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Peritoneal cavity is a route for gut-derived microbial signals to promote autoimmunity in NOD mice

Running Title: Peritoneal cavity and autoimmunity in NOD mice.

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Abstract

Macrophages play a crucial role in innate immune reactions, and Peritoneal Macrophages (PMs) guard the sterility of this compartment mainly against microbial threat from the gut. Type-1 Diabetes (T1D) is an autoimmune disease in which gut microbiota and gut immune system appear to contribute to disease pathogenesis. We have recently reported elevated free radical production and increased permeability of gut epithelium in non-obese diabetic (NOD) mice. Impaired barrier function could lead to bacterial leakage to the peritoneal cavity. To explore the consequences of impaired gut barrier function on extra-intestinal immune regulation, we characterized peritoneal lavage cells from young newly weaned NOD mice. We detected a rapid increase in the number of macrophages 1-2 weeks after weaning in NOD mice compared to C57BL/6 and BALB/c mice. Interestingly, this increase in macrophages was abrogated in NOD mice that were fed an anti-diabetogenic diet (ProSobee), which improves gut barrier function. Macrophages in young (5 week old) NOD mice displayed a poor TNF-α cytokine response to LPS stimulation, and high expression of Toll-like Receptor (TLR) signalling pathway negative regulator, Interleukin-1 Associated Kinase–M (IRAK-M), indicating prior *in vivo* exposure to TLR-4 ligand(s). Furthermore, injection of LPS intraperitoneally increased T-cell CD69 expression in pancreatic lymph node (PaLN), suggestive of T-cell activation. Leakage of bacterial components such as endotoxins into the peritoneal cavity may contribute to auto-reactive T-cell activation in the PaLN.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease, in which multiple factors contribute to the destruction of insulin producing β cells in the pancreas [1]. Several recent reports have highlighted the important role of diet and microbes [2, 3] in the early immunological events in T1D development. T1D is primarily mediated by T cells and B cells in the NOD mouse model [4-7].

However, innate immune cells including macrophages, dendritic cells, natural killer cells and natural killer T-cells also influence this disease. These cells are responsible for the primary immune response against invading pathogens and help safeguard the host from infections caused by these pathogens [8]. Macrophages are multifunctional; depending on the stimuli from their surroundings, naïve macrophages differentiate into pro-inflammatory or anti-inflammatory macrophages [9], which in turn also secrete pro- and anti-inflammatory cytokines. These cytokines influence the differentiation of T cells into Th1, Th2, Th17 or T regulatory cells [10-12]. T-cell differentiation also plays an important role in regulating glucose and lipid metabolism and tissue inflammation [13]. Macrophages in diabetes-prone experimental models are less efficient at clearing apoptotic cells compared to non-diabetes-prone models [14, 15]. Following continuous exposure to LPS, macrophages produce low levels of TNF-αi.e. become refractory to stimulation with LPS. Interleukin-1 receptor associated kinase-M (IRAK-M) is a negative regulator of TLR-signalling. It is induced by sustained TLR-signalling in monocytes and macrophages and is an important negative regulator of TLR-signalling [16-18].

We have recently observed increased production of reactive oxygen (ROS) species as a sign of inflammatory activity in the small intestine of young NOD mice in parallel with increased permeability of the gut [19]. To evaluate potential extra-intestinal manifestations of increased permeability and inflammation in the gut, we have investigated peritoneal lavage cells from NOD mice. We demonstrate that the number of macrophages in the peritoneal cavity increases rapidly in NOD mice after weaning, and that these macrophages show phenotypic characteristics of macrophages exposed to TLR-ligands *in vivo*. We also show enhanced plasma levels of LPS-binding protein suggesting translocation of gut microbes or their products into the systemic circulation, and activation of interferon-γ production in CD4 and CD8 T cells in pancreatic lymph node in response to peritoneal LPS challenge.

Methods

Mice

NOD mice (NOD/Mrk Tac, Taconic, USA) were bred and maintained under SPF conditions in the local animal facility of Turku University. For intracellular cytokine stainings, NOD/Shiltj mice (Jackson laboratories) were used. Age-matched C57BL/6 and BALB/c mice were used as healthy controls. All experiments were subjected to rules of the National Board of Animal Experimentation in Finland and performed under license ESLH-2009-02371/Ym-23. The Principles of Laboratory Animal Care (http://grants1.nih.gov/grants/olaw/ references/phspol.htm) were followed. NOD mice were either kept on normal chow diet (SDS, Witham, UK), or fed with infant formula for the whole study period (ProSobee, Mead Johnson Nutrition, Glenview, IL, USA). These two groups are hereafter referred to as 'NOD' and 'PNOD' mice, respectively.

Immune cell isolation

Peritoneal cells were isolated by flushing the peritoneum with 5ml ice-cold RPMI-1640 medium (Gibco, Grand Island, NY, USA) which was drawn back into the same syringe. Total number of cells retrieved was determined by calculating the concentration of cells/ml in the lavage and multiplying this with the distribution volume (5 ml injected). Single cell suspensions of pancreatic lymph node samples were acquired by pressing the isolated lymph nodes gently through a metal sieve.

Flow cytometry

Peritoneal cells were counted and analysed by flow cytometry after incubating the peritoneal cells with anti-mouse allophycocyanin- (APC) conjugated F4/80 (Biolegend) and FITC-conjugated CD11b (Immunotools, Friesoythe, Germany) antibodies. Surface expression of activation markers

was determined by incubating the cells with FITC-conjugated CD40 (Biolegend) or CD80 (eBioscience, CA, USA) and Phycoerythrin- (PE) conjugated CD86 (eBioscience).

Expression of adhesion molecule α4 integrin on peritoneal macrophages was analysed using FITC-conjugated anti-CD11b (Immunotools), allophycocyanin-conjugated anti-B220 antibody (Caltag Laboratories) (to exclude CD11b+ B-cells from the analysis) and PE-conjugated anti-integrin α4 antibody (AbD Serotec, Oxford, UK). CD69 activation marker was evaluated from pancreatic lymph node T and B cells by incubating the cells with FITC-conjugated CD4 (eBioscience) or CD8 (Immunotools) or APC-conjugated B220 (Biolegend) antibodies along with PE-conjugated CD69 (BD Bioscience). Cells were run for flow cytometry using FACS calibur (BD biosciences, New Jersey, USA) and analyzed using cell Quest (BD) software. In experiments where macrophages were used for functional (ELISA, qPCR and Western blotting) assays, peritoneal macrophages were sorted using FACS ARIA II flow cytometer (BD biosciences). The purity of macrophages used for functional assays was ≥96%.

For intracellular IFN-γ staining, lymphocytes from Pancreatic Lymph node (PaLN) were incubated in complete RPMI 1640 (supplemented with streptomycin and 10% FCS) with Cell Activation Cocktail (Biolegend) for 4 hr at 37 ºC in 5% CO_2. Stimulated cells were then washed and surface stained for 15 min in the dark at 4 ºC with FITC conjugated anti CD4 and allophycocyanin conjugated anti CD8 in FACS buffer. Non-specific binding was blocked with rat serum. The cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.5% saponin (Merck). For intracellular staining, PE conjugated anti IFN-γ and its isotype control were used (all staining antibodies were from Biolegend). Results were analyzed by BD Accuri C6 flow cytometer.

In *vitro* LPS stimulation of peritoneal macrophages

Macrophages purified by cell-sorting were plated on 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) at 200,000 cells / well in complete medium (RPMI 1640, 10% FCS, L-glutamine 2mmol/l, 100 U/ml penicillin and streptomycin). Cells were stimulated with 0.2, and 5.0 µg/ml LPS from Escherichia coli (Sigma-Aldrich, ST Louis, MO, USA), and the cell culture supernatants were collected after 48 hours and analysed for TNF-α using Luminex platform, and for IL-10 and IL-12 using a flow-cytometric bead assay (BD biosciences) kit. Samples were run using BD accuri (BD biosciences) and the analysis was performed using BD C6 Accuri software.

Western Blotting

Sorted macrophages were lysed using lysis buffer (50mM tris-HCL, pH 7.5, 150mM NaCl, 0.5% TX -100, 5% glycerol, 1% SDS, 1mM Na3v04, 10Mm NaF, and 1mM PMSF). Protein concentrations were measured using DC Protein Assay (Bio-Rad, USA) and 6xSDS sample buffer (0.5M Tris-HCl pH6.8, 28% glycerol, 9% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue) was added. 10% SDS-page gels were used to separate the proteins, which were then transferred to PVDF membrane. The membrane was incubated overnight at $+4^0$C with primary antibody raised in rabbit specific for IRAK-M (upstate, Merk Millipore, Billercia, Massachusetts), followed by HRP conjugated goat anti rabbit (HyTest Ltd, Turku, Finland) secondary antibody. Pierce developing solution (Pierce) was used for detection.

Plasma measurements of LPS

Blood samples were isolated aseptically by cardiac puncture and collected into tubes (Sarstedt, Karl-Schiller-Str, Germany) and centrifuged at 1500 RMP for 10 min on a bench-top centrifuge

(Thermo scientific, Waltham, MA, USA). Plasma samples were collected into sterile eppendorf -tubes and stored at -80 C until analysis. The samples were analysed for LPS by Enzyme linked Immunosorbent assay (LAL chromogenic Endpoint assay kit, Hycult biotech, Netherland) according to manufacturer's recommendations.

In vivo responses to LPS stimulation

To determine the responsiveness of peritoneal macrophages to LPS-stimulation age- and sex-matched NOD, BALB/c and C57BL/6 mice were injected *i.p,* with 10µg LPS from Escherichia coli (Sigma-Aldrich, ST Louis, MO, USA) in PBS buffer. The animals were sacrificed after 6 hours and PMs were collected by peritoneal lavage as described above. PMs were processed for flow cytometry and analysed for α4-integrin expression. To evaluate if LPS in peritoneal cavity affects T-cell activation in pancreatic lymph node, mice were injected with 30µg of LPS. Animals were sacrificed after 24 hours or 5 days and PaLN T cells were analysed for CD69 and intracellular IFN-γ expression, respectively.

Statistical analyses

Statistical analyses between groups were performed using unpaired Student's t-test with Welch correction or 1-way ANOVA and Bonferroni's multiple comparisons between the groups. Statistical significances are indicated with asterisk symbols: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Results

Macrophage numbers increase sharply in the peritoneal cavity of young NOD mice.

The total number of peritoneal macrophages (PMs) was analysed in NOD mice from peritoneal lavage specimens at different age groups as explained in Materials and Methods section. Age-

matched C57BL/6 and BALB/c mice were used as healthy control strains. No differences were observed at the age of 3 weeks. In NOD mice, a rapid increase between 3 and 5 weeks in peritoneal macrophages was detected compared with ProSobee-fed NOD mice and control strains (Figure 1). This dramatic increase in macrophages soon after weaning to normal chow (NC) diet but not to ProSobee diet suggests that NOD mice develop a sort of innate immune response extraintestinally. This correlates with our previously published data, showing improved intestinal homeostasis and gut barrier function in NOD mice fed anti-diabetogenic diets [19, 20].

Peritoneal macrophages in NOD mice display high levels of activation markers after weaning. We analysed the expression of activation markers CD40, CD80, and CD86 on PM to evaluate whether the increase in their numbers correlates with increased activation, indicative of an inflammatory response. We found that PM in NOD mice showed higher expression of CD40 and CD80 at the age of 3 weeks in comparison to control strain (Figure 2a, b, c), while the expression of CD40 and CD86 was significantly lower at 5 weeks age in comparison to control strain. In NOD mice, expression of both CD80 and CD86 was lower at 5 weeks compared to 3 weeks age. Interestingly, NOD mice weaned to antidiabetogenic diet showed less compromised expression of CD40, CD80 and CD86 at 5 weeks age (Figure 2 d, e, and f).

Peritoneal macrophages in NOD mice develop IRAK-M expression and become desensitized to LPS-stimulation.

IRAK-M protein expression analysis in PMs from 3 and 5 week old NOD, C57BL/6 and BALB/c mice demonstrated that the expression of IRAK-M is either very low or undetectable at the age of 3 weeks in all mouse strains. At 5 weeks, NOD mice but not the other mouse strains displayed a clear increase in expression of IRAK-M, the negative regulator of TLR-signalling (Figure 3a). To further

test if PM in NOD mice become tolerant to LPS between the time of weaning at 3 weeks and the age of 5 weeks, we cultured purified PMs from 3 and 5 week old NOD mice *in vitro* with or without addition of LPS. Supernatants from the cultures were collected and analysed for TNF-α and IL-10 and IL-12 secretion. At 3 weeks age NOD PM responded to a low and high concentration of LPS by 3- and 2.5-fold increase in secreted TNF-α, respectively. Interestingly, at 5 weeks age NOD PM responded to the same LPS concentrations only with 1.3-fold or no stimulation, respectively (Figure 3b). IL-10 levels increased similarly in response to LPS-stimulation both at 3 and 5 weeks age (Figure 3c). IL-12 expression was undetectable in both 3-wk and 5-wk old peritoneal macrophages (data not shown). These results indicate that PM in 5-week-old NOD mice have become tolerant to LPS stimulation.

Integrin-α4 expression on NOD peritoneal macrophages suggests defects in clearance. Macrophages were further analysed for expression of the adhesion molecule α4 integrin, since deficiencies in the regulation of adhesion molecules may interfere with macrophage trafficking and clearance of peritoneal inflammation. NOD peritoneal macrophages expressed less α4 integrin basally and displayed a blunted down-regulation of α4 surface expression following LPS-exposure (Figure 3d).

CD69 and IFN-γ expression is induced on pancreatic lymph node T-cells by intraperitoneal inflammation.

The accumulation of macrophages displaying signs of endotoxin tolerance in the peritoneal cavity guided us to examine the role of LPS in activation of T and B lymphocytes in PaLN. To investigate these effects, we injected intraperitoneally 30µg of LPS and isolated PaLN after 24 hours to analyse changes in CD69 expression on T- and B cells (Figure 4a, b, c). Interestingly, without any LPS

treatment CD69 expression was significantly higher in PaLN CD4 and CD8 T cells of NOD mice weaned to normal as compared to antidiabetogenic ProSobee diet. NOD mice exposed to intraperitoneal LPS showed a clear increase in T-cell CD69 expression (Figure 4a-c). In addition, intraperitoneal LPS promoted IFN-γ expression in PaLN T cells (Figure 4d,e). Thus, bacterial components in the peritoneum not only may affect peritoneal innate immune responses but also activate T cells in PaLN.

Levels of plasma LPS are increased in NOD mice.

Our results suggest that microbial components leak out from the gut into the peritoneal cavity in NOD mice, and subsequently cause T-cell activation in PaLN. To further examine the hypothesis that microbial components from the gut access extraintestinal compartments in NOD mice, we measured LPS levels in the plasma of NOD and age-matched C57BL/6 mice. LPS is an acute phase protein whose production is increased by microbial factors. Interestingly, a clear increase in plasma LPS levels was observed at 5 weeks of age compared to 3 week old NOD mice (Figure 5).

Discussion

In this study, we have shown that soon after weaning, a significant increase in the number of macrophages found in the peritoneal cavity occurs in NOD mice between weaning and 5 weeks age. Our phenotypic analyses revealed that the phenotype of these macrophages differed from peritoneal macrophages in control strains and in NOD littermates fed antidiabetogenic ProSobee diet. Their surface expression of activation markers, cytokine transcripts, intracellular IRAK-M kinase expression and their production of TNF-α in response to *in vitro* LPS-challenge suggested prior *in vivo* exposure to bacterial products. These results, together with elevated levels of LPS-binding

protein in plasma samples of NOD mice suggest that the inflammatory activity [19-21] and increased epithelial permeability previously detected in the gut of NOD mice leads to an innate immune response in the peritoneal cavity of NOD mice. Analysis of macrophage activation markers CD80 and CD86 showed that their expression decreased between the age of 3 and age 5 weeks in NOD mice, and this decrease was not detected in ProSobee-fed NOD mice. We therefore hypothesize that this down-regulation of macrophage activation markers in NOD mice might be due to their exposure to microbial products in the peritoneal cavity, which in turn could be due to increased gut permeability [19, 20]. Similar findings have been reported on sepsis patients, where the surface expression of CD86 has likewise been lower, while CD80 expression has remained unchanged on macrophages [22].

To further evaluate the possibility that the increase in numbers of peritoneal macrophages would be consistent with the notion of low-grade leakage of bacterial products, we analysed the expression of IRAK-M, , and *in vitro* response of macrophages to LPS-stimulation.. None of the strains expressed IRAK-M in peritoneal macrophages at the age of 3 weeks, but elevated levels of IRAK-M were observed in NOD peritoneal macrophages at 5 weeks. This coincides with the increased number of peritoneal macrophages at this age point in NOD mice. IRAK-M is a well-known negative regulator of TLR-signalling whose expression is upregulated by prolonged activation of TLR-signalling pathways [18]. Furthermore, *in-vitro* LPS-treated PMs from NOD mice produced reduced amounts of TNFα and a tendency to increased IL-10 levels at 5 weeks compared to 3 weeks age [23, 24]. These results indicate that peritoneal macrophages in NOD mice undergo cellular adaptation responses soon after weaning which are consistent with LPS tolerance [25].

In addition the levels of α4 integrin were investigated on peritoneal macrophages, as this integrin plays an important role in cell adhesion and migration and may thus contribute to the rate of clearance after inflammation [26]. Levels of α4 integrin were found to be significantly lower on NOD macrophages basally, but the transient down regulation of alpha4 integrin surface expression,

that normally follows LPS-treatment was blunted in NOD PM induction [27]. This suggests that the clearance of peritoneal macrophages may also be altered in NOD mice.

As we obtained evidence of elevated systemic levels of LPS-binding protein in NOD mice and of LPS-induced tolerance in peritoneal macrophages, we investigated whether elevated LPS in the peritoneal compartment could have any relevance to the autoimmune pathogenesis of T1D in the NOD mouse. Towards this, we tested the effects of intraperitoneal LPS injection on the phenotype of T cells in pancreatic lymph nodes (PaLN), the site where islet antigens are presented and autoreactive T cells are activated. We observed significant upregulation of the activation marker CD69 on both CD4 and CD8 T cells, and to a lesser extent, also on B cells. Furthermore, exposure to intraperitoneal LPS promoted Th1 and Tc1 differentiation in pancreatic lymph node, as both CD4 and CD8 T cells in PaLN developed higher IFN-γ expression. Interestingly, the levels of CD69 on T cells were lower in NOD littermates fed antidiabetogenic ProSobee diet, suggesting an association of this phenomenon with reduced gut permeability, microbial load and islet-autoimmunity [22]. As B cells are important also as antigen-presenting cells in TID in NOD mice, this together with previous observations [28, 29] implies that the route from peritoneal cavity into PaLN allows LPS in peritoneal cavity to diffuse in PaLN and activate antigen-presenting cells in the latter, with the consequence of promoting also T-cell activation. LPS can also activate peritoneal B1-cells which can migrate to PaLN [29]. Due to different migratory behaviour, we believe that peritoneal macrophages may not directly affect T-cell activation in PaLN, but could indirectly modify it by virtue of cytokines that they secrete.

In conclusion, our results suggest that inflammatory changes in the gut and enhanced gut permeability in young prediabetic NOD mice [19-21] lead to extra-intestinal manifestations which may be caused by leakage of bacterial products from the gut. These manifestations are characterised by increased numbers of macrophages in the peritoneal cavity with a phenotype suggesting adaptation to prolonged *in vivo* exposure to TLR-ligand(s), and T-cell activation and Th1 and Tc1

differentiation in pancreatic lymph node. Although it remains to be established if these phenomena are indispensable in beta-cell destruction, the tempering of these in NOD littermates fed antidiabetogenic diet suggest that these phenomena may provide a mechanism by which diabetes-permissive diet and inflammatory gut reactions promote islet-autoimmunity in NOD mice.

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Duality of Interest

The authors declare that there is no duality of interest associated with this manuscript.

Contribution Statement

RE, CA and AH share responsibility for the conception and design of the study and experiments, data analysis and writing the manuscript. SP, EMR, SZ performed some of the experiments and analysed data. All authors approve the final version of this manuscript to be published.

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Figure Legends

Figure 1. Rapid increase in macrophage numbers in the peritoneum of NOD mice and the effect of antidiabetogenic diet. Total macrophage numbers were calculated from peritoneal lavage samples at indicated ages in NOD (black circle), BALB/c (white square), C57BL/6 (white triangle), and PNOD (black diamond) mice. (PNOD = NOD mice weaned to antidiabetogenic ProSobee diet). Macrophages were stained for F4/80 expression and analysed by FACS (n=5 mice per group *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 2. **Co-stimulatory molecule expression on peritoneal macrophages**. Expression of CD40, CD80, CD86 activation markers on peritoneal macrophages (F4/80+ cells). Data are expressed as mean +/- SD of MFI (mean fluorescent intensity) values at a-c) 3 weeks age, and d-f) at 5 weeks age. At 5 weeks age, a group of NOD mice weaned to antidiabetogenic ProSobee (white bars) is included. NOD (black bars) mice weaned to normal chow express more activation markers on peritoneal macrophages at 3 weeks age compared to C57BL/6 (grey bars) mice, but the expression of activation markers in NOD tends to decline over the next 2 weeks. This is counteracted by ProSobee diet (n=3 to 4 mice per group).

Figure 3 NOD peritoneal macrophages show characteristics of LPS-tolerance at 5 weeks age. a) Peritoneal macrophages' IRAK-M expression was analysed by Western blotting from 3-week-old and 5-week-old NOD and control mice. Levels of b) TNF-α, and c) IL-10 were measured from *in-vitro* -stimulated peritoneal macrophages at 3weeks (white bars) and 5weeks (black bars) age. d) NOD peritoneal macrophages have lower α4-integrin expression and a weaker α4-decline in response to stimulation with LPS compared to controls. The bars show the relative expression of α4-integrin on macrophages in untreated NOD (black bar), BALB/c (white bar) and C57BL/6 (hatched bar) mice and the decrease (fold-change) in α4 expression for each mouse strain 6 hours after intraperitoneal injection with 10µg LPS. The asterisks indicate statistically significant differences ($p<0.001$) between NOD and BALB/c mice, as well as between NOD and C57BL/6 mice. N = 3-7 mice / group.

Figure 4. Antidiabetogenic diet reduced T cell activation from Pancreatic lymph nodes. Antidiabetogenic ProSobee diet associates with lower expression of CD69 on CD4+ and CD8+ T cells in pancreatic lymph node in NOD mice, and intraperitoneal LPS-injection results in CD69 upregulation in ProSobee-fed mice. Data represent MFI (mean fluorescence intensity) +/- SEM of a) CD4+ and b) CD8+ T cells, and of c) B cells in pancreatic lymph nodes of NOD (black bars) and ProSobee-fed NOD (white bars) mice, and in the same groups, i.e. NOD (grey bars) and ProSobee-fed NOD (striped bars) mice after LPS-injection. LPS-treatment promotes IFN-γ expression on d) CD4 and e) CD8 Tcells in pancreatic lymph node. The leftmost dot-plots represent intracellular staining using isotype control antibody, while middle and right dot-plots represent IFN-γ staining. Middle dot-plot is representative of CD4 (in d) or CD8 (in e) T-cell IFN-γ in untreated and the right dot-plot is representative of T-cell IFN-γ in LPS-treated NOD mice (grey bars: untreated; black bars: 5-day LPS-treated NOD mice). In all groups, n = 3-5 mice per group.

Figure 5. LPS levels in NOD mice are increased. Blood plasma samples of NOD and control C57BL/6 mice were analysed for LPS levels at indicted ages. Note the significant increase in LPS levels in NOD mice between 3 and 5 weeks age. (NOD (black bars) and C57BL/6 (hatched bars)). (Data represents n=3 to 6 mice per group).