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J Immunol published online 26 September 2012
http://www.jimmunol.org/content/early/2012/09/26/jimmunol.1200205

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/09/26/jimmunol.1200205.DC1

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Insulin Modulates the Inflammatory Granulocyte Response to Streptococci via Phosphatidylinositol 3-Kinase

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Group B streptococci (GBS; *Streptococcus agalactiae*) are a major cause of invasive infections in newborn infants and in patients with type 2 diabetes. Both patient groups exhibit peripheral insulin resistance and alterations in polymorphonuclear leukocyte (PML) function. In this investigation, we studied the PML response repertoire to GBS with a focus on TLR signaling and the modulation of this response by insulin in mice and humans. We found that GBS-induced, MyD88-dependent chemokine formation of PML was specifically downmodulated by insulin via insulin receptor-mediated induction of PI3K. PI3K inhibited transcription of chemokine genes on the level of NF-κB activation and binding. Insulin specifically modulated the chemokine response of PML to whole bacteria, but affected neither activation by purified TLR agonists nor antimicrobial properties, such as migration, phagocytosis, bacterial killing, and formation of reactive oxygen species. The targeted modulation of bacteria-induced chemokine formation by insulin via PI3K may form a basis for the development of novel targets of adjunctive sepsis therapy. The Journal of Immunology, 2012, 189: 000–000.

The immune system and the endocrine system are interwoven on many levels. A prominent example of this is insulin, which has been assigned regulatory properties both for the antimicrobial and the inflammatory response in infection (1–3). Insulin resistance is associated with increased susceptibility to invasive bacterial infections (4–7).

Group B Streptococcus (GBS; *Streptococcus agalactiae*) is particularly interesting in this context, because it produces invasive infection in two groups of patients with peripheral insulin resistance: newborn infants and patients with type 2 diabetes (8–11). GBS are mucocutaneous colonizers in 15–20% of all people and 10% of newborn infants. Invasive infection with this organism, however, is a comparatively rare event that affects only ~1% of all colonized infants (12–14). Containment of GBS at mucocutaneous surfaces is conceivable only if occasionally invading microorganisms are rapidly killed by phagocytes, to prevent bacterial dissemination and systemic inflammation. Polymorphonuclear leukocytes (PML) are key effector cells in several models of mucocutaneous bacterial infections (15–17). In response to both bacterial particles and specific TLR activation, PML initiate various pathogen-eliminating strategies, such as phagocytosis, the generation of reactive oxygen species, and prolonged survival [reviewed by Prince et al. (18)]. Multiple lines of evidence suggest that PML are targets of inflammatory control mediated by insulin. In patients with insulin resistance, inflammatory and antimicrobial PML functions have been found to be altered (1, 19). An immunomodulatory effect of insulin has been experimentally confirmed in mouse models (20). Furthermore, insulin therapy may modulate the outcome in sepsis, although this remains a controversial issue (21–24).

Although an association between diabetes and susceptibility to bacterial infections is widely accepted, the underlying molecular mechanisms are poorly understood. It remains to be established whether immunological alterations in insulin resistance are mainly due to changes in glucose provision, or whether the insulin receptor (IR) has further, glucose metabolism-independent, modulatory properties in cell autonomous inflammatory signaling.

In this study, we found that in PML, GBS induced substantial amounts of chemokines in a phagocytosis-independent fashion. In contrast to monocytes and macrophages, inflammatory cytokines such as TNF and IL-6 were poorly induced. Insulin specifically inhibited the chemokine response to GBS and other bacteria by modulating NF-κB binding via activation of PI3K. In contrast, insulin neither affected the PML response to purified TLR agonists nor directly altered antibacterial properties, such as formation of reactive oxygen species or chemotaxis.

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Materials and Methods
Reagents were obtained from Sigma-Aldrich, unless stated otherwise. PBS, DMEM, and trypsin were purchased from Cambrex. Low-endotoxin FBS was obtained from Hyclone. LPS derived from *Escherichia coli* strain 0111:B4 was purchased from Sigma-Aldrich and extracted twice by phenol chloroform, as described in Ref. 25. C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88-deficient mice (C57 BL/10) were generated as described in Ref. 26 and kindly provided by Shizuo Akira (Department of Biochemistry, Hyogo College of Medicine, Hyogo, Japan). Plasmids encoding for MyD88, IRAK1, and TRAF6 were a kind gift from Douglas Golenbock (Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA).

**Generation of heat-fixed GBS**

GBS type III strain COH1, initially isolated from a newborn infant with sepsis, has been previously described (27). Bacteria were grown on blood agar plates (Remel, Lenexa, KS). Bacterial colonies were removed from the plates after overnight culture and washed three times in PBS. The resulting bacterial suspension was used to inoculate culture medium (DMEM plus 10% FBS) and grown to midlog phase (absorption, 0.27–0.30). Subsequently, bacteria were harvested, washed, and suspended in pyrogen-free water at a concentration of 20 mg/ml (corresponding to ~1 × 10^10 organisms per milliliter as determined by CFU per milliliter). If indicated, GBS were employed live for stimulation. In all other cases, GBS were used as heat-fixed, lyophilized preparations (fixation at 80˚C over 1 h). For opsonization, fixed GBS were incubated with specific anti-GBS Abs for 30 min on a rotating platform. The preparations were essentially free of endotoxin, as described previously (28).

**PML isolation**

Cells were isolated from heparinized venous blood (5 μl per milliliter of blood) from healthy adult volunteers. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethics committee of the University Medical Center Freiburg (Protocol Permit Number: 282/11). Consent was documented for all participating volunteers. PMLs were prepared from centrifugation, using Percoll (density of 1.076 g/ml). Contaminating erythrocytes were lysed in ice-cold medium containing 155 mmol/l NH4Cl, 10 mol/l KHCO3, and 0.1 mmol/l EDTA, pH 7.4. Cells were washed and suspended in either RPMI 1640 medium with 10% FBS and 10 μg ciprofloxacin per milliliter, or HEPES-buffered saline solution (152 mmol/l NaCl, 6.0 mol/l KCl, 1.0 mol/l CaCl2, 1.0 mmol/l MgSO4, 1.2 mmol/l potassium phosphate, 20 mmol/l HEPES, 5.5 mmol/l glucose, and 0.5% [wt/vol] human serum albumin, pH 7.4). Both purity and viability of PML were typically >95%, as assessed by morphology and trypan blue staining.

**Mouse peritoneal PML**

This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123). The animal experimental protocol was approved by the ethics committee of the University Medical Center Freiburg (Protocol Permit Number: 35-9185.81/G-09/42). All animal experiments were planned and executed to minimize suffering. The 8-wk-old mice (MyD88 or C57BL6/J WT, The Jackson Laboratory) were infected i.p. with 2.5 ml of 3% thiglycollate solution (Remel). After 5–8 h, peritoneal exudate cells were harvested, as described previously (27). For further purification, peritoneal neutrophils were stained for CD11b (PE-Cy7–labeled anti-human CD11b Ab; eBioscience), Ly6G (FITC–labeled anti-human Ly6G Ab; BD Pharmingen), and CD115 (MCSF-R, primary biotin-labeled anti-human CD115 Ab, secondary Pacific blue-labeled streptavidin-linked Ab; eBioscience). Subsequently, cells were washed and subjected to FACS sorting. PML were identified by gating on CD11b+ cells and subsequently on the clearly defined Ly6G^hiCD115^+ population to obtain Ly6G^hiCD115^+ CD11b^hi neutrophils.

Cells were then washed and suspended in medium (RPMI 1640 medium containing 10% FBS and 10 μg ciprofloxacin per milliliter), counted with a hemocytometer, plated, and allowed to rest for at least 1 h.

**Inhibition of signaling cascades by chemical inhibitors, insulin, and Abs**

Cells were treated with insulin, an IR-specific Ab (directed against the α-subunit; Thermo Fischer Scientific, Fremont, CA), or isotype control (30 μg/ml) and/or the chemical inhibitors cytochalasin D, SB203580, wortmannin, and Ly294002 at indicated concentrations for 30 min prior to further treatment or stimulation. Cell activation was determined as indicated and described in the respective section.

**Transfection of RAW 264.7 macrophages, activation of luciferase reporter constructs, and determination of transcriptional gene activation**

RAW 264.7 cells were seeded into 96-well tissue culture plates at a density of 10^5 cells per well (DMEM with 10% FBS and ciprofloxacin at 10 μg/ml). The following day, cells were transfected with luciferase reporter constructs comprising minimal AP-1 and NF-κB promoters (Stratagene, La Jolla, CA) or human WT and mutant IL-8 promoters (provided by N. Mackman, The Scripps Research Institute, La Jolla, CA) with FireFlu (Roche), used per the manufacturer’s recommendations. In individual experiments, cells were cotransfected with a constitutively active Renilla luciferase reporter gene (Promega) to normalize for transfection efficacy. The following day, the cells were stimulated as indicated for 4–6 h or cotransfected with signaling protein expressing plasmids. Cells were lysed in passive lysis buffer (Promega), and reporter gene activity was measured in a luminometer (MicroLumat Plus; Berthold Detection Systems). In all cases, the data shown represent one of at least three separate, but similar, experiments, and are presented as the mean values of arbitrary light units ± SD of triplicate samples, unless SD was <1%.

**Measurement of inflammatory activity**

For determination of cytokine formation, cells were seeded in 96-well dishes (10^3 cells per well) in RPMI 1640 with 10% FBS plus 10 μg ciprofloxacin per milliliter and incubated for 16 h (37˚C, 5% CO2). If indicated, cells were treated with insulin and/or the indicated chemical inhibitor for 30 min. Then, cells were stimulated as indicated over 5 h. Supernatants were processed directly for determination of cytokines (ELISA; R&D Systems, Minneapolis, MN) or frozen until analysis. For determination of intracellular cytokines, cells were treated with 0.05% Triton X and subjected to ELISA measurement. Shown are representative results from three or more individual experiments in triplicate wells ± SD. For intracellular measurement of IL-8 formation by FACS, freshly isolated PML were incubated with the indicated Ab or a vehicle control for 30 min at 37˚C. Subsequently, insulin (2000 ng/ml) was added where indicated. After another 30 min of incubation, PML were stimulated as indicated for 1 h. Then, brefeldin A (GolgiStop) was added. Four hours later, cells were washed and fixed with 2% paraformaldehyde, permeabilized, and stained for intracellular IL-8 (anti-human IL-8 PE; BioLegend).

**Analysis of intracellular kinase activation**

Phosphorylation of MAPK and IκB was evaluated according to standard protocols. Briefly, lysates of stimulated cells were separated by SDS page and analyzed by Western immunoblotting using nitrocellulose membranes (HyClone, Erembodegem, Belgium) and Abs for phosphorylated p38 (Cell Signaling Technology, Beverly, MA), c-Jun (Cell Signaling Technology), phosphorylated Erk-1 (Cell Signaling Technology), and intracellular IκB (Santa Cruz Biotechnologies, Heidelberg, Germany). For analysis of cytosolic p65 phosphorylations, human PML were stimulated with GBS (10^9/ml) in the presence or absence of insulin (2000 ng/ml). After 20 min, cells were fixed, permeabilized, and intracellularly stained for phosphorylated p65 (primary Ab: rabbit anti-phospho-NF-κB p65 (Ser536) (Cell Signaling Technology); secondary Ab: donkey anti-rabbit Alexa Fluor 568 (Invitrogen, Carlsbad, CA)). Cells were analyzed by FACS for intracellular phospho-p65.

**Generation of nuclear extracts**

Human PML were stimulated as indicated and harvested (1500 rpm at 4˚C and strictly kept on ice. Cells were suspended in 100 μl hypotonic buffer, incubated for 15 min, suspended in 10% Nonidet P-40 buffer. Nuclei were collected by centrifugation (13,000 rpm, 5 min) and resuspended in 25 μl extraction buffer. After further incubation (15 min), nuclear lysates were collected (centrifugation at 21,200 rpm for 5 min) and stored at −20˚C until further processing.

**Analysis of NF-κB binding by EMSA**

For EMSA, 5′-biotinylated oligonucleotides were obtained from Biomers (Ulm, Germany), with the following sequences: 5′-GGGCAGGTCCGACCTGAGAC-3′; 5′-GGGTCCCTGAGAACGAGTCGAG-3′; 5′-GGTCCTGCCAAGGCCTTCTCGGA-3′. EMSA was performed with the Chemiluminescent Nucleic Acid Detection Module Kit (#89880; Pierce, Rockford, IL) according to the manufacturer’s instructions, with slight modifications. A 6% polyacrylamide gel was used
The gel was blotted onto a nylon membrane. DNA crosslinking was performed with 30 ml equilibration buffer before activation in luminol with peroxide. Analysis 100 m and 1200 m for an additional 1 h in 0.5 M MgCl2 and DTT (5 mM). Nuclear extracts were incubated for 15 min on ice before a master mix containing the indicated oligonucleotide, poly(dA-deoxycytidylic) (1 μg/μl; Pierce), and 10% BSA were added. Finally, 5 μl loading buffer (Pancoll + bromophenol blue) was added to each sample. Subsequently, the gel was loaded with 5 μg protein per lane and run at 90 V for 1.5 h, then 120 V for an additional 1 h in 0.5× Trisborate-EDTA. The gel was blotted onto a nylon membrane. DNA crosslinking was performed for 15 min on a UV table. The membrane was developed using 50 μl streptavidin-HRP. The membrane was washed and equilibrated for 5 min in 30 ml equilibration buffer before activation in luminol with peroxide. Analysis was performed by means of luminescence measurement.

**Analysis of nuclear translocation of NF-κB by fluorescence microscopy**

PML were seeded onto coverslips in 6-well plates (5 × 104/well) and rested for 2 h at 37˚C. Cells were then treated with insulin (2000 ng/ml) or a vehicle control for 30 min. Subsequently, cells were stimulated with GBS, as indicated. Cells were treated with TNF-α (25 ng/ml) or a vehicle control for 30 min. Subsequently, cells were cultured with or without FITC-labeled GBS (10⁸/ml) for 7 min. Then, cells were harvested and washed, and extracellular fluorescence was quenched with trypan blue. Cells were analyzed for intracellular fluorescence by FACs, using FlowJo software.

**Formation of reactive oxygen species**

Human PML were plated at a density of 10⁵ cells per well into a 96-well plate and incubated with GBS (10⁸ and 10⁶/ml) as indicated. Lucigenin was used to measure extracellular O₂⁻ over 90 min. Depicted is the mean fluorescence intensity from triplicate wells ± SD of one of at least three experiments.

**Chemotaxis**

PML migration was determined in a two-chamber system containing Fluoroblok inserts (Falcon; Becton Dickinson, San Jose, CA). Human PML (5 × 10⁶/ml) were labeled with calcine-AM for 30 min at 37˚C, washed twice, and suspended in HEPES buffer at a concentration of 2 × 10⁶/ml. Chemokine receptor antagonists (platelet-activating factor-IL-8, and C5a, all at 10 nM) in HEPES or HEPES alone were placed in the lower chamber of a 24-well plate (0.8 ml per well). Then, inserts (3-μm pore size) were placed on top and 0.3 ml PML suspension was pipetted into the inserts. Fluorescence in the lower compartment as a parameter of migrated PML was measured every 2.5 min for a total of 45 min. The maximal slope of migration was calculated over a 10-min interval. Migrational direction and speed of PML were assessed with the ibidi μSlide System (ibidi, Munich, Germany). An fMLF gradient was created, and PML pretreated with insulin or a vehicle, were loaded through the cell inlet. Slides were incubated at 37˚C for up to 1 h, and cell movement was assessed using a Nikon BioStation IM (Nikon Instruments, New York, NY). For image analysis, ImageJ software (W.S. Rasband, ImageJ, National Institutes of Health, Bethesda, MD) and the following plugins were used: Manual Tracking plugin (Fabrice Codiere, Institut Curie, Orsay, France) and Chemotaxis and Migration Tool (ibidi). Data were analyzed for migrational speed and direction. Depicted is one representative experiment.

**Phagocytosis**

Human PML were plated on a 24-well plate (2 × 10⁵ cells per well) and treated with insulin (2000 ng/ml) or vehicle for 30 min. Subsequently, cells were incubated with or without FITC-labeled GBS (10⁶/ml) for 7 min. Then, cells were harvested and washed, and extracellular fluorescence was quenched with trypan blue. Cells were analyzed for intracellular fluorescence by FACs, using FlowJo software.

**Results**

GBS induces chemokines in neutrophils in a MyD88-dependent but phagocytosis-independent fashion

The transcriptional regulation of inflammatory genes in mononuclear phagocytes stimulated by GBS has been studied in detail by us and others (27, 30–32). In contrast, requirements and consequences of PML stimulation by streptococci are incompletely understood. Alarminers in PML function, however, have been implicated in the specific susceptibility of infants and diabetic patients to invasive GBS infections (33). Accordingly, we first characterized the cytokine and chemokine response of human PML to GBS. We found that upon stimulation with GBS, PML produced high levels of IL-8 (Fig. 1A). In contrast, they did not form relevant amounts of IL-1β or TNF, the latter of which is potently induced in monocytes and macrophages. However, similar to GBS recognition in macrophages (32), recognition of GBS ssRNA was important for the IL-8 response by PML because specific digestion of ssRNA by RNase A substantially decreased IL-8 formation (Fig. 1B). Moreover, and similar to the situation in macrophages, the chemokine response of PML was largely dependent on MyD88, as determined for CXCL1/KC formation in sorted peritoneal PML from MyD88-deficient and wild type mice (Fig. 1C).
GBS phagocytosis and TLR recognition are tightly interrelated events synergizing in cytokine formation by macrophages (34, 35). However, we found that, in contrast to the situation in macrophages (Fig. 1D), human and mouse PML formed chemokines despite abrogation of GBS uptake by cytochalasin D or a vehicle control for 30 min. Then, cells were stimulated with GBS (10^9/ml) for 5 h. Supernatants were analyzed for TNF by ELISA and depicted as mean values ± SD from triplicate wells. (E) Human PML were treated with cytochalasin D or a vehicle control over a period of 30 min. After stimulation with GBS as explained above, IL-8 was determined by ELISA. In (A) to (E), means ± SD from triplicate wells are depicted. **p < 0.001 for all depicted differences. (F) Peritoneal PML were elicited 5 h after thioglycollate injection and sorted for Ly-6G^{high}-expressing cells. Cells were treated with cytochalasin D or a vehicle control over 30 min. Then GBS (10^9/ml) was added for 5 h. Finally, supernatants were analyzed for IL-8 by ELISA. Depicted are means ± SD from triplicate wells. (G) Ly-6G^{high} peritoneal PML from WT or MyD88^{−/−} mice were stimulated with FITC-labeled GBS 10^7/ml (black line) or a vehicle control (gray line) for 10 min. Next, extracellular fluorescence was quenched with trypan blue, and PML were analyzed for intracellular fluorescence by FACS. (H) Ly-6G^{high} mouse peritoneal PML were incubated with cytochalasin D plus FITC-GBS 10^9/ml for 10 min. Internalization of GBS was determined as indicated in (G). All experiments are representative of at least three independent experiments.

**FIGURE 1.** GBS induces predominantly chemokines in PML in a phagocytosis-independent fashion. Human PML were stimulated in triplicate wells with escalating doses of GBS (A) and RNAse-treated GBS (B) for 5 h. Supernatants were analyzed as indicated for IL-8, TNF, and IL-1β by ELISA. (C) FACS-sorted mouse peritoneal PML from WT or MyD88^{−/−} mice were stimulated with GBS organisms, as indicated, for 5 h. Then, supernatants were analyzed for CXCL1 by ELISA. Depicted are means ± SD from triplicate wells. (D) Mouse bone marrow-derived macrophages were pretreated with the actin polymerization inhibitor cytochalasin D or a vehicle control for 30 min. Then, cells were stimulated with GBS (10^9/ml) for 5 h. Supernatants were analyzed for TNF by ELISA and depicted as mean values ± SD from triplicate wells. (E) Human PML were treated with cytochalasin D or a vehicle control over a period of 30 min. After stimulation with GBS as explained above, IL-8 was determined by ELISA. In (A) to (E), means ± SD from triplicate wells are depicted. **p < 0.001 for all depicted differences. (F) Peritoneal PML were elicited 5 h after thioglycollate injection and sorted for Ly-6G^{high}-expressing cells. Cells were treated with cytochalasin D or a vehicle control over 30 min. Then GBS (10^9/ml) was added for 5 h. Finally, supernatants were analyzed for IL-8 by ELISA. Depicted are means ± SD from triplicate wells. (G) Ly-6G^{high} peritoneal PML from WT or MyD88^{−/−} mice were stimulated with FITC-labeled GBS 10^7/ml (black line) or a vehicle control (gray line) for 10 min. Next, extracellular fluorescence was quenched with trypan blue, and PML were analyzed for intracellular fluorescence by FACS. (H) Ly-6G^{high} mouse peritoneal PML were incubated with cytochalasin D plus FITC-GBS 10^9/ml for 10 min. Internalization of GBS was determined as indicated in (G). All experiments are representative of at least three independent experiments.

GBS phagocytosis and TLR recognition are tightly interrelated events synergizing in cytokine formation by macrophages (34, 35). However, we found that, in contrast to the situation in macrophages (Fig. 1D), human and mouse PML formed chemokines despite abrogation of GBS uptake by cytochalasin D (Fig. 1E, 1F, 1H). Why abrogation of phagocytosis rather increased chemokine formation in human PML remains to be established. Moreover, deficiency in MyD88 did not affect GBS phagocytosis, in contrast to its role in GBS-mediated chemokine formation (Fig. 1C, 1G). In summary, GBS induced predominantly chemokines in PML, and this response was not inhibited when phagocytosis was blocked.

**Insulin inhibits GBS-induced chemokine formation in PML in an IR-dependent fashion**

Next, we assessed the effect of insulin on the chemokine response of PML. We found that insulin dose-dependently inhibited the IL-8 release of human PML stimulated with GBS (Fig. 2A). This effect was observed for both heat-fixed (Fig. 2B, left panel) and live (Fig. 2B, right panel) bacteria. In resting cells, IL-8 is found in preformed vesicles (36). However, IL-8 release by GBS-stimulated PML predominantly resulted from de novo synthesis because it was blocked by actinomycin D (data not shown). In line with this notion, both intracellular and extracellular IL-8 was reduced by insulin in GBS-treated granulocytes (Fig. 2C). Furthermore, insulin inhibited transcription of IL-8, as determined by quantitative PCR (Fig. 2D). Similarly to human PML, mouse PML showed a decreased chemokine response. We analyzed CXCL1/KC, the mouse analog of the human chemokine functional IL-8 equivalent, because mice do not express a structural IL-8 homolog (Fig. 2E). Next, we wondered whether insulin exerted its effects via engagement of the IR or via binding to alternative sensors, such as the insulin-like growth factor receptors. First, we confirmed IR expression on human PML by flow cytometry, and IR function by insulin-induced glucose transport (data not shown). Blocking of insulin–IR interaction with a specific IR Ab blocked the inhibitory effect of insulin on IL-8 formation (Fig. 2F). Thus, insulin inhibited chemokine formation via direct interaction with the IR.
Insulin targets NF-κB for modulation of chemokine formation

Next we asked whether the insulin effect was specific for GBS or whether it generally modulated cellular activation by microbial stimuli. We found that insulin inhibited only the chemokine response to bacterial particles, whereas the response to the purified TLR ligands FSL-1 (TLR-2/-6 agonist) and flagellin (TLR-5) remained unaffected (Fig. 3A). Furthermore, insulin exerted its effect under both opsonizing and nonopsonizing conditions. Addition of fresh complement and a GBS type III specific antiserum shifted the dose response to the left by about one log, as compared with nonopsonic conditions (Fig. 3B).

The transcription factors AP-1, NF–IL-6, and NF-κB are critical for activation of the IL-8 promoter (Fig. 3C and Ref. 37). In this study, we determined the roles of NF-κB and AP-1 in IL-8 acti-

**FIGURE 2.** Insulin downregulates TLR-mediated cytokine induction in response to GBS. (A) Human PML were incubated with insulin at indicated concentrations for 30 min and stimulated in triplicates with escalating doses of heat fixed GBS for 5 h. Supernatants were analyzed for IL-8. (B) Human PML were treated with insulin and stimulated as indicated with either heat-fixed or live GBS for 5 h. In conditions stimulated with live GBS, ciprofloxacin was added after 2 h at a final concentration of 10 μg/ml. (C) Human PML were treated with insulin and stimulated as indicated. Supernatants were analyzed for extracellular cytokine release, and cellular lysates were prepared to determine intracellular cytokine concentrations. (A–C) Depicted are cumulative mean values from triplicate wells ± SD. (D) Human PML were incubated with insulin in triplicate wells and stimulated with escalating doses of heat-fixed GBS for 5 h. Samples were analyzed for RNA content by quantitative RT-PCR. (E) FACS-sorted mouse PML were incubated with insulin and stimulated with GBS for 5 h (triplicates), as indicated. Supernatants were analyzed for CXCL-1 by ELISA. Depicted are mean values from triplicate wells ± SD. (F) Human PML were incubated with 30 μg/ml of anti–IR-Ak or isotype control Ab. Subsequently, insulin or a vehicle control was added as indicated. Cells were then stimulated with heat-fixed GBS for 5 h, and IL-8 formation was determined by FACS (expressed as gMFI, geometric mean fluorescence intensity). ***p < 0.001.
vation by the GBS organism. A reporter assay employing the IL-8 promoter with established mutations for transcription factor binding revealed that NF-κB and AP-1 were both essential for IL-8 induction (Fig. 3D). Next, we determined the level of functional interference between the chemokine-inducing and insulin-dependent pathways. We analyzed ligand-independent activation of the ELAM promoter by heterologous expression of MyD88 and its downstream signaling intermediates (38). This ELAM–luciferase reporter construct comprises three NF-κB and one AP-1 binding sites. With this cellular model, we found that insulin inhibited NF-κB/AP-1 activation upon expression of MyD88 (Fig. 3E), IRAK-1 (Fig. 3F), and TRAF6 (Fig. 3G). Therefore, insulin interacted with NF-κB/AP-1 activation at the level of or downstream of TRAF6.

**Activation of MAPKs is not inhibited by insulin**

In earlier studies we found that MAPKs are important in GBS-induced cytokine formation by mononuclear phagocytes (27, 28, 30). With respect to AP-1 activation, the MAPK p38 is essential (39). Accordingly, we analyzed the effect of insulin on MAPK activation in human PML. We found that p38 phosphorylation was induced by insulin alone. This was further propagated by subsequent stimulation with GBS. In contrast, Erk, NF-κB, and JNK were not activated by insulin (Fig. 4A, Fig. 5C). GBS induced phosphorylation of ERK (Fig. 4A), but, in contrast to the situation in macrophages (27, 30), we did not detect GBS-induced cJun phosphorylation in PML (Fig. 4A). Because insulin alone induced p38 phosphorylation, we wondered whether p38 negatively regulated the chemokine response to GBS. Accordingly, PML were treated with insulin plus the p38 inhibitor SB203580. Subsequently, cells were stimulated with GBS and analyzed for IL-8 release. We found that inhibition of p38 phosphorylation plus insulin further inhibited GBS-induced IL-8. Therefore, under the applied conditions, p38 plays a role in IL-8 formation but does not appear to mediate the insulin-mediated inhibition of IL-8 (Fig. 4B).

**FIGURE 3.** The insulin-dependent downregulation of inflammatory cytokines is particle dependent. (A) Human PML were incubated with insulin in triplicate wells and stimulated with TLR ligands, as indicated. Supernatants were analyzed for IL-8. (B) Human PML were incubated with insulin and stimulated over 5 h with heat-fixed GBS, with or without opsonization by Abs raised against GBS capsular polysaccharide. Supernatants were analyzed for IL-8. Depicted are mean values from triplicate wells ± SD. (C) The human IL-8 promoter contains transcription factor binding sites for NF-κB, NF–IL-6, and AP-1. (D) RAW267.4 macrophages were transfected with a luciferase reporter plasmid comprising the IL-8 WT promoter sequence reporter constructs carrying point mutations in either the NF-κB (NF-κB–Mut) or the AP-1 (AP-1–Mut) binding site. Cells were stimulated with heat-fixed GBS over 5 h, and cellular lysates were analyzed by luminometry for reporter activity. (E–G) RAW267.4 cells were transfected with the NF-κB–dependent reporter Elam.luc-pCDNA, plus a constitutive Renilla-luciferase reporter. For activation of TLR-specific signaling, MyD88 (E), IRAK1 (F), TRAF 6 (G), or a parental plasmid (pCDNA) was coexpressed. After 16 h, luciferase activity was determined. All experiments are performed in triplicate wells and are representative of three or more independent experiments. ***p < 0.001.

**FIGURE 4.** GBS-mediated MAPK signaling is not altered by insulin. (A) Human PML (5 × 10⁶ per condition) were treated with insulin or a vehicle control and stimulated with GBS, as indicated. Cellular lysates were analyzed by Western blot for phosphorylated p38, cJun, or Erk. Data from one representative membrane out of three or more similar experiments are shown. (B) Human PML were treated with insulin, as indicated, and/or the p38 phosphorylation inhibitor SB203580. Cells were then stimulated with GBS (10⁷/ml) for 5 h. Supernatants were analyzed for IL-8. All experiments are representative of three or more independent experiments.
Insulin inhibits p65 phosphorylation and NF-κB–DNA binding

Because MAPKs were not essentially involved in mediating the insulin effect, we speculated that NF-κB activation was the target of insulin signaling. Accordingly, we first analyzed the influence of insulin on IκB degradation, the essential process in NF-κB liberation. GBS induced IκB degradation in human PML, however, this process was not modulated by insulin (Fig. 5A). Next, we analyzed nuclear localization of the NF-κB subunit p65 in human PML by confocal microscopy. As shown in Fig. 5B, GBS-induced nuclear translocation of p65 was inhibited by concomitant insulin treatment. Accordingly, whereas degradation of IκB, and therefore NF-κB liberation, was not altered by insulin, the presence of NF-κB in the nucleus was reduced by insulin. Because the nuclear presence of NF-κB depends on the on- and off-rate at its binding sites, we determined the binding of NF-κB in PML treated with GBS plus insulin by EMSA. We found that insulin indeed decreased DNA binding of NF-κB (Fig. 5C). DNA binding of NF-κB depends on aminoterminal serine phosphorylation of p65 (Ser536), the so-called transactivation. Accordingly, we tested whether insulin interfered with p65 phosphorylation induced by GBS. As shown in Fig. 5D, p65 is phosphorylated in PML stimulated with GBS. However, concomitant insulin treatment inhibits p65 phosphorylation. In conclusion, GBS induces iIk8 transcription in PML via MyD88, and insulin negatively regulates this process at the level of p65 phosphorylation and NF-κB binding. Insulin is well established as a potent activator of PI3K, both via direct interaction of the IR with the regulatory subunit p85 and via the IR substrate-1. Furthermore, PI3K has modulating properties for TLR signaling, with the majority of studies implicating a role in signal propagation (40, 41). Accordingly, we wondered whether insulin induces Akt phosphorylation as an endpoint of PI3K in PML and, if so, how signaling intermediates of classic TLR pathways were modified. We found that insulin alone induced phosphorylation of the established PI3K target Akt at residues Ser473 and Thr308 (Fig. 6A). To better understand the role of PI3K in inflammatory regulation of PML, we used the inhibitors wortmannin and Ly294002 to inhibit insulin-induced PI3K. We found that both inhibitors efficiently reverted the insulin-mediated decrease in IL-8 (Fig. 6B, 6C). Importantly, this effect of PI3K inhibition occurred when inhibitors were used in nanomolar concentrations, which did not interfere with transcriptional activation of IL-8 per se. In line with a regulatory role of PI3K in GBS-induced IL-8 formation, we found that the peroxisome proliferator-activated receptor-γ (PPARγ) agonist pioglitazone, which has previously been shown to activate PI3K, mimics the insulin effect (Fig. 6D) (42, 43). Thus, PI3K appeared to be the essential intermediate between IR activation and inhibition of GBS-induced transcriptional activation of IL-8.

Insulin does not alter antimicrobial properties of PML

The primary role of PML in the context of bacterial infections is the rapid recruitment to sites of microbial invasion and subsequent killing of bacteria. Accordingly, we wondered whether insulin affected these antimicrobial properties as well. First, we analyzed cellular adhesion to a variety of stimuli, as an initial step in PML recruitment from the blood to the site of infection, and found it not
Ly294002 (1 nM) were treated as indicated with insulin (2000 ng/ml), wortmannin (1 nM). Staining of total p38 was employed as loading control. The PPAR agonist pioglitazone (5 × 10^{-6}M as indicated) was added. Similarly, PML migration in response to rMLF, C5a, or IL-8 was not affected in a qualitative fashion. To better quantify migration, live cell imaging was employed. Re- petitive measurements over a period of up to 50 min did not show any differences between insulin-treated cells and the untreated control (Supplemental Fig. 1B, 1C). Mean velocity was 2.48 U/sec (SD 37.96) units for untreated and 3.55 U/sec (SD 40.72) units for insulin-treated cells. Accordingly, no significant difference was found between treated (6.05 × 10^{-3}) and untreated cells (6.05 × 10^{-3}) with respect to the Raleigh test for vector data (Supplemental Fig. 1B). Taken together, these results indicate that insulin does not interfere with chemotaxis. Next, we studied whether insulin influenced bacterial phagocytosis. PML were stimulated with FITC-labeled GBS for 7 min and analyzed for bacterial internalization by FACS. We did not find any difference between insulin-treated cells and vehicle controls (Supplemental Fig. 1D). In addition, the formation of reactive oxygen species by human PML and inducible programmed cell death were not modulated by insulin (Supplemental Fig. 1E and data not shown).

**Discussion**

Maintaining homeostasis in both the endocrine and the immune systems is critical for ensuring individual resistance against external stressors. It has long been appreciated that both systems are interdigitating, and that interference with one system, may negatively or positively affect the other. In this article, we report that insulin is involved in regulating the PML response to GBS, an important bacterial pathogen in states of insulin resistance. Insulin inhibits chemokine formation in a cell-autonomous fashion at the level of NF-κB activation. In contrast, antimicrobial properties of the same cell are left untouched. When bacteria such as GBS break the mucocutaneous barrier, resident cells such as keratinocytes, dendritic cells, and tissue macrophages are activated. Inflammatory signal activation upon physical contact between GBS and macrophages has been defined by us and others in considerable detail (reviewed in Refs. 44, 45). Phagocytosis and phagolysosomal processing of the bacteria are an absolute requirement for recognition of bacterial particles via their ssRNA and for subsequent signal induction (32). The host signaling molecules MyD88 and UNC-93B; the kinases JNK, p38, and ELK-1; and the transcription factors NF-κB, AP-1, and EGR-1 are pivotal downstream intermediates that lead to the formation of TNF and IL-6 (27, 30–32). Escalation of the response of resident phagocytes results in rapid recruitment of PML, which clearly dominate the cellular infiltrate of hematopoietic origin in the early stages of infection. We show in this article that, in contrast to macrophages, PML respond with a program biased towards chemokine formation and antimicrobial properties, whereas classical inflammatory cytokines (TNF, IL-1β, IL-6) are not substantially activated. Our observation on a restricted monokine response in human PML and mouse peritoneal PML is backed up by other studies, which found a similar response profile (45, 46). However, it contradicts a number of reports on TNF formation by PML in response to a variety of stimuli (e.g., Refs. 47, 48). It seems essential that in the assessment of cell-autonomous PML functions, the avoidance of any contamination with monocytes is utterly important because it will dramatically alter the inflammatory phenotype. Furthermore, the source from which mouse PML are isolated is essential. In contrast to PML isolated from peripheral blood or the peritoneal cavity, less committed bone marrow-derived PML, which are isolated by negative selection, are capable of forming TNF (M. Mergen, S. Kenzel, and P. Henneke, unpublished observations).

Moreover, we found that particle processing is not necessary for chemokine formation by PML. This is in sharp contrast to macrophages, which respond to GBS only, if the bacteria are processed via the phagosomal route. This seems particularly notable because recognition of GBS by both macrophages and PML requires the presence of bacterial ssRNA and MyD88. It remains to be established whether PML are capable of digesting GBS in the extracellular space through externalization of granular factors, thereby making ssRNA accessible for the PML recognition system. The MyD88-dependent chemokine formation in response to GBS and other bacterial particles was subject to IR-mediated modulation. Cell-autonomous transcriptional regulation by insulin was
program and that modulating it may have clinical implications. The predominance of an antibacterial and chemokine program in PML suggests a model in which PML are targeted to localize the infection through recruitment of further effector cells, rather than eliciting a systemic response. The specific conditions under which negative PML regulation via the IR has an impact on host defense against bacteria remain to be established. Furthermore, we currently do not know whether the repression of bacterial particle-induced chemokine production is advantageous or disadvantageous to the host. Notably, insulin concentrations in several tissues are higher than in plasma (61), although detailed information on human tissues such as skin is not available. It is conceivable that high tissue insulin concentrations may interfere with phagocytic host defense, thereby facilitating local bacterial infections. In contrast, attenuation of chemotactic PML properties by insulin may be beneficial under some circumstances because PML recruitment may affect tissue homeostasis. Importantly, insulin does not interfere with the motility of PML, hence PML remain chemokine responsive; at the same time, their contribution to the chemotactic milieu is dampened. How the modulation of normal human and mouse PML by insulin relates to the dysregulation of innate immunity in diabetes, with respect to both altered cytokine levels and depressed neutrophil function, is not clear at this stage (1, 19). In type 2 diabetes and neonatal insulin resistance, both hyperinsulinemia and hyperglycemia are found. Hyperglycemia itself may have a specific impact on clinical and cellular PML immunocompetence, such as that via posttranslational effector molecule glycosylation (1, 62, 63). Accordingly, both direct effects of insulin and indirect effects of glucose levels may underlie the adjuvant therapeutic effects of insulin in sepsis (24, 64–66).

The incomplete molecular understanding of how insulin regulates and poor glycemic control on role of neuglucopenia in haemolytic streptococci groups A, B, and G. J. Infect. Dis. 63: 8–16.


Disclosures

The authors have no financial conflicts of interest.

References


2. Martins, J. O., M. Ferracini, D. B. Anger, D. O. Martins, L. F. Ribeiro, J. P. Dias, and J. E. Krieger. 2006. Stimulation of innate immunity in diabetes, with respect to altered cytokine activation as a downstream event. PI3K is one of several regulators of TLR signaling, such as IRAK-M, MyD88s, A20, the SOCS proteins, and GSK3 (49–54). PI3K was originally characterized as a positive regulator of TLR ligands such as lipopolysaccharide-induced inflammation in mice. Shock 33: 75–237.


