Cathepsin K-Dependent Toll-Like Receptor 9 Signaling Revealed in Experimental Arthritis

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Cathepsin K–Dependent Toll-Like Receptor 9 Signaling Revealed in Experimental Arthritis

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Cathepsin K was originally identified as an osteoclast-specific lysosomal protease, the inhibitor of which has been considered might have therapeutic potential. We show that inhibition of cathepsin K could potently suppress autoimmune inflammation of the joints as well as osteoclastic bone resorption in autoimmune arthritis. Furthermore, *cathepsin K*$^{-/-}$ mice were resistant to experimental autoimmune encephalomyelitis. Pharmacological inhibition or targeted disruption of cathepsin K resulted in defective Toll-like receptor 9 signaling in dendritic cells in response to unmethylated CpG DNA, which in turn led to attenuated induction of T helper 17 cells, without affecting the antigen-presenting ability of dendritic cells. These results suggest that cathepsin K plays an important role in the immune system and may serve as a valid therapeutic target in autoimmune diseases.

Both innate and adaptive immune systems contribute to the inflammation seen in autoimmune diseases, but the molecular mechanism underlying this process is not completely understood (*1*, *2*). The cathepsins constitute a family of lysosomal cysteine proteases that were initially recognized as nonspecific scavengers of cellular proteins and that were also found to display cell type–specific functions (*3*, *4*). Cathepsins L and S are fundamental in processing of major histocompatibility complex (MHC) class II antigens and MHC class II trafficking and maturation (*3*, *4*). In contrast, cathepsin K is highly expressed in osteoclasts and is involved in degradation of bone matrices such as type I collagen (*5*). The loss-of-function mutation in the *cathepsin K* gene in humans causes pycnodysostosis, a rare genetic disorder characterized by impaired osteoclastic bone resorption (*6*). In mice, the targeted disruption of *cathepsin K* similarly results in the pycnodysostotic phenotype (*4*, *5*, *7*). Among matrix-degrading enzymes expressed in osteoclasts, cathepsin K is the only one for which an essential role in bone resorption has been clearly demonstrated in both mice and humans (*8*). Thus, cathepsin K remains a potential therapeutic target for the treatment of bone diseases such as osteoporosis and autoimmune arthritis, in which osteoclast activity is increased (*9*, *10*).

Through screening, we obtained a potent orally active cathepsin K inhibitor named NC-2300 (Fig. 1A and figs. S1 to S3), which suppresses osteoclastic bone resorption both in vivo and in vitro (figs. S1 and S4). Computer-assisted simulation of the cathepsin K/NC-2300 complex indicated that NC-2300 blocks the active-site cleft where Cys^{25} and His^{162} of cathepsin K form the catalytic site (Fig. 1B and fig. S5). To test the effects of the inhibitor on disease models, we treated adjuvant-induced arthritis (AIA) in rats with oral administration of NC-2300 and compared the results with the effects of alendronate, which is one of the bisphosphonate compounds used clinically as an inhibitor of osteoclastic bone resorption. Bone loss in arthritis occurs mainly in two forms: bone erosion at the inflamed joints and periarticular osteoporosis (*11*). Radiological analysis revealed that NC-2300, but not alendronate, markedly suppressed bone erosion (Fig. 1C), although bone mineral density analysis showed that both compounds had a comparable inhibitory effect on periarticular osteoporosis (fig. S6A). NC-2300 also ameliorated paw swelling (Fig. 1D) and improved locomotive activity (fig. S6B) without affecting the onset rate of arthritis. NC-2300 reduced inflammation even when administered after the onset of disease (fig. S7). These results indicate that cathepsin K also functions in cells other than osteoclasts, allowing it to participate in autoimmune inflammation.

In AIA, local injection of adjuvant stimulates antigen presentation by dendritic cells (DCs), leading to T cell autoimmunity, the production of inflammatory cytokines by macrophages, and osteoclast-mediated bone destruction (*9*, *12*, *13*). The adjuvant effects are mainly dependent on the pathogen-associated molecular patterns (PAMPs)–induced activation of Toll-like receptor (TLR) signaling (*14*, *15*). Therefore, we next analyzed the expression and function of cathepsin K in T cells, macrophages, and DCs. Cathepsin K mRNA was barely detected in nonadherent bone marrow (BM) cells or splenic T cells (Fig. 2A), and NC-2300 showed no effects on T cell activation (fig. S8A). Although macrophages have been reported to express cathepsin K (*4*), NC-2300 had no effects on the activation of BM-derived macrophages stimulated by PAMPs (fig. S8B). BM-derived DCs (BM-DCs) did express a detectable level of cathepsin K mRNA, although this was much lower than expression in osteoclasts (Fig. 2A). Nevertheless, cathepsin K activity was confirmed in DCs and was inhibited by NC-2300 (Fig. 2B).

To investigate whether cathepsin K has a role in antigen presentation in DCs, DCs were cultured with fluorescein isothiocyanate (FITC)–labeled ovalbumin. The uptake of ovalbumin-FITC was observed by flow cytometry in NC-2300–treated DCs as well as in nontreated cells (Fig. 2C). In addition, NC-2300–treated DCs stimulated proliferation of splenic T cells from ovalbumin-specific DO11.10 TCR transgenic mice to an extent similar to that of nontreated DCs (Fig. 2D). These results suggest that cathepsin K activity is not required for the antigen uptake, processing, or presentation by

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DCs. Considering the crucial role of cathepsins L and S in antigen presentation (*3*, *4*, *16*), these results also indicate that the effects of NC-2300 on these cathepsins are negligible.

Complete Freund's adjuvant (CFA) is composed of killed mycobacteria, which contain various PAMPs including TLR2, TLR4, and TLR9 agonists (*14*, *17*). Therefore, we tested the responses of DCs to these PAMPs with or without cathepsin K inactivation. Enzyme-linked immunosorbent assay revealed that the production of cytokines such as interleukin-12 (IL-12) and IL-23 by DCs was significantly inhibited by NC-2300 when stimulated with oligodeoxynucleotides containing unmethylated CpG motif (CpG: the TLR9 ligand), but not with Malp2 and peptidoglycan (PGN) (the TLR2 ligands) or lipopolysaccharide (LPS: the TLR4 ligand) (Fig. 2E). CpG-induced expression of IL-6, IL-12, and IL-23 in BM-DCs was down-regulated at the mRNA level (Fig. 2F), and IFN-β production by Flt3L-induced BM-DCs in response to CpG was suppressed by NC-2300 (Fig. 2F). These results suggest that cathepsin K plays an important role in the gene induction program regulated by TLR9 signaling.

Deoxyribonuclease treatment of the mycobacterial components in CFA greatly reduces the severity of AIA without affecting the induction efficiency, and this reduction is recovered by supplementation with CpG DNA (*18*, *19*), indicating that TLR9-mediated immune responses determine the severity of autoimmune inflammation. Because mycobacterial DNA can be detected weeks after CFA inoculation (*19*), CpG DNA may augment autoimmune inflammation throughout the course of arthritis. These observations lend support to the notion that cathepsin K–dependent TLR9 signaling contributes to autoimmune inflammation.

How does cathepsin K regulate TLR9 signaling? After being taken up into the cells, CpG DNA locates within the endosomal compartment, where CpG DNA binds to TLR9 (*14*, *18*). The binding of CpG with TLR9 results in the conformational change of TLR9 (*20*), leading to activation of MyD88 and downstream signaling such as the mitogen-activated protein kinase, interferon regulatory factor (IRF), and nuclear factor kappa B (NF-κB) pathways (*14*). Activation of these pathways leads to the production of inflammatory cytokines and up-regulation of cell-

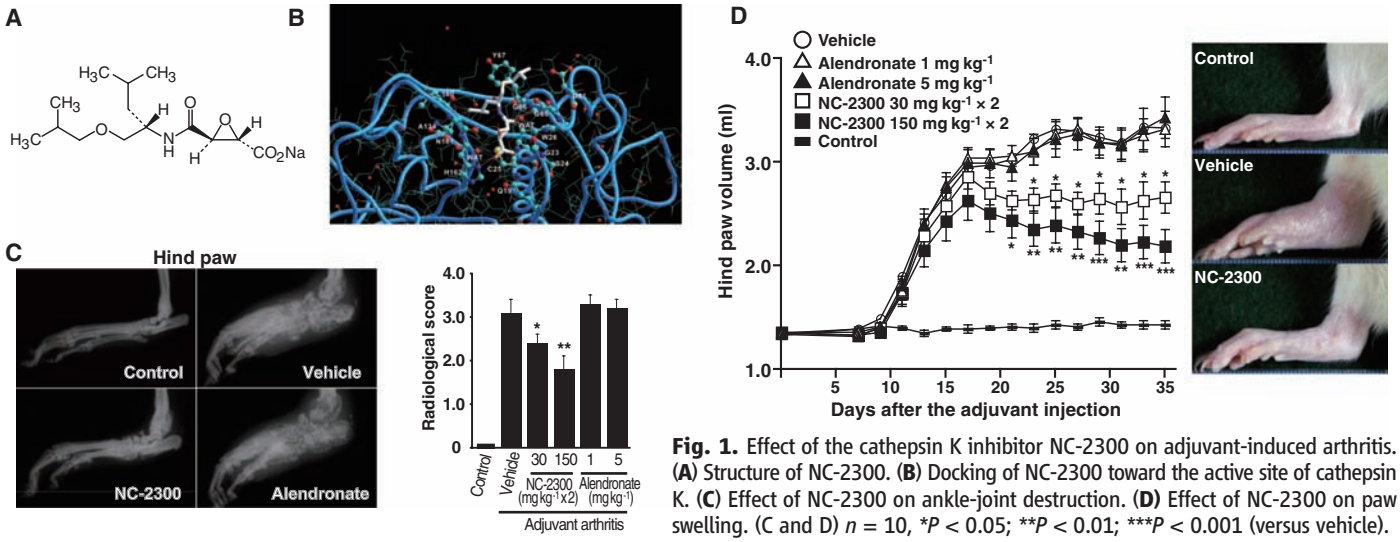

Fig. 1. Effect of the cathepsin K inhibitor NC-2300 on adjuvant-induced arthritis. (**A**) Structure of NC-2300. (**B**) Docking of NC-2300 toward the active site of cathepsin K. (**C**) Effect of NC-2300 on ankle-joint destruction. (**D**) Effect of NC-2300 on paw swelling. (C and D) $n = 10$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (versus vehicle).

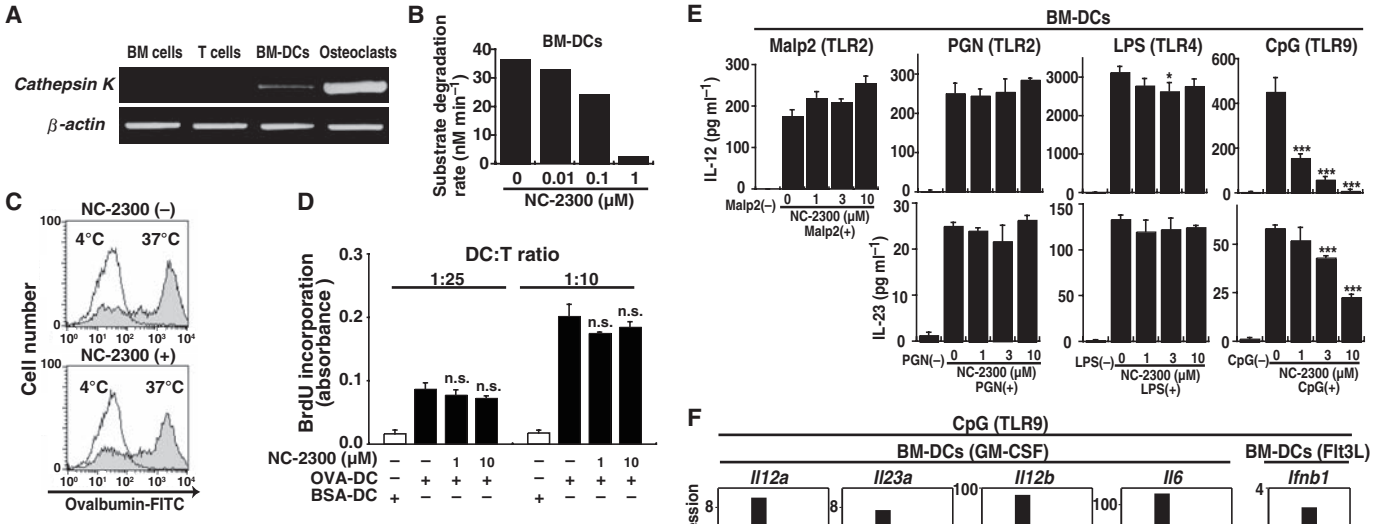

Fig. 2. Selective suppression of TLR9 response in dendritic cells (DCs) by cathepsin K inhibition. (**A**) Reverse transcription–polymerase chain reaction analysis of the cathepsin K mRNA. (**B**) Enzymatic activity of cathepsin K in BM-DCs. (**C**) Effect of NC-2300 on antigen uptake. (**D**) Effect of cathepsin K inhibition on the presentation of exogenous antigens by DCs. BrdU, bromodeoxyuridine; OVA, ovalbumin; BSA, bovine serum albumin; n.s., not significant. (**E**) Effects of cathepsin K inhibition on TLR-stimulated cytokine production. $^{*}P < 0.05$; $^{***}P < 0.001$. (**F**) Effect of NC-2300 on the cytokine mRNA expression in CpG-treated BM-DCs.

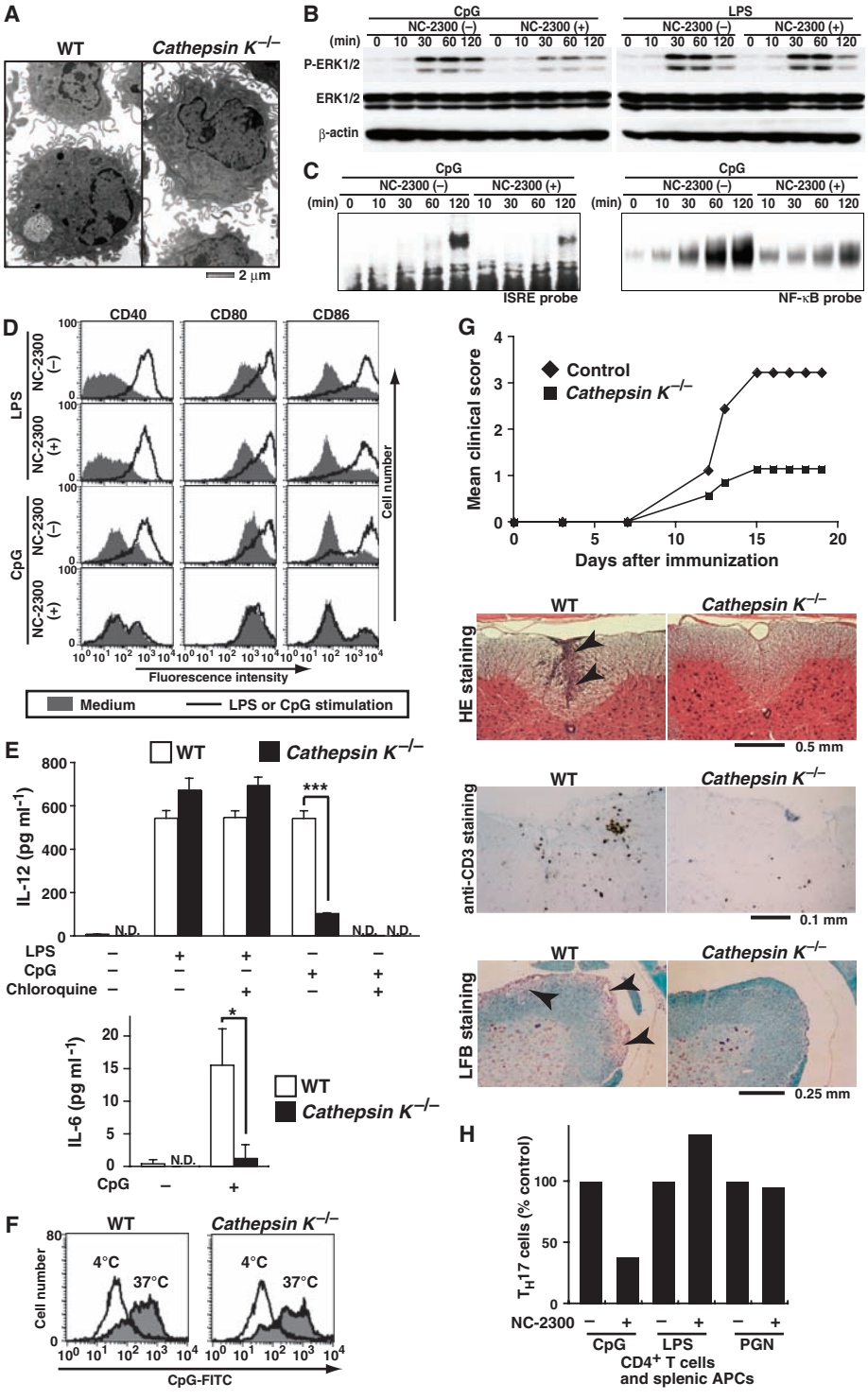

Fig. 3. Cathepsin K regulation of TLR9-mediated immune responses and autoimmune inflammation. (**A**) Electron micrographs of WT and *cathepsin* $K^{-/-}$ BM-DCs. (**B**) Suppression of ERK phosphorylation by NC-2300 in CpG-stimulated DCs. (**C**) Effect of NC-2300 on CpG-induced activation of transcription factors, IRF (left) and NF-κB (right) (electrophoretic mobility shift assay). ISRE, IFN-stimulated response element. (**D**) Impaired TLR9-mediated expression of cell-surface molecules in NC-2300–treated DCs. (**E**) Cytokine production in WT or *cathepsin* $K^{-/-}$ DCs in response to LPS or CpG. N.D., not detected. *$P < 0.05$; ***$P < 0.001$. (**F**) Uptake of CpG in *cathepsin* $K^{-/-}$ DCs. (**G**) Severity of EAE in *cathepsin* $K^{-/-}$ mice. Clinical scores (top). Lumbar spinal cord sections stained with hematoxylin and eosin (HE), immunostained for CD3, and stained with luxol fast blue (LFB) (bottom). Arrowheads indicate inflammatory cellular infiltrates (HE) and demyelinated areas (LFB). (**H**) Cathepsin K inactivation results in defective T_H17 polarization in response to CpG, but not to LPS or PGN.

surface molecules such as CD40, CD80, and CD86 in DCs (*2*, *21*). Compounds that block endosomal acidification such as chloroquine inhibit CpG-driven signaling (*14*, *18*). A normal number of BM-DCs were generated in BM cells derived from *cathepsin* $K^{-/-}$ mice (*5*, *22*), and they exhibited a normal morphology (Fig. 3A), suggesting that cathepsin K is dispensable for the DC differentiation. CpG-induced, but not LPS-induced, phosphorylation of extracellular-signal regulated kinases 1 and 2 (ERK 1/2) in DCs was suppressed by cathepsin K inactivation (Fig. 3B). CpG-induced activation of IRF and NF-κB was also suppressed by cathepsin K inactivation in DCs (Fig. 3C). In addition, NC-2300 reduced expression of CD40, CD80, and CD86 in DCs (Fig. 3D). CpG-induced, but not LPS-induced, production of cytokines such as IL-12 and IL-6 in *cathepsin* $K^{-/-}$ DCs was significantly suppressed (Fig. 3E). Thus, cathepsin K inactivation leads to the blockade of essentially all the downstream pathways of TLR9 signaling in DCs, suggesting that cathepsin K plays a critical role in the signaling events proximal to TLR9. However, the endocytosis of CpG was not affected in *cathepsin* $K^{-/-}$ DCs (Fig. 3F) and the endosomal acidification was not inhibited by NC-2300 (fig. S9), whereas chloroquine suppressed the cathepsin K activity in DCs (fig. S10).

It remains to be elucidated how cathepsin K regulates CpG-TLR9 signaling in the endosome, but the results of pharmacological inhibition indicate that the proteolytic activity of this enzyme is crucial for the mechanism. It is conceivable that cathepsin K may be involved in the degradation of proteins that inhibit the interaction between CpG and TLR9, or cathepsin K–mediated proteolysis may result in the conformational change of TLR9 that augments its signal transduction (*20*). Because fluorescence resonance energy transfer analysis showed that the CpG-induced conformational change of TLR9 was not affected in human embryonic kidney (HEK) 293 cells by NC-2300 (fig. S11), we cannot rule out the possibility that cathepsin K degrades a cytoplasmic protein that modifies the proximal TLR9 signaling. The observation that *cathepsin* $K^{-/-}$ DCs responded normally to TLR3 or TLR7/8 stimulation suggests that the role of cathepsin K is TLR9-specific in DCs (fig. S12).

To clearly demonstrate that cathepsin K plays a critical role in autoimmune inflammation in an osteoclast-independent manner, we subjected *cathepsin* $K^{-/-}$ mice to experimental autoimmune encephalomyelitis (EAE), in which TLR9 signaling plays an important role (*23*, *24*). The frequency of the onset of EAE was not different between control and *cathepsin* $K^{-/-}$ mice, but the severity of the paralytic symptoms was much lower in the *cathepsin* $K^{-/-}$ mice than in control mice (Fig. 3G, top). Histological analysis of spinal cords demonstrated a marked decrease in inflammation (Fig. 3G, HE staining), T cell infiltration (Fig. 3G, anti-CD3 staining), and demyelination (Fig. 3G, LFB staining) in *cathepsin* $K^{-/-}$ mice. Because T helper 17 (T_H17) cells play an essential role in the autoimmune inflammation in EAE (*25*), we examined the effect of cathepsin K inactivation on the ability of DCs to induce

T_H17 cells. The ability of DCs to induce T_H17 cells was markedly inhibited by cathepsin K inactivation when stimulated with CpG, but not with LPS or PGN (Fig. 3H). Taken together with the results on the role of cathepsin K in CpG-induced cytokine expression in DCs, the impaired induction of T_H17 cells by cathepsin K inactivation was caused, at least in part, by the reduced DC expression of cytokines that are involved in the induction and expansion of T_H17 cells such as IL-6 and IL-23 (*25*, *26*).

Our results show that cathepsin K, which was thought to be an osteoclast-specific enzyme, plays a critical role in the immune system. Cathepsin K functions under the acidified conditions in the endosome, where engagement of CpG by TLR9 occurs, and plays an important role in the signaling events proximal to TLR9. Thus, careful attention should be paid to the side effects of cathepsin K inhibitors on the immune system in the treatment of osteoporosis, whereas they may have dual benefits in the treatment of autoimmune arthritis, the pathogenesis of which is dependent on both DCs and osteoclasts (*9*).

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Supporting Online Material

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Systemic Leukocyte-Directed siRNA Delivery Revealing Cyclin D1 as an Anti-Inflammatory Target

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Cyclin D1 (CyD1) is a pivotal cell cycle–regulatory molecule and a well-studied therapeutic target for cancer. Although CyD1 is also strongly up-regulated at sites of inflammation, its exact roles in this context remain uncharacterized. To address this question, we developed a strategy for selectively silencing CyD1 in leukocytes in vivo. Targeted stabilized nanoparticles (tsNPs) were loaded with CyD1–small interfering RNA (siRNA). Antibodies to β_7 integrin (β_7 I) were then used to target specific leukocyte subsets involved in gut inflammation. Systemic application of β_7 I-tsNPs silenced CyD1 in leukocytes and reversed experimentally induced colitis in mice by suppressing leukocyte proliferation and T helper cell 1 cytokine expression. This study reveals CyD1 to be a potential anti-inflammatory target, and suggests that the application of similar modes of targeting by siRNA may be feasible in other therapeutic settings.

RNA interference (RNAi) has emerged as a powerful strategy for suppressing gene expression, offering the potential to dramatically accelerate in vivo drug target validation, as well as the promise to create novel therapeutic approaches if it can be effectively applied in vivo (*1*). Cyclin D1 (CyD1) is a key cell cycle–regulating molecule that governs the proliferation of normal and malignant cells (*2*, *3*). In inflammatory bowel diseases, colon-expressed CyD1 is aberrantly up-regulated in both epithelial and immune cells (*4*, *5*). Although CyD1 has also been implicated in promoting epithelial colorectal dysplasia and carcinogenesis, it is not clear whether leukocyte-expressed CyD1 contributes directly to the pathogenesis of inflammation and whether it might serve as a therapeutic target.

To address these questions, we used RNAi silencing of CyD1 in an experimental model of intestinal inflammation. A major limitation to the use of RNAi in vivo is the effective delivery of siRNAs to the target cells (*6*, *7*). RNAi in leukocytes, a prime target for anti-inflammatory therapeutics, has remained particularly challenging, as these cells are difficult to transduce by conventional transfection methods and are often disseminated throughout the body, thus requiring systemic delivery approaches (*8*). One possibility is to use integrins, which are an important family of cell-surface adhesion molecules, as targets for siRNA delivery (*8*). Specifically, we have shown that antibody-protamine fusion proteins directed to the lymphocyte function-associated antigen–1 (LFA-1) integrin selectively delivered siRNAs to leukocytes, both in vitro and in vivo (*8*). However, whether an integrin-directed siRNA delivery approach can induce

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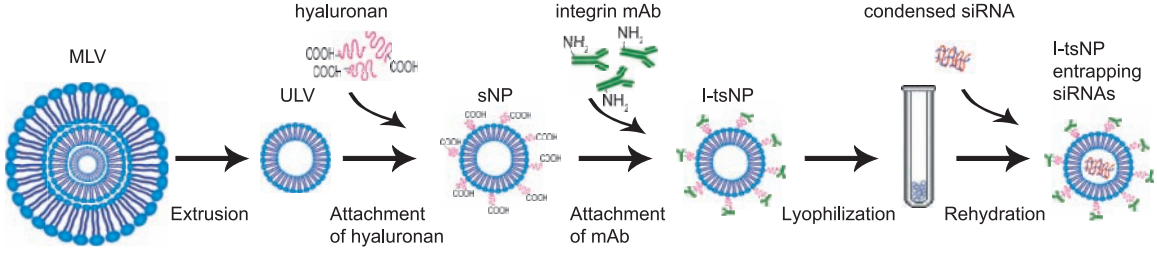

Fig. 1. The processes involved in generating I-tsNPs. Multilamellar vesicle (MLV) [prepared as described in (*9*)] is extruded to form a unilamellar vesicle (ULV) with a diameter of ~100 nm. Hyaluronan is covalently attached to DPPE in the ULV. A monoclonal antibody (mAb) to the integrin is covalently attached to hyaluronan, generating I-tsNP. siRNAs are entrapped by rehydrating lyophilized β_7 I-tsNP with water containing protamine-condensed siRNAs.