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ORAL MICROBIOLOGY
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Short communication

Gene targeting demonstrates that inducible nitric oxide synthase is not essential for resistance to oral candidiasis in mice, or for killing of *Candida albicans* by macrophages *in vitro*

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Introduction: Oral candidiasis is caused by opportunistic infections with the yeast *Candida albicans*. Previous studies have demonstrated important roles for innate immunity and T helper type 1-mediated inflammatory reactions in recovery from infection, with macrophages and neutrophils as key effector cells. Both effector cell types use the inducible isoform of nitric oxide synthase (iNOS) to generate candidacidal molecules, but it is not clear whether nitric oxide (NO) is an absolute requirement for candidacidal effector activity.
Methods: In this study we directly investigated the role of iNOS-derived NO in resistance to murine experimental oral candidiasis, using iNOS knockout mice.
Results: Knockout mice were no more susceptible to oral candidiasis than wild-type controls. Bone marrow-derived macrophages from the knockout mice killed *C. albicans* yeasts efficiently *in vitro*, and were still able to produce nitrites in an iNOS-independent manner, albeit less efficiently than wild-type controls. There were no significant differences in local mucosal production of interleukins 6, 12, 17A, or 23, interferon-γ, or transforming growth factor-β 24 h after oral challenge with *C. albicans*.
Conclusion: These data suggest that iNOS-derived NO is not required for resistance to oral candidiasis *in vivo*, and that bone marrow-derived macrophages may have iNOS-independent means of generating reactive nitrogen species.

Key words: *Candida albicans*; innate immunity; iNOS; oral candidiasis; oral infection

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Candida albicans is a commensal fungus that undergoes pathogenic transformation in immunocompromised hosts, such as patients with human immunodeficiency virus infection/acquired immune deficiency syndrome, patients who have undergone organ transplant, or those who have received head and neck radiation therapy or chemotherapy. Oral candidiasis is the most common manifestation of *C. albicans* infection (28, 31). Studying disease progression in cytokine knockout mice and other disease models has revealed critical roles for innate immunity and T helper type 1 cell-mediated

responses in host defense against oral candidiasis (1, 12, 14, 17). Sites of fungal invasion are characterized by a neutrophil-rich and $CR3^+$ phagocyte (predominantly macrophage)-rich inflammatory infiltrate (7), and functional inactivation of these cells significantly increased oral fungal carriage in different mouse strains (15), suggesting an important role for inflammatory effectors in the response to oral *C. albicans* infection.

One of the key candidacidal functions of activated phagocytes is the respiratory burst: the strong induction of oxidative metabolic pathways leading to a surge in production of reactive oxygen and nitrogen intermediates, which kill or inhibit fungal growth by producing protein and lipid adducts, and DNA breaks. The respiratory burst can be induced by pathogen-associated molecular pattern receptor (PAMP-R) signaling, and can be augmented by inflammatory cytokines, such as interferon-γ (IFN-γ), interleukin-17 (IL-17), IL-6, and tumor necrosis factor-α (5, 26, 29). Macrophages and neutrophils from both humans and mice use three oxidative enzyme systems to kill *C. albicans*: phagocyte oxidase (PHOX), which directly produces superoxide anion (O_2^-); myeloperoxidase (MPO), which uses hydrogen peroxide to produce hypochlorous acid (HOCl) and hydroxyl radical (·OH); and inducible nitric oxide synthase (iNOS/NOS2/macNOS), which catalyzes L-arginine oxidation, producing a nitric oxide free radical (NO). None of the immediate end products of the respiratory burst – O_2^-, HOCl, ·OH, or NO – are directly candidacidal *in vitro*; rather, it is thought that they are cross-reactive precursors for true candidacidal radicals, with most evidence pointing toward peroxynitrite ($ONOO^-$) as the major candidacidal product of the respiratory burst (19, 33, 34). NO facilitates cross-reactivity with both the MPO and PHOX systems, and importantly, is the rate-limiting component required for *C. albicans* killing *in vitro* (6, 33).

The immunoregulatory functions of iNOS-derived NO extend beyond direct killing: NO is able to regulate the expression of more than twenty cytokines, largely including, but not limited to, inflammatory cytokines such as IL-6, IL-12, and IFN-γ (5, 22), and in this way influences both innate and adaptive response pathways. For many cytokines the underlying mechanisms are not known, but in some cases this involves posttranslational modification of upstream regulatory proteins (reviewed in ref. 5).

NO has been implicated in the host response to oral candidiasis. Its concentration in saliva increased in response to oral *C. albicans* infection (10), and oral carriage in mice treated with the competitive iNOS inhibitor, *N*[G]-monomethyl-L-arginine (MMLA), persisted longer than in wild-type controls. Saliva from the treated mice was less efficient at killing *C. albicans in vitro*, and this could be reversed by treatment with an NO scavenger (10). MMLA treatment reduced IL-4 messenger RNA (mRNA) in regional lymph nodes of *C. albicans*-infected mice, and anti-IL-4 antibody treatment reduced saliva and lymph node NO production, with both treatments also increasing oral fungal loads (10, 11), suggesting the existence of a positive feedback loop between IL-4 and NO. However, our studies in $IL\text{-}4^{-/-}$ mice have shown that IL-4 is not necessary for resistance to oral candidiasis (18), and that IL-4 mRNA is not induced by fungal challenge in our infection model (16). Therefore there are some deficiencies in the literature concerning the exact role of iNOS-derived NO in oral candidiasis. The present study was designed to clarify the involvement of NO in resistance and responses to oral *C. albicans* challenge using $iNOS^{-/-}$ mice, which have been successfully used to explore the functions of iNOS-derived NO in other infection models (24, 27).

$iNOS^{-/-}$ mice are not susceptible to experimental oral candidiasis

Based on previous *in vitro* and *in vivo* studies with competitive iNOS inhibitors and NO donors (10, 19, 33, 34), we hypothesized that professional phagocytes in the oral mucosa may require iNOS-derived NO to kill phagocytosed *C. albicans* yeasts, and that activated phagocytes may require NO to respond appropriately to mucosal invasion. To investigate this, we established an experimental oral candidiasis infection model in $iNOS^{-/-}$ mice. We have previously shown that there is a good correlation between oral yeast carriage and the histological incidence of oral lesions containing *C. albicans* yeast and hyphae, and inflammatory infiltrate (14, 15, 18), so in this study we used oral yeast carriage as a marker of infection status and resistance. A minimum of four $iNOS^{-/-}$ mice and wild-type counterparts [$iNOS^{+/+}$; C57BL/6J × 129 background (24)] were infected with live *C. albicans* as previously described (18) using a passive mode of oral inoculation of mice under anesthesia (day 0). Oral yeast carriage was monitored during the infection by counting the colony-forming units (CFU) from oral cavity swabs on days 1, 4, 8, and 14 postinfection. We noted that there was greater inter-mouse variation in the oral carriage rates with both strains ($iNOS^{+/+}$ and $iNOS^{-/-}$) compared to other strains tested previously (such as non-backcrossed C57BL/6J and BALB/c-based strains) (18), even on days 1 and 4 postinfection, when eight and six mice were used respectively (Fig. 1). Oral fungal loads in $iNOS^{-/-}$ mice were not significantly different from those in $iNOS^{+/+}$ mice, suggesting that although the iNOS-mediated NO burst is unmistakably an important candidacidal feature of activated phagocytes (33), and iNOS-derived NO is a candidacidal component of saliva (10), it is not required for resistance in otherwise immunocompetent mice.

There are a few possible explanations for the discrepancy between this result and the results of Elahi et al., who showed that mice treated with the iNOS inhibitor MMLA developed increased oral fungal burdens (10). First, the studies employed different mouse strains: BALB/c (10) and C57B/6J × 129. While both are regarded as resistant and show similar infection phenotypes (18), they may depend on iNOS-mediated defense to different degrees. Second, mice in our study were genetically deficient in iNOS-derived NO, whereas the other study was based on mice treated with MMLA, an L-arginine analogue (10). L-Arginine analogues have been used extensively to investigate NO

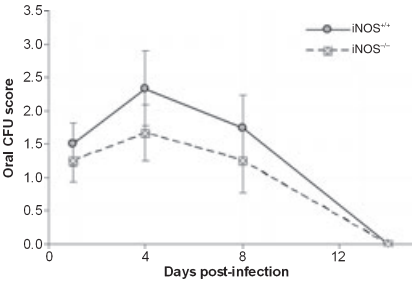

Fig. 1. The effect of inducible nitric oxide synthase (iNOS) deficiency on oral *Candida albicans* burden in mice. The $iNOS^{+/+}$ and $iNOS^{-/-}$ mice were inoculated orally with live *C. albicans* yeasts, and the fungal burden was monitored at four time-points over 2 weeks by counting colony-forming units (CFUs) from oral cavity swabs on Sabouraud's agar plates, which were then assigned a score as previously described (14): 0, no detectable yeast; 1, 1–10 CFU/plate; 2, 11–100 CFU/plate; 3, 101–1000 CFU/plate; 4 ≥ 1000 CFU/plate. Data shown are means ± SEM from at least four mice at each time point.

in phagocyte biology; however, they also affect the synthesis of polyamines by ornithine decarboxylase. This is potentially problematic because polyamines are associated with the pathogenesis of several different microbes, and competitive inhibition of ornithine decarboxylase has been shown to enhance *C. albicans* yeast proliferation (32). Therefore it is possible that MMLA treatment may worsen *C. albicans* infections independent of its actions on iNOS. Finally, the issue of differing infection models is worth noting. We used a non-traumatic mode of oral *C. albicans* inoculation, whereas Elahi et al. used an abrasive method, involving trauma to the oral mucosa (11). The immunocompetent oral mucosa is sufficient to resist candidal invasion, and comprises multiple innate defenses including tissue-resident T cells, phagocytes, and epithelial cells, and also humoral factors such as β-defensins and histatins, all of which are candidacidal (8, 9) and NO-independent. Therefore with passive inoculation, phagocyte-derived and salivary NO may be redundant in mediating fungal clearance, whereas the abrasive mode [representative of candidiasis involving physical trauma, e.g. denture stomatitis (13)] is likely to augment fungal invasion and create inflammation, a process with substantial macrophage involvement, and so may have a greater dependence on NO. Nevertheless, our experiments clearly and consistently showed that iNOS$^{-/-}$ mice do not have increased susceptibility to oral candidiasis.

iNOS deficiency does not hinder macrophage anticandidal effector activity

Although phenotypic analysis of iNOS$^{-/-}$ mice showed that iNOS was not critical for resistance to oral candidiasis, we continued our analysis of the mice to investigate whether iNOS-derived NO still played some role (albeit non-essential) in local phagocytic defense against *C. albicans* yeasts. Bone marrow-derived macrophages (BMDM) were isolated from iNOS$^{-/-}$ and iNOS$^{+/+}$ mice (21), and assayed for their abilities to phagocytose and kill *C. albicans* yeasts *in vitro* using flow cytometry-based assays; this was described previously (21, 30), see also the legend to Fig. 2 and supporting information **Appendix S1**. Interestingly, iNOS$^{-/-}$ BMDM phagocytosed a significantly greater proportion of yeasts than BMDM from the iNOS$^{+/+}$ controls (Fig. 2A), suggesting that NO may inhibit or suppress *C. albicans* phagocytosis, but ultimately this did not affect the candidacidal activity of the macrophages (Fig. 2B). There was no significant difference in *C. albicans* killing by iNOS$^{-/-}$ and iNOS$^{+/+}$ BMDM, suggesting that iNOS-derived NO is either not involved or is redundant in *C. albicans* killing, at least in the C57BL/6J × 129 strain.

Given that iNOS-derived NO is a critical component of the phagocytic respiratory burst (6, 33), we conducted a nitrite assay to see whether the BMDM were using an alternative, iNOS-independent pathway for the production of reactive nitrogen metabolites. BMDM were isolated as above, and either cultured alone, or with *C. albicans* and recombinant IFN-γ to stimulate and augment respiratory burst, respectively. As shown in Fig. 2(C), untreated macrophages from iNOS$^{+/+}$ mice produced a small amount of nitrite, whereas those from iNOS$^{-/-}$ mice produced negligible amounts. In response to IFN-γ and *C. albicans* activation, there was a surge in nitrite production from iNOS$^{+/+}$ BMDM, but the iNOS$^{-/-}$ macrophages only produced around half this amount ($P < 0.0001$). Therefore iNOS abrogation suppressed, but did not completely inhibit, the induction of nitrite synthesis from BMDM, suggesting that iNOS-independent pathways for nitrite synthesis may be functional in response

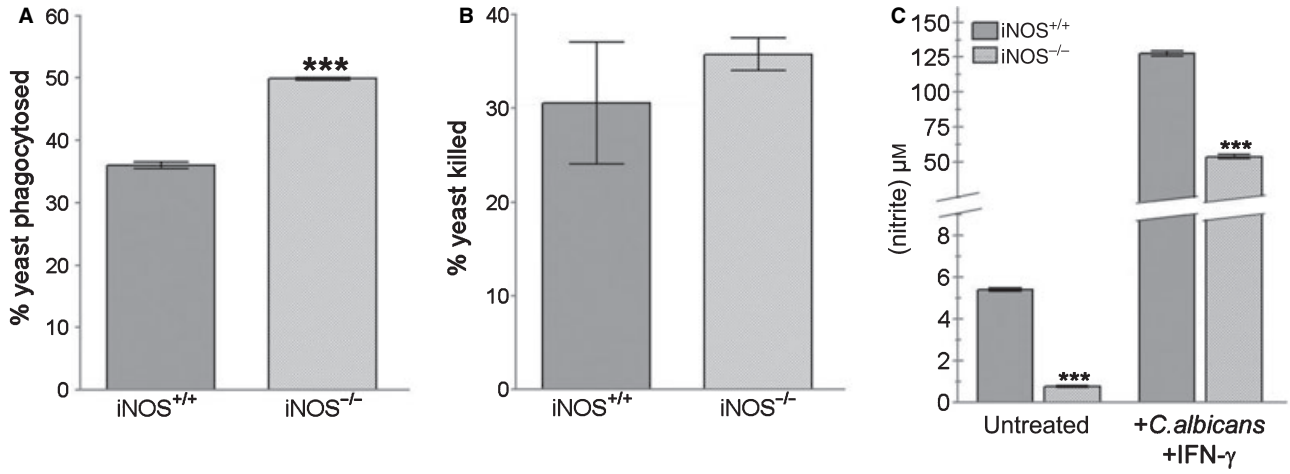

Fig. 2. Inducible nitric oxide synthase (iNOS) deficiency does not hinder macrophage effector activity or completely abrogate nitrite production. (A) *In vitro Candida albicans* phagocytosis assay. Bone marrow-derived macrophages (BMDM) were isolated from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice as described previously (30), incubated with fixed, fluorescein isothiocyanate (FITC) -labeled *C. albicans* yeasts, and then with ethidium bromide. FITC-positive yeasts were counted by flow cytometry, and double-positive cells were subtracted to distinguish phagocytosed from adherent yeasts. Data shown are means ± SD from three separate experiments, using macrophages pooled from four mice per group. (B) *In vitro C. albicans* killing assay. Live, FITC-labeled *C. albicans* yeasts were incubated with BMDM from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, then macrophages were lysed to release any phagocytosed yeasts. Dead yeasts were then labeled with propidium iodide. The percentage of yeasts killed was determined using flow cytometry as the fraction of double-positive events (dead yeasts, distinguished from dead macrophages by the FITC label) compared to the total FITC-positive events. Data shown are means ± SD from three separate experiments, each using BMDM pooled from four mice as for part (A). (C) Nitrite production by BMDM from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. BMDM were pooled from three iNOS$^{+/+}$ and three iNOS$^{-/-}$ mice, cultured alone (untreated) or in the presence of live *C. albicans* and interferon-γ to stimulate respiratory burst. Nitrites produced in the culture supernatants were measured relative to sodium nitrite concentration standards using a colorimetric reaction assay. Data shown are means ± SD from eight assay replicates of the pooled macrophages. The *y*-axis is divided for clarity. The statistical significance of differences between iNOS$^{+/+}$ and iNOS$^{-/-}$ was determined using Student's two-tailed *t*-tests; ***$P < 0.0001$.

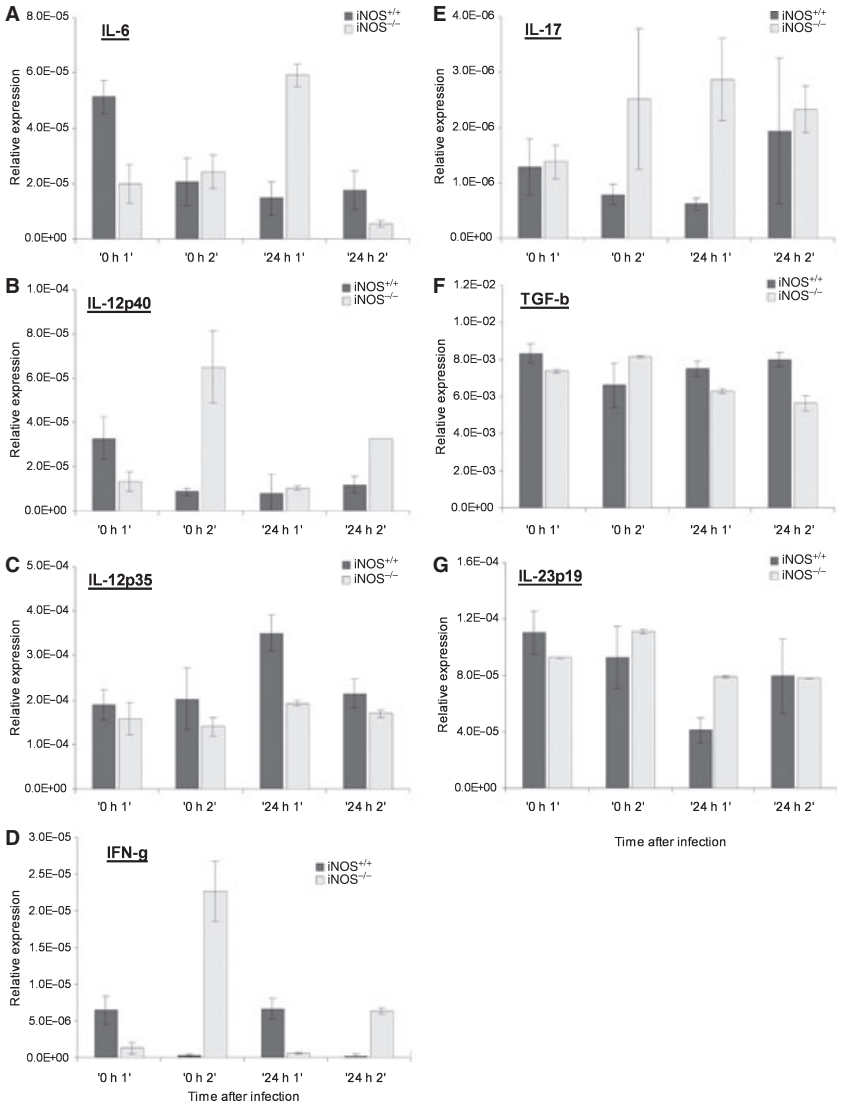

Fig. 3. Expression of messenger RNAs encoding confirmed and putative regulators of inducible nitric oxide synthase (iNOS) is not acutely altered in *Candida albicans*-infected iNOS$^{+/+}$ or iNOS$^{-/-}$ mice. Oral tissues (pooled buccal, lingual, palatal, and gingival mucosae) were harvested from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (two per time-point) either before or 24 h after oral infection with live *C. albicans* yeasts (0 h 1, 0 h 2, 24 h 1, and 24 h 2 for each genotype). Total RNA was extracted from each sample, then analyzed for expression of cytokine mRNAs by real-time reverse transcription–polymerase chain reaction, using 18S ribosomal RNA as the internal control. Data shown are relative expression means (test mRNA : 18S ratio) ± SD for each cytokine tested: (A) interleukin-6 (IL-6); (B) IL-12/23 p40 subunit (IL-12/23p40); (C) IL-12 p35 subunit (IL-12p35); (D) interferon-γ (IFN-γ); (E) IL-17; (F) transforming growth factor-β (TGF-β); (G) IL-23 p19 subunit (IL-23p19).

to *C. albicans*, and that this could, at least partly, explain why BMDM from iNOS$^{-/-}$ mice are able to kill *C. albicans* yeasts *in vitro*. These results are consistent with a study by Balish et al. (2) using a mixed peritoneal macrophage–granulocyte population from iNOS$^{-/-}$ mice, but they contradict the results of Vazquez-Torres and Balish (33), in which *C. albicans* killing by iNOS$^{-/-}$ macrophages was significantly impaired. Interestingly, both our study and that by Balish et al. used C57BL/6-based strains, whereas Vazquez-Torres and Balish used BALB/c. It is noteworthy that BALB/c mice also showed increased fungal loads when orally challenged with *C. albicans* in an independent study (10); the possibility that these mice depend on iNOS-derived NO to different degrees is an important consideration for experimental design in the future.

Another experimental design issue that could influence assays of macrophage functional activity in response to *C. albicans* is how they are treated after isolation. In our study, and another (2), which both found no significant difference in the candidacidal activity of macrophages from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, killing was assessed in the absence of other stimuli. In contrast, macrophages in the Vazquez-Torres and Balish study were treated with bacterial lipopolysaccharide before exposure to yeasts *in vitro* (33). Purified lipopolysaccharide activates Toll-like receptor 4 (TLR4) (4), whereas intact *C. albicans* yeasts comprise multiple distinct PAMPs that activate additional PAMP-R (e.g. TLRs 2 and 9, dectin-1, CR3, mannose-R, DC-SIGN) leading to activation of multiple signaling pathways, many of which overlap and cross-regulate each other (20, 29). Therefore it is possible that preincubation with purified lipopolysaccharide may generate activated macrophages with sets of effector characteristics different from those exposed to *C. albicans* alone. In fact, one may expect the more complex *C. albicans* antigen to engage multiple effector pathways in macrophages, which would be consistent with our finding that macrophages from the iNOS$^{-/-}$ mice produce nitrites in an iNOS-independent manner (Fig. 2C). We believe that incubation with *C. albicans* alone is more representative of a fungal infection.

iNOS deficiency does not alter early expression of inflammatory cytokines

We extended our investigation of immune response mechanisms activated by *C. albicans* to include analysis of the expression of several cytokines previously implicated in NO-mediated immunoregulation: IL-6, IL-12 (p40 and p35 subunits), and IFN-γ (5). We also investigated whether iNOS-derived NO regulates the IL-17 axis, because IL-17 has recently been implicated in early mucosal inflammatory responses (23), and has been shown to activate iNOS transcription and enhance NO responses (26). Therefore we wanted to explore the hypothesis that IL-17 could be involved in an early positive feedback loop that augments local inflammation in response to *C. albicans*. Synthesis of IL-17 by tissue-resident T cells is potentiated by IL-6, transforming growth factor-β (TGF-β), and IL-23 in mice (3, 25), so we also included TGF-β and IL-23 (p40 and p19 subunits) in our analysis.

iNOS$^{+/+}$ and iNOS$^{-/-}$ mice were inoculated orally with live *C. albicans* yeasts as described above, then were sacrificed 24 h after infection, a time-frame that was selected to capture the early immediate response in the oral mucosa. Tongues and

oral tissues (buccal, gingival, and palatal mucosae) were isolated separately from infected and uninfected mice (two mice each), then total RNA was extracted from the tissues and analysed by quantitative reverse transcription–polymerase chain reaction for the expression of cytokine mRNAs (see supporting information **Appendix S1** for reagents, methods, and instrumentation). All cytokine mRNAs were quantified relative to 18S ribosomal RNA. We did not find significant changes in the expression of any of the cytokines tested in iNOS$^{+/+}$ mice between 0 and 24 h postinfection, nor were there any significant differences between these wild-type mice and their iNOS$^{-/-}$ counterparts in uninfected or infected mice (Fig. 3), suggesting that *C. albicans* does not alter their mRNA expression levels within a 24-h period, and that iNOS abrogation does not alter this. Expression of IL-17 mRNA was barely detectable in the oral mucosa tissue, which was reflected in the relatively large error associated with the means at several data points. We also noted a relatively large degree of inter-mouse variability in the uninfected and/or postinfection expression levels of a few cytokines in particular (e.g. IFN-γ and IL-12/23p40), and have therefore shown the data for individual mice separately to illustrate this.

Concluding remarks

To date this is the first study to directly and specifically investigate the role of NO in oral candidiasis *in vivo*. Our study shows that there is no significant impact of iNOS abrogation on oral candidiasis susceptibility in mice, or on the successful phagocytosis and killing of *C. albicans* yeasts by macrophages *in vitro*, nor is there any effect on local expression of IL-6, IL-17, IL-23, IL-12, IFN-γ, or TGF-β messages in oral mucosal surfaces within 24 h of oral *C. albicans* challenge. We propose that although iNOS-derived NO may be an important molecule in inflammation, the lack of any noticeable oral candidiasis disease phenotype in iNOS$^{-/-}$ mice could be the result of redundancy in innate candidacidal effector mechanisms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Methodological details to assist in the replication of experiments (Word document).

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