

Dendritic cells from X-linked hyper-IgM patients present impaired responses to *Candida albicans* and *Paracoccidioides brasiliensis*

Otávio Cabral Marques, MSc,^{a,g} Christina Arslanian, BSc,^a Rodrigo Nalio Ramos, MSc,^a Mariana Morato, MSc,^a LenaFriederike Schimke, MD,^{a,g} Paulo Vitor Soeiro Pereira, MSc,^a Sonia Jancar, MD, PhD,^a Janaína Fernandes Ferreira, MD,^b Cristina Worm Weber, MD,^c Gisele Kuntze, MD,^d Nelson Augusto Rosario-Filho, MD, PhD,^e Beatriz Tavares Costa Carvalho, MD,^f Patricia Cruz Bergami-Santos, PhD,^a Mary J. Hackett, BSc,^g Hans D. Ochs, MD,^g Troy R. Torgerson, MD, PhD,^g Jose Alexandre Marzagão Barbuto, MD, PhD,^{a,*} and Antonio Condino-Neto, MD, PhD^{a,*} São Paulo, Fortaleza, Caxias do Sul, and Curitiba, Brazil, and Seattle, Wash

Background: Patients with X-linked hyper-IgM syndrome (X-HIGM) due to CD40 ligand (CD40L) mutations are susceptible to fungal pathogens; however, the underlying susceptibility mechanisms remain poorly understood.

Objective: To determine whether monocyte-derived dendritic cells (DCs) from patients with X-HIGM exhibit normal responses to fungal pathogens.

Methods: DCs from patients and controls were evaluated for the expression of costimulatory (CD80 and CD86) and MHC class II molecules and for their ability to produce IL-12 and IL-10 in response to *Candida albicans* and *Paracoccidioides brasiliensis*. We also evaluated the ability of *C. albicans*- and *P. brasiliensis*-pulsed mature DCs to induce autologous T-cell proliferation, generation of T helper (T_H) 17 cells, and production of IFN- γ , TGF- β , IL-4, IL-5, and IL-17.

Results: Immature DCs from patients with X-HIGM showed reduced expression of CD80, CD86, and HLA-DR, which could be reversed by exogenous trimeric soluble CD40L. Most important, mature DCs from patients with X-HIGM differentiated by coculturing DCs with fungi secreted minimal amounts of IL-12 but substantial amounts of IL-10 compared with mature DCs from normal individuals. Coculture of mature DCs from X-HIGM patients with autologous T cells led to low

IFN- γ production, whereas IL-4 and IL-5 production was increased. T-cell proliferation and IL-17 secretion were normal. Finally, *in vitro* incubation with soluble CD40L reversed the decreased IL-12 production and the skewed T_H2 pattern response.

Conclusion: Absence of CD40L during monocyte/DC differentiation leads to functional DC abnormalities, which may contribute to the susceptibility to fungal infections in patients with X-HIGM. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: CD40 ligand deficiency, fungal infections, dendritic cells, X-linked hyper-IgM syndrome, primary immunodeficiency

Most primary immunodeficiency disorders are characterized by an unusual susceptibility to particular infectious pathogens. In general, the class of pathogen points toward the type of immunological defect that is present; for example, extracellular bacterial pathogens are typically associated with a humoral immune deficiency, whereas the occurrence of opportunistic infections raises the suspicion of a cellular immune deficiency. Fungal infections are found predominantly in patients with innate, combined, or more complex immune disorders such as chronic granulomatous disease, myeloperoxidase deficiency, congenital neutropenias, defects in the IFN- γ /IL-12 axis, hyper-IgE syndromes, severe combined immunodeficiency, or Wiskott-Aldrich syndrome.¹

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that initiate and modulate the immune response.² They are present in most tissues and recognize different pathogen-associated molecular patterns.³ After DCs recognize, capture, and process antigens, they mature and display large numbers of HLA-DR-peptide complexes on their cell surface, thereby instructing antigen-specific T cells to trigger immune responses. This phenomenon is also observed during fungal infections.⁴

X-linked hyper-IgM syndrome (X-HIGM), caused by mutations in the *CD40 ligand* (*CD40L*) gene, is unique in that it primarily affects the development of normal humoral immunity but also predisposes affected patients to fungal infections without a well-defined cellular immune defect.⁵⁻⁹ CD40L (CD154) is inducible and expressed on the surface of activated CD4⁺ T cells or released in a soluble form.^{10,11} It has pleiotropic

From ^athe Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo; ^bthe Albert Sabin Hospital, Fortaleza; ^cthe Pediatric Allergy and Immunology Clinic, Caxias do Sul; ^dthe Pequeno Príncipe Hospital, Curitiba; ^ethe Department of Pediatrics, Federal University of Paraná Medical School, Curitiba; ^fthe Department of Pediatrics, Division of Allergy-Immunology and Rheumatology, Federal University of São Paulo, São Paulo; ^gthe Department of Pediatrics, University of Washington School of Medicine and Seattle Children's Hospital, Seattle.

*These authors contributed equally to this work.

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo—grant 2008/06635-0 to O.C.M., grant 2008/55700-9 to A.C.N., and grant 2009/54599-5 to J.A.M.B.—and by the Jeffrey Modell Foundation.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication April 6, 2011; revised September 11, 2011; accepted for publication October 11, 2011.

Corresponding author: Antonio Condino-Neto, MD, PhD, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, 1730 Lineu Prestes Ave, São Paulo, SP 05508-000, Brazil. E-mail: condino@icb.usp.br. 0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2011.10.026

Abbreviations used

APCs: Antigen-presenting cells
 CD40L: CD40 ligand
 DCs: Dendritic cells
 iDCs: Immature DCs
 mDCs: Mature DCs
 PTX3: Pentraxin 3
 sCD40L: Soluble CD40 ligand
 X-HIGM: X-linked hyper-IgM syndrome

functions in the immune response, activating both B cells and APCs. *In vivo* and *in vitro* murine studies demonstrate that CD40L is involved in IL-12 production by APCs, which express CD40 constitutively, and enhances the expression of MHC class I and class II molecules and costimulatory molecules CD80 and CD86, which cooperate for T-cell-mediated immunity coordinated by IFN- γ .^{12,13}

Previous work has demonstrated defective postthymic T-cell maturation and effector function in human CD40L deficiency,¹⁴ but the mechanistic basis of these defects remains to be clarified. Because DCs play a critical role in the development of T-cell-mediated immune responses, and defective T-cell function contributes to susceptibility to opportunistic infections such as fungal infections, we hypothesized that DC function could be impaired in patients with X-HIGM and affect T-cell responses. The aim of this work was, therefore, to investigate DC function in patients with CD40L deficiency.

METHODS**Subjects**

We studied 5 Brazilian patients (age range: 4–18 years) from 5 unrelated families with a clinical and genetic diagnosis of X-HIGM according to the criteria of the International Union of Immunological Societies Expert Committee on Primary Immunodeficiencies.¹⁵ In addition to recurrent bacterial infections, all the patients presented with fungal infections (*Pneumocystis jiroveci* pneumonia, candidiasis, or paracoccidioidomycosis) that were either uncommon or particularly severe. The patients were never neutropenic, neither immediately before nor during the fungal infections. Informed consent was obtained from all patients or from their parents, and blood was collected under institutional guidelines. The study was approved by the Ethics Committee of the Institute of Biomedical Sciences, University of São Paulo. All data obtained from the patients were compared with data obtained from young adult healthy male controls ranging in age from 22 to 29 years.

DC generation

Immature DCs (iDCs) were obtained as described by Barbuto et al.¹⁶ Briefly, PBMCs were isolated from heparinized blood after Ficoll-Hypaque sedimentation and adherent monocytes were cultured for 5 days in the presence of GM-CSF and IL-4. The nonadherent cells, mainly lymphocytes, were stored at -80°C to be used in the lymphocyte stimulation assays. Mature DCs (mDCs) were obtained by activating the adherent iDCs with *Paracoccidioides brasiliensis* (cultivated as previously described),¹⁷ *Candida albicans* (a clinical isolate from Sanset Medical Laboratory, Mogi das Cruzes, São Paulo, Brazil), or soluble CD40L (sCD40L) (1 $\mu\text{g}/\text{mL}$) (Invitrogen, Carlsbad, Calif) for 48 hours. *P. brasiliensis* and *C. albicans* were heat killed at 60°C for 60 minutes, counted, and adjusted to a DC/fungus ratio of 1:10. After 2 days of incubation, cell viability was determined by Trypan blue exclusion and mDCs were harvested and phenotyped. Based on the expression pattern of HLA-DR, CD14, CD11c, CD40, CD80, CD86, and CD83, this approach was successful in generating homogeneous populations of iDCs and mDCs.

Phenotyping of DCs

iDCs or mDCs were removed from the 6-well cell culture plates and washed with PBS-containing 0.5% BSA. The expression of Toll-like receptor 2 (TLR2), TLR4, CD14, CD40, CD86, CD80, CD83, and MHC class I and II molecules was assessed with cell surface staining by using specific, fluorophore-conjugated monoclonal antibodies (Becton Dickinson, Mountain View, Calif) and analyzed as previously described.¹⁸

Lymphocyte stimulation assay

To measure the ability of mDCs to activate autologous T lymphocytes, the adherent iDCs and *C. albicans*– or *P. brasiliensis*–pulsed mDCs were harvested by using cold medium and counted; their viability was determined by Trypan blue exclusion as previously described.¹⁴ iDCs or mDCs were cocultured with autologous T cells at a ratio of 1:10 (DCs/lymphocytes) for 5 days and T-cell proliferation and cytokine production (IFN- γ , IL-4, IL-5, IL-17, and TGF- β) were determined. Prior to coculture, lymphocytes were stained with 5 mM 5,6-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, Ore) following the manufacturer's instructions. After labeling, lymphocytes were cocultured either with iDCs or with *P. brasiliensis*– or *C. albicans*–pulsed mDCs at a ratio of 1:10 (DCs/lymphocytes), for 5 days at 37°C in 5% CO_2 , in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in a final volume of 300 μL of RPMI 1640, supplemented with 10% FCS. Immediately before flow cytometry, cells were stained with anti-CD4 and anti-CD8 (Becton Dickinson).

Evaluation of $\text{T}_\text{H}17$ function

$\text{T}_\text{H}17$ cells were analyzed as previously described.¹⁹ Briefly, PBMCs and nonadherent cells containing mainly lymphocytes from patients and healthy controls were stimulated overnight with 10 ng/mL of phorbol 12-myristate 13-acetate plus 1 $\mu\text{g}/\text{mL}$ of ionomycin (Sigma-Aldrich, St Louis, Mo) and *C. albicans*–pulsed DCs at a ratio of 1:10 (DCs/lymphocytes), respectively. For both situations, the cells were cultivated in the presence of GolgiPlug (BD Biosciences, San Jose, Calif). After cell-surface staining with phycoerythrin-conjugated anti-CD4 (eBioscience, San Diego, Calif), cells were fixed, permeabilized (Cytofix/Cytoperm, BD Biosciences), and stained with Alexa Fluor 647–conjugated anti-IL-17-A (eBioscience). Flow cytometric studies were performed on a BD FACSCanto II Cytometer and analyzed by using FlowJo software (Treestar, Inc, Ashland, Ore). The IL-17 release is described below.

Analysis of cytokine and pentraxin 3 release

DC culture supernatants were evaluated for the presence of pentraxin 3 (PTX3), IL-12p70, and IL-10 after 48-hour activation by *C. albicans* or *P. brasiliensis*. T cell/DC culture supernatants were evaluated for the presence of IL-17, TGF- β , IFN- γ , IL-4, and IL-5 after 5 days of coculture. PTX3 and cytokine levels were measured by using ELISA, according to the manufacturer's instructions (Becton Dickinson).

Statistical analysis

Statistical significance was assessed by using the nonparametric Mann-Whitney test. Data were expressed as median and 25th and 75th percentiles. The statistical analyses were performed by using the GraphPad PRISM 4.03 software (GraphPad Software, San Diego, Calif), and differences with a *P* value of less than .05 were considered significant.

RESULTS

Because of the essential role that DCs play as professional APCs in triggering cell-mediated immune responses that are essential for controlling fungal infections, we generated monocyte-derived DCs from patients with X-HIGM and studied their differentiation and function.

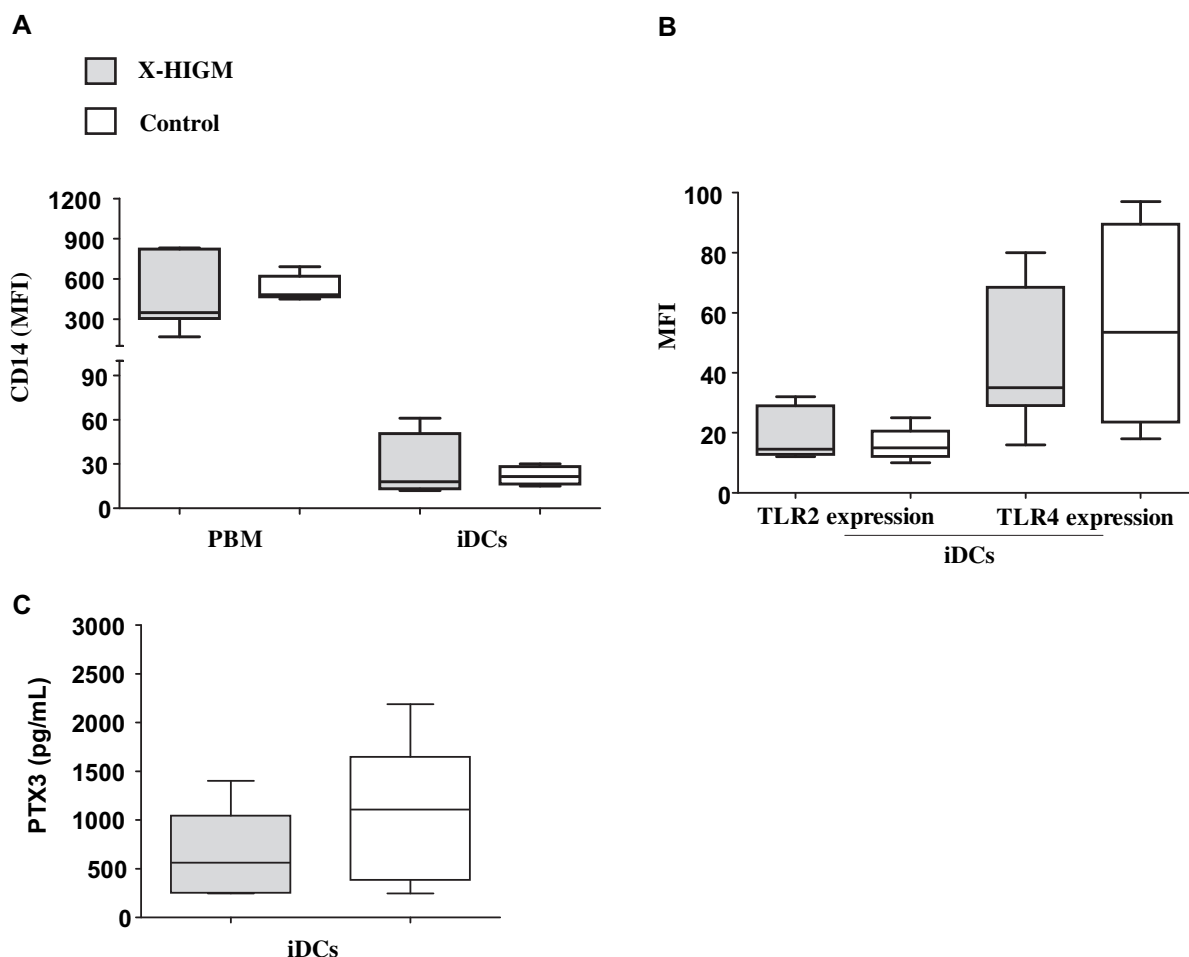


FIG 1. Normal expression and secretion of pattern recognition receptors by iDCs from patients with X-HIGM. Flow cytometric analysis of iDCs derived from peripheral blood monocytes (PBMs) in the presence of GM-CSF and IL-4 showed the expected CD14 downregulation (**A**). X-HIGM iDCs demonstrate normal expression of TLR2 and TLR4 (**B**) and secrete normal amounts of PTX3 compared with normal controls (**C**) ($P < .05$; $n = 5$, Mann-Whitney test). MFI, Mean fluorescence intensity.

iDCs from CD40L-deficient patients express normal TLR2, TLR4, and CD14 but reduced CD80, CD86, and HLA-DR that can be reversed by treating iDCs with sCD40L

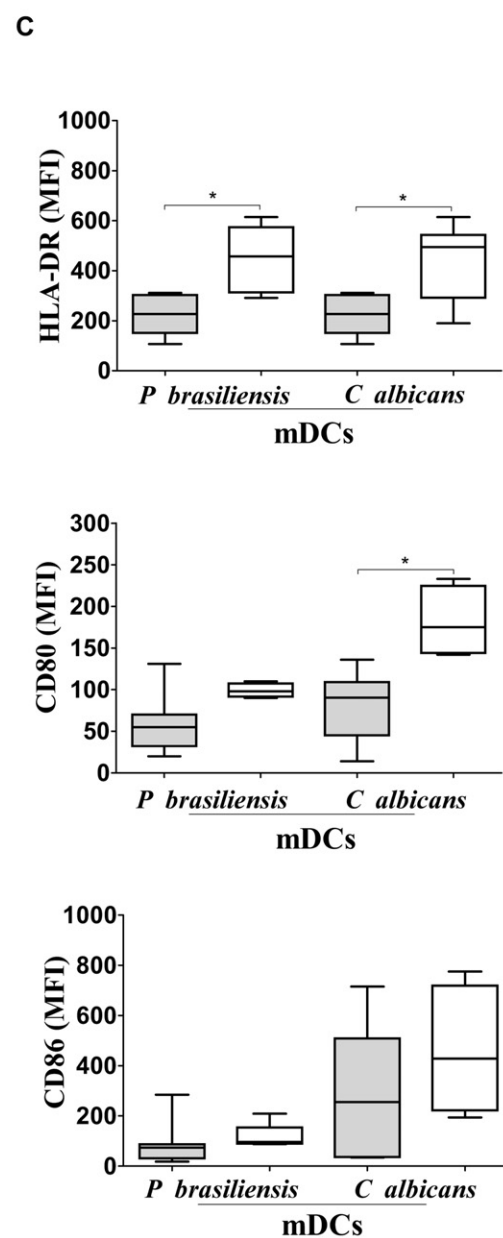
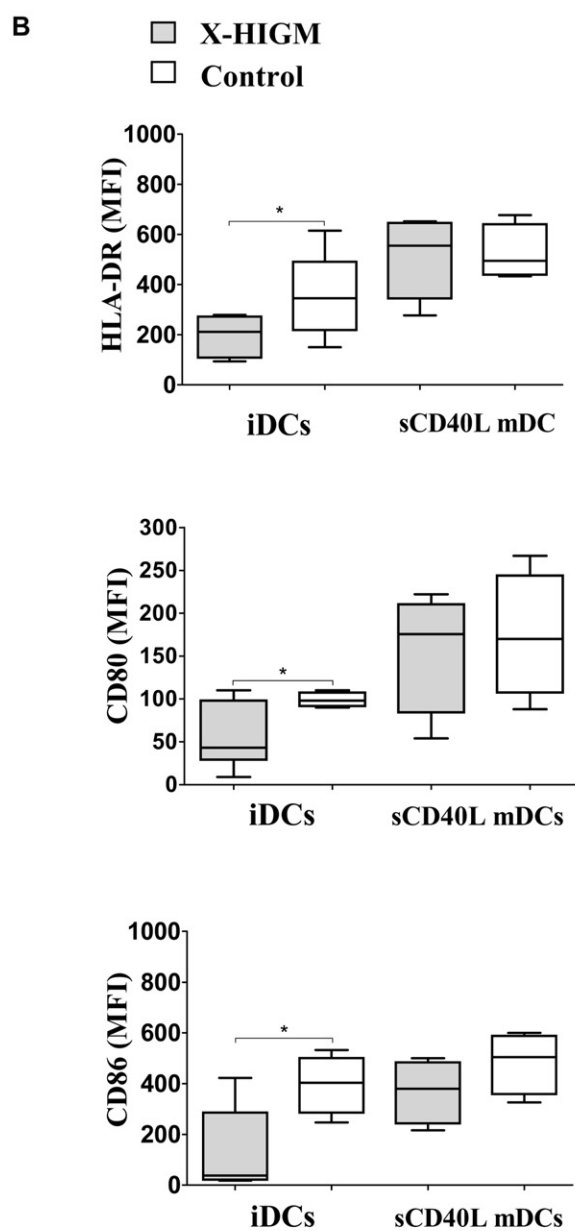
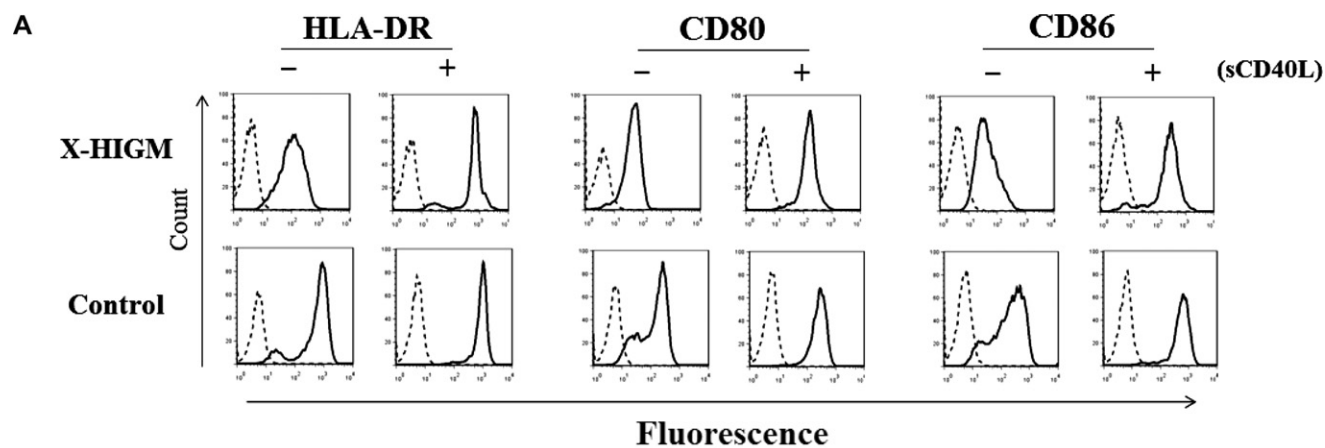
Because of previous reports suggesting an important role for CD14 and TLRs 2 and 4 in the immune response against fungi,²⁰⁻²² we evaluated their expression on peripheral blood monocytes and iDCs by flow cytometry using specific antibodies. iDCs from CD40L-deficient patients showed the expected downregulation of CD14 as in healthy controls (Fig 1, A). Expression of TLR2 and TLR4 was also similar among patients and controls (Fig 1, B). PTX3, the unique humoral pattern-recognition receptor that plays a nonredundant function in innate resistance to fungal infections, is predominantly produced by DCs and is regulated in part by CD40L-CD40 signaling.^{23,24} We therefore investigated the production of PTX3 by iDCs from patients with X-HIGM, which was similar to that in normal controls (Fig 1, C).

Because CD40L has been reported to modulate the expression of CD80, CD86, and HLA-DR,¹⁰ we analyzed the expression of these molecules. iDCs from patients with X-HIGM showed

reduced levels of HLA-DR, CD80, and CD86 expression compared with normal controls (Fig 2, A and B). The importance of CD40L-CD40 interaction for the phenotype of DCs was confirmed by the fact that the addition of sCD40L to the culture up-regulated costimulatory (CD80/CD86) and HLA-DR molecules on mDCs from patients with X-HIGM to normal levels (Fig 2, A and B).

To determine whether maturation of iDCs increases the expression of costimulatory and HLA-DR molecules, we stimulated iDCs with heat-killed *C albicans* or *P brasiliensis* for 48 hours. The resulting mDCs from patients with X-HIGM showed persistently decreased expression of HLA-DR and CD80 molecules compared with normal controls (Fig 2, A and B), but the expression of CD86 although somewhat reduced was not significantly affected (Fig 2, C). Notably, after stimulation with *P brasiliensis*, CD86 expression was downregulated on mDCs from patients with X-HIGM and from healthy controls, an observation that agrees with previous reports.²⁵

In addition, normal expression of CD40, CD83, and HLA-ABC molecules was found on the surface of mDCs from patients with X-HIGM (data not shown).



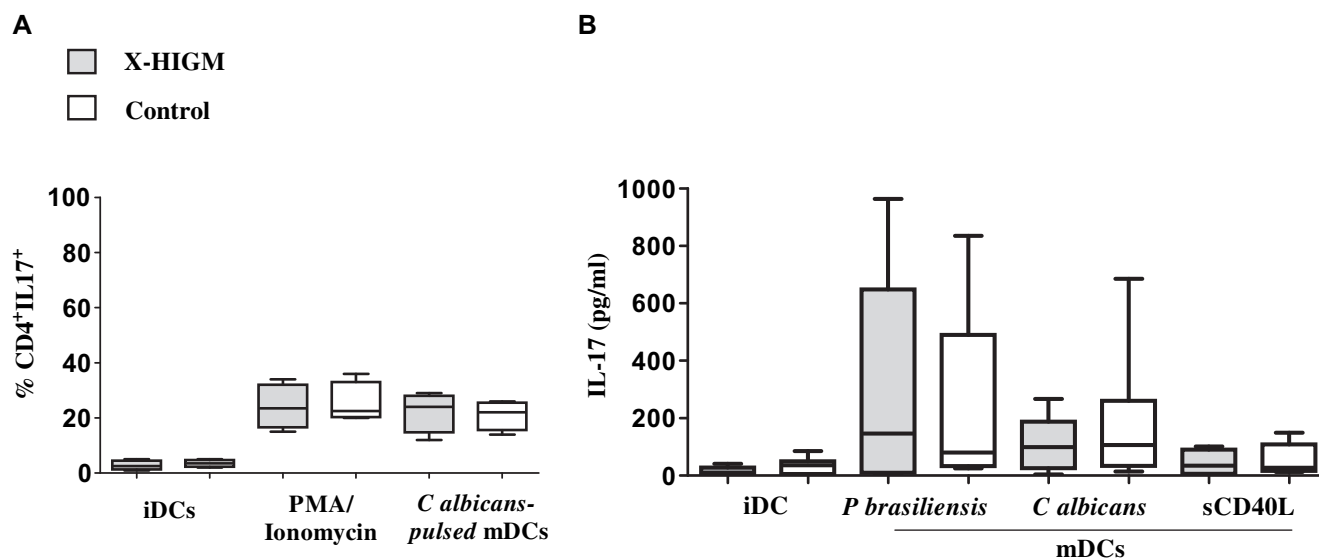


FIG 3. DCs from CD40L-deficient patients induced normal T_H17 cell generation. Patients with X-HIGM showed a percentage of T_H17 cells similar to that in normal controls after stimulation of T cells with iDCs, phorbol 12-myristate 13-acetate plus ionomycin, or *C. albicans*-pulsed mDCs (**A**). The levels of IL-17 in cocultures of T cells with *C. albicans*- or *P. brasiliensis*-pulsed mDCs from patients with X-HIGM were also similar to those of normal controls (**B**) ($P > .05$ in all situations; $n = 5$, Mann-Whitney test). PMA, Phorbol 12-myristate 13-acetate.

DCs from CD40L-deficient patients promote normal generation of T_H17 cells

Recent studies demonstrated a crucial role for IL-17 in the human immune response against *C. albicans*,²⁶ and experiments in mice suggest that CD40L-CD40 interaction is involved in the regulation of IL-17 production.^{27,28} To address the possibility that a defect in the generation of T_H17 cells underlies the susceptibility of patients with X-HIGM to fungal pathogens, we quantified the percentage of T_H17 cells and the secretion of IL-17 in the coculture of patient DCs with autologous T cells. We detected a similar percentage of T_H17 cells between patients with X-HIGM and normal controls after activation of PBMCs with phorbol 12-myristate 13-acetate and ionomycin (Fig 3, A). No differences were observed in the percentage of T_H17 cells when T cells were stimulated by mDCs pulsed with *C. albicans* from CD40L-deficient patients (Fig 3, A). In order to assess whether DCs from patients with X-HIGM are able to induce the secretion of IL-17 by T_H17 cells, we measured IL-17 levels in coculture supernatants of *C. albicans*- or *P. brasiliensis*-pulsed mDCs with autologous T cells (Fig 3, B). IL-17 levels were similar in coculture supernatants from patients with X-HIGM and normal controls. Addition of sCD40L did not increase IL-17 production (Fig 3, B).

mDCs from CD40L-deficient patients show imbalanced production of IL-12/IL-10

We tested the ability of *P. brasiliensis*- or *C. albicans*-pulsed mDCs from patients with X-HIGM to produce IL-12 and IL-10.

After 48 hours of culture, X-HIGM mDCs produced significantly less IL-12 (Fig 4, A) and significantly more IL-10 compared with the amounts produced by mDCs from normal control subjects (Fig 4, B). sCD40L was however able to restore IL-12 production by X-HIGM DCs to levels similar to those in normal controls (Fig 4, A).

DCs from CD40L-deficient patients induce similar levels of lymphocyte proliferation compared with normal controls

We tested the ability of DCs from CD40L-deficient patients to induce autologous T-cell proliferation. After 5 days of coculture, cells were harvested and the proliferation of CD4⁺ and CD8⁺ T cells was evaluated by flow cytometry using a 5,6-carboxyfluorescein diacetate succinimidyl ester dye-dilution approach. Lymphocyte proliferation induced by autologous X-HIGM and normal control iDCs was similar for CD4⁺ and CD8⁺ T cells (Fig 5, A and B). Similar results were obtained when autologous T cells were cocultivated with *P. brasiliensis*- or *C. albicans*-pulsed mDCs (Fig 5, A and B).

mDCs from CD40L-deficient patients induce a T_H2-skewed T-cell response

CD40L has been shown to be important for IL-12/IFN- γ (T_H1) production in response to different opportunistic pathogens.²⁹ Notably, the protective immune response against fungi induced by IL-12/IFN- γ can be suppressed by IL-4, IL-5, and IL-10

FIG 2. DCs from CD40L-deficient patients express reduced CD80, CD86, and HLA-DR levels that can be restored to normal levels by stimulation with sCD40L. Flow cytometric analysis of iDCs showing that cells from patients with X-HIGM express reduced levels of HLA-DR, CD80, and CD86 (**A** and **B**). mDCs incubated with sCD40L show expression of costimulatory and HLA-DR molecules at levels similar to those in normal controls (Fig 2, A and B). mDCs incubated with *C. albicans* and *P. brasiliensis* exhibit reduced expression of HLA-DR and CD80 but normal levels of CD86 (**C**) relative to normal controls. Significant differences are denoted by asterisk ($P < .05$; $n = 5$, Mann-Whitney test). MFI, Mean fluorescence intensity.

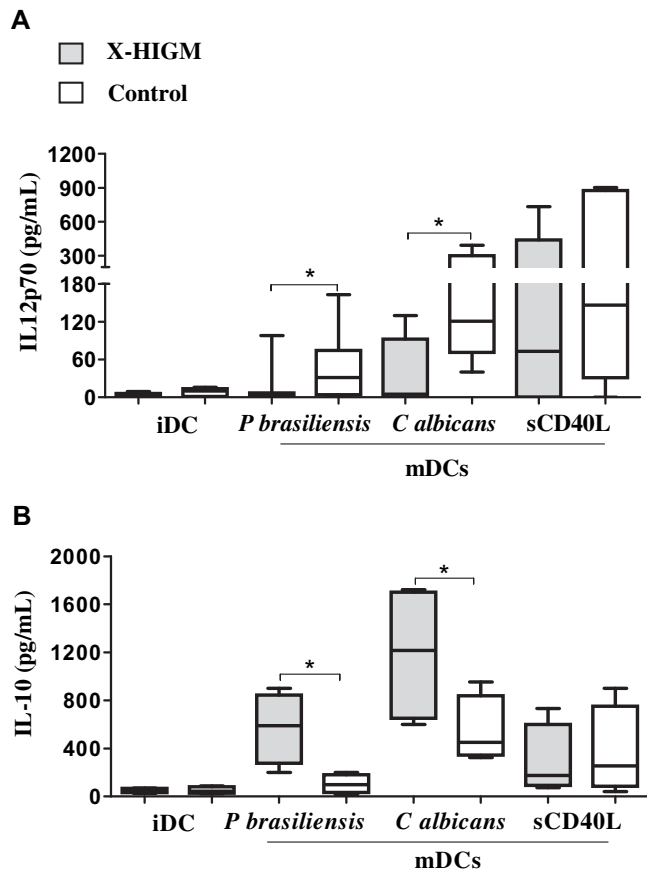


FIG 4. IL-12 and IL-10 production by X-HIGM mDCs. mDCs from patients with X-HIGM secrete decreased IL-12 but increased IL-10 in response to *P brasiliensis* and *C albicans*. After sCD40L stimulation of X-HIGM mDCs, IL-12 and IL-10 secretions were restored to a pattern similar to that observed in healthy controls (**A** and **B**). Significant differences are denoted by asterisk ($P < .05$; $n = 5$, Mann-Whitney test).

(T_H2 pattern).³⁰ To determine whether the differences observed in mDC cytokine production lead to altered APC function, we evaluated T-cell cytokine production by using an autologous coculture system. We observed that coculture of *P brasiliensis*- and *C albicans*-pulsed mDCs from CD40L-deficient patients with the non-adherent cell fraction containing mainly T cells led to lower IFN- γ production (Fig 6, A) and higher IL-4 and IL-5 production compared with normal controls (Fig 6, B and C). However, although somewhat increased, the levels of IL-4 were not statistically affected when the T cells were cocultured with *P brasiliensis*-pulsed mDCs from CD40L-deficient patients (Fig 6, B). Nevertheless, the cytokine patterns were similar in X-HIGM and control cocultures when mDCs were stimulated with sCD40L, strongly suggesting that the CD40L-CD40 interaction contributes to the balance of T_H1 and T_H2 cells (Fig 6). In contrast, we observed no significant difference in TGF- β production in cocultures of *P brasiliensis*- or *C albicans*-pulsed mDCs and autologous T cells from patients with X-HIGM and controls (data not shown).

The skewed T_H2 pattern response of CD40L-deficient patients can be reversed by exogenous sCD40L

To further address the role of CD40L in balancing T_H1 / T_H2 responses, we determined whether sCD40L could reverse the

impaired IFN- γ production observed in response to *C albicans* in patients with X-HIGM. As shown in Fig 7, stimulation of X-HIGM T-cell/mDC cocultures with exogenous sCD40L and *C albicans* caused normal production of IFN- γ and IL-4.

DISCUSSION

To better understand whether impaired cross-talk between APCs and T cells contributes to the observed T-cell dysfunction in CD40L deficiency, we investigated the differentiation and function of DCs from patients with X-HIGM. We found that iDCs from these patients have normal expression of TLR2 and TLR4, normal production of PTX3, and CD14 downregulation that was similar to that observed in cells from normal controls. However, iDCs from these patients had reduced cell-surface expression of CD80, CD86, and HLA-DR. When DCs were activated with *C albicans* or *P brasiliensis*, X-HIGM mDCs were found to have reduced CD80 and HLA-DR expression, and most important, exhibited markedly reduced IL-12 production and increased IL-10 production when compared with normal controls. Coculture of mDCs with autologous T cells resulted in significantly reduced IFN- γ production whereas IL-4 and IL-5 production was increased. In contrast, the ability to induce CD4⁺ and CD8⁺ T cells to proliferate, and generate TGF- β and IL-17, was comparable in mDCs from patients with X-HIGM and normal controls. These observations suggest that the maturation of DCs in patients with X-HIGM is partially impaired and may directly contribute to their susceptibility to fungal infections. The possibility that the differences we observed between patients with X-HIGM (age range: 4-18 years) and young adult controls are age related is unlikely since no differences in the phenotype and function of DCs and T-cell-mediated immunity were observed between children older than age 3 years and adults.³¹

HLA-DR and CD80/CD86 expressed on professional APCs such as DCs bind to the T-cell receptor and to CD28 on T cells to provide the costimulatory signals required to induce T-cell activation.³² The reduced expression of HLA-DR and CD80/CD86 by iDCs and mDCs derived from CD40L-deficient patients suggests defective costimulatory signaling and as a result, ineffective T-cell activation. Our data suggest that exposure to CD40L during the development of monocytes and DCs contributes to appropriate expression of molecules that are crucial for T-cell-derived activation and costimulation.¹³ These findings are also in accordance with those from Fontana et al¹⁸ who demonstrated reduced expression of CD80/CD86 and HLA-DR on iDCs from CD40-deficient patients.

Recent studies in humans revealed a crucial role for IL-17 in the immune response to *C albicans*, a process that requires APC-T-cell cross-talk.³³ Although no relationship between CD40L-CD40 interaction and T_H17 polarization has been described in humans, this issue has been addressed recently in mice, with contradictory results. Katzman et al²⁸ reported that the absence of CD40L-CD40 interaction results in a markedly increased percentage of IL-17-producing cells, whereas Iezz et al²⁷ observed reduced numbers of T_H17 cells. Interestingly, we found a normal percentage of T_H17 cells and IL-17 secretion in X-HIGM, suggesting that in humans, CD40L-CD40 interaction is either not involved in the generation of T_H17 cells or other yet unknown mechanisms compensate for the absence of CD40L-CD40 interaction. In addition, we found normal phosphorylation of STAT3, a transcription factor essential for T_H17 cell

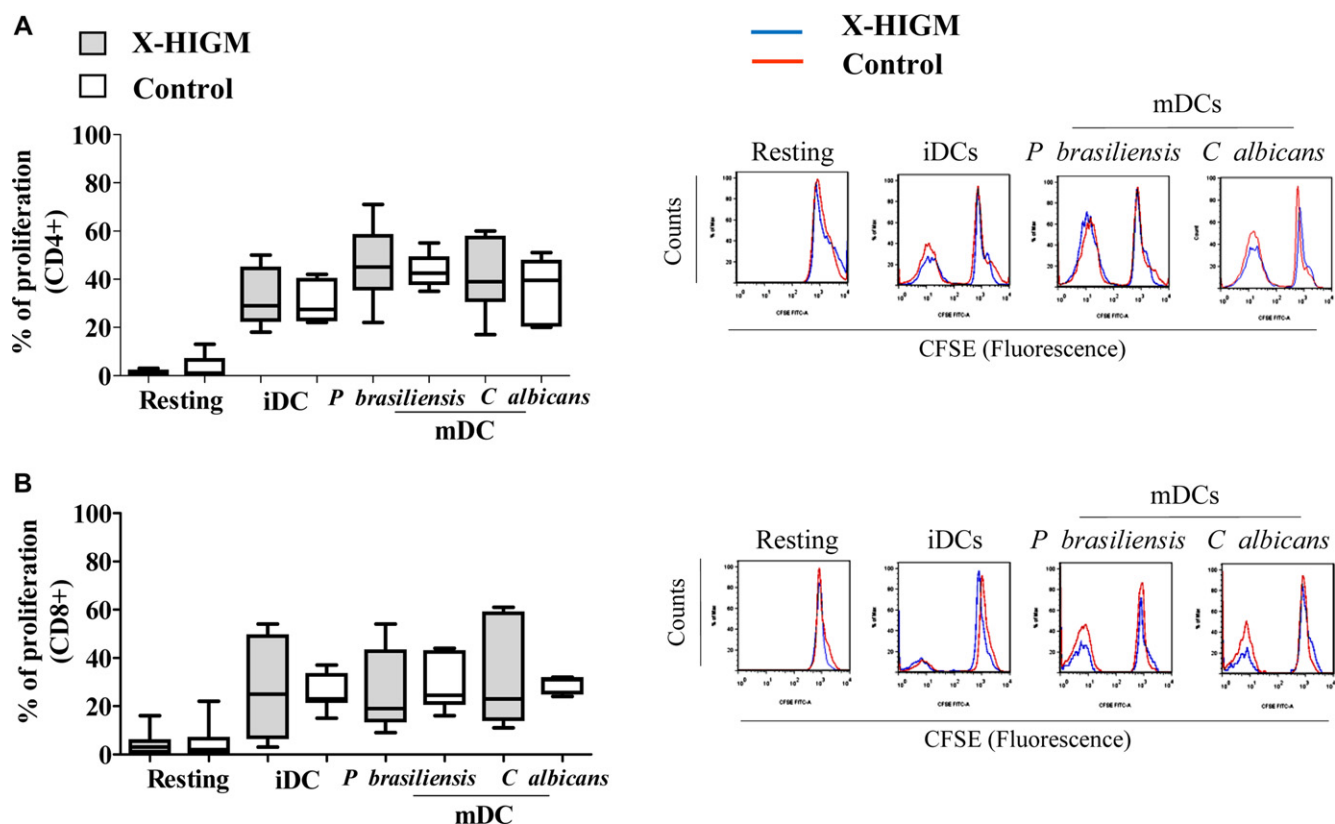


FIG 5. X-HIGM DCs induce normal CD4⁺ and CD8⁺ T-cell proliferation in coculture. iDCs and *P brasiliensis*- or *C albicans*-pulsed mDCs from patients with X-HIGM (blue) showed a normal capacity to induce CD4⁺ and CD8⁺ T-cell proliferation compared with healthy control cells (red) (A and B) ($P > .05$ in all situations; $n = 5$, Mann-Whitney test). CFSE, 5,6-Carboxyfluorescein diacetate succinimidyl ester.

differentiation,¹⁹ in CD4⁺ T cells in the cocultures of *C albicans*-pulsed mDCs with T cells from patients with X-HIGM (data not shown).

DCs are essential for the initiation of specific immune responses to fungal infections by producing IL-12 and inducing the generation of IFN- γ , which is essential for cell-mediated immune responses.⁴ Production of IL-12 by DCs can be initiated either by a T-cell-dependent pathway primarily induced by CD40L-CD40 interaction or by a T-cell-independent pathway induced by direct recognition of microbial products through pattern recognition receptors.³⁴ Our findings that DCs from CD40L-deficient patients produce markedly reduced amounts of IL-12p70 but higher concentrations of IL-10 in response to stimulation with *C albicans* and *P brasiliensis* strongly suggest that CD40L has a nonredundant role in inducing IL-12p70 production and downregulating IL-10.

Previously, a T_H2 pattern of cytokine production (predominance of IL-4, IL-5, and IL-10 and decreased IL-12 and IFN- γ) has been associated with increased susceptibility to fungal infections in humans.³⁰ Furthermore, antifungal immune responses coordinated by IFN- γ can be suppressed at both the inductive and effector levels, particularly by T_H2 inhibitory cytokines (IL-10, IL-4, IL-5).³⁰ Our data strongly suggest an essential role for CD40L-CD40 interaction in driving the protective cellular immune response against fungal infections. As expected from the decreased IL-12 and increased IL-10 production by mDCs, the generation of IFN- γ by X-HIGM T cells in coculture was

markedly reduced. In addition, IL-4 production in response to *C albicans* and IL-5 production in response to *C albicans* and *P brasiliensis* were increased in cultures of patient cells. The restoration of a normal pattern of cytokine production by sCD40L stimulation of the X-HIGM DCs confirms the important role of CD40L-CD40 interactions in these physiologic processes. Notably, sCD40L does not affect the proliferation of CD4⁺ or CD8⁺ T cells, T_H17 generation, or TGF- β production when T cells from patients with X-HIGM were cocultured with autologous DCs.

Our data support and extend observations made by other groups, who used different *in vitro* systems to address the involvement of CD40L-CD40 in driving the T-cell-mediated immune response. Jain et al¹⁴ demonstrated reduced IL-12 production by PBMCs from CD40L-deficient patients when stimulated with PHA, LPS, anti-CD3, or *Staphylococcus aureus*, Cowan's strain I. Similarly, Subauste et al³⁵ demonstrated impaired IL-12 and IFN- γ production by *Toxoplasma gondii*-stimulated PBMCs from 3 patients with X-HIGM, which could be reversed by sCD40L. Furthermore, they demonstrated that neutralization of IL-12 almost completely ablated the stimulatory effect of CD40L on IFN- γ production. Similar data were obtained by Fontana et al¹⁸ who evaluated DCs from 2 patients with CD40 deficiency and found low levels of IL-12 but high levels of IL-10 in the supernatants of DC cultures. In addition, LPS + IFN- γ -activated mDCs from CD40-deficient patients induced impaired allogenic T-cell immune response, which was reversed by blocking IL-10 with an anti-IL-10 neutralizing antibody.

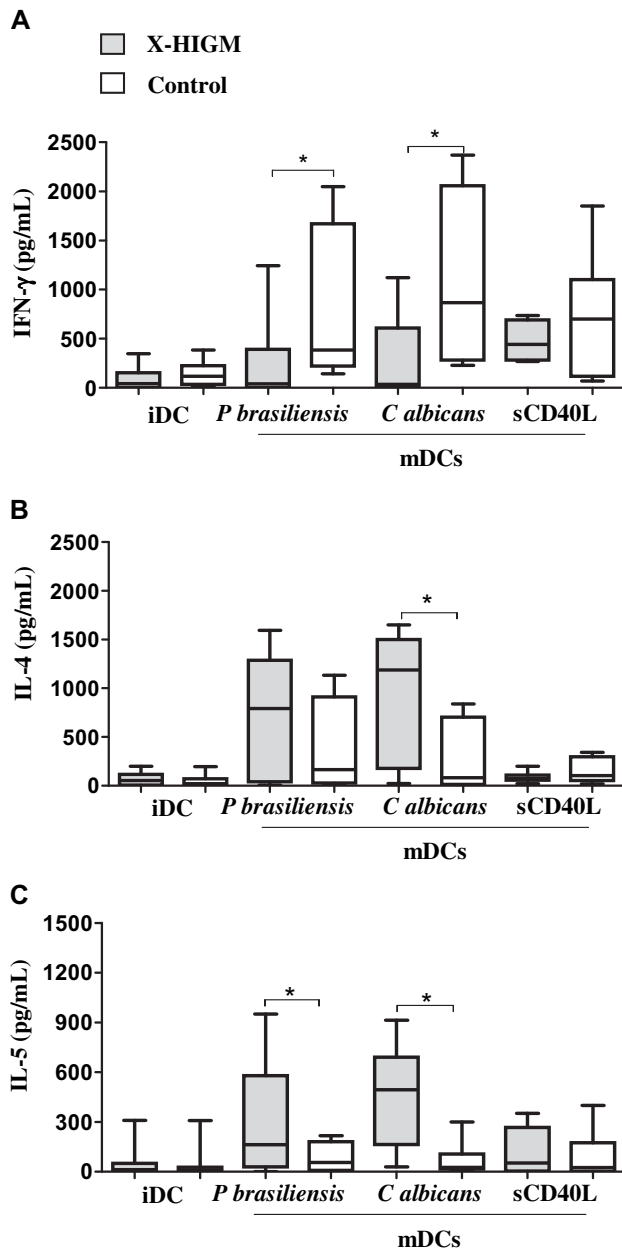


FIG 6. In cocultures, mDCs from CD40L-deficient patients skew T-cell response to a T_H2 pattern. Lower levels of IFN- γ and higher IL-4 and IL-5 production (A-C) were detected in cells from patients with X-HIGM. This was reversed to a pattern similar to that in normal controls when X-HIGM mDCs were matured in the presence of sCD40L. Significant differences are denoted by asterisk ($P < .05$; $n = 5$, Mann-Whitney test).

In conclusion, the functional impairment of DCs from patients with X-HIGM shown here provides potential pathophysiologic insights to explain the increased fungal susceptibility observed in patients with defective CD40L-CD40 signaling. Our data suggest that the impaired interaction of DCs and T cells results in ineffective T-cell-mediated immune responses without affecting T-cell proliferation as demonstrated by coculture experiments of autologous DCs and T cells. Finally, impaired CD40L-CD40 interaction during the development of monocytes and DCs affect their differentiation and costimulatory receptor expression, skewing the induced T-cell responses to a T_H2 pattern.

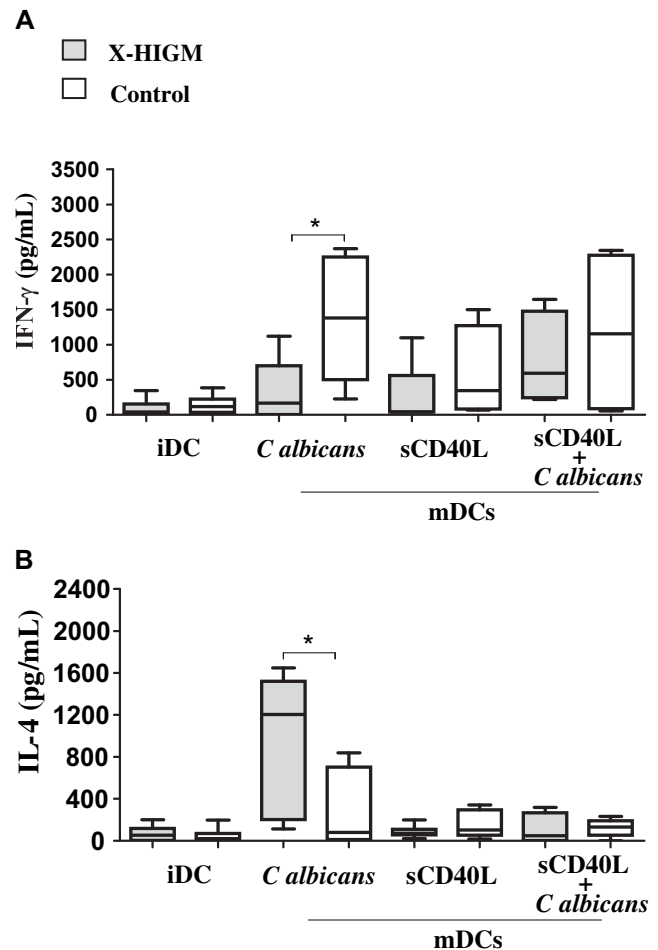


FIG 7. The imbalanced production of IFN- γ and IL-4 is reversed by exogenous sCD40L. Supernatants of T cells cocultured with *C. albicans*-pulsed mDCs from patients with X-HIGM showed reduced level of IFN- γ , which was upregulated by exogenous sCD40L (A), while increased level of IL-4 was downregulated (B). Significant differences are denoted by asterisk ($P < .05$; $n = 5$, Mann-Whitney test).

We thank FAPESP, CNPq, and the Jeffrey Modell Foundation for financial support and the patients and their families for their participation in this study. We are also grateful to Prof Vera L.G. Calich, PhD, and Claudia Feriotti, MSc, for the donation of *P. brasiliensis*.

Clinical implications: This work suggests that the absence of CD40L impairs DC differentiation and may contribute to the increased susceptibility of patients with X-HIGM to life-threatening fungal infections.

REFERENCES

1. Antachopoulos C, Walsh TJ, Roilides E. Fungal infections in primary immunodeficiencies. *Eur J Pediatr* 2007;166:1099-117.
2. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245-52.
3. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J Immunol* 2001;166:249-55.
4. Romani L. Immunity to fungal infections. *Nat Rev Immunol* 2004;4:1-23.
5. Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 1993;259:990-3.

6. Aruffo A, Farrington M, Hollenbaugh D, Li X, Milatovich A, Nonoyama S, et al. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* 1993;72:291-300.
7. DiSanto JP, Bonnefoy JY, Gauchat JF, Fischer A, de Saint Basile G. CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. *Nature* 1993;361:541-3.
8. Fuleihan R, Ramesh N, Loh R, Jabara H, Rosen RS, Chatila T, et al. Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci U S A* 1993;90:2170-3.
9. Korthauer U, Graf D, Mages HW, Briere F, Padayachee M, Malcolm S, et al. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* 1993;361:539-41.
10. Hollenbaugh D, Grosmaire LS, Kullas CD, Chalupny NJ, Braesch-Andersen S, Noelle RJ, et al. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO J* 1992;11:4313-21.
11. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci U S A* 1992;89:6550-4.
12. Dieu MC, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, et al. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* 1998;188:373-86.
13. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994;179:1109-18.
14. Jain A, Atkinson TP, Lipsky PE, Slater JE, Nelson DL, Strober W. Defects of T-cell effector function and post-thymic maturation in X-linked hyper-IgM syndrome. *J Clin Invest* 1999;103:1151-8.
15. Notarangelo LD, Fischer A, Geha RS, Casanova JL, Chapel H, Conley ME, et al. Primary immunodeficiencies: 2009 update. *J Allergy Clin Immunol* 2009;124:1161-78.
16. Barbuto JA, Ensina LF, Neves AR, Bergami-Santos P, Leite KR, Marques R, et al. Dendritic cell-tumor cell hybrid vaccination for metastatic cancer. *Cancer Immunol Immunother* 2004;53:1111-8.
17. Loures FV, Pina A, Felonato M, Calich VL. TLR2 is a negative regulator of Th17 cells and tissue pathology in a pulmonary model of fungal infection. *J Immunol* 2009;183:1279-90.
18. Fontana S, Moratto D, Mangal S, De Francesco M, Vermi W, Ferrari S, et al. Functional defects of dendritic cells in patients with CD40 deficiency. *Blood* 2003;102:4099-106.
19. Renner ED, Rylaarsdam S, Anover-Sombke S, Rack AL, Reichenbach J, Carey JC, et al. Novel signal transducer and activator of transcription 3 (STAT3) mutations, reduced T(H)17 cell numbers, and variably defective STAT3 phosphorylation in hyper-IgE syndrome. *J Allergy Clin Immunol* 2008;122:181-7.
20. Netea MG, Van Der Graaf CA, Vonk AG, Verschuere I, Van Der Meer JW, Kullberg BJ. The role of Toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J Infect Dis* 2002;185:1483-9.
21. Villamon E, Gozalbo D, Roig P, O'Connor JE, Ferrandiz ML, Fradelizi D, et al. Toll-like receptor 2 is dispensable for acquired host immune resistance to *Candida albicans* in a murine model of disseminated candidiasis. *Microbes Infect* 2004;6:542-8.
22. Wang JE, Warris A, Ellingsen EA, Jorgensen PF, Flo TH, Espevik T, et al. Involvement of CD14 and Toll-like receptors in activation of human monocytes by *Aspergillus fumigatus* hyphae. *Infect Immun* 2001;69:2402-6.
23. Doni A, Michela M, Bottazzi B, Peri G, Valentino S, Polentarutti N, et al. Regulation of PTX3, a key component of humoral innate immunity in human dendritic cells: stimulation by IL-10 and inhibition by IFN-gamma. *J Leukoc Biol* 2006;79:797-802.
24. Garlanda C, Hirsch E, Bozza S, Salustri A, De Acetis M, Nota R, et al. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* 2002;420:182-6.
25. Mamoni RL, Blotta MH. Flow-cytometric analysis of cytokine production in human paracoccidioidomycosis. *Cytokine* 2006;35:207-16.
26. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science* 2011;332:65-8.
27. Iezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, Kopf M. CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4⁺ T cells. *Proc Natl Acad Sci U S A* 2009;106:876-81.
28. Katzman SD, Gallo E, Hoyer KK, Abbas AK. Differential requirements for Th1 and Th17 responses to a systemic self-antigen. *J Immunol* 186:4668-73.
29. Romani L, Puccetti P, Bistoni F. Interleukin-12 in infectious diseases. *Clin Microbiol Rev* 1997;10:611-36.
30. Romani L. The T cell response against fungal infections. *Curr Opin Immunol* 1997;9:484-90.
31. Upham JW, Lee PT, Holt BJ, Heaton T, Prescott SL, Sharp MJ, et al. Development of interleukin-12-producing capacity throughout childhood. *Infect Immun* 2002;70:6583-8.
32. Sperling AI, Bluestone JA. The complexities of T-cell co-stimulation: CD28 and beyond. *Immunol Rev* 1996;153:155-82.
33. Vautier S, Sousa Mda G, Brown GD. C-type lectins, fungi and Th17 responses. *Cytokine Growth Factor Rev* 2011;21:405-12.
34. Takenaka H, Maruo S, Yamamoto N, Wysocka M, Ono S, Kobayashi M, et al. Regulation of T cell-dependent and -independent IL-12 production by the three Th2-type cytokines IL-10, IL-6, and IL-4. *J Leukoc Biol* 1997;61:80-7.
35. Subauste CS, Wessendarp M, Sorensen RU, Leiva LE. CD40-CD40 ligand interaction is central to cell-mediated immunity against *Toxoplasma gondii*: patients with hyper-IgM syndrome have a defective type 1 immune response that can be restored by soluble CD40 ligand trimer. *J Immunol* 1999;162:6690-700.