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Diuron modulates the DNA methylation status of the *ILT7* and *TRAIL/TNFSF10* genes and decreases the killing activity of plasmacytoid dendritic cells

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Abstract

Background: Plasmacytoid dendritic cell (pDC) is described as the Swiss knife of immune system. Thus, the understanding of aberrant epigenetic reprogramming of genes governing the pDC functionality by pollutants appears such as an attractive research point.

Results: Our study has investigated the effect of Diuron (an herbicide) on the pDC-killing activity towards cancer cells. Thus, we observed that the Diuron exposure of pDC promotes a context of global DNA hypomethylation, which is associated with a phenotype of decrease of the killing activity of pDC towards cancer cells. At molecular level, our data associated the Diuron-induced global DNA hypomethylation with the elevated expression of *TET2*, an epigenetic player involved in DNA demethylation processes, and the decrease of the pDC-killing activity with the decrease of *TRAIL* expression and the increase of *ILT7* expression.

Conclusions: Thus, our article reports that a pollutant (Diuron) induces an epigenetic reprogramming of a subtype of immune cell (pDC), which decreases its killing activity towards tumors cells. In some context, this mechanism might be conducive to the initiation of pathologies.

Keywords: DNA methylation, Plasmacytoid dendritic cells, *TET2*, Diuron

Background

Originally described in human lymph nodes in the 1950s plasmacytoid dendritic cells (pDCs) are a rare type of immune cells since these cells constitute < 0.4% of peripheral blood mononuclear cells (PBMC) [1, 2]. Despite this relative rarity, pDC plays a crucial role in immune response since they link the innate and adaptive immune systems [3–5]. Thus, pDC has potential multifaceted roles in the pathogenesis of autoimmune diseases, allergy, cancer and human immunodeficiency virus (HIV) infection [6]. Consequently, the understanding of aberrant

epigenetic reprogramming of certain genes governing functionality of pDC by pesticides appears such as an attractive research point. Besides literature reports that certain genes involved in pDC functionality can be epigenetically regulated by DNA methylation process. As example, pDCs can act as tolerogenic cells when expressing the programmed death 1 ligand (PD-L1) [7], and the PD-L1 expression can be regulated by the DNA methylation status of its promoter [8]. pDC-killing activity can be mediated by *TRAIL* [9] and Granzyme-B, which expression can be epigenetically regulated [10–12]. The *ILT7* expression, in which the interaction with *BST2* assures an appropriate TLR response by pDCs during viral infection and participates in pDC-tumor crosstalk, can be also epigenetically regulated by DNA methylation [13, 14].

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Diuron is a substituted urea compound used as herbicide and antifouling. Consequently, the Diuron toxicity can affect all the aquatic ecosystem (fish, algae, oyster, etc.) but also human health. Thus, literature reports that Diuron modulates the methylome of oysters [15–17], and affects the multixenobiotic resistance activity in Zebrafish [18]. Reports also mention a health risk for population exposed to Diuron [19–23].

The current study analyzed the putative effect of a Diuron exposure on the pDC-killing activity via the investigation of the DNA methylation status of genes regulating this activity.

Methods

Cell culture and exposure

The plasmacytoid dendritic cell line CAL-1 (Dr. T Maeda, Nagasaki University, Japan) has been derived from a blastic plasmacytoid dendritic cell neoplasm (BPDCN) patient [24]. These cells were cultured with RPMI 1640 supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin, and maintained at 37 °C in a humidified atmosphere containing 5% CO₂-air. Cells were exposed to Diuron (Santa Cruz, France) dissolved in dimethyl sulfoxide, (DMSO, Sigma). Control cells were exposed to 0.1% DMSO.

DNA extraction, 5mC ELISA and qMSRE

DNA extract is performed using QIAamp DNA Mini QIAcube Kit and QIAcube (Qiagen, France).

The quantification of 5-methylcytosine is performed using 5mC DNA ELISA kit (Zymo, Ozyme, France) according to the manufacturer's instruction.

qMSRE combines the use of methylation-sensitive restriction enzyme and real-time PCR. Briefly, 20 ng of DNA was digested or not by Hpy188III and HpyCH4IV. Then, 5 µL of digested or not mix solutions were used to perform qPCR using the Rotor-Gene SYBR Green PCR Kit and Rotor-Gene Q as real-time thermocycler (Qiagen, France). Primers are: ILT7-S: TGTGAGGACCCTGGACTTCCTTTT, ILT7-AS: CAGCCTGGGCAACAA GAACAAA, TRAIL-S: ATGCCTGTAATCCCAGCA CGTT and TRAIL-AS: GGTTTTACCATGTAGGCC AGGCT. The methylation level for any amplified region can be determined using the following equation: Percent Methylation = $100 \times 2^{-\Delta Ct}$ where ΔCt = the average Ct value from the digested reaction minus the average Ct values from the reference/undigested reaction. If DNA is methylated, Hpy188III and HpyCH4IV will not be able to digest DNA, and the DNA region will be amplified by PCR.

RNA extraction and RT-qPCR

RNA extract is performed using RNeasy Mini QIAcube Kit and QIAcube (Qiagen, France). RT-qPCRs are performed using QuantiTect Reverse Transcription Kit, Rotor-Gene SYBR Green PCR Kit, QuantiTect Primer Assays and Rotor-Gene Q as real-time thermocycler (Qiagen, France). Normalization of gene expression was performed using RPLP0. The $^{-2\Delta\Delta Ct}$ method was used to calculate the fold change of mRNA expression between two conditions.

Protein extraction and TET2 ELISA

Protein extracts were obtained using RIPA Lysis and Extraction Buffer (Thermo Scientific, France) in accordance with the manufacturer's instructions. TET2 ELISAs were performed according to the manufacturer's instructions (MyBiosource, MBS9317739, USA).

siRNA transfection

In a six-well culture plate, 2×10^5 cells were incubated for 24 h at 37 °C in a CO₂ incubator. Then, 60 pmol of siRNA was added with 100 µL of siRNA transfection medium and the cells incubated for 7 h at 37 °C in a CO₂ incubator (Santa Cruz, France). Without removing the siRNA, 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration was added and cells incubated for 24 h. Then, cells were cultured for 48 h in normal culture medium. Thus, analyses were realized ~72 h after the siRNA transfection. The following siRNA were used: siRNA-A (control) (sc-37007, Santa Cruz) and TET2 (sc-88934, Santa Cruz, France).

Cytotoxicity assays

CAL-1 cells (7×10^5) were seeded overnight in a 24-well plate in 2 mL medium devoid of Diuron. A total of 1.1 mL medium was replaced with 100 µL FBS-free RPMI medium containing 12.5 µg/mL CpG B or Ctrl CpG B (Invivogen, France). The ability of pDCs to kill tumor cells was assessed in a classic Europium-TDA release assay (DELFI; PerkinElmer), according to manufacturer's instructions. Briefly, tumor cells were labeled with the fluorescence-enhancing ligand BATDA, which was released into the supernatant after cytolysis. Tumor cells or PBMC and pDC were then co-cultured for 4 h at indicated E/T ratios. Supernatants were collected and the released Europium-TDA release was counted using a fluorometer. Target cells were treated with 1% NP40 and sonicated as a measure of maximal release. Target cells incubated without effector cells were used to measure spontaneous release. Percent of specific lysis was calculated using the following equation: % specific

lysis = [experimental release – spontaneous release] / [maximum release – spontaneous release] × 100.

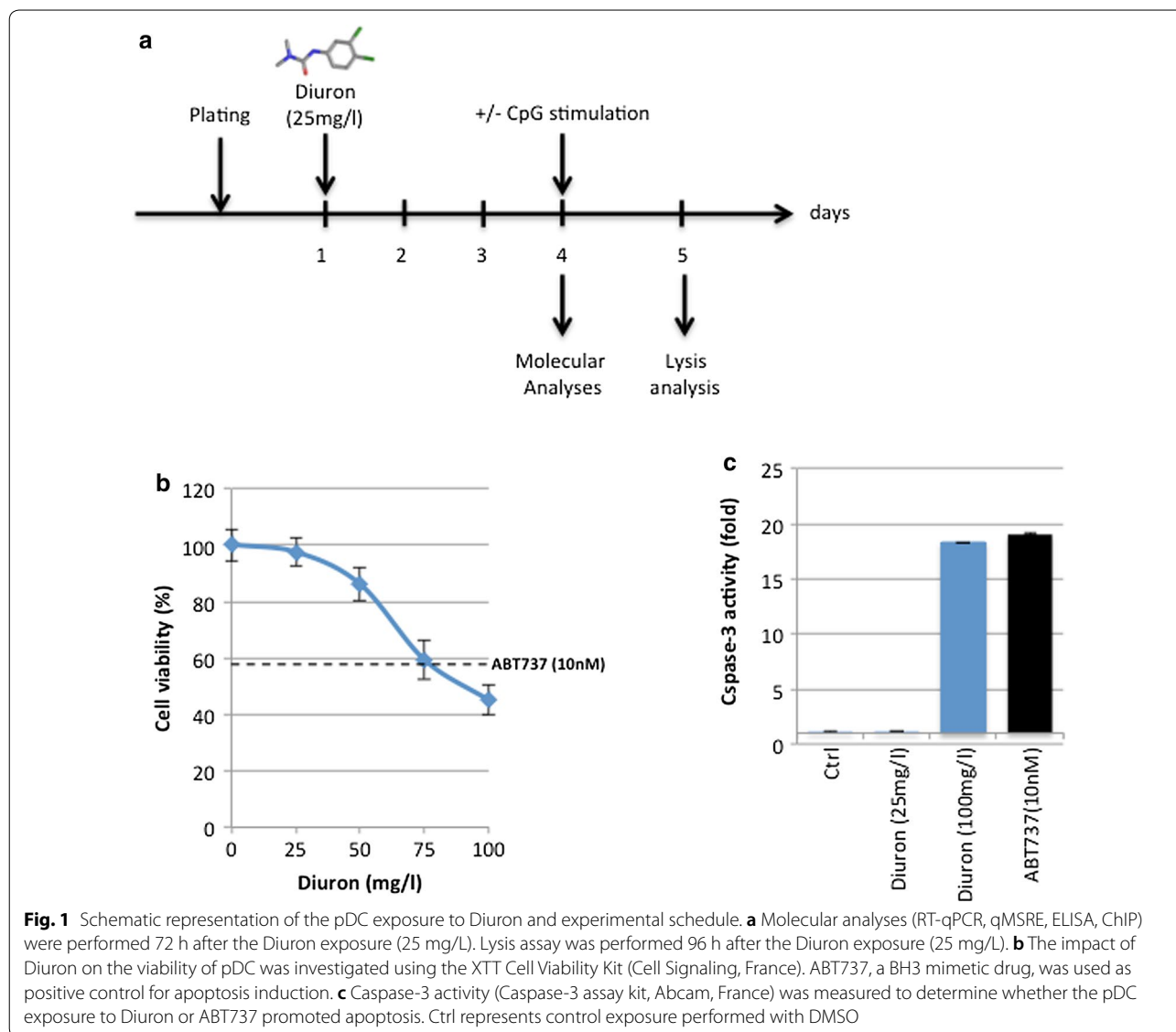
Chromatin immunoprecipitation (ChIP) experiments

Briefly, cross-linking step was performed by adding 37% formaldehyde to a final concentration of 1% [12]. Cells were incubated on a rotator for 10 min at room temperature (RT), and formaldehyde was quenched by adding 1.25 M glycine to a final concentration of 125 mM. Cells were rocked for 5 min at RT, washed with cold PBS, and flash-frozen in liquid nitrogen. Cells were lysed in buffer containing 1% SDS, 5 mM EDTA, and 50 mM Tris-HCl (pH 8.0) with freshly added protease inhibitor. The chromatin was fragmented to 200–500 bp with a Bioruptor plus sonicator (Diagenode, France) at 4 °C.

ChIP experiments were performed using ChIP-IT kit (Active Motif, France). The ChIP-grade Anti-CTCF (Abcam#Ab70303, France) and IgG (Abcam#AB2410, France) were used. DNAs eluted from ChIP were applied to real-time qPCR analysis using a QuantiFast SYBR green qPCR (Qiagen, France) on the Rotor-Gene Q (Qiagen, France). ChIP-quantitative PCR enrichment of target loci was normalized to input DNA.

Statistical analysis

All experiments were done at least in triplicates. Significance of the differences in means was calculated using Student *t* test. The probability level for statistical significance was *p* < 0.05 throughout the study.



Results

Diuron exposure promotes the TET2-mediated DNA hypomethylation of pDC

Literature reports that Diuron affects the global DNA methylation level [15–17]; we first investigated whether Diuron exposure could affect the global DNA methylation level of the pDC-like cell line CAL-1. For this purpose, CAL-1 cells were exposed to 25 mg/L of Diuron for 72 h since this herbicide and four of its metabolites had a total concentration as high as 100 mg/L in plasma and urine [25] (Fig. 1a). The dose of 25 mg/L of Diuron was also chosen since it had no effect on the cell viability, on contrary to ABT737, a BH3 mimetic drug-inducing apoptosis [26, 27] (Fig. 1b). We also observed that the dose of 25 mg/L of Diuron was unable to promote Caspase-3 activation, on contrary to ABT737 (Fig. 1c). The dose of 25 mg/L of Diuron being devoid of toxicity/apoptogenicity and “compatible” with a dose observed in human blood, we decided to continue our study with this dose.

The global DNA methylation level was here estimated by ELISA quantifying the presence of 5-methylcytosine (5mC). Thus, we noted that Diuron exposure decreased the percentage of 5mC (Fig. 2a). In other terms, Diuron promotes the global DNA hypomethylation of pDC.

RT-qPCR performed to quantify the mRNA encoding for the major DNA methylation players shown that the Diuron exposure promoted the TET2^{mRNA} overexpression (Fig. 2b). Our study also indicated that the Diuron

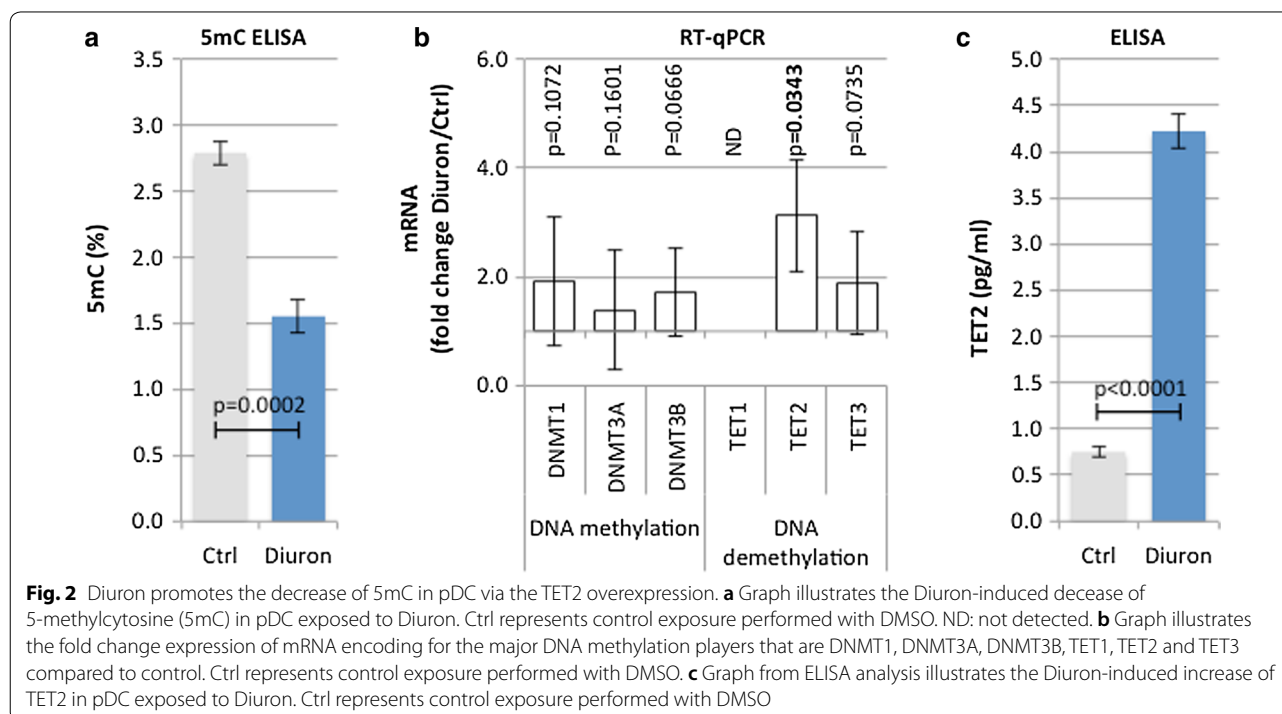
exposure promoted the TET2 protein overexpression (Fig. 2c).

Diuron exposure of pDC decreases the killing activity of these cells

To analyze the impact of the Diuron-induced DNA hypomethylation on the pDC-killing activity, we have compared the cell lysis activity of CpG-stimulated pDC previously exposed or not to Diuron (Fig. 1). The calculation of the cell lysis percentage indicated that the Diuron exposure decreased the killing activity of these cells toward several tumor cells such as glioblastoma cells (Fig. 3a), breast cancer cells (Fig. 3b), and ovarian cancer cells (Fig. 3c), but not toward PBMC (Fig. 3d).

The Diuron-mediated decreased killing activity of pDC is associated with the modulation of the methylation status of ILT7 and TRAIL

Several molecular actors play a crucial role in the killing activity of pDC. In our study, we have focused our analysis on PD-L1, ILT7, TRAIL and Granzyme-B, i.e., on genes whose expression can be epigenetically regulated. The RT-qPCR analyses comparing the CpG-stimulated pDC pre-exposed or not to Diuron indicated that Diuron exposure increased the ILT7^{mRNA} expression and decreased the TRAIL^{mRNA} expression. No expression changes were observed for PD-L1^{mRNA} and Granzyme-B^{mRNA} (Fig. 4a). ELISA experiments



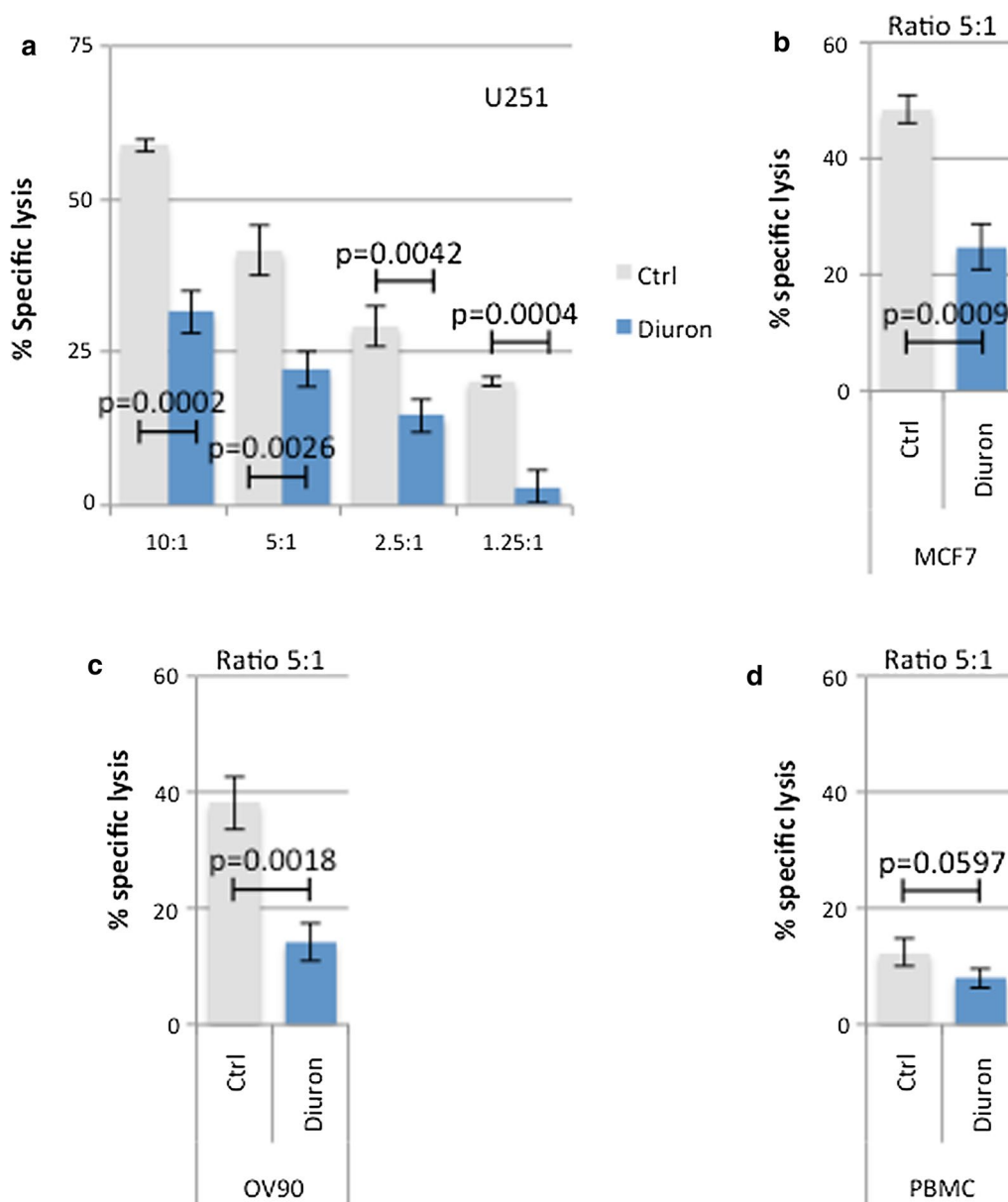
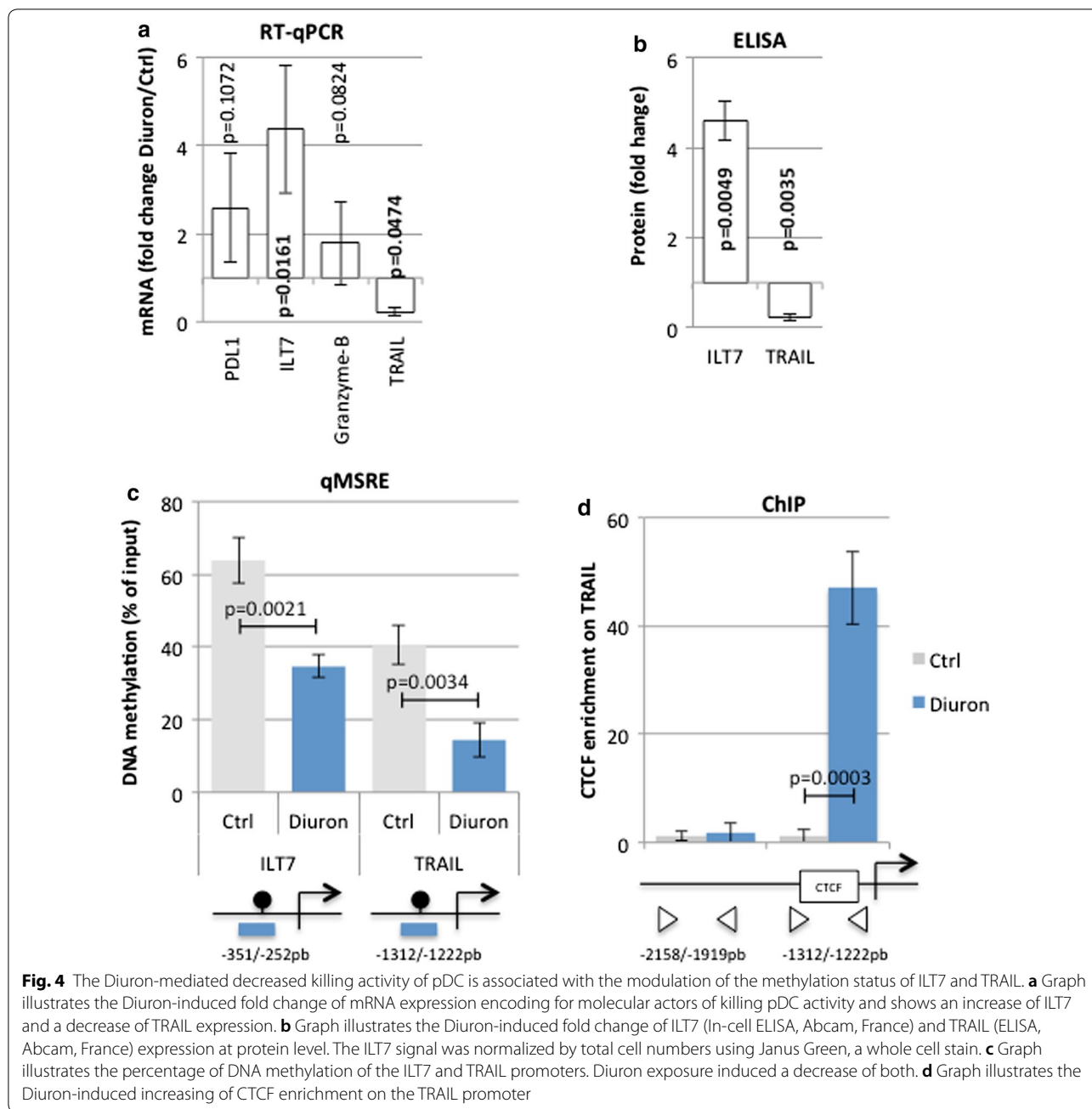


Fig. 3 Diuron exposure of pDC decreases the killing activity of these cells. After a pre-exposure to Diuron or DMSO (Ctrl), CAL-1 cells were stimulated by CpG for 36 h, and were co-cultured with U251 (a), MCF (b) OV90 (c) cells and PBMC (d) for another 4 h. The percentage of specific lysis was determined by cytotoxicity assay. Specific lysis (%) = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100

indicated that Diuron exposure increased the ILT7 expression and decreased the TRAIL expression (Fig. 4b). qMSRE investigating the methylation level of the TRAIL and ILT7 promoters shows an hypomethylation in pDC pre-exposed to Diuron (Fig. 4c). Thus, our data associated with the DNA hypomethylation of the ILT7 promoter with a gain of mRNA expression in

pDC pre-exposed to Diuron, which agrees closely with the dogma: “DNA demethylation promotes a gain of transcription”. Paradoxically, our data indicated that the DNA hypomethylation of the TRAIL promoter is associated with a loss of mRNA expression in pDC pre-exposed to Diuron. By analyzing the hypomethylated region of TRAIL promoter with the CTCFBS



prediction tool, we noted that this region included putative binding sites for the transcriptional repressor CTCF. CHIP experiments confirmed that CTCF was recruited on the Diuron-induced hypomethylated region of TRAIL promoter (Fig. 4d). Taken together, these data support the idea that the Diuron-mediated decreased killing activity of pDC is associated with the modulation of the methylation status of ILT7 and TRAIL.

siRNA TET2 abrogates the cascade of events promoting decreased killing activity of pDC

Our data suggest that TET2 plays a crucial role in the Diuron-mediated decreased killing activity of pDC via the modulation of the methylation-mediated expression of ILT7 and TRAIL. Based on this finding, we next analyzed the impact of the siRNA-induced TET2 down-regulation on the TET2 expression (ELISA), the global level of 5-methylcytosine (5mC, ELISA), the methylation level of ILT7 and TRAIL promoters (qMSRE), the

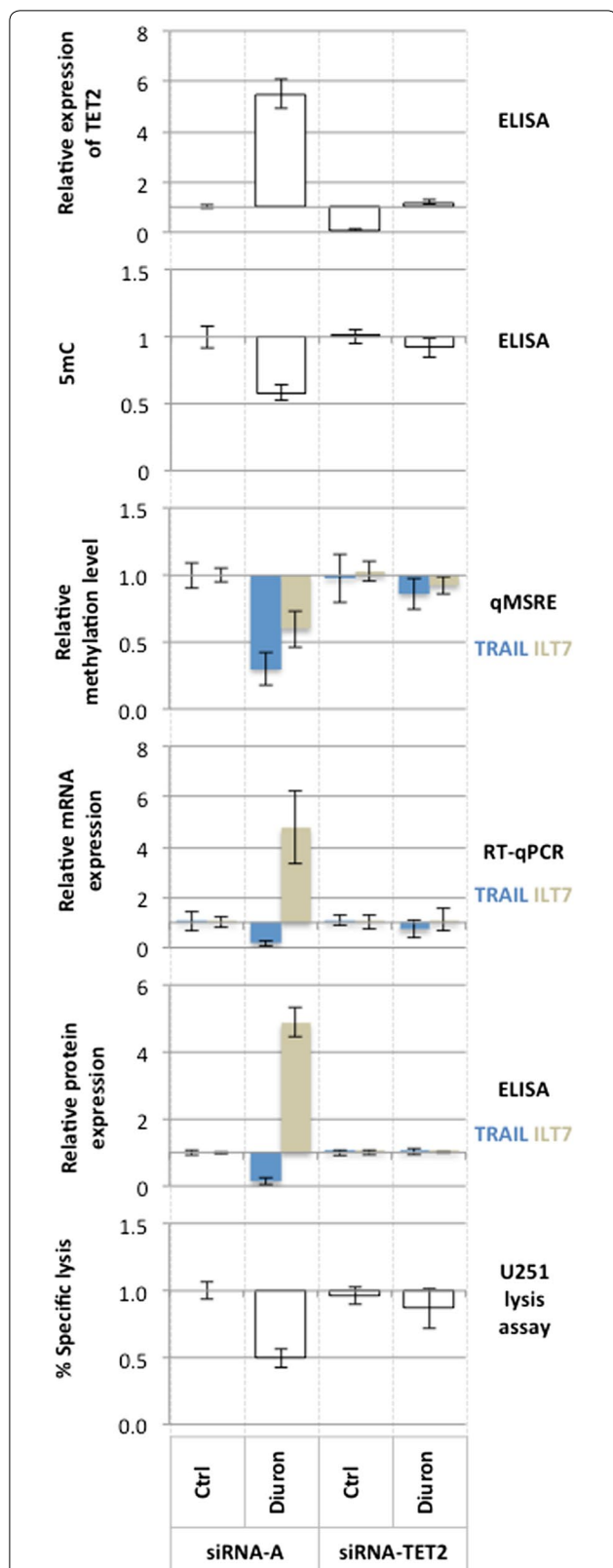


Fig. 5 Impact of the siRNA-mediated TET2 down-regulation on the cascade of events promoting decreased killing activity of pDC. Each parameter was calculated relatively to the siRNA-A/Ctrl condition

expression of TRAIL and ILT7 at mRNA (RT-qPCR) and protein (ELISA) levels and on the lysis toward U251 cells. As expected, we noted that the siRNA-induced TET2 down-regulation abrogated the decreased killing activity of pDC by abolishing the ILT7 overexpression and the TRAIL down-regulation occurring in a context of local and global DNA hypomethylation (Fig. 5).

Discussion

Due to its role of Swiss knife of immune system (such as described by Karrich et al. [28]), the understanding of aberrant epigenetic reprogramming of genes governing functionality of pDC by pesticides appears such as an attractive research point. Our data indicated that the Diuron exposure decreased the killing activity of pDC towards three different cancer cell types (glioblastoma, breast cancer, and ovarian cancer) but not toward PBMC. Thus, it appears, in some way, that the Diuron exposure impoverishes the immunosurveillance concept by acting on one effective unit killing pre-cancerous and/or cancerous cells: the pDC. To date, few other publications report that Diuron affects the immune system via a process of immunotoxicity [29]. Domingues et al. report that rat exposure to Diuron presented a decrease in macrophages spreading, but no change was observed regarding the phagocytosis index [30].

Our data also identified a molecular pathway associated with the decrease of pDC-killing activity since the Diuron exposure promotes the TET2 overexpression, which promotes the TRAIL down-expression and the ILT7 overexpression in a context of local and global DNA hypomethylation. Besides, the dual observation of the loss of TRAIL expression (a cytokine-promoting cell death [31]) and the overexpression of ILT7 (whose interaction with BST2 at the surface of tumor cells promotes the immune tolerance [13]) is consistent with the idea that the Diuron exposure decreased the killing activity of pDC.

By incriminating a TET2-mediated mechanism in the Diuron-mediated reduction of the pDC-killing activity, our data are one of the first to show that an environmental pollutant can affect the immunity via an epigenetic mechanism. Indeed, literature reports that environmental pollutants can affect the immunity, but no reports (at our knowledge) clearly incriminate an epigenetic player in this process. Taylor et al. report that Ziram (a broad-spectrum fungicide) activates mitogen-activated protein kinases and decreases cytolytic protein levels in human natural killer cells [32]. Lepeule et al. reports that the subchronic exposure to traffic-related pollutants was associated with significantly reduced lung function in the elderly and that epigenetic mechanisms related to inflammation and immunity may influence these associations

[33]. However, the authors of this article did not clearly identify the involved molecular epigenetic mechanism in their observation.

Classically, the promoter DNA hypomethylation is associated with an increase of gene expression [34, 35]. Of course, certain individual cases refute this dogma. Our study illustrates one of these individual cases since the TRAIL DNA hypomethylation is associated with its down-regulation. Our experiments support paradoxically situation by showing that the hypomethylated area of TRAIL recruits CTCF, a transcriptional repressor. More general, it is easy and intuitive to think that the DNA methylation status of the different gene areas (promoter, regulator, enhancer, super-enhancer, insulator, and others) could have distinct effects on genes expression.

The health outcomes resulting from environmental exposure(s) are highly varied and remarkably complex. In this article, we report that Diuron, an herbicide, induces an epigenetic reprogramming of a subtype of immune cells, pDC, which decreases its killing activity towards tumors cells. In some context, this mechanism might be conducive to the initiation of pathologies (such as cancer) via a process of “pollutants-induced alteration of immune surveillance”. Besides, Nadeau et al. report that increased exposure to AAP (Ambient Air Pollution) is associated with hypermethylation of the Foxp3 locus, impairing Treg-cell function and increasing asthma morbidity [36].

Conclusion

Our study tends to reinforce the converging evidences supporting the fact that Diuron may be a potential tumorigenic substance. This effect was direct such as already reported to the bladder [14, 15], urothelial [16], skin [17, 18] and mammary [15–19] carcinogenesis or indirect via the decrease of immunosurveillance phenomenon. However, in the absence of study in human, our study should not be considered as an absolute proof of the guilt of Diuron in the occurrence of cancer but as a scientific rationale incriminating a potential cellular mechanism that may be at the origin of the initiation of cancer.

Abbreviations

pDC: plasmacytoid dendritic cell; PD-L1: programmed death 1 ligand; TRAIL: TNF-related apoptosis-inducing ligand; TET: ten–eleven translocation; PBMC: peripheral blood mononuclear cells.

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Authors' contributions

PFC and SP designed and coordinated the project. JB, MPJ, AN, and PFC performed all experiments. FMV, GBC, SP and PFC interpreted and discussed the data. PFC wrote the first version of the manuscript and all the authors reviewed and approved it. All the authors read and approved the final manuscript.

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Availability of data and materials

The datasets obtained and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicting interests.

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