The inhibitory effects of these cytokines were less observed in the co-culture system than in the RANKL–M-CSF system (Figs. 1, B and C, and 2 A). We infer that osteoblasts may provide membrane-bound RANKL and stimulate costimulatory signals for RANKL simultaneously, enabling the strong cell–cell contact between osteoblasts and osteoclast precursor cells and preventing the access of T cells or inhibitory cytokines to osteoclast precursor cells.

Previous observations that IL-12 and IL-18, which drive Th1 differentiation, both inhibit osteoclastogenesis via IFN-γ or GM-CSF (43, 44), and that IL-10, which is released from Th2 cells, also negatively regulates osteoclastogenesis (45) further support the negative role of Th1 and Th2 cells on osteoclastogenesis. In contrast, Th17 cells stimulated by IL-23 promote osteoclastogenesis mostly through production of IL-17 (Fig. 3, A and C). Therefore, the osteoclastogenic ability of Th17 cells does not require cell–cell contact with osteoclast precursor cells, but additional membrane-bound mediators such as RANKL and CD40L may also contribute (46, 47). IL-17 is known to act on the osteoclastogenesis-supporting cells to induce RANKL (25). It should be noted that the effect of IL-17 is not limited to this direct effect on the osteoclastogenesis-supporting cells. IL-17 facilitates local inflammation by recruiting and activating immune cells, which leads to an abundance of inflammatory cytokines such as TNF-α and IL-1 (9, 10). The inflammatory cytokines enhance RANKL expression on osteoclastogenesis-supporting cells and activate osteoclast precursor cells by synergizing with RANKL signaling. A relatively high expression of RANKL on Th17 cells may also participate in the enhanced osteoclastogenesis (Fig. 3 E). Collectively, Th17 cells can be called an osteoclastogenic Th subset not only because Th17 cells have positive effects on osteoclastogenesis in vitro, but also because they tip the balance of the microenvironments in favor of osteoclast differentiation.

It is worth noting that Th17 cells do not induce osteoclastogenesis in the absence of osteoblasts. This strongly suggests that RANKL expressed on Th17 cells alone is not sufficient to induce osteoclastogenesis, although this is partly because even Th17 cells produce a small amount of IFN-γ, which counterbalances the RANKL action. To understand the role of RANKL on T cells in more detail, we need mice of T cell–specific ablation of the *RANKL* gene, which are currently unavailable. But it is conceivable that RANKL expressed on adherent cells such as osteoblasts has more potent effects than that expressed on T cells. This mechanism may also explain why osteoclasts are formed only in the bone microenvironments, but it currently remains to be clarified. We consider the following explanations: (a) T cell expression of membrane-bound RANKL, which is more osteoclastogenic than the soluble form (48), is very low compared with that on osteoblasts; (b) costimulatory signals provided specifically by osteoblasts (12, 27) are missing in T cells; and (c) cell adhesion induces specific signals including those mediated by integrins, which are also important for osteoclastogenesis (49).

In our study, T reg cells had no apparent effect on osteoclastogenesis in vitro (Fig. 1 G). However, their function in the regulation of bone metabolism should be investigated in vivo considering the recent finding that the development of Th17 cells and T reg cells is coordinately regulated (10, 35, 36).

The importance of the IL-23–IL-17 axis in the autoimmune inflammation has been demonstrated in a variety of models of autoimmune diseases such as arthritis and encephalomyelitis (23, 24, 38). In arthritis models, *IL-17$^{-/-}$* mice were protected from the development of destructive arthritis (24), whereas collagen-induced arthritis is exacerbated in IFN-γ receptor–deficient mice (21, 22). The specific role of IL-23 compared with IL-12 in the development of arthritis has been clearly demonstrated by a genetic study using mice deficient in p19 and p35 (23). Based on these observations, the IL-23–IL-17 axis inducing Th17 cells, rather than the IL-12–IFN-γ axis inducing Th1 cells, is critical for the development of autoimmune arthritis. Our study also provides evidence that the IL-23–IL-17 axis plays a critical role even in a model of bone loss induced by local inflammation that is independent of autoimmunity (Fig. 4 D), suggesting that the IL-23–IL-17 axis is not only essential for the onset phase, but also for the destruction phase of autoimmune arthritis characterized by the T cell–mediated activation of osteoclastogenesis. Thus, Th17 cells, an osteoclastogenic subset, have profound relevance in the bone damage that takes place in autoimmune arthritis. The identification of T cell subsets in the synovium of arthritis is a challenging issue of great importance that should be pursued in a future study. Considering the strong inhibitory effects of Th1 cells on osteoclastogenesis, Th17 cells may be overwhelmingly dominant and the colocalization of Th1 cells is unlikely, at least under the microenvironments in which osteoclastogenesis efficiently occurs. The positive correlation between IL-23 and RANKL expression in the synovium of RA patients further suggests the importance of IL-23 in the regulation of local osteoclastogenesis through IL-17 (Fig. 5 A). Despite the importance of TGF-β and IL-6 in the initiation of Th17 development (10, 35, 36), Th17 cells can be obtained in an IL-23–stimulated culture system without adding exogenous TGF-β/IL-6, suggesting that the endogenous level of TGF-β/IL-6 may suffice for the initiation and that osteoclastogenic activity of Th17 cells is mainly determined by IL-23 under certain pathological conditions.

For the treatment of RA, there are several drugs available, most of which were developed to modulate immune reactions. The antirheumatic drugs are effective in treating pain and inflammation, but patients still fairly frequently have to undergo joint replacement surgery because of the progressive bone damage despite long-term treatment with antirheumatic drugs. Therefore, it is clinically an urgent issue to establish a method to prevent such persistent bone destruction (3). Although rheumatologists are now aware of the great impact that anti-TNF therapy has had on the management of RA (50), it is still not determined whether all patients respond

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to the therapy or, indeed, whether bone destruction will be completely prevented by it. Recent progress in understanding the mechanism of bone loss in RA has provided promising new strategies, one of which is an anti-RANKL antibody directly suppressing RANKL-mediated osteoclastogenesis (51). As we have demonstrated a new role of Th17 in the context of bone damage in RA, the significance of the IL-23–IL-17 axis extends beyond the simple initiation or development of the autoimmunity. Because osteoclastogenic Th17 cells link the autoimmune inflammation to bone damage, inhibition of this axis has the potential of a doubly beneficial impact on RA, i.e., in the context of both the immune and skeletal systems, and thus appears to be an ideal therapeutic strategy for ameliorating the bone destruction associated with T cell activation.

MATERIALS AND METHODS

Mice. $Ifngr1^{-/-}$ (28), $Stat6^{-/-}$ (30), $Il17^{-/-}$ (37), and $Il23a^{-/-}$ mice (38) were described previously. All the mice were maintained under specific pathogen-free conditions and were backcrossed to C57BL/6 mice. All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

Analysis of bone phenotype and LPS-induced bone destruction. The mice were subjected to histomorphometric and microradiographic examinations as described previously (27). 8-wk-old mice were injected with 25 mg/kg body weight LPS (Sigma-Aldrich) subperiosteally in the calvarial bone. After 5 d, calvarial bones were analyzed as described previously using decalcified paraffin sections (14).

In vitro assays for osteoclast differentiation and function. In vitro osteoclast differentiation was described previously (27, 52). For the RANKL–M-CSF system, we cultured BMCs with 10 ng/ml M-CSF (R&D Systems) for 2 d and used them as BMMs. The cells were cultured with 50 ng/ml RANKL (PeproTech) and 10 ng/ml M-CSF for 3 d, and TRAP$^+$ multinucleated (more than three nuclei) cells were counted. The co-culture of osteoblasts derived from mouse calvarial cells and BMCs was performed in the presence of 10^{-8} M VitD$_3$ (Wako) and 10^{-6} M PGE$_2$ (Wako) for 7 d. For the assessment of the bone-resorbing function of osteoclasts, we cultured osteoclast precursors on a hydroxy appatite–coated disc (Osteologic; BD Biosciences). After the culture period, the cells were washed away as described in the manufacturer's protocol by 6% NaOCl and 5.2% NaCl.

Th cell differentiation. CD4$^+$ T cells were purified from the spleen using a magnetic sorter and anti-CD4 microbeads (MACS; Miltenyi Biotec). The purity of the CD4$^+$ T cells was >95%. These CD4$^+$ T cells were stimulated with a plate-bound anti-CD3 mAb and anti-CD28 mAb (1 μg/ml each) for 3 d in the presence of (a) 10 ng/ml IL-12 and 10 μg/ml anti–IL-4 mAb for the Th1 cells, (b) 10 ng/ml IL-4 and 10 μg/ml anti–IFN-γ mAb for the Th2 cells, and (c) 10 ng/ml IL-23 along with 10 μg/ml each of anti–IFN-γ and anti–IL-4 mAbs for the Th17 cells. When indicated, the T cells were added to the culture system with 1 μg/ml anti-CD3 mAb for restimulation. All the antibodies were purchased from BD Biosciences except for the anti-RANKL mAb (provided by H. Yagita, Juntendo University School of Medicine, Tokyo, Japan). Recombinant IL-17 and the other cytokines were purchased from Genzyme and R&D Systems, respectively. T reg cells were purified using a MACS CD4$^+$CD25$^+$ Regulatory T Cell Isolation kit.

Analysis of mRNAs expressed in RA synovial tissues. Synovial tissues were obtained at the time of total knee arthroplasty from five patients (age range, 55–70 yr) who fulfilled the American College of Rheumatology criteria and gave informed consent (16). The experiments were performed with the approval of the institutional ethical committee. The tissues were minced and homogenized in Sepasol-RNA (Nacalai Tesque), and total RNA was extracted and purified according to the manufacturer's protocol.

GeneChip analysis and quantitative RT-PCR. Total RNA (15 μg) was used for cDNA synthesis by reverse transcription followed by the synthesis of biotinylated cRNA through in vitro transcription. After cRNA fragmentation, we performed hybridization with a mouse A430 GeneChip (Affymetrix, Inc.) (31). We performed quantitative RT-PCR using a LightCycler (Roche), as described previously (52). The following primers were used: *IL23A*: 5'-CTGCTTGCAAAGGATCCACC-3' (sense), 5'-TTGAAGCGGAGAAGGAGACG-3' (antisense); *IL12A*: 5'-AGCC-TCCTCCTTGTGGCTA-3' (sense), 5'-TGTGCTGGTTTTATCTT-TTGTG-3' (antisense); *IL12B*: 5'-TCACAAAGGAGGCGAGGTT-3' (sense), 5'-ATGACCTCAATGGGCAGACTC-3' (antisense); and *RANKL*: 5'-AACCAGATGGGATGTCGGTGGCATTA-3' (sense), 5'-AGCGAT-GGTGGATGGCTCATGGTTAG-3' (antisense). The level of mRNA expression was normalized with that of *GAPDH* expression in Fig. 5 A.

Statistical analyses. All data were expressed as the mean ± SEM ($n = 4$, unless otherwise indicated). Mann-Whitney U test was used for statistical analyses (*, $P < 0.05$; **, $P < 0.01$), and comparisons were made between each sample and the control (not treated with T cells/cytokines or WT mice).

Online supplemental material. Fig. S1 shows the effect of recombinant IL-4 on osteoclast precursor cells derived from WT or $Stat6^{-/-}$ mice in the RANKL–M-CSF system. Fig. S2 shows the list of genes whose expression was increased by IL-4 in osteoclast precursor cells (GeneChip analysis). Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20061775/DC1.

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Mast cells contribute to initiation of autoantibody-mediated arthritis via IL-1

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Notes:

Mast cells contribute to initiation of autoantibody-mediated arthritis via IL-1

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Mast cells are immune sentinels that participate in the defense against bacteria and parasites. Resident within the joint, mast cells become activated in human rheumatoid arthritis and are implicated in the pathogenesis of experimental murine synovitis. However, their arthritogenic role remains undefined. Using a model of autoantibody-induced arthritis, we show that mast cells contribute to the initiation of inflammation within the joint by elaboration of IL-1. Mast cells become activated to produce this cytokine via the IgG immune complex receptor FcγRIII. Interestingly, mast cells become dispensable for the perpetuation of arthritis after delivery of IL-1, highlighting the contribution of this lineage to arthritis induction. These findings illuminate a mechanism by which mast cells can participate in the pathogenesis of autoimmune inflammatory arthritis and provide insights of potential relevance to human rheumatoid arthritis.

cytokine | mouse model | synovitis

Rheumatoid arthritis (RA) is a destructive inflammatory disease of the joints affecting up to 1% of the population (1). Although disease pathogenesis remains obscure, evidence implicates T and B cells as well as innate immune elements (2). Recently, therapeutic success with B cell depletion and appreciation of the diagnostic utility of antibodies against citrullinated peptides has renewed interest in the role of antibodies in this disease, highlighting previous research on the prevalence of circulating and synovial immune complexes as well as abnormal IgG glycosylation in patients with RA (3–6).

To understand the mechanisms whereby antibodies may cause arthritis, attention has turned to animal models of autoantibody-mediated joint disease. One of these is the K/BxN model, which shares with RA a dependence on T cells, B cells, and certain cytokines as well as clinical features, including symmetry, a distal-to-proximal gradient of severity, and the formation of erosive pannus within the joint (7). K/BxN arthritis results from polyclonal IgG antibodies against the autoantigen glucose-6-phosphate isomerase, whose pathogenic effector pathways include the C5a receptor (CD88), the IgG receptor FcγRIII, and the IL-1 receptor IL-1R1 (8–12). TNF can contribute but is not absolutely required. At the cellular level, depletion of neutrophils abrogates disease. Strikingly similar pathogenic pathways characterize other murine models of arthritis, including collagen-induced arthritis, suggesting that these mechanisms reflect a general pathway to arthritis (13).

Recently, studies in the K/BxN model have demonstrated a pathogenic contribution by mast cells (14–16). Mast-cell-deficient mice (W/W^v and Sl/Sl^d) are resistant to arthritis, whereas engraftment of W/W^v animals with wild-type mast cells restores susceptibility. However, the pathways by which mast cells participate in antibody-mediated arthritis remain incompletely defined.

Investigating the role of the mast cell in K/BxN serum-transfer arthritis, we found that a brief course of exogenous IL-1 could bypass the requirement for mast cells in W/W^v mice. Engraftment of cultured IL-1$^{-/-}$ mast cells into these mast-cell-deficient animals confirmed that mast cells are an obligate source of this cytokine in our experimental system. Interestingly, although arthritis in IL-1$^{-/-}$ mice provided with a pulse of exogenous IL-1 was transient, arthritis in mast-cell-deficient W/W^v mice treated similarly exhibited a normal course, highlighting the contribution of mast cells in disease initiation. Using in vitro studies and in vivo reconstitution experiments, we show that production of IL-1 by mast cells results from ligation of FcγRIII. Together, these findings demonstrate that mast cells can provide a "jump start" for IgG-mediated inflammatory joint disease via IL-1.

Results

Essential Role of IL-1 in K/BxN Arthritis. In other models of inflammation initiated via mast cells, TNF has emerged as the cytokine of critical importance (17–19). However, a substantial fraction of TNF-null animals administered K/BxN serum develop arthritis (12), implying that other mast cell mediators are operative in this context. Because previous work has shown that IL-1R1-null animals are resistant to K/BxN arthritis (12), we turned our attention to IL-1 as a proinflammatory cytokine of potential mast cell origin (20). Using animals genetically deficient in IL-1 (21), we confirmed that serum-transferred arthritis exhibits a striking dependence on IL-1 (Fig. 1 A–D). Furthermore, a brief course of IL-1β together with arthritogenic K/BxN serum restored robust, albeit transient, arthritis in the mutant animals (Fig. 1E). This finding defines an ongoing role for IL-1 in synovitis and demonstrates the ability of recombinant IL-1β to substitute functionally for endogenous IL-1 in supporting joint inflammation.

Mast Cells Are an Obligate Source of IL-1 in K/BxN Arthritis in W/W^v Mice. Hypothesizing that mast cell elaboration of IL-1 was an important event, we reasoned that administration of IL-1 might restore arthritis sensitivity to mast-cell-deficient W/W^v mice (14). As shown in Fig. 2A, a short course of IL-1 could fully complement mast cell deficiency in W/W^v animals and restore a normal disease course; IL-1 administered in the absence of serum resulted in no arthritis (n = 8 in two experiments; data not shown). Histologic evaluation for the presence of mast cells
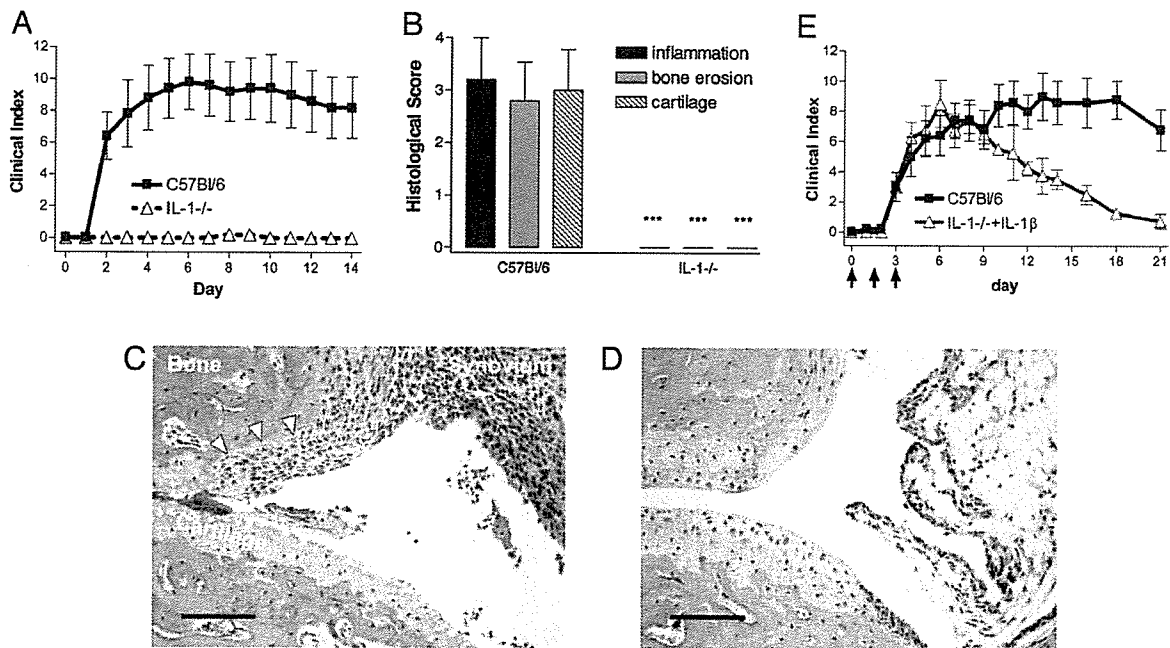
Fig. 1. IL-1 restores K/BxN arthritis susceptibility to IL-1-deficient mice. (*A*) Wild-type vs. IL-1$^{-/-}$ (*n* = 5 mice per group; *P* < 0.001). The results shown are representative of three experiments. (*B*) Histomorphometric quantitation of ankle tissues (*n* = 5 mice per group, ***, *P* < 0.001). (*C* and *D*) Representative ankle sections from wild-type (*C*) and IL-1$^{-/-}$ (*D*) mice. Arrowheads indicate erosions. (Scale bars, 100 μm.) (*E*) K/BxN serum administered to wild-type or IL-1$^{-/-}$ animals also given IL-1β (arrows) (*n* = 4 or 5 mice per group; *P* < 0.001). The results shown are representative of three experiments. All error bars indicate SEM.

remained negative in all W/W^v animals throughout the experiment. Because mast cells may contain preformed TNF (22) and because they participate in initiation of inflammation in other models via elaboration of this cytokine, we tested the ability of recombinant TNF to bypass the requirement for mast cells in arthritis-resistant W/W^v mice. Unlike IL-1, TNF administered at or significantly above doses that effectively complement mast cell deficiency, modulate systemic lymphocyte activation, and induce adhesion molecule expression on distant endothelium (18, 23, 24) could not complement mast cell deficiency in W/W^v mice (Fig. 2*B*). To further demonstrate the specificity of this IL-1 reconstitution effect, we assessed the ability of IL-1 to induce arthritis in mast-cell-sufficient CD88 (C5aR)-null mice (11). Administration of IL-1 failed to restore arthritis susceptibility in CD88$^{-/-}$ mice, illustrating that IL-1 cannot reverse all causes of resistance to K/BxN serum transfer arthritis (Fig. 2*C*).

To directly establish that mast cells are indeed an obligate source of IL-1 in K/BxN serum-transfer arthritis induction, we used a genetic approach. Wild-type and IL-1$^{-/-}$ bone-marrow-

derived mast cells (BMMC) were engrafted into W/W^v mice in which K/BxN serum was administered to induce arthritis (14). As shown in Fig. 3 *A* and *B*, mock-engrafted W/W^v mice were resistant to arthritis, whereas mice engrafted with wild-type mast cells developed arthritis similarly to WBB6 littermate controls. In contrast with wild-type mast cells, IL-1$^{-/-}$ mast cells could not promote arthritis, despite synovial mast cell engraftment equivalent to wild-type BMMC (Fig. 3 *C* and *D*). These results confirm that mast cells can contribute to disease induction via elaboration of IL-1.

Evidence That IL-1 Is Produced by Mast Cells at the Site of Inflammation. Where do the mast cells responsible for IL-1 elaboration reside? Our group has previously demonstrated that K/BxN serum-induced degranulation of mast cells is observed in the joints and not at other anatomic sites (14). This result is consistent with the absence of pathology outside the joints in K/BxN serum-transferred arthritis (8) and may be related to the joint specificity of serum-induced vascular leak (25). However, as previously reported (26),
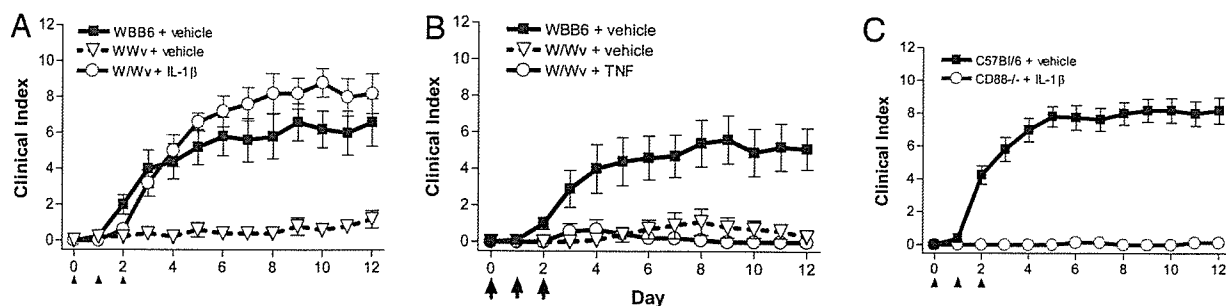
Fig. 2. Exogenous IL-1 restores susceptibility to arthritis in mast-cell-deficient mice. (*A*) W/W^v and control mice administered K/BxN serum and either IL-1β or vehicle (black arrows) (*n* = 5 mice per group). The data shown are representative of two experiments (*P* < 0.001 for W/Wv and IL-1β vs. vehicle; *P* was not significant vs. WBB6). (*B*) W/W^v and control mice administered K/BxN serum and 2.5 μg of TNF (*n* = 5), 7.5 μg of TNF (*n* = 4), or vehicle (black arrows). The data shown were pooled from two experiments (*P* < 0.001). (*C*) Administration of K/BxN plus IL-1β (arrows) to C5aR$^{-/-}$ mice. The results shown were pooled from two experiments (*n* = 16 B6 and 7 C5aR$^{-/-}$ animals; *P* < 0.001). All error bars indicate SEM.

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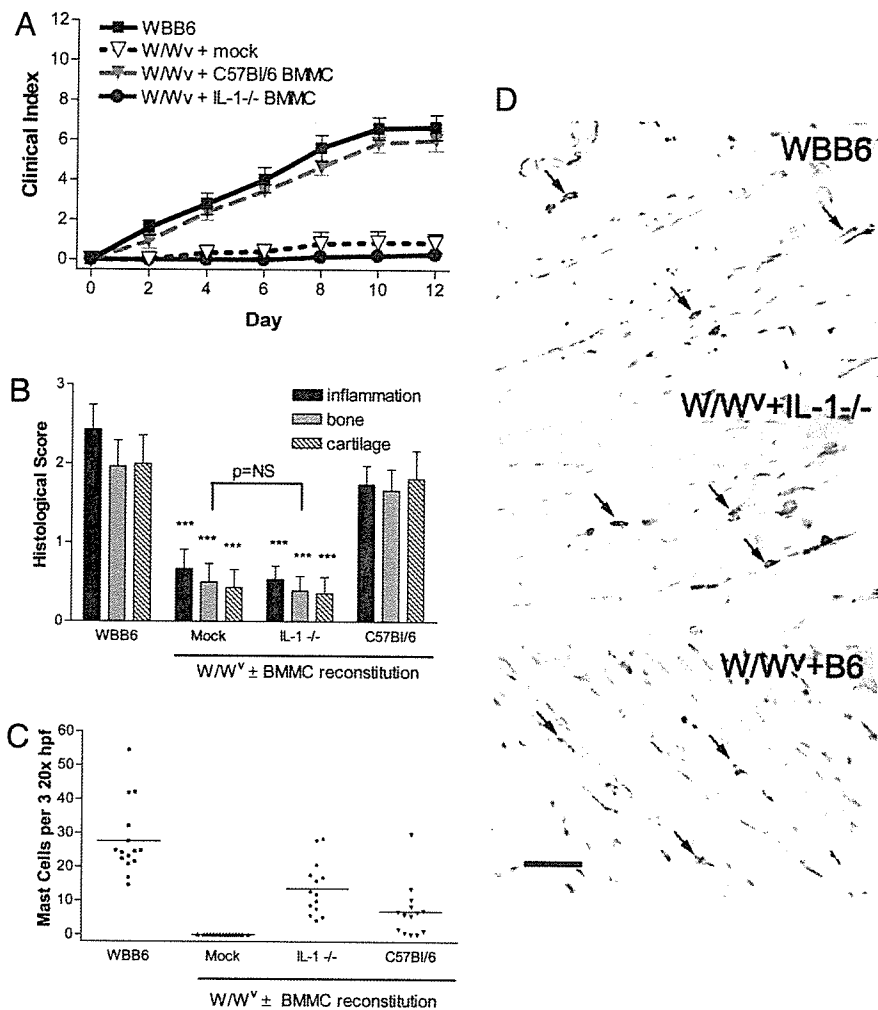
Fig. 3. Mast cells participate in arthritis via elaboration of IL-1. (*A*) Four-week-old W/W^v mice were engrafted with B6- or IL-1-deficient BMMC and administered K/BxN serum. Error bars indicate SEM. The results shown were pooled from three experiments (*n* = 14–15 per group; *P* < 0.001, IL-1$^{-/-}$ vs. B6 engraftment). (*B*) Histologic scoring of arthritis. ***, *P* < 0.001. (*C*) Efficacy of synovial mast cell engraftment (*P* not significant, IL-1$^{-/-}$ vs. B6). (*D*) Engrafted B6 and IL-1$^{-/-}$ synovial mast cells exhibit normal morphology and distribution. Arrows indicate mast cells; V, vessel. (Scale bar, 50 μm.)

mast cells abnormally populate the spleens of W/W^v mice reconstituted with BMMC, providing a potential source of systemic IL-1 that could confound the interpretation of our engraftment experiments. To address this possibility, we injected engrafted and unengrafted W/W^v mice with K/BxN serum and harvested serum and splenic tissue 4.5 h later, a time at which exogenous IL-1 (where administered) was readily detectable (Fig. 4*A*). Irrespective of engraftment status, administration of K/BxN serum did not result in significant systemic levels of IL-1 in any mouse at this time point (Fig. 4*A*). Furthermore, splenic IL-1 protein and mRNA levels provided no evidence that mast cells in the spleen were being activated to generate IL-1, despite a >100-fold excess of splenic mast cells in reconstituted W/W^v animals compared with controls (Fig. 4 *B–D*). By contrast, mice treated with i.p. LPS or with K/BxN serum followed 4 h later by IL-1, as per our IL-1 repletion protocol, demonstrated prominent levels of systemic and splenic IL-1.

To further confirm the irrelevance of splenic mast cells, we performed splenectomies on BMMC-engrafted W/W^v mice and found that splenectomized and sham-splenectomized animals demonstrated similar susceptibility to arthritis (Fig. 4*E*). We conclude that splenic mastocytosis does not confound the interpretation of the W/W^v engraftment experiments. Rather, absence of measurable circulating IL-1 supports the hypothesis that

mast cells local to the joint are the relevant source of this cytokine in synovial inflammation.

Mast Cells Elaborate IL-1 upon Stimulation via Fcγ Receptors. If IL-1 from synovial mast cells is a key mediator in arthritis, how do anti-glucose-6-phosphate isomerase antibodies induce production of this cytokine? The demonstration of circulating and joint-resident immune complexes as well as the genetic requirement for C5aR and FcγRIII all point to the role of immune complexes in disease initiation (15, 27–29). Murine mast cells express FcγRIII and may be stimulated to degranulate and elaborate eicosanoids via this receptor in culture, although activation is constrained by the inhibitory receptor FcγRII (30, 31). To examine the capacity of mast cells to produce IL-1 upon specific ligation of FcγRIII, we generated BMMC from FcγRII$^{-/-}$ mice (32). Immune complex stimulation was mimicked by mAb cross-linking of FcγRIII. As shown in Fig. 5*A*, FcγRII$^{-/-}$ BMMC responded to FcγRIII ligation with brisk degranulation and production of IL-1.

To confirm an *in vivo* role for IgG Fc receptor-mediated mast cell activation in this model, we engrafted mast cells deficient in Fc receptors (FcRγ$^{-/-}$) into W/W^v mice (33). Despite synovial engraftment to equivalence with wild-type BMMC, these mast cells proved unable to confer arthritis [Fig. 5*B* and
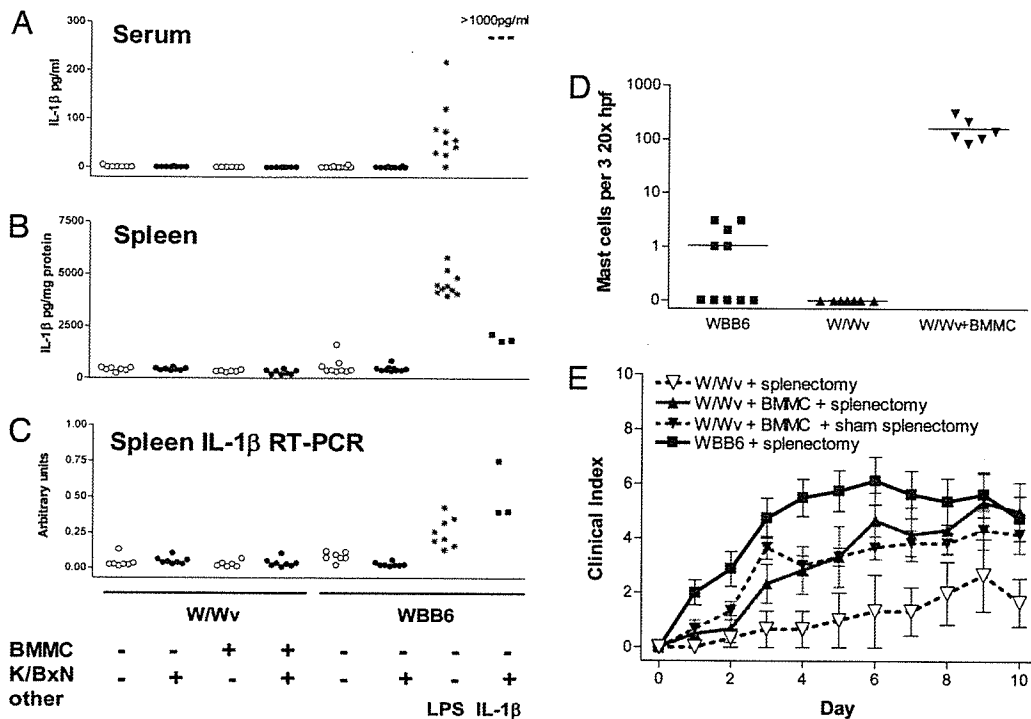
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Fig. 4. Splenic mast cells do not participate in the initiation of arthritis. (A–C) Serum (A) and splenic (B and C) IL-1β production 4.5 h after administration of 150 μl of ×1 K/BxN serum or 50 μg of LPS. Where administered, IL-1β (2.5 μg) was given 4 h after serum injection (*n* = 3; otherwise, *n* = 6–10 mice per group pooled from two experiments). (D) Quantitation of splenic mast cells. (E) BMMC engrafted (15–16 weeks) W/Wv and control mice were treated with splenectomy or sham splenectomy and allowed 2–3 weeks of recovery time before arthritis induction (*P* not significant, W/Wv plus BMMC plus sham vs. splenectomy). Error bars indicate SEM (*n* = 5–8 per group and *n* = 3 W/Wv unengrafted animals).

supporting information (SI) Fig. 6]. The FcRγ chain is shared by FcεRI, FcγRI, FcγRIII, and the recently described FcγRIV (34). Previous genetic studies have found that FcγRI is dispens-

able for arthritis induction, whereas *in vitro* assays demonstrate that FcγRIV does not bind the IgG1 subclass responsible for disease in the K/BxN model (7, 11, 34). To exclude participation
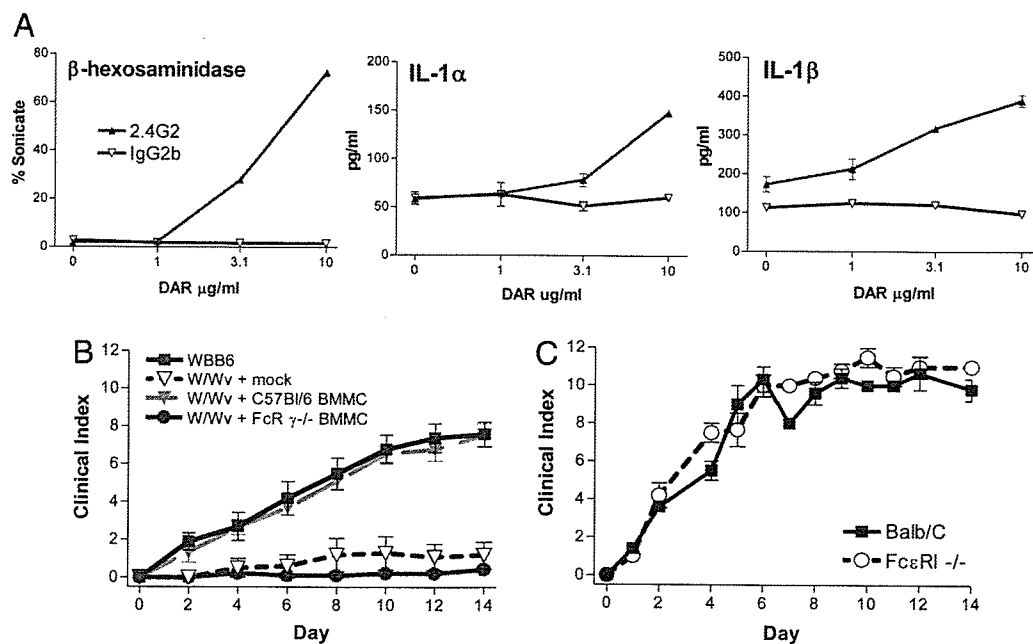
Fig. 5. Mast-cell production of IL-1 is mediated by FcγRIII. (A) FcγRII-null BMMC incubated with anti-FcγRII/III antibody 2.4G2 or isotype control followed by cross-linking donkey anti-rat (DAR) F(ab′)2. Results are the mean from duplicate wells and are representative of three independent experiments. (B) W/Wv mice engrafted with BMMC from B6- and FcγR-null mice and administered arthritogenic K/BxN serum. The results shown were pooled from two experiments (*n* = 10 mice per group; *P* < 0.001, B6 vs. FcγR−/− BMMC engrafted). (C) FcεRI-deficient and control BALB/c mice administered K/BxN serum (100 μl per injection). The results shown were pooled from two experiments (*n* = 5 mice per group; *P* not significant). All error bars indicate SEM.

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of FcεRI in arthritis pathogenesis, we administered K/BxN serum to mice deficient in the FcεRI α chain (35). These animals exhibited wild-type susceptibility to disease (Fig. 5C). Thus, consistent with our *in vitro* findings, these data confirm that mast cell dysfunction derives from lack of expression of FcγRIII, the IgG immune complex receptor shown previously to be critical for disease pathogenesis (11, 15, 29).

Discussion

Although many aspects of the etiology of RA remain obscure, substantial evidence has accumulated that antibodies contribute to disease pathogenesis. In seropositive RA, unlike in other inflammatory arthritides, such as gout or psoriatic arthritis, deposition of immune complexes is typically observed in synovium and cartilage, whereas joint effusions exhibit prominent consumption of complement (5, 36, 37). Similarly, circulating and synovial fluid immune complexes may be demonstrated in the majority of patients (5, 38). Intriguingly, autoantibodies are often present years before the onset of arthritis (39). What events convert a patient with serologic abnormalities into one with clinical disease? Using a mouse model of autoantibody-induced arthritis, we here demonstrate one potential pathway: immune complex stimulation of mast cells via a low-affinity IgG Fc receptor results in the elaboration of IL-1, potentiating the initiation of inflammation within the joint by pathogenic autoantibodies. If this role is bypassed, such as by administration of exogenous IL-1, mast cell deficiency no longer impedes the development of robust synovitis. Together, these results define a functional mast cell contribution to the initiation of autoimmune inflammation within the joint and identify IL-1 as a mast cell effector function in this activity.

This work further develops the understanding of the mast cell as immune sentinel (40). Mast cells are found constitutively in most tissues, clustered at sites where foreign invaders are first encountered, such as the skin, mucosal surfaces, and around blood vessels. They also reside proximate to vulnerable body cavities, such as the peritoneum and joint (41). Although equipped with a broad range of antimicrobial effector mechanisms, a major role of mast cells appears to be the mobilization of leukocytes and other immune elements upon the first signs of infection, a purpose for which they are uniquely suited given their capacity for rapid release of preformed and newly synthesized mediators (42). We postulate that the involvement of mast cells in arthritis represents a "down side" of this physiologic specialization: Monitoring the joint for bacterial invasion of the protein-rich but acellular and hypocomplementemic synovial fluid (36), mast cells may instead become activated by autoantibody-containing immune complexes, thereby participating in the pathogenesis of destructive synovitis.

These findings define a clear example of IgG-driven mast cell participation in organ-specific autoimmunity, extending earlier work on the activation of mast cells via Fcγ receptors in IgG-mediated anaphylaxis and cutaneous immune complex disease (31, 32, 43, 44). Although we find that similar mechanisms for mast cell activation are operative in the joint, we herein offer *in vivo* demonstration of a disease-provoking effector mechanism, IL-1 elaboration, downstream of FcγRIII-mediated mast cell activation. Although IL-1 is well appreciated as an important mediator in the pathogenesis of arthritis, the key source for this cytokine within the joint has generally been thought to be the synovial macrophage (45, 46). Although mast cells belong to the myeloid lineage, it was unexpected to find that they should represent an important source of IL-1 in arthritis. Taken together, these results demonstrate a role for mast cells in the chain of events linking adaptive immunity and the production of this potent innate proinflammatory mediator.

Our conclusions, in common with much work defining *in vivo* mast cell function, is derived from observations in BMMC-reconstituted W/Wᵛ animals (47). Impaired c-*kit* signaling in this strain has effects beyond the mast cell lineage, and it may be that the activity of the mast cell emerges with particular clarity in this genetic context. Recently, we have found that another mouse strain lacking mature tissue mast cells, C57BL/6-Kit^{W-sh}/ Kit^{W-sh} (Wˢʰ), that bears an inversion encompassing regulatory elements that modulate the tissue-specific expression of c-*kit* (48, 49) is susceptible to K/BxN serum-transfer arthritis, unlike the mast-cell-deficient W/Wᵛ and Sl/Slᵈ strains (P.A.N. and D.M.L., unpublished data). In preliminary experiments, we find that there exist cells in Wˢʰ animals that are functionally able to provide the arthritogenic activity supplied by mast cells in engrafted W/Wᵛ mice. Experiments designed to uncover the differences between these strains may yield important insights into mast cell physiology.

In light of discordance among mast-cell-deficient strains, it was especially important to consider the possibility that the apparent mast-cell-dependence of arthritis in reconstituted W/Wᵛ mice might be an artifact of IL-1-producing splenic mast cells that have no counterpart in normal physiology. However, an intact arthritic response in engrafted and splenectomized W/Wᵛ mice demonstrates that accumulated splenic mast cells play no discernable role in arthritis susceptibility (Fig. 4). Moreover, because administration of K/BxN serum does not result in measurable quantities of circulating IL-1, our data are consistent with a primary contribution by mast cells resident locally to the joint.

Our data highlight the importance of mast cells in the early phase of disease, yet this is unlikely to represent the whole story of mast cells in arthritis. In the model used in these studies, an initial pulse of arthritogenic autoantibodies results in a monophasic illness (8). By contrast, the generation of autoantibodies in RA is ongoing, and the disease runs a chronic course punctuated by recurrent flares. Whereas fluid from active joints commonly reveals histamine consistent with local mast cell degranulation (5, 50), it may be that targeting mast cells could limit flares by inhibiting recurrent waves of arthritis induction. Furthermore, mast cell numbers expand greatly in chronically inflamed joints, where they are capable of elaborating a range of growth factors, proangiogenic compounds, and other mediators with potentially substantial importance for the course of chronic arthritis (51). Although our results now delineate one discrete contribution of this lineage to inflammatory joint disease, the involvement of mast cells in the pathogenesis of RA is likely to be multifaceted and represents an important topic for further investigation.

Materials and Methods

Animals. C57BL/6J (B6), Kitᵂ/Kitᵂ⁻ᵛ (W/Wᵛ), WBB6F1, TNF⁻/⁻ (52), and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C5aR⁻/⁻ mice were a gift from Craig Gerard (Children's Hospital Boston) (53). FcεR1α⁻/⁻ mice on a BALB/c background were kindly provided by Jean-Pierre Kinet (Beth Israel Deaconess Medical Center, Boston, MA) (35). FcRγ⁻/⁻ and FcγRII⁻/⁻ mice were purchased from Taconic (Germantown, NY) (32, 33). IL-1⁻/⁻ and K/BxN mice were maintained as described (8, 21). Male mice 6–10 weeks of age were used for all experiments unless specified otherwise. All procedures were approved by the Dana–Farber Cancer Institute Animal Care and Use Committee.

Serum Transfer Protocol, Arthritis and Histologic Scoring, and Cytokine Reconstitution Experiments. Arthritis was induced in recipient mice by transfer of arthritogenic K/BxN serum on days 0 and 2 i.p. as described (8). For histologic scoring, ankle sections were scored in blinded fashion as described (54). For cytokine reconsistution, animals were administered recombinant murine IL-1β or TNF (R&D Systems, Minneapolis, MN)

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or vehicle by i.p. injection beginning 4 h after the initial dose of K/BxN serum.

BMMC Culture, Mast Cell Engraftment, and Assessment of Serum and Splenic IL-1β Protein and mRNA. BMMC were generated and engrafted as described (14). K/BxN serum (150 μl × 1), IL-1β (2.5 μg), LPS (50 μg; *Salmonella enterica* serotype abortus equi) (Sigma, St. Louis, MO) or mock treatment were followed 4.5 h later by blood and tissue harvest. Tissue lysates were prepared in cold lysis buffer (10 mM Tris, pH 7.4/150 mM NaCl/1% Triton X-100/0.05% SDS) containing PMSF (Sigma). IL-1β was assessed via ELISA (R&D Systems) and protein via Bradford assay. Tissue for RNA was snap-frozen, mechanically dispersed in RNase-inhibiting buffer, and lysed by using QiaShredder columns (Qiagen, Valencia, CA). RNA was extracted by using RNeasy (Qiagen). Quantitative RT-PCR was performed as described (55) and normalized to splenic RNA from a BALB/c mouse treated with 50 μg of LPS i.p. 6 h before being killed.

Splenectomy and *in Vitro* Stimulation of BMMC. Splenectomies were performed as described (56). Animals were allowed 2–3 weeks to recover from surgery before the initiation of arthritis. Cultured BMMC were preincubated overnight at 37°C in the anti-FcγRII/III antibody 2.4G2 or isotype control (BD Pharmingen, San Diego, CA), washed, and resuspended at 5×10^5 cells

per round-bottom well with cross-linking donkey anti-rat F(ab′)2 fragments (The Jackson Laboratory). After 6 h of incubation at 37°C, plates were gently centrifuged and supernatants were harvested for β-hexosaminidase determination as described (30). Cell pellets were resuspended in cold lysis buffer. IL-1α (BD Pharmingen) and IL-1β (R&D Systems) were determined by ELISA.

Statistical Analysis. Differences were considered significant at $P < 0.05$ by Student's t test. To compare curves, the area under the curve was calculated for each animal in an experimental set followed by Student's t test between groups (Prism 3.03; GraphPad Software, San Diego, CA).

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Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*

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Dectin-1 is a C-type lectin involved in the recognition of β-glucans found in the cell walls of fungi. We generated dectin-1-deficient mice to determine the importance of dectin-1 in the defense against pathogenic fungi. *In vitro*, β-glucan-induced cytokine production from wild-type dendritic cells and macrophages was abolished in cells homozygous for dectin-1 deficiency ('dectin-1-knockout' cells). *In vivo*, dectin-1-knockout mice were more susceptible than wild-type mice to pneumocystis infection, even though their cytokine production was normal. However, pneumocystis-infected dectin-1-knockout macrophages did show defective production of reactive oxygen species. In contrast to those results, wild-type and dectin-1-knockout mice were equally susceptible to candida infection. Thus, dectin-1 is required for immune responses to some fungal infections, as protective immunity to pneumocystis, but not to candida, required dectin-1 for the production of antifungal reactive oxygen species.

The C-type lectins form a group of proteins with a lectin-like carbohydrate-recognition domain in their extracellular carboxy-terminal domains[1]. Some C-type lectin family members recognize the carbohydrate structures of microbes as pathogen-associated molecular patterns, whereas other members, on natural killer cells, recognize endogenous ligands and discriminate self from nonself in a calcium-dependent way. Dectin-1 was first reported as a dendritic cell (DC)–specific type II C-type lectin receptor expressed as a 43-kilodalton membrane-associated glycoprotein, with a carbohydrate-recognition domain in its extracellular carboxyl terminus and an immunoreceptor tyrosine-based activation motif in its intracellular amino terminus[2,3]. Dectin-1 is also highly expressed on macrophages and neutrophils and is the receptor for β-1,3-linked and/or β-1,6-linked glucans (β-glucans)[4].

The β-glucans are important cell wall components of fungi and yeasts; they consist of a backbone of polymerized β(1→3)-linked β-D-glucopyranosyl units and β(1→6)-linked side chains[5] and are found in a wide-range of mushrooms, seaweeds, yeasts and pathogenic fungi. Although individual β-glucans are heterogeneous in terms of molecular weight, number of branches and helical construction, many β-glucans have immunological 'effector' activities *in vitro* and *in vivo*, and some are used beneficially to treat human diseases[6,7]. However, the mechanism of β-glucan-induced activity

has not been elucidated completely because it has been difficult to discriminate the responses caused by β-glucans from those caused by other pathogen-derived components that are often contaminants of β-glucan preparations.

Studies have suggested that dectin-1 is involved in the recognition of and host defense mechanisms against pathogenic fungi, including *Candia albicans* and *Pneumocyctis carinii*, because those fungi express β-glucans in their cell walls and cause cytokine production after infection[8,9]. As for the defense mechanisms against fungi, both the innate and acquired immune systems are thought to be involved[10]. T helper 1 cell–mediated immune responses and the opsonization of fungi and their incorporation into macrophages through Fc receptors are important immune responses to fungal infection in immunocompetent people; that is reflected in the fact that fungal infection causes morbidity and mortality only in immunocompromised patients[10,11].

Cytokines induced by fungi are also important in activating immune responses and phagocytic cells. The many Toll-like receptors (TLRs) are thought to be critically involved in the cytokine responses to fungi because, for example, the survival of *Tlr2*^{−/−} mice infected with *C. albicans* is much lower that of infected wild-type mice[12]; moreover, the cell walls of *C. albicans* can induce tumor necrosis factor (TNF) production by macrophages via TLR4 stimulation[13]. Although the mannose receptor (MRC1) has also been linked to the recognition

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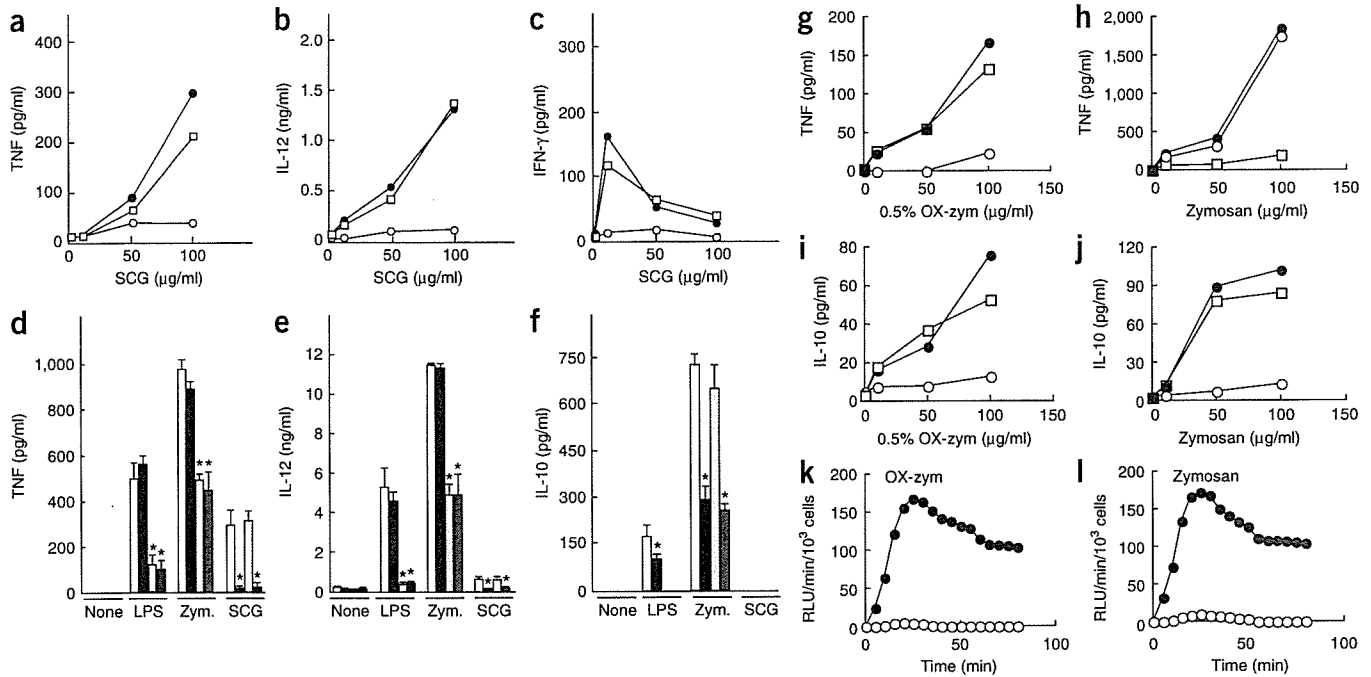
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Figure 1 Cytokine induction by β-glucans is dependent on dectin-1. (a–f) ELISA of cytokine concentrations in supernatants of dectin-1-wild-type, dectin-1-knockout Myd88^{+/+} and/or Myd88^{−/−} BMDCs cultured for 24 h with SCG (a–c; concentration, horizontal axes) or with LPS (20 ng/ml), zymosan (Zym.; 100 μg/ml) or SCG (100 μg/ml; d–f). Filled circles and white bars, dectin-1-wild-type, Myd88^{+/+}; open circles and black bars, dectin-1-knockout, Myd88^{+/+}; open squares and light gray bars, dectin-1-wild-type, Myd88^{−/−}; dark gray bars, dectin-1-knockout, Myd88^{−/−}. *, P < 0.01 (Student's t-test). Data represent mean (+ s.d.) of triplicate samples (d–f) and were reproducible in three independent experiments (a–f). (g–j) ELISA of cytokine concentrations in supernatants of wild-type (filled circles), dectin-1-knockout (open circles) or Myd88^{−/−} (open squares) thioglycollate-elicited macrophages cultured for 24 h with NaClO-oxidized zymosan (OX-zym) or zymosan (concentration, horizontal axes). (k,l) Luminol-enhanced chemiluminescence analysis of ROS produced by wild-type (filled circles) or dectin-1-knockout (open circles) thioglycollate-elicited macrophages stimulated with NaClO-oxidized zymosan (k) or zymosan (l). RLU, relative luciferase units. Similar results were obtained in one other independent experiment (g–l).

and phagocytosis of fungi via mannans expressed on fungal cell walls[14], Mrc1^{−/−} mice show normal responses to C. albicans and P. carinii infection[15,16]. In addition to those host molecules, the importance of dectin-1 in recognizing and responding to fungal β-glucans in vivo remains to be fully elucidated. Here we describe the generation of mice homozygous for deficiency of the gene encoding dectin-1 (called 'dectin-1-knockout mice' here) and experiments done to assess the function of dectin-1 in host defense against fungal pathogens. Dectin-1 signaling activated DCs and macrophages to produce cytokines and reactive oxygen species (ROS), and dectin-1 was important in protection against P. carinii but not C. albicans infection.

RESULTS

Dectin-1-knockout mice develop normally

We generated embryonic stem cells heterozygous for dectin-1 deficiency by replacing both exon 1, containing the translation start site, and exon 2 of the gene encoding dectin-1 with a neomycin-resistance gene (Supplementary Fig. 1 online). Dectin-1-knockout mice were born at the expected mendelian ratio, were fertile and showed no gross phenotypic abnormalities, including no alterations in lymphoid cell populations (data not shown). We did not detect dectin-1 mRNA in spleen cells from dectin-1-knockout mice (Supplementary Fig. 1), and there was no dectin-1 expression on the surfaces of dectin-1-knockout bone marrow–derived DCs (BMDCs; Supplementary Fig. 1), indicating that the gene encoding dectin-1 was correctly disrupted.

Dectin-1 is required for β-glucan activation of DCs

As dectin-1 is considered a receptor for β-glucans, we examined the responses of wild-type and dectin-1-knockout BMDCs to β-glucans. We used Sparassis crispa glucan (SCG), a soluble, β-1,6-branched β-1,3-glucan purified from the edible mushroom, because it is biologically active and is easily purified to homogeniety[17]. After stimulation of wild-type BMDCs with SCG, production of interleukin 12 (IL-12) and TNF was enhanced in a dose-dependent way (Fig. 1a,b). Production of interferon-γ (IFN-γ) was also stimulated by SCG, although production was suppressed at higher concentrations (Fig. 1c). In contrast, production of those cytokines was completely abolished in dectin-1-knockout BMDCs (Fig. 1a–c), indicating that dectin-1 is required for cytokine induction in BMDCs after β-glucan stimulation.

Zymosan, a polysaccharide particle from the cell wall of Saccharomyces cerevisiae, is one of most commonly used β-glucan-containing experimental agents, although it contains other components, including mannans, other glucans and chitins[18]. Studies have suggested that the induction of TNF and IL-12 in response to zymosan requires the 'collaboration' of dectin-1 and TLR2 (ref. 19) on the cell surface, followed by activation of the transcription factor NF-κB via MyD88 (refs. 20,21), an intracellular signal transducer required for TLR signaling[22]. To determine the relative requirements for dectin-1 and MyD88 in BMDC responses to zymosan and SCG and to the MyD88-dependent stimulus lipopolysaccharide (LPS), we assessed cytokine production in BMDCs from dectin-1-knockout and Myd88^{−/−} mice. Cytokine production induced by LPS was completely

dependent on MyD88 (**Figs. 1d–f**), as reported before[23]. In contrast, the SCG-induced production of TNF, IL-12 and IFN-γ was not affected at all by the lack of MyD88 (**Figs. 1a–f**). After stimulation with zymosan, in contrast, production of TNF and IL-12 was significantly reduced in Myd88^{-/-} BMDCs but not in dectin-1-knock-out BMDCs. Production of those cytokines, therefore, was dependent on TLR but not dectin-1 signaling. We also found that IL-10 production stimulated by zymosan was suppressed in dectin-1-knock-out BMDCs but not in Myd88^{-/-} BMDCs, suggesting that the induction mechanisms for IL-10 versus those for IL-12 and TNF are distinct (**Fig. 1f**). Zymosan-induced TNF and IL-12 production in dectin-1-knockout and Myd88^{-/-} double-deficient BMDCs did not differ significantly from that in Myd88^{-/-} single-deficient BMDCs (**Fig. 1d,e**), and IL-10 production was similar to that of dectin-1-knockout BMDCs (**Fig. 1f**). These results indicated that cytokine production via dectin-1 signaling is independent of MyD88 and that zymosan stimulates both dectin-1 and MyD88.

To further discriminate molecules that activate dectin-1 from those that activate MyD88, we compared the cytokine-stimulatory effect of NaClO-oxidized zymosan versus that of untreated zymosan on thioglycollate-elicited macrophages. The nitrogen content of zymosan is 1.81%, and it becomes 0.18% after treatment with 0.5% NaClO and 0.1 M NaOH; treating zymosan in that way results in a product composed mainly of β-glucans[24]. We found that although TNF was induced in both wild-type and Myd88^{-/-} thioglycollate-elicited macrophages by NaClO-oxidized zymosan in a dose-dependent way, production of TNF was abolished in dectin-1-knockout thioglycollate-elicited macrophages (**Fig. 1g**). In contrast, production of TNF was not affected in dectin-1-knockout thioglycollate-elicited macrophages treated with intact zymosan, whereas it was reduced in Myd88^{-/-} macrophages (**Fig. 1h**). Finally, we found that IL-10 induction was completely dependent on dectin-1 but not on MyD88, as with BMDCs (**Fig. 1i,j**). We next examined production of ROS, which is important for killing microbes that are phagocytosed by macrophages[25]. We found that production of ROS by thioglycollate-elicited macrophages was completely depended on dectin-1 for both zymosan and NaClO-oxidized zymosan (**Fig. 1k,l**). Our data collectively indicated that production of IL-10 and ROS is completely dependent on dectin-1, whereas TNF production by thioglycollate-elicited macrophages after zymosan treatment is mediated by molecules other than β-glucans that stimulate the MyD88 signaling pathway.

Dectin-1 signaling induces the SCG maturation of BMDCs

As stimulation by β-glucans has been suggested to be involved in the maturation of BMDCs[26], we next examined the cell surface expression of CD80 and CD40, maturation markers of DCs, after treatment of BMDCs with SCG. Immature BMDCs incubated for 24 h with SCG showed enhanced expression of both CD80 and CD40 (**Fig. 2**). However, there was no enhancement in dectin-1-knockout BMDCs, suggesting that dectin-1 is critical for SCG-inducted activation of BMDCs. To determine if the dectin-1-knockout BMDCs had an intrinsic 'maturation defect', we also treated them separately with LPS, which was sufficient to cause full maturation (at least by the criteria we used). In contrast to the results obtained with dectin-1-knockout BMDCs, Myd88^{-/-} BMDCs matured normally after stimulation with SCG, indicating again that activation of BMDCs via dectin-1 is independent of the MyD88 pathway.

T cell–mediated responses are normal in dectin-1-knockout mice

It has been reported that soluble recombinant dectin-1 can bind to the surfaces of T cells and promote proliferation induced by antibody to
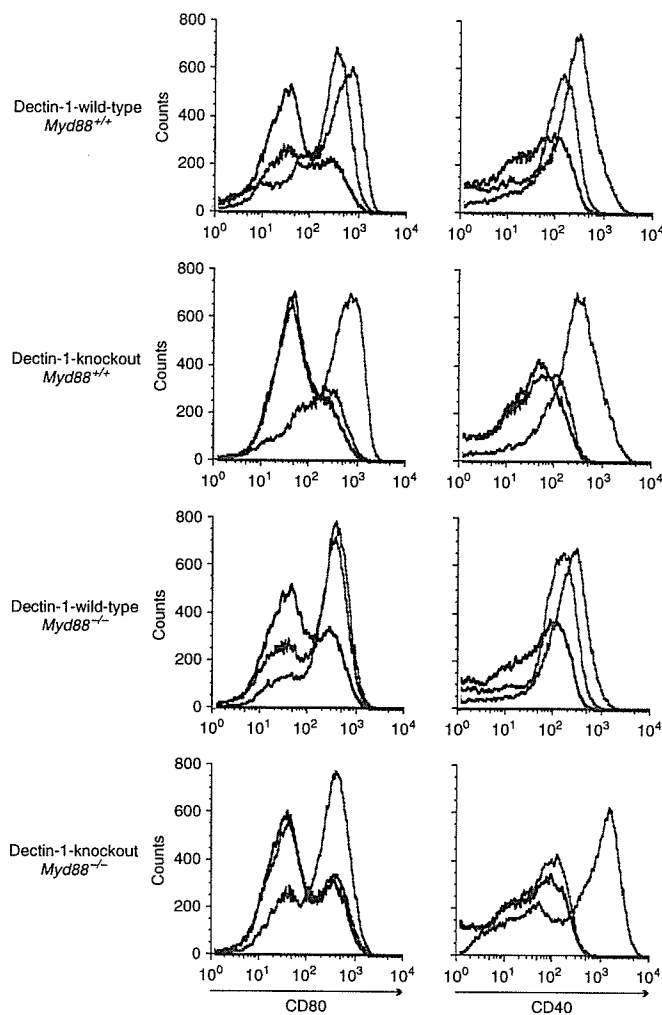

Figure 2 BMDC maturation is induced by β-glucans via dectin-1. Flow cytometry of the expression of CD80 and CD40 on CD11c+ BMDCs cultured for 24 h with SCG (100 μg/ml; red lines) or LPS (20 ng/ml; blue lines) or without any stimulation (black lines). Data are representative of three independent experiments.

CD3 (anti-CD3), suggesting the presence of an unknown endogenous dectin-1 ligand on T cells[2]. To evaluate that phenomenon in cells lacking dectin-1 expression, we next assayed proliferation. CD4+ T cells cultured with dectin-1-knockout DCs and monoclonal anti-CD3 had proliferative activity equal to that of wild-type DCs (**Fig. 3a**). Also, the antigen-presenting activity of ovalbumin peptide–loaded dectin-1-knockout DCs to DO11.10-transgenic CD4+ T cells was indistinguishable from the activity of wild-type DCs (**Fig. 3b**). Furthermore, production of antibodies to sheep red blood cells or to dinitrophenol–keyhole limpet hemocyanin was normal in dectin-1-knockout mice (**Fig. 3c** and data not shown), and delayed-type hypersensitivity responses to methylated bovine serum albumin were normal in dectin-1-knockout mice (**Fig. 3d**). These results indicated that a possible costimulatory effect of dectin-1 on T cell proliferation by means of T cell–expressed endogenous ligands (if they exist) is not essential.

Protection against *P. carinii* infection requires dectin-1

Because β-glucan, in addition to mannose-containing polysaccharides[27], is a chief cell wall component of *P. carinii*, we examined
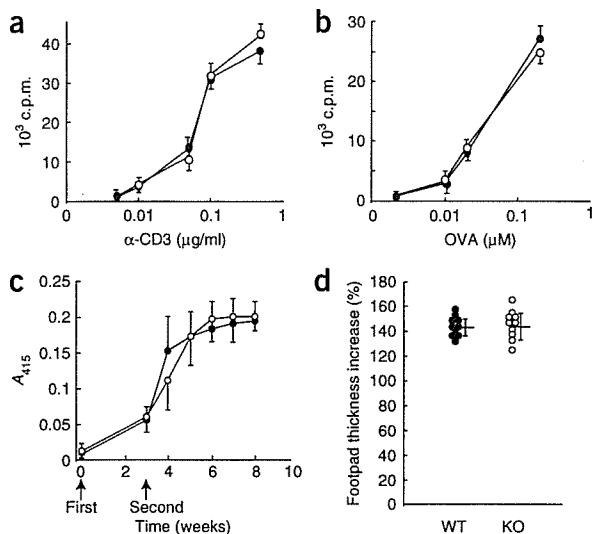
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Figure 3 Antigen presentation by dectin-1-knockout dendritic cells is normal. (a) Proliferation assay of wild-type CD4+ T cells cultured for 3 d with CD11c+ DCs from BALB/c dectin-1-wild-type or dectin-1-knockout mice in the presence of anti-CD3 before analysis of [^{3}H]thymidine incorporation. (b) Antigen-presenting activity of DCs, assessed as proliferation of CD4+ T cells from DO11.10-transgenic mice, cultured for 3 d with CD11c+ DCs from BALB/c dectin-1-wild-type or dectin-1-knockout mice in the presence of ovalbumin peptide (OVA) before analysis of [^{3}H]thymidine incorporation. Filled circles, dectin-1-wild-type; open circles, dectin-1-knockout. Data are mean (+ s.d.) of triplicate samples and are representative of three independent experiments (a,b). (c) ELISA of the production of antibodies to sheep red blood cells by dectin-1-wild-type and dectin-1-knockout mice at 21 d after secondary immunization, assessed as alkaline phosphatase activity (mean + s.d.). Upward arrows, first (left) and second (right) immunization. A_{415}, absorbance at 415 nm. Data are representative of one experiment. (d) Delayed-type hypersensitivity responses in dectin-1-wild-type mice (WT) and dectin-1-knockout mice (KO), assessed as increase in footpad thickness induced by methylated bovine serum albumin. Each circle represents an individual mouse; data represent mean (+ s.d.) for one experiment. Filled circles, dectin-1-wild-type ($n = 10$ mice in each); open circles, dectin-1-knockout ($n = 10$ mice in each).

the susceptibility of dectin-1-knockout mice to *P. carinii*. Dectin-1-knockout mice inoculated with 1.7×10^4 *P. carinii* cysts had many more organisms at 1 or 2 weeks after infection than did similarly infected wild-type mice, indicating that dectin-1-knockout mice are more sensitive (**Fig. 4a**). In contrast to those results early after infection, we did not detect *P. carinii* in either wild-type or dectin-1-knockout mice either 20 d or 4 months after infection (less than 1.5×10^2 cysts per mouse). We also infected nude mice, which lack T cells, in the same way and detected $2.6 \times 10^5 \pm 3.8 \times 10^5$ cysts in their lungs 4 months after infection, which indicated a critical function for T cell–mediated immune responses in the host defense against *P. carinii*. Thus, in the presence of an intact acquired immune system, dectin-1 is not required for protection against chronic infection by *P. carinii*.

As another means of assessing the importance of dectin-1 early after infection with *P. carinii*, we examined the susceptibility of immuno-compromised but otherwise normal wild-type and dectin-1-knockout mice. We gave the mice cortisone acetate twice weekly (2.5 mg/mouse), then infected them intranasally with *P. carinii* (6.4×10^5 cysts per

mouse) and counted cysts in the lungs 24 d later. Wild-type and dectin-1-knockout mice were equally sensitive to cortisone treatment, as the number of the white blood cells before and after treatment with cortisone was similar for those two strains (before treatment: wild-type, $11,700 \pm 1,600$ cells/µl, and dectin-1-knockout, $11,600 \pm 1,600$ cells/µl; after treatment: wild-type, 780 ± 580 cells/µl, and dectin-1-knockout, 880 ± 440 cells/µl). However, even though the two strains of mice were equally immunocompromised, dectin-1-knockout mice had significantly more cysts in their lungs ($2.5 \times 10^5 \pm 0.78 \times 10^5$ cysts per mouse) than wild-type mice did ($5.6 \times 10^4 \pm 4.2 \times 10^4$ cysts per mouse; **Fig. 4b**). These observations demonstrated that dectin-1 is important in host defense mechanisms against *P. carinii*.

P. carinii–induced ROS require dectin-1

Because TNF, IFN-γ and IL-12 are higher in *P. carinii*–infected mice[28] and because TNF and IFN-γ are reported to be important in protection against *P. carinii*[29], we measured cytokine production in dectin-1-knockout macrophages cultured with *P. carinii* cysts *in vitro*.
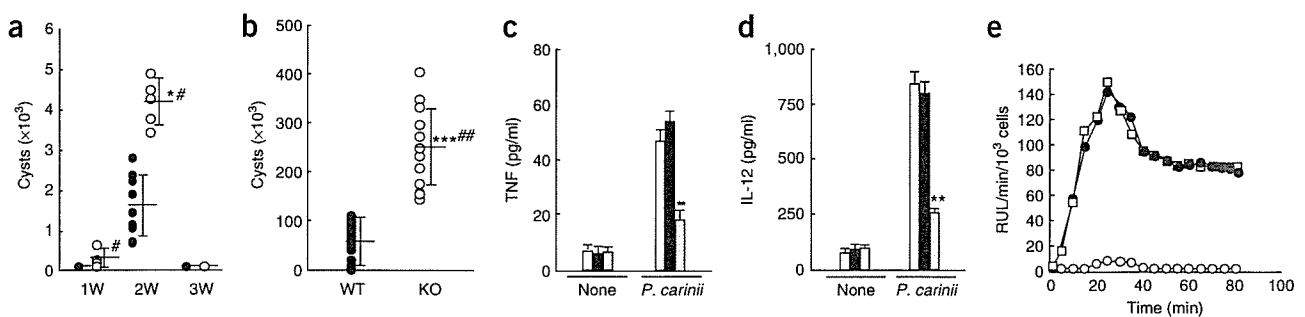

Figure 4 Dectin-1-knockout mice are more susceptible than dectin-1-wild-type mice to *P. carinii* infection. (a) *P. carinii* cysts in the lungs of dectin-1-wild-type mice (filled circles) and dectin-1-knockout mice (open circles) inoculated intranasally with 1.7×10^4 *P. carinii* cysts for 1 week (1W; wild-type, $n = 7$; knockout, $n = 4$), 2 weeks (2W; wild-type, $n = 9$; knockout, $n = 5$) or 3 weeks (3W; wild-type, $n = 5$; knockout, $n = 5$), determined by counting of toluidine blue O–positive cells. (b) *P. carinii* cysts in the lungs of dectin-1-wild-type mice (WT; $n = 21$) and dectin-1-knockout mice (KO; $n = 14$) inoculated intranasally with 6.4×10^5 *P. carinii* cysts and given 2.5 mg cortisone acetate per mouse twice weekly, determined 24 d after inoculation by counting of toluidine blue O–positive cells. *, $P < 0.05$, and ***, $P < 0.001$ (Mann-Whitney U test); #, $P < 0.05$, and ##, $P < 0.01$ (Student's t-test). Each circle represents an individual mouse (a,b); data represent mean (+ s.d.) and are from one experiment (a) or were reproduced in four independent experiments (b). (c,d) ELISA of TNF (c) or IL-12 (d) in supernatants from dectin-1-wild-type (white bars), dectin-1-knockout (black bars) or Myd88$^{-/-}$ (gray bars) alveolar macrophages (pooled from three mice) cultured for 24 h with (right) or without (left; None) *P. carinii* cysts. **, $P < 0.01$ (Student's t-test). Data represent mean (+ s.d.) from three wells and were reproduced in at least in three independent experiments. (e) Luminol-enhanced chemiluminescence analysis of ROS produced by wild-type (filled circles), dectin-1-knockout (open circles) or Myd88$^{-/-}$ (open squares) alveolar macrophages. Data were reproducible in three independent experiments.
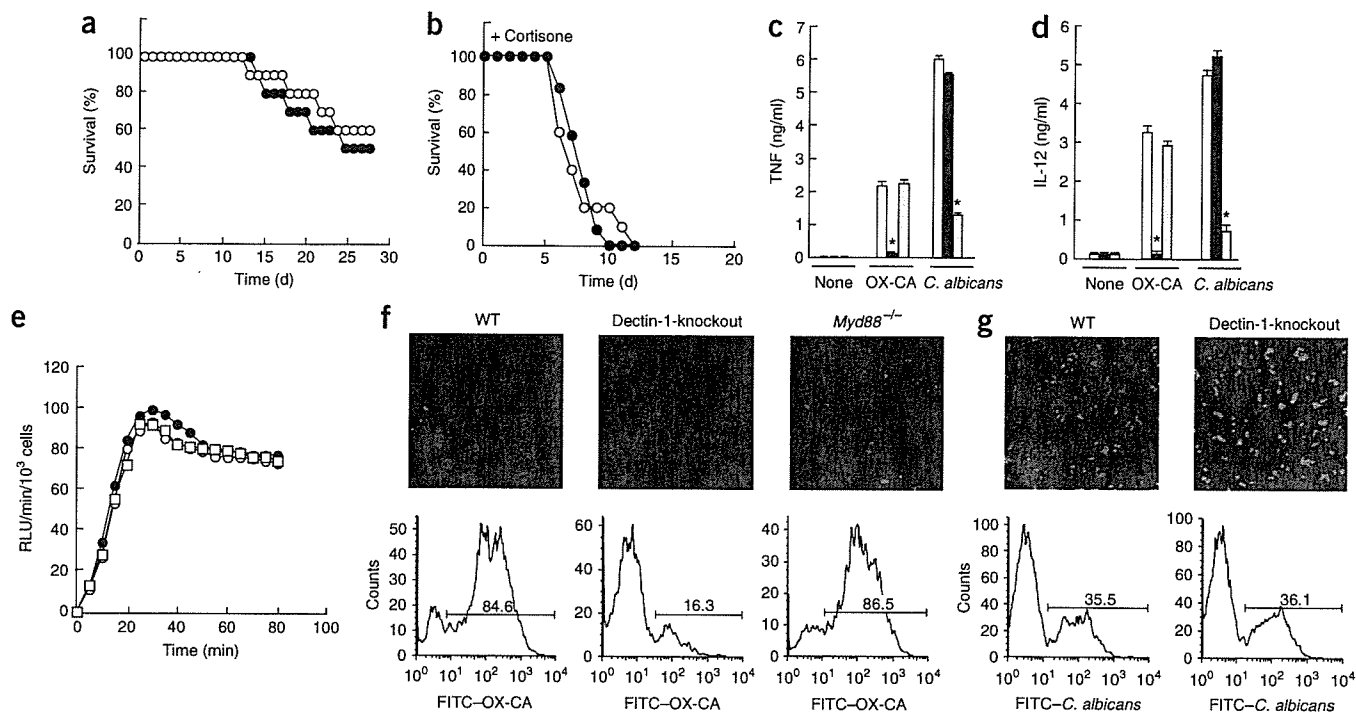
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Figure 5 Dectin-1 is not required for defense against *C. albicans*. (a) Survival of dectin-1-wild-type mice (*n* = 10; filled circles) and dectin-1-knockout mice (*n* = 10; open circles) on a C57BL/6J background, infected intravenously with 2 × 10⁵ *C. albicans*. (b) Survival of dectin-1-wild-type mice (*n* = 12; filled circles) and dectin-1-knockout mice (*n* = 10; open circles) on a BALB/cA background in an immunocompromised condition (+ Cortisone), after infection with 2 × 10⁵ *C. albicans*. (c,d) ELISA of cytokines in supernatants of dectin-1-wild-type (white bars), dectin-1-knockout (black bars) and *Myd88⁻/⁻* (gray bars) alveolar macrophages (C57BL/6J background) cultured for 24 h with NaClO-oxidized candida (OX-CA; 100 µg/ml) or *C. albicans* (multiplicity of infection, 10). Data represent mean (+ s.d.) of triplicate samples and were reproduced in three independent experiments. (e) ROS in supernatants of alveolar macrophages from dectin-1-wild-type (filled circles), dectin-1-knockout (open circles) or *Myd88⁻/⁻* (open squares) C57BL/6J mice; cells (pooled from three mice) were stimulated with *C. albicans*. *, *P* < 0.01 (Student's *t*-test). Data were reproducible in three independent experiments. (f,g) Confocal laser microscopy (top rows) and flow cytometry (bottom rows) of thioglycollate-elicited dectin-1-wild-type (WT), dectin-1-knockout and *Myd88⁻/⁻* macrophages cultured for 60 min together with FITC-labeled NaClO-oxidized candida (100 µg/ml; f) or with FITC-labeled *C. albicans* (1 × 10⁵ cells; g). For microscopy, coculture (FITC, green) was followed by macrophage staining with CM-Dil (red). For flow cytometry, coculture was followed by staining with phycoerythrin-conjugated anti–mouse CD11b (f) or with CM-Dil (g); numbers above bracketed lines indicate percent FITC⁺ cells in the CD11b⁺ or Dil⁺ cell population. Data are from one of two independent experiments (f,g).

Although production of TNF and IL-12 was not inhibited in dectin-1-knockout macrophages (**Fig. 4c,d**), cytokine production was much lower in *Myd88⁻/⁻* macrophages, indicating that cytokine induction by *P. carinii* is dependent on MyD88 signaling. In these conditions, we could not detect IL-10 production (data not shown). We also evaluated production of ROS. Although wild-type macrophages produced ROS after incubation with *P. carinii*, production of ROS was completely abolished in dectin-1-knockout macrophages incubated with *P. carinii* (**Fig. 4e**), indicating that dectin-1-induced signaling is essential for induction of ROS. In contrast, production of ROS in *Myd88⁻/⁻* macrophages was similar to that in wild-type macrophages (**Fig. 4e**), indicating that MyD88 signaling is not required for *P. carinii*–induced production of ROS. Thus, dectin-1 is crucial for the induction of ROS but not of TNF or IL-12 after infection with *P. carinii*.

Dectin-1-knockout mice are not more susceptible to *C. albicans*

To assess the effect of dectin-1 deficiency on systemic candidiasis, we infected mice intravenously with 2 × 10⁵ *C. albicans* cells per mouse and then monitored the survival of the mice. Unexpectedly, we detected no difference in the survival of dectin-1-knockout and wild-type mice (**Fig. 5a**), even after several doses of *C. albicans* (2 × 10⁴, 1 × 10⁵, 5 × 10⁵ or 1 × 10⁶ fungi per mouse all resulted

in similar survival; **Supplementary Fig. 2** online and data not shown). We also did not detect significant differences in fungal burdens in the lungs of dectin-1-knockout and wild-type mice after intratracheal administration of *C. albicans* (**Supplementary Fig. 2**). We also examined the sensitivity of dectin-1-knockout mice to *C. albicans* infection in immunocompromised conditions. When we treated mice with cortisone and infected them intravenously with 2 × 10⁵ *C. albicans*, there was no significant difference in the survival rates of dectin-1-knockout mice and wild-type mice (**Fig. 5b**). Thus, in contrast to the results we obtained after infection with *P. carinii*, dectin-1 is dispensable for the host defense against *C. albicans*.

C. albicans–induced ROS and cytokines do not require dectin-1

We next examined cytokine production in dectin-1-knockout macrophages after treatment with a β-glucan preparation purified from *C. albicans* or with live fungal cells. Although there was significant induction of TNF and IL-12 in wild-type macrophages stimulated with NaClO-oxidized candida, a particle-form β-1,6-branched β-1,3-glucan derived from *C. albicans*, production of those cytokines was completely abolished in dectin-1-knockout macrophages (**Fig. 5c,d**). In contrast, cytokine production was not reduced in dectin-1-knockout macrophages cultured with live *C. albicans*. Also, TNF and IL-12 production was not lower in *Myd88⁻/⁻* macrophages

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than in wild-type macrophages after induction with NaClO-oxidized candida, but it was significantly lower in $Myd88^{-/-}$ macrophages after infection with *C. albicans*, indicating that β-glucans are not the main inducer of cytokines after infection. In addition, production of ROS by dectin-1-knockout macrophages after infection with *C. albicans* was similar to that of wild-type macrophages, indicating that in contrast again to its function during *P. carinii* infection, dectin-1 is not required for *C. albicans*–induced production of ROS (**Fig. 5e**). MyD88 was also not involved in the induction of ROS by *C. albicans*.

Finally, we found that dectin-1-knockout macrophages did not bind NaClO-oxidized candida, whereas wild-type or $Myd88^{-/-}$ macrophages did efficiently bind that form of β-glucan (**Fig. 5f**). In contrast, dectin-1-knockout macrophages bound untreated *C. albicans* as efficiently as wild-type macrophages did (**Fig. 5g**), indicating that molecules other than β-glucan are required. These results indicated that dectin-1 is the only receptor for the β-1,3- and β-1,6-glucans derived from *C. albicans*, whereas other molecules are mainly involved in the induction of cytokines and ROS after infection of this fungus.

DISCUSSION

Here we have provided definitive evidence that dectin-1 is the sole receptor for β-glucans, that dectin-1 is required for production of cytokines and ROS from both DCs and macrophages after stimulation with β-glucans, that dectin-1 is required for DC maturation induced by SCG and that dectin-1 is required for optimal host defense against *P. carinii* but not *C. albicans*. We have demonstrated that TNF and IL-12 production was abolished in dectin-1-knockout BMDCs, alveolar macrophages and thioglycollate-elicited macrophages after stimulation with purified β-glucans such as SCG, NaClO-oxidized zymosan or NaClO-oxidized candida. $Myd88^{-/-}$ and wild-type BMDCs, in contrast, produced similar amounts of cytokines, indicating that MyD88 is not involved in dectin-1 signaling. We also found that dectin-1-knockout BMDCs and thioglycollate-elicited macrophages treated with zymosan produced normal amounts of TNF, whereas $Myd88^{-/-}$ cells produced much less, suggesting that zymosan-induced cytokine production is dependent on TLRs.

TNF production in BMDCs induced by either *P. carinii* or *C. albicans* was also unaffected by dectin-1 deficiency, although it was lower in $Myd88^{-/-}$ cells. Those observations are consistent with a published report that TLR2 and TLR6 signaling are important in zymosan-induced cytokine production in a macrophage cell line (RAW cells)[22]. However, we did find that zymosan-induced IL-10 production was much lower in dectin-1-knockout BMDCs and thioglycollate-elicited macrophages, which was consistent with a report that DCs deficient in Syk, a protein kinase 'downstream' of dectin-1, do not produce any IL-10 but instead produce IL-12 after stimulation with zymosan[30]. We also found that the binding of NaClO-oxidized candida to dectin-1-knockout thioglycollate-elicited macrophages was much lower than its binding to wild-type cells, whereas the binding of untreated *C. albicans* cells was not. Thus, living *P. carinii* and *C. albicans* induce cytokines, except IL-10, in a dectin-1-independent way, although those organisms express β-glucans that can activate dectin-1.

As only nude mice or cortisone-treated, immunocompromised wild-type mice are susceptible to *P. carinii*, it has been suggested that acquired immune responses are important mainly in the host defense mechanism. Our data that dectin-1-knockout mice were more susceptible than wild-type mice to *P. carinii* demonstrated that dectin-1 is required for defense against that pathogen. Because the production of cytokines such as IL-12 and TNF by dectin-1-knockout macrophages was similar to that of wild-type macrophages after

infection with *P. carinii*, those cytokines, which are required for T helper 1 cell induction and B cell activation, may not function in protection against that organism.

Notably, we found that production of ROS was completely abolished in dectin-1-knockout mice after infection with *P. carinii*, indicating that production of ROS is entirely dependent on β-glucan–dectin-1 signaling. Zymosan-induced production of ROS in thioglycollate-elicited macrophages was also completely dependent on dectin-1, consistent with a published study[20]. Those results contrast with results demonstrating that production of TNF and IL-12 is induced more efficiently through TLRs. ROS, which are produced by macrophages after microbe infection, are thought to be important in antifungal protection[25], because ROS modify fungal proteins, break nucleic acids and oxidize lipid components[31]. Our observations here suggested that dectin-1 is important in the host defense against *P. carinii* by inducing ROS-mediated killing of the organism.

Although possible involvement of dectin-1 in T cell activation has been suggested[2], we have shown here that dectin-1 is not involved in anti-CD3-induced T cell proliferation, antigen presentation of DCs, production of antibodies to sheep red blood cells and to dinitrophenol–keyhole limpet hemocyanin, or the delayed-type hypersensitivity reaction. Moreover, we have shown that the protective effect of dectin-1 against *P. carinii* was present in immunocompromised conditions. Therefore, it is unlikely that dectin-1 is involved in the host defense against *P. carinii* through activation of T cell–dependent immunity.

In contrast to the results obtained with *P. carnii*, we found no significant difference in the survival and fungal loads of wild-type and dectin-1-knockout mice after infection with *C. albicans* in either immunocompetent or immunocompromised conditions. We found that β-glucans on *C. albicans* did not significantly contribute to macrophage production of cytokines after infection with *C. albicans*, although dectin-1-knockout macrophages failed to produce cytokines in response to NaClO-oxidized candida purified from *C. albicans*. Those results suggest that β-glucans on live fungal surfaces are not efficiently accessible to dectin-1, which has been indicated in a published study[9]. Instead, we found that MyD88-dependent signaling induced cytokines after candida infection, which is consistent with published reports[10,12,13]. There was similar production of ROS in alveolar macrophages from dectin-1-knockout and wild-type mice after infection with *C. albicans*. That result contrasts with those obtained after *P. carinii* or zymosan treatment of dectin-1-knockout cells showing that production of ROS was completely dependent on the presence of dectin-1.

After *C. albicans* infection, production of ROS was also normal in $Myd88^{-/-}$ macrophages. Thus, other molecules in the cell walls of *C. albicans* might stimulate host cells in the absence of the binding of β-glucan to dectin-1. *C. albicans* indeed has a multilayered cell wall composed of an outer layer containing *N*- and *O*-linked mannosyl residues and an inner skeletal layer of β-glucans and chitins[32], and it has been reported that host cell recognition of *C. albicans* is mediated by three different receptor systems (MR, TLR4 and a dectin-1–TLR2 hetero-receptor complex) and that β-glucans contribute only marginally to cytokine production[32]. The structure of fungal cell walls and the accessibility of β-glucans, TLR ligands and mannans in the cell walls to their cognate receptors may be different for different pathogens. Therefore, different combinations of host receptors are probably needed for host defense mechanisms against different fungi. In conclusion, our results suggest the importance of dectin-1 for protection against *P. carinii* and indicate dectin-1 is a 'second' defense mechanism, in addition to TLRs, required particularly in the absence of acquired immune responses. Such a defense mechanism is probably critical in

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immunocompromised people, such people infected with human immunodeficiency virus, transplant recipients and patients with tumors.

METHODS

Mice. Dectin-1-knockout mice were generated by homologous recombination using the of embryonic stem cell line E14.1 (**Supplementary Methods** online). Dectin-1-knockout mice backcrossed for eight generations to BALB/cA mice (CLEA Japan) were used for *P. carinii* infection. For other experiments, unless stated otherwise in the figure legends, mice were backcrossed for eight generations to C57BL/6J mice (Nihon SLC). *Myd88^{-/-}* mice were also backcrossed for more than eight generations to C57BL/6J mice[33]. Male or female mice 5–10 weeks of age were used for experiments, and wild-type C57BL/6J mice or BALB/cA mice were used as controls. All mice were kept in specific pathogen–free conditions at the Center for Experimental Medicine, The Institute of Medical Science, The University of Tokyo. Experiments were done according to institutional guidelines and were approved by institutional committees.

Cell preparation and β-glucan sensitivity analysis. Bone marrow cells were removed from the femurs and tibiae of 6- to 8-week-old female mice and BMDCs were prepared as described[34]. SCG (100 μg/ml) or LPS (20 ng/ml) was added on day 5 after cultivation with granulocyte-macrophage colony-stimulating factor and IL-4. On day 6, culture supernatants were collected for cytokine titration and nonadherent cells were used in cell surface marker expression assays. For isolation of alveolar macrophages, mouse lungs were lavaged with prewarmed PBS supplemented with 0.5 mM EDTA using an intratracheal catheter. Cells were collected and seeded (1.0 × 10^{5} cells/ml), and nonadherent cells were removed 2 h later by washing in culture medium. Thereafter, NaClO-oxidized candida was added to the adherent cells, followed by culture for 24 h, and supernatants were then used for cytokine titration. For isolation of thioglycollate-elicited macrophages, mice were injected intraperitoneally with 2 ml of 4% (weight/volume) thioglycollate (Nissui). Then, 3 d later, peritoneal cells were collected by washing with PBS containing 0.5 mM EDTA. Total cells were then cultured for 2 d on a dish in RPMI 1640 medium supplemented with 10% (volume/volume) FCS; adherent cells were then used as thioglycollate-elicited macrophages.

Cytokine titration. Concentrations of IFN-γ, TNF, IL-10, and IL-12 were determined with a commercially available OptiEIA kit (BD Biosciences) according to the manufacturer's instructions.

Cell surface marker analysis. For analysis of cell surface markers on BMDCs, cells were cultured with or without stimulation as described above and then were analyzed by flow cytometry[35]. Antibodies used for detection were as follows: biotin-conjugated anti–mouse CD80 (16-10A1), biotin-conjugated anti–mouse CD40 (3/23), fluorescein isothiocyanate (FITC)–conjugated anti–mouse CD11c (HL3) and phycoerythrin-conjugated streptavidin were purchased from BD Bioscience, and biotin-conjugated monoclonal anti–mouse dectin-1 (RH1) was produced by immunization of rats with the recombinant carbohydrate-recognition domain of dectin-1. Fluorescence intensity was quantified with a FACSCalibur (BD) and data were analyzed with FlowJo software (Tree Star).

Preparation of β-glucans. SCG (β-1,6-branched β-1,3-glucan) was prepared from the fruit bodies of the edible mushroom *S. crispa* (Minahealth) as described[34]. NaClO-oxidized candida and insoluble β-1,6-branched β-1,3-glucans were prepared from *C. albicans* IFO1385 as described[36]. For conjugation with biotin, NaClO-oxidized candida (100 mg) was suspended in 5 ml of 0.1 M acetate buffer, pH 5.4. The suspension was mixed with 1.2 μmol sodium metaperiodate to partially oxidize branched 1, 6-linked glucosyl residues. The mixed suspension was further incubated for 1 h at 24 °C in the dark. NaClO-oxidized candida was washed with acetate buffer by centrifugation and then was mixed with 100 μg/ml of biotin (long arm) hydrazide (Vector Laboratories) dissolved in dimethysulfoxide and acetate buffer, followed by incubation for 1 h at 24 °C. The mixed NaClO-oxidized candida suspension was treated for 30 min with sodium borohydride. Biotin-conjugated NaClO-oxidized candida was washed with deionized water and then dried, with subsequent washing with ethanol, acetone and then diethyl ether. Biotin-labeled NaClO-oxidized candida was conjugated to FITC by incubation with streptavidin-FITC (BD Biosciences)

before use. NaClO-oxidized zymosan was prepared as described[24]. Zymosan (1 g) was suspended for 1 d at 4 °C in 100 ml of 0.1 M NaOH with 0.5% NaClO. The insoluble fraction (NaClO-oxidized zymosan) was then collected by centrifugation and was suspended in 1 ml of saline and was sonicated for 30 s.

Assay for production of ROS. Production of ROS was analyzed by luminol-enhanced chemiluminescence. Thioglycollate-elicited macrophages (5×10^{5} cells) isolated as described above were suspended in 1 ml culture medium and were incubated for 30 min at 37 °C. Aliquots (100 μl) were then placed in luminometer tubes and NaClO-oxidized zymosan (100 μg/ml) or zymosan (100 μg/ml) in 100 μl medium containing 100 μM luminol (Sigma) was added. Chemiluminescence was measured at 5-minute intervals with a luminometer (Lumat LB9507; Berthold Technologies). Chemiluminescence is expressed as relative luciferase units per minute per 1 × 10^{3} cells.

In vitro **binding assay.** Thioglycollate-elicited macrophages were collected from dectin-1-knockout mice, *Myd88^{-/-}* mice and wild-type C57BL/6J mice (Nihon SLC) as described above. Cells were adjusted to a concentration of 5 × 10^{5} cells/ml and 100 μl of the cell suspension was seeded onto a chamber slide system (Nalg Nunc International). Then, 2 h later, chambers were washed with culture medium and 100 μl of FITC-labeled NaClO-oxidized candida suspended in culture medium (100 μg/ml) or 100 μl of FITC-labeled *C. albicans* (1 × 10^{5} cells) was added, followed by culture for 60 min. Unbound, unphagocytosed cells were then washed, followed by staining with CM-DiI (octadecyl indocarbocyanine (DiI) with a thiol-reactive chloromethyl moiety; Molecular Probes) and visualization with a confocal laser-scanning microscopy system (Radiance 2100; Nihon BIO RAD). For flow cytometry, thioglycollate-elicited macrophages and FITC-labeled NaClO-oxidized candida or FITC-labeled *C. albicans* were incubated for 60 min at 37 °C in polypropylene tubes. Then, cells were washed three times and were stained with CM-DiI or phycoerythrin-conjugated anti–mouse CD11b (M1/70; BD Biosciences). Fluorescence intensity was quantified with a FACSCalibur (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

T cell proliferation assay. CD4^{+} T cells were isolated from lymph nodes and spleens of DO11.10 mice[37] or BALB/cA mice with an autoMACS (Miltenyi Biotec) after being stained with microbead-conjugated anti–mouse CD4 (Miltenyi Biotec) according to the manufacturer's instructions. DCs were also isolated with an autoMACS after being stained with microbead-conjugated anti–mouse CD11c (Miltenyi Biotec) from collagenase-digested spleens of dectin-1-knockout or dectin-1-wild-type mice on a BALB/cA background (fourth generation). CD4^{+} T cells (1 × 10^{5} cells) were cultured for 3 d in 96-well plates with DCs (1 × 10^{4} cells) in the presence of ovalbumin peptide (amino acids 323–339) or anti–mouse CD3 purified from the culture supernatants of 145-2C11 cells (CRL-1975; American Type Culture Collection). Cells were incubated with [^{3}H]thymidine (0.25 mCi/ml; Amersham) for 6 h before collection, then incorporated radioactivity was measured with a Microbeta counter (Pharmacia Biotech)[38].

Immunization of mice and measurement of antibody titers. Mice (fourth generation on a BALB/cA background) were immunized intraperitoneally with 1 × 10^{8} sheep red blood cells (Nihon Seibutsuzairyou) in PBS[39]. Blood samples were collected from tail veins both before immunization and 1 week after. Antigen-specific antibody titers were measured by enzyme-linked immunosorbent assay (ELISA) with plate-coated soluble sheep red blood cell antigens (2 mg/ml).

Delayed-type hypersensitivity response. Delayed-type hypersensitivity induced by methylated bovine serum albumin was assessed as described[40]. Footpad swelling was measured with a dial caliper and results were calculated as follows: footpad swelling (%) = (thickness of footpad injected with methylated bovine serum albumin) / (thickness of footpad injected with PBS) × 100.

***P. carinii* infection.** *P. carinii* was prepared from lung homogenates of BALB/c nude mice inoculated previously with *P. carinii*[41,42]. BALB/cA mice (dectin-1-wild-type or dectin-1-knockout) were inoculated intranasally with 1.7 × 10^{4} cysts and mice were then killed at 7 d, 14 d, 20 d or 4 months after infection; cysts in the lungs were then counted. For experiments in immunocompromised conditions, anesthetized mice were inoculated with 6.4 × 10^{5}

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cysts, followed by subcutaneous administration of cortisone acetate (Wako Pure Chemical Industries) at a dose of 2.5 mg per mouse twice weekly. *P. carinii* cysts in the lungs were counted as described[42], and total cysts on the smear were counted with a microscope after staining with toluidine blue O. The number of cysts per mouse was recorded. For *in vitro* cytokine production studies, alveolar macrophages at a density of 1×10^5 cells/ml were cultured for 2 h at 37 °C in 0.1 ml RPMI 1640 medium supplemented with 10% (volume/volume) FCS. After removal of nonadherent cells, adherent cells were incubated with 1×10^4 *P. carinii* cysts. Culture supernatants were then collected and cytokine concentrations were measured. Production of ROS was measured by luminol-enhanced chemiluminescence. Alveolar macrophages (4×10^5 cells) were isolated from dectin-1-knockout, $Myd88^{-/-}$ or wild-type C57BL/6J mice and were suspended in 1 ml of culture medium and incubated for 30 min at 37 °C. Aliquots (100 μl) were then placed in luminometer tubes and *P. carinii* (4×10^4 cysts) in 100 μl medium containing 100 μM luminol (Sigma) was added. Chemiluminescence was measured as described above.

C. albicans infection. *C. albicans* (18804; American Type Culture Collection) was grown for 36 h at 30 °C on potato dextrose agar plates (Eiken Kizai). For survival analysis, wild-type and dectin-1-knockout mice were infected intravenously with 1×10^6 or 5×10^5 *C. albicans* and then were monitored for 20 d. Production of ROS was determined by the luminal-enhanced test as described above using alveolar macrophages (5×10^4 cells) and *C. albicans* (5×10^4 cells). For FITC labeling, 5 ml of a suspension of *C. albicans* (1×10^6 cells/ml) were incubated for 30 min at 24 °C with 0.1 mg/ml of FITC (Sigma) in PBS, and then cells were washed five times with PBS.

Statistics. Statistical significance was determined with a Mann-Whitney U-test and Student's *t*-test for analysis of *P. carinii* infection. Student's *t*-test was used for analysis of ELISAs and ROS assays, and a χ^2 test was used for analysis of survival assays.

Note: Supplementary information is available on the Nature Immunology website.

AUTHOR CONTRIBUTIONS
S.S. mainly contributed throughout this work in collaboration with N.F., S.C. and K. Seki; H.K. and K. Sudo did embryonic stem cell culture and produced chimeric mice; Y.A. and N.O. made β-glucan preparations and did cytokine production experiments; T.F. did *in vivo P. carinii* experiments and provided *P. carinii* for the *in vitro* experiments; T.K., K.N. and K.K. did *in vivo C. albicans* experiments; S.A. provided $Myd88^{-/-}$ mice; and Y.I. supervised the study, designed the experiments and edited the draft paper.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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Nuclear Import of the Preintegration Complex Is Blocked upon Infection by Human Immunodeficiency Virus Type 1 in Mouse Cells[∇]

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Mouse cells do not support human immunodeficiency virus type 1 (HIV-1) replication because of host range barriers at steps including virus entry, transcription, RNA splicing, polyprotein processing, assembly, and release. The exact mechanisms for the suppression, however, are not completely understood. To elucidate further the barriers against HIV-1 replication in mouse cells, we analyzed the replication of the virus in lymphocytes from human CD4/CXCR4 transgenic mice. Although primary splenocytes and thymocytes allowed the entry and reverse transcription of HIV-1, the integration efficiency of the viral DNA was greatly reduced in these cells relative to human peripheral blood mononuclear cells, suggesting an additional block(s) before or at the point of host chromosome integration of the viral DNA. Preintegration processes were further analyzed using HIV-1 pseudotyped viruses. The reverse transcription step of HIV-1 pseudotyped with the envelope of murine leukemia virus or vesicular stomatitis virus glycoprotein was efficiently supported in both human and mouse cells, but nuclear import of the preintegration complex (PIC) of HIV-1 was blocked in mouse cells. We found that green fluorescent protein (GFP)-labeled HIV-1 integrase, which is known to be important in the nuclear localization of the PIC, could not be imported into the nucleus of mouse cells, in contrast to human cells. On the other hand, GFP-Vpr localized exclusively to the nuclei of both mouse and human cells. These observations suggest that, due to the dysfunction of integrase, the nuclear localization of PIC is suppressed in mouse cells.

A small animal model for AIDS would provide a valuable tool for the study of its pathogenesis and the evaluation of vaccine candidates and antiviral drugs. However, attempts to produce small animal models have been hampered thus far by species-specific host range barriers to infection by human immunodeficiency virus type 1 (HIV-1). CD4, the cellular receptor for HIV-1 (41, 49), was first identified as a host range barrier because mouse CD4 (muCD4) does not bind HIV-1 Env (46). Human CD4 (huCD4) transgenic (Tg) mice, however, were not susceptible to HIV-1 infection, suggesting the presence of additional barriers (47). Chemokine receptors were later identified as entry coreceptors (9, 22), but primary lymphocytes from mice transgenic for huCD4 and either huCXCR4 (70) or huCCR5 (13) exhibited little to no signs of productive infection.

Cyclin T1 (CycT1) is responsible for a transcriptional level barrier (3, 4, 26, 30, 58). CycT1 protein is a component of the TAK/pTEFb transcription factor complex (51, 78), and huCycT1 binds Tat and activates transcription from the promoter in the long terminal repeat (LTR). However, muCycT1 cannot

bind Tat. Nevertheless, introduction of the huCycT1 protein to rodent cells together with a mixture of human receptors was insufficient to induce productive viral infection (11, 52).

Additional barriers have been reported in the late steps of the viral life cycle (11, 27, 40, 42, 43, 53). These late-stage defects can be rescued by fusing HIV-1-infected rodent cells to uninfected human cells (11, 52), indicating that the defects are due to the lack of necessary factors in rodent cells rather than the presence of dominant inhibitors of HIV-1 replication. CRM1, a nuclear export factor that functions in association with Rev, and p32, a splicing inhibitor and Rev-binding protein, are suggested to be necessary late-phase factors (67, 83).

We previously produced Tg mice carrying the HIV-1 proviral genome in which the *pol* gene is deleted (HIV-Tg) (36). Although transgene expression in lymphoid tissues is barely detectable under normal physiological conditions, relatively high levels of p24 Gag protein were detected in the serum (up to 400 pg/ml) after injection of bacterial lipopolysaccharide (74). All mRNA species, including unspliced, singly spliced, and multiply spliced mRNAs were produced normally. Thus, once the viral genome is integrated into the host chromosome, viral genes are expressed at a reasonable efficiency even in mouse cells, suggesting that the major host range barriers are present in the early stage of infection (prior to viral DNA integration) rather than in the late stage. However, it is not yet known whether there are any additional host range barriers in the early steps.

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