Fumagillin Prodrug Nanotherapy Suppresses Macrophage Inflammatory Response via Endothelial Nitric Oxide

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ABSTRACT Antiangiogenesis has been extensively explored for the treatment of a variety of cancers and certain inflammatory processes. Fumagillin, a mycotoxin produced by Aspergillus fumigatus that binds methionine aminopeptidase 2 (MetAP-2), is a potent antiangiogenic agent. Native fumagillin, however, is poorly soluble and extremely unstable. We have developed a lipase-labile fumagillin prodrug (Fum-PD) that eliminated the photoinstability of the compound. Using αvβ3-integrin-targeted perfluorocarbon nanocarriers to deliver Fum-PD specifically to angiogenic vessels, we effectively suppressed clinical disease in an experimental model of rheumatoid arthritis (RA). The exact mechanism by which Fum-PD-loaded targeted nanoparticles suppressed inflammation in experimental RA, however, remained unexplained. We herein present evidence that Fum-PD nanotherapy indirectly suppresses inflammation in experimental RA through the local production of endothelial nitric oxide (NO). Fum-PD-induced NO activates AMP-activated protein kinase (AMPK), which subsequently modulates macrophage inflammatory response. In vivo, NO-induced AMPK activation inhibits mammalian target of rapamycin (mTOR) activity and enhances autophagic flux, as evidenced by p62 depletion and increased autolysosome formation. Autophagy in turn mediates the degradation of IkappaB kinase (IKK), suppressing the NF-κB p65 signaling pathway and inflammatory cytokine release. Inhibition of NO production by N^2-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, reverses the suppression of NF-κB-mediated inflammatory response induced by Fum-PD nanotherapy. These unexpected results uncover an activity of Fum-PD nanotherapy that may be further explored in the treatment of angiogenesis-dependent diseases.

KEYWORDS: angiogenesis · autophagy · nitric oxide · AMP-activated protein kinase · fumagillin prodrug · nanotherapy

Angiogenesis is the new blood vessel formation that accompanies normal development as well as many disease processes. Antiangiogenic therapy has been extensively explored for the treatment of solid tumors and other inflammatory conditions. For example, antiangiogenic drugs targeting the vascular endothelial growth factor (VEGF) pathway have offered improved survival benefits in some cancer patients; however the results are often modest,1,2 and most patients treated with anti-VEGF agents eventually develop resistance, leading to cancer progression.3 Macroautophagy (hereafter referred to as autophagy) is a physiologic process that clears defective cellular organelles in the maintenance of normal cell functions but also contributes to various pathological conditions.4 As defects in autophagy predispose to cancer and other inflammatory processes,5,6 stimulating autophagy with mTOR inhibitors or other autophagy enhancers may suppress cancer and limit inflammation. In fact, the combination of autophagy modulation and chemotherapy/antiangiogenesis is currently being explored for the treatment of various cancers.5

Fumagillin, a mycotoxin produced by Aspergillus fumigatus that inhibits methionine aminopeptidase 2 (MetAP-2),7,8 is a potent inhibitor of angiogenesis.9 Native fumagillin is highly hydrophobic and extremely photounstable, thus limiting its potential clinical translation. We have successfully developed a lipase labile fumagillin prodrug (Fum-PD), which eliminated the photoinstability of native fumagillin.10 Using an αvβ3-integrin-targeted perfluorocarbon (PFC) nanocarrier system, we delivered Fum-PD...
Figure 1. Schematic representation of Fum-PD PFC nanoparticle delivery mechanism. (A) Chemical formula of Sn-2 phospholipase labile Fum-PD. (B) Fum-PD FFC nanoparticle fuses with the targeted cell. Once inside the cell, Fum-PD is cleaved at the Sn-2 site by a phospholipase, such as PLA-2, releasing the active drug. Characteristics of PFC nanoparticles without and loaded with Fum-PD are presented on the right.
anti-inflammatory effect by actively suppressing the production of inflammatory cytokines.\textsuperscript{19,20} We hypothesized that antiangiogenic therapy with Fum-PD NPs increased NO production by endothelial cells (ECs) and that NO release following EC apoptosis suppressed the inflammatory response of nearby recruited macrophages. Consistent with this hypothesis, we observed increased NOS expression in the endothelial cells of paw sections obtained from mice treated with Fum-PD NPs, but not mice treated with Ctrl NPs (Figure 2H).

### Fum-PD Suppresses Macrophage Inflammatory Response through Nitric Oxide

To further confirm the hypothesis that Fum-PD enhanced NO production by ECs, we turned to an in vitro system. Mouse ECs (SVEC4-10) and day 5 thioglycollate-elicited primary peritoneal macrophages (MΦ) were individually exposed to increasing concentrations of Fum-PD (in DMSO) for 48 h, and the supernatants were assayed for NO release. Exposure of ECs to Fum-PD led to a dose-dependent release of NO (Figure 3A) and increased NOS expression (Figure 3B). In contrast, Fum-PD had no direct effects on inducible NOS (iNOS) expression and NO release by peritoneal MΦ (Figure 3A,B). Increased intracellular NO triggered EC apoptosis, as evidenced by positive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in cells that expressed high level of iNOS (Figure 3C).

To determine whether endothelial NO could modulate MΦ inflammatory activity, we cocultured thioglycollate-elicited peritoneal MΦ with ECs (1:1 ratio) in the presence of Fum-PD. We focused on TNF-\(\alpha\) levels for these in vitro assays since TNF-\(\alpha\) release was unique...
to peritoneal MΦ, whereas SVEC4-10 ECs released high levels of MCP-1 and IL-6 but no TNF-α (Figure S2). While Fum-PD had no direct effect on TNF-α release by MΦ (Figure 4A), exposure of EC–MΦ cocultures to Fum-PD led to a dose-dependent suppression of TNF-α release (Figure 4B). The anti-inflammatory activity of Fum-PD in EC–MΦ cocultures was recapitulated by the NO donor S-nitroso-N-acetyl-α-penicillamine (SNAP) [21], while the addition of Nω-nitro-arginine methyl ester (l-NAME, a nonspecific NOS inhibitor) reversed the suppressive effect of Fum-PD (and SNAP) on TNF-α release. Although l-NAME is known to inhibit NO synthesis, it also acts as a scavenger of hydroxyl radicals in the millimolar range. [22] However, ω-NAME, an isomer of l-NAME, can scavenge hydroxyl radicals at equal rates to l-NAME. [22] The fact that ω-NAME did not affect Fum-PD activity (Figure 4C) implied that it was the NOS inhibitory activity of l-NAME that contributed to the observed phenotype. Taken together these results strongly suggest that, in vitro, Fum-PD indirectly suppressed MΦ inflammatory cytokine release through an NO-dependent mechanism.

**Fum-PD-Induced NO Modulates Macrophage Activity in Vitro through AMPK Activation.** NO has been shown to activate a number of signaling pathways, including AMPK. [23,24] Several recent studies suggest that AMPK acts as a “central regulator” of inflammatory signaling in various cell types, macrophages in particular. [25–27] In addition, AMPK activation has been shown to reduce inflammation in vivo in several preclinical models. [26–30] To determine the mechanism by which Fum-PD induced NO-modulated macrophage inflammatory response, the activation status of AMPK in EC–MΦ cocultures in the presence of Fum-PD was examined. Fum-PD dose-dependently enhanced the phosphorylation of AMPK (Figure 4D). This activation of AMPK was mimicked by the addition of SNAP (Figure 4D). On the other hand, l-NAME reversed the Fum-PD-induced AMPK activation (Figure 4D). AMPK signaling has been shown to promote MΦ anti-inflammatory response by down-regulating NF-κB activation. [31] Consistent with previous studies, we observed reduced NF-κB p65 phosphorylation in the presence of Fum-PD and SNAP (Figure 4D). Fum-PD suppression of NF-κB-p65 phosphorylation was reversed with the addition of l-NAME (Figure 4D).

To further confirm that Fum-PD-mediated suppression of TNF-α release is AMPK-dependent, we cocultured EC–MΦ in the presence of Fum-PD and compound C (CC), a specific inhibitor of AMPK. [32] CC dose-dependently reversed the Fum-PD-mediated inhibition of TNF-α release (Figure 4E). Although mechanistic details regarding AMPK modulation of NF-κB signaling and cytokine production in macrophages remain undefined, some studies suggest that AMPK activation polarizes macrophages toward an anti-inflammatory phenotype.

In an oversimplification, macrophages can be loosely divided into two populations, the classically and alternatively activated macrophages. [33] Classically activated macrophages (M1) are generated through IFN-γ and TNF-α stimulation and secrete high levels of...
pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-23), while alternatively activated macrophages (M2) are induced by IL-4/IL-13 and promote matrix reorganization, wound healing, fibrosis, allergy, and immunomodulation.33 Alternatively activated M2 macrophages secrete IL-10 and TGF-β, among other mediators, and up-regulate the expression of arginase 1.34 We examined the release of IL-10 and mRNA expression of arginase 1 (M2 markers) in EC–MΦ cocultures exposed to Fum-PD and observed no difference in the level of IL-10 and a dose-dependent decrease in arginase 1 expression (Figure 4F,G). While Fum-PD did not definitively polarize macrophages toward an alternatively activated M2 phenotype in vitro, a reversal of the TNF-α/IL-10 ratio suggested that Fum-PD induced a shift in macrophage activity toward an anti-inflammatory phenotype.

**Fum-PD Nanotherapy Suppresses KRN Arthritis through Autophagy Modulation.** To determine whether NO modulated AMPK activation in vivo, KRN arthritic mice were again treated with Fum-PD NP. Increased AMPK phosphorylation was observed (Figure 5H), and this enhanced activity was reversed by L-NAME but not D-NAME (Figure 5I). Fum-PD NP treatment also inhibited the phosphorylation of mTOR (Figure 5H), which is known to be regulated by AMPK.35 Consistent with the in vitro studies, enhanced AMPK activation was accompanied by suppression of inflammatory cytokine profiles. However, neither IFN-γ (not shown) nor IL-10 (an M2 marker) was detected in the paw lysates of treated animals. In addition, the expression of CD206, the macrophage mannose receptor that is known to be up-regulated on alternatively activated M2 macrophages, was examined.36 We observed a predominance
of CD206\(^+\) macrophages in day-9 arthritic paws but no significant difference in the expression of this receptor in the synovial tissues of animals treated with Ctrl or Fum-PD NPs (Figure S3). As macrophage activation is plastic and reversible, the possibility that Fum-PD might have suppressed inflammation by inducing macrophages to differentiate toward an M2 phenotype in the early phases of disease (i.e., at a time when inflammatory M1 macrophages might predominate) could not be precluded.

We next considered whether AMPK-induced mTOR inhibition might also exert an anti-inflammatory effect in KRN arthritis through the induction of autophagy.\(^6,37,38\) Autophagy is now recognized as a major mechanism for regulating the secretion of cytokines and chemokines, especially in macrophages.\(^5\) Autophagy involves a set of evolutionarily conserved gene products known as Atg proteins that are required for the formation of autophagosomes. One of these critical proteins is microtubule-associated protein light chain 3 (LC3), a mammalian homologue of yeast Atg8. The cytoplasmic form of LC3 (LC3-I) is converted to LC3-II via lipidation and recruited to the autophagosomes.\(^35\) LC3-I to LC3-II conversion therefore serves as a reliable marker for autophagosome formation. We observed a significant increase in LC3 expression (Figure 6A), percentage of cells with punctated LC3 staining (Figure 6B,C), and conversion of LC3-I to LC3-II in arthritic compared to nonarthritic paws (Figure 6D). Collectively, these results suggest that autophagy was engaged in KRN arthritis.

To determine whether autophagy modulation underlies the mechanism by which Fum-PD-induced NO suppressed inflammatory arthritis, LC3 turnover in animals treated with Fum-PD NP, without or with l-NAME coadministration, was studied. The LC3 turnover assay is based on the observation that LC3-II is degraded by lysosomal enzymes when autophagosomes fuse with lysosomes to form autolysosomes.\(^39\) Indeed Fum-PD NP treatment significantly decreased LC3-II levels (Figure 7A,B). In addition, the protein p62, also known as SQSTM1/sequestome1, is selectively incorporated into autophagosomes and degraded by autophagy.\(^40\) The expression of p62 thus inversely correlates with autophagic activity. We noted a similar decrease in p62 expression (\(p < 0.05\)) with Fum-PD NP treatment (Figure 7A,B). Furthermore, increased LC3 co-localization with the lysosome marker LAMP-1 was appreciable (Figure 7C,D), further suggesting autolysosome formation and enhanced autophagic flux following Fum-PD NP treatment. Concomitant administration of l-NAME reversed the effects of Fum-PD NP on autophagic flux, confirming the critical role of NO in autophagy modulation (Figure 7).

Studies have shown that autophagy can modulate the transcription, processing and/or release of cytokines.\(^41\)
Consequently, mice deficient in specific Atg genes produce higher levels of cytokines and have been shown to be more susceptible to certain inflammatory diseases.6 Conversely, induction of autophagy inhibits NF-κB signaling, a pathway that plays a central role in the regulation of the cytokine response in RA.42 Consistent with these studies, we found that engagement of autophagy in KRN arthritis was accompanied by an increase in NF-κB-p65 phosphorylation (Figure 8A). On the other hand, enhanced autophagic flux (Figure 7A,B) following Fum-PD NP treatment led to marked reduction in IkappaB kinase alpha (IKKα) protein level and suppression of NF-κB-p65 phosphorylation (Figure 8B and Figure S4), all of which suggest a potential explanation for the global decrease in inflammatory cytokine levels observed in Figure 2C–F. Moreover, Fum-PD NP effects on NF-κB activity were NO-dependent (Figure 8C).

DISCUSSION

NO is produced by the conversion of l-arginine to l-citrulline by different NOS isoforms:43 endothelial NOS (eNOS, Nos3) is constitutively active in endothelial cells and controls key vascular functions; neuronal NOS (nNOS) is the predominant source of NO in neurons; and inducible NOS (iNOS, Nos2) is expressed in many different cell types, including macrophages and endothelial cells. Expression and activity of iNOS were shown to be up-regulated during inflammatory responses.19 Although NO is known to play a role in inflammation, there is no consensus concerning its contribution to disease and cytokine expression in inflammatory arthritis. Elevated levels of NO activity have been reported in serum, urine, and synovial fluid of RA patients.44–46 Subsequent studies in experimental arthritis models seemed to confirm the detrimental effects of NO and the benefits of NO inhibition on disease manifestation.47,48 On the other hand, evidence also suggests that NO can have beneficial properties, and the absence of iNOS or selective inhibition of iNOS aggravated rather than attenuated experimental arthritis.49,50 We observed that Fum-PD NP-induced NO exerted anti-inflammatory effects in the studies presented herein. Perhaps the contradictory reports on the effects of NO in inflammatory joint disease may be reconciled by recognizing that NO likely has different functions depending on the stage of the disease,51 the amount of NO generated or administered,52 and the tissues or cell types affected.53 As α/β3-targeted
Fum-PD-loaded nanoparticles homed specifically to angiogenic blood vessels, the local level of NO induced/released following Fum-PD nanoparticle exposure might be quite high but likely constrained to the neoendothelial cells or their immediate vicinity. While a small amount of NO may stimulate inflammatory cytokine/chemokine release, a large quantity of NO has been shown to suppress the inflammatory response.\(^\text{19}\)

NO has also been shown to activate AMPK through different mechanisms. A recent report suggested that, in response to nitrosative stress induced by NO, ataxiatelangiectasia mutated (ATM) kinase was activated and signaled to AMPK.\(^\text{54}\) Others suggested that nitrosative stress might also activate AMPK through a mechanism involving Ca\(^{2+}/\text{calmodulin-dependent protein kinase} (\text{CaMKK}) or the unfolded protein response (UPR).\(^\text{23,24}\) Regardless of the mechanism, we confirmed that Fum-PD activated AMPK in vitro and in vivo largely through a NO-dependent pathway, as L-NAME reversed the observed fumagillin-induced AMPK activation. The downstream consequences of AMPK activation are manifold and include the control of cell survival, activation, and metabolism.\(^\text{55}\)

In recent years the role of AMPK in macrophage polarization has received increased attention. Macrophage responses to environmental stimuli are remarkably plastic and can give rise to different populations of cells with distinct functions. This polarization, as it is termed, may fuel disease progression or promote resolution of inflammation.\(^\text{25}\) AMPK is thought to act as a “master switch” of macrophage polarization by

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**Figure 7.** Fum-PD NP increases autophagic flux in KRN arthritis. (A) Day-9 paw lysates derived from the different treatment groups in Figure 4 were blotted for LC3 and p62. Actin served as protein loading control. (B) Quantification: total LC3 (LC3-I and LC3-II) and p62 levels were first normalized to actin and expressed relative to Ctrl NP level, which was set at 1. Values represent mean ± SEM, \(n = 4\) or 5 mice per treatment group. (C) Increased LC3 (green) and LAMP-1 (red) co-localization (arrowheads) following Fum-PD NP treatment. The effect was reversed by L-NAME. (D) Quantification of LC3^+LAMP-1^+ cells. Values represent mean ± SEM, \(n = 4\) or 5 mice per treatment group. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). Scale bar = 20 μm.

**Figure 8.** Fum-PD NP treatment suppresses NF-κB activation in KRN arthritis. (A) Paws from nonarthritic and day-9 KRN serum-induced arthritic mice were stained for phospho (P)-p65 (orange). Scale bar = 200 μm. (B) Paw lysates from day-9 arthritic paws derived from different treatment groups were blotted for total IKK\(_\alpha\), P-p65, or total p65. (C) L-NAME, but not D-NAME, reversed Fum-PD NP-mediated IKK\(_\alpha\) degradation and suppression of p65 phosphorylation.
down-regulating NF-κB activation, although the exact mechanism by which AMPK inhibits NF-κB signaling remains unresolved.\textsuperscript{31,56–58} Our \textit{in vitro} data confirmed that Fum-PD-induced AMPK activation promoted an anti-inflammatory macrophage phenotype with significantly diminished M1 cytokines (TNF-α) but without enhanced IL-10 production or arginine 1 expression (both M2 markers). That fumagillin-induced AMPK activation \textit{in vivo} in KRN arthritis polarized macrophages toward an M2 phenotype was suggested but remains to be confirmed.

The activation of AMPK also leads to the inhibition of mTOR-dependent signaling activity.\textsuperscript{59} Inhibition of mTOR by AMPK activation (or by the addition of rapamycin) increases autophagy.\textsuperscript{60,61} In the KRN arthritis model, Fum-PD NPs generated a local nitrosative stress response, leading to the activation of AMPK and repression of mTOR activity, with subsequent increased autophagic flux. Although autophagy has been implicated in RA, the role of autophagy in disease progression has not been extensively explored. In a small clinical study the addition of the rapalogue everolimus to methotrexate has been extensively explored. In a small clinical study the addition of the rapalogue everolimus to methotrexate was associated with superior clinical responses. The results also suggest that close examination of the e ffects of Fum-PD nanotherapy on autophagy may yield unanticipated secondary benefits that can be exploited to improve antiangiogenic therapies in the treatment of various inflammatory disease processes.

**CONCLUSIONS**

In summary, the results herein suggest that antiangiogenic nanotherapy with Fum-PD NPs indirectly suppressed inflammatory cytokine production in the inflamed paws via the local production of (endothelial) NO, which likely acted on nearby recruited macrophages (Figure S5), leading to the activation of AMPK. NO-induced AMPK activation exerted a counter inflammatory response by modulating macrophage activity and enhancing autophagic flux, which in turn suppressed NF-κB-p65 activation and inflammatory cytokine release. Conversely, inhibition of NO production by L-NAME administered concomitantly with Fum-PD nanotherapy diminished AMPK activation and reversed the suppression of NF-κB-mediated inflammatory responses. The results also suggest that close examination of the effects of Fum-PD nanotherapy on autophagy may yield unanticipated secondary benefits that can be exploited to improve antiangiogenic therapies in the treatment of various inflammatory disease processes.

**METHODS**

**Chemical Reagents.** Unless otherwise listed, all solvents and reagents were purchased from Aldrich Chemical Co. (St. Louis, MO, USA) and used as received. Anhydrous chloroform and methanol were purchased from Aldrich Chemical Co. Perfluoroctyl bromide was acquired from Exfluor Inc. (Round Rock, TX, USA). A solution of C16-09:0 (COOH) PC 1-hexadecyl-2-azelaoyl-snglycero-3-phosphocholine followed by 4-dimethylaminopyridine and N,N'-dicyclohexylcarbodiimide was added to a solution of fumagillol in dry dichloromethane. The reaction mixture was stirred overnight at ambient temperature and then passed over a short pad of silica gel using EtOAc/mixture was stirred overnight at ambient temperature and then passed over a short pad of silica gel using EtOAc/hexane. The filtered solvent was removed in vacuo, and the oil residue was purified by column chromatography on SiO\textsubscript{2} using EtOAc/5% citric acid, brine, bicarbonate, and brine, dried with MgSO\textsubscript{4} and then concentrated \textit{in vacuo}. The crude product was purified with activated charcoal in acetone/trifluoroacetate and then filtered through a Celite pad. Yield: colorless solid, 59 mg (70%). HRMS found: M+ (283.3). A solution of C16-09:0 (COOH) PC 1-hexadecyl-2-azelaoyl-snglycero-3-phosphocholine followed by 4-dimethylaminopyridine and N,N'-dicyclohexylcarbodiimide was added to a solution of fumagillol in dry dichloromethane. The reaction mixture was stirred overnight at ambient temperature and then passed over a short pad of silica gel using EtOAc/hexane. The filtered solvent was removed in vacuo, and the oil residue was purified by column chromatography on SiO\textsubscript{2} using EtOAc/n-hexane for elution to yield the fumagillin prodruk (Fum-PD) compound as a pale yellowish solid. HR-MS found: 991.5475 (M + 2K – 2H).

**Preparation of Sn-2 Phospholipase Labile Fumagillin Prodrug.** Synthesis of the Sn-2 prodruk was accomplished in two steps: (1) saponifying fumagillin dicyclohexylamine salt to fumagillin; (2) esterifying the product with 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine and high-purity egg yolk phospholipids were purchased from Avanti Polar Lipids, Inc. Argon and nitrogen (Ultra High Purity: UHP, 99.99%) were used for storage of materials. Fumagillin dicyclohexylamine salt was provided by the National Cancer Institute. 3-phosphocholine (PAzPC) as previously. Briefly, fumagillin dicyclohexylamine salt in 1:1 methanol/water was treated with 35% NaOH, stirred in an ice bath for 2 h, warmed to room temperature, treated with another equivalent of 35% NaOH, and then stirred in an ice bath until the starting material was not detected by TLC (~4 h). After evaporating the methanol and resolubilizing in ethyl acetate, the mixture was extracted with 5% citric acid, brine, bicarbonate, and brine, dried with MgSO\textsubscript{4} and then concentrated \textit{in vacuo}. The crude product was purified with activated charcoal in acetone/trifluoroacetate and then filtered through a Celite pad. Yield: colorless solid, 59 mg (70%). HRMS found: M+ (283.3). A solution of C16-09:0 (COOH) PC 1-hexadecyl-2-azelaoyl-sn-glycero-3-phosphocholine followed by 4-dimethylaminopyridine and N,N'-dicyclohexylcarbodiimide was added to a solution of fumagillol in dry dichloromethane. The reaction mixture was stirred overnight at ambient temperature and then passed over a short pad of silica gel using EtOAc/hexane. The filtered solvent was removed in vacuo, and the oil residue was purified by column chromatography on SiO\textsubscript{2} using EtOAc/n-hexane for elution to yield the fumagillin prodruk (Fum-PD) compound as a pale yellowish solid. HR-MS found: 991.5475 (M + 2K – 2H).
was used for homing angiogenesis. The surfactant comixture of nanoparticles included ~97.6 mol % lecithin, 0.15 mol % α,β-β-ligand conjugated lipid, and 2.28 mol % Fum-PD (~0.5 mM). Nontargeted nanoparticles excluded the homing ligand and surfactant comixture was dissolved in chloroform and evaporated under reduced pressure, dried in a 40 °C vacuum oven overnight, and dispersed into water by probe sonication. The suspensions were combined with the perfluorocarbon, buffer, and glycercin with pH adjusted to 6.5 and homogenized with an S110 Microfluidics emulsifier (Microfluidics, Newton, MA, USA) at 20 000 psi for 4 min. The nanoparticles were aliquoted under a biohood into sterile vials and sealed under inert gas until use.

The α,β-β-integrin antagonist was a quinolone nonpeptide developed by Lantheus Medical Imaging (Billerica, MA, USA) and synthesized by Kereos (U.S. Patent 6 511 648 and related patents). The vitronectin antagonist was reported and characterized as the 111In-DOTA conjugate RP478 and cyan 5.5 homologue TA145. The homing specificity of the ligand was demonstrated and characterized with the Matrigel plug implanted in Rag2−/−Tg(12-loc2)182-Sato and C57BL/6 mice as there is an IC50 of 50 μM for the Mn2+-activated α,β-β-integrin. The nominal hydrodynamic diameter (Dh) of the α,β-β-targeted fumagillin prodrug PFC nanoparticle and control particles based on dynamic light scattering measurements (Brookhaven ZetaPlus, Brookhaven Instruments Corporation) in water was 252 nm with Pdi of 0.14 and zeta potential of ∼18 mV. Incorporation of prodrug at 2.28 mol % (~0.5 mM) within the surfactant comixture had negligible impact on particle sizes.

Arthritis Induction and Treatment. All mice were kept in a pathogen-free condition at Washington University Specialized Research Facility, and all the experiments were performed in compliance with federal laws and strictly according to protocols approved by the Division of Comparative Medicine at Washington University School of Medicine. Arthritis was induced using the KRN mouse model of inflammatory arthritis as previously described. Six- to eight-week-old male C57BL/6 mice (The Jackson Laboratory) were injected ip with 150 μL of KRN serum on day 0 to induce arthritis. Clinical manifestation of arthritis was assessed daily by an observer blinded to the treatment on a scale of 0–3 (0 = no swelling or erythema, 1 = slight swelling or erythema, 2 = moderate erythema and swelling in multiple instances, 3 = whole paw, maximum score of 12 per mouse). Change from day 0 to induce arthritis. Clinical manifestation of arthritis was assessed daily by an observer blinded to the treatment on a scale of 0–3 (0 = no swelling or erythema, 1 = slight swelling or erythema, 2 = moderate erythema and swelling in multiple instances, 3 = whole paw, maximum score of 12 per mouse).

In Vivo Cell Culture and Treatment. SEVC4 endothelial cells (obtained from the American Type Culture Collection) were cultured in DMEM supplemented with 10% fetal calf serum (Gibco) and streptomycin–penicillin solution at 37 °C in a 5% CO2 atmosphere. SEVC4 cells were plated in a 96-well plate at the density of 2 × 105 cells/well and treated with different concentrations of Fum-PD (in 0.1% DMSO) or 0.1% DMSO for 48 h, and supernatants were collected. NO release was detected with Griess reagent (Sigma-Aldrich) as previously described, and cytokines in cell culture supernatants were detected with cytometric bead array (CBA) using the mouse inflammatory kit (BD Bioscience).

Peritonitis was induced by ip injection of sterile thioglycollate (4 wt %/vol in 1 mL of sterile saline; Sigma-Aldrich).

On day 5, the mice were sacrificed by carbon dioxide exposure, and peritoneal cavities were lavaged with 8 mL of cold PBS. Cells were allowed to adhere on the surface of Petri dishes for 45 min at 37 °C and washed twice with cell culture media to remove nonadherent cells (macrophages) were detached with 5 mM EDTA in DMEM supplemented with 15% horse serum for 15 min at 37 °C. Cells were washed twice with cell culture media and seeded with or without SEVC4 cells at a ratio of 1:1 (2 × 105 cells of each cell type/well in 96-well plates). Macrophages or control were treated with different concentrations of fumagillin prodrug or SNAP (25 μg/mL). In some instances, cocultures were pretreated with L-NAME or n-NAME (20 μM) for 30 min followed by exposure to fumagillin prodrug for 48 h. Cytokine levels in cell culture supernatants were assessed with CBA. The cells in culture were also harvested, and cell lysates prepared for Western blotting analysis.

Immunohistochemistry and Immunofluorescence. The frozen sections (9 μm) of harvested paws were fixed in 4% PFA for 20 min at room temperature, incubated with rat anti-mouse CD3 (1:100, cat. 102507, Biolegend Technology), rabbit anti-mouse iNOS (1:400, cat. ab3523, Abcam), rat anti-Mac-3 monoclonal antibody (1:200, cat. CLB943AP, Cedarlane Laboratories), rabbit anti-mouse LC3B (1:100, cat. L7543, Sigma-Aldrich), phospho-p65 (phosphorylation site: S536, dilution 1:100, cat. 3033, Cell Signaling Technology), and rat anti-mouse LAMP-1 (1:100, cat. SC19992, Santa Cruz) followed by FITC-conjugated anti-rabbit or TRITC-conjugated anti-rat secondary antibody (1:100, Jackson Immunoresearch Laboratory). All images were visualized on a Nikon Eclipse microscope and acquired at the same exposure with QCapture software. Merged and single-color images were loaded into ImageJ software (http://rsb.info.nih.gov/ij) for analysis. Double (LAMP-1 ‘LC3’) positive cells or single positive (LC3) cells were marked with different colors, each group was tallied separately, and the ratio of double to single positive cells was calculated. Data were obtained from six to eight nonoverlapping fields per section and four to six sections per treatment. 3–5 mice per treatment.

NOS immunofluorescence was performed on SEVC4 cells or primary peritoneal macrophages that were treated with fumagillin prodrug for 48 h in LAB-TEK Permanox chambered slides (Thermo Fisher Scientific). Cells were fixed in acetonitrile at −20 °C for 5 min and incubated with rabbit anti-mouse iNOS (1:400, cat. ab3523, Abcam), followed by incubation with TRITC-conjugated anti-rabbit secondary antibody (1:100, Jackson Immunoresearch Laboratory). All images were visualized on a Nikon Eclipse microscope and acquired at the same exposure with QCapture software. All immunofluorescence grading was performed by an observer blinded to the treatment.

Apoptotic cells were detected using a DNA fragmentation (TUNEL) assay kit (Millipore) in which the addition of digoxin conjugated nucleotides to the 3' OH termini of the fragmented DNA strand was visualized by fluorescence microscopy with a FITC-labeled anti-digoxin antibody (1:100, Jackson Immunoresearch Laboratory).

Cytokine Analysis. Paws were homogenized in 1 mL of PBS with proteinase inhibitor cocktail and cleared by centrifugation. Cells were lysed in 100 μL of PBS with proteinase inhibitor cocktail and cleared by centrifugation. Concentrations of IFN-γ, IL-6, IL-10, and TNF-α in an equivalent volume of paw lysates, air pouch lavages, and cell culture supernatants were measured by CBA using the mouse inflammatory kit (BD Bioscience) according to the manufacturer’s instructions. Concentration of IL-1β in paw lysates was detected by using a commercially available ELISA kit (R&D System).

Western Blotting. Cell or paw lysates were prepared with 1% NP-40 lysis buffer containing proteinase inhibitor cocktail (Sigma) and 50 mM sodium fluoride (Sigma). Briefly, cells or paws were homogenized, sonicated in the lysis buffer, and cleared by centrifugation at 14 000 rpm for 10 min at 4 °C. Proteins from cell or paw lysates were quantified and equivalent amount fractionated by SDS-PAGE under reducing conditions. Membranes were blocked with anti-mouse LC3B (1:1000 cat. L7543 Sigma-Aldrich) or anti-β-actin (1:500, cat. sc-1615, Santa Cruz Biotechnology) antibody; antibody antibody anti-mouse phospho-AMPKa (Thr172, 1:1000, cat. 2353), AMPKα (1:1000, cat. 2603),
phospho-mTOR (Ser2448, 1:1000, cat. 5536), IKKα (1:1000, cat. 2682), phospho-p65 (Ser536, 1:1000, cat. 3033), or p65 antibody (1:1000, cat. B242) antibody from Cell Signaling Technology; and monoclonal mouse anti-mouse p62 antibody (1:1000, Cat. MAB312, Milligore). The blots were washed and incubated with HRP-conjugated donkey anti-goat or anti-rabbit IgG (1:2000, Jackson ImmunoResearch Laboratories) or HRP-conjugated mouse anti-mouse IgM (1:2000, μ-chain-specific; Jackson ImmunoResearch Laboratories). Bands were visualized using a SuperSignal Western blotting kit (Thermo Scientific). Briefly, Western blotting images were scanned into Carestream Molecular Imaging software. The band of interest was selected, and the average density was calculated. The intensity of the bands was normalized to that of β-actin.

RT-PCR. Cells from cocultures were harvested and placed in RNAwater stabilizing solution (Life Technologies) for at least 24 h prior to RNA processing. Total RNA was isolated using a Qiagen RNeasy mini kit (Qiagen Inc.) as recommended by the manufacturer. Two micrograms of RNA was used to synthesize cDNA by reverse transcription using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Life Technologies) on a Multigene thermal cycle system (Labnet International Inc., Edison, NJ, USA). The reverse-transcription product served as the template for real-time PCR analysis on a 7500 RT PCR system (Applied Biosystems) using fluorogenic primers for arginase 1 (Mm00440502_m1, Applied Biosystems) according to the manufacturer’s protocol. Results from each sample were normalized to the concentration of GAPDH mRNA measured in the same samples.

Statistics. Comparisons between two groups were performed by two-tailed, unpaired t test without correction. Grading data were analyzed with the γ° test. Comparisons between multiple groups (≥3) were performed by one-way ANOVA. Equality of variance assumption was tested, and Bonferroni’s correction for multiple comparisons was performed. Normality assumption appeared to be met, and tests for equality variance were conducted. The sample size (number of animals per treatment) appeared to be met, and tests for equality variance were conducted. The sample size (number of animals per treatment) appeared to be met, and tests for equality variance were conducted. The sample size (number of animals per treatment) appeared to be met, and tests for equality variance were conducted. The sample size (number of animals per treatment) appeared to be met, and tests for equality variance were conducted. The sample size (number of animals per treatment) appeared to be met, and tests for equality variance were conducted.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Supplementary Methods. Figure S1: Free Fum-PD is ineffective at suppressing iKN arthritis. Figure S2: Cytokine response in vitro following Fum-PD exposure. Figure S3: Alternatively activated M2 macrophages predominate in chronic phase of arthritis. Figure S4: Fumagillin nanochemistry-mediated NF-κB p65 suppression is NO-dependent. Figure S5: Endothelial-macrophage tight interactions in the inflamed synovium. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES


