

Highlights of the First International "Immunotherapy in Pediatric Oncology: Progress and Challenges" Meeting

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Summary: The first annual conference on immunotherapy in pediatric oncology was held in Bethesda, MD, from September 9 to 10, 2008 to discuss the state-of-the-art of immunotherapeutic strategies currently being explored in pediatric oncology. Major topics included targeting cell surface receptors, understanding and improving T-cell-based therapies, augmenting innate immune strategies, and enhancing graft-versus-leukemia for pediatric malignancies. As can be seen in the summaries of the individual presentations, significant progress has been made in developing preclinical models of pediatric tumors and a variety of novel immunobiologic therapies are approaching, or already in, the clinic. Although there is much excitement about the potential utility of these agents, a great deal of challenges lie ahead in improving the efficacy of each of these modalities and getting them to patients in a timely fashion. The resulting discussions will hopefully lead to new collaborations and insight for further translational and clinical studies.

Key Words: immunotherapy, cell surface receptors, T cells, innate immunity, graft-versus-leukemia

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Much progress has been made in understanding the tumor-immune interface. We now know that most tumors possess antigens that can be recognized by both the T-cell and B-cell arms of the immune system, and that natural killer (NK)-mediated antitumor effects can confer improved survival from malignant disease. At the same time, the earliest stages of tumor growth involve a dynamic interplay between the growing tumor and host immune responses that ultimately endow the tumor with immune-

evasive properties. Patients with progressive tumor growth often maintain the capacity to recognize their tumor, and ongoing immune responses can contribute to the control of minimal residual neoplastic disease, however, such responses are insufficient in most clinical settings.

Despite progress in the larger field of cancer immunobiology, we still know very little about the specific interactions between pediatric tumors and the immune system. Identification of optimal immune targets and translation of effective immune-based therapies for pediatric cancer await studies that specifically focus on the immunobiology of the host-tumor interface in pediatric cancer. In September 2008, the National Institutes of Health (NIH) Office of Rare Diseases, the National Cancer Institute (NCI)-Cancer Research Center of Excellence in Immunology and the NCI-Pediatric Oncology Branch sponsored a 2-day symposium aimed at bringing together researchers engaged in developing immune-based therapies for pediatric cancer. This summary highlights the translational and clinical work presented at the symposium followed by the individual abstracts submitted to this meeting.

TARGETING CELL SURFACE RECEPTORS ON PEDIATRIC TUMORS

Monoclonal Antibodies

Monoclonal antibodies (moAbs) represent the most effective form of immunotherapy for cancer to date. The administration of moAbs targeting CD20 and ErbB2 has substantially improved outcomes in adult lymphoma and breast carcinoma. However, pediatric lymphomas and leukemias rarely express CD20, and the ErbB2 gene is not amplified in pediatric cancers, rendering surface expression insufficient to induce antitumor effects with currently available moAbs. The GD2 antigen expressed on neuroblastoma, however, can be efficiently targeted with moAbs. 3F8 and hu14.18 are 2 anti-GD2 moAbs currently in clinical trials. The humanized 14.18 moAb has been conjugated to interleukin-2 (IL-2) (hu14.18-IL2) in an attempt to increase NK activation resulting in enhanced antibody-dependent cell-mediated cytotoxicity (ADCC).¹

Dr Paul Sondel (University of Wisconsin) presented results of a recent phase 2 trial through the Children's Oncology Group (COG) on hu14.18-IL-2, which seems to work in preclinical models through ADCC by NK cells. Although no responses were seen in patients with bulky disease, there were complete responses (CRs) observed in patients with either MIBG-positive or bone marrow-positive minimal residual disease (MRD), as predicted by preclinical models. In a preclinical murine model, major

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histocompatibility complex (MHC) expression was higher in tumors that escaped from NK-mediated immunotherapy. In contrast, tumors escaping from T-cell-mediated immunotherapy (FLT3 ligand) show down-regulation of MHC expression. Recent studies in mice indicate that combination of NK and T-cell-mediated approaches (hu14.18-IL2+FLT3 ligand) results in greater antitumor activity.² Future studies will seek to confirm activity of hu14.18-IL2 in neuroblastoma patients with low tumor burden and explore the activity of this agent with other cytotoxic or immunomodulatory agents.

Dr Nai-Kong Cheung (Memorial Sloan Kettering) presented results from the murine moAb 3F8, which also targets GD2, in the context of postinduction chemotherapy for neuroblastoma over the course of 2 decades. 3F8 seems to stay on tumors longer than other anti-GD2 moAbs, and induces 20% to 25% responses in the bone marrow as a monotherapy. 3F8 was given with subcutaneous granulocyte macrophage colony-stimulating factor (GM-CSF) to promote ADCC, which correlates with the FCGR2A (R/R and R/H) genotype polymorphism, in patients with refractory neuroblastoma. In an ongoing phase 2 trial, this combination induced CRs in the bone marrow in approximately 80% of patients,³ and approximately 40% CRs of MIBG⁺ disease. With 3F8, there was a survival advantage among patients receiving subcutaneous GM-CSF versus intravenous GM-CSF. Radiolabeling 3F8 with ¹³¹I for systemic disease does not add to long-term survival, although it seems to be effective as part of salvage regimen for patients with brain metastases.⁴

Dr Robert Seeger (Children's Hospital of Los Angeles) emphasized the importance of a diverse immunotherapy portfolio when attempting to target malignancies with immune-based therapies. This observation is especially pertinent as the method of escape from one mode of immunotherapy may be optimally targeted by another modality. For example, MHC-mediated down-regulation in response to T-cell-based therapies can render a tumor more receptive to NK-cell-mediated therapies. Hence, Dr Seeger has focused on combination therapies whereby moAbs are combined with agents that augment ADCC. Although NK cells alone do not cure mice with neuroblastoma, NK cells combined with the anti-GD2 moAb ch14.18 cured 70% of mice if started 7 days after tumor challenge, whereas waiting 21 days diminished the benefit. Their group was able to significantly improve immunotherapy of 21-day tumors if they added bolus and metronomic cyclophosphamide, zoledronic acid, and bevacizumab to NK plus moAb, which slowed tumor growth and prolonged survival in their model. He also mentioned how his group, and others, is using microarrays of neuroblastoma to determine prognosis.^{5,6}

Dr Alan Wayne (National Cancer Institute) discussed targeting CD22, which is highly expressed on the majority of blasts from patients with pediatric acute lymphoblastic leukemia (ALL), using BL-22, a recombinant immunotoxin composed of an anti-CD22 single chain variable fragment linked to modified *Pseudomonas* exotoxin. This agent reproducibly induces killing of ALL blasts in vitro, has been well tolerated in phase 1 and 2 trials in adults, and has proven efficacy against hairy cell leukemia in adults. Administration of BL22 to children in a phase 1 trial was well tolerated. Common reversible side effects included decrease in the serum albumin, increase in hepatic transaminases and microscopic proteinuria, as seen in

adults.⁷ About 13% of patients developed neutralizing antibodies. Clinical activity was noted at all dose levels. He noted that treating patients with MRD seemed to increase the exposure to immunotoxin, consistent with rapid binding by CD22-positive blasts. A second generation antibody called higher affinity (HA)-22 is currently being studied in a phase 1 trial at the NCI and St Jude Children's Research Hospital.

As we move forward with moAb technology, it seems there are some salient points that must be addressed. We have learned that combining moAb with cytokines may enhance the efficacy of these agents by promoting ADCC, but possibly at the price of additive toxicity, such as the vascular leak observed with IL-2. It seems that the amount of time the moAb spends in contact with its target is critical, and treating MRD may be a more fruitful approach than treating bulky disease because it increases the half-life of the moAb in the serum. Finally, licensing and proprietary issues associated with all of these technologies does restrict the ability to use these agents in combination with each other, and pharmaceutical companies need to share these compounds more freely so we can maximize our understanding of their immunobiology. For example, perhaps giving hu14.18 and 3F8 together may maximize targeting all GD2⁺ cells and lead to greater efficacy in neuroblastoma patients than as single agents.

Chimeric Antigen Receptors

MHC down-regulation may limit the therapeutic efficacy of tumor-directed T cells. Several groups are currently investigating approaches to use gene therapy to exploit the potent cytotoxic activity of T cells while avoiding the need for T cells to recognize cell-surface tumor-derived antigenic peptides in the context of MHC. This can be accomplished by genetically modifying T cells to express "chimeric antigen receptors" (CARs). These endow the T cell with a receptor that harnesses the antigen-recognition of an antibody to activate the T cells to expand, persist, and kill.

Dr Laurence Cooper (M.D. Anderson Cancer Center) discussed his group's use of T cells that have been genetically engineered to express a CAR that activates via CD3 and CD28 endodomains to target B-cell malignancies.^{8,9} The CAR is introduced using a *Sleeping Beauty* (SB) transposon/transposase system to integrate the transgene into the primary T cell. When the 2 SB DNA plasmids are electro-transferred together, there is a 71% improvement in CAR expression compared with when transposon-expressing CAR is introduced without transposase.¹⁰ Current work is focused upon improving signaling through the CAR and translating this technology into clinical trials.

Dr Xianzheng Zhou (University of Minnesota) also presented work on a CAR targeting CD19 developed through a SB transposon system, which he pointed out as providing stable integration at a low cost, and was less immunogenic than using a virus. It also might be safer because the integration is random rather than into the promoter region. His group modified both peripheral blood and umbilical cord blood-derived T cells to express a CD19 CAR/CD20 dual gene. In this instance, the anti-CD20 moAb rituximab can be used to enrich CARs administered to a patient. This CAR/CD20-modified T cell shows specific cytotoxicity against CD19⁺ cell lines, and prolongs survival in animals challenged with these tumors.¹¹

Dr Gianpietro Dotti (Baylor College of Medicine) presented the results of a phase 1 clinical trial in which patients with neuroblastoma received adoptive transfer of T lymphocytes genetically manipulated to express a CAR targeting GD2 expressed by neuroblasts. Baylor's group incorporated that CAR on virus-specific cytotoxic T lymphocytes (CTLs) such as Epstein-Barr virus-specific CTLs (EBV-CTLs) to enhance the *in vivo* persistence of these cells, as they can receive a complete activation by the engagement of their native $\alpha\beta$ -T-cell receptor with antigen-presenting cells expressing latent antigens of EBV.¹² Patients with neuroblastoma received both autologous EBV-CTLs and activated T lymphocytes (ATCs) genetically manipulated to express a CAR targeting GD2. Three different cell dose levels were used, and they found that the CAR-EBV-CTLs persisted longer and at higher levels in the peripheral blood than the CAR-ATCs. The treatment did produce tumor responses, including one CR.

Dr Michael Jensen (City of Hope) has focused efforts on developing CARs targeting IL13R α 2 that is expressed on medulloblastoma and most gliomas.^{13,14} He emphasized the importance of developing approaches to enhance the survival of these cells *in vivo* after adoptive transfer, and presented data suggesting that genetic transfer into starting populations of central memory cells may result in superior long-term survival than when nonselected T cells undergo gene transfer.¹⁵ Central memory T cells can be targeted by using EBV-specific T cells for gene transfer or by immunomagnetic enrichment for central memory populations before gene transfer.

As the field of CAR technology is a bit newer than moAbs, most of the current work has been in preclinical development and testing, and we have less phase 1 and 2 trial data to analyze. Although there is a great deal of promise for the agents presented, certainly other CARs have already been tested in children with neuroblastoma, demonstrating the potential for bringing these agents to the clinic.¹⁶ In addition, CARs that target other receptors are also in development that may offer new breakthroughs in lymphomas or medulloblastoma.^{17,18} As we have mastered integrating receptors for a variety of targets, like CD19 or GD2, using the *SB* or viral-based systems, identifying strategies that help these cells persist will be critical to the success of these therapies.

T-CELL-BASED THERAPIES FOR PEDIATRIC MALIGNANCIES

Several groups are involved in attempts to induce T-cell immunity toward pediatric tumors. Dr Crystal Mackall (National Cancer Institute) presented data from a recently completed phase 2 trial of consolidative immunotherapy that incorporated autologous adoptive cell transfer and dendritic cell (DC) vaccination for patients with high risk and recurrent pediatric sarcomas. The data demonstrated favorable overall survivals using autologous T-cell transfer after cytoreductive chemotherapy, but immune responses to peptides targeted with DC vaccines were modest.¹⁹ As a result, future studies are using tumor lysate-based vaccination, and newer approaches to DC maturation have been developed. A new phase 2 trial for Ewing sarcoma, rhabdomyosarcoma, or neuroblastoma combines regulatory T cell (T_{reg}) depletion, which in preclinical models has improved the effectiveness of adoptive immunotherapy, with rhIL-7 administration to

induce peripheral expansion of T cells and augment immune responses using tumor lysate-based vaccination. She also presented data demonstrating that rhIL-7 is capable of safe, transient T-cell expansion in humans associated with increased T-cell repertoire diversity.²⁰

Dr Stephan Grupp (University of Pennsylvania) presented a similar approach for consolidative immunotherapy that exploits lymphopenia to expand T cells collected at diagnosis from neuroblastoma patients after tandem autologous stem cell transplants (SCTs). In a phase 2 trial, CD3/CD28 costimulated/expanded T cells were given to patients at day+12 after SCT, and patients showed robust recovery of CD4⁺ and CD8⁺ T-cell counts. In a follow-up randomized pilot study, T cells were given at day+2 or day+90 to assess T-cell recovery and responses to pneumococcal vaccination given 12 and 60 days post-SCT. The cohort that received T cells on day+2 had protective pneumococcal responses, but some patients developed a self-limited engraftment syndrome clinically similar to graft-versus-host-disease (GVHD). Survivin is a potentially universal cancer antigen, and is an antiapoptotic protein expressed in high levels in neuroblastomas. The group has observed survivin-specific T cells in neuroblastoma patients,²¹ and future studies will aim to optimize a survivin-based, tumor-directed vaccine into this platform.

Dr Helen Heslop (Baylor College of Medicine) discussed how type 3 latency EBV⁺ lymphomas are very immunogenic such that adoptively transferred donor-derived T-cell lines specific for EBV, given after allogeneic SCT as a means for targeting EBV-associated lymphoproliferative syndrome, have generated sustained CRs in 11/13 patients. Further, complete protection was observed when EBV-specific T cells were prophylactically administered to 101/101 high-risk patients. These T cells can persist for up to 8 years in patients. However, treating immunocompetent patients is a greater challenge as their tumors have mechanisms of evasion in place. Her group manufactured polyclonal EBV-specific cytotoxic T cells and administered them to patients with advanced nasopharyngeal carcinoma in 2 phase 1 trials, and saw an overall response rate of 50% in patients with active disease. More recently, they used EBV-specific T cells that target the subdominant antigens LMP1 and LMP2 after chemotherapy or autograft in 24 patients with type 2 latency EBV⁺ lymphoma, with 12/13 of the high-risk patients remaining in remission up to 4.5 years after therapy and 9 of 11 patients with active disease having clinical responses.²²

Dr Richard O'Reilly (Memorial Sloan Kettering) presented substantial data demonstrating that WT1 is highly expressed in a variety of adult and childhood malignancies including gliomas, acute and chronic leukemia, Wilm tumor, desmoplastic small round cell tumor, and rhabdomyosarcoma. Moreover, several groups have demonstrated that WT1 is immunogenic and that immune responses toward WT1 can lyse tumor cells. Hence, Dr O'Reilly has constructed a pool of pentadeca- (15-mer) peptides that span the Wilm tumor antigen (WT1) that can sensitize donor-derived T cells for treatment of WT1⁺ leukemias.²³ His group has used epitope mapping to select and define 26 relevant epitopes that elicit a robust immune response. Using a xenograft tumor model with a WT1-expressing leukemia or solid tumor, they have shown that adoptive transfer of WT1-EBV transgenic T cells selectively causes regression of established tumors and prevents growth of new tumors when leukemias are infused at the

time of the lymphocyte infusion. A combined phase 1/2 trial is underway to look at infusing these transgenic T cells after a T-cell-depleted allogeneic SCT for patients with WT1-expressing leukemias or myelodysplastic syndrome.

Dr Bryon Johnson (Medical College of Wisconsin) presented a murine model of neuroblastoma that is under study to optimize approaches for consolidative immunotherapy. Using a syngeneic SCT platform, his group implants a luciferase-expressing tumor (AGN2a) on day-8, supplements the graft with added T cells on day+0 of transplant, then gives a vaccine consisting of irradiated AGN2a cells genetically modified to express the immune stimulatory molecules CD54, CD80, CD86, and CD137L on days+2, 7, and 14 post-SCT. They have found that vaccinating during the first 2 weeks post-SCT induces antitumor immunity capable of eliminating established tumors. He also has used T cells sensitized to tumor antigens, and demonstrated that these T cells improve antitumor efficacy. The tumor responses mediated by T cells sensitized to tumor antigens are CD8⁺ dependent and are improved after CD4⁺ depletion in part due to the loss of T_{regs}, but long-term CD8⁺ memory responses were also lost.²⁴ Selective elimination of T_{regs} similarly enhances tumor immunity but does not compromise CD8⁺ memory, suggesting that this may be a viable approach for generating potent long-term antitumor responses against neuroblastoma.²⁵

Dr John Ohlfest (University of Minnesota) presented data on targeting brain tumor stem cells (BTSCs) that form the therapy-resistant portion of gliomas. His group has found that BTSCs exhibit a heterogeneous expression of MHC class I or NK cell ligands and thus are difficult targets for immunotherapy.²⁶ He emphasized the substantial heterogeneity present within clinical tumors, and the potential for targeting of a smaller, and perhaps more homogenous, population of BTSCs as a means for immunologic targeting of the subset responsible for tumor growth. He presented work aimed at developing T-cell-based therapies for brain tumors. By combining administration of CpG nucleotides with a tumor lysate vaccine and intratumoral gamma interferon gene transfer in a murine model of glioblastoma multiforme, his group has demonstrated that gliomas can be made immunogenic, resulting in improved trafficking of effector cells, enhanced tumor cell lysis, and overall survival.²⁷ He is now moving these observations into a more clinically relevant canine model.

Dr Alex Huang (Case Western Reserve) presented studies using intravital 2-photon microscopy to identify critical chemokines that regulate T-cell trafficking to lymph nodes during the early stages of T-cell priming and memory generation. Using genetically engineered tumors, Dr Huang also demonstrated the enhancing effect of inflammatory chemokines in primary tumor rejection and in the generation of systemic antitumor immune responses that could ultimately be targeted in the clinical setting.^{28,29}

Collectively, T-cell-based therapies seem to be effective at expanding both CD4⁺ and CD8⁺ subsets, as well as promoting responses to infectious vaccines, but generation of T_{regs} seem to inhibit antitumor responses. Future studies will need to consider T_{reg} depletion or inhibition of T_{reg} function to be more effective. As observed with moAb trials, T-cell-based therapies may function better in the setting of MRD than in patients with gross residual disease. We are also finally developing strategies to move these

therapies past the blood-brain barrier to target brain tumors in a specific fashion.

AUGMENTING INNATE IMMUNITY FOR PEDIATRIC CANCERS

The innate immune system spans phagocytes, monocytes, macrophages, dendritic cells, and NK cells. Each of these cells exhibit effector function in their own right, but in addition, the responses of these innate immune cells often serve to initiate the adaptive immune system, which can result in long-term immunologic memory. Several groups are involved in activating the innate immune system to induce direct antitumor effects and/or to help initiate or augment adaptive antitumor immunity.

Toll-like Receptors

One approach to activate innate immunity is through the use of CpG oligodeoxynucleotides (ODNs) that mimic viral and bacterial DNA and therefore stimulate toll-like receptor (TLR)9 on innate immune cells and some tumor cells. Dr Kirk Schultz (British Columbia Children's Hospital) presented studies demonstrating that CpGs can increase the immunogenicity of ALL cells, resulting in enhanced T-cell-mediated reactivity.³⁰ When CpGs were administered in vivo to mice bearing ALL xenografts, antitumor effects were observed, related both to tumor-directed increases in immunogenicity and antitumor effects mediated by the innate immune system in vivo. In addition, depletion of NK cells by anti-asialo-GM1 treatment significantly reduced the in vivo antileukemic activity of CpG ODN.³¹ Clinical trials to evaluate the efficacy of CpG ODNs for leukemia are being developed.

Dr Bruce Blazar (University of Minnesota) presented promising data from murine models showing enhanced rejection of acute myelogenous leukemia (AML) in mice treated with CpGs as well. His group has shown that administering TLR9 agonists to naive mice 2 days before AML challenge allowed mice to tolerate at least 100 times a lethal tumor dose, but giving CpG ODNs later after syngeneic SCT allowed recipients to tolerate 10 times a lethal dose.³² After a major-mismatched allogeneic SCT, giving TLR9 agonists on day 0 accelerated GVHD,³³ but when it was used with a DLI 15 days after a T-cell-depleted SCT, TLR9 agonists enhanced survival to AML challenge.³² Separate class I and class II antigen major mismatch SCT models demonstrated that TLR9 agonists led to host antidonor rejection and low chimerism postallogeneic SCT.³³ Finally, a phase 1 trial of TLR7 agonist (852A) for refractory solid tumors was presented, and an increase in α interferon levels was observed along with enhanced NK cell function.³⁴ Preclinical allogeneic SCT models of TLR7/8 agonists given in the peri-SCT period also show accelerated GVHD and graft rejection, suggesting we should be cautious if TLR 7/8 or TLR9 agonists are to be used in humans early post-SCT.

Dr Gregor Reid (The Children's Hospital of Philadelphia) presented preliminary work showing that CpG ODNs enhanced survival in mice bearing syngeneic murine ALL. The death of leukemia cells in vivo was independent of the ability of ALL cells to respond directly to CpG ODNs, and correlated with the production of proinflammatory cytokines by the host.³¹ CpG ODN stimulated ALL cells also showed increased costimulatory molecule expression and skewed T cells toward a T_H 1 cytokine profile.^{30,35}

Exploitation of the Tumor Microenvironment for Therapeutic Benefit

Dr Eugenie Kleinerman (M.D. Anderson) summarized her work in developing new approaches to treat lung metastases in patients with osteosarcoma. She discussed the studies demonstrating improved survival in pet dogs with osteosarcoma that were treated with MTP-PE after surgery compared with those treated with surgery alone. She also gave the most recent update from the phase 3 COG trial in newly diagnosed osteosarcoma patients. This trial involved almost 700 patients and demonstrated a statistically superior long-term survival rate in those patients treated with MTP-PE together with either 3-drug or 4-drug chemotherapy compared to the group that received 3-drug or 4-drug chemotherapy without MTP-PE.³⁶ In addition, Dr Kleinerman presented her investigations using a human osteosarcoma lung metastasis nude mouse model which demonstrated that the expression of Fas on osteosarcoma cells correlates inversely with metastatic potential, and that up-regulating Fas expression resulted in the regression of established pulmonary metastases.^{37,38} The aerosol administration of IL-12 gene therapy,³⁹ gemcitabine,⁴⁰ or liposomal 9-nitro camptothecin (L-9NC),⁴¹ increased Fas expression in the pulmonary metastases with subsequent tumor regression. This effect was mediated by Fas ligand (FasL) in the lung.⁴⁰ Aerosol gemcitabine showed no activity in FasL-deficient mice, showing for the first time that the tumor microenvironment plays a critical role in therapeutic efficacy. These agents may have potential in the treatment of patients with relapsed disease. Aerosolized L-9NC is being combined with oral temozolomide in a clinical trial at M.D. Anderson.

Leonid Metelitsa and his colleagues (Children's Hospital of Los Angeles) have investigated the immune microenvironment of neuroblastomas by analyzing gene expression in primary tumors and studying the infiltration of innate cells into these tumors.⁴² He discussed why neuroblastoma patients with better survival have increased numbers of NKT cells in their tumors. Because neuroblastoma cells are CD1d⁻, he hypothesized that NKT cells may target the CD1d⁺ cells of the tumor microenvironment. Dr Metelitsa presented data that tumor-associated macrophages (TAMs) express CD1d and can be specifically recognized and killed by NKT cells. His group found that TAMs are the main source of IL-6 in primary tumors and metastatic bone marrows. IL-6 directly stimulates growth of neuroblastoma cells in vitro and in vivo, and expression of IL-6, IL-6R, and other TAM-related genes associates with poor survival. He concluded that killing of tumor-promoting TAMs may represent a novel mechanism of NKT cell antitumor activity.

NK Cell-based Therapies

NK cells represent another arm of the innate immune system that has been extensively studied in cancer immunobiology. They are attractive candidates for therapeutics as they do not require MHC expression by cancer cells and in fact, NK-mediated killing is increased when MHC expression is diminished. Recent studies have shed more light on the activating and inhibitory factors that control NK-mediated killing of cancer cells.

Dr Dario Campana (St Jude Children's Research Hospital) also presented data evaluating the potential of ex vivo-expanded NK cells for treating leukemias and solid tumors of childhood. He used a genetically modified cell

line (the leukemia cell line K562 transduced with CD137L and membrane-bound IL-15) to activate and expand human NK cells. A median expansion of more than 20-fold was observed after 7 days of culture, with no preferential expansion of any NK cell subset.⁴³ The NK cells generated in this culture system express gene expression profiles that are different than those expressed by primary or IL-2-activated NK cells. Expanded NK cells showed a high cytotoxicity to myeloid leukemias and even some pediatric solid tumors, including Ewing sarcoma, rhabdomyosarcoma, and neuroblastoma. Their cytotoxicity against ALL cells was relatively weak. To overcome this limitation, the group transduced these NK cells with a CAR against CD19 (anti-CD19-BB-z), which resulted in enhanced cytokine production of gamma interferon and GM-CSF, and markedly enhanced killing of ALL cells.⁴³

Dr A.C. Lankester (Leiden University, the Netherlands) presented a series of studies investigating NK receptor ligands on Ewing sarcoma cell lines. These studies demonstrated that Ewing sarcomas express ligands for activating NK receptors, and that these receptors are critical for NK-mediated lysis of Ewing sarcoma. Notably, even chemoresistant Ewing sarcoma cell lines are susceptible to NK-mediated lysis. The efficacy of lysis was enhanced with IL-15.⁴⁴

We are steadily improving our ability to develop clinical-grade cell based-therapies for refractory tumors. Stimulation of TLRs seems to yield durable antileukemic responses, but caution must be used in the setting of allogeneic SCT. Regarding cell-based therapies, artificial antigen presenting cells (APCs) are going to play a major role in the future in expanding these cells further ex vivo so that adequate doses can be delivered to patients in a timely and effective fashion. Additionally, studies involving the tumor microenvironment have demonstrated that effector cell trafficking to tumors is also a critical determinant of success, and must be dealt with simultaneously.

AUGMENTING GRAFT-VERSUS-LEUKEMIA FOR PEDIATRIC HEMATOLOGIC MALIGNANCIES

Allogeneic hematopoietic SCT arguably represents the most effective form of immunotherapy for hematologic neoplasms to date given that a large part of the curative effect of this procedure results from immunologically mediated graft-versus-leukemia (GVL). Dr Franco Locatelli (University of Pavia, Italy) presented the current state-of-the-art of allogeneic SCT for pediatric leukemia. Although it has been previously reported that the potency of GVL against ALL is less than for myeloid leukemias, Dr Locatelli presented data demonstrating that the occurrence of chronic GVHD is associated with a lower risk of leukemia recurrence in children with ALL.⁴⁵ Also, grade I/II/III acute GVHD improves survival for patients who undergo allogeneic SCT for ALL, preventing the incidence of cumulative leukemia relapse. Furthermore, while in adult patients, allogeneic NK cells seem to produce better disease-free survival for AML than for ALL, he presented data that in pediatric leukemia, ALL may be the better target. Biologic support to this observation is given by a recent paper documenting that in pediatric recipients of a haploidentical SCT, donor-derived alloreactive NK cells are generated and persist for many years.⁴⁶ Finally, provocative data were presented demonstrating that in the setting of haploidentical SCT, maternal SCT donors lead to

better survival as a result of both lower relapse rate and transplant-related mortality than paternal donors, even to female recipients.⁴⁷ This clinical observation raises the issue of transplacental trafficking of host antigens sensitizing the mother; the few T cells transferred with the graft (ie, a total of roughly 0.5 to 1 million cells), could undergo unopposed proliferation after transplantation by virtue of the absence of pharmacologic GVHD prophylaxis.

Dr R. Maarten Egeler (Leiden University, The Netherlands) presented data from the Dutch Oncology Children's Group on how MRD levels before SCT affect outcome for ALL patients.⁴⁸ His group is investigating if early tapering of cyclosporine, followed by incremental donor lymphocyte infusions (DLIs), could be safely performed in patients with no or low-grade GVHD. MRD detection assays on bone marrow were performed pre-SCT and patients were stratified into "MRD low" and "MRD high" cohorts. If patients were MRD low, there was no intervention, and their cyclosporine was tapered by day 100. If patients were MRD high, cyclosporine was tapered at week 4 or 5, and then 3 to 4 DLIs were given starting at weeks 9 to 10. The DLIs have not caused GVHD in any patients thus far. The results confirmed that pretransplant MRD did predict a higher rate of relapse after transplant, showed that the rapid taper and DLI could be performed safely and raised the prospect that such therapy may delay recurrence of ALL after transplant.

Dr Rupert Handgretinger (University of Tuebingen, Germany) presented data using immune-based therapies after haploidentical SCT for patients with hematologic malignancies. Whereas most investigators have used CD34 selection to prevent T-cell-mediated-GVHD in this setting, Dr Handgretinger illustrated the role for selective T-cell depletion, which leaves substantial numbers of NK cells and NK precursors within the grafts of adult reduced-intensity SCT patients.⁴⁹ Such grafts show greater NK expansion posttransplant, which may be associated with improved antitumor effects. They have learned that using G-CSF to mobilize CD34⁺ cells from donors may be deleterious on NK cells, and suggested exploring GM-CSF instead. He also presented the first clinical data on the ex vivo activation of NK cells with IL-15 and their subsequent infusion posttransplant. He also presented a large-scale method of depleting $\alpha\beta$ -T cells to enrich peripheral blood stem cells for NK cells and $\gamma\delta$ T cells.⁵⁰ Using this platform, he presented data infusing the moAb hu14.18 with NK cells or $\gamma\delta$ T cells for treating refractory neuroblastoma in a neuroblastoma mouse model.

Dr Dagmar Dilloo (Heinrich Heine University, Germany) presented approaches to enhance GVL in ALL. She has demonstrated that modulation of the ALL blast can also be used to augment GVL, and improved immunogenicity is seen after CD40 activation, via CD70 and CD80/86 up-regulation, resulting in substantial T-cell priming.^{51,52} As blockade of CD70 prevents effector T cell expansion and reduces cytotoxicity, strategies facilitating up-regulation of CD70 on APCs are critical for augmenting the quality of an antileukemic response against ALL. In a pilot clinical study for patients with relapsed ALL postallogeneic SCT, her group employed DCs pulsed with ALL cell lysate as a vaccine in combination with DLI, and induced responses in all patients, with some surviving long term. Future studies will aim to use CD40-activated ALL or lysate-pulsed professional DCs for T-cell priming, with manipulation of the CD70:CD27 axis serving as a means

for augmenting T-cell reactivity not only toward ALL, but also to a variety of different tumor antigens.

Dr A. John Barrett (National Heart, Lung, and Blood Institute) presented clinical work aimed at augmenting GVL by selective depletion of alloreactive T cells. Initial studies used CD25 immunomagnetic bead-based depletion after mixed lymphocyte cultures to deplete alloreactive T cells. More recent studies have exploited the differential capacity for activated versus resting T cells to extrude a photosensitizer, which can also profoundly and selectively deplete alloreactive T cells when exposed to light. Early clinical results with this approach show minimal GVHD after alloreactive T-cell depletion.⁵³ Future studies will build upon this platform, and seek to augment tumor-directed immune reactivity by combining alloreactive T-cell depletion with tumor antigen-based vaccines.

Dr Terry Fry (Children's National Medical Center) presented preclinical studies aimed at enhancing GVL using tumor vaccine-based approaches.⁵⁴ His data demonstrate a critical role for both thymic-dependent immune reconstitution after transplant in order for effective immunotherapy to be undertaken because of the risk of immunosuppressive GVHD when T-cell doses sufficient to induce meaningful antitumor responses are delivered via DLI. He also demonstrated potent immunosuppressive effects of GVHD on tumor-directed vaccines, thus emphasizing the importance of preventing GVHD if optimal effects of immune-based therapies are to be realized after allogeneic SCT.

The post-SCT environment seems to be a ripe platform for immunotherapy for already high-risk or refractory malignancies, and efforts seem to be headed toward making APCs better able to present tumor antigens, or making tumors better APCs themselves. In the allogeneic SCT setting, GVHD remains a significant obstacle, but a T-cell-depleted platform may allow both MHC-mismatched NK cell or delayed T-cell add-back therapies to be administered. Therapies that can abrogate GVHD without impacting antitumor effects also remain essential.

SUMMARY

Although preclinical and clinical testing of immune-based therapies have been pursued for a variety of malignancies, to our knowledge, this meeting reflected the first time clinical and translational immuno-oncologists met to exclusively discuss progress and opportunities pertaining to the use of immune-based therapies specifically for the treatment of childhood malignancies. A growing body of preclinical and clinical data supports the anticipated clinical benefit of certain immune-based treatments for these childhood diseases as clinical testing proceeds. Participants agreed that worldwide collaboration in the testing of these concepts will augment this progress, and has been aided by this meeting. As such, discussions are underway to convene a similar meeting, likely in approximately 2 years, and probably in Europe, to continue the development of a worldwide "community" of clinical-translational pediatric immuno-oncologists.

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Abstracts

1. MONOCLONAL ANTIBODIES

Development of hu14.18-IL2 for GD2+ Tumors

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The hu14.18-IL2 immunocytokine (IC) consists of the humanized 14.18 (hu14.18) antidisialoganglioside (GD2) monoclonal antibody (mAb) genetically linked to 2 molecules of human recombinant interleukin-2 (IL2). Anti-GD2 mAb and IL2 have been tested in a variety of diseases, including neuroblastoma and melanoma. Preclinical data show that hu14.18-IL2 is active in animal models of neuroblastoma, requires NK cell activity and can also induce T-cell reactivity, and is more effective when applied in animals with smaller amounts of established tumor. These preclinical data suggest that clinical activity may be possible in the presence of NK function, even if T cells are inhibited by prior chemotherapy, but would likely be most effective in the minimal residual disease setting. Phase 1 trials of hu14.18-IL2 have been published, and provided regimens for phase 2 testing. Phase 2 trials in adults with melanoma and in children with refractory neuroblastoma have recently been completed and reported at ASCO 2008 (Albertini et al¹ and Shusterman et al,² respectively). In the Children's Oncology Group (COG) phase 2 study of neuroblastoma (COG-ANBL0322), 0 of 13 evaluable Stratum 1 patients (those with bulky disease, measurable by CT or MRI) showed any response.

In contrast, 5 of 23 evaluable patients in Stratum 2 (disease detectable only by MIBG scanning and/or standard bone marrow histology) showed complete responses; 3 of these lasting over 1 year. Two additional Stratum 2 patients showed partial improvement, but did not meet protocol criteria for PR or CR. While not powered or designed to address this comparison, these data suggest that the response rate in Stratum 2 (5 of 23) may be different from that in Stratum 1 (0 of 13) ($P = 0.07$ 1-sided t test), as hypothesized from the preclinical data. Preclinical development includes combining hu14.18-IL2 with other antitumor agents that are not myelosuppressive or immunosuppressive, in order to develop it as a means to eradicate persistent microscopic disease remaining after chemotherapy. In addition, local intratumoral administration of hu14.18-IL2 in mice appears to provide far greater local antitumor effects against measurable tumors while still providing systemic effects. Next steps include obtaining additional data with hu14.18-IL2 treatment in Stratum 2 neuroblastoma patients to confirm antitumor efficacy in the MRD setting, as well as testing in the MRD setting following surgical resection for patients with melanoma. Consideration is also being given to clinical testing of hu14.18-IL2 with other agents showing synergy in the preclinical setting (chemotherapy, antivascular therapy, other immunotherapy).

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Radically Changing the Natural History of Metastatic Central Nervous System Cancer: A Pilot Study Incorporating Compartmental Intrathecal Radioimmunotherapy

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Background: Innovation in the management of brain metastases is needed. We evaluated the addition of compartmental intrathecal antibody-based radioimmunotherapy (cRIT) in patients with recurrent metastatic central nervous system (CNS) neuroblastoma following surgery, craniospinal radiation, and chemotherapy.

Methods: Of 48 patients treated for recurrent neuroblastoma metastatic to the CNS, 15 received a cRIT-containing salvage regimen incorporating intrathecal 131I-MoAb targeting GD2 (MoAb 3F8) or B7H3 (MoAb 8H9) following surgery and radiation. Most patients also received outpatient 3F8/GM-CSF immunotherapy, 13-cis-retinoic acid, and oral temozolomide for systemic control. Thirty-three patients received conventional treatments.

Results: Thirteen of 15 cRIT-salvage patients remain free of CNS neuroblastoma 6 to 58 months after CNS event, with 11 in complete remission. One patient died of infection at 22 months with no evidence of disease at autopsy, and 1 of lung and bone marrow metastases at 15 months. The cRIT-salvage regimen was well tolerated, notable for myelosuppression minimized by stem cell support ($n = 5$), and biochemical hypothyroidism ($n = 5$). One patient with a 7-year history of metastatic neuroblastoma is in remission from MLL-associated secondary leukemia. In contrast, 31 of 33 patients receiving conventional treatment have died, median time to death was 5.8 months for those with systemic and CNS disease and 11.5 months for those with isolated CNS relapses from the CNS event.

Conclusions: The cRIT-salvage regimen for CNS metastases was well tolerated by young patients, despite their prior history of intensive cytotoxic therapies. It has the potential to increase survival with better than expected quality of life.

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Developing Immune-based Therapies for Neuroblastoma: Treatment of Metastases of Drug-resistant Neuroblastoma Cells in NOD/SCID Mice With Human Natural Killer Cells and Anti-GD2 Antibody Combined With Cyclophosphamide, Zoledronic Acid, and Bevacizumab

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Background: Neuroblastomas that progress are clinically drug resistant. Activated natural killer (aNK) cells ± antitumor antibodies (mAb) can kill drug-resistant cells in vitro. However, the efficacy of aNK cells ± mAb against metastases formed by drug-resistant cells in vivo is unknown. We evaluated (1) aNK + anti-GD2 mAb ch14.18 therapy against metastases in NOD/SCID mice beginning 7 or 21 days after tumor cell injection (nondetectable and detectable disease with bioluminescent

imaging) and (2) aNK+mAb therapy beginning at 21 days combined with agents directed at tumor cells and the micro-environment.

Methods: aNK were generated by culturing NK cells with IL-2+IL-15. Intravenous injection of luciferase transfected CHLA-255 or CHLA-136 neuroblastoma cells into NOD/SCID mice provided models of drug-resistant metastases. End points were response and tumor growth (defined by bioluminescent imaging) and survival.

Results: When weekly therapy began 7 days after CHLA-255-luc injection, aNK alone had little effect whereas aNK+mAb cured 70% of mice ($P < 0.001$). However, weekly aNK+mAb treatment of both CHLA-255-luc and CHLA-136-luc, when begun at day 21, only modestly inhibited growth and prolonged survival. In contrast, aNK+mAb activity was dramatically increased in this setting if mice also received bolus (every 3wk) cyclophosphamide and weekly zoledronic acid and bevacizumab. The latter regimen, which was more effective than immunotherapy or chemotherapy alone, caused complete responses at days 28 and 35 ($P < 0.001$), slowed growth ($P < 0.001$), and prolonged survival ($P < 0.001$).

Conclusions: aNK+mAb is active against metastases formed by drug-resistant neuroblastoma cells in vivo. Adoptive transfer of aNK cells+mAb can cure mice with minimal disease (day 7) and can modestly prolong survival and decrease growth of established metastases (day 21). aNK+mAb therapy of established metastases is dramatically improved by adding bolus and metronomic cyclophosphamide, zoledronic acid, and bevacizumab.

Immunotoxin-based Targeting of Acute Lymphoblastic Leukemia

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Despite great progress in the treatment of childhood acute lymphoblastic leukemia (ALL), this malignancy remains the leading cause of cancer-related mortality in pediatrics. Additionally, current therapies are associated with a wide array of toxicities and substantial morbidity in long-term survivors. Novel approaches are needed to overcome resistance to and decrease side effects of standard ALL therapy. Since monoclonal antibodies (MoAb) were first generated against human differentiation antigens there has been the hope that MoAb-based reagents might be used to treat ALL. We have designed and tested a number of recombinant immunotoxins composed of an anti-CD22 single chain variable fragment (Fv) linked to modified *Pseudomonas* exotoxin (PE). These agents are highly active against ALL cell lines and primary patient blasts in vitro and they are synergistic with most standard chemotherapy agents tested. We recently completed a phase I clinical trial of RFB4(dsFv)-PE38 (CAT-3888 or BL22) in pediatric patients with ALL. BL22 was well tolerated and clinical activity was seen in many subjects. A successor study with a higher affinity anti-CD22 immunotoxin (CAT-8015 or HA22) is currently underway. These studies demonstrate proof-of-principle that recombinant immunotoxins can be safely and effectively applied to pediatric ALL.

2. CHIMERIC ANTIGEN RECEPTORS

Targeting ALL With Chimeric T-cell Receptors

Laurence J.N. Cooper. M.D. Anderson, Houston, TX. Adoptive transfer of antigen-specific T cells can be used to prevent and treat malignancies in mice and humans. Since immune

tolerance restricts the generation of T cells with sufficient functional avidity for desired tumor-associated antigens, we and others have redirected the specificity of T cells by the introduction of chimeric antigen receptors (CARs) that recognize cell surface molecules on malignant cells independent of the major histocompatibility complex. CARs are typically composed of an extracellular domain derived from the antigen-binding sequences of a monoclonal antibody and an endodomain including one or more T-cell activation motifs. One example is a CAR with specificity for CD19, a lineage molecule expressed on normal and malignant B cells. CD19-specific T cells are being evaluated in several proof-of-concept trials and these experiences provide a foundation for next-generation trials to clinically evaluate novel concepts developed in the laboratory as the cycle from bench to bedside is repeated. The new technologies to be discussed include (i) generation of clinical-grade artificial antigen-presenting cells that can sustain the outgrowth of CAR+ T cells, (ii) development of a CAR capable of delivering an antigen-dependent fully competent T-cell activation signal, (iii) avoidance of immunogenic sequences in CAR or ancillary transgenes, (iv) noninvasive imaging using positron emission tomography, and (v) combining nonviral gene transfer with improved integration efficiency of DNA *Sleeping Beauty* plasmids encoding for a CAR. It is envisioned that these platform technologies can be applied in a cost-effective manner so that multi-institution clinical trials can be undertaken to establish the therapeutic efficacy of CAR+ T cells for pediatric (and adult) malignancies.

Sleeping Beauty-mediated T-cell Therapy for CD19⁺ Lymphoid Malignancies

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We have previously reported that the nonviral *Sleeping Beauty* (SB) transposon system can mediate genomic integration and long-term reporter gene expression in human primary peripheral blood (PB) T cells. To test whether this system can be used to genetically modify both PB and umbilical cord blood (UCB) T cells as graft-versus-leukemia effector cells, an SB transposon was constructed to coexpress a single chain chimeric antigen receptor for human CD19, commonly expressed in B-ALL, and human CD20, a marker for in vitro selection of transfected T cells and a "suicide" gene for in vivo elimination by anti-CD20 mAb (Rituxan), when necessary. In preclinical studies, stable dual gene expression was confirmed in both T-cell types, permitting enrichment by positive selection with Rituxan. However, CD20⁺ primary human T cells but not CD20⁺ CEM T-cell line were resistant to Rituxan-mediated CDC and ADCC, suggesting that CD20 may not be suitable for a "suicide" approach. Nevertheless, the engineered CD4⁺ and CD8⁺ T cells both exhibited specific cytotoxicity against CD19⁺ leukemia and lymphoma cell lines as well as CD19 transfectants, and produced high levels of antigen-dependent T_H1 but not T_H2 cytokines. The in vivo adoptive transfer of genetically engineered T cells significantly reduced tumor growth and prolonged animal survival. We also directly compared the genomic integration efficiencies and transposition site preferences of SB, piggyBac, and Tol2 transposons in both PB and UCB T cells and found that piggyBac demonstrated the highest efficiency of stable gene transfer in PB T cells, whereas SB and Tol2 mediated intermediate and lowest efficiencies, respectively. Using recoverable cassette constructs derived from each transposon, we sequenced approximately 3000 integration sites in PB and UCB T cells from 2 different donors and found that integration sites by piggyBac and Tol2 were mainly localized near the transcriptional start sites, CpG islands, and DNase hypersensitive sites whereas SB integration sites were randomly localized. These results suggest that SB might be safer than piggyBac and Tol2 in T-cell engineering and imply that SB-mediated T-cell engineering for leukemia therapy could be moved to clinical trials.

Chimeric Antigen Receptor (CAR)-modified Virus-specific T Lymphocytes for Neuroblastoma

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Neuroblastoma is the commonest extracranial tumor of childhood. Children with advanced neuroblastoma have a poor outcome despite intensive therapy. Although, clinical remission can be achieved, relapse is common, especially in high-risk patients. Neuroblastoma can be susceptible to immunotherapy, and both monoclonal antibodies and vaccination have produced clinical responses. The adoptive transfer of T cells that have been genetically modified to express chimeric antigen receptors (CARs) targeting molecules selectively expressed by neuroblastoma cells is an additional approach. However, phase I clinical trials using adoptive transfer of primary T cells genetically modified to express CARs showed that the engineered cells persisted only for a limited period of time, likely because of incomplete activation—and consequent premature deletion—after engagement of their CAR by tumor-associated antigen. Expressing CARs in cytotoxic T lymphocytes (CTLs) with defined antigen specificity may improve the *in vivo* persistence of CAR-modified T cells. When antigen-specific CTLs are engrafted with CARs, they will receive appropriate costimulation through their native TCR ($\alpha\beta$ TCR) when this engages specific antigen-peptides presented by professional antigen-presenting cells (APC) in the context of the MHC complex. We have assessed this approach preclinically and now clinically. We use Epstein-Barr virus-specific CTLs (EBV-CTLs) expressing a CAR directed to the GD2 antigen, which is expressed at high levels on human neuroblastoma cells. In a phase I clinical trial, neuroblastoma patients simultaneously received autologous EBV-CTLs and autologous primary T cells, each expressing a distinguishable but otherwise identical GD2-CAR. EBV-CTLs expressing the CAR persisted longer and at higher levels in the peripheral blood of these patients than CAR primary T cells, and the CAR-EBV-CTLs remained functionally cytotoxic following engagement of either their native or chimeric receptors. Treatment produced tumor responses, including a complete remission. These observations support the benefits of activation through the native $\alpha\beta$ TCR by EBV antigens and costimulatory molecules on APC, and we will discuss how this approach can be further improved.

Engineering Central Memory-derived T Cells for Adoptive Therapy of Pediatric Malignancies

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Relapsed leukemias, neuroblastoma, and brain tumors, in aggregate, constitute the leading cause of cancer-related mortality in the pediatric age range. While many of these patients achieve a remission from conventional therapy, the persistence of minimal residual disease is a major barrier to improved long-term disease-free survival. Immunotherapy using antigen-specific mechanisms of tumor targeting is an attractive modality for targeting MRD. Our group has focused on the development of adoptive therapy for common pediatric tumor types and has engineered a series of chimeric receptors that when expressed by cytolytic T cells redirect their specificity to this group of tumors. Clinical trials have been initiated with CD19 and CD20-specific T cells for B lineage lymphoma/leukemia, neuroblastoma L1-CAM-specific T cells, and IL13Ra2 glioma-specific T cells. While clinical trials are in early phase, one major challenge to the field of adoptive therapy for cancer is to understand how the persistence of adoptively transferred T cells can be improved. Using a nonhuman primate model that closely recapitulates the methods for T-cell isolation, propagation, and genetic modification used in human trials, we observed that central memory-derived virus-specific effector cells grown to large numbers *ex vivo*—but not their effector memory counterparts—could persist following transfer, home to TCM

niches, and reestablish functional TCM and TEM viral immunity. Based on this fundamental insight, we are in the process of developing therapeutic platforms for pediatric applications that will use central memory-derived virus-specific T cells that are gene modified to express our tumor targeting chimeric antigen receptors. Virus-specific TCM enrichment will be accomplished by CD62L immunomagnetic selection followed by stimulation using an Ad5/35 vector encoding a CMV pp65/EBV EBNA3C fusion protein. Our CARs are packaged in SIN lentiviral vectors for high efficiency transduction. This platform is a closed system and is capable of generating clinical cell doses in 21 to 28 days. While disease-specific parameters will dictate the venue for adoptive therapy in children, cell products will likely be infused after some form of lymphodepletion either by chemotherapy, HSCT, or, in the future, by monoclonal antibodies. Finally, our preclinical primate model suggests that bispecific TCM's (virus-specific TcR x tumor-specific CAR) can be expanded *in vivo* by viral antigen stimulation and thus we are evaluating viral antigen-driven vaccine approaches for postadoptive transfer amplification of adoptively transferred T cells.

3. T-CELL-BASED THERAPIES FOR PEDIATRIC MALIGNANCIES

Exploiting and Inducing Changes in T-cell Homeostasis for Immune-based Therapy of Pediatric Cancer

Karen Cui, Martin Guimond, and Crystal L. Mackall. *Immunology Section, Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD.* Standard cytotoxic therapies for childhood cancer frequently induce lymphopenia that causes profound alterations in immune physiology. Lymphopenia can facilitate adoptive immunotherapies by increasing the expansion of antigen-specific T cells and increasing skewing toward weak tumor antigens. We have exploited this in patients with pediatric sarcomas by administering autologous lymphocyte infusions and dendritic cell vaccines to patients rendered lymphopenic by standard multiagent chemotherapy. Preliminary results demonstrate that this approach can induce immune responses in this patient population and that patients with high-risk metastatic and recurrent pediatric sarcomas treated in this manner experience relatively good clinical outcomes. However, several challenges remain that limit the effectiveness of this approach. First, regulatory T cells expand vigorously during lymphopenia, potentially limiting the capacity to respond to self antigens. Second, the lymphopenic milieu primarily supports CD8⁺ expansion but not antigen-specific CD4⁺ expansion, potentially limiting the effectiveness of tumor-based immunotherapies. Third, lymphopenia itself limits the capacity for epitope spreading and is associated with chronic immune dysfunction. We therefore seek to specifically harness the beneficial changes in immune physiology induced by lymphopenia in T-cell replete hosts by administering “targeted immunotherapies” comprising rhIL-7 and selective T_{reg} depletion. The results demonstrate that IL-7 therapy can induce homeostatic peripheral expansion in T-cell replete animals and humans, and thus augment immune-based therapies. Second, selective T_{reg} depletion, although incomplete and transient, substantially improves the effectiveness of adoptive immunotherapy in animal models. We conclude that the beneficial milieu afforded by lymphopenia can be harnessed in T-cell replete hosts by combining selected T_{reg} depletion with homeostatic cytokines in general and IL-7 in particular. Manipulating the host to provide a permissive milieu may enhance the effectiveness of tumor vaccines and/or adoptive immunotherapy for childhood cancer.

Exploiting Lymphopenia for Cell-based Therapies

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Chemotherapy-based approaches against pediatric cancers have reached limits of efficacy and tolerability. A tumor vaccine could

improve outcome, but must be delivered after therapies such as SCT, at a point of minimal residual disease, but when there is no recovery of T-cell function. Vaccine responses are absent for 6 to 12 months post-SCT. In a recently completed clinical trial, we tested the impact of post-SCT T-cell augmentation (TCA), where autologous CD3/CD28 activated costimulated T cells are delivered at varying times after stem cell infusion, on T-cell recovery and vaccine responses. These and similar novel T-cell engineering approaches have made the first real tests of cell therapies directed against tumors possible, but the ability to direct an effector response to cancers such as neuroblastoma (NB) remains an additional hurdle. In addition to adequate T-cell numbers and potential functionality, testing T-cell-based therapies requires a relevant antigen and an effector attack against the tumor. MHC class I is frequently absent on NB cells, a potential mechanism of immune evasion. Manipulation of class I expression on NB may, therefore, enhance T-cell targeting. We tested TCA in NB in 2 phases, in the context of a trial of high NB therapy consisting of induction chemotherapy followed by tandem SCT including total body irradiation in SCT no. 2, which has yielded a 3 year EFS of 55% in a large phase 2 study. Initially, TCA was given at day 12 post-SCT (D+12) in 10 patients. We then did a randomized pilot study (N = 30) of TCA at D+2 or at D+90, assessing CD4 and CD8 T-cell recovery, and giving a conjugate pneumococcal vaccine (Pevnar) on D+12 and D+60 to assess functional immune recovery. We assessed antitumor responses in T cells from the NB patients.

TCA given at D+12 or D+2 after a highly immunosuppressive tandem transplant results in rapid improvement in CD4 (Table 1) and CD8 T-cell counts. The D+2 TCA group displayed Pevnar response well above protective levels in almost all patients immunized (some as early as D+30), an effect not seen in the late TCA patients. Interestingly, 4 early TCA patients have also shown an engraftment syndrome, consisting of supranormal T-cell counts, fever, and a rash pathologically and clinically indistinguishable from GVHD, with 1/4 patients briefly receiving steroid therapy. This response is consistent with exuberant homeostatic expansion in the high cytokine and profoundly lymphopenic post-SCT environment.

CD4 Count Recovery After SCT and TCA

post-SCT	No TCA	D12 TCA	D2 TCA	Eng Syn
D0	0	10	5	0
D30	22	378	1500	1926
D60	87	560	931	1357

We then looked at antitumor T-cell responses. Eight of 9 HLA-A2+ patients with high-risk NB harbored T cells capable of cytotoxic and interferon- γ (IFN γ) responses directed against the universal tumor antigen survivin. Survivin-specific T cells kill NB, and the immunodominant response to NB by T cells is directed against survivin in CD107a assays. However, despite high-level survivin expression in 26/26 high-risk NB tumor biopsies we tested, we were unable to detect tumor infiltration by T cells, suggesting immune evasion. Treatment of 5 NB lines with IFN α significantly increased class I expression *in vitro*. Using human NB cell lines in a xenograft model, we observed that 3 daily doses of IFN α successfully up-regulated class I *in vivo* as well. To directly evaluate the influence of enhanced class I expression on the infiltration of T cells into NB tumors, we injected 50×10^6 CD3/28 expanded human T cells into immunodeficient mice with developing flank tumors. Expansion of injected T cells is seen in these mice, after which they received IFN γ . Increased class I was again detected on NB from IFN γ -treated mice, which correlated with the presence of tumor-infiltrating T cells.

This trial, together with our data showing survivin to be a tumor antigen relevant to T effector responses in NB, lead to a proposed study design where T-cell infusions restore cellular immunity, IFN γ is used to up-regulate class I and allow T-cell

targeting, and a survivin peptide vaccine currently in phase I is used to induce anti-NB immunity. All components of this design are available.

Targeting EBV in Pediatric Tumors

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Immunotherapy with antigen-specific T cells has the potential to produce antitumor activity without the toxicities seen with intensive chemotherapy. Our group has performed a series of clinical studies showing that cytotoxic T lymphocytes (CTLs) specific for Epstein-Barr virus (EBV) provide effective therapy for EBV-related lymphomas post-hematopoietic stem cell transplant (HSCT). We have administered polyclonal donor-derived EBV-specific CTLs to high-risk recipients after HSCT. A total of 114 patients have received CTLs, 101 as prophylaxis and 13 as therapy. Of the 13 patients treated with disease, 11 had sustained complete responses. None of the patients treated prophylactically subsequently developed lymphoma.

However, it is a greater challenge to treat tumors which arise in immunocompetent individuals and which possess tumor evasion mechanisms. Nasopharyngeal cancer (NPC), EBV-associated Hodgkin disease, and some non-Hodgkin lymphoma show type II latency expressing the subdominant EBV antigens EBNA1, LMP1, and LMP2, which may serve as targets for immunotherapy approaches. In NPC we have evaluated the safety and efficacy of autologous polyclonal EBV-specific CTL (EBV-CTL) in 2 phase I clinical trials treating 32 patients with advanced-stage NPC. Prior to adoptive transfer, 8 patients were in remission, 22 had active disease, and 2 had abnormal imaging studies of unknown significance. Seven of 8 patients in remission prior to CTL infusion remain in remission 6 to 64 months post-CTL. For the remaining 24 patients, the best overall response rate was 50% with 6 complete responses (CR/CRu), 2 partial responses, and 4 with stable disease during a median follow-up of 9 months (95% CI 2-16 mo). Of the 6 with a CR: 4 have been sustained for 2 to 4 years, and 2 relapsed more than 2 years post-CTLs. In studies, using polyclonal EBV-specific CTL in patients with relapsed EBV+ Hodgkin disease we also saw responses in around 30% of patients.

More recently we have administered CTL lines targeting the subimmunodominant antigens LMP1 and LMP2 which were generated using dendritic cells for initial stimulations then lymphoblastoid cell lines (LCL) both of which had been genetically modified to overexpress either LMP2 alone or LMP1 and LMP2 by transduction with an Ad5f35LMP2 (n = 16) or Ad5f35LMP1-I-LMP2 (n = 14) vector, respectively. Twenty-four patients with EBV+ Hodgkin disease and NHL have been treated on this dose escalation study. Twelve of 13 high-risk and/or multiply relapsed patients who received LMP-CTL as adjuvant treatment after chemotherapy remain in remission up to 4.5 years after CTL. Of 11 patients with detectable disease at the time of CTL infusion, 2 had progressive disease by 8 weeks and 9 had clinical responses (1 stable disease, 1 very good partial, and 7 complete responses).

We are now using gene transfer to modify CTLs to confer additional recognition specificities and to render CTLs resistant to inhibitory cytokines such as TGF- β produced by tumor cells.

Cellular Immunotherapy Targeting the WT-1 Tumor Antigen in Childhood Malignancies

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The Wilms tumor protein, WT-1, plays an essential role in the embryogenesis of the genitourinary system but normally expressed

postnatally only in the kidneys, gonads, pleuroperitoneal linings, and early hematopoietic precursors at very low levels. In contrast, WT-1 is differentially expressed at high levels in leukemic blasts in up to 80% of children with ALL, and in over 70% of cases with AML or advanced stages of MDS. In over 10% of AML, WT-1 mutations are also detected. High expression of WT-1 has been associated with a poor prognosis in AML and MDS. Persistent detection of WT-1 transcripts in children with ALL in remission has also been associated with a high risk of relapse. WT-1 is also differentially expressed in a series of mesoderm-derived tumors including serous adenocarcinoma of the ovary, DSRCT and mesothelioma, and in mutant form in Wilms tumor.

Recently, we have demonstrated that *in vitro* sensitization of T cells from normal donors with autologous DCs or EBV-BLCL loaded with a pool of overlapping 15-mer peptides spanning the sequence of WT-1, regularly induces the generation of IFN γ + CD8⁺ and/or CD4⁺ T cells specific for selected WT-1 peptides. Using a matrix of WT-1 peptide subpools, we have mapped and identified 26 new WT-1 epitopes eliciting responses. By then evaluating T-cell cytotoxic responses against targeted epitopes loaded on APCs sharing single HLA alleles with the donor, we have also determined the class I and class II HLA alleles presenting each epitope. The epitopes identified are also immunogenic in multiple donors sharing the presenting allele. Most importantly, all of the epitope specific CD8⁺ T cells and 60% of the CD4⁺ T cells generated are cytotoxic against WT-1 + leukemic cells coexpressing the presenting allele.

In NOD/SCID mice bearing WT-1 + and WT-1 – leukemia or solid tumor xenografts expressing different HLA alleles, adoptively transferred WT-1 specific T-cells selectively accumulate in and induce regressions of WT-1 + tumor coexpressing the presenting HLA alleles. In the assay of Lapidot and Dick, these T cells also prevent outgrowth of clonogenic leukemic cells in these animals.

Recently, we have initiated a phase I trial of WT-1 peptide pool sensitized donor-derived T cells for treatment of children and adults with WT-1 + leukemias or MDS who relapse or have persistent MRD following an allogeneic T-cell depleted HSCT. Since recipients of transplants depleted of T cells by our techniques do not require or receive posttransplant immunosuppression, the growth, disposition, and activity of adoptively transferred T cells is unfettered and can be closely monitored. Thus far, 2 patients have been treated on this protocol. These patients will be discussed. We have also initiated a phase I trial of autologous WT-1 reactive T cells in patients with relapsed WT-1 + ovarian cancer and hope to soon initiate trials in children and adults with DSRCT.

In summary, by sensitizing T cells with autologous APCs loaded with a pool of 15-mer peptides spanning the sequence of WT-1, we have been able to regularly generate IFN γ + cytotoxic T cells specific for WT-1 from normal donors and a proportion of tumor bearing patients irrespective of their HLA genotype. These T cells demonstrate significant HLA-restricted, WT-1 epitope-specific activity against WT-1 + tumors *in vitro* and in preclinical models, and are now being evaluated for clinical trials.

Effective Treatment of Established Neuroblastomas Using a Multifaceted Immunotherapeutic Approach

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While cell-based neuroblastoma vaccines developed in our laboratory can induce potent antitumor immunity in nontumor-bearing mice, they have been ineffective when used as single-arm therapy in tumor-bearing mice. We hypothesized that successful treatment of established tumors could be achieved by using a multifaceted immunotherapeutic approach that combines syngeneic hematopoietic stem cell transplantation (HSCT), adoptive T-cell transfer, and cell-based tumor vaccination immediately after transplantation. In the experimental design, mice bearing 7-day established AGN2a (neuroblastoma) tumors were given lethal total body irradiation followed by HSCT consisting of bone marrow

supplemented with T cells. On days 2, 7, and 14 after HSCT, the tumor-bearing mice were vaccinated with irradiated AGN2a cells that had been genetically modified to express the immunostimulatory cell surface proteins CD54, CD80, CD86, and CD137L (4-1BB ligand). We monitored tumor growth by using a combination of bioluminescent imaging and caliper measurements. Using this multifaceted immunotherapeutic approach, we have observed delayed tumor growth in virtually all treated mice, and we have been able to achieve 70% tumor-free survival when mice are given HSCT, T cells derived from tumor-vaccinated syngeneic donors (presensitized T cells), and vaccination. The T-cell transfer is crucial to the antitumor effect, and vaccination is also an important component as only 36% of mice given presensitized T cells only (no vaccine) survived long-term. Several of the mice given presensitized T cells without vaccination had late tumor recurrences that were not observed in vaccinated mice. This may reflect the requirement of a vaccine-based approach for the induction or maintenance of long-term immune memory, or that vaccination simply increases the magnitude of the antitumor response. Overall survival correlated with the frequency of IFN γ -producing, tumor-reactive CD8⁺ cells in lymphoid tissues of treated mice. When specific T-cell subsets were depleted in tumor-bearing hosts given the immunotherapy, 2 important observations were made. First, when CD4⁺ cells were depleted in mice given naive T-cell transfer at the time of HSCT, the antitumor effect was severely diminished. In contrast, CD4-depletion of mice given presensitized T cells resulted in better antitumor immunity, as reflected by 100% tumor-free survival; however, long-term antitumor responses (“memory”) in these mice were compromised. Second, more specific depletion of immune regulatory CD4⁺CD25⁺ cells from the adoptively transferred T cells resulted in better tumor immunity without compromising antitumor T-cell memory. In summary, our experimental results suggest that a multifaceted immunotherapeutic strategy including HSCT, T-cell adoptive transfer, and early posttransplant tumor vaccination can be effective for treating neuroblastoma.

Targeting Brain Tumor Initiating Cells With Immunotherapy

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Brain tumor initiating cells (BTICs) are a therapy-resistant, multipotent fraction of the bulk tumor mass that facilitate tumor recurrence. We isolated these cells from surgically resected gliomas and began to study their expression of MHC I and natural killer (NK) cell activating ligands. Only a subset of BTICs grown in neurosphere culture are able to up-regulate MHC I or NK cell ligands in response to ionizing radiation or treatment with IFN-gamma. These results shed insight onto the failure of immunotherapy to treat brain tumors, as only a fraction of the BTIC compartment is likely recognized by CD8⁺ T cells or NK cells. In order to overcome these mechanisms of resistance, we established a spontaneous mouse glioma model to study BTICs and their response to immunotherapy and radiation. We have isolated BTICs from these spontaneous mouse glioma that recapitulate many features of the human disease and are testing vaccination strategies that target BTICs. Based on results from murine studies and human neurosphere culture we are initiating a phase I study where pediatric brain tumor patients will receive vaccination with BTSC lysate grown under conditions shown to enhance immunogenicity.

Regulation of Inflammatory Chemokine Receptor, CCR5: its Role in Naive T-cell Memory Generation and Tumor Rejection

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Purpose of Study: Proper immune function relies on precise timing and sequence of individual cellular interaction *in vivo*. Using 2-photon microscopy, we have previously observed that inflammatory chemokines, CCL3 (MIP-1 α) and CCL4 (MIP-1 β), are critically involved in helper T-cell-dependent CD8⁺ T-cell memory generation. CD8⁺ T cells accomplish this by transient expression of the cognate chemokine receptor, CCR5. Our present research contains 3 specific aims: (A) investigate mechanisms of transient surface CCR5 expression on naive T lymphocytes in inflamed lymph node (LN); (B) interrogate functional consequences of naive T cells upon ligation of CCR5 prior to TCR engagement; and (C) examine efficacy of incorporating CCL3 and CCL4 into tumor vaccines to generate systemic antitumor immunity.

Methods Used: Naive (CD45.2⁺/CD62Lhi/CD44lo/CD69lo) T cells were transferred into congenic (CD45.1⁺) recipient mice previously immunized with CpG. Draining LNs were harvested at various times posttransfer to analyze T cells for CCR5 expression by flow cytometry. The same cells were also analyzed using histology, *in vivo* functional assays, and 2-photon microscopy. Concurrently, colon tumor cell line, CT26, was engineered to express CCL3 and/or CCL4. These cell lines were either injected live into naive recipient to monitor for primary tumor rejection, or irradiated and injected into recipient mice to boost antitumor immune response against subsequent lethal tumor challenge.

Summary of Results: We observed preferential translocation of preformed intracellular CCR5 pool to the cell surface of naive T cells within inflamed milieu, and that such enhanced expression can be induced by direct TCR contact with self-peptide/MHC. Naive cells with enhanced CCR5 expression exhibited increased expansion and effector function upon antigenic stimulation. Finally, tumors engineered to secrete CCL3 exhibited enhanced primary rejection and systemic antitumor memory T-cell immunity.

Conclusions Reached: Our study provided a novel role for inflammatory chemokines in immune memory generation. Future studies will involve proteomic and genomic profiling for factors critical in this process. We will also interrogate chemokine-assisted cellular recruitment, interaction, and functional development within tumor microenvironment. Enhanced understanding of these cellular and molecular events holds promise for rational design of immunotherapeutic strategies against cancer and immune-mediated disorders.

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4. TOLL-LIKE RECEPTORS

Immunostimulatory DNA (CpG ODN) as TLR9 Agonists for Immune Therapy of Pediatric Acute Lymphoblastic Leukemia

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Relapsed ALL is the fifth most common pediatric malignancy. Although the use of blood and marrow transplantation (BMT), currently the only available successful immune therapy for ALL, can achieve improved survival rates, the majority of patients fail, with relapse being the primary cause. Therapeutic options for ALL relapse after BMT are particularly limited with approaches to augment immune responses with donor leukocyte infusions yielding a very poor response. This may be partially due to the fact that ALL cells can develop immune resistance mechanisms similar to chemotherapy resistance. Recently, we have shown that the immunogenicity of human pre-B ALL cells can be increased after ligation of toll-like receptor-9 (TLR9) by immunostimulatory DNA-containing unmethylated CpG motifs (CpG ODN). This altered immunogenicity of ALL blasts stimulates increased anti-

ALL T-cell alloreactivity. CpG ODNs have the added benefit that they stimulate *in vivo* immunity mediated by NK cells, macrophages, plasmacytoid dendritic cells, and T_H1 responses. Moreover, we have recently reported the ability of CpG ODNs to induce immune activity against primary human ALL cells *in vivo*, using NOD-SCID mice. Repeated administration of CpG ODN into mice with established disease led to continued immune-mediated disease control and significantly improved survival. Unfortunately, relatively high *in vivo* dosages were required to attain these results. In addition, Seif et al has also shown that CpG ODNs can yield prolonged remissions in mice with ALL treated with standard chemotherapy. CpG ODNs can exacerbate graft-versus-host disease (GVHD) post-BMT and may increase recipient-mediated donor cell rejection post-BMT if given early after transplant. Thus, CpG ODNs have the potential to both improve the immunogenicity of leukemic blasts, induce *in vivo* activation of the innate immune system (NK cells and macrophages), and induce a T_H1 response in the adoptive immune system. We hypothesize that CpG ODNs have considerable potential for ALL therapy and should be evaluated in clinical trials. Plans for evaluation of CpG ODNs as therapy for relapsed ALL > 6 months post-BMT in patients with no cGVHD as well as for prophylaxis either post-BMT or chemotherapy are being developed.

In Vivo Effects of Toll-like Receptor Signaling on T-cell Responses for GVHD and GVL

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Cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODNs) are synthetic ODNs with unmethylated DNA sequences that mimic viral and bacterial DNA, and protect against infectious agents and tumor challenge. CpG ODNs bind to TLR9 expressed primarily on B cells, DCs, and NK cells. We have shown that CpG ODNs administered 2 days before the AML challenge allow mice to survive more than 100 times a lethal tumor dose. CpG ODNs protected against AML challenge in both syngeneic and allogeneic BMT recipients at both early and late time points after transplantation. In conjunction with donor lymphocyte infusion, CpG ODNs induce prolonged survival to AML challenge with 88% of mice surviving long-term. To determine whether early post-BMT CpG ODNs could be used to prevent AML recurrence post-BMT, CpG ODNs were given beginning at the peri-BMT period. CpG ODNs markedly accelerated graft-versus-host disease (GVHD) lethality by TLR9 ligation of host APCs, dependent upon host IFN γ . Imaging studies showed significantly more GFP(+) effector T cells in lymphoid and nonlymphoid organs. In engraftment studies, CpG ODNs promoted allogeneic donor BM rejection independent of host IFN γ , IL-12, or IL-6. CpG ODNs promoted BM rejection by ligation of donor BM, but not host, TLR9. CpG ODNs did not impair engraftment of TLR9^{-/-} BM unless wild-type CD11b⁺ myeloid, but not B lineage, BM cells were added to the donor inoculum. Similarly, the administration of a TLR7/8 agonist in non-BMT mice prior to AML challenge prolonged survival, while the peri-BMT administration of a TLR7/8 agonist also accelerated GVHD lethality and graft rejection. With these latter data, a phase 1 trial of a TLR7 agonist in cancer patients, including those with hematological malignancies, has been initiated. In aggregate, these data indicate that TLR agonists can be effective in stimulating immune system responses against AML in naive, non-BMT recipients. However, the increased risk of GVHD and graft rejection caused by peri-BMT TLR7/8 or TLR9 agonist

administration introduce a note of caution for human translation early post-BMT.

Control of Syngeneic Acute Lymphoblastic Leukemia In Vivo by Immunostimulatory DNA

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Acute lymphoblastic leukemia (ALL) is the second most common childhood cancer. While current therapies are very successful, relapsed patients are generally unresponsive to chemotherapy alone. Evidence that natural killer (NK) cells are involved in a posttransplant graft-versus-leukemia effect suggests agents targeting NK cell activation may be effective immune adjuvants in chemotherapy. Immunostimulatory DNA oligodeoxynucleotides containing CpG motifs (isDNA) stimulate antitumor innate immune activity by NK cells and macrophages and enhance subsequent T_H1 adaptive responses. isDNA are in clinical trials for several solid tumors in adults. We previously reported that isDNA stimulation of primary pediatric ALL cells enhances an allogeneic T_H1 T-cell response in vitro. We have also shown that isDNA stimulated NK cells mediate a significant reduction in leukemic burden of primary pediatric ALL xenografts in Nod-SCID mice. In this study, we investigated induction of anti-ALL activity by isDNA in a syngeneic setting, both as monotherapy and as a postchemotherapy adjuvant, using the E μ -RET transgenic mouse model of pediatric ALL. Significantly increased killing of E μ -RET leukemia cell lines by syngeneic isDNA-stimulated splenocytes was observed in a flow cytometric in vitro cytotoxicity assay. The difference between isDNA-treated and untreated controls became more pronounced at higher effector:target ratios ($P < 0.0001$). Cell depletion experiments revealed that both NK cells and macrophages significantly contributed to leukemia killing in vitro and in vivo. Monotherapy isDNA treatment of mice with a low leukemia disease burden yielded significantly prolonged tumor-free survival compared to PBS-treated mice ($P = 0.0052$). Interestingly, this early control of leukemia expansion was also achieved in isDNA-treated IFN-gamma-deficient mice. To assess the ability of isDNA to stimulate antileukemia activity in an immunosuppressed environment more relevant to potential clinical application, leukemic mice were treated with a standard pediatric chemotherapy induction and then given isDNA or PBS and followed for relapse. While these studies are still ongoing, preliminary data indicate a significant delay to relapse in the isDNA-treated groups. To our knowledge, this is the first demonstration of isDNA-induced anti-ALL activity in a syngeneic model and, combined with our previous studies, provides strong evidence for the potential utility of isDNA-based therapy for the treatment of relapsed and refractory pediatric ALL.

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5. EXPLOITATION OF THE TUMOR MICROENVIRONMENT FOR THERAPEUTIC BENEFIT

Biologic-based Therapy for Osteosarcoma

Eugenie S. Kleinerman, MD. *Children's Cancer Hospital at the University of Texas M. D. Anderson Cancer Center, Houston, TX.* The lung is the most common site for metastatic spread in patients with osteosarcoma (OS). Understanding the biologic characteristics that contribute to the ability of OS cells to grow in the lung microenvironment and how the microenvironment influences therapeutic efficacy may identify novel ways to treat OS patients with pulmonary metastases. Our investigations have demonstrated that the Fas/FasL signaling pathway plays a major role in the ability of OS cells to grow in the lung and their response to therapy.

Fas is a cell surface receptor that induces cell death following interaction with its ligand (FasL). This pathway is critical for the elimination of virus-infected cells by NK and cytotoxic T cells and in eliminating inflammatory cells at immune privileged sites such as the anterior chamber of the eye. FasL is constitutively expressed in the lung. Therefore, Fas expression on OS cells may play a pivotal role in the elimination of these tumor cells when they metastasize to the lung.

Using our experimental human OS lung metastasis nude mouse model and established mouse OS model, we demonstrated that Fas expression correlates inversely with metastatic potential. Osteosarcoma cells that express high levels of Fas are eliminated upon migration into the lung, whereas those with low or no Fas or those with a block in the Fas signaling pathway evade clearance from the lung. We demonstrated that the clearance of Fas⁺ cells is secondary to the constitutive expression of FasL in the lung using FasL-deficient *gld* mice. Fas⁺ OS cells formed lung metastases following IV injection in *gld* mice but not wild-type mice. Blocking the Fas signaling pathway also resulted in the ability of Fas⁺ OS cells to form lung metastases. Supporting the importance of Fas expression to the metastatic potential of OS cells is our finding that pulmonary metastases from OS patients are uniformly Fas⁻.

Using our mouse model systems we demonstrated that aerosol delivery of various agents including liposome-encapsulated 9-nitrocarnitine (L-9NC), gemcitabine, or IL-12 gene therapy up-regulated tumor Fas expression and resulted in the eradication of established OS lung metastases. The efficacy of aerosol therapy was severely compromised in mice that were FasL deficient. This is the first demonstration that the tumor microenvironment can influence therapeutic efficacy. Taken together these data indicate the importance of the Fas pathway in the metastatic process of OS and the therapeutic potential for targeting this pathway. Aerosol therapy aimed at up-regulating Fas expression resulted in the regression of established lung metastases. Aerosol delivery is particularly suited to OS as the lung is the most common and often the only organ for metastatic spread. Identifying agents that target the specific biology of the tumor cell or the microenvironment may lead to new therapeutic approaches.

NKT Cells in Neuroblastoma

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The levels of tumor-infiltrating or circulating natural killer T cells (NKTs) associate with long-term survival in neuroblastoma and other types of cancer. Although NKTs can be directly cytotoxic against CD1d-positive cells and mediate NK cell antitumorigenic activity in murine models, primary neuroblastomas (like the majority of solid tumors in humans) are CD1d-negative and not infiltrated with NKs. An alternative target for NKTs at the tumor site could be CD1d-positive cells of the tumor stroma. We found that CD1d-positive monocytes colocalized with NKTs to neuroblastoma xenografts in NOD/SCID mice. In response to tumor cell-conditioned medium, monocytes produced IL-6 that stimulated tumor cell proliferation in vitro and in a NOD/SCID murine model. Contrary to NKT-specific Valpha24-invariant TCR, RNA expression of monocyte/macrophage markers CD14 and CD16 as well as IL-6 and IL-6R in 129 primary MYCN nonamplified neuroblastomas inversely correlated with long-term event-free survival. High levels of IL-6 expression were also found in CD1d⁺ primary tumor-associated macrophages (TAMs) and bone marrow myelo-monocytic cells from neuroblastoma patients. Importantly, NKTs selectively killed monocytes pulsed with tumor cell lysate. The killing was CD1d-restricted since it was inhibited by anti-CD1d mAb. Furthermore, cotransfer of human monocytes and NKTs to tumor-bearing NOD/SCID mice decreased monocyte number at the tumor site compared to mice

transferred with monocytes alone. Therefore, killing of tumor-promoting TAMs may represent a novel mechanism of NKT cell antitumor activity.

6. NK CELL-BASED THERAPIES

NK-cell Therapies for Leukemias and Solid Tumors

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The generation of large numbers of human natural killer (NK) cells for cell therapy has been an elusive goal. We devised a method (NK activation and expansion system, NKAES) based on coculture with genetically modified leukemia cells (K562-mb15-41BBL line) produced in our laboratory with the intent of establishing long-term cultures of these cells. Seven-day cultures produced by this method showed a median 2160% expansion of CD56⁺ CD3⁻ NK cells from peripheral blood (range, 510% to 8660%; n = 34), with minimal or no expansion of CD3⁺ lymphocytes. By periodically replenishing the NKAES-generated cultures with K562-mb15-41BBL cells, we could expand the NK cell population up to 2 × 105-fold by 6 weeks of culture. NKAES-derived NK cells had a gene expression profile distinct from that of primary and IL-2 activated NK cells, and were more potent against acute myeloid leukemia (AML) cells. They could eradicate leukemia in murine models of AML and also exerted powerful cytotoxicity against Ewing sarcoma, rhabdomyosarcoma, and neuroblastoma cells. By contrast, their cytotoxicity against acute lymphoblastic leukemia (ALL) cells remained low. To overcome this resistance, we transduced NKAES-derived NK cells with chimeric receptors directed against CD19, a molecule widely expressed by malignant B cells including ALL. Expression of anti-CD19 receptors linked to CD3 ζ overcame NK resistance and markedly enhanced NK-cell-mediated killing of ALL cells. This result was significantly improved by adding the 4-1BB costimulatory molecule to the chimeric anti-CD19-CD3 ζ receptor: the cytotoxicity produced by NK cells expressing this construct uniformly exceeded that of NK cells whose signaling receptors lacked 4-1BB, even when natural cytotoxicity was apparent. Addition of 4-1BB was also associated with increased cell activation and production of IFN- γ and GM-CSF. The methods described here have now been implemented in a GMP-grade large-scale setting to support clinical protocols testing the feasibility and toxicity of infusing haploidentical expanded and genetically modified NK cells.

Towards NK-cell-mediated Immunotherapy in Ewing Sarcoma: Identification of Molecular Mechanisms

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Purpose: Despite current therapies, including high-dose chemotherapy, the prognosis of refractory and relapsed Ewing sarcoma (EWS) patients is poor. To explore the feasibility of natural killer (NK)-cell-mediated immunotherapy, the susceptibility of EWS to NK-cell-mediated cytotoxicity, with particular focus on chemo-resistant EWS cells, and the molecular mechanisms involved were investigated.

Methods: Expression of ligands for inhibitory and activating NK-cell-receptors was evaluated in a panel of chemo-sensitive and chemo-resistant EWS cell lines (n = 10) and primary tumors (n = 6) using flow cytometry and immunohistochemistry. Cytotoxicity was determined in chromium-release assays, using resting and IL

15-activated NK cells obtained from healthy donors or EWS patients at diagnosis. Blocking antibodies against specific ligands/receptors were used to study contribution of these molecules.

Results: All cell lines were lysed by resting allogeneic NK cells from healthy donors and to a lesser extent by NK cells from EWS patients at diagnosis, except for one of the chemo-resistant cell lines (CADO-ES). The efficacy of lysis was increased upon activation with IL 15, which also restored the difference in cytolytic activity between resting NK cells from healthy donors and EWS patients. Both cell lines and EWS tumors expressed ligands for the activating NK cell-receptors DNAM-1 and NKG2D. Cytotoxicity critically depended on these receptors, since blocking either of these receptors abrogated cytotoxicity of resting NK cells. IL 15-activated NK cells recognized EWS more efficiently, since only combined DNAM-1/NKG2D-blockade inhibited lysis. Induction or blockade of HLA class I did not significantly affect lysis, except for the chemo-resistant CADO-ES cell line. In the latter case, cytotoxicity by resting NK cells was critically dependent on loss of inhibition, since blocking antibodies against HLA class I reversed resistance to these cells. This finding was consistent with the observation of higher levels of HLA class I expression in this cell line compared to the other cell lines.

Conclusions: These results demonstrate that EWS cells, regardless of chemo-sensitivity, are generally susceptible to cytotoxicity by activated NK cells from either healthy controls and EWS patients. In future immunotherapeutic strategies, use of cytokine-activated NK cells may provide a promising additional treatment modality for EWS patients.

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7. AUGMENTING GRAFT-VERSUS-LEUKEMIA FOR PEDIATRIC HEMATOLOGICAL MALIGNANCIES

Graft-versus-Leukemia Induced by Graft-versus-Host Disease Early After Allogeneic Bone Marrow Transplantation in Children With Detectable Levels of Residual Acute Lymphoblastic Leukemia Prior to Transplantation

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To date, children with acute lymphoblastic leukemia are usually cured by chemotherapy alone. About a quarter of the patients however have either a highly chemoresistant form of leukemia or suffer from a relapse after the initial therapy. These patients receive an allogeneic stem cell transplantation (SCT) if a suitable donor is available. Based on previous studies,¹ patients with (high levels of) minimal residual disease (MRD) have a high probability of relapse after SCT. Therefore, additional (immuno) therapeutic modalities are warranted to improve outcome in these high-risk patients. A generally applicable strategy would be to optimize the antileukemic effect of the alloreactive response. The primary aim of the study was to investigate whether early tapering of CsA (4 to 5 wk after SCT) followed by incremental donor lymphocyte infusions at 6-week intervals could be safely performed. The secondary aim was to evaluate the impact of this intervention strategy on relapse rate. Each of the individual interventions was only performed if no GVHD grade II or more was present.

During 5 years, 50 patients were included in the study and were evaluated with a follow-up of 1 to 6 years. Eighteen of those had pre-SCT MRD levels $>1 \times 10^{-4}$, and were treated according to the intervention arm of the protocol. The remaining 32 patients, with pre-SCT MRD level $<1 \times 10^{-4}$ (12 patients), negative (11 patients), or not quantifiable (9 patients) were treated conventionally with CsA for 3 months.

The relapse-free and overall survival of the whole group was about 50% and comparable with historical results. The relapse-free survival of the MRDhi patients was 25% and of the MRDlo (= MRD $<1 \times 10^{-4}$ and negative) the survival was 60%.

The intervention strategy was relatively safe as it resulted in a slightly increased frequency of GVHD grade II or more (total frequency 40% vs. 12% in the nonintervention group) but in all of these patients the GVHD was controlled by standard medication (prednisone). Comparison of the relapse frequency of patients with GVHD grade II or more with that of all other patients revealed that there was no antileukemic effect of GVHD in itself.

Although the overall relapse-free survival of all patients in this study was not significantly different from the historical data, it was remarkable that in patients who received an intervention the relapses were delayed, that is, 5/11 occurred more than 1 year after SCT versus only 2/9 in the nonintervention group. Some unusual extramedullary (or combined) relapses occurred in the intervention group. Together these data suggest that the immunotherapeutic intervention may influence the timing and localization of relapses representative of an antileukemic effect, which was independent of the occurrence of GVHD.

The conclusion from this prospective study is that MRD levels pre-SCT predict the risk for relapse. Immune intervention, when carefully applied, can safely be given to recipients of an allogeneic stem cell transplant. Prolonged (immuno)therapeutic intervention posttransplant may be required for more effective prevention of leukemia relapse.

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Enhancing Graft Versus Leukemia in the Clinic

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Natural killer (NK) cells play a crucial role in the prevention of relapse of hematological malignancies after allogeneic stem cell transplantation. It has been shown in haploidentical transplantation in patients with ALL and AML that the risk of relapse is lower when grafted with stem cells from NK-alloreactive donors compared to NK-nonalloreactive donors. These grafts were composed of isolated CD34⁺ stem cells from mobilized parental donors. In order to retain NK cells, we have switched from CD34⁺ positive selection to the negative depletion of CD3⁺ T cells of mobilized grafts in haploidentical transplantation. With these grafts, large numbers of CD56⁺ NK cells are cotransplanted and more than 100 children with hematological malignancies as well as solid tumors have been treated with this approach.

In addition, the functional status of the NK cells is important and patients with a high persistent NK activity posttransplant have a lower risk of relapse compared to patients with a persistent low NK activity. Therefore, strategies that aim to augment the NK activity posttransplant should lead to a lower risk of relapse in pediatric leukemia. In order to activate the NK cells after haploidentical transplantation, we are clinically investigating several strategies. One is the additional and repetitive posttrans-

plant adoptive transfer of donor-derived NK-cells that have been activated ex vivo by overnight incubation with interleukin 15 (IL-15). The infusion of the IL-15-activated NK cells is followed by treatment with low-dose interleukin 2 to induce in vivo proliferation of the IL-15-activated NK cells and to augment their cytotoxicity. So far, 7 patients have been treated without major side effects. Another approach is the posttransplant repeated ex vivo stimulation of patients' donor-derived mononuclear cells (MNCs) by IL-15.

Since gamma/delta T lymphocytes have also been shown to exert an antileukemic effect posthaploidentical transplant, we are evaluating methods for the selective depletion of alpha/beta T lymphocytes from MNCs of mobilized donors for clinical application. This approach retains besides the NK cells large numbers of gamma/delta T cells in the graft. Since NK cells as well as gamma/delta cells can exert antibody-dependent cellular cytotoxicity (ADCC), we investigate whether the additional use of monoclonal antibodies directed against leukemia or solid tumor targets would increase the graft versus malignancy effect. Further prospective clinical studies have to show which strategies or combinations would be most beneficial to reduce the risk of relapse after transplantation.

Optimizing GvL Toward ALL

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As in some patients the GvL effect is insufficient to afford long-term protection following allogeneic hematopoietic stem cell transplantation (allo-HSCT) complementing concepts of immune modulation are currently pursued. In acute lymphoblastic leukemia (ALL), donor lymphocyte infusions (DLI) have proven less effective than in myeloid malignancies that have in part been attributed to the reduced immunogenicity of ALL blasts. We hypothesized that vaccine-mediated T-cell stimulation might serve to augment the frequency of leukemia-specific cytotoxic T lymphocytes (CTL) in ALL patients receiving DLI post-allo-HSCT. First we compared the T-cell stimulatory capacity of both leukemia-lysate pulsed dendritic cells and CD40-activated leukemic B-cell precursor blasts in vitro and could demonstrate effective leukemia-specific CTL generation in the presence of sufficient costimulation. In this context, triggering of CD27 on T cells by CD70 expressed on CD40-licensed antigen-presenting cells is known to augment the cytotoxic T memory pool. Indeed, on ALL-blasts both CD70 and CD80/86 up-regulated after activation via CD40 contribute to primary T-cell stimulation and cooperate in the prevention of T-cell anergy. In addition during generation of antileukemic T cells, blockade of CD70-mediated costimulation prevents effector cell expansion and reduces their cytotoxic capacity. Thus modulation of the CD70/CD27 pathway may represent a novel therapeutic approach for augmenting magnitude and quality of the antileukemic response in BCP-ALL. In a clinical pilot phase for relapsed ALL post-allo-HSCT, we then effectively employed DCs pulsed with leukemia cell lysate as a vaccine in combination with DLI to activate and expand GvL effectors. Clinically protective responses were induced in all 3 patients treated for ALL relapse post-HLA-matched allo-HSCT with 2 patients surviving long term.

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New Approaches for Separating GVH From GVL

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Given the high relapse rate of hematological malignancies following conventional allogeneic stem cell transplants (SCT) from

HLA identical siblings (especially in high-risk leukemia patients), it seems clear that relying on optimizing the “natural” graft-versus-leukemia (GVL) effect of SCT is inadequate. For this reason, a variety of approaches to augment GVL effects are under development in many centers. These strategies include methods to optimize immune recovery by reduction of T_{reg} function, selective elimination of graft-versus-host disease (GVHD)-reacting T cells, and methods to boost GVL effects with vaccines or the adoptive transfer of leukemia-specific lymphocytes. Our NHLBI allogeneic stem cell transplantation program has focused on the development of a GVHD-free SCT platform to optimize the administration of vaccines to boost GVL effects posttransplant.

Selective allodepletion (SD) is a strategy to eliminate host-reactive donor T cells from allografts to prevent GvHD while conserving useful donor immunity. We developed a semiclosed, GMP-quality, clinical scale SD process where donor-derived lymphocytes are stimulated with patient-derived T-cell antigen-presenting cells in an ex vivo mixed lymphocyte reaction. Alloactivated donor T cells preferentially retain the photosensitizer TH9402, rendering them susceptible to elimination by exposure to visible light in a photodepletion device (Kiadis Pharma Inc, The Netherlands). We initiated a clinical trial where HLA-identical sibling recipients with hematological (non-T cell) malignancies received a CD34-cell selected transplant (Miltenyi, Germany) containing less than 1×10^4 T cells/kg together with 5×10^6 /kg viable SD donor T cells on day 0, using an age-adapted, radiation-based preparative regimen (FluCyTBI). Low-dose cyclosporine was used as sole immunosuppression in the absence of acute GvHD (aGVHD). Eleven patients (9 at high risk for relapse), median age 43 years with acute leukemia, MDS, or mantle cell lymphoma (MCL) have so far been transplanted. The median follow-up is 228 days. Lymphocyte recovery was rapid and donor T-cell chimerism rapidly rose to 100%. Three patients developed skin aGVHD, but no grade III-IV aGVHD occurred. One patient, transplanted for refractory MCL relapsed 340 days after transplant and 1 died of infectious complications and GvHD 330 days after transplant after receiving an unmanipulated DLI. The goal of this trial is to determine whether cyclosporine is required posttransplant to prevent grade II-IV aGVHD by stepwise reduction in the duration of immunosuppressive treatment in successive cohorts.

Combining Vaccine Approaches With SD SCT to Enhance GVL: An immunosuppression-free transplant with adequate infusion of donor T cells should provide a platform for further boosting the GVL effect using vaccines. We have therefore begun to evaluate peptide vaccines against the antigens PR1 and WT-1 both expressed widely in leukemia. Two observations underpin the rationale of using vaccines to boost immune responses to leukemia after SCT. First, low frequencies of central memory cells to several leukemia-specific antigens (LSA) are detectable circulating in normal donors. Such donor-derived LSA-specific T cells expand posttransplant. Second, expansions of LSA-specific T cells occur very early after SCT during the period of “immune intolerance” suggesting that GVL could indeed be enhanced by posttransplant vaccination and facilitated by the strong lymphopenia provoked drive for lymphocyte expansion

after SCT. The opportunity to first vaccinate the donor prior to lymphocyte collection and then boost the response further by vaccinating the patient early after SCT would be best applied in conjunction with a selective depletion technique that permits robust immune recovery without the requirement for GVHD prophylaxis. In a safety study, a single vaccination with PR1 and WT-1 peptide together with montanide and GM-CSF adjuvants, either PR1-specific or WT-1-specific T-cell responses were induced in all 8 patients studied, with a brief fall in molecular markers of disease in patients with MDS. These results indicate that a variety of leukemia antigen peptide vaccinations can induce functional CTL responses associated with clinical improvement and justify the use of such relatively simple peptide-based vaccine approaches to boost GVL responses after SCT.

Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) as a Platform for Cancer Immunotherapy

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Allogeneic hematopoietic stem cell transplantation (HSCT) can be curative for a number of pediatric hematologic malignancies due, in part, to the graft versus tumor effect (GVT). However, relapse remains a major cause of poor outcomes. Efforts to enhance GVT can be effective at treating relapsed malignancy but often result in the induction of graft versus host disease (GVHD). Recent advancements in the field of cancer immunotherapy have led to success through manipulation of the autologous immune system but these results have been inconsistent and restricted to limited types of malignancy. One potential strategy to build on this progress is to incorporate tumor-directed immune-based therapies into allogeneic HSCT where the GVT effect may be active but insufficient. A major barrier to this approach is delayed immune recovery, particularly in the T-cell compartment. We have utilized allogeneic HSCT to treat pediatric patients with high-risk solid tumors following reduced-intensity conditioning resulting in rapid recovery of thymus-derived T cells by 3 months. In preclinical murine models, we have demonstrated that dendritic cell vaccines can effectively expand tumor-specific T cells following allogeneic HSCT but that even mild GVHD can inhibit these responses. We have identified interferon gamma ($IFN\gamma$) and STAT1 as contributing to the loss of tumor-specific vaccine responses due to GVHD. Interestingly, although lack of $IFN\gamma$ signaling on T cells results in diminished GVHD and loss of vaccine responses, manipulation of $IFN\gamma$ signaling on donor bone marrow-derived non-T cells abrogates GVHD and restores vaccine responses and tumor control. Importantly, the ability for these T cells to control tumor growth is enhanced in allogeneic recipients compared to syngeneic recipients despite quantitatively similar vaccine responses. Ongoing clinical efforts are combining allogeneic HSCT with tumor-directed vaccines in patients with high-risk hematologic malignancies.