

Impaired IFN- α secretion by plasmacytoid dendritic cells induced by TLR9 activation in chronic idiopathic urticaria

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Summary

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Background Understanding the early events of the immune response, through the activation of plasmacytoid dendritic cells (pDC) by Toll-like receptor (TLR)9-sensing, could contribute to the evaluation of immune dysregulation in chronic idiopathic urticaria (CIU).

Objectives We decided to investigate innate immunity in CIU and the mechanisms implicated in the modulation of interferon (IFN)- α production by pDC upon TLR9 activation.

Methods Patients with CIU ($n = 31$) and healthy control subjects (HC, $n = 36$) were enrolled in the study. Leucocytes cultured with the TLR9 ligand, CpG type A, or with inhibitory-oligodeoxynucleotide (ODN) were used to determine IFN- α secretion by enzyme-linked immunosorbent assay. Enumeration of pDC, intracellular IFN- α and signal transducers and activators of transcription protein (STAT) (1 and 4) phosphorylation were assessed by flow cytometry. TLR9 and regulatory factor-7 mRNA transcripts were evaluated by real-time polymerase chain reaction. Evidence of pDC in the skin lesions of patients was analysed with immunohistochemistry staining.

Results The findings show a decreased IFN- α secretion induced by CpG A by leucocytes, due to the diminished IFN- α expression on pDC in CIU. It was mediated by TLR9-activation since inhibitory-ODN further suppressed TLR9-induced IFN- α secretion. A normal pDC percentage and degree of activation by the expression of costimulatory molecules was observed in CIU, with the rare presence of pDC in the skin lesion. In addition, an increased constitutive STAT1 phosphorylation on nonstimulated lymphocytes and a downregulation of TLR9 mRNA transcripts after CpG A activation were verified in patients with CIU.

Conclusions The findings showed an innate immune response in CIU disturbed by impairment of the pDC response to TLR9 activation.

Innate immunity is the response of the body to various foreign pathogens by pathogen-associated molecular patterns (PAMPs), such as Toll-like receptors (TLRs), which trigger proinflammatory responses. Dendritic cells (DC) link innate and adaptive immunity, and can be distinguished by their ontogeny and their functional capacities in two major types; these are CD11c+ myeloid (mDC) and CD11c- plasmacytoid DC (pDC) cells.^{1,2} pDC, representing 0.2–0.8% of human peripheral blood mononuclear cells, express TLR7 and TLR9 that recognize viral and self nucleic acids within endosomal compartments and have the ability to produce exceptional amounts of IFN- α .³

The natural ligands for TLR9 are CpG motifs within microbial DNA.⁴ Synthetic CpG oligodeoxynucleotides have been identi-

fied, such as the CpG type A (ODN 2216), which is able to induce the production of large amounts of type I interferon (IFN) in pDC.⁵ The TLR9-mediated type I IFN induction in pDC is dependent on the adaptor protein MyD88, that subsequently forms a signalling complex with interleukin (IL)-1R-associated kinase (IRAK)1, IRAK4 and tumour necrosis factor receptor (TNFR)-associated factor 6.⁶ Essentially this event is required for the phosphorylation and activation of IRF7, a crucial factor for regulating IFN- α production, which is constitutively expressed in high amounts in pDC. On the other hand, to avoid excessive activation of TLR9 leading to inflammation or autoimmunity,⁷ specific DNA sequences containing the TTAGGGG motif have been reported present in mammalian telomeres where they are able to inhibit TLR9-mediated activation in

human as well as mouse cells.⁸ This inhibitory ODN could be a promising therapeutic option for autoimmune diseases.^{9,10}

pDC have been observed in inflammatory immunallergic dermatological disorders, in malignant cutaneous tumours and in cutaneous lesions of infectious origin.^{7,11} In the basal state pDC are absent from the skin, unlike mDC, which suggests that the presence of pDC in the skin in pathological conditions is the result of active recruitment. They seem to play a crucial role in the initiation of the pathological process of autoimmune diseases such as lupus erythematosus or psoriasis.^{7,12}

The investigation of the role of pDC in inflammatory disorders of the skin to confirm their ability to activate the TLR9 synthetic agonist could be an important strategy with potential therapeutic value. In this context, chronic urticaria (CU) is a persisting inflammatory disorder that lasts more than 6 weeks and is one of the most disabling types of urticaria.¹³ The main manifestation is urticarial lesions that may be accompanied by angioedema in 40% of patients.¹⁴ One-third of patients with CU have circulating autoantibodies against the α -chain of the high-affinity receptor for IgE (Fc ϵ RI), which allows them to be classified as presenting with chronic autoimmune urticaria.^{15,16} Other patients that have no obvious cause and are considered to have chronic idiopathic urticaria (CIU).¹⁴

The pathogenic mechanism of CIU remains unknown, although some studies have demonstrated that patients with CIU may have intrinsic abnormalities in basophils or mast cells.^{16,17} Another line of evidence suggested that the inflammatory process in CIU does not seem to be limited to the consequences of mast cell or basophil degranulation.^{18,19} Disturbed cytokine production has been described in CU, such as raised IL-4, IL-13 and IL-18 serum levels as well as factors related to the inflammatory process like neopterin and C-reactive protein.^{20–22} Moreover, supporting the concept of immunological dysfunction in CIU, an altered cytokine production by T cells, mainly of IL-17A and IL-10 production, has been seen regardless of skin reactivity to an autologous serum skin test (ASST).²³

Understanding the early stage of the immune response, through the innate immunity in CIU, could contribute to recognition of the immune dysregulation in this disease. We decided to investigate the ability of pDC in CIU to produce type I IFN after activation of oligodeoxynucleotides (ODNs) containing the immunostimulatory motifs CpG A at the cell-protein level, and to evaluate the expression of TLR9, signal transducers and activators of transcription protein (STAT 1 and 4) and regulatory factor-7 (IRF-7) mRNA transcripts. Also, we wished to verify the modulatory effect of inhibitory ODN in the activation of TLR9, to determine the number of pDCs among peripheral blood mononuclear cells and whether there is pDC recruitment to the skin lesions of patients with CIU.

Patients and methods

Study subjects

All patients with continuous CIU ($n = 31$; two males and 29 females; median age 52 years; age 22–65 years) were

selected from the Dermatological Outpatient Clinic of the Hospital das Clínicas de São Paulo (HC-FMUSP). These patients had weals daily for 6 weeks or longer, occurring at least four times a week. Patients with physical urticaria, urticarial vasculitis and food allergy were excluded, as previously described.¹⁷ None of the patients were taking corticosteroids or immunosuppressive drugs. Samples were taken from 36 individuals who were enrolled as healthy controls (HC; six males and 30 females, median age 44, aged 21–68 years). All the subjects gave written informed consent, and the study protocol was approved by the institutional ethics committee of the HC-FMUSP.

Oligodeoxynucleotides

The following phosphorothioate oligodeoxynucleotides (ODN) were used: CpG type A (ODN2216, 5'-GGGGGACGATCG TCGGGGG-3'); negative control (ODN2243 5'-GGGGGAG CAT GCTCGGGGG-3') and suppressive ODN (ODN A151, 5'-TTAGGGTTAGGGTTAGGGTTAGGG-3') synthesized by Eurofins MWG Operon (Huntsville, AL, U.S.A.). The bacterial endotoxin level was measured with the Limulus amoebocyte lysate assay and was < 0.03 endotoxin units mL^{-1} (Lonza, Walkersville, MD, U.S.A.).

Cytokine secretion

Fresh leucocytes were separated from EDTA blood with 5% dextran (Sigma-Aldrich, St Louis, MO, U.S.A.) sedimentation of erythrocytes. Leucocytes (5×10^5 cells/well) in 96-well microplates (Costar, Cambridge, MA, U.S.A.) were stimulated with varying molar concentrations (0.25 , 0.5 , 1.0 , $2.0 \mu\text{mol L}^{-1}$) of CpG (type A, 2216), negative control ODN (2243) in $200 \mu\text{L}$. In some experiments CpG suppressors (S, A151) were incubated together with CpG A ($1.0 \mu\text{mol L}^{-1}$ of each ODN) in RPMI 1640 with 5% fetal calf serum (RPMI-S, FCS, Hyclone; Gibco BRL, Sao Paulo, Brasil) for 48 h at 37°C in 5% CO_2 . Supernatants were collected, centrifuged, and analysed for IFN- α secretion by enzyme-linked immunosorbent assay (ELISA) (Endogen, Rockford, U.S.A.); the detection limit was 0.07 pg mL^{-1} .

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were isolated using a solution of Ficoll-Hypaque (Amersham Pharmacia Biotech, NJ, U.S.A.). For quantification of pDC, PBMC were stained with anti-BDCA-4 labelled phycoerythrin (PE) (Miltenyi Biotec, Bergisch Gladbach, Germany) and PE-Cy5 (cyanine 5)-labelled CD123 (IL-3Ra, 9F5), from BD Pharmingen (San Jose, CA, U.S.A.). For surface marker analysis of costimulatory molecules, PBMC were cultured with RPMI-S and CpG A ($2.0 \mu\text{mol L}^{-1}$) or ODN A ($2.0 \mu\text{mol L}^{-1}$) for 24 h at 37°C and 5% CO_2 . Cells were washed in phosphate buffer saline (PBS) or 0.5% bovine serum albumin (BSA), 0.1% sodium azide, and pDC were characterized as Lin/CD123+, using fluo-

rescein-isothiocyanate (FITC)-anti-lineage Cocktail1 (Lin1, a mix of CD3, CD14, CD16, CD19, CD20 and CD56), PE-Cy5-labelled CD123. Activation markers were evaluated using PE-CD40, CD80 or CD86 (BD Pharmingen, San Jose, CA, U.S.A.). For intracellular cytokine staining assays cells were activated with CpG A or ODN A ($2.0 \mu\text{mol L}^{-1}$) and $10 \mu\text{g mL}^{-1}$ brefeldin A (Sigma) was added to cells for the final 20 h of incubation. pDC were stained (Lin⁻/CD123⁺), fixed and permeabilized with buffer (Fix & Perm A and B, Invitrogen) and stained with antibody to PE-IFN- α (BD Pharmingen). After incubation, the cells were washed once in PBS with 0.5% BSA, processed with a Coulter EPICS XL-ML flow cytometer (Miami, FL, U.S.A.), and analysed with System II software.

Detection of STAT1 and STAT4 phosphorylation

PBMC were cultured with RPMI-S and CpG A ($2.0 \mu\text{mol L}^{-1}$) or ODN A ($2.0 \mu\text{mol L}^{-1}$) for 4 h at 37°C and 5% CO_2 . Cells were fixed with 4% paraformaldehyde for 10 min to 37°C , followed by 90% methanol for 30 min on ice. After washing, cells were incubated for 15 min at room temperature with $100 \mu\text{L}$ of PBS 2% FCS. The PBMC were washed with PBS 2% FCS and stained with alexa fluor 488-labelled anti-human STAT1pY701 (BD Phosflow, San Jose, CA, U.S.A.) or alexa fluor 488-labelled anti-human STAT4pY693 (BD Phosflow). At least 20 000 events of lymphocytes were acquired in the gate using a Coulter EPICS XL-ML flow cytometer.

Real-time polymerase chain reaction

Total RNA from PBMC was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, U.S.A.), and reverse transcription was done with a Sensiscript Reverse Transcriptase kit (Qiagen). Real-time PCR was performed with the following primers: albumin (sense 5'-ACAACAACATCCACAGCCAAGTGTC-3', antisense 5'-AAACTCATGGGAGCTGCTGGTT-3'); TLR9 (sense 5'-ACAACAACATCCACAGCCAAGTGTC-3', antisense 5'-AAGGC CAGGTAATTGTACGGAG-3'), IRF7 (sense 5'-TGGTCCTGGTG AAGCTGGAA-3', antisense 5'-GATGTCGTCATAGAGGCTGTTG G-3'). PCR was carried out in an Applied Biosystems 7300 Real-Time PCR system based on specific cytokine primers and general SYBR green fluorescence detection for 10 min at 94°C followed by 45 cycles of 15 s at 95°C , 30 s at 60°C and 30 s at 72°C . The relative quantitative expression was calculated as $2^{-\Delta\Delta\text{CT}}$.²⁴

Immunohistochemistry

Surgical specimens were fixed in alcohol/formalin/acetic acid and embedded in paraffin and sections were cut from 14 cases of confirmed CIU, deparaffinized in xylene and rehydrated with a descending alcohol series. Slides were incubated with anti-CD123 (eBioscience, San Diego, CA, U.S.A.) and with 'Post primary block' (NovoLink™ Max Polymer Detection System; Novocastra, Newcastle, U.K.), followed by

anti-mouse IgG (Novocastra) for 37 min at 37°C . Staining was completed with incubation with 3,3-diaminobenzidine tetrachloride (Sigma) followed by light counterstaining with Carazzi haematoxylin. Skin fragments from patients with lupus erythematosus were used as positive controls. Negative controls included parallel sections from adjacent tissue processed as described above, omitting the primary antibodies. From the 14 samples of skin from patients with CIU, only six samples could be analysed.

Statistical analysis

A Mann-Whitney U-test or Wilcoxon matched pairs test were used to compare variables between patients with CIU and healthy subjects. $P < 0.05$ was considered significant.

Results

Impaired cytokine secretion induced by TLR 9 activation in patients with chronic idiopathic urticaria

We evaluated the innate immunity in CIU regarding the ability to produce cytokines in response to the TLR9 ligand, CpG type A. Leucocytes were stimulated for 48 h with CpG A or ODN-control and IFN- α was measured in the supernatant.

Figure 1 shows that CpG A induces a significant increase in IFN- α secretion by leucocytes from the HC group and patients with CIU compared with the negative ODN controls. It is remarkable that IFN- α secretion mediated by TLR9 was hardly secreted by leucocytes from patients; this was significantly decreased compared with the HC group when 1 and $2 \mu\text{mol L}^{-1}$ of CpG A were used. Nonspecific IFN- α secretion was not induced by the ODN control.

Although very low levels of IFN- α secretion were induced by TLR9 activation in patients with CIU, we assessed the immunomodulatory effect of the suppressor ODN (ODN-S) on cytokine secretion induced by CpG A stimulation. This inhibitory ODN is able to prevent activation by TLR9.^{9,10} Figure 2 shows that ODN-S was able efficiently to inhibit IFN- α secretion in both HC and patients, achieving up to 98% inhibition. This finding confirmed that the impaired IFN- α secretion was due to TLR9 activation and not to a secondary effect.

The specificity of these results was obtained from the comparison with normal control individuals, although the ideal would be a comparison with controls with other disease.

Phosphorylation of STAT1 and STAT4 on lymphocytes from patients with chronic idiopathic urticaria

Next, we studied whether the altered IFN- α secretion induced by the TLR9 agonist in CIU could be related to the phosphorylation levels of STAT1 and STAT4. Both of them are related to the cellular response to IFN- α and IFN- γ .^{25,26} PBMC from patients with CIU and HC were stimulated with CpG A or control ODN for 4 h to evaluate phosphorylation of STAT1 and STAT4 by flow cytometry.

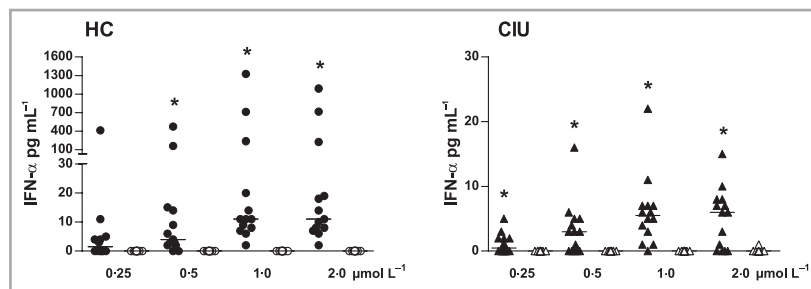


Fig 1. Impaired interferon (IFN)- α secretion induced by Toll-like receptor (TLR)9 ligand CpG A in patients with chronic idiopathic urticaria (CIU). Leucocytes from healthy control subjects (HC, $n = 13-15$) or patients with CIU ($n = 10-15$) were cultured at varying molar concentrations ($0.25, 0.5, 1.0, 2.0 \mu\text{mol L}^{-1}$) of CpG (type A, 2216, $\bullet$) or control oligodeoxynucleotide (ODN) ($\circ$) for 48 h at 37°C and 5% CO_2 . Supernatants were assessed for IFN- α secretion by enzyme-linked immunosorbent assay (ELISA). The horizontal line represents the median. * $P < 0.05$ when compared with control ODN.

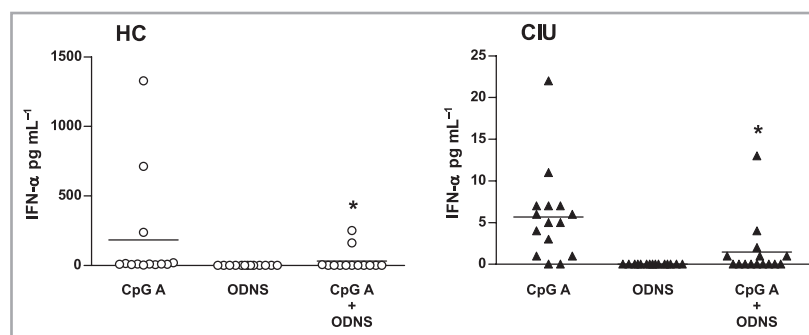


Fig 2. Inhibitory effect of oligodeoxynucleotide suppressor (ODN S) on the cytokine secretion induced by TLR9 ligand CpG A in patients with CIU. Leucocytes from healthy control subjects (HC, $n = 13-15$) or patients with CIU ($n = 10-15$) were cultured with CpG A plus ODN S (A 151, $1.0 \mu\text{mol L}^{-1}$ of each ODN) for 48 h at 37°C and 5% CO_2 . Supernatants were assessed for interferon (IFN)- α secretion by ELISA. The horizontal line represents the median. * $P < 0.05$ when compared with CpG A alone.

Histograms show the phosphorylation of STAT1 and STAT4 on lymphocytes, after stimulation with CpG A (Fig. 3). An increased percentage of STAT1 phosphorylation on nonstimulated lymphocytes was detected in patients with CIU (Fig. 3c), which was not observed for phosphorylated STAT4 (Fig. 3d). After CpG A stimulation, an increased percentage of STAT1 phosphorylation were detected in both patients and control, whereas only the cells from the HC group showed an increase in the percentage of phosphorylated cells for STAT4 (Fig. 3d). Evaluation of the intensity of phosphorylation STAT1 and 4 (Fig. 3e,f) revealed quite similar results shown as percentages. The data show an increased constitutive STAT1 phosphorylation on non-stimulated lymphocytes of patients with CIU, in contrast to a functional STAT phosphorylation induced by TLR9 activation.

Profile of activation of pDC induced by TLR 9 ligand in patients with chronic idiopathic urticaria

pDC after TLR9 stimulation are responsible for the rapid secretion of a large amount of type I IFN, thus we evaluated whether the impaired IFN- α secretion in TLR9-mediated activation

in CIU was due to an altered number of pDC or IFN- α secretion after CpG A stimulation.

Figure 4 shows that pDC percentage (CD123+ CD304+) on PBMC of patients with CIU (0.38 ± 0.14) was similar to that observed in HC (0.30 ± 0.12). In addition, the percentage of pDC (Lin-, CD123+) secreting IFN- α after CpG stimulation was significantly decreased in patients with CIU compared with control cells.

Furthermore, we evaluated the activation of pDC by costimulatory molecules expressed after TLR9 activation. Figure 5 shows the enhanced expression of CD40, CD80 and CD86 molecules on pDC mediated by TLR9 activation compared with baseline levels or with ODN-controls, in both HC and patients with CIU.

Downregulation of TLR 9 expression in chronic idiopathic urticaria

Figure 6 shows that TLR9 expression in PBMC from patients with CIU was significantly decreased at 8 h after stimulation with CpG A compared with that obtained in HC. This effect was transient as 24 h after stimulation a similar expression of TLR9 was found between groups. For IRF7 expression we

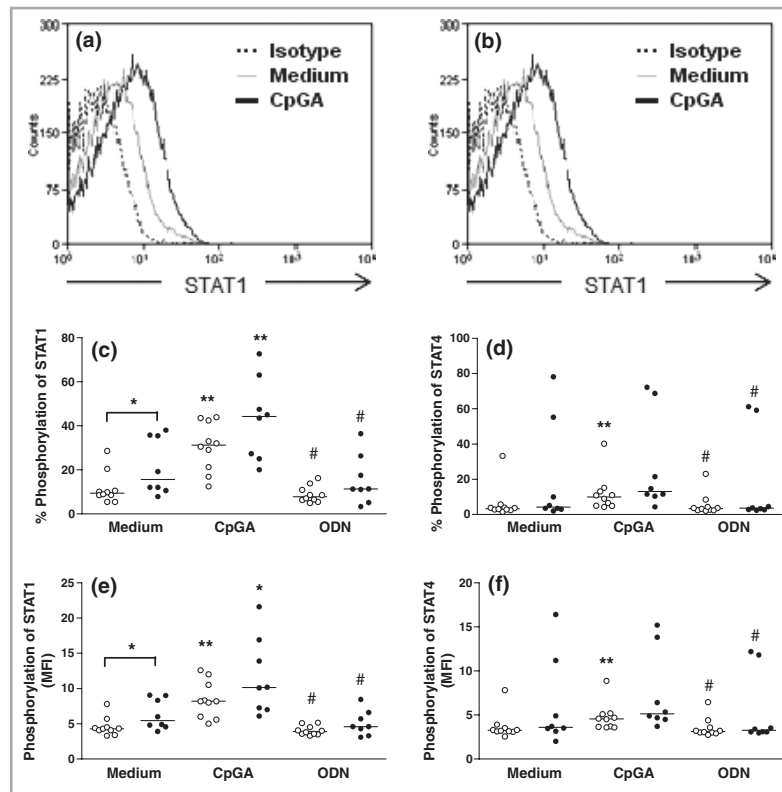


Fig 3. Phosphorylation of STATs induced by TLR9 ligand CpG A stimulation in patients with CIU. Peripheral blood mononuclear cells (PBMC) from healthy control subjects (HC) ($\circ$, $n = 10$) and patients with chronic idiopathic urticaria (CIU) ($\bullet$, $n = 8$) were cultured with CpG A ($2.0 \mu\text{mol L}^{-1}$) or control ODN for 4 h and assessed by flow cytometry. (a) Illustrative histograms of STAT1 and STAT4 phosphorylation gated on lymphocytes region; (b) percentage of STAT1 and (c) STAT4 phosphorylation induced by CpG A or control ODN, or mean fluorescence intensity (MFI) of (d) STAT1 and (e) STAT4 phosphorylation. The horizontal line represents the median. * $P < 0.05$, ** $P < 0.01$ when compared with unstimulated cells (medium); # $P < 0.05$ when compared with CpG A.

observed an increased expression at the time of CpG stimulation with PBMC from HC, but this effect was not verified in PBMC from patients with CIU.

These findings show that in CIU there is a normal pDC frequency in peripheral blood with an impaired intracellular IFN- α expression following CpG activation associated with a transient downregulation of TLR9 expression.

Plasmacytoid dendritic cells in skin lesions from patients with chronic idiopathic urticaria

Under steady state conditions, pDC are constantly released from bone marrow into the blood, from where they migrate into the T-cell-rich areas of secondary lymphoid organs such as lymph nodes through high endothelial venules (HEVs) or into inflamed skin.^{27,28} Thus, we evaluated the presence of pDC (CD123+) by immunohistochemistry in sections from skin lesions of CIU ($n = 6$). In all the samples analysed CD123+ cells were almost absent from the skin, though rarely some of the samples, as shown in Figure 7, showed the presence of CD123+ cells scattered along the cellular infiltrate of the skin of patients with CIU. In contrast, the skin of a patient with

cutaneous lupus erythematosus, a biopsy used as a positive control, showed a marked recruitment of CD123+ cells into the skin.

Discussion

In this work we have identified a disturbed innate immune response in CIU, related to TLR9-mediated activation and indicating a functional impairment of pDC. The findings showed a mild IFN- α secretion by pDC upon stimulation by CpG A, associated with a downregulation of TLR9 mRNA expression. In addition, a normal pDC percentage in the peripheral blood cells and rare pDC recruitment at the skin lesion in CIU was found. The alteration of innate immune response sensing through TLR9 by pDC supports the concept of immunological dysregulation in CIU.

We evaluated the innate immunity in CIU as an early step of the immune response, shown by the ability to respond to a TLR9 synthetic agonist. The results show a scarce IFN- α secretion by leucocytes activated with the agonist of TLR9, CpG type A, in CIU, which was confirmed by finding intracellular IFN- α expression on pDC. The cells expressing TLR9 are B

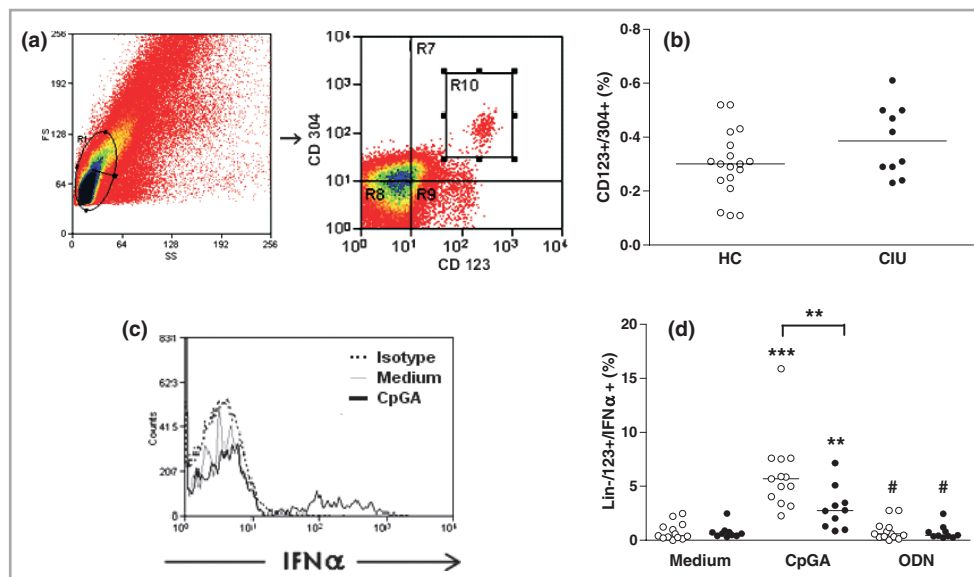


Fig 4. Altered pDC interferon (IFN)- α secretion induced by TLR9 ligand CpG A in patients with CIU. PBMCs from healthy control subjects (HC) ($\circ$, $n = 11-13$) and patients with chronic idiopathic urticaria (CIU) ($\bullet$, $n = 9-10$) were cultured with CpG A ($2.0 \mu\text{mol L}^{-1}$) or control ODN for 24 h, brefeldin A was added after 4 h and the culture analysed by flow cytometry. (a) Illustrative histogram of the lymphocyte gate region and (b) percentage of pDC (CD123+ CD304+); (c) Illustrative histogram of intracellular IFN- α expression in pDC (Lin-CD123+); (d) percentage of IFN- α expression in pDC. Horizontal line represents the median. $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with unstimulated cells (medium); $\#P < 0.05$ when compared with CpG A.

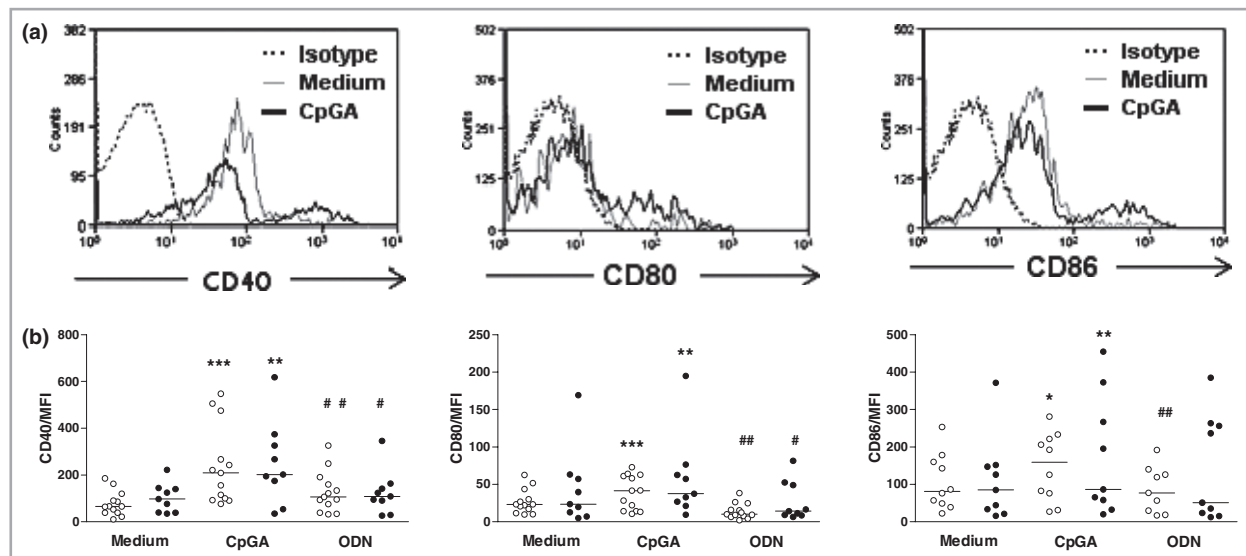


Fig 5. Costimulatory molecules expression on pDCs stimulated with TLR9 ligand CpG A. PBMCs from healthy control subjects (HC) ($\circ$, $n = 10-13$) and patients with CIU ($\bullet$, $n = 9$) were cultured with CpG A ($2.0 \mu\text{mol L}^{-1}$) or control ODN for 24 h and analysed by flow cytometry. Results represent the MFI of CD40, CD80 and CD86 by pDC (Lin-CD123+); (a) Representative phenotype of pDC from one of the healthy control subjects. (b) Activation/maturation marker expression on pDC. $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with unstimulated cells (medium); $\#P < 0.05$, $\#\#P < 0.01$ when compared with CpG A.

cells and pDC, whereas the CpG type A, a phosphodiester-linked sequence flanked by PS-linked poly-G ends, is known to be a potent inducer of IFN- α and IFN- γ but a weak activator of B cells. Moreover, in humans, this ODN stimulates pDC exclusively.²⁹

The inability to produce type I IFN upon CpG A stimulation in CIU could be a consequence of several mechanisms, possibly including an altered STAT phosphorylation. Evaluating STAT1 and STAT4, essential for mediating signalling by $\alpha/\beta/\gamma$ interferons,^{25,26} we verified that nonstimulated lymphocytes

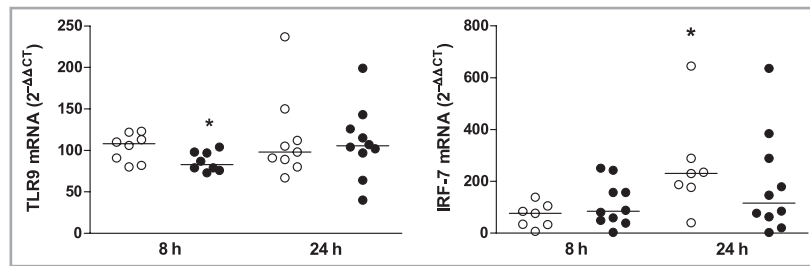


Fig 6. Decreased TLR9 mRNA expression in CIU. PBMCs from healthy control subjects (HC) ($\circ$, $n = 7-8$) and patients with CIU ($\bullet$, $n = 8-10$) were cultured with TLR9 ligand CpG A ($2.0 \mu\text{mol L}^{-1}$) for 8 or 24 h and analysed by real-time polymerase chain reaction. Unstimulated and stimulated cells were assessed for TLR9 and IRF7 mRNA expression and albumin as control gene. Horizontal line represents the median. * $p < 0.05$ when compared with HC after 8 h stimulation.

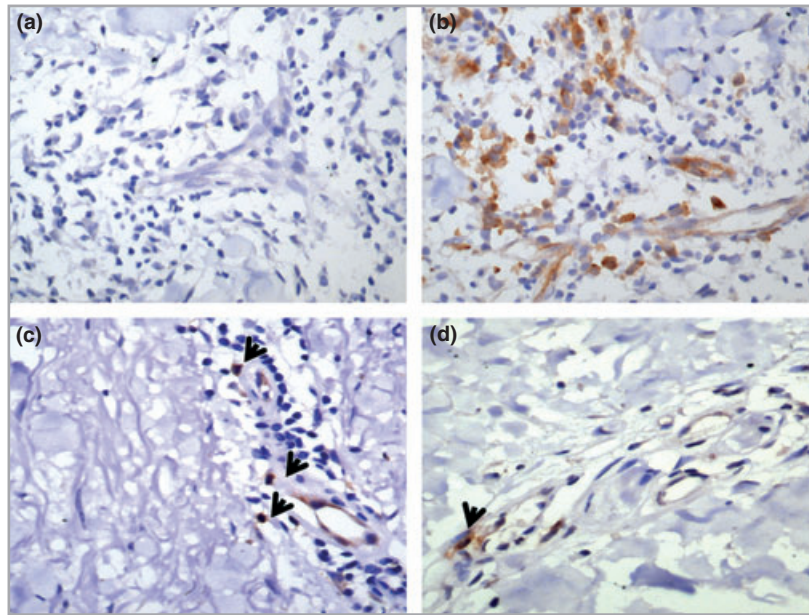


Fig 7. Evidence of pDC recruitment in skin lesions from patients with CIU. (a) Negative control, lupus erythematosus (LE), (b) positive control (LE), (c-d) CD123+ cells (arrows) on skin lesions of patients with CIU, ($\times 400$).

in patients with CIU exhibited an increased percentage of STAT1 phosphorylation. It is possible that the increase of STAT1 phosphorylation on lymphocytes could be due to the secondary effect of antihistamines, a drug that all patients took regularly, usually as an H1-antihistamine. It has been described that the histamine H4R antagonist could potentiate the phosphorylation of STAT1, mimicking the precursor responses of STAT1 α in the nonatopic group of patients.³⁰ Whether STAT1 phosphorylation on lymphocytes from patients with CIU is related to the use of antihistamines needs to be clarified.

The percentage of pDC in the peripheral blood mononuclear cells of patients with CIU and those of the healthy control group (0.3%) was similar. Moreover, no recruitment or scarce pDC to the skin lesions of patients with CIU was identified. In fact, pDC comprise 0.1% of total peripheral blood mononuclear cells,³¹ and are normally absent in the skin and

other peripheral nonlymphoid tissues. However, in the context of viral skin infections or skin injury,⁷ as well as during certain chronic inflammatory skin disorders such as psoriasis,²⁸ LE,³² and lichen planus,³³ pDC can migrate from the blood into the skin. One attractive candidate is chemerin for early pDC recruitment to damaged skin in the context of infection, injury and tumour invasion.³⁴ In contrast, in other skin diseases such as atopic dermatitis, pDC are not recruited to the inflamed tissue.³³

The recruitment of pDC to pathological tissues requires the release of cytokines and chemotactic factors as well as other signals associated with inflammation and tissue damage. In CIU, the source of chemokines may include mast cells and activated endothelial cells, probably not comprising the essential chemotactic factors for pDC recruitment to damaged skin. Furthermore, it is unknown whether in CIU the impaired type I IFN secretion by TLR9 stimulation could be compensated

somehow *in vivo* by other mechanisms. In fact, it is uncertain whether viral infectious agents are a cause of CIU, although some case reports of viral infections associated with CIU have been published, such as recurrent genital herpes simplex infections³⁵ and parvovirus B19.³⁶

TLR9 endosomal sensors of microbial and nucleic acids, and in some circumstances host factors such as anti-DNA antibodies and antimicrobial peptide LL-37, may facilitate the entry of self-DNA into the endosomes of pDC, triggering TLR9 to induce IFN- α type I responses.³⁷ This event may lead to constitutive activation of pDC, releasing large amounts of type I IFN that in turn may sustain B- and T-cell autoimmune responses. As an immunotherapeutic strategy for the treatment of murine autoimmune and inflammatory disease models inhibitory ODN has been explored, known to suppress activation by the TLR9 pathway.¹⁰ In humans, ODN suppressors inhibit type I IFN production after viral TLR9 stimulation by pDC.³⁸ We also assessed the effect of ODN suppressors in CIU, which are able further to inhibit the IFN- α secretion induced by TLR9 activation. The telomeric regions TTAGGG ODN responsible for the inhibitory effects of mammalian DNA do not bind to TLR9 directly, but bind to and prevent the phosphorylation of specific STATs, thereby inhibiting the signal transduction cascade needed to maintain inflammatory responses.³⁹

The downregulation of TLR9 mRNA expression after CpG A activation on PBMC from patients with CIU may have functional consequences when IFN- α secretion is reduced. Furthermore, it is possible that the downregulation of TLR9 may also interfere in the expression of IRF7. One hypothesis to explain the downregulation of TLR9 in pDC after CpG stimulation could be due to crosslinking of Fc ϵ RI. It has been described that the IgE cross-linking on immature pDC impairs innate immune function by suppressing IFN- α production in response to TLR9 activation by CpG.⁴⁰ Furthermore, impaired TLR9-dependent immune responses in human pDC are associated with allergic status and inversely correlated with Fc ϵ RI α expression and serum IgE levels.⁴¹ In fact, the levels of IgE serum levels in the cohort of patients with CIU enrolled in this study seemed to be positively correlated with Fc ϵ RI on basophils.¹⁷ Further investigation is needed to verify whether the IgE or autoantibodies against Fc ϵ RI in CIU may contribute to crosslinking of Fc ϵ RI in pDC, leading to a downregulation of TLR9. Furthermore, several other mechanisms could be implicated in the downregulation of pDC to produce IFN upon TLR9 activation, such as surface molecules or a direct effect of histamine. pDC surface receptors such as BDCA-2,⁴² ILT7⁴³ and NKp44⁴⁴ are regulatory receptors that downmodulate IFN production. Another possibility is that, due to the mast cell or basophil degranulation in CIU, the release of histamine into the circulation may regulate several cell types. In this sense, it has been described that human pDC, activated either by CpG or viral infection, also respond to histamine through H2 receptors, leading to a marked downregulation of IFN- α , which would explain the low levels of type I IFN frequently observed in atopic individuals.⁴⁵

To our knowledge, this is the first report showing that pDC in CIU have impaired IFN- α secretion through the activation of TLR9. The transient downregulation of TLR9 expression and altered IRF7 at the time of CpG activation, may together contribute to a decreased IFN- α secretion. The evaluation of the innate immune response to TLR ligands, mainly those on dendritic cells, can be exploited for therapeutic purposes.

What's already known about this topic?

- Chronic idiopathic urticaria shows a dysregulation of the immune response.

What does this study add?

- In this work we have identified a disturbed innate immune response in chronic idiopathic urticaria, related to a decrease in type I interferon production through TLR9-mediated activation by plasmacytoid dendritic cells, which was associated with downregulation of TLR9 expression.
- A search for a TLR ligand response of dendritic cells linking innate to adaptive immunity could be useful for therapeutic purposes.

References

- 1 O'Doherty U, Peng M, Gezelter S *et al.* Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* 1994; **82**:487–93.
- 2 Robinson SP, Patterson S, English N *et al.* Human peripheral blood contains two distinct lineages of dendritic cells. *Eur J Immunol* 1999; **29**:2769–78.
- 3 Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. *Nat Immunol* 2004; **5**:1219–26.
- 4 Bauer S, Kirschning CJ, Hacker H *et al.* Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* 2001; **98**:9237–42.
- 5 Hartmann G, Battiany J, Poeck H *et al.* Rational design of new CpG oligonucleotides that combine B cell activation with high IFN- α induction in plasmacytoid dendritic cells. *Eur J Immunol* 2003; **33**:1633–41.
- 6 Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; **11**:373–84.
- 7 Conrad C, Meller S, Gilliet M. Plasmacytoid dendritic cells in the skin: to sense or not to sense nucleic acids. *Semin Immunol* 2009; **21**:101–9.
- 8 Duramad O, Fearon KL, Chang B *et al.* Inhibitors of TLR-9 act on multiple cell subsets in mouse and man *in vitro* and prevent death *in vivo* from systemic inflammation. *J Immunol* 2005; **174**:5193–200.
- 9 Lenert P. Inhibitory oligodeoxynucleotides – therapeutic promise for systemic autoimmune diseases? *Clin Exp Immunol* 2005; **140**:1–10.
- 10 Klinman DM, Zeuner R, Yamada H *et al.* Regulation of CpG-induced immune activation by suppressive oligodeoxynucleotides. *Ann N Y Acad Sci* 2003; **1002**:112–23.

- 11 Sozzani S. Dendritic cell trafficking: more than just chemokines. *Cytokine Growth Factor Rev* 2005; **16**:581–92.
- 12 Banchereau J, Pascual V. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity* 2006; **25**:383–92.
- 13 Kaplan AP. Chronic urticaria: pathogenesis and treatment. *J Allergy Clin Immunol* 2004; **114**:465–74.
- 14 Kaplan AP, Greaves M. Pathogenesis of chronic urticaria. *Clin Exp Allergy* 2009; **39**:777–87.
- 15 Hide M, Francis DM, Grattan CE *et al.* Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N Engl J Med* 1993; **328**:1599–604.
- 16 Grattan CE. Basophils in chronic urticaria. *J Invest Dermatol Symp Proc* 2001; **6**:139–40.
- 17 Lourenco FD, Azor MH, Santos JC *et al.* Activated status of basophils in chronic urticaria leads to interleukin-3 hyper-responsiveness and enhancement of histamine release induced by anti-IgE stimulus. *Br J Dermatol* 2008; **158**:979–86.
- 18 Kaplan A. Inflammation in chronic urticaria is not limited to the consequences of mast cell (or basophil) degranulation. *Clin Exp Allergy* 2010; **40**:834–5.
- 19 Tedeschi A, Asero R, Lorini M *et al.* Plasma levels of matrix metalloproteinase-9 in chronic urticaria patients correlate with disease severity and C-reactive protein but not with circulating histamine-releasing factors. *Clin Exp Allergy* 2010; **40**:875–81.
- 20 Caproni M, Cardinali C, Giomi B *et al.* Serological detection of eotaxin, IL-4, IL-13, IFN-gamma, MIP-1alpha, TARC and IP-10 in chronic autoimmune urticaria and chronic idiopathic urticaria. *J Dermatol Sci* 2004; **36**:57–9.
- 21 Tedeschi A, Lorini M, Suli C *et al.* Serum interleukin-18 in patients with chronic ordinary urticaria: association with disease activity. *Clin Exp Dermatol* 2007; **32**:568–70.
- 22 Ciprandi G, De Amici M, Berardi L *et al.* Serum neopterin levels in spontaneous urticaria and atopic dermatitis. *Clin Exp Dermatol* 2011; **36**:85–7.
- 23 Dos Santos JC, Azor MH, Nojima VY *et al.* Increased circulating pro-inflammatory cytokines and imbalanced regulatory T-cell cytokines production in chronic idiopathic urticaria. *Int Immunopharmacol* 2008; **8**:1433–40.
- 24 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* 2001; **25**:402–8.
- 25 Sinigaglia F, D'Ambrosio D, Rogge L. Type I interferons and the Th1/Th2 paradigm. *Dev Comp Immunol* 1999; **23**:657–63.
- 26 Lankford CS, Frucht DM. A unique role for IL-23 in promoting cellular immunity. *J Leukoc Biol* 2003; **73**:49–56.
- 27 Shrestha S, Wershil B, Sarwark JF *et al.* Lesional and nonlesional skin from patients with untreated juvenile dermatomyositis displays increased numbers of mast cells and mature plasmacytoid dendritic cells. *Arthritis Rheum* 2010; **62**:2813–22.
- 28 Albanesi C, Scarponi C, Bosio D *et al.* Immune functions and recruitment of plasmacytoid dendritic cells in psoriasis. *Autoimmunity* 2010; **43**:215–19.
- 29 Krug A, Rothenfusser S, Hornung V *et al.* Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol* 2001; **31**:2154–63.
- 30 Horst B, Borck H, Diel J *et al.* The role of the histamine H(4) receptor in signal transduction of atopic and non-atopic human lymphocytes *ex vivo*. *Inflamm Res* 2006; **55** (Suppl. 1):S53–4.
- 31 McKenna K, Beignon AS, Bhardwaj N. Plasmacytoid dendritic cells: linking innate and adaptive immunity. *J Virol* 2005; **79**:17–27.
- 32 Vermi W, Lonardi S, Morassi M *et al.* Cutaneous distribution of plasmacytoid dendritic cells in lupus erythematosus. Selective tropism at the site of epithelial apoptotic damage. *Immunobiology* 2009; **214**:877–86.
- 33 Wollenberg A, Wagner M, Gunther S *et al.* Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. *J Invest Dermatol* 2002; **119**:1096–102.
- 34 Albanesi C, Scarponi C, Pallotta S *et al.* Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* 2009; **206**:249–58.
- 35 Zavar V, Godse K, Sankalecha S. Chronic urticaria associated with recurrent genital herpes simplex infection and success of antiviral therapy – a report of two cases. *Int J Infect Dis* 2010; **14**:e514–17.
- 36 Vuorinen T, Lammintausta K, Kotilainen P *et al.* Presence of parvovirus B19 DNA in chronic urticaric and healthy human skin. *J Clin Virol* 2002; **25**:217–21.
- 37 Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* 2008; **8**:594–606.
- 38 Peng WM, Yu CF, Allam JP *et al.* Inhibitory oligodeoxynucleotides downregulate herpes simplex virus-induced plasmacytoid dendritic cell type I interferon production and modulate cell function. *Hum Immunol* 2007; **68**:879–87.
- 39 Shirota H, Gursel I, Gursel M *et al.* Suppressive oligodeoxynucleotides protect mice from lethal endotoxic shock. *J Immunol* 2005; **174**:4579–83.
- 40 Schroeder JT, Bieneman AP, Xiao H *et al.* TLR9- and Fc epsilon RI-mediated responses oppose one another in plasmacytoid dendritic cells by down-regulating receptor expression. *J Immunol* 2005; **175**:5724–31.
- 41 Tversky JR, Le TV, Bieneman AP *et al.* Human blood dendritic cells from allergic subjects have impaired capacity to produce interferon-alpha via Toll-like receptor 9. *Clin Exp Allergy* 2008; **38**:781–8.
- 42 Dzionek A, Sohma Y, Nagafune J *et al.* BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med* 2001; **194**:1823–34.
- 43 Cao W, Rosen DB, Ito T *et al.* Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilon RI gamma inhibits Toll-like receptor-induced interferon production. *J Exp Med* 2006; **203**:1399–405.
- 44 Fuchs A, Cella M, Kondo T *et al.* Paradoxical inhibition of human natural interferon-producing cells by the activating receptor NKp44. *Blood* 2005; **106**:2076–82.
- 45 Mazzoni A, Leifer CA, Mullen GE *et al.* Cutting edge: histamine inhibits IFN-alpha release from plasmacytoid dendritic cells. *J Immunol* 2003; **170**:2269–73.