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L1CAM promotes enrichment of immunosuppressive T cells in human pancreatic cancer correlating with malignant progression

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ABSTRACT

Regulatory T cell (T-reg) enrichment in the tumor microenvironment is regarded as an important mechanism of tumor immune escape. Hence, the presence of T-regs in highly malignant pancreatic ductal adenocarcinoma (PDAC) is correlated with short survival. Likewise, the adhesion molecule L1CAM is upregulated during PDAC progression in the pancreatic ductal epithelium also being associated with poor prognosis. To investigate whether L1CAM contributes to enrichment of T-regs in PDAC, human $CD4^+CD25^+CD127^-CD49d^-$ T-regs and $CD4^+CD25^-$ T-effector cells (T-effs) were isolated by magnetic bead separation from blood of healthy donors. Their phenotype and functional behavior were analyzed in dependence on human premalignant (H6c7) or malignant (Panc1) pancreatic ductal epithelial cells, either exhibiting or lacking L1CAM expression. T cells derived from blood and tumors of PDAC patients were analyzed by flow cytometry and findings were correlated with

Abbreviations: CP, chronic pancreatitis; PBMC, Peripheral blood mononuclear cells; PDAC, pancreatic ductal adenocarcinoma; T-effs, $CD4^+$ effector T cells; T-effs[a], autologous T-effs; TGF-β1, Transforming-growth factor-beta 1; T-regs, regulatory T cells.

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clinical parameters. Predominantly T-regs but not T-effs showed an increased migration on L1CAM expressing H6c7 and Panc1 cells. Whereas proliferation of T-regs did not change in the presence of L1CAM, T-effs proliferated less, exhibited a decreased CD25 expression and an increased expression of CD69. Moreover, these T-effs exhibited a regulatory phenotype as they inhibited proliferation of autologous T cells. Accordingly, $CD4^+CD25^-CD69^+$ T cells were highly abundant in PDAC tissues compared to blood being associated with nodal invasion and higher grading in PDAC patients. Overall, these data point to an important role of L1CAM in the enrichment of immunosuppressive T cells in particular of a $CD4^+CD25^-CD69^+$-phenotype in PDAC providing a novel mechanism of tumor immune escape which contributes to tumor progression.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) belongs to the most malignant tumors with an overall 5-year-survival rate of ~2% (Siegel et al., 2013). Since the tumor is mostly diagnosed in an advanced stage, curative therapeutic options are limited to less than 20% of the patients. Moreover, PDAC exerts a profound radio- and chemotherapy resistance worsening therapeutic responses and prognosis of these patients (Schneider et al., 2005). Expression of the adhesion molecule L1CAM (CD171) increases during PDAC progression in the ductal epithelium (Sebens Müerköster et al., 2007; Geismann et al., 2009; Bergmann et al., 2010; Ben et al., 2010) being associated with poor prognosis of PDAC patients (Ben et al., 2010). Accordingly, L1CAM induces tumorigenicity of premalignant pancreatic ductal epithelial (H6c7) cells as well as migration, apoptosis resistance and metastasis of H6c7 and PDAC cells *in vitro* and *in vivo* (Sebens Müerköster et al., 2007; Geismann et al., 2009; Schäfer et al., 2012). L1CAM expression is induced by myofibroblasts (Geismann et al., 2009) being part of the pronounced desmoplastic reaction in chronic pancreatitis (CP) and PDAC (Kleeff et al., 2007). Besides myofibroblasts, the PDAC stroma is largely comprised of extracellular matrix proteins and immune cells, e.g. T cells (Kleeff et al., 2007). Given an immunosuppressive phenotype of the majority of tumor associated immune cells, their presence is regarded as an immune escape mechanism of the tumor. Additionally, immune cells might foster tumorigenesis by other mechanisms, e.g. by promoting angiogenesis, tumor cell migration and metastasis (Kleeff et al., 2007; Zou, 2005).

Accordingly, elevated levels of regulatory T cells (T-regs) have been identified in blood and tumors of PDAC patients being associated with poor prognosis (Liyanage et al., 2002; Hiroaka et al., 2006; Ikemoto et al., 2006). Similar to L1CAM, T-regs have been already detected in tissues of CP which represents a high-risk factor for PDAC (Hiroaka et al., 2006; Schmitz-Winnenthal et al., 2010). Accumulation of T-regs in tumors can be mediated e.g. by CCL5 or CXCL12 released by tumor or stromal cells (Zou et al., 2004; Tan et al., 2009), an altered addressin-expression on tumoral endothelial cells (Nummer et al., 2007) or the conversion of conventional T cells into T-regs through transforming growth factor-beta 1 (TGF-β1) (Moo-Young et al., 2009) overexpressed in CP and PDAC tissues, too (Farrow et al., 2002; Yen et al., 2002).

The T-reg's ability to suppress $CD4^+$ T effector cells (T-effs) is essential for the maintenance of peripheral tolerance, but also represents one major strategy of tumor immune evasion (Zou, 2005; Liyanage et al., 2002). T-regs are characterized by the constitutive expression of CD25 and the transcription factor forkhead FoxP3 (FoxP3) which are both widely used for the detection of T-regs (Liyanage et al., 2002; Hiroaka et al., 2006; Ikemoto et al., 2006). However, both markers are transiently expressed by activated T-effs, too, so that it is very likely that detection of $CD4^+CD25^+$ or $CD4^+Foxp3^+$ T cells does not exclusively mark T-regs. Consequently, functional analysis of T-regs might be impaired by contaminating T-effs and targeting of T-regs (e.g. by CD25-antibodies). Recently, other markers have been introduced more suitable for a better discrimination of T-effs and T-regs on the one hand and the isolation of untouched cells for functional analyses on the other hand. In detail, Kleinwietfeld et al. demonstrated that highly immunosuppressive T-regs completely lack expression of CD49d, the α-chain of the integrin VLA-4, and CD127 which is the α-chain of the IL-7 receptor (Kleinewietfeld et al., 2009). Thus, by removing $CD49d^+CD127^+$ cells from the pool of $CD4^+$ T cells $Foxp3^+$ T-regs are obtained free of contaminating, possibly activated $CD25^+$ T-effs and bound antibodies which might affect T cell function (Kleinewietfeld et al., 2009). Moreover, some studies in mice have described a novel subpopulation of T-regs with a $CD4^+CD25^-CD69^+$ phenotype lacking FoxP3 expression but exhibiting elevated secretion of IL-10 and TGF-β1 and clearly inhibiting proliferation of T-effs (Han et al., 2009; Sancho et al., 2005).

This study therefore aimed at improving the characterization of human T-regs and T-effs i) *ex vivo* in blood and pancreatic tissues of CP or PDAC patients, and ii) *in vitro* regarding the role of L1CAM based on an untouched isolation procedure for both T-cell populations and a direct coculture system using benign and malignant pancreatic ductal epithelial cells. Thereby, this study essentially contributes to a better understanding of how immunosuppressive T cells accumulate during initiation and progression of PDAC.

2. Material & methods

2.1. Cell lines and cell culture

H6c7 human pancreatic ductal epithelial cells (obtained from Prof. M.S. Tsao, Ontario Cancer Center, Toronto, Canada) lacking L1CAM expression and thereby resembling the conditions

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