GSK3β Inhibition Blocks Melanoma Cell/Host Interactions by Downregulating N-Cadherin Expression and Decreasing FAK Phosphorylation

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This study addresses the role of glycogen synthase kinase (GSK)-3β signaling in the tumorigenic behavior of melanoma. Immunohistochemical staining revealed GSK3β to be focally expressed in the invasive portions of 12 and 33% of primary and metastatic melanomas, respectively. GSK3 inhibitors and small interfering RNA (siRNA) knockdown of GSK3β were found to inhibit the motile behavior of melanoma cells in scratch wound, three-dimensional collagen-implanted spheroid, and modified Boyden chamber assays. Functionally, inhibition of GSK3β signaling was found to suppress N-cadherin expression at the messenger RNA and protein levels, and was associated with decreased expression of the transcription factor Slug. Pharmacological and genetic ablation of GSK3β signaling inhibited the adhesion of melanoma cells to both endothelial cells and fibroblasts and prevented transendothelial migration, an effect rescued by the forced overexpression of N-cadherin. A further role for GSK3β signaling in invasion was suggested by the ability of GSK3β inhibitors and siRNA knockdown to block phosphorylation of focal adhesion kinase (FAK) and increase the size of focal adhesions. In summary, we have, to our knowledge, demonstrated a previously unreported role for GSK3β in modulating the motile and invasive behavior of melanoma cells through N-cadherin and FAK. These studies suggest the potential therapeutic utility of inhibiting GSK3β in defined subsets of melanoma.

INTRODUCTION

Glycogen synthase kinase (GSK)-3β is a serine/threonine kinase that is present at the junction of the PI3K/AKT and Wnt signaling pathways (Cohen and Frame, 2001). Its activity is inhibited by AKT, which phosphorylates and inactivates the kinase (Cross et al., 1995). GSK-3β has a critical role in the regulation of canonical Wnt signaling by directly phosphorylating β-catenin, leading in turn to its proteasomal targeting and subsequent degradation (Yost et al., 1996). Although increased Wnt/β-catenin signaling has been implicated in oncogenesis (He et al., 1998; Camilli and Weeraratna, 2010), there is currently little evidence that inhibition of GSK3β contributes to melanoma progression (Chien et al., 2009; Arozarena et al., 2011).

In addition to its role in Wnt survival signaling, β-catenin is also expressed at adherens junctions, where it facilitates cell-cell adhesion through an association with E-cadherin and N-cadherin (Smalley et al., 2005; Harris and Tepass, 2010). Adherens junctions have been best studied in epithelial tissues in which they are known to be critical for the maintenance of cellular architecture (Lioni et al., 2007). Of relevance to melanoma, both melanocytes and skin keratinocytes express E-cadherin, and there is evidence that homotypic E-cadherin signaling constitutes a major regulatory mechanism of the “epidermal melanin unit” in normal skin (Hsu et al., 2000b; Haass et al., 2005). Loss of E-cadherin expression is often viewed as a first step in melanoma progression that allows transformed melanocytes to escape from local keratinocyte control (Li et al., 2001). In melanoma development, E-cadherin loss is typically accompanied by an increase in N-cadherin expression that facilitates tumor cell dissemination by increasing the interaction of melanoma cells with host
endothelial cells and fibroblasts, as well as increasing melanoma cell survival (Li et al., 2001, 2003).

Very little is currently known about the function of GSK3β signaling in melanoma. Here we present new data demonstrating that low constitutive levels of GSK3β signaling contribute to the oncogenic behavior of melanoma by regulating both N-cadherin expression and focal adhesion complexes. For these studies, we used a highly potent organometallic kinase inhibitor of GSK3β (IC₅₀ 0.3 nm) (Pagano et al., 2007; Atilla-Gokcumen et al., 2008) to show that inhibition of GSK3β signaling limits the motile and invasive behavior of melanoma cells through a mechanism associated with decreased Slug expression. We also provide evidence that inhibition of GSK3β abrogates the interaction of melanoma cells with host fibroblasts and endothelial cells.

RESULTS

GSK3β inhibition blocks the migration and invasion of melanoma cell lines

Western blotting revealed GSK3β to be constitutively phosphorylated at Ser9 in nearly all of the melanoma cell lines tested (Supplementary Figure S1A online). No correlation was noted between phospho-GSK3β expression and the presence of either BRAF or an NRAS mutation or PTEN expression (Supplementary Table S1 online and not shown). Treatment of melanoma cell lines with NP309 (300 nM) and another structurally unrelated GSK3β inhibitor (LiCl) led to increased β-catenin expression (Supplementary Figure S2 online), demonstrating the presence of an activated GSK3β pool. Analysis of melanoma lesions (n = 40) showed GSK3β and phospho-GSK3β to be expressed in both primary (5/16) and metastatic specimens (8/24). The strongest staining was noted to be focal and located to the leading edge areas of the tumor, where the tumor and stroma were interacting (Figure 1a–c; Supplementary Figure S3 online). In primary melanoma, the strongest GSK3β staining was located at the invasive front, with fewer primary samples exhibiting strong focal staining (2/16) than metastatic samples (8/24). As the leading edge is the area where invasion occurs, we next asked whether GSK3β signaling was required for melanoma cell migration and invasion.

NP309 prevents the migration and invasion of melanoma cell lines

Treatment of the WM793, 1205Lu, and WM9 melanoma cell lines with the GSK3β inhibitors NP309 and LiCl and small interfering RNA (siRNA) knockdown of GSK3β inhibited the motile behavior of melanoma cells in a scratch wound assay (Figure 2a and b; Supplementary Figure S4 online). NP309 and LiCl also prevented the invasion of 1205LuL, WM9, and WM793 melanoma cells in a modified Boyden chamber assay, as well as the invasion of spheroids into a collagen gel (Figure 2c and d; Supplementary Figure S5 online). Treatment of melanoma cells with NP309 for 24 hours did not affect the growth of the melanoma cells (Supplementary Figure S6 online), suggesting that the observed effects on invasion were not the result of reduced cell proliferation.

Inhibition of GSK3β signaling in melanoma cells reduces N-cadherin expression

Previous work from our group has shown that increased N-cadherin expression increases the migratory behavior of melanoma cells (Li et al., 2001). Treatment of melanoma cells with increasing concentrations of NP309 or LiCl led to biphasic effects upon the Ser33/Ser37/Thr41 phosphorylation of β-catenin (an increase at lower concentrations followed by a decrease at higher NP309 concentrations), an upregulation of total β-catenin expression (and its localization to the nucleus), and a reduction in N-cadherin expression (Figure 3a and b; Supplementary Figures S7 and S8 online). The effects of NP309 upon N-cadherin expression were GSK3β dependent, and could be recapitulated following siRNA knockdown of GSK3β expression (Figure 3c). We next used mass spectrometry to demonstrate that GSK3β inhibition did not posttranslationally modify N-cadherin (no changes were observed in ubiquitination, acetylation, phosphorylation, and methylation) (Supplementary Figure S9 online). Instead, it was noted that NP309 reduced N-cadherin expression.

Table: 1. Glycogen synthase kinase (GSK)-3β is focally expressed in melanoma specimens. (a) Representative immunohistochemical staining of an invasive primary melanoma and a melanoma brain metastases for expression of total GSK3β and phospho-GSK3β. Bar = 250 µm. Inset: arrows indicate focal expression of GSK3β. Bar = 100 µm. (b) The number of primary and metastatic melanoma specimens with high levels (+2/3) of focal staining for GSK3β. (c) High-power images of two melanoma metastases, showing increased levels of total GSK3β staining at the invasive front. M, melanoma; S, stroma.
at the messenger RNA level (Figure 3d), and decreased the expression of the epithelial-to-mesenchymal transition (EMT)-associated transcription factor Slug (Figure 3e). In agreement with the GSK3\(\beta\) inhibitor partly reversing the “EMT-like” state of melanoma cells, NP309 also reduced the expression of fibronectin at both the RNA and protein levels (Figure 3f and g). No changes were noted in the expression of E-cadherin, Vimentin, Twist, or Snail (Figure 3e and not shown).

Inhibition of GSK3\(\beta\) signaling prevents melanoma cell adhesion to fibroblasts and endothelial cells, and transendothelial migration

Previous work from our group demonstrated that N-cadherin is important for the interaction of melanoma cells with host fibroblasts and endothelial cells (Li et al., 2001; Smalley et al., 2005). In agreement with these findings, we observed that the pretreatment of Dil-labeled 1205Lu, WM793, and WM9 cells with NP309 or LiCl prevented adhesion onto a fully confluent monolayer of human skin fibroblasts and endothelial cells (Figure 4a and data not shown). These effects were partly N-cadherin mediated and could be recapitulated by siRNA knockdown of N-cadherin expression (Supplementary Figure S10 online), and partly rescued following the overexpression of N-cadherin (Figure 4b). Pharmacological inhibition and siRNA knockdown of GSK3\(\beta\) also inhibited the migration of 1205Lu, WM793, and WM9 melanoma cells through an activated, confluent endothelial cell monolayer (Figure 4c and d). Again, these effects were N-cadherin-dependent and could be rescued following the overexpression of N-cadherin (Figure 4e).

Inhibition of GSK3\(\beta\) increases the size of focal adhesions

Studies in colorectal and pancreatic cancer suggest that GSK3\(\beta\) modulates cell migration and invasion through the regulation of focal adhesion assembly (Kobayashi et al.,...
We next determined whether the inhibition of GSK3\(\beta\) also regulated focal adhesion kinase (FAK) activity. Using WM793 melanoma cells that express enhanced green fluorescent protein (EGFP)-FAK, and 1205Lu cells stained for total FAK, we demonstrated that NP309 treatment and siRNA knockdown of GSK3\(\beta\) increased the number of large focal adhesions as shown by increased staining for FAK (Figure 5), paxillin, and vinculin (Supplementary Figure S11 online).

NP309 inhibits FAK signaling in melanoma cells, leading to the inhibition of motility and invasion

Treatment of WM9, WM793, and 1205Lu melanoma cell lines with increasing concentrations of NP309 inhibited FAK
phosphorylation at the Tyr^{397} activation site (Figure 6a). A comprehensive mass spectrometry–based analysis of FAK phosphorylation demonstrated that GSK3β inhibition increased the phosphorylation of FAK at Ser^{843}, an inhibitory phosphorylation site (Jacamo et al., 2007; Supplementary Figure S12 online). The requirement for FAK in the motile and invasive behavior of melanoma cells was demonstrated by the ability of a FAK inhibitor (PF-228, 10 μM) to inhibit the movement of cells into a scratch wound and to reduce the invasion of 1205Lu, WM793, and WM9 melanoma cells in modified Boyden chamber and three-dimensional collagen-implanted spheroid assays (Figure 6b–d).

**DISCUSSION**

Strategies to prevent the metastatic spread of melanoma are currently lacking. Here, we demonstrate that the inhibition of GSK3β signaling limits the motile and invasive behavior of melanoma cells and prevents some of the host/tumor interactions required for the transit of melanoma through the dermal microenvironment and into the vasculature.
Since its initial identification as an enzyme involved in the regulation of glycogen synthesis in response to insulin signaling, GSK3β has been implicated in a wide range of physiological processes ranging from protein synthesis to subcellular protein localization (Cohen and Frame, 2001; Frame and Cohen, 2001). A role for GSK3β signaling in cancer development and progression is suggested by its role in the phosphorylation of cyclin D1 and its ability to control Wnt signaling through the phosphorylation and degradation of β-catenin (Diehl et al., 1998). GSK3β is negatively
regulated by the serine/threonine kinase AKT through its phosphorylation at Ser9 (Cross et al., 1995). Most melanomas are known to have constitutive activity in AKT that results from increased expression of AKT3, constitutive activation of receptor tyrosine kinase signaling, or through deregulation of the negative pathway regulator PTEN (Stahl et al., 2004; Tsao et al., 2004; Davies et al., 2008; Paraiso et al., 2011). Given that AKT inactivates GSK3β signaling, the potentially oncogenic role of GSK3β in melanoma has been little considered.

Immunostaining of melanoma tissue specimens showed that GSK3β was focally expressed and mostly localized at the leading edge, suggesting a role for this pathway in melanoma invasion. Although high, focal GSK3β expression was not restricted to melanoma metastases, it was noted that the strongest GSK3β staining observed in primary melanomas was located in nests of cells at the periphery of the tumor, where dermal invasion was occurring. In other tissue systems, there is already good evidence for the involvement of GSK3β in the control of cellular polarity and cytoskeletal architecture. In neuronal growth cones, GSK3β localizes to the invasive front and regulates the microtubule assembly required for cell motility and polarity (Eickhoff et al., 2002; Gartner et al., 2006). GSK3β signaling is also required for the formation of lamellipodia in migrating keratinocytes, as well as the directional motility of skin stem cells during wound healing (Koivisto et al., 2003; Wu et al., 2011).

Tumor development and metastasis requires the continual bidirectional interaction between host and tumor cells (Bhowmick et al., 2004a,b; Gaggioli et al., 2007). The progression of melanoma is often associated with a cadherin switch, in which E-cadherin expression is downregulated allowing nascent melanoma cells to escape from the control of the skin keratinocytes (Hsu et al., 2000a,b). Melanoma cells then change binding partners and associate instead with stromal fibroblasts and endothelial cells (Li et al., 2003). The interaction of melanoma cells with endothelial cells is likely to be particularly important when melanomas disseminate through the vasculature, such as during the seeding of metastases into the brain. Previous studies have indicated that the adhesion of melanoma cells to endothelial cells and their subsequent transendothelial migration is dependent upon homotypic N-cadherin binding (Li et al., 2001; Qi et al., 2005). In agreement with a role for GSK3β signaling in the regulation of N-cadherin expression, NP309 treatment blocked the adhesion of melanoma cells onto a confluent endothelial cell layer and prevented the transendothelial migration of melanoma cells. The role for N-cadherin in both of these processes was demonstrated by the ability of forced N-cadherin expression to rescue the inhibitory effects of NP309 upon adhesion and transendothelial cell migration.

From a mechanistic standpoint, GSK3β was found to regulate N-cadherin expression at the messenger RNA level, and GSK3β inhibition was associated with the decreased expression of Slug, a Snail-family transcription factor implicated in the metastatic behavior of melanoma cells (Gupta et al., 2005). The potential role of Slug down-regulation in the anti-migratory effects of GSK3β inhibition is supported by recent studies showing that overexpression of Slug in melanocytes increases N-cadherin expression, leading to an enhancement of cell motility (Shirley et al., 2012). Taken together, these data suggest a potential role for GSK3β signaling in the transcriptional program required for melanoma dissemination.

The disruption of homotypic N-cadherin signaling following GSK3β inhibition is likely to have other beneficial effects. The engagement of N-cadherin signaling in melanoma cells is known to activate the AKT pathway, leading to increased cell survival (Li et al., 2001). It is possible that the interaction of melanoma cells with fibroblasts through N-cadherin contributes to this survival as demonstrated by the reduced cisplatin-induced apoptotic response observed in melanoma cells following their adhesion to fibroblasts (Flach et al., 2011). The potential clinical relevance of these findings has already been suggested by the observation that inhibition of N-cadherin signaling enhances chemotherapy sensitivity in melanoma patients undergoing isolated limb infusion (Li et al., 2001; Beasley et al., 2011).

The coordinated movement of invading cells involves adhesion and membrane protrusion at the leading edge, and detachment and retraction at the trailing edge. As GSK3β was primarily expressed at the invasive front of melanoma specimens, we asked whether GSK3β was involved in melanoma cell adhesion. As our focus, we studied FAK, a kinase known to occupy a central position linking integrin-mediated adhesion to the downstream activation of key signaling pathways involved in cytoskeletal rearrangement and survival. It was observed that NP309 treatment inhibited phosphorylation of FAK at its activating autophosphorylation (Tyr397) site, and that a small-molecule FAK inhibitor (PF-228) also prevented both the migration of melanoma cells in a scratch wound assay and the invasion of the highly invasive 1205Lu and WM9 melanoma cell lines in Boyden chamber and three-dimensional spheroid assays. Although our studies did not demonstrate a direct link between GSK3β inhibition and inhibition of FAK phosphorylation at Tyr397, a detailed mass spectrometry analysis of FAK phosphorylation sites did reveal NP309 treatment to enhance FAK phosphorylation at Ser843, a site known to be associated with dephosphorylation of FAK at Tyr397 (Jacam et al., 2007). Studies are ongoing to identify the candidate serine/threonine kinase that directly phosphorylates FAK at Ser843 upon GSK3β inhibition.

GSK3β inhibition also led to an increase in the size of focal adhesions. Our results mirrored those observed in HeLa cells in which inhibition of GSK3β using both siRNA and pharmacological inhibitors impaired cell motility and increased the size and number of focal adhesions (Kobayashi et al., 2006). Links between inhibition of FAK and impaired motility have been reported by a number of other groups with FAK-null fibroblasts being shown to have an impaired migratory response associated with an increase in adhesion strength and the size and number of focal adhesions; the fact that constitutively activated FAK can partially rescue the motile behavior of cells in which GSK3β has been depleted has also been reported (Lic et al., 1995; Kobayashi et al., 2006).
To our knowledge, the role of GSK3β in both the motile behavior of melanoma cells and the interaction of melanoma cells with host fibroblasts and endothelial cells has never been previously reported. Further studies will be required to determine whether GSK3β inhibition is a viable strategy to limit the metastatic phenotype of melanoma in vivo.

**MATERIALS AND METHODS**

**Cell culture and growth inhibition**

Melanoma cells lines were grown as described in Paraiso et al., 2010. The identity of the cell lines was confirmed using the Coriell Institute (Camden, NJ) cell identity mapping kit.

**Drugs and inhibitors**

NP309 was synthesized and characterized for its anti-GSK3β activity as described in Pagano et al., 2007. PF-228 and LiCl were purchased from Sigma-Aldrich (St Louis, MO).

**Immunohistochemical staining of melanoma specimens**

Deidentified formalin-fixed paraffin-embedded tissue samples were obtained from the Moffitt Pathology archives under a written informed consent protocol approved by the Institutional Review Board of the University of South Florida under the Declaration of Helsinki Protocols and stained using the Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) (Paraiso et al., 2011). The rabbit primary antibody for p-GSK3β was from Cell Signaling Technology (Beverly, MA) and the antibody for total GSK3β was from Epitomics (Burlingame, CA). Staining was visualized using the Ventana Chromomap Redkit. Slides were analyzed by two independent observers and consensus scored on a scale from 0 to +3.

**Western blotting**

Proteins were extracted and blotted for as described in Smalley et al., 2007. The antibodies to phospho-GSK3β (Ser9), total GSK3β, phospho-FAK (Tyr 397), total FAK, phospho-β-catenin (Ser33/37/Thr41), and vimentin were from Cell Signaling Technology. The antibodies for fibronectin, N-cadherin, and total β-catenin were from BD Biosciences (San Jose, CA). The antibodies for Twist and SLUG were from Santa Cruz (Santa Cruz, CA), whereas the antibody for Snail was from Abcam (Cambridge, MA). In all cases, western blots were stripped once and probed for glyceraldehyde-3-phosphate dehydrogenase or actin (Sigma-Aldrich) to confirm even protein loading.

**RNA interference**

RNA interference experiments were performed as described in Paraíso et al., 2011. Cells were treated with 25 nM N-cadherin (Dharmacon, Lafayette, CO) and 20 nM GSK3β (Cell Signaling Technology). In addition, scrambled siRNAs at each concentration were also added as nontargeting controls.

**Three-dimensional spheroid assays**

Collagen-implanted spheroids were prepared using the liquid overlay method (Smalley et al., 2006) and were treated with 0.3 μM NP309 or LiCl (50 mM) for 24–72 hours before being analyzed using a Nikon-TS100 (Melville, NY) inverted fluorescence microscope and analyzed using ImageJ (NIH, Bethesda, MD).

**Fibroblast/endothelial cell adhesion assays**

Human skin fibroblasts (FF2554) or human vascular endothelial cells were seeded out to 100% confluency and left to grow overnight. 1205Lu, WM793, and WM9 melanoma cells were labeled with Dil before treatment with vehicle, NP309 (0.3 μM), GSK3 siRNA, or LiCl (50 mM) for 24 hours. Equal numbers of cells were then added to the confluent fibroblast/endothelial cell monolayers and allowed to adhere for 20 minutes before being washed four times with fresh media and quantified by counting five × 20 fields.

**Transendothelial cell migration assays**

Human vascular endothelial cells were plated onto transwell inserts and allowed to grow to confluence over 48 hours before being activated with TNF-α (10 ng/ml−1 24 hours). 1205Lu, WM793, and WM9 cells were pretreated with NP309, LiCl, or siRNA to GSK3β or scrambled control for 48 hours before being counted and tested for viability by trypan blue staining. A total of 25,000 melanoma cells were then Dil labeled and plated on top of the human vascular endothelial cell layer in serum-free media with fetal bovine serum-containing media added to the lower chamber. Following 1–4 hours of incubation, cotton swabs were used to remove nonmigratory cells before fixation (4% paraformaldehyde) and imaging with a Nikon Eclipse TS100 microscope.

**Adenoviral vector infections**

Cells were infected with 10–100 plaque forming units of adenovirus encoding for N-cadherin as described in Li et al., 2001.

**Scratch wound assays**

Confluent monolayers of melanoma cells (1205Lu, WM793, and WM9) were allowed to grow to confluence before being scratched with a p10 pipette tip. Cultures were treated with vehicle, NP309, LiCl (concentrations as above), or GSK3 siRNA (48 hours pretreatment) before being imaged (1–24 hours). Percentage wound closure was calculated using ImageJ.

**Modified Boyden chamber assays**

Invasion was measured in Chemotaxis Chambers (96-well format from Neuroprobe (Gaithersburg, MD)) following coating with Matrigel (BD, San Jose, CA). Briefly, cells were trypsinized, rinsed twice with PBS, resuspended in serum-free media, and were loaded on the upper chamber and allowed to invade through the Matrigel toward 10% fetal bovine serum for 20 hours. Noninvasive cells were removed, and the remaining cells were fixed and stained with Crystal Violet with absorbance being read at OD 560 nm. For loading control and to normalize for differences in cell proliferation, cells were allowed to grow for the same time as the incubation in the Boyden chambers, after which cells were stained with Crystal Violet. Cells were quantified at OD 560 nm after dye extraction, and this number was used to normalize the invasion value.

**EGFP-FAK-expressing cells**

The EGFP-coding region and multiple cloning site were amplified from pEGFP-C1 (Clontech, Mountainview, CA) and TOPO cloned into pENTR/D-TOPO (Invitrogen, Grand Isle, NY). Chicken FAK was subcloned from pBluescript-FAK (kindly provided by Dr Jihe Zhao, University of Central Florida Burnett School of Biomedical Sciences) into pENTR/D-TOPO/EGFP-MCS. The resulting pENTR/D-TOPO/
EGFP-FAK was shuttled into pLenti4/TO/V5-DEST (Invitrogen) using Gateway LR Clonase II (Invitrogen), which put the coding region in-frame with the vectors C-terminal V5-epitope and stop codons. pLenti4/TO/V5-GW/EGFP-FAK was packaged in HEK293FT cells using Invitrogen’s ViraPower Lentiviral Packaging Mix and protocol, with the substitution of 36 μl FuGENE HD (Roche, Nutley, NJ) for Lipofectamine 2000 for the transfection. Medium containing lentivirus particles was collected 72 hours post transfection and added to WM793TR cells (Abel and Aplin, 2010) for 72 hours followed by selection in Zeocin (100 μg/ml⁻¹).

Immunofluorescence staining
Cells (WM793 or WM793-FAK, and 1205Lu) were plated onto coverslips and treated for 24 hours before being fixed and permeabilized as previously described (Smalley et al., 2007) and imaged with a Leica (Lawrenceville, GA) confocal microscope at ×63. In some cases, slides were stained for paxillin, vinculin, or fibronectin (BD Pharmingen, San Jose, CA).

Quantitative real-time PCR
Cells were treated for 24 hours with 300 nM NP309 before RNA isolation. Total RNA was isolated using Qiagen’s RNeasy mini kit (Valencia, CA). The following TaqMan Gene Expression Assays primer/probes were used: Hs00983056_m1 (N-cadherin), Hs00365052 м1 (fibronectin), and P/N 4319413E (18S). The 18S data were used for normalizing BIM. Quantitative real-time PCR reactions were performed as previously described (Atilla-Gokcumen et al., 2008).

Statistical analysis
Unless otherwise stated, all experiments were performed at least three times. Data show mean values ± SEM. Significance was analyzed using a Student’s t-test with P<0.05 being considered significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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