INFECTION AND IMMUNITY, Feb. 2006, p. 1403–1406 Vol. 74, No. 2
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.2.1403–1406.2006

Killed *Candida albicans* Yeasts and Hyphae Inhibit Gamma Interferon Release by Murine Natural Killer Cells

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Received 29 July 2005/Returned for modification 30 August 2005/Accepted 26 October 2005

Killed yeasts and hyphae of *Candida albicans* inhibit gamma interferon secretion by highly purified murine NK cells in response to the Toll-like receptor ligands lipopolysaccharide and zymosan. This effect, which is also observed in the presence of NK-activating cytokines (interleukin-2 [IL-2], IL-12, and IL-15), may represent a novel mechanism of immune evasion that contributes to the virulence of *C. albicans*.

Natural killer (NK) cells constitute an important component of innate immunity, being able to limit viremia and tumor burden before the adaptive immune system can be activated. In addition, there is increasing evidence indicating that NK cells may also play a major role in the early defense against bacterial and fungal infections (2, 26). NK cells are able to inhibit the growth of *Cryptococcus neoformans* (14, 17), and the secretion of cytokines (granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha, and gamma interferon [IFN-γ]) by activated NK cells up-regulates the fungicidal activity of phagocytic cells, resulting in the growth inhibition of these agents (2, 10, 18). In addition, the interaction of lymphokine-activated killer cells and *Candida albicans* does not inhibit or kill the fungal pathogen by means of the lymphokine-activated killer cell lytic machinery, but through the secretion of cytokines which have stimulatory effects on phagocytic cells (2). Therefore, antifungal immunity mediated by NK cells appears to occur by the secretion of cytokines that activate phagocytic cells (1, 2). However, whether NK cells are stimulated directly by the fungus or in response to signals generated by activated bystander cells remains to be established (26).

The main pattern recognition receptors on the surfaces of phagocytic cells are the Toll-like receptors (TLRs), a family of evolutionarily conserved transmembrane proteins that function as sensors of infection and induce the activation of innate and adaptive immune responses (23, 24). There is evidence indicating that signal transduction mediated by TLRs is essential for murine resistance to disseminated *C. albicans* infection, basically through the induction of cytokine and chemokine release (4, 11, 16, 19–21, 28–30).

During recent years, the expression of TLRs in NK cells has been reported, and several data indicate that NK cells directly recognize and respond to pathogen components through TLRs (3, 5, 13, 15, 22). Moreover, TLR-induced stimulation of both dendritic cells and NK cells may play a critical role in inducing NK cells to select the best-fit immature dendritic cells and to facilitate their maturation, thus exerting a regulatory control on the early steps of innate immune responses against pathogens (7, 9, 22). Since the role of NK cells in resistance against many pathogens, including *C. albicans*, may be mediated by cytokine production, including IFN-γ production (2, 10, 18, 26), the aim of this work was to study the role of TLRs in triggering IFN-γ secretion by NK cells in response to yeasts and hyphae of *C. albicans*. Unexpectedly, our results reveal that fungal cells cause an inhibition of IFN-γ secretion by murine NK cells.

Murine NK cells were isolated from C57BL/6 mice (Harlan Ibérica), TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (C57BL/6 background; kindly provided by S. Akira, Osaka, Japan) (25), and DBA2/J mice (Jackson Laboratory). NK cells were purified from spleens by immunomagnetic cell sorting with MicroBeads (Miltenyi Biotec, Madrid, Spain) following a two-step procedure performed according to the manufacturer's instructions. Briefly, splenocytes were first depleted of T cells by using CD90 (Thy1.2) MicroBeads, and then NK cells were purified by positive selection using CD49b (DX5) MicroBeads. The purity of the DX5$^+$ CD90$^-$ NK cells was assessed by simultaneous labeling with a fluorescein isothiocyanate-labeled anti-NK1.1 monoclonal antibody (PK136; BD Pharmingen) and a phycoerythrin-Cy5-labeled anti-CD3 monoclonal antibody (145-2C11; BD Pharmingen) followed by flow cytometry analysis with an EPICS XL cytometer (Beckman Coulter). A homogeneous cell population was purified; viable NK cells represented >80% of the total cell population, whereas no T lymphocytes were detected. Cells were immediately cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Gibco, Barcelona, Spain) at a density of 1.2×10^6 cells per ml and challenged for 48 h with the indicated stimuli in the absence or presence of 200 U/ml interleukin-2 (IL-2), 5 ng/ml IL-12, or 5 ng/ml IL-15 (all from PeproTech EC, Madrid, Spain). The stimuli used were zymosan (3.84×10^6 particles per ml; Molecular Probes, Eugene, OR), lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (1 μg per ml; Sigma, Madrid, Spain), and two *C. albicans* ATCC 26555 forms, yeast and hypha forms, obtained as previously reported (12, 29). Briefly, starved yeast cells were inoculated (200 μg [dry weight] of cells per ml) into a minimal synthetic medium and incubated for 3 h at 28°C

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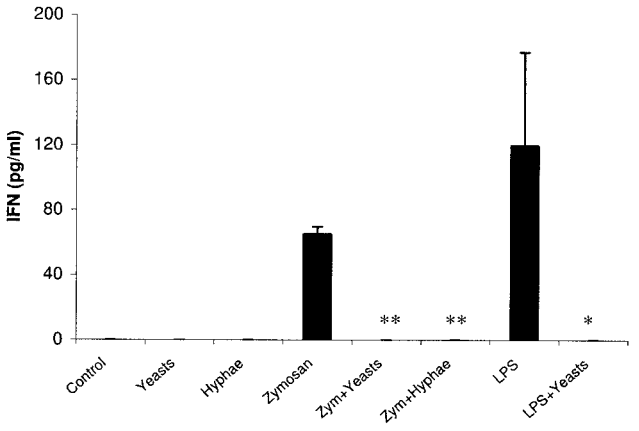


FIG. 1. Production of IFN-γ by purified NK cells from C57BL/6 mice in response to *C. albicans*. NK cells were cultured without (control) or with the indicated stimuli (150 μg [dry weight] of heat-killed *C. albicans* cells per ml). IFN-γ was quantified in the 48-h supernatants by ELISA. Means (± standard deviations [SD]) of duplicates from one representative experiment of two are shown. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to either the LPS- or zymosan-treated sample.

to obtain yeasts or at 37°C to obtain hyphae; >90% of the cells exhibited well-defined germ tubes (true hyphae) at 37°C, whereas only yeast cells were observed at 28°C. For heat inactivation, yeasts and hyphae were resuspended in phosphate-buffered saline (PBS) (20×10^6 cells/ml) and treated at 100°C for 1 h. For some experiments, yeasts were killed either by fixing the cells for 1 h with 4% paraformaldehyde (fixation buffer; eBioscience, San Diego, CA) (20×10^6 cells/ml) or by treatment with antimycotic agents (2.5 mg/ml amphotericin B and 240 U/ml nystatin) in PBS (20×10^6 cells/ml) for 72 h at room temperature. After treatments, fungal cells were extensively washed in PBS and brought to the desired cell density in complete cell culture medium. The completeness of killing was checked by plating on Sabouraud-dextrose agar. For some assays, *C. albicans* ATCC 26555 and PCA2 (6) and *Saccharomyces cerevisiae* SEY2101 (8) and 1403 (Spanish Type Culture Collection) were grown in YPD medium (1% Difco yeast extract, 2% peptone, 2% glucose) at 28°C until the late exponential growth phase and then inactivated as described above. The viability of NK cells after stimulation was measured by trypan blue staining and microscopic observation. IFN-γ was measured in cell-free culture supernatants by use of a commercial enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA). Student's two-tailed *t* test was used to statistically compare IFN-γ production levels.

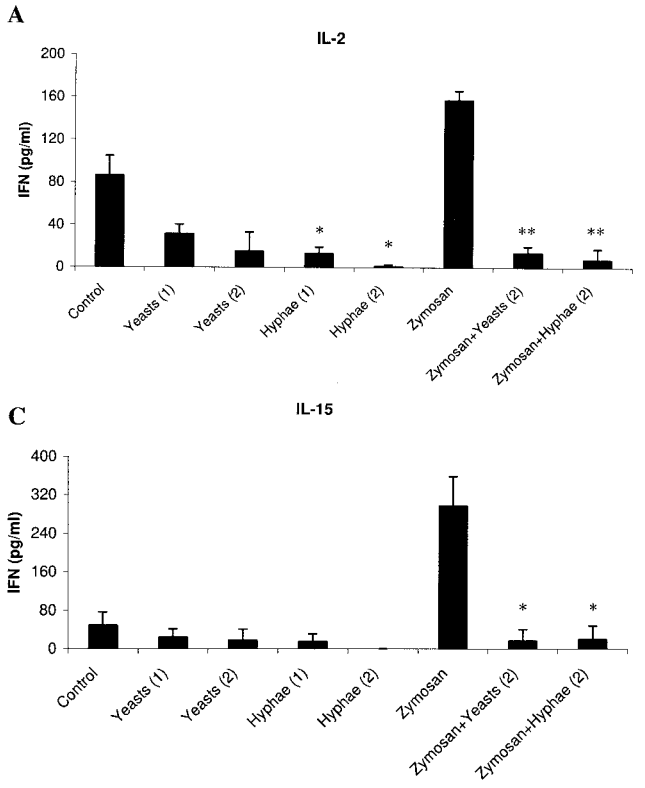
We first tested the ability of *C. albicans* cells to induce IFN-γ production by highly purified murine NK cells in the absence of exogenous cytokines. As shown in Fig. 1, both yeasts and hyphae failed to induce IFN-γ secretion; in contrast, NK cells exposed to zymosan or LPS produced detectable levels of IFN-γ, whereas a simultaneous challenge, with zymosan or LPS added to either yeasts or hyphae, resulted in the absence of detectable levels of IFN-γ. This result suggests that fungal cells inhibit IFN-γ production by NK cells in response to TLR2 and TLR4 ligands (zymosan and LPS, respectively). Next, we analyzed the response of NK cells to fungal stimuli (zymosan and *C. albicans* cells) in the presence of cytokines. IL-2, a potent NK cell activation factor, induced IFN-γ and synergized with zymosan (Fig. 2A). This IFN-γ production was again inhibited, in a dose-dependent manner, by *C. albicans* yeasts and hyphae. The reduced IFN-γ production by NK cells cultured in the presence of *C. albicans* was not a consequence of cell death, as the cell viability was similar to that of control NK cells (not shown). Similar results were obtained when NK cells were challenged in the presence of other NK-cell-activating cytokines, such as IL-12 and IL-15 (Fig. 2B and C). Since the yeast cell wall particle zymosan and heat-killed *C. albicans* cells showed opposite effects on IFN-γ production by NK cells, we tested the ability of the nongerminative low-virulence *C. albicans* PCA2 strain and two *S. cerevisiae* strains, SEY2101 and 1403, to induce IFN-γ production. Results indicated that all three heat-killed yeast strains were able to inhibit IFN-γ production by NK cells to similar extents in response to zymosan in the presence of IL-2 (Fig. 3).

Since the heat-killing process can result in changes to the fungal cell surface that could affect interactions with NK cells, we assayed the killing of *C. albicans* and *S. cerevisiae* cells by other methods (fixation with formaldehyde and antimycotic treatment) to determine whether they showed the same effect as heat-killed yeast cells on IFN-γ production by NK lymphocytes. The results indicated that all three types of killed yeasts were able to inhibit IFN-γ production by NK cells to similar extents in response to zymosan in the presence of IL-2. Average inhibition levels of 60 to 65% were observed for *C. albicans* ATCC 26555, with no statistically significant differences between heat-killed, fixed, and antimycotic-treated cells. A similar result was obtained with *S. cerevisiae* 1403 strain, with average inhibition levels of 75 to 85%.

We have previously described that TLR2 is the major receptor that activates macrophages in response to *C. albicans* (19, 29). Since we found that *C. albicans* cells inhibit IFN-γ production by NK cells, we investigated whether TLR2 might be involved in this effect. The results showed that fungal cells inhibited IFN-γ production by NK cells from $TLR2^{-/-}$ mice in response to LPS in the presence of IL-12 (about 65% inhibition for both yeasts and hyphae) as well as that by NK cells from $TLR4^{-/-}$ mice in response to zymosan (68% inhibition for yeasts and 75% inhibition for hyphae). Therefore, TLRs are not involved in the inhibition of IFN-γ production by NK cells in response to *C. albicans*.

The nature of the fungal ligand(s) involved in this inhibitory effect is unknown, although it may be a cell wall-associated molecule, present in whole cells and absent from zymosan particles, and is probably not a TLR2 ligand such as phospholipomannan (16). Similarly, the involved inhibitory NK-cell receptor is unknown. Assays performed with NK cells purified from DBA/2J mice from Jackson Laboratory, which lack surface expression of the CD94/NKG2A receptor, as they are naturally CD94 deficient (27), indicated that this receptor is not involved in mediating the inhibitory effect. *C. albicans* yeast cells caused 72% inhibition of IFN-γ production by NK cells from DBA/2J mice in response to zymosan in the presence of IL-2.

In parallel, we tested the effect of *C. albicans* cells on the proliferation of NK cells induced by IL-2. NK cells were incubated for 48 h in the presence of IL-2 prior to being pulsed



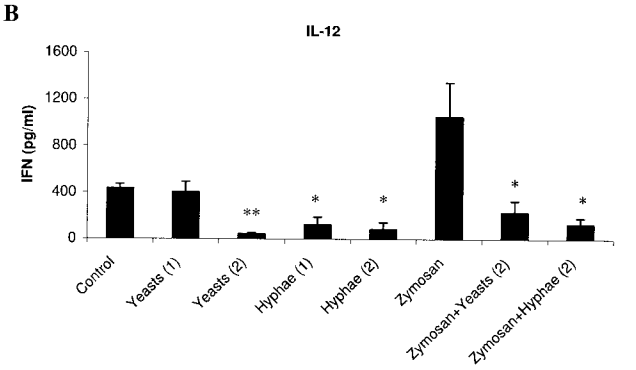


FIG. 2. Production of IFN-γ by purified NK cells from C57BL/6 mice in response to *C. albicans* in the presence of cytokines. NK cells were cultured without (control) or with the indicated stimuli (75 or 150 μg [dry weight] of heat-killed *C. albicans* cells per ml [samples 1 and 2, respectively]) in the presence of IL-2 (A), IL-12 (B), or IL-15 (C). IFN-γ was quantified in the 48-h supernatants by ELISA. The results depicted are mean values (± SD) of duplicates from one representative assay of three. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to either the control or zymosan-treated sample.

with 5-bromo-2′-deoxyuridine (BrdU) for 16 h. BrdU incorporation was measured by using a BrdU labeling and detection kit III (Roche, Mannheim, Germany). The results showed that *C. albicans* cells, both yeasts and hyphae, have no effect on the IL-2-induced proliferation of NK cells, as fungal cells neither synergize with IL-2 nor inhibit proliferation (not shown).

Despite the fact that the in vivo role of NK cells in the host defense against invasive bacterial or fungal infections is not unequivocally established, the observed in vitro inhibitory effect of *C. albicans* cells on NK-cell activation may be of biological relevance in vivo. Since the role of NK cells in resistance to *C. albicans* appears to be basically mediated by cytokine production, including IFN-γ production, which activates phagocytic cells (2, 18), inhibition of this production may favor the survival of the pathogen. In addition, this effect may also (i) inhibit IFN-γ production by NK cells in response to cytokines secreted by activated bystander cells (13) and (ii) disturb the cross talk between NK and dendritic cells, as the IFN-γ secreted by NK cells controls dendritic cell maturation and T-cell stimulatory activity (7, 9). Our finding may represent a novel mechanism of immune evasion, based on the inhibition of NK cells, that contributes to the virulence of *C. albicans.* The fact that *S. cerevisiae* and a nonvirulent *C. albicans* strain show the same effect on NK cells as the virulent *C. albicans* strain only indicates that all strains share the ligand(s) involved in this phenomenon, but it does not invalidate our hypothesis, as the virulence of *C. albicans* depends on a complex array of microbial virulence factors which are absent from nonvirulent *S. cerevisiae* strains.

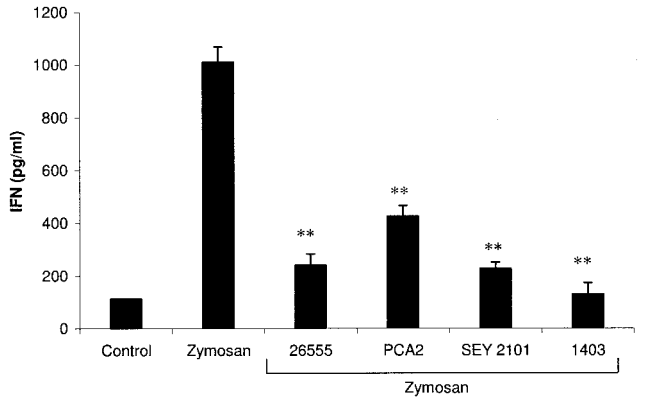


FIG. 3. Production of IFN-γ by purified NK cells from C57BL/6 mice in response to yeast strains in the presence of IL-2 and zymosan. NK cells were cultured in the presence of IL-2 without (control) or with zymosan and the indicated heat-killed yeast cells (150 μg [dry weight] per ml): *C. albicans* ATCC 26555, *C. albicans* PCA2, *S. cerevisiae* SEY 2101, and *S. cerevisiae* 1403. IFN-γ was quantified in the 48-h supernatants by ELISA. The results depicted are mean values (± SD) of duplicates from one representative assay of two. **, $P < 0.01$ compared to the zymosan-treated sample.

This work was supported by grants PI03/0647 and PI04/1472 (Ministerio de Sanidad y Consumo) and grupos 03-172 (Generalitat Valenciana), Spain. C.M. is the recipient of a fellowship from Ministerio de Educación y Ciencia, Spain.

We are grateful to Shizuo Akira for making the TLR4$^{-/-}$ and TLR2$^{-/-}$ mice available to us.

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Editor: A. Casadevall