TEXAS MEDICAL CENTER NASA/JOHNSON SPACE CENTER

COOPERATIVE AGREEMENT PROGRAM NCC 9-36, ROUND II

COVER SHEET FOR FINAL REPORT

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Name of Subcontractor:	Cherylyn A. Savary, Ph.D.
Title:	Assistant Professor
Institution:	University of Texas M.D. Anderson Cancer Center
Name of Project:	Role of Dendritic Cells in Immune Dysfunction
Amount of Grant:	~~$45,000~~

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**TEXAS MEDICAL CENTER NASA/JOHNSON SPACE CENTER
COOPERATIVE AGREEMENT NCC 9-36
Applications Subcontract Program, Round 2
Final Report**

Name of Institution: University of Texas M.D. Anderson Cancer Center
Name of Subcontractor: University of Texas M.D. Anderson Cancer Center
Title of Project: Role of Dendritic Cells in Immune Dysfunction (Savary)

Specific Aims:
1. Application of the NASA bioreactor to enhance cytokine-regulated proliferation and maturation of dendritic cells (DC).
2. Compare the frequency and function of DC in normal donors and immunocompromised cancer patients.
3. Analyze the effectiveness of cytokine therapy and DC-assisted immunotherapy (using bioreactor-expanded DC) in a murine model of experimental fungal disease.

Studies and Results:

Our investigations have provided new insight into DC immunobiology and have led to the development of methodology to evaluate DC in blood of normal donors and patients (1,2). Information gained from these studies has broadened our understanding of possible mechanisms involved in the immune dysfunction of space travelers and earth-bound cancer patients, and could contribute to the design of novel therapies to restore/preserve immunity in these individuals. Several new avenues of investigation were also revealed, one of which has been awarded funding through NRA-96-OLMSA-03. The results of studies completed during Round 2 are summarized below. Additional details are provided in the manuscripts and abstracts cited in the references and included in the Appendix.

AIM 1. Application of the NASA bioreactor to enhance cytokine-regulated proliferation and maturation of DC. In Round 1 we showed that the 3D environment of the NASA bioreactors (including the HARV, STLV and RCCS-D vessels) supported the generation and maturation of human DC from CD34$^+$ progenitors. Bioreactor-generated DC (3D-DC), however, displayed several differences compared to DC generated in standard 2D culture conditions (2D-DC), i.e., the proliferative and phagocytic activity of 3D-DC was reduced compared to 2D-DC. The 3D-DC though, displayed higher allostimulatory activity and higher levels of heat shock proteins (Hsp). According to current models, DC maturation progresses from a less mature stage, characterized by high antigen uptake/processing capabilities and low antigen-presenting function, to a more mature stage in which antigen uptake/processing capabilities are lost as antigen-presenting functions are upregulated. Based on this model, our results suggest that DC maturation may be enhanced in the NASA bioreactors. These data were presented at the annual meeting of the Biotechnology Cell Science Program Investigators Working Group (1997), and as a poster at the 13th Annual Meeting of the American Society for Gravitational and Space Biology (3).

During Round 2, we further analyzed the characteristics of human 3D-DC *versus* 2D-DC, and continued to explore ways to enhance DC generation in NASA bioreactors. During our studies, considerable interest arose in FLT3-ligand – a cytokine that substantially enhances DC

generation *in vivo* in mice, and that appears to have potential clinical application. It is known that FLT3L has little activity on its own, but can enhance the activity of other DC-promoting cytokines. We analyzed the effect of this agent on DC generation/maturation in the bioreactor. In agreement with our findings in Round 1, 3D-DC generated in the presence of a basic DC cytokine cocktail (GM-CSF+TNFα+stem cell factor [SCF]) displayed higher allostimulatory activity than 2D-DC (Fig. 1). Addition of FLT3L to this cocktail had a slight enhancing effect on the stimulatory activity of DC obtained from either culture condition. Using flow cytometry we found that, in comparison to 2D-DC, a higher proportion of 3D-DC generated with or without FLT3L, displayed CD86 costimulatory molecules (a mark of more mature/activated DC) (Fig. 2). FLT3L enhanced the proportion of CD86$^+$ in 2D cultures (Fig.

Fig. 1. Effect of FLT3L on the allostimulatory activity of DC. DC were generated from CD34$^+$ progenitors in standard 2D cultures or in NASA bioreactors (3D). Cultures were supplemented with GM-CSF, TNFα and SCF, with or without FLT3L. Proliferation was measured in a standard mixed lymphocyte reaction using ^{3}HTdR incorporation. Stimulator:responder ratio = 1:20.

2b), but by far, the highest proportion of CD86$^+$ DC was generated in 3D cultures supplemented with FLT3L (Fig. 2d). These results suggest that FLT3L promotes generation of more mature DC – a process which is further enhanced in the 3D environment of the bioreactors.

Another consistent finding in our investigations, was that the intensity of HLA-DR (MHC Class II) expression was reduced on 3D-DC regardless of the cytokine cocktail used to generate these cells (Table 1). This observation (in the 3D cultures only) runs counter to the general model of DC maturation in which HLA-DR is increased upon maturation. It is important to note, though, that this model is based primarily on results from 2D culture conditions. It is possible that HLA-DR expression
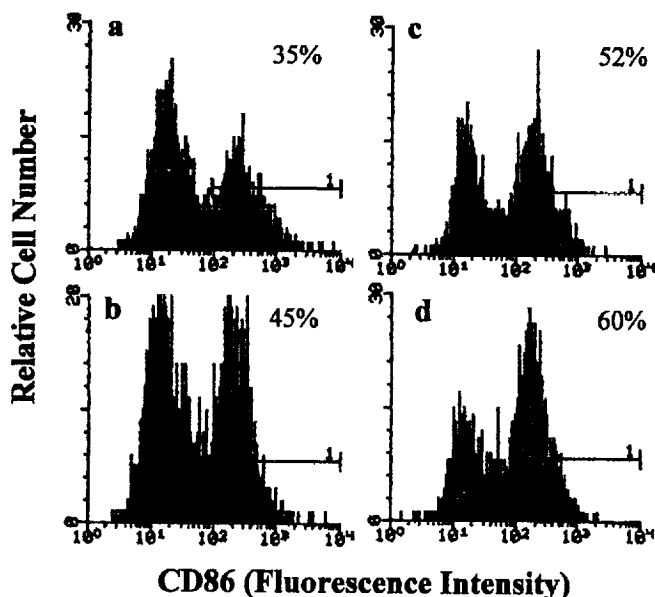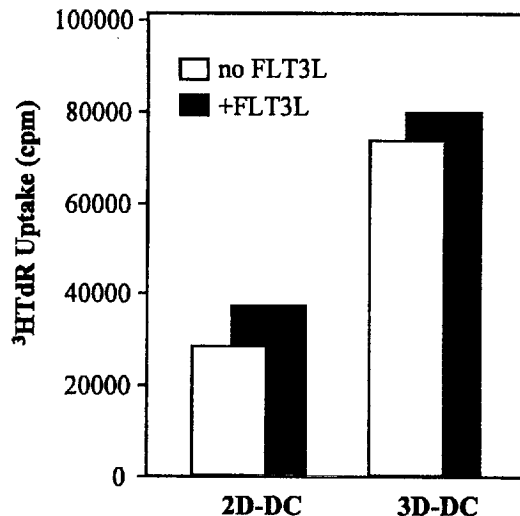

Fig. 2. Effect of FLT3L on expression of CD86 by DC. DC were generated from CD34$^+$ progenitors in standard 2D cultures (a and b) or in 3D NASA bioreactors (c and d). Cultures were supplemented with GM-CSF, TNFα and SCF alone (a and c), or together with FLT3L (b and d). Values represent percentage of CD86$^+$ cells.

Table 1. Reduced expression of HLA-DR by DC generated in NASA bioreactors.

Donor #	Density of HLA-DR (MFI)^a	
	2D-DC	3D-DC
1	1279	207
2	247	105
3	756	280
4	1910	1229
5	202	134

^{a}Values represent mean fluorescence intensity (MFI) of HLA-DR. MFI is a measure of antigen density.

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differs when DC are grown in 3D, or that some stress associated with simulated microgravity alters the assembly or transport of HLA-DR molecules.

HLA-DR plays a central role in antigen presentation to $CD4^+$ helper T cells. Lower expression of HLA-DR on antigen presenting cells could result in reduced stimulation of $CD4^+$ T cells, and consequently, a blunted immune response. Interestingly, Hsp have been shown to bind to HLA-DR subunits as the MHC Class II molecule is assembled in the cytoplasm. An intriguing possibility is that the Hsp induced in DC cultured in the bioreactor (3), might sequester HLA-DR within the cytoplasm and prevent transport of these molecules to the cell surface. Upregulation of stress proteins might reflect a protective response of DC against as yet undefined stressors, some of which might resemble those experienced in microgravity. Thus, understanding the role of Hsp in DC maturation might provide new insight into the molecular pathways that are disturbed in disease or in space. Some of these questions will be addressed as part of the NRA-96-OLMSA-03 grant. A manuscript describing the results of our studies of DC generation in the NASA bioreactor is currently in preparation.

AIM 2. Compare the frequency and function of DC in normal donors and immunocompromised cancer patients. Patients recovering from stem cell transp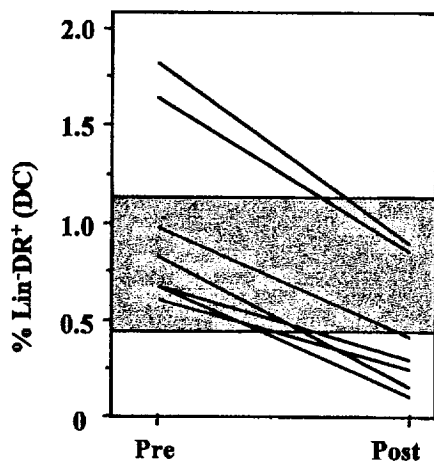lantation are immunocompromised and thus at high risk of infection. We hypothesized that a deficiency in DC might be an underlying cause of the lymphopenia experienced by these patients, since antigen presenting cells are required for the optimum activation and proliferation of T lymphocytes. In Round 1, we developed a multidimensional flow cytometric test to analyze DC in peripheral blood (1). We found that breast cancer patients (all undergoing some form of chemotherapy), and patients who were 2-6 months post stem cell transplantation, displayed a significantly lower proportion of circulating DC compared to normal donors (1). In Round 2, we extended this analysis to a group of sarcoma patients before and after chemo-immunotherapy. We found that these patients displayed normal, or above normal, levels of circulating DC prior to therapy, but that these leukocytes were severely decreased after a combination of chemotherapy+immunotherapy (Fig. 3). As is often

Fig. 3. Effect of chemotherapy on circulating DC. The percentage of DC (i.e. lineage negative, HLA-DR^+ cells) in blood of cancer patients was tested prior to (Pre) and after (Post) high dose chemotherapy + immunotherapy using a multidimensional flow cytometric technique (1,2). Shaded area represents range of circulating DC in normal donors. Lines represent 7 different patients.

the case in clinical studies, it was not possible to test the effects of chemotherapy or immunotherapy alone. However, the combined results from breast cancer patients (1) and sarcoma patients, suggest that any treatment which includes chemotherapy, can severely suppress DC. This could explain the lymphopenia and high susceptibility of these patients to infections (particularly fungal infections).

Interestingly, we found that functional DC could be generated from $CD34^+$ progenitors obtained from the patients described above. Figure 4 shows that these DC had potent allostimulatory activity. Such observations are important, since *in vitro* generated DC might
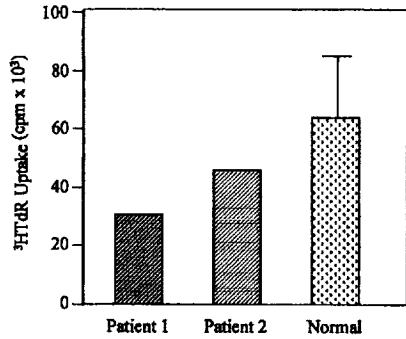
Fig. 4. Generation of allostimulatory DC from CD34 progenitors of cancer patients. DC were generated from blood of sarcoma patients and normal donors, and tested for the ability to stimulate proliferation of allogeneic T cells in a standard mixed lymphocyte reaction as described in Fig. 1.

eventually be reinfused to potentiate the immunity of patients post-therapy. Alternatively, antigen-stimulated DC could be used to stimulate autologous T cells *in vitro* for use in adoptive therapy.

We believe that such an approach might be used to enhance the immunity of bone marrow transplanted patients against fungi. To test this, we first investigated whether DC from normal donors could present fungal antigens to autologous T lymphocytes. It is now known that DC can be generated either from CD34 progenitors (DC_{34}) as described above, or from $CD14^+$ monocytes (DC_{Mo}) cultured for several days with GM-CSF+ IL-4. We tested both types of DC for their ability to present fungal antigens to autologous T lymphocytes. As seen in Fig. 5, both populations were effective in stimulating proliferation of autologous T lymphocytes to *Aspergillus fumigatus*. Stimulation was also observed for *Candida albicans* (data not shown).
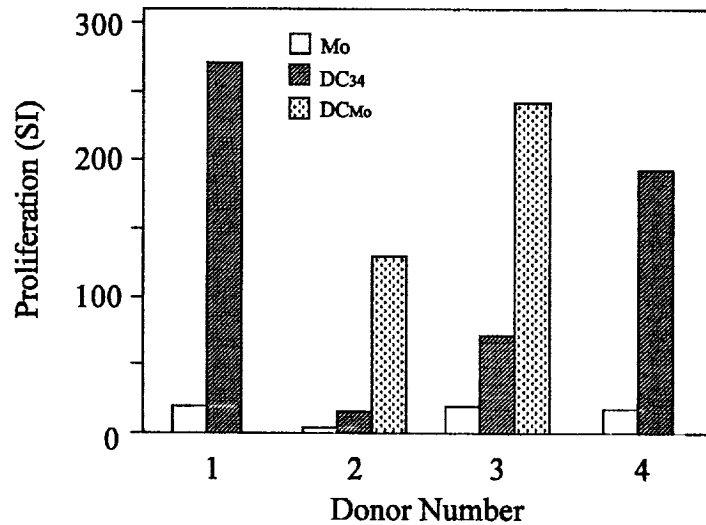

Fig 5. DC presentation of *A. fumigatus* antigens to autologous T lymphocytes. DC were generated from $CD34^+$ progenitors (DC_{34}) or from monocytes (DC_{Mo}) of normal donors, irradiated, cultured with heat-killed *A. fumigatus* conidia, and tested for the ability to stimulate proliferation of autologous T lymphocytes. Typical monocytes (Mo) were also tested as antigen presenting cells to illustrate the efficiency of DC. Bars represent a stimulation index (SI) where SI=cpm of antigen-stimulated lymphocytes/cpm of unstimulated lymphocytes.

More importantly, we next showed that in 3 of 4 stem cell transplanted patients tested, donor DC_{34} generated from blood collected pre-transplant, could potentiate the proliferative response of autologous post-transplant lymphocytes against *A. fumigatus* (Fig. 6). These results have important clinical implications, since there is presently no effective treatment for aspergillosis in severely immunocompromised patients. These data were presented at the annual
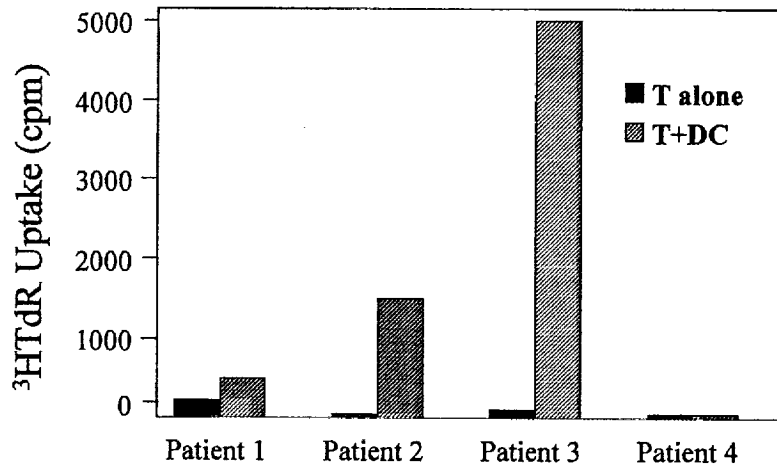
Fig 6. DC-mediated augmentation of antifungal response post stem cell transplantation. DC were generated from CD34$^+$ progenitors collected pre-transplantation, and tested for the ability to stimulate proliferation of post-transplant autologous T lymphocytes to *A. fumigatus*.

meeting of the Biotechnology Cell Science Program Investigators Working Group (1998), and as a poster at the Keystone Symposia Conference on Cellular and Molecular Biology of Dendritic Cells (4). A manuscript describing these results is near completion.

AIM 3. Analyze the effectiveness of cytokine therapy and DC-assisted immunotherapy (using bioreactor-expanded DC) in a murine model of experimental fungal disease. In Round 1 we observed some differences in DC generated from bone marrow of mice in 2D versus 3D conditions. Specifically, a higher proportion of NLDC-145$^+$ cells (a surface marker expressed by murine DC) was detected in 3D bioreactor cultures. It was our intention to compare the ability of 2D-DC and 3D-DC to protect mice from infection with *Aspergillus* or *Candida*. Unfortunately, our mouse colony at M.D. Anderson became infected with pinworms when we had completed only initial studies to determine the lethal dose of fungi in our C57BL/6 strain. These circumstances prevented us from continuing our *in vivo* tests. However, we continued to study murine DC through collaboration with Dr. Jagan Sastry at the M.D. Anderson facilities in Bastrop, TX.

We analyzed DC in murine lymph node tissues cultured by Dr. Sastry in standard 2D cultures (T flasks) and in the bioreactor. In contrast to the increased levels of marrow-derived DC observed in our own bioreactor studies, we found that DC from lymph nodes were often reduced in 3D cultures (see final report of Dr. J. Sastry). The observed differences could indicate differences in the degree of differentiation of these two DC subsets. According to current models, DC generated from marrow precursors, circulate in the blood and migrate into peripheral tissues. After encounter with antigen, the DC undergo maturation that allows them to migrate to areas of lymphocyte accumulation (e.g. lymph nodes) where they can interact with a large number of T lymphocytes, including antigen specific CTL. This maturation is believed to be terminal, and the DC may be destroyed upon interaction with the CTL. Murine marrow contains DC progenitors that are not likely to encounter antigen during culture. Therefore, it is expected that they may be longer-lived than the lymph node DC. This could explain the increased levels of DC observed in our own experiments. Lymph node DC, on the other hand, may be more

mature, may encounter CTL, and thus may be short-lived. It is possible that continued supplementation of CTL cultures with autologous DC could sustain or promote lymphocyte proliferation and function. It must also be considered that marrow and lymph node DC might be derived from different lineages of cells (e.g. myeloid vs lymphoid), that display different culture requirements or growth kinetics. The results of these studies are in a manuscript in preparation (5).

Significance. DC are considered to be the most potent of antigen presenting cells. Only recently though, has it been possible to expand this rare leukocyte subset *in vitro* in cytokine-supplemented cultures. To our knowledge, our studies are the first of their kind to generate and cultivate DC in a 3D environment. In Round 2, we have continued to improve this culturing environment by addition of FLT3L to our cocktail of growth factors. A long-term goal is to generate sufficient numbers of fully differentiated DC for *in vivo* supplementation/restoration of patients who we observed have low numbers of circulating DC. In Round 2 we showed that this may indeed be possible, since DC could potentiate the *in vitro* antifungal response of severely immunocompromised patients. Our data suggest that the bioreactor might represent a novel tool with potential clinical application, for generating fully mature DC.

We believe that the NASA bioreactor will continue to provide us with a unique opportunity to analyze in depth the cellular and molecular events associated with DC maturation from its earliest progenitor to terminal differentiation, as this entire process appears to be accomplished in the bioreactor. A clear understanding of DC immunobiology will be important to understanding the mechanism of DC dysregulation in cancer patients - information that might also give us clues about the mechanism of the blunting of immunity associated with spaceflight.

References Cited (Publications, abstracts, and manuscripts in preparation resulting from this research).

1. Savary CA, Grazziutti ML, Melichar B, Przepiorka D, Freedman RS, Cowart RE, Cohen DM, Anaissie EJ, Woodside DG, McIntyre BW, Pierson DL, Pellis NR, Rex JH. Multidimensional flow cytometric analysis of dendritic cells in peripheral blood of normal donors and cancer patients. Cancer Immunol. Immunother. 45:234-240, 1998.

2. Melichar B, Savary CA, Kudelka AP, Verschraegen C, Kavanagh JJ, Edwards CL, Platsoucas CD, Freedman RS. Lineage negative HLA-DR+ cells with the phenotype of undifferentiated dendritic cells in patients with carcinoma of the abdomen and pelvis. Cellular Immunol. 4:799-809, 1998.

3. Savary, C.A., Rex, J.H., Grazziutti, M.L., Tomasovic, S.P., Przepiorka, D., Pellis, N.R., Pierson, D.L. Characteristics of dendritic cell maturation in the NASA bioreactor. The Gravitational and Space Biology Bulletin, 11:68, 1997. Poster presented at the 13th Annual Meeting of the American Society for Gravitational and Space Biology, Washington, DC. Manuscript in preparation.

4. Savary, C.A., Grazziutti, M.L., Przepiorka, D., Braunschweig, I., Rex, J.H. Dendritic cell response to pathogenic fungi. Poster presentation, Keystone Symposia Conference on Cellular and Molecular biology of Dendritic Cells, Santa Fe, NM, 1998. Manuscript in preparation.

5. Sastry, K.J., Nehete, P.N., Savary, C.A. Microgravity adversely affects antigen-specific cellular immune responses. *In preparation.*

Funding resulting from these studies:

NRA-96-OLMSA-03 "Use of NASA Bioreactors in a Novel Scheme for Immunization Against Cancer"
        PI: C.A. Savary, Ph.D.; FY 1997 to FY 2000; $465,000

Other activities/accomplishments during Round 2:

Feb. 1998 - presented results of the present studies at the annual meeting of the Biotechnology Cell Science Program Investigators Working Group.

Student involvement in these studies -

        Monica Grazziutti, M.D., Clinical Fellow
        Luis Tome, M.D., Clinical Fellow
        Alysson Ford, summer student, King Foundation Summer Research Program
        Lee Aleksich, summer student, NASA Sharp Plus/Qem Program
        Manu Goyal, summer student; NASA Sharp Plus/Qem Program
                abstract of project accepted for presentation at the AJAS/AAAS conference (Appendix 3)