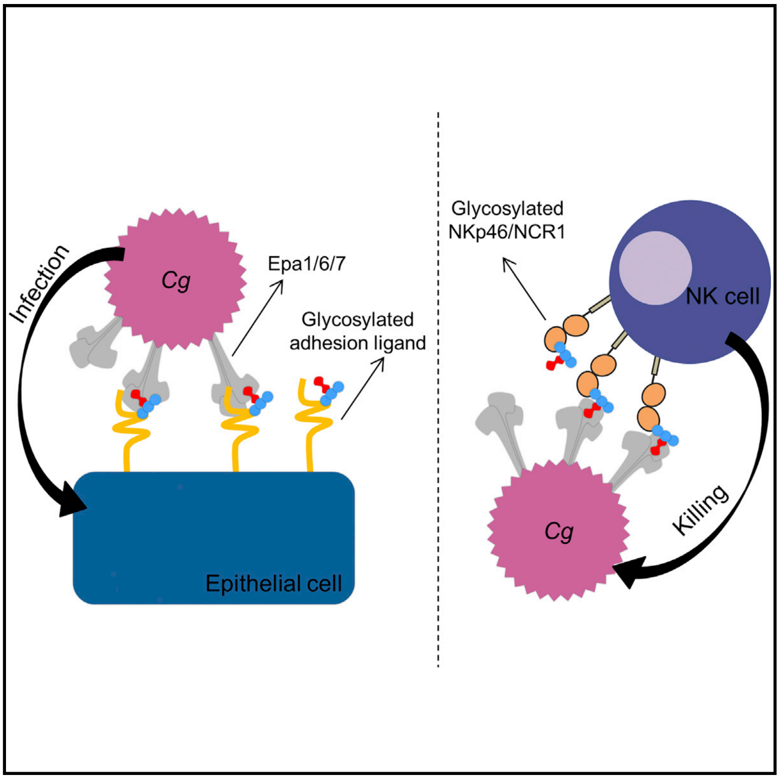
Short Article

Cell Host & Microbe

NK Cell Recognition of *Candida glabrata* through Binding of NKp46 and NCR1 to Fungal Ligands Epa1, Epa6, and Epa7

Graphical Abstract



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In Brief

Candida glabrata is an emerging fungal pathogen. Here, Vitenshtein et al. show that the NK cytotoxic receptor NKp46/NCR1 binds the fungal adhesins EPA1, EPA6, and EPA7 and further demonstrate that NCR1 is essential for clearing of systemic infection in vivo.

Highlights

- The human NK receptor NKp46 and mouse ortholog NCR1 bind *C. glabrata*
- NK cells from NCR1 knockout mice are compromised for *C. glabrata* killing in vitro
- NCR1 knockout mice have impaired clearance of systemic *C. glabrata* infection
- NKp46/NCR1 recognize *C. glabrata* by binding to the fungal Epa1, Epa6, and Epa7 adhesins

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NK Cell Recognition of *Candida glabrata* through Binding of NKp46 and NCR1 to Fungal Ligands Epa1, Epa6, and Epa7

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SUMMARY

Natural killer (NK) cells form an important arm of the innate immune system and function to combat a wide range of invading pathogens, ranging from viruses to bacteria. However, the means by which NK cells accomplish recognition of pathogens with a limited repertoire of receptors remain largely unknown. In the current study, we describe the recognition of an emerging fungal pathogen, *Candida glabrata*, by the human NK cytotoxic receptor NKp46 and its mouse ortholog, NCR1. Using NCR1 knockout mice, we observed that this receptor-mediated recognition was crucial for controlling *C. glabrata* infection in vitro and in vivo. Finally, we delineated the fungal ligands to be the *C. glabrata* adhesins Epa1, Epa6, and Epa7 and demonstrated that clearance of systemic *C. glabrata* infections in vivo depends on their recognition by NCR1. As NKp46 and NCR1 have been previously shown to bind viral adhesion receptors, we speculate that NKp46/NCR1 may be a novel type of pattern recognition receptor.

INTRODUCTION

Opportunistic fungal pathogens constitute a serious and rising health burden. Some of this burden is a consequence of modern medicine itself, notably the increased numbers of immunocompromised and elderly populations and the widespread use of antibiotics (Pfaller and Diekema, 2010). The predominant agents for disseminated fungal infections are Candida species (Lockhart et al., 2012; Pfaller and Diekema, 2007). One species, *Candida glabrata* (Cg), has emerged as a serious public health risk and has become the second most common cause of invasive candidiasis (Lockhart et al., 2012; Pfaller and Diekema, 2007). Of particular concern is the alarming increase in the number of *C. glabrata* strains resistant to first-line antifungal drugs of the azole and echinochandins classes (Alexander et al., 2013; Pfaller, 2012) or the emergence of multidrug-resistant strains (Alexander et al., 2013; Arendrup and Perlin, 2014; Pfaller et al., 2012).

Natural killer (NK) cells are lymphocytes of the innate branch of immunity. They employ a limited repertoire of germline-encoded receptors to target and eliminate cells infected by bacteria or viruses, as well as tumor cells (Arnon et al., 2004; Elboim et al., 2010; Gazit et al., 2006; Glasner et al., 2012, 2015; Gur et al., 2013, 2015; Jarahian et al., 2011; Lakshmikanth et al., 2009). Among the receptors that NK cells use to detect these pathogens are the natural cytotoxic receptors (NCRs) NKp30, NKp44, and NKp46. Of the NCRs, only NKp46 has a mouse ortholog, NCR1 (Biassoni et al., 1999; Moretta et al., 2001). Although targeting of cellular components by NKp46 and NCR1 is involved in a range of cellular pathologies (Glasner et al., 2012; Gur et al., 2010; Lakshmikanth et al., 2009), their cellular ligand remains unknown. Despite this, the viral hemagglutinin protein has been identified as the targeted ligand in many viruses, including influenza and Sendai viruses (Mandelboim et al., 2001), Poxviruses (Jarahian et al., 2011), and Newcastle disease virus (Jarahian et al., 2009). The viral hemagglutinins do not share any structural similarity; however, NKp46 and NCR1 effectively target this broad range of viral hemagglutinins through a sialic acid-based glycosylation (Arnon et al., 2004; Jarahian et al., 2009, 2011; Mandelboim et al., 2001).

Over 30 years ago, NK cells were shown to kill fungal cells directly (Murphy and McDaniel, 1982). Studies have shown that NK cells play an important role in combating fungal infections in vivo (Bär et al., 2014). Surprisingly, our knowledge concerning direct NK receptor-fungal ligand interactions remains extremely limited. No NK receptor recognition mechanisms are known apart from those mentioned in a recent paper, which discovered the recognition of *Candida albicans* and *Cryptococcus neoformans* by NKp30 (Li et al., 2013). However, we are still lacking answers to basic questions, such as are there other NK receptors that are important for fungal cell recognition. What is the fungal ligand that is bound by NK cells? Finally, does

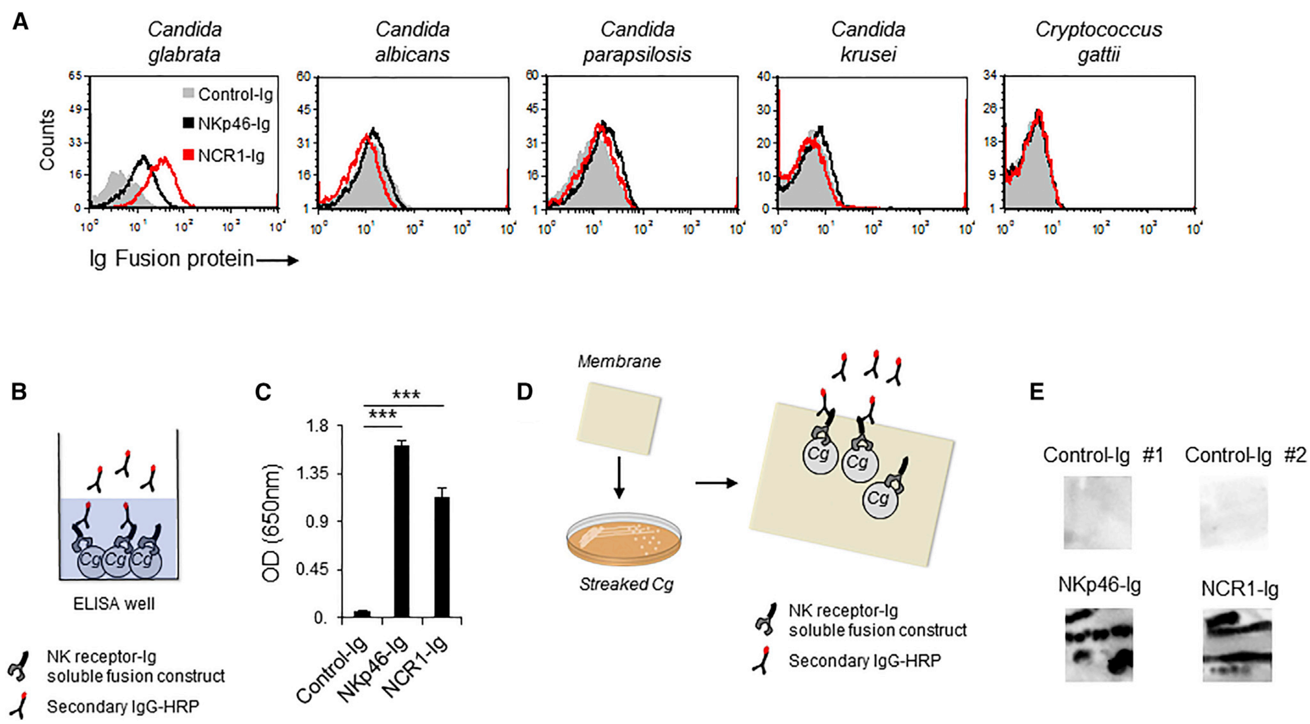

Figure 1. NKp46 and Its Mouse Ortholog NCR1 Bind Cg

(A) FACS staining of the binding of NKp46-Ig (black empty histogram) and NCR1-Ig (red empty histogram) to Cg and additional Candida and Cryptococcal strains compared to a control human NKG2D Ig fusion protein (Control-Ig, gray filled histogram).

(B) Diagram of ELISA technique used to test for binding of Cg (immobilized on the plate) to NKp46-Ig and NCR1-Ig.

(C) Binding of NKp46-Ig, NCR1-Ig, and control hNKG2D-Ig (Control-Ig) fusion protein to Cg, as determined by ELISA described in (B). Figure shows mean ± SD of triplicates; ***$p < 0.001$.

(D) Diagram that describes assays used to test binding of Ig fusion proteins to Cg blotted to membrane. In this assay, colonies of streaked Cg that were grown overnight on agar plates are blotted onto a nitrocellulose membrane (left). Binding of soluble Ig fusion proteins is then determined (right).

(E) Blot binding assay described in (D) was performed with NKp46-Ig, NCR1-Ig, and two control Ig fusion proteins, hNKG2D-Ig (Control-Ig #1) and mNKG2D-Ig (Control-Ig #2). Figures are representative of five (A) or three (C and E) experiments.

NK receptor recognition significantly contribute to in vivo clearance of disseminated fungal infections?

In the current study, we provide insights to these questions by describing a direct recognition of *C. glabrata* by the NK receptor NKp46 and its mouse ortholog, NCR1. We show this interaction to be of vital importance for killing *C. glabrata* (Cg) in vitro and for clearing systemic infection in vivo. Finally, we identify fungal ligands for an NK receptor by demonstrating that NKp46 and NCR1 recognize the *C. glabrata* virulence proteins: the adhesins Epa1, Epa6, and Epa7.

RESULTS

Human NK Receptors NKp46 and Mouse Ortholog NCR1 Bind Cg

To explore the possibility that NK receptors bind Cg, we initiated a fluorescence-activated cell-sorting (FACS) screening assay for Cg binding using a panel of soluble NK receptor fusion constructs composed of the extracellular portion of a given NK receptor fused to the Fc portion of human IgG1 antibody. One of the soluble receptors that demonstrated binding was the human NK cytotoxic receptor NKp46-Ig and, interestingly, also its mouse ortholog, NCR1-Ig (Figure 1A). No binding was observed to other Candida and Cryptococcus species (Figure 1A). Following this, we conducted three additional types of assays in order to confirm the NKp46/NCR1 binding. The first of these assays was an enzyme-linked immune-sorbent assay (ELISA)-based method, in which Cg cells were immobilized on ELISA plates and then probed with the soluble NKp46-Ig and NCR-Ig fusion proteins (Figure 1B). This assay successfully validated our initial observations by demonstrating binding of NKp46-Ig and NCR1-Ig to Cg (Figure 1C). The next system that was used to test the binding capacity of NKp46-Ig and NCR1-Ig was western blot assay. In this assay, Cg cells are grown on agar plates and then are lifted onto a nitrocellulose membrane, which was then probed with the soluble NK receptors to assess binding (Figure 1D). Once again, we saw that NKp46-Ig and NCR1-Ig demonstrated specific binding to whole Cg cells in this method compared to control Ig fusion receptors (Figure 1E).

NKp46 and NCR1-Dependent NK Cell Activation and Killing of Cg

The binding of NKp46 and NCR1 to Cg was also tested in a functional cellular-based reporting system. In this system, a construct composed of the extracellular domain of a receptor of choice is fused to the intracellular mouse zeta-chain domain

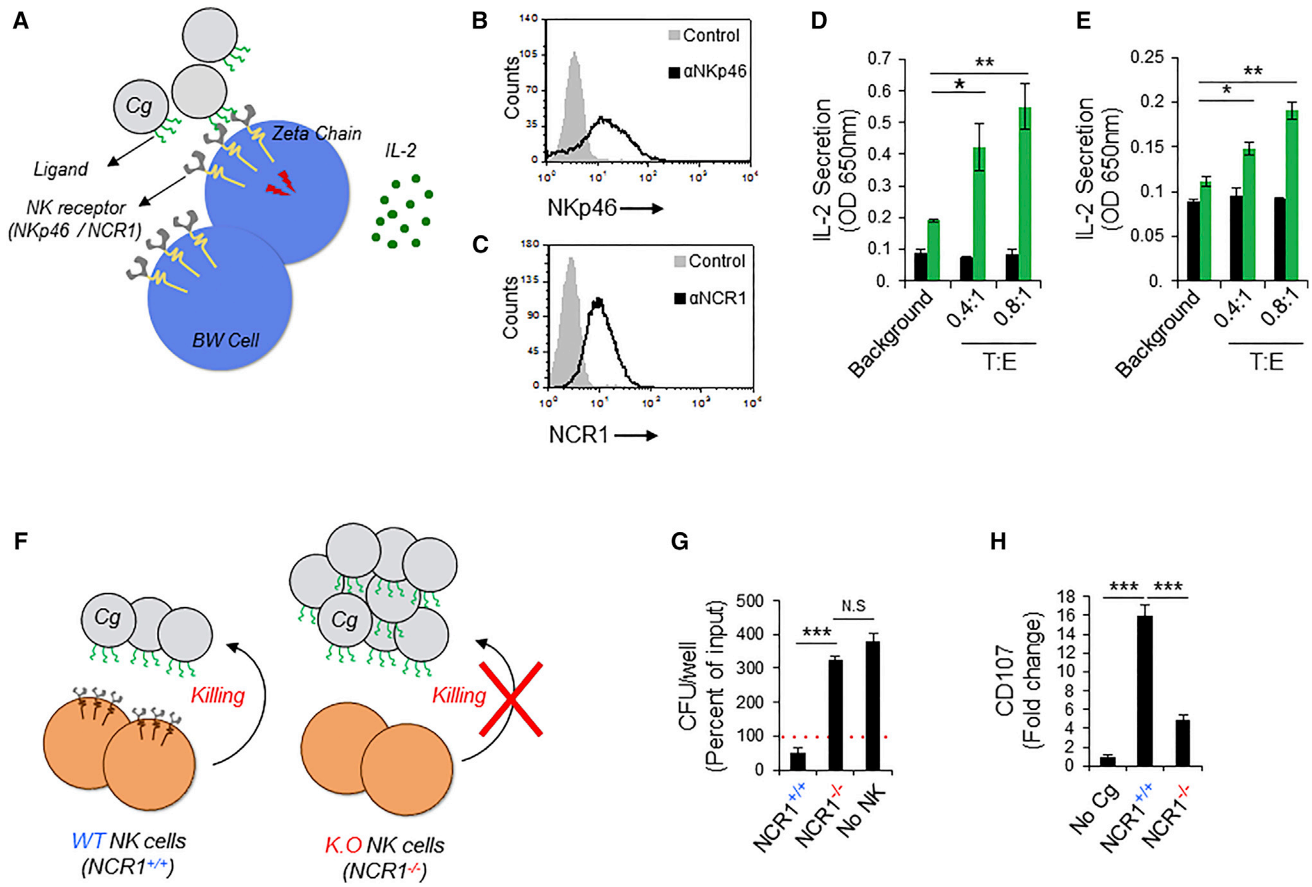

Figure 2. Functional Reporter and Killing Assay Confirms Binding and Elimination of Cg through NKp46 and NCR1

(A) A diagram that depicts the functional reporter system. The extracellular portion of an NK receptor to be tested is fused to intracellular mouse zeta chain and expressed in BW mouse thymoma cells. Cells are co-incubated with Cg and, upon ligation of the fusion receptor with a target ligand, an activation signal induces the BW cells to secrete IL-2 to the supernatant that is quantified by ELISA.

(B and C) FACS analysis of NKp46-zeta (B) and NCR1-zeta (C) fusion construct expression on BW cells transduced to overexpress these, respectively (black empty histograms). Background staining (filled gray histogram) was conducted with isotype-matched control IgG (Control).

(D and E) As described in (A), BW reporter assays were conducted with BW cells expressing NKp46-zeta (D) or NCR1-zeta (E) and were co-incubated with Cg at the depicted target/effector ratios (T:E) and shown in green. Background is the BW cells alone and control BW cells expressing NKp44-zeta construct are shown in black.

(F) Schematic representation summarizing in vitro killing assay, which was performed by co-culturing NK cells from wild-type ($NCR1^{+/+}$) and NCR1 knockout ($NCR^{-/-}$) mice with Cg.

(G) Quantification of Cg CFU following 12 hr of co-culturing with no NK cells or NK cells from wild-type or NCR1 knockout mice as described in (F). Numbers are percentage of initial input inoculum of 1,000 CFUs/well Cg cells indicated by red dotted line.

(H) Fold change in expression of CD107 on NK cells from (G). Figures are a representative of three (B and C) or seven (D and E) experiments conducted and are mean ± SD of triplicates (D, E, and H) or six replicates (G). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant.

and is transduced into the mouse BW thymoma cell line. This cell line is then co-cultured for 2 days with the binding partner to be tested, in this instance Cg. A productive interaction leads to IL-2 secretion (Figure 2A). For this assay, we used BW cells that were stably transduced with NKp46- and NCR1-zeta chain constructs (Figures 2B and 2C, respectively). Consistent with binding of Cg to NKp46 and NCR1, we observed that the NKp46/NCR1 BW cell transfectants were activated by Cg cells in a dose-dependent manner, compared to control cells that were unaffected (Figures 2D and 2E). We next set out to determine whether binding of NKp46/NCR1 is important for the antifungal activity of NK cells against Cg cells. To study this, we isolated NK cells from both NCR1 knockout ($NCR1^{-/-}$) and wild-type mice, co-cultured each with Cg, and then quantified the number of viable Cg cells (Figure 2F). We observed that NK cells from wild-type mice showed significant cytolytic activity against Cg cells, as there were less viable Cg cells (Figure 2G). Importantly, this killing was dependent on NCR1, since Cg numbers were significantly higher when co-cultured with NK cells from $NCR1^{-/-}$ mice as compared to NK cells from wild-type animals and were comparable to no NK cells (Figure 2G). Similarly, impaired NK cell activation was evident in the levels of cell surface expression of CD107, a marker for NK cell degranulation, which was markedly lower on $NCR1^{-/-}$ NK cells co-cultured with Cg compared to wild-type mice (Figure 2H).

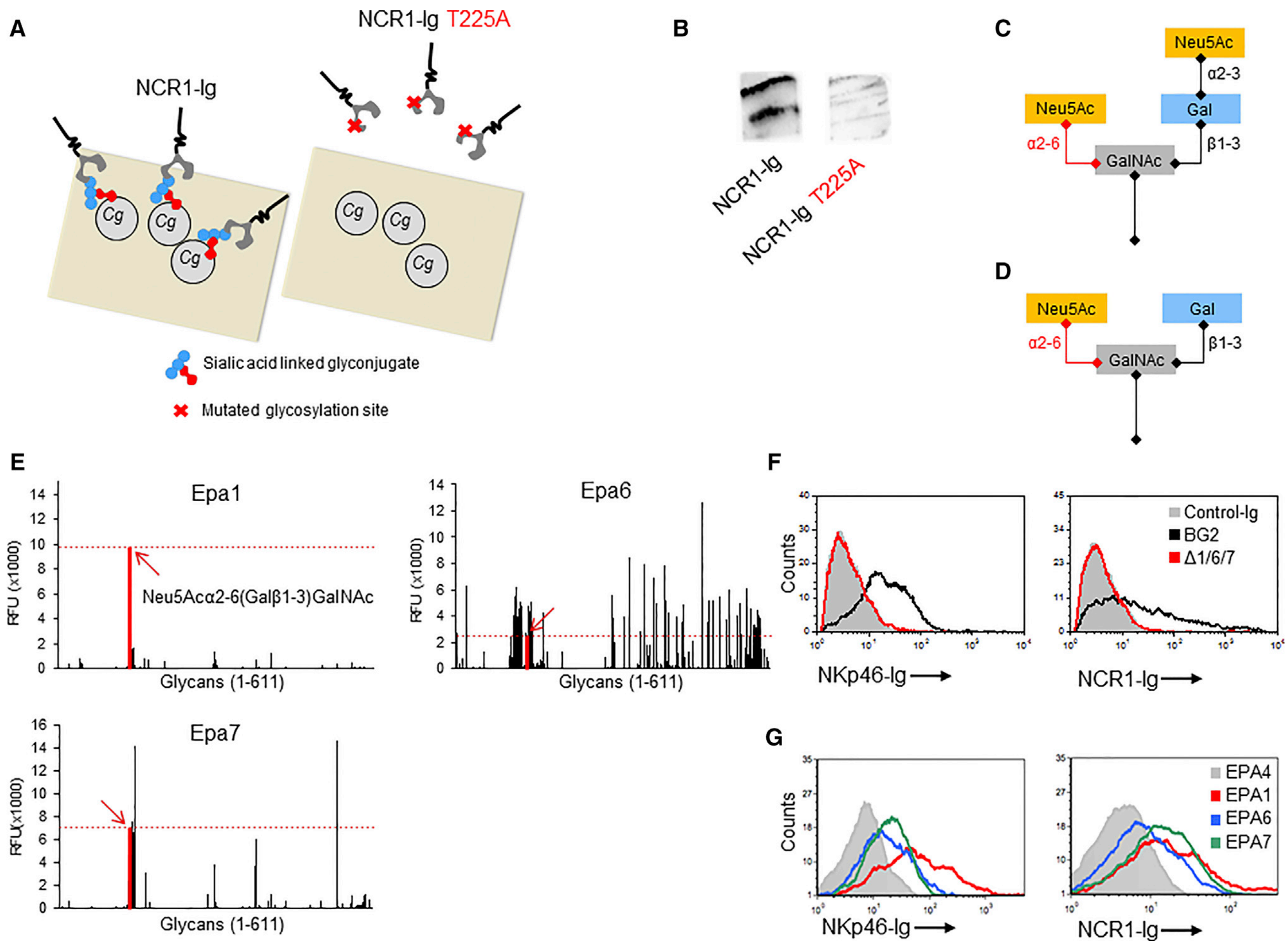

Figure 3. Epa1, Epa6, and Epa7 Are the Target Ligands Bound by NKp46 and NCR1

(A) A schematic depicting the binding assay performed in (B) using a wild-type NCR1-Ig (NCR1-Ig) and NCR1-Ig that was mutated in position 225 from threonine to alanine (NCR1-Ig T225A).

(B) Testing wild-type (NCR1-Ig) and glycosylation site-mutated (NCR1-Ig T225A) NCR1-Ig fusion proteins capacity to bind Cg cells blotted to nitrocellulose membrane.

(C and D) Schematic representation of the O-linked glycosylation on threonine 225 (C) and the glycosylated structure tested for binding to Cg Epas in (D). The glycoconjugate structure depicts sialic acid (Neu5Ac), Galactose (Gal), and N-Acetylgalactosamine (GalNAc) residues with the type of bond (α or β) and the carbon numbers that form it.

(E) Data from soluble Epa1, Epa6, and Epa7 binding to 611 different glycan derivative structures on a glycan array. Binding to each of the 611 glycan structures is shown as a vertical line and the binding to the sugar moiety shown in (D) was highlighted in red. The binding level was also depicted with a dotted line and an arrow. Bound protein was detected by Alexa 488-coupled anti-6His antibody fluorescence intensity (relative fluorescence units, RFUs).

(F) FACS analysis of NKp46-Ig (left) and NCR1-Ig (right) binding to parental Cg strain (BG2, black empty histogram), Cg deleted in *EPA1*, *EPA6*, and *EPA7* (Δ*epa1/6/7*, red empty histogram). Gray filled histogram is background staining with control hNKG2D-Ig fusion protein (Control-Ig).

(G) Analysis of NKp46-Ig (left) and NCR1-Ig (right) *Saccharomyces cerevisiae* expressing Cg *EPA* genes: *EPA1* (red empty histogram), *EPA6* (blue empty histogram), or *EPA7* (green empty histogram). Control staining was performed with *Saccharomyces cerevisiae* overexpressing Cg *EPA4* (filled gray histogram). Data (B, F, and G) are representative of three experiments. See also Tables S1, S2, and S3.

NKp46 and NCR1 Bind the Epa1, Epa6, and Epa7 Adhesins on the Surface of Cg

As mentioned, NKp46 and NCR1 have been implicated in binding to the influenza hemagglutinin protein. Moreover, the binding interaction has been shown to be dependent on a sialylated oxygen (O)-linked glycosylation on NKp46/NCR1, since receptors mutated for the O-glycosylated residue (T225A) are unable to bind hemagglutinin (Arnon et al., 2004; Glasner et al., 2015). We tested whether this O-linked glycosylation is also required for the binding of an NCR1-Ig to *C. glabrata* (Figure 3A). Strikingly, NCR1-Ig mutated on threonine 225 (Thr225) was unable to bind Cg (Figure 3B). This indicated that, similar to the binding of the influenza hemagglutinin lectin, NKp46 and NCR1 might bind to a lectin on the surface of Cg through the same sugar residue. The O-linked glycan on the NKp46/NCR1 Neu5Acα2-6 (Neu5Acα2-3Galβ1-3) GalNAc (Glasner et al., 2015; Mendelson et al., 2010) is shown in Figure 3C. In Cg, the *EPA* genes encode a major family of glycan-binding lectins, which mediate the attachment of the fungal cell to host cells. Previous studies of the Epa proteins (Maestre-Reyna et al., 2012; Zupancic et al.,

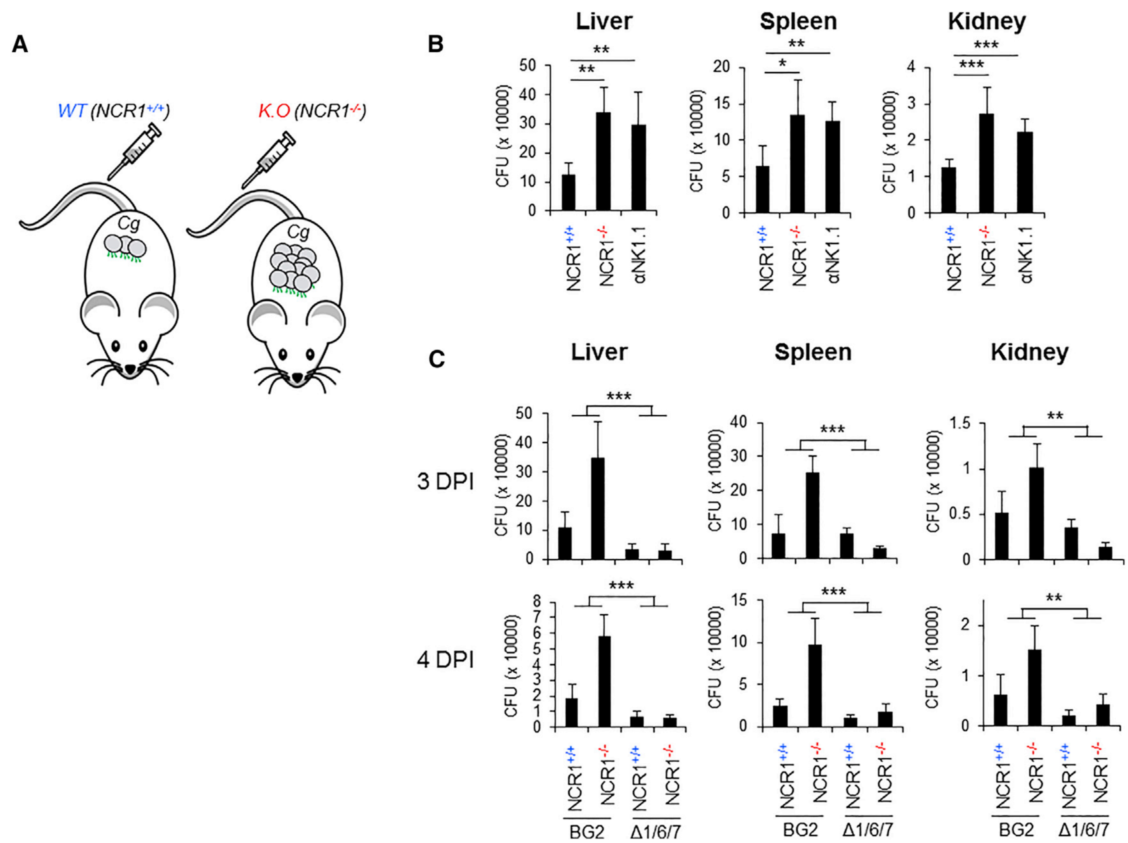

Figure 4. Clearance of Cg in Systemic Candidiasis In Vivo Is Impaired in NCR1$^{-/-}$ Mice and Dependent on Epa1, Epa6, and Epa7

Diagram depicting systemic model of Cg Candidiasis in vivo (A) performed in (B) and (C). 3 (B and C) and 4 (C) days following an i.v. injection of parental Cg (BG2) cells (B and C) and the *Δepa1/6/7* strain (Δ1/6/7) cells (C) to NCR1 wild-type (WT) and knockout (KO) mice and 4 days after injection of NK depleting antibody, αNK1.1 (B), the load of Cg for liver, spleen, and kidney was quantified by colony formation assay and expressed as CFUs per gram tissue. Data are mean ± SD of seven mice in each group and representative of two experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

2008) initially indicated that these might be potential candidates that could bind NKp46/NCR1, since the structures of the glycans they bound were similar in structure to the NKp46/NCR1 glycosylation. To identify the glycan structures that the Epas bind, we characterized the glycan-binding patterns of recombinant soluble proteins of the three major Cg Epas, Epa1, Epa6, and Epa7, to 611 glycan structures on a glycan array. One of the glycans on this array was highly similar to the glycosylation on the NKp46/NCR1 (Figure 3D). Interestingly, all three Epas demonstrate significant binding to this glycan (Figure 3E; Tables S1, S2, and S3). In fact, this glycan was the top-ranked glycan bound by Epa1 and ranked fourth from the top for glycans bound by Epa7. Although Epa6 demonstrated a broader specificity and was able to bind additional glycan structures, it also strongly binds Neu5Acα2-6(Galβ1-3) GalNAc and related glycans.

To test whether the Epa proteins were indeed the target ligands of NKp46 and NCR1, we analyzed the binding of NKp46-Ig and NCR1-Ig to Cg in which *EPA1*, *EPA6*, and *EPA7* were deleted (*Δepa1/6/7*). Interestingly, deletion of these three genes was sufficient to eliminate the binding of NKp46-Ig and NCR1-Ig to Cg (Figure 3F). To confirm this observation and to examine which of these three Epa adhesins NKp46 and NCR1 bind, we tested the binding of NKp46-Ig and NCR1-Ig to Epa1, Epa6, and Epa7 ectopically expressed in *Saccharomyces cerevisiae*. Using this system, we saw that NKp46-Ig and NCR1-Ig indeed bound directly to *S. cerevisiae* expressing any of Epa1, Epa6, or Epa7 but not to control *S. cerevisiae* expressing Epa4 (Figure 3G). These experiments identify fungal ligands for an NK cell receptor.

Clearance of Systemic Cg Infection In Vivo Is Dependent on Epa1, Epa6, and Epa7 Recognition by NCR1

We next sought to test the importance of NCR1 recognition of Epa1, Epa6, and Epa7 in vivo by intravenous (i.v.) injection of Cg in a non-lethal model of acute disseminated candidiasis (Arendrup et al., 2002; Brieland et al., 2001). Initially, we tested whether NCR1 has a significant role controlling Cg accumulation in this systemic infectious model. 3 days post-injection, the systemic fungal burden in wild-type and NCR1$^{-/-}$ mice was assessed by quantifying the number of Cg colony-forming units (CFUs) in their livers, spleens, and kidneys (Figure 4A). We observed that NCR1 is important for efficient clearance of systemic Cg infection, since NCR1$^{-/-}$ mice exhibited a significantly higher accumulation of Cg in all three sites of infection (Figure 4B). Furthermore, fungal burden levels were comparable to wild-type mice that underwent depletion of NK cells through administration of anti-NK1.1, indicating that NK cells are involved in Cg clearance (Figure 4B).

To determine whether the NCR1-mediated recognition of Cg was dependent on the Epa proteins, we performed additional in vivo experiments in which wild-type and NCR$^{-/-}$ mice were also infected with a Cg strain deleted for *EPA1*, *EPA6*, and *EPA7* (*ΔEPA1/6/7*). As in Figure 4B, we observed that the parental Cg strain (BG2) demonstrated a markedly exacerbated infection in NCR1$^{-/-}$ compared to wild-type mice (Figure 4C). Most importantly, however, when we used the *ΔEPA1/6/7* strain, a similar increase in Cg infectious load was not observed (Figure 4C). This indicated that this strain was not being targeted by NCR1 in the wild-type mouse due to the lack of its target ligands (Figure 4C). Despite demonstrating a relatively modest effect, these experiments were reproducible and show a crucial and direct role of NK cells in systemic control of Cg infections in vivo. Most importantly, we show that this process is dependent on NCR1 recognition of Epa1, Epa6, and Epa7.

DISCUSSION

In the current study, we identify the NK receptors NKp46/NCR1 as receptors that bind the fungal pathogen *Candida glabrata*. This study shows that binding of Cg by NK cell-activating receptors is crucial for clearing fungal infection in vivo. Importantly, we also identified three fungal ligands for NK receptors, the Cg adhesins Epa1, Epa6, and Epa7, and demonstrated that the interaction between Epa1, Epa6, and Epa7 and NCR1 is critical for clearance of Cg in vivo. *EPA1*, *EPA6*, and *EPA7* have been shown to encode the predominant mediators of binding in vitro to mammalian cells (Castaño et al., 2005; Cormack et al., 1999; Domergue et al., 2005).

The Epa proteins are lectins that bind glycans to mediate the first step of Cg infection (Maestre-Reyna et al., 2012; Zupancic et al., 2008). We show that the highest-affinity ligands for Epa1, Epa6, and Epa7 are glycans that are highly similar to those present on NKp46/NCR1 (Mendelson et al., 2010). NKp46 and NCR1 are structurally similar and share a conserved site for O-linked glycosylation at Thr225 (Glasner et al., 2015); mutating this site led to a loss of binding to Cg. Together with the fact that deletion of *EPA1*, *EPA6*, and *EPA7* eliminates binding of NKp46 and NCR1, these data strongly indicate that the binding of Epa1, Epa6, and Epa7 to NKp46/NCR1 is dependent on the O-linked glycosylation at Thr225.

Previous binding of hemagglutinin proteins by NKp46/NCR1 has been shown to be mediated by the sialic acid residues on the NKp46 glycosylations (Arnon et al., 2004; Bar-On et al., 2013; Mandelboim et al., 2001). However, sialic acids appear to play a different role with regard to Epa binding to its target glycan(s). We observed that the core structure that was bound by all three Epas was Galβ1-3GalNAc. Addition of a sialic acid to the terminal galactose residue (for example, glycan #244; Tables S1, S2, and S3) abolishes binding by Epa1, Epa7, and Epa6. In contrast, addition of a sialic acid (in 2-6 linkage) to the penultimate GalNAc, leaving the terminal galactose unmodified, are still strongly bound (Tables S1, S2, and S3). These data strongly suggest that binding to the NKp46/NCR1 by Epa1, Epa6, and Epa7 is driven primarily by the terminal unsubstituted galactose (rather than the sialic acid), and recognized glycans could include the glycan shown in Figure 3D, as well as potential variants, including asialo variants. Further studies will be required to clarify the native glycan structure(s) involved in this interaction.

As the site of the first encounter between host and pathogen, the host cell surface is the site of a constant arms race; the pathogens develop mechanisms of attachment and host cells develop evasion mechanisms. Glycosylation is an effective means to evade pathogen attachment mechanisms. In fact, all eukaryotic cells known to date are coated with complex glycosylation patterns (Varki and Sharon, 2009). Consequentially, a great majority of pathogens adhere to host cells through glycan targets, which they bind using lectins (Esko and Sharon, 2009). The present study highlights a mechanism developed by the host to elegantly counter this disadvantage by mimicking their targets on the NK receptors NKp46/NCR1.

NKp46/NCR1 targets a diverse range of viruses, including influenza, Sendai, Poxvirus, and Newcastle disease viruses, through their viral attachment receptor, the hemagglutinin lectin (Jarahian et al., 2009, 2011; Mandelboim et al., 2001).

The current study presents an additional example of NKp46/NCR1 targeting of pathogen glycan-binding attachment lectins, extending the recognition and protection beyond viruses and to fungi, an entirely different taxonomic kingdom. Our findings suggest that, in a sophisticated and elegant evolutionary countermeasure, NK cells have developed a similar glycosylation on the NKp46 and NCR1 receptors to mimic the cellular ligands of the viral hemagglutinins, the Cg Epa adhesins, and possibly other pathogen-associated lectins. Such a strategy would enable NK cells to target a conserved functional feature, rather than a direct molecular pattern, in a wide range of organisms. We thus speculate that NKp46/NCR1 may be a new type of pathogen recognition receptor. The host can modify cell surface glycosylations; however, microbes will always have the upper hand when it comes to adapting to infect the host due to their faster life cycle and adaptation mechanisms. This mechanism, whereby NK receptors express the same glycosylation as the host cells, levels out the playing field. Pathogens that successfully adapt to attach to their host target cell glycosylations would immediately be recognized by NK cells through such a bait glycosylation. NKp46-mediated recognition of Cg and the importance of NK cells in clearing systemic infection may also offer new potential clinical applications. Examples may include augmentation of NK immunity or use of NKp46-Ig as an opsonin to assist in precipitation and immune clearance of Cg. Exploring such options may be more relevant than ever due to the emergence of Cg as a serious health problem, particularly in light of the rapid appearance of resistance to current chemotherapeutic agents.

EXPERIMENTAL PROCEDURES

Cells and Mice

All Cg strains used in the paper were derived from BG2 (Cormack and Falkow, 1999). For ectopic expression of Epa1, Epa4, Epa6, and Epa7, *Saccharomyces cerevisiae* cells were used as previously described (Zupancic et al., 2008). Murine NK cells were prepared from BALB/c mice using the EasySep Mouse NK Cell Isolation Kit (STEMCELL). See also Supplemental Experimental Procedures.

FACS, BW Assays, and ELISA

FACS staining was performed using standard procedures. For BW assay, lag phase Cg cells were bound to 96-well plates. BW cells were then applied and co-cultured for 48 hr at 37°C. mIL-2 was quantified using ELISA. ELISA experiments were performed using standard procedures. Fusion proteins were applied at 0.05 μg/well and were detected by anti-human horseradish peroxidase (HRP) antibodies (Jackson ImmunoResearch Laboratories). See also Supplemental Experimental Procedures.

Blot

Cg cells were grown on Sabouraud agar plates for 48 hr and then absorbed into nitrocellulose membranes. Membranes were washed (PBS/tween 0.05%), blocked (5% milk/PBS), and stained with fusion proteins at 20 μg/mL 5% milk/PBS. Secondary staining (anti-human HR-conjugated secondary antibody [Jackson ImmunoResearch Laboratories]) was applied and membranes were developed. See also Supplemental Experimental Procedures.

In Vitro Killing Assay

Cg cells were co-cultured with purified mouse NK cells at effector/target ratio (E:T) 50:1 overnight at 37°C and plated on Sabouraud agar plates. Viable fungal colonies were counted 48 hr later as CFUs. See also Supplemental Experimental Procedures.

Disseminated Candidiasis Model of Infection

Protocol was based on Ferrari et al. (2009) with modifications. Each mouse was injected with 20×10^6 cells i.v. 3 days post-injection, mice were sacrificed and relevant tissues homogenized and plated. Cg colonies were counted after 48 hr and Cg burden was determined as CFUs/g tissue. See also Supplemental Experimental Procedures.

Glycan Arrays

Epa1, Epa6, and Epa7 ligand binding domains were prepared in HEK293 GnTI^{-} cells (Reeves et al., 2002). The Epa1, Epa6, and Epa7 N-terminal domains (Zupancic et al., 2008) were further refined by limited proteolysis. The corresponding Epa N-terminal domain gene sequences were cloned and made as soluble, secreted domains. Recombinant Epa proteins were purified using Ni-NTA resin (QIAGEN), eluted with binding buffer (BB) + 250 mM imidazole and verified using standard western blot. Epa proteins were screened for adherence to printed slide glycan arrays (version 5) developed by the Consortium for Functional Glycomics (http://functionalglycomics.org) as previously described (Blixt et al., 2004). See also Supplemental Experimental Procedures.

Statistical Analysis

Statistical studies were performed by Student's unpaired t tests (two-tailed) to compare differences among conditions. For in vivo experiments examining the role of Epa1, Epa6, and Epa7, two-way ANOVA was used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.09.008.

AUTHOR CONTRIBUTIONS

Conceptualization, A.V., B.P.C., and O.M.; Methodology, A.V., B.P.C., and O.M.; Investigation, A.V., Y.C.-A., R.Y., Y.B., B.I., N.S., O.B., L.D., M.G., C. Gur, A.G., C. Gomez, R.B.-A., and N.O.; Writing–Original Draft, A.V., B.P.C., and O.M.; Writing–Review & Editing, A.V., Y.C.-A., B.P.C., O.M., and R.Y.; Supervision, B.P.C. and O.M.; Project Administration, A.V., B.P.C., and O.M.

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