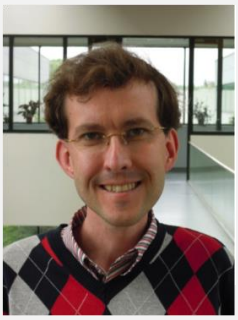
ImmunoTools *special* Award 2018



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Analysis of immunogenicity of cancer cells undergoing necroptosis *in vivo*

Introduction

In mammals, several forms of cell death can occur, including apoptosis and regulated necrosis. **In classical terms, cell death by apoptosis has been considered to be immunologically 'silent' or even tolerogenic**. In this regard it has been shown that an apoptotic process can dampen the immune response by caspase-mediated proteolytic cleavage and inactivation of "alarmin" cytokines such as IL1α and IL33 and the exposure of phosphatidylserine. However, the view of the immunological impact of apoptotic cell death has been considerably changed in the last years because apoptosis (at least in certain conditions) can induce significant immunological responses. These studies have **lead to emergence of the 'immunogenic cell death concept'**. Indeed, we and the others have shown that immunogenic apoptosis is characterized by the release of damage-associated molecular patterns (DAMPs), such as cell-surface exposure of calreticulin, secretion of ATP, and release of the chromatin-binding protein high-mobility group B1 (HMGB1), each of which interact with the phagocytic or scavenger receptors, e.g., LRP1 (calreticulin), purinergic receptors (ATP), and pattern-recognition receptors, such as TLR4 (HMGB1), respectively.

Besides apoptosis, **novel highly controlled cell death pathways are emerging**, which are gathered under an umbrella term **regulated necrosis**. Necroptosis is one of the forms of regulated necrosis, which has been recently described. **Necroptosis** is initiated when caspase-8 is inhibited or FADD or $FLIP_L$ are absent and it is transduced by the kinase activities of receptor interacting protein kinase-1 (RIPK1) and RIPK-3, eventually leading to the activation of mixed lineage kinase domain-like (MLKL) and plasma membrane permeabilization. It has been reported by several laboratories (including ours) that necroptotic cancerous cells can be perceived as a pro-inflammatory and immunogenic mode of cell death. Although this work highlights the relevance of immunostimulatory role of necroptosis, the immunogenic properties of necroptosis still needed to be fully elucidated.

It is now known that differences in the cellular microenvironment in cell culture models can cause deviations in cell response and behaviour. In this regard while 2D cell cultures (i.e. growing cells on flat surfaces) cannot reflect adequately the

physiological complexity of real tissue, **3D cell culture models are more accurate in representing the natural environment experienced by cells in the living organism**. In a 3D environment, cells behave and respond more like they would *in vivo* to internal and external stimuli. Therefore, we propose to evaluate the innate immune responses towards **cancerous cells undergoing necroptosis in a bioprinted 3D microenvironment *in vivo*.**

Project set up

In this work by using Tet-On inducible systems of cell death, we will analyze whether cancer cells undergoing necroptosis can induce attraction of the immune cells in vivo by using 3D bioprinted microenvironment.
In the project we will subcutaneously implant in mice 3D bio-printed constructs (developed and characterized in our lab) with pre-loaded cancerous cells caring Tet-On inducible cell death systems (CT26). The inflammatory reaction will be characterized by isolation of the constructs at different time points and we will identify the cells that will be attracted or become differentiated. The quality and quantity of the recruited host cells will be compared between the blank (without dying cells) and 3D bioprinted constructs loaded with necroptotic cells. The presence of distinct subsets of immune cells will be determined and a comparative analysis of the infiltration of the immune cells will be performed. Different subsets of DCs will be assessed looking at the markers CD11c, CD11b-FITC, MHCII, CD103, CD80-PE, CD86, CD40, CD8-APC; macrophages by using the following makers CD11b-FITC, F4/80, CD169, NK cells (NK-PE) and neutrophils by using CD11b FITC and Gr-1-APC. Also, the presence of $CD4^+$ and $CD8^+$ cells will be tested. Furthermore, lavage fluid will be screened for the presence of mouse IL-6 and TNFα using the ELISA kits. The cell death will be controlled by Annexin-V-FITC and Sytox Blue staining.

These experiments will give us a first clue if necroptotic cancerous cells are able to induce attraction of immune cells *in vivo* and thus if they are immunogenic.

For identification of attracted immune cells we will do the flow cytometry analysis by using the following **ImmunoTools** Abs. Of note, that the missing antibodies for example like CD11c, MHC-II and CD103 and others will be obtained from another suppliers.

ImmunoTools *special* AWARD for **Dmitri V. Krysko** includes 18 reagents

APC – conjugated anti-mouse CD4, CD8, CD45, Gr-1

FITC - conjugated anti-mouse CD3e, CD11b, Annexin-V

PE - conjugated anti-mouse CD80, NK-cells

PerCP - conjugated anti-human CD45

mouse ELISA-set (for one 96 plate): mouse IL-6, mouse TNF-alpha

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