T-bet-dependent expression of osteopontin contributes to T cell polarization

Mari L. Shinohara*†, Marianne Jansson*†‡, Eun Sook Hwang§¶‖, Miriam B. F. Werneck*†, Laurie H. Glimcher§‖, and Harvey Cantor*†**

*Department of Cancer Immunology and AIDS, Dana–Farber Cancer Institute, §Department of Immunology and Infectious Diseases, Harvard School of Public Health, and Departments of †Pathology and ‖Medicine, Harvard Medical School, 44 Binney Street, Boston, MA 02115

Contributed by Harvey Cantor, October 4, 2005

The osteopontin (Opn) glycoprotein has been implicated in diverse physiological processes, including vascularization, bone formation, and inflammatory responses. Studies of its role in immune responses has suggested that Opn can set the early stage of type-1 immune (cell-mediated) responses through differential regulation of IL-12 and IL-10 cytokine gene expression in macrophages. Although Opn has been suggested to play a role in the development of type-1 immunity, little is known about control of *Opn* gene expression. Here, we report that *Opn* gene expression in activated T cells, but not macrophages, is regulated by T-bet, a transcription factor that controls CD4$^+$ T helper (Th1) cell lineage commitment. We also find that T-bet-dependent expression of Opn in T cells is essential for efficient skewing of CD4$^+$ T and CD8$^+$ T cells toward the Th1 and type 1 CD8$^+$ T cells (Tc1) pathway, respectively. Taken together, these findings begin to delineate the genetic basis of Opn expression in T cells and further clarify the role of Opn in Th and Tc1 development.

genetic programming | T helper 1 development | type-1 immune response

The osteopontin (Opn) glycoprotein has been independently identified and studied by investigators from numerous scientific fields in view of its role in immune responses, vascularization, and bone formation through interactions with mononuclear, endothelial, and bone cells, respectively. Analysis of its contribution to immune responses has suggested that Opn expression can set the stage for protective type-1 immune responses after viral and bacterial infection through differential regulation of IL-12 and IL-10 cytokine production (1–3).

Studies of Opn-deficient (Opn$^{-/-}$) mice have indicated that Opn contributes to host resistance against diverse microbial pathogens including herpes simplex virus 1, *Listeria monocytogenes* (4), and rotavirus (5). Opn expression is also essential for effective Th1-dependent granuloma formation and a positive clinical outcome in patients suffering from mycobacterial infection (6), whereas ectopic Opn expression has been implicated in the granulomatous lesions of Crohn's disease (7). Dysregulated Opn expression has also been implicated in several autoimmune disorders, including murine experimental autoimmune encephalomyelitis (EAE) (8, 9), multiple sclerosis (10), rheumatoid arthritis (11), and atherosclerosis (12, 13). Dysregulated Opn expression has been correlated with excessive Th1 polarization of CD4$^+$ T cells in these disorders (7, 14) opening the possibility that *Opn* gene expression may directly contribute to Th1/type 1 CD8$^+$ T cells (Tc1) genetic programming. However, the genetic basis of Opn expression in Th-cell subsets is not well understood.

T-bet, a member of the T box family of transcription factors, is the master coordinator of gene expression in T cells that initiate type-1 immunity and is essential for Th1 cell polarization (15). Thus, T-bet deficiency reduces IFN-γ production by activated CD4$^+$ T cells and T cell antigen receptor (TCR)-transgenic CD8$^+$ T cells (OT-1) (16, 17). The rapid and specific induction of T-bet in developing Th1 but not in Th2 cells (15) is controlled by signals from the T cell receptor and the IFN-γ receptor/signal transducer and activator of transcription 1 transduction pathways (18, 19). The factors that contribute to the Th1 developmental pathway are broadly divisible into T-bet-dependent and T-bet-independent components. T-bet-dependent elements of the Th1 pathway include IFN-γ and IL-12 receptor β2, whereas T-bet-independent elements include IL-7, IL-15, IL-21, and IL-18. Although there is increasing evidence that Opn promotes Th1-dependent immune responses, we do not know whether Opn belongs to the T-bet-dependent or -independent component of this process. Here we investigate the potential role of T-bet in regulating *Opn* gene expression in activated T cells and macrophages. We find that Opn expression represents a unique aspect of the T-bet-dependent genetic pathway to type-1 immunity.

Materials and Methods

Animals and Reagents. Opn-deficient mice have been backcrossed onto B6 and BALB/c mice at least to 10 generations. T-bet-deficient (T-bet$^{-/-}$) mice have been backcrossed onto either B6 or BALB/c for seven generations. B6 2D2 transgenic mice were a gift from V. K. Kuchroo (Harvard Medical School). B6 Rag2$^{-/-}$ and B6 OT-1 mice were obtained from The Jackson Laboratory. Opn promoter-luciferase construct (pXP2) was a gift from D. Denhardt (Rutgers University, Piscataway, NJ). Recombinant mouse cytokines were purchased from Pharmingen. IFN-γ bioactivity was confirmed by a previously described method (20). Myelin oligodendrocyte glycoprotein (MOG) (residues 35–55) peptide (MEVGWYRSPFSRVVHLYRNGK) and OT-1 peptide (SIINFEKL) were synthesized at New England Peptide, Gardner, MA.

Cell Purification and Tissue Culture. T cells were purified by magnetic beads selection as described in ref. 9. In brief, single-cell suspensions were obtained, and cells were suspended in labeling buffer (2% FBS in PBS) at a concentration of 10^7 cells per ml. To purify total T cells, 10 μg/ml purified rat mAb for anti-B220 as well as anti-CD11b, anti-GR-1, and anti-NK1.1 or anti-DX5 (Pharmingen), each at 5 μg/ml, were incubated with the cells for 30 min at 4°C. For CD4$^+$ and CD8$^+$ T cell purification, anti-CD8α and anti-CD4 Abs, respectively, were added to the previously described Ab mixture. After being incubated and washed, Ab-bound cells were incubated with 50 μl/ml Dynabeads M-450 sheep anti-rat IgG (Dynal, Great Neck, NY) followed by magnetic separation. Cell purity was >90% as judged by enumeration of CD3ε^+CD4$^+$ and CD3ε^+CD8$^+$ cells by flow cytometry. Positive selection of dendritic cells (DCs), CD4$^+$, and CD8$^+$ T cells was carried out by using anti-CD11c,

Conflict of interest statement: No conflicts declared.

Abbreviations: EAE, experimental autoimmune encephalomyelitis; Opn, osteopontin; CTL, cytotoxic T lymphocyte; Th, T helper; TCR, T cell antigen receptor; OT-1, MHC class I-restricted OVA-specific TCR transgenic CD8$^+$ T cells; RV, retrovirus; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; DC, dendritic cells; Tc, CD8$^+$ T cells.

‡Present address: Department of Laboratory Medicine, Lund University, S-221 00 Lund, Sweden.

¶Present address: Division of Molecular Life Sciences and College of Pharmacy, Ewha Womans University, 11-1 DaeHyun-Dong, Seodaemun-Ku, Seoul 120-750, Korea.

**To whom correspondence should be addressed. E-mail: harvey_cantor@dfci.harvard.edu.

anti-CD4, or anti-CD8 MiniMacs beads (Miltenyi), respectively, according to the manufacturer's protocol. For peritoneal macrophage preparation, mice were i.p. injected with 1 ml of 3% thioglycollate (Sigma). Four days later, cells were harvested by peritoneal lavage using ice-cold PBS followed by plastic adherence for 1 h at 37°C, followed by washing three times with complete medium at 37°C. Adherent cells were removed from the plates by using Cell Stripper solution (Mediatech, Washington, DC) and were >95% positive for macrophage marker F4/80 staining. Complete medium consisted of RPMI medium 1640 supplemented with 10% FBS (Sigma), L-glutamine, 2-mercaptoethanol, and penicillin/streptomycin. Anti-CD3 Ab with or without anti-CD28 Ab were coated onto plates by incubating purified Abs at the indicated concentrations for overnight at 4°C. Plates were washed three times with 1× PBS before cells were plated.

T-Bet Retroviral Transduction. The GFP-RV-T-bet (RV, retrovirus) was constructed by inserting T-bet cDNA into GFP-RV bicistronic vector (15). The Phoenix-Eco packaging cell line was transfected as described in ref. 21. Purified primary T cells were activated with plate-bound anti-CD3 and anti-CD28 Ab (1 μg/ml each). Later (24 h), cells were infected with RV in culture medium containing 8 μg/ml polybrene (Sigma). The T cell culture plates were centrifuged at 480 × *g* for 45 min at room temperature. The medium was replaced 24 h after infection. Retrovirally transduced T cells were sorted by gating for GFP-positive cells and expanded until day 7 after primary activation. The cells were restimulated with plate-bound anti-CD3 Ab (1 μg/ml) for 24 h before harvesting the culture supernatants. As negative control, a GFP-RV mock vector lacking the *T-bet* gene was used.

ELISA Assays. All of the cytokine protein expression assays, with the exception of Opn, were carried out by using ELISA kits (Pharmingen or R & D Systems) according to the manufacturers' protocol. For detection of Opn by ELISA, EIA/RIA plate wells (Corning) were coated with purified AF808 anti-Opn Ab (R & D Systems) in coating buffer (0.1 M sodium carbonate, pH 9.5). Wells were blocked with ELISA dilution buffer (10% FCS in PBS) before samples were applied to wells. Detection was performed with biotinylated anti-Opn Ab (BAF808, R & D Systems) and a secondary detection Ab (avidin-horseradish peroxidase Ab, Pharmingen). Color development was achieved by using substrate solution (3,3′,5,5′,-tetramethylbenzidine substrate reagent set, PharMingen), and absorbance was determined by an ELISA plate reader (Molecular Devices) at 450 nm. Recombinant mouse Opn (441-OP, R & D Systems) was used as a protein standard in the Opn ELISA. The assays were carried out in triplicate wells.

RNA/cDNA Preparation and Real-Time PCR. After the cells were washed with PBS, total RNA was extracted from cells with an RNeasy kit (Qiagen, Valencia, CA). cDNA synthesis was initiated by priming total RNA with oligo(dT) and by reverse-transcribing with Moloney murine leukemia virus reverse transcription (Ambion, Austin, TX). Obtained cDNA was used for real-time PCR analyses on the Applied Biosystems 7700. QuantiTect SYBR Green PCR (Qiagen) was used to detect Opn and β-actin as an internal control. Opn primers were 5′-GCC TGT TTG GCA TTG CCT CCT C-3′ (forward) and 5′-CAC AGC ATT CTG TGG CGC AAG G-3′ (reverse). β-actin primers were 5′-TGT TAC CAA CTG GGA CGA CA-3′ (forward) and 5′-CTG GGT CAT CTT TTC ACG GT-3′ (reverse). Error bars indicate the maximum and minimum values calculated from SD and $-\Delta\Delta$Ct values of Opn and β-actin from triplicate PCR reactions as described in Applied Biosystems manuals.

Th1/Th2 (Tc1/Tc2) Polarization. Abs to cytokines and recombinant cytokines were added to the culture medium for T cell polarizing experiments. Th1/Tc1 culture medium included 5 ng/ml (0.4×10^9

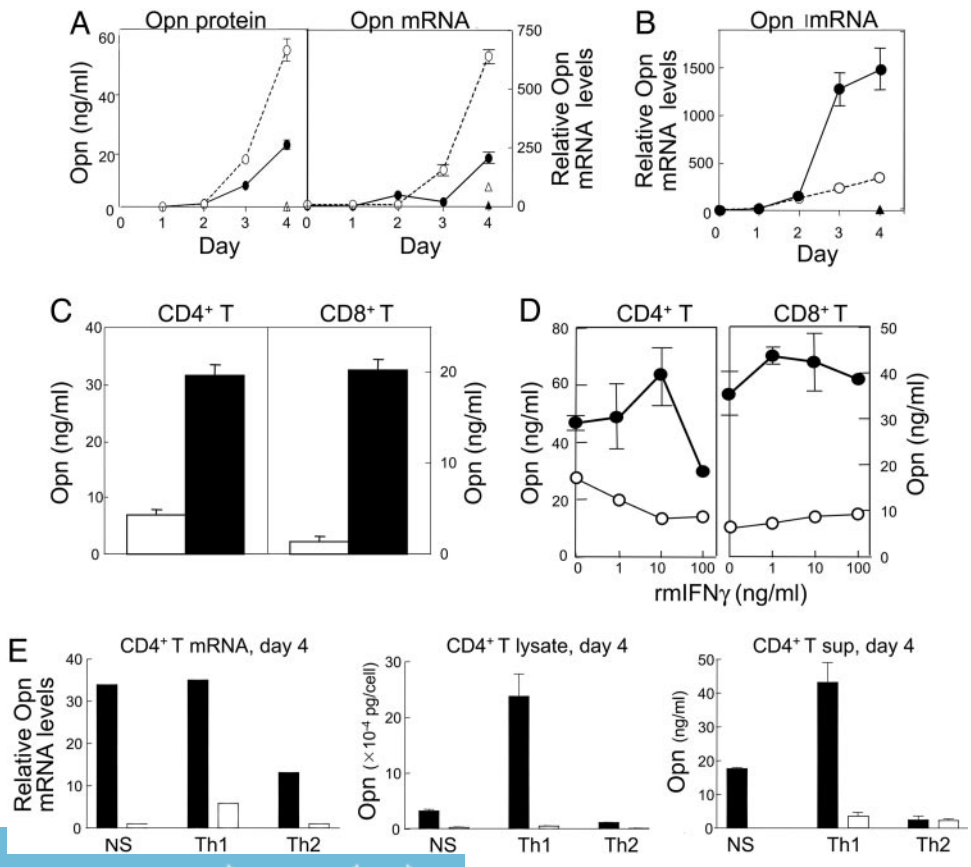

Fig. 1. T-bet up-regulates Opn production in activated T cells. (*A*) T cells were incubated (2.5×10^5 cells per ml) with (○ and ●) or without (△ and ▲) plate-coated 5 μg/ml anti-CD3 and 1 μg/ml anti-CD28 Abs, and daily levels of Opn protein and mRNA were determined by real-time PCR of cDNA samples from triplicates from $CD4^+$ T cells (○ and △) and $CD8^+$ T cells (● and▲). (*B*) Opn mRNA levels expressed by activated (●) and resting (▲) T-$bet^{+/+}$ (WT) T cells and activated (○) and resting (△) T-$bet^{-/-}$ T cells. Plate-coated CD3/28 Abs were used for T cell activation as described in *A*. (*C*) Levels of secreted Opn expressed by T-$bet^{+/+}$ (■) and T-$bet^{-/-}$ (□) T cells determined by ELISA 4 d after incubation as above with anti-CD3/28 Abs. (*D*) Effect of adding indicated concentrations of IFN-γ to Opn response of T-$bet^{+/+}$ (filled bars) and T-$bet^{-/-}$ (○) $CD4^+$ T or $CD8^+$ T cells on day 6. T cells were activated with plate-coated anti-CD3/28 Abs. (*E*) Purified $CD4^+$ T cells (10^6 cells per ml) from T-$bet^{+/+}$ (filled bars) and T-$bet^{-/-}$ (empty bars) mice with 3 μg/ml anti-CD3 Ab and 1 μg/ml anti-CD28 Ab. Culture supernatants and cells were harvested on day 4. Figures here are representative of at least three repeats.

to 2×10^9 units/mg) of recombinant mouse IL-12 and 10 μg/ml anti-IL-4 Ab (Pharmingen). Th2/Tc2 medium included 10 ng/ml (0.25×10^8 to 1×10^8 units/mg) of recombinant mouse IL-4 and 10 μg/ml each of anti-IFN-γ and anti-IL-12 Abs (Pharmingen).

Luciferase Assay. The assay was carried out as described in ref. 15. In brief, the Opn promoter (nucleotides -777 to $+79$) vector fused with a luciferase gene was cotransfected into EL-4 cells with the following vectors by electroporation: T-bet expression vector, pCMVβ (Clontech) vector expressing β-galactosidase as an internal control, and pcDNA vector (Invitrogen) as empty control to keep equal amounts of transfecting DNA among different samples. Luciferase activity was measured 48 h after transfection by using a luminometer. For indicated EL-4 cell samples, phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml) were added 24 h before cell harvest. Luciferase activity is shown as fold induction of the raw "L/G" values [raw luciferase activity (as relative light units) normalized to raw β-galactosidase activity (relative light units)] of T-bet-transfected cells compared to the raw L/G values of control vector-transfected cells.

Comparison of Opn mRNA and Secreted Protein Levels Between T Cells and Macrophages. T cells ($CD4^+$ and $CD8^+$) were purified from total spleen and lymph nodes (LNs). Macrophages were obtained by washing plate-bound peritoneal cells from mice injected with thioglycollate. T cells were seeded as 10^6 cells per ml on day 0 and activated with plate-bound 3 μg/ml anti-CD3 and 1 μg/ml anti-CD28 Abs. Nonactivated T cell samples were cultured in complete medium only. Macrophages were either activated with 30 ng/ml of LPS or unactivated as 2×10^6 cells per ml in complete medium. On day 5, live cells were selected and replated as 10^6 cells per ml for both T cells and macrophages. T cells previously activated were restimulated with plate-bound 3 μg/ml of anti-CD3 Ab. Macrophages previously stimulated with LPS were restimulated with the same concentration of LPS. Cells and culture supernatants were harvested 24 h after stimulation of T cells and macrophages.

EAE Induction with 2D2 TCR Transgenic Mice. Mice were backcrossed to B6 for 16 generations. MOG peptide in 20 μg complete Freund's adjuvant (Sigma) with 4 mg/ml of killed *Mycobacterium tuberculosis* (Difco) were injected s.c. over the flanks on day 0. Pertussis toxin (200 ng, List Biological Laboratories, Campbell, CA) was i.v. injected on day 0 and 2. Mice were monitored daily and assessed for clinical signs of disease in a blinded fashion according to the following criteria: 0, normal mouse without signs of disease; 1, limp tail; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; and 5, moribund state or death due to EAE. Mean clinical scores at separate days and mean maximal scores were calculated by adding scores of individual mice and dividing with number of mice in each group, also including mice not developing signs of EAE. Average day of onset was calculated by adding the first day of clinical signs of individual mice and dividing with number of mice in the group.

ELISA and Cell Proliferation Assays of LN Cells from EAE Mice. Total draining LN cells were harvested from day-16, EAE experimental mice and pooled for the experiments. The cells were plated as 2.5×10^6 cells per ml in a 96-well U-bottom plate with 10 μM of the MOG peptide. Culture supernatants for ELISA assays were harvested 65 h after culture setup. The cells were pulsed at 65 h with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per well and further incubated for 24 h for cell proliferation assay. The 2D2 T cell proliferation specific to the MOG peptide was detected by a β-counter (Wallac, Gaithersburg, MD) from triplicate wells.

Cytotoxic T Lymphocyte (CTL) Assay. B6 female mice were primed with irradiated 4×10^7 splenocytes i.p. The mice were boosted with irradiated 10^7 splenocytes by i.p 10 d later. Responder cells were total splenocytes and LN cells from female mice havested 2 days after boosting. As stimulator cells, male splenocytes were obtained and irradiated. For *ex vivo* CTL stimulation to prepare effector cells, responder and stimulator cells were mixed 1:1 and incubated with 10 units/ml of IL-2 at 37°C for 7 d. Live cells were separated by density gradient by using NycoPrep 1.077 (Axis-Shield) and used for effector cells. As target cells, male splenocytes were stimulated with 5 μg/ml ConA for 2 d and used after density gradient with NycoPrep 1.077. Effector and target cells were incubated at indicated ratios for 4 h in phenol red-free complete RPMI medium 1640 (Life Technologies) supplied with 5% FCS, L-glutamine, 2-mercaptoethanol, and penicillin/streptomycin. Cytotoxicity was evaluated by using CytoTox 96 nonradioactive cytotoxicity kit (Promega) by detecting lactose dehydrogenase release upon cell death. Reaction was carried out in quadruple wells.

Results

Opn Expression in T Cells Is Regulated by T-Bet. Because expression of the transcription factor T-bet is essential to Th1 and Tc1 development (15, 16), we investigated its role in *Opn* gene expression in activated T cells. Four days after stimulation both $CD4^+$ T

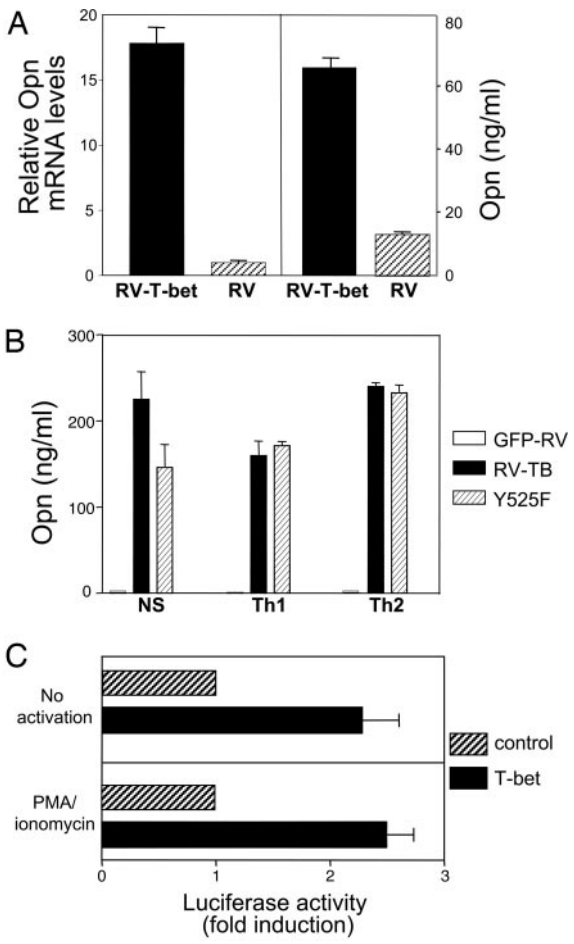

Fig. 2. T-bet induces Opn at the mRNA level. (*A*) Opn production was assessed after reconstituting T-bet expression in stimulated T-bet-deficient T cells. GFP-RV-T-bet (RV-T-bet) and negative control GFP-RV were used to transduce primary T-bet$^{-/-}$ T cells before activation with plate-bound 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 Abs. (*B*) $CD4^+$ T cells positively purified from T-bet$^{-/-}$ mice were stimulated and transduced with RV, RV-T-bet, or RV-T-bet Y525F. (*C*) Opn promoter (nucleotides -777 to $+79$) activity was assessed by luciferase assay with or without T-bet ectopic expression by using EL-4 cells. Values are averages of three independent experiments with error bars of mean ± SEM. Data are representative of multiple repeats.

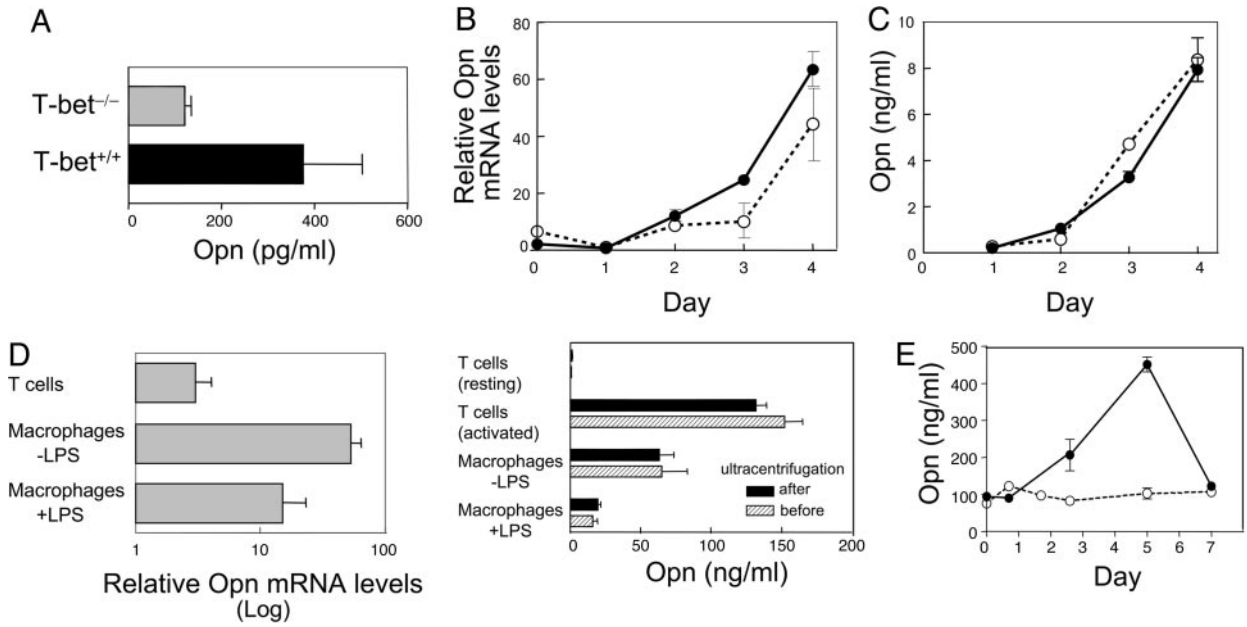

Fig. 3. T-bet does not regulate Opn in macrophages. (*A*) BALB/c splenic DCs from T-bet$^{+/+}$ and T-bet$^{-/-}$ mice were incubated (10^6 cells per ml) for 12 h, and Opn levels in supernatants were examined by ELISA. (*B* and *C*) Peritoneal macrophages isolated from naive BALB/c mice as described in *Materials and Methods*, plated (10^6 cells per ml) with 30 ng/ml of LPS. Levels of Opn mRNA (*B*) and secreted Opn protein in culture supernatants (*C*) are shown with T-bet$^{+/+}$ (●) and T-bet$^{-/-}$ (○) macrophages. (*D*) Levels of Opn mRNA and secreted protein were compared between T cells and macrophages (thioglycollate-elicited) on day 6. Live cells were selected by Nycodenz spin and plated on day 5 as the same concentration (10^6 per ml) for both cell types, as described in *Materials and Methods*. (*E*) The 2D2 TCR transgenic CD4$^+$ T cells were i.v. injected into B6 Rag2$^{-/-}$ mice (2×10^6 cells per mouse). Two weeks later, the mice were received 100 μg of MOG peptide by s.c. injection in complete Freund's adjuvant, and peripheral blood was collected at the indicated times for serum Opn measurements; three mice per group. Groups of recipients with (●) and without (○) 2D2 CD4$^+$ T cell transfer. Bars indicate mean ± SEM.

and CD8$^+$ T cells from B6 mice with plate-bound anti-CD3/CD28 Ab *in vitro*, culture supernatants and T cells contained substantial levels of supernatant Opn protein and Opn mRNA, respectively (Fig. 1*A*). Stimulation of T-bet-deficient T cells resulted in an approximate 5-fold reduction in Opn mRNA (Fig. 1*B*) and a 5- to 20-fold reduction of supernatant Opn in cultures of CD4$^+$ T and CD8$^+$ T cells (Fig. 1*C*). Reduced Opn expression by T-bet-deficient cells did not reflect the need for an IFN-γ intermediary, because provision of recombinant IFN-γ did not restore production of Opn (Fig. 1*D*). The T-bet dependence of Opn expression is congruent with the finding that Opn is overexpressed in Th1 cells and underexpressed in Th2 cells (Fig. 1*E*). Moreover, T-bet-deficient CD4$^+$ T cells expressed markedly reduced Opn RNA and protein (Fig. 1*E*) despite culture conditions that promote Th1 polarization (IL-12 plus anti-IL-4; see *Materials and Methods*), suggesting that Opn expression primarily depends on T-bet rather than Th1-polarizing conditions. The same conclusion was drawn with B6 (Th1-prone) or BALB/c (Th2-prone) mice (data not shown).

Because diminished Opn expression by T cells from T-bet-deficient donors might reflect T cell developmental defects, we asked whether acute reconstitution of T-bet expression *in vitro* might remedy defective Opn expression. Retroviral introduction of T-bet into T-bet-deficient CD4$^+$ T and CD8$^+$ T cells successfully reconstituted Opn expression at the mRNA and protein levels (Fig. 2*A*). Mutation of T-bet tyrosine Y525F reduces T-bet-dependent inhibitory activity for Th2 cytokines but has no effect on expression of the Th1 cytokine IFN-γ (or IL-2) (22). Analysis of the effects of transduction of T-bet-deficient CD4$^+$ T cells with RV-T-bet or RV-T-bet Y525F revealed that both RV-T-bet and RV-T-bet Y525F restored expression of the Th1 cytokine Opn (Fig. 2*B*). Opn transactivation by T-bet was examined by using a vector containing the Opn promoter (nucleotides −777 to +79) fused to a luciferase gene after transfection of EL-4 lymphoma cells with or without ectopic T-bet expression; the promoter activation was verified by luciferase activity. T-bet enhanced Opn promoter-driven luciferase activity by ≈2.5-fold with or without EL-4 cell activation by using phorbol 12-myristate 13-acetate/ionomycin (Fig. 2*C*), possibly reflecting cis-acting elements between −777 and + 79 nt in the 5′ region of the *Opn* gene.

To determine whether T-bet-dependent *Opn* gene expression is cell-type-specific, we evaluated Opn expression in T-bet-deficient DC and macrophages. T-bet$^{+/+}$ DC secreted 4-fold more Opn in 12-h culture supernatants than T-bet$^{-/-}$ DCs, suggesting that Opn expression by DC is at least partially dependent on T-bet (Fig. 3*A*). In contrast, the T-bet genotype of macrophages did not affect Opn expression at either the mRNA or protein levels (Fig. 3 *B* and *C*).

Activated T Cells Are a Potent Source of Extracellular (Secreted) Opn *in Vitro* and *in Vivo*. Unstimulated macrophages constitutively express high levels of Opn mRNA, which was not enhanced by LPS (Fig. 3*D*). However, Opn secretion by macrophages is relatively modest compared with that of activated T cells (Fig. 3*D*). The ratio of secreted Opn protein/Opn mRNA of T cells at day 5 was at least 100-fold greater than that of LPS-activated macrophages (secreted Opn/mRNA: T cells, 1,650 pg/ml/1 mRNA; macrophages, 180 pg/ml/15.6 mRNA), suggesting that activated T cells may use posttranslational mechanisms that allow more efficient Opn secretion compared to macrophages. Indeed, antigen-specific activation of 2D2 TCR transgenic CD4$^+$ T cells, reconstituted in Rag2$^{-/-}$ mice, led to a 5-fold increase in serum Opn levels 5 d after stimulation with MOG peptide (Fig. 3*E*), suggesting that activated T cells can play an important role in Opn levels during the immune responses *in vivo*.

Impact of Opn Deficiency on Th and Tc Responses. We then investigated the potential impact of Opn deficiency on Th and Tc polarization. The IFN-γ/IL-10 ratio of Opn-WT T cells was substantially higher than that of Opn-deficient cells with anti-CD3

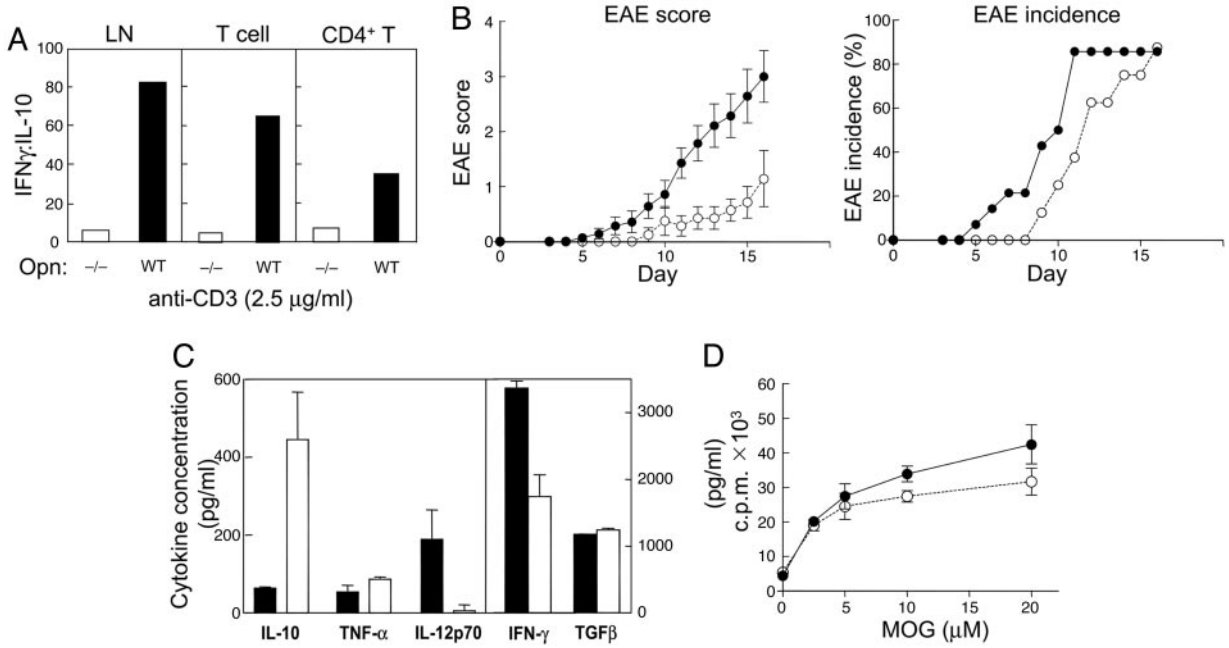

Fig. 4. Opn$^{-/-}$ T cells fail to elicit Th1 response. (*A*) Levels of IFN-γ and IL-10 were measured for day 4 cell culture supernatants. Cells of indicated types were plated as 2.5 × 10^5 cells per ml in precoated wells with 2.5 μg/ml of anti-CD3 Ab. Values show ratios of IFN-γ/IL-10 of ELISA. (*B*) EAE was induced in B6(N16)2D2$^+$ mice (Opn-WT/HZ, $n = 19$, ●; Opn deficient, $n = 14$, ○) as described in *Materials and Methods*. EAE scores are shown as mean ± SEM. (*C*) MOG-specific cytokine production by LN cells from EAE-induced Opn-deficient (□) and Opn WT/HZ (■) 2D2 TCR transgenic mice. (*D*) 2D2 CD4$^+$ T cell proliferation from the same set of Opn-WT (●) and Opn-deficient (○) cells in *C*.

activation (Fig. 4*A*), whereas development of EAE in Opn-deficient mice was blunted (Fig. 4*B*). The latter finding was based on disease intensity and incidence in B6 Opn-deficient mice generated after 16 generations of backcrossing to diminish the potential role of Opn-linked loci (23) before crossing with B6 2D2 TCR transgenic mice to allow a direct analysis of the CD4$^+$ T response to the autoantigenic MOG peptide. The T cell response of Opn-deficient 2D2 TCR-transgenic mice revealed a marked increase in IL-10 production and diminished IL-12 production (Fig. 4*C*), suggesting that Opn expression is essential for promotion of robust Th1 responses. Opn deficiency did not affect 2D2 CD4$^+$ T cell proliferation (Fig. 4*D*).

We next determined the impact of Opn on Tc1/Tc2 responses. Opn-deficient CD8$^+$ T cells displayed enhanced IL-10 responses (but no obvious defect in IFN-γ expression) with anti-CD3-dependent activation (Fig. 5 *A* and *B*) and with antigen-specific activation by using OT-1 peptide (Fig. 5*C*). We then analyzed the impact of Opn deficiency on anti-HY CTL responses after female mice were primed and boosted with irradiated male splenocytes, followed by *ex vivo* stimulation with irradiated male splenocytes. Compared with Opn$^{+/+}$ cells, 10-fold more Opn-deficient cells were required to achieve the same level of anti-HY cytotoxicity (percentage) (Fig. 5*D*).

Discussion

Opn is not a typical cytokine. Its activities are not confined to immune/inflammatory responses, and it is expressed in many cell types in addition to hematopoietic cells. Because its initial biochemical characterization in bony tissues, experimental findings have emphasized the wide range of its effects on diverse physiological processes including wound healing, bone formation, inflammation, and more recently, immune responses. The impact of Opn on type-1 immunity reflects its expression by macrophages, DCs, and activated T cells. The *Opn* gene is constitutively expressed in macrophages in the absence of deliberate immunization, where it may regulate cellular activation and nitric oxide synthesis in response to bacterial and viral products (24–26). In contrast, *Opn* gene expression in T cells is highly inducible upon signaling from the TCR (e.g., Fig. 1*A*). Although activator protein 1 and NF-κB have been implicated in up-regulation of *Opn* gene transcription in macrophages (27), the genetic basis of Opn expression in T cells and its role in T cell development is poorly understood. The studies presented here place the immunological role of Opn on a firmer genetic footing because they demonstrate the importance of the T-bet transcription factor in the induction of *Opn* gene expression and underline the importance of a T-bet-Opn axis in the events leading to Th1 and Tc1 polarization.

Opn reporter assays revealed that the *Opn* promoter region of nucleotides −777 to +79 includes T-bet transactivation site(s). Identification of the precise cis-acting element for T-bet binding in the *ifng* promoter has been difficult because of the possibility of multiple T-bet binding sites (28, 29). We also searched for possible T-bet binding sequences in the *Opn* promoter based on the Brachyury elements suggested by Cho *et al.* (29) and Soutto *et al.* (28), but the *Opn* promoter region of nucleotides −777 to +79 does not include the typical Brachyury sequences. Absence of a typical Brachyury sequence in the *Opn* promoter may account for the rather modest Opn transactivation by ectopic T-bet expression (2- to 2.5-fold, Fig. 1*D*) compared to a large degree of IFN-γ transactivation (15).

Macrophages express readily detectable Opn mRNA levels without overt stimulation and have been reported to represent the major cellular source of Opn. For example, although depletion of monocytes from cell culture reduced Opn levels after *Penicillium marneffei* stimulation, depletion of CD3$^+$ cells had little effect (25). Although macrophages and other cellular components of the innate immune system express nonclonal receptors that allow quick and vigorous reactions to microbial stimuli, T cells require clonal expansion by specific antigen to produce significant Opn responses. However, once expanded, the remarkable inducibility of Opn secretion by activated T cells can account for a substantial portion of the Opn response *in vivo* (e.g., Fig. 3 *D* and *E*).

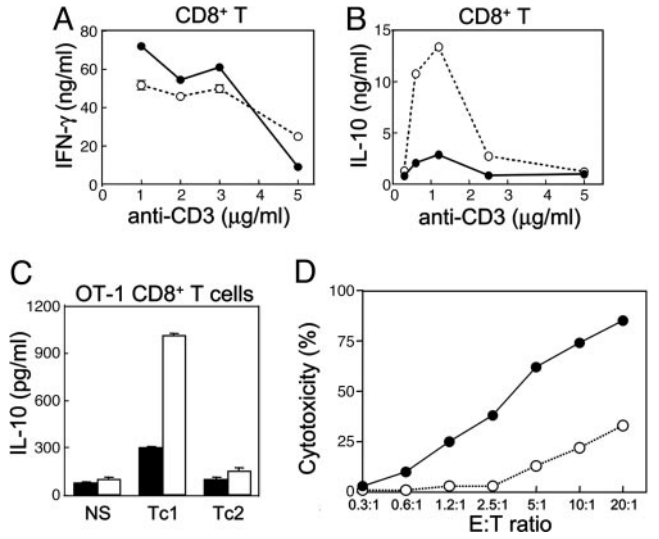

Fig. 5. Opn deficiency enhances IL-10 production and attenuates CTL cytotoxicity. (*A* and *B*) $CD8^+$ T cells (2.5×10^5 cells per ml) from Opn-WT (●) and Opn-deficient (○) mice were stimulated with plate-bound anti-CD3 Ab at the indicated concentrations and 1 μg/ml anti-CD28 Ab before culture supernatants were harvested on day 6. Levels of IFN-γ (*A*) and IL-10 (*B*) were assessed by ELISA. (*C*) Comparison of IL-10 production by Opn-WT (■) vs. Opn-deficient (□) T cells upon antigen-specific activation by DC-pulsed with OT-1 peptide. OT-1 $CD8^+$ T cells were purified from 16-generation mice backcrossed to B6. DCs were purified from $Opn^{-/-}$ mice and activated overnight with 10 μg/ml anti-CD40 Ab, followed by pulsing with OT-1 peptide (20 μg/ml). Peptides were washed off, and DCs were irradiated (3,000 rads) before setting up coculture with OT-1 cells. Levels of IL-10 were assessed from day-4 culture supernatants. (*D*) Comparison of $Opn^{+/+}$ (○) and $Opn^{-/-}$ (●) CTL cytotoxicity against male HY antigen. Results are representatives of triplicate experiments.

Our results that Opn-deficient 2D2 TCR transgenic mice displayed reduced development of EAE compared with Opn WT mice is consistent with the previous findings using non-TCR transgenic Opn-deficient mice backcrossed for six generations (8, 9). The potential influence of genetic loci linked to *Opn* (23) is unlikely, because the mice used for this study underwent 16 generations of backcrossing to B6, i.e., the *Opn* gene is directly involved in EAE pathogenicity.

Expansion of the T-bet-driven genetic programming of Th1/Tc1 cells to include Opn in addition to IFN-γ and IL-12Rβ2 provides an essential component of the cascade of genetic events that control T cell polarity after activation. However, our observations do not address the mechanism that translates Opn expression in Th1/Tc1 development. Previous studies have suggested that engagement of Opn receptors on macrophages by recombinant Opn may induce IL-12 production and inhibit IL-10 production (4). These findings are consistent with this observation that immune responses of mice lacking Opn are marked by enhanced IL-10 production. However, this may not fully account for defective Th1/Tc1 development of the mice because, for example, impaired development of CTL activity by Opn-deficient $CD8^+$ T cells is not remedied by provision of anti-IL-10 Ab or IL-12 (not shown), raising the possibility that the impact of Opn on Th1/Tc1 development may include factors that have not yet been defined.

We thank D. T. Denhardt for providing reagents, V. K. Kuchroo for supplying 2D2 mice, D. Laznik for technical assistance, and A. Angel for assistance with the manuscript and figures. This work was supported by National Institutes of Health Grants AI48125 and AI12184 (to H.C.), CA48126 and AI56296 (to L.H.G.), and T32 CA70083 (to M.L.S.); an Ellison Medical Foundation grant (to L.H.G.); an Arthritis Foundation fellowship (to E.S.H.); and a Juvenile Diabetes Research Foundation fellowship (to M.L.S.).

1. O'Regan, A. W., Nau, G. J., Chupp, G. L. & Berman, J. S. (2000) *Immunol. Today* **21,** 475–478.
2. Denhardt, D. T., Noda, M., O'Regan, A. W., Pavlin, D. & Berman, J. S. (2001) *J. Clin. Invest.* **107,** 1055–1061.
3. Weber, G. F. & Cantor, H. (2001) *Clin. Exp. Immunol.* **126,** 578–583.
4. Ashkar, S., Weber, G. F., Panoutsakopoulou, V., Sanchirico, M. E., Zawaideh, S., Rittling, S. R., Denhardt, D. T., Glimcher, M. G. & Cantor, H. (2000) *Science* **287,** 860–864.
5. Rollo, E. E., Hempson, S. J., Bansal, A., Tsao, E., Habib, I., Rittling, S. R., Denhardt, D. T., Mackow, E. R. & Shaw, R. D. (2005) *J. Virol.* **79,** 3509–3516.
6. Nau, G. J., Chupp, G. L., Emile, J. F., Jouanguy, E., Berman, J. S., Casanova, J. L. & Young, R. A. (2000) *Am. J. Pathol.* **157,** 37–42.
7. Sato, T., Nakai, T., Tamura, N., Okamoto, S., Matsuoka, K., Sakuraba, A., Fukushima, T., Uede, T. & Hibi, T. (2005) *Gut* **54,** 1254–1262.
8. Chabas, D., Baranzini, S. E., Mitchell, D., Bernard, C. C., Rittling, S. R., Denhardt, D. T., Sobel, R. A., Lock, C., Karpuj, M., Pedotti, R., *et al*. (2001) *Science* **294,** 1731–1735.
9. Jansson, M., Panoutsakopoulou, V., Baker, J., Klein, L. & Cantor, H. (2002) *J. Immunol.* **168,** 2096–2099.
10. Steinman, L., Martin, R., Bernard, C., Conlon, P. & Oksenberg, J. R. (2002) *Annu. Rev. Neurosci.* **25,** 491–505.
11. Xu, G., Nie, H., Li, N., Zheng, W., Zhang, D., Feng, G., Ni, L., Xu, R., Hong, J. & Zhang, J. Z. (2005) *J. Clin. Invest.* **115,** 1060–1067.
12. Matsui, Y., Rittling, S. R., Okamoto, H., Inobe, M., Jia, N., Shimizu, T., Akino, M., Sugawara, T., Morimoto, J., Kimura, C., *et al*. (2003) *Arterioscler. Thromb. Vasc. Biol.* **23,** 1029–1034.
13. Bruemmer, D., Collins, A. R., Noh, G., Wang, W., Territo, M., Arias-Magallona, S., Fishbein, M. C., Blaschke, F., Kintscher, U., Graf, K., *et al*. (2003) *J. Clin. Invest.* **112,** 1318–1331.
14. Renkl, A. C., Wussler, J., Ahrens, T., Thoma, K., Kon, S., Uede, T., Martin, S. F., Simon, J. C. & Weiss, J. M. (2005) *Blood* **106,** 946–955.
15. Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. & Glimcher, L. H. (2000) *Cell* **100,** 655–669.
16. Sullivan, B. M., Juedes, A., Szabo, S. J., von Herrath, M. & Glimcher, L. H. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 15818–15823.
17. Lugo-Villarino, G., Maldonado-Lopez, R., Possemato, R., Penaranda, C. & Glimcher, L. H. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 7749–7754.
18. Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., Nguyen, B. V., Gadina, M., Sher, A., Paul, W. E., *et al*. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 15137–15142.
19. Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. & Murphy, K. M. (2002) *Nat. Immunol.* **3,** 549–557.
20. Malu, S., Srinivasan, S., Kumar, M. P., Rajagopal, D., John, B. & Nandi, D. (2003) *J. Immunol. Methods* **272,** 55–65.
21. Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C. & Murphy, K. M. (1998) *Immunity* **9,** 745–755.
22. Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. & Glimcher, L. H. (2005) *Science* **307,** 430–433.
23. Blom, T., Franzen, A., Heinegard, D. & Holmdahl, R. (2003) *Science* **299,** 1845.
24. Scott, J. A., Weir, M. L., Wilson, S. M., Xuan, J. W., Chambers, A. F. & McCormack, D. G. (1998) *Am. J. Physiol.* **275,** H2258–H2265.
25. Koguchi, Y., Kawakami, K., Kon, S., Segawa, T., Maeda, M., Uede, T. & Saito, A. (2002) *Infect. Immun.* **70,** 1042–1048.
26. Lee, S. H., Seo, G. S. & Sohn, D. H. (2004) *Biochem. Biophys. Res.* **323,** 125–132.
27. Ogawa, D., Stone, J. F., Takata, Y., Blaschke, F., Chu, V. H., Towler, D. A., Law, R. E., Hsueh, W. A. & Bruemmer, D. (2005) *Circ. Res.* **96,** e59–e67.
28. Soutto, M., Zhang, F., Enerson, B., Tong, Y., Boothby, M. & Aune, T. M. (2002) *J. Immunol.* **169,** 4205–4212.
29. Cho, J. Y., Grigura, V., Murphy, T. L. & Murphy, K. (2003) *Int. Immunol.* **15,** 1149–1160.