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Hyperglycemia alters the inflammatory response of bone marrow derived macrophages

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SUMMARY Nod-Like Receptors (NLRs) are a type of immune receptor working to incite or dampen inflammation by means of downstream cytokine release. NLRP12 has been implicated in being a negative regulator of inflammatory responses by working in contrast to more well studied NLRs such as NLRP3. In the literature, the function of NLRP12 under normal glucose conditions is well documented. However, its ability to respond to pathogens under elevated levels of glucose, for example in diabetic conditions *in vivo* is not well characterized. Utilizing bone marrow derived macrophages cultured under varying glucose concentrations, this study examined the ability of NLRP12 to respond to antigen, utilizing a variety of ligands and bacterial strains. After exposure, the responses of both wild-type and NLRP12 deficient macrophages were evaluated by quantifying levels of secreted IL-1β and IFNγ as a measure of an inflammatory response using ELISA. The results of this study validate known scientific literature regarding NLRP12 as a negative regulator of inflammation, and additionally reveal that bone marrow derived macrophages cytokine response to *Salmonella enterica* is impaired under hyperglycemia, which could improve current understanding of *in vivo* diabetic immunity.

INTRODUCTION

Within the human gastrointestinal tract lives a diverse population of bacteria known as the gastrointestinal microbiota. Along with their crucial role in digestion, energy metabolism and vitamin synthesis, they also possess anti-pathogenic properties. The microbiota colonizes the gastrointestinal system beginning at birth and can be observed to take on its adult-like appearance around three years of age. The microbiota changes throughout the individual's lifetime with each exposure to new food, medication, and sickness (1). The gastrointestinal microbiota consists of two main phyla, Firmicutes (53.8%, largely Gram positive) and Bacteroidetes (46.1%, largely Gram negative). The ratio of these two phyla in the gut needs to be balanced with each other to maintain gastrointestinal health. Imbalances in this ratio (known as dysbiosis) caused by high fat diet correlate with metabolic disease such as Type 2 Diabetes Mellitus (T2DM) (2, 3). To avoid this, homeostasis is maintained by the immune system utilizing cells possessing pattern recognition receptors (PRR's) such as the Nod-Like Receptor (NLR).

The NLR is a mechanism of innate immunity which acts as a PRR to detect foreign antigen and neutralize it by means of a cytokine-induced inflammatory response. This is accomplished by macrophages, which secrete both pro- and anti-inflammatory cytokines to attract and activate a variety of immune cells (such as T cells and other macrophages) to the target area to neutralize the antigen. Many NLRs are known to have a pro-inflammatory function; however, NLRP12 has been demonstrated in literature to possess anti-inflammatory properties, especially regarding the gastrointestinal microbiota. It has been observed that

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NLRP12-deficient (-/-) mice (while simultaneously placed on a high fat diet) displayed significant dysbiosis compared to wild-type (WT) mice. The mice afflicted with dysbiosis also displayed correlated physiology associated with T2DM, such as increased weight gain and low-grade systemic inflammation (4, 5). Current literature largely demonstrates the capacity of NLRP12 to respond to antigen under normal concentrations of glucose (6, 7), but not hyperglycemia. In addition to confirming the known functions of NRLP12, hyperglycemia was surprisingly revealed to alter the pro-inflammatory cytokine secretion of bone marrow derived macrophages, specifically lowering secretion in response to commonly pathogenic strains of bacteria. Findings from this study can further contribute to a better understanding of immunity in patients afflicted with T2DM with elevated baseline glucose levels.

METHODS AND MATERIALS

Animal Model: Mice were maintained in specific-pathogen-free conditions at University of Massachusetts (UMass) Medical School, and all experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the UMass Medical School. All experiments were approved by the UMass Medical School IACUC and were performed according to ethical regulations regarding humane treatment of animals.

Cell Culture Media and Reagents: Cell culture reagents (DMEM, CellStripper, HEPES, glucose and Penicillin/streptomycin solution) were purchased from Lonza. LPS derived from *Escherichia coli* strain O26:B6 was purchased from eBioscience. Nigericin and alum were purchased from Sigma. WST-1 reagent was purchased from Roche. FBS was purchased from ATCC. ELISA kits and ancillary reagents to detect mouse IL-1β and IFN-γ were from R&D Systems.

Bacterial Strains and Growth Conditions: *Staphylococcus aureus* strains Newman and Newman ΔICA (generous gift of G. Pier, Harvard Medical School), *Yersinia pseudotuberculosis* strain YPIII (from UMass Medical School), and *Salmonella enterica* subspecies *bongori* (ATCC) were grown overnight in Brain Heart Infusion (BHI) broth at 37°C. The next day, bacteria were diluted in BHI supplemented with 12.5% glucose (to induce biofilm formation in *Staphylococcus aureus*, strain Newman) and then grown to log phase. Bacteria were diluted in DMEM without antibiotics for a Multiplicity of Infection (MOI) ratio of 10:1.

Bone Marrow Extraction: Two male C57/Bl6 mice (age 17 weeks) and two male NLRP12KO-245 mice (age 5 weeks) were obtained from the University of Massachusetts Chan Medical School and euthanized. Femur and tibia bones from both genotypes were aseptically extracted and cleaned using ethanol and flushed for bone marrow using sterile PBS. Resulting suspensions were then centrifuged at 600 x g for five minutes, and then resuspended in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 10% L929 supernatant, 1% penicillin/streptomycin solution (P/S) and 25mM HEPES (complete DMEM, cDMEM).

Cell Culture and Plating: Resuspended cells were plated into ten sterile plates per genotype with 9mL cDMEM and 1mL M-CSF containing L929 supernatant and incubated at 37°C and 5% CO_2 for five days. On day 3, cells were replenished with 10mL of cDMEM without removal of old media to protect the adhering cells, and glucose concentration was doubled to 9g/L in 50% of cultures to create hyperglycemic conditions. On day 5, all plates were rinsed with cDMEM, and bone marrow derived macrophages (BMDM's) were gently removed using 3mL CellStripper. BMDM's were incubated for five minutes at 37°C and 5% CO_2 and rinsed with 3mL cDMEM without L929 supernatant and centrifuged at 450 x g for 10 minutes. Cells were then resuspended in cDMEM without L929 supernatant and plated in tissue culture treated, flat-bottom 96 well plates at density of 1 x 10^6 /ml. Plates were then incubated overnight at 37°C and 5% CO_2.

BMDM Stimulation: On day 6, BMDM's were washed with un-supplemented DMEM to remove all antibiotics. BMDM's were then stimulated with a panel of known innate PRR ligands: LPS (100 ng/mL), Alum (125 µg/mL) and Nigericin (20 µg/mL). Stimulations with Alum and Nigericin were separated in two different groups: one with a two-hour 500 ng/mL LPS prime, and one without.

BMDM's were also stimulated with a panel of known pathogenic bacteria; *S. aureus* strains Newman and Newman ΔICA, *Y. pseudotuberculosis* (YPIII), and *S. bongori*. All bacterial stimulations were conducted using an MOI of 10 of log-phase grown bacteria, and after 3 hours were exposed to 50 ng/mL gentamycin for the remainder of the stimulation. All stimulations (both ligand and bacterial) were incubated for a minimum of six hours at 37°C and 5% CO_2 prior to cell supernatant collection for analysis.

Cytotoxicity: Cells were tested for viability on day 3 of differentiation using WST-1 reagent (Roche) according to the manufacturer's instructions. After incubation, absorbance was quantified by a Biotek Epoch plate reader using Gen 5 software at an optical density of 450 nanometers.

Cytokine Quantification and Analysis: Supernatants from the above stimulations were analyzed for levels of secreted IL-1β (immediately after 6 hours of stimulation) and IFNγ (48 hours post stimulation) using R&D Systems Mouse Duoset ELISA according to manufacturer's instructions. A standard curve of these results was generated using Microsoft Excel, and the resulting values were interpreted using unpaired T tests in GraphPad Prism for statistical validity.

RESULTS

Plating Efficiency. WT and NLRP12 -/- mice BMDMs were differentiated and cultured under the conditions mentioned above. To confirm cell viability and equal plating, cells were tested for metabolic activity by WST-1 reagent. The quantity of metabolically active BMDMs (both genotypes) in each glycemic condition was not statistically significant (Figure 1).

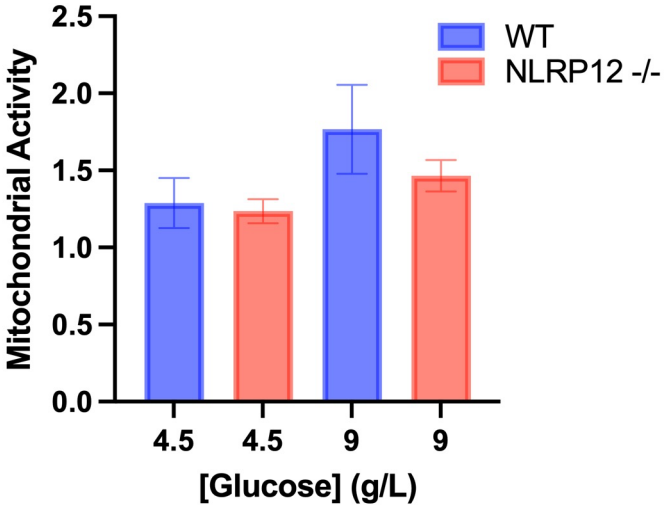

FIG. 1 Cell viability of bone marrow derived macrophages (BMDM). Cells were plated in 96-well microtiter plates at a density of 1 x 10^6 /ml in DMEM with either 4.5g/L or 9g/L glucose without L929 supernatant. On day 6, to assess cell viability of differentiated BMDM, a WST-1 assay of both genotypes and culture conditions was performed. Quadruplicate samples were quantified, and absorbance was measured. Groups were compared by one-way ANOVA and no significant differences between groups was determined, indicating an equal number of viable cells per well. Error bars represent standard error of mean (SEM).

NLRP12 Functions as a Negative Regulator of IL-1β in Response to Bacterial Stimulation. NLRP12 -/- BMDMs stimulated under normoglycemic conditions secreted significantly higher amounts of the pro-inflammatory cytokine IL-1β in response to bacterial stimuli compared to WT (Figure 2). When exposed to LPS ($p < 0.001$), *S. aureus* Newman ($p < 0.01$) and Newman ΔICA ($p = 0.0001$), NLRP12 -/- BMDMs secreted higher IL-1β levels compared to WT.

Hyperglycemia Triggers Pro-Inflammatory Cytokine Secretion. Hyperglycemic conditions triggered a higher IL-1β response ($p < 0.05$) in WT cells compared to knockout when stimulated with LPS with Alum (Figure 3).

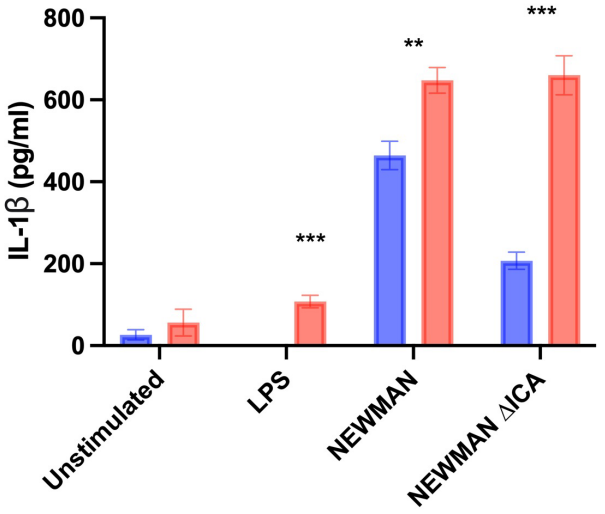

FIG. 2 Comparison of IL-1β secretion from WT and NLRP12-/- BMDM stimulated under normoglycemic conditions. Cells were stimulated with LPS (100 ng/mL), *S. aureus* Newman and Newman ΔICA at an MOI = 10 or left untreated (mock) for six hours. Antibiotics were added for the final 3 hours and removed as described in Methods. Cell supernatants were collected and IL-1β secretion was quantified using a sandwich ELISA. (*p <0.05, **p<0.01, ***p<0.001). Error bars represent standard error of mean (SEM).

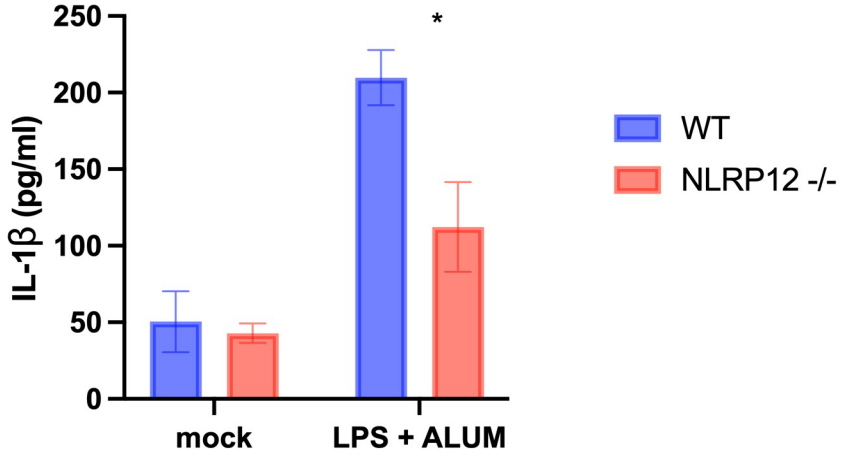

FIG. 3 Comparison of IL-1β secretion from WT and NLRP12-/- BMDM stimulated under hyperglycemic conditions. Cells were stimulated with LPS (500ng/mL) for two hours, and then alum (125 μg/mL) or left untreated (mock). Cell supernatants were collected after 48 hours of stimulation and IL-1β secretion was quantified using a sandwich ELISA. (*p <0.05, **p<0.01, ***p<0.001). Error bars represent standard error of mean (SEM).

Additionally, within the WT cells, hyperglycemic conditions triggered a higher IL-1β response in comparison to normoglycemic control (Figure 4). This was most observed when cells were stimulated with LPS with Alum ($p < 0.001$) and Newman ΔICA ($p < 0.0001$).

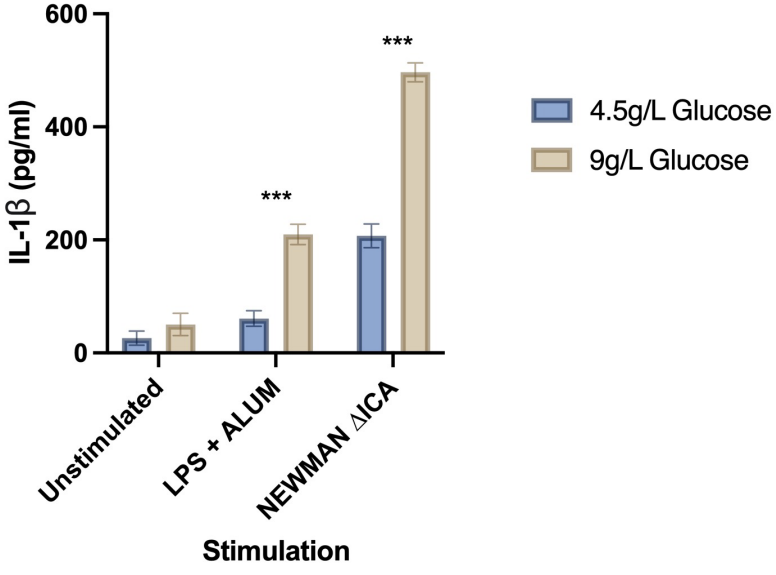

FIG. 4 Comparison of IL-1β secretion from WT BMDM's stimulated under normoglycemic and hyperglycemic conditions. Cells were stimulated with LPS (500ng/mL) for two hours, and then alum (125 μg/mL), *S. aureus* Newman ΔICA at an MOI = 10 or left untreated (mock) for six hours. Antibiotics were added for the final 3 hours and removed as described in Methods. Cell supernatants were collected and IL-1β secretion was quantified using a sandwich ELISA. (*p <0.05, **p<0.01, ***p<0.001). Error bars represent standard error of mean (SEM).

IL-1β was not the only cytokine affected however, as levels of IFNγ were also observed to be impacted in a similar manner. Compared to NLRP12 -/-, WT cells secreted higher levels of IFNγ when exposed to Newman ΔICA ($p < 0.001$) and *S. bongori* ($p < 0.01$) under hyperglycemic conditions (Figure 5).

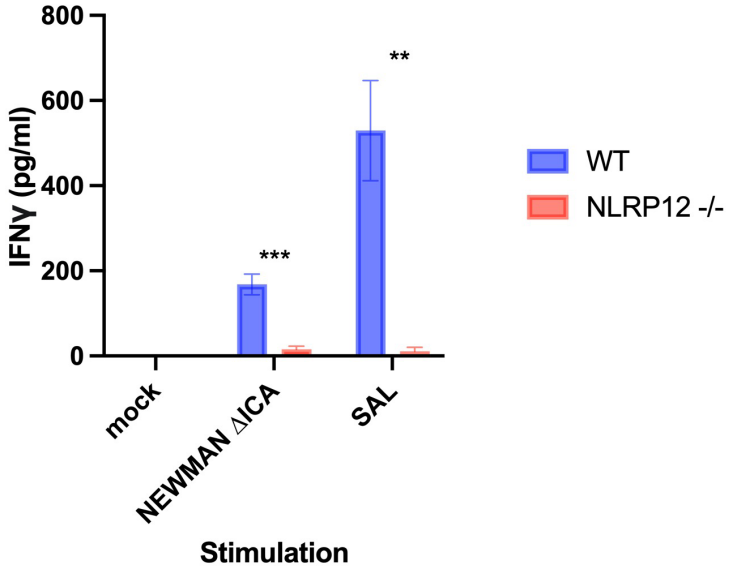

FIG. 5 Comparison of IFNγ secretion from WT and NLRP12-/- BMDM stimulated under hyperglycemic conditions. Cells were stimulated with *S. aureus* Newman ΔICA and *S. bongori* at an MOI = 10 or left untreated (mock). Antibiotics were added for the final 3 hours and removed as described in Methods. Cell supernatants were collected after 48 hours of stimulation and IFNγ secretion was quantified using a sandwich ELISA. (*p <0.05, **p<0.01, ***p<0.001). Error bars represent standard error of mean (SEM).

Again, within WT cells alone, cytokine secretion was similarly altered (Figure 6). When exposed to the same bacterial strains, WT BMDM's secreted significantly more IFNγ when exposed to Newman ΔICA ($p < 0.001$) and *S. bongori* ($p < 0.01$) compared to WT normoglycemic control.

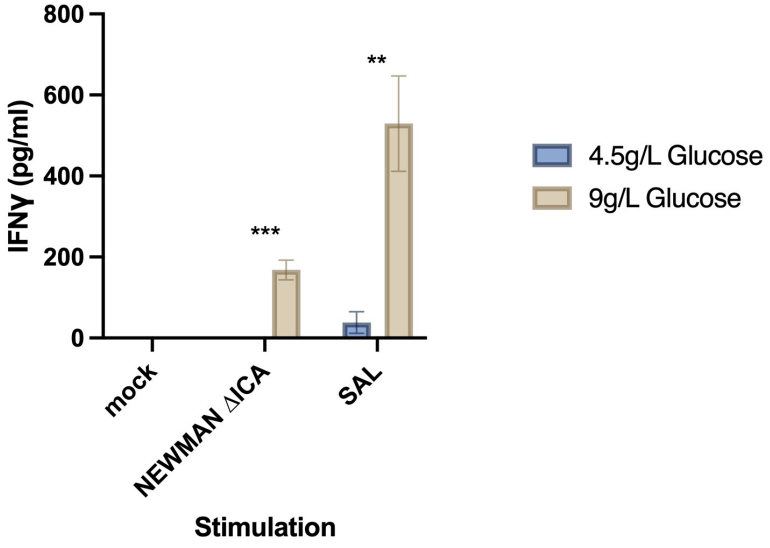

FIG. 6 Comparison of IFNγ secretion from WT BMDM's stimulated under normoglycemic and hyperglycemic conditions. Cells were stimulated with *S. aureus* Newman ΔICA and *S. bongori* at an MOI = 10 or left untreated (mock) for six hours. Antibiotics were added for the final 3 hours and removed as described in Methods. Cell supernatants were collected and IFNγ secretion was quantified using a sandwich ELISA. (*p <0.05, **p<0.01, ***p<0.001). Error bars represent standard error of mean (SEM).

Hyperglycemia Impairs IL-1β Secretion in Response to Bacteria Across Genotypes. Hyperglycemia significantly lowered both WT and NLRP12 -/- BMDM's IL-1β secretion in response to bacterial exposure (Figure 7). WT BMDM's secreted significantly lower ($p = 0.0001$) levels of IL-1β in response to *S. bongori* compared to normoglycemic control (Figure 7a). NLRP12 -/- BMDMs also saw a similar decrease in cytokine output when stimulated with *S. bongori* ($p = 0.0001$) as well as Newman ($p < 0.0001$) when glucose concentration was doubled (Figure 7b).

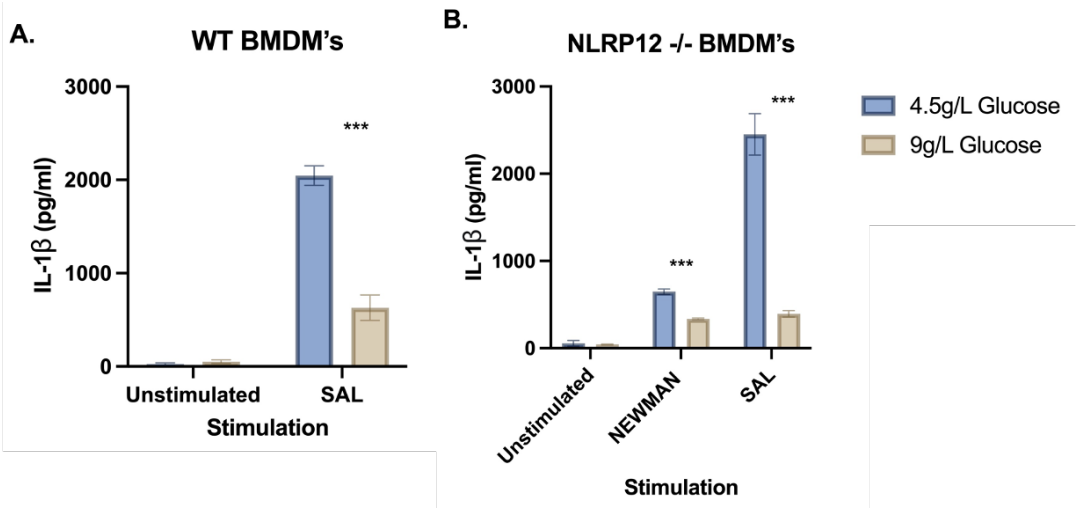

FIG. 7 Comparison of IL-1β secretion from WT and NLRP12-/- BMDM stimulated under normoglycemic and hyperglycemic conditions. WT (A) and NLRP12-/- (B) cells were stimulated with *S. aureus* Newman and *S. bongori* at an MOI = 10 or left untreated (mock) for six hours. Antibiotics were added for the final 3 hours and removed as in Methods. Cell supernatants were collected and IL-1β secretion was quantified using a sandwich ELISA. (*p <0.05, **p<0.01, ***p<0.001). Error bars represent standard error of mean (SEM).

DISCUSSION

NLRP12 is known to influence the gastrointestinal microbiota. NLRP12 -/- mice display increased low-grade inflammation when fed a high fat diet, which correlates with microbiota dysbiosis (4, 5). This change in composition has been implicated in the pathology of T2DM, in which patients have elevated baseline concentration of blood glucose compared to a non-disease state.

Deletion of NLRP12 was demonstrated to increase pro-inflammatory cytokine secretion upon stimulation. The major cytokine of effect was demonstrated to be IL-1β, with IFNγ being secreted as well. This may appear surprising at first, as macrophages are not classic IFNγ secretory cells. However, it is known that IL-1β activates factors which trigger T cell secretion of IFNγ in response to antigen (6). Additionally, no intracellular staining was done to ensure only macrophages were cultured and differentiated from the bone marrow samples. Therefore, these findings could potentially point to contamination of cultures by T cells. Overall, these findings are in agreement with and support known literature that NLRP12 acts to negatively regulate inflammation under normoglycemic conditions (7).

Hyperglycemic conditions place excess stress on macrophages, causing cytokine secretion independent of stimuli (8). This stress was demonstrated to override NLRP12's inflammatory regulation, agreeing with known literature. It is important to note however that stimuli choice may be a contributing factor to this. The selected stimuli are known NLRP3 ligands, thus the above observations may be indicative of activating a different inflammasome than originally intended.

Hyperglycemia was demonstrated to significantly lower the IL-1β response of both WT and NLRP12 -/- BMDMs to both Gram-positive and Gram-negative bacteria. This appears to be a novel finding, and reflects effect of hyperglycemic exposure for a total of three days. It is important to note exposure to hyperglycemia earlier or later in the monocyte differentiation process may produce different results, but that was not explored during this study.

Conclusions T2DM is a metabolic disease characterized by a disease state of baseline hyperglycemia. Based on the findings of this study, it can be hypothesized that a patient afflicted with T2DM may have an altered response to bacterial infection. Specifically, a patient may exhibit a significantly weaker IL-1β induced inflammatory response. As it is widely known, inflammation is used by the body to attract a wide variety of immune cells to neutralize infection. Therefore, the patient would potentially experience a weakened immune

response and be at increased risk for serious complications associated with *S. aureus* and *S. bongori* infection. Additionally, it can be hypothesized due to their baseline hyperglycemic inflammation (which has been shown to overwhelm the regulation of NLRP12), any additional antigen related inflammation would most likely be very difficult to resolve and could potentially worsen disease pathology and have a dangerous effect on any comorbidities the patient may also have. Further experiments would be necessary to validate these results both *in vitro* and *in vivo*.

Future Directions As this was a small-scale study, only 4.5 g/L (1x) and 9 g/L (2x) concentrations of glucose were examined during this study. Future trials should include a more thorough dose escalation study to observe at what specific concentration macrophage cytokine secretion becomes altered. Varying concentrations of ligands (or different ligands altogether) may also be desired to rule out unwanted NLR co-stimulation as well. It may also be beneficial to stimulate with a wider panel of known pathogenic bacteria to observe if cells are more susceptible to certain strains over others. Intracellular staining may also be desired to rule out stimulation of other immune cells, such as contaminating T cells. Further trials may be desired to add further statistical validity to the weakened cytokine response correlated to hyperglycemic culture conditions as well. Lastly, in order to more closely replicate clinical conditions, it may be desirable to duplicate this experiment utilizing human bone marrow derived macrophages or using an *in vivo* mouse model. This was beyond the scope of this project and this laboratory; thus, it was not conducted. However, once completed it would shed clearer light on the effect of hyperglycemia on the inflammatory response of wild type and NLRP12 deficient macrophages when presented with antigen.

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