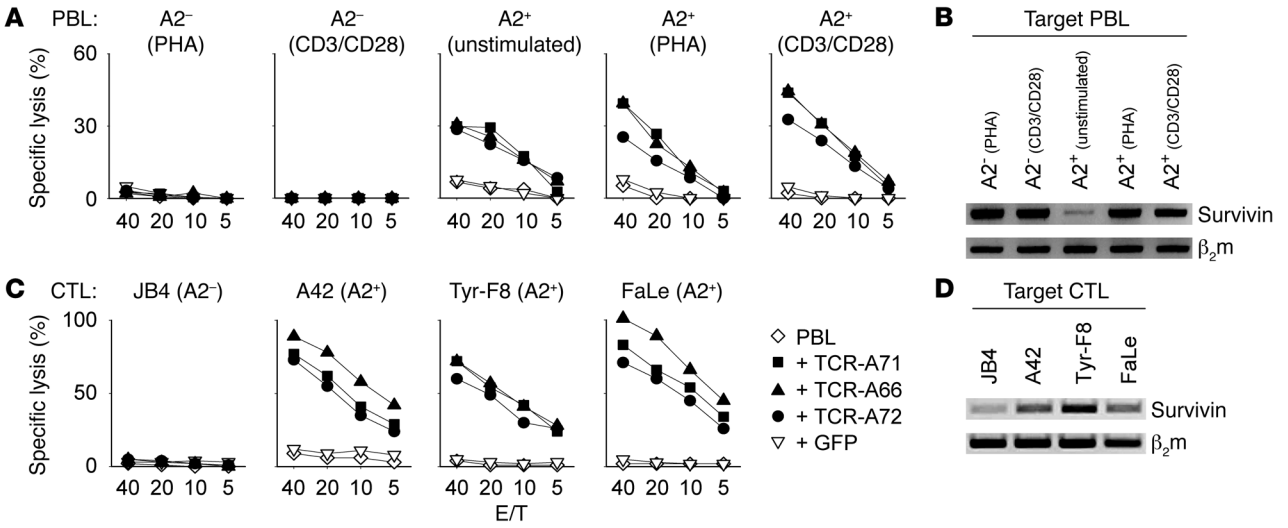

Figure 4

Cytotoxic activity of survivin-specific TCR-modified PBLs. The cytotoxicity of survivin-specific TCR-engineered PBLs using lymphocytes and T cell clones as target cells was determined in a standard 4-hour chromium release assay using varying E/T ratios, and results are presented as percent specific lysis. (**A**) Unstimulated or PHA- or CD3/CD28-activated lymphocytes of HLA-A2⁻ and HLA-A2⁺ donors were used as target cells at the given E/T ratios. (**B**) mRNA levels of survivin and β_2-microglobulin (β_2m) were analyzed by RT-PCR. (**C**) HLA-A2⁺ T cell clones with specificity for the TAA MART-1/melan-A (A42, ref. 28), tyrosinase (Tyr-F8, ref. 29), or Epstein Barr virus (FaLe) were assessed as target cells for TCR-modified effector cells. An alloreactive clone (JB4, ref. 30) derived from an HLA-A2⁻ donor served as a negative control. (**D**) Corresponding survivin mRNA levels of the T cell clones were determined and depicted as in **B**. Cytotoxicity data represent means of duplicates measured at each E/T ratio. These data are representative of 2–4 individual donors.

Therefore, the TAAs showing abundant mRNA levels (Figure 5, top quadrants) might also have the potential to generate targets for MHC-restricted fratricide.

Discussion

These studies revealed that transgenic expression of HLA-A2–restricted survivin-specific TCRs in activated PBLs led to massive apoptosis that was MHC restricted, since this only occurred in HLA-A2⁺ recipient lymphocytes. Expression of survivin in activated TCR-modified PBLs resulted in presentation of survivin-specific pMHC ligands and led to concurrent self-recognition and fratricide. MHC-restricted fratricide likely accounted primarily for our failure to expand TCR-engineered effector cells prepared using HLA-A2⁺ recipient lymphocytes, whereas expansion was readily achieved with HLA-A2⁻ recipient lymphocytes. Even though survivin-specific Tg-TCRs displayed excellent peptide sensitivities and good tumor cell recognition, which are important properties for selection of therapeutic Tg-TCRs, fratricide of HLA-A2⁺ recipient lymphocytes would preclude their use in TCR gene therapy, except in the clinical setting of HLA-A2–mismatched stem cell transplantation.

It has been reported previously that mouse T cells exposed to high levels of specific antigen can display anergy or even suicide through TCR-mediated induction of apoptosis (31, 32). Furthermore, suicide induced in human T cells by tumor cells expressing high levels of antigen altered antitumor immunity by eliminating high-affinity T cells (33). It is possible that TCR-induced suicide accounted for some of the apoptosis seen in the HLA-A2⁺ populations containing T cells expressing survivin-specific Tg-TCRs. However, the percentage of lymphocytes that underwent apoptosis was much higher than the fraction of T cells expressing a Tg-TCR; therefore, active fratricide clearly contributed to the death of Tg-TCR–negative lymphocytes. This contention was underlined by the demonstration that Tg-TCR effector cells had the capacity to directly kill both activated T cells and CTL clones of HLA-A2⁺ donors, irrespective of their particular TCR specificities.

Uncovering MHC-restricted fratricide helped to explain the loss of HLA-A2⁺ lymphocytes expressing survivin-specific Tg-TCRs over time and might also account for several other observations regarding survivin-specific T cells. After strong enrichment of T cells by survivin-multimer sorting and further culture, self-restricted T cell lines expressed only low percentages of CD8⁺ multimer⁺ T cells, whereas allorestricted lines retained high percentages of double-positive cells. Thus, it appears that expansion of survivin-specific self-restricted T cells was self-limited. We also noted early proliferation of self-restricted T cell clones in the majority of limiting dilution cultures, but T cell colonies showing robust proliferation over several weeks all proved to be nonspecific. We speculate that early clonal proliferation of survivin-specific clones may have occurred when low numbers of T cells were buffered by large numbers of feeder cells, but HLA-A2–restricted apoptosis may have hindered their later outgrowth. Because HLA-A2⁻ cells could not display the corresponding pMHC ligands, they were resistant to HLA-A2–restricted fratricide, and numerous HLA-A2–allorestricted survivin-specific T cell clones could be isolated. These results appear to be concordant with previous studies that described HLA-A2–restricted survivin-specific T cells that were propagated as T cell lines in vitro or detected in peripheral blood samples of cancer patients ex vivo, whereas survivin-specific T cell clones were difficult to obtain (9–18, 34).

Recently, one survivin-specific CTL clone that was isolated from an HLA-A2⁺ breast cancer patient recognized the same pMHC ligand as the Tg-TCR described here (18). This patient-

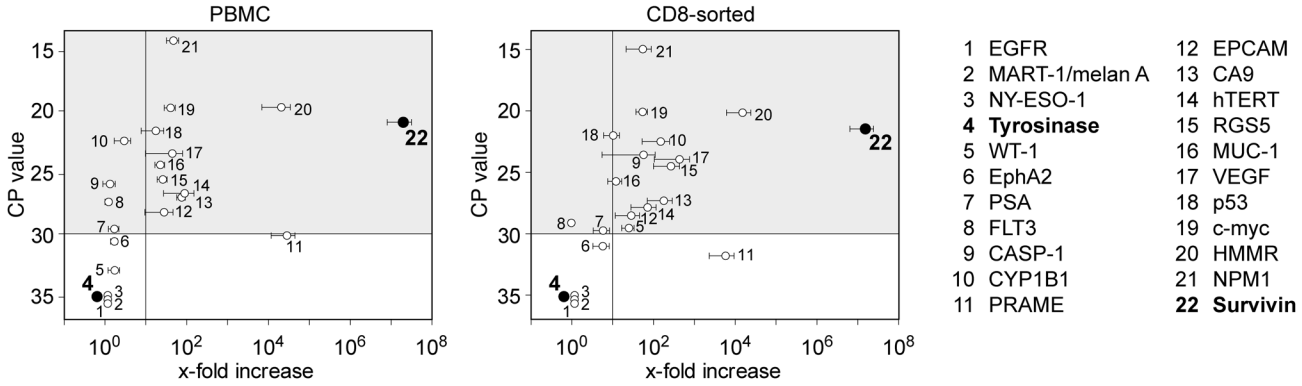

Figure 5

Analysis of mRNA levels for TAAs in activated T cells. Quantitative mRNA expression of 22 TAAs was performed using activated lymphocytes of 4 healthy donors using LightCycler technology, and the values are given as mean with SEM. For each donor, the TAA expression profile of nonactivated PBMCs and nonactivated enriched $CD8^+$ T cells was compared with that of CD3/CD28-activated PBMCs and $CD8^+$ T cells. Differences in mRNA levels detected in unstimulated versus stimulated cell populations were expressed as x-fold increases and are depicted on the *x* axis. The *y* axis represents the CP values, in order to demonstrate the overall prevalence of TAAs in activated cells. The CP value defines the cycle number in the logarithmic phase of the PCR where the product is the same in all the samples that are compared. Low CP values represent high levels of mRNA template, while high CP values indicate rare mRNA templates. An mRNA template with a CP value of less than 30 is considered rare. The housekeeping gene *18S* rRNA was processed as an internal control for normalization of samples. The data were statistically analyzed as described in Methods.

derived CTL clone was shown to recognize all $HLA\text{-}A2^+$ $survivin^+$ tumor cell lines in a small panel, with the exception of the FM-86 cell line. The authors surmised that FM-86 cells were not recognized due to disturbed pMHC ligand expression, since the tumor cells were found to have high levels of survivin mRNA. As shown here, this tumor cell line expresses relatively low levels of surface HLA-A2. We included FM-86 cells in our analyses and found that these tumor cells were recognized by effector cells transduced with each of the 3 Tg-TCRs; however, killing was less with effector cells expressing TCR-A71, the Tg-TCR that endowed the PBLs with the lowest functional avidity. The failure of the published patient CTL clone to kill FM-86 cells would be explained if it had a functional avidity decidedly lower than that of PBLs expressing TCR-A71. In addition, our Tg-TCRs were codon-optimized and modified to express murine constant regions, which imbued them with good surface expression and strong capacity to interact with tumor cells expressing low levels of pMHC ligand. Furthermore, it has been reported that TCR/CD3 expression or TCR signaling is frequently disturbed in patient-derived T cells, thereby hindering their capacity to recognize tumor cells. Such alterations may also have impacted the capacity of the patient-derived CTLs to recognize FM-86 tumor cells displaying low pMHC ligand density.

Because our survivin-specific TCRs were well expressed as transgenic proteins in activated recipient lymphocytes of $HLA\text{-}A2^-$ healthy donors, we could bypass deficits that impinge on expression, signaling, or function of patient-derived CTL clones. The 3 Tg-TCRs effected wide differences in functional avidity in recipient lymphocytes, varying by more than 4 orders of magnitude in peptide sensitivity. Based on analysis of numerous T cell clones (ref. 25 and D.J. Schendel, unpublished observations), we would classify TCR-A71 as having a relatively low affinity, whereas TCR-A72 had a very high affinity. Effector PBLs expressing TCR-A71 showed reduced recognition of FM-86 and KT-195-A2 tumor cells, which expressed the lowest levels of surface HLA-A2, indicating a relationship between T cell functional avidity and pMHC ligand density in efficacy of tumor cell recognition. It should be noted, however, that a correlation could not be drawn with respect to levels of survivin mRNA, since these tumor lines both showed high levels of survivin transcripts.

While our studies identified fratricide that was restricted by HLA-A2, it is also possible that T cells with adequate avidity could recognize additional survivin-derived peptides presented by other MHC molecules, leading to self-restricted fratricide even in $HLA\text{-}A2^-$ donors. The frequent failure to obtain self-restricted T cell clones specific for some self-peptides is often interpreted to be a consequence of deletional tolerance. Based on the results presented here, additional studies are warranted to explore the role of MHC-restricted fratricide in controlling the development of T cells specific for proteins that are well expressed in activated lymphocytes. The authors of two reports speculated that fratricide may have inhibited effective expression of a murine TCR specific for p53 in activated human lymphocytes (35) or limited expansion of T cells specific for hTERT (36), although direct experimental evidence of fratricide was not provided in these studies. On the other hand, other technical limitations could influence the expansion and isolation of such T cells.

The quantification of mRNA indicated that several other TAAs could potentially become targets for T cell–mediated fratricide, based on their high levels of expression in activated lymphocytes. In contrast, transcripts that were very rare, even upon T cell activation, would be less likely to generate pMHC ligands for self-restricted fratricide. This contention is supported by the failure of the high-affinity tyrosinase-specific TCR-T58 to induce widespread apoptosis in $HLA\text{-}A2^+$ lymphocytes. TCR-mediated fratricide specific for any TAA will be dependent on several factors, including protein expression, location, and turnover, as well as antigen processing and presentation of specific peptides by self-MHC molecules. Nevertheless, it would seem prudent to include analysis of RNA and protein expression in activated lymphocytes

as additional criteria in the selection process of candidate TAAs for development of TCR gene therapy.

MHC-restricted fratricide may also have consequences for tumor vaccine development, since this same mechanism could limit proliferation of high-avidity T cells in lymph nodes after vaccination with survivin or other TAAs that are expressed in lymphocytes (37). Some T cells might escape to the periphery, but substantial accumulation at tumor sites might again be self-limiting because of fratricide, thereby impacting clinical efficacy. Furthermore, MHC-restricted fratricide could have a local spreading effect, causing neighboring T cells with unrelated specificities to be eliminated due to presentation of target pMHC ligands, irrespective of their own TCR specificities. This contention is supported by our observation of high sensitivity of activated lymphocytes to killing by survivin-specific TCR-transduced PBLs, as well as the recognition of T cell clones of various specificities derived from HLA-A2$^+$ but not HLA-A2$^-$ donors.

Striving to attain effective antitumor immunity using TCR-transgenic effector T cells with high avidity might have the unintended consequence of causing MHC-restricted fratricide of other adaptive immune cells, if the TCR displays specificity for a pMHC ligand that is well expressed by activated lymphocytes. Our results emphasize that judicious selection of TAAs will be important for designing successful TCR gene therapies.

Methods

Cells. The cell lines Mel-624.38 (HLA-A2$^+$ [A2$^+$], survivin$^+$ [S$^+$]) (38), Mel-1379 (A2$^-$, S$^+$; M. Panelli, University of Pittsburgh, Pittsburgh, Pennsylvania, USA), UT-SCC-15 (A2$^+$, S$^+$; M. Schmitz, Technische Universität Dresden, Dresden, Germany), U-373 (A2$^+$, S$^+$; P.J. Nelson, Ludwig-Maximilians-Universität, Munich, Germany), KT-195 (A2$^-$, S$^+$; H. Gröner, Deutsche Krebsforschungszentrum, Heidelberg, Germany), and T2 (CRL-1992, ATCC) were cultured as described previously (25). KT-195-VC and KT-195-A2 are transfectants of KT-195, generated by transduction with retroviral vectors encoding GFP (MP71-iG) or HLA-A*0201 and GFP (MP71-A2iG). FM-86 cells were purchased from European Searchable Tumor Cell Bank and Database (University Tübingen, Tübingen, Germany) and cultured as described in ref. 39. The T cell clones A42 (28), FaLe (EBV-specific, generated in our facility using autologous B-LCL), JB4 (30), and Tyr-F8 (29) were cultured as described (30) and used as target cells 6 days after restimulation.

Generation of survivin peptide–specific T cells with RNA-pulsed DCs. The collection of blood and patient material was approved by the "Ethikkommission der Medizinischen Fakultät der Ludwig-Maximilians-Universität," Munich and donors gave informed consent. De novo priming of self-restricted and allorestricted peptide–specific T cells was performed using mature DCs, electroporated with 50 μg of survivin ivt-RNA, with or without 50 μg HLA-A2 ivt-RNA. The linearized plasmids pGEM4Z/survivin/A64 and pCDM8-HLA-A2 (E. Weib, Ludwig-Maximilians-Universität, Munich, Germany) were used as templates for RNA transcription. Survivin peptide–specific T cells were sorted using an HLA-A2-survivin$_{96–104[97L]}$ pentamer (survivin-multimer, ProImmune) and either expanded as bulk T cell lines or cloned in limiting dilution cultures (25).

Retroviral TCR gene transfer. TCR sequences of survivin-specific clones A71, A66, and A72 were determined, and TCRβ-2A-TCRα transgene cassettes were synthesized (GENEART) and integrated into MP71-PRE as previously described (25). To enhance surface expression, TCR constant regions were exchanged by their mouse counterparts (26) and transgene cassettes were codon optimized (27). Vector plasmids were used for production of retroviral particles and subsequent transduction of T cells (40).

FACS. Anti-CD8, anti–mouse TCRβ antibody detecting the mouse constant region of transgenic TCRβ (BD Biosciences — Pharmingen), anti–HLA-A2 antibody (AbD Serotec), survivin-multimer, and HLA-A2-CMVpp65$_{495–503}$ control multimer (CMV-multimer, D. Busch, Technische Universität, Munich, Germany) were used for analysis or sorting. Viability of TCR-modified PBLs was determined by incubation with 7-AAD (BD Biosciences — Pharmingen).

Cytotoxicity and IFN-γ release assays. Cytotoxic activities of bulk T cell lines, T cell clones, and TCR-modified PBLs were analyzed in standard 4-hour chromium release assays (25). For initial analysis at day 13 after the second unspecific stimulation, T cells were incubated with 1.5×10^3 survivin$_{96–104[97L]}$ (survivin, LMLGEFLKL) or influenza matrix protein$_{58–66}$ (flu, GILGFVFTL) peptide–loaded T2 cells (peptides: Metabion) as previously published (25). Bulk T cell lines were cocultured at various effector to target cell ratios (E/T ratios). For further analysis, allorestricted clones A71, A66, and A72 were analyzed by coculture either with 1×10^3 tumor cells (Mel-1379, Mel-624.38) or with flu peptide–loaded T2 cells (E/T, 10:1). Functional avidity of T cell clones and TCR-modified PBLs was determined by incubation with 1×10^3 survivin peptide–loaded T2 cells (10^{-12} to 10^{-5} M) at an E/T ratio of 10:1 for the T cell clones and 20:1 for TCR-modified PBLs. TCR-modified PBLs were cocultured with tumor cell lines, target PBLs or T cell clones using 2×10^3 target cells at the designated E/T. Specific, relative, and half-maximal lysis was calculated as described using duplicate samples at each E/T ratio or peptide concentration (25). Target PBL cultures were used directly after isolation or stimulated for 3 days using 100 IU/ml IL-2 (Chiron) and 5 μg/ml PHA (Roche) or anti-CD3, -CD28 antibody (40). TCR-modified PBLs (5×10^4) were incubated with given tumor cells at an E/T ratio of 2:1, and 24-hour supernatants were assessed by ELISA (BD Biosciences). aAPCs were generated as described previously (41), and 1×10^6 aAPCs were used either unloaded or loaded with 10^{-5} M survivin or flu peptide in cocultures with TCR-A72–transduced or unmodified PBLs at an E/T ratio of 1:2.

RT-PCR. Isolation of full-length RNA, cDNA synthesis, and PCR amplification of survivin and β_2-microglobulin sequences were performed as previously described (12, 40).

Real-time PCR for quantification of TAAs. To evaluate quantitative mRNA expression of TAAs, cryopreserved PBMCs of 2 donors as well as freshly drawn PBMCs of 2 donors were analyzed. For each donor, the TAA expression profile of nonactivated PBMCs and enriched CD8$^+$ T cells (CD8$^+$ T cell Isolation Kit II, Miltenyi Biotec) was compared with that of activated PBMCs and CD8$^+$ T cells. Activation of cells was performed as described above. Total RNA was extracted (TriReagent, Biozol), and equal RNA amounts were reverse transcribed using oligo(dT)$_{15}$ primer and AMV reverse transcriptase (First Strand cDNA Synthesis Kit for RT-PCR, Roche). Detection of TAA expression was performed using the LightCycler PCR Master Mix (Roche). Primers used for quantitative RT-PCR are listed in Supplemental Table 2. PCR amplification was performed with initial 10 minutes denaturation at 95°C, 35 cycles of amplification with 1 second at 95°C, 10 seconds at 56°C, and 25 seconds at 72°C except for HMMR (using 59°C as annealing temperature) and hTERT, survivin, tyrosinase (using a kit from Search-LC). Evaluation of results was done by directly plotting CP values (normalized by the housekeeping gene *18S* rRNA) as shown in Figure 5, as well as by conversion of the CPs into relative concentration of transcripts. Transcript levels obtained from nonactivated cells were set as 1 to determine an x-fold increase or decrease in transcripts in activated PBLs and CD8$^+$ T cells.

Statistics. The basis for data analysis for the different assays displayed in Figures 1–4 is provided in the individual figure legends. The values shown in Figure 5 represent mean values of transcript levels measured in cells of 4 donors, repeated in 2 independent experiments, with the exceptions that PSA was only measured in 3 donors and c-kit in only 2 donors.

The error bars represent SEM. The means and SEM were calculated using GraphPad Prism Software.

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Address correspondence to: Dolores J. Schendel, Institute of Molecular Immunology, Helmholtz Zentrum München, Marchioninistrasse 25, 81377 Munich, Germany. Phone: 49.89.7099301; Fax: 49.89.7099300; E-mail: schendel@helmholtz-muenchen.de.

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