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

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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.
Invariant natural killer T (iNKT) cells are a unique subset of T cells which use a restricted repertoire of T cell receptor $\alpha$- and $\beta$-chains ($V_\alpha 24-J_\alpha 281$ preferentially paired with $V_\beta 11$ 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$^{-/-}$ mouse [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\(_{α24}\) and V\(_{β11}\) 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\(_{α24Jα18}\) 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 significantly lower among CMC patients than healthy controls (supplementary figure 3 & supplementary tables 1&2).
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 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).

Taken together, these observations argue against a significant key role of 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 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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References

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+Vα14+Vβ11+ 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 with 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
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 fluorochrome-conjugated anti-CD3, anti-V_α14 and anti-V_β11. 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_α14^+V_β11^+ 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...
patients. The absolute numbers of peripheral blood iNKT cells were calculated by multiplying the following formula: \[ \% \text{ of iNKT cells (of lymphocytes)} \times \text{Lymphocyte counts from contemporaneous complete blood counts (No. of cells/ml of blood)} \]. Since contemporaneous 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.