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Title	Programmed cell death 1 inhibits inflammatory helper T-cell development through controlling the innate immune response
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Citation	Proceedings of the National Academy of Sciences of the United States of America (2013)
Issue Date	2013-09-16
URL	http://hdl.handle.net/2433/178757
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Type	Journal Article
Textversion	author

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Programmed Cell Death-1 inhibits inflammatory helper T cell development through controlling the innate immune response

Short title: PD-1 inhibits innate inflammation and autoimmunity

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Key words: Autoimmunity, tolerance, inflammation

ABSTRACT

Programmed Cell Death-1 (PD-1) is an inhibitory co-receptor on immune cells, and is essential for self-tolerance, as mice genetically lacking PD-1 (PD-1$^{-/-}$) develop spontaneous autoimmune diseases. PD-1$^{-/-}$ mice are also susceptible to severe experimental autoimmune encephalomyelitis (EAE), characterized by a massive production of effector/memory T cells against myelin auto-antigen, the mechanism of which is not fully understood. We found that an increased primary response of PD-1$^{-/-}$ mice to heat-killed mycobacteria (HKMTB), an adjuvant for EAE, contributed to the enhanced production of T-helper 17 (Th17) cells. Splenocytes from HKMTB-immunized, lymphocyte-deficient PD-1$^{-/-}$ RAG2$^{-/-}$ mice were found to drive antigen-specific Th17 cell differentiation more efficiently than splenocytes from HKMTB-immunized PD-1$^{+/+}$ RAG2$^{-/-}$ mice. This result suggested PD-1's involvement in the regulation of innate immune responses. Mice reconstituted with PD-1$^{-/-}$ RAG2$^{-/-}$ bone marrow and PD-1$^{+/+}$ CD4^{+} T cells developed more severe EAE, compared to the ones reconstituted with PD-1$^{+/+}$ RAG2$^{-/-}$ bone marrow and PD-1$^{+/+}$ CD4^{+} T cells. We found that, upon recognition of HKMTB, CD11b^{+} macrophages from PD-1$^{-/-}$ mice produced very high levels of IL-6, which helped promote naïve CD4^{+} T cell differentiation into IL-17-producing cells. We propose a model in which PD-1 negatively regulates anti-mycobacterial responses by suppressing innate immune cells, which in turn prevents auto-reactive T cell priming and differentiation to inflammatory effector T cells.

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INTRODUCTION

Autoimmune disease development is impacted by both genetic and environmental factors. Programmed death (PD)-1 is a type-I membrane protein that delivers inhibitory signals to immune cells upon the binding of its ligand, PD-L1 or PD-L2 (1). PD-1 has been shown to be important for self-tolerance, since spontaneous autoimmune diseases develop in PD-1^{-/-} mice (2-4). A single nucleotide polymorphism that affects PD-1 expression is associated with autoimmune diseases in humans, such as systemic lupus erythematosus (5), type I diabetes (6), rheumatoid arthritis (7), and multiple sclerosis (MS) (8), suggesting that PD-1 deficiency may be a genetic factor involved in the development of autoimmunity.

Experimental autoimmune encephalomyelitis (EAE) is a rodent model of T cell-mediated inflammatory disease in the central nervous system (CNS), causing demyelination, axonal damage and paralysis, and is a commonly used model for human MS. Previous reports suggested that PD-1 functions to attenuate EAE. PD-1 and its ligands were found to be strongly expressed on immune infiltrates in the CNS during the peak phase of EAE (9-11). In EAE studies, PD-1-deficient mice or the use of blocking antibodies that inhibit PD-1 engagement by ligands resulted in earlier disease onset, increased inflammatory infiltrates, and increased severity of clinical symptoms when compared to normal disease progression (10-16). It has been demonstrated that ligand engagement of PD-1 inhibits T cell activation, expansion, and cytokine production (17-19). Similarly, in EAE, PD-1 signaling in CNS-specific helper T cells may inhibit their expansion and secretion of inflammatory cytokines (10-12). Recently T-helper 17 (Th17) cells were shown to be involved in EAE, by producing IL-17 and GM-CSF (20, 21). Two

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reports showed that PD-1$^{-/-}$ mice mount an augmented Th17 response to EAE induction (14, 16). However, the fundamental mechanisms by which PD-1 regulates antigen-specific Th17 cell differentiation, expansion, and effector function in EAE remain to be understood.

To induce EAE, mice are immunized with myelin auto-antigens in an emulsion of mycobacterium tuberculosis (MTB)-derived adjuvants, causing a strong innate inflammatory response, leading to Th skewing (22). Curiously, recent studies showed that PD-1$^{-/-}$ mice exhibited an altered response to infection with mycobacteria, characterized by uncontrolled bacterial burden, massive production of cytokines, termed "cytokine storm," and early death (23-25). We wondered if this unique response of PD-1$^{-/-}$ mice to mycobacteria contributed to their Th response in EAE.

In this study, we took a combination of genetic and immunological approaches in which the innate response to MTB-derived adjuvant and antigen-specific T cell polarization were separately analyzed. The present data suggest that an enhanced innate response of PD-1$^{-/-}$ mice to MTB contribute to the susceptibility of these mice to severe EAE. We propose a novel function of PD-1 in controlling the basal state of the innate immune response, whose failure can cause the activation of adaptive immune responses, provoking autoimmunity.

RESULTS

Augmented EAE with sub-optimal immunization of PD-1$^{-/-}$ mice.

EAE in C57BL/6 mice is typically induced by immunizing the mice with MOG$_{35-55}$ peptide in an emulsion of CFA and 200-300 μg of additional heat-killed MTB (HKMTB), followed by two separate intraperitoneal injections of pertussis toxin (PTX), which is assumed to induce a strong innate immune response leading to MOG-specific T-cell priming. In agreement with previous reports (12, 14), the induction of EAE with this treatment resulted in accelerated disease progression in PD-1$^{-/-}$ mice, characterized by earlier disease onset and an earlier peak in disease activity (Fig. 1A).

 Since this treatment also caused prominent disease in PD-1$^{+/+}$ mice, we next examined the effects of suboptimal immunization with reduced adjuvants on both wild-type and mutant mice. PD-1$^{-/-}$ mice appeared to be susceptible to EAE by the suboptimal treatment. First, when PTX injection was eliminated from the treatment, the PD-1$^{-/-}$ mice developed a comparable disease as with full immunization, whereas PD-1$^{+/+}$ mice showed an attenuated response (Fig. 1B). PTX is known to open the blood brain barrier (26), inhibit Foxp3 expression on T cells (27), and enhance Th17 development in EAE (28). Our findings and a previous study by others (29) indicated that PTX is dispensable for the development of EAE in PD-1$^{-/-}$ mice. Next, when both PTX and the additional dose of HKMTB were eliminated, many PD-1$^{-/-}$ mice still developed EAE, while the PD-1$^{+/+}$ mice remained healthy (Fig. 1C). The severe EAE in PD-1$^{-/-}$ was well-correlated with the number of immune cells infiltrating in the affected brain (Fig. S1).

 CFA itself contains a low dose of HKMTB (50 μg/mouse). This small dose of HKMTB appeared to be required for EAE in PD-1$^{-/-}$ mice, because PD-1$^{-/-}$ mice that received MOG

in incomplete Freund's adjuvant did not develop EAE with or without PTX. These data indicated that PD-1$^{-/-}$ mice were susceptible to EAE by immunization with much lower doses of adjuvants.

Enhanced inflammatory cytokine production associates with EAE in PD-1$^{-/-}$ mice.
Th1 and Th17 cells, which typically produce IFN$-\gamma$ and IL-17, respectively, have been shown to be involved in EAE (30). To study the development of the two Th cell types during disease progression in PD-1$^{-/-}$ mice, we analyzed the MOG-specific effector T cell response at early and later phases of the disease. On day 8, purified CD4$^+$ T cells from the spleen of PD-1$^{-/-}$ mice produced significantly more IL-17 and IFN-γ than the cells from PD-1$^{+/+}$ mice, upon re-stimulation with MOG$_{35-55}$ in vitro (Fig. 2A). In response to suboptimal immunization conditions, the cytokine levels were lower, but the difference between PD-1$^{-/-}$ and PD-1$^{+/+}$ mice was still significant. These findings were consistent with the earlier onset, and the severe disease in PD-1$^{-/-}$ mice.

Next, we examined the T cell responses to MOG$_{35-55}$ on day 30, when the clinical symptoms have mostly receded. As shown in Fig. 2B, the PD-1$^{-/-}$ CD4$^+$ cells still produced significantly higher levels of IL-17 than PD-1$^{+/+}$ control cells; however, the IFN$-\gamma$ levels were indistinguishable. These results suggested that, in comparison to PD-1$^{+/+}$ mice, PD-1$^{-/-}$ mice generated a stronger memory response to both the standard and suboptimal EAE-inducing immunizations, and this response was associated with enhanced IL-17 production.

The innate response to HKMTB in PD-1$^{-/-}$ mice influences antigen-specific Th17 development.

Since PD-1$^{-/-}$ mice developed a MOG$_{35-55}$-specific Th17 response to EAE immunizations with low doses of HKMTB, PD-1$^{-/-}$ mice may have intrinsic properties to exhibit augmented response to HKMTB, which results in enhanced induction of Th17. As PD-1 is expressed on innate cells (31, 32) as well as T and B cells, PD-1 may regulate innate response that influences T cell activation. To address this point, we established a system in which we can separately evaluate the function of PD-1 in the innate and the acquired immune responses. First, we bred PD-1$^{-/-}$ mice onto the RAG2$^{-/-}$ background to exclude T and B cell responses (see Materials and Methods). Then, PD-1$^{-/-}$ RAG2$^{-/-}$or control PD-1$^{+/+}$ RAG2$^{-/-}$ mice were immunized with HKMTB alone in CFA to induce a genuine anti-HKMTB response in vivo. Spleen cells from the immunized mice were prepared and used to stimulate naïve OVA-specific TCR transgenic T cells (PD-1$^{+/+}$) under the various Th skewing conditions (see Materials and Methods).

We found that, when splenocytes from HKMTB-immunized PD-1$^{-/-}$ RAG2$^{-/-}$mice were incubated with PD-1$^{+/+}$ naïve OVA-reactive T cells under neutral condition, IL-17^{+} cells were produced more efficiently (average$\pm$SD of 22.5$\pm$3.627%) than when splenocytes from HKMTB-immunized PD-1$^{+/+}$ RAG2$^{-/-}$ mice (13.4$\pm$4.68%) were used (Fig3A 2nd columns; P=0.021 from 5 combined experiments). When exogenous IL-6 and TGF-β are added to the neutral condition (Th17 condition; Fig3A 4th columns), splenocytes from both background showed efficient induction of IL-17^{+} cells (PD-1$^{+/+}$ RAG2$^{-/-}$ vs. PD-1$^{-/-}$ RAG2$^{-/-}$: 34.33 $\pm$3.99 vs. 37.43$\pm$5.84, P=0.54). In contrast, splenocytes from both PD-

$1^{+/+}$ RAG2$^{-/-}$ and PD-1$^{-/-}$ RAG2$^{-/-}$ mice were similarly efficient in promoting the differentiation of IFN$-\gamma$-producing cells under Th1 skewing conditions (P=0.48 in no immunized and P=0.65 in immunized mice; 5 experiments) (Fig. 3B). We got essentially same data when we performed the same experiments with the mice in different genetic background (using PD-1$^{-/-}$ RAG2$^{-/-}$ in BALB/c background and naïve CD4^{+} cells from DO11.10 TCR Tg), suggesting the finding is general to PD-1$^{-/-}$ strain (Fig. S2). Also, splenocytes from HKMTB immunized PD-1$^{-/-}$ mice on RAG2-sufficient background were more potent in inducing Th17 than PD-1$^{+/+}$ counterpart (Fig. S3). It should be noted, however, that exogenous IL-6 and TGF-β were required for efficient Th17 induction (Th17 condition) when RAG2-sufficient splenocytes were used. It suggested that T cell and B cell responses in RAG2-sufficient mice somehow attenuate the primary innate anti-HKMTB-response. In any case, the current results indicated that the innate immune response of PD-1$^{-/-}$ mice, when exposed to mycobacteria, were more efficient at promoting a Th17 response, compared to PD-1$^{+/+}$ mice.

Innate immune cells of PD-1$^{-/-}$ mice causes enhanced Th17 and exacerbation of EAE

To directly determine contribution of enhanced innate response of PD-1$^{-/-}$ mice to HKMTB in the development of EAE, we reconstituted lethally irradiated wild-type C57BL/6 mice with bone marrow cells from PD-1$^{+/+}$ RAG2$^{-/-}$ and PD-1$^{-/-}$ RAG2$^{-/-}$ mice. Then, recipients received adoptive transfer of CD4^{+} T cell from PD-1$^{+/+}$ and were immunized with MOG$_{35-55}$ in the emulsion with CFA and HKMTB. We found that the mice reconstituted with PD-1$^{-/-}$ RAG2$^{-/-}$ bone marrow developed EAE with earlier onset and more severe clinical scores than the mice reconstituted with PD-1$^{+/+}$ RAG2$^{-/-}$ bone

marrow (Fig. 4A). The severe EAE in the PD-1$^{-/-}$ RAG2$^{-/-}$ bone marrow recipients were associated with significantly higher production of IL-17 in the recall response, compared to PD-1$^{+/+}$ RAG2$^{-/-}$ recipients (Fig. 4B). The data strongly suggested that PD-1 deficiency in the innate cells caused priming of CD4^{+} T cell from PD-1$^{+/+}$ into pathogenic Th17, leading to severe EAE.

CD11b^{+} macrophages from HKMTB-treated PD-1$^{-/-}$ mice efficiently induce Th17 cell polarization

We wanted to determine the innate immune cell population responsible for the enhanced Th17 induction in PD-1$^{-/-}$ mice. Since macrophages and some dendritic cells (DCs) express CD11b, we magnetically separated PD-1$^{-/-}$ splenocytes into the CD11b^{+} and CD11b^{-} populations, treated each of them with HKMTB in vitro, and then assayed their ability to induce Th17. We found that the CD11b^{+} population from PD-1$^{-/-}$ mice skewed naïve T cells into Th17 cells more efficiently than did CD11b^{+} cells from PD-1$^{+/+}$ mice, in a manner dependent on the added bacterial dose (Fig. 5A and 5B). In contrast, we detected little or no Th17 skewing by the CD11b^{-} population (Fig. 5B), suggesting that the antigen presenting cell (APC)s in the CD11b^{-} fraction (mostly B cells) were not involved in the Th17 skewing by PD-1$^{-/-}$ APCs. Further FACS-sorting of CD11b^{+} population into macrophage (CD11b^{+}) and DCs (CD11b^{+} CD11c^{+}) revealed that HKMTB-treated macrophages, but not of HKMTB-treated DCs promote Th17 skewing (Fig S4). Similar results were obtained when CD11b^{+} cells were prepared from PD-1$^{+/+}$ RAG2$^{-/-}$ and PD-1$^{-/-}$ RAG2$^{-/-}$ mice (Fig. 5C). These data suggested that CD11b^{+} macrophages in PD-1$^{-/-}$ mice influence Th17 development upon the recognition of

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HKMTB.

Enhanced production of IL-6 by CD11b$^+$ cells from PD-1$^{-/-}$ mice causes strong Th17 development

To examine the mechanism of the enhanced Th17 skewing by PD-1$^{-/-}$ macrophages, we measured the cytokine production from the CD11b$^+$ population from PD-1$^{-/-}$ mice after HKMTB treatment. Among the cytokines tested (IL-2, IL-4, TNF-α, IL-6, IL-17, IFN-γ, IL-10, IL-12 and IL-23), the PD-1$^{-/-}$ CD11b$^+$ cells produced more IL-6 in the presence of HKMTB than did the PD-1$^{+/+}$ CD11b$^+$ cells (Fig. 6A). Other cytokines detected, such as TNF-α (Fig. 6B) and IL-10 (Fig. 6C), were similar between CD11b$^+$ cells from PD-1$^{+/+}$ and PD-1$^{-/-}$ cells. In addition, this augmentation of IL-6 production by PD-1$^{-/-}$ cells was specific for HKMTB, as other gram-positive bacteria such as heat-killed listeria monocytogenes (HKLM), did not induce the production of significant levels of IL-6 from either PD-1$^{+/+}$ or PD-1$^{-/-}$ CD11b$^+$ cells (Fig. 6A). The difference in IL-6 production between bacterial strains may be due to differences in their ability to activate toll-like receptors, as well as other receptors, such as dectin, on the CD11b$^+$ cells.

Since IL-6 is important for Th17 induction, we confirmed that the IL-6 from HKMTB-treated PD-1$^{-/-}$ CD11b$^+$ cells functioned to induce the differentiation of IL-17-producing cells. As shown in Fig. 6D, the antigen-specific Th17 differentiation by HKMTB-treated CD11b$^+$ cells was inhibited in the presence of a blocking anti-IL-6 receptor antibody, in a dose-dependent manner. Taken together, these data suggested that PD-1 regulates the IL-6 production from macrophages to inhibit Th17 induction.

DISCUSSION

Here we found that the induction of EAE in PD-1$^{-/-}$ mice required considerably lower amounts of bacterial adjuvants compared to the EAE induction in PD-1$^{+/+}$ mice. In addition, PD-1$^{-/-}$ mice mounted an augmented Th-17 effector/memory response against myelin antigen. Although PD-1 has previously been shown to function on T cells, our in vitro and in vivo assays using naïve PD-1$^{+/+}$ T cells and lymphocyte-null PD-1$^{-/-}$ RAG2$^{-/-}$ mice suggested that PD-1 regulates Th17 differentiation indirectly, by suppressing the IL-6 production from innate immune cells.

Previous studies have shown that IL-6 is necessary for Th17 induction in EAE, as IL-6$^{-/-}$ mice (33) or treatment with an IL-6 blocking antibody (34) abrogates EAE. Although activated T cells can produce autocrine IL-6, the receptor for IL-6 is expressed only on naïve T cells and is down-modulated after activation (35, 36). Thus, T cells that are exposed to IL-6 early in their activation differentiate into pathogenic Th17 cells. Our experiments showed macrophages, but not DCs from PD-1$^{-/-}$ enhanced the induction of Th17 upon HKMTB treatment *in vitro* (Fig. S4). DCs are necessary for EAE development, but are not potent to produce IL-6 upon mycobacteria recognition at least *in vitro* (37, 38). Thus, although our data does not exclude PD-1's function on DCs *in vivo*, it suggest that CD11b^{+} macrophages are primary population that produces early IL-6 in response to HKMTB.

The massive production of IL-6 by PD-1$^{-/-}$ macrophages may explain the unique response of these mice to mycobacterial infection. Although the Th1 response is ideal for controlling MTB (39), infected PD-1$^{-/-}$ mice exhibit high serum IL-6 levels (23, 24), which may lead to an altered Th1 response and an augmented Th17 response. An

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augmented Th17 response induced by over-produced IL-6 not only inhibits efficient anti-mycobacterial immune responses, but also contributes to autoimmune-like diseases in mice. We conclude that PD-1 functions to control innate IL-6 production, which in turn suppresses auto-antigen-specific T cell responses. Our conclusions are consistent with a previous report that immunizing PD-1$^{-/-}$ mice with Bacille Calmette-Guerin (BCG) induces symptoms resembling Kawasaki disease, a vascular inflammatory disease associated with IL-6 production (40).

As PD-1 down-modulates the tyrosine phosphorylation of cytoplasmic proteins including Syk tyrosine kinase, through activation of non-receptor tyrosine phosphatase, SHP-2 (41), a direct function of PD-1 in macrophage activation could explain the augmented IL-6 production by PD-1$^{-/-}$ macrophages. However, we could not demonstrate the expression of PD-1 on the monocyte/macrophage population. This inability to reproduce results from a previous study in which PD-1 was detected on macrophages (32, 42) may be due to the high background binding of available anti-mouse PD-1 antibodies to this cell type. Indeed, the mAb used in previous research (42) was found to stain PD-1$^{-/-}$ macrophages as efficiently as PD-1$^{+/+}$ macrophages (our unpublished data). Thus, it is likely that the regulation of macrophage activation by PD-1 is not intrinsic to the macrophage. Since basal macrophage activation occurred in PD-1$^{-/-}$ RAG2$^{-/-}$ mice, this would suggest that non-T cells, possibly innate lymphoid cells, are responsible for the basal macrophage activation in these mice. For example, PD-1 is expressed on natural killer cells (ref (43) and our unpublished data) and regulates their cytokine production (44). In the absence of PD-1, natural killer cells may produce more cytokines during immune surveillance or upon infection, possibly affecting the activation status of

macrophages. <mark>How PD-1 regulates macrophage activation in cell-extrinsic manner is currently unclear and should be addressed in future studies.</mark>

In conclusion, we propose that PD-1 can regulate a magnitude of primary innate responses to certain microbes, and this regulation in turn inhibit the differentiation of self antigen-specific inflammatory T cells. Our findings provide a new perspective on the function of PD-1, which was previously well-established as a key molecule for the maintenance of immune tolerance.

MATERIALS AND METHODS

Mice. PD-1$^{-/-}$ mice were described before (45) and thoroughly backcrossed into C57BL/6 or BALB/c background (for 13 times). PD-1$^{-/-}$ and the littermate control (PD-1$^{+/+}$) were obtained from heterozygote (PD-1$^{+/-}$) to heterozygote breeding. DO11.10 TCR transgenic (Tg) mice and OT-II TCR Tg mice were obtained from Drs. K. Murphy (Washington University, St. Louis, MO) and K. Kabashima (Kyoto University, Japan), respectively. RAG2$^{-/-}$ mice were from Dr. F. Alt (Harvard Medical School, Boston, MA) and backcrossed into BALB/c or C57BL/6 background for 10 times. To generate mice doubly deficient for PD-1 and RAG2, we first mated PD-1 single KO with RAG2 single KO in the same genetic background. Resulting F1 generation (PD-1$^{+/-}$ RAG2$^{+/-}$) was intercrossed to obtain PD-1$^{-/-}$ RAG2$^{-/-}$ (double KO). Littermate PD-1$^{+/+}$ RAG2$^{-/-}$ mice from the intercross breeding were used as control. All mice were maintained under specific pathogen-free conditions and treated according to approved protocols by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Reagents. Neutralizing rat anti-IL-6 mAb (MR16-1) was kindly provided by Dr. T. Kishimoto (Osaka University, Japan) and Chugai Pharmaceutical Co. Ltd (Tokyo, Japan). Chrompure Rat immunoglobulin G (IgG) was from Jackson Immuno Research (West Grove, PA). Information of FACS antibodies, and peptide sequence are shown in supplementary materials.

EAE. Mice in were immunized subcutaneously with 0.2 ml of an emulsion containing 200 μg MOG$_{35-55}$ peptide, CFA, and 250 μg HKMTB. In addition, the mice received two intraperitoneal injections of 200 ng PTX at the time of, and 48 hours after immunization. In some experiments, "suboptimal" immunization conditions were used, in which HKMTB, PTX, or both were excluded. Mice were observed daily for clinical signs of disease, and EAE scores defined as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, paralysis of one hind limb; 4, paralysis of both hind limbs; 5, limited movement; 6, moribund or death. Scores are shown as the mean clinical scores for each experimental group. For re-stimulation assay, spleens were harvested 8 or 30 days after immunization, and CD4$^+$ T cell- enriched samples were obtained using anti-CD4 microbeads and the autoMACS system (Miltenyi). In a 1-ml volume, 2×10^6 of the isolated CD4$^+$ T cells were cultured with 2×10^6 mitomycin C-treated splenocytes from PD-1$^{+/+}$ mice in the presence of MOG$_{35-55}$ peptide (30 μg/ml). Supernatants were harvested 3 days later for cytokine analysis.

Antigen-specific Th skewing by HKMTB-immunized splenocytes. Mice were immunized subcutaneously with 0.2 ml of an emulsion containing 250 μg HKMTB and CFA. Eight days later, 1×10^6 splenocytes were cultured with 1×10^5 CD4$^+$ T cells purified from DO11.10 or OTII TCR Tg mice in the presence of 5 μg/ml OVA in 48-well plates. For Th-skewing, the following recombinant cytokines and antibodies were added to the cultures: Th1: IL-12 (20 ng/ml) and anti-IL-4 (10 μg/ml); Th17: human TGF-β1 (5 ng/ml), IL-6 (20 ng/ml), anti-IL-4 (10 μg/ml), and anti- IFN−γ (10 μg/ml); neutral conditions: anti-IL-4 (10 μg/ml) and anti-IFN−γ (10 μg/ml). Four days later, the cells

were re-stimulated with 10 ng/ml PMA and 1 µg/ml Ionomycin (Sigma-Aldrich) in the presence of 0.66 µl/ml BD Golgistop for 6 hours. After stimulation, the cells were washed in FACS buffer (PBS, 2% FCS, and 0.02% NaN_3) and stained for cell-surface markers. The cells were then treated with BD Cytofix/Cytoperm buffer, washed with BD perm/wash buffer twice, and subsequently stained with anti-cytokine antibodies. Samples were analyzed by FACSCanto II (BD).

Antigen-specific Th skewing by mycobacteria activated CD11b+ cells. Splenocytes were labeled with Miltenyi CD11b microbeads and subjected to 3 positive selections by autoMACS (Miltenyi). The purified cells contained approximately 95% CD11b+ cells. In a total volume of 200 µl, 3×10^5 of the isolated CD11b+ cells were stimulated with HKMTB or HKLM at 10 and 100 µg/ml. Three days later, cell culture supernatants were evaluated for cytokine production. For antigen-specific Th skewing, 1×10^6 CD11b+ cells were stimulated with HKMTB or HKLM at 10 and 100 µg/ml for 3 days. The stimulated CD11b+ cells were then cultured with 1×10^5 CD4+ T cells purified from OTII TCR Tg mice for 4 days in the presence of 5 µg/ml $OVA_{323-339}$ under neutral conditions. In some experiments, a rat anti-IL-6R blocking antibody or control rat IgG was added to the cultures. After a 4-day incubation, the development of Th1 and Th17 cells was examined by intracellular cytokine staining, as described above.

Cytokine analysis. Culture supernatants were stored at -80°c until the analysis. IL-12p70 and IL-23 (p19/p40) were determined by ELISA (eBioscience). The IL-2, IL-4, TNF-α,

IL-10, IL-6, IL-17 and IFN-γ levels were measured by the mouse Th1/Th2/Th17 CBA kit (BD) or ELISA (eBioscience), according to the manufacturer's protocols.

Statistics. Statistical significance between two groups was calculated using a two-tailed Student's t-test, and a P-value of <0.05 was considered to be statistically significant.

Acknowledgements

Author contributions

Y.R. and S.C. designed research, performed experiments, and analyzed data. Y.R., S.C. and T.H. wrote the paper.

Footnotes

The authors declare no competing financial interests.

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FIGURE LEGENDS

Fig. 1. Augmented EAE with sub-optimal immunization of PD-1^{-/-} mice. (A) EAE scores of PD-1^{+/+} (n=12) and PD-1^{-/-} (n=12) mice (C57BL/6 background) after immunization with MOG/CFA/HKMTB in the presence of PTX on day 0 and day 2. (B) EAE scores of PD-1^{+/+} (n=13) and PD-1^{-/-} (n=12) mice after immunization with MOG/CFA/HKMTB. (C) EAE scores of PD-1^{+/+} (n=13) and PD-1^{-/-} (n=11) mice after immunization with MOG/CFA. Data presented are combined from two independent

experiments (means±SEM). Statistical analysis was performed using a two-tailed Student's t-test. * P<0.05, and ** P<0.01.

Fig. 2. EAE in PD-1$^{-/-}$ mice associates with enhanced inflammatory cytokine production. (A) PD-1$^{+/+}$ and PD-1$^{-/-}$ mice (C57BL/6 background) were immunized with MOG/CFA/HKMTB/PTX (n=12), MOG/CFA/HKMTB (n=11), or MOG/CFA (n=6) and analyzed 8 days later. (B) PD-1$^{+/+}$ and PD-1$^{-/-}$ mice were immunized with MOG/CFA/HKMTB/PTX (n=7), MOG/CFA/HKMTB (n=7), or MOG/CFA (n=13) and analyzed 30 days later. (A,B) Sorted CD4$^+$ T cells were restimulated with MOG$_{35-55}$, and the cytokine concentrations were determined by ELISA. Statistical significance was determined by Student's t-test. NS P>0.05, * P<0.05, and ** P<0.01.

Fig. 3. Splenocytes from HKMTB-immunized PD-1$^{-/-}$ RAG2$^{-/-}$ mice facilitate Th17 induction in vitro. Splenocytes from non-immunized or HKMTB/CFA-immunized PD-1$^{+/+}$ RAG2$^{-/-}$ and PD-1$^{-/-}$ RAG2$^{-/-}$ mice (C57BL/6 background) were cultured with naïve CD4$^+$ T cells from OTII TCR Tg mice in the presence of OVA$_{323-339}$ (5 μg/ml) under conditions indicated. Development of the IL-17-producing (A) or IFN-γ-producing (B) CD4$^+$ T cells were examined by intracellular cytokine staining. Essentially same data were obtained from experiments immunizing PD-1$^{-/-}$ RAG2$^{-/-}$ mice (BALB/c background) and naïve CD4$^+$ cells from DO11.10 TCR Tg mice (BALB/c background) (Fig. S2).

Fig. 4. Non-lymphocyte population of PD-1$^{-/-}$ mice cause enhanced Th17 development and exacerbation of EAE. (A) Lethally irradiated C57BL/6 mice were reconstituted by bone marrow transplantation and adoptive transfer of CD4$^+$ cells as indicated. Mice were immunized with MOG/CFA/HKMTB and the EAE score evaluated. Data presented are combined from two independent experiments (total 18 mice included in each group) (mean±SEM). Statistical significance was determined by Student's t-test (* P<0.05, and ** P<0.01). (B) CD4$^+$ T cells were harvested at day 30 and re-stimulated by MOG$_{35-55}$ as indicated in materials and methods. IL-17 and IFN-γ were measured. Each point represents an individual mouse, with the black bar indicating the mean value.

Mice reconstituted with PD-1$^{-/-}$ RAG2$^{-/-}$ BM showed significantly higher IL-17 than PD-1$^{+/+}$ RAG2$^{-/-}$ counterpart by one-tailed Student's t-test. (* P=0.04)

Fig. 5. CD11b^{+} cells from PD-1$^{-/-}$ mice efficiently induce Th17 cell development upon HKMTB stimulation CD11b^{+} cells were purified from the splenocytes of PD-1$^{+/+}$ and PD-1$^{-/-}$ mice (A) or PD-1$^{+/+}$ RAG2$^{-/-}$ and PD-1$^{-/-}$ RAG2$^{-/-}$ mice (C) (all in C57BL/6 background) and were either untreated (ctrl), or stimulated by HKMTB (10 and 100 µg/ml). Three days later, the stimulated cells were co-cultured with CD4^{+} T cells from OTII TCR Tg mice in the presence of OVA$_{323-339}$ (5 µg/ml) for 4 days. IL-17-producing OTII^{+} CD4^{+} T cells (PD-1$^{+/+}$) were quantified by intracellular cytokine staining. (B) Summary of the data shown in (A). Note that CD11b^{-} cells from PD-1$^{+/+}$ and PD-1$^{-/-}$ mice did not induce Th17 by two independent experiments. Data shown of CD11b^{+} cells are mean±SD from six independent experiments (for ctrl and HKMTB 100 µg/ml condition) and four experiments (for HKMTB 10 µg/ml condition). Statistical significance was determined by Student's t-test. * P<0.05 and ** P<0.01.

Fig. 6. Enhanced production of IL-6 by CD11b^{+} cells from PD-1$^{-/-}$ mice causes strong Th17 development CD11b^{+} cells purified from the splenocytes from PD-1$^{+/+}$ and PD-1$^{-/-}$ mice (C57BL/6 background) were either untreated (ctrl) or stimulated with HKMTB (10 and 100 µg/ml), or HKLM (10 and 100 µg/ml). Three days later, the production of IL-6 (A), TNF-α (B), and IL-10 (C) was evaluated. Data presented are combined from three independent experiments (mean±SD). Significance was determined by Student's t-test. * P<0.05. (D) HKMTB-stimulated CD11b^{+} cells were co-cultured in the presence of OTII^{+}CD4^{+} T cells and OVA$_{323-339}$ together with rat IgG (200 ng/ml) or anti-IL-6R (as shown) under neutral condition for 4 days. IL-17-producing OTII^{+} CD4^{+} T cells were quantified by intracellular cytokine staining. Data shown are representative of three independent experiments.
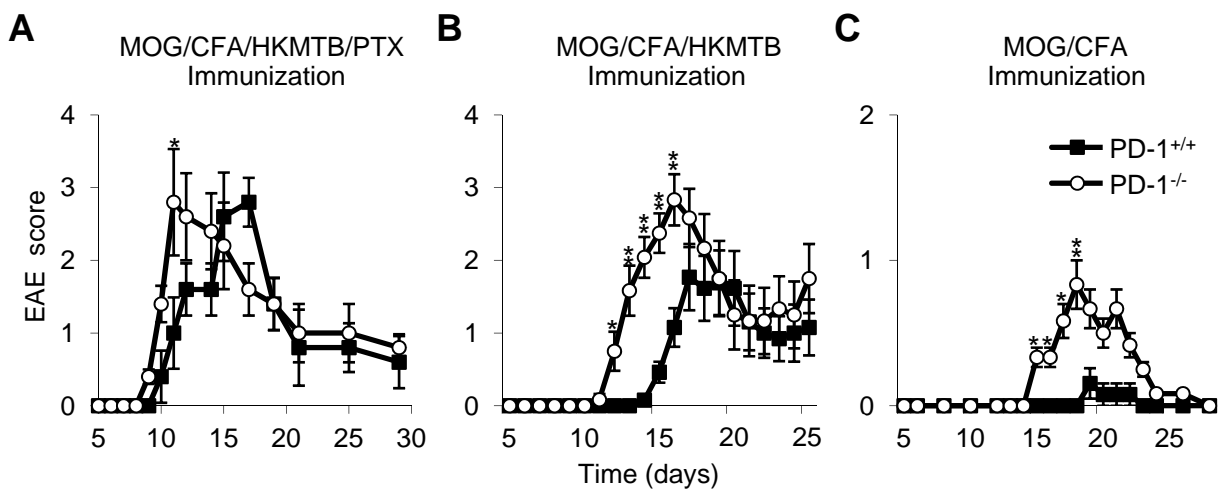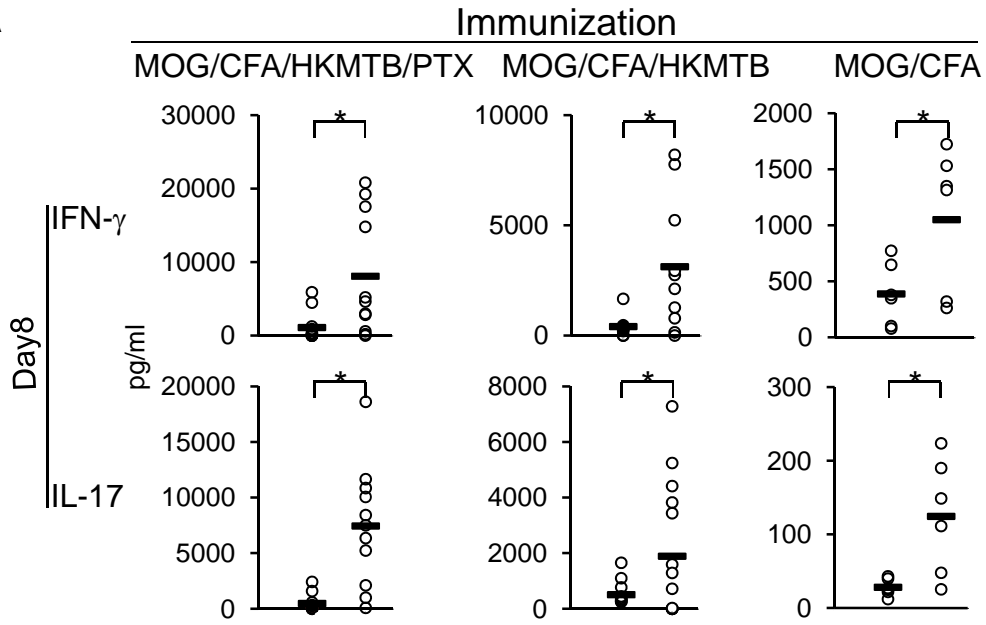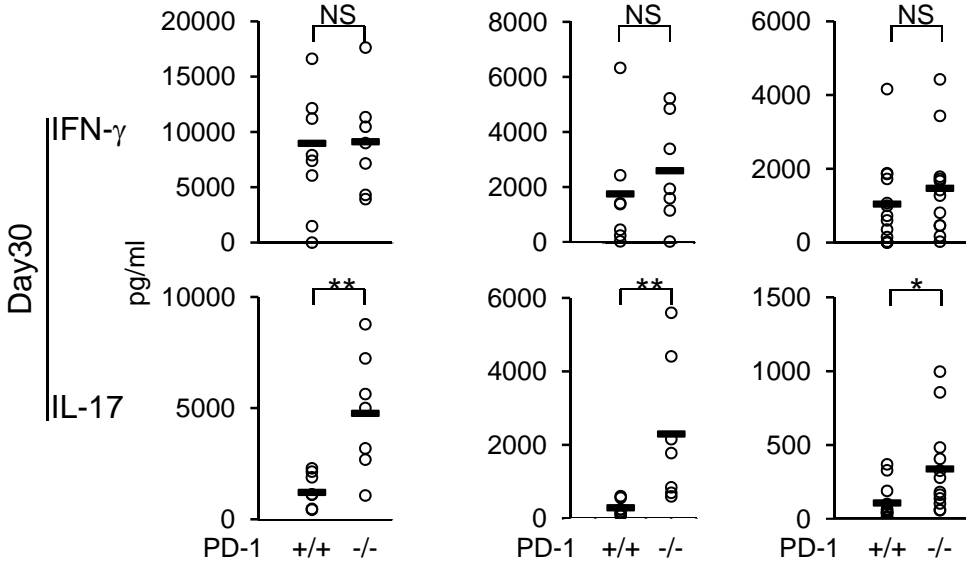
Fig. 1

A

MOG/CFA/HKMTB/PTX
Immunization

B

MOG/CFA/HKMTB
Immunization

C

MOG/CFA
Immunization

PD-1$^{+/+}$
PD-1$^{-/-}$

EAE score

Time (days)

Fig. 2

A

Immunization

MOG/CFA/HKMTB/PTX   MOG/CFA/HKMTB   MOG/CFA

Day8

IFN-γ

IL-17

pg/ml

B

Day30

IFN-γ

IL-17

pg/ml

PD-1   +/+   -/-   PD-1   +/+   -/-   PD-1   +/+   -/-

2

Fig. 3

A

Neutral | Th17 skewing
Immunization:

B

Th1 skewing

3

Fig. 4

A

MOG/CFA/HKMTB
Immunization

Mice reconstituted with
■ BM: PD-1$^{+/+}$ RAG2$^{-/-}$ ⎤ plus CD4: PD-1$^{+/+}$
○ BM: PD-1$^{-/-}$ RAG2$^{-/-}$ ⎦

B

IL-17

IFN-γ

Fig. 5

A

B

C

Fig. 6

A

IL-6 (pg/ml)

5000
4000
3000
2000
1000
0

ctrl HKMTB HKLM

B

TNF-α (pg/ml)

10000
8000
6000
4000
2000
0

ctrl HKMTB HKLM

C

IL-10 (pg/ml)

2000
1500
1000
500
0

ctrl HKMTB HKLM

mouse cells
■ PD-1⁺/⁺] CD11b⁺
□ PD-1⁻/⁻]
▨ PD-1⁺/⁺] CD11b⁻
▨ PD-1⁻/⁻]

D

Percent IL-17⁺ cells in total OTII⁺ CD4⁺

30
25
20
15
10
5
0

200	0.2	2	20	200	200	0.2	2	20	200	200	0.2	2	20	200	(ng/ml)
rat IgG	anti-IL-6R				rat IgG	anti-IL-6R				rat IgG	anti-IL-6R				
ctrl					HKMTB 10 μg/ml					HKMTB 100 μg/ml					

mouse cells
■ PD-1⁺/⁺] CD11b⁺
□ PD-1⁻/⁻]

6

Fig. S1

Fig. S1. Increased infiltrating cells to central nervous system in PD-1$^{-/-}$ EAE mice. PD-1$^{+/+}$ (n=9) and PD-1$^{-/-}$ mice (n=8) were immunized by MOG/CFA/HKMTB and analyzed 8 days later. Unimmunized PD-1$^{+/+}$ mice (n=3) were used as control. Cells were prepared as described in "supplementary methods" and referred to as total cells. (A) Data shown are representative FACS analysis of three independent experiments. (B) Calculated absolute numbers of each population was shown. Statistical significance was determined by two tailed Student's t-test. * * P<0.01, * P<0.05.
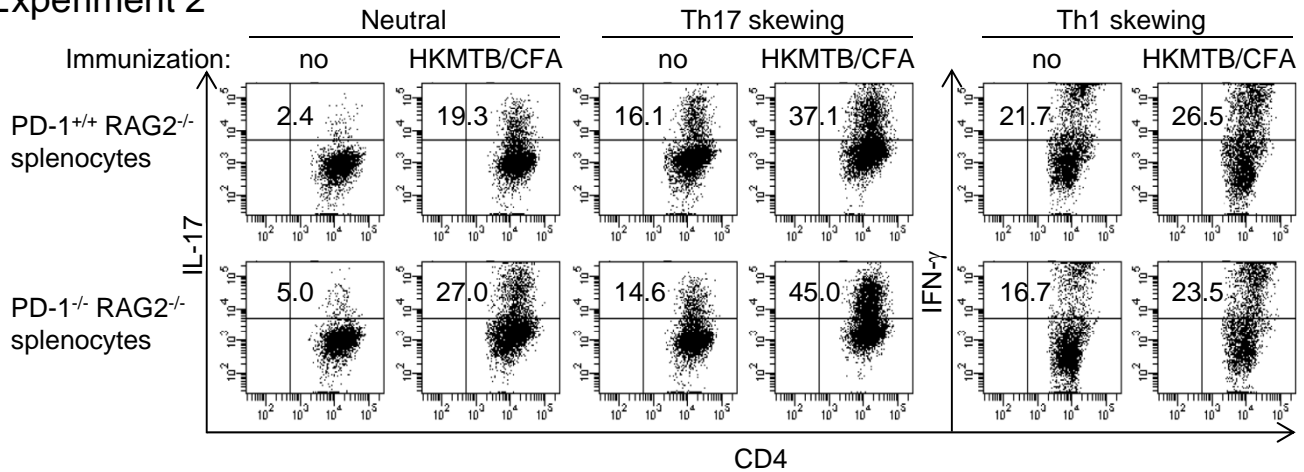
Fig. S2

Experiment 1
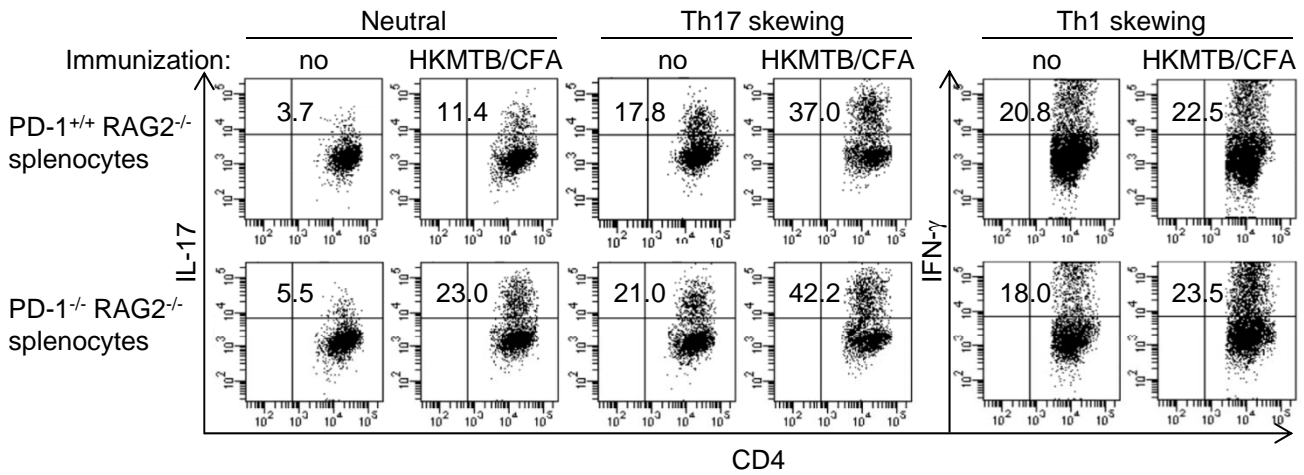
Experiment 2

Fig. S2. Splenocytes from HKMTB-immunized PD-1$^{-/-}$ RAG2$^{-/-}$ mice facilitate Th17 induction in vitro. Splenocytes from non-immunized or HKMTB/CFA-immunized PD-1$^{+/+}$ RAG2$^{-/-}$ and PD-1$^{-/-}$ RAG2$^{-/-}$ mice (BALB/c background) were cultured with naive CD4$^+$ T cells from DO11.10 TCR Tg mice in the presence of OVA$_{323-339}$ (5 μg/ml) under conditions indicated. Development of the IL-17-producing or IFN-γ-producing CD4$^+$ T cells were examined by intracellular cytokine staining. Data from two independent experiments were presented.
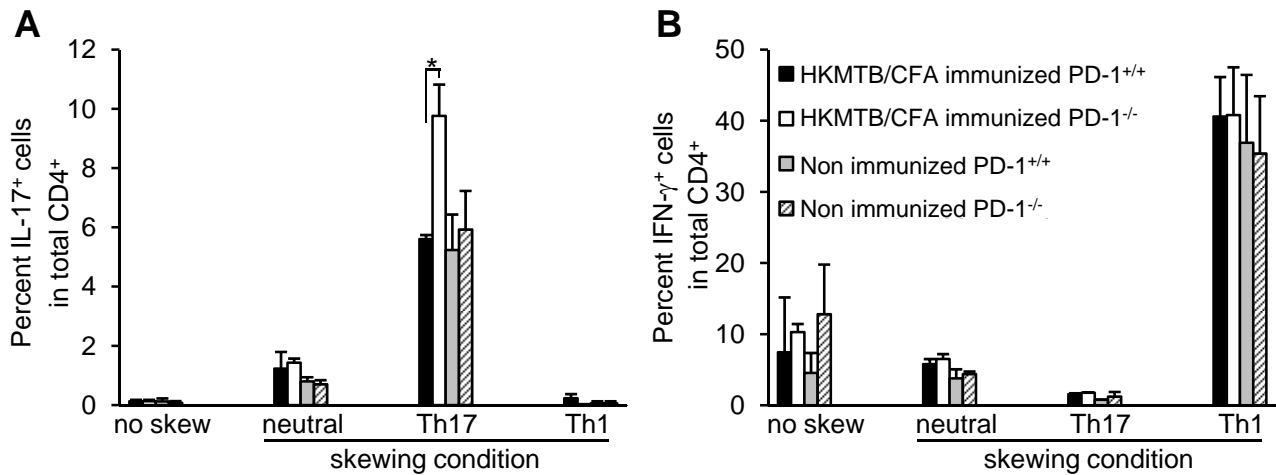
Fig. S3

Fig. S3. Splenocytes from HKMTB-immunized, RAG2 sufficient PD-1$^{-/-}$ mice facilitate Th17 induction in vitro. Splenocytes from non immunized or HKMTB/CFA immunized PD-1$^{+/+}$ and PD-1$^{-/-}$ mice (C57BL/6 background) were incubated with naive CD4$^+$ T cells from OTII TCR Tg mice in the presence of OVA (5 μg/ml) under no skew, neutral, Th17 skewing, or Th1 skewing conditions. Four days later, the IL-17-producing (A) or IFN-γ-producing (B) CD4$^+$ T cells were quantified by intracellular cytokine staining. Data presented are combined from two independent experiments (means±SD). Significance was determined by Student's t-test. * P<0.05.
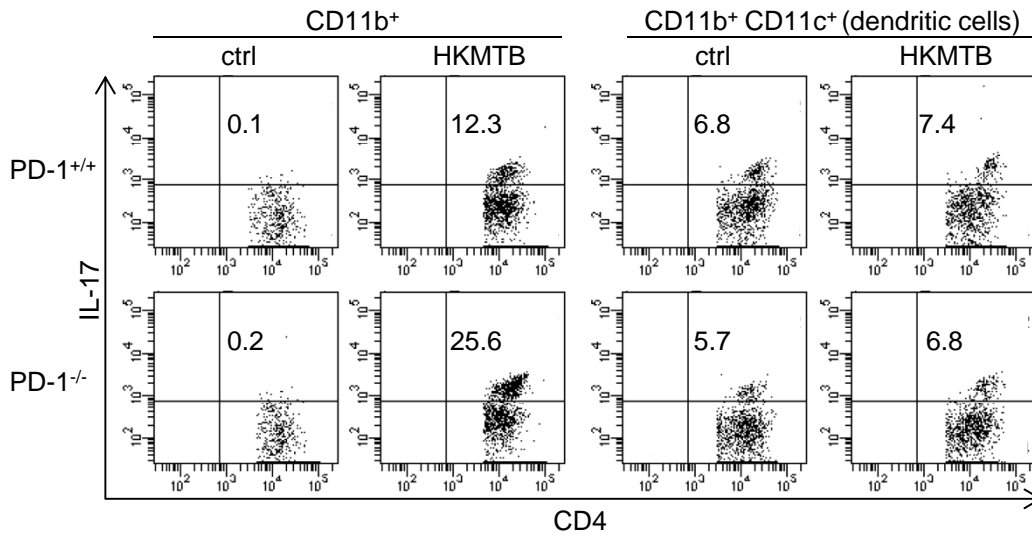
Fig. S4

Fig. S4. Dendritic cells are not involved in the HKMTB treated CD11b⁺-mediated Th17 polarization. FACS Aria sorted CD11b+ and DC (CD11b⁺ CD11c⁺) cells from splenocytes of PD-1⁺/⁺ and PD-1⁻/⁻ mice are treated by either ctrl (no stimulation) or HKMTB (100 μg/ml) and used in antigen-specific Th skewing in vitro. IL-17-producing OTII⁺ CD4⁺ T cells were quantified by intracellular cytokine staining. Data shown are representative of two independent experiments.

Supplementary Information
Peptides and FACS antibodies

Mouse myelin oligodendrocyte glycoprotein (MOG) $_{35-55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) and chicken OVA$_{323-339}$ peptide (ISQAVHAAHAEINEAGR) were synthesized by MBL (Nagoya, Japan). Incomplete Freund's adjuvant (IFA; Difco, Detroit, MI), complete Freund's adjuvant (CFA; Difco), heat killed Mycobacterium tuberculosis H37RA (HKMTB; Difco), heat killed listeria monocytogenes (HKLM; InvivoGen, San Diego, CA), and pertussis toxin (PTX; List Biological Laboratories, Campbell, CA) were used. Antibodies (Abs) for FACS analysis [anti-CD4 (RM4-5), anti-DO11.10 TCR (KJ1-26), anti-TCRVα2 (B20.1), anti-IFN-γ (XMG1.2), anti-IL-17 (TC11.18H10), anti-CD45 (30-F11), anti-CD11b (M1/70) and anti-CD11c (N418)] were from eBioscience (San Diego, CA) or Biolegend (San Diego, CA). Recombinant IL-6 and IL-12 were from Wako (Osaka, Japan), and human TGF-β1 was from Miltenyi Biotec (Tokyo, Japan). Neutralizing anti-IL-4 (1D11) and anti-IFN-γ (XMG1.2) antibodies were from Thermo Scientific (Yokohama, Japan).

Isolation of mononuclear cells from mouse brain

Brain was harvested and cells were prepared as previously described (1). Briefly, the tissue was minced, enzyme-digested and then subjected for percoll gradient separation. The cells in interface between 37% and 70% percoll were recovered, referred to as total cells and subjected for FACS analysis.

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