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An Angiogenic Switch in Macrophages Involving Synergy between Toll-Like Receptors 2, 4, 7, and 9 and Adenosine A_{2A} Receptors

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Adenosine A_{2A} receptor (A_{2A}R) agonists synergize with *Escherichia coli* (*E. coli*) LPS [toll-like receptor (TLR)4 agonist] to up-regulate vascular endothelial growth factor (VEGF) expression in murine macrophages. Here, we demonstrate that TLR2, TLR7, and TLR9, but not TLR3 and TLR5 agonists, also synergize with A_{2A}R agonists and adenosine to up-regulate VEGF, while simultaneously strongly down-regulating TNFα expression. In the absence of adenosine or A_{2A}R agonists, *Porphyromonas gingivalis* (*P. gingivalis*) LPS and PAM_3CAG (TLR2 agonists), resiquimod (R848) (TLR7 agonist), and non-methylated CpG DNA (TLR9 agonist) strongly up-regulate TNFα expression, with no effect on VEGF. In the presence of adenosine or A_{2A}R agonists, but not A_1R agonists, TLR2, 4, 7, and 9 agonists strongly up-regulate VEGF expression, while simultaneously down-regulating TNFα. C57BL/10ScN (TLR4 deletion mutant) macrophages produce TNFα in response to TLR2, 3, 7, and 9 agonists, but not the TLR4 agonist *E. coli* LPS. With adenosine or A_{2A}R agonists, TLR2, 7, and 9, but not TLR4 agonists, also synergistically up-regulate VEGF, while down-regulating TNFα expression. Polyinosinic-polycytidilic acid (poly(I:C)) (TLR3 agonist) stimulates TNFα expression in macrophages from both C57BL/10ScSn and C57BL/10ScN mice, but has little effect on VEGF expression in the presence of adenosine or A_{2A}R agonists. R-flagellins from *Serratia marcescens* (*S. marcescens*) and *Salmonella muenchen* (*S. muenchen*) do not stimulate TNFα expression in either C57BL/10ScSn or C57BL10/ScN mice, and have no effect on VEGF production in the presence of adenosine or A_{2A}R agonists. While adenosine and A_{2A}R agonists strongly down-regulate TNFα protein expression induced by TLR2, 3, 4, 7, and 9 agonists, TNFα mRNA and NF-κB activation are not reduced. We propose a novel signaling pathway in murine macrophages involving synergy between TLRs 2, 4, 7, and 9 and A_{2A}Rs, that up-regulates VEGF and down-regulates TNFα expression, thus acting as an angiogenic switch. This angiogenic switch may play an important role in ischemia when TLR agonists are present, providing an interface between innate immunity and wound healing. ***(Am J Pathol 2003, 163:711–721)***

Vascular endothelial growth factor (VEGF) potently stimulates both angiogenesis and vascular permeability.[1,2] We have shown previously that VEGF expression by murine macrophages is synergistically up-regulated by *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) acting through TLR4 receptors, and adenosine A_{2A} agonists acting through A_{2A}Rs.[3] Treatment of macrophages with 2-[p-(2-carboxylethyl)-phenylethyl amino]-5′-N-ethyl-carboxamido-adenosine (CGS21680) (a specific adenosine A_{2A}R agonist), or 5′-N-ethyl-carboxamido-adenosine (NECA) (a non-specific adenosine A_2R agonist), together with *E. coli* LPS, strongly up-regulates VEGF expression above the level induced by CGS21680 or NECA alone, while LPS alone does not induce VEGF expression. This synergistic up-regulation is independent of hypoxia, NO, and protein kinase-A and is stronger than that induced by hypoxia alone.[3]

Mammalian toll-like receptors (TLRs) are members of a family of proteins that resemble the *Drosophila* toll protein.[4,5] Toll plays a role in dorsal-ventral patterning in the developing fly embryo, and also plays a key role in regulating the innate immune response of adult flies to fungi.[4,5] In mammals, TLR receptors also play a key role in the innate immune response. TLR receptors respond to bacteria and bacterial products by transmitting a ligand-induced *trans*-membrane signal that induces the expression of cytokines such as TNFα, IL-1, IL-6, and IL-12 that

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are important in the host response to infection.[4,5] At least ten mammalian toll-like receptors have been described to date. TLR intracellular domains resemble that of the IL-1 receptor (IL-1R), and are termed toll/IL-1R (TIR) domains. Each TLR responds to conserved structural features of a particular group of bacteria or their products, acting as a pattern recognition receptor. TLRs have also been implicated in the recognition of viruses and fungi.[4,5] TLR2 and TLR4 mediate cellular activation by a variety of bacterial products, including gram-negative bacterial LPS, the mycobacterial glycolipid lipoarrabinomannan (LAM), bacterial lipoproteins, peptidoglycan, and zymosan.[6,7] Enterobacterial LPS is recognized by TLR4; however, LPS derived from *Leptospira interrogans* (*L. interrogans*) or *Porphyromonas gingivalis* (*P. gingivalis*) signal through TLR2. LAM, peptidoglycan, bacterial lipoproteins, and zymosan are recognized by TLR2. Recently, TLR5 has been shown to be the receptor for bacterial flagellins, which are a virulence factor recognized by the innate immune response system in organisms as diverse as flies, plants, and mammals.[8,9] Many pathogens, including bacteria, fungi, yeast, and nematodes, contain a characteristic DNA pattern, the CpG motif.[10,11] CpG motifs are non-methylated C-G dinucleotides flanked by two 5′ purines and three 3′ pyrimidines. In mammalian genomic DNA, these sequences are rare, and are generally methylated. TLR9 has been shown to be the receptor for non-methylated C-G rich DNA (CpG DNA).[10,11] The antiviral imidazoquinoline resiquimod (R-848) has been shown recently to be a TLR7 agonist in mice.[12] The synthetic double-stranded RNA (dsRNA) analog polyinosinic-polycytidylic acid (poly(I:C)), a viral RNA mimetic, has been shown recently to be a TLR3 agonist.[13] The TLR family of receptors thus appears to provide a critical interface between higher organisms and microorganisms, whereby microorganisms, or distinct components thereof, are recognized, signal cellular activation, and induce a host response against these organisms. TLR-dependent signaling pathways lead to the activation of the $I\kappa B\alpha$/NF-κB pathway, inducing the expression of genes that participate in innate immune responses, including many inflammatory cytokines and antimicrobial peptides.[4–6,14]

Adenosine, produced by many different tissues and cell types, is elevated in response to a variety of signals, including hypoxia and ischemia.[15–17] Interstitial concentrations of adenosine are normally in the region of 100 to 500 nmol/L.[18] The interstitial concentration of adenosine can increase dramatically in response to ischemia, hypoxia, and inflammation, to levels of up to 100 μmol/L.[15–17,19] Acting at both internal and external sites, adenosine modulates a variety of macrophage functions, including phagocytosis, antigen presentation, target cell killing, production of NO, IL-6, IL-10, IL-12, TNFα, and expression of MHC class-II molecules.[20–25] Adenosine mediates many of its effects through cell surface G-protein-coupled receptors. Four specific subclasses of adenosine receptors have been characterized to date, the A_1, A_{2A}, A_{2B}, and A_3 receptors.[26–29] Agonists of A_{2A}Rs down-regulate expression of TNFα and IL-12 in murine and human macrophages, and up-regulate expression of the anti-inflammatory cytokine IL-10.[22] Thus, adenosine acts as an anti-inflammatory agent, suppressing expression of inflammatory cytokines.

In this study, we demonstrate that the synergy between A_{2A}R agonists and *E. coli* LPS (a TLR4 agonist) that results in the up-regulation of VEGF production is not limited to TLR4, but can also be induced by TLR2, 7, and 9 agonists. This synergistic interaction between A_{2A}Rs and certain TLR receptors is also observed as a concurrent down-regulation of TNFα expression. Therefore, this interaction may represent an important interface between the innate immune response and the host response to injury, by providing an amplification mechanism between the host response to foreign organisms and their products, and the tissue response to ischemia. This mechanism involves a switch to the expression of the angiogenic growth factor VEGF, while simultaneously suppressing the expression of inflammatory cytokines. The concept of an angiogenic switch was first termed by Folkman and Hanahan in 1991, in the context of tumorigenesis.[30,31] We propose that the phenotypic change induced in macrophages by TLR agonists and adenosine A_{2A}R agonists represents an angiogenic switch that may play an important role in wound healing and ischemia, as well as in solid tumor development.

Materials and Methods

Reagents

5′-N-ethyl-carboxamidoadenosine (NECA), 2-[p-(2-carboxylethyl)-phenylethyl amino]- 5′-N-ethyl-carboxamidoadenosine (CGS21680), 2-chloro-N^6-cyclopentyl adenosine (CCPA), polymyxin B, and paclitaxel (taxol) were purchased from Sigma Chemical Co. (St. Louis, MO). Resiquimod (R848) was purchased from InvivoGen (San Diego, CA). Erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) was purchased from RBI (Natick, MA). Recombinant (r) flagellins from *Salmonella muenchen (S. muenchen*) and *Serratia marcescens* (*S. marcescens*) were prepared as previously described.[32] Purified, phenol/water-extracted *E. coli* LPS and *Porphyromonas gingivalis (P. gingivalis*) LPS were prepared as described previously.[33] PAM_3Cys (N-palmitoyl-S-[2,3-bis (palmitoyloxy)-(2R)-propyl]-(R)-cysteine) and PAM_3CAG (N-palmitoyl-S-[2,3-bis (palmitoyloxy)-(2R)-propyl]-(R)-cysteinyl-(S)-alanyl-glycine) were synthesized as described below. Poly(I:C), polyuridylic acid (poly(U)), and polydeoxyinosinic-polydeoxycytidylic acid (poly(dI:dC)) were purchased from Sigma. A phosphorothioate-stabilized CpG oligodeoxynucleotide (TCC-ATG-ACG-TTC-CTG-ATG-CT) (non-methylated CpG DNA) and a phosphorothioate-stabilized non-CpG oligodeoxynucleotide (GCT-TGA-TGA-CTC-AGC-CGG-AA) (non-CpG DNA) were synthesized by the Molecular Biology Core Facility at the New Jersey Medical School. All reagents (other than LPS) were tested for the presence of endotoxin using a Limulus Amebocyte Lysate kit (Associates of Cape Cod Inc., Falmouth, MA). A plasmid containing a 300-bp insert of

the murine TNFα cDNA cloned in the *Pvu*II restriction site was a gift of Genentech, Inc. (S. San Francisco, CA).

Synthesis of PAM_3Cys and PAM_3CAG

For the synthesis of PAM_3Cys and PAM_3CAG, reagents were purchased from Alfa Aesar (Ward Hill, MA) or Aldrich (Milwaukee, WI). N-Fmoc-protected alanine and the preloaded Wang resin were from NovaBiochem (Darmstad, Germany). All solvents were high grade and dry. N,N-dimethylformamide (DMF) was from EM Science-Merck (Gibbstown, NJ). Dichloromethane (DCM) was distilled over CaH_2 before use. ^1H NMR spectra were recorded in $CDCl_3$ at 29°C (internal standard Me_4Si, $\delta = 0.00$), using a Varian Inova 300 at 300 MHz. Negative ion matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra were recorded using an HP-MALDI instrument using gentisic acid as the matrix. N-palmitoyl-S-[2,3-bis (palmitoyloxy)-(2R)-propyl]-(R)-cysteine (PAM_3Cys) was synthesized as described earlier.[34] N-palmitoyl-S-[2,3-bis (palmitoyloxy)-(2R)-propyl]-(R)-cysteinyl-(S)-alanyl-glycine (PAM_3CAG) was assembled manually by conventional solid-phase peptide methodology[35] using Fmoc chemistry on a polystyrene resin functionalized with *p*-benzyloxybenzyl alcohol (Wang Resin). The resin preloaded with N-Fmoc-L-glycine (173 mg, loading 0.690 mmol/g, 0.12 mmol) was placed in a reaction vessel and pre-swollen in DMF (4 ml) for 1 hour. The DMF was removed by filtration and the Fmoc-group was subsequently cleaved by treatment with 20% piperidine in DMF (6 ml) for 15 minutes. The solvent was removed and the resin washed with DMF (3×6 ml). N-Fmoc-L-Ala-OH (75 mg, 0.24 mmol), PyBOP (130 mg, 0.24 mmol), HOBt (30 mg, 0.24 mmol), and DIPEA (84 μl, 0.48 mmol) were dissolved in DMF (2 ml) and added to the resin. The mixture was agitated for 4 hours (complete coupling as indicated by negative Kaiser test,[36] the solvent was removed, and the resin was thoroughly washed by DMF (6×6 ml). By repeating the cycle of Fmoc-group cleavage and coupling using PyBOP and HOBt as the activating agents, the compound was constructed by coupling successively N-fluorenyl methoxycarbonyl-S-[2,3-bis-(palmitoyloxy)-(2R)-propyl]-(R)-cysteine[37] (210 mg, 0.24 mmol), and then palmitic acid (60 mg, 0.24 mmol). The resin was prepared for cleavage by extensive washing with DMF (6×6 ml), DCM (6×6 ml) and methanol (6×6 ml), followed by drying under vacuum overnight. The PAM_3CAG was released from the resin by treating the dry resin with 95% trifluoracetic acid (TFA) aqueous. (10 ml) for 1 hour. The solvent was removed by filtration and the resin washed with TFA (2×5 ml). The filtrates were combined, evaporated and lyophilized from *tert*-butanol. The residue was purified by Sephadex LH-20 gel filtration (DCM-MeOH 1:1) to give PAM_3CAG (85 mg, 0.083 mmol, 69%) as a white powder. ^1H NMR ($CDCl_3$, selected data) δ 0.84 (m, 9H, CH_3CH_2), 1.31 to 1.20 (m, 72H, CH_2), 1.38 (d, 3H, CH_3CH), 1.54 (m, 6H, CH_2CH_2CO), 2.73 to 2.80 (m, 2H, $OCHCH_2S$), 2.90 to 2.93, (m, 2H, CH_2SCH_2CHNH), 4.50 to 4.51 (m, 1H, NH*CH*CO), 5.15 (m, 1H, O*CH*CH_2S); MALDI TOF MS: mass calculated for $C_{59}H_{111}O_9N_3S$: *m* 1038.5; *m/z* 1060.1 (M+Na). PAM_3Cys and PAM_3CAG were dissolved in THF, and then diluted at least 1000-fold in PBS at 72°C. with vortexing for 5 minutes.

Animals

C57BL/6J, C57BL/10ScSn (TLR4+/+), and C57BL/10ScN (TLR4 deletion mutant) mice (male, 7 to 12 weeks) were purchased from Jackson Laboratories and housed in the New Jersey Medical School animal facility. C57BL/10ScN mice have a deletion of the gene coding for TLR4, resulting in a null mutation that does not express TLR4, and are unable to respond to TLR4 agonists.[38–40] Mice were fed regular mouse chow and given access to drinking water *ad libitum*. All procedures described below were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey and carried out under the supervision of the facility veterinary staff.

Preparation and Treatment of Peritoneal Macrophages

Mice were injected intraperitoneally with 2.5 ml sterile Brewer's thioglycolate broth (4% w/v) (Difco Laboratories, Detroit, MI). Four days later, the mice were sacrificed and peritoneal exudate cells were harvested using phosphate-buffered saline (PBS). Cells were centrifuged at $300 \times g$ for 5 minutes at 4°C, washed twice with PBS, and re-suspended in RPMI containing 10% FCS, 100 μg/ml penicillin-streptomycin, and 2 mmol/L L-glutamine (RPMI-10%FCS). Cells were seeded into Falcon Multiwell 6-well tissue culture plates (Becton-Dickinson Labware, Franklin Lakes, NJ) (1.2×10^6 cells/well) in 2 ml medium. Dishes were then incubated at 37°C in a humidified incubator in 95% air/5% CO_2 overnight, to allow the cells to adhere. Non-adherent cells were removed by washing with serum-free RPMI, and the cells were re-fed with RPMI containing 1% FCS (RPMI-1% FCS). Eighteen hours following initial plating, the various test compounds (TLR agonists and adenosine receptor agonists) were added to the macrophages. To test for putative effects of contaminating endotoxin in the various TLR agonist preparations, polymyxin B (25 μg/ml) was added to the culture dishes with the various TLR agonist preparations. Conditioned media were harvested 24 hours following the addition of test compounds. Within each experiment, each test condition was assayed in duplicate. Each experiment was performed at least three times. Results of a typical experiment are shown in each figure, and are presented as means $\pm$ SD.

Assay of VEGF Protein Levels

VEGF protein in conditioned media was assayed using a sandwich ELISA kit (Quantikine M, R&D Systems, Minneapolis, MN), following the manufacturer's protocol. This assay detects murine VEGF in the range of 3 to 500

pg/ml. Samples with VEGF concentrations above this range were diluted with RPMI and re-assayed. All samples were assayed in duplicate. Results are presented as means ± SD.

Analysis of TNFα Steady-State mRNA Levels

Macrophages (10^7 cells/dish in 100-mm Falcon plastic tissue culture dishes) from C57BL/10ScSn mice were treated with NECA (1μM), either alone or in the presence of *E. coli* LPS (100 ng/ml), PAM_3CAG (10 μg/ml) or non-methylated CpG DNA (10 μg/ml). Control macrophages were incubated in medium alone. Total cellular RNA was isolated from the macrophages 2, 4, and 8 hours following addition of the various reagents, using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) as described previously.[3] Total RNA (10 μg) was resuspended in Formazol (MRC, Inc.) and analyzed by electrophoresis through 1.2% agarose/formaldehyde gels. The RNA was then electroblotted onto MagnaGraph nylon membrane (MSI, Inc., Westboro, MA). The 300-bp insert in the murine TNFα plasmid was excised with *Pvu*II, and labeled with α-^{32}P-dCTP (6000 Ci/mmol) (NEN, Boston, MA) using a random priming kit (Promega, Madison, WI). Membranes were pretreated and hybridized overnight in PerfectHyb Plus Hybridization buffer (Sigma) at 68°C. The blots were then washed once for 5 minutes at room temperature with 2X SSC, 0.1% SDS followed by two washes at 68°C with 0.5X SSC, 0.1%SDS, and a final wash with 0.1X SSC, 0.1% SDS. Membranes were then exposed to PhosphorImager cassette screens at room temperature overnight, and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Band densities were quantitated using the ImageQuant software analysis program (Molecular Dynamics).

Analysis of TNFα Protein Levels

TNFα levels in macrophage conditioned media were determined using a sandwich ELISA kit (R&D Systems), as described by the manufacturer. Each sample was assayed in duplicate for TNFα. Results are presented as means ± SD.

Analysis of NF-κB Activation

Preparation of Nuclear Extracts

Murine peritoneal macrophages were treated for 1 to 4 hours with TLR ligands [LPS (100 ng/ml), *S. marcescens* r-flagellin (100 ng/ml), non-methylated CpG DNA (10 μmol/L), or PAM_3CAG (10 μg/ml)], without or with NECA (1 μmol/L). The nuclear extracts were prepared as described previously with modifications.[41] Briefly, 20×10^6 cells were washed twice with ice cold PBS, harvested, centrifuged, and resuspended in 400 μl of ice cold buffer A [20 mmol/L Tris-Cl (pH 8.0) containing 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.5 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1 mmol/L $NaVO_3$, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml aprotinin, 0.5 mmol/L EDTA, 10 mmol/L NaF, and 10 mmol/L glycerol. The samples were left on ice for 15 minutes and the cells were lysed by adding Nonidet P-40 to a final concentration of 0.625% (v/v) and homogenizing. The nuclei were pelleted by centrifugation at 13,000 × *g* for 5 minutes at 4°C. The supernatant was carefully removed and the nuclei lysed by homogenizing in 25 μl of ice cold buffer B (same composition as buffer A except that concentration of KCl is 420 mmol/L). The samples were kept at 4°C on a shaker for 30 to 60 minutes, and then centrifuged at 13,000 × *g* at 4°C for 5 minutes. The supernatants (nuclear extracts) were stored at −80°C until use. The protein concentrations were determined by the Bradford method using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed as described[42] using 10 μg of nuclear extract protein and 0.2 to 0.5 ng of probe. The binding reaction (25 μl) contained 20 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L DTT, 10% glycerol, 5 μg BSA, 2 μg of poly(dI:dC), and 58.8 mmol/L KCl. The NF-κB wild-type and mutant double-stranded probes with the sequences 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-AGT TGA GGC GAC TTT CCC AGG C-3′, respectively, were synthesized at the NJMS Molecular Resource Facility, and were end-labeled by T4 polynucleotide kinase with ^{32}P-γATP (NEN). The binding reaction mixtures were incubated at room temperature for 20 minutes without the probe. Probes were then added and the reactions were incubated further at room temperature for 15 minutes. The reactions were then electrophoresed on 4% non-denaturing polyacrylamide gels. To determine the specifity of binding, 100-fold molar excess of unlabeled wild-type or mutant probe was added to the binding reaction 20 minutes before the addition of the radiolabeled probe. To determine the specific activated isoforms of NF-κB, specific antibodies to p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Binding reactions were incubated for 1 hour at room temperature in the presence of 2 μg specific antibody or non-specific antibody, before the addition of the radiolabeled probe. Rabbit or goat polyclonal antibodies sc-114 X and sc-109 X, specific for the subunits p50 and p65 respectively, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Unrelated rabbit polyclonal antibody specific to VEGF was obtained from NeoMarkers (Fremont, CA).

Assay of Macrophage Viability by the MTT Assay

To assess macrophage viability following incubation under the various test conditions, media were removed from each well and replaced with 1 ml MTT assay medium (RPMI-1640 without serum and phenol red, containing 50 μg/ml MTT). The plates were then incubated at 37°C for 2 hours. Medium was then removed, and the cells were washed with PBS. One ml of cold 95% ethanol was then

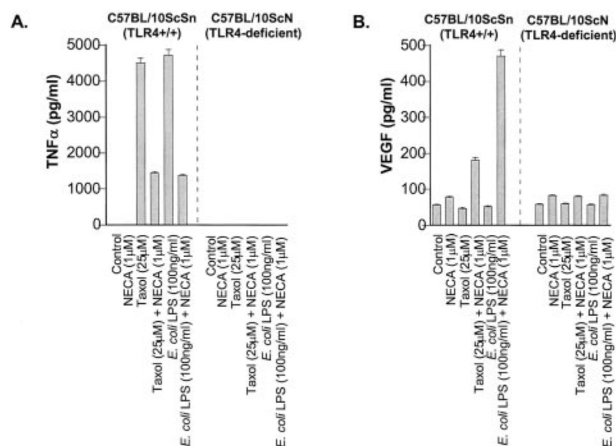

Figure 1. Effects of taxol on TNFα (**A**) and VEGF (**B**) production by macrophages from C57BL/10SnSc and C57BL/10ScN mice. Macrophages from either C57BL/10ScSn (TLR4+/+) or C57BL/10ScN (TLR4−/−) mice were treated with taxol, *E. coli* LPS, or NECA at the indicated concentrations, either alone or in combination. Test agents were added to the macrophage cultures 18 hours after plating, and conditioned media were harvested 24 hours later, and assayed for TNFα and VEGF levels. At least two replicates were studied for each condition, and each sample was analyzed in duplicate. Results are presented as means ± SD. An **asterisk** (*) indicates undetectable levels.

added, and the plates were incubated at 4°C for 5 minutes to allow the blue formazan product to dissolve. An aliquot (100 μl) of the solution from each well was then transferred to a 96-well plate, and absorption was read at 560 nm with a 690 nm reference wavelength.

Statistics

Values in the figures are expressed as means ± SD. Statistical analysis of the data were performed using Student's *t*-test or one-way analysis of variance followed by Dunnett's test, as appropriate.

Results

Effects of Taxol on Macrophage TNFα and VEGF Production

Taxol has been shown previously to mimic many of the effects of LPS on murine macrophages,[43–45] including induction of TNFα secretion, NO production, and NF-κB activation. The LPS-mimetic effects of taxol have been attributed to its shared use of TLR4. We therefore tested the effects of taxol alone and together with AR agonists on TNFα and VEGF expression in C57BL/10ScSn (TLR4+/+) and C57BL/10ScN (TLR4-deficient) macrophages.

Taxol alone strongly induced expression of TNFα in macrophages from C57BL/10ScSn mice (Figure 1A), and the A_2R agonist NECA strongly suppressed this effect. CCPA (A_1R agonist) had little effect. In macrophages from C57BL/10ScN mice, taxol failed to induce TNFα expression.

Taxol alone had no effect on VEGF production. In the presence of AR agonists, taxol stimulated VEGF production by macrophages from C57BL/10ScSn mice, although less strongly than *E. coli* LPS (Figure 1B). This effect was also absent in macrophages from C57BL/10ScN mice.

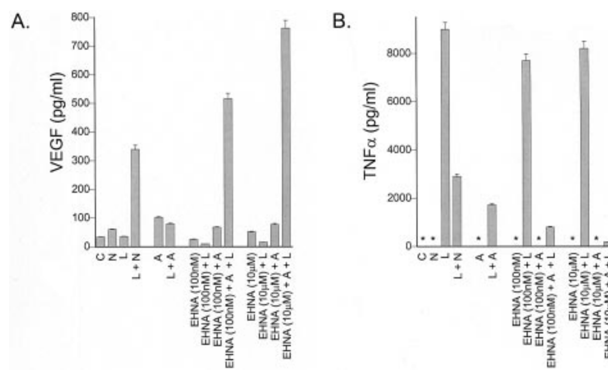

Figure 2. Effects of adenosine and an adenosine deaminase inhibitor (EHNA) on production of VEGF (**A**) and TNFα (**B**) by macrophages from C57Bl/10ScSn mice. Macrophages were treated with adenosine (A) (100 μmol/L), NECA (N) (1 μmol/L), LPS (L) (100 ng/ml), or EHNA at the indicated concentrations, either alone or in combination. Conditioned media were harvested 24 hours after addition of test agents and the TNFα and VEGF levels in the media were determined by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for TNFα and VEGF in duplicate. Results are presented as means ± SD. An **asterisk** (*) indicates undetectable levels.

Effects of Adenosine and an Adenosine Deaminase Inhibitor (EHNA) on Macrophage TNFα and VEGF Production from C57BL/10ScSn (TLR4+/+) Mice

Adenosine is very rapidly metabolized by macrophages by the action of adenosine deaminase, which converts adenosine to inosine.[46] To determine the effects of adenosine alone, in the presence of NECA, and/or *E. coli* LPS, macrophages were treated with the adenosine deaminase inhibitor EHNA. Adenosine alone (100 μmol/L) reduced TNFα, but had only a minor effect on VEGF production, either alone or in the presence of *E. coli* LPS (100 ng/ml) (Figure 2,A and B). In the presence of EHNA alone (100 nmol/L and 10 μmol/L), adenosine had little effect on VEGF production; however, in the presence of *E. coli* LPS (100 ng/ml), a strong induction of VEGF expression was observed with EHNA treatment.

Effects of TLR Agonists and Adenosine Receptor Agonists on Macrophage TNFα and VEGF Production from C57BL/10ScSn (TLR4+/+) Mice

Unstimulated macrophages from LPS-responsive C57BL/10ScSn mice produced undetectable levels of TNFα over 24 hours of incubation (Figure 3A). TNFα production was strongly stimulated by treatment with *E. coli* LPS (TLR4 agonist), *P. gingivalis* LPS and PAM_3CAG (TLR2 agonists), poly(I:C) (TLR3 agonist), R848 (TLR7 agonist), and non-methylated CpG DNA (TLR9 agonist). R-flagellins from *S. muenchen* and *S. marcescens* (TLR5 agonists) had no effect on TNFα production. In control experi-

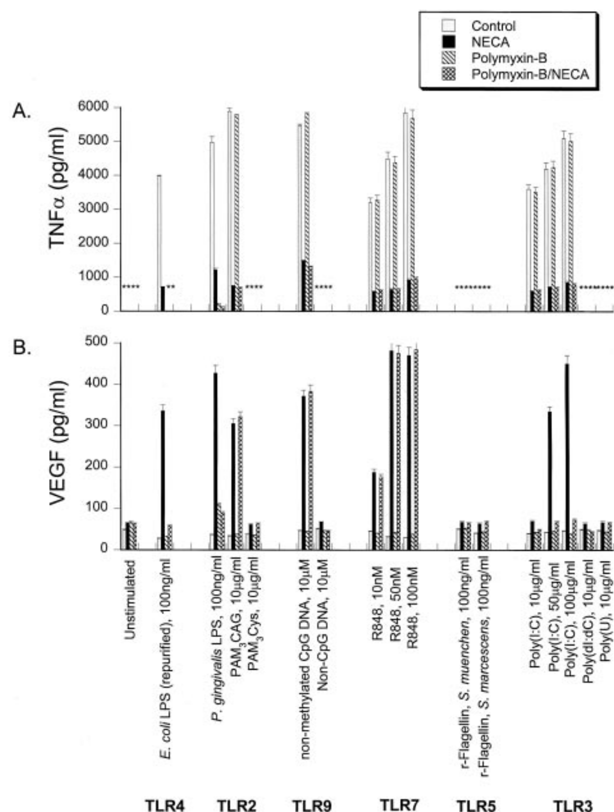

Figure 3. Effects of TLR agonists on TNFα (**A**) and VEGF (**B**) production by macrophages from C57BL/10ScSn (TLR4+/+) mice. Macrophages were treated with TLR agonists and controls at the indicated concentrations. NECA (an A_2R agonist) and polymyxin B were added to the cells at the same time as the TLR agonists. Conditioned media were harvested 24 hours after addition of test agents and the TNFα and VEGF levels in the media were determined by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for TNFα and VEGF in duplicate. Results are presented as means ± SD. The x-axis labels refer to both TNFα (**A**) and VEGF (**B**). The particular TLR studied in each group is indicated beneath the x-axis labels. An **asterisk** (*) indicates undetectable levels.

ments, these r-flagellins strongly induced TNFα expression in human monocytes, indicating that they are bioactive. PAM_3Cys, non-CpG DNA, poly(dI:dC), and poly(U) had no effect on TNFα production. NECA (an A_2R agonist) had no effect on TNFα production in unstimulated cells. However, NECA strongly inhibited TNFα production in macrophages treated with the TLR2, 3, 4, 7, and 9 agonists listed above (Figure 3A). CGS21680 (A_{2A}R agonist) had similar effects, while CCPA (A_1R agonist) had no effect (data not shown). Polymyxin B (25 μg/ml), an LPS antagonist, potently inhibited the effects of *E. coli* LPS in the induction of TNFα, and also blocked the effects of *P. gingivalis* LPS. Polymyxin B had no effect, however, on TNFα induction by PAM_3CAG, poly(I:C), R848, or non-methylated CpG DNA, indicating that the effects of these agonists were not due to LPS contamination. This conclusion is further supported by the effects of these agonists on macrophages from C57BL/10ScN (TLR4-deficient) mice (see below).

Unstimulated macrophages from C57BL/10ScSn control mice produced low levels of VEGF over 24 hours of incubation. Phenol/water-extracted *E. coli* LPS[33] slightly reduced basal VEGF expression. *P. gingivalis* LPS and PAM_3CAG (TLR2 agonists), poly(I:C) (TLR3 agonist), R848 (TLR7 agonist), and non-methylated CpG DNA (a TLR9 agonist) had little effect on basal VEGF expression (Figure 3B). R-flagellins from *S. muenchen* and *S. marcescens* (TLR5 agonists) also had no effect on VEGF production (Figure 3B).

To test whether TLR agonists synergize with A_{2A}R agonists to up-regulate VEGF expression in macrophages, the effects of these various TLR agonists in conjunction with NECA (A_2 agonist), CGS21680 (specific A_{2A} agonist), or CCPA (A_1 agonist), on VEGF production by murine macrophages were determined. The effects of NECA in this system are shown in Figure 3B. NECA synergistically up-regulated VEGF expression in the presence of *E. coli* LPS, *P. gingivalis* LPS, PAM_3CAG, R848, and non-methylated CpG DNA, but had little effect in the presence of PAM_3Cys, poly(I:C), poly(U), or r-flagellins. Similar results were obtained with CGS21680 (data not shown). CCPA (up to 10 nmol/L) had no effect in this system. Poly(I:C)(10 μg/ml) had little effect on VEGF production. At higher concentrations (50 to 100 μg/ml), induction of VEGF by poly(I:C) was observed; however, this effect was eliminated in the presence of polymyxin-B, suggesting that LPS rather than poly(I:C) was the contributing stimulus. This was supported by the failure of poly(I:C) to induce VEGF expression by macrophages from C57BL/ScN (TLR4−/−) mice (see below). These results thus indicate that agonists of TLR2, 4, 7, and 9 synergize with A_{2A}R agonists to up-regulate expression of VEGF, while TLR3 and 5 agonists do not. *E. coli* LPS re-purified by phenol/water extraction to remove lipoprotein impurities that might signal through TLR2 also induced strong up-regulation of VEGF expression in synergy with NECA. This confirms our previous conclusion that *E. coli* LPS synergizes with NECA by signaling through TLR4 receptors.[3] Polymyxin B (25 μg/ml) blocked the combined effect of *E. coli* LPS and NECA on the induction of VEGF and also blocked the effect of *P. gingivalis* LPS, but had no effect on the induction of VEGF by the TLR2, 7, or 9 agonists, indicating that, unlike poly(I:C), this effect was not due to the presence of contaminating LPS.

Effects of TLR Agonists and Adenosine Receptor Agonists on TNFα and VEGF Production by Macrophages from C57BL/10ScN Mice (TLR4-Deficient)

As anticipated, *E. coli* LPS did not induce TNFα expression in macrophages from LPS-unresponsive C57BL/10ScN mice. In contrast, *P. gingivalis* LPS, PAM_3CAG, poly(I:C), R848, and non-methylated CpG DNA all strongly induced TNFα expression in these TLR4-deficient macrophages (Figure 4A). This confirms that these agonists do not require TLR4 for the induction of TNFα expression, supporting the notion that they are working through their respective receptors. NECA treatment strongly reduced TNFα production by macrophages treated with these TLR2, 3, 7, and 9 agonists.

In the presence of adenosine A_2R agonists, *P. gingivalis* LPS, PAM_3CAG, R848, and non-methylated CpG DNA

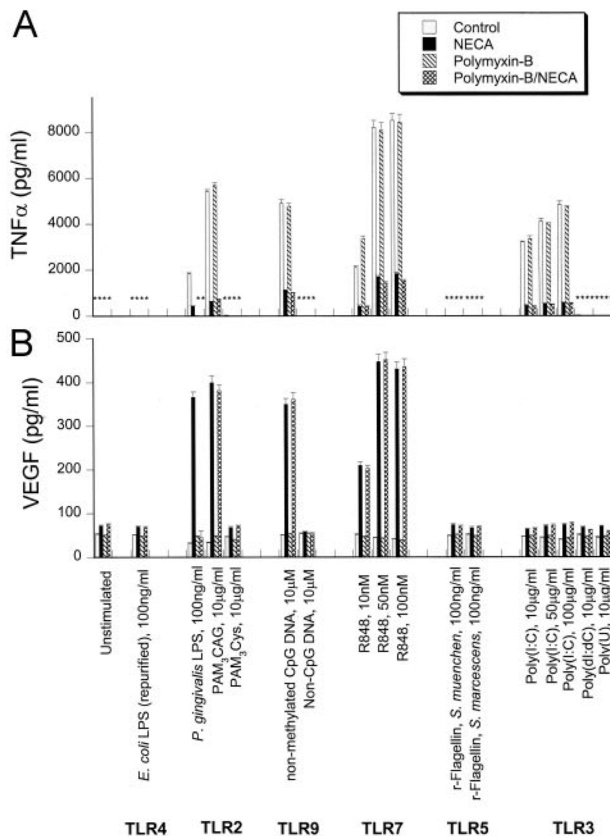

Figure 4. Effects of TLR agonists on TNFα (**A**) and VEGF (**B**) protein production by macrophages from C57BL/10ScN (TLR4-deficient) mice. Macrophages were treated with TLR agonists and controls at the indicated concentrations. NECA (an A_2R agonist) and polymyxin B were added to the cells at the same time as the TLR agonists. Conditioned media were harvested 24 hours after addition of test agents and the TNFα and VEGF levels in the media were determined by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for TNFα and VEGF in duplicate. Results are presented as means ± SD. The x-axis labels refer to both TNFα (**A**) and VEGF (**B**). The particular TLR studied in each group is indicated beneath the x-axis labels. An **asterisk** (*) indicates undetectable levels.

strongly induced VEGF expression, while PAM_3Cys, poly(I:C), poly(dI:dC), poly(U), and r-flagellins did not. Figure 3B shows the effects of NECA in this system. CGS21680 had similar effects, while CCPA was ineffective. This indicates that TLR2, TLR7, and TLR9 agonists can interact synergistically with A_2R agonists to induce VEGF expression, and that TLR4 is not required for this effect, with each TLR agonist signaling in this interaction through its own specific cognate receptor. As observed in control C57BL/10ScSn (TLR4+/+) mice, r-flagellins did not induce VEGF expression in TLR4-deficient mice. Also, poly(I:C) did not induce VEGF expression in these mice in the presence of NECA or CGS21680, confirming that the VEGF expression observed in C57BL/10ScSn mice was due to LPS contamination.

Effect of TLR Agonists and Adenosine Receptor Agonists on TNFα mRNA Expression

The effects of *E. coli* LPS (TLR4 agonist) on the steady-state level of TNFα mRNA 2 and 4 hours following stim-

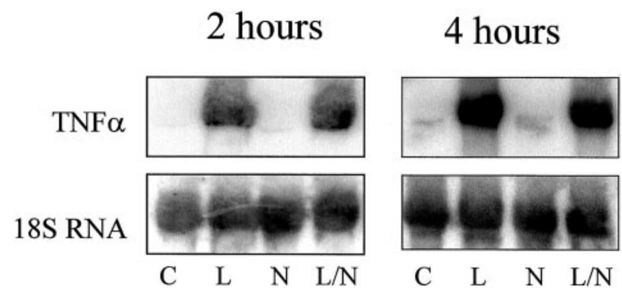

Figure 5. Northern blots showing the effect of *E. coli* LPS (TLR4 agonist) alone and with NECA (A_2R agonist) on the steady-state levels of TNFα mRNA in macrophages from C57BL/10ScSn mice. Macrophages were treated for 2 and 4 hours, total RNA isolated, and 10 μg total RNA electrophoresed per lane through 1.2% agarose/formaldehyde gels. The RNA was then transferred to nylon membranes, and hybridized with an α^{32}P-dCTP-labeled TNFα cDNA probe. Equal loading was confirmed by probing blots with a ^{32}P-labeled 18S ribosomal RNA probe. C, untreated cells; L, *E. coli* LPS (100 ng/ml)-treated cells; *N*, NECA (1 μmol/L)-treated cells; L/*N*, LPS/NECA-treated cells.

ulation are shown in Figure 5,A and B. *E. coli* LPS strongly induced the expression of TNFα mRNA 2 and 4 hours following stimulation of macrophages. By 8 hours following stimulation, TNFα mRNA levels had returned almost to baseline. NECA (1 μmol/L) had little effect on the expression of TNFα mRNA. Similar results were obtained with TLR2 and TLR9 agonists.

Effect of TLR Agonists and Adenosine Receptor Agonists on NF-κB Activation

The effects of TLR agonists and A_2R agonists on the activation of NF-κB are shown in Figure 6. Since TNFα mRNA levels peak in these cells at 2 to 4 hours following stimulation, macrophages were treated without or with TLR agonists and/or NECA for 1 and 2 hours in these experiments. Nuclear protein extracts were then analyzed for activated NF-κB by EMSA. NECA (1 μmol/L) alone did not activate NF-κB. *E. coli* LPS, *P. gingivalis* LPS, PAM_3CAG, R848, non-methylated CpG DNA, and poly(I:C) all strongly induced activation of NF-κB, while PAM_3Cys, *S. marcescens* r-flagellin, non-CpG DNA, poly-(dI:dC), and poly(U) did not (Figure 6A). NECA did not significantly reduce the levels of activated NF-κB in any TLR agonist treatment group. Activated NF-κB levels in cells treated with TLR agonists and NECA were similar to those observed in cells treated with TLR agonists alone. This is in marked contrast to the strong reduction in TNFα protein production induced by NECA in TLR agonist-treated cells (Figures 1, 2, and 3). Also, the presence of the p50 and p65 forms of activated NF-κB did not change in response to the NECA treatment. Specificity of the probe interaction was shown using an excess of unlabeled probe (Figure 6B). Also, a mutant probe did not bind the NF-κB proteins (Figure 6C). Similar results were observed at 1 and 2 hours following stimulation.

Discussion

In this study, we show that agonists of TLR2, 4, 7, and 9 synergistically up-regulate the expression of VEGF in

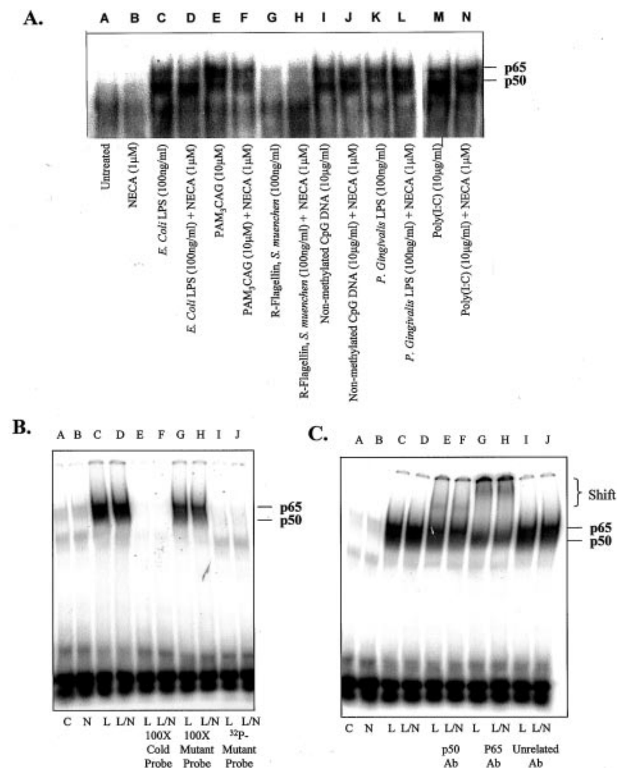

Figure 6. EMSAs showing the effects of TLR agonists alone and with NECA (A_2R agonist) on NF-κB activation in macrophages from C57BL/10ScSn mice. **A:** Macrophages were treated with TLR agonists and controls, with or without NECA for 1 hour (**lanes A–N**). Cells were harvested, nuclear protein extracts were prepared, and equal amounts of nuclear proteins were analyzed for NF-κB activation. **B:** Macrophages were treated with *E. coli* LPS (TLR4 agonist) for 1 hour, with or without NECA (1 μmol/L). Untreated cells are shown in **lane A**. Specificity of the activated NF-κB signal was determined by incubating in the presence of a 100-fold excess of unlabeled oligonucleotide as a competitor to inhibit the binding of NF-κB (**lanes E** and **F**) or 100X excess of a mutant probe (**lanes G** and **H**). Binding of the ^{32}P-labeled mutant probe was also assessed (**lanes I** and **J**). **C:** Macrophages were treated with *E. coli* LPS with or without NECA (1 μmol/L) for 1 hour. **Lane A:** Untreated control. **Lane B:** NECA alone. **Lane C:** LPS alone. **Lane D:** LPS + NECA. Supershift analysis using specific polyclonal antibodies to the p50 and p65 subunits of NF-κB are shown in **lanes E/F** and **G/H**, respectively, while the effects of a non-specific antibody are shown in **lanes I/J**. C, untreated cells; L, LPS (100 ng/ml)-treated cells; *N*, NECA (1 μmol/L)-treated cells; L/*N*, LPS/NECA-treated cells.

murine macrophages together with adenosine or agonists of A_{2A}Rs, while TLR3 and 5 agonists do not. At the same time, adenosine and A_{2A}R agonists strongly down-regulate TNFα expression. Together, we propose that the up-regulation of VEGF, combined with the down-regulation of TNFα, act as an angiogenic switch, shifting macrophages from an inflammatory to an angiogenic phenotype.

The synergistic up-regulation of VEGF by *E. coli* LPS through the TLR4 receptor, together with A_{2A}R agonists through A_{2A}Rs that we recently reported[3] was confirmed in this study, using *E. coli* LPS purified by phenol/water extraction to remove lipoprotein impurities that might function through TLR2.[33] The synergistic interaction between *E. coli* LPS and A_{2A}R agonists that results in the up-regulation of VEGF expression is critically dependent on TLR4 receptors.[3] Macrophages from C3H/HeJ mice did not exhibit the synergistic up-regulation of VEGF production induced in control mice by A_{2A}R agonists and *E. coli* LPS.[3] In the current study, we used macrophages from C57BL/10ScN mice, to determine whether TLR4 receptors are required for the synergistic interaction between A_{2A}Rs and various TLR agonists. C57BL/10ScN mice have a deletion of the gene coding for TLR4, resulting in a null mutation that does not express TLR4 protein.[38–40] In contrast to C3H/HeJ mice, which have a point mutation in the cytoplasmic domain of the TLR4 receptor that results in the inability of this receptor to function in LPS signaling,[47,48] these mice fail to express TLR4, which thus cannot function as a dominant-negative mutant. The specific requirement for TLR4 was indicated by the total lack of effect of *E. coli* LPS alone in inducing TNFα expression, or, in the presence of A_{2A}R agonists, of inducing VEGF expression in macrophages from C57BL/10ScN (TLR4−/−).

P. gingivalis LPS has been shown to be a TLR2 agonist in murine macrophages,[49,50] as is the synthetic lipopeptide PAM_3CAG.[51] Non-methylated CpG DNA has been shown previously to be a TLR9 agonist.[10,11] The antiviral imidazoquinoline resiquimod (R848) has been shown to be a TLR7 agonist in mice.[12] All these TLR agonists alone strongly stimulated TNFα production by macrophages from C57BL/10ScSn (TLR+/+) mice, as well as by macrophages from C57BL/10ScN (TLR4-deficient) mice, indicating that TLR4 is not required for their effect. These TLR agonists alone did not induce VEGF expression. However, synergistic up-regulation of VEGF expression by these TLR agonists was observed in the presence of NECA, an A_2R agonist, or CGS21680, a specific A_{2A}R agonist. CCPA, a specific adenosine A_1R agonist, had no effect on VEGF production, either alone, or in the presence of TLR2 receptor agonists. The effects of PAM_3CAG, non-methylated CpG DNA, and R848 were unaffected by polymyxin B, a specific antagonist of LPS,[52] again confirming that their effects were not due to LPS contamination. These results indicate that TLR2, 7, and 9 agonists synergize with A_{2A}Rs in a similar manner to that observed for TLR4 agonists.

Poly(I:C), a double-stranded RNA mimetic that has been shown recently to be an agonist of TLR3,[13] induced TNFα expression in macrophages from C57BL/10ScSn and C57BL/10ScN mice, indicating the specificity of the response. Polymyxin B had little effect on the production of TNFα by this TLR3 agonist. Poly(I:C) (10 μg/ml) had little effect on VEGF production in the presence of A_2R agonists in either mouse strain. At higher concentrations, however, induction of VEGF expression was observed. This induction was abolished by polymyxin B, suggesting that LPS was involved in this induction. This was confirmed by the lack of effect of poly(I:C) on VEGF expression in C57BL/10ScN mice. It thus appears that poly(I:C) does not induce VEGF expression in synergy with A_2R agonists, distinguishing its effects from those of TLR2, 4, 7, and 9 agonists.

Bacterial flagellins have been shown recently in human cells to be potent TLR5 agonists.[8,9] R-flagellins from *S. marcescens* and from *S. muenchen* failed to induce TNFα expression in either C57BL/10ScSn or C57BL/10ScN murine macrophages, and did not synergize with A_{2A}R agonists to induce VEGF expression. The failure of r-flagellins

to induce TNFα expression suggests either that TLR5 is not expressed at significant levels in murine macrophages, or that r-flagellins are ineffective in stimulating murine cells. We have screened for the presence of TLR5 mRNA in murine macrophages using RT-PCR, and have found only extremely low levels of this mRNA in comparison to TLR2, 3, 4, and 9, which are strongly expressed (Leibovich SJ, Pinhal-Enfield G, Fechner A, Elson G, manuscript in preparation). Both these r-flagellins strongly stimulate TNFα production in human monocytes (data not shown), indicating that they are indeed bioactive.

While VEGF production is strongly up-regulated by the synergy between A_{2A}Rs and TLRs 2, 4, 7, and 9, A_{2A}R agonists markedly down-regulate TNFα production induced by these agonists, as well as by taxol. NECA (1 μmol/L) treatment strongly reduced the expression of TNFα protein, but had little effect on TNFα mRNA levels. Induction of TNFα by taxol and by TLR agonists such as *E. coli* LPS, *P. gingivalis* LPS, PAM_3CAG, poly(I:C), R848, and non-methylated CpG DNA is mediated in large part through activation of the NF-κB-IκB pathway. We have observed that while production of TNFα is down-regulated by the A_2R agonist NECA, activation of NF-κB binding to its cognate nuclear DNA binding site is not affected. In a separate study,[53] we have found that, while adenosine suppresses TNFα protein expression in RAW264.7 cells transfected with a luciferase vector under the control of an NF-κB promoter, it does not suppress expression of luciferase. Together, this implies that the down-regulation of TNFα production and the up-regulation of VEGF expression by adenosine agonists involve alternative signal transduction pathways that do not directly regulate NF-κB activation.

Taxol is a diterpene isolated from the bark of the Western yew (*Taxus brevifolia*).[54] Taxol is an anti-tumor agent that acts at least in part by binding and stabilizing microtubules, thus blocking mitosis.[55,56] In addition, in murine, but not human macrophages, taxol acts as an LPS-mimetic, inducing secretion of TNFα, down-regulation of TNFα receptors, induction of LPS-inducible gene expression, and activation of NF-κB.[43,45,57] Recently, taxol signaling has also been shown to depend critically on MD-2, a protein that interacts with the extracellular domain of TLR4.[58,59] Taxol synergizes with A_{2A}R agonists in inducing VEGF expression, although less strongly than *E. coli* LPS. We are currently testing the requirements for MD-2 and CD14 in the signaling pathway(s) involved in these synergistic interactions.

VEGF is an important inducer of angiogenesis, acting through specific receptors on endothelial cells to induce neovascularization.[1] VEGF is also an important mediator of vascular permeability, modulating the exchange of solutes from the plasma to the extravascular tissues.[2] VEGF is thus an important mediator of both inflammation and repair, and is critical for the resolution of injury by the process of wound healing. Macrophages are a major source of VEGF in wound healing, as well is in chronic inflammation and cancer.[60–64] Hypoxia has been shown to be an important regulator of VEGF expression in several cell types.[65–67] Hypoxia induces VEGF expression in part via transcriptional up-regulation of VEGF mRNA through the hypoxia response element (HRE) in the VEGF gene, which binds the *trans*-acting transcription factor HIF-1.[68–70] In addition, major regulation of VEGF expression by hypoxia is mediated by stabilization of VEGF mRNA.[71,72] HIF-1 may also be involved in this pathway.[73] It is not yet clear whether regulation of HIF-1 levels plays a role in the up-regulation of VEGF induced by TLR agonists in synergy with A_{2A}R agonists. While hypoxia may play a role in regulating macrophage expression of VEGF in wound healing, the pathway we have demonstrated here does not require hypoxia, but rather involves a synergistic interaction between G-protein-coupled receptors (the A_{2A}Rs) and members of the TLR receptor family that act as pattern recognition receptors for foreign organisms and their constituents. Agonists of A_{2A}Rs have minor effects on VEGF production alone, and agonists of TLR receptors also show little effect on VEGF expression. Together, however, we show here that these dissimilar agonists induce a strong, synergistic up-regulation of VEGF expression.

Adenosine is rapidly metabolized extracellularly to inosine by the action of adenosine deaminase.[74] Adenosine accumulates to high levels (up to 100 μmol/L) extracellularly in ischemic situations, when blood flow to tissues is compromised, with intracellular breakdown of ATP resulting in the formation and release of adenosine.[15–17] To determine the effects of adenosine on macrophage expression of VEGF and TNFα, macrophages were incubated with adenosine in the presence of EHNA, a potent inhibitor of adenosine deaminase, to prevent the breakdown of adenosine to inosine. Under these conditions, strong synergy with *E. coli* LPS to induce VEGF expression and suppress TNFα expression was observed. This indicates that the effects of NECA and CGS21680 (both synthetic A_2R agonists) are representative of the effects of unmodified adenosine.

In the absence of infection, and thus of TLR agonists, macrophages may not be stimulated markedly by adenosine, and the expression of VEGF will not be strongly stimulated. In the presence of infectious agents, on the other hand, TLR agonists will synergize with adenosine to strongly stimulate the expression of VEGF by macrophages, while simultaneously suppressing the production of inflammatory cytokines such as TNFα. This will strongly promote vascular permeability in the area, and also stimulate angiogenesis. The TLR agonists may thus act in synergy with adenosine to stimulate a natural host mechanism that initiates repair responses, providing an interface between the innate immune response and wound healing, and acting as an angiogenic switch. The implications of a synergistic interaction between a product of ischemia (adenosine) and products of infectious organisms (TLR agonists) are far-reaching. Ischemic situations, such as those that exist in myocardial ischemia, atheromatous plaques, or ischemia in the brain, might behave very differently in the absence of a source of infection than in the presence of infectious agents or their products. It will be of interest to determine the effects of various TLR agonists on the outcome of ischemic injury in models of myocardial infarction, atherosclerosis, brain injury such as stroke, and other ischemic disease states.

In this context, it has been shown recently that *E. coli* LPS administered to rabbits on a hypercholesterolemic diet accelerates the development of atherosclerosis,[75] and that TLRs are expressed at high levels in cells in atherosclerotic plaques.[76] Similarly, *P. gingivalis* infection accelerates the progression of atherosclerosis in heterozygous apoliprotein-E-deficient mice.[77] It will also be of interest to study the effects of antagonists of A_{2A}Rs, and antagonists of TLR-mediated signaling in these experimental models.

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