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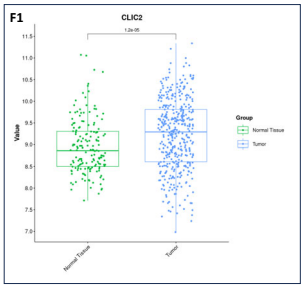
CLIC2 INFLUENCES MYELOID LINEAGE FATE MODULATING JAK/STAT SIGNALING

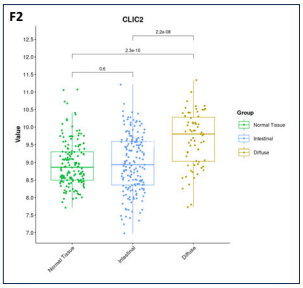
Francesco Albano[1,2,3], Pellegrino Mazzone[3,4], Giuseppina Di Paola[3,4], Giuseppe Cesta[1], Margherita Luongo[1], Claudia Sabato[1], Giovanni Calice[1], Sabino Russi[1], Simona Laurino[1], Giuseppe Patitucci[1], Chiara Balzamo[2], Valentina Pagliara[5], Giuseppina Amodio[5] and Geppino Falco[1,2,3]

[1] *Laboratory of Preclinical and Translational Research, IRCCS CROB Centro di Riferimento Oncologico della Basilicata, Rionero in Vulture (PZ), Italy*
[2] *Department of Biology, University of Naples Federico II, Naples (NA), Italy*
[3] *Biogem S.c.a.r.l., Istituto di Ricerche Genetiche "Gaetano Salvatore," Ariano Irpino (AV), Italy*
[4] *CaWUR s.r.l., organoids factory, Ariano Irpino (AV), Italy*
[5] *Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno, Baronissi (SA), Italy*

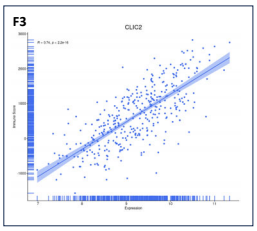
Gastric Cancer(GC) is a highly heterogeneous malignancy that could be divided in two great histotypes, intestinal (iGC) and diffuse GC (dGC). The latter is usually characterized by tumor cells that often spread out from the body of the stomach to the stroma; cancer cells are non-cohesive and often with a signet-ring appearance. Furthermore, dGC is associated with younger age patients, more aggressive progression, and low immunogenicity.

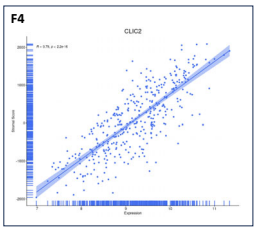
We screened The Cancer Genome Atlas (TCGA) for *CLIC2* gene expression in Stomach Adenocarcinoma (STAD) dataset. We found *CLIC2* to be upregulated in GC tumor samples (**F1**), with enhanced expression in dGC patients respect to iGC patients and healthy controls (**F2**).

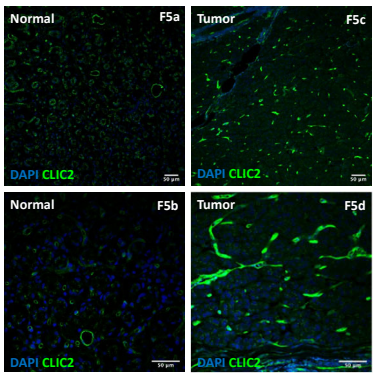




Furthermore, when we applied *Estimate* algorithm to TCGA STAD data, we found that *CLIC2* expression was strongly related to immune (**F3**) and stromal (**F4**) enrichment scores, but not to epithelial cells, suggesting that *CLIC2* was could not be directly expressed by the tumor itself, but by another cell type composing the tumor microenvironment (TME).

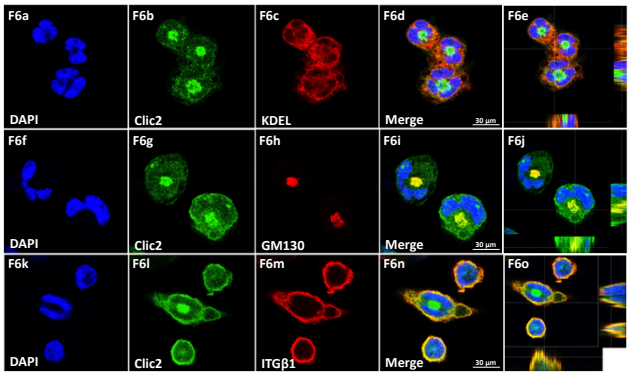


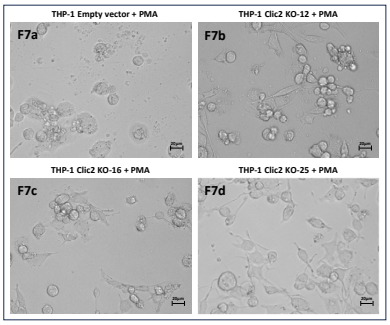




Clic2 IF performed on Normal (**F5a-b**) and GC samples (**F5c-d**), confirmed its high expression in GC biopsy compared to normal counterparts, and we could also appreciate as Clic2 signal could be retrieved in the *lamina propria*, just below the epithelial layer. Tissue Protein Atlas single cell next generation sequencing indicates *CLIC2* association with *Macrophages - immune response* cluster. Therefore, we decided to use THP-1 monocytes as in vitro cellular models as they differentiate when treated with PMA. We induced macrophages (MΦ) using low concentration of PMA, to not further polarize them to M1- or M2-like subsets.

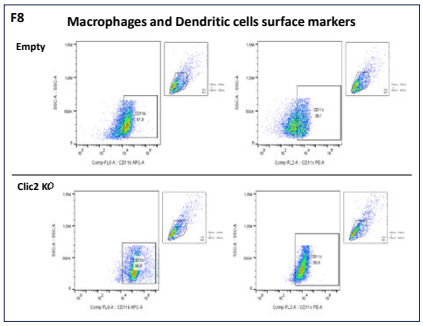
Clic2 IF on MΦ macrophages showed as its intracellular localization was primary associated with Golgi (**F6j**) and plasma membrane (**F6o**), but also with endoplasmic reticulum (**F6e**), therefore suggesting Clic2 to participate to the secretory pathway.

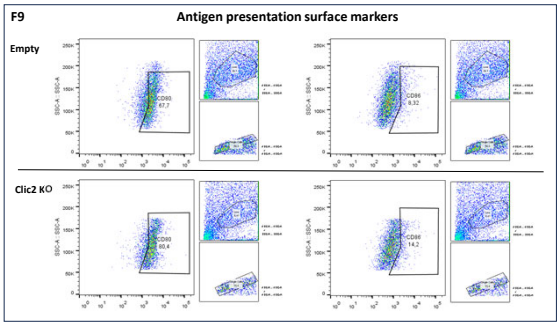




Next, we generated a stable Clic2 knock out THP-1 model using CRISPR/Cas9 to explore if Clic2 could impact monocyte to macrophages differentiation pathway. Interestingly, while empty vector MΦ macrophages displayed classical round morphology (**F8a**), Clic2-KO clones were spindly-shaped showing also membrane extroflections (**F8b-c-d**).

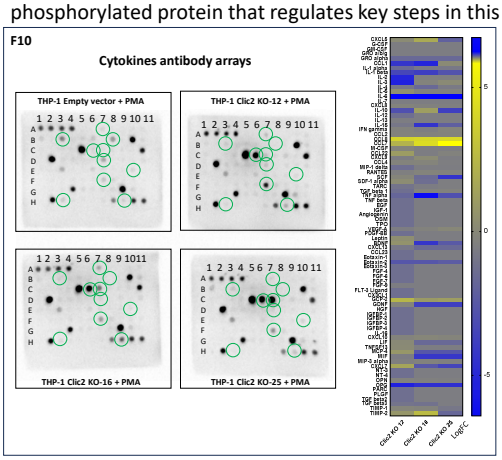
We further characterized Clic2-KO MΦ macrophages by membrane Immunophenotyping. We found increased CD11b/CD11c expression (**F8**) and increased CD80/86 expression (**F9**) in Clic2-KO macrophages, thereby indicating enhanced antigen presentation compared to control macrophages.

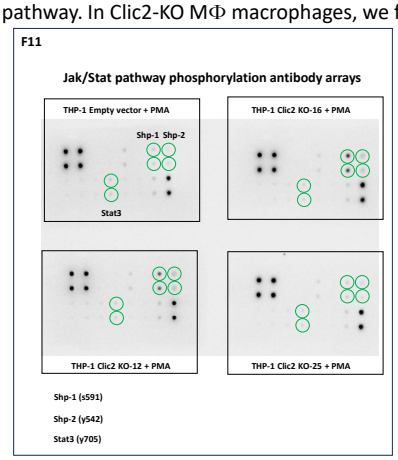


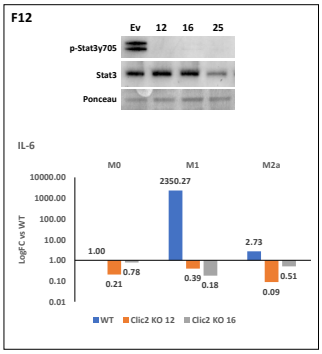


Macrophages also secrete cytokines that contribute to immune cells chemoattraction and lymphocyte activation or inhibition.

Cytokines profiling of MΦ macrophages (**F10**) revealed as Clic2-KO cells produced increased amounts of chemotactic cytokines together with reduced quantity of IL-6, one of the most characterized cytokines in the TME for its great support to the tumor growth. IL-6 secretion is regulated by Jak/Stat signaling, so we screened phosphorylated protein that regulates key steps in this pathway. In Clic2-KO MΦ macrophages, we found increased phosphorylation of Shp-1/Shp-2 and reduced Stat3 phosphorylation respect to controls (**F11**). Shp-1/Shp-2 balance Jak receptor activation/inhibition upon ligand binding, dephosphorylating targets to avoid abnormal stimulatory signals that could interfere normal cell processes. Clic2-KO MΦ macrophages showed impaired activation of Jak receptor as Stat3 phosphorylation was completely absent (**F12**). Furthermore, polarized Clic2-KO MΦ macrophages and controls to M1- and M2-like subsets, showed an impairment of Stat3-IL-6 axis in Clic2-KO cells (**F12**) while controls responded normally to differentiation.







Concluding, we describe a novel specific marker of dGC histotype that is expressed in the TME, furthermore we associate Clic2 for the first time to macrophages. Even if we still need to deepen information, we could hypothesize Clic2 overexpression in macrophages could support the tumorigenic and immunosuppressive TME of the dGC.