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13. ABSTRACT (Maximum 200) The major objective of this project is to establish a new modality for the treatment of breast cancer that employs the combination of chemokine gene-modified fibroblasts with breast tumor-pulsed dendritic cells (DC) to both recruit and/or concentrate from the periphery low frequency immune reactive T cells as well as to potently stimulate these effector cells once localized at the vaccination site. During the second year of this four-year project, studies were focused on three major areas either specified in the Statement of Work or in response to issues raised in the original Peer Review Panel Report: 1) to complete in vitro optimization of human DC generation and function; 2) to obtain high level chemokine cDNA expression in autologous breast cancer fibroblasts; and 3) to establish a relevant breast tumor model in mice to test effective vaccine strategies based on dendritic cells and chemokines. The first and third areas have been highly successful and have (to date) resulted in three (3) publications. We have also made new progress in the second area; we have moved away from retroviral supernatant transduction to particle-mediated (gene gun or "biolistics") gene transfer to obtain more reliable and higher levels of chemokine production by fibroblasts. Lastly, we have altered our original proposed plan of attack and provide alternative new approaches for Technical Objectives 1 and 3 as a result of certain unforeseen limitations in obtaining adequate tumor and blood samples from breast cancer patients, which are detailed. Collectively, the data and appended publications provided in this second annual report demonstrate steady progress and productivity toward meeting the overall goal of the funded research.				
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FOREWORD

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James F. Monti 09/21/98
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Introduction

Immunotherapies that have employed the adoptive transfer of lymphokine-activated killer (LAK) cells, tumor infiltrating lymphocytes (TIL), or tumor draining lymph node (TDLN) cells in combination with the systemic administration of interleukin-2 (IL-2) have resulted in the regression of several types of tumors in both humans and animals (1-4). In certain patients, responses have been observed in both renal cell carcinoma and melanoma. Few attempts, however, have been made to utilize this form of therapy for tumors of other histologic types, including those most prevalent in the overall patient population and often considered to be "poorly immunogenic". Breast cancer is an histology that falls within this category.

It has been shown that T cells from some patients with breast cancer can specifically react to autologous tumor as measured in vitro by either cytotoxic activity or the release of cytokines (5-8). This finding has recently led to the molecular identification of breast cancer-associated antigens or peptides, e.g., MUC-1 (6,7) and HER2/neu (8), that are recognized by autologous T cells from cancer patients. Although this rather new information raises the intriguing possibility for immune interventions in the treatment of breast cancer, the overall level and incidence of detectable T cell responses in these patients are rather low. This limitation could be due, in part, to: a) tumor-induced, active immune suppression (9) and/or defects in T cell signaling (10) in the cancer patient, which may actively down regulate the functional activity of antitumor effector cells. Recent data in rodents suggest that the latter deficit may be overcome by immunization with tumor cells that have been molecularly-engineered to secrete IL-2 (11); b) low incidence of antigen-reactive T cells. Although precursor CTL with activity against whole tumor cell targets or purified tumor peptides have been detected in peripheral blood and lymphoid tissue of some patients, by limiting dilution analyses their frequency is either absent or difficult to detect (12); and c) failure of host-derived T cells to recognize antigens on the tumor cell surface itself. In this regard, we have described significant inherent defects in antigen processing and presentation by certain human solid tumors that could be a crucial mechanism for their inability to stimulate the afferent arm of the immune response (13).

Recent attempts to improve upon immunotherapy for human cancers include the genetic-modification of TIL to express exogenous genes encoding for antitumor cytokines (1-2, 14) or new "chimeric" receptor genes to redirect tumor antigen specificity (15), as well as the utilization of CTL-defined tumor peptides (7,8,16) and gene-modified tumor cells (17-19) as immunogens to generate more potent TIL or TDLN or to impact directly on established metastatic disease by serving as "therapeutic vaccines". We have been investigating novel approaches to enhance the activity of tumor vaccines in order to increase the frequency of tumor-reactive T cells, to overcome tumor-induced immune suppression, and to stimulate potent primary and secondary responses to poorly-immunogenic tumors. Our recent studies have involved the use of either dendritic cells (DC) as potent antigen presenting cells (APC) or certain chemokines as potent immune cell chemoattractants.

Molecularly-engineered tumor cell vaccines have been shown in some published reports to be effective in reducing the size of preestablished tumor masses in rodents, but the issue of potency of tumor vaccination has become an important one. Most of the human clinical trials currently underway in gene therapy for a variety of different cancers involve introducing one of several cytokine genes into either tumor cells or autologous fibroblasts. The rationale for these trials is that, when reintroduced, these genetically modified cells will serve as sites of cytokine production and thus enhance immunity by several different mechanisms (depending on the cytokine of choice). To date, these include IL-2, IL-4, IL-7, IL-12, TNF- α , IFN- γ , and GM-CSF. However, the ability to induce tumor regressions or inhibit metastases by cytokine/tumor vaccines has been shown in a number of preclinical animal studies to be overcome by larger tumor cell inocula or by prolonging the period of time between tumor establishment and subsequent tumor/cytokine vaccination.

DC are highly potent APC of bone marrow origin (20,21), which have been shown to stimulate both primary and secondary T and B cell responses (22,23). Animal studies have indicated that DC are preferentially responsible for sensitization of naive T cells in their first exposure to antigen (24). Antigen distribution in

the host environment often favors uptake and presentation by DC rather than macrophages or B cells (25), and subsequent migration of primed DC to lymphoid organs enhances targeted presentation of antigens to the immune system (26). Antigen-pulsed DC have been used successfully in culture to sensitize naive mouse CD4⁺ T cells to a variety of antigens (27). When DC are cultured with exogenous proteins in vitro, presentation of antigen in a major histocompatibility (MHC) Class II context is favored (28), but a variety of treatments including peptide pulsing enables cultured DC to present antigen in a MHC Class I context as well (28,29). In addition, tumor-pulsed DC-rich preparations have been used successfully to treat established mouse tumors in vivo (30-32).

That MHC Class II-bearing APC primarily stimulate CD4⁺ T cells is of particular interest, since in several murine tumor models antitumor CD4⁺ T cells have proven capable of mediating tumor rejection or conferring protective immunity (33-35) and a human CD4⁺ T cell defined tumor antigen has been recently defined (36,37). In the animal models, the successful culture of antitumor CD4⁺ T cells has relied on immunization of rodents against a tumor or purified tumor protein and subsequent in vitro restimulation of sensitized T cells with macrophages or spleen cells pulsed with a purified tumor protein (34-38). Such strategies depend upon the availability of purified proteins; however, specific tumor rejection antigens are not yet known for many human tumors, including those of breast origin. Furthermore, the study of CD4⁺ T cells has been impeded because of the difficulty in expanding these cells in vitro. In contrast, it has proven far easier to expand antitumor CD8⁺ T cells, such as in the majority of human and mouse TIL studies. Recently, however, we have successfully developed a culture system to study the properties of antitumor CD4⁺ T cells that employ DC as APC. Our data have shown that relatively crude membrane preparations of tumor cells will suffice as sources of tumor antigen, avoiding the necessity for molecular identification of the tumor antigen for effective immunization (39-41). We have now identified for the first time specific CD4⁺ T cell reactivity to tumor cells both in the mouse and human (39-41). Moreover, methods are now available to generate sizable numbers of highly-enriched DC, both in humans and in rodents, by culturing progenitor cells in the presence of GM-CSF, TNF- α , and/or IL-4 (42-48). The establishment of DC cultures from the peripheral blood of adult patients has raised the very important possibility of now using these cells as immunotherapeutic agents for the treatment of breast cancer (30).

With respect to cytokine gene-modified tumor cells, much of the work to date has employed interleukins, interferons, TNF- α , and hematopoietic colony stimulating factors (17-19,49). Another class of cytokines which has more recently received attention, is the chemokines (or chemoattractant cytokines). Chemokines are essential for leukocyte trafficking and inflammatory processes and share structural similarities, including four conserved cysteine residues which form disulfide bonds in the tertiary structures of the proteins (50). Traditionally, the chemokine superfamily has been divided into two subgroups: C-X-C (where X is any amino acid) and C-C, according to whether an intervening residue space the first two cysteines in the motif (50). This structural distinction has been shown to delineate a general distinction in the biological properties of these molecules: most C-X-C chemokines are chemoattractants for neutrophils but not monocytes, whereas C-C chemokines appear to attract monocytes but not neutrophils (50). Of importance, the C-C group has also been shown to be chemoattractant to lymphocytes. For example, the C-C chemokine RANTES is a chemoattractant for memory T cells in vitro (51) and human macrophage inflammatory proteins-1 α and -1 β (MIP-1 α , MIP-1 β) have been found to be chemoattractant for distinct subpopulations of lymphocytes including naive T cells and B cells (52). Recent evidence suggests that the C-C chemokine MCP-1 induces T cell migration as well (53). It should also be noted that natural killer (NK) cells migrate vigorously in response to RANTES, MIP-1 α , and MCP-1 (50). In addition to chemoattraction, RANTES has been recently shown to activate T cells (54,55), and many of the C-C chemokine members increase the adhesive properties of the cells for which they are chemoattractant (50,56). The discovery of a new protein suggests that the superfamily now has an additional branch, the 'C' branch. Lymphotactin, a molecule isolated from pro-T cells, clearly lacks the first and third cysteines in the four cysteine motif, but shares a great deal of amino acid similarity at its carboxyl terminus with C-C chemokines (57,58). Of importance, lymphotactin is the only superfamily member to date to be selectively chemotactic to lymphocytes only, as it does not attract either monocytes or neutrophils (57,58).

We have examined one of the C-C chemokines RANTES for its properties in vivo using a murine tumor model (59). We have shown that immunogenic murine tumor cells that stably produce human RANTES chemokine after gene-modification lose their ability to form solid tumor masses in vivo. Furthermore, this loss of tumorigenicity correlates with in vitro chemoattraction of tumor-specific T cells and appears to be mediated in vivo by various host-derived immune cells since the tumorigenicity of RANTES-secreting cells is restored when CD8⁺ and CD4⁺ T cells are depleted or when murine macrophage migration is inhibited (59). Thus, this study represents the first analysis of the functions of RANTES as produced from an in vivo source, and shows that the chemoattractant properties of this chemokine for monocytes and T cells as predicted from in vitro assays using human cells appear to be broadly relevant in this in vivo murine model. Other C-C chemokines, namely MCP-1 and murine TCA3, have been recently shown to inhibit in vivo tumor growth as well (50, 60).

Given this background, this funded research proposal focuses on a series of studies to determine whether molecules potentially and selectively chemotactic for naive and memory T cell subsets can be used in conjunction with tumor-pulsed DC to provide a highly effective means of both detecting and augmenting the immune response to breast cancer.

Technical Objectives and Timelines

The following Technical Objectives and their corresponding timelines were specified in the original funded grant application:

1. To evaluate the capacity of human dendritic cells to detect T cell specific responses to autologous breast tumor in vitro (Months 1-48).
2. To generate high, stable chemokine producer cells by the introduction and expression of the relevant genes in human fibroblast preparations (Months 1-36).
3. To determine the capacity of the combination of chemokine-secreting cells and dendritic cells pulsed with autologous breast tumor to detect, attract, and augment specific, antigen-reactive T cells in vitro (Months 12-48).

Body (Methods, Results, Discussion)

A. Brief Summary of Results Reported in the Previous (Year 1) Annual Report:

1. We conducted studies to best generate "cytokine-driven" (GM-CSF, IL-4, and TNF- α) human DC obtained from peripheral blood as measured by yield, purity, phenotype, and function as originally proposed in Technical Objective 1.
2. We demonstrated that autologous DC could be successfully derived from the peripheral blood of advanced breast cancer patients, which, upon antigen pulsing, could stimulate potent tetanus toxoid- and KLH-specific proliferative responses by purified T cells obtained from these same patients.
3. The above findings considerably lessen the potential concern of a compromised immune system in advanced cancer patients (as a result of multiple chemotherapy/radiation therapy regimens that are also immunosuppressive) hampering attempts to clinically develop and utilize dendritic cell-based vaccines in our proposed breast tumor immunization approaches.

4. We have successfully expressed three (3) distinct chemokine cDNAs (i.e. RANTES, lymphotactin, MIP-1 β) constructed into the MFG-based retroviral vector as originally proposed in Technical Objective 2. Moreover, transduced fibroblasts produced significant levels of biologically active chemokine(s) by the introduced transgene(s).

B. Results for the Current (Year 2) Annual Report:

The research conducted during the second year of this four-year award continued to concentrate mainly on experiments proposed in Technical Objectives 1 and 2. Therefore, the laboratory effort has attempted to cohere to the original timetable (i.e. Statement of Work) provided in the grant application. All data figures and tables referred to in the text below are provided in the Appendix section of this annual report. Our publications (abstracts and manuscripts) to date as a direct outcome of the studies supported by DOD funding are listed in the Bibliography section. In addition, abstracts of these publications are provided in the Appendix section.

(a) Technical Objective 1: Our efforts in this aim continued to focus on completing the optimization of the generation/production of human dendritic cells (DC) to serve as potent antigen presenting cells (APC) in order to best detect and enhance low level specific T cell responses in vitro. We found that the addition of TNF- α to GM-CSF and IL-4 (at a critical timepoint of culture) resulted in both a significantly greater yield (at least two-fold) of DC and more potent antigen presenting function. These studies appear as publications in the Bibliography section as references 1 and 5.

We also attempted a series of initial experiments to begin to evaluate the capacity of human DC to detect T cell specific responses to autologous breast tumor in vitro. Here we have come up against obstacles that have hampered progress. We will first describe these obstacles and then provide our alternative approaches with relevant supportive preliminary data. From our studies reported in last year's annual report with the defined antigens TT and KLH, it became clear that successful "education" of autologous T cells by antigen-pulsed dendritic cells required multiple restimulations in vitro to elicit antigen-specific reactivity. Multiple in vitro restimulations of T cells in these cultures required adequate amounts of peripheral blood-derived T cells and DC as well as antigen(s), which have become unforeseen limitations as we moved our efforts away from the defined antigens TT and KLH to actual breast cancer tissue. Adequate amounts of tumor (to serve as lysates) for the pulsing of DC have required surgical removal of at least 1-2 grams of viable tumor from patients. Upon disaggregation of the solid tumor masses by enzymes, the viable yields of dispersed cells are low due to its fibrous nature. Moreover, adequate amounts of autologous T cells for multiple in vitro restimulations by antigen-pulsed DC have required patients to undergo a leukapheresis procedure to obtain a sufficient number of PBMC. To date, it has not been possible for us to obtain adequate amounts of both tumor and PBMC from the same donor breast cancer patient to conduct the experiments as originally outlined in Technical Objective 1. A compounding problem has been the low level of willingness of identified breast cancer patients to agree to provide surgical tumor specimens and undergo a follow-up leukapheresis for research purposes only (since there would be no treatment benefit to the patient). Nevertheless, we are committed to continue this arduous process of identifying suitable breast cancer patients for this in vitro work. In this regard, we will also attempt to establish and grow human breast tumors in SCID mice. Dr. Ethier, a co-investigator on this DOD project, has established a collaboration with Dr. Elizabeth Repasky's group at RPMI who has pioneered this successful technique (Sakakibara et. al, "Growth and Metastasis of Surgical Specimens of Human Breast Carcinomas in SCID Mice", Cancer J. Sci. Am. 2: 291-300, 1996). If successful in our attempts, we may be able to overcome the current limitation of adequate human breast tumor tissue to serve as lysates for pulsing onto DC.

Given the issues discussed above, we have pursued a new strategy that should allow for important progress to take place in a timely manner. As a source of tumor antigen(s) to pulse onto DC we will instead utilize known HER 2/neu and CEA peptides, which have been reported to elicit specific T cell reactivity in breast cancer patients. The peptides are detailed in Table 1. Our new strategy is to employ these peptides to generate specific CTL in vitro by restimulation with peptide-pulsed autologous DC. The CTL will then be

examined for reactivity against the relevant peptide-expressing target cells (i.e. T2) as well as HER 2/neu and/or CEA expressing human breast tumor cell lines. If peptide specific T cells are successfully generated, these effectors would then be tested for reactivity against breast tumor cell targets from patients. Since the latter would only serve as targets (and not as a source of antigen to pulse onto DC for the multiple T cell restimulations), adequate fresh surgical specimens of breast tumor should become available from HLA-A2+ patients. The chosen peptides (in Table 1) are indeed HLA-A2 restricted in their presentation by antigen presenting cells, necessitating the need for us to first screen the donors of leukapheresis (to serve as haplotype-matched sources of T cells and DC). To date, we have been successful in obtaining leukaphereses from 5 breast cancer patients (but who nevertheless have inadequate amounts of tumor). As shown by the FACS profiles in Figure 1 the frozen then thawed PBMC of 3 of these 5 patients were indeed HLA-A2+ upon monoclonal antibody staining. Thus, we have sufficient HLA-A2+ PBMC to both generate DC and provide autologous T cells. Moreover, three (3) HER 2/neu-expressing human breast cancer cell lines (denoted LSS, SUM 225, and SUM 229) were examined for HLA-A2 positivity. As shown by the FACS profiles in Figure 2, 2 of these 3 lines were indeed HLA-A2+ upon monoclonal antibody staining. Thus, we have relevant human breast tumor targets available to evaluate T cell activity against the known expressed peptides.

Our initial effort to generate specific CTL has been successful. In this experiment, DC were first generated from the PBMC of an HLA-A2+ breast cancer patient. As shown in Figure 3, these DC co-expressed high levels of CD86 and HLA-DR but were negative for the monocyte/macrophage marker CD14. T cells were obtained from the PBMC of this same patient. DC were pulsed overnight with the GP-2 peptide (20 µg/ml) and then used to stimulate the autologous T cells in the presence of IL-7 (10 ng/ml) for 1 week. These T cells were then restimulated twice more (7 days apart) with GP-2-pulsed DC plus additional IL-7. The T cells were then harvested and tested for CTL activity against GP-2-pulsed T2 target cells. As shown by the 2 separate experiments in Figure 4, these CTL mediated potent lysis (at relatively low effector-to-target ratios) of GP-2-pulsed, but not unpulsed, T2 target cells in a 4 hr. ⁵¹Cr-release assay. Given this success, we will now proceed to attempt to generate specific CTL to the other HER 2/neu and CEA peptides. In addition, we will also examine the capacity of these CTL to recognize and lyse the HLA-A2+ breast tumor cell lines as well as fresh tumor targets as they become available from patients. The successful generation of these CTL will also allow us to proceed with the experiments of Technical Objective 3, as originally proposed.

(b) Technical Objective 2: As stated in the Introduction, the primary goal of the funded application is to enhance the capacity of breast tumor-pulsed DC to trigger antitumor immune responses by including chemokine gene-modified fibroblasts to potently and selectively recruit specific immune cell subsets. The research directed in year two under Technical Objective 2 has continued to emphasize the construction of expression vectors containing the cDNAs encoding for five (5) distinct chemokines, transfection/transduction of fibroblasts, and analysis of chemokine production by molecular, ELISA, and/or functional assays. Our efforts continue to be highly successful. In addition to the originally proposed five (5) chemokines, we have also obtained chemokine cDNAs that have been just identified over the previous year by Albert Zlotnik and his colleagues at DNAX. These new chemokines include 6Ckine, Lungkine, Teck, GWCC, and MIP3. Of importance, these new chemokines have been shown to be highly selective in their recruitment of either DC or T cell subsets. These new chemokines will also be evaluated in our future studies outlined in Technical Objective 3.

As our efforts proceeded to utilize our MFG-based retroviral constructs to transduce fibroblasts with chemokine cDNAs, it has become apparent that our approach is not sufficient to provide reliable and efficient gene transfer into short-term, primary cultures of human fibroblasts from breast cancer patient skin biopsies (given slow cell doubling times in vitro). Thus, we have altered our strategy to utilize particle-mediated gene transfer. We have successfully introduced several chemokine cDNAs (including RANTES, lymphotactin, 6Ckine) into the expression plasmid pcDNA3.1. Using a biolistics device (BioRad), we have been highly successful in obtaining short-term lines of fibroblasts from breast cancer patients that secrete high levels of the transgene encoded chemokine for at least 7-10 days in culture. A representative experiment is shown in Figure 5. By ELISA, fibroblasts from two (2) separate breast cancer patients

produced between 30 and 40 ng of human RANTES per 1 million cells over a 24 hr. period for at least 4 days after the biolistics procedure. We are now optimizing this procedure by evaluating the parameters of distance, gold particle size, and pressure (psi) as a strategy to obtain even higher chemokine production levels.

Our successes to date in generating specific T cells to breast cancer-related peptides following stimulation by DC as well as producing autologous fibroblasts secreting chemokines should allow us in the upcoming year to embark on the studies proposed in Technical Objective 3.

(c) Additional Relevant Studies: One of the concerns raised by the reviewers in the Peer Review Panel Report was that by focusing only on in vitro studies "it is difficult to imagine how this recruitment will work in vivo". "...it is hard to imagine how one would make use of chemokine-secreting fibroblasts. Most cytokines act locally. Assuming the fibroblasts could secrete chemotactic cytokines, how or where would they be inserted into the patient to guarantee that appropriate T cells would be attracted to the tumor." In order to directly address these concerns and because our ultimate goal is to translate our findings into the design and execution of a clinical trial(s) in breast cancer patients, we have also embarked on murine studies utilizing a syngeneic breast tumor (denoted MT-901). These preclinical studies conducted over the past year are fully described in our recent publications (#3, #4, #6, #7 of the Bibliography section) and cite the support of DOD funding, including our most recent paper published in the *Proceedings of the National Academy of Sciences USA*; all abstracts are provided in the Appendix section.

As shown in Figures 6 and 7, we can now readily demonstrate that immunizations with tumor lysate-pulsed DC can mediate effective immune priming in vivo and can successfully treat established visceral lung metastases from the MT-901 breast carcinoma. These murine studies will now allow us to directly evaluate the therapeutic efficacy (and the operative mechanisms) of antigen-pulsed DC vaccines combined with chemokine-secreting fibroblasts in a relevant in vivo breast tumor model.

Lastly, we have embarked on additional human studies with direct relevance to breast cancer patients. We have begun to evaluate the potential of combining DC-based vaccines with bone marrow or peripheral blood stem cell transplantation (PBSCT) based on rationale that immunization with DC may have greater potential following transplant preparative regimens that reduce or eliminate active tumor-induced immunosuppression and lessen tumor burden. Because of the potent antigen presenting cell activity of DC, potential exists to bias the developing immune T cell repertoire towards higher frequency recognition of tumor-associated antigens through early immunization with tumor antigen(s)-pulsed DC post transplant. As shown in Figure 8 (see references #2 and #8 in the Bibliography section), we have been successful in obtaining both DC and CD34+ hematopoietic stem/progenitor cells with potent functional activity from the same leukapheresis collects from G-CSF-primed cancer patients in sufficient numbers for the purpose of combining PBSCT with DC-based immunization strategies for the treatment of breast cancer.

Conclusion/Significance

The significance of our research lies in the potential to develop a new, innovative molecular vaccine strategy for eventual use in breast cancer patients that employs chemokine gene-modified fibroblasts combined with tumor-pulsed dendritic cells to both recruit/concentrate relevant immune populations at the vaccination site (by secreted chemokines) as well as to activate the recruited T cells by potent presentation of tumor-associated antigens (by dendritic cells). This strategy may prove to be a highly effective means of both detecting and augmenting the immune response to poorly-immunogenic breast tumors that ultimately leads to tumor eradication.

Plans

The upcoming year will be the third year of the four-year support for this research project. Given the very promising dendritic cell-based vaccine results with the preclinical animal model utilizing the MT-901 breast tumor, we plan to directly evaluate *in vivo* for the first time the combined effect of added chemokine-gene modified fibroblasts on antitumor activity. We will continue our early, but very promising, *in vitro* studies of generating HER2/neu (and CEA) peptide-specific T cells generated by restimulation of antigen(s)-pulsed autologous DC *in vitro*. We will continue to optimize chemokine gene transfer into fibroblasts from breast cancer patients to achieve the highest level of production of the relevant chemokine(s). We will then initiate the combination approach of chemokine gene-modified fibroblasts and tumor-pulsed DC *in vitro* and begin to dissect the underlying mechanisms of antitumor T cell reactivities and chemoattraction observed (i.e. Technical Objective 3). We also hope to increase our knowledge of the mechanisms of T cell activation, recognition, and destruction of poorly-immunogenic tumors. Finally, we believe our human and murine experimental data will be sufficiently compelling to warrant the design and execution of a spin-off phase I clinical trial in patients with advanced breast cancer.

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TABLE 1

**HLA-A2 Restricted, CTL-Defined Peptides in
Human Breast Cancer**

Peptide	Sequence
Her-2/neu p369, E75	KIFGSLAFL
Her-2/neu p773	VMAGVGSPYV
Her-2/neu GP2	IISAVVGIL
CEA CAP-1	YLSGANLNL

Figure 1. FACS analysis of HLA-A2+ PBMC from leukapheresis of Breast cancer patients.

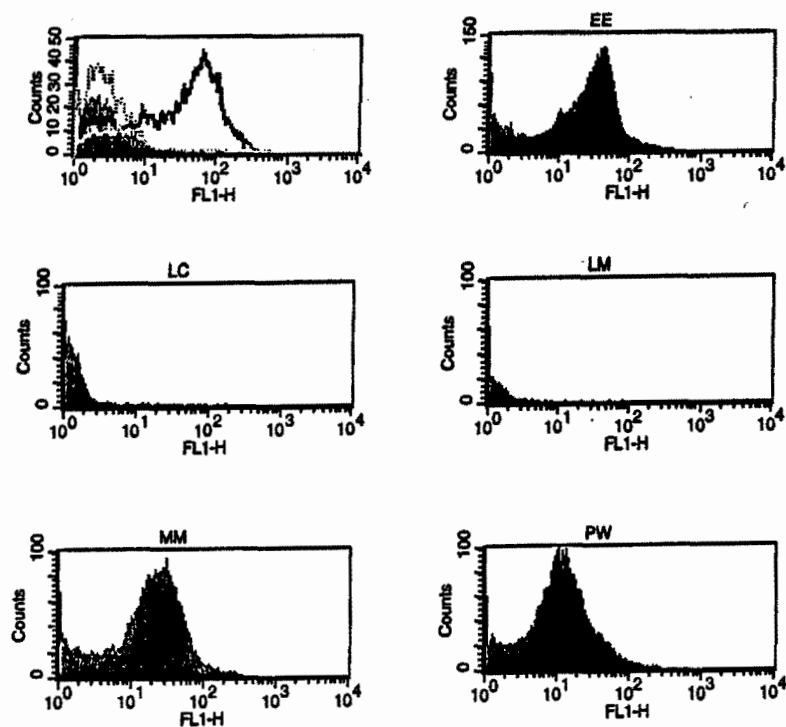


Figure 2. FACS analysis of HLA-A2+ Breast tumor cell lines.

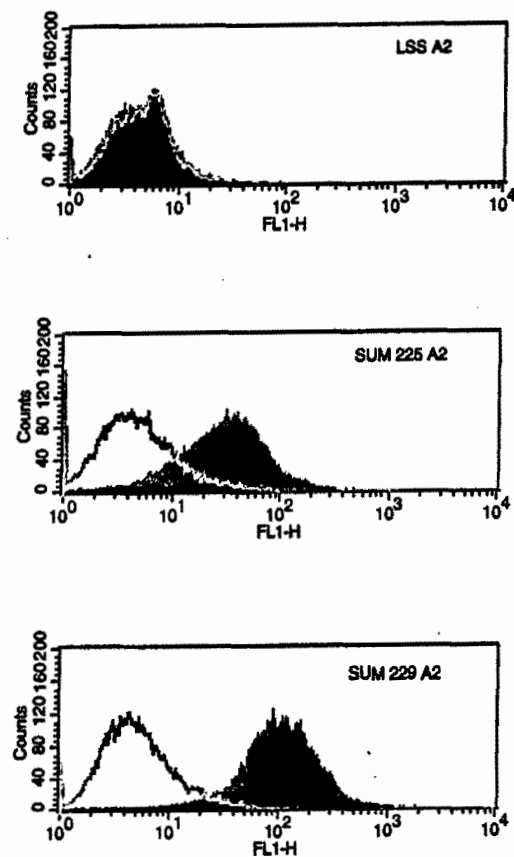


Figure 3. Generation of DC from PBMC of HLA-A2+ Breast cancer patient.

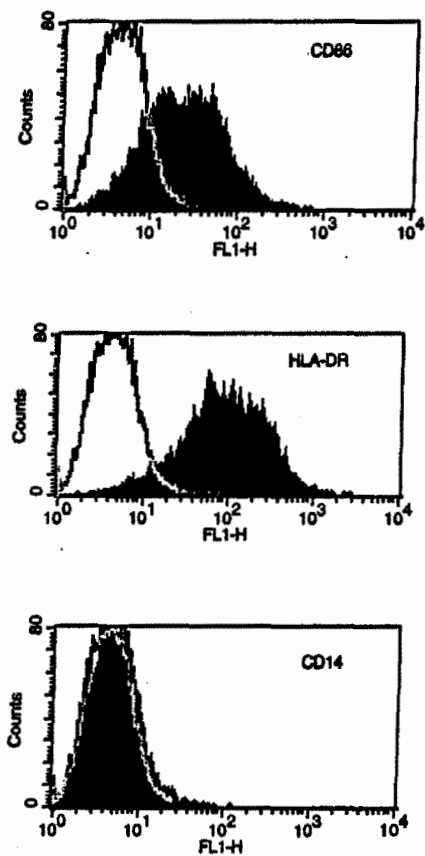


Figure 4. Generation of HER 2/neu peptide specific CTL by peptide-pulsed DC stimulation in vitro

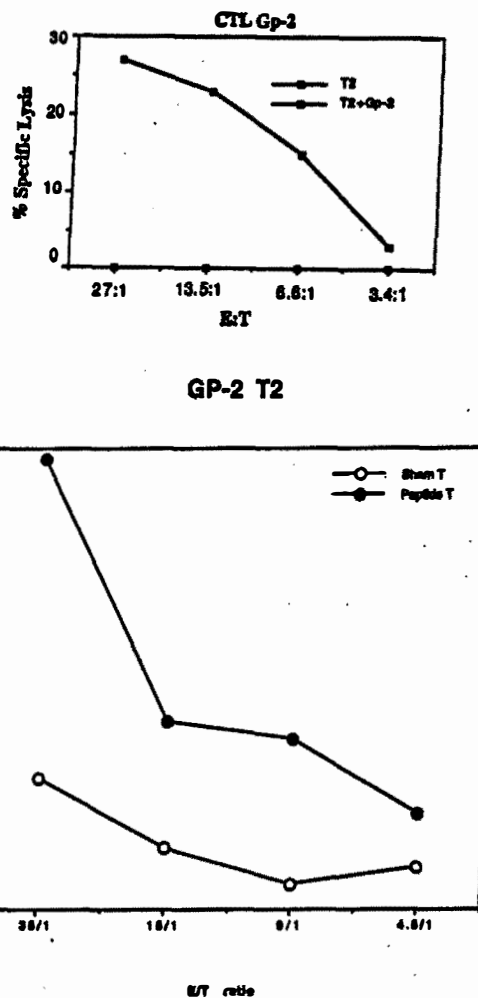
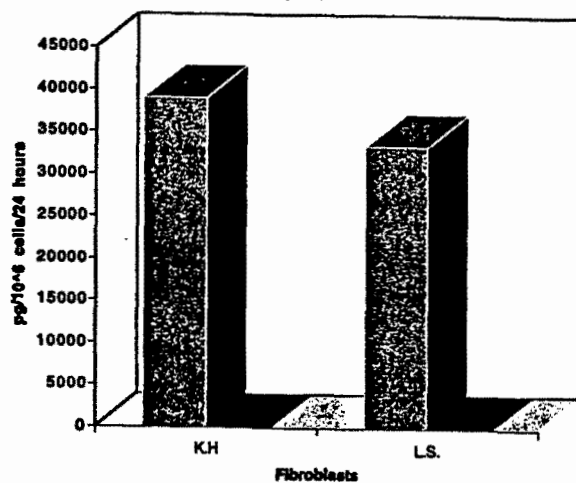


FIGURE 5
RANTES Expression by Fibroblasts from Breast Cancer Patients
Following Gene Therapy by Biolistic
(Day 4)



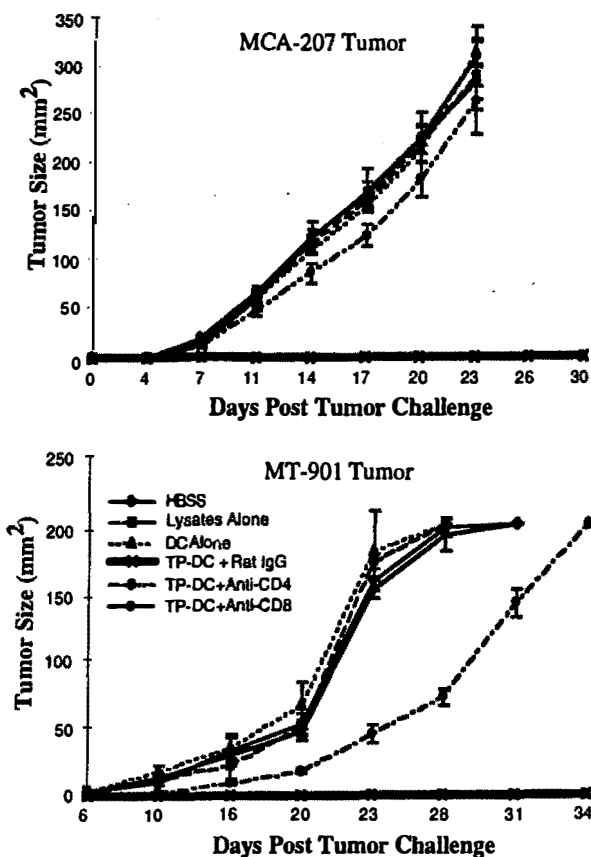


FIG. 6. Immunization with tumor lysate-pulsed DC protects B6 (Upper) and BALB/c (Lower) mice from a subsequent lethal challenge with syngeneic MCA-207 sarcoma and MT-901 breast carcinoma cells, respectively, and is T cell mediated. B6 and BALB/c mice were immunized s.c. with or without chronic depletion of CD4⁺ or CD8⁺ T cells, as described in *Materials and Methods*. Data are reported as the average tumor area $\pm$ SEM of five or more mice per group.

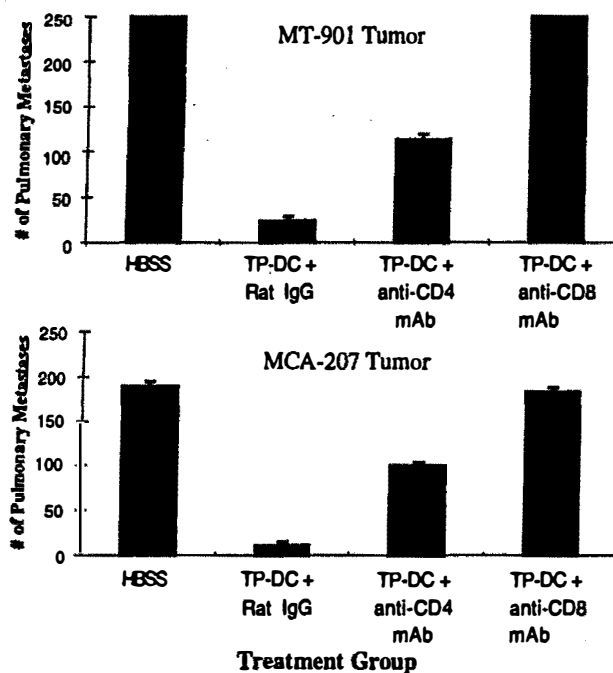
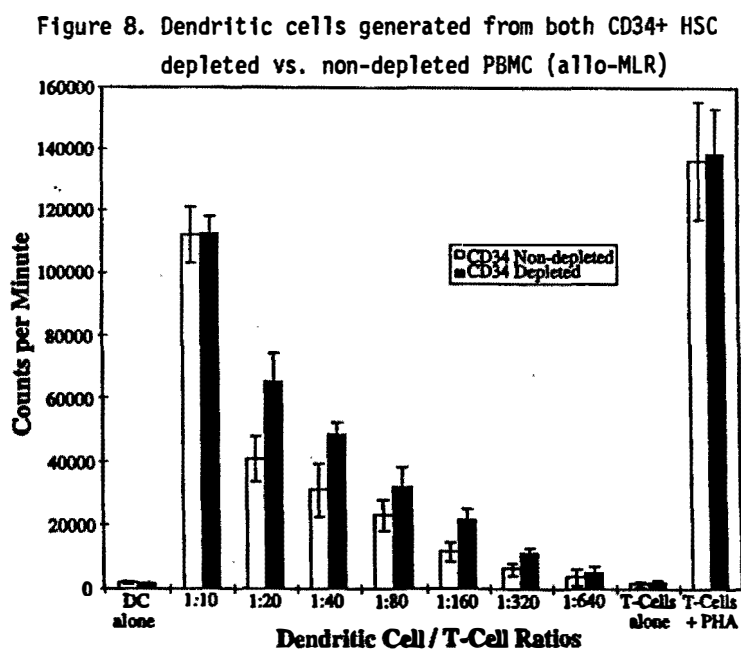


FIG. 7. Immunization with tumor lysate-pulsed DC mediates regression of established pulmonary metastases via host-derived T cells. Induction of tumor nodules and their treatment are detailed in *Materials and Methods*. Values represent mean number of metastases $\pm$ SEM of five or more mice per group.



2395

454-IV

Ex vivo expansion of myeloid and megakaryocytic progenitors using HS-5 stromal cell conditioned media plus promegapoietin. J. Lin, E. Nachtrieb, W. Bensinger, P.R. Streeter, J.G. Giri, C.M. Baum, J.P. Mc Keam and B. Roeklein. *Department of Transplantation Biology, Clinical Cryobiology, Fred Hutchinson Cancer Research Center, Seattle, WA; Searle R & D, Monsanto Co., St. Louis, MO.*

By combining conditioned media from a human bone marrow derived stromal cell line (HS-5CM) with the novel dual IL-3 and c-mpl receptor agonist Promegapoietin (PMP), we generate both myeloid and megakaryocytic progenitors measured as BFU-E, CFU-G/GM/M and CFU-MK, respectively. We previously demonstrated that PMP or KL yield maximal myeloid progenitor production from G-CSF mobilized peripheral blood CD34⁺ cells and that both treatments yield a production plateau at day 12. The expansion products differ in that increases in CFU-MK production are only detected with HS-5 plus PMP, with maximal production occurring on days 7-9. Moreover, expanded cells from PMP yield a 10-15 fold increase in megakaryocyte lineage committed cells (i.e., CD61 expressing cells). These observations are consistent with the expected biological properties of PMP. To further assess the potential of PMP in this system, a comparison was made between HS-5CM supplemented with PMP, the combination of IL-3 and TPO, or either molecule separately. Nucleated cell production was additive with the combination of IL-3 and TPO (83 fold) when compared to either factor separately. By contrast, the addition of PMP to HS-5 CM resulted in a synergistic effect on nucleated cell output, yielding a 122 fold increase in nucleated cells. HS-5CM supplemented with either PMP or the combination of IL-3 plus TPO resulted in the generation of similar numbers of myeloid progenitors and both combinations generated significantly more progenitors than HS-5CM supplemented with IL-3 or TPO alone. Further analysis revealed that the addition of either PMP or IL-3 plus TPO to HS-5CM generated cellular products containing similar percentages of CD61⁺ cells (10.4 ± 1.6 vs. 12.2 ± 1.4%, respectively). The products differed, in that 50% fewer PMP generated CD61⁺ cells had a ploidy ≥ 8N. These data indicate that treatment with PMP generates similar numbers of CD61⁺ cells. However, exposure to PMP yields fewer cells that have undergone terminal differentiation into endomitosis. Thus the combination of HS-5 plus PMP may be ideal for cell based therapies to reduce the duration and nadir of thrombocytopenia and neutropenia.

2396

455-IV

Optimized megakaryocyte expansion in a serum-free medium. Phil Lefebvre, Jane N. Winter, Judith G. Giri, Larry E. Kahn and Isaac Cohen. *Northwestern University, Chicago, IL; Searle R & D, St. Louis, MO.*

CD34⁺ cells were obtained from three hematopoietic sources, bone marrow (BM), peripheral blood progenitor cells (PB) and umbilical cord blood (UC). The cells were cultured for 12 days with 10 ng/ml thrombopoietin (TPO) and/or 10 ng/ml IL-3, or 200 ng/ml promegapoietin (PMP, Searle), a chimeric protein that is a multifunctional dual agonist of c-mpl and human IL-3 receptor. Cell expansion was evaluated in either human serum supplemented (HS) or serum-free liquid medium (SF) and megakaryocyte (MK) production measured. A synergistic increase in MK production in HS with co-addition of IL-3 and TPO was observed in both BM and PB (BM = 3.5 ± 0.5 × 10⁵ MK/ml ± SEM; PB = 1.7 ± 0.7 × 10⁵ MK/ml) when compared to TPO alone (BM = 0.4 ± 0.1 × 10⁵ MK/ml; PB = 0.1 ± 0.1 × 10⁵ MK/ml) or IL-3 alone (BM = 0.7 ± 0.1 × 10⁵ MK/ml; PB = 0.4 ± 0.2 × 10⁵ MK/ml). PMP induced an increase in MK production for BM and PB in HS that was equivalent to TPO + IL-3 (BM = 3.9 ± 0.5 × 10⁵ MK/ml; PB = 1.5 ± 0.7 × 10⁵ MK/ml). Culturing BM or PB cells in SF resulted in a two-fold increase of MK yield compared to HS. Umbilical cord MK production was more dependent on the culture medium used. The effect of TPO and IL-3 was synergistic in HS (26.6 ± 7.1 × MK/ml) compared to TPO alone (11.4 ± 1.5 × 10⁵ MK/ml) and IL-3 alone (4.4 ± 3.5 × 10⁵ MK/ml) but additive in SF (TPO + IL-3 = 18.2 ± 1.5 × 10⁵ MK/ml; TPO alone = 13.0 ± 1.0 × 10⁵ MK/ml, IL-3 alone = 2.2 ± 0.1 × 10⁵ MK/ml). The converse was true for PMP. Phenotypic analysis of the initially seeded CD34⁺ enriched cell population showed a higher frequency of CD34⁺/41⁺ cells in BM (4.0% ± 1.8%) than PB (1.6% ± 1.5%) or UC (1.8% ± 1.1%). In a collagen-based SF clonogenic assay with IL-3 and TPO, UC produced larger and significantly greater numbers of BFU-MK (2.7 ± 0.6) and CFU-MK (3.4 ± 0.9) colonies/seeded CD34⁺/41⁺ cell, than BM or PB. Comparable clonogenic activity was observed with PMP. Although BM CD34⁺ cells have a higher frequency of committed mature MK progenitors, UC CD34⁺ enriched cells have more proliferative potential due to their high percentage of immature MK progenitors and are therefore more productive in vitro. This study demonstrated that *ex vivo* expanded MKs can be obtained from bone marrow, umbilical cord or peripheral blood and may provide a thrombopoietic product for support of thrombocytopenic patients.

2397

456-IV

Ex vivo expansion of megakaryocytic colony-forming cells (Meg-CFC) from human umbilical cord blood (CB). I. Kashiwakura, D. Sotiropoulos and J.W. Adamson. *The New York Blood Center, New York, NY.*

CB is being used increasingly as a source of stem and progenitor cells for transplantation. However, a delay in the recovery of platelet counts seems to be a problem in many cases after CB transplantation. The average time for platelet counts to reach 20,000/μl is 52 days (range 29-121 days; data from the Placental Cord Blood Program of the New York Blood Center, P. Rubinstein, Director). An approach to this problem is to transplant *ex vivo* expanded progenitor cells from CB. In this project, we have studied *ex vivo* expansion of hematopoietic progenitor cells,

especially Meg-CFC, from CB-derived light-density (LD) cells or CD34⁺ cells in a serum-free liquid culture system supplemented with various cytokines, including IL-3, stem cell factor (SCF), IL-11, Flt3 ligand (FL) and thrombopoietin (TPO). To determine if these growth factors could generate Meg colonies directly, LD cells were plated in plasma clot culture at 3 × 10⁴ cells/ml and Meg colonies identified by immunofluorescence using an antibody to gp130. TPO alone stimulated 19 Meg colonies per 3 × 10⁴ LD cells. IL-11 and FL, alone or together, failed to support the growth of Meg colonies. When both FL and IL-11 were combined with TPO they gave rise to the highest number of Meg colonies (36 per 3 × 10⁴ LD cells). No synergistic or additive effect were observed by the addition of IL-3 and/or SCF to this combination. For the expansion experiments, LD or CD34⁺ cells were cultured at a concentration of 2 × 10⁵ or 1 × 10⁶/ml, respectively, with different cytokine combinations and the number of Meg-CFC determined at four to six day intervals (without medium exchange). In liquid culture of LD cells, the combination of FL + IL-11 + TPO resulted in a 100-fold expansion of Meg-CFC by day 8 to 12 of culture and about a 50-fold increase of total CFC, including granulocyte-macrophage CFC. However, this combination did not result in an increase in the total cell number. No growth factor by itself was capable of promoting expansion of CFC or total cell numbers. In the case of CD34⁺ cells, the combination of SCF + FL + IL-11 + TPO gave the greatest expansion (172 to 181-fold) of Meg-CFC by day 12 to 24. However, in comparison with the total number of Meg-CFC numbers generated in liquid culture, more Meg-CFC were recovered from the culture of LD cells as compared to the culture of CD34⁺ cells. Our results indicate that FL, along with IL-11 and TPO, are active in promoting both Meg-CFC growth and expansion in vitro. This simple expansion system, starting with LD cells, is capable of generating large numbers of Meg-CFC and could simplify expansion strategies which require preselection of CD34⁺ cells.

2398

457-IV

The delayed platelet recovery following selected PB CD34⁺ cells transplantation in children is probably due to a quantitative loss in CFU-MK number during immunoselection. M. Fouassier, N. Boiret, C. Rapatel, P. Halle, J. Kanold, J. Berger, P. Pireyre, V. Blanzat, J. Bonhomme, P. Travade, F. Demeocq and M. Berger. *S.E.H.M., Faculté de Médecine, Clermont-Ferrand, France.*

In a previous study we have analysed the capacity of immunoselected CD34⁺ PBSC to provide hematopoietic reconstitution after myeloablative therapy in 20 children with neuroblastoma (NB, n = 15) or other solid tumor (ST, n = 5) (Demeocq, ASH 1995). The myeloid recovery was similar (13 days) than the one observed after unpurified PBSC transplantation, while platelet recovery was delayed (35 days).

In order to assess effects of CD34⁺ cells immunoselection on megakaryocytic lineage, we have evaluated CFU-MK before and after selection in PBSC collection from 12 children with NB (7), other ST (4), acute lymphocytic leukemia (1). Leukapheresis products were processed using the Ceprate™ system (Cellpro). CD34⁺ cells were determined using flow cytometry. CFU-GM and BFU-E were scored in methylcellulose with growth factors. CFU-MK were assayed in a serum free methylcellulose medium as described by R. BERTHIER for bone marrow (Stem Cells, 1993, 11, 120) and modified to be available for cytopheresis products and CD34⁺ cells purified; the best cytokine combinations were: IL-6+TPO+SCF and IL-6+TPO+IL-3.

Yields of positive selection show a quantitative loss of progenitors more important for CFU-GM and BFU-E secondary to a clonogenicity decrease, subsequent to accessory cells depletion (manuscript submitted). CFU-MK clonogenicity and colony size were similar in harvest and in selected fraction. We observed a 68% loss in CFU-MK number (table 1).

Table 1	CD34 ⁺	CFU-MK	Clono.MK	CFU-GM	BFU-E
Median	10 ⁴ /kg	10 ⁴ /kg	10 ²	10 ⁴ /kg	10 ⁴ /kg
Before selection	9.42	19.29	3.16	117.67	59.72
	(0.39-29.64)	(3.27-78.40)	(0.37-21.92)	(9.90-595.35)	(1.24-385.88)
After selection	3.34	6.87	2.39	9.33	8.76
	(0.13-15.02)	(0.15-26.13)	(0.63-10.96)	(0.00-76.70)	(0.02-28.12)
Yield of selection	45.51%	31.98%		12.50%	9.24%
	(1.05-80.58)	(0.87-239.29)		(0.00-35.62)	(0.11-149.19)

Using the classical 2 × 10⁵ CD34⁺/kg threshold dose, we could propose a CFU-MK threshold dose at 4.1 × 10⁴/kg for hematopoietic reconstitution. Before selection, 11/12 (91.6%) patients have more than 4.1 × 10⁴ CFU-MK/kg and after selection only 6/12 (50%) were above this value. In conclusion, positive immunoselection induces a quantitative loss of CFU-MK which could be responsible for the delayed platelet reconstitution. We are evaluating the clinical interest of CFU-MK assay in selected PB CD34⁺ transplanted patients.

2399

458-IV

Dendritic cell-based vaccines in the setting of peripheral blood stem cell transplantation: CD34⁺ cell-depleted mobilized peripheral blood can serve as a source of potent dendritic cells. D. Choi, M. Walsh, S. Hoffmann, V. Ratanatharathorn, J. Uberti, A. Chang, K.T. McDonagh and J.J. Mulé (Intr. by L.A. Boxer). *Departments of Surgery and Internal Medicine, Blood Bank and General Clinical Research Center, University of Michigan Medical Center, Ann Arbor, MI.*

We are investigating the use of tumor-pulsed dendritic cell (DC)-based vaccines in the treatment of patients with advanced cancer. The potential of combining this strategy with PBSCT is currently being considered based on the rationale that

immunization with DC may have greater potential following transplant preparative regimens that reduce or eliminate active tumor-induced immunosuppression and lessen the tumor burden. Moreover, because of the potent antigen-presenting capacity of DC, potential exists to bias the developing immune T cell repertoire towards higher frequency recognition of tumor-associated antigens through early immunization post-PBSCT. In the current study, we evaluated the feasibility of obtaining both CD34⁺ hematopoietic stem/progenitor cells (HSC) and functional dendritic cells from the same leukapheresis collect in adequate numbers for both PBSCT and immunization purposes, respectively. Leukapheresis collections of mobilized peripheral blood (MPB) were obtained from normal donors receiving G-CSF (for allogeneic PBSCT) and from intermediate grade NHL or multiple myeloma patients receiving cyclophosphamide plus G-CSF (for autologous PBSCT). Enrichments of CD34⁺ cells were performed using the IsolexTM immunomagnetic bead cell separation device (Baxter Healthcare Corp., Deerfield, IL). We obtained enrichments of CD34⁺ cells of 87.0% $\pm$ 2.6% (n = 6) and 97.5% $\pm$ 2.7% (n = 5) from MPB of normal donor and cancer patients, respectively. Following separation, the "negative" fraction of MPB from normal and cancer patients contained, respectively, ~0.02% and ~0.00% CD34⁺ cells (i.e. levels below the detection by flow cytometry). This fraction of cells was then subjected to plastic adherence and the adherent cells were cultured for 7 days in GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) followed by an additional 7 days in GM-CSF, IL-4 and TNF- α (10 ng/ml) to generate DC. Harvested DC represented yields of 3.9% $\pm$ 1.6% and 4.1% $\pm$ 1.4% of the initial cells plated from normal donors and cancer patients, respectively and displayed high level expression of CD80, CD86, HLA-DR, CD11c, but not CD14. This phenotypic profile was similar to that of DC derived from non-CD34⁺ cell-depleted MPB. Moreover, DC generated from CD34⁺ cell-depleted MPB elicited potent anti-tetanus (stimulation index; S.I. = 81; at 10:1 DC:T cell ratio) as well as primary allogeneic (S.I. = 60; at 10:1 DC:T cell ratio) T cell proliferative responses in vitro, which were equivalent or slightly stronger than DC derived from non-CD34⁺ cell depleted MPB (S.I. = 49; at 10:1 DC:T cell ratio). Collectively, these results demonstrate the feasibility of obtaining HSC and DC from the same leukapheresis collect from normal donors and cancer patients in sufficient numbers for the purpose of combined PBSCT and immunization strategies. We have recently initiated a phase I clinical trial of the administration of escalating doses of tumor-pulsed DC alone in patients with advanced solid tumors.

2400

459-IV

In vitro expansion and characterization of dendritic cells derived from human CD34⁺ cells in static and continuous perfusion culture conditions. D. Soligo, N. Quirici, F. Servida, L. Caneva, G. Lamorte, F. Bertolini and G. Lambertenghi Dellilieri. *Bone Marrow Transplantation Unit, Ospedale Maggiore IRCCS, Milan, Fondazione Maugeri, Pavia and Fondazione Matarrelli, Milan, Italy.*

Dendritic cells (DCs), the major antigen presenting cells capable of stimulating resting T cells in the primary immune response, can be generated and expanded from bone marrow and mobilized peripheral blood CD34⁺ progenitor cells. We examined here the effects of different cytokine combinations and culture conditions on the expansion and modulation of cell surface antigens of CD34⁺ derived DCs. Cells with dendritic morphology and expressing HLA-DR, CD1a, S100 and CD83 were maximally expanded in serum free conditions with the addition of SCF, GM-CSF, TNF- α , TGF- β and Flt3 ligand (fold increase CD1a⁺ cells = 102 $\pm$ 32 after 2 weeks of culture). CD34⁺ cells were also grown under continuous flow conditions in an artificial capillary system, where a reduced depletion of oxygen and nutrients and dilution of inhibitory factors take place. After 14 days of culture, the expansion in total cell number was lower than that of static cultures (3.3 $\pm$ 2 vs 18.9 $\pm$ 4) but the percentage of cells expressing DC markers (CD1a⁺ and CD83⁺) and co-stimulatory molecules (CD80⁺ and CD86⁺) was considerably higher; on the other hand the % of CD14⁺ cells was significantly reduced (8.9 $\pm$ 2 vs 26 $\pm$ 13 in the static cultures). DCs expanded in flow cultures maintained low level of DC precursors (CFU-DC: 10.4 $\pm$ 1.2 vs 37 $\pm$ 12 in the starting CD34⁺ population) and of LTC-IC (19 $\pm$ 26 vs 470 $\pm$ 19) up to day 14. In addition, DCs generated in flow conditions stimulated the MLR more than cells grown in static cultures and than unmanipulated bone marrow mononuclear cells. By electron microscopy, cells grown in the continuous flow system showed an increased number of large cells with numerous dendritic processes and abundant multilamellar complexes. Cells expanded in these conditions were easily sorted on the basis of the light scatter properties into two fractions: the first consisted of "large cells" with increased internal complexity and containing a predominance of CD1a⁺, S100⁺, CD83⁺, CD80⁺, CD14⁺ cells, consistent with mature DC; the second fraction contained "small cells" either CD33⁺/CD14⁺ (monocytes), CD33⁺, CD15⁺ (granulocytes) or CD33⁺/CD13⁺/CD14⁺ (DC/monocytic precursors). DCs generated and selected with this method are homogeneous and pure and may be usefully utilized for immuno-therapeutic protocols.

2401

460-IV

In vitro differentiation of culture-derived dendritic cells obtained from CD34⁺ umbilical cord blood. S.L. Smith, M.V. Bennett, D.N. Liebowitz, R. Moldwin, T. Tyler, D.J. Vanderweele, T.M. Zimmerman and S.F. Williams. *University of Chicago, Department of Medicine, Section of Hematology/Oncology, Chicago, IL.*

Culture-derived dendritic cells were obtained from CD34⁺ umbilical cord blood (CB) cells following immuno-magnetic cell selection. CD34⁺ CB cells were cultured for up to 21 days in serum-free culture medium supplemented with 2% autologous plasma, GM-CSF, flt-2/flt-3 ligand, stem cell factor and TNF- α . Culture-derived CD34⁺ CB cells were evaluated for total cell expansion and dendritic cell expression using monoclonal antibodies with flow cytometry and immunocytochemistry methods. CB CD34⁺ cell achieved peak numbers of cells at 12 days of culture resulting in a 10-20x increase in total cells. Prior to culture (day 0), we found that

3-5% of CB CD34⁺ co-expressed CD86 but were negative for CD80, CD1a. On day 7 of culture, the percent of CD86 positive (+) cells had increased greater than 70% in conjunction with the co-expression of CD83. By day 14, more than 40% and 20% of the cells were CD80⁺ and CD1a⁺, respectively. Though cell numbers increased slowly after day 14 of culture, the culture-derived dendritic cells from CB CD34⁺ cells maintained high concentrations of both CD86 and CD80. In addition, culture-derived dendritic cells from CB CD34⁺ cells also expressed high concentrations of S100 (Langerhan cells) and X-11 (follicular dendritic cells). We also assessed for specific antigen functional activity by recognition of tetanus toxoid as presented by culture-derived dendritic cells to autologous T-lymphocytes. Using these culture conditions, we conclude from these in vitro studies that culture-derived dendritic cells from CB CD34⁺ cells differentiate by the sequential expression of CD86, CD83, CD80 and finally CD1a.

2402

461-IV

Ex vivo T-lymphopoiesis from umbilical cord blood. P. Wernet, G. Kögler, J. Fischer, Ch. Mahotka and H.G. Bender. *Bone Marrow Donor Center, José Carreras Cord Blood Stem Cell Program, Dept. of Gynecology and Obstetrics, Heinrich-Heine-University, Düsseldorf, Germany.*

In order to define the immunotherapeutic potential of the large quantity of naive and immature T cells in human cord blood, CB mononuclear cells were cultured within bioreactors in autologous serum containing 10ng/ml each of SCF, IL2, IL7 at a primary density of 10⁵ MNC/ml. This cytokine combination effectively shuts off any myeloid progenitor differentiation and proliferation. At day 0, +2, +5, +7 and +10 emerging T cell progenitors and subsets were analysed for differentiation and/or proliferation by three colour flow cytometry (CD34, CD117, CD25, CD3, CD4, CD8, TCR α/β). Total cell expansion was 10 fold after 10 days of culture. For already precommitted T-cells (CD4⁺, CD8⁺, CD3⁺) an 8fold expansion by proliferation was obtained. In striking contrast the regulatory T cell population (CD4⁺, CD8⁺, TCR α/β +) had expanded 1204 fold already after 7 days of culture. Most importantly the immature T cell subset (CD117⁺, CD25⁺, CD34⁺) had expanded 114 fold after 7 days. From these a predominantly TH2 population (INF γ β chain positive) could be differentiated and amplified at least 23 fold under these conditions. These purified subpopulations are subjected to molecular analysis of signalling pathways for T cell differentiation (p.e. Stat3) and of early acting genes such as Ikaros, Aiolos and Daedalus, which appear to regulate homeostasis in the hemo-lymphoid system by defining a complex regulatory protein network. This approach, in conjunction with stem cell transplantation, may open novel avenues for the development of effective T cell vaccines against viral infections (p.e. CMV, HHV8, EBV, HPV, HBV, HCV) and their associated malignancies.

2403

462-IV

B- and T-cells obtained from ex-vivo expanded bone marrow (BM) mononuclear cell (MNC) transplant recipients exhibit Ig H and TCRVB CDR3 repertoires similar to that seen after conventional hematopoietic stem cell transplantation. E. Gokmen, F.M. Raaphorst, C. Bachier, C.F. LeMaistre, T.E. Muller, R.D. Armstrong, D.H. Boldt and J.M. Teale. *University of Texas Health Science Center and South Texas Cancer Institute, San Antonio, TX; Aastron Biosciences, Inc., Ann Arbor, MI.*

B- and T cell repertoires derived from ex-vivo expanded hematopoietic progenitors after transplantation were analyzed at the molecular level using third complementarity determining region (CDR3) fingerprinting. Four patients underwent high dose chemotherapy and ex-vivo expanded BM MNC transplantation. Ex-vivo expansion was performed in continuous perfusion stromal-coated bioreactors (Aastron Biosciences, Inc.) in the presence of erythropoietin, GM-CSF/IL-3 and flt-3 ligand. Peripheral blood (PB) samples were collected at various times post-transplant up to 6 months. CD4⁺ and CD8⁺ T-cell fractions were obtained for T-cell repertoire analysis using immunomagnetic beads. The remaining CD4⁺ and CD8⁺ fractions or unseparated MNCs were used for B-cell repertoire analysis. Gene segments representing CDR3s were amplified in nested PCRs and separated on polyacrylamide gels according to size. The IgM CDR3 repertoires exhibited clonal diversity as complex as that seen in the recipients of conventional BM or PB stem cells. The IgM diversity correlated with the complexity of the multi-member VH3 family repertoire whereas the contribution of single-member VH6 family was limited early post-transplant. The VH6 repertoire became clonally diverse in one patient at 6 months. The IgG repertoires exhibited clonal diversity that appeared more complex when compared with that observed after conventional BM or PB stem cell transplants. Ig H CDR3 sizes were within adult range in all patients. Fingerprinting profiles of TCR CDR3s were also obtained by using each of the 26 TCRVB family specific primers. All VB families were represented among both CD4⁺ and CD8⁺ fractions. Few families were undetectable in individual patients without a bias against any particular family. Some families exhibited normal clonal diversity as early as 3 weeks post-transplant, while others showed limitations. There was suggestion of relative expansions in some families by darker bands observed in the profiles. These expansions were more common in the CD8⁺ fraction. No preference for a particular VB family was seen among clonally expanded bands. Interestingly, CD4⁺ T-cell repertoires appeared more complex than CD8⁺ T-cell repertoires. A normal CD4⁺ and near normal CD8⁺ repertoires were observed in a patient at 6 months post-transplant. The results show that the lymphopoiesis after ex-vivo expanded BM hematopoietic cell transplantation exhibits characteristics similar to what has been described after conventional transplants. Further studies and longer follow-up are needed to confirm improved IgG and CD4⁺ repertoires observed in this study and to determine whether lymphoid reconstitution is permanent.

Docetaxel was >1000-fold more potent in this activity. We conclude that the anticancer activity of individual cell cycle modulating agents may differ based on the molecular profile of individual cancers. (Supported in part by NCI #CA62505 and #CA63265, Lilly Research Labs and the Sheeter Foundation)

IMMUNOLOGY/PRECLINICAL AND CLINICAL 11: Immunological Approaches to Cancer Therapy

#4153 Expression of costimulatory molecules, B7-1(CD80) and B7-2 (CD86) on human gastric carcinoma. Koyama, S. Dept. of Internal Medicine, Inst. of Clinical Medicine, Univ. of Tsukuba, Tsukuba-City, Ibaraki 305, Japan.

Costimulation of T cells via B7-1 and B7-2 molecules on tumor has been shown to be important for eliciting cell-mediated antitumor immunity. We studied the expression of B7-1 and B7-2 in 24 cases of gastric carcinoma from primary locus, 16 cases of metastatic carcinoma from malignant ascites, 20 cases of benign gastric mucosa and 4 gastric carcinoma cell lines by two-color flow cytometry with mAbs, CD80 and CD86. The B7-1 and B7-2 molecules were expressed by 3 cell lines, and one cell line showed the predominant expression of B7-2 but not B7-1. Almost all patients with primary gastric carcinoma or benign gastric mucosa showed constitutive expression of the B7-1 and B7-2, revealing approximately 40% to 60% of mean positive cells. However, the percentage of B7-1 positive cells on poorly differentiated adenocarcinoma in primary lesions showed significantly lower than that of well-differentiated adenocarcinoma and normal mucosa. Furthermore, all of the metastatic carcinoma cells consistently revealed very low or undetectable levels of expression of B7-1 molecule, despite showing higher levels of expression of B7-2. Thus, it seems likely that reduced expression of B7-1 is correlated with grade of tumor differentiation, and tumor progression and metastasis. These results suggest that the B7-1 molecule on the gastric carcinoma is abrogated during the development of invasion and/or metastasis of tumor. Consequently, inadequate B7-1 costimulation may contribute to the escape of tumors from destruction by the host's immune system.

#4154 Tumor-derived heat responsive protein 12 (Hrp12): potential new neoplastic marker. Doerr, R.J., Samuel, J., Cohen, S.A. State University of New York at Buffalo, Buffalo General Hospital, Buffalo, NY, 14203 USA

Heat responsive proteins have been found to have a specific immuno-inhibitory effect in the liver. Heat-responsive protein 12 (Hrp12) was recently isolated and found to be surface bound. SDS-PAGE purification followed by sequencing of a cyanogen bromide digest of Hrp12 yielded an internal 20 amino acid polypeptide. Degenerate oligonucleotides made from this peptide were used to screen a cDNA liver library. A 1240 bp cDNA clone was obtained with an internal 529 bp open reading frame. Isolated hepatocytes and hepatoma cells were exposed to different heat shock temperature (39.5°C, 42.5°C, 44.5°C), total RNA extracted and northern analysis carried out. Immuno-histochemistry and immuno-fluorescence revealed that Hrp12 was expressed mainly in the liver's periportal (zone 1) region. Subcellular fractionation confirmed the presence of Hrp12 in the liver cytosol. In contrast to normal cells, Hrp12 was found on the cell surface of several colon-derived adenocarcinomas (MCA-38 and MCA-26) tumor cells. Functionally, Hrp12 inhibited protein synthesis, cell proliferation and rapidly declined in the liver after partial hepatectomy. Hrp12 is a new protein which may play a vital role in the normal liver and liver disease. This unique protein is surface bound and expressed on adenocarcinoma cell lines. Hrp12 can be utilized as a tumor marker in the liver.

#4155 Serum dependent downregulation of CD1a expression and specific CTL priming activity of human dendritic cells. Jasan, B., Navabi, H., Adams, M., Evans, A., and Mason, M. Velindre Hospital NHS Trust, Cardiff, University of Wales College of Medicine, Cardiff CF4 4XN, Wales, U.K.

CD1a has been used as a marker of functionally active dendritic cells (DC) grown in vitro from peripheral blood mononuclear cells (PBMC). Whilst optimising conditions for culturing DC from blood of cervical cancer patients, the serum source used in the culture medium was found to influence the level of CD1a expression. To examine the reproducibility of this effect, PBMC from 5 cancer (c) and 4 normal healthy (n) donors were cultured with GM-CSF/IL4 medium containing either fetal bovine serum (FBS), autologous serum (AS), or pooled AB serum (ABS), to obtain 8 day DC. No significant differences between the yields and the immunophenotype of cDC & nDC were noted. However, % CD1a expression was influenced significantly by cancer status and the serum source (p < 0.001 for both factors; multiple linear regression), being consistently high with FBS, moderate with AS, and low with ABS (see Table below).

	c1	c2	c3	c4	c5	n1	n2	n3	n4
FBS	79	90	95	83	90	89 (79)	93 (49)	98 (41)	90
AS	45	35	30	33	16	76 (38)	72 (14)	70 (9)	80
ABS	0	0	0	0	0	5 (14)	16 (0)	10 (0)	25

The % specific CTL activity induced by nDC against flu peptide-loaded 51Cr-labelled autologous EBV transformed B-cells, showed a parallel trend (figures in brackets). Hence, both homologous and autologous adult human serum as compared with foetal bovine serum, contain factor(s) capable of downregulating CD1a expression and specific CTL priming activity of DC. These data may be relevant in defining the optimum DC phenotype for use as a potential anti-cancer vaccine.

#4156 Comparative analysis of murine dendritic cells derived from spleen and bone marrow. Fields, R.C., Osterholzer, J.J., Fuller, J.A., Thomas, E.K., Geraghty, P.J., Mulé, J.J. University of Michigan, Ann Arbor, MI 48104

In order to improve upon preclinical tumor vaccine strategies that employ dendritic cells (DC), we have now compared short-term cultures of spleen (Spl) and GM-CSF/IL-4 stimulated bone marrow (BM) to determine if differences exist in phenotype and function of murine DC derived from primary and secondary hematolymphoid organs. Although cultures of BM contained a lower percentage of DC compared to Spl (20% vs. 50%), their capacity to stimulate a primary allogeneic MLR and to uptake fluorescent dextran was substantially greater. In addition, the overall yields of DC per animal was at least 2-fold greater from BM compared to Spl (4.3×10^6 vs. 1.5×10^6). Cultures of BM harvested at day 3, 6, or 9 stimulated comparable levels of primary allo-MLR on a per cell basis. However, there was a consistent loss (at least two-fold) of all cells occurring beyond day 6. Importantly, we also improved upon methods to rapidly obtain highly-enriched DC (>90%) from BM, which has obviated the reported prior need for complex antibody and complement treatments to remove contaminating mature T and B lymphocytes, Ia-bearing cells, and granulocytes before DC generation. In contrast, although similar purity of DC with similar phenotype and function could be obtained from the spleen, substantial loss in yield occurred (>90%), suggesting a further difference in DC between the two tissue sources. The overall yield of DC derived from Spl and BM cultures could be substantially increased (5-7 fold increase) by in vivo pretreatment of the donor animals with recombinant Flt3-L. Collectively, these studies demonstrate that notable differences exist in DC preparations derived from Spl vs. BM and that BM provides the preferred source of DC that can be rapidly enriched to high purity for use in further vaccine development.

#4157 Analysis of T-cell receptor (TCR) of autologous tumor-specific T cells induced by locoregional immunotherapy. Miyahara, E., Yamaguchi, Y., Hihara, J., Toge, T. Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-Ku, Hiroshima 734, Japan

We have been engaged in locoregional immunotherapy of malignant effusions (ME) with a biological response modifier, OK432, a strain of *Streptococcus pyogenes* A3. It is assumed that specific TCR may be involved in autologous tumor-specific T-cell response induced by OK432. We analyzed the TCR gene usage of the tumor-infiltrating lymphocytes (TIL) of ME before and after the administration of OK432. In 7 cases, the expression of TCRV β 20 was significantly higher after OK432 administration. In Case 1, autologous tumor killing was induced with in vivo OK432 administration and the cytotoxicity was abrogated by anti-TCRV β 20 antibody in vitro, and its TCRV β 20 included 3 clonotypes and one of these clonotypes (V β 20-1) expressed in other TILs of HLA-A24-positive patients. Locoregional administration of OK432 induced clonal expansion of TCRV β 20 by which cytotoxic T cell recognizes and kills autologous tumor cells, and strongly suggesting that OK432 may induce specific T-cell response cross-reactive with the autologous tumor cells. We consider for the future, the utilization of the V β 20-1 to search the responder to OK432 and the application of the V β 20-1 gene for gene therapy of cancer.

#4158 Clonally expanded gamma-chain T-cell receptor (TCR) transcripts are present in solid tumor specimens and peripheral blood from patients with epithelial ovarian carcinoma (EOC). W.J. Jung, J. Pappas, C.W. Helm, R.S. Freedman, C.D. Platsoucas. Temple Univ. Sch. Med., Philadelphia, PA 19140, Univ. Texas, M.D. Anderson Cancer Ctr, Houston, TX 77030.

To determine whether $\gamma\delta$ TCR+ T cells are clonally expanded in the peripheral blood and tumor infiltrating lymphocytes of patients with EOC, we amplified by PCR γ -chain transcripts using V γ 1 or V γ 11 specific primers and a C γ primer. The amplified transcripts were cloned and sequenced. Sequence analysis revealed substantial proportions of identical γ -chain transcripts of the V γ 1 (V γ 2, V γ 3, V γ 4 and V γ 5) and V γ 11 (V γ 9) subgroups in 4/4 solid tumor specimens from patients with EOC. γ -chain transcripts from peripheral blood from certain of these patients were also sequenced and contained substantial proportions of identical V γ 1 and V γ 11 transcripts. Certain clonally expanded transcripts were identical in both solid tumor specimens and peripheral blood. These results suggest that $\gamma\delta$ TCR+ T lymphocytes utilizing these transcripts may have undergone clonal expansion in vivo at the site of the tumor, in response to as yet unidentified antigen(s).

#4159 HLA-DR restricted T-helper type 2 CD4 cells exhibit strong in vivo and in vitro lytic activity. Abdel-Wahab, Z.A., DeMatos, P., Ward, F.E., Vervaeert, C., and Seliger, H.F. Duke University Medical Center, Durham, NC 27710.

A CD4+ T-cell line (DT366) was established by stimulating PBLs from a melanoma patient with the autologous melanoma cells DM366. These T-cells secreted large amounts of IL-4, but did not produce IL-2 or Interferon γ , indicating that they

1611

IRON TRANSPORT BY ISOLATED PHAGOLYSOSOMES (PL) OBTAINED FROM THE RAW264.7 MACROPHAGE (M ϕ) CELL LINE TRANSFECTED WITH RESISTANT OR SUSCEPTIBLE Nramp1. Donald E. Kuhn, William P. Lafuse and Bruce S. Zwilling. The Ohio State University, Columbus, OH 43210.

Resistance to Mycobacterial growth is mediated by a protein termed Nramp1, Natural Resistance Associated Macrophage Protein that has a transport protein motif. Nramp1 protein may act a transition metal transporter based on its homology to Mn⁺⁺ transporters in yeast and to Nramp2 an iron (Fe) transporter. Nramp1 is recruited to the PL membrane during phagocytosis. We investigated the transport of Fe into PL from the RAW264.7 M ϕ cell lines transfected with either Nramp1Gly-169 (resistant, R) or Nramp1Asp-169 (susceptible, S) alleles (provided by Jenefer Blackwell). PL were isolated by discontinuous sucrose gradients and incubated with 54-Fe-Citrate at 37°C. Fe uptake was linear for at least 15 minutes in PL isolated from cells with either the R or S alleles. However, PL isolated from the R transfected cells contained more than twice the amount of Fe incorporated by the PL isolated from the S transfected cells. When we measured the PL Fe in M ϕ that had been pre-labeled with 54-Fe-citrate prior to the addition of latex beads we found that the PL from R transfected cells contained up to 4X as much Fe as PLs from S transfected cells. These results are consistent with the possibility that Nramp1 functions as an Fe transporter and that transport of Fe into PL in M ϕ from R mice results in the increased reactive oxygen species via the Haber-Weiss reaction which may account for the increased antimycobacterial activity of these cells. Supported by NIH Grants HL59795, AI42901, MH54966.

1612

DIVERSITY OF CDw101 EXPRESSION ON EPITHELIAL DENDRITIC CELLS OF TONSIL AND SKIN: CORRELATION WITH T-CELL ACTIVATION. X.D. Wang, X.D. Ji, S.B. Wormsley, L-P Dong, G. Clark, C. Leung, Z. Chen, C-M Huang. PharMingen, San Diego, CA 92121.

The immunophenotype of dendritic cells (DC) is heterogeneous and highly dependent on tissue of origin and state of activation. Regardless of phenotype, however, to be an effective antigen presenting cell, the DC must not only display processed antigen in the context of MHC molecules, but must also activate T cells via membrane-expressed co-stimulatory molecules. We have studied the expression on DC of CDw101, a co-stimulatory molecule that is expressed on T cells, granulocytes, monocytes/macrophages and DC. Specifically, we have compared the expression of both CD1a (a commonly used DC marker) and CDw101 on DC found in the epithelial layers of both skin and tonsillar tissues using two murine monoclonals, H1149 (anti-CD1a) and V7.1 (anti-CDw101) and 2-color immunohistochemistry. Results on frozen sections demonstrated that while both CD1a and CDw101 were expressed on epithelial DC from tonsil, only CD1a, and not CDw101, was found on a morphologically similar DC population in the skin. Furthermore, the intensity of CDw101 staining in tonsillar epithelial DC correlated with the presence of well defined germinal centers, suggesting that the degree of T-cell activation could be influenced by the upregulation of CDw101 on nearby DC. Failure to upregulate CDw101 on DC in skin could potentially explain the eventual outgrowth of a cutaneous lesion and provide a target molecule for future immune modulating therapies.

MOUSE MODELS OF TUMOR IMMUNITY (1613-1616)

1613

GENERATION OF CEA PEPTIDE-SPECIFIC CYTOTOXIC T CELLS *IN VIVO* FROM HLA-A2.1K* TRANSGENIC MICE USING PEPTIDE-PULSED BONE MARROW (BM)-DERIVED DENDRITIC CELLS (DC). K.Y. Tsang, P. Correale, M. Zhu, H. Terasawa, P. Arlen, S. Zaremba and J. Schlom. (SPON: M. Nishimura). National Cancer Institute, Bethesda, MD 20892.

Previous studies have shown that a 9-mer carcinoembryonic antigen (CEA) peptide (CAP-1) selected to conform to human HLA class I-A2 motif can elicit cytotoxic T lymphocyte (CTL) responses in patients immunized with recombinant vaccinia CEA. In this investigation, we evaluated the use of HLA-A2.1K* transgenic mice to predict immunogenicity of CEA peptides in HLA-A2 individuals. DC were generated from HLA-A2.1 transgenic mice BM precursors, used as antigen presenting cells and pulsed with CAP-1. CAP-1-pulsed DC were then injected intravenously into HLA-A2.1K* transgenic mice. Spleen cells were removed from mice 10 days after immunization and restimulated *in vitro* with CAP-1 before being used as effector cells. We have observed that DC generated from BM cultures expressed high levels of class I and II MHC, together with CD11 β , CD80, CD86 and HLA-A2. Specificity of CTL activity was determined by cytotoxicity assays using CAP-1-pulsed Jurkat A2.1K* cells and C1R-A2 cells as targets. They lysed CAP-1-pulsed Jurkat A2.1K* and CAP-1-pulsed C1R-A2 cells. No lysis was observed when spleen cells isolated from mice immunized with CAP-1 in adjuvant were used as effector cells. These results indicate that CTL can be recruited and activated *in vivo* by peptide-pulsed DC. Furthermore, this study also suggests that the use of peptide-pulsed DC in HLA-A2.1K* transgenic mice may be an important model for the prediction and study of immunodominant peptides recognized by human T cells.

1614

DENDRITIC CELL VACCINE CONTAINING ENGINEERED PROSTATE ANTIGEN FUSION PROTEIN INDUCES AUTOIMMUNE PROSTATITIS AND INHIBITION OF TUMOR CELL GROWTH C.L. Ruegg, D.M. Yang, M.H. Shaper, P.H. Slagle and R. Laus. Molec. Immunol., Dendreon Corp., Mountain View, CA 94043

Immunotherapeutic approaches to the treatment of metastatic cancer stand to offer significant advantages over existing chemotherapy and radiation treatment regimens, however, efforts to date have met with very limited success. Herein we employ dendritic cells (DC) in combination with an engineered prostate antigen (PA) fusion protein (PA2024) to overcome immunotolerance to PA in an animal model and induce autoimmune prostatitis, a surrogate indicator of tumoricidal immunity. Both systemic administration of PA2024 in the absence of DC and PA in complete Freund's adjuvant elicited PA-specific antibodies but no sign of prostatic immune infiltration suggesting that a humoral response alone is not sufficient. Furthermore, coculture of PA-expressing prostate tumor cells with splenocytes from animals immunized by DC pulsed with PA2024, but not with PA alone nor control treatment, resulted in complete inhibition of tumor cell growth *in vitro* supporting such a rationale for tumor immunotherapeutic intervention. Current efforts are underway to evaluate analogous approaches in human trials.

1615

POTENT ANTITUMOR EFFECTS OF BONE MARROW-DERIVED DENDRITIC CELLS IN A MURINE MODEL OF BREAST CANCER. K. Candido, R. C. Fields, J. A. Fuller, and J. J. Muller. Department of Surgery, University of Michigan Medical Center, Ann Arbor, MI 48109-0666.

Dendritic cells (DC) derived from murine bone marrow (BM) cultured in GM-CSF and IL-4 can mediate potent primary allogeneic ML *in vitro* and can efficiently phagocytose high molecular weight dextran particles. We reported that whole tumor lysates can serve as effective immunogens to stimulate specific CD4⁺ T cell reactivity *in vitro* when processed and presented by APC. We have now determined the capacity of bone marrow-derived DC to induce antitumor responses in mice in a syngeneic model of a weakly-immunogenic mammary tumor (denoted MT-901). Cytokine-driven, BM-derived DC were pulsed overnight with freeze-thawed lysate of MT-901 tumor and used to immunize naive BALB/c mice by three subcutaneous immunizations seven days apart. Control groups of mice received either no treatment, tumor lysate alone, or unpulsed DC alone. Mice were then rechallenged with a lethal dose of viable MT-901 tumor. At day 28 after tumor injection, mice receiving DC pulsed with MT-901 lysate remained completely tumor free compared to those in the control groups ($p < 0.05$). The control mice all harbored large tumors of surface area greater than 200 mm². We also evaluated the effect of direct intratumoral injection of BM-derived DC. BALB/c mice bearing established (0.5 cm) MT-901 tumors were treated by direct injection with 1x10⁶ unpulsed DC administered four times. At day 19 after the first DC injection, mice receiving DC demonstrated significant inhibition of tumor growth compared to control tumors injected intratumorally with saline (60 mm² vs. 180 mm²; $p < 0.05$). Follow-up studies are now investigating the mechanism(s) of the *in vivo* therapeutic efficacy of DC. Collectively, these data demonstrate that cytokine-driven, BM-derived DC can be used in effective immunotherapeutic strategies for breast cancer.

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ELICITING T CELL IMMUNITY AGAINST A POORLY IMMUNOGENIC TUMOR BY IMMUNIZATION WITH DENDRITIC CELLS FUSED WITH TUMOR CELLS AS VACCINE. J. Wang, S. Saffold, G. E. Plautz, J. Krauss, S. Shu, and W. Chen. Center for Surgery Research, The Cleveland Clinic Foundation, Cleveland, OH 44195.

Dendritic cells (DCs) are the most effective antigen presenting cells and are being studied for use as adjuvants or antigen-delivery vehicles to elicit T cell-mediated antitumor immunity. This study examined whether fusion of DCs with syngeneic poorly immunogenic tumor cells as vaccine can be a useful strategy to elicit tumor-reactive T cells for adoptive immunotherapy. DCs isolated from bone marrow of B6 mice were fused with syngeneic B16 melanoma or RMA-S lymphoma cells by PEG. The fusion hybrids were positive of MHC class I, class II antigens, costimulatory molecules, as well as DCs and tumor-derived surface markers. The capability of the fused cells to process and present tumor-associated proteins was confirmed in experiments demonstrating that RMA-S/DC fused cells can be recognized and lysed by FMR-MuLV gag-specific CTL. Vaccination of B6 mice with irradiated B16/DC fusion preparations induced host protective immunity against challenge with low dose B16 tumor. Reduced tumor incidence and prolonged survival time were observed. Specific CTL activities against B16 tumor were detected from splenocytes of mice immunized with B16/DC. Adoptive transfer of T cells derived from B16/DC primed lymph nodes into B16 tumor-bearing mice greatly reduced the number of established pulmonary metastases, with or without *in vivo* administration of IL-2. The results demonstrated that fusion of DCs with syngeneic tumor cells can be used as an effective cancer vaccine for eliciting T cell-mediated immunity against poorly immunogenic tumors.

The Role of Tumor Necrosis Factor α in Modulating the Quantity of Peripheral Blood-Derived, Cytokine-Driven Human Dendritic Cells and Its Role in Enhancing the Quality of Dendritic Cell Function in Presenting Soluble Antigens to CD4⁺ T Cells In Vitro

By Bing-guan Chen, Yijun Shi, Jeffrey D. Smith, David Choi, James D. Geiger, and James J. Mulé

Because dendritic cells (DC) are critically involved in both initiating primary and boosting secondary host immune responses, attention has focused on the use of DC in vaccine strategies to enhance reactivity to tumor-associated antigens. We have reported previously the induction of major histocompatibility complex class II-specific T-cell responses after stimulation with tumor antigen-pulsed DC in vitro. The identification of in vitro conditions that would generate large numbers of DC with more potent antigen-presenting cell (APC) capacity would be an important step in the further development of clinical cancer vaccine approaches in humans. We have focused attention on identifying certain exogenous cytokines added to DC cultures that would lead to augmented human DC number and function. DC progenitors from peripheral blood mononuclear cells (PBMC) were enriched by adherence to plastic, and the adherent cells were then cultured in serum-free XIVO-15 medium (SFM) for 7 days with added granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). At day 7, cultures contained cells that displayed the typical phenotypic and morphologic characteristics of DC. Importantly, we have found that the further addition of tumor necrosis factor α (TNF α) at day 7 resulted in a twofold higher yield of DC compared with non-TNF α -containing DC cultures at day 14. Moreover, 14-day cultured DC generated in the presence of

TNF α (when added at day 7) demonstrated marked enhancement in their capacity to stimulate a primary allogeneic mixed leukocyte reaction (8-fold increase in stimulation index [SI]) as well as to present soluble tetanus toxoid and candida albicans (10- to 100-fold increases in SI) to purified CD4⁺ T cells. These defined conditions allowed for significantly fewer DC and lower concentrations of soluble antigen to be used for the pulsing of DC to efficiently trigger specific T-cell proliferative responses in vitro. When compared with non-TNF α -supplemented cultures, these DC also displayed an increased surface expression of CD83 as well as the costimulatory molecules, CD80 and CD86. Removal of TNF α from the DC cultures after 2 or 4 days reduced its enhancing effect on DC yield, phenotype, and function. Thus, the continuous presence of TNF α over a 7-day period was necessary to achieve the maximum enhancing effect observed. Collectively, our findings point out the importance of exogenous TNF α added to cultures of cytokine-driven human DC under serum-free conditions, which resulted in an enhanced number and function of these APC. On the basis of these results, we plan to initiate clinical vaccine trials in patients that use tumor-pulsed DC generated under these defined conditions.

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DENDRITIC CELLS (DC) are the most potent antigen-presenting cells (APC) distributed in many tissues of the body in humans and other species.^{1,2} DC can stimulate the primary activation of T cells due to their enhanced capacity of presenting immunogenic peptides in association with self-major histocompatibility complex (MHC) class I⁴ and class II molecules,⁵⁻⁸ expression of coreceptor molecules such as CD40, CD80, CD86,⁹ and ICAM-3,¹⁰ as well as production of cytokines such as interleukin-12 (IL-12).^{11,12} DC can also process both exogenous protein¹³ and intracellular protein derived from DNA transfection¹⁴ for presentation to T cells. Recently, it was reported that DC can directly modulate B-cell growth and differentiation via CD40 ligation¹⁵ and through the production

of soluble mediators such as IL-1, IL-6, and tumor necrosis factor α (TNF α), which have been shown to be produced by DC or DC-related cell lines.^{16,17}

We and others have shown that DC pulsed with tumor-associated antigen(s) in the form of whole cell lysates,⁵⁻⁷ peptides,^{1-4,18,19} proteins,²⁰ RNA,²¹ or DNA^{14,22} could initiate primary MHC class I- or class II-restricted T-cell responses that resulted in antitumor effects in vitro and in vivo. On the basis of these studies, attention has focused on the use of DC to enhance the host immune response to tumor-associated antigens in clinical vaccine strategies in humans with cancer.^{23,24} Thus, the identification of approaches that would generate large numbers of DC with more potent antigen-presenting capacity would be an important step in the further refinement of vaccine approaches based on DC.

There is general agreement that DC can be generated from bone marrow- and cord blood-derived CD34⁺ hematopoietic cell progenitors with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, and TNF α .^{25,26} In addition, DC have been derived from precursors in unfractionated^{27,28} and CD34⁺ cell-depleted²⁹ peripheral blood mononuclear cells (PBMC) as well as from CD14⁺ blood monocytes.^{17,30}

Various recombinant cytokines have been used for the in vitro generation of DC derived from several tissue sources (denoted cytokine-driven DC).^{1,2} In particular, the activity of TNF α in combination with GM-CSF with or without IL-4 has been studied. For example, the addition of TNF α to cultures has been shown to inhibit spontaneous apoptosis of DC,³¹ to

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Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses *in vitro* and *in vivo*

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ABSTRACT The highly efficient nature of dendritic cells (DC) as antigen-presenting cells raises the possibility of uncovering in tumor-bearing hosts very low levels of T cell reactivity to poorly immunogenic tumors that are virtually undetectable by other means. Here, we demonstrate the *in vitro* and *in vivo* capacities of murine bone marrow-derived, cytokine-driven DC to elicit potent and specific anti-tumor responses when pulsed with whole tumor lysates. Stimulation of naive spleen-derived T cells by tumor lysate-pulsed DC generated tumor-specific proliferative cytokine release and cytolytic reactivities *in vitro*. In addition, in two separate strains of mice with histologically distinct tumors, s.c. injections of DC pulsed with whole tumor lysates effectively primed these animals to reject subsequent lethal challenges with viable parental tumor cells and, important to note, also mediated significant reductions in the number of metastases established in the lungs. Tumor rejection depended on host-derived CD8⁺ T cells and, to a lesser extent, CD4⁺ T cells. Spleens from mice that had rejected their tumors contained specific precursor cytotoxic T lymphocytes. The use of whole tumor lysates as a source of tumor-associated antigen(s) for pulsing of DC circumvents several limitations encountered with other methods as well as provides certain distinct advantages, which are discussed. These data serve as rationale for our recent initiation of a phase I clinical trial of immunization with autologous tumor lysate-pulsed DC in adult and pediatric cancer patients.

Recent attempts to improve immunotherapy for cancer have included the genetic modification of tumor-infiltrating lymphocytes (TIL) to express exogenous genes encoding for either antitumor cytokines (1) or new “chimeric” receptors to redirect tumor antigen specificity (2). Moreover, cytotoxic T lymphocyte (CTL)-defined tumor peptides (3) and gene-modified tumor cells (4) have been used as immunogens to generate more potent TIL or tumor-draining lymph node cells or to impact directly on established metastatic disease by serving as “therapeutic vaccines.” We have been investigating approaches to enhance the activity of tumor vaccines to increase the frequency of tumor-reactive T cells and to overcome tumor-induced immune suppression (5, 6). Our recent studies have involved the use of dendritic cells (DC) as antigen-presenting cells (APC) in an attempt to stimulate both primary and secondary immune responses to poorly immunogenic tumors.

DC are of bone marrow origin, develop from myeloid (7) or lymphoid (8) precursors, and possess strong APC function. In this regard, DC have been shown to stimulate both primary and secondary T and B cell responses (9) and to internalize, process, and present antigens efficiently (7). DC pulsed with defined tumor-associated peptides or proteins as well as with

model “tumor” antigens have been shown to elicit potent antitumor T cell responses both *in vitro* and *in vivo* (10). We had reported earlier that murine epidermal Langerhans cells and splenic DC could present efficiently antigens associated with tumor cell lysates to primed CD4⁺ T cells *in vitro* (11, 12).

It is generally accepted that tumors growing *in vivo* naturally provide antigen(s) to APC either by shedding from the surface of viable cells or by fragmentation of dead tumor cells. Such processes elicit the induction of cellular immune responses by mechanisms that include “cross-priming” (13). The use of tumor cell lysates as a possible source of tumor antigen(s) for DC pulsing has several potential advantages, which include mimicking the physiologic processes by which a growing tumor induces an immune response (albeit low) *in vivo*. In practical terms, tumor lysates circumvent the need for viable fresh tumor cells and for the establishment of tumor cell lines *in vitro*, which for some human tumors (e.g., breast carcinoma) has been difficult, as well as avoid the necessity for molecular characterization of the tumor antigen(s) for effective immunization (11, 12). Because human cancers have been shown to elicit multiple specific immune responses in the autologous patient (14), the approach of using tumor lysates pulsed onto DC would offer the potential advantage of augmenting a broader T cell immune response to tumor-associated antigens that would not be obtained by pulsing DC with a single or perhaps several defined tumor peptides. This strategy potentially lessens the possibility of tumor escape by the broader elicited immune response yet increases the potential to trigger T cell reactivity to those particular antigens, which results in actual tumor regression *in vivo* (namely, “tumor rejection” antigens) (3, 15). In addition, greater potential exists for simultaneous presentation of “CTL-defined” and “T helper-defined” epitopes by virtue of a whole tumor lysate serving as the source for pulsing DC, which highly express both major histocompatibility complex (MHC) class I and II molecules. In this regard, although several distinct peptides have been identified in human tumors, few, to date, are MHC class II, CD4⁺ T cell-defined (3, 16). Yet, in several murine tumor models, the adoptive transfer of immune CD4⁺ T cells, when successfully elicited, mediates potent antitumor therapeutic effects *in vivo* (17). Moreover, certain melanoma vaccines generated from mechanical lysates stimulate both CD4⁺ and CD8⁺ T cell activity in immunized cancer patients (18).

Here, we investigated the capacity of bone marrow-derived, cytokine-driven DC pulsed with tumor lysates to elicit antitumor responses both *in vitro* and *in vivo* in two syngeneic murine tumor models. We show that tumor lysate-pulsed DC elicit specific proliferative and cytolytic T cell reactivities *in vitro*. In addition, immunization of mice with tumor lysate-pulsed DC

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Abbreviations: TIL, tumor-infiltrating lymphocytes; CTL, cytotoxic T lymphocyte; DC, dendritic cells; APC, antigen-presenting cells; CM, complete medium; MCA, 3-methylcholanthrene; MHC, major histocompatibility complex; FACS, fluorescence-activated cell sorter; CSF, colony-stimulating factor; IL, interleukin; MT, mammary tumor.

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PROOF

BONE MARROW

Comparative Analysis of Murine Dendritic Cells Derived from Spleen and Bone ~~MARROW~~ MARROW

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Summary: In order to improve upon preclinical tumor vaccine strategies that employ dendritic cells (DC), we now have compared short-term cultures of spleen- and GM-CSF/IL-4-stimulated bone marrow (BM) to determine if differences exist in phenotype and function of murine DC derived from primary and secondary hematolymphoid organs. Although cultures of BM contained a lower percentage of DC compared to spleen, their capacity to stimulate a primary allogeneic mixed leukocyte reaction (MLR) and to uptake fluorescent dextran was substantially greater. In addition, the overall yields of DC per animal was at least twofold greater from BM compared to spleen. Cultures of BM harvested at day 3, 6, or 9 stimulated comparable levels of primary allo-MLR on a per-cell basis. However, there was a consistent loss (at least twofold) of all cells occurring beyond day 6 as compared with cell yields from earlier time points. Importantly, we also improved on methods to rapidly obtain highly enriched DC (>90%) from BM, which has obviated the reported prior need for complex antibody and complement treatments to remove contaminating mature T and B lymphocytes, Ia-bearing cells, and granulocytes before DC generation. In contrast, although similar purity of DC with similar phenotype and function could be obtained from the spleen, substantial loss in yield occurred, suggesting a further difference in DC between the two tissue sources. The overall yield of DC derived from spleen and BM cultures could be substantially increased by in vivo pretreatment of the donor animals with recombinant Flt3-L. Collectively, these studies demonstrate that notable differences exist in DC preparations derived from spleen vs. BM and that BM provides the preferred source of DC that can be rapidly enriched to high purity for use in further vaccine development. **Key Words:** Dendritic cells—Antigen presenting cells—Immune priming—Immunotherapy—Vaccine.

INTRODUCTION

Dendritic cells (DC) are of bone marrow origin and develop from myeloid (1,2) or lymphoid (3–5) precursors. DC undergo a complex maturational process

whereby they acquire residence in peripheral tissues and become highly efficient at antigen uptake, processing, and presentation (6–10). Upon stimulation either by several distinct cytokines (11–17), bacterial products (1,2), or receptor ligation (18–20), DC differentiate into potent antigen presenting cells (APC) for naive lymphocytes and play a primary role in the induction of T cell-mediated immune responses. In a variety of settings, these specialized APC can induce both the generation and proliferation of specific CTL and TH cells through antigen presentation via MHC class I and class II molecules, respectively (21–25). As example, we (26–28) and others (29–31) have described the induction of either MHC class I- or class II-specific T cell responses fol-

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R. C. Fields and J. J. Osterholzer are equal contributors as first author.

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Dendritic Cell-Based Vaccines in the Setting of Peripheral Blood Stem Cell
Transplantation: CD34⁺ Cell-Depleted Mobilized Peripheral Blood Can Serve
as a Source of Potent Dendritic Cells[^]

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ABSTRACT

We are investigating the use of tumor-pulsed dendritic cell (DC)-based vaccines in the treatment of patients with advanced cancer. In the current study, we evaluated the feasibility of obtaining both CD34⁺ hematopoietic stem/progenitor cells (HSC) and functional DC from the same leukapheresis collect in adequate numbers for both peripheral blood stem cell transplantation (PBSCT) and immunization purposes, respectively. Leukapheresis collections of mobilized peripheral blood mononuclear cells (PBMC) were obtained from normal donors receiving G-CSF (for allogeneic PBSCT) and from intermediate grade non-Hodgkin's lymphoma (NHL) or multiple myeloma patients receiving cyclophosphamide plus G-CSF (for autologous PBSCT). High enrichment of CD34⁺ HSC was obtained using an immunomagnetic bead cell separation device. Following separation, the negative fraction of mobilized PBMC from normal donors and cancer patients contained undetectable levels of CD34⁺ HSC by flow cytometry. This fraction of cells was then subjected to plastic adherence and the adherent cells were cultured for 7 days in GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) followed by an additional 7 days in GM-CSF, IL-4, and TNF- α (10 ng/ml) to generate DC. Harvested DC represented yields of $4.1\% \pm 1.4\%$ and $5.8\% \pm 5.4\%$ of the initial cells plated from the CD34⁺ cell-depleted mobilized PBMC of normal donors and cancer patients, respectively, and displayed high level expression of CD80, CD86, HLA-DR, CD11c, but not CD14. This phenotypic profile was similar to that of DC derived from non-CD34⁺ cell-depleted mobilized PBMC. DC generated from CD34⁺ cell-depleted mobilized PBMC elicited potent anti-tetanus as well as primary allogeneic T cell proliferative responses in vitro, which were equivalent to DC derived from non-CD34⁺ cell-depleted

mobilized PBMC. Collectively, these results demonstrate the feasibility of obtaining both dendritic cells and CD34⁺ hematopoietic stem/progenitor cells from the same leukapheresis collects from G-CSF-primed normal donors and cancer patients in sufficient numbers for the purpose of combined PBSCT and immunization strategies.

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