FlowTex 2017 Emerging Scientist Abstract Book

Mass Cytometry phenotyping of Pediatric Cancer Cell Lines reveals changes in NK cell ligand Expression after IFNγ Treatment which alter Tumor Lysis by NK cells

Arianexys Aquino-López*, Eugenie S. Kleinerman*, Dean A. Lee*
*Department of Pediatric Research, UT M.D. Anderson Cancer Center

Natural Killer (NK) cells are a promising therapeutic alternative for cancer due to their capacity of targeting tumor cells without prior sensitization. Our laboratory developed an NK cell expansion protocol that generates large quantities of NK cells for patient infusion. NK cells expanded with our protocol have 20 times higher levels of IFNγ secretion than fresh NK cells. A better understanding of the NK-ligands present on target cells and how they can be affected by cytokines in the microenvironment (i.e. IFNγ) is necessary to help optimize NK cells as a therapy. IFNγ has been reported to upregulate inhibitory MHC class I. On the other hand, IFNγ can promote target cell cytolysis through Intercellular Adhesion Molecule 1 (ICAM-1) upregulation. ICAM-1 binding to LFA-1 in the NK cells leads to strong adhesion required for lysis by NK cells. In this study we evaluated 22 tumor cell lines from the Pediatric Preclinical Testing Program to determine the impact of IFNγ on their expression of NK cell activating and inhibitory ligands, death receptors, and adhesion molecules using mass cytometry. We also evaluated whether exposing tumor cells to IFNγ altered their sensitivity towards NK cell mediated lysis, and whether this correlated with changes in NK-ligand expression. Our results indicate that PDL-1, ICAM-1, HLA-ABC, HLA-DR and CD95 are upregulated after IFNγ treatment across a wide variety of tumors cell lines. Also we observed that the impact of IFNγ in NK cell mediated tumor lysis was variable. For some cell lines sensitivity was not affected, while other cell lines became more sensitive or more resistant to NK cell mediated lysis. We are currently investigating the molecular changes responsible for changes in NK cell mediated tumor lysis. Our data suggests that HLA-ABC and ICAM-1 are some of the key players in determining NK cell balance and tumor lysis.

Multi-parametric flow cytometry analyses for noninvasive prospective monitoring of changes in the local T cell infiltrates in cervical cancer patients undergoing chemoradiation therapy

Stephanie Dorta-Estremera*, Guojun Yang*, Lauren E. Colbert*, Rebecca Previs*, Patricia Eifel*, Anuja Jhingran*, Lois Ramondetta*, Stephen Hahn*, James Welsh*, Amir

Jazaeri*, Michael Frumovitz*, Ann Klopp*, Jagannadha Sastry*
*University of Texas M.D. Anderson Cancer Center

Background. Chemoradiation is thought to uncover relevant tumor antigens through tumor cell death, which enhances antigen presentation, and therefore, induces anti-tumor T cell responses. However, the recurrence rates after "successful" treatment in cervical cancer patients are significantly high, ranging from 30-40%, suggesting that in some patients their immune response becomes compromised. Whether chemoradiation changes the balance between effector T cells and regulatory T cells (Tregs) at the tumor site in cervical cancer patients is not well known. We sought to prospectively monitor the immune cell changes occurring at the site of the tumor in patients with cervical cancer using the noninvasive cytobrushing and multi-parametric flow cytometry.

Methods. Patients with newly diagnosed biopsy-proven cervical cancer were enrolled on a prospective protocol. Patients were treated with standard of care cisplatin-based chemotherapy and external beam radiation therapy delivered over 5 weeks followed by brachytherapy. Cervical brushings were collected from eight patients at baseline and after 1, 3, and 5 weeks of external beam radiation. Disease status at first follow up PET/CT and clinical exam categorized seven of the eight patients with complete response (NED) and one with recurrent disease (REC). Cells collected from the cervical brushings were stained with a 16-color panel of antibodies that included markers to identify T cell and dendritic cell subsets with activation and suppressor molecules. Changes in immune cell subsets were evaluated and calculated using matched-pair analysis with Wilcoxon rank sum test.

Results. For all patients, we observed a transient decrease in the percentage of total T cells (CD3+) as well as CD4+ and CD8+ subsets of T cells at week 1 followed by variable expansion over the course of treatment. The recovery of the T cell populations seems to correlate with an increase in Ki67+ phenotype. We also observed a decreasing trend for the ratio of CD8 to Tregs over the course of treatment suggesting the induction of an immunosuppressive microenvironment. The single patient with recurrent disease showed a decline of all T cell subsets and no further increase after 5 weeks of treatment.

Conclusion. By using the noninvasive cytobrushing procedure, we are able to discern that chemoradiation induces a transient decline in tumor infiltrating T cells followed by variable but positive expansion in NED patients. A failure to expand these populations after several weeks of treatment may relate to a higher risk of recurrence in cervical cancer patients. Future analyses with a higher cohort of patients are necessary to substantiate these findings.

Flow Cytometry-Based Measurement of Förster Resonance Energy Transfer for Detection of AKT Activity and Protein-Protein Interactions

Ondrej Havranek*, Man Chun John Ma*, Zhiqiang Wang*, Justin M. Comer*, Tomasz Zal^, and R. Eric Davis*
Departments of Lymphoma/Myeloma and Immunology^, UT M.D. Anderson Cancer Center*

Introduction. In cell lines of the germinal center B-cell (GCB) subtype of diffuse large B-cell lymphoma (DLBCL), our research showed that the B-cell receptor (BCR) is exclusively

engaged in tonic, antigen-independent signaling, which is known to serve principally to activate the phosphatidylinositol-3-kinase (PI3K)/AKT pathway. However, the effect of BCR knockout (KO) on proliferation of GCB-DLBCL lines was variable, which we hypothesized was due to a corresponding variable effect on AKT activity. To investigate this, we improved the AktAR2 AKT activity reporter developed by Zhang at al., based on Förster resonance energy transfer (FRET), and developed flow cytometry based methodology for FRET efficiency (E) detection.

Methods. CRISPR/Cas9 methods were used for BCR KO and knock-in (KI) to fuse fluorescent proteins (FP) to the ends of SYK (5') and CD79A (3'). The "three-cube FRET" method was used for confocal microscopy based E calculations. E determination by flow cytometry, using minimal and maximal FRET controls, was done with R scripts written to perform calculations. A transposon vector system was used for stable expression of reporters and individual controls.

Results. Phosphorylation of AktAR2 by AKT increases proximity between donor and acceptor FPs, Cerulean3 (C3) and cpVenus (cpV), and therefore E. We first modified AktAR2 by adding the 10 N-terminal amino acids from Lyn kinase to its N-terminus, for targeting to lipid rafts. Confocal microscopy showed strong membrane-localized fluorescence in C3 and cpV channels, and reduced E in BCR KO cells; e.g., in the OCI-Ly19 cell line, E was 24.1% in unmodified (WT) cells vs. 22.3% in BCR-KO cells (p=0.04). However, the low throughput of microscopy limited the ability to detect small differences in E. Assessing thousands of cells by flow cytometry also showed higher Lyn-AktAR2 E in WT forms of various GCB-DLBCL lines than in BCR-KO cells, with higher statistical significance, and the difference correlated highly ($r^2 = 0.83$) with BCR KO-induced reductions in proliferation of GCB-DLBCL lines. These results confirmed that the BCR in GCB-DLBCL cell lines makes a variable contribution to overall AKT activity, which we also found is proportional to the BCR surface density of each line. Since the LynAktAR2 reporter has a high E (31.3-36.4%) in the unphosphorylated state, we used a long flexible linker ("EV") to further separate the donor and acceptor FPs and improve the dynamic range of the reporter. The E of the unphosphorylated LynAktAR2-EV reporter dropped to levels around 11%, improving its dynamic range: the decrease in E after BCR KO increased from 2.2% to 6.5% in the SUDHL-6 cell line. Flow cytometry with the Lyn-AktAR2-EV reporter also showed expected dose-dependent reductions in AKT activity with small-molecule inhibitors of kinases mediating tonic BCR signaling: SYK (P505-15) and PI3K p110δ isoform (idelalisib). To detect protein-protein interactions, we used KI to fuse CFP variant mTurquoise2 (mT) to BCR component CD79A (+/- Y188F mutation) and cpV to SYK, and detected an E of 0.7% in cells with WT CD79A and 0.0% in cells with the Y188F mutation (p<0001). This implies that binding of SYK is dependent on CD79A Y188 phosphorylation in tonic BCR signal transduction. Finally, heterozygous KI fusing mT and cpV to separate alleles of CD79A enabled measurement of FRET between individual BCR units; as expected, E increased within minutes after BCR crosslinking.

Conclusions. Our methodology for flow cytometry-based FRET detection allows higher throughput, detection of small differences, and flexibility for analyzing reporters (including improved AktAR2), protein-protein interaction, or dose-response and kinetic studies.

Rejuvenation of Microglia in the Aged Central Nervous System

Edward C. Koellhoffer*^, John d'Aigle*, Diego Morales-Scheihing*, Akihito Urayama*, Louise D. McCullough*^+

*Department of Neurology, University of Texas Medical School, ^Graduate School of Biomedical Sciences, +Mischer Neuroscience Institute

Microglia, the resident innate immune cells of the central nervous system (CNS), play a crucial role in maintaining homeostasis of the brain and spinal cord. However, the phenotype of microglia change drastically with age. Microglia of the aged CNS demonstrate dystrophic morphology, decreased mobility, reduced phagocytosis of debris, and a primed phenotype. Together, this phenotype results in worse response to acute neurological injury and may play a role in the pathogenesis of many age-related neurological diseases. Understanding the molecular mechanisms that underlie the aging process of microglia will allow us to identify the factors responsible for this transition in phenotype with age and offer novel targets for interventional therapies. Our lab has previously shown that two histone modifiers, Jumonji Domain Containing 3 (Jmjd3) and Enhancer of Zeste Homologue 2 (Ezh2), antagonize each other in microglia polarization. Ezh2 is associated with H3K27me3 and pro-inflammatory polarization, while Jmjd3 demethylates this mark to H3K27me1 and is essential for anti-inflammatory polarization. We hypothesized that the transition of microglia to an aged phenotype is influenced by the periphery through epigenetic mechanisms and is reversible. We show that the aged brain has a higher H3K27me3:H3K27me1 ratio compared to young mice, and pro-inflammatory polarization of primary microglia is associated with increased deposition of H3K27me3. Additionally, pharmacological inhibition of Ezh2 abrogated both H3K27me3 deposition and up-regulation of pro-inflammatory genes. To determine if peripheral factors were responsible for the epigenetic dysregulation in the aged CNS and if these changes are reversible, we utilized a model of heterochronic parabiosis. In this model a young and aged mouse are surgically attached and come to share a common blood supply. Western blot analysis of whole brain cell lysates indicated that these epigenetic perturbations observed with age are reversible, with a rejuvenation effect of the H3K27me3:H3K27me1 ratio in aged heterochronic mice (an aged mouse attached to a young mouse) compared to aged isochronic surgical controls. Furthermore, using one GFP+ mouse and one wildtype mouse in each parabiotic pair, we were able to assess chimerism of peripheral organs in this model using flow cytometry, including blood, spleen, and bone marrow. Additionally, we used flow cytometry to assess markers of pro- and anti-inflammatory phenotypes of microglia. Together, our results suggest that Ezh2 and the H3K27me3 mark are central to the pro-inflammatory polarization of microglia. This epigenetic imbalance in favor of higher H3K27me3:H3K27me1 with aging is reversible through heterochronic parabiosis, suggesting that peripheral factors are responsible for these changes observed with age. Ongoing work including cell sorting microglia for downstream sequencing experiments, proteomic analysis of soluble plasma factors, and fecal microbiome studies aim to identify the specific factors responsible for this epigenetic dysregulation of the aged CNS.

A high-throughput multi-parameter flow cytometry-based approach for analysis of CAR T-cell cytotoxic function

Tamara Laskowski*, Natalya Belousova*, Radhika Thokala*, Ivan Liadi*, Amer Najjar*, Laurence J N Cooper*

Department of Pediatric Research, UT M.D. Anderson Cancer Center

Since the emergence of first results showing the efficacy of re-directed T-cells at targeting and eliminating tumor cells, the field of Immunotherapy has gained significant momentum. In 2013, Cancer Immunotherapy was named the breakthrough of the year, and various different strategies continue to be explored, all with the common goal of harnessing the immune system's intrinsic power to destroy tumors. The primary focus of these investigations has been on T-cells. With the increasing number of genome editing tools and cell expansion regimens available, primary T-cells can be rapidly and efficiently modified, and purified populations of genetically-enhanced cells can be enriched through in vitro culture. It becomes essential, therefore, to assess each modified T-cell population for critical processes associated with their response to tumor challenge, such as activation, cytotoxic potential, and persistence. An important assessment is whether or not a given T-cell modification enhances its tumor-specific cytotoxicity. To date, the most common analysis for measuring cytotoxicity by T- and NK cells is the Chromium-51 (51Cr) release assay. Developed in 1968, this assay determines cell lysis based on the amount of radioactive chromium that is released into the supernatant by dead or dying 51Cr-preloaded tumor cells. Chromium release assays, however, are often limited to a single time point readout, and are not very informative, only reporting on percent lysis of target cells.

In this work, we report our efforts toward developing a multi-parameter flow cytometry-based assay for assessment of in vitro T-cell-mediated anti-tumor cytotoxicity. Our experimental design allowed for interrogation of both tumor cells and effector T-cells in a co-culture setting. While tumor cells were assessed for expression of apoptosis markers and loss of viability, effector cells were analyzed for activation-induced cytotoxic cytokine production. By utilizing a high-throughput flow cytometry platform, we were able, in a very short time, to simultaneously analyze and compare the activity of various different CAR T-cell populations against the same tumor target, and at different time points during the assay. Our results demonstrate the feasibility and potential of high-throughput flow cytometry as a method for a more comprehensive analysis of cytotoxic function, while also highlighting the benefit of fast and efficient parallel screening of multiple CAR T-cell populations.

Characterization of mixed Th1/Tfh T cells in chronic malaria infection using multicolor Flow cytometry and SPICE analysis

Victor H. Carpio*, Michael M. Opata*, Marelle E. Montañez, Kyle D. Wilson*, Edrous Alamer*, Pinaki P. Banerjee^, Alexander L. Dent[+], and Robin Stephens*

UT Medical Branch, ^ Center for Human Immunobiology of Texas Children's Hospital, Baylor College of Medicine, [+]University of Indiana

Malaria kills almost one million people a year. Both macrophage activation and antibody production, driven by IFN-g+ (Th1) and IL-21+ (Tfh) CD4 T cell responses respectively, are necessary to control Plasmodium infection. In P. chabaudi infection, using SPICE analysis, we found that these two functions were combined in an IFN-g+IL-21+ T cell subset that also expresses the chemokine receptors of both lineages (CXCR3+CXCR5+). Using Imaging cytometry, we could also see both Th1 and Tfh transcription factors in the nucleus (T-bet+Bcl6+). This finding suggests an explanation for the delay of germinal center formation in malaria infection. Generation of these multifunctional Th1/Tfh cells is characteristic of chronic infections including M. tuberculosis, Leishmania, and LCMV Cl13. We showed that curing P. chabaudi-infected mice early greatly reduced the generation of mixed CXCR5+IFN-g+IL-21+ effector T cells, while promoting CXCR5-IFN-g+ single producers (Th1). Strikingly, deficiency of the Tfh lineage driver Bcl6 in T cells actually promoted Th1/Tfh, increasing T-bet expression in chronic but not acute infection, suggesting that these cells are not derived from Tfh. We are currently testing various other transcription factors to determine the molecular determinants of Th1/Tfh generation. Mice with a T cell-specific deletion of Bcl6 (TKO) cannot make GC-Tfh or germinal centers. Importantly, Bcl6 TKO were able to control parasite growth very well. This suggests that the increase in Th1/Tfh cells promotes the early control of parasite, though this will have to be tested. There is increasing concordance in the field that plasticity in Th cell cytokine production can be beneficial to the host.