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Dectin-1 diversifies *Aspergillus fumigatus*–specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation

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Pulmonary infection of mice with *Aspergillus fumigatus* induces concurrent T helper type 1 (Th1) and Th17 responses that depend on Toll-like receptor/MyD88 and Dectin-1, respectively. However, the mechanisms balancing Th1 and Th17 CD4 T cell populations during infection remain incompletely defined. In this study, we show that Dectin-1 deficiency disproportionally increases Th1 responses and decreases Th17 differentiation after *A. fumigatus* infection. Dectin-1 signaling in *A. fumigatus*–infected wild-type mice reduces IFN-γ and IL-12p40 expression in the lung, thereby decreasing T-bet expression in responding CD4 T cells and enhancing Th17 responses. Absence of IFN-γ or IL-12p35 in infected mice or T-bet in responding CD4 T cells enhances Th17 differentiation, independent of Dectin-1 expression, in *A. fumigatus*–infected mice. Transient deletion of monocyte-derived dendritic cells also reduces Th1 and boosts Th17 differentiation of *A. fumigatus*–specific CD4 T cells. Our findings indicate that Dectin-1–mediated signals alter CD4 T cell responses to fungal infection by decreasing the production of IL-12 and IFN-γ in innate cells, thereby decreasing T-bet expression in *A. fumigatus*–specific CD4 T cells and enabling Th17 differentiation.

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Abbreviations used: BALF, bronchoalveolar lavage fluid; DT, diptheria toxin; DTR, DT receptor; HPRT, hypoxanthine guanine phosphoribosyl transferase; ICCS, intracellular cytokine staining; ITAM, immunoreceptor tyrosine-based activation motif; MLN, mediastinal LN; TLR, Toll-like receptor.

Microbial infections induce expansion and differentiation of naive, pathogen-specific CD4 T cells into responder populations that restrict in vivo microbial growth and enable microbial clearance (Zhu et al., 2010). How and in which direction naive CD4 T cells differentiate vary with the microbial pathogen. Th1 cells control intracellular pathogens by producing IFN-γ, whereas Th2 cells contribute to helminth elimination by producing IL-4, IL-5, and IL-13, and Th17 cells protect against extracellular bacterial and fungal pathogens by making IL-17A and IL-22 (North and Jung, 2004; Anthony et al., 2007; Palm and Medzhitov, 2007). The transcriptional regulators T-bet, GATA-3, and RORγt control differentiation of naive CD4 T cells into Th1, Th2, and Th17 cells, respectively (Zhang et al., 1997; Zheng and Flavell, 1997; Szabo et al., 2000; Ivanov et al., 2006). IL-12 and IFN-γ induce T-bet expression in Th1 cells, whereas TGF-β and IL-6 support Th17 differentiation and RORγt expression (Szabo et al., 2003; McGeachy and Cua, 2008). Responding CD4 T cell populations cross-regulate each other, generally suppressing differentiation into

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other phenotypes once a dominant phenotype has become established. For example, IL-4 secreted by Th2 cells restricts Th1 and Th17 differentiation, whereas IFN-γ produced by Th1 cells suppresses Th2 and Th17 differentiation (Trinchieri, 2003; Harrington et al., 2005; Park et al., 2005). Similarly, IL-17A can suppress Th1 differentiation (O'Connor et al., 2009), and TGF-β promotes Th17 differentiation and inhibits Th1 and Th2 differentiation (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006).

Innate immune receptors bind microbial molecules and induce expression of cytokines that drive CD4 T cell differentiation. Toll-like receptors (TLRs) represent a major class of innate immune receptors that respond to microbial molecules and signal primarily through MyD88 (Medzhitov, 2007; Iwasaki and Medzhitov, 2010). For example, stimulation of DCs with purified TLR ligands induces IL-12 production and supports in vitro and in vivo differentiation of Th1 CD4 T cells (Schnare et al., 2001; Trinchieri, 2003). In addition to TLRs, several other innate immune signaling receptors contribute to cytokine induction and CD4 T cell differentiation in the setting of microbial infection. Dectin-1 is an innate immune receptor that mediates protection from fungal infection in mice (Saijo et al., 2007; Taylor et al., 2007; Werner et al., 2009) and humans (Ferwerda et al., 2009; Glocker et al., 2009; Cunha et al., 2010). Dectin-1 consists of an extracellular C-type lectin domain and an intracellular immunoreceptor tyrosine-based activation motif (ITAM)–like motif and responds to β(1,3)-glucan, a major cell wall component common to most fungal pathogens (Hohl et al., 2005; Brown, 2006; Saijo et al., 2007; Taylor et al., 2007). Binding of β-glucan by Dectin-1 activates canonical and noncanonical NF-κB signaling pathways via Syk-CARD9 and Raf-1, respectively (Gringhuis et al., 2009), and induces DC maturation and the production of chemokines and cytokines that direct CD4 T cell activation and differentiation (LeibundGut-Landmann et al., 2007; Gringhuis et al., 2009). DCs stimulated with purified β-glucan promote the development of Th17 cells (Brown, 2006; Acosta-Rodriguez et al., 2007; LeibundGut-Landmann et al., 2007) and produce IL-23, a cytokine which promotes the expansion and differentiation of Th17 cells (LeibundGut-Landmann et al., 2007; Gerosa et al., 2008; McGeachy et al., 2009; Ghoreschi et al., 2010).

The opportunistic fungal pathogen *Aspergillus fumigatus* is a prevalent environmental fungus that causes potentially lethal infections in immunosuppressed patients (Hohl and Feldmesser, 2007). Although humans inhale several hundred to thousands of *A. fumigatus* spores per day (Latgé, 1999), invasive disease is rare because of efficient and highly effective innate and adaptive immune responses that control fungal growth (Hohl et al., 2006). Th1 and Th17 CD4 T cells provide defense against *A. fumigatus* infection, and TLR/MyD88 and Dectin-1 signaling pathways likely contribute to their differentiation (Romani, 2004; Hohl et al., 2006). Germinating *A. fumigatus* spores activate TLRs and Dectin-1 (Hohl et al., 2005; Rivera et al., 2005; Steele et al., 2005; Gersuk et al., 2006) and induce robust recruitment of inflammatory cells to the lung. Although some experiments have demonstrated that Dectin-1– and TLR-mediated signals collaborate during early antifungal innate immune responses (Gantner et al., 2003), recent studies also indicate that ITAM-mediated signaling can suppress TLR-mediated signals (O'Neill, 2008; Wang et al., 2010).

Herein, we investigated the impact of TLR/MyD88- and Dectin-1–mediated signals on in vivo CD4 T cell differentiation after pulmonary infection with *A. fumigatus*. Loss of Dectin-1 signaling in infected mice markedly reduces Th17 and enhances Th1 differentiation of *A. fumigatus*–specific CD4 T cells. Analysis of responding T cells in WT and Dectin-1–deficient mice reveals an inverse correlation between the level of T-bet expression in responding CD4 T cells and the magnitude of the Th17 T cell response. Our hypothesis that Dectin-1–mediated signals enhance Th17 responses by suppressing Th1 differentiation is supported by the finding that T-bet–deficient CD4 T cells readily differentiate into RORγt$^+$, IL-17–producing Th17 cells in WT and Dectin-1–deficient mice. Dectin-1 signaling in *A. fumigatus*–infected mice inhibits innate IFN-γ and IL-12p40 production and IL-12 or IFN-γ deficiency or depletion of monocyte-derived DCs 2–4 d after *A. fumigatus* infection markedly decreases Th1 and commensurately increases Th17 responses, supporting the notion that Dectin-1 signaling liberates responding CD4 T cells from a Th1 fate and enables Th17 differentiation.

RESULTS

Dectin-1 deficiency diminishes *A. fumigatus*–specific Th17 and enhances Th1 responses

In previous experiments, we described the generation of *A. fumigatus*–specific CD4 TCR transgenic mice (Af3.16; Rivera et al., 2006), which enable in vivo experiments of fungus-specific CD4 T cell activation and differentiation. In vitro cultures under Th1 or Th17 skewing conditions demonstrate that naive Af3.16 TCR-tg T cells undergo Th1 or Th17 differentiation to the same extent as polyclonal CD4 T cells (Fig. S1). To determine the impact of in vivo TLR/MyD88- and Dectin-1–mediated innate receptor signaling in promoting *A. fumigatus*–specific CD4 Th1 and Th17 T cell differentiation in response to a pulmonary infection with live *A. fumigatus* spores, we adoptively transferred Af3.16 TCR-tg CD4 T cells into MyD88$^{-/-}$, Dectin-1$^{-/-}$, and control mice 1 d before infection. Differentiation of Th1 and Th17 cells was assessed in the airways 6 d after a pulmonary infection by intracellular cytokine staining (ICCS) for IFN-γ and IL-17A. Consistent with our previous experiments (Rivera et al., 2006), Th1 differentiation of Af3.16 TCR-tg CD4 T cells was significantly diminished in the absence of MyD88-derived signals (Fig. 1, A and C), whereas Th17 differentiation was preserved (Fig. 1, A and B). In contrast, in the absence of Dectin-1, Th17 differentiation was diminished (Fig. 1, A and B). Strikingly, diminished IL-17 production in Dectin-1$^{-/-}$ mice was accompanied by a significant increase in IFN-γ–producing, *A. fumigatus*–specific Th1 cells (Fig. 1 C). Thus, the absence of Dectin-1, in addition to decreasing Th17 differentiation, also

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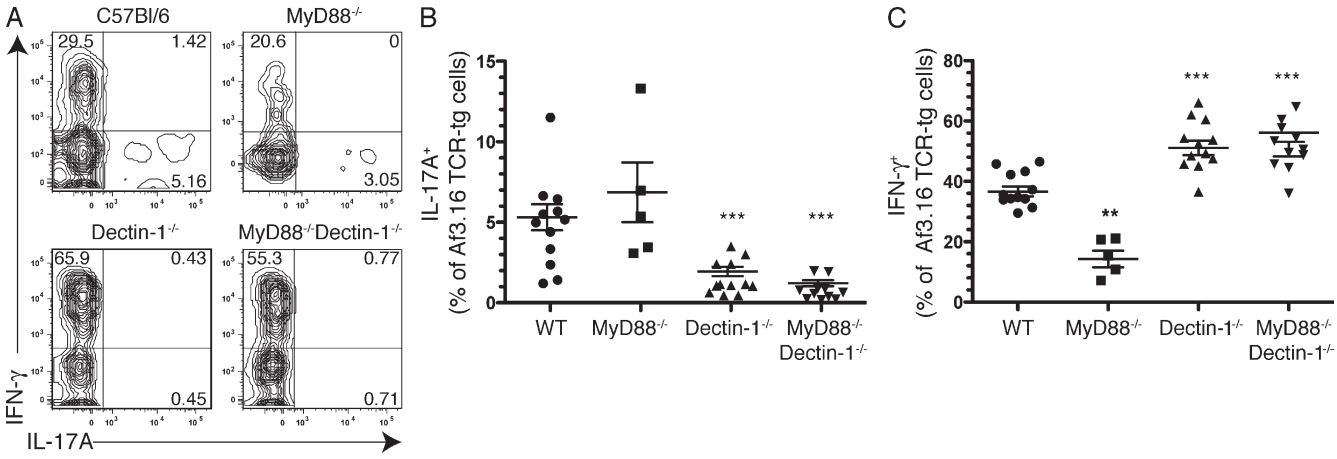

Figure 1. Diminished Th17 and enhanced Th1 differentiation in Dectin-1$^{-/-}$ mice. Af3.16-WT TCR-tg cells were adoptively transferred into MyD88$^{-/-}$, Dectin-1$^{-/-}$, Dectin-1$^{-/-}$MyD88$^{-/-}$, and control C57BL/6J mice 1 d before a pulmonary infection with live *A. fumigatus* spores. Af3.16 TCR-tg T cell differentiation was assessed by intracellular staining for IFN-γ and IL-17A of cells recovered from the airways (BALF) 6 d after infection. (A) Representative FACS plots of Af3.16-WT TCR-tg cells recovered from the BALF as indicated. (B and C) Frequency of IL-17A (B)– and IFN-γ–producing cells (C) among Af3.16 TCR-tg cells, as a measure of Th17 and Th1 differentiation, respectively. Each symbol represents one mouse. Data are cumulative of four separate experiments performed with two to four mice per group. Bars represent the mean with SEM. **, P ≤ 0.01; ***, P ≤ 0.001 compared with control mice.

resulted in enhanced Th1 differentiation. Dectin-1 deficiency also enhanced Th1 differentiation (Fig. 1 B) in MyD88$^{-/-}$ mice (Fig. 1, B and C), indicating that Dectin-1–mediated enhancement of Th1 differentiation does not depend on MyD88-mediated signals. These results suggest that Dectin-1 influences the ratio of fungus-specific Th1 and Th17 CD4 T cells by suppressing a dominant Th1 differentiation pathway.

The presence of viable *A. fumigatus* spores or the formation of fungal hyphae 6 d after infection was not significantly increased in MyD88$^{-/-}$, Dectin-1$^{-/-}$, or MyD88$^{-/-}$Dectin-1$^{-/-}$ as compared with control C57BL/6 mice (Fig. S2). Thus, changes in antigen load or expression are unlikely to account for changes in CD4 T cell differentiation in MyD88$^{-/-}$, Dectin-1$^{-/-}$, and WT mice.

T-bet suppresses Th17 differentiation, and in its absence, Th17 differentiation is Dectin-1 independent

The T-box transcription factor T-bet regulates Th1 differentiation. In the absence of T-bet, IFN-γ production by CD4 T cells is greatly diminished (Szabo et al., 2000, 2002). Moreover, enhanced IL-17 production has been observed in T-bet$^{-/-}$ mice, and several studies have suggested that T-bet suppresses Th17 differentiation (Harrington et al., 2005; Park et al., 2005; Mathur et al., 2006; Intlekofer et al., 2008; Guo et al., 2009). In contrast, recent experiments have demonstrated IL-23–mediated induction of T-bet^{+}RORγt^{+}Th17 CD4 T cells with enhanced encephalitogenic potential (Ghoreschi et al., 2010), suggesting that T-bet does not always suppress Th17 differentiation and that the role of T-bet in shaping CD4 T cell phenotypes is context dependent. To determine the role of T-bet in shaping CD4 T cell differentiation during *A. fumigatus* infection, we generated T-bet–deficient Af3.16 TCR-tg cells (Af3.16–T-bet$^{-/-}$). Af3.16–T-bet$^{-/-}$ TCR-tg cells that were adoptively transferred into WT-B6 mice differentiated into Th17 cells upon *A. fumigatus* infection (Fig. 2, A and B). Thus, T-bet induction is critical for Th1 differentiation in response to *A. fumigatus* infection. Moreover, Th17 differentiation of Af3.16–T-bet$^{-/-}$ TCR-tg cells occurred in Dectin-1$^{-/-}$, MyD88$^{-/-}$, and MyD88$^{-/-}$Dectin-1$^{-/-}$ mice (Fig. 2 B). Additionally, in the absence of T-bet expression, *A. fumigatus*–specific CD4 T cells produced minimal IFN-γ in all of the mice examined (Fig. 2 C). Thus, during *A. fumigatus* infection, T-bet acts in a T cell–intrinsic manner to promote IFN-γ expression and inhibit Th17 differentiation, with T-bet expression determining the in vivo ratio of responding Th1 to Th17 cells. T-bet and RORγt expression in Af3.16 TCR-tg CD4 T cells was mutually exclusive, with Af3.16-WT TCR-tg cells expressing T-bet and Af3.16–T-bet$^{-/-}$ TCR-tg cells expressing RORγt (Fig. 2 D). These findings are reminiscent of the established function of T-bet in inhibiting Th2 differentiation by suppressing GATA-3 expression (Hwang et al., 2005; Usui et al., 2006) and suggest that T-bet may similarly suppress RORγt expression in the context of a fungal infection.

Enhanced T-bet expression by Af3.16 TCR-tg cells primed in vitro with Dectin-1$^{-/-}$ DCs

Although the experiments presented in Fig. 2 suggest that Dectin-1–mediated signals enhance Th17 differentiation by inhibiting T-bet expression, it was unclear whether the absence of Dectin-1 signaling in antigen-presenting DCs affected T-bet expression in responding CD4 T cells. To address this issue, we performed in vitro priming experiments with naive Af3.16 TCR-tg cells and Dectin-1$^{-/-}$ or WT DCs infected with *A. fumigatus*. Naive Af3.16 TCR-tg CD4 T cells primed with Dectin-1$^{-/-}$ DCs up-regulated T-bet to higher

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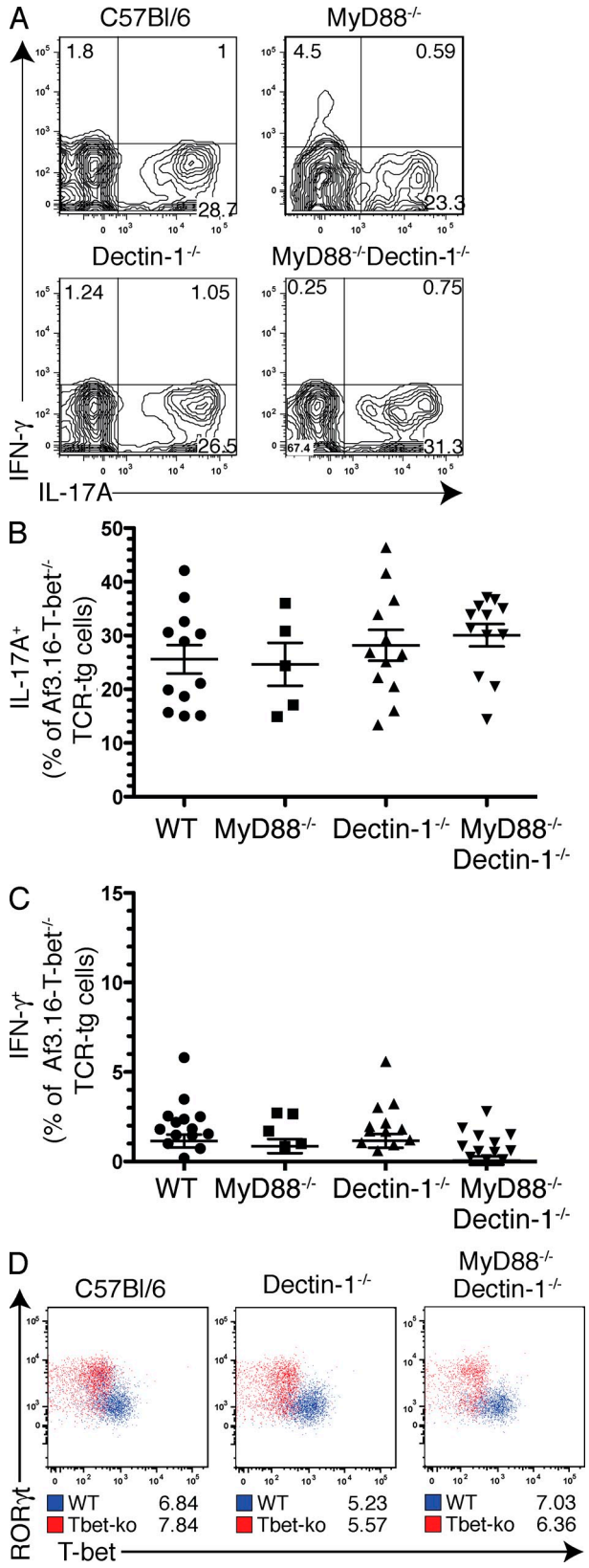

Figure 2. T-bet deficiency in fungus-specific CD4 T cells enhances Th17 differentiation. Af3.16-T-bet$^{-/-}$ TCR-tg cells were adoptively transferred into MyD88$^{-/-}$, Dectin-1$^{-/-}$, Dectin-1$^{-/-}$MyD88$^{-/-}$, and control C57BL/6J mice 1 d before a pulmonary infection with live *A. fumigatus* spores. Af3.16-T-bet$^{-/-}$ TCR-tg T cell differentiation was assessed by intracellular staining for IFN-γ and IL-17A of cells recovered from the airways (BALF) 6 d after infection. (A) Representative FACS plots of Af3.16-T-bet$^{-/-}$ TCR-tg cells recovered from the BALF as indicated. (B and C) Frequency of IL-17A (B)– and IFN-γ–producing cells (C) among Af3.16-T-bet$^{-/-}$ TCR-tg cells, as a measure of Th17 and Th1 differentiation, respectively. Each symbol represents one mouse. Data are cumulative of four separate experiments performed with two to four mice per group. Bars represent the mean with SEM. (D) Cells recovered from the BALF at day 6 after infection were analyzed for intracellular expression of RORγt and T-bet. FACS plots are for individual mice from each group and are representative of two separate experiments performed with five mice per group.

levels compared with T cells primed with control DCs (Fig. 3, A and B). Enhanced T-bet expression in Af3.16 TCR-tg cells primed by Dectin-1$^{-/-}$ DCs resulted in higher production of IFN-γ (Fig. 3 C) and lower IL-17A secretion (Fig. 3 D), as compared with Af3.16 TCR-tg primed by control DCs. These differences in fungus-specific CD4 T cell differentiation were not caused by altered priming capacity of Dectin-1$^{-/-}$ DCs, as demonstrated by equal IL-2 secretion (Fig. 3 E) and expression of activation markers (not depicted) by responding Af3.16 TCR-tg cells. T-bet deficiency resulted in enhanced IL-17A secretion by CD4 T cells primed in vitro with WT or Dectin-1$^{-/-}$ DCs (Fig. S3). These in vitro experiments support the notion that Dectin-1 signals in APCs such as DCs promote Th17 differentiation by modulating T-bet expression in responding CD4 T cells.

Distinct in vivo regulation of T-bet expression in responding CD4 T cells by MyD88 and Dectin-1

To quantify in vivo T-bet induction by TLR/MyD88- and Dectin-1–mediated signals, we cotransferred Af3.16-WT-Thy1.1$^{+/+}$ and Af3.16–T-bet$^{-/-}$Thy1.1/1.2 T cells into Dectin-1$^{-/-}$, MyD88$^{-/-}$, MyD88$^{-/-}$Dectin$^{-/-}$, and control mice. T-bet expression in *A. fumigatus*–specific CD4 T cells was measured in the mediastinal LNs (MLNs) and airways (bronchoalveolar lavage fluid [BALF]) by intracellular staining, and the amount of T-bet expression in Af3.16-WT TCR-tg cells relative to Af3.16–T-bet$^{-/-}$ cells was determined (Fig. 4 A). MyD88-mediated signals induced T-bet in the MLNs but not the airways (Fig. 4, B and C), which is consistent with our previous observations (Rivera et al., 2006). The frequency of T-bet–expressing, *A. fumigatus*–specific CD4 T cells was increased in the airways of Dectin-1$^{-/-}$ and MyD88$^{-/-}$Dectin-1$^{-/-}$ mice (Fig. 4, A and C) compared with WT mice, suggesting that Dectin-1 signaling in the WT hosts reduces Th1 differentiation by reducing T-bet expression in responding CD4 T cells. Because Th17 differentiation occurs in T-bet–deficient Af3.16 TCR-tg cells in the absence of Dectin-1–mediated signals (Fig. 2 B), we conclude that Dectin-1–mediated suppression of T-bet expression in responding *A. fumigatus*–specific CD4 T cells indirectly enables Th17 differentiation.

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Dectin-1–mediated signals diminish innate production of IFN-γ in the lung after infection with *A. fumigatus*

Concomitant in vitro stimulation of TLRs and Dectin-1 leads to enhanced IL-23 production and Dectin-1–dependent reduction in IL-12p70 secretion (Dennehy et al., 2009). Therefore, it is possible that in vivo IL-12p70 production is suppressed by Dectin-1 stimulation, resulting in decreased T-bet expression in responding CD4 T cells. Alternatively, increased expression of IL-23 upon Dectin-1 stimulation might directly reduce T-bet levels and thus promote Th17 differentiation. A third possibility is that Dectin-1 regulates the production of factors such as type I IFNs and/or IFN-γ, which can influence T-bet expression and Th1 differentiation. Therefore, we examined the induction of IL-12, IL-23, IFN-β, and IFN-γ expression in the lung of Dectin-1$^{-/-}$ and control mice at the peak of the innate response (Fig. 5). Although *A. fumigatus* infection induced transcription of these cytokines, IL-12p35 and IFN-β transcript levels were unaffected by the presence or absence of Dectin-1 (Fig. 5, A and B). In contrast, IL-12p40 transcripts were significantly increased in Dectin-1–deficient mice (Fig. 5 C), indicating that in WT mice, Dectin-1 signaling inhibits IL-12p40 transcription, which is consistent with previous experiments demonstrating that activation of the ITAM signaling pathway by FcγR stimulation suppresses IL-12p40 expression (Grazia Cappiello et al., 2001). Surprisingly, IL-23 expression was increased in Dectin-1$^{-/-}$ mice (Fig. 5 D), indicating that IL-23 induction by Dectin-1 does not, in this model, suppress T-bet expression in responding T cells. Rather, significantly enhanced IFN-γ production was observed in Dectin-1$^{-/-}$ as compared with control mice (Fig. 5 E), suggesting that in normal mice, Dectin-1 diminishes the amount of IFN-γ produced by innate immune cells.

A. fumigatus–specific Th17 differentiation is enhanced by IL-12p35 or IFN-γ deficiency in infected mice

IL-12p70 and IFN-γ induce T-bet expression in activated CD4 T cells, with IFN-γ promoting the initial induction of T-bet (Schulz et al., 2009). Our finding that Dectin-1 signaling suppresses in vivo IFN-γ and IL-12p40 expression led us to postulate that reduced IFN-γ and IL-12 levels diminish T-bet expression in *A. fumigatus*–specific CD4 T cells. To examine the role of IFN-γ, we adoptively transferred Af3.16-WT TCR-tg cells into IFN-γ$^{-/-}$ or WT control mice 1 d before pulmonary infection with *A. fumigatus* spores. The expansion of Af3.16 TCR-tg cells in the MLNs, their recruitment to the airways, and the control of fungal growth were similar in IFN-γ$^{-/-}$ and control mice (Fig. S4, A–C). In contrast, in the absence of IFN-γ production by the host, *A. fumigatus*–specific CD4 T cells failed to produce IFN-γ (Fig. 6 A), indicating impaired Th1 differentiation. Moreover, Af3.16-WT TCR-tg cells primed in IFN-γ$^{-/-}$ mice differentiated into Th17 cells (Fig. 6 B). Analysis of T-bet expression

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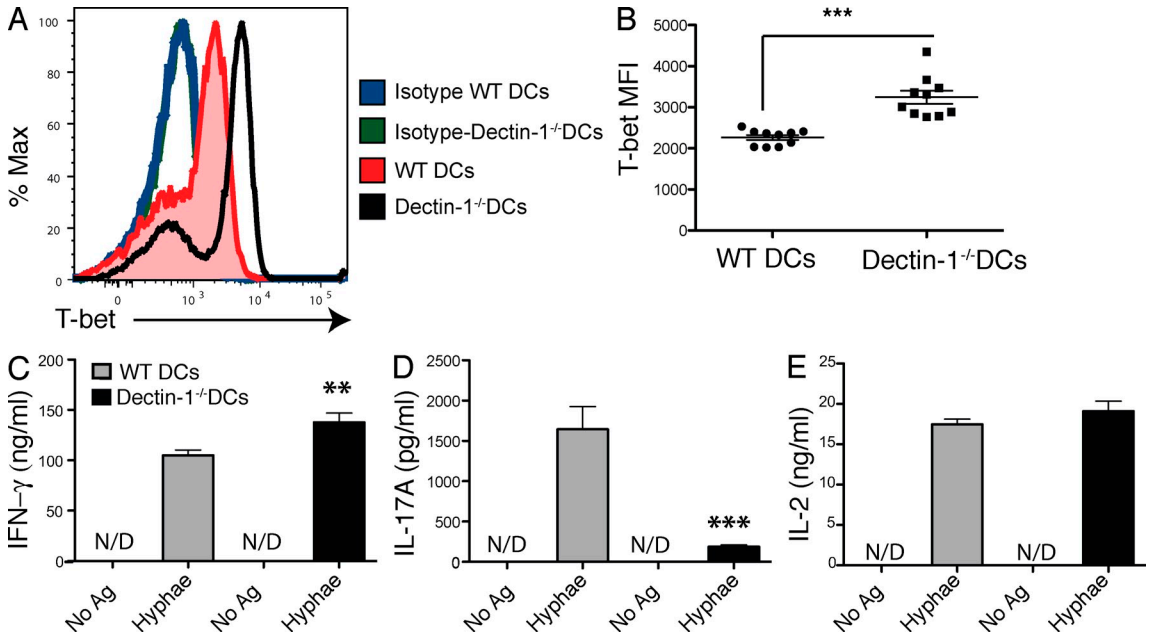

Figure 3. Enhanced expression of T-bet in Af3.16 TCR-tg cells primed in vitro with Dectin-1$^{-/-}$ DCs. Naive Af3.16 TCR-tg cells were cultured in vitro with FLT3L-induced DCs isolated from Dectin-1$^{-/-}$ or control C57BL/6J mice in the presence or absence of *A. fumigatus* hyphae. (A) T-bet expression was assessed by intracellular staining. Histograms shown are representative of 10 individual wells analyzed and are gated on CD4$^+$Vβ8.3$^+$ Af3.16 TCR-tg cells. Isotype control staining is shown. (B) The amount of T-bet expressed by Af3.16 TCR-tg cells primed with WT or Dectin-1$^{-/-}$ DCs as determined by T-bet mean fluorescence intensity (MFI) in gated Af3.16 TCR-tg cells. Each symbol represents one individual well per culture condition. Bars represent the mean with SEM. (C–E) Secreted IFN-γ (C) and IL-17A (D) at day 5 of culture or IL-2 (E) at day 2 of culture was measured by ELISA in 10 individual wells. Af3.16 TCR-tg cells were cultured with WT or Dectin-1$^{-/-}$ DCs with or without hyphae as indicated. Data shown are for one experiment representative of two separate experiments. Values shown are mean ± SEM. **, $P \leq 0.01$; ***, $P \leq 0.001$ compared with control WT DCs. N/D, no cytokine detected.

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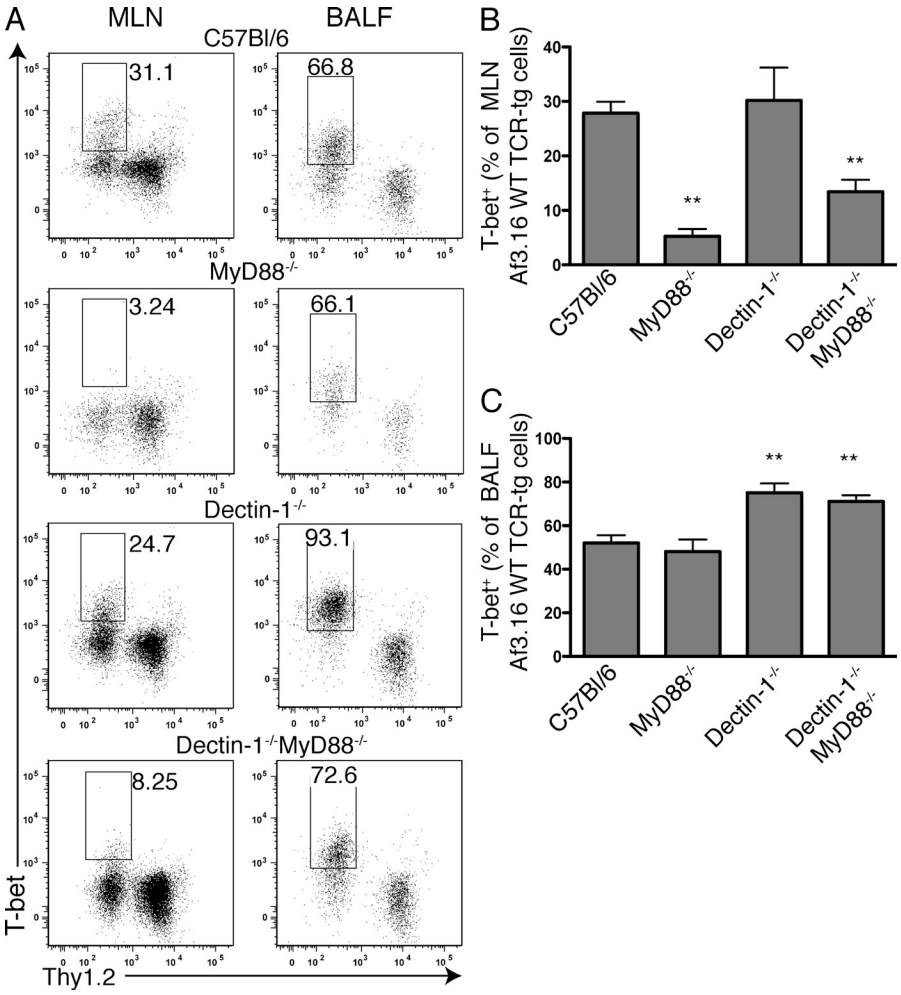

Figure 4. Distinct regulation of T-bet expression in fungus-specific CD4 T cells by Dectin-1 and MyD88. Congenically marked Thy1.2$^-$ Af3.16-WT and Thy1.2$^+$ Af3.16–T-bet$^{-/-}$ CD4 TCR-tg cells were co-transferred into MyD88$^{-/-}$, Dectin-1$^{-/-}$, Dectin-1$^{-/-}$MyD88$^{-/-}$, and control C57BL/6J mice 1 d before a pulmonary infection with live *A. fumigatus* spores. T-bet expression in TCR-tg cells was assessed 6 d after infection in the LNs (MLN) and airways (BALF) by intracellular staining. (A) FACS plots are gated on total Af3.16 TCR-tg cells. Percentages indicate the frequency of T-bet–positive cells among gated Af3.16-WT TCR-tg cells. FACS data are shown for one mouse per group and are representative of three separate experiments with three to four mice per group. (B and C) Frequency of T-bet–positive cells among Af3.16-WT TCR-tg cells in the MLNs (B) and airways (C) of the indicated recipient mice. Data presented in each plot are cumulative of three separate experiments with three to four mice per group. Error bars denote SEM. **, $P \leq 0.01$ compared with control mice.

by fungus-specific CD4 T cells in the MLNs and airways indicated that IFN-γ is required for T-bet induction at both sites (Fig. 6 C). Similar results were obtained in bone marrow chimeric mice reconstituted with IFN-γ$^{-/-}$ bone marrow (unpublished data). Because adoptively transferred Af3.16-WT TCR-tg cells were not deficient in IFN-γ production, these results indicate that bone marrow–derived cells provide IFN-γ, which promotes Th1 differentiation in responding CD4 T cells. Because T-bet deficiency drives *A. fumigatus*–specific CD4 T cells to differentiate into Th17 cells (Fig. 2), we conclude that enhanced Th17 differentiation in IFN-γ$^{-/-}$ mice results from diminished T-bet induction in responding CD4 T cells.

To determine the contribution of IL-12 to CD4 T cell differentiation during *A. fumigatus* infection, we adoptively transferred Af3.16-WT TCR-tg cells into IL-12p35$^{-/-}$ or control bone marrow chimeric mice. IL-12p35$^{-/-}$ mice supported the expansion of fungus-specific CD4 T cells in the MLNs, their recruitment to the airways, and the control of fungal growth to the same extent as control mice (Fig. S4, D–F). The results shown in Fig. 6 (D–F) demonstrate that absence of IL-12 in infected mice markedly decreases Th1 differentiation of responding Af3.16-WT TCR-tg cells (Fig. 6 D) while greatly enhancing Th17 differentiation (Fig. 6 E), with commensurate changes in T-bet expression (Fig. 6 F). Thus, similar to IFN-γ, IL-12 production by bone marrow–derived, host cells is essential for T-bet induction and suppression of Th17 differentiation.

Monocyte-derived DCs control the balance of Th1 versus Th17 differentiation by regulating T-bet expression in *A. fumigatus*–specific CD4 T cells

Our findings indicate that the level of T-bet induction determines the fate of *A. fumigatus*–specific CD4 T cells and that IL-12 and IFN-γ are essential for in vivo T-bet induction. DCs express Dectin-1, respond to TLR ligands, and express IL-12, which can enhance IFN-γ expression by other innate cells such as NK and NKT cells. In previous experiments, we used CCR2–diptheria toxin (DT) receptor (DTR) depleter mice to demonstrate that CCR2$^+$ monocyte-derived DCs are essential for *A. fumigatus*–specific CD4 T cell priming (Hohl et al., 2009). However, whether CCR2$^+$ monocyte-derived DCs also contribute to differentiation of CD4 T cells after *A. fumigatus* infection is unclear. Therefore, we adoptively transferred Af3.16 TCR-tg cells into CCR2-DTR mice 1 d before infection and treated infected mice with DT 2 or 4 d after infection. In contrast to monocyte depletion before or at the time of infection, depletion of CCR2$^+$ cells after infection did not significantly alter *A. fumigatus*–specific CD4 T cell expansion in the MLNs or recruitment to the lung (Fig. S5, A and B). Depletion of CCR2$^+$ cells 2 and

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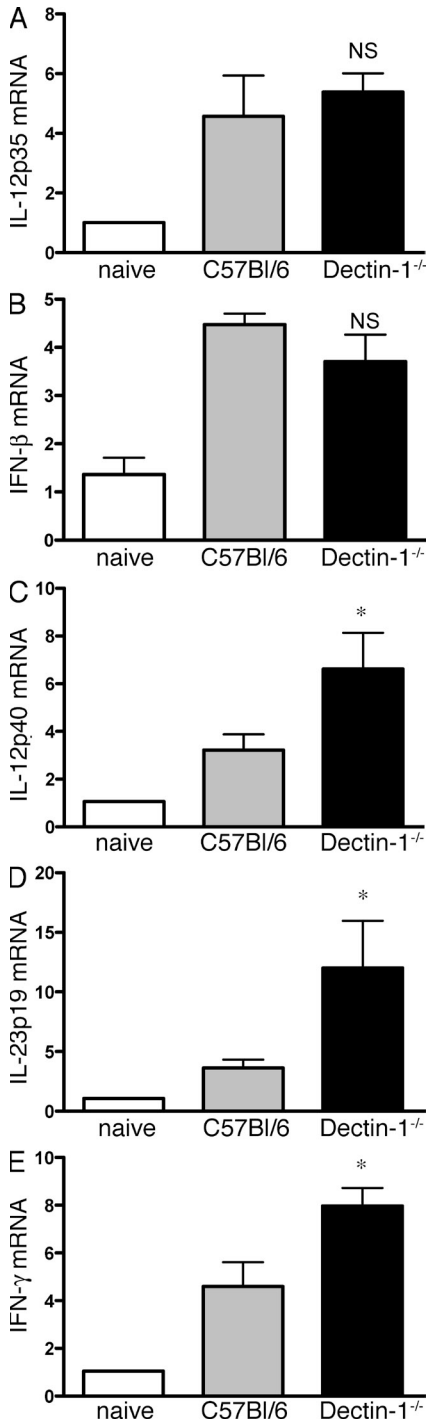

Figure 5. Enhanced IL-12p40 and IFN-γ expression in the lung of Dectin-1$^{-/-}$ mice 2 d after *A. fumigatus* infection. (A–E) Cytokine expression was analyzed in lung samples obtained from naive mice or from Dectin-1$^{-/-}$ and control C57BL/6J mice that had been infected 2 d earlier. Gene expression was assessed by quantitative RT-PCR using TaqMan probes and normalized to HPRT. Data shown are representative of two separate experiments performed with two to four mice per group. Bars are mean ± SEM of four mice per group. *, $P \leq 0.05$; NS, $P > 0.05$.

4 d after infection decreased the frequency of IFN-γ–producing cells (Fig. 7 A) and dramatically enhanced the frequency of IL-17A–producing cells (Fig. 7 B). Analysis of T-bet expression in fungus-specific CD4 T cells further revealed minimal T-bet expression when CCR2$^+$ monocytes were depleted, indicating that CCR2$^+$ monocytes or their derivative cells induce T-bet expression in responding CD4 T cells, and, in their absence, Th17 differentiation prevails.

Although CCR2 is expressed predominantly in inflammatory monocytes, other immune cells, including NK cells and monocyte-derived DCs, may also express CCR2 and thus may be depleted upon DT treatment of CCR2-DTR mice. To determine whether DCs restrict Th17 differentiation of *A. fumigatus*–specific CD4 T cells, we adoptively transferred Af3.16-WT TCR-tg cells into CD11c-DTR and control bone marrow chimeric mice 1 d before a pulmonary infection with *A. fumigatus* spores and depleted DCs 3 d after infection. Depletion of CD11c$^+$ cells 3 d after infection did not prevent expansion of Af3.16 TCR-tg cells in the MLNs or their recruitment to the lung (Fig. S5, C and D). However, depletion of CD11c$^+$ cells resulted in diminished Th1 (Fig. 7 D) and enhanced Th17 (Fig. 7 E) differentiation and was accompanied by reduced T-bet expression in *A. fumigatus*–specific CD4 T cells (Fig. 7 F). These results suggest that CCR2$^+$ monocyte-derived, CD11c$^+$ DCs, by responding to Dectin-1–mediated signals during fungal infection, control the balance of Th1 versus Th17 differentiation by modulating IL-12 and IFN-γ expression, which regulates T-bet expression in responding CD4 T cells and determines their fate as either Th17 or Th1 effectors.

DISCUSSION

Defense against infection with *A. fumigatus* requires efficient neutrophil recruitment and macrophage activation (Schaffner et al., 1982; Mircescu et al., 2009). *A. fumigatus*–specific CD4 T cells can contribute to this complex response by producing IL-17 to enhance neutrophil recruitment and IFN-γ to activate local macrophages. Our findings reveal a novel mechanism by which TLR/MyD88- and Dectin-1–mediated signals determine the balance of Th1 and Th17 T cells during *A. fumigatus* infection. Although previous experiments have demonstrated that Dectin-1–mediated signals promote differentiation of naive CD4 T cells into Th17 effector cells during infection with the yeast *Candida albicans* (LeibundGut-Landmann et al., 2007), in the context of a pulmonary mold infection, we demonstrate that Dectin-1–mediated signals decrease Th1 differentiation and thus enable default Th17 differentiation. We find that Th17 differentiation of *A. fumigatus*–specific CD4 T cells is markedly enhanced in IL-12– and IFN-γ–deficient mice or when responding T cells lack T-bet. Additionally, depletion of DCs after T cell priming also decreases Th1 differentiation and enhances Th17 differentiation. These results suggest that during *A. fumigatus* infection, lung DCs produce IL-12, which enhances IFN-γ expression and induces T-bet in responding T cells that have trafficked to the lung. Loss of DCs, IL-12, IFN-γ, or T-bet results in markedly

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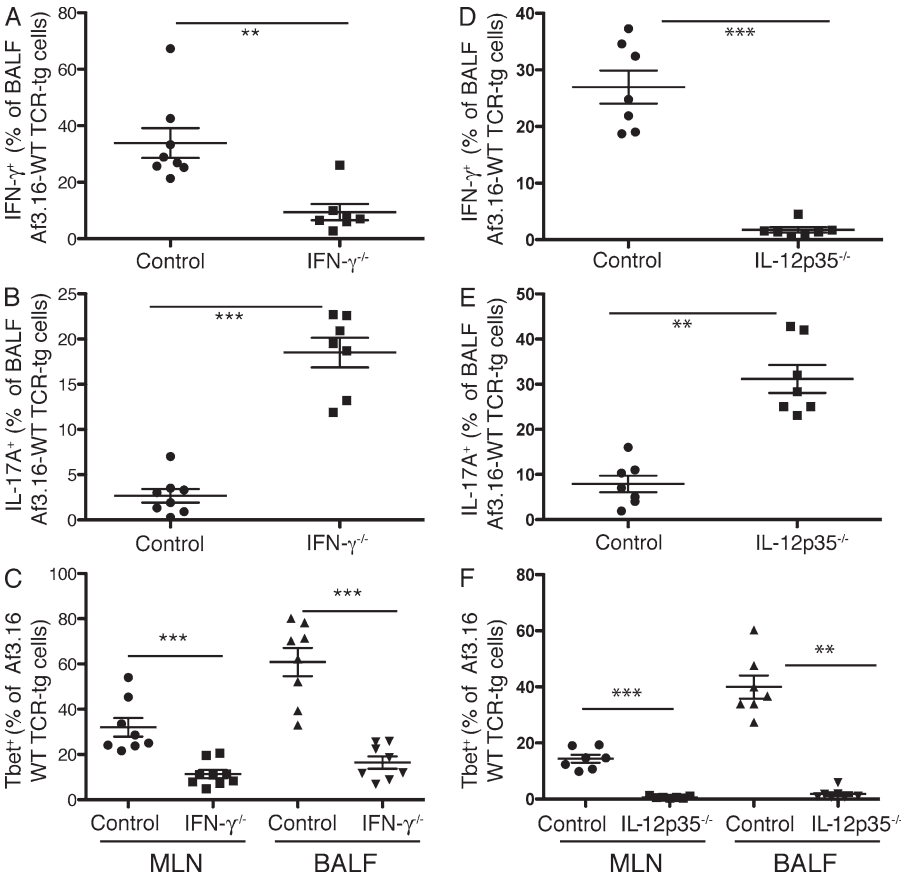

Figure 6. Enhanced *A. fumigatus*–specific Th17 differentiation in the absence of IFN-γ or IL-12 production by recipient mice. (A–C) Af3.16-WT TCR-tg cells were adoptively transferred into IFN-$\gamma^{-/-}$ or control mice 1 d before pulmonary infection with *A. fumigatus* spores. Af3.16 TCR-tg T cell differentiation was assessed by intracellular staining for IFN-γ and IL-17A in cells recovered from the airways (BALF) 6 d after infection. Each symbol represents one mouse. Data shown are cumulative of two separate experiments with three to four mice per group. Frequency of IFN-γ^+ (A), IL-17A$^+$ (B), and T-bet$^+$ (C) Af3.16-WT TCR-tg cells in BALF. (D–F) Af3.16-WT TCR-tg cells were adoptively transferred into chimeric mice that received either IL-12p35$^{-/-}$ or control C57BL/6J bone marrow 8 wk before. 1 d after adoptive T cell transfer, mice were infected with live *A. fumigatus* spores. Each symbol represents one mouse. Frequency of IFN-γ^+ (D), IL-17A$^+$ (E), and T-bet$^+$ (F) Af3.16-WT TCR-tg cells in BALF were analyzed by intracellular staining. Each symbol represents one recipient mouse. Data shown are cumulative of two separate experiments with three to four mice per group. (A–F) Bars represent the mean with SEM. **, $P \leq 0.01$; ***, $P \leq 0.001$

reduced Th1 responses and markedly enhanced Th17 responses. Our results demonstrate that innate immune signals mediated by Dectin-1 interfere with this cascade by decreasing IL-12 production, which results in commensurate decreases in IFN-γ expression and T-bet induction, thus limiting Th1 and enhancing Th17 responses. Dectin-1 signaling in *A. fumigatus*–infected mice does not completely inhibit Th1 differentiation but instead reduces T-bet sufficiently to enable some T cells to follow the Th17 differentiation pathway. This mechanism enables the host to mount a more effective and complex response that recruits and activates a broader range of antimicrobial cells to the site of infection.

Previous in vitro studies have shown that T-bet can inhibit Th17 differentiation of CD4 T cells (Harrington et al., 2005; Park et al., 2005; Mathur et al., 2006), and T-bet$^{-/-}$ mice have enhanced IL-17 production in experimental autoimmune encephalomyelitis and in some infection models (Park et al., 2005; Intlekofer et al., 2008; Guo et al., 2009). IFN-γ is a crucial inducer of T-bet and can also suppress Th17 differentiation (Szabo et al., 2000, 2002; Harrington et al., 2005; Mangan et al., 2006). Because T-bet induces IFN-γ production in CD4 T cells, it has been unclear whether T-bet$^{-/-}$ mice display enhanced Th17 differentiation caused by direct actions of T-bet or secondary to diminished IFN-γ production. Our experiments with Af3.16–T-bet$^{-/-}$ cells are consistent with a T cell–intrinsic role for T-bet in actively suppressing Th17 differentiation. The dominant role of T-bet is demonstrated by enhanced Th17 differentiation of Af3.16–T-bet$^{-/-}$ cells even in the absence of MyD88 and Dectin-1 signals (Fig. 2) and by the mutually exclusive expression of RORγt and T-bet (Fig. 2 D). Recent experiments have identified encephalitogenic Th17 cells that coexpress T-bet and RORγt (Ghoreschi et al., 2010), thus indicating that T-bet is not always suppressive of the Th17 differentiation program. The role of T-bet in Th17 differentiation appears to be dependent on the inflammatory context, with TGF-β suppressing T-bet and promoting differentiation of Th17 cells that do not express Th1 cytokines (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; McGeachy et al., 2007). In contrast, Th17 cells that are induced by IL-23, IL-6, and IL-1β in the absence of TGF-β can express both Th1 and Th17 transcription factors and cytokines (Ghoreschi et al., 2010). In our experiments, we did not detect Th1/Th17 hybrid CD4 T cells, suggesting that pulmonary TGF-β expression (Redington et al., 1997; Munger et al., 1999; Morris et al., 2003; Qian and Wahl, 2009; Wissinger et al., 2009) restricts Th17 differentiation after inhaled *A. fumigatus* infection.

Although Dectin-1 stimulation by fungal β-glucans is principally associated with the activation of innate immune responses (Hohl and Feldmesser, 2007), including induction of neutrophil and monocyte-recruiting chemokines and the production of inflammatory cytokines such as TNF, recent experiments have demonstrated that signals mediated by

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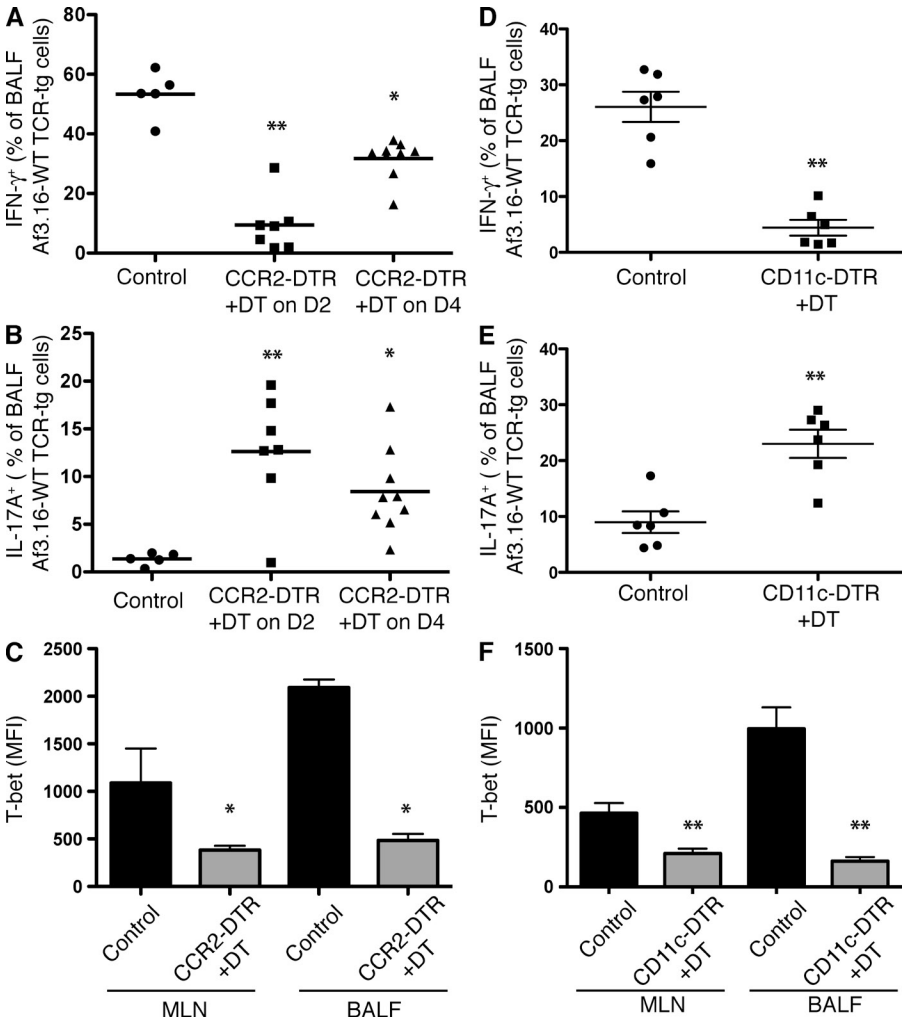

Figure 7. Monocyte-derived CD11c+ DCs inhibit Th17 differentiation of *A. fumigatus*–specific CD4 T cells. (A–C) Af3.16-WT TCR-tg cells were adoptively transferred into CCR2-DTR or control C57BL/6J mice 1 d before pulmonary infection with *A. fumigatus* spores. At days 2 or 4 after infection, mice were treated with DT to induce the selective depletion of CCR2+ cells. Fungus-specific CD4 T cell responses were analyzed 6 d after infection. Each symbol represents one mouse. Data shown are cumulative of two separate experiments. Frequency of IFN-γ (A)– and IL-17A–producing cells (B). (A and B) Bars represent the mean. *, $P \leq 0.05$; **, $P \leq 0.01$. (C) Mean fluorescent intensity (MFI) of T-bet expression in Af3.16-WT TCR-tg cells in the MLNs and airways. Bars are mean ± SEM of five to seven mice per group. *, $P \leq 0.05$. (D–F) Af3.16-WT TCR-tg cells were adoptively transferred into chimeric mice that received either CD11c-DTR or control C57BL/6J bone marrow 8 wk earlier. 1 d after adoptive T cell transfer, mice were infected with live *A. fumigatus* spores. Each symbol represents one mouse. Data are cumulative of two separate experiments. Frequency of IFN-γ (D)– and IL-17A–producing cells (E). (D and E) Bars represent the mean with SEM. **, $P \leq 0.01$. (F) Mean fluorescent intensity of T-bet expression in Af3.16-WT TCR-tg cells in the MLNs and airways. Bars are mean ± SEM of six mice per group. **, $P \leq 0.01$.

ITAM-containing receptors can inhibit TLR-mediated signaling (Wang et al., 2010). ITAM-mediated signals after β2 integrin ligation potently induce expression of IL-6 and TNF in the short term, but more prolonged stimulation induces expression of IL-10, SOCS3, ABIN-3, HES1, and A20, which inhibit TLR signaling at various stages (Wang et al., 2010). Ligation of FcγR, which also signals via an intracellular ITAM, reduces IL-12p40 expression, so it is likely that Dectin-1 signaling induced by prolonged β-glucan exposure in the lung decreases IL-12p40 production, perhaps by interfering with TLR stimulation. We were surprised that IL-23p19 expression was increased in the absence of Dectin-1 because previous studies demonstrated that Dectin-1 stimulation enhances IL-23 expression (LeibundGut-Landmann et al., 2007; Dennehy et al., 2009). These differences likely reflect the disparate mouse models that have been used to investigate Dectin-1–mediated defense against fungal infections. Pulmonary infection with *A. fumigatus*, a spore-forming mold, is certainly different than systemic infection with a yeast such as *C. albicans*, making direct comparisons between these types of infections difficult and the discovery of novel contributions of innate immune receptors to antifungal defense likely.

Our finding that T-bet is induced in CD4 T cells that traffic to the airways of MyD88- and Dectin-1–deficient mice indicates that other innate receptors contribute to *A. fumigatus*–specific CD4 T cell differentiation. Although β-glucan remains the major identified fungus-derived innate immune receptor ligand, our knowledge regarding the breadth of fungal ligands for innate immune receptors remains incomplete. For example, although TLR-2 and TLR-4 are known to respond to *A. fumigatus* and *C. albicans*, the fungal ligands for these receptors have yet to be identified (Romani, 2004; Hohl et al., 2006). Recognition of fungal cell wall components can also be mediated by other innate immune receptors, including Dectin-2, mannose receptor, and DC-SIGN (Taylor et al., 2004; Robinson et al., 2009; van de Veerdonk et al., 2009). Both mannose receptor and Dectin-2 have been found to influence CD4 T cell differentiation by promoting Th17 differentiation in response to *C. albicans* (Robinson et al., 2009; van de Veerdonk et al., 2009). Whether these receptors recognize *A. fumigatus* in vivo remains unclear, and further experiments will be required to assess the contribution of these receptors to *A. fumigatus*–specific CD4 T cell differentiation and antifungal immunity.

Our results suggest that Dectin-1–mediated signals promote Th17 differentiation indirectly by limiting Th1 differentiation.

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Dectin-1–mediated signals likely reduce the production of IL-12 by DCs and thus decrease IFN-γ production and T-bet expression in responding fungus-specific CD4 T cells. An alternate explanation for our findings is that enhanced Th1 differentiation in Dectin-1$^{-/-}$ mice is secondary to IL-17A deficiency. A study by O'Connor et al. (2009) in a model of CD4 T cell–induced colitis demonstrated IL-17A–mediated suppression of T-bet and Th1 differentiation in CD4 T cells. The suppressive effect of IL-17A was restricted to the early stages of Th1 differentiation and could not modulate already established T-bet levels (O'Connor et al., 2009). In contrast, during a pulmonary infection with *Francisella tularensis*, IL-17A was found to promote Th1 differentiation by acting on DCs and macrophages to promote IL-12 and IFN-γ production (Lin et al., 2009). Thus, it appears that the regulatory actions of IL-17A on Th1 differentiation can have opposite effects depending on cell type and time of exposure. Because Th1 and Th17 differentiation are occurring concurrently after inhalational infection with *A. fumigatus*, we believe it is unlikely that IL-17 production by responding Th17 T cells down-regulates T-bet expression in T cells destined to become Th1 T cells. This conclusion is supported by our finding of normal T-bet induction and Th1 differentiation in WT Af3.16 T cells cotransferred with T-bet$^{-/-}$ Af3.16 T cells (which express high levels of IL-17A; Fig. 4).

Recent experiments have identified a mutation in human Dectin-1 that leads to Dectin-1 deficiency (Ferwerda et al., 2009). Patients with this mutation have recurrent mucocutaneous Candidiasis and produce diminished amounts of IL-17 in response to stimulation with fungal cell wall components. Remarkably, this deficiency results from an ancient mutation that has been maintained at a relatively high prevalence in African populations (Ferwerda et al., 2009). Although our observation that Dectin-1 deficiency leads to increased Th1 differentiation is restricted to mice, our findings provide some grounds to speculate that Dectin-1 deficiency in humans might selectively provide benefits in an environment dominated by pathogens that require Th1 or Th2 responses for optimal host defense. Furthermore, the distinct contributions of Dectin-1 and MyD88 to T-bet expression and CD4 T cell differentiation identified in this study might contribute to the development of improved vaccination strategies against clinically important fungal pathogens.

MATERIALS AND METHODS

Mice. The generation of Dectin-1$^{-/-}$ was previously described (Saijo et al., 2007). Dectin-1$^{-/-}$MyD88$^{-/-}$ mice were generated by crossing Dectin-1$^{-/-}$ MyD88$^{-/+}$ to MyD88$^{-/-}$ mice originally obtained from S. Akira (Osaka University, Suita, Osaka, Japan). *A. fumigatus*–specific CD4^{+} TCR-tg mice (Af3.16) have been previously described (Rivera et al., 2006). Af3.16-Thy1.1$^{+/+}$ mice were bred to T-bet$^{-/-}$ mice to generate Af3.16–T-bet$^{-/-}$Thy1.1/1.2 TCR-tg mice. T-bet$^{-/-}$, IFN-γ$^{-/-}$, IL-12p35$^{-/-}$, and C57BL/6J control mice were purchased from The Jackson Laboratory. The previously described CD11c-DTR (Jung et al., 2002) and CCR2-DTR mice (Hohl et al., 2009) express a simian DTR under the control of the CD11c or CCR2 promoters and allow the selective depletion of CD11c^{+} or CCR2^{+} cells, respectively. For depletion experiments, mice were injected i.p. with 250 ng DT at various times after infection. To generate bone marrow chimeric mice, recipient mice were lethally irradiated and reconstituted with donor bone marrow cells and housed for at least 8 wk before adoptive T cell transfer and infection. All mice were bred and maintained under specific pathogen-free conditions at Memorial Sloan-Kettering Research Animal Resource Center. Sex- and age-matched controls were used in all experiments according to institutional guidelines for animal care. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center.

Infections, culture, and histology. *A. fumigatus* strain 293 is a clinical isolate and was originally provided by M. Anderson (The University of Manchester, Manchester, England, UK). The fungus was cultured on Sabouraud dextrose agar for 7–10 d before collection for pulmonary infections. Mice were infected intratracheally with 10^7 conidia/mouse as previously described (Rivera et al., 2006). This infection dose is sevenfold lower than the reported lethal dose for *A. fumigatus* in Dectin-1$^{-/-}$ mice (Werner et al., 2009) and the same dose previously reported for MyD88$^{-/-}$ (Rivera et al., 2006). The infection dose used did not lead to mortality by day 6 in any of the strains used in this study. The presence of viable *A. fumigatus* in infected mice was assessed by plating lung samples on Sabouraud agar plates. For histological examination, lungs were excised, immersed in 10% buffered formalin, paraffin embedded, and stained with modified Grocott's methanamine silver stain. Lung tissues were processed and analyzed at the Genetically Engineered Mouse Phenotyping Core Facility (Memorial Sloan-Kettering Cancer Center).

ICCS and flow cytometry. Staining for intracellular IFN-γ and IL-17A was performed on BALF cells that were stimulated with APCs and hyphal antigens as previously described (Rivera et al., 2006). ICCS was performed with reagents obtained from BD according to the manufacturer's instructions. Intracellular staining for T-bet and RORγt was performed on cells obtained from the MLNs or BALF without any further stimulation. For T-bet and RORγt staining, permeabilization was performed with eBioscience reagents and according to their protocol for foxp3 staining. Samples were stained with anti-CD4, Thy1.1, Thy1.2, and CD44 fluorescent antibodies obtained from BD. Anti–IL-17A and RORγt antibodies were obtained from eBioscience. Anti–T-bet, clone 4B10, was obtained from Santa Cruz Biotechnology, Inc. All samples were analyzed by flow cytometry on an LSR II (BD). Further flow cytometric analysis was performed with FlowJo software (Tree Star, Inc.).

Adoptive T cell transfers and in vitro T cell differentiation. Naive, CD4^{+} Af3.16 TCR-tg cells were isolated from LNs and spleen of Af3.16 TCR-tg mice using a CD4^{+} isolation kit from Miltenyi Biotec supplemented with anti-CD11c and anti-MHCII beads to eliminate transfer of APCs from TCR-tg mice. A total of 2×10^4 purified Af3.16 TCR-tg cells was injected intravenously into naive recipients 1 d before infection. For cotransfer experiments, a 1:1 ratio of purified Af3.16-WT-Thy1.1$^{+/+}$ to Af3.16–T-bet$^{-/-}$Thy1.1/1.2 was used and verified by flow cytometry before transfer. For in vitro experiments of CD4 T cell differentiation, cells were cultured according to standard procedures. In brief, purified CD4 T cells were cultured with irradiated splenocytes and anti-CD3 and anti-CD28 antibodies. For Th1 skewing conditions, cells were cultured in the presence of rm–IL-12p70 and anti–IL-4 antibodies. For Th17 skewing conditions, cells were cultured with anti–IL-4 and anti–IFN-γ antibodies together with recombinant IL-6, TGF-β, and IL-23. For in vitro priming experiments, DCs were purified with CD11c microbeads (Miltenyi Biotec) from the spleen of Dectin-1$^{-/-}$ and control B6 mice that had been injected 10 d earlier with a FLT3L-secreting cell line to induce in vivo expansion of mature DCs (Maraskovsky et al., 1996). Purified naive Af3.16 CD4 TCR-tg cells (5×10^4) isolated from Af3.16-WT or Af3.16–T-bet$^{-/-}$ mice were co-cultured with purified DCs at a 1:1 ratio in the presence or absence of *A. fumigatus* hyphae and the fungal growth inhibitor voriconazole for 5 d. Our previous experiments demonstrated that voriconazole has no effects on DCs or responding CD4 T cells (Van Epps et al., 2003). CD4 T cell responses were monitored by measuring

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cytokine secretion at various times after culture initiation. The production of IFN-γ (BD), IL-17A (eBioscience), and IL-2 (BD) was assessed by ELISA according to the manufacturer's instructions. The induction of T-bet was measured by intracellular staining with anti–T-bet antibodies (Santa Cruz Biotechnology, Inc.) on cells fixed with Foxp3 Fixation and Permeabilization buffers (eBioscience).

Gene expression. Cytokine expression was assessed by quantitative real-time PCR with TaqMan probes specific for mouse hypoxanthine guanine phosphoribosyl transferase (HPRT), IFN-γ, IFN-β, IL-12a, IL-12b, and IL-23a obtained from Applied Biosystems. RNA was isolated from the lungs of naive mice or Dectin-1$^{-/-}$ and control mice that were infected 2 d earlier. RNA isolation was performed with TRIZOL (Invitrogen), and cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (QIAGEN) according to manufacturer's instructions. Signals for each gene were normalized to HPRT transcript levels, and gene expression in infected mice relative to uninfected controls was quantitated by $\Delta\Delta C_t$ analysis.

Statistical analysis. All statistical analysis was performed with Prism software (GraphPad Software, Inc.). A p-value of <0.05 was considered significant. The Mann-Whitney nonparametric test was performed for all samples analyzed.

Online supplemental material. Fig. S1 shows that Af3.16 TCR-tg cells and polyclonal CD4 T cells have the same in vitro differentiation potential toward Th1 and Th17 lineages under standard skewing conditions. Fig. S2 shows normal restriction of *A. fumigatus* germination at 6 d after infection in mice with selective innate deficiencies. Fig. S3 shows that Af3.16–T-bet$^{-/-}$ TCR-tg cells have enhanced in vitro Th17 differentiation after culture with either WT or Dectin-1$^{-/-}$ FLT3L-induced DCs. Fig. S4 shows that IFN-γ$^{-/-}$ and IL-12p35$^{-/-}$ mice support equivalent expansion and trafficking of Af3.16 TCR-tg cells as compared with control C57BL/6J animals. Fig. S5 shows that depletion of CCR2^{+} DCs or CD11c^{+} cells at days 2 or 4 after infection does not significantly impair the expansion of Af3.16 TCR-tg cells in the MLNs or their trafficking to the lung. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100906/DC1.

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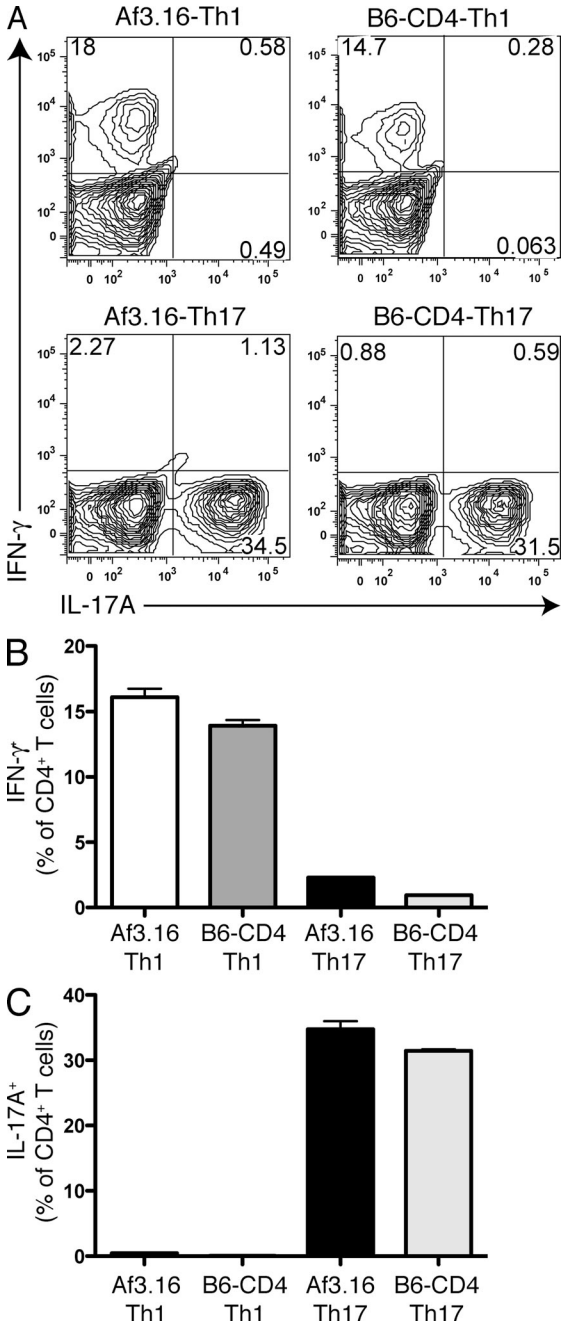

Figure S1. Normal in vitro differentiation of Af3.16 TCR-tg cells. Naive CD4 T cells were purified from Af3.16 TCR-tg mice or control C57BL/6 mice and stimulated in vitro under Th1 or Th17 skewing conditions using standard protocols. (A) Representative FACS plots of the frequency of IFN-γ and IL-17A among each cell population as labeled above each plot. (B) Cumulative data for the frequency of IFN-γ^+ cells among each cell population. (C) Cumulative data for the frequency of IL-17A^+ cells among each population. (B and C) Bars are mean ± SEM.

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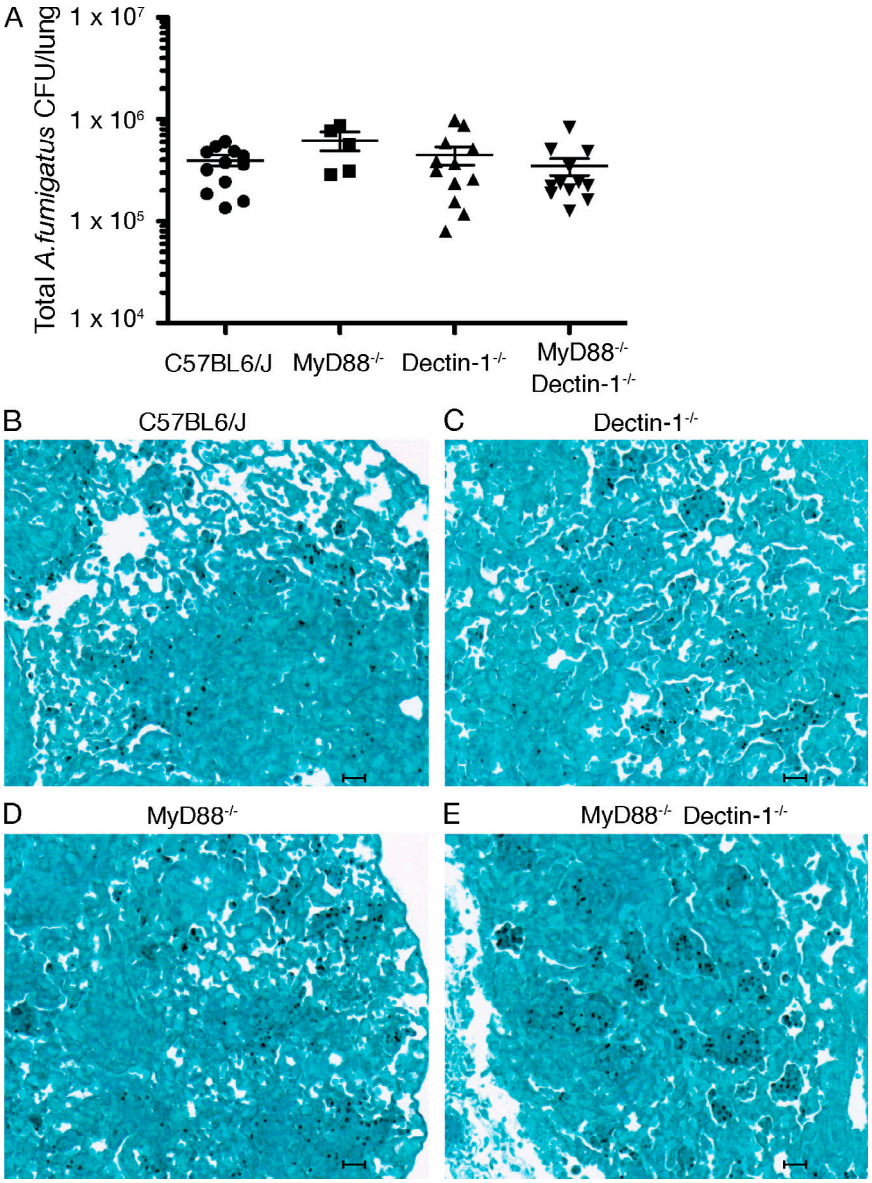

Figure S2. Normal restriction of *A. fumigatus* germination at 6 d after infection in mice with selective innate deficiencies. Dectin-1$^{-/-}$, MyD88$^{-/-}$, MyD88$^{-/-}$Dectin-1$^{-/-}$, and control mice were infected intratracheally with 1×10^7 live *A. fumigatus* spores 6 d before analysis. (A) Cumulative data for total *A. fumigatus* CFU cultured from the lung of each mouse strain analyzed. Each symbol represents one mouse. Mice shown are the same as depicted in Fig. 1. No statistically significant differences were detected. Bars represent the mean with SEM. (B–E) Representative pictures for Grocott's methanamine silver–stained lung sections from C57BL/6 control (B), Dectin-1$^{-/-}$ (C), MyD88$^{-/-}$ (D), and MyD88$^{-/-}$Dectin-1$^{-/-}$ mice (E). The whole lung was sectioned, and three separate sections were analyzed for each lobe. A total of four individual mice per group were analyzed and found to contain only ungerminated fungal conidia. Bars, 20 µm.

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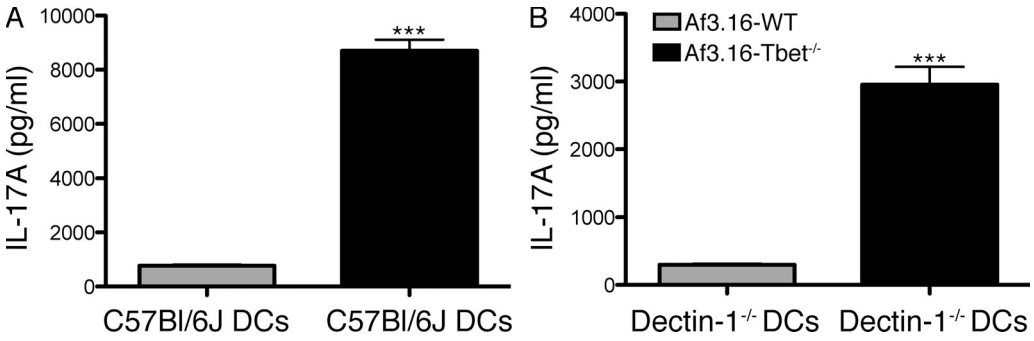

Figure S3. Enhanced in vitro Th17 differentiation of Af3.16–T-bet$^{-/-}$ CD4 TCR-tg cells. (A and B) Naive Af3.16 TCR-tg cells from WT or T-bet$^{-/-}$ mice were primed in vitro with FLT3L-induced DCs isolated from WT (A) or Dectin-1$^{-/-}$ mice (B). Cells were cultured for 5 d in the presence or absence of *A. fumigatus* hyphae and fungal growth inhibitor before analysis. The amount of secreted IL-17A was measured by ELISA. Values shown are mean ± SEM for 10 wells per condition. Data shown are representative of two separate experiments. ***, $P \leq 0.001$.

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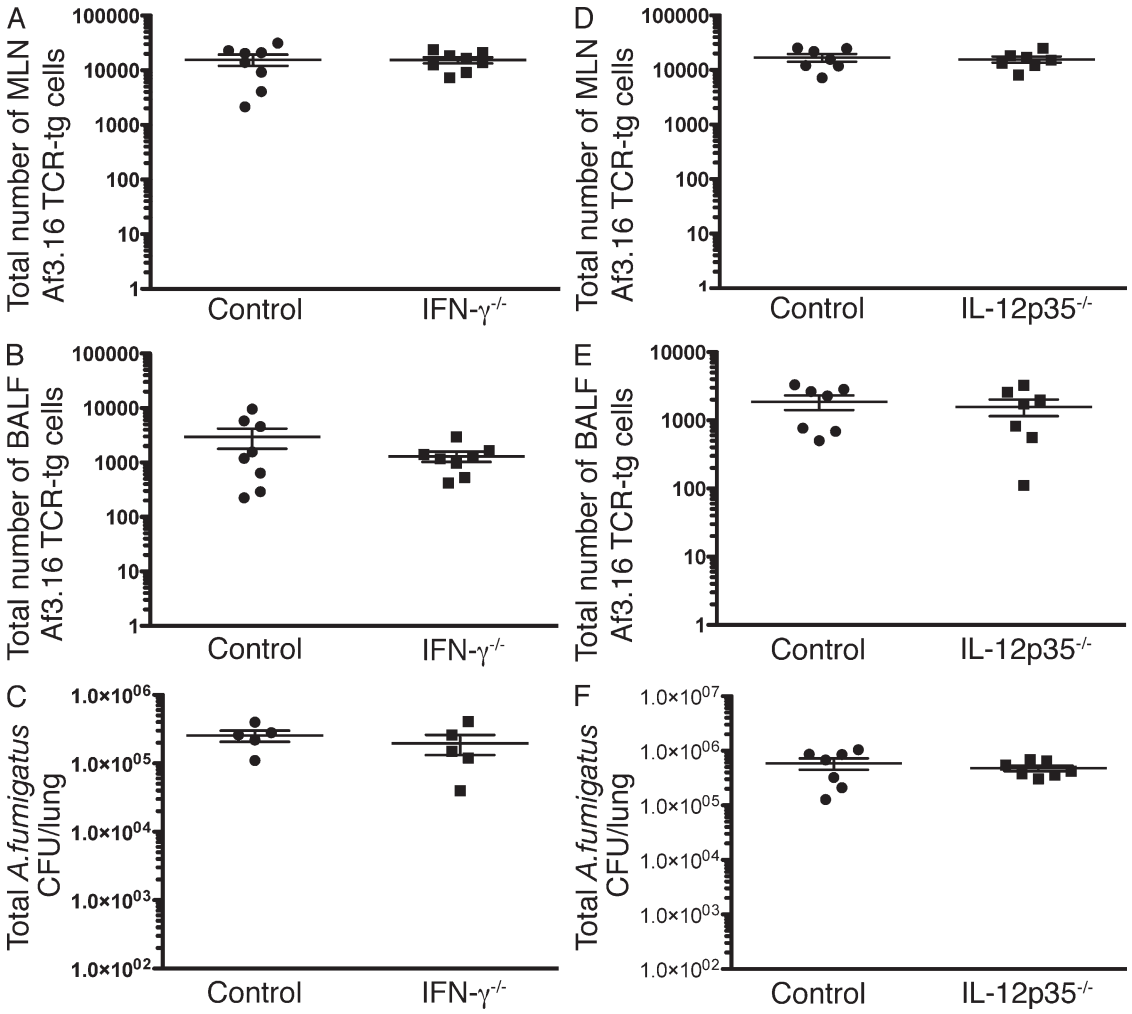

Figure S4. Normal expansion and recruitment of fungus-specific CD4 T cells in IFN-γ$^{-/-}$ and IL-12p35$^{-/-}$ mice. (A–C) Af3.16-WT TCR-tg cells were adoptively transferred into IFN-γ$^{-/-}$ or control mice 1 d before pulmonary infection with *A. fumigatus* spores. Each symbol represents one mouse. Data shown are cumulative of two separate experiments with three to four mice per group. (A) The expansion of Af3.16 TCR-tg cells in the MLNs was assessed at day 6 after infection by determining the total number of TCR-tg cells present in the MLNs. (B) Total number of fungus-specific CD4 TCR-tg cells that were recruited to the airways (BALF) 6 d after infection. (C) Total number of *A. fumigatus* colonies recovered in the lung of IFN-γ$^{-/-}$ and control mice 6 d after infection. (D–F) Af3.16-WT TCR-tg cells were adoptively transferred into chimeric mice that received either IL-12p35$^{-/-}$ or control bone marrow 8 wk before. 1 d after adoptive T cell transfer, mice were infected with live *A. fumigatus* spores. Each symbol represents one mouse. (D) Total number of fungus-specific CD4 TCR-tg cells that expanded in the MLNs of IL-12p35$^{-/-}$ or control mice 6 d after infection. (E) Total number of Af3.16 TCR-tg cells recovered from the airways of day 6 infected IL-12p35$^{-/-}$ and control mice. (F) Total number of *A. fumigatus* colonies recovered in the lung of IL-12p35$^{-/-}$ and control mice 6 d after infection. (A–F) Bars represent the mean with SEM.

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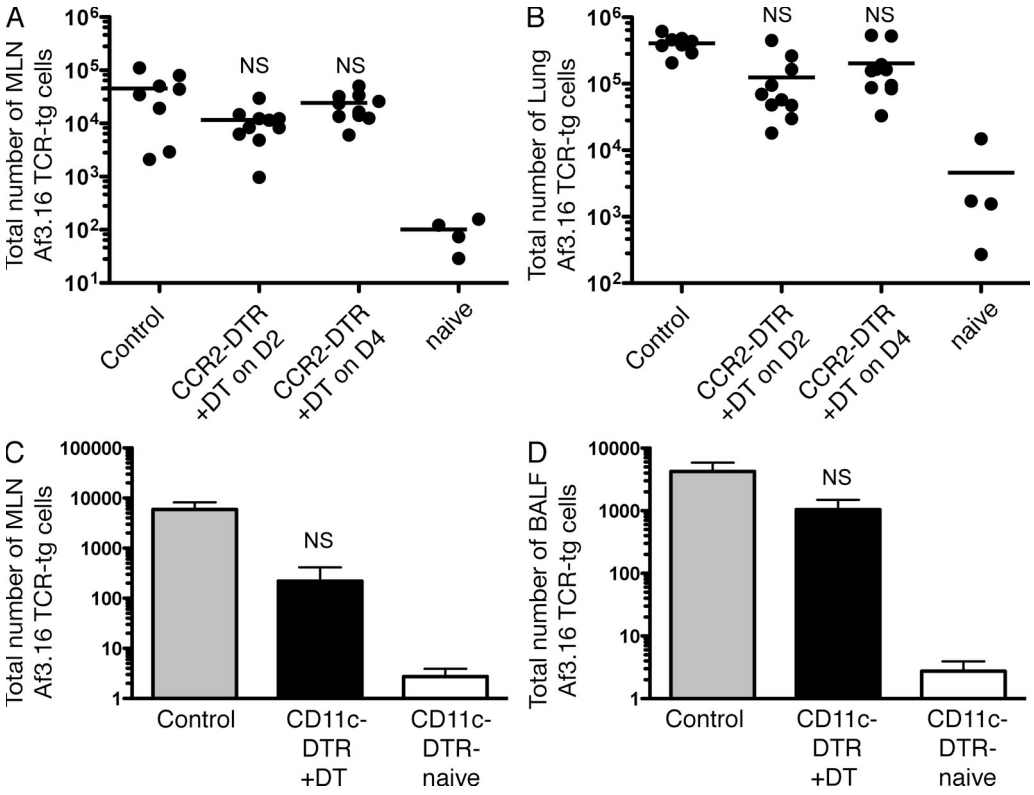

Figure S5. Unimpaired expansion of fungus-specific CD4 TCR-tg cells in mice depleted of monocyte-derived DCs at days 2–4 after infection. (A and B) Af3.16-WT TCR-tg cells were adoptively transferred into CCR2-DTR or control mice 1 d before a pulmonary infection with *A. fumigatus* spores. At days 2 or 4 after infection, mice were treated with DT to induce the selective depletion of CCR2$^+$ cells. Each symbol represents one mouse. Mice were analyzed at day 6 after infection. Bars represent the mean. (A) Total number of Af3.16 TCR-tg cells recovered from the MLNs. (B) Total number of Af3.16 TCR-tg cells recovered from the lung. (C and D) Af3.16-WT TCR-tg cells were adoptively transferred into chimeric mice that received either CD11c-DTR or control bone marrow 8 wk earlier. 1 d after adoptive T cell transfer, mice were infected with live *A. fumigatus* spores and treated with DT at day 3 after infection. Data are cumulative of two separate experiments. Bars are mean ± SEM of six mice per group. Total number of fungus-specific CD4 TCR-tg cells recovered from the MLNs (C) or airways (D) of mice infected 6 d earlier is shown. No statistically significant differences were observed in the mouse groups analyzed.

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