Utano Summer Immunology Forum 2008

Program and Abstract

Date: August 2(Sat) - 3(Sun), 2008
Place: Grandsoul Nara

Utano Summer Immunology Forum 2008

Date	August 2 and 3, 2008
Venue	Grandsoul Nara, 8-1 Matsui, Utano, Uda, Nara 633-2221, Japan
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Accommodation	Speakers: Grandsoul Nara guest house Audience: Hotel Sugi-no-yu
Foreign speakers	Rene van Lier (University of Amsterdam, The Netherlands) Eric Vivier (INSERM/CNRS, France) Mark Bonyhadi (Invitrogen Corporation, USA)
Domestic speakers	Akira Yamada (Kurume Univ. Sch. of Med., Kurume, Fukuoka) Haruo Sugiyama (Osaka Univ. Sch. of Med., Osaka) Tadao Ohno (Cell Medicine, Inc., Tsukuba) Shuji Nakamura (Hayashibara, Biochemical Labs, Inc., Okayama) Yasunobu Kobayashi (JB Therapeutics Inc., Tokyo) Yoshimasa Tanaka(Kyoto Univ. Sch. of Med., Kyoto) Yoshinobu Matsuo (Grandsoul Res. Inst. for Immunol., Inc., Uda, Nara)

Schedule

Day 1 August 2 (Sat)	
Session 1	Chair: Yoshinobu Matsuo
14:00 –	Opening Remarks Takuo Tsujimura (Grandsoul Nara)
14:10 – 14:45	Cell lines: *in vitro* models for the study of heterogeneity of human NK-like T cells Yoshinobu Matsuo (Grandsoul Res. Inst. for Immunol., Inc.)
14:45 – 15:20	Unique properties of cytotoxic regulatory T cell line, HOZOT, and its possibility for clinical application Shuji Nakamura (Hayashibara Biochemical Labs., Inc.)
15:20 – 15:40	Coffee
Session 2	Chair: Tadao Ohno
15:40 – 16:15	Personalized Peptide Vaccination for Advanced Cancer Akira Yamada (Dept. of Immunol., Kurume Univ. Sch. of Med.)
16:15 – 16:50	WT1 peptide-based Cancer immunotherapy Haruo Sugiyama (Osaka Univ. Graduate Sch. of Med.)
16:50 – 17:25	MOLECULAR BASIS OF CYTOTOXIC CD8+ T CELL DIFFERENTIATION Rene van Lier (Dept of Immunol., Univ. of Amsterdam)
18:00 –	Welcome reception: Grandsoul Nara Hall

Day 2 August 3 (Sun)	
Session 3	Chair: Akira Yamada
9:00 – 9:35	A novel assay for analyzing the function of NK cells and CTL using cell-based high content analysis system Yasunobu Kobayashi (JB Therapeutics Inc.)
9:35 – 10:10	Autologous formalin-fixed tumor vaccine (AFTVac) Tadao Ohno (Cell-medicine, Inc.)
10:10 – 10:30	Coffee
Session 4	Chair: Haruo Sugiyama
10:30 – 11:10	Definition, Traffic and Function of Natural Killer cells Eric Vivier (INSERM/CNRS)
11:05 – 11:45	Human γδ T cells: Application to cancer immunotherapy Yoshimasa Tanaka (Kyoto University)
Luncheon seminar	Chair: Keishi Tanigawa (Biothera Clinic)
11:45 – 12:15	Novel T cell expansion technologies for use in adoptive immunotherapy Mark Bonyhadi (Invitrogen Corporation)
12:15 –	Closing Remarks and excursion guide Yoshinobu Matsuo
Afternoon	Excursion to Hasedera Temple

Cell lines: *in vitro* models for the study of heterogeneity of human NK-like T cells

Yoshinobu Matsuo

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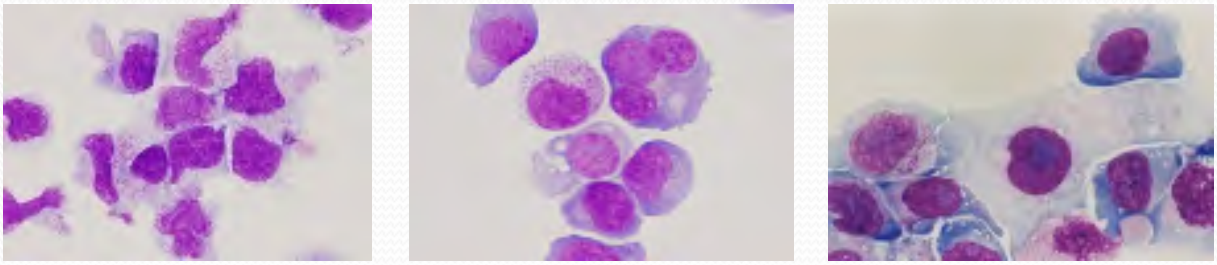
The relatively new cell type of NKT-cells (also termed NK-like T-cells) represents a subpopulation of T-cells that share some characteristics with NK-cells. We analyzed a series of seven NK-, five NKT- and five T-cell lines derived from leukemias and lymphomas using functional, immunophenotypic and genetic tools. All T-cell lines were negative for the presence of azurophilic granules, NK activity and Epstein-Barr virus (EBV). In contrast, 7/7 NK- and 4/5 NKT-cell lines displayed the azurophilic granules but only three of these combined twelve NK-/NKT-cell lines showed significant NK activity. EBV was found in 5/7 NK- and in 1/5 NKT-cell lines. As expected, T-cell lines were commonly positive for T-cell surface antigens and negative for NK-cell markers, and NK-cell lines vice versa; nevertheless, a number of immunomarkers were shared between T- and NK-cell lines. NKT-cell lines express T-cell, NK-cell and markers shared between T- and NK-cells.

Expression of the following thirteen transcription factors (TFs) was analyzed: AML1, CEBPa, E2A, ETS1, GATA1, GATA2, GATA3, IKAROS, IRF1, Pax5, PU.1, T-bet and TCF1 in cell lines tested above. Although expression profiles of these TFs was variable in the lineages, T-bet was positive for all NK- and NKT-cell lines and negative for all T-cell lines. In contrast to the expression of T-bet, TCF1 was negative for all NK- and NKT-cell lines, being positive for 4/5 T-cell lines. Expression analysis of TFs revealed that NK- and NKT-cell lines showed identical profiles, clearly distinct from those of the other cell lines.

In addition, peripheral blood derived “normal” γδT cell line HMGN-167 was established under standard culture containing IL-2 in the presence of Zoledronate. HMGN-167 was infected with HTLV-1, and found to be positive for CD2, CD3, CD4, CD25 and HLA-DR. The cells did not show NK activity in standard condition. RT-PCR revealed the Foxp3 expression. Although Treg cells with γδTcR type are not known, this γδT cell line might represent Treg cells with γδTcR.

The composite data gained on the present panels of cell lines allow for the operational definition of typical NK- and NKT-cell line profiles. Such cell lines will prove invaluable as informative models for studies of normal and neoplastic NK- and NKT-cell biology.



Morphology of expanded γδT cells derived from peripheral blood (left), HMGN-167 γδT cell line established from peripheral blood of patient with colon cancer (center) and MOTN-1 cell line established from peripheral blood of a patient with chronic T lymphocytic leukemia (right). The photomicrographs shown are representative fields from Wright-Giemsa-stained cytospin slide preparations. Note the prominent azurophilic granules in most cells.

Unique properties of cytotoxic regulatory T cell line, HOZOT, and its possibility for clinical application

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We have recently discovered a new type of regulatory T (Treg) cells in the human umbilical cord blood cells. Coculture of mononuclear cells of cord blood with mouse stromal cell lines resulted in the expansion of large granular lymphocyte-like T cell blasts. These cells have been characterized as a unique subset of T cells, and designated HOZOT, which signifies hozo-derived T cell lines (hozo means the navel or umbilicus in Japanese). HOZOT showed a Treg-like phenotype such as $FOXP3^+$, $GITR^+$, $CTLA\text{-}4^+$, and $CD25^{high}$, and also $CD4^+CD8^+$ double positive phenotype. HOZOT exerted dual functions as suppressor and cytotoxic T cells. Suppressor activity, which was evaluated in vitro by allogeneic MLR, seemed to be mediated through soluble factors distinct from IL-10 or TGF-β. Cytotoxic activity, which was originally observed against mouse stromal cells, have been shown against certain types of human tumor cells through a mechanism distinct from NK cells. HOZOT can produce high amounts of inflammatory cytokines, IFN-γ and RANTES, and anti-inflammatory one IL-10. In this talk, I will introduce HOZOT as promising cells both for the basic study on T cell biology and for clinical applications to treat immunological disorders or cancers.

Personalized Peptide Vaccination for Advanced Cancer

Akira Yamada

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Personalized selection of the right peptides for each patient could be a novel peptide-based immunotherapy for boosting anti-cancer immunity in many patients (pts). Here we describe immunological and clinical effects of a personalized peptide vaccine for advanced cancer pts who mostly failed the standard therapy. **Methods:** A total number of 211 advanced cancer pts with HLA-A24 (147 pts) or HLA-A2 (74 pts) positive including 60 hormone refractory prostate cancer (HRPC), 32 pancreatic cancer (PC), 28 colon cancer (CC), 21 brain tumor (BT), 15 gastric cancer (GC), 14 lung cancer (LC) and 41 other types of cancer (Others) pts were enrolled in a phase I/II clinical trial. Pre-vaccination peripheral blood mononuclear cells and/or plasma were measured for their CTL or IgG levels for each of the 14-20 candidate peptides which can induce HLA-A24 or A2-restricted CTL activity against cancer cells followed by weekly or bi-weekly subcutaneous administration of the top four peptides (3mg each) showing the strongest immune responses. The protocol was approved by each ethics committee. **Results:** Vaccination-related adverse events were mainly grade 1 or 2 skin reactions at the injection sites. In the post (6-8th)-vaccination samples, CTL responses were boosted in 131 of 199 (66%) pts tested, while IgG responses were boosted in 140 of 205 (68%) pts tested, respectively. Best clinical responses were 1 CR, 25 PR, 87 SD, and 98 PD. Objective responses were mostly obtained in urological and gynecological cancers or brain tumors by vaccination in the thigh or upper back regions, respectively. Median survival times in all pts are 12 months: 17 months in HRPC (A2 vs A24; 25 months vs 15 months), 8.3 in PC, 5 in CC, 11.2 in BT, 8 in GC, 23.9 in LC and 16.8 in Others, respectively. **Conclusions:** This personalized peptide vaccine could be recommended for further stages of clinical trials because of safety, boosting of immune responses, and potential clinical benefits. **Reference:** Itoh K, Yamada A. Personalized peptide vaccines: A new therapeutic modality for cancer. Cancer Sci. 2006, 97(10):970-976.

WT1 (Wilms' tumor gene) peptide-based Cancer immunotherapy

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In 2001, a phase I clinical study of cancer immunotherapy targeting the WT1 protein was performed for the first time for patients with leukemia, MDS, lung cancer, or breast cancer. Patients were intradermally injected with an HLA-A*2402-restricted, natural (CMTWNQMNL) or modified (CYTWNQMNL) 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant at 0.3, 1.0, or 3.0 mg per body at 2-week intervals, with toxicity and clinical and immunological responses. Toxicity consisted only of local erythema at the WT1 vaccine injection sites in patients with breast or lung cancer or AML with adequate normal hematopoiesis, whereas severe leukocytopenia occurred in MDS in which WBC was derived from WT1-expressing, transformed hematopoietic stem cells. Twelve of the 20 patients for whom the efficacy of WT1 vaccination could be assessed showed clinical responses. Four of eight patients with AML who had minimal residual disease for the assessment of clinical effect responded to three injections of WT1 vaccine in decrease in WT1 mRNA levels in peripheral blood. Three of the four patients are successively being injected at 2-4 week intervals over five years and enjoy complete remission until now. A new phase I/ II clinical study, in which WT1 vaccination was intensified and repeated every week at a dose of 3.0 mg / body of modified WT1 peptide for three months, were started from 2004, and safety of this study was confirmed. The phase II study to asses the clinical effect is being continued. Clinical effect of WT1 vaccination is satisfactory for glioblastoma with relapse. Three of four patients with multiple myeloma had clinical response. In one patient with minimal response, myeloma cells in bone marrow decreased from 86 to 28% after 12 WT1 vaccinations and bone lesions of ribs were improved. These results demonstrated that WT1 peptide-based immunotherapy should be a promising therapy for leukemias and solid tumors.

MOLECULAR BASIS OF CYTOTOXIC CD8+ T CELL DIFFERENTIATION

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A major problem in studying primary anti-viral immune responses in humans is that the moment of infection is generally unknown and that sequential sampling is impossible. We solved both issues by analyzing primary CMV responses in CMV-naïve recipients of allogeneic kidney grafts from CMV carriers. Although these patients receive immunosuppressive medication to prevent immune-mediated rejection of the transplanted organs, frequently the response to CMV is such that the virus can be suppressed without the need for anti-viral treatment. Using this model we have for the first time documented the *in vivo* development of virus-specific $CD4^+$ T cell (Rentenaar et al., *J Clin Invest*, 2000, 105: 541) and $CD8^+$ T cell responses in humans (Gamadia et al., *Blood*, 2003, 101: 2686).

The availability of longitudinal samples of patients experiencing a primary CMV infection opened the opportunity to perform for the first time a transcriptome analysis of virus-specific human CD8+ T cells as they develop from the naïve, via the expanded effector pool to the 'effector-type' fraction characteristic for CMV-specific T cell in the latency stage (Van Stijn et al., *J Immunol,* 2008, 180, 4550). Using Agilent® 40K micro-arrays we found that the expression of approximately 500 genes was significantly (3 donors were tested; $p<10^{-11}$) changed in the antigen-specific fraction. Among these were genes that were altered only at the peak of the $CD8^+$ T cell expansion (to this category belong genes involved in DNA replication and division), genes that were permanently down-regulated (i.e. CCR7, the chemokine receptor that directs naïve T cells to the lymph nodes) and genes that were persistently up-regulated (i.e. NK receptors and molecules involved in cytotoxicity such as granzymes).

One of the main goals of the micro-analysis was to identify genes that would function as 'master regulators' of $CD8^+$ T cell differentiation, specifically controlling the stable cytotoxic

potential. The micro-array database revealed that two members of the T-box family of transcription factors that have been implicated in the differentiation of mouse $CD8^+$ T cells (Intlekofer et al, Nature Immunology, 2005, 6: 1236), i.e. Tbx21 (or T-bet) and Eomes were strongly increased in CMV-specific human $CD8^+$ T cells. Moreover, as in mice, BLIMP-1, which was initially characterized as a factor that governs the terminal differentiation of activated B cells to plasma cells (Martins and Calame, *Ann Rev Immunol,* 2008, 26: 133) but has recently also shown to be a strong regulator of murine effector T cell differentiation (Martins et al., *Nature Immunology*, 2006, 7: 457 and Kallies et al., *Nature Immunology*, 2006, 7: 466), was induced in the virus-specific T cells (Hertoghs, manuscript in preparation). However, as none of the factors mentioned above showed a strict correlation with cytotoxicity, we searched the database for novel factors that might fulfil this criterion. A not yet described zinc finger domain molecule was found that has an over 80% homology to BLIMP-1 in the zinc finger domain. Based on this homology we called the molecule HOBIT (for Homologue of BLIMP- in T cells). Through alternative splicing the molecule can be expressed in three forms but only the form designated 'Large' has an alignment of its zinc finger domains similar to BLIMP-1. Strikingly, expression of HOBIT is primarily found in lymphocytes with cytolytic potential: $CD8^+CD45RA^+CD27^-$ and $CD4^+CD28^-$ T cells and NK cells. Extensive *in vitro* analyses showed that HOBIT expression levels are being down-regulated when either effector-type $CD8^+$ T cell or NK cells are mitogenically activated. An IL-2 dependent NK cell line upregulates the molecule when IL-2 is removed from the culture medium. Together, these data suggested that in analogy to BLIMP-1, HOBIT inhibits cell cycle progression. Indeed, overexpression of HOBIT-L in NK cell lines inhibited their growth. Functionally, overexpression of HOBIT-Large leads to a strong increase of IFNg production; its effects on cytotoxic activity are currently under investigation

A novel assay for analyzing the function of NK cells and CTL using cell-based high content analysis system

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In order to study functions of NK cells and CTL more efficiently and precisely, a novel assay system was established. We recently had an opportunity to try a state-of-the-art cell-based analysis system (CELAVIEW RS100, Olympus). This system is categorized into imaging cytometer, and automatically acquires and analyzes cellular images using a multi-titer plate. Using this system, we first tried cell image visualization and quantification with multi parameters of several cellular events in NK cells, including immunophenotyping, their cytotoxic activities and apoptosis-induction in target tumor cells.

With this system, the percentage of NK cells in PBMC, and their perforin and granzyme B expressions were readily determined in the similar way to FCM gaiting analysis. In cytotoxicity assay, living and dead target cells (K562) were also readily gated and enumerated as FCM-based cytotoxicity assay. We also observed the cleaved caspase-3 expression in cytoplasm of K562 cells, and the percentage of these apoptotic cells was increased in time- and E:T ratio-dependent manner. In some experiments, NK cells-K562 conjugation, the number of the cytolytic granules in each NK cell, and the polarization of these granules to the target cell-contact site were examined on the basis of CELAVIEW image analyzing strategy.

Thanks to the cell-based high content analysis system, multi parameters and cellular events of NK cells were assessed simultaneously, and we confirmed that this novel assay system is one of the useful tools for functional analyses in immune effector cell in more detailed. Using this system, we are now studying the function of CTL in PBMC prepared from cancer patients who had received peptide/DC-based vaccination therapy in our clinic. In this presentation, we will also show you some of our recent results in CTL analysis, particularly analysis of the ability of the cell to make cytokines in response to tumor-associated peptide antigen.

Autologous formalin-fixed tumor vaccine (AFTVac)

Tadao Ohno

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We have developed a novel cancer vaccine, consisting of autologous formalin-fixed tumor fragments and microparticulated adjuvant, named AFTVac. Background technology developed on *in vitro* human cytotoxic T lymphocyte cultures and their clinical efficacy will be described. AFTVac vaccination were performed by giving three five-site intradermal inoculations at biweekly or weekly intervals in clinical trials. The treatment was well tolerated, with only local erythema, induration, and low-grade fever being reported.

In a Phase I/IIa clinical trial to patients after resection of hepatocellular carcinoma (HCC), AFTVac induced longer time before the first recurrence than that in historical control patients operated in the same department. In the followed acedemic Phase IIb randomized clinical trial, the vaccination significantly improved the recurrence-free survival ($p=0.003$) and overall survival ($p=0.01$) rates in a median follow-up of 15 months.

A pilot study was performed to investigate the clinical responses to AFTVac by glioblastoma multiforme (GBM) patients. Of the 12 patients (8 recurrent and 4 primary disease but retained a visible tumor mass after resection), one showed a complete response, one showed a partial response, two showed minor responses, one had stable disease, and seven exhibited progressive disease. The median survival period was 10.7 months from the initiation of the AFTV treatment (24 months from the primary resection) but three of the five responders survived for 20 months or more after AFTV inoculation.

AFTVac will be promising against not only HCC and GBM but also other cancers since the formulation is applicable to any type of fixed cancer fragments such as paraffin-embedded cancer tissue.

Definition, Traffic and Function of Natural Killer cells

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Natural killer (NK) cells are effector lymphocytes of the innate immune system that control several types of tumors and microbial infections by limiting their spread and subsequent tissue damage. Recent research highlights the fact that NK cells are also regulatory cells and engage reciprocal interactions with dendritic cells, macrophages, T cells and endothelial cells. NK cells can thus limit or exacerbate immune responses. Three aspects of NK biology will be discussed.

First, the original definition of NK cells was based on their "natural" cytolytic response against tumor cells and virus-infected cells in the absence of specific immunization. However, "natural killer" neither reflects the education/maturation requirements before NK cells may kill, nor the entirety of their biological functions. In light of new functional assays, genetic models and genomics analysis, we propose to update definition of NK cells. This definition includes the phenotypical identification of NK cells as CD3-NKp46+ cells across mammalian species and functional features shared by subset of T effector/memory T cells.

Seoond, NK cells are widely distributed in lymphoid and non-lymphoid organs. They develop mostly in the bone marrow and go through a complex maturation process that leads to (i) the acquisition of their effector functions (ii) changes in their expression of integrins and chemotactic receptors (iii) redistribution from the bone marrow and lymph nodes to blood, spleen, liver and lung. We will review the current knowledge on the mechanisms of NK cell trafficking, and discuss novel data on NK cell localization in situ.

Finally, although NK cells might seem to be redundant in several conditions of immune challenge in humans, NK cell manipulation appears to hold promise in efforts to improve hematopoietic and solid organ transplantation, promote anti-tumor immunotherapy and control inflammatory and/or autoimmune disorders.

Human γδ T cells: Application to cancer immunotherapy

Yoshimasa Tanaka

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Human γδ T cells recognize pyrophosphomonoester antigens in a γδ -TCR dependent manner. They also exhibit potent cytotoxic activity against human tumor cells pretreated with nitrogen-containing bisphosphonates. Based on the structural analysis, we examined the effect of CDR3 length on the recognition of nonpeptide antigens by γδ T cells. In addition, we carried out a pilot study and a phase I/II clinical study on cancer immunotherapy utilizing γδ T cells activated with 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), an analog of isopentenyl pyrophosphate.

In order to scrutinize the mechanism for nonpeptide antigen recognition by γδ T cells, we employed a Jurkat gene transfer system and evaluated the role of CDR3 amino acid residues in the recognition by random alanine addition and substitution. It was clearly shown that the CDR3 length of TCR- γ chain was critical for the recognition, though TCR- δ CDR3 loops seemed not to be involved in the recognition.

To establish the safety of γδ T cell immunotherapy, we first assessed adverse events and clinical efficacy in patients with renal cell carcinoma infused with 2M3B1PP-stimulated γδ T cells together with IL-2. In 7 patients, the regimen was well tolerated and there were no significant adverse reactions attributable to γδ T cells.

On the basis of the pilot study, we extended the clinical trial and examined the safety and efficacy of administration of 2M3B1PP-activated γδ T cells, IL-2 and nitrogen-containing bisphosphonate. Seven of 10 patients enrolled were evaluable, with 1 patient being in CR, 5 in SD, and 1 in PD. The present results suggest that the combination of 2M3B1PP-primed γδ T cells and nitrogen-containing bisphosphonate is ideal for treatment of patients with renal cell carcinomas.

Luncheon seminar

Novel T cell expansion technologies for use in adoptive immunotherapy

Mark Bonyhadi

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Adoptive T cell-based immunotherapy has evolved over the past 20 years from a relatively simple lymphokine-activated killer (LAK) T cell approach to much more sophisticated approaches. Some of these approaches involve the expansion of tumor or virus-specific T cells from the circulation, isolation and expansion of tumor-infiltrating lymphocytes (TIL), isolation and expansion of regulatory T cells for the treatment of graft-versus-host disease (GVHD) or autoimmunity, as well as expansion of tumor-targeted gene-modified T cells. Translating these applications into practical therapies has been more difficult than originally envisioned as many of the methods used for growing T cells have resulted in T cells with diminished function, altered homing capabilities, and reduction in engraftment/long-term survival potential. Moreover, many of the tools and reagents traditionally used for growing T cells have not been designed with the long-term goal of making T cell therapy a practical GMP-compliant and economical process.

We have developed a variety of tools, reagents and protocols specifically aimed at making T cell expansion practical from both a GMP-compliant perspective, as well as from an economical perspective. In addition, these methods are ideal for preserving T cell function, facilitating T cell engraftment/survival, and preserving the T cell receptor (TCR) repertoire.

One such reagent is the ClinExVivo™ CD3/CD28 Dynabead®. This is a 4.5 micron superparamagnetic Dynabead to which antibodies directed against CD3 and CD28 have been covalently linked. The fully GMP clinical grade ClinExVivo CD3/CD28 bead has been used extensively to activate and expand T cells for clinical applications in the setting of cancer, GVHD, autoimmunity, post-stem cell transplant immune reconstitution, and HIV infection. This versatile platform has been used for the expansion of peripheral blood T cells, cord blood T cells, marrow-infiltrating lymphocytes (multiple myeloma), vaccine-primed tumor draining

lymph node T cells, as well as for the expansion of regulatory T cells. Protocols using this reagent have also been developed for the expansion of antigen-specific T cells and for the transduction and expansion of gene-modified T cells. A WAVE™ Bioreactor protocol has been developed to facilitate the rapid expansion of T cells where densities in excess of $2x10^7$ T cells are readily achieved, thereby offering a space- and media-saving approach to culturing large numbers of T cells. Also, a magnet for the large-scale selection and depletion of the magnetic beads has been developed for clinical applications. Data related to the expansion, function and characteristics of ClinExVivo CD3/CD28-activated T cells will be presented, in addition to various clinical data.

Finally, a new serum-free T cell expansion medium is under development. This medium is being formulated under GMP conditions and has been designed to support T cell activation and expansion in the absence of serum, or with reduced amounts of serum. Moreover, the medium has only a single animal-origin component (HSA), and supports T cell culture at very high density. The medium is intended for growing T cells for clinical applications, including commercialized T cell therapies. Data related to the performance of the medium in a variety of T cells expansion protocols will be presented.