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Cytomegalovirus Promotes Intestinal Macrophage-mediated Mucosal Inflammation through Induction of Smad7

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Abstract

Intestinal macrophages in healthy human mucosa are profoundly down-regulated for inflammatory responses (inflammation anergy) due to stromal TGF- β inactivation of NF- κ B. Paradoxically, in cytomegalovirus (CMV) intestinal inflammatory disease, one of the most common manifestations of opportunistic CMV infection, intestinal macrophages mediate severe mucosal inflammation. Here we investigated the mechanism whereby CMV infection promotes macrophage-mediated mucosal inflammation. CMV infected primary intestinal macrophages but did not replicate in the cells or reverse established inflammation anergy. However, CMV infection of precursor blood monocytes, the source of human intestinal macrophages in adults, prevented stromal TGF- β -induced differentiation of monocytes into inflammation anergic macrophages. Mechanistically,

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Author Contributions

EAD, PDS, LES and WJB designed the study. EAD conducted experiments and acquired data. RS and JG provided segments of human jejunum. LCM and LES assisted with the isolation of primary intestinal macrophages isolation and imaging of NF- κ B p65 by confocal microscopy. RG designed primers for CMV UL71 and assisted with the PCR detection of IE and UL71 and imaging of Smad7 by confocal. ML assisted with fluorescent *in situ* hybridization and confirmation of CMV infection in primary intestinal macrophages. MAB designed primers for detection of CMV IE1 in primary intestinal macrophages. MS propagated CMV TR stocks. JJS and MEN assisted with *M. tb* infection of THP-1 and primary intestinal macrophages. EAD, LES and PDS analyzed the data and wrote the manuscript.

Disclosures

The authors have no disclosures.

CMV up-regulated monocyte expression of the TGF- β antagonist Smad7, blocking the ability of stromal TGF- β to inactivate NF- κ B, thereby enabling MyD88- and NF- κ B-dependent cytokine production. Smad7 expression also was markedly elevated in mucosal tissue from subjects with CMV colitis and declined after antiviral ganciclovir therapy. Confirming these findings, transfection of Smad7 antisense oligonucleotide into CMV-infected monocytes restored monocyte susceptibility to stromal TGF- β -induced inflammation anergy. Thus, CMV-infected monocytes that recruit to the mucosa, not resident macrophages, are the source of inflammatory macrophages in CMV mucosal disease and implicate Smad7 as a key regulator of, and potential therapeutic target for, CMV mucosal disease.

Introduction

The gastrointestinal tract mucosa is a major site of opportunistic cytomegalovirus (CMV) infection, causing or exacerbating mucosal inflammation that may result in severe end-organ dysfunction¹⁻⁴. Although the clinical manifestations of mucosal CMV infection are well characterized, the mechanism by which CMV promotes mucosal inflammation is not known. After primary infection, CMV typically persists in the bone marrow, infecting progenitor myeloid cells that enter the circulation as latently infected blood monocytes⁵⁻⁷. CMV-infected monocytes that recruit into the intestinal lamina propria differentiate into infected macrophages that are positioned to contribute to CMV mucosal disease. Indeed, key features of CMV mucosal disease include foci of cells with characteristic CMV inclusions, accumulation of CMV-infected lamina propria macrophages^{8,9} and macrophage-derived pro-inflammatory cytokines¹⁰⁻¹², implicating a central role for intestinal macrophages in the inflammatory process. In sharp contrast, resident intestinal macrophages in healthy human intestinal mucosa are strictly non-inflammatory (inflammation anergic)¹³⁻¹⁵ and thus tolerant of bacteria and immunostimulatory products that normally elicit inflammatory responses¹⁶⁻¹⁹.

Inflammation anergy is induced when recruited monocytes, the source of intestinal macrophages after infancy²⁰⁻²², embed in the intestinal extracellular matrix (stroma) and encounter stromal products, including TGF- β , which drives Smad signaling and NF- κ B inactivation¹⁵. The consequence of these altered signaling pathways is potent down-regulation of TLR-inducible inflammatory responses, despite normal expression of TLRs and other pattern recognition receptors^{15,23,24}. Despite their down-regulated pro-inflammatory phenotype and function, resident intestinal macrophages retain potent phagocytic and microbiocidal activity, enabling the cells to provide non-inflammatory defense against microbes, a primary function of resident macrophages in healthy intestinal mucosa^{14,17,18}.

To address the paradox in which intestinal macrophages are pro-inflammatory in CMV mucosal disease, but profoundly inflammation anergic in healthy mucosa, we reasoned that CMV infection either reverses established inflammation anergy in intestinal macrophages or disrupts the development of inflammation anergy in monocytes that are infected by the virus prior to their recruitment into the mucosa. Exploring the impact of CMV infection on macrophage function, we recently reported that CMV infection significantly enhances blood

monocyte NF- κ B signal transduction and bacteria-induced cytokine release²⁵, raising the possibility that CMV similarly hijacks NF- κ B signaling in resident intestinal macrophages to promote inflammation in CMV-infected mucosa. Here, we show that CMV infection of monocytes blocked the development of stromal TGF- β -induced inflammation energy during monocyte-to-intestinal macrophage differentiation due to enhanced expression of the TGF- β antagonist Smad7. Our findings suggest a mechanism by which blood monocytes infected with CMV prior to their migration into the intestinal mucosa resist stromal TGF- β -mediated inflammation energy, thereby retaining their capacity to respond to bacteria and bacterial products with inflammatory cytokine production and implicate Smad7 as a novel therapeutic target in CMV mucosal inflammatory disease.

Results

Primary intestinal macrophages support CMV infection but not replication

Human blood monocyte-derived macrophages support productive CMV infection, as we²⁵ and others^{26–28} have shown, but whether primary human intestinal macrophages also support CMV infection and replication has not been determined. As shown in Fig. 1A, freshly isolated human intestinal macrophages were infected with increasing amounts of infectious CMV (TR strain) contained increasing amounts of CMV DNA beginning on day 4 post-infection. Internalization of the virus was confirmed by the presence of intracellular CMV DNA detected by fluorescence *in situ* hybridization (Fig. 1B), and early gene transcription was confirmed by the expression of CMV IE1 and UL71 transcripts (Fig. 1C). To determine whether CMV-infected intestinal macrophages also support viral replication, primary intestinal macrophages were infected with CMV, and viral DNA was quantified 2, 4 and 6 days later. CMV-infected intestinal macrophages did not support replication, whereas CMV-infected control cells, including human monocyte-derived macrophages, human foreskin fibroblasts (HFFs), and primary human intestinal fibroblasts showed a progressive increase in intracellular viral DNA over time (Fig. 1D). Furthermore, intestinal macrophages infected with CMV and exposed 6 days later to soluble *Escherichia coli* LPS (hereafter referred to as LPS) or infected with live *Mycobacterium tuberculosis* did not contain increased amounts of CMV DNA over time, although CMV-infected THP-1 macrophages showed a progressive increase in viral DNA (Fig. 1E). These findings indicate that intestinal macrophages support CMV infection, but not replication, even in the presence of extracellular or intracellular stimulation.

CMV infection does not reverse inflammation energy in primary intestinal macrophages

Among all resident tissue macrophages, intestinal macrophages are uniquely non-inflammatory due to stromal TGF- β down-regulation of NF- κ B activation^{14,15}. To determine whether CMV infection of intestinal macrophages reverses established inflammation energy and promotes bacteria-inducible inflammatory responses, primary human intestinal macrophages were CMV- or mock-infected for 4 days, stimulated with optimal concentrations of *Salmonella* Typhimurium flagellin (a TLR5 agonist, hereafter referred to as flagellin), LPS (a TLR4 agonist) or live *E. coli* (LF82, an adherent invasive strain associated with Crohn's disease), and then analyzed for pro-inflammatory cytokine production. As expected, mock-infected intestinal macrophages did not release IL-6, TNF- α

or IL-1 β in response to stimulation, and, importantly, CMV infection did not promote inducible cytokine release by the intestinal macrophages (Fig. 2A). In sharp contrast, mock-infected blood monocytes stimulated similarly with flagellin, LPS or *E. coli* released IL-6, TNF- α and IL-1 β , and CMV-infected monocytes released even larger amounts of the cytokines in a CMV concentration-dependent manner (Fig. 2B). Thus, CMV infection of primary intestinal macrophages did not reverse established inflammation anergy, indicating that resident intestinal macrophages infected with CMV lack the capacity to mediate CMV inflammatory disease.

CMV infection of precursor monocytes blocks the development of inflammation anergic macrophages

We next examined whether CMV infection of blood monocytes, the source of intestinal macrophages in adults^{20–22,29}, disrupts the development of inflammation anergy. Since CMV-infected blood monocytes are significantly more pro-inflammatory than mock-infected monocytes (Fig. 2B), we tested whether CMV infection of monocytes confers upon the cells the ability to resist stromal TGF- β -induced inflammation anergy using our established *in vitro* system that recapitulates the differentiation of monocytes recruited to the lamina propria stroma into intestinal macrophages^{14,15,30}. We first generated human intestinal stroma-conditioned media (S-CM) from isolated intestinal stroma using our previously reported protocol¹⁴ (Supplemental Fig. 1A). The S-CMs, which contained 9 key regulatory cytokines (Supplemental Fig. 1B), caused a dose-dependent decrease in monocyte production of flagellin- and LPS-induced TNF- α (Fig. 3A), resulting in inflammation anergic cells that we have shown to be phenotypically and functionally nearly identical to primary intestinal macrophages^{14,15}. Importantly, TGF- β blockade reversed S-CM-induced inflammation anergy¹⁴, implicating TGF- β as the key mediator of S-CM inhibitory activity.

To determine whether CMV infection of monocytes prevents S-CM down-regulation of inducible cytokine production, monocytes were CMV- or mock-infected for 24 to 96 h, exposed to an optimal dose of S-CM (Supplemental Fig. 1C) for 1 h to recapitulate recruitment into the mucosa, and then stimulated with flagellin in the continued presence of S-CM; control cells were exposed to media without S-CM. Monocytes infected with CMV for less than 48 h did not enhance flagellin-inducible cytokine (IL-6) production and were highly susceptible to stromal down-regulation (Fig. 3B). However, cells that were CMV-infected for 48 and 96 h released significantly more flagellin-induced IL-6 (or TNF- α , not shown) than mock-infected cells and were resistant to stromal down-regulation (Fig. 3B), indicating that at least 48 h of CMV infection was necessary to block inflammation anergy. CMV infection (48 h) also blocked stromal down-regulation of LPS-induced TNF- α protein and mRNA expression (Fig. 3C). In this connection, at least 48 h of CMV infection is required to enhance expression of TLR5, TLR4, and CD14 in infected monocyte-derived macrophages²⁵. Monocytes infected similarly with human herpesvirus 6 (HHV-6) for 48 h were highly susceptible to stromal down-regulation of IL-6 (Supplemental Fig. 2), suggesting that among the two betaherpesviruses that infect human monocytes, only CMV blocks inflammation anergy. Notably, monocytes that were first treated with S-CM, mimicking inflammation anergic resident intestinal macrophages, and exposed subsequently to CMV did not produce increased levels of LPS-induced IL-6 (or TNF- α , not shown) (Fig.

3D), similar to primary intestinal macrophages (Fig 2A). Together, these findings indicate that CMV infection of precursor blood monocytes blocks stromal down-regulation of inducible pro-inflammatory cytokine release.

CMV enhancement of inducible cytokine production is dependent on increased MyD88 expression

TLR ligation induces the dimerization of the TLR cytosolic Toll/IL-IR (TIR) domains, which activates the recruitment and binding of MyD88, triggering activation of the NF- κ B signal cascade³¹. Having shown that CMV enhances TLR inducible cytokine production by monocytes despite exposure to stromal products, we investigated whether CMV up-regulates the expression of MyD88, the master regulator of TLR signaling, in the presence of S-CM. As shown in Fig. 4, CMV infection up-regulated the constitutive and TLR4-induced expression of MyD88 mRNA (Fig. 4A) and protein (Fig. 4B) in the presence of S-CM, extending our previous finding of CMV-enhanced MyD88 expression in the absence of stromal products²⁵. Next, we determined whether increased MyD88 expression is required for CMV to enhance inducible cytokine production using monocytes transfected with control or MyD88 siRNA (Fig. 4C). CMV-infected monocytes transfected with control siRNA released significantly more LPS-induced TNF- α compared with mock-infected cells, even in the presence of S-CM, whereas CMV-infected monocytes transfected with MyD88 siRNA did not release increased amounts of TNF- α in the absence or presence of S-CM (Fig. 4D). Thus, CMV-mediated enhancement of inducible monocyte cytokine production in the absence and presence of stromal products is dependent, in part, on up-regulation of MyD88.

CMV infection of monocytes blocks stromal inactivation of NF- κ B signaling

During the differentiation of blood monocytes into macrophages in the intestinal lamina propria, exposure to stromal TGF- β inactivates NF- κ B, leading to profound down-regulation of TLR-induced cytokine production¹⁵. Since CMV enhances the inducible phosphorylation of NF- κ B p65 in human monocyte-derived macrophages²⁵, we investigated whether CMV blocks stromal TGF- β inactivation of NF- κ B p65. Compared with mock-infected cells, CMV infection significantly enhanced LPS-induced NF- κ B p65 phosphorylation in the presence of S-CM (Fig. 5A). Because the phosphorylation of NF- κ B p65 increases transcriptional activity, but not NF- κ B nuclear translocation or DNA binding³², we also examined the effect of CMV infection on LPS-induced NF- κ B p65 nuclear translocation in the presence and absence of down-regulatory stromal products. In mock-infected monocytes, LPS triggered NF- κ B translocation into the nucleus (Fig. 5B, 1st row panels), but when the cells were pre-treated with S-CM and then stimulated with LPS, NF- κ B p65 remained sequestered in the cytoplasm (Fig. 5B, 3rd row panels). In contrast, when the monocytes were infected with CMV and then stimulated with LPS, NF- κ B p65 translocated to the nucleus in the absence, as well as the presence, of S-CM (Fig. 5B, 2nd and 4th row panels, respectively), indicating that CMV infection blocks S-CM-induced inhibition of NF- κ B p65 nuclear translocation.

To further dissect the role of NF- κ B in CMV blockade of inflammation energy, we tested the ability of CMV infection to block stromal down-regulation of inducible cytokine release in

the presence of the NF- κ B inhibitor SN50, a cell-permeable peptide that carries the nuclear localization sequence (residues 360-369) of NF- κ B³³. SN50 is derived from the p50/NF- κ B1 subunit of NF- κ B and prevents the DNA binding of NF- κ B³³. As shown in Fig. 5C, a non-specific control peptide SN50M had no inhibitory effect on CMV-enhanced cytokine production, whereas NF- κ B inhibitor SN50 blocked CMV-enhanced inducible cytokine production in the absence as well as in the presence of S-CM. These findings indicate that CMV enhances NF- κ B p65 phosphorylation and translocation into the nucleus, despite the presence of down-regulatory stromal TGF- β , and that CMV enhancement of inducible cytokine production is dependent on NF- κ B activation.

CMV enhances expression of Smad7, the inhibitor of TGF- β signaling

Stromal TGF- β activates the Smad pathway, inducing expression of I κ B α , which sequesters NF- κ B in the cytoplasm and leads to inflammation energy in healthy human intestinal macrophages^{14,15}. The Smad pathway is regulated by Smad7, which after transcriptional activation and release into the cytoplasm associates with the TGF- β receptor to antagonize Smad signaling³⁴. To determine whether CMV infection disrupts stromal TGF- β -induced Smad signaling, we examined whether CMV enhances monocyte expression of Smad7, which is not expressed by normal human intestinal macrophages¹⁵. CMV infection of blood monocytes significantly increased the expression of Smad7 mRNA (Fig. 6A) and protein (Fig. 6B) compared with mock-infected monocytes. Using immunofluorescence and confocal microscopy, we next localized Smad7 in CMV- and mock-infected monocytes on days 1, 4, and 6 post-infection. As early as one day post-infection, CMV increased the proportion of cells with perinuclear and/or cytoplasmic localization of Smad7 compared with mock-infected cells, which contained Smad7 mainly in the nucleus. On days 4 and 6 post-infection, CMV induced cytoplasmic localization of Smad7 and increased the density of Smad7 and the proportion of cells that expressed Smad7 (Figs. 6C & 6D), consistent with CMV enhancement of Smad7 expression.

Extending the above *in vitro* findings, colonic tissue from two subjects with acute exacerbation of ulcerative colitis became available for further study. The mucosal tissue from the subject shown in Fig. 6E contained CMV inclusion cells (2.8×10^5 CMV DNA copies/biopsy, 3.2×10^5 CMV DNA copies/mL blood) and an accumulation of cells that expressed cytoplasmic Smad7 in areas where CMV DNA and infiltrating CD14⁺ monocytes were located (Fig. 6E, **upper panels**). Low numbers of CMV-immunoreactive cells were scattered in the lamina propria, which is typical of CMV colitis^{8,9,12}. The infected cells were associated with many Smad7-expressing cells, likely due to CMV-induced production of cytokines such as TNF- α , which we¹² and others¹⁰ have previously reported. In this connection, Smad7 expression is induced by activators of NF- κ B, including TNF- α and IL-1 β , in an NF- κ B p65/RelA-dependent manner³⁵. In contrast, colonic tissue from the same representative subject 5 months after ganciclovir therapy (4.4×10^3 CMV DNA copies/biopsy, 2.8×10^5 CMV DNA copies/mL blood) displayed much lower expression of Smad7 and fewer CD14⁺ cells (Fig. 6E, **lower panels**). These findings are consistent with the *in vitro* induction of Smad7 by CMV (Figs. 6A–D) and implicate Smad7 involvement in the potentiation of macrophage-mediated inflammation in CMV mucosal disease.

Inhibition of Smad7 in CMV-infected monocytes restores stromal TGF- β -induced down-regulation of inducible pro-inflammatory cytokine release

Because Smad7 blocks TGF- β signaling³⁴, and CMV infection of monocytes markedly enhances Smad7 expression, we reasoned that inhibition of Smad7 in CMV-infected monocytes would restore stromal TGF- β -induced inflammation energy. Therefore, we tested whether inhibition of Smad7 by a Smad7 antisense oligonucleotide would promote susceptibility to stromal TGF- β down-regulation of inducible cytokine release. Mock and CMV-infected monocytes were transfected with Smad7 sense or antisense oligonucleotides (Fig 7A) and then stimulated with flagellin in the presence or absence of S-CM (Fig 7B). Mock-infected cells transfected with Smad7 sense oligonucleotide were susceptible to S-CM down-regulation of inducible IL-6 production in an S-CM dose-dependent manner, whereas CMV-infected cells transfected with Smad7 sense oligonucleotide were resistant to S-CM down-regulation (Fig 7B). In sharp contrast, transfection with Smad7 antisense oligonucleotide caused CMV-infected cells to release markedly reduced levels of IL-6 (and TNF- α , not shown) in the presence of S-CM. Thus, the inhibition of Smad7 reversed CMV blockade of inflammation energy. These findings implicate Smad7 as a potential therapeutic target in CMV mucosal disease.

Discussion

Healthy human intestinal mucosa is characterized by the absence, or near absence, of inflammation and the presence of inflammation anergic lamina propria macrophages¹⁸. In contrast, CMV infection during immunosuppression is often complicated by severe mucosal inflammation and the accumulation of CMV-infected macrophages^{8,9} that express inflammatory cytokines¹⁰⁻¹². Here we used primary human intestinal macrophages and blood monocytes differentiated *in vitro* by stromal TGF- β into cells with an intestinal macrophage phenotype¹⁸ to elucidate the mechanism by which CMV promotes mucosal macrophage-mediated inflammation. We show that CMV entered and initiated early gene transcription in primary intestinal macrophages but did not replicate in, or induce pro-inflammatory cytokine release from, the cells. The inability of primary intestinal macrophages to support CMV replication is likely due to down-regulated NF- κ B activity. Indeed, NF- κ B activation is required to promote CMV late gene expression³⁷. We also have shown that down-regulation of NF- κ B activity in intestinal macrophages mediates refractoriness to productive HIV-1 infection³⁸. Taken together, these findings indicate that terminally differentiated intestinal macrophages are not permissive to productive CMV infection and that exposure to CMV does not reverse established inflammation energy.

In sharp contrast, CMV infection of monocytes, resulted in productive CMV infection and marked resistance to stromal TGF- β -mediated down-regulation of inflammatory function. Although CMV infection alone did not drive the release of inflammatory cytokines, the infection enhanced monocyte MyD88-dependent NF- κ B signaling and the induction of the TGF- β inhibitor Smad7, resulting in potent inducible cytokine production. Supporting these *in vitro* findings, Smad7 expression was markedly increased in the mucosal tissue of patients with CMV colitis and declined along with local CMV levels after antiviral therapy. Further, transfection of Smad7 antisense oligonucleotide into CMV-infected monocytes restored

monocyte susceptibility to stromal TGF- β down-regulation of inflammatory function, confirming that CMV-induced Smad7 is pivotal for CMV blockade of inflammation anergy. Thus, our findings show for the first time a mechanism by which CMV exploits monocyte NF- κ B and TGF- β signaling to promote the differentiation of mucosal macrophages capable of inflammatory cytokine release in response to tissue-invading bacteria and bacterial products.

Macrophages in major organs, including the brain, lung, liver, spleen and peritoneum, are derived before birth from yolk sac precursor cells and fetal liver hematopoietic stem cells and are maintained by longevity and limited self-renewal^{20,21}. Macrophages in the intestinal mucosa, however, are derived after infancy from circulating blood monocytes, which maintain the gut macrophage population by replenishment^{20–22}. CMV infection of monocytes increases their rate of adhesion and transmigration³⁹, possibly contributing to localization in the lamina propria stroma, and induces monocyte expression of genes implicated in macrophage activation²⁷. Thus, it is not surprising that the gastrointestinal mucosa, rather than other major organs, is the predominant site of CMV end-organ disease during immunosuppression. The recruitment of blood monocytes persistently infected with CMV to the intestinal mucosa delivers TLR ligand-responsive mononuclear phagocytes with enhanced inducible inflammatory activity to the gastrointestinal mucosa. In this location, the infected cells are positioned to interact with bacteria and immunostimulatory products that breach the epithelium. Among the cytokines released by the newly recruited and stimulated monocytes, TNF- α and IL-6 are not only inflammatory cytokines but mediators of viral reactivation and replication.^{6,40} Thus, our data implicate CMV-infected monocytes that migrate into the intestinal mucosa as the source of inflammatory macrophages in CMV mucosal disease.

CMV infection enhanced macrophage expression of the TGF- β antagonist Smad7 *in vitro*, leading to increased inducible cytokine release, thereby implicating Smad7 as a key mediator of CMV potentiation of mucosal inflammation. CMV infection also was associated with Smad7 expression *in vivo* in subjects with ulcerative colitis. Inhibition of Smad7 with a specific antisense oligonucleotide has been shown to suppress inflammatory pathways, reduce colitis in mice⁴¹ and induce clinical remission in patients with Crohn's disease⁴². In our study, transfection of a Smad7 antisense oligonucleotide into CMV-infected monocytes restored stromal TGF- β -induced inflammation anergy. Thus, targeted delivery of Smad7 antisense oligonucleotide could offer a new and safe therapeutic approach that circumvents the myelosuppression and drug-resistance that complicate current antiviral treatment regimens⁴³.

In summary, we have uncovered a mechanism by which CMV blocks the differentiation of inflammatory monocytes into inflammation anergic intestinal macrophages. We show that CMV hijacks this differentiation pathway in the presence of intestinal stromal products by enhancing MyD88 expression, promoting NF- κ B activation and inducing Smad7, thereby antagonizing stromal TGF- β -mediated inactivation of NF- κ B. CMV infection of precursor monocytes leads to the generation of TLR-responsive inflammatory macrophages resistant to down-regulatory stromal TGF- β , allowing the macrophages to react to invading bacteria and immunostimulatory products with a potent inflammatory cytokine response. Elucidation

of the mechanism by which CMV blocks inflammation anergy led us to identify the induction of Smad7 as a key mediator of CMV-induced inflammatory function. Together, these findings broaden our understanding of the pathogenesis of CMV mucosal inflammation and implicate Smad7 as a potential target for interdicting inflammatory responses by CMV-infected monocytes recruited into the intestinal mucosa.

Materials and Methods

Isolation and purification of human intestinal macrophages and blood monocytes

Macrophages were isolated from the mucosa by enzyme digestion and purified by counterflow centrifugal elutriation, as previously described⁴⁴. Blood monocytes from the same donors and additional healthy CMV-seronegative donors were purified by gradient segmentation followed by adherence to plastic⁴⁵, and the media was replaced with RPMI containing 10% human AB serum and antibiotics (complete RPMI). Adherent monocytes displayed morphological features (large, concave nuclei, phagocytic vacuoles) and the phenotype (CD13⁺, HLA-DR⁺, CD14⁺, CD11c⁺) of macrophages^{25,46}.

CMV preparation and quantification of viral stocks

A clinical strain of human CMV strain (TR, a gift from J. Nelson, Oregon Health and Sciences University, Portland OR) was propagated in human foreskin fibroblasts (HFFs). Virus was concentrated by centrifugation at 16,000×*g* for 2 h at 4°C²⁵, re-suspended in RPMI complete media, and frozen at -80°C. CMV titers were determined using our standard assay for the detection of early antigen fluorescent foci (DEAFF) in HFFs⁴⁷.

CMV, HHV-6 and *M.tb* infection of monocytes and/or intestinal macrophages

Cells were incubated with virus or media (mock infection) at a MOI of 1, 5, or 10 for 2 h, washed with RPMI, and then incubated for 2 to 14 days, depending on the experiment. Cells were treated with trypsin for 30 s to remove non-internalized adherent virus and then washed with RPMI plus fetal bovine serum to neutralize the trypsin. CMV infection was confirmed by immunofluorescence or qPCR for CMV DNA, as previously described⁴⁸. Expression of CMV immediate early (IE)1 and UL171 transcripts were quantified by PCR following conversion of RNA to cDNA, using IE1 forward (5' TCCTGCAGACTATGTTAAGG) and reverse (5' ATCTAAGGCATTCTGCAAACATCC) and UL171 forward (5' - AGTCACTAGTATGCAGCTGGCCCAGCGC) and reverse (5' - AGTCCCTCGAGCGCACTTGCTCTG) primers. HHV-6B strain Z-29 (a gift from M. Prichard, University of Alabama at Birmingham, Birmingham, AL) was propagated in cord blood lymphocytes, and virus stock solutions were prepared and cells were infected as previously described⁴⁹. In the indicated experiment, human intestinal macrophages and THP-1 macrophages were infected with *M. tb* (MOI 10), as previously described⁵⁰. Greater than 80% of mock- and CMV-infected cells remained viable throughout the infection time course, as determined by propidium iodide staining.

Preparation of stroma-conditioned media (S-CM)

Normal jejunum was depleted of epithelial and mononuclear cells, leaving the lamina propria extracellular matrix (stroma plus stromal cells), which was cultured in complete

RPMI (1 gm wet weight/mL) without serum for 24 h¹⁴. Culture supernatant was collected, filter-sterilized and stored at -80°C. Only S-CM containing <1.5RUunits/mL endotoxin determined by ELISA was used. S-CM was normalized to a total protein concentration of 500 µg/mL determined by ELISA, unless otherwise indicated. To induce inflammation energy *in vitro*, monocytes were incubated in S-CM for 1h. Culture of human blood monocytes with S-CM resulted in the differentiation of inflammation anergic macrophages that we have previously shown to be phenotypically and functionally similar to primary intestinal macrophages^{14,15}.

Detection of CMV by fluorescence in situ hybridization (FISH)

Cells were fixed in ice cold methanol, incubated for 10 min in 0.1 M triethanolamine, plus acetic anhydride, dehydrated with ethanol, hybridized for 1 h, then exposed to HCMV-specific digoxigenin (DIG)-labeled DNA probe at 37°C overnight. Cells next were treated with 50% formamide/SSC (saline-sodium citrate buffer) and equilibrated in 0.1% Triton X-100/PBS. Cells were blocked in 2% sheep serum/0.1% Triton X-100/PBS for 1 h before addition of anti-DIG-POD antibody 1:500 for 2 h at 37°C. Cells were stained with 0.3% sudan black/70% ethanol, counterstained in Hoechst 1:2000, then imaged by confocal microscopy.

Cytokine protein assay

Cells were exposed to media, *Salmonella* Typhimurium flagellin (1 µg/mL; InvivoGen, San Diego, CA), *Escherichia coli* K12 LPS (1 µg/mL; InvivoGen), or LF82 (adherent invasive *E. coli*, MOI 0.1, a gift from A. Darfeuille-Michaud, Institut Universitaire de Technologie, Clermont-Ferrand, France). After 18 h, supernatants were harvested, and levels of TNF-α, IL-1β, or IL-6 were measured by ELISA (eBioscience, San Diego, CA).

Gene expression analysis

Cells were stimulated with media, *S. Typhimurium* flagellin (1 µg/mL) or *E. coli* K12 LPS (1µg/mL) for 4 h, and total cellular RNA was extracted (Direct-zol RNA Kit, Zymo Research, Irvine, CA). First-strand cDNA was synthesized using the iScript cDNA synthesis Kit (BioRad, Hercules, CA). Genes were amplified in 25 µL mixtures containing TaqMan Universal PCR Master Mix and FAM/MGB-labeled primer probe sets for *TNFα*, *IL6*, *MyD88*, *Smad7* and control gene *GAPDH* (Life Technologies, Carlsbad, CA). Real-time PCR was run for 40 cycles (15 s 95° C, 60 s 60° C) on a Chromo4 PCR system (Biorad) and analyzed with Opticon Monitor™ software, version 3.1. Relative expression rates of target genes in stimulated *versus* unstimulated cells were calculated using the method of 2^{-CT} and presented as relative RNA expression.

Flow cytometry

Cells were fixed with Cytofix Fixation Buffer (BD Biosciences, San Jose, CA), permeabilized with Phosflow Perm Buffer III (BD Biosciences), then stained for 30min with optimal concentrations of PE-conjugated antibodies to p-NF-κB (Cell Signaling, Danvers, MA) or irrelevant antibodies of the same isotype and flouochrome. After staining, the cells

were washed, fixed in Cytofix Fixation Buffer (BD Biosciences), and analyzed by flow cytometry. Data were analyzed with FlowJo software version 10.0.

Immunohistochemistry

We used our previously-described immunofluorescence protocol¹⁴ to stain serial sections of formalin-fixed, paraffin-embedded tissue. Slides were incubated with either rabbit polyclonal anti-Smad7 (Santa Cruz Biotechnology), mouse monoclonal anti-CD14 (Cell Marque, Rocklin, California), or irrelevant isotype-matched antibody overnight at 4°C. Sections then were washed, incubated with anti-mouse or anti-rabbit secondary antibodies, washed, stained with DAPI, and visualized by confocal microscopy.

Protein detection

MyD88 and Smad7 expression were determined by Western blot using antibodies to MyD88 (1:200) or Smad7 (1:300; Santa Cruz Biotechnology) using our previously-described protocol²⁵. Blots were probed with horseradish peroxidase-conjugated secondary antibodies 1:5,000–1:10,000 in blocking buffer for 1 h, and bands were detected using the chemiluminescent method (SuperSignal West Dura, Thermo Scientific, Waltham, MA). Blots were stripped and re-probed with anti-actin (Santa Cruz Biotechnology) to ensure equal loading.

Confocal microscopy

Cells were fixed and permeabilized (20 min with Cytofix/Cytoperm; BD Biosciences), washed (Cytoperm Buffer; BD Biosciences), and then blocked with casein protein (DAKO; Carpinteria, CA) for 1 h. For detection of NF- κ B p65, cells were incubated with rabbit anti-NF- κ B p65 and mouse anti- HCMV p63-27, specific for IE1, or irrelevant antibody (0.05 mg/mL) for 90 min (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed and then incubated with donkey anti-rabbit IgG-FITC or anti-mouse IgG-PE (30 min, 1:500; Jackson ImmunoResearch Laboratories, West Grove, PA). For detection of Smad7, cells were incubated with rabbit anti-Smad7 or irrelevant antibody (overnight at 4°C, 1:50; Santa Cruz Biotechnology, Dallas, TX), washed, and then incubated with donkey anti-rabbit IgG-Cy (45 min, 1:500; Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were washed with PBS and counter-stained with DAPI, and then visualized by confocal microscopy.

Transfections

For MyD88 knock-down, silencer MyD88 siRNA (Ambion by life Technologies, Carlsbad, CA) or TYE Fluorescent-labeled transfection control siRNA (OriGene, Rockville, MD) were diluted to 0.28 μ M in 150 μ L RPMI complete. The standard complexation protocol was employed for Viromer GREEN (Lipocalyx, Weinbergweg, Germany), according to the manufacturer's instructions. Briefly, 0.28 μ M of MyD88 or control siRNA was added to 3 μ L Viromer GREEN, and the mixture was incubated at room temperature for 15 min. For each transfection, 25nM of siRNA plus Viromer GREEN mixture (10 μ L) was added to each well. MyD88 knock-down was confirmed by Western Blot, as described above. For Smad7 knockdown, phosphorothioate single-stranded oligonucleotides matching the region 107–

128 (5'-GCTGCGGGGAGAAGGGGCGAC-3') of the human Smad7 complementary DNA sequence was synthesized in the sense and antisense orientation (Eurofins Genomics, Louisville KY). On day 1 post-infection, mock- or CMV-infected cells were transfected with Smad7 antisense or sense oligonucleotides (2, 4, or 10 µg/mL) by lipofectamine in serum-free media for 4h, then media was removed and replenished with RPMI complete for 48h. Smad7 knock-down was confirmed by Western Blot, as described above.

NF-κB inhibition

Cells were treated with NF-κB inhibitor peptide SN50 (100 nM) or mock peptide (SN50M 100 nM; Milipore, Darmstadt, Germany) for 1h before exposure to media or S-CM (500 µg/mL), then stimulated with LPS (0.1 µg/mL) for 18h. Supernatants were collected for cytokine ELISAs.

Statistics

Data were analyzed using Graph Pad Prism software 135 version 5.03 with Student's *t* test or 1-way ANOVA with Tukey's or Dunnett's Multiple Comparison posttest, where appropriate. Data are expressed as mean ± SEM.

Study approval

Human blood and intestinal tissue specimens were obtained after written informed consent was provided under Institutional Review Board approval.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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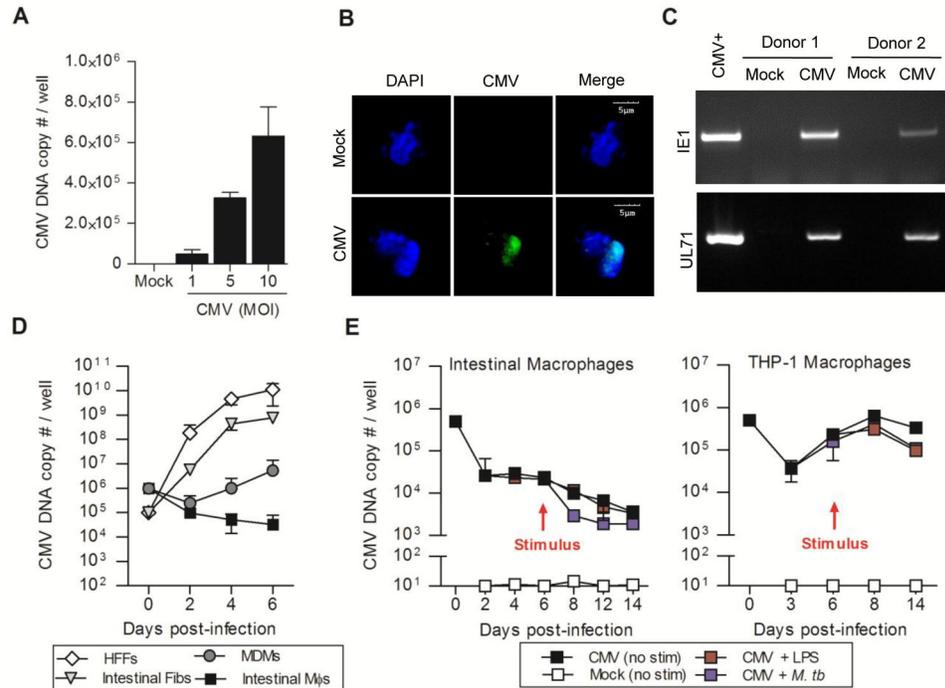


Figure 1. CMV infects but does not replicate in primary human intestinal macrophages
(A) Freshly isolated human intestinal macrophages were exposed to CMV at the indicated multiplicity of infection (MOI) or media (mock), and 4 days later CMV DNA was quantified by qPCR (n=3 donors). **(B)** CMV-infected (MOI 10) and mock-infected intestinal macrophages were analyzed on day 4 post-infection for internalized virus by fluorescence *in situ* hybridization (blue, DAPI-stained nuclei; green, CMV DNA; representative results, n=3 donors). Original magnification, 60x. **(C)** CMV-infected (MOI 5) and mock-infected intestinal macrophages were analyzed on day 4 post-infection for early CMV IE1 and UL71 gene transcripts by PCR (n=2 donors). **(D)** CMV-infected human foreskin fibroblasts (HFFs; MOI 0.5), human intestinal fibroblasts (Intestinal Fibs; MOI 0.5), human monocyte-derived macrophages (MDMs; MOI 1), and human intestinal macrophages (Intestinal MΦs; MOI 1) were analyzed on days 2, 4 and 6 post-infection for CMV DNA by qPCR (n=3 independent experiments). **(E)** CMV-infected (MOI 1) and mock-infected intestinal macrophages and CMV-infected (MOI 1) THP-1 macrophages were exposed to media (no stim), LPS (1 μg/mL) or *M. tb* (MOI 10) on day 6 post-infection, and CMV DNA was quantified subsequently on days 2, 3, 4, 6, 8, 12 and 14 by qPCR (representative results from 1 of 3 independent experiments). Data represent the mean ± SEM.

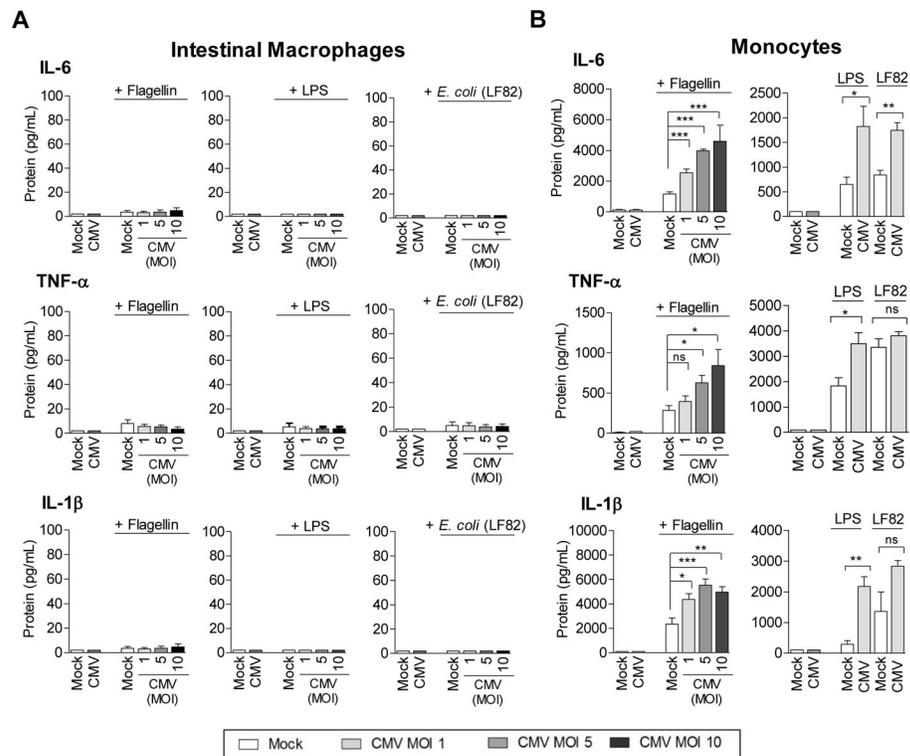


Figure 2. CMV infection of intestinal macrophages does not promote constitutive or inducible cytokine production

(A) Primary human intestinal macrophages and (B) blood monocytes were CMV-infected (MOI of 1, 5 or 10) or mock-infected, cultured for 4 days, and then treated with media, flagellin (1 $\mu\text{g}/\text{mL}$), LPS (1 $\mu\text{g}/\text{mL}$) or LF82 (adherent invasive *E. coli*) (MOI 0.1) for 18 h, and the culture supernatants were harvested and analyzed for IL-6, TNF- α , or IL-1 β (representative results from 1 of 3 donors; one-way ANOVA [Dunnett's multiple comparison's test, each group compared with mock plus stimulus]). Data represent the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

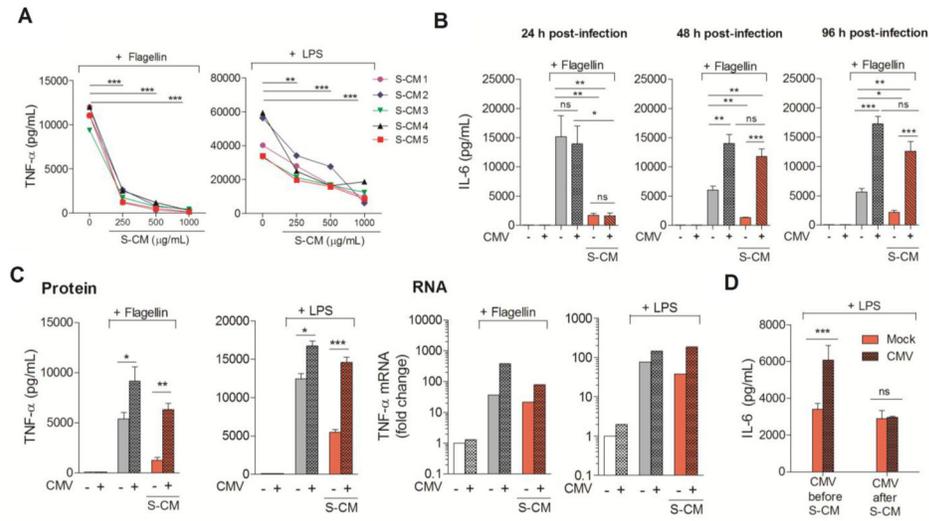


Figure 3. Pre-infection of monocytes with CMV blocks stromal down-regulation of inducible cytokine production

(A) Fresh blood monocytes were exposed to S-CM (0–1000 µg/mL) for 1 h, then flagellin or LPS (0.5 µg/mL) for 18h, and supernatants were analyzed for TNF-α (representative results from 1 of 4 donors, 3–5 S-CMs per donor); one-way ANOVA [Dunnett’s multiple comparison’s test, each group compared with 0 µg/mL S-CM plus stimulus]. (B) Monocytes were mock- or CMV-infected (MOI 5) for 24–96 h, exposed to media or S-CM (500 µg/mL) for 1 h, then flagellin (0.5 µg/mL) for 18 h, and supernatants were analyzed for IL-6 (representative results from 1 of 2 donors, 2 S-CMs per donor); one-way ANOVA [Tukey’s multiple comparison’s test]. (C) Monocytes were mock- or CMV-infected (MOI 5) for 48 h, exposed to media or S-CM (500 µg/mL) for 1 h, then flagellin or LPS (0.5 µg/mL) for 18 h. Supernatants were analyzed for TNF-α (representative results from 1 of 6 independent experiments using monocytes from 4 donors, 3–5 S-CMs per donor); Student’s *t* test), and total RNA was analyzed for TNF-α mRNA (representative from 1 of 3 donors, 1 S-CM per donor). (D) Monocytes were either (i) mock- or CMV-infected (MOI 5) for 96 h then exposed to CMV (500 µg/mL) for 1h or (ii) exposed to S-CM for 1 h then mock- or CMV-infected (MOI 5) for 96 h. Cells then were stimulated with LPS (1 µg/mL) for 18h, and supernatants were analyzed for IL-6 (representative from 1 of 3 donors, 1–2 S-CMs per donor); Student’s *t* test). Data represent means +/-SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.0001.

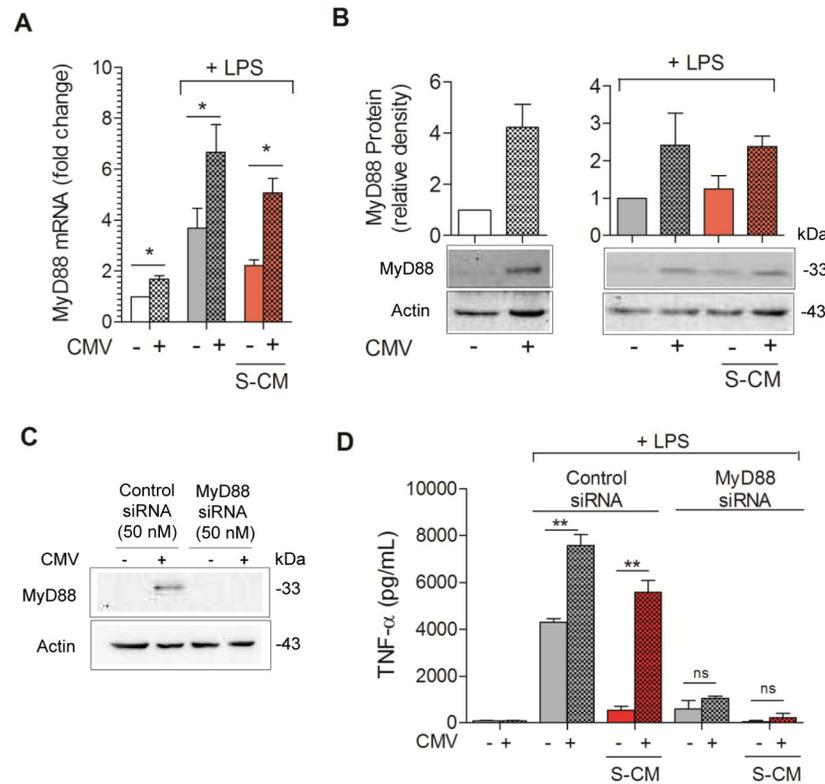


Figure 4. CMV potentiation of inducible cytokine production by monocytes is dependent on enhanced MyD88 expression

Blood monocytes were mock- or CMV-infected (MOI 5) for 3 days, exposed to media or S-CM (500 $\mu\text{g}/\text{mL}$) for 1 h, then exposed to LPS (1 $\mu\text{g}/\text{mL}$) or media for 4 h and analyzed for (A) MyD88 mRNA by qRT-PCR (representative results from 1 of 3 independent experiments using monocytes from 2 donors, 1–2 S-CMs per donor); Student's *t* test) * $p < 0.05$ and (B) MyD88 protein by Western blot (representative results using monocytes from 2 donors, 1 S-CM per donor) with relative density analysis (composite of 3 experiments). (C) Blood monocytes were transfected with control siRNA (50 nM) or MyD88-specific siRNA (50 nM), mock- or CMV-infected (MOI 5), harvested and analyzed 2 days post-infection for MyD88 protein by Western blot to confirm MyD88 knock down (representative results from 1 of 2 donors). (D) Blood monocytes were transfected with control siRNA (50 nM) or MyD88-specific siRNA (50 nM), mock- or CMV-infected (MOI 5) for 2 days and then stimulated with LPS (0.5 $\mu\text{g}/\text{mL}$) for 18 h, after which the culture supernatants were harvested and analyzed for TNF- α (representative results from 1 of 2 donors, 2 S-CMs per donor; Student's *t* test). Data represent the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

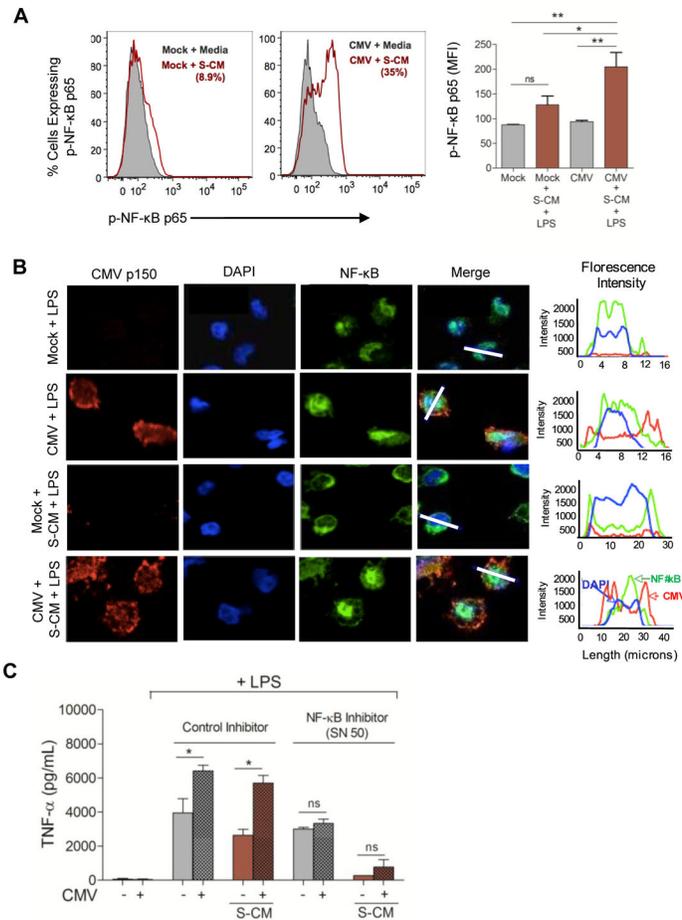


Figure 5. CMV blocks stromal inactivation of monocyte NF-κB p65

(A) Blood monocytes were mock- or CMV-infected (MOI 5) for 2 days, exposed to media or S-CM (500 μg/mL) for 1 h, stimulated with LPS (1 μg/mL) for 5 min and then analyzed for p-NF-κB p65 expression by flow cytometry (left and middle panels, representative donor, % expression; right panel, composite of 3 donors, mean fluorescence intensity (MFI); one-way ANOVA [Tukey's multiple comparison's test] * $p < 0.05$, ** $p < 0.01$). (B) Blood monocytes were mock- or CMV-infected (MOI 1) for 3 days, treated with media or S-CM (100 μg/mL) for 1 h and then stimulated with LPS (1 μL/mL) for 30 min. Cells were harvested and examined by confocal microscopy, and fluorescence histograms were generated to assess nuclear translocation of NF-κB p65 (green, NF-κB p65; blue, DAPI-stained nuclei; and red, pp150⁺ [UL32] CMV-infected cells; representative results from 1 of 3 independent experiments). (C) Blood monocytes were mock- or CMV-infected (MOI 5) for 2 days, treated with control inhibitor (SN 50M; 100 μM) or NF-κB inhibitor (SN 50; 100 μM), with or without exposure to S-CM (500 μg/mL) for 1 h, then exposed to LPS (0.5 μg/mL) or media for 18 h. Culture supernatants were harvested and analyzed for TNF-α (representative results from 1 of 2 donors, 2 S-CMs per donor; Student's *t* test). Data represent the mean ± SEM. * $p < 0.05$.

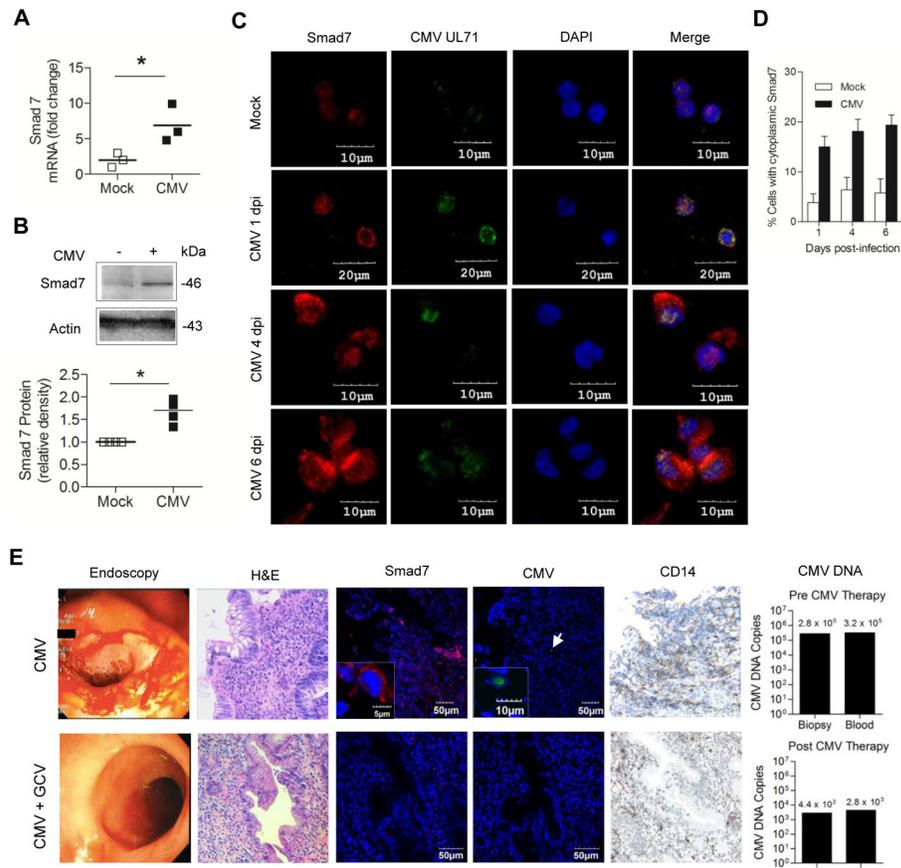


Figure 6. CMV infection of monocytes enhances Smad7 expression

Blood monocytes were mock- or CMV-infected (MOI 5) for 3 days and then analyzed for (A) Smad7 mRNA by qRT-PCR (n=3 experiments using monocytes from 3 donors; Student's paired *t* test) horizontal bar corresponds to the mean. **p* < 0.05; (B) Smad7 protein by Western blot (representative results from 1 of 4 independent experiments using monocytes from 2 donors) with relative density analysis (composite of 4 experiments); Student's paired *t* test) horizontal bar corresponds to the mean. **p* < 0.05; and (C) Smad7 localization in monocytes on days 1, 4, and 6 post-infection by immunofluorescence (red, Smad7; blue, DAPI-stained nuclei; green, UL71; Representative results from 1 of 5 experiments using monocytes from 2 donors). Original magnification, 60x. (D) Cytoplasmic localization of Smad7 was determined on day 1, 4, and 6 post-infection in mock- and CMV-infected cells. For each group, 4 different fields were chosen at random (20–50 cells per field), and the percentage of cells with cytoplasmic localization of Smad7 was calculated. Data are the mean \pm SD from analysis for each set of 4 fields. Original magnification, 20x (n=5 experiments). (E) Colon tissue specimens from a subject with ulcerative colitis and CMV infection before (**upper panels**) and after (**lower panels**) ganciclovir (GCV) therapy (n=2). Endoscopic images were obtained and biopsy consecutive sections were stained with hematoxylin & eosin and analyzed for Smad7 by immunofluorescence (red, Smad7; blue, DAPI-stained nuclei), CMV by fluorescence *in situ* hybridization (blue, DAPI-stained nuclei; green, CMV DNA in infected cells identified by white arrows), and CD14 (DAB). Original magnification for each 20x and insets 100x.

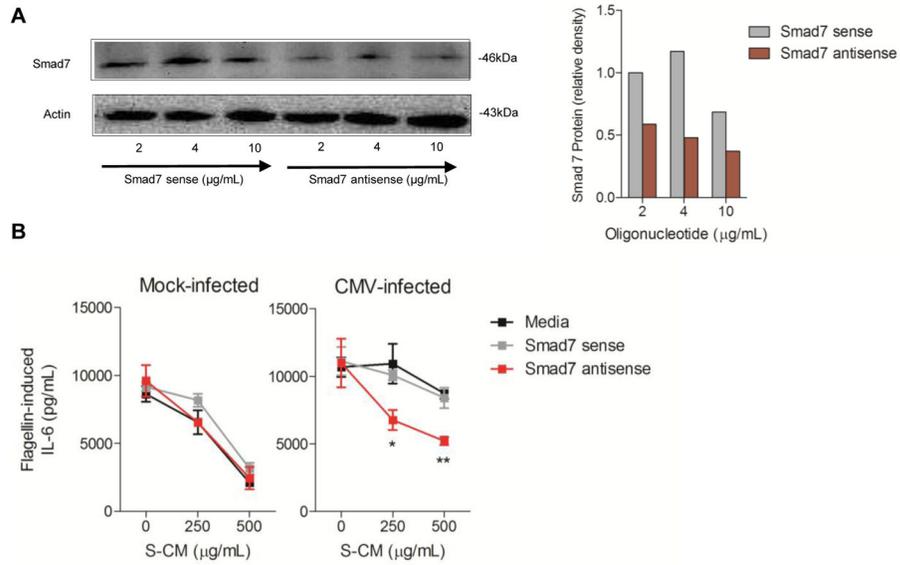


Figure 7. Smad 7 antisense oligonucleotide reverses CMV blockade of inflammation energy
 Blood monocytes were CMV-infected (MOI 5) for 1 day and then transfected with Smad7 sense or antisense oligonucleotides (2, 4 or 10 μg/mL). **(A)** Smad7 knock-down was confirmed 2 days later in CMV-infected cells by Western blot with relative density analysis (representative results from 1 of 2 independent experiments using monocytes from 2 donors). **(B)** CMV- and mock-infected monocytes were transfected 1 day post-infection with 4 μg/mL of Smad7 sense or antisense oligonucleotides, exposed to S-CM (0, 250 or 500 μg/mL) for 1 h, then flagellin (0.5 μg/mL) for 18 h, after which supernatants were analyzed for IL-6 (representative experiment, n=3, performed in duplicate with a single S-CM; one-way ANOVA [Tukey’s multiple comparison’s test]). Data represent the mean +/-SEM. For comparisons between cells transfected with antisense oligonucleotide and cells transfected with sense oligonucleotide or treated with media *p < 0.05 and **p < 0.01.