Invariant natural killer T (iNKT) cell deficiency in Chronic Mucocutaneous Candidiasis - a consequence ~~rather than~~or a cause?

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Key words: chronic mucocutaneous candidiasis (CMC), invariant natural killer T (iNKT) cells, autoimmune regulator gene

Abstract

Chronic mucocutaneous candidiasis (CMC) is a group of heterogeneous disorders characterised by primary selective susceptibility to chronic, recurrent *Candida* infections. The genetic defect of one subgroup of CMC patients have been identified as mutations of the autoimmune regulator (*AIRE*) gene. Recent data implicated the *AIRE* gene in iNKT cell development, raising the possibility that iNKT cells may be important in defending against *Candida* infections. In this study, we enumerated the circulating iNKT frequency in 22 CMC patients (9 with *AIRE* gene mutations) and 25 healthy controls. We also examined the effect of *Candida* stimulation on iNKT cells in vitro. Our data demonstrated that peripheral iNKT cell frequency is significantly reduced in CMC patients compared to healthy controls, regardless of their *AIRE* gene mutation status. Direct stimulation with *Candida* did not induce iNKT cell proliferation. Furthermore, circulating iNKT cell frequencies in some healthy controls were comparable to CMC patients. These observations suggest that iNKT cell deficiency is part of the CMC disease phenotype irrespective of ~~molecular aetiology~~*AIRE* gene mutations but does not appear to confer susceptibility to chronic *Candida* infections. We postulate that the reduced circulating iNKT cell frequency in CMC is a consequence rather than a cause of chronic *Candida* infections.

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Letter to the Editor

Invariant natural killer T (iNKT) cells are a unique subset of T cells which use a restricted repertoire of T cell receptor α- and β-chains (V_α24-J_α281 preferentially paired with V_β11 in humans) and express surface molecules that are usually found on natural killer cells. Unlike conventional T cells, iNKT cells recognise glycolipids presented by major histocompatibility complex class I-like molecules, CD1d. Data from rodent models implicate iNKT cells in the regulation of many immune responses [1] but their role in human diseases remains to be fully defined. Chronic mucocutaneous candidiasis (CMC) is a rare, heterogeneous group of conditions characterised by a primary selective susceptibility to persistent or recurring *Candida* infections [2]. A subset of CMC patients harbour mutations of the autoimmune regulator (*AIRE*) gene and also develop organ-specific autoimmune diseases and ectodermal dystrophy [3], hence termed Autoimmune Poly-Endocrinopathy Candidiasis Ectodermal Dystrophy (APECED) syndrome. Interestingly, Lindh et al recently reported that iNKT cell development was impaired in *aire*$^{-/-}$ mice and in 3 individuals with *AIRE* gene mutations [4]. In contrast, Pitt and colleagues showed that iNKT cell development is unimpaired in *aire*$^{-/-}$ mice [5] and Somech et al reported that children with Omenn syndrome have reduced transcriptional expression of the *AIRE* gene but normal iNKT cell frequency [6]. The reason for the discrepant findings is not clear. Furthermore, peripheral iNKT cell frequency in CMC patients without *AIRE* gene mutations has not been studied.

We enumerated the circulating iNKT cell frequency in 22 CMC patients including 9 with *AIRE* gene mutations, and 25 healthy controls (Table 1). The two groups were similar in

age and gender. None of the CMC patients were taking immuno-modulatory medications at the time of study. iNKT cells were defined by the co-expression of $V_{\alpha}24$ and $V_{\beta}11$ on $CD3^+$ lymphocytes using flow cytometry (gating strategy is shown in supplementary figure 1). This method has been demonstrated to correspond well with iNKT cell frequency determined by CD1d tetramers or a monoclonal antibody specific for the invariant CDR3 loop of the human canonical $V_{\alpha}24J_{\alpha}18$ chain, even at low numbers [7,8]. The frequency of circulating iNKT cells among $CD3^+$ lymphocytes in CMC patients was significantly lower compared to healthy controls (mean=0.018±0.003% versus 0.061±0.012%, p=0.008) regardless of the *AIRE* gene mutation status (Fig. 1a&b), indicating that reduced iNKT cell frequency is part of the CMC disease phenotype ~~irrespective of molecular aetiology~~. Serial measurements over a period of 6-12 months in 5 healthy volunteers and 3 CMC patients (Fig. 1c&d) showed that the iNKT cell frequency was stable over time. The absolute number of circulating iNKT cells was not specifically determined but since observations from routine clinical practice suggest that the lymphocyte counts of ~~all~~ CMC patients were usually within normal ranges, we anticipated that the absolute numbers of peripheral blood iNKT cells among CMC patients were also reduced compared to healthy controls. Consistently, we have estimated the absolute circulating iNKT cell numbers based on the contemporaneous absolute lymphocyte counts of CMC patients and mean reference lymphocyte counts of healthy volunteers, and showed that the absolute numbers of peripheral blood iNKT cells were also significant lower among CMC patients than healthy controls (supplementary figure 3 & supplementary tables 1&2).

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In this study, although the range of iNKT cell frequency among healthy individuals is wide, it is evident that the iNKT cell frequency was comparable between some healthy individuals and CMC patients suggesting that reduced circulating iNKT cell frequency *per se* ~~does not~~ is perhaps unlikely to confer susceptibility to *Candida* infections.

To further investigate whether iNKT cells may play a role in the protection against *Candida* infections, we stimulated control PBMC with total *Candida* extract and determined whether this induced iNKT cell expansion. As anticipated, stimulation of PBMC with an iNKT cell-specific agonist, α-galactosylceramide, induced marked expansion of iNKT cells after 10-12 days. In contrast, no apparent expansion of iNKT cells was observed in *Candida*-stimulated PBMC culture (Fig. 2a). In addition, co-culture of iNKT cell lines generated from 2 healthy individuals with total *Candida* extract in the presence of irradiated autologous PBMC also did not induce proliferation (Fig. 2b).

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Taken together, these observations argue against a ~~significant~~ key role ~~of~~ for iNKT cells in the protection against *Candida*, but raise the possibility that circulating iNKT cell deficiency in CMC patients is a direct or indirect consequence of persistent *Candida* infections. However, further experiments are needed to address this possibility. Consistent with this hypothesis, circulating iNKT cell frequency rises with age in healthy controls but not among CMC patients within our cohort but our sample sizes are small (supplementary figure 2). ~~This hypothesis may also account for the contradictory findings of iNKT cell development in *aire*$^{-/-}$ mice since the frequency of iNKT cells could be affected by their previous exposure to *Candida* infections.~~ It would be interesting to

monitor the circulating iNKT cell frequency ~~in *aire*$^{-/-}$ and~~ wild-type mice following repeated *Candida* infections and collect longitudinal data on iNKT cell frequency of healthy and CMC children.

In this study, iNKT cell frequency in the infected sites was not determined as no biopsy sample was available. This is because for CMC patients, *Candida* infections are usually localised to the gut and skin, and diagnosis is confirmed by culture so that biopsies of the infected sites are not indicated. Therefore, peripheral iNKT cell deficiency could be a consequence of preferential recruitment of iNKT cells to the sites of infection. However, we consider this a less attractive explanation because iNKT cells do not respond directly to *Candida* stimulation although an indirect role for iNKT cells in the defence against *Candida* remains possible. Furthermore, it does not adequately account for the observations that peripheral iNKT cell frequency of some healthy individuals is comparable to that of CMC patients. Determination of the activation states of the iNKT cells may be helpful in addressing these possibilities but requires a dedicated study in the future.

It has been suggested that different iNKT cell subsets with differential ability to skew cytokine production is a key mechanism by which iNKT cells regulate immune responses [9-11]. In this study, the frequencies of different iNKT cell subsets were not determined as we were limited by the amount of peripheral blood samples available, especially those from children. Further study dedicated to the study iNKT cell subsets and cytokine profiles in CMC patients are warranted.

To conclude, our data suggest that iNKT cell deficiency is part of the CMC disease phenotype irrespective of ~~*molecular aetiology*~~*AIRE* gene mutations ~~but does not appear to confer susceptibility~~and whether it is a cause or consequence ~~to~~ of chronic *Candida* infections remains to be determined.

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Acknowledgement

This work was supported by the JGW Patterson Foundation and the Primary Immunodeficiency Association. WFN also received salary support from the Arthritis Research UK. The authors would like to thank all the patients and healthy volunteers for taking part in this study.

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Figure Legends

Figure 1. *Circulating iNKT cell frequency of CMC patients is significantly reduced compared to healthy controls and is independent of the AIRE gene mutation status.* The frequency of peripheral blood iNKT cells was enumerated using flow cytometry. iNKT cells were identified by the co-expression of $CD3^+V_\alpha14^+V_\beta11^+$ cells. (a) The percentage of iNKT cells among lymphocytes was shown for all CMC patients (CMC, filled squares) and healthy controls (Controls, filled triangles). (b) The percentage of iNKT cells among lymphocytes was shown for CMC patients with *AIRE* gene mutation ($AIRE^{-/-}$, filled squares), without *AIRE* gene mutation ($AIRE^{+/+}$, unfilled squares) and healthy controls (Controls, filled triangles). Horizontal lines represent median values of the groups. *(c&d) Peripheral blood iNKT cell frequency is stable over time.* Circulating iNKT cell frequency was measured over a period of 6 to 12 months in 5 healthy volunteers (c) and 3 CMC patients (d).

Figure 2. *iNKT cells do not proliferate in response to Candida stimulation.* (a) $2x10^6$ PBMC from 3 healthy controls were stimulated total *Candida* extract (1.1 µg/ml protein or $1.1x10^5$ CFU/ml) or α-GC (100 ng/ml) for 12 days, the percentages of iNKT cells among lymphocytes were enumerated as earlier described at day 3, 5, 7, 10 and 12. A representative figure is shown. ***(b)*** An iNKT cell line, generated by 5 repeated stimulation with α-GC every 10-14 days, from a healthy individual was labelled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with total extracts of *Candida* or α-GC ~~(100 ng/ml)~~ in the presence of irradiated autologous PBMC for 12

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days. Dilution of CFSE signals among $6B11^+$ lymphocytes (which specifically identifies iNKT cells) were measured using flow cytometry. Similar findings were obtained with another iNKT cell line from a different individual.

Supplementary figure legends

Supplementary figure 1. *(a) The gating strategy and representative flow cytometric plots of iNKT cell frequency enumeration.* PBMC were stained with flurochrome-conjugated anti-CD3, anti-$V_{\alpha}14$ and anti-$V_{\beta}11$. The lymphocyte gate was set based on forward and side scattered characteristics. A minimum of 500,000 lymphocyte-gated events were collected for each sample. The number of $CD3^{+}V_{\alpha}14^{+}V_{\beta}11^{+}$ cells was expressed as a percentage of $CD3^{+}$ lymphocytes. Representative flow-cytometric plot of a healthy individual (left panel) and a CMC patient (right panel) together with the gating strategy are shown.

Supplementary figure 2. *Circulating iNKT cell frequencies of iNKT cells increase with age in healthy individuals but not in CMC patients regardless of their AIRE gene mutation status.* (a-b) The percentages of peripheral iNKT cells of healthy individuals (a) and CMC patients (b) were plotted against their ages. Correlation between iNKT cell frequency and age was analyzed using Pearson's correlation. The solid line and the dotted lines represent linear relationship and the 95% confidence intervals. (c) The percentages of lymphocytes of CMC patients were classified according to their age and *AIRE* gene mutation status: CMC patients with *AIRE* gene mutations who are adults (age>16 years, adult $AIRE^{-/-}$) or children (age≤16 years, Child $AIRE^{-/-}$), CMC patients without *AIRE* gene mutations who are adults (age>16 years, adult $AIRE^{+/+}$) or children (age≤16 years, Child $AIRE^{+/+}$). Horizontal lines represent median values of the groups.

Supplementary figure 3. *Estimated circulating iNKT cell numbers are reduced in CMC*

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patients. The absolute numbers of peripheral blood iNKT cells were calculated by multiplying the following formula: {[% of iNKT cells (of lymphocytes)] x [Lymphocyte counts from contemporaneous complete blood counts (No. of cells/ml of blood)]}. Since contemperanous complete blood counts were not performed in healthy volunteers and 8 CMC patients, for these subjects, the mean laboratory reference values of lymphocyte counts for healthy individuals (Comans-Bitter WM et al, 2006 (ISBN-10 90-73436-75-3)) were used instead of the measured lymphocyte counts. The estimated iNKT cell numbers of healthy volunteers were compared (Mann Whitney) with those of CMC patients, either for those with measured lymphocyte counts, or for the entire patient group.

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