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FINAL REPORT

While the notion of space travel and exploration has led to some interesting story telling, it has also motivated governments and private parties to spend billions of dollars in the pursuit of taking humans from Earth and into space. Over the last 50 years this effort has taught us that there are several limitations associated with humans leaving this planet. Obviously, the most prominent limitations relate to our biological and physical dependence on what the Earth has and space does not. These life-sustaining requirements include oxygen, food, water, protection from radiation, and perhaps more ambiguous, our dependence on gravity. While food, water, and oxygen can be taken along and measures can be taken to shield humans from some or most of space radiation, altered gravity remains a reality to be confronted in human space travel.

The biological and physiological effects on humans in microgravity have been well documented and result in a wide range of symptoms from muscle atrophy and bone loss to a lowered immune response (1). Fortunately, these symptoms are temporary and the recovery time generally correlates with the amount of time spent in space. Of course, this correlation continues to hold true considering the relatively short time periods that most astronauts spend in space. When long-term space travel is considered, questions arise concerning the human capacity to recover from or compensate for the negative biological effects of space. The major contributors to these deleterious consequences of space travel include the potential exposure to varying degrees of radiation, the increased stress levels associated space travel, and microgravity. The potential long term consequences are significant considering the round trip to and from our closest neighboring planet, Mars, would take close to a year and a half. Based on what is known about the immunological consequences of short-term space travel, it is difficult to imagine even the most prepared and healthiest of humans making such a journey.

Space travel can cause severe immunological effects due to factors such as space radiation, which can destroy sensitive immune cells; increased levels of stress-hormones that are known to lower immune responsiveness and, of course, microgravity (2,3). Deficiencies in immune function caused by microgravity are made clear in the diminished responsiveness of lymphocytes to appropriate stimuli as well as decreased cellular interaction between immune cells resulting in loss of normal regulatory events (4,5,6). In addition, there is evidence that some bacteria actually become more virulent and have higher mutation rates in microgravity (7). Under these conditions, long-term space travel could potentially result in the simplest of infections becoming severe and perhaps lethal in space.

Long-term microgravity-induced immunological consequences may be within a healthy human being’s capacity to endure and recover from. However, questions arise regarding the effects of microgravity on those with developing immune systems such as newborns, infants and pre-pubescent youth. The thymus is responsible for the development of most T cells and is highly active in the early stages of life and progressively diminishes until puberty is reached after which thymus structure and function deteriorates significantly.

T Cell Development

In mice, as well as humans, the stages of T cell development are well defined and characterized by the sequential expression of specific combinations of markers on the cell surface. These cell surface markers are essential for checkpoints and survival signals of pre-T cell intermediates as they migrate and differentiate in the thymus. The final end result of thymic development is the release of various lineages of naïve T cells that have made successful gene rearrangements and satisfied the rigorous demands of positive and negative selection. Collectively, these thymus-derived (T) cells can distinguish between an immense number of self and non-self antigens and upon activation can undergo clonal expansion in order to affect a myriad of adaptive immune responses.

Early progenitor cells migrate to the thymus from the bone marrow by following certain chemokine receptors (8). Once these progenitor cells arrive at the thymus, they begin their thymic ‘education’ by interacting with thymic stromal cells as well as the extracellular matrix, which regulate the process. These resident cells of the thymus provide essential survival signals for developing thymocytes including the cytokine IL-7. During the initial stages of development the thymocytes do not express either CD8 or CD4 and are therefore called double negative (DN) cells. The four intermediate stages making up the DN population (DN1-DN4) are characterized by differential expression of CD4 and CD25 (IL-2R alpha). (CD44 is also known as Pgp-1, which binds hyaluronic acid and mediates leukocyte adhesion).

The DN1 (CD44+CD25+) cell progresses to the DN2 (CD44+CD25+) cell, also called the pro-T cell. The pro-T cell then gives rise to the DN3 early pre-T cell (CD44+CD25-). It is during the DN3 stage that rearrangement of the TCR alpha chain gene begins. A successfully rearranged alpha chain is then stabilized by the pre-TCR alpha (pT alpha) chain in the subsequent DN4 late pre-T cell (CD44+CD25-). (If alpha chain gene rearrangement is unsuccessful or pT alpha is not expressed these cells will either die from neglect or continue down the alpha TCR lineage). The pre-TCR expressed by late pre-T cells is responsible for providing essential signals required for differentiation into CD4+CD8+ double positive (DP) cells, by a process called alpha-selection (9, 10). Once DP cells rearrange TCR? genes, replace pTalpha with TCRalpha chains and express CD4 CD8, they make their way to the cortical-medullary junction where they will undergo the highly discriminatory processes of positive and negative selection (11,12). Successful positive selection involves the survival of DP cells that express alphaTCR capable of recognizing low-affinity self-peptides presented in the context of major histocompatibility complexes (MHC) (13-15). This process ultimately results in vast amounts of mature T cells that can strongly bind foreign antigen in the context of MHC. Negative selection results in the clonal deletion of DP cells that recognize and strongly bind MHC presenting self-peptides, thereby eliminating potential auto-reactive T cells (16). In addition to other lineage pathway commitments (alpha T cells and NK T cells), the ultimate result of thymic “education” is the production and circulation of single positive (SP) CD4+CD8- or CD4-CD8+ mature T cells (12,13).

Selection and Pre-TCR Signaling

The role of pre-TCR in development is much more than that of pTalpha stabilizing the rearranged TCR-alpha chain gene product. Indeed pTalpha binding TCR-alpha is essential to promoting cell survival following this successful gene rearrangement event. Additionally, the pre-TCR has been implicated in several important roles in the DN to DP thymocyte transition including survival, expansion and differentiation (17). Unfortunately, the molecular signaling mechanism for pre-TCR is poorly defined and requires additional study in order to identify various events and interactions associated with expression and signaling.

The pre-TCR is made up of the alpha chain of TCR, the pre-Talpha chain (pTalpha and the CD3alpha,?,? and alpha signaling complex (18). The obvious difference between pre-TCR and typical ?? TCR is that, instead of a clonotypic TCR-alpha chain, the pre-TCR has an invariant pTalpha chain. The structural differences between the TCR–alphachain and pTalpha include the relative sizes of extracellular and intracellular domains as well as their respective protein
residue composition. Compared to TCR-alpha chain, the pTalpha chain has a smaller extracellular domain that includes several conserved charged residues and the intracellular domain of pTalpha is significantly longer and contains proline rich sequences (19). Studies suggest that these structural differences result in distinct pre-TCR abilities such as self-oligomerization of multiple pre-TCR facilitated by the charged residues of the pTalpha extracellular domain (20). In addition, studies using pTalpha transgenes introduced into pTalpha-/- mice showed that the proline-rich cytoplasmic tail is required for the signaling events as well as continuous internalization and degradation associated with the modulation of pre-TCR signaling (21, 25).

Most studies agree that pTalpha is essential for proper signaling and development (19-21). But, there is still much that is unknown with respect to the initiation of the pre-TCR signaling. Efforts to find an external ligand that binds pre-TCR have been fruitful and the generally accepted theory today is that pre-TCR signaling happens in a cell-autonomous, ligand-independent manner. Evidence to support this includes pTalpha’s ability to spontaneously form oligomers and the frequency of internalization of pre-TCR to lysosomes (22, 23). As for the details of pre-TCR signaling, there remains speculation regarding potential interactions. This includes a suggested bridging molecule required for oligomerization as well as an array of potential proteins that interact with the cytoplasmic tail or are involved downstream. Considering these unknowns, as well as evidence of ligand-independence and constitutive internalization of pre-TCR, this signaling mechanism remains rather obscure. Fortunately, current research continues to provide glimpses into pre-TCR signaling events by means of identifying the various kinases and transcription factors associated with pre-TCR (19,20,24).

The specific migratory pathway that T cell intermediates follow in the thymus is just as important as the signaling events within the cells. As in any phase of development, the microenvironment of the developing cell determines the type of external signals and stimuli the cell receives, thereby contributing to the eventual cell phenotype. In the case of progenitor cells entering the thymus, there is a specific migratory path associated with various stages of development, which are controlled by location specific expression of adhesion factors and chemo-attractants (26). In general, the DN (CD4-CD8-) intermediate cells migrate outward toward the subcapsular region of the thymus where they undergo the transition to DP (CD4+CD8+) intermediate cells and begin the migration inward through the cortex and medullary region. At the subcapsular region where the DN---DP transition occurs, there are essential events associated, or corresponding, with survival of DP cells via Delta-Notch interactions as well as the anti-proliferation effects of the TGFalpha-rich subcapsular region (26,27).

As mentioned previously, the events associated with the pre-TCR signaling mechanism are not well defined. However, the developmental stages when pre-TCR signaling is required and when it is not required are more clearly understood. For instance, much is known regarding the stage at which the invariant pTalpha chain is required for stabilizing the newly rearranged alpha chain and inducing autonomous signaling. Additionally, once normal ??TcR is expressed then pTalpha is no longer needed and at that point is severely down regulated. The down regulation of pTalpha actually begins much earlier since it has been shown that pre-TCR signaling itself terminates pTalpha transcription (20). One study has shown that fetal murine thymocytes are able to make this final transition from pre-TCR (pTalpha?) to ??TcR even outside of the thymus when left in overnight cell cultures (28). This extrathymic development transition also corresponds with the easily measurable phenotype transition of CD25+CD4- to CD25-CD4+, which is indicative of functional pre-TCR expression and signaling.

The validity and relevance of ground based microgravity models has often been questioned. Ideally, space biology research should be carried out in the microgravity of space. However, issues of cost and accessibility demand that research questions be first defined and tested using ground-based simulations, with the proviso that such studies provide reasonable clues to the effects of microgravity on biological processes. Ultimately, such results must be replicated in the microgravity of space. Even so, space-based research is also not an ideal environment: It is difficult to transport biological systems into the space environment, it is difficult to separate effects of microgravity from those of vibrations (of rocket exit and re-entry), and most importantly it is very difficult, if not currently impossible, to carry out sophisticated and detailed experiments on board the space station. For all these reasons, well-controlled simulations are still needed and provide a useful platform for examining the effects of vector-averaged, microgravity conditions on biological systems.

The microgravity organ culture dish system (MOCDS), previously developed in the labs of Gruener, DeLuca and in conjunction with Paragon Space Corporation, is an effective ground-based model for studying the effects of microgravity on tissue cultures (30). The MOCDS system has also been tested and cleared for spaceflight by the NASA Ames Research Center for further spaceflight in vitro studies (31). An important distinction to be made at this point is that while the term microgravity is used to describe the conditions associated with cinderotation using the MOCDS system, it is not true microgravity, but more accurately termed vector-averaged microgravity for reasons described in MOCDS methods.

Microgravity Research

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The MOCDS model has been used in our lab to specifically study the effects of microgravity on T cell development in the absence of other space-associated factors (stress-hormones, radiation, etc.). This model has previously been shown to effectively support fetal thymus organ cultures (FTOC), a technique used for several projects in our lab (30). We showed previously that in mouse FTOC subjected to this type of modeled microgravity (cinderotation), development of T cell intermediates was blocked (30, 31). Specifically, this block occurred at the DN (CD4-CD8-) --> DP (CD4+CD8+) T cell transition and was characterized by a loss of DP cells and increased frequency of the immature single positive (ISP) intermediate populations (31). The presence of ISP cells at a higher frequency indicates successful alpha-chain rearrangement but an inability to trigger sufficient CD8 and CD4 expression, which is normally achieved via successful pre-TCR signaling (32).

Potential reasons for this microgravity-induced, developmental block have been researched previously in our lab. These include the possibility that microgravity causes TNF-? induced apoptosis, abnormal IL-7 survival signaling, and disruption of normal CD3 signaling. Previous work in these areas collectively implicates a defect in the pre-TCR signaling checkpoint as the likely source of the block in T cell development. For instance, while apoptosis did not increase in cinderorated cultures, TNF-alpha levels were elevated, which is known to cause signal dampening in T cells (31,33). Also, pre-TCR signaling is required for the maintenance of IL-7 receptor (CD127) expression, which may explain the decreased levels of the receptor for CD127 on cinderorated immature single positive (ISP) cells (31,34). Finally, the ability to overcome this block by treatment of anti-CD3 monoclonal antibody validates the functionality of the CD3 signaling machinery but further implicates pre-TCR expression or function (31).
Since the results of this previous work support the idea that abnormal pre-TCR expression and signaling is causing or contributing to this developmental block, a major focus of this study is to determine the expression and function of the pre-TCR molecules of clinorotated cells. Both extracellular and intracellular flow cytometry are required to determine the expression of pre-TCR since pTalpha chain is expressed both externally and internally (19). While the pT? signaling mechanism remains largely undefined, testing the ability of clinorotated DN cells to progress just beyond the pre-TCR checkpoint to a CD25+CD44+ phenotype would allow an assessment of pre-TCR signaling function. This assay will be based on a study showing that cultured DN cells retain the capacity to progress past the pre-TCR checkpoint even outside of the thymus microenvironment (28).

However, focusing on one signaling molecule such as the pre-TCR is a rather confined approach to examining the effects of clinorotation on development. This approach is further complicated by the ambiguity of the pre-TCR signaling mechanism. Therefore, a major focus of this study was to look at the potentially broader structural effects that clinorotation has on the thymus tissue. This may help to see the effects of clinorotation as having a wide range of effects on the developmental process as opposed to focusing on limited cellular events. Histology of clinorotated and control thymus tissue was performed to examine the particular microenvironments (cortex and medulla) that are essential to T cell development, particular the developmental transition associated with the block in question.

RESULTS

Clinorotated Thymus Histology

Thymus tissue sections of 4-day stationary, motional and clinorotated fetal thymus organ cultures (FTOC) using the microgravity OC dish system (MOCDS) were processed and stained with haematoxylin and eosin (H&E). Clinorotated sections showed a loss of definition between the cortex and the medullary regions of the thymus. In addition, averaged cell counts of the cortical and medullary regions clinorotated tissue revealed a significant loss of cellularity in the thymic cortex. In fact, most of the samples show that the generally denser cortical region is virtually indistinguishable from the thymic medulla in the clinorotated thymus.

Pre-TCR Expression

Flow cytometry analysis was performed on lymphocytes from 4-day stationary, motional and clinorotated fetal thymus organ cultures in order to detect expression levels of pre-TCR via an antibody stain specific for the pTalpha chain. Antibody staining for other T cell specific markers included CD3?, CD4, CD8, ??TCR, and ?? TCR. Flow cytometric analysis of T cells stained for the pT? revealed varying results based on whether the staining was extracellular or intracellular. Standard extracellular staining for pTalpha did not reveal significant changes in preTCR expression on the cell surface due to clinorotation. However, when these cells were stained intracellularly there were some unexpected findings when comparing cells of clinorotated T cells to controls.

Extracellular pTalpha

While pre-TCR expression is known to be expressed at very low, and sometimes undetectable levels, on the cell surface, clinorotation showed no evidence that microgravity affects or alters normal in vitro pre-TCR extracellular expression (44).

Intracellular pTalpha

Representative flow of cells stained for CD8, CD4 and pTalpha show significant changes in levels of cell populations for CD8+ and pTalpha + cells, specifically decreases in CD8+cells and an increase in pTalpha + cells. The same was true when comparing CD4 and pTalpha populations. While the loss of CD4+ and CD8+ cells and increase of CD4- and CD8- cells was somewhat expected (being characteristic of the previously described block), the increase in pTalpha + cell populations when stained intracellularly, however, was rather unexpected. This increase in intracellular pTalpha was seen in all clinorotated samples, including CD8+ and CD8- cells as well as total percentage of cells.

Pre-TCR Signaling

In order to more definitively determine the functionality of pre-TCR signaling of T cells exposed to vector-averaged microgravity, clinorotated T cell intermediates that have not yet passed the pre-TCR signaling checkpoint, namely DN cells (CD4- CD8-), were sorted and cultured for 16 hours. After this period they were stained for developmental markers CD44 and CD25. The flow analysis of these samples indicates that pre-TCR maintains signaling function after clinorotation due to the progression of these cells past the CD44-CD25+ stage into the CD44-CD25- stage.

We have developed a microgravity organ culture dish system (MOCDS) that allows for the normal development of T cells from hematopoietic stem cell (HSC) progenitors. This system mimics the normal development of T cells outside of the body, away from stress hormones made during flight. The MOCDS can be used both on the clinostat and during spaceflight. The culture systems are easy to set up and manipulate which allows for a more detailed analysis. Using this specialized system, our work has revolved around studying the effects of vector-averaged gravity on the development of T cells. In our previous work that utilized MOCDS we demonstrated the gravity-dependence of T cell development. The results from this work suggest that the full sequelae of pre-TCR signaling may not be taking place in vector-averaged gravity, as indicated by a block at the immature single positive stage of development. Since then, we have set out to determine what the underlying mechanism was for this block. Our current focus has been on analyzing the cytokine profiles for our clinorotated cultures, specifically TNF-alpha and investigation of the exact point of blockage using pT-alpha and proliferation studies. Using the information obtained from these and our other studies, we aim to better understand, and hopefully reverse, the effects of clinorotation.

Task Objective: Determine the mechanism by which vector-averaged gravity blocks T cell development at the ISP developmental stage using organ culture, which preserves the 3-D microenvironment of the tissue.

Task Significance: This will allow for better understanding of the mechanism underlying decreased T cell development in vector-averaged gravity. This knowledge can be applied to reversing the effects of vector-averaged gravity on developing T cells. A functional immune system is critical if humans intend to reside in environments that have minimal gravity.
Rationale for HRP Directed Research:

GOALS & OBJECTIVES OF THE GRANT’S RESEARCH

The essence of this project was to examine the effects of real (i.e. space encountered) and simulated (i.e. through clinorotation) microgravity exposure on organ and cell development in the thymus gland of mice that serves as a model system for the development, maturation and well-being of the immune system in mammals, including humans. The potential impact of this study is the awareness that exposure to non-earth gravity fields is likely to produce alterations in the maturation and maintenance of the immune system thus resulting in a compromised system that could decrease astronauts’ resistance to infections and increase infectivity of bacteria and viruses which on earth might only produce minor issues. Considering especially the restricted environment of the International Space Station, the prolonged exposure of astronauts and cosmonauts to infectious agents, and the restricted availability of urgent medical care on board the ISS, issues of a compromised immune system might result in serious consequences.

To examine these issues, the following were goals set for this study:

* Determine whether pre T cell receptor (TCR) expression is affected in clinorotated organ cultures.
* Test the pre-TCR signaling function of clinorotated Double Negative (DN) T cell intermediates.
* Examine alterations in thymus cell architecture associated with clinorotation.
* Determine gene expression changes of thymocytes in space-flown vs ground control cells.
* Determine gene expression changes in thymocytes of space-flown vs ground control cells.

RESEARCH ACCOMPLISHMENTS

Relevance of Study to NASA Missions

Previous Findings & Current Results

Human spaceflight can result in an array of immunological consequences, some of which have been well documented. Previously, our lab discovered a block in T cell development (30) through the use of thymus organ cultures exposed to vector-averaged microgravity (clinorotation). This work implicated a developmental block in pre T cell receptor (pre-TCR) signaling checkpoint as a likely locus of this developmental impasse (30,31). Based on these earlier findings, we examined fetal thymic tissue and cells, respectively, using histology and flow cytometry in tissues subjected to vector-averaged (clinorotated) microgravity, with special attention to the following parameters: Thymic microenvironments, Pre-TCR expression and Pre-TCR signaling. Clinorotated thymic tissues fixed and stained with H&E revealed the loss of the normally well-defined cortical region as well as the loss of cellularity within this region. These finding implicate a developmental block associated with the disruption of the thymic cortex via an extracellularly transmitted process. Flow cytometric analysis showed an increase of cells expressing pT alpha+ intracellularly after clinorotation. These results indicate that that normal degradation and signaling pathways for pre-TCR may be disrupted. For the pre-TCR signaling assay, clinorotated cells that had yet to pass the pre-TCR checkpoint showed no evidence of deficient pre-TCR signaling after a 16-hour cell culture. Together, these results indicate that while pre-TCR expression may be abnormal, pre-TCR signaling is not functionally disrupted by vector-averaged microgravity. We also studied whether space flight affects gene expression in murine thymocytes. This was done by using a microarray analysis of spaceflown adult murine thymocyte RNA which revealed changes in the expression of genes associated with stress-responses as well as genes involved in thymocyte function and development. Stress response-related genes showing significant changes, of 1.9 fold or higher, included decreased expressions of heat shock protein 110 (hsp 110), prostaglandin E receptor 3 (ptger3), heat shock protein 90 (hsp90aa1), FK506 binding protein 4 (fkhp4) and an increased expression of RNA binding motif protein 3 (rbm3). The significant T-cell related genes with expression changes of 1.3 fold or higher all showed decreased expression and include the CXCL10 chemokine, CTLA-4, CD44, IL-7 and IFN-alpha2.

Our work has shed light on the signal dampening effects of microgravity exposure. By further understanding the underlying mechanism of this microgravity-induced effect, we could initiate a new line of research centered on the treatment and prevention of other thymocyte developmental problems, such as Type I diabetes. Many T cell developmental problems involve signaling defects similar to those seen in our microgravity work. For instance, microgravity exposure led to a significant increase in the production of TNF-alpha. Other work investigating the role of TNF-alpha during T cell development has shown that over expression of this cytokine may lead to deficient negative selection and hence the production of potentially autoreactive T cells.

The essence of this project was to examine the effects of real (i.e. space encountered) and simulated (i.e. through clinorotation) microgravity exposure on organ and cell development in the thymus gland of mice that serves as a model system for the development, maturation and well-being of the immune system in mammals, including humans. The potential impact of this study is the awareness that exposure to non-earth gravity fields is likely to produce alterations in the maturation and maintenance of the immune system thus resulting in a compromised system that could decrease astronauts’ resistance to infections and increase infectivity of bacteria and viruses which on earth might only produce minor issues. Considering especially the restricted environment of the International Space Station, the prolonged exposure of astronauts and cosmonauts to infectious agents, and the restricted availability of urgent medical care on board the ISS, issues of a compromised immune system might result in serious consequences.

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* Determine gene expression changes in thymocytes of space-flown vs ground control cells.

The following is a brief summary of the Project's results:

This research project has been completed. A detailed FINAL REPORT was submitted separately. In addition to histology, flow cytometry and cell fate analysis, we have also performed microarray studies to examine changes in gene expression of thymus tissues flown in STS 118. A major manuscript based on this research is in preparation.

Our goals were to:

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* Determine gene expression changes in thymocytes of space-flown vs ground control cells.

The following is a brief summary of the Project's results:

Human spaceflight can result in an array of immunological consequences, some of which have been well documented. Previously, our lab discovered a block in T cell development (30) through the use of thymus organ cultures exposed to vector-averaged microgravity (clinorotation). This work implicated a developmental block in pre T cell receptor (pre-TCR) signaling checkpoint as a likely locus of this developmental impasse (30,31). Based on these earlier findings,
we examined fetal thymic tissue and cells, respectively, using histology and flow cytometry in tissues subjected to vector-averaged (clinorotated) microgravity, with special attention to the following parameters: Thymic microenvironments, Pre-TCR expression and Pre-TCR signaling. Clinorotated thymic tissues fixed and stained with H&E revealed the loss of the normally well-defined cortical region as well as the loss of cellularity within this region. These findings implicate a developmental block associated with the disruption of the thymic cortex via an extracellularly transmitted process. Flow cytometric analysis showed an increase of cells expressing pTalpha. These results indicate that normal degradation and signaling pathways for pre-TCR may be disrupted. For the pre-TCR signaling assay, clinorotated cells that had yet to pass the pre-TCR checkpoint showed no evidence of deficient pre-TCR signaling after a 16-hour cell culture. Together, these results indicate that while pre-TCR expression may be abnormal, pre-TCR signaling is not functionally disrupted by vector-averaged microgravity. We also studied whether space flight affects gene expression in murine thymocytes. This was done by using a microarray analysis of spaceflown adult murine thymocyte RNA which revealed changes in the expression of genes associated with stress-responses as well as genes involved in thymocyte function and development. Stress response-related genes showing significant changes, of 1.9 fold or higher, included decreased expressions of heat shock protein 110 (hsp 110), prostaglandin E receptor 3 (ptger3), heat shock protein 90 (hsp90a1), FK506 binding protein 4 (fkbp4) and an increased expression of RNA binding motif protein 3 (rbm3). The significant T-cell related genes with expression changes of 1.3 fold or higher all showed decreased expression and include the CXCL10 chemokine, CTLA-4, CD44, IL-7 and IFN-alpha2.

REFERENCES CITED (in entire report)


Note that this was a new task created to cover the grant period with new PI, Dr. Raphael Gruener. Original PI, Dr. Dominick DeLuca, passed away on October 26, 2007. For research progress information prior to October 2007, see PI Dominick DeLuca.