Yeast zymosan, a stimulus for TLR2 and C-type lectin, induces regulatory antigen-presenting cells and immunological tolerance

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Emerging evidence suggests critical roles for APCs in suppressing immune responses. Here, we show that zymosan, a stimulus for TLR2 and C-type lectin, regulates cytokine secretion in DCs and macrophages to induce immunological tolerance. First, zymosan induces DCs to secrete abundant IL-10 but little IL-6 and IL-12(p70). Induction of IL-10 is dependent on TLR2- and C-type lectin-mediated activation of ERK MAPK via a mechanism independent of the activation protein 1 (AP-1) transcription factor c-Fos. Such DCs stimulate antigen-specific CD4+ T cells poorly due to IL-10 and the lack of IL-6. Second, zymosan induces F4-80+ macrophages in the splenic red pulp to secrete TGF-β1. Consistent with these effects on APCs, injection of zymosan plus OVA into mice results in OVA-specific T cells that secrete little or no Th1 or Th2 cytokines, but secrete robust levels of IL-10, and are unresponsive to challenge with OVA plus adjuvant. Finally, coinjection of zymosan with OVA plus LPS suppresses the response to OVA via a mechanism dependent on IL-10, TGF-β1, and lack of IL-6. Together, our data demonstrate that zymosan stimulates IL-10+ IL-12(p70) IL-6low regulatory DCs and TGF-β1 macrophages to induce immunological tolerance. These data suggest several targets for pharmacological modulation of immune responses in various clinical settings.

Introduction
Sensing a pathogen is the first, critical step in launching an immune defense. The immune system has evolved an elaborate system of pathogen surveillance, the so-called pathogen-recognition receptors (PRRs), which can recognize highly conserved molecular signatures contained in microbes and then decode this information to elicit an immune response (1–3). The prototypic examples of PRRs are the TLRs (4, 5) and the C-type lectins (6–8), which are expressed on and in DCs. Although DCs were initially thought to be important in initiating immune responses, more recent evidence points to a central role for them in tuning the quality of the immune response as well as in suppressing immune responses (1, 2). For example, there is now much evidence that triggering different TLRs on DCs results in distinct programs of gene expression and cytokine secretion that differentially regulate the type of adaptive immune responses (2, 4, 8–14). Furthermore, emerging evidence suggests that signaling through DC-SIGN, a C-type lectin, results in impaired DC maturation and antiinflammatory responses (15). Thus, determining the immunological consequences of triggering distinct PRRs on DCs might offer novel strategies for therapeutic immune intervention in various clinical settings. With this perspective, we embarked on a systematic screening of several PRR ligands for their effects on DCs and the adaptive immune response. In the present report, we describe the unique effects of zymosan, a yeast cell wall derivative which is recognized by C-type lectin-1, a C-type lectin receptor for β-glucans (16–18) expressed in murine (18) and human (19) DCs in conjunction with TLR2 (20). Our data suggest that zymosan induces regulatory DCs, which secrete abundant IL-10 but little or no IL-6 and IL-12(p70) and induce impaired T cell responses. Such regulatory DCs appear to be induced via activation of TLR2- and C-type lectin–dependent activation of ERK MAPK, which promotes IL-10 production. Furthermore, zymosan induces splenic F4-80+ macrophages to secrete TGF-β, another immunosuppressive cytokine. Consistent with this, injection of antigen plus zymosan into mice results in a transient but weak activation of antigen-specific T cells, which are resistant to further stimulation. Further, coinjection of zymosan with LPS plus antigen results in reduced antigen-specific T cell proliferation compared with LPS plus antigen injection alone. Thus zymosan appears to program DCs and macrophages to suppress immune responses via multiple mechanisms.

Results
Zymosan induces DCs to secrete robust IL-10 but little or no IL-12(p70) and IL-6. We first determined the responses of human and murine DCs to zymosan. Human monocyte-derived DCs cultured for 24 hours with either zymosan or LPS (a TLR4 ligand) were observed to upregulate costimulatory molecules CD80 and CD86 as well as the maturation marker CD83 (Figure 1A). There are presently con-
Flicting reports regarding the cytokine profiles induced by zymosan. Although previous work suggests that zymosan stimulates DCs to produce proinflammatory cytokines, including IL-12(p40) (20), more recent studies have shown that zymosan also stimulated DCs to produce robust IL-10 (21, 22). Therefore, it was important to determine the induction of these cytokines in our system. In human monocyte-derived DCs, while LPS induced abundant levels of IL-12(p70), IL-6, and IL-10, zymosan induced barely detectable levels of IL-12(p70) and lower levels of IL-6 but abundant levels of IL-10 (Figure 1B). This was consistent with data from the murine system, in which splenic CD11c+CD11b−CD8α− and CD11c+CD11b−CD8α+ DC subsets were isolated by flow cytometry and cultured in vitro with E. coli LPS or zymosan for 24–48 hours in the presence of a CD40-L-expressing cell line. Cytokines were analyzed by ELISA. Data are representative of 10 experiments. Data are representative of 18 experiments. (C) Murine, splenic CD11c+CD11b−CD8α−, and CD11c+CD11b−CD8α+ DC subsets were isolated by flow cytometry and cultured in vitro with E. coli LPS or zymosan for 24–48 hours in the presence of a CD40-L-expressing cell line. Cytokines were analyzed by ELISA. Data are representative of 10 experiments. (D) Immature, monocyte-derived DCs were cultured for 24–48 hours with laminarin (200 μg/ml) plus zymosan or with zymosan alone. IL-10 levels were measured after 24 hours by ELISA. Data are representative of 4 experiments. (E) CD11c+ murine splenic DCs were cultured with laminarin (200 μg/ml) plus zymosan and CD40-L–expressing fibroblasts. IL-10 levels were measured after 24 hours by ELISA. Data are representative of 7 experiments.

Figure 1
Zymosan induces IL-10 in human and murine DCs via a mechanism involving dectin-1. (A) Immature, monocyte-derived DCs were cultured for 24–48 hours with E. coli LPS (1 μg/ml) or zymosan (50 μg/ml), and flow cytometric analyses of the expression of the costimulatory molecules CD80 and CD86 and the maturation marker CD83 were performed. Filled histograms show isotype; open histograms represent marker. Data are representative of 10 experiments. (B) Secretion of cytokines in the culture supernatants was measured by ELISA. Data are representative of 18 experiments. (C) Murine, splenic CD11c+CD11b−CD8α−, and CD11c+CD11b−CD8α+ DC subsets were isolated by flow cytometry and cultured in vitro with E. coli LPS or zymosan for 24–48 hours in the presence of a CD40-L-expressing cell line. Cytokines were analyzed by ELISA. Data are representative of 10 experiments. (D) Immature, monocyte-derived DCs were cultured for 24–48 hours with laminarin (200 μg/ml) plus zymosan or with zymosan alone. IL-10 levels were measured after 24 hours by ELISA. Data are representative of 4 experiments. (E) CD11c+ murine splenic DCs were cultured with laminarin (200 μg/ml) plus zymosan and CD40-L–expressing fibroblasts. IL-10 levels were measured after 24 hours by ELISA. Data are representative of 7 experiments.

We then investigated the receptors through which these 2 microbial stimuli mediate their distinct effects. As observed previously, all the effects of LPS were mediated through TLR4 (data not shown). In the case of zymosan, 2 recognition receptors have been described. Dectin-1, a C-type lectin (16–19), is expressed on murine splenic DCs (18) and on human DCs (19) as well as on macrophages and neutrophils and mediates attachment and ingestion of zymosan. Dectin-1 recognition of zymosan appears...
to be dependent on β-glucans, which are a major component of the yeast cell wall, because laminarin (a soluble β-glucan from the brown seaweed Laminaria digitata) can block this recognition (17, 18). Consistent with this, zymosan-induced IL-10 production by human monocyte-derived DCs was significantly blocked by laminarin (Figure 1D). In mice, there was a significant diminution of IL-10 production when laminarin was used to block zymosan–dectin-1 interactions in DCs from wild-type mice (Figure 1E). In addition to dectin-1, TLR2 is also known to recognize zymosan (20, 24). DCs from TLR2-deficient mice produced lower levels of IL-10 in response to zymosan, and a further decrease in IL-10 production was observed when laminarin was used to block signaling via dectin-1 on TLR2-deficient DCs (Figure 1E). Taken together, these data suggest an important role for both dectin-1 and TLR2 in the induction of IL-10 in DCs.

Zymosan induction of IL-10 in DCs is dependent on TLR2- and dectin-1-mediated activation of ERK MAPK. We next sought to gain some insights into the intracellular cell-signaling networks within DCs that might mediate zymosan-induced IL-10 production. Previous work suggested that ERK MAPK is a potent mediator of TLR2-mediated IL-10 production by DCs (13, 14). Human and murine DCs cultured with zymosan expressed enhanced and sustained levels of ERK1 and 2 phosphorylation relative to DCs cultured with LPS (Figure 2, A and B). Our previous work suggests that Pam-3-cys, a synthetic TLR2 ligand, induces enhanced and sustained levels of ERK phosphorylation relative to TLR4 or TLR5 ligands (13, 14). The involvement of TLR2 in the induction of ERK phosphorylation in response to zymosan was demonstrated by lower levels being observed in TLR2-deficient DCs (Figure 2C). However, the induction of ERK phosphorylation by zymosan also appeared to be, at least in part, mediated by dectin-1, since laminarin could partially block this in wild-type DCs (Figure 2C). Crucial roles for the involvement of both dectin-1 and TLR2 in ERK phosphorylation were demonstrated by blocking dectin-1 receptors in TLR2-deficient DCs (Figure 2C).

We determined the role of ERK1 and 2 in IL-10 induction, using synthetic inhibitors of ERK activation as well as ERK1-deficient mice. In the presence of the synthetic inhibitor U0126, which inhibits MEK1 and 2, the upstream activators of ERK1 and 2, there was a profound impairment of IL-10 secretion by human DCs in response to zymosan (Figure 2D). Consistent with this, DCs from ERK1-deficient mice were significantly impaired in their ability to produce IL-10, and in the presence of U0126, there was a more profound reduction (Figure 2E). Therefore, zymosan appears to mediate IL-10 production in human and murine DCs via a mechanism involving ERK MAPK activation, and this is mediated through both TLR2 and dectin-1.
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Zymosan induction of IL-10 in DCs is not dependent on c-Fos. We next addressed the question of what downstream transcription factors might mediate the induction of IL-10 in DCs by zymosan. Our previous work suggests that the synthetic TLR2 ligand Pam-3-cys, which induces enhanced and sustained ERK activation, results in phosphorylation and stabilization of the activation protein 1 (AP-1) transcription factor c-Fos, which plays a critical role in regulating IL-12(p70) and IL-10 (13, 14). Therefore, we determined whether c-Fos also plays a role in regulating IL-10 induction by zymosan (Figure 3). Thus, DCs from wild-type or c-Fos–deficient mice were cultured with zymosan or Pam-3-cys (100 μg/ml) and CD40 ligand cells. IL-10 levels were assayed by ELISA. Data are representative of 3 experiments.

Zymosan induction of IL-10 in DCs is not dependent on c-Fos. CD11c+ murine splenic DCs from wild-type or c-Fos–deficient mice were cultured for 24 hours with zymosan or Pam-3-cys (100 μg/ml) and CD40 ligand cells. IL-10 levels were assayed by ELISA. Data are representative of 3 experiments.

IL-10 regulates the induction of TNF-α, IL-12, and IL-6 in DCs. IL-10 is known to be an antiinflammatory cytokine that inhibits the production of multiple, diverse inflammatory mediators from activated macrophages and DCs (25, 26). We thus determined whether the impairment of IL-6, IL-12, and other proinflammatory cytokines might be mediated by IL-10. DCs from IL-10–deficient mice were cultured in vitro with zymosan in the presence of a CD40L–expressing fibroblast. As shown in Figure 4, there was a profound enhancement of IL-12(p40), IL-12(p70), IL-6, and TNF-α in response to zymosan in IL-10−/− DCs relative to wild-type DCs. Thus, IL-10 plays a critical regulatory role in limiting proinflammatory cytokines in DCs.

Zymosan-treated DCs induce impaired activation of antigen-specific T cells in vitro via a mechanism dependent on IL-10 and lack of IL-6. Given the unique cytokine profile (high IL-10, low proinflammatory cytokines) produced by DCs in response to zymosan, it was important to evaluate the type of T cell response that zymosan-treated DCs induced. We thus determined the effects of zymosan-stimulated DCs on T cell priming and differentiation. CD11c+ splenic DCs from Flt3 ligand–injected mice were pulsed with either I-Aα–restricted OVA-peptide, OVA peptide plus LPS, or OVA peptide plus zymosan and then cultured with OVA-specific, MHC class II–restricted (I-Aα) transgenic αβ TCR T cells from OT-II mice (14). These OVA-specific T cells recognize the amino acid 323–339 peptide fragment (hereafter denoted as OVA323–339) from OVA. DCs pulsed with OVA, OVA plus zymosan, or OVA plus LPS were cultured with FACS-sorted, naive CD4+ T cells from OT-II mice for 2 days or 5 days, and T-cell proliferation was measured. As shown in Figure 5A, 5 days after primary stimulation, DCs pulsed with OVA plus LPS induced robust proliferation of OVA-specific T cells; in contrast, T cells stimulated with DCs pulsed with OVA alone or OVA plus zymosan induced weak T-cell proliferation. We then determined the consequences of restimulation of such T cells with anti-CD3 and anti-CD28. As shown in Figure 5B, T cells stimulated with DCs pulsed with OVA plus LPS could be efficiently restimulated; in contrast, T cells stimulated with DCs pulsed with OVA alone or OVA plus zymosan could not be efficiently restimulated. In fact, as observed
in the primary response (Figure 5A), T cells stimulated with zymosan-pulsed DCs proliferated even less than those stimulated with DCs pulsed with OVA alone (Figure 5B).

We then determined whether the impaired responsiveness of T cells stimulated with zymosan-pulsed DCs was caused by robust induction of IL-10 or by the lack of high levels of proinflammatory cytokines from such DCs (Figure 5C). Neutralizing the effects of IL-10 in the primary cultures with an antibody against the IL-10 receptor appeared to have little or no effect on the weak T cell proliferation induced by DCs pulsed with OVA plus zymosan. However, the addition of recombinant IL-6 induced high levels of proliferation similar to those induced by LPS. Interestingly, the addition of recombinant IL-6 in conjunction with anti–IL-10 receptor antibody appeared to act in a synergistic manner to enhance T cell proliferation. Thus, zymosan-pulsed DCs induce T cells that appear to be unresponsive to rechallenge with antigen in vitro.

Zymosan induces TGF-β–producing macrophages but few proinflammatory cytokines in vivo. We wished to determine whether zymosan could also induce T cell unresponsiveness in vivo. First, we determined whether i.v. or i.p. injections of zymosan or LPS resulted in upregulation of the activation markers CD80, CD86, CD40, and class II MHC. Consistent with our previous data (14), CD80, CD86, and CD40 on DCs in the spleens expressed greatly enhanced levels of CD80, CD86, CD40, and I-Aα within 4 hours of LPS injection, and these levels remained high up to 10 hours (Figure 6A). In contrast, zymosan was much less effective at inducing these molecules on either DC subset (Figure 6A). We also determined the kind of cytokines induced in the serum by these stimuli. LPS induced abundant levels of IL-12(p40), IL-12(p70), IL-10, IL-6, and TNF-α (Figure 6B). In contrast, zymosan induced little or no IL-12(p40), IL-12(p70), IL-6, or TNF-α (Figure 6B), consistent with its effects on DCs in vitro (Figure 1). However, it did induce significant levels of IL-10, albeit at lower levels than LPS, as well as robust levels of TGF-β, another potent antiinflammatory cytokine (27) (Figure 7A). It should be noted that the sera of untreated mice contain significant levels of TGF-β (27), but there was a very pronounced and significant induction of TGF-β by zymosan above this baseline level. The TGF-β present in the serum was biologically active as demonstrated by the phosphorylation of Smad-2 (Figure 7B). The question of exactly what cell types were induced to produce TGF-β by zymosan was addressed by 2 different approaches. First, spleen cells were isolated at 1, 4, or 10 hours from mice injected with zymosan and costained with TGF-β–TRITC and a number of other markers, including CD11b–FITC or F4-80–FITC. As indicated in Figure 7C, the majority of TGF-β–TRITC also stained positively for F4-80–FITC, suggesting that such cells were macrophages. Secondly, we performed confocal analysis of splenic sections from mice that had been injected with zymosan. These experiments also revealed that the principle producers of TGF-β were not DCs but rather a subset of F4-80+CD11b+ macrophages in the marginal zones and the red pulp of the spleens (Figure 7D).

Injection of zymosan plus antigen into mice results in antigen-specific T cell tolerance. Given the potent induction of the 2 antiinflammatory cytokines IL-10 and TGF-β and the weak induction of proinflammatory cytokines in vivo, we determined the type of adaptive immunity induced by zymosan in vivo. We addressed this question using OVA-specific, MHC class II–restricted (I-Aα), αβ TCR transgenic mice (OT-II mice) (14). In these mice, the CD4+ OVA-specific
T cells express Vα2 and Vβ5 and, as detailed above, recognize the amino acid 323–339 peptide fragment from OVA. TCR transgenic T cells were adoptively transferred into Thy-1 congenic B6.PL.Thy-1a (B6.PL) mice such that they constituted a small but detectable proportion of all T cells (14). In this system, the fate of OVA-specific, transgenic T cells was followed using the Thy-1.2 antibody, which stains the transferred cells but not the host cells. Cells with the phenotype Thy-1.2+CD4+Vα2+Vβ5+ are considered OVA-specific CD4+ T cells. In some of the experiments, we simply used Thy-1.2 in combination with CD4 to detect the OVA-specific T cells.

The reconstituted mice were injected with 50 μg of OVA323–339 peptide alone, OVA323–339 plus LPS, or OVA323–339 plus zymosan i.v. Injection of OVA323–339 alone did not induce any significant clonal expansion of the CD4+Thy-1.2 cells in the spleens of mice although these levels were consistently above those detected in reconstituted mice that had been left untreated, indicating that a limited amount of proliferation of the transferred T cells has taken place in response to OVA in the absence of any adjuvant (Figure 8, A and B). However, LPS significantly enhanced the clonal expansion of the CD4+Thy-1.2 cells; in contrast, zymosan induced a very weak response although this was still above the response to OVA peptide alone (Figure 8, A and B). There appeared to be no differences in the frequencies of CD4+‘Thy-1.2’CD25+ versus CD25- T cells between the various groups (data not shown).

Previous work has shown that productive T cell immunity is elicited only when the antigen is injected with an adjuvant and that injections of soluble antigens only result in a transient and abortive clonal expansion in which antigen-specific T cells cannot be efficiently restimulated in vitro with protein or peptide (14, 28). We thus examined the in vitro proliferative capacity of OVA-specific T cells from the various cohorts of mice by culturing single-cell suspensions of the spleen with varying concentrations of OVA. As shown in Figure 8C, mice that received OVA323–339 plus LPS had greatly enhanced responses compared with those that received OVA323–339 peptide alone; in contrast, mice injected with zymosan had responses that were scarcely different from those observed in mice injected with OVA alone (Figure 8C).

Cytokine production by antigen-specific T cells was measured by assaying the culture supernatants from the cultures described above for IFN-γ, IL-4, IL-5, and IL-10. There were significant differences between mice injected with OVA323–339 peptide alone, OVA323–339 plus LPS, or OVA323–339 plus zymosan (Figure 8E). In cultures from mice injected with OVA323–339 peptide alone, there was little, if any, response, whereas the combination of OVA323–339 peptide and zymosan induced a strong response. In contrast, the combination of OVA323–339 peptide and LPS induced a much weaker response than OVA323–339 peptide alone.

Figure 6
Zymosan is not a potent inducer of costimulatory molecules on splenic DCs or proinflammatory cytokines in vivo. To investigate the effect of zymosan in vivo, C57BL/6 mice were injected with PBS containing either 10 μg E. coli LPS or 25 μg or 100 μg zymosan. (A) Either 4 or 10 hours later, spleens were removed and a small portion digested with collagenase type 4 (1 mg/ml; Worthington Biochemical Corp.) in complete DMEM plus 2% FBS for 30 minutes at 37°C. The red blood cells were lysed and the cell suspension washed twice prior to analysis of cell surface–expressed activation markers by flow cytometry. (B) C57BL/6 mice were injected with PBS containing either 10 μg E. coli LPS or 25 μg zymosan. Blood samples were removed at 1, 4, and 10 hours and serum cytokines analyzed by ELISA.
IFN-γ, IL-4, IL-5, or IL-10. In contrast and consistent with previous reports (14, 28), in cultures from mice injected with OVA323-339 plus LPS, there were high levels of IFN-γ and low levels of IL-4 and IL-5. In striking contrast to this response, in cultures from mice injected with OVA323-339 plus zymosan, there were much lower levels of IFN-γ, IL-4, and IL-5 but high levels of IL-10 (Figure 8E). The fact that IL-10 is only produced in culture when OVA is present suggests that IL-10 induction is dependent on T cells. Therefore, unlike LPS, zymosan induces an antigen-specific T cell response characterized by very weak clonal expansion and in vitro proliferation as well as minimal production of Th1 and Th2 cytokines but high levels of IL-10. These characteristics appeared to be indicative of regulatory or tolerogenic responses. We tested this hypothesis further by determining whether injections of OVA323-339 plus zymosan would confer unresponsive-ness to a subsequent challenge with OVA323-339 plus IFA. Mice that previously received OVA323-339 plus LPS or no stimulus were competent at responding to OVA323-339 plus IFA. In contrast, mice that previously received OVA323-339 plus zymosan or OVA323-339 were unresponsive to OVA323-339 plus IFA, as evidenced by the negligible clonal expansion and IFN-γ and IL-2 secreted in culture (Figure 8G). Interestingly, there was some IL-10 production by the T cells in the zymosan group (Figure 8G). IL-4, IL-5, and IL-13 could not be detected in any of these cultures (data not shown). Therefore, these data provide evidence that injections of antigen plus zymosan into mice result in a T-helper response that is characterized by very weak clonal expansion and in vitro restimulation and negligible levels of Th1 or Th2 cytokines but high levels of IL-10. Furthermore, such T cells appear to be unresponsive to a subsequent rechallenge with the same antigen plus a gold-standard adjuvant, such as IFA. Further evidence for zymosan inducing unresponsive T cells was observed when LPS and zymosan were coinjected with OVA323-339. As Figure 8H shows, coinjection of LPS and zymosan resulted in T cells that underwent a reduced number of cell divisions compared with LPS-stimulated T cells (Figure 8H) and had reduced proliferation (Figure 8I). Taken together, these data suggest that an injection of antigen plus zymosan induces a state of immunological unresponsiveness in antigen-specific T cells.
Figure 8
Zymosan induces antigen-specific T cell tolerance in vivo and suppresses the OVA-specific response when coinjected with OVA plus LPS. (A) B6.PL mice reconstituted with OT-II TCR transgenic T cells were injected i.p. with class II–restricted OVA peptide, OVA\textsubscript{323–339} (50 μg) plus LPS (25 μg), OVA\textsubscript{323–339} (50 μg) plus zymosan (various doses), or OVA\textsubscript{323–339} alone (50 μg). Four days later, spleens were removed and clonal expansion of OVA\textsubscript{323–339}–specific T cells (A) and numbers of OVA\textsubscript{323–329}–specific CD4\textsuperscript{+} T cells per spleen (B) were determined by flow cytometry. (C–E) Spleens were isolated 4 days after immunization, and unfractionated spleen cells labeled with CFSE and restimulated in vitro with OVA\textsubscript{323–339} for 72 hours. Proliferation as measured by thymidine uptake (C) or CFSE labeling (D) and cytokine production by ELISA (E) were determined. Zymosan induced T cells that produced only high levels of IL-10 (box). Data are representative of 5 independent experiments. (F and G) Mice immunized as above with OVA\textsubscript{323–339} plus LPS or OVA\textsubscript{323–339} plus zymosan were rechallenged 10 days later with OVA\textsubscript{323–339} in IFA. Data are representative of 4 independent experiments. (H and I) B6.PL mice reconstituted with OT-II TCR transgenic T cells were injected with OVA\textsubscript{323–339} (50 μg) plus LPS (25 μg), OVA\textsubscript{323–339} (50 μg) plus zymosan (100 μg), OVA\textsubscript{323–339} and LPS (25 μg) plus zymosan (100 μg), or OVA\textsubscript{323–339} and LPS (25 μg) plus zymosan (100 μg). Four days later, spleens were removed and unfractionated splenocytes were restimulated with OVA\textsubscript{323–339} and (H) CFSE and (I) proliferation of OVA\textsubscript{323–339}–specific T cells determined.
Induction of immunological tolerance by zymosan is dependent on IL-10, TGF-β, and lack of IL-6. The potential importance of IL-10, TGF-β, and IL-6 in this induction of immunological unresponsiveness was evaluated in vivo studies using IL-10–knockout mice, in which TGF-β was neutralized with an anti–TGF-β antibody, IL-6 was supplemented, and the effect on OVA-specific immune responses induced by zymosan plus OVA was assessed (Figure 9). Blocking of TGF-β, addition of IL-6, and the absence of IL-10 in mice challenged with zymosan plus OVA each showed small increases in clonal expansion of OVA323–339–specific T cells (Figure 9A) and more impressive increases in the number of cell divisions by those T cells after in vitro restimulation (Figure 9B). The greatest increase, however, was observed when anti–TGF-β antibody and recombinant murine IL-6 were injected into IL-10/−/− mice challenged with zymosan plus OVA323–339. This injection regime resulted in the largest increase in clonally expanded OVA323–339–specific T cells compared with the zymosan plus OVA323–339–challenged C57BL/6 mice injected with isotype antibody (Figure 9A). Importantly, the combination of all 3 allowed T cells to undergo the same number of divisions that T cells from LPS plus OVA323–339 underwent upon restimulation in vitro (Figure 9B). Thus, the immunological unresponsiveness induced by zymosan in vivo can be abrogated by blocking TGF-β and IL-10 and by the addition of IL-6.

Discussion

In summary, these data suggest that the yeast cell wall, zymosan, previously considered to be a proinflammatory stimulus (20, 27), is a potent inducer of IL-10 and TGF-β as well as T cell unresponsiveness via multiple mechanisms. First, it induces regulatory-type DCs that produce few proinflammatory cytokines but abundant IL-10 and induce tolerogenic T cell responses. With regard to the mechanism of induction of IL-10, our data suggest a critical role for ERK activation after signaling through both TLR2 and dectin-1 via a mechanism that is independent of c-Fos. Second, zymosan induces abundant levels of biologically active TGF-β from splenic F4-80+ macrophages in the red pulp. Thus, the dual antiinflammatory effects of IL-10 and TGF-β in concert with diminished proinflammatory cytokines likely results in the induction of impaired T cell responses and tolerance. These antiinflammatory effects appear to be in contrast to numerous previous reports of the ability of zymosan to induce proinflammatory cytokines such as TNF-α from macrophage cell lines in vitro (20, 27, 29, 30) and to augment inflammatory responses in vivo (30–32). However, in contrast to these studies and in agreement with our study is recent work that suggests that zymosan is also a potent inducer of IL-10 from DCs (22, 33).
The question of which signaling pathway and transcription factors in DCs mediate IL-10 production is of great interest (Figure 10). Our recent work suggests that stimulation of human (13) or murine (14) DCs with the synthetic TLR2 ligand Pam-3-cys results in robust production of IL-10 and IL-6 but little IL-12(p70) (13, 14). Induction of IL-10 and impaired production of IL-12(p70) appear to be regulated by phosphorylation of ERK MAPK since blocking ERK signaling enhances IL-12(p70) while reducing IL-10 induction (13, 14). Furthermore, enhanced ERK induction appears to phosphorylate c-Fos, an AP-1 transcription factor, which represses IL-12 production in DCs (13, 14). However, zymosan, which signals through both TLR2 and dectin-1, appears to utilize an overlapping but distinct signaling pathway to mediate its distinctive effects. First, although zymosan is similar to Pam-3-cys in that it induces robust IL-10 and weak IL-12(p70), it differs in its failure to induce abundant IL-6. The mechanism underlying this difference is unclear, but one possibility is that IL-6 production by zymosan is regulated by some TLR2-independent signaling, perhaps via dectin-1. Consistent with this, our data suggest that laminarin results in an enhancement of IL-6 induction by zymosan-treated DCs, particularly at the lower doses of zymosan (Supplemental Figure 4). In contrast, DCs from TLR2-knockout mice produce reduced levels of IL-6. Thus, signaling via TLR2 and dectin-1 appear to exert opposing effects on IL-6 induction by zymosan at the lower doses. Second, although zymosan also induces robust ERK activation, c-Fos does not appear to be involved in regulating IL-12(p70) or IL-10 in zymosan-treated DCs since DCs from c-Fos–knockout mice do not show profound differences in these cytokines (Figure 3). Exactly what transcription factor(s) downstream of ERK might mediate IL-10 induction by zymosan is presently being investigated. Furthermore, which transcription factors might regulate the weak IL-6 and IL-12(p70) responses in zymosan-treated DCs is also being investigated. Whatever such factors might be, it is clear that IL-10 exerts a potent feedback regulatory mechanism that inhibits the production of IL-12, IL-6, and TNF-α, consistent with other studies (25, 26). Interestingly, 2 recent reports suggest that the adaptor protein Syk, which is involved in signaling via dectin-1 in macrophages (29) and DCs (33), mediates the induction of IL-10 (33). However, these reports did not describe the signaling events downstream of Syk. Our present data suggest that ERK activation occurs downstream of Syk and is consistent with similar findings in a different system (34).

Finally, it is intriguing to consider these data from an evolutionary perspective. It has been proposed that pathogens such as HIV, cytomegalovirus, hepatitis C virus, and Mycobacterium tuberculosis target another member of the C-type lectin family, DC-SIGN, to impair DC maturation and adaptive immunity (15, 16). One common characteristic of these pathogens is that they can cause chronic infection, during which the Th1/Th2/T regulatory balance is a critical determinant of pathogen persistence in the host. Thus, it is possible that zymosan induces IL-10 and TGF-β but suppresses IL-12 and IL-6 to disarm host immunity. However, since zymosan is not considered to be pathogenic in humans, the evolutionary significance of such an immune evasion mechanism may not be obvious. One possibility is that this mechanism may be equally beneficial for the host and the microbe. Thus, the initial, transient suppression of proinflammatory cytokines might permit the microbe to establish infection in the host, unhindered by the
host’s innate immune system — indeed, the observation that fungal pneumonia can occur after TNF-α ablation therapy underscores the critical role of proinflammatory cytokines in the control of infection (35, 36). Later, during an established infection, IL-10 and TGF-β may serve to keep the inflammation in check, thus avoiding immune pathology, which could be detrimental to the host. Thus, the persistent antiinflammatory response induced by the microbe may be beneficial for both the microbe and host. Such a mechanism may well operate in various chronic infections caused by pathogens, such as HIV, HCV, and M. tuberculosis. Whatever the evolutionary significance, our results highlight several molecular targets, such as TLR2, dectin-1, and ERK, in the therapeutic modulation of innate and adaptive immunity in the context of many pathologic states, such as autoimmunity, allergy, transplantation, and infectious diseases.

Methods

Mice. C57BL/6 mice were purchased from Jackson Laboratory. Male B6.PL mice were purchased from Jackson Laboratory or bred at the Rodent Vivarium of the Yerkes National Primate Center of Emory University. IL-10−/− mice (B6.129P2-Il10tm1Cgn/J) were purchased from Jackson Laboratory. B6.129/F1/Tac (B6.129 mice were purchased from Taconic). ERK1-knockout (ERK1 −/−) (37) mice on the C57BL/6 background were kindly provided by G. Landreth (Case Western Reserve University, Cleveland, Ohio, USA) and bred at the Yerkes Rodent Vivarium. c-Fos knockout mice were kindly provided by L. McCauley (University of Michigan, Ann Arbor, Michigan, USA). OT-II TCR transgenic mice (strain 426-6) (38), generated by W. Heath (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) and F. Carbone (Monash University, Melbourne, Victoria, Australia), were obtained from J. Kapp (Emory University) and bred at the Yerkes Animal Facility. All mice were aged 6–10 weeks. All animal studies were approved by the Institutional Animal Care and Use Committee of Emory University. For adoptive transfer studies, age-matched B6.PL recipients were given 2.5 × 10^6 OT-II transgenic T cells i.v.

Reagents. Zymosan (Sigma-Aldrich) was boiled for 30 minutes, washed extensively, and stored at −70°C. Highly purified Escherichia coli (Strain 25922) was a generous gift from T. Van Dyke. Recombinant human Fli3 ligand was expressed in CHO cells and purified at the Emory Vaccine Center. Recombinant GM-CSF and IL-4 were purchased from PeproTech. The 3T3-CD40 ligand fibroblasts (39) (a kind gift of P. Hwu, National Cancer Institute, Bethesda, Maryland, USA) were grown in complete DMEM plus 10% FBS into a confluent layer of irradiated CD40 ligand fibroblasts to induce CD40 ligand triggering. MAPK inhibitor U0126 (10 μM) or laminarin (200 μg/ml) was added prior to the addition of stimuli to the CD11c−/− enriched DCs. Cultures were incubated in a humidified atmosphere of 5% CO_{2} in air at 37°C for 48 hours. The culture supernatants were collected and assayed for the presence of IL-12(p70), IL-12(p40), IL-10, IL-6, and TNF-α.

In vitro cultures of murine DCs and T cells. CD11c−/− enriched DCs or CD11c+CD11b+ and CD11c+CD11b− DC subsets were plated with either LPS or zymosan in complete DMEM plus 10% FBS onto a confluent layer of irradiated CD40 ligand fibroblasts to induce CD40 ligand triggering. MAPK inhibitor U0126 (10 μM) or laminarin (200 μg/ml) was added prior to the addition of stimuli to the CD11c−/− enriched DCs. Cultures were incubated in a humidified atmosphere of 5% CO_{2} in air at 37°C for 48 hours. The culture supernatants were collected and assayed for the presence of IL-12(p40), IL-12(p70), IL-10, IL-6, and TNF-α. For in vitro T cell assays, CD11c+ enriched DCs were cultured with naive OT-II CD4+CD62L− and OVA peptide and either LPS (1 μg/ml) or zymosan (10 μg/ml). Various concentrations of recombinant murine IL-6 (PeproTech) or anti-IL-10 receptor antibody (BD Biosciences) were added to prepare wells. Either 2 or 5 days later, the T cells were restimulated with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) for an additional 3 days. Cultures were pulsed with 1 μCi [3H]thymidine for 12 hours, and incorporation of the radiolabeled DNA was measured by β-scintillation spectroscopy. Four days after in vivo priming with OVA peptide plus ligand, rbc-depleted spleen cells were cultured in triplicate in 96-well round-bottomed plates (1 × 10^5 cells/well) in complete DMEM plus 10% FBS together with different concentrations of OVA peptide. Proliferative responses were assessed after 72 hours of culture in a humidified atmosphere of 5% CO_{2} in air at 37°C. The cultures were pulsed with 1 μCi [3H]thymidine for 12 hours, and incorporation of the radiolabeled DNA was measured by β-scintillation spectroscopy. For cytokine assays, aliquots of culture supernatants were removed after 90 hours, pooled, and assayed for the presence of IL-2, IL-4, IL-5, IL-13, IL-10, and IFN-γ by ELISA.

In vivo blocking and addition of recombinant cytokines. OT-II T cells were transferred into C57BL/6 or IL-10−/− mice as described above. On the same day, anti-TGF-β antibody (150 μg per mouse; Harlan) or 500 ng recombinant murine IL-6 (PeproTech) was injected i.p. Mice were challenged with 50 μg OVA223-239 in PBS alone or PBS containing either 25 μg E. coli LPS or 100 μg zymosan the next day. Anti-TGF-β antibody or recombinant IL-6 were injected the same day and again 2 days later.

Measurement of cytokine production. IL-2, IL-4, IL-5, IL-6, IL-12(p40), IL-12(p70), IL-10, TNF-α, and IFN-γ in cell culture supernatants or serum...
were quantitated by ELISA antibody sets from BD Biosciences, and IL-13 was measured by an ELISA kit from R&D Systems. TGF-β was measured by an ELISA kit from BioSource International.

Isolation, culture, and phenotyping of human monocyte-derived DCs. CD14+ monocytes were enriched from peripheral blood mononuclear cells and cultured for 6 days with recombinant human GM-CSF at 100 ng/ml plus recombinant human IL-4 at 20 ng/ml. At day 6, the cultures consisted uniformly of CD1a+CD14 HLA-DR+CD11c+ cells, which were negative for CD83. These immature DCs were pulsed with LPS or zymosan for 24–48 hours. Phenotype was determined by flow cytometry using a FACSCalibur (BD Biosciences). Briefly, gated CD1a+CD14+CD11c+ HLA-DR+ DCs were analyzed for the expression of CD80, CD86, or CD83. Cytokine production was measured by ELISA.

Evaluation of ERK MAPK signaling. This was done using commercially available ELISA kits (BioSource International), or flow cytometry. Briefly, on day 6, immature, human monocyte-derived DCs (2 × 10^6) were cultured for the indicated times with the stimuli. ELISA assays were performed according to manufacturer instructions. Flow cytometric evaluation of phospho-ERK in DCs was determined by stimulating day-6, human monocyte-derived DCs for 15 minutes, 1 hour, and 4 hours with LPS or zymosan and then fixing the cells in 2% paraformaldehyde (10% ultrapure EM Grade; Polysciences Inc.) for 10 minutes at 37°C. After washing, the cells were permeabilized with freshly prepared 90% ice-cold methanol for 30 minutes on ice. Then the cells were washed twice in staining buffer (3% FCS in PBS) and labeled with phospho-ERK for 30 minutes on ice, then washed in staining buffer and labeled using FITC-labeled goat-anti-rabbit Ig. Flow cytometry was done on FACSCalibur. Detection of phospho-ERK1 and 2 was detected in murine CD11c+ in a similar fashion.

Detection of biological activity of serum TGF-β. Spleen cells were incubated with serum from mice previously challenged with PBS, LPS (25 μg/ml), or zymosan (25 μg/ml or 100 μg/ml). Lysates of cells treated with 2.5 ng TGF-β1/ml in RPMI medium with 0.5% fetal bovine serum were separated on 8% tris-glycine gels (Invitrogen Corp.). Immunoblotting was performed with an anti-phospho-Smad2, rabbit polyclonal antibody (Upstate USA Inc.) followed by a 1:10,000 dilution of goat-anti-rabbit secondary (Jackson ImmunoResearch Laboratories Inc.) and visualized with Super Signal (Pierce Biotechnology).

Immunohistological analysis of cells: cytoplasm. Single-cell suspensions of spleens were prepared as described elsewhere. Approximately 10^6 splenocytes were cultured for 6 hours with brefeldin A (BD Biosciences) per the manufacturer’s instructions. After blocking with Fc block (clone 2.4G2; BD Biosciences), surface staining was done using the FITC-labeled anti-mouse goat-anti-rabbit Ig. Flow cytometry was done on FACSCalibur. Detection of phospho-ERK1 and 2 was detected in murine CD11c+ in a similar fashion.

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