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TLR2 dependent induction of vitamin A metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits T_H-17 mediated autoimmunity

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Abstract

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 demonstrate that the different pathogen recognition receptors, 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 (RA) metabolizing enzyme Raldh2 and IL-10, and to metabolize vitamin A and stimulate $Foxp3^+$ T regulatory cells (Treg cells). RA acted on DCs to induce Socs3 expression, which suppressed activation of p38 MAPK and pro-inflammatory cytokines. Consistent with this, TLR2 signaling induced Treg cells, and suppressed IL-23 and T_H-17/ T_H-1 mediated autoimmune responses *in vivo*. In contrast, dectin-1 signaling mostly induced IL-23 and pro-inflammatory cytokines, and augmented T_H-17/ T_H-1 mediated autoimmune responses *in vivo*. These data define a new mechanism for the systemic induction of RA 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 responses1–5. Emerging

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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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evidence suggests that DCs are also vital in suppressing immune responses through the generation of anergic or regulatory T cells6. However, the mechanisms by which DCs can be programmed to induce regulatory T cells are poorly understood. DCs express several Toll-like receptors (TLRs) and the C-type lectins, which are critical in sensing and initiating immune response against pathogens7–9. Engagement of such receptors programs gene expression that orchestrate DC maturation and activation2,7,8. The types of cytokines secreted by the DCs can regulate the differentiation of $CD4^+$ T cells into T_H-1, T_H-2, T_H-17 or T regulatory (Treg) responses. For example stimuli that induce IL-12(p70) promote IFN-γ producing T_H-1 cells, stimuli that induce IL-10 favor T_H-2 or Treg responses, whereas stimuli that induce TGF-β, IL-6 and IL-23 promote T_H-17 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-gulcans10–15. 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 pro-inflammatory cytokines in macrophages and DCs14, as well as robust IL-10 production in DCs16–18. Consistent with this, our previous work demonstrates that zymosan conditions splenic DCs to secrete IL-10 and induce tolerogenic T cell responses18. Furthermore, zymosan is also known to induce splenic macrophages to secrete TGF-β18, a cytokine critical for the generation of regulatory T cells, as well as T_H-17 cells11,19–21. In contrast, other studies have shown that dectin-1 mediated signaling in DCs induces T_H-17 cells and IFN-γ producing T_H-1 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 Raldh2 expression in DCs via a mechanism dependent largely on TLR2-mediated activation of ERK MAPK. Raldh2 converts vitamin A derived retinal to RA, which acts in an autocrine manner to induce the expression of Socs3, and suppress activation of p38 MAPK and pro-inflammatory cytokines. Consistent with this, TLR2 signaling was critical for zymosan mediated induction of Treg cells, and suppression of T_H-1 and T_H-17 responses mediated autoimmunity *in vivo*; in the absence of TLR2 signaling, dectin-1 mediated signaling alone induced potent T_H-1 and T_H-17 responses and exacerbated autoimmunity.

Results

Mechanism of induction of vitamin-A metabolizing enzymes

Zymosan is known to induce both pro-inflammatory10–14, and anti-inflammatory cytokines in DCs and macrophages16–18. Our previous study demonstrated that zymosan stimulates regulatory DCs and macrophages, which produce IL-10 and TGF-β, respectively18. One important variable in the different studies is the source and type of DCs. Therefore, it was important to determine whether zymosan stimulated similar cytokine profiles in both *in vitro* generated bone marrow derived DCs (BM-DCs), as well as from splenic DCs. In agreement with published reports23,24, zymosan induced the pro-inflammatory cytokines IL-23, IL-6, IL-12 and TNF-α , as well as IL-10 from BM-DCs (Supplementary Fig. 1). In contrast, zymosan induced robust IL-10, and little IL-23, IL-6, IL-12 and TNF-α in splenic DCs *in*

vitro (Supplementary Fig. 2) and *in vivo* (Supplementary Fig. 3a, b). Thus, zymosan induces distinct cytokine profiles depending on the type DCs.

To obtain insights into how zymosan activates splenic DCs, we performed a microarray analysis. Zymosan induced robust expression of the anti-inflammatory gene *Il-10* and much lower expression of pro-inflammatory genes such *Il*-6, *Tnf-α* and *Il-12*, relative to LPS (data not shown). Surprisingly, zymosan also induced expression of genes involved in the biosynthesis of retinoic acid (RA) such as *Adh1* and *Aldh1a2* (Raldh2) (data not shown). This was unexpected as splenic DCs, unlike intestinal DCs25–30, are not thought to express these enzymes. Consistent with the microarray data, RT-PCR analysis demonstrated that splenic DCs constitutively express the RA 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). Importantly, zymosan stimulated an 80-fold increase in the expression of *Raldh2* mRNA (Fig. 1a). In contrast, BM-DCs stimulated with zymosan showed a modest increase in *Raldh2* mRNA expression compared to the splenic DCs stimulated with zymosan (Supplementary Fig. 4).

We then examined whether other TLR ligands could induce *Raldh2* in DCs. Neither LPS nor CpG induced *Raldh2* (Fig. 1b). However, other ligands specific for TLR2/TLR6, such as Pam-2-cys and FSL induced substantial expression of *Raldh2* (Fig. 1b). Consistent with this, injection of zymosan into mice induced robust expression of *Raldh2* mRNA in DCs (Fig. 1c). The expression of *Raldh2* protein in splenic DCs *in vivo* was confirmed by western blot and immunohistochemical analysis (Fig. 1d, e). These data demonstrate that *Raldh2* is robustly induced in splenic DCs upon stimulation with zymosan, or other TLR2/6 ligands.

Since zymosan signals via both TLR2 and dectin-110–12,14,16,18, 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). Consistent with this, DCs from *Tlr2*$^{-/-}$ mice showed a significant reduction in *Raldh2* expression upon zymosan injection *in vivo* (Fig. 1f). Curdlan, a β-glucan ligand, that is thought to be a specific ligand for dectin-116, induced *Raldh2*, albeit at a much lower level (12-fold, Fig. 1b) than zymosan. However, DCs from dectin-1-deficient mice displayed only a very modest reduction in *Raldh2* mRNA level 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 likely does not play a major role in inducing *Raldh2*.

We next investigated the downstream signaling pathways through which TLR2 induced Raldh2 expression. TLR2 stimulates rapid induction of ERK, which mediates IL-10 production by DCs16,18,23. 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 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 likely involved. In summary

therefore, zymosan induces *Raldh2* expression in DCs via TLR2-mediated activation of ERK, likely 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 *Il-10* mRNA preceded that of *Raldh2*, raising the possibility that IL-10 may promote *Raldh2* induction (Supplementary Fig. 5a). However, induction of *Raldh2* was unaffected in DCs from IL-10 deficient mice (Supplementary Fig. 5b). Conversely, blocking RA synthesis or RA signaling using RAR antagonists 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 WT DCs (Fig. 1i), but this induction was significantly reduced in *Tlr2*−/− DCs. Collectively, these results suggest an important role for TLR2 mediated ERK activation, in the induction of Raldh2 in splenic DCs upon stimulation with zymosan, or live yeast.

RA and IL-10 synergize to induce Treg cells

Next, we determined the role of RA and IL-10 in the induction of Treg cells. In the presence of TGF-β, RA can promote conversion of naïve T cells to Foxp3 expressing Treg cells25–27,29,30. Since zymosan induces enzymes involved in RA synthesis, we determined whether these DCs can metabolize vitamin A (retinol) to RA, and induce Treg cells. Splenic DCs were stimulated with zymosan, or LPS or curdlan for 10h, and then washed and pulsed with an 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 OVA18. After 4 days, 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 Tr1 cells, whereas in the presence of TGF-β, they induced both Treg cells, and Tr1 cells (Fig. 2a). Zymosan stimulated DCs did not induce robust T_H-1 or T_H-17 cells (Fig. 2a). In contrast, LPS stimulated DCs induced robust T_H1 responses, and curdlan stimulated DCs induced both T_H1 cells and T_H-17 cells (Fig. 2a). Interestingly, in contrast to splenic DCs, zymsoan treated BM-DCs induced T_H-17 cells (Supplementary Fig. 6).

The proportion of Treg cells stimulated by zymosan treated DCs was increased in the presence of retinol (Fig. 2b). To determine, whether this effect was mediated through RA synthesis, we stimulated DCs with zymosan, in the presence or absence of the Raldh inhibitor disulphiram, which inhibits RA synthesis by blocking its conversion from retinal25–30. After 10hrs, DCs were washed and cultured with naïve T cells in the presence of retinol and OVA. Inhibition of *de novo* RA synthesis in DCs suppressed zymosan-induced Treg cells (Fig. 2b). Interestingly, disulphiram also inhibited the frequency of Treg cells which were induced in the absence of zymosan (data not shown). This may reflect expression of low levels of Raldh1 in splenic DCs, in response to signals from activated $CD4^+$ T cells. This basal level of Raldh1 may play a role in converting pre-stored retinol to

retinal in splenic DCs. Nevertheless, taken together, these results suggest zymosan induces splenic DCs to express RA synthesizing genes and stimulate Treg cells.

Interestingly, zymosan-treated DCs from *Tlr2*$^{-/-}$ mice induced a significantly lower frequency of Treg cells compared to wild type DCs (Fig. 2c). Furthermore, zymosan stimulated DCs treated with an ERK inhibitor were compromised in their ability to induce Treg cells (Fig. 2d). However, addition of exogenous RA to the culture restored their ability to induce Treg 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 Treg cells.

In addition to the effects of RA on the induction of Treg cells, IL-10 was also observed to play a role, in experiments using a neutralizing antibody against IL-10 and its receptor (Supplementary Fig. 7a), and DCs from *Il-10*$^{-/-}$ mice (Supplementary Fig. 7b). However, addition of retinol to these cultures significantly increased the proportion of Treg cells (Supplementary Fig. 7b). Consistent with these results, blocking IL-10, or RA 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). 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 RA are interdependent. Importantly, inhibition of RA or IL-10 alone results in a pronounced diminution of Treg cells, suggesting that IL-10 and RA act synergistically to induce Treg cells.

Autocrine effects of RA and IL-10 on DCs induces Socs3

IL-10 exerts autocrine effects on DCs to suppress zymosan induced pro-inflammatory cytokines18. Given the synergistic effects of RA and IL-10 in stimulating Treg cells, we hypothesized that RA might exert a similar effect on DCs. Thus, we determined the expression of the RA nuclear receptors RARαβ and 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 pro-inflammatory cytokines. While zymosan-treated DCs produced little IL-6, IL-12, TNF-α, addition of retinol further reduced the levels 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 RA receptor antagonist (LE135/540). Addition of LE135/540 significantly increased the production of pro-inflammatory cytokines (Fig. 3b), compared to the retinol treated DCs. Furthermore, neutralization of IL-10 with an antibody (Fig. 3b), or DCs from *Il-10*$^{-/-}$ mice (Fig. 3c), produced enhanced levels of pro-inflammatory cytokines in response to zymosan; however the addition of retinol or RA receptor antagonists produced the same effects observed with wild type DCs (Fig. 3b & c). These results demonstrate that RA produced by zymosan-treated DCs can act in an autocrine manner to suppress the production of pro-inflammatory cytokines.

In exploring the mechanisms by which RA suppressed the production of pro-inflammatory 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; in contrast, addition of the RAR antagonist significantly reduced the *Socs3* mRNA expression (Fig. 3d). Thus *Socs3* is inducible upon zymosan stimulation and is partly dependent on RAR-mediated signaling. Consistent with this, knock-down of Raldh2 in DCs using siRNA significantly reduced the induction of *Socs3* in response to zymosan (Supplementary Fig. 9). Furthermore, IL-10 also enhanced the induction of *Socs3* expression by zymosan (Fig. 3e). DCs from *Il-10*$^{-/-}$ 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 increase in *Socs3* expression in DCs within 3 hours *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 RA on the activation of ERK and p38 MAPK. Blocking RAR-mediated signaling with LE135/540 led to a sustained activation of p38, but had no effect on ERK activation (Supplementary Fig. 10a, c). 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, RA signaling attenuates activation of p38 MAPK. To further address the role of *Socs3* in zymosan mediated suppression of pro-inflammatory cytokines we knocked-down *Socs3* in DCs using siRNA (Supplementary Fig. 11). DCs transfected with siRNA against *Socs3* produced significantly higher levels of pro-inflammatory cytokines upon zymosan treatment compared to DCs transfected with control siRNA (Supplementary Fig. 11). Collectively, these results show that RA meditated autocrine signaling is critical for the induction of Socs3, and for regulating the activity of p38 MAPK and pro-inflammatory cytokines, in zymosan-stimulated DCs.

TLR2 suppression of IL-23 and T_H-17 mediated autoimmunity

To determine whether zymosan induces Treg cells *in vivo*, we adoptively transferred naïve OT-II cells into wild-type, *Tlr2*$^{-/-}$ or *Il-10*$^{-/-}$ mice, and then immunized the mice with OVA alone, or OVA mixed with either zymosan, or curdlan or LPS. After 4 days, we analyzed the proliferation, and cytokine production of splenic OT-II T cells restimulated *in vitro*. Injection of zymosan + OVA resulted in a weak clonal expansion of antigen-specific T cells, relative to injection of OVA + LPS, or OVA + curdlan (data not shown). Consistent with this, stimulation with OVA+ zymosan resulted in a robust induction of antigen specific Treg cells and IL-10-producing Tr1 cells, compared to stimulation with OVA+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_H-17 responses in mice injected with curdlan. In contrast, mice injected with zymosan showed relatively weak T_H-1 and T_H-17 responses (Fig. 4a). In *Tlr2*$^{-/-}$ and *Il-10*$^{-/-}$ mice, induction of Treg 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 reduced IL-10 producing

cells (Fig. 4c). These data suggest that TLR2 mediated signaling is critical for the induction of both Treg cells and IL-10$^+$ Tr1 cells.

We next determined the therapeutic potential of zymosan stimulated Treg cells on autoimmune disease, using a mouse model of EAE. First, we immunized mice with myelin oligodendrocytes glycoprotein peptide, $MOG_{35–55}$ (MOG) emulsified in CFA, or MOG + zymosan, subcutaneously, on days 0 and 7. As previously described24, MOG + CFA showed onset of neurological impairment starting at around day 14 (Fig. 5a, left panel). In contrast MOG + zymosan induced a relatively attenuated and transient disease course (Fig. 5a, left panel). In *Tlr2*$^{-/-}$ mice, injection of MOG + zymosan resulted in as severe a disease as observed in the positive control, demonstrating a regulatory role for TLR2 (Fig. 5a, right panel). We next determined whether zymosan was capable of actively suppressing disease. Thus in a separate experiment, we immunized mice with MOG + CFA, and simultaneously injected zymosan, or curdlan. Control mice received PBS or curdlan, and displayed disease symptoms, starting around day 14 (Fig. 5b, left panel). In contrast, mice treated with zymosan developed significantly lower clinical scores compared to the PBS or curdlan treated mice (Fig. 5b, left panel). Consistent with a regulatory role for TLR2, zymosan induced enhanced disease progression in *Tlr2*$^{-/-}$ mice relative to wild type mice (Fig. 5b, right panel).

We then determined the phenotype of CNS infiltrated CD4$^+$ T cells at day 18. We observed that zymosan treatment resulted in enhanced induction of Treg cells and Tr1 cells, relative to mice treated 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 diminished frequency of Treg cells and Tr1 cells, and greatly enhanced T_H1 and T_H-17 responses (Fig. 5c). Thus, TLR2 mediated signaling is essential for the induction of Treg cells and Tr1 cells, and suppresses T_H-1 and T_H-17 responses.

IL-23 plays a pivotal role in the expansion of T_H-17 cells, and the pathogenesis of EAE. We thus evaluated the IL-23p19 mRNA expression in DCs isolated *ex vivo* from wild type versus *Tlr2*$^{-/-}$ mice (Fig. 5d). We also measured the serum cytokine levels 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 higher levels of 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 RA in zymsoan mediated suppression of EAE using *IL-10−/−* 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). Furthermore, *IL-10−/−* mice were also more susceptible to EAE compared to the wild type mice (Supplementary Fig. 12b). Even in *IL-10−/−* mice, injection of disulphiram resulted in a marked enhancement in the disease severity (Supplementary Fig. 12b). Similarly, wild type mice injected with RALDH inhibitor-encapsulated in microparticles, that specifically targets to antigen-presentig cells *in vivo* 31,32, displayed

enhanced EAE upon zymosan treatment compared to the mice injected with empty particles (Supplementary Fig. 12 c). These results collectively suggest both RA and IL-10 contribute to the suppressive effect of zymosan (Fig. 6).

Discussion

Several aspects of these findings deserve comment. The first concerns the mechanisms of generation and action of RA. Although recent studies have highlighted an important role for RA in the induction of Treg cells in the intestine26,27,33,34, 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 Treg cells. Dectin-1 signaling seems largely irrelevant, although the dependency on syk suggests that other syk-dependent receptors14 may act in concert with TLR2 to induce Raldh2. With respect to the target of RA, it acts directly on T cells25–30, but there is emerging evidence that RA can act also on DCs35–38. Retinoids signal via two groups of nuclear receptors, the RAR receptors (RARα,β. and γ) and the retinoid X receptors (RXRα, βand γ)39. Our data demonstrate that DCs express RARα, RARβ, RARγ and RXRα, and that signaling via RARs, is critical for enhanced Raldh2 expression. This is consistent with reports on other cell types that RA regulates its own synthesis through RAR receptor mediated signaling and RALDH expression40–44. Furthermore, Raldh2 suppresses pro-inflammatory cytokines in DCs via induction of Socs3, a well known regulatory of pro-inflammatory responses45–47.

The second point concerns the conflicting reports on the effects of zymosan on innate and adaptive responses. Zymosan induces pro-inflammatory responses from macrophages and DCs10–14,16,17, but also induces robust IL-10 from DCs16–18. Our work demonstrates that specific TLR2 ligands bias towards the Th2 pathway48,49, and that zymosan in particular induces tolerogenic responses18, which contrast with recent findings that zymosan induces T_H-17 responses23,24,50. One key variable in these studies is the type of DCs used. Our data suggest that splenic DCs produce robust IL-10, but little or no pro-inflammatory cytokines, while BM-DCs produce robust pro-inflammatory cytokines. Furthermore, zymosan induces splenic DCs to stimulate a predominantly Treg response, while it induces BM-DCs to stimulate a T_H-17 response. Consistent with this, BM-DCs expressed high levels of dectin-1 compared to splenic DCs, whereas TLR2 expression levels were comparable between BM-DCs and splenic-DCs (data not shown). Another variable is dose of zymosan. Stockinger *et al* 24 showed that injection of 500μg of zymosan induced T_H17 responses and EAE, but only transiently. Here, we observed that 100μg of zymosan, resulted in an active suppression of disease progression, and a striking reduction in the frequency of T_H-1/T_H-17 cells, and an enhancement in the frequency of Treg cells and Tr1 cells (Fig. 5). When we injected 500μg of zymosan, we could only see a mild and transient disease, of similar severity and kinetics observed with the lower dose (data not shown). The reasons for the difference between our study and those of Stockinger *et al*24 are not clear, but may lie with differences in the mouse colonies.

Finally, it is now clear that the immune system senses microbes not with a single innate immune receptor, but with a combination of several receptors51 . Combinatorial triggering of specific TLRs on DCs induces synergistic responses51, and cooperation between TLRs

and non TLRs is known9, but its impact on the 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 Treg 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 like tuberculosis, HIV, and HCV might exploit this balance. Therefore immune interventions against chronic infections should focus on strategies that not only enhance T_H-1/T_H-17 responses, but which simultaneously inhibit Treg cells. Furthermore, vaccine adjuvants that engage multiple innate receptors to simultaneously promote T_H-1/T_H-17 and Treg responses might induce effective, but safe immunity in humans.

Methods

Mice

C57BL/6, OT-II and B6.PL mice were from Jackson Laboratories. *Il-10*$^{-/-}$ mice (B6.129P2-Il10$^{tm1Cgn/J}$) were from Jackson Laboratories. Mice were maintained in specific pathogen–free conditions in the Emory Vaccine Center vivarium. All animal protocols were reviewed and approved by the Institute Animal Care and Use Committee of Emory University.

Purification of splenic DCs

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

TLR stimulation of DCs

CD11c$^+$ splenic DCs (10^6 cells ml^{-1}) were cultured for 24 h with Escherichia coli LPS (5 μg/ml), 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},) or curdlan (25 μg ml^{-1}). Supernatants were collected and ELISA measured cytokines. In some experiments, anti-IL-10 and anti-IL-10R (1 μg ml^{-1}) or disulphiram (100 nM) or LE 135/LE 540 (1 μM) were added for the duration of the stimulation.

In *vitro* cultures of murine DCs and T cells

For *in vitro* stimulation, purified splenic CD11c$^+$ DCs (10^6 cells ml^{-1}) were stimulated with LPS (5 μg ml^{-1}) or zymsoan (25 μg ml^{-1}) or curdlan (25 μg ml^{-1}) for 10 hrs and washed with media three times. In some experiments DCs were cultured with disulphiram (100 nM) or Erk inhibitor (100 nM) or LE135/LE540 (1 μM) for the duration of stimulation. Activated DCs (2 × 10^4) were washed, and then cultured together with naive CD4$^+$CD62L$^+$ OT-II CD4$^+$ T cells (10^5) and OVA (2 μg ml^{-1}) in 200 ml RPMI complete medium in 96-well round-bottomed plates. Supernatants were analyzed after 90 h and cells were collected and

analyzed directly or were restimulated after 90 h. In some experiments, 500 nM retinol (Sigma) and/or 1ng ml^{-1} TGF-β (R&D Systems) were added to cultures. Antibody against mouse IL-10 (JES5-16E3; Becton Dickinson), antibody against IL-10R (1B1.3a; Becton Dickinson), antibody against human TGF-β (MAB1835; R&D Systems) or rat IgG isotype control antibody (A95-1; Becton Dickinson) was added to cultures at a final concentration of 10 μg ml^{-1}. LE135/LE540 was added to some cultures at a concentration of 1 μM. For secondary restimulation, cells were collected after 90 h of primary culture, then were restimulated for 6 h with plate-bound antibody against CD3 (10 μg ml^{-1}; 145.2C11 from Becton Dickinson) and antibody against CD28 (2 μg ml^{-1}; 37.51 from Becton Dickinson) in the presence of brefeldin A (Becton Dickinson) for intracellular cytokine detection or were restimulated with OVA (2 μg ml^{-1}) for 48 h for analysis of proliferation and cytokine production in cell supernatants.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism. Mean clinical scores were analyzed using the Mann-Whitney non-parametric t test. The statistical significance of differences in the means + s. d. of cytokines released by cells of various groups were calculated with the Student's t-test (one-tailed).

Supplementary Material

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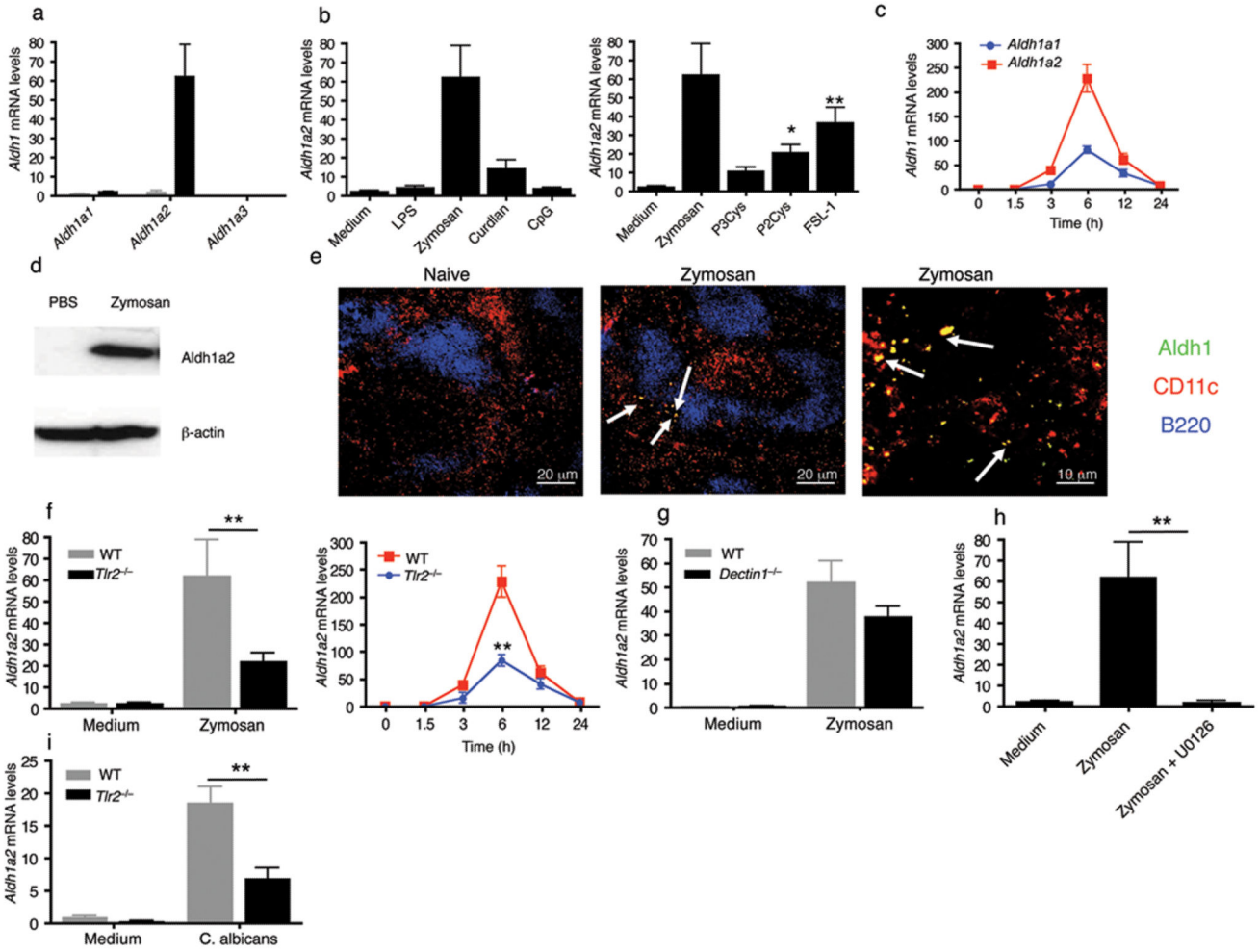

Figure 1.

Mechanism of induction of vitamin-A metabolizing enzymes in splenic DCs. (**a**) Expression of *Raldh* (*Aldh1a*) mRNA in splenic DCs cultured *in vitro* for 24 h with (black bars), or without (grey bars) zymosan. Expression of *Raldh* mRNA relative to expression of mRNA encoding glyceraldehyde phosphate dehydrogenase (GAPDH) was analyzed by quantitative RT-PCR in this and all figures below. (**b**) Induction of *Raldh2* (*Aldh1a2*) in splenic DCs cultured *in vitro* with TLR ligands or curdlan for 24 h. (**c**) Induction of *Raldh2* 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 panel: Splenic DCs from WT or *Tlr2*$^{-/-}$ mice were cultured in media alone or with zymosan and, 24 h later expression of *Raldh2* mRNA was analyzed. Right panel: C57BL/6 mice or *Tlr2*$^{-/-}$ were injected with zymosan and at various time points, splenic DCs were isolated, and expression of *Raldh2* mRNA was analyzed. (**g**) *Raldh2* induction is not dependent on dectin-1. Splenic DCs from wild type or *dectin-1*$^{-/-}$ mice were cultured in

media alone or with zymosan, and 24 h later expression of *Raldh2* mRNA was analzed. Difference between wild type and *dectin-1*$^{-/-}$ mice is not significant. (**h**) Induction of *Raldh2* is dependent on ERK MAPK signaling. Splenic DCs were cultured as described above, with or without U0126. (**i**) Induction of *Raldh2* in splenic DCs by *Candida albicans* is dependent on TLR2. Splenic-DCs from WT or *Tlr2*$^{-/-}$ mice were cultured in media alone or with *Candida albicans*. In all figures results are means + s. d. of 2 – 3 mice per group in one representative experiment out of two or three. *,$P < 0.05$; **,$P < 0.005$; ***$P < 0.0001$ in all figures.

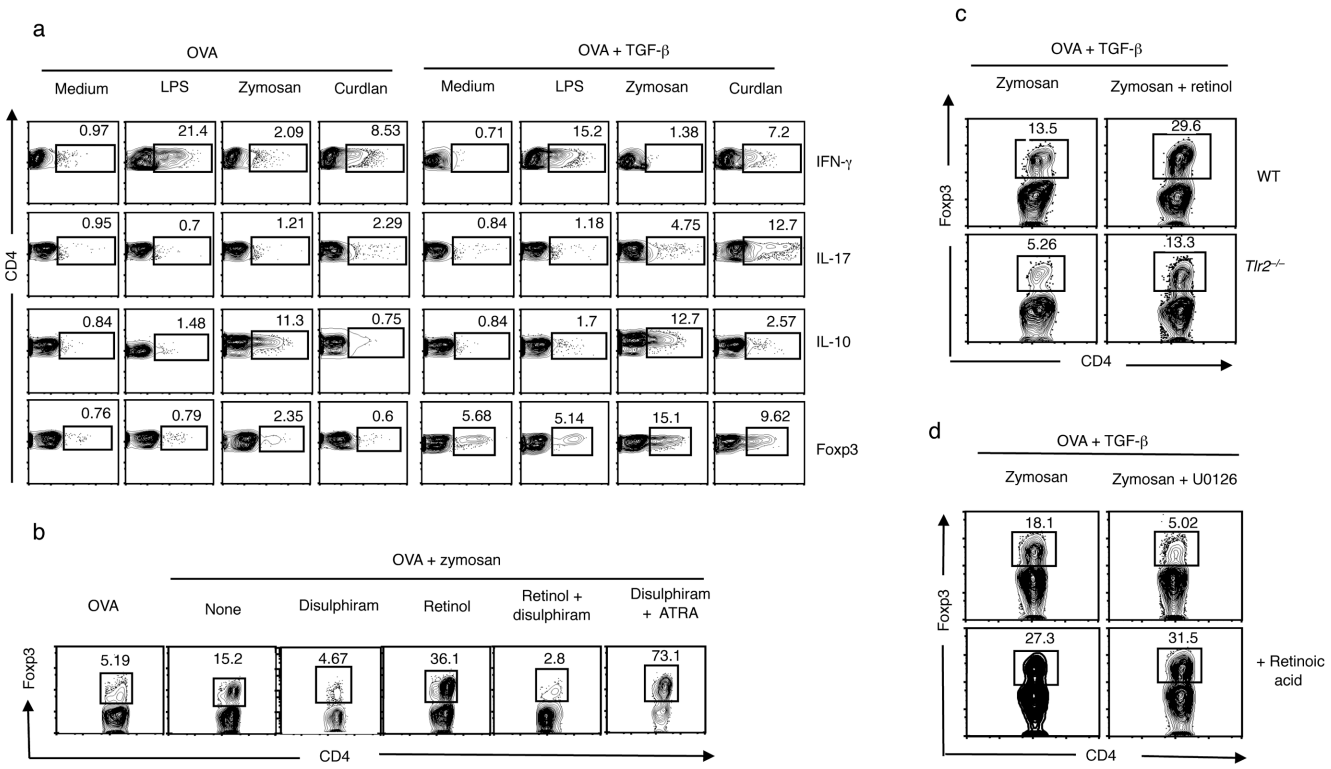

Figure 2.

RA and IL-10 synergize to induce Foxp3$^+$ Treg cells. (**a**) Zymosan stimulates splenic DCs to induce Treg cells. Splenic DCs were stimulated with zymosan or curdlan or LPS for 12 h, and washed and cultured with naïve (CD4$^+$CD62L$^+$) OT-II T cells with $OVA_{323–339}$ peptide (OVA) in the presence or absence of TGF-β. After 4 d, OT-II cells were restimulated for 6 h with plated bound antibodies to CD3 and CD28. Foxp3 expression and, intracellular production of IL-17, IFN-γ and IL-10 by CD4$^+$ T cell were assessed by intracellular staining and flow cytometry. Data are from one representative experiment of three. (**b**) Induction of Treg cells by zymosan stimulated splenic DCs is dependent on RA. Splenic DCs were stimulated with zymosan in the presence of disulphiram or vehicle for 10 h, and washed and cultured with naïve OT-II T cells with OVA and TGF-β in the presence or absence of retinol. After 4 d, Foxp3 expression by CD4$^+$ T cell was assessed by intracellular staining and flow cytometry. Data are representative of one experiment of three. (**c**) Induction of Treg cells by zymosan stimulated splenic DCs, is dependent on TLR2. Splenic DCs from wild type or *Tlr2*$^{-/-}$ mice were stimulated with zymosan for 10h, and washed and cultured with naïve OT-II T cells with OVA and TGF-β in the presence or absence of retinol. After 4 d, Foxp3 expression by CD4$^+$ T cell was assessed by intracellular staining and flow cytometry. Data are representative of one experiment of three. (**d**) Effect of blocking ERK activation in zymosan stimulated DCs on Treg induction. Data are representative of one experiment of three.

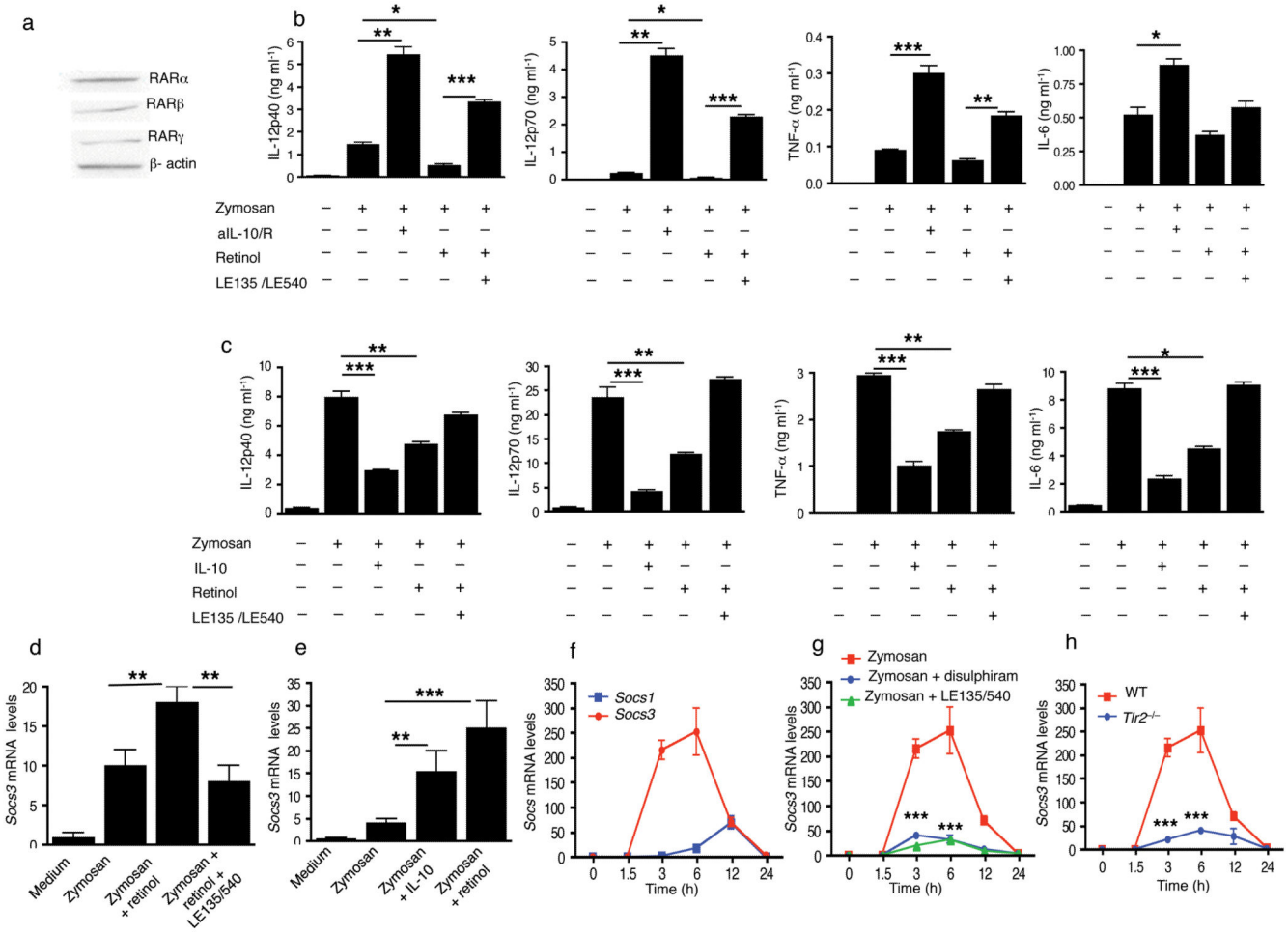

Figure 3.

RA and IL-10 exert autocrine effects on DCs to induce *Socs3* which regulates activation of p38 MAPK and pro-inflammatory cytokines. (**a**) Expression of RA nuclear receptors (RARs and RXRs) in splenic DCs by western blot. (**b, c**) Cytokines secreted in supernatants obtained after culture of splenic DCs from wild-type mice (**b**), and *Il-10*$^{-/-}$ mice (**c**), with zymosan for 24 h, in the presence or absence of antibodies against IL-10 receptor (aIL-10R) IL-10R, retinol or retinol plus LE135/ LE540, or IL-10. Representative of four experiments. (**d, e**) RA dependent induction of *Socs3* mRNA expression in splenic DCs from wild type (**d**) or *IL-10*$^{-/-}$ mice (**e**) stimulated with zymosan. (**f, g**) RA-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 expressions of *Socs1* and *Socs3* mRNA were analyzed by quantitative RT-PCR. (**h**) Induction of *Socs3* in splenic DCs *in vivo* is dependent on TLR2. Wild type or *Tlr2*$^{-/-}$ mice were injected with zymosan, splenic DCs isolated and mRNA expression of *Socs3* evaluated by RT-PCR. Representative of three experiments. **P* < 0.01; ***P* < 0.001; ****P* < 0.0001 in all figures.

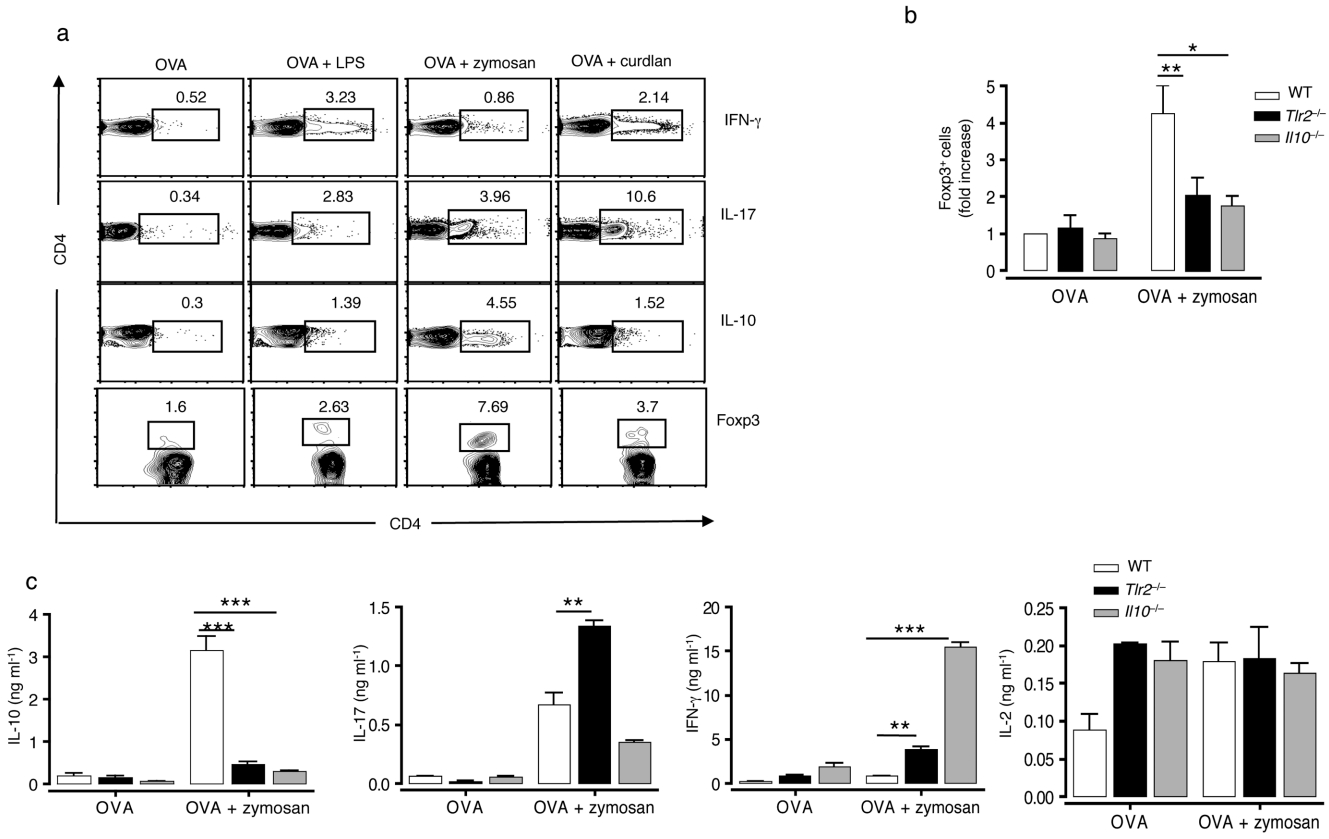

Figure 4.

Induction of antigen specific IL-10$^+$ Tr1 and Treg cells *in vivo*. (**a**) B6.PL (Thy1.1$^+$) mice reconstituted with OT-II TCR transgenic T cells were injected i.v. with class II–restricted $OVA_{323–339}$ peptide (OVA) alone, or OVA plus LPS, OVA plus zymosan, or OVA plus curdlan. Four days after challenge, the splenocytes were isolated and expression of Foxp3, IL-17, IFN-γ and IL-10 by CD4$^+$ T Thy1.2$^+$ cell was assessed by intracellular staining and flow cytometry. Data are from one experiment representative of two. (**b**) C57BL/6 or *Tlr2*$^{-/-}$ or *Il-10*$^{-/-}$ mouse were reconstituted with OT-II TCR transgenic T cells, and on the following day, injected with OVA or OVA plus zymosan. Five days later, splenocytes were isolated and induction of OVA specific Foxp3$^+$ T cells was assessed by intracellular staining and flow cytometry. Means + s. d. of 3 or 4 mice per group. (**c**) Splenocytes from immunized mice described in (**b**) were restimulated with OVA in culture for 48 h and cytokines in the supernatants were analyzed by ELISA. Means + s. d. of 3 or 4 mice per group. **P* < 0.01; ***P* < 0.001; ****P* < 0.0001 in all figures.

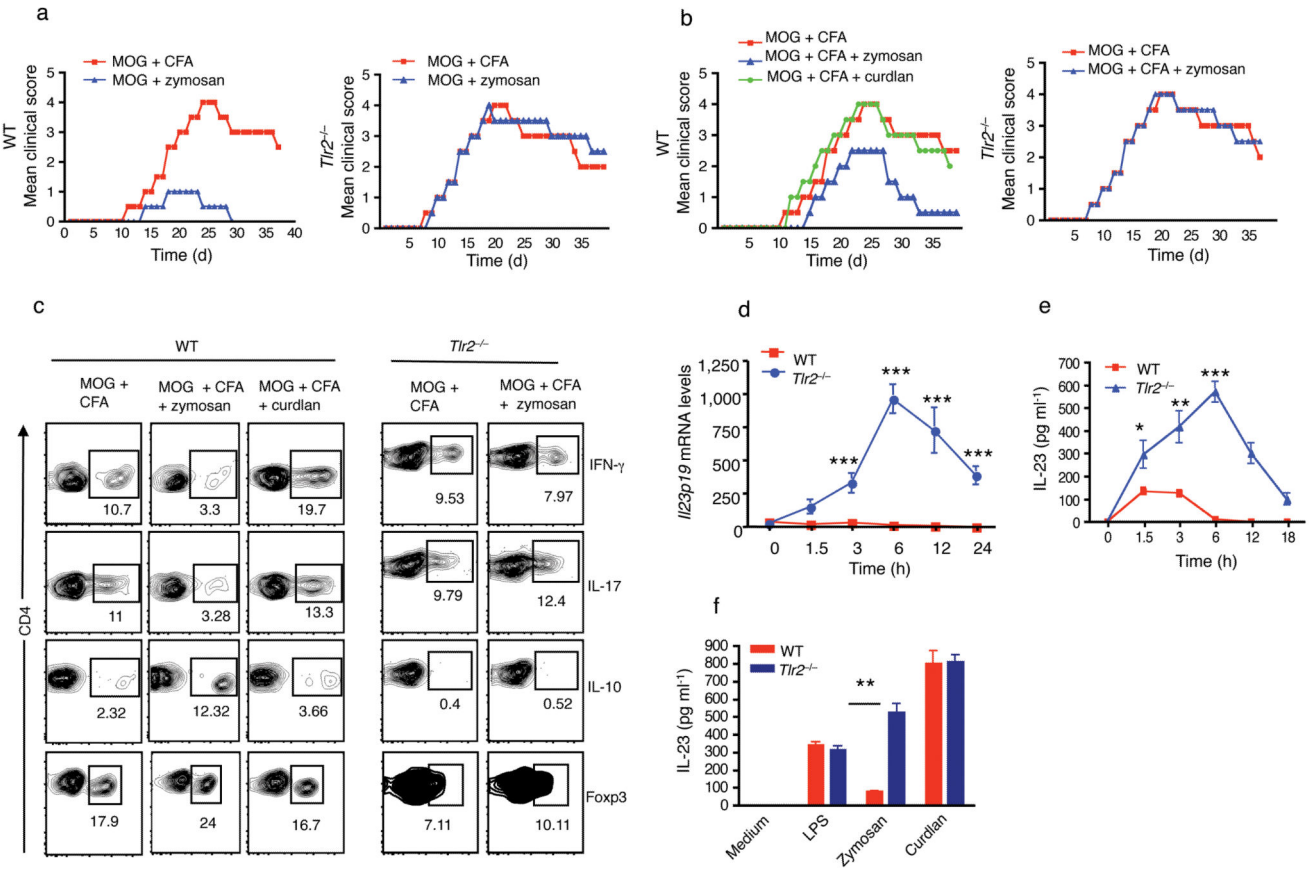

Figure 5.

Zymosan suppresses IL-23 and T_H-17 mediated EAE. (**a**) In wild type mice (left panel), immunization with MOG + zymosan resulted in substantially reduced EAE, relative to immunization with MOG + CFA. In *Tlr2*−/− mice, immunization with MOG + zymosan resulted in enhanced disease, relative to wild type mice ($P < 0.0001$). Representative experiment of two. (**b**) Wild type or *Tlr2*$^{-/-}$ mice were immunized s.c with MOG + CFA, MOG + CFA plus zymosan (i.v) or MOG + CFA plus curdlan (i. v), and monitored for disease. Injection of zymosan suppressed the severity of disease, relative to immunization with MOG + CFA alone, in wild type mice (left panel), but not in *Tlr2*$^{-/-}$ mice (right panel). Representative experiment of two. (**c**) Mononuclear cells were isolated from CNS tissue on day 18 after immunization and induction of IFN-γ, IL-17, IL-10 and Foxp3 was assessed by intracellular staining and flow cytometry, as described in Supplementary Methods, online. Representative experiment of two. (**d**) Wild type or *Tlr2*$^{-/-}$ mice were injected with zymosan, and expression of *Il23p19* mRNA in splenic DCs analyzed by quantitative RT-PCR. Representative of three experiments. (**e**) IL-23 induction in the serum of the mice described in (**d**) was assayed by ELISA. (**f**) TLR2 regulates IL-23 production in splenic DCs in response to zymosan. IL-23 secretion by splenic DCs from wild type or *Tlr2*$^{-/-}$ mice cultured *in vitro* with zymosan. *$P < 0.01$; **$P < 0.001$; ***$P < 0.0001$.

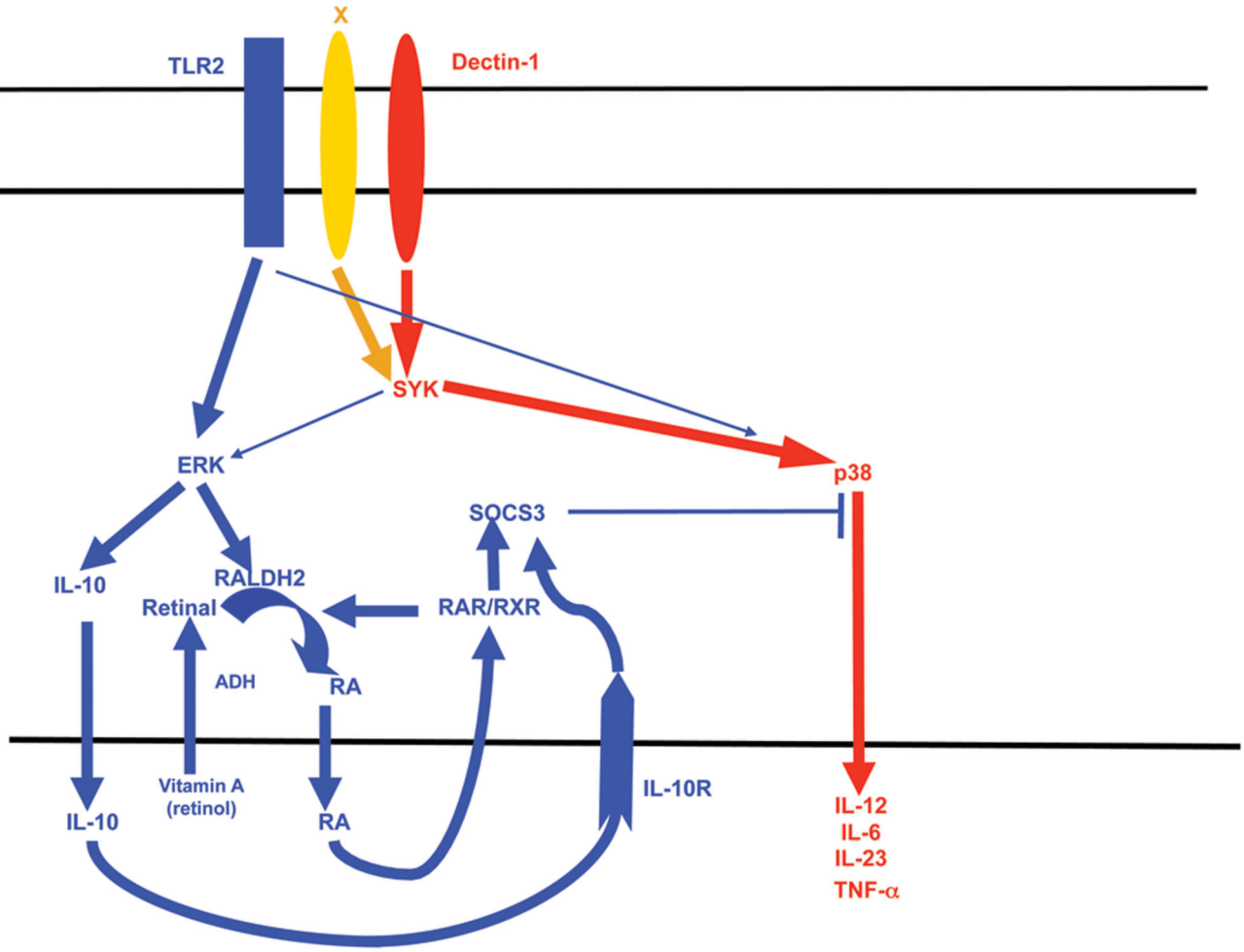

Figure 6.

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 play a major role in Raldh2 induction (thin arrow), although signaling via syk is critical, suggesting the involvement of an additional syk-dependent receptor (X). Thus, the combinatorial activation of TLR2 dependent ERK and syk, likely orchestrates induction of Raldh2. This results in the conversion of retinal to RA, which then exerts an autocrine effect on DCs via RAR/RXR to induce SOSC3, which suppresses activation of p38 MAPK and pro-inflammatory cytokines. In contrast, dectin-1 promotes induction of pro-inflammatory cytokines.