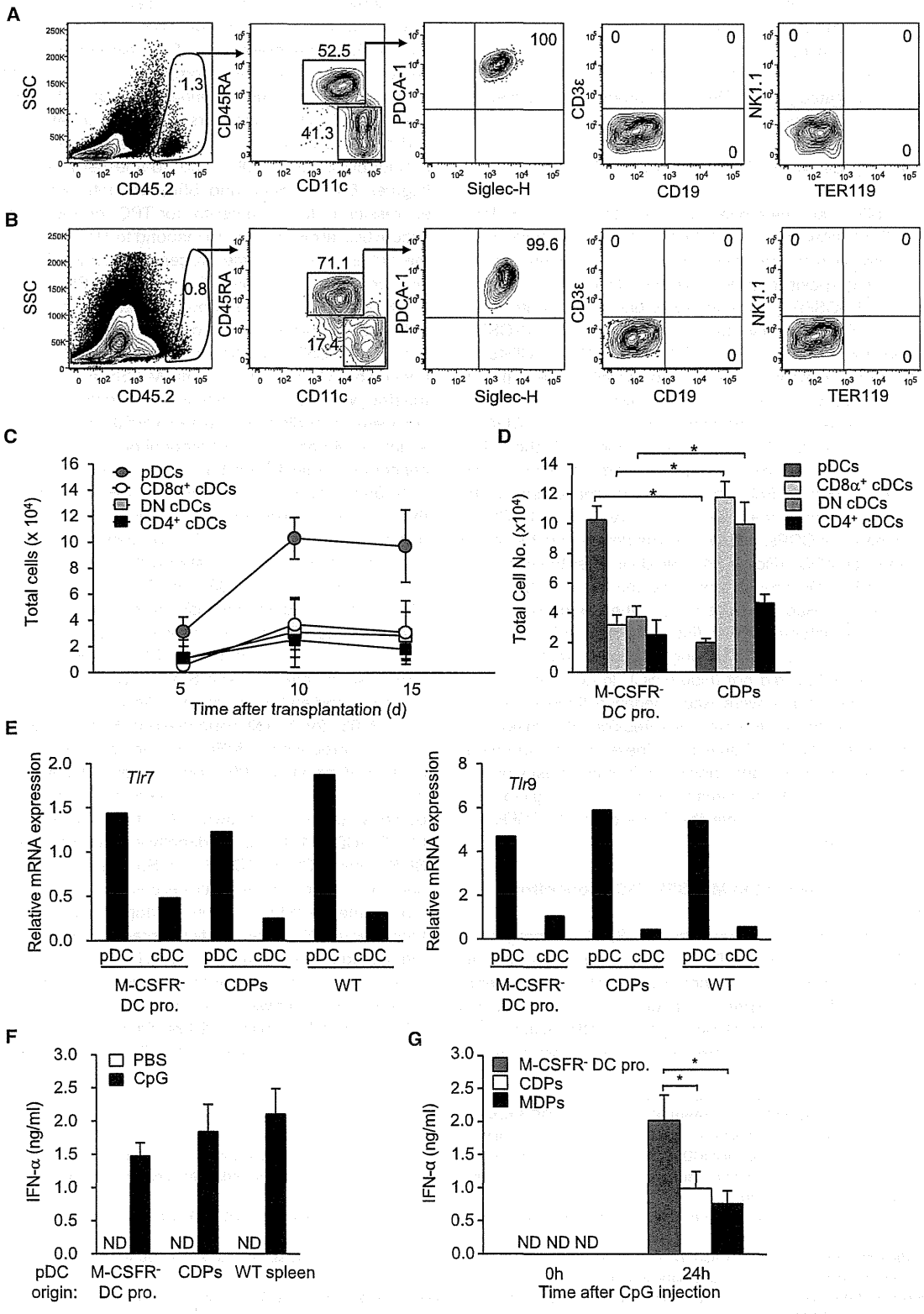

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M-CSFR$^-$ DC progenitors clearly expressed PDCA-1 as early as day 2 (data not shown) and started to express CCR9 on day 2. Its amount had increased on day 4, showing that CCR9$^-$ intermediate precursors give rise to pDCs (Figure S3H). These results collectively indicated that M-CSFR$^-$ DC progenitors have excellent pDC differentiation potential in vivo.

Rag1$^+$ pDCs Are Derived from the M-CSFR$^-$ DC Progenitors

Although pDCs are heterogeneous and 30%–40% of them express Rag1 (Pelayo et al., 2005), CDPs are entirely Rag1 negative, implying the presence of other DC progenitors that generate Rag1-positive pDCs (Reizis, 2010). To examine whether the M-CSFR$^-$ DC progenitors give rise to both Rag1-positive and Rag1-negative pDCs in vivo, 5 × 10^4 M-CSFR$^-$ DC progenitors or CDPs of *Rag1*$^{gfp/+}$ mice (CD45.1$^-$CD45.2$^+$), which express enhanced green fluorescent protein (EGFP) under the endogenous *Rag1* promoter (Kuwata et al., 1999), were transplanted into irradiated B6.SJL mice (CD45.1$^+$ CD45.2$^-$) (Figure 5). Of note, the majority of the CDPs (99.9%) and the M-CSFR$^-$ DC progenitors (97.9%) did not express Rag1 (Figure 5A). Consistent with these results, the D_H-J_H rearrangement was not detectable in the M-CSFR$^-$ DC progenitors or CDPs, although it was detected in these progenitor-derived pDCs (Figure S4). Ten days after the transplantation, M-CSFR$^-$ DC progenitors had given rise to a 2:1 ratio of Rag1-negative to Rag1-positive pDCs, whereas no Rag1-positive cDCs were detected in vivo (Figures 5B and 5F). Both pDC subsets expressed CCR9, although a small fraction of the Rag1-negative pDCs did not (Figure 5C). In contrast, most of the CDP-derived pDCs were Rag1 negative (Figures 5D and 5F) and, regardless of the Rag1 expression, the majority of pDCs expressed CCR9 (Figure 5E). These results suggested that both Rag1-negative and -positive pDCs are mostly derived from the M-CSFR$^-$ DC progenitors in vivo. No Rag1-positive cDCs were derived from either the CDPs or the M-CSFR$^-$ DC progenitors (Figure 5G).

Relationship between the M-CSFR$^-$ DC Progenitors and CDPs

Finally, the relationship between the M-CSFR$^-$ DC progenitors and CDPs was examined. Based on the findings that the M-CSFR$^-$ DC progenitors do not express M-CSFR, whereas MDPs and CDPs do express it (Figure 3A), that Flt3L and M-CSF promote pDC development from CDPs (Onai et al., 2007), and that Flt3L and thrombopoietin (TPO) promote pDC development from human CD34$^+$ cells (Chen et al., 2004), we cultured M-CSFR$^-$ DC progenitors, side by side with CDPs and MDPs, in the presence of Flt3L alone or Flt3L and M-CSF and/or TPO for 8 days. Compared with Flt3L alone, Flt3L together with TPO further enhanced pDC differentiation from the M-CSFR$^-$ DC progenitors, and Flt3L together with M-CSF and/or TPO significantly enhanced the differentiation of pDCs, but not cDCs, from CDPs, and, to a lesser extent, from MDPs (Figures 6A, 6B, S5A, and S5B). Of note, Flt3L induced the expression of Mpl, a receptor for TPO, on the DC progenitors (Figure 6C), allowing them to respond to TPO. Importantly, under this culture condition, the progenitors' *E2-2* mRNA expression was upregulated (Figures 6D, left, and S5C), whereas their M-CSFR expression was downregulated although the amount of M-CSFR downregulation by Flt3L and TPO was partial (Figures 6E and S5D), resulting in the M-CSFR$^-$ DC progenitor-like phenotype. Of note, when both M-CSF and TPO were added together with Flt3L, their enhancing effects on pDC differentiation were not additive. Consistent with these results, 3 days after an intra-BM transfer of DC progenitors, the M-CSFR$^-$ DC progenitors had maintained their surface phenotype, whereas the c-Kit and M-CSFR expression on CDPs and on nearly half of the MDPs was downregulated (Figure S5E). These results suggested that the M-CSFR$^-$ DC progenitors might be derived from CDPs that were stimulated with cytokines that upregulate E2-2 expression, i.e., M-CSF and TPO.

In addition, an upstream Flt3$^+$ progenitor might generate both CDPs and the M-CSFR$^-$ DC progenitors. To test this possibility, we focused on lymphoid-primed MPPs (LMPPs, also known as MPP4), which are Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^+$ and lack megakaryocyte and erythroid potential (Adolfsson et al., 2005; Wilson et al., 2008), for the following reasons. First, megakaryocyte and erythroid progenitors (MEPs) do not have DC developmental potential (Onai et al., 2006). Second, granulocyte and monocyte progenitors (GMPs) showed a very low pDC developmental potential (data not shown). We transplanted CFSE-labeled LMPPs (CD45.1$^-$CD45.2$^+$) directly into the BM of nonirradiated B6.SJL mice (CD45.1$^+$CD45.2$^-$). Soon after the LMPPs divided once, we identified daughter cells showing the surface phenotype of the M-CSFR$^-$ DC progenitors (Figure 7A) that retained their unique DC differentiation potential (Figures 7B and 7C). Interestingly, this was also the case for MDPs and CDPs (Figures 7A–7C). In this context, our data include the previously proposed differentiation pathways, i.e., the differentiation of GMPs and MDPs into CDPs (Liu et al., 2009). On the basis of these findings, we propose a new model for DC development, in which

Figure 4. In Vivo Differentiation Potential of M-CSFR$^-$ DC Progenitors

(A and B) Flow cytometric profile of the spleen (A) and BM (B) 10 days after i.v. transplantation of 5 × 10^4 double-sorted M-CSFR$^-$ DC progenitors from B6 mice (CD45.2$^+$) into irradiated B6.SJL mice (CD45.1$^+$). Progenies were stained for CD11c, CD45RA, PDCA-1, Siglec-H, CD3ε, CD19, NK1.1, and TER119.

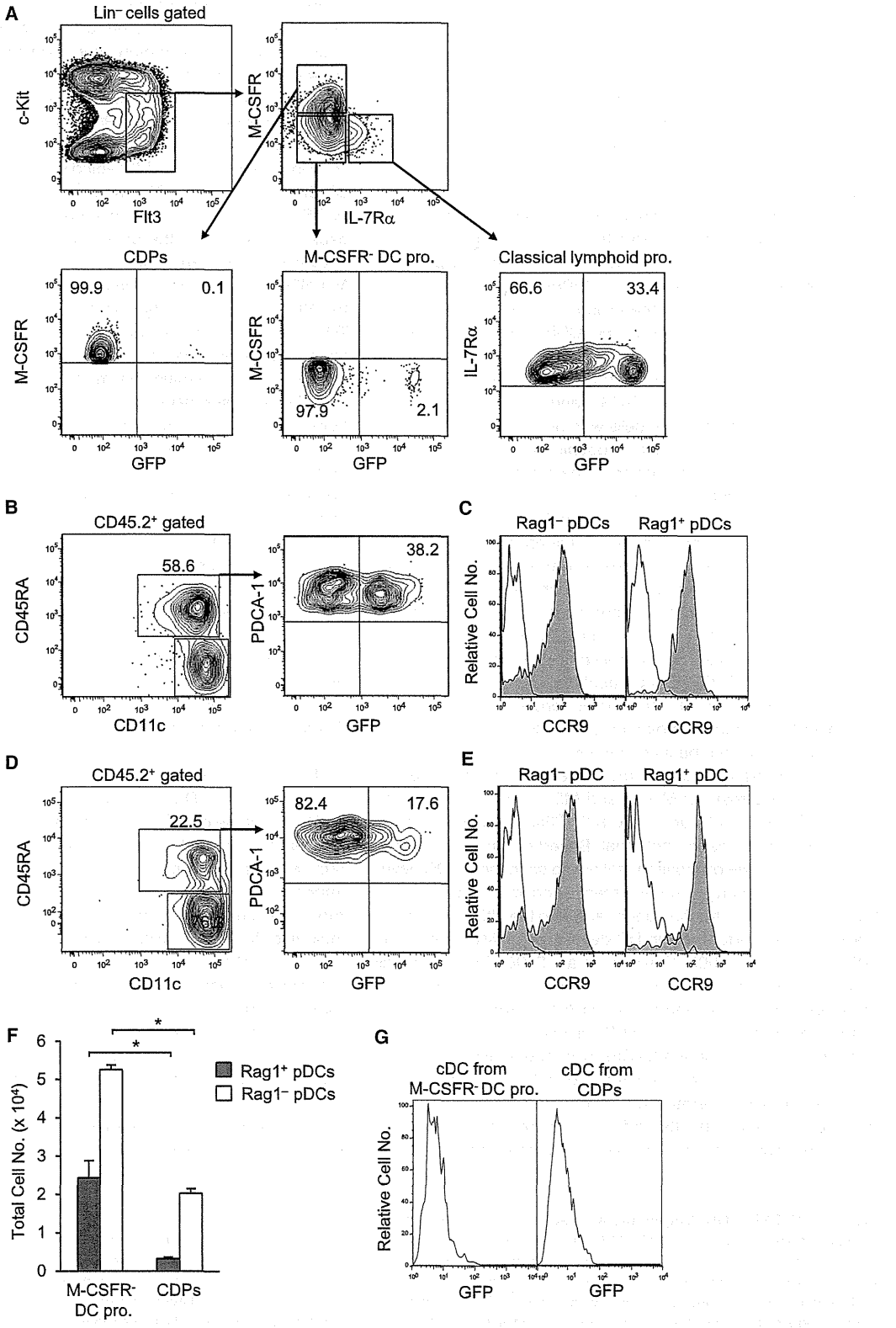
(C and D) Absolute cell numbers of DC subsets in splenic progenies from M-CSFR$^-$ DC progenitors at the indicated time points (C) and from M-CSFR$^-$ DC progenitors or CDPs at day 10 (D).

(E) Splenic pDC and cDC progenies were sorted, and the expression levels of *Tlr7* and *Tlr9* were analyzed by qPCR. Data are representative of two independent experiments.

(F) Ten days after transplantation, M-CSFR$^-$ DC progenitor (pro.)-derived pDCs were sorted from the spleen and stimulated with 1 μM CpG for 24 hr, and the IFN-α concentrations in the culture supernatants were determined by ELISA.

(G) Sorted M-CSFR$^-$ DC progenitor, CDPs, and MDPs were transplanted into lethally irradiated B6.SJL mice with rescue bone marrow. Ten days after transplantation, CpG + DOTAP was intravenously injected, and 24 hr later the serum IFN-α concentration was determined by ELISA. Data are representative of three independent experiments. ND, not detected.

Error bars in (C), (D), (F), and (G) show the mean ± SEM. *$p < 0.01$. See also Figure S3.



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M-CSFR$^-$ DC progenitors are derived from either CDPs or LMPPs (Figure S6).

DISCUSSION

Our findings extend the previous knowledge that DC-committed progenitors are confined to the Lin$^-$Flt3$^+$ fraction (D'Amico and Wu, 2003; Karsunky et al., 2003) to show that DC-committed progenitors belong to the Lin$^-$c-Kit$^{int/lo}$Flt3$^+$IL-7Rα^- fraction and that M-CSFR expression does not determine their presence. Thus, we propose that DC-committed progenitors consist of the M-CSFR$^-$ DC progenitors and CDPs, with the former probably arising downstream of the latter or LMPPs.

One could argue against the proposed relationship between the M-CSFR$^-$ DC progenitors and CDPs. When CDPs were transferred in vivo, they gave rise to large numbers of cDCs and few pDCs, seemingly arguing against the ex vivo findings that CDPs stimulated with M-CSF and/or TPO become the M-CSFR$^-$ DC progenitor-like cells with prominent pDC differentiation potential. In this context, progenitor transfer experiments might be appropriate to demonstrate each progenitor's own DC differentiation potential. However, it is uncertain whether progenitors transferred in vivo migrate to and settle in the "progenitor niche" where they are exposed to the appropriate cytokines and other ligands necessary for the conversion from CDPs to the M-CSFR$^-$ DC progenitors. Therefore, such transfer experiments have some technical limitations and might not demonstrate the physiological fate of the progenitor cells. In addition, our results showed that LMPPs are a possible upstream progenitor for both CDPs and the M-CSFR$^-$ DC progenitors.

Besides these two DC-committed progenitors, other DC-committed progenitor candidates are unlikely to be present in the Lin$^-$Flt3$^+$ fraction, because the Lin$^-$c-Kit$^{hi/+}$Flt3$^+$ population contains short-term HSCs, multipotent progenitors, earlier progenitors upstream of MDPs, and MDPs, and the rest of the Lin$^-$c-Kit$^{int/lo}$Flt3$^+$ cells are Lin$^-$c-Kit$^{int/lo}$Flt3$^+$M-CSFR$^-$IL-7Rα^+, with B cell differentiation potential. Based on previous findings and our results, we propose the following scenario for DC development. DC lineage commitment occurs in the Lin$^-$c-Kit$^{int/lo}$Flt3$^+$IL-7Rα^- fraction upon receiving the Flt3 signal, which plays a nonredundant role in DC development (McKenna et al., 2000; Onai et al., 2006; Waskow et al., 2008). Then, the cells receiving a second signal via M-CSFR or Mpl, a receptor for TPO, upregulate E2-2 and reciprocally downregulate M-CSFR, further committing them to the pDC lineage.

In this context, although M-CSF can induce pDCs and cDCs ex vivo and in vivo independently of Flt3L, the numbers of pDCs and cDCs generated are much reduced compared with the effect of M-CSF in Flt3L-sufficient conditions (Fancke et al., 2008), suggesting that the Flt3 signal is prerequisite for M-CSF to optimally influence DC development. In addition, M-CSF and TPO (and probably even other cytokines) function redundantly to upregulate E2-2. Therefore, mice lacking a single cytokine or its receptor gene may be unlikely to show impaired pDC development. In contrast to the M-CSF-induced downregulation of M-CSFR, the mechanism of TPO-induced M-CSFR downregulation remains unknown. The promoter region of M-CSFR contains an E-box, which is an E2-2-binding site (Ovchinnikov et al., 2010), implying that TPO-induced E2-2 might downregulate M-CSFR. In addition, DNA microarray analysis revealed that, except for M-CSFR, the expression amounts of receptors for known cytokines and ligands were comparable between the M-CSFR$^-$ DC progenitors and CDPs The microenvironments in the BM, i.e., the cells that physiologically secrete M-CSF and TPO, by which CDPs acquire E2-2 and the distinct set of transcriptional gene expressions associated with the pDC developmental potential, remains an important issue to be addressed.

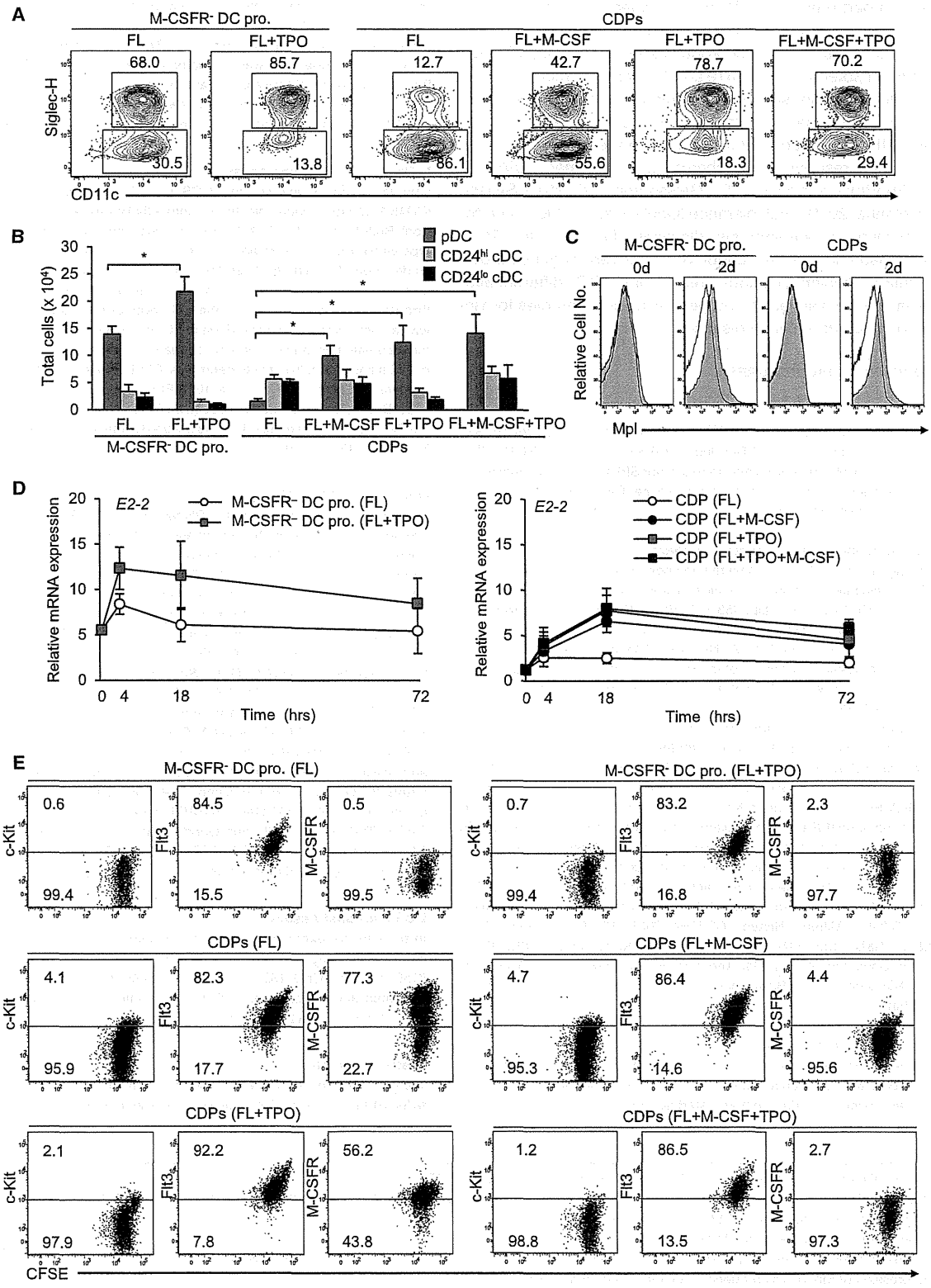
pDCs are heterogenous and can be divided into subpopulations based on the expression of Rag1 and CCR9 (Pelayo et al., 2005; Schlitzer et al., 2011). Because CDPs are uniformly negative for Rag1, the possibility of a partial lymphoid contribution to pDC development or the presence of some other source of pDCs that expresses Rag1 has been suggested (Reizis, 2010). The M-CSFR$^-$ DC progenitors described here gave rise to both Rag1-negative and -positive pDCs in vivo. In addition, consistent with a recent report identifying PDCA-1$^+$CCR9$^-$ cells as a pDC precursor (Schlitzer et al., 2011), the M-CSFR$^-$ DC progenitors gave rise to PDCA-1$^+$CCR9$^+$ pDCs via the PDCA-1$^+$CCR9$^-$ precursor stage. Interestingly, the PDCA-1$^+$CCR9$^-$ cells largely give rise to pDCs in the BM and liver, but they give rise to both pDCs and cDCs in other peripheral lymphoid organs, implying that their DC developmental potential is affected by tissue-derived cytokines that regulate the amounts of *E2-2* and *Id2* expression (Schlitzer et al., 2012).

The M-CSFR$^-$ DC progenitors gave rise to predominantly pDCs and some cDCs, indicating that the identity of the progenitors hasn't been definitively confirmed. In this context, because they express only low amounts of PDCA-1, it is technically impossible to clearly sort PDCA-1$^-$ and PDCA-1^{lo} cells. Furthermore, cell surface staining for more than 20 different markers including M-CSFR, MHC class II, CD11c, CD40, CX$_3$CR1, CD45RA, Ly49Q, CCR9, and Siglec-H and DNA microarray analysis revealed that the M-CSFR$^-$ DC progenitors do not distinctly express other surface markers, suggesting that the ability to identify progenitors with a unique pDC differentiation potential on the basis of surface markers is limited. Instead, because they express the highest levels of *E2-2* but still give rise to some cDCs, it is likely that the M-CSFR$^-$ DC progenitors represent a mixture of cells, the majority of which express *E2-2* and the rest of which express little or no *E2-2* and probably contain

Figure 5. M-CSFR$^-$ DC Progenitors as a Source of pDC Subsets

(A) Flow cytometric analysis of CDPs, M-CSFR$^-$ DC progenitor, and classical lymphoid progenitors (Lin$^-$c-Kit$^{int/lo}$Flt3$^+$M-CSFR$^-$IL-7Rα^+) for Rag1 expression. (B–G) Flow cytometric profile (B–E, G) and absolute numbers (F) of spleen progeny 10 days after i.v. transplantation of 5 × 10^4 sorted M-CSFR$^-$ DC progenitors (B, C) and CDPs (D, E) from *Rag1*$^{gfp/+}$ mice (CD45.2$^+$) into irradiated B6.SJL mice (CD45.1$^+$). The resulting pDCs were further analyzed for GFP (B, D) and CCR9 (C, E) amounts. The resulting cDCs were also analyzed for GFP amounts (G). Shaded histograms show CCR9 expression on Rag1$^-$ (left) and Rag1$^+$ (right) pDCs; open histograms represent results from corresponding isotype controls. Cell numbers are graphed in (F). Data are representative of three independent experiments. Error bars in (F) show the mean ± SEM. *$p < 0.01$.

See also Figure S4.



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at least in part previous CDPs with retained cDC differentiation potential; the former give rise strictly to pDCs, whereas the latter give rise to cDCs. To identify progenitors with a unique pDC differentiation potential will require an *E2-2* reporter mouse. Based on our findings, we suggest redefining CDPs to include both E2-2^{lo} and E2-2^{hi} cells regardless of M-CSFR expression.

Given that pDCs contribute critically to the induction of antiviral immune responses (Banchereau and Steinman, 1998; Liu, 2005; Shortman and Naik, 2007; Geissmann et al., 2010; Swiecki and Colonna, 2010), oral tolerance (Goubier et al., 2008), and the development of autoimmune diseases (Gilliet et al., 2008; Banchereau and Pascual, 2006), our discovery of genuine pDC progenitors, which provides insight into DC differentiation pathways, may also lead to progenitor-based therapies for viral infection and autoimmune disease.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (B6 mice, Clea), B6.*Cx$_3$cr1*$^{gfp/+}$ (Jackson) (Jung et al., 2000), B6.SJL-ptprc^a mice congenic at the CD45 locus (B6.SJL mice), and B6.*Rag1*$^{gfp/+}$ (Kuwata et al., 1999) mice were maintained in our SPF facility. All animal experiments were approved by the Institutional Animal Care Committee of Tokyo Medical and Dental University.

Cell Sorting and Flow Cytometric Analysis

BM lineage negative (Lin$^-$) cells were immunomagnetically pre-enriched with PE-Cy5-conjugated antibodies against lineage antigens including CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD19 (MB19-1), CD11c (N418), MHC class II (I-A and I-E; M5/114.15.2), CD11b (M1/70), Gr-1 (RB6-8C5), TER119 (TER119), NK1.1 (PK136) (all from BioLegend), and anti-Cy5-MicroBeads (Miltenyi Biotec). BM Lin$^-$ cells were then stained with FITC-anti-CD34 (RAM34), PE-anti-Flt3 (A2F10.1), PE-Cy7-anti-Sca-1 (D7), APC-anti-c-Kit (ACK2), Brilliant Violet 421-anti-IL-7Rα (A7P34), and Biotin-anti-M-CSFR (AFS-98) (all from BioLegend). Secondary labeling was performed with Streptavidin-APC-eFluor 780 (eBioscience). M-CSFR$^-$ DC progenitors as well as MDPs, CDPs, and LMPPs were sorted as Lin$^-$c-Kit$^{int/lo}$ Flt3$^+$M-CSFR$^-$IL-7Rα$^-$ cells, Lin$^-$c-Kit$^+$Flt3$^+$M-CSFR$^+$ cells, Lin$^-$c-Kit$^{int/lo}$ Flt3$^+$M-CSFR$^+$ cells, and Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^+$ cells, respectively, on a MoFlo instrument (Beckman Coulter) and analyzed on a FACSCanto II (BD Biosciences) in conjunction with FlowJo software (TreeStar). Antibodies against the following molecules were used for further phenotypic analysis of DC progenitors: MHC class II (I-A and I-E; M5/114.15.2), CD11c (N418), CD40 (1C10), and Siglec-H (eBio440c) (all from eBioscience), PDCA-1 (JF05-1C.2.4.1, Miltenyi Biotec), CD45RA (14.8, BD Biosciences), CCR9 (242503, R&D), Mpl (AMM2, Kyowa Hakko Kirin), and Ly49Q (NS34) (Toyama-Sorimachi et al., 2004). The cells were analyzed on a FACSCalibur or a FACSCanto II (BD Biosciences) in conjunction with FlowJo software (TreeStar).

Limiting-Dilution Analysis

Limiting-dilution assays were performed as described (Onai et al., 2007). Ac6 stromal cells were seeded at a density of 5 × 10^3 cells per well in 96-well flat-bottomed plates 1 day before starting coculture. Lin$^-$c-Kit$^{int/lo}$Flt3$^+$ M-CSFR$^-$IL-7Rα$^-$ cells were sorted and plated on irradiated Ac6 cells at a density of 100, 50, 10, 2, or 1 cells per well. Cells were cultured as described (Onai et al., 2007) and analyzed on day 12. The frequency of pDCs and/or cDCs derived from progenitors was evaluated on a FACSCanto II (BD) via "Loi de Poisson" statistics. Only wells containing more than 128 cells were considered positive. Statistics were calculated based on the mean values of each dilution step; the correlation coefficient for curve extrapolation was $r = 0.9706$.

In Vivo Reconstitution Assays

Fifty thousand double-sorted M-CSFR$^-$ DC progenitors from B6 mice (CD45.1$^-$CD45.2$^+$) were injected i.v. into lethally X-ray-irradiated (9 Gy, Faxitron) B6.SJL mice (CD45.1$^+$CD45.2$^-$). When irradiated, 2 × 10^5 recipient type whole BM cells were added to the injections. Intra-BMT was performed as described (Kushida et al., 2001). Fifty thousand double-sorted DC progenitors from B6 mice were suspended in 10 μl of PBS and carefully injected through a hole in the bone into the BM cavity of nonirradiated B6.SJL mice with a customized Ito microsyringe (Ito Corp.). Mice were killed 10 days after the reconstitution, and their splenic progenies were analyzed for the frequency of cDCs and pDCs. In some experiments, CFSE-labeled 2 × 10^5 M-CSFR$^-$ DC progenitors, CDPs, MDPs, or 1 × 10^5 MPPs were directly injected into the BM (intra-BMT) of nonirradiated B6.SJL mice. Three days after the transplantation, the BM progenies were resorted and cultured in the presence of irradiated Ac6, Flt3L, and TPO for 8 days.

Quantitative RT-PCR

Total RNA was extracted with the RNeasy Mini Kit (QIAGEN), and cDNA was synthesized with random hexamers and SuperScript III reverse transcriptase. For real-time PCR, cDNA products equivalent to the RNAs from 500 cells were amplified with a LightCycler480 SYBR Green I Master (Roche Diagnostics). The data were normalized to the amount of *gapdh* RNA expression in each sample. The primers used for real-time PCR were as follows: *E2-2* sense, 5′-TGAGATCAAATCCGACGA-3′ and antisense, 5′-CGTTATTGCTAGATCTTGACCT-3′; *Irf8* sense, 5′-AAGGGCGTGTTCGTGAAG-3′ and antisense, 5′-GGTGGCGTAGAATTGCTG-3′; *Sfpi1* sense, 5′-ATGCACGTCCTCGATACTC-3′ and antisense, 5′-TCTCACCCTCCTCCTCATCT-3′; *Spib* sense 5′-CACTCCCAAACTGTTCAGC-3′ and antisense, 5′-TGGGGTACGGAGCATAAG-3′; *Stat3* sense, 5′-TGGGTGGAAAAGGACATCAG-3′ and antisense, 5′-GGAATGTGGGGGTAGAGGTA-37; *Gfi1* sense 5′-CAAGAAGGCGCACAGCTA-3′ and antisense 5′-GGGCTCCATTTTGGACTC-3′; *Batf3* sense, 5′-AGACCCAGAAGGCTGACAA-3′ and antisense, 5′-CTGCACAAAGTTCATAGGACAC-3′; *Id2* sense, 5′-CATGAACGACTGCTACTCCAA-3′ and antisense, 5′-GTGATGCAGGCTGACGATAGT-3′; and *Gapdh* sense, 5′-TCCACCACCCTGTTGCTGTA-3′ and antisense, 5′-ACCACAGTCCATGCCATCAC-3′. Primers were synthesized by Operon Biotechnologies.

DC Functional Assays

In some in vivo experiments, CpG (D-19, 5 μg) in 50 μl HBSS was mixed with 30 μl DOTAP reagent (Roche) and 20 μl HBSS for 15 min at room temperature (CpG+DOTAP). CpG+DOTAP was injected into B6.SJL mice that were lethally irradiated and transplanted with DC progenitors and the serum IFN-α was evaluated by ELISA 24 hr after the injection.

Statistical Analysis

We evaluated the statistical significance of the obtained values by the two-tailed Student's t test. A p value of <0.05 was considered significant.

Figure 6. Relationship between the M-CSFR$^-$ DC Progenitors and CDPs

Sorted M-CSFR$^-$ DC progenitors and CDPs (2 × 10^4) were cultured in the presence of hFlt3L-Ig (FL) (100 ng/ml), hFlt3L-Ig + M-CSF (20 ng/ml), hFlt3L-Ig + human TPO (20 ng/ml), or hFlt3L-Ig + M-CSF (20 ng/ml) + hTPO (20 ng/ml).

(A) Flow cytometric profiles of the DC subsets.

(B) Absolute numbers of the pDC and cDC subpopulations (CD24^{hi} cDCs and CD24^{lo} cDCs) on day 8 of culture.

(C) Expression of Mpl on DC progenitors cultured with human Flt3L-Ig for 2 days.

(D) Relative *E2-2* mRNA expression in cytokine-stimulated DC progenitors at the indicated time points during culture.

(E) DC progenitors were labeled with CFSE and cultured as indicated for 2 days.

Data are representative of three independent experiments. Error bars in (B) and (D) show the mean ± SEM. *p < 0.01. See also Figure S5.

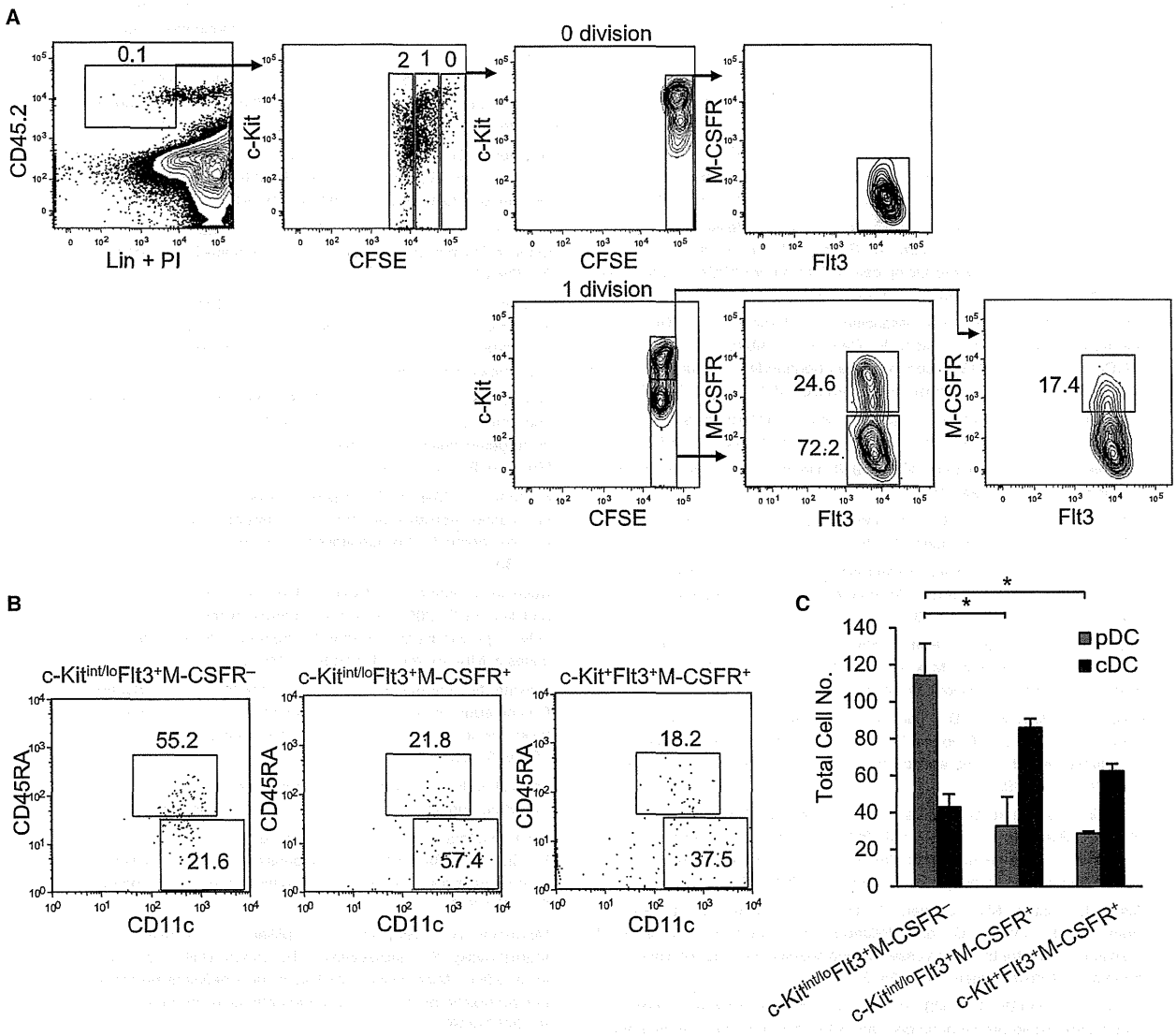

Figure 7. LMPPs Give Rise to M-CSFR⁻ DC Progenitors, CDPs, and MDPs In Vivo

Sorted and CFSE-labeled LMPPs (1×10^5 cells) were transplanted directly into the BM (intra-BMT) of nonirradiated B6.SJL mice.

(A) Representative flow cytometric profiles of BM progenies 3 days after intra-BMT are shown.

(B) Representative flow cytometric profiles of the progenies of sorted CFSE⁺LMPP-derived c-Kit$^{int/lo}$Flt3⁺M-CSFR⁻ cells, c-Kit$^{int/lo}$Flt3⁺M-CSFR⁺ cells, and c-Kit⁺Flt3⁺M-CSFR⁺ cells and were cultured in irradiated stromal cells Ac6 with Flt3-ligand and TPO for 8 days.

(C) Absolute numbers of pDC and cDC derived from different progenitors are shown. Error bars show the means ± SEM (n = 4 from two independent experiments). *$p < 0.01$.

See also Figure S6.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.04.006.

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Multiple therapeutic peptide vaccines consisting of combined novel cancer testis antigens and anti-angiogenic peptides for patients with non-small cell lung cancer

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Abstract

Background: Vaccine treatment using multiple peptides derived from multiple proteins is considered to be a promising option for cancer immune therapy, but scientific evidence supporting the therapeutic efficacy of multiple peptides is limited.

Methods: We conducted phase I trials using a mixture of multiple therapeutic peptide vaccines to evaluate their safety, immunogenicity and clinical response in patients with advanced/recurrent NSCLC. We administered two different combinations of four HLA-A24-restricted peptides. Two were peptides derived from vascular endothelial growth factor receptor 1 (VEGFR1) and 2 (VEGFR2), and the third was a peptide derived from up-regulated lung cancer 10 (URLC10, which is also called lymphocyte antigen 6 complex locus K [LY6K]). The fourth peptide used was derived from TTK protein kinase (TTK) or cell division associated 1 (CDCA1). Vaccines were administered weekly by subcutaneous injection into the axillary region of patients with montanide ISA-51 incomplete Freund's adjuvant, until the disease was judged to have progressed or patients requested to be withdrawn from the trial. Immunological responses were primarily evaluated using an IFN-gamma ELiSPOT assay.

Results: Vaccinations were well tolerated with no severe treatment-associated adverse events except for the reactions that occurred at the injection sites. Peptide-specific T cell responses against at least one peptide were observed in 13 of the 15 patients enrolled. Although no patient exhibited complete or partial responses, seven patients (47%) had stable disease for at least 2 months. The median overall survival time was 398 days, and the 1- and 2-year survival rates were 58.3% and 32.8%, respectively.

Conclusion: Peptide vaccine therapy using a mixture of four novel peptides was found to be safe, and is expected to induce strong specific T cell responses.

Trial registration: These studies were registered with ClinicalTrials.gov NCT00633724 and NCT00874588.

Keywords: Cancer vaccine, Multiple peptides, Prognosis, Non-small cell lung cancer

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Background

Lung cancer is the leading cause of cancer death in the world [1]. Despite the recent development of novel treatment modalities for patients with non-small cell lung cancer (NSCLC), survival rates are still unsatisfactory [2]. Furthermore, although molecular-targeted drugs are expected to cause fewer serious adverse events associated with the use of cytotoxic chemotherapeutic agents, but still cause some [3,4]. Therefore, the development of more effective and less toxic therapeutic modalities is eagerly awaited. In this regard, cancer immunotherapy is considered to be a promising option with minimum toxicity, but its effectiveness has not yet been proven to be superior to the presently available treatments. However, several ongoing clinical trials that are administering vaccines, such as MAGE-A3 or BLP25 for lung cancer as an adjuvant treatment or in a maintenance setting after standard chemotherapy, seem to be very promising [5,6]. Although these lung cancer trials have involved the administration of a single vaccine, a combination of multiple peptide vaccines has also been used in several types of solid cancer [7,8].

We have previously identified novel cancer-testis antigens, including up-regulated lung cancer 10 (URLC10; also called lymphocyte antigen 6 complex locus K [LY6K]) [9], TTK protein kinase (TTK) [10] and the cell division cycle associated gene 1 (CDCA1) [11], that were found to be expressed at very high levels in lung cancer using the genome-wide cDNA microarray method. We have also previously reported peptide vaccines that target VEGFR1 [12] and VEGFR2 [13]. To induce a higher level of cytotoxic T lymphocytes (CTLs), also known as cytotoxic T cells, that have direct cancer cell killing activity or block the blood supply to cancer cells, we attempted to combine the peptides derived from cancer-testis antigens, as well as those designed to induce an anti-angiogenic effect to achieve an effective response in patients with advanced NSCLC. In the current study we report on the safety of combination therapy involving multiple peptides and a possible improvement in patient prognosis.

Methods

Study design

We performed two phase I clinical trials using two different combinations of peptide vaccines. In the first trial, we administered peptides derived from URLC10, TTK, VEGFR1 and VEGFR2, and in the second trial we administered peptides derived from URLC10, CDCA1, VEGFR1 and VEGFR2. All peptides were restricted to HLA-A*2402. Fifteen HLA-24-positive patients with NSCLC who failed to respond to the standard therapy were enrolled in the three patient/dose/cohort phase I trial involving 0.5, 1 or 3 mg/body for each peptide (for trial 1), or 1 or 3 mg/body for each peptide (for trial 2). The clinical characteristics and treatment information for all patients enrolled in the study are summarized in Table 1. Vaccines were

Table 1 Patient clinical characteristics

Patients	Age/Gender (M/F)	Stage	Histology*	Lesion§	Performance status (ECOG)	Peptides†	Dose (mg)	Phase of treatment (Prior therapy**)
1	54/M	Recurrence	AD	LN, bone	2	L, T, R1, R2	0.5	5th (PLT, RT)
2	48/M	IIIB	AD	PM, effusion	2	L, T, R1, R2	0.5	5th (PLT)
3	65/M	Recurrence	AD	PM	2	L, T, R1, R2	0.5	6th (PLT, EGFR-TKI)
4	58/M	IV	AD	Primary, bone	2	L, T, R1, R2	1	4th (PLT)
5	60/M	IV	AD	Primary, LN	1	L, T, R1, R2	1	3rd (PLT)
6	47/M	IV	AD	Primary, LN, ADR	0	L, T, R1, R2	1	3rd(PLT, RT)
7	40/M	IIIA	AD	Primary, LN	1	L, T, R1, R2	3	3rd(PLT)
8	69/M	Recurrence	SQ	PM	1	L, T, R1, R2	3	3rd(PLT, RT)
9	65/M	Recurrence	AD	Dissemination	0	L, T, R1, R2	3	2nd(PLT, RT)
10	57/M	Recurrence	PLEO	LN	1	L, C, R1, R2	1	3rd(PLT, RT)
11	55/F	IIIB	AD	Primary, LN, effusion	2	L, C, R1, R2	1	5th(PLT, EGFR-TKI)
12	62/M	Recurrence	AD	PM	1	L, C, R1, R2	1	2nd(PLT)
13	68/F	IV	AD	Primary, bone, effusion	2	L, C, R1, R2	3	2nd(PLT)
14	39/F	IV	NSCLC	Primary, liver, bone	2	L, C, R1, R2	3	2nd(PLT, RT)
15	61/M	Recurrence	AD	PM, LN	1	L, C, R1, R2	3	5th(PLT, RT, EGFR-TKI)

*AD: adenocarcinoma; SQ: squamous cell carcinoma; PLEO: pleomorphic carcinoma; NSCLC: non-small cell lung cancer in which further histological determination was not possible.

§LN: lymph nodes metastasis; bone: bone metastasis; PM: pulmonary metastasis; effusion: malignant pleural effusion; Primary: primary tumor; ADR: adrenal gland metastasis; Dissemination: pleural dissemination; liver: liver metastasis.

† L: LY6K; T: TTK; R1: VEGFR1; R2: VEGFR2; C: CDCA1.

**PLT: platinum containing chemotherapy; RT: radiotherapy; EGFR-TKI: epidermal growth factor receptor tyrosine kinase inhibitor.

administered weekly and the sites of vaccination were rotated weekly. Administration was by subcutaneous injection into the patient's axillary region after mixing with incomplete Freund's adjuvant (IFA) Montanide ISA 51, SEPPIC until progression of the disease was observed, or until the patient declined the continuation of the vaccine treatment. Immunological responses were evaluated by means of INF-gamma ELISPOT assays. Every measurable lesion was evaluated using response evaluation criteria in solid tumors (RECIST) 1.0, and the toxicities caused by the vaccination therapy were assessed using Common Terminology Criteria for Adverse Events (CTCAE) version 3. These studies were approved by the ethical committee of Fukushima Medical University (trial 1 approval number: 554; trial 2 approval number: 810) and were registered with ClinicalTrials.gov (trial 1: NCT00633724; trial 2: NCT00874588). Written informed consent was obtained from all individuals. The trials were carried out in accordance with the Helsinki declaration on experimentation on human subjects.

Patient eligibility

Patients with an advanced or a recurrent non-small cell lung cancer who failed to respond to the standard therapy were enrolled in these two trials. Eligibility criteria were as follows: (1) patients who had an HLA-A*2402 allele evaluated using DNA genotyping; (2) adequate bone-marrow, cardiac, pulmonary, hepatic and renal functions including a white blood cell count of 1500-15000/mm^3, a platelet count of >75 000/mm^3, total bilirubin of < three times that of the institutional normal upper limit, levels of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase of < three times that of the institutional normal upper limits, and levels of creatinine of < two times the institutional normal upper limit; (3) no other therapy for lung cancer within 4 weeks prior to the initiation of the trial; (4) an ECOG performance status of 0–2; and (f) an age of ≥20 years. The exclusion criteria for patients participating in the two clinical trials were as follows: (1) pregnancy (including women of childbearing potential); (2) breast feeding; (3) bleeding disorder; (4) infections requiring antibiotics treatment; (5) concomitant treatment with steroid or immunosuppressant; and (6) decision of unsuitableness by principal investigator or physician-in-charge.

Peptides

The amino acid sequences of the peptides used were RYCNLEGPPI (URLC19-177), VYGIRLEHF (CDCA1-56), SYRNEIAYL (TTK-567), TLFWLLLTL (VEGFR1-770) and RFVPDGNRI (VEGFR2-169); these were expected to bind to an HLA-A24 molecule. These peptides were synthesized as GMP grade as described elsewhere [10-13]. The purity (>97%) and identity of the peptides were determined using analytical high-performance liquid chromatography and mass spectrometry analysis, respectively. Peptides were dissolved in dimethyl-sulfoxide at the concentration of 20 mg/ml and stored at −80°C.

Enzyme-linked immunospot (ELISPOT) assay

Specific CTL response was measured using an ELISPOT assay following *in vitro* sensitization. Frozen peripheral blood mononuclear cells (PBMCs) isolated from each patient were thawed, and the viability was confirmed to be more than 90%. 500,000 PBMC cells from each patient were cultured with 10 mg/ml of respective peptide and 100 IU/ml of IL-2 (Novartis, Emeryville, CA, USA) at 37°C for two weeks (each peptide was added to the culture medium on days 0 and 7). After CD4$^+$ cell depletion using a Dynal CD4-positive isolation kit (Invitrogen, Carlsbad, CA, USA), the IFN-γ ELISPOT assay was performed using a Human IFN-γ ELISpot PLUS kit (MabTech, Nacka Strand, Sweden) according to the manufacturers' instructions. Briefly, HLA-A*2402-positive B-lymphoblast TISI cells (IHWG Cell and Gene Bank, Seattle, WA, USA) were incubated with 20 mg/ml of each peptide overnight, then the peptide in the media was washed out to prepare the peptide-pulsed TISI cells as stimulator cells. Prepared CD4-negative cells were cultured with the peptide-pulsed TISI cells (2×10^4 cells/well) at the ratio of responder cells and stimulator cells (R/S ratio) of 1:1, 1:2, 1:4 and 1:8 on 96-well plates at 37°C overnight. Non-peptide-pulsed TISI cells were used as negative controls. To confirm the IFN-γ productivity, responder cells (2.5×10^3 cells/well) were stimulated with PMA (66 ng/ml) and ionomycin (3 mg/ml) without stimulator cells overnight, and then applied to the IFN-γ ELISPOT assay. All ELISPOT assays were performed in triplicate wells. The plates were analyzed using the automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology Ltd, Shaker Heights, OH, USA) and ImmunoSpot Professional Software Version 5.0 (Cellular Technology Ltd). The number of peptide specific spots was calculated by subtracting the spot number in the control well from the spot number in well with peptide-pulsed TISI cells. Antigen specific CTL responses were classified into 4 groups (−, +, ++ or +++) according to a previously reported protocol [14]. If the CTLs were indicated as +, we judged them as being positive in this study. The quality of our ELISPOT assay was ranked at the average level by the ELISPOT panel of Cancer Immunotherapy Consortium (CIC; http://cvc.assaymgmt.webbasix.com).

Flow cytometrical analysis

The presence of CTLs with peptide-specific T cell receptor was analyzed using a FACS-CantoII (Becton Dickinson, San Jose, CA, USA), using VEGFR1 or VEGFR2-derived epitope peptide-MHC dextramer-PE

Table 2 Summary of toxicity in Trial 1 using the TTK containing vaccine

Vaccine doses	0.5 mg (n=3)		1.0 mg (n=3)		3.0 mg (n=3)		Total patients (n=9)	
	Grade		Grade		Grade		(%)	
	1-2	3(4)	1-2	3(4)	1-2	3(4)		
Blood/bone marrow								
Anemia	1	0	1	0	2	0	3	(33%)
Leukopenia	0	0	1	0	0	0	1	(11%)
Constitutional symptoms								
Fatigue	1	0	2	0	1	0	4	(44%)
Gastrointestinal								
Nausea/vomiting	0	0	2	0	1	0	3	(33%)
Anorexia	0	1	2	0	0	0	3	(33%)
Constipation	0	0	1	0	0	0	1	(11%)
Dermatology/skin								
Rash	2	0	2	0	3	0	7	(77%)
Pruritus	0	0	1	0	2	0	3	(33%)
Reaction at the injection site	2	0	2	0	3	0	7	(77%)

(Immudex, Copenhagen, Denmark), CDCA1-derived epitope peptide-MHC pentamer-PE (ProImmune Ltd., Oxford, UK), or URLC10-derived epitope peptide-MHC tetramer-PE (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) according to the manufacturers' instructions. HIV-derived epitope peptide (RYLRDQQLL)-MHC dextramer, pentamer or tetramer-PE was used as a negative control. Briefly, cells were incubated with the peptide-MHC dextramer, pentamer or tetramer-PE for 10 min at room temperature, and then treated with FITC-conjugated anti-human CD8 mAb, APC-conjugated anti-human CD3 mAb, PE-Cy7-conjugated anti-human CD4 mAb, and 7-AAD (BD Pharmingen, San Diego, CA, USA) at 4°C for 20 min.

Table 3 Summary of toxicity in Trial 2 using the CDCA1 containing vaccine

Vaccine doses	1.0 mg (n=3)		3.0 mg (n=3)		Total patients (n=6)	
	Grade		Grade		(%)	
	1-2	3(4)	1-2	3(4)		
Blood/bone marrow						
Anemia	2	0	2	0	4	(67)
Thrombocytopenia	0	0	1	0	1	(17)
Hepatic						
Elevated AST	0	0	0	(1)	1	(17)
Elevated ALT	0	0	0	(1)	1	(17)
Constitutional symptoms						
Fatigue	0	0	3	0	3	(50)
Fever	1	0	1	0	2	(33)
Gastrointestinal						
Nausea/vomiting	0	0	2	0	2	(33)
Anorexia	0	0	2	0	2	(33)
Constipation	1	0	0	0	1	(17)
Dermatology/skin						
Rash	3	0	3	0	6	(100)
Pruritus	3	0	2	0	5	(83)
Reaction at the injection site	3	0	3	0	6	(100)

Table 4 Clinical outcome and immunological response

Patients	Vaccination course	RECIST	PFS† (DAY)	OS§ (DAY)	T cell response					After treatment
					LY6K	TTK	CDCA1	R1	R2	
1	1	PD	15	112	-	++		-	++	None
2	1	PD	29	36	-	+		-	++	None
3	1	PD	43	53	-	+		++	+	None
4	1	PD	33	33	-	-		-	-	None
5	2	PD	53	398	-	-		-	-	EGFR-TKI
6	5	SD	86	834	+	-		-	-	RT
7	1	PD	28	276	-	+		-	++	None
8	4	SD	476	476	+++	+++		+++	+++	None
9	25	SD	400	858	+++	+++		+++	++	None
10	9	SD	200	756	+++		+++	+	+++	EGFR-TKI
11	3	PD	60	265	+++		+++	-	+	None
12	19	SD	490	705*	+++		+++	+++	++	Cx
13	4	PD	53	282	++		++	+	-	None
14	6	SD	83	213	+++		+++	+++	+++	None
15	13	SD	316	571*	+++		+++	+	++	Immune**

*: patients still alive; **: another immunotherapy; †PFS: progression free survival; §OS: overall survival.

Statistical analysis

Statistical analysis for correlation between clinical response and reaction at the injection site (RAI) was performed Fisher's exact test. Overall survival rates were analyzed using the Kaplan-Meier method, and survival was measured in days from the first vaccination to death. Statistical significance of the survival period was analyzed using the log-rank test.

Results

Clinical characteristics of the enrolled patients

The clinical characteristics of the enrolled patients are summarized in Table 1. Eight advanced-stage patients and seven patients with recurrence after surgery were enrolled in the trials. The mean age of these patients was 56.5 years (±7.5 years). Twelve patients were diagnosed as having adenocarcinoma including two cases with sensitive EGFR mutations (Patients 5 and 12), and there was one patient with squamous cell carcinoma, one patient with pleomorphic carcinoma; the remaining patient was diagnosed as having non-histologically-specified non-small cell lung cancer. The patients had received at least one type of chemotherapy regime prior to enrollment as shown in Table 1.

Feasibility and adverse reactions

The toxicities observed in the 15 patients are summarized in Tables 2 and 3. There was no severe adverse event considered to be related to the vaccination except for local reactions at the injection sites. Although one patient revealed the elevation of hepatic transaminases equivalent to grade 4 toxicity, we judged that this was not due to the vaccine-related toxicity, but was caused by massive liver metastasis.

Monitoring of immunological responses and clinical response

PBMCs were obtained from all patients before the vaccine treatment and after every course (one course consists of four vaccinations), and in some patients every month after the vaccine treatment had been completed. Using these PBMCs, we analyzed the levels of peptide-specific CTL responses as shown in Table 4 and Additional file 1: Table S2. Immunological responses were found to be relatively weak in the 0.5 mg/body and 1 mg/body groups relative to the 3 mg/body group in Trial 1. Hence, in Trial 2 we deleted the 0.5 mg/body group and administered 1.0 and 3.0 mg/body. In the 3.0 mg/body group, four of a total of six patients in both of the trials revealed strong CTL responses for at least two kinds of peptides.

When we analyzed CTL induction according to performance status (PS), we only detected a strong CTL response in two out of the seven patients with PS 2, while we observed strong CTL responses in five out of the eight patients with PS 0 or 1. In addition, among the seven patients that showed strong CTL responses, six patients were judged as being in a stable condition using RECIST criteria for at least 2 months. On the other hand, among the eight patients who did not reveal a strong CTL response, seven patients showed rapid progression.

A representative case of stable disease is shown in Figure 1. Patient 8 had recurrent squamous cell carcinoma

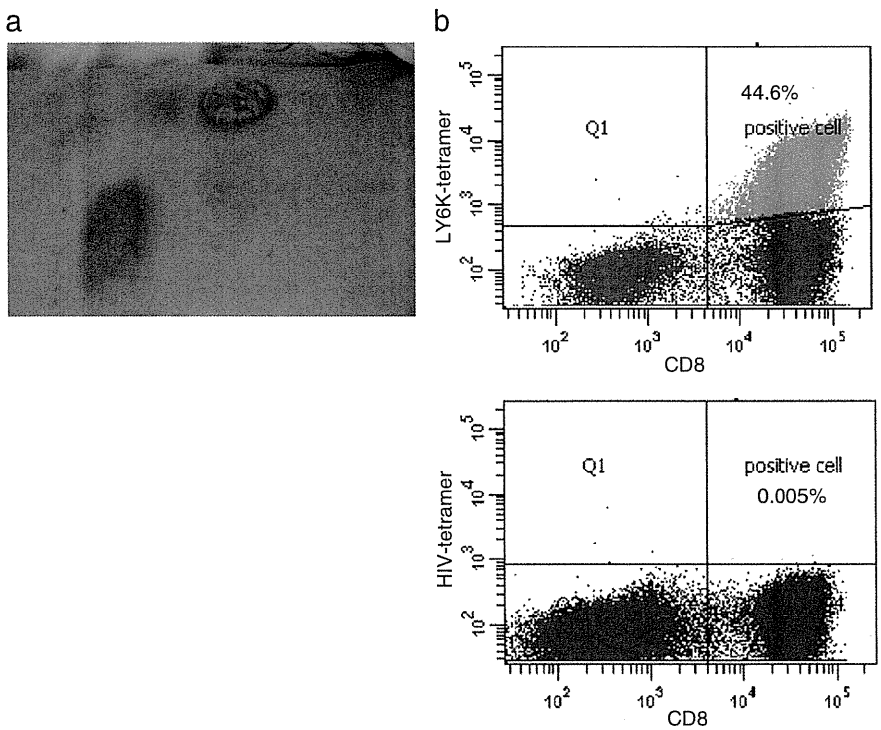

Figure 1 Strong injection site reaction in patient 8 with positive immune response. (**a**) Representative picture showing a positive immune reaction at the local injection site (axillary region in patient 8; Grade 2 reaction categorized using CTCAE). (**b**) HLA-tetramer assay showing a very high level of URLC10-specific CD8-positive cells (44.6% of CD8-positive cells) observed after the 4-month vaccine treatment in patient 8.

with pulmonary metastases. This patient showed relatively strong local reaction at the injection sites and tumors were maintained in a stable condition for 4 months (Figure 1a).

High levels of URLC10-specific CTLs (44.6% of CD8-positive cells) were identified after 4 courses of vaccination.

We also observed the relationship between delayed type hypersensitivity (DTH) as RAI and clinical responses. The stronger the RAI became, the better the clinical responses were, indicating that the RAI seems to be a good biomarker to predict the clinical response (Table 5).

Survival analysis

To clarify the prognostic factors in our vaccine treatment, we further analyzed the survival of patients as shown in Figure 2a, Additional 2: Figure S1 and Table 6. The 1-year survival rate was 58.3% and the median survival period was calculated as being 398 days (56.9 weeks). Sensitive EGFR mutations were found in patients 5 and 12. Patient 10 was treated with Erlotinib as the follow-up therapy, although this patient was found to have no EGFR mutation. The EGFR mutation in patient 5 was found after the vaccine therapy was terminated and was subsequently treated with an EGFR-tyrosine kinase inhibitor (EGFR-TKI). However, because of the poor PS, this patient did not tolerate EGFR-TKI. An EGFR mutation was also detected in patient 12 after the vaccine therapy, but this patient was also treated using cytotoxic chemotherapy because they wished to receive it.

Table 5 Reaction at injection site and clinical response

Clinical response	RAI: Grade 0	RAI: Grade 1	RAI: Grade 2
Stable disease	0	3	4
Progressive disease	2	6	0

Numbers shown: mean number of patients; RAI: reaction at injection site.
p=0.026 (Fisher's exact test).

As shown in Table 6, PS, CTL response and pre-treatment C-reactive protein (CRP) level (≥1.0 mg/ml) were indicated to be statistically significant prognostic factors (p=0.0004, 0.0176 and 0.0284, respectively). Since these three parameters were correlated with each other, further investigation of patients with good PS is essential in the evaluation of the contribution of CTL induction to good prognosis. The number of treatment regimens undergone before enrollment into the vaccine therapy also showed some tendency to influence overall survival (p=0.0629). No other laboratory and immunological parameter, including the proportion of regulatory T cells in PBMCs, was significantly correlated with patient survival.

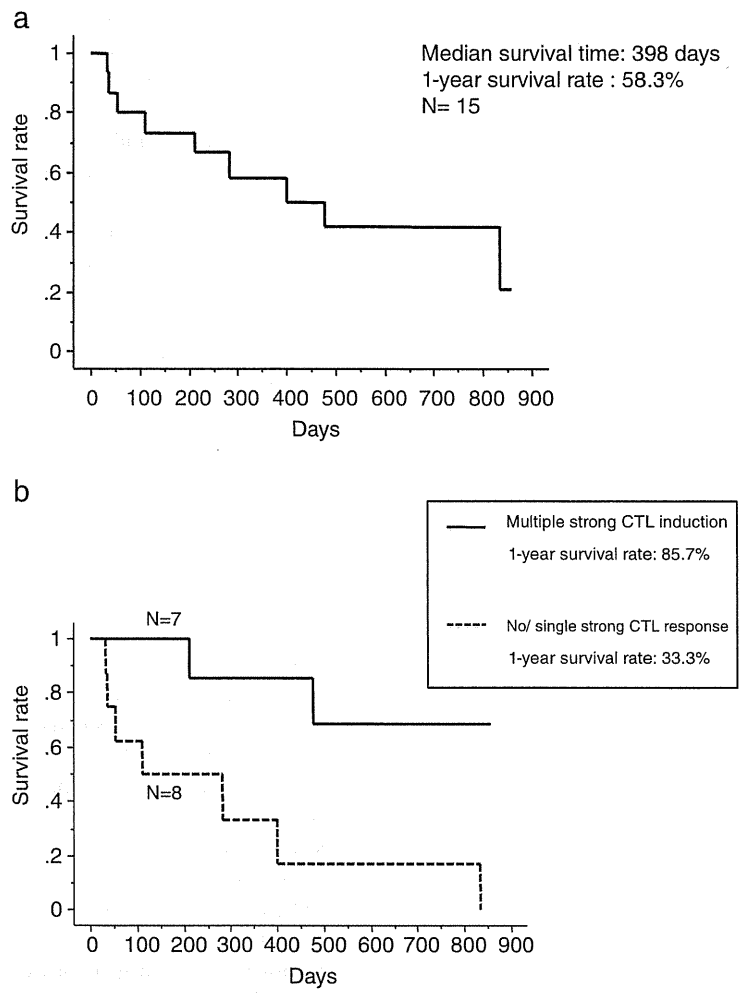

Figure 2 Survival analysis of patients. (**a**) Overall survival curve for the fifteen patients analyzed using the Kaplan-Meier method. The median survival time is 398 days and the 1-year survival rate is 58.3%. (**b**) Overall survival curve according to the CTL responses (Kaplan-Meier method). Patients with strong positive CTL responses (+++) to two or more peptides (n=7) had a significantly better prognosis than those revealing a strong CTL response to no or only one peptide (n=8, including several patients who had weak CTL responses with + or ++ against multiple peptides.) (p=0.0176 using the log-rank test). The 1-year survival rates for the group showing a CTL response with multiple peptides and those with no or a single peptide are 85.7% and 33.3%, respectively. As mention above the cutoff levels for CTL were set as (–, +, ++) vs. (+++) in survival analysis.

We also analyzed the relationship between patient survival and the number of peptides for which we observed CTL responses. As shown in Figure 2b, patients with CTL induction against multiple peptides had a significantly higher survival rate than those with CTL induction against a single peptide or no peptide, suggesting an advantage in using multiple peptides for cancer treatment.

Discussion

Among the large number of therapeutic cancer vaccine trials for solid tumors being conducted worldwide, most involve the administration of a single vaccine [15,16]. For lung cancer, two large phase 3 trials using MAGE-A3 or BLP25 are expected to be very promising (ClinicalTrials.gov NCT00480025 and NCT01015443) [5,6]. However, single vaccine therapies in these trials may have some disadvantages as compared with treatment involving a mixture of multiple peptides derived from multiple proteins; one important factor is that antigen expression occurs in a relatively limited proportion of tumors. For example, the expression of MAGE-A3 has been reported in only 40% of cases [17], and in only 24% of Japanese patients [18]. The other important issue is the frequency of CTL induction, the rate of which largely depends on the nature of individual antigens. In fact, two lung cancer studies reported previously shown CTL induction in only 20-53% of the cases treated with vaccines [6,19]. In this regard as recently reported, treatment using multiple vaccine therapy has

Table 6 Clinical and immunological parameters and patient survival

Parameter	1-year survival rate (%)	Median survival time (days)	P value
Total	**58.3**	**398**	
Age			
>=60y	71.4	476	
< 60y	50	213	0.4159
Sex			
Male	66.7	476	
Female	0	282	0.4797
Performance status			
0-1	100	834	
2	0	112	0.0004
Treatment line			
~2nd	72.9	834	
3rd~	42.9	112	0.0629
Reaction at injection site			
Strong	75.0	476	
Weak	50.9	398	0.5207
CTL			
Strong	85.7	-	
Weak	33.3	112	0.0176
Regulatory T (%)			
High	57.1	476	
Low	33.3	282	0.3856
C-reactive protein			
>=1.0	25.0	53	
< 1.0	71.6	834	0.0284
Hemoglobin			
Normal	57.1	834	
Low	56.3	398	0.891
Albumin			
Normal	57.1	834	
Low	62.5	398	0.8256
White blood cell count			
High	55.6	-	
Normal	66.7	398	0.7070
Neutrophile (%)			
High	75.0	834	
Low	38.1	282	0.1902
Lymphocyte (%)			
High	50.0	282	
Low	66.7	398	0.5006

some advantages owing to the possibility that CTL induction may be higher for one or more antigens [7,8]. Further in renal cell cancer, clinical benefits have been shown lately using a multiple peptide vaccination named IMA901, and a phase 3 study is currently ongoing [20]. In the present study, we have conducted a vaccine trial for lung cancer using multiple peptide vaccines, and observed that the specific CTL responses against one or more epitope peptides were very effective. In only two out of the 15 patients, no CTL induction was observed using any of the four peptides. Although we administered our vaccine treatment to the patients as a second line or later treatment, they achieved a median survival time of 398 days and a 1-year survival rate of 58.3%. Previous major second line trial data regarding NSCLC using a cytotoxic chemotherapeutic drug revealed a median survival time of about ~8 months and a 1-year survival rate of ~30% [21]. Hence, we expect that our vaccine formulation may contribute to an improvement in the prognosis of patients with NSCLC, although further investigation of survival benefit using a larger number of patients is required.

Peptide vaccines used in this trial included peptides that originated from VEGFR1 and VEGFR2 for targeting angiogenesis in tumors. Bevacizmab, an antibody targeting VEGF, has already been used to treat the advanced non-squamous type of NSCLC [22]. Although anti-angiogenic therapy alone does not have sufficient efficacy to induce tumor shrinkage [23], it may support the induction of a strong anti-tumor effect and/or contribute to improved patient survival when it is combined with other therapies [24,25]. Therefore, we considered that the combination of anti-angiogenic peptides with peptides derived from tumor-specific antigen-proteins may cause a synergistic clinical effect in patients with NSCLC. In addition, since HLA molecules are down-regulated in many types of advanced solid cancer including lung cancer [26,27], peptides targeting blood vessels in which HLA molecules are stably expressed should have some anti-tumor effect by reducing the blood supply to tumors.

In our vaccine trial, although we did not observe tumor shrinkage, we observed a possible survival benefit. "Clinical benefit without tumor shrinkage" is considered to be one of the characteristics of cancer vaccine treatment [28]. In fact, the guidance for therapeutic cancer vaccines released from the Food and Drug Administration (FDA) in the United States that was released in 2011 (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/UCM278673.pdf) mentioned that therapeutic cancer vaccine treatment can provide a survival benefit without evident tumor shrinkage. The FDA guidance further commented that "clinical progression that is asymptomatic and/or is not likely to result in life-threatening complications with further progression (e.g., central

nervous system (CNS) metastases or impending fractures from bony metastases) may not be sufficient reason for discontinuation of the administration of a cancer vaccine". Accumulating evidence has indicated the necessity of establishing novel criteria for the evaluation of clinical response in immunotherapy such as immune-related response criteria (irRC) [28]. Researchers have started using overall survival or relapse-free survival in recently conducted trials as endpoints in immunotherapy clinical trials.

Our data suggested that PS, CTL induction and pretreatment serum CRP level might be potential predictive markers for vaccine treatment. Extensive and systematic approaches regarding biomarker discovery for vaccine therapy have been carried out [29]. In addition, several prognostic factors possibly related to immunotherapy including clinico-pathological parameters or immunological parameters have been reported [30]. Some previous studies have implicated PS and CTL as good prognostic factors [31,32] in line with our findings. However, although our study has suggested that patients with a higher CRP level (≥1.0 mg/ml) had significantly shorter survival times than those with a lower CRP level, the usefulness of CRP as a prognostic marker has been controversial [33,34].

The US FDA guidance also suggests that cancer vaccine should be administered to patients at an earlier stage, at which the immune system has not been heavily damaged by cytotoxic anti-cancer drugs. In this regard, administration of vaccine therapy should be more appropriate as an adjuvant treatment after surgery, or as an early phase treatment after relapse of the disease in combination with or without chemotherapy.

In summary, we conducted phase I trials with multiple peptide vaccines for patients with NSCLC. These vaccine treatments were well tolerated and prolongation of patient survival owing to vaccine treatment might be expected. We believe that vaccine treatment using multiple peptides is likely to be very promising, although this should be validated by further advanced-phase clinical trials.

Additional files

Additional file 1: Table S2. Summary of Elispot assay data, before, post 1 course and post 2 course vaccination.

Additional file 2: Figure S1. (**A**) Overall survival analysis according to patient ECOG performance status. Patients with a good PS (PS: 0, 1) had a significantly higher survival rate than patients with a poor PS (PS: 2) ($p<0.0001$ using the log rank test). (**B**) Overall survival curve according to the CTL responses in the good PS group (PS: 0, 1) (Kaplan-Meier method). Patients with positive CTL responses to two or more peptides (n=5) had a relatively better prognosis than those revealing a CTL response to no or one peptide, although the difference was not significant (n=3; $p=0.09$).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HS participated as principle investigator of the study. HS, Mitsunori H, YS, TK, KI and MG participated in the design and coordination of the study, data acquisition and analysis and helped draft the manuscript. KT participated as the main coordinator and investigator regarding the immunological data analysis and evaluation. MF, TY, SM, NO, HY, TH, AY, JO and MH participated in the clinical data acquisition and evaluation, and helped draft the manuscript. All authors read and approved the final manuscript.

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Functional roles of tumor necrosis factor-related apoptosis-inducing ligand–DR5 interaction in B16F10 cells by activating the nuclear factor-κB pathway to induce metastatic potential

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been recognized as a promising target for cancer therapy because it can induce apoptotic cell death in tumor cells but not normal cells. Although TRAIL shows specific tumoricidal activity, resistance to TRAIL-induced apoptosis in some tumor cells has been considered a clinical obstacle of its application. It has been shown that TRAIL provides inflammatory signals that may contribute to the TRAIL-resistance of cancer cells; however, it is not known whether TRAIL itself is involved in malignant cancer cell behavior. In the present study, we examined the functional role of TRAIL in B16F10 mouse melanoma cells, which are totally insensitive to TRAIL-induced apoptosis. By establishing B16F10 cells stably expressing the nuclear factor-κB (NFκB)-luciferase reporter gene, we found that TRAIL can activate NFκB through its death receptor DR5 in B16F10 cells. Furthermore, TRAIL–DR5 interaction not only promoted malignant behaviors of B16F10 cells, such as cell proliferation and MMP-9 production, but also induced lung metastasis of B16F10 cells *in vivo*. These findings may imply a contrary role for the TRAIL–DR5 pathway in the inflammatory tumor microenvironment, in its ability to induce the metastatic potential of B16F10 melanoma cells instead of inducing apoptosis. (*Cancer Sci* 2013; 104: 558–562)

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also known as Apo2 ligand, is a type II transmembrane protein belonging to the TNF family[1,2] of cytokines that play important roles in inflammation and immunity.[3–6] It has been recognized as a promising target for cancer therapy, because TRAIL can induce apoptotic cell death in a variety of tumor cells but not in most normal cells.[7–9] Some studies have shown that this ligand has the potential to suppress the metastatic ability of cancer cells.[10,11] So far, two cell death-inducing receptors (TRAIL-R1/DR4, TRAIL-R2/DR5) and two non-cell death-inducing receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2) have been identified for TRAIL in humans; the latter two of these may act as decoys.[5–7,12,13] In mice, only one death-inducing receptor homologous to human DR5 (mTRAIL-R2/mDR5), and two potential decoy receptors have been identified.[4,14] These death receptors signal apoptosis through a Fas-associated death domain and the caspase-8-dependent pathway.[4,6,7,13,15,16] Moreover, the cytoplasmic regions of DR5 and mDR5 contain potential TNF receptor-associated factor (TRAF)-binding motifs, which may be responsible for NFκB and MAPK activation by this receptor.[13,15–18]

Although TRAIL has shown specific tumoricidal activity, some cancer cells are totally insensitive to TRAIL-induced apoptosis and such resistance may account for a clinical obstacle. Some studies have shown that the resistance to TRAIL-induced apoptosis is caused by lower expression levels of functional TRAIL receptors.[19–22] The B16F10 murine melanoma cell line is known to show resistance in spite of the high expression of mDR5 on the cell surface.[23,24] Therefore, the effects of TRAIL on B16F10 cells have not been comprehensively explored.

As a critical transcription factor for inflammation, NFκB regulates the expression of pro-inflammatory genes associated with invasion, angiogenesis, and metastasis.[25,26] Some reports indicated that the activation of NFκB maintains resistance to TRAIL-induced apoptosis.[19,27–29] However, it is not known whether the TRAIL pathway is involved in cancer cell behavior by providing inflammatory signals.

In the present study, we investigated the role of NFκB-mediated inflammatory signals in cancer progression, particularly through the TRAIL–DR5 receptor pathway in B16F10 melanoma cells. We found that TRAIL activated the NFκB pathway through DR5 in B16F10 cells and induced a tumor-promoting effect with MMP-9 production, proliferation ability *in vitro*, and also induced lung metastasis potential *in vivo*, instead of inducing apoptosis.

Materials and Methods

Reagents. Tumor necrosis factor-α was purchased from Peprotech (Rocky Hill, NJ, USA). The pGL4.32 (luc2P/NF-kappaB-RE/Hygro) vector and D-luciferin were obtained from Promega (Madison, WI, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Hygromycin B was obtained from Nacalai Tesque (Kyoto, Japan). Anti-TRAIL (N2B2) and anti-DR5 (MD5-1) antibodies were purchased from Biolegend (San Diego, CA, USA).

Cells. Mouse melanoma B16F10 cells were maintained in DMEM and Ham's F12 medium containing 10% bovine serum (Nissui, Tokyo, Japan). Mouse B lymphoma 2PK3 cells and 2PK3 expressing mouse TRAIL (TRAIL-2PK3) cells were cultured in RPMI-1640 (Nissui) containing 0.03% L-glutamine, 0.01 M HEPES, 0.2% $NaHCO_3$, and 10% bovine serum. To establish NFκB-mediated luciferase gene expressing B16F10 cells (B16F10 NFκB), B16F10 cells (5×10^5/well) were seeded in a 6-well plate and pGL4.32 vector was transfected using Lipofectamine 2000. The cells were selected with Hygromycin B (200 μg/mL) and cloned by limiting dilution.

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