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Role of Neutrophils in IL-17–Dependent Immunity to Mucosal Candidiasis

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Oropharyngeal candidiasis (OPC), caused by the commensal fungus Candida albicans, is an opportunistic infection associated with infancy, AIDS, and IL-17–related primary immunodeficiencies. The Th17-associated cytokines IL-23 and IL-17 are crucial for immunity to OPC, but the mechanisms by which they mediate immunity are poorly defined. IL-17RA–deficient humans and mice are strongly susceptible to OPC, with reduced levels of CXC chemokines and concomitantly impaired neutrophil recruitment to the oral mucosa. Paradoxically, humans with isolated neutropenia are typically not susceptible to candidiasis. To determine whether immunity to OPC is mediated via neutrophil recruitment, mice lacking CXCR2 were subjected to OPC and were found to be highly susceptible, although there was no dissemination of fungi to peripheral organs. To assess whether the entire neutrophil response is IL-17 dependent, IL-17RA−/− and IL-23−/− mice were administered neutrophil-depleting Abs and subjected to OPC. These mice displayed increased oral fungal burdens compared with IL-17RA−/− or IL-23−/− mice alone, indicating that additional IL-17–independent signals contribute to the neutrophil response. WT mice treated with anti–Gr-1 Abs exhibited a robust infiltrate of CD11b+Ly-6GlowF4/80− cells to the oral mucosa but were nonetheless highly susceptible to OPC, indicating that this monocyte influx is insufficient for host defense. Surprisingly, Ly-6G Ab treatment did not induce the same strong susceptibility to OPC in WT mice. Thus, CXCR2+ and Gr-1+ neutrophils play a vital role in host defense against OPC. Moreover, defects in the IL-23/17 axis cause a potent but incomplete deficiency in the neutrophil response to oral candidiasis. The Journal of Immunology, 2014, 192: 000–000.

Oropharyngeal candidiasis (OPC; thrush) is an opportunistic mucosal infection caused by the commensal fungus Candida albicans. C. albicans causes disease when exposure is combined with host susceptibility through immunodeficiency or a breach in normal barriers. A range of primary and secondary immunodeficiency syndromes are associated with susceptibility to OPC (1, 2), including the primary immunodeficiency hyper-IgE syndrome and a family of genetic diseases leading to chronic mucocutaneous candidiasis (3). Secondary immunodeficiencies are now the most common predisposing factor for mucosal Candida disease. OPC is well described in patients with HIV and is considered an AIDS-defining illness. Cancer chemotherapy, immunosuppression for transplant recipients, renal failure, and high-dose corticosteroids increase the risk for candidiasis.

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Abbreviations used in this article: ANC, absolute neutrophil count; CMC, chronic mucocutaneous candidiasis; OPC, oropharyngeal candidiasis.

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There is no knockout mouse system to study profound neutropenia, but alternate tools are available to examine the role of neutrophil recruitment and effector responses. Neutrophils, characterized by expression of Ly-6G (a component of the Gr-1 epitope), are regulated through the chemokine receptor CXCR2 (18–20). Neutrophils are the predominant CXCR2⁺ cell among blood leukocytes, with lower expression on mast cells, monocytes, macrophages, endothelial cells, and epithelial cells (21–23). In addition, several Abs (anti-Ly-6G and anti-Gr-1) are available to deplete neutrophils peripherally, albeit with variable degrees of efficiency in tissue and cell type specificity (24–27). To test the hypothesis that neutrophils are essential in host defense from OPC, we evaluated disease in CXCR2⁻/⁻ mice and Ab-mediated neutrophil depletion. In addition, we assessed redundancy in host defense systems with neutrophil depletion in mice deficient in IL-23 or IL-17RA. Our findings indicate a central role for CXCR2 and Gr-1⁺ neutrophils in the oral mucosa.

Materials and Methods

Mice

IL-23p19⁻/⁻ mice were provided by Genentech (South San Francisco, CA) and IL-17RA⁻/⁻ mice by Amgen (Seattle, WA) and bred in-house. All other mice were from The Jackson Laboratory (Bar Harbor, ME) on the C57BL/6 background unless otherwise noted. Mice were 6–9 wk old, and age- and sex-matched for all experiments unless otherwise noted. Protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and adhered to guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Ab-mediated neutrophil depletion

Mice were injected i.p. with mAb 24 h before and 2 d after inoculation with C. albicans (day 0). Anti-Ly-6G (clone 1A8; Bio X Cell, West Lebanon, NH) and isotype control (clone 2A3; Bio X Cell) were administered at a dose of 300 μg. Anti-Gr-1 (clone RB6-8C5; Bio X Cell) and isotype control (clone LTF-2; Bio X Cell) were administered at a dose of 80 μg. All cohorts not receiving mAbs were treated with PBS. The degree of neutrophil depletion was assessed in peripheral blood as follows: blood was collected by saphenous venous puncture or cardiac puncture and anti-coagulated in EDTA-containing tubes (Microvette Tubes, Sarstedt, Newton, NC, and Micropetainer Tubes, BD, Franklin Lakes, NJ). Total WBC counts were determined by visual enumeration after trypan blue exclusion. The percentage of neutrophils was determined by enumeration of a blood smear stained with Giemsa (Fisher Scientific, Fair Lawn, NJ). One hundred to 200 cells were counted per sample. The absolute neutrophil count (ANC) was determined by multiplying the percentage of neutrophils (mature and band forms) by total WBC.

Mouse model of OPC

OPC was performed as described previously (4, 28). In brief, mice were inoculated under anesthesia by placing a 0.0025-g cotton ball saturated with C. albicans in the oral cavity. Sham-inoculated mice were inoculated with a cotton ball saturated with sterile PBS. The oral cavity was swabbed immediately before each infection and streaked on yeast peptone dextrose agar to verify the absence of Candida. No antibiotics were administered. If indicated, control mice were immunosuppressed with cortisone acetate 225 mg/kg (Sigma-Aldrich) with baseline weight on day 0. Anti-Ly-6G and WT littermates at 6–9 wk of age were subjected to OPC on day 0. Sham-infected and WT mice treated with cortisone acetate were used as controls. Mice were sacrificed at 4 d, and fungal burden in tongue was assessed by plating and colony enumeration. Data are pooled from two independent experiments and geometric means are shown. **p < 0.01 comparing CXCR2⁺/⁻ and WT mice, ***p < 0.001 versus CXCR2⁺/⁻.

Flow cytometry

Tongue tissue was processed with an enzyme mixture (EDTA, HEPES, collagenase-2 [Worthington Biochemical, Lakewood, NJ], Dispase [Life Technologies, Invitrogen], DNase I [Applied Biochemical], and defined trypsin inhibitor [Life Technologies]). Tissue was mechanically homogenized and passed through a cell strainer to form a single-cell suspension. Abs were from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA) or Biolegend (San Diego, CA). One to 2 × 10⁶ cells were stained with CD11b-allophycocyanin (clone M1/70), Gr-1-FITC or Alexa Fluor 700 (AF700; clone RB6-8CS5), CD45-AF700 (clone 30-F11), Ly-6G-PE (clone 1A8), Ly-6C-PerCP-Cy5.5 (clone AL-21), CD115-AF488 (clone AFS98), CD11c-PerCP-Cy5.5 (clone HL3), and F4/80-PE-Cy7 (clone BM8). Isotype control Abs and unstained samples were included in the analyses. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo (Tree Star).

RNA extraction and real-time reverse transcriptase PCR

Frozen tongue was homogenized in RLT lysis buffer (RNAeasy Kit; Qiagen, Valencia, CA) with a GentleMACS Dissociator (M-tubes, RNA02 program; Valenzia, CA) followed by plating serial dilutions on yeast peptone dextrose agar to verify the absence of Candida. Inoculated mice were inoculated by placing a 0.0025-g cotton ball saturated with C. albicans in the oral cavity. Sham-inoculated mice were inoculated with a cotton ball saturated with sterile PBS. The oral cavity was swabbed immediately before each infection and streaked on yeast peptone dextrose agar to verify the absence of Candida. No antibiotics were administered. If indicated, control mice were immunosuppressed with cortisone acetate 225 mg/kg (Sigma-Aldrich) with baseline weight on day 0. Anti-Ly-6G and WT littermates at 6–9 wk of age were subjected to OPC on day 0. Sham-infected and WT mice treated with cortisone acetate were used as controls. Mice were sacrificed at 4 d, and fungal burden in tongue was assessed by plating and colony enumeration. Data are pooled from two independent experiments and geometric means are shown. **p < 0.01 comparing CXCR2⁺/⁻ and WT mice, ***p < 0.001 versus CXCR2⁺/⁻.

FIGURE 1. CXCR2 deficiency induces persistent susceptibility to OPC. (A) CXCR2⁻/⁻ mice are susceptible to OPC. CXCR2⁺/⁺ and CXCR2⁻/⁻ mice at 7–16 wk of age were subjected to OPC on day 0. Sham-infected and WT mice treated with cortisone acetate were used as controls. Mice were sacrificed at 4 d, and fungal burden in tongue was assessed by plating and colony enumeration. Data are pooled from two independent experiments and geometric means are shown. *p < 0.05 comparing CXCR2⁺/⁻ and WT mice at 7–16 wk of age. Mean and SEM are shown. (B) CXCR2⁻/⁻ mice show steady weight loss after inoculation. Mice were inoculated as in (A), and daily weight was shown as a percentage of day −1. Means and SEM are shown. (C) CXCR2⁻/⁻ mice show persistent weight loss after inoculation. Mice were inoculated as in (A), and daily weight was shown as a percentage of day −1. Means and SEM are shown. (D) CXCR2⁻/⁻ mice show persistent weight loss. Mice were inoculated as in (C), and daily weight was compared with day −1. Means and SEM are shown. (E) There is no dissemination of C. albicans from gastrointestinal tract to peripheral organs in CXCXR2⁻/⁻ mice. Four days after oral inoculation, CXCR2⁻/⁻ or WT mice ± cortisone acetate were evaluated for fungal loads in the indicated organs.
Miltenyi). RNA was extracted with RNAasy Kit. In all, 0.732 μg total RNA was used to synthesize cDNA with a SuperScript III First-Strand Synthesis System (Invitrogen). Relative quantification of indicated genes was determined by real-time PCR with SYBR Green (Quanta BioSciences, Gaithersburg, MD) normalized to GAPDH. Primers were from SA Biosciences (Qiagen). Results were analyzed on a 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA) in triplicate.

Statistics
Data were compared by Mann–Whitney U or unpaired Student t test using GraphPad Prism (La Jolla, CA) or Microsoft Excel. The p values <0.05 were considered significant. All experiments were performed a minimum of twice.

Results
CXCR2 deficiency induces persistent susceptibility to OPC
To assess the contribution of neutrophils to host defense from OPC, we used a mouse deficient in neutrophil recruitment because of knockout of the chemokine receptor CXCR2 (22, 29). CXCR2−/− mice exhibit abnormal neutrophil trafficking in response to the chemokines CXCL1, CXCL2, and CXCL5, all of which are up-regulated by IL-17 during oral C. albicans infection (22, 29–31). We subjected CXCR2−/− mice to OPC by sustained sublingual exposure (4, 28). In this model, WT mice are resistant to infection, but cortisone-treated or IL-17RA–deficient mice are susceptible (4, 28). Whereas heterozygous littermates were not susceptible to OPC, CXCR2−/− mice showed a mean oral fungal burden of 1.9 × 10³ CFU/g tongue tissue at 4 d after inoculation (Fig. 1A). CXCR2−/− mice also showed progressive weight loss after inoculation, with a mean of 16% weight loss on day 4 compared with baseline (Fig. 1B). To determine whether CXCR2−/− mice showed persistent susceptibility or only a delayed clearance, we inoculated CXCR2−/− mice and WT littermates with C. albicans on day 0, and oral fungal burden was assessed on days 3, 4, 6, and 8. CXCR2−/−

FIGURE 2. Elevated oral fungal load with preserved CD11b+ tongue cellular infiltrate in IL-17RA−/− mice after neutrophil depletion. (A) IL-17RA−/− mice treated with anti–Ly-6G had sustained neutropenia. IL-17RA−/− mice were treated with isotype or anti–Ly-6G Abs on days −1 and 2, and subjected to OPC on day 0. Sham- and Candida-inoculated WT mice served as controls. Peripheral blood neutrophil counts were assessed by visual smear examination on days 0, 2, and 4. Geometric means are shown. Dashed line indicates threshold for clinically significant neutropenia. (B) IL-17RA−/− mice treated with anti–Ly-6G have elevated oral fungal burdens. Mice were treated with Abs and infected as in (A). Fungal burden in tongue was assessed 4 d after inoculation. Geometric means are shown. ***p < 0.001. (C) IL-17RA−/− mice treated with anti–Ly-6G display elevated weight loss. Mice were weighed daily and weight loss was compared with baseline. Means and SEM are shown. (D) IL-17RA−/− mice treated with anti–Gr-1 exhibit profound neutropenia. IL-17RA−/− mice were treated with isotype or anti–Gr-1 Abs on days −1 and 2, and subjected to OPC on day 0. Controls included: sham- or Candida-inoculated IL-17RA−/− mice, IL-17RA−/− mice treated with PBS, Candida-inoculated WT mice, and cortisone-treated WT mice. Peripheral blood neutrophil counts were assessed as in (A). Geometric means are shown. Dashed line indicates threshold for clinically significant neutropenia. (E) IL-17RA−/− mice treated with anti–Gr-1 Abs show an elevated oral fungal burden. Mice were treated with Abs and inoculated as in (D). Fungal burden in tongue tissue was assessed after 4 d. ***p < 0.001. Mice were evaluated in two independent experiments. (F) IL-17RA−/− mice treated with anti–Gr-1 exhibited profound weight loss. Mice were weighed daily and weight loss was compared with baseline. (G) Candida-inoculated IL-17RA−/− mice show a CD11b+ cellular infiltrate in tongue that is maintained with anti–Gr-1 treatment. Mice were treated with Abs and inoculated as in (D). Tongue was harvested on day 4 and tongue homogenates were analyzed by flow cytometry.
mice had persistent oral fungal burden through day 8, in contrast with WT littermates that cleared the fungus fully by day 6 (Fig. 1C). Consistently, weight loss in CXCR2−/− mice was sustained through day 8, whereas WT mice returned to baseline (Fig. 1D). Although C. albicans was found in the mouth and gastrointestinal tract post-infection, CXCR2−/− mice did not show dissemination of yeast to peripheral organs such as kidney or brain, measured on day 4 after inoculation (Fig. 1E). Thus, CXCR2 signaling is essential for immunity to oral candidiasis, but not for barrier maintenance and prevention of dissemination.

**Neutrophil depletion augments OPC in IL-17/Th17−deficient mice**

To investigate the degree to which IL-17 signaling is responsible for regulation of neutrophils in OPC, we administered Abs to deplete neutrophils during the course of infection in IL-17RA−/− mice. IL-17RA−/− mice were treated with anti-Ly-6G (clone 1A8), which recognizes a highly expressed marker on neutrophils, 24 h before and 2 d postinfection. Anti-Ly-6G–treated mice were neutropenic throughout the experimental period, with ANCs <0.5 × 10⁶ neutrophils/ml (Fig. 2A). As expected, IL-17RA−/− mice treated with isotype Abs were susceptible to OPC, similar to untreated IL-17RA−/− (Fig. 2B). IL-17RA−/− mice treated with anti-Ly-6G showed significantly higher oral fungal burdens than isotype-treated mice (Fig. 2B). The anti-Ly-6G–treated mice also displayed more weight loss than isotype-treated mice (Fig. 2C).

To extend these findings, we treated IL-17RA−/− mice with an Ab directed against Gr-1 (clone RB6-8C5), which recognizes Ly-6G and, to a lesser extent, Ly-6C on mature granulocytes, monocytes (transiently during differentiation), and plasmacytoid dendritic cells (at low levels). Most mice treated with anti–Gr-1 demonstrated peripheral blood neutropenia (Fig. 2D). IL-17RA−/− mice treated with anti–Gr-1 exhibited a significant increase in oral fungal burden compared with isotype-treated mice 4 d after inoculation (Fig. 2E). Dissemination of C. albicans to the kidney was found in 10% of IL-17RA−/− mice treated with anti–Gr-1, but not in mice treated with isotype Abs, PBS, or anti-Ly-6G (data not shown). Treatment with isotype did not alter the susceptibility of IL-17RA−/− mice to OPC compared with PBS. Treatment with anti–Gr-1 resulted in increased weight loss in IL-17RA−/− after OPC challenge, similar to the weight loss observed in WT mice treated with high-dose cortisone (Fig. 2F). Collectively, these data show that the susceptibility to OPC caused by defective IL-17 signaling can be augmented by neutrophil depletion.

Although anti–Gr-1 and–Ly-6G can cause profound depletion of peripheral blood neutrophils, this has been shown to correlate poorly with neutrophil depletion in resident tissues (24). Accordingly, we assessed the effects of Ab treatment on neutrophil infiltration into tongue by flow cytometry. At 4 d after inoculation, WT mice showed a small but measurable CD11b+Gr-1low population (Fig. 2G). Inoculated WT mice treated with cortisone showed a tongue infiltrate of CD11b+Gr-1hi cells. Sham-treated IL-17RA−/− mice showed minimal infiltrate of cells staining positive for CD11b, Ly-6G, or Gr-1. IL-17RA−/− mice treated with isotype showed a robust infiltrate of CD11b+Ly-6Ghi and CD11b+Ly-6Glow cells. In inoculated IL-17RA−/− mice treated with anti–Gr-1, only the tongue infiltrate of CD11b+Ly-6Glow cells was observed, and these cells were Gr-1low (Fig. 2G). These data indicate that OPC induces a local CD11b+ cellular infiltrate that is preserved after treatment with anti–Gr-1 Abs, but the absence of CD11b+Ly-6Ghi neutrophils results in heightened susceptibility to OPC.

IL-23 is critical for the maintenance of IL-17−producing cells (32, 33), and deficiency in IL-23 results in susceptibility to OPC at levels indistinguishable from IL-17RA−/− mice (9). To determine whether neutrophil depletion in IL-23−/− mice followed a similar profile, we treated IL-23−/− mice with anti–Ly-6G 24 h before and 2 d after oral inoculation with C. albicans. Mice were neutropenic throughout the experimental period with peripheral ANC <0.5 × 10⁶ neutrophils/ml (Fig. 3A). Mice treated with anti–Ly-6G showed a significant increase in fungal burden on day 4 compared with IL-23−/− mice treated with isotype Abs (Fig. 3B). Similar to the IL-17RA−/− mice, IL-23−/− mice treated with isotype Abs exhibited a robust infiltrate of CD11b+Gr-1hi cells in tongue. IL-23−/− mice treated with anti–Ly-6G continued to have a CD11b+ population, but Gr-1 staining shifted to Gr-1low (Fig. 3C). Low numbers of CD11b+Ly-6Glow cells were present in sham-infected IL-23−/− and *Candida*–infected WT mice, whereas IL-23−/− mice treated with cortisone and inoculated with *C. albicans*...
showed a robust infiltrate of CD11b<sup>+</sup>Ly-6G<sup>hi</sup> and Gr-1<sup>hi</sup> cells (Fig. 3C). Together, these results show that deficiency in Th17 cells or IL-17 signaling does not fully eliminate the neutrophilic response to OPC.

**Susceptibility to OPC is induced in normally resistant WT mice with myeloid cell depletion**

Next, we assessed whether neutrophil depletion induced susceptibility in WT mice. WT mice were treated with isotype or anti–Ly-6G as described earlier. Peripheral ANCs demonstrated a transient neutropenia in anti–Ly-6G-treated mice, with recovery above 0.5 × 10<sup>6</sup> cells/ml by days 4–5 (Fig. 4A). Neutrophil-depleted mice showed a bimodal response to inoculation, with 86% of mice completely resistant to infection, whereas 14% were profoundly susceptible with high oral fungal burdens, sustained weight loss, and dissemination to kidney at days 4–5 (Fig. 4B and data not shown). The cohort treated with anti–Ly-6G experienced weight loss similar to isotype-treated mice (data not shown). There was no correlation between the degree of neutropenia and susceptibility to OPC. Isotype-treated mice had a large infiltrate of CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells, whereas anti–Ly-6G–treated mice showed an infiltrate of CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells at day 2. Both populations were absent in sham-inoculated WT mice (Fig. 4C).

Although anti–Ly-6G and anti–Gr-1 treatments cause similar phenotypes in IL-17RA<sup>−/−</sup> mice, their effects were different in WT mice. Anti–Gr-1 resulted in profound peripheral neutropenia on day 0, with some count recovery by day 2 (Fig. 5A). Unlike the anti–Ly-6G–treated mice, these animals were profoundly ill, with decreased activity and grooming seen as early as day 1. Mice were sacrificed on day 3 for humane reasons, at which time there was a significant difference in the oral fungal burden between isotype and anti–Gr-1–treated mice (Fig. 5B). At 2 d after inoculation, sham-treated WT mice treated with Gr-1 Abs showed minimal infiltration of leukocytes staining positive for CD11b and Ly-6G (Fig. 5Ci). WT mice treated with isotype Abs and inoculated with *Candida* had a dominant population of CD11b<sup>+</sup>Ly-6G<sup>hi</sup> cells and a smaller population of CD11b<sup>+</sup>Ly-6G<sup>low</sup> cells. At day 3, both populations were starting to wane as the infection cleared (data not shown). In WT mice treated with anti–Gr-1 and infected with *Candida*, a much larger percentage of the population consisted of CD11b<sup>+</sup>Ly-6G<sup>low</sup> cells (Fig. 5Cii). The CD11b<sup>+</sup> population was further characterized by staining for macrophage/monocyte markers including Ly-6C, F4/80, and CD115. Based on these markers, the cell infiltrate in infected mice (isotype treated) was consistent with neutrophils (CD11b<sup>+</sup>Ly-6G<sup>hi</sup>Ly-6C<sup>hi</sup>) and inflammatory monocytes (CD11b<sup>+</sup>Ly-6G<sup>low</sup>Ly-6C<sup>lo</sup>); Fig. 5Ciii). The neutrophil population, but not the inflammatory monocyte population, was depleted in anti–Gr-1–treated mice (Fig. 5Ciii). The inflammatory monocytes that remained could not orchestrate *Candida* clearance, because these mice were highly susceptible to OPC (Fig. 5B). Markers for monocyte-derived phagocytes were absent from this population, because these cells did not stain strongly for F4/80 or CD115 (Fig. 5C). We also looked for DCs by staining for CD11c, but no positive cells were observed (data not shown).

A Gr-1<sup>+</sup> or CD11b<sup>+</sup> cell capable of suppressing T cells has been described in other settings, including disseminated candidiasis (34–36). To determine whether the cellular influx into the tongue after oral *Candida* challenge suppressed the IL-17/Th17–mediated host defense, we examined expression of a panel of IL-17 signature genes known to be induced in tongue in infected mice (4, 22). These findings indicate that the tongue cellular influx does not appear to suppress IL-17 expression or activity.

**Discussion**

In this study, we demonstrate that Gr-1<sup>+</sup> neutrophils are vital for the elimination of *C. albicans* from the oral mucosa. Deficiency in the CXCR2 chemokine receptor results in persistent oral fungal burdens, weight loss, and augmented IL-17 signature gene expression in the oral tissue, although no dissemination to visceral organs was detectable. Ab-mediated neutrophil depletion also results in susceptibility to OPC in normally resistant WT mice. Susceptibility is profound and consistent in mice broadly depleted of myeloid cells.
with anti–Gr-1 mAb, and susceptibility is also induced with the more specific anti–Ly-6G mAb. In addition, an increase in susceptibility is induced in mice deficient in Th17 cells or IL-17 signaling upon Ab-mediated neutrophil depletion, indicating that IL-17 does not account for all of the Ly-6G/Gr-1–dependent host protection in the oral cavity. All susceptible mice showed a CD11b+ tongue infiltrate, with alterations in Ly-6G and Gr-1 positivity depending on the specific Ab treatment. It has been long assumed that neutrophils and CXC chemokines are the mediators of host defense at the oral mucosal surface, but the evidence supporting this assumption is surprisingly scant. This is, to our knowledge, the first study to directly demonstrate a role for CXCR2 in OPC and the role of neutrophils in the context of IL-23/IL-17 signaling deficiency.

CXCR2, as the central chemokine receptor for neutrophil recruitment, is considered a potential therapeutic target (37); thus, our data suggest a potential new infectious risk implication of blocking CXCR2. In the context of fungal infections, CXCR2 plays a host-protective role in a mouse model of periodontal disease, with CXCR2 deficiency resulting in a more severe phenotype of oral bone loss than IL-17 signaling deficiency alone (31). Depletion of neutrophils, the primary CXCR2-expressing cell type, with anti-Ly6G Abs resulted in two distinct phenotypes. The majority of mice were resistant to OPC, but a subset was profoundly ill with both oral and disseminated candidiasis (Fig. 4B). The more severe phenotype was observed in all anti–Gr-1–treated mice (Fig. 5B). It remains controversial in the field whether Ly-6G Abs are simply less efficient at depleting neutrophils than Gr-1 Abs, or whether an alternative Ly-6G cell type exists that compensates for neutrophils upon depletion. Our data are more consistent with the former model, because our staining experiments did not reveal any evidence for an alternative monocytic cell that might be functionally compensatory. In disseminated candidiasis, however, there is

**FIGURE 5.** Neutrophil depletion with anti–Gr-1 induces susceptibility to OPC in WT mice. (A) WT mice treated with anti–Gr-1 Abs have peripheral neutropenia. Mice were treated with isotype or anti–Gr-1 Abs on days −1 and 2, and subjected to OPC on day 0. Controls include WT sham-inoculated and IL-17RA−/− mice. Peripheral neutrophil counts were determined as in Fig. 2A. Dashed line indicates threshold for clinically significant neutropenia. (B) WT mice treated with anti–Gr-1 are highly susceptible to OPC. Mice were treated with Abs and inoculated as in (A). Fungal burden was assessed after 3 d. Mice were evaluated in two independent experiments, and geometric means are shown. ***p < 0.001. (C) Anti–Gr-1–treated WT mice lose neutrophils, but not inflammatory monocytes, in tongue. WT mice were treated with anti–Gr-1 Abs (i, iii) or isotype (ii) and inoculated with C. albicans (ii, iii) as in (A). Tongue was harvested on day 2 and analyzed by flow cytometry.

**FIGURE 6.** No evidence for suppression of IL-17 responses upon anti–Ly-6G or anti–Gr-1 treatment. WT mice were treated with isotype control Abs (n = 3–5 mice), anti–Ly-6G (n = 3), anti–Gr-1 (n = 5), or no Ab (n = 3) on day −1, and subjected to OPC on day 0. Controls include sham-inoculated WT mice (n = 3) or CXCR2−/− mice (n = 2). Tongue mRNA was evaluated for il17a (A), s100a9 (B), or cxcl5 (C) by real-time RT-PCR. Each sample was analyzed in triplicate, and bars represent means with SEM. *p < 0.05 compared with WT. n.d., Not detectable.
Therefore, it remains to be determined whether the compensatory phagocyte death after in murine versus human neutrophils (41).

It was unexpected to find that susceptibility to OPC in mice lacking IL-23 or IL-17 signaling could be augmented by neutrophil depletion. Although signals for neutrophil recruitment are mediated by IL-17 in the context of OPC, alternative mechanisms of neutrophil regulation and recruitment thus appear to be present when IL-17 signaling is perturbed. Cellular influx of CD11b+ cells into the tongue was observed in mice experiencing active infection (Figs. 2G, 3C), which may be because of TLRs, C-type lectin receptors, or other cytokotyks. In addition to mediating expression of neutrophil-recruiting chemokines, IL-17 regulates expression of antimicrobial peptides that exhibit antifungal activity, such as β-defensins and histatins (4, 42). IL-17 also mediates host defense through alterations in cytokine production and synergy with other proinflammatory effectors (43). The entirety of IL-17–mediated host protection to candidiasis presumably comes from the combined effects of all these facets of the immune system.

As noted, there are limited tools to study isolated states of neutropenia in mice. Historically, many studies used cyclophosphamide, whole-body irradiation or other chemotherapeutic agents that induce neutropenia, but these regimens also cause defects in other leukocytes and mucosal integrity. Selective depletion of neutrophils with mAbs is a useful approach, but it has limitations. Commercially available Abs such as anti–Gr-1 and anti–Ly-6G deplete mature neutrophils but may also affect eosinophils, monocytes, and dendritic cells. In addition, these Abs deplete mature neutrophils, but not the immature band forms, which are nonetheless fully functional. Therefore, in response to intact signals for neutrophil production in the bone marrow and/or neutrophil recruitment to the site of infection, an ongoing robust neutrophilic response may occur even in the face of continued Ab treatment. This is most evident in the affected tissue and is overlooked if only peripheral blood is evaluated to assess efficiency of depletion. In a prior study attempting to address the issue of neutrophils in OPC, a swabbing method was used as a qualitative measure of fungal infection (44). However, that approach does not accurately recover hyphal forms of the yeast that have invaded the mucosal tissue. This study advances the field by providing a highly quantitative evaluation of susceptibility to invasive mucosal Candida infection, the hallmark of OPC, in states of neutrophil/myeloid cell impairment.

The prevalence of immunodeficiency is on the rise, along with an increasing awareness of the risk for opportunistic infection in susceptible patients. In clinical medicine, morbidity and mortality caused by fungal infections are observed in patients with neutropenia. Early reports of primary immunodeficiency with isolated neutropenia focused on invasive rather than mucosal candidiasis, but more recent case reports of congenital neutropenia highlight susceptibility to oral candidiasis (45). Neutropenia can also be secondary to immunosuppressive medications, medication side effects, or autoimmunity. Notably, neutropenia is rarely an isolated immunodeficiency. Combination defects in branches of the immune system are common and are likely to become more so with the increasing use of selective immunomodulation with biologic agents. This study is directly relevant to the use of drugs currently in development that target IL-17 and the Th17 pathway (46–48). Along these lines, a recent analysis of IBD patients showed that anti–TNF-α therapy is linked to oral candidiasis (49). Our findings suggest that the addition of IL-17 blockade to neutropenia-inducing therapies is likely to lead to a substantially increased risk for mucosal candidiasis.

Acknowledgments

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Disclosures

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References


Corrections


There was an inadvertent error in the calculation of the absolute neutrophil count due to incorrect total WBC counts. The error does not change the conclusions of the paper.

In the Results section, in the second paragraph under the subheading Susceptibility to OPC is induced in normally resistant WT mice with myeloid cell depletion, the sentence “Anti–Gr-1 resulted in profound peripheral neutropenia on day 0, with some count recovery by day 2 (Fig. 5A)” should read “Anti–Gr-1 resulted in profound peripheral neutropenia on day 0, persisting through day 2 (Fig. 5A).”

A corrected panel for Fig. 5A is shown below. The new Fig. 5A shows a more profound neutropenia in mice treated with anti–Gr-1, which supports the conclusion that anti–Gr-1 treatment induces susceptibility to oropharyngeal candidiasis in wild-type mice due to neutrophil depletion. The new Fig. 5A also shows that there are normal peripheral neutrophil numbers in IL-17RA−/− mice.

The legend for Fig. 5A was correct as published and is shown below for reference.

**FIGURE 5.** Neutrophil depletion with anti–Gr-1 induces susceptibility to OPC in WT mice. (A) WT mice treated with anti–Gr-1 Abs have peripheral neutropenia. Mice were treated with isotype or anti–Gr-1 Abs on days −1 and 2, and subjected to OPC on day 0. Controls include WT sham-inoculated and IL-17RA−/− mice. Peripheral neutrophil counts were determined as in Fig. 2A. Dashed line indicates threshold for clinically significant neutropenia.

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