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Functional cloning of a gp100-reactive T-cell receptor from vitiligo patient skin

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Summary

We isolated gp100-reactive T cells from perilesional skin of a patient with progressive vitiligo with superior reactivity toward melanoma cells compared with tumor-infiltrating lymphocytes 1520, a melanoma-derived T-cell line reactive with the same cognate peptide. After dimer enrichment and limited dilution cloning, amplified cells were subjected to reverse transcription and 5' RACE to identify the variable TCR α and TCR β subunit sequences. The full-length sequence was cloned into a retroviral vector separating both subunits by a P2A slippage sequence and introduced into Jurkat cells and primary T cells. Cytokine secreted by transduced cells in response to cognate peptide and gp100-expressing targets signifies that we have successfully cloned a gp100-reactive T-cell receptor from actively depigmenting skin.

Keywords

T cell receptor; vitiligo; melanoma

Communication

The skin disease vitiligo provides a road map for immune responses in melanoma (Le Poole and Luiten, 2008). We can learn from autoimmune responses to melanocytes to create treatment opportunities for melanoma, where tumors arise from malignantly transformed melanocytes (Nordlund and Lerner, 1982). The progressive nature of depigmentation in vitiligo suggests autoimmune involvement, which is further supported by observations of

CD4⁺ and CD8⁺ T-cell infiltrates within a narrow margin surrounding depigmenting skin (Le Poole et al., 1996). Cytotoxic T lymphocytes (CTL) from marginal skin of a patient analyzed here displayed >16% reactivity to a single gp100-derivative peptide alone, suggesting that the vast majority of marginal skin-infiltrating T cells respond to melanocyte antigens (Oyarbide-Valencia et al., 2006). MART-1, tyrosinase, TRP-2, and others are also known to elicit CTL responses in vitiligo (Touloukian et al., 2001).

Paradoxically, vitiligo can also develop subsequent to a melanoma diagnosis. This is ascribed to immunogenic proteins expressed by both melanoma tumor cells and melanocytes (Byrne et al., 2011). In melanoma, skin-infiltrating CTL responsible for depigmentation are clonal replicas of those in the tumor (Becker et al., 1999). The initiative for generating antimelanocyte responses in the absence of tumor is likely different and includes a genetic predisposition to vitiligo (Jin et al., 2012).

In healthy individuals, T-cell receptors (TCRs) with high affinity for self-antigens are normally clonally deleted in primary lymphoid organs or develop into regulatory T cells (Tregs) (Moran et al., 2011). As Tregs are sparse in vitiligo skin, greater affinity of TCRs on Th1 T cells may leave room for relevant cytotoxic responses to develop in diseased skin (Klarquist et al., 2010). Indeed, others have isolated circulating MART-1 and tyrosinase-reactive T cells from patient blood and characterized their TCRs. Importantly, T cells from patients with vitiligo displayed greater avidity toward tumor cells than those from patients with melanoma (Palermo et al., 2005). The contribution of TCRs in this process implies a primary role in driving maladaptive immune responses, in which case TCRs from vitiligo skin may provide a superior source of TCRs to redirect a tumor patient's T cells following adoptive T-cell therapy (Sadelain, 2009). Indeed, T-cell avidity is defined at least in part by the affinity of the TCR (Stone et al., 2009).

In the current study, vitiligo T cells were sorted for reactivity to gp100_{209–217} and compared to tumor-infiltrating lymphocytes (TIL) regarding cytotoxicity toward melanoma cells. TCR amplicons were generated from clonal T cells and sequenced by 5' RACE, then introduced into a retroviral vector. The resulting construct was introduced into Jurkat cells, measuring recognition of the cognate peptide by HLA-dimer and decamer staining, as well as secretion of IL-2 in response to peptide.

Vitiligo skin biopsies were obtained from consenting adults with the approval of the University of Amsterdam and Loyola University Chicago Institutional Review Boards. To generate gp100_{209–217}-reactive T cells from vitiligo skin, two-mm punch biopsies from actively depigmenting, perilesional patient skin were maintained in Iscove's modified Dulbecco's modified Eagle's medium (Cambrex Bio Science, Verviers, Belgium) and 10% heat-inactivated human serum type AB (Cambrex Bio Science), with 20 U/ml IL-2 (Eurocetus, Amsterdam, the Netherlands), 5 ng/ml IL-15 (Strathmann Biotec AG, Duchefa, Bergisch Gladbach, Germany), 15 µg/ml gentamycin (Duchefa Biochemie B.V., Haarlem, The Netherlands), 2 mM L-glutamine (Gibco Invitrogen, Breda, the Netherlands), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco Invitrogen), and 50 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands). Anti-CD3/CD28 mAb-coated T-cell expander beads (DynaL Biotech-Invitrogen, Breda, the Netherlands) were added to promote

T-cell outgrowth. We subsequently combined these VIT1 T cells with HLA-A2+ fibroblasts that were pulsed with tyrosinase_{369–377} (YMDGTMSQV) or gp100-derived peptides, as well as with HLA-A2+ melanocytes as shown in Figure 1A–C. For these experiments, melanocytes and fibroblasts were isolated from foreskin tissue and HLA-A2 expression was validated by flow cytometry using FITC-conjugated antibody BB7.2 (BD Biosciences, San Jose, CA, USA). Clustering in response to gp100_{209–217} but not in response to tyrosinase peptide-pulsed fibroblasts was an indication that T cells within the culture were reactive with gp100; measuring IFN- γ secretion in response to peptide-pulsed T2 cells further confirmed that the majority of skin-derived T cells from this patient were reactive with gp100_{209–217} (not shown). Although TYR has been identified as a vitiligo susceptibility locus (Jin et al., 2012), this clearly does not translate into a consistent abundance of tyrosinase-restricted CTL infiltrating patient skin. In fact, earlier studies have already suggested that tyrosinase may not be the most immunogenic antigen to define T-cell specificity in vitiligo (Ogg et al., 1998).

The T cells were then combined with melanoma cells that either express or do not express gp100 in different effector target ratios. In F003 human melanoma cells, antigen loss was compensated for by introducing a gp100 expression vector described in prior studies (Stennett et al., 2004). Cytotoxicity was determined in chromium release assays, performed after pre-incubating adherent target cells with 4 μ Ci/well of ⁵¹Cr (PerkinElmer, Waltham, MA, USA) in 1% BSA in PBS. Washed target cells were co-incubated with effector T cells at indicated ratios for 20 h. A 2% saponin in culture medium was used to measure maximum target cell death, and medium alone was used to evaluate background release. Supernatant was combined with Microscint-40 (PerkinElmer) and measured in a PerkinElmer Scintillation counter. Percent cytotoxicity was measured in $[\text{cpm (in presence of T cells)} - \text{cpm (spontaneous)}] / [\text{cpm (total)} - \text{cpm (spontaneous)}] \times 100$. The results shown in Figure 1D support findings reported for circulating T cells from patients with vitiligo, suggesting that these T cells display superior reactivity toward melanoma cells when compared to T cells derived from patients with melanoma (Palermo et al., 2005).

We next sorted and pre-enriched for gp100-reactive T cells by flow cytometry using peptide-loaded dimers. To prepare this reagent, gp100_{209–217} 210 M (IMDQVPFSV) and HPV 16E7_{11–20} (YMLDLQPET) peptides (Pi Proteomics, Huntsville, AL, USA) were loaded into HLA-A2:Ig recombinant fusion proteins (BD Biosciences) as per manufacturer's instructions. Resulting pre-enriched gp100_{209–217}-reactive VIT1_{ds} T cells were subsequently cloned by limiting dilution, expanded, and probed for gp100_{209–217} reactivity in an IFN- γ ELISPOT assay (Mabtech, Mariemont, OH, USA). Clonal T cells were used in part to determine their affinity by measuring the amount of IFN- γ generated in response to increasing peptide concentrations presented by T2 cells via ELISAPRO kits (Mabtech) as per manufacturer's instructions. For these studies, T2 cells were obtained from the American Type Culture Collection (ATCC/CRL-1992) and maintained in RPMI 1640 medium (Mediatech, Manassas, VA, USA) with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 250 ng/ml amphotericin B, 100 U/ml penicillin, 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and 2 mM L-glutamine (Invitrogen). The results are shown in Figure 1E and indicate that among the sorted VIT1 population

which generated a half-maximum IFN- γ secretion in response to 4.6 ng/ml peptide, there are clones with higher and with lower EC₅₀.

Although one might instinctively select a clone with the lowest EC₅₀ to generate a TCR construct from, we know that TCR affinity is not a reliable predictor of T-cell avidity (Moore et al., 2009). Thus, different T-cell clones were expanded to identify their variable TCR α and TCR β sequences by 5' RACE (Li et al., 2013). Three clones were subjected to detailed analysis, with one revealing unambiguous sequences representative of both TCR subunits, and productive rearrangement of a single TCR α (13 of 14 sequencing reactions). The resulting sequence is shown in Figure 2A and cloned into a retroviral SAMEN CMV/SR α vector backbone as outlined in Figure 2B (Moore et al., 2009). TCR α and TCR β chains were joined through a viral 2A self-cleavage sequence, released from the pCR2.1 TA vector and inserted into the *NotI*- and *XhoI*-digested vector.

Once the skin-infiltrating lymphocyte TCR sequence SILv44 was identified, an antibody to the V β ₁₇ subunit was obtained to learn whether other clones within the population of VIT1_{ds} cells expressed the same receptor subunit. A subpopulation of VIT1_{ds} cells in the left image represents 31.8% of cells, which is notably more than the 16% of total VIT1 cells reactive with the gp100₂₀₉₋₂₁₇ peptide (Oyarbide-Valencia et al., 2006). Thus, it is very likely that one TCR was responsible for about a third of the reactivity with this single peptide among VIT1 cells. A potential sister clone derived from a T cell expressing the same TCR as clone 44 that gave rise to SILv44 is also shown in Figure 2C.

Resulting plasmids were transfected into Phoenix-A viral producer cells using lipofectamine 2000 (Invitrogen), and stable transfectants were selected using G418 sulfate (Gibco Life Technologies, Grand Island, NY, USA). Infectious particles were generated following transfection with the envelope gene from vesicular stomatitis virus. Virus production was confirmed by viral producing cell death through toxicity of secreted VSV-G. Phoenix viral producing cells and retroviral vector SAMEN CMV/SR α were described elsewhere (Lamers et al., 2013; Moore et al., 2009), and pVSV-g was obtained from Clontech Laboratories, Inc (Mountain View, CA, USA) and maintained in Dulbecco's modified Eagle's medium (Mediatech) with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 250 ng/ml amphotericin B, 100 U/ml penicillin, 100 μ g/ml streptomycin (Invitrogen), 4.5 g/l L-glutamine, 4.5 g/l glucose, and 4.61 g/l sodium pyruvate (Mediatech). Transfected Phoenix-A cells were maintained in the presence of 2 mg/ml G418 sulfate (Mediatech). This provided a platform for generating transduced Jurkat cells which could firmly establish the identification and functional expression of our SILv44 TCR in J76 cells. J76 is a subline of Jurkat cells without expression of endogenous TCR subunits that lack CD8 coreceptor expression (Heemskerk et al., 2003). Infectious particles were generated following transfection with envelope gene from vesicular stomatitis virus. Virus production was confirmed by cell death through toxicity of secreted VSV-G. For J76 cells transduction, plates were spun at 32 C with viral supernatants and 8 μ g/ml polybrene (Sigma) on 2 consecutive days. Resulting Jurkat cells were subjected to dimer and decamer analysis, and results are shown in Figure 3A, whereby $a > 50\%$ transduction efficiency was noted. Interestingly, recognition of transduced cells did not improve using decamer compared with dimer analysis.

Finally, we measured IL-2 secretion by transduced Jurkat cells by ELISA ($n = 4$). In cytokine release assays, effector T cells and a limiting number of 10^3 target cells were combined in U-bottom plates. Note that the absolute amount of secreted IL-2 was ~5-fold higher when the experiment was later repeated in the presence of 10 ng/ml PMA plus 25% of the amount of peptide used here (not shown). T2 target cells were peptide-pulsed for 2 h before adding effectors. Cocultures were incubated for 40 h before measuring cytokine release. In a one-way ANOVA, the amount of IL-2 secreted by transduced T cells varied significantly among groups at $***P < 0.001$. A Dunnett's multiple comparison post-test revealed that gp100-pulsed T2 cells elicited a significantly increased amount of IL-2 secretion compared with unpulsed T2 cells ($***P < 0.001$), whereas tyrosinase peptide-pulsed T2 cells did not. Primary melanocytes likewise elicited significant IL-2 secretion ($**P < 0.01$). The results shown in Figure 3B indicate that we have successfully identified, cloned, and expressed a gp100-reactive TCR originating from autoimmune vitiligo skin which may be of use for the treatment of melanoma. We further observed that when Jurkat cells were transduced to express either SILv44 or the coreceptor-independent and tyrosinase-reactive TCR TIL1383I, SILv44 required $a > 2$ -fold higher peptide concentration for half-maximum IL-2 secretion, with EC_{50} s of 310 and 686 nM, respectively. At a ~10-fold greater maximal IL-2 secretion for TIL1383I, we conclude that SILv44 requires CD8 coreceptor expression for full function (not shown).

Data obtained after introducing our TCR into primary human T cells to measure significant IFN- γ secretion by coreceptor⁺ T cells further suggest that the functionality of SILv44 can readily compete with that of previously described gp100-reactive TCRs generated from gp100 peptide-vaccinated patients with melanoma (Moore et al., 2009). Those preliminary data further showed that SILv44-transduced T cells respond to HLA-A2+ but not gp100-expressing HLA-A2- targets, again supporting the HLA-A2 restriction of the SILv44 TCR. To compare IFN- γ secretion by primary T cells from different donors, we subcloned the TCR into pQXCIN (Clontech Laboratories, Inc) to make use of the neomycin antibiotic selection marker and obtain a purified population of transgenic T cells before measuring cytokine secretion by ELISAPRO analysis (Mabtech). For T-cell transduction, PBMCs from 3 different donors were activated in culture medium for 3 days. Non-adherent cells were negatively sorted for CD8 (Stem Cell Technologies, Vancouver, Canada) and transduced twice, 1 day apart using the Retronectin-retrovirus method before selection in 1 mg/ml G-418 sulfate for 5 days. Transduced T cells were rapidly expanded using a 200-fold excess of 5000 rad irradiated feeders for 5 days. IFN- γ production by primary T cells from three different donors was compared in the absence and presence of 888A2 melanoma cells added 1:1 and found to be significantly elevated in response to these melanoma targets by a paired Student's t test $*P < 0.05$.

The target antigen for SILv44 is gp100, a glycoprotein responsible for structural integrity of melanosomes, important for melanin deposition and skin pigmentation (Watt et al., 2011). It is among the most immunogenic proteins expressed by melanoma cells, as the majority of T cells infiltrating melanoma tumors are reactive with either gp100 or MART-1 (Kwakami et al., 2000).

As this is the first gp100-reactive TCR identified from an autoimmune patient and notably the first one derived from involved skin, it will be of great interest to learn whether the TCR construct will define host T-cell reactivity in a manner that differs substantially from that of existing TCRs originally derived from patients with melanoma.

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Significance

We describe isolation of melanoma-reactive T cells and functional cloning of the first reported gp100-reactive T-cell receptor from affected tissue of a patient with progressive autoimmune vitiligo. Expression of the TCR in host T cells revealed specific recognition of the cognate peptide, suggesting the TCR construct may be therapeutic toward melanoma.

Figure 1.

VIT1 vitiligo T cells preferentially recognize gp100. VIT1 perilesional T cells were co-incubated 10:1 with (A) HLA-A2 Ff9931 P2 (fibroblasts) pulsed with peptides tyr₃₆₉₋₃₇₇ or (B) gp100₂₀₉₋₂₁₇, or with (C) Mc0006 P23 (melanocytes), showing selective reactivity toward gp100-presenting targets. Arrows: activated T-cell clusters. Skin-derived T cells from this patient with vitiligo were primarily reactive with gp100₂₀₉₋₂₁₇, as was later confirmed by IFN- γ ELISA. Scale bars 70 μ m. (D) Bulk cultured VIT1 vitiligo T cells display enhanced reactivity toward melanoma cells. VIT1 (filled symbols) and TIL1520 (open symbols) T cells were reacted with ⁵¹Cr-loaded 624.38, F003 melanoma cells, gp100-transduced F003, Ff9931 P2 primary fibroblasts, and chromium release was measured after 16 h, showing superior cytotoxicity of VIT1 cells toward gp100-expressing targets at 5:1 as well as 10:1 effector:target ratios. Sorted gp100-loaded HLA-A2 dimer-reactive T cells displayed superior reactivity toward targets as well. The experiment was performed twice with different target cells. (E) Dimers loaded with gp100₂₀₉₋₂₁₇ were used to sort VIT1_{ds} cells and cloned in part by limiting dilution. Reactivity to gp100 was confirmed by ELISPOT analysis and two different VIT1-reactive clones were included in a T-cell avidity assay to measure the amount of peptide required to induce half-maximum IFN γ secretion by each T-cell population. Clonal T cells displayed an avidity that was greater (clone 1) and smaller (clone 2) than the average dimer sorted VIT1_{ds} population.

Figure 2.

Variable regions of the TCR from T-cell clone SILv44 introduced into retroviral backbone SAMEN. (A) 5' RACE and sequencing revealed a TCR α sequence matching V α 8s1 and TCR β sequence matching V β 17s1A1T. Using the international ImMunoGeneTics (IMGT) information system, unique N regions sequences for the CDR3 regions were elucidated as shown. (B) The SAMEN retroviral backbone was used to introduce the SILv44 TCR α and TCR β sequences connected by a P2A self-cleavage linker sequence under the control of a hybrid CMV/MSV promoter in the 5' LTR region. (C) VIT1_{ds}- and VIT1-derived clone AH1 were subjected to TCR V β 17 staining and flow cytometry. Note 31.8% staining among the bulk culture. AH1 may be a sister clone of SILv44, expressing the same T-cell receptor.

Figure 3.

Functional expression of SILv44 in Jurkat cells. (A) Transduction efficiency measured by dextramer and dimer staining. Background APC fluorescence is measured in the middle panels. Note that approximately half of the cells were decamer or dimer reactive, indicating that the TCR α and TCR β subunits were expressed and recombined at the cell surface to recognize cognate peptide presented in the context of HLA-A2. (B) Jurkat cells generate IL-2 in response to gp100 but not tyrosinase peptide presented by T2 cells. Although secretion is low in the absence of added PMA to stimulate Jurkat activation, this experiment confirms functional expression of the SILv44 TCR in Jurkat cells. (C) IFN- γ secretion by T cells from 3 healthy donors transduced to express the SILv44 TCR, measured in response to 888A2 human melanoma cells. Secretion is normalized to and expressed as a percentage of PMA-ionomycin-treated T cells. Significant cytokine secretion in response to melanoma cells was revealed in a paired *t* test.