blood mononuclear cell samples were obtained from patients with metastatic melanoma undergoing monotherapy with ipilimumab via the Interdisciplinary Melanoma Cooperative Group at New York University Langone Medical Center. We isolated CD4 + T-cells and used a cytometric bead array assay following in vitro activation with anti-CD3, anti-CD28 antibodies to characterize cytokine expression profiles by quantifying IFN gamma, IL-2, IL-4, IL-6, IL-10, IL-17, and TNF-α at 5 time points during therapy. In total, 53 peripheral blood samples were included from 12 patients. To correlate cytokine profiles with CD4 + T-cell phenotypes in the intratumoral lymphocyte compartment, multiplex immuno-fluorescence was performed using CD4, CD8, CCR7, CD45RO, and FOXP3 antibodies on tumors before and after treatment with ipilimumab. RESULTS/ANTICIPATED RESULTS: Patients with evidence of clinical benefit (CB), as defined by having achieved partial response or stable disease, were compared with nonresponders (NR). All patients had an increase in IFN-γ, IL-2, and IL-10 secretion by CD4 + T-cells during ipilimumab therapy. NR had a statistically higher increase in all 3 cytokines. Mean IL-10 secretion was 22.3-fold higher compared with patients with CB (p value 0.0458; 95% CI = 0.6676–43.89). Mean IFN-γ secretion was 12.4-fold higher from baseline levels in NR compared with CB (p value 0.046; 95% CI = 0.3589–24.35). Mean IL-2 secretion was 6.9-fold higher in NR compared with CB (p value 0.032; 95% CI = 0.9688–12.75). There were no statistically significant differences seen in the secretion of IL-4, IL-6, IL-17, or TNF-α. Multiplex immunofluorescence for immune profiling of 20 pre and post treatment tumor biopsies is ongoing. We expect to see distinct intratumoral lymphocyte compartment changes which correlate with clinical response and the above described differential cytokine profiles. Specifically, we anticipate CB patients will have increased intratumoral effector T-cells and decreased regulatory T-cells when compared with their NR counterparts. DISCUSSION/SIGNIFI-CANCE OF IMPACT: Cytokine expression profiles of peripheral blood CD4 + T-cells have not been previously correlated with patient response in patients undergoing treatment with ipilimumab. We describe distinct secretion profiles for IFN-γ, IL-2, and IL-10 for CB Versus NR patients. NR had a statistically higher increase in IL-10, an inhibitory cytokine which typically indicates upregulation of regulatory T-cells and consequent immune escape. Increased secretion of IL-2 and IFN-γ suggests skewing towards a Th1 type, anti-tumor effector T-cell response; these cytokines increased with ipilimumab treatment in both patient groups. However, the mean increase was several fold higher in NR. Recent evidence suggests loss of the interferon gamma pathway in tumor cells confers resistance to anti-CTLA4 therapy. Chronic IFN-γ secretion is associated with an exhausted T-cell phenotype and impaired tumor rejection. Therefore, higher increases in IFN-γ secretion by CD4 + T-cells in NR suggest impaired IFN-γ dependent tumor rejection in these patients. Our findings suggest IFN-γ, IL-2, and IL-10 cytokine expression profiles can be useful as biomarkers for response to ipilimumab treatment.

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Chronic branched-chain amino acid ingestion aggravates hilar neuron loss in a rodent model of temporal lobe epilepsy

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OBJECTIVES/SPECIFIC AIMS: We previously developed a translationally relevant model of temporal lobe epilepsy (TLE) in which glutamine synthetase is irreversibility inhibited by methionine sulfoximine (MSO), resulting in spontaneous seizures and dentate hilar neuron loss. The objective of this study was to determine the effects of chronic BCAA ingestion on neuronal viability in the dentate hilus in the MSO model of TLE. METHODS/STUDY POPULATION: Sixteen rats were randomly divided into 2 groups: 8 rats drank a 4% aqueous solution of all 3 BCAAs (BCAA group) ad libitum for 31 days, and the other 8 rats drank regular water (control group) for the same period. After 10 days of drinking, a microinfusion cannula (Alzet osmotic pump, model 2004) was surgically implanted in the right dentate gyrus to continuously infuse MSO at a rate of 0.625 g/hour for 28 days. After 31 days of drinking, rats were perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in phosphate buffer. The brains were removed and fixed, sectioned on a Vibratome at 50-μm thickness, and were mounted on a gelatin-coated slides and stained with NeuN. Neuron counts in the hilar region were performed ipsilateral and contralateral to the infusion site using a stereological technique. RESULTS/ANTICIPATED RESULTS: Rats in the BCAA group had 37% fewer neurons in the ipsilateral dentate hilus than the control group ($5.8 \times 10^{-4} \pm 6.8 \times 10^{-5}$ vs. $8.9 \times 10^{-4} \pm 5.6 \times 10^{-5}$ cells, respectively, $p < 0.01$). Similarly, rats in the BCAA group had 39% fewer neurons in the contralateral dentate hilus than the control group ($5.0 \times 10^{-4} \pm 5.8 \times 10^{-5}$ vs. $7.0 \times 10^{-4} \pm 3.4 \times 10^{-5}$ cells, respectively, $p = 0.01$). DISCUSSION/SIGNIFICANCE OF IMPACT: This study demonstrates that chronic ingestion of BCAAs aggravates hilar neuronal loss in a translationally relevant rodent model of MTLE. This study gives important insight into how BCAAs may affect neuronal viability. Although the role of BCAAs on seizure activity is poorly understood, these results suggest that BCAAs may play an important role in neurochemical modulation and neurotoxicity.

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Aging-associated increases in platelet granzyme A regulate pro-inflammatory gene synthesis by monocytes

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OBJECTIVES/SPECIFIC AIMS: Platelets govern signal-dependent inflammatory responses by leukocytes. Although dysregulated inflammation is common in older adults, platelet-leukocyte signaling events and downstream inflammatory gene synthesis in aging is not known. METHODS/STUDY POPULATION: Highly-purified platelets and monocytes were isolated from healthy older (age > 60, n = 27) and younger (age < 45, n = 36) adults and incubated together in autologous and nonautologous conditions. Inflammatory gene synthesis by monocytes, basally and in the presence of activated platelets, was examined. Next-generation RNA-sequencing allowed for unbiased profiling of the platelet transcriptome in older and younger adults. Differentially expressed candidates in aged platelets were validated and recombinant granzyme A (in the presence and absence of TLR4 and Caspase-1 inhibition) identified putative ligands controlling inflammatory gene synthesis. RESULTS/ANTICIPATED RESULTS: In unstimulated or activated conditions, monocyte chemoattractant protein 1 (MCP-1) and interleukin-8 (IL-8) synthesis by monocytes alone did not differ between older and younger adults. However, in the presence of autologous activated platelets, monocytes from older adults synthesized significantly greater MCP-1 (867.150 vs. 216.36 ng/mL, $p < 0.0001$) and IL-8 (41.5 vs. 9.2 ng/mL, $p < 0.0001$) than younger adults. Nonautologous, or switch experiments, demonstrated that aged platelets were sufficient for upregulating MCP-1 and IL-8 synthesis by monocytes. Surprisingly, classic platelet proteins known to signal to monocytes and induce MCP-1 synthesis (p-selectin, RANTES, and PF4) were not increased in platelets from older adults. Using RNA-seq followed by validation via RT-PCR and immunoblot, we identified candidate platelet molecules increased in aging that mediate platelet-monocyte signaling and pro-inflammatory gene synthesis. We confirmed that granzyme A (GrmA), a serine protease not previously identified in platelets, is present in human platelets at the mRNA and protein level. GrmA is secreted by activated platelets in signal-dependent fashion. Moreover, GrmA in platelets is significantly increased in aging (~9-fold vs. younger adults). Blocking GrmA inhibited MCP-1 and IL-8 synthesis in older adults. Finally, we uncovered that platelet GrmA signaling to monocytes is regulated through TLR4 and Caspase-1. DISCUSSION/SIGNIFICANCE OF IMPACT: Human aging is associated with reprogramming of the platelet transcriptome. A previously unrecognized protein in platelets, GrmA, is increased in aging and causes increased MCP-1 and IL-8 gene synthesis by target monocytes in a TLR4 and Caspase-1 dependent mechanism. Increased platelet GrmA in aging may contribute to injurious inflammatory responses common in older adults.

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Endogenous reverse transcriptase (LINE-1) in human platelets regulates cell morphology and protein synthetic events

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OBJECTIVES/SPECIFIC AIMS: Endogenous RT (eRT) is necessary for the function of retrotransposons, elements that replicate via an RNA intermediate. One source of eRT activity is long interspersed elements (LINE). LINEs, of which there are several subgroups (L1, L2, L3), are retrotransposons that regulate cellular growth and gene expression. Given their diverse and important roles, we hypothesized that L1 elements regulate functional responses in megakaryocytes and platelets; a concept not yet examined in the field. METHODS/STUDY POPULATION: To study eRT in human platelets we used RT activity assays, PCR, and Western blot approaches. Furthermore, we used an RT-inhibitor to dissect the function of eRT, analyzed RT-dependent protein synthetic capacity, and immunoprecipitated RNA-DNA hybrids. RNA-DNA hybrids were also detected by means of ICC and automated analysis using CellProfiler software. RNA-DNA hybrids were validated by PCR and eRT

regulated synthesis of target proteins was analyzed using autoradiography and Western blot techniques. Platelets from patients with HIV+ were examined in parallel. RESULTS/ANTICIPATED RESULTS: We identified that highly purified, isolated platelets from healthy subjects possess eRT activity. eRT activity was blocked with the non-nucleoside RT inhibitor nevirapine at concentrations within the therapeutic drug range. L1 elements are bicistronic, containing 2 open reading frames (ORFs), ORF1 and ORF2. Thus, we next identified that human platelets express full-length L1 mRNA containing ORF1 and ORF2. In human platelets, eRT activity was localized to L1 protein containing ribonucleo particles. Platelet eRT reverse transcribed exogenous RNAs, a process inhibited by nevirapine, acting in trans using the 3′-UTR of exogenous mRNAs as a template. To dissect the function of eRT in platelets, we next examined cytoskeletal and protein synthetic events in the presence or absence of nevirapine. Inhibition of eRT in isolated platelets led to characteristically beaded platelets in appearance, strongly resembling bone marrow proplatelets. Parallel increases in platelet reactivity were also observed. As these changes occurred over hours, not minutes, we hypothesized that inhibition of eRT would affect platelet protein synthetic events. Consistent with this, RT inhibition resulted in upregulation of global platelet protein synthesis. We validated upregulation of the synthesis of specific proteins (mitofilin, p-selectin, and L26—a component of the 60S ribosomal subunit essential for mRNA translation). RNA-DNA hybrids, noncanonical nucleic acid structures that regulate gene expression, are enriched in regions where L1 is abundant. RNA-DNA hybrids were present in platelets and expression confirmed via differential digestion of RNAs (eg, with RNase A and RNAse I). Next-generation sequencing of pulled down (eg, immunoprecipitated) platelet RNA-DNA hybrids identified numerous differentially expressed transcripts and we focused on MAP1LC3B (LC3B), a primary regulator of autophagy. Hybrid sequencing results for LC3B were validated using qPCR and we confirmed that LC3B RNA binds to L1-encoded RNA binding protein. Platelets treated with nevirapine had increased total LC3B protein expression. As RT inhibition is an important mechanism to control HIV infection, we examined platelet morphology, activation, and LC3B expression in platelets from HIV+ subjects treated with nevirapine. HIV+ patients treated with RT inhibitors had higher numbers of platelets that were beaded in appearance at baseline, increased platelet reactivity, and differential LC3B expression compared with healthy controls. DISCUSSION/SIGNIFICANCE OF IMPACT: Taken together, these results demonstrate that platelets possess eRT activity that regulates platelet morphology, platelet hyperreactivity, and protein synthetic events. We postulate that eRT activity in platelets may be a new post-transcriptional regulatory checkpoint. Moreover, our findings have implications in HIV+ patients treated with RT inhibitors, where off-target effects may contribute to platelet activation and an increased risk of thrombosis.

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The role of estrogen receptor-β in the development of the early endometriotic lesion

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OBJECTIVES/SPECIFIC AIMS: To further elucidate the role of estrogen receptor β (ER-β) in the early endometriotic lesion attachment. METHODS/STUDY POPULATION: EECs were immortalized using a telomerase vector. Immortalized cells and parental cells were characterized by genotyping, and expression of ER-β as well as other epithelial cell markers. ER-β was knocked-down in immortalized EECs using lentivirus-mediated shRNA transduction. ER-β knockdown was confirmed by RT-qPCR and Western analysis. EEC cells with or without ER-β knockdown were used to assess their attachment to PMCs in an established in vitro assay (Lucidi, 2005). Results were analyzed with Student t-test. RESULTS/ANTICIPATED RESULTS: Genotyping using karyotype assay confirmed a normal chromosomal profile. Also positive staining for cytokeratin and lack of any staining with vimentin confirms the epithelial origin of these cells. ER-β knockdown has a significant decrease in attachment compared to control ($p = 0.02$). DISCUSSION/SIGNIFICANCE OF IMPACT: Primary and immortalized cells were 46XX, cytokeratin positive, and vimentin negative confirming their epithelial origin. ER-β knockdown has a significant decrease in attachment compared with control.

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Gut microbiome alterations in children undergoing hematopoietic stem cell transplantation

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OBJECTIVES/SPECIFIC AIMS: Aim 1: To compare microbiome diversity among patients who develop BSI post hematopoietic stem cell transplantation

(HSCT) and patients without BSI. Aim 2: To compare microbiome diversity among patients who develop moderate to severe acute GVHD post HSCT and patients without GVHD. Aim 3: To describe alterations in specific bacterial orders (Enterobacteriaceae, Clostridia, and Lactobacillales) in pediatric patients undergoing HSCT. METHODS/STUDY POPULATION: Next-generation sequencing of the hypervariable V3 region of the 16s rRNA gene isolated from stool specimens collected at baseline (start of preparative regimen to transplant day), early (day 7–14 post HSCT), and late (day 21–28 post HSCT) from 46 children was performed. Microbiome diversity was assessed by the Shannon index as well as UniFrac analysis, and compared among patients with and without GVHD/BSI. Baseline bacterial diversity was compared among patients by primary diagnosis, race, timing of antibiotic introduction and method of supplemental nutrition. RESULTS/ANTICIPATED RESULTS: Median age was 9 years (range 0.5–19.2 years). There were 36 patients with hematologic malignancies. Patients with nonmalignant disease had a characteristic pattern of microbiome diversity (and microbiota order distribution) at baseline that persisted throughout the first month of transplant ($p = 0.004$). Over all patients, there was an early and persistent drop in microbiome diversity throughout the transplant course. Early introduction of broad spectrum antibiotics (prior to transplant day) negatively impacted microbiome diversity ($p = 0.02$). There was no difference in microbiome diversity among patients who developed BSI, when compared with patients without BSI. Similarly, we did not find an association between microbiome diversity and the development of moderate to severe acute GVHD. Ongoing analysis is examining the individual variations in microbiome at the species level, and their relation to transplant characteristics and clinical outcomes. DISCUSSION/SIGNIFICANCE OF IMPACT: In our analysis, microbiome diversity decreased during HSCT, but in contrast to published data, mainly in adult HSCT populations, we found no association between gut microbiome diversity and GVHD or BSI. There are ongoing clinical trials in children and adults using probiotics in HSCT with the aim of decreasing GVHD. Further analysis of our data at the species level may further inform the relationship between gut microbiome alterations and HSCT complications in children and guide clinical interventions.

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PBX1 mRNA expression is a prognostic biomarker of and clinical indicator by RT-qPCR and RNAScope® in situ hybridization in neuroblastoma

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OBJECTIVES/SPECIFIC AIMS: (1) Correlate PBX1 mRNA expression as measured by RNAScope in situ hybridization, at an RNA number/cell measurement, Versus by RT-qPCR by the ddCt method. (2) Validate PBX1 mRNA expression in a second independent cohort of neuroblastoma tumor samples, and correlate with patient outcomes. We expect that PBX1 expression will correlate whether detected by RNAScope or by RT-qPCR. This work has the promise of validating a novel biomarker of disease severity, and for clinical translation as the RNAscope technology has been CLIA-certified for clinical use for other genes. METHODS/STUDY POPULATION: Primary neuroblastoma tumor samples were acquired through the Children's Oncology Group Tumor Bank, The Cooperative Human Tissue Network Tumor bank, and the Westmeade Tumor Bank (Westmeade, Australia), with patient outcomes annotated but sequestered until experiments are completed. RT-qPCR is performed using 1 µg total RNA isolated from each sample by Nucleospin RNA kit (Clontech), reverse transcribed by SuperScript VILO (ThermoFisher Scientific) and amplified using KiCqSTART SYBR Green qPCR mix (Sigma Aldrich). RNAScope was performed on sections of fresh frozen tumor, in triplicate, per manufacturer protocol (ACDBio) using company-designed probes. Statistical analyses performed using GraphPad Prism5. RESULTS/ANTICIPATED RESULTS: PBX1 mRNA expression as measured RNAScope correlated well with matched RT-qPCR values, with most PBX1 transcripts identified within the malignant cells and not in tumor stroma. Correlation with patient outcomes is ongoing (expected to be available at the time of presentation), but as the RNAScope values correlate with $R > 0.9$ with RT-qPCR values, we expect good correlation with outcomes in our primary data set and matching validation set. DISCUSSION/SIGNIFICANCE OF IMPACT: PBX1 mRNA expression is an accurate prognostic biomarker of outcome in low and intermediate-risk neuroblastoma, and testing on an additional validation set is planned based on thresholds established by RNAScope. RNAScope is a method readily translatable to clinical use and its inclusion in future clinical trials will be further studied. It provides an additional benefit that concomitant immunohistochemistry can also be performed. Analysis of high-risk neuroblastomas for responsiveness to retinoic acid based on PBX1 expression is planned.