Loss of miR-200 family in 5-fluorouracil resistant colon cancer drives lymphendothelial invasiveness in vitro

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Abstract

Invasive colorectal cancer is associated with poor prognosis requiring treatment with systemic chemotherapies usually including 5-fluorouracil. A consequence of prolonged treatment is the acquisition of resistance eventually resulting in the recurrence of highly metastatic cancer cells. To address the relationship between drug resistance and increased lymphatic metastatic potential, we used a 3D co-culture model of colon tumour cell spheroids of parent CCL227 cells and subclones with gradually increasing resistance against 5-fluorouracil. From each investigated cell line, homogeneous tumour spheroids were generated in the presence of methylcellulose yielding emboli of ∼700 µm diameter. When invasive, tumour spheroids disrupt the continuous lymphendothelial cell (LEC) layer and generate a ‘circular chemorepellent-induced defect’ (CCID), reminiscent of the entry gates through which tumour emboli intravasate lymphatic vasculature. Here we provide evidence that increasingly chemoresistant colon cancer spheroids were strongly associated with enhanced intravasative properties. In naïve CCL227 spheroids, miR-200 family members were released into exosomes thereby repressing the epithelial to mesenchymal transition-regulating transcription factors ZEB1 and SLUG in LEC. As a consequence of attenuated plasticity and migration of LEC, CCID formation was impaired. Loss of exosomal transferred miR-200c in resistant colon cells rendered LEC more susceptible to pro-migratory signals that were generated and directly transmitted by colon cancer spheroids. This observation indicates a common molecular axis in colon cancer and LEC where miR-200 family members act as regulators of ZEB proteins. The data support the notion that horizontal miR-200 signalling prevents the permeation of cells into adjacent epithelia and contributes to organ integrity.

Introduction

In accordance with the International Agency for Research on Cancer (IARC), ∼1.24 million new cases of colorectal cancer (CRC) are detected worldwide each year (1,2). The main reason for death of cancer patients is metastasis. The metastatic cascade includes local invasion of cancer cells, which is often associated with epithelial to mesenchymal transition (EMT), breaking through vessel structures (intravasation), survival within the systemic circulation, travelling through vessel structures to regional lymph nodes or the metastatic site (extravasation) and survival and outgrowth of metastatic colonies. In advanced stages of colon cancer, either with local invasion, regional lymph node or
distant metastasis, patients receive systemic therapies including oxaliplatin, irinotecan, cetuximab and 5-fluorouracil (5-FU) (3,4). A consequence of prolonged treatment with these drugs is the acquisition of resistance resulting in progression or the recurrence of highly metastatic cancer cells. This gain of chemoresistance was recapitulated in vitro by the colon cancer cell line CCL227 (SW620), which acquired increasing levels of drug resistance through long-term treatment with escalating doses of 5-FU. CCL227 was originally isolated from the lymph node metastasis of a colon adenocarcinoma.

Parent CCL227 cells were found to express microRNA (miR) such as the miR-200 family (miR-200a, 200b, 200c, 141). The miR-200 family negatively regulates ZEB proteins (ZEB1, ZEB2, SNAI, SLUG, TWIST), which are transcription factors involved in execution of EMT (5). microRNA actively shuttles within the cell via special nuclear exporters such as XPO5 (6). In addition, recent studies indicate that microRNA secretion is also mediated via vesicular/exosomal mechanisms (7).

Exosomes are 40–100 nm small membrane vesicles which are released by most cell types. Exosomes contain cytosolic components without organelles encapsulated by a lipid bilayer (8). Depending on the cell of origin, they contain varying molecular components such as proteins and nucleic acids and all types of RNA (9), sometimes actively transferred into exosomes (10). Depending on the cell of origin, they contain varying molecular components such as proteins and nucleic acids and all types of RNA (9), sometimes actively transferred into exosomes (10). A study indicated that microRNA produced by macrophages, encapsulated into exosomes and transferred to breast cancer cells, enhanced the invasive potential of the tumour cells (11). Therefore, microRNA can be transferred between different cell types and may alter gene expression in these recipient cells. We hypothesized that the exosomal transfer of CRC-derived miR-200 may also alter the response of the neighbouring stroma such as that of recipient lymphoid endothelial cells (LECs), and in return influences the progression of colon cancer.

We have previously shown that the invasiveness of breast cancer emboli through epithelial sheets was accompanied by an induction of ZEB1 in the LEC involved (12,13). Likewise, CRC is able to spread across the lymphatic vasculature (14). Therefore, CCL227 cell spheroids were investigated regarding their potential to disrupt the integrity of lymphatic cell walls and induce a ‘circular chemorepellent-induced defect’ (CCID) (12,13,15–17). CCID induced by spheroids generated from naïve CCL227 cells were compared with those induced by 5-FU resistant CCL227 spheroids (CCL227-R). We hypothesized that these may augment CCID formation in the adjacent LEC monolayer and hence increase the metastatic property owing to reduced miR-200 levels in the released exosomes with a subsequent de-repression of ZEB family members in LEC upon miR-200 uptake.

**Results**

**FU-resistance in colon cancer cells gradually enforces CCID formation in LEC**

Acquisition of resistance to chemotherapeutic drugs is associated with increased metastatic potential and poor prognosis (18). Here we analysed whether resistance of colon cancer cells to 5-FU enforces their competence to break through LEC barriers. We used a well-established three-dimensional in vitro assay consisting of tumour cell spheroids placed on a LEC monolayer (12,13,16) to study mechanisms supporting invasavation of naïve and increasingly 5-FU resistant CCL227 cells covering an IC50 range from 41 µM 5-FU in naïve CCL227 up to 7800 µM in the high-resistant cell line (19). We observed a highly significant difference in the formation of CCID within the LEC monolayer induced by spheroids of naïve, low- (CCL227-RL), medium- (CCL227-RM) or high-resistant (CCL227-RH) subclones of CCL227 (Fig. 1A and B). CCID induced by CCL227-RH spheroids increased by more than 50% when compared with those induced by naïve CCL227 spheroids. Interestingly, also CCL228 showed some ability to intravasate the lymphendothelial layer, which was 34% of the CCID formation observed in naïve CCL227 (data not shown).

From previous studies it is known that CCID may be triggered by 12(S)-HETE produced by cancer cell clusters (12). Using arachidonic acid as a substrate, ALOX12, ALOX15 and members of the CYP family in the tumour cells produce 12(S)-HETE. Under normal physiological conditions, 12(S)-HETE is secreted by macrophages and monocytes allowing their movement through endothelial barriers. Pathologically, 12(S)-HETE facilitates mammary tumour emboli intravasation into the lymphatic vasculature (12). Indeed, also 1 × 10⁶ CRC cells CCL227, CCL227-RL, CCL227-RM and CCL227-RH secreted between 12 and 37 ng/ml (38–116 nm) of 12(S)-HETE (data not shown). Consistently, inhibition of 12(S)-HETE by baicalein, an inhibitor of ALOX12/15, or proadifen, a pan-CYP inhibitor, reduced the formation of CCID (Fig. 1C).

**Down-regulation of miR-200 and expression of EMT-regulating transcription factors**

In addition to 12(S)-HETE, which is discharged into exosomes (20), we found microRNA of the miR-200 family at considerably high levels in the exosomes of naïve CCL227, which were dramatically reduced in the exosomes of CCL227-RH cells (21). miR-200a and miR-200b were not as efficiently down-regulated as miR-200c and miR-141, which were severely suppressed in cells with the resistance phenotype (Fig. 2A). miR-200c and miR-141 were reported to repress the ZEB family of transcription factors (22). The ZEB family is inducing EMT, confers a migratory phenotype (23) and hence, supports tumour progression. Since decreasing miR-200 levels in the resistant CCL227 clone were expected to de-repress ZEB, this urged us to study the expression of the ZEB family in the colon cancer cell lines CCL227, CCL227-RL, CCL227-RM and CCL227-RH by western blotting (Fig. 2B). In fact, the EMT promoting transcription factors ZEB1 and SLUG were up-regulated in increasingly resistant CCL227 clones causing the suppression of the target E-cadherin. SNAI and TWIST levels were unchanged in the distinct clones, whereas ZEB2 was not expressed. Taken together, decreasing levels of miR-200 family members in the resistant tumour cells correlated with de-repression of ZEB1 and suppression of E-cadherin thereby contributing to the plasticity of CCL227-RH cells.

**microRNAs are horizontally transferred into LEC**

It is known from mammary tumour cells that CCID formation involves an endothelial-to-mesenchymal transition process in LEC (13). Hence, it was investigated whether members of the miR-200 family were packed into exosomes and released by CCL227 cells (donors), were transferred horizontally to adjacent growing LEC (recipients) as a prerequisite for reprogramming LEC gene expression and de-repressing ZEB proteins there. To challenge our hypothesis of horizontal miR-200 transfer from CRC donors to LEC recipients, CCL227-RH cells were grown at the top side of the cell culture insert (3.0 µm membrane pore size) of a Boyden (transwell) chamber and LEC on the lower surface of the insert (see Fig. 3A and B). With this experimental set-up, a direct transfer of exosomes from the donors to the recipients can be achieved without direct cell–cell contact.
As assessed by RT-qPCR, the cellular expression of the miR-200 family members was low in LEC with Ct values >30. The high miR-200 expression in CCL227 is lost in the drug-resistant cells (Fig. 2A). Hence, CCL227-RH cells, expressing the lowest levels of miR-200, were transfected with precursors of the miR-200 family. As expected, the levels of the respective exosomal miR-200 strongly increased, up to 1000-fold (Fig. 3C). In the recipient LEC, which were grown on the under-surface of the cell culture insert in close vicinity to the transfected CCL227-RH donor cell line, miR-200a, miR-200b and miR-200c levels increased significantly within 24 h of separated co-culture (Fig. 3D), whereas miR-141 was hardly modulated in LEC. These observations indicate that miR-200 family members were differentially packed into colon cancer cell exosomes and that miR-200 are indeed horizontally transferred from cancer cells to the adjacent endothelium in vitro.

**Selected miRs-200 repress the constitutive levels of ZEB family members in LEC**

After demonstrating that miR-200 family members were transferred from cancer cells to LEC via exosomes the regulatory
function of miR-200 in recipient LEC was analysed. CCID formation is due to retraction of LEC (12, 24), which involves ZEB1 in LEC as well as endothelial-to-mesenchymal transition (13). This led us to investigate whether the family of ZEB transcription factors in LEC became inhibited by miR-200 family members and whether this would impact on the adhesion protein VE-cadherin, the endothelial equivalent of epithelial E-cadherin. For this purpose, 5 nM of each miR-200 precursor was separately transfected into LEC and the expression of ZEB1/2, SNAI, SLUG, TWIST and VE-cadherin were analysed by western blotting (Fig. 4A). Upon transfection, ZEB1 protein was repressed by all miR-200 family members. SNAI was suppressed by transfection of miR-200a, miR-200b and miR-141 but did not respond markedly to the transfection with miR-200c. On the other hand, TWIST protein was reduced upon transfection with miR-200c and miR-141 but did not respond to transfection with the other family members investigated here. The expression of SLUG in LEC was below the limit of detection (data not shown) and there was no relevant effect on ZEB2 and VE-cadherin. Hence, VE-cadherin was not directly regulated by ZEB1 or SNAI in LEC.

Since transfection of precursor miR-200 decreased ZEB1 expression in LEC, we next tested the impact of colon cancer-derived exosomes on the EMT transcription factor expression in LEC. Exosomes (100 µg protein equivalents) collected from naïve CCL227 cells and from CCL227-RH cells, were incubated with LEC for 100 min and analysed for the expression of VE-cadherin and ZEB-related family proteins (Fig. 4B). Exosomes from naïve CCL227 cells caused a decrease of ZEB1 in LEC, whereas exosomes from CCL227-RH did not have an effect on ZEB1 protein levels. Likewise, a similar effect was detectable for ZEB2, which was also negatively regulated via exosomal miR-200 family members transferred from naïve CCL227. The discrepancy, why ZEB2 was suppressed by exosomes from naïve CCL227 cells, whereas transfection of single miR-200 precursors was ineffective remained elusive. No change in expression was observed for transcription factor SNAI and TWIST (SLUG was below the limit of detection). These data demonstrate that exogenous miR-200 family members, either directly or transferred via exosomes, are able to modulate transcription factors associated with cellular plasticity in LEC.

**Proof of principle: reversing versus boosting malignancy**

In order to reverse the effect on CCID formation, we chose miR-200c because it was strongly suppressed in resistant cancer cells, transferred to LEC via exosomes and regulated ZEB1 and SLUG (Fig. 2B). The transfection of miR-200c precursor into the high-resistant colon cancer spheroids restored the miR-200c level similar to that of the parent cell line. In fact, miR-200c transfected CCL227-RH spheroids inhibited the miR-200c level similar to that of the parent cell line. This observation corroborated the conclusion that (i) the exosomal content directly influenced the intravasation process and (ii) provided evidence that a chemoresistant CRC phenotype was associated with significantly increased potential to intravasate through the lymphendothelial barrier. As the underlying mechanism, the EMT-regulating transcription factors ZEB1 and SLUG were regulated by microRNA, namely miR-200c, which is...
transferred horizontally from malignant colon cells to LECs by exosomes.

**Discussion**

In this study, a well-characterised colon cancer model with multiple levels of resistance against FU was used in vitro. This model showed several characteristics reminiscent of an EMT phenotype: morphologically by increased spindle-like cell morphology with a severe loss of E-cadherin at the transcriptional level (19) and the protein level (25). As putative other EMT-related factors, we investigated the expression of miR-200 family members and E-cadherin-regulating transcription factors, i.e. ZEB1, ZEB2, SNAI, SLUG and TWIST. In line with our previous observations, all members of the miR-200 family were suppressed in the chemoresistant colon cancer subclones. As a consequence of miR-200 family depletion, the E-cadherin blocking factors ZEB1 and SLUG were de-repressed, indicating a direct regulation in the chemoresistant colon cancer cells in vitro. An analogue mechanism was observed in the LEC model, where transfection of the miR-200 family resulted in decreased expression levels of the E-cadherin-regulating transcription factors ZEB1, SNAI and TWIST. A recent report underscores the role of SNAI expression in colon cancer metastasis (26). Interestingly, in our study SNAI remained unaffected in CCL227 subclones but was inhibited by miR-200a in LEC, thereby corroborating a role for SNAI in the metastatic progress and the contribution of horizontal modulation of stromal defence mechanisms. Although miR-141 affected SNAI expression, miR-141 was, however, not transferred to LEC via exosomes and did not play a role in our model.

The presented data indicate a parallel microRNA-driven regulation (particularly by miR-200c) of cellular migration of LEC, which was hypothesized to be enhanced by the chemoresistant colon subclones. In fact, the degree of cellular LEC migration as determined in the CCID assay was directly related to the degree of chemoresistance with the high-resistant colon subclones triggering more than 50% increase in LEC migration when compared with the parent cell line CCL227. This statistically significant effect was observed in the CCID assay when tumour cell spheroids were placed on top of a confluent layer of LEC and was independent of the spheroid density (no difference when Matrigel was used to generate spheroids).

The microRNA-200 family is known to play a key role in the EMT process (27,28). It was found that cells localized in the invasive front of colorectal tumours have lost miR-200 family members (23). These cells are more likely to intravasate into the blood or lymphatic system facilitated by a higher motility and reduced cell–cell contacts. Interestingly, in our model system the microRNAs located on chromosome 12 (miR-200c and miR-141) are stronger down-regulated than the microRNAs on...
chromosome 1 (miR-200a/b and miR-429). This might be caused by DNA methylation of the locus of miR-141 and miR-200c which was observed recently in vitro and in vivo (29). In line with our results, these microRNAs located on chromosome 12 are the focus of several studies as their putative targets are involved in EMT such as ZEB1, ETS1 and FLT1.

In search for the mediator of the observed LEC mobility upon contact with colon cancer spheroids, we analysed the role of exosomes as putative vehicles for microRNA transfer. The cellular expression of the miR-200 family members is low in LEC and in the drug-resistant phenotype. By adapted transwell experiments without direct cell–cell contact, transfer from microRNA via
exosomes from colon cells to LEC was favoured. CCL227-RH transfected with miR-200 family precursors released exosomes, which were selectively enriched in the different miR-200 family members. Upon exposure to exosomes released from the miR-200 transfected colon cancer cells, the cellular miR-200 expression level increased in LEC within 24 h to 2- to 14-fold levels (miR-200a/b/c). Once released, these exosomes specifically alter microRNA transcription in the target cells as miR-200 in LEC did not correlate directly with colon exosomal microRNA expression. This series of experiments demonstrate that microRNAs packed in cancer-derived exosomes may be horizontally transferred into LEC and alter the biological phenotype of the recipient cell. As an example, miR-200c reduced the CCID formation in LEC after exposure to transfected spheroids of resistant CCL227-RH cells strongly suggesting an exosomal contribution to LEC migration.

To prove the assumption that exosomes are able to alter gene expression of the recipient cell, isolated exosomes from naive and high-resistant colon cancer cells were incubated with LEC. The results indicate the direct effect of exosomes on ZEB1 and ZEB2. LEC treated with exosomes from naïve CCL227 cells had reduced ZEB1 and ZEB2 levels compared with LEC treated with exosomes from resistant cells.

Consistent with their low expression of miR-200 s LEC express rather high levels of ZEB1 and ZEB2 (compared with naïve CCL227 cells). Since exosomes derived from CCL227-RH cells are almost devoid of miR-200, ZEB1/2 present in LEC were exposed to these exosomes and remained at equal level with the untreated control. In contrast, miR-200-rich exosomes derived from naïve CCL227 cells repressed ZEB1/2 levels in LEC. This indicates that also CCID-generating components must be contained in the exosomes of resistant as well as of naïve CCL227 cells. miR-200 family members counteract this pro-metastatic components, whereas progressed or chemoresistant clones suppress miR-200. This might result in a full-blown malignancy of cancer cells that is imposed on the adjacent stroma causing the weakening of LEC–cell tightness, possibly through horizontal epigenetics.

12(S)-HETE is also known as endothelial retraction factor and influences the motility of LEC by regulating VE-cadherin and other motility factors (13). 12(S)-HETE is expressed at high levels in CCL227 clones and was shown to become packed into exosomes from MCF-7 cells (20). Modulating arachidonic acid- and 12(S)-HETE metabolism by pharmacological intervention, such as by baicalin and probasin, evidenced that 12(S)-HETE was an active factor in the formation of CCID, whereas the secretion of the miR-200 family was supporting lymphendothelial vascular integrity and caused the observed differences of naïve and high-resistant CCL227 clones on CCID formation. This was substantiated by reversing intravasation owing to transfection of miR-200c. As shown, CCID formation can be pharmacologically inhibited as well and, therefore, searching for inhibitory compounds that can be used in clinical indications, e.g. in high-risk patients, might be an important aspect. The screening of a panel of FDA approved drugs revealed that some of them unintentionally inhibited CCID formation as a secondary effect (30,31). The CCID assay, however, is a low throughput approach, whereas screening for up-regulation of miR-200 family members can be analysed by qPCR at high rate and speed suggesting that this approach might serve as a suitable read-out parameter for translational studies.

In summary, a chemoresistant phenotype in colon cancer cells was related to a significantly increased intravasative property through lymphendothelial barriers. Targeting the EMT-regulating transcription factors ZEB1 and SLUG, the cell surface molecule E-cadherin was in turn regulated by microRNA miR-200c, which was furthermore transferred horizontally from malignant colon cancer to LECs by exosomes. Thus, chemoresistance to 5-FU establishes an aggressive migratory phenotype in endothelial cells via loss of exosomal miR-200 that is otherwise transferred from naive donor CRC cells to the recipient LEC.

To date, there are no therapeutic approaches to prevent the metastatic process at early stages. With a putative benefit for patients at risk, this might be a strategy, which merits translational and clinical investigations.

Materials and Methods

Cell lines and culture conditions

The colon cancer line CCL227 (lymph node metastasis) was obtained from American Type Culture Collection (Rockville, MD, USA). The drug-resistant subclones of CCL227 were generated by continuous exposure of the cells to increasing concentrations of 5-FU (5 µM = CCL227-RL), 25 µM (CCL227-RM) and 125 µM 5-FU (CCL227-RH). The cells were cultured in RPMI 1640 medium (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Gibco), 25 mg/ml Gentamycin (Gibco) in a humidified atmosphere containing 5% CO₂ at 37°C. The resistant subclones were cultured in the presence of 5-FU. The telomerase-immortalized human LEC were cultured in EGM2 V medium (Lonza, Basel, Swiz) in a humidified atmosphere containing 5% CO₂ at 37°C

Generation of tumour spheroids

For spheroid (2000 cells/spheroid) generation, a suspension with a density of 200,000 cells/15 ml medium was prepared from the colon cell lines CCL227 and the resistant subclones. This suspension contained 0.3% methylcellulose. A total of 150 µl of this cell suspension was transferred into a 96 round bottom plate and centrifuged 15 min at 1200 rpm to collect the cells at the bottom.

Cells were incubated for 6 days at 37°C and 5% CO₂ to allow the formation of spheroids.

Circular chemorepellent-induced defects assay

For the CCID assay, LEC were grown in a 24-well plate to almost 100% confluence and stained for 1 h with a 1:5000 dilution of cyto tracker green (Molecular Probes, Invitrogen). Tumour spheroids were placed directly onto the LEC layer. The CCID formation of spheroids.

Western blotting and antibodies

The colon cancer cell lines or LEC were seeded in six-well plates and grown until 80% confluence. Afterwards, the cells were
was diluted (in 100 µl) to a

The cells or spheroids (6 days of cultivation) were transfected using siPORT NeoFX (Ambion, Life Technologies). The miR-200 microRNA transfection kit (Applied Biosystems, Life Technologies). Nanogram RNA was used for reverse transcription using the TaqMan PCR kit (Thermo Scientific, Portsmouth, IL, USA) was used to develop chemiluminescence on membranes and monitored by using Amersham Hyperfilm (GE Healthcare, Buckinghamshire, UK). The primary antibodies were ZEB1 (Santa Cruz; CA, USA), ZEB2 (Millipore, Merck, Darmstadt, Germany), E-cadherin (Abcam, Cambridge, England), VE-Cadherin (Beckman Coulter, Fullerton, CA, USA), SNAIL (Cell Signalling, Danvers, MA, USA), SLUG (Santa Cruz), TWIST (Abcam) and β-actin (Abcam).

Real-time PCR

The cells or spheroids were harvested after the transfection and the RNA was isolated using TRI reagent (Sigma Aldrich). Ten

RNA was isolated using TRI reagent (Sigma Aldrich). Ten

Real-time PCR

The cells or spheroids (6 days of cultivation) were transfected using siPORT NeoFX (Ambion, Life Technologies). The miR-200 family (200a, 200b, 200c, 141) was used as miR-precurors and was diluted (in 100 µl) to a final concentration of 5 nM or 20 nM in RPMI without serum and antibiotics. A Pre-miR negative control (Ambion) was used and referred as non-targeting control (ntc). A siPORT solution containing 10 µl siPORT per 100 µl RPMI was mixed 1:1 with the miRNA solution and incubated at room temperature for 10 min. The mixture was gently added to the spheroids in 2.3 ml RPMI. After 24 h the medium was changed and replaced by RPMI containing 10% calf serum and antibiotics.

Isolation of exosomes

The cells in suspension were twice washed with blank medium and seeded into flasks with medium containing exosome-free FCS. The cells were kept in culture for 2–3 days to allow the exosome production. Afterwards the supernatant was collected and centrifuged at 3000g for 15 min. The supernatant was transferred to a new 15 ml tube and per 5 ml supernatant 2 ml ExoQuick (System Biosciences, Mountain View, CA, USA) were used to precipitate the exosomes via refrigerating over night. The pellet was collected by centrifugation at 1500g, 4°C for 30 min. The supernatant was discarded and the pellet centrifuged for 10 min at 1500g, 4°C. The remaining liquid was aspirated and the pellet dissolved 10–100 µl water. The protein concentration was determined using a standard protein assay (Bio-Rad, Hercules, CA, USA).

Spheroid treatment with inhibitors

The spheroids were collected and washed twice with fresh medium to remove the methylcellulose. The different inhibitors were fresh prepared in DMSO such as the CYP inhibitor proadifen (stock 60 µM) and the arachonidate lipoxygenase (ALOX) inhibitor baicalein (100 nm stock) all from Sigma Aldrich and diluted to the indicated final concentrations by medium and incubated with the spheroids. Afterwards, the spheroids were washed twice with PBS pH 7.4 to exclude the spillover of the inhibitors to the LEC. Then, spheroids were transferred to 1 ml LEC medium, transferred to confluent LEC monolayers and incubated for 3 h at cell culture conditions, when CCID formation was measured.

Statistical analysis

A statistical analysis was performed using the GraphPad Prism 5 software (San Diego, CA, USA) as appropriate by a Student’s t-test or a non-parametric Mann–Whitney test.

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Conflict of Interest statement. The authors have no conflict of interest to declare.

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