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IL-13 attenuates early local CXCL2-dependent neutrophil recruitment for *Candida albicans* clearance during a severe murine systemic infection

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ABSTRACT

To investigate the role of IL-13 during a severe systemic *Candida albicans* infection, BALB/c control and *IL-13*$^{-/-}$ mice were examined for colony forming units (CFU) in the kidneys and survival days after intravenous infection. Proinflammatory mediators and cell recruitment into the tissue were measured by quantitative real-time PCR, a multiple ELISA system, and morphological cell differentiation. The *IL-13*$^{-/-}$ group exhibited a lower CFU number in the kidneys at 4 days and survived longer than the control mice, which was accompanied by significantly higher expression of C-X-C motif ligand 2 (CXCL2), IFN-γ, and polymorphonuclear neutrophils (PMNs) in the infected kidneys. By contrast, the expression of transforming growth factor β (TGF-β) and IL-17 A on day 10 were significantly higher in the control mice than in the *IL-13*$^{-/-}$ group. When using an intratracheal infection model, the *IL-13*$^{-/-}$ group recruited a greater number of PMNs in 6 h, with rapidly increased CXCL2 in the alveolar space. In vitro testing with cultured bone-marrow-derived cells demonstrated rapid CXCL2 mRNA upregulation at 3 h after contact with *C. albicans,* which decreased with recombinant IL-13 pretreatment, whereas rIL-13 retained TGF-β upregulation. In a murine model of *Candida* systemic infection, preexistent IL-13 limits both the rapid CXCL2 elevation and PMN aggregation in the target organ to suppress inflammatory mediators, which also attenuates local pathogen clearance within four days.

1. Introduction

Candida species are human commensal fungi on the gastrointestinal and urogenital luminal mucosae. This species causes deep septic mycosis in several organs, or mucocutaneous infection in compromised hosts bearing cancer, immunodeficiency, or during post-surgery, and in those receiving treatment at the intensive care unit (ICU) (Taube et al., 2004). Such patients are susceptible to blood stream infections (BSI) by the organism, and unlike with other fungal species, the kidney appears to be the most susceptible target organ to *C. albicans* BSI, according to several previous reports (Lionakis et al., 2012; Miyazato et al., 2009; Saijo et al., 2010). Among BSI cases in ICU patients, *C. albicans* is the third most causative pathogen, which can be a sign of a serious illness and an independent critical factor for mortality (Ostrosky-Zeichner and Pappas, 2006; Playford et al., 2008; Wisplinghoff et al., 2004; Montravers et al., 2006). Although reported cases are limited, primary respiratory infections caused by *Candida* species have been confirmed by autopsies of adult cancer patients (Haron et al., 1993), and in pediatric patients (Tan et al., 2004), which suggests that this pathogen may contribute to lower human respiratory infection. Understanding the pathophysiology of *Candida* infection is becoming increasingly important when considering immunosuppressed conditions due to the

Abbreviation: dpiv, days post intravenous infection; hpiv, hours post intravenous infection; iv, intravenous infection; hpin, hours post intranasal infection; CXCL2, C-X-C-motif ligand-2; TGF-β, transforming growth factor-beta; OD, optical density; iNOS, inducible nitric oxide synthesis; WT, wild type; BM, bone marrow; BSI, blood stream infection; ELISA, enzyme-linked immunosorbent assay; PDA, potato dextrose agar; BALF, Bronchoalveolar lavage fluids; Th1, T-helper type 1

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current advanced medical procedures and the increasing elderly population (Playford et al., 2008; Letterio et al., 2001).

In host innate immune responses to *C. albicans* infection, the number of PMN in peripheral blood cells and inflammatory mediators, such as IL-6, chemokines, IFN-γ (Zhang et al., 2016), inducible nitric oxide synthase (iNOS), and Th17 cells, are elements of host protection (Zhang et al., 2016; Yang et al., 2013; Wagener et al., 2017). Transforming growth factor (TGF)-β has biologically heterogeneous roles and is generally recognized as an anti-inflammatory mediator during acute inflammation (Letterio et al., 2001; Li and Flavell, 2008). However, in particular conditions, TGF-β likely has a role in establishing Th1 differentiation after the proinflammatory phase and in conferring resistance to the host against *Candida* infection (Spaccapelo et al., 1995). TGF-β has also been implicated in establishing the Th17 axis in the later chronic inflammatory phase than in the time when chemokines and proinflammatory mediators initially act (Campois et al., 2015). Th17 cells are an important factor in maintaining local inflammation or mucosal immune resistance via PMN supplementation to locally infected sites (Saijo et al., 2010). In addition to analogous role of chemokines and proinflammatory cytokines acting within a few hours post infection, IL-17 A is reported to potentiate later mucosal immunity against several microorganisms that include the *Candida* species (Ochs et al., 2009). In the murine infectious disease model, CXCL2, one of the chemokine superfamilies, also referred to as macrophage inflammatory protein (MIP)-2 and growth regulated protein (GRO) β, was investigated for its rapid chemotactic potential to attract PMN during both protective and pathogenic inflammation (Mizutani et al., 2014; Su et al., 2014). CXCL2 may be upregulated at the sites of host cell contact with bacterium and other microorganisms, including *C. albicans* (Yamamoto et al., 2004). Although CXCL2 has been widely investigated, the mechanism to control CXCL2 in the early phase after *C. albicans* infection is not fully understood.

IL-13 plays several pleiotropic roles including acting as a Th2 cytokine with exacerbating respiratory allergies and suppressing Th1 responses (Minty et al., 1993). Thus, inhibition of IL-13 is expected to alleviate diseases such as asthma; hence the reason for clinical trials of anti-IL-13 antibodies (Corren et al., 2011; Wenzel et al., 2007). IL-13 may also act as an inducer of TGF-β in a pulmonary fibrosis model (Lee et al., 2001) while promoting TGF-β synthesis. On the other hand, IL-13 protects against hyperoxic acute lung injury (Corne et al., 2000), severe septic shock in pediatric septic patients with high humoral levels of IL-13 that protect hosts from death (Blanco-Quiros et al., 2005), and suppresses proinflammatory cytokines in murine models injected with lipopolysaccharide (Muchamuel et al., 1997). In the last study referred to, IL-13 improved LPS-induced lethal endotoxemia only when rIL-13 was administered within 30 min after administration of LPS, but rIL-13 was not effective if it was employed after 1 h. This indicates that such Th2-type cytokine may have a critical role in protecting the host by avoiding an excessive immune reaction in the very early phase of the inflammatory stage.

With regard to the pathogenesis of infectious disease, reports of IL-13 are limited. Parasites were studied during the early 1990s, followed by echinococci (Zhang et al., 2016; Mizutani et al., 2014; McKenzie et al., 1998), and *Chlamydophila* or fungi has also been investigated. Thus, IL-13 was initially reported to potentiate the removal of nematodes, suggesting that it plays a protective role in nematode infection (McKenzie et al., 1998). In *Chlamydophila* respiratory infection models, the presence of IL-13 disturbed the elimination of *Chlamydophila* via allergic airway responses (Asquith et al., 2011). Recently IL-13 was reported as a protective factor in a bacterial pneumonia in a low dose intratracheal infection model in association with innate lymphocytes such as invariant natural killer cells and B-1B cells (Yamamoto et al., 2016). For models with *C. albicans* infection, IL-13 had a defensive role via promoting mannose receptors expressed on macrophages of the gastrointestinal tissue (Coste et al., 2008). Another protective role of IL-13 derived from $CD4^+$ T cells is studied for enhancing phagocytic function in association with IL-33-mediated M2 macrophage polarization (Tran et al., 2015). This study model resemble to the present study as treating with murine disseminated infection while we used a higher inoculum dose (8×10^5 CFU) with two different Candida strains for infection than their 1 to 3×10^5 CFU per mouse. In the present study, set of results by the relatively high-dose inoculum of *C. albicans* illustrated additional role and interpretation of IL-13. We postulated that even in response to the same microbe, by different inoculum burdens, the local molecular responses for pathogen elimination of IL-13 may act differently in association with particular inflammatory conditions. The current scope is to investigate the complemental role of IL-13 in early host resistance to the fungal pathogen with a higher dosage than reported, which may affect the acute phase of intensive local immune response to *C. albicans* blood stream infection.

2. Results

2.1. Early local inflammatory response associated with kidney fungal burden

To investigate whether IL-13 has a role in host resistance against severe *C. albicans* blood stream infection in vivo, the number of *C. albicans* CFU in the kidney, the typical target organ for this animal model (Lionakis et al., 2012; Miyazato et al., 2009; Xu et al., 2015), was examined at 4 days after intravenous infection. A few preliminary trials were conducted with mice according to gender and age, and neither factor affected kidney CFU, profiles for the production of cytokines, or their average survival duration. Inoculum was adjusted to induce a lethal dose (LD) 50 for the control BALB/c mice. Survival was censored for 12 days with body weight recorded daily. Administrations of different microbial strain components or their dosages greatly affect immunological responses on the same host (Saijo et al., 2010; Esvaran and Conway, 2016). Two independent *C. albicans*, ATCC18804 and another designated strain THK519 were employed for the testing kidney CFU by the same i.v. dose administration. At 4 days post intravenous infection (*dpiv*), the number of CFU in the *IL-13*$^{-/-}$ group was significantly lower compared to that in the control BALB/c group in the both strains (Fig. 1c, and d). In the survival experiment, in accordance with the greater kidney fungal burden, the control BALB/c mice succumbed to death from 5 to 11 *dpiv*, which was 4 days earlier than the *IL-13*$^{-/-}$ group, whose mice began to die at 9 *dpiv*. At the end of the censored period at 12 *dpiv*, 50% (8 of 16) of the *IL-13*$^{+/+}$ control group mice had died, whereas only 14% (2 of 14) of the IL-13 gene-disrupted mice did not survive. A reduction in weight may reflect early systemic inflammation caused by the infection (Fig. 1b). Although the *IL-13*$^{-/-}$ group showed greater weight loss compared to the BALB/c control group for the early 4 days, the *IL-13*$^{-/-}$ group survived for a significantly longer period than the controls and recovered their weight by 12 *dpiv* (Fig. 1b). We hypothesized that the *IL-13*$^{-/-}$ host manifested an intensive inflammatory response that conferred significant CFU reduction in the target organ and survived longer compared to the IL-13-competent BALB/c controls.

2.2. Susceptibility and inflammatory response within 4 days

When infected with certain bacterium or *Candida* species, C-X-C motif ligand2 (CXCL2) and KC are rapidly produced at the infected site, and both recruit phagocytic effector cells into the local site where pathogen exists within hours (Su et al., 2014; Yamamoto et al., 2004). IFN-γ produced by activated lymphocytes may act but usually after the chemokines. IFN-γ also enhances phagocytic killing, which in turn promotes intracellular nitrogen oxide upregulation (Campois et al., 2015; Schroppel et al., 2001), cooperating with several proinflammatory cytokines that are released by adjacent macrophages and $CD4^+$ T cells. Moreover, IFN-γ, as well as TNF-α, are also produced by the aggregated neutrophils themselves (Hatta et al., 2010). Both of

these mediator levels correlate to early PMN numbers and the degree of *C. albicans* CFU reduction at the site of the infected organs (Taube et al., 2004; Saijo et al., 2010; Spaccapelo et al., 1995). Phagocytic function of effector cells may be regulated in association with upstream monocytes and macrophages and IL-13, which is also critical for effective pathogen elimination (Wagener et al., 2017; Coste et al., 2008; Tran et al., 2015). Instead, TGF-β, IL-10, IL-4, and IL-13 are described as anti-inflammatory cytokines that may reduce rapid TNF-α and IFN-γ responses (Taube et al., 2004; Campois et al., 2015; Ochs et al., 2009). TGF-β has several biological roles, including the ability to inhibit and promote the differentiation of effector CD4 + Th cell subsets (Li and Flavell, 2008; Spaccapelo et al., 1995). The involvement of this multipotential and anti-inflammatory agent in *C. albicans* infection remains obscure (Spaccapelo et al., 1995; Campois et al., 2015).

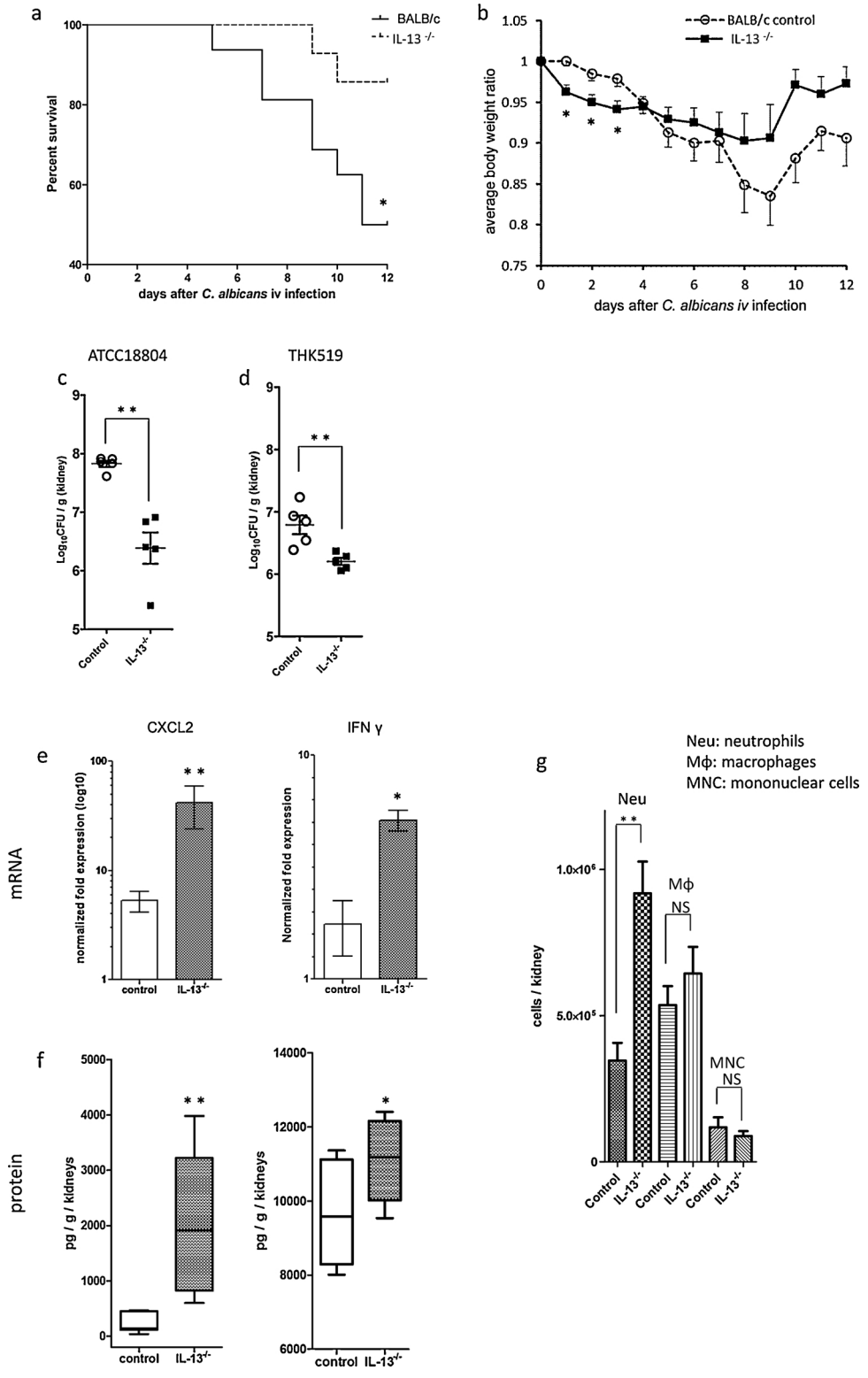

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Fig. 1. Role of IL-13 in *C. albicans* systemic infection in the early stage with inflammatory mediators.
a. Survival analysis of the BALB/c control (solid line; n = 16) and *IL-13*$^{-/-}$ groups (dotted line; n = 14) following intravenous infection with *C. albicans* with 8×10^5. The data shown is representative of two independent trials.
b. Mouse body weights were recorded daily after intravenous infection of the Fig. 1a. Average rates of each group are plotted, for the BALB/c control (open circle on solid line; n = 16), and the *IL-13*$^{-/-}$ group (closed square on dotted line; n = 14). Each raw weight was divided by the initial weight measured at day 0, the time of *iv* infection. Data after 4 *dpiv* the two average weights are not significant due to the wider variability than those within 4 days between morbid mice with decreased weights and recovered mice with gained weights.
c. and d. Kidney CFU examined at 4 *dpiv* with ATCC 18804 (c), and with THK519 (**d**) are plotted along the vertical log scale (log CFU/g). Open circle: wild type BALB/c control group (n = 5), vs closed square: *IL-13*$^{-/-}$ group (n = 4)
e. Upregulation of kidney mRNA for CXCL2 and IFN-γ at 5 *hpiv* for the control and *IL-13*$^{-/-}$ groups is shown as a bar graph with standard error. Open bar: wild type BALB/c control group (n = 5), vs gray bar: *IL-13*$^{-/-}$ group (n = 5).
f. Kidney CXCL2 and IFN-γ protein production at 4 *dpiv*, other proinflammatory agents of IL-1 β, TNF-α, KC, IL-6 (**h**), and anti-inflammatory factors of TGF-β, and IL-10 (**i**). Data are demonstrated as box-and-whisker diagrams illustrating the largest observation, upper quartile, median, lower quartile and smallest observation. Open bar: wild type BALB/c control group (n = 7), vs gray bar: *IL-13*$^{-/-}$ group (n = 7). Representative results are shown, and results for CFU, mRNA, and proteins (multiplex ELISA) were reproduced in at least two independent experiments.
g. Cell count and morphological differentiation was determined using modified Giemsa staining (Diff-Quick) for the kidney leukocytes collected at 4 *dpiv*. PMNs, macrophages, other mononuclear lymphocytes, and eosinophil numbers are expressed per group as a bar graph. Each bar shows the average cell count of the group and standard error.
(Statistical significances of *IL-13*$^{-/-}$ group vs BALB/c control; $*$ < 0.05, $**$p < 0.01. NS; no significant difference.).

To clarify the roles of IL-13 and the inflammatory mediators in the presented in vivo results, recruited PMNs in the kidney were enumerated by morphology of the infected kidney homogenates that were dissected at 4 *dpiv*. CXCL2 and IFN-γ, as well as other molecules that enhance PMN recruitment such as IL-1 β, IL-6, and TNF-α, were also measured using the multiple ELISA system for the supernatants of the homogenates. CXCL2 and IFN-γ protein concentrations and PMN number, among trafficked cellular population in the kidney, was significantly higher in the *IL-13*$^{-/-}$ host compared to the *IL-13*$^{+/+}$ controls (Fig. 1f, g). Importantly, considerable number of macrophages was observed in the aggregated cells after PMNs. The other inflammatory mediators except for IL-6, also exhibited significantly higher levels in the *IL-13*$^{-/-}$ host kidney than that in the control BALB/c mice (Fig. 1h). In both bacterial and *Candida* infections, a distinct influx of effector cells into the site of infection, and aggregation within hours is preceded (Farah et al., 2009) by the elevation of chemokines and proinflammatory proteins (Yamamoto et al., 2004). These proinflammatory mediators are reported to be upregulated in mRNA prior to protein production at the infected sites after contact with microorganisms then affect to systemic inflammatory response (Taube et al., 2004). Therefore, we quantified mRNAs at 5 h post intravenous infection (*hpiv*) in the current model to observe any early active kinetics. As shown in Fig. 1e, CXCL2 and IFN-γ mRNA were significantly more upregulated in the *IL-13*$^{-/-}$ group compared to that in the control BALB/c group. The degree of significances in upregulated mRNAs were correlated with each protein (Fig. 1f). Conversely, TGF-β protein levels were lower in the kidneys of *IL-13*$^{-/-}$ mice compared to the control group at 4 *dpiv* (Fig. 1i). Notably, IL-10 was also higher in the *IL-13*$^{-/-}$ hosts, despite its anti-inflammatory potential (Fig. 1i).

2.3. Susceptibility and inflammatory response at a later period of 10 days

Kidney CFU for later survived mice reached at 10 *dpiv*; was tested, however, there was no difference between both groups (Fig. 2b). At 10 *dpiv*, none of the examined inflammatory agents, including TNF-α, IFN-γ or IL-10, showed any difference between the control BALB/c and *IL-13*$^{-/-}$ mice except for IL-17 A (Fig. 2a, c). IL-17 A in the control group increased at 10 *dpiv*, whereas that in the *IL-13*$^{-/-}$ group did not. This observed increase in IL-17 A was significantly higher than that in the *IL-13*$^{-/-}$group (Fig. 2c).

2.4. CXCL2-dependent neutrophil numbers are critical to enhancing early C. albicans elimination

In the current systemic infection model of *C. albicans*, the presence of IL-13 resulted in an attenuation of rapid proinflammatory surges in the target organ (Fig. 1e,f,h). Among the measured inflammatory agents, CXCL2, protein and mRNA upregulation showed the most significant difference between the control and *IL-13*$^{-/-}$ hosts (Fig. 1e,f). The kinetics of such chemokine in response to microbes have also been studied in lung infection models in several related articles (Wagener et al., 2017; Asquith et al., 2011; Hatta et al., 2010). To confirm if the analogous role of IL-13 may apply to an airway-route infection model we conducted experiments using an airway route via nasal administration (*i.n.*) with the same background mouse and microorganism although *C. albicans* lung infection is not as common a disease condition as the current blood stream infection. The incidence of *Candida* CFU in BALF was significantly lower in the *IL-13*$^{-/-}$ mice compared to the control BALB/c mice at both earlier time points: 6 and 48 h post intranasal administration (*hpin)* (Fig. 3a). CXCL2 concentration at 6 *hpin* in BALF and trafficked phagocytic neutrophil count were significantly higher in the *IL-13*$^{-/-}$ mice compared to the control BALB/c mice (Fig. 3b, c). Other leukocyte fractions of BALF did not show any difference. These observations implied that preexisting IL-13 affected the attenuation of PMN influx into BALF at 6 *hpin* of *C. albicans* as demonstrated in the kidney (Fig. 1c, d, e, f and g).

2.5. Tests of IL-13 contribution to C. albicans killing and phagocytosis by cultured cells

In a resistance against *C. albicans* infection, phagocytic effector cells are macrophages and PMNs. PMN numbers were greater in *IL-13*$^{-/-}$ host kidney in the present study (Fig. 1g). Referenced studies demonstrated that IL-13 may promote phagocytic function of PMNs (Tran et al. (2015)). In the present mode, local IL-13 in the infected kidney was not elevated after *C. albicans* administration as described below in this section. Both macrophages and PMNs have a role for phagocytes, and PMN numbers were greater in the *IL-13*$^{-/-}$ host kidney, we postulated that macrophages dominate controlling PMN number as well as function at local sites. Therefore we considered that testing phagocytic activity on macrophages is more important than that on PMNs. It remains unclear whether the pre-exposure to IL-13 affects the killing activity of macrophages upon *C. albicans*. To test this, two in vitro experiments were conducted using different macrophages. One experiment included macrophage cells extracted from the peritoneal cavity (PEC) of the BALB/c control and *IL-13*$^{-/-}$ mice (Fig. 4a), and the other included J774.1 cell line macrophages (Fig. 4b). According to our preliminary measurements, naïve BALB/c mouse serum contained a considerable amount of IL-13, approximately 30 to 130 pg/ml. The amount varied regardless of the naïve state, which indicates that IL-13 may be produced under certain self-regulation (data not shown). However, similar to the findings of a previous investigation (Taube

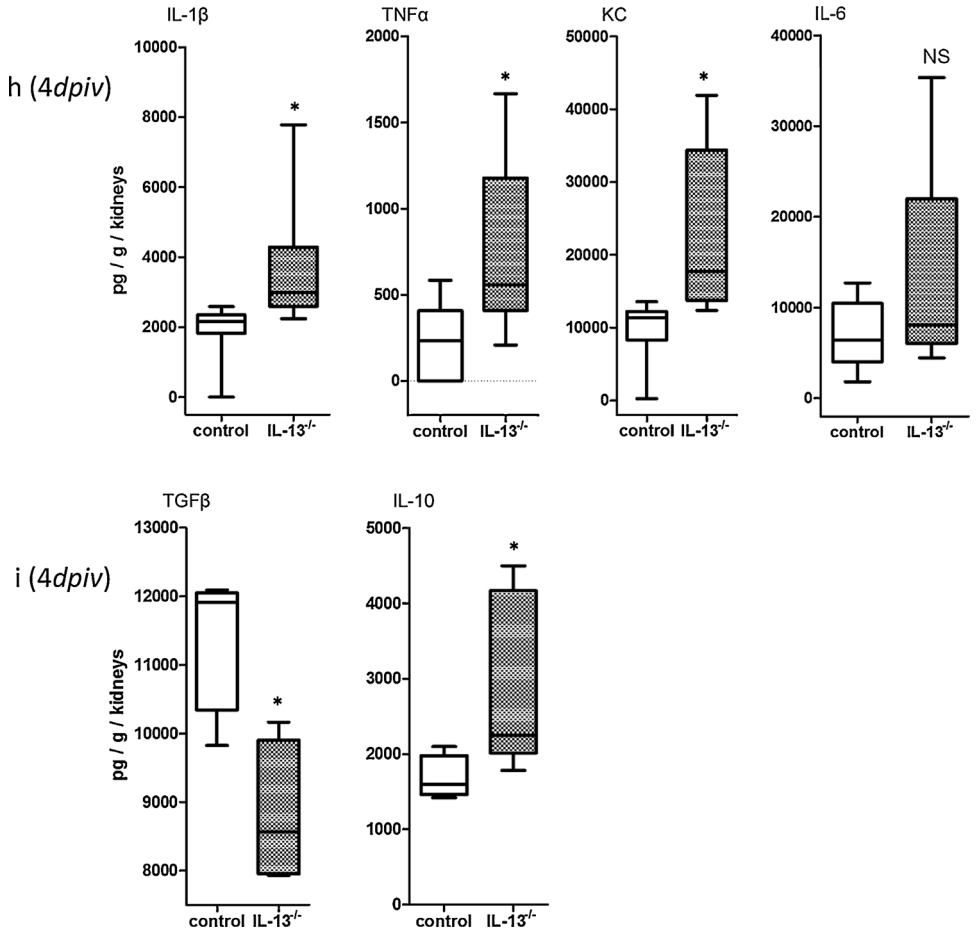

Fig. 1. (*continued*)

et al., 2004), IL-13 was not clearly elevated in either mRNA or protein in the present data derived from the infected organs after the *C. albicans* infection in both systemic methods (kidney) and methods via the airways (BALF) (data not shown). This observation indicates that, rather than post infection, preexisting IL-13 might critically affect the host response in the current in vivo model or in hypothesis, other lymphatic organs such as regional lymph nodes may produce IL-13 (Campois et al., 2015; Fallon et al., 2006; Jeon et al., 2007). Considering these preliminary observations, we tested the killing potential of extracted peritoneal lavage cells of the BALB/c control and *IL-13*$^{-/-}$ mice. As shown in Fig. 4a, no significant difference was observed in the *C. albicans* killing rate by the isolated macrophages between both groups of mice. In another disease condition, as seen in several allergy models, a considerable amount of IL-13 secretion has been reported after priming with a specific allergen like ovalbumin in the BALB/c mouse airway system (Taube et al., 2004; Mizutani et al., 2014; Jeon et al., 2007). Such conditions affect disease severity and pathogen elimination. Thus, to elucidate whether IL-13 has additional effects on standard macrophage cell line (*IL-13*$^{+/+}$) phagocytosis, another in vitro assay was performed using a J774.1 cell line (Hatsuzawa et al., 2009). In this assay, zymosan was used to clarify if a pure phagocytic mechanism was affected by a high concentration (5 ng) of additional IL-13 (Ochs et al., 2009). J774.1 cells with additional rIL-13 significantly reduced zymosan uptake rate. In summary, isolated PEC cells made no difference to the killing actions between control *IL-13*$^{+/+}$ and *IL-13*$^{-/-}$ mice (Fig. 4a). The standard macrophage cell line J774.1 revealed that a high concentration of IL-13 inhibited the pure phagocytic activity (Fig. 4b).

2.6. IL-13-dependent early cellular response using bone-marrow-derived cells

To further elucidate the early regulation of the IL-13-dependent inflammatory molecular kinetics that affect both local early CFU control and survival duration, cultured bone-marrow-derived (BM) cells of *IL-13*$^{-/-}$ (BALB/c) were examined for chemokine and cytokine responses upon contact with *C. albicans*. After preincubation with rIL-13 in physiological (40 pg to 1 ng/ml) and excessive (5 ng/ml) concentrations, mRNA of CXCL2, TGF- β, IFN-γ, and iNOS were tested using qRT-PCR since these molecules critically contribute to early host resistance via effector cell functions against *C. albicans* infection (Taube et al., 2004; Spaccapelo et al., 1995; Ochs et al., 2009; Farah et al., 2009). All Ct values of target genes were referenced to beta-actin expression. In cultured BM cells without microbes or stimulants, thus ⊿Ct of resting BM cells are exhibited as negative control (NC) culture cell conditions in. Tables 1 and Table 2. iNOS mRNA expressed at the lowest level shown as the widest delta (⊿) Ct (16.1–17.08) among four molecules. In contrast, a certain amount of TGF-β (⊿ Ct of 3.05–4.19) appeared to be expressed under naïve conditions (Table 2). IFN-γ expression showed second low levels and varied compared to other molecules as demonstrated in a wide SD range (1.35–2.12), and the CXCL2 expression level was between that of iNOS and TGF-β.

As shown in Fig. 5, the CXCL2 mRNA was rapidly upregulated approximately 40-fold compared to that of the control resting cells as early as 3 h following the initial contact with *C. albicans* cells. This rapid CXCL2 upregulation was clearly suppressed by recombinant (r) IL-13 in a concentration-dependent manner. CXCL2 upregulation at 3 h was more than 8 times higher than those at 24 h (up to 6 times compared to the NC), suggesting that this chemokine began to increase as early as

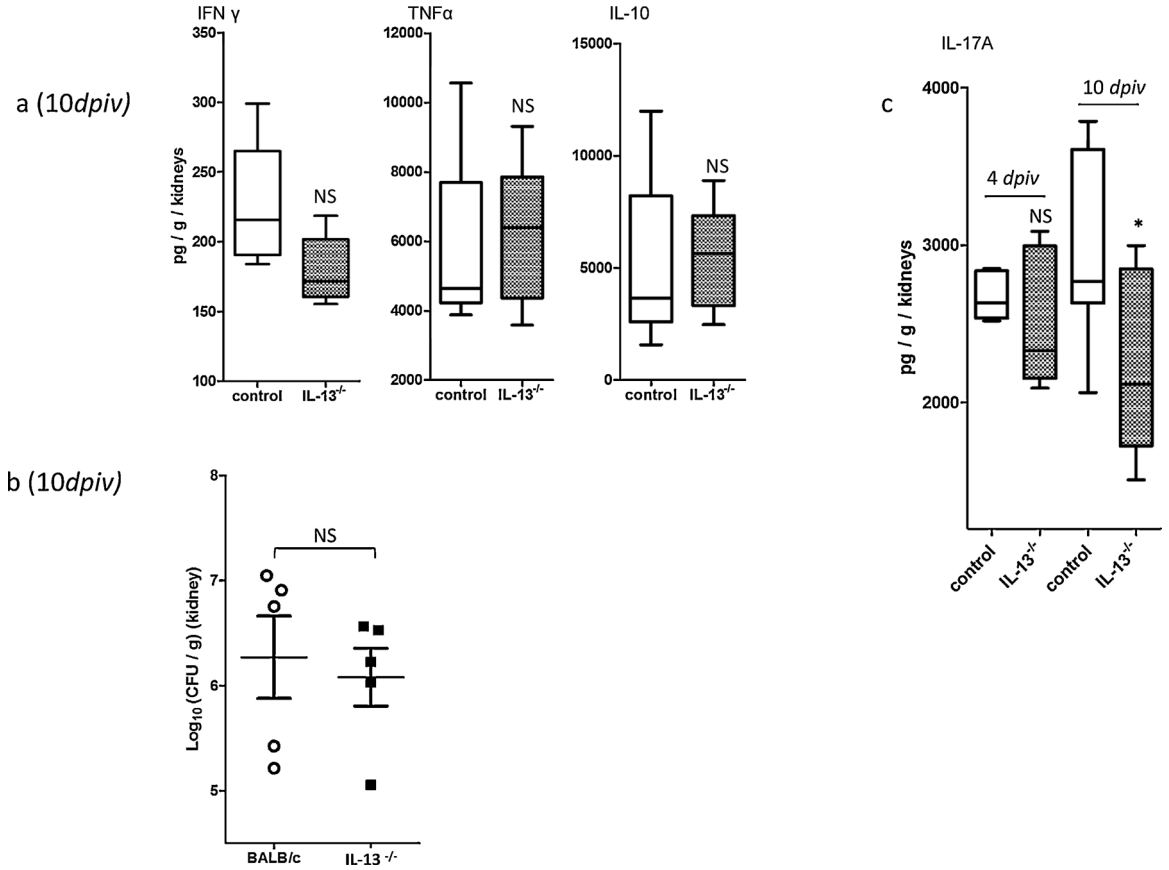

Fig. 2. Role of IL-13 at 10 days for survived mice after *C. albicans* systemic infection.
a. Of the survived mice at 10 dpiv, IFN-γ, TNF-α, and IL-10 were measured in the kidney homogenates.
b. Kidney CFU examined at 10 dpiv is shown. Open circle: wild type BALB/c control group (n = 5), vs closed square: *IL-13*$^{-/-}$ group (n = 5). All results represent three independent experiments.
c. IL-17 A at 4 and 10 dpiv are shown on the graph. Open bar: wild type BALB/c control group (n = 5), vs gray bar: *IL-13*$^{-/-}$ group (n = 5). (NS; no significant difference between control BALB/c and *IL-13*$^{-/-}$groups; * $p < 0.05$) Representative results are shown, and results for CFU, and proteins (multiplex ELISA) were reproduced in at least two independent experiments.

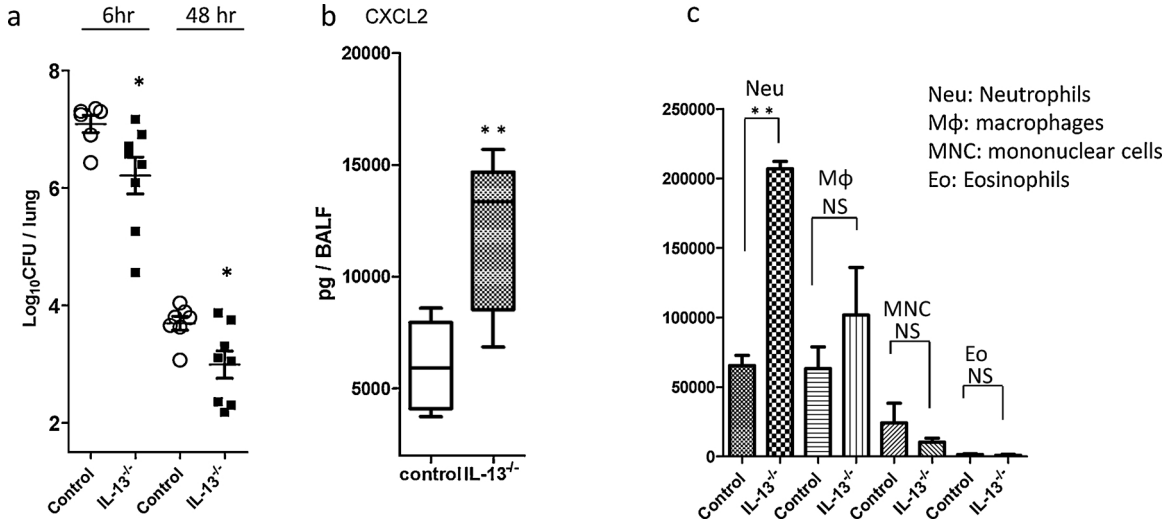

Fig. 3. Role of IL-13 with BALF analysis for phagocytes following *C. albicans* respiratory infection.
a. Six and 48 h post intranasal administration of 8×10^6 *C. albicans* CFU/mouse, BALF CFU was numerated for the control BALB/c (n = 7) and *IL-13*$^{-/-}$ group (n = 8).
b. CXCL2 concentration of BALF collected at 6 h.
c. Cell differentiation and cell count was determined using modified Giemsa staining (Diff-Quick) for the BALF collected at 6 hpin. PMNs, macrophages, other mononuclear lymphocytes, and eosinophil numbers are expressed per group as a bar graph. Each bar shows the average cell count of the group and standard error. (Statistical significances of *IL-13*$^{-/-}$ group vs BALB/c control; ∗$p < 0.05$, ∗∗$p < 0.01$. NS; no significant difference.).

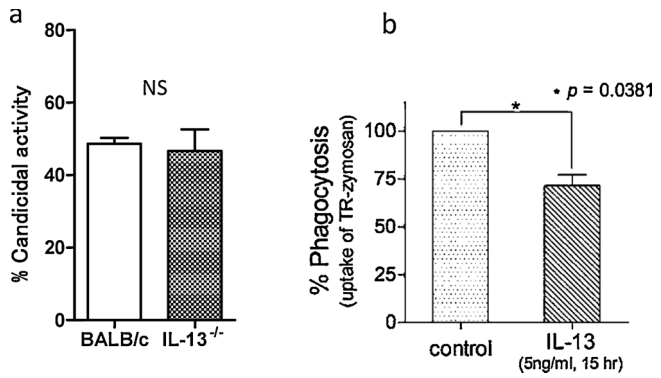

Fig. 4. *C. albicans* killing and phagocytosis assay dependent on IL-13.
a. Peritoneal lavage cells isolated from peritoneal cavity of control BALB/c and IL-13$^{-/-}$ mice were tested for the killing potential of the cells upon *C. albicans*. Each bar represents the average percentage of killed *C. albicans* by counting CFU on the PDA. The tests were reproduced three times.
b. J774.1 cell line was examined for their pure phagocytic action upon zymosan. Each bar represents the average percentage of zymosan uptake observed at 18 h after initial contact in J774.1 cells.

Table 1
Specifically-designed primers for qRT-PCR target genes, and β-actin, a reference gene. Primers used for Fig. 1e in vivo, and Fig. 5 and 6 in vitro, were originally designed using Primer 3 software.

Target		Sequence
I F Nγ	forward	AGCAAGGCGAAAAAGGATGC
	reverse	TCATTGAATGCTTGGCGCTG
CXCL2	forward	GCTGTCCCTCAACGGAAGAA
	reverse	CAGGTACGATCCAGGCTTCC
TGFβ	forward	GTCACTGGAGTTGTACGGCA
	reverse	GGGGCTGATCCCGTTGATTT
iNOS	forward	CAGCTGGGCTGTACAAACCTT
	reverse	CATTGGAAGTGAAGCGTTTCG
βactin	forward	ATGTGGATCAGCAAGCAGGA
	reverse	AAGGGTGTAAAACGCAGCTCA

Table 2
Messenger RNA expression of cultured control bone-marrow cells. Delta (⊿) threshold cycle (Ct) numbers were calculated as subtracted Ct; each target cDNA minus Ct of a common reference β-actin cDNA. Reference data shown was measured with cultured bone marrow cells incubated for 3 or 24 h without *C. albicans* and recombinant proteins.

	3 h		24 h	
	⊿C_T	SD	⊿C_T	SD
CXCL2	9.43	± 0.9	10.31	± 0.69
TGFβ	4.19	± 0.06	3.05	± 0.38
IFNγ	12.33	± 2.12	14.66	± 1.35
iNOS	17.08	± 0.49	16.1	± 0.48

3 h after contact with the pathogen compared to the other tested inflammatory agents. TGF-β was slightly upregulated at 3 h after initial contact with *C. albicans* regardless of the co-cultured rIL-13 concentration. However, this upregulation was attenuated by 24 h to 0.5 times of the NC wells without additional rIL-13, whereas 1 ng and 5 ng rIL-13 maintained the upregulation of TGF-β. TGF-β is generally recognized as an anti-inflammatory agent (Wi et al., 2012), but to be precise, it seems that TGF-β has various functions depending on its concentration and the type of cell present (Li and Flavell, 2008). Although contact with *C. albicans* upregulated the synthesis of several proinflammatory molecules, TGF-β synthesis was suppressed. This suppression was however reversed with IL-13 preincubation in a concentration gradient. No statistical significance was observed in the IFN-γ and iNOS mRNA kinetics in all media conditions, except that in the wells contacted with rIL-13 at a concentration of 5 ng /ml. In these condition iNOS mRNA was upregulated during 3–24 h, and that was explicit at 24 h, as elevating to more than 12,000 folds concentration (Fig. 5). This late iNOS upregulation among four inflammatory mediators might be reasonable as it represents an intracellular killing potential after initial phagocytosis (Wagener et al., 2017; Schroppel et al., 2001). Interestingly, 5 ng/ml rIL-13 greatly promoted iNOS mRNA levels at both 3 and 24 h, although such a strong additional rIL-13 effect was not seen in other present molecules. The kinetics of CXCL2 at 3 h and TGF-β at 24 h demonstrated a clear reversal in response to the pathogen contact along with the additional rIL-13 concentration gradients. IFN-γ mRNA was also upregulated moderately of less than 4 times of the NC at 3 h then slightly decreased to basal level at 24 h, without depending on the presence or absence of IL-13. LPS was added as a positive control independent of the other wells to which the pathogen had been in contact with. TGF-β was slightly downregulated in response to LPS at both 3 and 24 h.

2.7. TGF-β-dependent early mediators upregulated in bone marrow-derived cells

Based on the results shown in Fig. 5, we further attempted to observe the synergistic effects of rIL-13 and rTGF on BM cells (Fig. 6). The number of BM cells per well and multiplicity of infection (MOI) were identical (1:1) to those used in the experiment shown in Fig. 5; rTGF only and rTGF plus rIL-13 were added before contact with *C. albicans*. A single treatment with 5 ng rTGF enhanced CXCL2 upregulation at 24 h. Another single treatment with 1 ng rTGF maintained TGF mRNA upregulation compared to the wells without any recombinant proteins. Preincubation with rTGF plus rIL-13 resulted in TGF being slightly retained at both 3 and 24 h, IFN-γ enhancement at 3 h, and remarkably enhanced iNOS upregulation at 24 h.

2.8. Summary of IL-13-dependent early attenuation of CXCL2 and inflammatory cascades after C. albicans systemic infection

The pre-existing IL-13 critically attenuated CXCL2 upregulation in the infected kidneys in vivo and on BM cells in vitro, which may attenuate PMN recruitment in early host resistance against *C. albicans*. However, such IL-13 maintained TGF-β expression and upregulation both in vivo and in vitro meanwhile IL-17 A acted at equivalent levels between wild type and *IL-13*$^{-/-}$(Fig. 2c) by 4*dpiv*. According to previous studies and the presented results in vivo, TGF-β retained by IL-13 likely promotes IL-17 A upregulation, which may be important for *C. albicans'* elimination for maintaining necessary neutrophil trafficking at the *C. albicans* infected site (Fig. 7). As demonstrated in Fig. 1a,c and d, the high kidney CFU numbers at 4 *dpiv* of the control BALB/c mice were consistent with the early mortality of *C. albicans*-infected mice (Fig. 1a). This was likely associated with the attenuation of local early proinflammatory responses of CXCL2, IFN-γ, TNF-α, and PMN number (Fig. 1e, f, g, h). Instead, increased IL-17 A of kidneys in the control group at 10 *dpiv* might contribute to maintaining necessary inflammation with the help of IL-13 that may support local later resistance after 4*dpiv* against persistent *C. albicans* (Fig. 2b, c, Fig. 7).

3. Discussion

Rapid effector cell recruitment is highly involved in mammalian early innate response to microorganisms that may be beneficial in early host protection against various infectious diseases. Neutropenia renders the host susceptible to infection from several types of bacterium and fungi, such as *Candida* and *Aspergillus* spp. However, excessive PMN aggregation causes tissue injury, and PMN response could become a detrimental inflammatory loop during chronic autoimmune conditions as well as certain cases of allergic airway diseases (Mizutani et al.,

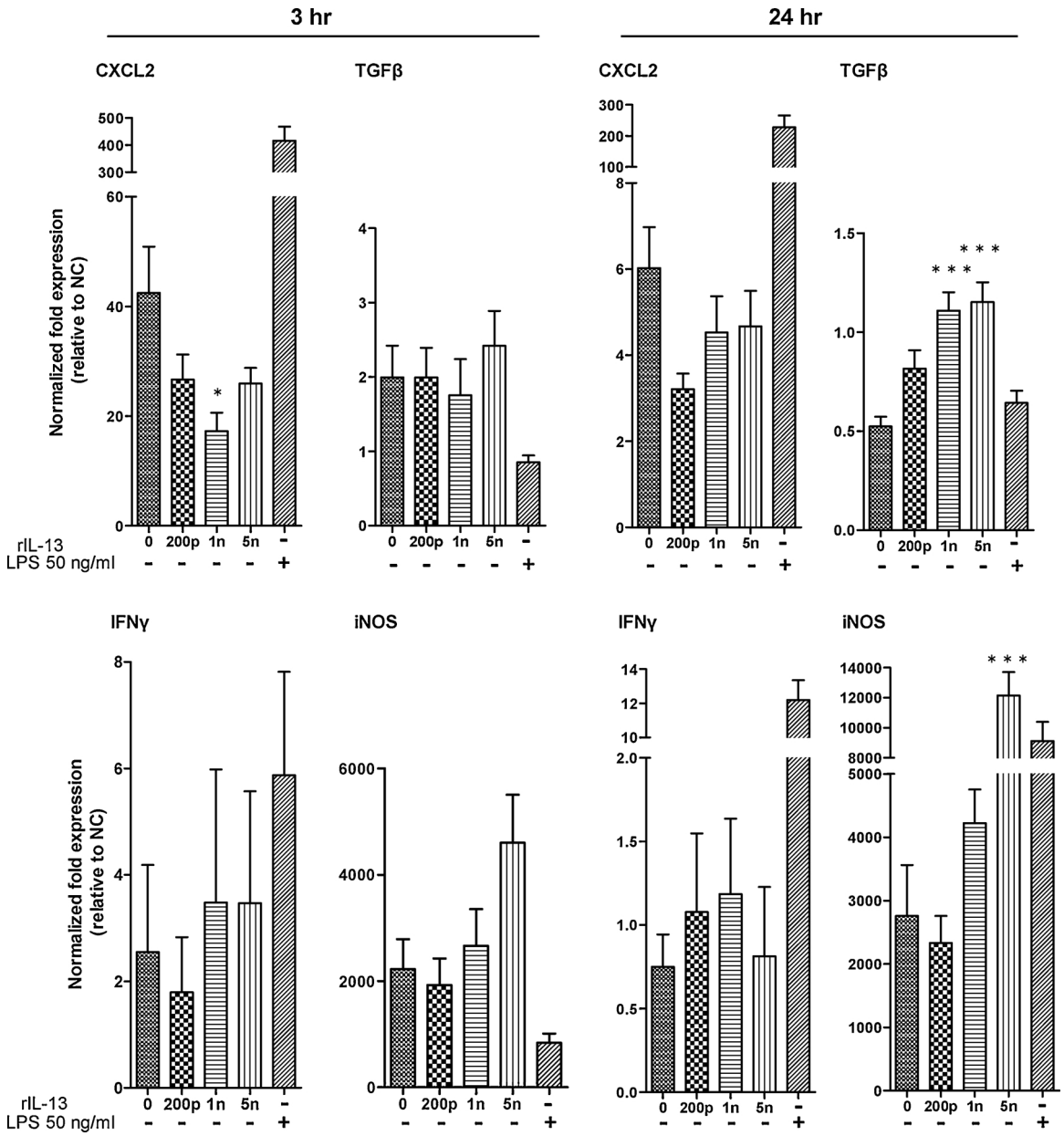

Fig. 5. IL-13-dependent regulation of inflammatory mediators in cultured bone-marrow cells following contact with *C. albicans.* Cultured BM cells were incubated at 6×10^5 cell / 600μl / well. Pre-incubation was performed with recombinant (r)IL-13 at each gradient concentration, and was allowed to stand for 24 h before contact with live *C. albicans* cells (MOI: 1:1). All incubating well conditions were studied in quadruplicate. At 3 h after contact with *C. albicans*, 200 μl medium (one-third of a well) was isolated, after gentle mixing of the well content for testing 3 h responses by ELISA and qRT-PCR. The remaining cells/medium were incubated until 24 h, then harvested and measured in the same procedure as the 3 h samples. In all in vitro experiments shown in Fig. 5 and 6, beta-actin was used as a house-keeping reference molecule. In cultured BM cells without microbes or stimulants, thus ⊿Ct of resting BM cells are exhibited as negative control (NC) culture cell conditions in Table 2. (∗ < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001).

2014; Jeon et al., 2007; De Paiva et al., 2011). Regarding clinical issues, *Candida* BSI is a major septic condition, in which the prognosis also depends on the efficacy of pathogen elimination and the complex balance of the host inflammatory response. Sepsis caused by microorganisms critically stimulates the host immune system, and may alter host responses and affect disease severity in both the emergency department and ICU (Lionakis et al., 2012; Blanco-Quiros et al., 2005).

Local contact with the pathogen in the infected organ triggers proinflammatory responses. IFN-γ, TNF-α, and CXCL2 chemokine then enhance the phagocyte-killing potential of microbes such as gram-positive and negative bacteria and *C. albicans*. Whereas, Th2 immune systems with anti-inflammatory cytokines, such as IL-10, IL-13, and TGF-β, are postulated to limit such inflammation. However the infectious condition with moderate dose against *C. albicans*, IL-13 may act on M2 macrophages to promote pathogen clearance (Tran et al., 2015). In the present study, under condition of relatively high-dose iv model, early upregulation of CXCL2 and Th1-related elements (Fig. 1) enhanced *Candida* elimination by promoting phagocyte cell aggregation; however, this rapid response was attenuated in an IL-13-competent group. The physiological concentration of IL-13 inhibited the rapid upregulation of CXCL2 in the BM cell assay in vitro (Fig. 5). Furthermore, a high concentration of IL-13 attenuated phagocytosis with zymosan, which was chosen for observation of the pure phagocytic function as an alternative to *C. albicans* in a cell-line culture (Fig. 4b). Generally, an intensive Th1 immune response likely occurs in certain bacterial or viral infections rather than fungi, and produces excessive

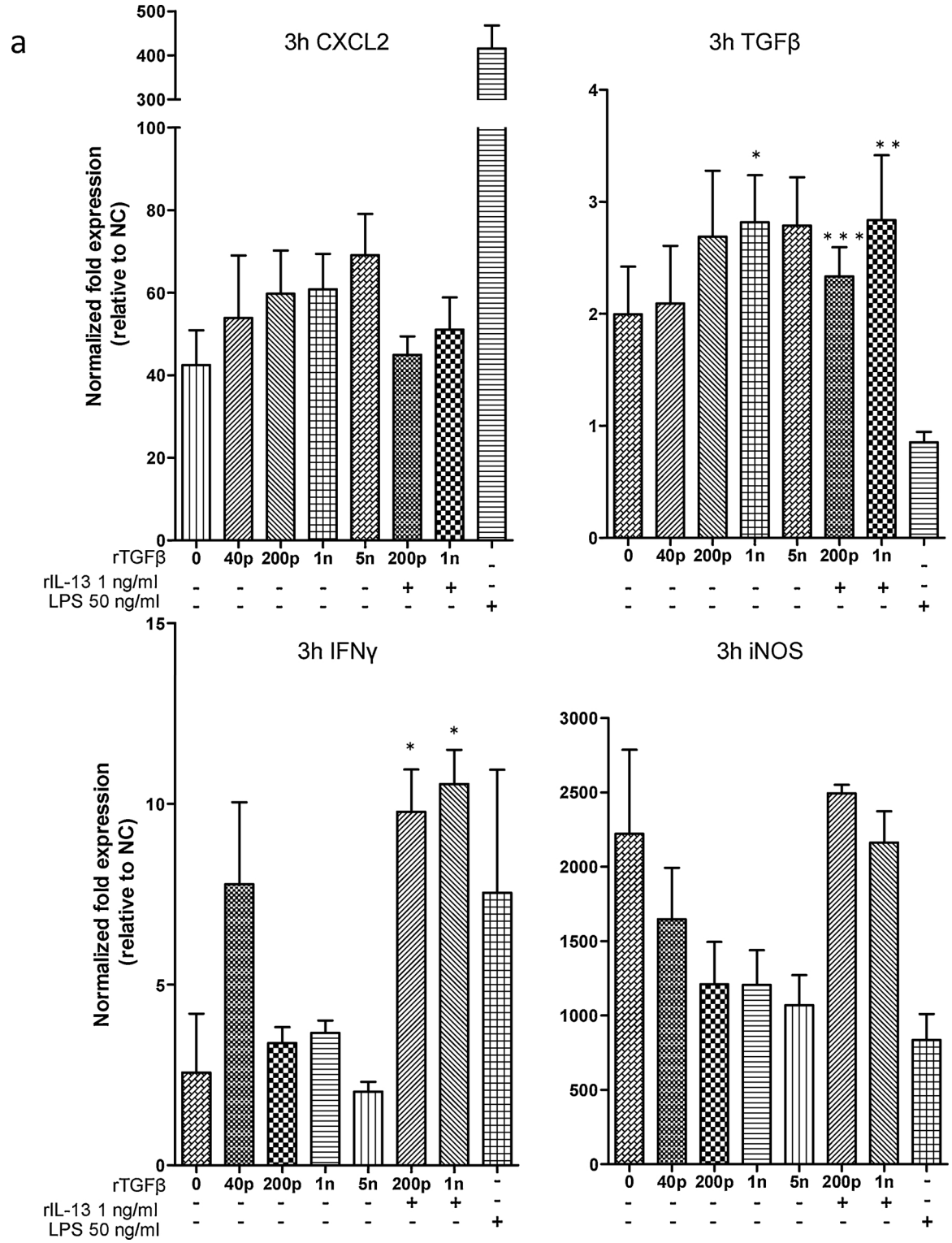

Fig. 6. Additive effects of TGF-β and IL-13 upon regulation of inflammatory molecules in cultured bone-marrow cells in contact with *C. albicans*. Cultured BM cells were incubated at 6×10^5 cell / 600μl / well. The response of the cells corresponding to each concentration gradient was measured by combining a mixture of rIL-13 single agent, rTGF single agent, and both for 24 h before contact with live *C. albicans* cells (MOI: 1:1). Other procedures were the same as those described in Fig 5. The responses at 3 h and 24 h were shown in **a.** and **b.**, respectively. (∗ < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001).

proinflammatory cytokines that cause hemodynamic instability, coagulation disorders, organ dysfunction, and septic shock, which is often associated with hemophagocytic syndrome, and can be detrimental or fatal to the host (Lionakis et al., 2012). The model in the current study also showed a significantly greater reduction in weight of the *IL-13*$^{-/-}$ host than in the weight of the control group during early days (Fig. 1b), most likely due to the inflammatory surge (Fig. 1e,f,h); however, the reduced weight was less than 10%. The general condition of the survived mice was improved after pathogen clearance. On the other hand, BALB/c control mice who succumbed to death also lost weight after 4

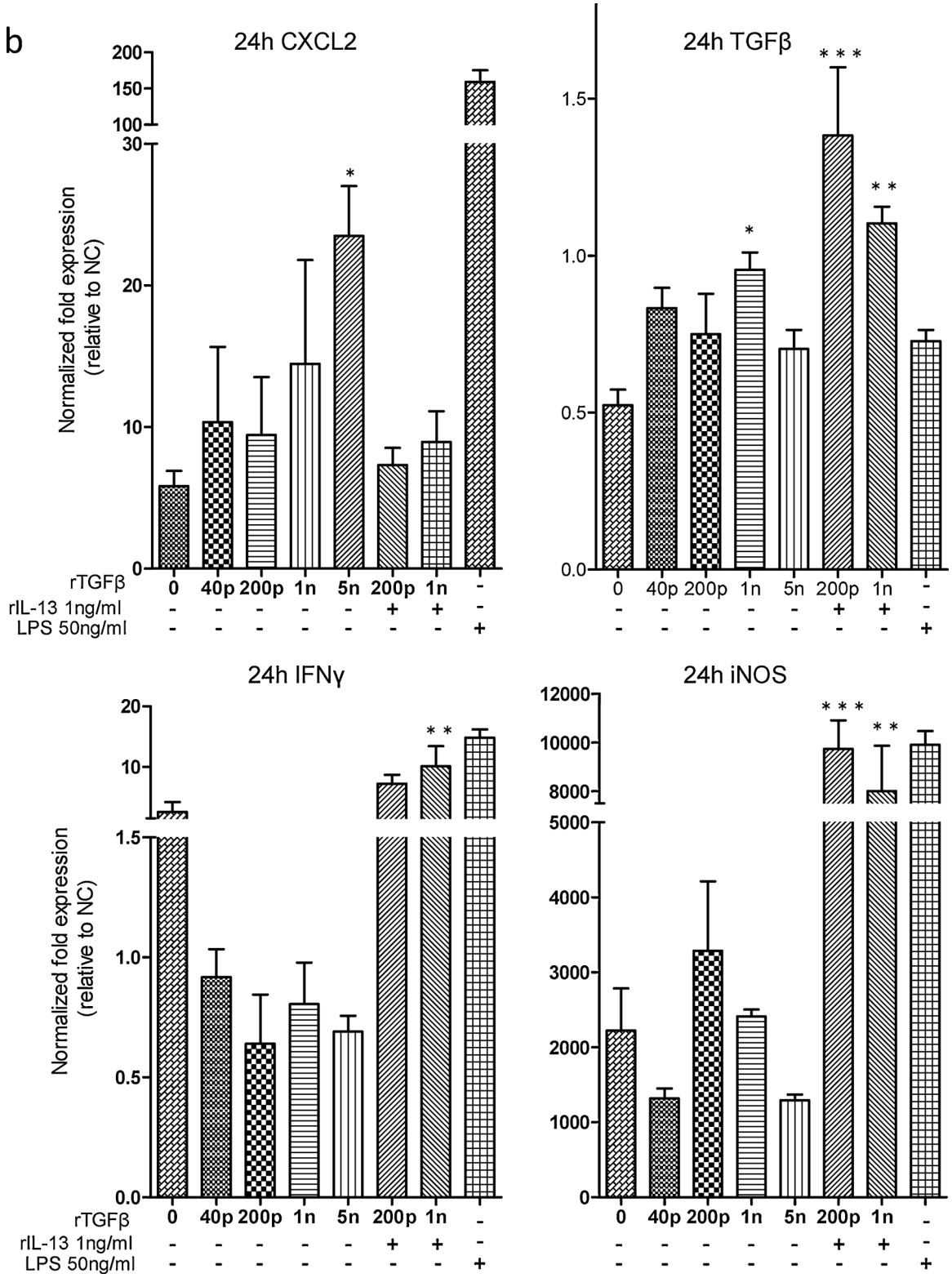

Fig. 6. (*continued*)

dpiv (Fig. 1b), which may be due to a pathogen load and persistent inflammation in the kidney (Fig. 2b). This finding implies that early promoted inflammation is beneficial to host recovery only when the response is precisely controlled. This milder reduction of weight in the early phase may be a feature of *C. albicans* infection, since the infectious diseases caused by other pathogenic bacterium may aggravate a higher inflammatory response and cause severe reduction in body weight than that observed in the present study.

An immune system with an excessive amount of Th2-arm causes immunoparalysis, and a compromised state can be induced, resulting in prolonged clinical symptoms, which leave the host unable to eliminate causative pathogens (Su et al., 2014). Although our *C. albicans* BSI did not induce apparent IL-13 production at least in the target organs, an allergic condition that causes excessive IL-13 production may induce

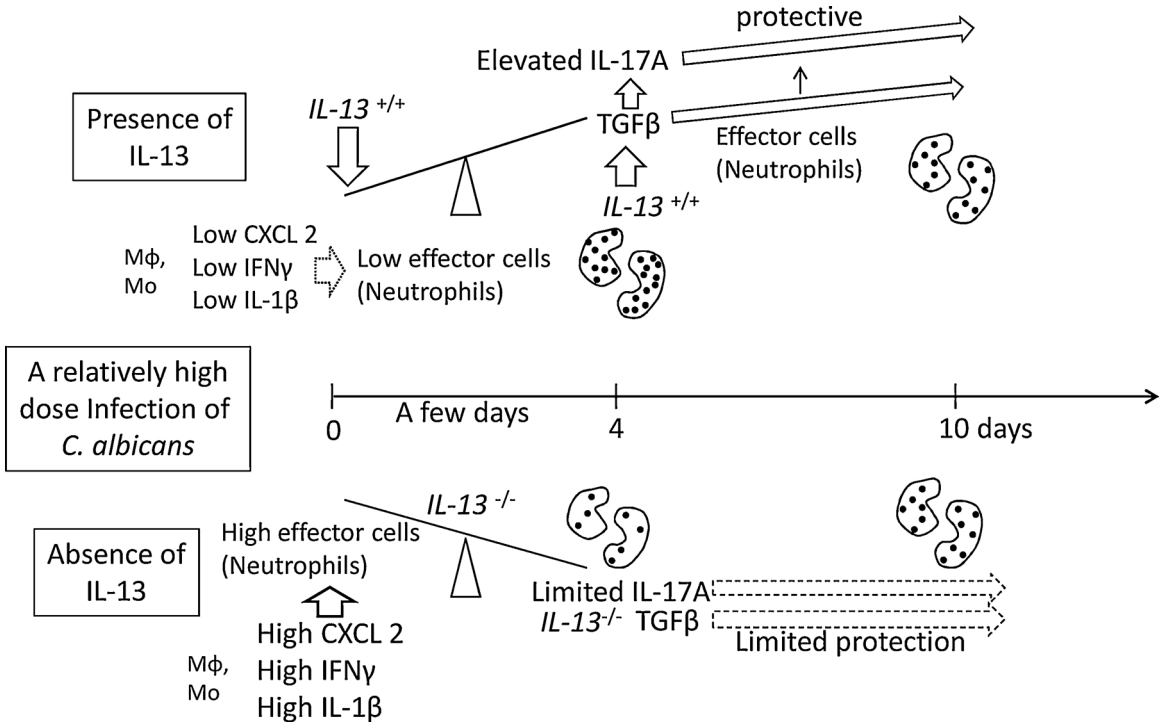

Fig. 7. Schematic host response dependent on IL-13 against *C. albicans* systemic infection. Host response to *C. albicans* systemic infection is illustrated in the schematic Fig. Due to preexisting IL-13, early upregulation of CXCL2, other proinflammatory agents, and the recruited number of phagocytic cells are less than in the *IL-13*$^{-/-}$ kidney. Furthermore, local CFU burden was greater in the control BALB/c group compared with the *IL-13*$^{-/-}$ group at 4*dpiv* when a high amount of *C. albicans* was infected *iv*. In later period than 4 *dpiv* instead in control BALB/c, IL-13-dependent TGF-β retains local IL-17 A production along with persistent *C. albicans* elimination to comparable level of *IL-13*$^{-/-}$ kidney.

unfavorable inflammation (Lionakis et al., 2012; Mizutani et al., 2014). In our in vitro results, attenuated phagocyte action in the J774.1 cell line pre-incubated with a high concentration of IL-13 (Fig. 4b) and synergistic enhancements of iNOS and IFN-γ by additive rIL-13 and rTGF (Fig. 6) may reflect that an excessive IL-13 hampers pathogen clearance. In a Th2-polarized state, prolonged inflammation and secondary infections can also lead to multiple organ failure (Leentjens et al., 2013; Puccetti et al., 1994); thus, Th1/Th2 balance is important in a clinical course of a disease. Results obtained from a system using BM cultured cells contacted with *C. albicans* also showed that IL-13 appeared to suppress CXCL2 production in a concentration-dependent manner within the physiological concentration range. IL-13 at high concentrations, on the contrary, is an excessive stimulus of many inflammatory molecules and seems to hinder the immediate exclusion of bacteria (Fig. 4,5).

It is reported that microorganism-specific component substances (chemical constituents) have the potential to shift immunomodulation toward Th2 or to suppress early Th1-related pro-inflammation. For instance, the molecular size of the chitin, a component of *C. albicans* cell structure, critically affects the polarization of host inflammatory cells that drive Th1, Th2, and Th17 cells (Wagener et al., 2017; Kogiso et al., 2011). Different strains of *Candida* spp. also stimulate heterogeneous carbohydrate receptor responses (Saijo et al., 2010). Albert et al. collected the blood of non-immunocompromised adult patients with ventilator-associated pneumonia (VAP). After the blood was stimulated by lipopolysaccharides (LPS), TNF-α in the blood was measured. They reported that the TNF-α response to LPS was lower in patients with *C. albicans* isolated of their respiratory tract specimen compared to patients whose respiratory specimens were free of *Candida* spp (Albert et al., 2014). This finding indicated that *Candida* colonization may alter the host immune condition, perhaps involving cascades via IL-13 and TGF-β to suppress proinflammatory responses, which appears to be analogous to the current results. How TGF-β acts in the etiology of various types of infectious diseases however remains unclear (Li and Flavell, 2008; Kaviratne et al., 2004).

With regard to analogous Th2 cytokine function of IL-13 against *Candida* infections, inhibition of macrophage phagocytosis by IL-4 has been reported (Varin et al., 2010). In the study, mice treated with murine-soluble IL-4 receptor cleared *C. albicans* from their kidneys. Moreover, proinflammatory cytokines were higher than those in the control groups (Puccetti et al., 1994). These results suggest that IL-4 also obstructs the clearance of *Candida* in a similar mechanism to the present results and those by other studies (Asquith et al., 2011). However, another study reported that IL-4 is required for the development of protective $CD4^+$ Th1 lymphocytes (Mencacci et al., 1998). Contrary to this, Dectin-1 expression by IL-13 involves the PPAR-gamma signaling pathway that promotes protection against this pathogen (Gales et al., 2010), which is logically inconsistent when we postulate that both types of Th2 cytokines, IL-4 and IL-13, may reduce host resistance to this pathogen. The differences in these results could be explained by the different *C. albicans* strains, hence their different carbohydrate structures, the amount (CFU) of infected *C. albicans* doses, mouse background, timing of observation after infection, or other unknown experimental conditions (Saijo et al., 2010). Although our present report did not reach the reason how host manifest opposite outcome by different dosages, our exhibited results were reproducible when equal to or higher than 8×10^5 *C. albicans* were loaded intravenously. Also our set of study did not examine direct phagocytic function of PMN with presence or absence of IL-13 since local IL-13 production was not elevated in the infected kidney.

In a model of primary bacterial pneumonia, host resistance measured by early lung CFU exhibited a distinct contrast between IL-4 and IL-13 single cytokine disrupted mice, respectively, in that the *IL-13*$^{-/-}$ mice only had a distinct defective protection to *S. pneumoniae* (Yamamoto et al., 2016). McKenzie et al. reported a difference in the excludability of parasitic gastrointestinal nematodes between IL-4 and

IL-13 disrupted mice, which provides further evidence for differentiated roles of IL-4 and IL-13 in infectious disease models, despite sharing the same receptor. They demonstrated that IL-13 had a unique potential to eradicate nematodes and stimulate more cytokines than IL-4 (McKenzie, 2000). IL-10, another anti-inflammatory Th2 cytokine was present in the kidneys, and was higher than 2000 pg/ml on 4 and 10 *dpiv* in both groups. This result further shows that IL-10 is involved in the balance between host and local inflammatory responses to *C. albicans*.

Although both IL-13 and TGF-β are recognized as anti-inflammatory cytokines to suppress acute inflammation, both act more heterogeneously in complex inflammatory cascades that are triggered by different types of microbes and host inflammatory mediators. Host responses also depend on various conditions. As seen in clinical reports, overproduction of IL-13 may abrogate airway inflammation or trigger anaphylactic shock in some disease conditions, and TGF may contribute to several interstitial inflammatory diseases (Letterio et al., 2001; Lee et al., 2001). Preceding local and systemic combinations of humoral mediators in naïve condition, such as IL-13 and TGF, affect inflammatory cascades in different phases on the infectious disease course (Li and Flavell, 2008; Muchamuel et al., 1997; Kaviratne et al., 2004). Generally, many microbial infections promote Th1 responses in infected organs, and Th2 and ant-inflammatory responses are likely suppressed in the acute phase. However, it is not clear how the Th2 response behind this acute phase affects the later individual course. Overall, local infection makes the host more proinflammatory, Th1-shifted than in their naïve condition, and promote to suppress anti-inflammatory agents (Taube et al., 2004). This may explain why IL-13 is barely increased in the infected kidney itself; although a certain amount of IL-13 exits in naïve mouse circulation. Very early circulating IL-13 that is deeply involved in the development and prognosis of subsequent systemic inflammation has been demonstrated in the shock model using LPS (Muchamuel et al., 1997).

As shown in Fig. 1, in the initial phase during *C. albicans* BSI, higher levels of CXCL2 and IFN-γ were observed in the *IL-13*$^{-/-}$ host compared with the control BALB/c. We believe that this was due to preexisting circulating IL-13, which may suppress an excessive inflammatory surge of CXCL2. The results indicate that such preexisting, but non-excessive, concentrations of IL-13 suppressed rapid chemokine upregulation, which in turn attenuated local *C. albicans* clearance in the kidney. This affected the early mortality of the control BALB/c group, but only when high amounts of *C. albicans* were loaded. IL-13 contributed to the suppression of cytokines and chemokines at 5 h in vivo (Fig. 1e) and 3 h in vitro (Fig. 5), which may primarily affect the scale of early effector cell recruitment. Without IL-13, the proinflammatory elements stimulate more PMN migration activity and inflammatory response to eliminate *C. albicans*. A similar mechanism was reported in a *Chlamydophila* infection model (Asquith et al., 2011); however, in this and other studies, no distinct analysis has been performed on the phases for proinflammatory agents in association with Th2-type cytokine (Campois et al., 2015). Increased kidney CXCL2 mRNA expression 5 *hpiv* in vivo after bloodstream infection, 3 h after contact with the fungi in BM cells in vitro (Fig. 5), and the inhibitory effect on CXCL2 chemokine by IL-13 have not yet been reported.

TGF-β has heterogeneous biological activities in metabolism, the endocrine system, and fibrotic tissue pathology, and is strictly regulated for homeostasis (Li and Flavell, 2008). In response to infectious diseases, TGF-β is released, and upregulated in the presented in vivo, and in vitro results. Other invasive *Candida* infection model demonstrated that, TGF-β has a protective immunogenicity (Letterio et al., 2001; Spaccapelo et al., 1995). TGF-β is also reported to have an independent function of Th17 cell establishment (Yang et al., 2013; Wagener et al., 2017) that may confer a more promising protective potential to hosts, especially when secondary infection occurs (Spaccapelo et al., 1995). TGF-β concentrations in the kidneys at 4 *dpiv* were significantly higher in the WT group compared to the *IL-13*$^{-/-}$ mice (Fig. 1i). TGF-β is known to promote the growth of fibroblasts, regulate T cell development, and attenuate phagocyte potential. The development of response to infectious disease has previously been examined in T cell-specific TGF-β receptor gene-disrupted mice (Li and Flavell, 2008). This cytokine was reported to suppress local phagocytic action; however, the response was concentration-dependent. Thus, TGF-β is associated with inflammation and inhibition of local phagocytes, as well as a pathogen-killing action. In a *C. albicans* BSI model, the median survival time of the mice intravenously injected with recombinant TGF-β was longer than that of the controls (Spaccapelo et al., 1995).

The positive links between IL-13 and TGF-β have been studied in bronchial asthma and pulmonary fibrosis (Lee et al., 2001). IL-13 induced subepithelial fibrosis of bronchial asthma by stimulating TGF-β (Jeon et al., 2007; Izuhara et al., 2006). In *Schistosoma mansoni* infection, expression of TGF-β mRNA did not differ between the *IL-13*$^{-/-}$ and wild type mice (Kaviratne et al., 2004), indicating that IL-13-dependent TGF-β has a specific role and association with the early production of TGF-β in *C. albicans* BSI.

To date, therapies for sepsis, which suppress a single target cytokine, have not been effective due to the complex networks of several mediators and biomarkers, including cytokines and chemokines. For instance, the administration of polyclonal anti-TNF-α antibodies in adult patients with severe sepsis, with or without septic shock, resulted in a decreased TNF-α serum concentration. However, the treatment yielded no clinical significance in mortality or serum IL-6 concentrations when compared with the placebo group (Bernard et al., 2014). Currently, cytokine removal, including immunonutrition or blood purification, has been implemented on a trial basis for cytokine adjustment in patients with sepsis. However, these therapies are not recommended in the 2012 Surviving Sepsis Campaign guidelines (Dellinger et al., 2013). Although the function of cytokines in patients with sepsis affects prognosis (Muchamuel et al., 1997), effective cytokine adjustment methods have yet to be established.

4. Conclusions

In the current study, the set of in vivo results of the survival course, kidney CFU, multiple ELISA assays in early phase post *C. albicans* BSI and in vitro BM cell assays with key cytokine treatments, demonstrated that IL-13 contributes to the suppression of early active CXCL2 after intravenous infection in vivo or BM cell contact with *C. albicans* in vitro, respectively. The lower number of CFU in the *IL-13*$^{-/-}$ group may reflect the early higher production of CXCL2 and proinflammatory cytokines than those in the WT group. This suppressed CXCL2 during the early phase may also affect the later persistency of CFU. IL-13 suppresses early CXCL2 production, but supports TGF-β synthesis from an early moment by 10 *dpiv* in the background. Supported TGF-β promotes later IL-17 A production. Therefore, the presence of IL-13 may still be important as the resistance against *Candida* by promoting IL-17 A in the later period of the systemic infection. Although M2 macrophage and IL-13 may promote effector cell phagocytosis (Tran et al., 2015) with moderate amount of administration, under conditions where a large number of *Candida* was administered, local accumulation of neutrophils via early CXCL2 upregulation is thought to predominantly affect prognosis according to the present results. The presented results are illustrating the early phase, and likely conflicting with IL-13-dependent host responses, between the early period with proinflammation and the late period by TGF-β and IL-17 A (Fig. 7), which may alter the clinical outcome during intensive therapy in the context of other underlying critical disease conditions.

5. Materials and methods

5.1. Mice

Wild-type (WT) BALB/c mice were obtained from CLEA Japan

(Tokyo, Japan). *IL-13*$^{-/-}$ mice originally generated at A.N. McKenzie, (Medical Research Council Laboratory of Molecular Biology, Cambridge University Biomedical Campus, Cambridge, UK), were provided to the animal center of our institute by Toshinori Nakayama, (Department of Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan), and bred in-house under a specific pathogen-free environment. These gene-disrupted mice compared with BALB/c controls were bred onto the BALB/c background for > 10 generations. The mice, matched for sex and age for 8 to 12 weeks, were used for a set of in vivo experiments. All the present animal studies were performed according to the guideline and approved by the animal research committee at Fukushima Medical University (Authorization number 26049).

5.2. Model of blood stream infection with Candida albicans

Candida albicans (ATCC18804, and THK519 (Saijo et al., 2010)), which had been cryopreserved at −70 °C, was cultivated at 30 °C in 5% CO_2 on potato dextrose agar (pearlcore® EIKEN CHEMICAL, Tochigi, Japan). After two days of incubation, live *C. albicans* was washed with sterile phosphate-buffered saline (PBS) twice, then re-suspended and adjusted to 8×10^6/ml with PBS. One hundred μl of the suspension was intravenously injected via the retro-orbital sinus vein after light anesthesia with isoflurane inhalation.

5.3. Cell differentiation and colony forming units in the kidney

After dissection, both kidneys were homogenized using a stainless mesh with 3 ml of chilled PBS. Subsequently, 100 μl of the homogenized kidneys were serially diluted 10 times, and each 100 μl of the dilution was inoculated on the potato dextrose agar (PDA) plates and cultured in an incubator (MCO-18AC SANYO, Tokyo, Japan) at 35 °C without CO_2. The colony forming units (CFU) were counted 48 h later.

The rest of the kidney samples were digested with 5% FBS RPMI containing 1 μg/ml collagenase (SIGMA-Aldrich) at 37 °C for 30 min, then passed through a 70-μm nylon mesh (AS ONE, Osaka, Japan), and the cell suspension was centrifuged at 300 g for 10 min. Cells were washed in 2% FBS PBS, suspended in 4 ml 36% Percoll (GE Healthcare, Tokyo, Japan), and softly overlaid on 4 ml 72% Percoll. After 30 min centrifugation at 900 g at 20 °C, the intercalated layer was collected slowly and washed in chilled PBS (Tran et al., 2015). The cell pellets were resuspended and the total cell number was counted. Following cytospinning and modified Giemsa staining (Diff-Quick), 600 cells per mouse kidney were used for calculating the cell differentiation using a microscope.

5.4. Airway infection with Candida albicans and BALF analysis

Lung infection was induced via nasal administration of a 50 μl *C. albicans* suspension that was adjusted to 1.6×10^7 CFU/ml PBS (8×10^5 CFU/mouse) after light anesthesia with isoflurane inhalation. At six hours post intranasal administration, the BALF were recovered with a tracheal cannulation after 900 μl of chilled PBS was gently flushed into the trachea to fill the lungs up. The procedure was repeated twice, 1.8 ml of chilled PBS was finally recollected and kept on ice. For the collected BALF samples, 100 μl of the fluid was serially diluted 10 times, and plated on PDA as described. The rest of the BALF was centrifuged 300 × g and the supernatants were kept frozen for multiple cytokine measurements. The cell pellets were resuspended and the total cell number was counted. Following cytospinning and modified Giemsa staining (Diff-Quick), 600 cells per mouse BALF were used for calculating the cell differentiation using a microscope.

5.5. In vitro bone marrow-derived cell assay

IL-13$^{-/-}$ BALB/c background mice bone marrow cells were collected by flushing out femurs and tibias with complete RPMI (RPMI 1640, 5×10^{-5} M 2-mercaptoethanol, 10% heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES, 100 μg/ml penicillin and 100 μg/ml streptomycin). Red blood cells were lysed with Ack buffer and leukocytes were washed through a 70 μm nylon cell strainer. The cells were incubated at 1×10^8 cell/ml in 16 ml of complete RPMI with 15 ng/ml recombinant murine (rm) GM-CSF (Wako, Osaka) at 37 °C5% CO_2. On day 5, the supernatants were replaced with new complete RPMI with rmGM-CSF. On day 8, after gentle washing in cold PBS, non-adherent cells were collected and seeded into 48 well plates at 6×10^5 cell / 600 μl / well for initiation of cell culture. A pre-incubation with either recombinant (r)IL-13 or rTGF-β or both of each gradient concentration was applied for 24 h before contact with live *C. albicans* cells (MOI: 1:1). All incubation well conditions were studied in quadruplicate. At 3 h after contact with *C. albicans*, 200 μl of total medium volume, which also includes one-third cells in a gently mixed well, were isolated, and then tested for 3 h responses. In order to observe the response at the 24 h point, the remaining cells were cultured until that time point, then harvested. All the samples were centrifuged (4 °C, 350 × g, 5 min) to separate the supernatant from the cell pellets, which were embedded in ISOGEN and kept frozen at -80 °C until cDNA synthesis, as described above.

5.6. Cytokine production

The rest of the homogenized kidneys were centrifuged (4 °C, 450 × g, 20 min). The supernatants kept at −70 °C were analyzed for cytokine production by using a multiple enzyme-linked immunosorbent assay (ELISA) (quantikine) kits manufactured with CXCL2, KC, TNF-α, IFN-γ, IL-6, IL-1β, and IL-17 A for the Luminex 2000 system. Mouse IL-10 DuoSet (R&D Systems) and multispecies TGF-β1 ELISA (Invitrogen, Camarillo, CA, USA) kits were used to measure the cytokines that were not included in the kit for the multiple assay combination system. A microplate reader (Multiskan FC Thermo SCIENTIFIC, Waltham, MA, USA) was used to measure the OD. The supernatant of BALF was measured for CXCL2 and other mediators after a similar centrifugation.

5.7. Quantitative real-time PCR

Mice were euthanized at the individual time points to obtain samples as indicated. After bleeding of the mice, PBS was systemically flushed into the portal vein to remove circulating blood, and both kidneys were removed. Then, 60 μl of the homogenized kidney in 3 ml of chilled PBS was used for RNA extraction. Peripheral blood mononuclear cells were separated from the blood by Lympholyte-Mammal (CEDARLANE, Ontario, Canada) according to the manufacturer's protocol.

RNA was extracted from the tissue or cells embedded in ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA was synthesized with a PrimeScript RT-PCR Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. A thermal cycler (Veriti ; Applied Biosystems, Foster City, CA, USA). SYBR Premix Ex Taq II (Takara Bio) was used for quantitative real-time (RT)-PCR using Step One Plus (Applied Biosystems, Foster City, CA, USA) with specifically designed primers for β-actin, a reference gene, and other primers for target molecules (Table 1). Control naïve BALB/c mice were sacrificed immediately after intravenous injection with 100 μl saline into the orbital sinus, and dissected. Normalized fold expression of cytokines and chemokines by $\Delta\Delta C_T$ was compared to the average expression of β-actin.

5.8. In vitro C. albicans killing assay

Peritoneal macrophages isolated from either the control or *IL-13*$^{-/-}$ mice (2×10^6 cells/ml) were seeded in the antibiotic-free media, then infected with 10^2 CFU/well of *C. albicans*. The same number of *C. albicans* suspended in antibiotic-free media were cultured alone in the same fashion and utilized as controls. Three hours after incubation, the

cells were lysed in 0.1% Triton X-100. Serial 10-fold dilutions of these fluids were plated on PDA agars. The number of *Candida* colonies was counted 48 h after incubation at 30 °C. The following formula was used to determine the candidacidal activity of macrophages: Candidacidal activity (%) = ([1 - test group CFU/control group CFU] x 100).

5.9. Non-opsonized Texas Red labeled zymosan (TR-zymo) uptake

J774 mouse macrophage-like cells were plated at a density of 0.7×10^6 cells in a 24-well plate and cultured overnight in the presence of recombinant IL-13 (final concentration 5 ng/ml). The cells were incubated for 1 h in the presence or absence of up to 30-fold excess of Texas Red-conjugated zymosan A particles (cat. No. Z2843; Invitrogen), and were then washed with PBS to remove the free particles. After quenching the fluorescence of non-internalized particles with trypan blue (0.25 mg/ml in PBS), cellular fluorescence was quantified in a Varioskan flash microplate reader (Thermo Fisher Scientific, MA, USA) at excitation 565 nm/emission 615 nm. Arbitrary fluorescence units were obtained by subtracting the fluorescence intensity observed in the absence of fluorescent zymosan particles from that observed in the presence of the particles.

5.10. Statistical analysis

Statistical analysis was carried out using the Mann-Whitney U test. A p-value of < 0.05 was considered statistically significant in Figs. 1–3. The statistical analysis in Figs. 5 and 6 used ANOVA one-sided test with Dunnett's multivariate comparison where the control was determined as the data of BM cells contact with live *C. albicans* without recombinant proteins or LPS (the left end bar). Data of the BM cells with LPS was shown as positive control, which was not included for the statistical analysis. All the results were analyzed with GraphPad Prism version 5 (GraphPad software, San Diego, CA, USA).

Conflict of interest

The authors do not have any conflict of interest.

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