molecular oral microbiology

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Early activation of the interleukin-23–17 axis in a murine model of oropharyngeal candidiasis

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Keywords: *Candida albicans*; interleukin 23; interleukin 17A; oropharyngeal candidiasis
Accepted 27 January 2010

SUMMARY

Candida albicans **is an oral commensal yeast that causes oropharyngeal candidiasis (OPC) in immunocompromised individuals. The immunological pathways involved in OPC have been revisited after the interleukin-17 (IL-17) pathway was implicated in fungal immunity. We studied immediate (<24 h) and adaptive (3–6 day) IL-12 and IL-23–17 pathway activation in naive p40$^{-/-}$ mice, which lack IL-12 and IL-23 and develop severe, chronic OPC upon oral inoculation with *C. albicans*. Macrophages from p40$^{-/-}$ mice were less efficient than C57BL/6J controls at killing *C. albicans in vitro* but very low numbers in the oral mucosae of infected C57BL/6J mice suggest that they are not critical *in vivo*, at least in this strain. Migration of macrophages to regional lymph nodes of infected p40$^{-/-}$ mice was impaired; however, dendritic cell migration was not affected. Recombinant IL-12 therapy provided only temporary relief from OPC, suggesting that IL-23 is required for full protection. In C57BL/6J mice, but not p40$^{-/-}$ mice, messenger RNAs encoding IL-23p19 and IL-17 were induced in the oral mucosa within 24 h of infection (6 ± 0.6 and 12 ± 2.7-fold). By day 6 of infection in C57BL/6J mice, IL-17A messenger RNA level had increased 5.1 ± 1.8 and 83 ± 21-fold in regional lymph nodes and oral tissues respectively. Ablation of p40 was associated with delayed or abrogated induction of IL-17A pathway targets (monocyte chemoattractant protein-1, IL-6 and macrophage inflammatory protein-2), and a lack of organized recruitment of neutrophils to the infected oral mucosa. Overall our data show that the IL-23–17A axis is activated early in the oral mucosae of immunologically naive mice with OPC.**

INTRODUCTION

Candida albicans is a commensal budding yeast that colonizes muco-cutaneous surfaces in the majority of healthy humans but causes oropharyngeal candidiasis (OPC) in individuals with compromised immune function. Significantly, there is a positive association between mucosal colonization and the deadly systemic manifestation of the disease (Martino *et al.*, 1994). Worldwide incidence has risen considerably since the 1980s in parallel with increases in organ transplants, chemotherapy and human immunodeficiency virus infections (Sims *et al.*, 2005). Mammalian immune response pathways activated in response to fungal infections are complex, and generally not well-characterized in comparison to bacterial and viral infections (Romani, 2004). Advances in our understanding of the host response pathways involved in OPC have clear clinical implications, and

will enhance our understanding of the molecular and cellular mechanisms involved in fungal immunity at mucosal surfaces.

Attention has recently focused on the interleukin-23–interleukin-17 (IL-23–17) axis and T helper type 17 (Th17) cells in host defence against infections with *C. albicans*, among other fungi including *Cryptococcus neoformans*, *Pneumocystis carinii* and *Aspergillus fumigatus* (Kleinschek *et al.*, 2006; Rudner *et al.*, 2007; Zelante *et al.*, 2007). IL-23 is a p19–p40 heterodimer produced by activated phagocytes, unique in that it shares the p40 subunit with IL-12. It is required for expansion and lineage commitment of the $CD4^+$ T-cell subset Th17, which secrete cytokines (including IL-17A, IL-17F and IL-22) involved in neutrophil-mediated immunity against extracellular pathogens. IL-23 is also involved in the early induction of IL-17 from innate immune cells such as $\gamma\delta$ T cells, neutrophils and macrophages (Ley *et al.*, 2006; O'Brien *et al.*, 2009). The IL-17 receptor complex [IL-17R; of which five subunits have been identified (Shen & Gaffen, 2008)] is expressed by many cell types, including leucocytes and epithelial, endothelial and stromal cells (Weaver *et al.*, 2007). IL-17R signalling activates a potent proinflammatory programme involving CXC chemokines and neutrophil recruitment, proinflammatory cytokines (e.g. IL-6) and antimicrobial proteins such as the β-defensins and S100A proteins (Ley *et al.*, 2006; Liang *et al.*, 2006; Matsuzaki & Umemura, 2007).

The immunological response to *C. albicans* is initiated by activation of pattern recognition receptors on resident phagocytes at the infection site and fungal virulence factors (Romani, 2004; Filler, 2006). In the oral mucosa, this engages $CD4^+$ T cells, macrophages and neutrophils; physical or functional depletion of these cell types in animal models, *in vitro* cell assays or disease increase the fungal burden (Farah *et al.*, 2000, 2001, 2002). Before the identification of Th17 cells (Harrington *et al.*, 2005), cell-mediated immunity was thought to be the mainstay for protection against OPC, based on increases in the levels of IL-12 and interferon-γ (IFN-γ) in the regional lymph nodes of experimental mice around 8 days following oral *C. albicans* challenge (Farah *et al.*, 2001), together with clinical and epidemiological data on patients with functional or physical $CD4^+$ T-cell defects (Saunus *et al.*, 2008). Additionally, $p40^{-/-}$ mice are susceptible to severe, chronic OPC, involving extensive hyphal penetration of the oral epithelium (Ashman *et al.*, 2004; Farah *et al.*, 2006). More recently however, *Candida*-specific Th17 cells from humans and mice were isolated and characterized (Acosta-Rodriguez *et al.*, 2007; LeibundGut-Landmann *et al.*, 2007; Zhou *et al.*, 2008), IL-17 pathway-specific knockout mice were found to be susceptible to both systemic and oropharyngeal *C. albicans* infections (Huang *et al.*, 2004; Conti *et al.*, 2009), and *Candida* antigen-treated dendritic cells (DCs) were shown to induce differentiation of Th17 cells *in vitro* (Acosta-Rodriguez *et al.*, 2007; LeibundGut-Landmann *et al.*, 2007). Conti and colleagues conducted OPC susceptibility studies in mice deficient in either Th1 or Th17 responses, and found that IL-23-deficient ($p19^{-/-}$) and IL-17 receptor-deficient ($IL\text{-}17RA^{-/-}$), but not IL-12-deficient ($p35^{-/-}$), mice were susceptible to OPC (Conti *et al.*, 2009). Involvement of the IL-17 pathway in the host response to OPC clarifies a few observations that were unaccounted for within the Th1/Th2 dichotomy: that deficiencies in the receptors for IL-12 and IFN-γ in humans, or for IFN-γ itself in mice, are not associated with overt susceptibility to OPC (Saunus *et al.*, 2008). Clinically, IL-17 pathway defects have been linked to syndromes with a mucocutaneous candidiasis component, including chronic mucocutaneous candidiasis (Eyerich *et al.*, 2008) and autosomal dominant hyper-immunoglobulin E (Job) syndrome (Ma *et al.*, 2008; Milner *et al.*, 2008).

In this study we investigated the IL-23–17 and IL-12 pathways in $p40^{-/-}$ mice to understand more about their relative contributions in the response of naive mice to OPC, and to clarify the mechanisms underlying the severe, chronic oral infection phenotype of $p40^{-/-}$ mice, which was described before the IL-17 pathway was implicated in fungal immunity (Farah *et al.*, 2006).

METHODS

Animals

Specific pathogen-free female C57BL/6J mice, 6–8 weeks old, were purchased from the Animal Resources Centre, Perth, Australia. Sex-matched and age-matched $p40^{-/-}$ mice also on the C57BL/6J background (Magram *et al.*, 1996) were obtained from the Monash Institute of Reproduction and Development, Monash University, Melbourne Australia, and

bred at the Herston Medical Research Centre, Brisbane, Australia, with regular polymerase chain reaction (PCR) -based genotyping. These mice undergo routine microbiological screening and do not harbour *C. albicans* in the gut. Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Queensland, and were carried out in accordance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 1997. Mice were housed in filter-top cages in a PC2 facility, and provided with food and water *ad libitum*.

Oral candidiasis model

Candida albicans isolate 3630, derived from a patient with cutaneous candidiasis, was obtained from the Mycology Reference Laboratory at the Royal North Shore Hospital, Sydney, Australia, and stored at −70°C in Sabouraud's broth/15% (volume/volume) glycerol. For use, yeasts were grown in Sabouraud's broth for 48 h at room temperature with continuous agitation. Blastospores were washed in phosphate-buffered saline (PBS) and adjusted to the appropriate concentration for use.

There is a good correlation between oral yeast carriage and histological incidence of oral lesions containing *C. albicans* yeast and hyphae, and inflammatory infiltrate (Farah *et al.*, 2001, 2002); for this reason, oral yeast carriage was assayed as a marker of infection status and resistance. Mice were inoculated orally with 1×10^8 live *C. albicans* yeasts in 20 μl sterile PBS. The severity of oral candidiasis over the following 6–21 days was assessed by monitoring oral yeast carriage at multiple timepoints after infection as previously described (Farah *et al.*, 2002).

Flow cytometry

Mice were sacrificed before or at various times after oral *C. albicans* infection, then the lymph nodes draining the oral cavity (submandibular and superficial cervical) or the oral tissues (pooled buccal, lingual, palatal and gingival mucosae) were dissected and placed immediately in RPMI-1640 medium (Invitrogen; Mulgrave, VIC, Australia) containing antibiotic/antimycotic (Ab/Am; Gibco®-Invitrogen, Mulgrave, VIC, Australia) at room temperature for about 1 h during transport from the animal facility to the laboratory. Dissected tissues were cut into pieces (~ 1 mm^3) using a scalpel, then pooled from three mice per genotype per timepoint. Oral tissues were incubated in digestion medium [RPMI-1640 (Invitrogen), 5% fetal calf serum (Gibco-Invitrogen), 1 × Ab/Am (Gibco-Invitrogen), 1 × monensin (eBioscience; San Diego, CA, USA), collagenase 1A (2 mg ml^{-1}; Sigma-Aldrich; Castle Hill, NSW, Australia) and hyaluronidase (100 U ml^{-1}; Sigma-Aldrich)] for 1.5 h at 37°C, with occasional pipetting to generate a single-cell suspension. Digested oral tissues or lymph nodes were processed through 250-μm and 70-μm mesh, pelleted at 400 ***g*** for 3 min at 4°C, and resuspended in flow cytometry staining buffer (eBioscience). The filtering process was necessary for oral tissues to remove clumps of surface epithelial cells; all samples were processed the same way in parallel.

Approximately 2×10^6 cells were incubated in 50 μl staining buffer containing 1 μg anti-CD16/32 antibody (eBioscience) and 2% fetal calf serum for 10 min on ice to block non-specific and Fc binding sites. Fifty microlitres of staining buffer containing primary antibodies against cell surface markers at appropriate concentrations was added, and incubated for 60 min on ice in the dark. Primary monoclonal antibodies used were: fluorescein isothiocyanate-conjugated α-F4/80 (1 μg per 2×10^6 cells; eBioscience 11-4801), phycoerythrin (PE) –conjugated α-CD11c (eBioscience 12-0114-82) and PE-α-neutrophil (20 μl per 2×10^6 cells; MCA771PE; Abd Serotec, Raleigh, NC, USA). After staining, cells were pelleted, washed three times in cold PBS, then resuspended in 200 μl cell dissociation buffer (Gibco-Invitrogen) on ice. Stained cells were analysed within 2 h on a BD LSRII flow cytometer (Becton Dickinson, North Ryde, Australia), with data collection from at least 10,000 events. Data were analysed using FACSDIVA software (V6.0; BD Australia, North Ryde, NSW, Australia). Forward scatter and side scatter were used to gate lymphocytes. Gates were established using unstained control samples (a 1 : 1 mixture of cells from wild-type and p40$^{-/-}$ mice).

Phagocytosis and killing assays

Macrophages were derived from bone marrow progenitor cells as previously described (Hu *et al.*, 2006). Before use, cells were stained with Giemsa

stain and examined under a light microscope for morphological confirmation of their identity and purity. Killing and phagocytosis of *C. albicans* yeasts was quantified using flow cytometry-based assays as previously described (Farah *et al.*, 2009). Flow cytometry was performed on a FACSCalibur flow cytometer (BD Australia) with CellQuest software (V3.1; BD Australia). For killing assays, recombinant murine (rm) IL-12 (0.005 μg ml^{-1}; R&D Systems; Minneapolis, MN, USA) and/or rmIFN-γ (2000 U ml^{-1}; R&D Systems) was added to phagocyte growth medium 1 h before the addition of yeast.

Recombinant IL-12 injection experiments

The $p40^{-/-}$ mice were divided into three groups, receiving intraperitoneal rmIL-12 (1 μg 200 μl^{-1} per mouse; R&D Systems) daily from day −5 to day 0 or day −5 to day 7 relative to the day of infection (day 0), or PBS daily from day −5 to day 7 to control for trauma. The dose was selected based on effective doses from other published studies (Zhou *et al.*, 1997; Decken *et al.*, 1998) and our own titration experiments.

Real-time reverse transcription–PCR

Oral tissues (buccal, palatal, gingival and lingual mucosa) or lymph nodes (superficial cervical and submandibular) were harvested before and at various times after oral infection with *C. albicans*, then homogenized in TRIZOL (Invitrogen), and treated to remove contaminating DNA using DNA-free™ (Ambion, Austin, TX) according to the manufacturer's instructions. Two or three mice were analysed separately by real-time reverse transcription (RT-) PCR per timepoint. Complementary DNA was synthesized from 3 μg total RNA using Superscript III (Invitrogen) with random hexamers (Promega, Alexandria, NSW, Australia) according to the manufacturer's instructions. Messenger RNAs (mRNAs) and the loading control 18S ribosomal RNA (rRNA) were quantified on a 7900 Sequence Detection System in triplicate, using SDS 2.2.3 software (Applied Biosystems, Mulgrave, VIC, Australia). Reactions contained SYBR green PCR master mix (Applied Biosystems), primers (see below) and template diluted appropriately in distilled H_2O. Cycling conditions were: 10 min (95°C), followed by 45 cycles of 15 s (95°C) and 1 min (59°C). Complementary DNA synthesis reactions conducted with no reverse transcriptase enzyme were included to control for genomic DNA contamination, and post-PCR melt analysis was conducted to ensure that the quantified PCR products were pure and free of non-specific amplicons. The comparative ΔΔCt method (Applied Biosystems) was used to determine cytokine mRNA expression relative to 18S rRNA, relative to the appropriate uninfected control treatment. Primers used were: 18S rRNA, 100 nM, F-cattggagggcaagtctgg, R-tcccaagatccaactacgagc; IL-12/23p40, 200 nM, F-actcacatctgctgctccac, R-cggagtaatttggtgcttca; IL-23p19, 300 nM, F-ccagcgggacatatgaatct, R-tgtgggtcacaaccatcttc; IL-17a, 300 nM, F-ccagggagagcttcatctgt, R-gctgagctttgagggatgat; Mcp-1 (Ccl2), 300 nM, F-agatgatcccaatgagtaggc, R-gcacagacctctctcttgagc; Mip-2 (Cxcl2), 300 nM, F-gtcaaaaagtttgccttgacc, R-ctcctttccaggtcagttagc; IL-6, 300 nM, F-ccggagaggagacttcacag, R-attgccattgcacaactcttt.

Statistics

Data were analysed using two-tailed Student's *t*-tests and GraphPad Prism software V4.0 (GraphPad Software Inc., CA, USA). *P* values are indicated in the respective figure legends.

RESULTS

Killing and phagocytosis of *C. albicans* are altered in macrophages from $p40^{-/-}$ mice

Others have shown that $p40^{-/-}$ macrophages have impaired effector functions and elevated production of transforming growth factor-β *in vitro* (Bastos *et al.*, 2002), hence a lack of p40 may affect their sensitivity to functional activation. Using a flow cytometry assay, we found that the ability of bone-marrow-derived macrophages from $p40^{-/-}$ mice to phagocytose *C. albicans* blastospores was not impaired (in fact there was an increase from 31.6 ± 0.9 to 36.1 ± 0.5) (Fig. 1A), but combined intra- and extracellular *C. albicans* killing activity was significantly reduced from 58.9 ± 6.8 to 29.1 ± 2.7% (Fig. 1B). Supplementation with either recombinant murine IL-12 or IFN-γ did not correct this; however, a combination of the two fully restored *Candida* killing activity to levels of macrophages from wild-type mice (Fig. 1C). This suggested impaired macrophage candidacidal activity

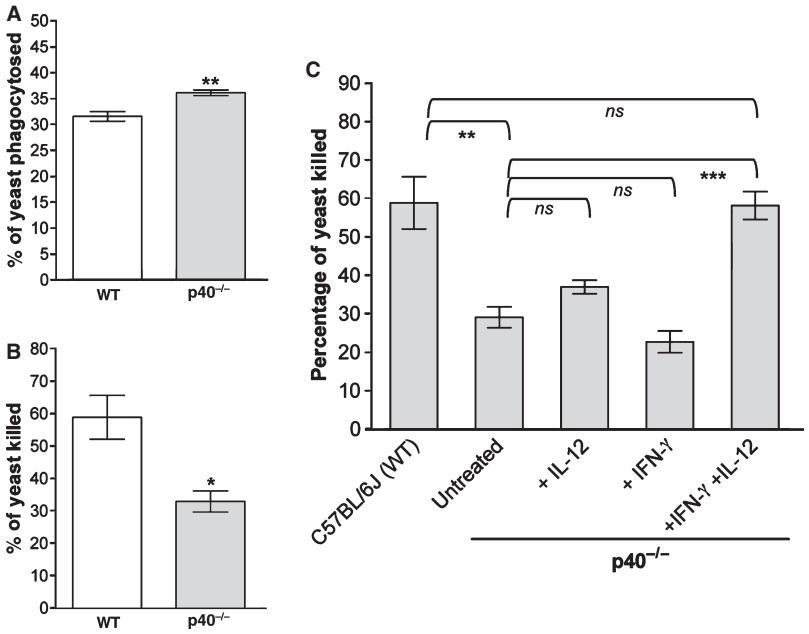

Figure 1 Phagocytosis and killing of *Candida albicans* by macrophages from p40$^{-/-}$ mice. (A) *In vitro C. albicans* phagocytosis assay. Bone-marrow-derived macrophages (BMDM) from wild-type C57BL/6J (WT) or p40$^{-/-}$ mice were incubated with fluorescein isothiocyanate (FITC) - labelled *C. albicans* yeasts, then with ethidium bromide (EtBr), and analysed by flow cytometry. The percentage of phagocytosed yeasts was calculated from the ratio of FITC-positive events to all events (FITC or EtBr). (B) *In vitro C. albicans* killing assay. Live *C. albicans* and BMDM were coincubated as above, then macrophages were lysed to release any phagocytosed yeasts. Dead yeasts were labelled with propidium iodide and counted by flow cytometry. (C) *C. albicans* killing by BMDM in the presence of supplemental cytokines. BMDM were isolated and assayed as in (B), except they were incubated with the recombinant cytokines indicated before yeast. All data shown are means ± the standard errors of the mean (SEM) using BMDM from at least three mice. Students' two-tailed *t*-tests were performed between test and control groups as indicated. ns, not significant; $^{**}P = 0.001$ to $P = 0.0001$; $^{***}P < 0.0001$.

could partly underlie the phenotype of p40$^{-/-}$ mice. To explore this idea further, we analysed the numbers of macrophages that infiltrate infected tissues of wild-type and p40$^{-/-}$ mice by flow cytometry. Despite the *in vitro* findings (Fig. 1), we were unable to detect significant numbers of F4-80-expressing macrophages in infected oral tissues of either wild-type or p40$^{-/-}$ mice, up to 6 days post-infection (data not shown), despite strong detection using the same protocol in lymphoid tissue (Fig. 2). We confirmed this result by immunohistochemical analysis of CD14 (most highly expressed by macrophages) in oral tissues of wild-type and p40$^{-/-}$ mice, and similarly could not detect a significant macrophage presence either before or after oral infection (data not shown). Therefore despite the fact that macrophages have lower candidacidal activity *in vitro*, their low frequency in the oral tissues of wild-type mice, even after infection, suggests that they may not be crucial candidacidal effector cells *in vivo*.

Macrophage and dendritic cell migration to regional lymph nodes upon oral infection with *C. albicans*

We have previously shown that systemic depletion of macrophages increases the oral fungal burden in experimental mice (Farah *et al.*, 2001). Given their low frequency in the infected oral mucosa (above), we investigated whether a failure of activated migration to regional lymph nodes (which would affect the efficiency of antigen presentation) could be involved. C57BL/6J and p40$^{-/-}$ mice were treated with PBS, or orally challenged with equal loads of *C. albicans* yeasts, and then regional lymph nodes were collected (24 h, 72 h and 6 days postinfection for the infected group). Lymphoid cell suspensions were prepared and stained with labelled antibodies against F4-80, then analysed by flow cytometry. In the uninfected state, macrophages represented 1.12 and 1.59% of the live lymphoid compartment of C57BL/6J

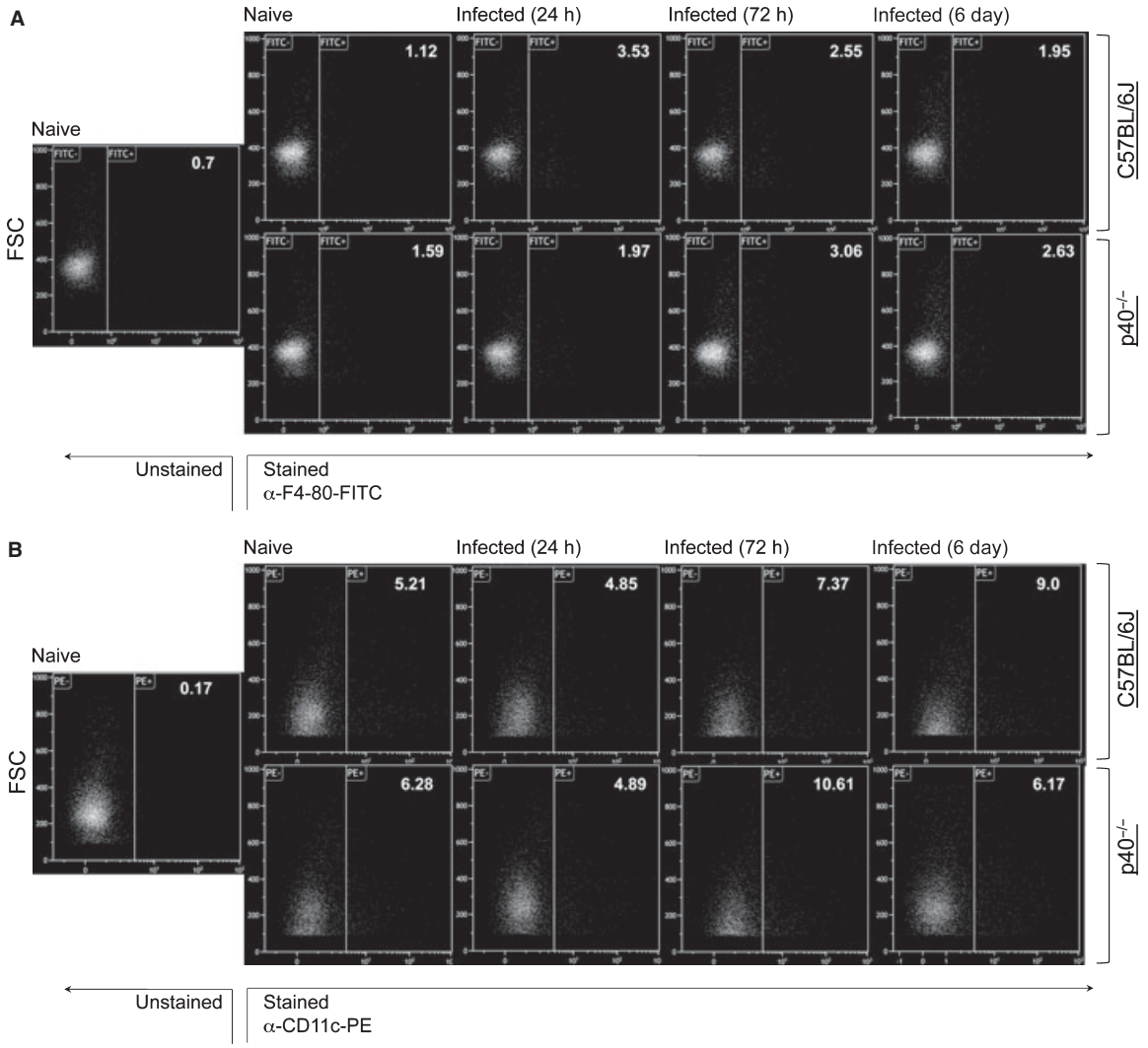

Figure 2 Migration of antigen-presenting cells to regional lymph nodes in wild-type and p40$^{-/-}$ mice upon oral infection with *Candida albicans*. Wild-type C57BL/6J or p40$^{-/-}$ mice (three per data-point) were orally infected with phosphate-buffered saline (naive) or *C. albicans* (infected), then sacrificed 24 h, 72 h or 6 days postinfection. Cell suspensions were prepared from the regional lymph nodes of the mice (submandibular and superficial cervical), labelled with either fluorescein isothiocyanate-conjugated α-F4-80 (A) or phycoerythrin-conjugated α-CD11c (B) antibodies for macrophages and dendritic cells respectively, then analysed by flow cytometry. Data shown are stained events, quantified as the percentage of live lymphoid cells.

and p40$^{-/-}$ mice respectively. In C57BL/6J controls, there was infiltration of macrophages to the regional lymph nodes within 24 h of infection (3.2-fold increase to 3.53% of the lymphoid population), which decreased over the following 5 days to 1.95% of lymphoid cells at day 6 (Fig. 2). In contrast, peak macrophage infiltration was delayed in p40$^{-/-}$ mice (3.06% at 72 h), and also lower in magnitude overall at the timepoints analysed. These results suggest that early migration of macrophages to regional lymph nodes upon stimulation with *C. albicans* in the oral mucosa is affected by p40 deficiency, but that migration is not completely inhibited.

Dendritic cells have been implicated in priming adaptive immune responses to *C. albicans* (d'Ostiani *et al.*, 2000; Romagnoli *et al.*, 2004; LeibundGut-Landmann *et al.*, 2007; Zelante *et al.*, 2007); therefore we also tested DC migration in parallel with the macrophage experiments using a PE-α-CD11c antibody. Overall there was an increase in the presence of DCs during the 6-day infection course, from 5.21 to 9.0% of the lymphoid compartment. We found no

overt reduction in DC infiltration of regional lymph nodes following infection in the p40$^{-/-}$ mice; in fact DC infiltration was higher at the 72-h timepoint (10.61% in p40$^{-/-}$ vs. 7.37% in C57BL/6J). Hence, antigen-presenting cell migration is not likely to be a major factor underlying the phenotype of the p40$^{-/-}$ mice.

Recombinant IL-12 therapy cannot fully correct the phenotype of p40$^{-/-}$ mice orally challenged with *C. albicans*

We tested the degree to which IL-12 deficiency contributed to OPC susceptibility in p40$^{-/-}$ mice *in vivo* by administering rmIL-12 before and after oral challenge with *C. albicans*, monitoring oral fungal load as a marker of disease severity. As shown in Fig. 3, rmIL-12 was able to relieve yeast carriage early in the infection, with fungal loads significantly lower than the untreated control at 24 h and 4 days after infection; however, treatment was not effective after day 4, with oral colony-forming units (CFUs) of treated mice equalling those of untreated controls by day 8. The two different injection schedules tested show that the short-term protection conferred was dependent on IL-12 being present before infection. The oral CFUs of treated mice at 24 h and 4 days postinfection are comparable to levels reproducibly observed in immunocompetent C57BL/6J mice (Farah *et al.*, 2006). These results suggest that IL-12 function is sufficient to resist infection at these early timepoints, but after 4 days IL-12-independent mechanisms are required. These results show that p40-dependent pathways other than those involving IL-12 are critical for full immunological defence against oral candidiasis in C57BL6/J mice.

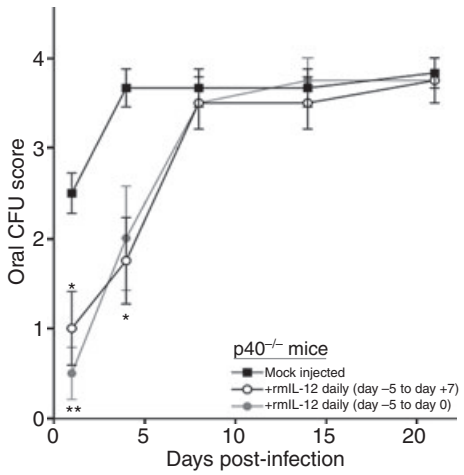

Figure 3 Oropharyngeal candidiasis in p40$^{-/-}$ mice receiving recombinant murine interleukin-12 (rmIL-12) therapy. p40$^{-/-}$ mice received daily intraperitoneal injections of either phosphate-buffered saline (mock), or rmIL-12 from day −5 to+7 or −5 to 0 relative to the day of oral infection with *C. albicans*. Oral yeast carriage was monitored on days 1, 4, 8, 14 and 21 after infection by counting colony-forming units (CFU) from oral cavity swabs. Data shown are means ± the standard error of the mean from four to six mice per group per timepoint. The significance of any differences to the mock-injected control at each timepoint was determined using Students' two-tailed *t*-tests: **P* = 0.01 to *P* = 0.001; ***P* = 0.001 to *P* = 0.0001.

Early, p40-dependent induction of IL-17 pathway genes in murine OPC

To directly examine whether cytokines in the IL-23–17A pathway are upregulated upon infection of the murine oral mucosa with *C. albicans*, we measured their expression by quantitative RT-PCR over the course of experimental oral infection. Consistent with the previously reported constitutive expression pattern of p35 in other tissues (Trinchieri, 2003), p35 mRNA levels did not significantly change over time in oral tissues of either C57BL/6J or p40$^{-/-}$ mice (Fig. 4A). In contrast, mRNAs encoding p40 and p19 (the subunits of IL-23) were significantly upregulated as early as 24 h postinfection (Fig. 4A; 10-fold and six-fold respectively). Not surprisingly, p40 mRNA levels did not change in p40$^{-/-}$ mice, but interestingly p19 induction was dependent on p40, suggesting that a feedback relationship may exist. The local, early increases in p40 and p19 expression correlated with IL-17A, which increased within the same time-frame and was sustained at high levels over the 6-day time-course (12-, 50- and 83-fold of uninfected oral tissue expression levels at 24 h, 72 h and 6 days postinfection, respectively). This effect was completely abrogated in p40$^{-/-}$ mice (Fig. 4B), which is consistent with dependence on IL-23.

Others have observed an increase in IL-17A expression in the cervical lymph nodes of C57BL mice with OPC, which increases further following *in vitro* stimulation of the T-cell receptor, suggesting that Th17 cells are involved in the response to OPC (Conti *et al.*, 2009). We assayed IL-17A mRNA expression

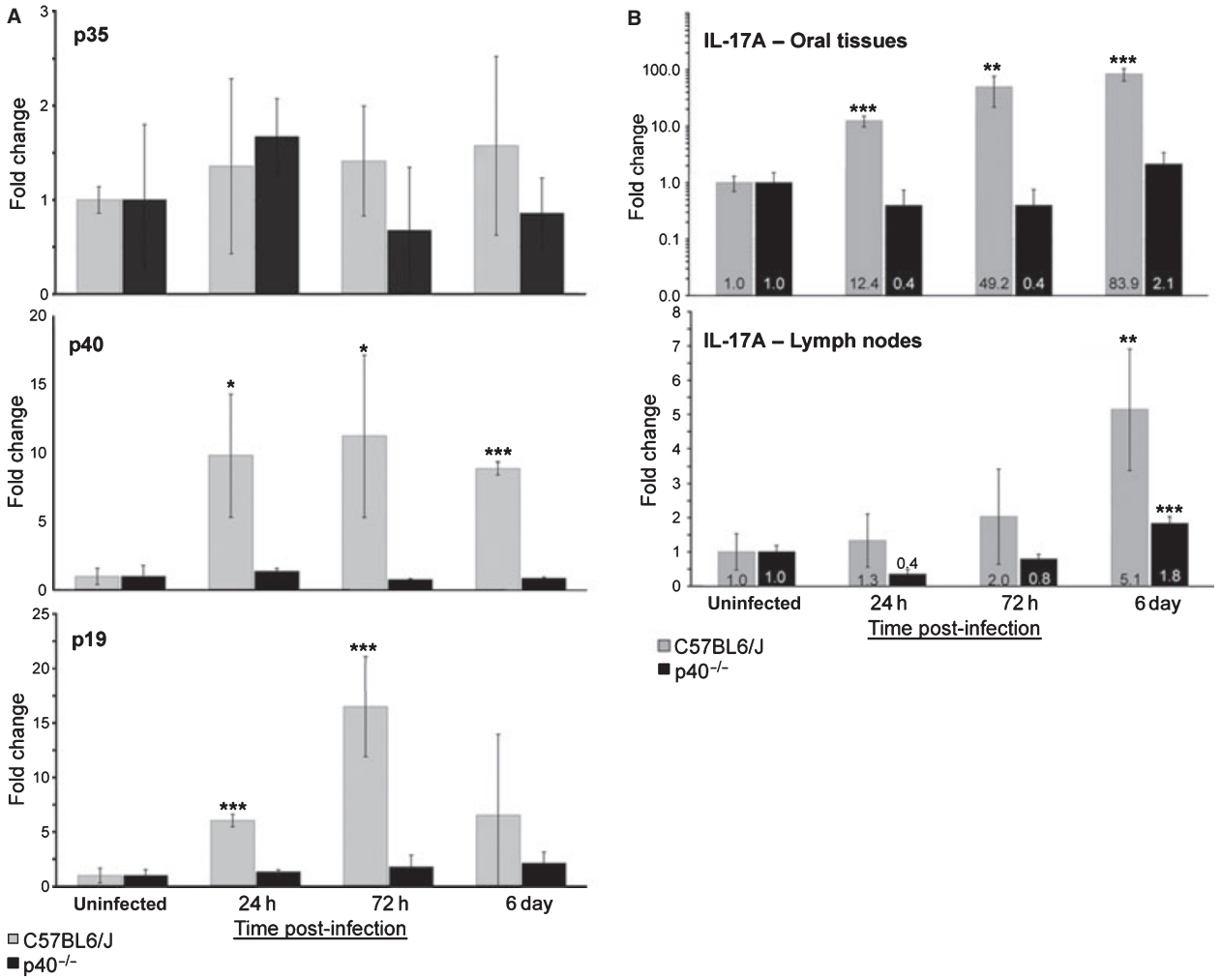

Figure 4 Expression of interleukins (IL-) 12, 23 and 17A in wild-type and p40$^{-/-}$ mice. Wild-type C57BL/6J and p40$^{-/-}$ mice were sacrificed either before (uninfected) or 24 h, 72 h or 6 days after oral infection with *Candida albicans*. Regional lymph nodes or oral tissues were dissected, and used to extract total RNA for real-time reverse transcription–polymerase chain reaction. p35, p40, p19 (A) and IL-17A (B) expression levels were determined relative to 18S ribosomal RNA, then expressed as fold changes relative to the levels measured in respective C57BL/6J or p40$^{-/-}$ uninfected control mice. Data were derived in triplicate from two or three mice per timepoint; means ± the standard deviation are shown. The statistical significance of any differences to the uninfected expression levels was calculated using Student's *t*-tests. **P* = 0.01 to *P* = 0.001; ***P* = 0.001 to *P* = 0.0001; ****P* < 0.0001.

in regional lymph nodes of C57BL/6J and p40$^{-/-}$ mice in parallel with oral tissue expression analysis. Expression of IL-17A increased at day 6 in controls (5.1 ± 1.8 -fold; Fig. 4). Interestingly there was a slight but significant, IL-23-independent increase at this timepoint in p40$^{-/-}$ mice (1.8 ± 0.2 -fold).

The functional effects of IL-17A are centred on induction of chemokines and inflammatory mediators from epithelial, inflammatory and stromal cells through paracrine signalling, which in turn results in chemotactic infiltration of neutrophils and other inflammatory cells. Some of these effects, including upregulation of IL-17 pathway chemokines and neutrophil microabscess formation, have already been established in murine OPC (Conti *et al.*, 2009). We therefore tested some of these functional effects of IL-17 pathway activation in our infection model. Using quantitative RT-PCR, we observed significant, transient increases in *Mcp-1*, *Mip-2* and *Il-6* mRNA levels in the oral tissues of C57BL/6J mice, which were either abrogated or delayed in p40$^{-/-}$ mice (Fig. 5).

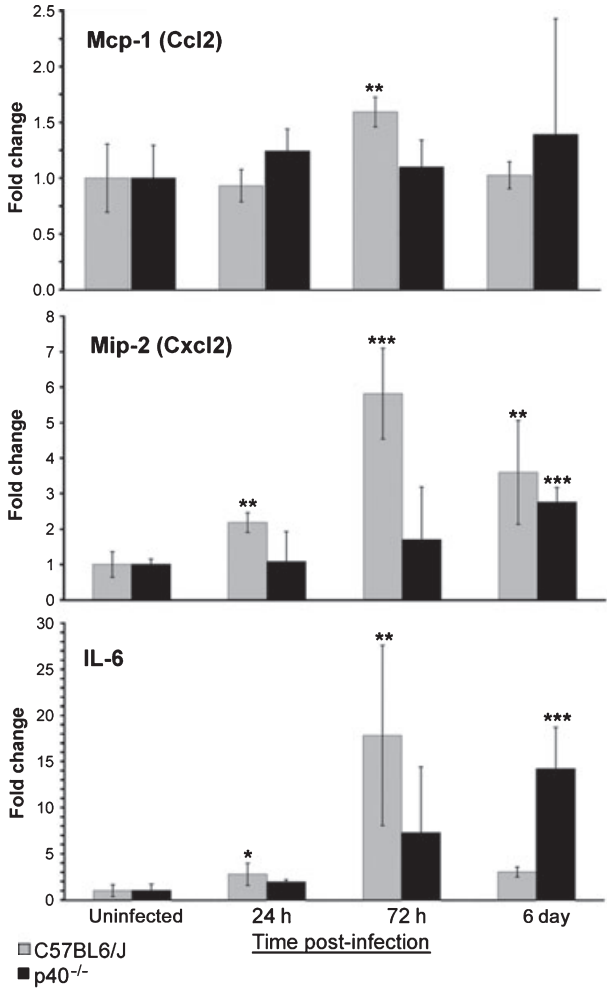

Figure 5 Local synthesis of messenger RNAs encoding cytokines and chemokines downstream of interleukin-17A (IL-17A) in wild-type and p40$^{-/-}$ mice. Wild-type C57BL/6J and p40$^{-/-}$ mice were sacrificed either before (uninfected) or 24 h, 72 h or 6 days after oral infection with *Candida albicans*. Oral tissues were dissected and used to extract total RNA for analysis of *Mcp-1*, *Mip-2* and *Il-6* messenger RNA expression relative to 18S ribosomal RNA by real-time reverse transcription–polymerase chain reaction. Relative expression is shown as fold change relative to respective C57BL/6J or p40$^{-/-}$ control mice. Data were derived in triplicate from two or three mice per timepoint; means ± the standard deviation (SD) are shown. The statistical significance of any differences to 'uninfected' expression levels was calculated using Students' two-tailed *t*-tests. **P* = 0.01 to *P* = 0.001; ***P* = 0.001 to *P* = 0.0001; ****P* < 0.0001.

Coordinated neutrophil infiltration in murine OPC is dependent on p40

We explored the chemokine expression analysis findings further by examining neutrophil recruitment to the oral tissues over the course of infection in C57BL/6J and p40$^{-/-}$ mice (Fig. 6). Pooled oral tissue cell suspensions were prepared from three C57BL/6J or three p40$^{-/-}$ mice in the uninfected state (Fig. 6; naive), and at 24 h, 72 h or 6 days after oral infection with *C. albicans*, then stained with a labelled polymorphonuclear leucoocyte-specific antibody, and counted by flow cytometry. There was infiltration of neutrophils into the infected oral tissues of C57BL/6J mice within 24 h of the infection, with neutrophil content increasing from 12 to 16.4% of live oral mucosal cells. Neutrophil numbers then normalized throughout the experiment, returning to 12.4% by day 6. In contrast, in p40$^{-/-}$ mice, neutrophil chemotaxis seemed to be dysregulated, with resting numbers almost twice those of wild-type controls (22.8%), and no pattern of recruitment followed by a return to baseline: neutrophil content was 17.3, 26.1 and 13.0% at 24 h, 72 h and 6 days postinfection, respectively. These results suggest that p40 is required for recruitment of polymorphonuclear leucoocytes to the oral mucosa upon oral infection with *C. albicans*, and that in the absence of baseline IL-23 and/or IL-17 expression, the local signals regulating resting neutrophil numbers could be impaired.

DISCUSSION

The immunological response to OPC involves oral epithelial cells and their antimicrobial secreted proteins (defensins, S100 proteins), phagocytic cells (neutrophils, macrophages and DCs), and also adaptive immunity. The cytokine pathways involved in this response have been revisited after discovery of the IL-23–17 pathway. Knockout mouse studies suggest that IL-17R-dependent processes are more critical for full recovery from OPC than IL-12-driven processes, but IL-12p35$^{-/-}$ mice still take around three times as long to recover from experimental OPC than their wild-type counterparts (Conti *et al.*, 2009), and IL-12 and IFN-γ increase in regional lymph nodes of BALB/c mice with OPC (Farah *et al.*, 2001), and also following reconstitution of nude mice with syngeneic lymphocytes correlating with recovery from OPC (Farah *et al.*, 2002). Indeed, *C. albicans* can induce both IL-12 and IL-23 in professional phagocytes (Acosta-Rodriguez *et al.*, 2007; LeibundGut-Landmann *et al.*, 2007; Gerosa *et al.*, 2008). To some degree this is dependent on yeast virulence factors; for example, hyphae preferentially bind different

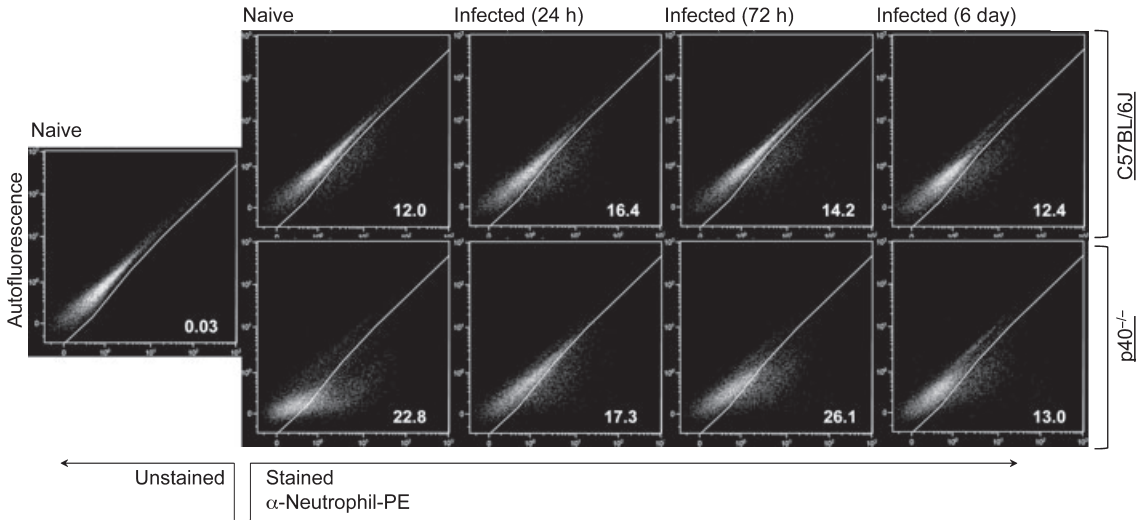

Figure 6 Recruitment of neutrophils to the oral mucosae of wild-type and $p40^{-/-}$ mice infected with *Candida albicans*. Wild-type C57BL/6J or $p40^{-/-}$ mice (three per data-point) were orally infected with phosphate-buffered saline (naive) or *C. albicans* (infected), then sacrificed 24 h, 72 h or 6 days postinfection. Cell suspensions were prepared from oral tissues of the mice, labelled with a phycoerythrin-conjugated α-neutrophil antibody, then analysed by flow cytometry. Data shown are stained events, quantified as the percentage of live, filtered mucosal cells.

PAMP-receptors to yeast (van der Graaf *et al.*, 2005), and preferentially induce production of IL-23 over IL-12 *in vitro* (Acosta-Rodriguez *et al.*, 2007). The relative contributions of IL-12 and IL-23-driven processes in the response to OPC are still not clear. In this study we investigated the IL-12 and IL-23 pathways in p40-deficient mice with OPC. Upon non-traumatic infection of the oral cavity with *C. albicans*, these mice develop chronic, severe OPC with extensive hyphal penetration of the oral epithelium, which can be reliably monitored using oral swabbing as a marker of disease severity (Farah *et al.*, 2002, 2006).

We studied macrophage activation as an example of an IL-12-regulated immune process, as systemic depletion of macrophages increases oral fungal burden in mice (Farah *et al.*, 2001), and also because macrophages are broadly implicated in priming Th1 responses. We detected a reduction in $p40^{-/-}$ macrophage candidacidal activity *in vitro* that could be fully restored by supplementation with both recombinant IL-12 and IFN-γ (Fig. 1), consistent with the idea that autocrine IL-12 and IFN-γ are involved in antifungal defence (Frucht *et al.*, 2001; Bastos *et al.*, 2004). Overall, the macrophage experiments suggest that both candidacidal effector function and activated migration (Fig. 2) are impaired by p40 deficiency in OPC, but that these functions are probably redundant *in vivo*. First, we could not detect a local oral mucosal macrophage population in naive or infected wild-type or $p40^{-/-}$ mice using two different antibodies and detection methods. Second, although we detected a p40-dependent activated migration event within 24 h of oral infection (Fig. 2A), DC migration was not overtly affected (Fig. 2B), and DCs have been strongly implicated in *C. albicans* antigen presentation (d'Ostiani *et al.*, 2000; Romagnoli *et al.*, 2004; LeibundGut-Landmann *et al.*, 2007; Zelante *et al.*, 2007). Even full replacement of IL-12 via recombinant protein administration did not completely rescue the phenotype of the $p40^{-/-}$ mice, although it did provide some protection initially, reducing oral fungal loads in the first 4 days of infection to levels usually observed in wild-type C57BL/6J mice (Farah *et al.*, 2006). Consistent with this, infected IL-$12p35^{-/-}$ mice take around three times as long to clear yeast from the oral tissues as wild-type controls, but they do eventually recover (Conti *et al.*, 2009).

Expression analysis confirmed strong, p40-dependent upregulation of IL-23 and IL-17A in the oral tissues of C57BL/6J mice (Fig. 4). Induction of IL-17A target genes (Fig. 5) and coordinated neutrophil recruitment (Fig. 6) were also impaired by p40 abrogation. IL-23/17A induction was obvious within 24 h of infection, raising the possibility that the IL-17 pathway could be activated independently of T-helper cellular immunity. This idea is supported by

profiling experiments showing that multiple IL-17 pathway activator and effector genes (e.g. IL-23, S100 proteins, Cxcl2, Cxcl5, Defb3) are induced in an IL-17-dependent fashion in the oral mucosae of formerly naive C57BL mice within 24 h of *C. albicans* infection (Conti *et al.*, 2009). At this stage we cannot confirm the cellular source(s) of early IL-17A in OPC, but the early timing of the increase in IL-17A expression raises the hypothesis that cells of the innate immune system may be an immediate source. The finding that oral *C. albicans* burden in $\alpha\beta$ T cells (includes Th17) is lower than in IL-17 pathway-specific knockouts ($p19^{-/-}$ and $IL\text{-}17RA^{-/-}$) (Conti *et al.*, 2009) would support this. Others have shown that IL-23 from professional phagocytes can induce a local IL-17 response, perhaps as the sole stimulus (Uhlig *et al.*, 2006; Matsuzaki & Umemura, 2007; Yu & Gaffen, 2008). Apart from Th17 cells, IL-17 can be produced in efficacious quantities by $\gamma\delta$ T cells, natural killer cells and granulocytes, which reside at mucosal surfaces (O'Brien *et al.*, 2009). Other examples of early activation and function of the IL-23–17 axis are also emerging: $IL\text{-}17RA^{-/-}$ mice have neutrophil recruitment and chemokine production defects, and are susceptible to systemic candidiasis and *Klebsiella pneumoniae* infections with noticeable pathology within a few days (Ye *et al.*, 2001; Huang *et al.*, 2004), which is possibly too early to involve an effective contribution from the adaptive immune system; and early IL-17 pathway activation also occurs in systemic and mucosal infections with certain intracellular and extracellular bacteria (Lockhart *et al.*, 2006; Shibata *et al.*, 2007; Zhang *et al.*, 2009). This idea should be explored more specifically in the future.

We also detected a fivefold increase in IL-17A mRNA in regional lymph nodes 6 days after oral infection (Fig. 4), which is consistent with a previous report that Th17 cells are involved in the host response to OPC (Conti *et al.*, 2009). *Candida*-specific memory Th17 cells have been identified in humans, where *C. albicans* is part of the oral and vaginal commensal flora (Acosta-Rodriguez *et al.*, 2007), and importantly, Th17 defects are associated with human mucosal candidiasis syndromes (Ma *et al.*, 2008; Milner *et al.*, 2008; Farah *et al.*, 2009). However, the importance of local, early IL-17 pathway activation has not been investigated in human disease.

Neutrophil microabscess formation is a well-documented histological feature of OPC in both humans and mice (Farah *et al.*, 2000; Conti *et al.*, 2009). Neutropenia predisposes to OPC, and also to invasive disease (Fidel, 2002). We hypothesized that neutrophil recruitment to the oral mucosa would be impaired in $p40^{-/-}$ mice, but instead observed a higher resting neutrophil presence, and no pattern of infiltration followed by a return to baseline as in the wild-type controls (Fig. 6), suggesting that chemotactic signals could be dysregulated and uncoordinated following infection. This is consistent with a previous histological report that neutrophils were fewer and appeared 'trapped in nests' in $IL\text{-}17RA^{-/-}$ and $IL\text{-}23p19^{-/-}$ mice with OPC (Conti *et al.*, 2009). Upon exposure to *C. albicans in vitro*, neutrophils are directly candidacidal, killing both phagocytosed and extracellular yeasts (Peltroche-Llacsahuanga *et al.*, 2000; Vonk *et al.*, 2002). Evidence suggests that cross-talk between neutrophils and oral epithelial cells enhances the candidacidal activities of both cell types (Schaller *et al.*, 2004; Dongari-Bagtzoglou *et al.*, 2005; Weindl *et al.*, 2007). Neutrophils are candidate sources of early IL-17 in OPC (Weaver *et al.*, 2007), and IL-17 potentiates their release of proinflammatory cytokines (Beklen *et al.*, 2009). Although IL-17 receptor isoform expression has not yet been characterized in either the healthy or *C. albicans*-infected oral epithelium, the ubiquitous expression pattern in other epithelial tissues (Weaver *et al.*, 2007) suggests that it is likely to be expressed in the oral epithelium. It is possible that IL-17 could be an early paracrine signal between neutrophils and oral epithelial cells involved in proinflammatory activation. Identifying the factors involved at this early stage of the infection should be a research priority because this could be targeted by specific drugs to enhance the local immune response in individuals with compromised immune function.

We agree with Pirofski and Casadevall that acute responses to oral *C. albicans* challenge in naive mice are probably not representative of OPC in humans where *C. albicans* is a commensal organism in the oral cavity (Pirofski & Casadevall, 2009). However, our results raise the possibility that activation of the IL-17 pathway in OPC may not be restricted to the adaptive immune system, and that innate paracrine IL-17 signalling in the oral mucosa could contribute to the homeostatic maintenance of commensalism. This

study also has important implications for the interpretation of results generated from IL-17 pathway knockout mice.

ACKNOWLEDGEMENTS

This work was funded by the Australian Dental Research Foundation. The authors acknowledge the technical assistance of and valuable discussions with Geoff Osborne and Virginia Nink at the UQ Qld Brain Institute Flow Cytometry Facility, and are grateful to Anthony Chan for assistance with immunohistochemical staining.

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