A metabolically engineered bacterium controls autoimmunity by remodeling the pro-inflammatory microenvironment

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Abstract

Immunotherapy has led to impressive advances in the treatment of autoimmune and pro-inflammatory disorders; yet, its clinical outcomes remain limited by a variety of factors including the pro-inflammatory microenvironment (IME). Discovering effective immunomodulatory agents, and the mechanisms by which they control disease, will lead to innovative strategies for enhancing the effectiveness of current immunotherapeutic approaches. We have metabolically engineered an attenuated bacterial strain (i.e., *Brucella melitensis* 16M $\Delta vjbR$, Bm $\Delta vjbR$) to produce indole, a tryptophan metabolite that controls the fate and function of regulatory T cells (T_{regs}). We demonstrated that treatment with this strain polarized M2 macrophages (M ϕ) which produced anti-inflammatory cytokines (e.g., IL-10) and promoted T_{reg} function; moreover, when combined with adoptive cell transfer (ACT) of T_{regs}, a single treatment with our engineered bacterial strain dramatically reduced the incidence and score of autoimmune arthritis and decreased joint damage. These findings show how a metabolically engineered bacterium can constitute a powerful vehicle for improving the efficacy of immunotherapy, defeating autoimmunity and reducing inflammation by remodeling the IME and augmenting T_{reg} function.

Main

Numerous studies have demonstrated promising results using immunotherapy to treat autoimmune and pro-inflammatory disorders¹⁻⁴. Nevertheless, despite advances in the field of immunotherapy, including regulatory T cell (T_{reg})-based therapy, the efficacy and benefits of these approaches remain less satisfactory due to limited *in vivo* T_{reg} expansion and persistence, the pro-inflammatory microenvironment (IME), and insufficient T_{reg} trafficking to inflamed sites. In addition, immune dysregulation of the IME contributes to disease⁵. Immunotherapeutic combinations may produce greater efficacy, and thus strategies that circumvent these barriers are urgently needed.

To address this need, we developed and tested an intervention that combined two innovations. First, although a therapeutic role for live attenuated bacterial vaccines in addressing infectious diseases is undeniable and appreciated in cancer immunotherapy, the use of bacterial agents to manage autoimmune and pro-inflammatory diseases remains limited. Second, a growing number of microbiota-specific products and metabolites have novel immunologic properties that constitute one mechanism whereby the microbiota influence host health and disease^{6,7}. We therefore developed a metabolically engineered bacterial vaccine to produce immunomodulatory metabolites that improve autoimmunity and inflammation.

For the bacterial vector, we selected an attenuated strain of *Brucella melitensis* that harbors a deletion in *vjbR*, a master regulator of virulence (BmΔ*vjbR*)⁸. Like other Gram-negative organisms, *Brucella* strains express a lipopolysaccharide (LPS) lacking endotoxin activity. Importantly, BmΔ*vjbR* has been shown to be safe in immunocompetent and

immunocompromised mice⁹, goats¹⁰, sheep¹¹, and non-human primates¹². We also have shown that BmΔ*vjbR* can combat cancer in a murine model by remodeling the tumor microenvironment (TME) to a pro-inflammatory state⁸. Moreover, when BmΔ*vjbR* treatment was combined with adoptive cell transfer (ACT) of tumor antigen (Ag)-specific CD8⁺ T cells, tumor growth and proliferation were dramatically impaired⁸. Conversely, in the current studies, we engineered BmΔ*vjbR* to express tryptophanase (*tnaA*); i.e., BmΔ*vjbR*::*tnaA*, to produce the tryptophan metabolite indole, a molecule that modulates the fate and function of T_{regs}¹³.

We have reported that indole, when used at a range of physiologic concentrations, suppresses several inflammatory characteristics in immune and non-immune cells, and also augments T_{reg} differentiation¹⁴. Consistent with our previous reports, we demonstrated that indole suppressed TNF-α production in CD11b⁺ spleen cells after *E. coli* LPS (eLPS) stimulation (**Fig. 1a & Fig. 1b**) and dampened their activation by suppressing Akt and ERK signaling pathways in response to microbial agonists (eLPS and heat-inactivated *Salmonella* Typhimurium [HKST]) (**Fig. S1a**). In addition, indole augmented the differentiation of naive CD4⁺CD25⁻ T cells into induced T_{regs} (iT_{regs}) measured by FoxP3 *in vitro* in a dose dependent manner (**Fig. 1c & Fig. S1f**). These findings were consistent with our earlier reports¹¹, and were comparable to results from studies using the microbiota metabolite butyrate, albeit with distinct dose-dependency¹⁵. Based on these findings, we hypothesized that indole would ameliorate immune-mediated inflammation in autoimmune and pro-inflammatory diseases.

Several lines of investigation demonstrated that indole reduces autoimmune responses in a murine collagen-induced arthritis (CIA) model. First, we showed that the severity of CIA was

significantly attenuated in indole treated mice, which exhibited clinical scores of 0.8 ± 0.2 (means $\pm$ SEM) at (Day 50), compared to 1.6 ± 0.5 in controls (**Fig. 1d**). However, a single dose of indole only showed a slight decrease in inflammation. Similarly, single dose treatment did not induce significant alterations in the infiltration of T_{regs} as assessed by confocal microscopy (**Fig. 1e & Fig. 1f**). These findings were in striking contrast to our *ex vivo* experimental findings, which indicated that indole significantly promoted the expansion of $CD4^+FoxP3^+$ T_{regs} and enhanced their activation by increased expression of PD-1 compared to the controls ($p < 0.001$), an immunosuppressive molecule, in cells derived from the mouse lymph nodes (LNs) and spleen (**Fig. 1g**). Based on our *in vivo* and *ex vivo* findings, we hypothesized that the sustained delivery of indole in a bacterial vector may greatly improve the durability of the molecule's immunomodulatory effects, resulting in an attenuation of autoimmunity and inflammation in CIA. To test this hypothesis, we engineered a safe live-attenuated bacterial strain ($Bm\Delta vjbR::tnaA$) to constitutively produce indole (**Fig. S1c, Fig. S1d & Fig. S1e**).

First, cytokine array profiling analyses showed that $Bm\Delta vjbR::tnaA$ induced the expression of IL-10 (**Fig. 2a & Fig. S2a**), which promotes the activities of T_{regs} and reduces autoimmunity and inflammation^{16,17}. Strikingly, $Bm\Delta vjbR::tnaA$ also significantly ($p < 0.01$) reduced the expression of additional pro-inflammatory cytokines like IL-6, IL-1 β and TNF- α in macrophages ($M\phi$) compared to $Bm\Delta vjbR$ parental strain (**Fig. 2a**). Second, we found that $Bm\Delta vjbR::tnaA$, when co-cultured with bone marrow-derived $M\phi$ (BMDMs), not only significantly reduced the total $CD4^+$ T cells ($p < 0.001$) but also reduced the production of the pro-inflammatory cytokines such as TNF- α and IFN- γ ($p < 0.001$) compared to the $Bm\Delta vjbR$ parental strain (**Fig. 2b**). Moreover, $Bm\Delta vjbR::tnaA$ promoted the expansion of T_{regs} and significantly enhanced their activity as

assessed by IL-10 production ($p<0.001$) and PD-1 expression ($p<0.01$) (**Fig. S2a**). Third, in the CIA model, a significant reduction in arthritis score and incidence was observed following treatment with *BmΔvjbR::tnaA*. This amelioration of autoimmunity and inflammation was further augmented when *BmΔvjbR::tnaA* treatment was combined with ACT of T_{regs} (**Fig. 2c**). Fourth, we observed significantly ($p<0.01$) reduced numbers of infiltrating inflammatory cells into the joints of mice treated with *BmΔvjbR::tnaA*. This effect was further enhanced by *BmΔvjbR::tnaA* treatment followed with ACT of T_{regs} compared to the controls ($p<0.001$) (**Fig. 2d**). Finally, mice treated with *BmΔvjbR::tnaA* showed reduced infiltrates in the joint as evidenced by H&E analysis and Safranin O (Saf-O) staining of knee cross-sections (60 days post collagen administration). Notably, these findings were further attenuated by addition of ACT of T_{regs} (**Fig. 2d**). There was also a significant reduction in the total $CD4^+$ T cell proportions and a dramatic increase in T_{reg} ($p<0.001$) proportions in mice treated with *BmΔvjbR::tnaA* compared to controls (**Fig. 2e**).

To identify the mechanism by which *BmΔvjbR::tnaA* might be acting, we conducted a multiparametric CyTEK analysis from the cells isolated from the joints of control, ACT with T_{reg} only, or ACT with T_{reg} plus *BmΔvjbR::tnaA* groups. We found the *BmΔvjbR::tnaA* reduced the proportion of B cells (**Fig. 2f, 2g, and Table S1**) in addition to promoting T_{reg} expansion. Overall, our results indicate that *BmΔvjbR::tnaA* not only remodels the IME and facilitates the expansion and suppressive function of T_{regs} but may also modulate B cell-mediated immunity in the CIA model.

Several strategies have been employed to enhance the efficacy of live attenuated bacterial vaccines. However, bacterial vaccines producing immunomodulatory metabolites that alter the immunological tolerance and the IME have not been previously reported. Our work features both conceptual and methodological innovations. First, this work provides the first description of a live attenuated vaccine whose metabolism has been reprogrammed to amplify anti-autoimmune/inflammation activity. Second, this study provides the first description of how combining a single dose of a live attenuated bacterial vaccination with ACT of T_{regs} can achieve potent therapeutic outcomes. Third, we demonstrate how the genetic tractability of BmΔ*vybR* can be exploited to engineer vaccines with novel properties. Although in this work, we focused on metabolic reprogramming of the bacterium, we envision that future iterations of the BmΔ*vybR*::*tnaA* vaccine will include engineered auto Ags to boost persistent auto Ag-specific T_{reg} responses. Fourth, this work showed that indole did not succeed as a stand-alone agent. However, this limitation was circumvented by delivery using our engineered bacterial vector. BmΔ*vybR*::*tnaA* may offer improved pharmacodynamics for indole by sustained production levels and effects *in vivo* versus indole alone. Moreover, we showed that the separate individual beneficial effects of BmΔ*vybR* and indole act synergistically *in vivo*. Finally, the natural localization of BmΔ*vybR* to leukocytes enabled BmΔ*vybR*::*tnaA* to provide improved targeted indole delivery and a higher local effective indole concentration in contrast to the untargeted and transient nature of indole as a single agent. In sum, our work presents an attractive new avenue for the development of anti-autoimmunity/inflammation vaccines.

Methods

Immunization with CIA

CIA was induced as described previously with minor modifications¹⁸. Briefly, male C57BL/6 mice were injected with an emulsion of 100 μ l of chick type II collagen (Chondrex; 100 μ g) in Complete Freund's Adjuvant (CFA; Chondrex) using a glass tuberculin syringe with 26-gauge needle. The mice were then assessed for development of joint inflammation and clinical arthritis score until Day 60.

Bacterial culture

Bm Δ *vjbR* or Bm Δ *vjbR::tnaA* were cultivated and prepared for experimentation as previously described⁸.

Engineering indole-producing Bm Δ *vjbR::tnaA* strain

To generate an indole producing attenuated Bm Δ *vjbR* strain, we cloned an *Escherichia coli* (*E. coli*) *tnaA* gene into a broad range bacteria expression plasmid (pBBR1MCS6Y)¹⁹ and transferred the plasmid to Bm Δ *vjbR*.

Bm Δ *vjbR::tnaA* treatment

CIA was induced in male C57BL/6 mice. On Day 7 after the CIA induction, mice were *i.v.* injected with 5.0×10^7 live Bm Δ *vjbR::tnaA* or PBS control. In the Bm Δ *vjbR::tnaA* + T_{reg} combinatorial treating experiments, mice (N=5) were adoptively transferred with 2.5×10^6 CD4⁺CD25⁺ T_{regs} derived from donor LNs and spleen of naive C57BL/6 mice, one week after the Bm Δ *vjbR::tnaA* administration.

Flow cytometric analysis

Cell staining and flow cytometric analysis were performed as described previously⁸ using the described labeling reagents. Briefly, surface and intracellular staining was performed on the single-cell suspensions and analyzed using LSR Fortessa cell analyzer (BD). The joints were also processed and stained similarly with antibodies listed in Table S2, and data was acquired on CyTEK aurora flowcytometer (Cytek Biosciences). For multiparametric analyses, the data were analyzed with FlowJo v10 and represented as heatmaps and tSNE plots.

Histology and immunofluorescence

Mice were humanely sacrificed on day 60 after induction of CIA, and tissue sections were analyzed as previously described¹⁸. Briefly, the hind foot paws and knees were removed and fixed in 10% formalin and decalcified in Formical-4 (Decal chemical, Tallman, NY). The fixed tissue sections were then stained with H&E and/or Safranin O fast green (Saf-O) stain. The H&E and Saf-O stained sections were then assessed by semiquantitative system of 0 to 4 as described previously.

Immunofluorescent staining and microscopy were performed on the deparaffinized sections by using FITC anti-mouse FoxP3 antibody (Ab) for T_{regs} and DAPI as nuclear stain.

Statistical Analysis

Student's *t*-test was performed for statistical analysis between the groups. All analyses were performed in GraphPad Prism v9. A *p* value of < 0.05 was considered statistically significant.

AUTHORS' CONTRIBUTIONS

JS, PDF, RCA and TF conceived and designed the experiments. JKD and FG performed the experiments and analyzed the data. JS, PDF, JKD, FG, RCA, TF, CH, SH, JAP, KSK and AJ wrote the manuscript and provided critical feedback. JS and PDF supervised the research. All the authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank Robbie Moore from COM-CAF, Malea Murphy from IMIL, Sankar P. Chaki from TAMH-CVM, and Elizabeth Bustamante from TAMU-HSC, and the Immunomonitoring Core facility at Houston Methodist Hospital-Texas Medical Center for their technical supports. This work was supported by funding from NIH (R01AI121180 and R01CA221867 to J.S., R01AI110642 to R.A., R01HD084339 to T.A.F., and R01AI141607-01A1 to P.D.F.), NSF DBI1532188, and NSF0854684 to P.D.F.

FIGURE LEGENDS

Fig. 1: Indole treatment dampens inflammation and promotes T_{reg} expansion and activity.

a, Indole reduces production of pro-inflammatory $TNF-\alpha$ in $CD11b^+$ cells. 0.25, 0.5, or 1.0 mM Indole was dissolved in DMF for the representative experimental flowcytometric analysis. b, Graphical representation of flow cytometric dot-plots derived from 3 independent experiments of HKST group. c, Indole promotes dose-dependent differentiation of T_{regs} ; Experiment (N=3) was performed under T_{reg} skew conditions ($[TGF-\beta] = 2 \text{ ng/mL}$, $[IL-2] = 100 \text{ U/mL}$). Th_0 control represents non- T_{reg} skew conditions. Butyrate was used as a control metabolite. d, Effects of indole alone on CIA in mice (N=5). e, Representative images of H&E, Safranin O (Saf-O) stained tissues, and confocal microscopy of knee tissues of CIA mice on day 60 post induction of arthritis. f, Quantitative analysis of H&E, Saf-O and T_{reg} infiltration from confocal microscopy sections of Control (Ctrl) and indole-treated mice. g, Flow cytometric dot-plot analysis of PD-1 and FoxP3 in indole-treated, *ex vivo* stimulated $CD4^+$ T cells derived from the LNs and spleen and activated with anti-CD3/CD28 Abs. Exposure to indole drives these cells towards higher T_{reg} phenotype by increased FoxP3 expression. Graphical representation of $CD4^+$ T cells (%) derived from the flow cytometric dot-plots of $CD4^+$ T cells exposed to indole. Graphical representation of $CD4^+$ FoxP3 $^+$ PD-1 $^+$ T cells (%) from the flow cytometric dot-plots. Data represent means $\pm$ SD. Student's *t*-test or Tukey's multiple comparisons test was applied for statistical analysis. *, **, ***: significance at $p < 0.05$, 0.01, 0.001.

Fig. 2: $Bm\Delta vjBR::tnaA$ significantly dampens inflammation and reduces arthritis in murine CIA model which is augmented by adoptive cell transfer (ACT) of T_{regs} .

a, Cytokine arrays were used to measure pro-inflammatory cytokines produced by control, *BmΔvjbR*, and *BmΔvjbR::tnaA* treated BMDMs. b, BMDMs were treated with either *BmΔvjbR::tnaA* or *BmΔvjbR* and then co-cultured with T cells derived from pooled LNs and spleen of C57BL/6 mice. Flow cytometric assays were then performed to measure IFN- γ and TNF- α of T cells. c, Arthritis score and arthritis incidence in CIA C57BL/6 mice from control (Ctrl, gray bar); *BmΔvjbR* (blue bar); *BmΔvjbR::tnaA* (pink bar) and *BmΔvjbR::tnaA* followed by ACT of T_{reg} (T_{reg} , red bar) (N=5 in each group). d, Representative images of H&E, Saf-O staining, and confocal microscopy from mouse knees on day 60 post CIA induction. Quantitative analysis of T_{reg} infiltration and inflammation scores from these mice are also shown. e, Cells from the LNs and spleen were collected from CIA-induced mouse groups (Ctrl, *BmΔvjbR::tnaA*, and *BmΔvjbR::tnaA* combined with ACT of T_{reg}). These cells were then stained and quantified by flow cytometry using markers for CD4⁺ T cells and intracellular staining of FoxP3 (T_{reg}). f, CIA-induced mice were treated with PBS (Ctrl), ACT of T_{reg} only (T_{reg} only; N=5), or *BmΔvjbR::tnaA* combined with ACT of T_{reg} (N=5). Cells from the knee and ankle joints were stained with 21 markers and measured by CyTEK aurora flow cytometry. Heatmap shows immune cell profiles in different treatment groups of mice (scale bar represents percentage of cell in each treatment group within each cell type). g, viSNE map shows the four subtypes of B cells differentially expressed in the treated group of mice. Data represent means $\pm$ SD. Student's *t*-test or Tukey's multiple comparisons test was applied for statistical analysis. *, **, ***: significance at $p < 0.05$, 0.01, 0.001.

Supplementary Figures

Fig. S1: Indole suppresses immune cell activation and *BmΔvjBR* is engineered to produce indole.

a, Indole (1mM) suppresses the Akt and ERK signaling pathways in CD11b⁺ cells in different post incubation time. Flow cytometric dot-plot analysis and graphical representation of Akt and ERK expression with time. b, Schematic diagram of murine CIA model used in all the subsequent studies. c, Schematic representation of the engineered *BmΔvjBR::tnaA* harboring a plasmid carrying a *tnaA* expression cassette. The indole biosynthesis pathway is depicted in the figure. TnaA catalyzes the conversion of tryptophan to indole. d, Engineered *BmΔvjBR::tnaA* produced high concentration of indole as shown in the mass-spectrometric analysis. e, Western blotting analysis of the expression of *tnaA* protein in the parental strain compared with the engineered *BmΔvjBR::tnaA* strain. Graphical representation of the comparative analysis of indole production by *BmΔvjBR* parental bacterial strain and the engineered *BmΔvjBR::tnaA* strain. f, Indole promotes significantly higher expansion of T_{regs} compared to butyrate at 1mM concentration as shown in the graphical analysis. Graphical data derived from flow cytometric representatives as shown in Fig. 1C. Data represent means ± SD. Student's *t*-test or Tukey's multiple comparisons test was applied for statistical analysis. *, **, ***: significance at *p*<0.05, 0.01, 0.001.

Fig. S2: *BmΔvjBR::tnaA* activates T_{regs} and suppresses B-cell mediated inflammation.

a, BMDMs infected with either *BmΔvjBR::tnaA* or *BmΔvjBR* were co-cultured with CD4⁺ T cells from mouse LNs and spleen and activated by using anti-CD3/CD28 Abs. Flow cytometric dot-plot assay shows that *BmΔvjBR::tnaA* treated BMDMs greatly promoted expression of FoxP3

and PD-1 and production of IL-10 in CD4⁺ T cells. The dot-plots are followed by graphical representation from 3 independent experiments. b, The total CD19 population is shown in the viSNE plots. Data represent means $\pm$ SD. Student's *t*-test or Tukey's multiple comparisons test was applied for statistical analysis. *, **, ***: significance at $p < 0.05$, 0.01, 0.001.

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Table S1

Population	Cell type	Ctrl	T _{reg}	BmΔ <i>vjbR</i> :: <i>tnaA</i> + T _{reg}
1	CD40L hi CD25 lo	8.6 %	18.7 %	72.7 %
2	CD25 hi Tim3 hi Ki67 hi	34.9 %	20.4 %	44.7 %
3	D45 lo CD44 mid	34.7 %	22.2 %	43.1 %
4	CD45 lo CD44 lo	34.3 %	27.9 %	37.8 %
5	CD44 hi CD160 mid	28.0 %	36.0 %	36.0 %
6	CD3 hi CD8hi CD25 lo	31.8 %	33.3 %	34.9 %
7	CD19 hi CD62L lo	32.7 %	33.6 %	33.7 %
8	CD4 hi CD25 lo	31.9 %	36.8 %	31.3 %
9	CD4 hi CD25 mid CTLA4 lo	37.0 %	32.0 %	31.0 %
10	CD25 hi PD-L1 hi	47.1 %	22.9 %	30.0 %
11	CD8 hi CD62L lo	38.2 %	32.4 %	29.4 %
12	CD19 hi CD40L hi	40.6 %	36.7 %	22.7 %
13	CD19 hi PD-1 mid CTLA4 hi	46.6 %	32.3 %	21.1 %
14	CD19 hi CD40L hi	32.1 %	47.4 %	20.5 %
15	CD25 hi CD140 hi	20.1 %	60.3 %	19.6 %
16	CCR7 hi PD-1 lo	53.1 %	28.0 %	18.9 %
17	CD4 mid PD-L1 hi	55.2 %	29.3 %	15.5 %
18	CD19 hi PD-1 lo CD40L lo	76.0 %	22.3 %	1.68 %

Supplementary Table 1. A list of different immune cell types which were differentiated using the tSNE analysis and their proportion in different treatment groups.

Table S2

Ab	Clone	Fluorophore	Source
Ghost Dye Red 710		Ghost Dye Red 710	Tonbo Biosciences
CD45	30-F11	FITC	Tonbo Biosciences
CD8a	53-6.7	BUV395	BD Biosciences
CD4	GK1.5	eFluor 450	Thermo Fisher Scientific
CD3	17A2	APC-Fire 810	BioLegend
CD19	1D3	BUV805	BD Biosciences
MHC II	M5/114.15.2	Violet Fluor 500	Tonbo Biosciences
CD160	7H1	APC	BioLegend
PD-L1	MIH5	Super Bright 780	Thermo Fisher Scientific
CTLA-4	UC10-4F10-11	PE-Cy7	Tonbo Biosciences
PD-1	29F.1A12	PE-Dazzle 594	BioLegend
Lag-3	C9B7W	BUV661	BD Biosciences
TIM-3	5D12/TIM-3	Brilliant Violet 605	BD Biosciences
CD44	IM7	PerCP	BioLegend
CD62L	HRL1	BUV737	BD Biosciences
Ki-67	B56	Brilliant Violet 711	BD Biosciences
CCR7	4B12	PE	BioLegend
CD69	H1.2F3	BUV563	BD Biosciences
CD25	PC61	Brilliant Violet 650	BioLegend
CD40L	MR1	BB700	BD Biosciences
CD28	37.51	BUV496	BD Biosciences

Supplementary Table 2: List of Abs used in CyTEK assay

Schematic representation of amelioration of CIA in C57BL/6 mice

The illustration depicts the process of amelioration of CIA in C57BL/6 mice by the engineered *BmΔvjbR::tnaA* bacterial strain. C57BL/6 mice may harbor a microbiome deficient in production of the metabolite indole. When challenged with the CIA, these mice rapidly develop arthritis due to dysfunctional T_{regs} and rapid expansion of CD4⁺ effector T cells. However, when *BmΔvjbR::tnaA* bacteria are administered to mice bearing CIA the T_{reg} activity is substantially increased and the pathogenic activity of CD4⁺ effector T cells is compromised, which results in amelioration of joint inflammation and improved synovitis. This activity is greatly augmented by adoptive transfer of T_{regs}.