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Authors: Chevalier MF, Bohner P, Pieraerts C, Lhermitte B, Gourmaud J, Nobile A, Rotman S, Cesson V, Martin V, Legris AS, Dartiguenave F, Gharbi D, De Leval L, Speiser DE, Nardelli-Haefliger D, Jichlinski P, Derré L

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Immunoregulation of Dendritic Cell Subsets by Inhibitory Receptors in Urothelial Cancer

Mathieu F. Chevalier[a#], Perrine Bohner[a#], Claire Pieraerts[a], Benoit Lhermitte[b], Jolanta Gourmaud[b], Antoine Nobile[b], Samuel Rotman[b], Valerie Cesson[a], Virginie Martin[a], Anne-Sophie Legris[a], Florence Dartiguenave[a], Dalila Gharbi[a], Laurence De Leval[b], Daniel E. Speiser[c], Denise Nardelli-Haefliger[a], Patrice Jichlinski[a], and Laurent Derré[a].

[a] *Department of Urology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland;*
[b] *Department of Pathology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland;*
[c] *Department of Oncology and Ludwig Cancer Research, University of Lausanne, Switzerland*
[#] These authors contributed equally as first authors

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Corresponding author: Laurent Derré
Dpt Urology, CHUV
IMU48/05/510
Bugnon 48
1011 Lausanne, Switzerland.
Phone: 021/314 03 73
Fax: 021/314 40 60
E-mail: Laurent.derre@chuv.ch

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Abstract:

Blockade of inhibitory receptors (IR), overexpressed by T-cells, can activate anti-tumor immune responses resulting in the most promising therapeutic approaches, particularly in bladder cancer, currently able to extend patient survival. Thanks to their ability to cross-present antigens to T cells, dendritic cells (DC) are an immune cell population playing a central role in the generation of effective anti-tumor T-cell responses. While function and expression of IRs have been mostly investigated in T cells, very few data are available for DC. Therefore, we analyzed whether DC may express IRs able to decrease their functions. For that purpose, we investigated several IR: PD-1, CTLA-4, BTLA, TIM-3 and CD160, in circulating $CD1c^+$ DC, $CD141^+$ DC and plasmacytoid DC (pDC) from healthy donors (HD) and urothelial cancer (UCa) patients. Different DC subsets expressed BTLA and TIM-3 but not other IRs. More importantly BTLA and TIM-3 were significantly upregulated in DC from blood of UCa patients. Locally, bladder tumor-infiltrating DC also overexpressed BTLA and TIM-3 compared to DC from paired non-tumoral tissue. Finally, i*n vitro* functional experiments showed that ligand-mediated engagement of BTLA and TIM-3 receptors significantly reduced the secretion of effector cytokines by DC subpopulations. Our findings demonstrate that UCa induces local and systemic overexpression of BTLA and TIM-3 by DC that may result in their functional inhibition, highlighting those receptors as potential targets for UCa treatment.

Patient summary: In this report, we investigated patients with urothelial carcinoma with regard to expression and function of a panel of inhibitory receptors by dendritic cells (DC), an immune cell subpopulation which is critical in the initiation of protective immune responses. We found high expression of BTLA and TIM-3 by blood and tumor DC, potentially mediating decreased DC function, suggesting that BTLA and TIM-3 might be new interesting targets for urothelial carcinoma treatment.

Take Home Message: The inhibitory receptors BTLA and TIM-3 are overexpressed on circulating and intratumoral dendritic cells in urothelial cancer patients, mediating reduction of dendritic cells function.

Main Text

Immune responses are tightly regulated by activatory and inhibitory receptors (IR), also called immune checkpoints. Engagement of IR, upon interaction with their cognate ligands, leads to dimming the T-cell receptor signaling, resulting in reduction of immune responses to antigen [1]. The expression of IR has been associated to T-cell exhaustion in autoimmune diseases, chronic infections and cancers and to the impotence of T-cells to eradicate tumors [2]. Over the past few years, the therapeutic use of humanized antagonist antibodies against IR or their ligands has shown unprecedented clinical results in patients with solid tumors, particularly in muscle invasive bladder cancer (MIBC) patients [1, 3], demonstrating the great potential of such approaches and leading to a breakthrough therapy designation by the American Food and Drug Administration. Albeit IR have been extensively studied in T-cell subpopulations, almost no data are available on IR expression and function by dendritic cell (DC) subsets in humans. DC are key players in the initiation and regulation of immune responses. Indeed, DC are able to uptake, process, and present tumor antigens to other immune cells, so that they are crucial to "prime"/activate anti-tumor T-cell responses eventually leading to tumor-cell killing. Notably, DC are thus prominent targets in cancer immunotherapy strategies [4]. Human circulating DC can be broadly categorized into two groups: $CD11c^{neg}CD123^{+}$ plasmacytoid DC (pDC) and conventional $CD11c^{+}CD123^{neg}$ DC (cDC) [5]. Among the cDC, two subpopulations have been identified according to the expression of CD1c (also known as BDCA-1) and CD141 (also known as BDCA-3 or thrombomodulin) [5]. $CD141^{+}$ DC have prominent capacities to cross-present antigen after its uptake and thus may play a major role for inducing anti-tumor immune responses [5, 6]. Here, we have conducted the first analysis of the expression and function of several well-known IR on DC subsets from healthy donors (HD) and urothelial cancer (UCa) patients.

Expression of Programmed cell Death-1 (PD-1; CD279), Cytotoxic T Lymphocyte Associated protein-4 (CTLA-4; CD152), B an T Lymphocyte Attenuator (BTLA; CD272), CD160 (BY55) and T-cell Immunoglobulin and Mucin-domain containing-3 (TIM-3; CD366) was first assessed by flow cytometry on circulating DC subpopulations from HD and UCa patients. pDC and conventional $CD1c^{+}$ and $CD141^{+}$ DC were identified using a combination of phenotypic markers (Supplementary Fig. 1) and expression of IR was determined. PD-1, CTLA-4 and CD160 were not expressed by any subtype of DC from HD or UCa patients (data not shown). In contrast, BTLA was observed in all DC subsets, albeit at a very low level in $CD1c^{+}$ DC and TIM-3 was only expressed by $CD1c^{+}$ and $CD141^{+}$ DC, in HD. Comparison to UCa patients showed that BTLA was significantly overexpressed by $CD141^{+}$ DC and pDC,

whereas only a slight increase of TIM-3 expression was observed in $CD141^+$ DC (Fig. 1A). This result suggests that bladder tumor microenvironment may increase BTLA and TIM-3 expression on DC. In order to have more insights into BTLA and TIM-3 expression by DC, we segregated the data from UCa patients in two groups, according to the stage of the disease (Supplementary Table 1): non-muscle invasive bladder cancer (NMIBC) and MIBC patients. A significantly higher expression of TIM-3 was only found in $CD141^+$ DC from MIBC patients as compared to HD, suggesting that UCa-mediated overexpression of TIM-3 is later than for BTLA, which was overexpressed in $CD141^+$ DC and pDC from both types of patients (Supplementary Fig. 2).

Seeking further evidence that BTLA and TIM-3 expression may be altered by the bladder tumor microenvironment, we analyzed their expression on tissue-infiltrating DC subpopulations from bladder tumor and paired non-tumoral adjacent tissue from surgical specimen recovered after cystectomy. Since DC subtypes from tissue are phenotypically different than circulating DC, we focused on $CD14^+CD11c^+$ and $CD14^{neg}CD11c^+$ tissue-infiltrating DC [7, 8] (Fig. 1B). Notably, we observed a significant overexpression of TIM-3 in both types of bladder DC and a higher frequency of $BTLA^+$ $CD14^{neg}CD11c^+$ bladder DC within tumor tissue (Fig. 1C). Similar results were obtained when comparing non-paired tissue samples (Supplementary Fig. 3).

We next sought to determine whether BTLA and TIM-3 expressed on DC are functional and could lead to an inhibition of DC function, as monitored by cytokine (IL-12, IL-1β and TNF-α) production, upon recombinant HVEM (BTLA ligand) or galectin-9 (TIM-3 ligand) binding. Thus, sorted $CD1c^+$ and $CD141^+$ DC were stimulated by polyionosinic-polycytidylic acid (Poly(I:C)) and pDC by CpG oligodeoxynucleotides (CpG ODN). In the presence of either HVEM or galectin-9, we observed a decrease of the cytokine secretion by $CD141^+$ DC (Fig. 2A). In addition, $CD1c^+$ DC produced less IL-12p70 and IL-1β upon galectin-9 ligation (Fig. 2B). In pDC, only IL-12p70 production was affected by the presence of HVEM (Fig. 2C). These data suggest a functional inhibition of DC subpopulations via BTLA/HVEM or TIM-3/galectin-9 interactions.

In this study, we present the first analysis of several IR on DC subsets from HD and UCa patients. We found that BTLA and TIM-3 are differentially expressed across circulating and tissue DC subsets. Moreover, our results indicate that UCa promotes the overexpression of BTLA and TIM-3 on DC, similarly to what has been described for T-cells [9, 10]. In contrast to the relatively well-known mechanisms of T-cell function dampening upon TIM-3/galectin-

9 binding, TIM-3 function in myeloid cells, particularly in DC, remains controversial. Indeed, while recent reports, in accordance with our results, show that TIM-3 may reduce DC functions, other reports also described a positive co-stimulatory effect of TIM-3 on DC upon galectin-9 ligation [11].

In our settings, we did not observe any PD-1 expression by circulating DC from either HD or UCa patients. It has been recently reported that mouse DC expressing PD-1 showed reduced function in ovarian and hepatocellular tumor models [12, 13]. Authors also observed PD-1^{+} tumor-infiltrating DC in human ovarian tumors. Thus, further investigation is warranted to determine PD-1 expression in bladder tumor DC.

Immune regulatory mechanisms are emerging as important targets to attenuate autoimmune diseases or enhance immune responses to tumors and infections. Therapies aimed at overcoming mechanisms of peripheral immune tolerance, in particular by blocking inhibitory checkpoints, offer the potential to generate anti-tumor activity, either as monotherapies or in synergy with other therapies [1] that directly or indirectly enhance presentation of tumor antigens to the immune system. Checkpoint blockade therapy targeting IR expressed by T-cells and DC simultaneously, such as BTLA and TIM-3, may therefore prove critical in the generation of a potent antitumor immune response, particularly in urothelial cancer.

Author contributions: Laurent Derré had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Derré.
Acquisition of data: Chevalier, Bohner, Pieraerts.
Analysis and interpretation of data: Chevalier, Derré.
Drafting of the manuscript: Derré.
Critical revision of the manuscript for important intellectual content: Derré, Nardelli-Haefliger, Chevalier, Speiser, Jichlinski, Bohner, Pieraerts, Cesson, Lhermitte, Rotman, Nobile, Gourmaud, De Leval, Legris, Dartiguenave.
Statistical analysis: Derré.
Obtaining funding: Derré, Jichlinski.
Administrative, technical, or material support: Chevalier, Bohner, Pieraerts Lhermitte, Cesson, Legris, Dartiguenave, Gharbi, Martin, Rotman, Nobile, Gourmaud, De Leval.
Supervision: Derré.

Other (specify): None.

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Figure legends:

Figure 1: Overexpression of BTLA and TIM-3 in circulating and tumor-infiltrating DC subsets. (A) Comparison of BTLA and TIM-3 expression in CD1c+, CD141+ and pDC from peripheral blood mononuclear cells (PBMC) of HD (n=15) and UCa patients (n=40). **(B)** Representative example of BTLA and TIM-3 labeling in $CD14^{+}CD11c^{+}$ and $CD14^{neg}CD11c^{+}$ DC infiltrating the bladder from MIBC patients. **(C)** Quantification of BTLA and TIM-3 expressed by bladder infiltrating DC. *p<0.05; **p<0.01. RFI: Ratio of mean-Fluorescence-Intensity of specific staining versus isotype Ig control, except for BTLA in $CD14^{neg}CD11c^{+}$ DC expressed in percentage of positive cells, since bimodal population was observed.

Figure 2: Functional inhibition of DC subsets by BTLA and TIM-3. Sorted circulating $CD141^{+}$ **(A)** and $CD1c^{+}$ **(B)** DC from HD were stimulated overnight by Poly(I:C) in the presence of plate bound Galectin-9, whereas sorted pDC **(C)** were activated by CPG in the presence of coated HVEM. After overnight incubation, IL-12p70, IL-1β and TNF-α secretion was determined in supernatant. *p<0.05.

Supplementary Figure legends:

Supplementary Figure 1: Identification of DC subsets by flow cytometry. Representative example of direct *ex vivo* HLA-DR, CD1c, CD141, CD123 staining after lineage (i.e. CD3, CD14, CD16, CD19, CD20, CD56), doublets and dead cell exclusion from PBMC of HD. pDC were identified as $Lin^{neg}HLA\text{-}DR^{+}CD123^{+}$, CD1c+ DC as $Lin^{neg}HLA\text{-}DR^{+}CD1c^{+}$ and $CD141^{+}$ DC as $Lin^{neg}HLA\text{-}DR^{+}CD141^{+}$.

Supplementary Figure 2: BTLA and TIM-3 expression in DC subpopulations from PBMC of HD, NMIBC and MIBC patients. Quantification of BTLA and TIM-3 expressed by CD1c+, CD141+ DC and pDC in PBMC of UCa patients according to invasiveness of the tumor. *p<0.05; **p<0.01.

Supplementary Figure 3: Overexpression of BTLA and TIM-3 in bladder-infiltrating DC subsets. Quantification of BTLA and TIM-3 expressed by $CD14^{+}CD11c^{+}$ and $CD14^{neg}CD11c^{+}$ DC from bladder tumor or non-tumor adjacent tissue from different patients. *p<0.05.

Supplementary materials and methods

Patient selection

Buffy coats from healthy subjects were purchased from the Blood Transfusion Center, Epalinges, Switzerland. Peripheral blood and bladder tissue from patient were obtained after written consent and full ethics approval (protocol #119/10). Bladder tissue samples were carefully collected by pathologist from tumor and non-tumoral adjacent tissue at a resection margin located at a mean distance of 2 cm from the tumor. Bladder cells were then fractionated as described previously [1] and were subsequently used for flow cytometry analysis. The pathological characteristics of patients are summarized in Supplementary Table 1.

Flow cytometry and cell sorting

The following antibodies to human proteins were used. From Biolegend: anti-CD1c-PerCP-Cy5.5, anti-CD123-PE-Cy7, anti-TIM-3-BV421, anti-CTLA-4-PE, anti-PD-1-BV421 and mouse IgG1-BV421. From Becton Dickinson: Lineage1 cocktail-FITC (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and anti-CD56) and anti-BTLA-PE. From Beckman Coulter: anti-CD160-PE. From Thermo Fisher Scientific: anti-HLA-DR-PE/TexasRed. From Miltenyi Biotec: anti-CD141-APC. From eBioscience: mouse IgG1-PE.

PBMCs were obtained by density centrifugation using Lymphoprep (Axis-Shieldy) and cryopreserved in RPMI1640 supplemented with 40% FCS and 10% DMSO. Surface labelling was performed for 20 min. at 4 C, as described previously [1]. Cells were then stained for dead cell exclusion with aqua live/dead stain kit (Life Technologies) according to the manufacturer's instructions. FcR Blocking Reagent (Miltenyi Biotec) was used during cells labelling to block unwanted binding of antibodies. Sample acquisition was performed on Gallios Flow-Cytometer (Beckman Coulter) and data were analyzed using the FlowJo Software (TreeStar).

For cell sorting (Astrios, Beckman Coulter), DC were magnetically enriched from 3-4x10^8 PBMC from healthy individuals using the Dynabeads® human DC enrichment kit (Thermo Fisher Scientific). Cells were subsequently stained and live Lin^{neg} $HLA\text{-}DR^+$ cells were sorted into the indicated subsets using Astrios cell sorter (Beckman Coulter).

Inhibition assay

Sorted DC subpopulations were activated by 1 μg/ml of poly (I:C) or ODN CpG (Pfizer/ Coley Pharmaceutical Group) and cultured with 10 μg/ml plate-bound recombinant human HVEM-Fc chimera (R&D Systems), soluble recombinant human Galectin-9 (Biolegend) or with Control-Fc (Ctrl) fusion protein (Mutated Thy-1-Fc, Enzo Life Sciences). After overnight incubation, culture supernatants were tested for the presence of IL-12p70, IL-1β and TNF-α by Cytokines Beads Array (BD Biosciences).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 and included unpaired (Supplemental Fig.2) or paired (Fig. 1C and 2) two-tailed Student's t-test or one-way ANOVA followed by Dunnett's test (Fig. 1A) for comparing the means of two or multiple groups, respectively. *p* values below 0.05 were considered statistically significant.

Supplementary references

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Supplementary Table 1. Characteristics of patients

Patient	All patients
Number of patients	40
Age, yr, median (IQR)	71 (62.25 – 75)
Sex, n (%)	
Male	32 (80)
Female	8 (20)
Tumor status, n (%)	
NMIBC	
pTa	14 (35)
pT1	6 (15)
MIBC	
pT2	7 (17.5)
pT3	11 (27.5)
pT4	2 (5)
Draining lymph nodes status	
N0	10 (50)
N1	5 (25)
>N2	1 (5)
NA	4 (20)
Adjuvant chemotherapy	5 (25)

NMIBC = Non Muscle Invasive Bladder Cancer
MIBC = Muscle Invasive Bladder Cancer
NA = Not Available

Figure 1

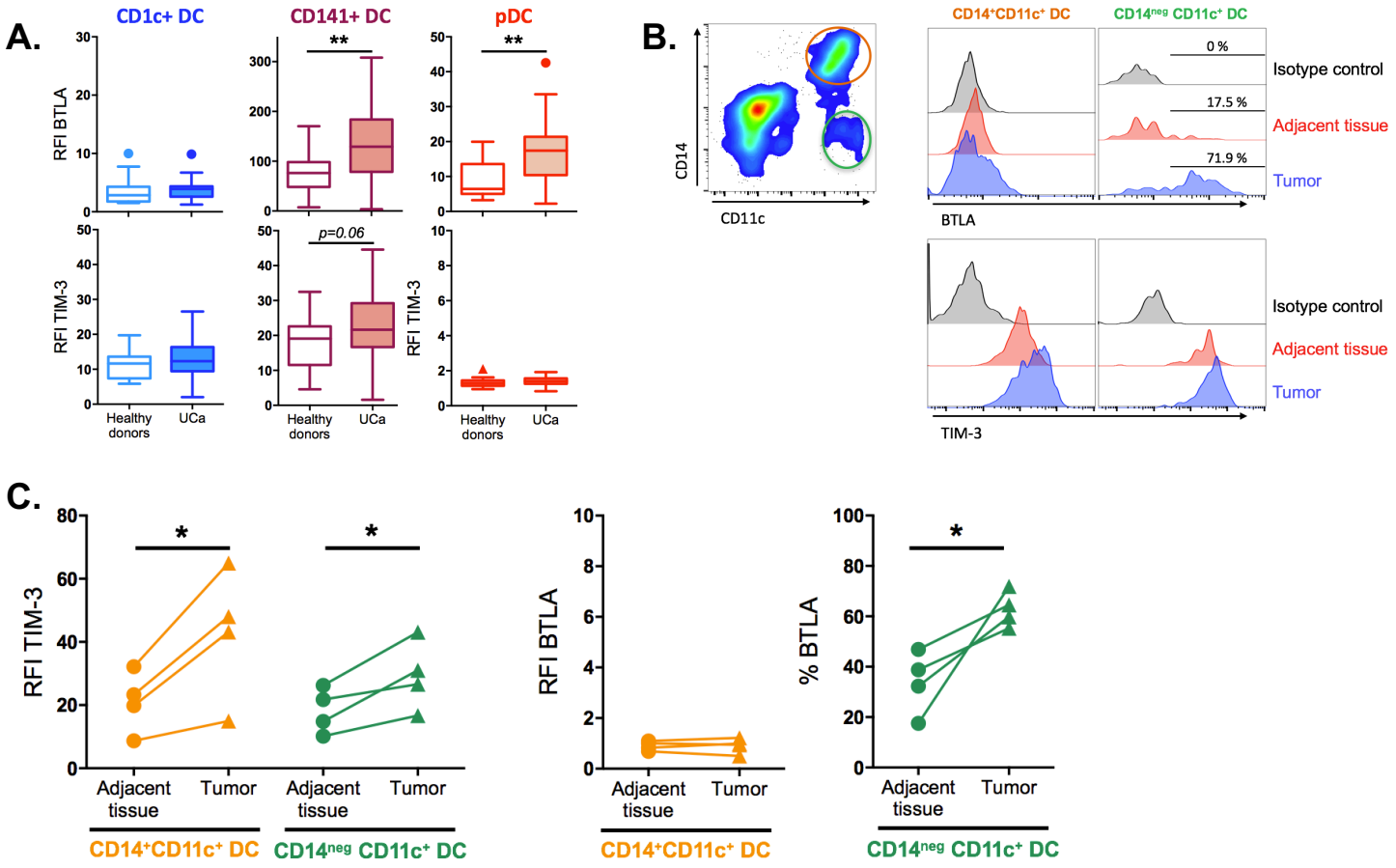
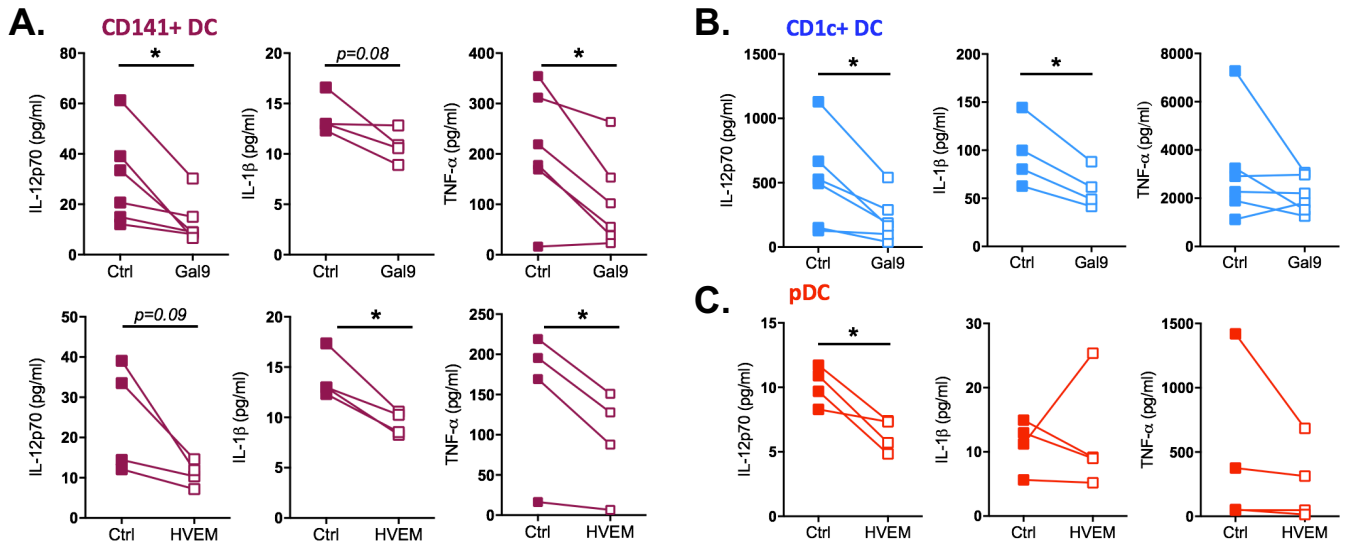
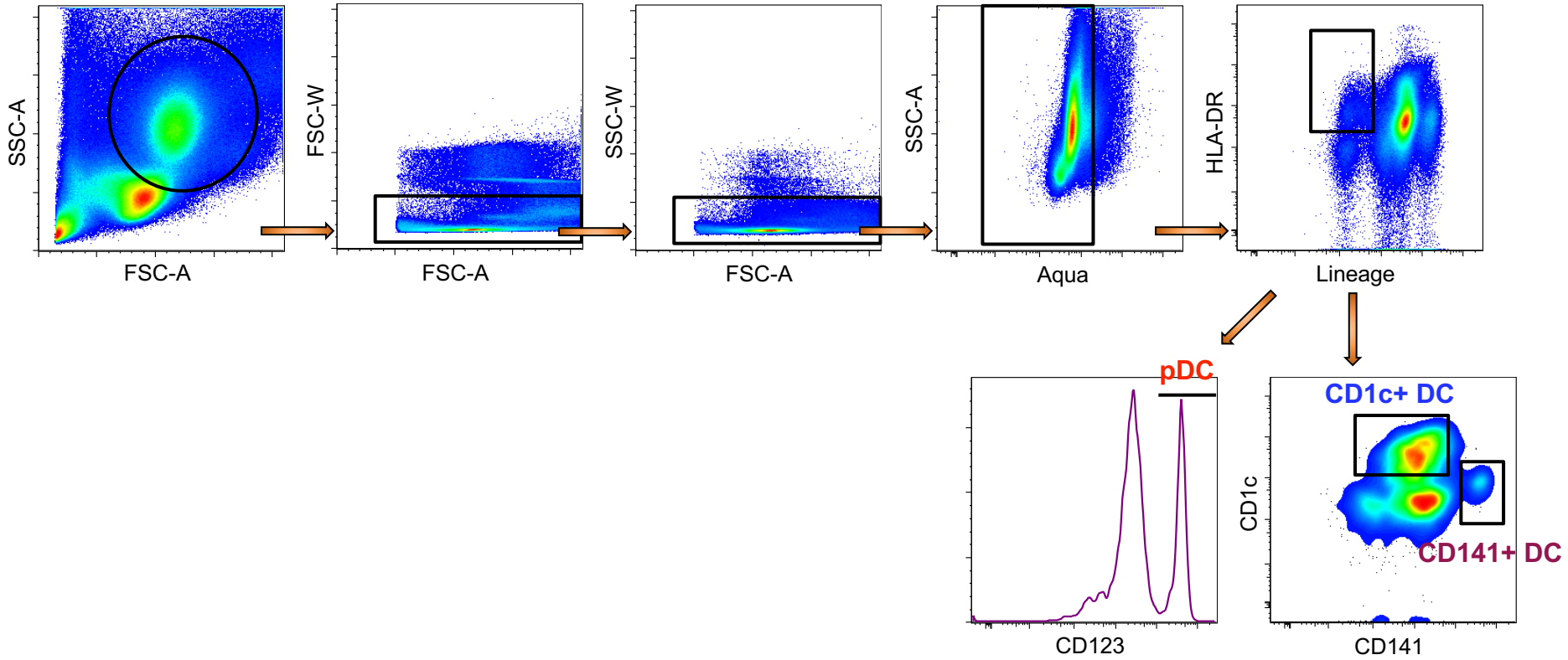

A.
CD1c+ DC
CD141+ DC
pDC
RFI BTLA
RFI TIM-3
**
**
p=0.06
Healthy donors
UCa
B.
CD14
CD11c
CD14+CD11c+ DC
CD14neg CD11c+ DC
0 %
17.5 %
71.9 %
Isotype control
Adjacent tissue
Tumor
BTLA
TIM-3
C.
RFI TIM-3
RFI BTLA
% BTLA
*
Adjacent tissue
Tumor
CD14+CD11c+ DC
CD14neg CD11c+ DC

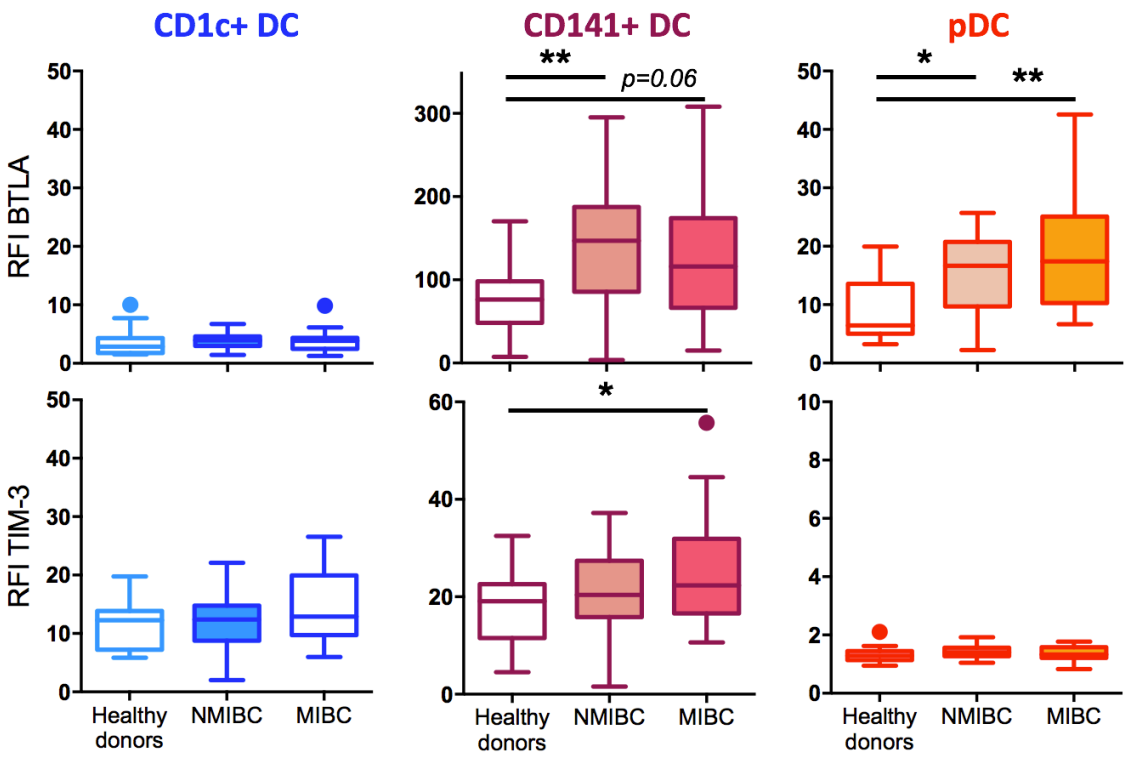
Figure 2


A.
CD141+ DC
IL-12p70 (pg/ml)
IL-1β (pg/ml)
TNF-α (pg/ml)
p=0.08
p=0.09
*
Ctrl
Gal9
HVEM
B.
CD1c+ DC
C.
pDC

Supplemental Figure 1 :



Supplemental Figure 2 :



Supplemental Figure 3 :