OTKA 114460 project closing report

The overarching goal of our studies funded by the current grant was to study the biology of melanoma-associated fibroblasts (MAFs). Our main focus was to describe interactions between MAFs and select immune cells, known to play a pivotal role in melanoma immunology.

**MAFs harbor stem cell properties similar to mesenchymal stem cells (MSCs)**

1. First we have shown that MAFs are rather similar to (MSCs) a cell type known to have potent immunoregulatory properties both in vitro and in vivo. MAFs, just like MSCs, were able to differentiate into osteogenic and adipogenic mesodermal lineages upon exposure to well-defined differentiation cocktails. Moreover, characteristic MSC cell surface markers like CD44, CD73, CD90 and CD105 were also detected on MAFs, while hematopoietic and endothelial markers such as CD45 and CD31 were absent.

**MAF interactions with CD8 cytotoxic T cells**

2. Our project has provided evidence that soluble factors released from MAFs- in contrast to dermal fibroblasts or DFs) impair various aspects of cytotoxic T cell function. We have shown that MAFs downregulate the expression of CD69 and granzyme B production in CD8+ T cells and inhibit their ability to eliminate target cells in a redirected in vitro killing assay. In addition, we also showed an increased expression of select immune checkpoint regulators (TIGIT and BTLA) in non-naïve/memory CD45RO+ T cells.

3. We found that MAFs exhibit a skewed spectrum of immune checkpoint regulators. The expression of cancer associated fibroblast-immune checkpoint receptor (CAF-ICR) ligands and the possible role of these molecules in CAF-mediated immunomodulation have been controversial. To analyze the question whether MAF could affect T cell ICR signaling, cell surface expression of several ICR ligands was compared between MAFs and DFs. We tested the presence and expression of Herpesvirus entry mediator (HVEM), Galectin-3, Galectin-9, Programmed death-ligand 1 (PD-L1), CD155 (poliovirus receptor) and V-domain Ig suppressor of T cell activation (VISTA). We found that compared to DFs, MAFs displayed increased amounts of the negative CTL regulators VISTA and HVEM.

4. Using molecular inhibitors of arginase, we showed that the TIGIT/BTLA increase in T cells is mediated by MAF-secreted arginase. To corroborate these findings, we transfected MAFs with a mammalian expression vector encoding the full-length human arginase 2 under the control of a CMV promoter and examined its effect on both BTLA and TIGIT. We found that forced expression of arginase in MAFs further increased BTLA and TIGIT on CD8+T cells. Taken together, these data indicate that MAF-mediated enhancement of BTLA and TIGIT expression on CD8+T cells is an L-arginase-dependent phenomenon.

5. Next we demonstrated that MAFs interfere with T cell signaling. Considering that impaired T cell function may be caused by lack of proper co-stimulation or other forms of aberrant signal transduction, we next analyzed if MAF supernatants influenced T cell signaling, required for full T cell activation and differentiation. We interrogated early (CD3ζ-chain activation, Tyr142-P), and downstream of T cell signaling events (NF-κB activation, Ser536-P) as well as a key transcription factor associated with late CD8+T cell differentiation (Runt-related transcription factor 3 or Runx3). MAFs suppressed NF-κB phosphorylation, while CD3ζ and Runx3 remained unaffected. Based on this observation, we next utilized phospho-kinase protein arrays to gain further insight into aberrant T cell signaling downstream of TCR. Although the majority of TCR-related kinase pathways remained functional, suppression of NF-κB activation was accompanied by increased ERK1/2 phosphorylation in MAF-exposed CD8+T cells. Taken together, these data suggest that there is an imbalance between aCD3-mediated TCR signaling and aCD28-provided co-stimulation in MAF CM-exposed T cells.

**MAF interactions with monocytes/macrophages**

6. Using double immunohistochemistry with macrophage and MAF specific antibodies (ionized calcium binding adapter protein 1 or Iba-1 and fibroblast activation protein or FAP respectively), we found macrophages in close proximity to MAFs in vivo in human metastatic melanoma samples.

7. Utilizing coculture systems between a monocyte/macrophage cell line (THP-1) or M1 and M2 differentiated primary macrophages derived from peripheral blood monocytes and MAFs, we have shown that MAFs induce an immunosuppressive phenotype in macrophages. This is highlighted by an increased secretion of one of the most potent known immunosuppressive cytokines: IL-10. This IL-10 increasing effect of MAFs on macrophages is time and dose dependent and can be further augmented by exposing MAFs to a melanoma supernatants (SK-MEL-28 and MALME-3 cell lines as well as primer melanoma cells), especially when melanoma cells were pretreated with chemotherapeutic agents such as dacarbazine, vemurafenib, dabrafenib or trametinib.

8. Using various molecular pathway inhibitors we found that the cyclooxygenase pathway as well as the enzyme indoleamine 2,3 dioxygenase played a critical role in the MAF-mediated IL-10 increase seen in macrophages.

9. Taking advantage of our clinical database we examined if there was any clinical correlation between the IL-10 inducing effect of MAFs and certain clinical parameters in melanoma patients. Preliminary data indicated that primary and metastatic MAFs from thicker melanomas (Breslow depth $>2$ mm) caused significantly more IL-10 output in macrophages as compared to their thinner counterparts (Breslow depth $< 2$ mm).

**MAF interactions with gamma-delta T cells**

10. It is well-known that gamma-delta T cells are able to kill tumor cells, but their interactions with tumor-associated fibroblasts is unexplored. From a clinical point of view, we found it interesting to investigate whether gamma-delta T cells are capable of killing MAFs, especially when activated. We enriched gamma-delta T cells from peripheral blood mononuclear cell fraction of healthy volunteers utilizing zoledronic acid/IL-2 stimulation, then cocultured these cells with MAFs.  We found that in this co-culture system, gamma-delta T cells were able to induce apoptosis in MAFs in the presence continued zolendronic acid stimulation.

11. The gamma-delta TCR target molecules (butyrophilin 3A1) BTN3A1 and BTN2A1 were expressed in MAFs (similarly to the melanoma tumor cell line-SK-MEL-28 control) and their levels seemed to be MAF donor specific. We are currently investigating the detailed mechanisms of the cytotoxic effect of gamma-delta T cells exerted on MAFs.