HEPATOBILIARY AND GENERAL SURGERY DEPARTMENT

PROJECT 1

Project title

"Development of a new diagnostic tool based on tumor-associated macrophages to refine the prognosis of patients undergoing hepatic resection for colorectal liver metastases: a prospective study"

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Abstract

Colorectal cancer is a major cause of mortality worldwide. Most patients develop colorectal liver metastases (CLM), and for such patients hepatectomy combined with chemotherapy may be curative. Nevertheless, in the era of precision medicine there is a critical need of prognostic markers to cope with the heterogeneity of CLM patients. Tumor-associated macrophages (TAMs) pave the way to tissue invasion and intravasation providing a nurturing microenvironment for metastases. The quantification of immune landscape of tumors has provided novel prognostic indicators of cancer progression, and the quantification of TAMs might explain the heterogeneity of CLM patients. Here, we will investigate the development of a new diagnostic tool based on TAMs with the aim to define the causative role of TAMs in CLM patients. This will open new clinical scenarios both for the diagnosis, therapy and prognosis, leading to the refinement of the therapeutic output in a personalized medicine perspective.

Main technical approaches

Methods and statistical analyses Liver perfusion and cell isolation by collagenase perfusion, minimizing the amount of leukocytes deriving from the peripheral blood. Multi-parametric flow cytometry: analysis and gating strategy will be performed. The acquisition will be dome on an LSR Fortessa analyser. Diva software v.6.2 will be used for data acquisition and analysis. RNA Sequencing: The cells will be isolated using a FACSAria III cell sorter. RNA-seq on bulk TAM populations will be performed using the Illumina NextSeq500 sequencer. The scRNA-seq libraries will be prepared using the Chromium Cell Solution platform by 10X GENOMICS. The recovered cells will then be sequenced with the Illumina NextSeq500 sequencer. We expect to recover approximately 50,000 raw reads per cell, covering 1000-1500 genes per cell. Statistical analysis and visualization of scRNA-seq data will be performed using the established pipelines Cell Ranger and Loupe Cell Browser by 10X GENOMICS. Digital pathology on FFPE specimens: multispectral fluorescent imaging on FFPE sections will

be performed using the Opal Multiplex technology by PerkinElmer and analyzed using segmentation algorithms (Matlab).
Metabolomics: MS-based untargeted and targeted metabolomics approaches (LC- or- FIA-MS/MS; multiple reaction monitoring MRM-MS) will be applied to capture metabolic state of the different TAM subtypes and analyzed by MetaboAnalyst 3.0. Molecular interaction networks will allow for the mapping of metabolites and genes/protein onto networks of interaction. Mass spectrometry based single reaction monitoring method (SRM-MS) will be used to simultaneously measure the abundance of the proteins involved in the regulation of the identified metabolic networks in each TAMs population. Thanks to the design of specific peptides for each protein included in the screening (selected by both in-house generated spectral library and PeptideAtlas database), we will be able to quantify the proteins of interest from the whole proteome digest of TAMs population.
Statistics: Statistical analyses will be carried out to find correlations among immune and clinical, pathological, surgical and follow-up data. Data will be analyzed using one-way ANOVA with Bonferroni's post-hoc tests, or Student t test, according to distribution of variables. Analyses will be carried out using the software IBM-SPSS and GraphPad Prism6.

Scientific references

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PROJECT 2

Project title

"At the borders between immune tolerance and autoimmunity: disclosing the mechanisms boosting the expansion of auto-reactive Natural Killer cells in Primary Biliary Cholangitis"

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Abstract

We recently described a unique subset of liver-resident Natural Killer (lr-NK) cells located within the hepatic sinusoids where they naturally interact with Kuppfer cells to provide an optimal immune-surveillance while keeping a certain threshold of immune tolerance. Our preliminary data showed that a significant fraction of this lr-NK cells become autoreactive and pathogenic relevant for killing autologous Biliary Epithelial Cells (BEC) in patients affected by primary biliary cholangitis (PBC), observing also that the decreased expression of TIR8 correlates with the expansion of auto-reactive NK cells.

This research proposal aims to characterize auto-reactive H-NK cells from end-stage livers of patients affected by PBC. By implementing the induced pluripotent stem cells (iPSCs) technology, we will also assess if lr-NK cells give rise to "memory-like" or auto-reactive H-NK cells when encountering auto-antigens presented by iPSC-derived autologous macrophages.

Main technical approaches

Aim 1: We will determine with a 18-color flow cytometry approach the frequencies of auto-reactive and tolerogenic lr-NK cells and c-NK compared to their circulating counterparts. To this end we will measure: i) the lysis of autologous BEC (CD107a degranulation assay); ii) the surface expression if KIRs mismatched with HLA-I. PBC patients undergoing liver transplant and donors of healthy liver wedges will undergo a full 4 digit HLA typing to determine the KIR-MHC-I mismatch. We will also analyze their activation, tissue residency and proliferation markers (HLA-DR, CD69 and Ki-67), repertoire of aNKRS and iNKRs regulating NK cell recognition of "self, intracellular levels of IFN-□, memory-like phenotype (CD57, NKG2C, KIRs) and differentiation states (Figure 1). For auto-reactive NK cell, we will also investigate the aNKRs involved in cell killing of autologous BEC and the expression of TIR8 as a possible checkpoint dysregulated in PBC.

Aim 2: H-NK and PB-NK cell precursors will be FACS-Sorted at single cell level and analyzed for gene expression by using the Fluidigm Biomark technology that allows the simultaneous quantification of 96 different transcripts by real time PCR in customized plates. We will determine transcript levels of transcription factors involved in NK cell ontogenesis (i.e. EOMES, ID2, IRF2, GATA 3, STAT-4, CD117, CRTH2, etc), of cytokines known to induce NK cell differentiation, activation and proliferation (IL-2, IL-7, IL-12, IL-18, IL-21), of markers that differentiate Innate Lymphoid Cells from NK cell precursors (i.e CD127, ROR□□, CRTH2, IL-22, IL-17, AhR, T-bet). Considering our preliminary data on the frequency of H-NK cell

precursors within LMNCs (0.1% - 0.6% for stages 1-3; 30-60% for stages 4-5) and PBMCs (0.1 %- 0.3 % for stages 1-3; 0.5 - 6 % for stage 4; 10-25% for stage 5), we expect to FACS-sort and analyze a minimal number of 1,000 cells. Normalization to a house-keeping gene is not required when performing single cell transcriptional profiling. At least 20 single cells will be sorted for each population and principal component and cluster analysis will be used to analyze the gene expression profile and identify trends in the data as a whole.
Aim 3: A small skin biopsy will be obtained from liver donors in order to generate iPSC-derived resting macrophages (M0) (Figure 2). We will FACS-sort lr-NK cells from liver perfusate resembling LMNCs and freeze two vials of at least 3 X 106 CD56bright lr-NK cells to be frozen in 2 vials. iPSC-derived M0 will be pulsed for 16 hours with PDC-E2 (PDC-E2 MO) and we will then assess their polarization status (M1 or M2). The 1st vial of “naïve” lr-NK cells will be thawed and co-cultured with PDC-E2 MO and IL-2 (200 IU/ml). We will then harvest NK cells at day 1, 3 and 7 and analyze their tissue residency markers (HLA-DR, CD69), proliferation rates (Ki67 and CFSE dilution), CD57pos/NKG2Cpos/KIRpos “memory-like” phenotype, intracellular IFN-□□□levels and cytolytic potential against autologous BEC (CD107a degranulation assay). We will also determine if "memory-like" phenotype can be retained in vitro by FACS-sorting proliferating lr-NK cells cultured with PDC-E2 MO and incubating them aone with either low (20 IU/ml) or high (200UI/ml) dose (200UI/ml) of IL-2 for 3 weeks. The 2nd vial of “naïve” NK cells will be then thawed and challenged with PDC-E2 MO. We will compare the phenotype, proliferation rate and effector functions (as detailed above) of the 2nd newly induced “memory-like” NK cells with those of the 1st set of lr-NK cells previously incubated with PDC-E2 MO and kept in cultures. The same experimental approach will be performed also with circulating CD56bright NK cells from the same donors to assess if the generation of “memory-like” NK cells is tissue specific.
Both parametric and non-parametric statistical analyses will be performed with Prism Software.

Scientific references

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