Unravel the mechanism of lactate-mediated suppression of dendritic cells in lung cancer and setting up a system to identify novel pathways of suppression.

Background and rationale

Cancer therapy has been recently revolutionized by the use of antibodies that target inhibitory receptors expressed on T cells and bystander cells, the so-called immune checkpoint inhibitors. This approach allows restoration of tumor specific T-cell responses, however the rate and the spectrum of responders is still relatively low. Therefore there is an urgent need to identify novel immunotherapeutic strategies to restore multiple arms of the immune response.
Recent evidences point to the importance of a competent antigen-presenting compartment to improve the efficacy of immunotherapy using checkpoint blockade (Salmon et al., 2016; Sanchez-Paulete et al., 2016). On the other hand it is also emerging that the most potent class of antigen presenting cells, dendritic cells (DCs), become compromised in the tumor microenvironment. For instance, DCs exposed to tumor-derived factors accumulate oxidized lipids that block cross-presentation (Herber et al., 2010; Ramakrishnan et al., 2014), possibly via XBP-1 mediated sensing of ER stress in the tumor microenvironment (TME) (Cubillos-Ruiz et al., 2015). Soluble cytokines such as IL-10 made by tumor-associated macrophages is involved in suppression of IL-12 production by DCs (Ruffell et al., 2014). Metabolites such as PGE2 present in the tumor microenvironment of breast cancer and melanoma respectively was also proposed to interfere with the antigen presenting functions of DCs (Zelenay et al., 2015). Despite these initial findings the major mechanisms causing suppression of DCs function in the tumor microenvironment are still poorly understood. Moreover, as mechanisms of suppression are likely to be tissue specific, it is important to address the question in a context specific manner.
During my undergraduate training I have set up the experimental system to induce and measure suppression of innate and adaptive DCs functions using a relevant cell culture system and factors derived from a KRAS/p53 driven lung cancer model. Using this system I have identified lactate as a major metabolite causing suppression of DCs functions in vitro and in vivo.

Specific aims:

During my PhD I intend to pursue two parallel research lines, based on the preliminary data and expertise that I have already acquired:
1. To investigate the mechanism by which lactate suppresses DCs functions in vitro and to explore the impact of lactate conditioning on the immune response in vivo.
2. To develop a functional screen to identify regulators of DCs suppression based on the assay that I have already developed.
The plan includes two distinct yet related approaches that will allow me to gain multiple skills and provides me with the opportunity to choose, if necessary, only the most promising approach.

Experimental plan

A. Explore the mechanism of lactate induced-suppression

My preliminary experiments showed that incubation of DCs with tumor-derived factors (supernatant of explanted lung tumors, TCM) induces suppression of inflammatory cytokine production and T cell activation (not shown). I have demonstrated that lactic acid is an abundant metabolite in two different TCM (fig 1A). DCs conditioned with pure lactic acid (LA-DCs) in doses similar to the amount contained in the TCM become unable to produce inflammatory cytokines, such as IFNα and IL12p70 after stimulation with PAMP/DAMP (Fig 1B, 1C). Moreover, DC conditioning with lactic acid induces an impairment of $CD8^+$ T cell activation ability, both *in vitro* and *in vivo* (Fig 1D, 1E). In sum, the data suggest that lactic acid in the tumor microenvironment is a major factor responsible of tumor suppression. The importance of lactic acid in suppression of T cells and NK cells is emerging, but nothing is presently known on its modulatory effects on the antigen-presenting compartment. For this reason I'm very keen to explore further the relevance and mechanisms of my findings.

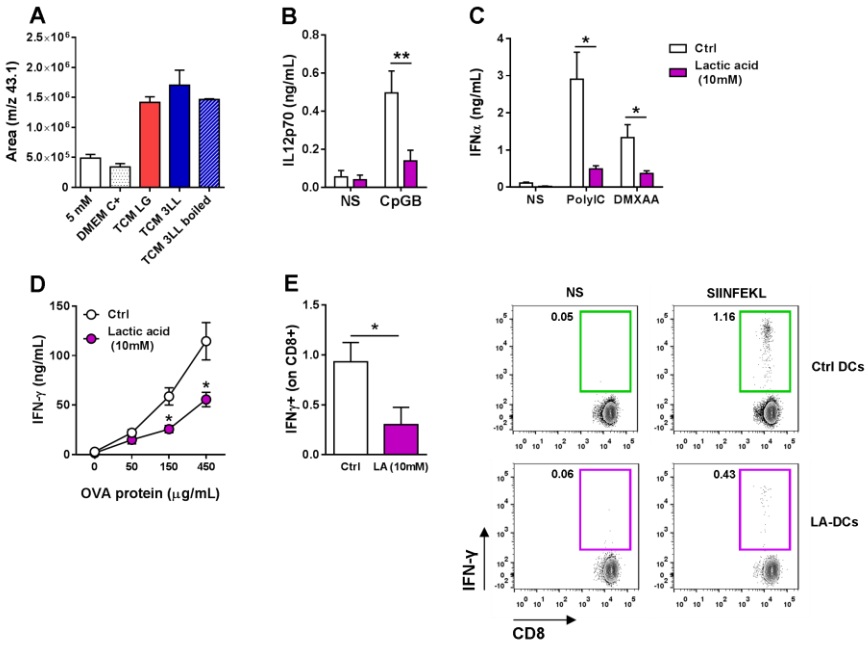

Figure1. Lactic acid in TCM induces suppression of DC functions. A) The presence of lactic acid in TCM has been measured by hydrophilic interaction chromatography (HILIC) and mass spectrometry. B-C) BMDCs are conditioned with lactic acid (10mM) for 24h and then stimulated with PAMP/DAMP on. Inflammatory cytokines production in measures by ELISA. Data represent the mean±SEM of 5 indipendent experiments. D) Conditioned or control BMDC are stimulated with OVA protein and then incubated with OTI T cells. The ability of DC to activate T cells has been evaluated by measuring T cell production of IFNγ after 48h of co-culture. E) BMDC±lactic acid are stimulated with CpGB (1μg/mL), PolyIC (1μg/mL) and OVA peptide (100nM) and injected in the footpad of C57BL/6 animals. After 14 days, lymph node were collected and *ex vivo* stimulated with OVA peptide to assess IFNγ production by flow cytometry. Data represent the mean±SEM of 2 indipendent experiment with 5 animals/group. Statistical significance is assessed by Student's T test. *p<005, **p<0.01

i. Understanding the intracellular mechanisms of lactic acid-induced DC suppression

Recent studies in $CD8^+$ T cells showed that an increased concentration of lactic acid in the tumor microenvironment (TME) causes a decrease in intracellular pH due to the symport of protons, via MCT which in turn blocks TCR signal transduction and T cell activation. (Brand et al., 2016)

We hypothesize that also in DCs, high lactate concentrations in the TME induces abnormal intracellular accumulation of lactate, associated with accumulation of protons inside the DCs, and a consequent decreased in intracellular pH. Two possible mechanisms could explain DC functional suppression:

1) acidic intracellular pH affects DCs functions, in particular lysosmal acidification and consequently the antigen degradation process;
2) increased intracellular levels of lactate blocks DC glycolysis, which has been associated to decreased DCs activation in other context (Everts et al., 2014).

To test the two hypotheses, I will perform the following *in vitro* experiments:

1) DCs will be treated with MCT inhibitors and lactic acid, and then stimulated with DAMP/PAMP to induce inflammatory cytokine production. If DC treatment with MCT inhibitors will restore the ability of LA-DCs to produce inflammatory cytokine, it would demonstrate that DC suppression is caused by the intracellular accumulation of lactic acid, via MCT.
2) In order to determine if the increased extracellular lactic acid causes an intracellular accumulation of protons, intracellular pH will be measured by using pH sensitive molecular probes (SNARF pH indicators, Life Technologies) and FACS analysis.
3) Intracellular ATP levels will be analyzed (ATP determination kit, Life Technologies) to test possible alterations in DC metabolism, in fact, the consequence of glycolysis block is a decreased intracellular ATP level.
4) The endocytic/lysosomal compartment pH will be measured by using pH sensitive molecular probes (pHrodo, Life Technologies) and FACS analysis.
5) LA-DCs antigen degradation ability will be tested by using fluorescent molecular probes, such as DQ-BSA, that is a fluorogenic substrate for proteases, emitting a bright fluorescence when degraded by proteases.
6) TLR signaling in LA-DCs will be analyzed by checking activation of adaptor molecules after stimulation with PAMP/DAMP.

ii. Explore the impact of lactate conditioning on the immune response in vivo

To assess the relevance of lactate in conditioning DCs function *in vivo*, I will target by CRISPR genome editing the gene encoding for the main enzyme involved in lactic acid production, lactate dehydrogenase A (LDHA) in a lung tumor cell line, already used in the laboratory (3LL-OVA). The obtained cells lines, 3LL-OVA^{LDHA-} and 3LL-OVA^{mock}, will be tested for equal proliferation and metabolism, and then will be injected subcutaneously in C57BL/6 mice. The following experiments will be perform:

1) FACS analysis at different time point to analyze the immune cells infiltrating the tumor with particular focus on DC subsets and CD8+ T cells.
2) Analyses of tumor-infiltrating DC (T-DCs) innate functions: T-DCs will be identified and isolated by FACS sorting, ex-vivo stimulated with PAMP/DAMP and their ability to produce inflammatory cytokines will be examined.
3) Analysis of tumor-infiltrating DC (T-DCs) adaptive functions: total dendritic cells (CD11c+) will be isolated from tumors, stimulated with the model antigen OVA and tested for their cross-presentation ability by FACS, quantifying the MHCI-OVA complex on DC cell surface.

4) To prove the relevance of DC function suppression by lactic acid in tumor growth, 3LL-OVA^{LDHA-} and 3LL-OVA^{mock} will be implanted in Baft3-/- animals, which the most efficient subset of DCs in cross-presenting antigens to CD8+ T cells.

B. Genome-wide sgRNA screen in CAS9 expressing DCs to identify novel mechanism of suppression.

During my undergraduate training I have established the conditions to induce suppression of DCs functions. Briefly, DCs incubated with tumor-conditioned medium (TCM) loose the ability to produce inflammatory cytokines (IL-12) and type-I interferon (IFN-I) in response to various stimuli. Here I intend to develop a system to perform a functional screen to identify genes involved in suppression.

Setting up the model for the screening will include several steps:

1) Constitutive CAS9 expression in immortalized hematopoietic progenitors

To overcome the limitations of primary cells that are difficult to manipulate we will employ a method already established in the lab whereby retroviral mediated expression of the transcription factor HoxB8 under estrogen control, in the presence of the growth factor Flt3L, generates immortal dendritic cells precursors. Upon estrogen removal, precursors are differentiated into FL derived DCs that maintain features of fresh bone marrow derived FL-DCs. We will immortalize DC precursors from the bone marrow of Cas9 *knock-in* mice (Platt et al., 2014) in order to obtain an abundant source of easy-to-transduce cells to induce genome editing by simply delivery sgRNA (HxC9).

2) Introducing a fluorescent reporter to monitor cytokine expression and isolate cells by cell sorting

To provide an easy system to select for cells expressing cytokines upon stimulation we will introduce a lentiviral vector expressing an NF-kB-GFP reporter into HxC9 (HxC9/NF-kB-eGFP). Cells transduced with the reporter plasmid will be selected and tested by flow for the ability to express green fluorescence upon stimulation with inducers of cytokine responses.

3) Library and screening protocol

A genome-scale library encoding 67.000 single-guide RNA (mouse GeCKOv2) cloned in lentiviral vector (LentiCRISPRv2, Genescript) will be used to infect HxC9/NF-kB-eGFP precursors. 20M cells/round will be infected and selected by puromycin. Upon selection cells will be differentiated into FL-DC by a 7-days culture in the presence Flt3L. FL-DCs will be exposed to TCM for 2hr and stimulated with innate agonists (poli:IC and CpG-B) for further 12 hrs.

According to our preliminary data exposure of DCs to TCM totally abolish cytokine gene expression in response to stimulation. Thus, control, not TCM-conditioned cells are expected to express high levels of EGFP reporter upon stimulation. In contrast, TCM conditioned cells will not express EGFP. Genome-edited cells that are still able to activate cytokine gene expression will express GFP after TCM conditioning (Fig2) and will be sorted by FACS. This "suppression-resistant population" will be subjected to deep sequencing to identify enriched sgRNA that will be used for subsequent rounds of selection.

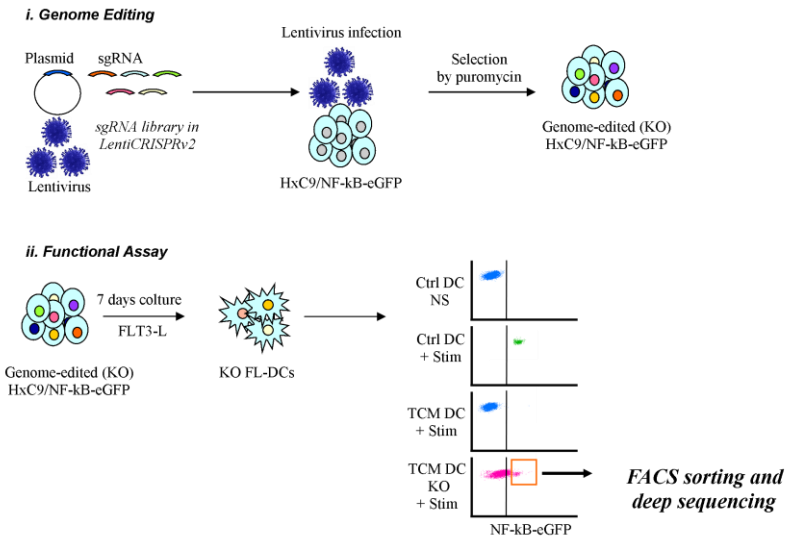

Figure2. Genome-wide sgRNA screen in CAS9 expressing DCs. A library of sgRNA cloned in LentiCRISPRv2 expression vector be used to infect HxC9/NF-kB-eGFP cells. Genome-edited HxC9/NF-kB-eGFP (DC KO) will be selected by puromycin and the resistant cells will be differentiated into DCs. To identify the genes that confer resistance to suppression by tumor conditioned medium genome edited cells (KO-FL DC) will be exposed to TCM and stimulated. Conditioned cells that continue to respond upon stimulation by inducing NF-kB-eGFP^{hi} will be sorted and sequenced.

Reference

Brand, A., K. Singer, G.E. Koehl, M. Kolitzus, G. Schoenhammer, A. Thiel, C. Matos, C. Bruss, S. Klobuch, K. Peter, M. Kastenberger, C. Bogdan, U. Schleicher, A. Mackensen, E. Ullrich, S. Fichtner-Feigl, R. Kesselring, M. Mack, U. Ritter, M. Schmid, C. Blank, K. Dettmer, P.J. Oefner, P. Hoffmann, S. Walenta, E.K. Geissler, J. Pouyssegur, A. Villunger, A. Steven, B. Seliger, S. Schreml, S. Haferkamp, E. Kohl, S. Karrer, M. Berneburg, W. Herr, W. Mueller-Klieser, K. Renner, and M. Kreutz. 2016. LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. *Cell metabolism*. 24:657-671.

Cubillos-Ruiz, J.R., P.C. Silberman, M.R. Rutkowski, S. Chopra, A. Perales-Puchalt, M. Song, S. Zhang, S.E. Bettigole, D. Gupta, K. Holcomb, L.H. Ellenson, T. Caputo, A.H. Lee, J.R. Conejo-Garcia, and L.H. Glimcher. 2015. ER Stress Sensor XBP1 Controls Anti-tumor Immunity by Disrupting Dendritic Cell Homeostasis. *Cell*. 161:1527-1538.

Everts, B., E. Amiel, S.C. Huang, A.M. Smith, C.H. Chang, W.Y. Lam, V. Redmann, T.C. Freitas, J. Blagih, G.J. van der Windt, M.N. Artyomov, R.G. Jones, E.L. Pearce, and E.J. Pearce. 2014. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nature immunology*. 15:323-332.

Herber, D.L., W. Cao, Y. Nefedova, S.V. Novitskiy, S. Nagaraj, V.A. Tyurin, A. Corzo, H.I. Cho, E. Celis, B. Lennox, S.C. Knight, T. Padhya, T.V. McCaffrey, J.C. McCaffrey, S. Antonia, M. Fishman, R.L. Ferris, V.E. Kagan, and D.I. Gabrilovich. 2010. Lipid accumulation and dendritic cell dysfunction in cancer. *Nature medicine*. 16:880-886.

Platt, R.J., S. Chen, Y. Zhou, M.J. Yim, L. Swiech, H.R. Kempton, J.E. Dahlman, O. Parnas, T.M. Eisenhaure, M. Jovanovic, D.B. Graham, S. Jhunjhunwala, M. Heidenreich, R.J. Xavier, R. Langer, D.G. Anderson, N. Hacohen, A. Regev, G. Feng, P.A. Sharp, and F. Zhang. 2014. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell*. 159:440-455.

Ramakrishnan, R., V.A. Tyurin, F. Veglia, T. Condamine, A. Amoscato, D. Mohammadyani, J.J. Johnson, L.M. Zhang, J. Klein-Seetharaman, E. Celis, V.E. Kagan, and D.I. Gabrilovich. 2014. Oxidized lipids block antigen cross-presentation by dendritic cells in cancer. *Journal of immunology*. 192:2920-2931.

Salmon, H., J. Idoyaga, A. Rahman, M. Leboeuf, R. Remark, S. Jordan, M. Casanova-Acebes, M. Khudoynazarova, J. Agudo, N. Tung, S. Chakarov, C. Rivera, B. Hogstad, M. Bosenberg, D. Hashimoto, S. Gnjatic, N. Bhardwaj, A.K. Palucka, B.D. Brown, J. Brody, F. Ginhoux, and M. Merad. 2016. Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity*. 44:924-938.

Sanchez-Paulete, A.R., F.J. Cueto, M. Martinez-Lopez, S. Labiano, A. Morales-Kastresana, M.E. Rodriguez-Ruiz, M. Jure-Kunkel, A. Azpilikueta, M.A. Aznar, J.I. Quetglas, D. Sancho, and I. Melero. 2016. Cancer Immunotherapy with Immunomodulatory Anti-CD137 and Anti-PD-1 Monoclonal Antibodies Requires BATF3-Dependent Dendritic Cells. *Cancer discovery*. 6:71-79.

Zelenay, S., A.G. van der Veen, J.P. Bottcher, K.J. Snelgrove, N. Rogers, S.E. Acton, P. Chakravarty, M.R. Girotti, R. Marais, S.A. Quezada, E. Sahai, and C. Reis e Sousa. 2015. Cyclooxygenase-Dependent Tumor Growth through Evasion of Immunity. *Cell*. 162:1257-1270.