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Allogeneic HLA-A*02–Restricted WT1-Specific T Cells from Mismatched Donors Are Highly Reactive but Show Off-Target Promiscuity

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T cells recognizing tumor-associated Ags such as Wilms tumor protein (WT1) are thought to exert potent antitumor reactivity. However, no consistent high-avidity T cell responses have been demonstrated in vaccination studies with WT1 as target in cancer immunotherapy. The aim of this study was to investigate the possible role of negative thymic selection on the avidity and specificity of T cells directed against self-antigens. T cell clones directed against the HLA-A*0201–binding WT1126–134 peptide were generated from both HLA-A*02–positive (self-HLA–restricted) and HLA-A*02–negative [nonself (allogeneic) HLA [allo-HLA]-restricted] individuals by direct ex vivo isolation using tetramers or after in vitro priming and selection. The functional avidity and specificity of these T cell clones was analyzed in-depth. Self-HLA–restricted WT1-specific clones only recognized WT1126–134 with low avidities. In contrast, allo-HLA–restricted WT1 clones exhibited profound functional reactivity against a multitude of HLA-A*02–positive targets, even in the absence of exogenously loaded WT1 peptide, indicative of Ag-binding promiscuity. To characterize this potential promiscuity, reactivity of the T cell clones against 400 randomly selected HLA-A*0201–binding peptides was investigated. The self-HLA–restricted WT1-specific T cell clones only recognized the WT1 peptide. In contrast, the allo-HLA–restricted WT1-reactive clones recognized besides WT1 various other HLA-A*0201–binding peptides. In conclusion, allogeneic HLA-A*02–restricted WT1-specific T cells isolated from mismatched donors may be more tumor-reactive than their autologous counterparts but can show specific off-target promiscuity of potential clinical importance. As a result of this, administration of WT1-specific T cells generated from HLA-mismatched donors should be performed with appropriate precautions against potential off-target effects. The Journal of Immunology, 2011, 187: 2824–2833.

The gene encoding the Wilms tumor protein (WT1) is expressed in human acute leukemia cells and chronic myeloid leukemia cells in blast crisis at significantly higher levels compared with normal hematopoietic cells, including CD34+ hematopoietic progenitors (1–4). T cells recognizing self-antigens (such as WT1) that are overexpressed in malignant cells are thought to contribute to antitumor reactivity after hematopoietic stem cell transplantation for the treatment of leukemia (5–7) and TCR gene transfer (12–14). Several immunogenic peptides derived from the WT1 protein have been characterized in the context of three different HLA class I molecules (HLA-A*01, HLA-A*02, and HLA-A*24), and T cell responses against these peptides have been described (8, 11, 15–19). A number of vaccination studies using WT1-derived peptides have been performed to boost in vivo WT1-specific T cell responses with the aim of enhancing immune control of leukemia (20–28). However, although the frequencies of WT1 tetramer-binding T cells generally increased and were accompanied by concomitant decreases in WT1 mRNA expression levels, no sustained clinical responses were observed. These results could indicate that patients with WT1-expressing tumors are capable of mounting T cell responses against WT1-derived Ags, but that these T cells are likely to be of insufficient avidity to eliminate the leukemic cells.

WT1-specific T cell lines and clones have been generated and characterized after in vitro stimulation and selection using various strategies (8–10, 15, 19, 29–32). The majority of T cells generated in these studies were capable of recognizing target cells exogenously loaded with the relevant WT1 peptides. However, marked recognition of primary leukemic cells presenting endogenously processed Ag has not been clearly demonstrated. Although the WT1 protein is considered to be a tumor-associated Ag due to its overexpression in malignant cells, expression is also observed in normal cells (3, 33–35), suggesting that it may function as a self-antigen. As thymic selection filters out thymocytes that express TCRs with high affinities for self-antigens presented in the context of self-MHC, this process may eliminate T cells with high avidity for WT1–self-MHC, thereby compromising immunotherapeutic approaches based on manipulation of the endogenous T cell repertoire. In contrast, thymic selection preserves or selects for clonotypes with low avidity for self-peptide–self-MHC complexes, and thus strategies designed to induce or boost WT1-specific T cell responses in...
the autologous or HLA-matched allogeneic setting are likely to result in the expansion of clonotypes with relatively low functional avidity for the target Ag. Indeed, although clinical responses associated with increased frequencies of WT1-specific T cells after different immunological interventions have been described by several groups (20–28), no convincing evidence for the induction of truly high-avidity T cells has been demonstrated in these patients. However, as the T cell repertoire does not undergo negative selection in response to peptides presented in the context of nonself (allogeneic) MHC (allo-MHC), allo-MHC recognition specific for a large repertoire of peptides can still be present in the postselection T cell compartment (36, 37). These T cells can exhibit high avidity for Ag, which has prompted efforts to induce tumor Ag-specific T cell responses across MHC barriers (8, 38–42). Such high-avidity antitumor activity could be transferred to third-party T cells using TCR gene transfer strategies, illustrating the intrinsically high affinity of these TCRs. However, in the HLA-mismatched situation, the reactive T cells have not undergone functional selection to prevent the recognition of other peptide bonds by the mismatched HLA molecule. Consequently, it is essential to exclude promiscuous recognition of other epitopes, as this could lead to harmful off-target reactivity.

In this study, we generated CD8+ T cell clones directed against the HLA-A*0201–restricted WT1126–134 peptide from both HLA-A*0201–positive and HLA-A*02–negative healthy individuals and from a patient after in vivo vaccination with the WT1126–134 peptide (22). Self-HLA–restricted WT1-reactive T cells isolated from HLA-A*0201–positive healthy donors and from an HLA-A*02–positive patient after in vivo vaccination were highly specific for the WT1–HLA-A*0201 complex but displayed low functional avidities, reflected by their inability to recognize endogenously processed Ag expressed by primary leukemic cells. In contrast, the nonself (allogeneic) HLA (allo-HLA)-restricted WT1-reactive T cell clones isolated from HLA-A*02–negative individuals showed not only variable functional avidities for the WT1–HLA-A*0201 complex, but these clones also demonstrated cross-reactivity with other peptides presented by HLA-A*0201. These results indicate that T cells generated from HLA-mismatched donors for use in adoptive cellular therapy or TCR gene transfer should be studied extensively for promiscuous recognition to prevent harmful off-target reactivity in patients expressing the mismatched HLA molecule.

Materials and Methods

Peptides and peptide–MHC class I tetramers

The HLA-A*0201–binding peptides of WT1 (RMFPNAPYL), PRAME (ALYVDSLFL), PR1 (VQLENVTV), HA-1 (VLHDDLLEA), and CMVpp65 (NLVPMMATV) were synthesized using standard solid-phase strategies (Department of Immunohematology, Leiden University Medical Center, Leiden, The Netherlands, or Biosynthesis, Lewisville, TX). Peptide–MCH class I tetramers were constructed as described previously (43, 44). The PRAME, PR1, HA-1, and CMV tetramers were used for control purposes.

A library of 400 randomly selected peptides eluted from HLA-A*0201 from EBV-LCL cells or predicted and confirmed to bind in HLA-A*0201 was constructed. HLA-A*0201 tetramers were generated with each of these peptides using UV peptide exchange technology (45). In addition, these peptide–MHC class I complexes were attached to latex beads to generate artificial Ag-presenting beads as described previously (46).

Culture conditions and cells

Peripheral blood was obtained from different individuals with informed consent. All experiments were approved by the local medical ethics committees. PBMCs were isolated by Ficoll-Isopaque separation and cryopreserved. Stable EBV-transformed lymphoblastoid B cell lines (EBV-LCL) and PHA blasts were generated using standard procedures. CD34+ precursor cells were isolated from PBMCs of healthy donors using magnetic CD34+ clinMACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. EBV-LCL cells, TAP-deficient T2 cells (CRL-1992; American Type Culture Collection), and K562 cells (CCL-243; American Type Culture Collection) were cultured in IMDM (Lonza, Basel, Switzerland) or RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% FBS (Lonza) supplemented with antibiotics and t-glutamine. EBV-LCL and K562 cells expressing HLA-A*0201 were generated by transduction with retroviral vectors encoding the HLA-A*0201 molecule as described previously (47, 48). Cells highly expressing HLA-A*0201 were sorted using a FACS Aria II (BD Biosciences) flow cytometer to generate a pure population of HLA-A*0201–expressing cells.

Monocytes were isolated from donor PBMCs using magnetic CD14 clinMACS beads (Miltenyi Biotec), according to the manufacturer’s instructions, and transformed into dendritic cells (DCs) by culturing for 2 d at a concentration of 1 × 10⁶ cells/ml in IMDM containing 10% prescreened human serum supplemented with 100 ng/ml GM-CSF (Novartis, Basel, Switzerland) and 500 IU/ml IL-4 (kindly provided by Schering-Plough, Innishannon, Cork, Ireland). Subsequently, the cells were matured for 2 d using a cytokine mixture containing 100 ng/ml GM-CSF, 10 ng/ml IL-1β (Cellgenix, Freiburg, Germany), 10 ng/ml IL-6 (Cellgenix), 10 ng/ml TNF-α (Boehringer Ingelheim, Alkmaar, The Netherlands), 500 IU/ml IFN-γ (Immukine; Boehringer Ingelheim), and 1 µg/ml PGE₂ (Sigma Aldrich, Zwijndrecht, The Netherlands) (49, 50).

T cells were cultured in IMDM containing 5% human serum, 5% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) supplemented with 100 IU/ml IL-2 (Proleukin; Chiron, Amsterdam, The Netherlands). For postisolation expansion of the T cells, a “feeder mix” was used that consisted of culture medium, 5× irradiated (50 Gy) allogeneic feeder cells, 0.5× irradiated (50 Gy) allogeneic EBV-LCL cells, 100 IU/ml IL-2, and 800 ng/ml PHA (Murex Biotech Limited, Dartford, UK.).

Flow cytometry and cell isolation

For flow cytometric analysis, T cells were stained with tetrarmers for 15 min at 37°C, followed by CD4/CD8 counterstaining for 15 min at 4°C. Cells were analyzed using a FACS Calibur, Canto II, or LSRII CellQuest software (BD), and FlowJo software (Tree Star, Ashland, OR).

For isolation, WT1-specific T cells were stained with HLA-A*0201–WT1 tetramers conjugated to allophycocyanin for 30 min at 4°C. Bulk enrichments were performed by magnetic bead separation using anti-allophycocyanin beads (Miltenyi Biotec) according to the manufacturer’s instructions. For the generation of T cell clones, single WT1 tetramer-positive CD8+ T cells were sorted at 4°C using a FACS Aria II (BD) flow cytometer into individual wells of U-bottom microtiter plates containing 100 µl feeder mixture. The T cell clones were subsequently expanded in T cell culture medium and resuspended at 2- to 3-wk intervals.

Generation of WT1-specific T cells

T cell clones specific for the WT1126–134 peptide (RMFPNAPYL) were generated from healthy HLA-A*0201–positive donors, HLA-A*02–positive patients after vaccination with the corresponding WT1 peptide (22), and from healthy HLA-A*02–negative donors.

For the generation of self-HLA–restricted WT1-specific T cells from healthy HLA-A*0201–positive donors, autologous mature monocyte-derived DCs were pulsed for 2 h at 37°C with 1 µM WT1 peptide, followed by three washing steps. CD14-depleted responder cells were cocultured for 10 d with irradiated (25 Gy) peptide-loaded DCs at a 1:1 responder to stimulator (R/S) ratio in T cell medium supplemented with 5 ng/ml hIL-7 (Biosource International, Camarillo, CA), 200 U/ml IL-2 (Proleukin; Chiron, Amsterdam, The Netherlands). After 10 d, a restimulation step was performed using peptide-loaded (1 µM) autologous PBMCs (R/S ratio 10:1). On day 20, the cell lines were stained with allophycocyanin-labeled HLA-A*0201–WT1 tetramers, single-cell sorted as described earlier, and expanded for further analysis.

For the isolation of in vivo–primed HLA-A*02–restricted WT1-specific T cells, samples from three HLA-A*02–positive patients who participated in a vaccination trial (PR1 vaccination trial [22]) were analyzed at selected time points at which increased frequencies of HLA-A*0201–WT1 tetramer-positive cells were detected. WT1-specific T cells were enriched directly ex vivo using allophycocyanin-labeled HLA-A*0201–WT1 tetramers and MACS bead (Miltenyi Biotec) isolation, nonspecifically expanded for 10 d, and then further enriched with a second round of selection. After another 10 d of culture, HLA-A*0201–WT1 tetramer-positive T cells were single-cell sorted as described earlier, and expanded for further analysis.

For the generation of allo-HLA-A*0201–restricted WT1-specific T cell lines from HLA-A*02–negative healthy donors, three different methods were used:
1) CD14-depleted responder cells were exposed to WT1_{126–134} peptide-loaded DCs generated from an HLA-A*0201–positive donor and fully matched at all HLA class I loci except HLA-A*0201 at an R/S ratio of 10:1 in T cell medium supplemented with 5 ng/ml IL-7. On day 10, CD4+ T cells were depleted using CD4 MACS beads (Miltenyi Biotec) and the CD8+ T cells were restimulated with peptide-loaded (1 μM) PBMCs from the HLA-A*0201–positive donor (R/S ratio 10:1). On day 20, HLA-A*0201–WT1 tetramer-positive T cells were single-cell sorted as described earlier and expanded for further analysis.

2) CD8+ T cells were selected using MACS beads (Miltenyi Biotec) and stimulated with irradiated (75 Gy) autologous HLA-A*0201–transduced EBV-LCL cells loaded with 5 μM WT1 peptide at a 10:1 R/S ratio in T cell medium supplemented with IL-7 (10 ng/ml), IL-12 (1 ng/ml), and IL-15 (2 ng/ml). Weekly restimulation was conducted with peptide-loaded EBV-LCL cells. After two rounds of stimulation, cells were further expanded using IL-2 (100 IU/ml). After the third and fourth stimulation, HLA-A*0201–WT1 tetramer-positive T cells were single-cell sorted as described earlier and expanded for further analysis.

3) WT1-specific T cells were enriched directly ex vivo using HLA-A*0201–WT1 tetramers and MACS beads (Miltenyi Biotec) isolation, nonspecifically expanded, and then enriched again as described earlier. Subsequently, HLA-A*0201–WT1 tetramer-positive T cells were single-cell sorted as described earlier and expanded for further analysis.

The TCR α-chain (TCRA V) and TCR β-chain (TCRBV) usage of the WT1-specific clones was determined using RT-PCR and sequencing as described previously (47, 51).

Functional analyses

The cytotoxic capacity of WT1-specific T cells was determined in standard 51Cr release assays (52) using T2 cells, EBV-LCL cells, or K562 cells exogenously loaded with different concentrations of the WT1_{126–134} peptide or control peptides (1 pM to 10 μM) as target cells. For analysis of IFN-γ production, 5000 T cells were cocultured either with 30,000 target cells (e.g., T2, EBV-LCL, or K562 cells) loaded with different concentrations of WT1_{126–134} peptide or control peptides (1 pM to 10 μM) or with artificial Ag-presenting beads. After 24 h, supernatants were harvested, and the concentration of IFN-γ was measured by ELISA (CLB, Amsterdam, The Netherlands, or BD OptEIA Human IFN-γ ELISA Set).

For intracellular cytokine staining, T cells were stimulated with HLA-A*0201–positive or HLA-A*02–negative EBV-LCL or T2 cells loaded with WT1 peptide or control peptides at an E:T ratio of 1:1. Brefeldin A and monensin (BD Biosciences) were added to prevent cytokine secretion and lysosomal acidification, respectively, and PE-conjugated anti–MIP-1β (BD) was added to assess Ag-specific CTL degranulation. Cells were incubated for 6 h at 37˚C, then stained for cell surface and intracellular Ags as described previously (53), and acquired on a Canto II flow cytometer. Data were analyzed using FlowJo software.
Results
WT1-specific T cell clones generated in the context of self-HLA exhibit low functional avidity

WT1-reactive T cell lines were generated in vitro from three HLA-A*0201–positive healthy donors (HD) by stimulation of CD14-depleted PBMCs with WT1 peptide-loaded autologous DCs followed by in vitro expansion. At day 20 after initial stimulation, CD8+ T cell frequencies of 0.01–0.4% were detected by staining with the HLA-A*0201–WT1 tetramer (data not shown). After flow cytometric cell sorting based on HLA-A*0201–WT1 tetramer staining, a total of 16 tetramer-positive T cell clones were isolated and expanded (designated HD-auto). Using the same strategy, 15 tetramer-positive T cell clones could be isolated and expanded from the peripheral blood of one of the patients after in vivo vaccination with the WT1126–134 peptide (22) (designated WT1-vacc.). As illustrated by the representative examples shown in Fig. 1A, the T cell clones isolated from both sources displayed equal levels of staining with the HLA-A*0201–WT1 tetramer [mean fluorescence intensities (MFIs) of tetramer staining of 428 ± 96 and 479 ± 81 for HD-auto and WT1-vacc. clones, respectively]. None of the T cell clones that stained with the HLA-A*0201–WT1 tetramer stained with control PRAME, CMVpp65, PR1, and HA-1 tetramers. Isolated clones showed differential TCRBV usage, as demonstrated using flow cytometric analysis (data not shown). The HD-derived WT1-reactive T cell clones showed modest functional activity against HLA-A*0201–positive T2 cells pulsed with 1–10 μM WT1 peptide, resulting in target cell lysis and low levels of IFN-γ production, as shown for two representative clones in Fig. 1B and 1C. The other clones that were generated showed similar low-avidity functional activity patterns. Clones generated ex vivo from the vaccinated patient only weakly recognized HLA-A*0201–positive targets pulsed with the highest (10 μM) concentration of WT1 peptide, resulting in marginal target cell cytotoxicity and IFN-γ production (representative examples shown in Fig. 1B, 1C). None of the clones recognized HLA-A*0201–positive leukemic cells presenting endogenously processed WT1 (data not shown), illustrating the low functional avidity of WT1 peptide-specific T cell clones isolated from HLA-A*02–positive patients and donors.

Generation of highly functional WT1-reactive T cell clones from HLA-A*02–negative donors

Three different methods were used to generate WT1-specific T cell clones from healthy HLA-A*02–negative donors. WT1-reactive T cell lines were generated by stimulation of CD14-depleted PBMCs from an HLA-A*02–negative donor with WT1 peptide-loaded DCs from an HLA-A*0201–positive donor, fully matched at all HLA class I loci except HLA-A*02. After expansion, HLA-A*0201–WT1 tetramer-positive CD8+ T cell frequencies approximating 5% were detected (data not shown), and 21 tetramer-positive T cell clones were generated by flow cytometric cell sorting (designated HD-allo-D). In addition, from five HLA-A*02–negative healthy donors, WT1-reactive T cell lines were generated by stimulating purified CD8+ T cells with autologous EBV-LCL cells transduced with HLA-A*0201 and loaded with WT1 peptide. After 2 wk of expansion, the observed frequencies of HLA-A*0201–WT1 tetramer-positive CD8+ T cells were 0.01–0.4% (mean fluorescence intensities of tetramer staining of 428 ± 96 for HD-auto and 479 ± 81 for WT1-vacc. clones, respectively). None of the T cell clones stained with the HLA-A*0201–WT1 tetramer stained with control PRAME, CMVpp65, PR1, and HA-1 tetramers. Isolated clones showed differential TCRBV usage. The HLA-A*0201–WT1 tetramer-negative healthy donors, WT1-reactive T cell lines were generated by stimulation of CD14-depleted PBMCs from an HLA-A*02–negative donor with WT1 peptide-loaded DCs and HLA-A*0201–positive EBV-LCL cells, as shown for two representative examples in Fig. 1C. WT1-reactive T cell clones were isolated from healthy HLA-A*02–negative donors using three different strategies: 1) stimulation with peptide-loaded allogeneic HLA-A*0201–positive DCs (HD-allo-D); 2) stimulation with peptide-loaded HLA-A*0201–transduced autologous EBV-LCL cells (HD-allo-C), and 3) by direct isolation using HLA-A*0201–WT1 tetramers (HD-allo-A). A, Representative HLA-A*0201–WT1 tetramer stained CD8+ T cell clones. Absence of staining with HLA-A*0201–CMVpp65 tetramer is shown as negative control. B, Cytotoxic activity of WT1-reactive CD8+ T cell clones against TAP-deficient T2 cells and HLA-A*0201–positive EBV-LCL cells in the absence (white bars) or presence (black bars) of exogenously loaded WT1 peptide (1 μM). Cytotoxicity was measured in standard 5-h51Cr release assays at an E:T ratio of 3:1 (n = 5, bars show mean percentages of lysis; error bars represent SDs). C, IFN-γ (IFNg) production by WT1-reactive CD8+ T cell clones after stimulation with T2 cells or HLA-A*0201–positive EBV-LCL cells loaded with 0 (white bars), 10 nM (light gray bars), 0.1 μM (gray bars), 1 μM (dark gray bars), or 10 μM (black bars) exogenously loaded WT1 peptide is shown at an E:T ratio of 1:3. None of the clones recognized HLA-A*0201–positive healthy donors, WT1-reactive T cell lines were generated by stimulating purified CD8+ T cells with autologous EBV-LCL cells transduced with HLA-A*0201 and loaded with WT1 peptide. After 2 wk of expansion, the observed frequencies of HLA-A*0201–WT1 tetramer-positive CD8+ T cell clones were 0.01–0.4% (mean fluorescence intensities of tetramer staining of 428 ± 96 for HD-auto and 479 ± 81 for WT1-vacc. clones, respectively). None of the clones stained with the HLA-A*0201–WT1 tetramer stained with control PRAME, CMVpp65, PR1, and HA-1 tetramers. Isolated clones showed differential TCRBV usage. The HLA-A*0201–WT1 tetramer-negative healthy donors, WT1-reactive T cell lines were generated by stimulation of CD14-depleted PBMCs from an HLA-A*02–negative donor with WT1 peptide-loaded DCs and HLA-A*0201–positive EBV-LCL cells, as shown for two representative examples in Fig. 1C. WT1-reactive T cell clones were isolated from healthy HLA-A*02–negative donors using three different strategies: 1) stimulation with peptide-loaded allogeneic HLA-A*0201–positive DCs (HD-allo-D); 2) stimulation with peptide-loaded HLA-A*0201–transduced autologous EBV-LCL cells (HD-allo-C), and 3) by direct isolation using HLA-A*0201–WT1 tetramers (HD-allo-A). A, Representative HLA-A*0201–WT1 tetramer stained CD8+ T cell clones. Absence of staining with HLA-A*0201–CMVpp65 tetramer is shown as negative control. B, Cytotoxic activity of WT1-reactive CD8+ T cell clones against TAP-deficient T2 cells and HLA-A*0201–positive EBV-LCL cells in the absence (white bars) or presence (black bars) of exogenously loaded WT1 peptide (1 μM). Cytotoxicity was measured in standard 5-h51Cr release assays at an E:T ratio of 3:1 (n = 5, bars show mean percentages of lysis; error bars represent SDs). C, IFN-γ (IFNg) production by WT1-reactive CD8+ T cell clones after stimulation with T2 cells or HLA-A*0201–positive EBV-LCL cells loaded with 0 (white bars), 10 nM (light gray bars), 0.1 μM (gray bars), 1 μM (dark gray bars), or 10 μM (black bars) exogenously loaded WT1 peptide is shown at an E:T ratio of 1:3. None of the clones recognized HLA-A*0201–positive healthy donors, WT1-reactive T cell lines were generated by stimulating purified CD8+ T cells with autologous EBV-LCL cells transduced with HLA-A*0201 and loaded with WT1 peptide. After 2 wk of expansion, the observed frequencies of HLA-A*0201–WT1 tetramer-positive CD8+ T cell clones were 0.01–0.4% (mean fluorescence intensities of tetramer staining of 428 ± 96 for HD-auto and 479 ± 81 for WT1-vacc. clones, respectively). None of the clones stained with the HLA-A*0201–WT1 tetramer stained with control PRAME, CMVpp65, PR1, and HA-1 tetramers. Isolated clones showed differential TCRBV usage. The HD-derived WT1-reactive T cell clones showed modest functional activity against HLA-A*0201–positive T2 cells pulsed with 1–10 μM WT1 peptide, resulting in target cell lysis and low levels of IFN-γ production, as shown for two representative clones in Fig. 1B and 1C. The other clones that were generated showed similar low-avidity functional activity patterns. Clones generated ex vivo from the vaccinated patient only weakly recognized HLA-A*0201–positive targets pulsed with the highest (10 μM) concentration of WT1 peptide, resulting in marginal target cell cytotoxicity and IFN-γ production (representative examples shown in Fig. 1B, 1C). None of the clones recognized HLA-A*0201–positive leukemic cells presenting endogenously processed WT1 (data not shown), illustrating the low functional avidity of WT1 peptide-specific T cell clones isolated from HLA-A*02–positive patients and donors.
A*0201–WT1 tetramer-positive cells ranged from 0.1 to 0.5%. By flow cytometric cell sorting based on tetramer staining, a total of 20 tetramer-positive T cell clones were generated (designated HD-allo-A). Finally, WT1-specific T cells were isolated directly ex vivo from HLA-A*02–negative donor PBMCs by direct enrichment of tetramer-positive cells with magnetic bead isolation followed by single-cell flow cytometric sorting. This resulted in the generation of 18 tetramer-positive T cell clones (designated HD-allo-A). Plating efficiency of growing CTL clones was similar for the tetramer-positive T cells generated according to the different strategies. Isolated clones showed differential TCRBV usage, as demonstrated using flow cytometric analysis. Oligoclonal populations were isolated from different individuals (data not shown). As illustrated in Fig. 2A, the clones isolated using the different strategies showed similar levels of staining with the HLA-A*0201–WT1 tetramer (MFI of tetramer staining of 460 ± 55, 385 ± 76, and 320 ± 88 for HD-allo-D, -C, and -A, respectively). No staining with the control HLA-A*0201 tetramers (PRAME, PR1, CMVpp65, and HA-1) was observed (Fig. 2A and data not shown).

The WT1 tetramer-positive T cell clones generated from HLA-A*02–negative donors showed substantial functional activity against HLA-A*0201–positive EBV-LCL cells and to a lesser extent against TAP-deficient T2 cells already in the absence of exogenously loaded WT1 peptide, resulting in target cell cytolysis (Fig. 2B) and IFN-γ production (Fig. 2C). For most clones, IFN-γ production in response to stimulation with T2 cells was increased when the T2 cells were pulsed with increasing concentrations of WT1 peptides. However, the marginal IFN-γ production by clone HD-allo-A38 appears to be reduced after exogenous WT1 peptide loading. Similar reactivity was seen against HLA-A*0201–positive CD34+ progenitor cells, PBMCs, PHA blasts, and primary human fibroblasts both in the absence or presence of exogenously loaded WT1 peptide (Fig. 2D). The other clones generated using the different selection strategies showed similar functional reactivity patterns (data not shown). None of the clones recognized control HLA-A*02–negative targets. However, induction of HLA-A*0201 expression by retroviral transduction resulted in recognition by the T cells (data not shown). This reactivity against a multitude of HLA-A*0201–positive targets in the absence of exogenously loaded WT1 peptides suggested off-target recognition of other peptides bound by HLA-A*0201.

Reactivity of HLA-A*0201–WT1 tetramer-positive T cell clones against WT1–HLA-A*0201 complexes presented on artificial Ag-presenting beads

To investigate the ability of the HLA-A*0201–WT1 tetramer-binding clones generated in the allo-HLA setting to exert WT1-specific functional activity, beads coated with different densities of HLA-A*0201 monomers loaded with WT1 or CMVpp65 peptides were used to stimulate the T cell clones. A self-HLA-restricted WT1-specific clone was used as a control. Fig. 3A shows that similar to the self-HLA–restricted clone, the allo-HLA–restricted T cell clones displayed a dose-dependent response to WT1–HLA-A*0201 complexes, illustrating that WT1-reactive T cell clones generated from HLA-A*02–positive and HLA-A*02–negative individuals showed HD-allo-A*0201–restricted WT1 reactivity. As a control, a T cell clone specific for CMVpp65 was stimulated with the same beads, resulting in CMVpp65-specific recognition (Fig. 3B). The level of IFN-γ production by the different WT1-reactive T cell clones in response to stimulation with the peptide–HLA-A*0201–coated beads was highly variable, indicating that the clones exhibited differential functional avidity for the WT1–HLA-A*0201 complex. Notably, this difference did not correlate with the HLA-A*02 status of the T cells. The marked difference between the profound reactivity against HLA-A*0201–positive EBV-LCL cells (Fig. 2B, 2C, black bars) and the marginal or virtually absent reactivity against WT1–HLA-A*0201 complexes presented in the cell-free bead system observed for the allo-HLA–restricted WT1 tetramer-positive clones HD-allo-C12 and -C9, respectively, suggests preferential recognition of other HLA-A*0201–binding peptides by these clones.

Differential competition of promiscuous recognition by exogenous WT1 peptide loading on TAP-deficient T2 cells

To analyze further the potential promiscuous peptide reactivity, we tested four different HLA-A*02–negative T cell clones with differential WT1-specific functional recognition for their reactivity against TAP-deficient T2 cells exogenously loaded with different concentrations of WT1 peptide. Because of their TAP deficiency, T2 cells only present a limited number of endogenously processed peptides bound to HLA-A*0201 on the cell surface. As shown in Fig. 4A, all clones showed modest baseline reactivity against
unpulsed T2 cells as reflected by IFN-γ production. T cell clones with high functional avidity for the WT1–HLA-A*0201 complex presented on beads (e.g., clones HD-allo-C10 and -C102) showed a dose-dependent increase in IFN-γ production in response to exogenous loading of increasing concentrations of WT1 peptide on the T2 cells, indicating that these clones recognized the WT1 peptide with higher avidity than the endogenously processed T2 peptides. In contrast, the clones that showed only marginal or virtually absent reactivity with the WT1–HLA-A*0201–coated artificial Ag-presenting beads (e.g., clones HD-allo-C9 and -C12) showed a dose-dependent decrease in IFN-γ production upon loading of the T2 cells with increasing concentrations of WT1 peptide. These data indicate that endogenously processed T2 peptides that are recognized by the clones with high avidity were out-competed for binding in HLA-A*0201 by the exogenously loaded WT1 peptides. A similar dose-dependent effect of exogenous WT1 peptide loading on T2 cells on the activation of the different T cell clones was reflected in analyses of the secretion of MIP-1β (Fig. 4B). Notably, although the four described T cell clones expressed similar TCRα V (TCRAV 17 according to international ImMunoGeneTics database nomenclature) and TCRβ V (TCRBV 6-5 according to international ImMunoGeneTics database nomenclature), clones HD-allo-C10 and -C102 differed from clones -C9 and -C12 at two positions in the TCRα CDR3 region (Fig. 4C), correlating with their differential reactivity patterns (Fig. 4A, 4B). These differential reactivity patterns against WT1 peptide-pulsed T2 cells was also observed for some of the clones selected using the other isolation strategies (data not shown).

Differential promiscuity of HLA-mismatched WT1-reactive T cell clones

To characterize further the promiscuous recognition pattern of the allo-HLA–restricted WT1 tetramer-binding T cells, a library of 400 HLA-A*0201–binding peptides was constructed, and fluorescently (PE and allophycocyanin) labeled HLA-A*0201 tetramers were generated using UV-exchange technology (45). Using flow cytometry, we tested 6 HLA-A*0201–WT1 tetramer-positive clones isolated from two different HLA-A*02–negative individuals for binding with this HLA-A*0201–peptide tetramer library. The autologous WT1-reactive clone HD-auto-2 was used as specificity control. As expected, the self–HLA-A*0201–restricted HD-auto-2 clone only recognized the HLA-A*0201–WT1 tetramer (Fig. 5C). In contrast, the allo–HLA-A*0201–restricted WT1 tetramer-positive clones exhibited high-avidity interactions with various other HLA-A*0201 tetramers in addition to the HLA-
Importantly, although all clones bound to the HLA-A*0201–WT1 tetramer, the patterns of promiscuous peptide recognition were different for the clones isolated from different individuals. Thus, the shared specificity of the TCRs for WT1–HLA-A*0201 is not predictive for the pattern of promiscuous peptide recognition. Notably, although the clones
HD-allo-C9/12 and C10/102 show amino acid differences in the CD3 region of the TCRV coding sequence and different functional avidity against WT1–HLA-A*0201–expressing targets, no differences were observed in their pattern of off-target promiscuous peptide recognition. For a selected number of peptides, the high avidity of the interaction was confirmed by functional analysis of IFN-γ production in response to artificial Ag-presenting beads coated with HLA-A*0201 monomers or T2 cells loaded with the peptides recognized in the library analysis (data not shown). Table I summarizes the sequences of the peptides that were recognized by the different allodestructive WT1–HLA-A*0201 reactive T cell clones. Peptide sequences were confirmed by mass spectrometry. Differential preference for especially the first amino acids of the peptides recognized by the T cell clones from the two individuals is suggested.

**Discussion**

In this study, we investigated in detail the functional avidities and peptide specificities of self-HLA–restricted and allo-HLA–restricted CD8+ T cell clones directed against the WT1126–134 peptide bound by HLA-A*0201. In both settings, T cells that stained brightly with the HLA-A*0201–WT1 tetramer were isolated. HLA-A*0201–WT1 tetramer-binding T cells generated in the context of self-HLA showed modest but specific functional activity against HLA-A*0201–positive targets pulsed with high concentrations of the WT1 peptide yet failed to recognize endogenously processed WT1 Ag. Of note, in contrast to previously reported findings (54), the intensity of HLA-A*0201–WT1 tetramer staining did not predict functional avidity. This likely reflects a “saturation threshold” at the concentrations used and may also explain the discrepancy between the in vivo frequencies of vaccine-induced WT1-specific T cells measured by tetramer staining and the lack of overt, lasting antitumor immune responses. In contrast, most HLA-A*0201–WT1 tetramer-binding T cells isolated in the allo-HLA setting showed profound reactivity against HLA-A*0201–positive target cells of different origins, including CD34+ progenitor cells, EBV-LCL cells, PBMCs, PHA blasts, primary human fibroblasts, and TAP-deficient T2 cells, even in the absence of exogenously loaded WT1 peptide. Using beads coated with WT1–HLA-A*0201 monomers as an artificial Ag-presenting system, we demonstrated that these clones showed variable levels of functional avidity for the WT1–HLA-A*0201 complex. Again, functional avidity did not correlate with the intensity of HLA-A*0201–WT1 tetramer staining. Competition experiments using T2 cells pulsed with various concentrations of the WT1126–134 peptide indicated differential avidity of the various T cell clones for WT1 and off-target endogenous T2 peptides bound by HLA-A*0201. Using a library of 400 randomly selected HLA-A*0201–binding peptides, we further demonstrated physical binding of these allo-HLA–restricted clones to non-WT1126–134 peptides in complex with HLA-A*0201 by tetramer staining. These peptide recognition patterns were confirmed functionally in stimulation assays using HLA-A*0201 monomer-coated beads. Furthermore, different HLA-A*0201–WT1 tetramer-positive T cell clones showed different patterns of peptide recognition. Thus, the observed cross-reactivity profiles did not correlate with the WT1 specificity of the TCR and therefore could not be easily predicted. Although others have been successful in generating T cells specific for self-antigens presented in the allogeneic setting (8, 38–41), none of our applied selection strategies resulted in the isolation of T cell clones that exhibited single WT1 specificity in the absence of promiscuous recognition of other peptides. Therefore, testing cross-reactivity is not a rare phenomenon among such high-avidity clones. In several of our experiments, we coisolated CMV-specific T cells that showed profound peptide-specific functional avidity, illustrating that our in vitro selection strategies did not result in deletion of high-avidity T cells. Apparently, the frequency of T cells with a promiscuous peptide recognition pattern is relatively high compared with the frequency of T cells showing single-peptide specificity in the HLA-mismatched setting. Murine studies by Felix et al. (55) demonstrated the generation of single-peptide–specific alloreactive T cells. However, in agreement with our findings, they also demonstrated the generation of relatively equivalent numbers of allorreactive T cells that responded specifically to multiple peptides. Other murine studies suggested that T cells that have not been tolerant for recognition of a particular MHC protein–self-peptide during thymic selection may often show promiscuous peptide recognition (56–58). This would suggest that a given TCR can engage multiple peptide–MHC complexes in slightly different orientations depending on the specific peptide. A similar phenomenon is shown for virus-specific T cells showing allorreactivity against other HLA molecules (36, 59). This may be influenced by the baseline affinity of the TCRs for MHC, for which are positively selected in the thymus, but may result in potentially dangerous promiscuous peptide specificity in the absence of proper negative selection.

In conclusion, our results demonstrate that allo-HLA–restricted WT1-specific T cells can show promiscuous off-target recognition of other peptides in the same HLA molecule, which likely reflects the absence of negative selection in the thymus. It is not yet clear whether all off-target allorreactivity detected in vitro translates into clinically significant adverse events (37). Nevertheless, our results suggest that all T cell lines and clones generated from HLA-mismatched donors for use in adoptive cellular therapy or TCR gene transfer should be extensively screened for promiscuous

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<tr>
<th>HD-Allo-C Clones</th>
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<td>RMFPNAPYL</td>
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This table lists the peptides recognized by allo-HLA–restricted WT1-reactive T cell clones isolated from two different individuals (HD-allo-C and HD-allo-D). The sequences are shown for the peptides that were recognized by the T cell clones after UV exchange into PE- and allophycocyanin-labeled HLA-A*0201 tetramers resulting in an MFI of >15 (HD-allo-C clones) or >35 (HD-allo-D clones) in flow cytometric analysis.
recognition and administered with appropriate precautions for potential off-target effects.

Disclosures

The authors have no financial conflicts of interest.

References


