RESEARCH ARTICLE

Role of the P2Y$_6$ receptor of UDP in the modulation of murine dendritic cell functions and Th1 polarisation of the immune response

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Abstract: Numerous studies have demonstrated the role of uridine diphosphate (UDP) and its P2Y$_6$ receptor in the inflammatory reaction and innate immunity. However, the importance of the P2Y$_6$ receptor in the adaptive immune response remains unclear. In this study, we demonstrate that the P2Y$_6$ receptor is functionally expressed in murine bone marrow dendritic cells (BMDC). UDP induced a Ca^{2+} transient in these cells that was decreased in P2Y$_6$-deficient mice. UDP also increased the endocytosis of fluorescein isothiocyanate-dextran (FITC-dextran) and amplified the secretion of interleukin 12-p70 (IL-12p70) induced by CpG; these responses were abolished in P2Y$_6$-deficient mice. *In vivo* experiments showed that the serum level of specific IgG2c after immunisation with ovalbumin was decreased in P2Y$_6$-deficient mice, while the level of specific IgG1 was unchanged. These data suggest that the P2Y$_6$-mediated effects of UDP on myeloid dendritic cells play a role in the *in vivo* Th1 skewing of the immune response.

Keywords: adaptive immunity, dendritic cells, UDP, P2Y$_6$ receptor

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1. Introduction

An increasing number of studies have shown that extracellular nucleotides and nucleosides modulate the inflammatory reaction and the immune response by a variety of mechanisms[1–3]. Adenosine triphosphate (ATP) and other nucleotides are now considered as danger signals or Damage Associated Molecular Patterns (DAMPs) that are released from injured or stressed cells by a variety of mechanisms including cell lysis, exocytosis of secretory granules and efflux through channels like connexins and pannexins[4,5]. Extracellular ATP modulates cell responses in an autocrine or paracrine way through the activation of the P2X receptors, which are nucleotide-gated ion channels, or of the G protein-coupled P2Y receptors[6,7]. ATP is degraded by ecto-nucleotidases into adenosine diphosphate (ADP) and later, adenosine, that also modulate the immune response through the P2Y$_{12}$ receptor and the A$_1$, A$_2$ and A$_3$ receptors, respectively[1,8].

Uridine nucleotides are also important and an increasing number of studies have demonstrated the role of uridine diphosphate (UDP) and its P2Y$_6$ receptor in

Role of the P2Y$_6$ receptor of UDP in the modulation of murine dendritic cell functions and Th1 polarisation of the immune response. © 2016 Dorothée Cammarata, et al. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses/by-nc/4.0/), permitting all non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

the inflammatory reaction and the immune response. The $P2Y_6$ receptor plays a role in the activation of monocytes/macrophages[9–12], microglial phagocytosis[13,14], secretion of various cytokines and chemokines from epithelial and other cells[15–21] and activation of microvascular endothelial cells during vascular inflammation[22]. The role of UDP and $P2Y_6$ receptors in adaptive immunity is less clear and apparently more complex. UDP has been shown to activate myeloid dendritic cells[23,24], but it is unclear if this action is mediated by the $P2Y_6$ receptor, since immature dendritic cells express the $P2Y_{14}$ receptor[25], which is also responsive to UDP[26,27]. The $P2Y_6$ receptor is also expressed in plasmacytoid dendritic cells, where UDP inhibits the production of interferon-α[28,29]. Expression of the $P2Y_6$ receptor has also been detected in activated $CD4^+$ lymphocytes[30,31]. It has been reported that activation of murine T cells by T cell receptor (TCR) stimulation is inhibited by a $P2Y_6$ antagonist[32], while a study on $P2Y_6$-deficient mice showed that the absence of $P2Y_6$ receptor on $CD4^+$ cells, but not antigen presenting cells, amplified the production of cytokines in a model of allergic pulmonary inflammation[31].

The aim of the present study was to clarify the roles of the $P2Y_6$ receptor in the activation of myeloid dendritic cells and their *in vivo* impact in a model of immunisation, using $P2Y_6$-deficient mice previously generated in our laboratory[10]. Our results show that the $P2Y_6$ receptor controls the biology of dendritic cells and that its expression is required for optimal IgG2c production *in vivo*.

2. Materials and Methods

2.1 Reagents

UDP, ionomycin, ovalbumin and fluorescein isothiocyanate-dextran (FITC-dextran, 40 kDa) were purchased from Sigma-Aldrich. Fluo-4AM was purchased from Invitrogen.

2.2 Animals

$P2Y_6^{-/-}$ mice that we previously generated were crossed with C57BL/6 mice for up to 10 generations[10]. All of the mice used in this study were spawned using heterozygous parents and housed in a sanitary protected animal facility.

2.3 Ethics Statement

All of the animal studies were authorized by the Animal Care Use and Review Committee of the Université Libre de Bruxelles (CEBEA IBMM protocol 44).

2.4 Dendritic Cells Generation

Bone marrow-derived dendritic cells (BMDC) were generated as previously described by Inaba *et al.*[33]. Briefly, bone marrow cells obtained from femur and tibia were cultured for 7 days in complete medium [RPMI 1640 supplemented with 5% FCS (foetal calf serum; MultiCell Technologies, Lincoln, RI), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercaptoethanol] supplemented with 20 ng /mL GM-CSF (granulocyte-macrophage colony-stimulating factor; Invitrogen). Culture medium was partly refreshed on day 3 and 5.

2.5 Isolation of RNA, Reverse Transcription and PCR

Total RNA was extracted using Tripure reagent (Roche Applied Science, Basel, Switzerland) and quantified on a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). After DNase I treatment (Fermentas, Hanover, Germany), RNA was reverse transcribed using the Revert Aid H Minus M-MuLV Reverse Transcriptase (Fermentas). PCR (29 cycles) were performed using the Qiagen Taq Core Kit. Primer sequences used were HPRT: forward 5′-CCTGCTGGATTACATTAAAGCACTG-3′, reverse 5′-GTCAAGGGCATATCCAACAACAAAC-3′ and $P2Y_6$: forward 5′-GGTAGCGCTGGAAGCTAATG-3′, reverse 5′-AGGAACACCAAATCTGGCA-3′.

2.6 $[Ca^{2+}]_i$ Measurement

$[Ca^{2+}]_i$ was measured in BMDC as described[34]. Briefly, freshly isolated BMDC were labelled with the calcium fluorescent dye Fluo-4-AM (5 µM) in 250 µM (1,2)-diphenyl-4-[phenylsulfinylethyl]-3,5-pyrazolidin edione and pluronic acid F-127 (100 µg/mL). Cell fluorescence was monitored at 37°C, using a Becton Dickinson FACScan Flow Cytometer. Data were analysed with the Winmdi software. Results were expressed as a ratio of the maximal response induced by 300 ng/mL ionomycin added 2 min after agonist stimulation.

2.7 Endocytosis

BMDC were stimulated as indicated below in the complete medium described earlier. FITC-dextran (1 mg/mL) and UDP (100µM) were simultaneously added to the cell culture and incubated at 37 °C for 30 min. After 3

washes with cold PBS [phosphate-buffered saline; supplemented with 0.1% bovine serum albumine (BSA) and 0.1% sodium azide], cells were labelled with phycoerythrin conjugated anti-CD11c antibody (eBioscience) and then analysed using a FC500 flow cytometer (Beckman Coulter). Cells incubated with the probe at 4 °C were used as background controls. Trypan blue (0.05%) was used to quench extracellular fluorescence. Data obtained are mean fluorescence intensity (MFI) of CD11c positive cell population.

2.8 IL-12p70 Measurement

BMDC were cultured for 24 h in complete medium with CpG ODN 1826 (250 ng/mL) with or without UDP (100 µM). Cell-free supernatants were harvested and IL-12p70 concentration was measured with the Ready-Set-Go enzyme linked immunosorbent assay kits (eBioscience).

2.9 Mice Immunization

Mice were injected subcutaneously at the base of the tail with 100 µL of an emulsion (1:1) of incomplete Freund's adjuvant (IFA; Sigma-Aldrich) and ovalbumin (100 µg/mice) and bled 14 days later. Serum was obtained after centrifugation of clotted blood (2–6 h at 4"°C). Specific anti-ovalbumin immunoglobulins (Ig) isotypes G1 and G2c were quantified by ELISA (LO-IMEX, Brussels, Belgium). Briefly, 96-well flat-bottom plates were coated with ovalbumin (5 µg/mL in PBS) overnight at 4"°C, to quantify ovalbumin-specific IgG. Wells were saturated with 1% BSA in PBS for 1 h at room temperature. Serum samples were diluted in PBS/ 0.5% BSA and incubated for 2 h at 37"°C. Specific IgG subclasses were detected using horseradish peroxidase-conjugated rat anti-mouse IgG1 or IgG2c antibodies (LO-IMEX, Belgium).

2.10 Statistical Analysis

Statistical tests were performed using Sigmat Plot, version 13.

3. Results

3.1 Functional Expression of the $P2Y_6$ Receptor in Dendritic Cells

We first examined the expression of the $P2Y_6$ receptor in BMDC. As shown in Figure 1A, in freshly isolated bone marrow cells, we did not detect any expression of the $P2Y_6$ mRNA. After 3 and 7 days of culture in the presence of GM-CSF (20 ng/mL), we

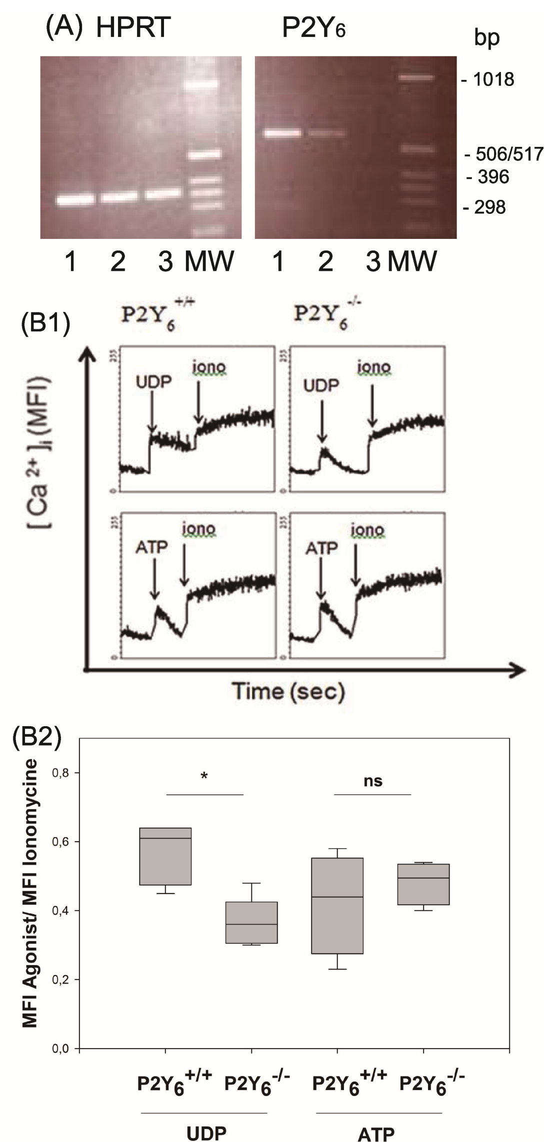

Figure 1. Expression of t he $P2Y_6$ receptor in BMDC. **(A)** RNAs of freshly isolated bone marrow cells (3) or of GM-CSF treated adherent bone marrow cells (2: 3 days; 1: 7days) were extracted and treated. RT-PCR was performed using specific primers for $P2Y_6$ or HPRT. bp: base pairs. **(B1)** $[Ca^{2+}]_i$ measurement in BMDC. Cells were loaded with Fluo-4-AM and fluorescence was registered by flow cytometry. After monitoring the basal fluorescence, cells were treated with UDP (100 µM) or ATP (100 µM) as positive control and fluorescence was recorded for 2 min. Then, cells were treated with ionomycin (300 nM) for norm alization. The dot plots (time versus FL1 fluorescence) shown are for each condition of one representative experiment out of two. **(B2)** Changes in intracellular calcium concentration in response of UDP or ATP in both genotypes. Each cell preparation has been tested in duplicates or triplicates. Results are shown as box-and-whisker plots of ratios of the maximal response measured after ionomycin treatment (median: bar into the box; most distant values: bars outside the box). $*p < 0.03$ using Holm-Sidak's test for multiple comparisons. ns: not significant.

detected by reverse transcription PCR (RT-PCR) a signal at the expected molecular weight (MW), reflecting the expression of the $P2Y_6$ mRNA. To check the functionality of the $P2Y_6$ receptor, we studied via flow cytometry the increase of cytoplasmic calcium concentration after UDP stimulation (Figure 1B). In wild type (WT) BMDC cells, we observed a marked elevation of $[Ca^{2+}]_i$ upon UDP (100 µM) stimulation. This UDP effect was reduced by 50% in $P2Y_6^{-/-}$ BMDC while the response to ATP stimulation was not affected. From these data, we can conclude that BMDC express a functional $P2Y_6$ receptor. The residual Ca^{2+} rise detected in $P2Y_6$-deficient mice might be due to the activation of the $P2Y_{14}$ receptor, a UDP-glucose receptor that is known to be sensitive to UDP in rodents[26] and to be expressed in human DCs[25].

3.2 Increased Antigen Uptake by BMDC Under $P2Y_6$ Receptor Stimulation

To characterize the effect of UDP and its receptor on endocytosis, we incubated $P2Y_6^{+/+}$ and $P2Y_6^{-/-}$ BMDC in complete medium containing FITC-Dextran known to be mainly captured by macropinocytosis[34]. The stimulation of wild type BMDC with UDP (100 µM) increased cell fluorescence of $CD11c^+$ cells by 2.36 ± 0.3-fold (mean ± SD of 7 BMDCs preparations analysed in 4 independent experiments). This UDP effect was abolished in $P2Y_6^{-/-}$ BMDC where mean cell fluorescence intensity of UDP-treated $CD11c^+$ cells was 1.05 ± 0.8-fold of non-treated cells (mean ± SD of 9 BMDCs preparations). The data of one representative experiment are shown in Figure 2.

The involvement of UDP in receptor-mediated endocytosis was also investigated using fluorescent ovalbumin (FITC-OVA) and CpG deoxynucleotide (FITC-CpG) as markers[35]. UDP enhanced the uptake of FITC-OVA by 1.21 ± 0.08 (mean ± SD of 6 BMDCs preparations analysed in 3 independent experiments) and that of FITC-CpG by 1.19 ± 0.07-fold (mean ± SD of 5 BMDCs preparations analysed in 2 independent experiments) (data not shown). Again, the stimulatory action of UDP was abolished in knockout BMDC (data not shown).

3.3 Increased Production of IL-12p70 by BMDC Under $P2Y_6$ Receptor Stimulation

IL-12p70 plays a key role in the DC-driven Th1 polarisation of T cells. UDP alone was unable to increase the secretion of IL-12p70 from BMDC (Figure 3). However, the CpG-triggered release of this cytokine

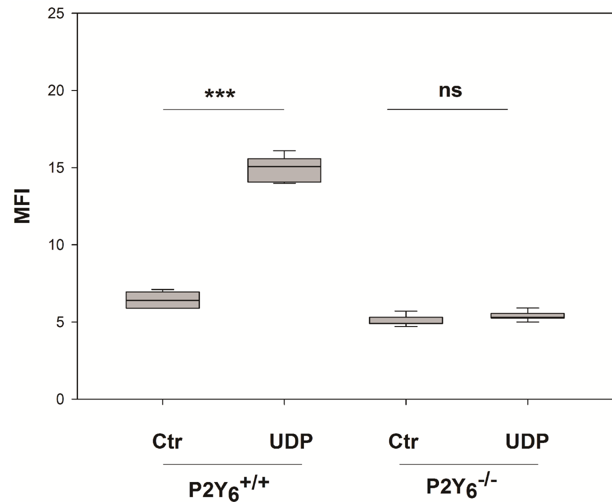

Figure 2. Effect of $P2Y_6$ activation on FITC-Dextran uptake by BMDC. WT and $P2Y_6^{-/-}$ BMDC were incubated with FITC-dextran (1 mg/mL) in the presence or absence of UDP (100 µM) for 30 min. Fluorescence of cells was recorded by flow cytometry. Results are shown as box-and-whisker plots of Mean of Fluorescence Intensity (MFI) of CD11c positive cells (median: bar into the box; most distant values: bars outside the box). The data are from one representative experiment out of 3. In this experiment, cell preparations from 2 WT mice and 3 from KO mice have been tested in duplicates or triplicates. ***$p < 0.001$ using the Holm-Sidak test for multiple comparisons. ns: not significant.

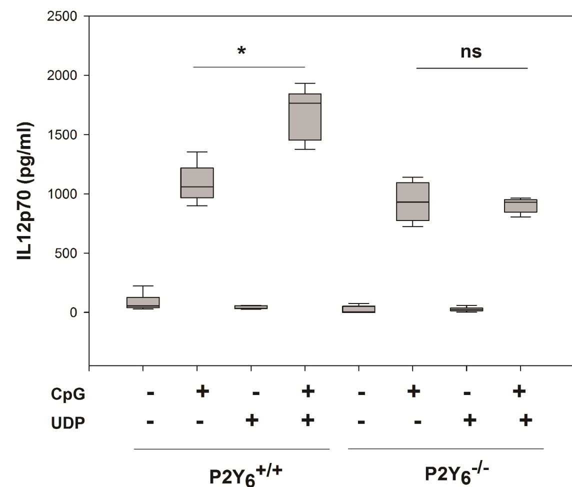

Figure 3. Effect of $P2Y_6$ activation on IL-12p70 production by BMDC. WT and $P2Y_6^{-/-}$ BMDC were incubated with CpG (250 ng/mL) in the presence or absence of UDP (100 µM) for 24 h. Supernatants were collected for measurement of IL-12p70 production by ELISA. Results are shown as box-and-whisker plots of the IL-12p70 concentration in the cells' supernatants (median: bar into the box; most distant values: bars outside the box). The data are from one representative experiment out of 3. In this experiment, cell preparations from 3 WT mice and 2 from KO mice have been tested in triplicates. *$p < 0.04$ using Dunn's test for multiple comparisons. ns: not significant.

by WT BMDC was amplified by 1.7 ± 0.6-fold (mean ± SD of 5 BMDC preparations). This UDP effect was abolished in $P2Y_6^{-/-}$ BMDC where the production of IL-12p70 by UDP+CpG-treated KO BMDC was 0.8 ± 0.2-fold of the control (mean ± SD of 6 B MDC preparations). The data of one representative experiment are shown in Figure 3.

3.4 Decreased Production of Anti-ovalbumin IgG2c in $P2Y_6^{-/-}$ Mice

To study the involvement of the $P2Y_6$ receptor in the adaptive immune response *in vivo*, we subcutaneously injected an emulsion of ovalbumin (OVA) in incomplete Freund's adjuvant (IFA) into wild type and $P2Y_6$ knockout mice. Fourteen days after immunization, we determined the concentration of different anti-OVA immunoglobulins. As shown in Figure 4A, the serum concentration of IgG1 anti-OVA was comparable in $P2Y_6^{-/-}$ and $P2Y_6^{+/+}$ mice, while the anti-OVA IgG2c concentration was significantly reduced in $P2Y_6^{-/-}$ mice (Figure 4B). These results suggest that the $P2Y_6$ receptor is involved in a Th1 skewing of the adaptive immune response.

4. Discussion and Conclusion

UDP has been shown previously to act on myeloid DCs, in which it induced a cytosolic Ca^{2+} transient[23,24] and chemotaxis[24]. However, it was not proven that these actions were mediated by the $P2Y_6$ receptor. They could have involved the $P2Y_{14}$ receptor that is also responsive to UDP[26,27] and is expressed on human immature myeloid DCs[25]. Our study with $P2Y_6$-deficient mice definitely proves the functional expression of $P2Y_6$ in myeloid DCs, resulting in increased antigen endocytosis and IL-12p70 secretion. This profile of action is clearly different from that of other nucleotide receptors expressed on DCs. In particular ADP also stimulates antigen endocytosis through the $P2Y_{12}$ receptor[8], but this action is not associated with a stimulation of IL-12p70 secretion. Actually, ADP decreased IL-12p70 production through its degradation into adenosine and activation of A_{2B} adenosine receptors[34,37]. In human myeloid DCs, the $P2Y_{11}$ receptor of ATP induces semi-maturation, characterised by an increased expression of co-stimulatory molecules but an inhibition of IL-12p70[38,39]. The $P2Y_2$ receptor of ATP and UTP is mainly involved in DCs chemotaxis[40], whereas the $P2X_7$ receptor of ATP induces the secretion of IL-1β[41,42].

The $P2Y_6$-mediated stimulatory effect of UDP on antigen endocytosis associated with increased IL-12p70 secretion is expected to amplify immunisation *in vivo* with a Th1 skewing. This is supported by the observation of a lower serum level of anti-ovalbumin IgG2c in $P2Y_6$-deficient mice immunised against ovalbumin, while the level of specific IgG1 was unaffected. Indeed, a Th2 response is associated with a switching to IgG1, while a Th1 response is associated with switching to IgG2a, or IgG2c in C57BL/6 mice[43].

We postulate that local injection of antigen in adjuvant leads to the local release of UDP driving through the $P2Y_6R$ a Th1 immune response. Therefore, agonists

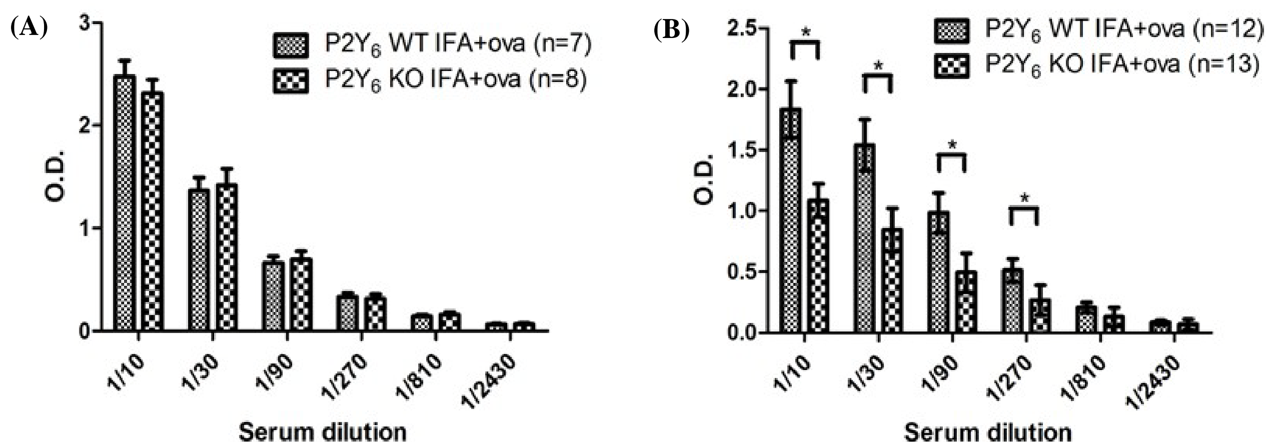

Figure 4. Immunoglobulin production in WT $P2Y_6$ and KO $P2Y_6$ mice. Mice were immunised subcutaneously with an emulsion of IFA and Ovalbumin. After fourteen days, mice were bled and specific anti-OVA IgG1 **(A)** or IgG2c **(B)** were determined in serum by ELISA. On the 14 (WT) and 15 (KO) mice immunized, 2 in each group did not respond in terms of IgG2c production (O.D. < 0); they have been discarded from the analysis. Data are expressed as mean ± SEM of O.D. from two **(A)** and three **(B)** independent experiments. *$p < 0.04$ using unpaired *t*-test (WT versus $P2Y_6$ KO are compared at the same dilution). *n* : number of mice.

of this receptor could play the role of adjuvant in case of vaccination directed against intracellular pathogens which require a Th1 cellular response of the immune system.

In conclusion, our study demonstrates that the $P2Y_6$ receptor of UDP is functionally expressed on murine myeloid dendritic cells, where it mediates a stimulation of antigen endocytosis and IL-12p70 secretion *in vivo*. This last action is expected to result in a Th1 skewing of the immune response, and this is supported by the decreased concentration of specific IgG2c in $P2Y_6$-deficient mice.

Author Contributions

Dorothée Cammarata, Abduelhakem Ben Addi, Eva D'Amico and Bernard Robaye were involved in the conception, design, data collection, assembly, analysis and interpretation. Dorothée Cammarata and Abduelhakem Ben Addi wrote the manuscript.

Conflict of Interest and Funding

The authors declare no commercial or financial conflict of interest.

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