Brief report: Immunobiology

Defect of plasmacytoid dendritic cells in warts, hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome patients

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Running title: Plasmacytoid dendritic cells in WHIM patients

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Abstract

Warts, Hypogammaglobulinemia, Infections, Myelokathexis (WHIM) syndrome is a genetic disease that is caused by heterozygous mutations of the \textit{CXCR4} gene. These mutations confer an increased leukocyte response to the CXCR4-ligand CXCL12, resulting in abnormal homeostasis of many leukocyte types, including neutrophils and lymphocytes. Analysis of the myeloid and plasmacytoid DC blood counts in WHIM patients revealed a striking defect in the number of pDCs as well as a partial reduction of the number of mDCs, compared to healthy subjects. Moreover, the production of interferon-alpha by mononuclear cells in response to \textit{Herpes simplex} infection, or after stimulation with the TLR9 ligand CpG, was undetectable in WHIM patients. Because pDCs play a key role in the defense against viruses, and their generation and motility are in part dependent on CXCR4, we hypothesized that the susceptibility of WHIM patients to warts is related to the abnormal homeostasis of pDCs.
Introduction

Warts, Hypogammaglobulinemia, Infections, Myelokathexis (WHIM) syndrome is an autosomal, dominant genetic disease that is characterized by severe/moderate neutropenia and leukopenia (despite the retention of mature neutrophils in the bone marrow, e.g., myelokathexis), hypogammaglobulinemia, recurrent respiratory infections and severe verrucosis, which are caused by common human papillomavirus (HPV) strains. WHIM is caused by heterozygous mutations at the C-terminus of the chemokine receptor protein CXCR4, and these mutations affect the intracellular signaling of the receptor in response to the ligand CXCL12. Because leukocytes that express CXCR4 have an increased chemotactic response to the CXCL12 ligand, these cells are retained in the bone marrow and lymphoid compartment, thereby reducing their blood counts. Although several studies have provided insight into the pathogenesis of WHIM syndrome, some clinical features of this genetic disorder remain unexplained. In particular, it is still unclear why WHIM patients develop verrucosis that affects both the hands and genitalia, which are extremely refractory to treatment.

Recent studies suggest that plasmacytoid dendritic cells (pDCs) might have a role in protecting against human papillomavirus (HPV) infection, as pDCs were present in the epidermis of patients diagnosed with chronic genital HPV infections. pDCs secrete the antiviral cytokine interferon-α (IFNα) in response to stimulation with HPV virus-like particles, and abnormalities in dendritic cell homeostasis have been implicated in various human diseases, including cancer, autoimmune disease, allergy and infection. Because pDC generation and trafficking among tissues is regulated by CXCR4, we investigated the number of circulating pDCs and their ability to secrete IFNα in WHIM patients.

Methods

Informed consent was obtained from all WHIM patients in keeping with the Declaration of Helsinki according to a protocol approved by the Hospital Ethical Committee (Spedali Civili, Brescia). EDTA-treated blood samples were collected from 5 WHIM patients (P1, P2, P3, P4 and P5) and healthy subjects, who were age matched with the patients. Immunophenotyping was performed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA) using the following anti-human antibodies (BD BioSciences, Milan, Italy): anti-CD14/anti-CD15/anti-CD19/anti-CD20-FITC.
(lineage cocktail), anti-CD1c-PE, anti-CD4-PerCP and anti-BDCA-2-APC. The DC subsets were then identified and gated based on CD1c and BDCA-2 surface marker expression. Myeloid DCs were defined as CD4+CD1c+BDCA-2- cells, and plasmacytoid DCs were defined as CD4+CD1c- BDCA-2+ cells 18.

Heparin-treated blood samples were collected from P1, P2, P3 and the controls, and peripheral blood mononuclear cells (PBMCs) were isolated using Lympholyte® H (Cedarlane, Ontario, Canada) density gradient centrifugation.

Formalin-fixed paraffin-embedded skin biopsies were obtained from a WHIM patient and ten control cases. For immunohistochemical staining primary antibodies to the following antigens were used: CD1a (Mouse IgG1, Clone 010, Dako, Glostrup, Denmark), CD207/Langerin (Mouse IgG2b, Clone 12D6, Vector Laboratories, Burlingame, CA, USA), MxA (Mouse IgG2a, kindly provided by Dr. O.Haller), BDCA2 (Mouse IgG1, Clone 124B3.13, Dendritics, Lyon, France), anti-HPV clone K1H8 (Dako) which recognizes a 57 kD capsid protein of HPV-119. All sections were counterstained with haematoxylin.

A two-tailed Mann Whitney U test (nonparametric analysis) was used for statistical comparison of the patients to the healthy controls. A P-value less than 0.05 was considered as significant.

Results and Discussion

To assess the effect of CXCR4 mutations on pDC homeostasis in WHIM patients, we performed a quantitative evaluation of the DC subpopulations (myeloid and plasmacytoid dendritic cells) in the peripheral blood of 24 healthy subjects and 5 WHIM patients bearing p.Arg334X or p.Gly336X mutations. As shown in Figure 1a, we observed a significant reduction in both DC subsets in the blood of WHIM patients in comparison to healthy subjects. In particular, the percentages of pDCs were markedly decreased in the patients (mean 0.032% ± 0.016%, range 0.01%-0.05%) compared to the healthy subjects (mean 0.53% ± 0.278%, p< 0.05, range 0.22%-1.11%). Additionally, the numbers of mDCs were reduced in WHIM patients (mean 0.2% ± 0.15%, range 0.06%-0.42%), although to a lesser extent, when compared to healthy subject levels (mean 0.99% ± 0.466%, p< 0.05, range 0.37%-2.39%) (Supplemental Table S1).

Because of the paucity of circulating mDC, we have evaluated cytokine production in DC derived in vitro from the monocytes isolated from blood of a WHIM patient and from a control subject. We specifically analyzed the production of IL-12, IL-6 and TNFα, which are secreted by mature monocyte-derived DCs after 24 hours of stimulation with LPS and IFNγ 21. Our results showed normal production of IL12 (p70), IL6 and TNFα by mature DCs from WHIM patients compared to
Because pDCs are the most potent secretors of interferon-α (IFNα) in response to viral stimuli, they are essential for the antiviral response. 22,23. To study the effect of the reduced pDC number in WHIM patients, we analyzed PBMC IFNα expression. We infected PBMCs derived from two WHIM patients and two control subjects with an increasing number of type 1 Herpes simplex virus particles (HSV1, from 10 up to 10,000 pfu/ml). Analysis of the IFNα supernatant concentration after 24 hours of culture showed that, with the lowest number of HSV1 copies, cytokine release by healthy PBMCs was already detectable. In contrast, WHIM-PBMC cytokine production remained undetectable, even after infection with 10,000 pfu/ml of HSV1 (Figure 1b). We subsequently analyzed the WHIM-PBMC IFNα production levels in response to CpG (5 µM), which stimulates pDCs by activating Toll-like receptor 9 (TLR9). Even under these experimental conditions, we observed a severe defect in IFNα production by WHIM-PBMC compared to the healthy, control PBMC (Figure 1c). These data suggest that WHIM patients have a severely impaired capacity to produce IFNα in response to TLR9 stimulation, which is likely due to the reduction of the pDC count.

Next, we analyzed the dermal mononuclear infiltrate of HPV-associated Verruca vulgaris from one WHIM patient (Fig. 2a and b) and from control subjects (Supplemental Figure S2 panel a). Dermal BDCA2+ pDCs were not detected in multiple step sections of skin biopsies of the WHIM patient but were present at variable extent in warts from control subjects (Fig 2e-f and Supplemental Figure S2 panel b). Because IFN-α secretion by pDC results in the expression of the anti-viral protein MxA, skin biopsies were stained with anti-MXA mAb. Remarkably, all control cases showed variable intraepithelial reactivity for MxA, whereas Verruca vulgaris from WHIM patient was completely negative (Fig. 2 g,h and Supplemental Figure S2 panels c and d). In contrast, intraepidermal CD1a+CD207+ Langerhans cells and dermal CD1a+ cells were detected in the biopsies from the WHIM patient as well as in control biopsies (Fig. 2c-d).

Taken together, our results demonstrate that the number of circulating plasmacytoid and myeloid dendritic cells is markedly reduced in WHIM patients. In particular, we show that the decline in pDC numbers is associated with a decreased production of IFNα in response to viral infection or CpG stimulation. We speculate that the high levels of CXCR4 expression by pDCs 12,15 [Tassone et al., data not shown], along with the increased responsiveness of WHIM patient leukocytes to CXCL12, may account for the low percentage of circulating pDCs in WHIM patients 3,5,6,24,25.
Because of the disturbed homeostasis of pDCs in WHIM patients, these cells may not be able to migrate to the skin, where they supply antiviral activity against HPV infection. This migration defect may lead to the development of severe, untreatable warts, which are typically observed in WHIM patients. However, the mechanism behind the immune response to HPV are not dependent upon the production of interferon by pDCs and might involve other cell types, including mDC and T cells.

Acknowledgements

This work was supported by grants from Telethon GGP07134, Fondazione Cariplo NOBEL Grant, EU Grant FP7 HLH-cure (project n. 201461), and PRIN 2007 n. 2007ACZMMZ_005 to RB.

Authorship Contributions

Laura Tassone performed the studies with the dendritic cells and wrote the manuscript; Daniele Moratto performed the flow cytometry analysis; William Vermi and Fabio Facchetti have performed immunohistopathology studies; Maria De Francesco studied the response of cells to herpes infection; Lucia D Notarangelo, Fulvio Porta, Vassilios Lougaris and Alessandro Plebani were in charge of the patients’ follow-up; and Raffaele Badolato supervised the project and helped write the manuscript.

Disclosure of Conflicts of Interest

The authors declare no conflicts of interest.
References


Figure 1. Evaluation of the pDC and mDC in WHIM patients.

(a) Percentages of mDCs and pDCs (calculated as the percentage of the lymphocyte population) in 5 WHIM patients compared to 24 healthy subjects. To identify pDCs and myeloid dendritic cells (mDCs) via flow cytometry, we utilized the following gating strategy. Side scatter and staining with anti-CD4-PerCP and anti-CD14/anti-CD15/anti-CD19/anti-CD20-FITC were used to exclude B cells, neutrophils and monocytes. (b) IFNα production was evaluated after HSV1 infection (dose ranging from 10 to 10,000 pfu/ml, for 24 h) (panel b) or CpG (TLR9 ligand, 5 μM for 24 h) stimulation (panel c) in PBMCs of 3 WHIM patients compared to healthy subjects. PBMCs (2.5 x 10^6/ml) were plated in U-bottomed 96-well plates and cultured with 5 μM CpG (ODN2216, InvivoGen, San Diego, CA) or infected with HSV1 (strain MacIntyre; from 10 up to 10,000 pfu/ml) for 24 h. IFNα levels in culture supernatants were determined using a VeriKine™ Human IFNα ELISA Kit (PBL InterferonSource, Piscataway, NJ) according to the manufacturer’s instructions. In panels b and c, results are presented as average of duplicate data; error bars indicate standard error values. Comparison of cytokine production by non parametrical analysis shows significant differences between WHIM patients and control subjects.

Figure 2. Dendritic cells in Verruca vulgaris. Sections of verruca vulgaris were obtained from a WHIM patient (panels a, b, c, d, f and h) and a control case (e and g) and stained for H&E (a), HPV (brown, b), CD1a (blue in c and brown d), CD207 (brown, c), BDCA2 (brown, e and f) and MxA (brown, g and h). Verruca vulgaris from WHIM patient P4 showed a moderate dermal mononuclear infiltrate (a) and contained HPV-infected keratinocytes (b). By immunohistochemistry intraepidermal CD1a⁺CD207⁺ Langerhans cells (c) and dermal CD1a⁺ cells (d) were detected in WHIM patient biopsy. Asterisks in panel c indicate the position of dermal papillae. Dermal BDCA2⁺ cells were detectable in control cases (e) but not seen in the patient biopsy f). Strong reactivity for MxA was observed in keratinocytes from a control biopsy (g and insert), whereas it was completely negative in WHIM patient biopsy (h). Magnification 40x (a, g and h; scale bar 500 μ), 600x (b; scale bar 20 μ) and 400x (c-f and insert in g; scale bar 50 μ).
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