Endosomal sequestration of TLR4 antibody induces myeloid-derived suppressor cells and reverses acute Type 1 Diabetes

Kathryn C.S. Locker1,2*, Kritika Kachapati3*, Yuehong Wu3, Kyle J. Bednar3, David Adams3, Carolyn Patel3, Hiroki Tsukamoto4, Luke S Heuer8, Bruce J. Aronow5,7, Andrew B. Herr1,6,7, and William M. Ridgway3,8

1 Division of Immunobiology, Cincinnati Children’s Hospital, Cincinnati, OH 45229, USA
2 Immunology Graduate Program, Cincinnati Children’s Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA.
3 Division of Immunology, Allergy and Rheumatology, University of Cincinnati College of Medicine, Cincinnati, Ohio, 45267, USA
4 Department of Pharmaceutical Sciences, School of Pharmacy at Fukuoka International University of Health and Welfare, Okawa, Fukuoka 831-8501, Japan
5 Division of Bioinformatics, Cincinnati Children’s Hospital, Cincinnati, OH 45229, USA
6 Division of Infectious Diseases, Cincinnati Children’s Hospital, Cincinnati, OH 45229, USA
7 Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, 45229, USA
8 Division of Rheumatology, Allergy and Clinical Immunology, University of California, Davis, CA 95616, USA

*These two authors contributed equally

Co-Corresponding authors: Andrew B. Herr, andrew.herr@cchmc.org; William M. Ridgway, wmridgway@ucdavis.edu, 451 Health Sciences Dr Suite 6515, Davis CA, 95616 Tel: 530 752 3877 Fax: 530 754 6047

Short Title: Anti-TLR4 antibody reverses acute T1D

Word Count: 3,975

Figures: 8
Abstract

We previously showed that treating NOD mice with an agonistic monoclonal anti-TLR4/MD2 antibody (TLR4-Ab) reversed acute type one diabetes (T1D). Here, we show that TLR4-Ab reverses T1D by induction of myeloid derived suppressor cells (MDSC). Unbiased gene expression analysis after TLR4-Ab treatment demonstrated upregulation of genes associated with CD11b+Ly6G+ myeloid cells, and downregulation of T cell genes. Further RNAseq of purified, TLR4-Ab treated CD11b+ cells showed significant upregulation of genes associated with bone marrow-derived CD11b+ cells and innate immune system genes. TLR4-Ab significantly increased percentages and numbers of CD11b+ cells. TLR4-Ab-induced CD11b+ cells, derived ex vivo from TLR4-Ab treated mice, suppress T cells, and TLR4-Ab-conditioned bone marrow cells suppress acute T1D when transferred into acutely diabetic mice. Thus, the TLR4-Ab-induced CD11b+ cells, by currently accepted definition, are MDSCs able to reverse T1D. To understand the TLR4-Ab mechanism we compared TLR4-Ab to TLR4 agonist LPS (which cannot reverse T1D). TLR4-Ab remains sequestered at least 48 times longer than LPS within early endosomes, alters TLR4 signaling and downregulates inflammatory genes and proteins including NFkB. TLR4-Ab in the endosome therefore induces a sustained, attenuated inflammatory response providing an ideal “second signal” for the activation/maturation of MDSCs that can reverse acute T1D.
Introduction

Type one diabetes (T1D) is a complex autoimmune disease triggered by genetic and environmental factors that ultimately results in the destruction of pancreatic islet beta cells. The progression of T1D is mediated by cellular responses of the innate and adaptive immune system. T cells are primarily responsible for destruction of the beta cells; however soluble and cellular mediators of the innate immune system have a crucial role in promoting T cell effector function. In T1D, tissue resident macrophages and dendritic cells are among the first to respond to beta cell insult and are essential for the retention of diabetogenic T cells during peri-insulitis (1-5). These antigen presenting cells (APCs) produce pro-inflammatory cytokines which drive lymphocytic infiltration of the islet and further promote priming and differentiation of destructive T effector cells (6-9). Despite the critical role of APCs in T1D pathogenesis, our ability to reverse acute T1D with agonistic anti-TLR4/MD2 antibody (TLR4-Ab) was surprising and the mechanism was unclear (10). Given the high expression of pathogen recognition receptors (PRRs) such as toll-like receptor 4 (TLR4) on APCs, our initial hypothesis was that TLR4-Ab induced “endotoxin tolerance”. However, the presence of downregulated, but still elevated amounts of inflammatory cytokines did not explain reversal of acute autoimmunity (11). Studies presented here provide further data on the mechanism of TLR4-Ab mediated reversal of T1D.

The TLR4 signaling axis plays a role in the recruitment of myeloid-derived suppressor cells (MDSCs) (12-15) and NFκB activation plays a role in MDSC expansion (16). MDSCs are morphologically and functionally heterogeneous but have a well-defined role in the
suppression of NK and T effector cells. Though they are most commonly studied in the context of cancer, MDSCs accumulate in many other disease models of chronic inflammation and autoimmunity, including T1D (17-19). MDSCs are unique in that they are not classically differentiated by growth factors, rather they are recruited in at least two steps: 1) mobilization from the bone marrow as immature myeloid cells, and 2) activation by chronic immune stimulation at a site of infection, inflammation, or tumor microenvironment (20). Here, we show induction of MDSCs with specific gene expression profiles by RNAseq, that suppress T cells in TLR4-Ab-treated NOD mice and reverse acute T1D. Furthermore, we establish how TLR4-Ab differentially activates APCs, compared to LPS, by sequestering in the early endosome. These results provide novel insight into the molecular mechanisms that underlie the mild agonist phenotype and reversal of acute T1D.

Methods

Mice and treatment with TLR4 Ab

NOD mice were bred and maintained and all procedures involving mice were conducted in accordance with IACUC guidelines at the University of Cincinnati Laboratory Animal Medical Services. The production and characterization of TLR4/MD-2 (Ctrl-Ab/TLR4-Ab) monoclonal antibodies was previously described (21). Prediabetic Female NOD mice were randomly assigned to either Ctrl-Ab or TLR4-Ab treatment groups. Diabetes was confirmed by blood glucose meter. After onset T1D, (blood glucose between 200-250mg/dL), mice were treated twice, one week apart with either Ctrl-Ab (5μg) or TLR4-Ab (5μg) injected intraperitonially.
Flow cytometry

Islet infiltrating hematopoetic cells (IIHC) and splenocytes were isolated from PBS and TLR4-Ab treated mice as described (22). These cells were treated with Fc-block (BD Biosciences), stained with the indicated antibodies and propidium iodine (PI), run on an LSRII machine (BD Biosciences) and analyzed using FlowJo software.

RNA-seq analysis and PCR Array

RNAseq was performed on splenocytes and on CD11b+ FACS-sorted cells from TLR4-Ab vs. Ctrl-Ab treated diabetic mice as previously described (23). RNA-seq analysis was carried out using Bowtie (24), Tophat2 (25) and the Cufflinks2 pipeline (26) as previously described. Differentially expressed gene signatures were identified using Audic Claverie tests (P < 0.05) and Student’s t-test (FDR < 0.05) followed by a two-fold change requirement. Gene Ontology/biological network analysis was carried out using ToppGene (27), and ToppCluster (28) and analysed in Cytoscape. For PCR arrays, cDNA was amplified on a StepOne real time PCR machine using the RT² Profiler™ PCR Array Mouse Innate & Adaptive Immune Responses plate (Qiagen) and RT² SYBR® Green qPCR Mastermix (Qiagen). Cₜ values were uploaded to the Qiagen GeneGlobe analysis website for analysis.

Bone marrow transfer experiments

NOD Bone marrow cells were cultured in the presence of either TLR4-Ab (250ng/ml) or Ctrl-Ab (250ng/ml) for 7 days. Cells were trypsinized and harvested for flow analysis and transfer. 2x10⁶ harvested cells were transferred into diabetic (BG > 200mg/dl) NOD recipients. Five separate experiments were done. The endpoint was BG > 500 mg/dl.
CD11b-mediated T cell suppression assay

NOD mice were treated twice with 10μg of TLR4-Ab or Ctrl-Ab. One week after the second treatment, splenic CD11b cells were purified using Mac beads. The cells were FACS sorted for CD11b+Gr1+ MDSC cells. CD4 cells were Mac purified from untreated NOD spleen and stained with Cell Trace Violet (Invitrogen). 100,000 CD4 T cells were cultured with 50,000 CD11b+Gr1+ MDSC cells and CD3/28 activation beads (Dynabeads). Control cells without MDSC were either left unstimulated or stimulated with CD3/28 beads. On day 3, the cells were stained with CD4 Percp (BD Bioscience), followed by viability dye eFluor 780 (Thermo Fisher) and analyzed by Facs Cantos and Flow Jo.

TLR4 trafficking and colocalization experiments.

Bone marrow was harvested from C57/B6 mice and cultured in complete DMEM (DMEM +10% FBS +1% Pen/Strep) with 40 ng/ml M-CSF for 5 days to generate bone marrow-derived macrophages (BMDMs). BMDMs were plated in 24 well plates at 2 x 10^6 cells/ml in complete DMEM with Fc-block (1:5000; Biolegend), then pulsed with TLR4-Ab (UT18; 100ng), Ctrl-Ab (UT15; 100ng), or LPS (100ng) for 5 minutes. BMDMs were stained in fix & perm (Invitrogen) with antibodies against CD11b (Biolegend), EEA1 (NovusBio), and UT18-/UT15-specific secondary antibodies (Biolegend). Cells stimulated with LPS were fixed and stained with TLR4-Ab to detect TLR4/MD2 localization. Internalization and colocalization was evaluated using ImageStream. TLR4/EEA1 colocalization was quantified using the bright detail similarity wizard. The internalization wizard generates an internalization erode score; scores greater than 1.0 indicate internalization of TLR4. IDEAS quantification data from 2 experiments (>5000 events, gated on CD11b+ cells) was imported to Graphpad Prism and P-values were calculated using a two-way ANOVA analysis.
**Western blots**

2 x 10⁶ RAW 264.7 cells (ATCC TIB-71) in complete DMEM were stimulated with UT18 (100ng), UT15 (100ng), or LPS (100ng) for various times, pelleted, washed, resuspended in 2x Laemmli sample buffer (Biorad), and sonicated. Proteins were separated by electrophoresis in a 4-20% polyacrylamide gel and transferred to nitrocellulose membranes, blocked with 5% BSA in TBST and probed with antibodies against P-ERK, P-p38 MAPK, P-JNK, IκBα, and GAPDH (Cell Signaling). After incubation with an HRP-conjugated secondary antibody (Cell Signaling), blots were incubated with ECL substrate (Thermo) and developed with film. Densitometry analysis of protein bands was quantified using Image J as described (29).

**Data analysis**

All statistical analysis was performed using GraphPad Prism version 6 for Windows (GraphPad software). Significance testing was done using either the unpaired t-test or Mann-Whitney tests for sample comparisons, and the Log-rank test for survival curve analysis.

**Data and Resource Availability**

Resources generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.
Results

TLR4-Ab reverses acute T1D in NOD mice.

We previously demonstrated that TLR4-Ab treatment of NOD mice with acute T1D results in reversal of T1D disease (in mice with initial BG up to 400 mg/dl) with significantly decreased blood glucose levels, recovery from weight loss, and a significantly improved islet infiltration score (10). Successful treatment was accompanied by significantly increased insulin area per insulin positive islet cell, reflecting larger insulin positive islets in treated mice compared to untreated or newly diabetic mice (10). We have subsequently studied a large cohort of NOD diabetic mice treated twice, one week apart, with TLR4-Ab vs. Ctrl-Ab and have focused our studies on the immunological consequences of treatment 1 week after the second antibody injection. TLR4-Ab successfully treated acute T1D in this group, confirming our previously published results (Figure 1). In the TLR4-Ab treated group, only 15% (7/46) of the mice progressed to end stage T1D within 2 weeks of T1D onset, compared to 53% (21/40 mice) progression in mice treated with Ctrl-Ab. Only 3 mice in the TLR4-Ab-treated group progressed to end stage (BG >500 mg/dl) T1D prior to the second dose, compared to 11 mice in the Ctrl-Ab-treated group. The final BG in the TLR4-Ab treated group was significantly less than in the Ctrl-Ab group (Figure 1). These results in a large cohort of treated mice support our prior findings of significant reversal of acute T1D with two treatments of an agonist TLR4-Ab.

TLR4-Ab treatment in vivo increases CD11b+ MDSCs in the spleen and islets of NOD mice.
Given that the vast majority of TLR4-Ab-treated mice achieve lower BG levels and reduced islet infiltration, we sought to immunophenotype the cells in the spleen and islets of TLR4-Ab-treated mice that may contribute to disease reversal. Splenocytes harvested from NOD mice 24 hours after the second TLR4-Ab treatment show increased numbers and percentages of CD11b+ cells as compared to splenocytes from PBS- or Ctrl-Ab-treated mice (Figure 2A, 2B). Within this CD11b+ population, increases in both monocytic and granulocytic myeloid-derived suppressor cell (MDSC) subsets were observed in TLR4-Ab-treated mice, in addition to increased macrophage and granulocyte (PMN) populations (Figure 2B). Splenocytes and intra-islet hematopoietic cells (IIHC) harvested 7 days after the final treatment still have significantly increased percentages of CD11b+ cells, and splenocytes have significantly increased numbers compared to their PBS counterparts (IIHC CD11b numbers are clearly increased but did not reach significance) (Figure 2C, 2D), suggesting that TLR4-Ab treatment results in the retention of these cells rather than transient recruitment and infiltration.

RNAseq data from TLR4-Ab-treated mice show upregulation of genes associated with CD11b+Ly6G+ cells and downregulation of genes associated with CD4+ T cells.

Given the longevity of the CD11b+ cell population in both the spleen and islet, we sought to characterize the transcriptional profile of these cells. RNA sequencing of splenocytes was performed to build cluster enrichment networks based on genes associated with biological processes and pathways that were significantly upregulated or downregulated in TLR4-Ab-treated mice compared to those treated with Ctrl-Ab. Splenocytes harvested one week after the second TLR4-Ab treatment have an upregulation of genes associated with CD11b+Ly6G+ cells, (granulocytic MDSCs), compared to their PBS- and Ctrl-Ab counterparts (Figure 3, Supplementary Table 1). Additionally, T cell-associated genes were down-
regulated in TLR4-Ab-treated mice, supporting the hypothesis that CD11b+ cells mediate suppressive effects on T cells *in vivo*.

**CD11b+ cells sorted from TLR4-Ab-treated mice display upregulation of genes associated with a bone marrow derived myeloid cell transcriptional profile.**

To determine the transcription profile of TLR4-Ab treated CD11b+ cells, CD11b+ splenocytes were FACS sorted from diabetic mice treated with either TLR4-Ab or Ctrl-Ab as in Figure 3 and analyzed by RNAseq. A heatmap was generated using 261 significantly upregulated genes in the TLR4-Ab treated group (Figure 4A), these genes were used to build a cluster enrichment network of significantly associated gene ontology categories (Figure 4B). The significantly upregulated genes were significantly associated with myeloid, CD11b+, bone marrow derived cells in co-expression atlas databases (Figure 4B, light green gene ontology categories). Furthermore, the first neighbors of the indicated GO co-expression atlas categories show a large proportion of the upregulated genes are linked to BM derived, CD11b+ gene signatures from the co-expression databases (Supplementary Figure 1). These results provide strong evidence that the TLR4-Ab induced increased CD11b+ population is derived from the bone marrow. Notably, the RNAseq data shows significant association to both granulocytic/neutrophil and macrophage pathways, and overall shows a significant association to activation of the innate immune system. Several novel gene families (e.g., Kinesins, Flotillins and Formyl Peptide receptors) are upregulated and will be addressed in the Discussion section.
CD11b+ Gr1+ cells sorted from TLR4-Ab-treated mice suppress T cell proliferation ex vivo.

Having met the criteria for the first step of MDSC generation (expansion and mobilization of immature myeloid cells) we next assayed whether TLR4-Ab could produce functionally active MDSCs and suppress T-cell activation. CD11b+ Gr1+ cells were therefore sorted from NOD mice one week after treatment with either TLR4-Ab or Ctrl-Ab, and co-cultured with donor-matched CD4+ T cells. T cells cultured with CD11b+Gr1+ cells from TLR4-Ab-treated mice demonstrated reduced proliferation compared to T cells cultured with Ctrl-Ab treated CD11b+ Gr1+ cells (Figure 4C+D). As MDSC share phenotypic markers with monocytes and neutrophils they are predominantly defined by their functional ability to suppress T-cell activation through metabolic paralysis (30-32). Our data demonstrates that highly purified CD11b+ Gr1+ cells from TLR4-Ab mice suppress T cell proliferation, further supporting our hypothesis that TLR4-Ab induces MDSCs.

CD11b+ cells generated from TLR4-Ab-treated bone marrow ameliorate progression of acute T1D.

Next, we tested the effect of TLR4-Ab-treated bone marrow-derived cells on acute T1D. All hematopoietic cell subsets except megakaryocyte-erythroid progenitors (MEPs) have been reported to express functional TLR4/MD2, with the highest expression observed on HSCs and GMPs (12). One theory of MDSC generation suggests a two-step model wherein immature myeloid cells are first expanded in the bone marrow and introduced into circulation, and second stimulated by chronic inflammation to become functionally suppressive MDSCs (16). We therefore tested whether TLR4-Ab-treatment of bone marrow was sufficient to generate a population of suppressor cells in vitro. Bone marrow grown in the presence of TLR4-Ab
yielded an enrichment of CD11b+ cells that express Ly6C and/or Ly6G, as compared to Ctrl-Ab treated bone marrow (Figure 5A), showing that TLR4-Ab alone was sufficient to drive differentiation of CD11b+ Gr1+ cells from bone marrow. TLR4-Ab-differentiated bone marrow cells transferred into acutely diabetic NOD recipient mice significantly reduced progression to end-stage T1D and final blood glucose levels compared to diabetic recipients that received Ctrl-Ab-treated bone marrow cells (Figure 5B+C). Thus, bone marrow derived, TLR4-Ab induced MDSCs can protect mice from progression of acute T1D.

**TLR4-Ab upregulates expression of inhibitory IκB and demonstrates an intermediate activation of MAPKs as compared to pro-inflammatory LPS.**

LPS, the prototypical TLR4 agonist, drives differentiation of HSCs and GMPs into monocytes and macrophages with an inflammatory phenotype (12), but the phenotype of cells generated from TLR4-Ab-conditioned bone marrow is markedly different. LPS, in contrast to TLR4-Ab, cannot reverse acute T1D (33). Given that TLR4-Ab can induce suppressor MDSCs, we next sought to understand how TLR4-Ab activates target cells differently at the molecular level compared to LPS. We quantified phosphorylation of key signaling adaptors in the NF-κB pathway in TLR4-Ab treated RAW cells. We observe an overall downregulation of P-JNK/SAPK, P-p38 MAPK, and P-ERK, as well as upregulation of IκB in cells stimulated with TLR4-Ab compared to LPS (Figure 6). Sustained expression of IκB is particularly interesting, since it blocks nuclear translocation of p50-/p65 (NF-κB) and the activation of genes that produce pro-inflammatory cytokines. These data suggest that TLR4 MyD88-dependent signaling is activated to a significantly lesser degree than with LPS. Furthermore, PCR array data from splenocytes harvested from NOD mice 1 week after TLR4-Ab treatment shows broad downregulation of multiple inflammatory genes compared to mice treated with
LPS (Figure 7). Interestingly, even one week after the last TLR4-Ab treatment, we still observe a significantly increased ratio of IκB:Nf-κB compared to splenocytes from LPS-treated mice (Figure 7). These results demonstrate a prolonged, suppressed inflammatory phenotype in TLR4-Ab cells compared to LPS, which may explain the inability of LPS to reverse acute disease. To further understand this critical difference in two TLR4 agonists, we next studied the effect of both treatments on TLR4 endosomal cycling.

**TLR4-Ab remains co-localized with EEA1 and sequesters TLR4/MD2 inside the endosome for at least 24 hours.**

Given the decreased NF-κB activation observed after TLR4-Ab treatment compared to LPS, we tested whether TLR4-Ab was inducing internalization of the TLR4/MD2 receptor complex. Receptor localization impacts the receptor’s accessibility to signaling mediators. In TLR4 signaling, while MyD88 signaling can be initiated at the cell surface and internally from the endosome, TRIF/TRAM signaling requires internalization of TLR4 to the endosome (34). For these experiments, we utilized imaging flow cytometry to perform internalization and co-localization analyses. In cells pulsed with TLR4-Ab, TLR4 co-localizes with early endosomal marker EEA1 within minutes (Figure 8). This data corresponds with a previous report that UT12, another TLR4/MD2-Ab agonist, induces internalization of TLR4 (35). Ctrl-Ab did not induce internalization of TLR4 in any experiments (Supplemental Figure 2). In contrast, cells pulsed with LPS also demonstrated internalization of TLR4 and as expected, LPS is quickly processed and TLR4 is recycled back to the cell surface within 30-60 minutes after the stimulation (Figure 8). Surprisingly, TLR4-Ab remains co-localized with EEA1 for a prolonged period of time, retaining TLR4 in the early endosome for at least 24 hours (Figure 8). The data in Figures 6-8 supports the hypothesis that TLR4-Ab initiates a prolonged,
chronic pro-inflammatory response by sequestering TLR4/MD2 within the endosome for a much longer time frame than the natural TLR4 agonist LPS. The effect of this sequestration is to alter downstream signaling events for an extended period of time (protein expression of key signaling molecules is altered by 120 minutes after treatment (Figure 6), while gene expression (Figure 7) is altered for at least one week after treatment) compared to LPS. The prolonged, but decreased inflammatory response compared to LPS is ideally suited to provide the “second signal” for MDSC activation of bone marrow mobilized immature myeloid cells.

Discussion

Our data provides novel insight into the mechanisms underlying TLR4-Ab reversal of acute T1D in NOD mice. An increase in the peripheral MDSC population (mostly likely derived from bone marrow precursors), evidence of T-cell immunosuppression, and recapitulation of disease protection through adoptive transfer are consistent with published standards for MDSC identification and functional characterization (36). Protection from T1D by MDSCs has been previously shown, but not reversal of acute T1D as we show here (17; 18). Human patients with T1D show an increase in the peripheral population of MDSCs, but they are not maximally suppressive (19; 37). Whitfield et al (19) describe weak T-cell suppressive ability of this population that is lost with addition of GM-CSF and IL-1β in culture. These results indicate that cells of an MDSC phenotype are induced in response to T1D, but that full licensing of their functional suppressive capacity has not been achieved. Unlike in cancer and infectious diseases which induce a strong MDSC response, autoimmune diseases induce a weak MDSC response (38). The evidence would therefore suggest that in T1D an MDSC
response is suboptimal and that amplification of MDSC number and suppressive ability has therapeutic potential.

MDSCs develop in response to chronic inflammatory conditions and require two signals to become functionally immunosuppressive. A primary myelopoietic signal (such as GM-CSF, G-CSF, CSF-1, IL-6, Notch ligands, and adenosine) expands and mobilizes the MDSC-precursor population from the bone marrow, while chronic inflammatory mediators provide a second signal critical for MDSC maturation (16). Clinical conditions in which strongly immunosuppressive MDSC are generated include postoperative stress (39), burns (40), and stroke (41) and these conditions leave patients susceptible to infections and sepsis. A common thread between these diverse pathologies is the presence of overt tissue damage and cellular stress that can provide a second signal necessary to activate and maintain the MDSC population. In autoimmunity, however, disease progression is gradual. In T1D the destruction of β-cells is regulated through highly controlled apoptotic processes, and reduction of beta cell numbers occurs over a period of years (42). Our hypothesis is that despite significant insulitis and chronic inflammation within the islet, sufficient to provide the first, myelopoietic signal and increase peripheral populations of MDSCs, the islets lack sufficient damage-associated signals to activate their suppressive potential and long-term survival. This scenario is consistent with the findings of Whitfield et al (19).

Our molecular data establish TLR4-Ab as a weak/moderate TLR4 agonist with a unique ability to induce sustained activation and sequestration of the receptor. The cellular response
is strong enough to result in cytokine production that is sustained for at least seven days (10), altered gene expression in immune cells for at least 7 days (Figure 7), and TLR4 sequestered in the early endosome for at least 24 hours (Figure 8). TLR4 is a unique innate immune receptor that has both inflammatory and tolerogenic functions; it can unleash a lethal cytokine storm during sepsis or can induce tolerance to protect against autoimmunity. The accessory molecules CD14 and MD-2 partly determine which response predominates; they aid in the recognition of LPS as well as influence two signaling pathway options, the classical MyD88/NFκB pathway or the alternative TRIF/IRF3 pathway. CD14 binds to LPS/TLR4 and brings them into lipid rafts where TIRAP/MyD88 signal via NFκB and initiate inflammation. MD2 binds LPS and dimerizes TLR4 to initiate endocytosis. In the endosome, the alternative TRAM/TRIF signaling pathway can activate the IRF3 transcription factor to initiate a Type 1 IFN response (34; 43). In the NOD T1D mouse model, deletion of MyD88 is protective, but only if TRIF is functional and the mice have a microbial community in the gut. If the mice are germ-free or if TRIF is knocked out the disease course is unchanged, implying activation of the alternative pathway is critical for protection (44-46). There is evidence that our TLR4-Ab binds to a region close to the LPS activation site (21) and we show here internalization and sequestration of the TLR4-Ab complex within the early endosomes. Our data shows not only trafficking of TLR4-Ab to the early endosome but prolonged endosome sequestration compared to LPS, suggesting enhancement of the alternative pathway. Taken together, our results suggest that TLR4-Ab is mimicking a DAMP-mediated tissue damage response, by sequestration in the endosome that provides prolonged second signals necessary for MDSC activation/generation.
We propose that cell surface binding of TLR4-Ab increases mobilization of bone marrow-derived myeloid cells (“signal one” in MDSC generation), and that prolonged sequestration of TLR4-Ab in myeloid cells (both systemically and in resident myeloid cells in the islet) provides the “second signal” for these cells to become differentiated/activated MDSCs. Furthermore, we have observed that acutely diabetic mice are most effectively treated when they receive at least two doses of TLR4-Ab. The initial TLR4-Ab dose may increase mobilization of immature MDSCs, while subsequent doses prolong endosomal sequestration and enhance generation and maintenance of mature, suppressive MDSCs which promote diabetogenic T cell suppression. TLR4-Ab would therefore be critical for maintaining this population of MDSCs, rather than simply initiating a transient recruitment of suppressor cells.

TLR4-Ab is attractive as a potential therapeutic as it reverses acute T1D even in advanced disease (10). Part of the therapeutic efficacy is likely the long half-life of the antibody that can perpetuate the second signal long enough to generate a substantial population of MDSCs. The RNAseq studies of purified CD11b+ cells treated with TLR4-Ab provide strong unbiased evidence that these cells are bone marrow derived. In addition, genes upregulated by TLR4-Ab involve both myeloid/granulocytic and monocyte associated gene subsets and pathways and are associated with genes upregulated in myeloid processes such as Myeloid Leukemia and Sepsis. Of note, the gene families associated with the upregulated genes include several novel families such as Kinesins, Flotillins and Formyl Peptide receptors. Understanding the role of such genes may provide novel mechanisms of MDSC action, which we will pursue in future studies. A limitation of our study is that we do not study mechanisms of TLR4-Ab in NOD APCS compared to non-diabetic strains (such as B6) or compared to congenic NOD mice protected from T1D. NOD macrophages are known to differ from other strains in
important ways that could affect TLR4 receptor processing and retention in the endosome; some of these mechanisms might contribute to the MDSC formation and T1D reversals seen here (47-49). These issues will need to be addressed in future studies. Overall, these studies have greatly enhanced our understanding of TLR4-Ab’s role in T1D reversal and identified a novel role for MDSCs in reinstating immune tolerance during acute T1D. Remedying the aberrant adaptive response by initiating profound changes to the underlying innate immune response is a promising approach for treating acute T1D and possibly other autoimmune diseases.

Author contributions

KL, KK, YW, KB, DA, CP, HT, LH, BA, performed experiments, analyzed data, made figures, wrote up results. AH, WMR: planned experiments, analyzed data, wrote paper.

Acknowledgements

This work was supported by NIH 1R21AI120084-01A1 (WMR).

WMR is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors declare no competing financial interests.
References

1. Lee KU, Kim MK, Amano K, Pak CY, Jaworski MA, Mehta JG, Yoon JW: Preferential infiltration of macrophages during early stages of insulinitis in diabetes-prone BB rats. Diabetes 1988;37:1053-1058
2. Hanenberg H, Kolb-Bachofen V, Kantwerk-Funke G, Kolb H: Macrophage infiltration precedes and is a prerequisite for lymphocytic insulinitis in pancreatic islets of pre-diabetic BB rats. Diabetologia 1989;32:126-134
3. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA: Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulinis and beta-cell destruction in NOD mice. Diabetes 1994;43:667-675
4. Dahlén E, Dawe K, Ohlsson L, Hedlund G: Dendritic cells and macrophages are the first and major producers of TNF-alpha in pancreatic islets in the nonobese diabetic mouse. J Immunol 1998;160:3585-3593
5. Graham KL, Sutherland RM, Manerring SI, Zhao Y, Chee J, Krishnamurthy B, Thomas HE, Lew AM, Kay TW: Pathogenic mechanisms in type 1 diabetes: the islet is both target and driver of disease. Rev Diabet Stud 2012;9:148-168
6. Green EA, Eynon EE, Flavell RA: Local expression of TNFalpha in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. Immunity 1998;9:733-743
7. Sen P, Bhattacharyya S, Wallet M, Wong CP, Poligone B, Sen M, Baldwin AS, Tisch R: NF-kB Hyperactivation Has Differential Effects on the APC Function of Nonobese Diabetic Mouse Macrophages. The Journal of Immunology 2003;170:1770-1780
8. Lee LF, Xu B, Miche SA, Beilhack GF, Warganich T, Turley S, McDevitt HO: The role of TNF-alpha in the pathogenesis of type 1 diabetes in the nonobese diabetic mouse: analysis of dendritic cell maturation. Proc Natl Acad Sci U S A 2005;102:15995-16000
9. Burrrack AL, Martinov T, Fife BT: T Cell-Mediated Beta Cell Destruction: Autoimmunity and Alloimmunity in the Context of Type 1 Diabetes. Front Endocrinol (Lausanne) 2017;8:343
10. Bednar KJ, Tsukamoto H, Kachapati K, Ohta S, Wu Y, Katz JD, Ascherman DP, Ridgway WM: Reversal of New-Onset Type 1 Diabetes With an Agonistic TLR4/MD-2 Monoclonal Antibody. Diabetes 2015;64:3614-3626
11. Itoh A, Ridgway WM: Targeting innate immunity to downmodulate adaptive immunity and reverse type 1 diabetes. Immunotargets Ther 2017;6:31-38
12. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, Makita K, Kincade PW: Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. Immunity 2006;24:801-812
13. Bunt SK, Clements VK, Hanson EM, Sinha P, Ostrand-Rosenberg S: Inflammation enhances myeloid-derived suppressor cell cross-talk by signaling through Toll-like receptor 4. J Leukoc Biol 2009;85:996-1004
14. Lei J, Wang Z, Hui D, Yu W, Zhou D, Xia W, Chen C, Zhang Q, Wang Z, Zhang Q, Xiang AP: Ligation of TLR2 and TLR4 on murine bone marrow-derived mesenchymal stem cells triggers differential effects on their immunosuppressive activity. Cell Immunol 2011;271:147-156
15. Ray A, Chakraborty K, Ray P: Immunosuppressive MDSCs induced by TLR signaling during infection and role in resolution of inflammation. Front Cell Infect Microbiol 2013;3:52
16. Condamine T, Mastio J, Gabrilovich DI: Transcriptional regulation of myeloid-derived suppressor cells. J Leukoc Biol 2015;98:913-922
17. Yin B, Ma G, Yen CY, Zhou Z, Wang GX, Divino CM, Casares S, Chen SH, Yang WC, Pan PY: Myeloid-derived suppressor cells prevent type 1 diabetes in murine models. J Immunol 2010;185:5828-5834
18. Hu C, Du W, Zhang X, Wong FS, Wen L: The Role of Gr1+ Cells after Anti-CD20 Treatment in Type 1 Diabetes in Nonobese Diabetic Mice. The Journal of Immunology 2012;188:294-301
19. Whitfield-Larry F, Felton J, Buse J, Su MA: Myeloid-derived suppressor cells are increased in frequency but not maximally suppressive in peripheral blood of Type 1 Diabetes Mellitus patients. Clin Immunol 2014;153:156-164
20. Gabrilovich DI, Nagaraj S: Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162-174
21. Bahrun U, Kimoto M, Tsukamoto H, Tsuneyoshi N, Kohara J, Fukudome K: Preparation and characterization of agonistic monoclonal antibodies against Toll-like receptor 4-MD-2 complex. Hybridoma (Larchmt) 2007;26:393-399
22. Pakala SV, Chivetta M, Kelly CB, Katz JD: In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. J Exp Med 1999;189:1053-1062
23. Huang W, Rainbow DB, Wu Y, Adams D, Shivakumar P, Kottyan L, Karns R, Aronow B, Bezerra J, Gershwin ME, Peterson LB, Wicker LS, Ridgway WM: A Novel Pkhd1 Mutation Interacts with the Nonobese Diabetic Genetic Background To Cause Autoimmune Cholangitis. J Immunol 2018;200:147-162
24. Langmead B, Trapnell C, Pop M, Salzberg SL: Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology 2009;10:R25
25. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL: TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biology 2013;14:R36
26. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L: Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 2012;7:562-578
27. Chen J, Xu H, Aronow BJ, Jegga AG: Improved human disease candidate gene prioritization using mouse phenotype. BMC Bioinformatics 2007;8:392
28. Kaimal V, Bardes EE, Tabar SC, Jegga AG, Aronow BJ: ToppCluster: a multiple gene list feature analyzer for comparative enrichment clustering and network-based dissection of biological systems. Nucleic Acids Res 2010;38:W96-102
29. Schneider CA, Rasband WS, Eliceiri KW: NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671-675
30. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, Mandruzzato S, Murray PJ, Ochoa A, Ostrand-Rosenberg S, Rodriguez PC, Sica A, Umansky V, Vonderheide RH, Gabrilovich DI: Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nature Publishing Group, 2016, p. 1-10
31. Veglia F, Sanseviero E, Gabrilovich DI: Myeloid-derived suppressor cells in the era of increasing myeloid cell diversity. Nature Reviews Immunology 2021;
32. Baumann T, Dunkel A, Schmid C, Schmitt S, Hiltensperger M, Lohr K, Laketa V, Donakonda S, Ahting U, Lorenz-Depiereux B, Heil JE, Schredelseker J, Simeoni L, Fecher C, Körber N, Bauer T, Hüser N, Hartmann D, Laschinger M, Eyerich K, Eyerich S, Anton M, Streeter M, Wang T, Schraven B, Spiegel D, Assaad F, Misgeld T, Zischka H, Murray PJ, Heine A, Heinenwölder M, Korn T, Dawid C, Hofmann T, Knolle PA, Höchst B: Regulatory myeloid cells paralyze T cells through cell–cell transfer of the metabolite methylglyoxal. Nature Immunology 2020;21:555-566
33. Caramalho I, Rodrigues-Duarte L, Perez A, Zelenay S, Penha-Gonçalves C, Demengeot J: Regulatory T cells contribute to diabetes protection in lipopolysaccharide-treated non-obese diabetic mice. Scand J Immunol 2011;74:585-595
34. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R: TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol 2008;9:361-368
35. Rajaiah R, Perkins DJ, Ireland DDC, Vogel SN: CD14 dependence of TLR4 endocytosis and TRIF signaling displays ligand specificity and is dissociable in endotoxin tolerance. Proceedings of the National Academy of Sciences 2015;112:8391-8396
36. Bronte V, Brandau S, Chen S-H, Colombo MP, Frey AB, Greten TF, Mandruzzato S, Murray PJ, Ochoa A, Ostrand-Rosenberg S, Rodriguez PC, Sica A, Umansky V, Vonderheide RH, Gabrilovich DI: Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nature Communications 2016;7:12150
37. Hassan M, Raslan HM, Eldin HG, Mahmoud E, Elwajed HAA: CD33(+) HLA-DR(-) Myeloid-Derived Suppressor Cells Are Increased in Frequency in the Peripheral Blood of Type1 Diabetes Patients with Predominance of CD14(+) Subset. Open Access Maced J Med Sci 2018;6:303-309
38. Veglia F, Perego M, Gabrilovich D: Myeloid-derived suppressor cells coming of age. Nat Immunol 2018;19:108-119
39. Kimura F, Shimizu H, Yoshidome H, Ohtsuka M, Miyazaki M: Immunosuppression following surgical and traumatic injury. Surg Today 2010;40:793-808
40. Schwacha MG, Scroggins SR, Montgomery RK, Nicholson SE, Cap AP: Burn injury is associated with an infiltration of the wound site with myeloid-derived suppressor cells. Cell Immunol 2019;338:21-26
41. Liesz A, Dalpke A, Mracsko E, Antoine DJ, Roth S, Zhou W, Yang H, Na SY, Akhisaroglu M, Fleming T, Eigenbrod T, Nawroth PP, Tracey KJ, Veltkamp R: DAMP signaling is a key pathway inducing immune modulation after brain injury. Journal of Neuroscience 2015;35:583-598
42. Von Herrath M, Sanda S, Herold K: Type 1 diabetes as a relapsing–remitting disease? Nature Reviews Immunology 2007;7:988-994
43. Tan Y, Kagan JC: Microbe-inducible trafficking pathways that control Toll-like receptor signaling. Traffic 2017;18:6-17
44. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA, Gordon JJ, Chervonsky AV: Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 2008;455:1109-1113
45. Burrows MP, Volchkov P, Kobayashi KS, Chervonsky AV: Microbiota regulates type 1 diabetes through Toll-like receptors. Proceedings of the National Academy of Sciences 2015;112:9973-9977
46. Androulidaki A, Wachsmuth L, Polykratis A, Pasparakis M: Differential role of MyD88 and TRIF signaling in myeloid cells in the pathogenesis of autoimmune diabetes. PLOS ONE 2018;13:e0194048
47. Serreze DV, Gaedeke JW, Leiter EH: Hematopoietic stem-cell defects underlie abnormal macrophage development and maturation in NOD/Lt mice: defective regulation of cytokine receptors and protein kinase C. Proc Natl Acad Sci U S A 1993;90:9625-9629
48. Stoffels K, Overbergh L, Giulietti A, Kasran A, Bouillon R, Gysemans C, Mathieu C: NOD macrophages produce high levels of inflammatory cytokines upon encounter of apoptotic or necrotic cells. J Autoimmun 2004;23:9-15
49. Steptoe RJ, Ritchie JM, Harrison LC: Increased generation of dendritic cells from myeloid progenitors in autoimmune-prone nonobese diabetic mice. J Immunol 2002;168:5032-5041
Figure Legends

**Figure 1. TLR4-Ab reverses acute T1D in NOD mice.** NOD diabetic mice (BG > 200 mg/dl) were treated twice, one week apart, with either TLR4-Ab (left) or Ctrl-Ab (right). “Final” BG measurements were taken one week after the second treatment. Red data points represent mice who reached endpoint BG (>500 mg/dl) before the second treatment. In the TLR4-Ab treated group, 7/46 = 15% progressed to endstage T1D and n=3 mice (red diamonds) became diabetic before the second dose. In the Ctrl-Ab treated group, 21/40 = 53% progressed to endstage diabetes at the 2-week timepoint; and n= 11 mice became diabetic before the second dose (red diamonds). P value calculated for the final BG between TLR4- and Ctrl-Ab treated groups.

**Figure 2. TLR4-Ab treatment in vivo increases CD11b+ MDSCs in the spleen and islets of NOD mice.** NOD mice were treated twice with TLR-4 Ab, Ctrl-Ab or PBS and splenocytes were harvested 1 day after the second treatment. **A:** Representative FACS gating for PBS vs. TLR-4 Ab vs. Ctrl-Ab treated splenocytes. **B:** Summary total numbers and percentages in indicated subsets for PBS (n=2), Ctrl-Ab (n=4) and TLR4-Ab (n=3) treated mice. Definitions of gating subsets as shown in “A”, with monocytic MDSC defined as (CD11b+, Ly6C<sup>hi</sup>, Ly6g+), and granulocytic MDSCs defined as (CD11b+, Ly6g+, Ly6c<sup>lo</sup>). *** p < 0.0001, ** p < .001 * P <.02. **C:** Representative plots of CD11b+ cells in diabetic mice treated with PBS or TLR4-Ab. Spleen (top) and intra-islet hematopoetic cells (bottom). **D:** CD11b+ cell percentages and numbers in spleen and islets one week after the second TLR4-Ab treatment of diabetic mice. n=4 mice in each group; cells harvested one week after second treatment. Unpaired t test comparing TLR4-Ab treatment to Ctrl-Ab (Figure 2B) or to PBS (Figure 2D) was used to determine statistical significance.

**Figure 3. RNAseq data from TLR4-Ab-treated mice show upregulation of genes associated with CD11b+Ly6G+ cells and downregulation of genes associated with CD4+ T cells.** NOD mice were treated at onset of T1D with either TLR4-Ab or Crtl-Ab, or were untreated. One week after the second treatment (or at the diagnosis of T1D, untreated group) splenocytes were taken, processed and analyzed by RNAseq. **A:** Heat map of gene expression between the 3 groups. **B**) Left side: The group of 10-fold differentially upregulated genes (n=32 genes) from TLR4-Ab treated mice compared to Ctrl-Ab treated mice was used to build
a cluster enrichment network based on biological processes, molecular functions, mouse knockout phenotypes and biological pathways. The red line highlights cell ontologies significantly associated with the differentially upregulated genes and indicates significant association with CD11b+ cells. Right side: differentially downregulated genes from TLR4-Ab treated mice (n=12 genes) compared to Ctrl-Ab treated mice were used to build a cluster enrichment network. The red line highlights T cell ontologies significantly associated with the downregulated genes.

Figure 4: CD11b+ cells sorted from TLR4-Ab-treated mice upregulate genes associated with bone marrow derived CD11b+ cells, and CD11b+GR1+ MDSCs suppress T cell proliferation ex vivo. CD11b+ splenocytes were FACS sorted from diabetic mice one week after the second treatment with either TLR4-Ab or Ctrl-Ab (n=3 mice per group). The cells were processed and analyzed by RNAseq as in Figure 3. A. Heat map of 261 significantly upregulated genes in TLR4-Ab treated CD11b+ cells compared to Ctrl-Ab treated. B. The genes from “A” were used to build a cluster enrichment network showing statistically significant associations between the various gene classification groups (molecular function, biological processes, cellular components, mouse phenotypes, gene families, Co-expression atlas, etc) and the upregulated genes. The gene classification categories are labelled with circular icons, and color coded. C. Representative MDSC suppression assay using sorted CD11bGr1+ cells from NOD mice treated with 10 µg TLR4-Ab vs Ctrl-Ab one week prior. Cells were first purified with MAC for CD11b+ cells, then sorted for CD11b+ Gr1+ cells. 100,000 CD4+T cells (purified using MAC columns and stained with CTV) and 50k sorted cells were used. Blue line = Ctrl-Ab, orange = TLR4-Ab, red= unstimulated cells. On the left are CD11b+Gr1+ sorted cells, on the right are CD11b+Gr1- sorted cells from the same mice. Each side used the same CD4+ cell donor. D. Mean + SEM of 5 suppression experiments using sorted CD11b+Gr1+ cells as in A.

Figure 5: CD11b+ cells generated from TLR4-Ab-treated bone marrow ameliorate progression of acute T1D in vivo. A. Bone marrow cells were prepared from NOD femur and tibia and cultured in the presence of either TLR-4 Ab (250ng/ml) or Ctrl-Ab (250ng/ml) for 5-7 days. Cells were assayed by FACS for CD11b+ Ly6g+ and Ly6c+ MDSCs. B. Bone marrow-derived cells were prepared as in A, then removed using trypsin EDTA and 2x10^6
cells were transferred into diabetic recipient mice. N=11 mice in both groups from 5 separate experiments. P value by Log-rank test and Kapler-Meier statistics. C. Initial and final mean BG in TLR4-Ab vs. Ctrl-Ab bone marrow-derived cell transfer groups. Final BG= 263 (TLR-4 group) vs 410 (Ctrl-Ab group); 4/11 TLR-4 Ab-treated mice were end stage diabetic (BG > 200) at endpoint vs. 10/11 Ctrl-Ab-treated mice. P value by unpaired T test.

Figure 6: TLR4-Ab upregulates expression of inhibitory IκB and demonstrates an intermediate activation of MAPKs as compared to pro-inflammatory LPS. RAW 264.7 cells were stimulated with LPS (100ng) or TLR4-Ab (100ng) and lysed at fixed intervals between 0-120 min. A: Lysates were tested by western blot for expression of activated TLR4 signaling mediators P-JNK, P-p38, P-ERK, and IκB. B: Densitometry analysis of protein bands from three individual experiments was performed using Image J, and fold change was calculated based on basal expression levels at time 0. P values calculated in Prism GraphPad: *p<0.05, **p<0.01, *** p <0.001, **** p<0.0001

Figure 7: Broad downregulation of inflammatory genes in NOD splenocytes treated with TLR4-Ab compared to LPS. NOD mice were treated with TLR4-Ab, LPS, or PBS. One week later splenocytes were harvested, RNA was isolated and converted to cDNA, and PCR array was performed using the RT2 Profiler PCR ARRAY Mouse Innate & Adaptive Immune Responses kit according to the manufacturer’s instructions. Fold Change was calculated as the ratio of experimental to control (delta CT) calculated using the Actb Housekeeping gene and the mean of the PBS control values. P values were calculated using unpaired t test in Prism GraphPad. *p<0.05, ** p<0.01, *** p<0.001, # p<0.15.

Figure 8: TLR4-Ab remains co-localized with EEA1 and sequesters TLR4/MD2 inside the endosome for at least 24 hours. BMDMs were pulsed with LPS (100ng) or TLR4-Ab (UT18; 100ng) for 5 minutes, washed, and then fixed at the noted time points. Cells were stained (CD11b, TLR4/UT18, EEA1) for imaging flow cytometry and 5000 events (gated on CD11b+ cells) were collected for each cell population. Representative images for A: LPS and B: TLR4-Ab stimulated BMDMs are shown for each time point. C: Internalization analysis generated using the IDEAS software provides a mean internalization erode score using CD11b as the surface marker and TLR4/UT18 as the internalization probe. TLR4 internalization scores greater than 1.0 indicate internalization of TLR4. P values calculated
in Prism GraphPad: * p<.05, **** p<0.0001. D: Bright detail similarity (BDS, median) measures pixel-by-pixel correlation between TLR4 and EEA1 in each image, effectively measuring co-localization of the two probes at each denoted time point. BDS greater than 1.0 indicates co-localization of TLR4 and EEA1. * p=0.014, ** p=0.0067. The quantification of (C) and (D) represent the data collected from 3 independent experiments.
Figure One

**TLR4-Ab Tx**

*P < 0.0001

**Ctrl-Ab Tx**
Figure Two

A. PBS

Ctrl-Ab

TLR-4 Ab

CD11b

Ly6C

Ly6G

FSc

B. Cell number (x10^6)

PBS

Ctrl-Ab

TLR4-Ab

CD11b

Monocyotic MDSC

Granulocytic MDSC

Macrophages

PMNs

Spleen

IHC

C. PBS

TLR-4 Ab

Spleen

IHC

D. Percentage CD11b+

Spleen

Islet

PMNs

Macrophages

Granulocytic MDSC

Monocyotic MDSC

**P= 0.027

**P= 0.01

***P= 0.016

*P= 0.01

*P= 0.14

Cell percentage

Cell number (x10^6)
Figure 4

A. Heatmap showing gene expression levels.

B. Gene co-expression network.

C. Flow cytometry analysis of CD11b+Gr1+ and CD11b+Gr1- cells.

D. Bar graph showing CTV dilution with statistical significance (P = 0.038).
Figure Five

A. Ctrl-Ab

B. TLR4-Ab

C. Percent not end-stage diabetes

*P = .05

For Peer Review Only

Diabetes

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/doi/10.2337/db21-0426/638275/db210426.pdf by guest on 19 January 2022
Figure Six

A.

| TLR4-Ab | LPS |
|---------|-----|
| 0'      | 0'  |
| 5'      | 5'  |
| 10'     | 10' |
| 15'     | 15' |
| 30'     | 30' |
| 45'     | 45' |
| 60'     | 60' |
| 90'     | 90' |
| 120'    | 120'|

- P-JNK
- P-p38
- P-ERK
- IkBa
- GAPDH

B.

- P-JNK/SAPK
- P-p38
- P-ERK
- IkBa
- GAPDH

*** LPS  **** TLR4-Ab

For Peer Review Only
Figure Seven

Surface Molecules

- CD40
- CD80
- CD86
- IFN-αR1
- CCR6
- ICAM1
- Ly2

Signaling Molecules

- Tyk2
- Jak2
- IRAK1
- ERK2
- NF-κB
- STAT3
- STAT4

Cytokines / Chemokines

- IL-18
- IL-1β
- Capase 1
- CCL5
- CXCL10
- CSF2

Fold Change

- TLR4-Ab
- LPS

For Peer Review Only
Figure Eight

A. LPS Stimulated

B. TLR4-Ab Stimulated

C. TLR4 Internalization

D. EEA1 Co-localization
Supplementary Figure 1. Genes associated with BM derived CD11b+ cells by gene ontology. This figure was derived as in Figure 4. Genes directly associated with BM derived CD11b+ cells in the co-expression atlas category are shown highlighted in yellow, linked directly to the 5 co-expression categories (also in yellow).
Supplementary Figure 2. Rapid internalization of TLR4 in response to LPS and subsequent return to the cell surface but no internalization in Ctrl-Ab treated cells. HEK-Blue cells (Invivogen) expressing TLR4/MD2/CD14 were pulsed with LPS (100ng), or Ctrl-Ab (100ng) for 5 min, washed and then fixed at the noted time points. Cells were stained (CD14, TLR4/SA15-21, EEA1) for imaging flow cytometry and 10,000 events were collected for each population. Representative images for (A) LPS- and (B)Ctrl-Ab-stimulated HEKBlue cells are shown for each time point.
Supplementary Table 1. Differentially expressed genes and gene ontologies (GOs) from Figure 3.

| Upregulated genes | Downregulated Genes |
|-------------------|---------------------|
| CEBPE             | LYPD6B              |
| TINAGL1           | ALS2CL              |
| ANKRD22           | TDRP                |
| GPR84             | TMIE                |
| CHI3L1            | ALPK1               |
| CD177             | RAMP3               |
| SLC4A4C1          | VIPR1               |
| OLFM4             | LEF1                |
| C15orf48          | RYR3                |
| LTF               | CNGA1               |
| LCN2              | ADH1C               |
| PRTN3             | AMPD1               |
| ICA1              |                     |
| OLR1              |                     |
| MS4A3             |                     |
| MAPK13            |                     |
| S100A8            |                     |
| ANXA1             |                     |
| CTSG              |                     |
| FPR2              |                     |
| PADI4             |                     |
| CDKN3             |                     |
| ELANE             |                     |
| UPP1              |                     |
| ANXA3             |                     |
| CAMP              |                     |
| ABCA13            |                     |
| RHOU              |                     |
| ORM1              |                     |
| CHIT1             |                     |
| 5-Sep             |                     |
| S100A9            |                     |
## Upregulated GOs

- **RAGE receptor binding**
- **neutrophil aggregation**
- **Myeloid Cells, GN.Arth.SynF, CD11b+ Ly6-G+, Synovial Fluid**
- abnormal innate immunity
- **B cells, MLP.BM, CD19- Igm- CD43+ CD24- AA4.1+ CD45R-**
- **Myeloid Cells, MF.BM, B220neg CD3neg Ly-6C/Glo CD115int**
- Neutrophil-specific genes up-regulated
- **B cells, MLP.FL, CD19- IgM- CD43+ CD24intermediate**
- abnormal immune cell physiology
- cytokine production
- altered susceptibility to bacterial infection
- **phagocytosis**
- **Genes down-regulated in CD133+**
- **Myeloid Cells, GN.Thio.PC, CD11b+ Ly6-G+, Peritoneal Cavity**
- **Genes up-regulated in bone marrow-derived macrophages**
- **endocytosis**
- **Network of differentially expressed myeloid genes.**
- neutrophil mediated immunity
- abnormal cell-mediated immunity
- **phospholipase A2 inhibitor activity**
- Genes significantly up-regulated in the blood mononuclear cells
- **intracellular ligand-gated ion channel activity**
- **Genes up-regulated in monocyte-derived dendritic cells**
- **Genes up-regulated in comparison of CD4 T**
- defense response to other organism
- defense response to bacterium
- **Genes down-regulated in monocyte-derived dendritic cell ...**
- Toll-like receptor 4 binding
- **arachidonic acid binding**
- abnormal neutrophil physiology
- **leukocyte migration**
- **Myeloid Cells, MF.Sbcaps.SLN, CD11b+ CD169+ F4/80-**
- response to bacterium
- leukocyte migration involved in inflammatory response
- **inflammatory response**
- abnormal adaptive immunity
- cytoplasmic, membrane-bounded vesicle
- abnormal hematopoietic system physiology
- **chitin binding**
- **Myeloid Cells, Mo.6C+II-.BM, B220neg CD3neg CD115+ Ly-6**
- **B cells, B.Pl.AA4-.BM, CD138+ AA4.1- CD43+, Bone marrow**
- icosanoid binding
- **Genes up-regulated in T cells: CD4**
- abnormal leukocyte physiology
- secretory vesicle
- **Up-regulated genes in myeloid progenitors immortalized**
- **Myeloid Cells, GN.Arth.BM, CD11b+ Ly6-G+, Bone marrow**
- abnormal response to infection
- abnormal granulocyte physiology
- **B cells, proB.CLP.BM, CD19- IgM- CD43+ CD24- AA4.1+ CD4**
- **Genes up-regulated in NKT cells versus CD8A.**
- **Genes up-regulated in CD8 T cells: central memory**
- interleukin-8 production

## Downregulated GOs

- **Genes up-regulated in CD4 T conv over-expressing GATA1**
- alpha beta T cells, T.8Nve.Sp, 4- 8+ 25- 62Lhi 44lo, 5p
- **Genes down-regulated in comparison of regulatory T cell**
- **Genes up-regulated in spleen B lymphocytes versus bone**
- **Genes down-regulated in CD4 T conv over-expressing IKZF**
- alpha beta T cells, T.4Pa.BDC, 4+ 8- BDC+, Pancreas, avg-2
- **Genes down-regulated in comparison of thymus regulatory**
- **Genes down-regulated in comparison of TregLP versus Tconv**
- **Genes down-regulated in comparison of lymph node**
- **alpha beta T cells, T.4Nve.MLN, 4+ 8- 25- 62Lhi 44lo,**
- **CD positive, CD4 Control, 4+8-B220-, Spleen,**
- **Genes up-regulated in dendritic cells stimulated by LPS**
- **Genes down-regulated in CXCR5+ BCL16+**
- **Genes down-regulated in comparison of regulatory T cell**
- alpha beta T cells, T.4Nve.MLN, TCRb CD44high CD122lo CD
- CD positive, CD4 Control, 4+8-B220-, Spleen,
- Cluster P4 of genes with similar expression profiles
- **Genes down-regulated in comparison of TregCD103-Klrg1**
- **Genes down-regulated in CD4 [GeneID=920] T conv**
- **Genes up-regulated in CD4 [GeneID=920] versus granulocytes**
- **Genes down-regulated in comparison of Treg**
- **Genes up-regulated in bone marrow-derived macrophages**
- **Genes up-regulated in comparison of TconvLN versus Treg**
- alpha beta T cells, T.8Nve.MLN, 4- 8+ 25- 62Lhi 44lo,**
- alpha beta T cells, T.4.PLN.BDC, 4+ 8- BDC+, Lymph Node