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Toll-like receptor 2–dependent induction of vitamin A–metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity

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Immune sensing of a microbe occurs via multiple receptors. How signals from different receptors are coordinated to yield a specific immune response is poorly understood. We show that two pathogen recognition receptors, Toll-like receptor 2 (TLR2) and dectin-1, recognizing the same microbial stimulus, stimulate distinct innate and adaptive responses. TLR2 signaling induced splenic dendritic cells (DCs) to express the retinoic acid metabolizing enzyme retinaldehyde dehydrogenase type 2 and interleukin-10 (IL-10) and to metabolize vitamin A and stimulate Foxp3$^+$ T regulatory cells (T_{reg} cells). Retinoic acid acted on DCs to induce suppressor of cytokine signaling-3 expression, which suppressed activation of p38 mitogen-activated protein kinase and proinflammatory cytokines. Consistent with this finding, TLR2 signaling induced T_{reg} cells and suppressed IL-23 and T helper type 17 (T_H17) and T_H1-mediated autoimmune responses *in vivo*. In contrast, dectin-1 signaling mostly induced IL-23 and proinflammatory cytokines and augmented T_H17 and T_H1-mediated autoimmune responses *in vivo*. These data define a new mechanism for the systemic induction of retinoic acid and immune suppression against autoimmunity.

It is now well established that a delicate balance between inflammatory versus regulatory responses underlies disease progression in many autoimmune disorders. DCs have emerged as central players in initiating and regulating adaptive immune responses[1–5]. Emerging evidence suggests that DCs are also vital in suppressing immune responses through the generation of anergic or T_{reg} cells[6]. However, the mechanisms by which DCs can be programmed to induce T_{reg} cells are poorly understood. DCs express several TLRs and the C-type lectins, which are crucial in sensing and initiating immune response against pathogens[7–9]. Engagement of such receptors programs gene expression that orchestrates DC maturation and activation[2,7,8]. The types of cytokines secreted by the DCs can regulate the differentiation of CD4$^+$ T cells into T_H1, T_H2, T_H17 or T_{reg} responses. For example, stimuli that induce IL-12 (p70) promote interferon-γ (IFN-γ)-producing T_H1 cells, stimuli that induce IL-10 favor T_H2 or T_{reg} responses and stimuli that induce transforming growth factor-β (TGF-β), IL-6 and IL-23 promote T_H17 differentiation.

Zymosan, a yeast cell wall derivative, is a complex microbial stimulus that is recognized by many innate immune receptors, including TLR2 and dectin-1, a C-type lectin receptor for β-glucans[10–15]. How signaling via both TLR2 and dectin-1 is integrated and influences adaptive immunity is poorly understood. Collaboration between TLR2 and dectin-1 results in the induction of proinflammatory cytokines in macrophages and DCs[14], as well as robust IL-10 production in DCs[16–18]. Consistent with this, our previous work shows that zymosan conditions splenic DCs to secrete IL-10 and induce tolerogenic T cell responses[18]. Furthermore, zymosan is also known to induce splenic macrophages to secrete TGF-β[18], a cytokine crucial for the generation of T_{reg} cells, as well as T_H17 cells[11,19–21]. In contrast, other studies have shown that dectin-1–mediated signaling in DCs induces T_H17 cells and IFN-γ producing T_H1 cells[22,23].

Here we show that TLR2 and dectin-1 mediate divergent programs of DC activation, resulting in distinct adaptive responses to zymosan. Thus, zymosan induces retinaldehyde dehydrogenase type 2 (Raldh2) expression in DCs via a mechanism dependent largely on TLR2-mediated activation of extracellular signal–regulated kinase (ERK) mitogen-activated protein kinase (MAPK). Raldh2 converts vitamin A–derived retinal to retinoic acid, which acts in an autocrine manner to induce the expression of suppressor of cytokine signaling-3 (Socs3) and suppress activation of p38 MAPK and proinflammatory cytokines. Consistent with this, TLR2 signaling is crucial for zymosan-mediated induction of T_{reg} cells and suppression of T_H1 and T_H17 responses mediated autoimmunity *in vivo*; in the absence of TLR2 signaling, dectin-1–mediated signaling alone induced potent T_H1 and T_H17 responses and exacerbated autoimmunity.

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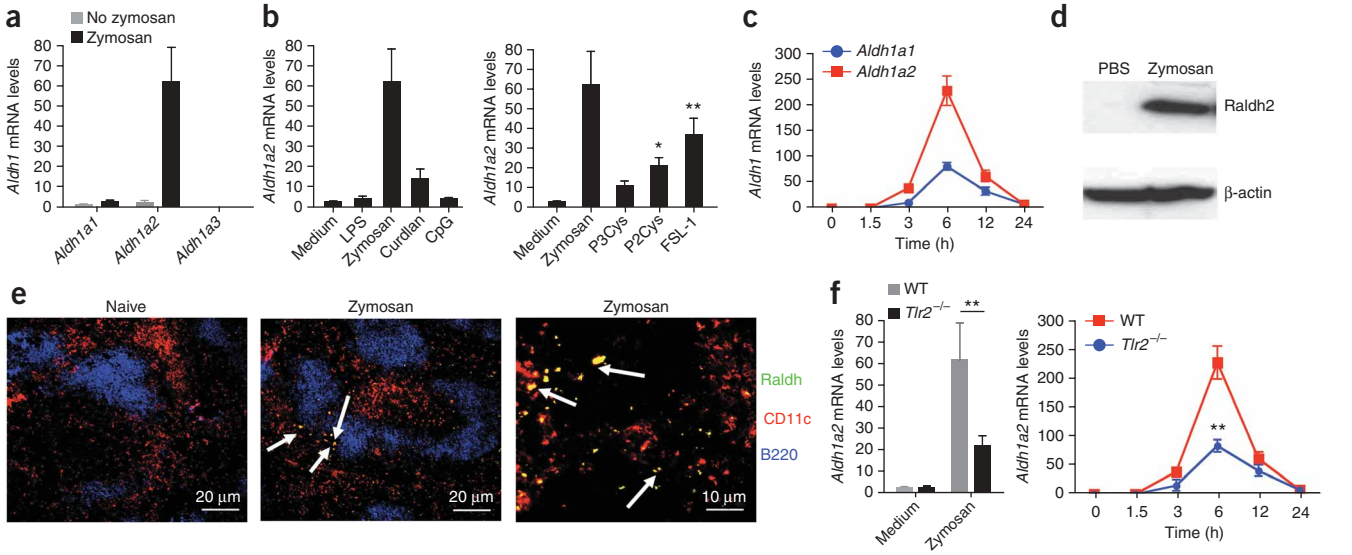

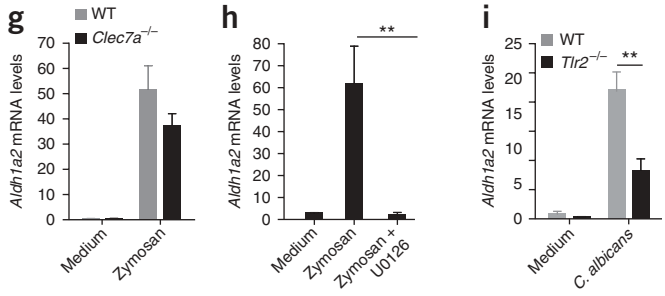

Figure 1 Mechanism of induction of vitamin-A metabolizing enzymes in splenic DCs. (**a**) Expression of *Aldh1* genes (encoding Raldh) mRNA in splenic DCs cultured *in vitro* for 24 h with or without zymosan. Expression of Raldh-encoding mRNA relative to expression of mRNA encoding glyceraldehyde phosphate dehydrogenase (GAPDH) was analyzed by quantitative RT-PCR in this and all figures. (**b**) Induction of *Aldh1a2* (encoding Raldh2) in splenic DCs cultured *in vitro* with TLR ligands or curdlan for 24 h. P3Cys, Pam-3-cys; P2Cys, Pam-2-cys; FSL, follistatin-like 1 ligand. (**c**) Induction of Raldh-encoding genes in splenic DCs *in vivo* in C57BL/6 mice injected with zymosan. (**d**) Western blot analysis of expression of Raldh2 protein in splenic DCs *in vivo*, 5 h after C57BL/6 mice were injected with zymosan. (**e**) Immunofluorescence microscopy of frozen tissue section of spleens of C57BL/6 mice injected with PBS or zymosan, fixed and stained with antibodies specific for mouse CD11c (red), Raldh (green) and B220 (blue). (**f**) Induction of Raldh2 is dependent on TLR2. Left, expression of *Aldh1a2* mRNA 24 h after splenic DCs from wild-type or *Tlr2*$^{-/-}$ mice were cultured in medium alone or with zymosan. Right, expression of *Aldh1a2* mRNA in splenic DCs taken at various time points from C57BL/6 or *Tlr2*$^{-/-}$ mice injected with zymosan. (**g**) *Aldh1a2* mRNA expression in splenic DCs from wild-type or *Clec7a*$^{-/-}$ (dectin-1–deficient) mice cultured for 24 h in medium alone or with zymosan. (**h**) Induction of *Aldh1a2* expression in splenic DCs cultured as described in **h** with or without U0126, an Erk inhibitor. (**i**) Induction of *Aldh1a2* by *C. albicans* in splenic DCs from WT or *Tlr2*$^{-/-}$ mice cultured in medium alone or with *C. albicans*. In all panels, the results are means ± s.d. of two or three mice per group in one representative experiment out of two or three. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

RESULTS

Mechanism of induction of vitamin A–metabolizing enzymes

Zymosan is known to induce both proinflammatory[10–14] and anti-inflammatory cytokines in DCs and macrophages[16–18]. Our previous study demonstrated that zymosan stimulates regulatory DCs and macrophages, which produce IL-10 and TGF-β, respectively[18]. One key variable in the different studies is the source and type of DCs. Therefore, it was necessary to determine whether zymosan stimulated similar cytokine profiles in both *in vitro*–generated bone marrow–derived DCs (BMDCs), as well as in splenic DCs. In agreement with previously published reports[23,24], zymosan induced production of the proinflammatory cytokines IL-23, IL-6, IL-12 and TNF-α as well as IL-10 from BMDCs (**Supplementary Fig. 1** online). In contrast, zymosan induced robust IL-10 production and little IL-23, IL-6, IL-12 and TNF-α production in splenic DCs *in vitro* (**Supplementary Fig. 2** online) and *in vivo* (**Supplementary Fig. 3a,b** online). Thus, zymosan induces distinct cytokine profiles depending on the type of DC.

To obtain insights into how zymosan activates splenic DCs, we performed a microarray analysis. Zymosan induced robust expression of the anti-inflammatory gene *Il10* and much lower expression of pro-inflammatory genes such *Il6*, *Tnfa*, *Il12a* and *Il12b*, relative to lipopolysaccharide (LPS), a potent immune modulator (data not shown). Zymosan also induced expression of genes involved in the biosynthesis of retinoic acid such as *Adh1* (encoding alcohol dehydrogenase 1) and *Aldh1a2* (encoding Raldh2) (data not shown). This was unexpected, as splenic DCs, unlike intestinal DCs[25–30], are not thought to express these enzymes. Consistent with the microarray data, RT-PCR analysis showed that splenic DCs constitutively express the retinoic acid–metabolizing enzymes Adh class I (*Adh1*), Adh class III (*Adh5*), and low levels of *Raldh1* (data not shown); upon zymosan treatment their expressions levels were further increased (data not shown). Notably, zymosan stimulated an 80-fold increase in the expression of *Raldh2* mRNA (**Fig. 1a**). In contrast, BMDCs stimulated with zymosan showed a modest increase in *Raldh2* mRNA expression compared to the splenic DCs stimulated with zymosan (**Supplementary Fig. 4** online).

We next examined whether other TLR ligands could induce *Raldh2* in DCs. Treatment with neither LPS (TLR4 ligand) nor CpG (TLR9 ligand) induced *Raldh2* (**Fig. 1b**). However, other ligands specific for TLR2 and TLR6, such as Pam-2-cys and follistatin-like 1 ligand induced substantial expression of *Raldh2* (**Fig. 1b**). Injection of zymosan into mice induced robust expression of *Raldh2* messenger RNA in DCs (**Fig. 1c**). The expression of Raldh2 protein in splenic DCs *in vivo* was confirmed by western blot analysis

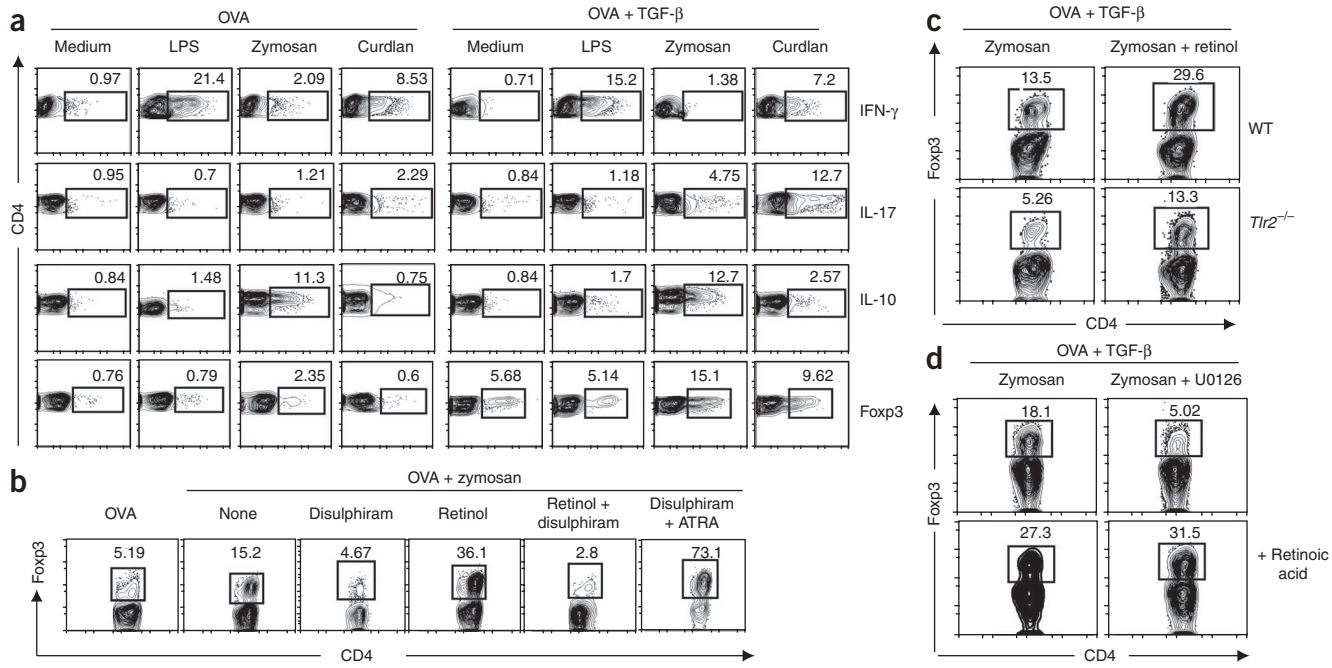

Figure 2 Retinoic acid and IL-10 work synergistically to induce Foxp3$^+$ T_{reg} cells. (**a**) Foxp3 expression and intracellular production of IL-17, IFN-γ and IL-10 by CD4$^+$ T cells, as assessed by intracellular staining and flow cytometry. Splenic DCs were stimulated with zymosan, curdlan or LPS for 12 h and washed and cultured with naive (CD4$^+$CD62L$^+$) OT-II T cells with OVA in the presence or absence of TGF-β. After 4 d, OT-II cells were re-stimulated for 6 h with plated bound antibodies to CD3 and CD28. (**b**) Foxp3 expression by CD4$^+$ T cells, as assessed by intracellular staining and flow cytometry 4 d after splenic DCs were stimulated with zymosan in the presence of disulphiram or vehicle for 10 h and washed and cultured with naive OT-II T cells with OVA and TGF-β in the presence or absence of retinol. ATRA, all-*trans* retinoic acid. (**c**) Foxp3 expression by CD4$^+$ T cells, as assessed by intracellular staining and flow cytometry 4 d after splenic DCs from wild-type or *Tlr2*$^{-/-}$ mice were stimulated with zymosan for 10 h and washed and cultured with naive OT-II T cells with OVA and TGF-β in the presence or absence of retinol. Data are representative of one experiment of three. (**d**) Effect of blocking ERK activation in zymosan-stimulated DCs on T_{reg} cell induction. All data are representative of one experiment of three. Numbers in FACS plots represent percentage of cells positive for the indicated protein.

and immunohistochemical analysis (**Fig. 1d**,**e**). These data indicate that Raldh2 is robustly induced in splenic DCs upon stimulation with zymosan or other TLR2 or TLR6 ligands.

Because zymosan signals through both TLR2 and dectin-1 (refs. 10–12,14,16,18), we determined whether induction of Raldh2 is dependent on both TLR2 and dectin-1. DCs from *Tlr2*$^{-/-}$ mice expressed lower levels of *Raldh2* mRNA compared to wild-type DCs upon zymosan stimulation (**Fig. 1f**). DCs from *Tlr2*$^{-/-}$ mice showed a significant reduction in *Raldh2* expression upon zymosan injection *in vivo* (**Fig. 1f**). Treatment with curdlan, a β-glucan ligand that is thought to be a specific ligand for dectin-1 (ref. 16), induced *Raldh2*, albeit to a much lower level (12-fold, **Fig. 1b**) than zymosan. However, DCs from dectin-1–deficient mice showed only a very modest reduction in *Raldh2* mRNA levels compared to the wild-type DCs upon zymosan stimulation (**Fig. 1g**), suggesting that the effects of curdlan may have been mediated in part by contaminants that triggered TLR2; thus, dectin-1 probably does not have a major role in inducing *Raldh2*.

We next investigated the downstream signaling pathways through which TLR2 induces Raldh2 expression. TLR2 stimulates rapid induction of ERK, which mediates IL-10 production by DCs[16,18,23]. Induction of *Raldh2* mRNA expression was largely abrogated by inhibitors against ERK (**Fig. 1h**). Additionally, DCs from *Erk1*$^{-/-}$ mice had substantially reduced *Raldh2* expression upon zymosan stimulation (data not shown). Furthermore, induction of *Raldh2* was also inhibited substantially by a spleen tyrosine kinase (syk) inhibitor (data not shown). Thus, *Raldh2* induction by zymosan is syk dependent but largely dectin-1 independent, suggesting that an alternative syk-dependent receptor is involved. In summary, therefore, zymosan induces *Raldh2* expression in DCs via TLR2-mediated activation of ERK, probably acting in concert with syk-dependent signaling via another receptor.

Next, we determined whether production of Raldh2 and IL-10 in DCs were interdependent. Notably, induction of *Il10* mRNA preceded that of *Raldh2*, raising the possibility that IL-10 may promote *Raldh2* induction (**Supplementary Fig. 5a** online). However, induction of *Raldh2* was unaffected in DCs from IL-10–deficient mice (**Supplementary Fig. 5b**). Conversely, blocking retinoic acid synthesis or retinoic acid signaling with antagonists to the retinoic acid receptor (RAR) had no effect on IL-10 production upon zymosan treatment (**Supplementary Fig. 5c**). Therefore, induction of IL-10 and *Raldh2* do not seem to be interdependent.

Finally, we explored whether the mechanisms of *Raldh2* induction observed with zymosan would also operate in response to a live yeast infection. Thus, we investigated whether the live pathogenic fungus *Candida albicans* triggered Raldh2 in splenic DCs. Stimulation of splenic DCs with live *C. albicans* induced *Raldh2* mRNA and protein in wild-type DCs (**Fig. 1i**), but this induction was significantly reduced in *Tlr2*$^{-/-}$ DCs. Collectively, these results suggest a major role for TLR2-mediated ERK activation in the induction of Raldh2 in splenic DCs upon stimulation with zymosan or live yeast.

Retinoic acid and IL-10 act synergistically to induce T_{reg} cells

Next, we determined the roles of retinoic acid and IL-10 in the induction of T_{reg} cells. In the presence of TGF-β, retinoic acid can

promote the conversion of naive T cells to T_{reg} cells expressing forkhead box P3 (Foxp3)[25–27,29,30]. Because zymosan induces enzymes involved in retinoic acid synthesis, we determined whether these DCs can metabolize vitamin A (retinol) to retinoic acid and induce T_{reg} cells. Splenic DCs were stimulated with zymosan, or LPS or curdlan for 10 h and then washed and pulsed with a major histocompatibility complex I-A^b–restricted ovalbumin peptide $OVA_{323–339}$ (OVA) and cultured with or without TGF-β, with naive OT-II $CD4^+$ T cells, which express a transgenic T cell receptor specific for the OVA peptide[18]. After 4 d, OT-II T cells were restimulated with antibodies against CD3 and CD28. Zymosan-treated DCs in the absence of TGF-β induced mostly IL-10–producing T regulatory type 1 (T_R1) cells, whereas in the presence of TGF-β they induced both T_{reg} cells and T_R1 cells (**Fig. 2a**). Zymosan-stimulated DCs did not induce marked T_H1 or T_H17 cell responses (**Fig. 2a**). In contrast, LPS-stimulated DCs induced marked T_H1 responses, and curdlan-stimulated DCs induced both T_H1 cells and T_H-17 cells (**Fig. 2a**). Notably, in contrast to splenic DCs, zymosan-treated BMDCs induced T_H17 cells (**Supplementary Fig. 6** online).

The proportion of T_{reg} cells stimulated by zymosan-treated DCs was increased in the presence of retinol (**Fig. 2b**). To determine whether this effect is mediated through retinoic acid synthesis, we stimulated DCs with zymosan in the presence or absence of the Raldh inhibitor disulphiram, which inhibits retinoic acid synthesis by blocking its conversion from retinal[25–30]. After 10 h, we washed the DCs were and cultured them with naive T cells in the presence of retinol and OVA. Inhibition of *de novo* retinoic acid synthesis in DCs suppressed zymosan-mediated induction of T_{reg} cells (**Fig. 2b**). Disulphiram also decreased the frequency of T_{reg} cells that were induced in the absence of zymosan (data not shown). This may reflect expression of small amounts of Raldh1 in splenic DCs in response to signals from activated $CD4^+$ T cells. This basal level of Raldh1 may have a role in converting prestored retinol to retinal in splenic DCs. Nevertheless, taken together, these results suggest zymosan induces splenic DCs to express retinoic acid–synthesizing proteins and stimulate T_{reg} cells.

Of note, zymosan-treated DCs from *Tlr2*$^{-/-}$ mice induced a significantly lower frequency of T_{reg} cells compared to wild-type DCs (**Fig. 2c**). Furthermore, zymosan-stimulated DCs treated with

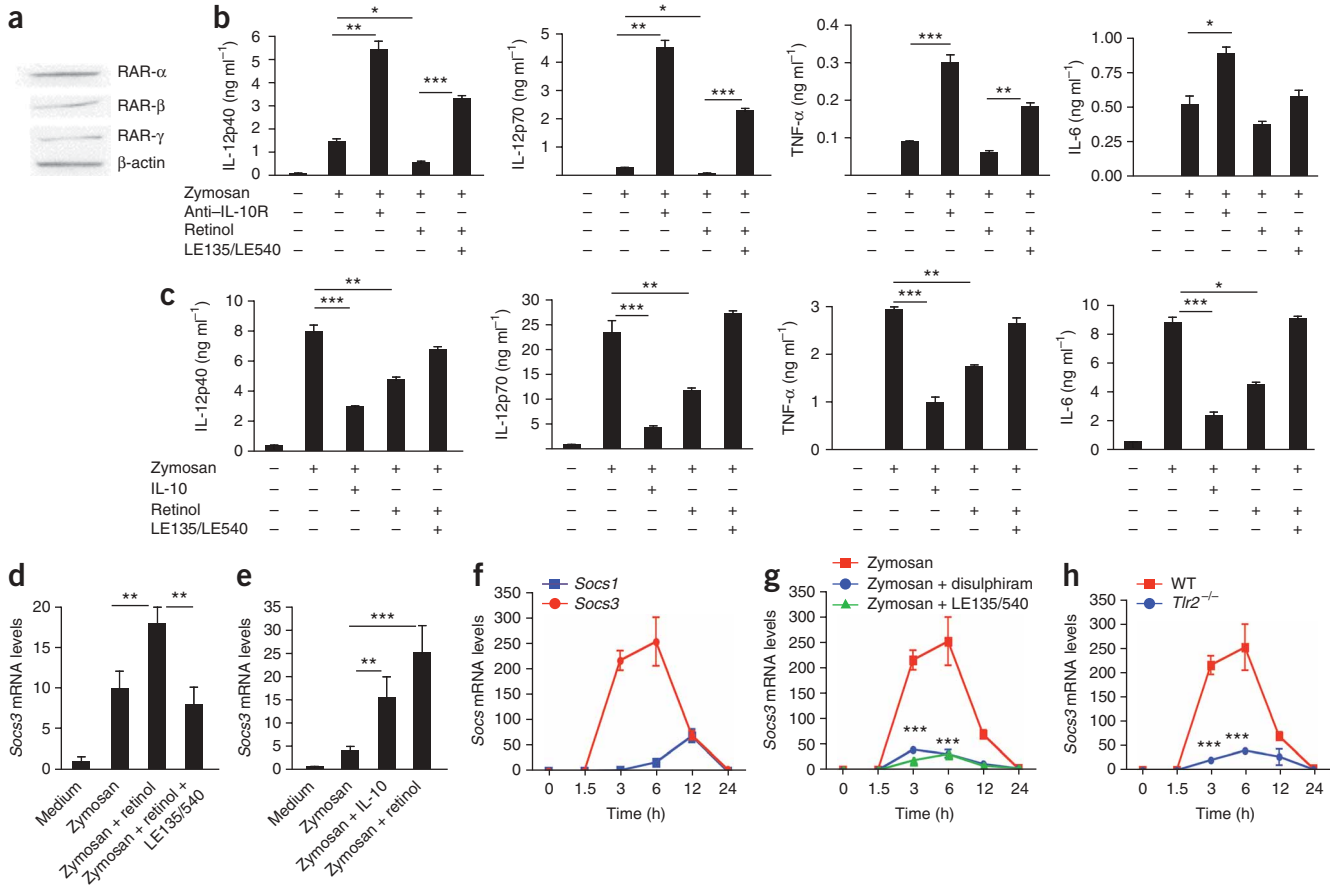

Figure 3 Retinoic acid and IL-10 exert autocrine effects on DCs to induce *Socs3*, which regulates activation of p38 MAPK and pro-inflammatory cytokines. (**a**) Expression of retinoic acid nuclear receptors (RARs and RXRs) in splenic DCs, as determined by western blotting. (**b**,**c**) Cytokine concentrations in supernatants obtained after culture of splenic DCs from wild-type mice (**b**) or *Il10*$^{-/-}$ mice (**c**) treated with zymosan for 24 h in the presence or absence of antibodies against IL-10 receptor (Anti–IL-10R), retinol or retinol plus LE135/LE540 or IL-10. Data are representative of four experiments. (**d**,**e**) Retinoic acid–dependent induction of *Socs3* mRNA expression in splenic DCs from wild-type (**d**) or *IL10*$^{-/-}$ mice (**e**) stimulated with zymosan. (**f**,**g**) Retinoic acid–dependent induction of *Socs3* in splenic DCs *in vivo*. C57BL/6 mice were injected with zymosan or zymosan plus disulphiram (**g**) or zymosan plus LE135/LE540 (**g**), and spleens were harvested at different time points. RNA was isolated from purified splenic DCs, and expression of *Socs1* and *Socs3* mRNA was analyzed by quantitative RT-PCR. (**h**) Induction of *Socs3* mRNA in splenic DCs from wild-type or *Tlr2*$^{-/-}$ mice were injected with zymosan, as evaluated by RT-PCR. Data are representative of three experiments. *$P < 0.01$; **$P < 0.001$; ***$P < 0.0001$. Error bars in **d**–**h** represent comparisons between *Socs3* mRNA and *Gapdh* mRNA.

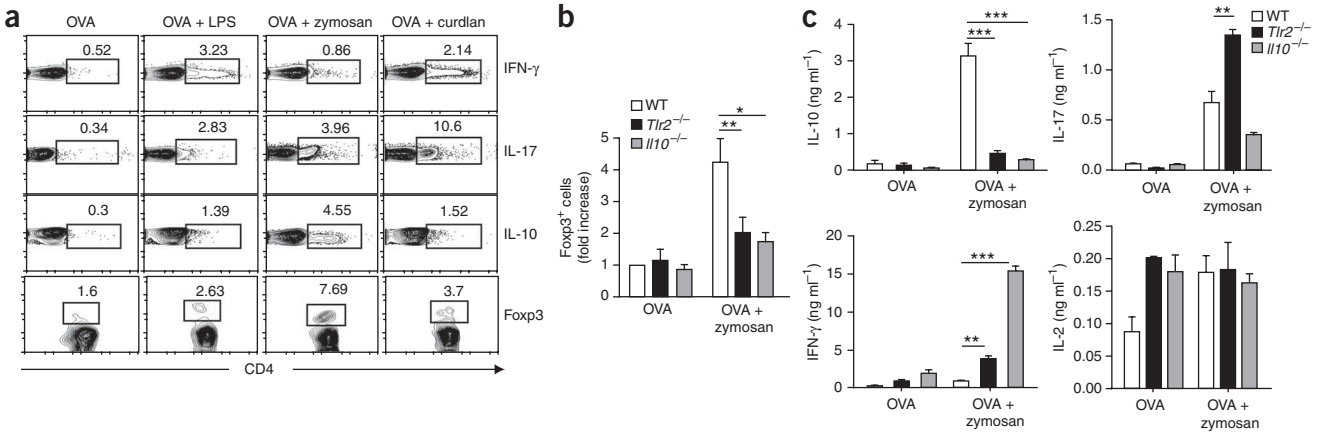

Figure 4 Induction of antigen-specific IL-10$^+$ T_R1 and T_{reg} cells *in vivo*. (**a**) Expression of Foxp3, IL-17, IFN-γ and IL-10 by CD4$^+$Thy1.2$^+$ cells, as assessed by intracellular staining and flow cytometry. B6.PL (Thy1.1$^+$) mice reconstituted with OT-II T cell receptor–transgenic T cells were injected intravenously with major histocompatibility complex class II–restricted OVA alone, OVA plus LPS, OVA plus zymosan or OVA plus curdlan. Four days after challenge, the splenocytes were isolated and analyzed. Data are from one experiment representative of two. (**b**) Induction of OVA-specific Foxp3$^+$ T cells, as assessed by intracellular staining and flow cytometry. C57BL/6, *Tlr2*$^{-/-}$ or *Il10*$^{-/-}$ mice were reconstituted with OT-II transgenic T cells and on the next day were injected with OVA or OVA plus zymosan. Five days later, splenocytes were isolated and analyzed. Data are means ± s.d. of three or four mice per group. (**c**) Cytokine concentrations in the supernatants of splenocytes isolated from the immunized mice described in **b** and re-stimulated with OVA in culture for 48 h, as analyzed by ELISA. Data are means ± s.d. of three or four mice per group. $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$.

an ERK inhibitor were compromised in their ability to induce T_{reg} cells (**Fig. 2d**). However, addition of exogenous retinoic acid to the culture restored their ability to induce T_{reg} cells (**Fig. 2c**). These results are consistent with the effects of TLR2-mediated ERK signaling in inducing Raldh2 in DCs (**Fig. 1f–h**). Thus, zymosan activates DCs via TLR2 to stimulate retinol-metabolizing enzymes, which induce T_{reg} cells.

In addition to the effects of retinoic acid on the induction of T_{reg} cells, IL-10 was also observed to have a role in experiments with a neutralizing antibody against IL-10 and its receptor (**Supplementary Fig. 7a** online) and DCs from *Il10*$^{-/-}$ mice (**Supplementary Fig. 7b**). However, addition of retinol to these cultures markedly increased the proportion of T_{reg} cells (**Supplementary Fig. 7b**). Consistent with these results, blocking IL-10 or retinoic acid–mediated signaling in DCs converts them from regulatory DCs to stimulatory DCs that induce enhanced T_H1 and T_H17 responses (**Supplementary Fig. 8a,b** online). These results demonstrate that IL-10–deficient DCs can metabolize vitamin A as efficiently as wild-type DCs and that synthesis of IL-10 and retinoic acid are interdependent. Inhibition of retinoic acid or IL-10 alone results in a pronounced diminution of T_{reg} cells, suggesting that IL-10 and retinoic acid act synergistically to induce T_{reg} cells.

Autocrine effects of retinoic acid and IL-10 on DCs

IL-10 exerts autocrine effects on DCs to suppress zymosan-induced proinflammatory cytokines[18]. Given the synergistic effects of retinoic acid and IL-10 in stimulating T_{reg} cells, we hypothesized that retinoic acid might exert a similar effect on DCs. Thus, we determined the expression of the retinoic acid nuclear receptors RAR-α, RAR-β and RAR-γ. We observed that all three receptors were expressed on DCs (**Fig. 3a**). We stimulated DCs with zymosan in the presence or absence of retinol and determined the induction proinflammatory cytokines. Although zymosan-treated DCs produced little IL-6, IL-12 and TNF-α, addition of retinol further reduced the expression of these cytokines (**Fig. 3b**). To determine whether this effect was dependent on RARs, we stimulated DCs with zymosan in the presence of retinol plus the RAR antagonist LE135/540. Addition of LE135/540 significantly increased the production of proinflammatory cytokines compared to addition of retinol alone (**Fig. 3b**). Furthermore, DCs with IL-10 neutralized with an antibody (**Fig. 3b**) or DCs from *Il10*$^{-/-}$ mice (**Fig. 3c**) produced increased amounts of proinflammatory cytokines in response to zymosan; however, the addition of retinol or retinoic acid receptor antagonists produced the same effects observed with wild-type DCs (**Fig. 3b,c**). These results indicate that retinoic acid produced by zymosan-treated DCs can act in an autocrine manner to suppress the production of proinflammatory cytokines.

In exploring the mechanisms by which retinoic acid suppresses the production of proinflammatory cytokines in zymosan-treated DCs, we observed an increase in the expression of *Socs3* in zymosan-treated DCs relative to the untreated DCs in our microarray analysis (data not shown). This was confirmed by RT-PCR (**Fig. 3d**). Addition of retinol to the culture further increased *Socs3* expression to approximately 20-fold above the control value; in contrast, addition of the RAR antagonist significantly lowered *Socs3* mRNA expression (**Fig. 3d**). Thus, *Socs3* is inducible upon zymosan stimulation and is partly dependent on RAR-mediated signaling. Consistent with this, knockdown of Raldh2 in DCs by small interfering RNA (siRNA) markedly reduced the induction of *Socs3* in response to zymosan (**Supplementary Fig. 9** online). Furthermore, treatment with IL-10 also enhanced the induction of *Socs3* expression by zymosan (**Fig. 3e**). DCs from *Il10*$^{-/-}$ mice stimulated with zymosan showed a significant reduction in *Socs3* expression, relative to wild-type DCs (**Fig. 3d,e**), but this defect could be corrected by the addition of exogenous IL-10 or retinol to the culture (**Fig. 3e**). Consistent with these *in vitro* observations, zymosan induced a significant ($P < 0.001$) increase in *Socs3* expression in DCs within 3 h *in vivo* (**Fig. 3f**). Furthermore, treatment of mice with disulphiram or LE135/540 reduced the level of *Socs3* expression *in vivo* upon zymosan injection (**Fig. 3g**). Induction of *Socs3* was dependent on TLR2, as zymosan induced much lower levels of *Socs3* in *Tlr2*$^{-/-}$ mice relative to wild-type mice (**Fig. 3h**).

Next, we determined the effect of retinoic acid on the activation of ERK and p38 MAPK. Blocking RAR-mediated signaling with

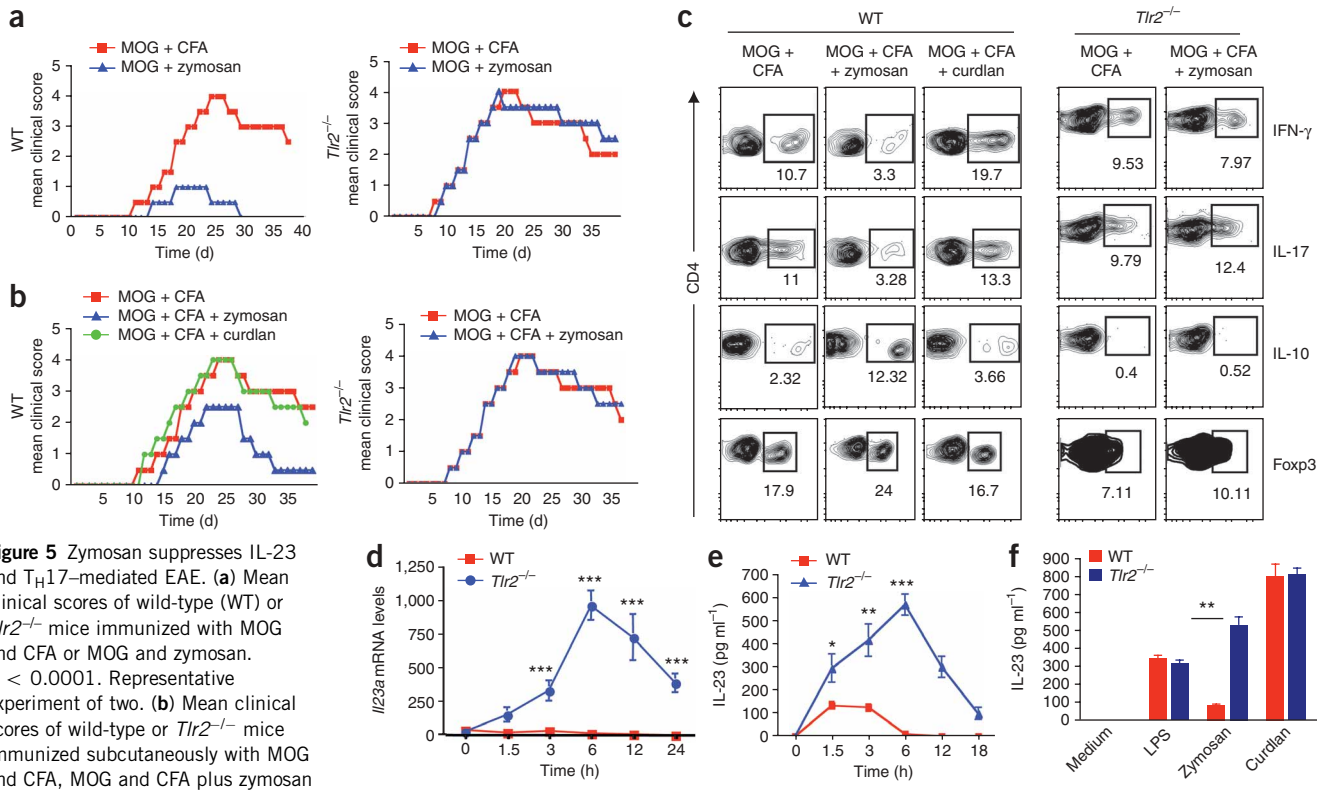

Figure 5 Zymosan suppresses IL-23 and T_H17–mediated EAE. (**a**) Mean clinical scores of wild-type (WT) or *Tlr2*$^{-/-}$ mice immunized with MOG and CFA or MOG and zymosan. $P < 0.0001$. Representative experiment of two. (**b**) Mean clinical scores of wild-type or *Tlr2*$^{-/-}$ mice immunized subcutaneously with MOG and CFA, MOG and CFA plus zymosan (intravenously) or MOG and CFA plus curdlan (intravenously). Representative experiment of two. (**c**) Induction of IFN-γ, IL-17, IL-10 and Foxp3 in mononuclear cells isolated from central nervous system tissue on day 18 after immunization, as assessed by intracellular staining and flow cytometry as described in the **Supplementary Methods** online. Representative experiment of two. (**d**) Expression of *Il23a* mRNA in splenic DCs from wild-type or *Tlr2*$^{-/-}$ mice injected with zymosan, as analyzed by quantitative RT-PCR. Data are representative of three experiments. (**e**) IL-23 induction in the serum of the mice described in **d**, as assayed by ELISA. (**f**) IL-23 secretion by splenic DCs from wild-type or *Tlr2*$^{-/-}$ mice cultured *in vitro* with zymosan. $^*P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

LE135/540 led to a sustained activation of p38 but had no effect on ERK activation (**Supplementary Fig. 10a,c** online). Furthermore, IL-10 deficiency also resulted in a marked effect on the sustained expression of p38 MAPK in DCs (**Supplementary Fig. 10d**). This suggests that, similar to IL-10 signaling, retinoic acid signaling attenuates activation of p38 MAPK. To further address the role of *Socs3* in zymosan-mediated suppression of proinflammatory cytokines, we knocked down *Socs3* in DCs with siRNA (**Supplementary Fig. 11** online). DCs transfected with siRNA against *Socs3* produced markedly higher levels of proinflammatory cytokines upon zymosan treatment compared to DCs transfected with control siRNA (**Supplementary Fig. 11**). Collectively, these results show that retinoic acid–meditated autocrine signaling is crucial for the induction of Socs3 and for regulating the activity of p38 MAPK and proinflammatory cytokines in zymosan-stimulated DCs.

TLR2 suppression of IL-23 and T_H17–mediated autoimmunity

To determine whether zymosan induces T_{reg} cells *in vivo*, we adoptively transferred naive OT-II cells into wild-type, *Tlr2*$^{-/-}$ or *Il10*$^{-/-}$ mice and then immunized the mice with OVA alone or OVA mixed with either zymosan, or curdlan or LPS. After 4 d, we analyzed the proliferation and cytokine production of splenic OT-II T cells re-stimulated *in vitro*. Injection of zymosan and OVA resulted in a weak clonal expansion of antigen-specific T cells relative to injection of OVA and LPS or OVA and curdlan (data not shown). Consistent with this, stimulation with OVA and zymosan resulted in a robust induction of antigen-specific T_{reg} cells and IL-10–producing T_R1 cells compared to stimulation with OVA and PBS or LPS or curdlan (**Fig. 4a**). Intracellular cytokine staining revealed robust induction of T_H1 responses in mice injected with LPS and induction of both T_H1 and T_H17 responses in mice injected with curdlan (**Fig. 4a**). In contrast, mice injected with zymosan showed relatively weak T_H1 and T_H17 responses (**Fig. 4a**). In *Tlr2*$^{-/-}$ and *Il10*$^{-/-}$ mice, induction of T_{reg} cells was markedly reduced relative to wild-type mice (**Fig. 4b**). Furthermore, in the knockout mice, zymosan induced enhanced T_H1 and T_H17 responses and induced a lower number of IL-10–producing cells (**Fig. 4c**). These data suggest that TLR2-mediated signaling is crucial for the induction of both T_{reg} cells and IL-10$^+$ T_R1 cells.

We next determined the therapeutic potential of zymosan-stimulated T_{reg} cells on autoimmune disease with a mouse model of experimental autoimmune encephalomyelitis (EAE). First, we immunized mice subcutaneously with myelin oligodendrocyte glycoprotein peptide ($MOG_{35–55}$; defined here as MOG) emulsified in complete Freund's adjuvant (CFA) or with MOG and zymosan on days 0 and 7. As previously described[24], mice treated with MOG and CFA showed onset of neurological impairment starting at around day 14 (**Fig. 5a**). In contrast, MOG and zymosan treatment induced a relatively attenuated and transient disease course (**Fig. 5a**). In *Tlr2*$^{-/-}$ mice, injection of MOG and zymosan resulted in as severe a disease as observed in the positive control, suggesting a regulatory role for TLR2 in autoimmune disease (**Fig. 5a**). We next determined whether zymosan is capable of actively suppressing disease. Thus, we immunized mice with MOG and CFA and simultaneously injected zymosan or curdlan. Control mice received PBS or curdlan and showed disease

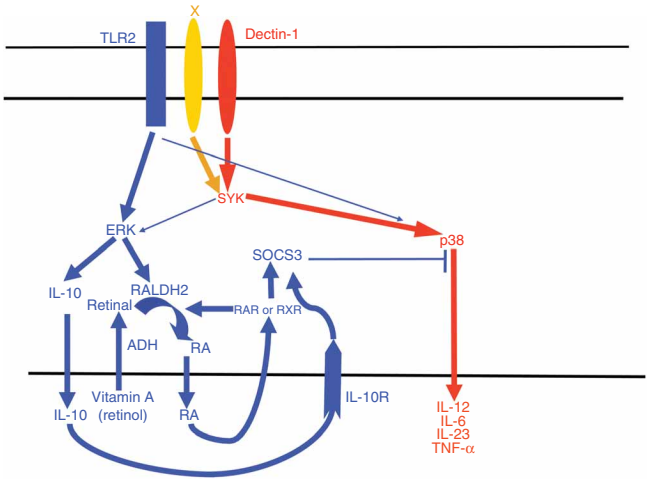

Figure 6 Mechanism of induction of Raldh2 in DCs. Innate sensing of zymosan via TLR2 efficiently (thick arrows) induces ERK activation and Raldh2. Dectin-1 does not have a major role in Raldh2 induction (thin arrow), although signaling via syk is crucial, suggesting the involvement of an additional syk-dependent receptor (X). Thus, the combinatorial activation of TLR2-dependent ERK and syk probably orchestrates induction of Raldh2. This results in the conversion of retinal to retinoic acid (RA), which then exerts an autocrine effect on DCs via RAR or RXR to induce SOSC3, which suppresses activation of p38 MAPK and proinflammatory cytokines. In contrast, dectin-1 promotes induction of proinflammatory cytokines.

symptoms starting around day 14 after immunization (**Fig. 5b**). In contrast, mice treated with zymosan developed significantly ($P < 0.001$) lower clinical scores compared to the PBS- or curdlan-treated mice (**Fig. 5b**). Consistent with a regulatory role for TLR2 in autoimmune disease, zymosan treatment induced enhanced disease progression in *Tlr2*$^{-/-}$ mice relative to wild-type mice (**Fig. 5b**).

We then determined the phenotype of CD4$^+$ T cells that had infiltrated the central nervous system at day 18 after immunization. We observed that zymosan treatment resulted in enhanced induction of T_{reg} cells and T_R1 cells relative to treatment with PBS or curdlan (**Fig. 5c**). In contrast, control or curdlan-treated mice showed a significant increase in the number of T_H1 and T_H17 cells compared to the zymosan-treated mice (**Fig. 5c**). In *Tlr2*$^{-/-}$ mice, there was a decreased frequency of T_{reg} cells and T_R1 cells and greatly enhanced T_H1 and T_H17 responses (**Fig. 5c**). Thus, TLR2-mediated signaling is essential for the induction of T_{reg} cells and T_R1 cells and suppresses T_H1 and T_H17 responses.

IL-23 plays a pivotal part in the expansion of T_H17 cells and the pathogenesis of EAE. We thus evaluated the *Il23a* mRNA expression in DCs isolated *ex vivo* from wild-type versus *Tlr2*$^{-/-}$ mice (**Fig. 5d**). We also measured the serum cytokine concentrations of IL-23 in wild-type and *Tlr2*$^{-/-}$ mice (**Fig. 5e**) upon zymosan injection. Zymosan induced much higher expression of IL-23 in *Tlr2*$^{-/-}$ mice relative to wild-type mice (**Fig. 5d,e**). Similarly, DCs isolated from *Tlr2*$^{-/-}$ mice produced significantly more IL-23 compared to wild-type DCs (**Fig. 5f**). Collectively, these results suggest that TLR2-mediated signaling suppresses IL-23 production in DCs (**Fig. 6**).

Finally, we investigated the relative roles of IL-10 and retinoic acid in zymosan-mediated suppression of EAE in *Il10*$^{-/-}$ mice and Raldh inhibitor. Injection of disulphiram resulted in a significant ($P < 0.01$) but transient enhancement in the acceleration of the disease (**Supplementary Fig. 12a** online). Furthermore, *Il10*$^{-/-}$ mice were also more susceptible to EAE compared to the wild-type mice (**Supplementary Fig. 12b**). Even in *Il10*$^{-/-}$ mice, injection of disulphiram resulted in a marked enhancement in the disease severity (**Supplementary Fig. 12b**). Similarly, wild-type mice injected with a Raldh inhibitor, encapsulated in microparticles, that specifically targets to antigen-presenting cells *in vivo*[31,32], showed worsened EAE upon zymosan treatment compared to the mice injected with empty particles (**Supplementary Fig. 12c**). These results collectively suggest that both retinoic acid and IL-10 contribute to the suppressive effect of zymosan (**Fig. 6**).

DISCUSSION

Although recent studies have highlighted a major role for retinoic acid in the induction of T_{reg} cells in the intestine[26,27,33,34], its role in systemic immune responses is poorly understood. The present data demonstrate that Raldh2 can be induced in splenic DCs via TLR2-dependent ERK signaling, which programs DCs to induce T_{reg} cells. Dectin-1 signaling seems largely irrelevant, although the dependence of Raldh2 induction on syk suggests that other syk-dependent receptors[14] may act in concert with TLR2 to induce Raldh2. Retinoic acid acts directly on T cells[25–30], but there is emerging evidence that retinoic acid can act also on DCs[35–38]. Retinoids signal via two groups of nuclear receptors, the RAR receptors (RAR-α, RAR-β and RAR-γ) and the retinoid X receptors (RXR-α, RXR-β and RXR-γ)[39]. Our data show that DCs express RAR-α, RAR-β, RAR-γ and RXR-α and that signaling via RARs is crucial for enhanced Raldh2 expression. This is consistent with reports in other cell types that retinoic acid regulates its own synthesis through RAR-mediated signaling and RALDH expression[40–44]. Furthermore, Raldh2 suppresses proinflammatory cytokines in DCs via induction of Socs3, a well known regulatory of proinflammatory responses[45–47].

There are also conflicting reports on the effects of zymosan on innate and adaptive responses. Zymosan induces proinflammatory responses from macrophages and DCs[10–14,16,17] but also induces robust IL-10 production from DCs[16–18]. Our work shows that certain TLR2 ligands bias T cell differentiation toward the T_H2 pathway[48,49] and that zymosan in particular induces tolerogenic responses[18], results that contrast with recent findings that zymosan induces T_H17 responses[23,24,50]. One key variable in these studies is the type of DCs used. Our data suggest that splenic DCs produce an abundance of IL-10, but little or no proinflammatory cytokines, whereas BMDCs produce robust proinflammatory cytokines. Furthermore, zymosan induces splenic DCs to stimulate a predominantly T_{reg} response, whereas it induces BMDCs to stimulate a T_H17 response. Consistent with this, BMDCs expressed high amounts of dectin-1 compared to splenic DCs, whereas TLR2 expression was comparable between BMDCs and splenic DCs (data not shown). Another variable is the dose of zymosan. Another group of researchers showed that injection of 500 μg of zymosan induced T_H17 responses and EAE, but only transiently[24]. Here we observed that injection of 100 μg of zymosan resulted in an active suppression of disease progression and a marked reduction in the frequency of T_H1 and T_H17 cells and an enhancement in the frequency of T_{reg} cells and T_R1 cells. When we injected 500 μg of zymosan, we could only see a mild and transient disease of similar severity and kinetics to that observed with the lower dose (data not shown). The reasons for the difference between our study and the other researchers'[24] are not clear, but they may lie with differences between the mouse colonies used.

Finally, it is now clear that the immune system senses microbes not with a single innate immune receptor, but with a combination of several receptors[51]. Combinatorial triggering of specific TLRs on DCs induces synergistic responses[51], and cooperation between TLRs and non-TLRs is known[9], but its impact on adaptive immunity is poorly

understood. The present data demonstrate that two receptors that sense the same microbe can mediate divergent innate and adaptive immune responses with distinct effects on disease progression. What evolutionary benefit might accrue to the microbe or to the host from these mixed signals? From the microbe's perspective, the induction of T_{reg} cells could represent an immune evasion strategy; from the host's perspective, a 'balanced' response could ensure immune defense without collateral damage caused by excessive inflammation. It is tempting to speculate that pathogens that cause chronic infections, such as *Mycobacterium tuberculosis*, HIV and hepatitis C virus, might exploit this balance. Therefore, immune interventions against chronic infections should focus on strategies that not only enhance T_H1 and T_H17 responses but that simultaneously inhibit T_{reg} cells. Furthermore, vaccine adjuvants that engage multiple innate receptors to simultaneously promote T_H1 and T_H17 and T_{reg} responses might induce effective but safe immunity in humans.

METHODS

Mice. We obtained C57BL/6, OT-II and B6.PL mice from Jackson Laboratories. We also obtained *Il10*$^{-/-}$ mice (B6.129P2-Il10$^{tm1Cgn/J}$) from Jackson Laboratories. We maintained the mice in specific pathogen–free conditions in the Emory Vaccine Center vivarium. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University.

Purification of splenic dendritic cells. In brief, we cut spleens from mice into small fragments and then digested the fragments with collagenase type 4 (1 mg ml^{-1}) in complete DMEM plus 2% FBS for 30 min at 37 °C. We washed the cells twice and enriched CD11c$^+$ DCs with the CD11c-specific microbeads from Miltenyi Biotech. The resulting purity of CD11c$^+$ DCs was approximately 95%.

Toll-like receptor stimulation of dendritic cells. We cultured CD11c$^+$ splenic DCs (1×10^6 cells per ml) for 24 h with *Escherichia coli* LPS (5 μg ml^{-1}), Pam-3-cys (100 ng ml^{-1}), Pam-2-cys (100 ng ml^{-1}), CpG dinucleotides (1 μg ml^{-1}), zymosan (25 μg ml^{-1}, Sigma) or curdlan (25 μg ml^{-1}, Wako). We collected the supernatants and used ELISA (Becton Dickinson and eBiosciences) to measure cytokine concentrations. In some experiments, we added antibody to IL-10 and antibody to IL-10R (1 μg ml^{-1}), disulphiram (100 nM, Sigma) or LE135/LE540 (1 μM, Wako) for the duration of the stimulation.

***In vitro* cultures of mouse dendritic cells and T cells.** For *in vitro* stimulation, we stimulated purified splenic CD11c$^+$ DCs (1×10^6 cells per ml) with LPS (5 μg ml^{-1}), zymosan (25 μg ml^{-1}) or curdlan (25 μg ml^{-1}) for 10 h and then washed them with medium three times. In some experiments, we cultured DCs with disulphiram (100 nM), Erk inhibitor (100 nM) or LE135/LE540 (1 μM) for the duration of stimulation. We washed activated DCs (2×10^4) and then cultured them together with naive CD4$^+$CD62L$^+$ OT-II T cells (1×10^5) and OVA (2 μg ml^{-1}) in 200 ml RPMI complete medium in 96-well round-bottomed plates. We analyzed supernatants after 90 h, and we collected cells and analyzed them directly or re-stimulated them after 90 h. In some experiments, we added 500 nM retinol (Sigma), 1ng ml^{-1} TGF-β (R&D Systems) or both to cultures. We added antibody to mouse IL-10 (JES5-16E3; Becton Dickinson), antibody to IL-10R (1B1.3a; Becton Dickinson), antibody to human TGF-β (MAB1835; R&D Systems) or rat IgG isotype control antibody (A95-1; Becton Dickinson) = to cultures at a final concentration of 10 μg ml^{-1}. We added LE135/LE540 to some cultures at a concentration of 1 μM. For secondary re-stimulation, we collected cells after 90 h of primary culture, and then re-stimulated them for 6 h with plate-bound antibody to CD3 (10 μg ml^{-1}; 145.2C11 from Becton Dickinson) and antibody to CD28 (2 μg ml^{-1}; 37.51 from Becton Dickinson) in the presence of brefeldin A (Becton Dickinson) for intracellular cytokine detection, or we re-stimulated them with OVA (2 μg ml^{-1}) for 48 h for analysis of proliferation and cytokine production in cell supernatants.

Statistical analyses. We conducted statistical analyses with GraphPad Prism software. We analyzed mean clinical scores with the Mann-Whitney nonparametric *t* test. We calculated the statistical significance of differences in the mean concencentrations ± s.d. of cytokines released by cells of various types with Student's *t* test (one-tailed).

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

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AUTHOR CONTRIBUTIONS

S.M. performed the experiments in the study, prepared the figures and helped write the paper. R.R. assisted S.M. with some of the experiments. J.D. performed some of the initial experiments. H.O. performed the immunohistology analysis. T.L.D. provided discussion and advice on some parts of the study. S.P.K. encapsulated disulphiram in microparticles. K.M.R. and B.D.E. assisted with the EAE experiments. B.P. conceived and supervised the study and, with S.M., wrote the paper.

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