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Xanthine Oxidoreductase Promotes the Inflammatory State of Mononuclear Phagocytes Through Effects on Chemokine Expression, PPARγ Sumoylation, and HIF-1α*

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Running Title: XOR Promotes the Inflammatory State of Mononuclear Phagocytes

The protective effects of pharmacological inhibitors of xanthine oxidoreductase (XOR) have implicated XOR in many inflammatory diseases. Nonetheless, the role played by XOR during inflammation is poorly understood. We previously observed that inhibition of XOR within the inflammatory mononuclear phagocytes (MNP) prevented neutrophil recruitment during adoptive transfer demonstrating the role of XOR in MNP mediated neutrophil recruitment. To further explore the role of XOR in the inflammatory state of MNP, we studied MNP isolated from inflammatory lungs combined with analyses of MNP cell lines. We demonstrated that XOR activity was increased in inflammatory MNP following insufflation of Th-1 cytokines *in vivo* and that activity was specifically increased by MNP differentiation. Inhibition of XOR reduced levels of CINC-1 secreted by MNP. Expression of PPARγ in purified rat lung MNP and MNP cell lines reflected both the presence of PPARγ iso-forms and PPARγ SUMOylation, and XOR inhibitors increased levels of SUMO-PPARγ in MNP cell lines. Both ectopic overexpression of XOR cDNA and uric acid supplementation reduced SUMO-PPARγ in MNP cells. Levels of the M2 markers CD36, CD206, and Arginase-1 were modulated by uric acid and oxonic acid, while siRNA to Sumo-1 or PIAS-1 also reduced Arginase-1 in RAW264.7 cells. We also observed that HIF-1α was increased by XOR inhibitors in inflammatory MNP and in MNP cell lines. These data demonstrate that XOR promotes the inflammatory state of MNP through effects on chemokine expression, PPARγ SUMOylation, and HIF-1α and suggest that strategies for inhibiting XOR may be valuable in modulating lung inflammatory disorders.

The protective effects of the pharmacological inhibitors of xanthine oxidoreductase (XOR), allopurinol and oxypurinol, have implicated XOR in a wide range of human inflammatory diseases and in animal models of inflammatory disease. Injury from inflammatory bowel disease (IBD) and the related Crohn's disease are reduced by XOR inhibitors (1,2); acute pancreatitis (3), lens induced uveitis (4), atherosclerosis (5), chronic heart failure (6,7), essential hypertension (8,9), and diabetic vascular injury (10-12) also exhibit inflammatory components that show improved function by treatment with XOR inhibitors. In the lung, inhibition of XOR is protective in chronic obstructive pulmonary disease airways (COPD) (13), ischemia reperfusion injury (14), acute lung injury (15,16), and other respiratory disorders that exhibit an inflammatory component (17). Significantly, microarray analysis identified XOR as a prominent molecular signature of sepsis induced systemic inflammation in many organs, including the lung (18). These diverse observations suggest that XOR may play a fundamental role in inflammation and inflammatory diseases.

While many cells of the tissue microenvironment, including vascular endothelial cells, tissue epithelial cells, leukocytes, or lymphocytes may be involved in the inflammatory response, mononuclear phagocytes (MNP) of the innate immune system play decisive roles in many inflammatory states. The MNP system includes lineage committed bone marrow precursors, macrophages, their monocyte precursors, and cells that are derived from this lineage including inflammatory and resident monocytes and macrophages found in the lung (19). In the lung, MNP and the MNP chemokine, MCP-1, contribute to the inflammation associated with COPD (20), and MNP and MCP-1 are involved in the pathogenesis of ventilator induced lung injury, acute lung injury, and the adult respiratory distress syndrome (ARDS) (21-26).

The contribution of MNP to inflammatory disorders is complex and not fully understood. Following the induction of inflammation in the lung, monocytes are recruited into the airspace where they rapidly differentiate into macrophages (27-29). Once established in the airspace MNP produce many pro-inflammatory mediators that contribute to inflammation and injury (15,21,24-26,30,31). MNP also respond to diverse activating signals in the inflammatory environment. Classically activated (M1) MNP are defined by their response to the bacterial lipopolysaccharide (LPS) and to the T

helper-1 (Th-1) cytokines IL-1 and/or IFN-γ and may contribute to the ensuing inflammation (32-35). On the other hand, alternatively activated (M2) MNP are defined by their response to interleukins-4 and 13 (IL-4, -13) and may exhibit anti-inflammatory properties that contribute to pathogen clearance and tissue repair (19,36).

Significantly, XOR has been found to comprise an essential component of innate immunity (37), to regulate leukocyte adhesion *in vivo* (38-40), and as a product of the MNP itself contribute to cytokine induced acute lung injury (15). Inhibition of XOR within the newly recruited inflammatory MNP prevented neutrophil (polymorphonuclear phagocytes or PMN) recruitment during adoptive transfer demonstrating a key role for XOR in MNP mediated PMN recruitment (15). Nonetheless, the role played XOR in MNP during inflammation is still poorly understood, and in the present experiments we hypothesized that XOR may regulate basic MNP functions that contribute to inflammation. Data shown here demonstrate that XOR promotes the inflammatory state of MNP in part through effects on chemokine expression, PPARγ sumoylation, and HIF-1α.

EXPERIMENTAL PROCEDURES

Reagents - Most reagents, buffers, substrates, and inhibitors were purchased from Sigma Chemical Company (St. Louis MO, USA). Recombinant human interleukin-1-β (IL-1; 201-LB), interferon-γ (IFN-γ; 285-IF-100), and recombinant human MCP-1 (279-MC) were purchased from R&D Systems (Minneapolis, MN). Sterile normal saline (0.9% NaCl, pH 6.0) was purchased from Baxter Health Care (Deerfield, Ill). Phorbol 12-myristate 13-acetate (PMA) was from Sigma (P8139). *E. coli* lipopolysaccharide (LPS) was from Sigma (L2880). MIG132 (Z-Leu-Leu-Leu-al) was purchased from Sigma (C2211) and prepared in DMSO as indicated by the supplier. Allopurinol (A8003) and oxypurinol (O6881) were from Sigma. Vitamin D_3 (Hoffman-La Roche, Nutley, NJ) was prepared in 100% ethanol. TGFβ1 (240-B) was purchased from R&D Systems . All-trans-retinoic acid (ATRA) was purchased from Sigma. The XOR inhibitor Y-700 was obtained from Dr. Atsushi Fukunari at Mitsubishi Pharma Corporation (Chiba, Japan) and prepared in sodium pyrophosphate as described (41). Rabbit polyclonal anti-PPARγ antibody (H-100; sc-7196), rabbit polyclonal anti-Arginase-1 (H-51; sc-20150), and rabbit polyclonal anti-GAPDH antibody (sc25778) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-HIF-1α antibody (NB100-123) was from Novus Biologicals (Littleton, CO). Rabbit monoclonal anti-SUMO-1 (ab32058) was from Abcam, Inc. (Cambridge, MA). Goat anti mouse IgG-HRP antibody (sc-2005) and goat anti rabbit IgG-HRP antibody (sc-2004) were purchased from Santa Cruz. The Immobilon Chemiluminescent horseradish peroxidase (HRP) detection kit (WBKLS0100) was from Millipore Corporation (Billerica, MA). FITC anti-mouse CD206 (123005) was from BioLegend (San Diego, CA.) and PE anti-mouse CD36 (12-0361) was from eBioscience (San Diego, CA.). Anti-SUMO-1 siRNA (sc-36574) and anti-PIAS-1 siRNA (sc-36220) were from Santa Cruz Biotechnology.

Animal Manipulations - Healthy male Sprague-Dawley rats (250-350g body weight, Sasco, Omaha, NE) were used in all studies. The Th-1 cytokines, 50 ng of recombinant rat IL-1β and 100 ng of IFN-γ in 0.5 mls of normal saline, were delivered intratracheally into the airway of rats previously anesthetized with ketamine/xylazine and lung tissues were harvested 5 or 24 hours later as described (15). Control rats were insufflated with normal saline alone. The use of rats in this study was approved by the University of Colorado Animal Care and Use Committee under the protocol number 21901907(08)1F.

Purification of MNP - Resident alveolar macrophages (RAM) were purified from the lungs of control rats by lavage as described (15). Circulating monocytes (CM) were purified from whole blood of control rats on percoll gradients (15). Alveolar MNP (A-MNP) comprising both the resident AM population and the newly recruited MNP were purified by lavage from lungs of rats insufflated with Th-1 cytokines 5 or 24 hours before. Cells were washed in RPMI medium and were then plated into flasks with RPMI medium and allowed to adhere for 1 hr. Non-adherent cells were removed, and the remaining, adherent cells were washed 4 times with PBS and harvested from the plates by scraping into PBS/EDTA (42). Interstitial MNP present in the lung tissue (I-MNP) were purified from the lungs of rats following removal of the A-MNP and therefore do not contain the resident AM population. Lungs were lavaged to obtain the A-MNP population, perfused blood free, and cells not released by lavage were prepared as described (43). Lung tissues were incubated under moderate agitation for 60 min in RPMI media containing 100 UmL^{-1} Collagenase Type 1 and 50 UmL^{-1} DNAse at 37°C. After incubation, the digested tissue was passed through a sterile sieve to remove tissue fragments, and the cells obtained in the flow through were adhered to flasks as described above. Adherent cells were suspended in

saline to a final concentration of 2 x 10^6 cells ml^{-1}. MNP comprised >95% of the cells recovered from adhesion as determined by microscopic examination of Wright's stained cells and M-CSF induced differentiation from both the A-MNP and the I-MNP preparations (15).

Cell Culture - Human U-937 cells (ATCC number CRL-1593.2), THP-1 cells (ATCC number TIB-202), and HL60 cells (University of Colorado Cancer Center, UCD, Aurora, CO) were grown in RPMI 1640 medium with 10% fetal bovine serum and 1X anti-biotic/anti-mycotic cocktail (GIBCO) at pH 7.4 and 37°C as indicated by the supplier. Cells were seeded in 12 well plates at 1 x 10^6 cells $well^{-1}$. NR8383 normal rat lung alveolar macrophage cell line was obtained from ATCC (CRL-2192) and grown exactly as specified by the supplier. The adherent RAW264.7 mouse monocyte/macrophage cell line was obtained from ATCC (TIB-71) and grown in DMEM with 10% fetal bovine serum and 1X anti-biotic/anti-mycotic cocktail (GIBCO) at pH 7.4 and 37°C as indicated by the supplier.

SDS-PAGE and Western Immunoblot Analysis - Whole cell lysates were either prepared as described previously (15) and the protein concentration determined by using the bicinchoninic acid assay (Sigma) or cells were disrupted in 2X Laemmli Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA; #161-0737), sonicated on ice twice for 15 seconds, and protein concentration determined using Pierce 660nm protein assay (Thermo Scientific, Rockford, IL). Aliquots containing 50 μg of protein were incubated with equal amounts of loading buffer (5% β-mercaptoethanol, 95% Laemmli loading buffer) for 10 mins at 37˚, then boiled for 5 minutes. Samples were then separated by electrophoresis on 7.5% SDS-PAGE or 4% to 15% gradient SDS-PAGE gels for 40 min at 100 V, transferred to PVDF membranes (Whatman, Inc, Albama, USA). Membranes were blocked overnight at 4° in 5% non-fat dried milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20. Membranes were then incubated with antibodies as indicated. Antigen-antibody complexes were detected by reaction with a chemiluminescent HRP Western blotting detection kit according to manufacturer's instruction (Millipore Corporation). Experiments were run in triplicate unless otherwise indicated, and representative immunoblots are shown. Band intensities were quantitated by scanning dosimetry with ImageQuant software.

Chemokine Quantitation – CINC-1, MCP-1, IL-10, and VEGF were determined either by ELISA (Enzyme-Linked ImmunoSorbent Assay) using kits from R&D Systems Inc. (CINC-1, RCN100) or by multiplex bead analysis using the Milliplex Rat Cytokine Kit from Millipore Corp. (Billerica, MA) (MCP-1, IL-10, VEGF). Data were quantitated on a Luminex 100 reader according to the manufacturer's instructions. VEGF was also measured using the rat specific Proteome Profiler Array (# ARY008, R&D Systems, Inc.).

Flow Cytometry - RAW 264.7 cells were grown as indicated, shifted into growth medium containing charcoal stripped and heat inactivated FBS and treated with 10 ng/ml IL-4 one hour after exposure to oxonic acid and/or uric acid at the indicated doses. Cells were harvested 24 hrs after treatment. Treated cells were collected into FACS running buffer (PBS with 2 mM EDTA and 0.5% BSA), washed twice in PBS, and were then fixed in 4% paraformaldehyde for 10 minutes. Cells were stained with anti-CD206 (50ng/10^5 cells) and anti-CD36 (2ng/10^5cells) fluorescently-conjugated primary antibodies in 200 ul running buffer for 20 minutes on ice. Samples were washed and data acquired on a MACSQuant flow cytometer (Miltenyi Biotec).

XOR Quantitation - Total XOR activity was determined at 293nm by quantitation of oxypurinol inhibitable uric acid synthesis using a Beckman-Coulter DU640 spectrometer, 1cm path length, and a molar extinction coefficient for uric acid of 12,600M^{-1} as previously described (44). All XOR activity data show nMol of Uric Acid/min/mg of total protein.

Statistical Analyses - Data are expressed as the mean and standard error of the mean and were assessed for significance using the Student's t-test or ANOVA. A p value of <0.05 was considered significant.

RESULTS

XOR Activity is Increased in A-MNP and I-MNP Following Insufflation of Th-1 Cytokines In vivo - XOR activity is rapidly increased in MNP purified from the bronchoalveolar lavage (BAL) of rats insufflated with the Th-1 cytokines IL-1β and IFNγ (15). In the present work, we found extremely low levels of XOR activity in resident alveolar macrophages (RAM) prior to cytokine insufflation, and undetectable levels in circulating monocytes (CM) from the same rats (Fig. 1A). In contrast, both alveolar MNP (A-MNP) and interstitial MNP (I-MNP) purified from rat lungs 24 hrs after Th-1 cytokine insufflation exhibited high levels of XOR activity (Fig. 1A).

Since MNP XOR activity was increased in both A-MNP and I-MNP populations, and was detected previously as early as 4 hrs following

cytokine insufflation, we hypothesized that XOR activity may be stimulated either by MNP differentiation per se or by exposure of RAM cells to Th-1 cytokines. To determine if XOR activity was increased in RAM cells by Th-1 cytokines, RAM cells were purified from the lungs of native, untreated rats and exposed to Th-1 cytokines *in vitro* for 24 hrs. In addition, XOR activity was measured in Th-1 cytokine stimulated normal rat alveolar macrophage cell line NR8383. The NR8383 cell line was derived from the rat RAM cell population and has been reported to exhibit many characteristics of normal RAM cells (45). We observed approximately 10 fold lower stimulation of XOR activity in RAM cells by the Th-1 cytokines IL-1β, IFNγ, IL-1β/IFNγ, or LPS (Fig. 1B; Table 1) than was observed in A-MNP or I-MNP from cytokine insufflated rats. Furthermore, Th-1 cytokine stimulation of NR8383 also exhibited approximately 10 fold lower stimulation of XOR activity than in A-MNP or I-MNP (Fig. 1C, Table 1).

XOR Activity is Increased by MNP Differentiation In vitro - To determine if XOR activity was increased by differentiation of MNP *per se*, we examined the effect on XOR of differentiation induced in U937 human myeloid cells *in vitro*. Unlike RAM that are fully differentiated macrophages, U937 cells exhibit monocyte-like characteristics and differentiate into macrophages by treatment with PMA or vitaminD3/TGFβ (46). Exposure to all trans retinoic acid (ATRA) promotes differentiation along the granulocytic lineage (47). In addition, since exposure of circulating monocytes to the chemokine MCP-1 is an early event in recruitment to an inflammatory site that may be involved in differentiation along the MNP lineage (48,49), we treated U937 cells with MCP-1 as well. We observed that XOR activity was increased in U937 cells by treatment with PMA, D3/TGFβ, or MCP-1 but not by exposure to ATRA (Fig. 2A). Increased XOR activity in U937 cells following PMA or MCP-1 treatment was both dose and time dependent (Fig. 2B, C). Thus, induction of macrophage differentiation or exposure to the MNP chemokine MCP-1 in human U937 cells increases XOR activity. These data demonstrate that XOR activity is increased in the inflammatory MNP by insufflation of Th-1 cytokines, and that differentiation *per se* and the MNP chemokine MCP-1 may both contribute to the increased XOR activity observed in the inflammatory MNP.

XOR Inhibition Reduces Levels of CINC-1 Secreted by Inflammatory MNP - To determine if XOR contributes to the inflammatory state of MNP we quantitated levels of CINC-1 and MCP-1 in rats insufflated with Th-1 cytokines. We observed that levels of CINC-1 and MCP-1 were elevated in both the serum and cell free lavage five hours after insufflation of Th-1 cytokines compared to levels measured from saline insufflated sham control rats (Fig. 3A). Inflammatory MNP harvested from the lungs of Th-1 cytokine insufflated rats were cultivated *in vitro*, and levels of CINC-1, MCP-1, and IL-10 secreted into the culture medium were measured over the course of 48 hours in the presence or absence of LPS. LPS increased the secretion of CINC-1 and IL-10 from inflammatory MNP over the course of 48 hrs *in vitro* (Fig. 3B), but did not significantly change secretion of MCP-1. CINC-1 secretion was inhibited by co-incubation with allopurinol or oxypurinol (Fig. 3C) even after exposure to LPS (Fig. 3D). No significant effect of allopurinol was observed on secretion of MCP-1 or IL-10. These data demonstrate that XOR contributes to the secretion of the PMN chemokine CINC-1 by MNP, and that XOR inhibition reduces CINC-1 secretion.

Expression of PPARγ in Purified Rat Lung MNP and Cultured Myeloid Lineage Cells - Regulation of MNP differentiation along an inflammatory (M1) pathway or an anti-inflammatory (M2) pathway (19,36) is mediated in part by the Peroxisome Proliferator Activator Receptor-γ (50), with high PPARγ promoting the M2 state. We purified RAM from untreated control rat lungs and both A-MNP and I-MNP from rats treated with Th-1 cytokines 24 hrs before. Western immunoblot of whole cell extracts revealed immunoreactive bands at 50/55 KDa, 62/67 KDa, and 78 KDa that were reactive to the anti-PPARγ antibody (Fig. 4A). Immunoreactive bands at 50/55 KDa and 62/67 KDa were reduced in both A-MNP and I-MNP compared to the RAM. Immunoreactive bands at 50/55 KDa, 62/67 KDa, and 78 KDa were all reduced by inclusion of purified recombinant PPARγ in the western immunoblot (data not shown). A similar pattern of immunoreactivity was obtained for several cultured myeloid lineage cells by reaction to the PPARγ antibody (Fig. 4B). Immunoreactive bands at 50/55 KDa and 62/67 KDa were predominant, with little evidence of a 78 KDa band, in each of the cultured MNP cell lines examined. U937 and THP-1 cells expressed similar levels of the bands at 50 and 55 KDa. Treatment of RAW264.7 cells with IL-4 resulted in marked increase in the immunoreactive band migrating at 62/67 KDa (Fig. 4C), suggesting that it may constitute a modified form of PPARγ induced by IL-4.

The Pattern of PPARγ Immunoreactive Material Reflects Both the Presence of PPARγ Isoforms and PPARγ Sumoylation - PPARγ is encoded

by a single gene in rats, mice, and humans that yields a primary translation product of 505 amino acids and 55 KDa in mass (51). Cleavage of 30 amino acids from the amino terminus of PPARγ2 (55 KDa) produces the PPARγ1 isoform (50 KDa). Our data indicate that U937 and THP-1 cells express both of these isoforms. PPARγ is subject to post-translational sumoylation at two sites in the primary translation product: at K107 in PPARγ2 and K77 in PPARγ1 and at K395 in PPARγ2 and K365 in PPARγ1 (51). While multiple sumoylation events of PPARγ have been described that may contribute to the increased mass of PPARγ (51), a single sumoylation adduct can add 12 KDa to the mass (52). To determine if the immunoreactive bands at 62/67 KDa reflect the presence of sumoylated PPARγ, we transfected RAW264.7 cells with anti-SUMO-1 siRNA or siRNA for the PIAS SUMO-1 conjugating enzyme (52,53). Cells were then treated with either IL-4, PMA and oxypurinol, or PMA and Y-700 (as described in Figure 6). Western immunoblots were performed on WCE 24 hrs after treatment using antibodies to PPARγ (Fig. 5). Scanning dosimetry revealed that exposure to anti-SUMO-1 siRNA reduced the immunoreactive band at 62/67 KDa relative to the scrambled siRNA control by 50%, while exposure to anti-PIAS siRNA reduced the same band by 75% (Fig. 5A, C). Furthermore, exposure of RAW264.7 cells to anti-SUMO-1 siRNA followed by treatment with IL-4 decreased both the 62/67 KDa immunoreactive band and decreased the free SUMO-1 band at 12 KDa (Fig. 5B). These data indicate that the anti-PPARγ immunoreactive band at 62/67 KDa contains a SUMO-1 adduct.

XOR Inhibitors Increase Levels of SUMO-PPARγ in Cultured Myeloid Lineage Cell Lines - To determine the effect of XOR inhibition on PPARγ or SUMO-PPARγ (S-PPARγ) in myeloid lineage cell lines, we exposed human U937, THP-1, and HL60 cells and the mouse RAW264.7 MNP cell line to the XOR inhibitors Y-700 or oxypurinol. After 1 hr cells were exposed to 30 nM PMA and 24 or 48 hrs later cell lysates were prepared. While western immunoblot analysis showed increases in PPARγ by PMA alone (Fig. 4B), we observed very little effect of PMA alone on SUMO-PPARγ in U937, THP-1, HL60, or RAW264.7 cells (Fig. 6). However, 24 hrs after treatment U937, THP-1, HL60, and RAW264.7 cells showed marked increase in levels of SUMO-PPARγ by both Y-700 and oxypurinol compared to control cells exposed to PMA alone. The increase in SUMO-PPARγ levels by XOR inhibitors occurred even in the absence of PMA stimulation. The increase in SUMO-PPARγ levels observed at 24 hrs in the presence of XOR inhibitors was frequently diminished by 48 hrs, although the basis for this effect is not presently understood.

Both Ectopic Overexpression of XOR cDNA and Uric Acid Supplementation Reduce SUMO-PPARγ in Stimulated Myeloid Lineage Cells - Further evidence that XOR was involved in modulating 62/67 KDa SUMO-PPARγ was obtained in three ways. First, to determine if XOR overexpression would block IL-4 induced SUMO-PPARγ, RAW264.7 cells were transfected with pCMV-Myc-XOR at various doses of input DNA. pCMV-Myc-XOR is a cDNA expression vector containing a functional XOR cDNA that is expressed under the control of the CMV constitutive promoter and produces ectopic overexpression of XOR (54). We observed that ectopic overexpression of XOR dose dependently reduced levels of SUMO-PPARγ in IL-4 treated RAW264.7 cells (Fig. 7A). Next, RAW264.7 cells were treated with IL-4 in the presence or absence of uric acid, the immediate product of XOR catalysis. Uric acid dose dependently reduced IL-4 stimulated SUMO-PPARγ at 62/67 KDa (Fig. 7B). Finally, U937 cells were exposed to PMA/Y-700 in the presence or absence of uric acid. Again, uric acid dose dependently reduced 62/67 KDa SUMO-PPARγ induced in U937 cells by PMA/Y-700 (Fig. 7C).

Arginase-1 Levels Are Modulated by Uric Acid, Oxonic Acid, and Anti-Sumo-1 or Anti-PIAS siRNA in RAW264.7 Cells - Induction of Arginase-1 by IL-4 may be specifically mediated by sumoylation of PPARγ (36,55), and modulation of IL-4 induced PPARγ sumoylation by uric acid (shown here) specifically predicts that uric acid will inhibit IL-4 induced Arginase-1. This prediction was tested in IL-4 treated RAW264.7 cells. IL-4 was found to increase Arginase-1 levels in a dose dependent fashion to 20 ng/ml, the highest dose tested (Fig. 8A). Transfection of RAW264.7 cells with anti-SUMO-1 or anti-PIAS siRNA reduced both the levels of 62/67 KDa Sumo-PPARγ and Arginase-1 (Fig. 8B), demonstrating that IL-4 induced Arginase-1 was dependent on sumoylation. To determine if XOR overexpression would also block IL-4 induced Arginase-1, RAW264.7 cells were transfected with pCMV-Myc-XOR at various doses of input DNA and treated with IL-4 for 24 hrs. Ectopic overexpression of XOR cDNA dose-dependently reduced Arginase-1 levels in IL-4 cells (Fig.8C). The effect of uric acid on IL-4 induced Arginase-1 was tested in two ways. In the first, the enzyme uricase that degrades uric acid to allantoin was inhibited with oxonic acid which then acts to elevate uric acid. Oxonic acid dose dependently inhibited IL-4 induced Arginase-1 (Fig. 8D). In the second, uric acid was added exogenously to IL-4 treated RAW264.7 cells in the presence of 50

uM oxonic acid to block uric acid degradation. Uric acid dose dependently decreased levels of IL-4 induced Arginase-1, an effect that was most pronounced at the higher doses of uric acid (Fig. 8E). These data demonstrate that ectopic overexpression of XOR or uric acid reduced levels of Arginase-1 induced by IL-4 treatment through a sumoylation dependent process.

The M2 markers CD36 and CD206 Are Also Modulated by Uric Acid and Oxonic Acid – CD36 and CD206 are additional markers of the alternative (M2) state of macrophage activation (36). RAW264.7 cells were exposed to oxonic acid or to uric acid in the presence of oxonic acid and were then treated with IL-4. Cells were analyzed by FACS 24 hrs later. As observed for levels of Arginase-1, both oxonic acid and uric acid dose dependently reduced levels of CD36 and CD206 detected by FACS (Fig. 8F).

HIF-1α is Increased by XOR Inhibitors in Inflammatory MNP and in Cultured Myeloid Lineage Cells - The Hypoxia Inducible Factor -1α (HIF-1α) is a transcription factor mediating both glycolytic and hypoxic effects in leukocytes. HIF-1α is subject to sumoylation that may affect both its stability and its transcriptional activity (56,57). To determine if XOR also modulated levels of HIF-1α in inflammatory MNP, we examined levels of HIF-1α in I-MNP purified from Th-1 cytokine insufflated lungs by western immunoblot. HIF-1α was undetectable in I-MNP immediately after isolation. However, HIF-1α levels in I-MNP showed both time and dose dependent accumulation when cells were treated *in vitro* with the proteosome inhibitor MIG132 (Fig. 9A). MIG132 dependent accumulation of HIF-1α in I-MNP occurred during both normoxic (21% O_2) and hypoxic (1% O_2) culture *in vitro* (Fig. 9B). Concurrent treatment of I-MNP with MIG132 and three different inhibitors of XOR (allopurinol, oxypurinol, or Y-700) resulted in marked increase in HIF-1α levels over MIG132 treatment alone for cells cultured in either normoxia or hypoxia (Fig. 9B). These data suggest that XOR may exert a suppressive or modulating effect on levels of HIF-1α in I-MNP purified from Th-1 cytokine insufflated lungs. Furthermore, exposure of I-MNP to Th-1 cytokines IL-1β, IFNγ, or LPS showed increased levels of HIF-1α in the presence of Y-700 that did not arise in the absence of Y-700 (Fig. 9C).

To determine if XOR exerted a similar modulating effect on HIF-1α in PMA differentiated U937 cells, U937 cells were differentiated with PMA and after 48 hrs treated with MIG132 and Th-1 cytokines in the presence or absence of Y-700. We observed that HIF-1α was nearly undetectable in differentiated and normoxically grown U937 cells treated with Th-1 cytokines in the absence of Y-700, while concurrent treatment with Y-700 resulted in increased levels of HIF-1α (Fig. 9D). Growth of the same cells in hypoxia resulted in significantly higher levels of HIF-1α for cells grown in the presence of Y-700 (Fig. 9D). Thus, concurrent treatment of PMA differentiated U937 cells with Th-1 cytokines in the presence of the XOR inhibitor Y-700 resulted in increased levels of HIF-1α that were not observed in the absence of Y-700. These data suggest that XOR may also exert a modulating effect on HIF-1α levels in PMA differentiated and Th-1 cytokine treated U937 cells as well.

Vascular endothelial growth factor (VEGF) is an important target of HIF-1 regulation. We observed that I-MNP showed marked accumulation of VEGF when cultured *in vitro* in normoxia over the course of 48 hrs (Fig. 9E). Cells exposed to MIG132 in the presence or absence of XOR inhibitors for either 6 or 24 hrs showed marked decrease in VEGF secretion. Since the profound suppression of VEGF by MIG132 may have obscured the effects of XOR inhibitors, cells were treated with Y-700 in the absence of MIG132 and secreted VEGF was measured in the cell free culture medium. Y-700 alone markedly suppressed secretion of VEGF.

DISCUSSION

XOR plays a central role in the function of innate immunity and inflammation, and it is an important mediator of many inflammatory diseases in humans (37,58). XOR is upregulated in many inflammatory models (15,18) where it shows particularly high levels of activity in the inflammatory MNP. Inhibition of XOR specifically reduces PMN recruitment to an inflammatory site *in vivo* (15,59), and inhibition of XOR specifically in the MNP, but not the PMN, modulates subsequent PMN recruitment (15,60). Thus, XOR contributes to the development of inflammation as a product of the newly recruited inflammatory MNP. This observation is important because the MNP themselves contribute to injury of the lung epithelium and XOR inhibitors may provide critical support of the inflamed lung (15,21,22). Despite the vast amount of literature published on the role of XOR in inflammatory disease and the broadly protective role afforded by XOR inhibitors, surprisingly little is known about the mechanisms by which XOR contributes to inflammation. Data shown in the present report demonstrate that XOR is both activated by MNP differentiation and contributes to the inflammatory state of the MNP. XOR was found

to promote the inflammatory state of the MNP by contributing to inflammatory chemokine secretion, regulation of PPARγ sumoylation, and HIF-1α stability.

XOR activity was markedly elevated in both A-MNP and I-MNP following insufflation of Th-1 cytokines in contrast to the very low activity observed in CM or RAM from untreated control rats. While XOR activity was increased by treatment of RAM or NR8383 normal rat macrophages with Th-1 cytokines, XOR activity failed to reach the levels observed in inflammatory MNP isolated from Th-1 cytokine insufflated rats. Thus, exposure of RAM to Th-1 cytokines alone could not account for the level of XOR activity observed in the inflammatory MNP. Furthermore, XOR activity was increased by agents promoting macrophage, but not granulocyte, differentiation in U937 cells. Treatment with vitaminD3/TGFβ or PMA increased XOR activity to levels approaching those observed in MNP from Th-1 cytokine insufflated rats. XOR activity was also increased by treatment with MCP-1, and this is important because MCP-1 is a central chemokine mediating the recruitment of monocytes to an inflammatory site including the lung (26,61) and may contribute to the inflammatory state of MNP (19,62,63). These data demonstrate XOR activity is markedly increased by agents promoting MNP differentiation along an inflammatory pathway.

Evidence that XOR contributes to the inflammatory state of MNP was derived in several ways. Insufflation of Th-1 cytokines in rats produced a rapid rise in both serum and lavage chemokines for PMN (CINC-1) and MNP (MCP-1). Both CINC-1 and MCP-1 were secreted by the MNP to a much greater extent than by the PMN isolated from the same rats (data not shown), and chemokine secretion was sustained after placing the MNP in culture. Significantly, CINC-1 levels were markedly elevated by treatment of the cultured MNP with LPS, and both uninduced and LPS induced CINC-1 was significantly reduced by co-incubation with XOR inhibitors. These data are consistent with and extend both our previous observations demonstrating that adoptive transfer of XOR inhibited MNP reduces PMN influx into the rat lung (15) and with data obtained in mice demonstrating the key role played by MNP in recruitment of PMN to the lung during inflammation (64-66). Consistent with the role of XOR in promoting the inflammatory state of MNP, we observed that the anti-inflammatory cytokine IL-10 was not modulated by inhibition of XOR.

Further evidence that XOR contributes to the inflammatory state of MNP was derived by analysis of PPARγ. PPARγ is a dual-function, ligand activated, member of the nuclear hormone receptor superfamily of transcription factors. Transcriptional activation by PPARγ requires both ligand binding and interaction with the retinoid X receptor (RXR). While transcriptional repression of inflammatory gene expression by PPARγ is well recognized, it is not yet fully understood (67,68). PPARγ expressed in the MNP plays an important role in regulating lung inflammation (69,70). RAM express high levels of PPARγ and this is down regulated by Th-1 cytokines (69). Down regulation of MNP PPARγ is associated with enhanced lung inflammation and injury (71-73), whereas activation or induction of MNP PPARγ is associated with an anti-inflammatory state characterized by reduced leukocyte infiltration, reduced inflammatory cytokine expression, and enhanced expression of anti-inflammatory cytokines (50,74-76). We observed an unexpectedly complex pattern of immunoreactive material on PPARγ western blots of freshly isolated rat MNP and from several cultured human and rodent MNP cells lines including U937, THP-1, HL-60, and RAW264.7 cells. Freshly isolated MNP from the rat exhibited immunoreactive material at 78 KDa, 62/67 KDa, and 50/55 KDa using an antibody to PPARγ, whereas we observed immunoreactive material predominantly at 62/67 KDa and 50/55 KDa in the cultured cell lines. In rats, mice, and humans PPARγ is encoded by a single gene that yields a primary translation product of 505 amino acids and 55 KDa in mass (51), and cleavage of 30 amino acids from the amino terminus of PPARγ2 (55 KDa) produces the PPARγ1 isoform (50 KDa). Treatment of RAW264.7 cells with IL-4 resulted in marked increase in immunoreactive material at 62/67 KDa suggesting that this higher molecular weight material reactive to the PPARγ antibody reflected a modified form of the primary translation product.

Sumoylation is one of a limited number of posttranslational modifications that would increase apparent molecular weight of PPARγ by 11 to 12 KDa (51). Sumoylation of PPARγ has been found to modulate its activity as a transcription factor (77,78), and sumoylation of PPARγ has been found specifically to mediate repression of pro-inflammatory genes (79-81). We used siRNA technology to determine if immunoreactive material at 62/67 KDa contained a SUMO adduct. Transient transfection of RAW264.7 cells with antisense RNA to both the PIAS-1 conjugating enzyme and SUMO-1 reduced immunoreactive material at 62/67 KDa that was induced in several ways, while antisense RNA to SUMO-2 did not affect this band (not shown). Levels of free SUMO-1 were likewise reduced by transfection with antisense RNA to SUMO-1. These

data indicate that the immunoreactive material at 62/67 KDa obtained by reaction to antibody against PPARγ also contains a SUMO-1 adduct. We infer, therefore, that freshly isolated RAM from the rat express high levels of SUMO-PPARγ at 62/67KDa, and that the inflammatory MNP present in the lung following induction of inflammation express very low levels of SUMO-PPARγ. These observations support the argument that SUMO-PPARγ expressed in the RAM modulates the inflammatory state of these cells, whereas the newly recruited inflammatory MNP express very little SUMO-PPARγ thereby contributing to their inflammatory state (79-81).

Evidence that XOR was involved in PPARγ sumoylation was obtained in three ways. First, inhibitors of XOR, in the presence or absence of PMA, increased levels of SUMO-PPARγ at 62/67 KDa in U937, HL-60, THP-1, and RAW264.7 cells. The increase in 62/67 KDa immunoreactive material was obtained with both Y-700 and oxypurinol, and similar results were obtained with allopurinol as well (data not shown). The consistent effect of the different inhibitors is important because the inhibitors have either different ROS scavenging properties (allopurinol and oxypurinol) that are distinct from inhibition of XOR *per se* and potentially confounding, or they have no detectable ROS scavenging properties (Y-700) (41). Second, ectopic overexpression of XOR cDNA dose dependently reduced levels of IL-4 induced 62/67 KDa SUMO-PPARγ. Third, uric acid, the principle catalytic product of XOR dose dependently reduced levels 62/67 KDa SUMO-PPARγ that were produced either by IL-4 stimulation of RAW264.7 cells or by treatment of U937 cells with Y-700 and PMA. In aggregate, these data demonstrate that XOR modulates levels of SUMO-PPARγ immunoreactive material at 62/67 KDa, and identify uric acid as an important component of this effect.

Modulation of PPARγ sumoylation explicitly predicts an inhibitory effect on Arginase-1 which is a canonical marker of alternatively activated (M2) macrophages that is specifically induced by IL-4 (36). We observed IL-4 dose dependent increases in Arginase-1 levels that were reduced by antisense RNA to both SUMO-1 and PIAS-1 using RAW264.7 cells, consistent with reports demonstrating the requirement of SUMO for PPARγ activation and PPARγ activation for induction of Arginase-1 (36,55,68,77). Ectopic overexpression of XOR cDNA dose-dependently blocked IL-4 induced Arginase-1. Furthermore, both uric acid and oxonic acid, a uric acid elevating reagent, dose dependently reduced Arginase-1 levels that were induced with IL-4. Furthermore, we also observed decreases in the M2 markers CD36 and CD206 by both oxonic acid and uric acid. These data confirm the involvement of XOR and uric acid in modulating markers of M2 polarized RAW264.7 cells.

The signaling pathways through which XOR activity modulates PPARγ sumoylation are unknown. XOR is an efficient source of both reactive oxygen and reactive nitrogen species, and both oxidative and nitrosative stress can regulate sumoylation (82-84). However, data shown here demonstrate that uric acid is at least one component of XOR biochemistry that modulates PPARγ sumoylation. XOR derived uric acid may exert proinflammatory effects at several levels. On the one hand, uric acid can activate the inflammasome to promote inflammation (59,85), promote T cell activation (86), activate dendritic cells (87), and as shown here modulate PPARγ sumoylation. Importantly, as shown here, these effects can arise at physiological levels of uric acid that are well below the point of crystallization and may therefore be distinct from the response to monosodium urate crystals (88). Uric acid can also serve as a scavenger of ROS, and this too may underlie its effects on PPARγ sumoylation and inflammation (37).

HIF-1α plays an essential role in the development of inflammation as a product of the MNP (89-91), and MNP HIF-1α is required for the expression of inflammatory cytokines in an LPS model of sepsis (90). Recent reports demonstrate that HIF-1α is also regulated by sumoylation. SUMO conjugation to HIF-1α both stabilizes the protein and reduces its activation function in transcription (56,57,92). Evidence that XOR is involved in regulating levels of HIF-1α protein was obtained using both I-MNP freshly isolated from Th-1 cytokine insufflated rat lungs and U937 cells. In the presence of the proteosome inhibitor MIG132, XOR inhibitors increased both normoxic and hypoxic levels of HIF-1α and increased the levels of HIF-1α induced by treatment with LPS, IL-1, or IFNγ. Again, the use of three different inhibitors of XOR suggests that the effect on HIF-1α protein levels is the result of XOR inhibition *per se*. We infer that in the uninhibited MNP XOR activity modulates levels of HIF-1α protein. Experiments are currently underway to determine whether the effect of XOR on HIF-1α protein levels reflects the effect of XOR on sumoylation of HIF-1α. However, consistent with this observation, we observed marked decrease in VEGF, a principal target of HIF-1 regulation, in the presence of MIG132 at 6 and 24 hrs after exposure, when HIF-1α levels were most increased. Furthermore, inhibition of XOR in the absence of

MIG132 alone reduced VEGF secretion from these cells.

Repair and rescue of the injured alveolar epithelium is a critical therapeutic objective for management of lung inflammatory disorders (93). While the relatively poor understanding of repair and injury has limited current treatment strategies, MNP are of considerable interest as therapeutic targets because they contribute to both injury and repair. During inflammation classically activated (M1) inflammatory MNP contribute to lung injury and PMN recruitment. On the other hand, alternatively activated (M2) MNP exhibit anti-inflammatory properties that may contribute to tissue repair. Experiments shown here demonstrate that XOR promotes the inflammatory state of MNP through effects on chemokine expression, PPARγ sumoylation, and HIF-1α, and that inhibitors of XOR reduce the inflammatory state. Thus, strategies that inhibit XOR may be important considerations for modulating lung inflammatory disorders.

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The abbreviations used are: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; VLI, ventilator induced lung injury; COPD, chronic obstructive pulmonary disease; MNP, mononuclear phagocyte; AM, alveolar macrophage; RAM, Resident Alveolar Macrophage; CM, Circulating Monocyte; A-MNP, alveolar MNP; I-MNP, Interstitial MNP; PMN, polymorphonuclear phagocyte or neutrophil; IL-1, interleukin-1; IFNγ, interferon-γ; PMA, phorbol 12-myristate 12-acetate; FACS, Fluorescent Activated Cell Sorting; WCE, Whole Cell Extract; XOR, xanthine oxidoreductase.

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FIGURE LEGENDS

Figure 1. **Inflammatory MNP express high levels of XOR activity.** A, Circulating monocytes (CM and resident alveolar macrophages (RAM) were purified from control untreated rats while alveolar MNP (A-MNP) and interstitial MNP (I-MNP) were purified from rat lungs 24 hrs after insufflation of the Th-1 cytokines, IL-1β and INFγ. XOR activity was determined from six preparations of each. Data show the mean and standard

deviation of XOR activity. Oxypurinol (150 uM) was included in separate assays to confirm the specificity of uric acid formation. B, RAM were purified from control untreated rats and placed into 12 well culture plates. After one hour cells were washed and either harvested immediately (Control, 0 hr) or exposed to vehicle, IL-1β (10 ng/ml), IFNγ (20 ng/ml), or LPS (1.0 ug/ml) in heat inactivated medium. Cells were harvested 24 hrs later and XOR activity determined. Data show the mean and standard deviation for six determinations. C, NR8383 rat AM cell line was cultured in 12 well plates at 1.0 x 10^6 cells/well and treated with vehicle, IL-1β (10 ng/ml), IFNγ (20 ng/ml), or LPS (1.0 ug/ml) as in panel B. Cells were harvested 24 hrs later and XOR activity determined. Data show the mean and standard deviation for six determinations.

Figure 2. **XOR activity is increased in U937 cells by differentiation along the macrophage pathway and by the MCP-1 chemokine.** A, U937 cells were cultured in 12 well plates at 1.0 x 10^6 cells/well and treated with PMA (30 nM), Vitamin D_3 (1×10^{-7} M)/TGFβ1 (1 ng/ml), ATRA (1 x 10^{-6}M), or MCP-1 (10 ng/ml) as shown. Cells were harvested and XOR activity determined 48 hrs after addition of each differentiation agent. Data show the mean and standard deviation of six determinations. B, U937 cells were grown as in panel A and treated with increasing dose of PMA. Cells were harvested and XOR activity determined 48hrs later. Data show the mean and standard deviation of six determinations. In addition, U937 cells were grown as in panel A and treated with PMA (30 nM) for the indicated times. Cells were harvested and XOR activity determined on freshly generated WCE. Data show the mean and standard deviation of six determinations. C, U937 cells were grown as in panel A and treated with increasing dose of MCP-1. Cells were harvested and XOR activity determined 48 hrs later. Data show the mean and standard deviation of six determinations. In addition, U937 cells were grown as in panel A and treated with MCP-1 (10 ng/ml) for the indicated times. Cells were harvested and XOR activity determined on freshly generated WCE. Data show the mean and standard deviation of six determinations.

Figure 3. **Allopurinol reduces secretion of CINC-1 but not MCP-1 or IL-10 from inflammatory MNP.** A, Rats were insufflated with either saline (Sham) or Th-1 cytokines (IL-1/IFN) and five hours later the cell free lung lavage and serum were collected and levels of CINC-1 and MCP-1 were measured by ELISA. *** $p<0.02$, ** $p<0.05$ using Students t-Test with n = six rats in each group. B, I-MNP were purified from rats insufflated with Th-1 cytokines 24 hrs before and cultured *in vitro* in the presence or absence of LPS at 1.0 ug/ml. Levels of secreted CINC-1, MCP-1, and IL-10 were measured over a period of 48 hrs from the time LPS added to the cells. C, D, I-MNP from Th-1 cytokine insufflated rats were purified and were either untreated (C) or treated with LPS (D) as in Panel B, but were incubated in the presence or absence of allopurinol or oxypurinol (150 uM). Levels of CINC-1, MCP-1, and IL-10 were determined 24 hrs later. ** $p<0.05$ using Students t-Test with n = three rats in each group.

Figure 4. **PPARγ is expressed in rat lung MNP and cultured myeloid lineage cells.** A, Western immunoblot of PPARγ from RAM of untreated control rats and from A-MNP and I-MNP purified from lungs of rats treated with Th-1 cytokines 24 hrs before. B, Western immunoblot of PPARγ from cultured myeloid lineage cells. Cells were either grown in the absence of PMA (30 uM) or treated with PMA for 24 or 48 hrs. C, Western immunoblot of PPARγ from RAW264.7 cells that were either treated with recombinant IL-4 (10 ng/ml) or saline for 24 hrs.

Figure 5. **Anti-PPARγ immunoreactive material at 62/67 KDa contains a SUMO-1 adduct.** A, RAW264.7 cells were plated at 0.5 x 10^6 cells/well on 12 well plates and the next day transfected with scrambled siRNA, anti-SUMO-1 siRNA, or siRNA for the PIAS SUMO-1 conjugating enzyme. After 24 hrs, cells were treated with either IL-4 (10 ng/ml), PMA and oxypurinol, or PMA and Y-700 (50 nM) as described in Figure 6. Western immunoblots were performed on WCE 24 hrs after treatment using antibodies to PPARγ. B, U937 cells were plated at 0.5 x 10^6 cells/well on 12 well plates and the next day were either left untreated (control no siRNA) or transfected with anti-SUMO-1 siRNA(siRNA SUMO-1), or nonspecific DNA (siRNA reagent control). After 24 hrs, cells were treated with PMA and oxypurinol and western immunoblots were performed on WCE 24 hrs after treatment using antibodies to PPARγ or SUMO-1. C, Bands at 62/67 KDa were quantitated by scanning dosimetry and then averaged over all treatments (IL-4, Oxy/PMA, and Y-700/PMA). Data were normalized to the untreated control samples and shown as % of the control band. Data show the normalized average and standard deviation for six determinations in each treatment group. ** $p< 0.05$, *** $p<0.01$ by Students t-test comparing Anti-SUMO-1 or Anti-PIAS to Scramble siRNA transfection.

Figure 6. **XOR inhibitors increase levels of SUMO-PPARγ in cultured myeloid lineage cell lines.** U937, THP-1, HL-60, and RAW264.7 cells were plated in 12 well plates at 1 x 10^6 cells/well and grown under normoxic conditions for one hour. Subsequently, cells were treated with Y-700 (50 nM) or oxypurinol (150 uM) for one hour and PMA (30 nM) was then added. Whole cell lysates were prepared 24 hrs or 48 hrs after exposure to PMA and western immunoblots were run sequentially with antibody to PPARγ and GAPDH. Representative blots are shown of three independent blots for each experiment.

Figure 7. **Ectopic overexpression of XOR or uric acid supplementation both reduce levels of SUMO-PPARγ in cultured myeloid lineage cell lines.** A, RAW264.7 cells were plated in 6 well plates at 0.5 x 10^6 cells/well and the next day were transfected with the indicated doses of pCMV-Myc-XOR. After 24 hrs, cells were shifted into standard medium with charcoal stripped and heat inactivated FBS and treated with recombinant mouse IL-4 (10 ng/ml). Whole cell lysates were prepared 24 hrs after exposure to IL-4 and western immunoblots were run sequentially with antibody to PPARγ and GAPDH. Representative blots are shown of three independent blots for each experiment. B, RAW264.7 cells were plated in 6 well plates at 1.0 x 10^6 cells/well. The next day cells were shifted into standard medium with charcoal stripped and heat inactivated FBS and treated with uric acid at the indicated doses in the presence of 50 mM oxonic acid. After one hour cells were treated with 10 ng/ml of IL-4. Cells were harvested 24 hours later, lysates prepared, and western immunoblots performed sequentially against PPARγ and GAPDH. C, U9376 cells were plated at a density of 1 x10^6 cells/well in six well plates. Cells were then treated with uric acid at the indicated doses and after one hour were exposed to PMA and Y-700 exactly as described in Figure 6. Cell lysates were prepared after 24 hrs and western immunoblots were performed sequentially against PPAR γ and GAPDH.

Figure 8. **Levels of the M2 markers Arginase-1, CD36, and CD206 are reduced by uric acid or oxonic acid.** A, RAW264.7 cells were plated at 0.5 x10^6 cells/well and the next day treated with recombinant mouse IL-4 from 0 to 20 ng/ml. WCE were prepared 24 hrs later and western blots performed with antisera to Arginase-1. B, RAW264.6 cells were plated as in panel A and the next day were either left untreated or were transfected with scrambled siRNA, anti-sumo-1 siRNA, or anti-PIAS siRNA. After 24 hrs cells were treated with IL-4 and 24 hrs later WCE prepared. Western immunoblots were performed using antisera to PPARγ, Arginase-1, or GAPDH. C, RAW264.6 cells were plated as in panel A and the next day were either left untreated or were transfected with pCMV-Myc vector alone or with pCMV-Myc-XOR in the presence of pCMV-Myc to fix the net input DNA at 3.0ug. After 24 hrs cells were treated with IL-4 and 24 hrs later WCE prepared. Western immunoblots were performed using antisera to Arginase-1 or GAPDH. Arginase-1 bands were quantitated by scanning dosimetry and normalized to the untransfected cells treated with IL-4. D, RAW264.7 cells were plated as in panel A and the next day treated with oxonic acid at the indicated doses. After one hour cells were treated with IL-4 and WCE prepared 24 hrs later. Western immunoblots were performed using antisera to Arginase-1 and GAPDH. E, RAW264.7 cells were plated as in panel A and the next day treated with uric acid at the indicated doses in the presence of oxonic acid (50 uM). After one hour cells were treated with IL-4 and WCE prepared 24 hrs later. Western immunoblots were performed using antisera to Arginase-1 and GAPDH. F, RAW264.7 cells were plated as in Panel A, shifted into charcoal stripped/heat inactivated medium, and treated with the indicated doses of oxonic acid alone or in combination with uric acid at the indicated doses. One hour later cells were treated with 10 ng/ml IL-4. Cells were harvested 24 hrs after treatment and analyzed by flow cytometry using antibodies to CD36 (PE conjugated) and CD206 (FITC conjugated).

Figure 9. **Inhibition of XOR activity increases levels of HIF-1α protein in rat inflammatory MNP and in PMA differentiated U937 cells.** A, I-MNP were purified from rat lungs 24 hrs following insufflation of Th-1 cytokines and were plated in 12 well plates at 1.0 x 10^6 cells/well and grown under normoxic conditions. Nonadherent cells removed by washing after one hour and cells were treated with the indicated doses of MIG132. Cells were harvested after 6 hrs, whole cell lysates prepared, and western immunoblots run with antibody to HIF-1α. In addition, washed, adherent cells were exposed to MIG132 (50 uM) for the indicated times, and western immunoblots were run on whole cell lysates and probed with antibody to HIF-1α. Blots were subsequently stripped and re-probed with antibody to GAPDH to control for protein loading. B, I-MNP were purified and plated as in panel A. Plates were placed in either normoxic (21% O_2, 5% CO_2) or hypoxic culture (1% O_2, 5% CO_2, 94% N). After one hour cells were treated with the XOR inhibitors allopurinol

(150uM), oxypurinol (150uM), or Y-700 (50 nM). One hour later cells were treated with MIG132 (50uM) and grown for 6 hrs. Whole cell lysates were then prepared and western immunoblots run with independent duplicate samples as indicated. Blots were first probed with antibody to HIF-1α and subsequently with antibody to GAPDH. Bands from western immunoblots were quantitated by scanning dosimetry and normalized to the signal obtained from the GAPDH blots. Data show the mean and standard deviation of duplicate samples. C, I-MNP were purified, plated, and grown in normoxia in the presence of MIG132 as in panel A. Cells were exposed to the XOR inhibitor Y-700 (50 nM) for one hour and subsequently treated with IL-1β (10 ng/ml), IFNγ (20 ng/ml), or LPS (1.0 ug/ml). Whole cell lysates were prepared and western immunoblots run after 24 hrs exposure to cytokines. Blots were run on triplicate samples and representative blots are shown. Bands from western immunoblots were quantitated by scanning dosimetry and normalized to the signal obtained from the GAPDH blots. Data show the mean and standard deviation of triplicate blots. D, U937 cells were plated in 12 well plates at 1 x 10^6 cells/well and treated with PMA (30 nM) for 48 hrs. Cells were then washed, the medium replaced, and cells were grown under normoxic or hypoxic conditions for one hour in the presence of 50 uM MIG132. Subsequently, cells were treated with Y-700 (50 nM) for one hour and cytokines added as above. Whole cell lysates were prepared after 24 hrs exposure to cytokine/MIG132 and western immunoblots run sequentially with antibody to HIF-1α and GAPDH. Representative blots are shown of three independent blots for each experiment. Bands from western immunoblots were quantitated by scanning dosimetry and normalized to the signal obtained from the GAPDH blots. Data show the mean and standard deviation of triplicate blots. E, I-MNP were purified and plated in normoxia as in panel B. Levels of VEGF were quantitated in the cell free supernatant over a period of 48 hrs in culture. In addition, cells were treated independently with MIG132 in the absence or presence of allopurinol, oxypurinol, or Y-700 as in panel B, and levels of VEGF were measured in the cell free supernatant 6 or 24 hrs after treatment. Data show the mean and standard deviation of three independent experiments. F, I-MNP were plated as above in the presence or absence of Y-700 (1.0 mM) and in the absence of MIG132. After 24 hrs cell free supernatants were collected and analyzed with the rat specific Proteome Profiler. Experiments were performed in quadruplicate and each cytokine was analyzed from two spots on each filter (boxed for VEGF), thus all data reflect 8 independent determinations for VEGF alone. Spots were quantitated as described by the supplier using an R&D transmission mode scanner and image analysis software from R & D. Data show the mean and standard deviation of light transmission signals (arbitrary units) from 8 spots for VEGF only and both Control and Y-700 groups and were normalized first to the mean positive control spots (A1, A2, A19, A20, D1, D2) and subsequently to the signal obtained from the Control samples which was thereby set at 1.00.

FIGURES

Figure 1

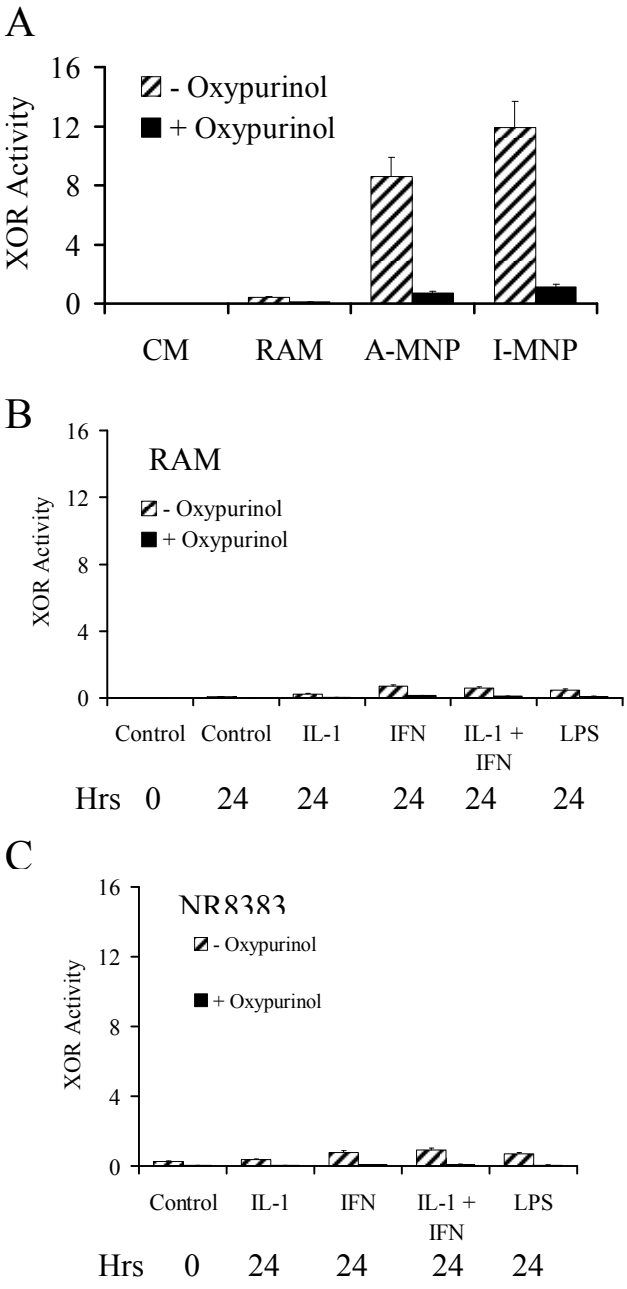


Figure 2

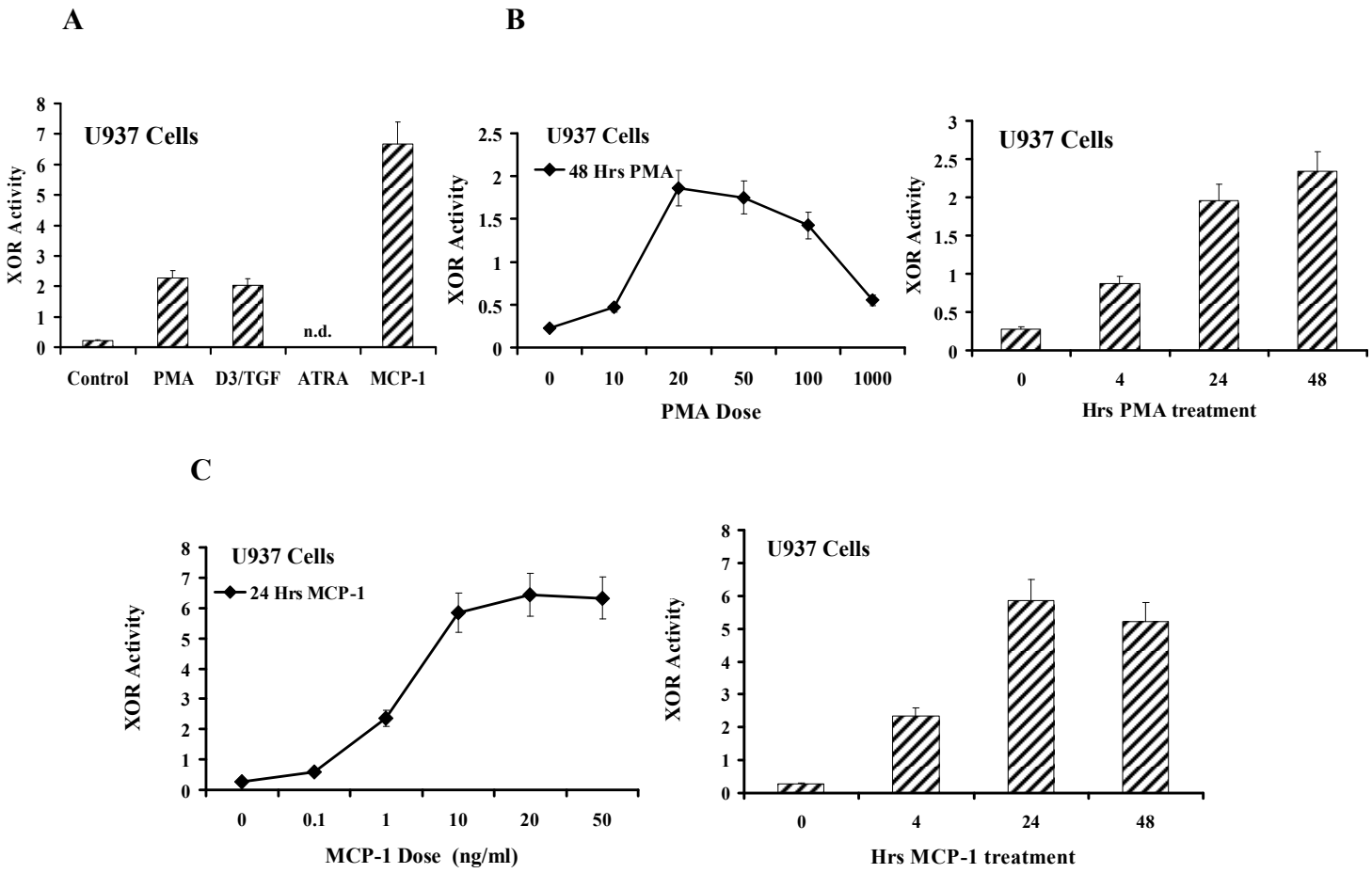

A
B
C
U937 Cells
XOR Activity
Control
PMA
D3/TGF
ATRA
MCP-1
n.d.
48 Hrs PMA
PMA Dose
Hrs PMA treatment
24 Hrs MCP-1
MCP-1 Dose (ng/ml)
Hrs MCP-1 treatment

Figure 3

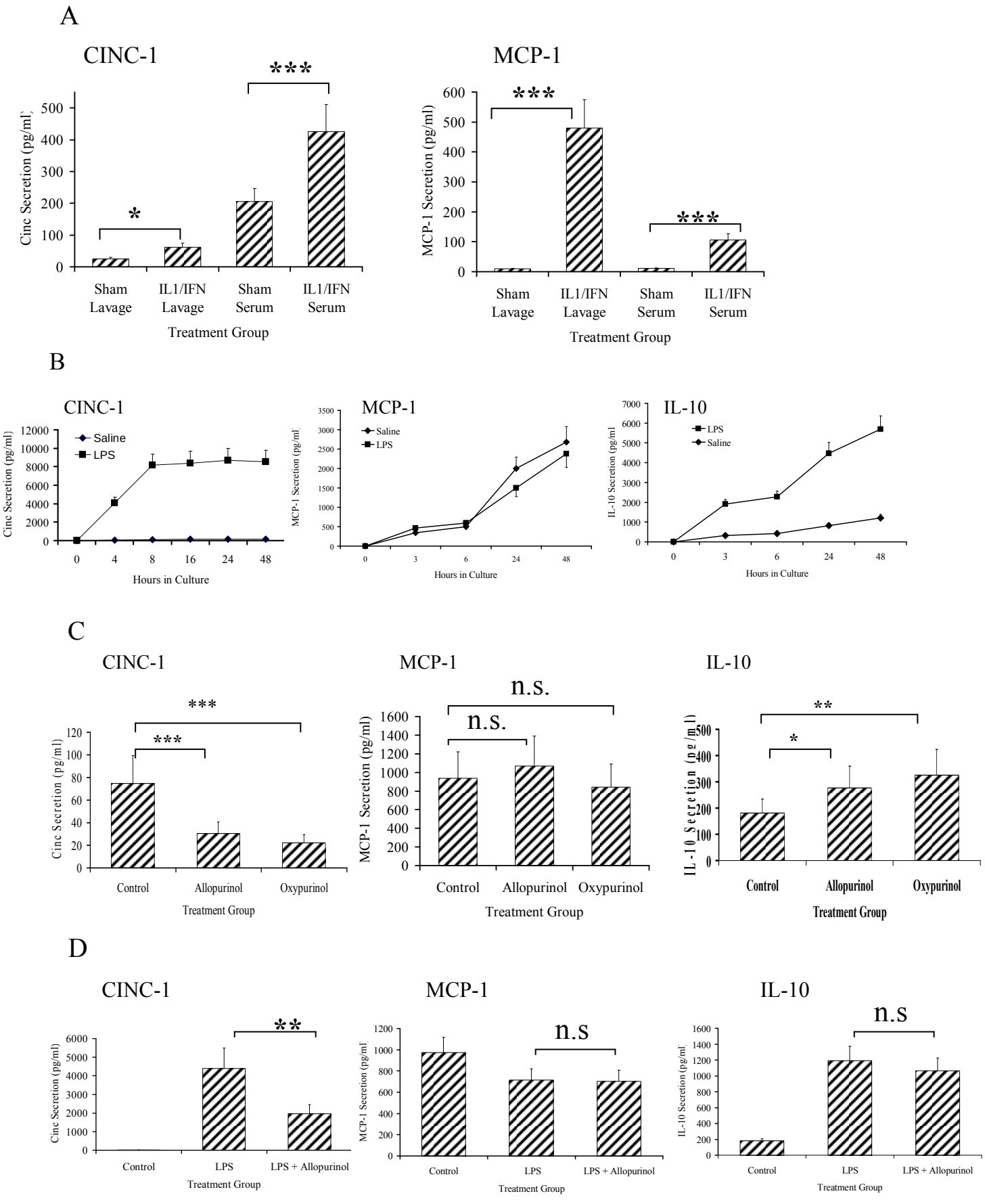

A
CINC-1
Cinc Secretion (pg/ml)
Sham Lavage
IL1/IFN Lavage
Sham Serum
IL1/IFN Serum
Treatment Group
MCP-1
MCP-1 Secretion (pg/ml)
Treatment Group
B
CINC-1
Saline
LPS
Cinc Secretion (pg/ml)
Hours in Culture
MCP-1
MCP-1 Secretion (pg/ml)
IL-10
IL-10 Secretion (pg/ml)
C
CINC-1
MCP-1
IL-10
n.s.
Control
Allopurinol
Oxypurinol
Treatment Group
D
CINC-1
MCP-1
IL-10
n.s
Control
LPS
LPS + Allopurinol
Treatment Group

Figure 4

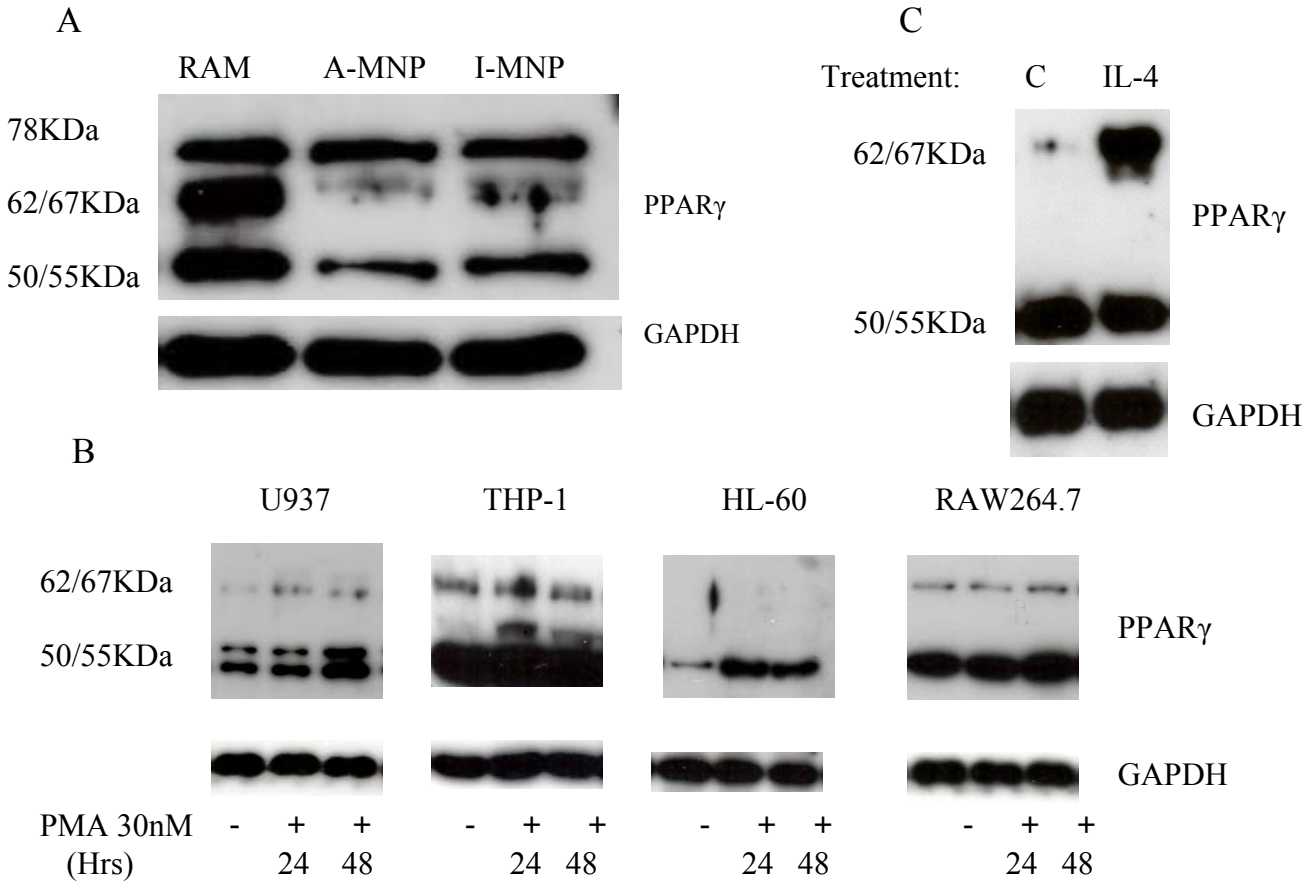

A
RAM
A-MNP
I-MNP
78KDa
62/67KDa
50/55KDa
PPARγ
GAPDH
C
Treatment:
C
IL-4
62/67KDa
50/55KDa
PPARγ
GAPDH
B
U937
THP-1
HL-60
RAW264.7
62/67KDa
50/55KDa
PPARγ
GAPDH
PMA 30nM
(Hrs)
- + +
24 48

Figure 5

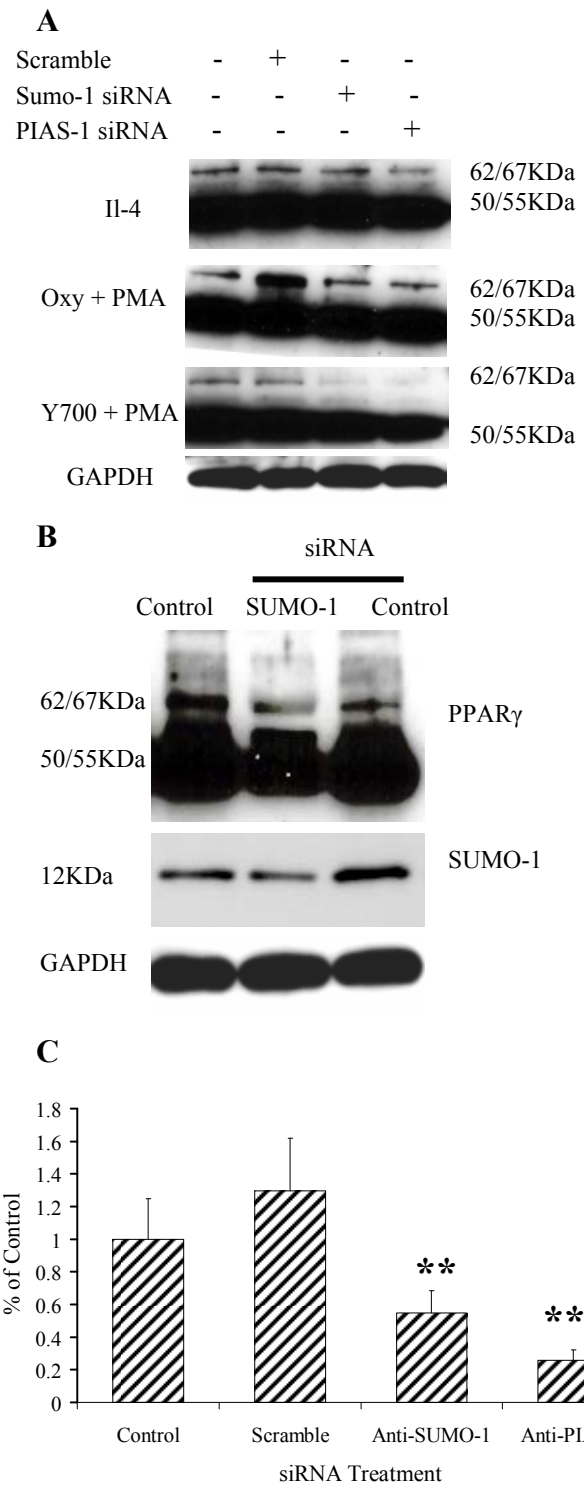

A
Scramble - + - -
Sumo-1 siRNA - - + -
PIAS-1 siRNA - - - +
Il-4
62/67KDa
50/55KDa
Oxy + PMA
62/67KDa
50/55KDa
Y700 + PMA
62/67KDa
50/55KDa
GAPDH
B
siRNA
Control
SUMO-1
Control
62/67KDa
50/55KDa
PPARγ
12KDa
SUMO-1
GAPDH
C
% of Control
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
***
Control
Scramble
Anti-SUMO-1
Anti-PIAS-1
siRNA Treatment

Figure 6

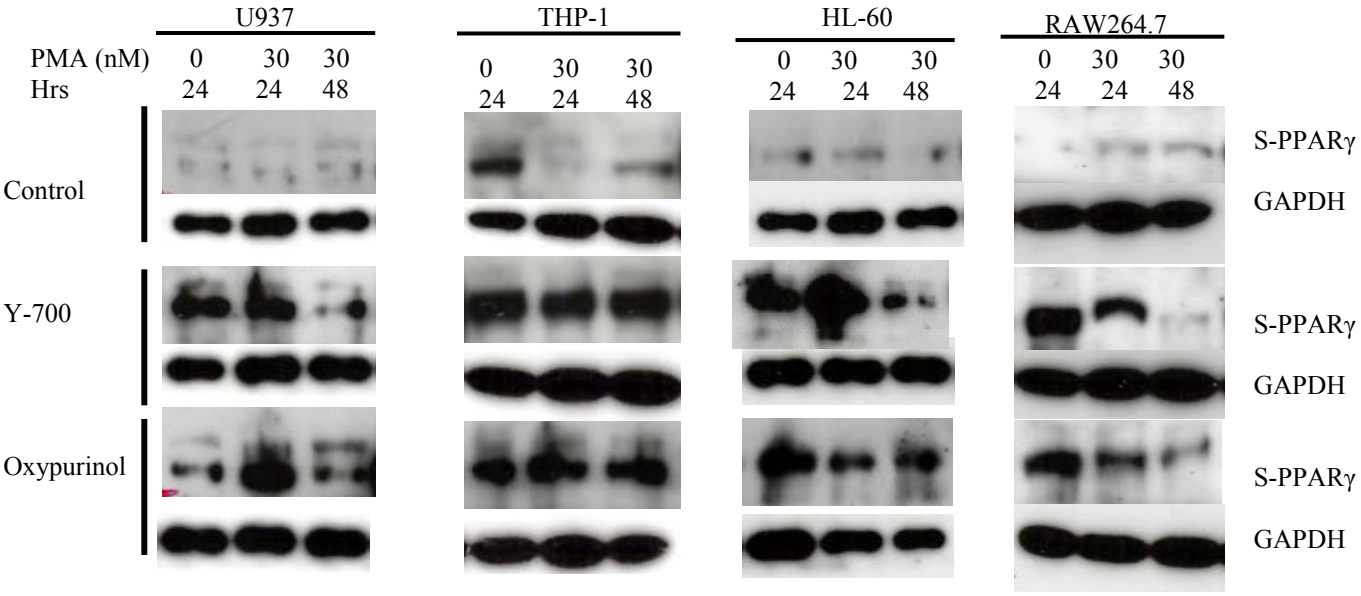

U937
THP-1
HL-60
RAW264.7
PMA (nM) 0 30 30
Hrs 24 24 48
Control
Y-700
Oxypurinol
S-PPARγ
GAPDH
S-PPARγ
GAPDH
S-PPARγ
GAPDH

Figure 7

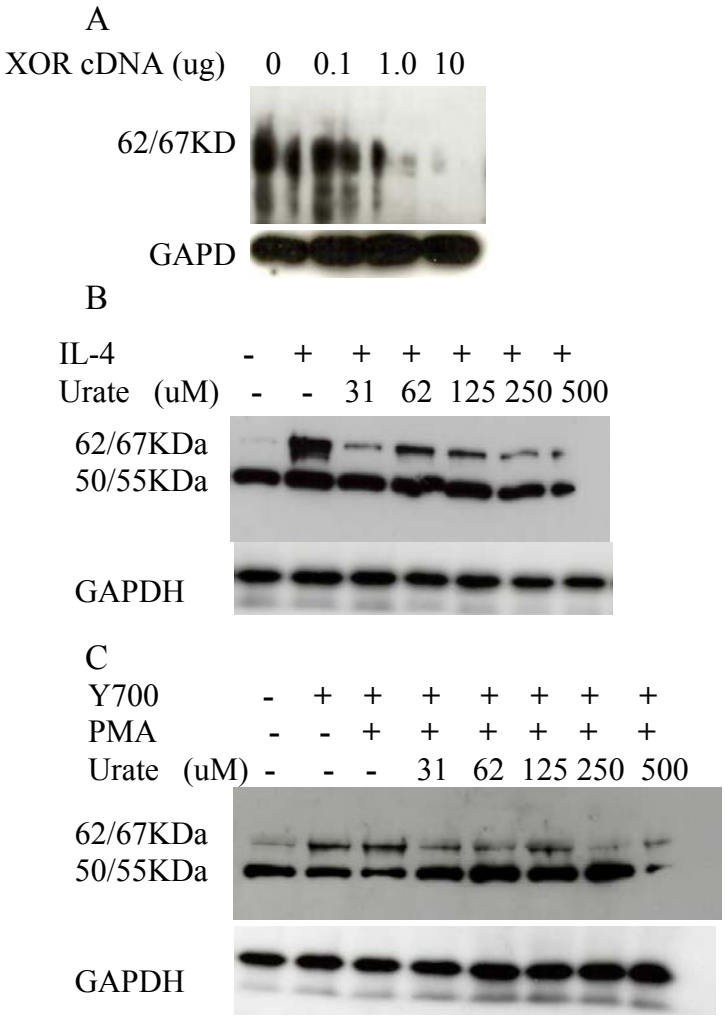

A
XOR cDNA (ug) 0 0.1 1.0 10
62/67KD
GAPD
B
IL-4 - + + + + + +
Urate (uM) - - 31 62 125 250 500
62/67KDa
50/55KDa
GAPDH
C
Y700 - + + + + + + +
PMA - - + + + + + +
Urate (uM) - - - 31 62 125 250 500
62/67KDa
50/55KDa
GAPDH

Figure 8

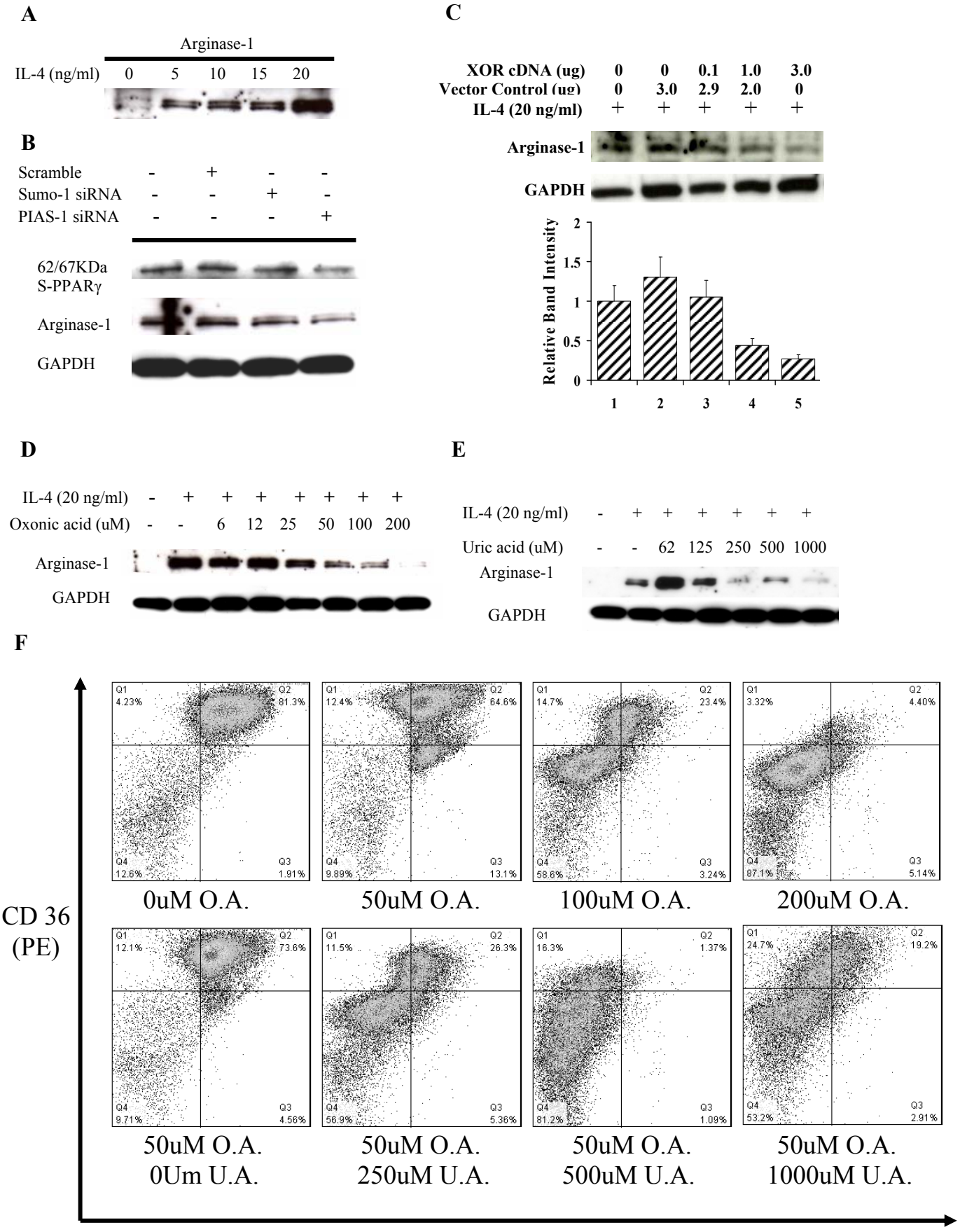
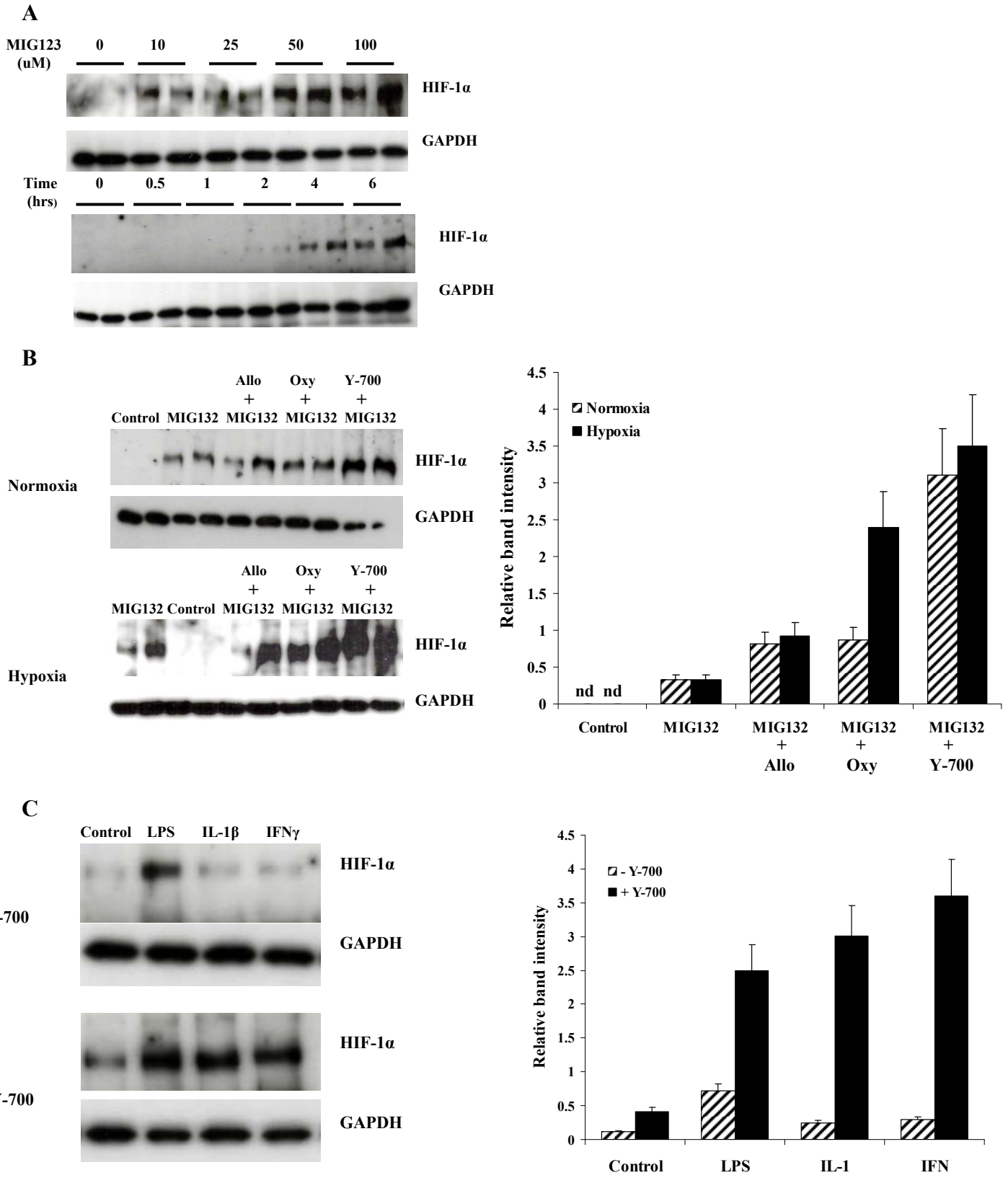
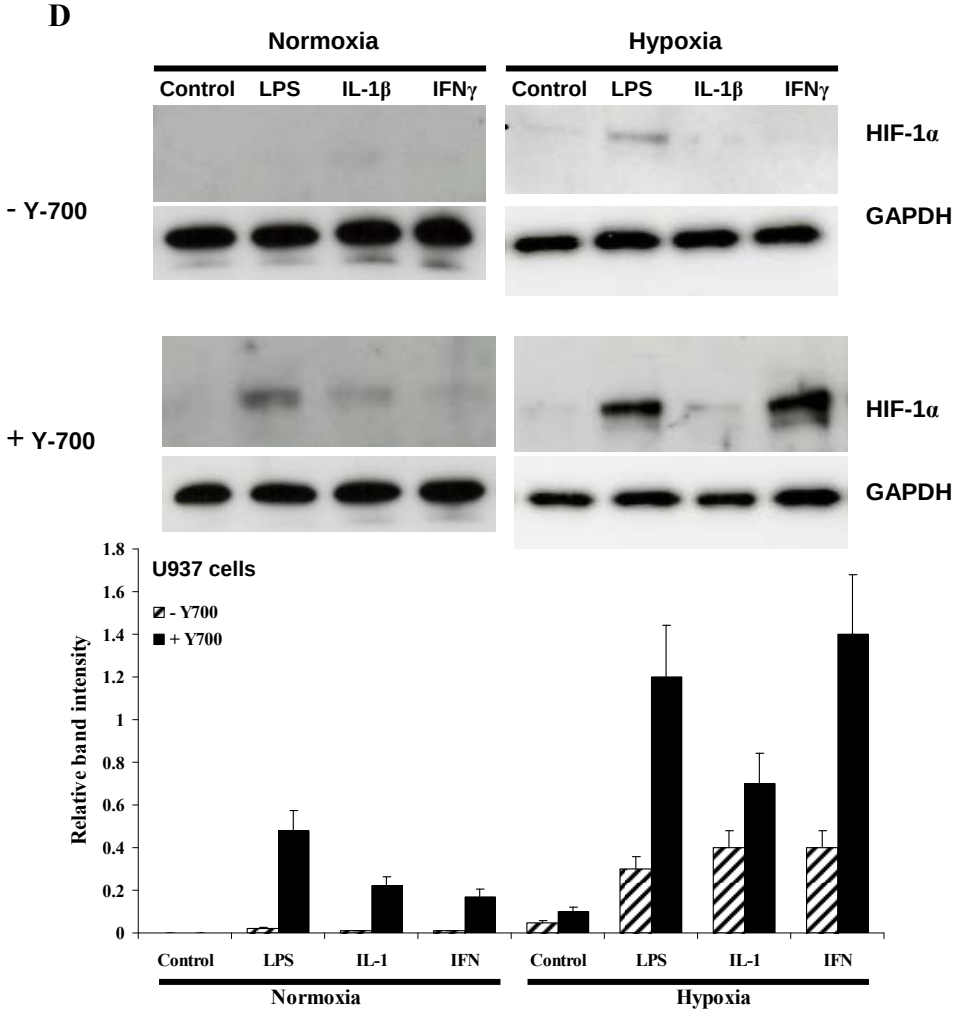

A
Arginase-1
IL-4 (ng/ml) 0 5 10 15 20
B
Scramble - + - -
Sumo-1 siRNA - - + -
PIAS-1 siRNA - - - +
62/67KDa S-PPARγ
Arginase-1
GAPDH
C
XOR cDNA (ug) 0 0 0.1 1.0 3.0
Vector Control (ug) 0 3.0 2.9 2.0 0
IL-4 (20 ng/ml) + + + + +
Arginase-1
GAPDH
Relative Band Intensity
2
1.5
1
0.5
0
1 2 3 4 5
D
IL-4 (20 ng/ml) - + + + + + + +
Oxonic acid (uM) - - 6 12 25 50 100 200
Arginase-1
GAPDH
E
IL-4 (20 ng/ml) - + + + + + +
Uric acid (uM) - - 62 125 250 500 1000
Arginase-1
GAPDH
F
CD 36 (PE)
Q1 4.23% Q2 81.3% Q4 12.6% Q3 1.91%
0uM O.A.
Q1 12.4% Q2 64.6% Q4 9.89% Q3 13.1%
50uM O.A.
Q1 14.7% Q2 23.4% Q4 58.6% Q3 3.24%
100uM O.A.
Q1 3.32% Q2 4.40% Q4 87.1% Q3 5.14%
200uM O.A.
Q1 12.1% Q2 73.6% Q4 9.71% Q3 4.56%
50uM O.A. 0Um U.A.
Q1 11.5% Q2 26.3% Q4 56.9% Q3 5.36%
50uM O.A. 250uM U.A.
Q1 16.3% Q2 1.37% Q4 81.2% Q3 1.09%
50uM O.A. 500uM U.A.
Q1 24.7% Q2 19.2% Q4 53.2% Q3 2.91%
50uM O.A. 1000uM U.A.
CD 206 (FITC)

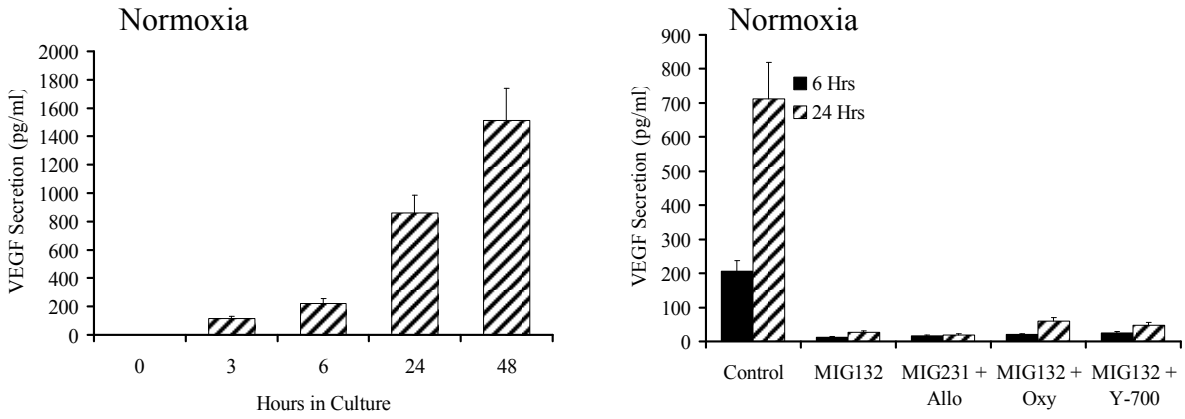
Figure 9


A
MIG123 (uM)
0
10
25
50
100
HIF-1α
GAPDH
Time (hrs)
0
0.5
1
2
4
6
HIF-1α
GAPDH
B
Allo + MIG132
Oxy + MIG132
Y-700 + MIG132
Control
MIG132
Normoxia
HIF-1α
GAPDH
MIG132
Control
Hypoxia
HIF-1α
GAPDH
Normoxia
Hypoxia
Relative band intensity
nd nd
Control
MIG132
MIG132 + Allo
MIG132 + Oxy
MIG132 + Y-700
C
Control
LPS
IL-1β
IFNγ
- Y-700
+ Y-700
HIF-1α
GAPDH
- Y-700
+ Y-700
Relative band intensity
Control
LPS
IL-1
IFN

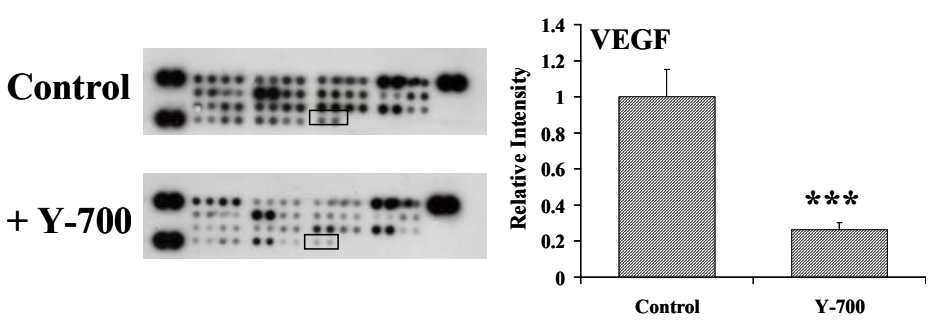
D


Normoxia
Hypoxia
Control
LPS
IL-1β
IFNγ
- Y-700
+ Y-700
HIF-1α
GAPDH
U937 cells
- Y700
+ Y700
Relative band intensity
Control
LPS
IL-1
IFN
Normoxia
Hypoxia

E


Normoxia
VEGF Secretion (pg/ml)
Hours in Culture
Normoxia
6 Hrs
24 Hrs
Control
MIG132
MIG231 + Allo
MIG132 + Oxy
MIG132 + Y-700

F


Control
+ Y-700
VEGF
Relative Intensity
***
Control
Y-700

TABLES

Table 1. XOR activity in RAM and NR8383 cells treated with Th-1 cytokines as described in the legend to Fig. 1.

	Control	IL-1β	IFNγ	IL-1β/ IFNγ	LPS
RAM	0.0794	0.238 ($p<0.01$)	0.695 ($p<0.01$)	0.596 ($p<0.01$)	0.488 ($p<0.01$)
NR8383	0.252	0.366 ($p<0.05$)	0.781 ($p<0.02$)	0.922 ($p<0.01$)	0.694 ($p<0.02$)