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## **TLR9 activation induces normal neutrophil responses in a child with IRAK-4 deficiency: involvement of the direct PI3K pathway**

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Running title: IRAK-4 deficiency and normal PMN TLR9 responses

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## Abstract

Polymorphonuclear neutrophils (PMN) play a key role in innate immunity. Their activation and survival are tightly regulated by microbial products via pattern-recognition receptors such as TLRs, which mediate recruitment of the IRAK complex. We describe a new inherited IRAK-4 deficiency in a child with recurrent pyogenic bacterial infections. Analysis of the *IRAK4* gene showed compound heterozygosity with two mutations: a missense mutation in the death domain of the protein (p.Arg12Cys) associated in cis with a predicted benign variant (p.Arg391His) and a splice-site mutation in intron 7 that led to the skipping of exon 7. A non-truncated IRAK-4 protein was detected by western blotting. The patient's functional deficiency of IRAK-4 protein was confirmed by the absence of IRAK-1 phosphorylation after stimulation with all TLR agonists tested. The patient's PMN showed strongly impaired responses (L-selectin and CD11b expression, oxidative burst, cytokine production, cell survival) to TLR agonists which engage TLR1/2, TLR2/6, TLR4, and TLR7/8; in contrast, the patient's PMN responses to CpG-DNA (TLR9) were normal, except for cytokine production. The surprisingly normal effect of CpG-DNA on PMN functions and apoptosis disappeared after pretreatment with PI3K inhibitors. Together, these results suggest the existence of an IRAK-4-independent TLR9-induced transduction pathway leading to PI3K activation. This alternative pathway may play a key role in PMN control of infections by microorganisms other than pyogenic bacteria in inherited IRAK-4 deficiency.

## Introduction

Polymorphonuclear neutrophils (PMN) play a key role in host defense against bacterial and fungal pathogens (1). They contribute to early innate response by rapidly migrating into inflamed tissues, where their activation triggers microbicidal mechanisms such as release of proteolytic enzymes and antimicrobial peptides, and rapid production of reactive oxygen species (ROS), in the so-called oxidative burst. PMN die spontaneously by apoptosis and are then recognized and phagocytosed by macrophages (2).

PMN directly recognize microbial products via pattern-recognition receptors such as TLRs. Human PMN have been reported to express all TLRs except TLR3 (3); TLR5 and TLR7 are weakly expressed (4). TLRs are members of the IL-1R superfamily, characterized by an intracytoplasmic Toll-IL-1 receptor (TIR) domain which mediates recruitment of the interleukin-1 receptor-associated kinase (IRAK) complex via TIR-containing adapter molecules such as MyD88. During formation of this complex, IRAK-4 is activated, leading to hyperphosphorylation of IRAK-1, which in turn induces the interaction of tumor necrosis factor receptor-associated factor 6 (TRAF6) with the complex. TRAF6 then triggers downstream signalling, and this results in NF- $\kappa$ B activation (5, 6). In addition, TLR engagement activates stress kinases such as MAP kinases, c-Jun NH<sub>2</sub>-terminal kinase (JNK), and phosphatidylinositol 3,4,5-triphosphate kinase (PI3K) in most cells, including PMN (7, 8). The PI3K pathway has variously been shown to regulate TLR-mediated inflammatory responses, through negative feedback functions (9, 10), or to enhance NF- $\kappa$ B nuclear translocation (11, 12). In particular, it was recently suggested that the PI3K signaling cascade occupies a central role in TLR2-induced activation of PMN (13).

PMN stimulation through TLRs causes an immediate defensive response, including modulation of adhesion molecule expression (L-selectin shedding and  $\beta$ 2-integrin

upregulation), production of an array of antimicrobial molecules (ROS and cytokines) (3, 13), and inhibition of apoptosis (4). The major role of the TIR-IRAK signaling pathway in immunity to infections by pyogenic bacteria is illustrated by the recent descriptions of children with inherited IRAK-4 deficiency associated with recurrent infections (14-22). The cells of these patients fail to respond to IL-1 and IL-18, and to the stimulation of at least five TLRs (TLR2, TLR3, TLR4, TLR5, TLR9).

Here we describe a case of inherited IRAK-4 deficiency related to new double heterozygous mutations generating a non functional IRAK-4 protein. We show that some PMN functions (adhesion molecule expression, ROS production, survival) which are critical for antimicrobial defenses, occur normally in response to CpG-DNA (TLR9), despite an impaired response to the other TLR agonists, suggesting the existence of a distinct TLR9-induced transduction pathway.

## Materials and Methods

### *Case report*

We investigated a 14-year-old boy with recurrent infections, osteomyelitis and cellulitis. He was born in July 1991 and was the second child of healthy unrelated parents. There was no family history of recurrent or severe infections, autoimmune disease, or lymphoma. His brother and sister were healthy. At age 15 days he developed a severe necrotic infection of his palate, due to *Pseudomonas aeruginosa*. Despite several surgical procedures he had velopharyngeal insufficiency and recurrent otitis media. Vaccines were normally tolerated. From the age of 5 years he had severe chronic otitis media, arthritis, and impetiginous infections of the face and limbs, usually after skin trauma. Local and systemic antibiotics were usually necessary to eradicate the infections. At age 9 years he underwent tympanoplasty for tympanic membrane perforation, which was complicated by retroauricular cellulitis due to *Staphylococcus aureus*, and severe impetigo of the face and hands, leading to graft loss. The procedure failed to close the tympanic perforation. At age 10 years he was hospitalized for cervical adenitis associated with fatigue and weight loss. The CRP was elevated, at 24 mg/ml. The PMN count was reduced at 920/mm<sup>3</sup> but chest radiograph and tuberculin test were normal. Serological tests for *Bartonella*, *Borrelia* and Lyme's disease were negative. Surgical biopsy showed non specific subacute lymphadenitis. The adenitis regressed on amoxicillin + clavulanic acid. At age 14 years he developed asthma and common verrucas. Chest radiography and computed tomography were normal. Inhaled steroid therapy and smoking cessation improved his bronchospasm. Prophylactic antibiotic therapy with sulfamethoxazole + trimethoprim was started and he underwent a second tympanic repair, without infectious complications.

His growth and development were normal. He had no severe viral or fungal infections or infections due to intracellular bacteria.

Immunological studies gave normal results (lymphocyte counts:  $CD3^+=2037/mm^3$ ,  $CD4^+=1328/mm^3$ ,  $CD8^+=798/mm^3$ ,  $CD19^+=798/mm^3$ ,  $CD16^+=321/mm^3$ ; IgA=1.27g/l; IgG=12.08g/l (IgG1=10g/l, IgG2=2.52g/l, IgG3=0.72g/l, IgG4=0.05g/l); IgM=1.38g/l) excepted for a still low number of PMN. The complement system was normal.

PMN migration was normal when tested with the under-agarose method, with and without fMLP ( $10^{-7}$  M) and activated serum (23), ruling out leucocyte adhesion deficiency (LAD). PMN phagocytosis of *Staphylococcus epidermidis* was normal, as was PMN chemiluminescence after stimulation with PMA (100 ng/ml), ruling out a chronic granulomatous disease.

### *Reagents*

The reagents and sources were as follows:

Ultrapurified LPS from *E. coli* serotype R515 (LPS) and synthetic macrophage-activating lipopeptide-2 (MALP-2) (Alexis, Lausen, Switzerland); R-848 and a synthetic palmitoylated mimic of bacterial lipopeptides (Pam3CSK4) (Invivogen, San Diego, CA); unmethylated CpG-DNA (HyCult Biotechnology, Lausen, Switzerland); hydroethidine (HE, Fluka, Buchs, Switzerland); N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA) and ionomycin (Sigma Chemical CO., St Louis, MO); SN50, SB203580, PD98059, genistein, wortmannin, rottlerin, and GF109203X (Calbiochem, La Jolla, CA); allophycocyanin (APC)-conjugated annexin V, 7-amino-actinomycin D (7-AAD), fluorescein (FITC)-anti-CD15, purified anti-L-selectin and FITC-conjugated goat anti-rabbit antibodies (Abs), phycoerythrin (PE)-conjugated anti-phosphorylated p38MAPK and ERK1/2 Abs, and cytometric bead array (CBA) kit (Pharmingen, Becton Dickinson, San Jose, CA); PE-

conjugated anti-CD45 Ab (Immunotech, Marseille, France); PE-conjugated anti-CD11b Ab (Dakopatts, Glostrup, Denmark); FITC-conjugated goat anti-mouse Ab (Nordic Immunology, Tilburg, The Netherlands); anti-IRAK-4, anti-phospho-IRAK-1 and anti-phospho-Bad (S136) Abs (Cell Signaling Technology, Beverly, MA); anti-Mcl-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA); TNF $\alpha$  and GM-CSF (R&D, Abington, UK); IL-18 (MBL, Tokyo, Japan).

#### *Incubation of whole blood with TLR agonists*

One-milliliter aliquots of fresh blood, collected on lithium heparinate (10 U/ml), were incubated at 37°C for various times with phosphate-buffered saline (PBS), IL-18 (500 ng/ml), or the following TLR agonists (reported to stimulate PMN functions) (4): LPS (10 ng/ml) (TLR4), MALP-2 (10 ng/ml) (TLR2/6), Pam3CSK4 (500 ng/ml) (TLR1/2), R-848 (10  $\mu$ g/ml) (TLR7/8), and CpG-DNA (100  $\mu$ g/ml) (TLR9). These optimal concentrations were determined in preliminary concentration-response experiments (personal data).

In some experiments samples were pretreated with the NF- $\kappa$ B inhibitor SN50 (100  $\mu$ g/ml) or kinase inhibitors at optimal concentrations previously determined in whole blood (wortmannin, 2500 nM; LY2940002, 25  $\mu$ M; GF109203X, 5 $\mu$ M; genistein, 100  $\mu$ M; PD98059, 50  $\mu$ M; SB203580, 25  $\mu$ M; rottlerin, 10  $\mu$ M) (4).

#### *Determination of adhesion molecule expression at the PMN and monocyte surface*

Whole-blood samples were either kept on ice or incubated at 37°C for 1 hour with PBS, IL-18 or TLR agonists as described above; TNF $\alpha$  (100 U/ml) was used as control. Samples (100  $\mu$ l) were then stained at 4°C for 30 minutes with PE-anti-human CD11b or purified anti-L-selectin Abs. To study L-selectin expression, samples were then washed with ice-cold PBS and incubated at 4°C for 30 minutes with FITC-goat anti-mouse Ab. Red blood cells were lysed with FACS lysing solution (Becton Dickinson, Mountain View, CA) and white blood



cells were resuspended in 1% paraformaldehyde-PBS. Nonspecific Ab binding was determined on cells incubated with the same concentration of an irrelevant Ab of the same isotype.

#### *NADPH oxidase activity in priming conditions*

Superoxide anion ( $O_2^{\cdot-}$ ) production was measured with a flow cytometric assay derived from the HE oxidation technique (24): Whole-blood samples (500  $\mu$ l) were loaded for 15 minutes with HE (1500 ng/ml) at 37°C and then incubated with PBS, IL-18 or TLR agonists as described above; TNF $\alpha$  (100 U/ml) was used as positive control; samples were then treated with PBS or  $10^{-6}$  M fMLP for 5 minutes. Red cells were lysed as described above and white cells were resuspended in 1% paraformaldehyde-PBS.

#### *Measurement of PMN apoptosis*

Apoptosis of PMN in whole blood was quantified by using annexin V and 7-AAD (an impermeant nuclear dye) as previously described (4, 25). Samples were incubated in 24-well tissue cultures plates at 37°C with 5% CO<sub>2</sub> for 8 hours with PBS, IL-18 or TLR agonists as described above; GM-CSF (1000 pg/ml) was used as anti-apoptotic control. Samples (100  $\mu$ l) were washed twice in PBS, incubated on ice with FITC-anti-CD15 and PE-anti-CD45 Abs for 15 minutes, and then with APC-annexin V for 15 minutes. After dilution in PBS (500  $\mu$ l), samples were incubated with 7-AAD at room temperature for 15 minutes and analyzed immediately by flow cytometry. PMN were identified as CD15<sup>high</sup> cells. Use of the combination of APC-annexin V and 7-AAD distinguishes between early apoptotic PMN (annexin V<sup>+</sup>, 7-AAD<sup>-</sup>) and late apoptotic PMN (annexin V<sup>+</sup>, 7-AAD<sup>+</sup>).

*Study of intracellular phospho-IRAK-1, phospho-p38MAPK, phospho-ERK1/2 and Bcl-2 family protein content by flow cytometry*

After incubation of whole blood with TLR agonists or PBS for various times at 37°C, leukocytes were permeabilized in 90% methanol as previously reported (4, 26). Cells were then stained with anti-IRAK-1 phosphospecific, anti-Mcl-1 or anti-Bad phosphospecific Abs for 1 hour at room temperature and washed once in PBS-2% HSA. Samples were then incubated for 30 minutes with FITC-goat anti-mouse or anti-rabbit Ab. Phospho-p38MAPK and phospho-ERK1/2 contents were studied by staining with PE-conjugated anti-phospho-p38MAPK and phospho-ERK1/2 Abs. After one wash, leukocytes were resuspended in 1% paraformaldehyde-PBS.

*Cytokine production by blood cells*

Blood PMN were isolated in LPS-free conditions in medium containing 9% Dextran T-500 (Pharmacia, Uppsala, Sweden) and 38% Radioselectan (Schering, Lys-Lez-Lannoy, France); the leukocyte suspension was then centrifuged on Ficoll-Paque medium (Pharmacia). The cell pellet was washed with PBS, and erythrocytes were removed by hypotonic lysis; PMN were further purified by negative selection with pan anti-human HLA class II-coated magnetic beads (Miltenyi Biotec) to deplete B lymphocytes, activated T lymphocytes and monocytes as previously described (27). Less than 0.5% of cells were positive by nonspecific esterase staining, and flow cytometry showed the absence of CD45<sup>+</sup>/CD14<sup>high</sup>, CD45<sup>+</sup>/CD3<sup>+</sup>, and CD45<sup>+</sup>/CD19<sup>+</sup> cells; this showed that the PMN were highly purified, without contaminating monocytes. In parallel, the mononuclear cell ring obtained after Ficoll-Paque centrifugation was treated with anti-CD14-coated magnetic beads (Miltenyi Biotec) to positively select monocytes.

Whole blood, pure PMN ( $5 \times 10^6$ /ml) or pure monocytes ( $5 \times 10^5$ /ml) were cultured for 18 hours at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  in 24-well tissue culture plates (Costar, Cambridge, MA) in RPMI 1640 culture medium (Sigma, St Louis, MO). TLR agonists, IL-1 $\beta$  and IL-18 were added to the culture medium. PMA (100 ng/ml) and PMA (100 ng/ml) + ionomycin ( $10^{-5}$  M) were used as positive controls. Supernatants were stored at  $-70^\circ\text{C}$  for no longer than 15 days before assay. IL-8, IL-6, IL-1 $\beta$  and TNF $\alpha$  were detected simultaneously in supernatants by using the human inflammatory cytokine cytometric bead array (CBA) kit (BD Pharmingen, San Diego, CA). The CBA working range was 20-5000 pg/ml for each cytokine.

#### *Flow cytometry*

We used a Becton Dickinson FACScalibur (Immunocytometry Systems, San Jose, CA) with a 15-mW, 488-nm argon laser and a 635-nm diode laser. PMN functions were analysed using CellQuest software. To measure apoptosis in whole blood, PMN were identified on the CD15/SSC dot plot and  $2 \times 10^5$  events were counted per sample. In other experiments, forward and side scatter were used to identify the PMN population and to gate out other cells and debris;  $10^4$  events were counted per sample. Plasma cytokine levels were analyzed with CBA software (BD Pharmingen).

### *Blot analysis of IRAK-4*

PMN were isolated and highly purified as described above. Suspensions of  $40 \times 10^6$  PMN/ml in PBS buffer were incubated with PBS or TLR agonists for 5 minutes and treated with 2.7 mM diisopropylfluorophosphate for 20 minutes at 4°C then pelleted at 400 g for 8 minutes at 4°C (28). The pellet was resuspended in Chaps solubilization buffer containing 50 mM Tris pH 7.5, 15 mM Chaps, 1 mM EDTA and antiproteases. The cells were incubated on ice and the suspension was then centrifuged at 1500 g for 5 minutes. Following SDS-PAGE on 10% acrylamide gels, the proteins were transferred to nitrocellulose filters. The filters were incubated for 1 hour at room temperature in 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (TBST) containing 5% (w/v) fat-free dried milk. The nitrocellulose membranes were incubated overnight with anti-IRAK-4 Ab at 1/500 dilution. Following 5 washes with TBST, the membranes were incubated with goat anti-mouse or goat antirabbit Abs conjugated to horseradish peroxidase. After 5 washes with TBST, the blots were revealed with a chemiluminescence method (ECL; Amersham Life Sciences, Arlington Heights, IL) following the manufacturer's instructions.

### *Genetic analysis*

The propositus and his parents underwent genetic analysis with their written informed consent. DNA and RNA were extracted from whole blood with Qiagen extraction kits following the manufacturer's instructions. The *IRAK4* coding sequence and intron-exon junctions were sequenced in the patient and his parents (PCR conditions and primers are available on request), using an ABI sequencing kit (Applied Biosystems, Foster City, CA) and a 3130xl DNA sequencer (Applied Biosystems). cDNA was analyzed after reverse transcription of the patient's RNA by PCR using sets of primers located in various exons (Ex6F cDNA: CTA CTG AAG AAC TGA AAC AGC AGT TTG A; Ex7F cDNA: GTT TAC ATG CCT AAT GGT TCA

TTG C, Ex11R cDNA: CGA CAT TGG CTA GCA CCA GAG TA). Forward primers were 6-FAM-labelled. Allele-specific amplification of cDNA was performed using modified oligonucleotides (the 3' end nucleotide is one LNA molecule from Proligo) (Ex10R LNA\_G: TAT CTA GCA ATA ACT GAG GTT CAC; Ex10R LNA\_A: TAT CTA GCA ATA ACT GAG GTT CAT ). Analysis of the fluorescent PCR products was done with a 310 DNA sequencer (Applied).

### *Statistical analysis*

Data are reported as means  $\pm$  SEM. Comparisons were based on ANOVA and Tukey's posthoc test, using Prism 3.0 software (Graph Pad Software).

## Results

### *IRAK4 mutations*

The patient's disease was characterized by recurrent infections due to extracellular pyogenic bacteria. Standard immunological studies gave normal results and major PMN defects (i.e. chronic granulomatous disease) were ruled out. As inherited IRAK-4 deficiency has been associated with recurrent infections (14-22), we analyzed the *IRAK4* gene in our patient.

DNA sequencing revealed three mutations: one missense mutation resulting in the substitution of arginine by cysteine at position 12 (c. 34 C>T; p.Arg12Cys), a second missense mutation at position 391 (c. 1170 G>A; p.Arg391His), and an intronic mutation at position +5 of intron 7 (G>T) (designated c.831 +5 G>T) (Figure 1, A and B). Analysis of DNA from the two parents showed that c. 34 C>T; p.Arg12Cys was inherited from the father, along with p.Arg391His, whereas c.831 +5 G>T was inherited from the mother (Figure 1A). Polyphen software (<http://tux.embl-heidelberg.de/ramensky/polyphen.cgi>) and SIFT software (<http://blocks.fhcrc.org/sift/SIFT.html>) both predicted a benign effect of the p.Arg391His substitution and detrimental effect of the p.Arg12Cys mutation. Indeed, the p.Arg12Cys mutation involves a highly conserved residue that is located in the external region of the death domain of the protein (Figure 1C). P.Arg391His affects a non conserved amino acid residue of the IRAK-4 kinase domain and is located near a previously described polymorphism (rs4251583, p. His390Arg). In addition, the p.Arg391His mutation does not alter mRNA splicing (data not shown). Although we cannot rule out the possibility that the presence of the two mutations on the same paternal allele has a detrimental effect on the protein function, our findings make it more likely that only p.Arg12Cys is deleterious and that p.Arg391His is a rare neutral variant.

RNA from the patient was further studied to assess the potential consequence of the c.831 +5 G>T mutation. For this purpose, primers were designed in exons 6 and 11 and PCR products from the patient's cDNA were analyzed onto ABI310 (Figure 2A). The presence of an abnormal band revealed that the intronic mutation resulted in a splice defect leading to the skipping of exon 7 and a predicted stop codon at position 249. Sequencing of the shortened PCR band confirmed the abnormal exon 6-8 junction (Figure 2B). The observation of a relative abundance of a shortened mRNA without exon 7 as compared with full-length mRNA (see figure 2) argued against marked mRNA nonsense-mediated decay and could thus theoretically lead to the production of a truncated protein ending at position 248 (p.Cys240MetfsX8).

The possibility that residual full-length RNA molecules were produced from the maternal allele was excluded by taking advantage of the patient's heterozygosity for the p.Arg391His variant in exon 10. cDNA from the patient was amplified using LNA modified primers with a C or a T at the 3' end (thus specific for the wild-type allele G, ex10R LNA\_G, or for the paternal allele A, ex10R LNA\_A, figure 3A). Maternal mRNA molecules were theoretically specifically amplified using ex10R LNA\_G and paternally derived mRNA, using ex10R LNA\_A. The specificity of the LNA primers was confirmed using the ex7F and ex10 LNA primers as shown in figure 3B. PCR amplification was only observed with the ex7F and ex10 LNA\_G primers in the control (homozygous for the wild-type allele, c.1170 G/G), whereas PCR amplification was positive in the patient using primers ex7F and ex10 LNA\_A and negative using primers ex7F and ex10 LNA\_G. These results confirmed that full-length mRNA molecules (containing exon 7) were exclusively produced from the paternal allele. They were confirmed by using Ex6F – ex10R LNA\_G, that amplified only exon 7-truncated mRNA, and full-length mRNA molecules using Ex6F – ex10R LNA\_A (Figure 3B). Together

these data confirmed that full-length mRNA molecules were exclusively produced from the paternal allele and thus carried the [p.Arg12Cys and p.Arg391His] mutations.

#### *Presence of a non functional IRAK-4 protein*

As expected, a band corresponding to an IRAK-4 protein of apparently normal molecular weight was detected by western blotting of the patient's PMN; no shortened protein was observed (Figure 1D). As the first step of IRAK-4 activity is the phosphorylation of IRAK-1 (29), we studied the functionality of IRAK-4 protein by analyzing the phospho-IRAK-1 content of intact PMN treated with TLR agonists in whole blood, by means of flow cytometry with a mouse anti-human phospho-IRAK-1 Ab. Incubation of whole blood from healthy controls with TLR agonists for 5 minutes significantly increased IRAK-1 phosphorylation as compared to PBS (Table I). In contrast, pretreatment of the patient's PMN with all the TLR agonists, including the TLR9 agonist, did not modify IRAK-1 phosphorylation as compared to PBS. This result suggested that IRAK-4 was non functional.

#### *Impaired PMN adhesion molecule expression and ROS production in response to IL-18 and TLR agonists, except for TLR9*

PMN dysfunctions have been reported in IRAK-4 deficiencies (15), especially in response to LPS (TLR4), contrasting with normal responses to TNF. We therefore analyzed the effect of a broad range of TLR agonists on adhesion molecule expression and ROS production by PMN. CD11b expression by resting patient's PMN was normal, in keeping with normal chemotaxis and with the absence of LAD (not shown). In controls, incubation of whole-blood samples with TNF $\alpha$ , GM-CSF, IL-18 and the following TLR agonists: LPS (TLR4), MALP-2 (TLR2/6), Pam3CSK4 (TLR1/2), R-848 (TLR7/8) and CpG-DNA (TLR9) induced a significant increase in CD11b expression (Figure 4A) and a significant decrease in L-selectin



expression (Figure 4B) related to activation-induced shedding of this molecule (30). In the patient, TNF $\alpha$  and GM-CSF stimulation induced normal CD11b expression and normal L-selectin shedding at the PMN surface as compared to PBS-treated samples. In contrast, after treatment of patient's samples with IL-18 and the following TLR agonists: LPS (TLR4), MALP-2 (TLR2/6), Pam3CSK4 (TLR1/2) and R-848 (TLR7/8), no significant increase in CD11b expression was observed as compared with the sample incubated with PBS; In addition, L-selectin was still detectable at the PMN surface, reflecting a defect in the shedding of this molecule. Surprisingly, however, the response to CpG-DNA (TLR9) was conserved (Figure 4, A and B).

In controls, pretreatment of whole blood with TNF $\alpha$ , GM-CSF, IL-18 or with the various TLR agonists, followed by stimulation with fMLP, a structural analog of bacterial metabolic products, strongly increased ROS production (Figure 4C). A similar increase in ROS production was also observed in the patient's PMN after pretreatment with TNF $\alpha$  or GM-CSF and stimulation with fMLP, ruling out defective priming of the phagocyte oxidative burst (31). This priming effect on the fMLP-stimulated PMN oxidative burst, which was also observed after treatment with CpG-DNA, was no longer detectable after incubation with the other TLR agonists or IL-18 (Figure 4C).

Although the effect of TLR agonists on adhesion molecule expression and ROS production by monocytes is far lower than with PMN, the patient's monocytes showed a pattern of responses similar to that of his PMN, with altered responses to TLR agonists except for CpG-DNA (not shown).

### *Impaired prolongation of PMN survival by IL-18 and TLR agonists, except for TLR9*

As PMN are usually very short-lived immune cells, prolongation of their lifespan by proinflammatory mediators is critical for their efficacy against pathogens (32). In keeping with previous reports (4, 33, 34), treatment of control PMN for 8 hours with GM-CSF, IL-18 and TLR agonists induced ~ 50 to 90% inhibition of PMN apoptosis in whole blood (total annexin V<sup>+</sup> cells); similar levels of inhibition were found in the early (annexin V<sup>+</sup>/7AAD<sup>-</sup> cells) and late stage (annexin V<sup>+</sup>/7AAD<sup>+</sup> cells) of PMN apoptosis (not shown). In contrast, neither IL-18 nor LPS (TLR4), MALP-2 (TLR2/6), Pam3CSK4 (TLR1/2) and R-848 (TLR7/8) were able to inhibit the patient's PMN apoptosis (percentage inhibition of PMN apoptosis ~ 0%), while GM-CSF induced a normal prolongation of PMN survival. In keeping with the results for adhesion molecule expression and ROS production, the effect of CpG-DNA (TLR9) on the patient's PMN apoptosis was conserved (Figure 4D).

We recently reported that the TLR-induced delay in PMN apoptosis was associated with modulation of Bcl-2 family members (4), with an increased level of the anti-apoptotic protein Mcl-1 and increased phosphorylation of the proapoptotic protein Bad, which have been reported to inhibit apoptosis (35). In our patient, while CpG-DNA (TLR9) induced a normal increase in Mcl-1 and phospho-Bad content, no modulation of either of these two proteins was observed after stimulation with the other TLR agonists, as compared to samples incubated with PBS (Table II).

### *Impaired cytokine production by PMN and monocytes in response to IL-18, IL-1 and all TLR agonists.*

In keeping with previous data on patients with IRAK-4 deficiency (15, 16), we found strongly impaired pro-inflammatory cytokine (IL-8) production in the supernatant of the patient's

whole-blood samples cultured with all TLR agonists, including CpG-DNA (Figure 5A). Similar results were observed for IL-6, IL-1 $\beta$  and TNF $\alpha$  production (not shown).

As cytokine production by whole blood cells mainly reflects synthesis by monocytes, the patient's monocytes and PMN were isolated and highly purified in order to analyze the individual response of the two cellular subpopulations to TLR agonists, and especially to CpG-DNA (TLR9). As expected, IL-8 production by control monocytes incubated with TLR agonists was far higher than that of control PMN. Neither monocytes (Figure 5B) nor PMN (Figure 5C) were able to produce significant amounts of IL-8 in response to LPS (TLR4), MALP-2 (TLR2/6), Pam3CSK4 (TLR1/2) nor R-848 (TLR7/8). IL-8 production by the patient's PMN and monocytes in response to CpG-DNA was also strongly diminished as compared to that of cells from a healthy control.

Finally, the parents' PMN exhibited normal responses (adhesion molecule expression, ROS production, delayed apoptosis, cytokine production) to all TLR agonists (not shown).

#### *Involvement of direct stimulation of the PI3K pathway in the preserved PMN responses to TLR9*

CpG-DNA induced normal responses by the patient's PMN in terms of survival, adhesion molecule expression and ROS production, despite the lack of functional IRAK-4. This strongly suggested that an alternative pathway, independent of the classic TLR9/IRAK-4 pathway, was involved in TLR9 signaling. We therefore examined the effects of various kinase inhibitors on L-selectin and CD11b expression at the PMN surface, as well as on PMN apoptosis in whole blood incubated with CpG-DNA. These inhibitors were used at optimal concentrations previously determined in whole blood (4). Pretreatment with inhibitors of conventional protein kinase C (GF109203X: 5  $\mu$ M), PKC $\delta$  kinase (rottlerin: 10  $\mu$ M) and tyrosine kinase (genistein: 100  $\mu$ M) had no effect on CpG-DNA-induced responses (not

shown). In contrast, pretreatment of the patient's whole blood with PI3K inhibitors (wortmannin and LY2940002) suppressed the effect of CpG-DNA on PMN responses. CpG-DNA-induced shedding of L-selectin led to a significant decrease in L-selectin expression at the PMN surface as compared to PBS-treated samples (Figure 6A); this decrease was totally abolished after preincubation of the patient's PMN with PI3K inhibitors (the MFI of the sample incubated with PI3K inhibitors + CpG-DNA was similar to that observed after treatment with PBS alone) (Figure 6A). CpG-DNA-induced modulation of L-selectin expression was also significantly reduced after preincubation with PD98059 (a ERK1/2 kinase inhibitor) or SB203580 (a p38MAPK inhibitor). Similarly, the CpG-DNA-induced increase in CD11b expression (reflected by the increase in stimulation index) (Figure 6B) was significantly reduced after pre-incubation with PI3K inhibitors as well as with ERK1/2 kinase and p38MAPK inhibitors.

Finally, in keeping with previous data (4), the CpG-DNA-induced increase in the percentage of cell survival (annexin V<sup>+</sup>/7-AAD<sup>-</sup> cells) was significantly reduced after preincubation of control samples with PI3K inhibitors and the NF- $\kappa$ B inhibitor SN50, while MAPKinase inhibitors did not affect PMN apoptosis (Figure 6C). In the patient's PMN, the inhibitory effect of PI3K inhibitors was conserved while the NF- $\kappa$ B inhibitor SN50, which strongly suppressed CpG-DNA-induced survival of control PMN, had no effect on TLR9-induced survival of the patient's PMN.

The PI3K inhibitor-induced reduction in PMN responses was also observed in healthy controls but at a lower level than in the patient (Figure 6, A, B and C). This result suggested a critical role of the direct TLR9/PI3K pathway in the patient's PMN responses.

The effect of ERK1/2 kinase and p38MAPK inhibitors on CpG-DNA-induced modulation of adhesion molecule expression observed in control and the patient's PMN strongly suggested the involvement of these kinases downstream of TLR9 activation. We therefore studied the

phospho-ERK1/2 and phospho-p38MAPK contents of intact PMN treated in whole blood, by means of flow cytometry. As shown in Figure 7, incubation of whole blood from controls and the patient with CpG-DNA significantly increased ERK1/2 and p38MAPK phosphorylation after 10 min as compared to PBS. This effect was significantly reduced by preincubation with PI3K inhibitors. Total ERK1/2 and p38MAPK content, measured in the same conditions, was not modified by treatment with CpG-DNA (data not shown).

## Discussion

We describe an inherited IRAK-4 deficiency in a patient with compound heterozygosity generating a non functional IRAK-4 protein. PMN functional responses (adhesion molecule expression, ROS production, survival and pro-inflammatory cytokine production) to MALP-2, Pam3CSK4, LPS, and R-848, which engage TLR2/6, TLR1/2, TLR4 and TLR7/8, respectively, were strongly impaired, as were PMN responses to IL-18 (whose receptor shares the same intracytoplasmic TIR domain). In contrast, the patient's PMN responses to CpG-DNA (TLR9) were normal, except for cytokine production, suggesting the existence, in parallel to the MyD88/IRAK-4-dependent pathway, of a distinct TLR9-induced transduction pathway regulating adhesion molecule expression, ROS production and survival.

The patient's PMN exhibited an impaired response to several agonists of the IL-1R family, and especially TLRs, while a normal response to other stimuli, including TNF, was observed. These results suggest that the patient has a defect in the common TIR signalling pathway, upstream of TRAF-6 and downstream of individual TIR membrane receptors. *IRAK4* gene analysis showed two compound heterozygous mutations in our patient. The maternally inherited mutation at position + 5 of intron 7 (G>T) was predicted to result in a protein of 248 amino acids, truncated of a large part of the kinase domain. However, no shortened band was observed on western blots with a polyclonal Ab directed against the whole IRAK-4 protein. These results suggest that the truncated protein resulting from the maternal mutation is degraded as exon 7 skipped mRNA molecules were not subject to drastic nonsense mediated decay. The two paternally inherited missense mutations, located in the death domain (p.Arg12Cys) and in the kinase domain of IRAK-4 (p.Arg391His), did not interfere with IRAK-4 synthesis: IRAK-4 protein was detected in the patient's PMN by western blot. However, none of the TLR agonists increased IRAK-1 phosphorylation, further demonstrating the non function of IRAK-4 protein. These results suggest that the p.Arg12Cys

missense mutation of the paternal allele may hamper protein-protein interactions via the death domain in the TIR pathway. Indeed, the p.arg12Cys mutation involves a residue that is located at the surface of the protein; this domain is thought to interact with the different ligands of IRAK-4 such as MyD88. This residue is highly conserved through evolution in IRAK-4 orthologous proteins including human, *Mus musculus*, *Bos taurus*, *Gallus gallus*, *Xenopus tropicalis*, *Danio rerio* and *Euprymna scolopes* (Supplementary data available on request) but is not conserved in paralogues of the IRAK family, suggesting that it participates to the specificity of interaction. It is therefore most likely that the substitution of this positively charged residue (arginine) by a neutral one (cystein) interferes either with the conformation of the IRAK-4 death domain or with the interaction of IRAK-4 with its partners, preventing the assembly of an active signaling complex following TLR activation. P.Arg391His, located in cis of the p.Arg12Cys substitution, was predicted to be benign with two software programs based on structural and amino acid conservation, and had no effect on mRNA splicing. A deleterious effect is thus unlikely, but a synergistic deleterious effect of the two missense mutations could not be excluded. The non function of IRAK-4 protein in our patient was also confirmed by the absence of significant IKK phosphorylation, which results from TLR pathway activation (not shown).

TLR9-induced responses, i.e. adhesion molecule expression, ROS production and survival, were normal in the patient's PMN, while cytokine production was lower than with control PMN. This suggests that different pathways may be involved in these functions; in particular, the IRAK-4 dependent pathway is necessary for TLR9-induced cytokine production, while other functions might use independent pathways. PI3Ks have been reported to enhance nuclear translocation of NF- $\kappa$ B through phosphorylation and activation of I $\kappa$ B-kinase and activation of MAPK, especially in TLR2-stimulated PMN (13); nevertheless, it is not known whether MyD88/IRAK-4 complexes are required for this pathway. We clearly

found that PI3K inhibitors (wortmannin and LY2940002) totally suppressed the effect of CpG-DNA on adhesion molecule expression and survival of our patient's PMN. These results suggest that TLR9 may be directly linked to PI3K, while the MyD88/IRAK-4 dependent pathway may be required for PI3K activation through the other TLRs. Such direct PI3K activation has been described for TLR2 in a human monocytic cell line (36).

The lipid products of PI3K – mainly phosphatidylinositol 3,4,5-triphosphate – induce translocation of Akt/PKB to the plasma membrane, where it is phosphorylated and activated by phosphatidylinositol 3,4,5-phosphate-dependent kinase (PDK1) (37). This pathway has been forwarded as a major mediator downstream of PI3K (38). In particular, Akt activation induces modulation of Bcl-2 family proteins such as Mcl-1 and phospho-Bad (39, 40), and could therefore be involved in the inhibitory effect of CpG-DNA on our patient's PMN apoptosis. In addition, it has been reported that class I PI3K catalytic subunits can lead to phosphorylation of ERK1/2 and p38MAPK (9, 10, 41, 42); activation of these signaling pathways has been implicated in the upregulation of CD11b expression (43, 44) and L-selectin shedding (45, 46) after PMN treatment with various inflammatory stimuli. In keeping with these data, we demonstrated that CpG-DNA-induced modulation of CD11b and L-selectin on the surface of our patient's PMN is partially inhibited by pharmacological inhibitors of ERK1/2 and p38MAPK; furthermore, we found a CpG-DNA-induced increase in phosphorylation of ERK1/2 and p38MAPK in the patient's PMN, which was reduced by PI3K inhibitors. These findings strongly suggest that IRAK-4-independent TLR9-induced PI3K activation leads to MAPK recruitment. As CpG-DNA has no direct effect on ROS production, the use of kinase inhibitors did not allow us to analyse the involvement of MAPK in the priming effect of CpG-DNA on the PMN oxidative burst in response to fMLP. Nevertheless, PI3K products have also been reported to exert their effects on the PMN



oxidative burst by activating downstream protein kinases such as Akt, which may directly phosphorylate components of the oxidase complex (47).

Taken together, our results suggest that activation of class I PI3K (PI3K (I) – PDK1/Akt/PKB) through TLR9, and subsequent recruitment of MAPK, could be an alternative pathway to the IRAK/IKK/NF- $\kappa$ B pathway involved in PMN adhesion, oxidative burst, and prolonged survival, which are major components of PMN functional activity. A schematic representation of the different pathways involved in PMN functions is proposed in Figure 8. Nevertheless, we cannot formally exclude the involvement of other, unidentified signalling pathways leading to CpG-DNA-induced PMN responses. In particular, there is evidence of a TLR9-independent pathway leading to downstream PI3K activation and CD11b upregulation in response to bacterial CpG-containing DNA in murine neutrophils. (44, 48). This pathway described in human PMN by Alvarez et al is MyD88-dependent and leads to IRAK-1 phosphorylation, suggesting the involvement of IRAK-4 in subsequent PI3K activation (44). However, we cannot formally exclude the possibility that the IRAK-4-independent activation of PI3K observed in our patient after CpG-DNA stimulation may be related to the existence of TLR9-independent mechanisms, thus implicating non-CpG molecular motifs in synthetic oligonucleotides.

IRAK-4-deficient patients suffer from pyogenic infections but are resistant to viruses, fungi and parasites, as well as many other bacteria. It has been speculated that cell-surface TLRs rapidly sense bacterial infections by recognizing bacterial cell wall constituents in the extracellular medium. In contrast, several lines of evidence suggest that molecular recognition of CpG-DNA occurs inside the cells (49). TLR9 might enter the phagosome from the endoplasmic reticulum (50) and bind bacterial DNA released into the phagosome following bactericidal processes. In addition, TLR9 was recently implicated in host defenses against intracellular pathogens (51, 52). Further studies are necessary to elucidate the role of direct

PI3K activation by TLR9 in the phagosome, relative to cell surface activation of the TLRs/IRAK-4-dependent pathway in defenses against microorganisms, and especially intracellular pathogens. Nevertheless, we clearly observed that CpG-DNA induced normal PMN functions in terms of adhesion molecule expression and survival in our IRAK4-deficient patient, suggesting that the IRAK-4 dependent pathway may be compensated for by the TLR9-dependent IRAK-4-independent pathway. This may account, at least in part, for the observed clinical improvement with age.

In conclusion, this study provides the first description of persistent TLR9-induced responses, critically involved in anti-microbial defenses, by PMN from a patient with inherited IRAK-4 deficiency. These results strongly suggest the existence of a TLR9 alternative pathway leading to PI3K activation independently of the classical MyD88/IRAK-4 pathway. This may explain the control of infections due to microorganisms other than pyogenic bacteria by PMN in patients with inherited IRAK-4 deficiency. Finally, our study emphasizes the importance of “lessons of nature” in understanding the role of the TLR in human defenses.

## **Acknowledgments**

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## Footnotes

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<sup>2</sup> Non standard abbreviations: IRAK: interleukin-1 receptor associated kinase; PMN: polymorphonuclear neutrophil; TIR: Toll-IL-1 receptor; TRAF6: tumor necrosis factor receptor-associated factor 6; LAD: leucocyte adhesion deficiency; HE: hydroethidin; fMLP: N-formyl-methionyl-leucyl-phenylalanine; APC: allophycocyanin; 7-AAD: 7-amino-actinomycin D;

## Figure legends

**FIGURE 1.** Genetic analysis of *IRAK4* gene and protein expression in the patient

A, Schematic representation of the *IRAK4* gene and alleles in the patient. Sequencing chromatograms obtained in our patient are shown.

B, Schematic representation of IRAK-4 protein, with the death domain and kinase domain. The positions of the mutations found in the patient are indicated.

C, Schematic representation of the IRAK-4 death domain (Protein Data Bank accession code 2A9I). This ribbon diagram was generated with PyMOL (DeLano Scientific, [www.pymol.org](http://www.pymol.org)). The Arg12 is shown as full surface amino-acid residue.

D, Expression of IRAK-4 by Western blotting

A total of  $2.5 \times 10^6$  cell equivalents were loaded in each well. Following SDS-PAGE, the proteins were transferred to nitrocellulose membranes and incubated with anti-human IRAK-4 Ab at 1/500 dilution overnight. The Western blots were revealed as described in Materials and Methods.

**FIGURE 2.** Mutation + 5 G>T in intron 7 induces exon 7 skipping

A, PCR amplification products using ex6F cDNA and ex11R cDNA primers and cDNA from a control and the patient were analysed onto ABI310 DNA sequencer. A normal PCR product size (647 pb) and a truncated one (532 pb) was observed in our patient.

B, Sequencing of the shortened band was performed using ex6F cDNA and ex11R cDNA non fluorescent primers.

**FIGURE 3.** Full length mRNA molecules are exclusively produced from the paternal allele. Allele specific PCR was performed using two different reverse LNA primers. Ex10R LNA\_G primer matches the wild type allele (c.1170 G allele) whereas Ex10R LNA\_A primer is specific of the variant allele (c.1170 A allele). The paternal mRNA contained the A allele and the maternal mRNA the G allele. The control used in this experiment was homozygous for the G allele. Fluorescent products obtained after PCR amplification of cDNA from the patient or the control were then analysed on a ABI310 DNA sequencer. Expected PCR product sizes are indicated. The 416-bp peak observed with the Ex6F-Ex10R LNA\_G set of primers corresponds to the exon 7-skipped form of mRNA.

**FIGURE 4.** Impaired PMN functions in response to IL-18 and TLR agonists, except for TLR9

A and B, Adhesion molecule expression at the PMN surface: whole-blood samples were incubated at 37°C for 1 hour with PBS, TNF $\alpha$  (100 U/ml), GM-CSF (1000 pg/ml), IL-18 (500 ng/ml) or with the following TLR agonists: LPS (10 ng/ml) (TLR4), MALP-2 (10 ng/ml) (TLR2/6), Pam3CSK4 (500 ng/ml) (TLR1/2), R-848 (10  $\mu$ g/ml) (TLR7/8), or CpG-DNA (100  $\mu$ g/ml) (TLR9). Samples were then stained with PE-anti-CD11b and purified anti-L-selectin antibodies at 4°C for 30 minutes.

Results are expressed as mean fluorescence intensity (MFI).

\* Significantly different from sample incubated with PBS ( $p < 0.05$ ).

C, PMN oxidative burst: whole-blood samples were pretreated with HE for 15 minutes at 37°C and then incubated with TNF $\alpha$ , GM-CSF, IL-18 or TLR agonists as described above, followed by fMLP stimulation ( $10^{-6}$  M, 5 minutes).

Results are expressed as a stimulation index (ratio of the mean fluorescence intensity of stimulated cells to that of unstimulated cells).

\* Significantly different from the sample incubated with PBS (stimulation index=1) ( $p < 0.05$ ).

D, PMN apoptosis: whole-blood samples were incubated in 24-well tissue cultures plates at 37°C with 5% CO $_2$  for 8 hours with PBS, GM-CSF, IL-18 or TLR agonists as described above. PMN were identified by using a FITC-anti-CD15 Ab. Apoptosis was quantified by staining with APC-annexin V and 7-AAD as described in Materials and Methods.

Results are expressed as the percentage inhibition of PMN apoptosis [ $1 - (\% \text{ of total annexin V}^+ \text{ PMNs in stimulated sample} / \% \text{ of total annexin V}^+ \text{ PMN in PBS-treated sample})$ ] x 100.

\* Significantly different from the sample incubated with PBS (percentage inhibition of apoptosis=0) ( $p < 0.05$ ).

Panel A to D: three independent experiments, each with a different healthy control.

**FIGURE 5.** Impaired cytokine production by blood cells in response to TLR agonists, IL-1 $\beta$  and IL-18.

Whole-blood samples (Panel A), isolated monocytes ( $5 \times 10^5$ /ml) (Panel B), and highly purified PMN ( $5 \times 10^6$ /ml) (Panel C) were incubated for 18 hours with PMA-ionomycin ( $10^{-7}$ M and  $10^{-5}$ M), PMA ( $10^{-7}$ M), IL-1 $\beta$  (50 ng/ml), IL-18 (500 ng/ml) or TLR agonists as described in the legend of figure 4.

IL-8 production was measured by using the human inflammatory cytokine cytometric bead array (CBA) kit.

\* Significantly different from the sample incubated with PBS ( $p < 0.05$ ) ( $n = 3$ , each experiment performed with a different healthy control).

**FIGURE 6.** Involvement of the putative IRAK-4-independent PI3K signalling pathway in the persistent PMN response to TLR9.

A and B, Effect of kinase inhibitors on CpG-induced modulation of adhesion molecule expression at the PMN surface: whole-blood samples were pretreated at 37°C with PBS, PI3K inhibitors (wortmannin: 2500 nM; LY2940002: 25 µM), MEK1/2 inhibitor (PD98059: 50 µM) or p38MAPK inhibitor (SB203580: 25 µM) for 15 minutes and then with PBS or CpG-DNA for 1 hour. L-selectin and CD11b expression at the PMN surface were then studied as described in the legend of figure 4. Results are expressed in MFI (L-selectin expression) and as a stimulation index (CD11b expression: ratio of the MFI of CpG-DNA-stimulated cells to that of unstimulated cells).

C, Effect of an NF-κB inhibitor (SN50) and kinase inhibitors on CpG-DNA-induced PMN survival.

Whole-blood samples were pretreated in 24-well tissue culture plates at 37°C in 5% CO<sub>2</sub>/air with PBS, SN50 (100 µg/ml), PI3K inhibitors (wortmannin: 2500 nM; LY2940002: 25 µM), MEK1/2 inhibitor (PD98059: 50 µM) or p38MAPK inhibitor (SB203580: 25 µM) for 1 hour. Samples were then incubated with PBS or CpG-DNA for 8 hours. Survival was quantified as described in the legend of figure 4. Results are expressed as the percentage of viable cells (annexin V<sup>-</sup>/7-AAD<sup>-</sup> cells).

\* Significantly different from the CpG-DNA-stimulated sample incubated with PBS alone instead of kinase inhibitors (p<0.05) (n=3, each experiment performed with a different healthy control).



**FIGURE 7.** Effect of CpG-DNA on intracellular ERK1/2 and p38MAPK phosphorylation.

Whole-blood samples were preincubated at 37°C in a water bath with PBS or PI3K inhibitors (wortmannin: 2500 nM; LY2940002: 25 µM) for 15 minutes and then with PBS or CpG-DNA (100 µg/ml) for 10 min. Phospho-p38MAPK and phospho-ERK1/2 contents were then measured by flow cytometry on methanol-permeabilized cells as described in Materials and Methods.

Results are expressed as the mean fluorescence intensity (MFI). Values obtained with an irrelevant Ab of the same isotype were subtracted.

Values are means ± SEM (n=3, each experiment was performed with a different healthy control).

\* Significantly different from samples incubated with PBS alone (p<0.05).

**FIGURE 8.** Scheme of CpG-DNA/TLR9-mediated cellular signalling in PMN.

**IRAK-4 dependent pathway.** Recruitment of the TIR domain activates IRAK-4-TRAF6-TAK1 complex formation. This leads to the activation of both MAPKs and IKK complexes, culminating in upregulation of transcription factors, including NF-κB. NF-κB activation leads to pro-inflammatory cytokine production and delays apoptosis. MAPK activation may be involved in the modulation of adhesion molecule expression at the PMN surface and in increased ROS production by primed PMN.

**Alternative IRAK-4-independent pathway.** Activation of class I PI3K (PI3K (I) – PDK1/Akt/PKB) through TLR9 could be an alternative to the IRAK/IKK/NF-κB pathway. Its activation could lead to 1) delayed apoptosis through independent modulation of Bcl-2 family proteins, and 2) recruitment of MAPKs involved in PMN adhesion and the oxidative burst.

Figure 1

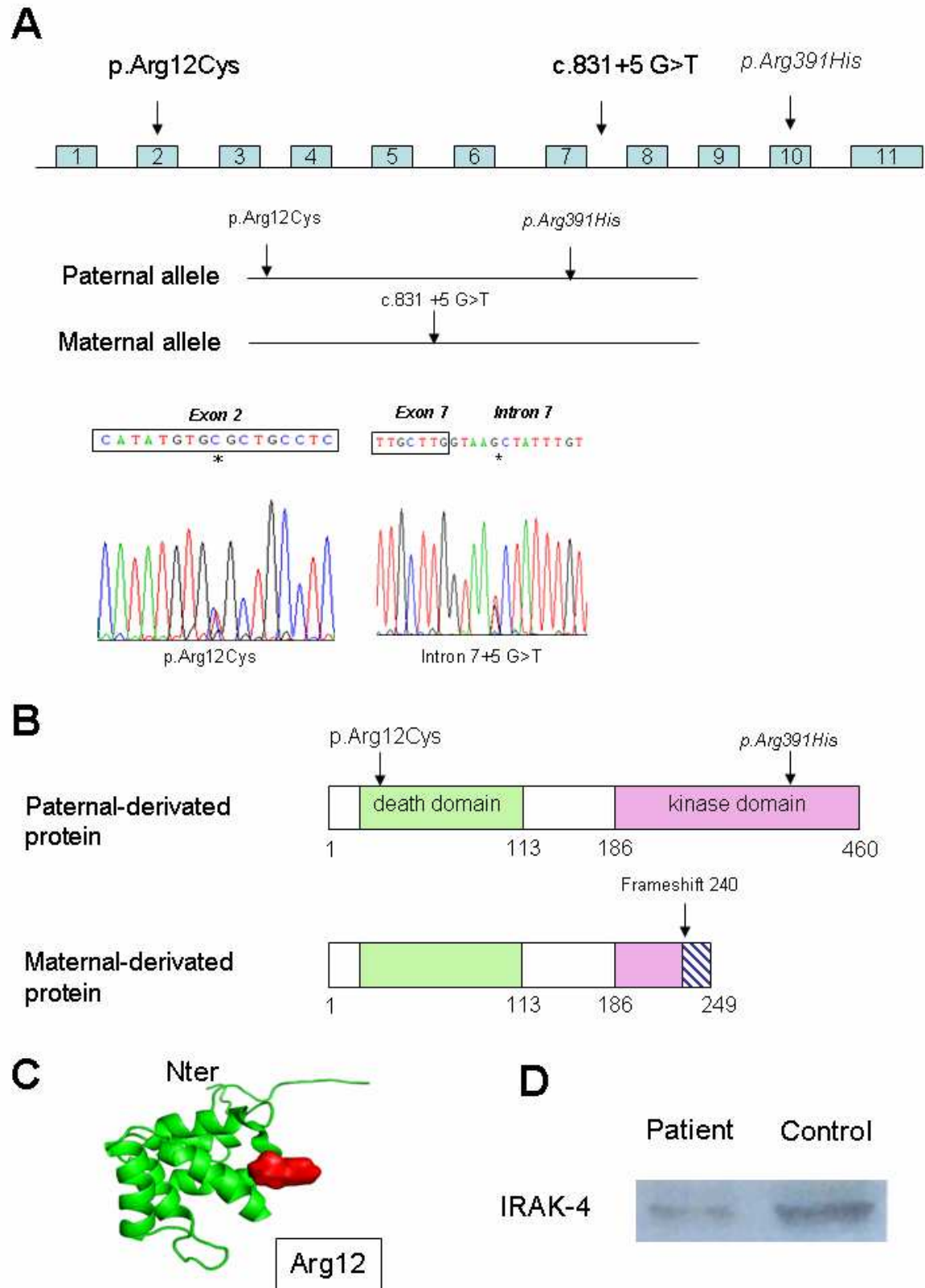
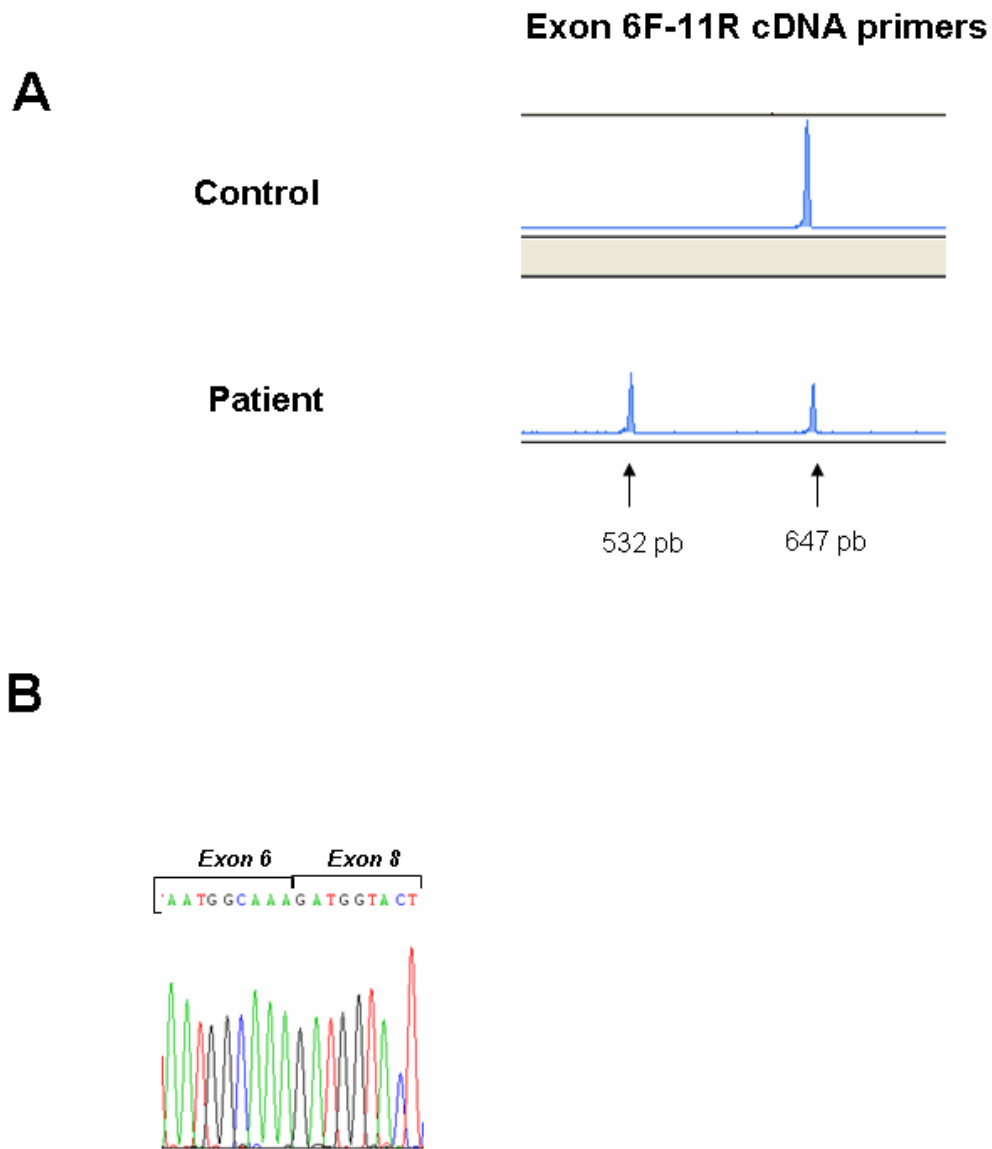
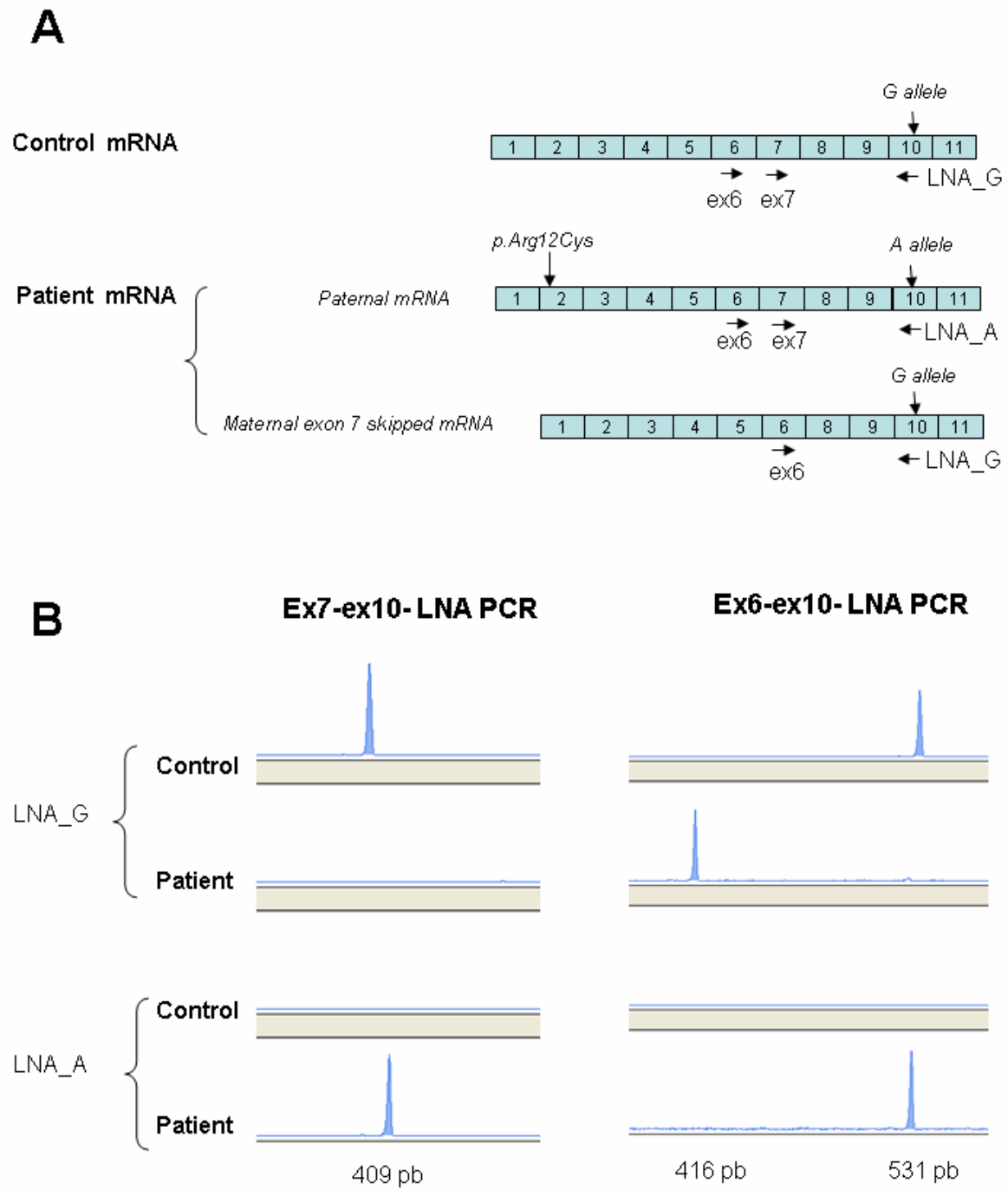


Figure 2



**Figure 3**



**Figure 4**

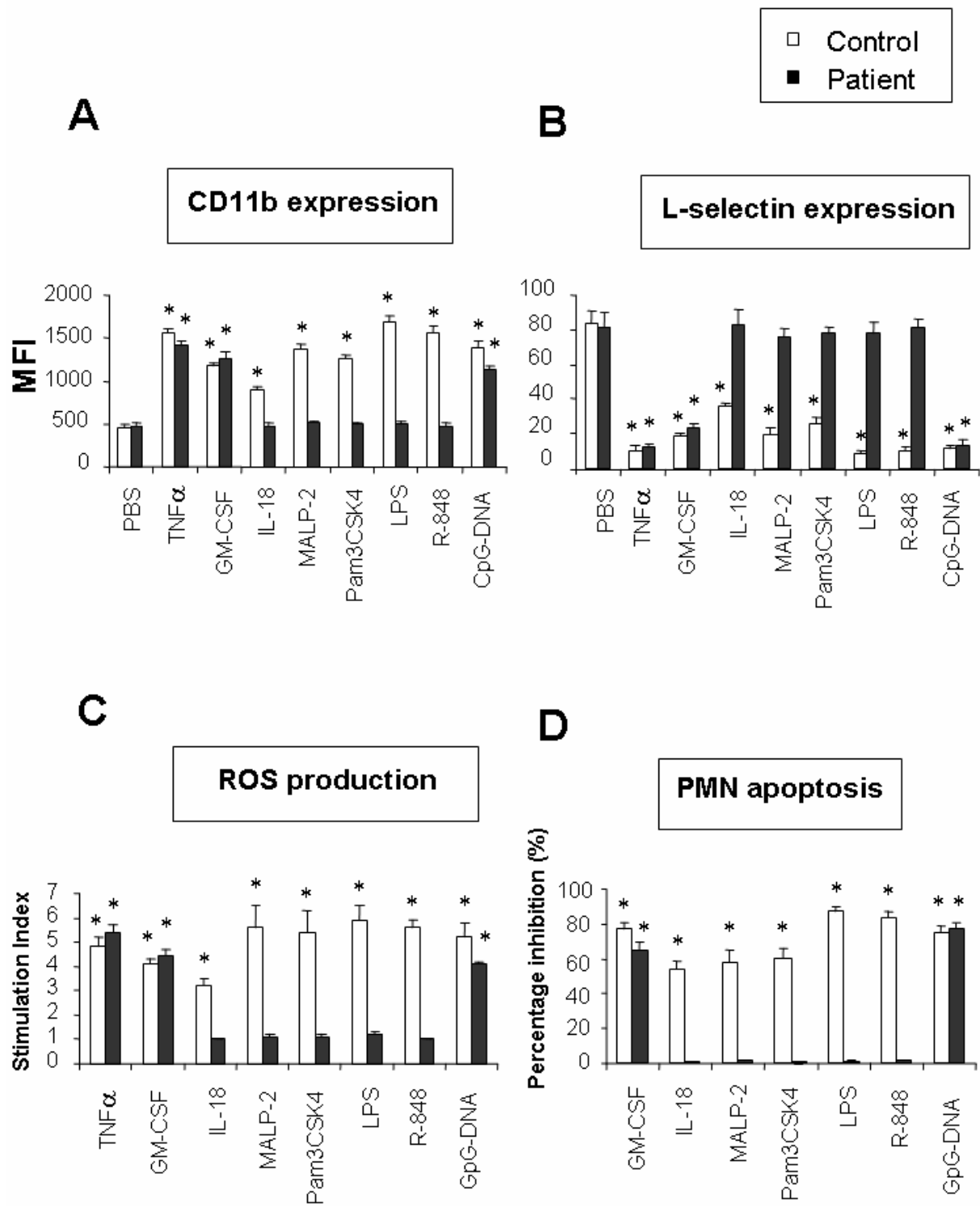


Figure 5

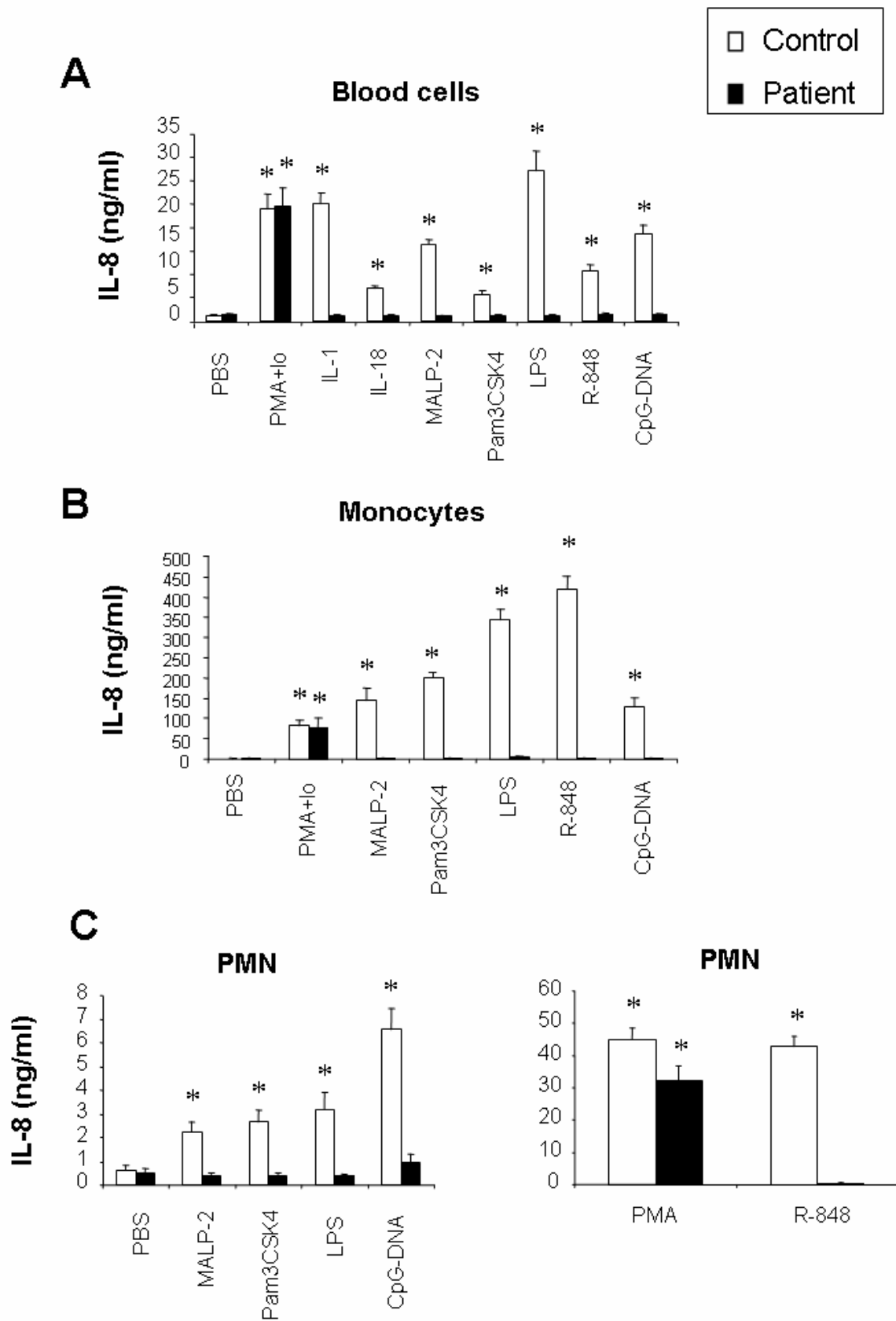


Figure 6

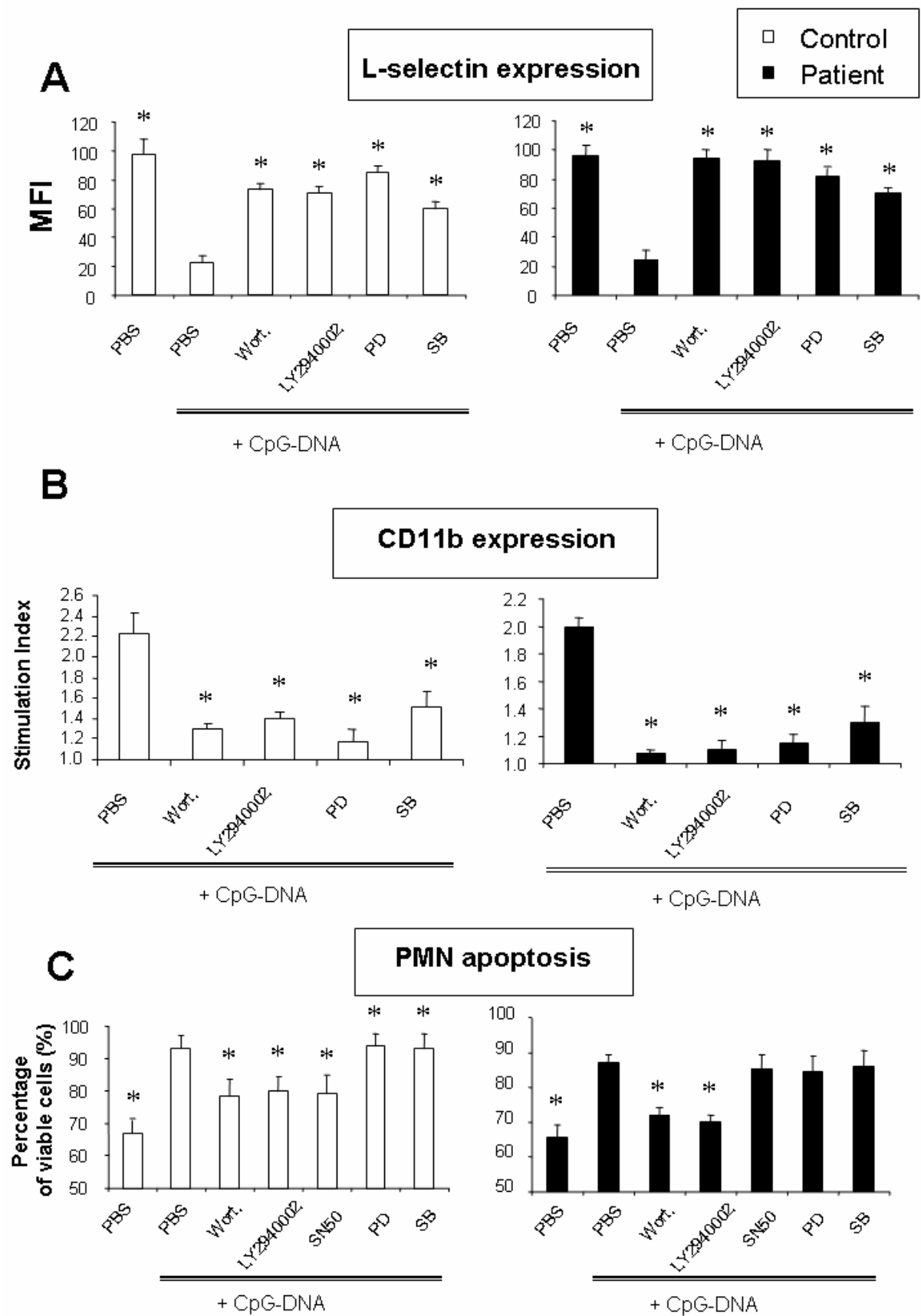
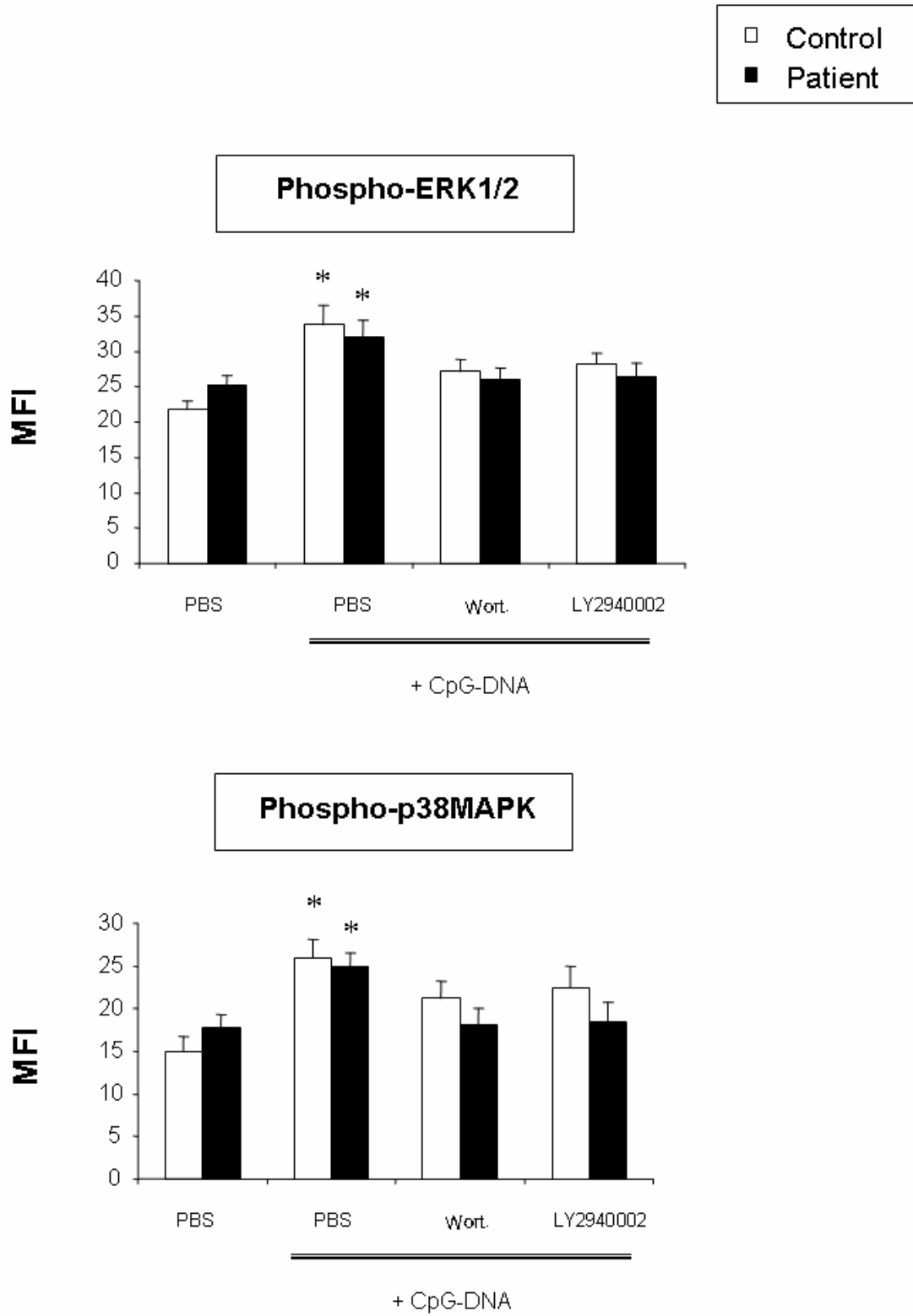


Figure 7





**Figure 8**

