INTRODUCTION

Transplantation of hematopoietic stem cells (HSC) has been applied successfully to restore bone marrow function and provide curative therapy for a number of previously untreatable diseases. The original HSC transplant programs were aimed at reconstituting hematolymphopoiesis in patients with immunodeficiency syndromes, aplastic anemia and acute leukemia. Encouraged by advances in myeloablative chemotherapy and immunosuppressive regimens, HSC transplant protocols were subsequently developed for many other hematological malignancies, solid tumors (breast, ovarian, neuroblastomas etc.) and more recently, for a host of metabolic, congenital and autoimmune disorders.

HSC transplantation has the capacity to reconstitute erythroid, myeloid and lymphoid functions. The pluripotent nature of HSC give rise to all blood cell types including immune cells. Patient pre-conditioning for stem cell transplantation includes a myeloablative elimination that eliminates most of the lymphoid cells as well as the lymphopoietic tissues. Thus, the reconstitution of the immune system and in particular the recovery of T cell function, is one of the most important goals of stem cell transplantation.

T cells are regarded as the key regulatory and effector cells for multiple cellular and humoral immune functions. The availability of monoclonal antibodies to lymphoid antigens has played a key role in the identification and characterization of functional T cell populations. Therefore the dissection of T cell responses has led to our current understanding of the functional heterogeneity among T cells and their associated phenotypes. Thus, the enumeration of T cells has evolved from T cell counting to subset profiling.

The purpose of this communication is to review the current understanding of reconstitution of immunocompetent cells and the methods used to assess their recovery.

T CELL BIOLOGY

Helper CD4 and suppressor CD8 lymphocytes were the first human T cell subsets characterized by immunophenotyping. These findings gained clinical relevance with the recognition of the effect of HIV infection on T cells and the importance of CD4 monitoring in the management of HIV/AIDS patients. CD4 phenotyping provides critical clinical information concerning diagnosis, prognosis, monitoring and evaluating the efficacy of developing therapies.

The significance of these findings in conjunction with the availability of monoclonal reagents and technological advances in flow cytometry led to an explosion in the research of T lymphocyte differentiation and functional development. This research has increased our understanding of T cell heterogeneity, the laboratory evaluation of T cells must be more comprehensive than the simple enumeration of CD4 and CD8 subsets.

IMMUNE RECONSTITUTION

Immune reconstitution following stem cell transplantation encompasses the recovery of the adaptive cells and functions of the immune system. Sustained and complete immune reconstitution is achieved in stages, many of which have been defined by the appearance of distinct cell populations.

LYMPHOCYTE SUBSETS

In order to evaluate T cell recovery, the first level of post-transplant monitoring consists of immunophenotyping the total T cell population and the helper (CD4) and suppressor (CD8) subsets (Table 1).
A study of the kinetics of CD4+ T cell recovery demonstrated that the first wave of cells is exclusively in the CD4+CD45RA+CD45RO- memory subset. These memory cells resulted from the expansion of donor-derived naïve/memory T cells present in the inoculum. However, repopulation of naïve CD4 T cells (CD4+CD45RA+CD45RO+) followed different kinetics. Naïve cells develop from T lineage committed stem cells which undergo differentiation in the thymus, and which repopulate a broad T cell repertoire. The effect of age on the naïve T cell repopulation is consistent with the decrease in thymic function with age. The fact that early expansion of CD4 cells was not dependent on patient age supports the notion that this expansion included mostly memory cells. This explains why young recipients recovered normal levels of naïve cells after the first year while for many older patients with low thymic function, it required years to detect any naïve cells.

Other studies showed below normal levels of naïve cells in adults 1.5 years after transplantation. Age dependence of naïve cell recovery has been shown for different transplant variables, such as source of stem cells,[19,20,21] and incidence of Graft Versus Host Disease (GVHD). It is important to note that these variables are all interrelated and it is often difficult to determine which variable is independent. Overall, the integrity of the thymic environment is likely to be the most important factor associated with the age-dependence of naïve T cell reconstitution.

Monitoring the recovery of naïve CD4+ T cell levels has become one of the standard methods for evaluating the efficacy of transplant protocols. Various studies have shown that the levels of naïve CD4 cells are higher when either cord blood or mobilized peripheral blood is the source of transplanted HSC as compared to bone marrow.[19,20,21] It is also believed that transplantation of relatively immature and naïve cord blood stem cells reduces the incidence of GVHD, thus the use of cord blood (CB) stem cells is being carefully evaluated. Naïve cell monitoring is a large component of this evaluation.

The expression of CD45RA and CD45RO was initially used as the basic phenotypic profile to distinguish naïve and memory T cell subsets, respectively. More recently, CD45RA and CD45RO were found to overlap in different functional subsets.[22] Thus the combination of CD8 or CD4 and CD45RA with a variety of other markers is necessary to further discriminate functional T cells. Table 1 describes both the basic and expanded profiles that have been used to define naïve, memory and effector T cell populations. As an example, expression of CD27 on CD8 CD45RA+ cells correlated with a functionally naïve population and loss of CD27 was seen when these cells became highly differentiated.[23] Figure 3 illustrates an example of this expanded phenotypic analysis. Similar results were found with other marker combinations.[24,25]

**Table 1**

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These data indicate that this first level of phenotypic screening has provided useful preliminary information on T lymphocyte recovery following HSC transplantation.
Another parameter that is increasingly useful in monitoring T cell reconstitution is T cell receptor (TCR) diversity. The ability of the immune system to protect an individual from a great variety of pathogens is dependent on a diverse TCR repertoire. Diversity is generated during T cell maturation in the thymus, by recombining TCR genes in the variable (V) regions.

**CDR3 Spectratyping**

CDR3 spectratyping analyzes the complexity of the complementarity-determining regions (CDR3) of the TCR Vβ. These regions are generated during gene rearrangement in the thymus and play a major role in conferring TCR diversity. The greater the size heterogeneity of CDR3 sequences demonstrated for each Vβ family, the more complex and diverse is the TCR repertoire. During the course of T cell reconstitution the number of peaks in a CDR3 spectragraph would be expected to increase as has been described in Roux et al.15 This study also demonstrated a strong correlation between the number of CDR3-complete Vβ families, and the number of CD4+CD45RA+ CD45R0- T cells. Of clinical importance, an increase in naïve cells and in their diversity was necessary for patients to respond to tetanus toxoid immunization. This finding may help define more effective reconstitution protocols. These data also suggest that the degree of diversity is indicative of the level of immune protection.

**T Cell Receptor Excision Circles (TREC)**

During thymic TCR gene rearrangement, DNA fragments are excised in the form of circular DNA or TRECs. TRECs are stable within the cell but do not replicate and are diluted during T cell proliferation. Quantitative detection of TRECs using PCR appears to be a sensitive measurement of thymic T cell output. T cells have the largest number of circles right after rearrangement, then get diluted out by cell division as cells get further in time from thymic emigration. This measurement has been used to monitor the emergence of naïve T cells after stem cell transplantation. TREC levels have been shown to correlate with the frequency of naïve CD4 T cells and with the recovery of proliferative responses.12
Phenotypic Analysis Of The Vβ Repertoire

It is now possible to measure the diversity of the T cell repertoire by detection of the distinct Vβ families of the TCR β chain using multiparametric flow-cytometric analysis. This approach can determine whether representation in the TCR repertoire is broad or skewed/biased. The broadest Vβ usage is indicative of the most intact immune system. The imbalance in naïve/memory T cells seen following stem-cell transplantation undoubtedly contributes to a bias in Vβ usage.

Cytometric analysis has previously limited antibody based T cell repertoire monitoring, because of the large number of cells needed to test more than 20 Vβ families. However a recent advance, which combined most of the available Vβ antibodies into 8 tubes has greatly improved this methodology and made it feasible to include it in the routine monitoring of immune reconstitution.

Figure 4 illustrates the use of Betamark™ flow cytometry to quantitate the expression of 24 distinct TCR Vβ families in functional T cell populations.

Flow cytometry offers a standardized method for the evaluation of TCR Vβ diversity that is easier, less expensive and faster than comparable molecular methods. In addition, multiparametric analysis permits the evaluation of TCR Vβ diversity within functional T cell subsets. This capability may contribute to better patient management in the evaluation of HSC transplantation and immune reconstitution.

IMMUNE FUNCTION/RESPONSE

The measurement of T cell responses after in vitro stimulation is often used to assess effector functions during immune reconstitution. Measurement of proliferative responses has traditionally been performed by measuring thymidine incorporation following polyclonal stimulation with mitogens (PHA, PWM) or anti-CD3 antibodies or specific antiviral and bacterial antigens.** In general, it has been shown that proliferative responses correlate with the generation of naïve T cells and a high degree of diversity as found using phenotyping or molecular methods. More recently, single cell analysis of proliferation using reagents such as BrdU, PCNA, Ki67 and CFSE has become popular in an effort to eliminate radioisotope-based protocols.**

Figure 4 Analysis of TCR Vβ diversity in functional T cell subsets. Twenty-four Vβ families were characterized within the naïve CD4+ T cell population as defined by expression of CD45RA and CD27. See Figure 2 for T cell subsets.

Figure 5 Analysis of Intracellular IFN-γ in Peripheral Blood Mononuclear Cells (PBMC). PBMC were analyzed for CD69 and IFN-γ co-expression following stimulation with Staphylococcal enterotoxin B (SEB) and anti-CD28. Sample preparation and staining was identical for the non-activated control. Samples were permeabilized with IntraPrep and stained with a 5-color antibody cocktail consisting of CD8/IFN-γ/CD3/CD27/CD4.
Multiparametric flow cytometry is the method of choice in assessing the activation responses of lymphocytes, as many post stimulation events consist of up-regulation of surface antigens and intracellular antigens that can be detected with monoclonal antibodies. Thus, measurement of CD69 and cytokine production hours after stimulation, or CD25, CD71, HLA-DR, etc. days after stimulation provides an indication of T cell functional status during immune reconstitution. An example of intracellular cytokine analysis is shown in Figure 5.

**SUMMARY**

Transplantation of hematopoietic stem cells (HSC) has become a common procedure for the treatment of a number of hematological, neoplastic, metabolic and genetic disorders. Immunoaphenotyping by multiparametric flow cytometric analysis has been very effective in defining functional subsets of lymphocytes and has allowed the phenotypic characterization of naïve/memory and effector cells. In addition, T cell diversity can be assessed by TCR Vβ analysis using flow cytometry. This capability has led to more comprehensive immunological monitoring of grafted patients. As a result, it is now apparent that one of the most critical parameters is the reappearance of newly generated and fully diverse naïve T cells, reflecting the availability of T lineage stem cells and the level of thymic function. Once an intact T cell compartment is phenotypically identified, functional integrity of the immune cells can be confirmed by measuring post-stimulation parameters which are indicative of responsiveness.

The improved ability to monitor the course of immune reconstitution will aid in designing therapeutic strategies for HSC transplantation and post-transplant management.

### References


# PRODUCT LISTING FOR IMMUNE RECONSTITUTION STUDIES

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## Hematopoietic Stem Cell Transplantation

Reconstitution of T Cell Immune Function