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

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ABSTRACT

Objectives. *Growth and differentiation factor 5 (GDF5), member of 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$^{Bp\text{-}J/Bp\text{-}J}$ mice and wild type (WT) mice to LPS.*

Methods. *Peritoneal macrophages from Gdf5$^{Bp\text{-}J/Bp\text{-}J}$ 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 an indirect luciferase assay using LNF-luc C3 cells. Gdf5$^{Bp\text{-}J/Bp\text{-}J}$ mice and WT mice were injected with LPS i.p. (30mg/kg) and LPS induced lethality was checked every 3 hours for 36 hours.*

Results. *Gdf5$^{Bp\text{-}J/Bp\text{-}J}$ macrophages showed no difference in TNF expression upon LPS stimulation measured by ELISA and by indirect luciferase assay. Gdf5$^{Bp\text{-}J/Bp\text{-}J}$ 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.*

Introduction

Growth and differentiation factor-5 (GDF5), is a member of the TGFβ superfamily. GDF5 has been implicated in limb development, and is known to play an important role in formation of joint and joint-associated structures (1, 2). Postnatally, GDF5 is expressed in different joint structures like articular cartilage, bone, synovium, tendons, ligaments, and menisci (2). Absence of Gdf5 leads to brachypodism in mice and a number of skeletal malformation syndromes in humans (3, 4). Brachypodism is induced by missense mutation in *Gdf5* gene, resulting in complete loss of function, inducing severe defects in the developing appendicular skeleton; in *Gdf5$^{Bp\text{-}J/Bp\text{-}J}$* the proximal interphalangeal joints are absent, bones in wrist and ankle are fused (3). Recently, an association was shown between osteoarthritis (OA) and a 5' UTR polymorphism in *GDF5* gene, suggesting that GDF5 may play an important role in arthritic diseases (5).

In addition, the role of GDF5 may reach beyond the musculoskeletal system. GDF5 appears to be present in a lipopolysaccharide (LPS) receptor cluster (6). LPS is a component of the outer membrane of Gram-negative bacteria and is a potent activator of monocytes and macrophages in the inflammatory response. Moreover, LPS triggers the secretion of many cytokines from macrophages including interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, which can lead to cardiovascular collapse and hemodynamic instability and eventually can result in a fatal sepsis syndrome. In general, recognition of LPS requires binding of LPS to the LPS-binding protein (LBP), so that the LPS-LBP complex can be recognized by CD14, a 55-kD glycosyl-phosphatidylinositol–linked, cell surface receptor. The CD14 receptor does not transverse the cell membrane, but mediates LPS responses by recruiting different signal-transducing molecules, such as heat-shock protein (Hsp)70, Hsp90, chemokine (CXC motif) receptor (CXCR)- 4 and GDF5 (6). Eventually, myeloid differentiation protein (MD)-2 and toll-like receptor (TLR)-4 are recruited into the activation cluster, leading to the activation of nuclear factor kappa B (NF-κB) and the production of proinflammatory cytokines.

Since GDF5 is part of the 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. In this study we compared the sensitivity of *Gdf5$^{Bp\text{-}J/Bp\text{-}J}$* mice and WT mice to LPS;

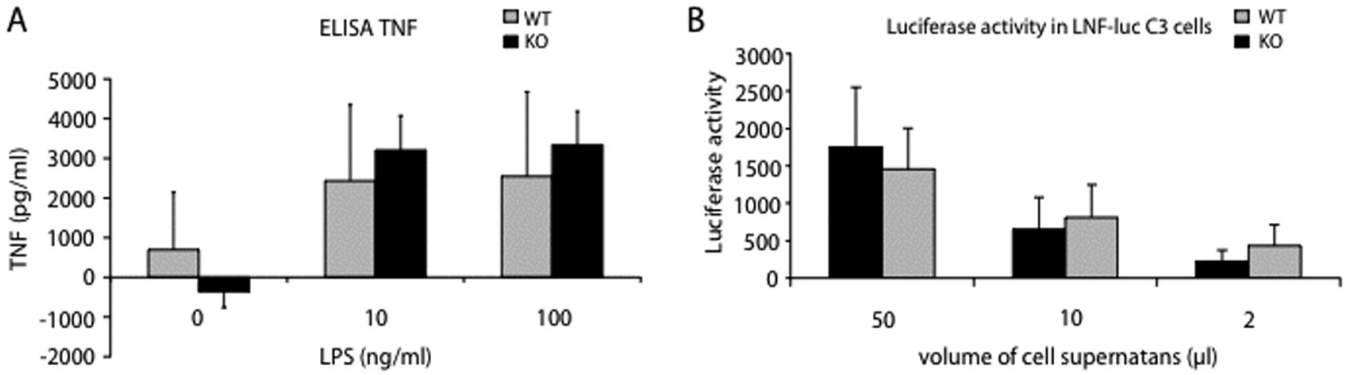

Fig. 1. *In vitro* LPS responsiveness of *Gdf5*$^{Bp\text{-}J/Bp\text{-}J}$ (KO) *and Gdf5*$^{+/+}$ (WT) macrophages. (A) Enzyme linked immunosorbent assay (ELISA) for TNF-α on supernatant of *Gdf5*$^{Bp\text{-}J/Bp\text{-}J}$ and WT macrophages stimulated with 0, 10 or 100 ng/ml LPS during 18h. Data are presented as mean ± stdev (ELISA performed in duplicate; n=3 per group/ condition). (B) Indirect luciferase assay, cell lysate of LNF lucC3 cells stimulated with 50, 10 or 2 µl of supernatant of *Gdf5*$^{Bp\text{-}J/Bp\text{-}J}$ and WT macrophages stimulated with 10 ng/ml LPS during 18h, in presence of luciferase substrate. Data are presented as mean ± stdev (n=3 per group).

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*$^{Bp\text{-}J/Bp\text{-}J}$ mice and wild type (WT) littermates (*Gdf5*$^{+/+}$) 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'-ATTTGTTCAGCCAGCGGCG-3') and reverse primer p2 (5'-CTCAAGAGGTGCGAT-GATCCAGTC-3'). Sequencing of PCR product was performed at the VIB Genetic Service Facility (Antwerp, Belgium) using reverse primer p2 to determine absence (WT) or presence of a single (HZ or *Gdf5*$^{Bp\text{-}J/+}$) or double (KO or *Gdf5*$^{Bp\text{-}J/Bp\text{-}J}$) cysteine insertion.

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

Peritoneal macrophages from *Gdf5*$^{Bp\text{-}J/Bp\text{-}J}$ mice and WT mice were collected 4 days after thioglycolate-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 10^6 cells/ml in RPMI-1640 (Gibco, Invitrogen, Merelbeke, Belgium) with 10% Fetal Bovine Serum (FBS) (Biochrom Ag, VWR, Leuven, 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

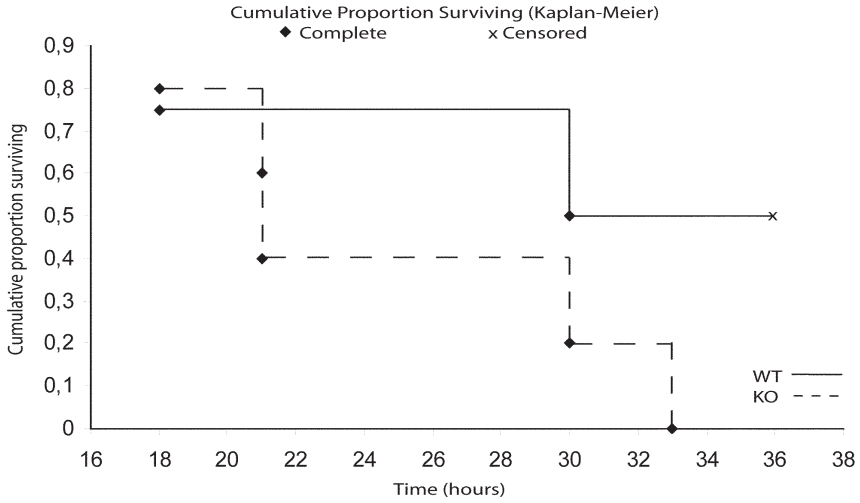

Fig. 2. *In vivo* LPS responsiveness of *Gdf5*$^{Bp\text{-}J/Bp\text{-}J}$ (KO (dashed line)) mice and *Gdf5*$^{+/+}$ (WT (full line)) mice. A Kaplan-Meier survival curve is shown, demonstrating the cumulative proportion of surviving mice after a certain amount of time. '◆' presents a mouse that died, '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$^{Bp\text{-}J/Bp\text{-}J}$* 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 substate and buffer, Promega Corporation, Madison, WI, USA).

In vivo *study on the effect of absence of Gdf5 on LPS Toll-like signaling*
Gdf5$^{Bp\text{-}J/Bp\text{-}J}$ 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$^{Bp\text{-}J/Bp\text{-}J}$ and WT macrophages*
Gdf5$^{Bp\text{-}J/Bp\text{-}J}$ 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$^{Bp\text{-}J/Bp\text{-}J}$* mice and WT mice, showed a dose-dependent response and no difference in response between *Gdf5$^{Bp\text{-}J/Bp\text{-}J}$* mice and WT mice (Fig. 1B) was found.

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

Discussion

In the present study, we demonstrated that *Gdf5$^{Bp\text{-}J/Bp\text{-}J}$* mice respond normally *in vitro* and *in vivo* to LPS stimulation. *Gdf5$^{Bp\text{-}J/Bp\text{-}J}$* 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 implicate that *Gdf5$^{Bp\text{-}J/Bp\text{-}J}$* mice and *Gdf5$^{Bp\text{-}J/+}$* 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-κB 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 CII degradation products (13). In rheumatoid arthritis, macrophages are increasingly activated via TLR-4 and they react over-responsively 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

1. FRANCIS-WEST PH, PARISH J, LEE K, ARCHER CW: BMP/GDF-signalling interactions during synovial joint development. *Cell Tissue Res* 1999; 296: 111-9.
2. MIKIC B: Multiple effects of GDF-5 deficiency on skeletal tissues: implications for therapeutic bioengineering. *Ann Biomed Eng* 2004; 32: 466-76.
3. STORM EE, HUYNH TV, COPELAND NG, JENKINS NA, KINGSLEY DM, LEE SJ: Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. *Nature* 1994; 368: 639-43.
4. LUYTEN FP: Cartilage-derived morphogenetic protein-1. *Int J Biochem Cell Biol* 1997; 29: 1241-4.
5. MIYAMOTO Y, MABUCHI A, SHI D *et al.*: A functional polymorphism in the 5' UTR of GDF5 is associated with susceptibility to osteoarthritis. *Nat Genet* 2007; 39: 529-33.
6. TRIANTAFILOU K, TRIANTAFILOU M, DEDRICK RL: A CD14-independent LPS receptor cluster. *Nat Immunol* 2001; 2: 338-45.
7. CACCESE RG, ZIMMERMAN JL, CARLSON RP: Bacterial lipopolysaccharide potentiates type II collagen-induced arthritis in mice. *Mediators Inflamm* 1992; 1: 273-9.
8. TERATO K, YE XJ, MIYAHARA H, CREMER MA, GRIFFITHS MM: Induction by chronic autoimmune arthritis in DBA/1 mice by oral administration of type II collagen and Escherichia coli lipopolysaccharide. *Br J Rheumatol* 1996; 35: 828-38.
9. YOSHINO S, OHSAWA M: The role of lipo-

polysaccharide injected systemically in the reactivation of collagen-induced arthritis in mice. *Br J Pharmacol* 2000; 129: 1309-14.
10. TERATO K, HARPER DS, GRIFFITHS MM *et al*.: Collagen-induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity* 1995; 22: 137-47.
11. TRIANTAFILOU M, TRIANTAFILOU K: Lipo-polysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Tr*ends Immunol* 2002; 23: 301-4.
12. LEE EK, KANG SM, PAIK DJ, KIM JM, YOUN J: Essential roles of Toll-like receptor-4 signaling in arthritis induced by type II collagen antibody and LPS. *Int Immunol* 2005; 17: 325-33.
13. KIM HA, CHO ML, CHOI HY *et al*.: The catabolic pathway mediated by Toll-like receptors in human osteoarthritic chondrocytes. *Arthritis Rheum* 2006; 54: 2152-63.
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.
15. KIM KW, CHO ML, LEE SH *et al*.: Human rheumatoid synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-2 and TLR-4 activation. *Immunol Lett* 2007; 110: 54-64.