function. This study aimed at a comparative analysis of CTLA-4⁺ cells between different tumor entities.

Materials and Methods To quantify CTLA-4⁺ cells, 4,582 tumor samples from 90 different tumor entities as well as 608 samples of 76 different normal tissue types were analyzed by immunohistochemistry in a tissue microarray format. Two different antibody clones (MSVA-152R and CAL49) were validated and quantified using a deep learning framework for automated exclusion of unspecific immunostaining.

Results Comparing both CTLA-4 antibodies revealed a clone dependent unspecific staining pattern in adrenal cortical adenoma (63%) for MSVA-152R and in pheochromocytoma (67%) as well as hepatocellular carcinoma (36%) for CAL49. After automated exclusion of non-specific staining reaction (3.6%), a strong correlation was observed for the densities of CTLA-4⁺ lymphocytes obtained by both antibodies ($r=0.87$; $p<0.0001$). The mean density of CTLA-4⁺ cells was 674 ± 1482 cells/mm² and ranged from 71 ± 175 cells/mm² in leiomyoma to 5916 ± 3826 cells/mm² in Hodgkin's lymphoma. Within epithelial tumors, the density of CTLA-4⁺ lymphocytes were higher in squamous cell (421 ± 467 cells/mm²) and urothelial carcinomas (419 ± 347 cells/mm²) than in adenocarcinomas (269 ± 375 cells/mm²) and renal cell neoplasms (256 ± 269 cells/mm²). A high CTLA-4⁺ cell density was linked to low pT category ($p<0.0001$), absent lymph node metastases ($p=0.0354$), and PD-L1 expression in tumor cells or inflammatory cells ($p<0.0001$ each). A high CTLA-4/CD3-ratio was linked to absent lymph node metastases ($p=0.0295$) and to PD-L1 positivity on immune cells ($p<0.0026$).

Conclusions Marked differences exist in the number of CTLA-4⁺ lymphocytes between tumors. Analyzing two independent antibodies by a deep learning framework can facilitate automated quantification of immunohistochemically analyzed target proteins such as CTLA-4.

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P02.07 CHARACTERIZATION OF THE TUMOR IMMUNE MICROENVIRONMENT OF PEDIATRIC POSTERIOR FOSSA A EPENDYMOMAS

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Background Ependymoma is the third most common brain tumor in children. At the moment, surgery and radiotherapy are the only effective treatments that can be offered, and despite this, a significant part of the patients relapse with no therapeutic salvage options. Therefore, new treatment modalities are needed. To develop immunotherapies for these children, knowledge of the tumor microenvironment is crucial. The current study aims to unravel the tumor immune microenvironment (TIME) of pediatric posterior fossa A (PFA) ependymomas.

Materials and Methods We used bulk RNA sequencing data of 22 pediatric ependymomas. We defined two groups, hereafter called PFA immune+ (PFAI+) and PFAI-, based on the RNA expression levels of the NanoString panel of Human PanCancer Immune Profiling genes. We performed gene set enrichment analysis and deconvoluted the bulk RNA samples with ependymoma-specific single-cell RNA sequencing datasets. To validate our findings on a protein level, we applied immunohistochemistry with antibodies recognizing tumor-infiltrating lymphocytes, tumor-associated macrophages and microglia.

Results Unsupervised hierarchical clustering of RNA expression of immune-related genes revealed two distinct PFA groups. Differential gene expression analysis showed that PFAI+ have a significantly higher expression of genes associated with immune functions, such as CD3E, CCR2, GZMA, CXCL9 and TRBC2. Accordingly, gene set enrichment analysis demonstrated that several immune pathways, including T-cell signalling, interferon-gamma response and TNF α signalling are enriched in PFAI+ ependymomas. RNA expression of immune checkpoints was also higher in PFAI+ tumors, indicating that these tumors might be more responsive to combinational therapies including immune checkpoint inhibitors. While immunohistochemistry showed low amounts of infiltrating CD3+, CD8+ and CD20+ cells, high numbers of CD163+ and HLA-DRA+ cells were detected. These cells were mainly located in regions of tumor necrosis. Increased amounts of CD4+ and CD8+ lymphocytes were present in PFAI+ tumors compared to PFAI- tumors. Deconvolution of the bulk RNA samples based on single-cell RNA sequencing data revealed an enrichment of myeloid cell populations, especially microglia and macrophages. Furthermore, PFAI+ tumors were found to contain significantly higher relative proportions of T-cells compared to PFAI- tumors (median of 3.76% for PFAI+ compared to 0.03% for PFAI-).

Conclusions We suggest that pediatric posterior fossa A ependymomas can be divided into two groups based on the expression of immune-related genes, in which PFAI+ ependymomas are characterized by higher RNA expression levels of these genes and greater amounts of tumor-infiltrating immune cells. Several techniques showed an enrichment of T-lymphocytes in the PFAI+ ependymomas relative to the PFAI- ependymomas.

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P02.08 THE ROLE OF FOXP3+ REGULATORY T CELLS AND IDO+ IMMUNE AND TUMOR CELLS IN MALIGNANT MELANOMA – AN IMMUNOHISTOCHEMICAL STUDY

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Background Although Malignant Cutaneous Melanoma (CM) is a highly immunogenic cancer, it can evade the immune

system by forming an immunosuppressive tumor microenvironment (TME). FoxP3+ Regulatory T cells (Tregs) and indoleamine-2,3-dioxygenase (IDO) are a part of the immunosuppressive TME in CM. In previous studies, IDO expression correlates with poor prognosis and greater Breslow's depth, but results concerning the role of FoxP3+ Tregs in CM have been controversial. Furthermore, the correlation between IDO and Tregs has not been substantially studied in CM, although IDO is known to be an important regulator of Tregs activity. To develop new therapeutic strategies, it is important to understand the role of immunosuppressive factors in CM.

Materials and Methods We investigated the associations of FoxP3+ Tregs, IDO+ tumor cells and IDO+ stromal immune cells with tumor stage, prognostic factors, and survival in CM. FoxP3 and IDO were immunohistochemically stained from 29 benign and 29 dysplastic nevi, 18 in situ -melanomas, 48 superficial and 62 deep melanomas and 67 lymph node metastases of CM. The number of FoxP3+ Tregs and IDO+ stromal immune cells was analysed quantitatively and the coverage and intensity of IDO+ tumor cells was evaluated semiquantitatively. Tumors were divided into IDO-negative and IDO-positive, containing less or more than 1% IDO+ melanoma cells of all tumor cells, respectively. P values equal to or less than 0.05 were considered statistically significant.

Results IDO+ stromal immune cells and FoxP3+ Tregs mainly accumulated in the areas with lymphocyte infiltration and thus resided mostly in the perilesional stroma. The number of FoxP3+ Tregs and IDO+ stromal immune cells were significantly higher in malignant melanomas compared with benign lesions. The increased expression of IDO in melanoma cells was associated with poor prognostic factors, such as recurrence, nodular growth pattern and increased mitotic count. Furthermore, the expression of IDO in melanoma cells was associated with reduced recurrence-free survival. We further showed that IDO-positive tumors contained significantly higher amounts of FoxP3+ Tregs and IDO+ stromal immune cells than IDO-negative tumors. However, the correlation between FoxP3+ Treg and IDO+ stromal immune cell counts was rather weak.

Conclusions Our results indicate that IDO expression is intimately involved in creating a TME conducive to tumor growth in CM. Thus, targeting IDO enzymatic pathway might be a worth of further studies in CM. Furthermore, we show that FoxP3+ Tregs appear to contribute to the immunosuppressive TME in CM, but their role may not be that critical to melanoma progression. The positive association of FoxP3+ Tregs with IDO+ melanoma cells, but not with IDO + stromal immune cells, indicates a complex interaction between IDO and Tregs in CM, which demands further studies. Support: Sigrid Juselius Foundation (S.P.-S.), Academy of Finland (S.P.-S.), The Paavo Koistinen Foundation (S.S.), Emil Aaltonen Foundation (S.S.) and North-Savo Cultural Foundation (S.S.).

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P02.09

HETEROMERIZATION OF UPA AND PAI-1 ENFORCES PRO-TUMORIGENIC NEUTROPHIL TRAFFICKING TO MALIGNANT TUMORS IN BREAST CANCER V/A VLDLR-DEPENDENT β 2 INTEGRIN CLUSTERING

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Background High tumor levels of urokinase-type plasminogen activator (uPA)-plasminogen activator inhibitor-1 (PAI-1) heteromers independently predict poor survival in early breast cancer. The pathogenetic role of this protein complex, however, remains largely obscure.

Material and Methods Neutrophil trafficking was analyzed in orthotopic (multi-channel flow cytometry) and heterotopic (ear; multi-channel *in vivo* microscopy) mouse models of 4T1 breast cancer, in a mouse peritonitis assay (multi-channel flow cytometry), as well as in the mouse cremaster muscle (multi-channel *in vivo* microscopy). Cytokine expression in tumors was determined by multiplex ELISA. Phenotypic and functional properties of primary mouse neutrophils, microvascular endothelial cells (cell line bEnd.3), macrophages (cell line RAW 264.7), and breast cancer cells (cell line 4T1) were characterized in different *in vitro* assays. uPA/PAI-1 expression and neutrophil infiltration in human breast cancer samples were assessed by RNA sequencing, immunohistochemistry, and ELISA.

Results and Discussion Here, we demonstrate that uPA-PAI-1 heteromerization multiplies the potential of the single proteins to attract pro-tumorigenic neutrophils. To this end, tumor-released uPA-PAI-1 activates peritumoral macrophages (VLDL receptor- and ERK/MAPK-pathway). This promotes neutrophil trafficking to cancerous lesions (enhanced β 2 integrin activation and clustering) and primes these immune cells towards a pro-tumorigenic phenotype (elevated neutrophil elastase expression), thus supporting tumor growth and metastasis. Blockade of uPA-PAI-1 heteromerization by a novel inhibitor effectively interfered with these events and prevented tumor progression.

Conclusions Here, we identified an already therapeutically targetable interplay between hemostasis and innate immunity that drives advanced stages of breast cancer as well as characterized the underlying mechanisms of this process. As a personalized immunotherapeutic strategy, blockade of uPA-PAI-1 heteromerization might be particularly beneficial for patients with highly aggressive uPA-PAI-1^{high} tumors. *This study was*