



## Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function

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from *Pdcd1*<sup>-/-</sup> mice contained antibodies specific for components of commensal bacteria, which indicated a breach of normal mucosal-systemic compartmentalization (fig. S4, A and C) (24). On the basis of the data presented here, we propose that the skewed gut microbial communities that result from the dysregulated selection of IgAs drive the expansion of self-reactive B and T cells. Our studies have implications for how modulation of PD-1 expression promotes tolerance or uncontrolled immune reactions leading to autoimmunity.

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#### Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6080/485/DC1  
Materials and Methods  
Figs. S1 to S4  
References (25–34)

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# Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function

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Exposure to microbes during early childhood is associated with protection from immune-mediated diseases such as inflammatory bowel disease (IBD) and asthma. Here, we show that in germ-free (GF) mice, invariant natural killer T (iNKT) cells accumulate in the colonic lamina propria and lung, resulting in increased morbidity in models of IBD and allergic asthma as compared with that of specific pathogen-free mice. This was associated with increased intestinal and pulmonary expression of the chemokine ligand CXCL16, which was associated with increased mucosal iNKT cells. Colonization of neonatal—but not adult—GF mice with a conventional microbiota protected the animals from mucosal iNKT accumulation and related pathology. These results indicate that age-sensitive contact with commensal microbes is critical for establishing mucosal iNKT cell tolerance to later environmental exposures.

The mammalian host immune system is broadly stimulated with the first exposure to microorganisms during neonatal life (1). The inner surfaces of the gastrointestinal tract and lungs are particularly affected because they are predominant sites of microbial contact (2). Epidemiologic observations further suggest that the immune effects of early-life microbial exposure are durable and persist into later life because they can be associated with prevention of diseases such as inflammatory bowel disease (IBD) and asthma (3–5).

Invariant natural killer T (iNKT) cells probably play an important role in the pathogenesis of ulcerative colitis (UC)—a major form of IBD—and in asthma (6, 7). Such cells recognize endogenous and exogenous lipid antigens presented by the nonpolymorphic major histocompatibility complex (MHC) class I-like protein CD1d and

secrete abundant amounts of proinflammatory cytokines such as interleukin-4 (IL-4) and IL-13 upon activation (8, 9). We therefore investigated age-dependent regulation of iNKT cells by use of microbes in mouse models of IBD and asthma.

We first examined the appearance of iNKT cells in tissues of 8-week-old germ-free (GF) and specific pathogen-free (SPF) Swiss-Webster (SW) mice. Relative and absolute numbers of iNKT cells were increased in GF mice in colonic lamina propria (LP) (Fig. 1, A to C). These differences in colonic iNKT cell numbers between GF and SPF mice were detectable after weaning and stable for life, suggesting early and persistent effects of the microbiota (Fig. 1D). iNKT cells were not increased in the ileal LP (ileum) of GF mice, and the liver, spleen, and thymus contained even fewer iNKT cells under GF relative to SPF conditions (fig. S1), which is consistent with a recent

report (10). GF C57BL/6 mice (B6) exhibited similar increases of iNKT cells in the colonic LP as well as the liver, in contrast to GF SW mice (fig. S2). Although increased in number, the iNKT cell expression of several activation and memory markers was unaltered in GF SW, relative to SPF, mice (fig. S3).

To examine the relevance of these findings, we investigated the susceptibility of GF and SPF mice to oxazolone-induced colitis, a model of intestinal inflammation that possesses features of UC and is dependent on IL-13 production by CD1d-restricted iNKT cells (11, 12). Although GF or germ-reduced mice exhibit exacerbated inflammation in an innate mouse model of colitis (13, 14), colitis is typically prevented under GF conditions in models dependent on an adaptive immune response (15). Surprisingly, GF mice were more sensitive to oxazolone-induced colitis, as revealed by severe weight loss, pathology, and a high mortality rate in contrast to SPF mice (Fig. 1, E to H). This was not due to overexpression of cell-surface CD1d expression on intestinal epithelial (fig. S4A) and hematopoietic cells (fig. S4B). Consistent with previous studies (11), the

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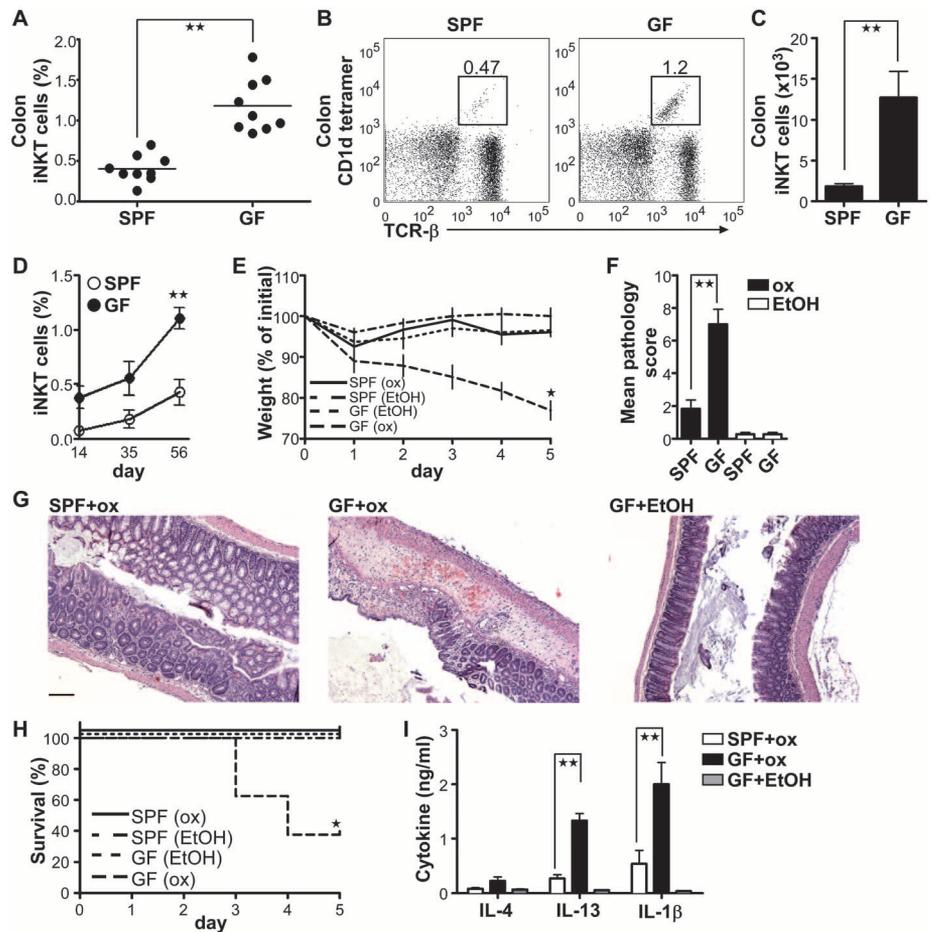
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colitis in GF mice was characterized by a marked increase in production of IL-13 and IL-1 $\beta$  in comparison with SPF mice (Fig. 1I).

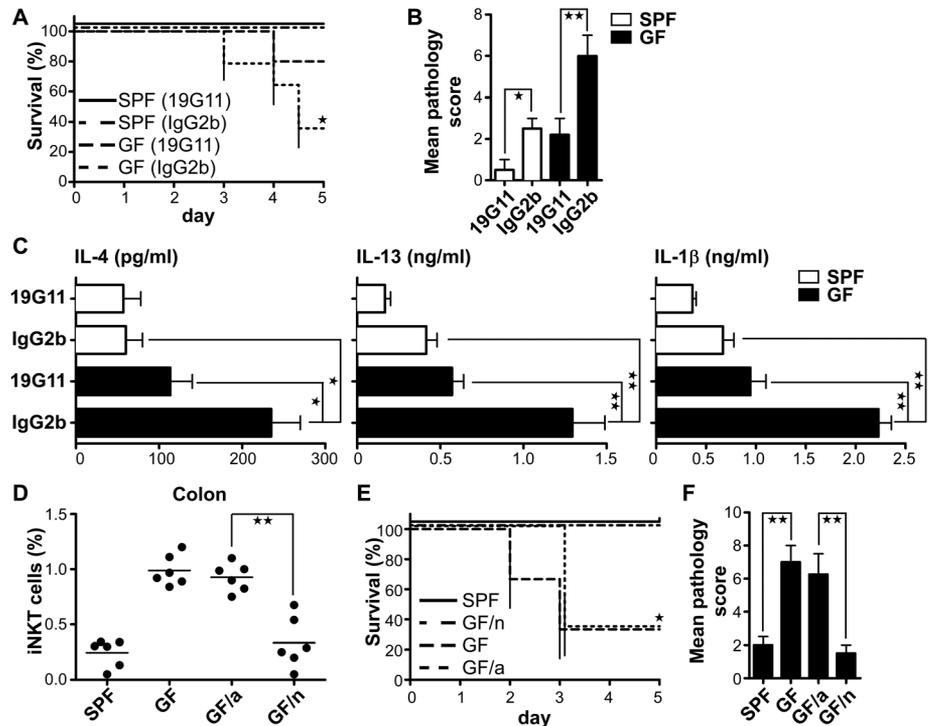
**Fig. 1.** Intestinal bacteria-dependent accumulation of colonic iNKT cells in GF mice leads to high mortality in oxazolone-induced colitis. (A to C) The percentage of CD1d tetramer–positive cells (iNKT cells) within the live lymphocytes from the lamina propria (LP) of gender-matched GF and SPF SW mice (7 to 8 weeks old) was analyzed by means of flow cytometry (A). Representative dot-plots are shown in (B), and the absolute number of iNKT cells is shown in (C). Each circle in (A) represents an individual mouse. (D) Percentages of colonic iNKT cells of live LP lymphocytes in gender-matched SPF and GF mice were analyzed at different ages by means of flow cytometry ( $n = 4$  mice per group). (E to H) Eight-week-old GF and SPF mice were monitored and scored after rectal challenge with 1% oxazolone (ox) or 50% ethanol for survival and body weight loss for 5 days. On day 5, the colons were collected and dissected for histological analysis ( $n = 5$  mice per group). (G) Scale bar, 50  $\mu$ m. (I) The concentration of IL-4, IL-13, and IL-1 $\beta$  in the supernatant of 24 hours–colon organ explant cultures determined by means of enzyme-linked immunosorbent assay (ELISA) on day five ( $n = 5$  mice per group). All data were obtained from three independent experiments with similar results. In all panels, error bars represent the SD. \* $P < 0.05$ , \*\* $P < 0.01$ , unpaired  $t$  test and \* $P < 0.05$ , log-rank test in (H).

To confirm the CD1d-restriction of this colitis in GF mice, we investigated the effects of CD1d blockade. Adult (8- to 9-week-old) GF and SPF

mice were treated with a blocking monoclonal antibody (Ab) specific for CD1d (19G11) or an isotype control Ab (16). Treatment of GF mice



**Fig. 2.** Microbial colonization during early life prevents the CD1d/iNKT cell-dependent high mortality in oxazolone colitis in GF mice. (A to C) Eight-week-old GF and SPF mice were treated once with 1 mg of 19G11 or isotype control IgG2b antibody before presensitization and rectal oxazolone challenge. (A) Survival after oxazolone challenge is shown. (B) On day 5, the colons were collected for histological analysis, and (C) the cytokine concentration in the supernatant of 24 hours–colon organ explant cultures was measured with ELISA ( $n \geq 4$  mice per group). (D) Colonic LP lymphocytes were analyzed for iNKT cells by flow cytometry. (GF/a) mice were GF mice that were exposed to SPF environmental conditions at the age of 5 weeks and maintained for 4 more weeks. (GF/n) mice were pups exposed to SPF conditions on their first day of life and maintained under SPF environmental conditions for 8 to 9 weeks. Each circle represents a mouse. (E to F) Colonized mice were treated as above with oxazolone ( $n = 5$  mice per group). All data were obtained from more than two independent experiments with similar results. Error bars indicate SD. \* $P < 0.05$ , \*\* $P < 0.01$ , unpaired  $t$  test and \* $P < 0.05$ , log-rank test in (A) and (E).



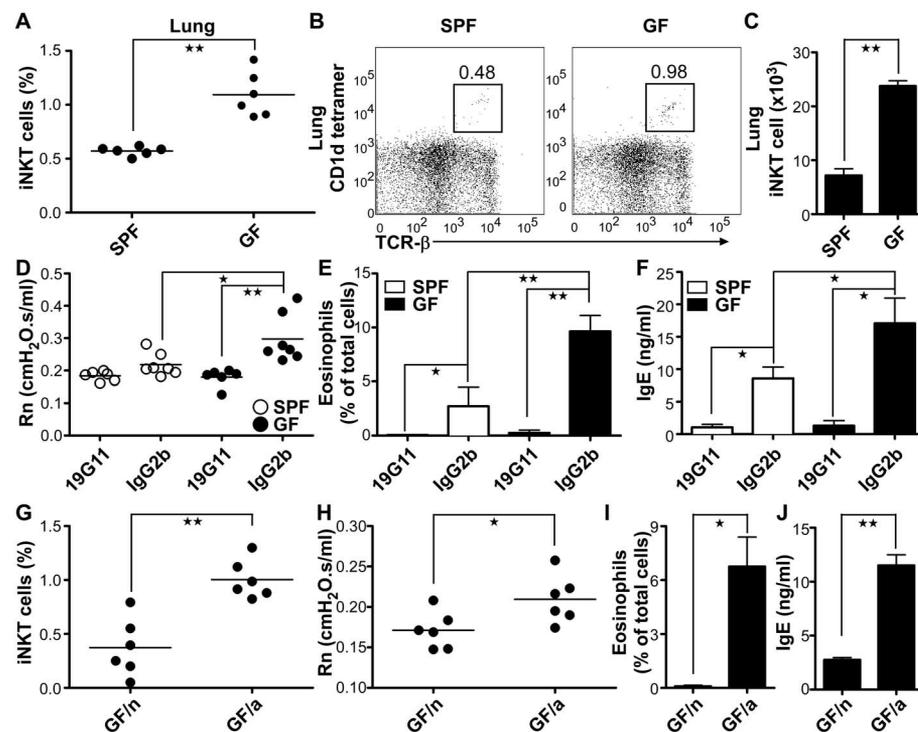
with 19G11, but not the isotype control, protected against colitis-induced mortality and associated pathology (Fig. 2, A to C, and fig. S4, C and D). Furthermore, CD1d blockade of GF or SPF mice did not lead to significant functional changes in dendritic cells (17) or B cells (18) as demon-

strated by stable IL-12 or IL-10 production (fig. S4, E and F), respectively.

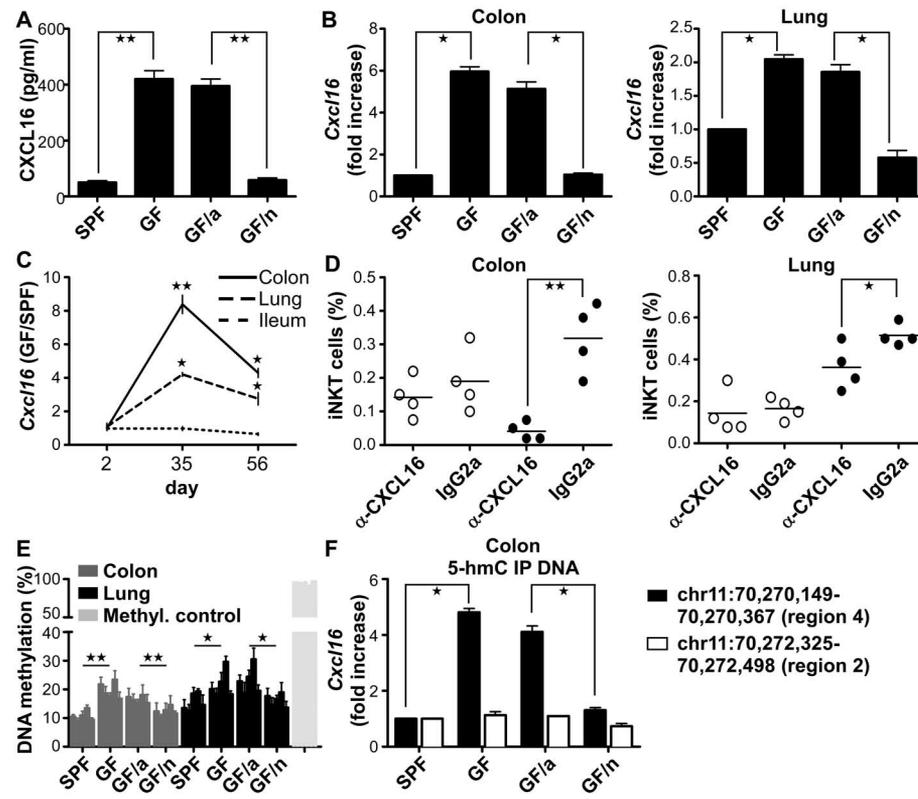
We next examined whether reestablishing microbiota in adult GF mice would normalize iNKT cell levels in the colon. Quite surprisingly, exposure of adult GF mice to SPF conditions (GF/a)

did not restore iNKT cells in the colon to the levels observed in SPF mice or reverse the mortality and severe pathology after oxazolone administration (Fig. 2, D to F). We therefore considered whether the ability to normalize iNKT cell levels and function in the colon was dependent on the

**Fig. 3.** The increased CD1d/iNKT cell-mediated allergic response sensitivity of GF mice is dependent on age of colonization. (A to C) Lymphocytes from lungs of 8-week-old mice in each group were analyzed for iNKT cells by means of flow cytometry (A). Representative dot-plot is shown in (B), and the absolute number of iNKT cells is shown in (C) ( $n = 6$  mice per group). (D to F) Age-matched mice from each group were treated once before OVA presensitization with 1 mg of 19G11 or IgG2b isotype control antibody and with 0.5 mg of 19G11 or IgG2b antibody before the first and the third serial OVA exposure. Twenty-four hours after the last challenge with 5% aerosolized OVA, the mice were analyzed for (D) airway resistance (Rn), (E) percentage of eosinophils of total BALF cells, and (F) IgE ( $n = 4$  mice per group). (G to J) Lung lymphocytes were analyzed for iNKT cells by means of flow cytometry of age- and gender-matched GF/n and GF/a mice. (H to J) Mice were presensitized with OVA and analyzed after the last serial OVA challenge as described above ( $n \geq 4$  mice per group). Each circle in (A), (D), (G), and (H) represents an individual mouse. All data were obtained from more than two independent experiments with similar results. Error bars indicate the SD. \* $P \leq 0.05$ , \*\* $P < 0.01$ , unpaired  $t$  test.



**Fig. 4.** Microbiota affects tissue specific iNKT cell accumulation by genetic modifications of *Cxcl16*. (A) The CXCL16 concentration in the serum of each group was measured with ELISA in SPF, GF, GF/a, and GF/n as described above ( $n = 4$  mice per group). (B) Colon and lung tissue samples were harvested from age-matched mice from each group and analyzed for *Cxcl16* expression ( $n = 4$  mice per group). (C) Colon, ileum, and lung tissue samples were analyzed from age-matched SPF and GF mice at different time points for *Cxcl16* expression ( $n = 5$  mice per group). (D) SPF (open circles) and GF (closed circles) newborn mice were treated three times a week intraperitoneal with 25  $\mu$ g of a neutralizing CXCL16 antibody ( $\alpha$ -CXCL16) or its isotype control IgG2a antibody and analyzed at the age of 2 weeks for iNKT cell percentages by means of flow cytometry. Each circle represents a mouse. (E) Analysis of DNA methylation of five CpG sites of *Cxcl16* by bisulfite pyrosequencing. For each group, the mean of DNA methylation of 5 CpG sites is shown as a percentage according to the methylated control (Methyl. control) ( $n \geq 3$  mice per group). (F) *Cxcl16* qPCR analysis of colonic DNA after performing a 5-hydroxymethylated DNA immunoprecipitation (5-hmC IP DNA) ( $n \geq 3$  mice per group). All data were obtained from at least two independent experiments with similar results. Error bars indicate the SD. \* $P \leq 0.05$ , \*\* $P < 0.01$ , unpaired  $t$  test.



age at which microbial contact occurred. Indeed, when we colonized pregnant GF female mice just before delivery and therefore exposed neonatal GF mice to SPF conditions on their first day of life (GF/n), we observed a complete normalization of iNKT cell levels that persisted even two months after colonization (Fig. 2D). Consistent with this, GF/n exhibited reduced susceptibility to oxazolone-induced colitis (relative to that of GF mice) two months after colonization, with a degree of severity identical to that observed in SPF animals, as shown by an analysis of mortality, weight loss, pathology, and cytokine production (Fig. 2, E and F, and fig. S5).

To verify that the early life events associated with the absence of normal microbial colonization on iNKT cell homeostasis were CD1d-dependent, we treated GF mice with 19G11 Ab for their first 6 weeks of life. 19G11, but not control Ab, treatment blocked accumulation of iNKT cells in all tissues examined and susceptibility to oxazolone-induced colitis in later life (fig. S6). Similar to GF mice, SPF mice (B6) born under germ-reduced conditions exhibited an increase in colonic iNKT cells and excessive oxazolone-induced colitis at 4 weeks of life (fig. S7). As predicted (11), *Cd1d*<sup>-/-</sup> and *Ja18*<sup>-/-</sup> mice, both of which lack iNKT cells, were not susceptible to oxazolone-induced colitis either under SPF or germ-reduced conditions (fig. S7).

Animal models of asthma demonstrate an important role for iNKT cells (19, 20), although the contribution to human disease remains controversial (21). We found that the lungs of GF SW and B6 mice contained significantly higher relative and absolute numbers of iNKT cells in comparison with that of the lungs of the respective SPF mice (Fig. 3, A to C, and fig. S8). Because commensal microbes may affect the induction of experimental asthma (22), we tested an ovalbumin (OVA)-driven allergic-asthma mouse model in GF mice. We observed that similar to the colon, GF mice developed an allergic airway response to OVA significantly greater than that observed in SPF mice, as demonstrated by increases in airway resistance, total bronchial alveolar lavage fluid (BALF) cell numbers, BALF eosinophilia, serum immunoglobulin E (IgE) levels, proinflammatory cytokine production in the BALF, and lung tissue eosinophil infiltration (Fig. 3, D to F, and fig. S9). The asthma in GF mice was CD1d- and antigen-dependent because elimination of the asthmatic response was only observed with 19G11, but not control, Ab treatment (Fig. 3, D to F, and fig. S9) of OVA-sensitized mice but not mice exposed to phosphate-buffered saline (PBS), which did not induce allergic asthma (fig. S10).

We therefore next investigated whether early-life exposure to a conventional microbiota also protects animals from CD1d-restricted inflammation in the lungs. As observed in the intestinal mucosa, neonatal (GF/n)—but not adult-life (GF/a)—exposure of GF mice to a conventional microbiota abrogated the increased accumulation of iNKT cells in the lungs and protected adult

mice in the allergic asthma model from the pathology (Fig. 3, G to I, and fig. S11).

The chemokine receptor CXCR6 on iNKT cells (23) and its ligand CXCL16, which is expressed at high levels by human epithelial cells (24) and increased in inflammation (25), plays an important role in iNKT cell homeostasis. Therefore, we examined the serum of GF and GF/a mice for the presence of CXCL16 and observed significantly higher levels than that observed in SPF and GF/n mice (Fig. 4A). This was due to significant increases of *Cxcl16* mRNA expression levels in the colon and lung—but not ileum, liver, and thymus—of GF and GF/a relative to SPF and GF/n mice (Fig. 4B and fig. S12A), and mainly immunolocalized to the epithelium (fig. S12B). Although *Cxcl16* mRNA levels in the colon, ileum, and lung were similar immediately after birth in the GF and SPF animals at the beginning of colonization, they increased significantly and specifically only in the colon and lungs of the GF mice during later life (Fig. 4C). These results suggest that microbial exposure provides signals that determine *Cxcl16* gene expression in specific tissues. GF and SPF mice, however, exhibited similar levels of *Cxcr6* mRNA expression in tissues and CXCR6 expression on the cell surface of iNKT cells (fig. S12, C and D).

CXCL16 was responsible for the accumulation of iNKT cells because treatment of newborn SPF and GF mice with a CXCL16-neutralizing Ab caused a decrease in iNKT cells in the colon and lung (Fig. 4D) but had no effect on iNKT cell levels in the ileum or thymus (fig. S13A). However, it was associated with an increased accumulation of iNKT cells in the liver (fig. S13A), suggesting CXCL16 blockade caused a redirection of iNKT cells to this organ. Consequently, Ab-to-CXCL16—but not isotype-treated GF mice were protected from oxazolone colitis-induced mortality and pathology (fig. S13, B to D).

We next examined the epigenetic content of the *Cxcl16* gene of GF and SPF mice. A region 5' of the *Cxcl16* gene of SW mice that contains five potential CpG sites was determined by means of bisulfite pyrosequencing to be hypermethylated in the colon and lungs (Fig. 4E)—but not in other tissues such as the spleen and liver (fig. S14)—under GF conditions as compared with SPF conditions. Colonization of neonatal—but not adult—GF mice with a conventional microbiota decreased the hypermethylation of the *Cxcl16* gene to SPF levels (Fig. 4E). Gene activation due to hypermethylation may occur as a consequence of specific types of environmental exposure (26). To confirm this, we treated SPF neonates on their first day of life by oral gavage with high doses of folic acid as previously described (27) so as to force *Cxcl16* methylation. Compared with PBS-treated control mice, folic acid administration resulted in elevated methylation of the *Cxcl16* gene in the colon, ileum, and lung; at least a three- to fourfold increase of *Cxcl16* mRNA expression in the same tissues; increased CXCL16 serum levels; and accumulation of iNKT cells in

these tissues as observed in GF mice (fig. S15). Alternatively, we investigated whether elimination of *Cxcl16* methylation in GF mice would reverse the elevation of CXCL16 expression by treating GF newborn littermates with the DNA methyltransferase inhibitor 5-Azacytidine (5-Aza). When examined at 2 weeks of life, 5-Aza treatment inhibited methylation and mRNA expression of *Cxcl16* in the colon, ileum, and lung (fig. S16, A and B), diminished the levels of CXCL16 in the serum, and reduced iNKT cells in these tissues (fig. S16, C and D). 5-Aza treatment had no effect on other cell populations such as CD45<sup>+</sup>CD11c<sup>+</sup> double positive cells (fig. S17).

Epigenetic marks provided by 5-hydroxymethylcytosine (5-hmC) incorporation into DNA have been suggested to be different from those provided by 5-methylcytosine (5-mC) and a distinct signature for elevated levels of transcription (28). We therefore performed DNA immunoprecipitation with a monoclonal 5-hmC Ab followed by quantitative polymerase chain reaction (PCR) analysis for *Cxcl16* in seven different regions of *Cxcl16* in DNA samples obtained from the colons of SPF, GF, GF/a, and GF/n mice. We observed that *Cxcl16* was highly increased in three out of the seven investigated regions (with region 4 as a representative) within the 5-hmC-modified DNA of GF and GF/a mice but not in the DNA acquired from SPF and GF/n mice (Fig. 4F). No differences were observed in the other four regions (with region 2 as a representative) and in the DNA samples obtained from the ileum demonstrating the colonic specificity for 5-hmC modification of *Cxcl16* (fig. S18). A map summarizing this information is provided in fig. S19.

Our studies indicate that CXCL16 is an age- and organ-dependent microbially regulated factor that modulates the quantities and function of iNKT cells in the colon and lungs and, consequently, susceptibility to tissue inflammation. The exact mechanism by which the microbiota regulates CXCL16 expression and thus iNKT cell accumulation in these organs is unknown, although it is independent of the Toll-like receptor adapter protein MyD88 (fig. S20). These observations are in accordance with previous epidemiological studies collectively known as the “hygiene hypothesis,” which proposes that early-life exposure to specific microbe-enriched environments decreases susceptibility to diseases such as IBD and asthma (3, 5, 29, 30), whereas its absence, as in antibiotic treatment during childhood, may have the opposite effect (31, 32). Our results suggest that CD1d-restricted iNKT cell pathways and their relationship to microbes play a central role in these aforementioned processes. Early-life microbial exposure elicits long-lasting effects on iNKT cells, and in their absence, later-life exposure to factors that stimulate these cells may induce an autoinflammatory response. These findings are predicted to extrapolate to humans, given the extensive similarities between the mouse and human CD1d and iNKT cell systems (33).

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## Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1219328/DC1  
Materials and Methods  
Figs. S1 to S20  
Tables S1 and S2  
References (34–37)

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# Analytic Thinking Promotes Religious Disbelief

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Scientific interest in the cognitive underpinnings of religious belief has grown in recent years. However, to date, little experimental research has focused on the cognitive processes that may promote religious disbelief. The present studies apply a dual-process model of cognitive processing to this problem, testing the hypothesis that analytic processing promotes religious disbelief. Individual differences in the tendency to analytically override initially flawed intuitions in reasoning were associated with increased religious disbelief. Four additional experiments provided evidence of causation, as subtle manipulations known to trigger analytic processing also encouraged religious disbelief. Combined, these studies indicate that analytic processing is one factor (presumably among several) that promotes religious disbelief. Although these findings do not speak directly to conversations about the inherent rationality, value, or truth of religious beliefs, they illuminate one cognitive factor that may influence such discussions.

Although most people fervently believe in God or gods, there are nonetheless hundreds of millions of nonbelievers worldwide (1), and belief and disbelief fluctuate across situations and over time (2). Religious belief and disbelief are likely complex, multi-determined, psychologically and culturally shaped phenomena, yet to date little experimental research has explored the specific cognitive underpinnings of religious disbelief (3, 4). Here we begin to address this important gap in the literature by applying a dual-process cognitive framework, which predicts that analytic thinking strategies might be one potent source of religious disbelief.

According to dual-process theories of human thinking (5, 6), there are two distinct but interacting systems for information processing. One

(System 1) relies upon frugal heuristics yielding intuitive responses, while the other (System 2) relies upon deliberative analytic processing. Although both systems can at times run in parallel (7), System 2 often overrides the input of system 1 when analytic tendencies are activated and cognitive resources are available. Dual-process theories have been successfully applied to diverse domains and phenomena across a wide range of fields (5, 6, 8, 9).

Available evidence and theory suggest that a converging suite of intuitive cognitive processes facilitate and support belief in supernatural agents, which is a central aspect of religious beliefs worldwide (10–13). These processes include intuitions about teleology (14), mind-body dualism (13), psychological immortality (15), and mind perception (16, 17). Religious belief therefore bears many hallmarks of System 1 processing.

If religious belief emerges through a converging set of intuitive processes, and analytic processing can inhibit or override intuitive processing,

then analytic thinking may undermine intuitive support for religious belief. Thus, a dual-process account predicts that analytic thinking may be one source of religious disbelief. Recent evidence is consistent with this hypothesis (4), finding that individual differences in reliance on intuitive thinking predict greater belief in God, even after controlling for relevant socio-demographic variables. However, evidence for causality remains rare (4). Here we report five studies that present empirical tests of this hypothesis.

We adopted three complementary strategies to test for robustness and generality. First, study 1 tested whether individual differences in the tendency to engage analytic thinking are associated with reduced religious belief. Second, studies 2 to 5 established causation by testing whether various experimental manipulations of analytic processing, induced subtly and implicitly, encourage religious disbelief. These manipulations of analytic processing included visual priming, implicit priming, and cognitive disfluency (18, 19). Third, across studies, we assessed religious belief using diverse measures that focused primarily on belief in and commitment to religiously endorsed supernatural agents. Samples consisted of participants from diverse cultural and religious backgrounds (20).

Study 1 was a correlational study with Canadian undergraduates ( $N = 179$ ). We correlated performance on an analytic thinking task with three related, but distinct, measures of religious belief. The analytic thinking task (6) contains three problems that require participants to analytically override an initial intuition. This task was designed to specifically measure analytic processing because an intuitive reading of each problem invites a quick and easy, yet incorrect, response that must be analytically overridden (Table 1). Furthermore, experimental manipulations known to induce analytic processing

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