

Treatment with incomplete Freund's adjuvant and *Listeria monocytogenes* delays diabetes via an interleukin-17-secretion-independent pathway

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Abstract. Non-obese diabetes (NOD) mice are widely used as an animal model in studies of type I diabetes (T1D). Treatment with complete Freund's adjuvant (CFA) in pro-diabetic NOD mice is known to inhibit disease progression by activating CD1d-specific natural killer (NK) T cells and inducing interleukin (IL)-17 secretion in innate immune cells. The aim of the present study was to examine the effect of incomplete Freund's adjuvant (IFA) and *L. monocytogenes* treatment on the development of T1D in NOD mice. This combined treatment of IFA and *L. monocytogenes*, a microbe that infects the liver and is primarily combatted by NK and cytotoxic T lymphocytes, was applied to mimic CFA treatment in pro-diabetic NOD mice. The combined IFA + *L. monocytogenes* treatment effectively delayed T1D development in the NOD mice. In contrast to CFA, the IFA + *L. monocytogenes* treatment did not induce T cells or innate immune cells to secrete IL-17. However, increased levels of regulatory T cells were detected. Furthermore, IFA + *L. monocytogenes* mice exhibited higher levels of IgG2a, although no notable T helper 1 cell response was observed when compared with the CFA or IFA control treated mice. Therefore, combined IFA + *L. monocytogenes* treatment was shown to delay T1D development in NOD mice via a novel mechanism, which was independent from the secretion of IL-17 by CFA-activated NKT cells.

Introduction

Non-obese diabetes (NOD) mice are one of the most commonly used animal models for the study of autoimmune diseases, and exhibit a susceptibility to the spontaneous development of autoimmune insulin-dependent diabetes mellitus (1). A

number of factors are associated with the development of diabetes in NOD mice, including the release of self-reactive cytotoxic T lymphocytes (CTL) from negative selection in the thymus (central tolerance) and the loss of regulatory T (Treg) cell function (peripheral tolerance) (2). In addition, natural killer (NK) and NKT cells play a critical role in the progression of type I diabetes (T1D) (3-5).

In 1990, adjuvant immunotherapy was first reported to be effective in preventing the development of T1D in pro-diabetic NOD mice (6), and subsequent studies (7,8) suggested that complete Freund's adjuvant (CFA) therapy was effective in treating new-onset NOD mice (9), although the underlying mechanisms remain unclear. Numerous studies have attempted to reveal the underlying immunotherapy mechanism and have found that adjuvant treatment in mice induces the differentiation of regulatory cell populations *in vivo*, inhibiting the onset of T1D (10-12). Mice treated with immunological adjuvants exhibit an altered ratio of T helper (Th)1 and Th2 cells, which promotes an antibody response, but prevents Th1-mediated auto-reactive T cell responses to pancreatic β -cell surface antigens (13). Furthermore, upregulation of pro-inflammatory cytokines, including tumor necrosis factor (TNF)- α , has been reported following adjuvant treatment (14). A previous study found that NOD mice treated with the bacillus Calmette-Guerin (BCG) vaccine were protected against T1D (15). CFA and the BCG vaccine contain inactivated *M. tuberculosis*; however, incomplete Freund's adjuvant (IFA) does not contain *M. tuberculosis*. Since IFA is unable to prevent T1D development in NOD mice as effectively as CFA (16,17), *M. tuberculosis* has been hypothesized to play an important role in the modulation of the immune response in cases of T1D.

A previous study demonstrated that the pro-inflammatory cytokine, interleukin (IL)-17, plays a critical role in the pathogenesis of T1D in NOD mice (18). In addition, treatment with CFA or *M. tuberculosis* has been reported to induce IL-17 expression. However, this increase in IL-17 expression was produced primarily by CD8⁺ (19) or $\gamma\delta$ T cells (20), rather than CD4⁺ Th17 cells. Further studies have indicated that NKT cells are involved in CFA-mediated protection against T1D in NOD mice via the activation of NK cells (21), which are the primary source of interferon (IFN)- γ in the pro-diabetic NOD mice (12,22). Mechanism studies show that these NKT cells are activated directly by *M. tuberculosis*, possibly via

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CD1d recognition of specific long fatty acyl chains (23) on the surface of *M. tuberculosis*. Activated NKT cells, including V α 19 NKT cells, produce IL-17 and other immunoregulatory cytokines, such as IL-4, -10 and IFN- γ (24).

In the present study, NOD mice were treated with a combined therapy of IFA and inactivated *L. monocytogenes*, a microbe that often infects the liver in humans and mice and whose elimination is in part dependent on activated invariant NKT cells (25). Heat-killed *L. monocytogenes* has been previously used as an adjuvant to induce strong Th1 responses in mice (26). *L. monocytogenes* shares numerous characteristics with *M. tuberculosis*; however, *L. monocytogenes* cannot induce IL-17 secretion in NKT cells as effectively as *M. tuberculosis*. The effects of a combined IFA + *L. monocytogenes* treatment on the development of T1D was investigated in a NOD mouse model.

Materials and methods

Mice and immunizations. A total of 108 female NOD mice (aged five weeks; 17–20 g) were purchased from Shanghai Animal Laboratory Center (Shanghai, China) and housed in the East Hospital of Tongji University (Shanghai, China). Mice were immunized by a hypodermic injection into their back with one of the three treatments. The IFA + *L. monocytogenes* group mice received heat-killed *L. monocytogenes* (10^8 bacteria/mouse) in 100 μ l IFA. A second group was injected with CFA, while a third IFA-only group received a control injection containing no bacteria. Another 10 mice were administered twice with IFA + *L. monocytogenes* immunization with the same dose at 5 weeks and 8 weeks of age. Blood sugar levels were measured every three days following immunization and the mice whose blood sugar levels were >11.8 mmol/L were defined as positive for T1D. All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of East Hospital of Tongji University. (Shanghai, China).

Fluorescence-activated cell sorting and intracellular staining. Cytokine secretion in the lymphocytes was analyzed using Cytofix/Cytoperm™ Plus (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. Spleen cells were collected and incubated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA), 5 μ M calcium ionophore A23187 (Sigma-Aldrich) and GolgiStop™ (BD Biosciences) at 37°C for 4 h. Surface staining was performed using anti-CD3e-PerCP/Cy5.5 antibodies (BioLegend, Inc., San Diego, CA, USA) for 20 min at 4°C. Cells were subsequently permeabilized with Cytofix/Cytoperm™ solution for 20 min at 4°C, and intracellular cytokine staining was performed with anti-IL-17A-Alexa Fluor 647 (cat. no. 560224; BD Biosciences) and phycoerythrin (PE)-IFN- γ antibodies (cat. no. 557735; BD Biosciences). For Treg staining, spleen cells were fixed and stained using anti-T cell receptor (TCR) b-fluorescein isothiocyanate (cat. no. 553171; BD Biosciences), anti-CD25-PE (cat. no. 553075; BD Biosciences) and intercellular anti-Foxp3-Alexa Fluor 647 (cat. no. 560402; BD Biosciences) antibodies. Antibodies were used in a 1:100 dilution (BioLegend) or 1:50 dilution (BD Biosciences), according to the manufacturer's instructions.

Antibody levels in the blood serum. Total levels of IgG, IgG1 and IgG2a were examined by ELISA. In brief, 96-well plates (Nunc; Thermo Fisher Scientific, Waltham, MA, USA) were coated with 300 ng/well goat anti-mouse IgG antibodies (Life Technologies, Grand Island, NY, USA) in phosphate-buffered saline (PBS) and incubated overnight at 4°C. After blocking with 5% skim milk in PBS-Tween-20, the plates were incubated for 1 h at 37°C with serially-diluted serum samples. Following three washes with PBS-Tween-20, the samples were reacted with sheep anti-mouse IgG, IgG1 or IgG2a antibodies conjugated to horseradish peroxidase (BD Biosciences). Plates were developed by adding tetramethylbenzidine (Endogen®; Pierce Biotechnology, Inc., Rockford, IL, USA) and incubating in the dark. The reaction was stopped using 1 mol/L H₂SO₄, and the optical densities (OD) were read at 450 nm using an ELISA reader (Thermo Fisher Scientific). ELISA end-point titers were expressed as the reciprocal of the highest sample dilution that yielded an OD two times the mean value of the blank control.

ELISA analysis of the expression of IL-17. A total of 1×10^6 lymphocytes were collected from pancreas-draining lymph nodes and cultured with RPMI-1640 medium with 10% FCS and 200 U/ml mouse IL-2 (cat. no. CYT-370; ProSpec-Tany Technogene Ltd. East Brunswick, NJ, USA) in 96-well plates with plate-coated 0.5 μ g/ml functional anti-CD3e (cat. no. 16-0031-81; eBioscience, Inc., San Diego, CA, USA) and 0.5 μ g/ml soluble CD28 (cat. no. 16-0821-81; eBioscience, Inc.) for two days in the presence of recombinant mouse IL-2 (40 U/ml, R&D Systems, Minneapolis, MN, USA). The expression of IL-17 in the medium was detected using a Mouse IL-17A Platinum ELISA kit (cat. no. M17AF0; eBioscience Inc.), according to the manufacturer's instructions.

Statistical analysis. Data were analyzed using GraphPad Prism® 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and are expressed as the mean \pm standard deviation. Differences between the groups were analyzed by one-way analysis of variance with a post-test comparison using the t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IFA and *L. monocytogenes* treatment in pro-diabetic NOD mice delays T1D development. T1D development in NOD mice is associated with numerous factors, including age, diet and living environment. Blood sugar levels of >11.8 mmol/L were observed in the mice aged 8–10 weeks (data not shown). As previously reported (6), treatment with CFA in pro-diabetic (five-week old) NOD mice was able to effectively block T1D development, which was confirmed in the present study. In order to determine the effects of alternative immune therapies, heat-killed *L. monocytogenes* (10^8 bacteria/mouse) was used instead of *M. tuberculosis* in CFA. The results indicated that IFA + *L. monocytogenes* was unable to totally prevent T1D development, as >50% of the mice became diabetic within 12 weeks of treatment, while in the CFA treatment group, this figure was <30% (Fig. 1). However, increased blood sugar levels were not detected in the IFA + *L. monocytogenes* group

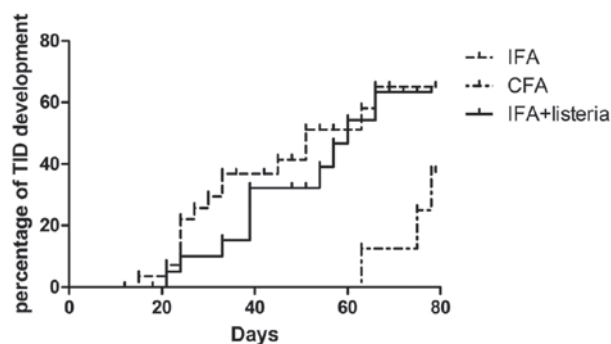


Figure 1. Percentage of T1D development in the different adjuvant-treated NOD mice groups. IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; T1D, type I diabetes; NOD, non-obese diabetes.

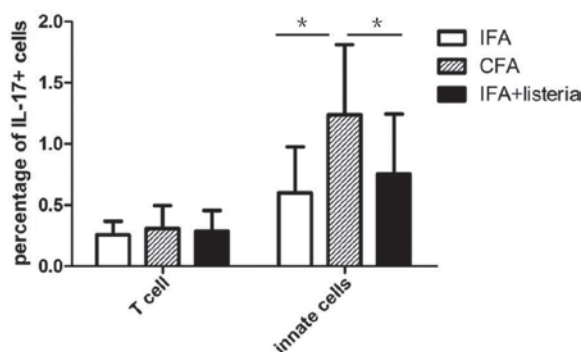


Figure 2. Percentage of IL-17⁺ T cells and innate cells in the different adjuvant-treated NOD mice groups. *P<0.05. IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; IL, interleukin; NOD, non-obese diabetes.

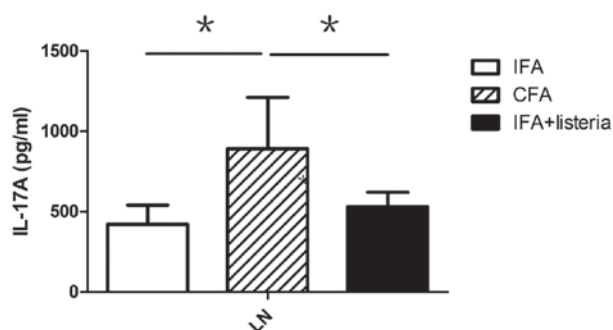


Figure 3. IL-17 secretion in the pancreatic draining LNs from the different adjuvant-treated NOD mice groups. *P<0.05. IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; IL, interleukin; NOD, non-obese diabetes; LN, lymph nodes.

mice until they were 12 weeks-old (seven weeks after treatment), suggesting that IFA + *L. monocytogenes* treatment resulted in a delayed disease progression when compared with the IFA-only group. Another 10 pro-diabetic NOD mice received a second administration of IFA + *L. monocytogenes* at three weeks following the initial immunization (age, eight weeks), in order to study whether an additional immunization was able to further delay T1D development. However, no significant change in disease progression was observed in the twice-immunized mice when compared with the once-immunized mice (data not shown).

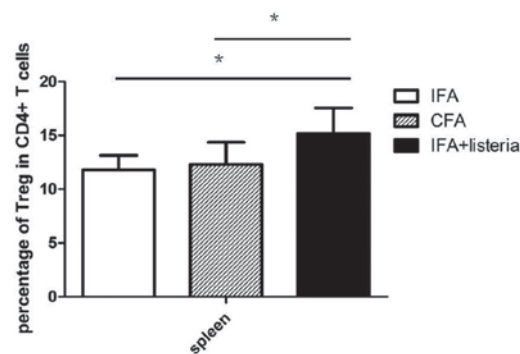


Figure 4. Treg cell populations in the spleens of mice treated with different adjuvants. *P<0.05. IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; Treg cell, regulatory T cell.

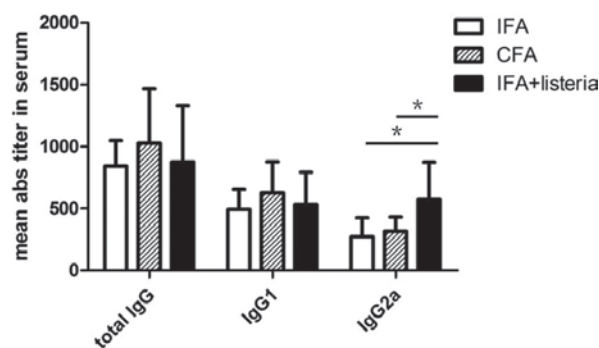


Figure 5. Abs titer in the serum of mice treated with different adjuvants. *P<0.05. IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; Abs, antibodies.

IFA + L. monocytogenes treatment has no effect on IL-17-producing T cell populations. Spleen cells were collected three weeks after adjuvant treatment and were incubated with PMA/calcium ionophore. The percentage of IL-17-producing spleen cells was between 0.2 and 0.5%, which was relatively low and no significant difference was observed between the CFA, IFA + *L. monocytogenes* and IFA-only control groups (data not shown). Spleen cells were separated using the T cell-specific surface marker, TCR β , into TCR β ⁺ T cells and TCR β ⁺B220⁺ innate cell populations, which consisted primarily of macrophages, dendritic and NK cells. It is generally accepted that $\gamma\delta$ T cells are innate immune cells, despite expressing the TCR on their surface. CFA treatment induced significant IL-17 secretion in the innate immune cells when compared with the IFA + *L. monocytogenes* and IFA-only control groups (Fig. 2). Furthermore, adjuvant treatment appeared to have no influence on the TCR β ⁺ T cell population, since no statistically significant difference was observed between the three groups. In addition, IFN- γ expression was compared among the groups, and adjuvant treatment was shown to induce a notable increase in IFN- γ expression in T cells of the IFA-only control group. However, no significant difference in IFN- γ expression was observed in the T cells of the CFA and IFA + *L. monocytogenes* groups (data not shown).

CFA treatment induces strong IL-17 expression in the pancreatic draining lymph nodes. Considering that IL-17 is a pro-inflammatory cytokine that is primarily involved in

local inflammation, the expression of IL-17 in the pancreatic draining lymph nodes was analyzed. Lymphocytes from pancreatic draining lymph nodes were collected from eight week-old NOD mice (three weeks after treatment) and cultured *in vitro* for two days, after which secreted IL-17 was captured by plate-coated antibodies. ELISA results revealed that CFA treatment induced strong IL-17 expression in the pancreatic draining lymph nodes when compared with the IFA + *L. monocytogenes* and IFA-only groups (Fig. 3). The source of this extra IL-17 expression in the CFA group remains unclear, but it may have been secreted by Th17 and/or innate immune cells, such as NKT cells.

IFA + L. monocytogenes treatment promotes Treg proliferation and increases IgG2a levels in the blood serum. IFA + *L. monocytogenes* treatment delayed disease progression, but did not alter the secretion of cytokines, including IL-17, in the innate immune response, which indicates that alternative mechanisms were involved. The Treg cell populations in the spleen of the mice were analyzed and the IFA + *L. monocytogenes* group mice were found to exhibit a significantly higher percentage of Treg cells in the CD4⁺ T cell population when compared with the CFA and IFA-only groups (Fig. 4). Treg cells are the major immunoregulatory cells in the development of T1D in NOD mice; thus, the increased Treg cell population was hypothesized to be associated with the protective effects of the IFA + *L. monocytogenes* treatment against T1D. In addition, Th1 and Th2 responses to the treatment were assessed via serum antibody-subtype analysis, which revealed no change in the total serum levels of IgG. However, the IFA + *L. monocytogenes* group mice exhibited increased levels of the IgG2a subtype when compared with the CFA and IFA-only groups (Fig. 5). In summary, the results indicated that treatment with IFA + *L. monocytogenes* had a notable effect on T cell differentiation and antibody responses during the development of T1D.

Discussion

In the present study, pro-diabetic NOD mice were treated with IFA + *L. monocytogenes*, which was found to delay the development of T1D. However, the treatment was unable to inhibit disease progression indefinitely. The levels of the cytokines, IL-17 and IFN- γ , were examined in T cells and innate immune cells in all three groups. CFA was found to induce the expression of IL-17 in innate immune cells; however, IFA + *L. monocytogenes* treatment induced only a small increase in IL-17 expression levels when compared with the IFA-only control group. No statistically significant differences were observed in the levels of IL-17-producing T cells and IFN- γ -producing Th1 cells between the CFA and IFA + *L. monocytogenes* groups. CFA treatment induced the production of IL-17 in innate immune cells, including NKT and $\gamma\delta$ T cells, which is consistent with previous studies investigating the effects of CFA on T1D development in NOD mice (27). In the present study, IFA + *L. monocytogenes* treatment delayed disease progression, but did not induce IL-17 secretion in T cells and innate immune cells, which suggests that alternative mechanisms may be involved in *L. monocytogenes*-mediated protection against T1D.

No significant difference was observed between the groups in the levels of IFN- γ -producing Th1 and Th17 cells; therefore, the levels of Treg cells were analyzed. Treg cells are widely considered to play a critical role in the regulation of autoimmune pathologies, such as T1D (28). The results showed that the percentage of Treg cells in the IFA + *L. monocytogenes*-treated mice was higher compared with the CFA and IFA-only groups. Although treatment with CFA and IFA-only had no effect on thymic Treg levels, the IFA + *L. monocytogenes* treatment contained components, such as the cell wall and microbial DNA, which effectively activated innate immune cells. This activation was hypothesized to induce local pro-inflammatory cytokine secretion through Toll-like receptor signaling pathways, altering T cell differentiation and promoting the proliferation of Treg cells. However, the data from the current study are not sufficient to confirm whether the IFA + *L. monocytogenes* treatment increased the Treg cell population via this mechanism.

The levels of IgG antibody isotypes in the blood serum were analyzed, and the IFA + *L. monocytogenes* group mice were found to exhibit increased levels of IgG2a when compared with the CFA and IFA-only groups. However, no statistically significant difference was observed in the other antibody subtypes. These results indicate that IFA + *L. monocytogenes* treatment altered the Th1/Th2 balance in NOD mice, inducing the production of IgG2a antibodies, which is closely associated with the Th1 response.

In conclusion, treatment with IFA + *L. monocytogenes* was observed to delay disease progression in pro-diabetic NOD mice. The mechanisms underlying this *L. monocytogenes*-specific protection differed from those involved in CFA treatment, since *L. monocytogenes* did not induce IL-17 secretion in innate immune cells. However, IFA + *L. monocytogenes* treatment was shown to affect the Th cell subsets. Mice treated with IFA + *L. monocytogenes* exhibited increased levels of Treg cells and IgG2a antibodies in the blood serum, indicating a marked antibody response when compared with the CFA or IFA-only treated mice. However, the mechanisms by which these T cell subsets affect disease progression remain unclear, and further experimental data are required.

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