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TNFRp55 modulates IL-6 and nitric oxide responses following *Yersinia* lipopolysaccharide stimulation in peritoneal macrophages

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ABSTRACT

While cytokines are major regulators of macrophage activation following host–pathogen interactions, they also act to limit inflammation to avoid tissue damage. In previous studies we reported the development of progressive *Yersinia enterocolitica*-induced reactive arthritis (ReA) in mice lacking the tumor necrosis factor receptor p55 (TNFRp55). In this work, we analyzed the response of *TNFRp55*$^{-/-}$ macrophages to *Y. enterocolitica* antigens. We found higher concentration of nitric oxide (NO) in *TNFRp55*$^{-/-}$ compared to wild-type macrophages in response to heat-killed *Yersinia* (HKY) and *Yersinia* outer membranes (OM). Moreover, Toll-like receptor (TLR)4 expression was increased in OM-stimulated *TNFRp55*$^{-/-}$ versus wild-type (WT) macrophages. Accordingly, NO production was inhibited in TLR4-deficient macrophages following stimulation with OM, suggesting that LPS may function as a major OM component implicated in these responses. Thus, augmented NO production together with enhanced expression of inducible nitric oxide synthase (iNOS) and higher IL-6 production, may provide a pro-inflammatory setting in *Yersinia* LPS-stimulated *TNFRp55*$^{-/-}$ macrophages. Augmented synthesis of NO and IL-6 was prevented by treatment with Polymyxin B, or by exposure to a specific NF-κB p65 oligonucleotide antisense, indicating the involvement of TLR4-mediated NF-κB activation in the unleashed pro-inflammatory response triggered by TNFRp55 deficiency. Thus, TNFRp55 modulates macrophage functions in response to *Yersinia* LPS stimulation through mechanisms involving NO, IL-6 and NF-κB pathways, suggesting an essential regulatory role of TNF via TNFRp55 signaling.

Introduction

The ability to recognize a wide range of endogenous and exogenous ligands and to respond appropriately has placed macrophages as central cells able to influence homeostasis, inflammation as well as host defenses in innate and acquired immunity (Geissmann et al. 2010). Monocytes leave the unique environment of the bone marrow and access the blood and peripheral tissues, where they are exposed to a plethora of environmental signals, including cytokines, chemokines and soluble immunoregulatory factors, which are capable of impacting their phenotypic and functional characteristics (Geissmann et al. 2010). Tissue macrophages are characterized by their ability to phagocytose a wide variety of pathogenic microorganisms making them accessible for recognition by effector T cells (Silva 2010). Moreover, highly activated macrophages are the major effectors of tissue damage during autoimmune diseases (Fairweather and Cihakova 2009), and macrophage-derived cytokines are critical mediators of a variety of chronic inflammatory diseases (Simmonds and Foxwell 2008). Yet, macrophages also contribute to tissue homeostasis by producing a wide array of regulatory mediators (Silva 2010).

Tumor necrosis factor (TNF) is a pleiotropic cytokine that plays pivotal roles in the initiation and orchestration of inflammatory responses (Feldmann et al. 2004). Signaling pathways triggered by TNF have been found to be instrumental in activating macrophages in the protective host response to *Yersinia enterocolitica*, a Gram-negative extracellular bacteria, responsible of food-borne acute and chronic gastrointestinal diseases (Di Genaro et al. 2003). However, the precise biological mechanisms underlying the effects of TNF in *Yersinia* infection or sequelae of this infection,

Abbreviations: HKY, heat-killed *Yersinia*; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; OM, outer membrane; PMB, Polymyxin B; ReA, reactive arthritis; RNI, reactive nitrogen intermediates; SON, *Y. enterocolitica* O:3 disrupted by sonication; TLR, Toll-like receptor; TNFRp55, TNF receptor p55; WT, wild-type.

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including reactive arthritis (ReA), are still uncertain. TNF exerts its biological activities via two distinct receptors, namely TNFRp75 and TNFRp55; the latter being responsible of most cytokine effects (Peschon et al. 1998; Vandenabeele et al. 1995). Although TNF is a major proinflammatory mediator, increasing evidence indicates that this cytokine has also immunosuppressive effects (Aggarwal 2003; Kim and Moudgil 2008). In previous work, we demonstrated that TNFRp55-mediated immune mechanisms prevent evolution to ReA following oral infection with *Y. enterocolitica* O:3 as *TNFRp55*$^{-/-}$ mice developed severe and chronic arthritis when compared to wild-type (WT) mice (Di Genaro et al. 2007). Yet, the cellular and molecular mechanisms underlying this increased pro-inflammatory effect remain uncertain.

Outer membranes (OM) of Gram-negative bacteria have been shown to modulate a variety of macrophage functions, including cytokine production (Jubier-Maurin et al. 2001). Lipopolysaccharides (LPS) are integral components of this OM which initiate pro-inflammatory signaling pathways following binding to monocytes and macrophages (Liu et al. 2001). We demonstrated that *Yersinia* OM contain relevant antigens which may play essential roles in *Yersinia*-induced ReA in *TNFRp55*$^{-/-}$ mice (Di Genaro et al. 2007). However, it remains unclear whether OM components induce hyperactivation of *TNFRp55*$^{-/-}$ macrophages.

Toll-like receptor (TLR)2 and TLR4 have been found to be the key signaling molecules which mediate biological responses of OM components. LPS binding to TLR4 and lipoprotein binding to TLR2 trigger macrophage activation and release of pro-inflammatory mediators such as TNF and IL-6, reactive oxygen species (ROS) and nitric oxide (NO) (Schmitt et al. 2009; Beutler et al. 2003).

In this study, we investigated the modulation of cytokine responses and NO pathway in TNFRp55-deficient macrophages exposed to *Y. enterocolitica* O:3 antigens and examined the main OM components and TLRs associated with these pro-inflammatory responses. Our findings suggest that the TNF-TNFRp55 signaling pathway modulates the proinflammatory response of macrophages following *Yersinia* infection, including IL-6 secretion and NO production. This effect involves engagement of the TLR4/NF-κB signaling pathway. Collectively, our findings provide further support for the involvement of macrophages as key cellular components of the aberrant arthritogenic response observed in *TNFRp55*$^{-/-}$ mice following *Yersinia* infection.

Materials and methods

Mice

TNFRp55$^{-/-}$ (C57BL/6) were obtained from the Max von Pettenkofer Institute, Munich, Germany. TLR4 deficient mice (*TLR4*$^{-/-}$) were kindly provided by Dr Mariana Maccioni (CIBICI, National University of Cordoba, Argentina). C57BL/6 WT mice were purchased from the Faculty of Veterinary Sciences of the National University of La Plata (Argentina). Breeding colonies were established at the Animal Facility of the National University of San Luis (San Luis, Argentina). Mice were kept under specific-pathogen-free conditions in positive-pressure cabinets (EHRET, Emmendingen, Germany) and provided with sterile food and water *ad libitum*. Male mice (6–8-week old) were used for the experiments. At least two independent experiments were carried out with three to four mice per group.

Bacteria strain and Yersinia antigen preparation

Y. enterocolitica O:3 strain MHC 700 was kindly provided by Dr. Georg Kapperud (Department of Bacteriology, Oslo, Norway). Bacteria were cultured overnight in Luria broth at 26 °C, harvested during the log phase, and frozen in 1 ml aliquots at −80 °C. Heat-killed *Yersinia* (HKY) consisted in a twice autoclaved bacterial suspension (1×10^{10} bacteria/ml), in which the absence of bacteria growth was tested by plating on Mueller–Hinton agar and incubation at 26 °C for 48 h. OM were obtained from the whole *Y. enterocolitica* O:3 disrupted by sonication (SON) as described by Michiels et al. (1990). HKY and OM were obtained from bacteria cultured overnight at 26 °C and then at 37 °C for 3 h. LPS was extracted from a *Y. enterocolitica* O:3 culture at 26 °C by phenol-water method, purified, resuspended in pyrogen-free saline solution and quantified as previously described (Di Genaro et al. 2000). Protein concentration was determined by the Lowry method in OM and LPS preparations. Different OM preparations showed protein concentrations ranging from 1000-2500 μg/ml. LPS contained less than 0.5% protein and did not show bands in Coomasie blue-stained SDS-PAGE gels, although characteristic LPS bands were observed in silver-stained SDS-PAGE gels (Tsai and Frasch 1982).

Stimulation of peritoneal macrophages

Elicited peritoneal macrophages were obtained from different groups of mice 4 days after intraperitoneal inoculation with 4% thioglycollate broth. Cells were harvested using 5–10 ml of sterile pirogen-free saline, washed twice by centrifugation at 200 × *g* for 10 min at 4 °C and resuspended in Dulbecco's modified Eagle's medium (DMEM) (HyClon) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicine. This suspension was then added to each well of a 24-well culture dish to obtain 2×10^6 cells/well. Cells were then incubated at 37 °C in a humidified atmosphere under 5% CO_2 for 24 h, and after washing three times with saline, adherent cells were incubated without (unstimulated) or with HKY (1×10^6, 1×10^7 and 1×10^8 bacteria/ml), *Yersinia* OM (5, 10 and 50 μg), *Yersinia* LPS (10, 100 and 1000 ng) or zymosan (10 μg) (Sigma, San Louis, USA). Culture supernatants were obtained and immediately used for NO determination, or stored at −20 °C until cytokine measurement.

NO measurement and iNOS expression

Nitrite production, as the stable end product of NO metabolism, was measured in the culture supernatants from 24 h unstimulated and stimulated macrophages using the Griess reagent (Green et al. 1982). Supernatants from duplicate cultures were assayed in duplicate and concentrations shown as μM calibrated against solutions of known nitrites concentrations. Fifty microliters of supernatants were mixed in 96-well plates with 100 μl of 1.5% sulfamide in 1 N chloride acid and 5.5 nM naphtylenediamide. A standard curve (0–40 μM) of $NaNO_2$ in saline was prepared and read together with the samples at 550 nm in a microplate reader (Bio-Rad, New York, USA).

iNOS expression was studied by Western blot analysis of lysates obtained from *Yersinia* LPS-stimulated macrophages as described (Correa et al. 2003). Cell extracts were loaded at the same protein concentration on a 12% SDS-PAGE. Resolved proteins were transferred onto nitrocellulose membranes (Bio-Rad New York, USA). which were blotted with a rabbit polyclonal antibody against iNOS (Santa Cruz Biotechnology, USA) diluted 1:1000, or β-actin (I-19, Santa Cruz, California, USA). After incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody, color was developed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Unstimulated macrophage lysates were used as controls. Equal loading was confirmed by using Ponceau S staining and β-actin immunoreactivity.

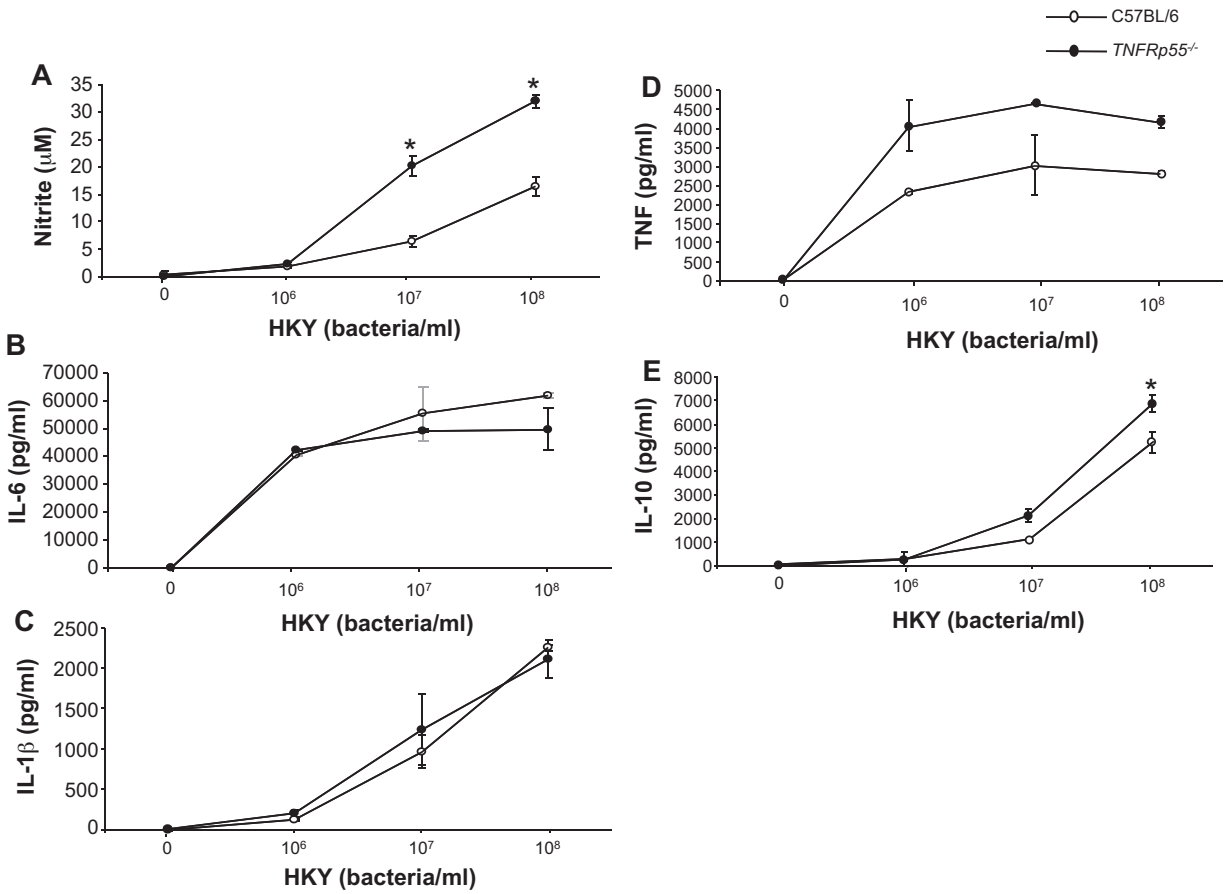

Fig. 1. Increased NO responses induced by HKY in *TNFRp55*$^{-/-}$ macrophages. Peritoneal macrophages from WT (C57BL/6 WT) and *TNFRp55*$^{-/-}$ mice were stimulated with heat killed *Yersinia* (HKY) during 24 h. A: Nitrite production in supernatants of stimulated or unstimulated macrophages from different strains. Asterisks (*$p < 0.01$) indicate significant differences compared to unstimulated macrophages. B, C, D and E: IL-6, IL-1β, TNF and IL-10 in supernatants from *TNFRp55*$^{-/-}$ and WT macrophages. Data were obtained from 3 to 4 mice of each strain and are representative of two independent experiments. Error bars indicate SD.

ELISA for cytokine determination

Mouse IL-6, IL-1β, TNF, and IL-10 were quantified in culture supernatants by using a capture ELISA kit (eBioscience, San Diego, USA) according to the manufacturer's instructions. Supernatants from unstimulated cells were used as controls.

RNA extraction and quantitative RT-PCR

Total cellular RNA was isolated from the cells using TRIzol (Invitrogen, Carlsbad, USA) and reverse-transcribed using oligodT as primers. Primers specific for IL-6, IL-1β, TNF and GAPDH (Invitrogen, USA), and SYBR Green PCR Master Mix (Applied Biosystems, USA) were used. Analysis was performed using ABI Prism 7500 (Applied Biosystems). The content of the cDNA samples was normalized to GAPDH as endogenous gene, and relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blotting for detection of TLR4

Cell lysates were prepared and supplemented with a protease inhibitor cocktail (P 8340, Sigma). The total protein concentration in cell lysates was measured by using a Qubit-iT Protein assay kit (Invitrogen), according to the manufacturer's instructions. The samples were read by using a Qubit fluorometer (Invitrogen). The samples were resolved by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% semi-skimmed milk in PBS-0.05% Tween and probed with rabbit anti-mouse TLR4 (clone M-300, Santa Cruz) or β-actin (I-19, Santa Cruz). The blots were visualized by using peroxidasse-conjugated anti-rabbit IgG (Sigma) and chemiluminescent substrate (Millipore, Billerica, USA). The blots were finally exposed to Kodak BioMax Light film (Sigma).

NF-κB p65 in LPS-induced TLR4 activation

Macrophages (2×10^6 cells/well) were incubated with *Yersinia* LPS (100 ng) in presence or absence of 15 μg Polymyxin B (PMB) (Sigma) or 2 μM NF-κB p65 antisense oligonucleotide (5′-GAGGGGAAACAGATCGTCCATGGT-3′), p65 sense oligonucleotide (5′-ACCATGGACGATCTGTTTC-3′), or p65 nonsense oligonucleotide (5′-GTACTACTCTGAGCAAGGA-3′) for 24 h. Culture supernatants were obtained for NO and IL-6 determinations as described above.

Statistical analysis

Differences between groups were tested for significance by using the Student *t* test for unpaired samples (two-tailed). A *p* value less than 0.05 was considered statistically significant. All experiments were repeated at least twice showing comparative results.

Results

HKY augments NO responses in TNFRp55$^{-/-}$ *macrophages*

To investigate the mechanisms underlying the arthritogenic response in *TNFRp55*$^{-/-}$ mice, we examined the ability of

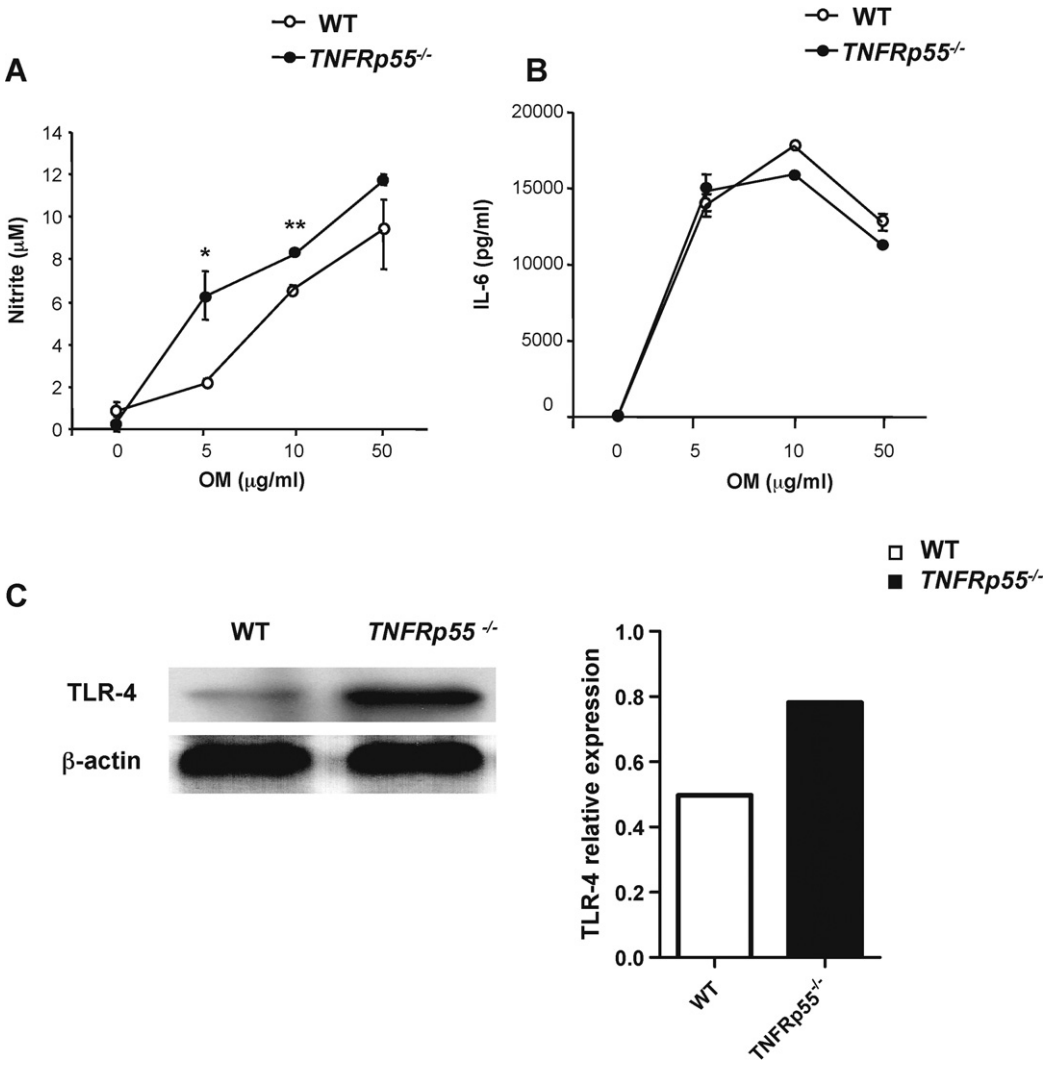

Fig. 2. Increased NO responses in *TNFRp55*$^{-/-}$ macrophages are mediated by *Yersinia* OM antigens. Peritoneal macrophages from WT (C57BL/6 WT) and *TNFRp55*$^{-/-}$ mice were stimulated with *Yersinia* outer membrane (OM) proteins A: Nitrite production was measured in supernatants from WT and mutant macrophages. B: IL-6 in supernatants from mutant and WT macrophages. Asterisks ($^{*}p < 0.05$ and $^{**}p < 0.01$) indicate statistically significant differences among strains. Error bars indicate SD. For A and B, the data were obtained from 3 to 4 mice per strain and are representative of two independent experiments. C: TLR4 expression by Western blot in *Yersinia* OM-stimulated WT or *TNFRp55*$^{-/-}$ macrophages. Values are expressed in arbitrary units as the ratio of TLR4 to the corresponding β-actin levels. Data are from 3 mice examined in one representative of two independent experiments.

macrophages from this mouse strain to generate pro-inflammatory mediators upon bacterial stimulation. For this purpose, we stimulated WT and *TNFRp55*$^{-/-}$ macrophages with different numbers of HKY. Remarkably, HKY induced significantly higher NO production in macrophages lacking TNFRp55 compared to those obtained from WT mice following stimulation with 1×10^7 or 1×10^8 HKY ($p < 0.01$) (Fig. 1A). Although high concentrations of IL-6, IL-1β and TNF secretion were detected in supernatants of 1×10^6 to 1×10^8 HKY-stimulated WT and *TNFRp55*$^{-/-}$ macrophages, differences in cytokine concentrations between these mouse strains did not reach statistical significance (Fig. 1B–D). Real time PCR was performed to examine the concentrations of IL-6, IL-1β and TNF mRNA. In accordance with the cytokine secretion, we demonstrated no statistically significant differences at the transcriptional level between WT and *TNFRp55*$^{-/-}$ macrophages (data not shown). Moreover, IL-10 was slightly higher in *TNFRp55*$^{-/-}$ macrophages after stimulation with 1×10^8 HKY ($p < 0.05$) (Fig. 1E). These results suggest a selective regulatory role of TNFRp55 signaling in macrophage-derived NO production upon stimulation with complete HKY.

Increased NO responses are mediated by OM antigens in TNFRp55$^{-/-}$ *macrophages*

To determine the nature of *Yersinia* antigens capable of stimulating NO production under TNFRp55 deficiency, we stimulated WT and *TNFRp55*$^{-/-}$ macrophages with *Yersinia* OM. Stimulation with OM (5 or 10 μg) induced higher NO production in *TNFRp55*$^{-/-}$ compared to WT macrophages ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 2A). In contrast, OM induced high concentrations of IL-6 in both WT and *TNFRp55*$^{-/-}$ macrophages; yet this effect fail to reach statistically significant differences in mutant compared to WT strains (Fig. 2B), similarly to the results obtained following HKY stimulation. Notably, OM-induced NO production in *TNFRp55*$^{-/-}$ macrophages correlated with higher TLR4 expression when compared with WT macrophages (Fig. 2C). The central role of TLR4 in the recognition of LPS, which is a major Gram-negative OM component, suggested that LPS-TLR4 signaling may be involved in the higher NO production observed in *TNFRp55*$^{-/-}$ macrophages. To examine this possibility, we analyzed nitrite production in OM-stimulated *TLR4*$^{-/-}$ macrophages and compared this effect with the

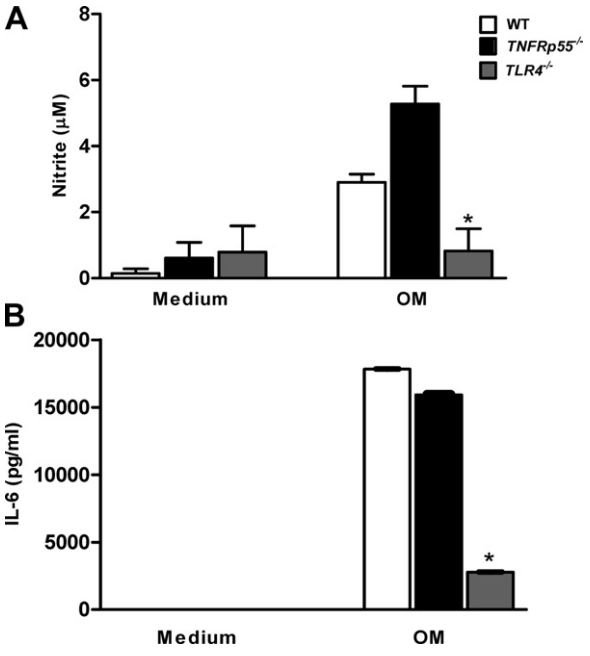

Fig. 3. TLR4 mediates NO and IL-6 production in *TNFRp55*$^{-/-}$ macrophages. Peritoneal macrophages from WT (C57BL/6 WT), *TNFRp55*$^{-/-}$ and *TLR4*$^{-/-}$ mice were stimulated with *Yersinia* outer membrane (OM) (10 μg) during 24 h. A: Nitrite production was measured in supernatants from WT and mutant macrophages. B: IL-6 in supernatants of stimulated macrophages from WT and mutant strains. Unstimulated macrophages (medium) were used as controls. Asterisks (*$p < 0.05$) indicate significant differences between *TLR4*$^{-/-}$ compared with *TNFRp55*$^{-/-}$ and WT macrophages. Error bars indicate SD. Data were obtained from 3 to 4 mice per strain and are representative of two independent experiments.

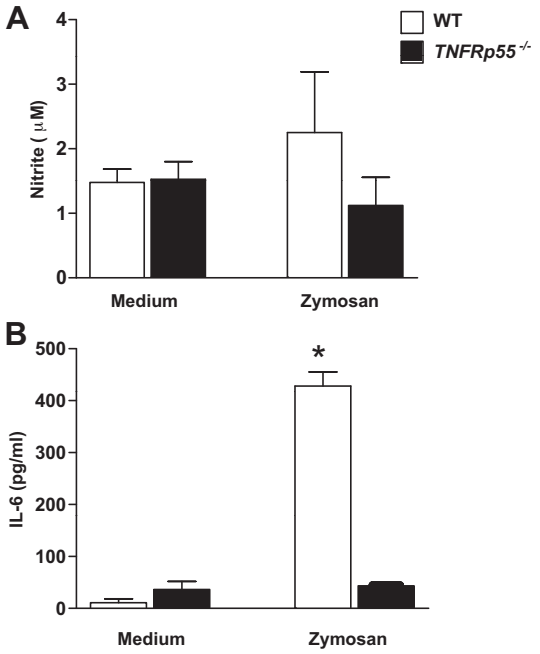

Fig. 4. A TLR2 agonist does not activate NO and IL-6 responses in *TNFRp55*$^{-/-}$ macrophages. Peritoneal macrophages from WT (C57BL/6 WT) and *TNFRp55*$^{-/-}$ mice were stimulated with zymosan (10 μg) for 24 h. A: Nitrite production was measured in supernatants from WT and mutant macrophages. B: IL-6 in supernatants of stimulated macrophages from WT and mutant strains. Unstimulated macrophages (medium) were used as controls. Error bars indicate SD. Asterisks (*$p < 0.01$) indicate significant differences compared with unstimulated macrophages. The data were obtained from 3 mice and are representative of two independent experiments.

response of *TNFRp55*$^{-/-}$ macrophages. Interestingly, not only nitrite secretion (Fig. 3A) but also IL-6 secretion (Fig. 3B) were significantly reduced in *TLR4*$^{-/-}$ macrophages compared with *TNFRp55*$^{-/-}$ and WT macrophages following *Yersinia* OM stimulation ($p < 0.05$ and $p < 0.0001$, respectively). These results suggested that both nitrite and IL-6 secretion are induced following OM LPS stimulation through TLR4 signaling.

To further determine whether other OM components may contribute to NO and IL-6 production, we treated *TNFRp55*$^{-/-}$ macrophages with zymosan, a TLR2 agonist which may recapitulate the effects triggered by porine and lipoproteins present in *Yersinia* OM. Notably, stimulation with zymosan did not induce NO and IL-6 production in *TNFRp55*$^{-/-}$ macrophages, as the concentrations of NO and IL-6 were similar in supernatants of zymosan-stimulated versus unstimulated *TNFRp55*$^{-/-}$ macrophages (Fig. 4A and B). In contrast, WT macrophages secreted higher concentrations of IL-6 after zymosan stimulation ($p < 0.05$) (Fig. 4B). Thus, TLR4 but not TLR2 ligands mediates the increased NO and IL-6 production in *TNFRp55*$^{-/-}$ macrophages.

Yersinia LPS mediates higher NO and IL-6 production in TNFRp55$^{-/-}$ *macrophages*

To confirm the role of *Yersinia* LPS as a trigger of high NO production by *TNFRp55*$^{-/-}$ macrophages, we stimulated *TNFRp55*$^{-/-}$ and WT macrophages with different concentrations of *Yersinia* LPS. As shown in Fig. 5A *TNFRp55*$^{-/-}$ macrophages produced higher concentrations of NO following stimulation with *Yersinia* LPS (100 and 1000 ng) as compared with WT macrophages ($p < 0.01$ and $p < 0.005$, respectively). This effect was accompanied by an increased expression of inducible nitric oxide synthase (iNOS) as shown by Western blot analysis (Fig. 5B). In contrast to *Yersinia* HKY and OM, direct stimulation with LPS not only promoted NO synthesis, but also induced higher concentrations of IL-6 in supernatants of *TNFRp55*$^{-/-}$ macrophages ($p < 0.001$, $p < 0.05$ and $p < 0.01$ for 10, 100 and 1000 ng, respectively) (Fig. 5C).

To determine whether the observed effects were specific to LPS and were not mediated by a potential contaminant in LPS preparation, we stimulated *TNFRp55*$^{-/-}$ macrophages with LPS (100 ng) in the presence of polymixin B (PMB; 15 μg), a specific inhibitor of LPS-induced TLR4 activation. Notably, exposure to PMB successfully prevented LPS-induced increase of NO and IL-6 in *TNFRp55*$^{-/-}$ macrophages ($p < 0.05$ and $p < 0.001$, respectively, versus *TNFRp55*$^{-/-}$ macrophages exposed to LPS alone) (Fig. 6A and B), thus confirming the specificity of *Yersinia* LPS stimulation. Thus, specific interactions between *Yersinia* LPS and TLR4 favor a 'pro-inflammatory macrophage status' in *TNFRp55*$^{-/-}$ macrophages.

NF-κB mediates the pro-inflammatory activities of TNFRp55$^{-/-}$ *macrophages*

To further elaborate on the mechanisms underlying NO and IL-6 production from *TNFRp55*$^{-/-}$ macrophages, we investigated the role of NF-κB, as this transcription factor regulates gene expression and secretion of proinflammatory mediators (Baeuerle and Henkel 1994). In addition, TLR activation, including TLR4, promotes nuclear translocation of NF-κB mainly through the canonical pathway (Kawai and Akira 2007). The heterodimer p65/p50 is the most common NF-κB complex activated through TLR signaling pathways. Given the pro-inflammatory profile imprinted by TNFRp55 deficiency, we next studied whether NF-κB may be involved in NO and IL-6 production by LPS-stimulated *TNFRp55*$^{-/-}$ versus WT macrophages. In an attempt to control IL-6 and NO secretion from *TNFRp55*$^{-/-}$ macrophages, antisense oligodeoxynucleotides targeting the p65 subunit of NF-κB were used based on their

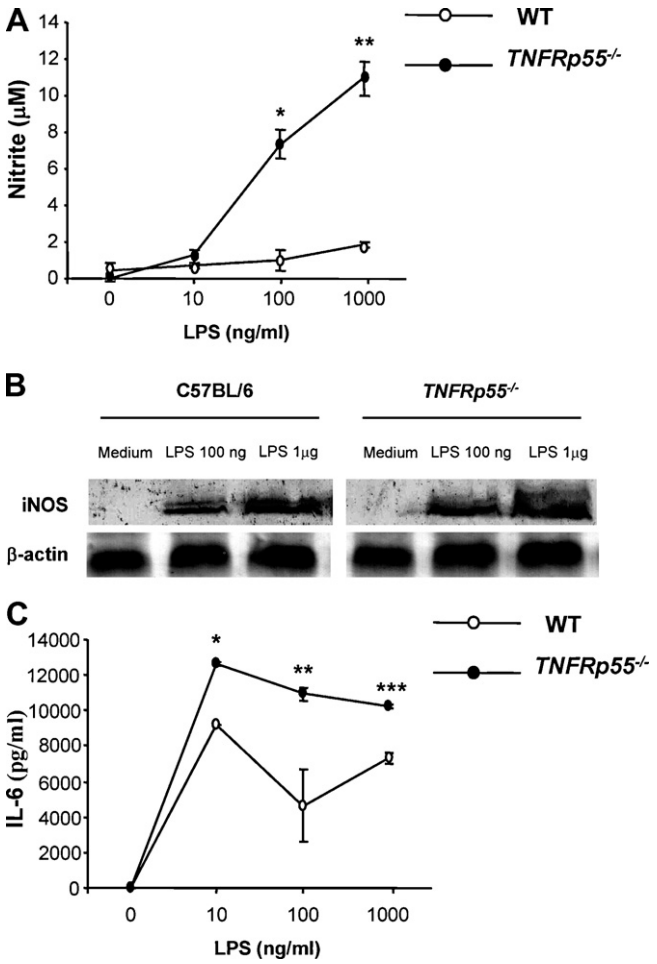

Fig. 5. *Yersinia* LPS induces higher NO and IL-6 productions in *TNFRp55*$^{-/-}$ macrophages. Peritoneal macrophages from WT (C57BL/6 WT) and *TNFRp55*$^{-/-}$ mice (2×10^6 cells/well) were stimulated with different *Yersinia* LPS concentrations during 24 h. A: Nitrite production was measured in supernatants from WT and mutant macrophages. Asterisks ($^*p < 0.01$ and $^{**}p < 0.005$) indicate significant differences. B: Western blot analysis of iNOS expression in lysates of unstimulated, and *Yersinia* LPS (100 ng or 1 μg)-stimulated macrophages using a rabbit polyclonal antibody against iNOS. Equal loading was confirmed by β-actin immunoreactivity. C: Culture supernatant IL-6 levels were determined by ELISA. Asterisks ($^*p < 0.001$, $^{**}p < 0.05$ and $^{***}p < 0.01$) indicate significant differences compared with unstimulated macrophages. Error bars indicate SD. Data were obtained from 3 to 4 mice per strain and are representative of two independent experiments.

ability to suppress NF-κB activation. Direct transfer of 'naked' antisense oligonucleotides can be achieved through passive uptake (Morishita et al. 2004). This experimental approach has often been used in different settings (Morishita et al. 2004; Deng et al. 2000; Ikeda et al. 2004; Schlaak et al. 2001; Neurath et al. 1996). Oligonucleotides were synthesized with a phosphorothioate backbone to improve resistance to endonucleases as previously described (Deng et al. 2000). In addition, a control mismatched oligonucleotide was prepared. Notably, stimulation of macrophages with *Yersinia* LPS in the presence of an NF-κB-p65 antisense oligonucleotide which substantially blocked NF-κB-p65 nuclear translocation and binding to consensus sequences, significantly decreased both NO and IL-6 production, as compared with cultures performed in the presence of a control sense or nonsense oligonucleotide ($p < 0.05$) (Fig. 6C and D). Thus, TNFRp55 deficiency confers a pro-inflammatory status to *Yersinia* LPS-stimulated macrophages through mechanisms involving NO and IL-6 secretion, as well as activation of the NF-κB signaling pathway (Fig. 7).

Discussion

The paradoxical role of TNF receptors acting as amplifiers, silencers or modulators of inflammatory responses remains poorly understood. In the present study, we found that TNFRp55-deficient macrophages are hyperactivated to secrete common pro-inflammatory mediators such as NO and IL-6 following stimulation with *Yersinia* antigens, suggesting that TNFRp55 signaling negatively regulates inflammatory pathways such as those mediated by TLRs (Fig. 7). Accordingly, increasing evidence has been accumulated in support of the regulatory or inhibitory attributes of pro-inflammatory cytokines (Kim and Moudgil 2008; Kelchtermans et al. 2008). In this regard, our recent studies highlighted an immunoregulatory role for TNFRp55 signaling in modulating Th1 and Th17 differentiation (Eliçabe et al. 2010). Thus, TNF like many other cytokines and mediators may display paradoxical pro- or anti-inflammatory effects. In this regard, we have recently demonstrated a 'double-edge' immunoregulatory effect of galectin-1, an endogenous glycan-binding protein, in modulating the pro-inflammatory or inhibitory activity of macrophages and dendritic cells (Correa et al. 2003; Barrionuevo et al. 2007; Ilarregui et al. 2009).

In this study we found that TNFRp55-deficient macrophages respond to HKY stimulation by synthesizing NO, suggesting that a key antimicrobial effector system such as reactive nitrogen intermediates (RNI) (Degrandi et al. 2009) is still operational in macrophages lacking *TNFRp55*. Accordingly, Endres et al. demonstrated the presence of RNI in *Listeria*-infected *TNFRp55*$^{-/-}$ macrophages (Endres et al. 1997). However, we detected higher doses of NO production following *in vitro* stimulation with *Yersinia* antigens in *TNFRp55*$^{-/-}$ versus WT macrophages, clearly indicating that, at least in this model, TNFRp55 signaling may act as a critical regulatory pathway. In contrast, the amounts of IL-6, TNF and IL-1β found in culture supernatants of HKY-stimulated *TNFRp55*$^{-/-}$ macrophages were similar to the amounts secreted by WT macrophages, suggesting that TNFRp55 deficiency results in selective pro-inflammatory phenotypes. In addition, in the absence of TNFRp55 signaling, a negative feed-back loop mediated by IL-10 was activated only at high HKY concentration being close to that found in WT macrophages. However, while *Yersinia* OM stimulation showed similar effects on IL-6 secretion, direct stimulation with *Yersinia* LPS induced higher levels of both NO and IL-6 in *TNFRp55*$^{-/-}$ macrophages. These results indicate that other TLR agonists present in HKY and OM may mediate TNFRp55-independent signals leading the cytokine secretion to the levels reached by WT macrophages. Since it is well known that several factors of *Yersinia* are regulated by temperature, and HKY and OM were prepared from *Yersinia* cultured with temperature switch from 25 °C to 37 °C, the presence of theses factors could also influence cytokine secretion when HKY or OM were used as stimuli. Thus, TNFRp55 may control *Yersinia*-induced NO production through diverse TLR signaling pathways, while both NO and IL-6 production are controlled by TNFRp55 when TLR4 is activated. Remarkably, increased NO production was in accordance with higher iNOS expression in the absence of TNFRp55, suggesting that TNFRp55 may regulate iNOS activity and consequently RNI in *Yersinia*-stimulated macrophages. To our knowledge this is the first study documenting the regulatory role of TNFRp55 in macrophages in response to *Yersinia* antigens.

Expression of TLR4 on macrophages regulates the magnitude of the response to LPS (Arranz et al. 2008). In the present study, TLR4 was expressed at high levels in *TNFRp55*$^{-/-}$ macrophages, and this expression was induced in response to *Yersinia* OM stimulation. This up-regulated expression may operate as an innate immune mechanism to facilitate bacterial antigen recognition (Dennis et al. 2009). Moreover, OM-stimulated *TLR4*$^{-/-}$ macrophages showed strong reduction in both nitrite and IL-6 secretion compared with

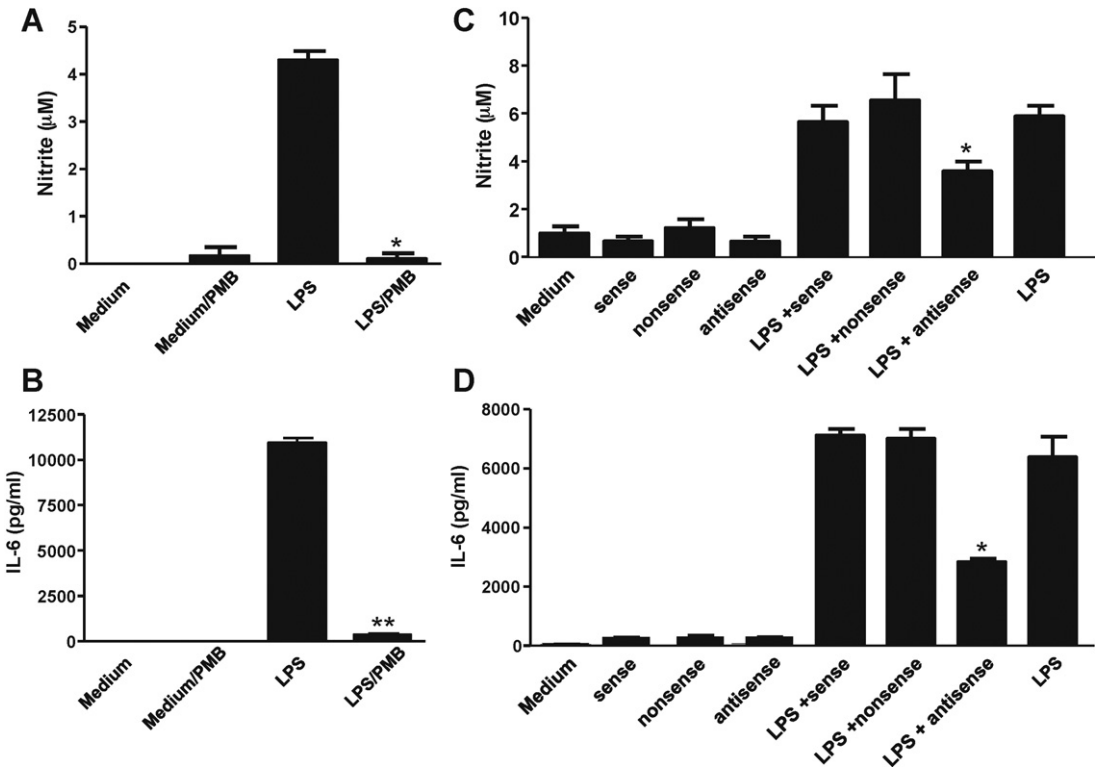

Fig. 6. TLR4 signaling and NF-κB mediates hyperactivation of *TNFRp55*$^{-/-}$ macrophages. Peritoneal macrophages from *TNFRp55*$^{-/-}$ mice were stimulated with *Yersinia* LPS (100 ng) for 24 h in the presence or absence of 15 μg Polymyxin B (PMB) (A and B) or 2 μM NF-κB p65 antisense oligonucleotide (OGN), control sense or nonsense OGN (C and D). A and C: Nitrite production was measured in supernatants from macrophages exposed to different treatments. Asterisks indicate significant differences compared with LPS-stimulated macrophages ($^{*}p < 0.05$). B and D: IL-6 in supernatants of macrophages obtained from different strains exposed to different treatments. Asterisks indicate significant differences compared with LPS-stimulated macrophages ($^{*}p < 0.05$ and $^{**}p < 0.001$ in D and B, respectively). Data were obtained from 3 to 4 mice per strain and are representative of two independent experiments. Error bars indicate SD.

TNFRp55$^{-/-}$ macrophages. Taken together, these results indicate that TLR4 is a major pathway activated by OM which is considerably up-regulated in the absence of TNFRp55.

Sensing of LPS determines innate immune responses to Gram-negative bacterial infection (Freudenberg et al. 2008). In order to determine the level of specificity of LPS-TLR4 interactions in our system, we co-cultured LPS with PMB which resulted in a dramatic reduction of NO and IL-6 secretion by LPS-stimulated *TNFRp55*$^{-/-}$ macrophages. Thus, the TLR4 pathway is considerably dysregulated in response to *Yersinia* LPS in TNFRp55-deficient macrophages. This result indicates that TNFRp55 may act as a negative regulator of TLR4 signaling, thus supporting previously published data (Zakharova and Ziegler 2005). Upon ligand interaction with TLRs, intracellular signaling occurs via adaptor proteins, including MyD88, TIRAP/Mal, TRIF and TRAM, which induce a downstream signaling cascade that culminates in nuclear translocation of NF-κB (Doyle and O'Neill 2006; Kawai and Akira 2007). Therefore, we investigated whether silencing of the NF-κB-p65 subunit might impair NO and IL-6 production in *TNFRp55*$^{-/-}$ macrophages. Remarkably, silencing of NF-κB-p65 expression resulted in substantial reduction of NO and IL-6 production by *TNFRp55*$^{-/-}$ macrophages. Importantly, introduction of a p65 antisense oligonucleotide inhibited NF-κB activation as previously demonstrated in other models (Deng et al. 2000; Ikeda et al. 2004; Schlaak et al. 2001; Neurath et al. 1996).

However, we cannot exclude the possibility that TNF, through specific interaction with TNFRp75, might play a role in the hyperactivation of *TNFRp55*$^{-/-}$ macrophages, and that this cytokine might be responsible of the higher concentrations of NO and IL-6 production in response to *Yersinia* LPS. However, given that stimulation of TNFR2 signaling itself induces TNF production (Wajant et al. 2003), and we found similar concentrations of this cytokine in supernatants of both LPS-stimulated *TNFRp55*$^{-/-}$ and WT macrophages, we postulate that this cytokine may not influence the hyperactivation of *TNFRp55*$^{-/-}$ macrophages in response to *Yersinia* LPS. Taken together these findings are consistent with the results observed by Notley et al. (2008), and our recent results (Eliçabe et al. 2010) demonstrating a regulatory role for TNFRp55 on cytokine production following arthritogenic responses.

Finally, the higher concentrations of IL-6 production detected in *Yersinia* LPS-stimulated *TNFRp55*$^{-/-}$ macrophages may be associated with our previous *in vivo* results demonstrating the increased susceptibility of *TNFRp55*$^{-/-}$ mice to *Yersinia*-induced ReA (Di Genaro et al. 2007). Furthermore, higher concentrations of IL-6 were detected in the joints of these mice which showed a severe chronic synovitis (Eliçabe et al. 2010). These results are also consistent with those obtained in other arthritis models (Iwanami et al. 2008). IL-6, together with TGF-β and IL-23, participates in the differentiation of Th17 cells which are major contributors to inflammatory and autoimmune disorders (Ouyang et al. 2008). Remarkably, we also found higher concentrations of IL-17 in the joints of *TNFRp55*$^{-/-}$ mice (Eliçabe et al. 2010), suggesting a critical role of TNFRp55 signaling in attenuating IL-6-dependent Th17 differentiation *in vivo*. Given that macrophages are a major source of IL-6, the present study contributes to partially dissect the underlying mechanisms involved in the development of severe ReA in *Yersinia*-infected *TNFRp55*$^{-/-}$ mice, supporting a central role for macrophages in this process (Di Genaro et al. 2007).

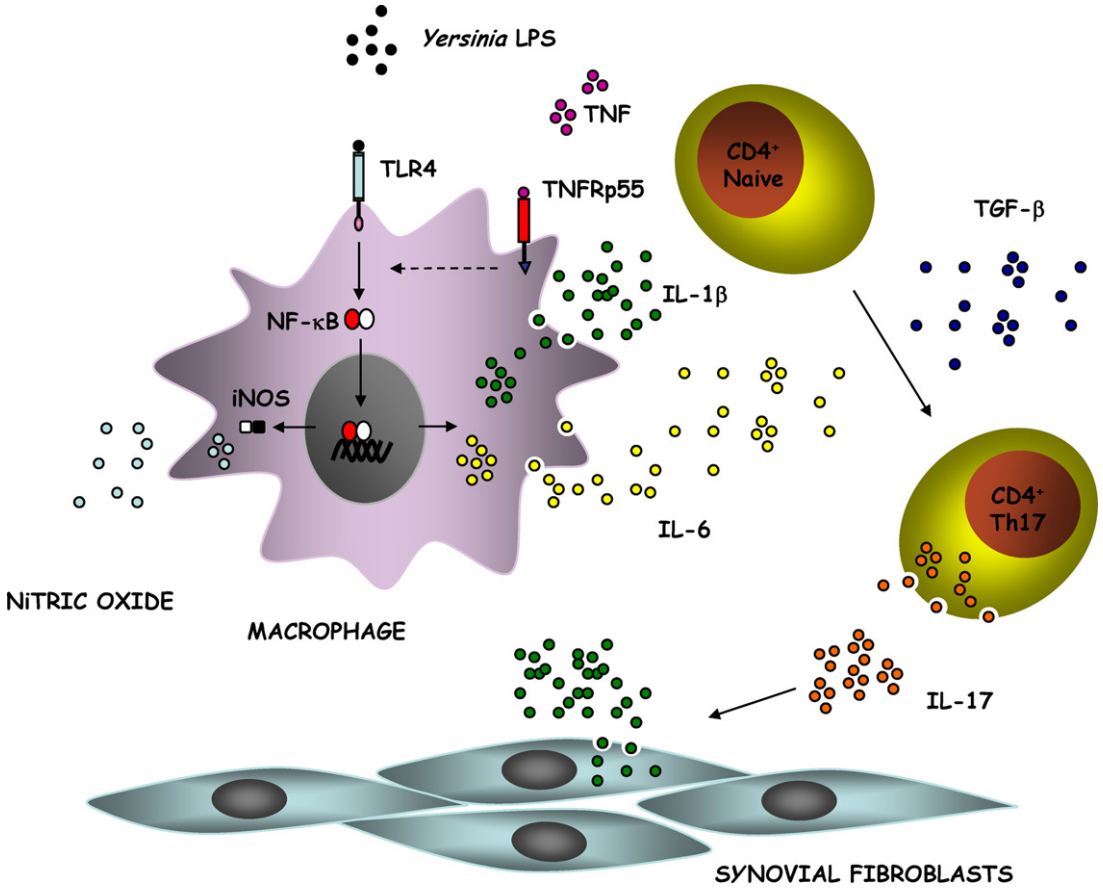

Fig. 7. Hypothetical mechanism underlying TNFRp55 signaling in macrophages. TNFRp55 signaling may control the response of macrophages to LPS stimulation by controlling the production of pro-inflammatory mediators like nitric oxide (NO) and IL-6 in response to TLR4 activation. In the absence of TNFRp55, uncontrolled secretion of IL-6 favors Th17 differentiation then contributes to chronic joint inflammation during reactive arthritis. This proposed mechanism may provide an alternative explanation for the effects of TNFRp55 deficiency in macrophage hyperactivation, suggesting that TNFRp55 signaling may limit macrophage activation and attenuate *Yersinia*-induced reactive arthritis.

In summary, although the molecular mechanisms involved in TNFRp55-dependent modulation of TLR4 signaling remain uncertain, our results support a dynamic cross-talk between these apparently independent pathways, demonstrating that TLR4 modulation by TNFRp55 occurs in macrophages in response to *Yersinia* LPS stimulation. Our findings support the concept of a regulatory role for TNFRp55 signaling in the modulation of macrophage physiology with critical implications in a diversity of inflammatory disorders including *Yersinia*-induced ReA.

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