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# Immune recognition of *Streptococcus pyogenes* by dendritic cells

***Running title:*** MyD88-dependent *S. pyogenes*-induced DCs activation

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**ABSTRACT**

*Streptococcus pyogenes* is one of the most frequent human pathogens. Recent studies have identified dendritic cells (DCs) as important contributors to host defense against *S. pyogenes.* The objective of this study was to identify the receptors involved in immune recognition of *S. pyogenes* by DCs. To determine whether Toll-like receptors (TLRs) were involved in DCs sensing of *S. pyogenes*, we evaluated the response of bone marrow-derived DCs obtained from mice deficient in MyD88, an adapter molecule used by almost all TLRs, following *S. pyogenes* stimulation. Despite of the fact that MyD88$^{-/-}$ DCs did not differ from wild type DCs in their ability to internalize and kill *S. pyogenes*, the up-regulation of maturation markers such as CD40, CD80 and CD86 and the production of inflammatory cytokines such as IL-12, IL-6 and TNF-α was dramatically impaired in *S. pyogenes*-stimulated MyD88$^{-/-}$ DCs. These results suggest that signaling trough TLRs is the principal pathway by which DCs sense *S. pyogenes* and become activated. Surprisingly, DCs deficient in signaling through each of the TLRs reported as potential receptors for gram-positive cell components such TLR1, TLR2, TLR4, TLR9 or TLR2/6 were not impaired in the secretion of proinflammatory cytokines and up-regulation of costimulatory molecules after *S. pyogenes* stimulation. In conclusion, our results excluded a major involvement of a single TLR or the heterodimer TLR2/6 in *S. pyogenes* sensing by DCs and argue for a multimodal recognition in which a combination of several different TLRs–mediated signals are essential for a rapid and effective response to the pathogen.

## INTRODUCTION

*Streptococcus pyogenes* (Group A streptococcus) is an important human pathogen that causes a wide spectrum of diseases, ranging from mild infections such as pharyngitis and superficial skin infections, to severe invasive diseases such as necrotizing fasciitis and sepsis (23). Invasive streptococcal infection is a significant cause of morbidity and mortality (27). Protection from primary streptococcal infection results from innate rather than adaptive immune responses (11, 24) and we have recently shown that dendritic cells (DCs) play an important role in host defense against *S. pyogenes* (22).

Dendritic cells (DCs) are important components of the innate immune system that sense microbial products and initiate immune responses against pathogens (33). Upon recognition of microbes, DCs undergo a maturation process characterized by the production of inflammatory cytokines and chemokines as well as by the up-regulation of costimulatory molecules (3). *In vitro* studies examining the interaction of *S. pyogenes* with DCs have clearly demonstrated the rapid induction of maturation and activation of human (41, 42) as well as murine DCs (22) after exposure to this pathogen. DCs are the main *in vivo* source of IL-12 during infection and this response is critical for the development of protective immune responses against infection with *S. pyogenes* (22). Despite the important contribution of DCs to the initiation of innate immune responses against *S. pyogenes*, nothing is known about the mechanism of immune recognition of *S. pyogenes* by these important antigen-presenting cells.

Recognition of microbes by DCs is mediated by pattern recognition receptors (PRRs), including the toll-like receptor (TLR) family (19, 20, 34). TLRs recognize the presence of microbial pathogens via the detection of conserved microbial structures called pathogen-associated molecular patterns (PAMPs) (1, 2, 6, 36, 37). For example, TLR4 has been reported to function as a receptor for lipopolysaccharide (LPS), an integral component of the outer membranes of gram-negative bacteria (31, 38). TLR2 is reported to be specialized to recognize lipoprotein and lipoteichoic acid (LTA) from diverse species of bacteria (26, 35,

43). TLR6, in combination with TLR2, recognizes zymosan and peptidoglycan (29, 39). TLR9 and TLR5 have been shown to recognize bacterial derived CpG DNA (14) and flagellin (12), respectively. The interaction of TLRs with their target motifs initiates a cascade of intracellular signaling events that culminates in the activation of NFκB and the production of pro-inflammatory cytokines, chemokines and co-stimulatory molecules that are important for host defenses (1, 4, 36). Upon recognition of microbial components, these TLRs associate with several adaptor molecules such as myeloid differentiation factor 88 (MyD88), critical for downstream signaling events (25, 28).

Based on previous studies reporting that several constituents of *S. pyogenes* can be recognized by the TLR family of receptors (29, 18), we hypothesized that this receptor family might be involved in the recognition of the whole microorganism. To investigate this, we evaluated the responses of DCs isolated from animals that lack the common adapter molecule MyD88, which is required for almost all TLR activation cascades, to stimulation with *S. pyogenes*. Our results show that the MyD88 signaling pathway is crucial for *S. pyogenes* induction of maturation and secretion of proinflammatory cytokines by DCs suggesting the participation of TLRs in bacterial sensing. However, using DCs from mice deficient in the expression of TLR1, TLR2, TLR4, TLR2/6 or DCs pretreated with an inhibitor of the TLR9, we have excluded a major involvement of each of these specific TLRs in DC sensing of *S. pyogenes* microorganisms. Taken together, these observations probably reflect the involvement of multiple TLR pathways triggered by different bacterial components in the activation of DCs by *S. pyogenes*. These multimodal recognition may be essential for a rapid and effective response to the invading pathogen.

## MATERIALS AND METHODS

***Bacterial strains and culture conditions.*** The *S. pyogenes* strain KTL3 (M1 type), initially isolated from the blood of a patient with streptococcal bacteremia, has been previously described (32). The *S. pyogenes* strain 90-226, a serotype M1 strain cultured from the blood of a patient with sepsis and the M protein-deficient *emm1*:: Km isogenic mutant constructed by insertional inactivation of *emm1* with the *aphA-3* (8) were kindly provided by P. Cleary (University of Minnesota Medical School, Minneapolis, MN, USA). Stock cultures were maintained at -70°C and were cultured at 37°C in Todd-Hewitt broth (Oxoid, Basingstoke, UK), supplemented with 1% yeast extract (THY). Bacteria were collected in mid-log phase, washed twice with sterile PBS, diluted to the required inoculum and the number of viable bacteria was determined by counting colony-forming units (CFU) after diluting and plating in blood agar plates (GIBCO, Paisley, UK) containing 5% sheep blood.

***Mice.*** C57BL/6 and TLR2$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). MyD88$^{-/-}$ and TLR4$^{-/-}$ mice were obtained from the Max Planck Institute for Infection Biology (Berlin, Germany). TLR1$^{-/-}$ and TLR2/6$^{-/-}$ mice were originally from the Centre National de la Recherche Scientifique (CNRS, Paris, France) and kindly provided by T. Ebensen (HZI). These mice were housed in a pathogen-free animal facility at the HZI. Mice were maintained under standard conditions and according to institutional guidelines. All experiments were approved by the appropriate national ethical board (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany).

***Isolation and culture of bone marrow-derived DCs.*** For the generation of bone marrow-derived DCs, bone marrow was removed from the tibias and femurs of 5- to 10-wk-old mice. Following red cell lysis and washing, progenitor cells ($10^6$/ml) were resuspended and plated in RPMI 1640 containing 10 ng/ml of recombinant mouse GM-CSF (granulocyte-macrophage

colony-stimulating factor) (Sigma, St. Louis, MO, USA) and 10 ng/ml of IL-4 (R & D Systems, Minneapolis, MN, USA). DCs were cultured for 6 days at 37°C in 5% C02 and were gently washed and fed with fresh medium and cytokines on days 2 and 4. On day 6, the DCs were used for infection experiments.

***Experimental infection of DCs.*** DCs were infected with the corresponding *S. pyogenes* strain at a multiplicity of infection (MOI) of 10 bacteria per DC and incubated for 2 h in antibiotic-free medium. DCs were washed twice with PBS to remove unbound bacteria and pure fractions of DCs were obtained by labeling cells with anti-CD11c MACS beads (Miltenyi Biotec Inc., Bergisch-Gladbach, Germany) and passed twice through a $MACS^{+}$ selection column (Miltenyi Biotec Inc.). The cells purified from the column were routinely >95% CD11c+, as determined by flow cytometry. Cells were further incubated in the presence of antibiotics for additional 24 or 48 hours.

For inhibition of TLR-9, DCs were pretreated with ODN2088 (5 µM) (Invivogen, San Diego, CA, USA) for 2 hours prior to infection or stimulation.

The TLR9 activator ODN1826 (0,5 µM) (Invivogen, San Diego, CA, USA) was used to stimulate DCs through the TLR9.

DCs stimulated with 2 µg/ml of ultra pure LPS from *Escherichia coli* (Invivogen, San Diego, CA, USA) were used as a control.

***FACS Analysis of Dendritic Cells .*** DCs were harvested at 48 h after infection, washed, incubated for 5 min with anti-CD16/32 antibodies to block the Fc receptor and double stained with phycoerythrin (PE)-conjugated anti-CD11c and fluorescein isothiocynate (FITC) conjugated anti-CD40, anti-CD80, and anti-CD86 antibodies along with their isotype controls (BD PharMingen) for 30 minutes. Labeled-DCs were washed and analyzed on a FACScalibur (Becton Dickinson, Erembodegem-Aalst, Belgium).

***Cytokines assay.*** The levels of IL-12, IL-6 and TNF-α were determined in the supernatant of *S. pyogenes*-stimulated, LPS-stimulated and unstimulated DCs collected at 48 h of culture by ELISA. Briefly, polystyrene microtiter plates (Costar, Fernwald, Germany) were incubated overnight at 4 °C with purified rat anti-mouse anti-IL-12 or anti IL-6 antibodies (BD Pharmingen) diluted in coating buffer. Wells were blocked with 3% bovine serum albumin-PBS and sample supernatants, serum samples and standards (serial concentrations of recombinant murine IL-12 or IL-6) were added and incubated overnight at 4°C. Bound cytokine was detected with either biotinylated rat anti-mouse IL-12 or IL-6 antibodies (BD Pharmingen), followed by streptavidin-peroxidase conjugate, and developed with ABTS. The optical densities of samples and standards were measured at 405 nm with a correction wavelength of 650 nm. The levels of TNF-α were determined by using the BD OptEIA$^{TM}$ mouse TNF-ELISA Set (mono/poly) (BD Biosciences, San Diego, CA, USA) according to the manufacture's instructions.

***RNA isolation, reverse-transcription polymerase chain reaction(RT-PCR), and quantitative real-time PCR.*** Total RNA was isolated from purified *S. pyogenes*-infected, LPS-stimulated and unstimulated DCs at 24 h of culture. DCs were resuspended in RLT-Buffer and homogenized by using QIAshedder columns (QIAGEN). Total RNA was isolated with the RNeasy® Mini Kit (QIAGEN) according to the manufacturer's protocol and cDNA synthesis was performed using a Gibco RT-PCR kit following the manufacturer's instructions. The *ß-actin* was used as internal reference gene for the RT-PCR. *Rsp-9* was used as housekeeping gene for the quantitative real-time PCR. The following oligonucleotides were generated: *ß-actin* 5'- TGG AAT CCT GTG GCA TCC ATG AAA C – 3' and 5' - TAA AAC GCA GCT CAG TAA CAG TCC G – 3'; *rsp-9* 5' - CTG GAC GAG GGC AAG ATG AAG C – 3' and 5' - TGA CGT TGG CGG ATG AGC ACA – 3'; *IL-12p40*: 5'- CGT GCT CAT GGC TGG

TGC AAA G - 3' and 5' - CTT CAT CTG CAA GTT CTT GGG C - 3'. Cycling conditions for PCR amplification were 15 sec at 94°C, 30 sec at 58°C, and 45 sec at 72°C for 30 cycles. For quantitative real time-PCR, amplification of rsp-9 and IL-12p40 cDNA was performed using the rotor gene cycler (Corbett Research, Cambridgeshire, UK) and SYBR® green PCR Master Mix (Applied Biosystems). Templates used to generate the standard curve for rsp-9 and IL-12p40 real time-PCR were produced by amplification of full-length cDNA, which was then subcloned into the TOPO PCR2.1 cloning vector (Invitrogen). The resulting plasmids were prepared using the QIAprep® Spin Miniprep-Kit (QIAGEN) following the manufactor´s instructions and then used to prepare a tenfold dilution series of amplification standard. For the amplification standards, cycle threshold (Ct) was plotted against $Log_{10}$ copy number to obtain the standard curve used to calculate cDNA copy numbers. Cycle threshold values for IL-12p40 were normalized to the value for β-actin expression. The relative expression levels of stimulated DCs were compared to the expression levels of unstimulated DCs. Data are presented below as fold changes in mRNA in infected or stimulated DCs compared to the mRNA in unstimulated control DCs.

***Immunofluorescence microscopy.*** For double immunofluorescence of extracellular/intracellular bacteria**,** DCs were applied to sterile coverslips and infected with *S. pyogenes* for 2 h. Coverslips were then rinsed to remove unbound cells, and adherent cells were fixed with 4% formaldehyde. For double immunofluorescence staining, extracellular bacteria were stained with polyclonal rabbit anti-*S. pyogenes* antibodies, followed by Alexa green-conjugated goat anti-rabbit antibodies (Sigma-Aldrich, Germany). After several washes, cells were permeabilized by 0.025% Triton X-100 in PBS, washed again, and intracellular bacteria were stained by anti-*S. pyogenes* antibodies, followed by Alexa red-conjugated goat anti-rabbit antibodies (Sigma-Aldrich). The fluorescence images were obtained with a Axiophot microscope (Zeiss, Oberkochen, Germany) and analyzed using

AxioVision Release 4.5 software (Zeiss). Intracellular bacteria will then appear in red while extracellular *S. pyogenes* will appear in yellow-green.

***Gentamicin protection assay.*** DCs were infected with *S. pyogenes* (10:1 MOI) for 2 h. DCs were then washed to remove unbounded bacteria and were further incubated in the presence of gentamicin (100 µg/ml) at 37°C in 5% $CO_2$ to kill extracellular bacteria. After different periods of time, DCs were washed and disrupted with $dH_2O$ to release intracellular bacteria. The resulting suspension was serially diluted and bacterial numbers were determined after plating onto blood agar.

***Preparation of S. pyogenes DNA.*** Overnight cultures of *S. pyogenes* were centrifuged and the obtained pellet resuspended in Sucrose-TES buffer followed by addition of lysozyme and mutanolysin and 1 h incubation at 37°C. RNAse and Proteinase K were added and the mixture was incubated for 15 minutes at 37°C. After adding 500µl of 10% SDS-solution and incubating for 1 h, the DNA was isolated by Phenol-Chloroform extraction. The DNA fraction was then precipitated overnight with Sodium Acetate and Ethanol at –20°C. After washing with Ethanol, the DNA was resuspended in TE buffer. Quantification was performed by photometric measuring at 260nm. Bacterial DNA was stored at 4°C.

## RESULTS

### Effect of MyD88-deficiency on DC maturation.

To determine the role of the MyD88 signaling pathway in *S. pyogenes*-induced maturation of DCs, the expression of CD40, CD80, and CD86 was then determined in the surface of DCs differentiated from bone marrow obtained from either C57BL/6 (wild type DCs) or MyD88$^{-/-}$ (MyD88$^{-/-}$ DCs) mice at 48 h after *S. pyogenes* infection by flow cytometry analysis. While wild type DCs significantly increased expression of CD40, CD80, and CD86 upon infection (Fig. 1, left histograms), *S. pyogenes*-stimulated MyD88$^{-/-}$ DCs completely failed to upregulate the surface expression of these molecules (Fig. 1, right histograms). These results indicate that *S. pyogenes* induces DC maturation through a MyD88-dependent pathway.

### MyD88 is essential for *S. pyogenes*-induced IL-12 production from DCs.

We have recently shown that DCs are the major source of IL-12 during *S. pyogenes* infection (22). Therefore, we investigated the role of MyD88 signaling in the production of IL-12 by DCs in response to *S. pyogenes.* Wild type and MyD88$^{-/-}$ DCs were infected for 2 h with *S. pyogenes* and further cultured in the presence of antibiotics. Supernatant of DCs stimulated with LPS were used as control. The levels of IL-12 were measured in the supernatants at 48 h after infection by ELISA determination of IL-12p40. As shown in Fig. 2A, secretion of IL-12p40 was completely abolished in MyD88$^{-/-}$ DCs infected with *S. pyogenes*. To test whether the impaired capacity of MyD88$^{-/-}$ DCs to release IL-12 in response to *S. pyogenes* is caused by a lack of induction of IL-12 gene expression, we compared the levels of mRNA for IL-12p40 subunit, which is induced in DCs after activation (40), in *S. pyogenes*-infected and uninfected wild type and MyD88$^{-/-}$ DCs. Absence of MyD88 resulted in suppression of IL-12p40 transcription as measured by reverse transcription PCR (Fig. 2B) as well as real time-PCR (Fig. 2C). These results indicate that *S. pyogenes* induction of IL-12p40 gene is completely MyD88-dependent.

**The secretion of TNF-α and IL-6 by DCs in response to *S. pyogenes* is MyD88-dependent.**

We next examined if MyD88 was involved in the production of other inflammatory cytokines such as TNF-α and IL-6. These cytokines were detected by ELISA in the supernatants of uninfected and *S. pyogenes*-infected wild type or MyD88$^{-/-}$ DCs, at 48 h after *in vitro* infection. Wild type DCs or MyD88$^{-/-}$ DCs stimulated with LPS were used as controls. The production of both TNF-α (Fig. 3A) and IL-6 (Fig. 3B) in response to *S. pyogenes* infection was drastically reduced in MyD88$^{-/-}$ DCs compared with wild type cells. These data indicate that *S. pyogenes*-induced production of proinflammatory cytokines in DCs was TLR-mediated and dependent on MyD88.

**Uptake and killing of internalized *S. pyogenes* by DCs is independent of MyD88 expression.**

It has been reported that activation of the TLR signaling pathway by bacteria regulates phagocytosis at multiple steps including internalization and phagosome maturation (5). Therefore, we assessed whether DCs that lack MyD88 expression differed from normal DCs with respect to the internalization of *S. pyogenes* by using double immunofluorescence microscopy to discriminate between extracellular and intracellular-located bacteria. In contrast to the impressive phenotype of these cells with respect to the induced activation of inflammatory cytokines, MyD88 expression did not affect the ability of DCs to internalize *S. pyogenes,* since the uptake of *S. pyogenes* by MyD88$^{-/-}$ DCs (Fig. 4B) was comparable to that of wild type DCs (Fig. 4A). Similarly, wild type and MyD88$^{-/-}$ DCs did not differ in their ability to kill internalized *S. pyogenes* as shown by the kinetics of bacterial killing displayed in Fig. 4C.

**Signaling through Toll-like receptor 2 is not absolutely required for *S. pyogenes*-induced activation of DCs.**

The finding that DCs response to *S. pyogenes* was MyD88-dependent prompted an effort to define which TLRs were involved in *S. pyogenes*-induced DCs activation. Since gram-positive cell-wall products such as lipoteichoic acid (LTA) and peptidoglycans have been implicated in the activation of DCs through the TLR2 (26), we first investigated the participation of TLR2 on DC sensing of *S. pyogenes*. DCs from TLR2$^{-/-}$ as well as from wild type C57BL/6 control mice were infected with *S. pyogenes* for 2 h and further incubated after removal of unbound bacteria. Maturation markers were determined in *S. pyogenes*-stimulated and non-stimulated DCs after 48 h of culture. The obtained results show that both, TLR2$^{-/-}$ as well as wild type DCs up-regulated the expression of maturation markers upon exposure to *S. pyogenes*, indicating that a functional TLR2 was not absolutely required for this process. A representative histogram showing up-regulation of CD40 in DCs from wild type (left histogram) and TLR2$^{-/-}$ (right histogram) mice is depicted in Fig. 5A. Similar results were observed when expression of other surface markers (CD80 and CD86) was examined (data not shown).

In addition, examination of cytokines secretion revealed that wild type and TLR2$^{-/-}$ DCs produced comparable amounts of IL-12p40 in response to stimulation with *S. pyogenes* (Fig. 5B). Thus, *S. pyogenes* is clearly able to induced IL-12p40 production by DCs by signaling though TLRs other than the TLR2.

It has been recently reported (30) that streptococcal M protein, a classical virulence determinant of *S. pyogenes*, interacts with TLR2 on human blood monocytes, thereby inducing the production of high amounts of proinflammatory cytokines. To investigate the relative contribution of M protein-TLR2 receptor interaction to the overall production of IL-12p40 by DCs, we compared the levels of IL-12p40 induced in wild type and TLR2$^{-/-}$ DCs by a *S. pyogenes* mutant strain deficient in the expression of M protein versus that by the *S.*

*pyogenes* parental strain. The IL-12p40 response induced by the M protein-deficient strain was roughly equivalent to the corresponding wild type strain, irrespective of the integrity of TLR2 expression by the DCs (Fig. 5B). Additionally, the pattern of secretion of other inflammatory cytokines such as IL-6 did not differ between wild type and TLR2$^{-/-}$ DCs after stimulation with the parental or the M protein-deficient strains of *S. pyogenes* (Fig. 5C).

It has been reported that TLR2 posses the ability to co-localize with TLR6 and may function as a heterodimeric receptor in response to ligation with the appropriate microbial product (29). Accordingly, we compared maturation and cytokine secretion between DCs derived from bone marrow of mice deficient in the expression of both TLR2 and TLR6 after *in vitro* exposure to *S. pyogenes*. Again, no significant differences in the expression of maturation markers (data not shown) were seen. IL-12p40 production was also equivalent between wild type and TLR2/6$^{-/-}$ DCs (Fig. 6).

**Inhibition of Toll-like receptor 9 does not affect the ability to *S. pyogenes* to induce activation of DCs.**

Bacteria-derived DNA containing CpG motifs can also induce cytokine production and DC maturation through TLR9 (14). DNA from *S. pyogenes* has been reported to contain stimulatory motifs that induce activation of human lymphocytes (7). To determine whether DNA isolated from *S. pyogenes* could stimulate IL-12 production through the stimulation of TLR9 signaling, DCs were stimulated with *S. pyogenes* DNA in the absence or presence of the TLR9 inhibitor ODN 2088, and the secretion of IL-12p40 was determined at 48 h of culture. The production of appreciable levels of IL-12p40 by DCs stimulated with *S. pyogenes* DNA compared with unstimulated DCs demonstrated that bone marrow DCs actively responded to this agent (Fig. 7A). *S. pyogenes* DNA-induced IL-12p40 production by DCs is dependent on TLR9 since the pretreatment of DCs with the TLR9 inhibitor ODN 2088 significantly attenuated IL-12p40 production by DNA-stimulated DCs (Fig. 7A).

To determine the relative contribution of TLR9 to IL-12p40 production by whole *S. pyogenes* microorganisms, DCs were stimulated either with *S. pyogenes* or with ODN 1826 as positive control, either in the presence or absence of the TLR9 inhibitor ODN 2088. The results show that, in contrast to what was observed with purified DNA, inhibition of TLR9 did not affect the production of IL-12p40 by DCs in response to *S. pyogenes* stimulation. Therefore, these data ruled out a primary role of TLR9 signaling for DC activation by *S. pyogenes*.

A major contribution of TLR1 and TLR4 in immune recognition of *S. pyogenes* by DCs was also discarded after evaluating the response of DCs isolated from mice deficient in the expression of either TLR1 or TLR4 (data not shown).

## DISCUSSION

DCs play an important role in host defense against *S. pyogenes* (22). In this report, we attempted to identify the receptors involved in innate immune recognition of *S. pyogenes* by DCs. To determine the possible contribution of TLRs in this process, we tested the requirement for the TLR-associated adapter molecule MyD88 in maturation and production of cytokines by DCs after exposure to *S. pyogenes.* MyD88 has been shown to be a critical signaling molecule for most TLRs as well as IL-1R family responses (25). After recognition of a pathogen-associated molecule, TLRs associate with MyD88 and induce a cascade of signaling events leading to NF-κB activation and subsequent transcription of genes encoding proinflammatory cytokines such as TNFα, IL-6, or IL12 (36). Using DCs deficient in the expression of MyD88, we show here that the induction of DC maturation and production of cytokines in response to *S. pyogenes* infection is entirely dependent on the MyD88-mediated signaling pathway. Our results are in agreement with previously published reports suggesting that the production of inflammatory cytokines, and in particular IL-12, by DCs is completely abolished in the absence of MyD88 (19, 21). The observation that *S. pyogenes*-mediated activation of DCs was MyD88-dependent suggested that one or multiple TLRs were involved. However, contrary to our expectations, DCs lacking either TLR1, TLR2, TLR4, TLR2/TLR6 or inhibited in TLR9 signaling, retain their ability to maturate and to produce inflammatory cytokines in response to *S. pyogenes* infection. Therefore, despite the indications that TLRs mediate the response of DCs to *S. pyogenes*, we failed to identify a specific TLR with a predominant impact on this response.

These observations indicate that immune recognition of *S. pyogenes* is not likely to occur through recognition of a single microbial target molecule by a single TLR. Instead, innate immune cells will be exposed to multiple bacterial components capable of engaging different TLR family members. Thus, we hypothesize that TLRs 1, 2, 4, 6, and 9 may all be involved in the immune sensing of *S. pyogenes* through the recognition of lipoproteins, petidoglycans,

LTA (29, 38), and bacterial DNA (14). Deletion of an individual TLR can, therefore, be compensated for signaling triggered through the others. To cope with the complexity of sensing pathogens, the innate immune system probably evolved to respond via multiple interactions to maximize activation. Simultaneous or sequential activation of multiple TLRs can also be important for tailoring the inflammatory responses to be effective against specific microbial pathogens.

According to our observations with *S. pyogenes* and DCs, Henneke and colleagues (15, 16) have reported that macrophages deficient in MyD88 expression are significantly impaired in the immune response to Group B streptococcus. However, macrophages isolated from mutant mice with targeted deletions of the individual TLR 1, 2, 4, 6 or 9 did not show a significantly decreased cytokine response to this pathogen, thus excluding all of the TLRs that have been reported as potential receptors for cell components of gram-positive bacteria (15, 16).

It is noteworthy that, in general, the impact of TLR deletion on microbial infection phenotypes is often not severe. Thus, although LPS from *E. coli* is recognized by TLR4, mice deficient in TLR4 do not display enhanced susceptibility to infection with this pathogen (10, 13). A similar situation has been observed with *Listeria monocytogenes*, which can induce inflammatory signaling through TLR2, but mice deficient in TLR2 are not more susceptible to infection (9). These observations further demonstrate the complex interactions between a pathogen and innate immune cells, in which a single receptor cannot be assigned a sole recognition molecule and the induction of all components of the inflammatory response.

Another conclusion from this study is that the uptake and killing of *S. pyogenes* by DCs was unaffected in the absence of MyD88 suggesting that, in these settings, phagocytic and killing mechanisms of DCs do not require TLR-mediate signaling for their induction.

In summary, our results indicate that immune recognition of *S. pyogenes* by DCs is highly complex and requires a coordinated interplay between MyD88-independent events involving phagocytosis and antigen-processing as well as MyD88-dependent signaling pathways

involved in the induction of DC maturation and cytokine production. Adding an additional level of complexity, the data presented here argues for a multimodal detection of *S. pyogenes* by DCs involving several TLR signaling pathways, resulting in a complex triggering of proinflammatory responses that impacts on the nature and the intensity of the ensuing immune response. The evolution of such a multi-receptor, integrated system for sensing pathogens is crucial for innate immune defenses to prevent pathogens from easily subverting recognition by mutating or deleting critical TLRs ligands.

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## FIGURE LEGENDS

**FIGURE 1.** *S. pyogenes*-induced maturation of DCs is MyD88-dependent. Bone marrow-derived DCs from C57BL/6 (left histograms) and MyD88$^{-/-}$ (right histograms) mice were infected for 2 h with *S. pyogenes* and further incubated for 48 h. Uninfected DCs were used as a control. DCs were collected, stained with PE-labeled monoclonal anti-mouse CD11c antibody and FITC-labeled monoclonal anti-mouse CD40, CD80 or CD86 antibodies, and subjected to flow cytometry analysis. CD11c+ cell populations were gated and analyzed for CD40, CD80, and CD86 and expression. Thin lines represent uninfected DCs; thick lines represent DCs infected with *S. pyogenes*. Results are representative of four independent experiments.

**FIGURE 2.** DCs from MyD88$^{-/-}$ mice show impaired *S. pyogenes*-induced IL-12 responses *in vitro*. Purified bone marrow-derived DCs from C57BL/6 or MyD88$^{-/-}$ mice were infected *in vitro* with *S. pyogenes* for 2 h and further incubated in the presence of antibiotics. (A) IL-12p40 was measured in the supernatant of *S. pyogenes*-infected (hatched bars) or uninfected (white bars) DCs by ELISA at 48 h after infection. DCs stimulated with 2 μg/ml of LPS were used as a control (black bars). Bars are means ± SD of triplicates. Similar experiments were performed at least three times. (B) RT-PCR amplification of IL-12p40 cDNA in unstimulated, LPS-stimulated, and *S. pyogenes*-infected DCs isolated from wild type C57BL/6 (left) or from MyD88$^{-/-}$ (right) mice. *β-actin* expression served as a loading control. Plasmids containing the sequences encoding for IL-12p40 or β-actin were used as positive controls and reactions in the absence of cDNA were used as negative controls. (C) Fold changes in the ratio of IL-12p40 mRNA to β-actin mRNA in *S. pyogenes*-infected or LPS-stimulated DCs compared to those in unstimulated control DCs isolated from either wild type C57BL/6 (black bars) or MyD88$^{-/-}$ mice (white bars).

**FIGURE 3.** Production of TNF-α (A) and IL-6 (B) in response to *S. pyogenes* in wild type and MyD88$^{-/-}$ DCs. DCs from wild type C57BL/6 or MyD88$^{-/-}$ mice were infected with S. pyogenes or stimulated with 2 μg/ml LPS and further cultured for 48 h. Concentrations of TNF-α (A) and IL-6 (B) in the culture supernatants were measured by ELISA. The results are shown as the mean ± SD of three experiments. *, $p < 0.05$ by F-test.

**FIGURE 4.** *In vitro* phagocytosis and killing of *S. pyogenes* by DCs isolated from wild type C57BL/6 or MyD88$^{-/-}$ mice. (A, B) Double immunofluorescence staining of *S. pyogenes*-infected DCs isolated from wild type C57BL/6 (A) or MyD88$^{-/-}$ (B) mice showing the intracellular/extracellular location of *S. pyogenes* within the DCs. Extracellular bacteria are yellow-green and intracellular bacteria are red, DNA in the nucleus is stained in blue. (C) Kinetic of *S. pyogenes* killing by DCs isolated from wild type C57BL/6 (squares) or MyD88$^{-/-}$ mice (triangles). DCs from indicated mice were infected with *S. pyogenes* at a MOI of 1:10 for 2 h and further incubated in the presence of gentamicin (100 μg/ml) at 37°C in 5% $CO_2$ to kill extracellular bacteria. The numbers of viable intracellular-located *S. pyogenes* were determined after different periods of time by disrupting infected DCs with $dH_2O$ and plating onto blood agar. Each point represents the mean value± SD of four independent experiments.

**FIGURE 5.** *S. pyogenes* induced maturation and production of cytokines in DCs deficient in the expression of TLR2. (A) Flow cytometry analysis for detection of CD40 expression on DCs derived from bone marrow of wild type C57BL/6 (left histograms) or TLR2-/- (right histograms) mice uninfected (thin line) or after infection with *S. pyogenes* (thick line). DCs were infected with *S. pyogenes* for 2 h and further cultured in the presence of antibiotics for 48 h. Then DCs were stained with PE-conjugated anti-CD11c and FITC-conjugated anti-CD40 antibodies and analysed by FACS. (B and C) Induction of IL-12p40 (B) and IL-6 (C) in

DCs derived from bone marrow of C57BL/6 or TLR2-/- mice by wild type *S. pyogenes* (black bars) or the isogenic *S. pyogenes* mutant strain deficient in the expression of M1 protein (hatched bars). Cytokines were determined in the supernantant of uninfected and infected DCs after 48 h of culture. Bars represent the mean ± SD of three experiments. *, $p < 0.05$ by F-test.

**FIGURE 6.** Secretion of IL-12p40 by DCs derived from bone marrow of wild type C57BL/6 or TLR2/6$^{-/-}$ mice after infection with *S. pyogenes* (hatched bars) or stimulation with LPS (black bars). DCs were infected with *S. pyogenes* for 2 h and further cultured in the presence of antibiotics for 48 h. IL-12p40 was determined in the supernatant of untreated and treated DCs at 48 h of culture. Bars represent mean ± SD of three experiments. *, $p < 0.05$ by F-test.

**FIGURE 7.** *S. pyogenes* DNA stimulation of IL-12p40 secretion by DCs is dependent of TLR9 but stimulation of DCs IL-12p40 secretion by the whole *S. pyogenes* microorganism is TLR9-independent. (A) DCs isolated from bone marrow of C57BL/6 mice were pretreated or not with the TLR9 inhibitor ODN 2088 for 2 h, followed by stimulation with 50 μg/ml of purified *S. pyogenes* DNA for 48 h and analysis of IL-12p40 by ELISA. Data are mean ± SD of triplicates. *, $p < 0.05$, F-test. (B) DCs were pretreated or not with the TLR9 inhibitor ODN 2088 for 2 h prior to stimulation with the TLR9 agonist CpG ODN 1826 or infection with *S. pyogenes* and further cultured for 48 h. IL-12p40 was determined in the supernatants by ELISA. Data are mean ± SD of triplicates. *, $p < 0.05$, F-test.

1

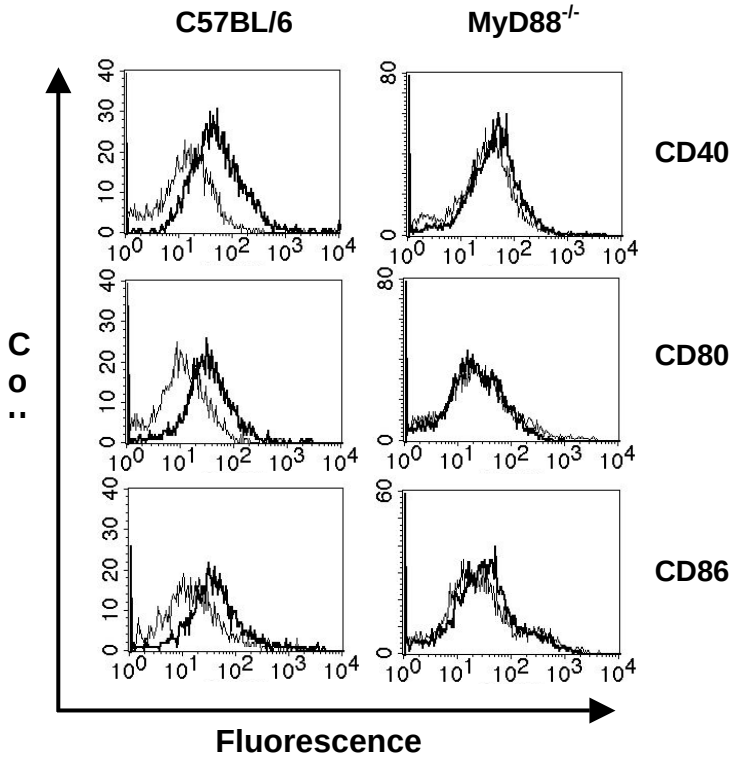



**Figure 1**

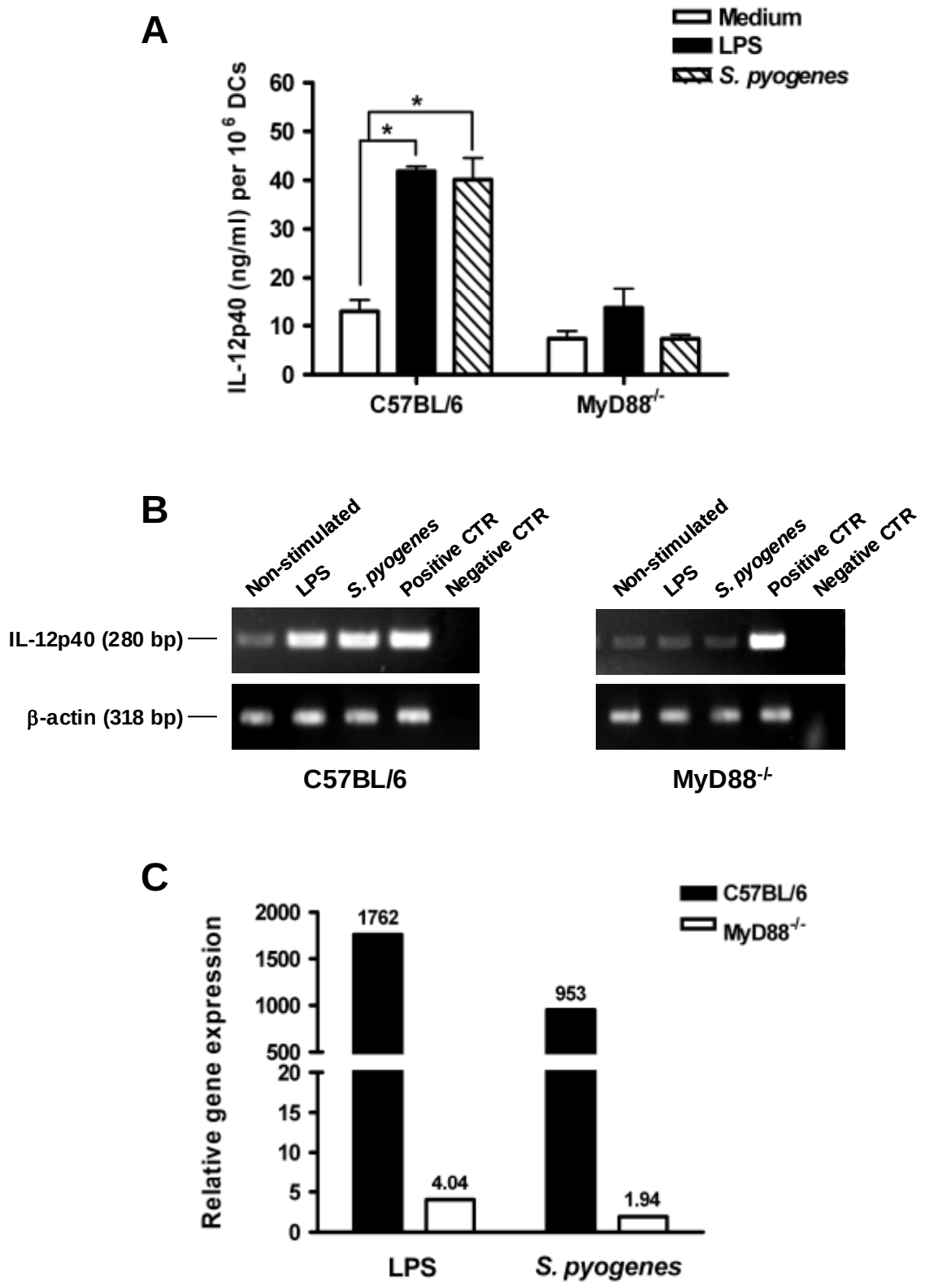

A
Medium
LPS
S. pyogenes
IL-12p40 (ng/ml) per 10^6 DCs
60
50
40
30
20
10
0
*
*
C57BL/6
MyD88-/-
B
Non-stimulated
LPS
S. pyogenes
Positive CTR
Negative CTR
IL-12p40 (280 bp)
β-actin (318 bp)
C57BL/6
MyD88-/-
C
C57BL/6
MyD88-/-
Relative gene expression
2000
1500
1000
500
20
15
10
5
0
1762
4.04
953
1.94
LPS
S. pyogenes

**Figure 2**

1

1

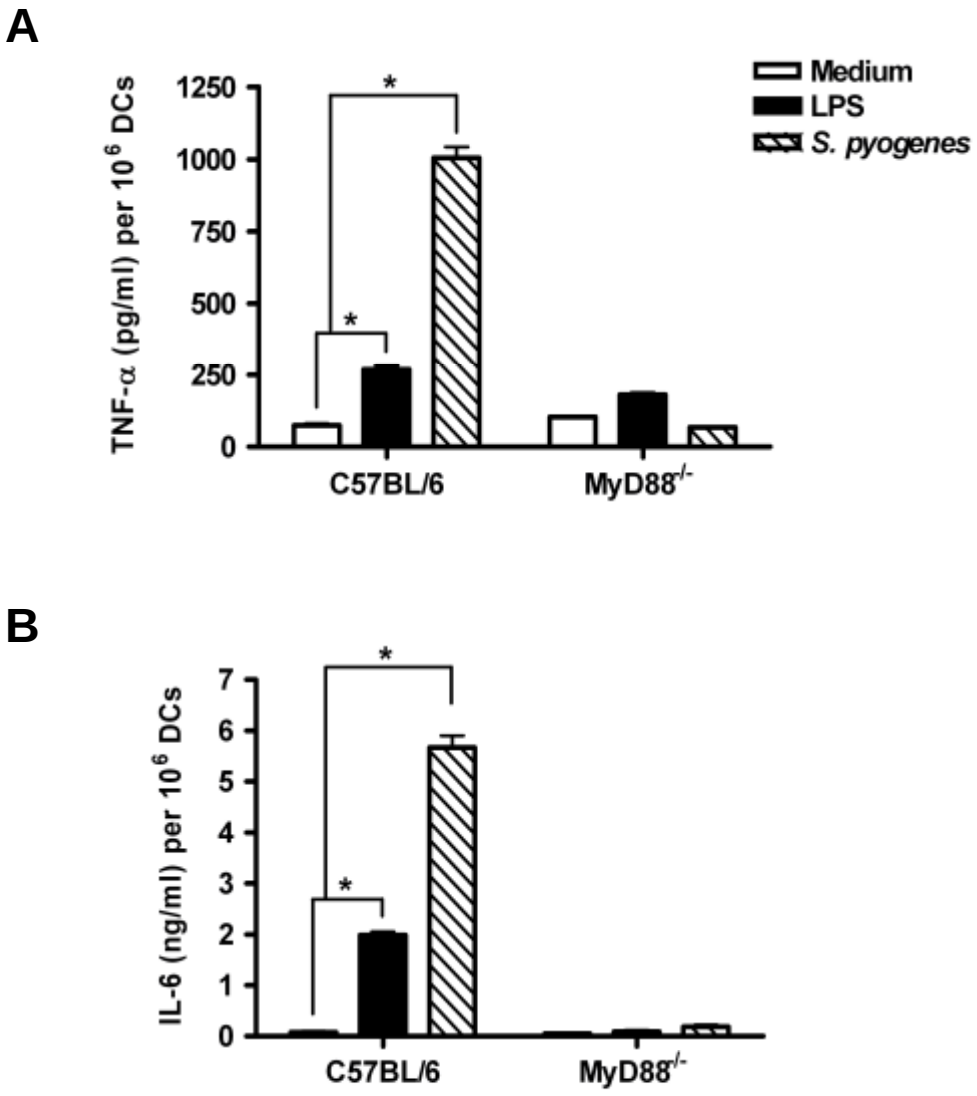
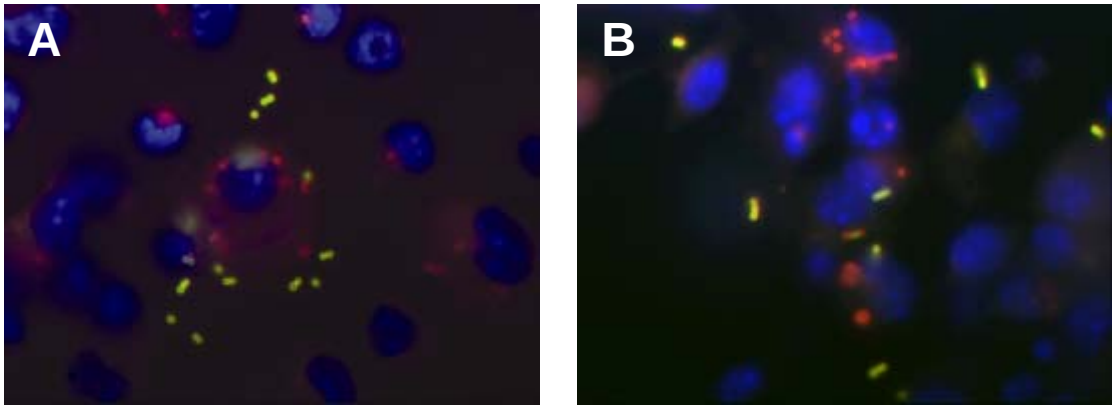

A
Medium
LPS
S. pyogenes
TNF-α (pg/ml) per 10^6 DCs
1250
1000
750
500
250
0
*
*
C57BL/6
MyD88-/-
B
IL-6 (ng/ml) per 10^6 DCs
7
6
5
4
3
2
1
0
*
*
C57BL/6
MyD88-/-

**Figure 3**

1


A
B

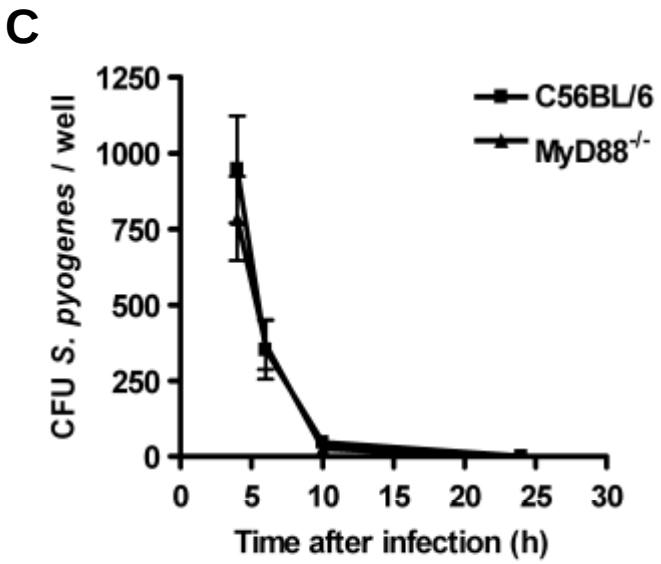

C
1250
1000
750
500
250
0
CFU S. pyogenes / well
0
5
10
15
20
25
30
Time after infection (h)
C56BL/6
MyD88-/-

**Figure 4**

1

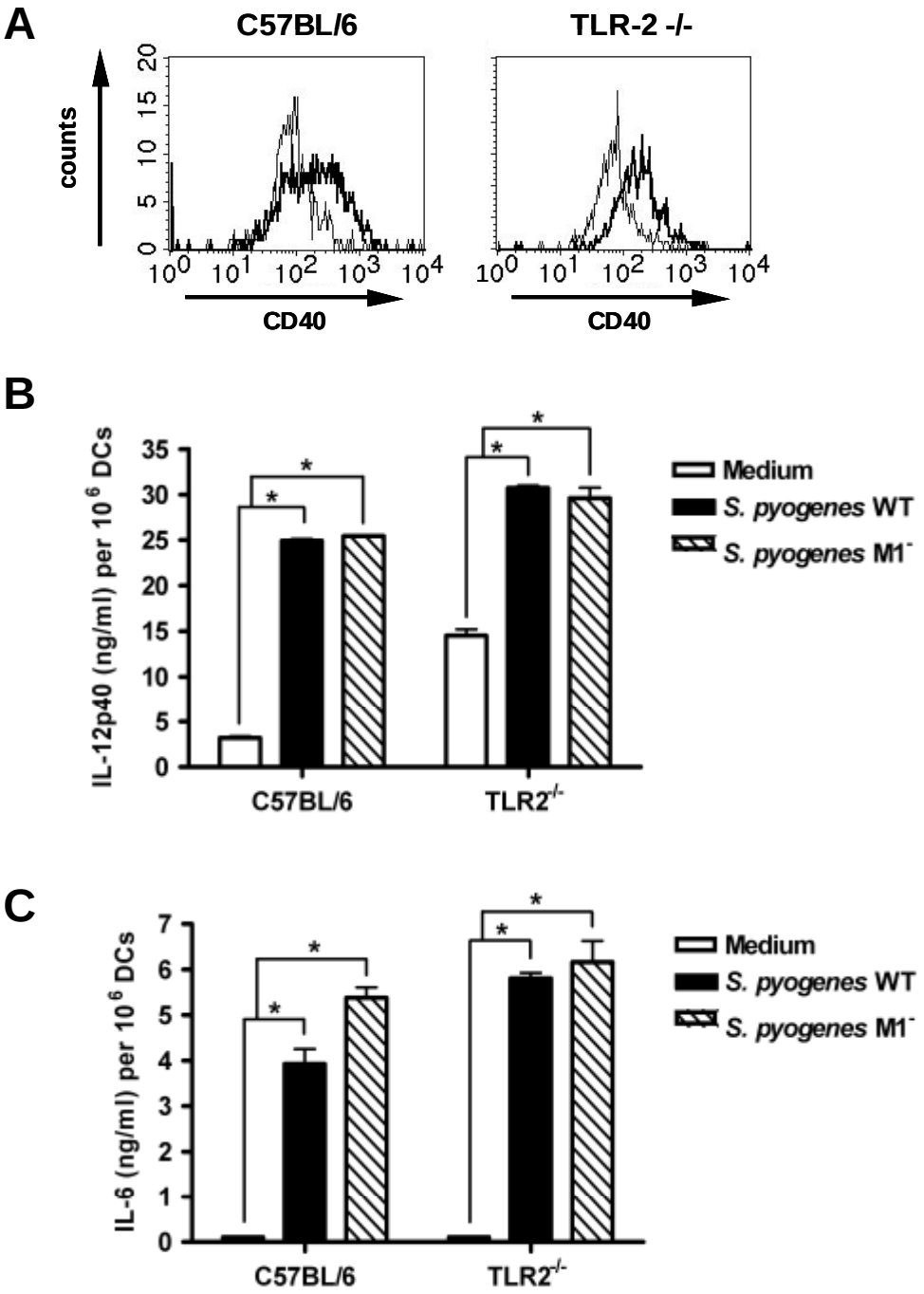

A
C57BL/6
TLR-2 -/-
counts
0 5 10 15 20
$10^0$ $10^1$ $10^2$ $10^3$ $10^4$
CD40
B
IL-12p40 (ng/ml) per $10^6$ DCs
0 5 10 15 20 25 30 35
C57BL/6
TLR2$^{-/-}$
Medium
S. pyogenes WT
S. pyogenes M1$^-$
C
IL-6 (ng/ml) per $10^6$ DCs
0 1 2 3 4 5 6 7
C57BL/6
TLR2$^{-/-}$
Medium
S. pyogenes WT
S. pyogenes M1$^-$

**Figure 5**

1

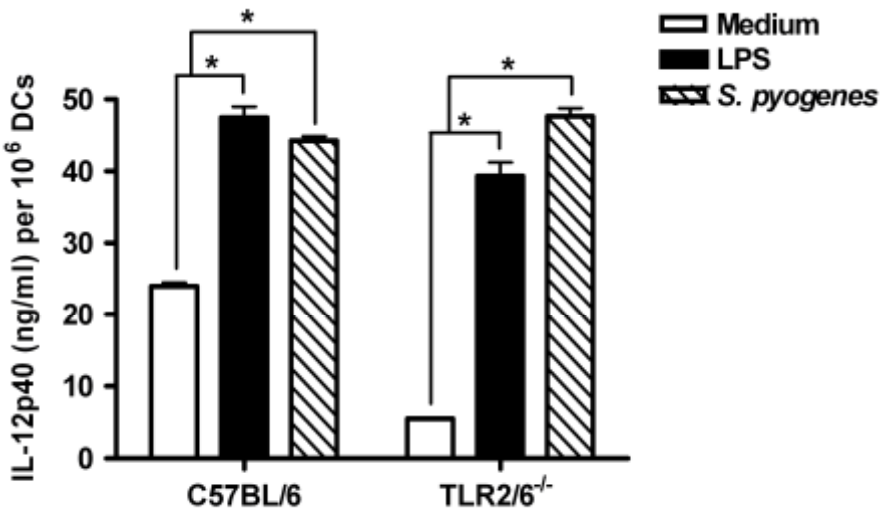

Medium
LPS
S. pyogenes
IL-12p40 (ng/ml) per 10^6 DCs
0
10
20
30
40
50
C57BL/6
TLR2/6^-/-
*
*
*
*

**Figure 6**

1

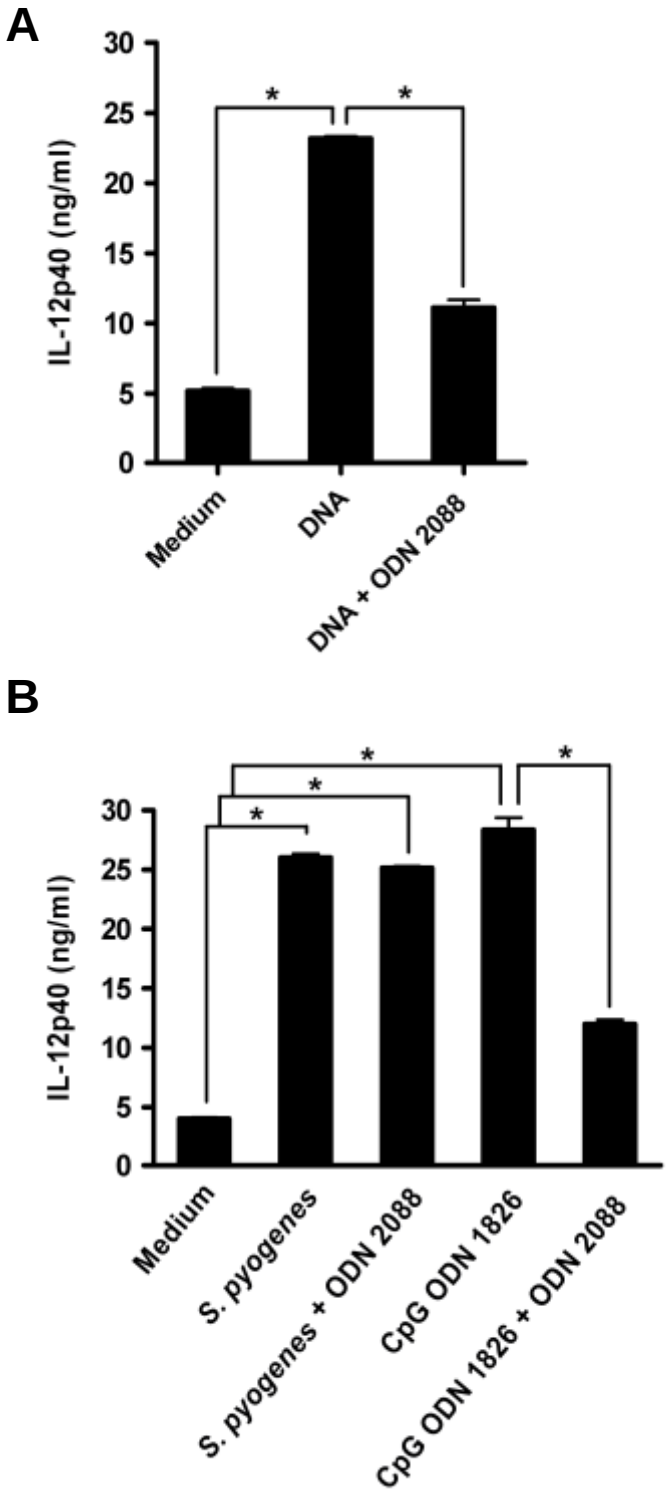

A
IL-12p40 (ng/ml)
30
25
20
15
10
5
0
*
*
Medium
DNA
DNA + ODN 2088
B
IL-12p40 (ng/ml)
30
25
20
15
10
5
0
*
*
*
*
Medium
S. pyogenes
S. pyogenes + ODN 2088
CpG ODN 1826
CpG ODN 1826 + ODN 2088

**Figure 7**