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# Functional characterization of *Mycobacterium tuberculosis* Rv2969c membrane protein

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ABSTRACT

Identifying *Mycobacterium tuberculosis* membrane proteins involved in binding to and invasion of host cells is important in designing subunit-based anti-tuberculosis vaccines. The Rv2969c gene sequence was identified by PCR in *M. tuberculosis* complex strains, being transcribed in *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG. Rabbits immunized with synthetic peptides from highly specific conserved regions of this protein produced antibodies recognizing 27 and 29 kDa bands in *M. tuberculosis* lysate, which is consistent with the molecular weight of the Rv2969c gene product in *M. tuberculosis* H37Rv. Immunoelectron microscopy revealed the protein was localized on the bacillus surface. Four and three specific high activity binding peptides (HABPs) to the A549 alveolar epithelial and U937 monocyte cell lines were found, respectively. Two of the HABPs found inhibited *M. tuberculosis* invasion of A549 cells, suggesting that these peptides might be good candidates to be included in a multiepitopic, subunit-based anti-tuberculosis vaccine.



*Mycobacterium tuberculosis* bacillus, the major causative agent of tuberculosis, is responsible for the death of 2 million annually and the infection of other 9 million people around the world. This disease's prevalence has increased over the past few years due to co-infection with the Human Immunodeficiency Virus (HIV) and the appearance of multi-drug and extensive-drug resistant strains; which, added to the financial and practical limitations of directly observed therapy strategies (DOTS) [1], has increased the need for a much deeper understanding of this microorganism's biology for developing new cost-effective therapies and vaccines.

Designing a multiepitopic, subunit-based, chemically-synthesized anti-tuberculosis vaccine requires characterizing protein mechanisms involved in invasion and infection of target cells. Studies carried out during the last 10 years following publication of the *M. tuberculosis* genome [2] have led to identifying different membrane proteins expressed during certain infection stages or under specific culture conditions by using transcriptomic [3,4] or proteomic [5,6] analysis tools.

The infection of epithelial cells and macrophages in the alveoli is an obligated step for mycobacteria dissemination to other body tissues [7] and involves the interaction of several surface receptors with bacteria, most of which have been slightly studied to date [8]. Studies of non-polarized pulmonary epithelial cells (HEp-2) only have revealed the existence of at least five *M. tuberculosis* proteins binding to biotinylated cell fractions, among which the already characterized adhesion heparin-binding hemagglutinin (HBHA) has been identified [9].

Rv2969c protein's presence on bacterial membrane has been confirmed by membrane proteomic profile studies [5]. Even though, it was not immunogenic in T-cell activation assays [6] suggesting an immunological silent state. Bacterial dormancy models in mice have shown that Rv2969c gene is not over-expressed in intracellular persistent stages [3]; moreover, it has been demonstrated that this gene is expressed equally both in cultured microorganisms and in those phagocyted by naive or IFN-$\gamma$ activated macrophages [4], suggesting that its role is not linked to the intraphagolysosomal lifestyle.

This work involved detecting the presence of the Rv2969c gene and its transcripts in *M. tuberculosis* complex (MTC) strains and clinical isolates, and then characterizing its HABPs involved in binding to and invasion of monocytes (U937) and epithelial cells (A549) using synthetic peptides with the specific purpose of assessing their potential as possible components of a multiepitopic, subunit-based, synthetic anti- tuberculosis vaccine.

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## Materials and methods

*Bioinformatics analysis. Mycobacterium tuberculosis* Rv2969c protein sequences were obtained from the Tuberculist Webserver (http://www.genolist.pasteur.fr/TubercuList/). Protein alignments were done with BLAST and protein features and GRAVY scores were calculated by using the PROTPARAM tool (http://www.us.expasy.org/tools/protparam.html) [10]. Signal sequences, non-classical secretion, transmembrane domains, and *O*-glycosylation sites were predicted

*Mycobacterial species and strains*. The ATCC and Trudeau Mycobacterial Collection were the sources of the mycobacterial species and strains used. Ten *M. tuberculosis* clinical isolates were obtained from different patients coming from different regions of Colombia and attending the TB program at either the San Juan de Dios Hospital or the Santa Clara Hospital, in Bogotá, Colombia. All Mycobacterium species were grown for 5–15 days in Middlebrook 7H9 supplemented (OADC) (BBL, Becton Dickinson, USA).

*DNA extraction, PCR, and DNA sequencing.* Genomic DNA was extracted from MTC strains broth by using UltraClean™ Microbial DNA Isolation Kit (MOBIO Inc., Carlsbad, CA). A 1 μl gDNA was used as amplification template for the Rv2969c protein-encoding gene. Amplification primers were designed based on the GenBank reported genomic sequence (Accession No. NC_000962.2) as follows: E8-F (5′-GGCACCACCATCGTC-3′) and E10-R (5′-TCTGTGCCATT GACCCG-3′). The PCR mixture included: 1U Taq polymerase (Biolase) (Bioline Inc., London), 1× Taq polymerase reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 μM of each primer. Under the following conditions: initial DNA denaturing at 94 °C for 5 min, followed by 25 cycles of: 1 min annealing at 59 °C for E8-F/E10-R, 45s extension step at 72 °C and 1 min denaturing at 94 °C. A final extension cycle was performed at 72 °C for 5 min. Negative control included PCR mixture with water instead of DNA. The amplified products were visualized in 1% agarose gel electrophoresis stained with SYBR safe (Invitrogen, Eugene, Oregon). Positive amplicons were purified using a Wizard preps kit (Promega, Madison, WI) and sequenced in an automatic sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA), the sequencing strategy involved forward and reverse specific primers for the Rv2969c gene from *M. tuberculosis* H37Rv strain and the 10 clinical isolates.

*RNA isolation and RT-PCR*. Two volumes of Trizol (Invitrogen, Carlsbad, CA) were then added and extraction was carried out following all manufacturer's instructions. Total RNA was quantified by GeneQuant spectrophotometer (Pharmacia Biotech, Piscataway, NJ), treated with DNAseI (Invitrogen, Carlsbad, CA) and used as template for the RT-PCR employing the primers designed for the Rv2969c and *rpo*B genes, the latter was used as a transcription positive control, following the Super Script III One Step RT-PCR (Invitrogen, Carlsbad, CA) manufacturer instructions. *M. tuberculosis* H37Rv treated with DNAseI was used as negative control for cDNA synthesis.

*Rabbit immunization* . Five-hundred microgram of the 25666 ($CG^{141}$VADESIEAFRRFHAALFSKD$^{160}$GC) peptide polymerized by adding cysteine at the amino and carboxi terminus were used for inoculating two New Zealand strain rabbits on days 0, 20, and 40. Polymerized peptide was emulsified in Freund's Incomplete Adjuvant. Final bleeding was carried out on day 60. All animal handling was done in accordance to the Fundación Instituto de Inmunología's bioethics committee guidelines.

*Cell culture*. The U937 (ATCC No. CRL-1593.2) and A549 (ATCC No. CCL-185) cell lines were cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logon, UT), at 37 °C and 5% $CO_2$. Cells were dislodged then collected and washed with PBS.

*Binding assays*. 20-mer non-overlapping peptides derived from the protein encoded by the Rv2969c gene were synthesized [11] and a Tyr-residue was added at the N-terminal of those peptides which had no Tyr-residue in their sequences to allow $_{125}$I-radiolabeling [12,13]. Purified peptides were radio-labeled according to previously described techniques [13–16]. Briefly, 0.3 μmol chloramine-T and 3.2 μL $Na^{125}I$ (100 mCi/ml, MB Biomedicals) were added to 5 μl peptide solution (1 μg/μl), incubated for 15 min at 18 °C and then adding 0.18 μmol sodium metabisulphite. The radio-labeled peptide was purified on a Sephadex G-10 packed column (Pharmacia, Uppsala, Sweden).

A549 and U937 cells ($1.5 \times 10^6$) were incubated with increasing radio-labeled peptide concentrations (0–950 nM), in the presence or absence of unlabeled peptide (40 μM) for 90 min at 4 °C. This reaction mixture was passed through a 60:40 dioctylphthalate-dibutylphthalate cushion and spun at 4500 g for 3 min. Cell-associated radioactivity was quantified. Specific binding values were obtained by subtracting non-specific binding from total-binding values [12]. Any peptide showing a specific binding activity $\geqslant$1% was considered to be a HABP [17] (Fig. 3).

For saturation assays, A549 and U937 cells ($1.2 \times 10^6$) were incubated with radio-labeled HABPs at 0–3000 nM concentrations, in the presence or absence of unlabeled peptide (30 μM). The curves obtained were analyzed by saturation and Hill analyses [14,16].

*SDS–PAGE* and immunoblotting. A total of 500 μg/ml *M. tuberculosis* protein sonicate was separated in a discontinuous SDS–PAGE system, then transferred to nitrocellulose membrane by semidry blotting and cut into strips [18]. Commercial molecular weight markers (NEB, Inc. Beverly, MA) were used for calibration. The strips were incubated with 1:100 (v/v) diluted sera obtained from rabbits immunized with peptide 25666, in TBST (0.02 M Tris–HCl pH 7.5, 0.05 M NaCl, 1% Tween 20) and 5% skimmed milk. After washing five times with TBST, strips were incubated for 1 h with 1:3000 alkaline phosphatase conjugated anti-rabbit IgG antibody (ICN Biomedicals, Costa Mesa, CA) and the reaction was developed with NBT/BCIP (KPL, Gaithersburg, MD).

*Immunoelectron microscopy*. Immunoelectron microscopy (IEM) studies were carried out on a Phillips CM 10 TEM. Briefly, *M. tuberculosis* H37Rv wet pellet were fixed with a 4% paraformaldehyde–0.5% glutaraldehyde solution for 2 h at 4 °C. After being fixed, the pellet was dehydrated in graded-ethanol (50–100%) and then embedded in LR- white hard-grade resin (Sigma) for 4 days at 40 °C. Forty nanometer thin sections were cut and mounted on 300 mesh nickel grids. The grids were incubated in a saturated sodium metaperiodate solution for 1 h at 20–22 °C for antigen retrieval. Grids were then floated sections-down in a beaker containing 0.01 M sodium citrate buffer for 15 min at 80 °C. After 1 h of blocking in Tris-buffered saline (TBS) (0.05 M Tris in isotonic saline, pH 7.6) containing 0.05% BSA, grids were incubated in either 1:10 (v/v) or pure rabbit polyclonal serum for 1 h at 37 °C. Following a TBS-0.025% Tween 20 wash, grids were immersed in a 1:50 (v/v) dilution of 5 nm gold-labeled anti-rabbit IgG (Sigma) for 1 h at 20–22 °C. Grids were then washed with TBS-Tween and fixed in 2.5% glutaraldehyde. Fifteen minutes incubation in 1% uranyl acetate followed fixation. Grids were washed with distilled water and dried at 20–22 °C before observation [19].

*Invasion inhibition assay using HABPs*. The biological relevance of HABPs identified in Rv2969c was evaluated [20]. In brief, $1 \times 10^6$ A549 cells suspended in incomplete RPMI 1640 medium were incubated for 1 h in the presence of 50 μM, 100 μM or 200 μM identified A549-HABPs, before adding $1 \times 10^7$ previously stained *M. tuberculosis* H37Rv bacilli with 20× SYBR Safe (MOI 1:10) to a 200 μl final volume. They were incubated at 37 °C for 2 h with constant shaking and then placed in 20 μg/ml amikacin-supplemented medium (ICN Biomedicals, Costa Mesa, CA) for 30 min to kill off

extra-cellular bacteria. Cells were pelleted at 500 g for 5 min, washed twice, then fixed in 1% paraformaldehyde (Merck, Darmstadt, Germany) in RPMI 1640 at 4 °C for 1 h, and finally washed and resuspended in RPMI 1640. The cells were counterstained with freshly-prepared 3% Methylene Blue (Merck, Germany) 5 min before flow cytometry and then incubated at 20–22 °C. Invasion negative control was carried out in the presence of 30 μM colchicine (SIGMA, St. Louis, MO) as inhibitor added to A549 cells under the same conditions described. The inhibitors were maintained throughout the 2 h invasion period. Uninfected epithelial cells were discriminated from infected epithelial cells according to light FL1 characteristics. Two tails Student's *t*-test was used for verifying possible differences between peptide treatments and control not pre-incubated with peptides or colchicine.

## Results and discussion

### *Molecular analysis of the Rv2969c gene*

The presence of Rv2969c in MTC (*M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*) strains was confirmed by the amplification of a 587 bp region from this gene (Fig. 1A). A similar amplification band was also seen in 10 *M. tuberculosis* isolates from patients suffering pulmonary and extra-pulmonary tuberculosis (data not shown). Sequence analysis of all clinical isolates PCR amplicons revealed no sequence variation suggesting that this protein was conserved. TBLASTN analysis found similar (although not identical) genes in the pathogenic *M. ulcerans* and opportunistic *M. avium* species, which indicates that this gene is present in related species and may possibly play an important role in *M. tuberculosis* pathogenesis.

RT-PCR was used to evaluate Rv2969c gene transcription under culture conditions in MTC strains, finding an amplification band of the expected length (bp) in *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG. There was low transcription in *M. bovis*, *M. africanum*, and *M. microti* species as evidenced by the amplification band's low intensity (upper part of Fig. 1B). The constitutive *rpo*B gene used as positive transcription control amplified a 360 bp fragment in cDNA from these species when using the same cDNA concentration used for Rv2969c RT-PCR (lower part of Fig. 1B).

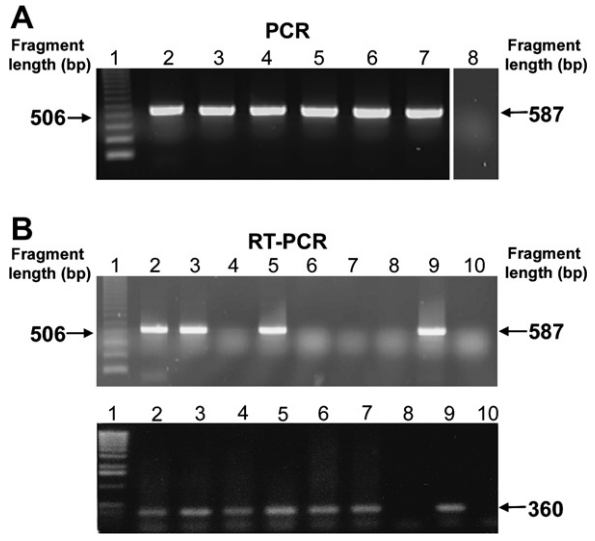


**Fig. 1.** Rv2969c gene identification and transcription assessment for MTC strains. (A) Gel showing the Rv2969c 587 bp PCR amplicon on mycobacterial genomic DNA. Lane 1, MWM (1 Kb Gibco); 2, *M. tuberculosis* H37Rv; 3, *M. tuberculosis* H37Ra; 4, *M. bovis*; 5, *M. bovis* BCG; 6, *M. africanum*; 7, *M. microti*; 8, PCR negative control. (B) Upper part. 587 bp amplicon of Rv2969c gene by RT-PCR from MTC strains' cDNA. 1, Length marker (1kb Gibco); 2, *M. tuberculosis* H37Rv; 3, *M. tuberculosis* H37Ra; 4, *M. bovis*; 5, *M. bovis* BCG; 6, *M. africanum*; 7, *M. microti*; 8, Negative control (*M. tuberculosis* DNA treated with DNAseI); 9. PCR positive control (*M. tuberculosis* H37Rv DNA); 10, PCR negative control. Lower part. RT-PCR for *rpo*B gene (RNA polymerase, β-subunit) as positive transcription control in the same strains (360 bp fragment).

### *Anti-peptide Rv2969c rabbit antibodies specifically recognized 27 and 29 kDa bands on M. tuberculosis sonicate*

The synthetic peptide 25666 ($CG^{141}$VADESIEAFRRFHAALFSK $DY^{160}$GC) belonging to Rv2969c protein sequence was used for immunizing rabbits. This peptide was chosen based on its hydrophilicity plot, bearing in mind that this sequence is expected to be exposed on protein surface. Pre-immune and post-third immunization sera were used to verify the translation and presence of this protein in *M. tuberculosis* sonicate. Rabbit pre-immune sera did not recognize *M. tuberculosis* proteins (lanes 1 and 3, Fig. 2A). On the contrary, sera from rabbits immunized with peptide 25666 elicited antibodies that specifically recognized two (27 and 29 kDa) bands close to the theoretical protein's molecular weight (26.8 kDa) (lanes 2 and 4, Fig. 2A). The Rv2969c amino acid sequence has 15 potential *O*-glycosylation sites as predicted by YinOYang 1.2 server (http://www.cbs.dtu.dk/services/YinOYang/), which could explain the recognition of the 29 kDa band by both obtained sera. The second serum slightly recognized 50 and 60 kDa bands also.

### *Surface detection of Rv2969c by immunoelectron microscopy*

Rabbit sera against peptide 25666 (shadowed in Fig. 3), which specifically recognized the *M tuberculosis* protein Rv2969c by Western blot, was used for immunolabeling and immunoelectron microscopy (IEM). IEM showed 5 nm colloidal gold particles located on *M. tuberculosis* H37Rv surface in the microphotography of a longitudinal cut through the bacterium (Fig. 2B). This result supported the notion that this gene is being expressed and that the expressed protein is located on the cell surface.

All the data here presented strongly suggest that the Rv2969c gene is present in the MTC and that this gene is being transcribed and expressed in *M. tuberculosis*. The protein encoded by this gene is a highly conserved protein, which has been shown to be localized on the mycobacterial surface as shown by our IEM studies and previous proteomic analyses [5,6].

### *HABP binding to U937 and A549 cells*

Taking into account that there is no evidence of protein Rv2969c biological function, we were interested in determining whether regions of this protein specifically interacted with U937 and A549 cells (these cell lines have been widely used as a model for studying *M. tuberculosis* infection) [6,21]. For this purpose synthetic peptides, covering the complete Rv2969c protein sequence were tested in A549 and U937 binding assays.

Four (11188, 11189, 11194, and 11198) and three (11188, 11189, and 11192) specific high activity binding peptides (HABPs) to the A549 and U937 cell lines were found, respectively. Peptides 11194 and 11198 exclusively bound to A549 cells, while peptide 11192 exclusively bound to U937 cells (Fig. 3). All identified HABPs displayed saturable binding. Dissociation constants (Kd), Hill coefficients and specific binding sites number for the A549 and U937 HABPs are shown in Table 1. All found HABPs showed positive cooperativity in their interaction with host receptors.

Two of the identified HABPs bind to both cell types, further studies will allow determining whether a common receptor is found for alveolar epithelial cells and monocytes. Greater availabil-

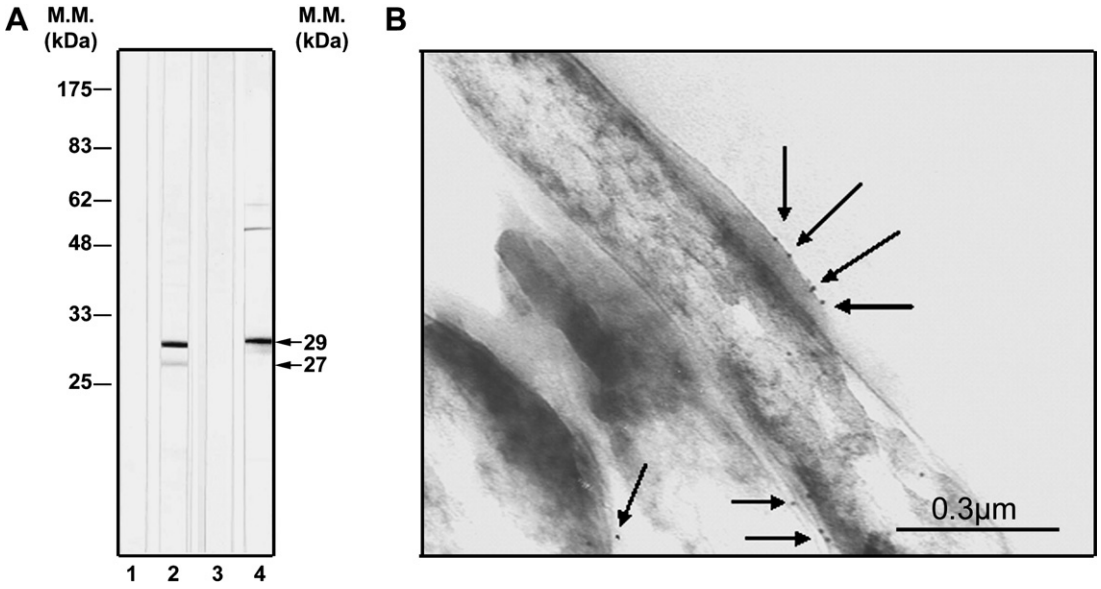


**Fig. 2.** Rv2969c expression and subcellular localization. (A) Western blot analysis of sera (pre-immune in lanes 1 and 3, post-3rd immunization in lanes 2 and 4) obtained from two rabbits inoculated with peptide 25666, against *M. tuberculosis* sonicate. (B) Rv2969c protein localization in *M. tuberculosis* H37Rv membrane as assessed by immunoelectron microscopy. Gold-labeled particles can be observed on mycobacterial surface with a 1:10 dilution of rabbit immune serum. No labeling was observed in control experiments when pre-immune serum was used (data not shown).

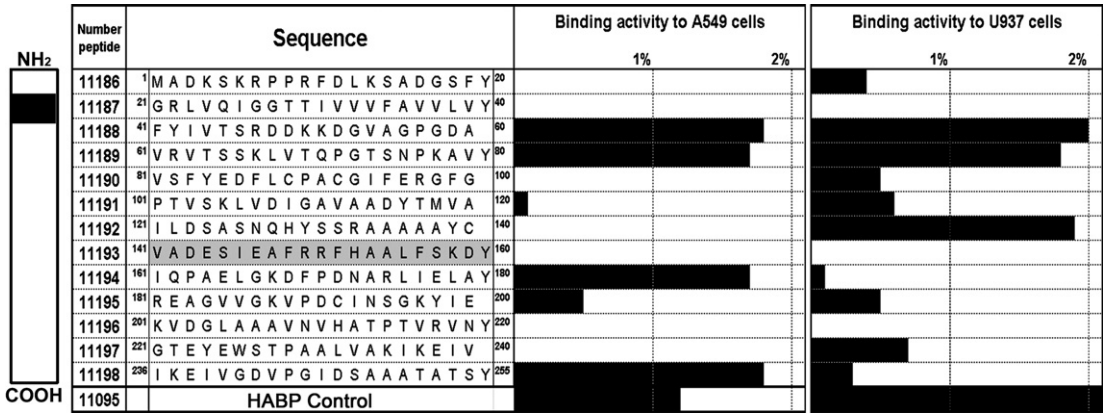


**Fig. 3.** A549 and U937 cell binding profiles for Rv2969c derived peptides. Each black bar represents the specific binding graph's slope. Peptides with slope $\geqslant 1\%$ were considered as HABP. The shaded peptide was the one used in its polymeric form to immunize rabbits and produce the polyclonal antibodies. Rv2969c transmembrane topology is presented on the left-hand side. Black shows the transmembrane domain present in the Rv2969c sequence.

**Table 1**
Binding properties of Rv2969c HABPs estimated from saturation curves

| Cell line | HABP | Kd (nM) | Hill coefficient | Specific binding sites |
|---|---|---|---|---|
| A549 | 11188 | 670 | 1.4 | 642,000 |
| | 11189 | 1250 | 1.7 | 2,250,000 |
| | 11194 | 620 | 1.3 | 1,300,000 |
| U937 | 11188 | 204 | 1.5 | 325,000 |
| | 11189 | 180 | 1.6 | 102,000 |
| | 11192 | 171 | 1.6 | 96,000 |

ity of HABP binding sites was identified on A549 compared to U937 cells.

*Inhibition of M. tuberculosis invasion of A549 cells by HABPs*

Three different concentrations of previously identified HABPs were used for verifying HABP biological relevance, thereby seeking to inhibit *M. tuberculosis* invasion of epithelial cells (A549). It was found that two of the A549 HABPs identified for the Rv2969c protein (11188 and 11198) inhibited *M. tuberculosis* invasion of this cell line at all concentrations used. Fig. 4 shows density plots displaying an example of the data obtained at the peptide concentrations tested. HABP inhibitory ability was also observed to be increasingly higher when peptide concentrations increased. 11198 presented an invasion inhibition effect at 200 µM which was even greater than that exhibited by positive control (30 µM colchicine) ($P$ value $\leqslant 0.05$) (Fig. 4).

The functional A549 cell binding assay revealed the existence of two peptides able to inhibit bacterial invasion of this epithelial cell line, thereby suggesting their relevance in recognizing and invading the A549 cell line. Bearing in mind the lower number of specific binding sites found for HABP 11188 on A549 cells (Table 1), the percentage of inhibition reached at greater concentrations did not vary compared the one at 100 mM possibly due to cellular receptor saturation.

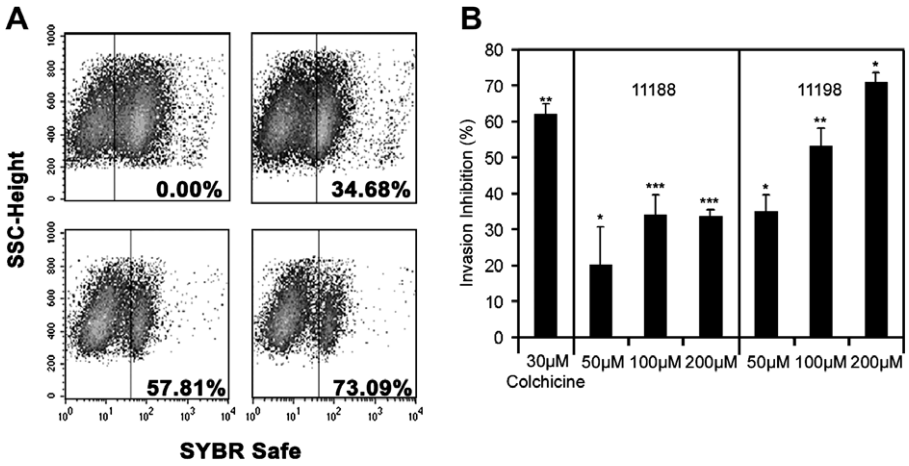


**Fig. 4.** Invasion inhibition assays. (A) Density plots showing the invasion inhibition assays. Top left, Invasion without peptide. Top right, Invasion after pre-incubation with 100 μM peptide 11188. Bottom left, invasion after pre-incubation with 100 μM peptide 11198. Bottom right, invasion after pre-incubation with 200 μM peptide 11198. (B) Inhibition percentages with different concentrations of peptides 11188 and 11198. The results correspond to average inhibition percentage calculated for each treatment ±SD. $^{*}P$ value $\leqslant 0.05$, $^{**}P$ value $\leqslant 0.01$, $^{***}P$ value $\leqslant 0.001$.

Regarding colchicine control, HABP 11188 inhibited invasion by 50% at 100 and 200 μM and by around 30% at lesser concentration. It was observed that HABP 11198 had 30–70% invasion inhibition at different concentrations; this HABP presented a dose-dependent inhibition pattern. HABP 11198 presented 50–100% inhibition, emphasizing that this Rv2969c protein HABP had the highest invasion inhibition ability.

*Bioinformatics analysis*

Different bioinformatics tools were used for predicting Rv2969c cell localization as well as this protein's transmembranal topology. Even though SignalP did not predict the existence of a signal peptide, the high GRAVY value (0.106), the predicted non-classical secretion pathway (SecP score:0.789), and the existence of a possible transmembranal helix in the complete Rv2969c amino acid sequence suggested its presence on bacterial membrane.

In the present study, host binding regions of the membrane protein Rv2969c (corresponding to HABPs 11188 and 11198) have been identified and characterized, which represents an initial step in the rational selection of components for a subunit-based multiepitopic vaccine candidate against tuberculosis. Blocking antibodies or a cellular immune response directed against such regions (more exactly against the sequences directly involved in invasion of type II alveolar epithelial cells) will allowed to reducing bacterial load in the lungs and hampering systemic expansion of infection, since it has been shown that invasion of alveolar epithelium is a required prior stage for bacterial blood vessel translocation [7]. Future studies aimed at testing the identified HABPs immunogenicity and protection-inducing ability in tuberculosis animal models would confirm its potential as an anti-tuberculosis vaccine candidate.

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