

Prevention of type 1 diabetes through infection with an intestinal nematode parasite requires IL-10 in the absence of a Th2-type response

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Helminth infection can prevent type 1 diabetes (T1D); however, the regulatory mechanisms inhibiting disease remain largely undefined. In these studies, nonobese diabetic (NOD) IL-4^{-/-} mice were infected with the strictly enteric nematode parasite, *Heligmosomoides polygyrus*. Short-term infection, 5–7 weeks of age, inhibited T1D onset, as late as 40 weeks of age. CD4⁺ T-cell STAT6 phosphorylation was inhibited, while suppressed signal transducer and activator of transcription 1 phosphorylation was sustained, as were increases in FOXP3⁻, CD4⁺ T-cell interleukin (IL)-10 production. Blockade of IL-10 signaling in NOD-IL-4^{-/-}, but not in NOD, mice during this short interval abrogated protective effects resulting in pancreatic β -cell destruction and ultimately T1D. Transfer of CD4⁺ T cells from *H. polygyrus* (Hp)-inoculated NOD IL-4^{-/-} mice to NOD mice blocked the onset of T1D. These studies indicate that Hp infection induces non-T-regulatory cells to produce IL-10 independently of STAT6 signaling and that in this Th2-deficient environment IL-10 is essential for T1D inhibition.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease where pancreatic β cells, which produce insulin, are selectively destroyed. Various immune cells have been implicated, including CD4⁺ T cells, CD8⁺ T cells, and innate immune cells. These cells can mediate a tissue-damaging inflammatory Th1-type immune response, characterized by elevations in the transcription factor T-bet, interleukin (IL)-12, interferon- γ (IFN- γ), and phosphorylation and subsequent dimerization of the transcription factor, signal transducer and activator of transcription 1 (STAT1).^{1–4} Both genetic and environmental factors appear to contribute to susceptibility as identical twins show only a 40–60% concordance rate for T1D onset.⁵ Furthermore, the incidence of T1D is increasing in more developed countries at rates greater than can be explained by genetic changes.^{6–8} One possible environmental explanation, referred to as the hygiene hypothesis,^{9,10} suggests that vaccination and better sanitation, resulting in decreased chronic parasitic infections, can impair the development of robust immune regulatory networks, favoring autoimmune inflammatory reactions. Consistent with this possibility, a number of studies have now shown that chronic

parasitic helminth infection in the nonobese diabetic (NOD) mouse, which spontaneously develops T1D, can dramatically reduce the onset of diabetes.^{11–14} A number of components of the helminth-induced immune response, including both cell surface and secreted molecules, have been proposed as potential agents that down modulate the Th1-type inflammatory response that mediates β -cell destruction.^{4,15} Most recently, infection of NOD-IL-4^{-/-} mice with *Litosomoides sigmodontis* remarkably showed that the development of diabetes is still prevented despite blockade of the Th2-type response.¹⁶ As yet, the mechanism of helminth-induced Th2-independent control of T1D remains unclear.

Chronic infection of NOD mice with the intestinal nematode parasite, *Heligmosomoides polygyrus*, can prevent T1D through blockade of the inflammatory Th1-type response resulting in pancreatic β -cell destruction.^{11,13} This is consistent with previous studies indicating that intestinal immune responses can modulate harmful inflammation contributing to T1D^{17,18} and suggests that oral helminth administration may be a basis for future treatments of T1D. As *H. polygyrus* (Hp) is strictly enteric and adults reside indefinitely in the host intestinal lumen, it may

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continuously release stimuli that control the harmful inflammatory response causing β -cell destruction. The immune response to Hp is characterized by a potent polarized Th2-type response with pronounced elevations in IL-4 and IL-13.^{19,20}

In the present study, we examined whether short-term infection with *H. polygyrus* can prevent subsequent development of T1D and also examined potential mechanisms of Hp-induced immune protection in NOD-IL-4^{-/-} mice. Our studies demonstrated that infection with Hp for only 2 weeks (5–7 weeks of age) was sufficient to induce long-term protection from T1D in NOD and NOD-IL-4^{-/-} mice, where STAT6 phosphorylation, an indicator of IL-4R signaling, was effectively blocked. Inhibition of IL-10 for weeks 5–7 of age was sufficient to prevent Hp-induced control of insulinitis and T1D development in NOD-IL-4^{-/-} mice but not NOD mice, while combined blockade of IL-4 and IL-10 signaling inhibited Hp-induced prevention of insulinitis in NOD mice. These studies indicate that Hp infection can control development of T1D through independent mechanisms involving either IL-4 or IL-10.

RESULTS

Hp infection markedly reduces T1D and β -islet cell infiltration in NOD-IL-4-deficient mice

Previous studies have demonstrated that infection with the intestinal nematode parasite, Hp, can prevent T1D in female NOD mice.^{11,13} To examine whether enteric Hp-induced control of T1D was IL-4-dependent, we orally inoculated 5-week-old female NOD and NOD-IL-4^{-/-} mice with Hp L3 and assessed serum glucose levels every 2 weeks until 40 weeks of age. As shown in **Figure 1a**, 100% of untreated NOD mice developed diabetes compared with only 10% of Hp-inoculated NOD mice. Similarly, 85% of untreated female NOD-IL-4^{-/-} mice developed diabetes by 40 weeks of age compared with 15% of Hp-inoculated NOD-IL-4^{-/-} mice (**Figure 1b**). Lymphoid infiltration of the islet cells progresses from mild peri-insulinitis to severe insulinitis ultimately resulting in islet cell destruction and T1D. Histological analysis of β -cell infiltration was performed with Hp-inoculated NOD-IL-4^{-/-} mice and untreated NOD-IL-4^{-/-} mice at 13 weeks of age, a time point when insulinitis is readily detectable but has not yet resulted in total β -cell destruction. As shown in **Figure 1c** and **d**, Hp inoculation of NOD-IL-4^{-/-} mice at 5 weeks of age markedly reduced both invasive insulinitis and more mild peri-insulinitis.

As previously reported in Hp-inoculated NOD mice,¹¹ Hp infection of NOD-IL-4^{-/-} mice generally reduced inflammatory infiltrates without obvious selective changes in specific innate or adaptive immune cell populations (data not shown). Consistent with recent studies,¹⁶ serum IgE and IgG1 elevations were effectively blocked in Hp-inoculated NOD-IL-4^{-/-} mice (**Supplementary Figure 1s** online). Furthermore, serum IgG2a elevations, which are dependent on IFN- γ ,^{21,22} were not increased, suggesting that immune deviation towards an alternative Th1-type response did not develop. As shown in **Supplementary Figure 1s** online, increases in B-cell surface MHCII, which is IL-4 dependent in Hp-inoculated mice,^{23,24} were also blocked.

Cytokine gene expression signature is altered by 2 weeks after Hp inoculation in NOD-IL-4^{-/-} mice

The finding that the development of insulinitis and diabetes was effectively inhibited in Hp-inoculated NOD-IL-4^{-/-} mice raised the possibility that components of this helminth-induced response that regulate harmful Th1-type inflammatory responses may remain intact even in the absence of the cardinal Th2 cytokine, IL-4. Previous studies have shown that Hp stimulates a potent response between 1–2 weeks after Hp inoculation in WT mice.^{20,25} Pancreatic lymph nodes (PLNs), mesenteric lymph nodes (MLNs), and spleens were examined for cytokine gene expression in NOD-IL-4^{-/-} mice at 2 weeks after Hp inoculation. As shown in **Figure 2**, significant decreases in T-bet, IFN- γ , and IL-12 were observed in secondary lymphoid tissues, particularly those draining the pancreas (PLN) of Hp-inoculated NOD and NOD-IL-4^{-/-} mice. In contrast, elevations in the Th2 cytokines IL-4 (primers used for messenger RNA analysis outside of altered DNA in KO construct), IL-5, and IL-13 were markedly depressed in draining lymph nodes of Hp-inoculated NOD-IL-4^{-/-} mice compared with inoculated NOD mice. Intriguingly, the regulatory cytokine, IL-10, was markedly elevated in lymph nodes from Hp-inoculated NOD and NOD-IL-4^{-/-} mice compared with untreated controls, suggesting that this component of the immune response to Hp is IL-4 independent. Elevations in transforming growth factor- β messenger RNA were not detected in either the PLN or the MLN, but were increased in the spleen of Hp-inoculated NOD-IL-4^{-/-} mice, while expression of FoxP3 was generally elevated after Hp inoculation.

CD4⁺ T-cell STAT6 and STAT1 phosphorylation in lymphoid tissues

STAT1 phosphorylation following IFN- γ signaling and STAT6 phosphorylation following IL-4R signaling are important steps in the activation of Th1 and Th2 cells, respectively.²⁶ Recent studies have shown that flow cytometric analysis of STAT6 and STAT1 phosphorylation on CD4⁺ T cells can be effectively used as markers of IL-4/IL-13 (both of which signal through the type 2 IL-4R) signaling^{25,27} and IFN- γ signaling,^{25,28} respectively. NOD and NOD-IL-4^{-/-} mice were inoculated with Hp at 5 weeks of age, and phospho-STAT1 and phospho-STAT6 levels in CD4⁺ T cells isolated from lymphoid tissues were assessed 2 weeks later at 7 weeks of age. As shown in **Figure 3a**, CD4⁺ T-cell phospho-STAT6 was markedly upregulated in the PLN, MLNs, and spleen of Hp-inoculated NOD mice. In contrast, CD4⁺ T-cell phospho-STAT6 expression was not elevated in draining lymph nodes of Hp-inoculated NOD-IL-4^{-/-} mice (**Figure 3a**). Analysis of IFN- γ -dependent CD4⁺ T-cell STAT1 phosphorylation showed marked reductions in the PLNs, MLNs, and spleens of Hp-inoculated NOD and NOD-IL-4^{-/-} mice (**Figure 3b**), indicating that Hp-induced downregulation of IFN- γ signaling in NOD-IL-4^{-/-} mice was suppressed even in the absence of STAT6 signaling. Isotype control staining was <0.2% (data not shown; also see **Supplementary Figure 3s** online).

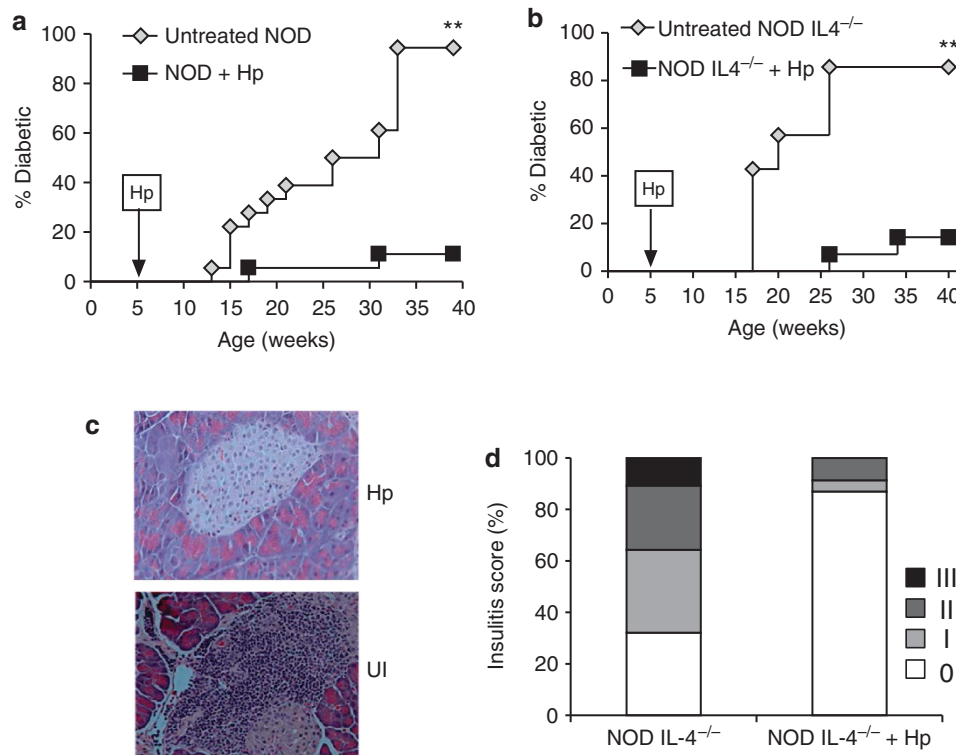


Figure 1 Helminth-mediated control of type 1 diabetes is interleukin (IL)-4 independent. Nonobese diabetic (NOD) (16/treatment group) and NOD-IL-4^{-/-} (14/Hp treated; 7/untreated) mice were orally inoculated with 200 *H. polygyrus* (Hp) L3 at 5 weeks of age. Blood glucose levels were monitored every 2 weeks and mice with values >200 mg dl⁻¹ on two consecutive occasions were considered diabetic. Percentages of treatment groups with diabetes were plotted against age in (a) NOD mice and (b) NOD-IL-4^{-/-} mice. In separate experiments, NOD-IL-4^{-/-} mice were inoculated with Hp as described in a and islet cells were examined for lymphoid infiltration at 13 weeks. (c) Representative islets from Hp-inoculated and untreated NOD-IL-4^{-/-} mice. (d) Pancreata isolated from 13-week-old NOD-IL-4^{-/-} mice were scored using the following scale: grade 0 (no insulinitis)=0% infiltration; grade I (peri-insulinitis)=1–10% infiltration; grade II (moderate insulinitis)=11–50% infiltration; and grade III (severe insulinitis)= >50% infiltration. We typically counted 50–100 islets per experiment from six mice depending on the number of islets that were present in the sections. All data are representative of two independent experiments. ***P*<0.01. UI, uninfected.

Short-term Hp infection effectively controls pancreatic β -cell destruction and development of T1D

Our finding that Hp inoculation of NOD and NOD-IL-4^{-/-} mice markedly downregulated components of the Th1-type inflammatory immune response by 2 weeks after infection raised the possibility that this may be a sufficient treatment window to modulate the development of disease. By 8 days after inoculation, all Hp parasites reside in the lumen and complete expulsion is achieved by oral administration of the anti-helminthic drug, pyrantel pamoate.^{29,30} At 5 weeks of age, NOD and NOD-IL-4^{-/-} mice were inoculated with Hp and 2 weeks later (7 weeks of age) all parasites were expelled by oral administration of pyrantel pamoate. Pyrantel pamoate was similarly administered to uninfected control groups to exclude any unexpected effects of this drug treatment; pyrantel pamoate-treated NOD mice showed similar development and kinetics of T1D to untreated NOD mice. Mice in the different groups were then followed for 40 weeks or until diabetes was diagnosed. As shown in **Figure 4a** and **b**, NOD and NOD-IL-4^{-/-} mice infected with Hp from age 5–7 weeks showed inhibition of diabetes at 40 weeks, with 80% of untreated NOD and NOD-IL-4^{-/-} mice developing T1D

compared with 10% of Hp-inoculated NOD mice and 5% of Hp-inoculated NOD-IL-4^{-/-} mice. We also evaluated pancreatic insulinitis in these experiments at 13 weeks after inoculation, as also shown in **Figure 4c** and **d**. Short-term Hp infection led to inhibition of severe and moderate insulinitis in pancreatic islets with a similar pattern to that seen with our long-term infection model (see **Figure 1d**).

Autoantigen-specific T cells show increased IL-10 and decreased IFN- γ after Hp inoculation in NOD-IL-4^{-/-} mice

Autoantigen (AA)-specific CD4 T cells are known to contribute to β -cell destruction, in part through IFN- γ production.³¹ PLNs from NOD and NOD-IL-4^{-/-} mice either untreated or inoculated with Hp for 2 weeks (5–7 weeks of age) were assessed, using an enzyme-linked immunosorbent spot (ELISPOT) assay, for responsiveness to previously described β -cell AAs.^{32–35} As shown in **Figure 5**, cell suspensions cultured with anti-CD3Ab or β -cell Ags from control NOD (**Figure 5a**) and NOD-IL-4^{-/-} (**Figure 5b**) mice showed marked increases in IFN- γ at 7 weeks of age while Hp-inoculated groups showed decreased IFN- γ and markedly increased IL-10 secretion. These findings suggest that

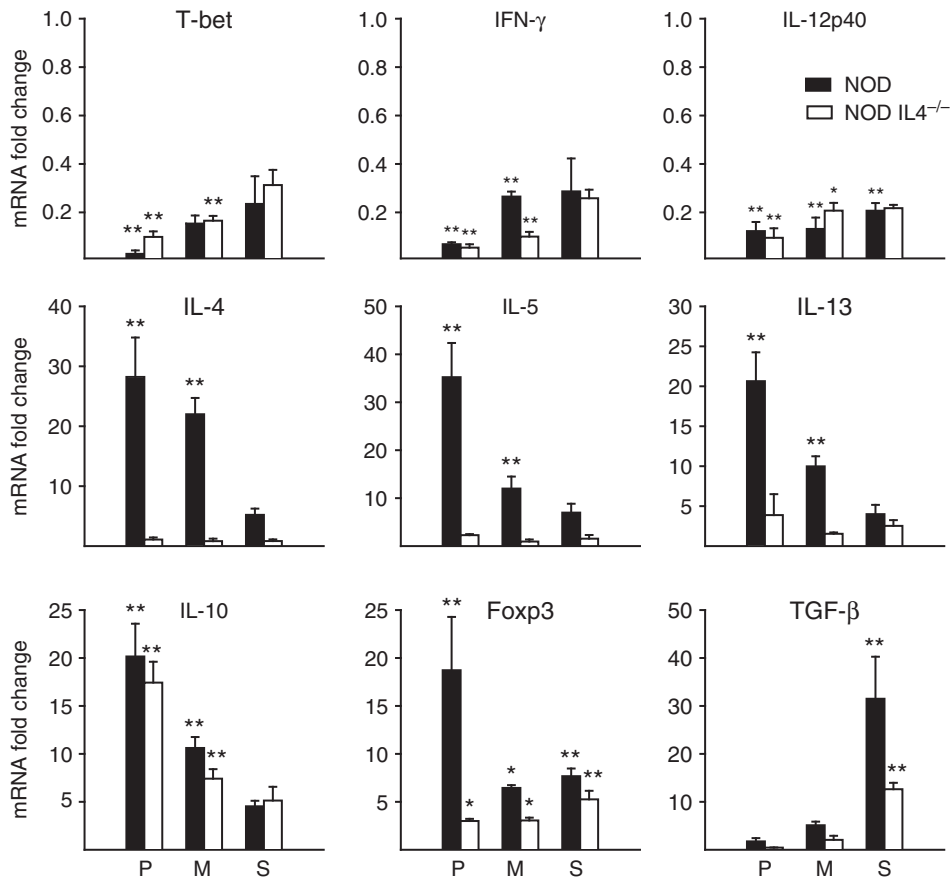


Figure 2 Elevations in Th2 cytokines were blocked while interleukin (IL)-10 elevations were sustained, and Th1-type cytokines and transcription factors remained inhibited in *H. polygyrus* (Hp)-inoculated nonobese diabetic (NOD)-IL-4^{-/-} mice. NOD and NOD-IL-4^{-/-} mice were inoculated with 200 Hp L3 at 5 weeks of age and lymphoid tissues (pancreatic lymph node (P), mesenteric lymph node (M), and spleen (S)) were harvested 2 weeks later. All gene expression data were determined by quantitative real-time PCR, and are expressed as fold changes relative to untreated controls. Data are shown as the mean \pm s.e.m. of four different mice per treatment group and are representative of two independent experiments. **P* < 0.05, ***P* < 0.01 compared with untreated NOD or NOD-IL-4^{-/-} mice.

short-term Hp infection causes IFN- γ -producing autoreactive Th1 cells to instead produce IL-10 and that this remodeling effect occurs independently of IL-4.

CD4⁺ effector T-cell phenotype is the major source of IL-10

Our findings that infection of NOD and NOD-IL-4^{-/-} mice with Hp for only a 2-week interval was sufficient to block pancreatic β -cell destruction, and T1D suggested that potent, sustained IL-4-independent immune regulatory mechanisms were initiated during this brief time interval. The pronounced elevation of IL-10 in Hp-inoculated NOD and NOD-IL-4^{-/-} mice raised the possibility that IL-10-producing cells may be controlling the development of the Th1-type response and subsequent β -cell destruction. To further investigate IL-10-expressing cell populations, CD4⁺ T cells were electronically sorted (99% pure) from pooled samples from PLNs, MLNs and spleens of Hp-inoculated NOD-IL-4^{-/-} mice. As shown in **Figure 6a**, marked increases in CD4⁺ T-cell IL-10 expression was observed relative to either CD4⁻ T cells or total cell suspensions from untreated NOD-IL-4^{-/-} mice. To specifically examine whether T-regulatory cells or T-effector cells

were expressing IL-10, magnetic bead-isolated CD4⁺ T cells from NOD-IL-4^{-/-} mice were electronically sorted for CD127 (IL-7R)^{hi} and CD127^{lo} populations. Using high-speed cell sorting (FACS Aria), CD4⁺ T cells were sorted into CD127^{lo} (>99% pure) and CD127^{hi} (95% pure) cell populations. CD127^{hi} cells expressed 33–60-fold increases in IL-10 compared with 3–8-fold increases in CD127^{lo} cells relative to cell suspensions from uninfected mice. (**Figure 6b**). Elevations in IL-5 and IL-13 were at most less than threefold while IFN- γ was generally decreased (data not shown). Previous studies have shown that FoxP3⁺, CD4⁺ T-regulatory cells are predominantly CD127^{lo} while CD4⁺ T-effector cells are CD127^{hi}.^{36,37} To examine whether this is also the case in Hp-inoculated NOD-IL-4^{-/-} mice, sorted CD4⁺, CD127^{hi}, and CD127^{lo} cells (**Supplementary Figure 2s** online) were analyzed for FoxP3 gene expression using quantitative fluorescent real-time (RT)-PCR. As shown in **Figure 6c**, FoxP3 gene expression was primarily expressed in the CD127^{lo} population. Fluorescence-activated cell sorting (FACS) analysis showed that cytoplasmic FoxP3 was primarily expressed in CD4⁺, CD127^{lo} T cells (**Figure 6d**), corroborating our findings with FoxP3 gene expression. Cytoplasmic IL-10

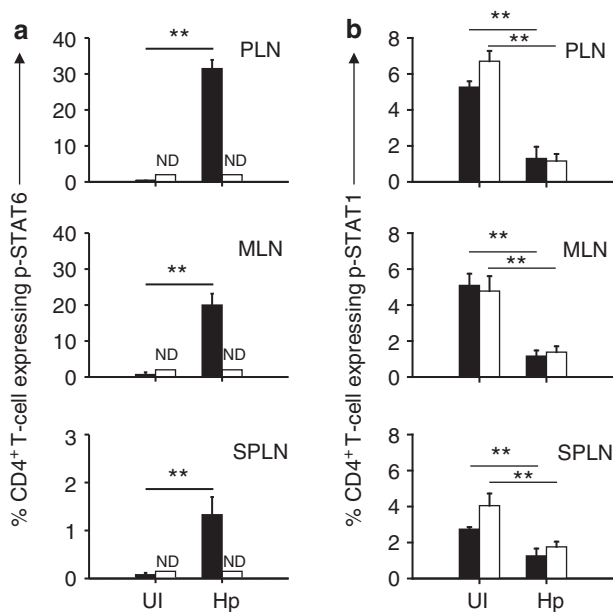


Figure 3 Decreased CD4⁺ T-cell signal transducer and activator of transcription 1 (STAT1) phosphorylation was sustained in *H. polygyrus* (Hp)-inoculated nonobese diabetic (NOD)-interleukin (IL)-4^{-/-} mice even without elevations in CD4⁺ T-cell STAT6 phosphorylation. NOD (■) and NOD-IL-4^{-/-} (□) mice were inoculated with Hp at 5 weeks of age and 2 weeks later cell suspensions from draining lymph nodes were stained for CD4 and phosphorylated STAT6 (pY641) or phosphorylated STAT1 (pY701). Flow cytometric analysis was performed on stained cell suspensions from pancreatic lymph nodes (PLN), mesenteric lymph nodes (MLN), and spleen (SPLN) of uninfected (UI) and Hp-inoculated mice. Percentage of CD4⁺ T cells expressing (a) pSTAT6 or (b) pSTAT1 are shown as the mean ± s.e.m. of four different mice per group. These data are representative of two independent experiments. ***P* < 0.01. ND, not detected.

protein expression was also assessed and found to be increased in CD4⁺, CD127^{hi} T cells (Figure 6e). To corroborate these results, IL-10 ELISPOT assays were performed on sorted CD4⁺, CD127^{hi}, and CD4⁺, CD127^{lo} subpopulations from Hp-inoculated NOD IL-4^{-/-} mice. As shown in Figure 6f, in either unstimulated sorted cells or following restimulation with either anti-CD3 or β-islet cell AA, IL-10 was primarily expressed in the CD127^{hi} population, although IL-10 was also expressed at lower levels by conventional T-reg cells. These findings indicate that IL-10 is predominantly expressed by a CD127^{hi}, FoxP3⁻ CD4⁺ T cell following Hp infection.

Blocking IL-10R signaling preferentially enhances Th1-type cytokine expression and CD4 T-cell STAT1 phosphorylation in NOD-IL-4^{-/-} mice after Hp inoculation

To directly examine whether IL-10 was contributing to down modulation of the Th1-type response shortly after Hp inoculation, blocking anti-IL-10R Ab was administered. NOD and NOD-IL-4^{-/-} mice were inoculated with Hp and injected intravenously with either IL-10R blocking antibody (1B1.3A) or IgG1 isotype control antibody at 5 weeks of age. At age 7 weeks, draining lymph nodes and spleen were collected and assessed for

Th1-type cytokines and transcription factors. As shown in Figure 7, blocking IL-10R signaling in Hp-inoculated mice resulted in increases in Tbet, IFN-γ, and IL-12B gene expression in both NOD and NOD-IL-4^{-/-} mice. However, substantially more pronounced increases were observed in Hp-inoculated NOD-IL-4^{-/-} mice, suggesting that other factors associated with IL-4 production in the NOD mice provided significant control of the Th1-type response in the absence of IL-10R signaling. Similarly, CD4 T-cell STAT1 phosphorylation was markedly increased in draining lymph nodes and spleen of Hp-inoculated NOD-IL-4^{-/-} mice, but not inoculated NOD mice, administered blocking anti-IL-10R Ab (Supplementary Figure 3s, B online); pSTAT1 was also increased in the corresponding CD4⁻ cell population. Isotype control Ab staining was < 0.2% (Supplementary Figure 3s, C online).

Blocking IL-10R during short-term Hp infection blocks protective effect in NOD-IL-4^{-/-} but not NOD mice

Our observation that enteric Hp infection for 2 weeks was sufficient to control islet destruction suggested that immune regulatory mechanisms activated during this period were sufficient to control disease. Our findings that Hp-induced IL-10 could control mediators of the autoreactive Th1-type response 2 weeks after Hp inoculation raised the possibility that IL-10 might have an important role during this short interval in remodeling immune function to control β-cell destruction. To test this possibility, NOD and NOD-IL-4^{-/-} mice were inoculated with Hp at 5 weeks and administered IL-10R antibody or isotype control Ab intravenously 1 day before Hp inoculation and every 2 days thereafter for two weeks. Inoculated NOD and NOD-IL-4^{-/-} mice given antibody were either treated with an anti-helminthic agent 2 weeks after inoculation (age 7 weeks) or not treated, resulting in chronic infection. Prevention of insulinitis was not reduced in groups given short-term infection compared with groups with chronic infection. NOD-IL-4^{-/-} mice inoculated with Hp for 2 weeks and administered anti-IL-10R Ab developed pronounced increases in β-cell destruction (Figure 8a). We further assessed serum glucose levels every 2 weeks until 36 weeks of age for similarly treated NOD-IL-4^{-/-} mice to further examine the effect of β-cell destruction on the occurrence of diabetes. As shown in Figure 8b, 71% (experimental treatment 1) and 86% (experimental treatment 2) of two groups of Hp-inoculated NOD-IL-4^{-/-} mice administered anti-IL-10R Ab (age 5–7 weeks) developed diabetes by 36 weeks of age compared with 11% of Hp-inoculated NOD-IL-4^{-/-} mice administered the IgG1 isotype control. These findings suggest that IL-10 is essential for Hp-induced control of β-cell destruction and subsequent development of T1D in the absence of IL-4, but in IL-4-sufficient mice other pathways can compensate for IL-10 blockade.

To examine whether short-time simultaneous blockade of IL-4 and IL-10 signaling in NOD mice also blocked Hp-mediated control of T1D, NOD mice were inoculated with Hp at 5 weeks of age and administered blocking anti-IL-4 (11B11) Ab and anti-IL-10R Ab from weeks 5–7. As shown in Figure 8c, analysis of insulinitis at 13 weeks of age showed that only combined

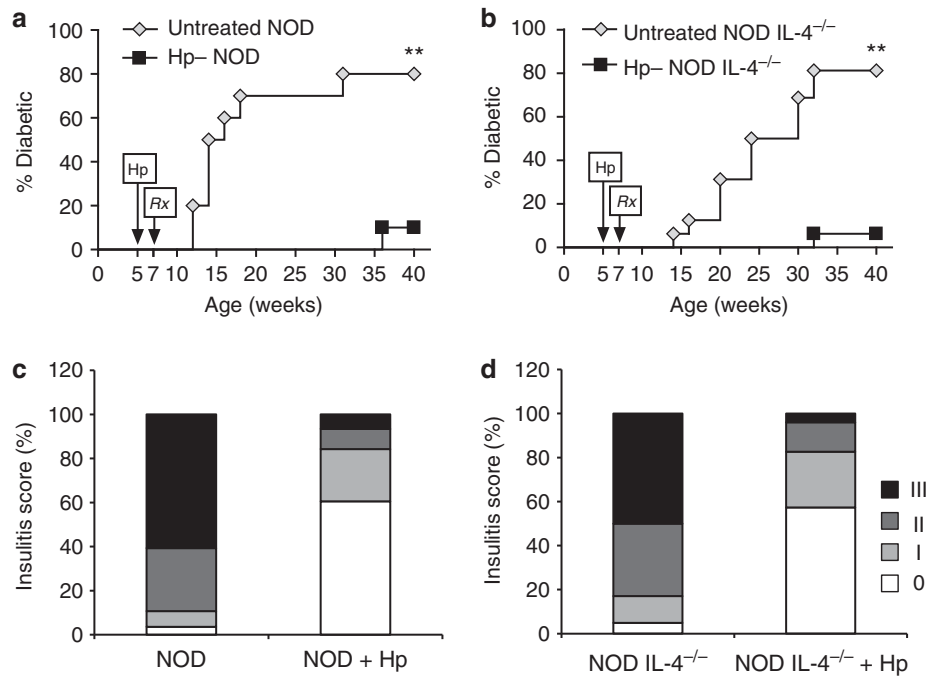


Figure 4 Short-term infection with *H. polygyrus* (Hp) inhibited the development of type 1 diabetes in nonobese diabetic (NOD) and NOD-interleukin (IL)4^{-/-} mice. NOD (10/treatment group) and NOD-IL-4^{-/-} (16/treatment group) mice were inoculated with Hp at 5 weeks of age, and at 7 weeks worms were expelled with the anti-helminthic drug, pyrantel pamoate, which was also administered to uninfected controls. Blood glucose levels were monitored every 2 weeks and mice with values >200 mg dl⁻¹ on two consecutive occasions were considered diabetic. Percentages of treatment groups with diabetes were plotted against age in (a) NOD mice and (b) NOD-IL-4^{-/-} mice. In a separate experiment, (c) NOD and (d) NOD-IL-4^{-/-} mice were inoculated with Hp at 5 weeks, treated with pyrantel pamoate at 7 weeks, and examined for islet cell lymphoid infiltration at 13 weeks and scored as described in **Figure 1d**. Uninfected controls were also administered pyrantel pamoate. This experiment was repeated twice with similar results. ***P*<0.01.

blockade of IL-4 and IL-10 was sufficient to abrogate Hp-induced inhibition of insulinitis, while administration of either anti-IL-4 or anti-IL-10R Ab alone had little effect. These additional experiments corroborate our findings with Hp-inoculated NOD-IL-4^{-/-} mice administered anti-IL10R Ab.

CD4⁺ T cells from Hp-infected NOD-IL-4^{-/-} mice can prevent T1D onset

Our findings that Hp-induced IL-10 could control T1D inflammation and that CD127^{hi} CD4⁺ T cells were the major IL-10 source suggested the possibility that transfer of CD4⁺ T cells from Hp-inoculated mice could confer T1D protection. Magnetic bead-sorted CD4⁺ T cells or CD4⁻ cells (5×10⁷ cells) from lymph nodes of Hp-inoculated NOD-IL-4^{-/-} mice were adoptively transferred to recipient NOD mice at 5 weeks of age. CD4⁻ cells included cells that did not bind the magnetic column during CD4 cell sorting; subsequent flow cytometric analysis showed that this population primarily included CD8 cells and B cells (data not shown). Serum glucose levels were assessed every 2 weeks until 35 weeks of age. As shown in **Figure 8d**, only 16% of recipient NOD mice with CD4⁺ T-cell transfer developed diabetes compared with 83% of recipient NOD mice with CD4⁻ cell transfer and 100% of NOD mice with no adoptive transfer of cells. These findings suggest that CD4⁺ T cells from short-term Hp-infected NOD-IL-4^{-/-} mice, with a deficient Th2-type response, can effectively prevent the development of T1D.

DISCUSSION

These studies demonstrate that short-term (age 5–7 weeks) enteric Hp infection is sufficient to block the development of T1D as late as 40 weeks of age. During this short-time interval, Hp-induced IL-4- and IL-10-producing T cells develop and act independently to modulate the immune response and prevent T1D onset. These findings indicate why in previous studies blockade of IL-4 or IL-10 alone had little effect on inhibiting helminth-induced prevention of T1D. They suggest that the potency of helminth-induced immune responses in controlling inflammatory diseases may be partly due to the simultaneous activation of multiple and, in some cases, redundant immune regulatory pathways.

Our results are consistent with a model where *H. polygyrus* infection triggers IL-10-producing T cells, which down modulate STAT1 phosphorylation and the Th1-type response, thereby inhibiting autoimmune destruction of pancreatic beta cells and the development of T1D. As IL-10 blockade inhibited helminth-induced prevention of T1D in NOD-IL-4^{-/-}, but not in NOD-IL-4^{+/+} mice IL-4-dependent down-modulatory effects can also control T1D, even in the absence of IL-10. Our results demonstrating abrogation of Hp-induced control of β-islet destruction following short-term combined treatment of NOD mice with blocking anti-IL-4 and anti-IL-10R Abs corroborates our studies in NOD-IL-4^{-/-} mice. Taken together, these findings indicate that the immune response that develops

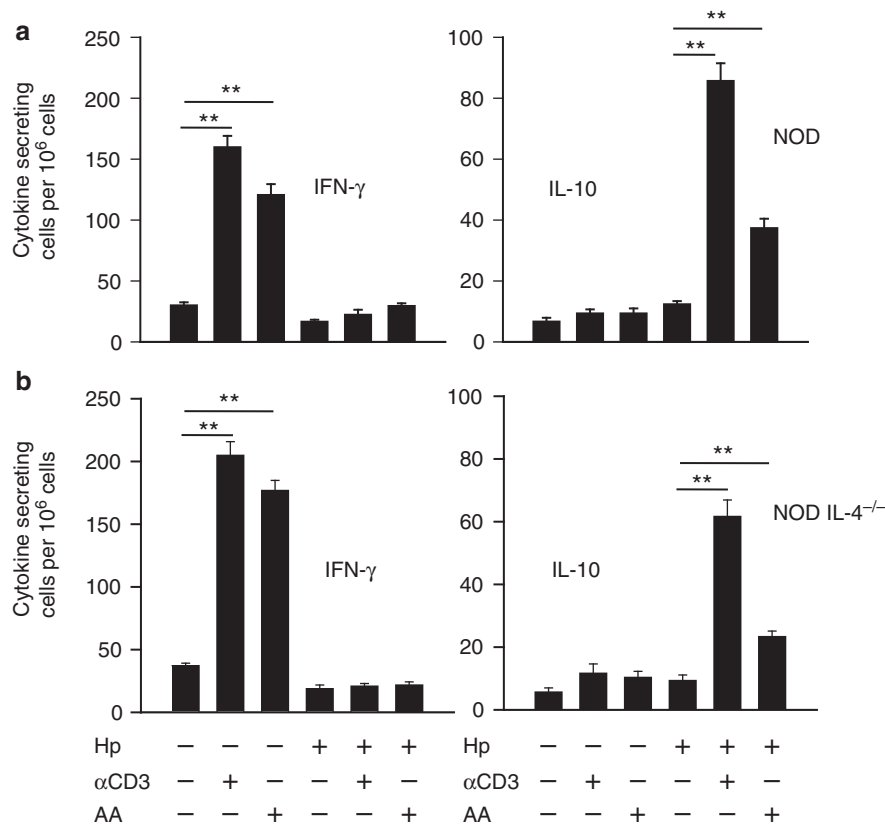


Figure 5 *H. polygyrus* (Hp) inoculation deviated the autoantigen-specific T-cell response from interferon (IFN)- γ to interleukin (IL)-10 production. Nonobese diabetic (NOD) and NOD-IL-4^{-/-} mice were inoculated with Hp at 5 weeks of age. Two weeks later, draining pancreatic lymph nodes were collected and either stimulated with anti-CD3 antibody or a mixture of islet-specific autoantigens (AA) for 48 h after which IFN- γ - and IL-10-secreting cells were assessed by enzyme-linked immunosorbent spot assay in (a) NOD and (b) NOD-IL-4^{-/-} mice. Data are expressed as the mean and s.e. of five mice/treatment group. Similar results were obtained in two independent experiments. ** $P < 0.01$.

following helminth infection triggers the independent activation of IL-4 and IL-10 regulatory pathways, either of which can effectively control the development of T1D. Identification of additional important regulatory pathways involved in helminth-induced control of T1D may also require simultaneous inhibition of multiple redundant pathways. An important exception may be transforming growth factor- β , which has recently been shown to be essential for prevention of T1D in *Litosomoides*-infected NOD mice.¹⁶ It will be important in future studies to examine whether different helminthes may elicit different dominant immunoregulatory pathways. Also, recent findings that the helminth-induced Th2-type immune response can also enhance wound healing³⁸ suggest additional potential ameliorative effects during diabetes.

The redundancy in these IL-4- and IL-10-dependent regulatory pathways, that can control pancreatic islet destruction, may help to explain why previous studies have found little effect of IL-10 blockade on the development of T1D in helminth-infected NOD mice.^{11,16,39} These results also suggest that during helminth infection, IL-10-mediated regulation can develop independently of IL-4-mediated regulatory pathways. This finding may help to explain previous studies indicating that helminth-induced Th2-type responses

can control allergy-associated Th2-type responses,⁴⁰ as the latter response may not include an effective IL-10 response.

IL-10 can be produced by many immune cell types, including B cells, Th1, Th2, and Treg cells, depending on the particular experimental system.⁴¹⁻⁴³ Although CD4⁺, CD127^{lo}, and FoxP3^{hi} cells did express some IL-10, our findings indicate that following Hp infection the predominant source of IL-10 was CD4⁺, CD127^{hi}, and FoxP3^{lo} T cells. This phenotype is consistent with a T-helper effector phenotype, as conventional Treg cells typically express high FoxP3 and low CD127.³⁶ Our observation that increased IL-10 was associated with decreased IFN- γ following AA or anti-CD3 Ab *in vitro* stimulation of lymphocytes from helminth-infected mice raises the possibility that autoreactive effector T-helper cells deviate from a harmful Th1 phenotype to a beneficial IL-10-producing phenotype. This remodulation of the Ag-specific T-effector cell phenotype would essentially replace a deleterious autoreactive T-cell population with a population producing the potent regulatory cytokine IL-10, as recently suggested with naturally occurring CD4 T cells from human diabetic patients.⁴⁴ Alternatively, T-regulatory 1 cells have also been shown to express high levels of IL-10 and low FoxP3,^{45,46} making this population an additional candidate:

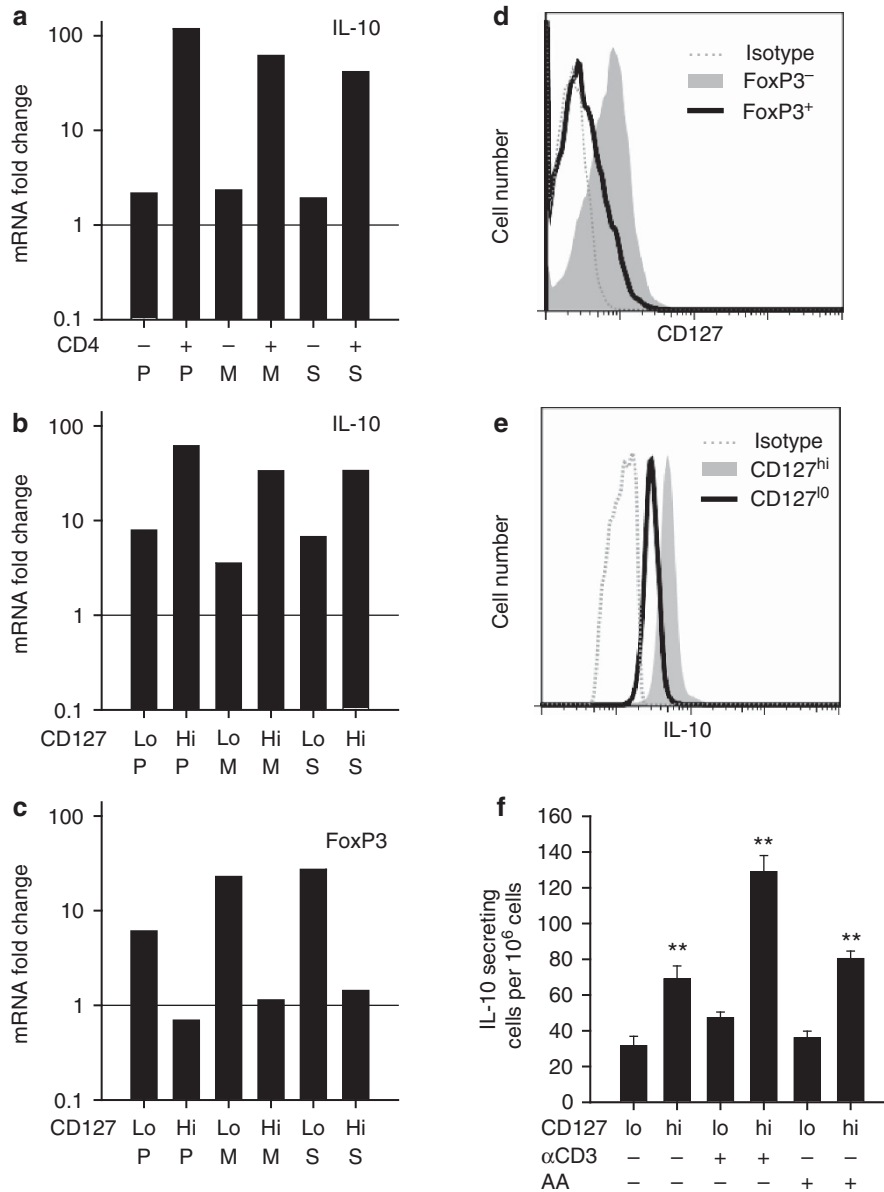


Figure 6 CD4⁺ CD127^{hi} T cells are the major source of interleukin (IL)-10 in *H. polygyrus*-inoculated nonobese diabetic (NOD)-IL-4^{-/-} mice. NOD-IL-4^{-/-} mice (5/treatment group) were inoculated with *H. polygyrus* at 5 weeks of age and 2 weeks later pooled cell suspensions from draining lymph nodes were collected. (a) CD4⁺ and CD4⁻ cells from pooled samples from either pancreatic lymph nodes (P), mesenteric lymph nodes (M), or spleen (S) from *H. polygyrus*-inoculated NOD-IL-4^{-/-} mice were isolated by magnetic bead cell sorting and analyzed for IL-10 gene expression. (b and c) CD4⁺ cells were stained with anti-CD4 and anti-IL-7 receptor (CD127) Abs and electronically sorted for CD4⁺ cells expressing high or low levels of CD127. Sorted populations were analyzed for (b) IL-10 and (c) FoxP3 gene expression. (d) Cells pooled from pancreatic lymph nodes and mesenteric lymph nodes were analyzed for CD127 (IL-7R) in CD4⁺ FoxP3⁺ and CD4⁺ FoxP3⁻ cells. CD4⁺ cells sorted for CD127^{hi} and CD127^{lo} cells were also analyzed for (e) intracellular IL-10, and (f) IL-10 secretion by enzyme-linked immunosorbent spot assay after stimulation with anti-CD3Ab or islet-specific autoantigens (AA). All gene expression data are expressed as fold changes relative to untreated controls, and similar results were obtained in two independent experiments. ***P*<0.01 compared with CD127^{lo} cell population.

T-regulatory 1 cells may actually be an end-stage differentiation state of Th1 cells.⁴²

It should also be considered that our *in vitro* culture system may have biased against conventional T-regulatory cells. Although this possibility cannot be formally excluded, it should be noted that previous studies have shown that Treg cells from NOD mice are responsive to the AAs used⁴⁷ and also to anti-CD3, even in the absence of IL-2.⁴⁸ Taken together, our *in vivo*

and *in vitro* analyses suggest that conventional T-regulatory cells are not the predominant source of IL-10 during Hp infection in the draining lymph nodes. It is also possible that IL-10-producing T-regulatory cells are localized to the beta islet cells after Hp inoculation; previous studies have identified T-regulatory cells in the islet at early stages of insulinitis in NOD mice.⁴⁹ However, few CD4⁺ T cells are actually observed in the beta islet cells of Hp-inoculated mice;¹¹ it may, however, be

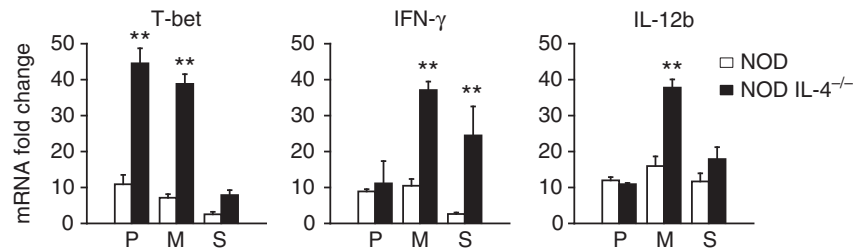


Figure 7 Dual blockade of interleukin (IL)-4 and IL10R blocked suppression of Th1 inflammation. Nonobese diabetic (NOD) and NOD-IL-4^{-/-} mice (5 mice/treatment group) were inoculated with *H. polygyrus* (Hp) and administered α IL-10R Ab (clone 1B1.3a) or IgG1 isotype control Ab at 5 weeks of age. Two weeks later, pancreatic lymph node (P), mesenteric lymph node (M), and spleen (S) were analyzed for T-bet, interferon- γ (IFN- γ), and IL-12 messenger RNA (mRNA) by quantitative real-time PCR. Data are expressed as fold changes of group receiving anti-IL-10R Ab (1B1.3) over group receiving isotype control. The experiments were repeated two times with similar results, and a statistically significant difference between the two different groups was denoted by ** $P < 0.01$.

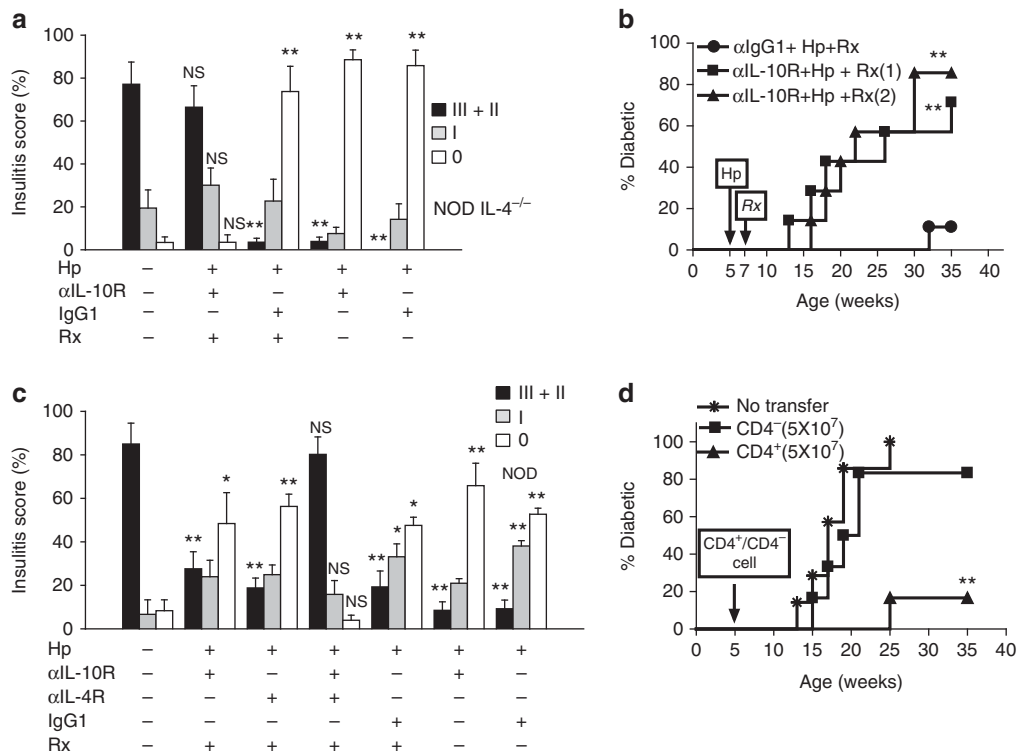


Figure 8 Inhibition of interleukin (IL)-10 signaling blocks helminth-induced prevention of type 1 diabetes in nonobese diabetic (NOD)-IL-4^{-/-} mice while transferred CD4⁺ T cells from infected NOD-IL-4^{-/-} mice conveys protection. **(a)** NOD-IL-4^{-/-} mice (5 mice/treatment group) were inoculated at age 5 weeks with *H. polygyrus* (Hp). Treatment groups included drug-induced worm expulsion at 7 weeks, resulting in short-term infection, or chronic infection and treatment with anti-IL-10R Ab or isotype control. Pancreata from all treatment groups were collected at 13 weeks of age and assessed for insulinitis. **(b)** NOD-IL-4^{-/-} mice were inoculated with Hp at 5 weeks and administered anti-helminth Rx at 7 weeks, and either treated with anti-IL-10R Ab (two separate groups, each with seven mice/treatment group) or isotype ctrl Ab (eight mice/treatment group). All groups were monitored for diabetes up to age 36 weeks as described in Figure 4a. **(c)** NOD mice were Hp inoculated and Rx treated, as in a, and separate groups administered anti-IL10R Ab, anti-IL-4 Ab, both, or isotype control Ab. Insulinitis was assessed at age 13 weeks. **(d)** CD4⁺ and CD4⁻ T cells were isolated from mesenteric lymph nodes and pancreatic lymph nodes of NOD-IL-4^{-/-} mice at age 7 weeks (2 weeks after infection), and transferred to uninfected recipient NOD mice age 5 weeks and monitored for diabetes. Experiments were repeated two times with similar results. A statistically significant difference between the two different groups was denoted by * $P < 0.05$, ** $P < 0.01$. NS, not significant.

of interest in future studies to isolate and characterize these cell populations.

The presence of Hp only during age 5–7 weeks was sufficient to block T1D onset in both NOD and NOD-IL-4^{-/-} mice. These findings suggest a critical temporal window when introduction of exogenous agents, in this case enteric Hp infection, could

change the nature of the immune response, influencing disease development several months later. We now demonstrate that Hp infection downregulates Th1-type cytokines and associated STAT1 phosphorylation, an effect sustained well after the 2-week infection window. Previous studies have suggested that STAT1 phosphorylation can contribute to pancreatic beta-cell

destruction,^{50,51} and STAT1 islet deficiency in an experimentally induced T1D murine model that is protected against initial graft failure.³ Adoptive transfer of IL-10-producing CD4⁺ T cells from 7-week-old Hp-inoculated NOD IL-4^{-/-} mice to 5-week-old NOD recipient mice inhibited T1D onset. These findings indicate that the Hp-induced immune milieu in Th2-deficient NOD mice can modulate CD4⁺ T-cell function such that it can sustainably block severe autoimmune disease even in the potentially dysregulated immune environment of recipient uninfected NOD mice.

These findings thus indicate that Hp infection, in the absence of a Th2-type response, can still induce T cells to produce IL-10, which can have potent inhibitory effects on pancreatic β -cell destruction and T1D development, even when infection occurs for a short interval and is restricted to the small intestine. Our findings of redundant and independent mechanisms raise the possibility that multiple pathways may need to be blocked to identify important contributing protective mechanisms induced by helminthes. This multicomponent paradigm with built-in redundancy may become increasingly significant as we begin to dissect modulation of inflammation by infectious agents.

METHODS

Mice. Four-week-old female NOD mice were purchased from Jackson Laboratories, Bar Harbor, ME. A breeder pair of NOD-IL-4^{-/-} mice (NOD.129P2 (B6)-IL4^{tm1Cgn/DvsJ}) were purchased from Jackson Laboratories and used to establish a colony in the specific pathogen-free facility in the Center for Comparative Medicine at New Jersey Medical School. The studies reported here conformed to the principle for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education and Welfare (National Institute of Health) guidelines for the experimental use of animals and were approved by the IACUC at NJMS.

Parasite infection, glucose monitoring, and antibiotics, anti-helminth, and blocking antibody treatments. Five-week-old female NOD and NOD-IL4^{-/-} mice were inoculated orally with 200 infective third-stage (L3) Hp larvae using a rounded gavage tube as previously described.²⁹ Mice were screened for blood glucose levels every 2 weeks using a Bayer contour blood glucose monitoring system (Bayer Healthcare LLC, Mishawaka, IN), and were considered diabetic when glucose levels reached >200 mg dl⁻¹ on two consecutive occasions. Animals were also evaluated daily for clinical signs of diabetes (e.g., weight loss), morbidity, and mortality.

Five-week-old female NOD and NOD-IL4^{-/-} mice were inoculated orally with 200 infective Hp L3 and, in short-term infections, after 2 weeks treated with the anti-helminth agent pyrantel pamoate (First Priority, Elgin, IL) at a dose of 1 mg per mouse. One week after drug treatment, fecal egg counts were taken from treated and untreated mice, as previously described,²⁹ to confirm complete expulsion. In chronic infection, mice were not administered the anti-helminth drug. In certain experiments, groups of NOD mice were also administered 500 μ g anti-IL-10RA mAb (1B1.3A) (BioXCell, West Lebanon, NH) alone, 500 μ g anti-IL-4 mAb (Clone: 11B11) (BioXCell) alone, or in combination for every 2 days from age 5–7 weeks. Control groups were similarly administered the IgG1 isotype control antibody (BioXCell).

Flow cytometry. Single-cell suspensions, prepared from MLNs, PLNs, inguinal lymph nodes (non-draining lymph nodes), and spleen, were collected from mice at the times indicated. Cell preparations were treated with Fc Block (BD Pharmingen, San Diego, CA) and stained with different combinations of antibodies as follows: anti-B220-FITC, anti-B220-PerCP, anti-MHCII-I-A(d)-FITC, anti-CD8-PE, anti-CD8-PerCP,

anti-CD4-APC, anti-CD4-PerCP, anti-CD127(IL-7R)-FITC, and anti-CD25-APC (all from BD Pharmingen). Foxp3 staining was performed using eBioscience reagents (eBioscience, San Diego, CA) according to the manufacturer's protocol. Phosphorylation of STAT6 at tyrosine 641 (pY641) and pY701 of STAT1 was detected by intracellular staining with PE-conjugated anti-phospho-STAT6 and STAT1, using PhosFlow Fix Buffer I and Perm Buffer III reagents (all from BD Pharmingen) according to the manufacturer's instructions. For IL-10 cytoplasmic staining, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and permeabilised in 0.5% saponin (Sigma-Aldrich, St Louis, MO), before staining with PE-conjugated rat anti-mouse IL-10 (BD Pharmingen). All antibody incubations were performed at 4°C for 30 min (isotype controls were included). Cells were immediately acquired on a FACSCalibur or LSRII flow cytometer (BD-Pharmingen), and analyzed using FlowJo software (Treestar, Ashland, OR).^{11,23}

Quantification of serum immunoglobulins. Total serum IgG1, IgG2a, and IgE levels were measured using an enzyme-linked immunosorbent assay, as previously described.²³

Histology. Pancreata were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, and evaluated as previously described.¹¹ Briefly, islets with infiltrates occupying 10% or more of their areas were identified as positive for insulinitis. Islets were scored using the following scale: grade 0 (no infiltrates), 0% infiltration; grade I (peri-insulinitis), 1–10% infiltration; grade II (moderate insulinitis), 11–50% infiltration; and grade III (severe insulinitis), >50% infiltration. We typically counted ~50–100 islets per experiment from six to eight mice depending on the number of islets that were present in the sections.

ELISPOT. IL-10, IFN- γ , and IL-4 ELISPOT were performed as previously described.¹¹ Briefly, lymph node cell suspensions were restimulated *in vitro* for 48 h either in the presence of 10 μ g ml⁻¹ anti CD-3 ϵ (BD Pharmingen, San Diego, CA) or with a mixture of β -islet AA peptides (a gift from Dr David H. Wagner, Jr, The Webb-Waring Center, University of Colorado Denver School of Medicine, Aurora, CO) including 50 μ g ml⁻¹ of β -memb Ag peptide (EKahrPIWARMdAKK) and 50 μ g ml⁻¹ INS-B 9–23 peptide (SHLVEALYLVCGERG), which have previously been shown to stimulate AA-specific CD4 T cells.^{32–35}

Sorting of CD4⁺ T cells/CD4⁺ CD127 (IL-7R)^{hi/lo} cells after Hp inoculation. For CD4⁺ T-cell sorting, single-cell suspensions of lymph nodes were prepared from Hp-inoculated NOD-IL-4^{-/-} mice, and CD4⁺ and CD4⁻ cells were isolated using anti-CD4 micro beads (Miltenyi Biotec, Auburn, CA), as previously described.⁵² The sorted CD4⁺ cells were stained with CD4PE and CD127 (IL-7R) FITC and sorted into CD127 high and low populations using either the FACS Vantage SE TM with the FACSDiVa upgrade cell sorter (Becton Dickinson, Mountain View, CA) or state-of-the-art high-speed cell sorter FACS Aria, (Becton Dickinson). The sorted cells were analyzed for gene expression, intracellular IL-10 by FACS analysis, and IL-10 secretion by ELISPOT.

Cytokine gene expression by RT-PCR. PLNs, MLNs, and spleen were collected from mice at the times indicated. Total RNA was extracted from tissue or sorted cells using either the Trizol or *RNAqueous*[®] Kit (Ambion, Foster City, CA), respectively, and then reverse transcribed. RT-PCR kits (PE Applied Biosystems, Foster City, CA), specific for individual cytokines or ribosomal RNA, were used to quantify differences in gene expression. All data were normalized to constitutive ribosomal RNA values and expressed as mRNA fold changes relative to untreated controls, as previously described.^{52,53}

Statistical analysis. A statistical analysis for life tables was performed using GraphPad Prism 4 for Windows software version 4.03 (GraphPad Software, La Jolla, CA). For life table analysis, we used the

Log-rank test to compare survival curves. Statistical analyses of results from RT-PCR, ELISPOT, and insulinitis scoring were accomplished with Sigma plot version 11.0 (Systat Software, San Jose, CA). For comparisons of three or more treatment groups, we use a one-way analysis of variance and a Tukey *t*-test analysis for individual comparisons ($P < 0.05^*$); $P < 0.01^{**}$).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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