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Human Plasmacytoid Dendritic Cells Sense Lymphocytic Choriomeningitis Virus-Infected Cells *In Vitro*

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We previously reported that exosomal transfer of hepatitis C virus (HCV) positive-strand RNA from human Huh-7 hepatoma cells to human plasmacytoid dendritic cells (pDCs) triggers pDC alpha/beta interferon (IFN-α/β) production in a Toll-like receptor 7 (TLR7)-dependent, virus-independent manner. Here we show that human pDCs are also activated by a TLR7-dependent, virus-independent, exosomal RNA transfer mechanism by human and mouse hepatoma and nonhepatoma cells that replicate the negative-strand lymphocytic choriomeningitis virus (LCMV).

Interferons (IFNs) are key mediators of the innate immune response to many viruses, including hepatitis C virus (HCV) (1) and the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) (2). Accordingly, HCV and LCMV have evolved mechanisms to block IFN induction in the infected cell (1, 2). Nevertheless, HCV and LCMV infections strongly induce IFN and IFN-stimulated gene (ISG) expression *in vivo* (3–7). Recently, we reported that Huh-7 cells infected with HCV or containing a subgenomic HCV replicon can trigger alpha/beta IFN (IFN-α/β) production *in vitro* by exosomal transfer of positive-strand HCV RNA to cocultured human peripheral blood-derived plasmacytoid dendritic cells (pDCs) in a Toll-like receptor 7 (TLR7)-dependent manner without infecting them (1, 8, 9). Here, we have extended those observations to a negative-strand RNA virus. The broad host cell range of LCMV allowed us to show that human pDCs can be activated by a wide variety of infected human and mouse cell lineages, a process that required cocultivation of pDCs and infected cells but no infection of pDCs.

LCMV is a noncytolytic enveloped virus with a bisegmented negative-strand RNA genome (1, 2, 10, 11). LCMV causes a long-term chronic infection in its natural host, the mouse. Human infections occur through mucosal exposure to aerosols or by direct contact of abraded skin with infectious material (3–7, 11). LCMV infection of humans can result in severe disease that in some cases can be fatal (12). LCMV infection of mice is associated with an initial burst of type I interferon produced in large part by infected dendritic cells (DCs) (7, 13–15). However, LCMV nucleoprotein (NP) efficiently blocks interferon regulatory factor 3 (IRF3) activation and thus IFN production in LCMV-infected cells (16). This might explain why only a small fraction of LCMV-infected dendritic cells produce IFN in the infected mice (7). Interestingly, however, IFN production also occurs in pDCs in the spleen in the absence of active LCMV replication, suggesting that pDCs can sense LCMV infection independently of virus production (7). Thus, in this study we asked if pDCs can sense LCMV-infected cells by a mechanism similar to that described for sensing of HCV-infected cells (8, 9).

Blood was collected from healthy adult human volunteers after informed consent was obtained according to procedures approved by the Scripps Research Institute Human Research Committee. In a first set of experiments, we infected Huh-7.5.1c2 cells, a subclone of the human hepatoma Huh-7 cell line that is highly permissive for HCV infection (17), with LCMV (Armstrong strain) (multiplicity of infection [MOI] = 0.1) 3 days before coculture with human peripheral blood-derived pDCs as described previously (9). The supernatant harvested after 24 h of coculturing LCMV-infected Huh-7.5.1c2 cells (2×10^5) with human pDCs (2×10^4) contained up to 100 ng/ml of IFN-α (Fig. 1A, lane 5). This was ≥10-fold higher than the amount of IFN-α produced by pDCs that had been cocultured with Huh-7.5.1c2 cells infected by the cell culture-adapted HCV JFH-1 D183 variant (9, 18) (Fig. 1A, lane 4), which correlated with the relative intracellular viral RNA levels in the HCV- and LCMV-infected cells (Table 1). Interestingly, similar amounts of IFN-α were produced in pDC cocultures with cells infected with a single-cycle recombinant LCMV (scrLCMVΔGP/GFP [33]) that cannot produce infectious virus (Fig. 1A, lane 6), suggesting that production of LCMV infectious progeny was not required to trigger IFN-α production by the pDCs. Notably, inoculation of human pDCs with a high dose (MOI = 10) of LCMV for 24 h in the absence of Huh-7 cells did not trigger IFN-α production in the pDCs (Fig. 1A, lane 7). Likewise, pDCs did not produce IFN-α after incubation with the cell culture supernatant (Fig. 1A, lane 8) of the LCMV-infected Huh-7.5.1c2 cells used for the coculture shown in lane 5 of Fig. 1A. These results indicated that production of IFN-α by pDCs did not require that they be infected by LCMV. Human pDCs incubated for 3 days with infectious LCMV were negative for LCMV nucleoprotein (NP) expression by fluorescence-activated cell sorter (FACS) analysis (data not shown), indicating that human pDCs are not likely to be productively infected by LCMV *in vitro*. It is noteworthy that pDC IFN-α production levels were equally robust when infected Huh-7.5.1c2 cells or infected parental Huh-7 cells were used (Fig. 1A, lane 10). Importantly, neither Huh-7 cells nor Huh-7.5.1c2 cells produced IFN-α themselves either before or

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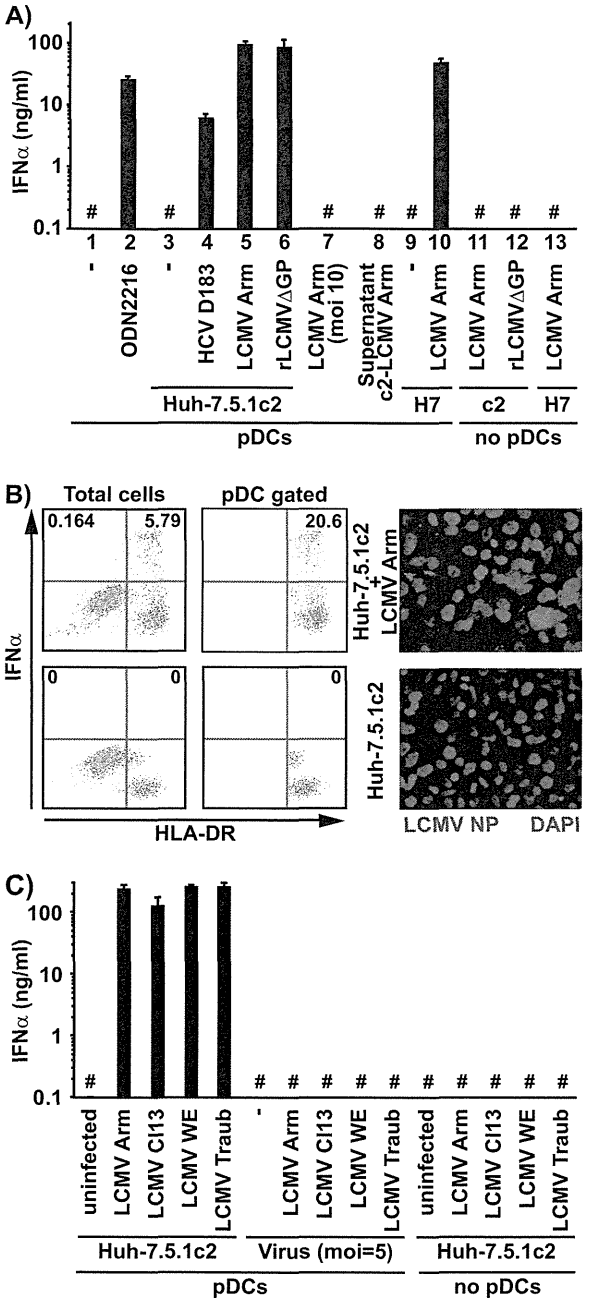

FIG 1 LCMV-infected Huh-7-derived cells trigger IFN-α production in cocultured human peripheral pDCs. (A) Huh-7.5.1c2 or Huh-7 cells (2×10^5) infected with LCMV or HCV D183 (18) at MOI = 0.1 3 days earlier were cocultured with 2×10^4 freshly purified human peripheral pDCs in wells of a 96-well round-bottom plate for 24 h before IFN-α was quantified in the coculture supernatant by enzyme-linked immunosorbent assay (ELISA) as described previously (9). c2, Huh-7.5.1c2 cells; H7, Huh-7 parental cells. (B) Cocultures grown as described for panel A were fixed and analyzed by FACS as described previously (9). All cells are shown in the left panels, and cells gated for HLA-DR and CD123 (pDCs) are shown in the right panels. Huh-7.5.1c2 cells infected with LCMV as described for panel A were analyzed by immunofluorescence as described previously (9) using an anti-NP MAb (1.1.3 [34]) and a secondary Alexa 555-conjugated goat anti-mouse antibody (Invitrogen). (C) IFN-α was quantified in the supernatant of cocultures of Huh-7.5.1c2 cells infected with different LCMV strains (39, 40) with pDCs as described for panel A. #, below the limit of detection of the IFN-α ELISA (36 pg/ml). Error bars represent means ± standard deviations (SD) ($n = 3$).

after LCMV infection (Fig. 1A, lanes 11 to 13), suggesting that IFN-α production reflects activation of the cocultured human pDCs. This was confirmed by FACS analysis of cocultures of human pDCs and LCMV-infected Huh-7.5.1c2 cells (9) after staining for pDC markers HLA-DR (allophycocyanin [APC]-mouse anti-HLA-DR; eBioscience) and CD123 (phycoerythrin [PE]-Cy7-mouse anti-CD123; Biolegend) and intracellular IFN-α (PE-mouse anti-IFN-α; Miltenyi, Auburn, CA). Approximately 20% of the cocultured HLA-DR-positive (HLA-DR^+) pDCs but none of the LCMV-infected HLA-DR^- Huh-7.5.1c2 cells produced IFN-α (Fig. 1B) even though all of the Huh-7.5.1c2 cells were infected, as shown by LCMV NP-specific immunofluorescence (IF) analysis using an anti-NP monoclonal antibody (MAb) (1.1.3 [34]) as described previously (9) (Fig. 1B). Levels of infectious virus-independent pDC activation were similarly robust for all four different strains of LCMV tested (Fig. 1C). Together, these results demonstrate that LCMV-infected human Huh-7-derived hepatoma cells are sensed by human pDCs that respond by producing IFN-α even more strongly than when they are stimulated by HCV JFH1-infected cells (9). The lack of IFN-α production by human pDCs directly exposed to infectious LCMV virions strongly suggested that, in similarity to their response to HCV-infected cells, they likely responded to something other than the virus particles themselves.

Next, we asked whether production of IFN-α by human pDCs in response to coculture with LCMV-infected cells was also related to the exosome-mediated mechanism by which they respond to HCV-infected cells (8, 9). Human pDC activation by LCMV-infected Huh-7.5.1c2 cells was inhibited by the TLR7-specific antagonist IRS661 (Fig. 2A), suggesting that activation of pDCs is mediated by TLR7 as we have previously described for HCV (8, 9) and as has also been observed in the spleen of LCMV-infected mice (15). Likewise, as previously described for HCV (9), human pDC activation by LCMV-infected cells was cell-cell contact dependent since cultivation of LCMV-infected Huh-7.5.1c2 cells and human pDCs in transwell chambers did not result in detectable levels of IFN-α production by the pDCs (Fig. 2B). Next, we performed a series of experiments to determine whether LCMV-infected cell-mediated pDC activation might be exosome dependent. The two structurally unrelated exosome release inhibitors GW4869 (10 μM) and spiroepoxide (20 μM) strongly reduced the ability of LCMV-infected Huh-7.5.1c2 cells to trigger IFN-α production by human pDCs (Fig. 2C) without affecting intracellular LCMV RNA levels in the Huh-7.5.1c2 cells (data not shown). Furthermore, cytochalasin D (0.1 μg/ml), an inhibitor of actin-dependent endocytosis (35) that does not affect LCMV infection (36), completely blocked LCMV-infected cell-mediated IFN-α production by pDCs (Fig. 2C). In control experiments, GW4869, spiroepoxide, and cytochalasin D had little or no effect on TLR7 agonist (resiquimod)-triggered IFN-α production by pDCs (Fig. 2C). These results suggest that exosome release from infected cells and active endocytosis by the pDCs are required for pDC stimulation by LCMV-infected cells. These findings, together with the observation that supernatants of scrLCMVΔGP/GFP-infected cells that do not produce infectious virus nevertheless contain membrane-protected LCMV RNA (data not shown), suggest that LCMV RNA is likely to be transferred to pDCs via exosomes as we have previously described for HCV (8). These findings are consistent with the notion that, in similarity to the situation described for HCV (8, 9), human pDCs sensed LCMV-infected hepatoma

TABLE 1 Cell lines and viruses triggering IFN-α production by pDCs

Cell line	Reference(s)	Species	Cell of origin	Virus	Virus strain/genotype	Infection (log GE/μg RNA)[a]	IFN-α log (ng/ml)[b]
Huh-7.5.1c2	17, 19, 20	Human	Hepatocyte	HCV	JFH1/2a	7	1
				LCMV	Armstrong	8	2
					Cl13	8	2
					WE	8	2
					Traub	8	2
					rLCMVΔGFP	8	2
Huh-7	20, 21	Human	Hepatocyte	HCV	JFH1/2a	7	1
				LCMV	Armstrong	8	2
HepG2	22	Human	Hepatocyte	LCMV	Armstrong	8	2
HepG2.2.15	23	Human	Hepatocyte	HBV	ayw	7	ND[f]
				HBV + Res[c]	ayw/TLR7-agonist	7	1
				HBV + LCMV	ayw/Armstrong	7/8	1–2
Hep3B	22	Human	Hepatocyte	LCMV	Armstrong	8	2
HeLa	24	Human	Cervix	LCMV	Armstrong	8	1
HEK293T	25, 26	Human	Embryonic kidney	LCMV	Armstrong	8	ND
PHH[d]		Human	Liver	LCMV	Armstrong	7	1
AML12	27	Mouse	Hepatocyte	LCMV	Armstrong	8	0
NIH 3T3	28	Mouse	Embryonic fibroblast	LCMV	Armstrong	7	1
CV-1	29	Monkey	Kidney	LCMV	Armstrong	nt[e]	ND
LMH D2	30–32	Chicken	Hepatocyte	DHBV	DHBV3	8	ND
				DHBV + Res[c]	DHBV3/TLR7-agonist	8	1
				DHBV + LCMV	DHBV3/Armstrong	8/8	1

[a] Approximate magnitude of intracellular viral RNA content in log genome equivalents per μg of total cellular RNA (log GE/μg RNA) at the start of the coculture with human pDCs.
[b] Approximate magnitude of IFN-α production [in log(ng/ml)] after 24 h of coculture of the infected cell line with human pDCs.
[c] Cocultures of HepG2.2.15 and LMH D2 cells and human pDCs were simultaneously treated with 50ng/ml of the TLR7 agonist resiquimod (Res).
[d] Freshly isolated primary human hepatocytes (PHH); Life Technologies, Carlsbad, CA.
[e] nt, not tested.
[f] ND, not detected (<36 pg/ml).

cells by a short-range exosome-mediated and TLR7-dependent mechanism.

Unlike HCV, LCMV has a broad host cell range in terms of both type and species, which enabled us to determine if the ability to trigger IFN-α production by human pDCs could be extended to other cell types and species. Coculture of human pDCs with either LCMV-infected human cervical epithelium-derived (HeLa) or human hepatoma-derived (HepG2 and Hep3B) cells triggered strong IFN-α production by the human pDCs (Fig. 3A). In contrast, coculture of human pDCs with LCMV-infected human embryonic kidney 293T cells did not result in production of IFN-α, though 293T cells were infected at levels similar to those of all the other cell lines (Fig. 3B). Importantly, human pDCs did not produce IFN-α when cocultured with the uninfected cell lines or when incubated with supernatants of the LCMV-infected cell lines; neither was it produced by LCMV infection of any of the cell lines examined (Fig. 3A). Next, we determined whether freshly prepared cultures of primary human hepatocytes (PHHs) (Life Technologies, Carlsbad, CA) (1.25×10^5 cells per well in 48-well plates) infected with LCMV would also be capable of triggering IFN-α production by cocultured human blood-derived pDCs. As shown in Fig. 4A, 1.25×10^5 PHHs in a 48-well plate (maintained according to the manufacturer's instructions) infected with LCMV Arm for 3 days (d3) or 4 days (d4) triggered IFN-α production by 1×10^5 cocultured human blood-derived pDCs whereas the uninfected PHHs did not. Importantly, the LCMV-infected PHH cells did not produce IFN-α despite containing high levels of LCMV RNA (Fig. 4B) and despite most of the PHH cells being positive for LCMV NP (Fig. 4C), suggesting that pDCs, rather than PHH cells, were the source of IFN-α in the cocultures (Fig. 4A). These results demonstrated that sensing of virus-infected cells by human pDCs is not restricted to Huh-7-derived cells but also extends to cells of nonhepatic human origin (i.e., HeLa cells), to other human hepatoma-derived cell lines (HepG2 and Hep3B), and, most importantly, to primary human hepatocytes.

Since mice are the natural host of LCMV, we asked if LCMV-infected mouse cell lines would trigger IFN-α production by human pDCs. Both LCMV-infected murine hepatoma (AML12) and fibroblast (NIH 3T3) cells triggered IFN-α production by cocultured human pDCs, while the corresponding uninfected cells did not (Fig. 5A). Furthermore, the LCMV-infected cells themselves did not produce IFN-α, indicating that the pDCs in the cocultures were the source of the IFN-α (Fig. 5A). Interestingly, we observed substantial differences in the magnitude of IFN-α production by pDCs depending on the LCMV-infected cell type used in the coculture, although the levels of LCMV infection were similar in all cell lines tested (Fig. 5B) as determined by LCMV-NP-specific immunofluorescence and reverse transcription-quantitative PCR (RT-qPCR) using LCMV NP-specific primers (LCMV_NP-up [GTTGCGCATTGAAGAGGTCGG] and LCMV_NP-lo [CCAACCACAGAACGGGCAGT]). This suggested that there may be cell type- and species-specific differences in the efficiency of viral RNA transfer to pDCs.

Table 1 shows a complete list of the IFN-α responses shown by human pDCs during coculture with LCMV-infected cells of dif-

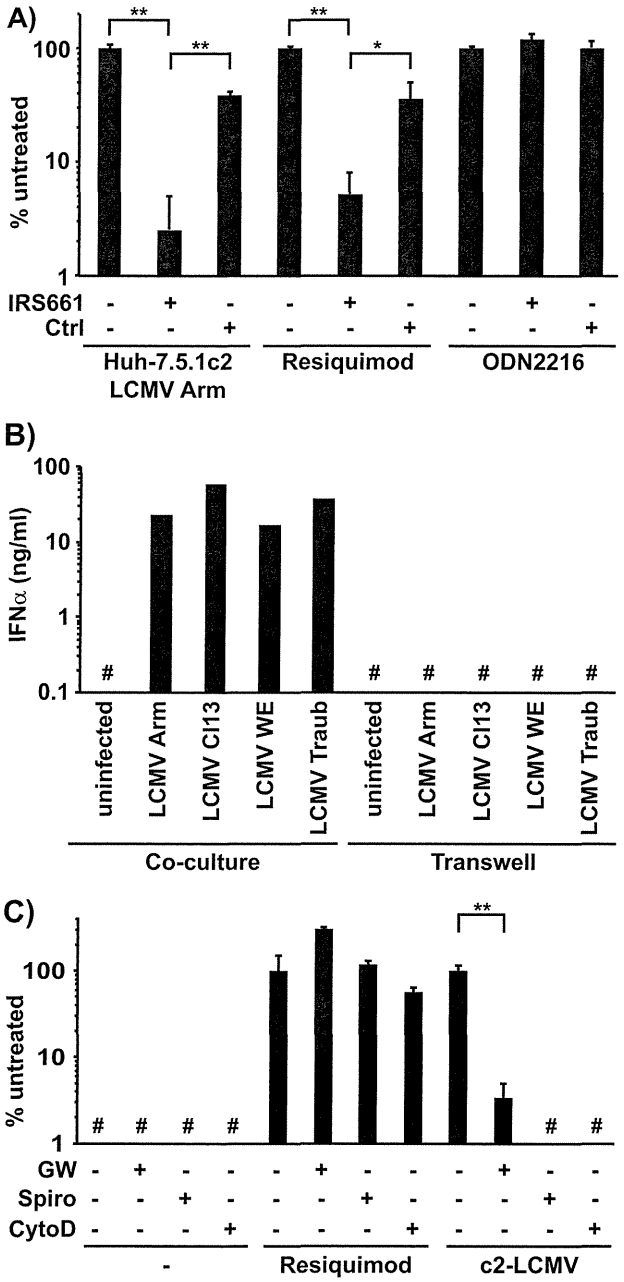

FIG 2 Mechanism of activation of pDCs by LCMV-infected Huh-7.5.1c2 cells. (A) Human peripheral pDCs were cocultured with LCMV Armstrong-infected Huh-7.5.1c2 cells or incubated with a TLR7 (Resiquimod) or TLR9 (ODN2216) ligand and left untreated or treated with a TLR7 antagonist or a control oligonucleotide (Ctrl) exactly as described previously (9). IFN-α production is shown as a percentage of the untreated control level in each group. (B) IFN-α production was quantified by ELISA in the supernatant of cocultures of LCMV-infected Huh-7.5.1c2 cells and pDCs set up exactly as described in the legend to Fig. 1A but either seeded together on top of the membrane of transwell chambers (Coculture) or separated by the membrane (Transwell). Data of individual wells are shown. (C) Human peripheral pDCs (2×10^4) were cocultured with 6.7×10^3 LCMV Armstrong-infected Huh-7.5.1c2 cells or incubated with a TLR7 agonist (Resiquimod) and left untreated or treated with the exosome release inhibitors GW4869 (GW, 10 μM) and spiroepoxide (Spiro, 20 μM) or the endocytosis inhibitor cytochalasin D (CytoD, 0.1 μg/ml) as described previously (8). IFN-α production is shown as a percentage of the untreated control group level. #, below the limit of detection of the IFN-α ELISA (150 pg/ml). Error bars represent means ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (paired Student's t test).

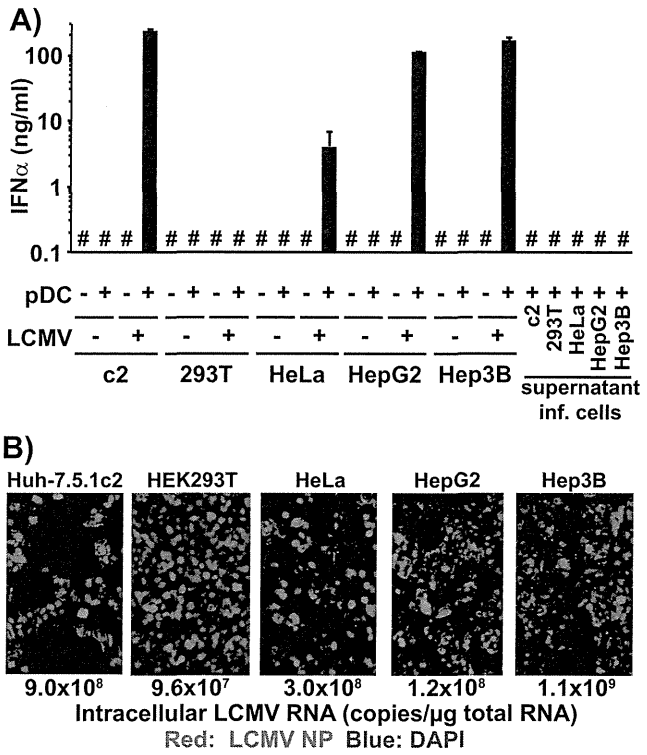

FIG 3 LCMV-infected human cell lines other than Huh-7 trigger IFN-α production in cocultured pDCs. (A) Quantification of IFN-α production in cell culture supernatants of uninfected or LCMV Armstrong-infected cells (LCMV +/−) cocultured or not with human peripheral pDCs (pDC +/−) set up as described for Fig. 1A. Alternatively, 2×10^4 pDCs were incubated with the supernatant of LCMV-infected cells collected 3 days after LCMV inoculation. #, below the limit of detection of the IFN-α ELISA (36 pg/ml); inf, infected. Error bars represent means ± SD ($n = 3$). (B) Analysis of LCMV infection 3 days after LCMV Armstrong inoculation (MOI = 0.1). LCMV-infected cells were visualized by LCMV NP-specific immunofluorescence. Intracellular LCMV RNA levels were determined by LCMV-specific RT-qPCR and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels as described previously (9).

ferent origins and species. Interestingly, human hepatoma cells (HepG2.2.15 [37]) that replicate hepatitis B virus (HBV) and secrete infectious virions did not trigger IFN-α production by pDCs, and the same was true for chicken hepatocyte-derived cells (LMH D2 [38]) that replicate the duck hepatitis B virus (DHBV) (Table 1). For both systems, however, the failure to trigger pDC activation seemed to be virus specific, since superinfection of the same cells with LCMV resulted in strong IFN-α production by human pDCs. Furthermore, pDCs cocultured with HBV- and DHBV-producing HepG2.2.15 and LMH D2 cells were fully able to produce IFN-α in response to the TLR7 agonist resiquimod (Table 1) compared to resiquimod stimulation of human pDCs only (data not shown), suggesting that neither HBV- nor DHBV-infected cells impaired the ability of pDCs to produce IFN-α in response to TLR7 ligation.

While we were unable to detect IFN-α production in *in vitro* cocultures of murine splenic pDCs and LCMV-infected mouse or human cell lines (data not shown), it is well documented that LCMV infection triggers IFN-α production by pDCs *in vivo* in mice (7, 15). Interestingly, it has recently been shown *in vivo* that the majority of IFN-α-producing pDCs in the mouse spleen early

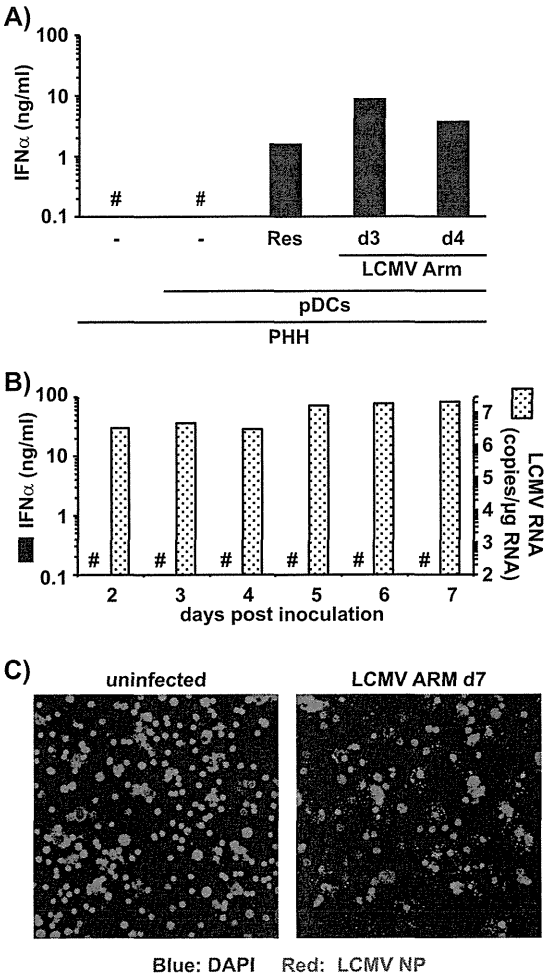

FIG 4 LCMV-infected primary human hepatocytes (PHHs) trigger IFN-α production in cocultured pDCs. (A) Quantification of IFN-α production in cell culture supernatants of uninfected PHHs cocultured (20 h) or not with human peripheral pDCs and cocultures of human pDCs with PHHs infected with LCMV (MOI = 0.1) for 3 days (d3) or 4 days (d4). #, below the limit of detection of the IFN-α ELISA (36 pg/ml); Res, resiquimod. Single wells were analyzed. (B) Analysis of LCMV infection and IFN-α production of PHHs at different time points after LCMV Armstrong inoculation (MOI = 0.1). Intracellular LCMV RNA levels were determined by LCMV-specific RT-qPCR and normalized to GAPDH mRNA levels as described previously (9). #, below the limit of detection of the IFN-α ELISA (36 pg/ml). (C) LCMV-infected PHHs were visualized by LCMV NP-specific immunofluorescence 7 days postinoculation.

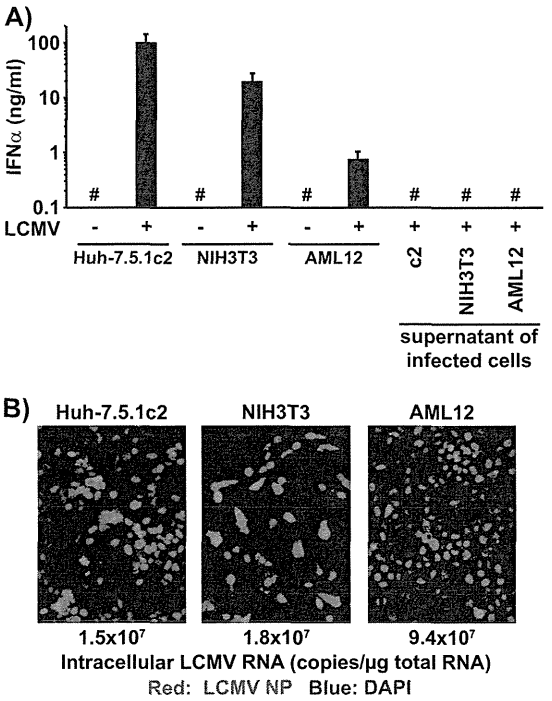

FIG 5 LCMV-infected murine cell lines trigger IFN-α production in cocultured human peripheral pDCs. (A) IFN-α production in cell culture supernatants of uninfected or LCMV Armstrong-infected cells (LCMV +/−) cocultured with human peripheral pDCs (pDC +/−) set up exactly as described for Fig. 1A. Alternatively, pDCs were incubated with the supernatants of LCMV-infected cells collected 3 days after LCMV inoculation and set up exactly as described for Fig. 1A. #, below the limit of detection of the IFN-α ELISA (36 pg/ml). Error bars represent means ± SD (n = 3). (B) Analysis of LCMV infection 3 days after LCMV Armstrong inoculation (MOI = 0.1). LCMV-infected cells were visualized by LCMV NP-specific immunofluorescence. Intracellular LCMV RNA levels were determined by LCMV-specific RT-qPCR and normalized to GAPDH mRNA levels as described previously (9).

after LCMV Cl13 infection are not productively infected and it was suggested that those pDCs might sense infected cells by a mechanism that is independent of intrinsic virus replication in pDCs, e.g., by the sensing of LCMV-infected cells (7). The results reported here might explain how those pDCs sense LCMV infection *in vivo* in the mouse spleen. Together with our previous studies employing analysis of the responsiveness of human pDCs to activation by HCV-infected cells (8, 9), the results presented here support the concept that the ability of noninfected pDCs to direct a strong IFN-α response upon sensing infected cells might be a general mechanism by which the host can circumvent the ability of viruses to block innate signaling in productively infected cells and thus mount efficient innate immune responses that have the potential to control viral infection.

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REFERENCES

1. **Heim MH.** 2013. Innate immunity and HCV. J. Hepatol. **58**:564–574. http://dx.doi.org/10.1002/hep.26227, http://dx.doi.org/10.1016/j.jhep.2012.10.005.
2. **Borrow P, Martínez-Sobrido L, de la Torre JC.** 2010. Inhibition of the type I interferon antiviral response during arenavirus infection. Viruses **2**:2443–2480. http://dx.doi.org/10.3390/v2112443.
3. **Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, Lemon SM, Lanford RE.** 2004. Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. J. Virol. **78**:13779–13792. http://dx.doi.org/10.1128/JVI.78.24.13779-13792.2004.
4. **Bigger CB, Brasky KM, Lanford RE.** 2001. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. J. Virol. **75**:7059–7066. http://dx.doi.org/10.1128/JVI.75.15.7059-7066.2001.
5. **Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV.** 2002. Genomic analysis of the host response to hepatitis C virus infection. Proc. Natl. Acad. Sci. U. S. A. **99**:15669–15674. http://dx.doi.org/10.1073/pnas.202608199.

6. **Lee LN, Burke S, Montoya M, Borrow P.** 2009. Multiple mechanisms contribute to impairment of type 1 interferon production during chronic lymphocytic choriomeningitis virus infection of mice. J. Immunol. **182:** 7178–7189. http://dx.doi.org/10.4049/jimmunol.0802526.
7. **Macal M, Lewis GM, Kunz S, Flavell R, Harker JA, Zuniga EI.** 2012. Plasmacytoid dendritic cells are productively infected and activated through TLR-7 early after arenavirus infection. Cell Host Microbe **11:** 617–630. http://dx.doi.org/10.1016/j.chom.2012.04.017.
8. **Dreux M, Garaigorta U, Boyd B, Décembre E, Chung J, Whitten-Bauer C, Wieland S, Chisari FV.** 2012. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. Cell Host Microbe **12:**558–570. http://dx.doi.org/10.1016/j.chom.2012.08.010.
9. **Takahashi K, Asabe S, Wieland S, Garaigorta U, Gastaminza P, Isogawa M, Chisari FV.** 2010. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. Proc. Natl. Acad. Sci. U. S. A. **107:**7431–7436. http://dx.doi.org/10.1073/pnas.1002301107.
10. **Meyer BJ, de la Torre JC, Southern PJ.** 2002. Arenaviruses: genomic RNAs, transcription, and replication. Curr. Top. Microbiol. Immunol. **262:**139–157. http://dx.doi.org/10.1007/978-3-642-56029-3_6.
11. **Buchmeier MJ, De La Torre JC, Peters CJ.** 2007. Arenaviridae: the viruses and their replication, p 1791–1827. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5 ed. Lippincott Williams & Wilkins, Philadelphia, PA.
12. **Peters CJ.** 2006. Lymphocytic choriomeningitis virus—an old enemy up to new tricks. N. Engl. J. Med. **354:**2208–2211. http://dx.doi.org/10.1056/NEJMp068021.
13. **Merigan TC, Oldstone MB, Welsh RM.** 1977. Interferon production during lymphocytic choriomeningitis virus infection of nude and normal mice. Nature **268:**67–68. http://dx.doi.org/10.1038/268067a0.
14. **Zuniga EI, Liou L-Y, Mack L, Mendoza M, Oldstone MBA.** 2008. Persistent virus infection inhibits type I interferon production by plasmacytoid dendritic cells to facilitate opportunistic infections. Cell Host Microbe **4:**374–386. http://dx.doi.org/10.1016/j.chom.2008.08.016.
15. **Jung A, Kato H, Kumagai Y, Kumar H, Kawai T, Takeuchi O, Akira S.** 2008. Lymphocytoid choriomeningitis virus activates plasmacytoid dendritic cells and induces a cytotoxic T-cell response via MyD88. J. Virol. **82:**196–206. http://dx.doi.org/10.1128/JVI.01640-07.
16. **Martínez-Sobrido L, Zúñiga EI, Rosario D, García-Sastre A, de la Torre JC.** 2006. Inhibition of the type I interferon response by the nucleoprotein of the prototypic arenavirus lymphocytic choriomeningitis virus. J. Virol. **80:**9192–9199. http://dx.doi.org/10.1128/JVI.00555-06.
17. **Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M.** 2007. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature **449:**919–922. http://dx.doi.org/10.1038/nature06205.
18. **Zhong J, Gastaminza P, Chung J, Stamataki Z, Isogawa M, Cheng G, McKeating JA, Chisari FV.** 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. J. Virol. **80:**11082–11093. http://dx.doi.org/10.1128/JVI.01307-;06.
19. **Blight KJ, McKeating JA, Rice CM.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. **76:** 13001–13014. http://dx.doi.org/10.1128/JVI.76.24.13001-13014.2002.
20. **Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J.** 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. **42:**3858–3863.
21. **Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. **102:**9294–9299. http://dx.doi.org/10.1073/pnas.0503596102.
22. **Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB.** 1979. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature **282:**615–616.
23. **Sells MA, Chen ML, Acs G.** 1987. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. U. S. A. **84:**1005–1009. http://dx.doi.org/10.1073/pnas.84.4.1005.
24. **Scherer WF, Syverton JT, Gey GO.** 1953. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix.. J. Exp. Med. **97:**695–710.
25. **DuBridge RB, Tang P, Hsia HC, Leong PM, Miller JH, Calos MP.** 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. **7:**379–387.
26. **Graham FL, Smiley J, Russell WC, Nairn R.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–74. http://dx.doi.org/10.1099/0022-1317-36-1-59.
27. **Wu JC, Merlino G, Fausto N.** 1994. Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor alpha. Proc. Natl. Acad. Sci. U. S. A. **91:**674–678. http://dx.doi.org/10.1073/pnas.91.2.674.
28. **Todaro GJ, Green H.** 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. **17:**299–313. http://dx.doi.org/10.1083/jcb.17.2.299.
29. **Jensen FC, Girardi AJ, Gilden RV, Koprowski H.** 1964. Infection of human and simian tissue cultures with rous sarcoma virus. Proc. Natl. Acad. Sci. U. S. A. **52:**53–59. http://dx.doi.org/10.1073/pnas.52.1.53.
30. **Kawaguchi T, Nomura K, Hirayama Y, Kitagawa T.** 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res. **47:**4460–4464.
31. **Condreay LD, Aldrich CE, Coates L, Mason WS, Wu TT.** 1990. Efficient duck hepatitis B virus production by an avian liver tumor cell line. J. Virol. **64:**3249–3258.
32. **Gong SS, Jensen AD, Chang CJ, Rogler CE.** 1999. Double-stranded linear duck hepatitis B virus (DHBV) stably integrates at a higher frequency than wild-type DHBV in LMH chicken hepatoma cells. J. Virol. **73:**1492–1502.
33. **Rodrigo WW, de la Torre JC, Martínez-Sobrido L.** 2011. Use of single-cycle infectious lymphocytic choriomeningitis virus to study hemorrhagic fever arenaviruses. J. Virol. **85:**1684–1695. http://dx.doi.org/10.1128/JVI.02229-10.
34. **Emonet SF, Garidou L, McGavern DB, de la Torre JC.** 2009. Generation of recombinant lymphocytic choriomeningitis viruses with trisegmented genomes stably expressing two additional genes of interest. Proc. Natl. Acad. Sci. U. S. A. **106:**3473–3478. http://dx.doi.org/10.1073/pnas.0900088106.
35. **Gottlieb TA, Ivanov IE, Adesnik M, Sabatini DD.** 1993. Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells. J. Cell Biol. **120:**695–710. http://dx.doi.org/10.1083/jcb.120.3.695.
36. **Borrow P, Oldstone MB.** 1994. Mechanism of lymphocytic choriomeningitis virus entry into cells. Virology **198:**1–9. http://dx.doi.org/10.1006/viro.1994.1001.
37. **Sells MA, Zelent AZ, Shvartsman M, Acs G.** 1988. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. J. Virol. **62:**2836–2844.
38. **Gong SS, Jensen AD, Wang H, Rogler CE.** 1995. Duck hepatitis B virus integrations in LMH chicken hepatoma cells: identification and characterization of new episomally derived integrations. J. Virol. **69:**8102–8108.
39. **Dutko FJ, Oldstone MB.** 1983. Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. J. Gen. Virol. **64**(Pt 8)**:**1689–1698. http://dx.doi.org/10.1099/0022-1317-64-8-1689.
40. **Ahmed R, Salmi A, Butler LD, Chiller JM, Oldstone MB.** 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. J. Exp. Med. **160:**521–540.

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Retroviral vectors for homologous recombination provide efficient cloning and expression in mammalian cells

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ABSTRACT

Homologous recombination technologies enable high-throughput cloning and the seamless insertion of any DNA fragment into expression vectors. Additionally, retroviral vectors offer a fast and efficient method for transducing and expressing genes in mammalian cells, including lymphocytes. However, homologous recombination cannot be used to insert DNA fragments into retroviral vectors; retroviral vectors contain two homologous regions, the 5′- and 3′-long terminal repeats, between which homologous recombination occurs preferentially. In this study, we have modified a retroviral vector to enable the cloning of DNA fragments through homologous recombination. To this end, we inserted a bacterial selection marker in a region adjacent to the gene insertion site. We used the modified retroviral vector and homologous recombination to clone T-cell receptors (TCRs) from single Epstein Barr virus-specific human T cells in a high-throughput and comprehensive manner and to efficiently evaluate their function by transducing the TCRs into a murine T-cell line through retroviral infection. In conclusion, the modified retroviral vectors, in combination with the homologous recombination method, are powerful tools for the high-throughput cloning of cDNAs and their efficient functional analysis.

1. Introduction

A variety of gene cloning strategies have been established and utilized for the cloning of cDNAs to analyze the biological functions of their protein products. These cloning strategies can be divided into two major categories: the viral and non-viral methods [1,2]. In the viral methods of transducing cDNAs into mammalian cells, retroviral, adenoviral, adeno-associated viral and herpes simplex viral vectors are often used [3]. Retroviral vectors have an advantage in the transduction of cDNAs over the non-viral conventional methods, as they efficiently and stably integrate the cDNAs into the genome of the cells. The integrated cDNA is stably expressed, and the functions of its protein products can then be analyzed. A large repertoire of well-characterized-retroviral vectors has been developed during the last decade and used for the treatment of human diseases [4,5].

Ligation-dependent cloning is most commonly employed to introduce DNA fragments into vectors [6,7]. However, ligation-dependent cloning often requires multiple rounds of enzyme treatments and purification of both the inserts and vectors. Furthermore, the limited number of appropriate restriction enzyme sites for the insertion of the DNA into the vector DNA limits the flexibility of the vectors in constructing recombinant molecules. These processes hamper high-throughput cloning. In contrast, homologous recombination technologies enable the seamless insertion of any DNA fragment into any desired position [8,9]. Although the homologous recombination method offers several advantages for high-throughput cloning, it cannot be applied to vectors that contain homologous sequences within them, as homologous recombination preferentially occurs between those sequences, and the DNA of interest cannot be inserted [10]. Therefore, the homologous recombination method cannot be applied to retroviral vectors because they contain two homologous long terminal repeats (LTR).

In this study, we designed a modified retroviral vector into which PCR-amplified DNA fragments can be selectively and efficiently inserted using homologous recombination. We cloned TCR cDNA prepared from a large number of human single T cells into the retroviral vector using homologous recombination and analyzed the functions of these cDNAs in a high-throughput manner.

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2. Materials and methods

2.1. Vector construction

The pMX-CH vector was constructed from the pMX vector (kindly provided by Dr. Kitamura, University of Tokyo) [2] by inserting the human IgG Cγ1 gene and the *Bacillus subtilis sacB* gene encoding levansucrase. In *Escherichia coli*, the expression of *sacB* in the presence of sucrose is lethal [11]. Briefly, the pCMVtag1 vector (Agilent technology) was modified at the multiple cloning site (MCS), and the *sacB* gene was inserted into the modified MCS (pCMVtag1-SacB) [12]. Constant region of human IgG cDNA was then inserted into the pCMVtag1-SacB vector. The DNA fragment containing the *sacB* and *Cγ* genes was removed from the pCMVtag1-CH vector by digestion with BamHI and NotI and was inserted into the pMX vector to construct the pMX-CH vector. To prepare the pMX-KmAmpR-TCR-Cα or Cβ vector, the ampicillin resistance gene (*AmpR*) from the pMX vector was first substituted with the kanamycin resistance gene (*KmR*) from the pCMVtag1 vector (Promega) (pMX-Km). The *AmpR* gene was amplified from pBR322 (Promega) by PCR and was then inserted into the BstXI site of the pMX-Km vector (pMX-KmAmpR). To prepare the DNA fragment containing the *sacB* gene and the constant region of TCRα (Cα) or TCRβ (Cβ), the Cα and Cβ cDNAs were amplified from human T cells by RT-PCR and were inserted into the NruI and NotI sites (pCMVtag1-Cα and pCMVtag1-Cβ, respectively) [13]. DNA fragments containing the *sacB-Cα* and *sacB-Cβ* genes were prepared from the pCMVtag1-Cα and pCMVtag1-Cβ plasmids and were inserted into the BamHI and NotI sites of the pMX-KmAmpR vector to construct the pMX-KmAmpR-TCR-Cα and Cβ vectors, respectively. Expression vectors for the human CD3γ, CD3δ, CD3ε, ζ and CD8 cDNAs were constructed by linking these cDNAs with the 2A sequence from the foot-and-mouth disease virus, as previously described (2A-hCD3-hCD8 vector) [14]. All human CD3γ, CD3δ, CD3ε, ζ and CD8 cDNAs were purchased from Origene.

2.2. Preparation of human peripheral blood lymphocytes and HLA typing

Human experiments were performed with the approval of the Ethical Committee at the University of Toyama. Informed consent was obtained from all subjects. Peripheral blood lymphocytes (PBLs) were isolated from heparinized blood samples by density gradient centrifugation using Ficoll-Hypaque (Immuno-Biological Laboratories). Screening for HLA-A24 haplotype positivity was performed by staining the PBLs with FITC-conjugated anti-HLA-A24 (MBL). The cells were then analyzed by flow cytometry.

2.3. Cell lines

RPMI 1640 and DMEM media (Wako Pure Chemical) were supplemented with 10% fetal bovine serum (Biowest), 100 μg/ml streptomycin and 100 U/ml penicillin. Human CD8 (hCD8)-expressing TG40 cells [15] were kindly provided by Dr. Ueno (Kumamoto University) with permission from Dr. Saito (Riken) and were maintained in RPMI 1640 medium. The retroviral packaging cell lines, PLAT-E and Phoenix-A, were kindly provided by Dr. Kitamura (University of Tokyo) and by Dr. G. Nolan (Stanford University), respectively, and were maintained in DMEM medium. The 2A-hCD3-hCD8-293T cells were established by transducing the 2A-hCD3-hCD8 vector into HEK293T cells (purchased from ATCC).

2.4. Antibody and MHC tetramer staining

EBV-specific T cells were stained with PE-conjugated HLA-A*2402/peptide tetramers. The amino acid sequences of the HLA-A*2402-restricted EBV peptides are as follows: TYPVLEEMF (BRLF-1 198-206), DYNFVKQLF (BMLF-1 320-328), IYVLVMLVL (LMP2 222-230), RYSIFFDYM (EBNA3A 246-254) and TYSAGIVQI (EBNA3B 217-225). All tetramers were purchased from MBL. The FITC-conjugated anti-human CD8 antibody (MBL), APC-conjugated anti-human CD3ε antibody (TONBO Biosciences), biotin-conjugated anti-murine CD3ε antibody (eBioscience) and APC-conjugated streptavidin (eBioscience) were used for flow cytometry.

2.5. Single-cell RT-PCR

Single-cell RT-PCR of the *TCR* gene from human T cells was performed using the single-cell 5′-RACE method as previously described [16]. The PCR products were analyzed by either direct sequencing or sequencing after subcloning into an expression vector. The TCR repertoire was analyzed using the IMGT/V-Quest tool (http://www.imgt.org/) [17].

2.6. Homologous recombination reaction in the competent cells

Homologous recombination in the competent cells was performed according to the instructions of the manufacturer (GENE BRIDGES). Briefly, competent cells harboring the pRedET expression plasmid were mixed with the pMX-KmAmpR-TCR-Cα or Cβ vector that was linearized by NruI digestion to remove *sacB* gene and the amplified cDNAs encoding TCR-Vα or Vβ, respectively. After transformation of the competent cells with the mixtures, the competent cells were streaked onto LB agar plates containing 4% sucrose and 100 μg/ml ampicillin. After incubation at 37 °C overnight, ampicillin-resistant *E. coli* cells were expanded. Plasmid DNAs were purified, digested with BamHI and NotI, and separated by agarose gel electrophoresis.

2.7. Retroviral transfection

The cDNAs encoding the TCR α or β chain were independently inserted into the pMX-KmAmpR-TCR-Cα or pMX-KmAmpR-TCR-Cβ plasmids, which were then transfected into the retroviral packaging cell line PLAT-E using FuGENE 6 (Roche). The culture supernatant from the transfected PLAT-E cells was collected 72 h after transfection and was added to hCD8-TG40 cells along with polybrene (Sigma–Aldrich). The transfection was monitored by the cell surface expression of murine CD3ε. For the retroviral transduction of the TCR cDNAs into 2A-hCD3-hCD8 293T cells, the recombinant retroviruses were produced using the packaging cell line Phoenix-A and infected with 2A-hCD3-hCD8 293T cells as described above.

3. Results

3.1. Undesirable homologous recombination between the 5′- and 3′-LTRs in the pMX retroviral vector

To insert cDNA fragments into the retroviral vector using homologous recombination, we first constructed a retroviral vector with the human IgG constant region (Cγ) and the *sacB* gene (Fig. 1A). For examining the cDNA insertion into the vector using homologous recombination, we amplified the V_H genes encoding human IgG V_H from single human B cells using the 5′-RACE method. The amplified V_H genes were mixed with the linearized vectors and introduced into the competent cells containing the pRED-ET plasmid [9], which induces homologous recombination in these

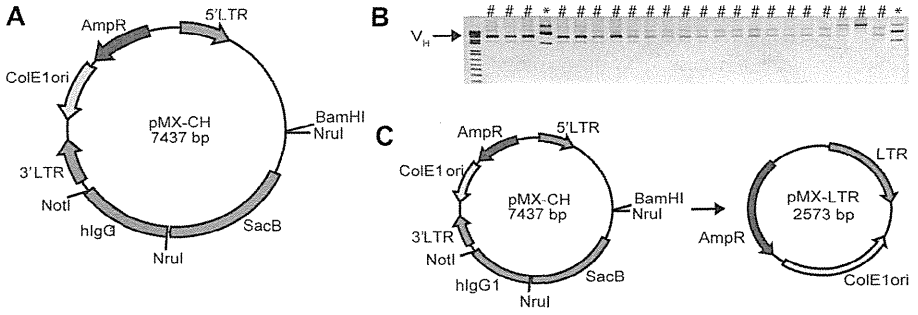

Fig. 1. Undesirable homologous recombination in the conventional retroviral pMX vector. (A) Map of the pMX-CH vector. The pMX-CH vector contains the *sacB* gene and the gene encoding the constant region of human IgG (B) Analysis of the homologous recombination products generated by the pMX-CH vectors. The PCR-amplified variable region of the antibody heavy chain (V_H) from human single B cells was inserted into the pMX-CH vector using homologous recombination. Plasmid DNA was prepared from the transformed colonies, digested with the BamHI and NotI restriction enzymes, and analyzed by agarose gel electrophoresis. The black arrow shows the target V_H gene. The asterisks indicate the pMX-CH vectors with the properly inserted V_H gene. "#" indicates vectors resulting from unsuccessful homologous recombination. (C) Scheme of unsuccessful homologous recombination in the pMX-CH vector. See the sequence analysis in Supplementary Fig. 1. The pMX-LTR results in the pMX- CH vector following unsuccessful homologous recombination.

cells [12]. The transformed cells were cultured on LB plates including ampicillin. Plasmid DNA from the ampicillin-resistant colonies was prepared, and the insertion of the V_H gene was examined by the restriction enzyme digestion. As shown in Fig. 1B, only 8% of the ampicillin-resistant cells contained pMX-Cγ vectors into which the V_H genes were properly inserted, whereas the rest of the colonies did not. Sequence analysis of the plasmid DNA that did not contain the V_H gene revealed that it only contained a single *LTR*, and the sequence between the 5′-LTR and 3′-LTR was deleted (Supplementary Fig. 1). These results demonstrated that homologous recombination preferentially occurred between the 5′- and 3′-LTRs of the pMX-C_H vectors, as illustrated in Fig. 1C.

3.2. Modification of the pMX retroviral vector for the insertion of DNA fragments using homologous recombination

In conventional retroviral vectors, *E. coli* selection markers, such as AmpR or KmR genes, are located adjacent to the *Col-E1* replication origin (*ori*). Consequently, homologous recombination between the 5′- and 3′-LTRs in these retroviral vectors resulted in the production of the vector shown in Supplementary Fig. 1. We assumed that the frequency of homologous recombination between the 5′- and 3′-LTRs was much higher than that of homologous recombination between the retroviral vectors and the target DNA fragments when the recombination reaction was conducted between linearized retroviral vectors and the target DNA fragments because the distance between the 5′- and 3′-LTRs is stochastically much shorter than the distance between the vector and the target DNA fragment.

To specifically select the *E. coli* cells that contained the retroviral vector into which the target DNA was properly inserted, we constructed a retroviral vector in which the *E. coli* selection marker gene was located adjacent to the insertion site of the target DNA fragment by homologous recombination (Fig. 2A). When we inserted the target DNA into the modified retroviral vector using homologous recombination, three possible products may be produced. When homologous recombination occurs between the 5′-LTR and 3′-LTR, the products (i) and (ii) in Fig. 2A will be produced. When homologous recombination properly occurs between the vector and the target DNA fragment, then product (iii) will be produced. Product (i) contains the *ori* but lacks the *AmpR* gene, and the *E. coli* harboring this product fail to grow in medium containing ampicillin. Product (ii) contains the *AmpR* gene but lacks the *ori*, and the *E. coli* harboring this product fail to grow in the medium because the plasmid DNA cannot be replicated. Only the *E. coli* harboring product (iii) can grow in the medium containing ampicillin, as this product contains both the *ori* and the *AmpR* gene. Therefore, we may obtain only the proper product (iii) through homologous recombination. According to this hypothesis, we inserted the *AmpR* gene into the pMX-Km vector, in which the ampicillin resistance gene was replaced with the kanamycin resistance gene at a site between the 3′-LTR and the multiple cloning site (pMX-KmAmpR) (Fig. 2B).

3.3. High-throughput cloning using the modified retroviral vectors with homologous recombination

To confirm the applicability of the vector for cloning by homologous recombination, we inserted the *sacB* gene and the constant region of either human TCRα or TCRβ into the vector (pMX-KmAmpR-TCR α/β) (Fig. 3A). We amplified the V region cDNA of either the TCRα or TCRβ gene from single human $CD8^+$ T cells using the 5′-RACE method (Fig. 3B) and inserted them into the *Nru*I-linearized pMX-KmAmpR-TCR α/β vector using homologous recombination, as described in Section 2. We picked the resultant ampicillin resistant colonies, grew them in medium containing ampicillin and prepared the plasmid DNA. We then digested the plasmid DNA with restriction enzymes to examine the proper insertion of the TCR Vα (TRAV) or Vβ (TRBV) cDNA. Of the ampicillin resistant cells, 100% and 81.3% of the competent cells contained the vectors in which the TRAV- and TRBV-cDNAs were properly inserted, respectively (Fig. 3C). These results showed that the pMX-KmAmpR-TCR α/β vectors enabled the efficient cloning of the TCR gene using homologous recombination.

3.4. Usefulness of the pMX-KmAmpR-TCR α/β vectors for the functional evaluation of the cloned TCR cDNAs in a high-throughput and comprehensive manner

To evaluate the usefulness of the vector for high-throughput cloning, we cloned the TCR genes from Epstein-Barr virus (EBV)-specific $CD8^+$ T cells derived from HLA-$A24^+$ latent healthy donors. We detected the EBV-specific $CD8^+$ T cells using a HLA-A*2402-restricted tetramer mixture of the five EB virus epitopes (BRLF-1, BMLF-1, LMP2, EBNA3A and EBNA3B) and then single-cell sorted the tetramer-positive cells from 10 donors whose frequencies of EBV tetramer-positive cells were more than 0.06% of the $CD8^+$ T-cell population. We amplified 444 pairs of TRAV and TRBV cDNAs from the sorted single cells using the 5′-RACE method and cloned the TCR α and β cDNAs into the pMX-KmAmpR-Cα or pMX-KmAmpR-Cβ vectors, respectively. Next, to determine the antigen-specificity of the cloned TCRs, we retrovirally transferred the

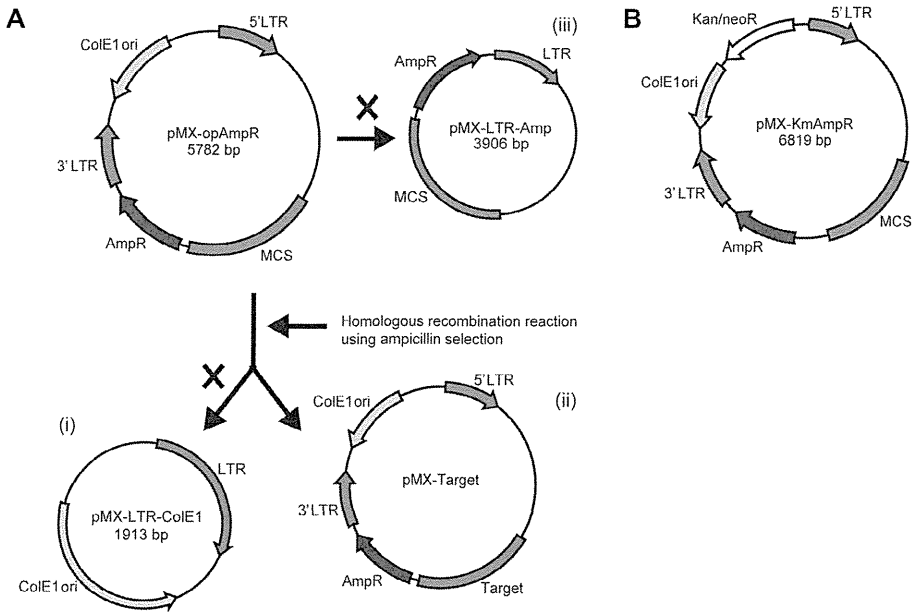

Fig. 2. Schematic illustration of the modified retroviral pMX vector. (A) Schematic illustration of the homologous recombination using the pMX-opAmp vector. (i) and (ii) Products of homologous recombination between the 5′- and 3′-LTRs. (iii) Product of homologous recombination between the target gene and the vector. (B) The pMX-Km vector contains the *AmpR* gene in the opposite position of the Col-E1 ori relative to two LTRs (pMX-KmAmpR).

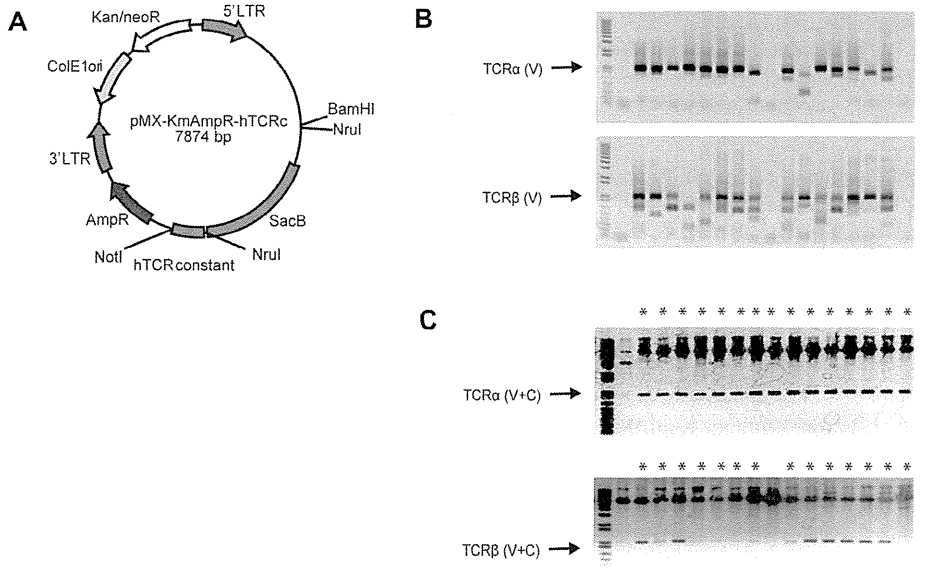

Fig. 3. Cloning of PCR-amplified cDNAs into the modified retroviral pMX vector through homologous recombination. (A) Schematic illustration of the modified pMX-KmAmpR vector for TCR cloning. (B) Amplified products of the variable regions of the TCRα or β genes from single human T cells. The amplified products were analyzed by agarose gel electrophoresis. Black arrow shows the target TCR-Vα (upper) or Vβ (lower). (C) Cloning of the PCR-amplified TCR-Vα or Vβ genes into the pMX-KmAmpR-TCR-Cα/Cβ using homologous recombination. The PCR-amplified TCR-Vα or Vβ were cloned into the pMX-KmAmpR-TCR-Cα or Cβ vectors using homologous recombination, respectively. Plasmid DNA was prepared from ampicillin-resistant colonies, digested with BamHI and NotI and separated by agarose gel electrophoresis. The black arrows show TCRα (upper) or TCRβ (lower), respectively. Asterisks indicate the plasmid that underwent successful homologous recombination.

TCR cDNAs into the TG40 cell line, which does not express endogenous TCR, and stained them using the EBVpep/HLA-A∗2402 tetramer mixture. Ninety-five percent of the TCRs that were expressed on TG40 cells bound the tetramer (data not shown). The analysis of the cloned TCRs showed that the repertoire of the EBV-specific TCRs was highly restricted; in particular, Vβ5 was frequently used with TRBV (Fig. 4A). Furthermore, the number of T cell clones obtained from each donor was inversely correlated with the percentage of tetramer$^+$ CD8$^+$ T cells (Fig. 4B), suggesting that the specific clones were expanded in each donor to regulate EBV latency.

During flow cytometric analysis, we found that some TCRs were hardly expressed on TG40 cells. The TG40 cells expressed only mouse CD3 molecules. We speculated that some human TCRs were difficult to associate with mouse CD3 molecules. To examine this possibility, we transduced two groups of human TCRs into the HEK293T-hCD3 cells that expressed human CD3 molecules: Ones that could be expressed on a large proportion of TG40 cells and ones that were hardly expressed on TG40 cells. Flow cytometric analysis (Fig. 4C) revealed that similar percentages of HEK 293T-hCD3 cells that were transduced with TCRs of each group

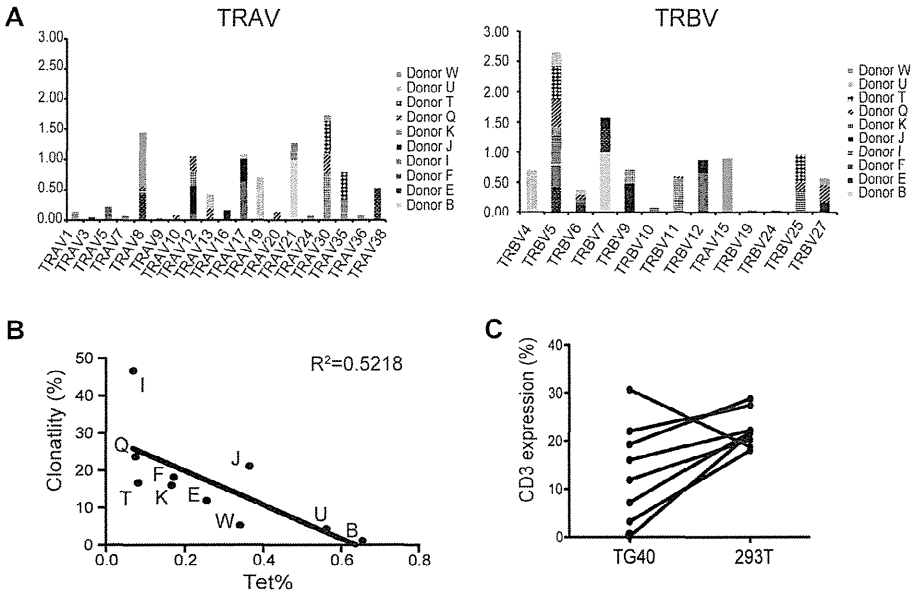

Fig. 4. Functional analysis of the cloned TCRs using the retroviral transduction method. (A) Comprehensive repertoire analysis of the EBV-specific TCRs from 10 latent healthy donors. The V regions of TCRα/β were obtained from single CD8$^+$ T cells that were stained with EBVpep/HLA-A*2402 tetramer mixture and were cloned into the pMX-KmAmpR-TCR-Cα/Cβ vectors using homologous recombination. The EBV-specificity of the cloned TCRα/β pairs was analyzed as described in Section 2. The frequency of the TCR repertoire (%) was calculated using the following formula: Frequency of TCR repertoire (%) = (the number of cloned TCR)/(the number of analyzed T cell clones) × 100. (B) Relationship between the number of cloned TCRs and the percentage of EBV-specific tetramer-positive cells in the CD8$^+$ T cells of 10 latent healthy donors. Clonality (%) was calculated using following formula: Clonality (%) = (the repertoire number)/(the number of analyzed T-cell clones) × 100. "R^{2}" shows the index correlation. The index correlation was calculated using the GraphPad Prism6 software. (C) Comparison of the cell surface expression of retrovirally-transduced TCRs. Representative TCRs of two groups: Ones that could be expressed on a large proportion of TG40 cells and ones that were hardly expressed on TG40 cells were transduced into 2A-hCD3-hCD8-293T cells and the cell surface expression of the TCRs was analyzed.

expressed human CD3 on the cell surface. The results showed that the expression of some human TCR molecules on the cell surface was affected by the species of the CD3 molecules.

4. Discussion

This study was performed to construct a retroviral vector in which the gene of interest can be inserted by homologous recombination and that is suitable for high-throughput cloning. Retroviral vectors contain 5′- and 3′-LTRs of more than 600 base pairs that exhibit sequence homology of more than 99%. Consequently, it is extremely difficult to insert a target gene into a retroviral vector because homologous recombination preferentially occurs between the LTRs rather than between the target gene and the vector [9,10]. To overcome this difficulty, we integrated the DNA replication origin *Col E1 ori* and the *E. coli* selection marker at opposite positions relative to the 5′- and 3′-LTRs. Because both elements are necessary for *E. coli* to grow, only *E. coli* that harbor a retroviral vector containing the properly inserted target DNA fragment can grow in the selection medium.

Using our modified retroviral vectors, we cloned 444 pairs of TRAV and TRBV cDNAs from EBV-specific CD8$^+$ T cells and analyzed their antigen-specificity and function [16]. Numerous studies on the TCR repertoire of antigen-specific T cells have been performed using conventional analysis methods, such as a FACS-based method with a panel of mAbs specific to each TCR β (TRB) V gene family product [18] or PCR-based methods with a panel of TRBV-specific primers [19,20]. Additionally, our group and others have reported a single cell RT-PCR protocol that allows for the simultaneous identification of the CDR3 α and CDR3 β transcripts of TCRs in human [13] and mice [21]. However, these protocols could not retrieve the TCR α/β pairs and analyze their functions, including antigen-specificity. In contrast, the retroviral vector reported here enabled the high-throughput and comprehensive cloning of TCRs using homologous recombination and efficient confirmation of the antigen-specificity of the cloned TCRs through retroviral transduction.

We used TG40 cells that expressed murine CD3 to analyze the human TCRs. It is worth noting that some human TCRs are difficult to express on the cell surface in association with the murine CD3 molecules, whereas they can be efficiently expressed on the cell surface in association with human CD3 molecules. The TCRα/β heterodimer is expressed in association with the CD3γ, CD3δ, CD3ε and ζ molecules [22,23]. A remarkable feature of the transmembrane domains of these receptor components is the presence of nine basic/acidic residues. Mutations of some of these polar residues resulted in a loss of receptor expression at the cell surface, demonstrating that these polar residues are essential for the expression of TCR-CD3 at the cell surface [24,25]. In contrast, our data showed that the V regions of the TCRα/β heterodimer also affected TCR-CD3 expression at the cell surface, at least in the case of the human TCR-murine CD3 complex.

In conclusion, target cDNA can be cloned into our modified retroviral vector using homologous recombination, making this vector a powerful tool for high-throughput cloning and the functional analysis of cDNA products.

5. Conflict of interest

E.K., H.K., T.O., M.H., and A.M., have a patent regarding the modified retroviral vector described in this work.

Acknowledgments

We would like to thank S. Hirota for technical assistance and K. Hata for secretarial work. The retroviral pMX vector and the PLAT-E cell line were kindly provided by T. Kitamura (University of Tokyo). The hCD8-expressing TG40 cell line was kindly provided by T. Ueno and C. Motozono (Kumamoto University), with permission from T. Saito (Riken), and the Phoenix-A cell line was kindly

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.049.

References

[1] H. Okayama, P. Berg, A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells, Mol. Cell. Biol. 3 (1983) 280–289.
[2] T. Kitamura, Y. Koshino, F. Shibata, T. Oki, H. Nakajima, T. Nosaka, H. Kumagai, Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics, Exp. Hematol. 31 (2003) 1007–1014.
[3] M.A. Kay, J.C. Glorioso, L. Naldini, Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics, Nat. Med. 7 (2001) 33–40.
[4] M. Onodera, D.M. Nelson, A. Yachie, G.J. Jagadeesh, B.A. Bunnell, R.A. Morgan, R.M. Blaese, Development of improved adenosine deaminase retroviral vectors, J. Virol. 72 (1998) 1769–1774.
[5] S.H. Kim, S.S. Yu, J.S. Park, P.D. Robbins, C.S. An, S. Kim, Construction of retroviral vectors with improved safety, gene expression, and versatility, J. Virol. 72 (1998) 994–1004.
[6] J. Wrammert, K. Smith, J. Miller, W.A. Langley, K. Kokko, C. Larsen, N.Y. Zheng, I. Mays, L. Garman, C. Helms, J. James, G.M. Air, J.D. Capra, R. Ahmed, P.C. Wilson, Rapid cloning of high-affinity human monoclonal antibodies against influenza virus, Nature 453 (2008) 667–671.
[7] T. Tiller, E. Meffre, S. Yurasov, M. Tsuiji, M.C. Nussenzweig, H. Wardemann, Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning, J. Immunol. Methods 329 (2008) 112–124.
[8] M.D. Hamilton, A.A. Nuara, D.B. Gammon, R.M. Buller, D.H. Evans, Duplex strand joining reactions catalyzed by vaccinia virus DNA polymerase, Nucleic Acids Res. 35 (2007) 143–151.
[9] Y. Zhang, F. Buchholz, J.P. Muyrers, A.F. Stewart, A new logic for DNA engineering using recombination in *Escherichia coli*, Nat. Genet. 20 (1998) 123–128.
[10] Y. Zhang, J.P. Muyrers, G. Testa, A.F. Stewart, DNA cloning by homologous recombination in *Escherichia coli*, Nat. Biotechnol. 18 (2000) 1314–1317.
[11] P. Gay, D. Le Coq, M. Steinmetz, T. Berkelman, C.I. Kado, Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria, J. Bacteriol. 164 (1985) 918–921.
[12] A. Jin, T. Ozawa, K. Tajiri, T. Obata, S. Kondo, K. Kinoshita, S. Kadowaki, K. Takahashi, T. Sugiyama, H. Kishi, A. Muraguchi, A rapid and efficient single-cell manipulation method for screening antigen-specific antibody-secreting cells from human peripheral blood, Nat. Med. 15 (2009) 1088–1092.
[13] T. Ozawa, K. Tajiri, H. Kishi, A. Muraguchi, Comprehensive analysis of the functional TCR repertoire at the single-cell level, Biochem. Biophys. Res. Commun. 367 (2008) 820–825.
[14] A.L. Szymczak, C.J. Workman, Y. Wang, K.M. Vignali, S. Dilioglou, E.F. Vanin, D.A. Vignali, Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector, Nat. Biotechnol. 22 (2004) 589–594.
[15] T. Ueno, H. Tomiyama, M. Fujiwara, S. Oka, M. Takiguchi, Functionally impaired HIV-specific CD8 T cells show high affinity TCR-ligand interactions, J. Immunol. 173 (2004) 5451–5457.
[16] E. Kobayashi, E. Mizukoshi, H. Kishi, T. Ozawa, H. Hamana, T. Nagai, H. Nakagawa, A. Jin, S. Kaneko, A. Muraguchi, A new cloning and expression system yields and validates TCRs from blood lymphocytes of patients with cancer within 10 days, Nat. Med. 19 (2013) 1542–1546.
[17] V. Giudicelli, D. Chaume, M.P. Lefranc, IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis, Nucleic Acids Res. 32 (2004) W435–440.
[18] K. Bieganowska, P. Hollsberg, G.J. Buckle, D.G. Lim, T.F. Greten, J. Schneck, J.D. Altman, S. Jacobson, S.L. Ledis, B. Hanchard, J. Chin, O. Morgan, P.A. Roth, D.A. Hafler, Direct analysis of viral-specific CD8+ T cells with soluble HLA-A2/Ta11–19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy, J. Immunol. 162 (1999) 1765–1771.
[19] M. Saito, G.P. Taylor, A. Saito, Y. Furukawa, K. Usuku, J.N. Weber, M. Osame, C.R. Bangham, In vivo selection of T-cell receptor junctional region sequences by HLA-A2 human T-cell lymphotropic virus type 1 Ta11–19 peptide complexes, J. Virol. 75 (2001) 1065–1071.
[20] N. Eiraku, R. Hingorani, S. Ijichi, K. Machigashira, P.K. Gregersen, J. Monteiro, K. Usuku, S. Yashiki, S. Sonoda, M. Osame, W.W. Hall, Clonal expansion within CD4+ and CD8+ T cell subsets in human T lymphotropic virus type I-infected individuals, J. Immunol. 161 (1998) 6674–6680.
[21] P. Dash, J.L. McClaren, T.H. Oguin 3rd, W. Rothwell, B. Todd, M.Y. Morris, J. Becksfort, C. Reynolds, S.A. Brown, P.C. Doherty, P.G. Thomas, Paired analysis of TCRalpha and TCRbeta chains at the single-cell level in mice, J. Clin. Invest. 121 (2011) 288–295.
[22] R.D. Klausner, J. Lippincott-Schwartz, J.S. Bonifacino, The T cell antigen receptor: insights into organelle biology, Annu. Rev. Cell Biol. 6 (1990) 403–431.
[23] L.P. Kane, J. Lin, A. Weiss, Signal transduction by the TCR for antigen, Curr. Opin. Immunol. 12 (2000) 242–249.
[24] R.S. Blumberg, B. Alarcon, J. Sancho, F.V. McDermott, P. Lopez, J. Breitmeyer, C. Terhorst, Assembly and function of the T cell antigen receptor. Requirement of either the lysine or arginine residues in the transmembrane region of the alpha chain, J. Biol. Chem. 265 (1990) 14036–14043.
[25] T. Rutledge, P. Cosson, N. Manolios, J.S. Bonifacino, R.D. Klausner, Transmembrane helical interactions: zeta chain dimerization and functional association with the T cell antigen receptor, EMBO J. 11 (1992) 3245–3254.

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A novel system for cloning human TCRs

Cutting short the way to TCR-based anticancer therapy

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Keywords: gene therapy, hTEC10, single-cell RT-PCR, T-cell receptor

T-cell receptor (TCR)-based gene immunotherapy has emerged as a promising approach for the treatment of multiple malignancies. We have recently reported an efficient system for the cloning and functional evaluation of TCR-coding cDNAs. This system, which we named hTEC10, allows for the determination of TCR antigen specificity in less than 10 days, and may therefore constitute a fast and powerful platform for the development of new TCR-based anticancer therapies.

T cell receptor (TCR)-based gene immunotherapy is a promising strategy for the treatment of various cancers. Despite its great potential, this approach is still limited to specific tumor-associated antigens (TAAs) and to patients bearing common MHC class I alleles. Generally, characterizing the genes that encode TAA-specific TCRs requires the establishment of TAA-specific T-cell clones, which can take up several months. Furthermore, the screening of large amounts of T-cell clones is laborious. Recently, we have developed a system that allows for the cloning of genes encoding TCR α/β pairs from single TAA-specific T cells and the functional analysis of their antigen-specificity in less than 10 d. We named this system hTEC10, for "human TCR efficient cloning system within 10 days" (**Fig. 1**).[1]

Using hTEC10, we obtained 73 and 126 α fetoprotein (AFP)-specific TCRα/β-coding cDNA pairs from 2 hepatocellular carcinoma patients who had been successfully treated with an AFP-targeting peptide vaccine. The sequencing of the TCR-coding genes revealed that 199 TCRα/β cDNA pairs were categorized into 3 and 4 cDNA clones, respectively. The functional characterization of 7 AFP-specific TCRs identified one (clone 1–14, obtained from a single T-cell clone out of 199 AFP-specific T-cell clones available) that mediated robust cytotoxic effects against target cells pulsed with AFP-derived peptides. This result suggests that T-cell clones bearing high-affinity TAA-specific TCRs are very rare even among the peripheral blood lymphocytes of patients who had been successfully treated by TAA-targeting vaccines. Using conventional methods, minor T-cell populations such as clone 1–14 would be lost during culture, since large T-cell populations would take over and expand preferentially. Thus, our method is suitable for exploring very small populations of antigen-specific T cells that conventional screening methods may overlook, significantly increasing the possibility to obtain optimal TCR-coding cDNAs for TCR-based gene therapy.

Until recently, numerous studies on the TCR repertoire of antigen-specific T cells have been performed by flow cytometry, based on a panel of monoclonal antibodies specific for TCR β variable fragment (TRBV),[2] or by PCR-based methods, using a panel of TRBV-specific primers.[3] These methods characterize the TRBV regions of TCRs at the population level, but fail to provide insights into the TCR α variable fragments (TRAVs) as well as into the TRBV regions at a single-cell level. Thus, so far we have not been able to measure the true extent of the clonal diversity within $CD8^+$ cytotoxic T lymphocyte (CTL) populations isolated from cancer patients. In this context, we and others had reported single-cell RT-PCR protocols that permit the simultaneous characterization of the sequences encoding complementarity-determining region 3 (CDR3) α and β in human[4] and mouse[5] TCRs. However, these protocols cannot identify TCR α/β pairs, confirm their antigen specificity nor examine their ability to promote cytotoxic effector functions. In contrast, the hTEC10 system may provide us with a new way to analyze the TCR repertoire, as it supplies information of both the TCR α and β chains at the single-cell level and can assess their functional profile. In addition, hTEC10 may provide a useful means to assess the efficacy of anticancer vaccination.

For cancer immunotherapy to be efficient, hence resulting in tumor eradication in vivo, cytotoxic T cells expressing a TCR of sufficiently high avidity are required. In this context, Johnson and colleagues selected CTLs that displayed TAA-specific TCRs with sufficient affinity to induce tumor regression among more than 600 different TAA-specific T cells.[6] To obtain T cells with a sufficient avidity to eliminate tumors in vivo, Nauerth and collaborators have recently developed a new assay based on reversible MHC streptamers, allowing for the assessment of the dynamic dissociation

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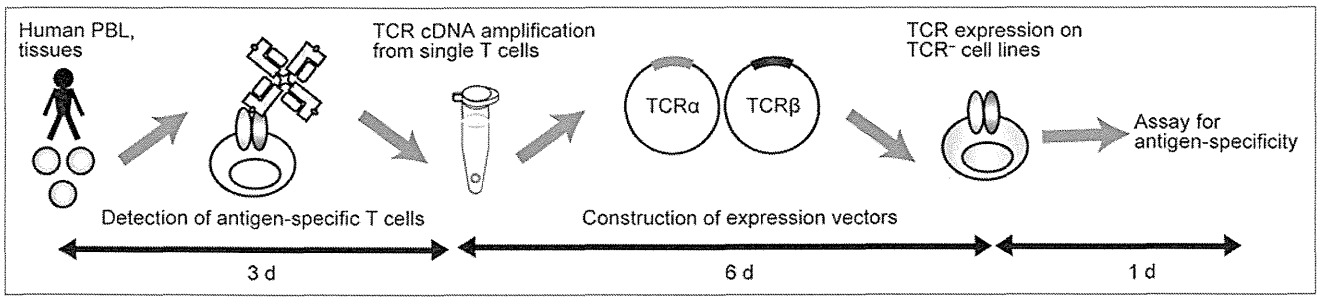


Figure 1. The hTEC10 system. Briefly, the cDNAs coding for human T-cell receptor (TCR) α and β chain are amplified from single T cells and cloned into an expression vector, which is then used to transduce the TCR- T-cell line TG40. The antigen specificity of the TCR can be assessed by staining TCR-expressing TG40 cells with MHC tetramers or by monitoring CD69 expression. Of note, the entire procedure can be performed in less than 10 d. Reproduced with permissions from ref. 1.

(K_{off} rate) of fluorescently labeled, peptide-loaded MHC class I monomers from TCRs expressed on the surface of living T cells.[7] The assay enables a simple, quantitative and reproducible measurement of the K_{off} rate as a reliable indicator of TCR binding avidity. The combination of the hTEC10 system with this new method may provide us with a valuable approach to selectively retrieve high-affinity T-cell clones for TCR-based gene therapy. Another possible strategy to generate high-affinity TCRs is the genetic alteration of TCR-coding genes. Preliminary clinical trials have already demonstrated that genetically enhanced TCRs can indeed confer improved on-target effector functions to T cells. In this scenario, the hTEC10 system might also contribute by supplying several TCR-coding sequences as starting point for genetic engineering.

Concerning the adverse effect of high-avidity TCRs, 2 patients receiving affinity-enhanced melanoma antigen family A3 (MAGEA3)-specific T cells have recently died owing to the cross-reaction of adoptively transferred lymphocytes with a protein expressed in the pulsating cardiac tissue.[8] Furthermore, T cells expressing TCRs whose affinity is much higher than the physiological one have been shown to mediate increased off-target activity at the expenses of on-target effector functions.[9] Thus, methods are needed that allow for refining the affinity/avidity of TCRs to optimal levels, ensuring robust on-target effector functions in the absence of severe side effects.

Finally, in combination with MHC multimer-based staining protocols, the hTEC10 system can detect and retrieve TCR α/β cDNA pairs from $CD8^+$ T cells that secrete specific cytokines or express the activation marker tumor necrosis factor receptor superfamily, member 9 (TNFRSF9, best known as CD137 or 4–1BB) upon stimulation with antigenic peptides. Our findings indicate that the hTEC10 system can be used to isolate T cells from cancer patients without the identification of the corresponding TAAs. Briefly, T cells are stimulated with cancer cells followed by the isolation of interferon γ (IFNγ)-secreting or CD137-expressing $CD8^+$ T cells. Once the TCR-coding sequences of these cells are cloned, their antigen-specificity can be examined by analyzing the response of TCR-transduced T cells against malignant cells. This protocol extends the applicability of the hTEC10 system from the cloning of TCRs with known antigen specificity to the retrieval of TCRs of unknown antigen specificity. In conclusion, the hTEC10 system may provide a fast and powerful approach for the development of novel paradigms of TCR-based gene therapy against cancer.

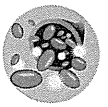
Disclosure of Potential Conflicts of Interest

Authors have applied a patent of hTEC10 system.

References

1. Kobayashi E, Mizukoshi E, Kishi H, Ozawa T, Hamana H, Nagai T, Nakagawa H, Jin A, Kaneko S, Muraguchi A. A new cloning and expression system yields and validates TCRs from blood lymphocytes of patients with cancer within 10 days. Nat Med 2013; 19:1542-6; http://dx.doi.org/10.1038/nm.3358; PMID:24121927
2. Bieganowska K, Höllsberg P, Buckle GJ, Lim DG, Greten TF, Schneck J, Altman JD, Jacobson S, Ledis SL, Hanchard B, et al. Direct analysis of viral-specific CD8+ T cells with soluble HLA-A2/Tax11-19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. J Immunol 1999; 162:1765-71; PMID:9973440
3. Eiraku N, Hingorani R, Ijichi S, Machigashira K, Gregersen PK, Monteiro J, Usuku K, Yashiki S, Sonoda S, Osame M, et al. Clonal expansion within CD4+ and CD8+ T cell subsets in human T lymphotropic virus type I-infected individuals. J Immunol 1998; 161:6674-80; PMID:9862696
4. Ozawa T, Tajiri K, Kishi H, Muraguchi A. Comprehensive analysis of the functional TCR repertoire at the single-cell level. Biochem Biophys Res Commun 2008; 367:820-5; http://dx.doi.org/10.1016/j.bbrc.2008.01.011; PMID:18191637
5. Dash P, McClaren JL, Oguin TH 3rd, Rothwell W, Todd B, Morris MY, Becksfort J, Reynolds C, Brown SA, Doherty PC, et al. Paired analysis of TCRα and TCRβ chains at the single-cell level in mice. J Clin Invest 2011; 121:288-95; http://dx.doi.org/10.1172/JCI44752; PMID:21135507
6. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 2009; 114:535-46; http://dx.doi.org/10.1182/blood-2009-03-211714; PMID:19451549
7. Nauerth M, Weißbrich B, Knall R, Franz T, Dössinger G, Bet J, Paszkiewicz PJ, Pfeifer L, Bunse M, Uckert W, et al. TCR-ligand koff rate correlates with the protective capacity of antigen-specific CD8+ T cells for adoptive transfer. Sci Transl Med 2013; 5:192ra87; http://dx.doi.org/10.1126/scitranslmed.3005958; PMID:23825303
8. Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, Litzky L, Bagg A, Carreno BM, Cimino PJ, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood 2013; 122:863-71; http://dx.doi.org/10.1182/blood-2013-03-490565; PMID:23770775
9. Schmid DA, Irving MB, Posevitz V, Hebeisen M, Posevitz-Fejfar A, Sarria JC, Gomez-Eerland R, Thome M, Schumacher TN, Romero P, et al. Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. J Immunol 2010; 184:4936-46; http://dx.doi.org/10.4049/jimmunol.1000173; PMID:20351194

Asai et al. Journal of Hematology & Oncology 2014, 7:3
http://www.jhoonline.org/content/7/1/3



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LETTER TO THE EDITOR Open Access

Adoptive transfer of genetically engineered WT1-specific cytotoxic T lymphocytes does not induce renal injury

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Abstract

Because WT1 is expressed in leukemia cells, the development of cancer immunotherapy targeting WT1 has been an attractive translational research topic. However, concern of this therapy still remains, since WT1 is abundantly expressed in renal glomerular podocytes. In the present study, we clearly showed that WT1-specific cytotoxic T lymphocytes (CTLs) certainly exerted cytotoxicity against podocytes *in vitro*; however, they did not damage podocytes *in vivo*. This might be due to the anatomical localization of podocytes, being structurally separated from circulating CTLs in glomerular capillaries by an exceptionally thick basement membrane.

Keywords: Immunotherapy, WT1, Podocytes, Cytotoxic T lymphocytes

Findings

Because WT1 is expressed in leukemia cells, including leukemia stem cells, the development of cell-mediated immunotherapy targeting WT1 has been an attractive translational research topic [1,2]. However, concern still remains about adverse events resulting from damage to normal tissues mediated by cytotoxic T lymphocytes (CTLs), since WT1 is also expressed in some lineages of normal cell as well as leukemia cells.

It is well known that WT1 is abundantly expressed in renal glomerular podocytes (or visceral epithelial cells) and that dysfunction of podocytes results in severe renal failure [3]. In addition, it has been recently reported that podocytes have functions of professional antigen-presenting cells [4]. Therefore, it seems important to clarify whether WT1-specific CTLs do not exert cytotoxicity against podocytes. In the present series of experiments, we examined in detail the cytotoxic effect of WT1-specific CTLs against podocytes using *in vitro* and *in vivo* systems.

Methods

WT1-specific and HLA-A*24:02-restricted CTLs were generated by *T-cell receptor* (*TCR*) gene transfer using the novel retrovirus vector [5] into peripheral blood $CD8^+$ T cells, as described previously [6]. We used a mouse podocyte cell line, MPC-5 [7], as the target cells, since there is a high homology between the human and mouse WT1 amino acid sequences, and $WT1_{235-243}$ (CYTWNQMNL), which is the epitope of our WT1-specific CTLs, is completely conserved between the two species. The MPC-5 cells were transfected with the *HLA-A*24:02* gene, as described previously with a slight modification [8]. As shown in Figure 1A, *HLA-A*24:02* gene-transduced mouse podocytes expressed HLA-A24:02 molecules on their surface. We named this cell line MPC-5-A24.

*HLA-A*24:02*-transgenic mice were produced as reported previously [9]. All *in vivo* experiments were approved by the Ehime University animal care committee. As shown in Figure 1B, HLA-A24:02 molecules were expressed in the tissues of these transgenic mice, including glomeruli. HLA-A*24:02-transgenic mice were subsequently injected intravenously with 2.5×10^6 WT1-specific and HLA-A*24:02-restricted CTLs or non-gene-modified $CD8^+$ T cells (control CTLs). As we reported previously

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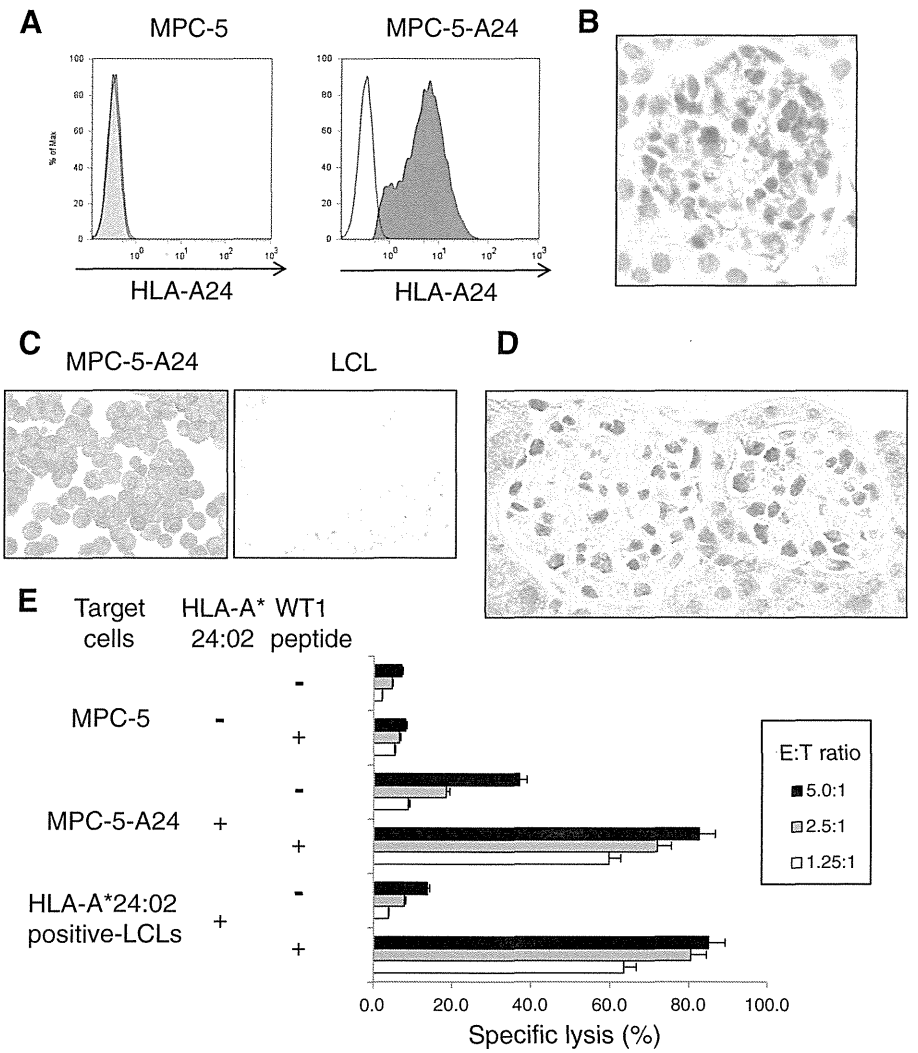

Figure 1 Cytotoxicity of WT1-specific and HLA-A*24:02-restricted CTLs against podocytes. (A) Expression of HLA-A24:02 on the *HLA-A*24:02* gene-transduced mouse podocyte cell line, MPC-5 (MPC-5-A24). Flow cytometric analysis was performed using anti-HLA-A24:02 monoclonal antibody (One Lambda, Canoga Park, CA, USA). **(B)** Expression of HLA class I in the glomerulus of a *HLA-A*24:02*-transgenic mouse (original magnification ×400). Immunohistochemistry was performed using an anti-HLA class I framework monoclonal antibody (Abcam, Cambridge, UK). **(C)** Expression of WT1 in the *HLA-A*24:02* gene-transduced mouse podocyte cell line, MPC-5-A24. MPC-5-A24 cells and LCL were stained with a rabbit anti-human and mouse WT1 polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (original magnification ×400). Notably, WT1 is abundantly expressed in MPC-5-A24 cells but not LCL. **(D)** Expression of WT1 in the glomerulus of a HLA-A*24:02-transgenic mouse (original magnification ×400). Immunohistochemistry was performed using a rabbit anti-human and mouse WT1 polyclonal antibody (Santa Cruz Biotechnology). **(E)** Cytotoxicity of WT1-specific and HLA-A*24:02-restricted CTLs against MPC-5, MPC-5-A24, and HLA-A*24:02-positive LCLs in the presence or absence of WT1 peptide at various effector:target cell ratios.

[6,10], the dose of *TCR* gene-engineered T cells used in the present study is enough to show anti-leukemia effect *in vivo*. Mice that had received WT1-specific CTLs and control CTLs were sacrificed after 7 days, and the presence of tissue damage was examined morphologically. Trafficking of WT1-specific CTLs in *HLA-A*24:02*-transgenic mice was examined using *luciferase* gene-transfected CTLs in a bioluminescence imaging assay as reported previously [10]. Serial acquisition of luciferase photon counts using luciferin was carried out on days 1, 3, and 6 using AEQUORIA (Hamamatsu Photonics, Hamamatsu, Japan), and analyzed using AQUACOSMOS software (Hamamatsu Photonics).

Results

As shown in Figure 1C, WT1 appeared to be abundantly expressed in the *HLA-A*24:02* gene-transduced mouse podocyte cell line, MPC-5-A24. We also confirmed that WT1 was abundantly expressed in podocytes of *HLA-A*24:02*-transgenic mice. (Figure 1D). Figure 1E shows

the cytotoxicity of WT1-specific and HLA-A*24:02-restricted CTLs against various target cells. WT1-specific CTLs showed strong cytotoxicity against $WT1_{235-243}$ peptide-loaded but not -unloaded HLA-A*24:02-positive LCLs. Notably, WT1-specific CTLs apparently exerted cytotoxicity against MPC-5-A24, and their cytotoxicity against $WT1_{235-243}$ peptide-loaded MPC-5-A24 appeared to be higher than that against WT1 peptide-unloaded MPC-5-A24. In contrast, WT1-specific CTLs did not show cytotoxicity against WT1 peptide-loaded or -unloaded MPC-5. These results showed that WT1-specific CTLs can lyse podocytes in an HLA-restricted manner through recognition of the WT1 epitope that is naturally processed from WT1 protein in podocytes and presented on the cell surface in the context of HLA class I molecules.

We monitored in detail the renal function of *HLA-A*24:02*-transgenic mice following transfer of WT1-specific CTLs. Body weight loss and severe proteinuria were not observed in mice that had received WT1-specific CTLs (data not shown). As shown in Figure 2A, lymphocyte infiltration or glomerular injury was not detectable morphologically in WT1-specific CTL-transferred mice. Also, damage of other organs, including pleura, was not detectable (data not shown). Finally, we examined the kinetic distribution of WT1-specific CTLs in HLA-A*24:02-transgenic mice. As shown in Figure 2B, WT1-specific and HLA-A*24:02-restricted CTLs did not accumulate in kidneys.

Discussion

The present *in vitro* and *in vivo* studies clearly showed that WT1-specific CTLs indeed exerted cytotoxicity against renal glomerular podocytes in an HLA-restricted manner; *in vivo*, however, podocytes were able to escape from the cytotoxicity of WT1-specific CTLs. This might be due to the anatomical localization of podocytes, being located outside the capillaries of the glomerulus. Because podocytes are completely separated from capillaries in which CTLs are circulating by a thick glomerular basement membrane which can inhibit the pass of blood

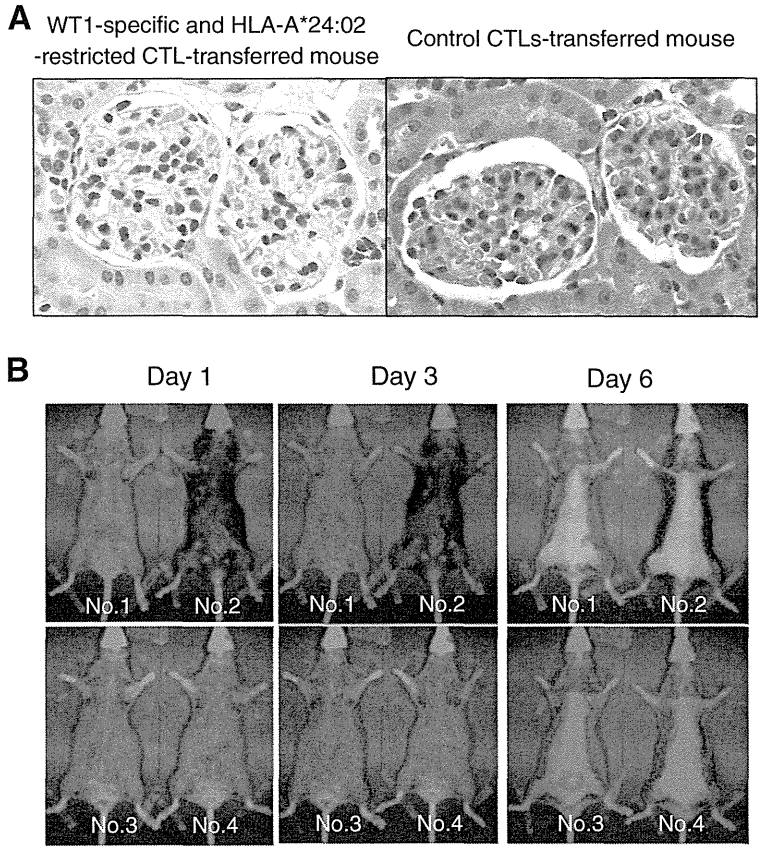

Figure 2 Monitoring of renal damage in *HLA-A*24:02*-transgenic mice following transfer of WT1-specific and HLA-A*24:02-restricted CTLs. (A) Histopathology of the glomeruli of *HLA-A*24:02*-transgenic mice that had received WT1-specific and HLA-A*24-restricted CTLs and control CTLs. (hematoxylin-eosin stain; original magnification x400). Notably, lymphocyte infiltration and tissue damage are not detectable in the glomerulus of the WT1-specific CTL-transferred mouse. **(B)** Trafficking of WT1-specific and HLA-A*24:02-restricted CTLs in *HLA-A*24:02*-transgenic mice. Four mice were transferred with WT1-specific and HLA-A*24:02-restricted CTLs. Notably, CTLs have not accumulated in specific organs, including the kidneys.

cells and even serum protein, CTLs cannot come into contact with podocytes under normal condition. However, in the patients with glomerulonephritis, the permeability of the glomerular basement membrane increases, resulting in proteinuria; therefore, CTLs may infiltrate through the basement membrane and damage podocytes. Therefore, in conclusion, adoptive transfer of WT1-specific CTLs in patients without renal failure is likely safe; however, it should be performed cautiously in patients with proteinuria.

Abbreviations

CTLs: Cytotoxic T lymphocytes; TCR: T-cell receptor.

Competing interests

The authors declare no competing interest.

Authors' contributions

HA, SK, TO, YM, and FO performed experiments and analyzed data. HF designed research and performed experiments. NK, SO, JM, KK, HI, and HS provided materials and discussed the experimental results. YA provided materials and performed experiments. MY designed research, wrote the manuscript, and provided financial support. All authors read and approved the final manuscript.

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References

1. Anguille S, van Tendeloo VF, Berneman ZN: **Leukemia-associated antigens and their relevance to the immunotherapy of acute myeloid leukemia.** *Leukemia* 2012, **26**:2186–2196.
2. Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM, Duerkopp N, Roberts IM, Pogosov GL, Ho WY, Ochsenreither S, Wölfl M, Bar M, Radich JP, Yee C, Greenberg PD: **Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients.** *Sci Transl Med* 2013, **5**:174ra27.
3. Morrison AA, Viney RL, Saleem MA, Ladomery MR: **New insights into the function of the Wilms tumor suppressor gene WT1 in podocytes.** *Am J Physiol Renal Physiol* 2008, **295**:F12–F17.
4. Goldwich A, Burkard M, Olke M, Daniel C, Amann K, Hugo C, Kurts C, Steinkasserer A, Gessner A: **Podocytes are nonhematopoietic professional antigen-presenting cells.** *J Am Soc Nephrol* 2013, **24**:906–916.
5. Okamoto S, Mineno J, Ikeda H, Fujiwara H, Yasukawa M, Shiku H, Kato I: **Improved expression and reactivity of transduced tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR.** *Cancer Res* 2009, **69**:9003–9011.
6. Ochi T, Fujiwara H, Okamoto S, An J, Nagai K, Shirakata T, Mineno J, Kuzushima K, Shiku H, Yasukawa M: **Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety.** *Blood* 2011, **118**:1495–1503.
7. Mundel P, Reiser J, Zúñiga Mejía Borja A, Pavenstädt H, Davidson GR, Kriz W, Zeller R: **Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines.** *Exp Cell Res* 1997, **236**:248–258.
8. Akatsuka Y, Goldberg TA, Kondo E, Martin EG, Obata Y, Morishima Y, Takahashi T, Hansen JA: **Efficient cloning and expression of HLA class I cDNA in human B-lymphoblastoid cell lines.** *Tissue Antigens* 2002, **59**:502–511.
9. Miyahara Y, Naota H, Wang L, Hiasa A, Goto M, Watanabe M, Kitano S, Okumura S, Takemitsu T, Yuta A, Majima Y, Lemonnier FA, Boon T, Shiku H: **Determination of cellularly processed HLA-A2402-restricted novel CTL epitopes derived from two cancer germ line genes, MAGE-A4 and SAGE.** *Clin Cancer Res* 2005, **11**:5581–5589.
10. Miyazaki Y, Fujiwara H, Asai H, Ochi F, Ochi T, Azuma T, Ishida T, Okamoto S, Mineno J, Kuzushima K, Shiku H, Yasukawa M: **Development of a novel redirected T-cell-based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia.** *Blood* 2013, **121**:4894–4901.

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Adoptive transfer of genetically modified Wilms' tumor 1–specific T cells in a novel malignant skull base meningioma model

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Background. Meningiomas are the most commonly diagnosed primary intracranial neoplasms. Despite significant advances in modern therapies, the management of malignant meningioma and skull base meningioma remains a challenge. Thus, the development of new treatment modalities is urgently needed for these difficult-to-treat meningiomas. The goal of this study was to investigate the potential of build-in short interfering RNA-based Wilms' tumor protein (WT1)–targeted adoptive immunotherapy in a reproducible mouse model of malignant skull base meningioma that we recently established.

Methods. We compared *WT1* mRNA expression in human meningioma tissues and gliomas by quantitative real-time reverse-transcription polymerase chain reaction. Human malignant meningioma cells (IOMM-Lee cells) were labeled with green fluorescent protein (GFP) and implanted at the skull base of immunodeficient mice by using the postglenoid foramen injection (PGFi) technique. The animals were sacrificed at specific time points for analysis of tumor formation. Two groups of animals received adoptive immunotherapy with control peripheral blood mononuclear cells (PBMCs) or WT1-targeted PBMCs.

Results. High levels of *WT1* mRNA expression were observed in many meningioma tissues and all meningioma cell lines. IOMM-Lee-GFP cells were successfully implanted using the PGFi technique, and malignant skull base meningiomas were induced in all mice. The systemically delivered WT1-targeted PBMCs infiltrated skull base meningiomas and significantly delayed tumor growth and increased survival time.

Conclusions. We have established a reproducible mouse model of malignant skull base meningioma. WT1-targeted adoptive immunotherapy appears to be a promising approach for the treatment of difficult-to-treat meningiomas.

Keywords: adoptive immunotherapy, cranial nerve, skull base meningioma, Wilms' tumor 1.

Recent advances in T cell immunology and gene transfer have enabled adoptive tumor immunotherapy using genetically engineered T cells in clinical medicine.[1] Among a number of tumor-associated antigens, Wilms' tumor gene product 1 (WT1) is one of the most promising and universal target antigens for tumor immunotherapy. WT1-peptide vaccines have been the most widely evaluated, because they are easy to produce and are well tolerated in clinical trials with minimal toxicity.[2,3] However, whether vaccine therapies induce sufficient amounts of effector cells to kill solid tumors in vivo is an issue that remains to be addressed. In contrast, the adoptive transfer of ex vivo–expanded effector cells could be more advantageous than vaccination, given the greater control of tumor-specific effector cell numbers. Thus, adoptive T cell immunotherapy using WT1-specific T cell receptor (TCR) gene transfer is an alternative direct approach. To increase the effectiveness of TCR gene therapy, we have recently developed a novel vector system that can selectively express target antigen–specific TCR, in

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which expression of endogenous TCR is suppressed by built-in short-interfering RNAs (siRNAs), named as siTCR vector.[4] By using this siTCR vector, we previously generated WT1-specific/HLA-A*2402–restricted T cells with enhanced antitumor cytotoxicity.[4,5]

Meningiomas are the most commonly diagnosed of all primary intracranial neoplasms, constituting ~30% of all primary tumors (Central Brain Tumor Registry of the United States, 2010). Approximately 75% of meningiomas are benign (World Health Organization [WHO] grade I), 20%–35% are atypical (WHO grade II), and 1%–3% are anaplastic/malignant (WHO grade III).[6,7] Despite significant advances in modern therapies, surgical resection remains the treatment of choice for many patients with meningiomas.[8–10] However, some histologically benign meningiomas often recur and become difficult to treat.[11] Moreover, grade II and III meningiomas have high recurrence rates after surgical or radiosurgical management.[12–14] In addition to the intrinsic biology, tumor location is also an important determinant of patient outcome. Skull base is a common site of origin for meningiomas. Complete resection of skull base meningioma is often not possible without a high risk of morbidity and mortality, given its surgical inaccessibility and proximity to vital brain structures, such as the cranial nerves. Cranial nerves are delicate nerves that arise directly from the brain, and meningiomas have a tendency to involve and infiltrate cranial nerves.[15] The management of malignant meningioma and skull base meningioma remains a challenge, and development of new treatment modalities is urgently needed for these difficult-to-treat meningiomas.

In this study, we examined the expression of WT1 antigen in meningioma tissues and found a high level of *WT1* mRNA expression in a majority of the tissues, compared with malignant gliomas. The evidence prompted us to develop adoptive transfer of WT1-specific TCR gene-engineered T cells targeting meningioma cells. In vitro studies revealed that TCR-transduced peripheral blood mononuclear cells (PBMCs) were able to secrete interferon-γ (IFN-γ) and lyse meningioma cells in an HLA-A*2402–restricted manner. To evaluate the efficacy of adoptive transfer of TCR-transduced PBMCs in meningioma in vivo, we developed a clinically relevant skull base model of malignant meningioma encasing the trigeminal nerve using the postglenoid foramen injection (PGFi) technique. To the best of our knowledge, this is the first report to describe the efficacy of adoptive immunotherapy by using genetically modified WT1-specific PBMCs in a meningioma model.

Materials and Methods

PBMCs

Whole blood samples were obtained from healthy donors who gave their informed consent. Whole blood was then diluted with the equal volume of phosphate-buffered saline (PBS) and FICOLL and centrifuged at 1600 rpm for 30 min. The buffy coat with PBMCs was carefully aspired. PBMCs were cultured in GT-T503 (Takara Bio, Shiga, Japan) supplemented with 1% autologous plasma, 0.2% human serum albumin, 2.5 mg/mL fungizone (Bristol-Myers Squibb, Tokyo, Japan), and 600 IU/mL interleukin-2 (IL-2). PBMCs obtained from the same donor and same blood sample were used to generate gene-modified PBMCs (GMCs) and non–gene-modified PBMCs (NGMCs).

Construction of Retroviral Vector and Retroviral Transduction

TCR genes were cloned from the HLA-A*2402–restricted $WT1_{235-243}$–specific $CD8^+$ CTL clone TAK-1.[16–18] Partial codon optimization was performed by replacing the C_α and C_β regions with codon-optimized TCR C_α and C_β regions, respectively.[4] Partially codon-optimized TCR-α and TCR-β genes were integrated into a novel vector encoding small-hairpin RNAs that complementarily bind to the constant regions of endogenous TCR-α and TCR-β genes (WT1-siTCR vector).[4]

PBMCs were stimulated with 30 ng/mL OKT-3 (Janssen Pharmaceutical, Beerse, Belgium) and 600 IU/mL IL-2 and transduced using the RetroNectin-Bound Virus Infection Method, in which retroviral solutions were preloaded onto plates coated with RetroNectin (Takara Bio), centrifuged at 2000 × *g* for 2 h, and rinsed with PBS. The procedure was repeated twice on days 4 and 5 after the initiation of PBMC culture. PBMCs were applied onto the preloaded plate.[4] The transduced PBMCs were cultured for a total of 10 days. Control PBMCs (NGMCs) and TCR-transduced PBMCs (GMCs) were stored frozen in liquid nitrogen, thawed, and cultured in GT-T503 supplemented with 1% autologous plasma, 0.2% human serum albumin, 2.5 mg/mL fungizone, and 600 IU/mL IL-2 for 2 days to use in all the experiments below.

Cell Lines

The human meningioma cell lines IOMM-Lee (HLA-A*2402/0301),[19] HKBMM (HLA-A*2402/1101),[20] and KT21-MG1 (HLA-A*0207/1101)[21] were used. IOMM-Lee was kindly provided by Dr. Anita Lai (University of California at San Francisco, CA), and HKBMM and KT21-MG1 were from Dr. Shinichi Miyatake (Osaka Medical University, Osaka, Japan). The T2A24 cell line was derived from the T2 cell line, which is deficient in TAP transporter proteins, after transfection with the HLA-A*2402 complementary DNA (cDNA). The human embryonic kidney cell line GP2-293 was obtained from the American Type Tissue Culture Collection (ATCC; MD). All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin. Cell lines were grown at 37°C in a humidified atmosphere of 5% carbon dioxide. HLA-A genotyping was performed using polymerase chain reaction (PCR) sequence-based typing (SRL, Tokyo, Japan).