

Defective trained immunity in patients with STAT1-dependent chronic mucocutaneous candidiasis

Short title: Trained immunity in CMC and HIES patients

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Keywords: trained immunity, CMC, HIES

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/cei.12642

Abstract

Patients with *STAT1*-dependent chronic mucocutaneous candidiasis (CMC) and patients with *STAT3*-dependent hyper IgE syndrome (HIES) display defects in Th17 cytokine production capacity. Despite this similar immune defect in Th17 function, they show important differences in the type of infections to which they are susceptible. Recently, our group reported differential regulation of STAT1 and STAT3 transcription factors during epigenetic reprogramming of trained immunity, an important host defense mechanism based on innate immune memory. We therefore hypothesized that *STAT1* and *STAT3* defects have different effects on trained immunity and this may partly explain the differences between CMC and HIES regarding the susceptibility to infections. Indeed, while trained immunity was normally induced in cells isolated from patients with HIES, the induction of innate training was defective in CMC patients. This defect was specific for training with *Candida albicans*, the main pathogen encountered in CMC, and it involved a type II interferon-dependent mechanism. These findings describe the role of STAT1 for the induction of trained immunity, and may contribute to the understanding of the differences in susceptibility to infection between CMC and HIES patients. This study could also provide directions for personalized immunotherapy in patients suffering from these immunodeficiencies.

Introduction

Chronic Mucocutaneous Candidiasis (CMC) and hyper immunoglobulinemia E syndrome (HIES) are rare primary immunodeficiencies, both characterized by defective Th17 cytokine production; this immunological defect is mainly caused by *STAT1* and *STAT3* mutations, respectively (1-5). More rarely, mutations in *IL-17F* or *IL-17RA* have also been described in CMC patients (6), and *DOCK8* mutations in autosomal recessive HIES (7). Despite the similarities between CMC and HIES concerning genetic and immunological defects, important phenotypic differences distinguish these clinical entities regarding susceptibility to infections. While CMC patients suffer mainly from *Candida* and dermatophyte infections, and only later during life from respiratory tract infections (8), HIES patients have a strongly increased susceptibility to skin and respiratory tract infections, especially with *Staphylococcus aureus* and *Streptococcus pneumoniae*, while their fungal complications are less prominent (4, 9-12). The immunological substrate for these differences in susceptibility is not known. Other striking differences are the skeletal abnormalities in HIES and the autoimmune endocrinopathy in CMC (13, 14).

Recently, a novel host defense mechanism has been proposed, consisting of long-term enhanced biological activity of innate immune cells, which was termed *trained immunity* (15, 16). Trained immunity relies on epigenetic reprogramming of monocytes or macrophages, and represents a *de-facto* memory of the innate immune response (17, 18). The epigenetic and metabolic substrate of trained immunity has been lately described (19, 20). Interestingly, these studies uncovered differential regulation of *STAT1* and *STAT3* during induction of trained immunity and innate immune tolerance (19). This finding led us to hypothesize that the

induction of trained immunity may be differentially regulated or defective in patients suffering from CMC and/or HIES.

In the present study we explored the trained immunity responses in peripheral blood mononuclear cells isolated from either CMC or HIES patients. We demonstrate that CMC patients display a defective induction of trained immunity by *Candida albicans*, while this was normal in HIES patients. This defect in CMC patients is specific for *Candida* and is mediated through an interferon- γ (IFN γ)-dependent pathway. These findings might explain the incapacity of CMC patients to clear fungal infections, and may lead to further understanding the differences between these two complex immunodeficiency disorders.

Materials and Methods

Patients and healthy volunteers

Blood samples were collected from five autosomal dominant CMC patients harboring mutations in the *STAT1* gene, three HIES patients with *STAT3* mutations, and from age- and gender-matched healthy volunteers who did not suffer from infectious or inflammatory diseases. Blood was collected by venipuncture into 10 mL EDTA tubes (367525; BD). The study was approved by the Arnhem-Nijmegen Ethical Committee, and the participants gave written informed consent.

Reagents and microorganisms

Culture medium used was RPMI 1640 Dutch modifications (Sigma-Aldrich) supplemented with 1% gentamicin, 1% L-glutamine and 1% pyruvate (Life Technologies, Nieuwekerk, Netherlands). Particulate *C. albicans* β -1,3-glucan was isolated and purified as previously described (21). *C. albicans* ATCC MYA-3573 (UC 820) (22) was grown overnight in Sabouraud broth at 29⁰C; cells were harvested by centrifugation, washed twice, and resuspended in PBS. *Candida* conidia were heat killed for 30 min at 95 ⁰C. Other reagents were obtained as follows: LPS (*E. coli* serotype 055:B5, Sigma-Aldrich) with an additional purification step (23), CD56 MicroBeads were purchased from Miltenyi Biotec. Ustekinumab (CNTO 1275) was purchased from Janssen Biotech, Inc. Nanogam (IgG, human normal immunoglobulin, IVIg) was purchased from Sanquin and IL-18BP was kindly provided by Prof. Charles Dinarello (University of Colorado).

Stimulation experiments

Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (24). PBMCs were counted on a Beckman Coulter Z1 Particle Counter and adjusted to 5×10^6 cells/ml. The trained immunity protocol was performed as described (17): in brief, 100 μ l of the PBMC suspension were added per well in 96-well round-bottom plates, and pre-incubated for 24 h with either RPMI, β -glucan (1 μ g/ml) or heat-killed *Candida* conidia 10^4 CFU/ml, at 37 °C and 5% CO₂. After preincubation, cells were washed with warm PBS and let to rest in RPMI supplemented with 10% human pool serum for 5 days (period during which media was refreshed on day 3). Thereafter, on day 6, cells were subjected to a second stimulation of cytokine production with various stimuli (RPMI, LPS 10 ng/ml, or *Candida* 10^5 CFU/ml) in a final volume of 200 μ l. After another 24 hours, supernatants were collected and stored at -20 °C until assayed.

To investigate whether IFN- γ inhibitors affect *Candida* training, PBMCs were preincubated for 1 h prior to the first stimulation with IgG control, Ustekinumab or IL-18BP in a final concentration of 10 μ g/ml. Subsequently, RPMI or *Candida* were added to the cells with the inhibitors for an additional 24 h. Thereafter, PBMCs were washed with PBS and further incubated for 5 days in culture medium supplemented with 10% pooled human serum. On day 6, the trained macrophages were subjected to a second stimulation, for 24 h, with either LPS or *Candida*. The supernatants were collected and stored at -20°C until assessed.

To assess the role of NK cells in this process, NK cells were isolated via positive selection with CD56 isolation beads from Miltenyi Biotec conform manufacturer's instructions. Thereafter, the CD56 positive cells were stimulated for 48 hours with heat-killed *Candida* (10^6 CFU/ml) or heat-killed *S. aureus* (10^7 CFU/ml). PBMCs depleted of NK cells were further included in training experiments, being trained with *Candida* (10^4 CFU/ml) as described above and restimulated with either LPS (10 ng/ml) or *Candida* (10^5 CFU/ml). After

24 hours of cell stimulation, supernatants were collected and stored at -20 °C until the amounts of TNF- α and IL-6 were assayed.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF- α (R&D Systems, Abingdon, UK), and IL-6 (Sanquin, Amsterdam, Netherlands) were measured 24 h after the second stimulation in cell culture supernatants with ELISA, according to the manufacturer instructions. The concentration of IFN- γ (Sanquin, Amsterdam, Netherlands) was measured in cell culture supernatants at 48h after the stimulation.

Statistical analysis

Results from at least two sets of experiments with a minimum of four volunteers were pooled and analyzed in GraphPad. The data are presented as the means $\pm$ SEM and Wilcoxon signed-rank test was used to compare differences between groups. The level of significance was set at $*p < 0.05$.

Results

PBMCs isolated from patients with CMC, but not HIES, display defective *Candida*-mediated trained immunity

As previously shown, PBMCs of patients who suffer from CMC due to *STAT1* mutations stimulated for 24 h with heat-killed *Candida* were able to produce normal amounts of TNF α and IL-6 compared to the healthy controls (2). We therefore tested whether trained immunity could be triggered by *Candida*. PBMCs from five CMC patients and nine healthy donors that were exposed to β -glucan, *Candida* and RPMI (negative control) for 24 h. On day 6 after recovery, cells were subjected to a second exposure to LPS, *Candida* or RPMI (negative control) for an additional 24 h. Interestingly, while PBMCs from CMC patients could be trained by β -glucan (**Suppl. Fig. 1**), they could not be trained by *C. albicans* (**Fig. 1**).

In contrast, PBMCs isolated from HIES patients showed a normal degree of training with both *Candida* (**Fig. 2**) and β -glucan (**Suppl. Fig. 2**), as shown by the enhanced production of TNF α and IL-6 upon restimulation with either LPS or *Candida*.

Defective IFN- γ production by NK cells from CMC patients

The defective trained immunity in PBMCs from CMC patients suggests that *STAT1*, but not *STAT3*, is crucial for *Candida*-induced trained immunity. IFN- γ is a proinflammatory cytokine produced by NK- and T-cells that induces activation of both innate and adaptive immunity against candidiasis. Because IFN- γ acts through an IFNGR- and *STAT1*-dependent signaling pathway, and has been previously reported to epigenetically reverse immune tolerance (25), we hypothesized that it may be involved in the defective trained immunity in CMC. Interestingly, while purified NK cells from HIES patients produced similar amounts of

IFN- γ as the healthy controls (**Fig. 3A**), IFN- γ production was significantly diminished in CMC patients (**Fig. 3A**), suggesting defective NK cell function in patients suffering of CMC only. Moreover, when PBMCs from both groups of patients were depleted of NKs and subsequently trained with *Candida*, not only the cells from patients with CMC showed a defect in training, but NK-depleted cells from HIES were also not able to respond to a similar extend as PBMCs from healthy controls (**Fig. 3B**), demonstrating an essential role for the NK-cell-derived IFN- γ production for the induction of training in HIES patients.

Of importance, depletion of NK cells in PBMCs from healthy volunteers did not abolish their capacity to mount a trained immunity response (**Fig. 3C**), suggesting that in healthy volunteers T-cell-derived IFN- γ is able to complement for the loss of NK-cell function. The defective Th1-cell function in CMC and HIES patients leads however to a strong dependency on NK-cell-derived IFN- γ .

Inhibiting IFN- γ production by blocking IL-12 and IL-23 with Ustekinumab, or by blocking IL-18 with IL-18BP, significantly decreased the training by *Candida* in PBMCs from healthy controls, both for TNF α and IL-6 (**Fig. 4**). Of note, the training was not abolished by IgG control (**Fig. 4**). Moreover, the inhibition was significant only for *Candidia* training and *Candidia* restimulation, and not for LPS restimulation (data not shown).

Discussion

In this study we demonstrated that PBMCs from CMC patients failed to mount *Candida*-induced trained immunity. This process was caused by *STAT1* defects and mediated through NK-cell-dependent production of IFN- γ .

The findings of the present study are relevant at several levels. Firstly, they point to an important immunological difference between patients with CMC and HIES. As both these immunodeficiencies are caused by genetic defects in STAT molecules, and have a similar immunological defect in proper activation of Th17 pathway (1, 3), it remained unexplained why their susceptibility to infection is different. The identification of specific defects in *Candida*-induced trained immunity in CMC, but not HIES patients, represents a first clue for the severe susceptibility to fungal infections in CMC, compared with the less prominent susceptibility to fungal infections in HIES.

Secondly, the discovery of trained immunity defects in CMC patients, and not in HIES, provides important insights into understanding the communication between important players in host defense. Through *in vitro* experiments with NK cells and NK cell depletions, we concluded that T- and NK cell-derived IFN- γ plays a central role in potentiating trained immunity in a paracrine and possible endocrine manner. Although trained immunity has been initially described to be induced in monocytes, it should be expected that important interactions should take place with other cell types. The first of such interactions is described here, with NK-cell derived IFN- γ playing a central role in potentiating trained immunity in a paracrine and possible endocrine manner. We found that while the role of NK-cells is redundant in healthy individuals with normal T-cell function, the release of IFN- γ by NK cells

rescues the training in HIES patients that have defective T cell IFN- γ production. In contrast, CMC patients that display both defective T- and NK-cell production of IFN- γ explain defective trained immunity. A role for NK-cells in the induction of trained immunity has been also recently described after BCG vaccination (26).

The role of IFN- γ and STAT1 for the induction of trained immunity is in line with previous observations. We have described differential regulation of STAT1 and STAT3 during induction of trained immunity and immune tolerance (19), data that provided the initial impulse to study these mechanisms in CMC and HIES. Furthermore, IFN- γ has been demonstrated to reverse the effects of LPS tolerance, the 'opposite' of trained immunity, specifically by activating *Brg1*, which in turn remodels chromatin facilitating the access of transcription factors (25). IFN- γ exerts its biological activity through activation of intracellular pathways containing Jak1, Jak2 and STAT1 (27). The role of STAT1 signaling for trained immunity may thus be explained through its role in the type II IFNGR pathway. In line with this, a recent study demonstrated that activation of STAT-1 by IFN- γ correlates with multiple histone modifications such as H3K4me2, H3K4me3, H3K9me3 and H3K36me3 (28).

It remains unclear why β -glucan is still able to train cells from CMC patients, as it has been proposed that the mechanism through which *Candida* trains immune cells is through β -glucan/Dectin-1 (17). However, recent data suggesting that other cell wall *Candida* structures, such as chitin, are also important for trained immunity induced by the whole fungus (Rizzetto L, personal communication) may represent one possible explanation. One limitation of the study that should be acknowledged is the limited number of patients. Unfortunately, this was unavoidable due to the rare nature of both CMC and HIES diseases.

Although the *in vitro* model of training PBMCs is a simplified system, it does provide a direct assessment of the propensity of PBMCs from diverse individuals to be trained. However, this is the first study reporting defective trained immunity in CMC and normal training effects in HIES patients. Thereby this study enhances our understanding of specific immunological disturbances in each of these complex immunodeficiencies. Moreover, it provides new openings in the design of novel immunotherapeutical strategies towards a personalized treatment of CMC and HIES.

Acknowledgements

This work was supported by European Union ALLFUN (FP7/2007 2013, HEALTH-2010-260338) (Fungi in the setting of inflammation, allergy and autoimmune diseases: Translating basic science into clinical practices “ALLFUN”). M.G.N and J.Q. were also supported by a Vici grant of the Netherlands Organization of Scientific Research (to M.G.N.). F.vd.V was supported by a Veni grant of the Netherlands Organization of Scientific Research. D.C.I., J.Q., T.S.P., J.W.M.v.d.M., F.L.v.d.V. and M.G.N. designed and analyzed the experiments. D.C.I. performed the experiments. J.Q. and T.S.P. contributed to some of the experiments. D.C.I., L.A.B.J, F.L.v.d.V., J.W.M.v.d.M., and M.G.N. wrote the manuscript, and all authors contributed to the manuscript preparation. M.G.N. supervised the project.

Conflicts of interest.

The authors declare no conflict of interest.

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Figure legends

Figure 1: Defective trained immunity in CMC patients.

PBMCs from healthy volunteers and CMC patients were trained for 24 hours by incubation with culture medium or low concentrations of *C. albicans* (10^4 CFU/ml). Thereafter the stimulus was removed and cells were kept in RPMI with 10% human serum. On day 6, a second stimulation with LPS (10 ng/ml) or *C. albicans* (10^5 CFU/ml) was performed for an additional 24 h. TNF α and IL-6 levels were measured in cell culture supernatants after the second stimulation. $n = 9$ healthy controls, and $n = 5$ CMC patients. The data are presented as the means $\pm$ SEM. HC: healthy control; CMC: Chronic Mucocutaneous Candidiasis; LPS: Lipopolysaccharide; *C.alb.*: *Candida albicans*.

Figure 2: Normal trained immunity in HIES patients.

PBMCs from healthy volunteers and HIES patients were trained for 24 hours with culture medium or to very low concentrations of *C. albicans* (10^4 CFU/ml). At day 6, a second stimulation with LPS (10 ng/ml) or *C. albicans* (10^5 CFU/ml) was performed for an additional 24 h. TNF α and IL-6 levels were measured in cell culture supernatants after the second stimulation. $n = 9$ healthy controls, and $n = 3$ HIES patients. The data are presented as the means $\pm$ SEM. HC: healthy control; HIES: Hyper-IgE syndrome; LPS: Lipopolysaccharide; *C.alb.*: *Candida albicans*.

Figure 3: NK-dependent production of IFN- γ mediated trained immunity.

(A) Defective NK cells in CMC patients. NK cells from two CMC and two HIES patients were isolated with CD56 positive beads and thereafter stimulated with either *C. albicans* or *S. aureus*. IFN- γ was measured in cell culture supernatants after 48 hours of incubation. (B) Defective training of PBMCs depleted of NK cells in CMC and HIES patients. PBMCs from one CMC and two HIES patients were first depleted of NK cells and then trained for 24 hours with culture medium or to very low concentrations of *C. albicans* (10^4 CFU/ml). At day 6, a second stimulation with *C. albicans* (10^5 CFU/ml) was performed for an additional 24 h. TNF α and IL-6 levels were measured in cell culture supernatants after the second stimulation. The data are presented as the means $\pm$ SEM. HC: healthy control; CMC: Chronic Mucocutaneous Candidiasis; HIES: Hyper-IgE syndrome; *C.alb.*: *Candida albicans*. (C) Training of PBMCs from healthy controls depleted of NK cells. PBMCs from healthy controls were trained for 24 hours with culture medium or to very low concentrations of *C. albicans* (10^4 CFU/ml), after which they were stimulated as described above. $n = 6$, 3 independent experiments. The data are presented as the means $\pm$ SEM. LPS: Lipopolysaccharide; *C.alb.*: *Candida albicans*.

Figure 4: IFN- γ -dependent *Candida*-induced training.

Before training, PBMCs were preincubated for 1 h with IgG (isotype control) (10 μ g/ml), Ustekinumab (10 μ g/ml) or IL-18BP (10 μ g/ml). Thereafter, 100 μ l of culture medium or *C. albicans* (10^4 CFU/ml) was added to the cell suspension, without removing the inhibitors, and further incubated for an additional 23 h. At day 6, macrophages were subjected to a second stimulation with LPS (10 ng/ml) or *C. albicans* (10^5 CFU/ml) for another 24 h. TNF α and IL-6 levels were measured in cell culture supernatants after the second stimulation. Data are

presented as mean $\pm$ SEM (n = 5, three independent experiments), *p<0.05. Wilcoxon signed rank test was used to detect significant differences. U: Ustekinumab; *Ca.*: *Candida albicans*.

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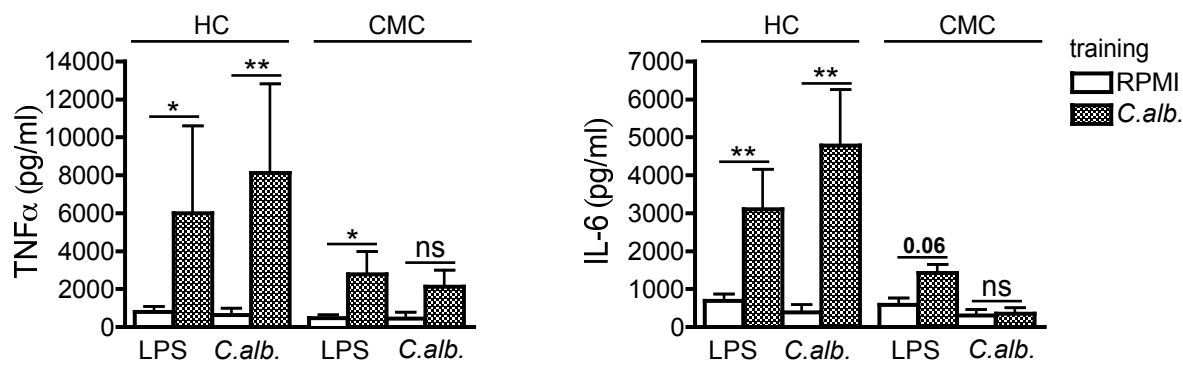


Figure 1

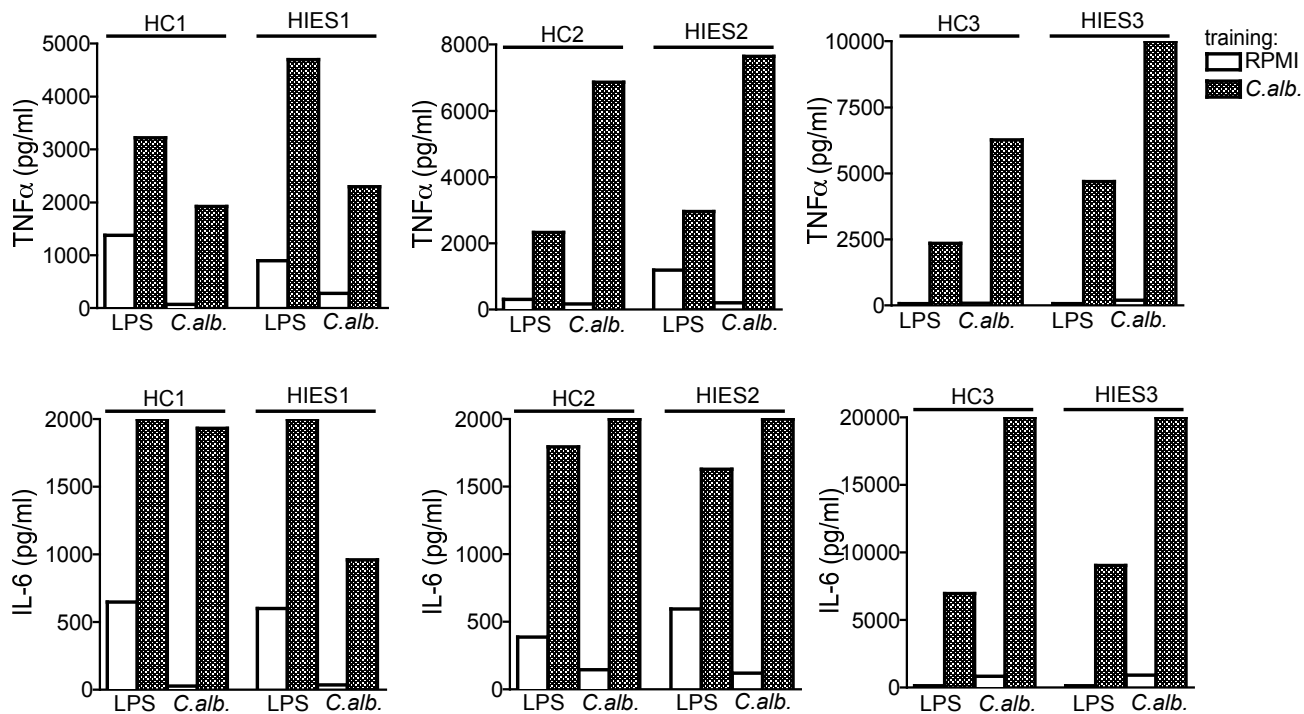
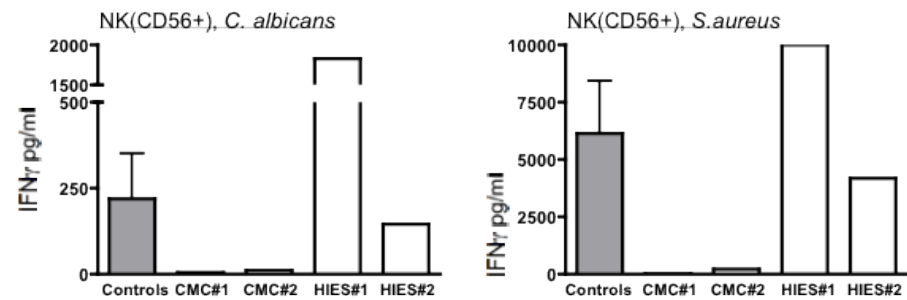
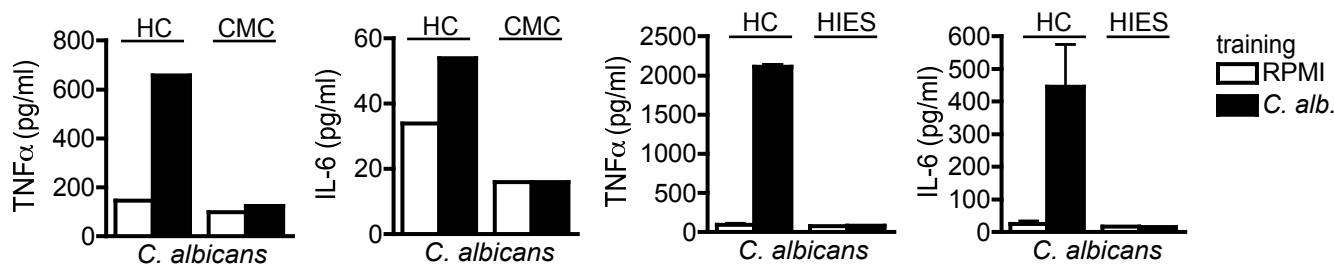


Figure 2

A: Deficient IFN γ production by NKs from CMC patients



B: Deficient training of PBMCs depleted of NKs from CMC and HIES



C: Normal training of PBMCs from healthy controls depleted of NK cells

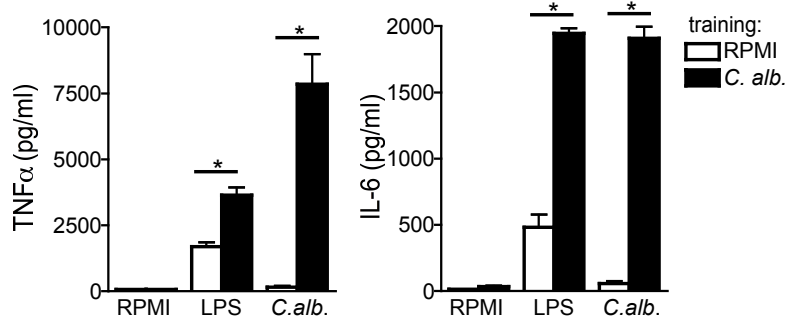


Figure 3

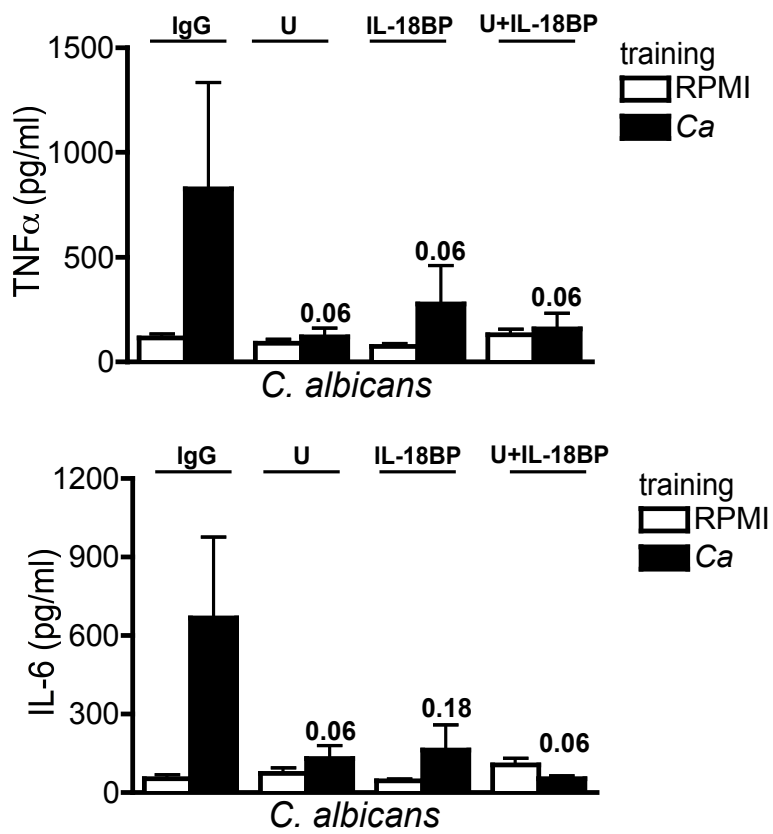
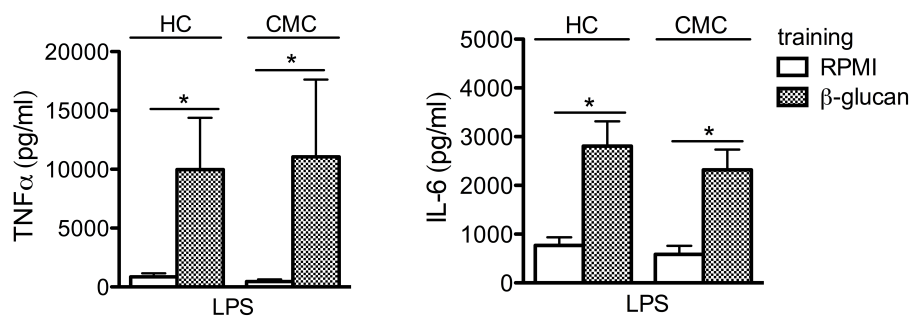
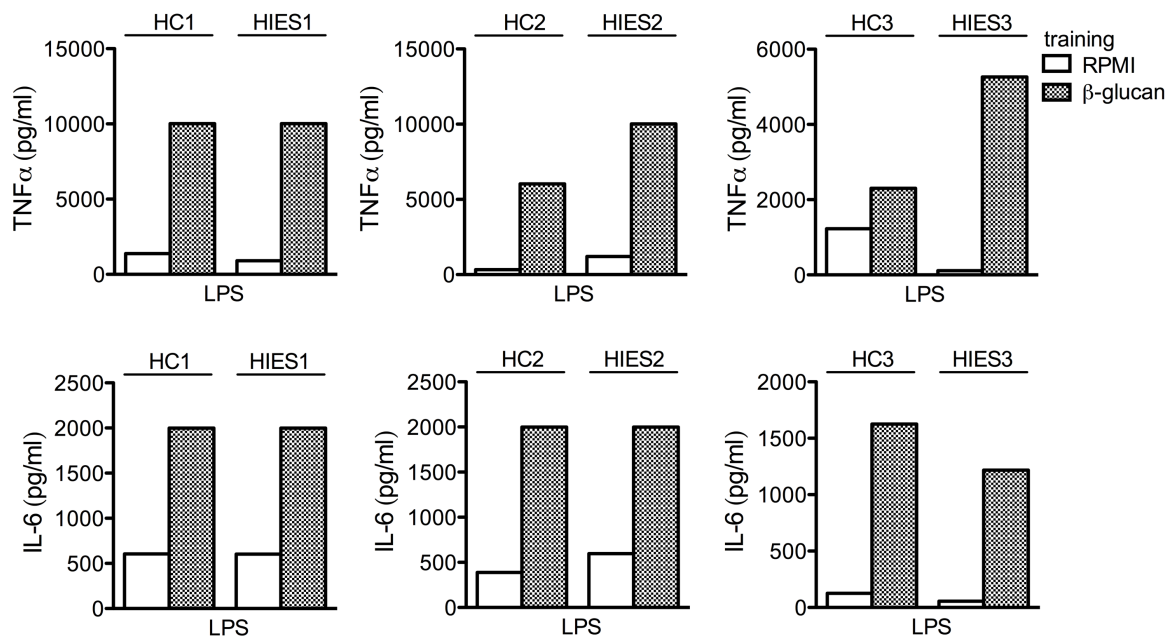


Figure 4



Supplementary Figure 1
PBMCs from healthy volunteers and CMC patients were trained for 24 hours with culture medium or β-glucan (10 μg/ml). On day 6, a second stimulation with LPS (10 ng/ml) was performed for an additional 24 h. TNFα and IL-6 levels measured were measured in cell culture supernatants after the second stimulation. n = 9 healthy controls, n = 5 CMC patients, bars indicate mean ±SEM. CMC: Chronic Mucocutaneous Candidiasis; LPS: Lipopolysaccharide.



Supplementary Figure 2

PBMCs from healthy volunteers and HIES patients were trained for 24 hours with culture medium or β-glucan (10 μg/ml). On day 6, a second stimulation with LPS (10 ng/ml) was performed for an additional 24 h. TNFα and IL-6 levels measured were measured in cell culture supernatants after the second stimulation. $n = 3$ healthy controls, $n = 3$ CMC patients, bars indicate mean ± SEM. HIES: Hyper-IgE syndrome; LPS: Lipopolysaccharide.