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Osteoprotegerin Expression in Dendritic Cells Increases With Maturation and Is NF-κB-Dependent

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Abstract Dendritic cells (DC) comprise a unique leukocyte population which controls primary immune responses. Recent studies indicate that DC express osteoprotegerin (OPG), a secreted tumor necrosis factor receptor homolog, which regulates DC survival, monocyte chemotaxis, and B cell development and function by ligating TNF family member receptor activator of NF-κB ligand (RANKL). The precise regulators of OPG expression in DC have not been investigated. In this study, we assessed OPG mRNA steady state levels by Northern blot analysis and OPG protein secretion by an immunoassay in monocyte-derived DC of different maturation, and the effect of different cytokines and hormones on OPG expression. OPG was upregulated with maturation of DC, whereas pretreatment of DC with $1\alpha,25(OH)_2$ vitamin D_3, tamoxifen, or dexamethasone, agents that inhibit differentiation of DC, decreased OPG expression. In vivo, OPG was found to be colocalized with mature $CD83^+$ DC in human tonsils by immunofluorescence confocal microscopy analysis. Furthermore, OPG was upregulated by TNF superfamily members TNF-α, anti-CD40, and RANKL, and by ligands of the Toll-like/IL-1 receptor family including IL-1β, double-stranded RNA (poly I:C), or lipopolysaccharide (LPS), all of which induce maturation of DC. Gene silencing by small interfering RNA (siRNA) directed against transcription factor NF-κB abrogated the expression of OPG as demonstrated by real-time PCR. In summary, we describe that the expression of OPG by DC increases with maturation and is NF-κB-dependent, possibly regulating immune responses in lymphoid tissues. J. Cell. Biochem. 100: 1430–1439, 2007.

Key words: antigen-presenting cells; dendritic cells; immune system; NF-κB; osteoprotegerin

Dendritic cells (DC) constitute a heterogeneous population of antigen-presenting cells, that initiate and regulate immunity by the activation of naïve B and T cells but also maintain T cell tolerance to self antigens [Steinman et al., 2003]. DC have sentinel functions in infectious diseases, immune disorders, and vaccination strategies [Banchereau et al., 2000]. They differentiate from $CD34^+$ hematopoietic stem cells or from $CD14^+$ monocytes into immature DC, which migrate to peripheral non-lymphoid tissues, sites where DC are challenged by pathogen-derived molecules or inflammatory cytokines. Exogenous antigens are internalized, processed, and presented on the DC surface by MHC II molecules, or alternatively by MHC I molecules, while DC migrate to local lymph nodes and mature phenotypically. Mature DC downregulate their endocytic capacity but upregulate the expression of surface-bound costimulatory or MHC II molecules and synthesize high levels of bioactive cytokines such as IL-12, which enhances innate and adaptive immunity [Banchereau and Steinman, 1998]. Ligands of the TNF or

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Toll-like/IL-1 families of receptors are essential regulators of DC maturation, signals which converge to the activation of transcription factors such as NF-κB [Rescigno et al., 1998; Neumann et al., 2000].

OPG, a member of the TNF receptor family, is a soluble glycoprotein which has initially been described as a decoy receptor for receptor activator of NF-κB ligand (RANKL), the principal regulator of osteoclast functions such as differentiation, activation, maturation, and survival [Simonet et al., 1997; Hsu et al., 1999]. OPG has also been assigned functions in the immune system, since DC from OPG-deficient mice stimulate allogeneic T cells more efficiently, show an impaired development of B cells, and exhibit defects in antibody isotype class switch during primary immune responses [Yun et al., 2001]. OPG is widely produced by a variety of tissues and cells, including the heart, arteries, lymph nodes, B lymphocytes, and DC [Simonet et al., 1997; Yun et al., 1998]. In DC, OPG is upregulated by ligating CD40, by lipopolysaccharide (LPS), and by estrogens [Yun et al., 1998; Bengtsson et al., 2004]. However, the precise mechanisms and stimuli that influence OPG expression in DC have remained unclear. We hypothesized that OPG is regulated by various cytokines of the TNF and Toll-like receptor stimulator family and found that OPG is regulated in a maturation- and NF-κB-dependent manner.

MATERIALS AND METHODS

Isolation of DC

DC were generated from blood monocytes as described previously [Romani et al., 1996]. In brief, peripheral blood mononuclear cells derived from whole blood of healthy volunteers were prepared by density gradient centrifugation, resuspended in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; Allgaeu BioTech Service, Goerisried, Germany), and subsequently incubated in a humidified atmosphere at 37°C, 5% CO_2 at 5×10^6 cells/ml in a tissue-grade petri dish to allow monocytes to adhere to the bottom. Nonadherent cells were removed after 2 h by washing, and the adherent cell fraction was further cultured in complete medium supplemented with GM-CSF (1,000 U/ml) and IL-4 (800 U/ml) (both BD Pharmingen, San Diego, CA) for 7 days. To retard maturation of DC, cells were pretreated with $1\alpha,25(OH)_2$ vitamin D_3 (10^{-8} M; Biomol, Plymouth, PA), tamoxifen (10^{-6} M; Sigma), or dexamethasone (10^{-8} M; Sigma) for 7 days. Medium and cytokines were replaced at days 4 and 7. For full maturation, DC were supplemented by TNF-α, IL-1β, IL-6 (1,000 U/ml each; all from R&D Systems, Minneapolis, MN), and prostaglandin E_2 (PGE_2; 10^{-8} M; Sigma) for another 3 days, or immature DC were stimulated by anti-CD40 (10 μg/ml; BD Pharmingen), RANKL (1 μg/ml; R&D Systems), double-stranded RNA (poly I:C; 20 μg/ml; Sigma), or lipopolysaccharide (LPS; 1 μg/ml; Sigma) for another 3 days.

Northern Blot Analysis

Total cellular RNA was extracted from DC using the RNeasy Mini Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany). Extracts were probed with radiolabeled human OPG cDNA insert, followed by hybridization and stringent washing. Hybridization with human β-actin cDNA verified that equal amounts of RNA were loaded.

Enzyme-Linked Immunosorbent Assay (ELISA) for OPG

Conditioned medium was harvested from cultured DC and centrifuged to remove debris. OPG protein concentrations were determined in triplicates with an immunoassay from Immundiagnostik (Bensheim, Germany) following the instructions of the manufacturer. The assay has a lower limit of detection of 0.5 pmol/L, the intraassay coefficient of variation (CV) is between 8% and 10%, and the interassay CV is between 12% and 15%.

Flow Cytometry

DC were analyzed for cell surface expression of leukocyte markers by fluorescence-activated cell sorting (FACS) analysis. Flow cytometry was performed with primary antibodies against CD54, CD83, CD86, and HLA-DR labeled with fluorescein isothiocynate, phycoerythrin, or isotype control by use of a FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany). Mouse $IgG_1\kappa$ isotype control antibody was purchased from Sigma, all other antibodies were purchased from BD Pharmingen. A minimum of 20,000 cells per sample were analyzed.

Gene Silencing and Real-Time PCR

The interfering RNA against the p105 subunit of NF-κB and irrelevant control small

interfering RNA (siRNA) were purchased from Qiagen. Transfection was performed with SiLentFect lipid reagent from Bio-Rad (Hercules, CA), and the medium changed after 24 h. Real-time PCR analysis was performed using the Qiagen one step RT-PCR kit, additionally SybrGreen I was added to the samples. The master mix contained 1 × PCR buffer, 1 × Q-solution, 10 mM deoxynucleotides (equimolar dA/C/G/TTP), 0.6 μM forward primer, 0.6 μM reverse primer SYBR Green I, 1 μl Qiagen OneStep RT-PCR enzyme mix, and H_2O containing equilibration solution (Bio-Rad, Munich, Germany) to a total of 25 μl per reaction when added to 2.5 μl of template. The PCR was carried out using the Bio-Rad iCycler system, cycling conditions were 50°C for 30 min, 95°C for 15 min, 40 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s. A melting curve analysis was run after the final amplification period via a temperature gradient from 55 to 94°C in 0.5°C increment steps measuring fluorescence at each temperature for a period of 10 s. The relative expression of OPG and NF-κB transcripts was calculated as the ratio between the level of the respective gene and the level of GAPDH (for nucleotide sequences of primer pairs see Table I). Using the Bio-Rad iQ iCycler system software, the threshold (Ct) at which the cycle numbers were measured was adjusted to areas of exponential amplification of the traces. The ΔΔ-method was used to determine comparative expression level by applying the formula $2^{(-\Delta Ct\ gene - \Delta Ct\ GAPDH)}$ as described previously [Pfaffl, 2001].

Immunohistochemistry, Immunofluorescence Staining, and Confocal Microscopy

Human tonsil specimens were obtained from patients undergoing tonsilectomy after informed consent. The material was waste material left over after the operation. Specimens were subjected to conventional staining (hematoxylin and eosin), immunohistochemistry, and immunofluorescence staining. Specimens were snap-frozen in liquid nitrogen immediately after surgical excision and embedded in tissue freezing raisin (Leica, Nussloch, Germany). Specimens were cut in 6 μm transverse sections, mounted on poly-L-lysine-coated slides and fixed with acetone for 10 min. For immunohistochemistry, serial sections of each specimen were preincubated with 5% normal goat serum in PBS, then incubated over night in a humidified chamber at 4°C with different monoclonal mouse antibodies against the following antigens: anti-human CD11c (clone KB90; 10 μg/ml), anti-human CD13 (clone WM-47; 6 μg/ml), anti-human CD68 (clone KP1; 15 μg/ml), anti-human CD83 (clone HB15e; 20 μg/ml), anti-human CD86 (clone BU63; 10 μg/ml) (all from DakoCytomation, Glostrup, Denmark), anti-human OPG (clones 69146.11 and 69127.11; 12.5 μg/ml), and anti-human RANKL (anti-TRANCE, clone 70525.11; 25 μg/ml) (both from R&D Systems). Ensuing incubations were carried out with biotinylated goat anti-mouse antibodies, streptavidin-alkaline phosphatase-conjugate, Fast Red staining (all reagents from Sigma), and Mayer's hemalum counterstaining. Slides were covered with glycerol-gelatine and cover slips, finally examined by light microscopy. For immunofluorescence staining, sections were preincubated with 5% normal rabbit serum in PBS, then incubated over night in a humidified chamber at 4°C with monoclonal mouse antibodies against human CD11c, CD13, CD68, CD83, and CD86 at the concentrations described above. Then, sections were incubated with biotinylated anti-human OPG polyclonal goat antiserum (15 μg/ml; R&D Systems) for 2 h at RT. Ensuing incubations were carried out with Cy3-conjugated goat anti-mouse antiserum (3.5 μg/ml; Jackson Immuno Research, West Grove, PA), followed by FITC-conjugated Streptavidin (2.5 μg/ml; BD Pharmingen). Slides were covered with glycerol in TBS, pH 8.4, and cover slips, then examined by conventional and confocal immunofluorescence (LSM 510 microscope with AxioVision LE Rel. 4.5

TABLE I. Nucleotide Sequences of Primer Pairs

Gene		Oligonucleotide sequences	Annealing temperature (°C)
OPG	Forward	5′-AAA gCA CCC TgT AgA AAA CAC A-3′	60
	Reverse	5′-gTT gCC gTT TTA TCC TCT CTA C-3′	
NF-κB	Forward	5′-ggA AAC CAT ATg AgC CAg Ag-3′	60
	Reverse	5′-CTC ATA gTT gTC CAT AAg Tg	
GAPDH	Forward	5′-gAA ggT gAA ggT Cgg AgT C-3′	60
	Reverse	5′-gAA gAT ggT gAT ggg ATT TC-3′	

software; Zeiss, Oberkochen, Germany). All negative controls were conducted by substituting the primary antibody by an irrelevant antibody or PBS, respectively.

Statistical Analysis

Each in vitro experiment was repeated at least three times, values are expressed as the mean ± SD of triplicate measurements of individual DC cultures with data from representative experiments shown. Student's paired *t*-test was used to evaluate differences between the sample of interest and its respective control. For analysis of time courses and dose responses, multiple measurement ANOVA was performed. A *P*-value of <0.05 was considered statistically significant. Standard software from StatView 5.0 (SAS Institute, Cary, NC) was used for statistical analyses.

RESULTS

Antigen-presenting DC were treated with TNF-α, IL-1β, IL-6, and PGE_2 added simultaneously to achieve full DC maturation in order to study OPG expression over the course of DC differentiation. OPG expression by DC increased with maturation as evident from comparison of immature DC on day 7 after isolation with mature DC on days 9–15 (Fig. 1). Upregulation of OPG mRNA was paralleled by increased OPG protein in the supernatant of DC as assessed by an ELISA system (Fig. 1).

Since these experiments suggested that OPG expression could depend on maturity of DC, we next assessed OPG expression after preincubation of DC with various agents known to inhibit or delay DC maturation such as $1\alpha,25(OH)_2$ vitamin D_3, tamoxifen, or dexamethasone [Piemonti et al., 1999; Komi and Lassila, 2000; Griffin et al., 2001]. Therefore, DC were differentiated with a cytokine combination comprising TNF-α, IL-1β, IL-6, and PGE_2 as described previously [Romani et al., 1996]. FACS analysis demonstrated the downregulation of DC maturation and activation markers such as CD54, CD83, CD86, or HLA-DR after pretreatment with the respective agents (Fig. 2A). DC expressed lower OPG mRNA steady state levels (Fig. 2B) and secreted lower concentrations of OPG protein (Fig. 2C) which correlated with the degree of maturity. Whereas pretreatment with the glucocorticoid dexamethasone almost completely inhibited and $1\alpha,25(OH)_2$ vitamin D_3 pretreatment substantially decreased OPG expression, tamoxifen pretreatment only moderately diminished OPG expression on the mRNA level but not protein secretion, correlating with its slight change in DC surface markers on FACS analysis.

For detection of DC-derived OPG in vivo, we analyzed human tonsils by immunohistochemistry and immunofluorescence, which contain both mature and immature DC (Fig. 3). OPG protein was found to be colocalized with areas of $CD83^+$ (mature) DC, whereas CD83 was not detected in areas negative for OPG. $CD83^+$ DC in human tonsils frequently expressed the costimulation marker CD86, the maturation and activation markers CD11c and CD13 as previously demonstrated on activated tonsil DC, and the monocytic lineage marker CD68 [Takahashi et al., 1998; Summers et al., 2001]. Confocal microscopy revealed that all CD markers lined the outer side of the DC cytoplasmic membrane. OPG was found in the

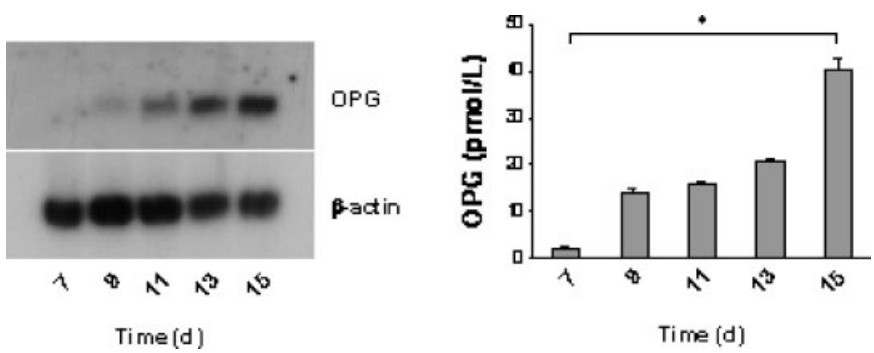

Fig. 1. OPG mRNA expression and protein secretion is upregulated in DC in a maturation dependent manner. Immature DC were treated with cytokines to achieve full maturation (TNF-α, IL-1β, IL-6, and PGE_2 added simultaneously) starting at day 7 after isolation of cells for the indicated time points. $^*P<0.001$ (ANOVA). DC, dendritic cells; OPG, osteoprotegerin; PGE_2, prostaglandin E_2.

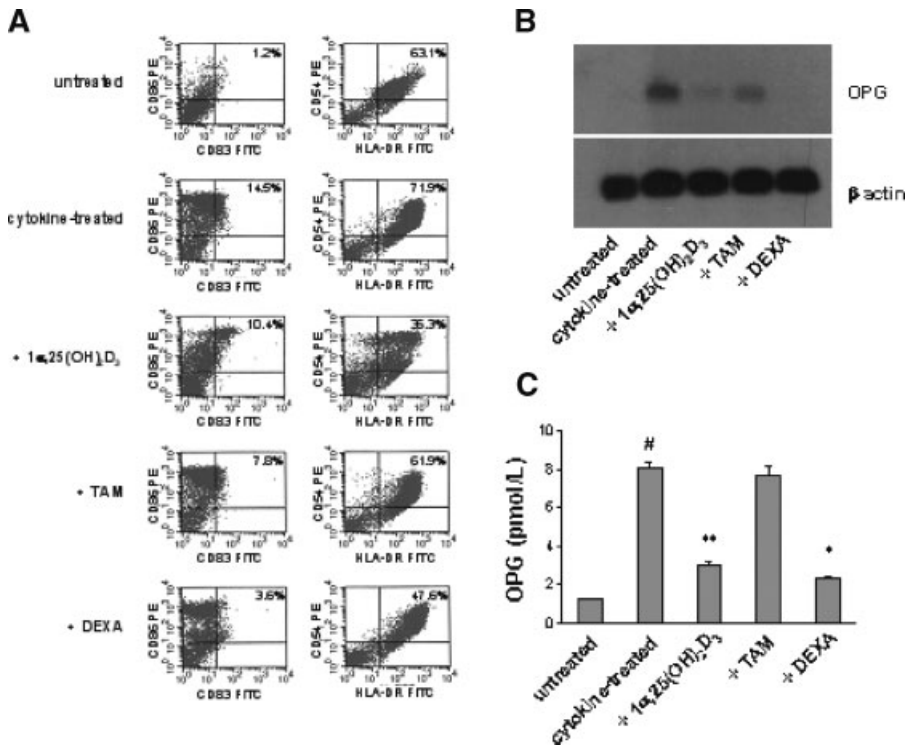

Fig. 2. Inhibitors of DC maturation were tested for their ability to influence OPG production by DC. Monocytes were incubated with GM-CSF and IL-4 for 7 days to achieve immature DC in parallel to $1\alpha,25(OH)_2$ vitamin D_3 (10^{-8} M), tamoxifen (10^{-6} M), or dexamethasone (10^{-8} M). Subsequently, immature DC were stimulated with cytokines (TNF-α, IL-1β, IL-6, and PGE_2 added simultaneously) for 72 h, and studied for OPG expression. **A**: FACS analysis of DC surface markers to assess maturity of DC. **B**: Northern blot analysis for OPG mRNA expression by DC. **C**: Immunoassay for OPG protein secretion by DC. $^{\#}P < 0.005$ compared to untreated DC; $^{*}P < 0.005$, $^{**}P < 0.001$ compared to cytokine-treated DC (Student's *t*-test). DC, dendritic cells; DEXA, dexamethasone; OPG, osteoprotegerin; PGE_2, prostaglandin E_2; TAM, tamoxifen; $1\alpha,25(OH)_2D_3$; $1\alpha,25(OH)_2$ vitamin D_3.

cytoplasm of $CD83^+$, $CD86^+$, $CD11c^+$, $CD13^+$, and $CD68^+$ DC. However, OPG was not expressed by all $CD11c^+$, $CD13^+$, or $CD68^+$ cells. OPG was also localized extracellularly adjacent to DC. All isotype controls were completely negative. These findings indicate that OPG is also expressed by mature DC in vivo.

To further characterize potential mediators of OPG expression in these antigen-presenting cells, immature DC were stimulated with various cytokines. SW579, a thyroid carcinoma cell line, and ligation of CD40, which has been shown to upregulate OPG expression in DC, were used as positive controls. Of the agents tested, treatment with TNF-α and IL-1β increased OPG expression (Fig. 4), whereas PGE_2 or IL-6 did not alter OPG mRNA expression but modestly stimulated OPG protein secretion by DC (Table II). In addition, neither lovastatin, a HMG-CoA reductase inhibitor known to influence DC functions, nor LL-37, a chemotactic antimicrobial peptide, stimulated OPG expression (data not shown).

We further assessed the ability of different members of the TNF superfamily and Toll-like receptor stimulating cytokines to influence OPG expression by DC. Members of the TNF family including TNF-α, RANKL, and ligation of CD40 by a monoclonal antibody (which mimics CD40L activity) increased expression of OPG by DC (Fig. 5A). Furthermore, stimulators of Toll-like receptors such as IL-1β, lipopolysaccharide (LPS), or double-stranded RNA (poly I:C) had also a stimulatory effect on OPG expression by DC as TNF family members, with a synergistic effect of TNF-α and IL-1β (Fig. 5B).

Since the transcription factor NF-κB has been shown to be involved in DC maturation and differentiation processes, we next studied the influence of NF-κB on OPG expression by using gene silencing. Introducing siRNA directed against the p105 subunit of NF-κB into DC effectively abrogated production of NF-κB

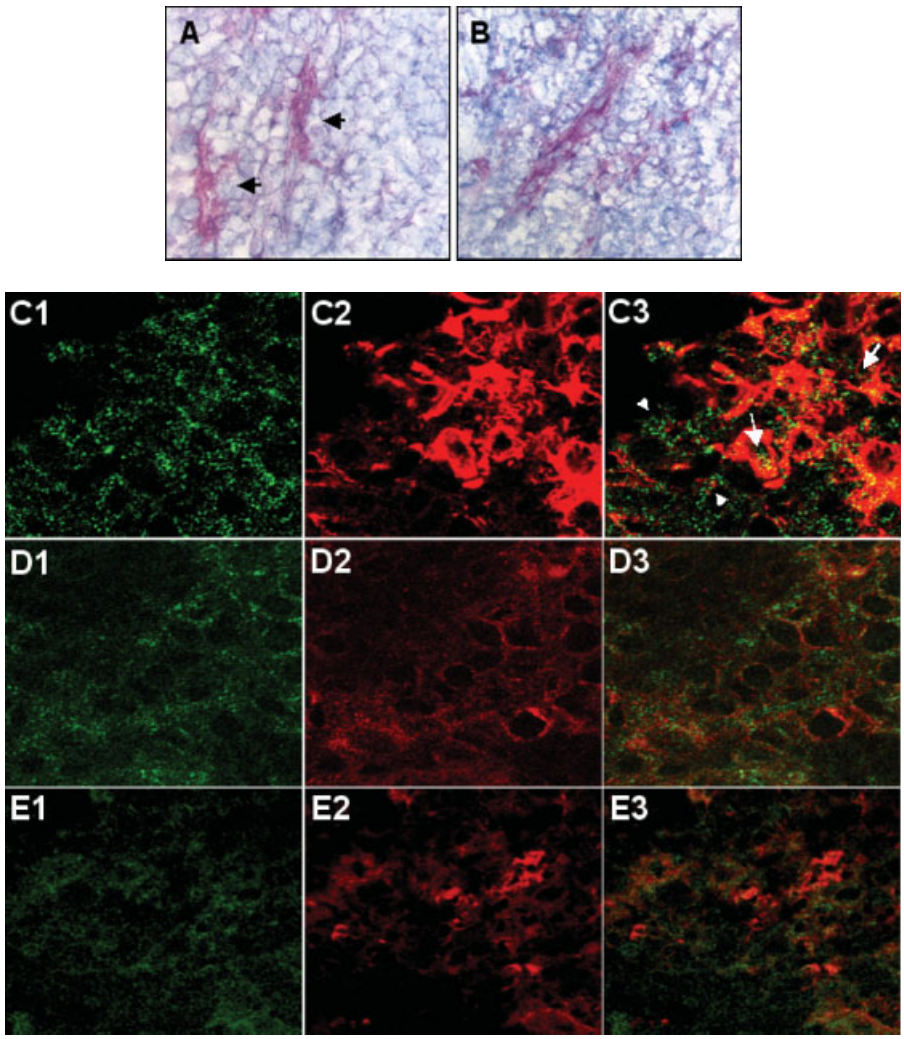

Fig. 3. Immunohistochemistry and immunofluorescence confocal microscopy of human tonsils for OPG protein and DC surface markers. Immunohistochemical analysis of serial sections of a human tonsil specimen using antibodies directed against OPG (**A**) and CD83 (**B**), visualized by streptavidin-alkaline phosphatase-conjugate, Fast Red staining, and Mayer's hemalum counterstaining (arrows: red color indicates positive staining, 400×). The interdigitating, $CD83^+$ (mature) DC were found to express OPG. $CD83^+$ DC also expressed the costimulatory molecule CD86, the monocytic lineage marker CD68, and the tonsil DC maturation markers CD11c and CD13 (data not shown). **C–E**: Immunofluorescence staining for OPG (green; C1, D1, E1), CD 83 (red; C2), CD 86 (red; D2), and CD68 (red; E2) analyzed by confocal microscopy (630×; A1-3: 3× zoom, D1-3 and E1-3: no zoom). By overlay technique, yellow areas indicate the exact match of green and red fluorescence (panels C3, D3, and E3). CD markers lined the cytoplasmic membrane and the thin cytoplasmic processes of DC (C2, D2, E2). OPG was found in the cytoplasm of $CD83^+$ (arrows in C3), $CD86^+$, and $CD68^+$ DC and extracellularly (arrowheads in C3). DC, dendritic cells; OPG, osteoprotegerin.

peaking 2 days after siRNA treatment (Fig. 6A), paralleled by almost complete inhibition of OPG expression by DC (Fig. 6B).

DISCUSSION

In this study, we show that DC, the most potent antigen-presenting cells of the immune system which are required for the initiation of a primary immune response by activating naïve T cells, express OPG, a soluble glycoprotein involved in the regulation of bone metabolism, immune responses, and vascular homeostasis [Hofbauer and Schoppet, 2004], in a maturation- and NF-κB-dependent manner.

OPG acts as a decoy receptor for T cell-derived RANKL, which limits the stimulatory effects of RANKL in the immune system such as activation of mature DC [Wong et al., 1999]. Studies in transgenic, $RANKL^{-/-}$, or $OPG^{-/-}$ mice have

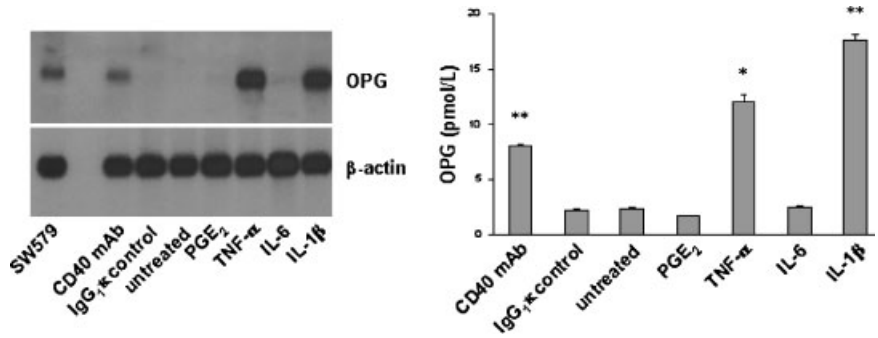

Fig. 4. DC were studied by Northern blot analysis for OPG mRNA expression and by an ELISA system for OPG protein secretion. Immature DC were stimulated by various agents for 72 h and studied for OPG expression, including CD40-triggering by a monoclonal antibody. A thyroid carcinoma cell line (SW579) was used for positive control, β-actin served as house keeping gene. $^{**}P < 0.001$ and $^{*}P < 0.005$ compared to untreated DC by Student's *t*-test. CD40 mAb, monoclonal antibody against CD40; DC, dendritic cells; $IgG_1\kappa$, isotype control antibody; OPG, osteoprotegerin; PGE_2, prostaglandin E_2.

revealed a pivotal role of the RANKL/OPG cytokine system in regulating the immune system and bone metabolism [Bucay et al., 1998; Kong et al., 1999; Yun et al., 2001]. While mice with a disrupted RANKL gene (or $RANK^{-/-}$ mice) exhibit defects in early differentiation of T and B lymphocytes and lack lymph nodes, OPG is thought to negatively regulate B cell lymphopoiesis, to alter DC-B cell interactions, and to influence the magnitude of T cell responses in lymph nodes. Furthermore, $OPG^{-/-}$ mice have an impaired IgG isotype switching, which suggests a role for OPG in the development of efficient antibody responses [Yun et al., 2001]. Of note, a dysbalance in the RANKL/OPG cytokine network may lead to autoimmune disease, as evident in the IL-2-deficient animal model of autoimmunity in which mice develop spontaneous osteopenia and colitis caused by unopposed RANKL effects subsequently promoting intestinal DC survival in vivo, that can be reversed by administration of exogenous OPG [Ashcroft et al., 2003]. Recruitment of monocyte-derived macrophages to lymph nodes from peripheral tissues is under control of cytokines such as monocyte chemoattractant protein (MCP)-1 [Palframan et al., 2001]. In a recent study, OPG has been described to induce monocyte chemotaxis [Mosheimer et al., 2005], which may represent another function of OPG derived from mature DC in lymph nodes.

TABLE II. Stimulation of DC for 72 h With Different Cytokines at Various Concentrations

Cytokine exposure (dose)	OPG secretion (mean ± SD, normalized to control, %)	*P*-value
PGE_2 (M)		
0	100 ± 10.8	<0.05
10^{-10}	92 ± 0.8	
10^{-9}	117 ± 19.2	
10^{-8}	133 ± 1.7	
TNF-α (U/ml)		
0	100 ± 0	<0.0001
10	440 ± 90	
100	1480 ± 70	
1,000	3390 ± 350	
IL-6 (U/ml)		
0	100 ± 12.8	<0.001
10	164 ± 21	
100	196 ± 16.8	
1,000	131 ± 12.8	
IL-1β (U/ml)		
0	100 ± 37.1	<0.0001
10	260 ± 35.8	
100	738 ± 43.7	
1,000	993 ± 22.3	

OPG was measured in the supernatant of cells by an ELISA system. OPG in the supernatant of untreated cells was set as 100%. The values represent mean values ± SD of triplicate measurements. Statistical analysis by ANOVA. PGE_2, prostaglandin E_2.

We found that OPG is expressed in mature DC, but not in immature DC, and that expression is a function of the maturation state of DC. In contrast, pretreatment of DC with $1\alpha,25(OH)_2$ vitamin D_3, tamoxifen, or dexamethasone decreased OPG expression, underlining that OPG expression is coupled to DC maturity. Furthermore, OPG protein colocalized with $CD83^+$ DC in human tonsils, suggesting that mature DC in lymphoid tissues also represent a source of OPG in vivo. A variety of hormones or steroids including $1\alpha,25(OH)_2$ vitamin D_3 [Griffin et al., 2001], tamoxifen [Komi and Lassila, 2000], or dexamethasone [Piemonti et al., 1999] have been demonstrated to negatively modulate DC differentiation. In

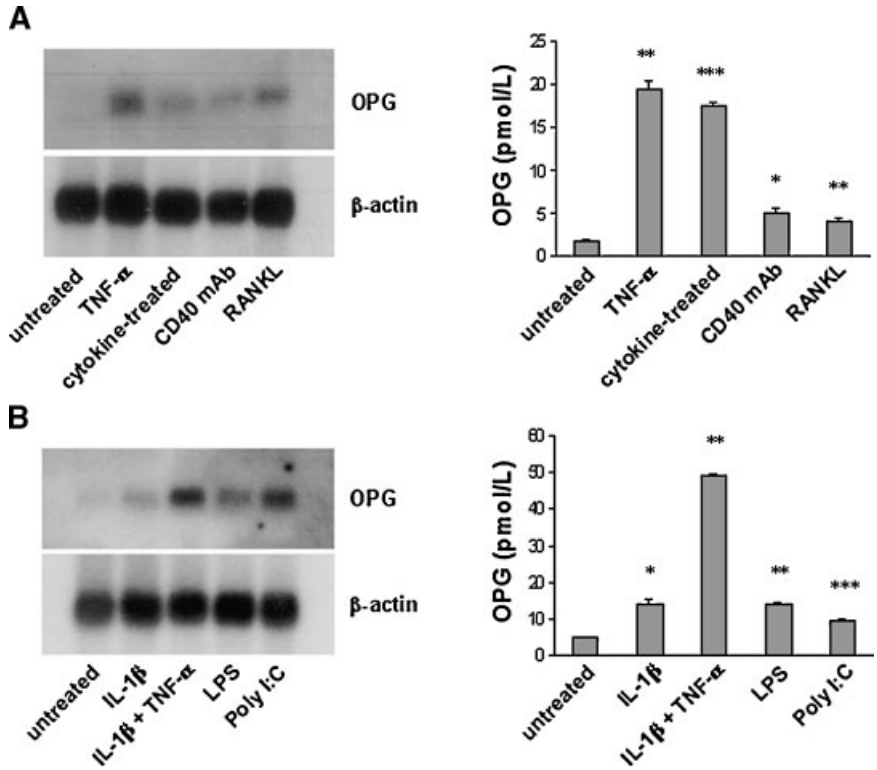

Fig. 5. Immature DC were stimulated by various ligands of the TNF and Toll-like/IL-1 families of receptors for 72 h. **A**: Northern blot analysis and ELISA for OPG mRNA expression and protein secretion after stimulation of DC by TNF-α, cytokines to achieve full maturation of DC (TNF-α, IL-1β, IL-6, and PGE_2 added simultaneously), CD40 mAb, or RANKL. **B**: Treatment of DC by IL-1β, LPS, or poly I:C also resulted in upregulation of OPG by DC. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ compared to untreated DC by Student's *t*-test. CD40 mAb, monoclonal antibody against CD40; DC, dendritic cells; LPS, lipopolysaccharide; OPG, osteoprotegerin; PGE_2, prostaglandin E_2; poly I:C, double-stranded RNA; RANKL, receptor activator of NF-κB ligand.

contrast, lipopolysaccharides [Rescigno et al., 1998] and double-stranded RNA [Cella et al., 1999], both of which act by binding to Toll-like/IL-1 receptors, have been shown to induce maturation of DC. We identified a number of TNF family cytokines and Toll-like receptor stimulators known to promote DC maturation to induce OPG expression in DC in vitro.

Of interest, OPG knock-out mice exhibit vascular calcification of the aorta and renal arteries [Bucay et al., 1998], a finding which has attributed OPG a protective role in the vascular system, and endothelial cells and smooth muscle cells have been characterized as a source of OPG in vessels [Malyankar et al., 2000; Hofbauer et al., 2001]. In advanced carotid plaques, DC have been detected that exhibit a mature phenotype [Yilmaz et al., 2004], and OPG is upregulated in carotid atherosclerosis endarterectomy specimen [Golledge et al., 2004]. Thus, DC may represent another important source for OPG production in the vascular system.

To characterize downstream signaling pathways of OPG expression in DC, we studied NF-κB in DC and found that targeting this transcription factor by siRNA abrogates OPG expression in DC. Accordingly, OPG is upregulated in endothelial cells following $\alpha_v\beta_3$ ligation by osteopontin [Malyankar et al., 2000] or in intestinal epithelial cells in a NF-κB-dependent fashion [Toruner et al., 2006].

The control of the lifespan of DC, which is a critical determinant of immunity, is incompletely understood, and regulation of DC survival has a key regulatory function in the immune system. Triggering of CD40 on DC by interaction with T cell-derived CD40L or RANKL binding to its receptor RANK expressed on DC is thought to promote DC survival [Wong et al., 1997; Miga et al., 2001], and DC transduced with an adenoviral vector expressing RANKL survived significantly longer than control DC [Yurkovetsky et al., 2006]. In another recent study, a subtype of DC has been shown to express both RANKL and RANK which seems to

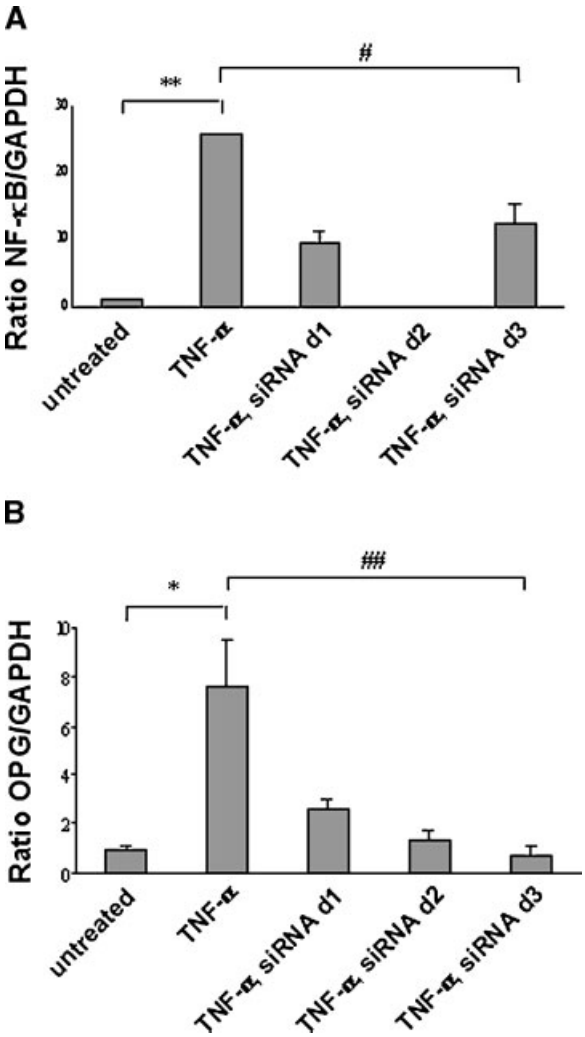

Fig. 6. Blockade of NF-κB downregulates OPG expression in DC as assessed by real-time PCR. Immature DC were stimulated with TNF-α for 3 days to induce expression of OPG and treated with siRNA against the p105 subunit of NF-κB for the whole time (d3), the last 48 h (d2), or the last 24 h (d1) of TNF-α-treatment. **A**: Downregulation of the transcription factor after RNA silencing of NF-κB. **B**: Concomitant with the downregulation of NF-κB, OPG expression is abrogated. $^{*}P < 0.01$ and $^{**}P < 0.005$ for untreated vs. TNF-α (Student's *t*-test), $^{\#}P < 0.005$ and $^{\#\#}P < 0.001$ comparing all TNF-α treated DC groups (ANOVA). DC, dendritic cells; OPG, osteoprotegerin; siRNA, small interfering RNA.

regulate their longevity, although these DC downregulate RANKL with maturation, rendering cells dependent on exogenous RANKL [Cremer et al., 2002]. In this context, OPG derived from mature DC may interfere with this pathway by ligating RANKL in an autocrine or paracrine fashion, thereby limiting the lifespan of DC and thus protecting against undue immunity as in autoimmune diseases. In summary, we describe NF-κB-dependent OPG expression in mature but not immature DC, which may represent a mechanism to modulate T and B cell functions or a mechanism to regulate the longevity of DC.

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