The WSX-1 pathway restrains intestinal T-cell immunity

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Abstract

Mechanisms regulating intestinal T-cell accumulation during inflammation have considerable therapeutic value. In this study, LPS increased Staphylococcus aureus enterotoxin A-specific T cells in the gut through induction of IL-12 family members. Mice deficient in IL-12 (p35⁻/⁻) favored Th17 differentiation in lamina propria, whereas mice lacking both IL-12 and IL-23 (p40⁻/⁻) produced significantly fewer Th17 cells. However, serum analysis revealed that IL-27p28 was much higher and sustained following LPS injection than other IL-12 family cytokines. Strikingly, WSX-1 (IL-27Rα) deficiency resulted in log-fold increases in lamina propria Th17 cells without affecting Th1 numbers. These results may be explained by increased expression of α4β7 on WSX-1-deficient T cells after immunization. WSX-1-deficient regulatory T cells (Tregs) were also perturbed, producing more IL-17 and less IL-10 than wild-type Tregs. Thus, IL-27 blockade may provide a new pathway to improve mucosal vaccination.

Keywords: differentiation, lamina propria, LPS, superantigen

Introduction

The activation of antigen-presenting cells (APCs) through TLRs leads to their release of IL-12 family members, which substantially impact T-cell responses. IL-12 (p35/p40) preferentially stimulates IFN-γ production from T cells while IL-23 (p19/p40) stimulates IL-17A (IL-17) (1–3). Thus, genetic deficiency of p35, p19 or p40 results in decreased levels of T-cell-derived IFN-γ, IL-17 or both, respectively (1, 4). Accordingly, IL-12 and IL-23 are characterized as Th1 and Th17 cytokines, respectively.

The third member of the IL-12 family, IL-27, is a heterodimer of subunits named p28 and Epstein–Barr virus-induced gene 3 (EBI3) and signals through a receptor complex containing WSX-1/TCCR and gp130 (5, 6). IL-27 is a T1,1-type cytokine since it stimulates IFN-γ production from T cells while inhibiting IL-17 (5, 7–9). In addition, IL-27 has anti-inflammatory properties and suppresses T-cell hyperactivity following infection with Toxoplasma gondii, Mycobacterium tuberculosis and Leishmania donovani (10–12), possibly due to its ability to induce IL-10 in Th1 cells (13–15). EBI3 can also associate with p35 to form the anti-inflammatory cytokine IL-35, which is produced by regulatory T cells (Tregs) (16). Since LPS can induce all five genes within the IL-12 family (17, 18), this may explain its ability to support both Th1 and Th17 responses in vivo (19, 20). The balance of cytokines produced upon LPS stimulation, which could impact T-cell polarization, has not been carefully examined in vivo, and tissue micro-environments such as the intestine versus lymphoid tissue could be differentially regulated. Therefore, we studied the contribution of IL-12 family cytokines toward intestinal T-cell priming following peripheral immunization with LPS.

Materials and methods

Mice

C57BL/6, IL-12p35⁻/⁻ and IL-12/23p40⁻/⁻ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and Taconic Farms (Oxnard, CA, USA). Mice maintained in the animal facility at The University of Connecticut Health Center were kept under specific pathogen-free conditions and handled in accordance to National Institutes of Health federal guidelines. WSX-1⁻/⁻ mice [ref. (21); 92% C57BL/6 background] were maintained at Taconic Farms.
Immunization

Reagents were diluted in PBS and injected intraperitoneally (i.p.) in a total volume of 0.2 ml. Staphylococcal enterotoxin A (SEA; Toxin Tech, Sarasota, FL, USA) was injected at 1 μg per mouse, followed by LPS derived from *Salmonella typhimurium* (45–50 μg; Sigma–Aldrich; St Louis, MO, USA) 18 h later. LPS doses were determined from titration studies on individual batches to find the amount providing maximal T-cell survival.

Tissue processing

Spleens were crushed through nylon mesh cell strainers (Falcon/BD Biosciences, San Jose, CA, USA) and treated with ammonium chloride to lyse RBCs. Isolation of intestinal lamina propria lymphocytes was based on a previous report (22) with some modifications. The small intestine was excised from mice followed by removal of Peyer’s patches and fat. The tissue was then flushed with balanced salt solution (BSS), sliced open longitudinally, cut into ~1 cm pieces and washed with Ca/Mg-free BSS. Tissue was stirred at 37°C in Ca/Mg-free BSS containing 5 mM EDTA and 0.15 mg ml⁻¹ dithioerythritol, with the supernatant removed. Then, tissue was incubated at 37°C in BSS containing 1 mM CaCl₂, 1 mM MgCl₂, 0.3 mg ml⁻¹ collagenase (Sigma–Aldrich) and 0.1 mg ml⁻¹ DNase I (Sigma–Aldrich). Supernatants from collagenase-treated tissue were poured over cell strainers and spun down at 1400 r.p.m. The cells were then fractionated on a 44% and 67% percoll gradient (Amersham Biosciences; Piscataway, NJ, USA), with lymphocytes partitioning at the interface.

Serum cytokines

Blood was taken from tail veins of mice at 1.5, 3, 6 and 9 h following injection of 120 μg LPS. Blood was kept on ice for >30 min followed by centrifugation at 13 000 r.p.m. and 4°C for 20 min, with serum partitioning as the upper fraction. Levels of IL-27p28 and IL-23p19/p40 complex were determined with ELISA kits from R&D Systems (Minneapolis, MN, USA) and eBioscience (San Diego, CA, USA).

Cell culturing

For *in vitro* re-stimulation, 1 million cells were cultured for 5 h at 37°C in 0.2 ml complete tumor medium, consisting of MEM with fetal bovine serum (FBS), amino acids, salts and antibiotics. Cells were cultured with phorbol myristate acetate (PMA) (50 ng ml⁻¹; Calbiochem, Gibbstown, NJ, USA) plus ionomycin (1 μg ml⁻¹; Invitrogen), SEA (1 μg ml⁻¹) and brefeldin A (BFA; 5 μg ml⁻¹; Calbiochem), as indicated, and stained intracellularly for cytokines.

Cell staining and flow cytometry

The following mAbs were purchased from eBioscience: Allophycocyanin-conjugated α4β7 integrin, IL-17A and rat single positive for IFN-γ (T₃,17) or DP. (C) Total numbers of T₃,17, T₃,1 and DP cells in each tissue. Data are combined from three experiments with n = 8 and displayed as mean ± SEM. Asterisks represent significant statistical differences compared with WT mice, as determined by two-tailed Student’s *t*-tests (*P* < 0.05).
IgG2a; PE-conjugated CD62L, CD103 and Foxp3; FITC-conjugated CD44; Alexa-700-conjugated IL-10 and rat IgG2a. The following mAbs were purchased from BD Biosciences: biotinylated TCR Vβ3; Pacific Blue-conjugated CD4; PerCP-conjugated CD4, CD90.1 and streptavidin; PE-conjugated TCR Vβ3; FITC-conjugated CD4, CD8b.2, IFN-γ and rat IgG1. Allophycocyanin-conjugated CXCX3 was purchased from Biolegend (San Diego, CA, USA).

Surface and intracellular staining was performed as described previously (23). Briefly, cells were re-suspended in staining buffer consisting of BSS, 3% FBS and 0.1% sodium azide. Non-specific binding was blocked by a solution containing mouse serum, human IgG and the anti-Fc mAb 2.4G2 (24), followed by incubation with fluorescently conjugated mAbs on ice for 30 min. For intracellular staining, the Foxp3 staining buffer set from eBioscience was used. Flow cytometry was conducted on BD LSR II flow cytometers, with data analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis
Two-tailed Student’s t-tests were performed with P < 0.05 representing a significant statistical difference. The type of variance was determined by F-tests, with F > 0.05 corresponding to equal variance and F < 0.05 corresponding to unequal variance.

Results

Divergent roles for IL-12p35 and IL-12/23p40 in determining the balance of Th subsets following immunization
Staphylococcus aureus synthesizes enterotoxins that stimulate massive cytokine responses associated with a variety of inflammatory-based diseases in humans (25). SEA induces TCR Vβ3+ T cells to undergo clonal expansion followed by peripheral deletion unless LPS is co-injected (26). This LPS-rescuing effect is observed in lymph nodes, spleen, liver, lung, bone marrow and small intestinal lamina propria (LP) (19, 23, 27). Although Th1 cells predominate in most tissues, Th17 cells are found in LP and the mechanism regulating this process is unknown. We hypothesized the balance of IL-12 family members induced through SEA and LPS immunization determines T-cell polarization and their accumulation into mucosal tissues.

C57BL/6 (wild type, WT), IL-12p35−/− and IL-12/23p40−/− mice obtained from Jackson Laboratory were immunized i.p. with SEA at time 0 and LPS 18 h later. On day 7, the frequency and numbers of CD4+ Vβ3+ T cells were assessed in LP and spleen by flow cytometry. Conventional T cells were distinguished from Tregs by intracellular staining for the transcription factor Foxp3. The total number of Foxp3-negative CD4+ Vβ3+ T cells were >80% lower in the LP of p40−/− mice compared with WT, while a 40% reduction was observed in p35−/− mice (Fig. 1A, left). In spleen, T-cell numbers were modestly reduced by ~30% in the absence of either IL-12 subunit. Therefore, p40 mediated the accumulation of specific CD4 T cells in the intestine. In contrast, numbers of Foxp33+ CD4+ Vβ3+ Tregs were unaffected by IL-12 deficiency (Fig. 1A, right).

To analyze effector cytokine production, cells were re-stimulated with SEA in vitro and stained for intracellular IL-17 and IFN-γ, with Foxp3+ cells excluded from analysis. In the LP of WT mice, ~2.6% of Foxp3-negative CD4+ Vβ3+ T cells were identified as Th17 (IL-17 single positive; Fig. 1B). In the absence of p35, Th17 levels increased to 7.5%. In contrast, the Th17 percentage was significantly lower in p40−/− mice (Fig. 1B). Th1 differentiation was not affected by p40, but p35−/− mice generated fewer Th1 cells in LP (IFN-γ single positive; P = 0.056; Fig. 1B), demonstrating that p35 influences the Th17:Th1 balance following immunization. A similar trend was observed in spleen with Th17 percentages increased in p35−/− mice and decreased p40−/− mice (Fig. 1B). Total numbers of cytokine-producing cells correlated very well with percentages (Fig. 1C). Although the number of double-positive cells (DP; IL-17+ IFN-γ+) was not affected by p35, significant reductions were observed in the tissues of p40−/− mice.

LPS potently induces IL-27 in vivo
By comparing p35−/− with p40−/− mice, we could distinguish between effects caused by IL-12 versus IL-23. Based on these data, we postulated that the related cytokine IL-27 may also contribute to LPS adjuvanticity and found that i.p. injection of LPS potently increased serum levels of IL-27p28 (Fig. 2). The most significant increase was observed between 1.5 and 3 h, when p28 levels peaked at 3.1 ng ml−1. Interestingly, p28 was sustained for at least 9 h (Fig. 2). By comparison, the kinetics of IL-23 induction covered a much

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narrower time frame, with levels approaching 0.5 ng ml\(^{-1}\) at 3 h (Fig. 2). Thus, LPS was a much more potent inducer of the Th1 cytokine IL-27 than the Th17 cytokine IL-23.

The early levels of IL-27p28 were similar to that observed for IL-12p70: at time 0, background levels (146 ± 61 pg ml\(^{-1}\)) of IL-12p70 were detected in serum. At 3 h after LPS injection, statistically significant (\(P = 0.00587\)) amounts of IL-12p70 were detected (2626 ± 607 pg ml\(^{-1}\)), and this declined by 6 h although the levels were still statistically significant compared with time 0 (\(P = 0.0487\), 638 ± 176 pg ml\(^{-1}\)). Therefore, the expansion and contraction kinetics of IL-12p70 in the serum slightly preceded those of IL-27p28.

**WSX-1 inhibits Th17 conversion following LPS-based immunization**

To determine the role of IL-27 signaling in LPS adjuvanticity, WT and WSX-1\(^{-/-}\) mice were immunized with SEA and LPS, with tissues examined on day 7. In these experiments, both strains were acquired from Taconic Farms. Significantly more CD4\(^+\) V\(\beta\)3\(^+\) T cells were recovered from the LP of WSX-1\(^{-/-}\) mice (Fig. 3A). This increase was selective for the intestinal mucosa since increases were not observed in spleen.

To determine the balance of Th subsets, cells were re-stimulated in vitro with PMA plus ionomycin, SEA or nothing and then stained for intracellular cytokines. Cells from WSX-1\(^{-/-}\) mice had decreased percentages of IFN-\(\gamma\) cells and increased percentages of IL-17\(^+\) cells compared with WT (Fig. 3B). In the LP of WSX-1\(^{-/-}\) mice, over 35% of the Foxp3-negative CD4\(^+\) V\(\beta\)3\(^+\) T cells produced IL-17, versus an average of 5% in WT. When total cell numbers were calculated, WSX-1 deficiency yielded a massive 37-fold increase in Tn17 cells compared with WT (Fig. 3B). This effect was very selective since Tn1 cells numbers were not significantly impacted by WSX-1. In spleen, WSX-1 deficiency resulted in 13-fold more Tn17 cells. Importantly, LPS was required for this effect since the splenic Tn17 frequency was ≲1% in WT and WSX-1\(^{-/-}\) mice that were treated with SEA alone or left untreated (data not shown). In spleen, significantly more Foxp3\(^+\) V\(\beta\)3\(^+\) T cells also produced IL-17 in the absence of WSX-1, correlating with fewer IL-10 producers (Fig. 3D). Altogether, LPS-induced Tn17 differentiation was potently blocked by IL-27.
inhibited by WSX-1. Finally, in the steady state LP, i.e., with no immunization, WSX-1-/- mice had increased IL-17 producers (data not shown).

In addition to its effects on CD4 T-cell priming, SEA activates endogenous CD8 T cells expressing Vβ3. The total number of CD8+ Vβ3+ T cells was significantly increased in the LP of WSX-1-/- mice compared with WT (Fig. 4, left). When analyzing cytokine production, gating on the CD4-negative Vβ3+ T cells revealed increased numbers of IFN-γ producers in the absence of WSX-1. In comparison with the spleen, where nearly half of the CD8 T cells produced IFN-γ upon re-stimulation, only 4% were capable of IFN-γ production in the LP. Thus, in comparison with other tissues, the intestinal LP is less conducive for IFN-γ production from both CD4 and CD8 T cells. It is notable that neither WT nor WSX-1-/- CD8 T cells produced IL-17 following immunization (data not shown). Therefore, the inhibitory effect of WSX-1 on IL-17 production was limited to CD4+ T cells.

**Discussion**

T<sub>n</sub>17 cells contribute to immunity although uncontrolled responses are also associated with autoimmune etiologies [reviewed in ref. (29)]. We show that IL-27 receptor signaling restrains T<sub>n</sub>17 induction following LPS-based immunization (Fig. 3). This effect was observed in spleen but was most prominent in the intestine where WSX-1 deficiency resulted in a 37-fold increase in T<sub>n</sub>17 numbers. Perhaps, this is related to the higher steady-state levels of T<sub>n</sub>17 cells observed in the LP of WSX-1-/- mice [ref. (30) and data not shown].

We favor the hypothesis that WSX-1 inhibits T<sub>n</sub>17 priming in the spleen and their subsequent migration to intestinal LP. The milieu in the gut appears to be particularly suited for T<sub>n</sub>17 outgrowth and/or maintenance in the absence of WSX-1 signaling. Although intestinal microbiota may support T<sub>n</sub>17 differentiation by stimulating IL-23 production from LP-resident dendritic cells (DCs) (31), IL-27 is induced within the intestine upon infection with *Trichuris muris* or during Crohn’s disease (32–34). Our data support earlier findings that one function of IL-27 is to limit IL-17-mediated pathology (7, 8, 10–12). Although we did not study the relative contributions of WSX-1 signaling in T cells versus DCs, another study found that WSX-1-/- DCs were hyperinflammatory to LPS, producing elevated levels of IL-12/23p40, EBI3, IL-12p70, delta4 and tumor necrosis factor (35). Future experiments will be required to determine if IL-27 inhibits T<sub>n</sub>17 priming in our model through its direct action on T cells or DCs.

LPS induced high serum levels of IL-27p28, which peaked at 3 h and were sustained to 6 h (Fig. 2). The 1 ng ml<sup>−1</sup> level observed at 9 h suggests that IL-27 exerts its function over a broad time frame following its induction. In contrast, IL-23 levels were much lower (0.46 ng ml<sup>−1</sup>) and transient, nearly returning to baseline by 9 h (Fig. 2). The dominant effect of IL-27 over IL-23 could explain why LPS generates robust T<sub>n</sub>1, T<sub>n</sub>2 responses as LPS.

**IL-27 blocks T<sub>n</sub>17 polarization and gut homing**

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had decreased numbers of splenic Th1 cells (data not shown), LPS stimulation in vivo bypasses the requirement for IL-27 in Th1 differentiation and may confer a powerful survival signal that is not seen with infections. Although further studies are necessary to identify the IL-27-independent Th1 factors, possible candidates include IL-12 (Fig. 1B), IL-18 (39) or other products downstream from the TLR4–TRIF pathway (40).

CD8+ Vβ3+ T cells did not produce IL-17 following SEA treatment in the presence or absence of WSX-1 (data not shown). CD8 T cells are a source of IL-17 in humans and mouse models of infection and contact hypersensitivity (41–46). Although antigen stimulation in vivo is not sufficient to generate IL-17-producing CD8 T cells, factors that down-regulate T-bet and Eomesodermin within this population may be involved (47). We found that WSX-1 blocked the accumulation of IFN-γ+ CD8 T cells in the LP but not spleen, suggesting a negative influence for IL-27 on intestinal CD8 T cell responses (Fig. 4; CD4-negative Vβ3+). In contrast, other models have found IL-27 to potentiate IFN-γ production and anti-tumor activity from CD8 T cells (48–51). Thus, the effects of IL-27 on specific cell populations are highly context dependent and warrant investigations on how different PAMPs or routes of immunization influence immunity. For example, intranasal immunization with SEA preferentially recruited CD8 T cells to lung, while the spleen and mesenteric lymph nodes harbored greater levels of CD4 T cells (52). The possibility for IL-27 to influence T-cell migration remains an important consideration for translational research.

**Fig. 5.** WSX-1 suppresses intestinal homing phenotype on T cells following LPS-based immunization. WT (closed bars) and WSX-1−/− (open bars) mice were immunized with SEA at time 0 and LPS 18 h later, with tissues analyzed on day 7. Representative dot plots gated on CD4+ Vβ3+ T cells show expression of integrin α4β7 or CXCR3 in small intestinal LP (A) and spleen (B). (C) Charts show percentage of gated cells staining high for surface markers. Data are from same experiments used for Fig. 3 and displayed as mean ± SEM, with asterisks representing statistically significant differences between WT and WSX-1−/− mice (P < 0.014).
The genes encoding individual IL-12 subunits differentially impacted T<sub>eff</sub> polarization. Whereas p35 blocked T<sub>eff</sub> commitment, p40 was required for the accumulation of T<sub>eff</sub> cells in both spleen and LP (Fig. 1). This suggests that the balance of IL-12 family members determines T<sub>eff</sub> polarization rather than absolute levels. In addition to forming IL-12, p40 can pair with p19 to form IL-23, and p40 also forms homodimers [reviewed in ref. (53)]. In our system, the phenotype of p40<sup>−/−</sup> mice was very similar to that of p19<sup>−/−</sup> mice following immunization [Fig. 1 and ref. (19)], suggesting that IL-23 rather than p40 homodimers supports the accumulation of intestinal T<sub>eff</sub> cells. Our data correspond with an earlier finding that p40, but not p35, is required for T<sub>eff</sub> cell accumulation in vivo (4).

Since p35 and p40 were both required for T<sub>eff</sub> cell accumulation in the LP (Fig. 1C), IL-12 appears to be important for intestinal T-cell survival and/or recruitment. Expression of the T-cell homing markers α4β7 and CXCR3 were slightly reduced in IL-12-deficient mice, corresponding with a slight increase in CD62L expression (data not shown). This suggests a positive role for IL-12 in extra-lymphoid T-cell migration, although its impact on homing markers was not striking. In contrast, T-cell expression of α4β7 was significantly increased in the spleen and LP of WSX-1<sup>−/−</sup> mice, indicating that IL-27 can negatively influence T-cell homing to the intestine (Fig. 5). This hypothesis is supported by earlier findings showing that migration of CD8 T cells to the gut is mediated by beta 7 integrins (54) and that α4β7 expression on CD4 T cells is associated with their rapid egress from lymphoid tissue (20). Interestingly, CXCR3 expression was unaffected by WSX-1 deficiency (Fig. 5C), corresponding with a normal T<sub(reg) </sub>1 cytokine response (Fig. 3C). Therefore, IL-27 may control multiple facets of T<sub>eff</sub> responses including priming and intestinal migration (Figs 3 and 5). Currently, it is not understood if IL-27 regulates the longevity of T<sub>eff</sub> responses.

The impact of IL-27 on Treg development and function is not well understood. Although IL-27 promotes Foxp3 expression through STAT1 activation (55), IL-27 does not increase T-cell populations are found to co-express Foxp3 and ROR<sup>γ</sup>T, the signature transcription factors for Tregs and T<sub>eff</sub> cells, respectively (59–61). As IL-27 signaling inhibits ROR<sup>γ</sup>T expression (62), it is predicted to tip the balance in favor of Treg development. In support, antigen-specific Foxp3<sup>+</sup> CD4 T cells produced less IL-10 and more IL-17 following immunization in the absence of WSX-1 signaling (Fig. 3D). Future studies are necessary to address the effects of IL-27 on Treg function.

Recently, mice from Taconic Farms were shown to have more T<sub>eff</sub> cells in the gut than Jackson mice due to the presence of segmented filamentous bacteria (63, 64). In our experiments, gene-deficient mice were always compared with WT from the same vendor, and the WSX-1<sup>−/−</sup> strain was obtained from Taconic Farms. The extent to which commensal organisms are driving genetic phenotypes is an interesting question. It is notable, however, that increased T<sub>eff</sub> numbers were observed in the gut of IL-12p35<sup>−/−</sup> mice that were purchased from Jackson Laboratory (Fig. 1) and therefore should not contain segmented filamentous bacteria in their microbiome. Overall, this suggests that commensal-dependent and IL-12 family-dependent mechanisms contribute to the balance of T<sub>reg</sub> subsets in gut mucosa.

The role of IL-27/WSX-1 signaling in inflammatory bowel diseases is context dependent. Although WSX-1<sup>−/−</sup> mice are protected from a chronic low-dose dextran sulfate sodium (DSS)-induced colitis (65), IL-27R<sup>−/−</sup> mice have worse disease during high-dose DSS treatment, which results in a rapid onset of colitis (30). In the high-dose model, pathology correlated with increased IL-17 levels (30). Chronic colitis caused by IL-10 deficiency was also found to be IL-27R dependent (66). Interestingly, EB13<sup>−/−</sup> mice that cannot produce IL-27 were protected from an acute TNFα-driven colitis model (67). We favor the hypothesis that IL-27 is therapeutic during T<sub>eff</sub>17-mediated colitis but pathologic during T<sub>reg</sub>1 or T<sub>reg</sub>2-mediated colitis. The finding that IL-27 polymorphisms are associated with human inflammatory bowel diseases warrants further investigations on its regulatory versus inflammatory functions (68).

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