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Interleukin-27 Priming of T Cells Controls IL-17 Production In *trans* via Induction of the Ligand PD-L1

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DOI 10.1016/j.immuni.2012.03.024

SUMMARY

Interleukin-27 (IL-27) is a key immunosuppressive cytokine that counters T helper 17 (Th17) cell-mediated pathology. To identify mechanisms by which IL-27 might exert its immunosuppressive effect, we analyzed genes in T cells rapidly induced by IL-27. We found that IL-27 priming of naive T cells upregulated expression of programmed death ligand 1 (PD-L1) in a signal transducer and activator of transcription 1 (STAT1)-dependent manner. When cocultured with naive CD4$^+$ T cells, IL-27-primed T cells inhibited the differentiation of Th17 cells in *trans* through a PD-1-PD-L1 interaction. In vivo, coadministration of naive TCR transgenic T cells (2D2 T cells) with IL-27-primed T cells expressing PD-L1 inhibited the development of Th17 cells and protected from severe autoimmune encephalomyelitis. Thus, these data identify a suppressive activity of IL-27, by which CD4$^+$ T cells can restrict differentiation of Th17 cells in *trans*.

INTRODUCTION

Interleukin-27 (IL-27) is a member of a family of heterodimeric cytokines with critical immunoregulatory properties (Cox et al., 2011; Cua et al., 2003; Villarino et al., 2003). IL-27 was initially identified as being important for inducing expression of T-bet and enhancing Th1 cell differentiation. Later, it was appreciated that IL-27, which shares the EBI3 subunit with IL-35, has essential, nonredundant immunosuppressive actions (Collison et al., 2010; Owaki et al., 2005; Tong et al., 2010; Yoshida et al., 2001). Specifically, mice lacking the IL-27 receptor (IL-27R) develop lethal inflammation upon infection with a variety of pathogens. For instance, during infection with *Trypanosoma cruzi*, *Il27ra*$^{-/-}$ mice show severe pathological changes resulting from enhanced Th1 and Th2 cell responses (Hamano et al., 2003). Similarly, *Il27ra*$^{-/-}$ mice infected with *Toxoplasma gondii* develop uncontrolled tissue damage mediated by activated Th1 cells and increased IL-17 production (Stumhofer et al., 2006; Villarino et al., 2003).

Various mechanisms of IL-27 have been suggested to explain its immunosuppressive potency. IL-27 can directly inhibit inflammatory cytokine production in activated T cells in vitro (Villarino et al., 2006; Yoshimura et al., 2006). In particular, IL-27 has been suggested to diminish Th17 cell differentiation by inhibiting the expression of RORγt (Diveu et al., 2009). In experimental autoimmune encephalomyelitis (EAE), mice deficient in IL-27R develop more severe Th17 cell-associated neuropathology, whereas treatment of wild-type mice with IL-27 can constrain Th17 cell differentiation and abolish development of EAE (Batten et al., 2006; Fitzgerald et al., 2007).

In addition to inhibiting the production of IL-17, IL-27 enhances the production of the immunosuppressive cytokine IL-10 in various T cell subsets (Batten et al., 2008; Diveu et al., 2009; Stumhofer et al., 2007). Importantly, IL-27 works together with TGF-β to drive the differentiation of IL-10-producing regulatory type 1 T cells (Tr1) through induction of aryl hydrocarbon receptor (AhR), c-Maf, IL-21, and ICOS (Apetoh et al., 2010; Awasthi et al., 2007; Murugaiyan et al., 2009; Pot et al., 2009). IL-27 has also been reported to amplify TGF-β-induced FoxP3 expression in a STAT1-dependent manner (Ouaked et al., 2009); however, other work has argued that IL-27 negatively regulates FoxP3 expression (Neufert et al., 2007). Adoptive transfer of *Il27ra*$^{-/-}$ T cells was associated with reduced pathology and enhanced Treg cell differentiation in a model of autoimmune colitis (Cox et al., 2011).

IL-27 also has different effects on non-T cell lineages. For instance, IL-27 can enhance IFN-γ expression in NK cells (Chiyo et al., 2005), whereas IL-27 can suppress LPS-induced cytokine production by dendritic cells (DCs) in vitro. However,

the underlying mechanism of the latter has not been elucidated (Wang et al., 2007).

Despite its important actions, relatively little was known about the genes that are directly regulated by IL-27. Herein, we show that naive T cells express receptors of IL-27. Among the genes upregulated by IL-27 was *Cd274*, which encodes PD-L1. IL-27-dependent induction of PD-L1 on naive cells inhibited IL-17 production in an untreated population of differentiating Th17 cells, and the development of Th17 cell-mediated autoimmune encephalomyelitis was ameliorated by coadministration of IL-27-treated naive T cells that express PD-L1. Thus, these data point to a mechanism by which IL-27 can exert its ability to suppress IL-17-mediated pathology.

RESULTS

IL-27 Inhibits Early IL-17 Induction

Previous work has shown that IL-27 inhibits Th17 cell differentiation (Batten et al., 2006; Diveu et al., 2009; Stumhofer et al., 2006). Consistent with these findings, we found that IL-17 production was suppressed when IL-27 was present early in Th17 cell differentiation (Figure S1A available online). However, when added 24 hr or later after initial activation, it was ineffective (Figure S1A). To explain these results, we first considered that the effects of IL-27 might be a reflection of receptor expression.

IL-27R comprises a heterodimer consisting of two subunits encoded by the *Il27ra* gene and the *Il6st* gene (Pflanz et al., 2002, 2004). Consistent with previous work (Chen et al., 2000), naive T cells express both subunits and *Il27ra* was downregulated upon activation of $CD4^+$ T cells (Figure S1B). This IL-27R expression was functional as assessed by IL-27-dependent STAT activation (Figures S1C–S1E; Stumhofer et al., 2007). IL-27 readily induced STAT1 activation in naive $CD4^+$ T cells but induced relatively less STAT3 phosphorylation. This contrasted with the action of IL-6, which induced roughly equivalent levels of STAT1 and STAT3 phosphorylation. In summary, naive T cells express the IL-27R and functionally respond to this cytokine.

IL-27-Primed Naive T Cells Inhibit Th17 Cell Differentiation In *trans*

Because IL-27 had the capacity to exert its effect on naive $CD4^+$ T cells, we wondered whether it was necessary for IL-27 to act directly on nascent Th17 cells or whether it could modulate naive cells that might be able to inhibit development of Th17 cells in *trans*. Therefore, we primed naive $CD45.2^+CD4^+$ T cells with IL-27 for 3 hr, washed the cells to remove the cytokine, and then added these cells to naive $CD45.1^+CD4^+$ T cells cultured under Th17 cell-polarizing conditions (Figure S1F). In the presence of untreated naive $CD45.2^+CD4^+$ T cells, more than 15% of nonprimed $CD45.1^+CD4^+$ T cells became IL-17 producers (Figure 1A). When IL-27-primed $CD4^+$ T cells were added to developing Th17 cells, IL-17 production was significantly inhibited (by 40%–60%) (Figures 1A and 1B; $p = 0.0062$, two-tailed Student's t test), regardless of whether the polarizing conditions were IL-6 and TGF-β or IL-6, IL-1β, and IL-23 (Figures S1G and S1H). Because we added IFN-γ neutralizing antibody to the Th17 cell-polarizing conditions, this reduction of IL-17 producers was not due to the ability of IL-27 to promote IFN-γ production (Owaki et al., 2005; Yoshida et al., 2001). Because IL-6 can also activate STAT1, we assessed the effect of IL-6-primed naive T cells in *trans*. Addition of IL-6-primed naive T cells had no effect on the production of IL-17 by bystander nascent Th17 cells ($CD45.1^+CD4^+$) (Figures 1A and 1B). The inhibition of Th17 cell development by IL-27-primed naive T cells was also effective when DCs were included (Figures S1I and S1J). However, global T cell activation was not impaired by IL-27-primed naive cells, as shown by the fact that proliferation of bystander $CD4^+$ T cells was unaffected (Figure S1K). Priming with IL-27 did not influence Th1 cell differentiation as evidenced by IFN-γ production in $CD45.1^+CD4^+$ T cells (Figure S1L), indicating that the effect was specific for IL-17 production.

Because it has been reported that the inhibitory effect of IL-27 on T cell differentiation can be mediated by DCs (Wang et al., 2007), we wondered whether IL-27 priming of DCs might also constrain Th17 cell differentiation. However, we found that this was not the case. In the absence of IL-27-primed T cells, IL-27 priming of $CD11c^+$ DCs to T cells had no effect on IL-17 production by $CD45.1^+CD4^+$ T cells (Figures S1M and S1N). Accordingly, we noted minimal expression of *Il27ra* on freshly isolated, nonactivated $CD11c^+$ cells (Figure S1O). Taken together, naive T cells primed with IL-27 can inhibit IL-17 production, even if the T cells have not been directly exposed to this cytokine.

IL-27-Primed Naive T Cells Inhibit Th17 Cell Differentiation In Vivo

To assess whether IL-27-primed naive $CD4^+$ T cells can attenuate the development of Th17 cells in vivo, we primed naive $CD45.2^+CD4^+$ T cells from OVA-TCR-transgenic (OT-II) mice with either IL-27 or no cytokine for 3 hr and transferred these cells along with unprimed naive $CD45.1^+$ OT-II T cells into mice. Mice were immunized with OVA peptide and CFA, and cytokine expression in directly isolated T cells was measured by intracellular staining 7 days after immunization. Consistent with our results in vitro, we did not find a difference in the absolute number of $CD45.1^+CD4^+$ T cells that expanded in vivo, suggesting that proliferation was not impaired (Figure 1C). Importantly though, the relative and absolute numbers of IL-17-producing $CD45.1^+$ T cells were greatly diminished in mice that received IL-27-treated naive $CD45.2^+$ T cells, but not in immunized mice that received unprimed naive $CD45.2^+$ T cells (Figures 1D and 1E). The effect of IL-27 priming was limited to Th17 cell differentiation, as shown by the fact that there was no difference in the relative or total numbers of $CD45.1^+CD4^+$ T cells that produced IFN-γ (Figure 1F). Thus, naive T cells primed with IL-27 can exert their inhibitor effect on IL-17 production in other T cells in vivo.

Administration of IL-27-Primed Naive $CD4^+$ T Cells Ameliorates Development of Autoimmune Disease

Based on our findings, we reasoned that exposure of naive Th cells to IL-27 could be another important mechanism by which IL-27 acts to limit IL-17-dependent pathology. Therefore, we next used the model of EAE in which Th17 cells are thought to be key in terms of immunopathogenesis (Langrish et al., 2005). We adoptively transferred untreated or IL-27-primed

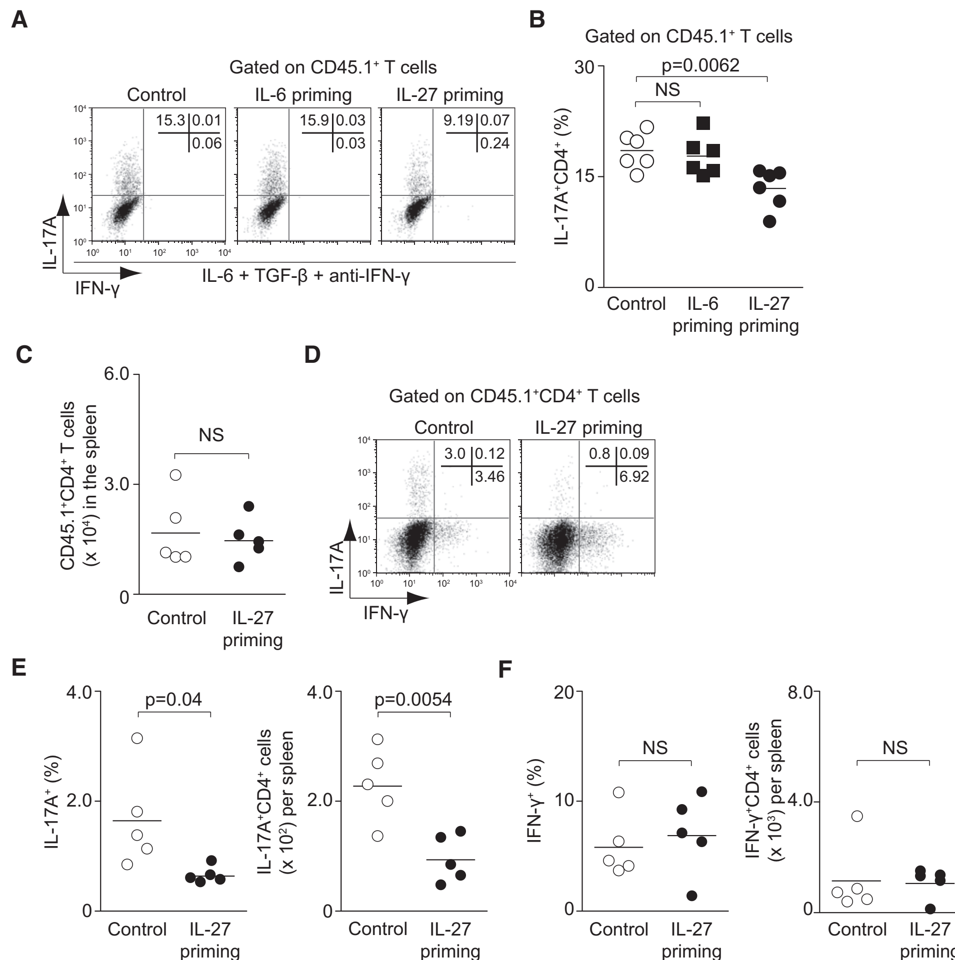

Figure 1. IL-27-Primed Cells Inhibit Th17 Cell Differentiation In *trans*

In brief, naive CD45.2$^+$CD4$^+$ T cells were primed with the indicated cytokines. After washing, cells were mixed with naive CD45.1$^+$CD4$^+$ T cells and cultured with IL-6, TGF-β, and anti-IFN-γ for 3 days.

(A and B) IL-17A and IFN-γ production in CD45.1$^+$CD4$^+$ T cells was assessed by intracellular staining. Representative intracellular staining is shown for Th17 cells (A). Pooled data from six independent experiments are provided in (B) (mean values, NS, not significant).

(C–F) Production of IFN-γ and IL-17A in CD45.1$^+$ OT-II CD4$^+$ T cells was analyzed by flow cytometry. Total numbers of CD45.1$^+$ OT-II CD4$^+$ T cells are provided in (C). Representative results from two individual mice are shown in (D) and pooled data from two independent experiments are provided in (E) and (F) (mean values; NS, not significant).

non-TCR transgenic naive T cells with or without naive TCR transgenic T cells that recognize MOG peptide (2D2 T cells) into *Rag2*$^{-/-}$ mice. After transfer, mice were immunized with MOG and CFA and followed for the development of clinical signs (Figure S2A). As expected, mice that received primed T cells alone without transgenic T cells did not develop disease. Control mice that received unprimed naive T cells together with naive 2D2 T cells developed severe disease with an average score of 3.3 (Figure 2A). In sharp contrast, mice that received IL-27-primed naive T cells together with 2D2 T cells developed a significantly milder disease, manifested by an average score of 2.0 ($p < 0.001$, two-way ANOVA). Cumulative disease scores from each mouse also showed significant decrease in the mice that received IL-27-primed naive T cells together with 2D2 T cells (Figure S2B; $p < 0.0001$, two-tailed Student's t test). To ascertain that IL-27 priming was independent of TCR specificity, we primed OT-II T cells, transferred along with naive 2D2 T cells, and found that they too limited pathology (Figures S2C and S2D). Again, consistent with the notion that IL-27-cultured naive cells did not globally suppress immune responses, the absolute numbers of CD4$^+$ T cells infiltrating in the CNS were not significantly different between groups (Figure 2B). Strikingly, the protection from severe EAE in mice, which received IL-27-primed naive T cells, was associated with significant suppression in the absolute numbers of IL-17$^+$ T cells in the CNS (Figures 2C, 2D, and S2E; $p = 0.0112$, two-tailed Student's t test). In contrast, the numbers of IFN-γ^+ T cells, IL-10$^+$ T cells, or FoxP3-expressing T cells were not different between the two groups (Figures 2C, 2D, and S2E). These data suggest that IL-27-primed naive T cells act to selectively inhibit Th17 cell differentiation in vivo and modulate IL-17-mediated autoimmune disease.

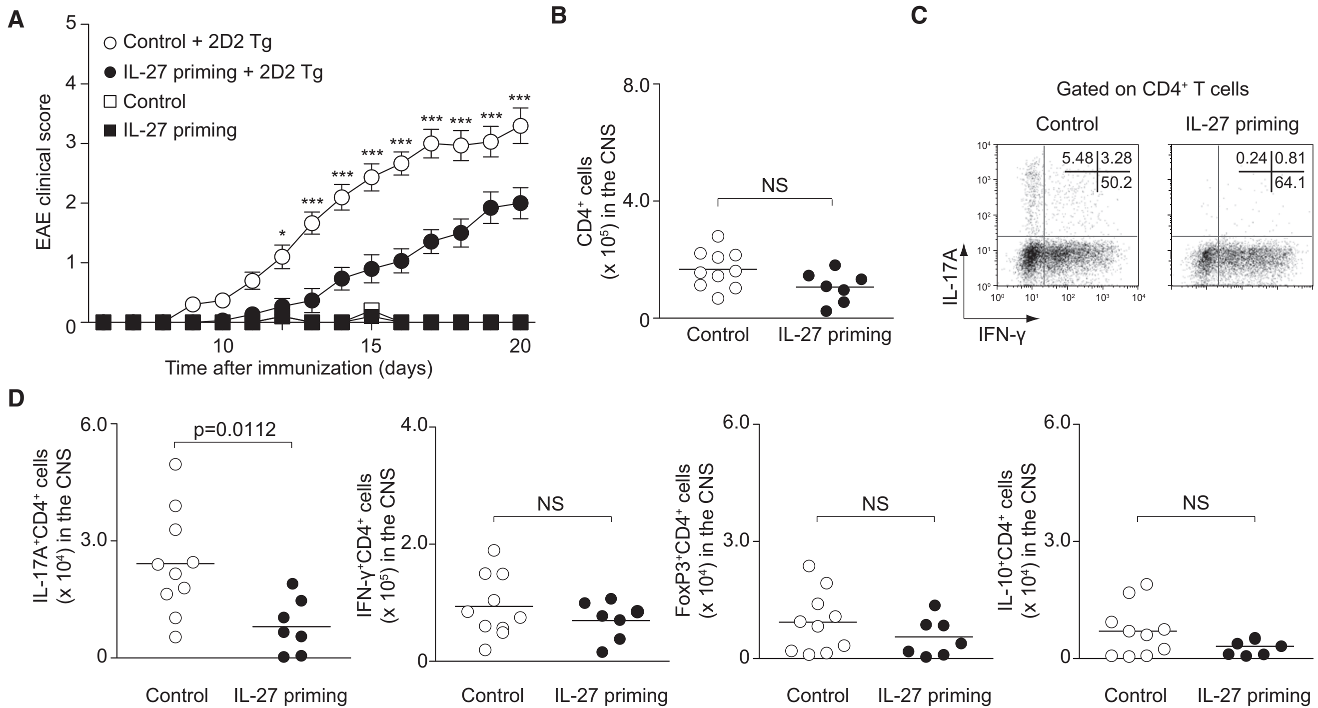

Figure 2. IL-27-Primed Naive CD4+ T Cells Inhibit Pathogenicity of T Cells In *trans*

In brief, naive CD4+ T cells were primed with or without IL-27 and transferred with or without sorted naive TCR transgenic 2D2 CD4+ T cells into *Rag2*$^{-/-}$ mice to induce EAE, which were immunized with MOG peptide in CFA.

(A) Data provided represent mean ± SEM of the EAE clinical score of 40 mice pooled from 3 independent experiments (***$p < 0.001$, *$p < 0.05$).

(B–D) CNS-infiltrating CD4+ T cells from recipient mice of two groups from (A) were analyzed on day 20 after immunization. Absolute numbers (B), representative flow cytometric analysis of IL-17A and IFN-γ (C), and absolute numbers of subsets of CD4+ T cells (D) are provided from two independent experiments (mean values; NS, not significant).

IL-27 Rapidly Induces PD-L1 Expression on Naive CD4+ T Cells

To define potential mechanisms by which IL-27 priming inhibited Th17 cell differentiation of nonprimed cells, we analyzed global gene expression in naive CD4+ T cells stimulated with IL-27 for 3 hr. We found that 375 genes showed a >2-fold difference in unstimulated versus IL-27-stimulated naive T cells. Because IL-6 priming was able to activate STAT1 and STAT3 but was incapable of inhibiting Th17 cell differentiation in *trans*, we compared genes induced by IL-27 or IL-6. IL-6 stimulation resulted in 648 genes with a >2-fold difference compared to unstimulated naive T cells (Figure 3A). One of the genes significantly increased in IL-27-stimulated cells was *Cd274*, which encodes PD-L1, a key immunosuppressive molecule that constrains immune responses (Table S1; $p = 0.0014$; Francisco et al., 2010). Of note, this gene was not induced by IL-6 (Figures 3A, 3B, and S3A), and the expression of other related costimulatory molecules was not affected (Figure S3A). The ability of IL-27 to induce PD-L1 at the level of protein expression was confirmed (Figure 3C).

Of note, IL-27 did not induce PD-L1 expression in CD11c+ DCs (Figure 3D), a cell type that lacks the expression of the *Il27ra* (Figure S1O). In contrast, IL-27 enhanced PD-L1 expression on CD8+ T cells (Figure 3D). In addition, we found no difference in expression of the immunosuppressive cytokines *Il10* or *Ebi3*, a component shared by IL-27 or IL-35, between unstimulated T cells and IL-27-stimulated T cells (Figure S3B). These results indicate that IL-27 rapidly induces PD-L1, affecting naive T cells but not other subsets.

Requirement for IL-27 Signaling for Optimal Induction of PD-L1 in *T. gondii* Infection

To assess the relevance of IL-27 in controlling PD-L1 expression in vivo, we employed a model in which IL-27 is important for controlling immunopathology (Villarino et al., 2003). We found that expression of PD-L1 on CD4+ T cells was significantly lower in *Ebi3*$^{-/-}$ mice (Figure 3E; $p = 0.0182$, $p = 0.0073$, two-tailed Student's t test). However, the effect was selective for T cells; PD-L1 expression on other immune cells remained unaffected (Figure 3F).

PD-L1 Engagement Underlies the Immunosuppressive Effect of IL-27-Primed Naive T Cells

We next sought to examine whether the induction of PD-L1 on IL-27-primed naive CD4+ T cells was the principal component of the inhibition of bystander Th17 cell differentiation. To this end, we first investigated the effect of the addition of neutralizing PD-L1 antibody to cocultures of IL-27-primed naive CD4+ T cells (CD45.2+) added to developing Th17 cells (CD45.1+). The addition of PD-L1 neutralizing antibody (Figure 4A, bottom) completely reversed the effect of IL-27-primed naive CD4+ T cells to inhibit Th17 cell differentiation in nonprimed CD4+ T cells (Figures 4A and 4B).

To confirm the nonredundant role of PD-L1 in mediating the in *trans* inhibition of Th17 cell development by IL-27-primed CD4$^+$ T cells in vivo, we assessed the effect of T cells from mice that lack this ligand (Latchman et al., 2004). Specifically, untreated or IL-27-primed CD4$^+$ T cells from wild-type or *Cd274*$^{-/-}$ mice were transferred along with 2D2 T cells into recipient mice that were immunized with MOG peptide. As demonstrated before in Figure 2A, IL-27-primed naive T cells ameliorated EAE severity (Figures 4C, top, and S4A). Importantly, this protection by IL-27-primed naive T cells was completely lost in the absence of PD-L1 (Figures 4C, bottom, and S4A). Although similar numbers of absolute CD4$^+$ T cells and IFN-γ-producers were present in the CNS in all groups, the number of IL-17 producers was significantly reduced when IL-27-primed wild-type cells were administered (Figures 4D–4F; $p = 0.0189$, two-tailed Student's t test). Again, administration of *Cd274*$^{-/-}$ T cells had no effect on the number of IL-17-producing cells in the CNS (Figures 4E and 4F).

If the immunosuppressive effect of IL-27 priming was mediated by upregulation of PD-L1, it should be possible to mimic this effect simply by adding this ligand to differentiating Th17 cells in vitro. Consistent with our hypothesis, we found that inclusion of soluble PD-L1 fusion protein also resulted in suppression of IL-17 expression comparable to that observed with IL-27 (Figures 5A and 5B). Th17 cell differentiation was not affected by addition of a control Ig fusion protein (IgG1 Fc). The effect of soluble PD-L1 during Th17 cell differentiation was specific for IL-17, as shown by the fact that IFN-γ expression in Th1 cells was not affected by exogenous PD-L1 (Figures 5A and 5B). In addition, inhibiting PD-1-PD-L1 interactions with neutralizing antibodies reversed the inhibition of IL-17 production by the PD-L1 fusion protein (Figures 5C and 5D). In summary, the inhibitory effect of IL-27-primed T cells is dependent upon PD-L1 expression.

PD-L1 Engagement Inhibits Th17 Cell Differentiation Independently of FoxP3

Previous studies have indicated that PD-1-PD-L1 interactions induce expression of FoxP3 in CD4$^+$ T cells (Francisco et al., 2009). We therefore considered the possibility that this might be an explanation underlying the inhibitory effect of PD-L1 on IL-17 production. However, neither IL-27 nor PD-L1 directly affected FoxP3 expression in CD4$^+$ T cells cultured under Th17 cell-polarizing conditions (Figures 5A and S5A). To confirm that PD-L1 exerted its immunoregulatory function independently of FoxP3, we examined whether PD-L1 inhibited IL-17 production in T cells from mice with mutation of *Foxp3* (Scurfy). Equivalent inhibitory effects of IL-27 on IL-17 production were evident in T cells from Scurfy mice, arguing for a FoxP3-independent mechanism (Figures S5B and S5C). Similarly, the inhibitory effect of recombinant PD-L1 was the same in cells from wild-type and Scurfy mice. Thus, PD-L1 engagement inhibits IL-17 production in a FoxP3-independent manner.

The *Cd274* Locus Is Accessible in Naive CD4$^+$ T Cells and Is Directly Regulated by STAT1

We next sought to determine how IL-27 might be acting to regulate PD-L1 on naive CD4$^+$ T cells. First, we hypothesized that the *Cd274* gene locus should be accessible in naive Th cells, as indicated by the fact that IL-27 rapidly induced PD-L1 expression. Indeed, by chromatin immunoprecipitation and massive parallel sequencing, we could identify that the *Cd274* promoter was characterized by H3 lysine 4 trimethylation, whereas the *Pdcd1lg2* promoter did not show this positive epigenetic modification (Figure S6A; Wei et al., 2009).

Next, because IL-27 induces phosphorylation of STAT1 and STAT3, we stimulated naive CD4$^+$ T cells from wild-type mice and mice deficient in STAT1 or STAT3. We found that induction of *Cd274* by IL-27 was completely abolished by the absence of STAT1 but was unaffected by the absence of STAT3 (Figures 6A, top, S6B, and S6D). IL-6 had no effect on PD-L1 in *Stat1*$^{-/-}$ T cells (Figure S6C). However, in *Stat3*$^{-/-}$ T cells, in which IL-6 induced unopposed activation of STAT1, this cytokine did induce expression of PD-L1 (Figures 6A, bottom, S6C, and S6D). The importance of STAT1 in regulating PD-L1 expression was confirmed by stimulation of cells with other STAT1-activating cytokines. Like IL-27, type I and type II interferons induced PD-L1 expression on naive CD4$^+$ T cells (Figure 6B). However, the effect of these ligands was not selective; type I and type II interferons (IFNs) also upregulated PD-L1 expression on cells that failed to respond to IL-27, including B cells, DCs, and CD11b$^+$ cells (Figure 6B).

One other important target of STAT1 is *Tbx21*, which encodes T-bet, the master regulator of Th1 cells (Lighvani et al., 2001). To explore whether STAT1 indirectly regulates PD-L1 through induction of T-bet, we first assessed the induction of PD-L1 in cells from mice lacking T-bet. However, we found that this transcription factor had no effect on induction (Figure S6E). We next investigated the possibility that STAT1 directly binds to the *Cd274* promoter in naive T cells. Binding of STAT1 to the *Cd274* promoter, which contains GAS and ISRE elements, was noted after stimulation of cells with IL-27. In contrast, minimal binding was observed to the *Cd274* intronic region (Figure 6C). Thus, the epigenetic modifications of the *Cd274* locus in naive T cells and the direct binding of IL-27-induced STAT1 help explain the rapid expression and function of PD-L1 on naive T cells. Because IFNs induced PD-L1 expression on naive CD4$^+$ T cells (Figure 6B), we wondered whether IFNs, like IL-27, had the capability to inhibit Th17 cell differentiation in *trans*. In fact, we found that IFN-γ priming of T cells inhibited in vitro Th17 cell differentiation to a similar degree as did IL-27 priming (Figures 6D and 6E). To assess whether IFN-γ-primed naive CD4$^+$ T cells can also limit IL-17-dependent pathology in vivo, we adoptively transferred untreated or IFN-γ-primed naive CD4$^+$ T cells along with 2D2 T cells into *Rag2*$^{-/-}$ mice, which were immunized with CFA and MOG peptide. Again, control mice that received unprimed naive T cells together with naive 2D2 T cells developed severe disease with an average score of 3.5 (Figures 6F and S6F). In contrast, mice that received IFN-γ-primed naive T cells together with 2D2 T cells developed a significantly milder disease, manifested by an average score of 1.95 ($p < 0.001$, two-way ANOVA).

In summary, STAT1 directly binds the *Cd274* promoter region and induces its transcription.

STAT1 Is Critical for Inhibition of Th17 Cell Differentiation In Vivo by IL-27-Primed Naive T Cells

The in vivo physiological relevance of the STAT1-dependent PD-L1 induction on naive CD4$^+$ T cells was investigated in a model of

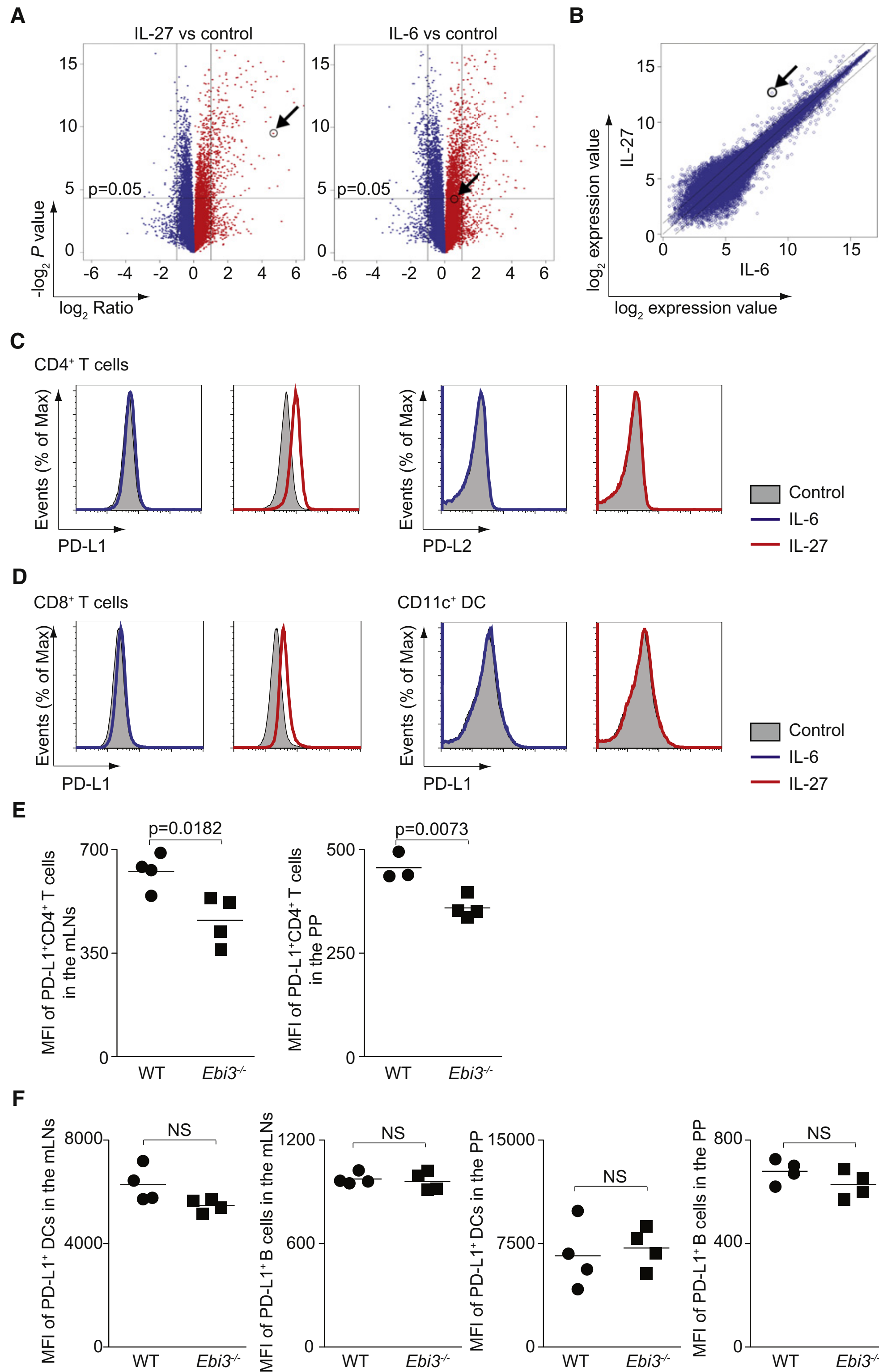

Figure 3. IL-27 Priming Rapidly Induces PD-L1 on Naive CD4$^+$ T Cells

(A–C) Naive CD4$^+$ T cells were cultured in medium alone (control) or stimulated with IL-6 or IL-27 for 3 hr before analyzing differential gene expression by microarray. The arrow indicates *Cd274* expression. Volcano plots depict differential gene expression induced by IL-27 or IL-6 compared to control (A). Comparison of IL-27- and IL-6-dependent gene expression is shown as a scatter plot (B).

(C and D) Naive CD4$^+$ T cells (C) and naive CD8$^+$ T cells or freshly isolated CD11c$^+$ DCs (D) were stimulated as in (A) and expression of PD-L1 and PD-L2 was determined by flow cytometry.

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systemic inflammation in which IFNs and other cytokines are elaborated (Karaghiosoff et al., 2003). To this end, we injected lipopolysaccharide (LPS) into wild-type and STAT1-deficient mice and assessed the PD-L1 expression. We found that PD-L1 expression was induced by naive $CD4^+$ T cells after LPS injection in wild-type mice, but the effect was blocked in *Stat1*$^{-/-}$ mice (Figures 7A and 7B). To confirm the importance of STAT1-dependent PD-L1 regulation, we next examined whether STAT1 was required for the immunosuppressive function of IL-27-primed naive T cells and their ability to inhibit bystander cells in *trans*. We therefore adoptively transferred IL-27-primed wild-type *and Stat1*$^{-/-}$ naive $CD4^+$ T cells along with 2D2 T cells into *Rag2*$^{-/-}$ mice, which were then immunized with CFA and MOG peptide. We found that STAT1 deficiency abrogated the ability of IL-27 priming to attenuate autoimmune disease (Figures 7C and S7A).

In summary, the ability of IL-27-primed T cells to limit immune-mediated pathology is dependent upon STAT1.

DISCUSSION

Previous work has established that IL-27 has critical, nonredundant functions as an immunosuppressive cytokine (Batten et al., 2006; Kastelein et al., 2007; Stumhofer et al., 2006; Villarino et al., 2003). Herein, we report an unexpected, direct action of IL-27 on naive Th cells that allows primed cells to limit IL-17-mediated pathology in *trans*. Our data show that IL-27 induces PD-L1 in a STAT1-dependent manner, which in turn inhibits Th17 cell differentiation in bystander cells. This effect limits IL-17-dependent pathology but has no effect on global T cell activation or Th1 cell differentiation. Thus, the data point to an important mechanism of action of IL-27.

Multiple mechanisms have been identified by which IL-27 can suppress immune responses. It is recognized that IL-27 inhibits Th17 cell differentiation by acting directly upon differentiating $CD4^+$ T cells via induction of T-bet and downregulation of Rorγt (Batten et al., 2006; Diveu et al., 2009; Stumhofer et al., 2006). We found this to be the case as well. There was a trend in reduction of IL-17 expression in $CD4^+$ T cells exposed to IL-27 for 3 hr. But we did not examine this further, because direct versus indirect effects could not be discerned. The mechanism described in the present study is distinct from this mode of inhibition in that differentiating $CD4^+$ T cells were not directly exposed to IL-27 and yet a profound effect was still noted. In the conditions we employed, naive T cells were exposed to IL-27, after which the cytokine was removed, and thus the inhibitory effect of IL-27 was mediated indirectly.

Another immunosuppressive action of IL-27 is mediated by the production of IL-10 (Batten et al., 2008; Diveu et al., 2009; Stumhofer et al., 2007). IL-27, in combination with TGF-β, has been reported to generate IL-10-producing regulatory T cells characterized by the expression of c-Maf, IL-21, ICOS, and AhR (Apetoh et al., 2010; Awasthi et al., 2007; Pot et al., 2009). Our short-term stimulation of IL-27 did not induce IL-10 in naive $CD4^+$ T cells (Figure S3B); however, this is not to say that IL-27-induced IL-10 production is not an important part of IL-27's action. Nonetheless, in our system the inhibitory effect of IL-27 was largely reversed by elimination of PD-L1 on T cells, arguing that this is a functionally important mechanism.

One of the striking findings of the present study was that IL-27 rapidly induced PD-L1 on naive $CD4^+$ and $CD8^+$ T cells. To our knowledge, this has not been recognized previously to be an action of IL-27. Within 3 hr of exposure to this cytokine, mRNA of PD-L1 could be seen. Furthermore, we have demonstrated that this is functionally relevant in blocking IL-17 production both in vitro and in vivo. The PD-1-PD-L1 pathway is now recognized to be important in the maintenance of peripheral tolerance (Chen, 2004; Francisco et al., 2010; Freeman et al., 2000). Mice lacking PD-L1 do not develop spontaneous autoimmune disease, but its deficiency exacerbates disease in EAE, autoimmune arthritis, and autoimmune diabetes (Chen, 2004; Fife et al., 2006; Hamel et al., 2010; Latchman et al., 2004). The relevance of this pathway in human disease is substantiated by the association of polymorphisms of *CD274* (which encodes PD-L1) with Graves' disease and autoimmune Addison's disease (Hayashi et al., 2008; Mitchell et al., 2009). Conversely, upregulation of PD-1 in $CD8^+$ T cells contributes to exhaustion of reactive T cells in mice with chronic viral infection (Barber et al., 2006). Similarly, high expression of PD-1 by HIV-specific $CD8^+$ T cells was found in HIV-infected individuals and was associated with high viral load and exhaustion of HIV-specific $CD8^+$ T cells (Trautmann et al., 2006).

In some settings, engagement of PD-L1 with PD-1 generates FoxP3-expressing Treg cells (Francisco et al., 2009). However, in our system, we saw no evidence of FoxP3 induction either in naive cells directly primed with IL-27 or in cocultured bystander cells under Th17 cell-polarizing condition (data not shown). Multiple stimuli can induce PD-L1 expression on T cells and antigen-presenting cells. The promoters of the genes encoding mouse and human PD-L1 exhibit interferon-sensitive response elements, IFN regulatory factor-1 (IRF-1) binding sites, and gamma interferon-activated sites (Lee et al., 2006). Accordingly, PD-L1 can be induced in DCs by type I and type II IFNs (Schreiner et al., 2004; Yamazaki et al., 2002), although we found that nonactivated DCs do not express IL-27 receptors and thus are not responsive to this cytokine basally. As reported herein, STAT1 activation is of particular importance for PD-L1 upregulation by naive T cells. These data argue that IL-27 is not unique in its ability to induce PD-L1. In fact, this was the case. We found that IFNs also induced PD-L1 on naive $CD4^+$ and $CD8^+$ T cells. Moreover, T cells primed with IFN-γ also inhibited Th17 cell induction in *trans*, just as did cells primed with IL-27. However, it is notable that the effect of IL-27 is more circumscribed compared to type I and type II IFNs. In contrast to IL-27, which acts predominantly on $CD4^+$ and $CD8^+$ T cells, IFNs induce PD-L1 expression on a wide variety of cells.

(E and F) Wild-type or *Ebi3*$^{-/-}$ mice were orally administered *T. gondii* and sacrificed on day 9. Mesenteric lymph node (mLN) and Peyers patch (PP) cells were gated on $CD4^+$, TCR-β^+, $CD8^-$, and $Foxp3^-$ cells. PD-L1 expression is shown as percentages of $CD4^+$ T cells and by geometric mean channel fluorescence (E). DCs were gated on $CD3^-$, NK1.1$^-$, $CD19^-$, and CD11c^{hi}. B cells are gated on $CD3^-$, NK1.1$^-$, and $CD19^+$ (F). The experiment shown is representative of two separate experiments (mean values; NS, not significant).

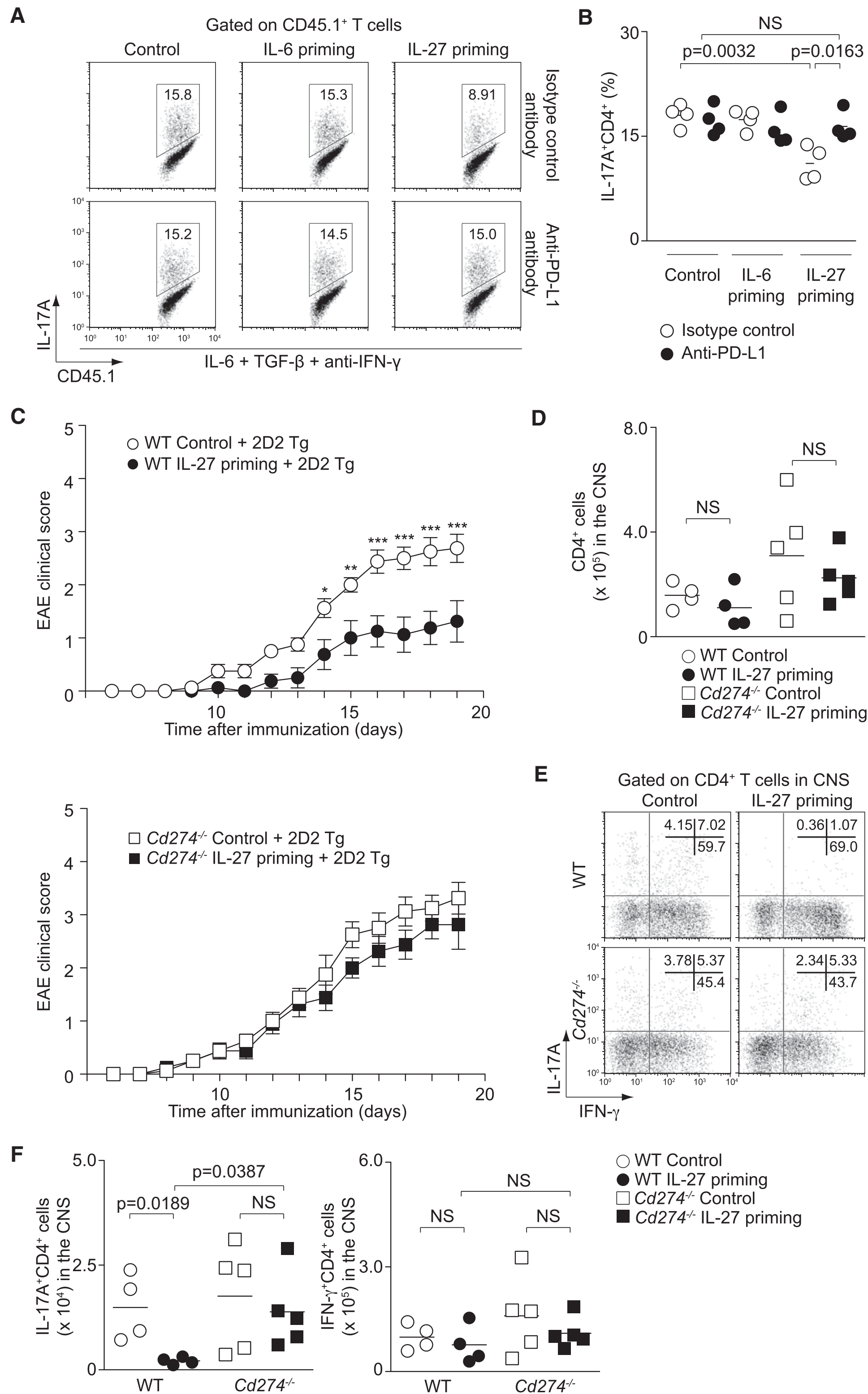

Figure 4. IL-27-Induced Expression of PD-L1 on Naive CD4+ T Cells Inhibits IL-17 Production In *trans*

(A and B) Naive CD45.2+CD4+ T cells were primed with IL-6 or IL-27 or cultured in medium alone. After 3 hr, cells were washed and cocultured with naive CD45.1+CD4+ T cells under Th17 cell-polarizing conditions with an isotype control antibody (top) or PD-L1 antibody (bottom). After 3 days, IL-17A protein expression of CD45.1+CD4+ T cells was analyzed by intracellular staining. Representative intracellular staining is depicted in (A) and pooled data from four separate experiments with mean values are shown in (B) (NS, not significant).

Activation of T cells via the antigen receptor also upregulates PD-L1 expression (Yamazaki et al., 2002). However, the findings presented in the present study argue that in the course of an inflammatory immune response, IL-27 can induce PD-L1 upregulation in T cells that are not activated by cognate antigen and thereby limit immunopathology. With respect to the relative proportion of bystander cells expressing PD-L1 needed to attenuate Th17 cell differentiation of activated CD4 T cells, it is obviously difficult to directly extrapolate the relevance of in vitro conditions. Nonetheless, the proportion of naive $CD4^+$ and $CD8^+$ T cells that can upregulate PD-L1 in response to IL-27 greatly exceed the proportion of antigen-specific CD4 cells. We also found that the *Cd274* promoter was accessible in naive Th cells, as characterized by the presence of H3 lysine 4 trimethylation associated with the absence of H3 lysine 27 trimethylation, further supporting the notion that PD-L1 can be rapidly induced. Moreover, our results with *T. gondii* infection substantiate the claim that this mechanism is relevant in vivo. However, this is not to say that induction of PD-L1 on other cells by other cytokines is irrelevant. This is not the case at all, because PD-L1 is inducible in a wide variety of cells where it has critical actions. The present study simply points out that cytokine-dependent induction of PD-L1 on T cells can influence the differentiation of antigen-activated $CD4^+$ T cells.

In summary, our study highlights that naive $PD\text{-}L1^+CD4^+$ T cells induced by IL-27 can limit the effect of pathogenic IL-17-producing Th17 cells in vitro and in vivo. It is conceivable that the strategy of IL-27 pretreatment of naive T cells ex vivo with subsequent adoptive transfer might be used therapeutically to attenuate autoimmune disease.

EXPERIMENTAL PROCEDURES

Mice and Media

C57BL/6J, B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II, C57BL/6-Tg (Tcra2D2, Tcrb2D2)1Kuch/J (TCR(2D2)), and B6.129S6-*Tbx21*^{tm1Glm}/J (*Tbx21*$^{-/-}$) mice were purchased from Jackson Laboratory; B6.129S6-*Rag2*^{tm1Fwa} (*Rag2*$^{-/-}$) mice, B6.SJL-*Ptprc*^{a}/BoyAiTac (CD45.1) mice, and *Foxp3*^{sf} mice were from Taconic; and *Stat3*$^{fl/fl}$ mice were from D. Levy (Lee et al., 2002) and bred with CD4-Cre^+ Tg mice. *Stat1*$^{-/-}$ mice were also from D. Levy (Durbin et al., 1996). OT-II Tg mice were bred with B6.SJL-*Ptprc*^{a}/BoyAiTac (CD45.1) mice in our laboratory. FoxP3-enhanced green fluorescent protein (eGFP) reporter mice have been reported previously (Bettelli et al., 2006). EBI3-deficient (*Ebi3*$^{-/-}$) mice were from Lexicon Pharmaceuticals, Inc. (The Woodlands, TX). They were generated by Lexicon Pharmaceuticals for Centocor Research and Development and provided by Centocor. PD-L1-deficient (*Cd274*$^{-/-}$) mice were generated by A. Sharpe (Latchman et al., 2004). All animal studies were performed according to the NIH guidelines for the use and care of live animals and were approved by the Institutional Animal Care and Use Committee of NIAMS. All cells were cultured in RPMI medium with 10% (vol/vol) FCS, 2 mM glutamine, 100 IU ml^{-1} of penicillin, 0.1 mg ml^{-1} of streptomycin, and 20 mM HEPES buffer, (pH 7.2–7.5) (all from Invitrogen) and 2 mM β-mercaptoethanol (Sigma-Aldrich).

Cell Isolation and Differentiation

$CD4^+$ T cells from spleens and lymph nodes of 6- to 8-week-old mice were purified by negative selection and magnetic separation (Miltenyi Biotec) followed by sorting of naive $CD4^+CD62L^+CD44^-CD25^-$ population by FACSAria II (BD). For natural T regulatory population, GFP^+ cell were sorted with FACSAria II (BD). $CD8^+$ T cells, $B220^+$ cells, $CD11c^+$ DCs, or $CD11b^+$ macrophages from spleens of 6- to 8-week-old mice were purified by magnetic separation (Miltenyi Biotec). In some experiments, those cells were stimulated with IL-6 (20 ng ml^{-1}), IL-27 (20 ng ml^{-1}), IFN-α (10,000 U ml^{-1}), IFN-β (10,000 U ml^{-1}), or IFN-γ (20 ng ml^{-1}) for 3 hr (all from R&D Systems). Naive $CD4^+$ T cells were activated by plate-bound anti-CD3 (10 μg ml^{-1}; eBioscience) and soluble anti-CD28 (1 μg ml^{-1}; eBioscience) in media for 3 or 4 days either under neutral conditions or with IL-6 (20 ng ml^{-1}, R&D Systems) plus human TGF-β1 (2 ng ml^{-1}, R&D Systems) and IFN-γ neutralizing antibodies (10 μg ml^{-1}, BDPharmingen) for Th17 cell-polarizing condition or IL-12 (20 ng ml^{-1}, R&D Systems) and IL-4 neutralizing antibodies (10 μg ml^{-1}, BioXCell) for Th1 cell-polarizing condition. For coculture experiments, we incubated naive $CD45.2^+CD4^+$ T cells with $CD45.1^+$ T cells at a ratio of one to one, because we found in preliminary experiments that this was optimal. In some experiments, we added IL-6 (20 ng ml^{-1}), IL-27 (20 ng ml^{-1}), PD-L1 (5 μg ml^{-1}), recombinant human IgG1 Fc (5 μg ml^{-1}) (all from R&D Systems), PD-L1 neutralizing antibody (10 μg ml^{-1}, Abcam (MIH6)), or isotype control antibody (10 μg ml^{-1}, Abcam).

Flow Cytometry

Expression of cytokines and transcription factors was assessed by intracellular staining. The following antibodies were used. For cell surface staining: anti-CD4-PerCPCy5.5, anti-CD4-APC, anti-CD44-FITC or -PE, anti-CD25-PE, or -APC, anti-Vβ5.1/5.2-FITC, anti-Vα2-PE, anti-Vβ11-FITC, CD45.1-PerCPCy5.5, anti-PD-L1-PE, anti-PD-L2-PE, and anti-CD3-FITC (all BD). Anti-CD62L-PE-Cy7, anti-CD11c-PECy7, anti-CD19-eFlour450, and anti-NK1.1-FITC were purchased from eBioscience. Anti-TCR-β-Alexa Fluor 700 was purchased from BioLegend. Anti-CD8a-PE-Texas Red was purchased from Abcam. For intracellular staining: anti-IFN-γ-FITC, anti-IL-17A-APC, Foxp3 eFluor450, and anti-FoxP3-FITC (all eBioscience). Anti-IL-4-APC, anti-IL-10-PE, anti-IFN-γ-PE, anti-pSTAT1-Alexa Fluor 647, and anti-pSTAT3-Alexa Fluor 647 were purchased from BD. For intracellular staining, cells were stimulated for 4 hr with PMA and ionomycin with the addition of brefeldin A (GolgiPlug; BD). Afterward, cells were fixed with 4% formyl saline, permeabilized with 0.1% saponin buffer, and stained with fluorescent antibodies before analyzing on a FACS Calibur (BD). Events were collected and analyzed with Flow Jo software (Tree Star).

In Vivo Generation of Th17 Cells

Sorted naive $CD45.2^+$ OT-II $CD4^+$ T cells were primed with or without IL-27 (20 ng ml^{-1}) for 3 hr. After washing, 5.0×10^5 cells were transferred together with sorted naive $CD45.1^+$ OT-II $CD4^+$ T cells (1.0×10^5 cells) intravenously into C57BL/6J mice. After adoptive transfer, recipient mice were immunized subcutaneously with 50 μg OVA323-339 peptide (Peptides International) in CFA. Seven days after immunization, spleens were harvested and T cells were analyzed by flow cytometry for surface marker and intracellular cytokine production.

Experimental Autoimmune Encephalomyelitis

Sorted naive T cells from C57BL/6J, TCR(OT-II) transgenic, *Cd274*$^{-/-}$, or *Stat1*$^{-/-}$ mice were primed with or without IL-27 (20 ng ml^{-1}) or IFN-γ (20 ng ml^{-1}) for 3 hr. After washing, 2.5×10^6 cells were transferred intravenously together with sorted naive $V\beta11^+$ T cells (5.0×10^5 cells) into *Rag2*$^{-/-}$ mice. After adoptive transfer, recipient mice were immunized subcutaneously with 100 μg MOG35-55 peptide (Peptides International) in CFA on day 0. These mice were scored daily according to the criteria as described previously (Ghoreschi et al., 2010). On day 19 or 20, mononuclear cells were isolated from the spinal cord of a subset of mice, counted, and analyzed for cytokine expression. Cumulative disease scores were calculated as the area under the curve for clinical score of each individual mouse as described previously (Greve et al., 2004).

(C) Data provided represent mean ± SEM of the EAE clinical score of 32 mice pooled from 2 independent experiments (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).
(D–F) CNS-infiltrating $CD4^+$ T cells from recipient mice of two groups from (C) were analyzed on day 19 after immunization. Absolute numbers (D), representative flow cytometric analysis of IL-17A and IFN-γ (E), and absolute numbers of subsets of $CD4^+$ T cells (F) are provided (mean values; NS, not significant).

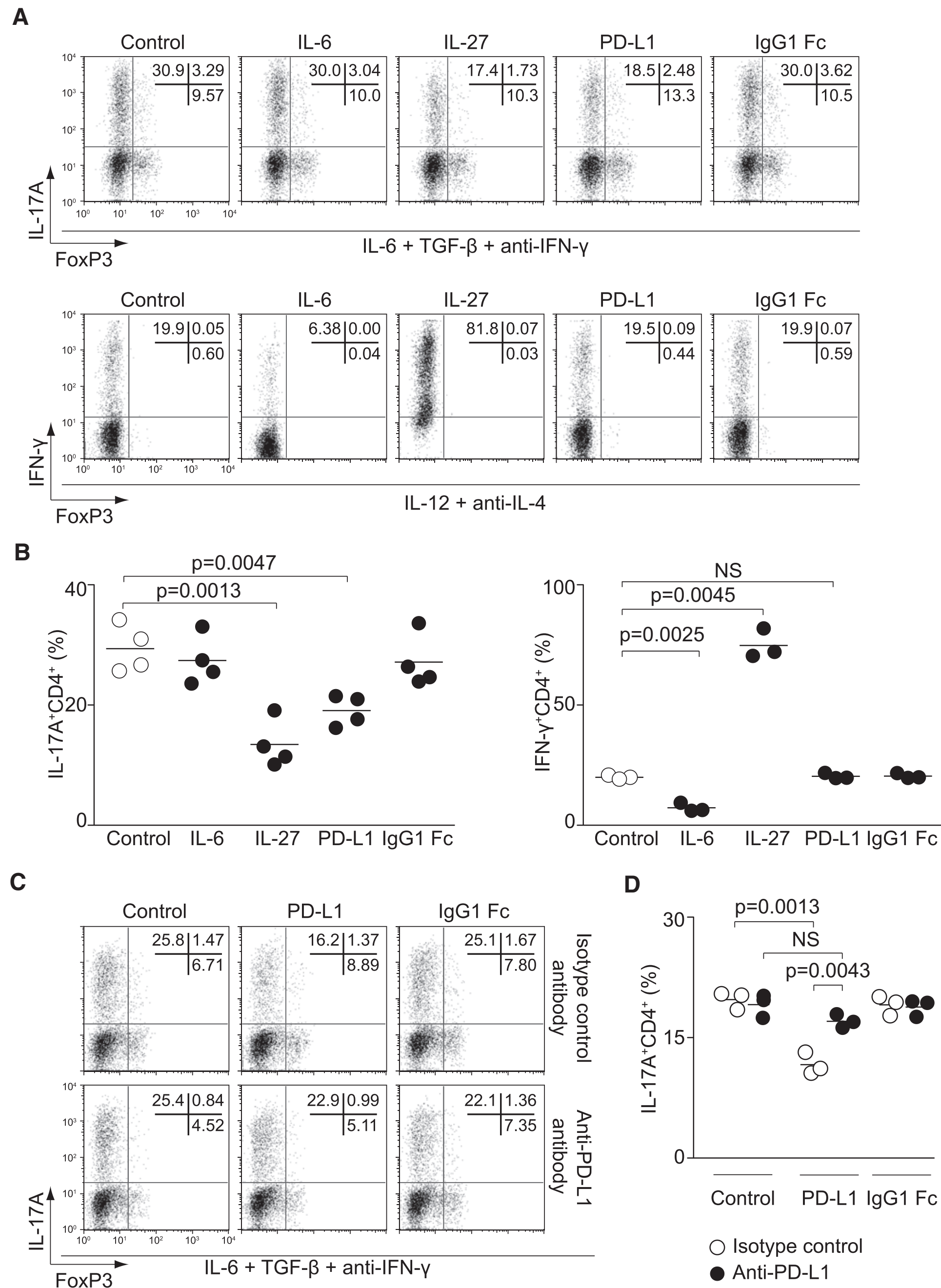

Figure 5. Soluble PD-L1 Affects Th17 Cell Differentiation

(A and B) Naive CD4$^+$ T cells were cultured with IL-6, TGF-β, and anti-IFN-γ (Th17 cell-polarizing conditions, top) or IL-12 and anti-IL-4 (Th1 cell-polarizing conditions, bottom) for 3 days with PD-L1 or IgG1-Fc as a control. IL-27 and IL-6 were added as additional controls. IL-17A, IFN-γ, and FoxP3 protein expression were analyzed by intracellular staining. Representative flow cytometry plots are depicted in (A) and pooled data from three (Th1 cell-polarizing condition) or four (Th17 cell-polarizing condition) individual experiments with mean values are shown in (B) (NS, not significant).

(C and D) Naive CD4$^+$ T cells were cultured under Th17 cell-polarizing conditions with PD-L1 or IgG1-Fc in the presence of PD-L1 antibody (bottom) or isotype control antibody (top) for 3 days. IL-17 and FoxP3 protein expression were analyzed by intracellular staining. Representative intracellular staining is depicted in (C) and pooled data from three individual experiments with mean values are shown in (D) (NS, not significant).

Microarray Data Collection and Analysis

The microarray was performed and analyzed as described previously (Ghoreschi et al., 2010). Approximately 10 μg of RNA was labeled and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) according to the manufacturer's protocols. Expression values were determined with GeneChip Operating Software (GCOS) v1.1.1 and MAS5 method. Downstream analyses were performed with Partek Genomics Suite software and the statistical package R.

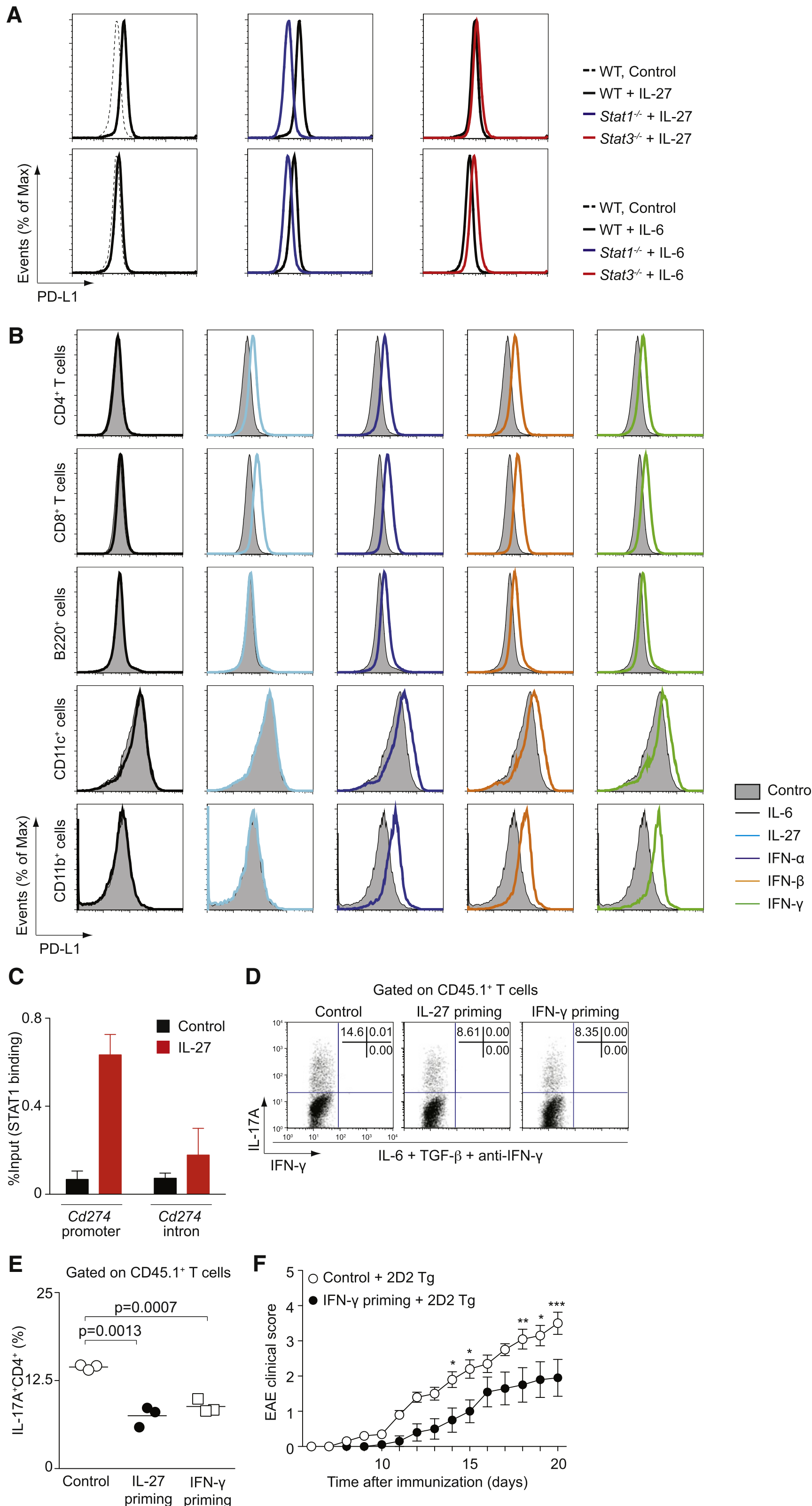

Figure 6. Rapid Induction of PD-L1 by IL-27 Is Directly Mediated by STAT1

(A) Naive CD4+ T cells from *Stat1*$^{-/-}$, *Stat3*$^{-/-}$, or wild-type control mice were stimulated with the indicated cytokines for 3 hr. Surface expression of PD-L1 is shown.

(B) Naive CD4+ T cells, freshly isolated CD8+ T cells, B220+ cells, CD11c+ cells, and CD11b+ cells were stimulated with indicated cytokines for 3 hr and expression of PD-L1 was determined by flow cytometry. Representative data are provided from two separate experiments.

(C) Naive CD4+ T cells were stimulated with IL-27 for 30 min. Fixed cells were immunoprecipitated with STAT1 antibody. Eluted DNA was analyzed by quantitative PCR (mean ± SD). Representative data are provided from two separate experiments.

(D and E) In brief, naive CD45.2+CD4+ T cells were primed with the indicated cytokines. After washing, cells were mixed with naive CD45.1+CD4+ T cells and cultured with IL-6, TGF-β, and anti-IFN-γ for 3 days. IL-17A and IFN-γ production in CD45.1+CD4+ T cells was assessed by intracellular staining. Representative intracellular staining is shown for Th17 cells (D). Pooled data from three independent experiments are provided in (E) (mean values).

(F) Data show mean ± SEM of the EAE clinical score of 20 mice pooled from 2 independent experiments (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

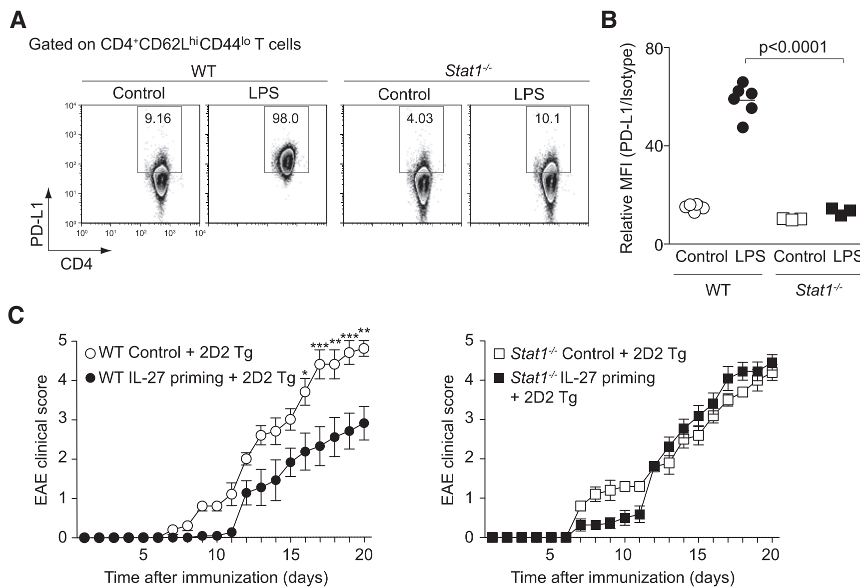

Figure 7. STAT1 Is Critical for Induction of PD-L1 and Inhibition of Th17 Cell Differentiation In Vivo

(A and B) Wild-type or *Stat1*$^{-/-}$ mice received an intraperitoneal injection of LPS with PBS. One day after injection, spleens were harvested and CD4$^+$ T cells were analyzed by flow cytometry for PD-L1 expression. Representative results are shown in (A) and pooled data from two independent experiments are provided in (B) (mean values).

(C) Data show mean ± SEM of the EAE clinical score of 32 mice pooled from 2 independent experiments (***p < 0.001, **p < 0.01, *p < 0.05).

T. gondii Infections

The ME49 strain of *T. gondii* was maintained in Swiss Webster and CBA/CaJ, and tissue cysts were prepared as described (Villarino et al., 2003). For infections, mice were administered 10 tissue cysts orally and sacrificed within 14 days postinfection. All experiments were conducted according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Chromatin Immunoprecipitation

ChIP was performed as described previously (Ghoreschi et al., 2010). Sorted naive CD4$^+$ T cells were activated with or without IL-27 (20 ng ml^{-1}) for 30 min followed by cross-linking for 10 min with 1% formaldehyde. The cells were harvested and lysed by sonication. After preclearing with protein A agarose beads (Upstate), cell lysates were immunoprecipitated with anti-STAT1 (Cell Signaling) overnight at 4°C. After washing and elution, cross-links were reversed at 65°C for 4 hr. The eluted DNA was purified, and samples were analyzed by quantitative-PCR with custom-designed primers and probes via a 7500 real-time PCR system (Applied Biosystems). Primers spanning the promoter region of *Cd274* and the intronic region of *Cd274* are described in Supplemental Experimental Procedures. The Ct value of each sample was normalized to the corresponding input value.

LPS Challenge

Mice received an intraperitoneal injection of 1 μg of LPS (*Escherichia coli* 0111:B4 LPS; Sigma L2630) in 200 μl of PBS. 18 hr after injection, T cells isolated from spleens were analyzed by flow cytometry for surface marker.

Statistics

For statistical analysis, all p values in clinical scores of EAE experiments were calculated with a two-way ANOVA. All others were calculated with a two-tailed Student's t test.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE38375.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2012.03.024.

ACKNOWLEDGMENTS

We thank M. Pelletier (Autoimmunity Branch, NIAMS), J. Simone, J. Lay (Flow Cytometry Section, NIAMS), and the NIAMS LACU staff for their excellent technical support. We thank A. Villarino for critically reading this manuscript and providing helpful suggestions. This work was supported by the Intramural Research Programs of NIAMS, NIAID, the JSPS Research Fellowship for Japanese Biomedical and Behavioral Researchers at NIH (K.H. and H.T.), Istituto Pasteur-Fondazione Cenci-Bolognetti (G.S.), NIH R01 AI 42334 (C.A.H.), NIH R01 AI 40614 (A.H.S.), and NIH R37 AI 38310 (A.H.S.). K.H. and K.G. designed, performed, analyzed, and interpreted all the experiments and wrote the manuscript. X.-P.Y., H.T., A.L., G.S., A.O'H.H., C.D.D., L.M.F., Q.C., and M.T. helped with performing experiments. H.-W.S. and G.V. interpreted the microarray experiments and ChIP-seq data. Y.K., A.H.S., and C.A.H. contributed to the experimental design and data interpretation and made helpful suggestions. J.J.O'S. contributed to experiment design, analyzed and interpreted all acquired data, and helped to write the manuscript.

Received: July 12, 2011
Revised: December 27, 2011
Accepted: March 21, 2012
Published online: June 21, 2012

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