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Molecular Diagnosis of Sezary Syndrome Using Quantitative PCR on 5 Genes

Louise C. Showe, Ph.D., the Wistar Institute, Philadelphia, PA

We previously reported the identification of a small number of genes using cDNA arrays that accurately diagnosed patients with Sézary Syndrome (SS). We now report the development of an assay using quantitative real-time polymerase chain reaction (QRT-PCR) that uses expression values for just 5 of the informative genes, STAT4, GATA-3, PLS3, CD1d and TRAIL. QRT-PCR data accurately classified (100 %) our 17 patients with high blood tumor burden and 12 healthy controls. The same 5 genes were then tested on an additional 56 unique samples from 48 distinct SS patients with blood tumor burdens of 5-99% and an additional 69 samples from healthy controls. The accuracy on these new samples was 90. To test the specificity of these genes for peripheral disease we also assayed 13 samples from patients with Mycosis Fungoides with no detectable peripheral involvement, 3 patients with Atopic Dermatitis with severe erythroderma and 1 SS patient in remission with no evidence of circulating tumor cells. All samples were classified as non-SS patients. These results are the first to demonstrate that quantitative PCR on a selected number of critical genes can be employed to molecularly diagnosis SS, may be useful for detecting peripheral disease in MF patients and to monitor response to therapy.

The Life Cycle and Immunosuppressive Nature of Cutaneous T Cell Lymphoma

Carole L. Berger and Richard L. Edelson, Yale School of Medicine, Department of Dermatology, New Haven, CT

The growth of cutaneous T cell lymphoma (CTCL) is stimulated by an interaction with immature dendritic cells (DC) presenting self antigen in class II MHC to the clonal T cell receptor (TCR) of the malignant CD4 T cells. CTCL cells proliferate and up-regulate the phenotypic and functional characteristics of regulatory T cells (Treg) after encountering DC loaded with apoptotic CD4 T cells. Treg CTCL cells express CD25, CTLA-4, FoxP3 and secrete IL10 and TGF- β . Treg CTCL cells suppress normal T cell proliferation and production of IL2 and IFN- γ . The suppression is not mediated through mechanisms operative in conventional Treg cells including direct contact or secretion of the cytokines IL10 or TGF- β . CTCL cell suppression can be partially reversed by anti-CTLA-4 antibodies and patients with advanced disease have increased levels of soluble CTLA-4 in their serum, indicating a role for CTLA-4 in mediating suppression. One source of antigen that drives CTCL cell assumption of a Treg profile derives from heat shock proteins (HSP). Heat shock enhances CTCL cell development of a Treg phenotype. DC loaded with GRP78 protein stimulate CTCL cells to become Treg and BE2 antibody to GRP78, a molecule expressed on the CTCL cell surface, can inhibit Treg conversion. Quercetin, a

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drug that inhibits HSP, also partially prevents adoption of a Treg phenotype in CTCL cells. Therefore chronic inflammation and increased expression of HSP may play a role in induction of Treg CTCL cells. Antibodies and drugs that reverse the immunosuppressive profile of CTCL may have a therapeutic benefit.

Fas and FasL mediated apoptosis: Role in pathogenesis and treatment of CTCL?

Madeleine Duvic, MD, Xiao Ni, MD, Chunlei Zhang, MD, Dept of Dermatology, University of Texas MD Anderson Cancer Center, Houston, Texas

Programmed cell death (apoptosis) plays a critical role in deleting autoreactive T-cells in the thymus and in limiting T-cell inflammatory responses to antigen or superantigen(s) mediated by activation induced cell death (AICD). Defective AICD of skin homing CD4+CD45Ro+ T-cells is hypothesized to allow their accumulation in cutaneous T-cell lymphoma lesions. In addition, functional Fas L expression on malignant T-cells could eliminate bystander tumor infiltrating CD8+ lymphocytes (TILs) capable of controlling disease progression through cytotoxicity. To further study the role of Fas & Fas L in CTCL, we studied MF lesions and paired normal skin from 21 patients, three CTCL cell lines (HH,MJ,Hut78), and normal control and Sezary patients' PBMC by immunohistochemistry staining, immunofluorescence with confocal microscopy, RT-PCR, western blotting, and flow cytometry. MF lesions from all stages express Fas and FasL. There was an inverse correlation between high Fas+ CD45Ro+ tumor cells and lack of CD8+ cytotoxic cells in skin lesions. CD8+ cells remaining in areas containing FasL+ tumor cells were tunnel positive, suggesting bystander induced apoptosis. CTCL lines also express Fas and FasL. CTCL lines were resistant to CD3 Mab stimulation, but there were variable degrees of apoptosis/AICD following exposure to Fas mAb (CH11). Surface expression of FasL was absent initially and was delayed following exposure to CD3 Mab by as much as 72 hrs in the most resistant Hut78 line. Hut78 cells were best effectors for killing Fas+ Jurkat T-cell targets, There was a strong correlation between cellular FasL and Jurket cell killing among the 3 lines ($r=0.961$). Blocking antibodies to FasL (NOK2) and to Fas (ZB4) and also exposure to bexarotene (10 uM) either blocked or abrogated Jurkat cell killing by Hut78 cells. Sezary PBMCs, but not normal PBMCs from healthy donors, also functioned well as effectors in this assay. These data suggest that a defective Fas/FasLapoptosis pathway and decreased AICD allow accumulation of tumor cells while functional FasL on tumor cells can help to remove the cytotoxic CD8+ anti-tumor response. The Fas/FasL pathway and the mitochondrial stress induced pathway provide potential key targets for develop more effective, novel therapeutic approaches for CTCL.

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Chemokines in CTCL: Biology and Therapeutic Opportunities

Sam T. Hwang, MD, PhD, Dermatology Branch, CCR, NCI. Bethesda, MD

Chemokines were originally defined as small chemotactic proteins that bound 7-transmembrane spanning, G-protein-coupled receptors. It is clear, however, that chemokines and their receptors play diverse roles in cell migration, adhesion, infectious diseases, and cancer growth and metastasis. In addition to their ability to mediate directional migration, chemokines also activate key prosurvival pathways, including PI3K and AKT. With regard to CTCL, the chemokine receptors CCR4, CCR7, CCR10, CXCR4, and CXCR3 have demonstrated to be expressed by the malignant T cells in mycosis fungoides (MF) and Sezary syndrome (SS), where they may be important for localization and survival of malignant T cells. The chemokine ligand of CCR4 known as TARC/CCL17 has been shown to be elevated in the skin and sera of patients with advanced MF. The interaction of CCR10 with its epidermal ligand, CTACK (CCL27), may promote localization of skin-homing T cells to skin and survival of malignant T cells within the cutaneous environment. CCR10-transduced Jurkat leukemia cells were shown to survive for more than 6 weeks in the skin of SCID mice whereas their control counterparts could not. Furthermore, in a B16 melanoma system, CCR10-transduced B16 cells exposed to CTACK were far more resistant to apoptosis induced by GP100-peptide specific cytolytic T lymphocytes (CTL) than the same cells in the absence of CTACK. Chemokines may also be useful in developing targeted therapy for CTCL. Chemokine-tumor antigen fusion proteins bind to specific chemokine receptors present on immature dendritic cells and facilitate antigen processing and cross-presentation of the tumor antigen, resulting in efficient immune responses against tumors *in vivo*. Moreover, other fusion proteins composed of chemokines and toxins may be useful as specific drugs that target and kill tumor cells bearing the appropriate cognate receptors. In summary, chemokines and their receptors may play key roles in the homing and survival of malignant T cells. Chemokine receptor antagonists as well as novel chemokine-based fusion proteins may be useful in target therapy for the cutaneous T cell lymphomas.

Cytotoxic T Lymphocyte Antigen-4 (CTLA4) Biology and Targeted Immunotherapy

John Janik, Metabolism Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland

Cytotoxic T lymphocyte antigen-4 (CTLA4) is one of the major negative regulators of the adaptive immune response. The opposing roles of CD28 and CTLA4 in modulation of T cell responses provides an important area of investigation in cancer immunotherapy. Inhibition of signaling through CTLA4 is predicted to augment T cell immune responses and anti-CTLA4 alone is effective in producing tumor regression and lasting tumor immunity in immunogenic tumor models. In non-immunogenic tumors

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however anti-CTLA4 alone is ineffective and additional signals typically vaccines are required to produce tumor regression. Based on these pre-clinical models, the fully antibody MDX-010 which inhibits signaling through CTLA4 entered clinical trials in 2000. Complete tumor regressions are observed in about 10-15% of patients with renal cell cancer and melanoma. Significant autoimmune toxicity is observed in patients treated with MDX-010 and tumor responses are more likely to occur in the setting of autoimmunity. The most common autoimmune toxicities include diarrhea, skin rash, ocular inflammation and hypophysitis but occasional autoimmune responses to lung, liver, kidney, brain, joints and tendons have been observed and suggest that any tissue could be susceptible to autoimmune attack. We evaluated MDX-010 in eleven patients with non-Hodgkin's lymphoma and have observed tumor regressions in patients with mantle cell and follicle center cell lymphoma with an overall response rate of 27%. Only a single patient experienced significant autoimmune toxicity that required discontinuation of therapy. The study has been expanded to include other histologies of non-Hodgkin's lymphoma, including cutaneous T cell lymphoma.

Gene Expression Abnormalities in Mycosis Fungoides/Cutaneous T Cell Lymphoma

Henry K. Wong, Department of Dermatology, Henry Ford Hospital, Detroit, MI.

Our lab has been interested in identifying and studying molecular defects in Mycosis Fungoides(MF), a malignancy of CD4 T cells that home to the skin. The pathogenesis of this malignancy remains unclear, and to gain better insights into pathogenic mechanisms that may contribute to the development of this disease, we have focused on studying mechanisms that contribute to defective gene expression in T cells in MF (CTCL). We have identified the CTLA-4 as a gene that is abnormally regulated in MF, as well as identified multiple defects in the ability to upregulate inducible cytokine genes, both Th1 and Th2 cytokine genes. To more comprehensively identify gene expression abnormalities in MF/CTCL, we have performed oligomicroarray profiling of purified T cells from Sezary patients and compared the gene expression profile to that of normal T cells. We have identified genes upregulated in purified T cells of MF patients relative to normal memory T cells and have identified genes which were downregulated in the purified T cells of MF patients relative to control memory T cells. The microarray results were validated with qRT-PCR. These findings indicate that MF T cells express novel genes involved in immune modulation, proliferation and differentiation and simultaneously lose the ability to express another subset of genes found in control memory T cells.

To investigate the mechanism for the aberrant gene expression in CTCL, we focused on the control of the T-plastin gene, which is abnormally increased in Sezary T cells. Plastins are proteins that have actin bundling functions with 2 major isoforms, L-plastin and T-plastin. There is tissue specific distribution of

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plastins with T-plastin restricted to epithelial and mesenchymal cells whereas L-plastin is found in hematopoietic cells. The significance of T-plastin in CTCL is not known, however its expression in T cells is highly abnormal. In analysis of PBMC and T cells from patients with SS, we have confirmed the high expression of T-plastin in SS T cells. SS PBMC have greater than 500 fold higher expression of T-plastin than normal. In analysis of cell lines that express T-plastin, expression is readily detected in adherent cells such as human 293 cells and HeLa cells. T-plastin is not detected in T cell line such as Jurkat T cells or lymphoid cells such as CEM. However cell lines derived from SS such as Hut102 and HH have higher levels of T-plastin expression. This suggests the presence of cell-type specific factors that control the expression of T-plastin gene.

In summary, there are multiple gene expression abnormalities that develop in T cells in MF. Studying these gene expression abnormalities may provide insight into the defects that may lead to the development of MF and may provide approaches to normalize T cell gene expression in the treatment of MF/CTCL.

Multimodality Immune Modifying Therapy for Advanced Cutaneous T-Cell Lymphoma

Alain H. Rook, University of Pennsylvania, Philadelphia, PA.

Although indirect, substantial recent evidence supports the importance of the host immune response in the control of disease progression in CTCL, even among patients with Sezary syndrome. Thus, a multimodality approach has been utilized to simultaneously augment the cellular immune response while also targeting the malignant T-cells for apoptotic death. Efforts to enhance antitumor immunity incorporate agents to optimize both the afferent immune response (antigen presentation/dendritic cell differentiation and activation) as well the efferent response (cytolytic T-cell/NK cell activity). In this presentation I will review both classes of agents that are currently available for clinical practice (interferons, GM-CSF, G-CSF, retinoids, fusion toxins) as well as agents in clinical development (Toll receptor agonists, interleukin-12, CD40 ligand) in addition to pro-apoptotic agents (photopheresis, retinoids). Using combinations of these agents for the treatment of Sezary syndrome has resulted in greater than an 80% response rate in regard to skin and blood disease. Therefore, this has been the preferred approach at our center prior to the introduction of therapeutics that lead to the down modulation of host immunity (chemotherapy, nucleoside analogues, HDAC inhibitors, anti-CD4 antibodies).

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Transimmunization for Cutaneous T Cell Lymphoma: A Phase I Study

Michael Girardi, Yale School of Medicine, Department of Dermatology, New Haven, CT

Extracorporeal photochemotherapy (ECP) is a widely used immunotherapy for cutaneous T cell lymphoma (CTCL). It involves four sequential steps: conversion of blood monocytes into dendritic antigen presenting cells (DC) by repetitive adherence and disadherence to plastic surface; reinfusion of the new DC; presumed in vivo loading of the new DC with apoptotic malignant leukocytes; expansion of the anti-tumor CD8 T cell pool. To improve ECP's efficacy by enforcing ex vivo contact between the apoptotic malignant cells and new DC, prior to reinfusion, a single-center, open-label, Phase I clinical study of a revised procedure, referred to as "Transimmunization" was conducted in patients with biopsy-proven stage IB or more advanced CTCL. Twenty-seven subjects were treated monthly for 3 to 5 months, alone or in combination with radiotherapy. For those receiving Transimmunization alone, there was an overall diminution in infiltrative lesions in 11 (55%) of 20 patients. In the 12 leukemic CTCL patients, there was a significant mean reduction of 50.1% in the circulating malignant cells, as determined with family-specific anti-T cell receptor V β monoclonal antibodies ($P \leq 0.021$). Because this therapy permits the synchronous induction and tumor loading of DC, with minimal toxicity, Transimmunization merits further investigation in CTCL and other malignancies.

Extracorporeal Photopheresis (ECP) Induces the Generation of Tolerogenic Dendritic Cells and Regulatory T Cells

Frank Strobl, Kim A. Campbell, Janine Huber, Amy Krutsick, David Peritt, Agatha Schwarz, Akira Maeda, and Thomas Schwarz

Research & Clinical Development, Therakos, Inc., Exton, Pennsylvania USA

Department of Dermatology and Allergy, University Clinics Schleswig-Holstein, Campus Kiel, Kiel, Germany

Extracorporeal photopheresis (ECP) involves the clinical reinfusion of autologous apoptotic peripheral blood leukocytes that have been exposed ex vivo to 8-methoxypsoralen (8-MOP) and UVA light. ECP is approved for the palliative treatment of cutaneous T cell lymphoma (CTCL). The biological mechanism of action of ECP, however, remains unresolved. The prevailing hypothesis is that ECP induces a cytotoxic T cell response against the malignant clone of T cells in CTCL. Recent studies in animal models, however, suggest that delivery of apoptotic cells may actually regulate immune responses through the down regulation of antigen-presenting cell (APC) function, the modulation of cytokines, and the generation of regulatory T cells. When co-incubated with ECP-treated cells, activated dendritic cells produce reduced levels of proinflammatory cytokines, such as IL-12, while TGF β levels were modestly increased. Activation of CD4⁺ T cells in the presence of allogeneic dendritic cells and ECP-treated cells

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promotes generation of a population of T cells that can suppress proliferation of, and IFN γ production by, naïve syngeneic T cells. To confirm these findings *in vivo*, we employed a murine contact hypersensitivity model. ECP-treated or control spleen and lymph node cells from mice sensitized with the hapten dinitrofluorobenzene (DNFB) were injected intravenously into naïve recipients. Compared to controls, mice that received ECP-treated cells demonstrated significantly less ear swelling following sensitization and challenge with DNFB. Suppression of ear swelling was specific for DNFB and cell-mediated, as demonstrated by the ability to transfer DNFB tolerance to naïve mice, which could appropriately respond to the unrelated hapten oxazalone. Transfer of this tolerance was abrogated by depletion of either CD4⁺ or CD25⁺ T cell populations. Collectively, these results suggest that delivery of ECP-treated cells promotes the generation of tolerogenic dendritic cells and regulatory T cells that are capable of modulating immune responses.

CpG Immunotherapy in CTCL

Youn H Kim MD, Department of Dermatology, Multidisciplinary Cutaneous Lymphoma Group, Stanford University School of Medicine, Stanford, CA.

Synthetic oligodeoxynucleotides consisting of unmethylated CpG motifs (CpG ODNs) recognized by TLR9 have potent stimulatory effects of both innate and adaptive immunity. CpG ODNs have been investigated for its therapeutic immune effects in lymphoma using pre-clinical tumor models and in human subjects. Balb/c mice implanted with A20 B-cell lymphoma cells were treated with CpG 1826 +/- chemotherapy or radiation. Mice that were treated with CpG plus chemotherapy or radiation had much improved response than those treated with chemotherapy or radiation alone. This therapeutic effect in mice required a direct intra-tumoral injection of CpG. Effective response was dependent on presence of CD8 T-cells, but not CD4 T-cell or B-cells. Most importantly, this therapeutic effect was dependent on the presence of TLR9 receptor either in the host or on the tumor cells. These animal model findings formed the basis for an on-going clinical trial at Stanford using CpG 7909 plus radiation in patients with nodal B-cell lymphoma or CTCL. CpG 7909 is a B-Class CpG ODN that has been optimized for potent modulation of human immune functions by direct activation of B-cells and plasmacytoid DCs. A multi-center phase I/II study has been conducted using CpG 7909 as monotherapy in patients with CTCL. CpG 7909 was administered as weekly sc injection at rotating sites mostly of non-tumor areas for 24 wks. In the initial phase I/II segment, the dose was escalated from 0.08 to 0.36 mg/kg, where total of 28 subjects were treated. Clinical anti-tumor responses began within a few wks and reached best response of PR (6/28) or CR (3/28) by 4-20 wks. CpG 7909 was well-tolerated mostly with Gr 1-2 injection site reactions or flu-like symptoms. Dose of ≥ 0.16 mg/kg resulted in the best clinical response and was the

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basis for the 10 mg vs. 25 mg dose stratification for the phase II component of the study. The results from the phase II study are forthcoming.

Gene Delivery-Based Immunotherapy for CTCL

Mirjana Urosevic, Department of Dermatology, University Hospital Zurich, Zurich, Switzerland

Immunotherapy using gene delivery of cytokine genes has shown beneficiary effects in modifying anti-tumor response. Intratumoral cytokine gene transfer is suppose to provide locally sustained transgene (cytokine) expression levels with less systemic side effects as with recombinant cytokines. Cutaneous T-cell lymphomas have been successfully treated with interferons (IFNs) that counterbalance the Th2-skewed cytokine dysbalance. Intratumoral injection of TG1042, a non-replicating recombinant adenovirus with a human IFN- γ cDNA insert, allows high local levels of IFN γ without severe toxicity due to systemic delivery. We therefore undertook a phase I/II multicentric trial of repeated, intratumor injection of TG1042 in patients with advanced primary cutaneous T-cell (CTCL) and B-cell (CBCL) lymphoma. To date, 39 patients, out of which 32 CTCL, have been included in the study. Irritation at the injection site, flu-like syndrome and fatigue were the most common side effects, mostly mild and transient. Transgene-derived IFN- γ mRNA was detected in injected lesions. Gene expression analysis of treated lesions revealed profound immune activation together with the up-regulation of IFN γ -inducible genes. Local clinical response has been observed in 17 (including 9 complete responses [CR]) out of 31 evaluable patients. 13 global responses (7 CR) out of 30 evaluable patients have been observed. These results demonstrate that TG1042 is well tolerated and presents a potential significant benefit for the treatment of different cutaneous lymphoma subtypes, including CTCL.

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Mimotope Vaccines for Immunotherapy of Cutaneous Lymphoma

Peter Walden and Wolfram Sterry, Department of Dermatology, Venerology and Allergy, Clinical Research Group Tumor Immunology, Charité – Universitätsmedizin Berlin, Humboldt University, Schumannstr. 20/21, D-10117 Berlin, Germany

We employed a combinatorial peptide library approach to design mimotopes of T cell epitopes associated with cutaneous T lymphomas. This approach involves positional scans with randomized OX₈ nonapeptide libraries to identify active amino acids for every sequence position and combination of these amino acids to potential mimotopes. The active mimotopes selected from the resulting peptide sets were used to analyze frequencies, phenotypes and functional capacities of tumor-specific CD8⁺ T cells in CTCL patients. The frequencies ranged up to 1.4% of the CD8⁺ T cells in peripheral blood and up to 20% in tumor lesions. Most tumor-infiltrating T cells had an effector-memory phenotype (CD45RA⁻/CD45RO⁺/CCR7⁻) but lacked expression of perforin, a key effector molecule of the antigen-triggered cytolytic machinery. A part of the cells expressed the activation markers CD69, HLA-DR and CD95 but were negative for CD25, CD30 and the transferrin receptor, CD71. The tumor-infiltrating T cells, thus, seem to be functional inactive or anergic. In contrast, tumor-specific T cells in the peripheral blood were functional active and, upon stimulation with the mimotopes, produced IFN-gamma. The fine-specificities of T cells induced with mimotopes varied with the peptide sequence and the individual donor for the peripheral white blood cells, and differed from the fine-specificities of the original T cell clones isolated from the tumor. The mimotopes, thus, can recruit and activate a different repertoire of tumor-specific T cells than what is naturally addressed by the tumor cells. They may, thereby, help to overcome immune tolerance to the tumor and tumor-related anergy. In an initial therapeutic mimotope vaccination trials with two CTCL patients, several-fold increases of the frequencies of tumor-specific T cells and tumor regression were induced. Therapeutic vaccination, thus, appears to be a promising treatment option for cutaneous lymphomas.

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Dendritic Cell Immunotherapies for CTCL

Louis D. Falo, Jr., MD, PhD, Department of Dermatology, University of Pittsburgh School of Medicine.

Dendritic Cell (DC) based immunization can induce potent antigen specific T-cell immunity, and DC based immunotherapies are being developed for a broad range of human malignancies. Generation of durable, tumor-specific cellular immunity is a rationale goal for the immunotherapy of CTCL, and is likely to require immunization strategies capable of inducing both tumor-specific CD8+ T-cell immunity and Th1 skewed CD4+ helper T-cell responses. Ongoing studies demonstrate that DC vaccines can induce these cellular responses, and the improvement of DC-based immunotherapies is the focus of considerable effort. Here, we discuss our ongoing efforts to develop an effective DC adoptive transfer-immunotherapy for CTCL. We focus on efforts to address major challenges in tumor immunotherapy including the preclinical and clinical development of strategies to define and deliver tumor antigens, engineer DC function, and overcome tumor induced immunosuppression. Our preclinical efforts to extend results from these studies to the development of "next generation" in vivo targeted DC vaccines will be discussed.

Does Adjuvant Alpha-Interferon Improve Outcome When Combined with Rotational Total Skin Irradiation (RTSEI) for Mycosis Fungoides?

Roberge D, Muanza T, Blake G, Shustik C, Freeman CR, Departments of Oncology (Division of Radiation Oncology) and Medicine (Division of hematology), McGill University, Montreal, Quebec, Canada.

Purpose: To review the McGill experience with adjuvant alpha-interferon (IFN) in the treatment of mycosis fungoides.

Patients & Methods: From 1990-2000, 50 patients with mycosis fungoides were treated with RTSEI. 32 patients were treated with RTSEI alone (Group I) and 18, with RTSEI and IFN (Group II). Median RTSEI dose was 35 Gy. In Group II IFN was given subcutaneously at 3×10^6 units 3x per week 2 weeks prior to start of RTSEI, concurrently and then adjuvantly for 6 months. Group I included 17 males and 15 females with a median age of 61 years (range 31-84). Group II included 12 males and 6 females with a median age of 51 years (range 24-81). Clinical stage was IA, IB, IIA, IIB, III, IVA in 2, 9, 4, 9, 1 and 7 patients of Group I and 0, 3, 3, 6, 4 and 2 patients of Group II.

Results: Median follow-up for living patients was 70 months. All patients responded to treatment. CR rate was 60 % in group I and 59% in group II. Actuarial 5-year OS was 52% for group I and 39% for group II (p= 0.7). Acute grade II-III dermatitis was observed in all patients. Fever, chills or myalgia were exclusively seen in 28% of group II patients.

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Conclusions: Concurrent IFN and RTSEI is feasible, with acceptable toxicity. Although the groups were not matched for disease stage, the addition of IFN did not appear to increase CR rate or OS.

Idiotype Vaccine and Dose-Adjusted EPOCH-Rituximab Treatment in Untreated Mantle Cell Lymphoma

Wyndam Wilson, Medical Oncology Branch, CCR, NCI. Bethesda, MD

Idiotype (ID)-vaccine may eradicate minimal residual disease (MRD) in MCL pts with intact cellular immunity. Effects of rituximab (R) on Id immune responses are unknown, but animal models suggest B-cell depletion may enhance cellular responses. Dose-adjusted EPOCH-R was administered q3 weeks x 6, followed by 5 cycles of Id-vaccine beginning at least 12 weeks later. Id was isolated/scaled using hybridoma technology and conjugated to KLH. Pts received Id-KLH/GM-CSF x 5 over 6 months. Characteristics of 26 planned pts are median (range) age 57 (22-73) and PS 1 (0-2), stage IV (96%) and blastic histology (15%). Disease sites include bone marrow (92%), colon (90%: 18/20 evaluated), splenomegaly (42%: 3/11 resected), and leukemic phase (23%: ALC > 5000/ μ l). All 26 pts completed DA-EPOCH-R with 92% CR/CRu and 8% PR and 11 of 12 (92%) pts with blood MCL became flow negative. Vaccine was administered to 25 pts. At 24 months median follow-up, OS is 100% and EFS is 50%. The proliferation gene expression signature model for CHOP treatment of MCL (Cancer Cell 3:185, 2003) did not predict outcome with a median EFS of 24 and 25 months, respectively, for high and low proliferation (Fig 1). Pre-DA-EPOCH-R and pre-vaccine, the median (range) CD4 cells were 584 (276-2309) and 361 (124-1055) ($p_2=0.0005$ for paired differences), respectively, and CD8 cells were 302 (130-3577) and 377 (75-1034) ($p_2=0.23$). B-cells were 615 (133-70498) pre-DA-EPOCH-R, 0.72 (0-1460) pre-vaccine ($p_2=0.0005$), and 42 (1-2225) post-vaccine. KLH humoral responses occurred in 9/13 pts, and were delayed compared to follicular lymphoma pts receiving vaccine after chemotherapy without R. KLH response developed 5-10 mos after the last vaccine in 3 pts. Post-vaccine, but not pre-vaccine, PBMC from 16/22 (73%) pts recognized autologous tumor cells as demonstrated by TNF, GM-CSF and/or IFN γ ELISPOT. Significant numbers of IFN γ spot forming cells (SFC) were detected in the post-vaccine PBMC (23-140 SFC/100,000 PBMC) as compared to pre-vaccine PBMC (3-18 SFC/100,000 PBMC). Anti-MHC Class I and Class II antibodies inhibited cytokine production suggesting both CD4+ and CD8+ T cells were involved in anti-tumor immune responses; some T cells specifically lysed autologous tumor cells. DA-EPOCH-R can achieve a MRD state while maintaining T-cells and outcome is not predicted by tumor proliferation. Delayed KLH responses suggest B-cell depletion affects early humoral responses, but Id-KLH/GM-CSF vaccine can induce T-cell responses in most MCL patients following rituximab. These results demonstrate that severe B-cell depletion does not impair T-cell

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priming in humans. It is justifiable to administer vaccines in the setting of B-cell depletion, but additional vaccine boosts may be necessary for optimal humoral responses.

TRAF1 Expression Reliably Distinguishes LyP from Primary or Secondary cALCL

Chalid Assaf, Skin Cancer Center Charité, Charité-Universitätsmedizin Berlin

Lymphomatoid papulosis (LyP), primary cutaneous anaplastic large cell lymphoma (cALCL) and cutaneous infiltrates of systemic anaplastic large cell lymphoma (sALCL) are CD30+ lymphoproliferative disorders of the skin that overlap clinically, histopathologically, immunophenotypically, and genetically but differ considerably in their biologic behavior. In particular, lesions of LyP regress spontaneously, while those of cALCL and sALCL persist and often progress with a devastating clinical outcome. Therefore, in contrast to patients with cALCL, LyP patients do not benefit from an aggressive radio- and/or chemotherapeutic approach. In this study, we investigated the expression of the tumor necrosis factor receptor (TNFR)-associated factor 1 (TRAF1). TRAF1 is an intracellular signal transduction component of TNFR, e.g. CD30. We generated a novel TRAF1 antibody that recognizes a formalin-resistant epitope. This antibody was applied in 90 cases of cutaneous CD30+ lymphoproliferations and 15 cases of nodal CD30+ ALCL. The antibody strongly labels CD30+ cells of LyP (42/49 cases, 84 %). In contrast, primary and secondary cutaneous ALCL showed weak to negative TRAF1 staining like the tumor cells of nodal ALCL. The data indicate that TRAF1 expression reliably distinguishes LyP from primary or secondary cALCL. This is of crucial diagnostic importance and has a strong impact on early decision-making in the treatment of patients with cALCL or LyP.

Evaluation of T-reg Population and CD25-Targeting Approaches for Treg Depletion in CTCL.

Larisa Geskin, Soon You Kwon, Timothy Patton, Sue McCann, Lauren Campbell, Kristina Paley and Louis D. Falco, Jr. Department of Dermatology, University of Pittsburgh School of Medicine.

Regulatory T-cells (Tregs) have been implicated in the progression of cancer and in resistance to immunotherapies. Initially, we sought to evaluate and compare Treg populations in patients with CTCL and in healthy volunteers. Patients with advanced disease (stages IIB-IVA) were enrolled in the study under IRB guidelines. Patients were subdivided into 3 groups: responders to current therapy (no evidence of disease or minimal clinical disease); non-responders without peripheral blood involvement (progressive or active skin disease); and active disease with peripheral blood involvement (Sezary Syndrome, SzS). Using multicolor flow cytometry, Tregs were defined as the CD4+CD25+FoxP3+ population. In patients without evidence of disease or with minimal disease at the time of evaluation, Treg levels were comparable to those of healthy volunteers. Patients with progressive skin disease had

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markedly elevated levels of cells with a Treg phenotype (mean 13.5%, compared to 3% healthy volunteers). Patients with profound leukemic involvement, demonstrated intermediate levels of Treg cells (mean 6%). In all but one SzS patient (N=8) T-regs resided within nonmalignant population (malignant clone defined by TCR-Vbeta expression). Interestingly, in one patient, the malignant population included a significant portion of CD4+CD25+FoxP3+ cells (60% of the tumor population), suggesting that a subset of tumor cells expressed a Treg phenotype.

We further sought to determine the effects of denileukin diftitox (ONTAK) on regulatory T cells (Tregs) in Cutaneous T cell Lymphoma (CTCL) patients. CD25 is a low affinity IL-2 receptor transiently expressed on activated lymphocytes, but also highly and constitutively expressed on Tregs. In Mycosis Fungoides (MF), the utilization of CD25 as a target molecule is complex because it is a malignancy of mature memory CD4+ lymphocytes that frequently expresses CD25. ONTAK is an IL-2-diphtheria recombinant fusion protein (DAB₃₈₉IL-2) that is postulated to exert a therapeutic effect in MF by targeting and eliminating CD25 expressing tumor cells, though its mechanism of action has not yet been conclusively established.

Patients undergoing routine therapy with ONTAK were enrolled in this study under IRB regulations. Peripheral blood was obtained before the first course of ONTAK, immediately after the first course (day 5), and 3 weeks after the first course (day 19). Using multicolor flow cytometry, we phenotypically identified T-regs as cells co-expressing CD4, CD25, and FOXP3 markers. We found that ONTAK therapy significantly depleted Tregs during initial administration in the patient naïve to the drug. This depletion was completely reversed in one month. However, for patients receiving ongoing ONTAK therapy Tregs numbers were lower overall than in ONTAK naïve patients.

In conclusion, patients with progressive and active disease have the highest levels of T-regs as compared to normal volunteers. In SzS patients the overall numbers of T-regs may appear lower due to tumor expansion, but are high relative to the population of non-malignant T-cells. Denileukin diftitox (ONTAK) transiently depletes Tregs in CTCL patients, as evident by a decrease in CD4+CD25+FOXP3+ population by flow cytometry. This study provides proof of principle that Tregs can be depleted from the peripheral blood of CTCL patients, and represents a clinically feasible strategy to accomplish this goal in conjunction with other immunotherapeutic approaches, including DC-based immunotherapy.

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Lenalidomide in Patients with Cutaneous T-cell Lymphoma: Preliminary Data of a Single Center Phase II Trial.

*Christiane Querfeld, MD^{1,3}, Timothy M. Kuzel, MD^{2,3}, Joan Guitart, MD^{1,3}, and Steven T. Rosen, MD^{2,3}.
¹Department of Dermatology and ²Department of Medicine, Division of Hematology/Oncology, ³Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Background: Mycosis fungoides (MF) and the leukemic variant Sézary syndrome (SS) represent the most common type of cutaneous T-cell lymphoma (CTCL) comprising 50% of all cutaneous lymphomas. CTCL presents a unique challenge in that no treatment has been shown to prolong survival and patients ultimately develop advanced or persistent disease that is refractory to standard treatment options. Lenalidomide, an oral immunomodulatory thalidomide analogue, is currently being used in clinical trials to treat various hematological malignancies and solid tumors. The immunomodulatory properties such as T-cell co-stimulation with induction of Th1 cytokine production and cytotoxic activity along with anti-angiogenic, anti-proliferative, and pro-apoptotic properties provided the rationale to use this agent in patients with MF/SS. Preliminary data from this ongoing phase II trial are reviewed. **Methods:** Twelve patients have been enrolled between April and December 2005. Eleven patients are evaluable for response and toxicity. Patients received 25 mg lenalidomide daily for 21 days with 7 days rest of a 28-day cycle. Response was assessed after every cycle using Composite Assessment (CA) of Index Lesion Disease Severity for skin lesions, absolute Sézary cell count for quantification of circulating malignant lymphocytes and/or CT scans for measurement of adenopathy or visceral disease. **Results:** The median patient age was 61 years (range, 47-66) and patients had received a median of 6 prior treatment regimens (range, 2-9). Preliminary results indicate that a total of 4 patients have achieved a partial response (defined as a CA ratio less than or equal to 0.5 with no new clinically abnormal lymph nodes, no progression of existing clinically abnormal lymph nodes, and no new cutaneous tumors) after 1 to 3 cycles of therapy. Eight patients have experienced minor responses such as regression of cutaneous tumor lesions and cervical lymphadenopathy in one patient each, and skin improvement from initial generalized erythroderma to less severe erythema with less scaling. Six patients developed progressive disease by developing new lesions and/or recurrence of indicator lesions. Interestingly, most patients experienced an initial flare during the first cycle. A re-growth of former hair loss was observed in 4 patients. The most common side effects were anemia, fatigue, burning sensations, pruritus, and lower leg edema, and were usually mild (grade I or II) at presentation. Two patients developed grade III neutropenia requiring treatment with G-CSF in one patient. One patient discontinued treatment after one cycle because of neurological symptoms (slurred speech) possibly related to the study drug. **Conclusions:** In our study lenalidomide has shown efficacy in heavily pretreated patients with advanced MF/SS and a mild toxicity

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profile. Accrual is ongoing.

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Improved Generation of Anti-Tumor Immunity by Antigen Dose Limitation

*J.D. Shofner, J.G. Vasquez, C.L. Berger, R.L. Edelson, Yale University,
Dept. Derm., New Haven, CT*

Since the malignant cells of cutaneous T cell lymphoma (CTCL) display immunogenic antigens derived from the clonal T cell receptor (TCR), CTCL offers a model for the refinement of anti-tumor immunization methodology. To produce a clinically meaningful anti-tumor response, induction of a cytotoxic anti-CTCL response must be maximized while induction of a suppressive T regulatory cell (Treg) response is minimized. We previously demonstrated that processing of apoptotic CTCL cells by autologous dendritic cells (DC) can lead to either CD8 anti-CTCL responses or Treg induction, with Treg induction being favored when the number of apoptotic cells in co-culture is high. In this study, we determined whether that balance could be shifted towards a positive anti-CTCL response by doing the opposite: lowering the ratio of apoptotic CTCL cells to DC.

CTCL cell apoptosis was produced by engagement of the TCR by anti-CD3 antibody affixed to magnetic beads. Passage of unfractionated blood mononuclear cells from leukemic CTCL patients through these columns efficiently produced apoptotic malignant cells as a source of tumor antigens. The physical perturbation inherent in passage through the same columns simultaneously induced blood monocytes to differentiate into DC, which could internalize and process the produced apoptotic CTCL cells. Purified CD8 T cells were added overnight, phenotyped and analyzed by flow cytometry.

At low levels of apoptotic cell/DC loading ($<1.15 \pm 0.42$), CD8 T cells increased perforin expression ($8.1\% \pm 2.3$ to $10.1\% \pm 1.9$), and mediated a 1.74X increase in apoptotic T cell death ($p \leq 0.05$). Absolute CD8 cell counts ($27.8 \times 10^4 \pm 6.5$ to $38.8 \times 10^4 \pm 4.0$, $p \leq 0.03$) increased significantly upon co-culture with loaded DC compared to controls. A dose dependent increase in CD3+/CTLA-4+ cells was found as the number of apoptotic cells ingested by DC increased ($p=0.0008$), with a high apoptotic cell/DC ratio favoring suppression via Treg generation. Induced Treg CTCL cells suppressed CD8 perforin expression. These findings suggest that the ratio of tumor antigen to DC is an important determinant of whether a cytotoxic anti-tumor or suppressive immune response is generated. A high ratio favors Treg generation while a lower ratio favors generation of a potent anti-tumor CD8 response.