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Dectin-1/IL-15 Pathway Affords Protection against Extrapulmonary *Aspergillus fumigatus* Infection by Regulating Natural Killer Cell Survival

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Keywords
Aspergillus fumigatus · Dectin-1 · IL-15 · NK cells

Abstract
Aspergillus fumigatus is a ubiquitous, yet potentially pathogenic, mold. The immune system employs innate receptors, such as dectin-1, to recognize fungal pathogens, but the immunological networks that afford protection are poorly explored. Here, we investigated the role of dectin-1 in anti-*A. fumigatus* response in an experimental model of acute invasive aspergillosis. Mice lacking dectin-1 presented enhanced signs of inflammation, with increased production of inflammatory cytokines and neutrophil infiltration, quickly succumbing to the infection. Curiously, resistance did not require T/B lymphocytes or IL-17. Instead, the main effector function of dectin-1 was the preservation of the NK cell population in the kidneys by the provision of the cytokine IL-15. While the depletion of NK cells impaired host defense in wild-type mice, IL-15 administration restored antifungal responses in dectin-1-deficient mice. Our results uncover a new effector mechanism for dectin-1 in anti-*Aspergillus* defense, adding an alternative approach to understand the pathophysiology of this infection.

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Introduction

Aspergillus spp. are one of the most common fungi in the environment and essentially saprophytic microorganisms, but some species, such as *A. fumigatus*, are potentially pathogenic, able to cause a wide range of manifestations according to the immune status of the individual, ranging from mild allergic reactions to life-threatening invasive disease [1], due to the compromise of systemic organs as renal failure [2, 3]. Because of its ubiquitous nature, we are daily exposed to considerable amounts of fungal cells, yet, in normal conditions, we neither develop

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signs of infections nor experience overt inflammation, which suggests our immune system developed ways to balance efficient antifungal defense without deleterious inflammatory activation.

Most of our knowledge about the immunity to *A. fumigatus* was derived from immunosuppressed models, most commonly pharmacological immunosuppression [4], which, although valuable to mimic clinical conditions, might blunt the fully understanding of basic immune mechanisms as the administration of drugs may become a confounding factor for the investigation of the host-pathogen interaction. Regarding the contribution of the innate immunity to antifungal response, pattern recognition receptors from the C-type lectin (CLR) family, as dectin-1 (gene symbol, *Clec7a*) and dectin-2 (gene symbol, *Clec4n*), may be considered essential players due to their ability to recognize fungal structures, particularly carbohydrate-rich domains [5]. Indeed, the anti-*Aspergillus* role of dectin-1 in the lungs has been extensively explored, where it was shown to regulate the local inflammatory function of phagocytes [6–9].

However, considering the key role played by dectin-1 in anti-*Aspergillus* defense suggested by genetic studies that identified *Clec7a* polymorphisms as risk factors for developing severe aspergillosis [10–12], the receptor may also be critical for host defense in extrapulmonary locations. Since *A. fumigatus* is potentially invasive, able to cause disseminated disease, dectin-1 might also contribute to host defense in the periphery, yet this is still a poorly explored topic and possible effector mechanisms remain unknown. Here, we aimed to explore the effector mechanisms regulated by dectin-1 in extrapulmonary anti-*Aspergillus* defense.

Materials and Methods

Mice

Dectin-1-deficient (*Clec7a*$^{-/-}$), dectin-2-deficient (*Clec4n*$^{-/-}$), dectin-1/dectin-2-deficient (*Clec7a*$^{-/-}$–*Clec4n*$^{-/-}$), IL-17A/IL-17F-deficient (*Il17a*$^{-/-}$–*Il17f*$^{-/-}$), and rag2-deficient (*Rag2*$^{-/-}$) mice were used in C57BL/6J genetic background [13]. C57BL/6J wild-type (wt) mice were acquired from CLEA Japan (Tokyo, Japan). All mice were maintained under specific-pathogen free conditions with gamma ray-sterilized diet and acidified tap water (0.002 N HCl) ad libitum.

Fungal Strain and Inoculum Preparation

Aspergillus fumigatus MYA4609 strain (American Type Culture Collection, VA, USA) was used in this study. Fungal cultures were maintained in Sabouraud Dextrose Agar (BD, NJ, USA) at 30°C with weekly subcultures. For conidia production, colonies were seeded on Potato Dextrose Agar (PDA, Eiken Chemical, Tokyo, Japan) and incubated at 30°C for 5 days. Conidia were harvested in 0.1% Tween 80/PBS solution by gentle scraping, filtered through a 70 μm cell strainer (BD, NJ, USA) and resuspended in PBS, being kept at 4°C until use. For assays using swollen conidia or hyphae, fungal cells were incubated in Sabouraud Dextrose Broth (Glucose 20 g/L, Peptone 10 g/L, pH 5.6) for 3 h (swollen conidia) or 6 h (hyphae) at 37°C.

In vivo Infection

Animals were inoculated by intravenous (i.v.) route (lateral caudal vein) with 1×10^6 conidia cells in 100 μL of PBS. Animal weight and survival were monitored daily.

For organ analysis, mice were euthanized by cervical dislocation, and tissues were perfused with ice-cold PBS. Organs were harvested and weighted. For fungal burden and cytokine assays, organs were macerated in PBS through mesh sieves. Dilutions of the macerates were plated on PDA plates (Eiken Chemical, Tokyo, Japan), incubated at 30°C for 2 days, and recovered colonies-forming units (CFU) were counted. Fungal burden was expressed as CFU per gram of organ. The macerates were centrifuged at 14,000×*g* for 5 min, and supernatants were collected and stored at −80°C for cytokine analysis.

For histopathological analysis, animals were infected as described above. After euthanasia, animals were perfused with ice-cold PBS followed by 10% formalin solution. Harvested organs were fixed in 10% formalin solution and stored in 70% ethanol. Tissues were embedded in paraffin and stained by routine hematoxylin-eosin or periodic Schiff acid staining methods. Images were acquired by optical microscopy (BZ-9000 microscope, Keyence, IL, USA).

For assays with NK cell depletion, wt mice were treated intraperitoneally (i.p.) with 200 μg doses of *InVivo*Plus anti-mouse NK1.1 antibody (clone PK136) or *InVivo*Mab mouse IgG2a isotype control, unknown specificity (clone C1.18.4) (Bio X Cell, VT, USA) as described by [14] and infection was performed as shown in Figure 4d. For IL-15 treatment, *Clec7a*$^{-/-}$ mice received i.p. 0.5 μg doses of recombinant murine IL-15 (Peprotech, NJ, USA) after being infected as shown in Figure 6e. PBS (vehicle) was used as sham control.

Flow Cytometry Analysis

For cell phenotyping analysis, kidneys were harvested as described above, minced, and incubated in 10 mg/mL of Collagenase (from *Clostridium histolyticum*, Sigma-Aldrich, MO, USA) and 10 mg/mL of DNase (Sigma-Aldrich, MO, USA) in RPMI-1640 medium (Fujifilm Wako, Osaka, Japan) for 30 min at 37°C/5% CO_2. Enzymes were inactivated by addition of 10% inactivated Fetal Bovine Serum (Biosera, Nuaillé, France)/RPMI-1640 medium (Fujifilm Wako, Osaka, Japan). The organ suspension was filtered through a 70 μm cell strainer (BD, NJ, USA), and cells were centrifuged at 700×*g* for 5 min. Pelleted cells were submitted to Percoll 40/75% gradient (Percoll™, GE Healthcare, Uppsala, Sweden), centrifuged at 1,500×*g* for 30 min, and mononuclear cells (Percoll 40/75 interphase) were collected and stained for FACS analysis. The list of antibodies used in this work is shown in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000527188).

Data were acquired in a FACS Verse flow cytometer (8-color configuration, BD, NJ, USA) and analyzed in the software FlowJo (v.10.7.1 for Mac OS X, BD, NJ, USA). Gates were determined by

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the fluorescence minus one (FMO) method. Representative gating strategies for the analysis are shown in online supplementary Figure 8.

Tracking of IL-15-Producing Cells

Mice were infected with *A. fumigatus* intravenously as described and kept for 2 days. On the analysis day, mice received 0.25 mg of Brefeldin A (Tocris/Bio-Techne Japan, Tokyo, Japan) intravenously (from a 20 mg/mL stock solution in DMSO dissolved in 200 μL of PBS) and kept for 6 h, when the animals were euthanized, and the kidneys harvested after being perfused with PBS. Mononuclear cells were collected as described above, and IL-15 was stained by indirect intracellular staining. Samples were acquired in a FACS Verse flow cytometer (8-color configuration, BD, NJ, USA) and analyzed in the software FlowJo (v.10.7.1 for Mac OS X, BD, NJ, USA).

Peritoneal Recruitment of Neutrophils

Animals were inoculated with 1×10^7 conidia cells in 500 μL of PBS by intraperitoneal route. After 24 h, mice were euthanized, and the peritoneal cavity was washed with 5 mL of cold PBS. Cells in the peritoneal lavage were stained for FACS analysis.

Neutrophil Isolation and Infection

Neutrophils were isolated from bone marrows as described [15]. Briefly, bone marrows were extracted from femur and tibia, and red cells were removed by osmotic shock with hypotonic solution. Cells were submitted to percoll gradient density centrifugation (Percoll 62.5%, 1,000×*g*, 30 min), and neutrophils were recovered in the lower phase. Neutrophil's identity ($CD11b^+Ly6G^+$) was confirmed by flow cytometry, and cell purity was >90%.

After isolation, neutrophils were plated on 96-well plates and allowed to adhere for 45 min at 37°C and 5% CO_2. Cells were then incubated with *A. fumigatus* for 3 h. Cells were lysed with 1% Triton X-100 solution, and dilutions were plated on PDA plates. Recovered CFU were counted for determination of viable fungal cells. As reference to inoculum size, conidia cultured without neutrophils was run in parallel. Neutrophils were also co-cultured over glass microslides with fungal cells for 6 h and stained with Neat Stain Hematological Stain Kit (Polysciences Inc., PA, USA) and analyzed by optical microscopy (BZ-9000 microscope, Keyence, IL, USA).

Bone Marrow-Derived Macrophages

BMDMs were generated from bone marrows harvested from femur and tibia of wt, *Clec7a*$^{-/-}$, *Clec4n*$^{-/-}$, or *Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ mice and cultured in 10% inactivated Fetal Bovine Serum (Biosera, Nuaillé, France)/RPMI-1640 medium (Fujifilm Wako, Osaka, Japan) supplemented with 20 ng/ml GM-CSF (Invitrogen, CA, USA) at 37°C and 5% CO_2 for 7 days (cells received additional supplemented medium on day 4). On day 7, adherent cells were harvested in cold 0.02% EDTA/PBS solution and plated on 96 well (1×10^5 cells/well). After overnight resting, cells were incubated with swollen conidia (multiplicity of infection 1:1) and incubated for 24 h, when supernatants were harvested, centrifuged at 14,000×*g*/5 min, and stored at −80°C for cytokine analysis. Macrophage identity and expression of CLRs were confirmed by FACS phenotyping ($CD11b^+F4/80^+$).

Cytokine Measurements

Cytokines (except IL-15) were quantified in organ macerates by BD Cytometric Bead Array assay, according to the manufacturer's instructions. Data were acquired in FACS Verse (BD, NJ, USA) and analyzed in the software FCAP Array (v.3.0.1; BD, NJ, USA). The detection limits were as follows: IL-1β = 1.9 pg/mL; IL-6 = 1.4 pg/mL; IFN-γ = 0.5 pg/mL. Any values below the limit of detection were normalized as half the nominal value of the detection limit. IL-15 was quantified by sandwich ELISA using a commercially available kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Statistics

Statistical analyses were performed in the software GraphPad Prism (v. 9 for OSX, GraphPad Inc., CA, USA). The statistical test and the number of individuals/replicates in each experiment are disclosed in the figure legends. A *p* value below 0.05 was considered as statistically significant for all analyses.

Results

Dectin-1 Is Essential for Extrapulmonary Anti-Aspergillus Response

Based on the current understanding of the natural history of invasive aspergillosis, the infection initiates by the inhalation of conidia, when the fungus colonizes the lungs and, whether able to transpose the local defenses, can reach the bloodstream and go to extrapulmonary sites, leading to the disseminated form of the disease [16]. Yang et al. [17] showed that the immunosuppressor cyclophosphamide, commonly used in experimental models of aspergillosis [4], also represses dectin-1 expression, which hinders investigations about its role in the host defense. Thus, to initially assess the contribution of dectin-1 to anti-*Aspergillus* response, we infected immunocompetent wt mice and animals lacking dectin-1/dectin-2 (*Clec7a*$^{-/-}$–*Clec4n*$^{-/-}$ double knockouts) through the intratracheal (i.t.) route and followed their outcome (shown in online suppl. Fig. 1).

In agreement with previous reports [6, 7], *Clec7a*$^{-/-}$–*Clec4n*$^{-/-}$ mice did show enhanced fungal colonization in the lungs, but no remarkable extrapulmonary dissemination was noticed (shown in online suppl. Fig. 1b). However, when we accompanied their evolution, these animals not only did not develop severe disease, presenting any weight loss or remarkable alterations in other clinical parameters (shown in online suppl. Fig. 1a, b), as they cleared the infection as efficiently as the wt counterparts (shown in online suppl. Fig. 1c). Thus, albeit dectin-1 might regulate the anti-*Aspergillus* defense in the lungs in the first stages of the infection, its deficiency can be compensated by other clearance mechanisms.

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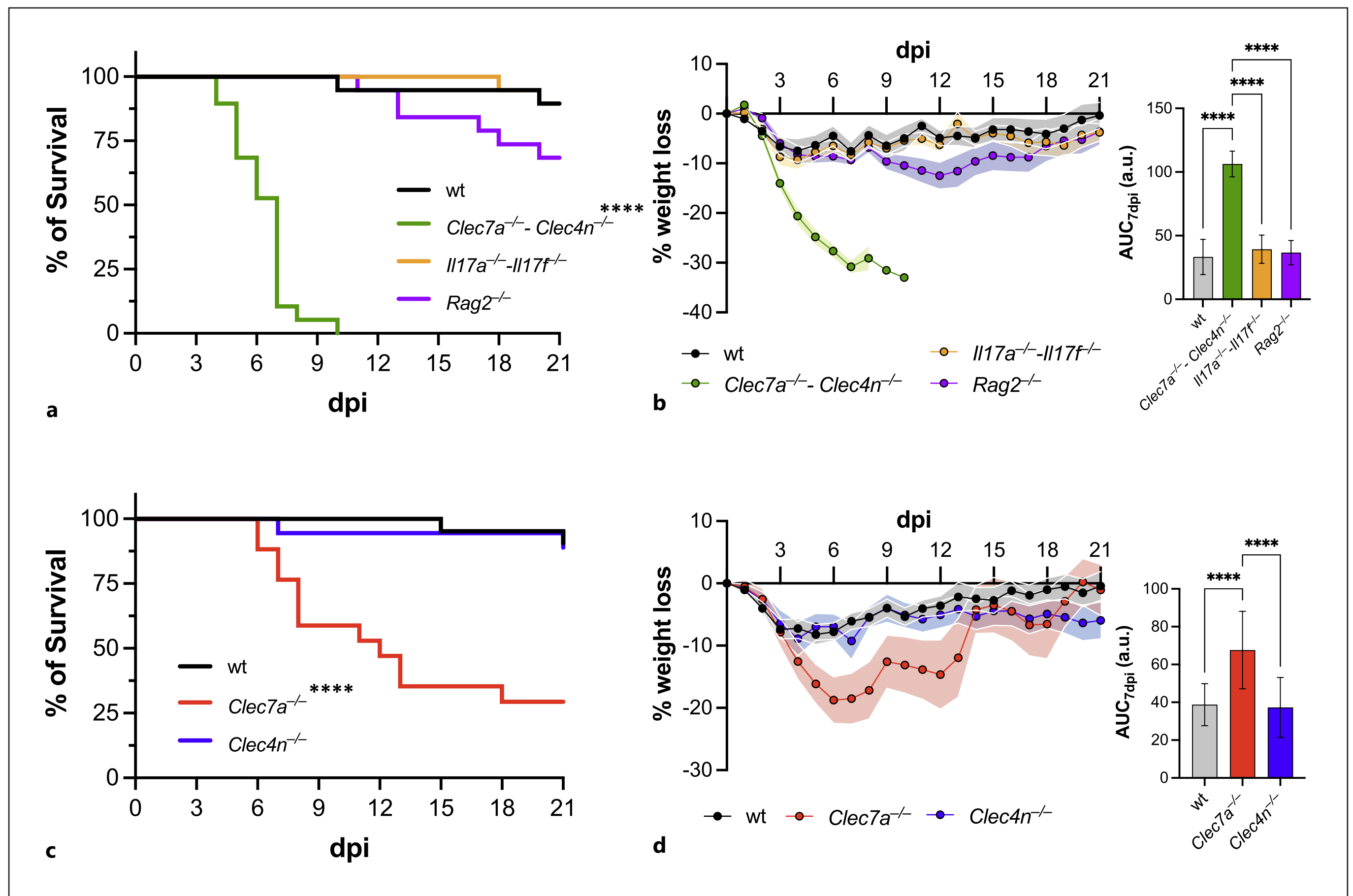

Fig. 1. Dectin-1 is essential for survival against *A. fumigatus* infection while T/B lymphocytes and IL-17 are dispensable. **a**, **b** wt, *Clec7a*$^{-/-}$ *Clec4n*$^{-/-}$, *Il17a*$^{-/-}$*Il17f*$^{-/-}$, and *Rag2*$^{-/-}$ mice were infected intravenously with 1×10^6 *A. fumigatus* conidia. Body weight and survival were monitored 21 days post infection (dpi). **c**, **d** wt, *Clec7a*$^{-/-}$, and *Clec4n*$^{-/-}$ mice were infected intravenously with 1×10^6 *A. fumigatus* conidia. Body weight and survival were monitored 21 dpi. **a**, **c** Survival plots, Log-Rank test: ****$p < 0.0001$. **b**, **d** Body weight loss (BWL) plots and corresponding area under the curve for the first week, data shown as mean ± s.e.m (shadowed area in BWL plots). One-way ANOVA and Fisher's LSD test: ****$p < 0.0001$.

We hypothesized that dectin-1 could be more critically involved in another step of the infection pathogenesis whether the fungus has already crossed the pulmonary barrier. To test this assumption, but because the i.t. cannot be used for successful infection, we opted for an i.v. inoculation route, to bypass the resistance of the lung barrier, and followed the outcome of the infected animals (shown in Fig. 1a, b). Astonishingly, in this model of extrapulmonary aspergillosis, *Clec7a*$^{-/-}$–*Clec4n*$^{-/-}$ mice were very susceptible, promptly succumbing to the infection, whereas the wt counterparts retained their resistant phenotype.

When dectin-1 and dectin-2 were assessed individually, only *Clec7a*$^{-/-}$ mice succumbed to the infection, whereas mice lacking dectin-2 performed as well as wt animals (shown in Fig. 1c, d). Although *Clec7a*$^{-/-}$ mice start to die during the second week postinfection, the response driven by dectin-1 in the first week of infection was key to determine the animals' fate. Thus, we observed that *Clec7a*$^{-/-}$–*Clec4n*$^{-/-}$ and *Clec7a*$^{-/-}$ mice show a significant weight loss in this period (shown in Fig. 1d). Therefore, the contribution of dectin-1 to anti-*Aspergillus* response is even more pronounced outside the lungs than in the primary site of exposure.

Lymphocytes and IL-17 Are Dispensable in Extrapulmonary Aspergillosis

The antifungal role of IL-17 response in *A. fumigatus* infection is controversial: while some authors report a protective contribution in the lungs [6, 18], other works suggest it worsens the infection pathology [19, 20]. But

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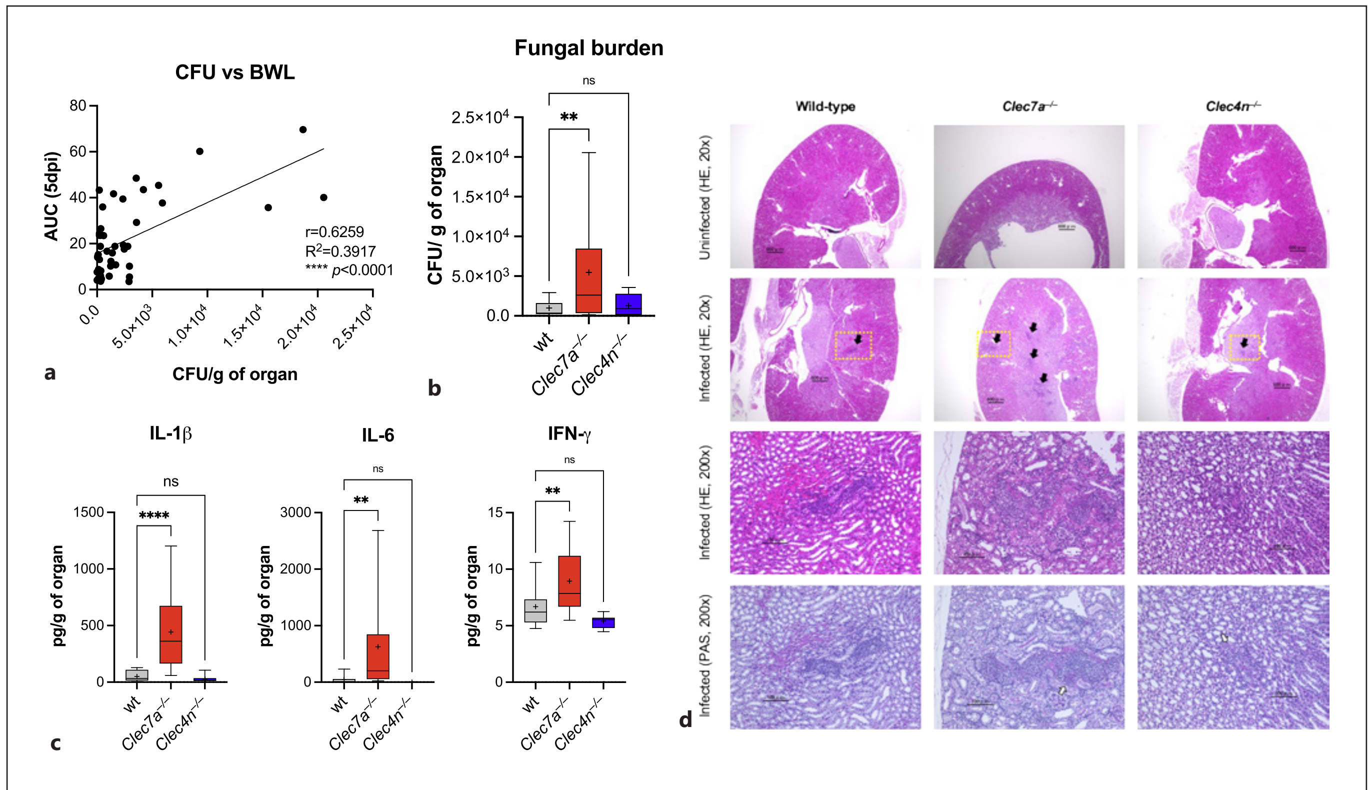

Fig. 2. *Clec7a−/−* mice display enhanced fungal colonization and inflammation. wt, *Clec7a*$^{-/-}$, and *Clec4n*$^{-/-}$ mice were infected intravenously with 1×10^6 *A. fumigatus* conidia, and organ analysis was performed 5 days post infection (dpi). **a** Pearson correlation between fungal burden and the accumulated body weight loss during the first week (expressed as area under the curve, AUC). **b** Fungal burden in kidneys macerates, expressed as the number of colony-forming units per gram of tissue. **c** Cytokine levels for IL-1β, IL-6, and IFN-γ in kidney macerates, expressed as pg of cytokine per gram of tissue. Data shown in **a** and **c** are expressed as boxplots (line indicates median and +, mean values). One-way ANOVA and Fisher's LSD test: ns, not significant; $^{**}p < 0.01$; $^{****}p < 0.0001$. N = 15–16 animals/group, data pooled from three independent experiments. **d** Micrographs of kidney sections stained with hematoxylin-eosin (HE, first three rows) or periodic acid Schiff (PAS, bottom row) methods: arrows point to inflammatory foci with presence of polymorphonuclear cells and fungal structures. Yellow delimited section was magnified in the bottom two rows. Data representative of 4 mice per group.

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considering CLRs can shape the adaptive immunity toward an T_H17 profile [21], we explored next whether the protective effect of dectin-1 here could be linked to IL-17. By comparing the performance of mice lacking T/B cells (*Rag2*$^{-/-}$ knockout) or IL-17A/F (*Il17a*$^{-/-}$–*Il17f*$^{-/-}$ double knockouts), we observed that neither deficiency in lymphocytes nor in the cytokines had significant effect in the outcome of the infection, as both groups performed as well as the wt mice (shown in Fig. 1a, b). These data suggest that dectin-1 triggers key antifungal effector mechanisms beyond the induction of a classical adaptive response.

Remarkably, we observed the same results with a clinical isolate of *A. fumigatus* (IFM 47439 [22]) as shown in online supplementary Figure 2. Thus, while neither lymphocytes, IL-17 nor dectin-2 were essential to allow survival against *A. fumigatus* IFM 47439, dectin-1 is still required for host defense, ruling out bias due to strain-specific characteristics.

Clec7a$^{-/-}$ *Mice Display Heightened Inflammation*

We next aimed to understand how dectin-1 contributes to anti-*Aspergillus* defense. Interestingly, we noticed that the renal fungal burden correlated with the weight loss in those animals (shown in Fig. 2a), indicating that the infection severity is linked to ineffective fungal clearance. As expected, *Clec7a*$^{-/-}$ mice presented a higher fungal burden compared to wt and *Clec4n*$^{-/-}$ counterparts (shown in Fig. 2b). Although we also observed colonization of the spleen, dectin-1/-2 deficiency showed no correlation with the fungal burden there (shown in online suppl. Fig. 3a).

Because CLRs are important initiators of the inflammatory response [23], we presumed that the observed phenotype in *Clec7a*$^{-/-}$ mice could be attributed to a defective inflammatory response. Surprisingly, however, mice lacking dectin-1 presented an enhanced, instead of reduced, inflammation, illustrated by a heightened production of IL-1β, IL-6, and IFN-γ (shown in Fig. 2c). In addition, *Clec7a*$^{-/-}$–*Clec4n*$^{-/-}$ mice also displayed higher fungal burden and inflammatory markers (shown in online suppl. Fig. 4a, b).

In accordance with the cytokine profile, the histopathological analysis of the kidneys showed an increased number of inflammatory foci in *Clec7a*$^{-/-}$ mice (shown in Fig. 2d), surrounding fungal structures. Because the histological features of most of the cells in these inflammatory infiltrates were suggestive of polymorphonuclear cells, we analyzed their phenotype by flow cytometry. Indeed, the immune cell composition of *Clec7a*$^{-/-}$ mice kidneys was characterized by a higher neutrophil infiltrate ($CD11b^+Ly6G^+$) on day 5 (shown in Fig. 3a). Renal populations of macrophages and dendritic cells, however, were not altered (data not shown). Curiously, if we administer *A. fumigatus* i.p., which is a known model to trial phagocyte recruitment, neither the lack of dectin-1 nor dectin-2 altered significantly the cell composition in the peritoneal lavage (shown in Fig. 3b), although neutrophil recruitment in response to i.p. administration of *C. albicans* is known to be dectin-1 dependent [24]. Thus, the contribution of dectin-1 to phagocyte recruitment is dependent on the type of pathogen involved, and it is not an intrinsic feature of the receptor.

Neutrophils display strong antifungal activities due to their immunochemical arsenal, but they can also cause excessive, even lethal, host damage through the same mechanisms [25]. We considered next whether *Clec7a*$^{-/-}$ mice presented defective neutrophil responses, being unable to eliminate the pathogen albeit their high numbers in the infiltrates. But, when we incubated bone marrow-purified neutrophils with different morphologies of *A. fumigatus* (resting conidia, swollen conidia, and hyphae) and analyzed fungal survival, no significant differences could be detected in their killing ability (shown in Fig. 3c), as cells from wt, *Clec7a*$^{-/-}$ and *Clec4n*$^{-/-}$ genotypes seemed to interact identically with the fungal structures in vitro (shown in Fig. 3d).

Hence, our data suggest that the main extrapulmonary function of dectin-1 against *A. fumigatus* does not rely on canonical inflammatory responses. Without dectin-1, the fungal burden is not controlled, which promotes a deleterious inflammatory environment with excessive neutrophil influx that can lead to host death before pathogen clearance and infection resolution. Thus, dectin-1 might regulate an alternative antifungal mechanism which allows *Aspergillus* elimination instead of triggering strong inflammatory reactions.

NK Cell Survival Is Compromised by Dectin-1 Deficiency

The kidneys harbor a heterogeneous population of immune cells, particularly those belonging to the lymphoid lineage as T and NK cells [26]. While our previous data on *Rag2*$^{-/-}$ mice (shown in Fig. 1a) ruled out the involvement of classical lymphocytes, experiments performed with *Scid-Il2rγ*$^{-/-}$ mice pointed to a high susceptibility profile in response to *A. fumigatus* infection (shown in online suppl. Fig. 5), indicating the participation of other lymphoid cells. Thus, we investigated whether NK cells could be involved, since there is strong evidence in the literature associating defects in the NK cell pool to the

Fig. 3. Neutrophil recruitment is enhanced in vivo by dectin-1/-2 deficiency, response in vitro is not affected. **a** Quantification of neutrophil infiltrate in infected kidneys by flow cytometry analysis. wt, *Clec7a*$^{-/-}$, and *Clec4n*$^{-/-}$ mice were infected intravenously with 1×10^6 *A. fumigatus* conidia and, 5 days post infection (dpi), organs were harvested and submitted to enzymatic digestion for isolation of mononuclear/immune cells. Neutrophils were characterized and expressed as percentage (or cell counts) of $CD11b^+/Ly6G^+$ cells within the live leukocyte population ($CD45^+$/Live gate), representative dot plots are shown in parallel. $N = 6$ animals/group, data pooled from two independent experiments. **b** Quantification of neutrophil infiltrates in peritoneal cavity by flow cytometry analysis. wt, *Clec7a*$^{-/-}$, and *Clec4n*$^{-/-}$ mice were inoculated intraperitoneally with 1×10^7 *A. fumigatus* conidia and cells in peritoneal lavage were analyzed after 24 h. $N = 10$–11 animals/group, data pooled from three independent experiments. Data from **a** and **b** are expressed as boxplots (line indicates median and +, mean values). One-way ANOVA and Fisher's LSD test: ns, not significant; ****$p < 0.0001$. **c** Fungicidal ability of bone marrow-purified neutrophils challenge in vitro with *A. fumigatus* conidia (multiplicity of infection 100 neutrophils per fungi) for 3 and 6 h. Data are expressed as the percentage of reduction in the number of colony-forming units recovered compared to fungal cells cultured without neutrophils as inoculum reference. Data expressed as mean ± SD from three independent experiments. **d** Representative images of neutrophil/*A. fumigatus* interaction (multiplicity of infection 1 neutrophil per 1 conidium) showing neutrophils phagocytosing conidia and concentrating around germlings after 6 h of interaction. i.p., intraperitoneally.

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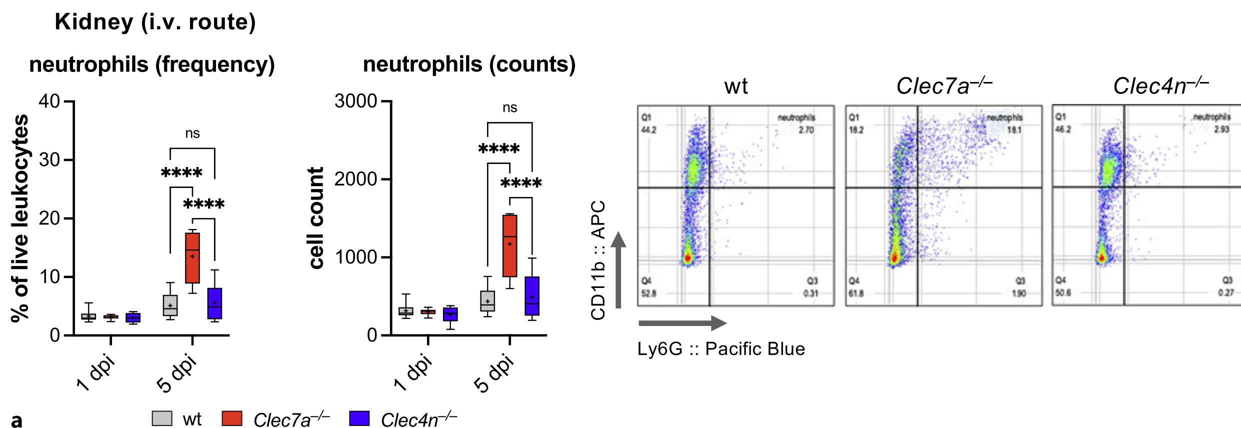

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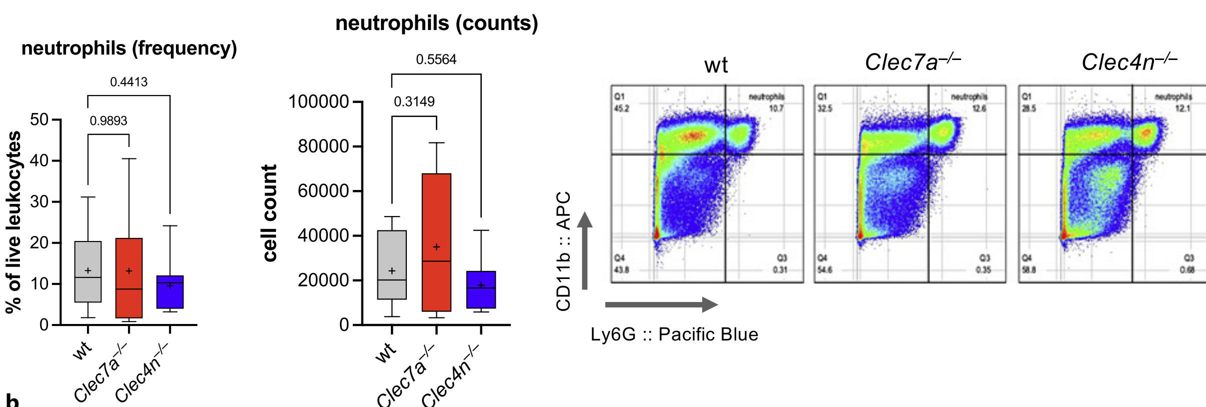

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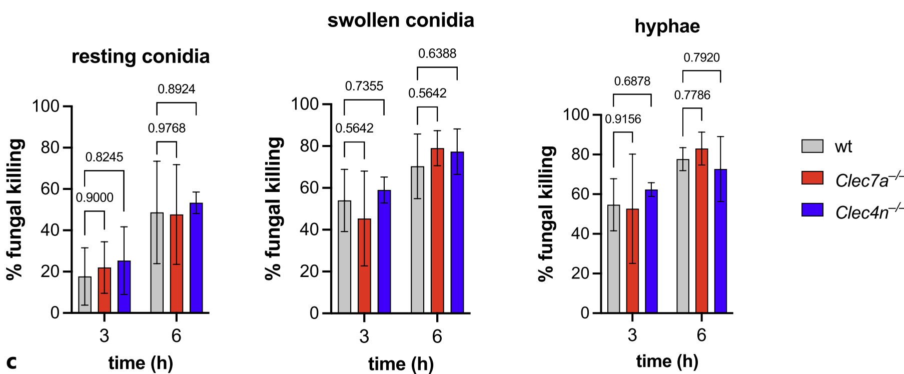

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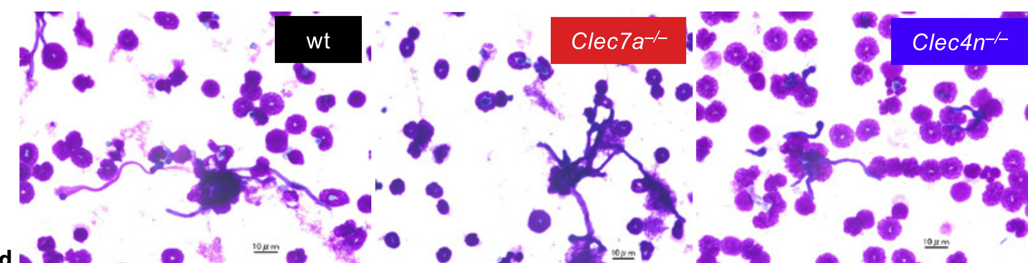

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development of invasive fungal disease [27–29] and that NK cells display direct antifungal activities against a plethora of fungi, including *Aspergillus* spp. [30].

Strikingly, we did observe that mice lacking dectin-1 showed a significant reduction in the NK cell pool in response to *A. fumigatus* infection (shown in Fig. 4a). Most of the lymphoid cells in the kidneys are tissue-resident and, in accordance, no changes in CD69 expression (an activation marker expressed by resident lymphocytes) were detected (shown in Fig. 4b). However, we noted that NK cells from *Clec7a*$^{-/-}$ mice lost expression of CD94 (shown in Fig. 4b), an inhibitory receptor whose downmodulation is associated to increased cell death [31]. Indeed, a higher proportion of dead NK cells was detected in these knockout mice (shown in Fig. 4c).

To confirm whether NK cells are required for anti-*Aspergillus* defense in our model, we performed antibody-mediated depletion of those cells in wt mice and assessed their response (shown in Fig. 4d). As expected, depletion of NK cells compromised the survival of the mice, favored an increased weight loss (shown in Fig. 4e), and fungal burden (shown in Fig. 4f). Thus, it is likely the main working mechanism is the maintenance of NK cell population.

Aspergillus-Induced IL-15 Is Dectin-1 Dependent

Because expression of dectin-1 in NK cells is known to be minimal, if any [32, 33], the receptor might influence the cellular pool indirectly, regulating the production of essential cell factors. To investigate how the survival of NK cells is being compromised by dectin-1 deficiency, first, the death profile of those cells in infected animals was characterized by annexin V/PI assay (shown in Fig. 5a). Curiously, most of the dead cells in dectin-1-deficient mice presented an apoptotic profile, suggesting the lack of pro-survival signals.

Among the immune components involved in NK cell biology, a key cytokine for their survival and maintenance is IL-15 [34], and therefore, we assessed its levels in kidney macerates. Interestingly, IL-15 levels were significantly reduced by dectin-1 deficiency but were unaffected in *Clec4n*$^{-/-}$ mice, compared to the wt group (shown in Fig. 5b and online suppl. Fig. 3c). In addition, IL-15 levels correlated negatively with the fungal burden in the kidneys (shown in Fig. 5c), pointing to a protective role for this cytokine.

Domínguez-Andrés et al. (2017) [35] showed that type I interferon-dependent production of IL-15 by splenic monocytes is involved in the anti-*Candida* response, allowing NK cell activation, which then promotes neutrophils recruitment and fungal clearance. Although our data also points to IL-15 in anti-*Aspergillus* response, different mechanisms are likely involved. Aside from our previous result that neutrophil infiltrates are enhanced in *Clec7a*$^{-/-}$ kidneys (shown in Fig. 3a), the spleens do not rely on dectin-1/-2 for the response, considering neither the splenic fungal burden nor their IL-15/IFN-β levels were altered among the groups (shown in online suppl. Fig. 3b). In accordance, splenic IL-15 showed no association with the fungal burden and, therefore, could not explain *Clec7a*$^{-/-}$ and *Clec7a*$^{-/-}$–*Clec4n*$^{-/-}$ mice increased susceptibility (shown in online suppl. Fig. 3c).

The mature form of IL-15 is produced by different cell types, but macrophages are a primary source in many tissues [36], and we investigated whether their IL-15 production could be dectin-1 dependent. Thus, we tracked the main source of *Aspergillus*-induced IL-15 in our model by phenotyping the mononuclear cell fraction in the kidneys from infected mice (shown in Fig. 5d). Interestingly, *A. fumigatus*-driven IL-15 production was limited to Dectin-1 expressing cells, which we further

Fig. 4. Renal NK cells are dependent on dectin-1 and are required for anti-*A. fumigatus* defense. wt, *Clec7a*$^{-/-}$, and *Clec4n*$^{-/-}$ mice were infected intravenously with 1×10^6 *A. fumigatus* conidia, and analysis was performed at 5 days post infection (dpi). **a** Quantification of NK cell population in infected kidneys. Organs were harvested and submitted to enzymatic digestion for isolation of mononuclear/immune cells. NK cells were characterized by flow cytometry analysis and expressed as percentage (or cell count) of CD3ε$^-$/NK 1.1$^+$ cells within the live leukocyte population (CD45$^+$/Live gate), representative dot plots are shown in parallel. **b** Expression of CD69 and CD94 by NK cells, data are expressed as median fluorescence intensity (MFI) values. **c** Percentage of dead NK cells. Data from **a–c** are shown as boxplots (line indicates median and +, mean values). One-way ANOVA and Fisher's LSD test: ns, not significant; **$p < 0.01$; ****$p < 0.0001$. $N = 6$ animals/group, data pooled from two independent experiments. **d–f** Depletion of NK cells. wt mice were submitted to NK cell depleting antibody (or isotype control) treatment as depicted in **d** and infected with 1×10^6 *A. fumigatus* conidia. Survival and weight loss were monitored. **e** Survival, body weight loss (BWL), and area under the curve (AUC, first week) plots for the groups. **f** Fungal burden in kidneys macerates at 5 dpi is expressed as number of colony-forming units per gram of tissue. Data from **e** are shown as mean ± SEM (shadowed area in BWL plots) and in **f** as boxplots (line indicates median and +, mean values), pooled from 4 independent experiments. Unpaired *t* test: *$p < 0.05$; ****$p < 0.0001$ or Log-Rank test: *$p < 0.05$ (survival). $N = 15$–16 animals/group or $N = 6$ animals/group (survival). i.p., intraperitoneally.

(For figure see next page.)

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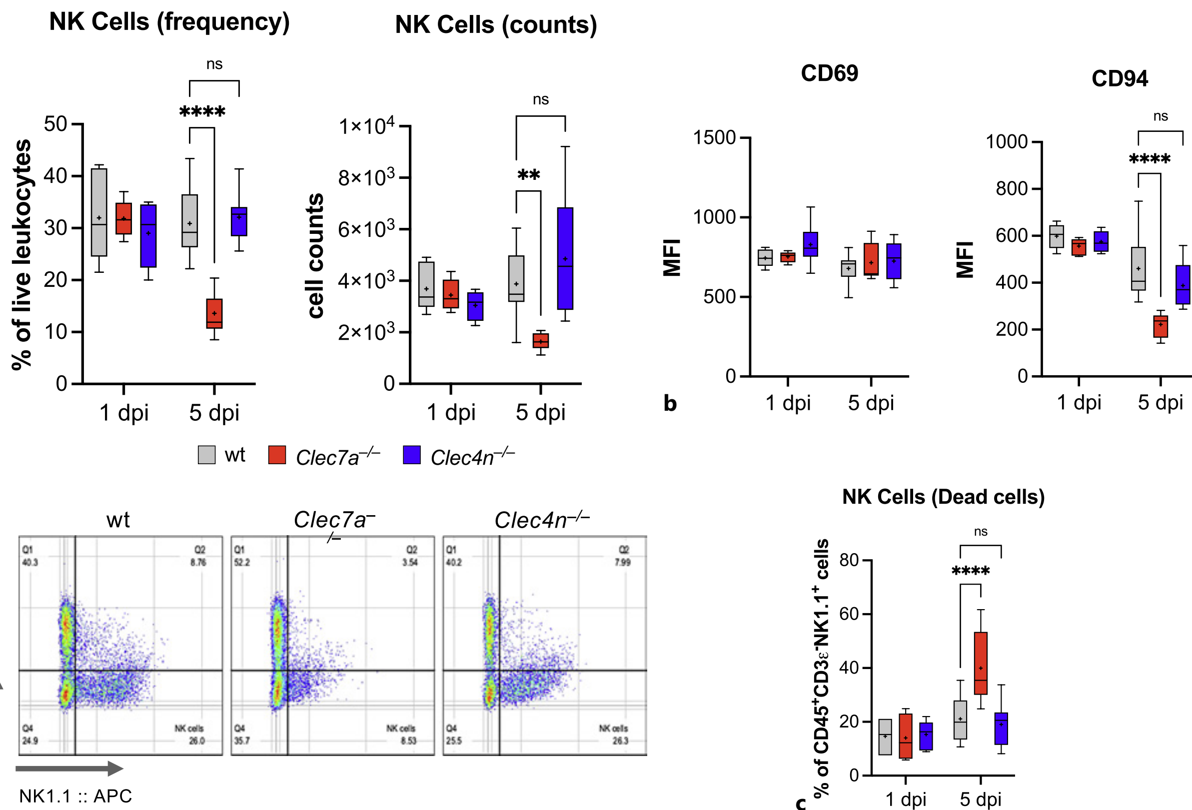

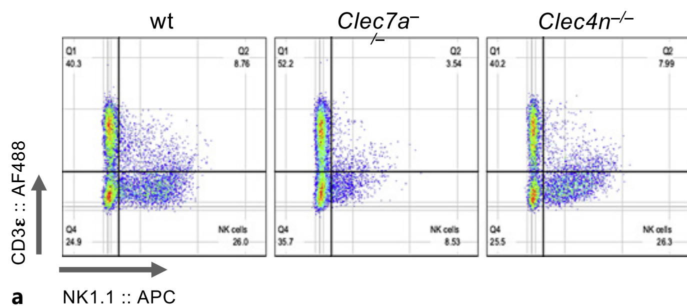

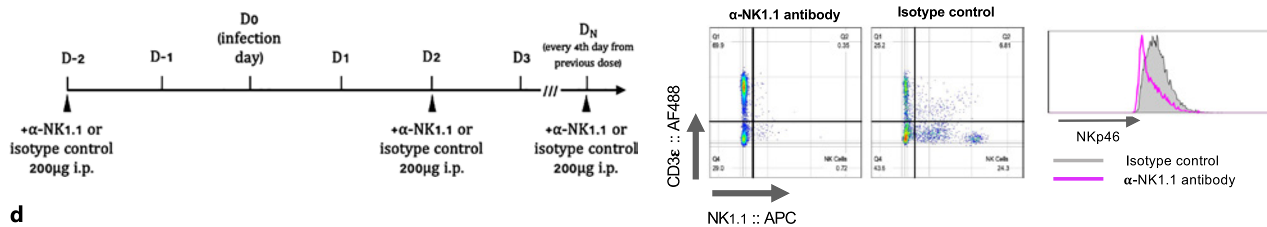

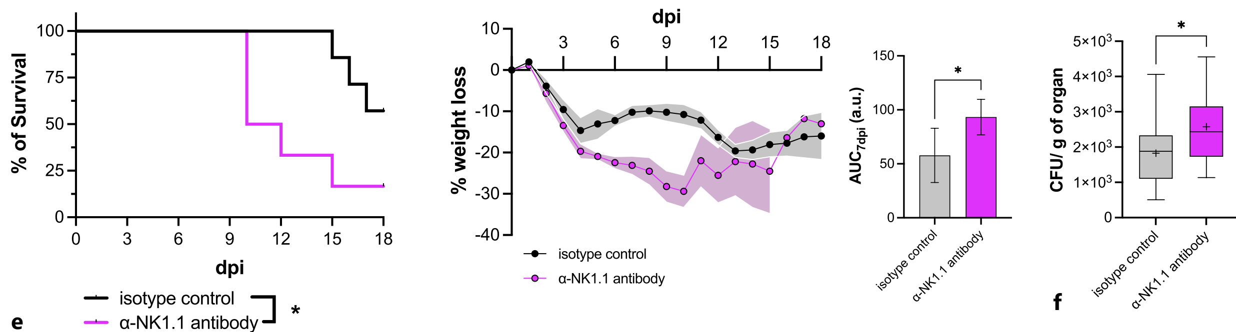

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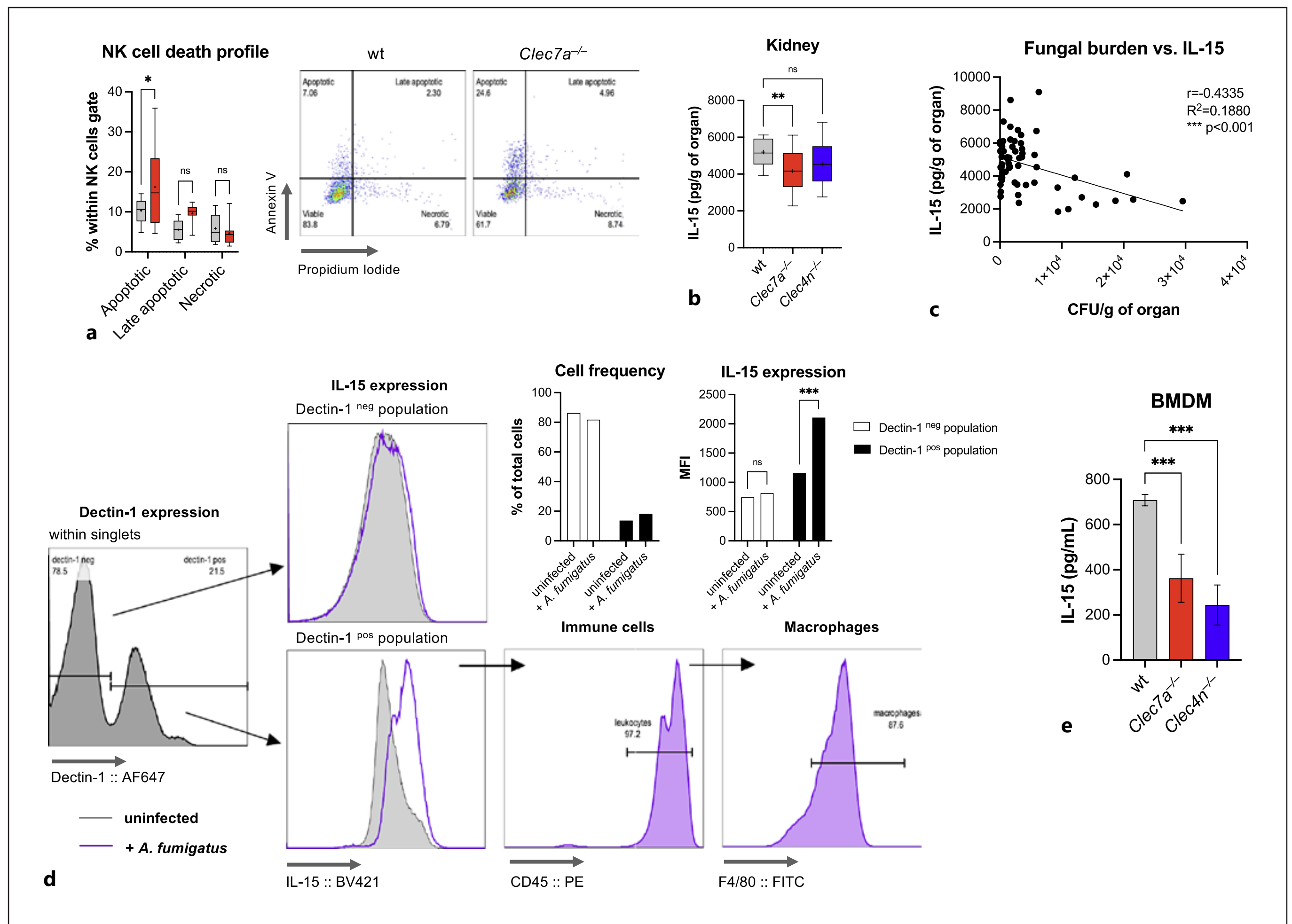

Fig. 5. Dectin-1 drives IL-15 production in response to *A. fumigatus*. **a** NK cell death profile was assessed by Annexin V/Propidium iodide staining. Mice were infected intravenously with 1×10^6 *A. fumigatus* conidia and, 5 days post infection (dpi), organs were harvested and submitted to enzymatic digestion for isolation of mononuclear/immune cells, $N = 8$ animals/group. Two-way ANOVA and Fisher's LSD test: $*p < 0.05$. **b** IL-15 levels were measured in kidney macerates from wt, *Clec7a*$^{-/-}$ and *Clec4n*$^{-/-}$ mice after 5 days of being infected intravenously with 1×10^6 *A. fumigatus* conidia, data expressed as pg of IL-15 per gram of tissue. $N =$ 15–16 animals/group, data shown as boxplots (line indicates median and +, mean values) were pooled from three independent experiments. One-way ANOVA and Fisher's LSD test: ns, not significant; $**p < 0.01$. **c** Pearson correlation between fungal load and IL-15 levels. **d** Tracking of IL-15 in vivo. Mice (wt) were infected intravenously with 1×10^6 *A. fumigatus* conidia, and analysis was performed in kidney suspensions 2 days post infection by flow cytometry. $N = 2–4$ animals/group. Mean ± SEM. One-way ANOVA and Fisher's LSD test: $***p < 0.001$. **e** BMDMs generated from wt, *Clec7a*$^{-/-}$ and *Clec4n*$^{-/-}$ mice were cultured with *A. fumigatus* conidia and IL-15 levels were measured in the supernatants. Data were shown as mean ± SD from three independent experiments. One-way ANOVA and Fisher's LSD test: $***p < 0.001$. CFU, colonies-forming units.

identified as macrophages (F4/80 expressing cells). To confirm this finding, we incubated *A. fumigatus* with BMDMs and measured IL-15 production in the supernatants. As expected, macrophages derived from *Clec7a*$^{-/-}$ mice were impaired in the cytokine secretion in response to fungal stimulation (shown in Fig. 5e), indicating the cytokine is a downstream product of dectin-1 activation.

Curiously, *Clec4n*$^{-/-}$ cells were also defective in the cytokine production in vitro (shown in Fig. 5d), suggesting that dectin-2 might still act as a sensor for *A. fumigatus* as already observed by others [37]. Hence, we sought to confirm whether these CLRs were indeed able to directly recognize our strain of *A. fumigatus* since antigen masking is a common evasion strategy for this pathogen [38]. First, we employed chimeric proteins

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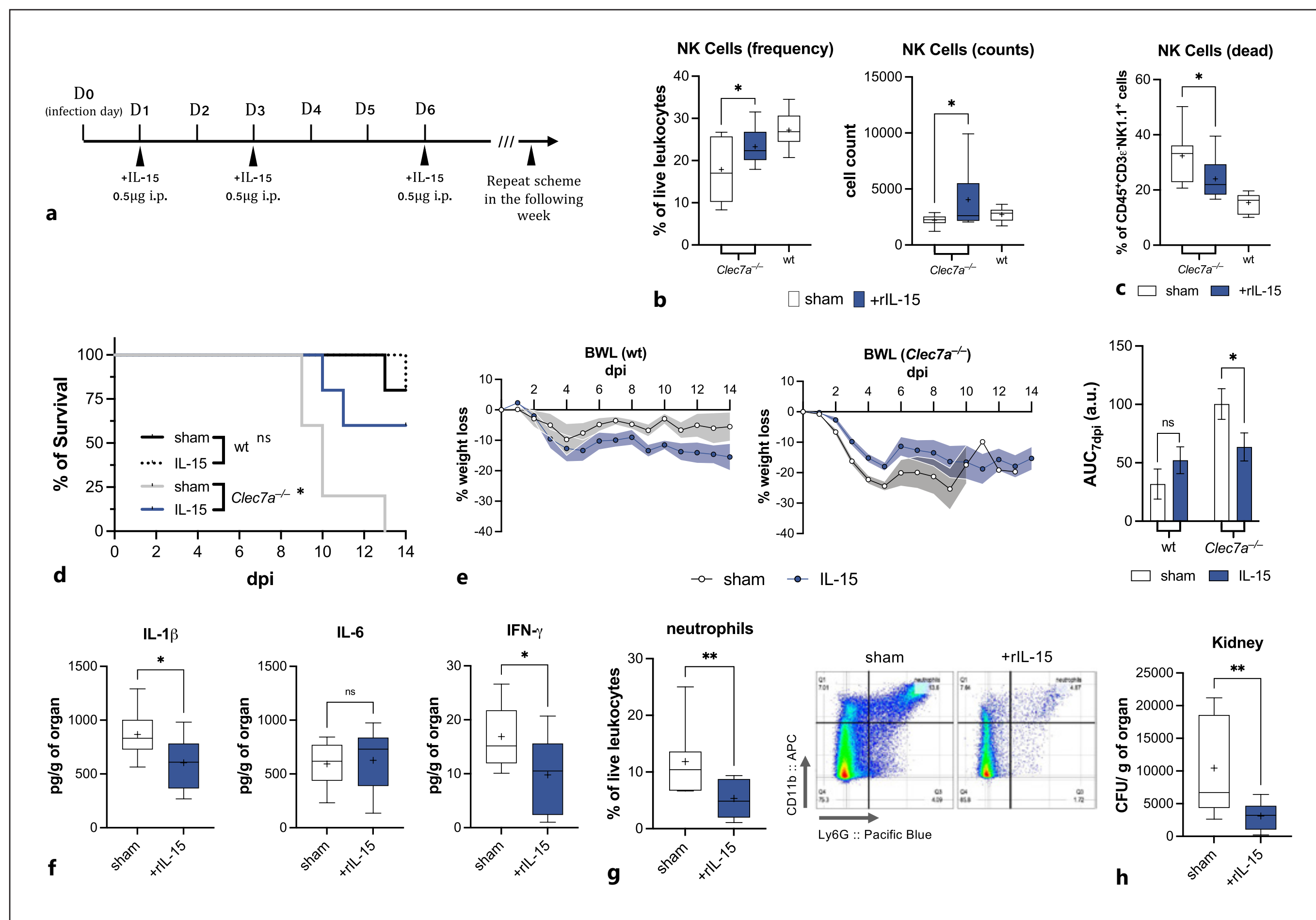

Fig. 6. IL-15 treatment restores anti-*Aspergillus* response to *Clec7a*$^{-/-}$ mice. **a** Scheme for the treatment of *Clec7a*$^{-/-}$ mice with recombinant IL-15 after *A. fumigatus* infection. **b** Increase in the NK cell population 5 days post infection (dpi). NK cell frequency and cell counts were analyzed by flow cytometry, identified as CD3ε^{-}/NK 1.1^{+} cells within the live leukocyte population (CD45^{+}/Live gate). **c** Percentage of dead NK cells. Data shown as boxplots (line indicates median and +, mean values). One-way ANOVA and Fisher's LSD test: *$p < 0.05$; **$p < 0.01$. **d** Survival of IL-15 treated mice. $N = 8$ animals/group, Log-Rank test: *$p < 0.05$. **e** Body weight loss and area under the curve (AUC, first week) plots. Data shown as mean ± SEM (shadowed area in BWL plots). Unpaired t test: *$p < 0.05$. $N = 8$ animals/group. **f** Cytokine levels for IL-1β, IL-6, and IFN-γ in kidney macerates of *Clec7a*$^{-/-}$ mice expressed as pg of cytokine per gram of tissue. **g** Neutrophil influx in the kidneys of *Clec7a*$^{-/-}$ mice at 5 dpi. Neutrophils were analyzed by flow cytometry, identified as CD11b^{+}Ly6G^{+}cells within the live leukocyte population (CD45^{+}/Live gate). **h** Fungal burden in kidneys macerates harvested from of *Clec7a*$^{-/-}$ mice at 5 dpi is expressed as number of colony-forming units per gram of tissue. **f–h** Data shown as boxplots (line indicates median and +, mean values). Unpaired t test: ns, not significant; *$p < 0.05$, **$p < 0.01$. $N = 7–11$ animals/group, pooled from two independent experiments.

(where the CLR extracellular/binding domain was fused to the Fc portion of human IgG) in an ELISA assay. As a second approach, we used 43-1 reporter cells (NFAT-GFP reporter gene) that were engineered to express murine dectin-1 or dectin-2 fused with FcRγ chain – thus, receptor activation would lead to GFP expression, detected by flow cytometry. As expected, in both assays direct binding to *A. fumigatus* was confirmed (shown in online suppl. Fig. 6a, b).

Next, we inquired the expression of dectin-1 and dectin-2 in kidney macrophages. Strikingly, renal macrophages do not express significant levels of dectin-2, only dectin-1, unlike other macrophages populations, as BMDMs or peritoneal macrophages (shown in online suppl. Fig. 6c). To validate this result, we consulted the public database Expression Atlas [39] and confirmed that, both in humans and mice, while dectin-1 expression occurs in a diverse set of tissues, dectin-2 is more

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restricted and negligible in the kidneys (shown in online suppl. Fig. 6d). Thus, dectin-2 seems to be dispensable in our model because it is not expressed substantially in the target organ albeit its intrinsic ability to recognize *A. fumigatus*.

IL-15 Treatment Restores Antifungal Defense to Clec7a$^{-/-}$ *Mice*

Whether IL-15 is downstream of dectin-1 activation, cytokine supplementation would compensate for the receptor deficiency. Thus, to confirm this assumption, we treated the mice with recombinant IL-15 after fungal inoculation (shown in Fig. 6a). As expected, cytokine treatment restored the NK cell population (shown in Fig. 6b), reducing the pool of dead cells (shown in Fig. 6c). Consequently, IL-15-treated *Clec7a*$^{-/-}$ mice displayed enhanced survival (shown in Fig. 6d) and were more protected from weight loss (shown in Fig. 6e).

In agreement with the better fitness to the infection, they also showed reduced levels of IL-1β and IFN-γ (shown in Fig. 6g) and reduced infiltration of neutrophils (shown in Fig. 6h), suggesting amelioration of the inflammatory condition. Most importantly, the fungal burden was significantly reduced by the treatment (shown in Fig. 6h), pointing to the restoration of the antifungal response. Therefore, IL-15 is a downstream mechanism of dectin-1 activation, maintaining the NK cell pool for the efficient anti-*Aspergillus* response.

Discussion/Conclusion

In this study, we uncovered a new effector mechanism for dectin-1 in the host defense against *A. fumigatus* based on the provision of IL-15 and maintenance of the NK cell pool. Because acute tissue inflammation can be as harmful to the host as an insufficient response [40], efficient fungal clearance mechanisms need to conciliate fungicidal activity with mild inflammatory collateral damage.

Albeit some authors reported death of dectin-1 knockout animals by i.t. infection with a higher fungal inoculum [6, 9], it should be pointed that they still did not observe fungal colonization outside the lungs [6], suggesting that those mice might have succumbed due to local pulmonary failure instead of disseminated disease. In fact, the requirement of a massive infectious dose to achieve lethality in animals lacking dectin-1 through i.t. route, in contrast to their remarkable susceptibility by an i.v. challenge that we described here (up to 80x higher if compared to the model described by Dutta et al. [9]), reinforces our idea that dectin-1 main contribution occurs outside the lungs. Thus, albeit dectin-1 is still a player for pathogen restriction in the pulmonary barrier [41, 42], it is not a key gatekeeper at this interface.

Reinforcing this notion, it is interesting to notice that human data about dectin-1 genetic polymorphisms and invasive aspergillosis observed this link in immunosuppressed subjects (as cancer or post organ transplantation patients) [12], albeit they lacked a former history of *Aspergillus* infections previously to their immunodepression. Because clinical immunosuppression (pharmacological or secondary to infections as by HIV) is not lymphocyte-specific, as other immune cells, as NK cells, or even the expression of immune receptors can be affected either [43–46], it is likely that these depressive states weaken the pulmonary defenses which facilitate the fungus to transpose the lungs. It is at this point that dectin-1 response becomes essential, and those with susceptible gene variants are prone to severe invasive disease. It can also explain why *Rag2*$^{-/-}$ mice were not more susceptible either, unlike immunosuppressed patients, since classical lymphocytes *strictu sensu* may not be critical in primary infection compared to innate immunity components.

Apart from classical lymphocytes, NK cells are lymphoid cells recently recognized by their antifungal potential [30], displaying direct *Aspergillus* killing activity [47]. Although Soe et al. [48] showed that NK cells treated with the dectin-1 antagonist laminarin present lower killing activity, hinting a direct role of dectin-1 in fungal recognition by those cells, Zhu et al. [49] reported, in the opposite direction, that laminarin can increase NK cell cytotoxicity, prompting the expression of activation markers, granzyme B and perforin. Since the expression of dectin-1 in NK cells is considerably low [32, 33] and laminarin may display off-target effects in parallel to dectin-1 blockade, the working mechanism behind those findings remains to be investigated.

Instead of a cell-intrinsic action, our observations that dectin-1 can induce IL-15 production in response to *A. fumigatus* stimulation indicate that the receptor allows the NK cell pool to respond and adapt to environmental cues. Hence, in a scenario of infection where NK cells are demanded, the host boosts its IL-15 production in response to pathogen molecules recognized by innate immune receptors. Reversely, without dectin-1 participation, the steady-state input of IL-15 does not fulfill the new requirements of the NK cell population to assure an optimal response. Interestingly, human data on single nucleotide polymorphisms in the IL-15 gene have already been suggested as a susceptibility factor for the

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development of chronic cavitary pulmonary aspergillosis [50], indicating this cytokine is an important player in anti-*Aspergillus* defense.

IL-15 is considered a key cytokine for NK cell development, maintenance, and functionality. Most of its action relies on three signaling pathways: MAPK cascade (Ras/Raf/MAPK axis), STAT5 cascade, and mTOR pathway (PI3K/AKT/mTOR axis), which regulate different steps in the differentiation and metabolic program of this population [51, 52]. Because NK cells are heavily dependent on IL-15 inputs, a fine balance in the cytokine pool needs to be maintained. While NK cell proliferation rate is directly proportional to IL-15 concentration at normal conditions, insufficient cytokine provision favors their prompt contraction by altering the balance between pro- and antiapoptotic proteins [51]. Nevertheless, excessive and chronic exposure to IL-15 can lead ultimately to NK cell exhaustion [53], which needs to be considered for translating those findings into clinical use.

It was surprising, however, that *Clec7a*$^{-/-}$ mice displayed higher neutrophil influx since neutropenia is a known risk factor for aspergillosis [54]. Indeed, neutrophils are still required for host defense in our model, too, since antibody-mediated neutropenia was also lethal (shown in online suppl. Fig. 7). Notwithstanding, dectin-1 did not influence the infection outcome in this context, as both groups (neutropenic wt and neutropenic *Clec7a*$^{-/-}$ mice) are equally susceptible but displaying different kinetics than non-neutropenic mice. These data reinforce the notion that immunosuppression is a strong bias for the study of basic immune mechanisms (although important for clinical investigations) since it can mask the contribution of innate receptors.

The dysregulated activity of neutrophils can be as harmful as its absence, causing tissue damage and compromising the organ function [55]. Thus, in a disbalanced environment where the NK cell population is compromised, the host may only rely on stronger inflammatory cells, which promote an excessive and deleterious inflammatory milieu, leading to host death before the efficient fungal clearance.

Even though defects in the effector function of *Clec7a*$^{-/-}$ neutrophils have been described by others [6], in contrast to our observations; Gazendam et al. [56] indicated that the main pathway for fungal binding to neutrophils relies on the recognition of opsonins (as antibodies or complement proteins) instead of dectin-1. Curiously, Shepardson et al. [57] uncovered an important role for dectin-1 mediated recognition under hypoxia conditions, which affect the β-glucan exposure in the *Aspergillus* surface, suggesting that the degree of dectin-1 contribution in neutrophil response is also influenced by environmental conditions in addition to cell-intrinsic variables.

Many works indicate that the way the immune system deals with *Aspergillus* infections is heavily determined by the site of the disease. For example, in models of localized infection, as *Aspergillus*-induced keratitis, neutrophils were shown to be key for fungal clearance in a dectin-2/IL-17-dependent way [58]. Recently, in a model of pulmonary aspergillosis, plasmacytoid dendritic cells were shown to regulate phagocyte recruitment in a chemokine-dependent fashion [59]. Here, we highlight the contribution of dectin-1/IL-15/NK cells crosstalk. Considering that we are daily exposed to *Aspergillus* spp., our immune system may have acquired different strategies to control the pathogen to maximize the preservation of homeostasis. Hence, localized infections may benefit from a strong, yet limited, inflammatory reaction (as a localized influx of neutrophils), whereas a "leakage" to extrapulmonary sites may benefit from mild responses to minimize the collateral damage.

In summary, we proposed a new effector mechanism for dectin-1 in anti-*A. fumigatus* immunity. By allowing the recognition of *Aspergillus* structures, dectin-1 drives the production of IL-15, preserving the local NK cell population, which promotes fungal clearance without triggering an excessive inflammatory reaction. Future works may provide promising insights into the relationship between CLRs and NK cell function in human infection, particularly considering that NK cell-based therapies are almost a reality for anti-tumoral protocols [60] and could be translated for invasive aspergillosis therapy.

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Statement of Ethics

Experiments were conducted in accordance with the "Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the Jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology" (Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006). The protocols have been

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reviewed by the Institutional Animal Care and Use Committee from Chiba University and approved under the process numbers A2-39 and A2-40 (animal experimentation), BT29-14 (biosafety), and G29-6 (recombinant DNA experiments).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

FSY Yoshikawa and S Saijo designed the study and wrote the paper. FSY Yoshikawa, M Wakatsuki, K Yoshida, R Yabe, and S Torigoe performed experiments. S Yamasaki and GN Barber provided reagents and discussed the results. S Saijo supervised the study.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request at saijo@faculty.chiba-u.jp.

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