

1

CROSSTALK BETWEEN TUMOR CELLS AND ENDOTHELIUM RECRUITS T LYMPHOCYTES TO THE TUMOR MICROENVIRONMENT ENHANCING TUMOR GROWTH IN VIVO

Vita S Salsman^{1,2,3}, Kwong-Hon Chow^{1,2,3}, Donald Shaffer^{1,2,3}, Huseyin Kadikoy^{1,2,3}, Xiao-Nan Li^{2,3}, Claudia Gerken^{1,2,3}, Laszlo Perlaky^{2,3}, Leonid S. Metelitsa^{1,2,3,4}, Xiuhua Gao^{1,2,3,4}, Meena Bhattacharjee⁵, Karen Hirschi^{1,4}, Helen E Heslop^{1,2,3,6}, Stephen Gottschalk^{1,2,3,4}, Nabil Ahmed^{1,2,3}

¹Center for Cell and Gene Therapy, ²Texas Children's Cancer Center, Departments of ³Pediatrics, ⁴Immunology, ⁵Pathology and ⁶Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA

Cancer cells can live and grow if they succeed in creating a favorable niche that often includes elements from the immune system. We show here that T lymphocytes consistently infiltrate the primary brain cancer, medulloblastoma and indeed enhance the growth of cancer cells implanted in the brain of experimental animals. We demonstrate that T lymphocytes are attracted to tumor deposits only if the tumor cells have interacted with tumor vascular endothelium. Macrophage Migration Inhibitory Factor (MIF) is the key chemokine molecule secreted by tumor cells which induces the potent T lymphocyte attractant Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) from the tumor vascular endothelial cells, which in turn creates a chemotactic gradient for receptor (CCR3) bearing T lymphocytes. Based on our model, manipulation of this pathway could have important therapeutic implications.

Presenting author Email: vssalsma@txccc.org

2

INHIBITING STAT1 AS A PLATFORM FOR GVHD RESISTANCE

Christian M Capitini, Shannon M Larabee, Haiying Qin, Young K Song, Javed Khan, Crystal L Mackall, Terry J Fry

Pediatric Oncology Branch, National Cancer Institute, Bethesda, MD

T cell depleted (TCD) allogeneic bone marrow transplantation (alloBMT) can minimize Graft-versus-host-disease (GVHD) but render the recipient immune incompetent, necessitating delayed lymphocyte infusions (DLIs) to improve T cell reconstitution and prevent relapse. We have previously demonstrated that TCD gamma interferon (IFN γ) receptor-deficient bone marrow prevents DLI-induced GVHD after major histocompatibility complex (MHC)-matched, minor histocompatibility antigen(mHA)-mismatched alloBMT. Since STAT1 is the main signaling molecule for IFN γ , we hypothesized that STAT1-deficient bone marrow could prevent GVHD. We also sought to determine if this protection occurs after T cell replete (TCR) mHA-mismatched alloBMT (129?B6 x C3H.SW) as well as after TCD MHC-mismatched alloBMT (129?C3H.HeNcr) with DLI. STAT1-deficient bone marrow prevents GVHD after TCD mHA-mismatched alloBMT, allowing the host to tolerate high doses of wild-type. alloreactive DLI. Interestingly, STAT1-deficient bone marrow did not prevent GVHD after TCR mHA-mismatched alloBMT nor after TCD MHC-mismatched alloBMT, implying the timing and severity of GVHD can overcome GVHD resistance. TCD IRF9-deficient bone marrow does not prevent GVHD, implying signaling through the IFN-alpha (IFN α) receptor (which also uses STAT1) is not critical. Lastly, after TCD mHA-mismatched alloBMT, recipients of STAT1-deficient bone marrow show a marked expansion of plasmacytoid dendritic cells (pDCs) that show increased IFN- α production. Gene expression profiling of pDCs from recipients of STAT1-deficient marrow also show increased expression of S 100A8 and S 100A9, suggesting a tolerogenic phenotype. In summary, absence of STAT1 mediates GVHD resistance after TCD mHA-mismatched alloBMT and allows for the administration of high doses of T cells to enhance Graft-versus-leukemia effects and immunocompetence.

Presenting author Email: capitinic@mail.nih.gov

3

ADOPTIVE IMMUNOTHERAPY USING EPHA2-SPECIFIC T CELLS FOR THE TREATMENT OF GLIOBLASTOMA MULTIFORME

Kevin Chow, Vita Salsman, Claudia Gerken, Yvonne Kew, Robert Grossman, Suzanne Powell, Nabil Ahmed, Stephen Gottschalk

Center for Cell and Gene Therapy, Texas Children's Cancer Center, Interdepartmental Program in Translational Biology and Molecular Medicine, Baylor College of Medicine, Departments of Neurosurgery and Pathology, The Methodist Hospital, Houston, Texas

Background: Glioblastoma multiforme (GBM) is the most common and most aggressive primary brain tumor in humans. In children, these tumors often occur in the brainstem, making standard therapies even less effective than in adults. New targeted treatments are needed to improve outcomes, and adoptive immunotherapies have the potential to fulfill this need. Genetic modification of T cells with chimeric antigen receptors (CARs) enables the rapid generation of T cells that can recognize and kill tumors with specific tumor antigens. Erythropoietin-producing hepatocellular A2 (EphA2) receptor has been identified as an antigen that is overexpressed in many solid tumors including GBM. The aim of this study was to 1) generate a CAR specific for EphA2 and express it in T cells, and 2) determine if EphA2-specific T cells can selectively recognize and kill EphA2-positive GBM *ex vivo* and *in vivo*.

Methods: EphA2.CAR was generated by fusing the antigen-binding domain of an anti-EphA2 antibody (4H5) to the signaling domain of a T-cell receptor. T cells were retrovirally transduced to express the EphA2.CAR (EphA2-specific T cells). The *ex vivo* efficacy of the EphA2-specific T cells was determined by their ability to proliferate and secrete cytokines in response to EphA2-positive tumor cells, and to kill EphA2-positive targets in cytotoxicity assays. The *in vivo* efficacy of EphA2-specific T cells was tested for their ability to induce tumor regression in an orthotopic xenograft murine model of GBM.

Results: EphA2.CAR was successfully expressed on T cells as determined by FRCS. Expression of EphA2 receptor on target cells was confirmed by Western blot. EphA2-specific T cells were able to recognize EphA2-positive tumors as evidenced by their production of high levels of IFN- γ and IL-2. EphA2-specific T cells were also able to kill EphA2-positive tumors, whereas nonspecific T cells could not. *In vivo*, EphA2-specific T cells were able to induce regression of established GBM tumors and increase overall survival of the treated mice.

Conclusions: We successfully generated an EphA2.CAR that is able to direct T cells to tumors that overexpress the EphA2 receptor. EphA2-specific T cells were able to recognize EphA2-positive GBM, secreting immunostimulatory cytokines in response and killing the tumor. EphA2-specific T cells were also able to cause regression of established GBM xenografts in an orthotopic murine model. EphA2 has emerged as an important tumor antigen for immunotherapy, and targeting this receptor using EphA2.CAR-modified T cells shows promise for the treatment of GBM and other solid tumors.

Presenting author Email: kxchow@txccc.org

4

COMBINED EPIGENETIC AND IMMUNE-BASED THERAPIES TARGETING MAGE-A4 FOR RELAPSED/REFRACTORY HODGKINS LYMPHOMA

Conrad Russell Y. Cruz¹, Ulrike Gerdemann¹, Jessica A. Shafer¹, Ann M. Leen¹, Cliona M. Rooney¹, Anas Younes², Terzah M. Horton³, Helen E. Heslop¹, Catherine M. Bollard¹

¹Center for Cell and Gene Therapy, Baylor College of Medicine/The Methodist Hospital/Texas Children's Hospital, Houston TX ²University of Texas MD Anderson Cancer Center, Houston TX ³Department of Pediatrics, Baylor College of Medicine, Houston TX

Patients with relapsed Hodgkins Lymphoma (HL) have limited options for long-term cure. We have shown that infusion of cytotoxic T-cells (CTL) targeting EBV induced complete remissions in EBV+ HL patients. Unfortunately, —70% of relapsed HL tumors are EBV-negative. Thus, we investigated the tumor antigen MAGE-A4 as a potential target for CTL therapy. MAGE-A4 expression can be enhanced in MAGE-A4-negative HL tumors by epigenetic modification using clinically available demethylating agents. We first verified whether MAGE-A4-specific CTL could be expanded from autologous and allogeneic donors. Dendritic cells from healthy donors (n=9) or HL patients (n=3) were pulsed with overlapping peptides spanning the MAGE-A4 protein. Analysis of the stimulated T cells using IFN γ ELISPOT assays showed high specificity against MAGE-A4. In addition, these cells specifically killed MAGE A4+ tumor cells as measured by chromium release assays. CI L lines generated from healthy donors were predominantly CD8+ and 4/9 of these lines showed specificity for a unique epitope (aa266-285, NPARYEFLWGPRALAETSYV). In contrast, 4/6 T-cell lines derived from HL patients were predominantly CD4+, and showed a range of MAGE-A4 epitope specificities. We next explored the effect of combining MAGE-CTL with the epigenetic-modifying demethylating agent 2-deoxy5azacytidine. Increased expression of MAGE-A4 in tumor cells was demonstrated using Q-PCR and immunohistochemistry. Moreover, analysis of patient peripheral blood, showed increased numbers of MAGE-A4-specific T cells following epigenetic therapy. These results suggest that MAGE-A4-specific T cell immunotherapy, coupled with epigenetic therapy with decitabine, is a novel and practical approach for the treatment of refractory Hodgkins Lymphoma.

Presenting author Email: rxacruz@txccc.org

5

MULTIVIRUS-SPECIFIC T CELL IMMUNOTHERAPY TO PREVENT OR TREAT INFECTIONS OF ALLOGENEIC STEM CELL TRANSPLANT RECIPIENTS

Ulrike Gerdemann, Juan F. Vera, Tamara Tripic1, Malcolm K. Brenner, Helen E. Heslop, Cliona M. Rooney, Ann M. Leen

Center for Cell and Gene Therapy, Baylor College of Medicine, The Methodist Hospital, and Texas Children's Hospital, Houston, TX, USA

Viral infections cause morbidity and mortality in allogeneic HSCT recipients. Our group has generated trivirus-specific cytotoxic T lymphocytes (CTL) targeting EBV, CMV and Adv using adenovector-modified monocytes and EBV-LCLs as antigen presenting cells (APCs) and shown that even small numbers of infused CTLs ($2 \times 10^5/\text{kg}$) are effective and protective in vivo. However, broader implementation of this therapy is limited by production costs, complexity and generation time. To overcome these limitations we have developed a new, GMP-compliant production strategy using DCs nucleofected with DNA plasmids encoding the viral antigens IE1, pp65 (CMV), EBNA1, LMP2, BZLF1 (EBV) and Hexon, Penton (Adv) as APCs for T-cell stimulation. With this approach we can reproducibly generate multivirus-specific CTL lines comprised of both CD4+ and CD8+ T cells, which produce multiple effector cytokines (IL2, IFN- γ , TNFa) and kill viral antigen-expressing targets with minimal/no alloreactivity as assessed by Cr51 release. Addition of the cytokines IL-4 and IL-7 at culture initiation enhances CTL proliferation and sustains the breadth of reactivity in our multiantigen-specific CTL lines while culture in a gas permeable cell culture device (G-Rex) supports maximal CTL expansion and ensures production of sufficient cell numbers for infusion with a single stimulation. By implementing these changes we can now produce multivirus CTL in 2 rather than 12 weeks, reducing costs >90%. Our approach, extendable to additional viruses, should be of value for prophylactic and treatment applications for high-risk allogeneic HSC1' recipients and the safety and potency of these CTL will be assessed in a Phase I/II clinical trial.

Presenting author Email: uxgerdem@txccc.org

6

MULTI-TUMOR-ANTIGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTES FOR CANCER IMMUNOTHERAPY

Ulrike Gerdemann, Juan F. Vera, Conrad R. Cruz, Tamara Tripie, Anne S. Christin , Helen E. Heslop , Malcolm K. Brenner , Catherine M. Ballard, Cliona M. Rooney, Ann M. Leen

Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children Hospital and The Methodist Hospital, Houston.

Tumor immunotherapy using in vitro-expanded cytotoxic T lymphocytes (CTLs) can effectively treat virus-associated malignancies such as EBV+ve Hodgkins Lymphoma (HL). However, extension to non virus-associated malignancies has been complicated by the low frequency of circulating T-cells reactive against tumor-associated antigens (TAA), which are often anergized or tolerized in vivo. We have overcoming these limitations by activating T-cells ex vivo using dendritic cells pulsed with pepmixes (overlapping peptide libraries) spanning selected TAA in presence of the Th1-polarizing cytokines IL12 and IL27, pro-survival/proliferative cytokines IL7 and IL15 and the Treg inhibitory cytokine IL-6. The use of whole antigen as a stimulus increases the range of suitable patient HLA polymorphisms beyond those matched to single peptides. Further, to reduce the risk of tumor immune evasion we chose to simultaneous target multiple TAAs. We were consistently able to generate multi-lymphoma TAA-CTLs directed against SSX2, Survivin, and MAGEA4 from healthy donors and HL patients. The lines were polyclonal, comprising CD4-1-/CD8+ T cells, with simultaneous reactivity against the stimulating antigens, and killed autologous pepmix-pulsed targets, HLA-matched Hodgkins cell lines and autologous tumor as demonstrated by cytotoxicity and co-culture assays. Finally we demonstrated that lymphoma-TAA CTLs could be retrovirally transduced with a chimeric antigen receptor (CAR) targeting CD19, expressed on lymphoma, to further increase the breadth of specificity of the TAA-CTL, without loss of native antigen specificity. This study shows the successful generation of CTLs targeting multiple TAAs simultaneously, which can be used as a platform for a variety of genetic modifications improving survival, migration and function.

Presenting author Email: uxgerdem@txccc.org

7

EXPANSION OF CLINICAL-GRADE T CELLS EX VIVO USING ARTIFICIAL ANTIGEN PRESENTING CELLS

Hillary N Gibbons¹, Carrie Yuen, M.D.¹, Harjeet Singh, Ph.D.¹, Helen Huls¹, Carl H June, M.D.^{2,3}, Laurence JN Cooper, M.D., Ph.D.¹

¹Pediatrics, M.D. Anderson Cancer Center, Houston, TX, United States, 77054; ²Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, Philadelphia, PA, United States, 19104 and ³Abramson Cancer Research Institute, University of Pennsylvania Medical Center, Philadelphia, PA, United States, 19104.

Patients who rapidly recover their absolute lymphocyte count (ALC) after ablative therapy have improved disease-free and event-free survival. Therefore, we developed a clinically viable method to numerically expand T cells of desired phenotypes from peripheral blood mononuclear cells (PBMC) and umbilical cord blood (UCB) for infusion after ablative therapy to aid ALC recovery. We hypothesized that the culturing environment could be altered to improve the T cell expansion and alter expanded T cell phenotype. To rapidly expand polyclonal T cells we developed, in collaboration with the University of Pennsylvania, a bank of clinical-grade artificial antigen presenting cells (aAPC) derived from K562 were genetically modified to express the T-cell costimulatory molecules CD86, and 41BB-L, and a membrane bound version of IL-15. Co-expression of the high affinity Fe receptor, CD64, on aAPC enabled loading of clinical grade CD3-specific mAb (OKT3) which provides a proliferative signal upon cross-linking CD3. The T-cell to aAPC ratio was systematically varied (from 10:1 to 1:2) and expanded T-cell populations were enumerated and evaluated for viability and phenotype assessed by flow cytometry. After 21 days, the T cells expanded up to 11104fold. Interestingly, the cultures containing the lowest number of aAPC (10:1) yielded more CD8+ T cells, while the culture with the highest number of aAPC (1:2) yielded more CD4+ T cells. The aAPC were able to support the expansion of T-cell subpopulations containing central memory T cells (CCR7+CD45RA^{neg}) and effector memory T cells (CCR7^{neg}CD45RA^{neg}), with higher stimulation resulting in statistically more central memory T cells than the lower stimulation. This difference was in part due to the relative difference of CD4+ T cells in these cultures, as CD4+ T cells contained a higher fraction of central memory than CD8+ T cells, however higher stimulation also resulted in more CD8+ central memory T cells than lower stimulation. To evaluate function, the T cells were activated and found to produce high amounts of IFN γ and granzyme B, moderate amounts of IL2, IL4, IL17, IL21 and perform, and minimal amounts of IL10 and TGF β . In conclusion, these data demonstrate that OKT3-loaded aAPC can be used at defined ratios to efficiently propagate CD8 or CD4 T cells which contain desired subsets.

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Presenting author Email: hgibbons@mdanderson.org

8

CYTOTOXIC T LYMPHOCYTES (CTL) SPECIFIC FOR CMV, ADENOVIRUS, AND EBV CAN BE GENERATED FROM NAIVE T CELLS FOR ADOPTIVE IMMUNOTHERAPY

Patrick J. Hanley¹, Sharon Lam¹, Russell Cruz¹, Ann M Leen¹, Gail J Demmler¹, Helen E Heslop¹, Cliona M Rooney¹, Malcolm K Brenner¹, John R Rodgers¹, Elizabeth J Shpall², Catherine M Bollard¹

¹Baylor College of Medicine, Houston, Texas; ²University of Texas, MD Anderson Cancer Center, Houston, Texas

Adoptive transfer of virus-specific T-cells can effectively restore anti-viral immunity after stem cell transplant. However, standard protocols for eliciting virus-specific T-cells from adult seropositive donors have been ineffective when applied to cord blood (CB) or virus-naïve adult donors. We tested alternative techniques for eliciting primary T-cell responses in vitro. Culturing APC transduced with an Ad5f35pp65 vector and cytokines IL-2,7,12 and 15, we generated T-cells specific for multiple viruses from CB and CMV-seronegative (CMVneg) donors. 9 CB-derived T-cell lines contained 87% (range 81-94) CD8+ and 26% (12-40) CD4+ T-cells. Specificity was determined in 51Cr release and IFN- γ ELISPOT assays against CMVpp65, adenovirus, and EBV targets. Results showed mean spot forming cells (SFC) following incubation with CMVpp65(209;range 45-694), Adhexon(74;0-128), and EBV(157;23-291) targets. Further, CMVpp65-specific T-cells expanded from 7 CMVneg adult donors showed mean 142(38-410) SFC to CMVpp65. CB and CMVneg-derived T-cells derived only from naïve(CD45RA+CCR7+) T-cells and recognized “unconventional” CMVpp65 epitopes as identified by overlapping pp65 peptide pools using IFN- γ ELISPOT, suggesting an inherent difference between naïve and memory T-cell responses to CMV. We have now opened a clinical trial using CB-derived virus-specific CTL for the prevention and treatment of viral infection after CB transplant. To date, we have treated one CBT recipient with CMV reactivation and showed a marked decrease in viral load corresponding with a rise in CMVpp65 T-cells in the peripheral blood as detected by IFN- γ ELISPOT assay. In conclusion, virus-specific T-cells can be generated from naïve sources for clinical use and may be beneficial for these highest risk patients.

Presenting author Email: pxhanley@txccc.org

9

ESTABLISHING OPTICAL IMAGING PLATFORMS TO MONITOR IMMUNE SURVEILLANCE AND ANTI-TUMOR PRIMING IN SITU

Jay Myers, Rachel Lion, Deborah Barkauskas, Kathryn Davis, Agne Petrosiute, Alex Y. Huang

Division of Pediatric Hematology - Oncology, Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, Ohio

Proper immune function relies on continuous surveillance of host tissues by immune cells. Timing and host tissue microenvironment in which antigen recognition occurs greatly influence the final outcome. Using intravital 2-photon microscopy (IVM), we have previously observed that inflammatory chemokines, CCL3 (MIP-1 α) and CCL4 (MIP-1 β), are critically involved in the orchestration of primary immune response initiation and in the helper T cell-dependent CD8⁺ T cell memory generation¹. Our current research contains three specific aims: A) To characterize dynamic immune surveillance of self- and tumor-associated antigens by immune cells; B) to examine the efficacy of incorporating CCL3 and CCL4 into model tumor vaccines to enhance anti-tumor immunity; and C) to develop longitudinal imaging platforms for the interrogation of these and other cellular responses in peripheral tissues, including lymph nodes (LN), CNS, and the bone marrow of live experimental animals. Mouse models of colon tumor (CT26; H-2d) and medulloblastoma (MMI; H-2b) were fluorescently labeled to allow real-time in vivo tracking by 2-photon microscopy. These tumor cell lines were also engineered to express CCL3 and/or CCL4 with or without chicken ovalbumin expression. These cell lines were injected into naive immuno-competent recipients, or irradiated and injected into recipient mice to boost anti-tumor immune response against subsequent lethal doses of wild type tumor challenge. Antigen-specific and antigen non-specific immune responses within the primary tumor implants and LN metastasis were then subjected to direct visualization under IVM. These responses were further characterized by tissue histology, in vivo functional studies, and in vitro cellular characterizations. Monitoring of CNS tumor development was accomplished by utilizing a chronic cranial bone window. We are able to quantify the duration of TCR / self-MHC surveillance in LN and tissues using IVM. Furthermore, tumors engineered to secrete CCL3 / CCL4 exhibited enhanced primary rejection and systemic anti-tumor memory T cell immunity. Finally, we are now able to monitor tumor development and related host immune and tissue responses on a single-cell level in the same experimental animal over a period of days to weeks using implanted windows. These and other exciting data will be presented at the Immunotherapy Conference in October. Our studies provide a potential role for inflammatory chemokines in generating anti-tumor immunity. Establishing novel optical imaging techniques for a variety of tissues promises to further enhance our understanding of current and future immunotherapeutic strategies against cancer and immune-mediated disorders. ¹Castellino F, Huang AY et al. 2006. Nature 440: 890-895,

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Presenting author Email: alex.y.huang@case.edu

EXPRESSION OF IL-7 AND IL-15 AS MEMBRANE-BOUND MOLECULES ON TUMOR-SPECIFIC T CELLS FOR ENHANCED COSTIMULATION

Lenka V. Hurton¹, Harjeet Singh¹, Simon Olivares¹, Dean A. Lee², Richard E. Champlin³, Laurence J.N. Cooper²

¹Pediatrics-Research, Children's Cancer Hospital, The University of Texas M.D. Anderson Cancer Center, Houston, TX; ²Pediatrics-Patient Care, Children's Cancer Hospital, The University of Texas M.D. Anderson Cancer Center, Houston, TX; ³Stem Cell Transplantation and Cellular Therapy, The University of Texas M.D. Anderson Cancer Center, Houston, TX

The genetic modification of T cells with a chimeric antigen receptor (CAR) enables retargeted specificity to a select cell surface tumor-associated antigen. Despite continued modifications to the CAR endodomains to provide T cells with additional signaling, CAR-dependent T-cell activation remains insufficient with respect to in vivo persistence, which has been a limiting factor to achieving an optimal therapeutic response. To improve T-cell survival, and thus improve anti-tumor efficacy, investigators have infused soluble recombinant cytokines such as interleukin (IL)-2 and IL-7. Deleterious effects are associated with the systemic use of IL-2 and IL-15, and clinical-grade IL-7 has limited availability. As IL-7 and IL-15 are important in T cell homeostatic expansion during lymphopenia and maintenance of memory T cell subsets, we hypothesized that membrane-bound cytokines mIL7 and mIL15 could provide added costimulatory signals to CD19-specific CAR-modified T cells. Multiple transposition of Sleeping Beauty DNA plasmids for mIL7, mIL15, and CD19-specific CAR was accomplished by electro-transfer into primary human T cells. The mIL7-modified T cells could be numerically expanded ex vivo on K562-derived artificial antigen presenting cells bearing CD19+ without additional soluble cytokine supplementation, while mIL15-modified T cells required exogenous IL-21 to achieve optimal expansion. T cells expressing mIL7, mIL15, and CAR exhibited preferential outgrowth, while CAR+ T cells lacking membrane-bound or soluble cytokine supplementation did not sustain antigen-specific proliferation. The mIL7+CAR+ T cells and mIL15+CAR+ T cells exhibited redirected specific lysis of CD19+ tumor targets. Verification of mIL7 and mIL15 signaling in the modified T cells was assessed via phosphorylation of signal transducer and activator of transcription 5 (STAT5). These cells expressed markers of central- and effector-memory phenotype, with mIL7 inducing more of a central memory phenotype. These results suggest that mIL7+CAR+ T cells and mIL15+CAR+ T cells may exhibit enhanced persistence in vivo and thus should be tested for improved therapeutic potential in clinical trials.

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Presenting author Email: lhurton@mdanderson.org

IN VITRO STUDIES ON THE IMPACT OF TLR2 AGONISTS ON THE CYTOTOXIC POTENTIAL OF CHEMOTHERAPY AND IMMUNE RESPONSES IN PRE-B ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Nina Rolf^{1,2}, Amina Karirninia¹, Sabine Ivison¹, Kirk R. Schultz^{1,2}

¹Child & Family Research Institute, Childhood Cancer and Blood Research Group, Vancouver, BC, Canada; ²Division of Hematology/Oncology/BMT, Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver, BC, Canada

Background: Currently, prognosis is poor for patients with relapsed pre-B-ALL. Although endosomal Toll-like receptor (TLR) 9 agonist, CpG-ODN, induces significant anti-tumor immune effects, surface-expressed TLR2 receptors are more abundant on pre-B-ALL cells, suggesting TLR2 agonists may be better immune activators. As it has been shown that stimulation with TLR agonists can increase leukemia chemosensitivity, combining TLR2 agonists and chemotherapy may offer improved outlooks for relapsed pre-B-ALL patients.

Objective: We tested the ability of TLR2 agonists (TLR2/6: Pam2CSK4, TLR2/1: Pam3CSK4 to 1) activate NFkB; 2) induce apoptosis 3) augment cell immunogenicity and 4) lower the effect concentrations of chemotherapeutics for pre-B-ALL.

Methods: Blasts pre-treated with TLR2 agonists were examined as follows: First by flow cytometry for 1) activation of main TLR signaling mediator NFkB; 2) AnnexinV/7AAD; and 3) induction of costimulatory molecules. Secondly, cells were tested for their ability to stimulate proliferation (MLR) and for sensitivity to different chemotherapeutics.

Results: NFkB activation studies revealed Pam2CSK4 as a more rapid inducer of TLR2 signaling than Pam3CSK4. While both agonists increased blast immunogenicity to a similar extent, Pam3CSK4 induced more direct apoptosis after 6/24/48hr than Pam2CSK4 (36/66/81% vs. 14/16/17%, resp.). Pam3CSK4 pre-treatment lowered effective killing concentrations of both Doxorubicin and Asparaginase.

Conclusion: Despite more rapid kinetics of NFkB activation by Pam2CSK4, Pam3CSK4 may be a more promising agonist for TLR2-based pre-B-ALL immunotherapy. Treatment with both chemotherapy and Pam3CSK4 could increase destruction of blasts at lower chemotherapy concentrations, and may also improve the induction of lasting anti-blast immunity for the prevention of relapse.

Presenting author Email: sabine.ivison@gmail.com

IMPROVING T-CELL IMMUNOTHERAPIES BY CO-TARGETING TUMOR CELLS AND THEIR SUPPORTIVE STROMA USING GENETICALLY ENGINEERED T-CELLS

Sunitha Kakarla^{1,5}, Lisa Wang², David Rowley³, Klaus Pfizenmaier⁴, Stephen Gottschalk^{2,5}

¹Interdepartmental Program in Translational Biology and Molecular Medicine, Departments of ²Pediatrics and ³Cell and Molecular Biology, Baylor College of Medicine; ⁴University of Stuttgart, Germany; ⁵Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital, The Methodist Hospital, Houston TX USA.

Adoptive T-cell therapy has had considerable success in effectuating antitumor responses, however complete eradication of bulky disease is rarely observed. This limited efficacy is most likely due to the tumor stroma, which is not targeted by tumor-specific T cells. Cancer associated fibroblasts (CAFs), the central component of the tumor stroma, secrete inhibitory factors and nutrient depleting enzymes that are detrimental to effector T-cell function. In addition, CAFs promote angiogenesis and secrete extracellular matrix components, which act as a physical barrier. CAFs express fibroblast activation protein (FAP); a membrane bound serine protease, which is an attractive immunotherapeutic target. The aim of this project was to generate FAP-specific T cells and determine if targeting the tumor stroma with FAP-specific T cells has antitumor effects.

Methods: To generate FAP-specific T cells we took advantage of chimeric antigen receptors (CARs), which consists of antigen-specific single chain variable fragments (scFv) linked to T-cell receptor signaling domains. Using this approach we generated 2 CARs, which are specific for human FAP (hFAP) or human and murine FAP (mhFAP). T cells expressing hFAP-CARs or mhFAP-CARs were generated by retroviral transduction (hFAP- or mhFAP-T cells). Ex vivo, efficacy of hFAP- and mhFAP-T cells was determined by their ability to 1) secrete cytokines in coculture experiments with FAP-positive tumor or stroma cells, and 2) kill FAP-positive targets in cytotoxicity assays. To test in vivo, if selective targeting of FAP on tumor stroma prevents the development of human tumors in a xenograft model, we took advantage of lymphoblastoid cell lines (LCL), which are FAP-negative.

Results: hFAP-CARs or mhFAP-CARs were successfully expressed on T cells as judged by FACS analysis. hFAP- and mhFAP-T cells recognized FAP-positive human tumor and stroma cells as judged by cytokine production and cytotoxicity assays. To evaluate if targeting the tumor stroma, prevents the development of tumors, FAP-negative, luciferase-expressing LCLs, were mixed with hFAP, mhFAP-, or nontransduced T cells prior to the s.c. injection into flanks of SCID mice. While LCL tumor readily established in mice injected with LCL/hFAP-T cells or LCL/NTT cells; LCL tumor growths was 10 to 100 slower in mice injected with LCL/mhFAP-T cells as judged by serial bioluminescence imaging.

Conclusions: Our preliminary results indicate that targeting FAP-positive stroma with T cells greatly delays the development of tumors in human xenograft models. Thus, targeting the tumor stroma with FAP-specific T cells has the potential to improve current immunotherapy approaches for cancer.

Presenting author Email: sxkakarl@txccc.org

13

TARGETING THE TIMOR ANTIGEN CD56 WITH T CELLS

Denise Kellar, B.S.¹, Sonny Ang, Ph.D.¹, Simon Olivares, B.S.¹, Helen Huls, B.S.¹, Laurence Cooper, M.D./Ph.D.¹

¹Experimental Pediatrics, University of Texas M.D. Anderson Cancer Center, Houston, TX

1. Introduction: Some candidate tumor-associated antigens (TAAs) are also expressed on T cells limiting the use of T cells to target such molecules. CD56 is an attractive TAA due to expression on a diverse set of malignancies, yet CD56 is also upregulated on activated T cells. We hypothesized that a chimeric antigen receptor (CAR) could be generated with specificity for CD56 and tested whether CD56-specific CAR⁺ T cells could be propagated using our artificial antigen presenting cells (aAPCs).

2. Aims and Methods: Primary human T cells were electrotransferred with either a CD56-specific CAR (CD56RCD28) or a CD19-specific CAR (CD19RCD28) and CAR⁺ T cells were selectively propagated on γ -irradiated CD56⁺ or CD19⁺ aAPCs, respectively, in the presence of exogenous recombinant IL-2 and IL-21. We compared the ability to express the CD56RCD28 with the CD19RCD28, as measured by expansion kinetics and phenotype. Next we determined if CD56⁺CD56RCD28⁺ T cells are capable of lysing allogeneic targets versus autologous CD56⁺ T cells with and without the CAR.

3. Results and Conclusions: There were no significant differences between the expression of CD56 and the expansion kinetics of CD19RCD28⁺ T cells compared with CD56RCD28⁺ T cells, which infers that the CD56-specific T cells were not lysing themselves (autolysis). The CD56RCD28⁺ and CD19RCD28⁺ T cells were phenotypically similar and were functional as demonstrated by CD56-specific redirected cytotoxicity against a panel of allogeneic CD56⁺ tumor cells. An isolated CD56⁺ fraction in CD56-specific T cells maintained CD56 expression. Significantly, the CD56⁺CD56RCD28⁺ T cells were resistant to autolysis by CD56RCD28⁺ T cells in a chromium release assay while autologous CD56⁺ T cells and NK cells were lysed. To conclude, our data demonstrate that we can express the CD56RCD28 CAR on T cells despite the co-expression of CD56 and these genetically modified T cells can target tumor cells.

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Presenting author Email: dlkellar@mdanderson.org

IMMUNE RESPONSE AFTER CEREBRAL ENGRAFTMENT OF HUMAN NEURONAL CELLS IN PATIENTS WITH HUNTINGTON'S DISEASE: CONSEQUENCES FOR PEDIATRIC NEURONAL CELL TRANSPLANTATION PROGRAMS

Simone Krebs^{1,2}, Michael Trippel¹, Thomas Prokop¹, Talib N. Omer¹, Bernard Landwehrmeyer³, Wolfgang A. Weber², and Guido Nikkhah¹

¹Department of Stereotactic and Functional Neurosurgery, Faculty of Medicine, University of Freiburg, Germany. ²Department of Nuclear Medicine, Faculty of Medicine, University of Freiburg, Germany; ³Department of Neurology, Faculty of Medicine, University of Ulm, Germany

Children diseases of the central nervous system, particularly those of genetic, metabolic, or inflammatory etiology, are associated with neural degeneration or dysfunction. Neuronal cell transplantation is under consideration to treat some of these disorders. Furthermore, many children who are treated for brain tumors experience significant long-term deficiencies in motor functions. In some, the therapy might also require neural cell replacement, a challenge not regarded as amenable to cell replacement treatment. The allogeneic graft material in human brain raises questions of immune response.

Very little data is available how the human brain reacts immunologically to cell transplantation over time. Anti HLA-antibodies may act as an early indicator of a slowly emerging rejection that is not yet manifested clinically. We measured in patients with Huntington's disease immune responses as assessed by the presence of anti HLA-antibodies to allogeneic intra-striatal transplantation of fetal neurons. The patients received standard triple immunosuppressive therapy for one year after stereotactic intervention. Out of 10 patients, without anti HLA-antibodies prior to engraftment, five developed anti HLA-antibodies of class I and II. The time between transplantation and development of HLA antibodies was variable, with detection either immediately after engraftment, during immunosuppressive therapy, or three and 24 months after prophylactic immunosuppressive therapy was stopped. The antibodies disappeared in three patients when immunosuppression was reinstated. Development of anti HLA-antibodies had no immediate effect on the initial favorable clinical response.

The findings show that there is indeed a significant number of patients who may develop antibodies to allogeneic cell transplantation into the brain parenchyma. The triggering of anti HLA-antibodies may not lead to immediate graft rejection, a possibility that cannot be excluded. As the time of onset of immune response is unpredictable, long term prophylactic immune suppression cannot be justified. Instead regular assessment of blood for anti HLA-antibodies for indefinite long periods appears mandatory.

Given the ongoing cell transplantation studies for cerebral palsy, Batten disease and future cell transplantation programs for children with brain tumors, the results are of significant implications.

Presenting author Email: simone.krebs@uniklinik-freiburg.de

EXPANSION OF REGULATORY T CELLS WITH LOW-DOSE IL-2 FOR GRAFT-VERSUS-HOST DISEASE PROPHYLAXIS AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Alana Kennedy-Nasser, Stephanie Ku, Walailuk Chaiyarat, Benjamin Tzou, April Durett, Aaron Foster, Barbara Savoldo, Eric Yvon, Helen Heslop, George Carrum, Rammurti Kamble, Malcolm Brenner, Robert Krance, Catherine Bollard

Center for Cell and Gene Therapy, Texas Children's Hospital, The Methodist Hospital, Baylor College of Medicine

CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells (Tregs) are of increasing interest in hematopoietic stem cell transplantation (HSCT) as a preventive measure against the development of graft-versus-host disease (GvHD). In humans, it has been reported that HSCT recipients with low Treg numbers in the donor graft have increased rates of GvHD. In the past, our group and others have used IL-2 therapy post HSCT in an attempt to enhance antitumor immunity. Retrospectively, it has been determined that patients who received IL-2 therapy had higher levels of FoxP3⁺ T cells (i.e. Tregs) than controls. Therefore, it is plausible that IL-2 may lead to the preferential in vivo expansion of Tregs, thereby preventing GvHD. We have initiated a phase II clinical trial to evaluate the efficacy and toxicity of using ultra low-dose IL-2 injections following allogeneic HSCT to promote Treg expansion in vivo and prevent acute GvHD. We have also initiated clinical trials to evaluate Treg reconstitution post HSCT in patients not receiving IL-2 to serve as control populations. Thus far, 11 patients with hematologic malignancies have received ultra low-dose IL-2 (100,000 to 200,000 units/m² subcutaneously three times weekly) post transplant beginning from day +7 to +28 for 6 to 12 weeks. Median age at time of HSCT was 14 years (range, 6 to 56 years). Seven patients received grafts from matched siblings and four from alternative donors. All recipients of alternative donor grafts received in vivo T-cell depletion with Campath 1-H. By flow cytometry, all patients demonstrated a rise in the percentage of CD4⁺ CD25⁺ FoxP3⁺ Tregs by 6 weeks following initiation of IL-2 therapy with mean of 5.1% (range, 0 to 11.0%) pre IL-2 to a mean of 13.5% (range, 4.4% to 31.1%) post IL-2 treatment. In comparison to the control population, a statistically significant rise in Treg percentages was observed at 1 month post HSCT in patients receiving IL-2 (1month: 17.0% vs 6.8%, p=0.009; 3 months; 11.3% vs 6.9 %, p=0.09). Preliminary functional analyses of CD4⁺ CD25^{bright} cells demonstrate suppression in thymidine uptake assays. No grade 3 or 4 toxicities occurred while on IL-2. No patients developed > grade I acute GvHD. One patient developed chronic GvHD two months after stopping IL-2. Nine of 11 patients are in complete remission. In conclusion, low-dose IL-2 is relatively well-tolerated and may expand a CD4⁺ CD25⁺ FoxP3⁺ Treg population in vivo, and further work is ongoing to corroborate these findings.

Presenting author Email: sxkul@txccc.org

EXPANSION OF T CELLS TARGETING MULTIPLE ANTIGENS OF CMV, EBV, AND ADENOVIRUS TO PROVIDE BROAD ANTIVIRAL SPECIFICITY AFTER STEM CELL TRANSPLANT

Sharon Lam, Patrick J Hanley, Donald R Shaffer, Conrad Russell Y Cruz, Stephanie Ku, Benjamin Tzou, Ann M Leen, Hao Liu, Gail Demmler-Harrison, Cliona M Rooney, Helen E Heslop, Stephen Gottschalk, Catherine M Bollard

Center for Cell and Gene Therapy, Texas Children's Hospital, The Methodist Hospital, Baylor College of Medicine

Hematopoietic Stem Cell Transplantation is the treatment of choice for many common malignancies. However, recipients are susceptible to viral infections after transplant. Anti-viral drugs can be effective, but are toxic and can become drug-resistant. Another option is the administration of virus-specific donor-derived cytotoxic T lymphocytes (CTL). Using a chimeric adenovirus-CMVpp65 vector to modify APC, we generated CTL specific for EBV, CMVpp65 and Adenovirus (Multivirus-specific CTL) from healthy donors. We have infused 26 patients with multivirus-specific CTL and have shown protection against all 3 viruses in vivo without significant toxicity. However, while all patients have been protected against EBV and Adenovirus, >10% of patients developed CMV reactivation after CTL infusion, suggesting that targeting a single CMV antigen may not be entirely protective. Therefore we sought to develop a GMP-compliant strategy to generate CTL targeting two CMV antigens while not losing the breadth of specificity to EBV and adenovirus. CTL were generated by transducing monocytes (first stimulation) and then EBV-LCL with an Ad5f35-IE-1-I-pp65 vector. Resultant T-cells recognized CMV pp65 (mean:330;range:47-995) and IE-1 (mean:154; range:11-505), Adenovirus hexon (mean:189;range:30-465) and penton (mean:109; range:1-353), and EBV-LCL (mean:79; range:9-301) as measured by IFN- γ ELISPOT and pentamer analysis. CTL were also able to lyse EBV, CMV, and adenovirus targets in cytotoxicity assays and recognized a breadth of epitopes within CMV antigens IE-1 and pp65 as well as Ad and EBV. In conclusion, we have developed a strategy to expand multivirus CTL targeting at least 2 antigens from EBV, CMV, and adenovirus thus increasing the breadth of the antiviral response.

Presenting author Email: sxlam@texaschildrens.org

NOVEL ANTIGEN-SPECIFIC EXPANSION OF T CELLS TRANSDUCED WITH A CD19 CHIMERIC ANTIGEN RECEPTOR

Daniel W. Lee¹, James N. Kochenderfer², Christoph Rader², Rimas J. Orentas¹, Crystal L. Mackall¹

¹Pediatric Oncology Branch, ²Experimental Transplantation and Immunology Branch, National Cancer Institute, Bethesda, MD

Relapsed ALL remains a difficult therapeutic challenge. Chimeric antigen receptors (CARs) are genetically engineered molecules expressed in transduced T lymphocytes. CARs express both a target-binding motif and TCR signals needed for T-cell activation and may include costimulatory domains. To date, CARs have been expanded ex vivo using antigen-independent techniques. This project sought to develop antigen-specific expansion of cells transduced with a CD19-specific CAR by employing artificial APCs (aAPCs) and to explore whether the method of expansion impacts functionality of CD19-CAR T-cells. aAPCs used in these studies express the high affinity Fc receptor (CD64) and the costimulatory molecule CD137L (aAPC-41BBL). We created an Fc-CD19 fusion protein that when loaded onto the aAPCs, engages the CAR and induces antigen-specific activation. Briefly, OKT3 and IL-2 activated T-cells were transduced with CD19CAR, control CAR (Her2-specific), or non-transduced (Mock) and maintained in IL-2. Then, three different expansion protocols (EPs) were used. Transduced T-cells were expanded with A) irradiated aAPC-41BBL loaded with anti-OKT3, B) irradiated aAPC-41BBL loaded with rFc-CD19 or C) irradiated allogeneic PBMC feeder cells with anti-OKT3. Functionality was assessed in a 4-hour ⁵¹Cr release assay against four distinct CD19+ ALL cell lines, K562 cells, and K562 stably transfected with CD19 (K562-CD19) or NGFR (K562-NGFR). Results demonstrate similar significant levels of CD19-specific cytotoxicity at E:T ratios as low as 2.5:1, regardless of the EP used (20-40% lysis of all CD19+ targets when expanded by OKT3/aAPC, 40-60% lysis with rFc-CD19/aAPC, and 35-45% lysis with allogeneic feeders and OKT3). To evaluate cytotoxicity in culture, expanded CD19-CAR T-cells were co-cultured with ALL cell targets for four days. Surviving ALL cells, as determined by CD22+ staining, was determined. NALM6 and K562-CD19 cells were entirely eliminated even at an E:T ratio of 2.5:1 by CD19-CAR T-cells, regardless of EP, and not by any of the three Mock EPs. These results demonstrate that the cytolytic potential of CAR-transduced T-cells is similar, regardless of whether expansion occurs via CD3 signaling or the CAR itself. Interestingly, we did observe substantial NK-mediated killing, which correlated with CD56+ cell content and was eliminated by cold target inhibition using K562 cells. Studies are underway to determine whether differences in NK killing vary with expansion protocol. In summary, Fc-bearing aAPCs combined with CAR specific Fc fusion proteins provide a potential off-the-shelf reagent for antigen specific expansion of T-cells with CARs. This could overcome variable transduction efficiencies and allow administration of a more homogenous population of CAR-specific T-cells.

Presenting author Email: leed3@mail.nih.gov

NK-CELL ACQUISITION OF CHEMOKINE RECEPTORS FROM ENGINEERED ANTIGEN PRESENTING CELLS

Srinivas S. Somanchi, Anitha Gururajan, Laurence J. N. Cooper, Dean A. Lee

Division of Pediatrics, UT MD Anderson Cancer Center, Houston, TX.

Natural Killer (NK) cells are able to extract fragments of cell membrane from antigen presenting cells through the immunological synapse, functionally incorporating the membrane receptors contained therein by a process called trogocytosis. Recently it was demonstrated that NK cells can acquire functional CCR7 from dendritic cells through this process and migrate in response to chemokines (CCL19 and CCL21). We investigated whether this process could be used to transiently modify NK cells ex vivo without genetic intervention. In previous work, we developed a K562-based artificial antigen presenting cell (aAPC) expressing membrane-bound IL-21 (K562-c19-mIL21) which enables robust NK-cell expansion, and determined that NK cells did not express detectable CCR7 during this process. To investigate trogocytosis in this system, we genetically modified K562-c19-mIL21 to express membrane-bound CCR7 (K562-c19-mIL21CCR7) using the Sleeping Beauty transposon/transposase system. After 24 hours of co-culture the NK cells cultured with K562-c19-mIL21CCR7 demonstrated marked surface expression of CCR7 compared to NK cells cultured on K562-c19-mIL21 (Figure 1a). In kinetic experiments using three independent donors, CCR7 peak uptake occurred at 24 hours (Figure 1b), followed by a decline that corresponded with the loss of aAPCs in the cultures due to lysis by NK cells. This demonstrates that NK cells can be transiently modified during in vitro expansion to bear receptors that are otherwise not a normal part of their transcriptional repertoire. We are currently establishing the functionality of trogocytosed receptors in vitro and in vivo, and investigating the kinetics of CCR7 persistence after removal of the aAPCs. However, even transient expression as demonstrated might be sufficient for bestowing novel NK-cell migration ability in vivo in response to chemokine signaling, giving the engineered NK cells ability to reach desired tissue targets.

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Presenting author Email: dalee@mdanderson.org

T-CELL THERAPY FOR COMPANION CANINES WITH B-CELL NON-HODGKIN LYMPHOMA

Colleen M. O'Connor¹, Sabina Sheppard⁴, Helen Huls¹, Mark Johnson⁴, Carl June⁵, Suzanne Craig³, Dean A. Lee¹, Richard E. Champlin², Heather M. Wilson⁴, Laurence J N Cooper¹

¹Division of Pediatrics, University of Texas MD Anderson Cancer Center, Houston, Texas; ²Department of Stem Cell Transplantation and Cellular Therapy, University of Texas MD Anderson Cancer Center, Houston, Texas; ³Department of Veterinary Medicine & Surgery, University of Texas MD Anderson Cancer Center, Houston, Texas; ⁴Small Animal Clinical Sciences, College of Veterinary Medicine, Texas A&M University, College Station, Texas; ⁵Abramson Family Cancer Research Institute and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

Introduction: Canine cancer provides a clinically relevant immunotherapy model for human malignancies because of their large size, intra-species genetic diversity, human genetic similarity, and spontaneously occurring tumors which develop inside an intact immune system. Canine lymphoma is genetically and physiologically similar to human Non-Hodgkins Lymphoma (NHL). Lymphocyte recovery after chemotherapy is a predictor for improved survival in human clinical trials for NHL. We have adapted this model to assess whether restoring depleted canine CD3⁺ T-cell pools can potentiate chemotherapy through immune reconstitution.

Methods: T cells derived from NHL⁺ canines were propagated in the presence of IL-2 and IL-21 on El-irradiated OKT3-loaded K562-derived artificial presenting cells (aAPC). Autologous PKH-26 labeled CD3⁺CD8⁺ T cells were intravenously infused post CHOP into six patients using an intra-patient dose escalation scheme.

Results: Infused T cells were detectable in peripheral blood for at least 35 days post infusion and their overall percentage increased after each dose, demonstrating engraftment and in vivo persistence. Infused T cells showed tumor trafficking capacity, as they were detected in the tumor 10 days post infusion, correlating with CCR7 expression pre-infusion. Two negative prognostic factors, increased CD4:CD8 ratios and transforming growth factor- β (TGF- β serum levels, were decreased in six and three canines, respectively, post infusion.

Conclusions: These data demonstrate that autologous T cells can be numerically expanded to clinically-sufficient numbers on OKT3-aAPC and safely used to reconstitute the immune system after chemotherapy-induced lymphopenia. Their ability to traffic to secondary lymphoid tissue supports the next trial infusing graded doses of T cells genetically modified to express a chimeric antigen receptor, specific for canine CD20. The same aAPC are currently being used in human clinical trial. The model for using out-bred canines is justified not only to improve their survival, but as a model that may inform human immunotherapy with genetically manipulated T-cells.

Presenting author Email: coconnor@mdanderson.org

CHIMERIC ANTIGEN RECEPTOR (CAR) DESIGN BASED ON ALK-SPECIFIC SCFV

Rimas Orentas¹, Anton Wellstein², Sneha Ramakrishna¹, Richard Morgan³, Crystal Mackall¹

¹Pediatric Oncology Branch, CCR, National Cancer Institute, Bethesda, MD; ²Pharmacology and Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC; ³Surgery Branch, CCR, National Cancer Institute, Bethesda, MD

The protein ALK (CD246) is receptor tyrosine kinase expressed during embryonic development, primarily in the nervous system. ALK was originally described as an intracellular fusion protein with nucleophosmin in anaplastic large cell lymphoma (ALCL). In neuroblastoma, ALK mutations have been linked to familial inheritance of the disease. Unlike hematological malignancies, full-length ALK is re-expressed on the cell surface of pediatric solid tumors and contains activating mutations. Work in the Wellstein lab has identified the ALK ligand-binding domain using a phage display methodology to block the binding of pleiotropin to ALK. We used the single chain scFV that was generated against this region of ALK as the starting point for the construction of a CAR that featured the ALK-specific single chain Fv fragment linked to T lymphocyte-derived transmembrane and signaling domains, or which also included the CH2CH3 regions of IgG, as a means to extend the scFV-derived sequences further away from the surface of transduced T cells. Preliminary data demonstrates that the extended CH2CH3 conformation is active against two neuroblastoma cell lines, while the single Fv-expressing construct has diminished activity. By altering the domain-structure, T cell-derived signaling motifs, and potentially the antigen-combining region of the scFV we seek to establish general principles of CAR design. Once optimized, patient-derived T cells transduced with a retroviral vector expressing the ALK-specific CAR can be utilized in immunotherapy trials for ALK(+) tumors.

Presenting author Email: Rimas.Orentas@nih.gov

IL-15, UNLIKE IL-2, SUPPORTS EFFECTOR T CELLS PROLIFERATION AND FUNCTION EVEN IN THE PRESENCE OF REGULATORY T CELLS

Serena Perna , Biagio De Angelis, Dania Pagliara, Lan Zhang, Cliona Rooney, Helen Heslop, Malcolm Brenner , Gianpietro Dotti, Barbara Savoldo

Center for Cell and Gene Therapy, Texas Children's Hospital, The Methodist Hospital, Baylor College of Medicine

Adoptive T-cell immunotherapies are safe and clinically effective in cancer patients. Clinical responses strongly correlate with T-cell survival in vivo. Systemic administration of recombinant IL-2 is frequently used to sustain T-cell persistence. However, although effective, IL-2 induces side effects and expansion of regulatory T cells (Tregs). We have now explored whether IL-15, able to sustain T-cell expansion and function, shares with IL-2 the unwanted effect on Tregs. We used Epstein-Barr Virus (EBV)-specific cytotoxic T lymphocytes (CTLs) as a model. The inhibitory activity of Tregs was assessed using CFSE-based assays (to evaluate the inhibition of proliferation), and co-culture experiments with lymphoblastoid cell line (LCLs) (to assess the inhibition of effector function), with or without IL-2 (25IU/mL) or IL-15 (2.5ng/mL). T-cell proliferation was inhibited by Tregs alone (from 56%6% to 20%5%, $p < 0.05$) or IL-2+Tregs (from 76%3% to 53%3%, $p < 0.05$), but not in the presence of IL-15+Tregs (from 81%3% to 74%3%, $p = 0.1$). Similarly, the anti-tumor activity of EBV-CTLs was significantly impaired by the presence of Tregs alone (residual LCLs increased from 31%13% to 55%13%, $p < 0.05$) or IL-2+Tregs (from 16%8% to 36%14%, $p < 0.05$) but not by IL-15+Tregs (from 76%6% to 11%7%, $p = 0.2$). Our study suggests that IL-15, unlike IL-2, can relieve effector cells from the Tregs inhibition. We are currently exploring potential mechanisms of this observation by studying the effects of IL-2 and IL-15 directly on each component (Tregs vs effector cells). Preliminary data suggest that IL-15 does not directly revert the inhibitory properties of Tregs, but preferentially enhance T-cell proliferation and anti-tumor activity.

Presenting author Email: sxperna@txccc.org

ROLE OF STAT3 IN EX-VIVO EXPANSION AND ACTIVATION OF NK CELLS

Prasad V. Phatarpekar¹, Shiguo Zhu, MD, Ph.D², Cecele J. Denman¹, Srinivas S. Somanchi, Ph.D¹, Vladimir Senyukov, Ph.D¹, Anitha Gururajan¹, Laurence J.N. Cooper, MD, Ph.D¹, Dean A. Lee, MD, Ph.D¹

¹Pediatrics, UT MD Anderson Cancer Center, Houston, TX; ²Institute of Medical Science, Shanghai Jiao Tong University, Shanghai, China;

Introduction: Activation and expansion of patient- or donor-derived NK cells under laboratory conditions to a sufficiently large number is a pre-requisite for the success of NK adoptive immunotherapy against cancer. We previously described robust ex vivo expansion and activation of NK cells by co-cultivation with K562-based artificial antigen presenting cells (aAPC) genetically modified to express membrane bound IL-21 (mIL-21). This aAPC induces significantly higher NK cell expansion than K562 expressing membrane bound IL-15 (mIL-15). Although, IL-21 and IL-15 activate multiple STATs, STAT3 is pre-dominantly activated by IL-21, while IL-15 pre-dominantly activates STAT5.

Aims and Methods: The superior performance of IL-21 in NK cell expansion and the central role of STAT3 in IL-21 induced signaling led us to hypothesize that activation of STAT3 plays a critical role in NK cell expansion and their anti-tumor activity. To test our hypothesis, purified NK cells from human blood samples were expanded with K562 bearing either mIL-21 or mIL-15 in the presence and absence of small molecule STAT3 inhibitors, JSI-124 or S31-201. Anti-tumor cytolytic activity of NK cells was assessed for their ability to lyse K562 targets using the calcein release assay. Expression of NK cell surface receptors and phosphorylated STAT3 was evaluated by flow-cytometry.

Results and Conclusions: Inhibition of STAT3 strongly suppressed the expansion and cytolytic activity of NK cells induced by either cytokine. Expression of NKG2D, a primary activating receptor of NK cells, was reduced in NK cells treated with JSI-124. Analysis of phosphorylated STAT3 showed increased levels of activated STAT3 in NK cells stimulated with mIL-21 compared to mIL-15. Our findings indicate that STAT3 activation plays a major role in NK cell expansion and activation. Work is in progress to understand the mechanistic details of signaling downstream of STAT3.

Presenting author Email: pvphatarpekar@mdanderson.org

**IMMUNOTHERAPY APPROACHES FOR ACUTE LYMPHOBLASTIC LEUKEMIA:
CLUES FROM PRE-LEUKEMIA STUDIES**

Gregor SD Reid, Alix E Seif, Stephan A Grupp

Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, PA

While the peak age for pediatric B cell precursor (BCP) acute lymphoblastic leukemia (ALL) onset is 4 years, abnormal cells that will eventually give rise to disease can be detected in blood taken from patients at birth. Interestingly, similar abnormal cell populations can also be detected in blood taken at birth from many children who do not go on to develop ALL. Identifying the factors that influence the fate of these abnormal cells may reveal strategies for the prevention of BCP-ALL progression and recurrence. A protective effect from early-life infection has been suggested by several large epidemiological studies of ALL. Using E-RET (RFP) transgenic mice, which harbor an abnormal BCP population and develop ALL between 3 and 9 months of age, we demonstrate a significant delay in disease onset following immune modulation during the pre-leukemic period. Specifically, the administration of toll-like receptor-9 ligand, CpG ODN, reduced the size of the pre-leukemic cell population in peripheral blood of treated mice ($p=0.028$) and significantly delayed disease onset ($p<0.0001$). While no mice in the control group survived beyond 250 days of age, several CpG ODN-treated mice remained disease free at one year of age and showed no sign of an expanded BCP cell population at sacrifice. The CpG ODN-mediated delay in disease onset was dependent on the presence of IFN- γ , a result consistent with our finding that IFN- γ -deficient RFP mice develop leukemia at an earlier age than wild-type RFP mice ($p<0.0001$). Cell depletion studies suggested that T cells may be the relevant source of the IFN- γ , as pre-leukemic cell populations were significantly elevated in the spleen ($p<0.001$) and blood ($p<0.01$) of T cell-depleted mice compared to controls, and this correlated with earlier disease in the T cell-depleted group ($p<0.0007$). However, as production of IL-7 is elevated in lymphopenic settings, such as following T cell depletion, we also evaluated the influence of IL-7 on pre-leukemic cells in RFP mice. The *in vivo* blockade of IL-7R α signaling induced a rapid and sustained eradication of pre-leukemic cells (studies are currently ongoing to determine if this correlates with a delay in disease onset). Taken together, these results suggest that IFN- γ and IL-7 exert opposing influences on pre-leukemic cell fate prior to ALL development. The manipulation of these immune influences may provide a strategy to prevent disease recurrence by reducing the survival of early-occurring pre-leukemic cells and the residual post-chemotherapy clones that give rise to relapsed disease.

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Presenting author Email: reidg@email.chop.edu

GENERATION OF CYTOKINE INDUCED KILLER (CIK) CELLS FOR ADOPTIVE IMMUNOTHERAPY

Eva Rettinger¹, Selim Kui¹, Bernhard Voss¹, Ivonne Natmann³, Hermann Kreyenberg¹, Martina Anzaghe¹, Andre Willasch¹, Zyrafete Kui¹, Ulrike Koehl¹, Thomas Klingebiel¹, Simone Fulda^{2,3}, Bader Peter¹

¹University Children's Hospital of Frankfurt/Main and Department of Pediatric Hematology, Oncology and Hemostaseology, Goethe-University Frankfurt/Main, Theodor-Stern-Kai 7,60590 Frankfurt/Main, Germany; ²Institute for experimental cancer research in pediatrics, Goethe-University Frankfurt/Main, Komturstasse 3a, 60528 Frankfurt/Main, Germany; ³University Children's Hospital of Ulm and Department of Pediatric Hematology and Oncology, Eythstrasse 24,89075 Ulm, Germany

Haploidentical stem cell transplantation (HSCT) has become an important treatment modality for children and adolescents with high risk leukemia and is also more frequently used as a therapeutic tool for patients with solid malignancies like rhabdomyosarcoma (RMS) and Ewing's sarcoma. Cytokine-induced killer (CIK) cells may serve as an alternative approach to adoptive donor lymphocyte infusions (DLI) for patients with acute leukemia or solid malignancies relapsing after HSCT. We therefore investigated the feasibility of enhancing CIK cell-mediated cytotoxicity without increasing the risk for graft versus host disease (GvHD). Interleukin (IL)-2 stimulated, conventional CIK cells showed potent cytotoxicity against alveolar (RH4, RMS13), embryonal RMS (TE671, RD) and Ewing's sarcoma (RH1) cell lines. In parallel, CIK cells demonstrated excellent cytolytic capacity against T-ALL cell line MOLT-4 and subtype M4 AML cell line THP-1, but low cytotoxicity against precursor B (pB)-ALL cell line Tanoue and Bcl-2 transfected Jurkat cells, indicating that antiapoptotic Bcl-2 family proteins may contribute to resistance towards CIK cell-mediated killing. To enhance cytotoxic capacity of IL-2 stimulated CIK cells against less susceptible targets, CIK cells were activated using IL-15 or cytokine combination. IL-15 activation significantly enhanced cytolytic capacity of CIK cells whereas their alloreactive potential remained low. We could show that cytotoxicity of IL-15 activated CIK cells was mediated via activating NK receptor NKG2D. In conclusion, these findings provide first evidence that IL-15 expanded CIK cells may offer an improved pre-emptive immunotherapeutic approach for patients suffering from leukemia or solid malignancies who face impending relapse after HSCT.

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Presenting author Email: eva.rettinger@kgu.de

IN VITRO STUDIES ON THE IMPACT OF TLR2 AGONISTS ON THE CYTOTOXIC POTENTIAL OF CHEMOTHERAPY AND IMMUNE RESPONSES IN PRE-B ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Nina Rolf^{1,2}, Amina Kariminia¹, Sabine Ivison¹, Kirk R. Schultz^{1,2}

¹Child & Family Research Institute, Childhood Cancer and Blood Research Group, Vancouver, BC, Canada; ²Division of Hematology/Oncology/BMT, Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver, BC, Canada

Background: Currently, prognosis is poor for patients with relapsed pre-B-ALL. Although endosomal Toll-like receptor (TLR) 9 agonist, CpG-ODN, induces significant anti-tumor immune effects, surface-expressed TLR2 receptors are more abundant on pre-B-ALL cells, suggesting TLR2 agonists may be better immune activators. As it has been shown that stimulation with TLR agonists can increase leukemia chemosensitivity, combining TLR2 agonists and chemotherapy may offer improved outlooks for relapsed pre-B-ALL patients.

Objective: We tested the ability of TLR2 agonists (TLR2/6: Pam2CSK4, TLR2/1: Pam3CSK4 to 1) activate NFκB; 2) induce apoptosis 3) augment cell immunogenicity and 4) lower the effect concentrations of chemotherapeutics for pre-B-ALL. **Methods:** Blasts pre-treated with TLR2 agonists were examined as follows: First by flow cytometry for 1) activation of main TLR signaling mediator NFκB; 2) AnnexinV/7AAD; and 3) induction of costimulatory molecules. Secondly, cells were tested for their ability to stimulate proliferation (MLR) and for sensitivity to different chemotherapeutics.

Results: NFκB activation studies revealed Pam2CSK4 as a more rapid inducer of TLR2 signaling than Pam3CSK4. While both agonists increased blast immunogenicity to a similar extent, Pam3CSK4 induced more direct apoptosis after 6/24/48hr than Pam2CSK4 (36/66/81% vs. 14/16/17%, resp.). Pam3CSK4 pre-treatment lowered effective killing concentrations of both Doxorubicin and Asparaginase.

Conclusion: Despite more rapid kinetics of NFκB activation by Pam2CSK4, Pam3CSK4 may be a more promising agonist for TLR2-based pre-B-ALL immunotherapy. Treatment with both chemotherapy and Pam3CSK4 could increase destruction of blasts at lower chemotherapy concentrations, and may also improve the induction of lasting anti-blast immunity for the prevention of relapse.

Presenting author Email: nrolf@cw.bc.ca

TARGETING CD70 POSITIVE MALIGNANCIES WITH T CELLS EXPRESSING A CD27-ZETA CHIMERIC ANTIGEN RECEPTOR REVEALS IMPORTANT COSTIMULATORY ROLE OF CD27

Donald Shaffer, Barbara Savoldo, Zhongzhen Yi, Kevin Chow, Gianpietro Dotti, David Spencer, Shannon Kenney, Cliona Rooney, Stephen Gottschalk

Center for Cell and Gene Therapy; Texas Childrens Cancer Center; Interdepartmental Program in Translational Biology and Molecular Medicine, Baylor College of Medicine, Houston, TX

Background: Redirecting T-cells to cell surface antigens with chimeric antigen receptors (CARs) is an attractive strategy to overcome common tumor immune evasion mechanisms such as down regulation of MHC class I molecules or defects in the antigen processing machinery. While clinical studies with T-cells expressing CD20- and CD19-specific CARs in lymphoma patients have shown promising results, CD19 and CD20 are widely expressed in the hematopoietic system increasing the risk of unwanted side effects. The goal of this project is to develop a more specific approach for hematological malignancies targeting CD70, a tumor necrosis family member. CD70 is expressed on a broad spectrum of hematological malignancies, however in contrast to CD19 and CD20, its expression is restricted. Preclinical studies in animal models using monoclonal antibodies have validated CD70 as an immunotherapeutic target and the aim of this study is to generate a CD70-specific CAR for adoptive immunotherapy approaches.

Methods: A CD70-CAR was constructed by fusing the full-length CD70 receptor (CD27) to the signaling domain of the T-cell receptor zeta chain. T-cells expressing CD70-CARs were generated by retroviral transduction. Coculture assays were used to determine the secretion of IFN- γ and IL-2 in response to tumor cell lines or primary tumors that overexpress CD70. In vitro lysis of CD70+ targets was determined in cytotoxicity assays. Coimmunoprecipitation and flow cytometry were used to determine the costimulatory capacity of the CD70 receptor, and a murine SCID xenograft model was used to determine in vivo anti-tumor activity.

Results: After coculture with CD70+ cell lines or patients tumors, CD70-CAR T-cells produced significantly more IFN- γ and IL-2 than control T-cells, CD70-CAR T-cells killed non-Hodgkins lymphoma, Hodgkins lymphoma, leukemia, and myeloma cells that express CD70 at high levels. Co-IP studies revealed that the CD27 costimulatory domain of the CD70 receptor retains its ability to associate with its downstream adaptor protein TRAF2, costimulating T-cells and enhancing their viability and cytolytic function in comparison to T cells expressing CD70-CARs in which the costimulatory signaling moiety of CD27 was deleted. Murine xenograft studies demonstrated significant in vivo antitumor activity of CD70-CAR T-cells.

Conclusions: We have successfully engineered a CD70-CAR that simultaneously redirects T-cell specificity to CD70 and provides a costimulatory signal to T-cells via its CD27 endodomain. Targeting CD70 using genetically modified T-cells may be an attractive new immunotherapeutic approach for the treatment of CD70+ malignancies with minimal off target effects.

Presenting author Email: drshafer@txccc.org

ADMINISTRATION OF TUMOR-SPECIFIC CYTOTOXIC T LYMPHOCYTES (CTL) ENGINEERED TO RESIST TGF-B TO PATIENTS WITH EBV-ASSOCIATED LYMPHOMAS

Catherine Bollard¹, Gianpietro Dotti¹, Stephen Gottschalk¹, Benjamin Tzou¹, Stephanie Ku¹, Egli Liu¹, Andrea Sheehan², Martha Mims¹, Hao Liu¹, Adrian Gee¹, Malcolm Brenner¹, Helen Heslop¹, Cliona Rooney¹

¹Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas; ²Department of Pathology, Texas Children's Hospital, Houston, Texas

EBV+ Hodgkin's and some non-Hodgkin's Lymphomas in immune-competent hosts utilize immune evasion strategies to evade host T-cell responses. These include limiting expression of EBV to subimmunodominant antigens LMPI and LMP2, and production of the immunosuppressive cytokine TGF-. We first tested the hypothesis that CTL enriched for specificity to LMP antigens would have efficacy in EBV+ lymphoma patients. LMP-CTL were generated using APCs genetically modified to overexpress either LMP2, or LMP2 and inactive LMPI, of which 16 and 20 patients were recipients, respectively. Of the evaluable patients, 16 of 17 high-risk patients receiving LMP-CTL as adjuvant treatment remain in remission. 11 of 16 (78%) patients with detectable disease at the time of infusion had clinical responses (median: 1.5 years). However, 36% of patients with active disease failed CTL therapy suggesting the importance of additional tumor immune escape mechanisms. We next tested the hypothesis that infused CTL can be rendered resistant to TGF- β . LMP-CTL from 7 patients with relapsed EBV+ HL were transduced with a retroviral vector encoding a dominant-negative TGF- type H receptor (DNR). 3 patients have been treated with DNR-LMP-CTL without toxicity. DNR-CTL have been detected by Q-PCR in the peripheral blood of all patients for up to 12 months post-infusion. One patient attained a CR, one had a PR and one mixed clinical response. Hence, immunotherapy with CTL targeting LMP antigens is well-tolerated and can induce durable clinical responses. Furthermore, for patients with incomplete responses to CTL, the use of DNR-transduced CTL may be beneficial.

Presenting author Email: bxtzou@txccc.org

EFFECTIVE KILLING OF PEDIATRIC NEUROECTODERMAL TUMORS AND SARCOMAS BY T-CELLS ARMED WITH ANTI-HER2, ANTI-EGFR, OR ANTI-GD2 X CD3-BISPECIFIC ANTIBODIES

Maxim Yankelevich¹, Archana Thakur², Steven Buck¹, Yaddanapudi Ravindranath¹, Lawrence Lum²

¹Wayne State University, Children's Hospital of Michigan, Detroit, Michigan; ²Wayne State University, Barbara Ann Karmanos Cancer Institute, Detroit, Michigan.

Immunotherapy directed against tumor-specific targets, such as GD2, Her2, or EGFR can become an effective adjuvant strategy for high-risk pediatric cancers as was demonstrated in the studies with chimeric antigenic receptor-transduced T-cells. We tested another strategy of redirecting activated T-cells against tumor-associated targets using bispecific antibodies (BiAB). Herceptin (anti-Her2), Cetuximab (anti-EGFR), and 14.G2a (anti-GD2) were each heteroconjugated to anti-CD3 to produce BiAB capable of binding to activated T-cells (ATC). ATC from 2 normal subjects were armed with 50 ng of Her2Bi, EGFRBi, or GD2Bi/106 cells and tested in Cr51 release cytotoxicity assay with 10:1 or 25:1 E:T ratios. Both Her2 and EGFR-Bi-armed ATC exhibited high levels of specific cytotoxicity against medulloblastoma (Dayo), Ewing Sarcoma (HS822, HS863), and osteosarcoma (HOS, U2-OS) cell lines: the % of cytotoxicity was significantly higher compared to unarmed ATC or ATC armed with irrelevant BiAB. FACS analysis demonstrated detectable but low levels of Her2 expression in all medulloblastoma, Ewing sarcoma, and osteosarcoma cell lines. Only neuroblastoma and osteosarcoma cell lines expressed high levels of GD2. GD2-mediated cytotoxicity was more dependent on the rate of antigen expression: although Her2-negative neuroblastoma cell lines were not killed efficiently by Her2Bi-armed ATC, GD2Bi-ATC demonstrated significantly higher cytotoxicity against these neuroblastoma and GD2-high expressing osteosarcoma (U2-OS) cell lines. Our data demonstrated the capacity of Her2Bi and EGFRBi armed ATC to effectively kill medulloblastoma, Ewing sarcoma, and osteosarcoma cell lines. Therapy with antigen-specific BiAB-armed ATC may represent a promising immunotherapeutic approach for pediatric neuroectodermal tumors and sarcomas.

Presenting author Email: yankelevic@gmail.com

USE OF ACTIVATING RECEPTORS EXPRESSED BY 4-1BBL/IL-15 ACTIVATED NATURAL KILLER (NK) CELLS TO ERADICATE ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Hua Zhang¹, Kristin Baird¹, Bruce L. Levine³, Nga Voong², Alan S. Wayne¹, Carl H. June³, Crystal L. Mackall¹

¹Pediatric Oncology Branch, ²Experimental Transplantation and Immunology Branch, NCI, Bethesda, MD, 20892, ³Abramson Family Cancer Research Institute, Philadelphia, PA 19104

NK Killer cell immunoglobulin-like receptors (KIRs) and their HLA ligands play critical roles in maintaining NK cell tolerance, while providing surveillance against pathogens and malignant transformation. In previous studies, expression of KIR ligands has been presented as a mechanism of tumor immune escape from NK cell mediated killing leading to the prediction that a mismatch or absence of KIR ligands on target cells is prerequisite for effective NK cell mediated cytotoxicity. In this study, IL15R α +K562-based aAPCs expressing 4-1BBL + rhIL15 were used to activate and expand peripheral NK cells. Compared to resting NK cells, 4-1BBL/IL15-activated NK cells showed significantly enhanced cytotoxicity against a multitude of tumor targets and KIR signaling showed minimal effects on the lysis by 4-1BBL/IL15-activated NK cells. Rather, lysis was predominantly correlated with expression levels of natural cytotoxicity receptors (NCRs including NKp30, p44, p46, etc.). NCR expression varied from 0% on resting NK cells, to 15-72% on 4-1BBL/IL15-activated NK cells. We noted peak levels of expression 7 to 10 days after stimulation with 4-1BBL/IL15 aAPCs with diminished levels on Days 12 to 14. Tumor susceptibility correlated with levels of NCR ligand expression as evidenced by binding to NCR-Igs. Finally, tumor lysis could be significantly reduced by NCR-blocking fusion proteins, and lysis of ALL cells was completely eliminated when NCR-Igs were added to the cell culture. We conclude that IL15/4-1BBL activated NK cells mediate potent tumor lysis that is primarily dependent upon NCR signaling, the NCR expression levels provide a biomarker for potent KIR independent cytotoxicity, and that the development of approaches to optimize NCR expression on NK cells may enhance the effectiveness of adoptive NK cell therapies. An IND has been filed to administer IL15/4-1BBL NK cells to patients with high risk hematopoietic and solid tumors following MHC matched allogeneic stem cell transplantation.

Presenting author Email: zhanghu@mail.nih.gov

GENERATION, CHARACTERIZATION AND FUNCTIONAL ASSESSMENT OF WT1 PEPTIDE-SPECIFIC T CELLS FOR ADOPTIVE IMMUNOTHERAPY

Martina Anzaghe¹, Gerrit Weber², Miriam Stais¹, Eva Rettinger¹, Selim Kuçi¹, Günther Richter³, Petra Skrablin⁴, Björn Kloke⁵; Winfried Wels⁵, Thomas Klingebiel¹ and Peter Bader¹

¹Goethe University, Hospital for Children and Adolescents III, Division of Stem Cell Transplantation Frankfurt, Germany; ² Hematology Branch, National Heart, Lung and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, MD; ³ Hospital for Children of the Technical University München, Division of Functional Genomics and Transplantationbiology München, Germany; ⁴German Red Cross Blood Donor Service Baden-Württemberg-Hessen, Institute of Transfusion Medicine and Immune Hematology, Department of Transplantation Immunology and Immunogenetics, Frankfurt, Germany; ⁵Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus, Frankfurt, Germany

Allogeneic stem cell transplantation (SCT) has become an important treatment option for patients with leukemia and solid tumors. Nevertheless, relapse is the major reason for treatment failure. Hence, development of adoptive immunotherapy strategies in order to prevent relapse, attracted increased notice in the recent years. Tumor associated antigens (TAA) such as WT1 are highly expressed in leukemia, solid tumors and soft tissue sarcomas, providing an attractive target for anti-cancer immunotherapy. In this study we investigated feasibility of WT1 peptide-specific T cells. CD8⁺ cytotoxic T cells (CTLs) were isolated and co-cultivated with WT1 p126-loaded autologous antigen presenting cells (APC) with subsequent weekly restimulation. Analysis via pentamer stain showed donor-dependant expansion of WT1 p126-specific T cells ranging from 0.6-24% after 21 days of cultivation. Subcloned WT1 peptide specific T cells were analysed regarding their potential to lyse peptide loaded T2 cells and WT1-expressing tumor cell lines, respectively. WT1 peptide specific T cells showed specific IFN- γ production in ELISPOT assays and efficiently lysed peptide loaded T2 cells as well as breast cancer cell line MDA-MB-231. To improve clinical applicability we evaluated IFN- γ secretion assay and Streptamer technology for the isolation of WT1 peptide-specific T cells. WT1 p126-specific T cells showed specific IFN- γ production by intracellular staining and IFN- γ producing cells consisting of CD8⁺CD3⁺ T cells could be isolated by IFN- γ secretion assay from stimulated CD8⁺ T cells. Streptamer technology analysis showed that 0.5-2% WT1 p126-specific T cells could be isolated from CD8 T cells via Streptamer technology and T cells remain functional regarding IFN-g production and cytotoxic activity. Regarding donor dependant expansion capacity we tried to optimize priming conditions. Application of a mixture of WT1 peptides p37, p126, p187 and p235 increased population of IFN- γ producing cells 2 fold. Furthermore, we stimulated CTLs via in vitro generated autologous dendritic cells. For this, we isolated CD14⁺ monocytes from healthy volunteer donors and cultivated cells with GM-CSF and IL-4 to generate immature dendritic cells (iDC). On day 5 IFN- γ was added to culture medium to obtain an activated mature phenotype (mDC). Priming of CTLs with peptide-loaded mDCs resulted in activation of WT1 peptide-specific T cells. Functional evaluation and cytotoxic efficiency indicated potential clinical impact of in vitro generated WT1 peptide specific T cells after allogeneic stem cell transplantation.

Presenting author Email: Martina.Anzaghe@kgu.de