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# Different tumor necrosis factor $\alpha$ antagonists have different effects on host susceptibility to disseminated and oropharyngeal candidiasis in mice

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Tumor necrosis factor  $\alpha$  is important for the host defense against intracellular pathogens. We tested the effect of mouse analogs of human TNF- $\alpha$  antagonists, the rat anti-mouse TNF- $\alpha$  monoclonal antibody (XT22) and the soluble mouse 75 kDa TNF- $\alpha$  receptor fused to the Fc portion of mouse IgG1 (p75-Fc), on the susceptibility of mice to hematogenously disseminated candidiasis (HDC) and oropharyngeal candidiasis (OPC). Both XT22 and p75-Fc significantly reduced mice survival, increased kidney fungal burden, and reduced leukocyte recruitment during HDC. However, only XT22 significantly increased the oral fungal burden and reduced leukocyte recruitment during OPC. This result suggests that XT22 and p75-Fc affect host susceptibility to different types of *Candida albicans* infections by different inhibitory mechanisms.

Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) plays a major role in various immune responses.<sup>1</sup> From various studies, TNF- $\alpha$  is known to play a key role in the recruitment of neutrophils to the site of infection<sup>2</sup> and also to modulate the phagocytic activity of both neutrophils and macrophages.<sup>3</sup> Also, studies with TNF- $\alpha^{-/-}$  mice show that TNF- $\alpha$  is required for the normal host defense against hematogenously disseminated candidiasis (HDC) and oropharyngeal candidiasis (OPC).<sup>4,5</sup> Thus, it is expected that the host will become significantly susceptible to various infections when TNF- $\alpha$  is neutralized by TNF- $\alpha$  antagonists. Recent studies suggest that the incidence of invasive fungal infections has significantly increased among patients who are receiving TNF- $\alpha$  antagonists. Although the risk of candidal infections in these patients is lower than the risk of other infections by other dimorphic fungi, there are several reports of candidiasis in patients on TNF- $\alpha$  antagonist therapy. *Candida* esophagitis has occurred in the patients who received infliximab for the treatment of Crohn disease,<sup>6</sup> and transplant patients who received infliximab for graft-vs.-host-disease had a significant incidence of HDC and also other non-*Candida* invasive fungal infections.<sup>7,8</sup> Disseminated candidiasis has also occurred in a patient with rheumatoid arthritis who was treated with etanercept.<sup>9</sup>

As part of the normal microbiota on mucosal surfaces, *Candida albicans* may be likely to cause serious infection in

patients who receive TNF- $\alpha$  antagonists. However, the precise effects of TNF- $\alpha$  antagonism on susceptibility to invasive *Candida* infections have not been delineated. Here, we tested the effect of murine analogs of two TNF- $\alpha$  antagonists, infliximab and etanercept, which have been clinically used to treat various autoimmune diseases. We focused on the effect of TNF- $\alpha$  antagonists in the mouse models of HDC and OPC. The therapeutic TNF- $\alpha$  antagonists chosen for this study are known to have different mechanisms of action. Infliximab is a monoclonal antibody that neutralizes TNF- $\alpha$  activity by binding with high affinity to membrane-bound TNF- $\alpha$ .<sup>10</sup> Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of p75 TNF- $\alpha$  receptor (TNFRp75) fused to the Fc portion of human IgG1.<sup>11</sup> Thus the different mechanisms of action of these two therapeutic agents may result in different outcomes in the treatment of autoimmune diseases as well as in increasing the host susceptibility to candidiasis. To mimic the effect of infliximab in murine model, the rat anti-murine TNF- $\alpha$  monoclonal antibody MP6-XT22 (XT22) was used as previously described.<sup>12</sup> To mimic the effect of etanercept, the soluble mouse 75 kDa TNF- $\alpha$  receptor fused to the Fc portion of mouse IgG1 (p75-Fc) was used.<sup>12</sup> Since XT22 and p75-Fc also demonstrate different TNF- $\alpha$  binding patterns and pharmacokinetics<sup>9</sup> similar to infliximab and etanercept action in humans,<sup>13</sup> it was expected that

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the effectiveness of XT22 and p75-Fc in the mouse models of HDC and OPC would also vary and result in different susceptibility to candidal infection.

Male BALB/c mice weighing 18–20 g (National Cancer Institute) were used in all experiments. XT22 was obtained from DNAX Research Institute (now part of Schering-Plough Biopharma) and p75-Fc was provided by Amgen, Inc.<sup>14</sup> To test the effects of TNF- $\alpha$  antagonists in HDC, the mice were divided into four groups: XT22 group treated with XT22, p75-Fc group treated with p75-Fc, a control group for XT22 receiving rat IgG (National Cell Culture Center), and another control group for p75-Fc receiving phosphate-buffered saline (PBS), which was used as the diluent for the p75-Fc protein. The XT22 group was divided into five subgroups that were injected intraperitoneally with 40, 20, 10, 5, and 2.5 mg/kg XT22 diluted in PBS. The p75-Fc group was also divided into five subgroups that were injected subcutaneously with 5, 2.5, 1.25, 0.625, and 0.375 mg/kg p75-Fc diluted in PBS. Eight mice per each subgroup were treated on days -5 and -1 relative to infection as described previously.<sup>12</sup> To initiate HDC, the mice were inoculated with  $10^5$  blastospores of *C. albicans* SC5314 in 0.5 mL saline via tail vein injection and monitored for survival for 14 d as previously described.<sup>15</sup> **Figure 1A** demonstrates that neutralization of TNF- $\alpha$  with both XT22 (top) and p75-Fc (bottom) significantly increased the susceptibility of mice to HDC ( $P < 0.05$  when compared with anti-murine IgG and PBS controls). This result suggests that both TNF- $\alpha$  antagonists significantly increased the susceptibility of mice to HDC and that the concentrations of XT22 and p75-Fc chosen for this study had a similar immune suppressive effect in this mouse model.

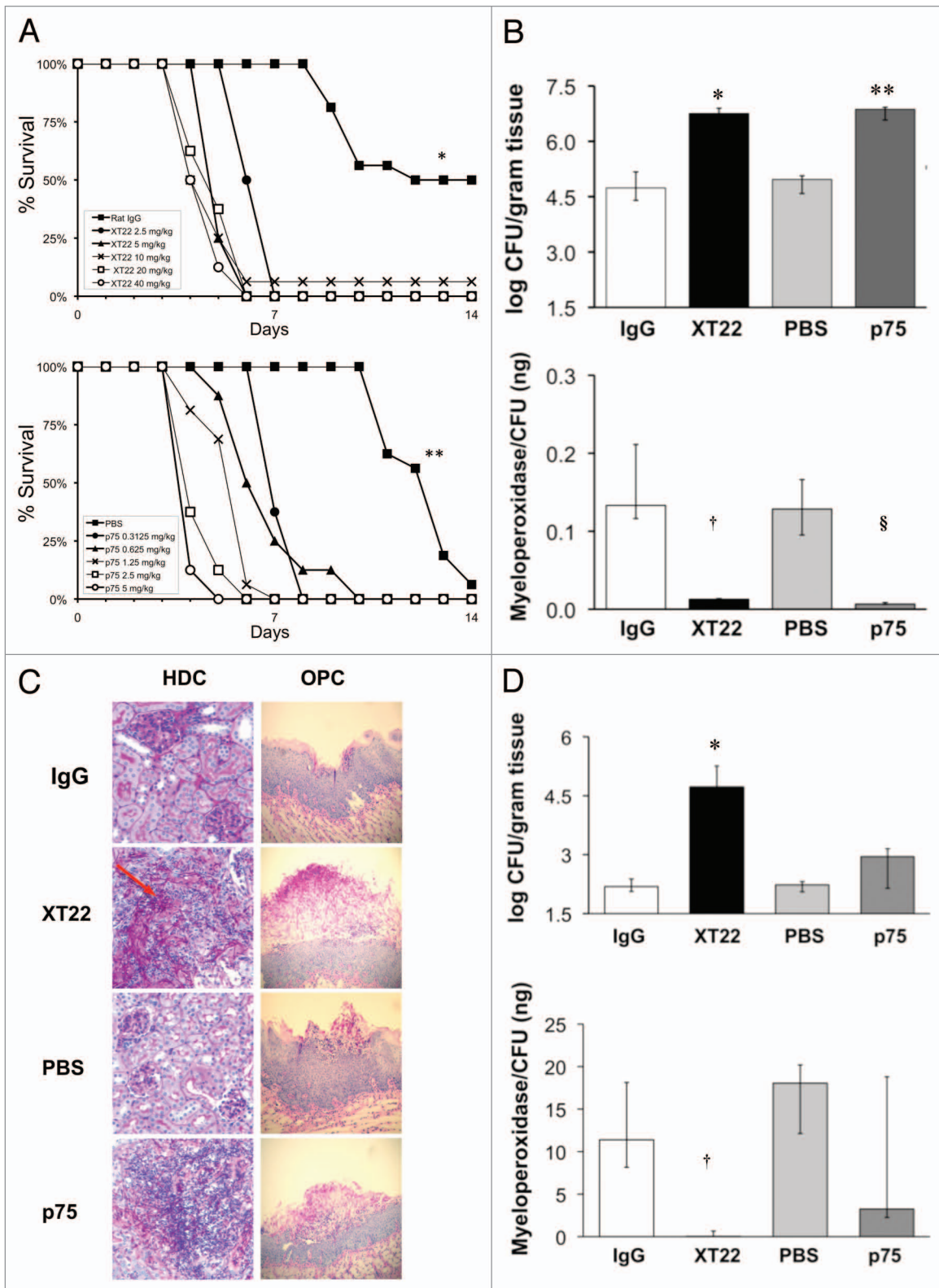
To determine the effects of the two TNF- $\alpha$  antagonists on the kidney fungal burden, an additional seven mice per subgroup were infected and treated with 10 mg/kg XT22 and 2.5 mg/kg p75-Fc, both of which demonstrated similar effects on susceptibility to HDC as shown in **Figure 1A**. The mice were sacrificed at 1 d after infection and their kidneys were quantitatively cultured as previously described.<sup>15</sup> Portions of the kidneys were fixed in formalin, followed by 70% ethanol, and stained with periodic acid schiff (PAS) for histopathological analysis. As shown in **Figure 1B** (top), the kidney fungal burden of mice treated with either XT22 or p75-Fc was significantly increased when compared with the respective control mice. The kidney fungal burden in the control mice that did not receive the TNF- $\alpha$  antagonists

was between log 4.5 to log 4.7, whereas the kidney fungal burden in mice treated with either TNF- $\alpha$  antagonists was 2 logs higher than the controls ( $P < 0.05$ ).

To measure neutrophil recruitment into the kidneys, the myeloperoxidase (MPO) level in the kidney homogenates was determined by a commercial ELISA (Hycult BT) following the manufacturer's protocol. MPO is most abundantly expressed in neutrophils and the level of MPO is used as an indirect measurement of neutrophils recruitment to the infected site as described in previous studies.<sup>16–18</sup> To measure the impact of the TNF- $\alpha$  antagonists on neutrophil influx relative to the number of invading microorganisms, we expressed the results in terms of ng MPO per CFU as previously described.<sup>19</sup> As shown in **Figure 1B** (top right), both TNF- $\alpha$  antagonists caused a significant reduction in the kidney MPO level relative to the fungal burden as compared with the untreated control mice ( $P < 0.05$ ). This result suggests that treatment with XT22 or p75-Fc reduced neutrophil recruitment into the infected kidney. The histopathology of kidneys of mice in the different treatment groups are shown in **Figure 1C**. They also demonstrate that there were significantly more lesions containing fungal hyphae (shown as red filaments) in the mice treated with either XT22 or p75-Fc. In addition, the number of neutrophils (shown as blue dots) recruited to the infection site with both TNF- $\alpha$  antagonists was notably reduced when compared with the control groups. The finding that neutrophil recruitment to the infection site was significantly diminished in mice receiving either XT22 or p75-Fc indicates that both antagonists effectively inhibited functional TNF- $\alpha$ . These results also suggest that TNF- $\alpha$  governs renal neutrophil recruitment in response to fungal infection during HDC. The relatively few neutrophils that are recruited when TNF- $\alpha$  is neutralized are incapable of preventing fungal proliferation in the kidneys, and thus the severity of HDC is increased. To confirm our finding, direct measurement of the decreased neutrophil influx in mice treated with TNF- $\alpha$  antagonists should follow in future studies using more accurate immunological assays such as flow cytometry.

We also tested the effect of TNF- $\alpha$  antagonists in the mouse model of OPC. Our previous study showed that the molecular mechanisms by which host cells interact with fungal cells are different in in vitro models of HDC vs. OPC.<sup>20</sup> Thus, we hypothesized that the two different TNF- $\alpha$  antagonists may have different effects on the host defense against OPC in mice. To

**Figure 1** (See opposite page). (A) TNF- $\alpha$  neutralization shortens the survival of mice with hematogenously disseminated candidiasis (HDC). Eight mice per different concentration of anti TNF- $\alpha$  antagonists were treated with the indicated doses of XT22 or p75-Fc at days -5 and -1, and then inoculated intravenously with  $10^5$  *C. albicans* SC5314 strain. The survival of mice treated with all doses of XT22 or p75-Fc was significantly shorter than that of the control mice (\* $P < 0.05$  by the Log Rank test). (B) Both XT22 and p75 significantly increased kidney fungal burden while decreasing leukocyte recruitment. (Top) The kidney fungal burden of mice with HDC was significantly increased by both XT22 and p75-Fc (\* and \*\*  $P < 0.05$  by Wilcoxon Rank Sum test when compared with IgG and PBS, respectively). (Bottom) Both TNF inhibitors caused a significantly reduced MPO content of the kidneys relative to the CFUs (\* and \*\*  $P < 0.05$  compared with their relative control by Wilcoxon Rank Sum test). MPO and CFU values are average and standard deviation of individual kidneys from seven mice with HDC. (C) PAS staining of the kidneys of mice with HDC (left) and the tongues of mice with OPC (right) demonstrates increased fungal burden but not increased leukocyte accumulation in mice treated with TNF inhibitors. Arrows indicate *C. albicans* hyphae and surrounding leukocytes. Images were taken 4 d after infection with the mice given 40 mg/kg XT22 and 10 mg/kg p75-Fc and representing the actual abscess found on the histopathology analysis. (D) XT22 caused a significant increase in oral fungal burden and reduced oral leukocyte accumulation in OPC. (Top) In mice with OPC, treatment with XT22 (40 mg/kg) resulted in 2.5 log greater oral fungal burden compared with control mice after 5 d of infection (\* $P = 0.004$ ). In contrast, treatment with p75-Fc (10 mg/kg) only increased oral fungal burden by 0.7 log ( $P = 0.3176$ ). (Bottom) XT22 caused significant reduction in oral MPO levels relative to the number of CFUs (\* $P = 0.004$ ). Seven mice per different group were used and all numeric values are average and standard deviation from three biological replicates.



**Figure 1.** For figure legend, see page 626.

test the effects of TNF- $\alpha$  antagonists on susceptibility to OPC, seven mice per group were given with 40 mg/kg XT22, 10 mg/kg p75-Fc, rat IgG, and PBS on days -5 and -1 relative to infection,

as those concentrations were found to have similar immune suppressive effects during HDC. The mice were also given with 50 mg/kg cortisone acetate at days -1, 1, and 3 relative to infection

to establish adequate oral infection with countable CFU in the mouse model (Park, unpublished data). All procedures to initiate OPC were followed as previously described.<sup>21</sup> After 5 d of infection, 7 mice per group were sacrificed, and their oral tissues were quantitatively cultured as previously described.<sup>21</sup> Interestingly, only XT22 caused a significant increase in oral fungal burden and also showed significant reduction in oral leukocyte recruitment relative to the oral fungal burden. As shown on **Figure 1D** (top), XT22 treatment resulted in 2.5 log greater oral fungal burden compared with the control group after 5 d of infection ( $P = 0.004$ ). In contrast, p75-Fc treatment only increased oral fungal burden by 0.7 log compared with the control group and this difference was not statistically significant ( $P = 0.3176$ ). It was also notable that there was a 1.7-log greater fungal burden in the XT22 treated group compared with the p75-Fc group ( $P = 0.002$ ). There was also a significant reduction in oral MPO levels relative to the number of CFUs in mice treated with XT22 ( $P = 0.004$ ) as shown on **Figure 1D** (bottom), suggesting that XT22 treatment significantly reduced neutrophil recruitment to the site of infection. The oral histopathology results are shown in **Figure 1C** (right). They did not clearly correspond to the oral fungal burden but did demonstrate that there were a significant number of lesions containing fungal hyphae (shown as red filaments) in mice treated with either XT-22 or p75-Fc. In addition, there was greater damage to the oral tissues in mice treated with XT22 as compared with the control groups. These results suggest that only XT22, but not p75-Fc, has significant negative impact on the host immune defense against *C. albicans* infection in the oropharyngeal cavity. Collectively, these results indicate that XT22 and p75-Fc have different effects on the immune response in the mouse model of HDC vs. OPC.

In summary, this study recapitulates the importance of TNF- $\alpha$  in protecting the host from opportunistic fungal infection. Mice treated with XT22 and p75-Fc, two TNF- $\alpha$  antagonists with different inhibitory mechanisms, become more susceptible to disseminated *C. albicans* infection. A recent study

reported a 95% correlation between organ fungal burden and neutrophil influx in mice infected with *C. albicans*.<sup>22</sup> Our data suggest that the increased fungal burden in mice treated with both TNF- $\alpha$  antagonists was due to decreased neutrophil influx into the kidney.

Interestingly, only treatment with XT22, but not p75-Fc increased host susceptibility to OPC. Plessner et al. have previously described the mechanistic difference between TNF- $\alpha$  antagonists and their roles in the mouse model of tuberculosis.<sup>19</sup> They found that systemic TNF- $\alpha$  neutralization was equivalent between the anti-TNF- $\alpha$  antibody and the receptor fusion molecule in the tuberculosis murine model, which is similar to what we have discovered in murine model of HDC with XT22 and p75-Fc. However, the receptor fusion molecule penetrated poorly into the tuberculous granulomas compared with the anti-TNF- $\alpha$  antibody.<sup>23</sup> Thus, it is possible that XT22 may increase susceptibility to OPC more than p75-Fc because XT22 may achieve higher levels in the oral mucosa and/or cause greater inhibition of TNF- $\alpha$  activity in this anatomic site. This regional difference in mechanism of action may explain the variation in the TNF- $\alpha$  agonists' effectiveness in the treatment of various autoimmune diseases and in inducing susceptibility to pathogenic agents at different sites of infection. Future studies to address the molecular mechanisms by which XT22 and p75-Fc TNF- $\alpha$  agonists increase host susceptibility to *Candida* infection should follow to clarify this finding.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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