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Dramatic improvement of DC-based immunotherapy against various malignancies

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1. ABSTRACT

Dendritic cells (DCs) play a crucial role in maintaining the immune system. Though DC-based cancer immunotherapy has been suggested as a potential treatment for various kinds of malignancies, clinical efficacies are still insufficient in many human trials. To identify the causes of the low efficacies, we paid attention to their numbers and how they are activated. We proved that DCs' antitumor effect depends on their number and the way they are activated. We here established a possible breakthrough, a simple cytokine-based culture method to realize a log-scale order of functional murine/human DCs. Moreover, we demonstrated that DCs activated by replication-deficient recombinant Sendai virus (rSeV) were dramatically more effective than that seen in the use of current DC vaccine for immunotherapy against malignancies. Our study could overcome these problems and would improve treatment of malignancies.

2. INTRODUCTION

Dendritic cells (DCs) are unique antigen-presenting cells that can efficiently stimulate innate as well as acquired immune responses against pathogens and endogenous cancers. DC-based immunotherapy has been expected as a new therapeutic modality for cancers and infectious diseases in the past decade. However, relatively limited efficacies have been reported in their clinical studies. Complex issues, a consequence of too many variables in current clinical studies, must be solved in order to standardize DC-immunotherapy, including DC subtypes, antigen targeting *in vivo*, doses of DCs, and so on. Among these parameters, the possible critical issues underlying DC immunotherapy are that the stimuli that activate DCs for a sufficient antitumor effect are still unknown, and that the limited numbers of DCs (roughly 10^6 to 10^8 DCs) are available from each patient in clinical studies, even via frequent aphereses for DC progenitor collection. Thus there

is a strong desire for activation stimuli to generate activated DCs showing optimal antitumor effect *in vivo*. Moreover, the establishment of technology to increase the number of DCs should make the process of DC manipulation less invasive and improve quality control in industrial production. More importantly, experimental studies on the use of dermal tumor and lung metastasis models revealed that DC-based immunotherapy showed a significant dose-response; the optimal dose in both models was at least 10^6 DCs/30g, roughly equivalent to 10^9 DCs per patient according to the weight ratio. Therefore, increasing the number of DCs is expected to improve the efficacy of DC-based immunotherapy in clinical settings. In this review article, we introduce our studies, which overcome a part of these issues of DC-based immunotherapy for malignancies.

3. DENDRITIC CELLS

DCs were first described in the mid-1970s by Ralph Steinman, who observed in the spleen a subpopulation of cells with a striking dendritic shape (1-3). DCs are bone marrow-derived cells of both lymphoid and myeloid stem cell origin. Murin DC was found to be differentiated from Monocyte-Dendritic cell Progenitor (MDP), and $Lin^-/ckit^+/M\text{-}CSFR(CD115)^+$ was a common feature of cells that were able to differentiate into DC (4-6). In humans, it was reported that GM-CSF receptor alpha $(CD116)^{low}$ cells differentiate into macrophages and that $CD116^{high}$ cells differentiate into DCs (7). However, the mechanisms underlying this differentiation remain unclear.

DCs are antigen-presenting cells (APCs) with the unique ability to take up and process antigens in peripheral blood and tissues. These cells are the most potent and professional APCs and determine either Th1 or Th2 polarization of naive T cells. The cells have PAMPs (pathogen-associated molecular patterns) recognition systems using Toll-like receptors (TLRs) (8), endocytotic activity, and antigen-presenting activity stronger than any other APCs (macrophages, monocytes, or B cells). They subsequently migrate to draining lymph nodes, where they present antigen to resting lymphocytes.

4. DCS IN THE IMMUNE SYSTEM

DCs work as a conning tower of the immune system and influence other immunocytes. Cytotoxic T-lymphocyte (CTL) is a subset of T cells, which can kill cells that are infected with viruses (and other pathogens), damaged, or dysfunctional (9). Naive CTLs are activated when their T-cell receptor (TCR) strongly interacts with a peptide-bound MHC class I molecule of APC. Once activated, the CTL undergoes a process called clonal expansion in which it gains functionality and then divides rapidly to produce effector cells. Activated CTL will then search for cells bearing unique MHC Class I + peptide. When exposed to these target cells, effector CTL releases perforin and granulysin, which form pores in the target's plasma membrane, causing it to burst or lyse. DC has a cross-presentation pathway. Although APCs except for DCs present foreign antigens only with MHC Class II to $CD4^+$ T cells, DCs can present foreign antigen with MHC Class I to $CD8^+$ CTLs. Importantly, signaling through CD40 on DCs mediates the assistance of T-cells in generating CTLs by cross-priming (10).

DCs can induce three types of effector $CD4^+$ T helper cell (Th) responses. The Th1 response is characterized by the production of Interferon (IFN) - gamma, which activates CTLs and induces cell-mediated immunity, and the Th2 response is characterized by the release of Interleukin 4 (IL-4) and leads to humoral immunity. Th17 cells are a newly discovered subset of T helper cells producing interleukin 17 (IL-17), and they are considered developmentally distinct from Th1 and Th2 cells; excessive amounts of the cell are thought to play a key role in autoimmune disease (11, 12). Th1 responses are more effective against intracellular pathogens such as viruses and bacteria that are inside host cells or tumor cells, while Th2 responses are more effective against extracellular bacteria or parasites. Not IL-12p40 or IL-12p35 but IL-12p70 produced by DCs strongly induces the Th1 response. Claudia Rieser *et al.* had reported that prostaglandin E2 (PGE2) activates human DCs to produce IL-12 (13), and Paweł Kalin´ski *et al.* revealed that PGE2 was a selective inducer of IL-12p40 production and an inhibitor of bioactive IL-12p70 (14). Consequently, DCs activated by PGE2 produced IL-23 (IL-12p40 + IL-12p19) and caused Th17 cell expansion (15).

Most clinical studies to date use monocyte-derived DCs (md-DCs). When cultured in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (GM/IL-4), the cells develop into immature DCs over a period of 3 to 5 days, and can be further matured by a subsequent one- to two-day culture period using various stimuli. Additionally, cord blood (CB) or peripheral blood (PB) $CD34^+$ cells are used to generate DCs by culture in some cytokine mixtures (16). The character of the obtained cells depends on the materials and the culture methods used. In humans, three distinct subtypes of DCs have been delineated based on studies of skin DCs (17), DCs generated *in vitro* from $CD34^+$ hematopoietic progenitors (18), and blood DC precursors (19). Langerhans cells (LCs) and interstitial DCs emerge in cultures from $CD34^+$ progenitors and $CD11c^+$ blood precursors in the presence of GM-CSF and either IL-4 or TNF-alpha (19-21). LCs require TGF-beta (22) and arise from either a $CD11c^+CD14^+$ monocyte or a $CD11c^+CD14^-$ precursor, whereas interstitial DCs arise from $CD11c^+CD14^+$ precursors that can also differentiate into macrophages in the presence of only M-CSF (19-21, 23). Plasmacytoid DCs are a third type of DC and are so named because at the ultrastructural level they resemble Ig-secreting plasma cells. Plasmacytoid DCs are characterized by a unique phenotype, $CD11c^-$ $CD4^+CD123^+CD45RA^+HLA\text{-}DR^+$, and possess the unique ability to secrete large amounts of IFN-alpha/beta upon viral stimulation (24-27).

5. DC VACCINES FOR VARIOUS MALIGNANCIES - CURRENT STATUS AND PROBLEMS

A DC vaccine is defined as DCs loaded with a tumor-associated antigen such as hall tumor lysate, supernatant of tumor lysate, or a designed peptide (e.g., WT-1, MUC1) (28). DC-based immunotherapy requires a

system in which DCs pulsed with antigens induce Th1 and activate CTLs, and these effectors execute malignancies. In addition, our data suggested that the main effector of antitumor immunity is $CD4^+$ T cells and NK cells (29). Upon administration, the vaccine is thought to induce an antigen-specific T-cell response and NK activation against the tumor. To be effective as an APC, the MHC molecules of a DC must be loaded with antigenic cargo. Peptides can be endogenously loaded onto MHC molecules after proteolytic processing of endocytosed tumor lysates or recombinant protein.

The efficacies of peptide-pulsed DC immunotherapy in mice were reported in 1995 (30), followed by the first clinical studies of DC vaccine in 1996 (B-cell lymphoma) (31) and 1998 (melanoma) (32). These studies showed sufficient efficacies (about 30%). Based on these clinical studies, many cases of DC-based immunotherapy against various malignancies were reported. However, Rosenberg *et al.* showed lower-than-expected efficacy of DC immunotherapy (about 7.1%) (33). Nestle *et al.*, who reported DC vaccine for melanoma in 1998, also showed insufficient efficacy of the treatments (34). The uncertainty of clinical efficacy might mainly be nonconstancy of quality control for *ex vivo*–generated DCs, though other reasons are involved; a description of vaccine preparation according to GMP guidelines, description of patient characteristics, description of trial design, clear documentation and definition of clinical response, description of clinical outcome of all patients, and description of immunological measures before and after vaccination (35). We have attempted to improve the efficacies of DC-based immunotherapy for cancers.

6. SOLUTION FOR LOW EFFICACY

To maximize clinical efficacy, we should standardize the most effective DC subtypes, the optimal conditioning and activation stimuli (cytokine cocktail, CD40L, or OK-432), the optimal route of administration (s.c., i.v., or i.t.), the optimal antigen (tumor lysate, peptide, or RNA), the optimal dose and frequency of DC vaccinations, and so on. Based on our studies, we proposed that 1) the optimal activation stimuli and 2) the dose of administered DCs were especially important.

6. 1. Stimuli

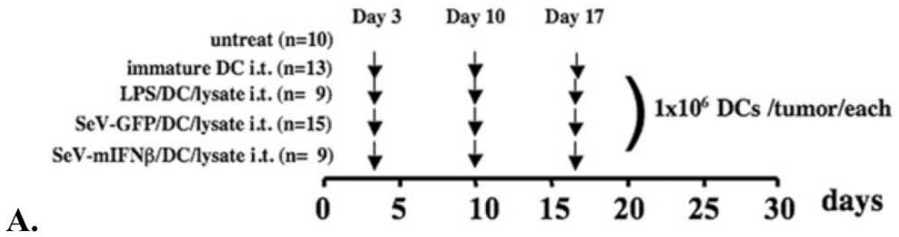
Some authors have reported that, despite high expression levels of co-stimulatory molecules, long-matured DCs failed to produce inflammatory cytokines (36) and decreased DC-T cell contact duration (37), suggesting that the long maturation period exhausted the DCs. During extensive assessment of the mechanisms of immune responses against Sendai virus (SeV), we found that *ex vivo* infection of SeV in immature DCs induced maturation and activation spontaneously in only one hour. SeV, a member of the Paramyxoviridae family, has a nonsegmented negative-strand RNA genome and makes use of sialic acid residue on surface glycoprotein or asialoglycoprotein, which is present as a receptor on most cell types (38, 39). As SeV uses a cytoplasmic transcription system, it can mediate gene transfer to a cytoplasmic location, avoiding possible malignant transformation due to genetic alteration of host cells (40, 41); this is a safety advantage of SeV. We showed that i.t. administration of replication-competent SeV-modified DCs (rSeV/DCs) induced a dramatically efficient antitumor effect on established tumors *in vivo*, an effect comparable to that seen with DCs treated with LPS that was well-known as a strong DC stimulator irrelevant to clinical use, and that antitumor immunity against an IFN-beta-sensitive tumor, a B16 melanoma, was strongly enhanced by the use of rSeV/DCs expressing a foreign IFN-beta gene (42) (Figure 1). Using DCs expanded by the FS36 system, these results were seen.

Unknown mechanisms underlie treatment efficacy. We showed a case where SeV/DC activated NK cells more sufficiently than other matured DCs did (unpublished data). There are reports that support this mechanism (43-45). It was reported that NK cells need 1) a license to kill (46, 47) and 2) transpresentation of IL-15 from DCs (48, 49). And whether or not an NK cell kills a cell was determined by the balance of activating/inhibitory signals (50). Moreover, recognition of hemagglutinins on virus-infected cells by NKp46 activates lysis by NK cells (43-45). SeV/DC might play strong roles in these mechanisms.

6. 2. Dose of administered DCs

Experimental studies on the use of dermal tumor and lung metastasis models revealed that DC-based immunotherapy showed significant dose-response even though rSeV/DCs were administered (Figure 2); the optimal dose in both models was at least 10^6 DCs/30g, roughly equivalent to 10^9 DCs per patient according to the weight ratio (51, 52). However, limited numbers of DCs (roughly 10^6 to 10^8) are available from each patient in clinical studies, even via frequent aphereses for DC progenitor collection. The ultimate needs for sufficient numbers of functional DCs in the clinical setting, in the view of therapeutic efficacy as well as industrial production, have forced us to develop an efficient and representative method for the mass production of functional DCs.

We established and optimized a culture method for DC expansion in mice using a two-step culture with a cytokine cocktail (FS36; Flt3-L, SCF, IL-3, and IL-6). Briefly, bone marrow cells were harvested from femurs and tibias, and lineage antigen-positive (CD45R, CD5, CD11b, Gr-1, TER119, 7/4) cells were removed using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell Technologies, Canada). For the expansion, these lineage-negative cells were cultured under FS36 in RPMI 1640 medium for several weeks (first step). Subsequently, expanded cells were cultured under GM/IL-4 for one week (second step). To the best of our knowledge, this is the first demonstration that murine functional DCs can be efficiently expanded *ex vivo* by more than 3 logs (Figure 3A). We proved that the expanded DCs had properties that were required to obtain therapeutic gain--such as expression of molecules needed for antigen presentation, endocytotic activity, potential for inflammatory



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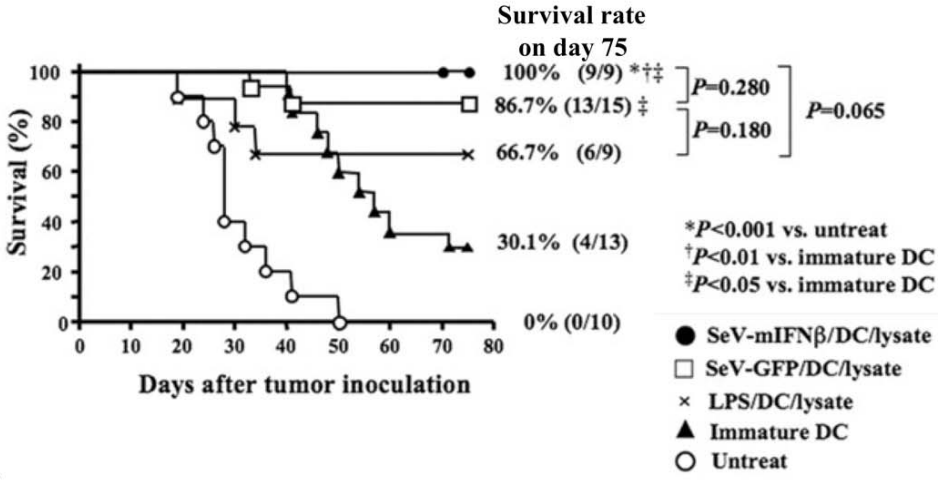

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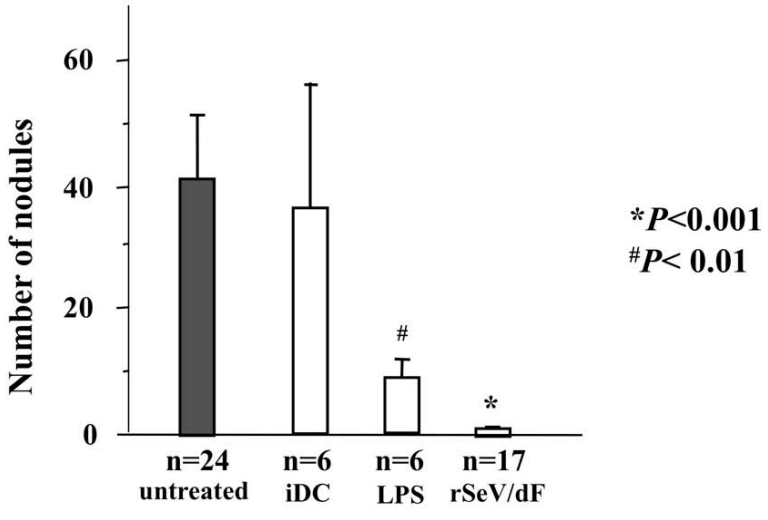

C.

Figure 1. rSeV-activated DCs showed significant efficacies. A). Experimental design to assess the stimulator-dependent antitumor effect of DCs. The figure is adopted from reference number 42. B). Survival curve of the mice bearing B16F1 melanoma treated with various DCs. Survival was significantly prolonged in the SeV/DC groups. The figure is adopted from reference number 42. C). Antimetastatic activity via bolus intravenous injection of various DCs. Female C3H/He mice (7 weeks old) were intravenously vaccinated with 1 x 10^6 of rSeV or LPS-activated DCs or immature DCs once via the tail vein. Two days later, 1 x 10^6 osteosarcoma LM8 cells were inoculated intravenously. Seventeen days later, themice were sacrificed and macroscopically recognized nodules on the surface of the bilateral lungs were counted. The figure is adopted from reference number 53.

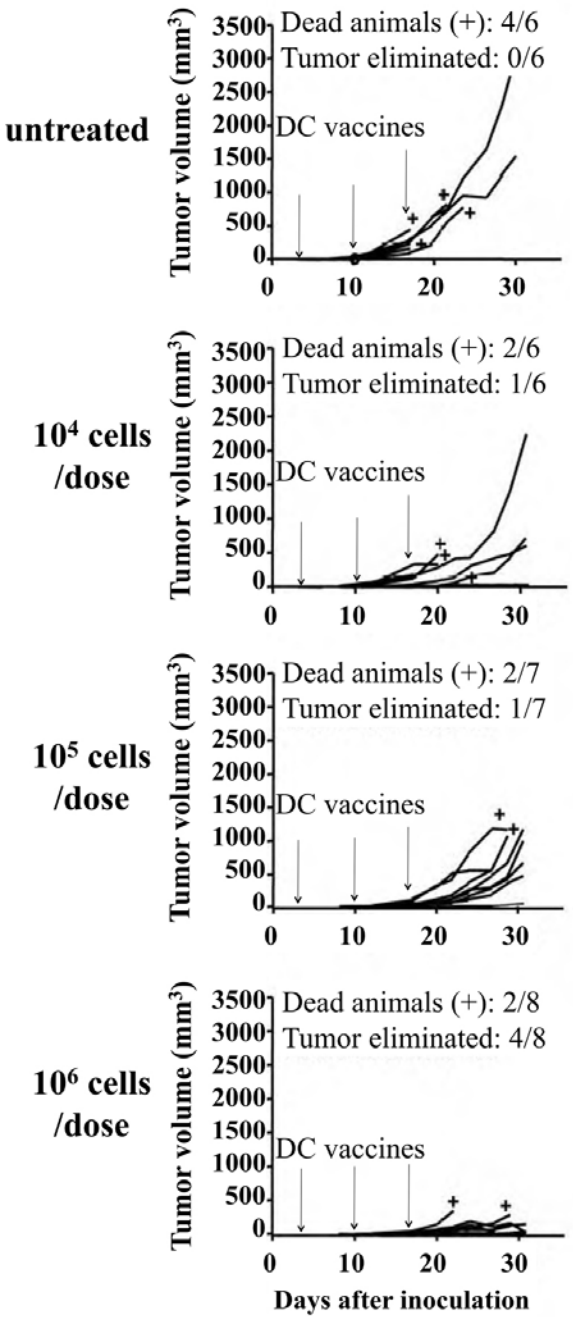

Figure 2. The efficacies depend on the number of administered DCs. Three days after intradermal inoculation of B16F10 melanoma, various amounts of ts-rSeV/dF-DCs were injected weekly via an intratumoral route. Thereafter, the tumor volume was measured. Lines on the panels indicate time courses of tumor volume in individual animals. Apparent dose-efficacy response was seen in the tumor volume of B16F10 melanoma, and 4 of 8 animals that received 10^6 DCs per dose showed complete tumor elimination. The + indicates animals that died during observation. The figure is adopted from reference number 51.

cytokine/chemokine production, and stimulation activity for allogenic T-cell proliferation--and also showed significant prevention of hypodermic tumor and experimental lung metastasis *in vivo* (53). However, this culture method couldn't be extended to human DC expansion.

We then established and optimized a new culture method for human DC expansion: 5-weeks' expansion and differentiation under GM-CSF and SCF (GM/SCF); and a two-step culture method: 4-week's expansion and differentiation under GM/SCF followed by one week of cultivation under GM/IL-4. The expansion rates varied according to the cell sources. The cells showing greater expansion were CB $CD34^+$ cells; they increased approximately 100,000-fold after 5 weeks of culture, and >80% of expanded cells expressed CD11c (Figure 3B). Thus, by this method, 100–1,000 times more $CD11c^+$ cells were obtained than under conventional procedures (unpublished data). As are seen in conventional DCs, these expanded DCs showed dendrites after maturation and endocytotic activities. Expanded DCs also expressed HLA-DR, adhesion molecules, and co-stimulatory molecules and produced inflammatory cytokines/chemokines as well as conventional DCs did. Functionally, the mixed lymphocyte reaction (MLR) assay revealed that expanded DCs could stimulate allogenic T-cell proliferation to the same extent as conventional DCs. Furthermore, to the best of our knowledge, this is the first demonstration that human functional DCs derived from $CD34^+$ cells can be efficiently expanded *ex vivo* by more than 5 logs. Moreover, we have succeeded in expanding DCs from PBMC by an easy new method: expansion rate = ~ $1x10^7$ DCs from 1 ml PB (patent pending). We expect that this technology will be able to contribute largely to both basic and clinical research of human cancer immunotherapy. DC expansion technology will improve cancer therapies and alleviate patients' burden of apheresis.

7. A NEW APPROACH TO THE TREATMENT OF MALIGNANCIES

We show schematic representation of our studies (Figure 4). We have investigated other approaches to the treatment of malignancies using SeV-activated DCs. CTLs were found to be very important for showing the efficacy of DC-based immunotherapy, but our data suggested that CTLs were not required to prevent lung metastasis. To protect the lung from cancer metastasis with DC vaccination, CD4-positive T cells and NK cells must exist spontaneously. Furthermore, we showed that this antimetastatic effect was sustained for over 3 months, even when administered DCs were already cleared from the lung and organs related to the immune system. Although NK cell activity had already declined to baseline at the time of tumor inoculation, Ab-mediated depletion studies revealed that CD4 cells as well as the presence of, but not the activation of, NK cells were crucial to the prevention of lung metastasis. These results are the first demonstration of the efficient, sustained, NK/CD4-cell-dependent inhibition of lung metastasis via bolus administration of virally activated DCs. Our results

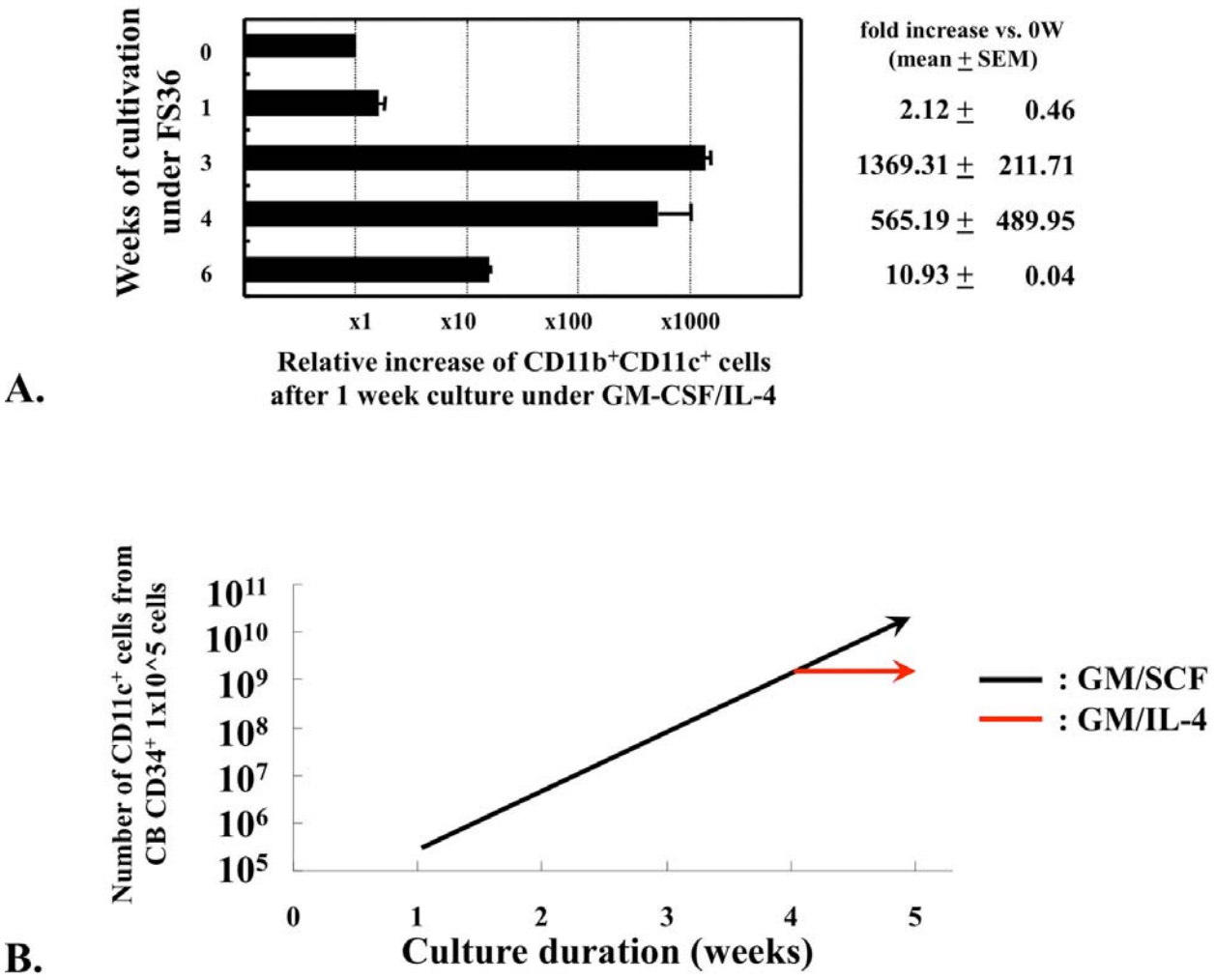

Figure 3. Log-scale expansion of murine/human DCs. A). Murine; bar graph indicating relative increase of $CD11b^+CD11c^+$ DCs yielded after 1-week cultivation under GM/IL-4 in use of expanded hematopoietic progenitors at each time point. The data include three independent experiments. Note that 3-weeks' expansion was optimal, over 3 logs, for efficient production of $CD11b^+CD11c^+$ DCs. The figure is adopted from reference number 53. B). Human; kinetics of the number of CD11c-positive cells from $1x10^5$ cord blood CD34-positive cells under GM-CSF and SCF (black line) and under GM-CSF and IL-4 (red line). The figure is under preparation.

may suggest a potential new mechanism of DC-based immunotherapy for advanced malignancies (29).

Malignant ascitis (MA) is a highly intractable and immunotherapy-resistant state of advanced gastrointestinal and ovarian cancers. Using a murine model of MA with CT26 colon cancer cells, we here determined that the imbalance between the vascular endothelial growth factor-A/vascular permeability factor (VEGF-A/VPF) and its decoy receptor, soluble *fms*-like tyrosine kinase receptor-1 (sFLT-1), was a major cause of MA's resistance to dendritic cell (DC)-based immunotherapy. We found that the ratio of VEGF-A/sFLT-1 was increased not only in murine but also in human MA, and that rSeV/dF-mediated secretion of human sFLT-1 by DCs not only augmented the activity of DCs themselves but also dramatically improved the survival of tumor-bearing animals associated with enhanced CTL activity and its infiltration to peritoneal tumors. These findings were not seen in immunodeficient mice, indicating that a VEGF-A/sFLT-1 imbalance is critical for determining the antitumor immune response by DC-vaccination therapy against MA (unpublished data).

Moreover, SeV-loaded tumor peptides made DCs efficiently present antigens with MHC class I. Thus, by selecting loaded genes according to tumor status, SeV/DCs will show the potential to treat various malignancies.

8. PERSPECTIVES

DC-based immunotherapy has proven to be feasible and effective in some patients, and the cases of this therapy has been increasing. However, complex issues must be solved to standardize DC-immunotherapy. The

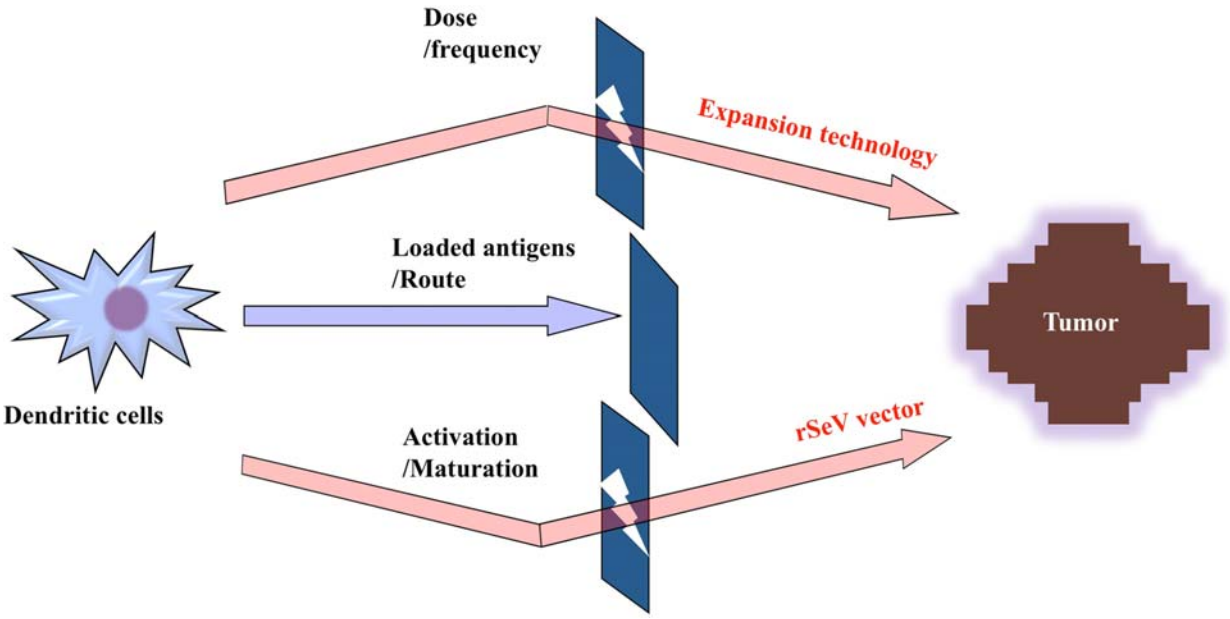

Figure 4. Schematic representation of our studies. Our new technologies overcome some of the issues of DC-based immunotherapy for malignancies.

lack of clinical effectiveness of currently available DC-vaccines should not be interpreted to mean that DC-vaccine approaches reach an investigational limit. Rather, it emphasizes the need for profound changes in the application of this approach.

We have established new methods to solve the possible critical issues underlying DC-based immunotherapy. Recombinant SeV vectors activate DCs for a sufficient antitumor effect and the DC expansion method yielded sufficient number of DCs. Moreover, we revealed that $CD4^+$ T cells and NK cells must exist spontaneously to protect the lung from cancer metastasis with DC vaccination. Then, based on these new technologies and the new findings, we should optimize DC-vaccine and enhance the development of the immunotherapy. In addition, future clinical studies should utilize standard criteria for clinical response and require validation in increased numbers of patients.

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