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1   **Immunoregulation of Dendritic Cell Subsets by Inhibitory Receptors**
2   **in Urothelial Cancer**
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4   Mathieu F. Chevalier[a#], Perrine Bohner[a#], Claire Pieraerts[a], Benoit Lhermitte[b], Jolanta
5   Gourmaud[b], Antoine Nobile[b], Samuel Rotman[b], Valerie Cesson[a], Virginie Martin[a], Anne-
6   Sophie Legris[a], Florence Dartiguenave[a], Dalila Gharbi[a], Laurence De Leval[b], Daniel E.
7   Speiser[c], Denise Nardelli-Haefliger[a], Patrice Jichlinski[a], and Laurent Derré[a].
8

9   [a] *Department of Urology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland;*
10   [b] *Department of Pathology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland;*
11   [c] *Department of Oncology and Ludwig Cancer Research, University of Lausanne, Switzerland*
12   [#] These authors contributed equally as first authors
13

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16   **Corresponding author:** Laurent Derré
17   Dpt Urology, CHUV
18   IMU48/05/510
19   Bugnon 48
20   1011 Lausanne, Switzerland.
21   Phone: 021/314 03 73
22   Fax: 021/314 40 60
23   E-mail: Laurent.derre@chuv.ch
24

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28 **Abstract:**

29

30 Blockade of inhibitory receptors (IR), overexpressed by T-cells, can activate anti-tumor

31 immune responses resulting in the most promising therapeutic approaches, particularly in

32 bladder cancer, currently able to extend patient survival. Thanks to their ability to cross-

33 present antigens to T cells, dendritic cells (DC) are an immune cell population playing a

34 central role in the generation of effective anti-tumor T-cell responses. While function and

35 expression of IRs have been mostly investigated in T cells, very few data are available for

36 DC. Therefore, we analyzed whether DC may express IRs able to decrease their functions.

37 For that purpose, we investigated several IR: PD-1, CTLA-4, BTLA, TIM-3 and CD160, in

38 circulating CD1c$^+$ DC, CD141$^+$ DC and plasmacytoid DC (pDC) from healthy donors (HD)

39 and urothelial cancer (UCa) patients. Different DC subsets expressed BTLA and TIM-3 but

40 not other IRs. More importantly BTLA and TIM-3 were significantly upregulated in DC from

41 blood of UCa patients. Locally, bladder tumor-infiltrating DC also overexpressed BTLA and

42 TIM-3 compared to DC from paired non-tumoral tissue. Finally, *in vitro* functional

43 experiments showed that ligand-mediated engagement of BTLA and TIM-3 receptors

44 significantly reduced the secretion of effector cytokines by DC subpopulations. Our findings

45 demonstrate that UCa induces local and systemic overexpression of BTLA and TIM-3 by DC

46 that may result in their functional inhibition, highlighting those receptors as potential targets

47 for UCa treatment.

48

49 *Patient summary:* In this report, we investigated patients with urothelial carcinoma with

50 regard to expression and function of a panel of inhibitory receptors by dendritic cells (DC), an

51 immune cell subpopulation which is critical in the initiation of protective immune responses.

52 We found high expression of BTLA and TIM-3 by blood and tumor DC, potentially

53 mediating decreased DC function, suggesting that BTLA and TIM-3 might be new interesting

54 targets for urothelial carcinoma treatment.

55

56 *Take Home Message:* The inhibitory receptors BTLA and TIM-3 are overexpressed on

57 circulating and intratumoral dendritic cells in urothelial cancer patients, mediating reduction

58 of dendritic cells function.

59

60

61 **Main Text**

62   Immune responses are tightly regulated by activatory and inhibitory receptors (IR), also

63 called immune checkpoints. Engagement of IR, upon interaction with their cognate ligands,

64 leads to dimming the T-cell receptor signaling, resulting in reduction of immune responses to

65 antigen [1]. The expression of IR has been associated to T-cell exhaustion in autoimmune

66 diseases, chronic infections and cancers and to the impotence of T-cells to eradicate tumors

67 [2]. Over the past few years, the therapeutic use of humanized antagonist antibodies against

68 IR or their ligands has shown unprecedented clinical results in patients with solid tumors,

69 particularly in muscle invasive bladder cancer (MIBC) patients [1, 3], demonstrating the great

70 potential of such approaches and leading to a breakthrough therapy designation by the

71 American Food and Drug Administration. Albeit IR have been extensively studied in T-cell

72 subpopulations, almost no data are available on IR expression and function by dendritic cell

73 (DC) subsets in humans. DC are key players in the initiation and regulation of immune

74 responses. Indeed, DC are able to uptake, process, and present tumor antigens to other

75 immune cells, so that they are crucial to "prime"/activate anti-tumor T-cell responses

76 eventually leading to tumor-cell killing. Notably, DC are thus prominent targets in cancer

77 immunotherapy strategies [4]. Human circulating DC can be broadly categorized into two

78 groups: $CD11c^{neg}CD123^{+}$ plasmacytoid DC (pDC) and conventional $CD11c^{+}CD123^{neg}$ DC

79 (cDC) [5]. Among the cDC, two subpopulations have been identified according to the

80 expression of CD1c (also known as BDCA-1) and CD141 (also known as BDCA-3 or

81 thrombomodulin) [5]. $CD141^{+}$ DC have prominent capacities to cross-present antigen after its

82 uptake and thus may play a major role for inducing anti-tumor immune responses [5, 6]. Here,

83 we have conducted the first analysis of the expression and function of several well-known IR

84 on DC subsets from healthy donors (HD) and urothelial cancer (UCa) patients.

85   Expression of Programmed cell Death-1 (PD-1; CD279), Cytotoxic T Lymphocyte

86 Associated protein-4 (CTLA-4; CD152), B an T Lymphocyte Attenuator (BTLA; CD272),

87 CD160 (BY55) and T-cell Immunoglobulin and Mucin-domain containing-3 (TIM-3; CD366)

88 was first assessed by flow cytometry on circulating DC subpopulations from HD and UCa

89 patients. pDC and conventional $CD1c^{+}$ and $CD141^{+}$ DC were identified using a combination

90 of phenotypic markers (Supplementary Fig. 1) and expression of IR was determined. PD-1,

91 CTLA-4 and CD160 were not expressed by any subtype of DC from HD or UCa patients

92 (data not shown). In contrast, BTLA was observed in all DC subsets, albeit at a very low level

93 in $CD1c^{+}$ DC and TIM-3 was only expressed by $CD1c^{+}$ and $CD141^{+}$ DC, in HD. Comparison

94 to UCa patients showed that BTLA was significantly overexpressed by $CD141^{+}$ DC and pDC,

95 whereas only a slight increase of TIM-3 expression was observed in CD141$^+$ DC (Fig. 1A).

96 This result suggests that bladder tumor microenvironment may increase BTLA and TIM-3

97 expression on DC. In order to have more insights into BTLA and TIM-3 expression by DC,

98 we segregated the data from UCa patients in two groups, according to the stage of the disease

99 (Supplementary Table 1): non-muscle invasive bladder cancer (NMIBC) and MIBC patients.

100 A significantly higher expression of TIM-3 was only found in CD141$^+$ DC from MIBC

101 patients as compared to HD, suggesting that UCa-mediated overexpression of TIM-3 is later

102 than for BTLA, which was overexpressed in CD141$^+$ DC and pDC from both types of patients

103 (Supplementary Fig. 2).

104 Seeking further evidence that BTLA and TIM-3 expression may be altered by the bladder

105 tumor microenvironment, we analyzed their expression on tissue-infiltrating DC

106 subpopulations from bladder tumor and paired non-tumoral adjacent tissue from surgical

107 specimen recovered after cystectomy. Since DC subtypes from tissue are phenotypically

108 different than circulating DC, we focused on CD14$^+$CD11c$^+$ and CD14^{neg}CD11c$^+$ tissue-

109 infiltrating DC [7, 8] (Fig. 1B). Notably, we observed a significant overexpression of TIM-3

110 in both types of bladder DC and a higher frequency of BTLA$^+$ CD14^{neg}CD11c$^+$ bladder DC

111 within tumor tissue (Fig. 1C). Similar results were obtained when comparing non-paired

112 tissue samples (Supplementary Fig. 3).

113 We next sought to determine whether BTLA and TIM-3 expressed on DC are functional

114 and could lead to an inhibition of DC function, as monitored by cytokine (IL-12, IL-1β and

115 TNF-α) production, upon recombinant HVEM (BTLA ligand) or galectin-9 (TIM-3 ligand)

116 binding. Thus, sorted CD1c$^+$ and CD141$^+$ DC were stimulated by polyionosinic-polycytidylic

117 acid (Poly(I:C)) and pDC by CpG oligodeoxynucleotides (CpG ODN). In the presence of

118 either HVEM or galectin-9, we observed a decrease of the cytokine secretion by CD141$^+$ DC

119 (Fig. 2A). In addition, CD1c$^+$ DC produced less IL-12p70 and IL-1β upon galectin-9 ligation

120 (Fig. 2B). In pDC, only IL-12p70 production was affected by the presence of HVEM (Fig.

121 2C). These data suggest a functional inhibition of DC subpopulations via BTLA/HVEM or

122 TIM-3/galectin-9 interactions.

123 In this study, we present the first analysis of several IR on DC subsets from HD and UCa

124 patients. We found that BTLA and TIM-3 are differentially expressed across circulating and

125 tissue DC subsets. Moreover, our results indicate that UCa promotes the overexpression of

126 BTLA and TIM-3 on DC, similarly to what has been described for T-cells [9, 10]. In contrast

127 to the relatively well-known mechanisms of T-cell function dampening upon TIM-3/galectin-

128 9 binding, TIM-3 function in myeloid cells, particularly in DC, remains controversial. Indeed,
129 while recent reports, in accordance with our results, show that TIM-3 may reduce DC
130 functions, other reports also described a positive co-stimulatory effect of TIM-3 on DC upon
131 galectin-9 ligation [11].

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

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

146

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

149

150 *Study concept and design:* Derré.
151 *Acquisition of data:* Chevalier, Bohner, Pieraerts.
152 *Analysis and interpretation of data:* Chevalier, Derré.
153 *Drafting of the manuscript:* Derré.
154 *Critical revision of the manuscript for important intellectual content:* Derré, Nardelli-
155 Haefliger, Chevalier, Speiser, Jichlinski, Bohner, Pieraerts, Cesson, Lhermitte, Rotman,
156 Nobile, Gourmaud, De Leval, Legris, Dartiguenave.
157 *Statistical analysis:* Derré.
158 *Obtaining funding:* Derré, Jichlinski.
159 *Administrative, technical, or material support:* Chevalier, Bohner, Pieraerts Lhermitte,
160 Cesson, Legris, Dartiguenave, Gharbi, Martin, Rotman, Nobile, Gourmaud, De Leval.
161 *Supervision:* Derré.

162   *Other (specify): None.*

163

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165   interests and relationships and affiliations relevant to the subject matter or materials discussed

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175

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209

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 $Lin^{neg}HLA-DR^+CD123^+$, CD1c+ DC as $Lin^{neg}HLA-DR^+CD1c^+$ and $CD141^+$ DC as $Lin^{neg}HLA-DR^+CD141^+$.

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 $3\text{-}4\text{x}10^8$ PBMC from healthy individuals using the Dynabeads® human DC enrichment kit (Thermo Fisher Scientific). Cells were subsequently stained and live $\text{Lin}^{neg} \text{HLA-DR}^+$ 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
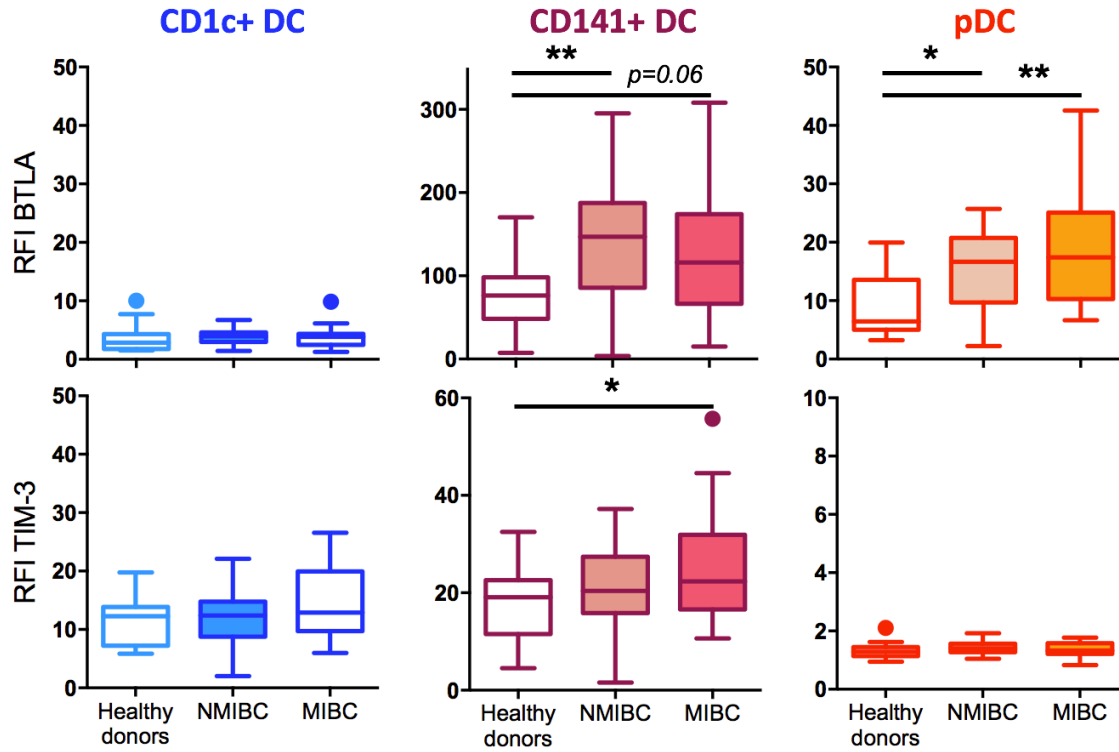MIBC = Muscle Invasive Bladder Cancer
NA = Not Available

Figure 1
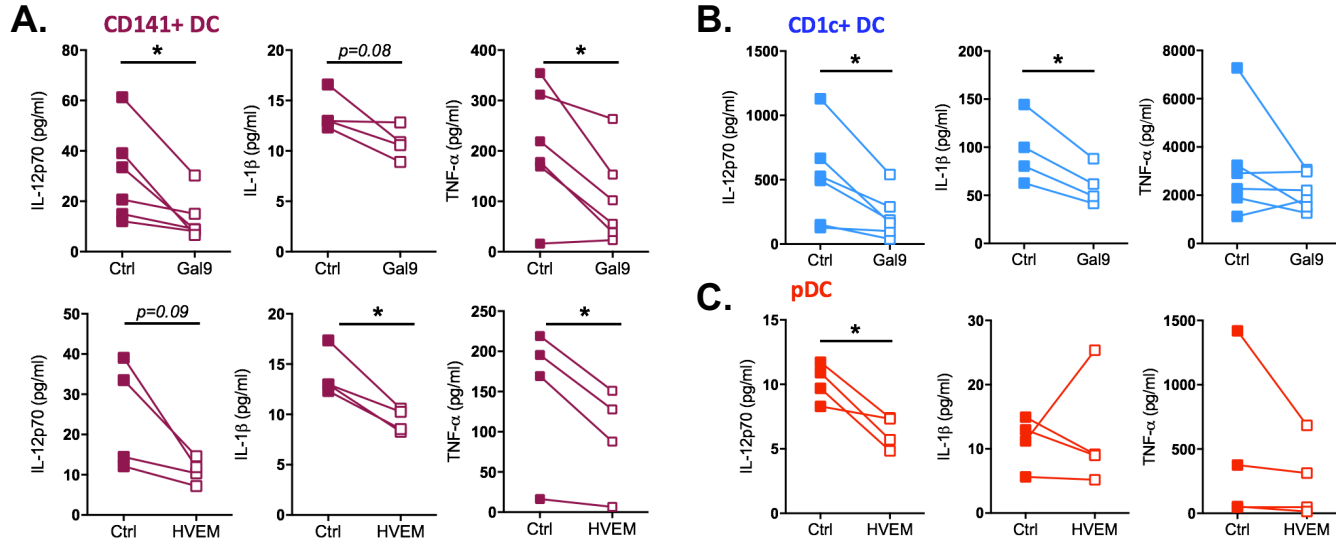
Figure 2

Supplemental Figure 1 :
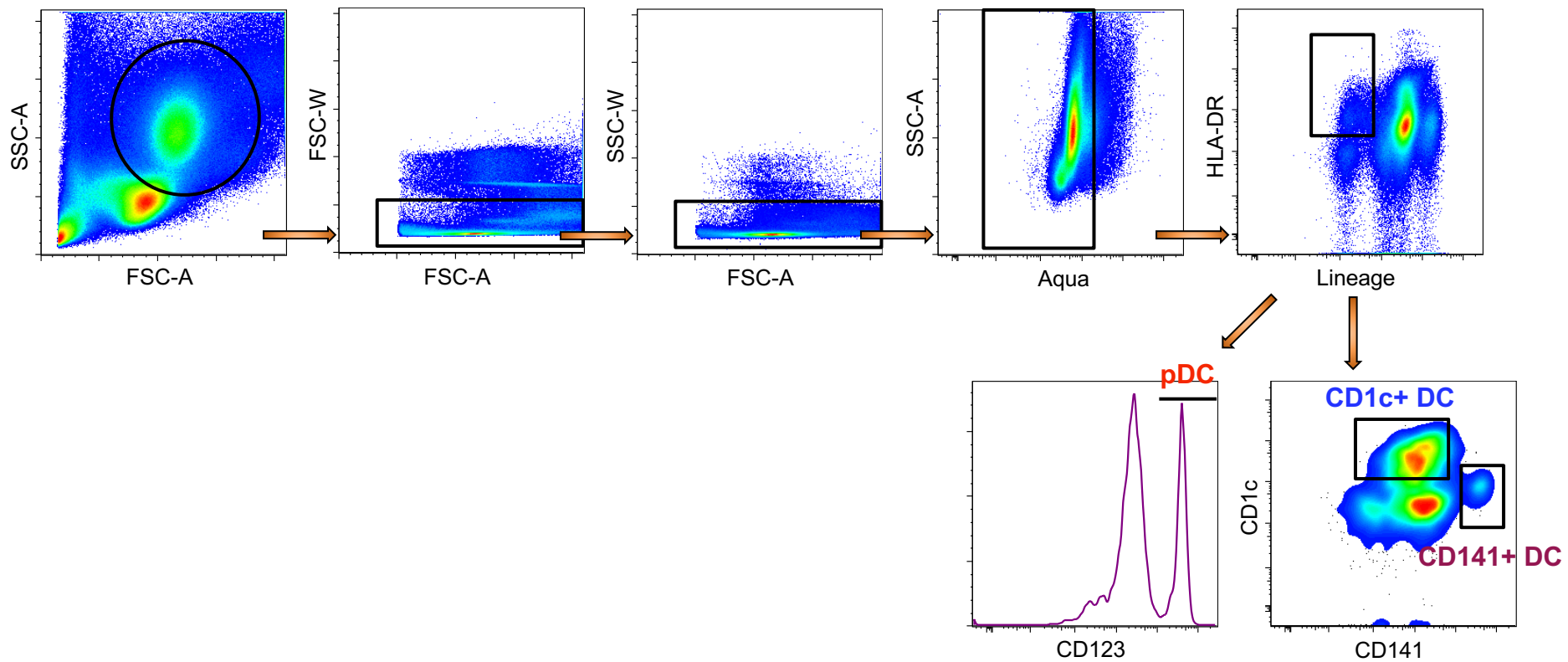
Supplemental Figure 2 :

Supplemental Figure 3 :