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Notes and Insights

The helminth-derived peptide, FhHDM-1, reverses the trained phenotype of NOD bone-marrow-derived macrophages and regulates proinflammatory responses

The concept of "trained immunity" proposes that exposure to ligands of microbial pathogens imprints ("trains") macrophages with an inflammatory memory, endowing them with the capacity to respond faster and stronger to future stimuli [1]. These long-lasting effects are attributable to the increased methylation of histones, which facilitates a quicker and amplified transcription of proinflammatory cytokines (notably TNF and IL-6) in response to immune challenges. Concurrent with the remodeling of the epigenetic landscape is an alteration in metabolism toward a requirement for glycolysis to fuel the epigenetic machinery, and subsequent proinflammatory functions [1, 2]. Unlike the adaptive immune response, the trained innate response is not antigen-specific, evolving to provide general protection against future infections [1]. However, the activation of trained innate immune cells can also be induced by endogenous sterile stimuli, such as metabolites, fatty acids, and glucose, which can cause immune-mediated disease [3].

Although initiating events for type-1 diabetes (T1D) have not been fully defined, viral and bacterial infections have long been implicated as disease triggers [4]. Additionally, macrophages from T1D patients display enhanced proinflammatory responses, a phenotype that is necessary for the initiation of autoimmunity [5] and is characteristic of a trained macrophage. We propose that in T1D patients, myeloid cells in the bone marrow are trained by a previous bacterial/viral infection or an altered gut microbiota. Recruitment of these trained macrophages to the islets, induced by beta (β)-cell stress signals, would subsequently mediate an amplified proinflammatory response, triggering disease initiation. The ongoing interaction between macrophages and β-cells would perpetuate a cycle of inflammation, culminating in the activation of autoreactive T-cells and accelerated β-cell loss. Therefore, preventing, or reversing, the centrally trained macrophage phenotype may mitigate the initiation of autoimmunity, and T1D.

Exploring this premise, we investigated whether the bone-marrow-derived macrophages (BMDMs) from untreated nonobese diabetic (NOD) mice, but not from nondiabetic BALB/c mice, displayed the hallmarks of innate immune cell training [3]. These data revealed, for the first time, that the histone H3K4 in BMDMs from untreated NOD mice at 6 and 11 weeks of age was both mono (H3K4me1)- and tri-(H3K4me3)-methylated (Fig. 1A; Supporting information S1). In comparison, BMDMs of age-matched BALB/c mice showed no detectable methylation of the same site. This suggests that the immune progenitor cells in the bone marrow of NOD mice were indeed trained and that this status was sustained as mice aged, thus driving the inflammatory, disease-promoting phenotype of macrophages.

Correlating with this increase in histone methylation, the glycolytic activity in NOD BMDMs that were unstimulated ex vivo was higher than that of BALB/c BMDMs, regardless of whether the BMDMs were resting (Fig. 1B), stimulated with glucose, or functioning at maximal metabolic activity (Fig. 1B and C). Furthermore, although the maximal glycolytic flux of macrophages from NOD and BALB/c was equal after LPS stimulation (Fig. 1B and C), there was a significant difference in the immediate response to glucose stimulation. Glycolytic activity in NOD BMDMs stimulated ex vivo with LPS increased to maximal levels within minutes of glucose exposure, to greatly exceed that of LPS-treated BALB/c macrophages (Fig. 1B and C). Therefore, NOD BMDMs were metabolically primed for an immediate and enhanced response to stimulation. This outcome was validated by the significant increase in IL-6 and TNF secretion in response to LPS in NOD BMDMs, as compared with BALB/c BMDMs (Fig. 1D and E).

These data provide a mechanistic framework to explain the long-term association of T1D with previous viral infections [4]. Whether viral proteins are directly training the macrophages, or if the resulting imbalance in the gut microbiome and associated bacterial translocation, provides the activating signal remains to be elucidated. However, as the NOD mice were housed in pathogen-free conditions, it was likely that alterations to gut microbiota initiated the training of macrophages observed in this study. In NOD mice there is a higher disease penetrance in females, which has been attributed to altered gut microbiota [6]. Furthermore, analyses of the microbiota between newborn NOD female mice and non-autoimmune strains confirmed an association between gut dysbiosis, proinflammatory responses, and diabetogenesis [6].

The association between a previous infection and/or an altered gut microbiota with aberrant proinflammatory responses is becoming widely associated with seemingly disparate immune-mediated pathologies such as diabetes, asthma, cardiovascular disease, and mental health disorders [7]. Given the evidence presented here, a trained innate immune response

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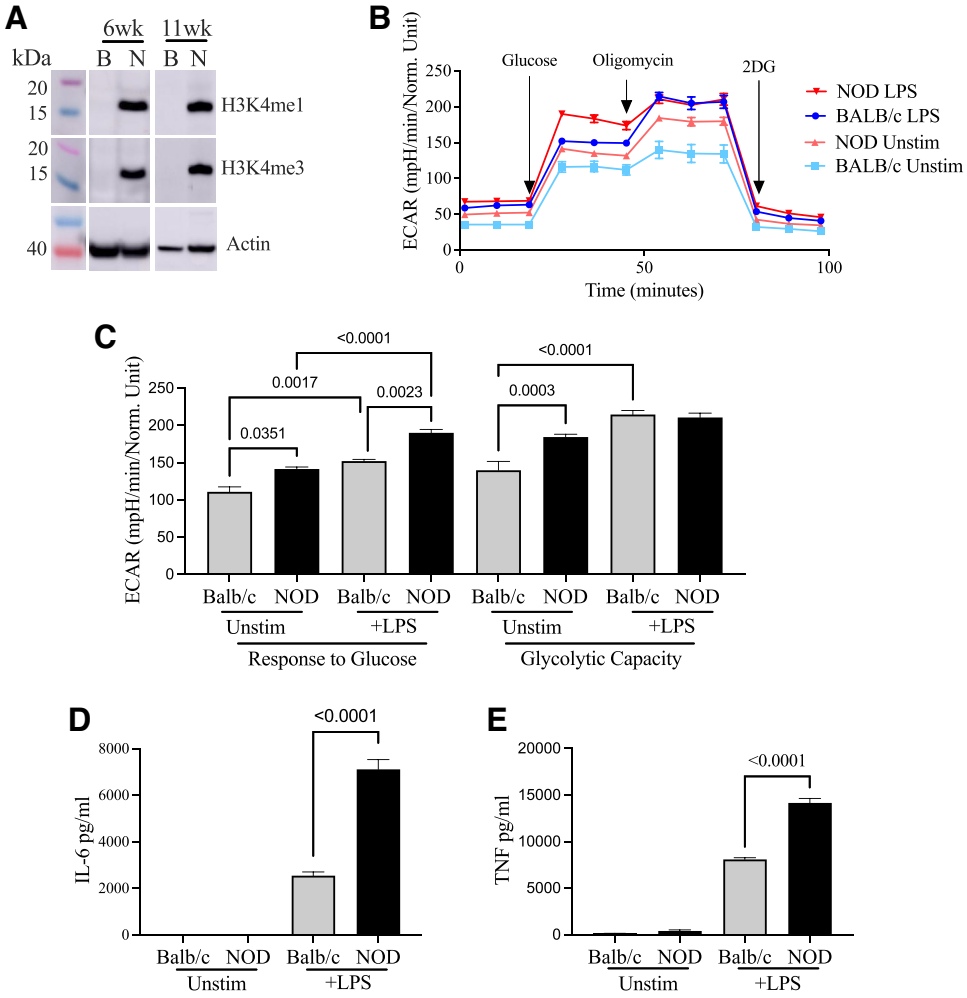

Figure 1. Bone-marrow-derived macrophages from NOD mice display the characteristics of a trained immune cell. (A) Macrophages were differentiated from the bone marrow of age-matched BALB/c (B) or NOD (N) mice at 6 and 11 weeks of age. Protein lysates were normalized by protein concentration and the methylation status of H3K4 was analyzed by western blot using primary antibodies specific for H3K4me1 and H3K4me3. The protein loading of each sample was illustrated by the detection of actin. The data shown is representative of $n = 5$–10 mice of each strain at each time point. (B) Macrophages were differentiated from the bone marrow of age-matched BALB/c or NOD mice at 11 weeks of age and were either unstimulated (Unstim) or stimulated with LPS for 24 h. The glycolytic activity was determined by measuring the extracellular acidification rate (ECAR) and the (B, C) immediate and maximum glycolytic capacity of macrophages after subsequent treatment with glucose, oligomycin, and 2-deoxy-glucose (2-DG) (representative of three independent experiments of $n = 5$). (D) Secreted TNF and (E) IL-6 protein were quantified by ELISA (representative of two independent experiments of $n = 5$). Data is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

may be the common initiating event among these conditions. Equally, there is a well-established inverse correlation between infection with parasitic worms (helminths) and the prevalence of the same broad range of immune-mediated diseases [8]. This outcome is explained by their need to survive long-term in their mammalian hosts to complete their life cycle. Accordingly, helminths have evolved mechanisms to regulate the host's protective innate proinflammatory response such that their presence is tolerated and the tissue damage they cause is repaired. Millennia of endemic exposure to these tolerated infections with parasitic worms has imprinted a regulatory influence on the human immune response, which controls the development of trained inflammatory responses, to prevent immune-mediated disease and to facilitate the homeostatic resolution of infection-fighting inflammation, after pathogen clearance [8, 9]. Thus, exploiting these regulatory mechanisms employed by helminths offers a new approach for the treatment of immune-mediated diseases, such as T1D.

We have previously shown that the administration of a peptide (FhHDM-1) secreted by the parasitic worm, *Fasciola hepatica*, to NOD mice at a time-point coincident with the initiation of β-cell directed autoimmunity, prevented T1D [10]. Remarkably, only six intraperitoneal (i.p.) injections of FhHDM-1 delivered over 12 days permanently prevented T1D.

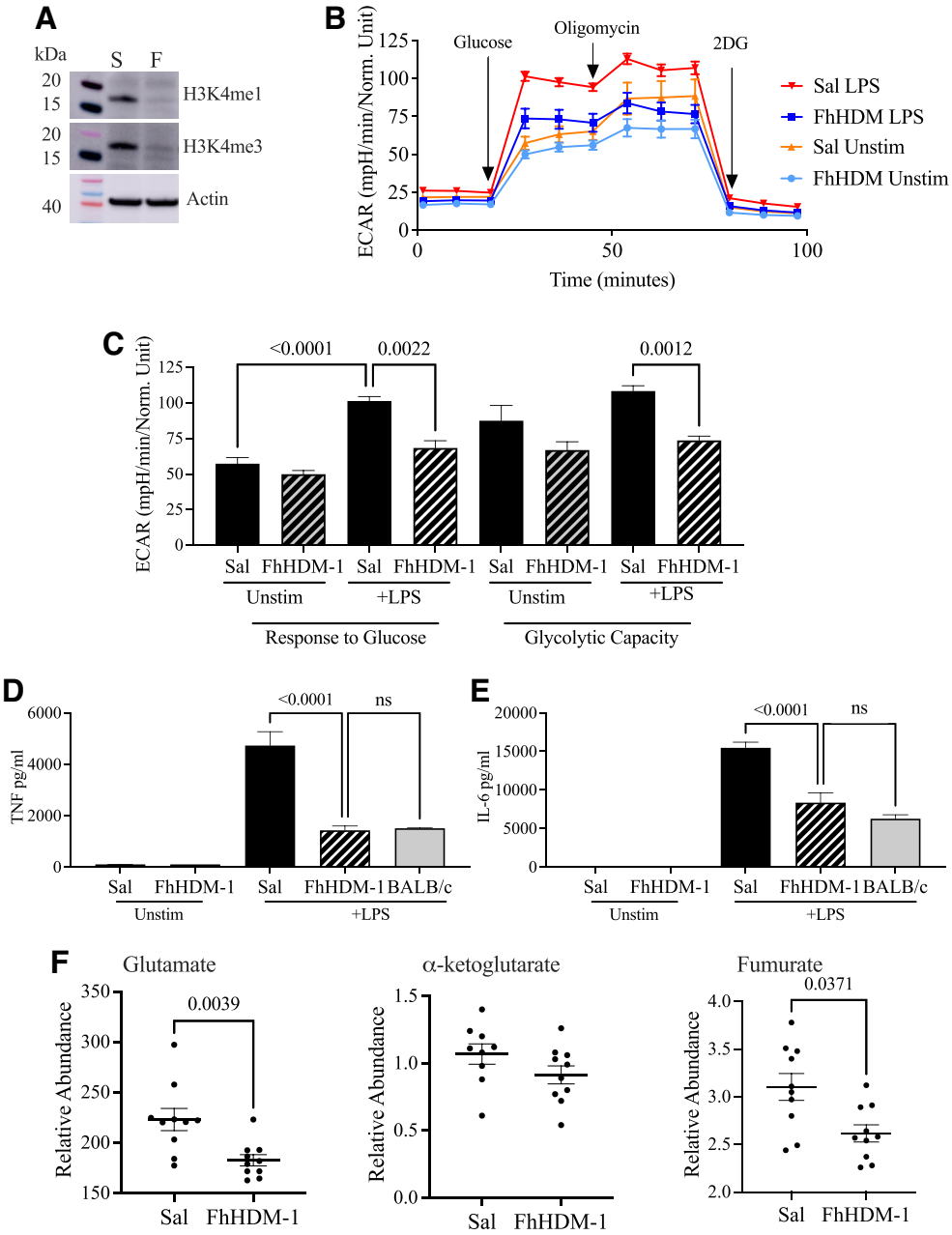

Figure 2. Administration of FhHDM-1 to NOD mice reversed the methylation of H3K4 and regulated the proinflammatory response to LPS. (A) Macrophages were differentiated from the bone marrow of NOD mice 3 weeks after the final administration of either saline (S) or FhHDM-1 (F). Protein lysates were normalized by protein concentration and the methylation status of H3K4 analyzed by western blot using primary antibodies specific for H3K4me1 and H3K4me3. The protein loading of each sample was illustrated by the detection of actin. The data shown is representative of $n = 10$ of each strain of mouse. (B) Macrophages were differentiated from the bone marrow of NOD mice previously treated with either saline or FhHDM-1, were either unstimulated (Unstim) or stimulated with LPS for 24 h. The glycolytic activity was determined by measuring the extracellular acidification rate (ECAR) and the (C) immediate and maximum glycolytic capacity of macrophages after subsequent treatment with glucose, oligomycin, and 2-deoxy-glucose (2-DG) (representative of two independent experiments of $n = 5$). (D) Secreted TNF and (E) IL-6 protein were quantified in the culture media by ELISA and compared with BMDMs isolated from age-matched BALB/c mice treated with saline and stimulated with LPS ex vivo (representative of two independent experiments of $n = 5$). (F) Intracellular levels of Glutamate, α-KG, and Fumarate in BMDMs derived from mice that had received six injections of either Saline or FhHDM-1 3 weeks earlier ($n = 10$) was measured by liquid chromatography-mass spectrometry after metabolite extraction. Data is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test, or a student *t*-test as appropriate.

This protective effect was attributed to a preferential interaction with macrophages and a regulation of their capacity for proinflammatory responses. Subsequent examination of macrophages treated with FhHDM-1 in vitro revealed a switch in metabolism to a dependence on oxidative phosphorylation that was driven by an increase in glutaminolysis. These metabolic changes mediated a concomitant reduction in glycolytic flux and a decrease in TNF and IL-6 production [11]. As the metabolites produced through the catabolism of glutamine have been functionally linked to the methylation (fumarate) and demethylation (α-ketoglutarate [KG]) of histones [12], it seems plausible that FhHDM-1 is altering the epigenetic imprint that mediates central immune training, to consequently regulate immune responses and prevent T1D. Such alterations in FhHDM-1-treated macrophages offer a mechanism by which the long-term prevention of disease was attained by a short-term treatment, implying that the reversal of innate immune training was underpinning the beneficial effects of FhHDM-1 treatment in all disease models.

Having established that immune training of macrophages was driving the aberrant proinflammatory immune response, and thus likely disease onset of NOD mice, this model was used to test this hypothesis of the mechanism of action of FhHDM-1. The previous treatment regime that prevented T1D in NOD mice was employed [10]. Thus, FhHDM-1 (or saline) was delivered i.p. on alternate days, for a total of six injections, to NOD mice beginning at 6 weeks of age. At 11 weeks of age (3 weeks posttreatment) bone marrow was harvested, macrophages were differentiated, and H3K4 methylation was determined. As hypothesized, the BMDMs of NOD mice that had received FhHDM-1 (Fig. 2A and B) displayed a significant reduction in the methylation of histone H3K4 (Fig. 2A; Supporting information S1). Furthermore, the glycolytic activity in response to LPS in BMDMs of FhHDM-1 treated NOD mice was reduced, as compared with those from NOD mice that received saline alone (Fig. 2B and C). Correlating with these changes, the production of TNF and IL-6 in response to LPS was suppressed in BMDMs from FhHDM-1 treated NOD mice, as compared with those given saline alone (Fig. 2D and E). These data validated the hypothesis that FhHDM-1 was reversing the trained immune response imprinted on BMDMs of NOD mice (as seen in Fig. 1A).

Treatment of mice with FhHDM-1, as compared with saline, resulted in a significant reduction in the levels of glutamate in BMDMs, concurring with our previous discovery that FhHDM-1 drives a switch to glutaminolysis in macrophages. Furthermore, the relative abundance of the downstream metabolites of α-KG (no significant change between treatments) and fumarate (significantly decreased in FhHDM-1 treated mice), which leads us to hypothesize that α-KG produced by the metabolism of glutamate, is utilized as a co-factor for demethylases, preventing its further oxidation, and thus reducing the abundance and availability of fumarate as a co-factor for methylases. As a result, the methyl markers on histones are removed and the expression of marked genes are regulated.

Notably, the administration of FhHDM-1 to NOD mice had no effect on the glycolytic activity of unstimulated BMDMs (Fig. 2B and C), suggesting the specific regulation of only LPS stimulated, and not resting, macrophages. Furthermore, the proinflammatory response to bacterial LPS was not abolished in macrophages from FhHDM-1 treated NOD mice, but rather restored to levels observed in BMDMs from immune-competent BALB/c mice (Fig. 2D and E). This observation indicates a subtle fine-tuning of macrophage immune responses by FhHDM-1, rather than a switch to an anti-inflammatory phenotype or complete immune suppression.

These findings suggest that FhHDM-1 can recreate the ancestral microenvironment in which innate immune training evolved, and thus restore the balance between activation and regulation of inflammation. While a causal link to disease outcomes, and a deeper understanding of the molecular mechanisms that mediate these effects in vivo will be required, the data presented here supports a potential, and previously unexplored, therapeutic strategy for T1D, and likely other autoimmune/inflammatory disorders.

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Abbreviations: **αKG**: alpha-ketoglutarate · **β**: beta cells · **BMDM**: bone-marrow-derived macrophages · **FhHDM-1**: *Fasciola hepatica* helminth defense molecule-1 · **H3K4**: lysine 4 of histone H3 · **ip**: intraperitoneal · **NOD mice**: nondiabetic obese mice · **T1D**: type 1 diabetes

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