Absence of GDF5 does not interfere with LPS Toll-like receptor signaling

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ABSTRACT

Introduction

Growth and differentiation factor-5 (GDF5), member of the TGFβ superfamily, has been implicated in limb development, and is known to play an important role in joint formation. Its absence leads to brachypodism in mice and a number of skeletal malformation syndromes in humans. Recently, an association was shown between osteoarthritis and a 5′ UTR polymorphism in GDF5 gene. In addition, the role of GDF5 may reach beyond the musculoskeletal system. GDF5 appears present in a lipopolysaccharide (LPS) receptor cluster. Absence of GDF5 may limit the response to LPS. This may have consequences for immune responses and macrophage function in general, and for arthritis in particular.

Here we compared the sensitivity of Gdf5−/− mice and wild type (WT) mice to LPS.

Methods. Peritoneal macrophages from Gdf5−/− mice and WT mice were stimulated for 18h with LPS (0, 10 or 100ng/ml). The supernatant was collected and TNF release was measured by ELISA and by indirect luciferase assay using LNF-luc C3 cells.

Results. Gdf5−/− macrophages showed no difference in TNF expression upon LPS stimulation measured by ELISA and by an indirect luciferase assay. Gdf5−/− mice died upon a lethal dose of LPS, as is seen in WT controls.

Conclusion. Absence of Gdf5 appears not to affect the LPS response. Mice with a reduced expression of Gdf5 can be used in disease models which are dependent on LPS boost.

Key words: GDF5, LPS, arthritis, arthritis models.

Competing interests: none declared.
in vitro, by determining TNF release of macrophages after stimulation with LPS, in vivo, by an LPS induced lethality assay.

Material and methods

Mice and genotyping

The Gdf5-brachypodism (Bp-J) allele occurs spontaneously in A/J strain mice (Jackson Laboratory, Main, USA). Intercrossed breeding with Swiss/CD1 mice (Janvier, Le Genest Saint Isle, France) in our animal facility (Proefdierencentrum, Leuven, Belgium), introduced the Bp-J-allele in CD1 background. From 5th generation onwards, Gdf5<sup>Bp-J:<i>Bp-J</i></sup> mice and wild type (WT) littermates (Gdf5<sup><i>Bp-J</i></sup>/+) were used. Mice were genotyped by PCR on genomic DNA from tail biopsies, amplifying a 155bp product surrounding the insertion site using forward primer p1 (5'-ATTGGTTCAGCCAGCGGCG-3') and reverse primer p2 (5'-CTCAAAGGTTGCAAGGTCGAGTC-3'). Sequencing of PCR product was performed at the VIB Genetic Service Factory (Antwerp, Belgium) using reverse primer p2 to determine absence (WT) or presence of a single (HZ or Gdf5<sup>Bp-J</sup>/+) or double (KO or Gdf5<sup>Bp-J:<i>Bp-J</i></sup>) cysteine insertion.

In vitro study on the effect of absence of Gdf5 on LPS Toll-like signaling

Peritoneal macrophages from Gdf5<sup>Bp-J:<i>Bp-J</i></sup> mice and WT mice were collected 4 days after thioglycollate-induced peritonitis (1ml 10% thioglycollate medium/PBS i.p.) in PBS (Lonza, Verviers, Belgium), followed by red blood cells lysis (50mg KHCO3, 414 mg NH4Cl, 10 μl 0.5M EDTA and MilliQ till 50 ml). Cells were resuspended at 1x 10<sup>6</sup> cells/ml in RPMI-1640 (Gibco, Invitrogen, Merelbeke, Belgium) and 200 μl of cells were plated in each well of a 24-well plate. Two hours after adherence, cells were stimulated for 18h with different concentrations of LPS E. coli in serum-free RPMI-medium (0, 10 or 100ng/ml) (in triplicate) (Sigma-Aldrich, Bornem, Belgium). Supernatant was collected and cell viability was checked by trypan blue (Fluka, Sigma-Aldrich) staining. TNF release in supernatant was measured by ELISA (Biosource, Nivelles, Belgium), as described by the manufacturer, and by an indirect luciferase assay using LNF-luc C3 reporter cells (kind gift from R. Beyaert, University of Gent, Gent, Belgium). LNF-luc C3 cells were cultured in a 24 well plate till 90% confluence in DMEM (Gibco, Invitrogen) with 10% FBS, cells were washed and stimulated for 24h with

Figure 2

Cumulative Proportion Surviving (Kaplan-Meier)

WT
KO

Fig. 2. In vivo LPS responsiveness of Gdf5<sup>Bp-J:<i>Bp-J</i></sup> (KO (dashed line)) mice and Gdf5<sup><i>Bp-J</i></sup> (WT (full line)) mice. A Kaplan-Meier survival curve is shown, demonstrating the cumulative proportion of surviving mice after a certain amount of time. ‘♦’ presents a mouse that died, ‘x’ presents a mouse that did not die before the end of the experiment was reached (n=5 animals/group; Gehan-Wilcoxon p<0.35).
serum-free medium (DMEM/0% FBS) or with different amounts (50, 10 or 2μl) of supernatant from Gdf5−/−Bp-J mice and WT LPS-stimulated macrophages. After stimulation, cells were washed and lysed in 150μl PBS/0.05% Triton (Sigma-Aldrich) by two freeze/thaw cycles at -80°C for 15 min. Luciferase activity was measured in 10 μl of cell lysate with the Victor 2 multi-task luminometer (Perkin Elmer Optoelectronics, Fremont, CA, USA) and luciferase assay reagent (Luciferase assay substrate and buffer, Promega Corporation, Madison, WI, USA).

In vivo study on the effect of absence of Gdf5 in LPS Toll-like signaling Gdf5−/−Bp-J mice and WT mice were injected with a lethal dose of LPS (Sigma-Aldrich) i.p. (30mg/kg) and lethality was checked every 3 hours for 36 hours.

Results
In vitro LPS responsiveness of Gdf5−/−Bp-J and WT macrophages Gdf5−/−Bp-J macrophages showed no difference in TNF-α expression upon LPS stimulation (10 and 100 ng/ml) as measured by ELISA and by indirect luciferase assay in comparison with WT macrophages (Figs. 1A and B).

Stimulation of macrophages with 10 and 100 ng/ml did not induce cell death, as demonstrated by trypan blue staining (data not shown), but increased the secretion of TNF-α in the supernatant; however no dose-dependent difference could be distinguished between 10 and 100 ng/ml LPS by ELISA (Fig. 1A).

When LNF-lucC3 cells, TNF-reporter cells, were stimulated with 50μl supernatant of 10 ng/ml and 100 ng/ml LPS stimulated macrophages a significantly increased luciferase activity was seen in comparison with control situation (medium alone) or 50μl supernatant of 0 ng/ml LPS stimulated macrophages (data not shown). Stimulation of LNF-lucC3 cells with different amounts (50, 10, 2 μl) of supernatant from 10 ng/ml LPS stimulated macrophages from Gdf5−/−Bp-J mice and WT mice, showed a dose-dependent response and no difference in response between Gdf5−/−Bp-J mice and WT mice (Fig. 1B) was found.

In vivo LPS responsiveness of Gdf5−/−Bp-J and WT mice Gdf5−/−Bp-J mice died even earlier than WT mice probably due to lack of resistance or loss of weight. These data suggest that Gdf5−/−Bp-J mice are good responders to LPS (Fig. 2).

Discussion
In the present study, we demonstrated that Gdf5−/−Bp-J mice respond normally in vitro and in vivo to LPS stimulation. Gdf5−/−Bp-J mice show a normal macrophage function and normal immune responses in presence of LPS. Since LPS-toll-like receptor signaling plays an important role in the induction and progression of arthritic diseases and/or their models, these data imply that Gdf5−/−Bp-J mice and Gdf5−/+ mice can be used in arthritis models that are dependent on LPS boost.

LPS has been proven to be an important adjuvant in arthritic models. In collagen induced arthritis (CIA) LPS accelerates the onset of disease and exacerbates the clinical severity scores [7]. LPS enhances the antibody production and T-cell responses to collagen type II (CII), and induces a more chronic arthritis that progresses spontaneously (8). Moreover, when CIA is gradually subsided, LPS markedly reactivates arthritis on a dose-related fashion (9), suggesting that LPS may play an important role in the reactivation of inflammatory joint diseases such as rheumatoid arthritis in humans.

In antibody CIA a single injection of LPS reduces the threshold values of the arthritogenic dose of mAb and the number of mAb required for inducing arthritis (10).

LPS evokes different responses by activating a complex signaling cascade. LPS binds to LBP, whereupon this complex can be recognized by CD14. CD14 will mediate the LPS responses by recruiting different signal-transducing molecules, such as heat-shock protein (Hsp)70, Hsp90, CXCR4 and GDF5 (6, 11). GDF5 might exert its effect by working directly or indirectly on the cellular constituents of the vascular cell wall or by inducing angiogenesis and thereby contributing to septic shock; however no prove of principle was shown (11). Eventually, MD-2 and TLR-4 are recruited into the activation cluster, leading to the activation of NF-kB and the production of proinflammatory cytokines.

TLR-4 is shown to be the signaling receptor for LPS, in a complex where the signal-transducing molecules help in bringing the LPS to the receptor. TLR-4 plays a pivotal role in the pathogenesis of arthritis, involving different cell types. Tlr-4−/− mice fail to develop a severe arthritis (12). In contrast, OA patients have chondrocytes with an increased expression of TLR-4 at their cell surface and stimulation with LPS results in an increased expression of catabolic factors and an increased release of proteoglycans and CI degradations products (13). In rheumatoid arthritis, macrophages are increasingly activated via TLR-4 and they react over-responsive to LPS stimulation compared to healthy macrophages (14). In addition, it is shown that RA synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-4 activation (15).

References
9. Yoshino S, Ohsawa M: The role of lipo-


14. KOWALSKI ML, WOLSKA A, GRZEGORCZYK J *et al.*: Increased responsiveness to toll-like receptor 4 stimulation in peripheral blood mononuclear cells from patients with recent onset rheumatoid arthritis. *Mediators Inflamm* 2008; 132732.